UNIVERSITY OF CALIFORNIA, SAN DIEGO

Abl-dependent tyrosine phosphorylation of the RNAPII-CTD increases binding affinity with the active miRNA Microprocessor complex

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by

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The thesis of Aaron Tsai is approved, and it is acceptable

in quality and form for publication on microfilm and electronically:

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I dedicate this thesis to my mother and father,

for their endless love and support.

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Acknowledgments

I would first like to thank Dr. Wang for providing me with this precious opportunity to participate in her lab. During the time in this lab, I have been trained with not only the basic skills required for basic research, but also skills needed to ask scientific questions, design experiments, and interpret results. Dr. Wang has always provided me with valuable guidance throughout this project, and has continued in guiding me to become a better scientist.

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ABSTRACT OF THE THESIS

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Professor Jean Y. J. Wang, Chair

During RNA transcription, the Carboxyl-terminal domain (CTD) of RNA Polymerase II (RNAPII) is phosphorylated on its serine-5 (Ser-5) positions to trigger elongation and RNA 5'-capping. In addition, CTD is also phosphorylated on the serine-2 (Ser-2) positions, which stimulates RNA splicing and polyadenylation. In the case of DNA damage, Abl kinase is activated to induce Tyrosine-1 (Tyr-1) phosphorylation on

CTD. However, the exact role and function of the Tyr-1 phosphorylation during DNA damage has yet to be understood. Here we discovered interactions between CTD peptides and various Drosha-associated proteins (DAPs) by a mass spectrometry pulldown experiment. By applying a number of in vitro binding assays, we identified that CTD interacts with DiGeorge syndrome critical region 8 (DGCR8), one of the key and necessary proteins in the Microprocessor complex responsible for miRNA biogenesis. We also showed that the CTD-DGCR8 interaction can be enhanced in the presence of active Abl kinase. Our results demonstrated a possible outcome followed by the CTD Tyr-1 phosphorylation during DNA damage, which is to recruit the active Microprocessor complex and initiate miRNA biogenesis.

I:

Introduction

DNA Damage-Induced Cell Death

Genomic DNA is constantly under threats of DNA damaging by environmental and physiological agents, such as UV exposure from the sun. To ensure the integrity of genomic DNA, an elaborated system of DNA repair pathways within the damage tolerance is available via DNA replication machinery [1]. Upon DNA damage, an immediate responded transduction network becomes activated to stimulate DNA repair, and to activate cell cycle checkpoints. The series of immediate responses attempt to self-preserve the genome and to ultimately achieve the survival of the damaged cell [2]. An alternative delay response towards persistent DNA damage is to induce senescence or cell death through apoptosis [2]. The delay response aims to reduce the harmful effects, and to protect the organism by minimizing potential damage.

Exposure to Genotoxin, such as some anti-cancer agents used in cancer therapy, can cause DNA modification and create critical DNA lesions. These DNA lesions can obstruct normal functions of the DNA, such as transcription and DNA replication [3]. Previous studies have shown that DNA lesions can trigger apoptosis as a self-protecting mechanism [2, 3].

Abl Kinase

In this lab we have discovered that nuclear Abl tyrosine kinase can be activated by genotoxins, such as cisplatin, to induce apoptosis [3]. Abl has been previously identified as a proto-oncogene that contains oncogenic potential [4]. On the contrary, studies from this lab have also shown that Abl induces apoptosis via a p53 and its related p73

transcription factor dependent pathway, and therefore it acts as a pro-apoptotic, or anti-oncogenic factor [5]. In addition to the p53 and p73 dependent apoptotic pathway, Abl has also been shown to induce tyrosine phosphorylation on RNA polymerase II (RNAPII) at its Carboxyl-terminal domain (CTD) [6-8]. By utilizing its SH2 kinase domain and the CTD-interacting domain (CID), Abl is capable of phosphorylating RNAPII at its CTD on the tyrosine residues, which located on the first position of the CTD heptad repeats. Moreover, mutant Abl lacking CID is able to auto-phosphorylate itself, but unable to phosphorylate on the CTD [6-8].

