



universität  
wien

# DIPLOMARBEIT

Titel der Diplomarbeit

Functional Genomics and Proteomics of  
Virus-Induced Innate Immunity Pathways

durchgeführt am

Research Center for Molecular Medicine  
of the Austrian Academy of Sciences

angestrebter akademischer Grad

Magistra der Naturwissenschaften (Mag. rer.nat.)

Verfasserin / Verfasser:	Evelyn Dixit
Matrikel-Nummer:	0309442
Studienrichtung (lt. Studienblatt):	Molekulare Biologie
Betreuerin / Betreuer:	Dr. Tilmann Bürckstümmer

Wien, am 28.10.2007



## Danksagungen

Ich bedanke mich ganz besonders bei Giulio Superti-Furga für die Möglichkeit, meine Diplomarbeit zu einem großartigen Thema am Ce-M-M- anfertigen zu dürfen und für seine Unterstützung bei der Planung meiner beruflichen Zukunft.

Im gleichen Maße danke ich Tilmann Bürckstümmer für die hervorragende Betreuung, die angenehme Zusammenarbeit und das hohe Maß an Freiraum und Eigenverantwortung.

Zudem bedanke ich mich bei Jacques Colinge für die ausführliche Erklärung der statistischen Analysen und bei Gerhard Dürnberger für die geduldige Unterstützung in allen bioinformatischen Belangen. Wer hätte je gedacht, dass ich das Ende eines Excel Spreadsheets sehen werde?

Im übrigen danke ich allen Kollegen am Ce-M-M- für ihre Hilfsbereitschaft und die freundliche Arbeitsatmosphäre.

Außerdem möchte ich an dieser Stelle Tilmann Bürckstümmer, Adriana Goncalves, Agnes Gstöttenbauer und Giulio Superti-Furga meinen Dank für die Ausdauer beim Korrekturlesen dieser Arbeit aussprechen.

Nicht zuletzt danke ich Sunil, dass er mich geduldig durch alle Höhen und Tiefen meiner Studienzeit begleitet hat.



<b>1</b>	<b>INTRODUCTION</b>	<b>1</b>
1.1	The immune system protects from infectious disease	1
1.1.1	Innate immune system	1
1.1.2	Adaptive immune system	3
1.1.3	Common and distinct features of innate and adaptive immune responses	4
1.2	Innate immunity to viral infections	5
1.2.1	Toll-like receptors 3, 7, 8 and 9 are membrane-associated nucleic acid receptors	7
1.2.1.1	TLR3	8
1.2.1.2	TLR7, TLR8 and TLR9	9
1.2.2	Retinoic acid-inducible gene I-like helicases are cytosolic dsRNA receptors	10
1.2.2.1	RIG-I	10
1.2.2.2	Other RIG-I-like helicases	12
1.2.3	Cell-type specific differences in type I interferon production	12
1.2.4	DAI is a cytosolic receptor for DNA	13
<b>2</b>	<b>AIM OF THE STUDY</b>	<b>14</b>
<b>3</b>	<b>MATERIALS AND METHODS</b>	<b>15</b>
3.1	Molecular biology	15
3.1.1	Plasmids	15
3.1.2	Cloning of candidate 4	15
3.1.3	Site-directed mutagenesis	15
3.2	Cell biology	16
3.2.1	Cell lines and cell culture	16
3.2.2	RNA interference	17
3.2.3	Stimulation of cells	17
3.2.4	Transient transfection	18
3.2.5	Generation of stable cell lines by retroviral gene transfer	18
3.3	Protein biochemistry and immunological assays	19
3.3.1	Immunofluorescence	19
3.3.2	Cell lysates for protein gels	19

## Table of contents

---

3.3.3	SDS-PAGE .....	20
3.3.4	Western blots .....	21
3.3.5	Silverstaining .....	21
3.3.6	RNA resins .....	22
3.3.7	DNA resins .....	22
3.3.8	Pull-down experiments .....	23
3.3.9	Tandem affinity purification .....	24
3.3.10	Mass spectrometry analysis .....	25
3.4	Genomic analyses .....	26
3.4.1	RNA extraction .....	26
3.4.2	Microarray analysis .....	26
3.4.3	Quantitative PCR.....	26
<b>4</b>	<b>RESULTS .....</b>	<b>28</b>
4.1	Generation of a list of candidate proteins .....	28
4.1.1	Pull-down experiments identify nucleic acid binding proteins .....	28
4.1.2	Microarray analyses reveal nucleic acid-regulated genes.....	30
4.1.3	Merging the proteomics and genomics datasets.....	31
4.2	Confirmation of microarray data by quantitative PCR.....	35
4.3	Validation of functional relevance of candidates .....	37
4.3.1	Evaluation of all candidates in parallel.....	37
4.3.1.1	Effect of candidate silencing on polyI:C-stimulated IFN $\beta$ induction.....	37
4.3.1.2	Effect of candidate silencing on polydAdT:dTdA-stimulated IFN $\beta$ induction .....	42
4.3.2	“Educated guess”-approach.....	43
4.3.2.1	Expression of candidate 4 is nucleic acid- and IFN $\beta$ -inducible .....	43
4.3.2.2	Candidate 4 is a perinuclear protein .....	45
4.3.2.3	Overexpression of candidate 4 reduces IFN $\beta$ induction in response to DNA stimulation .....	46
4.3.2.4	Interactors of candidate 4 .....	47
4.3.2.5	Overexpression of catalytically inactive mutants of candidate 4 still reduces DNA-induced IFN $\beta$ induction .....	48
<b>5</b>	<b>DISCUSSION.....</b>	<b>50</b>

5.1	List of candidate proteins .....	51
5.2	Candidates affecting polyI:C-induced IFN $\beta$ induction .....	53
5.3	Candidates affecting polydAdT:dTdA-induced IFN $\beta$ induction .....	54
5.4	Alternative approaches to determine the functional relevance of candidates.....	54
5.5	Candidate 4.....	56
5.6	Conclusion .....	57
<b>6</b>	<b>SUMMARY.....</b>	<b>59</b>
<b>7</b>	<b>ZUSAMMENFASSUNG .....</b>	<b>61</b>
<b>8</b>	<b>REFERENCES .....</b>	<b>63</b>
<b>9</b>	<b>ABBREVIATIONS .....</b>	<b>66</b>





# 1 Introduction

## 1.1 The immune system protects from infectious disease

Multicellular organisms have developed diverse mechanisms to fight foreign invaders. The complexity and specificity of these mechanisms have increased during evolution. Mammals defend themselves against constant challenges by viruses, bacteria, fungi and parasites using a sophisticated immune system that consists of a network of soluble molecules, specialized cells, tissues and organs. The components of the immune system work together in a coordinated manner to prevent the entry of infectious agents or of foreign substances in general into the body. If microbes enter the host despite these protective measures, the immune system reacts by mounting an immune response, which is a collection of processes that aim to kill and clear the invading pathogen.

The immune system is divided into two branches: the innate and the adaptive immune system. Innate and adaptive immunity are interdependent, and therefore the cooperation between the two is crucial for mounting an appropriate immune response to various pathogens. [1]

### 1.1.1 *Innate immune system*

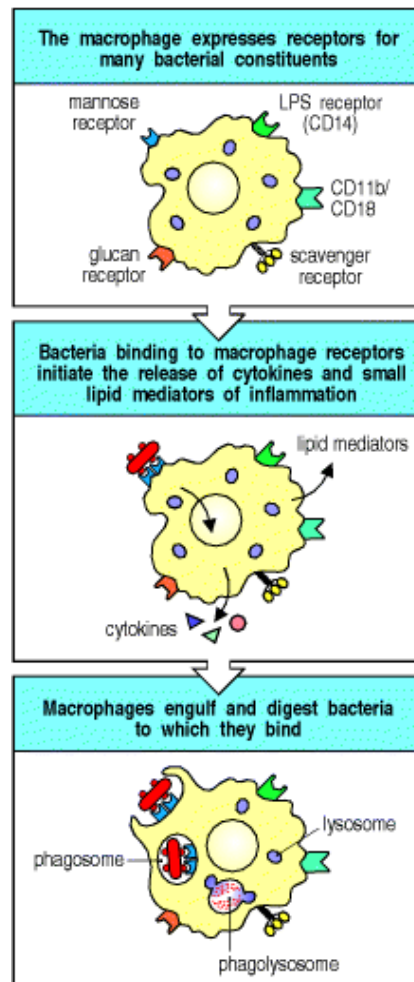
The innate immune system is the first line of defense against invading pathogens. It prevents infections and often eliminates microbes before the adaptive immune response is mounted. In addition, it stimulates and modulates the quality of the subsequent adaptive immune responses. The components of the innate immune system are epithelial barriers, circulating effector cells i. e. phagocytes and natural killer (NK) cells, the complement system and other soluble mediators.

Epithelial barriers physically prevent entry of pathogens into the host organism and often secrete chemical substances that interfere with microbial growth. For example, lysozyme degrades the bacterial cell wall component peptidoglycan and is secreted in tears and saliva. Antibiotic peptides known as defensins protect gut and respiratory epithelia.

The phagocytes including neutrophils, macrophages and dendritic cells display various germ line-encoded pathogen recognition receptors (PRRs) that recognize highly conserved

## Introduction

molecular structures shared by large groups of microbes. These so-called pathogen-associated molecular patterns (PAMPs) are usually essential for the survival or pathogenicity of microbial pathogens, but are not present on mammalian cells. Examples for PAMPs include lipopolysaccharide (LPS) of gram-negative bacteria, DNA sequences rich in unmethylated CG dinucleotides (CpG-DNA) as found in bacteria or viruses, and viral double-stranded RNA (dsRNA). Some PRRs like the macrophage mannose receptor promote endocytosis upon ligand recognition resulting in the internalization of a microbe by phagocytosis. Phagocytosed microbes are eventually killed by reactive oxygen and nitrogen species when the phagosome fuses with the lysosome to form the phagolysosome. Other PRRs like Toll-like receptors (TLRs) activate a signaling cascade leading to the induction of inflammatory cytokines [2] that recruit more cells to the infection site and stimulate the adaptive immune response. A schematic overview on phagocyte functions is displayed in figure 1.



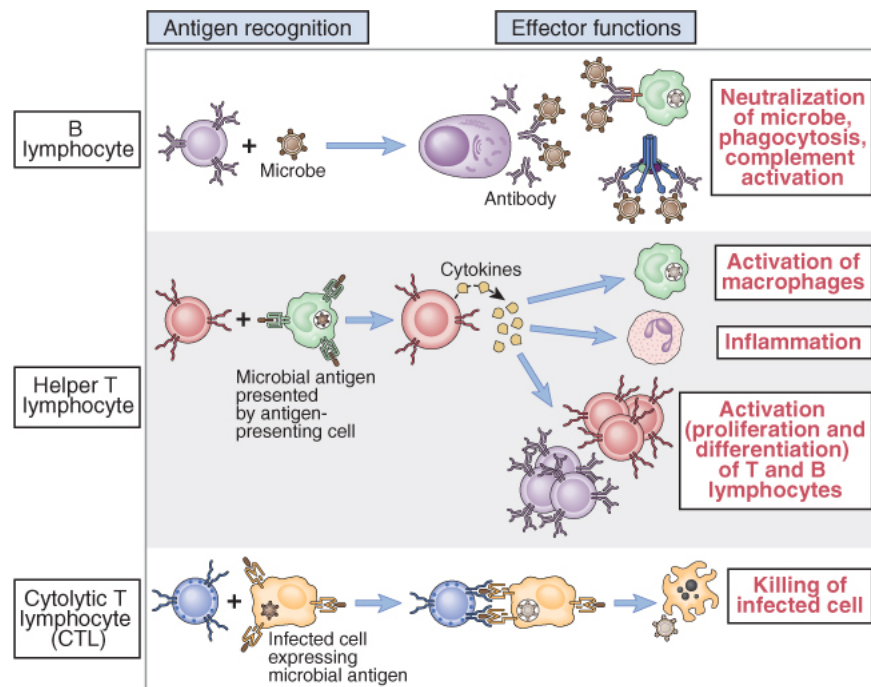
**Figure 1. Macrophages phagocytose microbes and produce inflammatory cytokines.** Phagocytes like macrophages express a variety of germ line-encoded PRRs. Here five such receptors are illustrated – the LPS receptor CD14, the complement receptor CD11b/CD18, the macrophage mannose receptor, the scavenger receptor and the glucan receptor. The mannose receptor mediates phagocytosis of microbes. Ingested microbes are killed when the phagosome fuses with the lysosome. Other phagocyte receptors lead to the production of various inflammatory cytokines [3].

Natural killer cells trigger apoptosis in host cells infected with viruses or intracellular bacteria, and tumor cells.

Soluble factors of the innate immune system include the blood plasma proteins of the complement system, a proteolytic cascade that ultimately leads to lysis of microbes, and a variety of inflammatory cytokines like tumor necrosis factor  $\alpha$  (TNF $\alpha$ ), type I interferons (IFN $\alpha$  and IFN $\beta$ ) and many others.

### 1.1.2 Adaptive immune system

The most prominent components of the adaptive immunity are B cells (lymphocytes matured in the bone marrow) and T cells (lymphocytes matured in the thymus) that mediate two types of adaptive immune responses, called humoral and cellular immunity respectively. Humoral immunity is the principal defense mechanism against extracellular microbes and their toxins. B cells secrete antibodies that constitute the effectors of humoral immunity as they recognize microbial antigens, neutralize the infectivity of microbes, and target extracellular microbes for elimination by various mechanisms e. g. ingestion by phagocytes or lysis by complement proteins (figure 2).



**Figure 2. Effector mechanisms of adaptive immune cells.** B cells recognize soluble antigens and differentiate into antibody secreting plasma cells. Helper T cells recognize antigens displayed by antigen presenting cells e. g. macrophages and secrete cytokines. Cytolytic T cells recognize antigens on infected cells and kill them [1].

## Introduction

---

Cellular immunity targets intracellular microbes such as viruses as well as certain bacteria that survive and proliferate inside host cells, and are therefore inaccessible for antibodies. T cells are the mediators of cellular immunity. Cytolytic T lymphocytes kill cells that are infected with microbes, whereas helper T lymphocytes instruct phagocytes of the innate immune system e. g. macrophages to kill ingested microbes. Moreover, helper T cell-secreted cytokines cause inflammation and stimulate proliferation and differentiation of T and B cells.

The hallmarks of adaptive immunity are described below. i) Specificity: Lymphocyte receptors and thus adaptive immune responses are specific for distinct antigens or even structural details of antigens, so-called epitopes. ii) Diversity: Lymphocyte receptors are expressed clonally. Thus each lymphocyte displays receptors with a single antigen specificity. However, the diversity of an individual's lymphocyte population, the lymphocyte repertoire, is very large (approximately  $10^7$  to  $10^9$ ). Receptor diversity is generated by somatic rearrangement of gene fragments during B and T cell maturation. iii) Memory: Secondary immune responses are faster, stronger, and qualitatively different in comparison to primary immune responses, because the respective lymphocyte clone expands after the primary encounter with an antigen and long-lived memory cells are generated. iv) Specialization: Immune responses are tailored to each pathogen to maximize efficiency. v) Self-limitation: Once the antigen has been cleared, immune responses eventually decline until homeostasis is reached due to regulatory mechanisms. vi) Nonreactivity to self: The immune system distinguishes between its own (self) and foreign (nonself) antigens. It responds to nonself antigens, but tolerates self antigens in healthy individuals. [1]

### ***1.1.3 Common and distinct features of innate and adaptive immune responses***

Innate immunity is phylogenetically older than adaptive immunity and can be found in insects and plants, while mechanisms of adaptive immunity only occur in vertebrates. In contrast to lymphocyte receptors, innate immune receptors are not clonally expressed and recognize common structures on microbes. As their specificity is genetically determined, their diversity is limited and estimated to be in the range of  $10^2$  to  $10^3$ .

The innate immune response is mounted within hours after infection, whereas adaptive immune responses take 3 – 5 days to develop, since appropriate lymphocyte clones need

to expand and differentiate into effector cells. During this time the innate immune system contains the infection or even clears it. The innate immune response is fast, but its quality remains unchanged, whereas the adaptive immune response is optimized during the course of an infection. Moreover, the adaptive immune system is capable of building an immunological memory and therefore improves its response with repeated exposures to a given antigen.

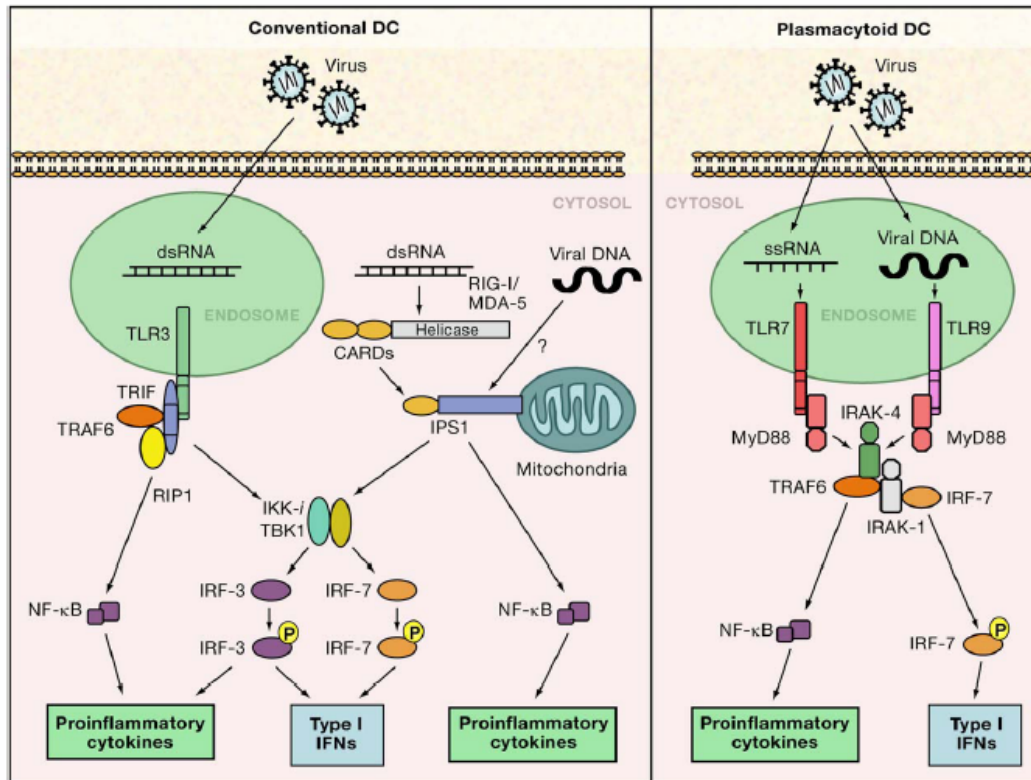
Immunologists have focussed their research interests on adaptive immune responses for many decades, while innate immunity was considered to be of minor importance for immune functions and thus has been neglected. The seeming unspecificity and primitiveness of innate immunity due to its ancient origin may explain the lack of attention. During the last 10 years the perspective has changed as research is revealing the powerful role of the innate immune system. Studies on the septic shock causing agent LPS that is produced by all gram-negative bacteria and leads to  $\text{TNF}\alpha$  release by macrophages led to the identification of TLR4 as its receptor. Further investigations revealed the specificities of the remaining members of the TLR family. Ever since the ground breaking studies on TLR function, innate immunity has been a fast growing research area. In contrast to earlier assumptions, recent findings demonstrate that innate immunity is crucially important for the host immune defense and for driving adaptive immunity.

## 1.2 Innate immunity to viral infections

The prerequisite for an antiviral innate immune response is the detection of viruses by PRRs of innate immune cells. Viruses are typically sensed by the presence of their genomes consisting of either DNA or RNA, but there are also PRRs that recognize other PAMPs like glycoproteins on the viral surface. Several nucleic acid sensors exist, for example the membrane-associated TLRs 3, 7, 8 and 9 as well as various cytosolic receptors. Engagement of these receptors with their respective ligands triggers a signaling cascade that ultimately leads to the production of cytokines and chemokines (figure 3).

The central event of the antiviral immune response is the induction and secretion of type I interferons ( $\text{IFN}\alpha$  and  $\text{IFN}\beta$ ) that establish an antiviral state in infected and adjacent cells and promote adaptive immune responses.  $\text{IFN}\beta$  production is tightly controlled by extracellular and intracellular signals. The concerted action of the transcription factors

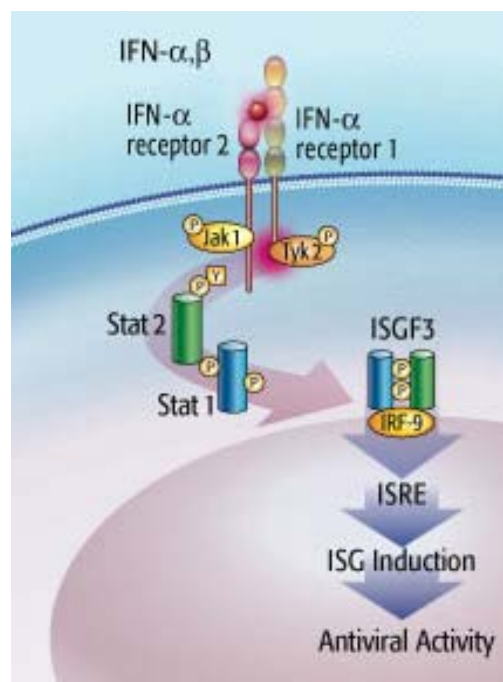
## Introduction



**Figure 3. Antiviral innate immunity pathways.** Various PRRs specifically recognize nucleic acids to sense viral infection. This figure illustrates the membrane associated TLRs 3, 7, and 9 as well as the cytosolic receptors RIG-I and MDA-5. The characteristics of these receptors and their signaling pathways are described below. Ultimately nucleic acid stimulation leads to the induction of type I IFNs and inflammatory cytokines.

activator protein 1 (AP-1), nuclear factor  $\kappa$ B (NF- $\kappa$ B) as well as interferon regulatory factors 3 and 7 (IRF3 and IRF7) is required for transcriptional activation of the IFN $\beta$  gene. AP-1 is activated by phosphorylation of the stress kinases Jun N-terminal kinase (JNK) and p38. The activity of the IRFs and NF- $\kappa$ B is regulated by their subcellular localization. In unstimulated cells, the inhibitor of NF- $\kappa$ B (I $\kappa$ B) binds to NF- $\kappa$ B dimers and sequesters them in an inactive form in the cytosol. Viral infection results in activation of the I $\kappa$ B kinase (IKK) complex that phosphorylates I $\kappa$ B targeting it for polyubiquitylation and subsequent proteasomal degradation. Free NF- $\kappa$ B dimers translocate to the nucleus and activate their target genes e. g. IFN $\beta$ . Inactive IRF3 and IRF7 are also retained in the cytosol. Upon viral challenge the non-canonical IKKs, TANK binding kinase 1 (TBK1) or IKK-i, phosphorylate IRF3. Phosphorylation causes a conformational change and dimerization of IRF3 that allow nuclear translocation and transcription factor activity. AP-1, IRF3 and NF- $\kappa$ B bind to their respective binding sites in regulatory sequences of the IFN $\beta$  gene and participate in the formation of a multiprotein enhancer complex that remodels chromatin in the promoter region and allows transcriptional initiation of IFN $\beta$ .

