

MASTERARBEIT

Regulation and role of RNA Pol II CTD phosphorylation in the transcription cycle of the Nos2 gene

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2 ZUSAMMENFASSUNG

Listeria monocytogenes ist eines der am besten charakterisierten und am meisten untersuchten intrazellulären Pathogene. Dieses Gram positive Bakterium wird oft verwendet, um das angeborene Immunsystem und die Abwehr gegen intrazelluläre Mikroben zu untersuchen. Es verursacht schwere Infektionen bei immunsupprimierten Individuen. Intravenös injizierte Bakterien in einem Mausmodell finden sich unverzüglich in der Milz und in der Leber wieder, und auf der zellulären Ebene gehören Makrophagen zu den ersten infizierten Zellen. *L. monocytogenes* ist in der Lage, aus dem Phagosom des infizierten Makrophagen zu entkommen, sich im Cytosol zu vermehren und auf benachbarte Zellen auszubreiten.

Unser Labor untersucht, die Reaktion des angeborenen Immunsystems auf Listerien-Infektionen, und wie die Effektoren der Immunabwehr reguliert werden. Viele antimikrobielle Gene in infizierten Zellen werden von NF κ B und dem Typ-I-interferon/ISGF3-Weg reguliert. Zu dieser Gruppe der koregulierten Gene gehört auch *Nos2*, das die induzierbare Stickstoffmonoxid-Synthase (iNOS) kodiert.

Mein Projekt konzentrierte sich auf Phosphorylierungsereignisse an der carboxyterminalen Domäne (CTD) der RNA-Polymerase II (RNA Pol II) während des Transkriptionszykluses des *Nos2* Gens. Die CTD ist aus einer sich oftmals wiederholenden siebenstelligen Aminosäuresequenz aufgebaut. Modifikationen, im Speziellen Phosphorylierungen dieser Domäne sind unerlässlich für RNA-Pol II Prozessivität sowie die Assemblierung von Proteinen, die an der pre-mRNA-Prozessierung beteiligt sind. Die beiden Cyclin-abhängigen Kinasen Cdk7 und Cdk9 phosphorylieren die CTD bei jeweils Serin 2 und Serin 5 während der Transkriptionsinitiation. Allerdings gibt es wenige Informationen über Veränderungen und Funktion dieser Modifikationen während der Elongation. Darüber hinaus ist bekannt, dass auch Serin 7 von Cdk7 und -9 phosphoryliert wird. Es ist aber unklar, ob dies bei der Initiation, Elongation oder Termination der Transkription eine Rolle spielt.

Meine Studie zeigt, dass die Phosphorylierung von Serin 2 in BMDMs, stimuliert mit *Listeria*-abgeleiteten Signalen Richtung 3' Ende des *Nos2* Gens stark ansteigt. Im Gegensatz dazu ist Serin 5 stark am Transkriptionsstart (TSS) phosphoryliert, dies stimmt mit seiner bekannten Rolle in der Trennung der RNA Pol II vom Promotor überein. Interessanterweise bleibt das Niveau bis zum 3'-Ende des *NOS2* Gens konstant. Außerdem konnte ich zeigen, dass Serin 7 Phosphorylierung am TSS signifikant erhöht ist aber unmittelbar danach stark abnimmt. Es könnte eine wichtige Rolle bei der Initiation, der Übergang zur Elongation oder bei der Rekrutierung von Spleißfaktoren spielen. Der zweite Teil der Arbeit beschreibt eine Klonierungsstrategie zur Schaffung

eines Fusionsgenes aus der p50-Untereinheit von NFκB und einer der Cyclin-abhängigen Kinasen, Cdk7 oder Cdk9. In der Folge könnten mit diesen Fusionskonstrukten Untersuchungen zur Rolle von NFκB bei der Rekrutierung von CTD-Kinasen an den Promoter des NOS2 Gens durchgeführt werden.

3 ABSTRACT

Listeria monocytogenes is one of the best characterized and widely studied intracellular pathogens. This Gram-positive bacterium is often used to investigate the innate immune system and the defense against intracellular microbes. It causes severe infections in immune-compromised individuals. In a mouse model, intravenously injected bacteria are found in the spleen and liver almost instantly and at the cellular level, macrophages are among the first cells to be infected. *L. monocytogenes* is able to escape and replicate in the cytosol of infected macrophages and to spread to neighbouring cells.

Our lab investigates how the innate immune system responds to *Listeria* infection and how the effectors of host defense are regulated. Many antimicrobial genes in infected cells are regulated by NFkB and the type I interferon/ISGF3 pathways. Among these the *Nos2* gene, which encodes inducible nitric oxide synthase (iNOS), is co-regulated by these two pathways.

My project focused on phosphorylation events at the carboxy-terminal domain (CTD) of RNA polymerase II (RNA Pol II) during the transcription cycle of the *Nos2* gene. The CTD is formed by a large number of amino acid heptarepeats. Modification, in particular phosphorylation is required for RNA Pol II processivity as well as the association of proteins involved in pre mRNA processing. The two cyclin-dependent kinases Cdk7 and Cdk9 phosphorylate the CTD heptarepeats at, respectively, serine 5 and serine 2 during transcriptional initiation. However, there is little information about the fate of these modifications during elongation. In addition, serine 7 is known to be modified by phosphorylation as well, but it is unclear whether this occurs predominantly during initiation, elongation or even termination of transcription.

My study shows that the phosphorylation level of serine 2 in BMDMs stimulated with *Listeria*-derived signals increases towards the 3' end of the *Nos2* gene. In contrast, serine 5 is strongly phosphorylated at the transcription start (TSS), in line with its known role in promoter clearance, but its level stays constant up to the 3' end of the *Nos2* gene. My results also show that serine 7 phosphorylation is highest at the TSS but decreases strongly during transcription into the gene body. It may play a role in initiation, the transition to elongation, or in splicing factor recruitment.

The second part of my thesis describes a cloning strategy aimed at creating fusion genes of the NFkB subunit p50 with either of the cyclin dependent kinases Cdk7 or Cdk9. In subsequent studies these fusion constructs will allow examining the role of NFkB in recruiting CTD kinases to the *Nos2* promoter.

4 INTRODUCTION

4.1 *LISTERIA MONOCYTOGENES AND THE INNATE IMMUNE SYSTEM*

4.1.1 *Listeria monocytogenes*

Listeria monocytogenes (*L. monocytogenes*) is a ubiquitous, gram-positive, intracellular pathogen, which is a causative agent of foodborne Listeriosis. It can grow and reproduce inside the host's cells and is one of the most virulent food-borne pathogens with 20 to 30 percent of clinical infections resulting in death (Farber J.M., 1991).

L. monocytogenes is usually ingested with raw, contaminated food. This bacterial infection is a major threat to pregnant women and immune-compromised persons with symptoms of fetal abortion, neonatal death, sepsis and meningitis. (Farber J.M., 1991) (Ramaswamy V., 2006).

While *L. monocytogenes* multiplies with short generation times and remarkable resistance to low temperatures on soil or contaminated food, replication upon infection of mammalian hosts is predominantly inside cells. Macrophages are entered via phagocytosis, whereas parenchymal or epithelial cells are forced into uptake by *Listeria* virulence proteins (Todar K., 2012). To maintain this intracellular lifestyle, the bacterium has a handful of mechanisms to take advantage of host processes to grow and spread. Listeriolysin O (LLO), a pore forming protein helps the bacteria to escape vacuoles of the host. Once in the cytosol, the protein actin assembly-inducing protein ActA helps *L. monocytogenes* to spread to neighbouring cells (Portnoy D.A., 2002).

Easy handling and its intracellular life style have turned *L. monocytogenes* into a popular and well-studied model organism to investigate basic aspects of cell biology, intracellular pathogenesis, and innate and adaptive immunity. The microbe has been instrumental in studying the principles of cell-mediated immunity against intracellular pathogens (Kernbauer E., 2012), (Portnoy D.A., 2002).

In mice, intravenously injected bacteria are rapidly cleared from the bloodstream and predominantly infect the spleen and liver. (Serbina N.V., 2003). On a cellular level, *L. monocytogenes* first appear in macrophages and then spread to hepatocytes in the liver. In the spleen both marginal zone macrophages and myeloid dendritic cells are important for the transfer of bacteria to the white pulp. The bacteria stimulate a cell-mediated immune response that includes the production of TNF α , interferons, macrophage activating factors and a cytotoxic T cell response (Cossart P., 2011), (K., 2014).

4.1.2 Adherence, invasion and cell spreading

L. monocytogenes is first recognized by surface receptors of macrophages, like TLRs, which leads to the formation of endo- or phagosomal compartments containing the detected pathogen. The bacterium is able to survive the phagocytosis-associated respiratory burst due to its ability to produce catalase and superoxide dismutase (Todar K., 2012). Subsequently the bacterium escapes from the phagosome before phagolysosome fusion occurs, mediated by the toxin LLO, which also acts as a haemolysin (K., 2014), (Pamer E.G., 2004).

For the intracellular life cycle of *L. monocytogenes*, additional virulence mechanisms are necessary: *L. monocytogenes* spreads directly from cell to cell without being exposed to the extracellular environment. Essential for this is ActA, a protein encoded and used by *L. monocytogenes* to move through a mammalian host cell. ActA promotes the polymerization of actin on the bacterial surface. Surrounded by actin filaments, the bacteria can survive and multiply. The growing actin filaments are like a driving force pushing the bacteria across the cytoplasm until they reach the surface. Then, the host cell is induced to form slim, long protrusions containing living *L. monocytogenes*. Those cellular projections are engulfed by neighboring cells, including non-professional phagocytes such as parenchymal cells. With this mechanism, a direct cell-to-cell spread of *L. monocytogenes* in an infected tissue may occur without an extracellular stage (Todar K., 2012), (Farber J.M., 1991), (Kocks C., 1992).

4.1.3 Innate immune response to *L. monocytogenes*- a small overview

In the case of *L. monocytogenes*, both, innate and adaptive immune defenses play a crucial role in controlling the infection of the intracellular bacterium. The outcome of the infection depends strongly on the effectiveness of the fine-tuned host immune system.

Innate immune defense against *L. monocytogenes* shows a stepwise activation with sequential triggering of receptors and downstream signalling pathways. This network is very complex, scientists have started to slowly decipher it, though. (Pamer E.G., 2004).

4.1.4 Important inflammatory cytokines in *L. monocytogenes* infection

One of the most important cytokines to fight *L. monocytogenes* infection is IFN γ . The type II interferon is induced by IL-12 and IL-18, which are produced by infected macrophages. IFN γ is produced by innate lymphocytes such as NK cells and by dendritic cells. It activates macrophages and neutrophils to kill *L. monocytogenes* by increased lysosome activity, generation of reactive oxygen species and nitric oxide (NO) (Zenewicz L.A., 2007), (Seki E., 2002).

Like IFN γ , type I interferons (IFN-I, mainly IFN α/β) are produced during *L. monocytogenes* infection (Zenewicz L.A., 2007), (Havell E.A., 1986). These cytokines are best known for their crucial role in the innate antiviral response. Whereas IFN-I protect from viral infection, mice deficient in either type I interferon receptor (IFNAR) or in IFN regulatory factor (IRF3) have decreased resistance against *L. monocytogenes* (O'Connell R.M., 2004) (Pietras E.M., 2006).

4.1.5 Response of phagocytes

Neutrophils are rapidly recruited after infection by cytokine IL-6 and other factors and they secrete chemokines such as CCLs and MCP-1, which guide macrophages to the site of infection. When it comes to innate immune response to *L. monocytogenes*, macrophages were always in the focus of interest in immunology, due to the fact that they are one of the first cells coming in touch with *L. monocytogenes* and that the bacterium replicates primarily within them (Pamer E.G., 2004) (Zenewicz L.A., 2007). In response to infection, macrophages secrete TNF α and IL-12 (Havell E.A., 1987). These cytokines stimulate NK cells to produce IFN γ , leading to macrophages activation as described above (Zenewicz L.A., 2007).

4.1.6 Clearance of *L. monocytogenes* from infected mice

During a typical *L. monocytogenes* infection, bacteria proliferate in vivo for 2 to 3 days and then, upon induction of an antigen-specific CD8⁺ T cell response, are cleared from the spleen and liver (Busch D.H., 1999).

In infected macrophages *L. monocytogenes* infection triggers two distinct, temporally separated waves of gene induction. The earlier wave induces genes that are dependent on TLR signalling from the plasma membrane and from endosomal compartments. The NF κ B pathway is very dominant in this early response. It is not reliant on the invasion of cells by living bacteria. To activate this pathway in in vitro experiments, it is possible to use heat-killed *Listeria*, which present the relevant microbe-associated molecular patterns. In contrast, the second wave of induced genes is dependent on *L. monocytogenes* escaping from the phagolysosome. These genes include type I interferons, as well as interferon-induced genes (ISG) (Zenewicz L.A., 2007).

LLO deficient *L. monocytogenes* or heat-killed *Listeria* are not able to leave the phagosome and trigger neither IFN β induction nor IFN-I receptor signalling through the JAK/STAT pathway (Stockinger et al. 2002) (Mary O'Riordan, 2002).

4.1.7 Toll-like receptor signalling and activation of NF κ B pathway

Toll-like receptors (TLRs) are one of the mechanisms the innate immune system uses to recognize pathogens. They recognize specific microbe/pathogen-associated molecular patterns (interchangeably called MAMPS or PAMPS) (Akira S., 2004). These receptors are divided in 2 groups, depending on their cellular localization and the PAMP ligands they bind. On one hand there are TLR1, TLR2, TLR4, TLR5, TLR6 and TLR11, which mainly recognize microbial membrane components (lipids, lipoproteins and proteins) and are expressed on the cell surface. Among these TLR2 is involved in the recognition of a wide range of PAMPs, including some presented by *L. monocytogenes* (Torres D., 2004).

On the other hand there are TLRs (TLR3, TLR7, TLR8, TLR9 and TLR13) which are only expressed in intracellular vesicles e.g. ER, endosomes or endolysosomes and recognize microbial nucleic acids (Kawai T., 2010). Endosomal TLR may contribute to the innate response against *L. monocytogenes* as well (Biondo S.C., 2012). After sensing PAMPs, a variety of signalling pathways are activated, classified as Myd88 and/or TRIF-dependent according to the use of the adapter proteins linking them to TLRs (Torres D., 2004), (Kawai T., 2010). These cascades consequently trigger several downstream events like the activation of MAPK and nuclear transcription factors, e.g. NF κ B, which induce the production of pro-inflammatory cytokines and antigen-presentation-related genes. Collectively these genes and their products form the innate immune response and stimulate the emergence of adaptive immunity (Takeuchi O., 2010), (Wang C., 2013), (Akira S., 2004), (Zenewicz L.A., 2007). They include genes required for the production of microbicide effector mechanisms. An important example is nitric oxide (NO), produced by the inducible nitric oxide synthase (iNOS) (Kawai T., 2010) (Wink D.A., 2010) (Kleinert H., 2004).

Transcription factor NF κ B is of paramount importance for the early cellular response to invading pathogens, including *L. monocytogenes*. NF κ B activation occurs when its inhibitory subunit, I κ B, is phosphorylated and subsequently degraded by the I κ B kinase (IKK) complex that is formed by NEMO, IKK α and IKK β subunits. Both TRIF and MyD88-dependent TLR signalling activates IKK activity and causes NF κ B activation. In the case of *L. monocytogenes* early NF κ B activation occurs through MyD88-dependent signalling, most likely downstream of TLR2 (S. Stockinger and T. Decker, unpublished results). Consistently, MyD88-deficient mice are more susceptible to *L. monocytogenes* infection (Seki E., 2002).

