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„Transcriptional regulation by mediator-associated kinases CDK8 and CDK19“

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1. Abstract

Gene expression in immune responses is a highly complex and well-orchestrated process. An altered regulation of gene transcription may lead to disease. Despite extensive studies over the last decades, transcription regulation remains incompletely understood. In particular, mechanisms that quantitively regulate the transcriptional output remain underexplored.
The kinases CDK8 and CDK19 can reversibly associate with the versatile Mediator complex, a key component of the transcription machinery, and were lately revealed as key regulators in transcriptional regulation. The effects of CDK8 are broad and range from changes in chromatin to phosphorylation of transcription factors such as STAT1. Recent research of our lab disambiguated the roles of CDK8 and its paralog CDK19 in IFN-γ-driven immune responses. Both kinases were regulating the quantity of transcription of IFN-γ target genes. However, as both of them either up or downregulate a large subset of genes, the exact mechanisms underlying the effects of CDK8 and CDK19 remain elusive.
In this study we confirmed that CDK8 is the key driver of IFN-γ-induced phosphorylation of STAT1 at S727 in MEFs. We further confirmed that pharmacological inhibition of the mediator kinase function results in lower induction of the IFN-γ target genes *Irf1* and *Gbp2.* A flexible cell-based system combining mediator kinase inhibition with an inducible CDK8 knockout was optimized for ChIP experiments. Subsequently, ChIP experiments probing the occupancy of STAT1, total RNA polymerase II (Pol II), Ser 2-phosphorylated (pS2) Pol II and Ser 5-phosphorylated (pS5) Pol II were conducted. Our data suggests that mediator kinase activity regulates pS5 Pol II abundance in the gene body of the IFN-γ target gene *Irf1* and Pol II abundance at GAS of *Gbp2.* Future work employing ChIP-Seq experiments will provide a broader and more detailed understanding of the role of the mediator kinases in IFN-γ-regulated transcription.

2. Introduction

2.1 Interferons

The immune system is a host defense system relying on well-structured biological processes within an organism that protects against disease upon different pathogenic stimuli like viruses, bacteria and other harmful foreign substances. These processes are orchestrated by small soluble peptides called cytokines which are produced by a variety of cells of the innate and adaptive immunity. Cytokines play a crucial role in cell signaling and immunomodulation and have been shown to be involved in autocrine, paracrine and endocrine signaling. Upon encounter of a pathogen with a host cell, pathogen-associated molecular patterns (PAMPs) are recognized by pattern recognition receptors (PRRs) on the host cell surface[1]. PRRs can be found either in the cytosol or associated with cellular or endosomal membranes. They can be divided into four major sub families: The Toll-like receptors (TLRs), the nucleotide-binding oligomerization domain (NOD) – Leucin Rich Repeats (LRR) – containing receptors (NLR), the retinoic acid-inducible gene 1 (RIG-1) – like receptors (RLRs), and the C-type lectin receptors (CLRs)[2,3].

The interaction of the PRRs with PAMPs activates the innate immune response against pathogenic agents and further leads to the establishment of a strong adaptive immune response.

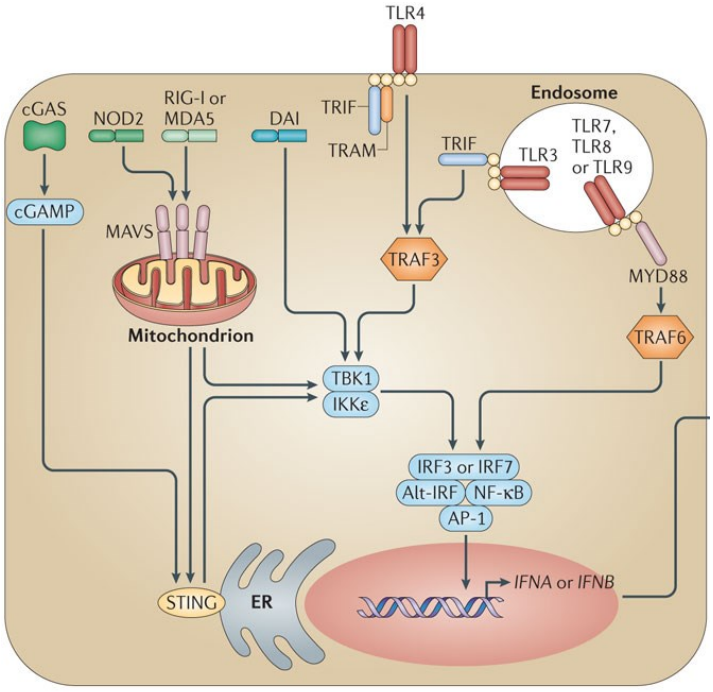
Interferons (IFNs) belong to the family of cytokines and are considered being pro-inflammatory. Based on the type of receptors through which they signal, they have been classified into three major types: Type I IFNs (IFN-α, IFN-β, IFN-δ, IFN-κ, IFN-ε, IFN-τ, IFN-ω), Type II IFNs (IFN-γ) and Type III IFNs (IFN-λ1/λ2/λ3/λ4)[4,5]. All three classes are considered antiviral and antibacterial agents and exhibit immunomodulatory properties. However, the interferon production must be tightly controlled as an over-production of them would cause excessive inflammatory responses and host injury, leading to interferonopathies. Interferonopathies are an expanding group of complex genetic disorders characterized by disturbance of homeostasis due to overexpression of IFNs[6].

2.1.1 Type I Interferons – relevance, production and signaling

Each type I IFN is encoded by a single gene with the exception of IFN-α, which comprises 13 subtypes in humans[7]. While IFN-α/β are the best understood type I IFNs and can be produced by almost all somatic nucleated cells, specialized immune cells known as plasmacytoid dendritic cells produce the vast majority of IFN-α[7].

The production of IFN-α/β is triggered by association of PAMPs with either cytosolic or membrane bound PRRs during an infection. The cytosolic PRRs include the RNA helicase RIG-I and the melanoma differentiation-associated gene 5 (MDA5). They can recognize RNA and are highly associated with type I IFN induction[8]. Likewise, some receptors like the DNA-dependent activator of IFN-regulatory factors (DAI), the DEAD box helicases and the cytosolic GAMP synthase (cGAS) recognize DNA motifs in the cytosol and are also tightly linked to type I IFN production[8]. Finally, NOD1 and NOD2, two members of the NLR-family, recognize nucleic acids and other ligands in the cytosol as well and are therefore also leading to IFN-α/β production[8].

In addition to the above mentioned cytosolic receptors, PAMPs can also be detected outside of cells via TLR-4, which recognizes LPS, or in the endosome via TLR-3/7/8/9 to activate pathways that lead to IFN-α/β



(*McNab,* F., Mayer-Barber, K., Sher, A., Wack, A. & O'Garra, A. Type I interferons in infectious disease. Nature Reviews Immunology vol. 15 87–103 (2015).

Figure 1 **IFN-α and IFN-β production during infection**
Production of type I interferons is triggered by association of PAMPs with either cytosolic or membrane bound PRRs: RIG-I and MDA5 recognize RNA originating from viruses, DAI and cGAS recognize DNA motifs in the cytosol, NOD2 recognizes nucleic acids and other ligands in the cytosol, TLR-4 recognizes LPS on the cell surface and TLR-3/7/8/9 PAMPs in the endosome. Most of the pathways converge in activation of IRF3 and IRF7, NF-κB and AP-1, ultimately leading to production of IFN-α and IRF-β[8].
IRF3/IRF7, interferon regulatory factors; cGAS, cytosolic GAMP synthase; RIG-I, retinoic acid inducible gene 1; MDA5, melanoma differentiation-associated gene 5; NOD2, NOD-containing protein 2; DAI, DNA dependent activator of IRFs; TLR4, toll-like receptor 4; TRIF, TIR domain-containing adaptor protein; TRAM, TLR adaptor molecule; MAVS, mitochondrial antiviral signaling proteins; STING, stimulator of IFN genes; MYD88,myeloid differentiation primary response protein 88; TRAF3/6, TNF receptor associated factor; TBK1, TANK binding kinase; IKKε, IκB kinase ε; NF-κB, nuclear factor κB; AP1, activator protein 1

production (Figure 1)[8,9]. Downstream of the above mentioned receptors, the expression of IFN-α/β is induced by the activation of certain transcription factors, including interferon regulatory factors (IRFs), NF-κB and AP-1[10]. The most fundamental IRFs to induce type I IFN expression are IRF3 and IRF7. The initial transcription of the IFN-α/β genes totally relies on IRF3. This event, in turn, triggers transcription of IRF7, which orchestrates a positive feedforward loop[8]. This results in a second, strong wave of IFN-α/β gene expression. NF-κB is most likely required as a cofactor for IRF3 and IRF7 in this pathway. The main driver kinases in the type I IFN pathway are IkB-kinase-ε (IKKε) and TANK-binding kinase 1 (TBK1), as they phosphorylate IRF3 and IRF7[8,11].

A key sensor causing type I IFN production is the cyclic GMP-AMP synthase (cGAS). It is capable of recognizing DNA of either pathogenic or self-origin. Upon binding to double-stranded DNA, cGAS catalyzes the synthesis of a cyclic dinucleotide (referred to as cGAMP) from the precursor molecules ATP and GTP. This dinucleotide in turn stimulates the sensor STING, which induces the recruitment of the protein kinase TBK1 and IRF3. Phosphorylation of IRF3 by TBK1 promotes oligomerization of IRF3, followed by its translocation to the nucleus where it activates expression of the IFN-α/β genes together with the co-factor NFκB[12–14].

The RNA sensor RIG-I signals through the adaptor mitochondrial antiviral signaling protein (MAVS) to activate TBK1[15]. TLR3 and TLR4 use the well-known adaptor TRIF, TLR7 and TLR9 the myeloid differentiation primary response protein 88 (MYD88) to activate TBK1[16].

Once IFN-α/β are produced, they can trigger an immune response by binding a transmembrane receptor termed IFN-α/β receptor (IFNAR) comprising of two subunits IFNAR1 and IFNAR2 (Figure 2). The two distinct kinases tyrosine kinase 1 (Tyk1) and Janus kinase 1 (Jak1) are associated with IFNAR1 and IFNAR2, respectively[17]. Upon binding of IFN-α/β, the receptor dimerizes and the receptor-associated kinases undergo cross-phosphorylation which causes their activation[17]. The activated kinases further phosphorylate the receptor to promote dimerization of STAT1 and STAT2 via phosphorylation of tyrosine 701. The new formed dimeric molecule is able to translocate to the nucleus and associates with IRF9 to form a trimolecular complex called interferon-stimulated gene factor 3 (ISGF3)[17]. This complex binds to IFN-stimulated response elements (ISRE) on the DNA and promotes expression of several hundred interferon stimulated genes (ISGs) which allow the cell to establish an anti-viral state[8].

In addition to the STAT1-STAT2 heterodimer being formed in response to type I IFNs, other cytokines can cause formation of the STAT1-STAT1 homodimer and the STAT3-STAT3 homodimer. Although both of them bind to gamma-activated sequences (GASs), the STAT1-STAT1 homodimer acts as a pro-inflammatory complex, whereas the STAT3-STAT3 homodimer indirectly suppresses pro-inflammatory gene expression by the induction of as-yet-unknown repressors[8,17].

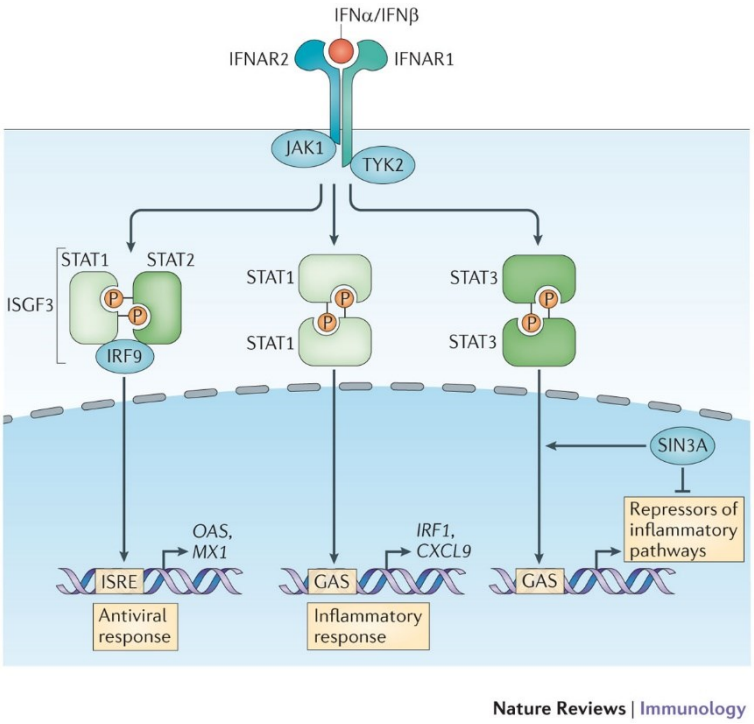


Figure 2 **Downstream signaling of type I interferons during infection**
Prior produced IFN-α and IFN-β can bind IFNAR, which dimerizes upon cytokine binding, ultimately leading to dimerization of STAT1 and STAT2. The new formed dimeric molecule translocates to the nucleus and associates with IRF9[17]. This complex can bind a region termed ISRE, which in turn promotes expression of several hundred genes which allow the cell to establish an anti-viral state[8]. Other dimeric molecules like STAT1-STAT1 and STAT3-STAt3 homodimers can lead to an in inflammatory response by either binding ISRE or GAS. IFNAR1/2, Interferon α receptor 1, TYK2, tyrosine kinase 1; JAK, Janus kinase 1; PI3K, phosphoinositide 3 kinase; MAPKs, mitogen activated protein kinases; STAT1/2, signal transducer and activator of transcription 1/2; ISRE, interferon stimulated response element; ISG, interferon stimulated gene; GAS, gamma-activated sequence (picture from Ivashkiv et.al.,2014[17]).

Type I IFNs have a pronounced impact on the development of innate and adaptive immune responses. To confirm the impact of type I IFNs on the innate immunity, it was shown that murine dendritic cells (DCs) undergo phenotypic maturation upon exposure to type I IFNs *in vivo* and *in vitro*[18]. DCs derived from the bone marrow of mice lacking a receptor for type I IFNs showed reduced surface expression of costimulatory molecules and an impaired ability to stimulate naive T-cell proliferation[18].

Type I IFNs have also been shown to aid clonal expansion and effector functions of $CD8^+$ T-cells as well as facilitate gene expression relevant for $CD8^+$ T-cell differentiation[19].

Previous reports also suggested, that type I IFNs affects B-cells only through their effects on DCs, yet it was shown that B-cells are in fact direct targets of type I IFN-mediated activation[20].

2.1.2 Type II Interferons – relevance, production and signaling

Interferon gamma (IFN-γ) is the only member of the type II IFN family and a cytokine that is critical for both innate and adaptive immunity against viral and bacterial infections[21]. Contrary to the type I IFN family, whose members get produced by almost all somatic nucleated cells, only NK cells, $CD4^+$ Th1 T-cells and some $CD8^+$ cells contribute to the production of IFN-γ. While under certain *in vitro* conditions in the early immune response, IFN-γ mRNA transcripts and protein can also be found in the macrophage lineage and in dendritic cells, they do not appear to be relevant IFN-γ producers in vivo[22]. Naive $CD4^+$ and $CD8^+$ T-cells can transcribe the gene encoding for INF-γ in a maturation process, dependent on certain transcription factors and chromatin topology within the *Ifng* locus[23]. Antigen presenting cells (APCs) can recognize PAMPs by membrane bound PRRs[24]. This activates the expression and secretion of IFN-γ by NK cells. Additionally, other cytokines like IL-18, TNF-α and type I IFNs are also known to be potent activators of IFN-γ synthesis[25]. Once IFN-γ is secreted, it can mount an immune response by binding its transmembrane IFN-γ receptor (IFNRG) comprising of two subunits IFNGR1 and IFNGR2 (Figure 3)[26]. Two phospho-tyrosine-kinases, termed Janus kinase 1 (Jak1) and Janus kinase 2 (Jak2) are tightly associated with IFNGR1 and IFNGR2, respectively. Upon binding of the cytokine, the receptor dimerizes and the two kinases cross-phosphorylate themselves. This leads to recruitment of STAT1 into close proximity of Jak2, which in turn catalyzes phosphorylation of STAT1 on tyrosine residue 701 (Y701 STAT1)[26]. This event leads to formation of STAT1-STAT1 homodimers that are able to translocate to the nucleus where they bind gamma activated sites (GASs, TTTCCNGGAA) on the DNA. Consequently, this drives expression of a set of primary and secondary response genes to establish an anti-pathogenic state in the cell[26].

It is important to note that the cyclin-dependent kinase 8 (CDK8) was shown to phosphorylate STAT1 within the regulatory trans-activation domain (TAD) on S727. This CDK8-mediated STAT1 phosphorylation positively or negatively regulated over 40 % of IFN-γ-responsive genes[27].

Nonetheless, full-fledged activation of STAT1 requires phosphorylation on both tyrosine 701 and serine 727[28]. However, S727 phosphorylation can also be promoted independently of CDK8 by the p38 mitogen-activated kinase upon treatment with stress inducing agents like LPS, TNF-α or UV irradiation[28].

NK cells constitutively express basal levels of IFN-γ mRNA, allowing for rapid secretion of the potent cytokine in case of infection. To fine tune the NK cell response, a complex network of activation and inhibition receptors underlies this cell system. Activating receptors can bind to molecules mostly found on infected and damaged cells. The inhibitor receptors counteract the activating receptors and prevent the NK cell from damaging healthy cells, establishing a precisely regulated system. The inhibitory receptors contain immunoreceptor tyrosine-based inhibitory motifs (ITIMs) that can signal via certain phosphatases like SH2-containing protein tyrosine phosphatase 2 (Shp2) to interfere with intracellular kinase signaling[29]. Like all cytokines, IFN-γ shows strong immunomodulatory effects by upregulating both classes of major histocompatibility complexes (MHC) and activating macrophages by enhancing their phagocytosis. It is also able to control the differentiation of $CD4^+$ cells into Th1 effector cells[30]. Despite the beneficial aspects of IFN-γ, an excess of the cytokine has been associated with severe diseases like inflammatory bowel disease[31].

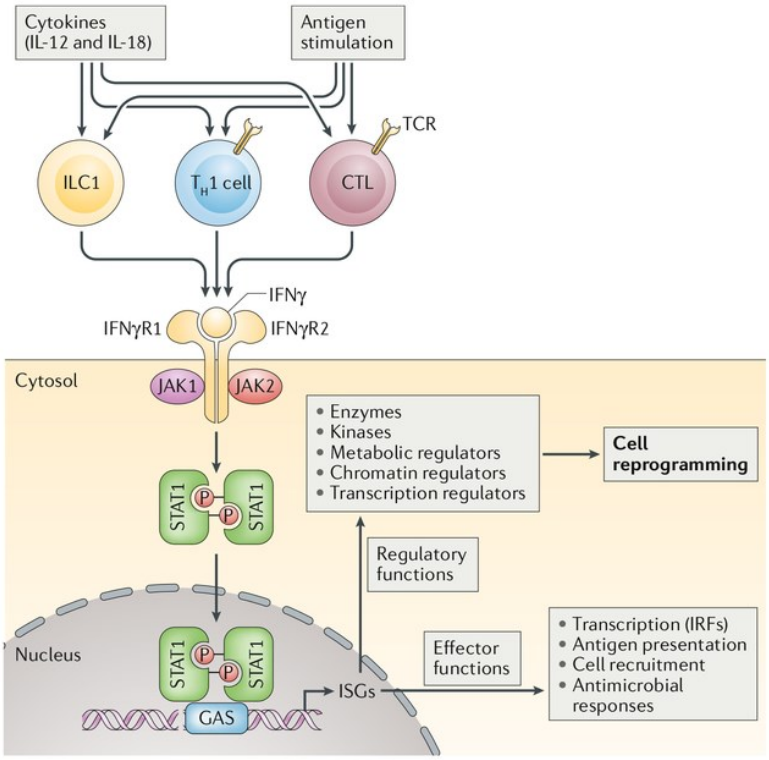


Figure 3 **IFN-γ production and signaling during infection**
Interferon-λ is produced by some immune cells and binds to the IFNGR receptor. This leads to JAK/STAT signaling cascades, ultimately resulting in ISG induction and establishment of various cellular functions including antimicrobial responses[26]. IL-12/18; Interleukin 12/18; ILC1, innate lymphoid cell 1; TH1, T-helper cell; TCR, T cell receptor; CTL, cytotoxic T lymphocytes; IFNGR, interferon-γ receptor; JAK1/2, Janus kinase 1/2; STAT1, signal transducer and activator of transcription; GAS, gamma activated sequence.

Ivashkiv, L. B. IFNγ: signalling, epigenetics and roles in immunity, metabolism, disease and cancer immunotherapy. Nature Reviews Immunology vol. 18 545–558 (2018).

2.2 Eukaryotic transcription

Eukaryotes have three different types of RNA polymerases, each having distinct roles and properties. One of the members of the family is the RNA polymerase II (RNA Pol II), which transcribes protein-coding genes most non-coding RNAs such as micro RNAs (miRNAs), small nuclear RNAs (snRNAs) and small nucleolar RNAs (snoRNAs)[9,32]. The RNA polymerase I transcribes ribosomal RNA (rRNA), the RNA Polymerase III transcribes transfer RNA (tRNA) and 5S rRNA, making them both of crucial importance for ribosome biogenesis and translation[32]. The above-mentioned properties make the enzymes an indispensable part of the cell, as it allows for quick adaptation to changes in the environment. The process of transcription is tightly regulated and will be elaborated in the next chapters.

2.2.1 Initiation and promoter escape

Initiation of transcription is a well-structured regulated process, ranging from the formation of the pre-initiation complex (PIC) up to productive elongation of RNA Pol II (Figure 4). The core promoter, a region in the immediate vicinity of the transcription start site (TSS) of protein-coding genes displays a highly conserved sequence termed the TATA-box (5´-TATAWAWAR-3´)[33]. This consensus sequence is recognized by the TATA-binding protein (TBP) which is, next to TBP-associated factors (TAFs), part of a multiprotein general transcription factor called TFIID. Subsequently, the protein TFIIA associates with the upstream region of the TATA-box, leading to stabilization of the DNA-TFIID complex[33]. Nonetheless, it was shown that the presence of TFIIA is not required for basal levels of transcription. TFIIB then enters the complex through bipartite interaction with both TBP and DNA to create a binding-hub for the RNA polymerase II – TFIIF complex[34]. TFIIB positions RNA pol II near the transcription start site (TSS) whereas TFIIF prevents unwanted interactions and aids TFIIA in stabilizing the macromolecular complex[35]. To facilitate the transition from the core PIC (RNA polymerase II – TFIIA/B/F-complex) to the closed PIC, addition of TFIIE and TFIIH is necessary. TFIIE recruits TFIIH to the PIC, stimulates the DNA-dependent ATPase activity of TFIIH and is thought to be involved in DNA melting at the promoter[36]. TFIIH possesses many subunits, of which some have been shown to be involved in processes other than transcription. Important ones are the ATPase XPB, helicase XPD and the kinase module CDK/-Cyclin-H-MAT1[37]. The ATPase XPB subunit enables, together with the helicase XPD, ATP-dependent opening of the promoter DNA at the transcription start site

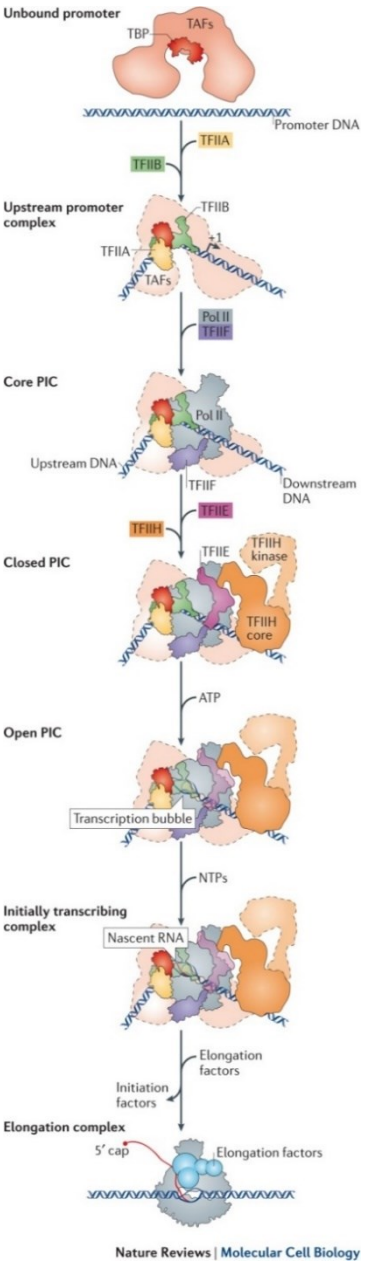


Figure 4 **Transcription initiation to productive elongation**
TAFs, TATA-associated factors; TBP, TATA-binding protein; TFIIA/B/E/F/H, transcriptional factor II A/B/E/H; PIC, pre-initiation complex; ATP, adenosine tri-phosphate; NTPs, nucleotide tri-phosphates.

Sainsbury, S., Bernecky, C. & Cramer, P. Structural basis of transcription initiation by RNA polymerase II. Nature Reviews Molecular Cell Biology vol. 16

and is therefore of vital importance in the process of transcriptional initiation and formation of the so-called open PIC[38]. RNA pol II can now initiate transcription, but has to break contacts with the PIC in a process called promoter escape. RNA pol II promoter escape occurs after 12-13 base pairs (bps) downstream of TSS and is heavily supported through mechanisms involving CDK7-dependent phosphorylation of RNA pol II C-terminal domain (CTD) on S5 (Figure 5)[38]. The CTD of RNA pol II contains 52 heptad repeats of the general consensus sequence Y1-S2-P3-T4-S5-P6-S7 [38,39]. Following promoter escape, RNA pol II pauses after generating a transcript of 20-60[40] bases in length. This pausing serves as an important mechanism to regulate general transcription in terms of timing and magnitude[38]. It has been shown that selective inhibition of the TFIIH-associated kinase CDK7 leads to a strong increase in promoter-proximal pausing at many genes[38,41]. In addition, the DRB sensitivity inducing factor (DSIF) complex is a well-established regulator of proximal promoter pausing as well[42]. Moreover, the important regulatory complex of RNA pol II, called negative elongation factor (NELF), comprising of four subunits, helps together with DSIF to stabilize RNA pol II during pausing[40,42]. However, recent data suggest that NELF might regulate other transcription steps than RNA pol II pausing. Instead, new findings indicate that TFIID is the key factor in the establishment of paused RNA pol II[43,44]. Latest research revealed the cyclin dependent kinase 8 (CDK8) as a negative regulator in polymerase II pausing, as it promotes pause release during the IFN-γ response[45]. CDK8 will be subject to further analysis in chapter 2.4. In addition, it is worth mentioning that the Mediator complex stabilizes and regulates the entire PIC assembly.

2.2.2 Elongation

The positive transcription elongation factor B (P-TEFb) is required to counteract the effects of negative elongation factors and allows the polymerase to shift its state towards productive elongation[46]. P-TEFb contains a CDK9 catalytic subunit with the corresponding cyclin T1 or T2[46]. It acts, like CDK7, on the CTD of RNA pol II and phosphorylates S2 as well as DSIF and NELF (Figure 5). This leads to reversion of the negative effects caused by DSIF and NELF and allow RNA pol II to enter the productive elongation state[46]. The transcript cleavage stimulatory factor (TFIIS) is necessary to rescue RNA pol II from an arrested state, a phenomenon that can occur during productive elongation. It does so by endonucleolytically cleaving the nascent RNA and generating a new 3´ - end that is properly aligned with the catalytic site[46,47].

