The CDK8 Module: Molecular Architecture and the Role of the C-terminus in Regulation of kinase activity

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Abstract

The CDK8 protein found within humans is a unique cyclin-dependent kinase as it is often found in a four-protein complex with MED12 and MED13, lacks a phosphorylatable threonine in its activation loop and has an extended C-terminus. The goal of this project is to determine whether the CDK8 C-terminus is acting as an alternate activation loop to regulate the kinase function of CDK8. To test this hypothesis, a unique type of cross-linking was performed on the CDK8 module (CDK8, CCNC, MED12, MED13) and truncation mutants of CDK8 were created to test the activity of the kinase. The results suggest that instead of the CDK8 C-terminus acting as a traditional activation loop, it is instead playing a more structural role by binding to MED12 and/or MED13. The cross-linking experiment also suggests a more structural role for the CDK8 C-terminus from identified intramolecular cross-links from the C-terminal domain of CDK8 to the rest of CDK8. Since MED12 is required for CDK8 kinase activity, the CDK8 C-terminus may be activating the kinase by interacting with and possibly binding MED12. This is an interesting finding, as it was largely believed that the CDK8 C-terminus was playing a regulatory role rather than a structural role in activating the kinase. Future experiments are proposed to further test this finding and find a more specific mechanism for the binding of MED12 to the C-terminus of CDK8.

Chapter I. Introduction

The Mediator Complex

Within the human body, the process of transcription is a regulated process that involves a variety of different proteins and enzymes (Figure 1). One of the most important enzymes responsible for controlling transcription is RNA polymerase II, commonly referred to as pol II. Pol II requires further regulation from a variety of proteins, which assemble into a pre-initiation complex (PIC). Within this large complex, there is a smaller component known as Mediator, which is generally required for activated transcription (where DNA is copied into RNA by pol II), and whose subunit composition has been shown to vary (Poss, Ebmeier & Taatjes, 2013). The Mediator complex has been shown to stabilize or facilitate the formation of the PIC and the knockout of various Mediator subunits has been shown as embryonic lethal (Poss, Ebmeier & Taatjes, 2013). Additional studies of Mediator in humans suggest that it may also have post-recruitment activation of pol II, and thus does more than merely recruit pol II to the site of transcription activation (Taatjes, 2010).

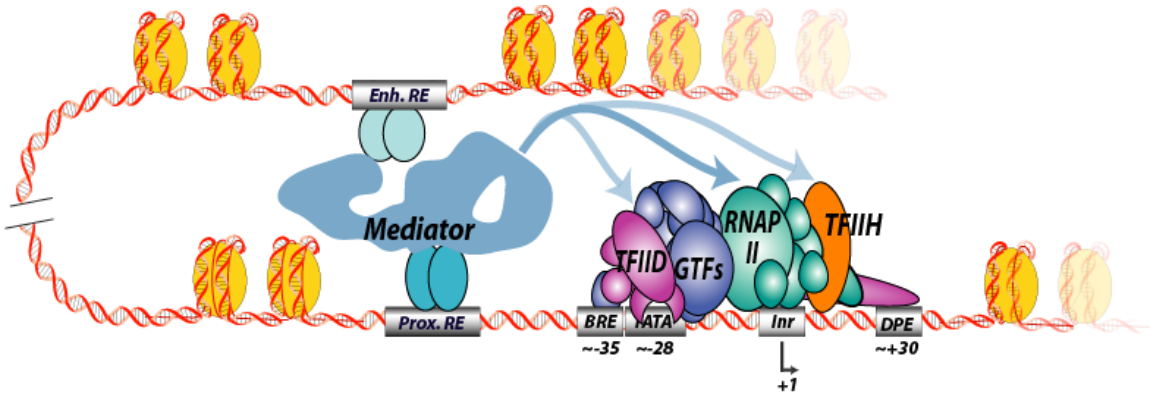


Figure 1. Mediator helps to activate transcription. A model of the general transcription process, showing the important proteins and enzymes involved from the Mediator, RNA polymerase II (RNAP II), general transcription factors (GTFs), TFIID, and TFIIH.

The human Mediator complex consists of twenty-six subunits and is 1.2 MDa in size, which makes it a very large complex (Table 1) (Taatjes, 2010). The general structure consists of a head module, a body portion, and a leg and it seems that pol II assembles around the head module when assembled with the Mediator complex (Figure 2) (Taatjes, 2010). In humans, Mediator can be inactivated, whereby it can no longer bind with pol II, when Mediator assembles with the CDK8 module. The CDK8 module can inhibit Mediator from binding to pol II by causing a structural shift, adding a "foot" module onto Mediator (Figure 3). This, in turn, may repress transcription in cells by blocking the assembly of the pol II complex into the PIC (Taatjes, 2010) and therefore, there is unique interest in the role of the CDK8 module in relation to human Mediator.

Mediator subunit	Molecular weight (kDa)	Mediator subunit	Molecular weight (kDa)
MED1	220	MED21	19
MED4	36	MED22	16
MED6	33	MED23	130
MED7	34	MED24	100
MED8	32	MED25	92
MED9	16	MED26	70
MED10	16	MED27	37
MED11	13	MED28	20
MED14	150	MED29	24
MED15	105	MED30	25
MED16	95	MED31	18
MED17	78	CDK8	55
MED18	28	CCNC	34
MED19	26	MED12	240
MED20	23	MED13	250

Table 1. Mediator subunits and their corresponding molecular weights. The CDK8 module subunits are shown in red. (Poss, Ebmeier & Taatjes, 2013).

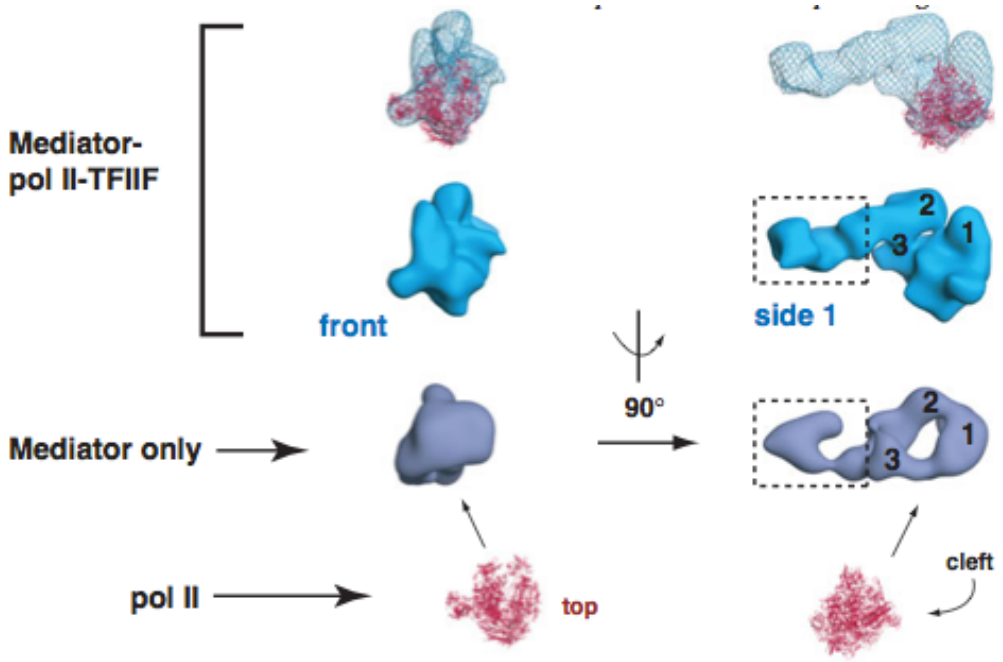


Figure 2. Mediator-pol II causes structural changes in Mediator. A schematic outlining how Mediator structurally changes after pol II binding. Pol II is shown in red and can be seen binding the head region of the Mediator complex. (Taatjes, Ebmeier & Taatjes, 2013)

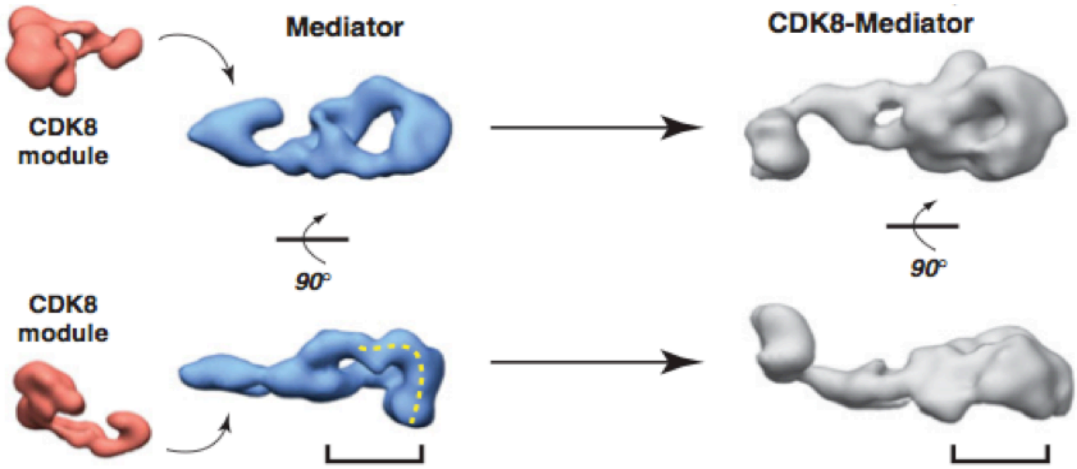


Figure 3. CDK8-Mediator Inhibits pol II Binding. CDK8 module-Mediator binding appears to occlude pol II-Mediator binding by an allosteric mechanism. EM structures of Mediator and CDK8-Mediator are shown (Taatjes *et al,* 2002). The lower panel shows "bottom" views of each complex. The bracket shows the general region occupied by pol II upon binding human Mediator, and the corresponding position in the CDK8-Mediator complex. (Poss, Ebmeier & Taatjes, 2013).

The CDK8 Module and Human Mediator

A distinct form of Mediator, which is of primary interest to this project, is the CDK8-Mediator complex. The CDK8 module is made up of four different subunits: CDK8, cyclin C (CCNC), MED12, and MED13. The CDK8 module reversibly associates with human Mediator and its function appears to switch off transcription by phosphorylating cyclin H, a subunit within TFIIH (Akoulitchevl et al., 2000), which is a general transcription factor in the PIC. Knockout of CDK8 has been shown to be embryonic lethal in mice, while CDK8 has been identified as a colon cancer oncogene and its kinase activity is required for oncogenesis (Firestein et al., 2008; Morris et al. 2008). Based on these studies, it can be seen that the kinase activity of CDK8 plays an important biological role in humans, but it remains less clear how the kinase activity is more specifically regulated.

The CDK8 module has also been shown to block Mediator binding to the pol II C-terminal domain (CTD) by binding to Mediator and causing a structural shift that alters the pol II CTD binding site (Figure 3) (Poss, Ebmeier & Taatjes, 2013). When Mediator can no longer bind to pol II, transcription initiation is repressed, whereby pol II stops copying DNA into RNA. Additionally, CDK8 has been shown to phosphorylate the CTD of pol II, separately from Mediator (Knuesel et al., 2009; Ebmeier & Taatjes, 2010; Näär et al., 2002; Elmlund et al., 2006). CDK8 can phosphorylate the pol II C-terminal domain, which then disrupts Mediator binding due to the CTD becoming hyperphosphorylated and transcription becomes repressed in-vitro (Knuesel et al., 2009; Ebmeier & Taatjes, 2010; Näär et al., 2002; Elmlund et al., 2006). Overall, the CDK8 module as a whole has been linked to a variety of biologically important functions, but there are several ways in which CDK8 is unique among kinases.

An interesting point is that CDK8 can exist separately, apart from MED12 and MED13, with only CCNC (Knuesel et al., 2009). Looking at the dimer form, CDK8-CCNC is modestly conserved, at the primary amino acid level, in yeast in comparison to humans. In yeast, the CDK8-CCNC dimer is instead known as Srb10-Srb11. Both yeast and human CDK8 modules have shown to reversibly bind Mediator, both have been shown to be able to repress and activate DNA-binding transcription factors by phosphorylation, and both bind Mediator through their respective MED13 subunits (Knuesel et al., 2009; Tsai et al., 2013). The kinase activity of both yeast and human CDK8 modules is also very similar, as yeast CDK8 has been shown to phosphorylate different activators to alter their activity or stability and can phosphorylate the CTD of RNA pol II to repress transcription (Hengartner et al., 1998). Finally, there is some sequence homology between human and yeast CDK8 module subunits (Poss, Ebmeier & Taatjes, 2013), again suggesting a biological importance to the CDK8 module since it can be found, in varied forms, across evolutionarily divergent organisms

Out of the four proteins that make up the CDK8 module, of primary interest are CDK8 and MED12. MED12 and CDK8 have both been proven to interact with a variety of DNA-binding transcription factors (Poss, Ebmeier & Taatjes, 2013), suggesting the ability of the CDK8 module to act independently of Mediator. Notably, MED12 was shown to be required for CDK8 activity in human cells, as the three-protein complex containing CDK8, CCNC, and MED12 was active when testing kinase function, while the three-protein complex instead containing MED13 was not active (Figure 4) (Knuesel et al., 2008). As well, MED12 has been implicated in prostate cancer, as well as drug resistance in certain cancer types (Huang et al., 2012; Barbieri et al, 2012), making it an important subunit to study in relation to the CDK8

module. Yet, what is not known at this time is how the CDK8-CCNC dimer interacts with MED12 and MED13, which may be related to a unique part of CDK8, its extended C-terminus.