IFN $\beta$  in turn acts in an autocrine and paracrine manner on neighbouring cells. It binds to the IFN $\alpha/\beta$  receptor (IFNAR) that signals via the Janus kinase (JAK)/ signal transducer and activator of transcription (STAT) pathway. STAT1 and 2 proteins form a heterotrimeric complex with IRF9 called IFN-stimulated gene factor 3 (ISGF3) that transcriptionally activates interferon stimulated genes (ISGs) by binding to the corresponding regulatory sites, the IFN stimulated response elements (ISRE). Among the ISGs are the members of the IFN $\alpha$  family which create a positive feedback loop ensuring a robust interferon response. Most ISGs are effectors of the innate immune response to viruses as they establish an antiviral state in host cells that interferes with viral replication (figure 4). Examples of such effector proteins are double-stranded RNA-activated protein kinase (Pkr) and 2'-5' oligoadenylate synthetase (Oas3). Pkr, activated when bound to dsRNA, a common viral replication intermediate, phosphorylates and thus inactivates eukaryotic translation initiation factor 2 (eIF2 $\alpha$ ), thereby inhibiting protein translation within infected cells. Oas3 is also stimulated by dsRNA. It polymerizes special oligomers that activate the endoribonuclease RNaseL to cleave viral as well as cellular RNAs [4, 5].



**Figure 4. Type I interferons induce an antiviral state in the cell.** Secreted IFN $\beta$  binds to the IFN $\alpha/\beta$  receptor in an autocrine and paracrine fashion and signals via the JAK/STAT pathway to activate ISGs that inhibit various stages of virus replication.

### 1.2.1 Toll-like receptors 3, 7, 8 and 9 are membrane-associated nucleic acid receptors

12 members of the TLR family (TLR1 – 12) have been identified in mammals so far [6]. They are expressed on various types of immune cells, most prominently though on

## Introduction

---

macrophages and dendritic cells (DC). However, certain nonimmune cells like fibroblasts and some epithelial cells also express TLRs. All TLRs are integral membrane glycoproteins that share a tripartite structure consisting of an N-terminal extracellular domain with variant numbers of leucine-rich repeats (LRR), a single transmembrane region, and an intracellular Toll/interleukin-1 receptor (TIR) domain. The extracellular domain confers specificity for PAMPs, whereas the TIR domain mediates downstream signaling by recruiting cytosolic adaptor proteins [7].

TLR signaling is achieved by either the MyD88-dependent pathway or the MyD88-independent pathway. In the latter pathway TRIF (TIR domain containing adaptor inducing interferon-beta) functions as an adaptor instead of MyD88 (myeloid differentiation primary response gene 88). All TLRs, except for TLR3, signal in a MyD88-dependent manner [8]. Only TLR3 and TLR4 recruit TRIF as an adaptor [9]. Differences in adaptor usage by TLRs result in activation of distinct signaling pathways and transcription factors and therefore in a different outcome in terms of gene expression [8].

The TLRs for nucleic acid detection - TLRs 3, 7, 8 and 9 - are found almost exclusively in endosomes with their TIR domain facing the cytosol, whereas all other TLRs are cell surface receptors. TLR3 is specific for dsRNA, murine TLR7 and human TLR8 detect single-stranded RNA (ssRNA), and TLR9 recognizes CpG-DNA.

### *1.2.1.1 TLR3*

TLR3 is the receptor for dsRNA, a very potent inducer of IFN $\beta$ . dsRNA represents the genome of dsRNA viruses, is generated as a replication intermediate of ssRNA viruses, or originates from symmetrical transcription of DNA viruses. TLR3 is expressed in conventional dendritic cells (cDCs), but not in plasmacytoid DCs (pDCs). It is also expressed in macrophages, in several epithelial cell types, and in astrocytes in the brain. While in cDCs TLR3 is found in endosomes, epithelial cells display it on the cell surface. TLR3 expression is dsRNA and type I IFN-inducible [6].

What are the signaling events downstream of TLR3? Upon stimulation TLR3 triggers a signaling cascade that leads to the activation of the transcription factors NF- $\kappa$ B, IRF3, and IRF7, consequently leading to the induction of IFN $\beta$  and proinflammatory cytokines. TLR3 relays the signal via a single TIR domain containing adaptor called TRIF (TIR-domain containing adaptor inducing interferon-beta). In contrast to all other TLRs, TLR3 only



signals through TRIF. TRIF recruits TRAF6, RIP1, and TRAF3, in which TRAF6 and RIP1 activate the canonical IKK, while TRAF3 is responsible for activation of the non-canonical IKKs TBK1 and IKK-i [10].

### *1.2.1.2 TLR7, TLR8 and TLR9*

TLR7 and TLR8 are receptors for ssRNA and the antiviral imidazoquinoline compounds imiquimod and R848. TLR9 is specific for DNA sequences rich in unmethylated CG dinucleotides, also designated as CpG-DNA for cytosine phosphatidyl guanosine. While CpG-DNA is highly methylated in eukaryotic cells, it remains largely unmethylated in bacterial and viral genomes. Synthetic CpG-oligonucleotides (CpG-ODN) act species-specific and are divided into 2 classes: type A/D and type B/K. The A/D type of CpG-ODNs contains a phosphodiester CpG motif that is flanked by phosphorothioate-modified polyG stretches at the 5' and 3' ends and potently induces IFN $\alpha$  in pDCs, but not cDCs. The B/K type of CpG-ODNs are short CpG containing sequences with a phosphorothioate backbone throughout that do not induce type I IFN in pDCs, but inflammatory cytokines in other cell types like macrophages [6].

TLR7, TLR8, and TLR9 are highly expressed in pDCs. Stimulation of these TLRs with their respective ligands results in massive IFN $\alpha$  secretion in pDCs, but not in cDCs. Interestingly, TLR7, TLR8, or TLR9 mediated IFN $\alpha$  production of pDCs depends on MyD88 as an adaptor. MyD88 mainly leads to activation of NF- $\kappa$ B and secretion of proinflammatory cytokines when recruited to other TLRs. However, in the case of TLR7, TLR8, and TLR9 in pDCs, MyD88 recruits a complex consisting of IL-1R-associated kinase 4 (IRAK-4), IRAK-1, TNFR-associated factor-6 (TRAF6), TRAF3, IKK $\alpha$ , and IRF7. Out of these complex components IRAK-1, TRAF3, and IKK $\alpha$  regulate IRF7 activation independent of the non-canonical IKKs TBK1 and IKK-i.

The ability of pDCs to secrete high levels of IFN $\alpha$  in response to TLR7, TLR8, or TLR9 stimulation presumably depends on the constitutively high expression of IRF7 in this cell type, as IRF7 is essential for IFN $\alpha$  transcription. Alternatively, TLR ligands may remain in endosomes for longer periods of time in pDCs than in cDCs [10].

## Introduction

---

### ***1.2.2 Retinoic acid-inducible gene I-like helicases are cytosolic dsRNA receptors***

#### ***1.2.2.1 RIG-I***

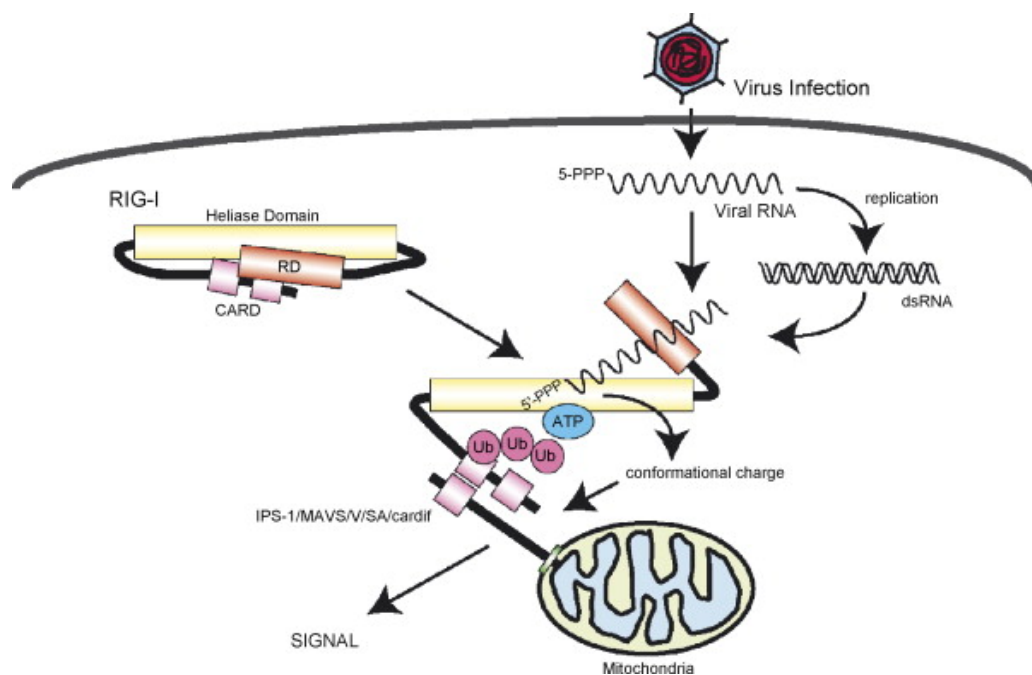
During replication of most viruses intracellular dsRNA accumulates in the cell and triggers type I interferon production. Due to its localization in the endosomal membrane TLR3 detects extracellular dsRNA that has reached the endosome by the endocytotic pathway, but fails to recognize cytosolic dsRNA.

Does a TLR3-independent pathway for detection of cytosolic dsRNA exist? In 2004, the first cytosolic receptor for dsRNA was reported, namely the DExD/H box-containing helicase retinoic acid-inducible gene I (RIG-I). RIG-I was identified by screening an expression cDNA library for those cDNAs that enhance IRF reporter activity in response to polyI:C stimulation [11]. RNAi of RIG-I demonstrated its role in antiviral immunity, as neither IRF3 nor NF- $\kappa$ B could be activated upon viral challenge. In agreement with this finding, induction of IFN $\beta$  and interferon-stimulated genes was abolished in RIG-I<sup>-/-</sup> MEFs in response to several RNA viruses.

Recognition of viral dsRNA by RIG-I is accomplished by its helicase domain. Apart from the helicase domain, RIG-I contains two N-terminal caspase recruitment domains (CARDs) and a C-terminal repressor domain (RD) [12]. RIG-I signaling activity tightly regulates the CARD domains. This is illustrated by the finding that overexpression of full length RIG-I does not stimulate IFN $\beta$  expression in absence of a stimulus, whereas overexpression of the N-terminal part consisting of the CARD domains is sufficient to do so [4]. In resting cells the RD domain associates with the CARD domains, thereby keeping RIG-I in an autoinhibitory state. Presumably, ligand binding to the RIG-I helicase domain causes a conformational change that liberates the CARD domains from the RD domain and allows signal transmission [12]. Recently, it was reported that ubiquitination of the RIG-I CARD domain by the E3 ubiquitin ligase tripartite motif protein 25 (TRIM25) enhances RIG-I-mediated signaling upon viral infection (figure 5) [13].

How does activated RIG-I relay the signal? Four studies independently described a protein named IFN $\beta$  promoter stimulator 1 (IPS-1) [14], mitochondrial antiviral signaling protein (MAVS) [15], virus induced signaling adaptor (VISA), [16] or CARD adaptor inducing IFN $\beta$  (Cardif) [17] as the signaling component immediately downstream of RIG-I. MAVS is a 62

kDa protein that consists of an N-terminal CARD-like domain followed by a proline-rich domain and a C-terminal transmembrane domain targeting it to the outer mitochondrial membrane. Overexpression of MAVS induces the transcription factors NF- $\kappa$ B, IRF-3, as well as IRF7, and as a result, type I IFN expression. On the other hand, knockdown of MAVS expression abolishes IFN induction upon viral infection. In addition, the IKKs responsible for activation of NF- $\kappa$ B and IRF-3 are not activated in the absence of MAVS. Finally, overexpression of MAVS protects cells from the cytopathic effects of a VSV infection, whereas RNAi of MAVS makes them more susceptible to killing by the virus. These findings demonstrate the essential role of MAVS in antiviral innate immunity [4].



**Figure 5. Activated RIG-I relays the signal to MAVS.** In resting cells RIG-I activity is autoinhibited by an intramolecular association of the RD domain. Stimulation with viral dsRNA causes a conformational shift. Thus the RD domain dissociates from the CARD domains which allows signal transmission to MAVS by a CARD-CARD interaction [18].

Interestingly, MAVS is a mitochondrial protein and thus presents the first link between innate immunity and mitochondria. Mislocalization of MAVS to other subcellular compartments abolishes MAVS function, whereas a truncated form consisting of only the CARD domain and the transmembrane domain is sufficient for IFN $\beta$  induction.

Epistasis studies position MAVS downstream of RIG-I and upstream of various proteins known to be involved in viral responses such as the non-canonical IKK TBK1. RIG-I is believed to recruit its adaptor MAVS by CARD-CARD interaction. While binding of the two proteins was shown in overexpression studies, interaction between endogenous RIG-I and MAVS in response to viral infection remains to be demonstrated [4, 15]. Due to the fact that MAVS acts upstream of the canonical and non-canonical IKKs, it coordinates several

## Introduction

---

signaling pathways that lead to NF- $\kappa$ B as well as IRF activation. The early steps of RIG-I signal transduction are summarized in figure 5 [18].

### *1.2.2.2 Other RIG-I-like helicases*

By homology to RIG-I two closely related proteins were found. Melanoma differentiation-associated gene 5 (MDA-5) shares 23% and 35% of its amino acids with the RIG-I CARD and helicase domains respectively, and like RIG-I positively regulates IFN $\beta$  induction. In contrast Lgp2, lacks the CARD domain and acts as a negative regulator of the pathway. It competes with RIG-I and MDA-5 for dsRNA binding, but devoid of a CARD domain, it is incapable of transmitting signals [19]. These three proteins are grouped into the family of RIG-I-like helicases (RLHs).

Comparison of antiviral immunity in MDA-5 or RIG-I deficient mice revealed that MDA-5 is stimulated by the synthetic analogue for dsRNA polyinosinic acid • polycytidylic acid (polyI:C), whereas RIG-I does not respond to polyI:C, but to in-vitro transcribed dsRNA. Furthermore it was shown that MDA-5 and RIG-I recognize different sets of viruses. While MDA-5 interferes with picornavirus infection, RIG-I is essential for the detection of paramyxoviruses, influenza virus, and Japanese encephalitis virus [20]. The reason for the difference in specificity is explained by the finding that RIG-I does not recognize dsRNA molecules per se, but rather a 5' triphosphate on ssRNA molecules typical for most viral RNAs that may or may not form double-stranded structures. This modification is also found in in-vitro transcripts. In contrast, eucaryotic RNAs are protected by a 7-methyl-guanosine cap and picornaviral RNA is covalently linked to the viral protein VPg [21].

Due to the co-evolution of pathogens and the host innate immune system, viruses developed strategies to interfere with immune responses. A prominent example is hepatitis C virus (HCV) whose NS3/45 protease cleaves MAVS and the TLR3 adaptor TRIF to inhibit IFN $\beta$  induction [6]. The V proteins of paramyxoviruses associate and thus inactivate MDA-5 signaling [22].

### *1.2.3 Cell-type specific differences in type I interferon production*

dsRNA is a widespread viral PAMP. Therefore its recognition by TLR3 should be essential for the host's antiviral response. However, the physiological role of TLR3 was unclear as susceptibility to many viral infections such as vesicular stomatitis virus (VSV), lymphocytic

choriomeningitis virus (LCMV), or murine cytomegalovirus (MCMV) was not affected in TLR3<sup>-/-</sup> mice. To investigate the relative contributions of TLR3 and RIG-I signaling to antiviral innate immunity, RIG-I<sup>-/-</sup> and TRIF<sup>-/-</sup> MyD88<sup>-/-</sup> cells were compared for their IFN responses upon viral challenge. IFN $\beta$  induction after infection with Newcastle disease virus (NDV, a ssRNA virus) was abrogated in RIG-I deficient mouse embryonic fibroblasts (MEFs) and resulted in increased viral yield. However, TRIF<sup>-/-</sup> MyD88<sup>-/-</sup> MEFs, lacking all TLR signaling, displayed the same antiviral activity as wild-type cells. IFN $\beta$  and IFN $\alpha$  production of cDCs also depended on RIG-I. In contrast, induction of both interferons was abolished in MyD88 deficient pDCs arguing for an essential role of TLRs in this cell type. Therefore, the RIG-I and TLR pathways are non-redundant and cell type specific [6, 23].

### **1.2.4 *DAI is a cytosolic receptor for DNA***

Earlier studies had provided evidence for the existence of DNA receptors distinct from TLR9 [24, 25], before DNA-dependent activator of IFN-regulatory factors (DAI) was very recently identified as the first cytosolic DNA receptor. DAI contains 3 DNA-binding domains arranged in tandem, 2 of which had been already described as left-handed Z-form DNA-binding domains. It was shown that DAI overexpression results in earlier and stronger type I IFN induction in response to DNA, but not RNA stimulation. Reciprocally, RNAi of DAI resulted in reduced IFN induction and less IRF3 dimerization after DNA stimulation. This was confirmed by the observation that cells, where DAI had been silenced are more susceptible to infection with the DNA virus herpes simplex virus-1 (HSV-1), but not with the RNA virus Newcastle disease virus (NDV). Moreover, DAI seems to recruit TBK1 and IRF3 upon DNA stimulation according to co-immunoprecipitation studies [26].

## 2 Aim of the study

Viral infection is detected by diverse receptors of the innate immune system that recognize conserved molecular patterns and trigger various immune responses including production of type I interferon. Knowledge on the signaling pathways that mediate interferon production and the understanding of the complex cross-talk of sensing, transmitting and effecting are still incomplete. Only recently in 2004, the role of nucleic acid receptors other than TLRs began to emerge with the identification of RIG-I and MDA-5. When this project was started in November 2006, various lines of evidence supported the existence of more, yet unidentified nucleic acid sensors that are independent of TLR-signaling, such as receptors for unmethylated CpG-rich sequences [25] or B-form DNA [27]. The latter was revealed to be DAI in July 2007 illustrating the rapid pace at which innate immunity research is currently evolving. Therefore, the goal of my diploma thesis was to identify new nucleic acid sensors implicated in antiviral innate immunity by a combined genomics and proteomics approach.

The central hypothesis of this thesis was that nucleic acid receptors bind to nucleic acids *and* are transcriptionally regulated by nucleic acid stimulation. Pull-down experiments with immobilized nucleic acids followed by mass spectrometric analysis were performed to identify nucleic acid binders, while genes, whose expression is regulated by nucleic acids, were determined by microarray analyses. By these means 2 datasets - the proteomics dataset including nucleic acid binders and the genomics dataset consisting of genes regulated by nucleic acids - were generated.

Based on the before mentioned hypothesis, proteins that belonged to both the proteomics and the genomics datasets had to be filtered in order to compile a list of candidate proteins.

Once a list of 24 candidate proteins had been generated, the functional relevance of each candidate for antiviral innate immunity needed to be assessed. As the key event of the innate immune response against viruses is the production of type I interferon, we determined which candidates are essential for interferon induction upon nucleic acid stimulation. For this purpose real-time PCR had to be established as a read-out system for transcriptional activation of type1 interferons as well as other cytokines.

## 3 Materials and methods

### 3.1 Molecular biology

#### 3.1.1 *Plasmids*

pDONR201 (Invitrogen)

Gateway donor vector containing attP sites

pRV NTAP (GS 2xT) Gw (CeMM)

Gateway destination vector with attR sites for N-terminally TAP-tagged proteins. The TAP-tag consists of protein G, followed by two TEV protease cleavages sites, streptavidin binding protein, and a myc-epitope.

pIE N-HA (CeMM)

Gateway destination vector with attR sites for N-terminally HA-tagged proteins

#### 3.1.2 *Cloning of candidate 4*

Candidate 4 was reversely transcribed and amplified from RAW264.7 cell RNA using the SuperScript One-Step RT-PCR System for Long Templates (Invitrogen) and candidate 4-specific primers flanked by sequences required for the subsequent Gateway cloning (Invitrogen) procedure. The Gateway technology is a cloning method based on the site-specific recombination properties of the bacteriophage  $\lambda$ . Cloning was carried out according to the manufacturer's instructions. Briefly, in the BP reaction, the candidate 4 PCR product and the donor vector were recombined using their attB and attP sites respectively, and as a consequence, the candidate 4 entry clone containing attL sites was generated. In the following LR reaction the attL sites of the entry clone and the attR sites of the destination vector recombined, giving rise to the candidate 4 expression clone. Successful cloning was verified by restriction digest and DNA sequencing.

#### 3.1.3 *Site-directed mutagenesis*

To generate a catalytically inactive mutant of candidate 4 a point mutation resulting in an amino acid change was introduced in the wild-type sequence using the QuikChange Site-

## Materials and methods

---

Directed Mutagenesis Kit (Stratagene) according to the manufacturer's recommendations. Primers that contain the desired mismatch were designed and the candidate 4 entry clone served as a template for the subsequent PCR reaction. Next, the original non-mutagenized plasmid strands were removed by DpnI restriction digestion. This enzyme cleaves dam-methylated DNA from bacterial origin, but not unmethylated DNA from PCR amplifications. The modified entry clone was used for the Gateway LR reaction as described in 3.1.1.

### 3.2 Cell biology

#### 3.2.1 Cell lines and cell culture

HEK293 (DMSZ)

Human embryonic kidney cell line

HEK293gp (Prof. Herbert Strobl, Medical University of Vienna)

Human embryonic kidney cell line stably expressing the retroviral core protein (gag) and the retroviral polymerase (pol) and thus used as a packaging cell line

RAW264.7 (Prof. Thomas Decker, University of Vienna)

Murine macrophage cell line

RAW NTAP candidate 4

Murine macrophage cell line stably expressing N-terminally TAP-tagged candidate 4

Cell lines listed above were cultured in DMEM (Invitrogen) supplemented with 10% fetal bovine serum (Invitrogen) as well as 100 U/ml penicillin and 100 µg/ml streptomycin (Invitrogen) at 37°C in a 5% CO<sub>2</sub> humidified incubator. Cells were maintained at subconfluency by splitting them three times a week using trypsin (Invitrogen) for HEK cell lines or dissociation buffer for RAW cell lines.

