KINASE-DEPENDENT AND -INDEPENDENT ROLES OF CDK8 IN STAT1-REGULATED TRANSCRIPTION

Research Report

Research report describing the experiments carried out between 08.09.2016 and 14.12.2016 in the laboratory of Prof. Dylan Taatjes at JSCBB - University of Colorado Boulder.

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INTRODUCTION

CDK8 is a positive regulator of the Interferon gamma (IFN-γ) response (Bancerek, 2013). We showed that CDK8 is the long sought-after kinase introducing the serine phosphorylation on the serine residue 727 in the transactivation domain of Stat1, which is necessary for full biological activity. Moreover we showed that CDK8 has additional gene-specific regulatory roles in the IFN-γ pathway. The aim of my project is to figure out the kinase-dependent and -independent mechanisms how CDK8 regulates IFN-γ-induced transcription.

To address this question we developed a set of tools to distinguish between kinase-dependent and -independent function of CDK8. The most straightforward way to inhibit kinase activity is to use an inhibitor specific against the kinase of interest. In case of CDK8 there are meanwhile specific inhibitors available (Dale et al., 2015, Pelish et al., 2015). All these inhibitors share one important limitation, since there exists a CDK8 paralogue called CDK19. CDK19 shows high similarity to CDK8, especially within the kinase domain. Because of this all so far developed CDK8 inhibitors also target CDK19. To date the function of CDK19 is not known, it was shown to interact with the Mediator complex and similar to CDK8 it is organized in a submodule with Cyclin C, Med12 and Med13 (Sato et al., 2014). Because of the difficulty to distinguish between these two kinases, it is not known whether they have distinct functions in transcription regulation (Galbraith et al., 2013, Tsutsui et al., 2013).

Knowing the limitations of CDK8 inhibitors we developed an analogue sensitive CDK8 mutant (CDK8as). The principle is to exchange the gatekeeper residue of the ATP binding pocket to a smaller amino acid, which still allows binding of ATP to the pocket but also allows binding of bulky ATP-analogues. Under normal conditions the kinase activity is not affected, in presence of an ATP-analogue, e.g. NM-PP1, the CDK8as kinase is inhibited without influencing other kinases in the cell. This approach was already successful used to study the kinase activities of CDK7, another cyclin-dependent kinase involved in transcription regulation (Larochelle et al., 2006). We successfully introduced the CDK8as in HAP cells and verified the inhibition of the kinase by the ATP-analogue NM-PP1. Before introducing the kinase into other cell types, the idea was to characterize the CDK8as in detail using in vitro kinase assays that are established in the Taatjes

lab. Using this method the kinase activity of Wt CDK8 and CDK8as can be compared. Moreover the inhibition of the CDK8 kinase activity in the presence of the ATP-analogue or other CDK8 inhibitors can be determined in a cell-free environment.

To further determine the function of CDK8 kinase activity, the HAP cell line is as well a useful tool. Although they are no classical immune cells and are therefore not the optimal system to study immune responses, the aim was to look at the serum response. It was published by Donner et al. (2010) that CDK8 knockdown using shRNA reduces gene expression of serum response genes, especially on the level of transcription elongation. It is not known if this regulation is kinase-dependent or -independent since the analysis was done after CDK8 knockdown. Moreover a possible role of CDK19 was not addressed in this pathway. The comparison between CDK8 kinase inhibition by using the ATP-analogue and the CDK8 knockout HAP cells allows to distinguish between kinase-dependent and -independent effects. Moreover using the also available CDK19 knockout HAP cells, the effects of CDK19 in serum response can be analyzed. In addition the presence of the kinase itself without its kinase activity can be addressed by blocking the remaining kinase in the knockout cell line with Cortistatin A.

To get more insights into the transcription regulation of the IFN-γ signaling response by the kinase activity of the Mediator kinases, the GRO-Seq method is the state-of-the-art approach. This analysis allows to determine the position of the RNA Polymerase II (RNA Pol II) genome wide at different time points after IFN-γ stimulation. By blocking the kinase activity using Cortistatin A (Pelish et al., 2015) the step of transcription that involves Mediator kinase activity can be determined. The goal was to optimize the GRO-Seq protocol established in the Taatjes and Dowell lab (Allen et al., 2014) for our cell type of interest and to prepare GRO-Seq libraries for downstream sequencing.

KINASE ASSAYS TO CHARACTERIZE THE CDK8as MUTANT

In our analogue-sensitive CDK8 (CDK8as) expressing cell line we could already show that the serine phosphorylation of Stat1 is reduced upon inhibition of CDK8 by adding the ATP-analogue NM-PP1. In the absence of the ATP-analogue, the kinase function of CDK8as is not affected, leading to serine phosphorylation levels of Stat1 that are comparable to Wt cells.

To further characterize the CDK8as mutation in more detail, we planned to analyze the mutated kinase using in vitro kinase assays, to have a clean system in which we can compare Wt CDK8 to CDK8as kinase activity in absence and presence of the ATP-analogue.

Since CDK8 is organized in the CDK8 submodule of Mediator, consisting of CDK8, Cyclin C, Med12 and Med13 and it is only functional within this submodule, it is necessary to express and purify the whole complex from insect cells in order to perform the kinase assays.

The Wt CDK8 module was already available in the Taatjes lab from a previous collaboration (Bancerek et al., 2013). For our purpose we had to express the CDK8as containing module with Cyclin C, Med12 and Med13.

Cloning procedure for the analogue sensitive CDK8

At the time I started in the lab, the analogue sensitive mutation of CDK8 was successfully introduced into the Wt CDK8 construct and the mutated sequence was in the pACO plasmid. I started by performing the maxi prep for the CDK8as in the pACO vector using the E.Z.N.A. Fast Filter Plasmid Maxi Kit (Omega). To avoid any contaminations of the sample, sterile filtered water and sterile components were used throughout the whole procedure. After the elution in 1.5 ml water the sample was concentrated. Therefore 1/10 volume of sodium acetate and 1 volume of isopropanol were added and incubated 10 minutes at room temperature. After a 15 minutes centrifugation at maximum speed at 4°C, the pellet was washed once with 75 % EtOH. The pellet was resuspended in the appropriate volume of water to get the final concentration of 1 µg/µl.

This plasmid was send to the insect cell facility at the Anschutz Medical Center in Denver to determine the protein expression.

In parallel the insert was amplified from the pACO plasmid using the primers CDK8ShpACOFwd and CDK8ShpACORev listed in Table 1. The product from the PCR reaction was gel purified, the band of the according size (1395 bp) was cut out and cleaned up using QIAquick Gel Purification Kit (Qiagen). The product was then treated with the restriction enzymes EcoRI and XbaI for an hour at 37°C, followed by heat inactivation at 65°C for 20 minutes.

Table 1: Primer sequences to amplify the CDK8as construct

Primer	Sequence (5'-3')
CDK8ShpACOFwd	GGCAGCGAATTCATGGACTATGACTTTAAAGTGAAG
CDK8ShpACORev	CGACTATCTAGATCAGTACCGATGTGTCTGATGTGAG

Restriction enzyme digest:

- 1 µg DNA
- 5 µl CutSmart (NEB)
- 1 µl per enzyme
- x µl water up to 50 µl

The vector pACEBacI (Figure 1) was treated with the same restriction enzymes, afterwards it was CIP (NEB) treated for an hour at 37°C. To ligate the CDK8as insert into the pACEBacI vector, the ligation was performed at 16°C overnight.

Ligation mix:

- 10 µl vector
- 2 µl insert
- 2 µl 10x Ligation buffer
- 5 µl water
- 1 µl DNA ligase

Vector only control was done using 7 µl water without adding the insert.

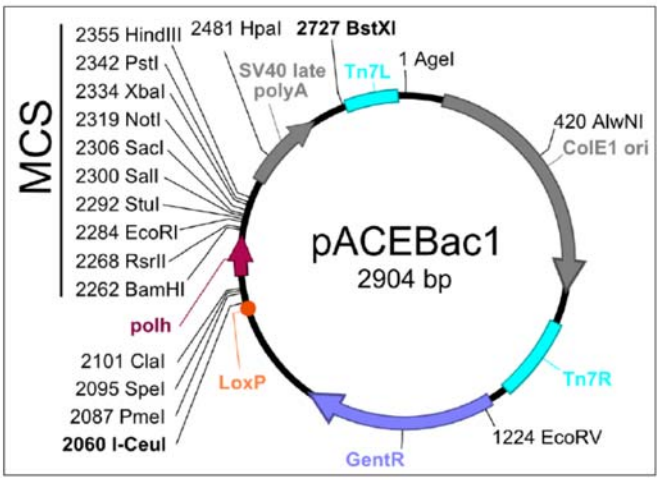

Figure 1: pACEBacI plasmid used for cloning of the CDK8as construct. The restriction enzymes EcoRI and XbaI were used for ligation.

After ligation the plasmid was transformed into E. coli. Therefore 40 µl of competent bacteria per transformation were thawed on ice and 2 µl of the ligation product and a non-digested vector control were added. After incubation for 30 minutes on ice, the bacteria were heat shocked for 45 seconds at 42°C. Following the recovery for 2 minutes on ice, 960 µl LB were added per sample and the tubes were shaken at 37°C for an hour. Finally the samples were plated on pre-warmed agar plates and incubated overnight at 37°C. Afterwards colonies were picked and inoculated into 5 ml LB for growing an overnight culture for mini prep.

The mini prep was done using the E.Z.N.A. Plasmid DNA Mini Kit (Omega) according to the manufacturer's protocol. Afterwards a control digestion using AccI was performed for 1 hour using 1 µg DNA, 5 µl Cutsmart buffer and 1 µl enzyme in 50 µl total. 10 µl of the digestion product was mixed with 2 µl DNA loading buffer and was run on a 1 % agarose gel. As shown in Figure 2 the pattern showed no digestion with AccI, therefore the digestion was resumed for another two hours, unfortunately without improving the result.

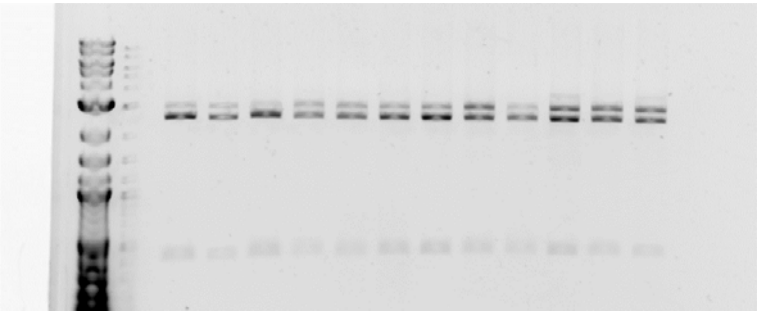

Figure 2: Control digestion of the plasmids after mini prep using AccI. 12 samples were analyzed for successful ligation of CDK8as into the pACEBacI plasmid. First lane marker, followed by one free lane and then the 12 ligation products were loaded.

To repeat the ligation of the CDK8as insert into the pACEBacI vector the insert was once more amplified using the CDK8ShpACOfwd and CDK8ShpACOrev primers. The bands of the expected length were cut out and gel purified. After the digestion of the plasmid and insert, the plasmid was CIP treated and the insert was purified using the QIAquick PCR purification kit (Qiagen).

The ligation was again set up overnight at 16°C and transformation was done into competent E. coli. This time 100 µl of competent cells were used per transformation. 18 colonies were picked for mini prep. The control digestion with AccI showed 2 samples containing the right pattern and were send for sequencing.