4.1.8 Induction of type I interferon

Interferons are cytokines produced and released by host cells in response to the presence of all classes of pathogens or to tumour cells. They ensure communication between cells to trigger the protective defences of the immune system. Interferons are divided into three types, depending on their cell surface receptor. Type I IFNs bind to the IFN α receptor (IFNAR), while type II IFNs bind to the IFN- γ receptor (IFNGR) (Decker T., 2005). Type III IFNs IFN- λ or interleukin-28/29 exert their action through a receptor complex distinct from the type I IFNs but they show IFN-like activities (Ank N., 2006). While type I and II interferons (IFNs α β and γ) generally complement each other in the emergence of protective immunity, they may have opposing effects on immune resistance to pathogenic bacteria in some cases. Importantly IFN γ plays a protective role in infections with *L. monocytogenes* or *Mycobacterium tuberculosis*, whereas IFN α and - β intensify these infections and decrease innate resistance (Rayamajhi M., 2010).

Contrasting the NF κ B pathway, type I IFN production in response to *L.monocytogenes* infection

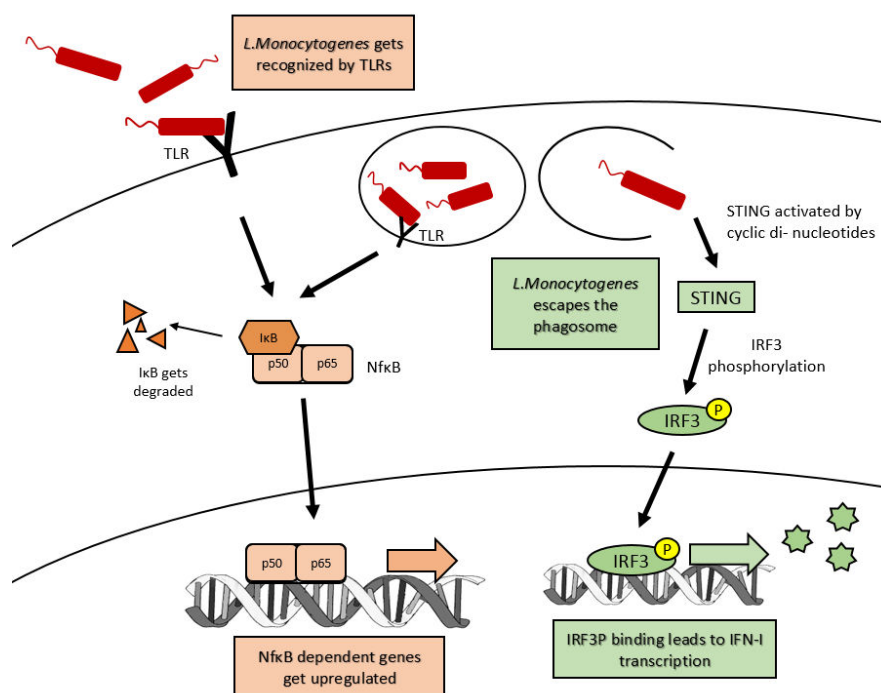


Figure 1. Recognition of *L. monocytogenes* by an infected macrophage. TLR recognize *L. monocytogenes* at the cells surface and in the phagosome, activating NF κ B, which leads to up-regulation of NF κ B dependent genes. When the bacteria escape the phagosome, IRF3 gets phosphorylated and induces IFN-I transcription.

is the result of a cytoplasmic signalling cascade. Recent findings suggest an important role for cyclic dinucleotides, either released by the bacteria or synthesized when the cellular cyclic GMP-AMP synthase (cGAS) binds to bacterial DNA (Witte C.E., 2012), (Sauer J.D., 2011). Cyclic dinucleotides interact with their sensor protein STING, which leads to activation of a downstream signalling cascade involving TBK1 and IRF3. IRF3 is phosphorylated by TBK1 (tank-binding kinase 1) and migrates to the nucleus, where it leads to the expression of IFN β (Burdette D.L., 2011),

(O'Connell R.M., 2004), (O'Connell R.M., 2004), (Decker T., 2005). Surprisingly, type I IFN production increases the bacterial burden of infected mice (Zenewicz L.A., 2007). In the absence of type I interferon signalling due to the lack of IFNAR receptors or IRF3, mice are more resistant to *L. monocytogenes*. (Auerbuch V., 2004), (O'Connell R.M., 2004) (Stockinger et al. 2009).

4.1.9 Activation of the JAK/STAT pathway by type I IFN

JAK/STAT (Janus kinase/ signal transducer and activator of transcription) pathways transmit extracellular polypeptide signals through transmembrane-receptors directly to promoters of target genes in the nucleus. In mammals, this pathway is mainly a mechanism to transduce a wide range of growth factors and cytokines, which play a crucial role in the regulation of the immune system (Aaronson D.S., 2002) (Rawlings J.S., 2004).

The three main components of the JAK/STAT signalling pathway are a cytokine receptor, the Janus kinase (JAK) and Signal Transducer and Activator of Transcription (STAT).

The Jak family comprises 4 members:

- Janus kinase 1 (JAK1)
- Janus kinase 2 (JAK2)
- Janus kinase 3 (JAK3)
- Tyrosine kinase 2 (TYK2)

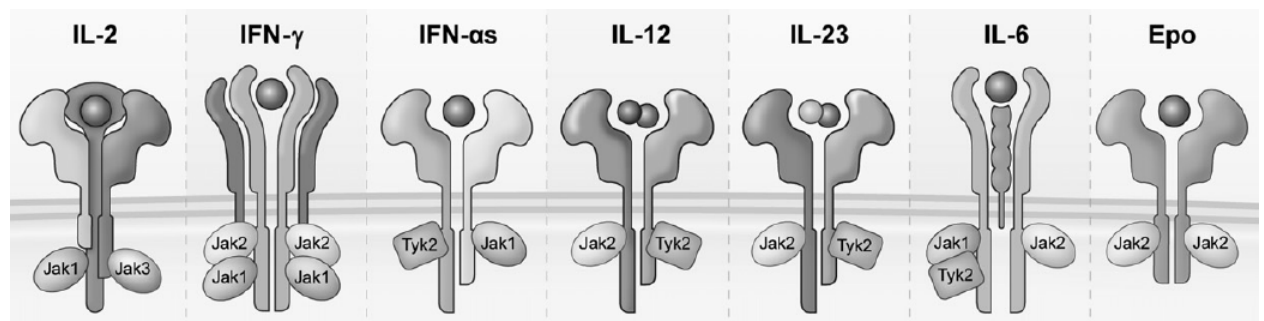


Figure 2. Different cytokine receptors and their interacting JAKs (O'Shea J.J., 2012)

Of these, JAK1 and JAK2 are involved in type II interferon (interferon-gamma) signalling, JAK1 and TYK2 are involved in type I interferon signalling (Kisseleva T., 2002), (Aaronson D.S., 2002), (Stark G.R., 2012).

In mammals, 7 different STATs (STAT1-4, STAT5A and STAT5B, STAT6) are found. They are different in tissue distribution and their roles in cytokine signalling. STAT proteins are inactive in unstimulated cells and are localized in the cytoplasm. The first step of this signalling cascade is the extracellular binding of the ligand to the receptor. In the case of cytokine receptors (e.g. IFNAR, IFGR or IL6R), Jaks are associated with the intracellular domain which contains conserved

box1 and box2 motifs. Ligand binding activates the Jaks by autophosphorylation or crossphosphorylation, followed by phosphorylation of receptor tyrosines. These phosphorylated residues are STAT docking sites. When STATs are bound, phosphorylation is the consequence, which leads to their homo- or hetero-dimerization (Heinrich P.C., 2003), (Rawlings J.S., 2004).

Different STATs are activated by distinct groups of cytokines:

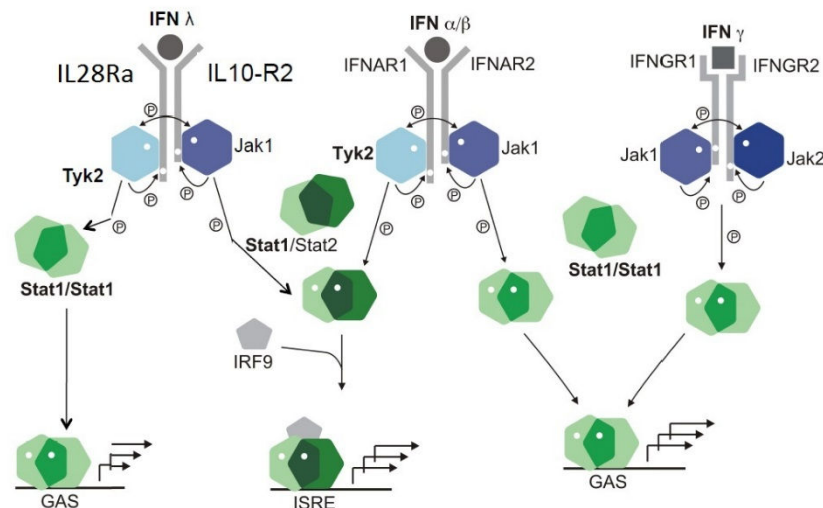


Figure 3. JAK/ STAT signalling in response to interferons (modified from <http://www.jak-stat.at/index.php?id=26> group decker)

The dimerized, activated STATs translocate to the nucleus and bind to promoter regions. STAT1 homodimers bind to a DNA sequence called GAS (gamma activated site) and STAT1, STAT2 and IRF9 (ISGF3) complexes bind to ISRE (Interferon-Stimulated Response Element) sites to drive gene expression (Aaronson D.S., 2002), (Kisseleva T., 2002), (Decker T., 2005).

Relevant for the project of my thesis is JAK/STAT activation by type-I interferons during *L. monocytogenes* infection. The secreted IFN β binds to IFNAR receptor, a receptor which binds type I interferons including IFN α and IFN β . It is a receptor composed of two subunits referred to as IFNAR1 and IFNAR2. They are constantly associated with the Janus tyrosine kinases Tyk2 and JAK1. If IFN β binds to the IFNAR receptor, JAKs phosphorylate each other and the intracellular part of the receptor, followed by the recruitment of STAT1 and STAT2 and binding to the phosphorytyrosine- based motif. A single tyrosine of the STATs is phosphorylated, which leads to a dissociation and dimerization of these two factors. The heterodimer associates with IRF9 and builds the interferon stimulated gene factor 3 (ISGF3). ISGF3 complex translocates to the nucleus and binds to ISRE sequence, found in the promoter of type I interferon stimulated genes (ISGs) (Kisseleva T., 2002) (Decker T., 2005).

4.1.10 Genes expressed in macrophages after *L. monocytogenes* infection

After the infection by *L. monocytogenes*, NFκB and type I interferon pathways are activated, to induce gene expression:

- 1) NFκB dependent pathway -> NFκB dependent genes are upregulated
- 2) JAK/STAT pathway activated -> type I IFN-dependent genes (ISG) are expressed

After *L. monocytogenes* infection, there is also a third group of genes, which is co-regulated by NFκB and type I interferon/ISGF3 pathways. An important member of this group, *Nos2*, is expressed in macrophages after *L. monocytogenes* infection.

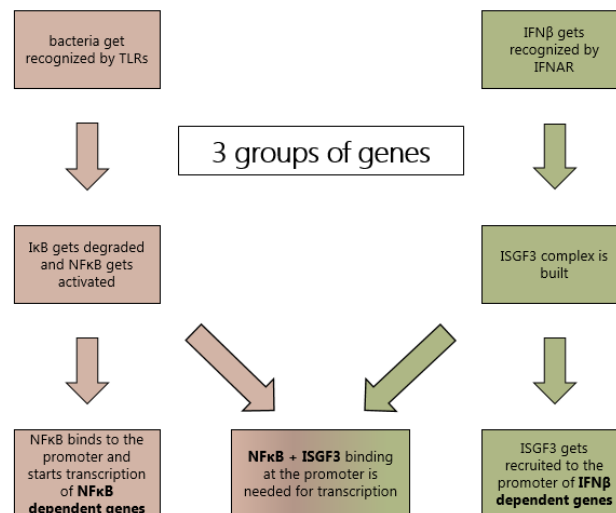


Figure 4. 3 important groups of genes that play a crucial role in immune defense and are activated in macrophages after *L. monocytogenes* infection.

Our group previously demonstrated that both stimuli are necessary for the expression of iNOS. On the one hand, ISGF3 is essential for TFIID and RNA polymerase II (RNA Pol II) recruitment. On the other hand, NFκB contributes to RNA Pol II recruitment and is essential for the binding of TFIID, pTEFb and the establishment of histone modifications like histone 3 trimethylation at lysine 4 (H3K4me3) or the acetylation of histones 3 and 4 (Hac) (Wienerroither, unpublished data, 2013/2014), (Farlik M., 2010).

4.2 *iNOS* AND THE *Nos2* GENE

The *Nos2* gene encodes a nitric oxide synthase, which is inducible by LPS and certain cytokines. NO is a reactive free radical, which acts as a biologic mediator in several processes, including neurotransmission, antimicrobial and antitumoral activities. (geneID, 2014), (Fang F.C., 2004), (Utainsincharoen P., 2004), (Bogdan C., 2001). Inducible nitric oxide synthase (iNOS), along with neuronal nitric oxide synthase (nNOS) and endothelial nitric oxide synthase (eNOS), catalyse the generation of nitric oxide from L-arginine and molecular oxygen.

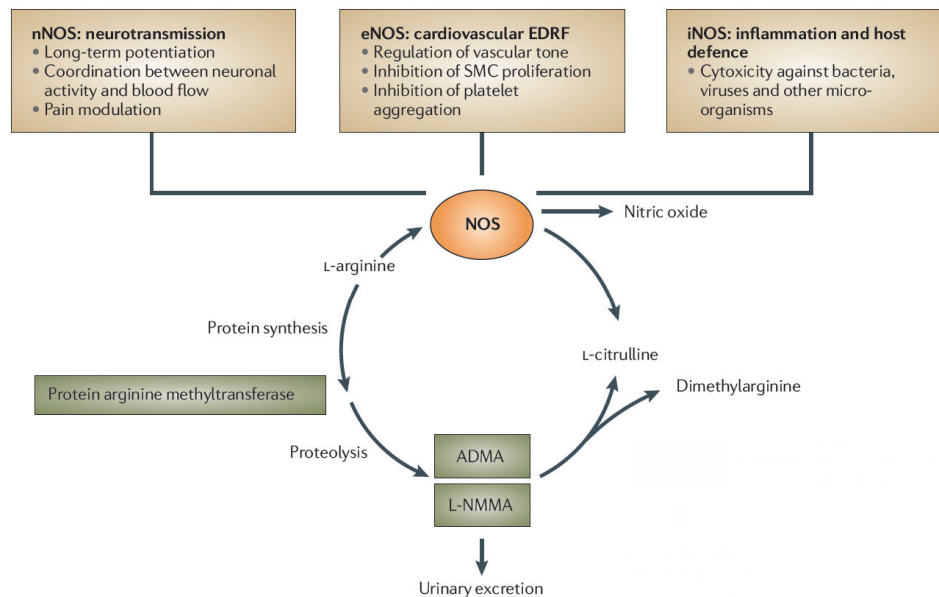


Figure 5. The ADMA–NOS pathway. Mammalian nitric oxide (NO) synthesis is catalysed by three isoforms of nitric oxide synthase (NOS) that have different tissue distributions: neuronal NOS (nNOS), endothelial NOS (eNOS) and inducible NOS (iNOS). L-arginine is the substrate for all three isoforms of NOS (James Leiper, 2011)

The translation of *Nos2* gene and the iNOS expression is regulated positively by transcription factors including NFκB, IFNγ and IFNβ (signalling through the JAK-STAT cascade) and HIF-1. TGF-β and IL-4, -10 and -13 inhibit the expression (genewiki, 2013) (genecards, 2013).