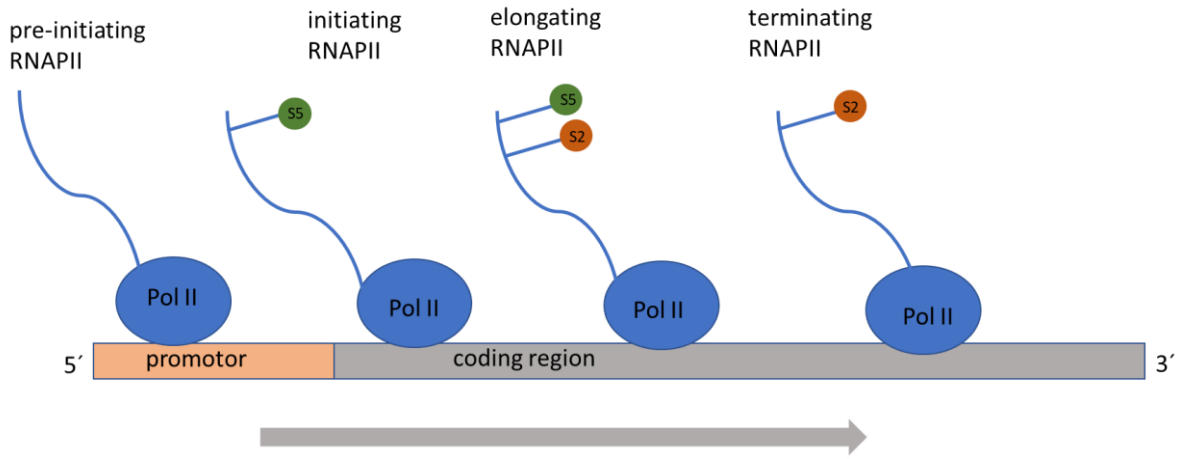


Figure 5 **Schematic simplified view of CTD-phosphorylation of RNA polymerase II during transcription**
S5 phosphorylation is catalyzed by CDK7, which supports promoter escape and shift towards productive elongation during transcription. S2 phosphorylation is catalyzed by CDK9, a subunit of P-TEFb. The S2 phosphorylated form of the RNA pol II, together with several other elongation transcription factors seem to result in more efficient recognition, cleavage and polyadenylation of mRNA and is more situated towards the end of transcription[36].

Coupled to the elongation, other important events like capping, polyadenylation and splicing ensure the proper expression of a certain gene. The capping of the 5´- ends of nascent pre-mRNAs is accomplished by an enzyme containing both RNA triphosphatase and guanylyl transferase activities. In order for the enzyme to exert its catalytic properties, the CTD of RNA pol II has to be phosphorylated specifically on S5. This event is, as stated above, catalyzed by CDK7. In addition, splicing is thought to require hyperphosphorylated S2 pol II CTDs as it recruits several key splicing factors to the elongation complex[46,48].

In order to polyadenylate the nascent mRNA 3´- end, lots of well-coordinated enzymes are necessary. The S2 phosphorylated form of the RNA pol II CTD, together with several other elongation transcription factors seem to result in more efficient recognition, cleavage and polyadenylation of mRNA[46].

2.2.3 Termination

RNA pol II termination can be elicited through different pathways, two of them being the poly(A)-dependent pathway and the Sen1-dependent pathway[49]. As the name implies, the poly(A)-dependent pathway is functionally dependent on the 3´- end maturation polyadenylation event of the nascent transcript. In order to properly initiate 3´- end maturation, RNA pol II associates with both the cleavage and polyadenylation specificity factor (CPSF) and the cleavage stimulatory factor (CstF)[9,49]. Upon transcription of the AAUAAA sequence, CPSF recognizes this motif and binds to a GU-rich site. The XNR2 endonuclease cleaves at the poly A site in a 5´- 3´ manner causing Pol II displacement from the mRNA[9,49].

An alternative RNA pol II termination pathway represents the Sen1-dependent pathway. It terminates mostly non-coding RNAs and was first discovered in *S. cerevisiae.* The helicase Sen1 is proposed to terminate transcription by catalytically unwinding the RNA-DNA hybrid in the active site[49].

2.3 The Mediator complex

As described in chapter 2.2, expression of most non-coding RNA genes and all protein coding genes is controlled by an enzyme called RNA polymerase II (RNA pol II). However, the enzyme functions not on its own and is dependent on and regulated by a macromolecular assembly called pre-initiation complex (PIC)[50]. The PIC consists of TFIIA, TFIIB, TFIID, TFIIE, TFIIF, TFIIH, RNA pol II and ultimately the Mediator complex, which was the last PIC component to be discovered[50,51]. Elegant studies in yeast revealed that activator-dependent transcription *in vivo* and *in vitro* needs a factor directly associated with the RNA pol II[52,53]. This factor was termed the Mediator complex, subsequently purified[54] and was regarded a general transcription factor (GTF) later[55]. The multi-protein complex serves as an endpoint of signaling pathways and regulates RNA pol II transcription[56].

2.3.1 The mediator complex – dynamic subunit composition and regulation

Through a combination of biochemical and structural approaches the approximately 4 MDa[51] Mediator complex was divided into four distinct modules called “head”, “middle”, “tail” and “kinase”[57] (Figure 6). The Mediator complex regulates gene expression on a global level and is considered a general transcription factor[55]. However, in contrast to other transcription factors, the Mediator complex displays high structural plasticity due to variable subunit composition. Whereas the “head”, “middle” and “tail” module of the Mediator complex form a stable core unit comprising 26 subunits, the cyclin dependent kinase 8 (CDK8) module can reversibly associate with the core Mediator complex[58]. It consists of the Mediator complex subunit 12 (MED12), the Mediator complex subunit 13 (MED13), CDK8 and its corresponding cyclin C (CCNC)[58]. The kinase CDK8 will be discussed in more detail in chapter 2.4. The MED1 and MED26 subunits are not present in all Mediator complex isolates and are likely to be located between the “middle” and the “tail” module[59]. Interestingly, post-transcriptional modifications (PTMs) seem to have a crucial impact on regulation of the Mediator complex function. 125 modifications within 17 subunits were identified in *S. cerevisiae,* one example

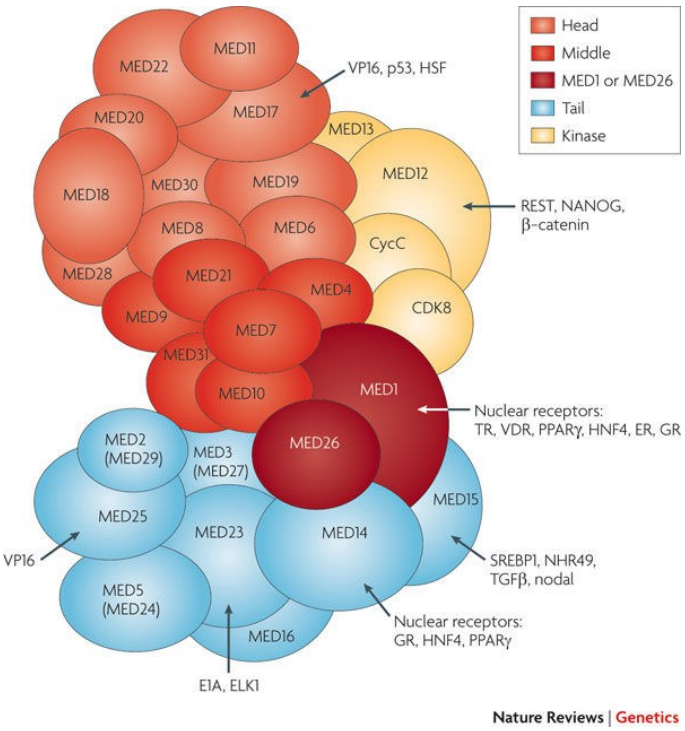


Figure 6 **Mediator complex – structure and subunit composition**
The mediator complex is divided into four distinct modules called “head”, “middle”, “tail” and “kinase”[57] and displays high plasticity due to the kinase module (yellow) reversibly associating with the whole complex when needed. Many key components interact with various subunits of the Mediator complex, ultimately leading to fine-tuning of transcription.
ER, estrogen receptor; GR, glucocorticoid receptor; HNF4, hepatocyte nuclear factor; NHR49, nuclear hormone receptor 49; PPARγ, peroxisome proliferator-activated receptor-γ; SREBP1, sterol regulatory element-binding protein 1; TGFβ, transforming growth factor-β; VDR, vitamin D3

Malik, S. & Roeder, R. G. The metazoan Mediator co-activator complex as an integrative hub for transcriptional regulation. *Nature Reviews Genetics* vol. 11 761–772 (2010).

being the phosphorylation of the “tail” subunit MED15, which seems to be required for suppression of stress-induced changes during non-stress conditions[56]. Despite PTMs as a regulatory driver mechanism, the Mediator complex can also be regulated through binding of specific microRNAs targeting specific subunits. An example for this is shown in a recent publication, where the miRNAs 378 and 378* are targeting MED13 causing metabolic dysregulation in mitochondria[60].

2.3.2 The mediator complex – versatile regulator of many functions

Despite the apparent function of the Mediator complex – regulating gene expression through a versatile set of transcription factors – other distinct roles of Mediator complex subunits have been elucidated. An example being the subunit MED15, which was shown to be essential for transforming growth factor beta (TGFβ) signal transduction in *Xenopus laevis*[61]. In addition, MED12 has been revealed to operate together with the protein regulator of cytokinesis 1 (PRC1) to silence pivotal developmental genes in pluripotency[62]. According to further studies, the subunit MED8 interacts with the Elongin BC complex and associates with Cullin2 (Cul2) and RING-box protein 1 (Rbx1) to reconstitute a ubiquitin ligase[63].

As mentioned above, the apparent functionality of the Mediator complex lies in its pleiotropic effects on genome wide transcription. The different subunits of the Mediator complex are, to a certain degree, involved in regulating different set of genes[51]. As the Mediator complex itself lacks the ability to sequence-specifically bind DNA, it relies on transcription factors to establish a connection. Thus, the link between Mediator subunits and regulation of different sets of genes derives from the fact that different transcription factors bind different Mediator complex subunits[51]. In the following sub-chapter, the role of the Mediator complex in the different stages of gene expression gets discussed in more detail.

2.3.3 The mediator complex – pivotal coordinator of gene expression

A. Chromatin remodeling and DNA looping

In eukaryotes, DNA is twined around nucleosomes to form a compressed, more dense form of genomic DNA called chromatin. In general, chromatin is said to inhibit gene expression, as formation of the PIC is topologically restrained. Interestingly, the Mediator complex was

shown to interact with two well-known chromatin remodeling complexes: The Switch/Sucrose Non-Fermentable (SWI/SNF) complex and the chromodomain-helicase-DNA-binding protein 1 (CHD 1). Thus, the Mediator complex facilitates nucleosome displacement and consequently successful PIC assembly[64,65]. To further substantiate these findings, the cyclin dependent kinase 8 (CDK8) was shown to phosphorylate the histone H3S10, which is strongly tied to transcriptional activation. Together with the histone acetyltransferase GCN5L, CDK8 seems to facilitate an open chromatin state[66].

Proteins can regulate gene expression not only by binding to sites proximal to their target genes but also by binding to distal sites. This can be accomplished by organizing the DNA in three dimensional manner in a process called DNA looping, which functions as an additional layer of gene regulation[67]. DNA looping enables interactions between linearly separated DNA sequences and seem to be of vital importance for driving cell-type specific gene expression[68]. The mediator complex plays an important role in formation and/or stabilization of looped DNA in eukaryotes, as the interaction of the Mediator complex with cohesin in a ncRNA-dependent manner enhances chromatin structure and general transcription[58,69].

B. PIC-formation

One of the most basic functions of the Mediator complex is its ability to stabilize and/or facilitate PIC assembly, which was proven by early studies in yeast and human cells[70–73]. Its central role in PIC formation is best reflected by the fact that every PIC subunit (TFIIA, TFIIB, TFIID, TFIIE , TFIIF, TFIIH and RNA pol II) is either physically and/or functionally connected to the Mediator complex[51]. In this chapter, some of the interesting interactions between certain PIC subunits with the Mediator complex are explained in more detail. Some studies were able to demonstrate functional coordination between the Mediator complex and the well-known PIC factor TFIID, as the Mediator complex was shown to orchestrate TFIID binding to the DNA[74]. Another study revealed an elegant interplay of the Mediator complex and TFIID at inducible genes under control by the metal regulatory transcription factor 1 (MTF-1)[75]. Interestingly, the Mediator complex serves as a checkpoint for gene activation as well as TFIID activity. Upon stimulation of cells with various metals in the absence of the transcription factor MTF-1 the cells display aberrant transcriptional activity due to an inactive state of TFIID at the promoter. In presence of MTF-1, the Mediator complex associates with the promoter and renders TFIID active[75].

Other studies demonstrate the dependence of the Mediator complex on recruitment of TFIIB, TFIID and TFIIE to gene promoters[76,77].

TFIIE and TFIIH play crucial roles in eukaryotic transcription as elaborated in chapter 2.2.1. TFIIH recruitment to the PIC is dependent on TFIIE, which is thought to be involved in DNA melting at the promoter[36]. TFIIH is a 10 - subunit complex that possesses an extensive range of different enzymatic properties, including ATPase, helicase and kinase activities. It should be noted that the Mediator complex binds the unphosphorylated RNA pol II CTD. CDK7, the kinase within TFIIH, phosphorylates the RNA pol II CTD which was shown to disrupt the CTD-Mediator binding, facilitating transcriptional transition into elongation[78]. Recent studies reveal a close functional interplay between the Mediator complex and TFIIH in the early stages of transcription[79]. In the presence of the Mediator complex, TFIIH associates with the PIC in an inactive state, causing the Mediator complex to bind to RNA pol II CTD. TFIIH is then able to transition into its activate form through autocatalytic ATPase activity, leading to dissociation of the Mediator complex of the RNA pol CTD, facilitating entry into elongation[79].

C. Paused RNA pol II and shift towards productive elongation

The phenomenon of paused RNA pol II in eukaryotes has been subject of discussion in chapter 2.2.1. The regulated release of paused RNA pol II into productive elongation has emerged as a major mechanism of transcriptional activation[80]. In a study from 2011 by Takahashi *et al.*, the Mediator complex subunit MED26 was identified to play a pivotal role in above mentioned pause release[80]. It is proposed to function as a docking site for transcription elongation factors. In addition, MED26 can function as a molecular switch that interacts first with TFIID in the PIC and later on exchanges TFIID for complexes containing P-TEFb to promote productive elongation[80]. This is in agreement with recent findings by Taatjes *et al.,* who found that RNA pol II pausing was lost upon replacement of the TFIID complex with TATA-binding protein (TBP)[44]. In accordance with this data, PRO-seq experiments revealed widespread disruption of RNAPII pausing upon acute depletion (t = 60 min) of TFIID subunits in human cells[44].

Moreover, the PIC factor TFIIF is of vital importance to promote shift towards productive elongation by counteracting RNA pol II pausing. According to recent studies, the negative regulator of TFIIF Gdown1/POLR2M is remodeled by the Mediator complex, which leads to RNA pol II pause release and elongation[81,82].

As the CDK8 module can reversibly associate with the Mediator complex, it is important to note that CDK8, but not CDK19 was recently shown to promote RNA polymerase II pause release during the IFN-γ response, as the inhibition of CDK8 significantly increased the pausing index[45].

2.4 Cyclin-dependent kinase 8

While the broad family of cyclin-dependent kinases were thought to only regulate checkpoint control during the cell cycle, they were later found to are also being implicated in neuronal function and transcription[83]. CDKs are catalytic subunits of a family of heterodimeric serine/threonine kinases and need a specific cyclin to exert their enzymatic properties. The discovery of CDK8 and its corresponding cyclin C dates back to 1995, where the kinase-cyclin pair was discovered as being a part of the RNA polymerase II holoenzyme[84]. Although experiments showed the importance of the kinase-cyclin pair for general transcription *in vivo*[84]*,* further studies on CDK8 have been neglected for the next few years. With the advent of high-throughput research methods in the last years, the amount of publications ascribing important roles to CDK8 increased.

2.4.1 Mediator kinase module

CDK8 is part of a 4-protein complex termed the Mediator kinase module, which consists of MED12, MED13, Cyclin C (CCNC) and CDK8[51]. Interestingly, during the evolution of vertebrate's genes, a paralogous complex comprising MED12L (MED12), MED13L (MED13) and CDK19 (CDK8) has emerged due to gene duplication events. CDK8 and CDK19 are highly similar (77% amino acid sequence identity) paralogs and each appears to associate in a mutually exclusive fashion with the Mediator kinase module[45]. The Mediator kinase module can reversibly associate with the MED13 subunit of the Mediator complex[51]. Thus, CDK8 and CDK19 are therefore collectively considered being Mediator-associated kinases. Although being highly similar paralogs, CDK8 and CDK19 seem to exhibit different functions, which will be discussed in chapter 2.4.3 in more detail.

2.4.2 CDK8 – both positive and negative regulator

Historically, CDK8 has been described mostly as a transcriptional repressor[85]. A common mechanism by with CDK8 negatively regulates transcription seems to be the binding of CDK8 via MED13 to the Mediator complex, which leads to inhibition of the interaction between

RNA pol II and the Mediator complex.

Experiments revealed a precise mechanism of CDK8 as a negative regulator of transcription, as it was shown to phosphorylate the RNA pol II CTD, which disrupts the interaction between the Mediator complex and the polymerase[85,86,87]. Moreover, CDK8 can phosphorylate cyclin H, which inhibits TFIIH, subsequently leading to transcriptional repression[88].

In contrast to the above-mentioned repressive properties of CDK8, evidence of positive regulation by the enzyme has also been growing over the last couple of years. Chromatin immunoprecipitation (ChIP) studies spanning the whole genome showed that SRB10 (CDK8 homolog in yeast) is present on both active and inactive genes *in vivo*[85,89,90]. A study from 2010 describes CDK8 as a positive co-regulator of p53 target genes, which underlines its impact on alternative stress responses such as cell-cycle arrest and apoptosis[91]. Moreover, at study from 2009 shows that CDK8 drives a cycle of Smad utilization and disposal, representing an integral part of canonical BMP and TGF-β pathways[92]. Another study found that CDK8 is recruited by Mastermind (MAM) to phosphorylate the Notch ICD to activate responsive genes[93].

Moreover, CDK8 seems to regulate transcription of inflammatory genes upon stimulation of TLR9. Under special conditions, CDK8 colocalized with NF-κB at the promoter regions of certain genes, therefore positively regulating their transcription[94]. To further substantiate the findings mentioned above, CDK8 was shown to phosphorylate the histone H3S10, which is strongly tied to transcriptional activation. Together with the histone acetyltransferase GCN5L, CDK8 seems to facilitate an open chromatin state[66]. Latest research revealed CDK8 as a key regulator in polymerase II pausing, as it promotes pause release during the IFN-γ response[45].

CDK8 is without doubt both a negative and positive regulator of transcription. A good example for this dual-edged behavior is that CDK8 phosphorylates the trans-activation domains (TADs) of STAT1, STAT3 and STAT5. In case of STAT1 the phosphorylation event takes place at S727 and causes activation of STAT1, thereby playing an essential role in the antiviral response[27]. This CDK8-mediated STAT1 phosphorylation positively or negatively regulated over 40 % of IFN-γ-responsive genes[27].

2.4.3 CDK8 and its paralog CDK19

CDK19, emerged in vertebrates due to gene duplication events. CDK19 displays high sequence similarity to CDK8, including near identical kinase domains as well as cyclin interaction domains[95]. CDK19 is part of an analogous CDK19 kinase module, which consists of the MED12 and MED13 paralogs MED12L and MED13L. In contrast to CDK19, MED12L and MED13L are more divergent from their counterparts, as they only exhibit 59% and 53% sequence identity, respectively[95]. A recent study from our lab revealed CDK8, and not CDK19 as the major IFN-γ-activated STAT1 kinase in both mouse and human cells[45]. Due to a versatile toolbox including inducible knockout of CDK8 with 4OHT treatment, knockdown of CDK19 with siCDK19 and specific kinase inhibitors, it was possible to decouple the effects of CDK8 and CDK19. Evidently, CDK8 uses its kinase functionality to drive the IFN-γ antiviral response, whereas CDK19, which impacts IFN-γ signaling as well, does this kinase-independently and might exhibit scaffolding properties[45].

3. Aims

CDK8 and CDK19 are important but mechanistically distinct drivers of the IFN-γ response; CDK8 acts as a kinase in Pol II pause release while CDK19 functions as a kinase-independent scaffold. The precise mechanisms how these two kinases upregulate or downregulate IFN-γ-induced transcription remain to be elucidated.

The scientific objective of this study was to characterize mediator kinase-mediated changes in chromatin recruitment of Pol II, pS2 Pol II and pS5 Pol II. Specifically, the study included the following aims:

- Test/optimize the experimental system for ChIP
- Impact of CDK8 on IFN-γ regulated genes
- Test feasibility of ChIP against flag-tagged CDK8
- Effects of mediator kinase activity on recruitment of Pol II and CTD phosphorylated Pol II
- Establishment of CDK19 Western blot in MEFs and BMDMs

4. Experimental system used in this study

The cell-based system has been generated in our laboratory and described by Steinparzer *et al.* in 2019[45].

Throughout the study, solely mouse embryonic fibroblasts (MEFs) were used. The experimental framework is based on the CreERT/loxP system, a method widely used to induce conditional deletion in cells. The system consists of a recombinase termed Cre that recognizes and recombines a pair of target sequences called *LoxP.* Therefore, it is a site-specific recombinase technology and can be precisely controlled through tamoxifen treatment. All cell lines used in this study have their CDK8 gene flanked by above mentioned *loxP* sites. MEFs carrying only the endogenous flanked CDK8 gene are termed CDK8fl MEFs in this study. In addition, two other cell lines are based on the CreERT/loxP background: CDK19 KO MEFs (cells that lack endogenous CDK19) and CDK8 WT flag MEFs (cells that carry an additional exogenous flag-tagged CDK8). For more details on these cell lines see Material and Methods. In all cell lines endogenous CDK8 can be knocked out conditionally through tamoxifen treatment. Additionally, kinase function of both CDK8 and CDK19 can be impaired with the kinase inhibitor Senexin A. Lastly, silencing RNA can be used to knock down CDK19 in CDK8fl MEFs and CDK8 WT flag MEFs. Together, this allows for various combinations of treatments, leading to different effects that can be studied in vitro (Figure 7).

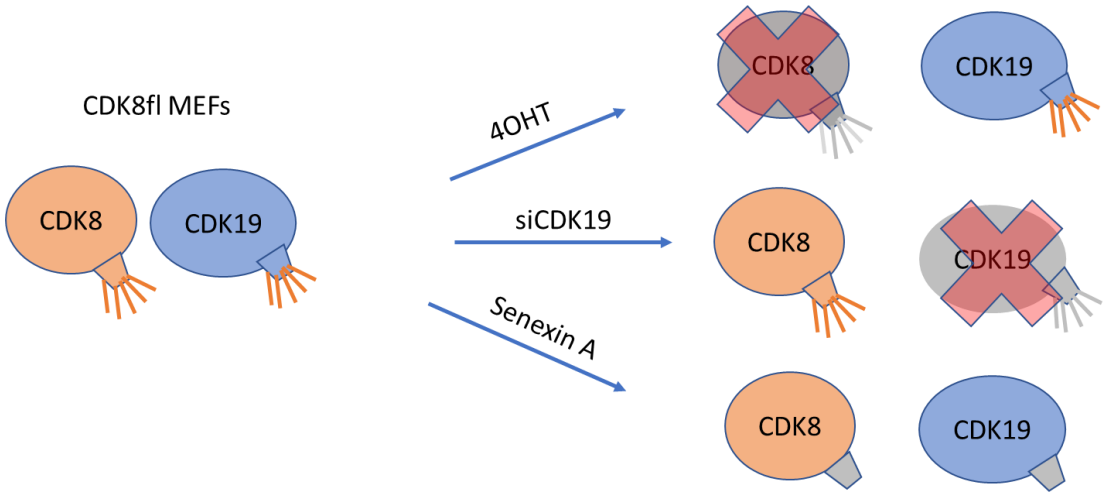


Figure 7 **Experimental system used in this study**
Cells can be either treated with tamoxifen (4OHT) to knock out CDK8, treated with siRNA to knock down CDK19 or treated with Senexin A to inhibit CDK8 and CDK19 kinase function. This allows for combinatorial experimental approaches to create different setups that can be further studied in vitro.

5. Results

5.1 CDK8 regulates expression of IFN-γ induced genes and phosphorylates STAT1 on Serine 727 in MEFs

STATs are key players in the immune system which control gene expression on transcriptional level. They can be modified in many ways at various positions. For example, the phosphorylation of a tyrosine residue leads to STAT homo- or heterodimers translocating to the nucleus, which further regulates transcription.

Next to this well described tyrosine phosphorylation, STATs can also be modified on serine residues, originally described for S727 of STAT1 and STAT3 by the Darnell laboratory[27,96]. TAD phosphorylation has been intensively studied in the canonical JAK-STAT pathway, in which STAT phosphorylation of both tyrosine and serine are of vital importance[97,98].

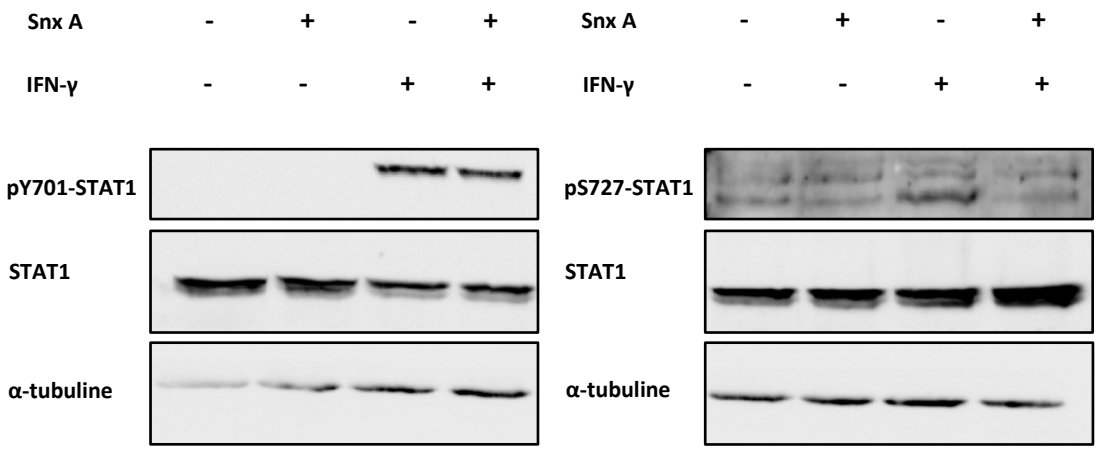


Figure 8 **IFN-γ induced pS727-STAT1 is inhibited by Senexin A in CDK8fl MEFs**

MEFs were treated for 15 minutes with the mediator kinase inhibitor Senexin A (10 µM), followed by 45 minute stimulation with IFN-γ. Protein extracts were generated and analyzed via western blot with antibodies against pY701-STAT1, pS727-STAT1, total STAT1 and tubulin as loading control. The two bands visible in STAT1 represent its two isoforms alpha and beta.

The mediator kinase CDK8 was identified by our laboratory to be the main kinase involved in Ser727 STAT1 phosphorylation and is therefore considered a key regulator in antiviral responses. As such, CDK8 represents a promising target for therapeutic modulation of cytokine responses[27]. To test the reliability of our reagents, we initially examined the induction and inhibition of phosphorylation of S727 using MEFs treated for 15 minutes with the mediator kinase inhibitor Senexin A followed by stimulation with IFN-γ afterwards. Senexin A was able to block IFN-γ induced S727 phosphorylation of STAT1 in MEFs at a final concentration of 10 µM. The tyrosine phosphorylation on residue 701 (pY701) was not affected by the inhibitor (Figure 8).