Dissociation buffer      0,54 M KCl  
                                 0,06 M sodium citrate



### 3.2.2 RNA interference

For each candidate siRNA SMARTpools targeted at the NCBI RefSeqs (NM\_XXXXXX) corresponding to the peptides found in the proteomics dataset were purchased from Dharmacon. siRNAs were dissolved according to the manufacturer's instructions to obtain a 20  $\mu$ M solution. Expression of endogenous candidate proteins in RAW 264.7 cells was silenced by 2 rounds of transfection on consecutive days using 50 nM siRNA: The day before transfection  $1 \times 10^5$  cells/well were seeded on a 6-well plate. 500  $\mu$ l serum-free DMEM were mixed with 5  $\mu$ l siRNA stock and 15  $\mu$ l HiPerfect (Qiagen). The mix was incubated 5-10 min at room temperature to allow for complex formation and then added dropwise to the cells covered with 500  $\mu$ l culture media. 6 h after transfection 1 ml media was added to the cells. This procedure was repeated on the following day. The day after the RNAi treatment cells were stimulated.

### 3.2.3 Stimulation of cells

polyI:C, polydAdT:dTdA and salmon sperm DNA (SSD) were obtained from Sigma, dissolved in water and tested for endotoxin using the QCL-1000 Chromogenic LAL Endpoint Assay (Cambrex). Invitrogen synthesized the mouse-selective CpG-oligodeoxynucleotide (CpG-ODN) 1826 5'-tccatgacggttcctgacggtt-3' as a phosphorothioate that was resuspended to obtain a 1 mM solution. Lipopolysaccharide from *E. coli* serotype 0111:B4 (LPS) was purchased from Sigma and prepared as a 1 mg/ml stock. Imiquimod was obtained from Invivogen and stored as a 20 mM stock. Mouse IFN $\beta$  was purchased from R&D systems as a  $1 \times 10^6$  U/ml solution. Unless stated otherwise cells were stimulated with 10  $\mu$ g/ml polyI:C, 1  $\mu$ g/ml polydAdT:dTdA or SSD, 1  $\mu$ M CpG-ODN, 1  $\mu$ g/ml LPS, 50  $\mu$ M imiquimod, and 100 U/ml IFN $\beta$ .

For stimulation of cytosolic nucleic acid receptors, RAW264.7 cells on 35 mm dishes were transfected with the indicated amounts of RNA or DNA. PolyI:C was transfected using HiPerfect (Qiagen): the appropriate amount of polyI:C in a total volume of 100  $\mu$ l serum-free DMEM was mixed with 12  $\mu$ l HiPerfect. The mix was incubated 5-10 min at room temperature to allow for complex formation and then added dropwise to the cells. For DNA-stimulations Lipofectamine 2000 (Invitrogen) was used as a transfection reagent: first, 10  $\mu$ l Lipofectamine 2000 were diluted in 250  $\mu$ l serum-free DMEM and incubated for 5 min at room temperature. Next, the required amounts of polydAdT:dTdA, SSD, or CpG-ODN were diluted in 250  $\mu$ l serum-free DMEM. Then 250  $\mu$ l diluted Lipofectamine 2000

## Materials and methods

---

were mixed with 250 µl diluted DNA. After a 20 min incubation at room temperature the transfection mix was added dropwise to the cells.

When stimulation of membrane-associated nucleic acid receptors was required, or for other than nucleic acid treatments, stimuli were directly added to the culture media.

After indicated stimulation periods either cell lysates or RNA extracts were prepared.

### **3.2.4 *Transient transfection***

HeLA cells were transfected using Polyfect (Qiagen) according to the manufacturer's instructions. In brief,  $1 \times 10^4$  cells/well were seeded on an 8 chamber culture slide (BD Falcon) one day before and transfected with 0,5 µg plasmid DNA and 4 µl Polyfect in a total volume of 50 µl. 24 h later cells were stained for immunofluorescence.

### **3.2.5 *Generation of stable cell lines by retroviral gene transfer***

As pantropic viruses were generated, the entire procedure was carried out under S2 conditions.

In order to produce recombinant viruses containing the gene of interest, HEK293gp cells were transfected using Polyfect (Qiagen). For this purpose cells were seeded at 70% confluency on 10 cm dishes. 6 h later 8 µg of TAP-construct DNA and 2 µg plasmid DNA coding for the viral envelope protein VSV-G were diluted in a total volume of 300 µl serum-free DMEM and mixed with 100 µl Polyfect. After a 5 - 10 min incubation period at room temperature the transfection mix was diluted to 1,4 ml with media and added to the cell culture dish containing the packaging cells with 7 ml media. The next day the medium was replaced by 6 ml fresh medium. Two days after transfection the medium containing recombinant viral particles was harvested and centrifuged at 250xg for 3 min. The supernatant was filtered to remove packaging cells and added to subconfluent RAW 264.7 on a 35 mm dish that had been incubated with 5 µg/ml polybrene (Sigma) for 5 min to facilitate the fusion of the virus with the cell membrane. Another 6 ml medium were added to the packaging cells to allow further virus production for a 2<sup>nd</sup> infection procedure the next day. After recovery from viral infection, cells were sorted for highly GFP-positive cells (TAP constructs contain GFP under an internal ribosomal entry site as a marker) by preparative FACS (Dieter Prinz, St. Anna Kinderspital) to enrich for transduced cells.

### 3.3 Protein biochemistry and immunological assays

#### 3.3.1 *Immunofluorescence*

Cells were washed with PBS (Invitrogen), fixed with ice cold absolute ethanol (Sigma) for 2 min, and washed with PBS again. Then cells were permeabilized with 0,5 % Triton-X 100 (Sigma) in PBS for 5 min and blocked with 3 % BSA (Sigma) in PBST for 10 min. Next, the slide was incubated with anti-HA.11 antibody (Covance) diluted 1:1000 in 0,1 % Triton-X 100 in PBS for 1 h. After washing with 0,1 % Triton-X 100 in PBS the samples were incubated with Alexa 594 goat anti-mouse IgG (Molecular Probes) diluted 1:500 in 0,1 % Triton-X 100 in PBS for 40 min. Finally the slide was washed with 0,1 % Triton-X 100 in PBS and nuclei were stained with 300 nM DAPI (Sigma) in PBS for 10 min. The slide was rinsed briefly with water and a coverslip was mounted with mowiol (Calbiochem).

PBST                      0,1% Tween 20  
                                  in PBS

#### 3.3.2 *Cell lysates for protein gels*

To prepare lysis buffer, IP buffer was supplemented with the following protease inhibitors immediately before use: 1 mM PMSF (Sigma), 5 µg/ml TLCK (Roche), 10 µg/ml TPCK (Biomol), 1 µg/ml leupeptin, 1 µg/ml aprotinin and 10 µg/ml soybean trypsin inhibitor (all Roche). 50 µl or 100 µl chilled lysis buffer were added per well of a 24 or 6 well plate, respectively. After 2 min incubation on ice, lysed cells were rinsed from the dishes and collected in a tube. The crude extract was centrifuged for 10 min at 13000 rpm at 4°C to pellet insoluble material. The supernatant is the cell lysate. If normalization of protein amounts among samples was required, the total protein concentration was determined by a Bradford assay (Bio-Rad) according to the manufacturer's instructions using known amounts of BSA (Sigma) as reference.

IP buffer                      50 mM Tris pH 7,5  
                                  150 mM NaCl  
                                  5 mM EDTA  
                                  5 mM EGTA  
                                  1% NP-40

## Materials and methods

---

### 3.3.3 SDS-PAGE

An aliquot of cell lysate corresponding to 50-100 µg total protein was mixed with an appropriate amount of 4x SDS sample buffer and heated at 95° for 3 min. PageRuler Prestained Protein Ladder (Fermentas) was used as a molecular weight marker. Marker and samples were loaded on a discontinuous polyacrylamide gel consisting of a 10 % separating gel and a 5 % stacking gel:

10% separating gel	
30% acrylamide/ bisacrylamide (Sigma)	10 ml
gel buffer 1	7,5 ml
water	ad 30 ml
Temed (Sigma)	30 µl
10% APS (Sigma)	300 µl
5% stacking gel	
gel buffer 2	20 ml
Temed (Sigma)	30 µl
10% APS (Sigma)	200 µl

The PAGE was run at 60 or 90 mA depending on gel size until the running front left the gel. Protein gels were further analyzed by Western blotting or silverstaining.

4x SDS sample buffer	200 mM Tris- HCl pH 6,8 40% (v/v) glycerol 8% (w/v) SDS brom phenol blue 1,4 M β- mercaptoethanol
Gel buffer 1	1,5 M Tris-HCl pH 8,8 0,4% SDS
Gel buffer 2	0,125 M Tris-HCl pH 6,8 0,1% SDS 5 % acrylamide/ bisacrylamide
Running buffer	50 mM Tris-HCl pH 8,5 380 mM glycin 7mM SDS

### 3.3.4 *Western blots*

Proteins were transferred onto a Protran nitrocellulose membrane (Whatman Schleicher & Schuell) using a semi-dry blotting apparatus. For assembly of the blotting sandwich 2 sheets of filter papers soaked in transfer buffer were placed on the positively charged anode at the bottom of the apparatus, followed by the membrane, the polyacrylamide gel containing negatively charged proteins, and another 2 layers of wet filter paper. The blot was run at 1 mA/cm<sup>2</sup> for 1 h. When the transfer of the proteins to the membrane was completed, unspecific binding sites were blocked with blocking solution for at least 15 min.

#### a) Blots for TAP-tagged proteins

The membrane was incubated with IRDye 800-conjugated anti-myc antibody (Rockland) in blocking solution for 1 h.

#### b) Candidate 4 blots

The membrane was incubated with a mouse candidate 4-specific antibody diluted 1:2500 in blocking solution for 1 h. After washing the membrane 3 times with PBST, it was incubated with an Alexa Fluor 680-labeled goat anti-mouse antibody (Invitrogen) for 30 min.

Blots were washed again 3 times and scanned using the Odyssey Infrared Imaging System (Li-Cor Biosciences)

PBST	0,1% Tween 20 in PBS
Blocking solution	5% Blotting Grade Blocker Non-Fat Dry Milk in PBST
Transfer buffer	2,5 mM Tris 15 mM glycine 10 % (v/v) methanol

### 3.3.5 *Silverstaining*

First the gel was incubated in fixation solution for 1 h at room temperature. Then it was washed in 30% ethanol twice for 20 min and in water once for 20 min. After sensitizing the gel for 1 min with 0,02% Na<sub>2</sub>S<sub>2</sub>O<sub>3</sub> solution and washing with water three times for 20 sec, it was incubated with cold silver nitrate solution for 20 min. Next, the gel was washed

## Materials and methods

---

again with water three times for 20 sec. At last the gel was incubated in developer solution until the desired staining intensity was reached. Incubation for at least 5 min in 5% acetic acid stopped the staining.

Fixation solution	40% ethanol 10% acetic acid
Silver nitrate solution	0,2% AgNO <sub>3</sub> 0,007% formaldehyde
Developer solution	3% Na <sub>2</sub> CO <sub>3</sub> 0,018% formaldehyde

### 3.3.6 RNA resins

PolyU agarose, polyC agarose, polyA and polyI were purchased from Sigma. A volume of about 10 µl lyophilized polyU agarose or polyC agarose were dissolved in 400 µl RNase-free water and incubated on ice for 10-15 min to allow the agarose to swell. After washing the resins twice with wash buffer they were resuspended in 2 ml of wash buffer each. PolyA and polyI were diluted in RNase-free water to a final concentration of 2 mg/ml and heated to 65°C. 2 ml of polyA were added to 1ml of polyU agarose. 2 ml of polyI were added to 1 ml of polyC agarose. Each of the four samples (polyC, polyI:C, polyU, polyA:U) was supplemented with 40 U of RNasin Ribonuclease Inhibitor (Promega). PolyI:C and polyA:U were incubated on the rotary wheel at 4°C over night. The next day, all four samples were washed three times with TAP default lysis buffer.

Wash buffer	50 mM Tris/HCl pH 7,5 100 mM NaCl
TAP default lysis buffer	50 mM Tris/HCl pH7.5 5 % glycerol 0,2 % NP-40 1,5 mM MgCl <sub>2</sub> 100 mM NaCl

### 3.3.7 DNA resins

PolydA:dT and polydAdT:dTdA were obtained from Sigma. 10 U of polydA:dT or polydAdT:dTdA were dissolved in 1 ml water each and then denatured by heating to 95°C. For annealing of the complementary strands the DNA solutions were allowed to cool down slowly to room temperature. In order to use these dsDNAs as affinity reagents they were

labelled with biotin. For the labelling reaction 450  $\mu$ l dsDNA, 50  $\mu$ l 10x restriction enzyme buffer 2, 16,6  $\mu$ l 1 mM dATP, 8,4  $\mu$ l 1 mM dUTP-biotin, and 5  $\mu$ l 50 U/ $\mu$ l Klenow DNA polymerase (all New England Biolabs) were mixed and incubated at 25°C for 1 h. The mouse-selective CpG-oligodeoxynucleotide (CpG-ODN) 1826 5'-tccatgacggttctgacggtt-3' was synthesized as a biotinylated phosphorothioate by Invitrogen.

Next, the biotin-labelled DNAs were incubated with Ultralink Immobilized Streptavidin Plus resin at 4°C for 1 h (Pierce) to immobilize polydA:dT, polydAdT:dTdA, or CpG-ODN on agarose beads. The resins were washed three times with TAP default lysis buffer.

TAP default lysis buffer	50 mM Tris/HCl pH7.5
	5 % glycerol
	0,2 % NP-40
	1,5 mM MgCl <sub>2</sub>
	100 mM NaCl

### **3.3.8 Pull-down experiments**

Immediately before use, TAP default lysis buffer was supplemented with protease inhibitors as follows: 0,4 mM Na<sub>3</sub>VO<sub>4</sub>, 20 mM NaF and 1 tablet/ 50 ml Complete Mini Inhibitor Cocktail (Roche). 4x10<sup>8</sup> RAW264.7 cells were lysed in 12 ml TAP default lysis buffer as the cell extract should be very concentrated (>20mg/ml; here: 28mg/ml). In order to prevent RNA degradation, cell extracts for RNA pull-downs were supplemented with 25 U/ml RNasin Ribonuclease Inhibitor (Promega). 2 ml lysate were incubated with each of the immobilized RNAs or DNAs for 2 h on a rotary wheel at 4°C. The resins were washed with 10 ml TAP default lysis buffer containing protease inhibitors. Bound proteins were eluted by boiling in 50  $\mu$ l 2x SDS sample buffer (Fluka) and submitted to mass spectrometry analysis (Dr. Keiryn Bennett, Mass Spectrometry Department, CeMM).

TAP default lysis buffer	50 mM Tris/HCl pH7.5
	5 % glycerol
	0,2 % NP-40
	1,5 mM MgCl <sub>2</sub>
	100 mM NaCl

## Materials and methods

---

### 3.3.9 *Tandem affinity purification*

5x10<sup>8</sup> RAW cells stably expressing candidate 4 were harvested by scraping them in 10 ml PBS (Invitrogen) supplemented with 1mM EDTA (Sigma). The cell suspension was centrifuged at 300 x g for 5 min at 4°C. Cells were resuspended in 10 ml PBS and centrifuged again. Finally, the pellet was frozen in liquid nitrogen and stored at – 80°C for further use.

The cell pellet was resuspended in approximately 5 ml TAP default lysis buffer supplemented with 0,4 mM Na<sub>3</sub>VO<sub>4</sub>, 20 mM NaF and 1 tablet/ 50 ml Complete Mini Inhibitor Cocktail (Roche) immediately before use. To ensure complete lysis the suspension was incubated for 30 min on ice and then centrifuged at 15 000 x g for 15 min at 4°C. Next, the supernatant was centrifuged at 100 000 x g for 1 h at 4°C. The supernatant from the 2<sup>nd</sup> centrifugation step constitutes the lysate, 150 µl of which were spared for Western blotting.

200 µl Rabbit IgG Agarose suspension (Sigma) were washed twice with lysis buffer and centrifuged at 2000 rpm for 1 min. The lysate was combined with 200 µl washed IgG bead suspension and incubated for 2 h at 4°C on a rotating wheel. After completion of the incubation time the beads were pelleted at 600 rpm for 2 min at 4°C. 90 µl supernatant were taken for Western blotting. Subsequently the beads were washed with 10 ml lysis buffer and 5 ml TEV buffer at 4°C.

Protein complexes were eluted by TEV cleavage, adding 400 µl TEV protease solution (CeMM) and incubating at 16°C for 1 h. The eluate was collected by gravity flow. The beads were rinsed with another 400 µl TEV buffer to quantitatively elute protein complexes. This fraction was combined with the initial eluate. 40 µl of 800 µl TEV eluate were spared for Western blotting.

150 µl Ultralink Immobilized Streptavidin Plus resin (Pierce) were washed twice with TEV buffer and centrifuged at 2000 rpm for 1 min. The TEV eluate was combined with 150 µl washed streptavidin bead suspension. After incubation for 1 h at 4°C on a rotating wheel, protein complexes bound to the beads were pelleted at 600 rpm for 2 min. 90 µl supernatant were saved for Western blot analysis. Next, the beads were washed with 10 ml TEV buffer.



Finally the protein complexes were eluted from the streptavidine resin by addition of 400  $\mu$ l saturated biotin solution and incubation at 16°C for 5 min. The remaining streptavidin beads are boiled in 50  $\mu$ l 2x SDS sample buffer for 3 min. The biotin eluate as well as the boiled bead fraction were submitted to mass spectrometry analysis (Dr. Keiryn Bennett, Mass Spectrometry Department, CeMM).

TAP default buffer	50 mM Tris/HCl, pH7.5 5% glycerol 0.2% NP-40 1.5mM MgCl <sub>2</sub> 100mM NaCl
TEV buffer	10 mM Tris/HCl, pH 7.5 100 mM NaCl 0.5 mM EDTA
Saturated biotin solution	10 mM Tris 10 mM NaCl add spatula tip biotin to 10 ml buffer immediately before use

### **3.3.10** *Mass spectrometry analysis*

TAP samples were analyzed by one-dimensional SDS-PAGE using NuPAGE 4 – 12% bis-Tris gels (Invitrogen) followed by silver staining. Specific bands and/or regions of interest were excised from the gel and digested *in situ* with modified porcine trypsin (Promega). Trypsically-digested samples were analyzed by data-dependent nanocapillary reversed-phase LC-MSMS using customized 75  $\mu$ m inner diameter columns packed with C18 3  $\mu$ m diameter Reprosil beads (Maisch) on a nanoLC system (Agilent Technologies) coupled to a quadrupole time-of-flight (QTOF) mass spectrometer (QTOF Ultima, Waters). Proteins were identified by automated database searching (Mascot Daemon, Matrix Science) against the International Protein Index protein sequence database (IPI, European Bioinformatics Institute, [www.ebi.ac.uk/IPI/](http://www.ebi.ac.uk/IPI/)). Results from the database search were parsed into EPICenter (Proxeon Biosystems) for automated validation and protein grouping based on the number of shared peptides identified by MSMS. Criterion for a positive protein identification was identification of a minimum of 2 peptides as there is a relationship between abundance of peptides and peptide counts.

### 3.4 Genomic analyses

#### 3.4.1 RNA extraction

RNA was purified using the RNeasy Mini Kit (Qiagen) in combination with QIAshredder columns (Qiagen) following the manufacturer's recommendations. If real-time PCR was to be performed RNA was digested with DNase (Fermentas) to eliminate a potential genomic DNA contamination. RNA quantity was determined by measuring the absorbance at 260 nm in a spectrophotometer. RNA quality was verified by running 5 µl of RNA sample after DNase digestion diluted 1:1,5 in loading buffer on a 1 % agarose (Sigma) gel in TAE, and checking for integrity of the 28S and 18S rRNA bands.

Loading buffer	8 ml formamide
	20 µl 0,5 M EDTA
	1,98 ml RNase-free water
TAE	40 mM Tris
	40 mM acetic acid
	1 mM EDTA

#### 3.4.2 Microarray analysis

RNA samples were applied to the GeneChip Mouse Genome 430 2.0 Array (Affymetrix) (Dr. Martin Bilban, KIMCL, AKH Wien). The microarray data was computed using the software tool "Significance Analysis of Microarrays" (SAM) [28] in order to identify regulated genes (Gerhard Dürnberger, Bioinformatics Department, CeMM)

#### 3.4.3 Quantitative PCR

Reverse transcription was carried out according to the manufacturer's recommendation. In brief, 1 µg RNA and 0,5 µg oligo(dT)<sub>18</sub> primer (Fermentas) in a total volume of 11 µl were heated for 5 min at 70°C, then chilled on ice. Next, 4 µl 5x reaction buffer (Fermentas), 2 µl 10 mM dNTPs, 20 U RNasin ribonuclease inhibitor (Promega), and water were added to reach a final volume of 19 µl. The mix was incubated at 37°C for 5 min. 1 µl 200 U/µl RevertAid™ M-MuLV Reverse Transcriptase (Fermentas) was added to each reaction and incubated at 42°C for 1 h. The enzyme was heat-inactivated at 70°C for 10 min.

Real-time PCR was performed with iTaq SYBR Green Supermix with ROX (Bio-Rad) as detection chemistry using the ABI PRISM 7700 Sequence Detection System (Applied Biosystems) or the Rotor-Gene 6500 (Corbett). Primer sequences are listed below:

Gene	Species	Primer pair	
IFN $\beta$	Mouse	Forward primer (5'-3')	TCAGAATGAGTGGTGGTTGC
		Reverse primer (5'-3')	GACCTTTCAAATGCAGTAGATTCA
HPRT	Mouse	Forward primer (5'-3')	CGCAGTCCCAGCGTCGTG
		Reverse primer (5'-3')	CCATCTCCTTCATGACATCTCGAG
CycB	Mouse	Forward primer (5'-3')	CAGCAAGTTCCATCGTGTCAAGG
		Reverse primer (5'-3')	GGAAGCGCTCACCATAGATGCTC

For each reaction, 10  $\mu$ l iTaq SYBR Green Supermix, 0,5  $\mu$ l 10  $\mu$ M primer mix, 4,5  $\mu$ l water and 5  $\mu$ l 1:20 diluted cDNA were combined by hand or using the CAS-1200 liquid handling system (Corbett). PCRs were run with the following thermoprofile: Initial denaturation at 95°C for 3 min; then 40 cycles of 94°C 30 sec, 60°C 15 sec and 72°C 30 sec. Melting curves were checked after each run to detect potential primer dimers and contaminations. Results were calculated from  $C_t$  values by the  $2^{-\Delta\Delta C_t}$  method [29] using either CycB or HPRT as a reference.