In a C57BL/6 genetic background iNOS deficiency does not reduce innate resistance to *L. monocytogenes* infection (Michael U Shiloh, 1999). In fact, one study suggested that iNOS contributes to pathogen spread (Cole C., 2012). Other studies showed that NO production reduces the survival of infected cells (Stockinger S., 2008), (Stockinger S., 2002), (O’Riordan M., 2002). Taken together these findings emphasize the need for a tight control of iNOS expression. Consistently, expression of iNOS in *L. monocytogenes*- infected macrophages is under stringent regulation of two signalling pathways: on the one hand NFκB is necessary, as it binds to the proximal promoter of *Nos2*, on the other hand binding of the ISGF3 complex at the distal promoter after IFNβ stimulation is essential for iNOS expression.

4.3 SERINE PHOSPHORYLATION AT THE CTD OF RNA POLYMERASE II

4.3.1 RNA Pol II and its carboxy-terminal domain

Regulation of transcription and the synthesis of mRNA is one of the fundamental processes of life. In eukaryotic organisms, RNA Pol II and a large number of transcription factors are responsible for these functions. The RNA Pol II is built up by 10- 12 subunits (12 in human and yeast) (Hahn S., 2005). In mammals Pol II subunits can be divided into 3 groups: subunits of the core domain: Rpb1, 2, 3, and 11, subunits shared between all three nuclear polymerases: Rpb5, 6, 8, 10, and 12, and subunits specific to Pol II but not obligatory for transcription elongation: Rpb4, 7, and 9 (Hahn S., 2005).

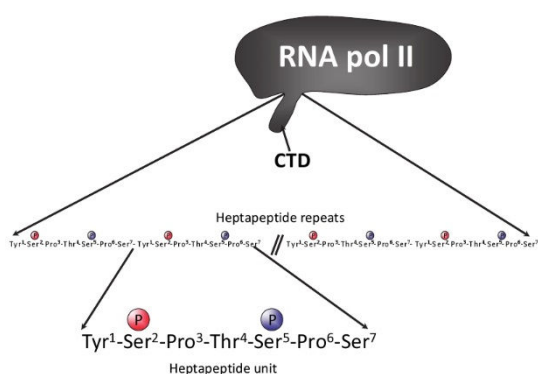


Figure 6: RNA polymerase II cyrboxyterminal domain consists of heptapeptide repeats (Hall J.A., 2011).

The biggest subunit of RNA Pol II, Rpb1, has a carboxy-terminal domain (CTD), which undergoes modification during the transcription cycle. This C-terminal heptapeptide region typically consists of up to 52 repeats of the sequence Tyr-Ser-Pro-Thr-Ser-Pro-Ser. These heptapeptide repeats are highly conserved and also exist in other organisms. In yeast, for example, the amino acid sequence is the same, but the CTD is only built up by 26 repeats (Morris D.P., 2005). The more complex

the organism, the more repetitions, thus more opportunities for modifications are given.

In recent years, there has been an increasing interest in the dynamic modifications of the carboxy-terminal domain during gene transcription. It coordinates the recruitment of factors that play a crucial role in transcription regulation and pre-mRNA processing (Heidemann M., 2012) (Hsin J.P., 2012). Partial deletion of the CTD can be tolerated up to a certain extent, but the entire deletion is lethal in mice, *Drosophila* or yeast (Egloff S., 2008). Interestingly, the C-terminal domain of RNA Pol II functions independently of Rpb1. It neither needs to interact with the core region of this subunit nor has to be physically connected. The Rpb1 CTD can be transferred to e.g. Rpb4 or Rpb6, where the transferred CTDs have reduced phosphorylation levels but recruit CTD binding factors normally (Suh H., 2013).

4.3.2 Modification of the CTD of RNA Pol II

All amino acids in the CTD of Rpb1 of the RNA Pol II are potential targets for post-translational modifications, which play a crucial role in transcription and post-transcriptional RNA processing, including 5'-capping, transcription initiation, splicing, termination and 3'-end formation. These

modifications are dynamic and reversible and can be found on several positions of the heptapeptide and in numerous amounts. The composition and the number of modifications are referred as CTD code and can be read by different transcription factors (TFs) and pre mRNA modifying proteins (Cho E.J., 1997), (Egloff S., 2012), (Morris D.P., 2005), (Emily Brooke, 2009).

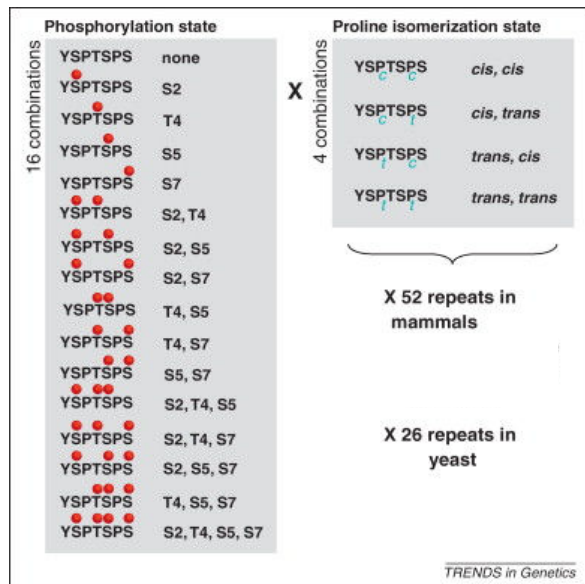


Figure 7. Potential modifications of the RNA polymerase II carboxy-terminal domain (CTD) heptapeptide during transcription. All the possible serine phosphorylation and proline isomerization combinations are shown (Egloff S., 2012).

Phosphorylation is the dominant form of these modifications and occurs mainly at tyrosine, threonine and at one or several serines, while the two prolines can undergo isomerization between cis- and trans- conformation. Additionally, serine and threonine can be glycosylated. Ubiquitination is also a possible modification. Theoretically, all amino acids of the CTD can be modified but little is known about their role or influence. (Heidemann M., 2012) (Morris D.P., 2005).

These different modifications have an enormous combinatorial potential. Some CTD “codes” are related to different phases of mRNA transcription, but not all of them are linked to a special stage or are known to have a precise function, yet. Mutation of single amino acids within the C-terminal domain of Rpb1 can have a serious impact on transcription rate, failure in splicing factor recruitment or even viability. For example, Thr4 mutants (Thr4 was exchanged to Ala) lead to lethality in mammalian cells. In contrast to this, mutants are viable in yeast (Hintermair C., 2013), (Heidemann M., 2012). When serine 2 is mutated to alanine, the RNA Pol II is still able to transcribe, but it fails to recruit splicing components. This is a clear hint that serine 2 phosphorylation is important for splicing and termination factor recruitment (Bo G., 2012).

The mutation of different amino acids of the CTD heptarepeats influences the viability of mammalian cells. Serine 2, serine 5 and serine 7 mutants have a severe growth defect and after 4 days, a strong decrease in cell number was observed (Hintermair C., 2013).

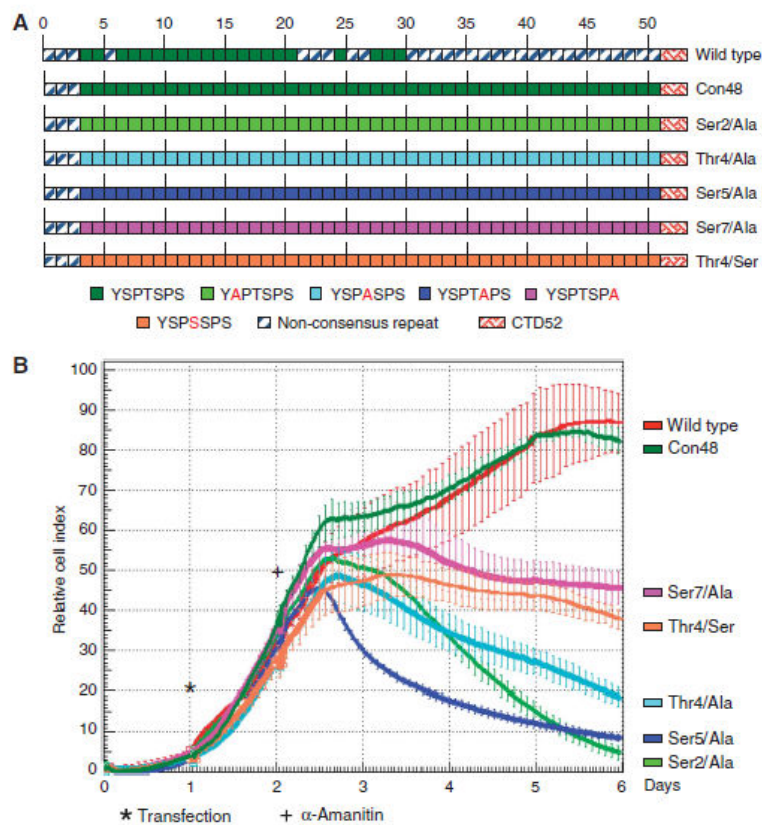


Figure 8. Viability of cells expressing CTD mutants. Fig. 8 A Overview of analysed CTD mutants. Fig. 8 B HEK293 cells were transfected with an expression vector for *Rpb1* carrying a point mutation for α -amanitin resistance (wild type). CTD mutants carry 48 consensus repeats (Con48), or have replaced specific residues in all consensus repeats by alanine (Serine 2, Threonin 4, Serine 5, Serine 7) or serine (Threonin 4). Twenty-four hours after transfection, α -amanitin was added (2 μ g/ml) and cell growth rates were monitored for 4 days in the xCELLigence system (Roche), error bars show standard deviation of 3 replicates. (Hintermair C, 2013)

All these published data show how indispensable the carboxy-terminal domain and even single amino acids are in guaranteeing correct transcription, pre mRNA processing and survival of the cell.

4.3.3 Serine phosphorylation: Role in transcription and controversy in literature

Phosphorylation is predominantly found at serine 2 or serine 5, but also occurs at tyrosine1 and in mammals as well at serine 7 (Morris D.P., 2005). Cyclin dependent kinases like Cdk7 and Cdk9 play a fundamental role in transcription initiation as enzymes responsible for the phosphorylation of serine 5 and 2, respectively.

Serine 2 phosphorylation

Serine2 phosphorylations (serine 2P) first appear when RNA Pol II resides at the transcription start (TSS) and increase towards the end of the gene. They occur when RNA Pol II enters the productive elongation phase and it needs the previous phosphorylation of serine 5. The kinases, responsible for the phosphorylation at serine 2, are: Cdk9 (which is a part of pTEFb, a positive transcription elongation factor), Cdk12 and maybe Cdk13 as well. Knock-out or inhibition of one of these kinases might be hard to interpret, since other factors play a role in phosphorylation, too. Serine 2P is crucial in transcription elongation and in pre mRNA splicing, just as in termination factor recruitment. Serine 2P at the 3'-end of the gene also occurs simultaneously with the recruitment of histone modification and other 3'-end RNA processing factors. To remove the phosphorylation at serine 2 at the CTD in mammals, the phosphatases Fcp1 and Cdc14 are necessary (Egloff S., 2012), (Heidemann M., 2012), (Bo G., 2012).

Serine 5 phosphorylation

Serine 5 gets phosphorylated (Serine 5P) by either Cdk7 (a part of the general transcription factor TFIIF which plays a role in the building of the pre-initiation complex at the TSS) or Cdk8 (Egloff S., 2012). This phosphorylation normally occurs at the promoter and decreases towards the end of the gene. Serine 5P is associated with promoter release and it is also required for the recruitment of the guanylyltransferase, which adds the 5'-cap structure to the nascent transcript. It also plays a role in histone modification and chromatin remodelling. Set1, a histone methyl transferase important for H3K4 tri-methylation, interacts with serine 5P of RNA Pol II. Phosphatases, which are responsible for removing the phosphorylation at serine 5 residue again, are RPAP2, Scp1, Ssu72, Cdc14 (Morris D.P., 2005), (Phatnani H.P., 2006), (Heidemann M., 2012), (Egloff S., 2012).

Serine 7 phosphorylation

Cdk7 and Cdk9 phosphorylate serine7 of the carboxy-terminal domain of Rpb1. Serine 7 phosphorylation (Serine 7P) is known to be necessary for correct transcription of snRNAs, some protein coding genes and for 3'-processing of the transcript. In the case of snRNA, serine 2 and serine 7P is required for the interaction of the integrator with the CTD of Rpb1 (Egloff S., 2010) (Egloff S., 2012). The integrator is an evolutionary conserved multiprotein mediator which is necessary for the correct processing of snRNAs (Baillat D., 2005). Additionally, serine 7P facilitates serine 5 dephosphorylation in the transcription cycle of snRNA (Egloff S., 2012).

In protein coding genes, the phosphorylation at serine 7 occurs early in the transcription like at serine 5. Interestingly, the change from serine at position 7 to an alanine does not reduce gene expression, but it blocks the processing of the nascent RNA transcript (Corden J.L., 2007), (Heidemann M., 2012). Although a high level of serine 7P correlates with an increased transcription rate, the role of this modification at the CTD of RNA Pol II has yet to be clarified.

Notably, serine 7P is specifically enriched over introns. This leads to the conclusion that serine 7P plays a potential role in co-transcriptional assembly or disassembly of splicing complexes (Kim H., 2010). Liu et al could show that TCERG1, a transcription elongation regulator that interacts with the splicing factors and the transcribing RNAPII, needs hyper-phosphorylation on all three serine residues for binding. In this case serine 7P enrichment at intron-rich regions could be important for adaptor protein recruitment, such as TCERG1, coupling transcribing RNAPII with splicing factors to regulate co-transcriptional splicing (Liu J., 2013). It was also shown that serine 7P has a stimulating effect on the binding of pTEFb by priming the C-terminal domain of Rpb1 for further phosphorylations at serine 2 (Czudnochowski N., 2012). Other data show that the serine 7P varies in a gene-specific way, with discrete peaks at 5'- ends, 3'- ends or both. Serine 5P and serine 2P are normally very uniform when comparing different genes (Egloff S., 2012), (Heidemann M., 2012).

Almost all data describing the role of serine 7P are collected in yeast or mammals with a focus on housekeeping genes. Therefore it will be interesting to take a closer look at genes displaying complex regulation, such as those of the immune system that are up-regulated after infection.

Controversy in the literature

Publications about modifications of the CTD of RNA Pol II during transcription agree that serine 2P peaks at the end of the gene and serine 5P is at its highest level at the beginning of the gene. On the contrary, the opinions about serine 7P levels and function differ widely. While some argue that serine 7P peaks around the transcription start (Heidemann M., 2012) others claim that serine 7P peaks at the end of the gene and is important for RNA 3' end formation and termination (Egloff S., 2012).

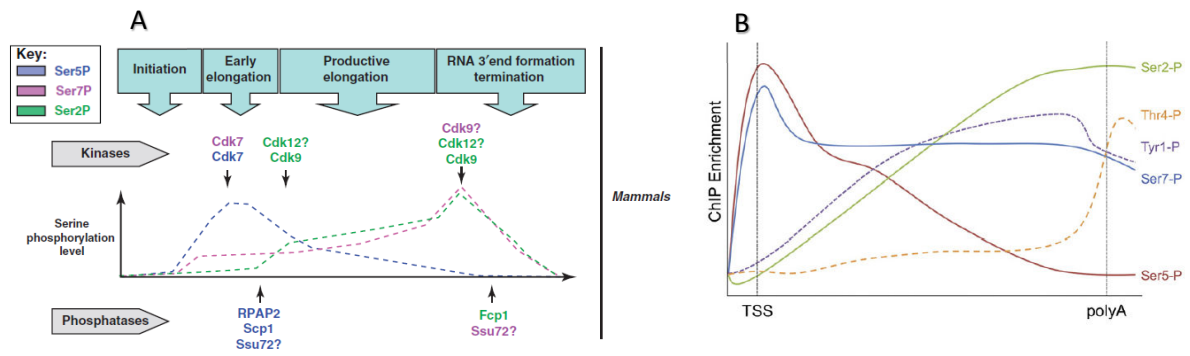


Figure 9. Controversy in literature: consensus in serine 2 and serine 5P levels during transcription in housekeeping genes. The opinions about serine 7P differ widely (Heidemann M., 2012) B, (Egloff S., 2012) A;

5 AIM OF THE PROJECT

Our group is interested especially in the transcriptional regulation of genes of the innate immune defense, which are co-regulated by the STAT and NFκB pathways. This group of genes includes *Nos2*, which encodes iNOS and plays a crucial role in the innate immunity.