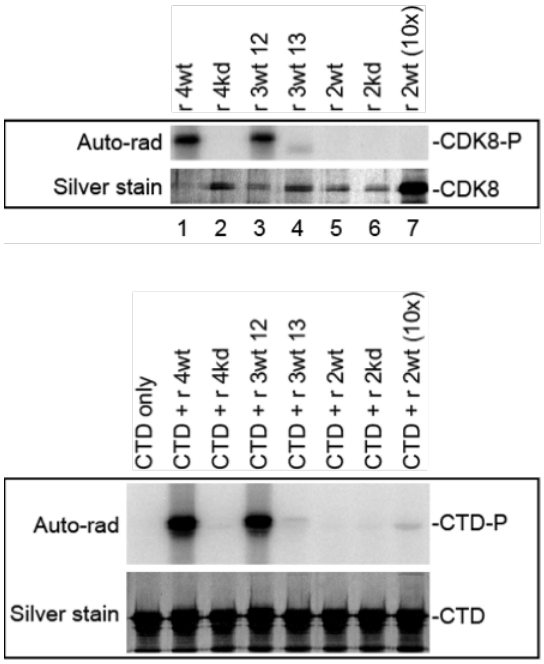


Figure 4. MED12 is required for kinase activity. Kinase assays of the complexes, either wild-type (wt) or kinase dead (kd), meaning the kinase activity is inactive. The second figure additionally shows how the kinase activity of CDK8 can phosphorylate the CTD, again with the different complexes (Knuesel et al., 2008).

In nature, there are a variety of cyclin dependent kinases (CDKs) that often share many structural features, such as an N-terminal lobe that binds different cyclins (Schneider et al., 2011). As well, many CDKs are auto-phosphorylated at a conserved threonine (T) residue in their activation loops, often referred to as a T-loop (Johnson et al., 1996). A typical mechanism is that once the T residue is phosphorylated in the activation loop, a structural shift is initiated that causes the loop to become less ordered and move away from the substrate binding pocket, allowing the substrate to bind (Figure 5) (Johnson et al., 1996).What is unique about CDK8 is its lack of this T residue and in the place of threonine, CDK8 contains and aspartate (D) in its activation loop (Figure 6). Another unique feature of CDK8 is that it has an extended C-

terminus, not found in lower eukaryotes, the purpose of which is unknown but may play an important role in the activation of CDK8 (Figure 7). This activation role is supported by previous data that suggests residue T411, in the CDK8 CTD, may be phosphorylated in the active kinase and thus acting as an alternate activation loop (Knuesel, 2009).

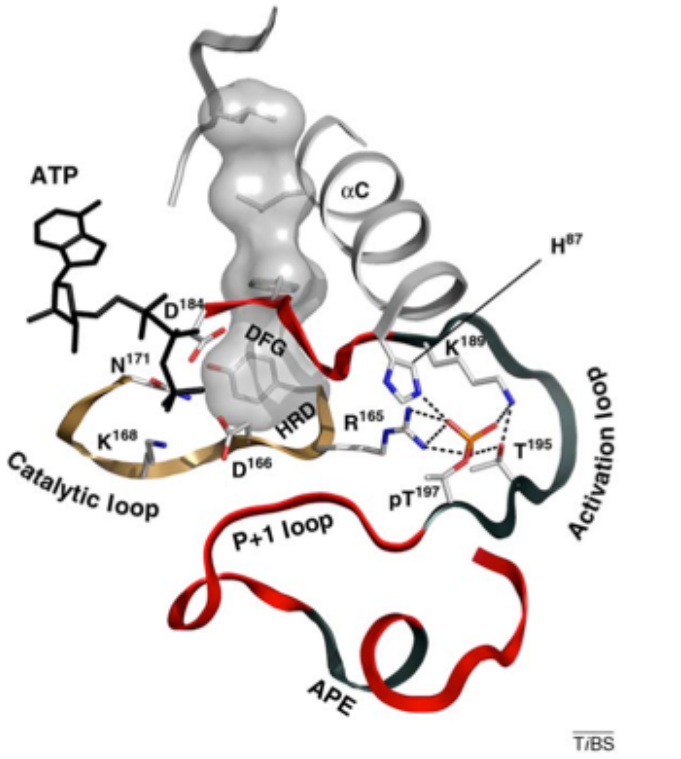


Figure 5. Activation loop of a general protein kinase. The activation loop contains the primary phosphorylation site (T^{197}). The P+1 loop forms a pocket that forms a pocket to accommodate other phosphorylated residues (Taylor and Kornev, 2010).

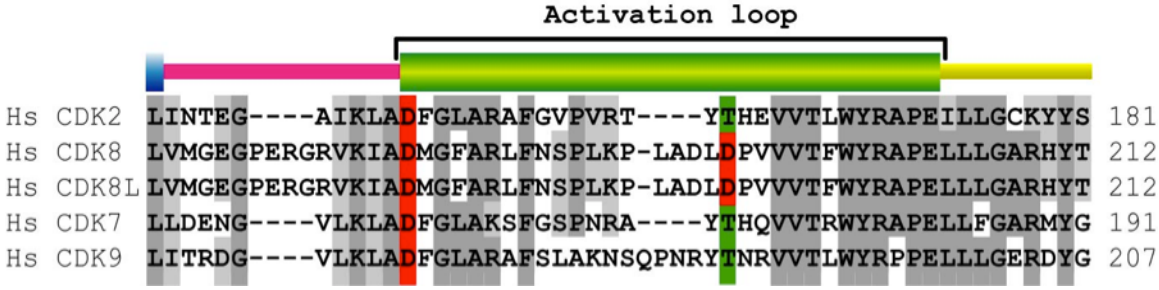


Figure 6. Alignments of the activation loop regions in various CDKs. The activation loop T, t160 within CDK2 (green), is conserved among all CDKs except CDK8 and its paralogs. CDK2 T160 aligns with D304 in yeast CDK8 and D173 in human CDK8 and CDK8L (red). Hs: *Homo sapiens*. (Knuesel, 2009).

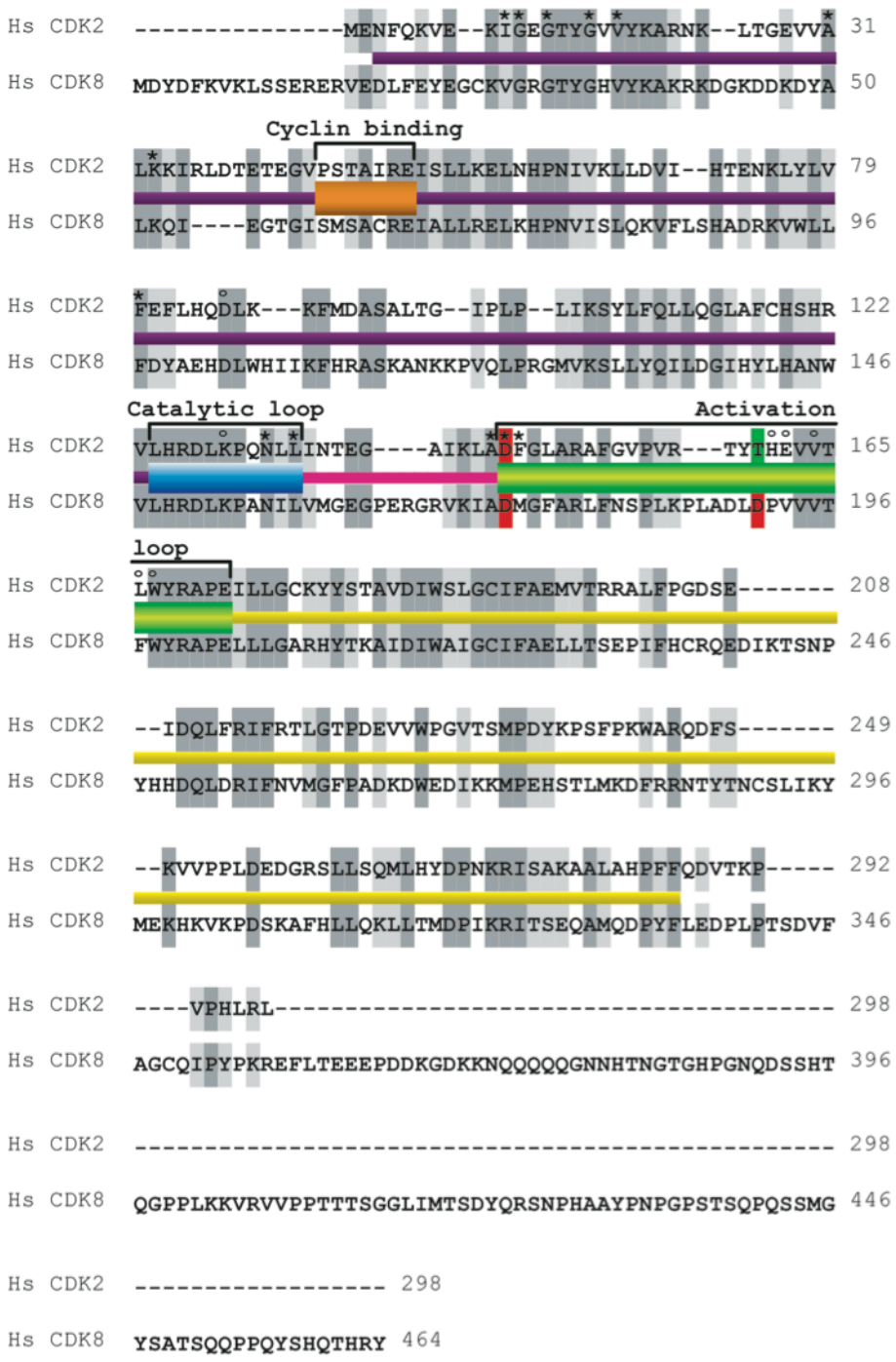


Figure 7. Sequence alignment of CDK2 and CDK8 with CDK domains color-coded. Identical residues are shaded dark grey and similar residues are shaded light grey. Note the extended C-terminal domain of CDK8, 117 residues longer than the CTD of CDK2. Alignments were done using ClustalW (Knuesel, 2009).

Hypothesis and Goals

For this project, there are two different goals, both related to determining the function of the extended C-terminus of CDK8, since it is rather unique among CDKs and it's structure has not been able to be determined with crystallography. The first goal is to determine the architecture and molecular interfaces within the CDK8 module as the current data lack the structure of the entire module. The second is to determine the regulatory role, if any, for the CTD of CDK8.

The first part of the project was to analyze the CDK8 module with a cross-linking/mass spectrometry (CXMS) method that would be able to identify any links between CDK8 and CCNC and MED12. The CXMS methodology is discussed more thoroughly in Chapter II. If cross-links were identified, it would point to the possibility that the C-terminus is important for binding CCNC and/or MED12. This information could show insights into the molecular architecture of the CDK8 module, as well as any possible interfaces within the module with the CTD of CDK8. The second part of the project was to look at the C-terminus in terms of its possible function in regulating kinase activity. Since CDK8 lacks the usual T-loop, due to presence instead of an aspartate (D) residue, which cannot be phosphorylated (Figure 6) it may have an alternate activation loop and be phosphorylated at a different residue, like T411.

For this project, to address the possible mechanisms that the C-terminus may be involved in for activation of the kinase, there were multiple hypotheses. The first hypothesis was that CDK8 contains an alternate activation loop that is found in its extended C-terminus. The second hypothesis was that MED12 interactions with CDK8:CCNC facilitate the activation of the kinase. My predictions for this project were then that the cross-linking with MED12 and CDK8:CCNC would identify critical residues for kinase activation of CDK8. As well, if there is

any site that needs to be phosphorylated for the kinase to be active, by truncating the C-terminus, CDK8 will become active since the active site will no longer be blocked. The following experiments and data address these hypotheses and goals.

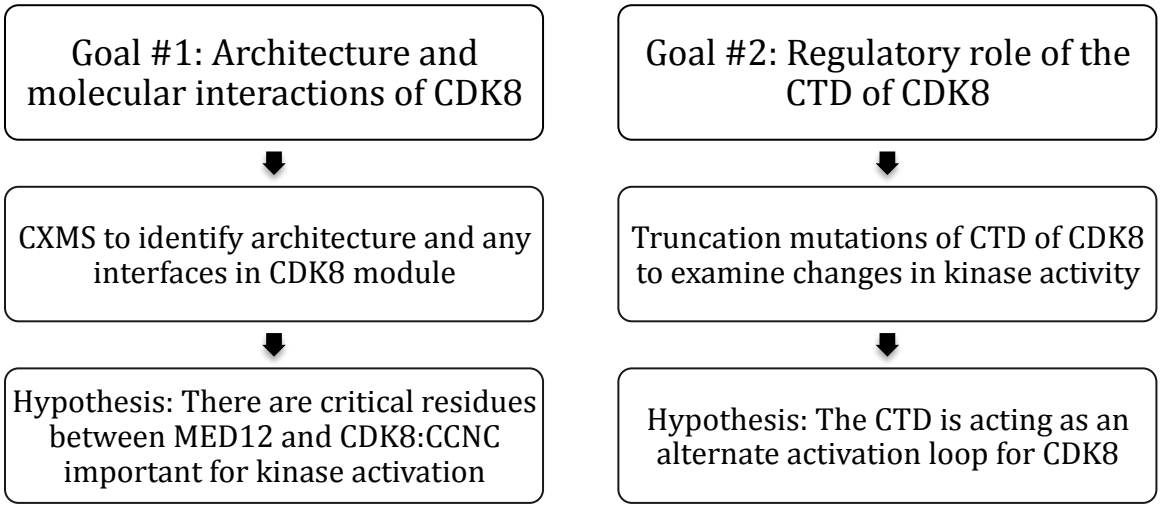


Figure 8. The overall structure and goals of the project for easy reference.

Chapter II. Molecular Architecture of CDK8 Module

A. Introduction

Cross-linking/Mass Spectrometry Methodology (CXMS)

For one part of my thesis project, I worked in close collaboration with the Institute for Systems Biology and the lab of Dr. Jeff Ranish to figure out interactions within the CDK8-CCNC two-protein module and the CDK8-CCNC-MED12 three-protein module. The crystal structure of the CDK8-CCNC dimer is fairly well known (Schneider et al., 2011), so when looking at the dimer, the initial goal was to prove that using this CXMS method was viable for this protein complex as the CXMS data could be compared to the known crystal structure. The Ranish lab uses different cross-linkers, and for this particular project the cross-linker is Bis(sulfosuccinimidyl) suberate (BS^3) (Figure 9). BS^3 works by forcing lysine groups, which are amino acids with nitrogen in them, to form a bond, or cross-link, if the residues are approximately within 11.4 Å. Then mass spectrometry (MS) is used to separate the two cross-linked residues by their mass and charge, which allows the two residues to individually be identified by how they broke apart in MS (Luo et al., 2012). Then each identified residue can be matched to its location on the protein in question from its primary sequence. This information can then tell the lab how many protein interactions there are between two different proteins, like CDK8 and CCNC, and therefore give us structural interfaces within the protein dimer. The same method can then be applied to larger modules, particularly the three-protein module with MED12.