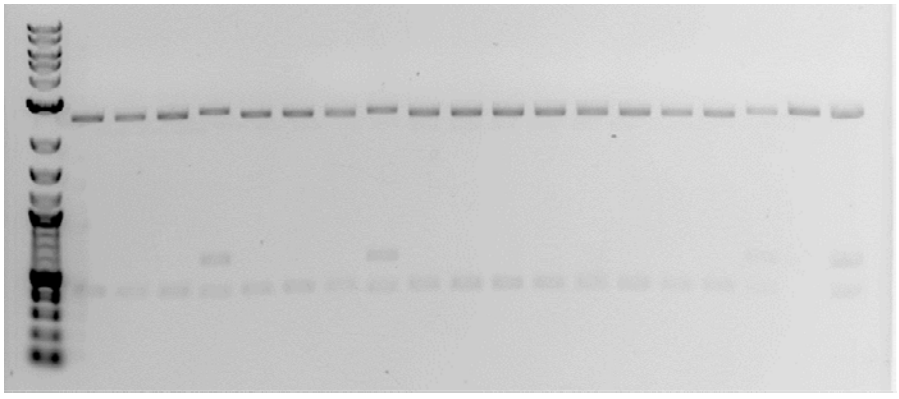

Figure 3: Control digestion of 18 colonies using the restriction enzyme AccI. First lane marker, followed by the 18 samples. The samples in lane 5 and 9 show the expected pattern pointing towards a successful ligation of the CDK8as into the pACEBacI plasmid.

The sequencing results revealed a 600 bp missing part in the CDK8as insert. Because of this observation the cloning procedure was started all over from the original Wt CDK8 sequence. The

Wt sequence was amplified and ligated into pACEBacI vector. Before setting up the ligation all components - insert and plasmid were purified using the QIAquick PCR purification kit. This additional purification step led to a ligation efficiency of 100 %. Afterwards site directed mutagenesis was performed (Table 2), followed by DpnI treatment for 3 hours. 2 µl of the sample were directly used for transformation of competent bacteria.

Site directed mutagenesis setup:

- 10 µl Phusion buffer
- 1 µl dNTPs
- 2.5 µl forward primer (10 mM)
- 2.5 µl reverse primer (10 mM)
- 1 µl template
- 32.5 µl water
- 0.5 µl Phusion Polymerase

Table 2: PCR setup for site directed mutagenesis

Step	Duration	Temperature	
Denaturation	30 sec	98°C	
Denaturation	10 sec	98°C	30x
Annealing	30 sec	68°C	
Extension	3 min	72°C	
Final Extension	10 min	72°C	

Mini prep was done from the overnight cultures and sent for sequencing. To finish the cloning the last step was to transposition our insert into a bacmid. Therefore bacmid expressing cells were used. The selection consisted of three antibiotics in parallel, tetracycline, kanamycin and gentamycin. Additionally there was a blue-white screening of the colonies to verify the transposition of our insert into the bacmid. The positive colony was picked for maxi prep and the bacmid was send off to the insect facility. At the facility the cells were first only transfected with the CDK8as bacmid and the cells were send back to us to verify that CDK8 is present in these cells. Unfortunately we could not detect CDK8 protein in the cell lysate. Most probably the bacmid got fragmented during the maxi prep. To avoid the fragmentation another precipitation method was used to isolate the bacmid from the bacteria. Therefore cells were grown overnight and pelleted

by centrifugation at 3200 g for 8 minutes at 4°C. The pellet was resuspended in 330 µl of Solution I (15 mM Tris-HCl, pH=8; 10 mM EDTA; 100 µg/ml RNase A) and transferred into 1.5 ml tubes. 330 µl Solution II (0.2 N NaOH, 1 % SDS) were added followed by a 5 minutes incubation at room temperature. To the mix 460 µl KOAc (3 M, pH=5.5) were added dropwise, incubated 5 minutes on ice and centrifuged for 15 minutes at 13200 g at room temperature. The supernatant was transferred into a 2 ml tube containing 900 µl isopropanol using a high-viscosity P-1000 tip. After gently mixing the sample was incubated 15 minutes on ice followed by centrifugation for 20 minutes at 13200 g at 4°C. The pellet was washed once with 500 µl 70 % EtOH and resuspended in 40 µl TE buffer. After measuring the concentration the plasmid was sent off to the insect facility.

This time the test pellet contained CDK8as protein as shown in Figure 4. Immediately a co-transfection of all four CDK8 submodule components was set up at the insect cell facility, one with Wt CDK8 and one with analogue sensitive CDK8.

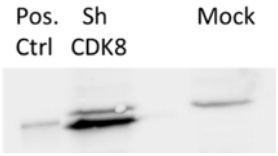

Figure 4: Western blot analysis of transfected insect cells. As positive control the previously purified Wt CDK8 submodule was used. The analogue sensitive CDK8 presence was confirmed using the Santa Cruz antibody. Sh CDK8 = CDK8as

Purification of the CDK8 submodule

To purify the CDK8 submodule, the His-tag on one of the Med subunits was used. For the whole cell extracts 3.75 ml lysis buffer were added to each insect cell pellet and the pellets were thawed in a cold water bath for 10 minutes. Afterwards the pellets were dounce homogenized 25x in a pre-chilled douncer. The samples were centrifuged for 30 minutes at maximum speed at 4°C. Before binding the whole cell extracts to the beads, the Ni2+ resin was washed 1x with 5 ml lysis buffer. Afterwards a 50 % slurry was prepared. The beads were divided equally between the cleared whole cell extracts with a total of 200 µl slurry per protein purification. The binding was done overnight on a nutator at 4°C.

To remove unspecific bound proteins, the beads were washed 2x with 0.5 Wash buffer, 1.0 Wash buffer and HGKE buffer. For the elution 100 µl elution buffer were added and incubated for 30 minutes at 4°C. After the centrifugation the supernatant was transferred to a new tube and two more elution rounds were performed.

The samples were then loaded on a glycerol gradient from 15 % to 40 % glycerol. The gradients were centrifuged in a Falke lab ultracentrifuge at 50000 rpm for 6 hours at 4°C. The gradient was fractionated into 100 µl aliquots and sub aliquoted for analysis by silver staining and western blot.

Lysis buffer:

- 5 mM Imidazole
- 0.5 M NaCl
- 0.2 M Tris-HCl, pH=7.9
- 1 % Triton-X 100
- 2 mM MgCl2
- PMSF, Benzamidine 1x

0.5 Wash buffer:

- 5 mM Imidazole
- 0.5 M NaCl
- 0.2 M TrisHCl, pH=7.9
- 1 % Triton-X 100
- PMSF, Benzamidine 1x

1.0 Wash buffer:

- 25 mM Imidazole
- 1 M NaCl
- 0.2 M Tris-HCl, pH=7.9
- 1 % Triton-X 100
- PMSF, Benzamidine 1x

HGKE buffer:

- 25 mM Imidazole
- 70 mM KCl
- 25 mM HEPES, pH=7.6

- 15 % glycerol
- 0.1 mM EDTA
- % Triton-X 100
- PMSF, Benzamidine 1x

Elution buffer:

- 0.3 M Imidazole
- 70 mM KCl
- 25 mM HEPES, pH=7.6
- 15 % glycerol
- 0.1 mM EDTA
- % Triton-X 100
- PMSF, Benzamidine, DTT 1x

The silver stains were not conclusive, therefore Western blot analysis was performed using aliquots taken at different steps during the purification and from the fractions 11 - 15 of the glycerol gradient were the module should be present. The used antibodies were CDK8 (Santa Cruz), Med12 (Bethyl) and Med13 (Bethyl). As shown in Figure 5 the CDK8 subunit can be detected in both complexes in the lysate, flow through and the eluate. In the fractions from the glycerol gradient only Wt CDK8 can be detected.

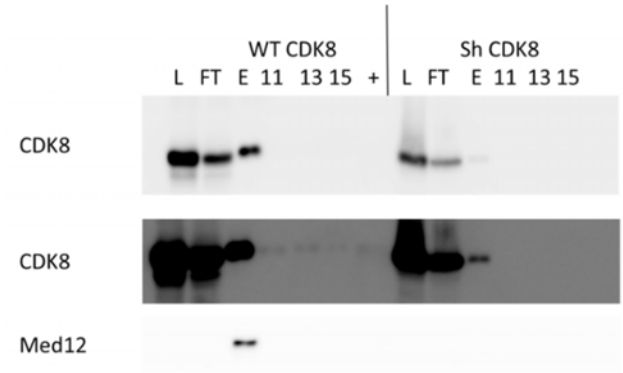

Figure 5: CDK8 presence during different steps of the submodule purification. Western blot analysis was done using antibodies against CDK8 and Med12. L - Lysate; FT - Flow through; E - Eluate; 11, 13, 15 - Fractions from the glycerol gradient. Sh CDK8 = CDK8as

To further verify this observation, another western blot analysis was performed, running the Wt CDK8 and CDK8as samples from fraction 13 directly next to each other. As shown in Figure 6 CDK8, Med12 and Med13 can only be detected in the purification from the Wt submodule but are not present in the analogue sensitive CDK8 purification.

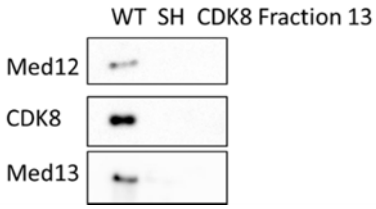

Figure 6: Direct comparison of CDK8 submodule components in Wt and Sh CDK8 purifications. Fraction 13 from the glycerol gradient was analyzed for the presence of Med12, Med13 and CDK8. Only the Wt submodule is present after the purification. SH CDK8 = CDK8as

One explanation is that the stoichiometry of the components was not ideal, since we had to skip the determination of the viral titer for CDK8as out of time reasons. Another more likely explanation is that the transfection didn't work since we can't detect the tagged subunit as well. This points toward a not existing complex formation in the CDK8as submodule transfection.

The experiment will be repeated by a member of the Taatjes lab. Based on another experiment, that showed a way more efficient purification of the CDK8 Wt submodule using a Glu-tag on CDK8, this CDK8 tag will be introduced to the CDK8as form before repeating the transfection procedure. Since the cloning steps are already optimized, the procedure should be straight forward. The kinase assays will be done using CDK8 Wt and CDK8as modules in parallel with Stat1 and RNA Pol II CTD as targets. The kinase activity of the two kinases will be compared and the inhibition of the CDK8as in the presence of the ATP-analogue NM-PP1 and the CDK8 inhibitor Cortistatin A will be determined.

EFFECT OF CDK8 AND CDK19 ON SERUM RESPONSE IN HAP CELLS

Knockdown studies using shRNA against CDK8 in human HCT116 cells showed an influence of CDK8 on the gene expression of serum response genes (Donner et al., 2010). The regulation was shown to occur at the level of transcription elongation and does not affect the RNA Pol II recruitment to the target genes. So far it was not distinguished if this regulation is due to the kinase activity of CDK8 or if the effects are kinase-independent. Moreover the function of the CDK8 paralogue CDK19 was not addressed during the serum response.