To elucidate the role of CDK8 in transcriptional regulation of interferon-stimulated genes (ISGs), we analyzed the gene expression levels of *Irf1*, a primary response gene and *Gbp2,* a secondary response gene after Senexin A treatment. MEFs were stimulated with IFN-γ for 0, 2 and 4 hours and were either treated with the kinase inhibitor Senexin A or left untreated. The primary response gene interferon regulatory factor 1 (*Irf1)* was the first member of the interferon regulatory transcription factor (IRF) family identified. *Irf1* regulates expression of target genes by binding to an interferon stimulated response element (ISRE) on their respective promoters[99]. As can be seen in Figure 9A, stimulation with IFN-γ for 2 and 4 hours leads to an immediate upregulation of *Irf1* expression in MEFs after 2 hours, followed by strong upregulation after 4 hours of stimulation. Mediator kinase inhibition with Senexin A leads to reduced expression of *Irf1* at both 2 and 4 hours, however, the effect seems to more pronounced after 4 hours of IFN-γ stimulation.

In contrast to the primary response gene *Irf1*, stimulation with IFN-γ leads to elevated levels of the secondary response gene *Gbp2* only after 4 hours. After 2 hours of IFN-γ stimulation, *Gbp2* levels are not significantly elevated, whereas after 4 hours a 10-fold increase in gene expression can be observed. The inhibition of CDK8 by Senexin A leads to reduced expression of *Gbp2* at both timepoints, however, after 4 hours of stimulation the effect is more pronounced (Figure 9B).

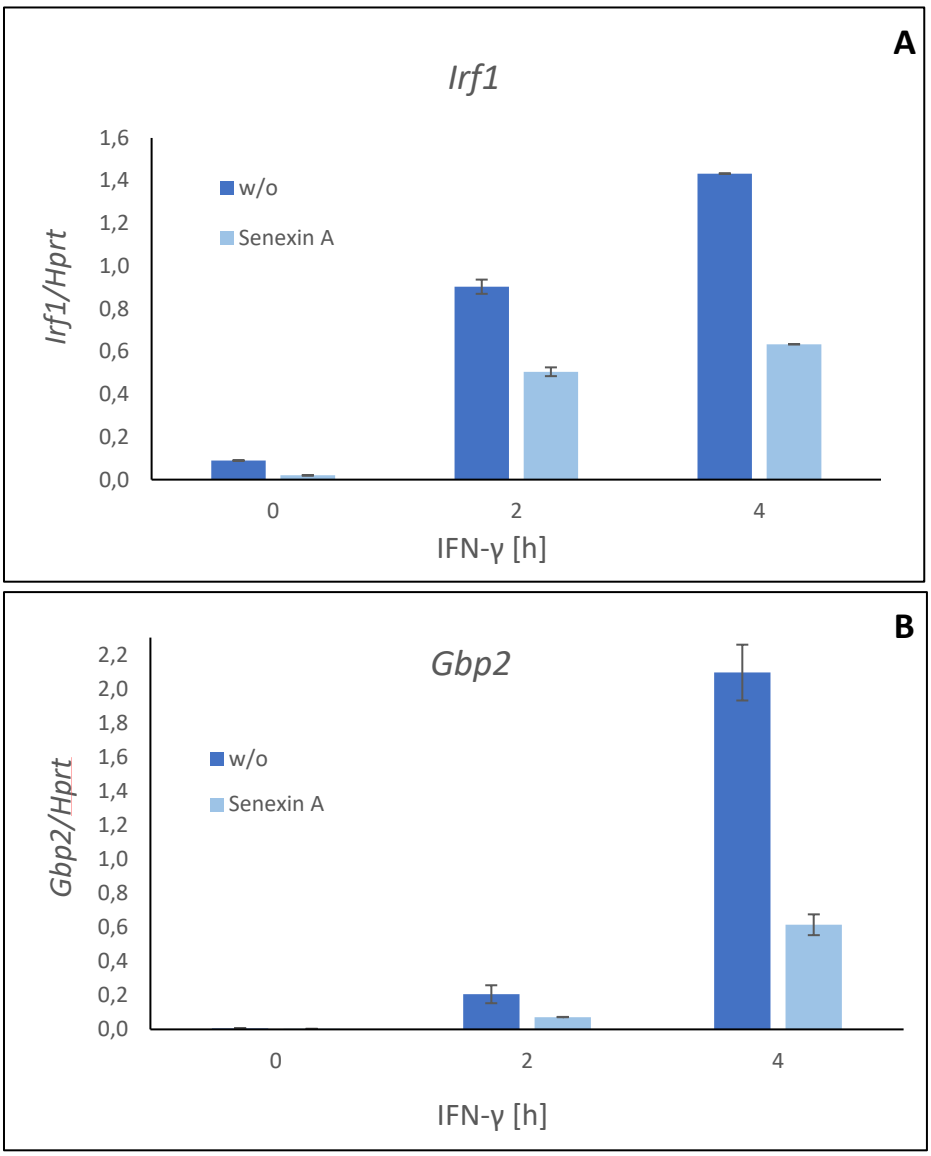


Figure 9 **CDK8 regulates expression of IFN-γ stimulated genes (ISGs) in CDK8fl MEFs**
CDK8fl MEFs were treated with Senexin A for 15 min, followed by stimulation with IFN-γ for 0, 2 and 4 hours or left untreated. RNA was isolated and qPCR was used to assess gene expression of the primary response gene *Irf1* **(A)** and the secondary response gene *Gbp2* **(B)**. The housekeeping gene *Hprt* was used for normalization. Error bars represent SDs of technical replicates (n=2). Inhibition of kinase function downregulates expression of both *Irf*1 and *Gbp2*.

In general, CDK8 kinase inhibition in MEFs with Senexin A leads to downregulation of the primary and secondary response genes *Irf1* and *Gbp2*. (Figure 9A/B).
Given that CDK8 and not CDK19 was identified by our lab to exert kinase activity, these data indicate that CDK8 is phosphorylating STAT1 at S727[27]. Furthermore, pharmacological inhibition of mediator kinase activity has a variable effect on gene expression of interferon stimulated genes when comparing different cell types. This ascribes cell and gene specific roles to mediator kinases in regards to IFN-γ regulated gene expression of ISGs.

5.2 Tamoxifen induced CDK8 knockout in MEFs leads to reduction of pS727 and shows no impact on STAT1 driven expression of interferon stimulated genes

MEFs used in this study (CDK8fl) can be treated with tamoxifen (4-OHT) to create an inducible knockout of CDK8. To confirm that CDK8 is responsible for pS727-STAT1, cells were treated with 4-OHT for 72 hours, followed by stimulation with IFN-γ for 45 minutes.
As can be seen in Figure 10, 4-OHT treatment for 72 hours leads to full depletion of CDK8. In line with our expectations, the inducible knockout of CDK8 has an impact on pS727-STAT1 and no impact on total STAT1 and pY701-STAT1.

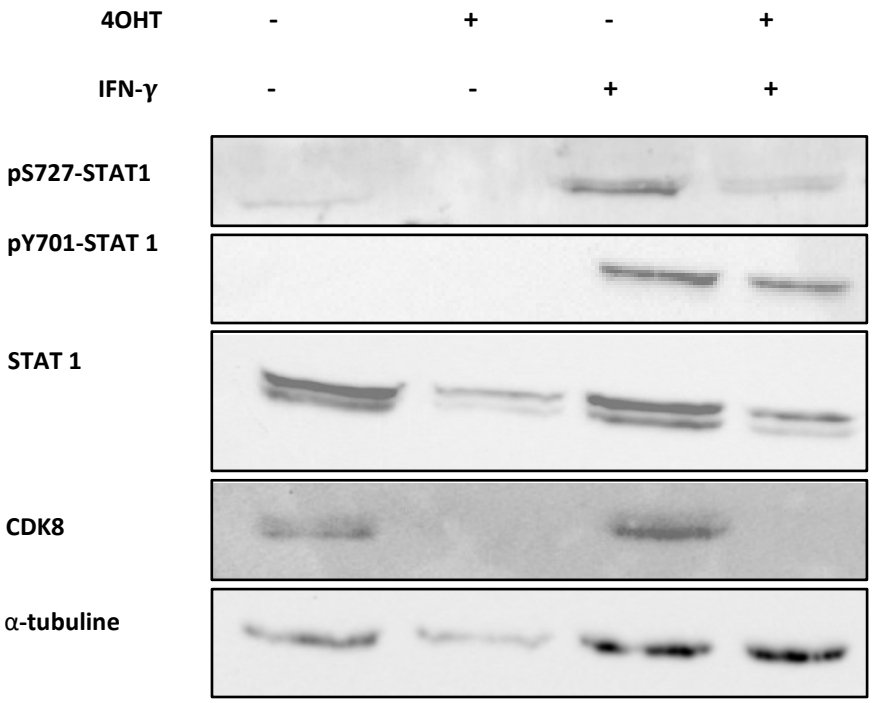


Figure 10 **Tamoxifen induced knockout in CDK8fl MEFs leads to reduction of pS727-STAT1**
CDK8fl MEFs were treated 3 hours with tamoxifen (10 µg/ml) to induce CDK8 knockout. Cells were then kept in culture for 72 hours and subsequently treated with IFN-γ for 45 minutes. Whole cell extracts were generated and analyzed via western blot with antibodies against pY701-STAT1, pS727-STAT1, total STAT1, CDK8 and tubulin as loading control. The two bands visible in STAT1 represent its two isoforms alpha and beta. Tamoxifen clearly blocks pS727-STAT1 phosphorylation by knocking CDK8 out, indicating that CDK8 mediates canonical phosphorylation of pS727-STAT1 in CDK8fl MEFs.

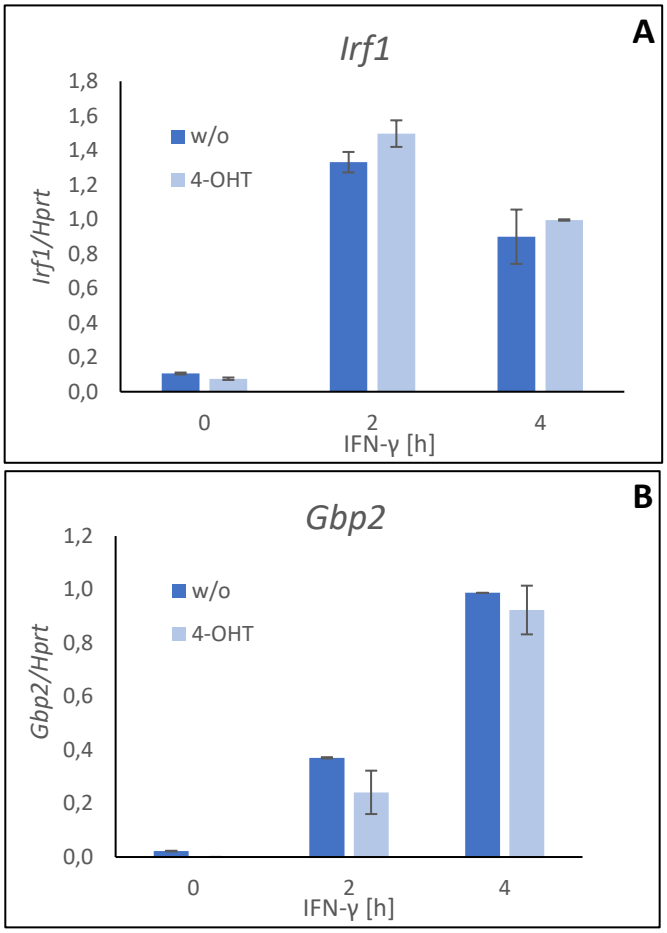


Figure 11 **Tamoxifen treated cells show no impact on STAT1 driven Interferon-γ stimulated genes** CDK8fl MEFs were treated 3 hours with tamoxifen (10 µg/ml) to induce CDK8 knockout. Cells were then kept in culture for 72 hours and subsequently treated with IFN-γ for 0, 2 and 4 hours. RNA was isolated and qPCR was used to assess gene expression of the primary response gene *Irf1* **(A)** and the secondary response gene *Gbp2* **(B).** The housekeeping gene *Hprt* was used for normalization. Error bars represent SDs of technical replicates (n=2). Interestingly, the inducible KO of CDK8 does not show impact on gene expression levels of both *Irf1* and *Gbp2.*

To further understand and elucidate the role of CDK8 in transcriptional regulation of interferon-stimulated genes (ISGs), MEFs were treated 72h with tamoxifen, causing full depletion of CDK8 and stimulated with IFN-γ for 0h, 2h and 4h.

Then, the gene expression of the primary response gene *Irf1* and the secondary response gene *Gbp2* was assessed with qPCR (Figure 11 A/B). Interestingly, the inducible knockout of CDK8 does not seem to have an impact on gene expression levels of both *Irf1* and *Gbp2.* It is worth mentioning that RNA-seq experiments from our lab by *Steinparzer et al.* showed reduced expression of *Gbp2* in cells lacking CDK8[45]. Therefore, results from this study regarding *Gbp2* expression upon inducible CDK8 knockout cannot be considered plausible. Nevertheless, it is suggested to repeat this experiment to disregard technical errors that might have happened.

5.3 Testing ChIP in the experimental system

Chromatin immunoprecipitation (ChIP) is used to investigate whether specific proteins of interest are associated with specific genomic regions, such as transcription factors on promoters. To test ChIP in the experimental system used in this study, MEFs were treated with IFN-γ for 0, 1 and 2 hours. The ChIP was directed against 4 different regions of interest, namely the *Irf1* transcription start site (TSS), the *Irf1* Intron 8, the *Irf1* untranslated region (UTR) and a negative control where no transcription is taking place. The regions aim to give a good representation of the *Irf1* gene, ranging from the transcription start site to the 3´ untranslated region. In addition, two isotype controls were included as negative controls to measure non-specific background signal caused by immunoglobulins binding non-specifically to Fc receptors.

5.3.1 Establishment of ChIP for STAT1, total Pol II, pS2 Pol II and pS5 Pol II

5.3.1.1 STAT1

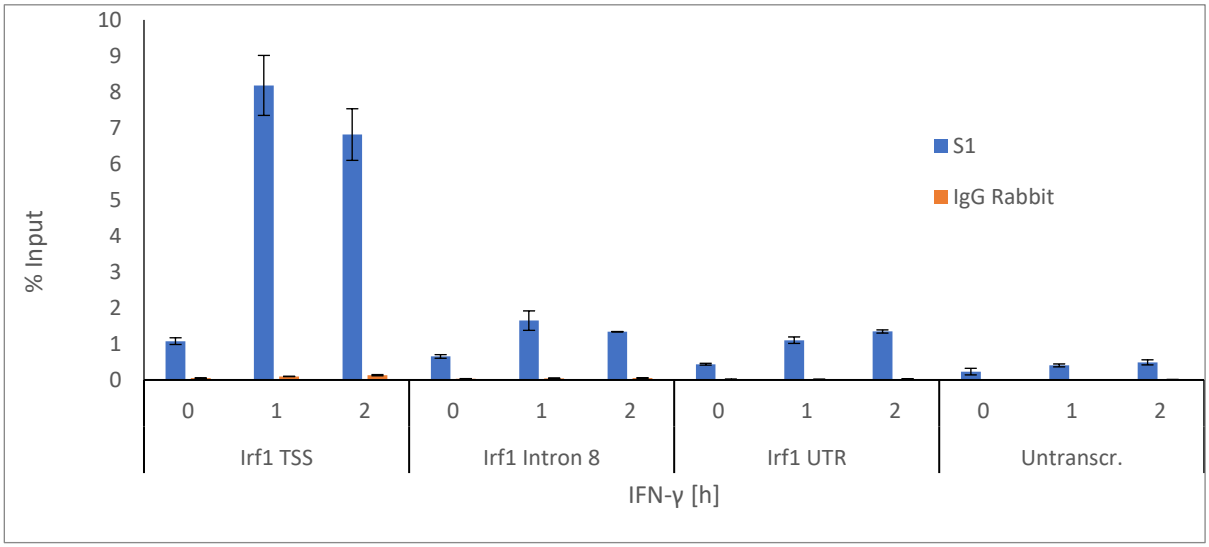


Figure 12 **STAT1 ChIP**
Cells were treated with IFN-γ (10ng/ml) for 0, 1 and 2 hours. Subsequently, cells were harvested and ChIP was conducted (Materials and Methods 7.5). STAT1 recruitment to *Irf1* TSS, Irf1 Intron 8, *Irf1* 3´-UTR was examined by using STAT1 antibodies (Material and Methods 7.6, 7.7). The immunoprecipitated DNA was analyzed by qPCR. Signal values are depicted in % of Input. Error bars represent SDs of technical replicates (n = 2). In addition, isotype controls were used to discriminate against non-specific background signal caused by immunoglobulins.
STAT1 gets recruited to the promoter of *Irf1* and its abundance declines in the gene body. The negative control shows no binding of STAT1.

Upon IFN-γ stimulus, STAT1 undergoes phosphorylation and homodimerization, which causes its translocation to the nucleus and further lead to transcription of interferon stimulates genes (ISGs) to help establish an antiviral state in the cell. As can be seen in Figure 12, the expected behavior of STAT1 upon IFN-γ stimulus is reflected in the ChIP results (Figure 12).

STAT1 accumulates at the TSS of the primary response gene *Irf1* upon stimulation with IFN-γ and the signal weakens in the gene body and further downstream in the 3´UTR.

The isotype controls yield very low signal, indicating a low non-specific background signal caused by immunoglobulins binding non-specifically to Fc receptors.

5.3.1.2 total Pol II

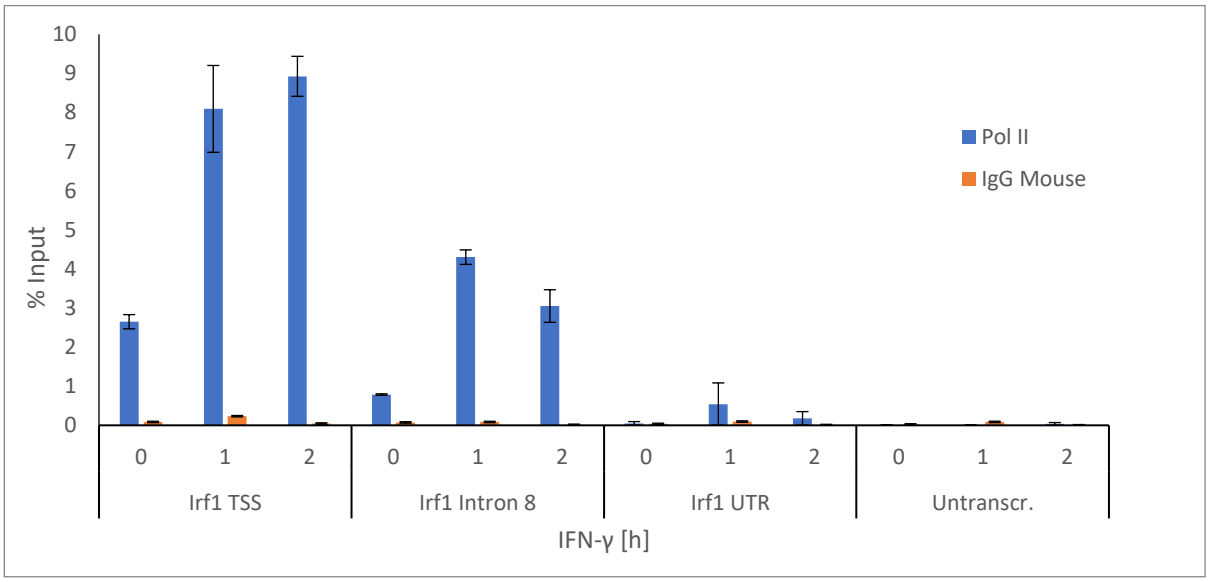


Figure 13 **Pol II ChIP**

Cells were treated with IFN-γ (10ng/ml) for 0, 1 and 2 hours. Subsequently, cells were harvested and ChIP was conducted (Materials and Methods 7.5). STAT1 recruitment to *Irf1* TSS, *Irf1* Intron 8, *Irf1* 3´-UTR was examined by using RNA pol II antibodies (Material and Methods 7.6, 7.7). The immunoprecipitated DNA was analyzed by qPCR. Signal values are depicted in % of Input. Error bars represent SDs of technical replicates (n = 2). In addition, isotype controls (orange) were used to discriminate against non-specific background signal caused by immunoglobulins.

In line with expectations, Pol II is most abundant at the TSS of *Irf1* and declines further downstream of the gene. Induction of transcription with IFN-γ treatment leads to Pol II recruitment to DNA. The negative control shows no polymerase DNA interaction at all.

To further test the experimental system, the recruitment of Pol II to the TSS of the gene *Irf1* was examined. Using the same IFN-γ stimulation times as above, as well as an isotype control to check for unwanted binding, ChIP was conducted.

Living up to our expectations, Pol II is mainly located at the TSS of the primary response gene after stimulation with the cytokine IFN-γ.

Across the *Irf1* gene, ranging from the promoter to the 3´ UTR, Pol II is less abundant in regions further downstream of the transcription start site. Pol II is, in line with our assumptions, not recruited to the non-transcribed region, further underlining the benefit of using this DNA region as a negative control (Figure 13).

5.3.1.3 pS5 Pol II and pS2 Pol II

Pol II has, next to its fundamental enzymatic properties, also the ability to coordinate co-transcriptional processing. It associates with a vast amount of enzymes and other regulators through its C-terminal domain (CTD)[100]. The CTD of Pol II contains 52 heptad repeats of the general consensus sequence Y1-S2-P3-T4-S5-P6-S7[38,39].

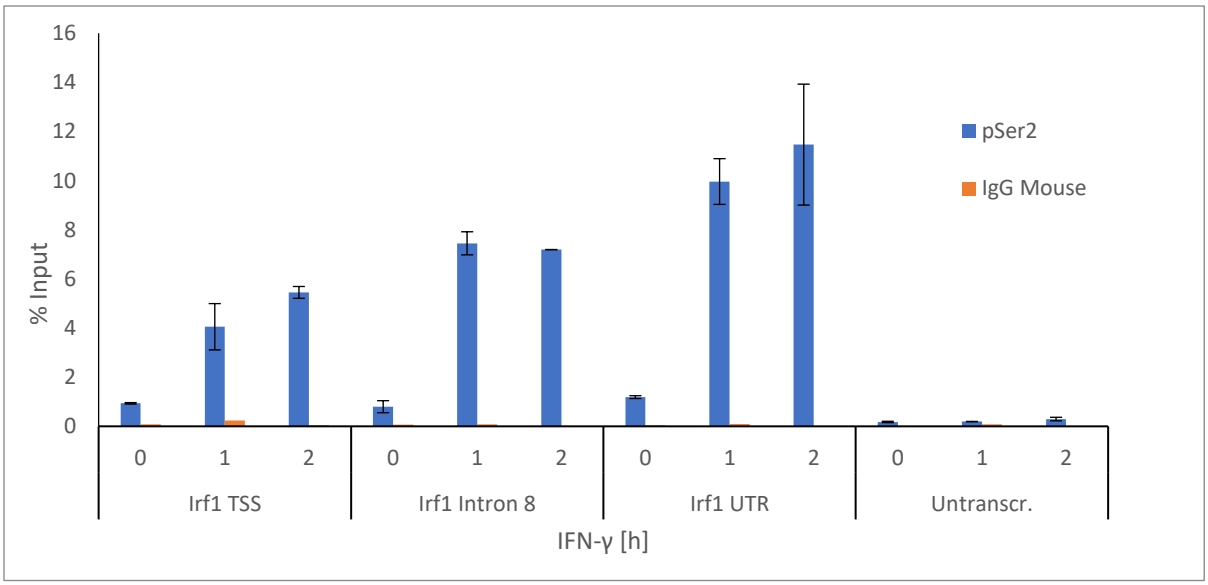


Figure 14 **pS2 Pol II ChIP**
Cells were treated with IFN-γ (10ng/ml) for 0, 1 and 2 hours. Subsequently, cells were harvested and ChIP was conducted (Materials and Methods 7.5). pS2 Pol II recruitment to *Irf1* TSS, *Irf1* Intron 8, *Irf1* 3´-UTR was examined by using pS2 Pol II antibodies (Material and Methods 7.6, 7.7). The immunoprecipitated DNA was analyzed by qPCR. Signal values are depicted in % of Input. Error bars represent SDs of technical replicates (n = 2). In addition, isotype controls (orange) were used to discriminate against non-specific background signal caused by immunoglobulins.
As expected, the abundance of pS2 Pol II inclines towards the end of the *Irf1* gene.

The CTD can be post-translationally modified, resulting in combinatorial patterns (CTD code) that are recognized by factors acting in orchestration with the transcriptional cycle[100]. P-TEFb contains a CDK9 catalytic subunit with the corresponding cyclin T1 or T2[46].

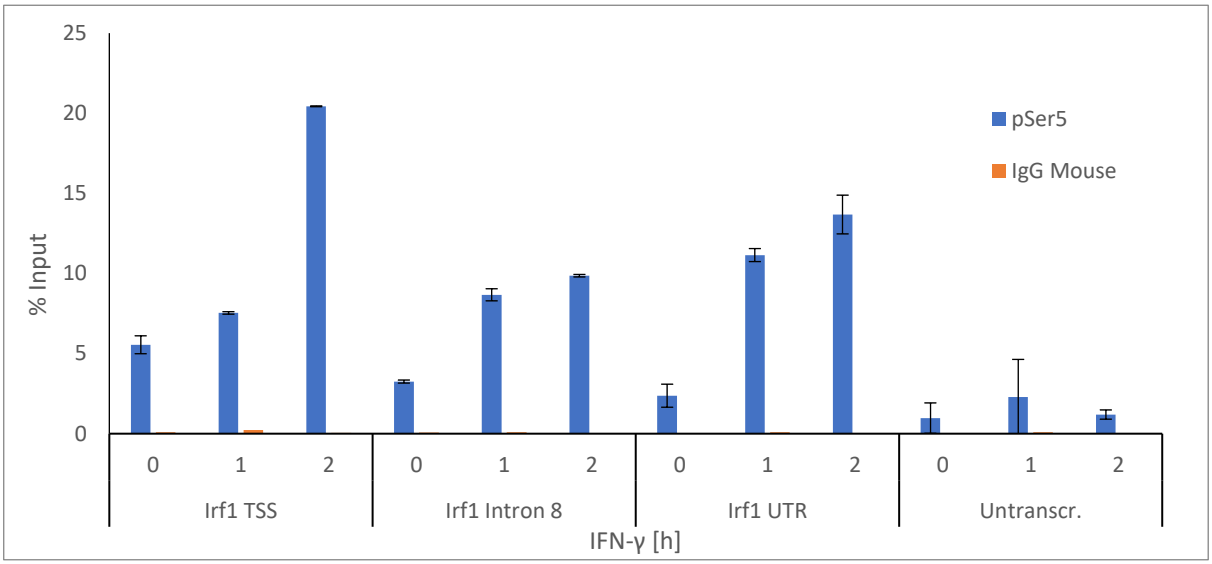


Figure 15 **pS5 Pol II ChIP**
Cells were treated with IFN-γ (10ng/ml) for 0, 1 and 2 hours. Subsequently, cells were harvested and ChIP was conducted (Materials and Methods 7.5). pS5 Pol II recruitment to *Irf1* TSS, *Irf1* Intron 8, *Irf1* 3´-UTR was examined by using pS5 Pol II antibodies (Material and Methods 7.6, 7.7). The immunoprecipitated DNA was analyzed by qPCR. Signal values are depicted in % of Input. Error bars represent SDs of technical replicates (n = 2). In addition, isotype controls (orange) were used to discriminate against non-specific background signal caused by immunoglobulins.
As expected, the abundance of pS5 Pol II declines towards the end of the *Irf1* gene and is mostly found at the start of the ISG *Irf1.*

It acts, like CDK7, on the CTD of Pol II and phosphorylates S2 of the CTD. This allows Pol II to enter the productive elongation state Generally, pS2 Pol II is more situated towards the end of a gene, as can be clearly seen in Figure 14.
Pol II promoter escape occurs after 12-13 base pairs (bps) downstream of TSS and is supported through mechanisms involving CDK7-dependent phosphorylation of Pol II of CTD on serine 5[38]. As depicted in Figure 15, pS5 Pol II is most abundant at regions near the promoter and declines toward the 3´ end of the gene *Irf1*.