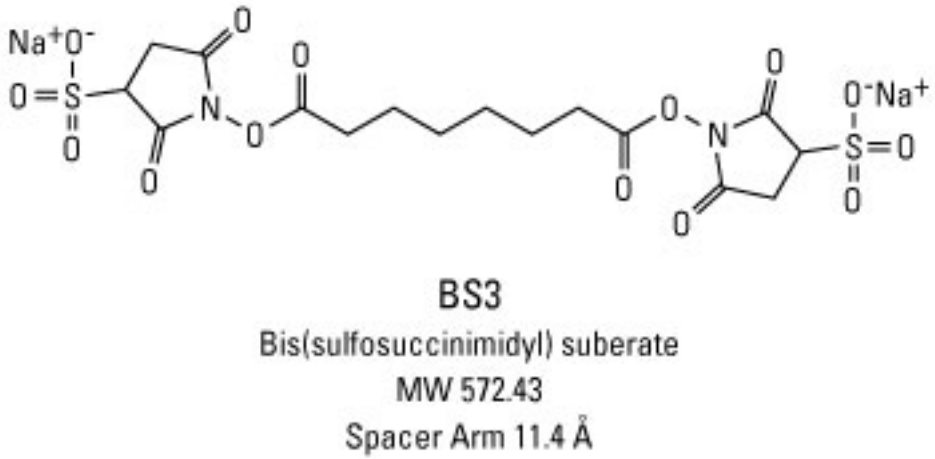


Figure 9. The structure of the cross-linker group, BS[3]. The Ranish Group uses this for CXMS (Thermo Fisher Scientific, 2014)

As mentioned above, the goal is to pay close attention to the extended C-terminus of CDK8 to determine if this part of the protein is linked in any way to either CCNC or MED12, which would give a structural purpose to this C-terminus. As well, it may provide insights into the molecular architecture and organization of the entire CDK8 module, which is largely unknown.

B. Results

Purification of CDK8-CCNC Dimers and CDK8-CCNC-MED12 Modules

The first step of the cross-linking process is to purify the CDK8:CCNC complex in order to provide pure samples to the Ranish Lab. Initially, the methods used to purify the dimer with CDK8 and CCNC was attempted with *E.coli*, but this was stopped as the dimer was shown to be inactive in a kinase assay. The next attempt was to instead purify the dimer from insect cells. The pellets were spun down in lysis buffer, then the supernatant was removed after spinning down the cells, and the dimer was purified from the whole cell extract (WCE) using glu-glu peptide. In

order to estimate the concentration of the dimer that would be purified from the insect cells, previous purifications of the CDK8-CCNC dimer (with the point mutation T411E) were quantified using a SYPRO Ruby gel stain (Figure 10). Then, purifications were compared using a silver stain to estimate how close the concentrations were to the T411E mutant (Figure 11) and a western was run to ensure that both CDK8 and CCNC were present and no MED12 or MED13 proteins were present (Figure 12).

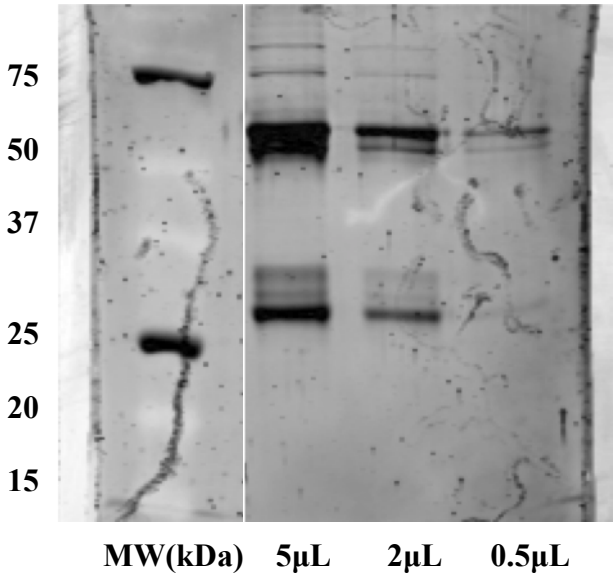
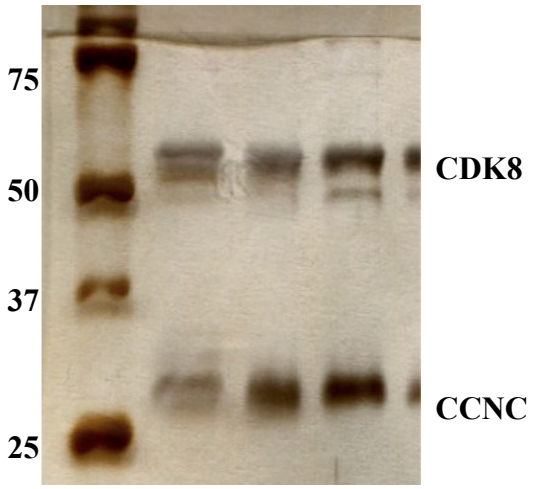


Figure 10. A SYPRO gel of CDK8-CCNC T411E mutant. Used to quantify the approx. concentration of the dimer from insect cell purifications. 5µL, 2µL, and 0.5µL of the purification were added. The top bands (around 55kDa) are CDK8 bands and the bottom bands (around 33kDa) are CCNC. The determined CCNC concentration was 0.602 µM.



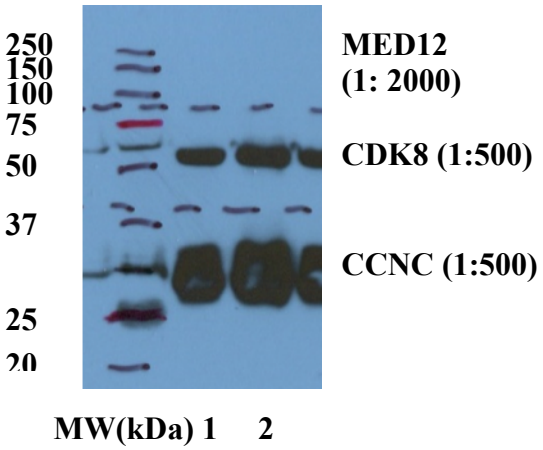


Figure 11 (left). A silver stain of the CKD8:CCNC purified elutions. Lane 2 is 1µL of CDK8:CCNC, Lane 3 is 2µL of CDK8:CCNC. The purification was estimated to produce a concentration of CCNC at 0.602µM. **Figure 12 (right). A western of the CDK8:CCNC elutions**. Used to ensure the presence of CDK8 and CCNC only. Lane 1 is 1µL of CDK8:CCNC, lane 2 is 2µL of CDK8:CCNC.

Once the purification was shown to be successful, the elutions were concentrated and sent to the lab of Dr. Jeff Ranish at the Systems Biology Institute in Seattle, WA. A very similar protocol was followed for the 3-protein module, containing CDK8, CCNC, and MED12. Again, the purifications were tested with a western (Figure 13) to ensure the presence of each of the 3-protein module components and no MED13.

In order to ensure a 1:1:1 molar ratio for each module component, a glycerol gradient was used to try and separate out any free CDK8-CCNC dimer that was not linked with MED12 (Figure 14). Multiple gradients were used to amass the largest amount of CDK8-CCNC-MED12 module and the estimated concentration, from a SYPRO Ruby, for MED12 was 0.05µM. The samples were then combined, concentrated, and a buffer exchange was performed and the sample was shipped to the Ranish lab for analysis.

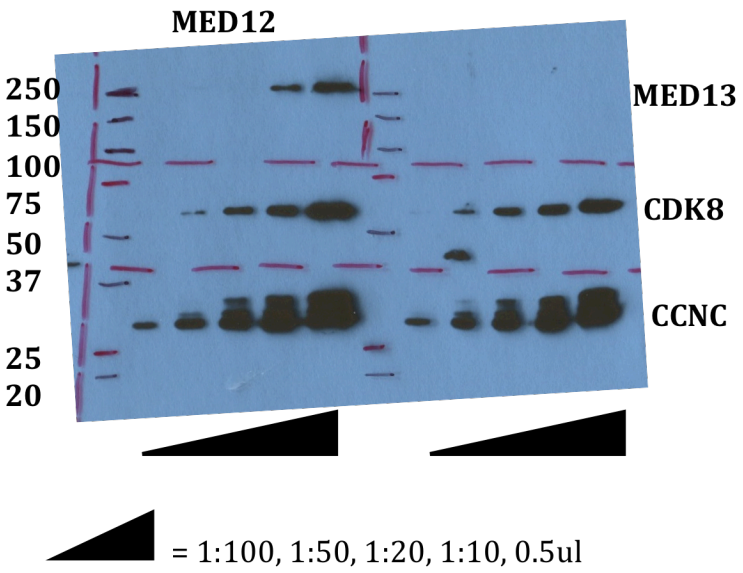


Figure 13. A Western of the 3wt purification. Shows the presence of MED12, CDK8, and CCNC but no MED13.

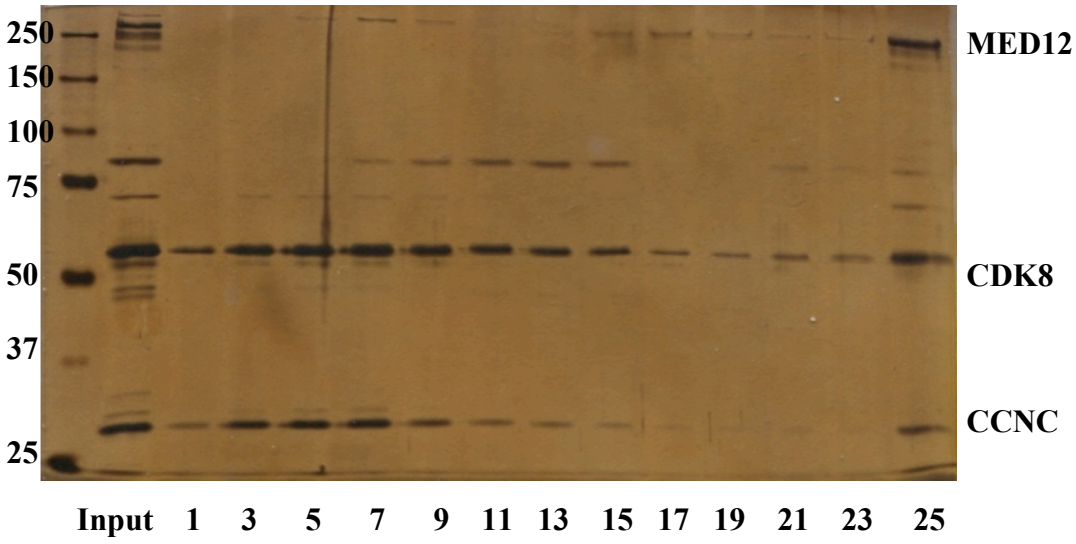


Figure 14. A silver stain of MED12, CDK8, and CCNC. This gel shows the odd fractions of a 15-30% gradient. The components become concentrated in the very last fraction (25). From a SYPRO Ruby gel, the concentration of MED12 was estimated to be 0.05μM in the final fraction.

Cross-linking Data for the CDK8-CCNC Dimer and CDK8-CCNC-MED12 Module

The samples for both the CDK8-CCNC dimers and CDK8-CCNC-MED12 modules were run through the mass spectrometry procedure used by the Ranish lab. The results are laid out with each line connecting two residues that were shown to be cross-linked. For the CDK8-CCNC module (Figure 15), the sequence of CCNC is laid out on the top and the CDK8 sequence is on the bottom. For the CDK8-CCNC-MED12 module, the very top figure represents CCNC, CDK8 is laid out in the middle, and MED12 is the very bottom figure (Figure 16).

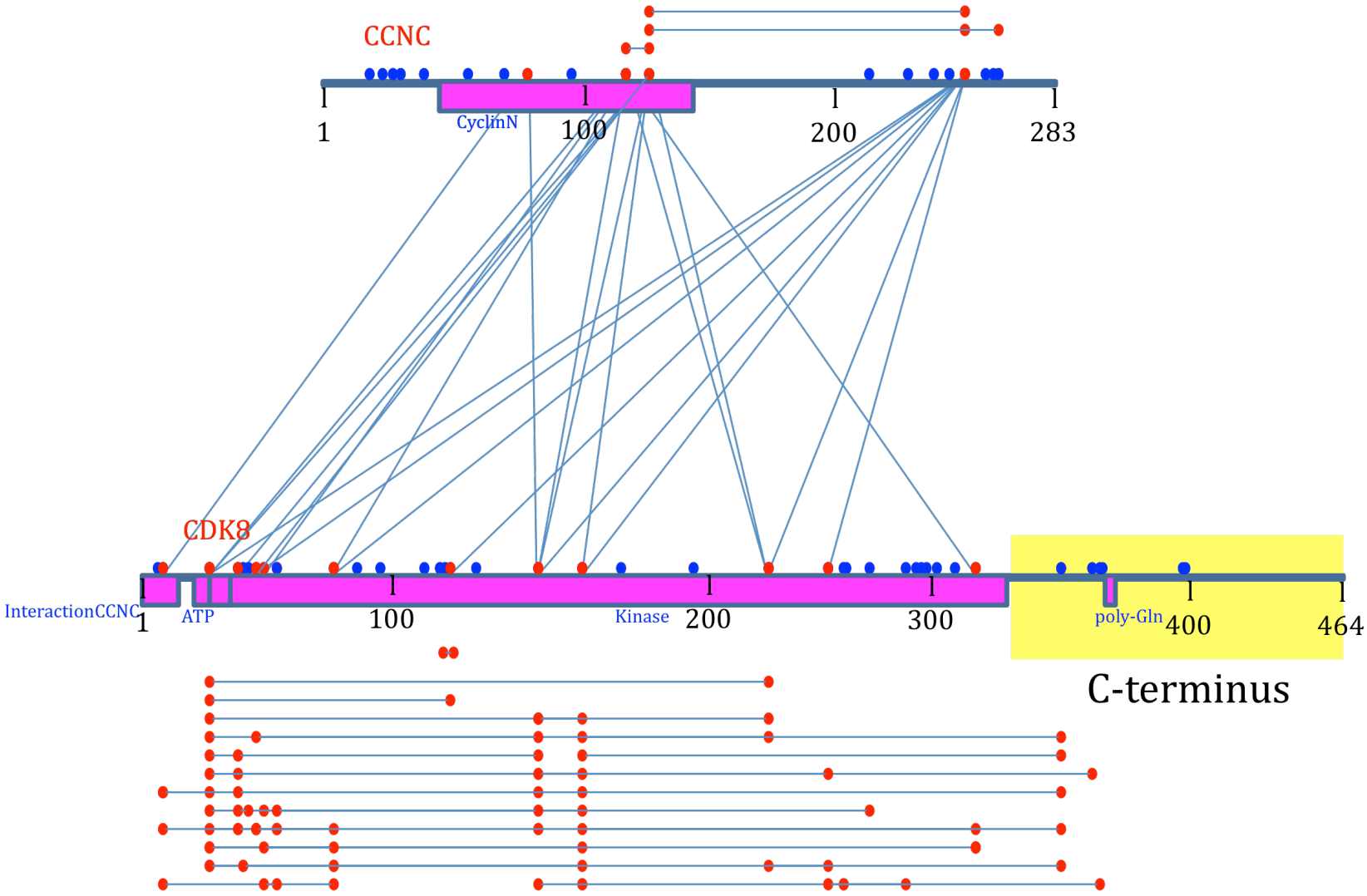


Figure 15. CXMS Data from CDK8-CCNC dimers. This demonstrates what the cross-linking data received from the Ranish lab with residues from cyclin C (CCNC – top) interact with residues from CDK8 (bottom). The lines connecting the top to the bottom show the cross-links. Red dots indicated cross-linked residues, blue indicates residues that were not cross-linked, but could have been. The blue lines that are connected by red dots represent intramolecular cross-links between two residues on the same protein.