In the myeloid leukemia-derived haploid cell line (HAP cells) we established a set of tools to analyze CDK8 kinase-dependent and -independent functions. Initially used just as a proof-of-principle approach to verify the functionality of the analogue sensitive CDK8 mutation, the additionally available CDK8 and CDK19 knockout clones in this cell line offer a toolbox to analyze gene expression under various conditions. Looking at our main pathway of interest, the IFN-γ stimulation, the inhibition of the CDK8 kinase activity and the CDK8 knockout leads to a decrease of the serine phosphorylation of Stat1 to basal levels. Due to the fact, that these cells are no real immune cells, the effects on gene expression of IFN-γ-induced genes are not conclusive. Since CDK8 was shown to have a role in serum response, we analyzed the gene expression of selected serum induced genes in different settings - under CDK8 kinase inhibition (NM-PP1), inhibition of CDK8 and CDK19 kinase activity through Cortistatin A (CA) and in cells lacking CDK8 or CDK19.

Methods

Protein isolation

Cells were washed 2x with ice cold PBS and scraped into 70 µl Frackelton buffer. After lysis on ice for 5 minutes the samples were centrifuged for 5 minutes at maximum speed at 4°C. The supernatant was transferred to a new tube and 6x SDS Loading buffer was added in a 1:2 ratio. The samples were boiled for 5 minutes at 95°C before freezing at -20°C until further processing.

Frackelton buffer:

- 10 mM Tris base
- 30 mM Tetrasodiumpyrophosphate
- 50 mM NaCl
- 50 mM NaF
- 1 % Triton-X-100

Directly before protein isolation following inhibitors were added to the Frackelton buffer: Sodium fluoride, sodium orthovanadate, aprotinin, pepstatin A, beta-glycerophosphate, sodium pyrophosphate, benzamidine, PMSF and leupeptin

6x SDS Loading buffer:

- 375 mM TrisHCl, pH=6.8
- 6 % SDS
- 0.03 % Bromophenol Blue
- 48 % Glycerol
- 9 % 2-Mercapthoethanol

Western blot

9 % acrylamide gels were used as separating gels. The gels were run for 1 ½ hours at 100 V. For the wet transfer the sandwich consisting of the cassette with the green filter papers, 2 whatman papers at each side of the membrane and gel was prepared. The transfer was done at 100 V for 1 hour. Afterwards the membrane was washed 3x in TBS-T and blocked with 5 % milk for an hour. The primary antibody was directly added into the milk and incubated overnight at 4°C on the shaker. After 3x TBS-T washings the membrane was incubated with the HRP-conjugated secondary antibody for 1 hour followed by 3x TBS-T washes. For the detection the chemiluminescence detection solutions were mixed and added on the membrane for 1 minute. Pictures were taken using the ImageQuant LAS-4000 machine.

RNA isolation

RNA was isolated using 500 µl Trizol (ThermoFisher Scientific) per 6 cm dish. 150 µl Chloroform were added and after vortexing the samples were centrifuged for 5 minutes at room temperature.

The aqueous phase was transferred into a new tube and 1 volume isopropanol and 1/10 volume 5 M NaCl was added. After 10 minutes incubation at room temperature, the samples were centrifuged for 30 minutes at maximum speed at 4°C. Following 2x 75 % EtOH washes the pellets were resuspended in 30 µl water and frozen at -80°C for further processing.

cDNA synthesis

Since pre-mRNA and mRNA gene expression levels were analyzed, the isolated RNA was treated with DNase from the RNase-Free DNase Set (Qiagen) for 15 minutes at room temperature. Afterwards the RNA cleanup was done using the RNeasy Mini Kit (Qiagen) according to the manufacturer's protocol. For cDNA synthesis the RevertAid First Strand cDNA Synthesis Kit (ThermoFisher Scientific) was used, either with random primers for pre-mRNA transcription or with OligodT primers for mRNA transcription.

qRT-PCR

The analysis was done by qRT-PCR using the SYBR Select Master Mix (ThermoFisher Scientific) with primers specific for either pre-mRNA or mRNA of selected target genes (Table 3,

Table ***4***).

Table 3: PCR program for gene expression analysis using the SYBR Select Master Mix

Step	Duration	Temperature	
UDG activation	2 min	50°C	
UP activation	2 min	95°C	
Denature	10 sec	95°C	40x
Anneal	30 sec	Tm	
Extend	30 sec	72°C	
Denature	15 sec	95°C	
Final extension	1 min	72°C	

PCR reaction setup:

- 7.5 µl Master mix

- 0.045 µl forward primer
- 0.045 µl reverse primer
- 2.41 µl water
- 5 µl cDNA

Table 4: Primer sequences used for analysis of selected serum response genes

Target gene	Orientation	Sequence (5'-3')
Jun	fwd	CCTCAACGCCTCGTTCCTC
	rev	TTACTGTAGCCATAAGGTCCGCT
JunB	fwd	TGCACTAAAATGGAACAGCCCT
	rev	CGTAGCTGTGTATGAGTCGTCGT
Hprt	fwd	TGTGTGCTCAAGGGGGGC
	rev	CGTGGGGTCCTTTTCACC
pre-Fos	fwd	CCACCTGTGTCCGGAACC
	rev	GAAGTTGGCACTGGAGACGG
pre-Egr1	fwd	ACCCGTGCCGTTCCAGA
	rev	ATCTCTCGCGACTCCCCG
pre-Egr3	fwd	ACTCGGTAGTCCATTACAATCAGATG
	rev	AGGTCGTCCCCTCCTCCTC

Results

The first step was to determine the right concentration and timing for the ATP-analogue treatment of the analogue sensitive CDK8 HAP clone and to verify efficient inhibition of the mutated kinase by Cortistatin A. As shown in Figure 7 reduction of the incubation time down to 30 minutes pre-treatment already reduces the phosphorylation of Stat1 S727 to basal levels. Moreover CA efficiently blocks the serine phosphorylation in these cells.

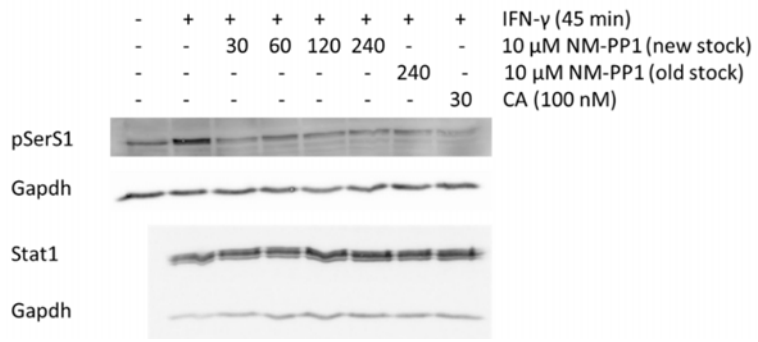

Figure 7: Inhibition of Stat1 S727 phosphorylation in CDK8as HAP cells by addition of the ATP-analogue NM-PP1 and the inhibitor Cortistatin A (CA). The serine phosphorylation is reduced to basal levels upon treatment with NM-PP1 for a timeframe between 30 and 240 minutes and upon treatment with Cortistatin A for 30 minutes.

The reduction of the ATP-analogue concentration was not successful as shown in Figure 8, therefore the following experiments were done using 10 µM NM-PP1. As time for the pre-treatment with 1-NM-PP1 1 hour was chosen, to perform the ATP-analogue and CA treatment for the same time span.

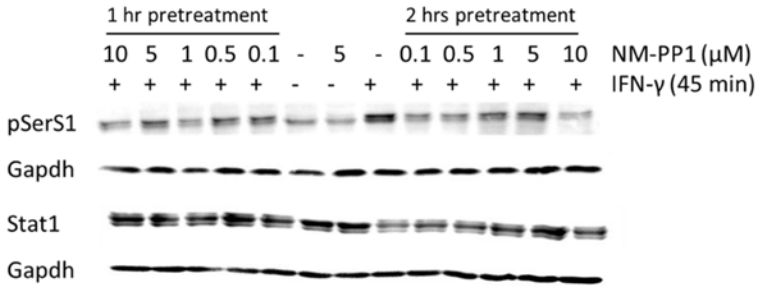

Figure 8: 10 µM NM-PP1 are necessary for efficient inhibition of the CDK8as kinase. Reducing the amount of NM-PP1 added for 1 or 2 hours before IFN-γ stimulation leads to incomplete kinase inhibition.

To test the serum response of the HAP cell line initial experiments in Wt HAP cells were performed. The cells were starved for 40 hours in IMDM medium without FBS. After this time FBS was added to the medium to 20 % FBS final.

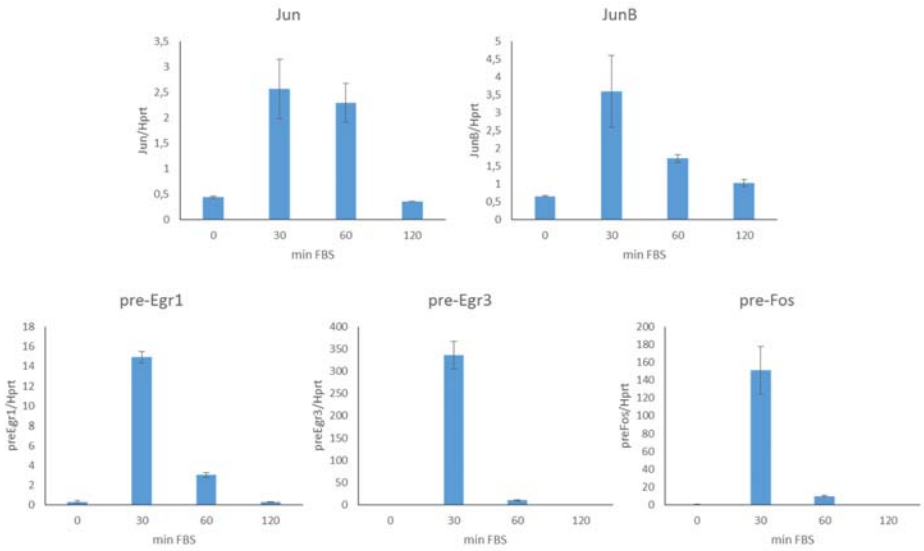

Figure 9: Time course after FBS stimulation in HAP Wt cells. The cells were starved in medium without FBS for 40 hours, followed by FBS stimulation for 0, 30, 60 and 120 minutes. Selected target genes were analyzed by qRT-PCR. For mRNA levels the gene expression of *Jun* and *JunB* was determined, as pre-mRNA targets *Egr1*, *Egr3* and *Fos* were analyzed. The values were normalized to the house keeping gene *Hprt*.

As shown in Figure 9 after FBS treatment Wt HAP cells showed the expected induction of target genes. The time course of the response to FBS stimulation was already pronounced after 30 minutes with an efficient downregulation visible especially on the pre-mRNA level already after 1 hour of FBS treatment.

To determine the kinetics of gene expression after FBS treatment in more detail, a shorter time course was done in Wt HAP cells. As shown in Figure 10 the mRNA levels of the serum response target genes *Jun* and *JunB* peak around 50 minutes after treatment, whereas the pre-mRNA levels peak already at 20 minutes and decrease afterwards.