# 4 Results

## 4.1 Generation of a list of candidate proteins

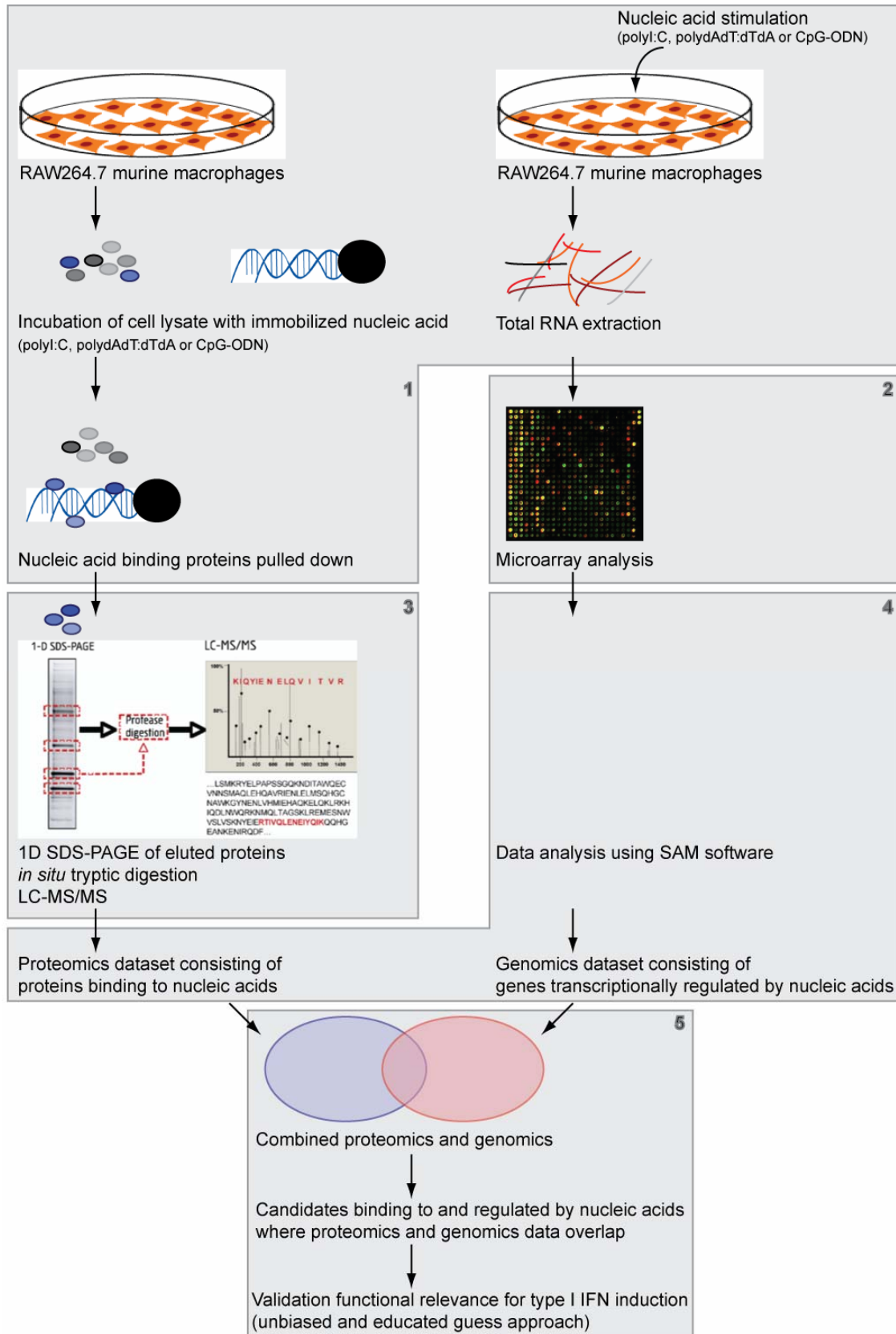
In order to identify novel components of innate immunity signaling in response to nucleic acid stimulation a combined proteomics and genomics approach was utilized. 3 different nucleic acids were tested, namely polyI:C, polydAdT:dTdA, and CpG-ODN. All of these compounds have been shown to be potent inducers of IFN $\beta$  secretion. PolyI:C is the synthetic analogue for dsRNA, which is the ligand for the membrane-associated TLR3 as well as the cytosolic receptor MDA-5. PolydAdT:dTdA is believed to adopt a B-form configuration in solution and mimics double-stranded DNA (dsDNA), whose cytosolic receptor DAI was recently identified, but had been unknown at the start of this project. CpG-ODNs are known to activate TLR9 signaling in a species-specific manner. Even though receptors are known for these ligands, the existence of even more yet unidentified ones is likely. The identification of such receptors was the goal of this project. The experimental flow as well as the major contributors to each task are outlined in figure 6.

### 4.1.1 *Pull-down experiments identify nucleic acid binding proteins*

To study which proteins bind to nucleic acids, pull-down experiments were performed using 3 different nucleic acids immobilized on agarose beads: PolyI:C and polydAdT:dTdA, the synthetic analogues for dsRNA and dsDNA respectively, as well as CpG-ODN. Cell lysate prepared from the murine macrophage cell line RAW264.7 was incubated with the various immobilized nucleic acids and bound proteins were identified by mass spectrometry. Four polyI:C, two CpG-ODN and one polydAdT:dTdA pull-downs were analyzed.

Incubation of cell lysates with polyC served as negative control for the polyI:C pull-down. PolyC does not induce IFN $\beta$  production and therefore proteins that bind to polyC alone would not be of interest. For the DNA pull-downs streptavidin beads were used as negative control, because the corresponding nucleic acids were coupled to agarose beads by biotin-streptavidin interaction.

The polyI:C pull-down in particular revealed a vast number of interacting proteins (data not shown). Among about 300 proteins we found transcription factors as well as splicing



**Figure 6. Overview of the experimental setup for this diploma project.** 1, Initial pull-down experiments and RNA preparations for microarray analyses (Dr. Tilmann Bürckstümmer, Innate Immunity Group, CeMM). 2, Microarray analysis (Dr. Martin Bilban, KIMCL, AKH Wien). 3, Mass spectrometry analysis (Dr. Keiryn Bennett, Mass Spectrometry Department, CeMM). 4, Bioinformatics (Gerhard Dürnberger, Bioinformatics Department, CeMM) 5, Functional relevance of candidates (Evelyn Dixit, Innate Immunity Group, CeMM).

## Results

---

factors, which demonstrates that the proteins identified by this approach specifically bind to nucleic acids. More importantly, proteins with well established roles in innate immunity to viral infection were pulled down as well. For example the classical effectors of the viral dsRNA response 2'-5' oligoadenylate synthetase (Oas3), double-stranded RNA-activated protein kinase (Pkr), and PKR-activator A (Prkra) were identified with 32, 19, and 2 peptides, respectively. Moreover, the cytoplasmic sensors of viral infection, the helicases RIG-I, MDA-5, and Lgp2, were identified with 8, 3, and 7 peptides, respectively.

The pull-down data demonstrates that among the nucleic acid binders are proteins with functional relevance for innate immunity signaling. How could we filter selectively for proteins that are involved in the recognition of nucleic acids and as a consequence elicit an IFN $\beta$  response? Our hypothesis was that nucleic acid receptors not only bind to nucleic acids, but are also transcriptionally regulated by them. In this scenario up-regulation of a nucleic acid binding protein would create a positive feedback loop resulting in enhanced IFN $\beta$  production, which is a common principle in cytokine regulation.

### **4.1.2 *Microarray analyses reveal nucleic acid-regulated genes***

In order to find out which genes are transcriptionally regulated by nucleic acids, RAW264.7 cells were stimulated with polyI:C, polydAdT:dTdA, or CpG-ODN. After the treatment total RNA was extracted and alterations in gene expression in comparison to untreated cells were analyzed using a microarray (Dr. Martin Bilban, KIMCL, AKH Wien). Regulated genes were determined by the software tool "Significance Analysis of Microarrays" (SAM) (Gerhard Dürnberger, Bioinformatics Department, CeMM). The data indicates that regulated genes are functionally relevant for innate immunity processes, supporting the initial hypothesis. This is illustrated below for dsRNA signaling pathways (table 7A and B). Both known cytosolic dsRNA receptors RIG-I and MDA-5 as well as the negative regulator of this pathway, Lgp2, are upregulated in response to polyI:C treatment (table 7A). Expression of the membrane-associated counterpart for dsRNA recognition, TLR3, is strongly increased, but also downstream signaling components like the TLR3 adapter TRIF and several IRF family members are induced by polyI:C stimulation (table 7B). Interestingly, polydAdT:dTdA upregulates dsRNA signaling molecules much like polyI:C treatment does. This finding may be due to the fact that both stimuli lead to IFN $\beta$  production and thus result in induction of the same IFN-regulated genes. CpG-ODN stimulation seems to have a minor impact on the regulation of dsRNA signaling

components. A possible explanation might be that CpG-ODNs affect type I IFN production in a different manner than polyI:C or polydAdT:dTdA do.

Table 7A

Ligand Sensors	polyI:C TLR3/RIG-like helicases		CpG TLR9		polydAdT:dTdA DNA receptors	
	4 h	6 h	4 h	6 h	4 h	6 h
<b>RIG-I</b>	3,81	3,48	1,42	1,89	3,22	5,44
<b>MDA-5</b>	6,07	6,41	1,88	2,26	5,36	7,45
<b>Lgp2</b>	4,13	5,78	1,49	2,74	3,53	4,33
<b>MAVS</b>	0,80	0,95	1,10	1,32	0,88	1,66
<b>TBK1</b>	1,39	1,33	1,10	1,32	0,88	1,66

Table 7B

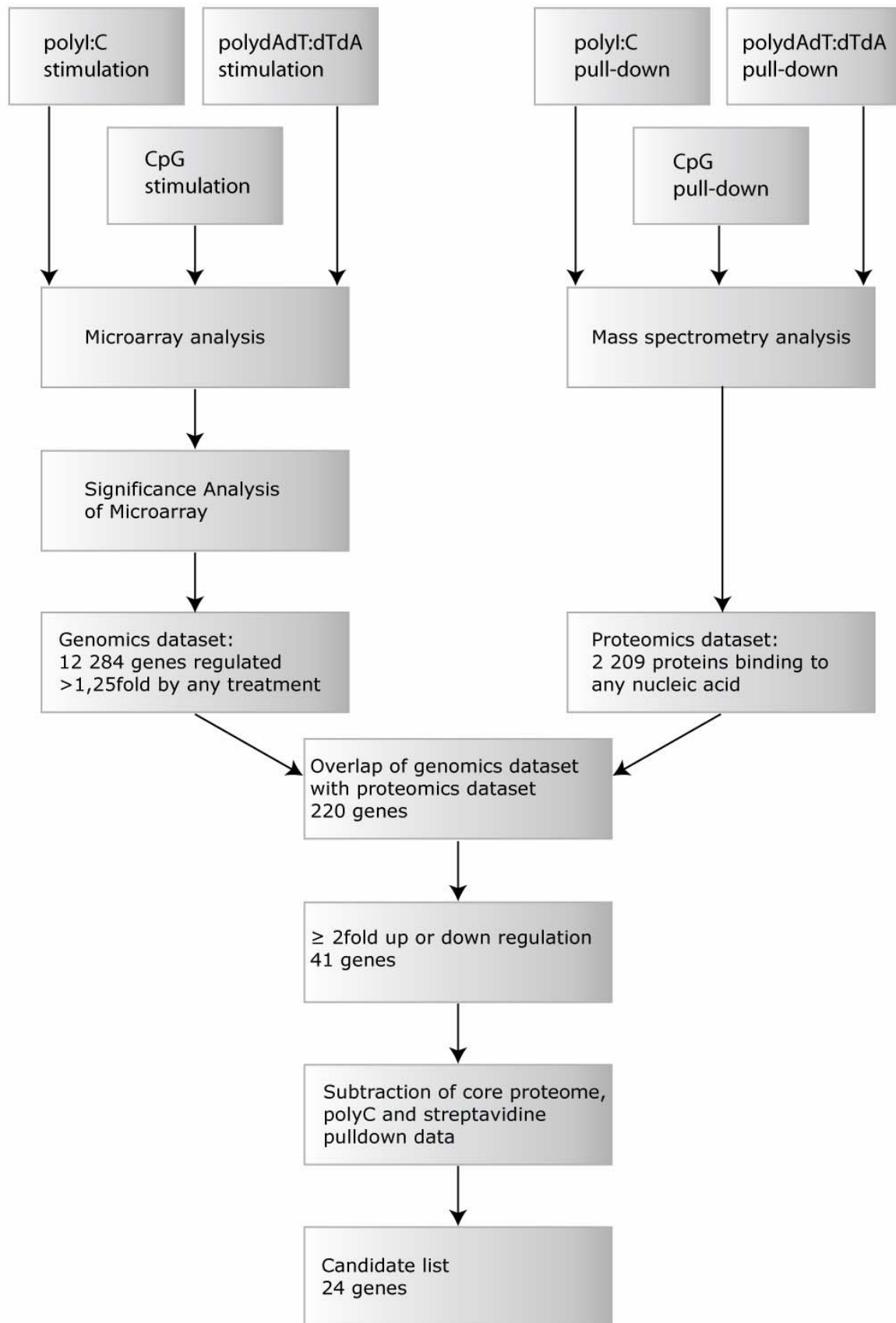
Ligand Sensors	polyI:C TLR3/RIG-like helicases		CpG TLR9		polydAdT:dTdA DNA receptors	
	4 h	6 h	4 h	6 h	4 h	6 h
<b>TLR3</b>	8,27	10,51	1,63	1,87	8,81	18,24
<b>TRIF</b>	0,95	1,93	2,01	3,02	0,95	1,82
<b>TBK1</b>	1,39	1,33	1,10	1,32	0,88	1,66
<b>TRAF6</b>	0,85	1,10	1,08	1,31	0,97	0,93
<b>IRF1</b>	4,66	2,67	0,92	0,87	7,43	2,59
<b>IRF2</b>	2,17	1,53	1,02	0,90	2,13	1,94
<b>IRF3</b>	0,90	0,97	0,99	1,04	1,04	0,83
<b>IRF7</b>	2,89	3,84	1,47	1,67	3,12	2,01
<b>IRF8</b>	1,95	1,72	2,21	1,80	2,38	1,29
<b>IRF8</b>	1,63	1,95	1,82	2,03	2,05	1,35

**Table 7A and B. Regulated genes are functionally relevant.** RAW264.7 cells were stimulated with 10 µg/ml polyI:C, 1 µM CpG-ODN, or 1 µg/ml polydAdT:dTdA for 4 and 6 h. Total RNA was analyzed for expression changes using a microarray. Fold changes relative to untreated cells are displayed. dsRNA signaling for A. cytosolic receptors and B. Membrane-associated TLR3.

#### 4.1.3 Merging the proteomics and genomics datasets

In order to find out which genes encode nucleic acid binding proteins and are regulated by nucleic acids, the information obtained from both approaches - microarrays and pull-downs - needed to be combined. Therefore two datasets were generated, the genomics and the proteomics dataset (figure 8). For the genomics dataset all genes that were found to be regulated by polyI:C, polydAdT:dTdA, or CpG-ODN were pooled into one large

## Results



**Figure 8. Flowchart of the bioinformatics procedure to generate candidate list.** Proteomics and genomics data were combined to identify nucleic acid binders that are transcriptionally regulated by nucleic acids.



dataset composed of 12 285 genes regulated >1,25fold. We proceeded similarly to generate the proteomics dataset. 2209 proteins identified in any of the four polyI:C, the two CpG-ODN, and the polydAdT:dTdA pull-downs were combined in one dataset. Thus the genomics dataset consists of all genes that are *regulated by* any of the 3 nucleic acids tested, while the proteomics dataset includes all proteins that *bind to* any of the 3 nucleic acids.

Next, we searched for genes that were present in both the proteomics and the genomics dataset i. e. in the intersection of these two sets. To this end the pull-down data was mapped onto the microarray dataset. This analysis was performed with a Perl program developed in-house using annotation data provided by Affymetrix (Gerhard Dürnberger, Bioinformatics Department, CeMM) and revealed 220 genes in the overlap of the genomics and proteomics datasets.

In the following step we discarded all genes whose transcription is less than twofold up or down regulated, because it may be difficult to prove biological relevance for these genes despite the fact that less than twofold regulation is statistically significant. 41 out of the 220 genes met this criterion.

At last, proteins found in the negative controls, i. e. polyC and streptavidin pull-downs, and in the core proteome were subtracted. PolyC is the complementary strand to polyI which together constitute polyI:C. While polyI:C is a potent inducer of IFN $\beta$ , stimulation with polyC does not have this effect and therefore serves as negative control. The DNAs polydAdT:dTdA and CpG-ODN were immobilized on streptavidin beads after biotinylation. The streptavidin pull-down allows discrimination between proteins that bind specifically to DNA and those that stick to streptavidin or the beads themselves. The most abundant proteins in the cell represent the core proteome that is identified by analyzing lysates of untreated RAW264.7 cells. As these proteins are present in such large amounts they are likely to be co-purified with specific interactors. Therefore the core proteome may be subtracted from mass spectrometry analyses. Even though the most prevalent proteins are usually not regulated and are likely to be contaminants, the inherent problem of this approach is that false negatives might be excluded. Being aware of this pitfall, we decided that we would rather lose a candidate than have a higher background noise. After subtraction of negative controls we ended up with a list of 24 candidate proteins (table 9).

## Results

Table 9

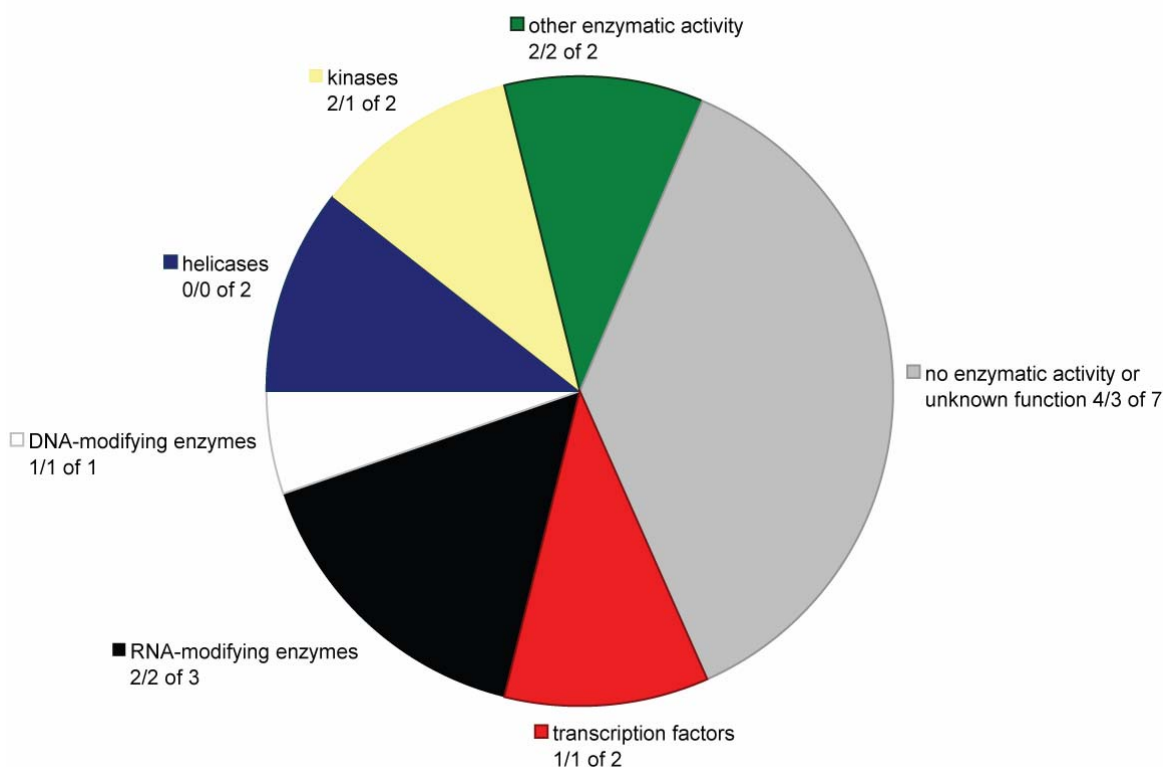
Candidates	Fold change						Peptide count		
	polyI:C		CpG		poly dAdT:dTdA		polyI:C	CpG	poly dAdT:dTdA
	4 h	6 h	4 h	6 h	4 h	6 h			
1	25	20	5	4	33	31	3/4	6/10	-
2	8	13	2	3	8	14	2	-	-
MDA-5	6	7	2	2	5	8	3	-	-
3	5	7	2	3	4	7	2	-	-
RIG-I	4	4	-	2	3	6	7/3/8/5	-	-
4	4	4	2	2	5	3	-	2/4	-
5	4	4	2	2	4	2	3/2	5/7	-
Pkr	3	3	2	2	3	3	19/11/8/6	-	-
IKK-i	2	3	3	3	-	-	-	3	-
Oas3	2	2	-	-	2	3	32/8/18/12	-	-
6	2	2	-	-	2	3	3/2	-	-
7	-	2	-	-	2	3	1/8/3	-	-
8	2	2	-	-	2	3	2	-	-
9	-	-	-	-	-	-3	2	-	-
10	2	2	-	-	2	3	13	-	-
11	2	2	-	-	2	3	2	-	-
12	-	-	-	-	-2	-3	2	-	-
13	-	-	-3	-2	-	-	-	12	-
14	-	-2	-3	-	-3	-3	14/2	-	-
15	-	-	-	-	-	-2	14/5/10/6	3	-
16	-	-	-2	-	-	-2	2/4	-	-
17	-	-	-2	-	-	-	17/3	-	-
18	-	-	-	-	-	-2	2	-	-
19	-	-	-2	-	-	-2	8/3/3/3	5/7	-

**Table 9. List of candidates.** Summary of the 24 candidates including their fold change of expression after 4 or 6 h nucleic acid stimulation and the number of peptides counted for each protein in the respective nucleic acid pulldown. Four polyI:C, two CpG-ODN, and one polydAdT:dTdA pull-downs were analyzed.