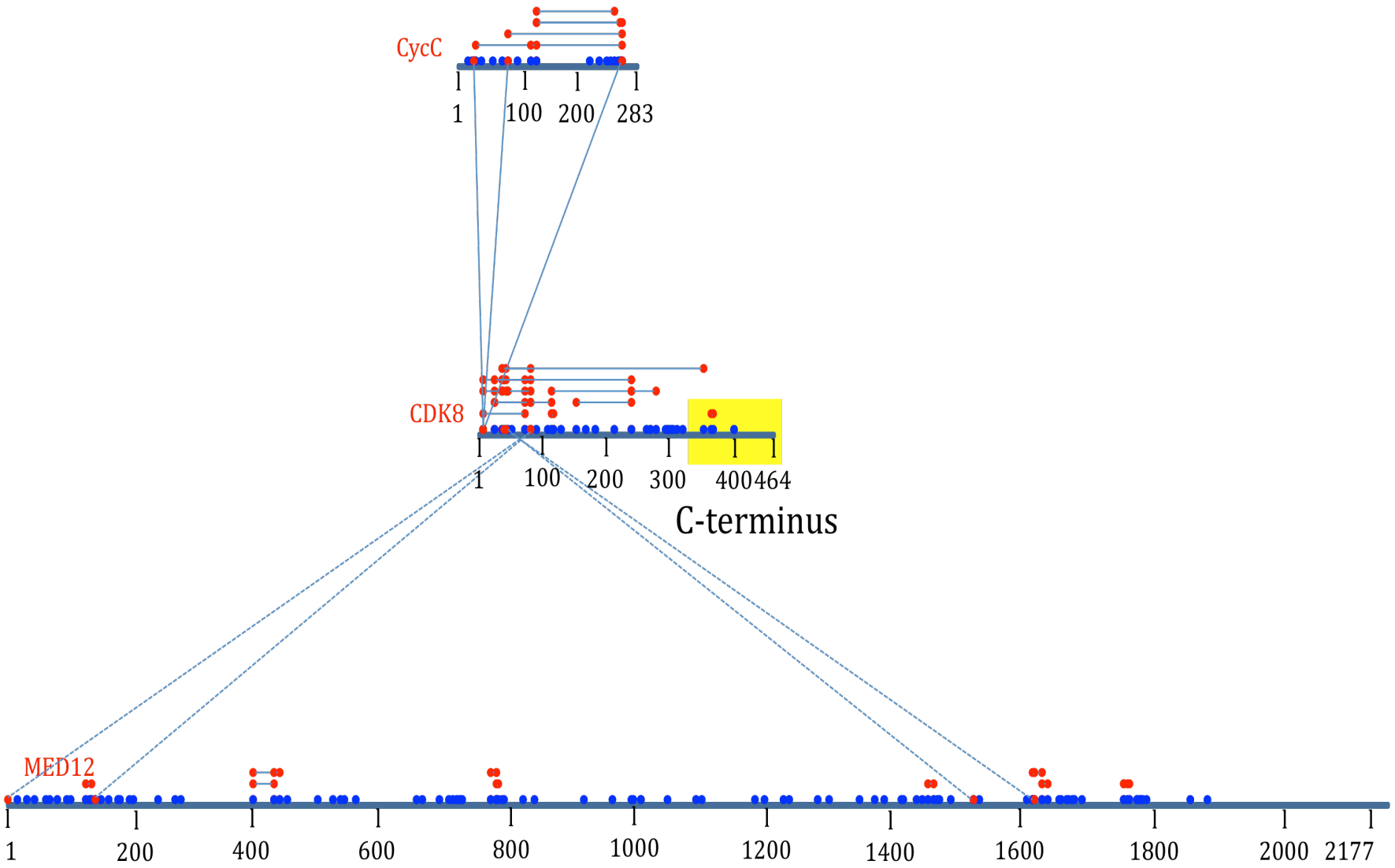


Figure 16. CXMS Data for CDK8-CCNC-MED12. The very top figure is CCNC's primary sequence, the middle is CDK8's sequence, and the very bottom, and longest, sequence is MED12. Again, the blue dots represent residues that could be cross-linked while red dots represent residues that have been cross-linked. The lines between the proteins represent which residues were cross-linked, and the lines above the sequences represent all intramolecular cross-links.

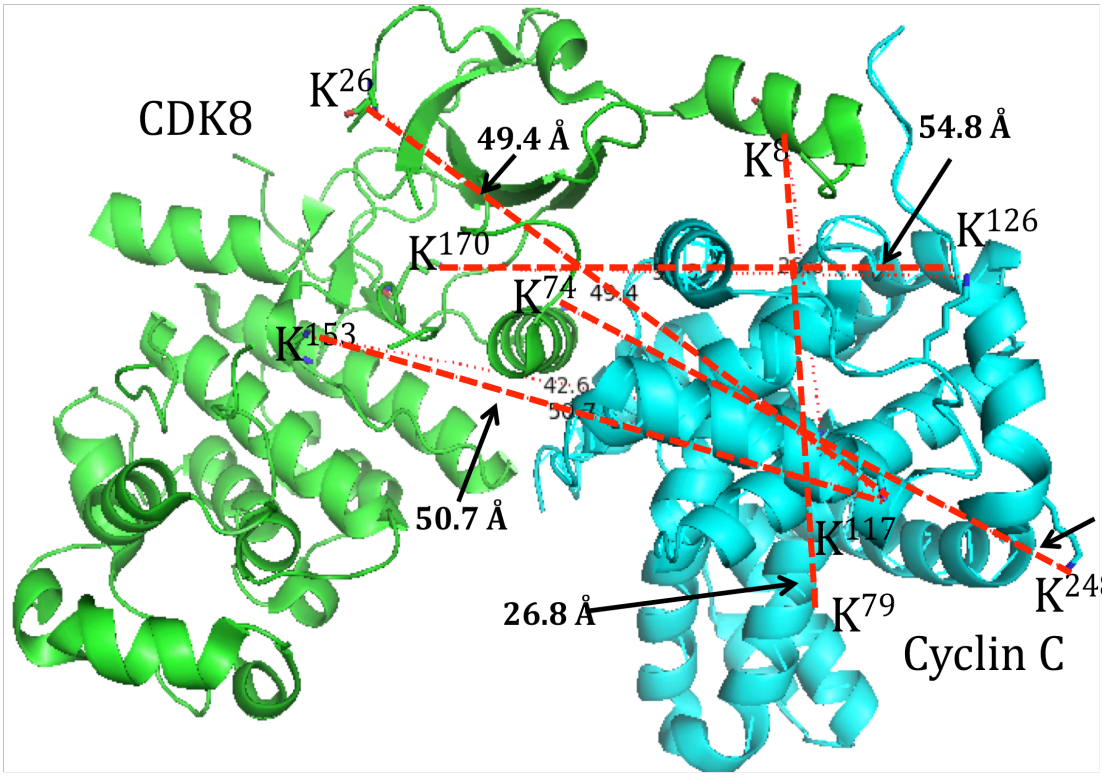
C. Discussion

Cross-linking with CDK8:CCNC

One of the main purposes of cross-linking the CDK8:CCNC dimer was to verify that the CXMS method could be used to cross-link the CDK8 module. Based on the results, the method does work as the cross-linking data identifies some interactions that were previously identified from the crystal structure of CDK8:CCNC (Table 2, Figure 17).

CDK8 Residue	CCNC Residue	CDK8 Residue	CCNC Residue
153	79	47	117
8	79	322	117
119	248	26	126
153	248	170	126
44	248	153	126
74	248	242	126
26	248	37	126
242	248	44	126
170	248	281	248
265	248	355	248
74	248	355	117
26	117	8	117
242	117	355	126
153	117		

Table 2. CXMS Identified Residues for CDK8-CCNC Dimer. This table shows all of the 27 identified cross-links from the CXMS data. The pink highlighted residues are those with high-confidence (81%) and the yellow highlighted residues are low-confidence.



CDK8 Residue	CCNC Residue	Distance (Å)
8	79	26.8
44	248	62.4
74	248	52.6
26	117	49.4
153	117	50.7
170	126	54.8

Figure 17. CXMS Intermolecular Data with the crystal structure of CDK8-CCNC. CDK8 is green with CCNC blue and the cross-linked lysine (K) residues labeled along with the length (in Å) of the cross-link. The table lists the cross-linked residues, along with the distances between the residues.

The cross-links depicted in Figure 17 do not represent all of the identified cross-links but show the capability of using the CXMS methodology to identify intermolecular cross-links in the CDK8-CCNC dimer. This then shows that the same methodology could be used to identify cross-links between CDK8, CCNC, and MED12. It is important to note, however, that the spacer length for the cross-linker used, BS^3, is only 11.4 Å, while the cross-links identified are all longer than the spacer length. There could be multiple possibilities for these results, such as

multiple BS^3 cross-linkers being linked between two lysines, increasing the length of the cross-link. As well, it could be that the structure of the dimer is more mobile than the crystal structure suggests, creating longer lengths between residues (Rappsilber, 2010). Still, overall, there was great success in cross-linking between the heterodimer that proved the efficacy of the CXMS methodology.

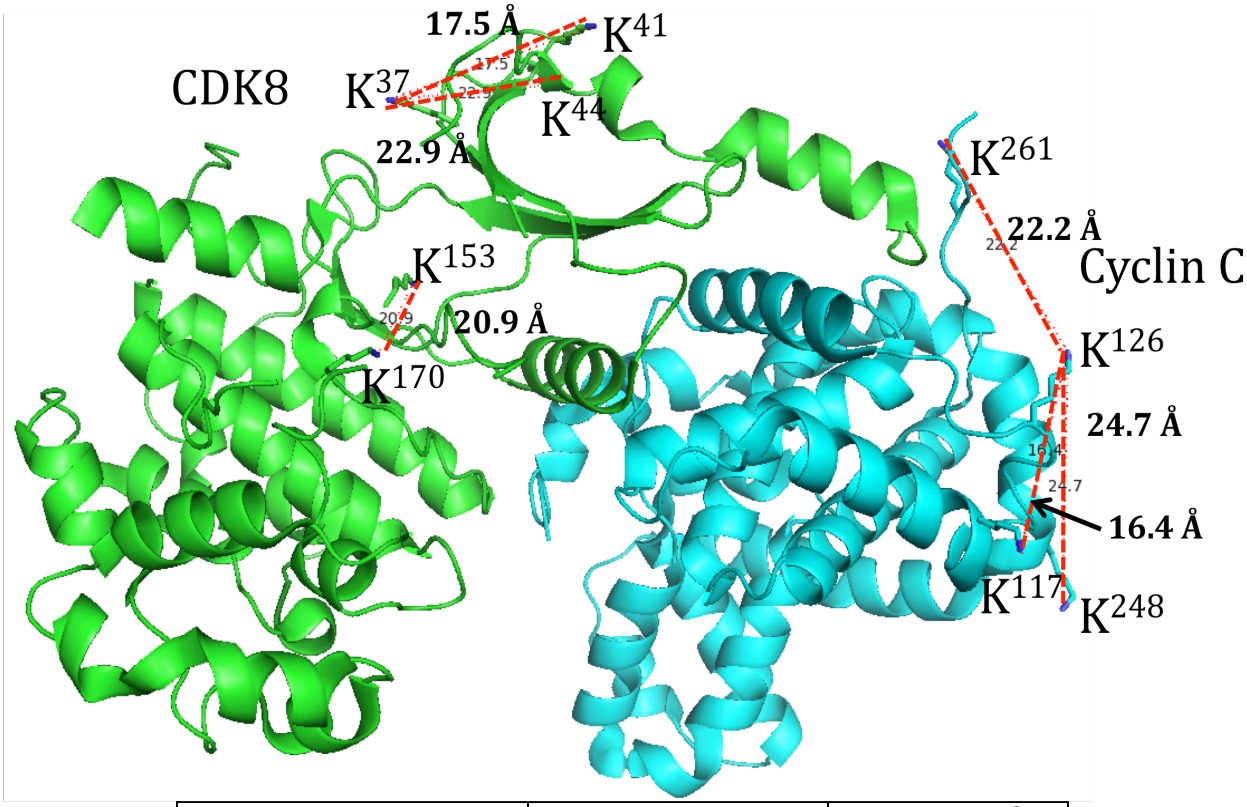
As well, the cross-linking data showed that there quite a few intramolecular cross-links between the two proteins (Table 3, Table 4, Figure 18), suggesting that there may be a lot of contacts within CDK8 that may play an important role in the structure and function of the kinase. Looking closer at the C-terminus of CDK8, however, shows no cross-links at any of the possible residues: 370, 371, 402, or 403. This is not surprising as it is well known that cyclin C binds at the N-terminal lobe rather than the C-terminus (Schneider et at., 2011). Nevertheless, by proving the efficacy of the CXMS method, this allowed for the next step of purifying the three-protein complex, with CDK8, CCNC and MED12.