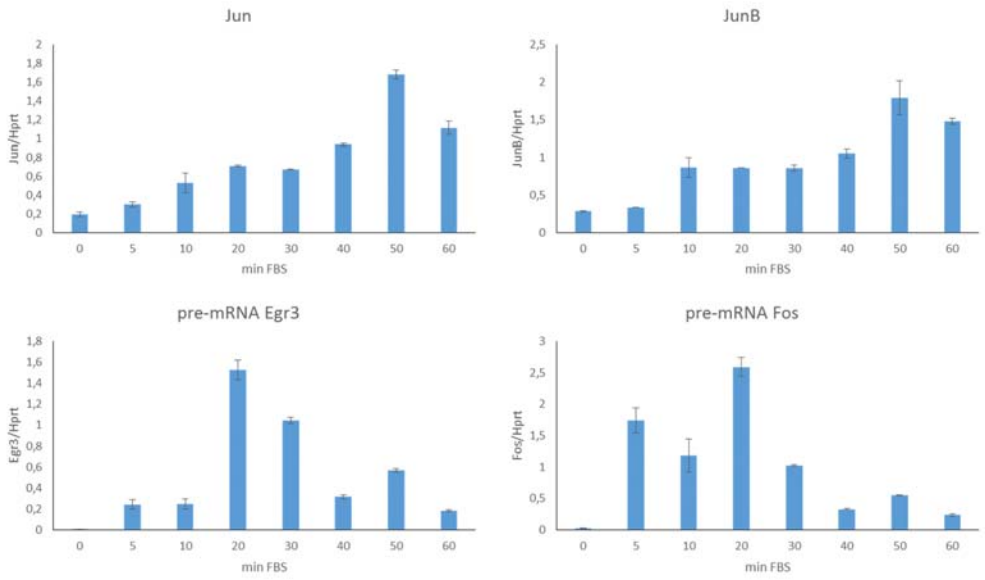

Figure 10: Detailed time course with time points between 0 and 60 minutes FBS stimulation in Wt HAP cells. The cells were starved in medium without FBS for 40 hours, followed by FBS stimulation for several time points. Selected target genes were analyzed by qRT-PCR. The expression values were normalized to the house keeping gene *Hprt*.

Based on these results for further analysis the time points 0, 20, 40 and 60 minutes after FBS treatment were analyzed. As shown in Figure 11 treatment of the CDK8as HAP clone with the ATP-analogue NM-PP1 shows some effects on the early time point of mRNA levels of *Jun* and *JunB* but not at later time points or on pre-mRNA levels. Therefore it is important to repeat this experiment to rule out a problem of this specific sample.

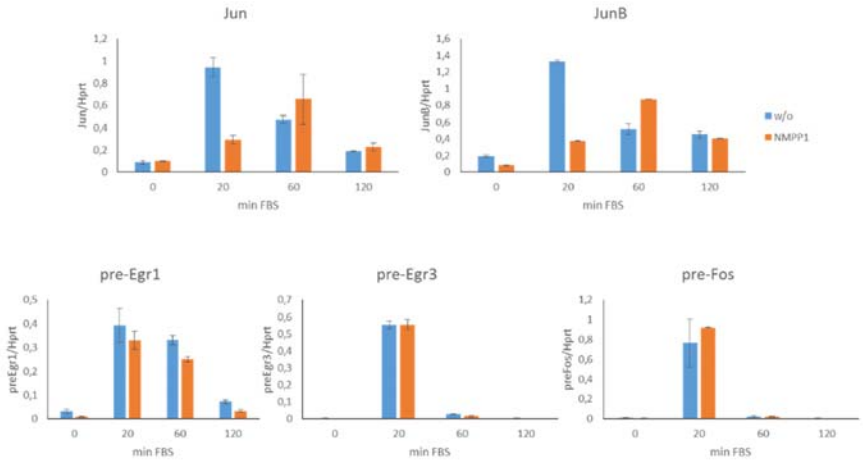

Figure 11: Treatment of CDK8as HAP cells with NM-PP1 shows effects at early time points. Cells were starved for 40 hours in medium without FBS followed by 1 hour pre-treatment with 10 µM NM-PP1. FBS treatment was done for 0, 20, 60 and 120 minutes and gene expression of selected target genes was analyzed by qRT-PCR. For normalization the housekeeping gene *Hprt* was used.

For the second trial of FBS treatment in CDK8as HAPs the cells were pre-treated with NM-PP1, CA or a combination of both. As shown in Figure 12 the effects of the inhibitor treatment and the kinase inhibition do not show a clear phenotype.

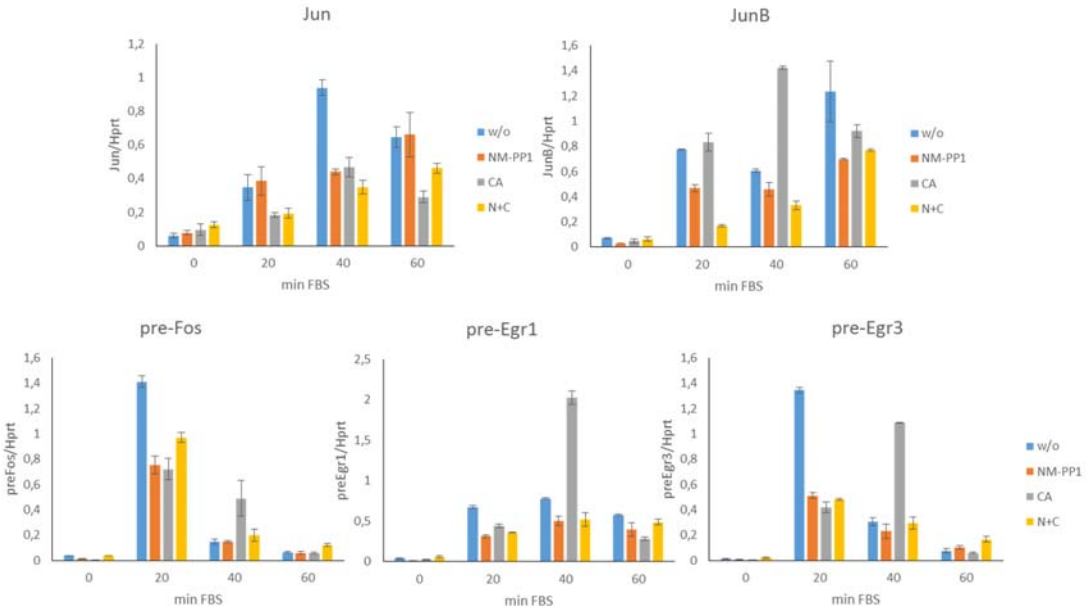

Figure 12: Effect of kinase inhibition on serum response in CDK8as HAP cells. The cells were starved for 40 hours in medium without FBS. The cells were pre-treated for 1 hour with 10 µM of the ATP-analogue (NM-PP1), 100 nM Coristatin A (CA) or a combination of both. FBS treatment was done for 0, 20, 40 and 60 minutes. Gene expression of selected target genes was analyzed using qRT-PCR. For normalization the housekeeping gene *Hprt* was used.

To prove the efficiency of the ATP-analogue, CA inhibition and the treatment with both inhibitors in this setup, the cells were once more stimulated with IFN-γ and proteins were isolated. As shown in Figure 13 the serine phosphorylation of Stat1 was reduced to basal level in all three conditions.

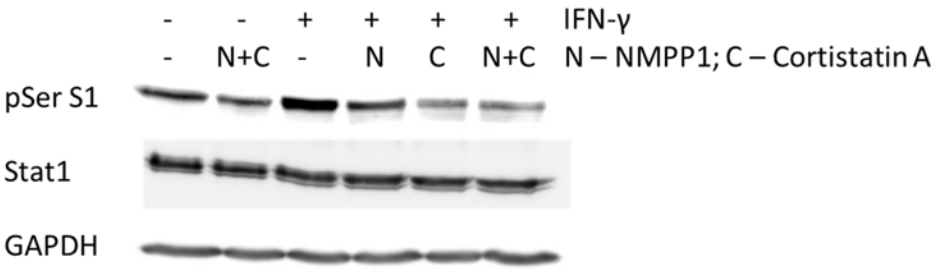

Figure 13: Pre-treatment with NM-PP1, Cortistatin A and a combination of both inhibits IFN-γ-induced serine 727 phosphorylation of Stat1. CDK8as HAPs were treated with 10 µM NM-PP1 and 100 nM CA for 1 hour, followed by stimulation with IFN-γ for 45 minutes. Whole cell extracts were prepared and analyzed by Western blot. Total Stat1 levels and GAPDH was used as loading control.

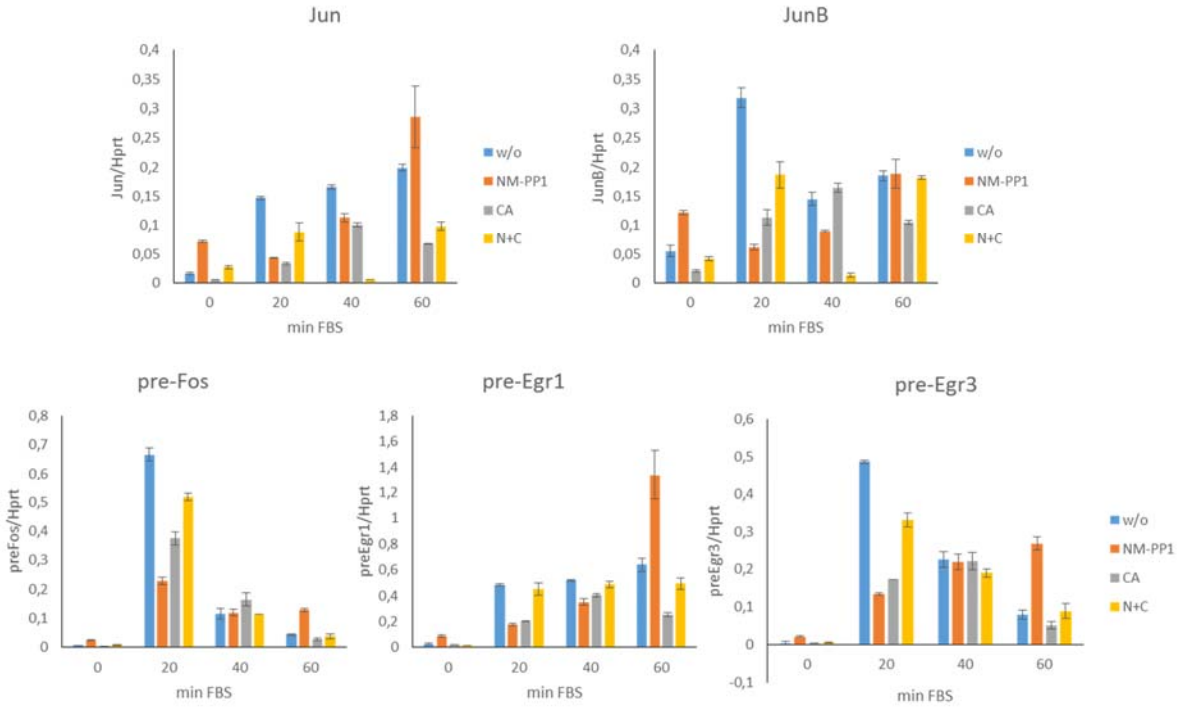

Figure 14: Second replicate of the effect of kinase inhibition on serum response in CDK8as HAP cells. The cells were starved for 40 hours in medium without FBS. The cells were pre-treated for 1 hour with 10 µM of the ATP-analogue (NM-PP1), 100 nM Coristatin A (CA) or a combination of both. FBS treatment was done for 0, 20, 40 and 60 minutes. Gene expression of selected target genes was analyzed using qRT-PCR. For normalization the housekeeping gene *Hprt* was used.

Figure 14 shows an effect of CDK8 kinase inhibition by NM-PP1 and by CA on the early time points after FBS treatment and less effects on later time points. There is no clear phenotype visible that would point to a difference between ATP-analogue treatment and CA treatment. In the first scenario only CDK8 kinase function is blocked, in the second case both kinases CDK8 and CDK19 are kinase-inactive.