C-Terminal Repeated Domain (CTD)

CTD is consisted of heptad amino acid residues with the consensus sequence repeat of Tyr-Ser-Pro-Thr-Ser-Pro-Ser for 26 times in the yeast RNA Pol II and for 52 times in the mammalian RNA Pol II. Among the seven amino acid residues, Tyr-1, Pro-3, Ser-5, and Pro-6 are conserved among all repeats [9]. While CTD is not required for mRNA synthesis, it is critical for cell survival, because it interacts with essential proteins which process mRNA during transcription [10]. CTD is served as a platform to recruit various enzymes and factors that are responsible for the transcriptional processing of nascent RNA, including capping, splicing, and polyadenylation of RNA transcripts [11]. The CTD platform can thus easily change shape and orientation based upon its phosphorylated conditions on Tyr-1, Ser-2, and Ser-5 positions mediated by different protein kinases, including the previously mentioned Abl kinase on Tyr-1 [12]. In general, RNA polymerase II with an unphosphorylated CTD is found in the transcription initiating

complex at the promoters; whereas the CTD-phosphorylated RNA polymerase II is found in the transcription elongation complex located at the actively transcribing region of a gene [13]. To begin transcription, the protein kinase in TFIIH phosphorylates CTD at its Ser-5 position to stimulate transcription elongation and RNA 5‘capping. Then, as CTD gets phosphorylated at the Ser-2 position, the stimulation of RNA splicing and polyadenylation are both in motion. Finally, CTD is de-phosphorylated and recycled back to the promoter after the dissociation of nascent RNA [13]. As mentioned earlier, tyrosine phosphorylation of CTD by the active Abl kinase can be induced during DNA damage. However, the exact purpose and mechanism behind the role of tyrosine phosphorylation of CTD and its regulation are still largely unknown.

Biogenesis of miRNA

MicroRNAs (miRNAs) are small single-stranded RNAs that inhibit translation by inducing degradation of messenger RNAs (mRNAs) via mostly the binding to mRNA’s 3’ -untranslated region (3‘UTR) [14]. The biogenesis of miRNA begins by RNA transcription catalyzed by RNA Polymerase II. The crude transcript, known as primary-miRNA (pri-miRNA), is generally a few kilo-bases in length, and contains unique stem-loop structures that can be recognized and bound by active miRNA Microprocessor complex [15]. Once bound, Drosha, a type III RNase, acts as the effector of Microprocessor complex, and cleaves the pri-miRNA strand, thereby releasing a small hairpin shaped RNA known as the pre-miRNA [16]. From here the pre-miRNA is transported out to cytoplasm via the interaction with exportin-5. Once it arrives in

cytoplasm, the pre-miRNA can be further processed by Dicer, which is also a RNase, to generate a ~22 nucleotides long RNA duplex [14]. Finally, one of the two 22 nucleotides strands is loaded onto the RNA induced silencing complex (RISC) as the mature effector miRNA, while the other strand gets degraded [17].

miRNA Microprocessor Complexes

It has been shown that the Microprocessor complex exists in two forms. The larger Microprocessor complex is composed of multiple classes of RNA-associated proteins, including various of RNA binding proteins, heterogenous nuclear ribonucleoproteins, and Ewing sarcoma family proteins. Although the larger Microprocessor complex is consisted of Drosha, yet it appears to be catalytically inactive. [15]. On the other hand, the active Microprocessor complex is consisted of only Drosha and its critical co-factor protein named DiGeorge Critical Region 8 (DGCR8).

DGCR8, also known as Pasha in other literatures, was first identified as a Drosha-interacting protein through immunoprecipitation (IP) from human cells. Experiments have shown that when DGCR8 is absent from miRNA processing, pri-miRNA level accumulates, whereas pre-miRNA and mature miRNA levels decline [18]. Such results suggest that DGCR8 plays an essential role in the processing of pri-miRNA, regardless of its inability to directly cleave RNAs. The human DGCR8 is a ~85 kilo-Dalton protein containing 773 amino acids. It consists of two double-stranded RNA Binding Domain (dsRBD), as well as a small region at the C-terminus end that interacts directly with Drosha. Experiments done by employing various DGCR8 mutant constructs have shown

that the region of its first 275 amino acid residues is responsible for nuclear localization. In addition, DGCR8 also contains of a WW domain near the center region [19]. Unlike the other identified domains within DGCR8, the role which the WW domain acts on DGCR8 is much less understood. A recent publication has reported that the WW domain of DGCR8 can act as a platform for extensive dimerization interaction among two DGCR8 proteins [20].

II:

Results

Discovery of interactions between Drosha-associated proteins (DAP) and pY1pS5-CTD peptides

To investigate the roles played by different phosphorylated states of CTD in RNA transcription, Dr. Gabriel Pineda, a former post-doctoral investigator from our lab, collaborated with a proteomics lab at UCSD in 2008. He conducted a pull-down experiment to identify CTD-associated proteins using MUDPIT mass spectrometry system. By applying biotinylated-phospho-CTD peptides of different phosphorylated states to the nuclear extracts of HeLa cells, a number of proteins were shown to display differential bindings to differentially phosphorylated CTD-peptides (table. 1). An especially intriguing result from this mass spectrometry assay reveals that the pY1pS5-phosphorylated CTD peptide specifically pulled-down a group of 13 proteins that have been previously found to associate with Drosha as part of the miRNA Microprocessor complex [15].