5.3.2 Flag-CDK8 ChIP is inconclusive

CDK8 is known to play a pivotal regulatory role in IFN-γ driven gene expression. Latest research revealed CDK8 to be a key regulator in polymerase II pausing, as it promotes pause release during the IFN-γ response. As previous work showed, detecting CDK8 with ChIP yielded very low signal in general. In order to still investigate CDK8 and its recruitment to promoters of IFN-γ regulated genes, MEFS harboring an additional exogenous flag-tagged CDK8 were used this time. The immunoprecipitation was targeted against the flag-tag and

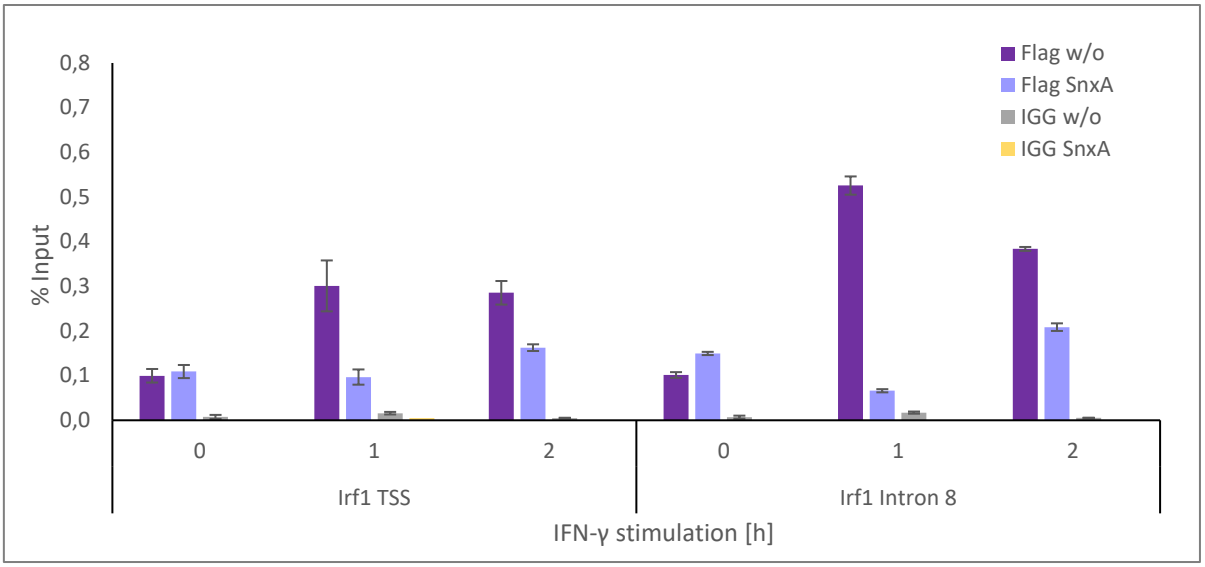


Figure 16 **Flag – CDK8 ChIP is inconclusive**
Flag CDK8 MEFs were treated with IFN-γ (10ng/ml) for 0, 1 and 2 hours, prior being treated with Senexin A (10µM) for 15 minutes. Cells were harvested and ChIP was conducted (Materials and Methods 7.5). Flag CDK8 recruitment to *Irf1* TSS and *Irf1* Intron 8 binding was examined by using anti-flag antibodies (Material and Methods 7.6, 7.7). The immunoprecipitated DNA was analyzed by qPCR. Signal values are depicted in % of Input. Error bars represent SDs of technical replicates (n = 2). In addition, isotype controls (grey and yellow) were used to discriminate against non-specific background signal caused by immunoglobulins. ChIP against Flag-CDK8 remains inconclusive, as the general signal is very low. Therefore, no clear-cut results regarding the influence of Senexin A on CDK8 recruitment to DNA can be drawn.

not the CDK8 protein itself. The MEFs were treated with 10µM Senexin A to inhibit mediator kinase function prior to stimulation with IFN-γ for 0, 1 and 2 hours. This time, the ChIP was directed against two genomic regions of the primary response gene *Irf1,* namely the transcription start site (TSS) and a region in the intron 8 of *Irf1.* The latter serves as a negative control, as CDK8 binding is not expected. In addition, two isotype controls were added as negative controls to measure non-specific background signal caused by immunoglobulins binding non-specifically to Fc receptors. As can be seen in Figure 16, the Flag-CDK8 ChIP still remains inconclusive. The general signal is very low and not distinguishable from the intron 8 region. The Senexin A treated cells yield generally lower signal compared to the untreated ones, but given the fact that the general signal is very low, no conclusion regarding CDK8 and its recruitment to DNA can be drawn.

Collectively, the data in Chapter 5.3 clearly displays that ChIP against STAT1, Pol II and it's CDT phosphorylation's pS2 and pS5 is established. All isotype controls yield a low signal, indicating very low non-specific background signal of the antibodies used in the experiments. The CDK8-Flag ChIP yields low signal, therefore making ChIP-Seq in the future unlikely.

To further shed light on CDK8 and its exact role in transcription, a similar ChIP was conducted using additionally Senexin A to inhibit kinase function in MEFs (Chapter 5.4).

5.4 A ChIP suggests that mediator kinase activity regulates pS5 Pol II abundance at TSS of the IFN-γ target gene *Irf1*

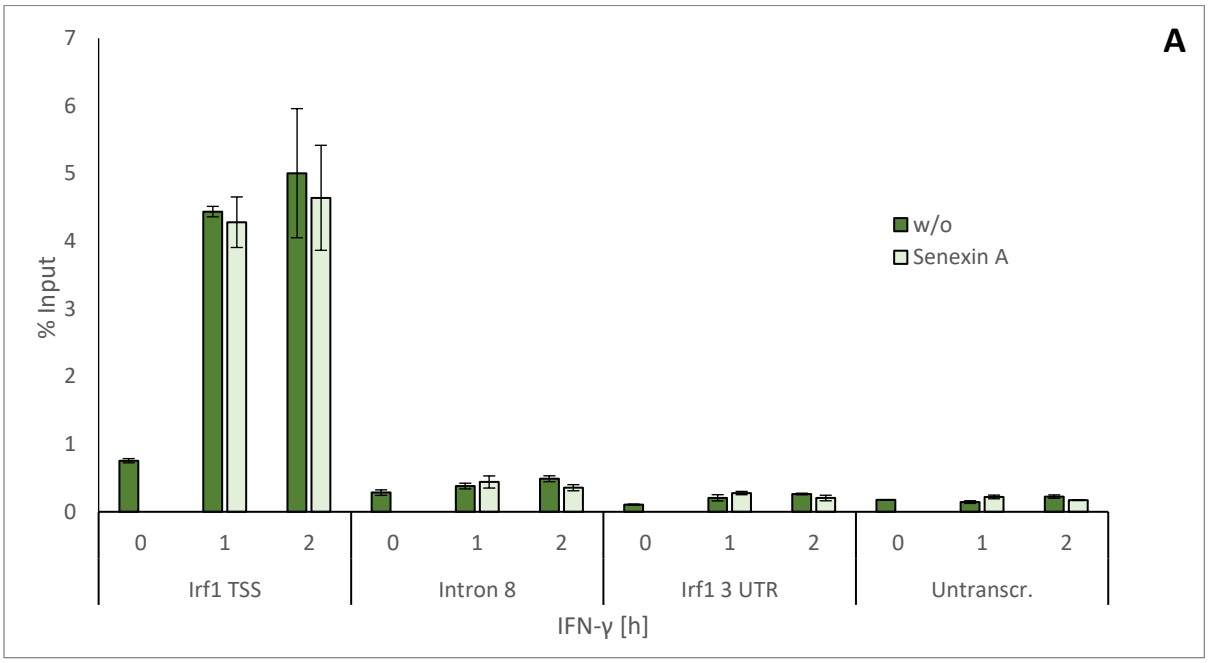
As ChIP experiments were technically working in our *in vitro* system, further research could be conducted. To investigate the role of the mediator kinase CDK8 in transcriptional regulation in more depth, abundance of STAT1 and core enzymes like Pol II on certain gene regions were measured in MEFs treated with and without Senexin A and stimulated with IFN-γ. As in the other experiments, the JAK-STAT pathway was initiated by addition of IFN-γ for 0, 1 and 2 hours. In order to gain statistic relevance, the whole ChIP experiment explained in this chapter was repeated 3 times (marked with A/B/C) in total. All three ChIP experiments were conducted under the same condition to increase reproducibility. The only difference being repetition B where MEFs used for the experimental procedure have been in culture 3 weeks longer than the others. Therefore, results of this repetition should be treated with caution. Beyond that, the samples at timepoint 1 hour in the third repetition (C) of the ChIP against Pol II might have been swapped and are therefore subject for repetition in the future and should be met with skepticism.

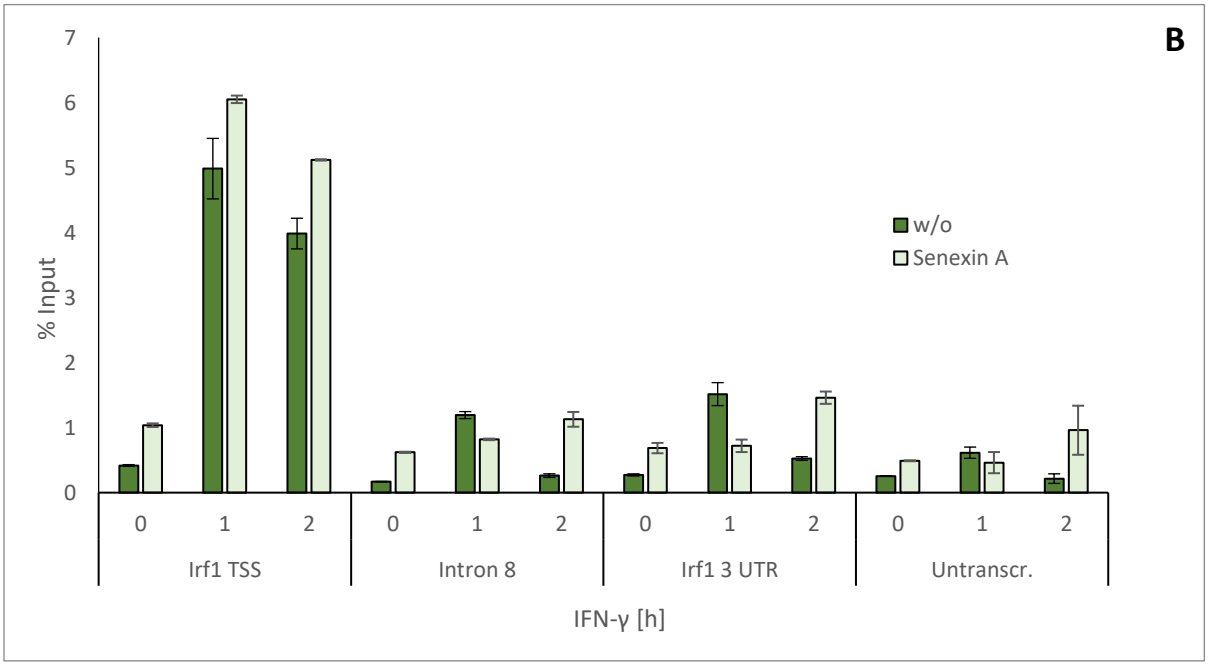
5.4.1 Inhibition of mediator kinase CDK8/19 with Senexin A has no impact on STAT1 recruitment to *Irf1* transcription start site

STAT1 is known to bind gamma activated sequences (GAS) in promoter regions of primary and secondary response genes upon IFN-γ stimulus. As CDK8 was revealed as a core transcriptional coordinator and regulator for interferon stimulated genes (ISGs), the impact of CDK8 on the recruitment of STAT1 to promoter regions of IFN-γ driven genes has been an area of great interest. To address this question, MEFs were stimulated with the potent cytokine IFN-γ for 0, 1 and 2 hours, prior being treated with the kinase inhibitor Senexin A. 4 regions were investigated with ChIP for binding of the transcription factor STAT1: *Irf1* transcription start site (TSS), *Irf1* Intron8, *Irf1* 3´UTR and a negative control. Throughout all three ChIP repetitions (17 A/B/C), STAT1 binding is most abundant at the TSS of the primary response gene *Irf1*.

This is in accordance with common knowledge, as STAT1 binding in the gene body is not expected. Interestingly, inhibition of kinase function does not seem to have an impact on

STAT1 recruitment to the TSS of our gene of interest in the first two repetitions of the experiment (Figure 17 A, 17B), but in the third repetition a strong decrease in STAT1 abundance at the promoter region of *Irf1* can be observed (Figure 17 C). This can be seen as an outlier, as many other ChIP experiments done in this study show no influence of Senexin A on STAT1 recruitment to the promoter of *Irf1* as well.





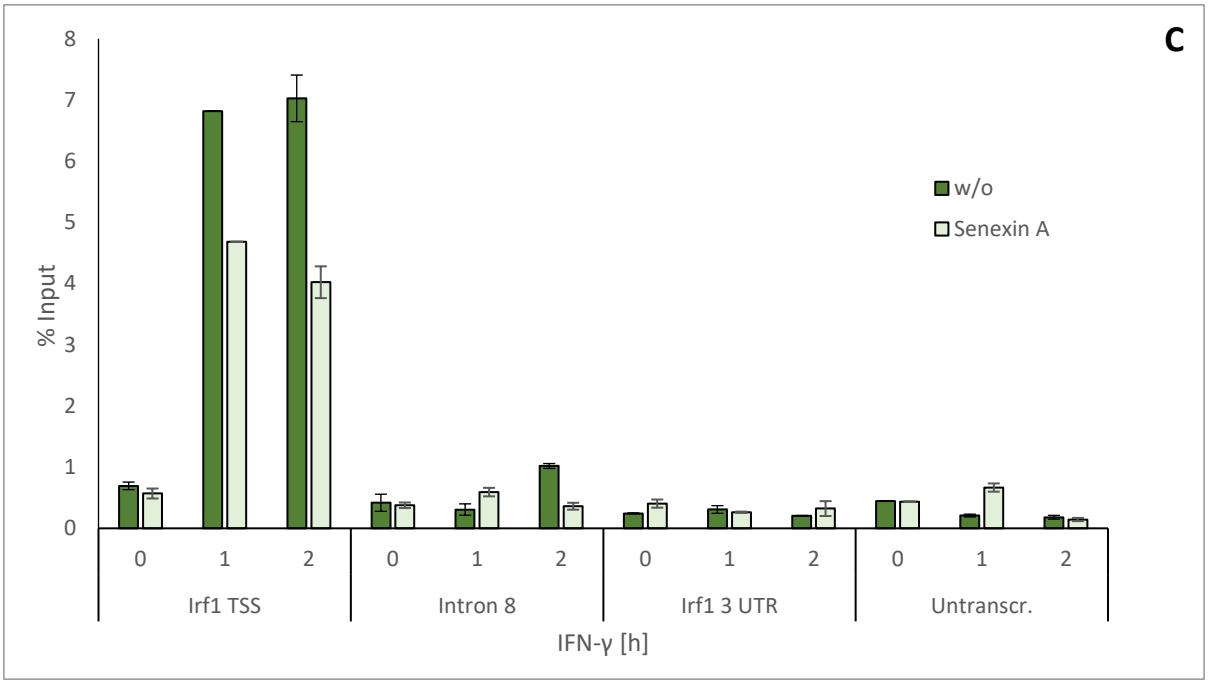


Figure 17 **Inhibition of mediator kinase CDK8/19 with Senexin A does not appear to affect STAT1 recruitment to *Irf1* transcription start site**

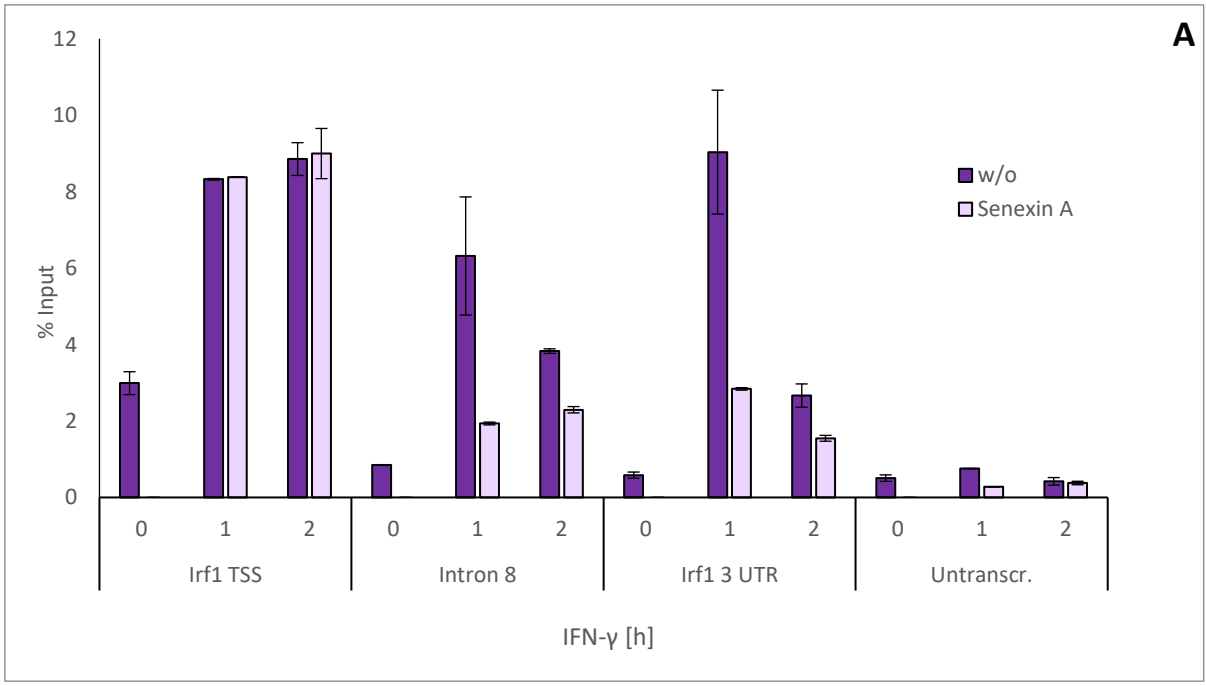
CDK8fl MEFs were treated with IFN-γ (10ng/ml) for 0, 1 and 2 hours, prior being treated with Senexin A (10µM) for 15 minutes. Subsequently, cells were harvested and ChIP was conducted (Materials and Methods 7.5). STAT1 recruitment to *Irf1* TSS, *Irf1* Intron 8, *Irf1* 3´-UTR was examined by using STAT1 antibodies (Material and Methods 7.6, 7.7). The immunoprecipitated DNA was analyzed by qPCR. Signal values are depicted in % of Input. Error bars represent SDs of technical replicates (n = 2). A heterochromatic region was used as negative control. Due to human error, timepoint 0h Senexin A in **(A)** is missing. The ChIP was repeated 3 times **(A/B/C).** STAT1 binding is most abundant at the TSS of *Irf1* in all three repetitions.

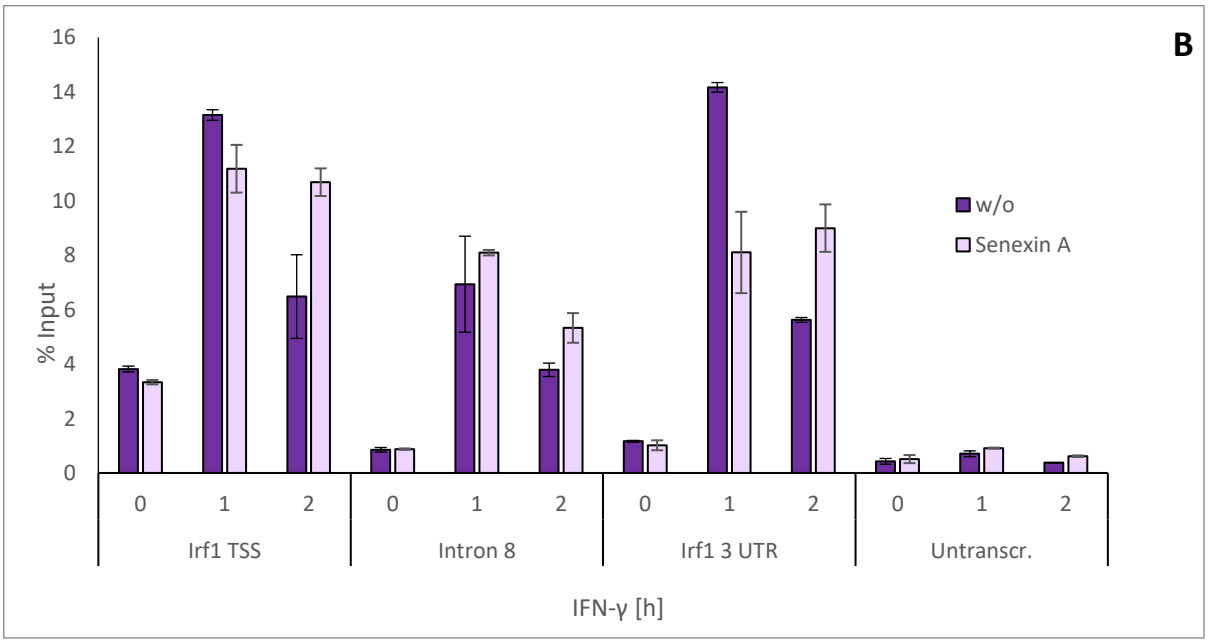
As can be seen in **(A)** and **(B)**, STAT1 recruitment to TSS is not impacted by kinase inhibition with Senexin A. However, in **(C)** Senexin A treatment leads to a decrease of STAT1 abundance at the TSS. This can be seen as an outlier, as many other ChIP experiments done in this study show no influence of STAT1 recruitment to the promoter of *Irf1* as well.

5.4.2 Mediator kinase inhibition does not appear to influence IFN-γ-induced Pol II recruitment to TSS; the effects on recruitment to the gene body are unclear

As CDK8 was shown to regulate expression of many IFN-γ driven genes either up or down, the assumption that Pol II recruitment is affected by mediator kinases is evident. The next target of the ChIP experiment described in this chapter was therefore Pol II in cells treated with the inhibitor Senexin A prior to IFN-γ stimulation. The first experiment showed no difference in Pol II recruitment to the start site of the gene *Irf1*, but a notable drop of it in the gene body when kinase function was impaired (Figure 18 A). As revealed by our laboratory, CDK8 promotes pause release during transcription in the IFN-γ response[45]. If the kinase function of CDK8 is impaired, this pause release might be delayed, resulting in less Pol

II in the gene body. Not coinciding with the first experiment, the second repetition shows no decrease of Pol II the gene body under kinase inhibition conditions (Figure 18 B). Regarding the third repetition, the samples at time point 1 hour with Senexin A treatment and without might have been swapped by accident during the experimental procedure (Figure 18 C). This would indicate a decrease of polymerase II abundance at the TSS and the gene body of *Irf1.* The decreased abundance in the gene body can be explained by pause release mediated by CDK8. If the kinase activity is compromised, the pause release might be delayed, which leads to lower abundance of polymerase II in regions downstream of the TSS of *Irf1.*





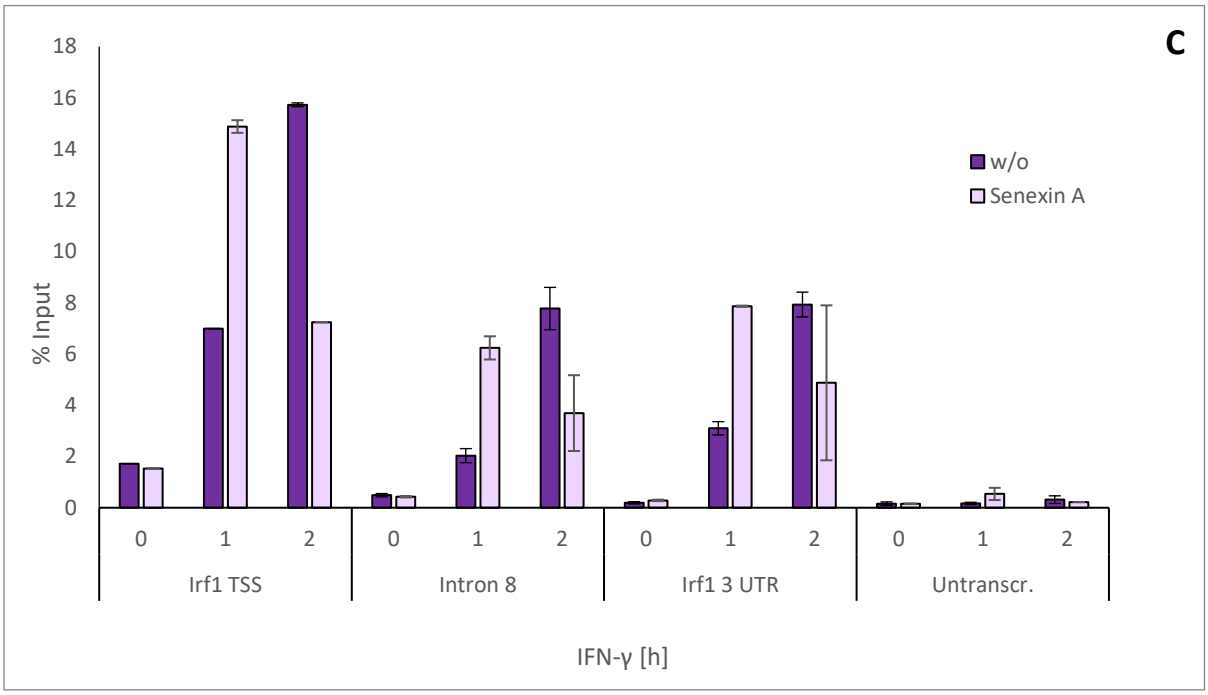


Figure 18 **Total Pol II abundance upon mediator kinase inhibition remains inconclusive**

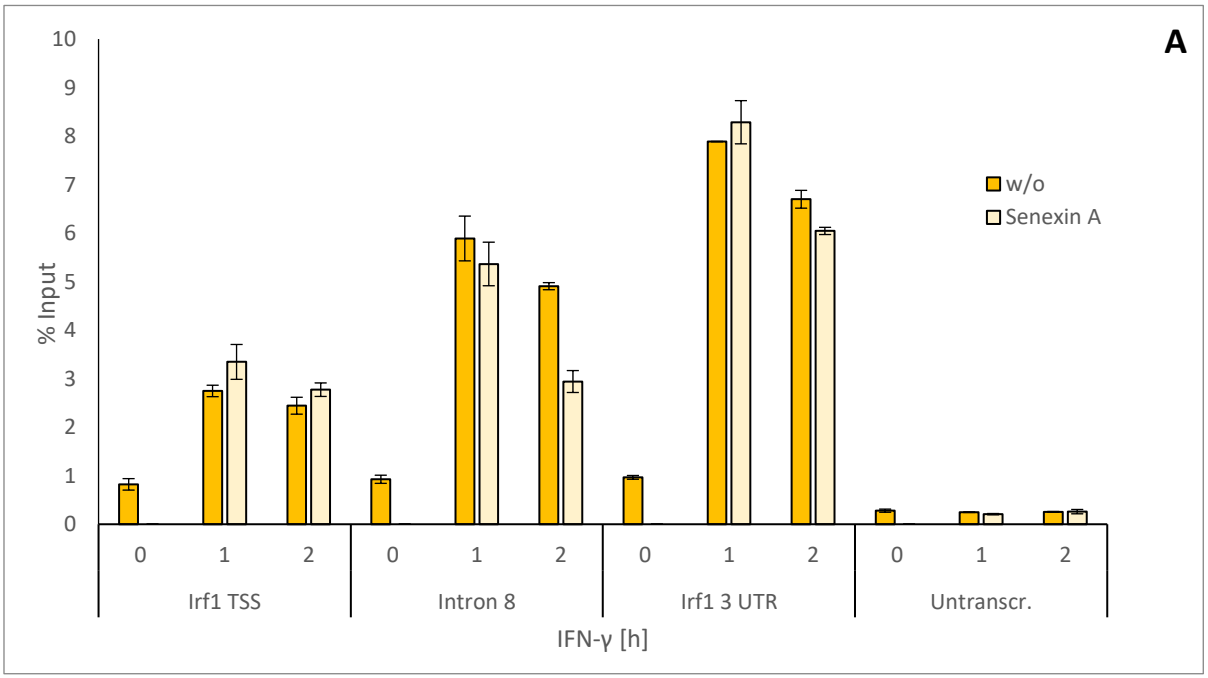
CDK8fl MEFs were treated with IFN-γ (10ng/ml) for 0, 1 and 2 hours, prior being treated with Senexin A (10µM) for 15 minutes Subsequently, cells were harvested and ChIP was conducted (Materials and Methods 7.5). Pol II recruitment to *Irf1* TSS, *Irf1* Intron 8, *Irf1* 3´-UTR was examined by using Pol II antibodies (Material and Methods 7.6, 7.7). The immunoprecipitated DNA was analyzed by qPCR. Signal values are depicted in % of Input. Error bars represent SDs of technical replicates (n = 2). A heterochromatic region was used as negative control. Due to human error, timepoint 0h Senexin A in **(A)** is missing. The ChIP was repeated 3 times **(A/B/C). (A)** The first experiment showed no difference in RNA polymerase II recruitment to the start site of the gene *Irf1*, but a notable drop of it in the gene body when kinase function was impaired. **(B)** Not coinciding with the first experiment, the second repetition shows no decrease of total polymerase II in the gene body under kinase inhibition conditions. Samples at 1-hour Senexin A of **(C)** might have been swapped, thus leading to inconsistent results.