CDK8 Residue	CDK8 Residue	CDK8 Residue	CDK8 Residue
119	26	26	322
52	52	115	119
153	153	170	37
153	52	170	8
153	8	170	355
153	37	170	74
153	44	170	26
153	26	170	170
153	295	170	242
153	242	170	47
153	170	170	322
153	265	242	26
37	41	242	265
37	8	47	74
37	44	322	322
44	74	271	271
355	39	119	119
355	37	119	355
355	44	52	8
74	8	153	355
26	39	153	322
26	52	355	355
26	281	355	370
26	8	35	47
26	37	26	44
26	367	115	355
26	355	47	52
26	74	322	355

Table 3. CXMS Identified Intramolecular cross-links in CDK8. The high-confidence cross-links are highlighted in pink (90%) and low-confidence cross-links are highlighted in yellow.

CCNC Residue	CCNC Residue
248	248
117	248
117	126
126	248
126	261
248	117

Table 4. CXMS Identified Intramolecular cross-links in CCNC. The high-confidence cross-links are highlighted in pink (70%) and low-confidence cross-links are highlighted in yellow.



CDK8 Residue #1	CDK8 Residue #2	Distance (Å)
153	170	20.9
37	41	17.5
37	44	22.9
CCNC Residue #1	**CCNC Residue #2**	**Distance (Å)**
117	126	16.4
126	248	24.7
126	261	22.2

Figure 18. CXMS Intramolecular Data with the crystal structure of CDK8-CCNC. Intramolecular cross-links identified from the CXMS methodology plotted onto the crystal structure of CDK8-CCNC. CDK8 is green and CCNC is green and the cross-linked lysines are labeled. The table lists the cross-linked residues and the distances between residues.

Cross-linking with MED12

The reason for cross-linking data the 3-protein complex, CDK8:CCNC:MED12, is to determine the architecture of the module to identify any interfaces, with close attention paid to any possible contacts between the C-terminus of CDK8 and MED12. Since MED12 is required for the kinase activity of CDK8 (Knuesel et al., 2009), it would be interesting to see any interactions between MED12 and CDK8 that would elucidate how MED12 is activating the

kinase. From the data, the initial indication seems to be that there are no cross-links present between the C-terminus of CDK8 and MED12. Yet, the links that the Ranish group was able to identify were all links deemed not “confident”. This suggests that MED12 was underrepresented in the samples and so it was hard to identify cross-links with the protein. This may stem from the fact that it is hard to get a solid stoichometric ratio between MED12 and CDK8:CCNC due to MED12’s large size, which is hard to express in insect cells. Sample may also have been lost in the process of concentrating the sample and performing a buffer exchange.

Even though there were no MED12-CDK8 C-terminus cross-links that were found from this initial cross-linking data, there are a couple possibilities to explain these results, beyond lack of enough sample. One possibility is that the CDK8 C-terminus is binding MED13 instead. Then MED13 could then be binding to MED12, connecting the module together. If this were occurring, there would be no cross-links between the C-terminus of CDK8 and MED12. Another possibility is that the CDK8 C-terminus is not binding MED12 and so this CXMS methodology cannot be used to determine any important residues in the CDK8 C-terminus that may be helping to activate the kinase via interaction with MED12. Ultimately, this initial data suggests that there are no cross-links in the C-terminus of CDK8 and MED12, but more sample needs to be sent to verify these results. This will be done without a concentration step, as it has just recently been found to be unnecessary to achieve “high-confidence” cross-links.

Yet there were some cross-linked residues that can be discussed. The cross-links that were identified with a low amount of certainty were between CDK8 residues 41 and 44 with MED12 residues 1622 and 1526 respectively. This corresponds to the “front” end of the CDK8 module in the N-terminus, suggesting this could be where MED12 is interacting with CDK8. As well, intramolecular links were also identified within the MED12 module, indicating some

presence of cross-links between relatively close residues, for example between MED12 residue 1453 and residue 1463. Since these residues are not very far apart, they could be indicative of an intramolecular connection or merely two closely positioned residues. Still, the identified cross-links do suggest that this method can be used, with more MED12, to identify further cross-links between the three proteins.

Future Directions

Beyond the immediate direction of purifying more CDK8:CCNC:MED12 complexes to send for more cross-linking data, there are more experiments that can be done to look at interactions between CDK8's C-terminus and other proteins in the module. One will be to purify and concentrate the whole CDK8 module with MED13 to identify and cross-links between all four proteins, with close attention still paid to the C-terminus of CDK8. The CDK8 C-terminus is of particular interest, as its purpose is not defined and it may be involved in interactions with MED12 or MED13, which would be indicated by cross-links. This experiment could also highlight possible residues that are interacting between MED12 and MED13 and MED13 and CDK8, beyond the CDK8 C-terminus. Since the structure of the full module is not well known, these data could give details about how each protein is interacting with the other proteins in the full module, which would be new and useful information.

Other data, from similar CXMS data with the CDK8-Mediator complex, done by Ben Allen in the Taatjes lab, indicates high-confidence cross-links, on CDK8 residues 44 and 74, with MED13. Interactions were also found between MED12 and MED13, so one possibility is that CDK8 is interacting with MED13 and not MED12, so the three-protein module with MED13 could also be purified and sent to CXMS to get a closer look at those interactions. Close

attention still needs to be paid to the notion that the CDK8 C-terminus could be interacting with the rest of the CDK8 module, somehow causing a conformational change that could then allow for CDK8 interactions with MED12 and MED13. This complicated mechanism remains another option for the function of the CDK8 C-terminus and further CXMS experiments and data will be helpful in figuring the architecture out for the CDK8 module.

The experiments mentioned above are possible future directions for continuing to use this CXMS method to gain insights into the CDK8 module intra- and inter-molecular interactions and how these interactions could be affect the kinase activity of CDK8.

Chapter III. Role of CDK8-CTD in Regulation of Kinase Activity

A. Introduction

Kinase Activity of CDK8

CDK8, along with CCNC, MED12, and MED13, affects transcription by interacting with Mediator, which negatively affects Mediator interaction with pol II (Knuesel et al., 2009; Ebmeier & Taatjes, 2010; Näär et al., 2002; Elmlund et al., 2006). CDK8 has been shown to also phosphorylate cyclin H, which inhibits TFIIH and represses transcription (Akoulitchev et al., 2000). The kinase activity of CDK8 is required for oncogenesis in colon cancer, based on mouse models (Firestein et al., 2008). These examples indicate that the kinase activity of CDK8 plays an important role in transcription regulation, which is then dependent on the other subunits, mainly MED12 and MED13. MED13 was shown to be critical for allowing the CDK8 module to interact with Mediator, allowing the module to repress transcription (Knuesel et al., 2009). More importantly for this project, it was found that MED12 is required for the kinase activity of CDK8 by showing that the CDK8:CCNC:MED12 module was almost as active as the wild-type CDK8:CCNC:MED12:MED13 module (Figure 19) (Knuesel et al., 2008). Ultimately, while it is known that MED12, and to a lesser extent, MED13, play an important role in the kinase activity of CDK8, the mechanisms of their roles within the CDK8 module are not well known.

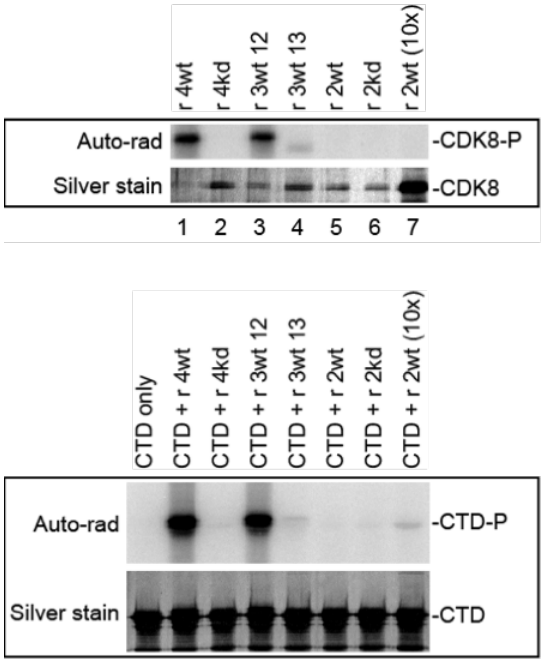


Figure 19. MED12 is required for kinase activity. Kinase assays of the complexes, either wild-type (wt) or kinase dead (kd), meaning the kinase activity is inactive. The second figure additionally shows how the kinase activity of CDK8 can phosphorylate the CTD, again with the different complexes (Knuesel et al., 2008).

B. Results

Cloning of CDK8 CTD Truncation Mutants

Initially, the goal was to clone the truncation mutants with Polymerase Chain Reaction (PCR) and digesting the preferred CDK8 sequences and insert the new CDK8 sequence into the pACO vector. The restriction endonucleases chosen were XbaI and EcoRI-HF due to their presence on the CDK8 sequence in the pACO vector. Yet, this method of cloning did not prove to be successful and instead the method used was site directed mutagenesis and a stop codon was put into the CDK8 sequence to terminate the protein after residue 357 and residue 403. These two truncations were chosen as residue 357 is roughly where similarities end with other CDK alignments for the C-terminus in yeast Cdk8 (Srb10) (Figure 20) and residue 403 is the end of density from the known crystal structure of the human CDK8-CCNC dimer (Schneider et al.,

2011). As well, the CDK8 protein up until residue 403 is known to be stable and bind CCNC, giving more support as to choosing this residue to truncate the C-terminus.

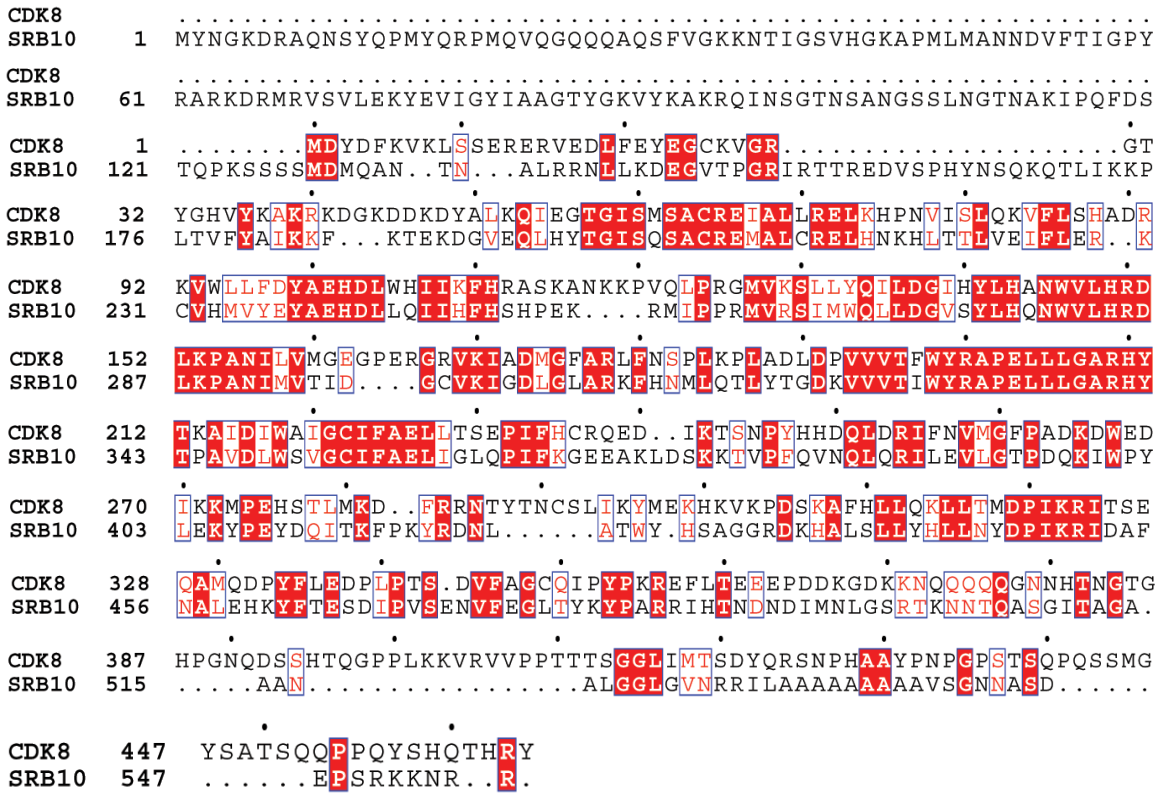


Figure 20. CDK8 alignment (human) with Srb10 (yeast). Red-highlighted residues are exact matches between sequences, while red-lettered residues are similar residues between each sequence.

The sequence was cloned in the pGEX vector, which is optimized for *E.coli*, and then inserted into the pACO vector, which is used for insect cell expression. This method proved to be successful, as determined by restriction digestions with additional restriction sites that were also cloned in. In CDK8(1-357), the stop codon inserted was TAA and an SspI site (AATATT) was added into the sequence. In CDK8(1-403), the stop codon was also TAA and a PmlI site (CACGTC) was added into the sequence. An analytical digestion (Figure 21) shows that both mutations were successfully inserted into the pGEX vector and the DNA was PCRed out, ligated into the pACO vector, and purified using a Maxi-prep and finally sent to UC Denver for insect

cell baclovirus infection. The cloned sequences were also then verified with sequencing data to ensure the stop codon was inserted after each appropriate residue.