The result from Dr. Pineda's mass spectrometry pull-down experiment prompted further investigations in order to confirm and to understand more on how the CTD peptides interact with the miRNA Microprocessor complex. In other words, which of the Drosha-associated proteins (DAP) are responsible for the direct interaction with the CTD peptides under different phosphorylated conditions.

Interaction between CTD and Ewing sarcoma family proteins (EWS) with or without the presence of Abl Kinase is not clearly observed

We began this project by isolating the most likely candidates among DAPs, and to investigate their involvement in the interaction with differentially phosphorylated states of CTD peptides. The first DAP chosen and acquired for this investigation was the Ewing sarcoma family protein (EWS). EWS was among the DAPs pulled-down by pY1pS5-phosphorylated CTD peptide in Dr. Pineda's mass spectrometry experiment, and it is known to be part of the larger miRNA Microprocessor complex [15].

To confirm the interaction between CTD and EWS, a CTD tagged with pGLUE tag, which consists of a Strept-tag, a HA-tag, a TEV recombinant site, and a calmodulin binding site (pGLUE-CTD) [21], as well as an EWS fused with the GST-tag (gst-EWS) were amplified and prepared. In addition, an active Abl kinase (Abl-PP-NES) was also constructed to apply for the induction of tyrosine phosphorylation in selected samples in order to differentiate levels of interaction among the unphosphorylated- and the Tyr-1 phosphorylated CTD with EWS. We then carried out an immunoprecipitation (IP) assay using an EWS-specific antibody coupled to protein A/G beads on whole-cell extracts from 293T cells over-expressing pGLUE-nCTD and gst-EWS, with or without the presence of active Abl. (figure1a, lane 2 & 3). Samples without the over-expression of gst-EWS were also tested as negative controls (figure 1a, lane 1 & 4). Anti-EWS-beads bound fractions were then subjected to a Western Blot analysis by EWS-specfic antibody to confirm the presence of EWS. As shown in figure 1b, over-expressed gst-EWS appeared at 120kD on lane 2 and 3. Furthermore, endogenous EWS was also detected by

EWS specific antibody in all lanes at ~85kD. After the presence of EWS was confirmed, the duplicate pulldown fractions were subjected to another Western Blot analysis by HA-specific antibody in order to find the presence of pGLUE-nCTD. As shown in figure 1c, the presence of pGLUE-nCTD was not clearly observed as part of the EWS-specific antibody pulldown in all lanes, with or without the presence of active Abl.

An alternative method to confirm the pulldown results was by comparing the Western Blot analysis of the starting whole-cell lysate (input) to the first flow-through wash from the immunoprecipitated fraction immediately after incubation (FT). The decreasing level of the signals from the input to the respective FT could be considered as a estimate to the pulldown level. In this assay, the pulldown efficiency of EWS-specific antibody against both the over-expressed gst-EWS and the endogenous EWS could be determined as ~25 percent (figure 1d). More importantly, the FT fractions of pGLUE-nCTD showed no decrease when compared to the respective inputs, (figure 1e), which reflected similarly to the earlier observation of the pulldown fractions of pGLUE-nCTD.

Immunoprecipitation binding assay shows interaction between the CTD and DGCR8s; such binding is also positively influenced by active Abl kinase

Previous in vitro experiments have shown that the active Microprocessor complex interacts with pri-miRNAs through the dsRBDs of DGCR8. Moreover, DGCR8 binds to pri-miRNA hairpins before Drosha [18, 22]. Based on these observations, it is logical to suggest a connection between DGCR8 and CTD, which is responsible for RNA transcriptional processing as part of RNA Pol II. To test this hypothesis, we carried out an

immunoprecipitation (IP) assay using anti-Flag Affinity Gel on whole-cell extract from 293T cells over-expressing pGLUE-nCTD and Flag-tagged DGCR8 (Flag-DGCR8), with or without the presence of active Abl kinase (figure 2a lane 2 & 3). To further confirm the binding activity influenced by Abl, we incubated one of the cell samples in Imatinib (Gleevec) (figure 2a lane 4), which inhibits the kinase activity of Abl proteins [23]. To include a positive control, the Flag IP was also conducted on a whole-cell extract sample over-expressing pGLUE-nCTD and Flag-tagged CTD-Interacting Domain (Flag-CID), known to interact with CTD (figure 2a lane 5). To include a negative control, each of the five samples had a counterpart, which the pGLUE-nCTD was replaced by a empty pGLUE vector (figure 2a lane 6-10). Finally, all the anti-Flag gel bound fractions were subjected to a Western Blot analysis by HA-specific antibody to find the presence of pGLUE-nCTD.