To further clarify the role of these two kinases in the serum response pathway and to determine a possible phenotype, the HAP CDK8 and CDK19 knockout cells were analyzed.

As shown in Figure 15 there is a reduction of gene expression levels on pre-mRNA and also mRNA levels of the selected serum response target genes. This reduction is visible in both knockout cells which points to an essential function of each of these two kinases in the pathway.

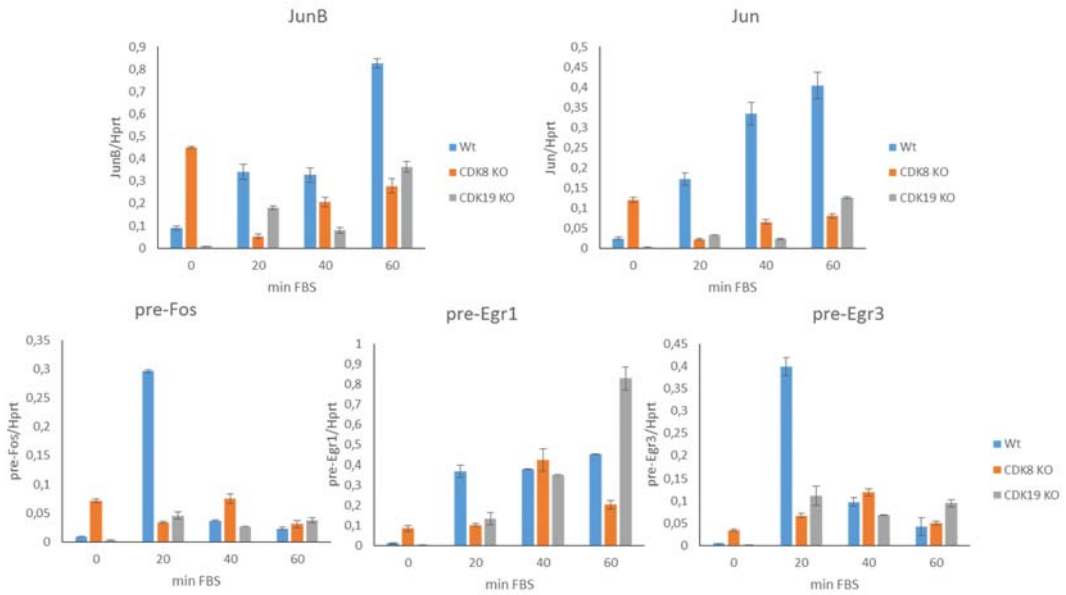

Figure 15: Gene expression of serum induced genes in CDK8 and CDK19 knockout cells. Wt, CDK8 and CDK19 knockout cells were serum starved for 40 hours following FBS treatment for 0, 20, 40 and 60 minutes. Changes in gene expression were analyzed using qRT-PCR with primers for selected target genes. For normalization the house keeping gene *Hprt* was used.

To further analyze the knockout cells Cortistatin A was used to inhibit the kinase activity of the remaining kinase in the HAP knockouts. As shown in Figure 16 treatment of the CDK8 knockout cells or the CDK19 knockout cells with CA does not increase the influence on gene expression of the selected serum response genes.

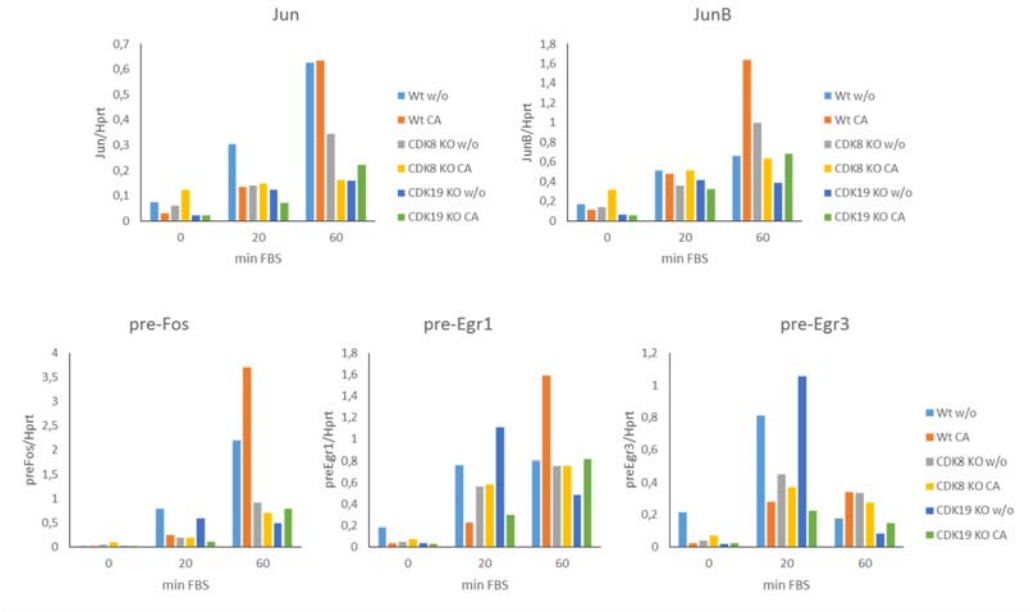

Figure 16: Effect of kinase knockout and kinase inhibition on serum stimulation. Wt, CDK8 and CDK19 knockout cells were starved for 40 hours in medium without FBS. Pre-treatment with 100 nM Cortistatin A (CA) was done for 1 hour followed by FBS treatment for 0, 20 and 60 minutes. Gene expression changes were analyzed using qRT-PCR and the levels were normalized to the house keeping gene *Hprt*.

Discussion

The data show only a slight influence of kinase inhibition on the gene expression of selected serum response genes. The lower expression after kinase inhibition is only seen at early time points and there is no difference if only CDK8 is inhibited using the sensitive analogue CDK8 cells and the ATP-analogue NM-PP1 or if both Mediator kinases are inhibited by Cortistatin A.

In contrast the FBS treatment in CDK8 and CDK19 knockout HAP cells shows a strong reduction of the gene expression upon serum treatment in comparison to the Wt cells. Since the reduction is comparable in both knockout settings it seems that the presence of both kinases is essential. If they act in a synergistic way could only be answered by looking at the expression levels in CDK8/CDK19 double knockouts or by analyzing the serum response in the knockout cells after silencing of the second kinase. Moreover additional kinase inhibition in each knockout cell line does not increase the effect on gene expression upon serum stimulation, which goes in line with only slight effects observed in case of kinase inhibition by Cortistatin A.

GRO-SEQ ANALYSIS IN MEF CELLS

We showed that the serine phosphorylation of Stat1 on the serine residue 727 influences the expression of IFN-γ target genes in a gene-specific way. Moreover additional effects on gene expression are observed upon CDK8 knockdown which can be either kinase-dependent, e.g. the phosphorylation of other factors besides Stat1, or kinase-independent. To determine the kinase-dependent effects on transcription in more detail it is necessary to look on ongoing transcription rather than on steady state mRNA levels. Therefore, a method established by the Lis laboratory, called Global Run-On sequencing (GRO-Seq) was used (Core et al., 2008).

GRO-Seq is the state-of-the-art method to get insight into RNA Pol II position on genes and to distinguish between pausing and elongation. The main principle is to stop transcription at a point of interest and isolate the cell nuclei. During this step the RNA Pol II stays bound to the Chromatin and after adding nucleotides, reaction buffer and increasing the temperature to 30°C the transcription proceeds. Since one of the added nucleotides is labelled (Br-UTP), the newly synthesized RNA can be captured using Anti-BrdU-Antibodies conjugated to agarose beads. After several washes to remove the unlabeled RNA, the labeled fraction is eluted from the beads and analyzed by sequencing.

Blocking the CDK8 kinase activity by using Cortistatin A (CA) and following stimulation with IFN-γ, GRO-Seq allows to determine the distribution of the RNA Pol II on IFN-γ target genes at different time points of the stimulation. We hope we can clarify the mechanism how CDK8 kinase activity influences the transcription of IFN-γ-induced genes by generating a GRO-Seq dataset.

Optimization of the GRO-Seq protocol for MEFs

The underlying protocol which was used to prepare GRO-Seq libraries was published by Hah et al. (2011). Here the optimization steps to perform the procedure in immortalized MEFs are explained in detail.

To perform the GRO-Seq experiment in my cell type of interest, first intact nuclei need to be isolated. The commonly used buffers for nuclei isolation (Allen et al., 2014) did not lead to intact nuclei isolation from MEFs. Therefore the isolation procedure was changed to slightly different buffer compositions, lower centrifugations speeds and the freezing step of the nuclei was skipped.

Nuclei isolation from immortalized MEFs

Cells were washed 2x with ice cold PBS, followed by trypsinization for 5 minutes at room temperature. The cells were collected in 10 ml cold PBS and centrifugation was performed for 5 minutes at 270 g. The pellets were resuspended in 10 ml Lysis buffer and incubated for 5 minutes on ice, followed by a centrifugation for 10 minutes at 170 g. The cell pellets were resuspended in 1 ml Reaction buffer and centrifuged again for 10 minutes at 170 g. Afterwards pellets were pooled in a total volume of 100 µl Reaction buffer and nuclei were counted.

Lysis buffer:

- 10 mM Tris-HCl, pH = 7.5
- 5 mM MgCl2
- 10 mM NaCl
- 0.5 % (v/v) NP-40

The inhibitors DTT, NaMeta, Benz, PMSF and SUPERase-In were added fresh.

Reaction buffer:

- 20 mM TrisHCl, pH = 7.5
- 10 mM MgCl2
- 150 mM KCl
- 20 % (v/v) Glycerol
- SUPERase-In

Determination of the fragment length during Run-on reaction

After establishing the nuclei isolation from immortalized MEFs, the length of the produced transcript was determined. Therefore nuclei were isolated, treated with RNase A during the isolation procedure, to remove all RNA fragments that are already transcribed by the RNA Pol II

and the Run-on was performed by adding nucleotides of which one was radioactive labelled. The fragments were in the end analyzed on a Urea gel.

After lysis of the nuclei in 10 ml Lysis buffer, nuclei were resuspended in 1 ml Lysis buffer and incubated with 5 µl RNase A for 30 minutes on ice. Afterwards the nuclei were washed 3x in 10 ml Lysis buffer with 5 µl SUPERase-In per sample. The centrifugation steps were done for 10 minutes each at 170 g. After the three washes the isolation procedure was performed as described above.

For the Run-on $1.25x10^6$ nuclei were used in 25 µl reaction buffer.

Setup per reaction:

- 5.5 µl Reaction buffer
- 1.25 µl rGTP (10 mM)
- 0.5 µl rCTP (10 mM)
- 1.25 µl rATP (10 mM)
- 1.25 µl Br-UTP (10 mM)
- 0.02 µl DTT

0.25 µl SUPERase-In, 12.5 µl 2 % Sarkosyl and 2.5 ^{32}P CTP were added to the mix and pre-warmed to 30°C. Afterwards 25 µl nuclei were added and Run-on reaction was carried out for 5, 7 and 10 minutes.

The RNA was isolated using 500 µl Trizol and 100 µl Chloroform, followed by 1 volume Phenol-Chloroform and 1 volume chloroform isolation, the RNA was prepped using 100 % Ethanol and 1 µl glycoblue at -20°C. Afterwards the samples were centrifuged for 30 minutes and washed twice with 75 % EtOH. Finally the pellets were resuspended in 20 µl DEPC water and were purified using P30 columns to remove remaining nucleotides.