The 24 candidates include 5 molecules known to be implicated in the innate immune response to nucleic acids. These proteins are RIG-I and MDA-5, the cytosolic receptors for dsRNA, IKK-i, an activating kinase of the transcription factor IRF3, as well as the interferon-stimulated genes Pkr and Oas3. The presence of these known pathway components in the candidate list demonstrates the validity of the approach. Except for IKK-i the above mentioned proteins have been reported to directly interact with nucleic

acids. Whether IKK-i binds indeed to nucleic acids remains to be shown. Alternatively, it is possible that the pull-down experiments also identified proteins that form complexes with nucleic acid binders and thus are co-purified, which is conceivable for IKK-i.

Among the remaining 19 candidates we find 10 well characterized proteins, 6 proteins with little available information, and 3 very poorly annotated ones. Some candidates are known to be implicated in innate immune responses, whereas others have not yet been described to be involved in immunological processes. Regarding enzymatic activity we found 3 RNA modifying enzymes, 1 DNA modifying enzyme, 2 kinases, and 2 helicases. Moreover, 2 transcription factors are included in the list of candidates (figure 10). Most of the candidates are polyI:C binding proteins, few interact with CpG-ODN, but none were pulled down with polydAdT:dTdA as summarized in table 9.



**Figure 10. The candidates are functionally diverse proteins.** Among the candidates we find RNA and DNA-modifying enzymes, helicases, kinases, transcription factors, and proteins without enzymatic activity or unknown function. The figure illustrates how many candidates belong to each category. Furthermore, the number of well annotated proteins and of those with a reported role in immune processes is indicated, e. g. 4/3 of 7: Out of 7 proteins 4 are well annotated and 3 are implicated in immunology.

## 4.2 Confirmation of microarray data by quantitative PCR

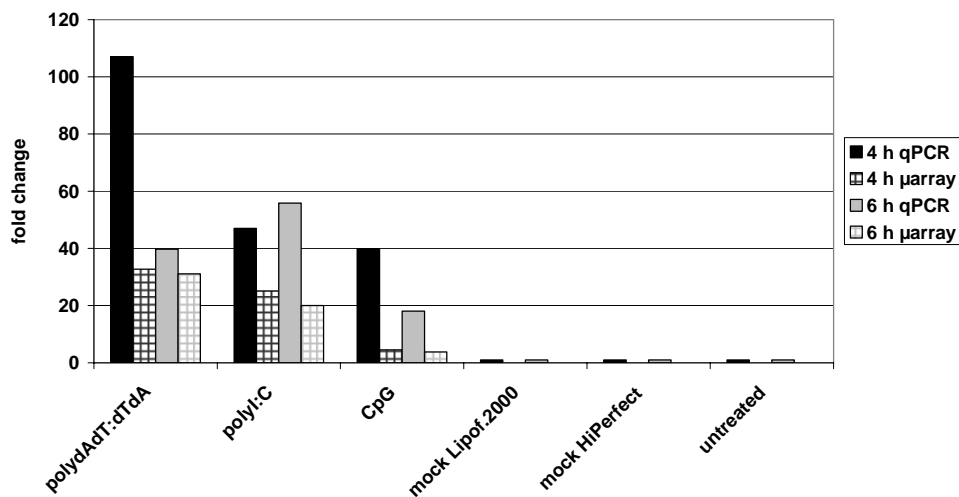
To validate the genomics dataset, the expression change of 6 randomly selected candidates in response to nucleic acid stimulation was analyzed by quantitative PCR. RAW264.7 cells were stimulated with polyI:C, polydAdT:dTdA, or CpG-ODN for 4 h and 6

## Results

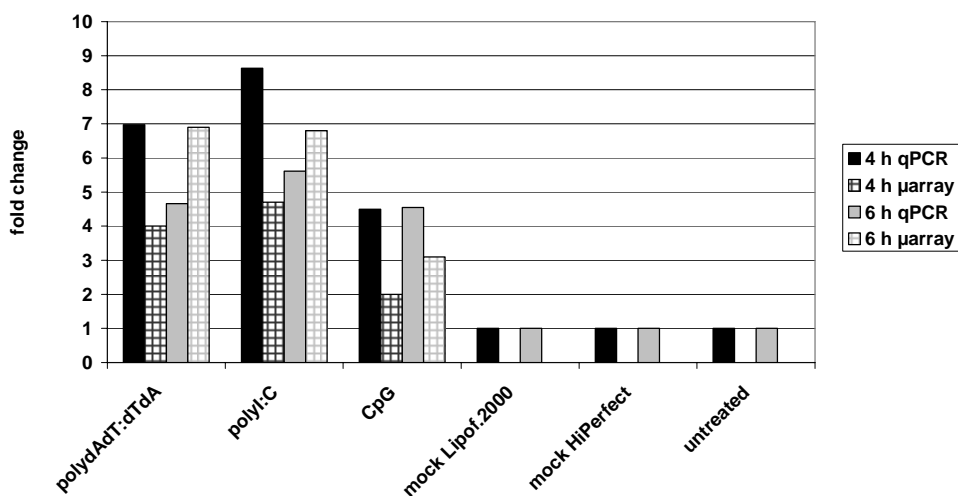
h, RNA was extracted, reversely transcribed in cDNA, and real-time PCR performed. Results for 3 candidates are shown in figure 11.

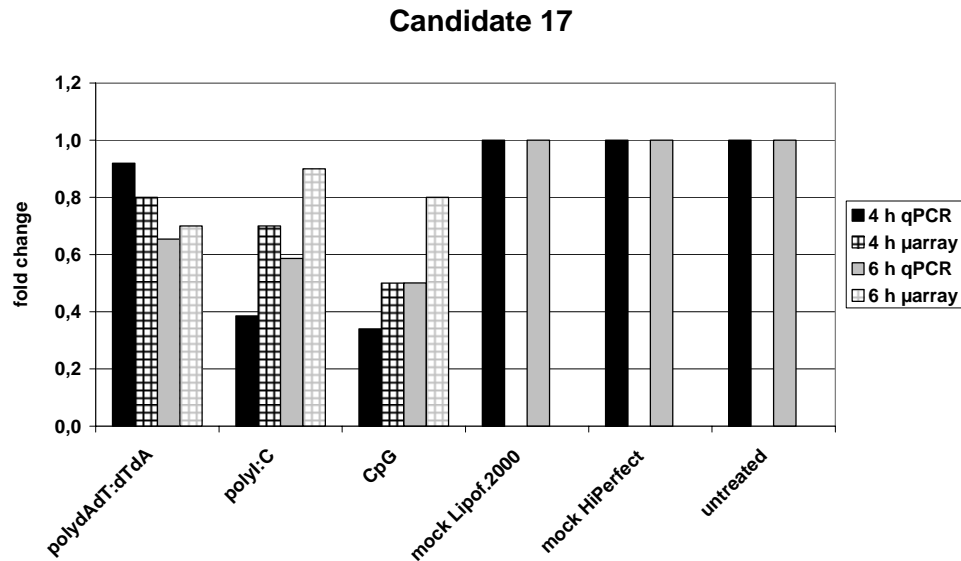
Both methods, microarray analysis and quantitative PCR, detected similar changes in expression levels for the 6 candidates tested (3 examples are shown), despite having used RNA from two different biological experiments. In general we noticed that reduced expression of candidates in response to nucleic acid stimulation is more difficult to reproduce than their induction, however we have no explanation for this observation. Overall these findings argue for the reliability of the genomics dataset that is an integral part for generation of the candidate list.

### Candidate 1



### Candidate 3





**Figure 11. Comparison of transcriptional activation of candidates as measured by microarray analysis and quantitative PCR.** RAW264.7 cells were stimulated by transfection of 1  $\mu$ g/ml polydAdT:dTdA or 10  $\mu$ g/ml polyI:C, or by addition of 1 $\mu$ M CpG-ODN to the media. 4 and 6 h later RNA was extracted, cDNA prepared, and real-time PCR performed using gene-specific primers for candidate 1, 3, or 17. Results are normalized to the respective control.

### 4.3 Validation of functional relevance of candidates

After having generated a list of proteins with potentially yet unknown implications in the innate immune response, the candidates had to be tested for their functional relevance. Two complementary research avenues were pursued in parallel: i) Effects on IFN $\beta$  induction in response to nucleic acid stimulation after silencing of each candidate were evaluated. ii) One promising candidate was chosen based on a thorough literature search and its role for IFN $\beta$  activation was investigated in more depth.

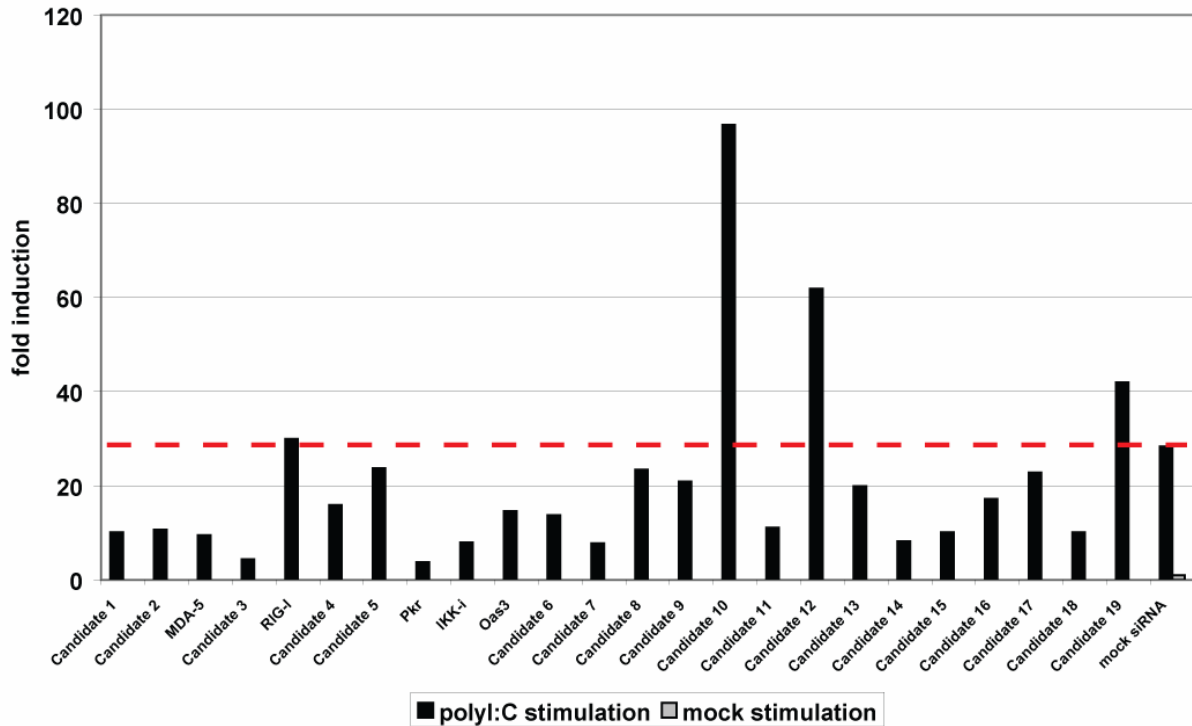
#### 4.3.1 Evaluation of all candidates in parallel

##### 4.3.1.1 Effect of candidate silencing on polyI:C-stimulated IFN $\beta$ induction

As nucleic acid stimulation results in type I IFN secretion, we wanted to test the effect of each candidate on IFN $\beta$  induction. Therefore we targeted each candidate with a gene-specific siRNA pool. 24 h after RNAi treatment, RAW264.7 cells were stimulated by polyI:C transfection for 4 h, and IFN $\beta$  induction relative to unsilenced RAW264.7 cells was determined by quantitative PCR. Two biological experiments were performed and each

## Results

cDNA was analyzed twice by real-time PCR. One representative experiment is shown in figure 12.



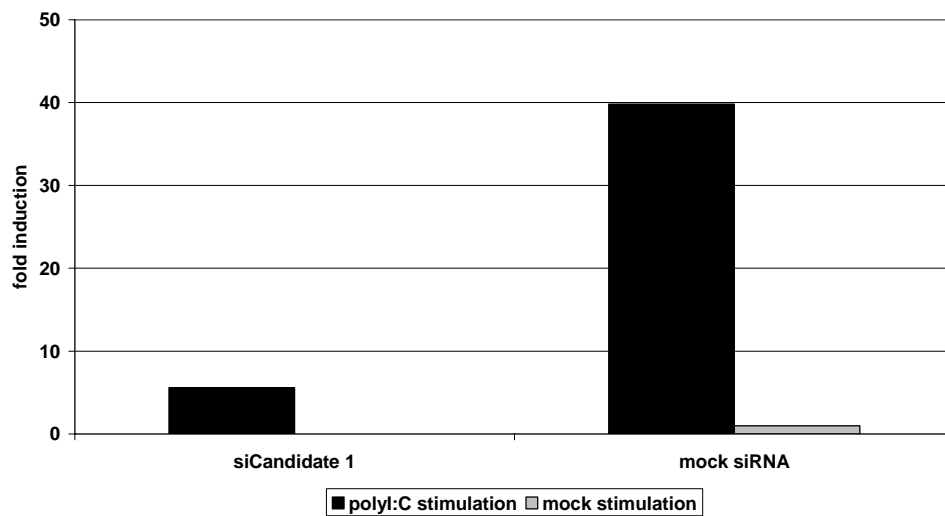
**Figure 12. Functional relevance of candidates for polyI:C-induced IFN $\beta$  production.** siRNA treated cells were stimulated by transfection of 10  $\mu$ g/ml polyI:C. Transcriptional activation of IFN $\beta$  was determined by real-time PCR. Data is normalized to mock siRNA transfected mock stimulated cells. The red dashed line indicates the normal level of IFN $\beta$  induction in mock siRNA transfected polyI:C stimulated cells, i. e. the threshold.

PolyI:C stimulated cells that were not treated with any siRNA reflect normal IFN $\beta$  induction. This threshold is indicated by a red dashed line in figure 12. If silencing of a candidate results in decreased IFN $\beta$  induction relative to the threshold, this protein is positively involved in the IFN $\beta$  response. Conversely, increased IFN $\beta$  induction after knock down of a candidate indicates a negative regulator.

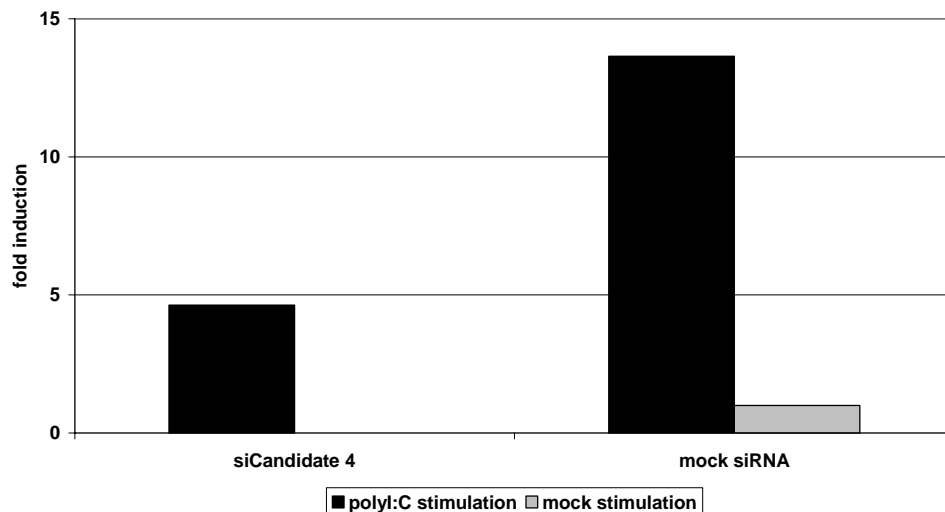
Most importantly, the 5 candidates with an established role in IFN $\beta$  induction, showed the expected effects, which validates the experimental setup. It is noteworthy that knock down of MDA-5, but not RIG-I had an effect, even though both proteins are receptors for viral dsRNA which was believed to be mimicked by polyI:C. However, it was reported that RIG-I rather recognizes 5' triphosphates in RNA molecules than dsRNA per se. The exact structural detail recognized by MDA-5 is not known yet. This result confirms that polyI:C is not the activating ligand for RIG-I, even though RIG-I binds to polyI:C. The data reveals mostly positive regulators among the other 19 candidates.

The knock down efficiency is a key aspect of the experimental setup. Due to the selection procedure all candidates are transcriptionally regulated by nucleic acids. To verify that an induction does not overrule the silencing of a candidate, the knock down efficiency was determined for a subset of candidates by real-time PCR using candidate-specific primers. Knock down efficiency for the tested candidates ranges between 60 and 90% (figure 13). Therefore, we concluded that the silencing efficiencies are similar for the remaining candidates. This interpretation implies that unchanged IFN $\beta$  levels in the candidate evaluation data are not due to failed RNAi, but to functional irrelevance of candidates.

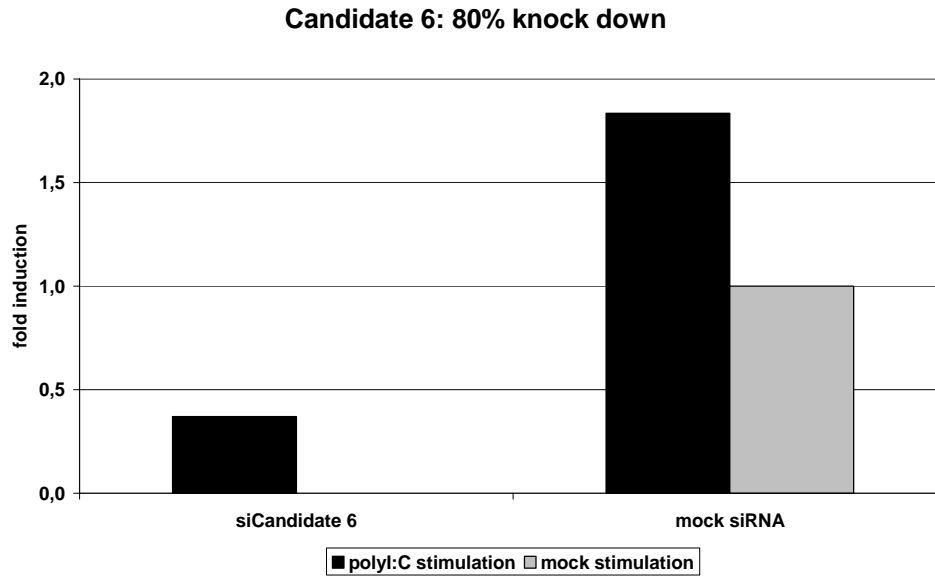
**Candidate 1: 86% knock down**



**Candidate 4: 66% knock down**



## Results



**Figure 13. Knockdown efficiencies of candidates 1, 4 and 6 after polyI:C stimulation.** The same cDNAs as in figure 12 were used. Relative amounts of candidate 1, 4 and 6 transcripts were determined by real-time PCR. Data is normalized to mock siRNA transfected mock stimulated cells. Samples of candidate siRNA transfected mock stimulated cells were not tested.

In order to determine the significance of the candidate evaluation data all 4 datasets were submitted to statistical analysis (Dr. Jacques Colinge, Bioinformatics Department, CeMM). In summary, the original  $C_t$  values from one real-time PCR run consisting of two replicates of each gene of interest (goi) and each reference gene (ref) were bootstrapped. This means all possible combinations of goi and ref were formed. Bootstrapping yielded four datapoints for each of the samples, i. e. 24 silenced (siCandidate) and the non-silenced (mock siRNA) polyI:C-stimulated samples. This data was analyzed twofold, namely by pairwise comparison for each dataset separately and by analysis of variance (ANOVA) for all four datasets simultaneously (table 14).

For pairwise comparison, the Wilcoxon test was performed on all pairs of silenced and non-silenced samples within one dataset. The Wilcoxon test is a non-parametric hypothesis test, i. e. it does not assume Gaussian distribution of data within a population and it is based on the hypothesis that two samples - silenced and non-silenced - are the same. This hypothesis is proven wrong, when the two samples are different from each other. The likelihood of this event to occur by coincidence is given by the p-value. If a silenced sample differed from a non-silenced sample with a p-value smaller than 0,0035, a successful event was scored. This procedure was applied to all 4 datasets. Knockdown of a given candidate was defined to have a significant effect on polyI:C-stimulated IFN $\beta$  induction, if at least 3 successes were counted within 4 datasets.



Table 14

Candidate	Pairwise comparison		ANOVA
	Successes	Relevance of candidate	Relevance of candidate
1	3	+	+
2	inversion	-	-
MDA-5	3	+	+
3	inversion	-	-
RIG-I	inversion	-	-
4	3	+	-
5	3	+	+
Pkr	4	+	+
IKK-i	4	+	+
Oas3	4	+	+
6	4	+	+
7	4	+	+
8	2	-	-
9	2	-	-
10	inversion	-	-
11	4	+	+
12	inversion	-	-
13	4	+	+
14	4	+	+
15	4	+	+
16	4	+	+
17	3	+	+
18	4	+	+
19	inversion	-	-

**Table 14. Statistical analysis of functional relevance of candidates for polyI:C-induced IFN $\beta$  production.** Summary of results from pairwise comparison and ANOVA. +: candidate is functionally relevant, -: candidate is not relevant, inversion: knockdown of a candidate resulted in significant, but contradictory alterations of IFN $\beta$  expression and thus candidate is categorized as not relevant.