As shown in figure 2b, intensive bands appeared from lane 2 through lane 5, and from lane 7 through lane 9. Those bands were later disregarded as they appeared in all samples, including the negative controls. However, pGLUE-nCTD pulldown was observed in lane 2, where DGCR8 and CTD were co-expressed. The intensity of pGLUE-nCTD bands increased when Abl was also co-expressed (figure 2b lane 3); then they decreased slightly when the sample was incubated in Imatinib (figure 2b lane 4). Furthermore, the results were confirmed by the positive control as the CTD pulldown by CID was also observed (figure 2b lane 5).

As a result to the potential contamination from Flag gel pulldown assay, a reverse pulldown strategy was developed by running an IP using Streptavidin beads to pulldown

pGLUE-nCTD. The Streptavidin beads bound fractions were then subjected to a Western Blot analysis by Flag-specific antibody to detect Flag-DGCR8 and Flag-CID. As shown in figure 2c, DGCR8 pulldown was observed when it was co-transfected with pGLUE-CTD; with an increase in band intensity in the presence of Abl (figure 2c lane 2 & 3). In this assay, however, the change in CTD-DGCR8 interaction level caused by Imatinib could not be clearly observed (figure 2c lane 4). Finally, the CID pulldown by CTD was again observed (figure 2c lane 5).

In vitro binding assay of differentially phosphorylated CTD-peptides shows interaction between DGCR8 and unphospho-CTD, pY1-CTD, and pY1-pS5-CTD

Previous IP results have indicated interaction between DGCR8 and CTD in which an even higher binding affinity could be observed in the presence of active Abl kinase. To further determine the effect demonstrated by Abl, an in vitro binding assay was carried out using differentially phosphorylated CTD-peptides, which were also Biotin tagged. To quantify the interactions, each of the peptides were titrated to 1 ng, 10 ng, 100 ng, 1 μg, and 10 μg. They were then incubated in the whole cell lysate over-expressed with Flag-DGCR8. After an hour of incubation, the peptides were pulled from the mixture using Streptavidin beads. Finally, the pulldown fractions were subjected to a Western Blot analysis by Flag-specific antibody to detect DGCR8 pulldown. As a trial run, Unphospho-CTD and pY1pS5-CTD peptides of each titrations were incubated in the whole cell lysate over-expressing Flag-DGCR8. As shown in figure 3a, the Flag-DGCR8 pulled-down by unphospho-CTD peptides saturated at 1 μg titration; whereas in the

pY1pS5-CTD peptides, DGCR8 pulldown saturated at 1ng titration. This result preliminarily suggested that the pY1pS5-CTD-DGCR8 binding has a higher affinity than the unphospho-CTD-DGCR8 binding. In addition, both samples showed no bands in the 10 μg titration, suggesting that such peptide concentration could be over-saturated.

In order to acquire a more complete picture of the interactions between DGCR8 and the CTD peptides, all six available peptides, including: unphospho-CTD, pY1-CTD, pY1pS5-CTD, pS2-CTD, pS5-CTD, and pY1pS2-CTD, were employed to carry out the same binding assay with Flag-DGCR8 in 1 ng, 10 ng, 100 ng, and 1 μg titrations. DGCR8 pulldown was observed in samples with unphospho-CTD, pY1-CTD, and pY1pS5-CTD in an intensifying fashion in accordance to the increase of peptide concentration (figure 3b). On the contrary, samples with pS2-CTD, pS5-CTD, and pY1-pS2-CTD showed no clear increase among different titrations, and the band intensities dispersed at a random fashion (data not shown). After plotting each results on a scatter chart, Unphospho-CTD pulldown of DGCR8 appeared to be a 2nd-order reaction curve; whereas pY1-CTD and pY1pS5-CTD pulldown of DGCR8 appeared to be linear curve (figure 3c).