The fragment length analysis was done using a 6 % Urea gel, which was run for 1 hour 30 minutes at 1500 V max, 35 A max. 1 µl of sample was mixed with 10 µl FBL loading buffer. After the run the gel was dried for 1 hour and the exposure was done overnight (approximately 16 hours). As shown in Figure 17 all three Run-on times led to the same fragment length of approximately 50 bases. Since the marker unfortunately did not work the fragment length was determined by comparison to other 6 % gels that were performed in the lab.

The fact, that the fragment size does not increase with longer Run-on times is different to the results from other cell types, but was reported also from other people working with MEFs (unpublished data).

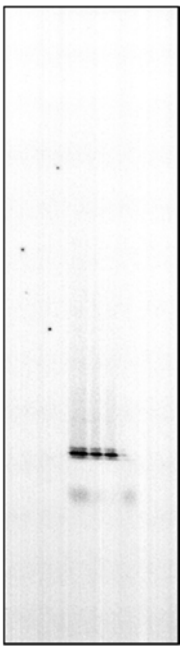

Figure 17: RNA gel showing the fragment length of newly synthesized RNA during different Run-on reactions. The Run-on reaction was performed for 5 (lane 1), 7 (lane 2) and 10 minutes (lane 3) in the presence of ^{32}P CTP. The samples were analyzed on a 6 % urea gel.

The samples were further analyzed using the scintillation counter. 1 µl per sample, 1 µl of ^{32}P CTP and 1 µl blank were put into 1 ml scintillation counting buffer. The scintillation values in Table 5 represents the results already seen in the RNA gel. After 5 minutes Run-on the ^{32}P CTP levels are higher than after the longer time points.

Table 5: Scintillation counts from samples after different Run-on times

Sample	Scintillation count
Blank	32.80
32P CTP	739599.94
5 minutes Run-on	1141.00
7 minutes Run-on	524.20
10 minutes Run-on	502.40

GRO-qRT-PCR to determine efficient pulldown of labelled RNA fragments

Since the fragment length obtained by Run-on in MEFs was shorter as expected from other cell types, it was important to verify a successful pulldown of the labelled fragments by using BrdU antibodies coupled to agarose beads (Santa Cruz Biotechnology). Therefore nuclei were isolated, the Run-on reaction was performed and one round of bead binding was done. Afterwards the RNA was precipitated and cDNA was synthesized. The analysis was done using qRT-PCR and primers for target genes of interest.

The procedure was based on a nature protocols publication from Roberts et al., 2015. The nuclei isolation was carried out as described above. For the Run-on reaction $3x10^6$ nuclei were used in 100 µl reaction buffer.

Reaction mix per sample:

- 28.9 µl Reaction buffer
- 5 µl of each NTP 10 mM (rATP, rCTP, rGTP, Br-UTP)
- 0.1 µl 1 M DTT

1 µl SUPERase-In and 50 µl 2 % Sarkosyl were added to the mix and pre-warmed to 30°C. After adding of 100 µl nuclei the Run-on was performed for 5 minutes. The reaction was stopped by adding 1 ml Trizol, followed by RNA isolation as described previously.

The Br-UTP-antibody agarose beads were blocked for an hour in Blocking buffer before adding the RNA to the beads. After incubation of the RNA together with the beads for 30 minutes at room temperature, the beads were washed 3x with PBS-T. To elute the RNA 500 µl Trizol were directly added to the beads and RNA was precipitated as before. The pellets were resuspended in 11 µl water. 2 µg per sample were used for cDNA synthesis which was done as written in the protocol (Roberts et al., 2015). Pulldown of labelled RNA after Run-on reaction was analyzed by qRT-PCR using SYBR Select PCR master mix and primers specific for Irf1 and Gbp2 (Table 6).

Table 6: qRT-PCR primers used to analyze efficient RNA pulldown after Run-on using Br-UTP

Target gene	Direction	Sequence (5'-3')
Irf1	fwd	TCTCGGGCATCTTTCGCTTC
	rev	TCTGCATCTCTAGCCAGGGT
Gbp2	fwd	TCAGAGACAAAAAGAAACCACCC
	rev	AATGAGGCACATGGGTTCCG
Hprt	fwd	TGAGCCTTCCTATGGGTCTG
	rev	AGAGGTCCTTTTCACCAGCA

As shown in Figure 18 the pulldown of the interferon gamma target genes Irf1 and Gbp2 was successful after the Run-on reaction.

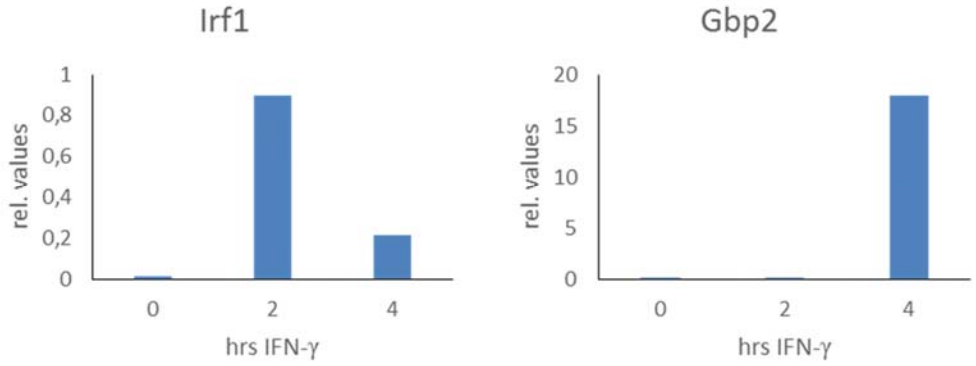

Figure 18: Pulldown of labelled RNA after Run-on reaction shown by qRT-PCR analysis. The cells were stimulated with IFN-γ for 0, 2 or 4 hours. After nuclei isolation the Run-on reaction was performed and the labelled RNA was captured using BrdU-antibodies coupled to agarose beads. Efficient pulldown was determined by qRT-PCR analysis, values were normalized to the housekeeping gene Hprt.

Optimization of RNA fragmentation for GRO-Seq

Before preparing the library for GRO-Seq it is necessary to fragment the RNA isolated after the Run-on reaction, to just pulldown the labelled fragments of a length between 100-200 bases. This is necessary to be able to determine the position of the RNA Pol II on the gene of interest at the time of nuclei isolation.

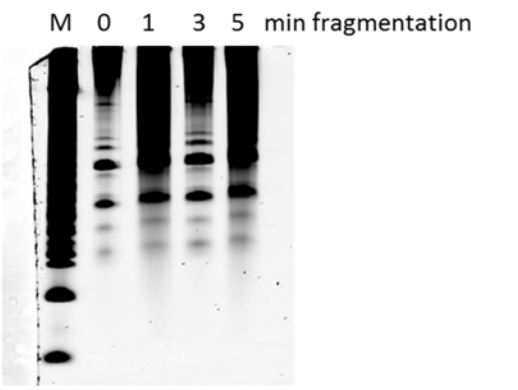

Figure 19: Fragmentation of RNA samples using the NEBNext Magnesium RNA Fragmentation Module. The Run-on RNA was incubated with the fragmentation module for 0, 1, 3 and 5 minutes. The samples were analyzed on a 6 % urea gel.

First the NEBNext Magnesium RNA Fragmentation Module was used to fragment the Run-on samples according to the manufacturer's protocol. For the trial different incubation times (0, 1, 3 and 5 minutes) were tested and analyzed on a 6 % Urea gel. As shown in Figure 19 none of the treatments worked, all samples were unfragmented. To rule out that the fragmentation buffer underwent too many freezing/thawing cycles, a new kit was ordered. Figure 20 shows that independent of the fragmentation kit used, none of the RNA samples was fragmented.

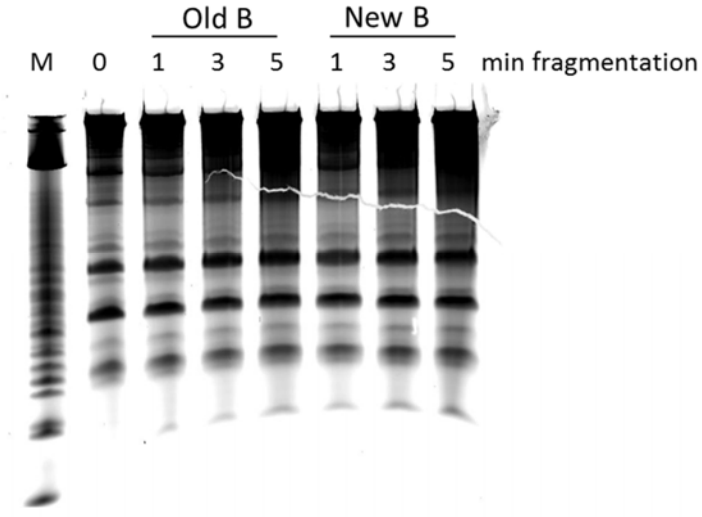

Figure 20: Fragmentation of Run-on RNA using two different lots of the NEBNext Fragmentation module. Samples were either incubated with the old or the new vial of the fragmentation kit for 0, 1, 3 and 5 minutes. The fragmentation efficiency was analyzed using a 6 % urea gel.

To overcome this problem another RNA fragmentation kit from Ambion was used. The incubation was performed according to the kit protocol for 0, 4, 6, 8 and 10 minutes. The samples were analyzed using a MOPS Formaldehyde gel. For the gel 1 g agarose was melted in 100 ml water. After cooling 10 ml of 10x MOPS (0.4 M MOPS, 0.1 M Sodium acetate, 0.01 M EDTA) and 10 ml formaldehyde were added. The samples were mixed 1:1 with 2x RNA loading dye. The gel was run at 150 V for 60 minutes. As shown in Figure 21 also the fragmentation with this kit failed.

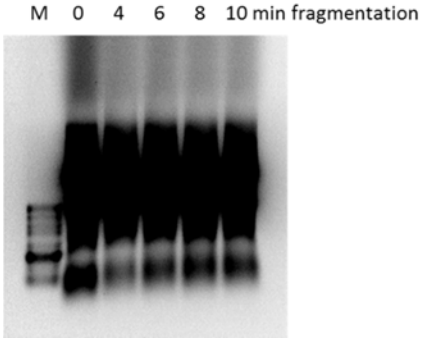

Figure 21: Fragmentation of Run-on RNA using the Ambion Fragmentation Reagents. The RNA samples were incubated with the reagent for 0, 5, 6, 8 and 10 minutes. The samples were analyzed using a MOPS formaldehyde gel.

To rule out an effect of the very clean HPLC water used for the experiment, normal RNA isolated from MEFs was used and either dissolved in HPLC water or distilled (DI) water. The samples were incubated with the NEBNext Magnesium RNA fragmentation module (5 and 10 minutes) or the Ambion fragmentation reagents (5, 10 and 20 minutes).