Many details about transcription of the *Nos2* gene, and especially about the regulation at the promoter were brought to light by previous projects in our lab. It was shown that the recruitment of the general transcription factor TFIID is mediated by NFκB. The association of the pTEFb-complex with the promoter is dependent on the same pathway. This means that the cyclin dependent kinases Cdk7 and Cdk9, which are necessary for CTD modifications of the RNA Pol II, are NFκB-dependent, due to the fact that they are part of TFIID and pTEFb. Serine 5P is needed for the following serine 2P, which are both necessary for transcription initiation and the transition to the elongation phase. (Farlik M., 2010), (Wienerroither S., 2014).

Modifications of the carboxy-terminal domain of RNA Pol II play a crucial role, not only in initiation, but also in all other phases of the transcription cycle. This project attempts to investigate the serine 2 and serine 5P over the entire *Nos2* gene as well as taking a closer look to at serine 7P.

Furthermore, this research seeks to address whether NFκB alone is sufficient to recruit Cdk7 and Cdk9 or if there are other, unknown factors involved that are activated by TLRs or a downstream component of the signalling cascade. In order to demonstrate this, a cloning experiment was devised and initiated to fuse the p50 subunit of NFκB and one of the cyclin-dependent kinases.

Specific aims of my project

- 1) Investigate the phosphorylation of serine 7 at the carboxy-terminal domain of the RNA Pol II during *Nos2* transcription.
- 2) Compare serine 2, -5 and -7 phosphorylation over the entire *Nos2* gene
- 3) Examine whether serine 7P, like serine 2 and serine 5 phosphorylation, requires NFκB
- 4) Create fusion proteins of the p50 subunit of NFκB and one of the cyclin dependent kinases (Cdk7 and Cdk9)

6 RESULTS

6.1 INDUCTION OF GENE EXPRESSION BY THE TYPE I IFN AND NFκB PATHWAYS

The first set of analyses examined gene induction by IFNβ and by NFκB signalling, stimulated by treatment of macrophages with heat-killed *Listeria* (hKL). HkL were added either alone or combined with IFNβ and iNOS expression was determined by Q-PCR.

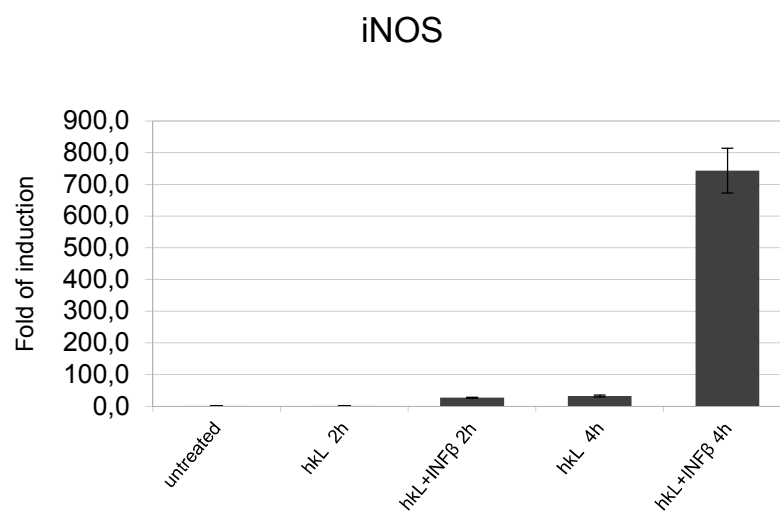


Figure 10. *iNOS* expression in macrophages after stimulation with hKL or hKL + IFNβ for 2hrs or 4hrs. Data represent normalized mRNA expression as determined by qPCR.

As expected, stimulating with hKL alone did not induce iNOS expression. Adding IFNβ and hKL strongly increased iNOS expression after 4 hours (Fig. 11). Macrophages infected with *L. monocytogenes* show a similar level of expression, but at a later time-point because during *L. monocytogenes* infection IFNβ has to be produced first to trigger iNOS expression.

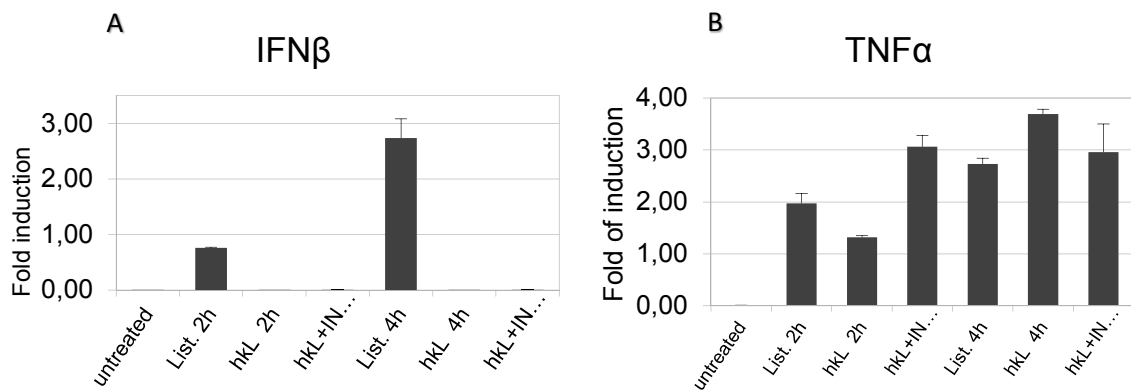


Figure 11. *IFN β and TNF α expression in macrophages.* BMDMs were infected with *L. monocytogenes*, or stimulated with either hKL or a combination of hKL and IFN β for 2 and 4h. Expression of IFN β and TNF α mRNA was determined by qPCR.

To demonstrate that IFN β expression requires infection with viable, cytoplasmic *L. monocytogenes* and is poorly stimulated by hKL with- or without additional IFN β , macrophages were infected or subjected to treatment with hKL alone or in combination with IFN β (fig 11 A). In accordance with previous results (Stockinger 2002), IFN β expression occurred exclusively in the infected cells. By contrast, expression of the TNF- α gene, a known target of the NF κ B pathway, occurred both after infection and stimulation with hKL.

6.2 PHOSPHORYLATION OF THE RNA POLYMERASE CTD AT THE *NOS2* GENE

To initiate the analysis of RNA Pol II CTD phosphorylation, the kinetics of iNos expression were determined and correlated with the presence of RNA Pol II in the gene body. Figure 12 shows that maximal expression levels were obtained 4hrs after treatment with combined hKL+ IFN β and the levels declined thereafter to reach near baseline levels at 20hrs. Consistent with this, the binding of RNA Pol II to exons 2, 7, 17 and 26 as determined by chromatin immunoprecipitation (ChIP) was maximal at 4hrs and decreased at 6hrs after hKL+ IFN β treatment (representatives are shown in figure 13). As expected (Farlik 2010), hKL or IFN β alone recruited comparably little RNA Pol II to *Nos2* exons. Based on these data, 4hrs of hKL+ IFN β treatment were chosen for the ChIP analysis of RNA Pol II CTD phosphorylation.

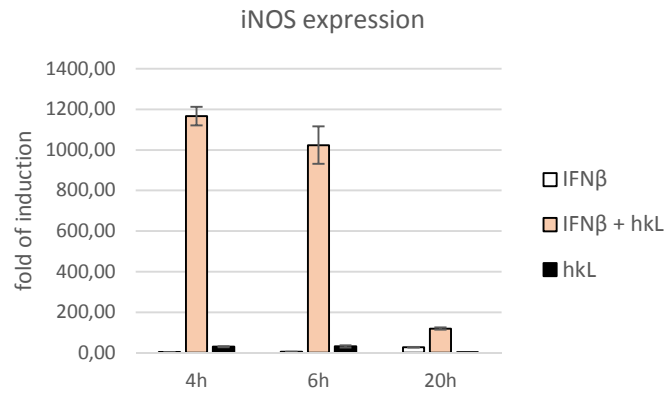


Figure 12. **iNOS expression in BMDMs.** Cells were treated with IFN β , IFN β + hkl and hkl for 4h, 6h and 20h. The figure shows a representative experiment. Expression of iNOS mRNA was determined by qPCR.

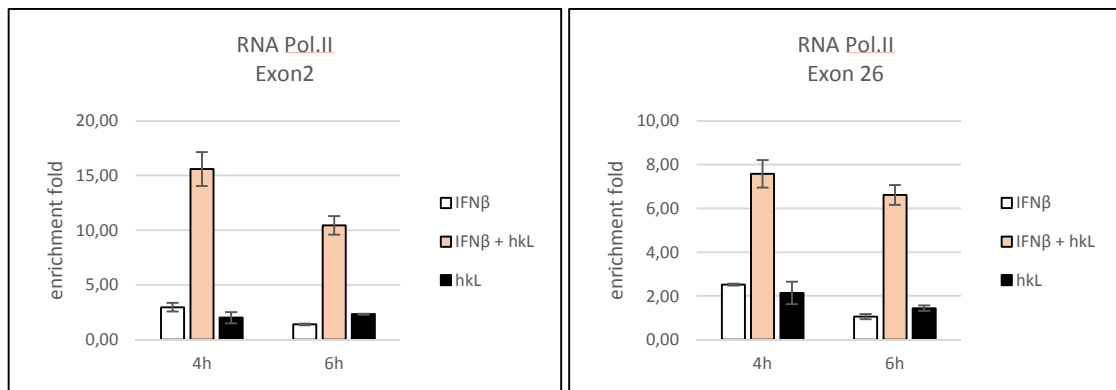


Figure 13. **RNA Pol II recruitment and distribution at the Nos2 gene** after IFN β /hkl treatment in BMDM over the entire Nos2 gene (TSS, Exon 8 and Exon 17 not shown). BMDMs were stimulated for 4h and 6h with IFN β , hkl or both followed by ChIP with antibodies to Pol II. Data were analysed by qPCR. Different exon regions of the Nos2 gene were amplified. Representative experiments are shown.

6.2.1 Normalization and analysis of the ChIP data

To be able to analyse the ChIP data and compare experiments with each other, it was necessary to perform several normalization steps. In every ChIP experiment, the data needs to be normalized to the input. Additionally, the data obtained after stimulation of the cells were normalized to the sample of the unstimulated cells to get a fold enrichment value. Every single dataset was normalized to the TSS value as well. This minimizes the technical error and biological fluctuations of different ChIP experiments. It thus becomes possible to compare 5 independent experimental replicates. To determine the level of CTD phosphorylation, the amount of RNA Pol II must be set in relation to the phosphorylation of different serines. Otherwise, the amount of phosphorylation would only reflect the amount and distribution of polymerase at the Nos2 gene. The normalization procedure is depicted in fig. 14.

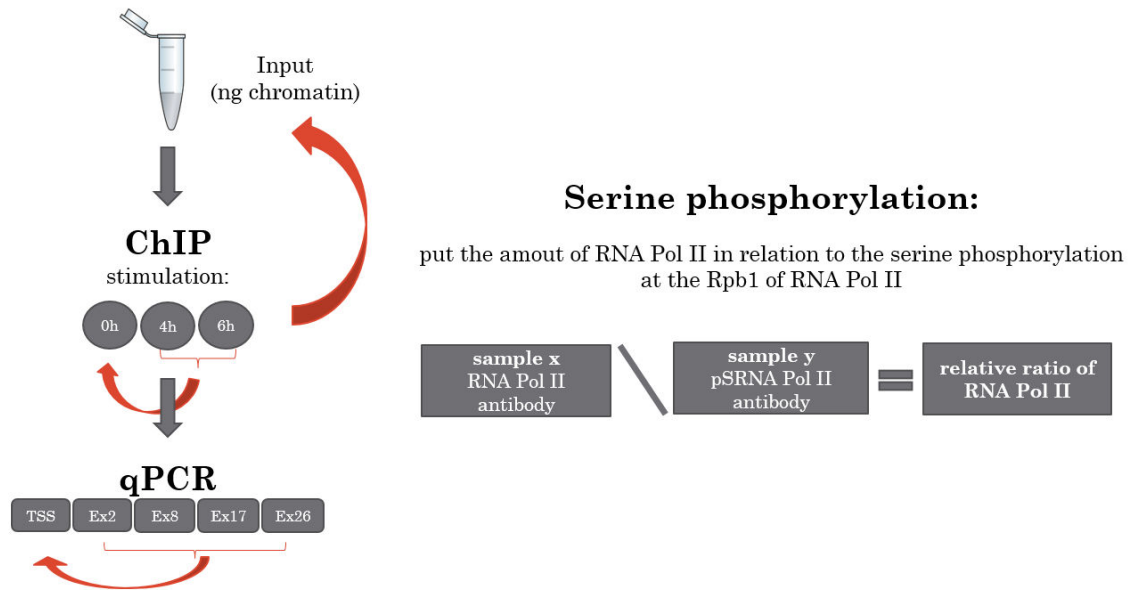


Figure 14. **Determination of the pSPol II/Pol II ratio** to obtain the degree of CTD phosphorylation. ChIP data were normalized to the input as well as to the unstimulated samples. To compensate fluctuation of different ChIP experiments individual experiments were normalized to values obtained at the TSS. For analysis of serine phosphorylation the results were put in relation to the amount of RNA Pol II.

6.2.2 RNA Pol II distribution at the Nos2 gene

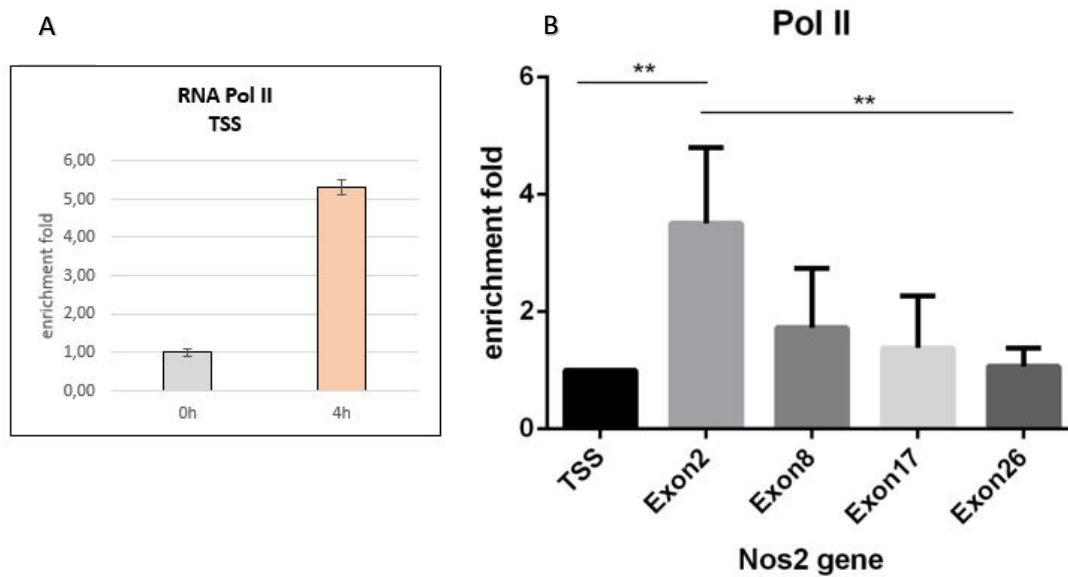


Figure 15. **RNA Pol II enrichment at the Nos2 transcription start site (TSS)** after IFN β + hK α L treatment in BMDMs and **RNA Pol II distribution over the gene**. BMDMs were stimulated for 4h with IFN β + hK α L followed by ChIP with antibodies to Pol II. Fig. 15 A) shows a representative experiment for the enrichment at the TSS. Fig.15 B) depicts means and standard errors from 5 independent biological replicates. Individual experiments were normalized to the value obtained at the TSS to even out fluctuations between different ChIP experiments. Data were analysed by qPCR. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ns, not significant;

Fig.15 A demonstrates a clear enrichment of RNA Pol II at the proximal promoter of the Nos2 gene 4 hours after induction with IFN β and hK. After normalization to the unstimulated sample, a 5-time enrichment of RNA Pol binding is observed.