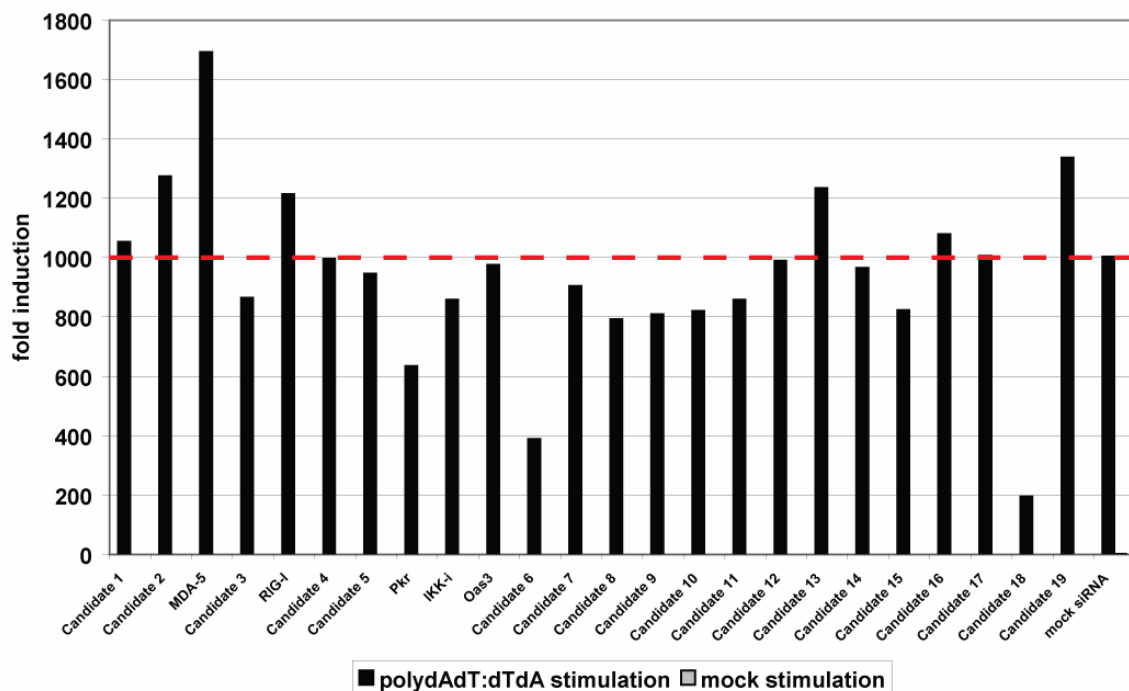
ANOVA enables analysis of the 4 datasets simultaneously. This statistical method corrects the trends of datasets and takes the variability of the biological experiment into account. For ANOVA, the non-parametric Friedman test was performed. If knockdown of a candidate resulted in IFN $\beta$  levels differing from the threshold with a p-value smaller than 0,05, the candidate was considered to be functionally relevant for IFN $\beta$  induction by polyI:C.

## Results

The statistical analysis confirms the interpretation of results for the 5 control candidates. Knockdown of MDA-5, but not RIG-I affects polyI:C-induced IFN $\beta$  induction. For RIG-I the silenced samples were indifferent from the non-silenced samples or showed significant, but opposite effects. All candidates whose knockdown resulted in such an inversion were classified as non-relevant. In three datasets silencing of MDA-5 lowered IFN $\beta$  induction significantly and once an opposite, but insignificant trend was observed. The other three controls qualified unambiguously as positive regulators of the IFN $\beta$  response. Out of the 19 candidates 12 have positive effects on IFN $\beta$  induction, while the other 7 have no significant impact. The functionally relevant candidates are 1, 6, 7, 11 and 13 - 18. Candidate 4 passed statistical analysis by pairwise comparison, but did not meet the requirements with ANOVA.

### 4.3.1.2 Effect of candidate silencing on polydAdT:dTdA-stimulated IFN $\beta$ induction

The polydAdT:dTdA evaluation was performed as described in 4.3.1.1, except cells were stimulated with polydAdT:dTdA. One biological experiment was carried out and the cDNA was analyzed twice by real-time PCR. The results of one of the PCR runs is shown in figure 15.



**Figure 15. Functional relevance of candidates for polydAdT:dTdA-induced IFN $\beta$  production.** siRNA treated cells were stimulated by transfection of 1  $\mu$ g/ml polydAdT:dTdA. Transcriptional activation of IFN $\beta$  was determined by real-time PCR. Data is normalized to mock siRNA transfected mock stimulated cells. The red dashed line indicates the threshold.

Neither RIG-I nor MDA-5 were expected to have an effect in this experimental setup, whereas IKK-i as activating kinase of IRF3 might be implicated. In this regard the results show contrary trends. However, it is unknown, if the 5 control candidates are involved in polydAdT:dTdA-stimulated IFN $\beta$  induction.

As the candidate list does not contain any polydAdT:dTdA-binding protein, it is conceivable that only 2 candidates, 6 and 18, show an impact on polydAdT:dTdA-induced IFN $\beta$  induction. These proteins may have a general effect on gene expression like expected of a splicing factor or could be far downstream components of IFN $\beta$  induction such as transcription factors. However, more biological experiments are required to identify candidates with significant effects by statistical analysis.

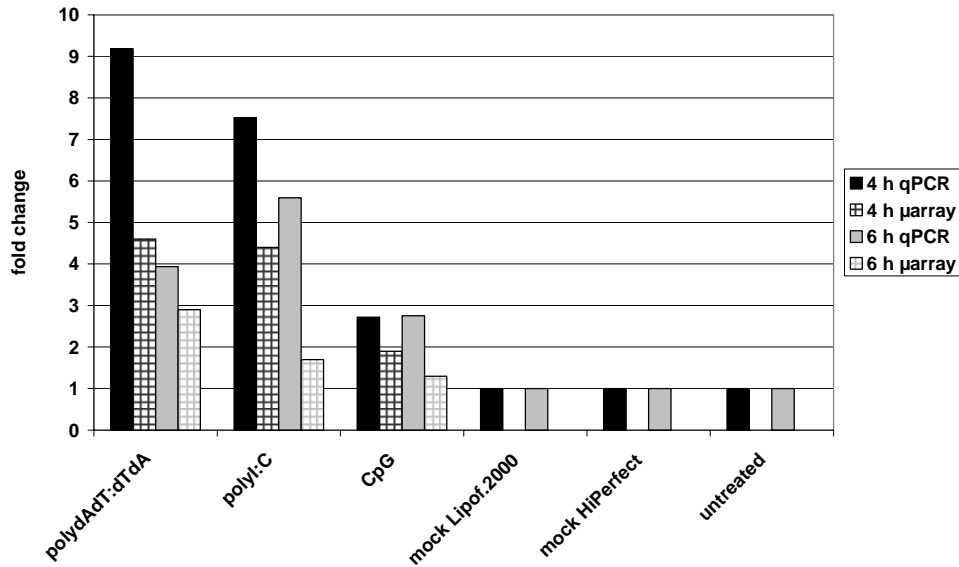
#### **4.3.2**     *“Educated guess”-approach*

A comprehensive literature search revealed specifically interesting properties of candidate 4, which encouraged us to study this protein in more detail. Candidate 4 is a DNA-modifying enzyme that, if inactivated by mutation, leads to a severe disease with an inflammatory component. Candidate 4 knock-out mice have been reported to show an inflammatory phenotype as well. Moreover, a concise hypothesis on the mechanism of action of candidate 4 has been proposed, but not been proven, yet. In this model candidate 4 would modify DNA in the cytosol and thereby modulate the IFN $\beta$  response.

##### *4.3.2.1*    *Expression of candidate 4 is nucleic acid- and IFN $\beta$ -inducible*

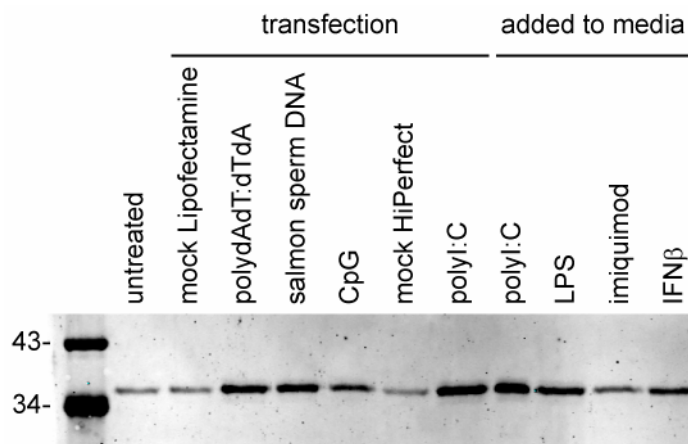
To study which stimuli induce the transcription of candidate 4 RAW264.7 cells were treated with polyI:C, polydAdT:dTdA or CpG-ODN for 4 h and 6 h, RNA was extracted, reversely transcribed in cDNA, and real-time PCR was performed. The results indicate that candidate 4 is induced by both polydAdT:dTdA and polyI:C about 4 to 8 fold depending on the time point, and by CpG to a lesser extend (figure 16).

## Results



**Figure 16. Candidate 4 transcription is activated by nucleic acids.** RAW264.7 cells were stimulated by polydAdT:dTda or polyI:C transfection (1  $\mu$ g/ml and 10  $\mu$ g/ml, respectively), or by addition of 1  $\mu$ M CpG-ODN to the media. 4 and 6 h later RNA was extracted, cDNA prepared and real-time PCR performed using gene-specific primers for candidate 4. Results are normalized to the respective control and displayed in comparison to the corresponding microarray data.

Next, we tested candidate 4 induction on the protein level. RAW264.7 cells were stimulated by transfection of polydAdT:dTda, salmon sperm DNA, CpG-ODN and polyI:C as well as by addition of polyI:C, LPS, imiquimod and IFN $\beta$  to the media. 24 h after treatment lysates were prepared and analyzed by Western blotting for candidate 4 expression (figure 17).



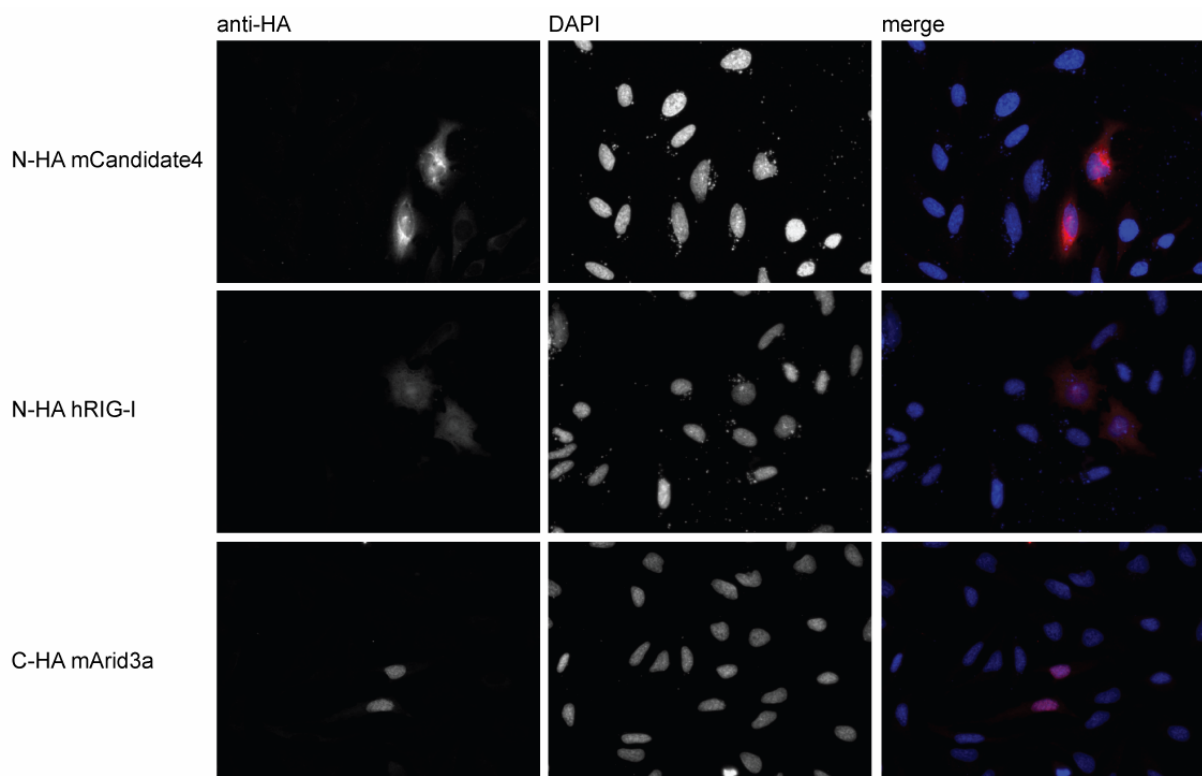
**Figure 17. Candidate 4 expression is induced by various stimuli.** RAW264.7 cells were stimulated by transfection of 500 ng/ml polydAdT:dTda or salmon sperm DNA, 1  $\mu$ M CpG-ODN or 10  $\mu$ g/ml polyI:C, or by addition of 10  $\mu$ g/ml polyI:C, 1  $\mu$ g/ml LPS, 50  $\mu$ M imiquimod or 100 U/ml IFN $\beta$  to the media. Lysates were prepared 24 h after treatment and 50  $\mu$ g total protein were analyzed by Western blotting using a candidate 4-specific antibody.

The Western blot shows that candidate 4 is induced by all tested nucleic acids, but most strongly by polyI:C regardless if transfected, thus stimulating signaling via cytosolic receptors, or added to the media, consequently activating membrane-associated TLR3

signaling. As polyI:C stimulation leads to IFN $\beta$  production, elevated candidate 4 expression may be due to secreted IFN $\beta$  that stimulates cells in an autocrine fashion. This is supported by the fact that IFN $\beta$  treatment leads to an increased production of candidate 4 as well. Moreover, candidate 4 expression is stimulated by LPS signaling through TLR4, but not by the TLR7 agonist imiquimod.

#### 4.3.2.2 Candidate 4 is a perinuclear protein

Databases frequently list candidate 4 as a nuclear protein due to a membrane domain that is predicted to target it to the nuclear envelope. However, it has been reported that candidate 4 is part of a complex that is endoplasmic reticulum-associated. The proposed mechanism of action requires candidate 4 to modify DNA in the cytosol in order to modulate the type I IFN response. If candidate 4 indeed was a nuclear protein, its localization would interfere with this hypothesis and thus make it irrelevant. Therefore we analyzed the subcellular localization of candidate 4 by immunofluorescence of transiently transfected HeLa cells (figure 18).



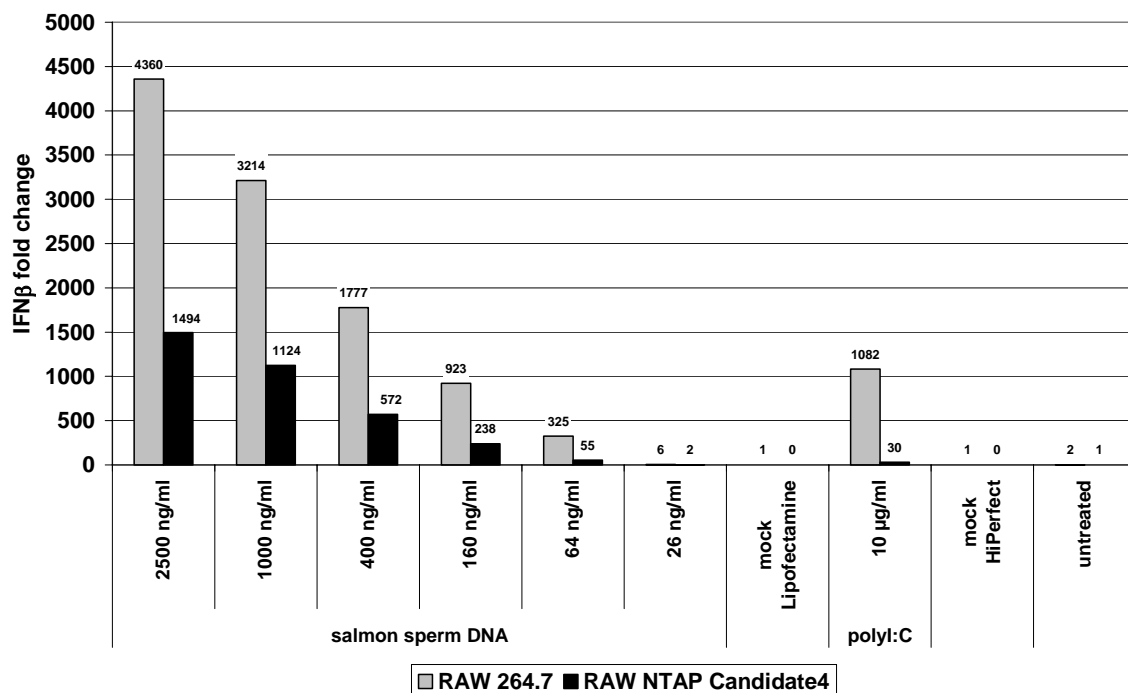
**Figure 18. Candidate 4 is a perinuclear protein.** HeLa cells were transiently transfected with HA-tagged versions of candidate 4, Arid3A or RIG-I. 48 hours after transfection immunofluorescent staining was performed using an HA-specific primary antibody and an Alexa 594-labeled secondary antibody. Nuclei were visualized by DAPI staining.

## Results

Arid3a is a nuclear protein which is reflected by a staining pattern that co-localizes with DAPI fluorescence. RIG-I is a cytosolic protein and yielded diffuse staining throughout the cytoplasm. Even though candidate 4 and RIG-I stain differently, the immunofluorescence data indicates that candidate 4 is predominantly found in the cytoplasm in close proximity to the nucleus, which is in agreement with the proposed model.

### 4.3.2.3 Overexpression of candidate 4 reduces IFN $\beta$ induction in response to DNA stimulation

Next, we investigated the role of candidate 4 on the IFN $\beta$  response upon DNA stimulation. A stable RAW264.7 cell line overexpressing an N-terminally TAP-tagged version of candidate 4 was generated and stimulated with increasing concentrations of polydAdT:dTdA (data not shown) or salmon sperm DNA for 4 h. Transcriptional activation of IFN $\beta$  was determined by real-time PCR (figure 19).



**Figure 19. Overexpression of candidate 4 decreases IFN $\beta$  induction.** RAW264.7 cells and RAW cells stably overexpressing candidate 4 were stimulated by transfection of indicated concentrations of salmon sperm DNA as well as polyI:C. IFN $\beta$  induction 4h after stimulation was measured by real-time PCR. Data is normalized to mock stimulated RAW264.7 cells.

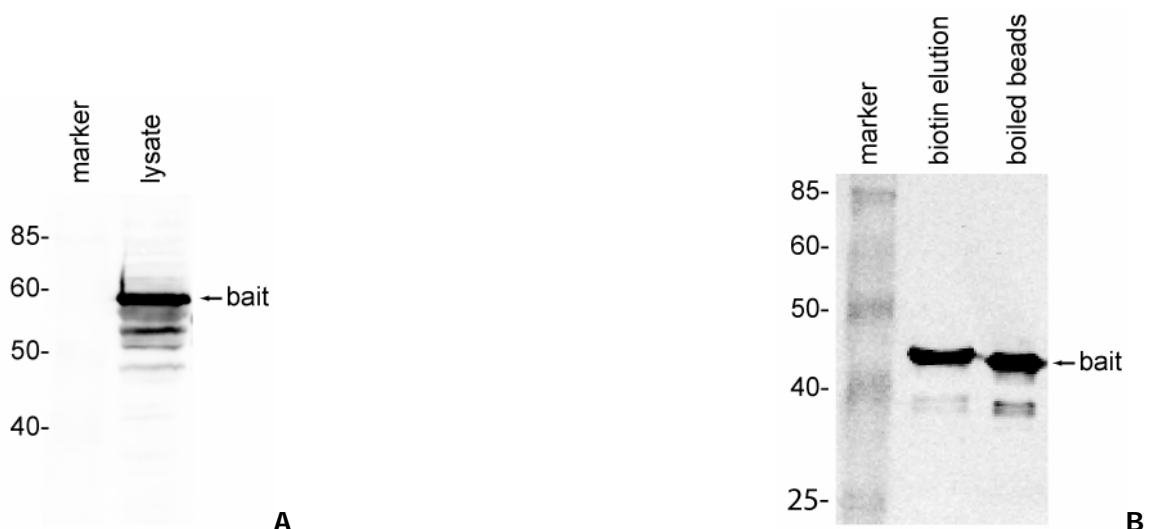
IFN $\beta$  induction in response to DNA is reduced by 66 (2500 ng/ml DNA) to 80% (64 ng/ml DNA) in cells overexpressing candidate 4 in comparison to RAW264.7 cells. Interestingly, polyI:C-stimulated IFN $\beta$  induction is decreased by 98%. This finding indicates either a

generalized effect of candidate 4 on IFN $\beta$  expression or an unspecific effect that does not require the catalytic properties of candidate 4.

#### 4.3.2.4 Interactors of candidate 4

To study the mechanism of action by which candidate 4 is involved in the regulation of the IFN $\beta$  response upon nucleic acid stimulation we sought to identify interactors of candidate 4 by tandem affinity purification (TAP) [30]. Analysis of protein complexes by TAP requires fusion of the TAP-tag consisting of protein G, the Tobacco Etch Virus (TEV) protease cleavage site and streptavidin binding protein (SBP) with the protein of interest. This fusion protein will then serve as bait during the TAP procedure. Therefore an N-terminally tagged version of candidate 4 was cloned and used to generate a stable RAW264.7 cell line. Lysates were prepared and subjected to TAP. Aliquots of lysate and eluate were analyzed by Western blotting to determine the quality of the purification (figure 20).

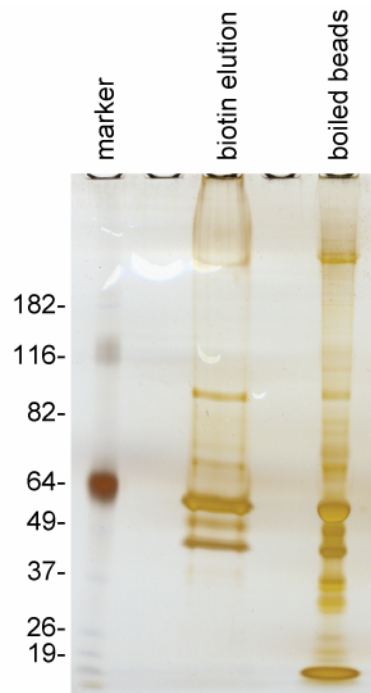
The bait was detected in the lysate and the eluates. In the lysate the bait appeared as a 60 kDa protein, whereas in the eluates as a 45 kDa protein. This size difference is due to the fact that the TEV protease cleaves off the protein G moiety (20 kDa) of the TAP tag. This result indicated that the purification was performed successfully. As the specific elution of complexes was incomplete, both fractions biotin elution and boiled beads were submitted to mass spectrometry.



**Figure 20. Comparison of lysate and eluate of the NTAP candidate 4 TAP.** 70  $\mu$ g total protein of the lysate and 2  $\mu$ l of the eluate were loaded on a protein gel. For the subsequent Western blot analysis a myc-specific antibody was used to probe for NTAP candidate 4 as all TAP constructs contain a myc-epitope.