Immunoprecipitation assay of Flag-tagged DGCR8 and YAP (both WW domain containing proteins) shows positive CTD pulldowns, which can be detected by CTD antibodies targeting different CTD biochemical properties

The in vitro binding assay of differentially phosphorylated CTD-peptides demonstrated promising results showing the interactions between DGCR8 and

unphospho-CTD, pY1-CTD, and pY1pS5-CTD peptides. However, to repeat this result was proven to be more difficult than originally expected due to the unstable nature of these synthetic peptides. Therefore an alternative assay was needed to further confirm the interactions between DGCR8 and differentially phosphorylated conditions of CTD. We decided to resort back to the immunoprecipitation binding assay of Flag-tagged proteins. In order to differentiate the phosphorylated conditions of each CTD pulled-down by different proteins, multiple CTD-specific antibodies, which target different phosphorylated conditions, were utilized to detect the pulldown fraction of Flag-tagged proteins on the whole cell lysates over-expressing Flag-DGCR8 and pGLUE-nCTD. Similar to the previous immunoprecipitation assays, Flag-CID was co-transfected with pGLUE-nCTD as a positive control. Additionally, a WW domain containing protein, Yes-associated protein (YAP), was also co-transfected with pGLUE-nCTD. Since DGCR8 also contains a WW domain, this additional WW domain-containing-protein was applied in this assay to help us explore the possibility of a WW domain directed interaction with the CTD (figure 4a).

To ensure the success of this immunoprecipitation assay, the blot showing whole-cell-lysate (input) of each Flag-tagged proteins and its corresponding flow-through are shown in figure 4b. Based on this blot, Flag-gel pulldown of both the Flag-YAP and the Flag-CID had pulldown efficiencies greater than 99%. In addition, the Flag-DGCR8 pulldown efficiency was over 50% (figure 4b).

After each Flag-tagged protein pulldown was confirmed, all eight pulldown samples were loaded identically into four gels, which were subsequently converted into

four separate Nitrocellulose blots. Each of the identical blots was probed separately in different CTD-specific antibodies available in our lab, including: Ser-2 CTD antibody (Ser2) that detects Ser-2 phosphorylations on CTD peptides; Ser-5 CTD antibody (Ser5) that detects Ser-5 phosphorylations on CTD peptides; phosphorylated-CTD antibody (anti-phospho); and unphosphorylated-CTD antibody (R-CTD). As shown in figure 4c, Flag pulldown of Flag-CID showed positive pulldown of CTD, which were detected by both the Ser2 and Ser5 antibodies. This pulldown also showed little change with or without the presence of active Abl kinase. Additionally, the CTD pulldown by Flag-CID was also detected vaguely by the anti-phospho antibody. Flag pulldown of Flag-DGCR8 showed positive pulldown of CTD by the detection of anti-phospho antibody. The band intensity also increased in the presence of active Abl kinase. It is also interesting to note that, as shown on the bottom part of lane six, a relatively stronger band representing a possible fragmented CTD peptide was observed in the presence of active Abl kianse. Lastly, the R-CTD antibody detected the presence of CTD in only the pulldown fractions of Flag-YAP. Additionally, the CTD pulldown by Flag-YAP remained unchanged with or without the presence of active Abl kinase (figure 4d).

Table 1. Drosha complex components pulled-down by pY1pS5 phosphorylated CTD peptide

Family	Name	DAP name	CTD	pY1-CTD	pS5-CTD	pY1pS5-CTD
DEAD/H- box						
	DDX1	DAP100	No	No	No	Yes
	DDX3X	DAP88	No	No	No	Yes
	DDX5	DAP70	No	No	No	Yes
	DHX15	DAP105	No	No	No	Yes
DSRM						
	ILF2	DAP125	No	No	No	Yes
	ILF3	DAP54	No	No	No	Yes
hnRNP						
	hnRNPD-L	DAP40a	No	No	No	Yes
	hnRNPH1	DAP55	No	No	No	Yes
	hnRNPM	DAP85	No	No	No	Yes
	hnRNPU	DAP130	No	No	No	Yes
RRM/ZnFRBZ						
	EWS	DAP90	No	No	No	Yes
	TAF15	DAP60	No	No	Yes	Yes
RRM						
	TDP-43	DAP52	No	No	No	Yes

HeLa nuclear extracts were reacted with each of the four different CTD peptides of four heptad repeats (28 aa), either unphosphorylated (CTD), phosphorylated at the four tyrosines (pY1-CTD), the four serine-5's (pS5-CTD) and doubly phosphorylated (pY1pS5-CTD).