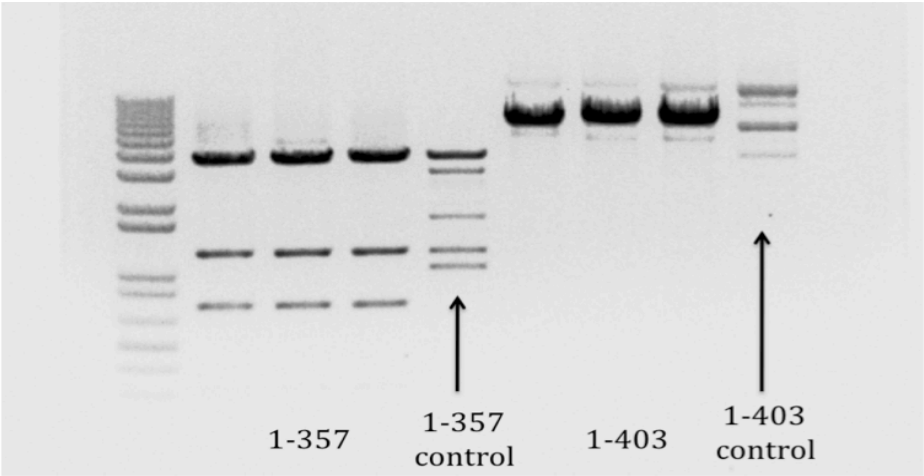


Figure 21. An analytical digestion of truncation mutants, 1-357 and 1-403. The first three lanes for each mutant represent different DNA sequences isolated from different *E.coli* colonies that were chosen. The difference between the control and digested lanes show that the truncations were successful.

Expression of Mutant CDK8 Modules

Each CDK8 truncation mutant (1-357 and 1-403) was sent to UC-Denver for insect cell expression in a 2-protein form with CDK8:CCNC and its 4-protein form with CDK8:CCNC:MED12:MED13 and all of the modules were purified using the glu-glu antibody purification protocol. SYPRO Ruby gels (Figure 22) were used to confirm successful purification and to determine the concentrations of each of the modules. For CDK8(1-357) in a complex with CCNC, its concentration was 1.04μM based on CDK8 and in its 4-protein form (CDK8-CCNC-MED12-MED13), its concentration was 0.11μM based on CDK8. For CDK8(1-403), its concentration in complex with CCNC was 2.57μM based on CDK8 and in its 4-protein (CDK8-CCNC-MED12-MED13) form its concentration was 0.26μM based on CDK8.

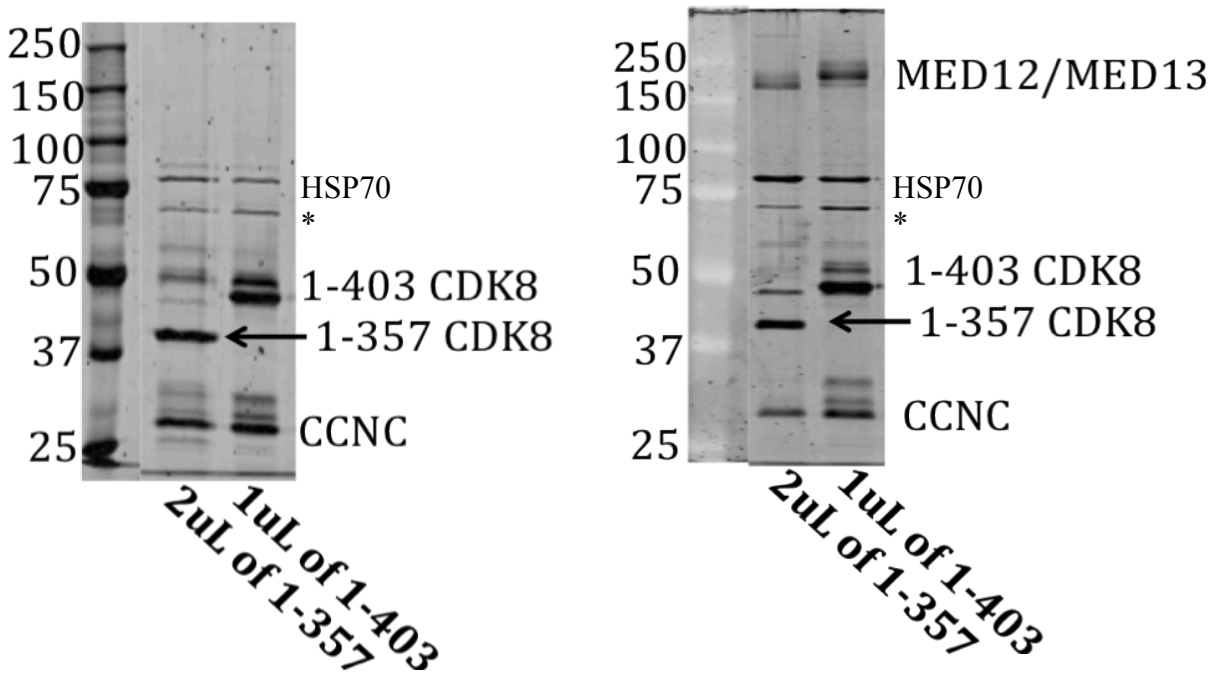


Figure 22. The SYPRO Ruby gels of CDK8 (1-357) and CDK8(1-403). The left is purified CDK8-CCNC dimers and the right is purified CDK8:CCNC:MED12:MED13.

Further verification was used to ensure that all CDK8 modules were present in their 4-protein form (Figure 21). For the CDK8(1-403) truncation mutant, all four subunits were present based on CCNC, MED12, and MED13 western blot signals. In the CDK8(1-357) truncation mutant MED12 and MED13 were not found, but CCNC and CDK8 were still present (Figure 23). CDK8 in its truncated form cannot be verified with a western because the antibody used binds the C-terminus of CDK8, which is no longer present so a SYPRO Ruby gel is used as verification. Both the western blot and SYPRO Ruby gels verify successful purification of the truncation mutants from whole cell extracts and the concentrations were used to determine kinase loads in the kinase assays

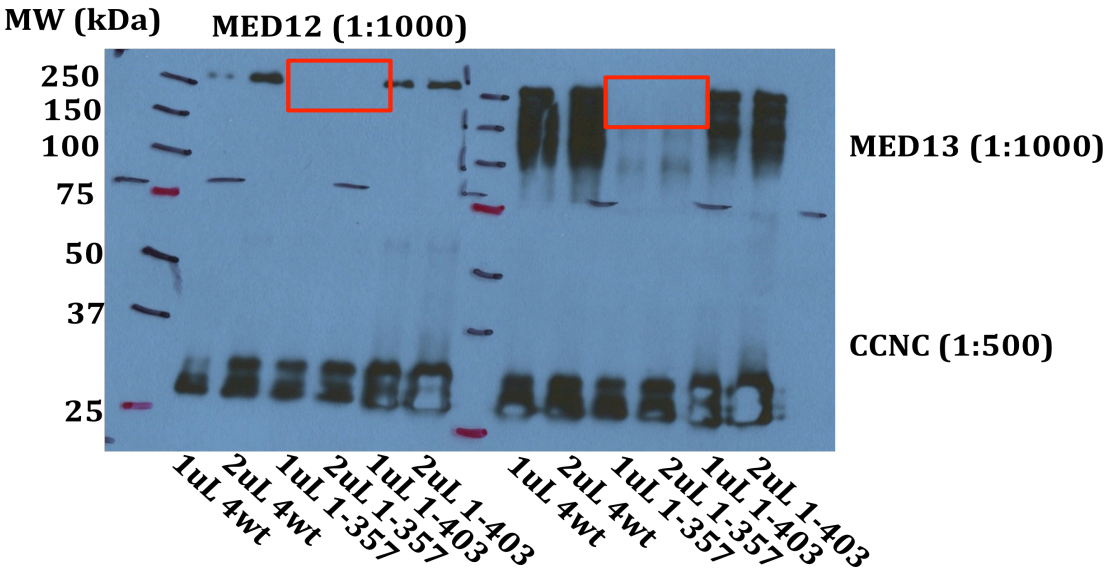


Figure 23. Western blot of CDK8 Truncation Mutants. This shows the presence of all four proteins in the wild-type module and Δ403 module, but no MED12 or MED13 in the Δ357 module (highlighted in red). All primary antibody dilutions are indicated and the same secondary antibody, rabbit, at a 1:2000 dilution.

Kinase Assay data between wild-type and mutant CDK8 modules

After the CDK8 truncation mutants were purified, kinase assays were run for the 2-protein and 4-protein modules to determine the activity of the mutated CDK8 proteins. The pol II CTD was used in all of the kinase assays, as it was previously proven that CDK8 phosphorylates the CTD in-vitro (Knuesel et al., 2009). All of the kinase samples used were added in relative amounts to the wild-type modules so that direct comparisons could be made between each of the three protein complexes.

The first kinase assay with the two-protein complexes of CDK8:CCNC (Figure 24) showed kinase activity in the wild-type CDK8 module and comparable activity with the CDK8(1-403) truncation mutant but no activity was seen with the CDK8(1-357) truncation mutant, even at high concentrations. The second kinase assay with the four-protein complexes with CDK8:CCNC:MED12:MED13 (Figure 25) shows similar results to the two-protein

complex kinase assay. There is activity in the wild-type module, 2-4x reduced activity in the CDK8(1-403) truncation mutant, and greatly reduced activity in the CDK8(1-357) module. Each kinase assay was replicated at least twice to ensure the validity of the results.

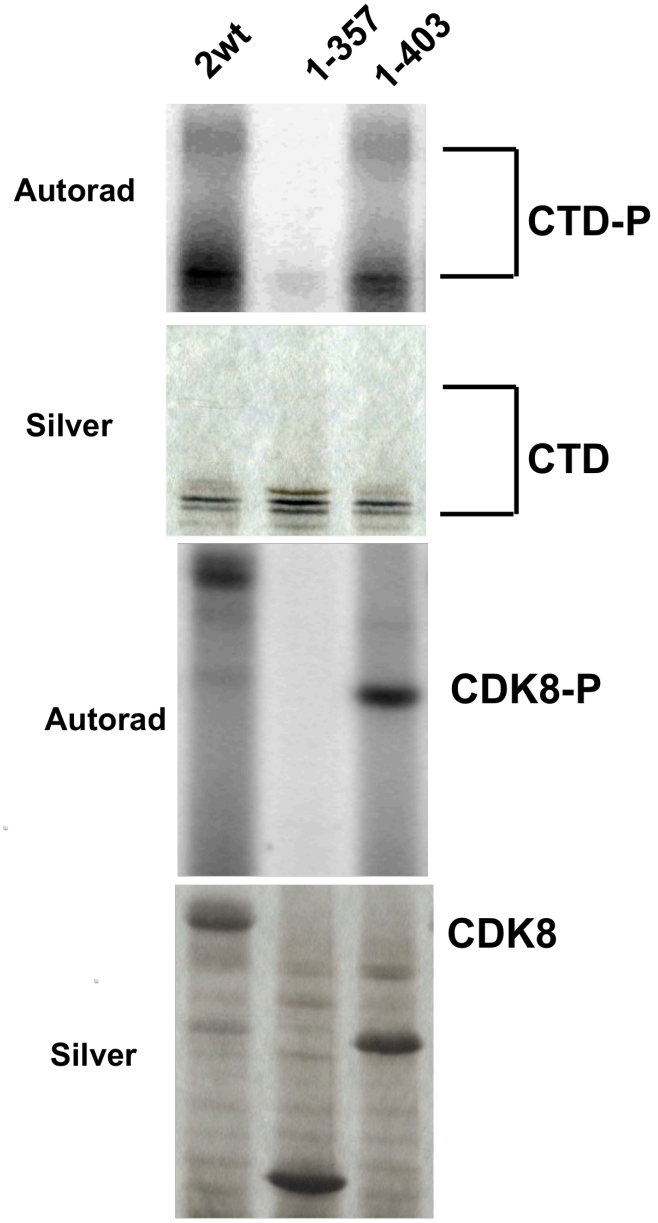


Figure 24. Kinase assays from CDK8:CCNC Truncation Mutants. This diagram shows the silver and autorad signals from the kinase assays. The silver shows the amount of protein in the sample and the autorad signals indicate the kinase activity of CDK8 as it auto-phosphorylates and phosphorylates the CTD.

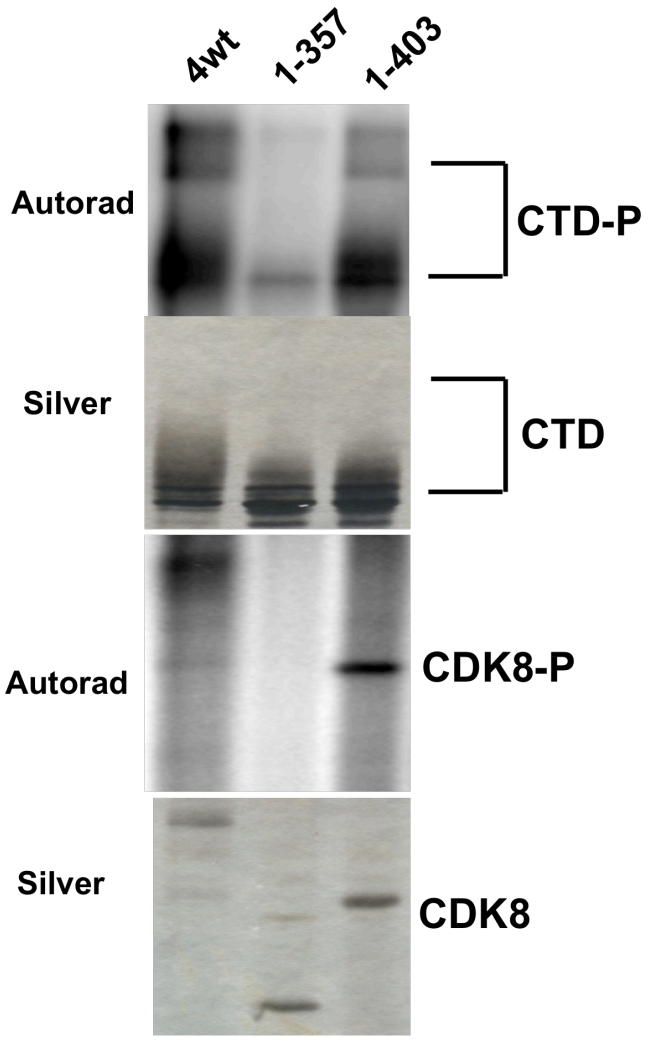


Figure 25. Kinase assays from CDK8 Module Truncation Mutants. This diagram shows the silver and autorad signals from the kinase assays done with CDK8:CCNC:MED12:MED13 normal module and with truncated CDK8. Again the silver shows the amount of protein in the samples and the autorad signals are indicative of the activity of CDK8 as it auto-phosphorylates and phosphorylates the CTD.