## Results

In order to visualize the proteins that were co-purified with the bait, the eluate was analyzed by silver staining (Melanie Planyavsky, Mass Spectrometry Department, CeMM) of an SDS- PAGE (figure 21)



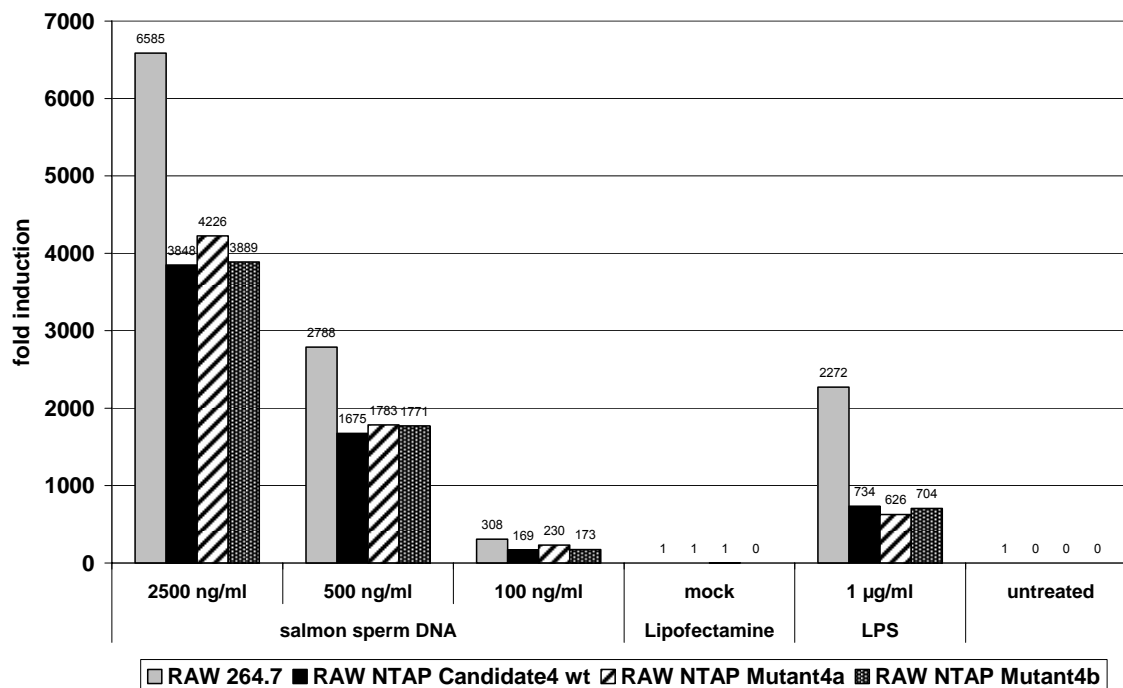
**Figure 21. NTAP candidate 4 interactors isolated by TAP.** Silver staining of an SDS-PAGE loaded with the NTAP candidate 4 eluate shows several potential interactors that were co-purified with the bait.

The silver gel illustrated that there are several potential interactors that co-purify with candidate 4. The individual bands were isolated from the gel and are currently identified by mass spectrometry.

### *4.3.2.5 Overexpression of catalytically inactive mutants of candidate 4 still reduces DNA-induced IFN $\beta$ induction*

As described in 4.3.2.3 overexpression of candidate 4 decreased IFN $\beta$  levels in response to DNA, but also polyI:C stimulation which might indicate an unspecific or indirect effect. To address this issue two catalytically inactive point mutants of candidate 4 were cloned. One of the introduced point mutations was reported to be a disease causing mutation. If the observed effect depended on the protein's enzymatic activity, it should be abolished when inactive mutants are overexpressed. Therefore the experiment was repeated to compare 4 cell lines side by side: 3 RAW cell lines expressing either wild-type candidate 4, mutant 4a or mutant 4b and the parental RAW264.7 cell line (figure 22).





**Figure 22. Overexpression of wild-type and inactive candidate 4 affect IFN $\beta$  induction in an identical manner.** RAW264.7 cells and RAW cells stably overexpressing candidate 4 wild-type, mutant 4a or 4b were stimulated by transfection of indicated concentrations of salmon sperm DNA as well as LPS. IFN $\beta$  induction 4h after stimulation was measured by real-time PCR. Data is normalized to mock stimulated RAW264.7 cells.

Overexpression of candidate 4 wild-type showed an overall weaker reduction of IFN $\beta$  transcriptional activation in comparison to the previous experiment. More importantly, overexpression of either of the two inactive mutants did not reverse this effect. These results suggest that the effect of overexpression of candidate 4 is not as strong as we had originally hoped and that the effect is independent of the suggested catalytic activity of the protein. Such a catalytic activity-independent effect could imply poor specificity of candidate 4 in the process or a general dominant-interfering effect that occurs also with the catalytically incompetent forms. Thus, more experiments are needed to conclusively dismiss this candidate as non-specific or, alternatively, elucidate its mode of action in the nucleic acid-sensing pathway.

# 5 Discussion

The goal of this project was to identify novel nucleic acid receptors based on the hypothesis that several more than the few identified so far should exist and that nucleic acid receptors bind to nucleic acids and are transcriptionally regulated by nucleic acids. Thus a combined proteomics and genomics approach was utilized to generate a list of candidate proteins.

For this purpose nucleic acid binding proteins were identified by pull-down experiments using 3 different nucleic acids immobilized on agarose beads: polyI:C mimicking dsRNA, polydAdT:dTdA for B-form DNA and CpG-ODN as synthetic analogue for bacterial or viral unmethylated CG dinucleotides. The purified nucleic acid interactors were analyzed by mass spectrometry to reveal their identity. Due to the large quantity of identified nucleic acid binding proteins, a filtering criterion had to be introduced. Our assumption was that proteins that are functionally relevant for nucleic acid detection, e. g. a receptor, would be transcriptionally regulated in response to nucleic acid stimulation. This additional selection process has two advantages: On one hand it decreases the number of potential receptors; on the other hand it increases the probability of proteins that are functionally relevant for innate immunity signaling in response to nucleic acid treatment. Therefore alterations in expression patterns in response to stimulation with the before mentioned 3 nucleic acids were assessed by microarray analysis. Regulated genes were determined using the software tool SAM. Once two datasets - one proteomics set including all proteins binding to any nucleic acid tested and one genomics set consisting of genes regulated by any of the three nucleic acids - had been established, we looked for proteins that belonged to both datasets. In the end we obtained a list of 24 candidates binding to and being regulated by either polyI:C, polydAdT:dTdA or CpG-ODN.

Next the nucleic acid-sensing candidates were assessed in two ways in parallel. For a side by side evaluation of all candidates, the effect of candidate silencing on transcriptional activation of IFN $\beta$  after nucleic acid stimulation was determined. Complementary to this, we chose to investigate one particular candidate, namely the DNA-modifying enzyme candidate 4, in more depth.

## 5.1 List of candidate proteins

19 candidate proteins and 5 proteins with established roles in antiviral innate immunity - RIG-I, MDA-5, IKK-i, Oas3 and Pkr - constitute the list of candidates. The presence of these five proteins in the candidate list validates our approach and facilitates quality control during subsequent experiments.

The 19 candidate proteins that were identified as potential components of nucleic acid signaling pathways are quite diverse in terms of their enzymatic activity, their domain structure and the extent of annotation. While for some proteins there is barely any information available, others are well characterized proteins, some of which have been reported to be implicated in immunological processes.

Nucleic acid receptors are estimated to account only for a small fraction of nucleic acid binding proteins. Thus we expected to find among our candidates not only receptors, but also proteins involved in nucleic acid binding further downstream of ligand recognition, and proteins whose function is not restricted to innate immunity responses. The latter category of candidates includes transcription factors, splicing factors or proteins involved in DNA replication, repair and recombination. All these proteins are nucleic acid binding proteins. If they are transcriptionally regulated by nucleic acid or IFN $\beta$  stimulation, we would find them in our candidate list. Pkr represents a downstream effector of antiviral immunity. It contains two dsRNA binding motifs suggesting its role as a nucleic acid receptor. However, its RNA binding domains serve to regulate kinase activity that allows interference with viral replication. While Pkr apparently is not the receptor that triggers the initial wave of IFN $\beta$  and thus is not involved in the early events of IFN $\beta$  induction, it contributes to the amplification of type I IFN production at a later stage of the innate antiviral immune response. Another scenario is illustrated by IKK-i. This non-canonical IKK does not contain any nucleic acid binding domain, nor has it been reported to be a nucleic acid binder, but still it is part of the candidate list. One possible explanation is that IKK-i forms a complex with an actual nucleic binder and thus is co-purified. Which protein it was binding to, is unknown. It may be in the candidate list, if its expression changes in response to nucleic acid stimulation.

## Discussion

---

The polyI:C pull-downs yielded by far the most interactors, followed by CpG-ODN pull-downs and the polydAdT:dTdA pull-down. In fact, a surprisingly small number of proteins bind to polydAdT:dTdA. In contrast to polydAdT:dTdA, polydA:dT neither assumes the confirmation of B-form DNA when in solution, nor stimulates type I interferon secretion. Therefore polydA:dT serves as a negative control. When eluates from a polydA:dT and a polydAdT:dTdA pull-down were compared side by side on a silver stained protein gel, the staining pattern was identical for the most part. As some differences were apparent in the gel region corresponding to proteins larger than 64 kD, only this region rather than the whole lane was submitted to mass spectrometry analysis. The low number of identified polydAdT:dTdA binders is reflected in the composition of the candidate list. 12 candidates are polyI:C binders and regulated by polyI:C, which is only true for 3 candidates in the case of CpG-ODN. On top of this 3 more candidates are regulated by and bind to both nucleic acids, polyI:C and CpG-ODN. The remaining candidates bind to polyI:C, but are regulated by a different nucleic acid, which is polydAdT:dTdA in 5 instances and CpG in one case. PolydAdT:dTdA, polyI:C and to a lesser extent CpG-ODN result in IFN $\beta$  secretion that in turn modulates gene expression. Therefore, we speculate that the genomics dataset mainly consists of IFN $\beta$  inducible genes. Based on this assumption, we are not concerned if proteins bind to a different type nucleic acid than the one they are regulated by.

However, the fact that none of the candidates binds to polydAdT:dTdA was considered disturbing. To counteract the imbalanced contribution of polydAdT:dTdA binding proteins to the candidate list, meanwhile three more polydAdT:dTdA pull-downs were performed and regardless of the abundance of purified interactors visible on silver stained gels the whole lane was analyzed by mass spectrometry. Additional polydAdT:dTdA-binding proteins were blended with the proteomics dataset and the described bioinformatics procedure was applied to obtain an updated candidate list for future experiments. The new list contains 28 candidates, eight of which are polydAdT:dTdA-binding proteins. Six of the polydAdT:dTdA binders are also transcriptionally regulated by polydAdT:dTdA.

The absence of TLRs from the list is probably due to the lysis conditions that were applied for the initial pull-down experiments. As TLRs are integral membrane proteins, a higher detergent concentration may be required to solubilize them. On the other hand proper ligand recognition by TLRs might require additional proteins that stabilize the interaction

with nucleic acids which are available under physiological conditions, but not in cell lysates.

Is DAI among the candidates? The recently identified cytosolic DNA receptor, DAI, is not among the candidates, because it was not detected in any of the performed pull-downs. Due to its specificity for dsDNA, DAI was neither pulled-down with polyI:C nor CpG-ODN. Moreover, it was not identified in the original polydAdT:dTdA pull-down, because the 44 kDa protein DAI ran below the cut-off for mass spectrometry analysis of 64 kDa. However, it was not found in any of the additional pull-downs, where all purified proteins were analyzed, either. DAI expression in resting cells is very low. As pull-down experiments were performed with lysates of unstimulated cells, the amount of purified DAI may have been below the detection limit of the mass spectrometry set-up. Alternatively, there may be cell-specific differences regarding DNA receptors. DAI was shown to act in fibroblasts (MEFs and L929) and kidney cells (HEK293T), but other proteins than DAI may serve as DNA receptors in macrophages, i. e. RAW264.7 cells as used in this study.

Even though our approach failed to identify DAI as a candidate due to technical or biological reasons, the properties of DAI further validated the validity of our approach: DAI is a DNA binding protein and it is strongly induced in response to DNA stimulation.

## 5.2 Candidates affecting polyI:C-induced IFN $\beta$ induction

In order to determine the functional relevance of candidates on polyI:C-stimulated IFN $\beta$  induction, endogenous candidate expression was knocked down using a pool of gene-specific siRNAs before cells were stimulated with polyI:C. If the knockdown resulted in statistically significant alterations of transcriptional activation of IFN $\beta$ , the candidate was considered to be functionally relevant. MDA-5, but not RIG-I, IKK-i, Oas3 and Pkr are essential for IFN $\beta$  production upon polyI:C treatment, which is in agreement with previous studies. Oas3 and Pkr do not trigger IFN $\beta$  induction, but amplify the response once it has been initialized. Thus the lack of amplification due to silencing of endogenous Pkr and Oas3, may result in reduced IFN $\beta$  levels. Based upon this data we concluded that the experimental setup was valid. Among the remaining 19 candidates, 12 candidates are functionally relevant and 7 candidates have either controversial or non-significant effects.

## Discussion

---

The high number of potentially relevant candidates was unexpected. The goal of the “filtering” process was to reduce the number of candidates before detailed functional studies had to be started. As it is not feasible to characterize 12 candidates in depth, additional and more stringent selection criteria may need to be applied in order to make an accurate decision on further research strategies.

### **5.3 Candidates affecting polydAdT:dTdA-induced IFN $\beta$ induction**

The functional relevance of candidates on polydAdT:dTdA-induced IFN $\beta$  induction was investigated in analogy to the above described polyI:C evaluation. However, as only one biological experiment was performed, the statistical significance of the data could not be determined. Considering the composition of the candidate list consisting of mainly polyI:C binders but no polydAdT:dTdA interactors, it is conceivable that very few if any candidates are functionally relevant for IFN $\beta$  production in response to dsDNA stimulation. In fact, the preliminary data obtained so far suggests only two candidates, 6 and 18, as positive regulators of IFN $\beta$  induction.

Nevertheless further biological experiments as well as technical repeats of the relative quantification of IFN $\beta$  transcripts are required for statistical analysis of the data. Moreover, the new polydAdT:dTdA binding candidates identified by analysis of additional pull-down experiments need to be tested as well.

### **5.4 Alternative approaches to determine the functional relevance of candidates**

How can the accuracy of candidate evaluation be improved? Due to the initial selection procedure, the expression of all candidates is regulated by nucleic acid stimulation to various degrees. For a 4 h polyI:C stimulation alterations in candidate expression range between slight downregulation and 25fold upregulation. Overall, most candidates are upregulated in response to nucleic acid treatment. Thus a major drawback of the RNAi approach is that the candidate expression level affects the knockdown efficiency to a certain, but among all candidates variable extent. In the worst case, induction of a

candidate might rescue the knock down. Therefore it might be interesting to relate the knock down efficiency of each candidate to its effect on IFN $\beta$  induction as candidates with a strong knockdown for a given change in IFN $\beta$  induction may be less promising than those with a weaker knockdown. However, knockdown efficiency can only be determined at the transcriptional level as antibodies are not available for all candidates, while the analysis of knockdown efficiency on the protein level would be most appropriate for this approach.

In order to circumvent this issue, dominant negative versions of candidates could be generated and overexpressed before nucleic acid stimulation. On the downside, the generation of dominant negative mutants is time-consuming and difficult for poorly characterized proteins and the precise mode of action of the interfering mutants often remains elusive.

Alternatively, assessing which candidates selectively affect IFN $\beta$  induction could help to better focus on the functional relevance of candidates, as production of type I interferons is the key event in antiviral immunity. Both TNF $\alpha$  and IFN $\beta$  induction depend on the activation of the transcription factor NF- $\kappa$ B, but only IFN $\beta$  induction requires activation of IRF3 and IRF7. Thus candidates that have an effect on IFN $\beta$ , but not TNF $\alpha$  induction, are more likely to be essential for signal transduction in response to viral infection.

The five control proteins in the candidate list are associated with two phases of host defence against viruses: While RIG-I, MDA-5 and IKK-i are signaling molecules that lead to IFN $\beta$  induction, Oas3 and Pkr are effectors whose expression is stimulated by IFN $\beta$  secretion. In addition to their effector functions that interfere with viral replication, Oas3 and Pkr amplify type I interferon induction by an unknown mechanism, but they are not involved in the initial events of IFN $\beta$  induction. Only candidates that trigger IFN $\beta$  production qualify as potential nucleic acid receptors. In order to subdivide the candidates into the 2 classes of IFN $\beta$  inducers or IFN $\beta$ -induced effectors, wild-type cells need to be compared to cells deficient in IFN signaling, e. g. cells lacking functional interferon receptor or Jak/Stat signal transduction. The read-out for such epistasis studies can either be the abundance of IFN $\beta$  transcripts determined by quantitative PCR, or measurement of the IFN $\beta$  promoter activity by a reporter gene assay after knock down of candidates and nucleic acid stimulation. Performance of reporter gene assays in parallel to quantitative

## Discussion

---

PCRs would have the advantage of having a second read-out to substantiate the data, but would require to switch from murine macrophages to more easily transfectable cells like MEFs.

Epistasis studies as described above may also be carried out in a more detailed manner. Overexpression of signaling components typically has the same effect as activation of the corresponding pathway with an external stimulus, e. g. overexpression of MAVS results in IFN $\beta$  production. If knockdown of a candidate results in decreased IFN $\beta$  induction in MAVS overexpressing cells, this candidate acts downstream of MAVS. Conversely, silencing candidates upstream of MAVS would not interfere with IFN $\beta$  production due to MAVS overexpression. In analogy, this procedure would allow to position candidates relative to TBK-1 and IKK-i as well.

Conduction of a screen in the fruitfly *Drosophila melanogaster* using the first genome-wide transgenic RNAi library [31], constructed in Vienna, represents another, directly functional approach to validate the relevance of those candidates that have a fly orthologous gene. The library covers about 90% of the predicted protein-coding sequences in the *Drosophila* genome. Each of the approximately 22 000 transgenic lines carries a transgene consisting of an inverted repeat of a short gene fragment under control of the trans-acting UAS element. In order to achieve a conditional knockdown in the tissue of interest, these transgenic lines are crossed with strains expressing GAL4 in a tissue-specific manner thereby creating a functional GAL4-UAS expression system: The transcription factor GAL4 is expressed in the tissue of interest, binds to the UAS element and thus drives transcription of the transgene yielding a long dsRNA hairpin that has been shown to trigger RNAi in *Drosophila*. Candidate validation in *Drosophila* requires identification of orthologous candidate proteins, and expression of corresponding transgenes in fly immune cells. Conditional gene silencing would reveal, if a given candidate is essential for immunity against infection in *Drosophila*.

### 5.5 Candidate 4

In addition to the parallel evaluation of all candidates, we chose one candidate, number 4, in order to be able to perform more in-depth validation. Why was candidate 4 selected for follow-up studies? Once the list of candidates had been generated, the decision was



based on an “educated guess”, i. e. an extensive literature search was performed. Candidate 4 specifically evoked our interest, as it had been recently reported that a mutation inactivating candidate 4 leads to a severe disease. Despite being caused by a genetic defect, its phenotypic presentation is reminiscent of a congenital infection. Furthermore, a model has been proposed on how the DNA-modifying enzyme candidate 4 would lead to abnormal immunological parameters, one of which being raised type I interferon levels in the serum. According to this hypothesis, candidate 4 would modify DNA in the cytosol of e. g. dying cells and thereby modulate the subsequent interferon response.

In order to test this hypothesis candidate 4 was cloned, and a cell line stably expressing candidate 4 was generated. If the hypothesis was true, overexpression of candidate 4 should lead to decreased IFN $\beta$  induction upon DNA stimulation. Comparison of parental cells with the overexpressing cells confirmed this hypothesis initially. However, further experiments with catalytically inactive mutants of candidate 4, wild-type candidate 4 and parental cells, showed that the previously observed effect is not due to the enzymatic activity of candidate 4. The finding that candidate 4 overexpression also affects polyI:C- and LPS-stimulated IFN $\beta$  production, despite being a DNA-modifying enzyme, further argued for a possible unspecific effect. Notably, knockdown of candidate 4 affects IFN $\beta$  induction in the polyI:C evaluation while it does not seem to have an impact in the polydAdT:dTdA evaluation. Overall, the results regarding candidate 4 are such that no definitive conclusions are allowed within the time frame of this diploma work.

The possibility exists that the observed effect was due to the fact that stable cell lines generated by retroviral gene transfer were compared to parental cell lines. The retroviral infection may have interfered with the ability to induce IFN $\beta$  in response to various stimuli. Thus using mock transduced cells instead of parental cells would be appropriate for a fair comparison. More experiments are planned in the laboratory to reach the decision point of whether to continue with the candidate or discard it.

## 5.6 Conclusion

This thesis describes how 24 candidates were identified as potential components of innate immunity signaling in response to nucleic acid stimulation, and tested for their functional relevance for IFN $\beta$  induction. The list was obtained by combination of proteomics and

## Discussion

---

genomics, where the proteomics branch identified proteins that bind to nucleic acids, which is a key property of a nucleic acid receptor. Could one imagine other ways to obtain a list of nucleic acid binding proteins? Simple Modular Architecture Research Tool (SMART) lists roughly 100 domains with and without enzymatic activity involved in nucleic acid binding including helicase domains as present in RIG-I and MDA-5 and the DNA binding Zalpha domain in DAI. Both, the proteomics and the domain dataset, can be merged with the genomics dataset in order to find nucleic acid binding proteins whose expression is regulated by nucleic acids. While the limitations of the proteomics approach are of technical nature, such as sensitivity of mass spectrometry equipment and co-purification of unspecifically binding proteins, the domain approach depends on the curation quality of the database. Furthermore, the virtual approach can only identify nucleic acid binders that contain annotated nucleic acid binding domains, whereas it fails to identify proteins with unknown nucleic acid binding domains or proteins lacking a clear and detailed domain annotation. For example the CARD domain of RIG-I is not annotated in the SMART database. In addition, databases may contain incorrect information, e. g. the Zalpha domain of DAI is listed as an RNA binding domain, even though it is a DNA binding domain.

This diploma work has led to the identification of several candidate genes likely to be involved in the intracellular recognition of nucleic acids and in the signaling to innate immunity pathways. Further work will be needed in order to further validate all of the candidates. The ambitious attempt to obtain conclusive data on a single chosen candidate on top of the parallel evaluation has yielded contradictory results and proven to elude the time-frame offered by a diploma thesis. Thus, also in the case of the hand-picked "super"-candidate, number 4, more work will be needed. As this thesis was finalized, we obtained news from an international conference on innate immunity (last week of October 2007) that a leading innate immunity laboratory has reported the identification of the protein corresponding to candidate 4 as a major new player in the cellular process leading to the recognition of foreign nucleic acid. Thus, this diploma work may have provided the basis for a whole new research avenue on this protein.