Fig.15 B shows pooled data from five independent biological replicates, normalized to the value obtained for binding to the TSS. A clear enrichment of Pol II at the exon2 after stimulation is observed. Towards the end of the gene, RNA Pol II binding decreases.

6.2.3 Serine 2P of the Pol II CTD at different Nos2 exons

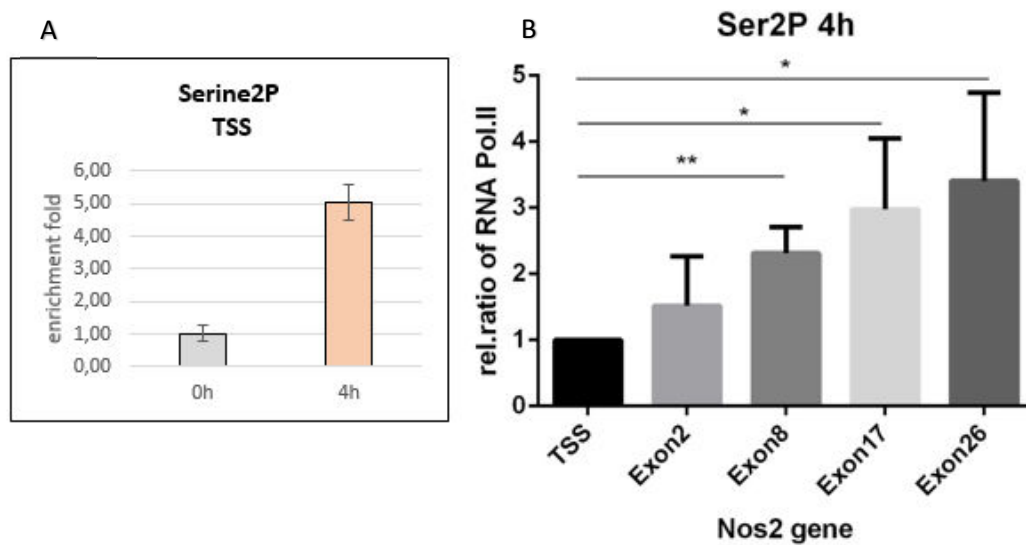


Figure 16. **Serine 2P at the transcription start site (TSS) and CTD phosphorylation levels over the entire gene after IFN β + hK treatment.** BMDMs were stimulated for 4h with IFN β + hK followed by ChIP with antibodies to Pol II phosphorylated at CTD serine 2. Fig. 16 A) shows a representative experiment for the enrichment at the TSS. Fig.16 B) depicts means and standard errors from 5 independent biological replicates. Individual experiments were normalized to the value obtained at the TSS to even out fluctuations between different ChIP experiments. Data were analysed by qPCR. *, P < 0.05; **, P < 0.01; ***, P < 0.001; ns, not significant;

Fig. 16 A shows the enrichment of RNA Pol II phosphorylated at CTD serine 2P at the TSS of the Nos2 gene after 4 hours of induction with IFN β and hK. After normalization to the unstimulated sample, a 5-time enrichment of the phosphorylation at serine 2 is detected. In Fig. 16 B, the pooled data from five independent biological replicates, which were normalized to the TSS and to the amount of RNA Pol II are presented. A stepwise increase of phosphorylated serine 2 towards the end of the gene is noticed. This is in line with previous studies on housekeeping genes (Heidemann M., 2012) (Egloff S., 2012), where serine 2P increases with decreasing distance of the polymerase to the 3'-end of the gene.

6.2.4 Serine 5P of the Pol II CTD at different Nos2 exons

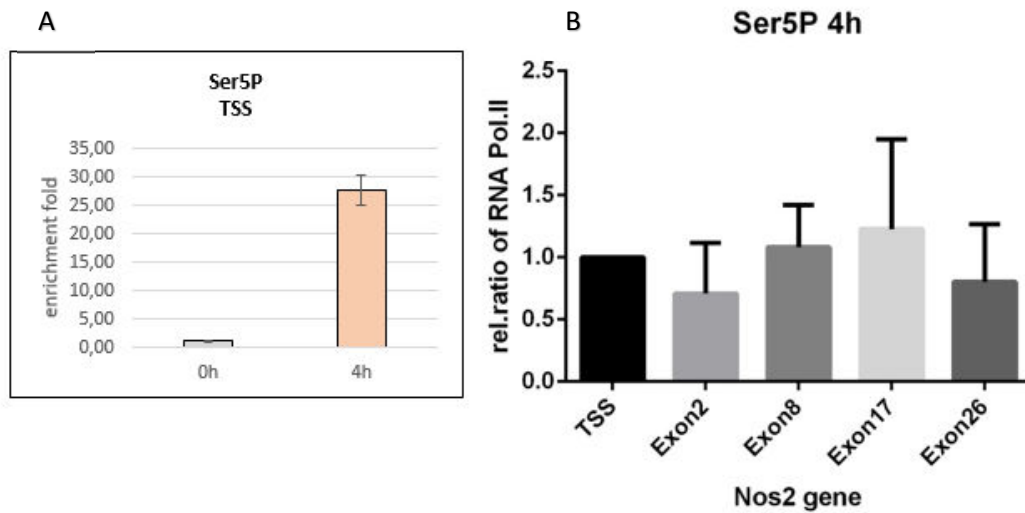


Figure 17. Serine 5P at the transcription start site (TSS) and phosphorylation levels over the entire gene after IFN β + hKL treatment. BMDMs were stimulated for 4h with IFN β + hKL followed by ChIP with antibodies to Pol II phosphorylated at CTD serine 5. Fig. 18 A) shows a representative experiment for the enrichment at the TSS. Fig. 18 B) depicts means and standard errors from 5 independent biological replicates. Individual experiments were normalized to the value obtained at the TSS to even out fluctuations between different ChIP experiments. Data were analysed by qPCR. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ns, not significant;

Fig. 17 A suggests that Nos2 TSS-bound RNA polymerase II is strongly phosphorylated at CTD serine 5 after 4 hours of induction with IFN β and hKL. Fig 17 B reveals that, after pooling data from 5 independent biological replicates, there is no significant change in the phosphorylation level at serine 5 across the Nos2 gene, i.e. the relative amount of serine 5P corresponds to that of the TSS bound RNA polymerase II. These data do not agree with the results for housekeeping genes, where serine 5 at the TSS is higher than elsewhere in the gene. Towards the end of the house-keeping genes serine 5P decreases successively (Heidemann M., 2012).

6.2.5 Serine 7P of the Pol II CTD at different Nos2 exons

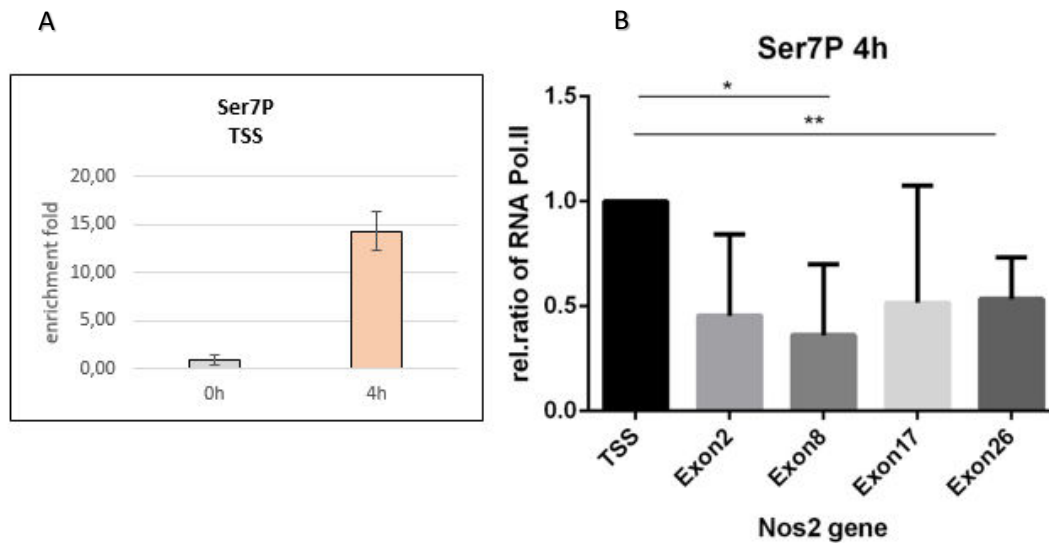


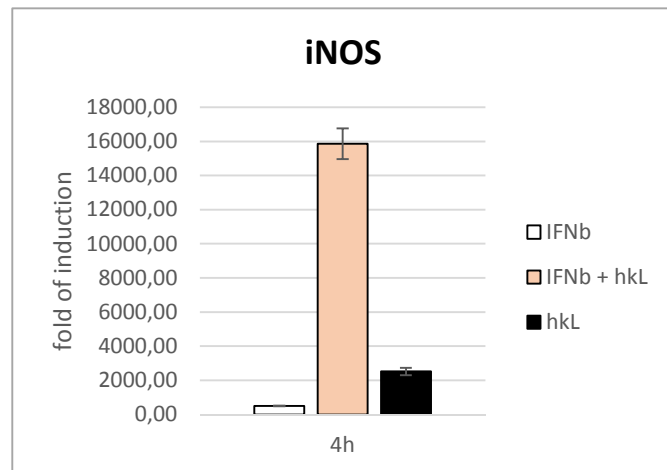
Figure 18. **Serine 7P at the transcription start site (TSS) and phosphorylation levels over the whole gene after IFN β + hKL treatment.** BMDMs were stimulated for 4h with IFN β + hKL followed by ChIP with antibodies to Pol II phosphorylated at CTD serine 7. Fig. 18 A) shows a representative experiment for the enrichment at the TSS. Fig. 18 B) depicts means and standard errors from 5 independent biological replicates. Individual experiments were normalized to the value obtained at the TSS to even out fluctuations between different ChIP experiments. Data were analysed by qPCR. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ns, not significant;

Fig 18 A shows that CTD phosphorylation at serine 7 is also very prominent when analyzing the Nos2 TSS-bound Pol II after 4 hours induction with IFN β and hKL. Fig 18 B demonstrates the pooled data of 5 independent biological replicates, which are normalized to the values obtained at the TSS and to the amount of recruited RNA Pol II. A significant decrease of serine 7 occurs upon elongation of the transcript into the Nos2 gene body. Serine 7P stays moderately high up to the 3' end of the gene.

6.3 iNOS EXPRESSION IN THE RAW 264.7 MACROPHAGE CELL LINE

The aim of the project initiated here is to express a p50/CDK7 or p50/CDK9 fusion protein in RAW 264.7 macrophages and examine whether the fusion proteins can substitute for NF κ B function. As a preliminary experiment, I had to show that the iNOS expression in RAW 264.7 and primary cells is comparable. As can be seen from Fig. 29, the synergistic effect of hKL and IFN β on Nos2 expression is as pronounced as in BMDMs.

Figure 19. *iNOS* expression in RAW 264.7 cells. Induction of *iNOS* after stimulation of the RAW 264.7 macrophages for 4h with hKL and IFN γ . Expression of *iNOS* mRNA was determined by qPCR.



6.4 CLONING STRATEGY FOR A NF κ B P50 AND CDK7 OR CDK9 FUSION GENE

6.4.1 Cloning strategies

The vector pLVX- TRE3G-ZsGreen1 from the LentiviralTet-On 3G Inducible Expression Systems by Clontech was used for all cloning strategies (see above for materials).

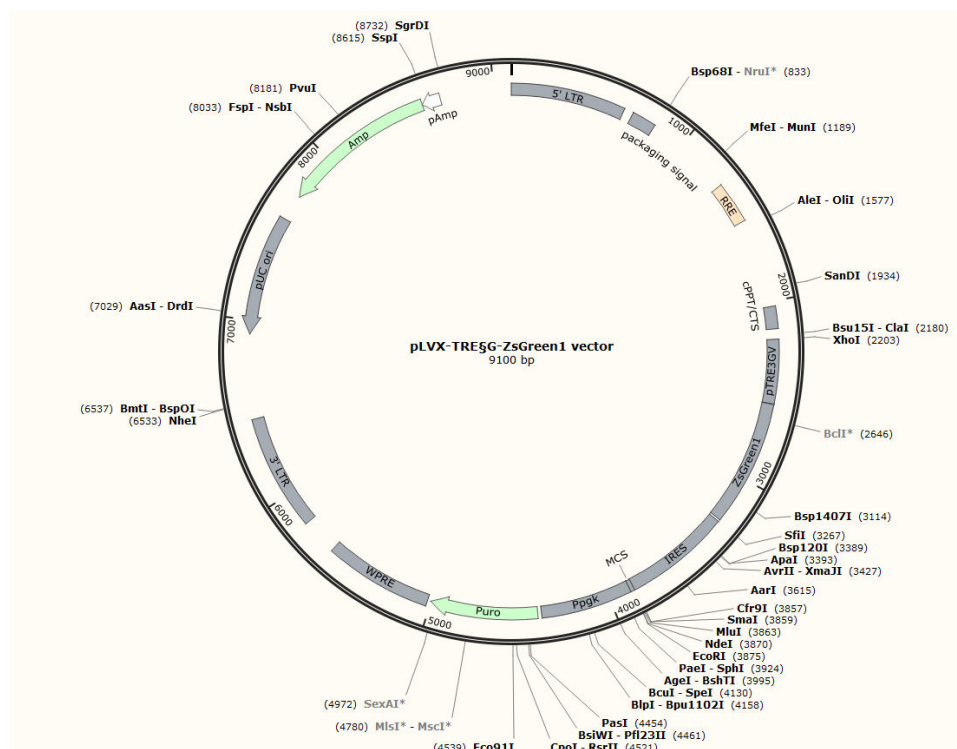


Figure 20. vector pLVX- TRE3G-ZsGreen1 from the Lentiviral Tet-On 3G Inducible Expression Systems (Clontech), used for all further cloning experiments.

6.4.1.1 1st cloning strategy

The first strategy for cloning was to produce the fusion gene (p50- Cdk7 or p50- Cdk9) by two-step PCR, followed by cloning of the whole fused insert into the vector. First, a PCR amplifying p50 (with NdeI and NotI restriction sites), Cdk7 and Cdk9 (with NotI and EcoRI restriction sites) was carried out. This was followed by digestion with the appropriate enzymes for the flanking restriction sites and a ligation to fuse the two PCR products. A second PCR was done only with the p50 forward primer and the Cdk7/Cdk9 reverse primer to amplify the whole Insert, followed by normal cloning procedure. This approach was not successful and therefore abandoned.