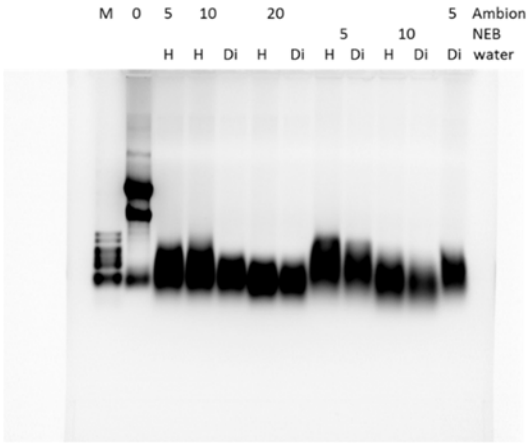

Figure 22: Fragmentation trial with normal RNA samples diluted in HPLC water or distilled water. Samples were incubated for the incubated timeframes with the Ambion or the NEB fragmentation reagents. H - RNA diluted in HPLC water; Di - RNA diluted in distilled water. Analysis was performed using a MOPS formaldehyde gel.

As shown in Figure 22 the fragmentation of normal RNA worked in all settings, pointing towards a role of the components of the Run-on reaction setup as inhibitors of the fragmentation of RNA samples.

Before trying to change the buffer compositions for the Run-on reaction, the different ways of RNA isolation were compared. Therefore Run-on reactions were performed followed by different RNA precipitation methods. As shown in Figure 23 all samples were isolated with Trizol and Chloroform, either followed by direct Isopropanol precipitation or Phenol/Chloroform and Chloroform cleanup followed by Ethanol or Isopropanol precipitation in presence and absence of additional salt. These different settings show clearly that the inhibition of RNA fragmentation does only occur after precipitation using ethanol. Fragmentation of Run-on RNA samples after isopropanol precipitation gives the expected results of fragments around 100 bases length. Furthermore we could show that for normal RNA samples the precipitation method does not make any difference, with these samples the fragmentation works in every setting.

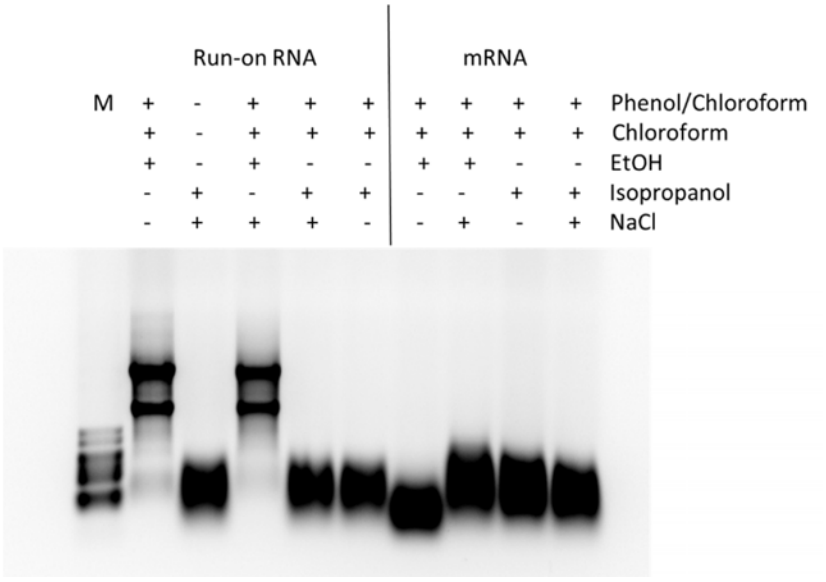

Figure 23: RNA precipitation methods and their effects on the Ambion fragmentation kit efficiency. Run-on RNA and total RNA were isolated using Trizol and chloroform followed by different precipitation methods. RNA was fragmented using the Ambion Fragmentation Reagents for 10 minutes. Samples were analyzed on a MOPS formaldehyde gel.

GRO-Seq library preparation

After solving the fragmentation problem, the pre-tests for GRO-Seq were successfully completed and the first replicate for the sequencing experiment was prepared. The setup was 0, 30, 90 and 240 minutes IFN-γ stimulation in presence or absence of 100 nM Cortistatin A (CA). After the elution of the labelled RNA from the beads, the sequencing library was prepared using the NEBNext Ultra Directional RNA Library Prep Kit according to the manufacturer's protocol. The library quality was analyzed using the TapeStation D1000 that is used to determine fragment lengths of libraries between 35 and 1000 base pairs. Figure 24 shows a representative picture for all samples, except for the 30 minutes time point treated with CA.

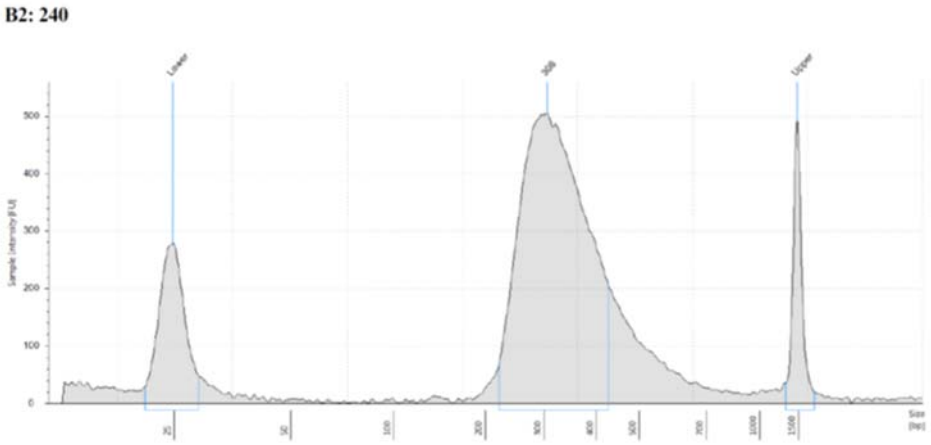

Figure 24: TapeStation D1000 analysis of library samples from GRO-Seq. Representative sample from the first replicate.

The sample with 30 minutes IFN-γ stimulation after CA treatment was way lower concentrated than the other samples (Figure 25). This can be unfortunately due to almost every step during the 4 days GRO-Seq protocol or also due to a problem during the library preparation.

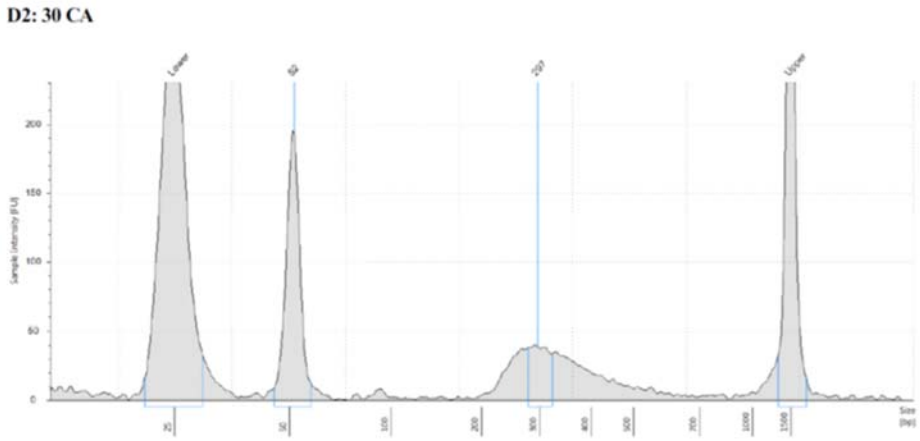

Figure 25: TapeStation D1000 result for the 30 minutes IFN-γ time point pre-treated with Cortistatin A (30 CA).

Since in some samples there was a peak visible around 50 bp which indicates the presence of primer dimers, another cleanup round using AMPure XP beads was done. The following TapeStation analysis showed that the cleanup worked for all samples except for 30 min IFN-γ CA treated. Due to the already low starting amount this sample was almost lost after the additional cleanup. Therefore the 30 minutes time point was repeated during the preparation of the second sequencing replicate.

The second biological replicate was prepared similar to the first one. After the library preparation the samples were again analyzed using the TapeStation. As shown in Figure 26 for the second time the concentration of the 30 minutes time point with CA was way lower than in all other samples.

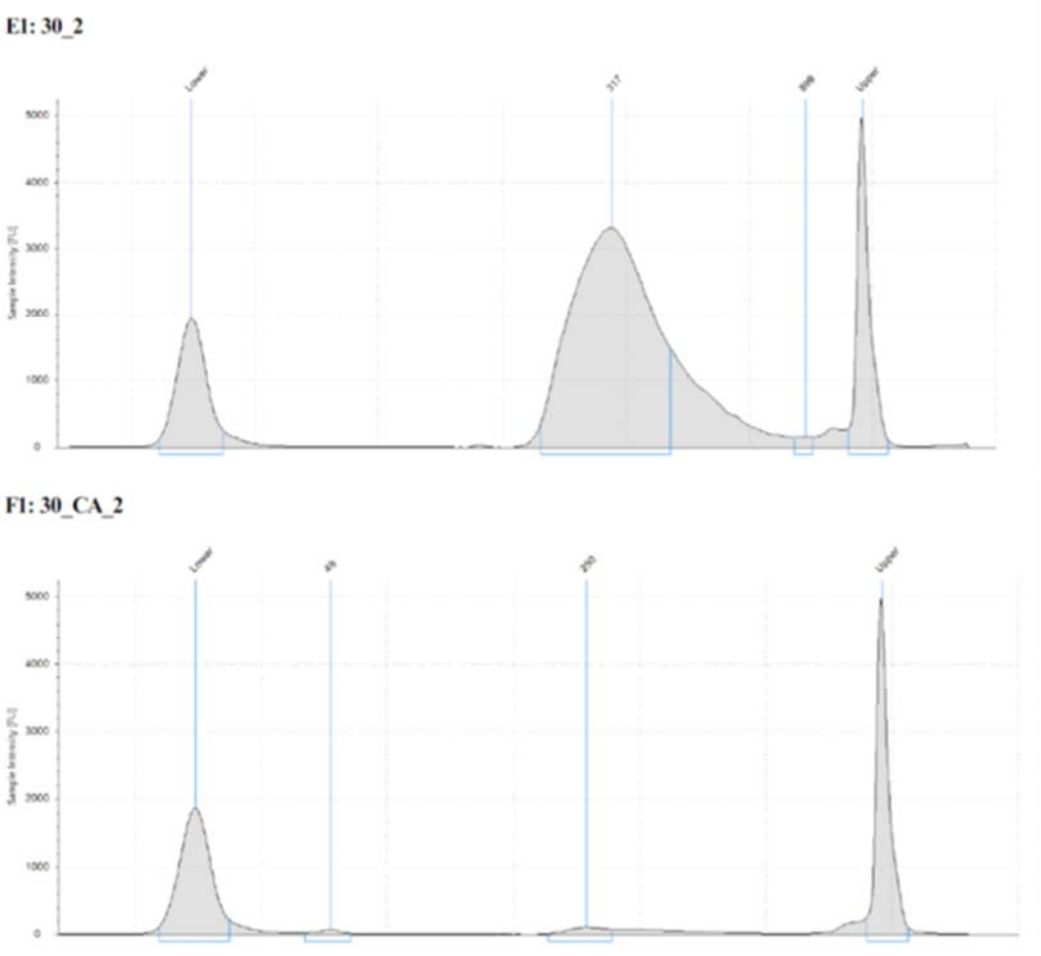

Figure 26: TapeStation D1000 results for the second replicate. Again the 30 minutes time point - upper panel - shows the representative result for all samples, except for the Cortistatin A treated 30 minutes time point shown in the lower panel.

To rule out an effect of Cortistatin A on transcription, another two replicates from this time point only were made, using the double amount of nuclei for the Run-on reaction to make sure not to be just below the detection limit. Therefore $10x10^6$ cells were used per Run-on reaction and the reaction volume was doubled. The other steps of the protocol were carried out as for the previously prepared libraries.