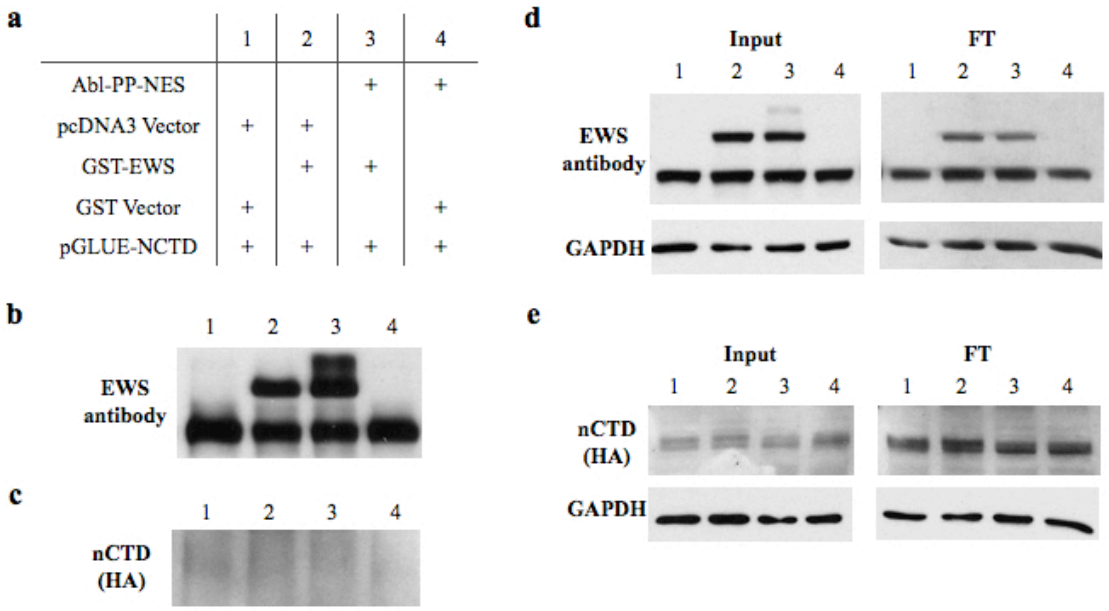

Figure 1. Ewing sarcoma family protein does not directly bind to CTD with or without the presence of active Abl kinase. (a) Transfection table for the EWS-CTD interacting assay. (b) Autoradiograph of blot probed with EWS-specific antibody. Top bands in lane 2 & 3 are overexpressed gst-EWS, and the bottom bands in all four lanes are endogenous EWS. (c) Autoradiograph of blot probed with HA-specific antibody. No clear HA-tagged CTD was observed. (d) Autoradiograph of blot probed with EWS-specific antibody on equal input cell lysate and flow-through wash. Bottom bands are GAPDH shown as loading control. (e) Autoradiograph of blot probed with HA-specific antibody on equal input cell lysate and flow-through wash. Similarly, bottom bands are GAPDH shown as loading control.

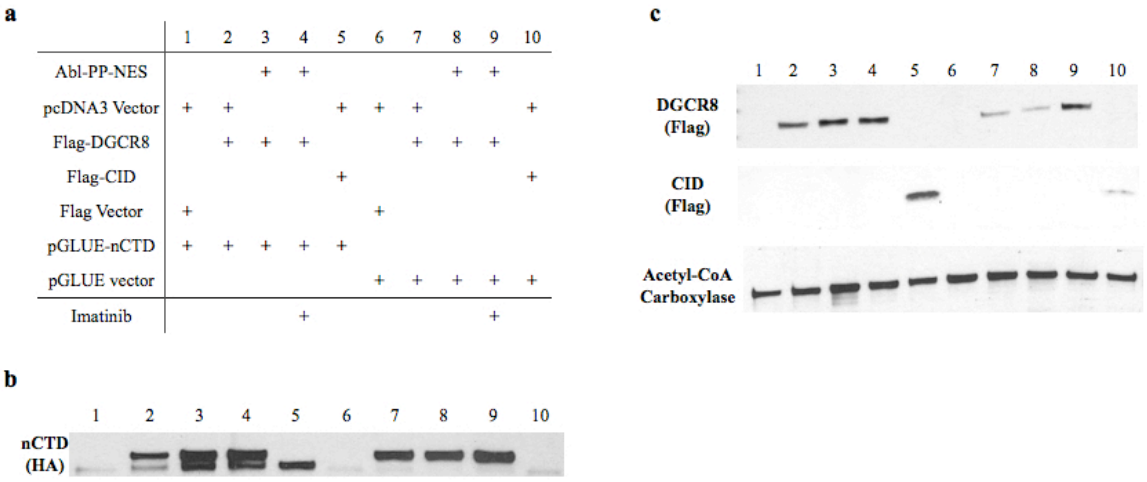

Figure 2. In vitro binding assay shows CTD-DGCR8 interation, which is positively influenced by Abl kinase. (a) Transfection table for the CTD-DGCR8 interacting assay. (b) Autoradiograph of blot probed with HA-specific antibody on Flag-gel pulldown fractions. (c) Autoradiograph of blot probed with Flag-specific antibody on Streptavidin bead pulldown fractions. Autoradiograph of blot probed by Acetyl-CoA Carboxylase-specific antibody is shown as IP loading control.