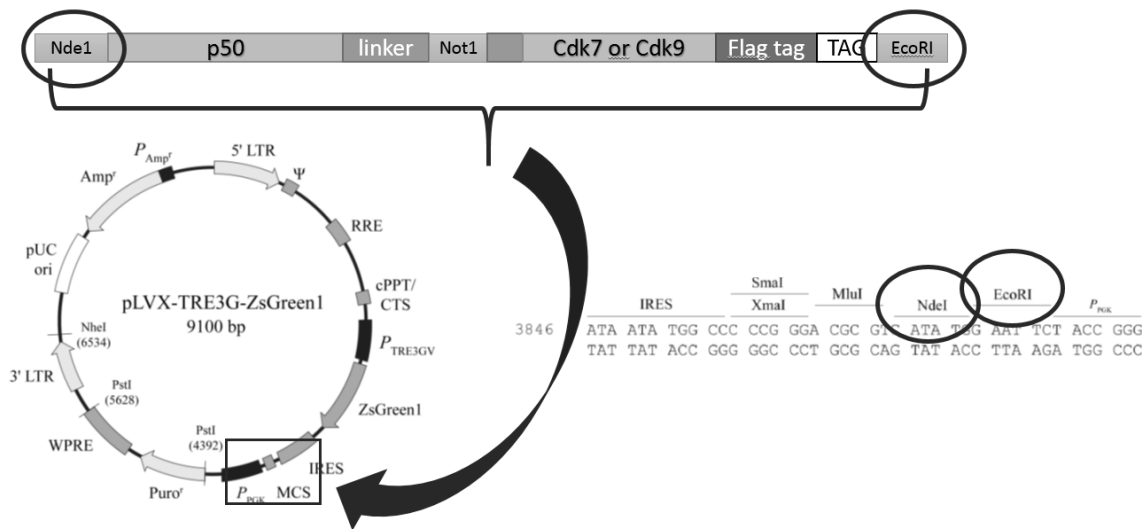


Figure 21. 1st cloning strategy (modified from www.clontech.com pLVX-TRE3G-ZsGreen1 Vector Information)

6.4.1.2 2nd cloning strategy

1st step: Insert the gene for the NFκB p50 subunit into the pLVX-TRE3G-ZsGreen1 vector.

For this attempt, new primers with new restriction sites were constructed (primer sequences can be found in the materials).

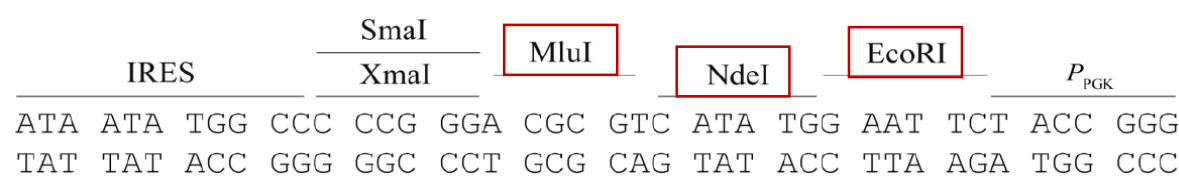


Figure 22. multiple cloning site of pLVX-TRE3G-ZsGreen vector (clontech, 2014); red frames show restriction sites used for 2nd cloning strategy;

2nd step: Insert one of the two cyclin dependent kinases genes (Cdk7 or Cdk9) into the pLVX-TRE3G- p50 ZsGreen1 vector (shown in Fig. 24).

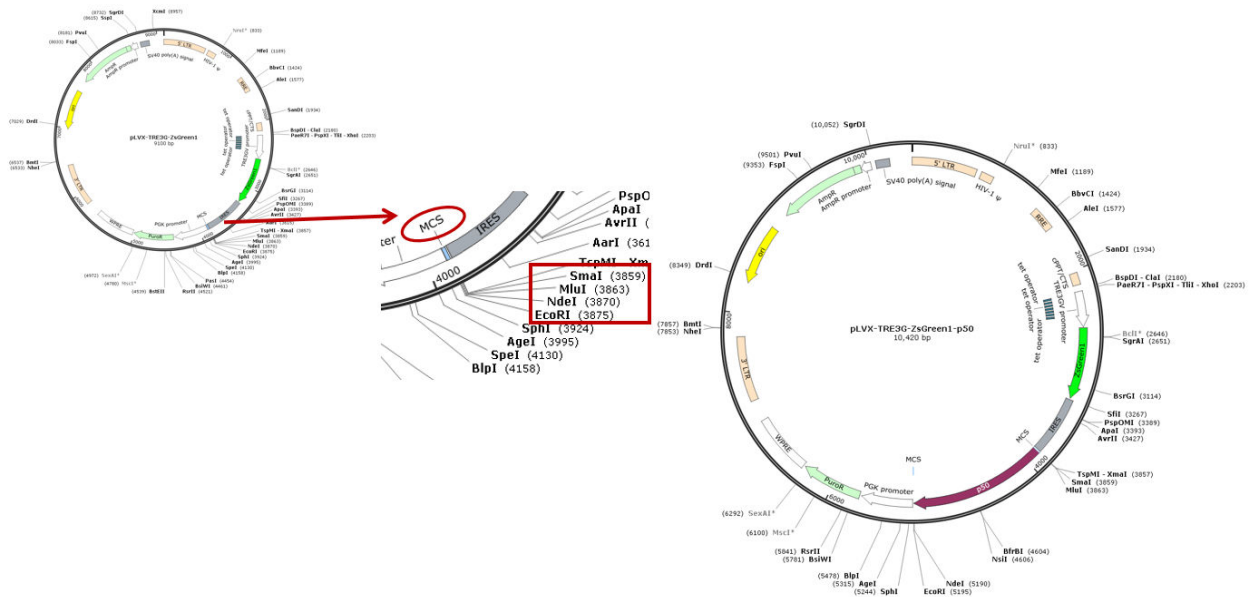


Figure 23: 1st step of 2nd cloning strategy; p50 with the flanking restriction sites MluI + NdeI will be cloned into the pLVX-TRE3G-ZsGreen1 vector.

After insertion of the p50 gene, the next step is to clone one of the 2 cyclin dependent kinases into the pLVX-TRE3G-ZsGreen1-p50 vector (shown in Fig. 25). This approach was similarly unsuccessful.

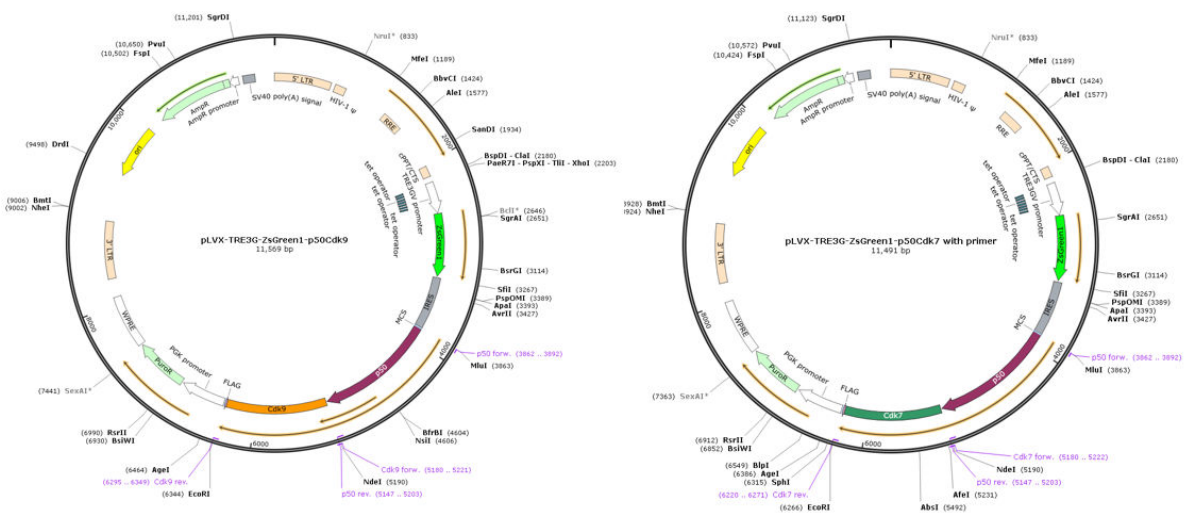


Figure 24. 2nd step of 2nd cloning strategy: Insertion of Cdk7 or Cdk9 into the pLVX-TRE3G-ZsGreen1-p50 vector using enzymes NdeI and EcoRI;

6.4.1.3 3rd cloning strategy

A new cloning strategy was developed following the consideration that the restriction sites for the 2nd strategy were in too close proximity. Further, MluI restriction activity was found insufficient.

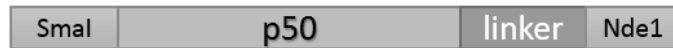


Figure 25. new construct for 3rd cloning strategy for cloning into pVLX-TRE3G-ZsGreen1 vector

A new forward primer for the p50 gene was developed containing a new restriction site (SmaI) (sequence see above under materials). The restriction sites are further distant, therefore the restriction enzymes should be more active. Because SmaI is a blunt-end cutter, the ligation conditions had to be adjusted.

6.4.1.4 4th cloning strategy



Figure 26. new construct of 4th cloning strategy for cloning into pVLX-TRE3G-ZsGreen1 vector

Because the 3rd cloning strategy did not work out, a new strategy was devised. This time the amplification product was cut by one restriction enzyme and cloned in an intermediate vector with an NdeI site. The intermediate vector can be any mammalian expression vector. I used the mammalian expression vector pcDNA3.1 mycBioID (Kyle Roux Lab/sequence available on addgene).

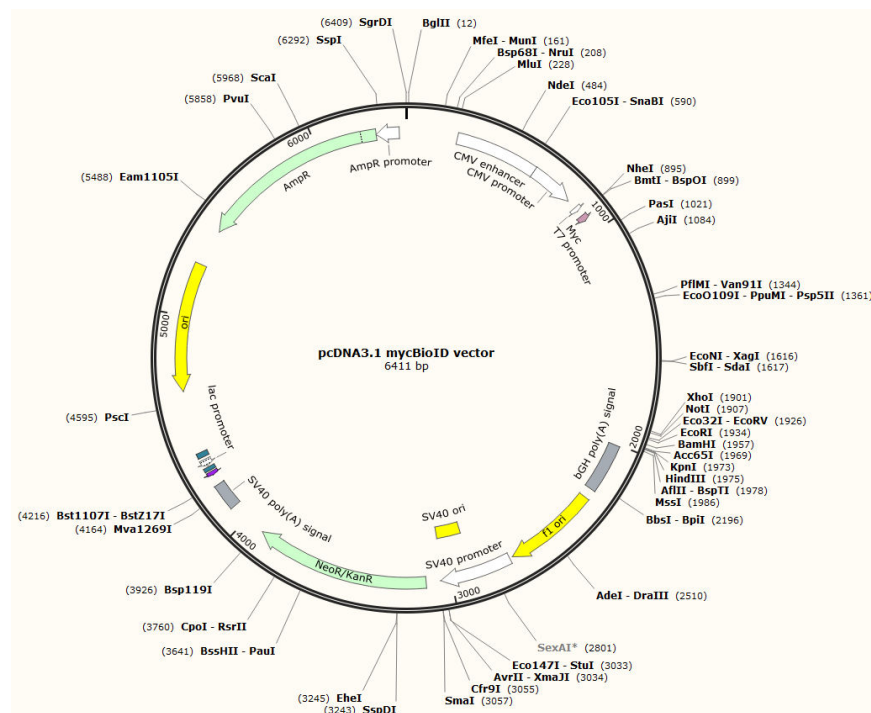


Figure 27. mammalian expression vector: pcDNAmycBioID vector by Kyle Roux Lab/sequence available on addgene

6.4.2 Optimization of the PCR protocol

Pre- calculated temperature for the primer was not always giving satisfying PCR products, therefore several troubleshooting steps were performed. To optimize the PCR, a set-up gradient

should show the best annealing temperature for the primer. To enhance annealing, 5% DMSO was added, which is known to increase efficiency. The usage of DMSO could lead to alterations in annealing temperature. These optimizations aside, different cDNA templates (cDNA and specific primed cDNA) and different concentration of starting material (50ng, 100ng, and 200ng cDNA) are used. At an early stage of polymerization, a 2nd PCR is performed, using the product from the first round as template. To be sure to get enough PCR product the cycle number was increased to 40 and the elongation time was extended to 2min 30 sec as suggested in the manual of Pfu polymerase (Pfu Pol) suggested for a product around 1,3kb (thermoscientific, 2013) (Roux K.H., 2009).

6.4.3 Sequencing data

The insert amplified with Pfu Pol was sequenced in order to check for point mutations and correctness of the product. Sequencing data showed the correct products, allowing to proceed to the next step of cloning.

6.4.4 Optimization of digestion and ligation step

For digestion, different restriction enzymes (conventional and fast-digest;Thermoscientific) were used. Because digestion, especially of the PCR products, was problematic, a several optimization steps were necessary. First, efficiency of the enzymes was tested and particularly MluI, NotI and NdeI were found to exert low activity. NdeI after 2 hours showed roughly 75% of cleavage, requiring 6-8 extra bases flanking the restriction site. As a consequence, new primers were designed. MluI cuts only 25% of its DNA substrate after 2h and 50% after 20h. NotI needs a big flanking region and is also problematic when used for only 2h.

Different attempts were performed such as digestion of different amounts of template, various enzyme amounts (1-2µl) and different incubation periods (1h, 5h or o/n).

To examine the successful digestion, the fragments were analyzed on a 1% agarose gel. The vector was cut properly with conventional enzymes as well as with FD enzymes. Regarding the PCR products, it was not apparent if the digest was complete or not.

To avoid these difficulties, fast-digest (FD) enzymes of Thermoscientific were used, which all work in the same buffer and should cut within 1 hour. Also those enzymes did not work.

Sadly, no other enzymes within the MCS could be used because they cut inside one of the two inserts – CDK7 or CDK9.

In addition, troubleshooting of the Ligation was realized, since it was not clear whether digestion or other steps in the cloning procedure are the central problem. T4 Ligase (Thermoscientific) was used in different concentrations. The molar ratio was used to find the right molar ratio of insert and vector. The concentration and the size were considered to calculate different ratios (1:2, 1:5, 1:7, 1:10 and 1:15). The recommended minimum amount of DNA was applied. Several ligation conditions were tried: 45min – 1h/RT at the bench or o/n at 4°C or 16°C. Unfortunately, this optimization experiments did not solve the problem.

6.4.5 Insertion of the p50 gene into a mammalian expression vector

After trying several cloning strategies, which unfortunately did not deliver the desired results, I decided to clone p50 into a mammalian expression vector as an intermediate step. This vector allows an assessment of p50 expression and function in a transient transfection experiment before proceeding to clone the gene into a lentiviral vector.

7 CONCLUSION & OUTLOOK

Recent discoveries show that the modification of the carboxy-terminal domain of the biggest subunit of the RNA Pol II is critical for transcription and the regulation of gene expression (Brookes E., 2009). Different phases and processes, like initiation, elongation and recruitment of essential factors for pre- mRNA processing are influenced by the so called “CTD code”. Different modifications have different impact on the successful process of transcription. Serine phosphorylation is very important in initiation and termination factor recruitment (Heidemann M., 2012), (Egloff S., 2012), (Egloff S., 2008).

Since most of the scientific work was done on housekeeping genes, it was tempting to take a closer look at genes, which play a crucial role in immune defence and are not constitutively expressed. An important focus in our lab is on the transcriptional regulation of genes of the innate immune defense, which are co-regulated by STAT and NFκB pathways. *Nos2*, which encodes iNOS, belongs to this group of genes and its regulation may be paradigmatic for Stat/NFκB co-operation. Previous experiments in our lab have shown that cyclin dependent kinase 7, which is a part of the general transcription factor TFIIF, and CDK9, an important part of pTEFb, are recruited by the NFκB pathway and play a crucial role in iNOS transcription initiation.

7.1 PHOSPHORYLATION AT SERINE 2, 5 AND 7 RESIDUES AT THE *NOS2* GENE

All studies performed previously examined phosphorylation of the Pol II CTD at serines 2 and 5 in the process of transcriptional initiation. No information concerning its phosphorylation status at different steps of elongation was available. Moreover, the regulation of Serine 7P had not been addressed. My goal was to investigate the phosphorylation level of all 3 serines over the entire gene and to determine whether the phosphorylation profile during the transcription cycle differs from housekeeping genes.