## 6 Summary

The innate immune system is the first line of defense against invading pathogens. Innate immune cells such as macrophages express pattern recognition receptors (PRRs) that detect conserved structures shared by many microbes, so-called pathogen-associated molecular patterns (PAMPs). Viruses are typically sensed by the presence of their genomes. Various PRRs are implicated in virus detection and trigger a signaling cascade that leads to the secretion of type I interferon (IFN $\alpha$  and IFN $\beta$ ). Type I interferons are essential for antiviral immunity, as they limit virus replication and stimulate the adaptive immune system. However, the knowledge on the signaling pathways leading to interferon induction is still incomplete, and while the first cytosolic nucleic acid sensors are being identified, evidence for the existence of more, yet unknown receptors accumulates.

Therefore, the aim of my diploma thesis was to identify novel nucleic acid sensors implicated in antiviral innate immunity. The central hypothesis was that nucleic acid receptors bind to nucleic acids *and* are transcriptionally regulated by nucleic acid stimulation. To this end we chose a combined proteomics and genomics approach. Two datasets were generated: The proteomics dataset consists of nucleic acid binding proteins that were identified by pull-down experiments with immobilized nucleic acids and subsequent mass spectrometry analysis. The genomics dataset includes genes that are regulated by nucleic acids as determined by microarray analysis. Based on the before mentioned hypothesis, proteins that belonged to both datasets were selected to compile a list of 24 candidate proteins. Among these 24 candidates are five proteins with an established role in nucleic acid signaling e. g. the receptors for double-stranded RNA (dsRNA), RIG-I and MDA-5, whose presence in the candidate list validates the approach.

Once the candidate list had been generated, the microarray data was confirmed for selected candidates by real-time PCR.

In order to assess the functional relevance of each candidate for antiviral innate immunity, the effect of candidate silencing on nucleic acid-stimulated IFN $\beta$  induction was measured by real-time PCR. The five control candidates showed the expected effects and 12 out of the 19 remaining candidates positively regulate IFN $\beta$  induction by polyI:C, the synthetic analogue of dsRNA. Thus this thesis provided the basis for further research leading to the identification of additional nucleic acid receptors.

## Summary

---

In parallel to the RNAi-based evaluation of candidates, the DNA-modifying enzyme candidate 4, was investigated in more detail. Candidate 4 is a IFN $\beta$ -inducible, perinuclear protein. When inactivated by mutation, candidate 4 has been reported to cause a severe disease with an inflammatory component. Contradictory results were generated regarding its role in DNA-mediated IFN $\beta$  induction. Therefore, further studies are needed to elucidate its mechanism of action.

## 7 Zusammenfassung

Das angeborene Immunsystem stellt die erste Verteidigungslinie gegen eindringende Pathogene dar. Zellen des angeborenen Immunsystems wie z. B. Makrophagen exprimieren Mustererkennungsrezeptoren (pattern recognition receptors), die konservierte Strukturen vieler Mikroorganismen, sogenannte Pathogen-assoziierte molekulare Muster (pathogen-associated molecular patterns), erkennen. Viren werden vorwiegend durch die Anwesenheit ihrer Genome detektiert und lösen eine Signaltransduktionskaskade aus, die zur Sekretion von Typ I Interferonen ( $\text{IFN}\alpha$  und  $\text{IFN}\beta$ ) führt. Typ I Interferone sind essentiell für die Ausbildung antiviraler Immunität, da sie die Replikation des Virus hemmen und die spezifische Immunabwehr stimulieren. Allerdings sind die Signaltransduktionswege, die zur Interferoninduktion führen, nicht vollständig geklärt, und während die ersten cytosolischen Nukleinsäuresensoren entdeckt werden, häufen sich die Indizien für die Existenz weiterer, noch unbekannter Rezeptoren.

Das Ziel dieser Diplomarbeit war, neue Nukleinsäurerezeptoren, die maßgeblich zur angeborenen antiviralen Immunantwort beitragen, zu identifizieren. Im Mittelpunkt des Projekts stand die Hypothese, dass Nukleinsäurerezeptoren an Nukleinsäuren binden *und* von Nukleinsäuren transkriptionell reguliert werden. Folglich wurde eine Kombination aus Proteomik und Genomik als experimenteller Ansatz gewählt. Zwei Datensätze wurden generiert: Der Proteomikdatensatz enthält Proteine, die an Nukleinsäuren binden. Zu diesem Zweck wurden Pulldown- Experimente mit immobilisierten Nukleinsäuren durchgeführt um Nukleinsäure- bindende Proteine zu isolieren, die anschließend mittels Massenspektrometrie identifiziert wurden. Gene, deren Expression durch Nukleinsäurestimulation transkriptionell reguliert werden, wurden mithilfe von Microarrays erfasst und repräsentieren den Genomikdatensatz. Basierend auf der eingangs erwähnten Hypothese, wurden die Proteine, die in beiden Datensätzen vorhanden waren, in eine Kandidatenliste aufgenommen. Zu den 24 Kandidaten zählen fünf Proteine, deren Funktion innerhalb der Nukleinsäuresignaltransduktion bereits bekannt ist, wie z. B. die Rezeptoren für doppelsträngige RNA (dsRNA) RIG-I und MDA-5. Die Tatsache, dass diese fünf Proteine Teil der Kandidatenliste sind, validiert den experimentellen Ansatz.

Nachdem die Kandidatenliste erstellt worden war, wurden die Microarray- Ergebnisse mittels real-time PCR bestätigt.

## Zusammenfassung

---

Weiters wurden die Kandidaten auf ihre funktionelle Relevanz für die Entstehung einer Nukleinsäure-stimulierten antiviralen Immunantwort untersucht. Zu diesem Zweck wurde die Auswirkung des Silencings der einzelnen Kandidaten durch RNA- Interferenz auf IFN $\beta$ -Induktion aufgrund Stimulation mit Nukleinsäuren mittels real-time PCR bestimmt. Die fünf Kontrollkandidaten zeigten die erwarteten Effekte, und zwölf der restlichen 19 Kandidaten hatten einen positiven Einfluss auf die IFN $\beta$  Induktion, wenn mit dem synthetischen dsRNA- Analogon polyI:C stimuliert wurde. Im Rahmen dieser Diplomarbeit wurde somit die Basis für weiterführende Untersuchungen zur Identifikation von Nukleinsäurerezeptoren geschaffen.

Parallel zu der auf RNA- Interferenz basierenden Evaluation der Kandidaten wurde das DNA- modifizierende Enzym Kandidat 4 genauer untersucht. Kandidat 4 ist ein IFN $\beta$ -induzierbares, perinukleäres Protein. Mutationen, die eine Inaktivierung von Kandidat 4 nach sich ziehen, bewirken eine schwere Erkrankung mit entzündlicher Komponente. Welche Rolle Kandidat 4 bei DNA- stimulierter IFN $\beta$ - Induktion spielt, konnte aufgrund widersprüchlicher Ergebnisse nicht geklärt werden. Weitere Versuche sind notwendig um die Funktion von Kandidat 4 zu verstehen.

## 8 References

1. Abbas and Lichtman, *Cellular and molecular immunology*. 2005: Elsevier Saunders.
2. Medzhitov, R. and C. Janeway, Jr., *Innate immunity*. N Engl J Med, 2000. **343**(5): p. 338-44.
3. Janeway, T., Walport and Shlomchik, *Immunobiology : The immune system in health and disease*. 2001: Garland Publishing.
4. Seth, R.B., L. Sun, and Z.J. Chen, *Antiviral innate immunity pathways*. Cell Res, 2006. **16**(2): p. 141-7.
5. Meylan, E., J. Tschopp, and M. Karin, *Intracellular pattern recognition receptors in the host response*. Nature, 2006. **442**(7098): p. 39-44.
6. Akira, S., S. Uematsu, and O. Takeuchi, *Pathogen recognition and innate immunity*. Cell, 2006. **124**(4): p. 783-801.
7. Dunne, A. and L.A. O'Neill, *Adaptor usage and Toll-like receptor signaling specificity*. FEBS Lett, 2005. **579**(15): p. 3330-5.
8. O'Neill, L.A., K.A. Fitzgerald, and A.G. Bowie, *The Toll-IL-1 receptor adaptor family grows to five members*. Trends Immunol, 2003. **24**(6): p. 286-90.
9. Takeda, K. and S. Akira, *TLR signaling pathways*. Semin Immunol, 2004. **16**(1): p. 3-9.
10. Takeuchi, O. and S. Akira, *Signaling pathways activated by microorganisms*. Curr Opin Cell Biol, 2007. **19**(2): p. 185-91.
11. Yoneyama, M., et al., *The RNA helicase RIG-I has an essential function in double-stranded RNA-induced innate antiviral responses*. Nat Immunol, 2004. **5**(7): p. 730-7.
12. Saito, T., et al., *Regulation of innate antiviral defenses through a shared repressor domain in RIG-I and LGP2*. Proc Natl Acad Sci U S A, 2007. **104**(2): p. 582-7.
13. Gack, M.U., et al., *TRIM25 RING-finger E3 ubiquitin ligase is essential for RIG-I-mediated antiviral activity*. Nature, 2007. **446**(7138): p. 916-920.
14. Kawai, T., et al., *IPS-1, an adaptor triggering RIG-I- and Mda5-mediated type I interferon induction*. Nat Immunol, 2005. **6**(10): p. 981-8.
15. Seth, R.B., et al., *Identification and characterization of MAVS, a mitochondrial antiviral signaling protein that activates NF-kappaB and IRF 3*. Cell, 2005. **122**(5): p. 669-82.
16. Xu, L.G., et al., *VISA is an adapter protein required for virus-triggered IFN-beta signaling*. Mol Cell, 2005. **19**(6): p. 727-40.

## References

---

17. Meylan, E., et al., *Cardif is an adaptor protein in the RIG-I antiviral pathway and is targeted by hepatitis C virus*. *Nature*, 2005. **437**(7062): p. 1167-72.
18. Yoneyama, M. and T. Fujita, *RIG-I family RNA helicases: Cytoplasmic sensor for antiviral innate immunity*. *Cytokine Growth Factor Rev*, 2007. **18**(5-6): p. 545-51.
19. Yoneyama, M., et al., *Shared and unique functions of the DExD/H-box helicases RIG-I, MDA5, and LGP2 in antiviral innate immunity*. *J Immunol*, 2005. **175**(5): p. 2851-8.
20. Kato, H., et al., *Differential roles of MDA5 and RIG-I helicases in the recognition of RNA viruses*. *Nature*, 2006. **441**(7089): p. 101-5.
21. Pichlmair, A., et al., *RIG-I-mediated antiviral responses to single-stranded RNA bearing 5'-phosphates*. *Science*, 2006. **314**(5801): p. 997-1001.
22. Andrejeva, J., et al., *The V proteins of paramyxoviruses bind the IFN-inducible RNA helicase, mda-5, and inhibit its activation of the IFN-beta promoter*. *Proc Natl Acad Sci U S A*, 2004. **101**(49): p. 17264-9.
23. Meylan, E. and J. Tschopp, *Toll-like receptors and RNA helicases: two parallel ways to trigger antiviral responses*. *Mol Cell*, 2006. **22**(5): p. 561-9.
24. Stetson, D.B. and R. Medzhitov, *Recognition of cytosolic DNA activates an IRF3-dependent innate immune response*. *Immunity*, 2006. **24**(1): p. 93-103.
25. Ishii, K.J., et al., *A Toll-like receptor-independent antiviral response induced by double-stranded B-form DNA*. *Nat Immunol*, 2006. **7**(1): p. 40-8.
26. Takaoka, A., et al., *DAI (DLM-1/ZBP1) is a cytosolic DNA sensor and an activator of innate immune response*. *Nature*, 2007. **448**(7152): p. 501-5.
27. Sanjuan, M.A., et al., *CpG-induced tyrosine phosphorylation occurs via a TLR9-independent mechanism and is required for cytokine secretion*. *J Cell Biol*, 2006. **172**(7): p. 1057-68.
28. Tusher, V.G., R. Tibshirani, and G. Chu, *Significance analysis of microarrays applied to the ionizing radiation response*. *Proc Natl Acad Sci U S A*, 2001. **98**(9): p. 5116-21.
29. Livak, K.J. and T.D. Schmittgen, *Analysis of relative gene expression data using real-time quantitative PCR and the 2<sup>(-Delta Delta C(T))</sup> Method*. *Methods*, 2001. **25**(4): p. 402-8.
30. Burckstummer, T., et al., *An efficient tandem affinity purification procedure for interaction proteomics in mammalian cells*. *Nat Methods*, 2006. **3**(12): p. 1013-9.



31. Dietzl, G., et al., *A genome-wide transgenic RNAi library for conditional gene inactivation in Drosophila*. *Nature*, 2007. **448**(7150): p. 151-6.

## 9 Abbreviations

<b>ANOVA</b>	Analysis of variance	<b>IRAK-4</b>	IL-1R-associated kinase 4
<b>AP-1</b>	Activator protein 1	<b>IRFs</b>	Interferon regulatory factors
<b>APS</b>	Ammonium persulfate	<b>ISGs</b>	Interferon stimulated genes
<b>BSA</b>	Bovine serum albumin	<b>ISRE</b>	IFN stimulated response element
<b>CARD domain</b>	Caspase recruitment domain	<b>JAK</b>	Janus kinase
<b>Cardif</b>	CARD adaptor inducing IFN $\beta$	<b>JNK</b>	Jun N-terminal kinase
<b>cDC</b>	conventional dendritic cell	<b>LCMV</b>	Lymphocytic choriomeningitis virus
<b>cDNA</b>	complementary DNA	<b>LPS</b>	Lipopolysaccharide
<b>CpG-ODN</b>	cytidine-guanosine dinucleotide containing oligo-deoxynucleotide	<b>LRR</b>	Leucine-rich repeat
<b>DAI</b>	DNA-dependent activator of IFN-regulatory factors	<b>MAVS</b>	Mitochondrial antiviral signaling protein
<b>DAPI</b>	4',6-diamidino-2-phenylindole	<b>MCMV</b>	Murine cytomegalovirus
<b>dATP</b>	Deoxyadenosine triphosphate	<b>MDA-5</b>	Melanoma differentiation-associated gene 5
<b>DMEM</b>	Dulbecco's Modified Eagle Medium	<b>MEFs</b>	Mouse embryonic fibroblasts
<b>DNA</b>	Deoxyribonucleic acid	<b>MyD88</b>	Myeloid differentiation primary response gene 88
<b>DNAse</b>	Deoxyribonuclease	<b>NDV</b>	Newcastle disease virus
<b>dNTP</b>	Deoxynucleotide triphosphate	<b>NF-<math>\kappa</math>B</b>	Nuclear factor $\kappa$ B
<b>dsDNA</b>	double-stranded DNA	<b>NK cells</b>	Natural killer cells
<b>dsRNA</b>	double-stranded RNA	<b>NTAP</b>	N-terminally TAP tagged
<b>dUTP-biotin</b>	Biotinylated deoxyuracil triphosphate	<b>Oas3</b>	2'-5' oligoadenylate synthetase
<b><i>E. coli</i></b>	<i>Escherichia coli</i>	<b>oligo(dT)<sub>18</sub></b>	Single-stranded 18-mer oligo- deoxythymidine
<b>EDTA</b>	Ethylenediamine-tetraacetic acid	<b>PAGE</b>	Polyacrylamide gel electrophoresis
<b>eIF2<math>\alpha</math></b>	Eukaryotic translation initiation factor 2 $\alpha$	<b>PAMP</b>	Pathogen-associated molecular pattern
<b>ER</b>	Endoplasmic reticulum	<b>PBS</b>	Phosphate-buffered saline
<b>FACS</b>	Fluorescence-activated cell sorting	<b>PCR</b>	Polymerase chain reaction
<b>FCS</b>	Fetal calf serum	<b>pDC</b>	plasmacytoid dendritic cell
<b>GFP</b>	Green fluorescent protein	<b>Pkr</b>	Double-stranded RNA-activated protein kinase
<b>HA</b>	Hemagglutinin	<b>PMSF</b>	Phenylmethanesulfonyl fluoride
<b>HCV</b>	Hepatitis C virus	<b>polydA:dT</b>	Polydeoxyadenylic-thymidylic acid, ds homopolymer
<b>HEK293</b>	Human embryonic kidney cell line	<b>polydAdT:dTdA</b>	Poly(deoxyadenylic-thymidylic) acid, ds alternating copolymer
<b>HSV-1</b>	Herpes simplex virus- 1	<b>polyI:C</b>	Polyinosinic-polycytidylic acid, double-stranded homopolymer
<b>IFN</b>	interferon		
<b>IFNAR</b>	IFN $\alpha/\beta$ receptor		
<b>IgG</b>	Immunoglobulin G		
<b>IKK</b>	I $\kappa$ B kinase		
<b>IPS-1</b>	IFN $\beta$ promoter stimulator 1		

## Abbreviations

---

<b>Prkra</b>	PKR-activator A	<b>STAT</b>	Signal transducer and activator of transcription
<b>PRR</b>	Pattern recognition receptor		
<b>RAW264.7</b>	Murine macrophage cell line	<b>TAP</b>	Tandem affinity purification
<b>RD</b>	Repressor domain	<b>TBK1</b>	TANK binding kinase 1
<b>RIG-I</b>	Retinoic acid-inducible gene I	<b>TEMED</b>	N,N,N',N'- Tetramethylethylenediamine
<b>RLHs</b>	RIG-I-like helicases		
<b>RNA</b>	Ribonucleic acid	<b>TEV</b>	Tobacco etch virus
<b>RNAi</b>	RNA interference	<b>TIR domain</b>	Toll/interleukin-1 receptor domain
<b>rRNA</b>	Ribosomal RNA	<b>TLCK</b>	Na-Tosyl-Lys-chloromethylketone
<b>RT</b>	Reverse transcription	<b>TLR</b>	Toll-like receptor
<b>SAM</b>	Significance analysis of microarrays	<b>TNF<math>\alpha</math></b>	Tumor necrosis factor $\alpha$
<b>SBP</b>	Streptavidin binding protein	<b>TPCK</b>	Tosyl-L- phenylalanin- chloromethylketon
<b>SDS</b>	Sodium dodecyl sulfate	<b>TRAF6</b>	TNFR-associated factor-6
<b>siRNA</b>	Small interfering RNA	<b>TRIF</b>	TIR domain containing adaptor inducing interferon-beta
<b>SMART</b>	Simple modular architecture research tool	<b>TRIM25</b>	Tripartite motif protein 25
<b>SSD</b>	Salmon sperm DNA	<b>Tris</b>	Tris(hydroxyamino)methane
<b>ssRNA</b>	Single-stranded RNA	<b>VISA</b>	Virus-induced signaling adaptor
		<b>VSV</b>	Vesicular stomatitis virus

# CURRICULUM VITAE

## Evelyn Dixit



Date of birth            November 23<sup>rd</sup>, 1976  
Citizenship            Austria

### EDUCATION

November 2006 –    Research Center for Molecular Medicine, Vienna, Austria  
                                 Laboratory of Dr. Giulio Superti-Furga,  
                                 Master's thesis: Functional proteomics and genomics of virus-induced  
                                 innate immunity pathways

October 2003 –      University of Vienna, Austria  
                                 Combined Bachelor's and Master's program for Molecular Biology  
                                 Areas of study: immunology, cell biology and neuroscience

1987 – 1998            Secondary and post-secondary education, Vienna, Austria  
                                 Graduation from high school cum laude in 1995  
                                 Graduation from biochemistry school magna cum laude in 1998

### WORK EXPERIENCE

August 2004 – October 2006

Student internships and part-time positions

---

- Laboratory of Dr. Erwin Wagner, Institute of Molecular Pathology, Vienna, Austria
- Laboratory of Dr. Barry Dickson, Institute for Molecular Biotechnology, Vienna, Austria
- Laboratory of Dr. Giulio Superti-Furga, Center for Molecular Medicine, Vienna, Austria
- Laboratory of Dr. Jan-Michael Peters, Institute of Molecular Pathology, Vienna, Austria

# CURRICULUM VITAE

## Evelyn Dixit

March 2002 – August 2003

SangStat Medical Corporation, Fremont, CA, USA

Research Associate II

---

Mechanism of action of RDP58 – effects of an anti-inflammatory peptide on cell signaling

- Optimization of electroporation and lipofection protocols for various cell lines and primary cells
- Investigation of transcription factor activation using luciferase reporter assays
- Determination of the phosphorylation status of p38, Erk1,2 and JNK1,2 MAPKs
- Establishment of cytokine profiles in response to cell-specific stimuli for a variety of cell types

February 1999 – February 2002

Baxter Healthcare, Orth/Donau, Austria

Research Technician

---

Recombinant proteins as a therapeutic option for hemophiliacs

- Molecular biology (PCR, standard cloning)
- Generation of stable cell lines
- Purification and characterization of recombinant proteins from cell culture supernatants
- Responsibility for GMP implementation within the department according to FDA and corporate guidelines

July 1998 – January 1999

University of Veterinary Medicine, Dept. of Biochemistry, Vienna, Austria

Technician

---

- Extraction of steroid hormones from biological sources and routine ELISA testing

# CURRICULUM VITAE

## Evelyn Dixit

### PUBLICATIONS

under maiden name of Evelyn Muhr

- [Topical application of a novel immunomodulatory peptide, RDP58, reduces skin inflammation in the phorbol ester-induced dermatitis model.](#)  
De Vry CG, Valdez M, Lazarov M, **Muhr E**, Buelow R, Fong T, Iyer S.  
J Invest Dermatol. 2005 Sep;125(3):473-81.
- [A fully recombinant partial prothrombin complex effectively bypasses fVIII in vitro and in vivo.](#)  
Himmelspach M, Richter G, **Muhr E**, Varadi K, Turecek PL, Dorner F, Schwarz HP, Schlokot U.  
Thromb Haemost. 2002 Dec;88(6):1003-11.

### SCHOLARSHIPS & AWARDS

- [Scholarship for special study achievements \(2007\)](#)  
For performance during the academic year 2005/06 awarded by the University of Vienna
- [Scholarship for special study achievements \(2006\)](#)  
For performance during the academic year 2004/05 awarded by the University of Vienna
- [Baxter Values in Action Award \(2000\)](#)  
For exemplary work and attitude  
A prize given to 50 out of 45,000 Baxter employees worldwide each year
- [Baxter Technical Award \(1999\)](#)  
For achievements as a group regarding the project "Recombinant FEIBA™: An Innovative Concept Redesigning the Coagulation Cascade"