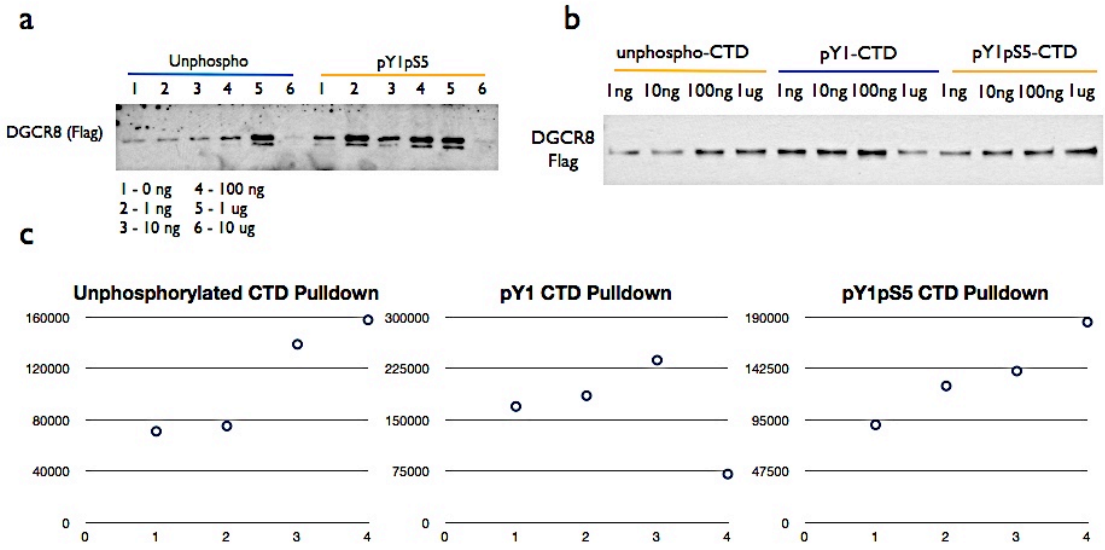

Figure 3. Immunoprecipitation assay shows interaction between DGCR8 and differentially phosphorylated CTD peptides, including unphospho-CTD, pY1-CTD, and pY1pS5-CTD. (a) Autoradiograph of blot probed with Flag-specific antibody on streptavidin pulldown fractions involving unphospho-CTD peptides and pY1pS5-CTD peptides in the amount of 0, 1 ng, 10 ng, 100 ng, 1 μg, and 10 μg. (b) Autoradiograph of blot probed with Flag-specific antibody on streptavidin pulldown fractions involving unphospho-CTD peptides, pY1-CTD peptides, and pY1pS5-CTD peptides in the amount of 1 ng, 10 ng, 100 ng, and 1 μg. (c) Scatter charts showing results based on band intensities from previous autoradiograph showing the increase of DGCR8 pulldown with unphospho-CTD peptides, pY1-CTD peptides, and pY1pS5-CTD peptides.

a

	1	2	3	4	5	6	7	8
Abl-PP-NES					+	+	+	+
pcDNA3	+	+	+	+				
Flag Vector	+				+			
Flag-DGCR8		+				+		
Flag-YAP			+				+	
Flag-CID				+				+
pGLUE-NCTD	+	+	+	+	+	+	+	+