Collectively, the data regarding Pol II recruitment to different regions across the *Irf1* gene is inconclusive. The effect of mediator kinase inhibition is indecisive in respect to the impact on RNA polymerase II abundance. Taking into account that for repetition two (B) the cells used remained in cell culture slightly longer than the others and that the samples at time point 1 hour in repetition three (C) might have been swapped, reduction in recruitment of Pol II in the gene body might be feasible.

5.4.3 Senexin A does not appear to change occupancy of CTD phosphorylation pS2 of Pol II, however more data is needed for conclusive results

The C-terminal domain of Pol II acts as a central binding hub for several important factors crucial for guaranteeing smooth operation of transcription. Those factors recognize different post-translational modifications (PTMs) on different amino acid residues embedded in the CTD. One example of this modifications contributing to the CTD-code is phosphorylated serine at position two in the heptad repeats of the CTD. Its phosphorylation allows Pol II to enter the productive elongation state. In general, pS2 Pol II is more situated towards the end of a gene. The increase of pS2 Pol II abundance towards the 3´UTR of the *Irf1* gene can be seen in all of the three ChIP experiments (Figure 19). However, no effect of Senexin A can be observed throughout the three ChIP experiments.

In line with our expectations, RNA polymerase II phosphorylated at serine 2 has a higher abundance downstream of the TSS of *Irf1*. Regarding the effect of kinase inhibition with Senexin A, no clear result can be derived. Collectively, CDK8 might have a regulatory effect on RNA-polymerase II serine 2 phosphorylation, however the tendency still remains inconclusive.



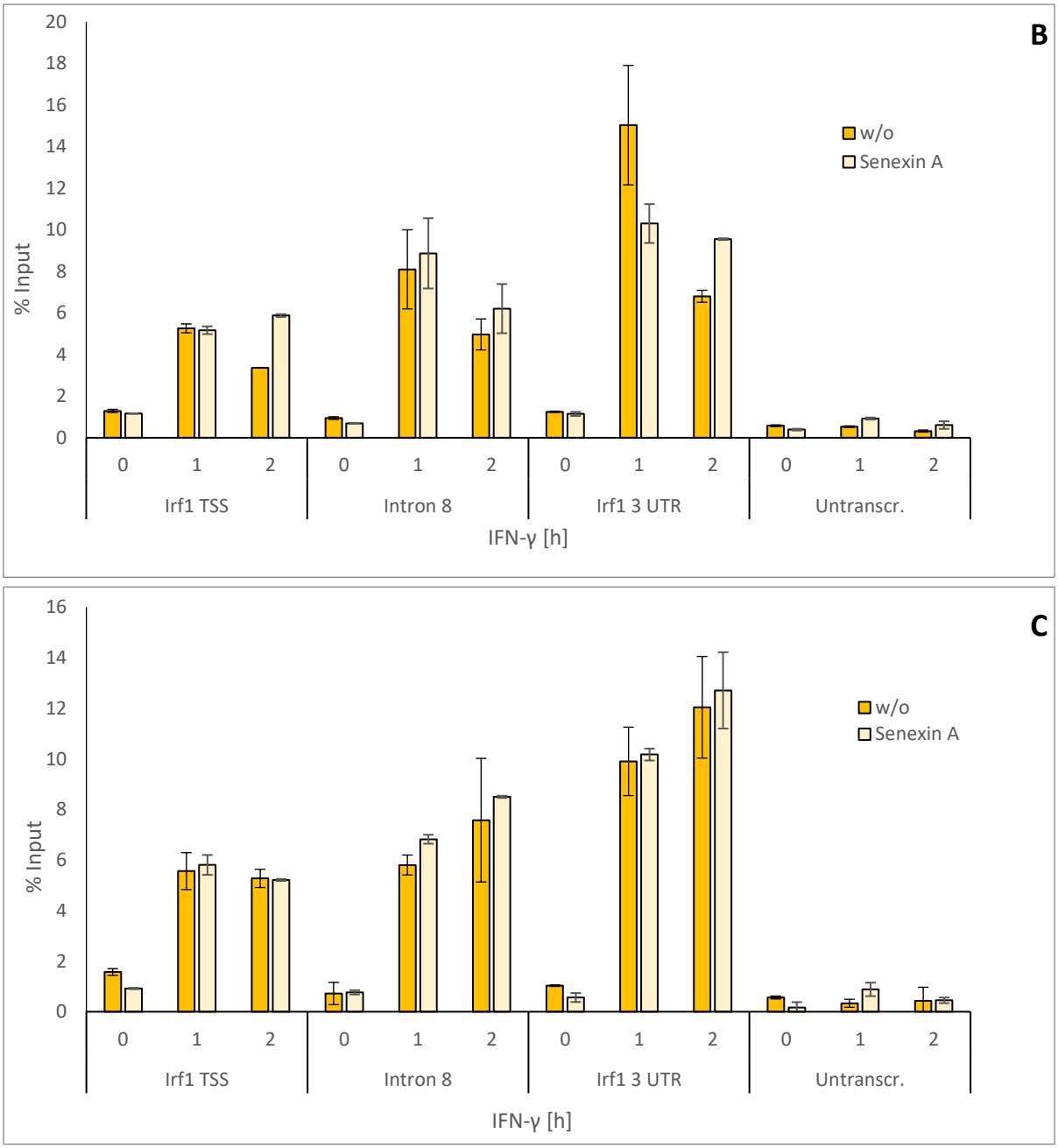
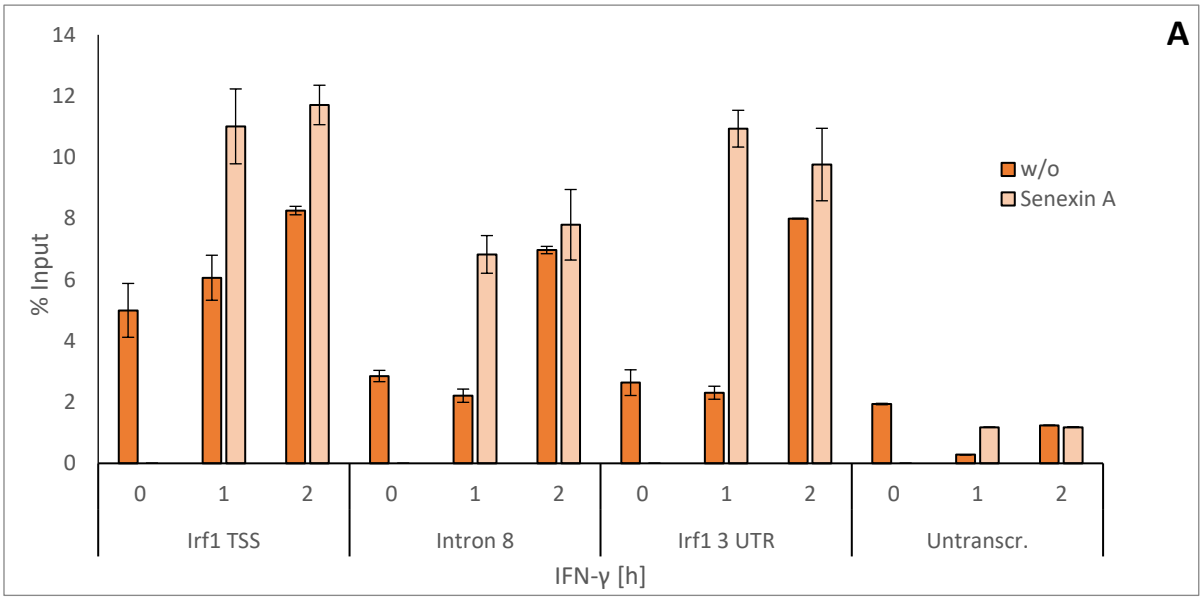


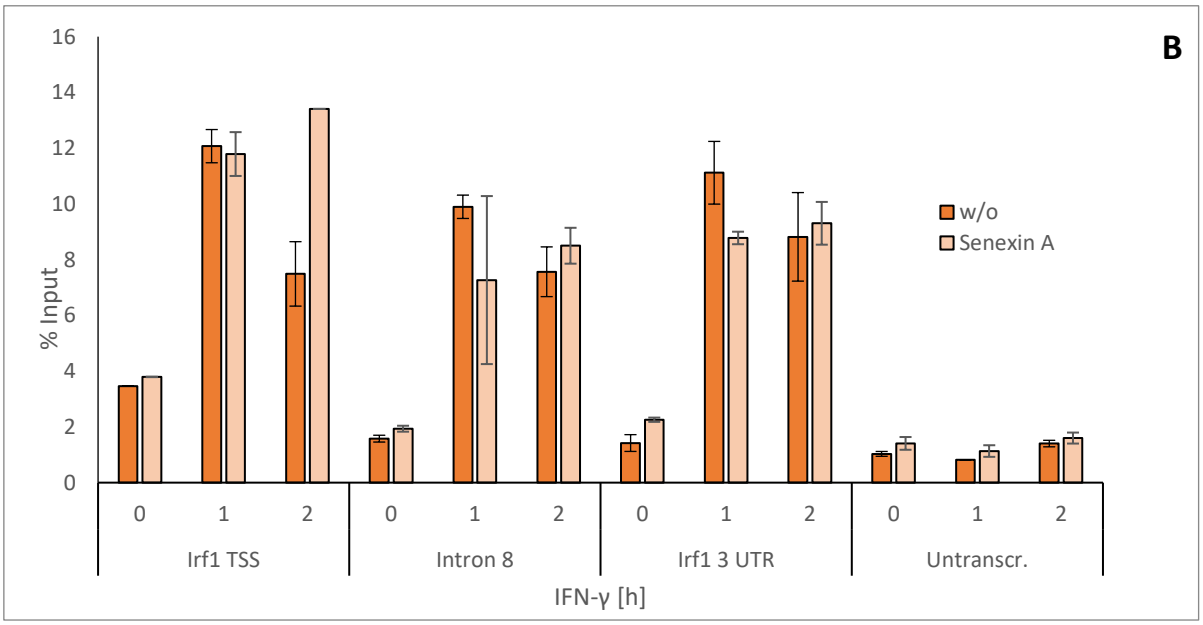
Figure 19 **Senexin A does not appear to change occupancy of CTD phosphorylation pS2 of Pol II, however more data is needed for conclusive results**

CDK8fl MEFs were treated with IFN-γ (10ng/ml) for 0, 1 and 2 hours, prior being treated with Senexin A (10µM) for 15 minutes Subsequently, cells were harvested and ChIP was conducted (Materials and Methods 7.5). pS2 pol II recruitment to *Irf1* TSS, *Irf1* Intron 8, *Irf1* 3´-UTR was examined by using pS2 pol II antibodies (Material and Methods 7.6, 7.7). The immunoprecipitated DNA was analyzed by qPCR. Signal values are depicted in % of Input. Error bars represent SDs of technical replicates (n = 2). A heterochromatic region was used as negative control. The ChIP was repeated 3 times **(A/B/C).** In all three repetitions, RNA polymerase II phosphorylated at serine 2 declines towards the end of the gene. Nevertheless, no clear effect of Senexin A on pS2 pol II can be observed.

5.4.4 Inhibition of mediator kinase increases abundance of pS5 Pol II at TSS after 2-hour IFN-γ stimulation and in the gene body after 1-hour IFN-γ stimulation

The phosphorylation on Serine 5 is of utmost importance in transcriptional regulation. Pol II promoter escape occurs after 12-13 base pairs (bps) downstream of TSS and is heavily supported through mechanisms involving CDK7-dependent phosphorylation of Pol II C-terminal domain (CTD) on serine 5. Consistent with our expectations, this behavior is reflected in the ChIP results throughout all three repetitions (Figure 20 A/B/C), as higher abundance of pS5 Pol II is situated at the start of the gene *Irf1.*





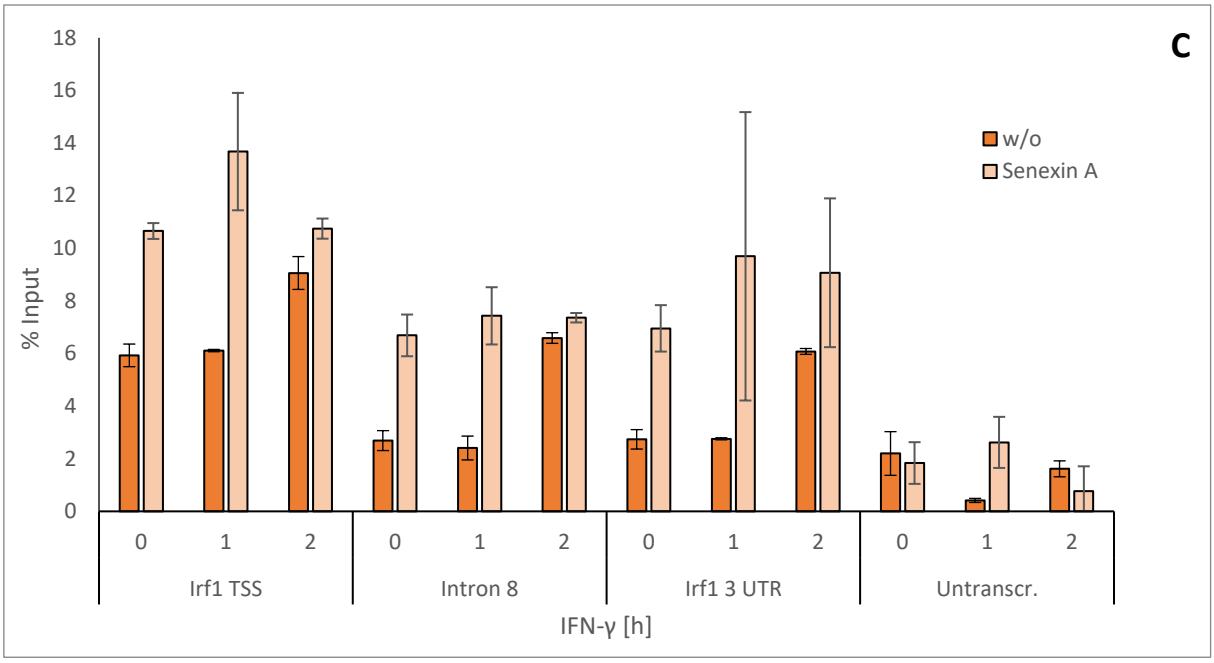
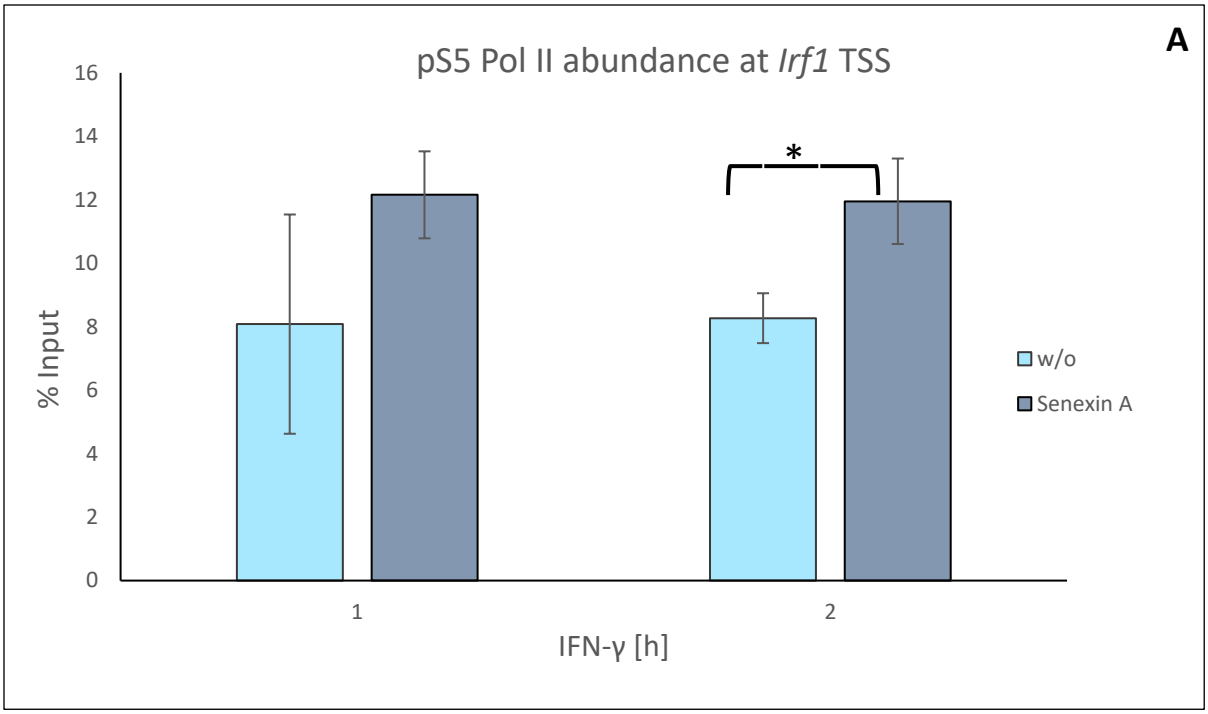


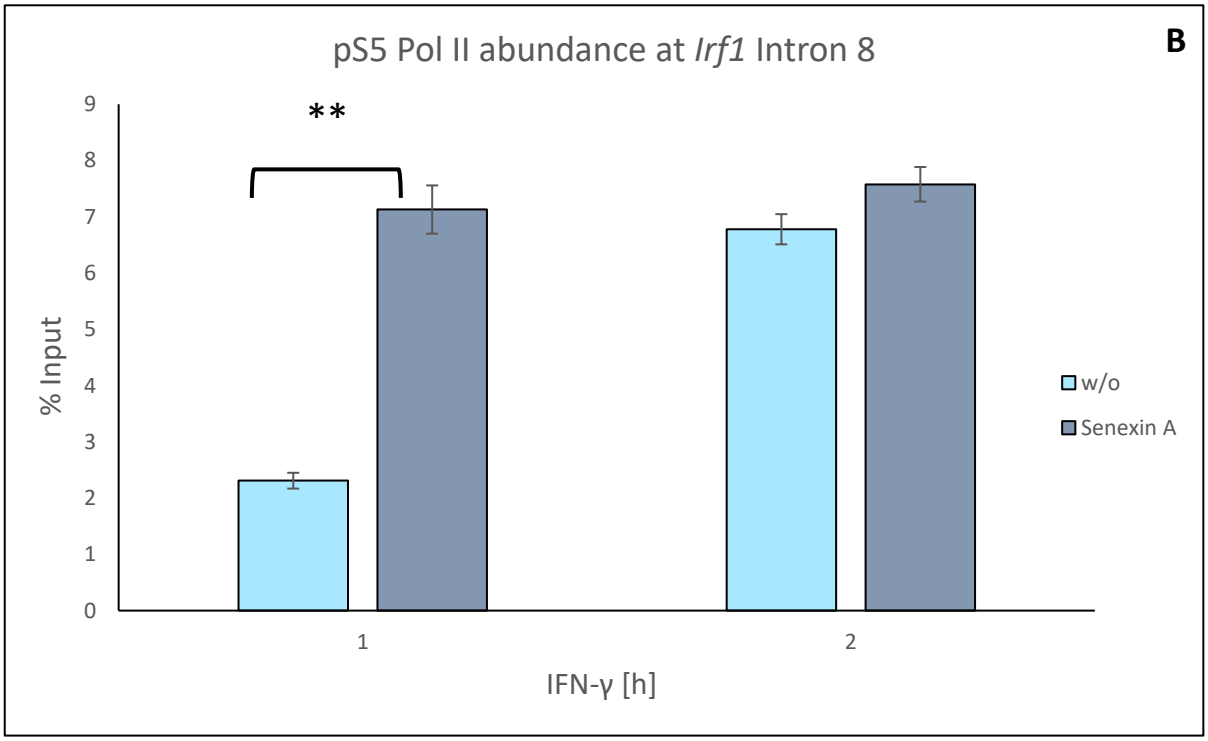
Figure 20 **pS5 Pol II abundance on *Irf1* in MEFs**

CDK8fl MEFs were treated with IFN-γ (10ng/ml) for 0, 1 and 2 hours, prior being treated with Senexin A (10µM) for 15 minutes. Subsequently, cells were harvested and ChIP was conducted (Materials and Methods 7.5). pS5 pol II recruitment to *Irf1* TSS, *Irf1* Intron 8, *Irf1* 3´-UTR was examined by using pS5 pol II antibodies (Material and Methods 7.6, 7.7). The immunoprecipitated DNA was analyzed by qPCR. Signal values are depicted in % of Input. Error bars represent SDs of technical replicates (n = 2). A heterochromatic region was used as negative control. The ChIP was repeated 3 times **(A/B/C)**. Due to human error, timepoint 0h Senexin A in **(A)** is missing. In **(A)** and **(C)** an increase, especially after 1 hour of IFN-γ stimulation of Serine 5 phosphorylated form of polymerase II can be observed. Contrary to this result, **(B)** shows no clear effect of kinase inhibition on pS5 pol II recruitment to DNA.

Subsequently, data from all (A/B/C) repetitions was pooled and pS5 Pol II occupancy at TSS and in gene body was analyzed. Statistical analysis revealed a significant increase of pS5 Pol II at the *Irf1* TSS after 2 hours of IFN-γ stimulation (Figure 21A).

When pooling repetitions A and C, a significant increase after 1 hour of stimulation can be observed at Intron 8 and 3´UTR (Figure 21 B/C)


A
pS5 Pol II abundance at *Irf1* TSS
% Input
16
14
12
10
8
6
4
2
0
*
1
2
IFN-γ [h]
w/o
Senexin A


B
pS5 Pol II abundance at *Irf1* Intron 8
% Input
9
8
7
6
5
4
3
2
1
0
**
1
2
IFN-γ [h]
w/o
Senexin A

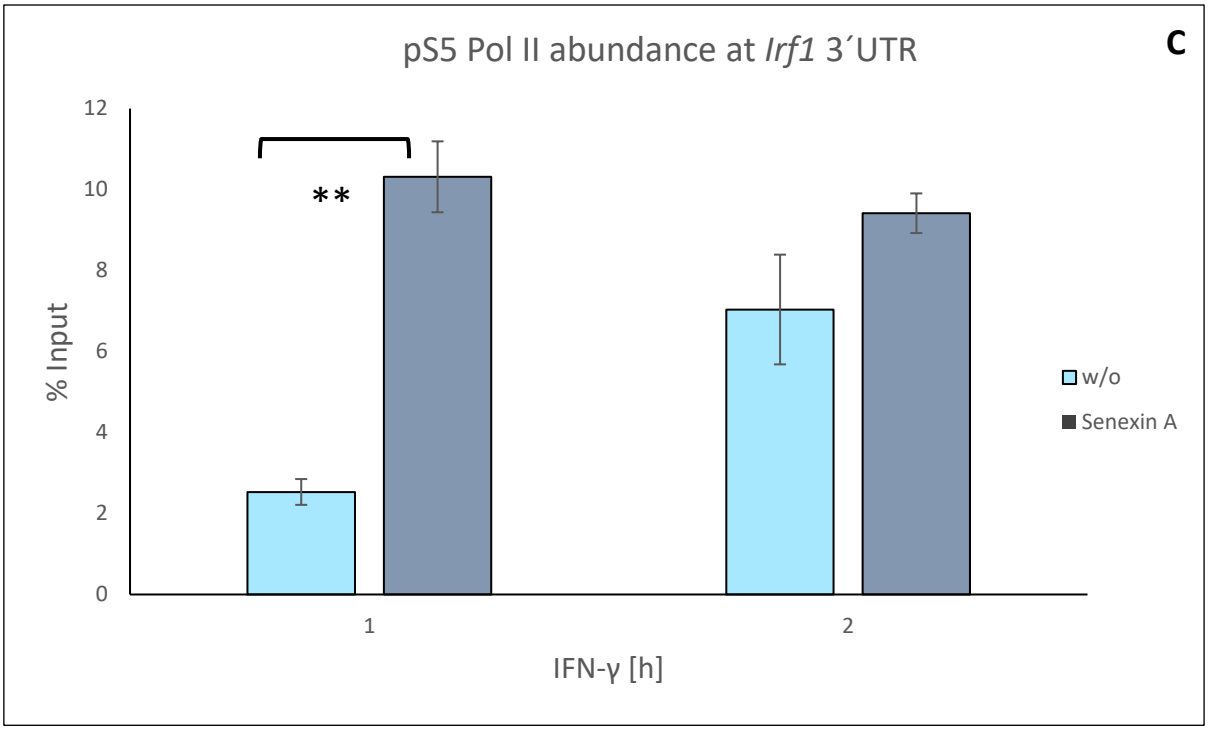


Figure 21 **Inhibition of mediator kinase significantly increases abundance of pS5 Pol II at TSS after 2-hour IFN-γ stimulation and in the gene body after 1-hour IFN-γ stimulation**

(A) SDs from ChIP experiments (Figure 20 A/B/C) were pooled completely. Signal values are depicted in % of Input. Error bars represent SDs of ChIP repetitions (n = 3). Unpaired multiple t test, $p < 0,05$

(B) SDs from ChIP experiments (Figure 20 A/B/C) were pooled (repetition A and C). Signal values are depicted in % of Input. Error bars represent SDs of ChIP repetitions (n = 2). Unpaired multiple t test, $p < 0,001$

(C) SDs from ChIP experiments (Figure 20 A/B/C) were pooled (repetition A and C). Signal values are depicted in % of Input. Error bars represent SDs of ChIP repetitions (n = 2). Unpaired multiple t test, $p < 0,001$

5.5 ChIP experiment probing STAT1, Pol II, pS2 Pol II and pS5 Pol II occupancy suggest that mediator kinase activity regulates Pol II recruitment to GAS of the IFN-γ target gene *Gbp2*

IFN-γ induces a large set of genes collectively termed interferon stimulated genes (ISGs). A subfamily of ISGs are guanylate-binding proteins (GBPs). They include dynamin-related large GTPases important for regulation of cell proliferation and for resistance to certain infections[101]. Gbp2, encoded by the gene secondary response *Gbp2,* was shown to exhibit antiviral activity against the influenza virus as well as promoting oxidative killing of pathogens[101]. Collectively, *Gbp2* provides host protection against different pathogen classes and is therefore considered being a crucial part in IFN-γ induced establishment of an anti-viral state in the cell.