The current findings add substantially to our understanding of transcription regulation at the *Nos2* gene. The results demonstrate that the maximum recruitment of RNA Pol II to the *Nos2* promoter was observed after stimulation with hK_L and IFNβ. All over the gene, it is apparent that both stimuli are needed for transcription, due to the results of previous projects of our lab. An obvious enrichment of Pol II at the Exon2 after stimulation can be observed. This could be a sign for a pausing situation.

Taken together, the results after analysing the phosphorylation levels of the CTD of RNA Pol II with ChIP suggest that serine 2, 5 and 7 play an important role in transcription of iNOS, but the results deviate from those published for housekeeping genes.

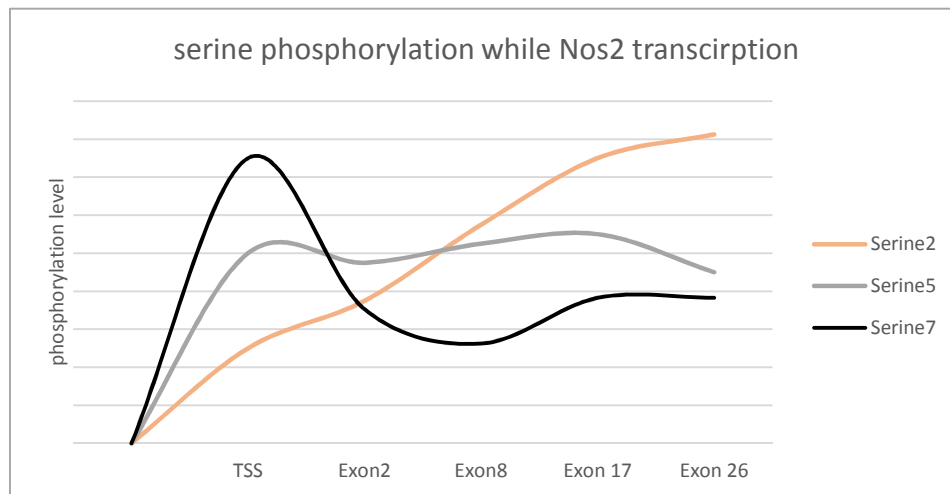


Figure 28. Phosphorylation levels of serine residues at the CTD of RNA Pol II during Nos2 transcription. Serine 2, 5 and 7 are shown. Serine 2P increases strongly towards the end of the gene, whereas serine 5P is strong at the TSS, but does not change significantly. Serine 7P is highly phosphorylated at the promoter, but the phosphorylation decreases rapidly after the TSS.

Serine 2 shows a phosphorylation pattern, which looks quite familiar to the one, which is known for housekeeping genes. Serine 2P rises downstream of the TSS, suggesting that this phosphorylation event continues during elongation. This supports the assumption that serine 2P is regulated in the same way in housekeeping genes as in Nos2. This phosphorylation mark seems to be essential and has the same function for transcription and termination factor recruitment as in constitutively transcribed genes like GAPDH.

Serine 5P at Nos2 gene shows a different pattern in comparison with the published data for housekeeping genes. It is phosphorylated at the TSS by Cdk7 and is known to be required for subsequent serine 2P (Liu J., 2013) (Egloff S., 2012). As for other genes, serine 5P at the pol II CTD is associated with promoter clearance and the recruitment of the guanylyl transferase for mRNA capping. Only when both serine 2 and serine 5 are phosphorylated, elongation starts. Over the whole gene there are no significant changes of the level of serine 5P marks. The strong enrichment at the TSS indicates that serine 5P is needed mainly for transcription initiation. Reduced levels in the gene body suggest that the Pol II CTD is actively dephosphorylated at serine 5 during elongation.

Serine 7, like serine 2 and 5, is a target of dynamic modifications during transcription by RNA Pol II (Heidemann M., 2012). With regard to the Nos2 gene, I could show that serine 7P peaks at the

transcription start and decreases rapidly thereafter. It stays moderately phosphorylated until the end of the gene.

The strong phosphorylation at the beginning might be important for the recruitment of pTEFb, the complex containing the catalytic subunit and CTD serine 2 kinase Cdk9. This assumption is in line with published data, showing that serine 7P primes the CTD for binding of pTEFb and consecutive phosphorylation at serine 2 (Czudnochowski N., 2012).

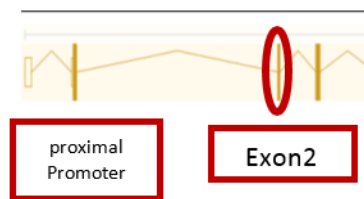


Figure 29. Intron DNA amplified between promoter and 1st Exon at the Nos2 promoter (modified from <http://www.ensembl.org>)

Serine 7P is also known to be enhanced over intron regions. Reportedly it plays a role in splicing factor recruitment (Kim H., 2010). If this applies to the Nos2 gene the high level of serine 7P at the transcription start may be influenced by the fact that the primers used to amplify this region are close to a great intronic region between the promoter and exon 2.

By contrast the other primer pairs, amplify regions which are not as close to intronic DNA. A possible conclusion could be that the level of serine 7P at the 5'-end of the gene is smaller than suggested by the data.



Figure 30. Exon/Intron structure of the 3'-end of the Nos2 gene and primers chosen for ChIP analysis. (modified from <http://www.ensembl.org>)

Serine 7P, in concert with serine 2P and serine 5P, plays a role in the recruitment of TCERG1, the transcription elongation regulator1, which plays a role in elongation and splicing factor recruitment (Liu J., 2013). The large intron region after the promoter could necessitate TCERG1 recruitment for co-transcriptional splicing.

The results of this research support the idea that the modification of the CTD of RNA Pol II, especially phosphorylation at serine 2, serine 5 and serine 7 differ between highly regulated genes like Nos2 and housekeeping genes. Future studies using the same experimental set up could be used to investigate other similarly regulated genes in order to find a common modification pattern and its relationship to transcriptional control.

8 MATERIALS & METHODS

8.1 MATERIAL

8.1.1 Mice and cell lines

C57BL/6 mice (WT; 6-8 weeks of either gender) were used for isolating bone marrow for the differentiation and cultivation of bone marrow derived macrophages (BMDM).

The RAW 264.7 cell line represents leukemic mouse macrophages.

8.1.2 Media

- Dulbecco's Modified Eagle's Medium (DMEM)+ AB (Penicillin and Streptomycin) + 10 % fetal calf serum (FCS) 10% + 10 % L-Cond.were used for bone marrow differentiation into macrophages.
- DMEM + AB (Penicillin and Streptomycin) + 10%FCS (50ml) + were used for cultivating RAW 264.7 cell line
- LB medium with or without Ampicillin was used for growing *E.coli*
1l LB medium: 10 g tryptone, 5 g yeast extract, and 10 g NaCl were dissolved in deionized water and the pH was adjusted to 7.0 using NaOH. For plates 15g agar/1l medium was added. The medium was sterilized by autoclavation.
When antibiotics were needed for selection the solution was cooled down to approximately 55°C, and antibiotics were added. (50µg / mL of Ampicillin or Kanamycin). The medium as well as the plates can be stored at +4°C.
- BHI for growing *L. monocytogenes*
37g Brain- Heart- Infusion- powder was dissolved in ddH2O for 1l medium and sterilized by autoclavation. For plates 1% agar/1l medium was added.

8.1.3 Bacteria

The *L. monocytogenes* strain LO28 was used to produce heat killed *L. monocytogenes* (hkl). Hkl was utilized to induce the NFκB part of the investigated pathway.

For the cloning competent *E.coli* TOP10 were used.

8.1.4 Interferon β

250 U/ml of recombinant mouse Interferon Beta from pbl interferon source was used for stimulation of the BMDMs as well as RAW 264.7 cells.

8.1.5 Antibodies against

- RNA Pol II polyclonal IgG, 200µg/ml, Santa Cruz Biotechnology
- Serine2P polyclonal IgG, 200µg/ml, Bethyl
- Serine5P polyclonal IgG, 200µg/ml, Bethyl
- Serine7P monoclonal, IgG, Millipore
- p65 polyclonal IgG, 200µg/ml, Santa Cruz Biotechnology
- p50 polyclonal IgG, 200µg/ml, Santa Cruz Biotechnology
- Cdk7 polyclonal IgG, 200µg/ml, Santa Cruz Biotechnology
- Cdk9 polyclonal IgG, 200µg/ml, Santa Cruz Biotechnology
- Stat1 polyclonal IgG, 200µg/100µl, Santa Cruz Biotechnology

8.1.6 Primers

primers for analyzing the ChIP

Nos2 dist.: annealing temp.: 60°C

forward: 5'- CCAACTATTGAGGCCACACAC 3'

reverse: 5'- GCTTCCAATAAAGCATTCACA -3'

Nos2 prox.: annealing temp.: 61°C

forward: 5'- TGTAAGTTGTGACCCTGGCAGCA - 3'

reverse: 5'- AAGCACACAGACTAGGAGTGTTCA - 3'

IRF1 (3' UTR): annealing temp.: 62°C

forward: 5'-TGCTGTGCTGGTCTTG-3'

reverse: 5'- CCTTCATTCCGTCCTGTCCTT-3'

Nos2 Exon2: annealing temp.: 65°C

forward: 5'- ACGGAGAACGTTGGATTTGGA -3'

reverse: 5'- TGAGAACAGCACAAGGGGTTT -3'

Nos2 Exon8: annealing temp.: 65°C

forward: 5'- TTCACAGCTCATCCGGTACG -3'

reverse: 5'- AACTCCAAGGTGGCAGCATC -3'

Nos2 Exon17: annealing temp.: 65°C

forward: 5'- AGTTCTGCGCCTTTGCTCAT -3'

reverse: 5'- AGTTCGTCCCCTTCTCCTGT -3'

Nos2 Exon26: annealing temp.: 62°C

forward: 5'- CTGGCCAATGAGGTACTCAGC -3'

reverse: 5'- CTGGAAGAAATAGTCTTCCACCTG -3'

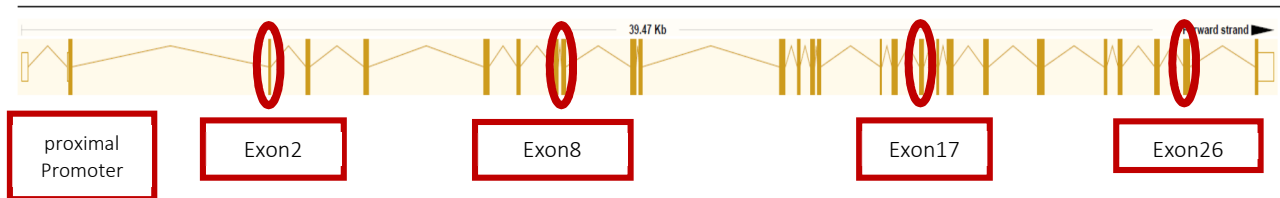


Figure 31. Primer distribution at the Nos2 gene for qPCR analysis after ChIP for investigating serine phosphorylation at the CTD of Pol II (modified from <http://www.ensembl.org>)

primers for PCR-mediated amplification from cDNA:

p50 (subunit of NFκB):

forward: 5'- GCGACGCGTCGTATGGCAGACGATGATCCCTAC - 3'

with MluI restriction site at the 5'- end

forward: 5'- GCGCCCGGGCGAATGGCAGACGATGATCCCTAC - 3'

with SmaI restriction site at the 5'- end

forward: 5'-GGGCCTTCCATATGGACTTCCATGGCAGACGATGATCCC- 3'

with NdeI restriction site at the 5'- end

reverse: 5'- GGCAGACGACATATGTGCGGACGACGCCGAGGCTGAAGCGCCATGGGTGACCCCTGC - 3'

NdeI restriction site

Cdk7:

forward: 5'- TCGTCCGCACATATGTCGTCTGCCGCTGTGGACGTGAAGTCTC - 3'

NdeI restriction site

reverse: 5'- GAATTCCTACTTGTTCATCGTCATCCTTGTAAATCAAAAATGAGCTTCTTGGGC - 3'

EcoRI restriction site

Cdk9:

forward: 5'- TCGTCCGCACATATGTCGTCTGCCGCAAGCAGTACGACTCG - 3'

NdeI restriction site

reverse: 5'- GAATTCCTACTTGTTCATCGTCATCCTTGTAAATCGAAGACACGTTCAAATTCCGTC - 3'

EcoRI restriction site

primers for cDNA preparation (specific priming):

p50: 5'-GCCATGGGTGACCCCTGC-3'

Cdk7:5'-AAAAATGAGCTTCTTGGG-3'

Cdk9:5'-CCGTCTGGTTGGTGGTGG-3'

8.1.7 Cloning system

A lentiviral Tet- ON system was used. I decided to use the Lentiviral Tet-On 3G Inducible Expression Systems sold by Clontech. This is a two-vector lentivirus format for inducible expression in eukaryotic cells.

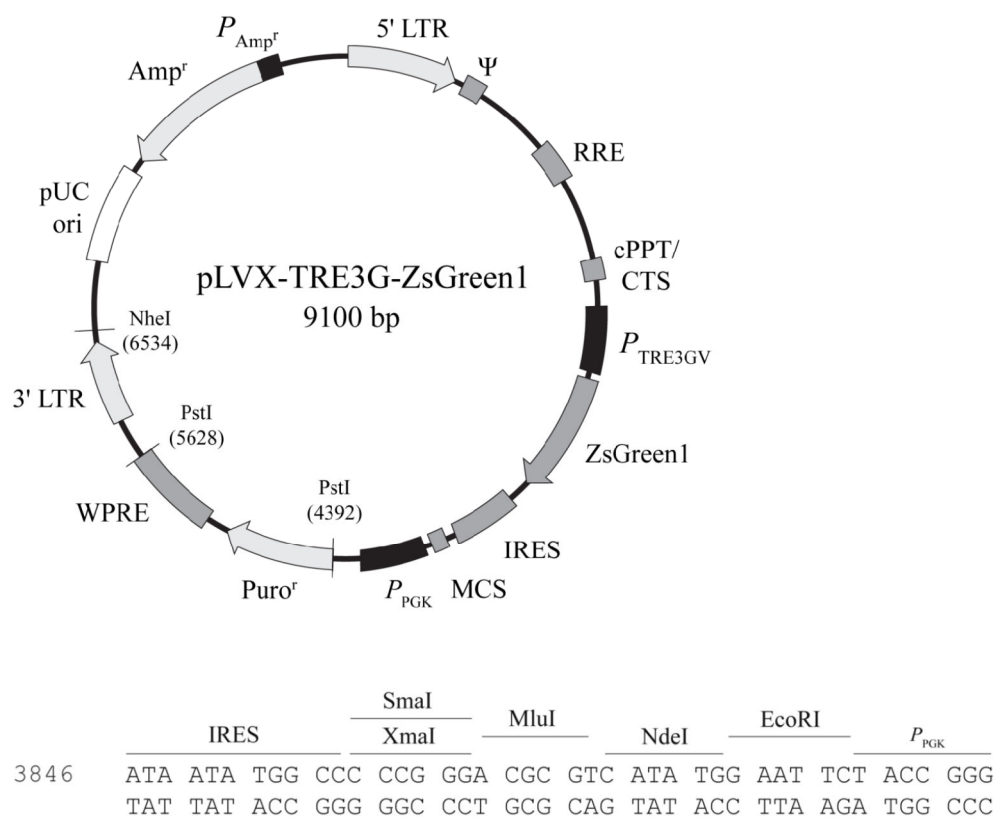


Figure 32. Vector map and multiple cloning site (MCS) of pLVX – TRE3G – ZsGreen vector (clontech, 2014)

8.1.8 Restriction enzymes

All enzymes for digestion reaction were purchased from Thermoscientific. Both FastDigest and conventional enzymes were used.