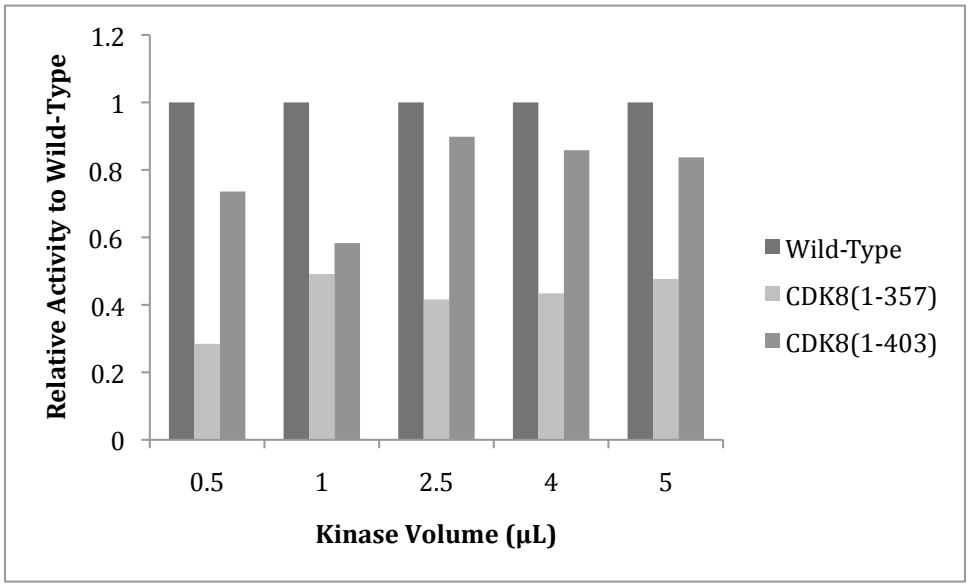


Figure 26. Relative Activity of Truncation Mutants in CDK8 Module with pol II CTD. For this quantification (n=1).

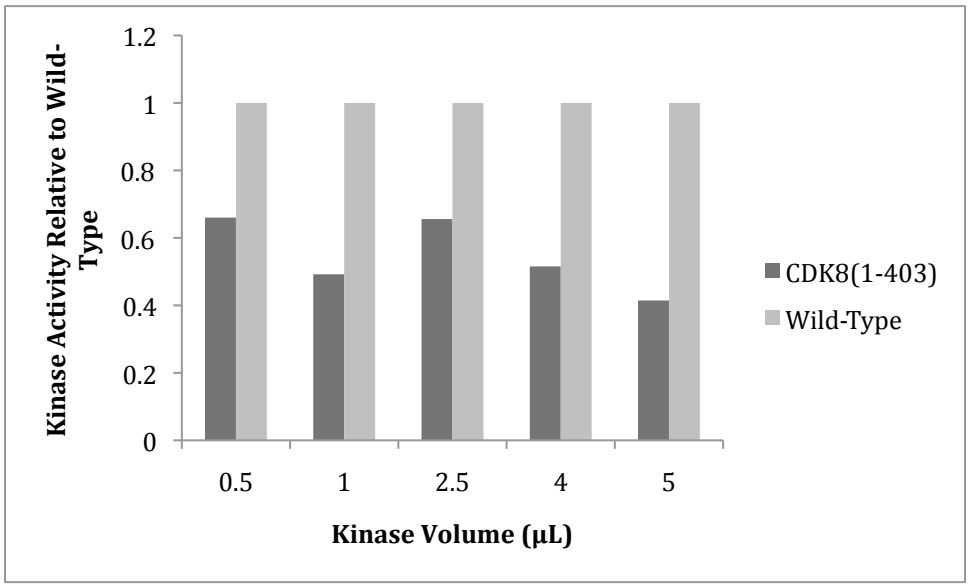


Figure 27. Relative Activity of Truncation Mutants in CDK8 Module with CDK8. The average activity of the CDK8(1-403) truncation mutant compared to the wild-type module based on kinase activity (CDK8) for the four-protein modules. CDK8(1-357) was omitted due to the lack of a visible band of activity to quantify (n=1).

C. Discussion

Regulatory role of CDK8 C-terminal domain (CTD) in CDK8-CCNC

From both of the kinase assays that were done with the CDK8 truncation mutants, differences can be seen in activity in comparison to the wild-type complexes. Since CDK8 is a kinase that phosphorylates other proteins, the auto-rad signal is indicative of how active the kinase is, with a stronger signal corresponding to higher levels of activity. Based on SYPRO Ruby gels, where the amount of protein was quantified from both the CDK8 and CCNC bands, equal amounts of protein were loaded as close as possible to be able to make inferences about the amount of activity in relation to the amount of kinase.

In the 2-protein module, it can be seen that the wild-type CDK8:CCNC auto-phosphorylates and also phosphorylates the CTD (Figure 24). The CDK8(1-403) truncation mutant shows comparable amounts of protein in relation to the CDK8 and pol II CTD, but has slightly less activity from its less intense band on the auto-rad, looking both at the CDK8-P and CTD-P bands (with the P standing for phosphorylated bands). Then looking at the CDK8(1-357) truncation mutant, there is much less activity in comparison to CDK8(1-403) and the wild-type CDK8-CCNC complex when looking at the pol II CTD-P bands and practically no activity in the CDK8-P region. This finding is quite interesting as it seems that the CDK8(1-357) truncation mutant is no longer able to auto-phosphorylate and phosphorylates the pol II CTD to a much lesser extent. This loss of auto-phosphorylation could be linked to either the last remaining auto-phosphorylatable site being between residue 357 and 403 or it could be linked to the possible differences in the truncation mutant's ability to bind MED12. Thus, the next step was to look at the truncation mutants in the full protein module.

Regulatory role of CDK8 C-terminal domain (CTD) in CDK8 Module

In the 4-protein CDK8 module, there should be increases in activity with the CDK8(1-357 truncation mutant, since it is known that MED12 is required for kinase activity (Knuesel et al., 2009). Looking at the silver stained gel, there are comparable amounts of kinase (CDK8) and of the pol II CTD in each of the samples, allowing for comparisons to be made between the different complexes (Figure 25). The kinase assay shows this in the wild-type CDK8 module, with dramatic increases in the pol II CTD-P signal and CDK-P signal, all coming from the addition of MED12 to the complex. In comparison, the CDK8(1-403) truncation mutant in the context of the 4-subunit CDK8 module shows decreased activity in the pol II CTD-P bands and in the CDK8-P bands, and it was estimated to be 2-4x less active (Figure 26, Figure 27). Then looking at the CDK8(1-357) complex, there is much less activity in the pol II CTD-P bands and practically no activity in the CDK8-P region. Again, there is a loss of auto-phosphorylation between the two CDK8 truncation mutants and introducing MED12 back into the CDK8 module did not increase kinase activity. Yet it was important to verify that all four proteins were present.

A western was then run to verify how well the wild-type and truncation mutants were binding MED12 and MED13 (Figure 23). From the western, it was found that the wild-type and CDK8(1-403) complexes were able to bind MED12 and MED13 in comparable amounts but that the CDK8(1-357) complex was apparently unable to bind either MED12 or MED13. This can help to explain the lack of activity in the kinase assay of the four-protein modules as MED12 was not present in the CDK8(1-357) samples and MED12 activates CDK8 kinase activity (Knuesel et al., 2008).

Significance of Data

From the kinase data and western blots, it appears that the CDK8 C-terminus is not acting as a traditional kinase activation loop. The original hypothesis was that increased levels of activity would be seen in the truncation mutants as the CDK8 C-terminus would no longer be able to block the active site of the kinase. Yet, from the kinase assays, decreased levels of CDK8 kinase activity are seen rather than increased levels, indicating that the CDK8 C-terminus is not acting as a traditional activation T-loop. Still, other possibilities could be that chopping off over 100 residues in the CDK8(1-357) truncation mutants could be causing a slight disorder in the structure of the protein, particularly near the C-terminus end, that could be affecting activity. As well, it could be that there is a residue located between residue 357 and 403 that needs to be phosphorylated that could be causing the C-terminus to be acting as a non-traditional activation loop, since there is a loss of auto-phosphorylation between CDK8(1-403) and CDK8(1-357) (Figure 22). Yet, of more immediate interest is the fact that CDK8(1-357) is unable to bind MED12 and MED13.

More directly comparing the activity of the CDK8(1-357) truncation mutant in the CDK8-CCNC complex to the 4-protein CDK8 module, the activity levels are fairly similar when looking at the pol II CTD-P bands (Figure 26). This would be expected since CDK8(1-357) is unable to bind MED12 and indicates that the CDK8(1-357) protein is structured enough to phosphorylate the pol II CTD to some extent. Thus, this suggests that the CDK8 C-terminus is instead playing a structural role and is binding MED12 and/or MED13 to the CDK8 module. This is an extremely interesting finding as it was not clear that the CDK8 C-terminus was playing any structural function in the module, due to its inability to be crystallized with CCNC

(Schneider et al., 2009) and the lack of cross-links found with MED12 in previous experiments. Yet, at this point, the cross-linking data are inconclusive due to a lack of sample.

From this information, I propose two possible models for how the C-terminus of CDK8 may be influencing MED12 binding. One model is that the CDK8 C-terminus is binding, intramolecularly, with the rest of CDK8 and/or CCNC, causing a structural shift in the dimer that would allow MED12 and MED13 to bind to CDK8-CCNC (Figure 28). Therefore, when the CDK8 C-terminus is truncated, that structural shift does not occur and so MED12 and MED13 can no longer bind the CDK8-CCNC dimer. The second model is that the CDK8 C-terminus is directly binding MED12 and/or MED13 (Figure 29), so when the CDK8 C-terminus is removed, MED12 and/or MED13 can no longer interact with the CDK8-CCNC dimer. These two models could be how the C-terminus of CDK8 is influencing MED12 binding, but there are still other possibilities beyond these two mechanisms.

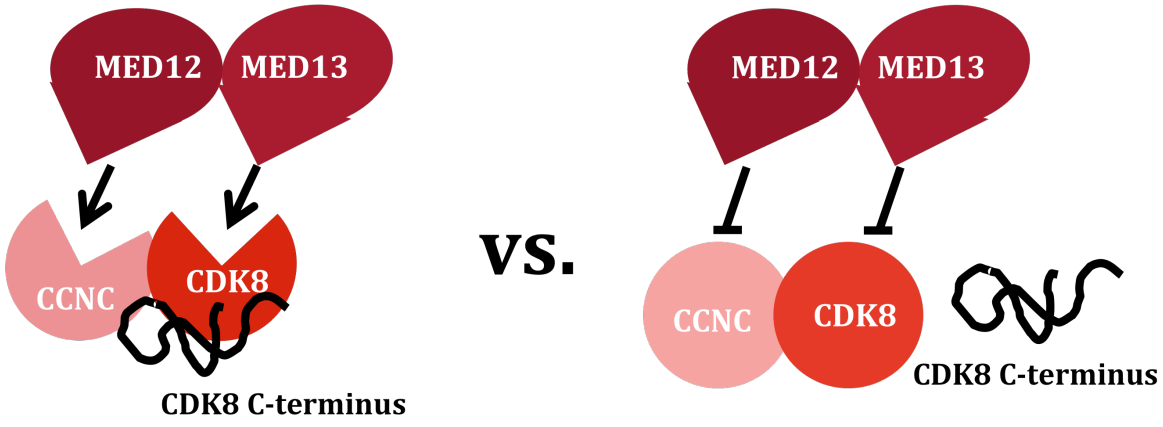


Figure 28. One possibility of the CDK8 C-terminus influencing MED12 binding. This model shows how the CDK8 C-terminus could be interacting with CDK8 and/or CCNC, causing a structural shift that would allow MED12/MED13 to bind. When the C-terminus is removed, MED12/MED13 can no longer interact with CDK8 and CCNC.

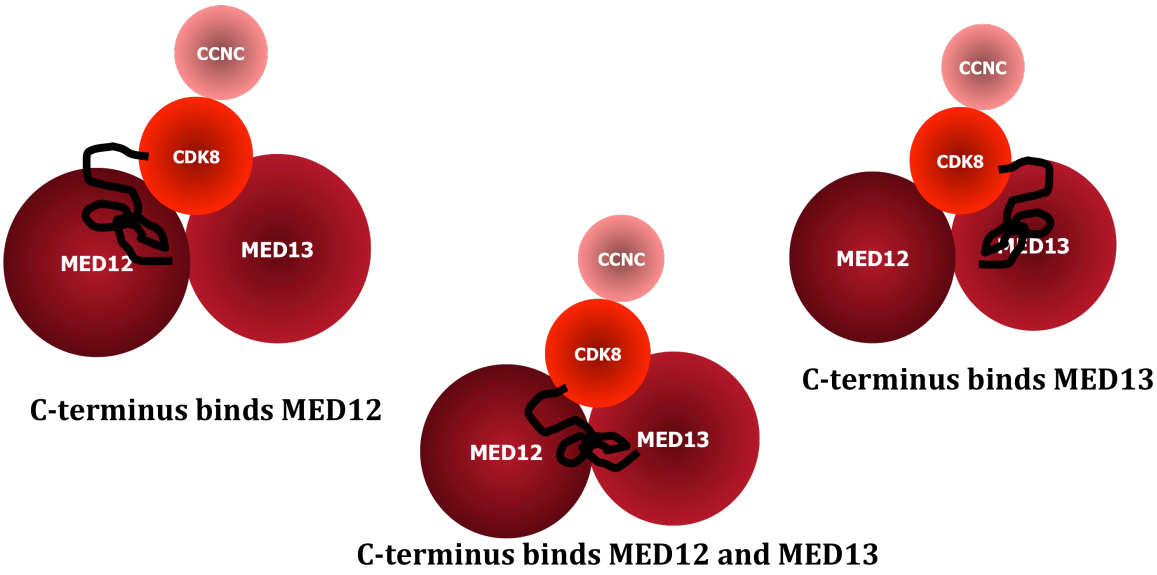


Figure 29. Second possibility for the CDK8 C-terminus influencing MED12 binding. This model shows how the CDK8 C-terminus could be directly binding MED12, MED13, or both proteins. So when the CDK8 C-terminus is removed, MED12 and/or MED13 can no longer interact with MED12 and MED13.