In this chapter, the samples derived from the Senexin A ChIP repetition A (Figure 17/18/19 A) were examined in regards to STAT1, total Pol II, pS2 Pol II and pS5 Pol II recruitment to the *Gbp2* gamma-activated sequence (GAS) as well as to a transcriptionally inactive heterochromatic region serving as a negative control (see Chapter 5.3 and 5.4). It is worth noting that this Chapter shows data from one ChIP experiment.

5.5.1 Mediator kinase inhibition does not appear to impact STAT1 recruitment to *Gbp2* GAS upon IFN-γ stimulation

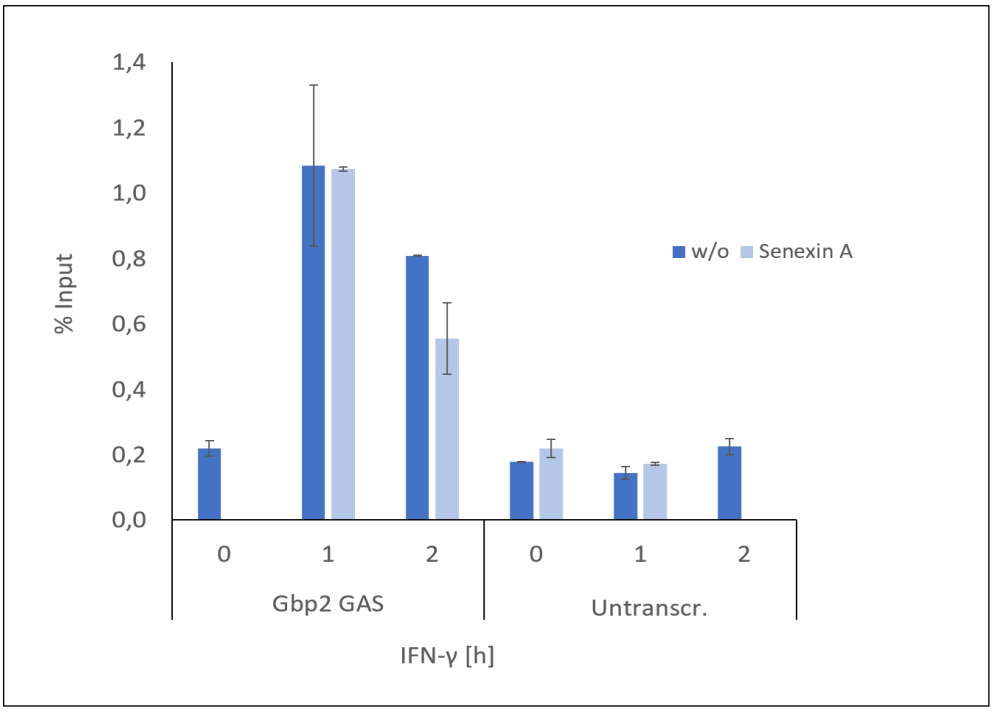


Figure 22 **Mediator kinase inhibition does not appear to impact STAT1 recruitment to *Gbp2* GAS upon IFN-γ stimulation** CDK8fl MEFs were treated with IFN-γ (10ng/ml) for 0, 1 and 2 hours, prior being treated with Senexin A (10µM) for 15 minutes. Subsequently, cells were harvested and ChIP was conducted (Materials and Methods 7.5). STAT1 recruitment to *Gbp2* GAS was examined by using STAT1 antibodies and compared to *Irf1* (Figure 10.A, same samples used) (Material and Methods 7.6, 7.7). The immunoprecipitated DNA was analyzed by qPCR. Signal values are depicted in % of Input. Error bars represent SDs of technical replicates (n = 2). A heterochromatic region was used as negative control. Due to human error, timepoint 0h Senexin A is missing.

STAT1 is known to play a pivotal role in IFN-γ induced signaling, by binding to promoters of interferon stimulated genes (ISGs). As expected, addition of IFN-γ causes an increase of STAT1 abundance at the *Gbp2* GAS.

Although the general signal of STAT1 abundance at the *Gbp2* GAS is low compared to the *Irf1*

TSS (Figure 17 A), a similar fold-change from 0h to 1h upon IFN-γ stimulation can be observed (Figure 22).

5.5.2 Inhibition of mediator kinase with Senexin A causes decreased Pol II abundance on *Gbp2* gamma activated sequence (GAS)

To elucidate the function of the mediator kinases CDK8 and CDK19, the influence of Senexin A on RNA polymerase II recruitment to the GAS region of the secondary response gene *Gbp2* was assessed. As with *Irf1,* IFN-γ stimulation leads to recruitment of Pol II to the promoter region of *Gbp2.* After 2 hours of cytokine stimulation, Pol II abundance declines. Beyond that, the treatment with the kinase inhibitor Senexin A seems to downregulate the abundance of RNA polymerase II (Figure 23). In comparison to *Irf1* the general signal is low, however, the fold-change after stimulation is reproducible. In chapter 5.4.2 a decrease in Pol

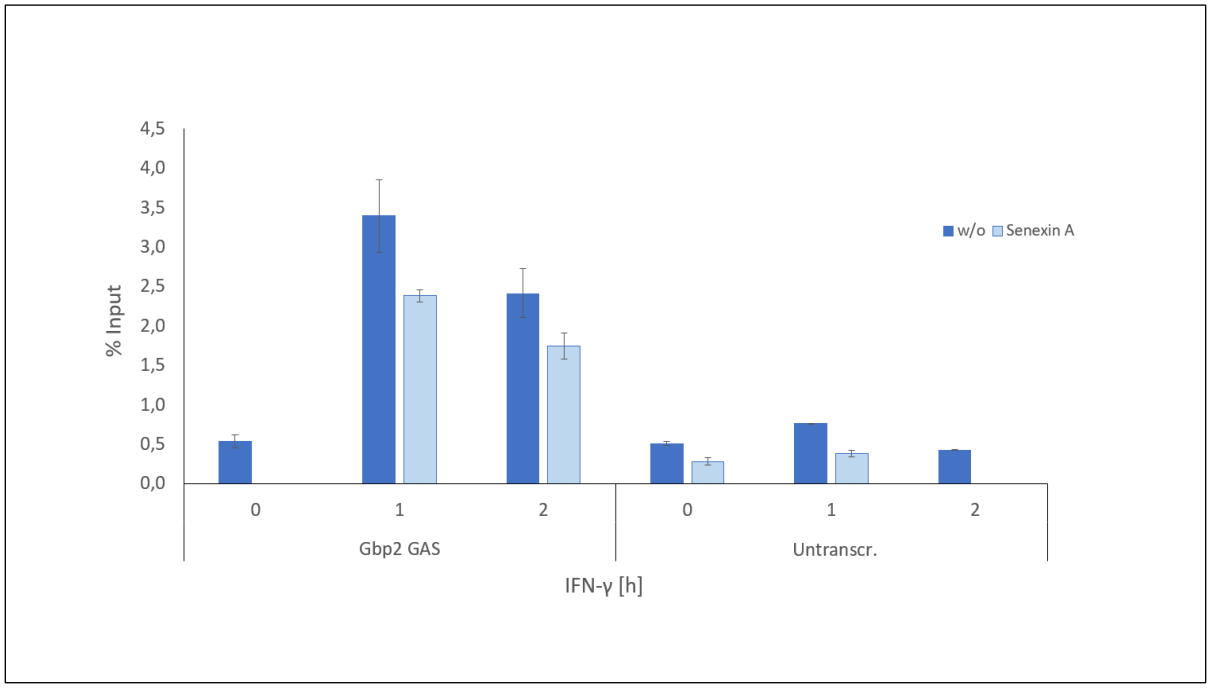
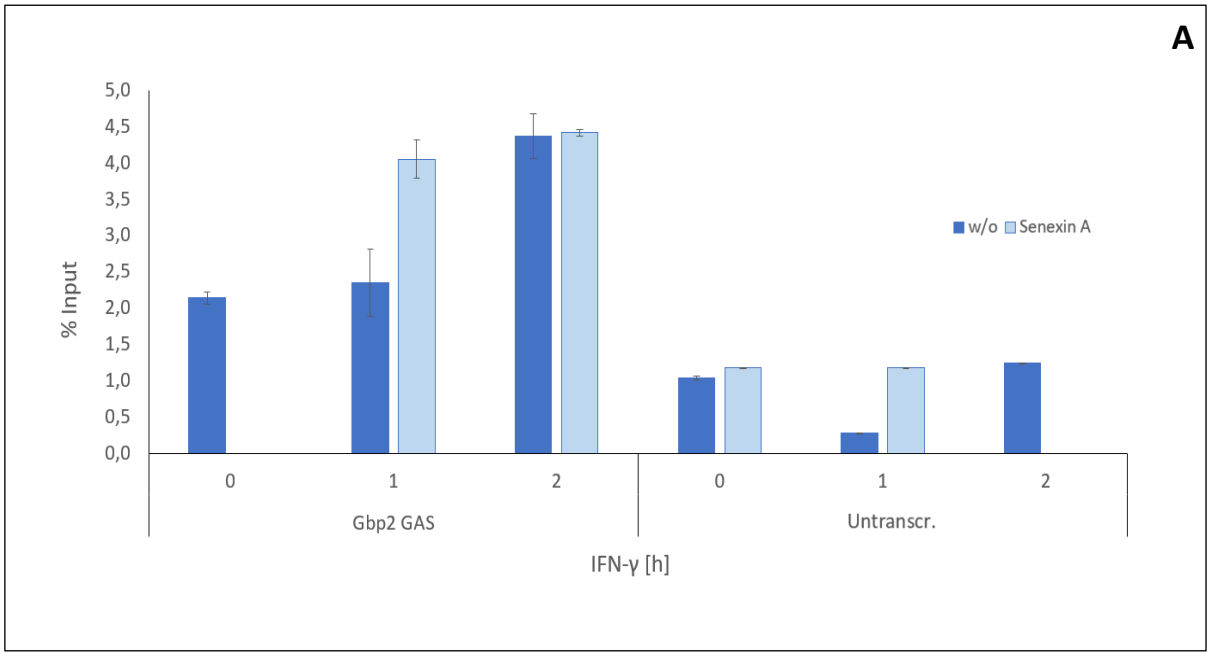


Figure 23 **Mediator kinase inhibition with Senexin A impacts RNA polymerase II abundance on *Gbp2 GAS***
CDK8fl MEFs were treated with IFN-γ (10ng/ml) for 0, 1 and 2 hours, prior being treated with Senexin A (10µM) for 15 minutes. Subsequently, cells were harvested and ChIP was conducted (Materials and Methods 7.5). RNA polymerase II recruitment to *Gbp2* GAS was examined by using pol II antibodies (Material and Methods 7.6, 7.7). The immunoprecipitated DNA was analyzed by qPCR. Signal values are depicted in % of Input. Error bars represent SDs of technical replicates (n = 2). A heterochromatic region was used as negative control. Due to human error, timepoint 0h Senexin A is missing. After stimulation with IFN-γ, a strong recruitment of Pol II to the *Gbp2* GAS sequence can be observed. Beyond that, the treatment with the kinase inhibitor Senexin A seems to downregulate the abundance of Pol II at the GAS region of *Gbp2.*

II abundance after kinase inhibition in the gene body was shown. To get a complete picture, it is therefore advised to investigate regions of the gene body of *Gbp2* in the future. The same negative control used for *Irf1*, namely a transcriptionally inert heterochromatic region, has been utilized for *Gbp2.*

5.5.3 Senexin A does not appear to change occupancy of pS2 Pol II and pS5 Pol II, however more data is needed for conclusive results



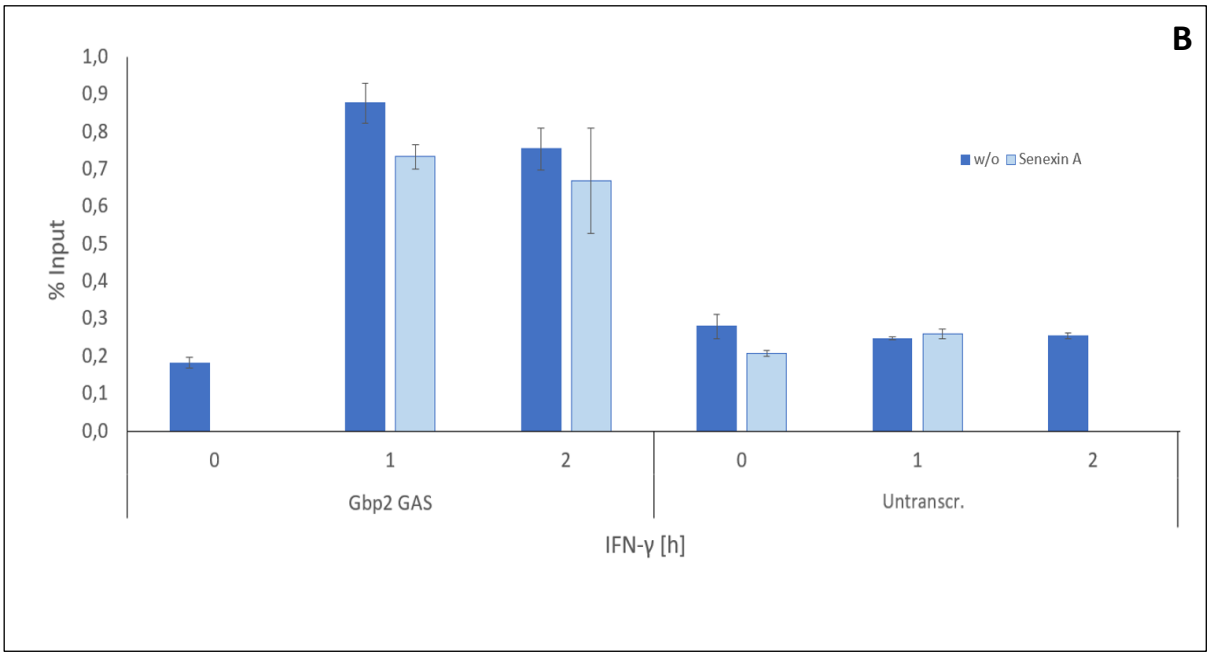


Figure 24 **Impact of Senexin A induced mediator kinase inhibition on CTD phosphorylation's pS2 and pS5 remains inconclusive**

CDK8fl MEFs were treated with IFN-γ (10ng/ml) for 0, 1 and 2 hours, prior being treated with Senexin A (10µM) for 15 minutes. Subsequently, cells were harvested and ChIP was conducted (Materials and Methods 7.5). pS2 and pS5 RNA polymerase II recruitment to *Gbp2* GAS was examined by using respective antibodies **(A and B)** (Material and Methods 7.6, 7.7). The immunoprecipitated DNA was analyzed by qPCR. Signal values are depicted in % of Input. Error bars represent SDs of technical replicates (n = 2). A heterochromatic region was used as negative control. Due to human error, timepoint 0h Senexin A is missing. **(A)** pS2 Pol II increases upon cytokine stimulus, effect of Senexin A on pS2 Pol II at *Gbp2* GAS remains inconclusive. **(B)** pS5 Pol II increases upon cytokine stimulus, however, in line with pS2 Pol II, no clear-cut effect of kinase inhibition on pS5 Pol II abundance at *Gbp2* GAS can be observed.

The C-terminal domain (CTD) of RNA polymerase acts as a central binding hub in transcriptional regulation. The CTD can be post-translationally modified, resulting in combinatorial patterns (CTD code) that are recognized by factors acting in orchestration with the transcriptional cycle[100]. Two crucial phosphorylation's are pS2 and pS5, which are catalyzed by CDK9 and CDK7, respectively. On the one hand, Pol II tends to be more in its pS5 form at the start of a gene, as serine 5 phosphorylation was shown to aid promoter escape. On the other hand, pS2 Pol II is more abundant towards the middle and end of a gene, as it is known to help in the transition towards productive elongation.
In regards to the secondary response gene *Gbp2*, no impact of mediator kinase inhibition by Senexin A on abundance of pS2 Pol II and pS5 Pol II at the gamma activated sequence (GAS) of *Gbp2* can be observed (24 A/B). Like in all other *Gbp2* ChIP experiments, the signal is low, but the fold change after induction with IFN-γ is reproducible in comparison to *Irf1.*

5.6 Establishment of Western blot against CDK19 in MEFs and BMDMs

The paralog of CDK8, termed CDK19, emerged in vertebrates due to gene duplication events. CDK19 displays high sequence similarity to CDK8, including near identical kinase domains as well as cyclin interaction domains[95]. CDK19 is part of an analogous CDK19 kinase module, which consists of the MED12 and MED13 paralogs MED12L and MED13L[95]. A recent study from our lab revealed CDK8, and not CDK19 as the major IFN-γ-activated STAT1 kinase in both mouse and human cells[45]. Evidently, CDK8 uses its kinase functionality to drive the IFN-γ antiviral response, whereas CDK19 regulates IFN-γ responses in kinase-independent ways[45].

As the western blot against CDK8 was already possible, optimization of antibodies against CDK19 in MEFs and BMDMs was necessary. To initiate optimization, the CDK19- antibody from Sigma Aldrich (SAB 4301196) was tested under conditions that have been proven optimal for CDK8 in the past (Materials and Methods, 7.3.3.4)

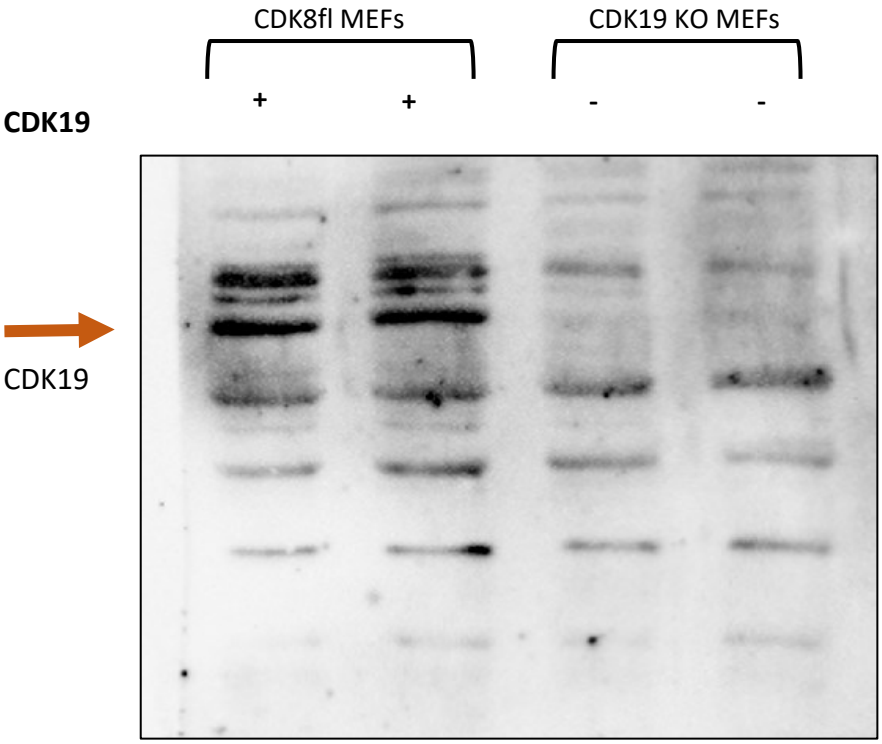


Figure 25 **CDK19 can be detected in CDK8fl MEF background, however, antibody specificity is low** CDK8fl MEFs and CDK19 KO MEFs were harvested without treatment or stimulation and proteins were isolated. CDK19 band is clearly detectable in CDK8fl MEFs, however a lot of unspecific bands can be observed. To detect CDK19, SAB4301196 from Sigma Aldrich was used. For more information and details about conditions used see Material and Methods.

5.6.1 CDK19 can be detected in MEFs and bone marrow derived macrophages (BMDMs) with SAB 4301196

In Figure 24, MEFs endogenous CDK19 (CDK8fl MEFs) were compared to CDK19 Crispr KO. In line with our expectations, CDK19 KOs shows no detection of CDK19, whereas endogenous CDK19 in CDK8flMEFs is clearly visible (Figure 25). Although the antibody detects CDK19 properly, a lot of unspecific binding can also be observed. In order to address and solve the problem of unspecific bands, the conditions of the western blot with SAB 4301196 have been optimized. In the end, conditions described in more detail in Material and Methods (7.3.3.4) have been found optimal and led to clear results.

MEFs containing CDK19 KOs and BMDMs from our colleague Luisa Morelli were tested under conditions specified above. As can be retrieved from Figure 25, the unspecific bands vanished and the endogenous CDK19 band is clearly visible. CDK8fl MEFs containing endogenous CDK19 were compared to CDK19 Crispr generated knockouts and various BMDMs, two of them treated with tamoxifen, were additionally tested under the newly found optimal conditions (Figure 26).

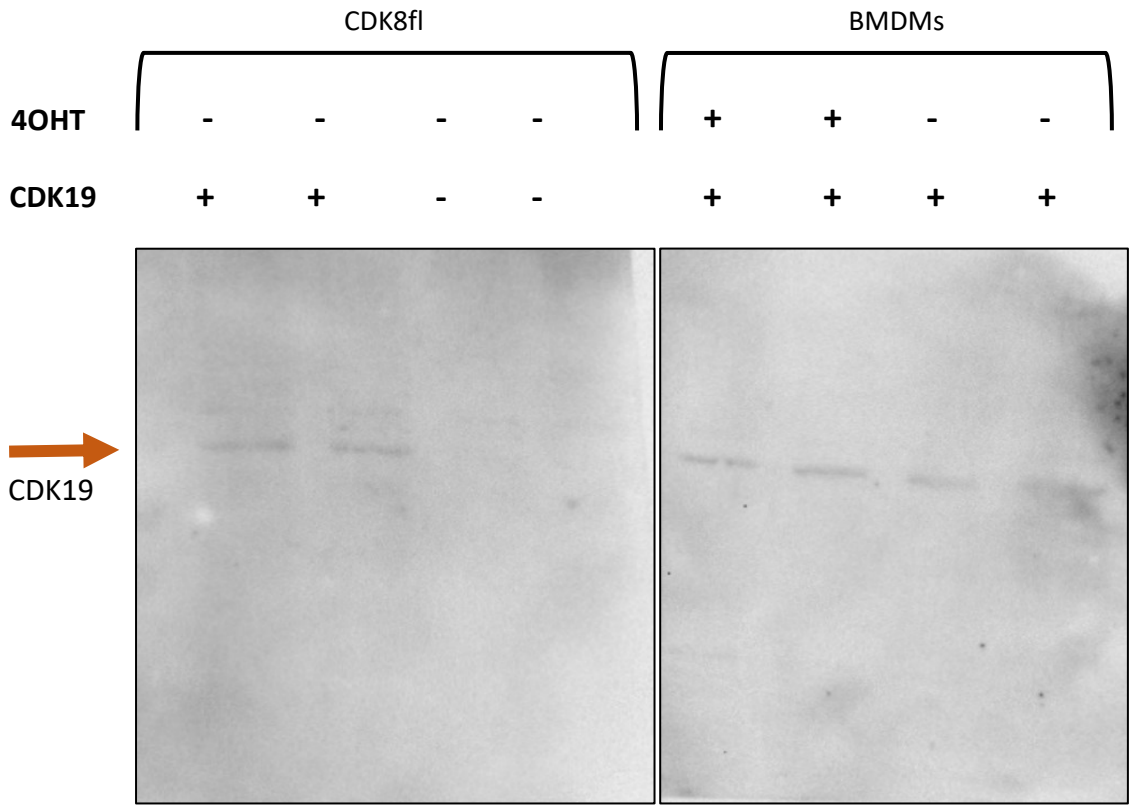


Figure 26 **CDK19 can be detected in MEFs and BMDMs with SAB 4301196**
CDK8fl MEFs were harvested and protein isolation was performed thereafter. Whole cell extracts from BMDMs were received from our colleague Luisa Morelli. Two of them had been treated with tamoxifen (not important for the purpose of this experiment). CDK19 can be detected in CDK8 MEFs and BMDMs, no unspecific bands visible. To detect CDK19, SAB4301196 from Sigma Aldrich was used. For more information and details about conditions used see Material and Methods.

To conclude, the antibody SAB 4301196 was optimized in terms of western blot conditions and showed specific binding in MEFs and BMDMs.

6. Summary and Discussion

The mediator kinase CDK8 is a crucial player in IFN-γ signaling and was shown to phosphorylate STAT1 on S727, which plays an important role in transcription[27]. CDK8 regulates a large set of genes, some of them being down and some of them upregulated. This provides an additional layer to control and fine-tune IFN-γ driven gene expression, ultimately establishing an antiviral state in the cell.

In line with published data, our experiments show that CDK8 is phosphorylating S727 of STAT1 in MEFs. In this study, the gene expression of two interferon stimulated genes, namely *Irf1* and *Gbp2*, was assessed. Mediator kinase inhibition led to a decrease of expression of both *Irf1* and *Gbp2* and to a decrease of pS727-STAT1. Published data from *Steinparzer et al.* compared gene expression of IFN-γ regulated genes in WT MEFs and S727A MEFs[27]. They found that *Irf1* was equally expressed and expression of *Gbp2* was reduced in cells lacking S727[27]. Our data regarding *Gbp2* is in line with these findings. RNA-seq data published in 2019 by *Steinparzer et al.* shows a reduction in both *Gbp2* and *Irf1* upon mediator kinase inhibition, whereby *Gbp2* expression is more reduced[45]. These findings are in line with the gene expression data generated in this study.

As expected, the conditional knockout of CDK8 led to reduction of pS727-STAT1 in CDK8fl MEFs. However, gene expression of the interferon stimulated genes *Irf1* and *Gbp2* was not impacted by the induced knockout of CDK8. In contrast, published RNA-seq data from our laboratory shows reduction of *Gbp2* upon either mediator kinase inhibition or knockout of CDK8. Therefore, results from this study regarding *Gbp2* expression upon CDK8 iKO cannot be considered plausible. A possible explanation is that RNA-Seq is more accurate than qRT-PCR. Thus, it is suggested to repeat this experiment to disregard technical errors that might have happened.

ChIP experiments directed against CDK8 had been proven complicated in the past, possibly explained by steric hindrance. CDK8 resides inside the Mediator complex and might therefore be topologically occluded from antibodies. Besides that, association of CDK8 with chromatin might be too short-lived to generate a robust ChIP signal.

However, as the recruitment of CDK8 to DNA is a topic of great interest, an attempt to ChIP against Flag-tagged CDK8 was conducted. Unfortunately, the signal values were below background, rendering the whole ChIP inconclusive. To shed light on this issue, it is suggested to try different anti-flag antibodies and possibly different tags in the future.

After successful establishment of ChIP in our cell-based system, the scientific objective of this study was to characterize mediator kinase-mediated changes in chromatin recruitment of STAT1, total Pol II, pS2 Pol II and pS5 Pol II. In particular, abundance of stated components at the TSS, the Intron 8 and the 3`UTR of *Irf1* was probed. MEFs were stimulated with IFN-γ and treated with and without Senexin A to inhibit mediator kinase activity. To address the significance of the findings, the ChIP experiment was repeated three times under the same conditions (A, B and C) and statistical analysis was carried out. It should however be noted that MEFs used in repetition B remained in cell culture 3 weeks longer than in repetition A and C.

Our data suggests that inhibition of mediator kinase activity has no impact on STAT1 recruitment to *Irf1* TSS. STAT1 occupancy at the TSS of *Irf1* is not altered in repetition A and B, however in C, a decrease in STAT1 abundance at the promoter region of *Irf1* can be observed. This can be seen as an outlier, as other ChIP data acquired in the course of this study shows no influence of mediator kinase activity on STAT1 recruitment to *Irf1* TSS. Furthermore, mediator kinase inhibition does not appear to influence IFN-γ-induced Pol II recruitment to TSS of *Irf1.* In repetition A and B, mediator kinase inhibition does not change Pol II occupancy. In contrast, data from repetition C shows self-contradicting results regarding an effect of Senexin A on Pol II recruitment to TSS. This might therefore be caused by technical mistakes, e.g. swapping of samples at 1 hour of IFN-γ treatment.