As shown in Figure 27 this time the concentration of all library samples was comparable and these newly produced replicates of the 30 minutes time point can be used to replace the previous ones during sequencing.

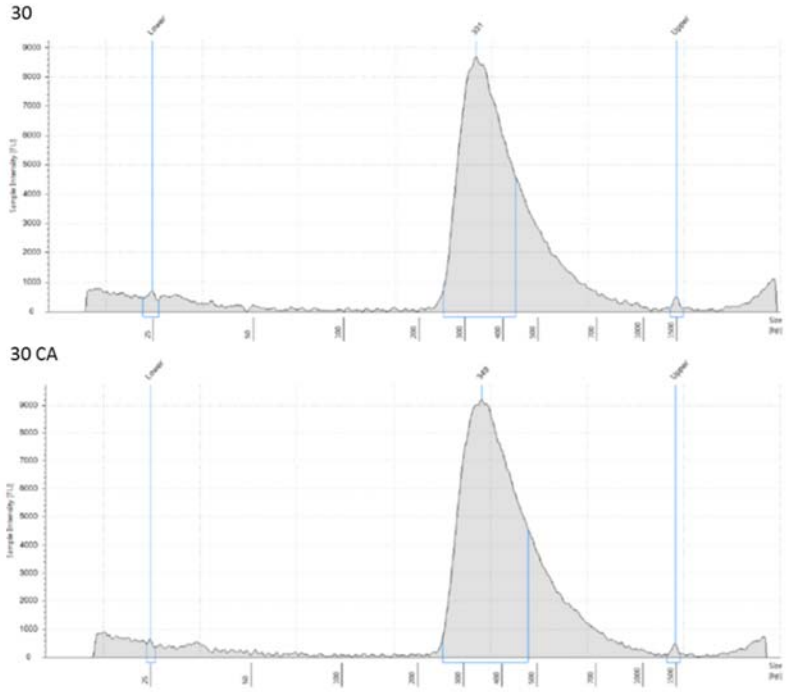

Figure 27: Representative picture from the TapeStation D1000 results of the additional replicates of the 30 minutes time point.

The samples from replicate 1 were sent for sequencing to the Genomics and Microarray Core at the University of Denver. Two lanes in total with 4 multiplexed samples per lane were sequenced on a HighSeq 4000 system with Single Read 50. The results were available upon my return to Vienna and will be analyzed in detail within the next months.

RNA-SEQ SAMPLE PREPARATION FROM MEF CELLS

To complete the picture of transcription regulation by CDK8 kinase activity after IFN-γ stimulation, RNA-Seq samples were prepared in presence and absence of the CDK8 inhibitor Cortistatin A. The experiment was done in 3 biological replicates.

For each replicate MEFs were grown on 15 cm dishes at a confluency around 70 %. Treatment with IFN-γ was done for 6 hours after pre-treatment with 100 nM CA for 1 hour or DMSO as a negative control. RNA was isolated by scraping the cells in 7 ml Trizol reagent per dish. At this step the samples were aliquoted into 7x1 ml aliquots and frozen at -80°C for further processing.

Analysis of RNA-Seq samples

For the RNA precipitation 200 µl Chloroform were added to 1 ml Trizol and the samples were centrifuged for 5 minutes. The supernatant was transferred into a new tube and 1 volume isopropanol and 1/10 volume 5 M NaCl were added. After 10 minutes incubation at room temperature, the samples were centrifuged for 30 minutes at maximum speed at 4°C. 2 washes with 75 % EtOH were followed by resuspension of the pellets in 30 µl water.

1 µg per sample was used for reverse transcription using the RevertAid First Strand cDNA Synthesis Kit (ThermoFisher Scientific). cDNA synthesis setup was done as stated in the manufacturer's protocol. qRT-PCR analysis was performed using primers specific for Irf1 and Gbp2 to determine the gene expression of these IFN-γ target genes (Table 7).

Table 7: Primers used for qRT-PCR analysis of IFN-γ-induced genes

Target gene	Direction	Sequence (5'-3')
Irf1	fwd	CCGAAGACCTTATGAAGCTCTTTG
	rev	GCAAGTATCCCTTGCCATCG
Gbp2	fwd	TGCTAAACTTCGGGAACAGG
	rev	GAGCTTGGCAGAGAGGTTTG
Hprt	fwd	GGATTTGAATCACGTTTGTGTCAT
	rev	ACACCTGCTAATTTTACTGGCAA

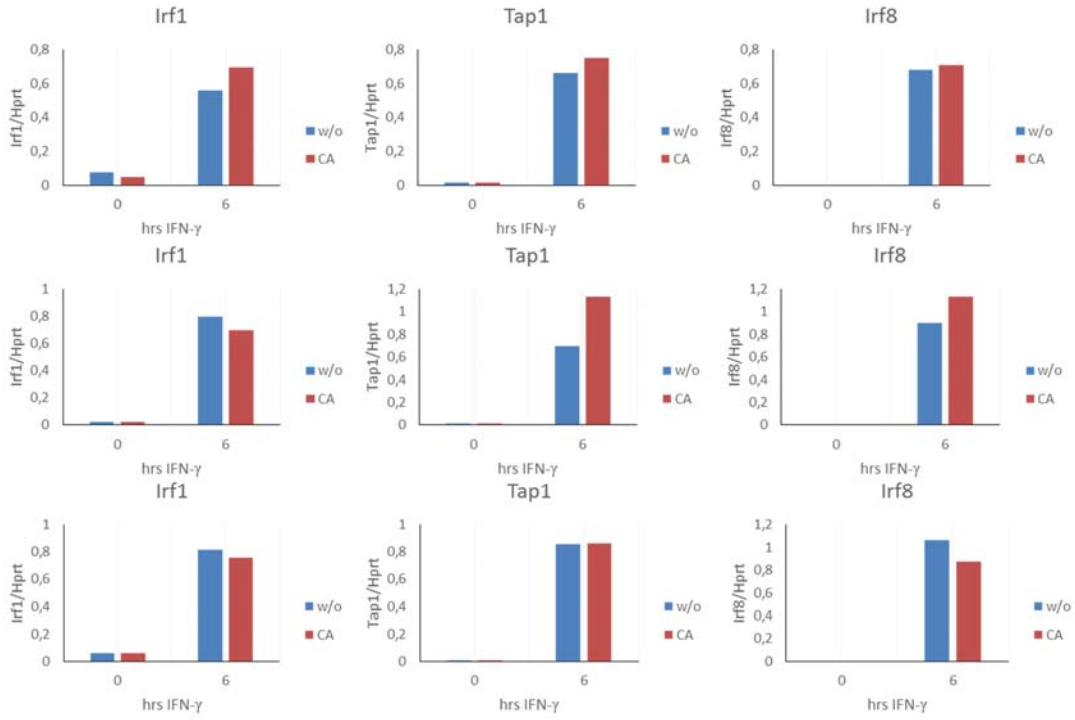

Figure 28: qRT-PCR analysis of selected IFN-γ target genes. MEFs were pre-treated for 1 hour with 100 nM Cortistatin A (CA) followed by 6 hours stimulation with IFN-γ. Gene expression of the IFN-γ-induced genes Irf1, Gbp2 and Irf8 was analyzed. Every line represents one replicate.

As shown in Figure 28 the gene expression of the replicates is very similar for both genes and the samples can be used for sequencing.

Sample preparation for RNA-Seq library

Therefore another Trizol aliquot was precipitated and the samples were afterwards treated with DNase from the RNase-free DNase Kit (ThermoFisher Scientific). RNA cleanup after DNase treatment was done using the RNeasy Mini Kit (Qiagen) according to the manufacturer's protocol. After elution in 30 µl water, the samples were kept on ice and directly dropped off at the sequencing facility. All samples passed the quality control, the values of the analysis are shown in Table 8. The library preparation was done at the Genomics and Microarray Core (Anschutz Medical Campus, Denver), preparing the library after ribo-depletion. Two lanes were used for the

samples with all 12 samples multiplexed on both lanes. The sequencing results will be analyzed within the next months in Vienna.

Table 8: Quality control of RNA-Seq samples handed in for library preparation

Sample	RIN	28S/18S	Concentration [ng/µl]
0_1	9.8	3.2	571
0_CA_1	9.6	3.3	504
6_1	9.7	3.3	468
6_CA_1	10.0	3.1	283
0_2	9.8	3.3	270
0_CA_2	9.9	3.2	194
6_2	9.7	3.1	593
6_CA_2	9.7	1.8	64
0_3	9.8	3.4	400
0_CA_3	9.8	3.3	463
6_3	10.0	3.3	199
6_CA_3	10.0	3.4	477

DISCUSSION

During my three month research stay in the Taatjes lab I addressed three different questions to get more insight into the kinase-dependent roles of CDK8 during IFN-γ signaling and during serum response. First of all, for the biochemical characterization of our established analogue sensitive CDK8 mutant, the cloning procedure for the expression of the mutant kinase in insect cells was optimized. Therefore we used different insect cell expression plasmids, purification steps of the insert and vector components and different precipitation methods to get in the end an intact bacmid suitable for insect cell expression. Unfortunately we could not get a purified submodule with the CDK8as submodule. The purification method itself worked since we got a nicely purified Wt CDK8 module. Unfortunately it seemed that there was a problem during the CDK8as module transfection, since we could not detect any part of the module after the glycerol gradient. The procedure will be repeated by a colleague from the Taatjes lab, also including a different tag during the cloning procedure, to even more efficiently purify the CDK8 submodule. The complete CDK8 subunit will be used to compare Wt CDK8 kinase activity to the mutant kinase activity and to test the inhibition of the CDK8as mutant with the ATP-analogue and the known CDK8/CDK19 inhibitor Cortistatin A.

To address the kinase-dependent and -independent effects of CDK8 on serum response, gene expression patterns of the analogue sensitive CDK8 HAP cells and full CDK8 and CDK19 knockout HAP cells were compared. There was a slight inhibition of selected serum response genes upon CDK8 kinase inhibition by the ATP-analogue, the extend was comparable to combined CDK8 and CDK19 kinase inhibition by Cortistatin A. The effect on gene expression in CDK8 and CDK19 knockout cells was strongly pronounced, but there was no difference between the two knockouts observed. Additionally blocking the kinase of the remaining kinase in the knockout cells did not show additional effects on gene expression. It seems that CDK8 and CDK19 in these cells are both essential for the serum response, since the lack of one already dramatically reduces gene expression. The figure out if they act synergistically the analysis of double knockouts missing CDK8 and CDK19 would be necessary. Since both kinases have a similar influence on the gene

expression upon serum stimulation it is for us not the right system to analyze possible differences of CDK8 and CDK19 on transcriptional regulation.

To get insights into the mechanism how Mediator kinases regulate transcription upon IFN-γ stimulation, the GRO-Seq protocol was successfully optimized to isolate newly transcribed RNA from MEF cells. The isolation of intact nuclei was established as well as the optimal Run-on time, efficient pull down of the labelled RNA and the fragmentation of the isolated RNA to the desired length. The prepared dataset consisting of GRO-Seq and RNA-Seq data will hopefully allow a detailed comparison of IFN-γ-induced genes. The aim is to determine differences in RNA Pol II position on IFN-γ regulated genes in the presence and absence of the CDK8/19 inhibitor Cortistatin A. This will allow conclusions about the step of transcription regulated by CDK8/19 kinase activity in response to IFN-γ.

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