Restriction enzyme	Restriction site
Smal	5'...C C C↓G G G...3' 3'...G G G↑C C C...5'
MluI	5'...A↓C G C G T...3' 3'...T G C G C↑A...5'
NdeI	5'...C A↓T A T G...3' 3'...G T A T↑A C...5'
EcoRI	5'...G↓A A T T C...3' 3'...C T T A A↑G...5'
BamHI	5'...G↓G A T C C...3' 3'...C C T A G↑G...5'

Figure 33. Restriction enzymes used for cloning.

8.1.9 Enzymes and materials used for PCR, ChIP and cloning

Product	company	ID number	
SYBR GoTaqqPCRMasternmix	Promega	A6002	1,000 × 50µl reactions
Pfu- Polymerase	Thermo sceintific	EP0571	100U
T4 DNA Ligase	Thermo sceintific		
FastAPthermosensitive alkaline phosphatase	Thermo sceintific	EF0654	300U
dNTPs	5Prime	Deoxynucleotide Mix, PCR grade	
RNase (DNase free)	Thermoscientific		
ProteinaseK	Thermo sceintific	#EO0491	
Chelex 100% (Biotechnology Grade Chelex 100 Resin)	Bio-Rad	143-2832	100g
Complete, EDTA-free (Protein inhibitor cocktail tablets)	Roche	05 056 489 001	3x20 tabl.

8.1.10 Kits

Kit	company	usage
PCR prufication kit	5Prime	Clean up PCR samples directly or after digestion and FastAP treatment without gel separation
Gel extraction mini kit	5Prime	Clean up samples from agarose gels after PCR, digestion or control digestions
PerfectPrep Spin Mini Kit	5Prime	Miniprep of plasmids from bacteria after successful transformation
NucleoSpin RNAII/Nucleid acid + protein purification	Macherey- Nagel	Isolate RNA from stimulated and unstimulated cells to determine gene expression

8.2 METHODS

8.2.1 Cell culture

Standard conditions for cultivating eukaryotic cells are 37°C, 5%CO₂ and 95% humidity.

Cultivating bone marrow derived macrophages (BMDMs)

Bones of mice were isolated, crushed in a pre-sterilized mortar and filtered through a cell strainer (70µm). The cell suspension was centrifuged at 1300 rpm for 5 min and the pellet was dissolved in DMEM + AB (Penicillin and Streptomycin) + 10% FCS + 10% L-Cond. The Bone marrow cells were cultivated on culture dishes and incubated at 37°C. 3 days later the cells were fed with medium with the same additives. After 10 days, the fully differentiated macrophages were used for experiments (Baccarini M., 1985).

Cultivating RAW 246.7

RAW 246.7 were seeded as follows to obtain approximately 80% confluence in 2-3 days:

1. 150 mm dishes: 5×10^6 /20 ml medium
2. 100 mm dishes: 2×10^6 /10 ml medium
3. 60 mm dishes: 5×10^5 /5 ml medium
4. 6-well dishes: 3×10^5 /well/3 ml medium

The maximum passage number was 24 passages, thereafter significant changes in transfection efficiency have been observed (lipidmaps/protocols, 2004).

Freezing and thawing of cells

Freezing: the cells (RAW 246.7 or BMDM) were detached from the dish, transferred to a falcon tube and centrifuged at 1300rpm for 5min. The supernatant was discarded and the cells were resuspended in 90% FCS + 10%DMSO. The cell suspension was transferred to a cryotube and quickly transferred to -80°C. For longer storage the cells were put into liquid N₂.

Thawing: Cells were quickly thawed by re-suspending in prewarmed medium. The cells were transferred to a Falcon tube and centrifuged at 1300rpm for 5min. The supernatant was removed and the cells were again resuspended in medium and plated on a 10cm dish.

8.2.2 Heat killed *L. monocytogenes*

Bacteria were incubated at 70°C for 20min. Subsequently, complete loss of viable cells was ascertained by plating on BHI medium.

8.2.3 mRNA extraction and cDNA preparation

To analyze the expression level of cytokine and enzyme-encoding genes that play a crucial role in the immune response, the mRNA from untreated and treated macrophages was isolated as described below.

Table 1: treatment of macrophages for mRNA isolation

Method	Macrophages/well	IFN β	hkL
mRNA extraction	1 x 10 ⁶ cells in 6cm dish	250U	50 MOI

The NucleoSpin RNAII/Nucleid acid + protein purification kit from Macherey- Nagel was used for RNA extraction. The amount of RNA was analyzed with the Nanodrop method.

For cDNA preparation 10 μ l sample and 1 μ l Oligos were used. They were placed on the heating block for 10min at 70°C. 8 μ l Mastermix (2 μ l dNTPs, 2 μ l H₂O, 4 μ l buffer) was added and incubated at 37°C for 10 min, followed by the addition of 1 μ l rev. transcriptase and incubation at 42°C for 60min. Finally, the samples were heated up to 70°C for 10min.

Preparation of cDNA with specific priming was performed likewise, to troubleshoot bad PCR amplification (primer see above).

8.2.4 Chromatin Immunoprecipitation

Chromatin immunoprecipitation (ChIP) is a technique to investigate protein–DNA interactions. Combined with next generation sequencing it is used to map DNA target sites for transcription factors and other chromosome-associated proteins and histone modifications. The function of a protein or transcription factor in gene expression can be partly specified by the location of that protein along a specific gene sequence. In recent years, ChIP technology has developed rapidly. It is suitable to determine association of proteins located within 2A of DNA (Spencer V.A., 2003), (Das P.M., 2004), (Collas P., 2010).

Seeding the cells

After counting the cells, ~2.0 - 2.5 x 10⁷ BMDMs or ~1.7 x 10⁷ RAW cells were seeded on 15cm dishes.

Treatment of the cells

The cells were treated with hkL and/or IFN β and treatment was stopped by adding the cross linking solution as described below.

Table 2: treatment of macrophages for ChIP experiments

Method	macrophages	IFN β	hKL
ChIP	- - 2,5 x 10⁷ BMDMs in 20 ml (14mm dish) - 1,8 x 10⁷ RAW cells in 20ml	250U	50 MOI (600 μ L for 20ml)

Reversible cross-linking

DNA and proteins are commonly cross-linked with formaldehyde (which is heat-reversible) to covalently attach proteins to target DNA sequences. For this we added Formaldehyde to the medium ($\rightarrow$ 540 μ L/20ml of 37% stock) and incubated 10 minutes at 37°C.

To stop the crosslinking reaction, glycine was added to a final concentration of 0.25M ($\rightarrow$ 1ml/20ml of 2.5M) and the samples were left on a shaker at RT.

Washing and lysing of the cells

The cells were washed twice in PBS and then scraped in 5 ml PBS. Afterwards they were centrifuged 5minutes at 4°C at 600g. 4ml of washing buffer I was added, followed by a centrifugation step and after sucking off the supernatant, wash buffer II was added. Both washing solutions were kept for 10 minutes on the cells. The samples were centrifuged as before and the pellet was resuspended in 750 μ L lysis buffer and the samples were kept o/n at +4°C (rotation). All buffers contained protease inhibitors. In this experiment, PMSF (1:100 from the 10mM stock in isopropanol) was used together with protease inhibitor cocktail tablets (30 μ L/ml) (Roche).

Sonication

The samples were sonicated with the Bioruptor Standard from diagenode, 27 cycles for BMDMs and 10 cycles for RAW 264.7 at high frequency. Then, the samples were centrifuged twice 30min at 10°C at 13.000 rpm. The amount of DNA in the supernatant was measured with the nanodrop method (PEQlab).

Chelex

To analyze the size of the fragments received from the sonication, Chelex DNA purification was carried out followed by gel electrophoresis.

25-30 μ L chromatin were used and 100 μ L 10% chelex were added, vortexed, and finally removed from the Falcon tube. The samples were incubated 10 minutes on a thermomixer at 99°C and 1400rpm. Afterwards, they were cooled down at the bench for approximately 10min. 1 μ L RNase

(Fermentas special without DNase) was added and the samples were incubated 30min at RT. Subsequently, 2µl ProteinaseK was added and the samples were incubated on a thermomixer at 55°C for 60min and 300rpm. Subsequently, the samples were centrifuged 5 min at 13200 rpm and 75µl of the samples was mixed with 15µl orange loading dye and loaded on a 2% agarose gel (10- 15min at 120V).

Binding of the antibodies

For the next steps, 25-30µg DNA per sample were used. The input (25-3ng; calculated after measurement with the nanodrop) was taken away and stored in the fridge for later use. The other samples were diluted 1:10 with dilution buffer (with inhibitors). Later on, the antibodies were added and the samples were rotated o/n at 4°C.

Preparation of the beads

45µl beads (Dynabeads Protein G from Invitrogen (30mg dynabeads/ml in PBS, pH 7,4) per sample were used. The beads were washed three times with dilution buffer and then dilution buffer with a final concentration of 0,1mg/ml BSA was added. The beads were rotated o/n at +4°C and thereafter combined with the samples, followed by further rotation for 3hrs at 4°C. Afterwards several washing steps were performed sequentially with the following buffers:

1ml was added (rotate samples 10 minutes at 4°C after each step):

- RIPA
- high salt
- LiCl
- 2x TE

Elution

400µl fresh elution Buffer were added to the samples and they were shaken for 40min at 1400rpm and RT.

Reverse crosslinking

The samples were transferred to fresh Eppendorf tubes and 20µl 4M NaCl were added. They were shaken at 300rpm at 65°C o/n.

Digestion of proteins

2µl Proteinase K, 8µl EDTA 0.5M pH 7.5 and 16µl IM TRIS pH 6.5 were added and the samples were shaken again (850rpm) at 55°C for 1 hour.

Phenol extraction of nucleic acids

Phase lock gel tubes from 5 prime were used to perform a phenol extraction. The samples were transferred into the phase lock tubes and 500µl Phenol-ChloroformIsoamyl Alcohol (PCI) was added. The samples were centrifuged 15min at full speed.

The water phase was transferred to new Eppendorf tubes and 40µl 3M CH₃COONa pH 5.2, 1µl Glycogen and 1ml 96% EtOH (uvg.) were added. The samples were stored on -20°C for at least 3 hours or o/n. Afterwards, they were centrifuged at full speed for 45 min (4°C). The pellets were washed with 70% ice cold EtOH and again centrifuged 15min at full speed (4°C). The samples were dried and afterwards diluted with 200µl H₂O.

ChIP buffers and inhibitors:

Inhibitors:

PMSF: use 1:100 (stock: 100mM in isopropanol)

Protease inhibitor: 50ul/ml (stock: 1 tablet in 2ml H₂O)

Buffers:

Wash I (400ml)		Wash II (400ml)	
10% Triton-X100	10ml	4M NaCl	20ml
0.5M EDTA	8ml	0.5M EDTA	0.8ml
0.5M EGTA	0.4ml	0.5M EGTA	0.4ml
1M HEPES	4ml	1M HEPES	4ml

Dilute in H₂O and sterile filter

Dilution Buffer (400ml):		RIPA buffer (400ml)	
4M NaCl	16.7ml	4M NaCl	15ml
1M Tris pH 8.1	6.68ml	1M Tris pH 8.0	20ml
0.5M EDTA	0.96ml	20% SDS	2ml
10% Triton-X100	44ml	10% NaDOC (fresh)	20ml
10% SDS	0.4ml	10% NP40	40ml

Dilute in H₂O and sterile filter

High Salt (400ml)		LiCl buffer (400ml)	
4M NaCl	50ml	1M LiCl	100ml
1M Tris pH 8.0	20ml	1M Tris pH 8.0	20ml
20% SDS	2ml	10% NaDOC (fresh)	20ml
10% NP40	40ml	10% NP40	40ml

Dilute in H₂O and sterile filter

TE buffer (400ml)		SDS-Lysis Buffer (200ml)	
1M Tris pH 8.0	4ml	10%SDS	20ml
0.5M EDTA	0.8ml	0,5M EDTA	4ml
		Tris pH8	10ml

Dilute in H₂O and sterile filter

Elution Buffer (5ml): 1ml 10% SDS
0.5ml 1M NaHCO₃ (0,168g/2ml H₂O)
50ul 1M DTT

qPCR for analysis of the ChIP samples

For analysing the ChIP samples real-time quantitative PCR (qPCR) was used. qPCR was performed with Eppendorf Realplex mastercycler and the SYBR GoTaq qPCR Mastermix (Promega). The same setup was used for all PCR protocols, except the annealing temperatures were changed according to the primer.

Table 3: qPCR conditions for analysing the ChIP

Step of PCR	temperature	time	Cycles
Initialization	95°C	2min	1x
denaturation	95°C	15sec	40x
annealing	dependent on primer pair	15sec	
elongation	72°C	20sec	
Measurement of SYBGR at 88°C to avoid primer dimers	88°C	30sec	
melting curve	60°C → 95°C	10min	1x

DNA cloning

8.2.5 Competent cells

E.coli TOP10. 1 colony of a LB plate was inoculated into 20 ml LB and grown O/N. From this culture, 200µl were taken and inoculated in 20ml fresh LB media. Bacteria were grown at 37°C, 2-3 hours at 260 rpm until they reached 0,45-0,5 OD. Then they were cooled down on ice for 5 min. followed by centrifugation at 3000rpm for 5 min at 4°C. The supernatant was carefully removed and the bacteria were resuspended in 5 ml of TFB I buffer and incubated on ice for 30-60 min. Next, they were centrifuged at 2000 rpm for 5 min at 4°C. The supernatant was removed again and TOP10 were resuspended in 2ml TFB II, which was previously cooled down on ice. The cells were distributed into 100 µl aliquots. Afterwards, they were shock-frosted in liquid nitrogen and stored at -80°C. All steps were performed under sterile conditions.

used buffers:

TFBI		TFBII	
KOAc	30mM	MOPS	10mM
MnCl ₂	50mM	CaCl ₂	75mM
KCl	100mM	KCl	10mM
CaCl	10mM	Glycerol	15 (w/V)%
Glycerol	15 (w/V)%	pH: 7.0	
pH: 5.8 (with AcOH)		Total volume: 50ml	
total volume: 50ml			

8.2.6 Digestion and ligation

For digestion, conventional or fast digest (FD) enzymes from Thermoscientific were used. For the reaction, the reaction mixtures were used as recommended by the supplier. For ligation, T4 DNA ligase from Thermoscientific was used. To find the right proportion of vector and insert, the correct molar ratio was calculated using size and concentration of the components. Different ratios were tested (1:5, 1:10, 1:15) and a negative control (ligation mixture without insert) was included to test for vector re-ligation.

8.2.7 Transformation of competent E. coli TOP10

10 µl of the ligation reaction was utilized to 50 µl competent TOP10 E. coli and incubated on ice for 20 min. The bacteria were heat-shocked for 80 seconds at 42° C, then put back on ice for 2-3mins. 200 µl fresh LB (without antibiotics) were added and put to 37°C while shaking for 1 hour (200 rpm). Afterwards bacteria were centrifuged at 13.000 rpm for 1 min (or 8000g/5 min), the supernatant was discarded, 50 -60 µl LB was left in the tube. This residual amount is then plated on LB-agar containing the appropriate antibiotic (in this experiment: Ampicillin). Bacteria were incubated at 37° C O/N (16-18hours).

8.2.8 MiniPrep

Single bacterial colonies were picked from the LB-plate after O/N incubation and inoculated into 5ml LB-medium. They were incubated at 37° C while shaking O/N. Plasmid isolation was performed the next day with PerfectPrep Spin Mini Kit from 5PRIME.

8.2.9 Gel- electrophoresis

To check for successful fragmentation by sonication for the ChIP experiments, a 2% agarose gel was run for optimal separation in the range of approx. 200bp at ~120V for 10-20min.

In order to assure correct digestion, fragment or vector size, a 1% agarose gel (referring to the size between 1 -9 kb) was used, which was run at ~120V for 30-40 min. If necessary, bands were cut out after electrophoresis and purified with a gel extraction kit (5Prime).

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