The effects of Pol II recruitment to the gene body of *Irf1* are unclear, however, mediator kinase inhibition might decrease Pol II abundance in the gene body. Data from repetition A suggests a reduction in Pol II recruitment to the gene body. When assuming that in repetition C the 1-hour timepoint sample was swapped by mistake, a decline in Pol II in the gene body upon mediator kinase inhibition can also be observed. However, in repetition B, no such effects can be confirmed. Taken together, mediator kinase inhibition does not appear to influence Pol II recruitment to TSS, but a trend towards reduction in the gene body was observed. Thus, effects of mediator kinase on Pol II recruitment are possible. Published data from our laboratory showed that CDK8 promotes pause release of Pol II[45]. These findings could explain the lower abundance in the gene body, as inhibition of mediator kinases might lead to delayed pause release and ultimately less Pol II in the gene body. Nevertheless, more data is needed for decisive results.

Next to STAT1 and Pol II, the effects of mediator kinase inhibition on pS2 Pol II and pS5 Pol II occupancy was an important question. As such, Senexin A does not appear to change occupancy of pS2 Pol II in all repetitions A, B and C. However, more experiments are needed to obtain conclusive results.

Our study shows that inhibition of mediator kinase with Senexin A causes significantly increased abundance of CTD phosphorylation pS5 of Pol II at TSS of *Irf1* after 1 hour of IFN-γ stimulation in all three repetitions.

Having in mind that passage number of MEFs in repetition B was higher compared to A and C, data acquired from repetition B can be seen as an outlier. Therefore, data on pS5 Pol II recruitment was pooled only from repetition A and C. This reveals a significant increase of pS5 Pol II occupancy upon mediator kinase inhibition at *Irf1* Intron 8 and 3´UTR after 2 hours of IFN-γ stimulation.

If we assume that pS5 is indeed altered upon mediator kinase inhibition the following explanation can be considered:

Possible mechanisms of action might be that mediator kinases interact with S5 phosphatases during transcription, leading to removal of the pS5 modification. When kinase activity is inhibited, a phosphatase controlled by mediator kinases, might not be able to remove the pS5 PTM. This could explain the high abundance of pS5 Pol II upon mediator kinase inhibition in repetition A and C.

Collectively, the high abundance of pS5 Pol II suggest a disturbance in transcriptional dynamics. This could explain the low Pol II abundance in repetition A and C and is in line with reduced gene expression of *Irf1*. In accordance, Gro-Seq data from *Steinparzer et al.* shows lower abundance of Pol II in the gene body of *Irf1* when mediator kinase activity is inhibited[45].

In addition to *Irf1,* the GAS region of *Gbp2* was tested for occupancy of STAT1, total Pol II, pS2 Pol II and pS5 Pol II to further characterize mediator kinase-mediated changes in chromatin recruitment. Importantly, samples of ChIP repetition A from Chapter 5.4 were used and ChIP was done once.

In line with data from TSS *Irf1,* STAT1 recruitment to GAS of *Gbp2* is not affected by mediator kinase activity. Moreover, data from this study suggests that inhibition of mediator kinase activity with Senexin A causes decreased Pol II recruitment to GAS of *Gbp2*

To amend Pol II data, pS2 Pol II and pS5 Pol II chromatin recruitment upon mediator kinase inhibition was topic of interest. As such, Senexin A does not appear to alter pS2 Pol II and pS5 Pol II occupancy at the GAS site of *Gbp2.*

Collectively, our ChIP targeting *Gbp2* reveals that mediator kinase impairment has an effect on Pol II recruitment to the GAS site of *Gbp2.* Effects of Senexin A on STAT1, pS2 Pol II are pS5 Pol II are unclear and need more data to be conclusive. For future experiments it is suggested to target a region residing in the gene body of *Gbp2.* This might reveal more details on mediator kinase regulation.

Lastly, a Western blot against CDK19 has been successfully established for MEFs and BMDMs (exact conditions listed in Material and Methods).

In terms of a broader outlook, ChIP experiments with the inducible KO of CDK8 as well as with CDK19 KOs probing STAT1, total RNA polymerase II and its CTD phosphorylation´s should be conducted for different ISGs in the future. Using our experimental system, different combinations, for example Senexin A in combination with tamoxifen, leaving only CDK19 scaffolding properties, should be tested for STAT1, Pol II, pS2 Pol II and pS5 Pol II chromatin recruitment.

As ChIP is now established in our experimental system, future work should be dedicated to ChIP-Seq experiments. This would amend our published RNA-seq data and provide a genome-wide view on mediator kinases and their role in transcription.

7. Material and Methods

7.1 Culture of immortalized cell lines

CDK8fl MEFs	Mouse embryonic fibroblasts	CDK8 fl/fl CreERT2/+ immortalized MEFs

CDK19 KO MEFs	Mouse embryonic fibroblasts	CDK19 KO Clone 9 from CDK8 fl/fl CreERT2/+ immortalized MEFs

CDK8 WT flag MEFs	Mouse embryonic fibroblasts	CDK8 WT flag expressing cells from CDK8 fl/fl CreERT2/+ background

7.1.1 MEFs culture

Cells were cultured in Dulbecco´s Modified Eagle´s Medium – high glucose (4500mg/L glucose, L-glutamine, sodium bicarbonate, without sodium pyruvate liquid, sterile filtered) supplemented with 10% Fetal Calf Serum (FCS), 100µg/ml streptomycin and 100 U/ml penicillin[9]. FCS and DMEM was stored at 4°C.

7.1.2 Cell culture procedures

If not stated otherwise, all procedures were carried out under a hood (HERASAFE 2030i) and possible centrifugation was conducted using the Eppendorf Centrifuge 5804 R. Three different dish sizes were used (6cm, 10cm, 15cm), with the maximum volume of them being 5ml, 10 ml and 20 ml, respectively. Cells were incubated under a constant temperature of 37 °C and 5% CO_2[9].

To split cells, plates were washed with sterile PBS (5-10 ml) followed by trypsin to detach them. Depending on the plate diameter (6cm, 10cm, 15cm) different amounts of trypsin (0.5 ml, 1ml, 1.5ml) was added. Trypsinization took place at 37 °C for 5 minutes and was stopped by addition of DMEM medium, followed by transfer onto new dishes.

To count cells, cells were washed with sterile PBS, followed by trypsinization at 37 °C for 5 minutes. To get rid of unwanted trypsin, ~ 5ml of medium was added, followed by centrifugation at 1000rpm for 3 minutes at RT. Supernatant was aspirated and pellet was resuspended in 10ml of medium. 10µl of suspension was mixed together with the azo dye 10µL of trypan blue. 10µL of mixture was applied to Neubauer-improved chamber and live cell number was calculated using the following formula: Concentration = Number of cells x 10000 / Number of squares.

To freeze cells, a ~ 80% confluent plate was washed in PBS and trypsinized for 5 minutes in the incubator, followed by centrifugation at 1000rpm for 3 minutes. Supernatant was aspirated, pellet was resuspended in 900 µL of FCS and 100µL of dimethyl sulfoxide (DMSO) in cryo-tubes. The samples were immediately put on ice and subsequently stored at -80 °C. If cells needed to be deep-frozen in liquid nitrogen for long term storage, cells were kept at -80 °C for 72 hours before transferring them into liquid nitrogen.

To thaw cells, cryo-tubes were quickly thawed by warming them up in a 37 °C water bath. Afterwards, cells were added, drop by drop, in a falcon tube containing ~ 8 ml of pre-warmed medium. The drops dispersed upon contact with the medium surface, leading to a clean distribution of cells. Then cells were centrifuged for 5 minutes at 1000rpm at RT, resuspended in fresh warm medium and plated onto one 6 cm dish with 5 ml of appropriate medium. The following days, medium was frequently changed to get rid of dead cells, followed by transfer onto 10 cm dish.

7.2 Cellular manipulations

All cellular manipulations were performed under the hood in cell culture.

7.2.1 Stimulations

To induce JAK-STAT signaling, the cells were stimulated with 10ng/ml final concentration of recombinant mouse interferon-gamma (rmIFN-γ) from Gibco (stored at -80 °C) under the hood in cell culture for the desired time frame stated in the experiments or for 45 minutes in case of protein analysis by western blot.

7.2.2 Kinase inhibition

To inhibit kinase function if mediator kinases CDK8/19, cells were treated 15 minutes prior to IFN-γ stimulation with 10 µM final concentration of Senexin A (stored at -20 °C).

7.2.3 CDK8 iKO: 4-hydroxy tamoxifen treatment

To induce CDK8 KO in CDK8fl MEFs, cells were treated with 4-hydroxy tamoxifen (4OHT) at a final concentration of 10 µg/ml for three hours. After treatment, cells were split and left for recovery for 72 hours, before further procedures were conducted

7.3 Methods for protein isolation and detection

7.3.1 Generation of whole cell extracts

Prior to protein isolation, $3x10^6$ cells were seeded on 6 cm plates the day before. Cells were stimulated with IFN-γ for 45 minutes and if needed treated with Senexin A or tamoxifen as described in Chapter 7.2. Thereafter, the 6 cm dishes were transferred on ice. While keeping them as close to the ice as possible, the medium was aspirated and the cells were washed two times in 4 °C PBS. After the last washing step, dishes were slightly tilted and aspirated again after 2 minutes to get rid of all the PBS. Subsequently, 100 µl of Frackelton Buffer (recipe see chapter 7.8) supplemented with 10mM Na_3VO_4 (=100x stock solution), 1 pill cOmplete™ EDTA-free protease inhibitor cocktail (=100x stock solution) and 1M Dithiothreitol (=1000x stock solution) was added to each 6 cm dish. Afterwards, cells were scraped with the use of a rubber scraper, centrifuged 5 minutes at 4 °C at 13200rpm and transferred into new Eppendorf tube. Subsequently, SDS sample buffer (composition see Chapter 7.8) was added in ratio 2:1 (Lysate: SDS). Reaction tubes were removed from the ice and boiled at 95 °C for 5 minutes in thermo blocks, followed by a quick centrifugation to push the liquid out of the lids. Samples were kept on ice for 30 minutes before used for protein analysis via western blot or, if used later, stored at -20 °C.

7.3.2 SDS-polyacrylamide gel electrophoresis (SDS PAGE)

Whole cell extracts of cells were prepared and harvested as described in chapter 7.3.1. The gels were either made fresh or made prior to the experiment and kept at 4 °C in a sealed plastic bag containing some water to prevent it from drying out.
Either way, stacking and separating gels were prepared as follows:

Separating gel (10%)		Stacking gel (5%)	
50% acrylamide	0,8 ml	30 % acrylamide	0,25 ml
1,5M Tris (pH= 8,8)	1 ml	0,5M Tris (pH=6,8)	0,5 ml
ddH_2O	2,2 ml	ddH_2O	1,25 ml
10 % SDS	40 µl	10 % SDS	20 µl
20% APS	12 µl	20% APS	6 µl
TEMED	8 µl	TEMED	4µl

To cast the gel, equipment from Hoefer was composed and checked for leakage with ddH_2O. Separating gel was poured into the casting chamber and covered with isopropanol on top for 15 minutes. Subsequently, the isopropanol was removed carefully and the stacking gel was added. Immediately after, a thin comb was inserted. As for the separating gel, polymerization took approximately 15 minutes. Afterwards, the gel was put in electric running chambers by Hoefer (Mighty small II for 8x7 cm gels). The chamber was filled up with 1x SDS Running Buffer (Chapter 7.8), slots were signed and comb was removed.
5 µl of marker (PageRuler Plus Prestained Protein Ladder, Thermo Scientific) and 15 µL of whole cell extracts were, prior to being shaken, loaded. Settings for one gel were: 200 V, 20 mA at RT. Running process was stopped when blue line ran out of chamber. If two gels were used, mA were doubled to 40 mA.

7.3.3 Immunoblotting

7.3.3.1 Semi-dry blot

After running of gel was over, the separating gel was removed and the stacking gel was rinsed in ddH_2O. For protein transfer onto nitrocellulose membrane, the trans-blot SD semi-dry transfer cell of Bio-Rad was used. Whatman paper and nitrocellulose membrane were, if not stated otherwise, cut to 7,5 x 9 cm and assembled as follows:

5 Whatman paper were soaked in Anode 1 buffer and placed in the middle of the apparatus. Subsequently, 3 pieces of Whatman paper were soaked in Anode 2 buffer and squarely put on top of the stack. The nitrocellulose membrane (GE HealthCare) was soaked in Anode 2 buffer (not touched with gloves, only forceps) and carefully placed on the stack.

The gel was then put on top of the stack and air bubbles were gently removed with wet gloves to prevent ripping. In the end, 5 pieces of Whatman paper were soaked in Cathode buffer and placed on top of the stack. Access liquid was gently rolled out with a falcon tube, chamber was closed and run at 25 V (not more!) and 60 mA per gel for 60-80 minutes.

7.3.3.2 Membrane blocking and antibody incubation

Nitrocellulose membrane was rinsed in ddH_2O and incubated in Ponceau stain for 1-4 minutes. Subsequently, membrane was washed three times 10 minutes in 1x TBST (Chapter 7.8) All steps were performed under constant longitudinal shaking at RT. To block, either 15 % non-fat dry milk (Fixmilch instant) or 5% BSA in 1x TBST (Sigma-Aldrich) was added to each membrane at RT for 1-1,5 hours. After sufficient blocking of the nitrocellulose membrane, it was washed three times in 1x TBST again. Incubation with 10 ml prior prepared primary antibody diluted in 5% BSA in TBST and 0,05% NaN_3, see antibody list chapter 7.7) was performed over night at 4 °C[9]. On the next day, the primary antibody was poured back into the falcon and the membrane was washed three times in 1x TBST, followed by incubation with appropriate secondary detection antibodies diluted to a final concentration between 1:4000 and 1:10000 in 5% non-fat dry milk for 30 minutes at RT (see antibody list chapter 7.7). Subsequently, the secondary antibody was removed and the membrane was once again washed in 1x TBST three times for 10 minutes each. Detection of protein was performed by using the ChemiDocTM MP Imaging system from Bio-Rad with ~ 400 µl 1:1 mixture of solution A and B from GE Healthcare - Amersham™ ECL Western Blotting Detection Reagents Select™ or Prime™.

7.3.3.3 Stripping of membranes

Following scan and detection of proteins, membranes can be re-used for detection of different proteins (same size possible) by stripping it. To do so, membrane was incubated and washed two times in stripping buffer (see chapter 7.9), followed by proper rinsing in ddH_2O for 1 min. Afterwards, the membrane was washed three times in 1x TBST for 10 minutes each, followed by blocking and incubation with primary antibodies as described in chapter 7.3.3.2.

7.3.3.4 Special Western blot conditions

In the course of CDK19 optimization for the CDK19 SAB4301196 in MEFs and BMDMs the following conditions have been established: Primary antibody dilution 1:500, secondary antibody 1:10000, blocking in 20 % milk and primary antibody incubation for only 2 hours on RT. For all other antibodies, the following conditions were met: Primary antibody dilution 1:1000, secondary 1:4000, blocking with 10-15% milk or 5% BSA for 1 hour and primary antibody incubation over night at 4 °C.

7.4 Gene expression analysis

7.4.1 RNA isolation with Trizol

For the following steps filter tips and high-grade ethanol was used. As for the protein isolation, $3x10^6$ cells were seeded on 6 cm plates overnight. Cells were put on ice and washed 2x with pre-cooled (4°C) PBS. At the last washing step, cell dishes were slightly tilted for ~ 1 minute, followed by aspiration to completely get rid of the medium. 500 µl Trizol (QIAzol Lysis reagent, 79306 Qiagen) was added onto the plates and cells were scraped thoroughly into new Eppendorf tubes. Subsequently, 150 µl chloroform was added and tubes were heavily vortexed for ~ 15 seconds till the color turned pastel pink. Eppendorf tubes were then centrifuged for 5 minutes at 13200 rpm at RT. To precipitate RNA, 1 volume of isopropanol and 1/10 volume of 5 M NaCl was added to the supernatant. The samples got inverted a couple of times and were left for incubation 10 minutes at RT. Thereafter, samples were centrifuged 30 minutes at 13200 rpm at 4°C and the resulting pellet was washed in 75 % ethanol. In the end, the pellet was air-dried under the hood for 5 minutes. RNA was resuspended in 50 µl nuclease-free water and stored at -80°C.

7.4.2 Reverse transcription

The concentration of RNA was measured using the NanoDrop Spectrometer. The amount of sample needed for 1 µg was calculated and mixed together with 100 pmol Oligo dT primer and water. To destroy possible RNA-secondary structures, samples were incubated in the Thermo Block for 5 minutes at 70°C. Thereafter, samples were put on ice and 4 µl of 5x Reaction buffer, 2 µl 10 mM dNTPs and 2 µl of nuclease free water was added. For pre-incubation, samples were put on 37°C for 5 minutes. To start reverse transcription and cDNA synthesis, 1 µl of Mu-MLV Revert Aid Transcriptase (200u/µl) was added and reaction was

incubated for 1 hour at 42°C. To stop the reaction, cDNA was incubated at 70°C for 10 minutes. If not used any further, cDNA was stored at -20°C.

7.4.3 Reverse transcription quantitative PCR (RT-qPCR)[9]

For each sample, the following PCR mixture was prepared:

GoTaq qPCR Mastermix (Promega)	7,5 µl
Forward primer + Reverse primer	0,09 µl
Nuclease-free water	2,41 µl
1/10 diluted cDNA	5 µl

The cDNA samples were each mixed together as described above and were transferred into twin-tec real-time 96 PCR plates from Eppendorf. For the standard curve a mixture of all samples diluted 1:2, 1:4, 1:8 and 1:16 in nuclease free water was made. Each reaction was done in duplicates and measured with the CFX96 Touch Real-Time PCR Detection System in accordance to following PCR temperature program for 40 cycles:

initial denaturation	**denaturation**	**annealing**	**elongation**	**melting curve**
95°C	95°C	56,5 - 60°C	72°C	58°C-95°C (0,5°C increment)
5 min	15 sec	20 sec	20 sec	18,5 min

7.5 Chromatin immunoprecipitation (ChIP) for MEFs

Cells used for experiments were seeded on 2x15 cm dishes per experimental timepoint until ~ 80 % confluency was reached. If needed, cells were then stimulated and treated for cellular manipulations as described in more detail in chapter 7.2.

7.5.1 Crosslinking of DNA and proteins and lysis of cells

The medium was thoroughly aspirated and cells were covered in 20 ml PBS at RT. Crosslinking of proteins and DNA was achieved by addition of 540 µl 37% formaldehyde (final 1%) to 20 ml PBS. Thereafter, cells were put on shaker for 10 minutes at RT. To stop the crosslinking process, 1 ml of 2.5 M glycine (final 125mM) was added to the dishes and cells were shaken again for 10 minutes at RT. After addition of glycine, a visual color shift to yellow was observed. Next steps were all performed on ice. PBS-formaldehyde-glycine mixture was aspirated and cells were washed thrice with pre-cooled PBS (4°C). For the last washing step, 15 cm plates were slightly tilted and aspirated again after ~ minute to completely get rid of PBS. Afterwards, cold PBS was supplemented with 100x Pille (one pill cOmplete^{TM} EDTA-free protease inhibitor cocktail in 1 ml of nuclease-free water) 200mM PMSF (=200x stock solution). 5 ml of supplemented PBS was added to each plate and cells were scraped into falcon tubes (replicates were pooled).
The cells were then centrifuged for 5 minutes at 1500 rpm at 4°C. The resulting pellet was resuspended in 5 ml WASH I solution, supplemented with 100x Pille and 200mM PMSF (=200x stock solution) (see Chapter 7.8) and kept on ice for 15 min. Every 5 minutes falcons were inverted a couple of times. Thereafter, cells were centrifuged again for 5 minutes at 1500 rpm at 4°C. Subsequently, the resulting cell pellet was resuspended in WASH II, supplemented with 100x Pille and 200mM PMSF (=200x stock solution) (see Chapter 7.8) and kept on ice for 15 minutes. As already mentioned above, falcons were inverted a couple of times every 5 minutes. Afterwards, cells were centrifuged again for 5 minutes at 1500 rpm at 4°C. Lastly, the pellet was resuspended in 800 µl freshly prepared SDS lysis buffer (for 10 ml – 1ml 10% SDS, 200 µl 0,5 M EDTA, 500 µl 1 M TRIS HCl pH 8, 100 µl Pille, 50 µl 200mM PMSF and nuclease free water to reach a total volume of 10 ml) and transferred into Eppendorf tubes. Subsequently, samples were incubated under constant turning at 4°C for one hour.

7.5.2 Sonication of DNA

In ChIP, DNA needs to be sheared on average into 500 base pair length fragments. To do so, samples were transferred into 15 ml Bioruptor Tubes from Diagenode and a small amount of sonication beads from the same company was added. Then the samples were put into the Bioruptor, which was pre-cooled to 4°C, and the following settings were used:
15 seconds on, 45 seconds off, 3 cycles.
The fragmented DNA was carefully decanted into new Eppendorf tubes (no up and down pipetting!) and samples were centrifuged 2x15 minutes at 13200 rpm and 8°C. Each time, the supernatant was transferred into new Eppendorf tubes. The samples were put on ice for ~ 30 minutes before DNA concentration with NanoDrop (absorbance of 260 nm and blanked to SDS lysis buffer) was assessed. 30 µl per sample was taken as sonication control and 25 µg of sample as input, both stored at 4°C in a metal rack. For each antibody used, 25 µg of sample was diluted in dilution buffer (recipe see Chapter 7.8) supplemented with 100x Pille (one pill cOmplete^{TM} EDTA-free protease inhibitor cocktail in 1 ml of nuclease-free water) and 200mM PMSF (=200x stock solution) to obtain a dilution between 1:10 and 1:20 (500-1300 µl dilution buffer). The specific antibodies needed for the experiment were then added to the respective diluted samples (see Chapter 7.7). Dynabeads Protein G from Invitrogen by Thermo Fisher Scientific were washed three times with supplemented dilution buffer in the magnetic rack and incubated in 0,1% BSA in dilution buffer, together with the ChIP samples, at 4°C over night under constant turning on the wheel.

7.5.3 Immunoprecipitation

The next day, 40 µl of blocked beads were added to the samples and incubated for 4 hours at 4°C on the turning wheel. Subsequently, the beads were washed every 10 minutes in the magnetic rack using different buffers in the following order at 4°C: 1x RIPA buffer, 2x High Salt buffer, 1x LiCl buffer and 2x TE buffer. To elute the immune complexes from the beads, 400 µl of freshly made elution buffer (for 5 ml – 1 ml 10 % SDS, 0,5 ml 1 M $NaHCO_3$,50 µl 1 M DTT and 3,5 ml of nuclease-free water) was added to the samples, followed by heavy shaking at 1400 rpm at RT for1 hour. Afterwards, the supernatant was transferred into new Eppendorf tubes, 400 µl of elution buffer was added to Input and Sonication samples (stored at 4°C in metal rack) and to reverse crosslinking, 20 µl of 4M NaCl was added to eluates, vortexed immediately and incubated at 65°C overnight.

7.5.4 DNA precipitation

After reverse crosslinking overnight, proteins were degraded by proteinase K treatment. Per sample 8µl of 0,5 M EDTA, 16 µl of 1 M TRIS HCl pH 6,5 and 2 µl of Proteinase K (stock 20 mg/ml) was added and incubated for 1 hour at 66°C under slight shaking at 850 rpm. Afterwards, samples were transferred into pre-centrifuged (1000 rpm, 15 seconds) 5PRIME Phase Lock Gel tubes by QuantaBio and 400 µl phenol-chloroform-isoamyl was added per tube. Samples were thoroughly shaken by hand and subsequently centrifuged for 5 minutes at 12000 rcf at RT. The aqueous phase containing DNA was decanted into new Eppendorf tubes and DNA was precipitated upon addition of 0,8 ml 96% ethanol, 40 µl 3M pH 5,2 sodium acetate and 1 µl of the co-precipitant glycogen (20mg/ml stock). Samples were then vortexed and incubated at -20°C for at least two hours. Thereafter, samples were centrifuged at 4°C, 13200 rpm for 45 minutes. The newly formed, small pellet was washed 2-3 times in 70 % ethanol and air-dried thereafter for 10 minutes under the hood.
The pellet was then resuspended in 100 µl nuclease-free water and incubated 10 minutes at 55°C to get rid of the residual ethanol, followed by storage at -20 °C.
In contrast to normal samples, sonication controls were resuspended in 30 µl nuclease-free water. Thereafter, RNA was digested using RNAse A (10mg/ml, Thermo Scientific) for 30 minutes at RT. To check uniform shearing of DNA, samples were, together with 6 µl Orange loading dye, analyzed on an 1% agarose gel (20 minutes, 100V).