Other possibilities could be that the CDK8 C-terminus is binding MED13, which then causes some shift allowing MED12 to then bind to the module. This possibility is further indicated by CXMS data from Ben Allen (graduate student in the Taatjes lab), where two "high-confidence" cross-links were found between MED13 and CDK8. Another possibility could be that the CDK8 C-terminus undergoes from being unstructured to structured as it binds MED12, not allowing it to be crystallized in the 2-protein module. Ultimately, there are multiple possibilities to how the CDK8 C-terminus is interacting with MED12 in activating the kinase, but this is a promising finding in determining the function of the C-terminus of CDK8.

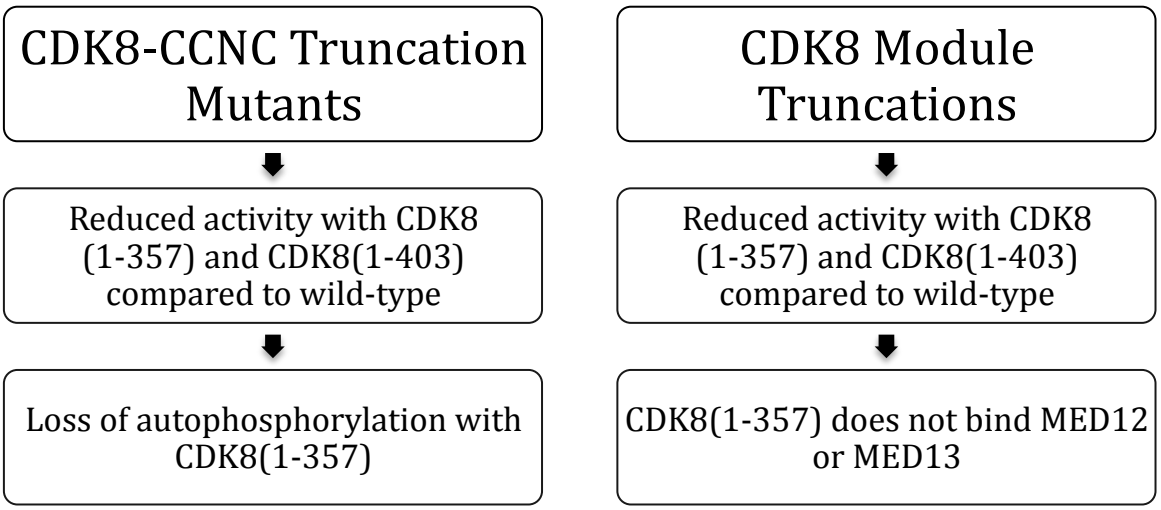


Figure 30. A summary of results with the CDK8 truncations in relation to the kinase activity of CDK8.

Future Directions

In order to get a better idea of how the C-terminus may be vital for binding MED12 and/or MED13 to the CDK8 module, there are a variety of experiments that could be done. One of the first experiments that could be done would be to run a western blot of whole cell extract (WCE) from insect cells producing the truncation mutant CDK8(1-357), to ensure that MED12 and MED13 are present so that we know that the lack of MED12 and MED13 in the purified protein samples is related to loss of CDK8's C-terminus. Another future experiment would be to express GST tagged CDK8 residues 357 to 464, which represents the entire C-terminus of CDK8. By binding the CDK8 C-terminus to glutathione beads and pouring whole cell extract from insect cells that express the entire CDK8 module (MED12, MED13, CDK8, CCNC) and looking at what the CDK8 C-terminus pulls down could produce interesting data. If, for example, the CDK8 C-terminus (residues 357-464) were able to pull down MED12 and/or MED13, this would be a strong indication that the CDK8 C-terminus is binding either one or both. Very similarly, one other possibility would be to express CDK8:CCNC and then

MED12:MED13 separately in insect cells and then combine the whole cell extracts to see if the two protein dimers are able to bind one another to form complete CDK8 modules. The same could also be done for the CDK8 truncation mutants in the 2-protein module to see if either CDK8(1-403) or CDK8(1-357) could bind MED12:MED13, which again could prove that the C-terminus is required for binding MED12 and/or MED13.

Additionally, the CDK8 truncation mutants could be sent to Dr. Ranish for CXMS and looking at how truncating the C-terminus may affect cross-links in the dimer, trimer, and full CDK8 module. If the CDK8 C-terminus is required for binding any of the other proteins in the module, changes in the cross-links, as compared to the wild-type modules that have been or will be cross-linked, could indicate a structural role for CDK8's C-terminus

Another interesting experiment could be to switch the C-terminus sections of CDK8 with CDK19. CDK8 and CDK19 are paralogs and form their own subfamily of CDKs, which is informative of their closely related nature (Manning, 2014). As well, they share a very similar primary sequence (92%) apart from their C-terminal regions (Figure 31) (Galbraith et al., 2013), which could alter how each CDK interacts with other protein complexes (Sato et al., 2004). As well, CDK19 has been shown to be able to form modules, similarly to CDK8 (Daniels et al., 2013). Therefore, an interesting experiment would be to switch the C-terminus of CDK8 with the C-terminus of CDK19 and do purifications of the 4-protein module. This may help to determine whether it is the sequence of the C-terminus that is required for interaction with MED12 or if it is somehow a particular interaction within the full protein module with CDK8 and MED13 that is allowing the CDK8 C-terminus to bind MED12. As well, the CDK8 C-terminus could be binding MED13, which could be causing additional conformational changes to allow the binding of MED12. Overall, there are multiple experiments that could be performed in the future to try and

get a better picture of how the CDK8 C-terminus may be activating the kinase activity of CDK8 by interacting with MED12.

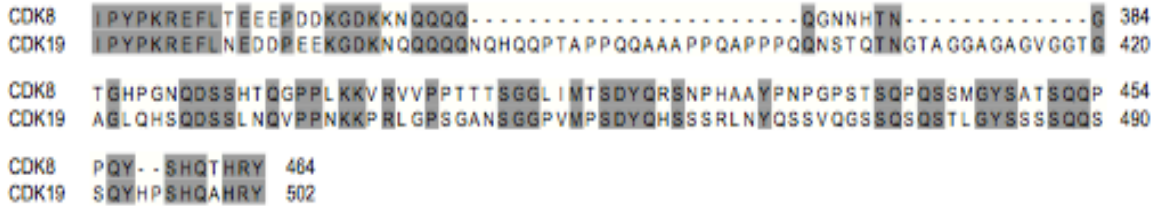


Figure 31. **The C-terminus alignments of CDK8 and CDK19.** Shows how the sequences differ in the C-terminal regions of both CDKs (Galbraith et al., 2013).

Chapter IV. Experimental Methods and Procedures

Expression of CDK8:CyclinC and CDK8:CyclinC:MED12 in insect cells

The expression has already been done by a specialized lab, the University of Colorado Protein Production, Monoclonal Antibody, Tissue Culture Core in Denver, from which the insect cells are ordered after being created in the Taatjes Lab. The insect cell pellets were then lysed to separate the soluble and insoluble proteins. A pestle is used to crush the cells open with pressure and then the solution is spun down at 55,000 RPM for 45 minutes to pellet the cells. The solution is then collected in 2mL aliquots.

Purification of the CDK8 subcomplexes and truncation mutants

33 µL of Glu-Glu peptide is added to 125 µL of glutathione fast-flow beads and 33 for every 8 mL of whole cell extract (WCEG). The peptide binds overnight. The WCEG is added to the beads and binds for 4 hours at 4°C. The beads are spun down, the solution is removed, and then the beads are washed five times with 0.5M HEGN and three times with 0.15M HEGN. Glu peptide is then added in the same amount as the dry bead volume (100 µg for 8 mL) to compete with the protein for binding, forcing the CDK8 subcomplex to elute off the column. Two elutions are done for 45 minutes at 4°C and then a third elution at half of the bead volume is done for 30 minutes at 4°C. The elutions are run through a spin filter at 4°C at 12,000 RPM to remove any beads. Samples are stored at -80°C.

Western Blots

A 9% polyacrylamide gel is run to completion and then transferred onto a nitrocellulose gel This is done with a Western blot. A gel is run and then transferred onto a nitrocellulose gel in

transfer buffer (10% 10x running buffer, 10% MeOH, 80% MiliQ H_2O) at 100 V and .25 Amps. The gel is cut above 75 kDa to probe for MED12 , between 75kDa and 50kDa for CDK8 and below 50 kDa for cyclin C (CDK8=55 kDa, Cyclin C=33 kDa, MED12=243 kDa). The gel is washed in 5% milk in TBS-T to block the gel to decrease background signals. Primary antibodies are added to each gel section (CDK8 and cyclin C are added in 1:500 dilution = 10 µL, MED12 is 1:1000 = 5µL). The antibody is left overnight in 5 µL of 5% milk at 4°C. The gel is washed in TBS-T and then the secondary antibody is added (CDK8=5µL of anti-goat, cyclin C, MED12=2.5µL of anti-rabbit) for 1 hour at room temperature in 5% milk. The gel is then developed with a chemiluminescent agent to create phosphorescence of he proteins and then using photo paper, an image of the gel is developed.

SYPRO Ruby Staining for Quantitative Determination of Protein Amount

A 9% polyacrylamide gel is run and the gel is first fixed with a solution containing 50% methanol and 7% acetic acid for 30 minutes. Then the gel is stained with 60 mL of SYPRO Ruby gel stain and is shaken overnight at room temperature and must be covered in foil to prevent light exposure. The gel is then washed with a solution containing 10% methanol and 7% acetic acid and is washed in 100 mL for 30 minutes. An image is then taken using a Typhoon scanner and ImageJ software is used to calculate the amount of each protein (CDK8, cyclin C, MED12).

Concentration of Protein Samples

For the CDK8:CyclinC:MED12 complex, a glycerol gradient is run with 30%, 20%, and 15% glycerol layers to achieve greater 1:1:1 concentrations of each subunit. The gradient is poured into a 2mL centrifuge tube and then a small sample of the three-protein complex is

poured on top (200µL). The gradient is spun for 6 hours at 4°C at 55,000 RPM and 50-100µL fractions are collected.

The elution samples are run through a size cut-off filter (30K) and the sample is concentrated 5X per spin at 14,000 RPM at 4°C for between 2-4 minutes. The buffer is also exchanged with a modified 0.15M HEGN to remove any possible contaminants for the cross-linking. The sample is concentrated 50-100x and then stored at -80°C until they are shipped to the Ranish Lab.

Chemical cross-linking and mass spectrometry

For detailed methods, please refer to Appendix 1 and (Luo, Fishburn, Hahn & Ranish, 2012).

Single Direct Mutation with Stop Codon (TAA)

The primary sequence for the CDK8 protein has been previously determined in the lab. Two mutants were created, referred to as 1-357 K8 and 1-403 K8. The wild-type CDK8 protein is typically 464 amino acid residues long. The C-terminus was truncated at residue 357 since CDK8 in yeast is not as long and lacks the extended C-terminus. The truncation at 403 was determined as it is where the density from the crystal structure ends, suggesting the start of the c-terminus arm, and this mutant was also shown to bind cyclin C.

For each mutant, a primer was created to introduce both a stop codon at either 357 or 403, along with a restriction enzyme site. The sequences are listed below, with the stop codon in italics and the restriction enzyme in bold.

The 357sense sequence (Restriction Enzyme SspI):

GTCAAATCCCTTACCCAAAACGAGAAT**AATATT***TAA*CGGAAGAAGAACCTGATGAC
AAAGG

The 403sense sequence (restriction enzyme PmlI):

CACACACAGGGACCCCCGTTGAAGAAA*TAA***CACGTG**AGAGTTGTTCCTCCTACCACT
AC

The primers are used to perform a polymerase chain reaction (PCR) to amplify the sequence of DNA from the CDK8 sequence we are trying to insert into the DNA vector. The full-length DNA is then digested with the restriction enzymes (EcoRI-HF and XbaI) to cut out the full-length CDK8 sequence. The digestion is run on an agarose gel to separate the bands, along with the PCR sequences of the truncated DNA. The bands are cut out of the gel and the DNA is purified using kits from Omega Bio-Tek using the manufacturer's protocol for gel purification. The purified DNA plasmid is then added onto XL-10 *E.coli* cells to transform the bacteria. The *E.coli* are grown on plates with ampicillin overnight. A single cell culture is then collected, grown into a 3mL culture, then grown in a 1L culture, and then spun down.

The cells are lysed and the DNA collected using the manufacturer's protocol from the Omega Bio-Tex mini-prep kit. The DNA is digested with the added restriction enzymes (SspI and PmlI then run on an agarose gel to determine the accuracy of the DNA plasmid. The DNA is then sent to GeneWiz for sequencing and if accurate, the DNA is then transformed into bacteria once more and then the DNA plasmid is purified using the manufacturer's protocol from the Omega Bio-Tek protocol from the maxi-prep kit. The plasmid is then sent to University of Colorado Protein Production, Monoclonal Antibody, and Tissue Culture Core in Denver to be viralized into insect cells.

Kinase Assays of CDK8 Mutants in CDK8-CCNC dimers and CDK8 Module

Based on the concentrations determined from a SYPRO Ruby gel, varying amounts of the mutants will be loaded onto a large gel. The reaction buffer consists of 25mM Tris at pH 8.0, 100mM KCl, 2mM DTT, 100μM ATP (cold), 10mM $MgCl_2$, and 2.5μCi of ATP (hot). CTD (C-terminal domain) is added, along with 100mM KCl, and the kinase component (CDK8 mutants). The reaction is incubated at 30°C for 1 hour and then run on a 9% polyacrylamide gel.

A silver staining technique is used to stain the gel. Shake the gel in 50% methanol for 10 minutes, 5% methanol for 10 minutes, 3.5μM DTT for 5 minutes, followed by 10 minutes in silver nitrate solution (250mL water, 250mg silver nitrate, 25μL formaldehyde). The gel is washed a few times, for no more than 10 seconds, in water. Rinse the gel briefly with sodium carbonate solution (500mL of cold water, 15g sodium carbonate, 250μL formaldehyde) and then pour the whole solution onto the gel. Once the gel is thoroughly developed, quench with citric acid monohydrate and then rinse the gel in a large volume of water

Then a storage PhosporScreen (GE Healthcare) is used, along with a Typhoon Scanner, to view the activity of the radioactively labeled subunits.

Chapter V. Works Cited

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