7.5.5 qRT-PCR readout analysis

For the qPCR analysis, the Input samples were diluted 1:20 and the Kappa Sybr Green Mastermix was used as follows:

Kappa Sybr Green Mastermix	10 µl
Forward primer + Reverse primer	0,16 µl
Nuclease-free water	4,84 µl
DNA	5 µl

The DNA samples were each mixed together as described above and were transferred into twin-tec real-time 96 PCR plates from Eppendorf. For the standard curve a mixture of one input sample diluted 1:2, 1:4, 1:8 and 1:16 in nuclease free water was made. Each reaction was done in duplicates and measured with the CFX96 Touch Real-Time PCR Detection System in accordance to following PCR temperature program for 40 cycles:

initial denaturation	**denaturation**	**annealing**	**elongation**	**melting curve**
95°C	95°C	56,5 - 60°C	72°C	58°C-95°C (0,5°C increment)
5 min	15 sec	20 sec	20 sec	18,5 min

7.6 Primer list

7.6.1 Expression analysis of IFN-γ induced genes

Gene	Primer name	Sequence	Annealing temperature
Irf1	*Irf1* forw.	5´-CCGAAGACCTTATGAAGCTCTTTG-3´	60 °C
	Irf1 rev.	5´-GCAAGTATCCCTTGCCATCG-3´	
Gbp2	*Gbp2* forw.	5'-TGCTAAACTTCGGGAACAGG-3'	60 °C
	Gbp2 rev.	5'-GAGCTTGGCAGAGAGGTTTG-3'	
Hprt	*Hprt* forw.	5'-GGATTTGAATCACGTTTGTGTCAT-3'	60 °C
	Hprt rev.	5'-ACACCTGCTAATTTTACTGGCAA-3'	

7.6.2 qChIP analysis of IFN-γ induced genes

Gene	Primer name	Sequence	Annealing temperature
Irf1	*Irf1* TSS forw.	5´-TCCCGCTAAGTGTTTAGATTTC-3´	61 °C
	Irf1 TSS rev.	5´-TTCGGTTCGGCTTAGACTG-3´	
Irf1	*Irf1* Intron 8 forw.	5´-TGCCTAGTTGCTTGTCTCTG-3´	61,5 °C
	Irf1 Intron8 rev.	5´-CTCCTGTGTGTCGCTGTC-3´	
Irf1	*Irf1* 3´UTR forw.	5´-TGCTGTGCTGCTGGTCTTG-3´	61,5 °C
	Irf1 3´UTR rev.	5´-CCTTCATTCCGTCCTGTCCTTC-3´	
Gbp2	*Gbp2* GAS forw.	5´-AGTGGTGCTAAAATTGTTGTGG-3´	61 °C
	Gbp2 GAS rev.	5´-AGAAAGGAAGGAGAAAGATGGG-3´	
untranscr. region	untranscr. forw.	5´-TCAGGCATGAACCACCATAC-3´	56,5 °C
	untranscr. rev.	5´-AACATCCACACGTCCAGTGA-3´	

7.7 Antibody list

7.7.1 Primary Antibodies Western blot

Name	Blocking	Cat Number	Company	Species	Dilution
pS727-STAT1	BSA	/	Kovarik et al., 1999	rabbit	1:1000
pY701-STAT1	BSA	9167S	Cell signaling	rabbit	1:1000
STAT1	milk	D4Y6Z/14995S	Cell signaling	rabbit	1:1000
CDK8	BSA	4101S/6398	Cell signaling	rabbit	1:1000
CDK19	milk	SAB4301196	Sigma-Aldrich	rabbit	1:500
CDK19	milk	SAB1302333	Sigma-Aldrich	rabbit	1:500
Tubulin	milk	T9026	Sigma-Aldrich	mouse	1:5000

7.7.2 Secondary Antibodies Western blot

Name	diluted in	Company	Species	Dilution
Peroxidase AffiniPure Anti-Rabbit IgG (H+L)	milk	Jackson ImmunoResearch	donkey	1:4000-1:10000
Peroxidase AffiniPure Anti-Mouse IgG (H+L)	milk	Jackson ImmunoResearch	donkey	1:4000-1:10000
Peroxidase AffiniPure Anti-Goat IgG (H+L)	milk	Jackson ImmunoResearch	donkey	1:4000-1:10000

7.7.3 ChIP antibodies

Name	Cat number	Company	Species	ChIP amount
RNA-pol II	ab817	abcam	mouse	10 µl
RNA-pol II S2	A300-654A	Bethyl	rabbit	3.5µl
RNA-pol II S5	AB5408	abcam	mouse	5 µl
RNA-pol II	AB26721	abcam	mouse	10 µl
IgG rabbit isotype control	3900S/DA1E	Cell signaling	/	2,5 µl
IgG mouse isotype control	M5284	Sigma-Aldrich	/	25 µl
IgG goat isotype control	O26202	Thermo Fisher	/	2,5 µl
Flag	A190-101A	Bethyl	goat	3 µl

7.8 Reagent/Buffer list

7.8.1 Cell culture

Dulbecco´s modified eagle medium (DMEM, 500ml)
10 % FCS (Gibcon)
100 U/ml penicillin
100 µg/ml streptomycin

Freezing medium
FCS (Gibcon)
10 % DMSO

7.8.2 Protein isolation / Western blot

Stripping Buffer (1l)
15,02g Glycine
8,77g NaCl
0,1ml Tween20
Adjust pH = 2,5, fill up to 1l with ddH_2O, autoclave immediately

10x Western running buffer (1l)
30,29g Tris base
144,13g Glycine
10g SDS
Fill up to 11l with ddH_2O, autoclave immediately

Cathode buffer (1l)
5,25g Hexa-aminocaproic acid
200ml Methanol
0,1g SDS
fill up to 1l with ddH_2O

Anode 1 buffer (1l)
36,34g Tris base
200ml Methanol
Adjust pH =10,4, fill up to 1l with ddH_2O

Anode 2 buffer (1l)
0,3g Tris base
200ml Methanol
Adjust pH =10,4, fill up to 1l with ddH_2O

Frackelton buffer (1l)
1,2g Tris base
13,4g Tetrasodiumphosphate
2,9g NaCl
2,1g NaF
10 ml (1%) Triton-X-100
Adjust pH =7,1, fill up to 1l with ddH_2O

10x TBST (1l)
12,11g Tris base
87,66g NaCl
5ml Tween20
Adjust pH =8, fill up to 1l with ddH_2O

SDS sample buffer (20ml, 4x)
5ml 0,5M Tris-HCl pH = 6,8
2,5ml Glycerol
4ml 10% SDS
2ml Beta-Mercaptoethanol
0,2 ml 0,05% Bromophenol blue
6,5ml ddH_2O

Ponceau Stain
0,1% Ponceau S stain
5% Trichloroacetic acid (TCA)
0,05% SDS

7.8.3 Chromatin immunoprecipitation

Wash 1 (400ml)
10ml 10% Triton X-100
8ml 0,5M EGTA
4ml 1M HEPES
fill up to 400ml with ddH_2O, filter sterile

Wash 2 (400ml)
20ml 4M NaCl
0,8ml 0,5M EGTA
0,4ml 1M EGTA
4ml 1M HEPES
fill up to 400ml with ddH_2O, filter sterile

Dilution buffer (400ml)
16,7ml 4M NaCl
6,7ml 1M Tris HCl pH = 8,1
0,96ml 0,5M EDTA
44ml 10% Triton X-100
0,4ml 10% SDS
fill up to 400ml with ddH_2O, filter sterile

RIPA buffer (400ml)
15ml 4M NaCl
20ml 1M Tris HCl pH = 8,0
4ml 10% SDS
20ml 10% NaDOC (fresh)
4ml concentrated NP4O
fill up to 400ml with ddH_2O, filter sterile

High Salt buffer (400ml)
50ml 4M NaCl
20ml 1M Tris HCl pH = 8,0
4ml 10% SDS
40ml 10% NP4O
fill up to 400ml with ddH_2O, filter sterile

LiCl Wash buffer (400ml)
100ml 1M LiCl
20ml 1M Tris HCl pH = 8,0
20ml 10% NaDOC
40ml 10% NP40
fill up to 400ml with ddH_2O, filter sterile

TE buffer (500ml)
5ml 1M Tris HCl pH = 8,0
1ml 0,5M EDTA
fill up to 400ml with ddH_2O, filter sterile

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9. References

1. Van De Veerdonk, F. L., Gresnigt, M. S., Romani, L., Netea, M. G. & Latgé, J. P. Aspergillus fumigatus morphology and dynamic host interactions. *Nature Reviews Microbiology* vol. 15 661–674 (2017).

2. Walsh, D., McCarthy, J., O'Driscoll, C. & Melgar, S. Pattern recognition receptors-Molecular orchestrators of inflammation in inflammatory bowel disease. *Cytokine and Growth Factor Reviews* vol. 24 91–104 (2013).

3. Amarante-Mendes, G. P. *et al.* Pattern recognition receptors and the host cell death molecular machinery. *Frontiers in Immunology* vol. 9 2379 (2018).

4. HAGBERG, N. The Role of Plasmacytoid Dendritic Cells and Natural Killer Cells in Systemic Lupus Erythematosus. in (2014).

5. Dembic, Z. Cytokines of the Immune System. in *The Cytokines of the Immune System* 123–142 (Elsevier, 2015). doi:10.1016/b978-0-12-419998-9.00005-5.

6. Eleftheriou, D. & Brogan, P. A. Genetic interferonopathies: An overview. *Best Practice and Research: Clinical Rheumatology* vol. 31 441–459 (2017).

7. Schneider, W. M., Chevillotte, M. D. & Rice, C. M. Interferon-stimulated genes: A complex web of host defenses. *Annual Review of Immunology* vol. 32 513–545 (2014).

8. McNab, F., Mayer-Barber, K., Sher, A., Wack, A. & O'Garra, A. Type I interferons in infectious disease. *Nature Reviews Immunology* vol. 15 87–103 (2015).

9. Vcelkova, T. Transcription regulation by CDK8 and CDK19. (2017).

10. Kalliolias, G. D. & Ivashkiv, L. B. Overview of the biology of type I interferons. *Arthritis Research and Therapy* vol. 12 S1 (2010).

11. Honda, K., Takaoka, A. & Taniguchi, T. Type I Inteferon Gene Induction by the Interferon Regulatory Factor Family of Transcription Factors. *Immunity* vol. 25 349–360 (2006).

12. Ishikawa, H. & Barber, G. N. STING is an endoplasmic reticulum adaptor that facilitates innate immune signalling. *Nature* **455**, 674–678 (2008).

13. Tanaka, Y. & Chen, Z. J. STING specifies IRF3 phosphorylation by TBK1 in the cytosolic DNA signaling pathway. *Sci. Signal.* **5**, ra20 (2012).

14. Li, X. *et al.* Cyclic GMP-AMP Synthase Is Activated by Double-Stranded DNA-Induced Oligomerization. *Immunity* **39**, 1019–1031 (2013).

15. Fang, R. *et al.* MAVS activates TBK1 and IKKε through TRAFs in NEMO dependent and independent manner. *PLOS Pathog.* **13**, e1006720 (2017).

16. Kawasaki, T. & Kawai, T. Toll-like receptor signaling pathways. *Frontiers in Immunology* vol. 5 461 (2014).

17. Ivashkiv, L. B. & Donlin, L. T. Regulation of type i interferon responses. *Nature Reviews Immunology* vol. 14 36–49 (2014).

18. Montoya, M. *et al.* Type I interferons produced by dendritic cells promote their phenotypic and functional activation. *Blood* **99**, 3263–3271 (2002).

19. Keppler, S. J., Rosenits, K., Koegl, T., Vucikuja, S. & Aichele, P. Signal 3 Cytokines as Modulators of Primary Immune Responses during Infections: The Interplay of Type I IFN and IL-12 in CD8 T Cell Responses. *PLoS One* **7**, e40865 (2012).

20. Coro, E. S., Chang, W. L. W. & Baumgarth, N. Type I IFN Receptor Signals Directly Stimulate Local B Cells Early following Influenza Virus Infection. *J. Immunol.* **176**, 4343–4351 (2006).

21. Ding, G. *et al.* IFN-γdown-regulates the PD-1 expression and assist nivolumab in PD-1-blockade effect on CD8+ T-lymphocytes in pancreatic cancer. *BMC Cancer* **19**, 1053 (2019).

22. Thäle, C. & Kiderlen, A. F. Sources of interferon-gamma (IFN-γ) in early immune response to Listeria monocytogenes. *Immunobiology* **210**, 673–683 (2005).

23. Jacca, S. *et al.* Interferon Gamma-Mediated BoHV-4 Replication Restriction in Bovine Endometrial Stromal Cells Is Host IDO1 Gene Expression Independent and BoHV-4 IE2 Gene Expression Dependent1. *Biol. Reprod.* **91**, (2014).

24. Suryawanshi, A., Tadagavadi, R. K., Swafford, D. & Manicassamy, S. Modulation of inflammatory responses by Wnt/β-catenin signaling in dendritic cells: A novel immunotherapy target for autoimmunity and cancer. *Frontiers in Immunology* vol. 7 460 (2016).

25. Lieberman, L. A. & Hunter, C. A. The role of cytokines and their signaling pathways in the regulation of immunity to Toxoplasma gondii. *Int. Rev. Immunol.* **21**, 373–403 (2002).

26. Platanias, L. C. Mechanisms of type-I- and type-II-interferon-mediated signalling. *Nature Reviews Immunology* vol. 5 375–386 (2005).

27. Bancerek, J. *et al.* CDK8 Kinase Phosphorylates Transcription Factor STAT1 to Selectively Regulate the Interferon Response. *Immunity* **38**, 250–262 (2013).

28. Sadzak, I. *et al.* Recruitment of Stat1 to chromatin is required for interferon-induced serine phosphorylation of Stat1 transactivation domain. *Proc. Natl. Acad. Sci. U. S. A.* **105**, 8944–8949 (2008).

29. Papamichail, M., Perez, S. A., Gritzapis, A. D. & Baxevanis, C. N. Natural killer lymphocytes: Biology, development, and function. *Cancer Immunology, Immunotherapy* vol. 53 176–186 (2004).

30. Schoenborn, J. R. & Wilson, C. B. Regulation of Interferon-γ During Innate and Adaptive Immune Responses. *Advances in Immunology* vol. 96 41–101 (2007).

31. Bouma, G. & Strober, W. The immunological and genetic basis of inflammatory bowel disease. *Nature Reviews Immunology* vol. 3 521–533 (2003).

32. Carter, R. & Drouin, G. Structural differentiation of the three eukaryotic RNA polymerases. *Genomics* **94**, 388–396 (2009).

33. Sainsbury, S., Bernecky, C. & Cramer, P. Structural basis of transcription initiation by RNA polymerase II. *Nature Reviews Molecular Cell Biology* vol. 16 129–143 (2015).

34. Liu, D. *et al.* Solution structure of a TBP-TAF(II)230 complex: Protein mimicry of the minor groove surface of the TATA box unwound by TBP. *Cell* **94**, 573–583 (1998).

35. Fishburn, J. & Hahn, S. Architecture of the yeast RNA polymerase II open complex and regulation of activity by TFIIF. *Mol. Cell. Biol.* **32**, 12–25 (2012).

36. Lee, T. I. & Young, R. A. Transcription of Eukaryotic Protein-Coding Genes. *Annu. Rev. Genet.* **34**, 77–137 (2000).

37. Adamczewski, J. P. *et al.* MAT1, cdk7 and cyclin H form a kinase complex which is UV light-sensitive upon association with TFIIH. *EMBO J.* **15**, 1877–1884 (1996).

38. Rimel, J. K. & Taatjes, D. J. The essential and multifunctional TFIIH complex. *Protein Science* vol. 27 1018–1037 (2018).

39. Corden, J. L., Cadena, D. L., Ahearn, J. M. & Dahmus, M. E. A unique structure at the carboxyl terminus of the largest subunit of eukaryotic RNA polymerase II. *Proc. Natl. Acad. Sci. U. S. A.* **82**, 7934–7938 (1985).

40. Kwak, H. & Lis, J. T. Control of Transcriptional Elongation. *Annu. Rev. Genet.* **47**, 483–508 (2013).

41. Ebmeier, C. C. *et al.* Human TFIIH Kinase CDK7 Regulates Transcription-Associated Chromatin Modifications. *Cell Rep.* **20**, 1173–1186 (2017).

42. Adelman, K. & Lis, J. T. Promoter-proximal pausing of RNA polymerase II: emerging roles in metazoans. *Nature reviews. Genetics* vol. 13 720–731 (2012).

43. Aoi, Y. *et al.* NELF Regulates a Promoter-Proximal Step Distinct from RNA Pol II Pause-Release. *Mol. Cell* **78**, 261-274.e5 (2020).

44. Fant, C. B. *et al.* TFIID Enables RNA Polymerase II Promoter-Proximal Pausing. *Mol. Cell* **78**, 785-793.e8 (2020).

45. Steinparzer, I. *et al.* Transcriptional Responses to IFN-γ Require Mediator Kinase-Dependent Pause Release and Mechanistically Distinct CDK8 and CDK19 Functions. *Mol. Cell* **76**, 485-499.e8 (2019).

46. Zhou, Q., Li, T. & Price, D. H. RNA Polymerase II Elongation Control. *Annu. Rev. Biochem.* **81**, 119–143 (2012).

47. Fish, R. N. & Kane, C. M. Promoting elongation with transcript cleavage stimulatory factors. *Biochimica et Biophysica Acta - Gene Structure and Expression* vol. 1577 287–307 (2002).

48. Yoh, S. M., Cho, H., Pickle, L., Evans, R. M. & Jones, K. A. The Spt6 SH2 domain binds Ser2-P RNAPII to direct Iws1-dependent mRNA splicing and export. *Genes Dev.* **21**, 160–174 (2007).

49. Kuehner, J. N., Pearson, E. L. & Moore, C. Unravelling the means to an end: RNA polymerase II transcription termination. *Nature Reviews Molecular Cell Biology* vol. 12 283–294 (2011).

50. Dannappel, M. V., Sooraj, D., Loh, J. J. & Firestein, R. Molecular and in vivo Functions of the CDK8 and CDK19 Kinase Modules. *Front. Cell Dev. Biol.* **6**, (2019).

51. Poss, Z. C., Ebmeier, C. C. & Taatjes, D. J. The Mediator complex and transcription regulation. *Critical Reviews in Biochemistry and Molecular Biology* vol. 48 575–608 (2013).

52. Thompson, C. M., Young, R. A. & Fink, G. R. *General requirement for RNA polymerase II holoenzymes in vivo (transcription initiation/conditional mutants)*. *Biochemistry* vol. 92 (1995).

53. Flanagan, P. M., Kelleher, R. J., Sayre, M. H., Tschochner, H. & Kornberg, R. D. A mediator required for activation of RNA polymerase II transcription in vitro. *Nature* **350**, 436–438 (1991).

54. Boyer, T. G., Martin, M. E. D., Lees, E., Ricciardi, R. P. & Berk, A. J. Mammalian Srb/mediator complex is targeted by adenovirus E1A protein. *Nature* **399**, 276–279 (1999).

55. Kornberg, R. D. Mediator and the mechanism of transcriptional activation. *Trends in Biochemical Sciences* vol. 30 235–239 (2005).

56. Miller, C. *et al.* Mediator phosphorylation prevents stress response transcription during non-stress conditions. *J. Biol. Chem.* **287**, 44017–44026 (2012).

57. Yin, J. W. & Wang, G. The Mediator complex: A master coordinator of transcription and cell lineage development. *Dev.* **141**, 977–987 (2014).

58. Allen, B. L. & Taatjes, D. J. The Mediator complex: A central integrator of transcription. *Nature Reviews Molecular Cell Biology* vol. 16 155–166 (2015).

59. Malik, S. & Roeder, R. G. The metazoan Mediator co-activator complex as an integrative hub for transcriptional regulation. *Nature Reviews Genetics* vol. 11 761–772 (2010).

60. Carrer, M. *et al.* Control of mitochondrial metabolism and systemic energy homeostasis by microRNAs 378 and 378*. *Proc. Natl. Acad. Sci. U. S. A.* **109**, 15330–15335 (2012).

61. Kato, Y., Habas, R., Katsuyama, Y., Näär, A. M. & He, X. A component of the ARC/mediator complex required for TGFβ/nodal signalling. *Nature* **418**, 641–646 (2002).

62. Papadopoulou, T., Kaymak, A., Sayols, S. & Richly, H. Dual role of Med12 in PRC1-dependent gene repression and ncRNA-mediated transcriptional activation. *Cell Cycle* **15**, 1479–1493 (2016).

63. Brower, C. S. *et al.* Mammalian mediator subunit mMED8 is an Elongin BC-interacting protein that can assemble with Cul2 and Rbx1 to reconstitute a ubiquitin ligase. *Proc. Natl. Acad. Sci. U. S. A.* **99**, 10353–

10358 (2002).

64. Lin, J. J. *et al.* Mediator coordinates PIC assembly with recruitment of CHD1. *Genes Dev.* **25**, 2198–2209 (2011).

65. Sharma, V. M., Li, B. & Reese, J. C. SWI/SNF-dependent chromatin remodeling of RNR3 requires TAFIIs and the general transcription machinery. *Genes Dev.* **17**, 502–515 (2003).

66. Knuesel, M. T., Meyer, K. D., Donner, A. J., Espinosa, J. M. & Taatjes, D. J. The Human CDK8 Subcomplex Is a Histone Kinase That Requires Med12 for Activity and Can Function Independently of Mediator. *Mol. Cell. Biol.* **29**, 650–661 (2009).

67. Vilar, J. M. G. & Saiz, L. DNA looping in gene regulation: From the assembly of macromolecular complexes to the control of transcriptional noise. *Current Opinion in Genetics and Development* vol. 15 136–144 (2005).

68. Sanyal, A., Lajoie, B. R., Jain, G. & Dekker, J. The long-range interaction landscape of gene promoters. *Nature* **489**, 109–113 (2012).

69. Lai, F. *et al.* Activating RNAs associate with Mediator to enhance chromatin architecture and transcription. *Nature* **494**, 497–501 (2013).

70. Wu, S.-Y., Zhou, T. & Chiang, C.-M. Human Mediator Enhances Activator-Facilitated Recruitment of RNA Polymerase II and Promoter Recognition by TATA-Binding Protein (TBP) Independently of TBP-Associated Factors. *Mol. Cell. Biol.* **23**, 6229–6242 (2003).

71. Ranish, J. A., Yudkovsky, N. & Hahn, S. Intermediates in formation and activity of the RNA polymerase II preinitiation complex: Holoenzyme recruitment and a postrecruitment role for the TATA box and TFIIB. *Genes Dev.* **13**, 49–63 (1999).

72. Koleske, A. J., Buratowski, S., Nonet, M. & Young, R. A. A novel transcription factor reveals a functional link between the RNA polymerase II CTD and TFIID. *Cell* **69**, 883–94 (1992).

73. Cantin, G. T., Stevens, J. L. & Berk, A. J. Activation domain-mediator interactions promote transcription preinitiation complex assembly on promoter DNA. *Proc. Natl. Acad. Sci. U. S. A.* **100**, 12003–12008 (2003).

74. Johnson, K. M., Wang, J., Smallwood, A., Arayata, C. & Carey, M. TFIID and human mediator coactivator complexes assemble cooperatively on promoter DNA. *Genes Dev.* **16**, 1852–1863 (2002).

75. Marr, M. T., Isogai, Y., Wright, K. J. & Tjian, R. Coactivator cross-talk specifies transcriptional output. *Genes Dev.* **20**, 1458–1469 (2006).

76. Hwa, J. B., Yun, K. K. & Roeder, R. G. Human mediator enhances basal transcription by facilitating recruitment of transcription factor IIB during preinitiation complex assembly. *J. Biol. Chem.* **281**, 15172–15181 (2006).

77. Baek, H. J., Malik, S., Qin, J. & Roeder, R. G. Requirement of TRAP/Mediator for Both Activator-Independent and Activator-Dependent Transcription in Conjunction with TFIID-Associated TAFIIs. *Mol. Cell. Biol.* **22**, 2842–2852 (2002).

78. Søgaard, T. M. M. & Svejstrup, J. Q. Hyperphosphorylation of the C-terminal repeat domain of RNA polymerase II facilitates dissociation of its complex with mediator. *J. Biol. Chem.* **282**, 14113–14120 (2007).

79. Malik, S., Molina, H. & Xue, Z. PIC Activation through Functional Interplay between Mediator and TFIIH. *J. Mol. Biol.* **429**, 48–63 (2017).

80. Takahashi, H. *et al.* Human mediator subunit MED26 functions as a docking site for transcription elongation factors. *Cell* **146**, 92–104 (2011).

81. Jishage, M. *et al.* Transcriptional regulation by pol II(G) involving mediator and competitive interactions of gdown1 and tfiif with pol II. *Mol. Cell* **45**, 51–63 (2012).

82. Cheng, B. *et al.* Functional association of gdown1 with RNA polymerase II poised on human genes. *Mol. Cell* **45**, 38–50 (2012).

83. Malumbres, M. & Barbacid, M. Mammalian cyclin-dependent kinases. *Trends in Biochemical Sciences* vol. 30 630–641 (2005).

84. Liao, S.-M. *et al. A kinase-cyclin pair in the RNA polymerase II holoenzyme*.

85. Galbraith, M. D., Donner, A. J. & Espinosa, J. M. CDK8: A positive regulator of transcription. *Transcription* vol. 1 4–12 (2010).

86. Hengartner, C. J. *et al.* Temporal regulation of RNA polymerase II by Srb10 and Kin28 cyclin-dependent kinases. *Mol. Cell* **2**, 43–53 (1998).

87. Knuesel, M. T., Meyer, K. D., Bernecky, C. & Taatjes, D. J. The human CDK8 subcomplex is a molecular switch that controls Mediator coactivator function. *Genes Dev.* **23**, 439–451 (2009).

88. Akoulitchev, S., Chuikov, S. & Reinberg, D. TFIIH is negatively regulated by cdk8-containing mediator complexes. *Nature* **407**, 102–106 (2000).

89. Zhu, X. *et al.* Genome-Wide Occupancy Profile of Mediator and the Srb8-11 Module Reveals Interactions with Coding Regions. *Mol. Cell* **22**, 169–178 (2006).

90. Andrau, J. C. *et al.* Genome-Wide Location of the Coactivator Mediator: Binding without Activation and Transient Cdk8 Interaction on DNA. *Mol. Cell* **22**, 179–192 (2006).

91. Donner, A. J., Szostek, S., Hoover, J. M. & Espinosa, J. M. CDK8 Is a Stimulus-Specific Positive Coregulator of p53 Target Genes. *Mol. Cell* **27**, 121–133 (2007).

92. Alarcón, C. *et al.* Nuclear CDKs Drive Smad Transcriptional Activation and Turnover in BMP and TGF-β Pathways. *Cell* **139**, 757–769 (2009).

93. Fryer, C. J., White, J. B. & Jones, K. A. Mastermind recruits CycC:CDK8 to phosphorylate the Notch ICD and coordinate activation with turnover. *Mol. Cell* **16**, 509–520 (2004).

94. Yamamoto, S. *et al.* Mediator cyclin-dependent kinases upregulate transcription of inflammatory genes in cooperation with NF-κB and C/EBPβ on stimulation of Toll-like receptor 9. *Genes to Cells* **22**, 265–276 (2017).

95. Fant, C. B. & Taatjes, D. J. Regulatory functions of the Mediator kinases CDK8 and CDK19. *Transcription* **10**, 76–90 (2019).

96. Wen, Z., Zhong, Z. & Darnell, J. E. Maximal activation of transcription by statl and stat3 requires both tyrosine and serine phosphorylation. *Cell* **82**, 241–250 (1995).

97. Decker, T. & Kovarik, P. Serine phosphorylation of STATs. *Oncogene* vol. 19 2628–2637 (2000).

98. Li, W. X. Canonical and non-canonical JAK-STAT signaling. *Trends in Cell Biology* vol. 18 545–551 (2008).

99. Kovarik, P., Castiglia, V., Ivin, M. & Ebner, F. Type I interferons in bacterial infections: A balancing act. *Front. Immunol.* **7**, 1–8 (2016).

100. Jasnovidova, O. & Stefl, R. The CTD code of RNA polymerase II: A structural view. *Wiley Interdisciplinary Reviews: RNA* vol. 4 1–16 (2013).

101. Praefcke, G. J. K. Regulation of innate immune functions by guanylate-binding proteins. *International Journal of Medical Microbiology* vol. 308 237–245 (2018).

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10. Supplementary – Zusammenfassung

Die Genexpression in der Immunantwort ist ein hochkomplexer Prozess, der auf verschiedenen Ebenen beeinflusst werden kann. Trotz umfangreicher Studien in den letzten Jahrzehnten sind viele Mechanismen nach wie vor ungeklärt. Die Kinasen CDK8 und CDK19 können reversibel mit dem Mediator-Komplex assoziieren und wurden kürzlich als wichtiger Bestandteil der Transkriptionsmaschinerie identifiziert. CDK8 ist in der Lage, die Transkription durch eine Vielzahl von Mechanismen zu beeinflussen. Aktuelle Forschungsarbeiten unseres Labors haben die Rolle von CDK8 und seinem Paralog CDK19 in der IFN-γ Immunantwort beschrieben. Da beide jedoch eine große Teilmenge von Genen entweder hoch- oder herunterregulieren, bleiben die genauen Wirkungsmechanismen im Dunkeln. In dieser Studie wird der Effekt der CDK8-mediierten Phosphorylierung S727 von STAT1 in murinen embryonalen Fibroblasten bestätigt. Darüber hinaus zeigen wir, dass bei der Hemmung der Mediator-Kinase-Funktion die Expression der IFN-γ-induzierten Gene *Irf1* und *Gbp2* herunterreguliert wird. Der Knock-Out von CDK8 zeigte keinen Einfluss auf die Expression ebengenannter Gene.

Im Zuge dieser Studie wurde ChIP in unserem *In-vitro*-System etabliert. Wir konnten zeigen, dass bei Inhibition der Mediator-Kinasen pS5 Pol II im Genkörper hochreguliert wird. Unsere Daten deuten darauf hin, dass Mediator-Kinasen ohne ihre enzymatischen Eigenschaften zu einem Ungleichgewicht während der Transkription und folglich zu niedrigeren Expressionsleveln von Genen wie *Irf1* und *Gbp2* führen. Zukünftige Arbeiten sollen unser experimentelles System nutzen um die Rolle von CDK8 und CDK19 weiter zu entschlüsseln. Des Weiteren sollte so bald wie möglich ein ChIP-Seq Experiment innerhalb unseres Systems durchgeführt werden. Dies würde ein tieferes Verständnis über Mediator-Kinasen zulassen und daher Möglichkeiten eröffnen, diese in der nahen Zukunft als Ziele in der Krebsbehandlung einzusetzen.