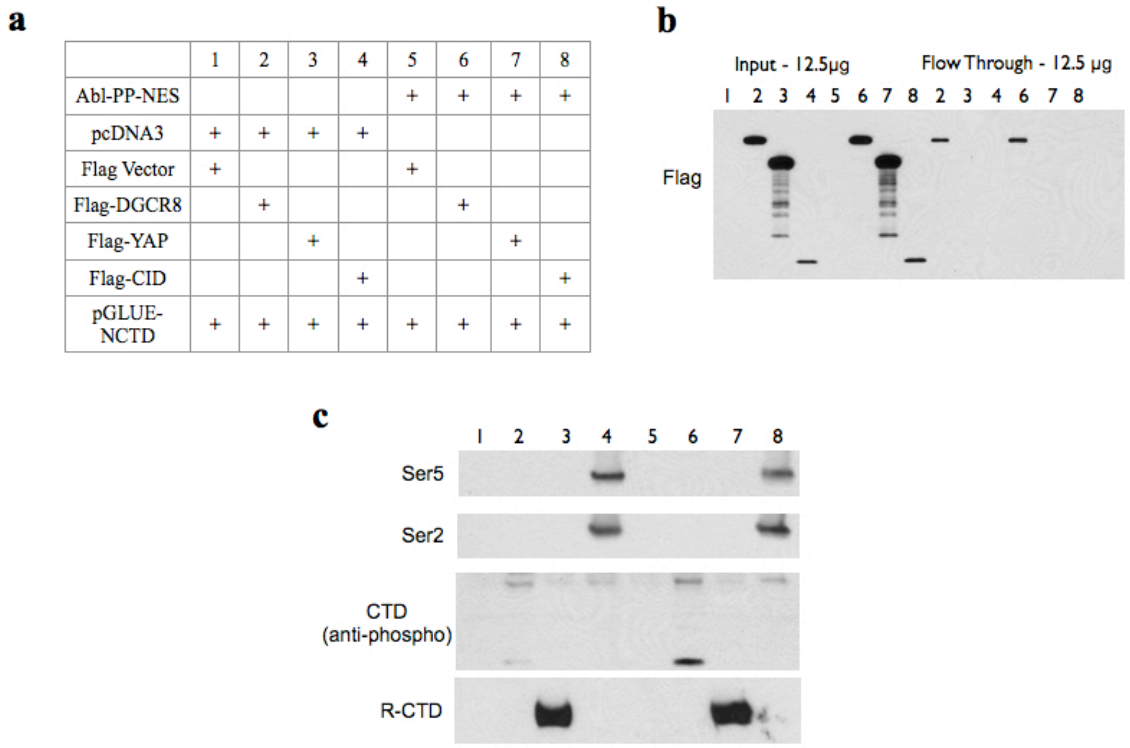

Figure 4. In vitro binding assay shows that the CTD pulled-down by CID, YAP, and DGCR8 were detected by CTD antibodies of different target specificities. (a) Transfection table for the immunoprecipitation assay featuring Flag-tagged CID, YAP, DGCR8 and pGLUE-nCTD. (b) Autoradiograph of blot probed with Flag-specific antibody. The blot shows input lanes containing Flag-DGCR8, Flag-YAP, and Flag-CID, as well as their corresponding flow-through lanes. (c) Autoradiograph of blots probed with Ser-5 and Ser--2 phosphorylation-specific-CTD antibodies. Additionally, autoradiograph showing blots probed with phosphorylated-specific-CTD antibody (anti-phospho) and unphosphorylated-specific-CTD antibody (R-CTD) are shown.

III:

Discussion

The functions of Ser-5 and Ser-2 phosphorylation on the CTD during RNA transcription have been well established by many literatures. In contrast, the roles played by Tyr-1 phosphorylation on the CTD is still largely unknown. While it had been shown that Abl kinase can be activated during DNA damage to induce Tyr-1 phosphorylation on CTD, and ultimately triggers apoptosis in a p53-dependent pathway, the underlying mechanism on how these pathways are interconnected remains relatively uncertain [6-8]. Using a mass spectrometry assay, this lab had demonstrated a possible link between the pY1pS5-CTD and many proteins of the miRNA Microprocessor complex. In this project, we have identified DGCR8, a key player in the miRNA biogenesis, as a viable candidate for the interaction between CTD and the Microprocessor complex. Moreover, we have investigated the role Tyr-1 phosphorylation of CTD plays in the CTD-Microprocessor complex interaction.

Before the beginning of this project, the mass spectrometry experiment conducted by Dr. Pineda prompted many intriguing questions on the possible connection between pY1pS5-CTD and the Microprocessor complex. This project began as an attempt to study the mass spectrometry result more specifically. A mass spectrometry generally pulls down more than the proteins involved in direct interaction. Therefore we had to assume that many of the proteins pulled-down in this particular assay were results of indirect interactions. Our first goal was to identify the direct protein interaction between CTD and the Microprocessor complex. On top of that, we were to confirm the validity of the mass spectrometry results using more specific methods.

By conducting immunoprecipitation assays in a system of over-expressed proteins, we now have established that CTD does not directly interact with EWS, a protein that is part of the inactive form of Microprocessor complex, with or without the presence of active Abl kinase (figure 1c). In this project, we over-expressed active Abl kinase in the experimental system to phosphorylate CTD on its Tyr-1 position. However, it is also important to note that the active Abl kinase can phosphorylate tyrosine residues on other proteins as well. Since the activation of Abl kinase results in apoptotic pathway during DNA damage, one of our hypotheses was that miRNA biogenesis is induced as a precursor to the apoptotic pathway. Although EWS is only one of the many proteins belongs to the inactive form of Microprocessor complex, the negative result had us begin forming our hypothesis instead on the idea that an induction of miRNA biogenesis occurs in the event of Abl activation. With this hypothesis in mind, a protein involved in the active form of Microprocessor would be a more likely candidate to interact with the CTD during DNA damage and upon Abl activation. Thus we shifted our focus to DGCR8, a critical co-factor of Drosha that is known to involve in the interaction with RNA transcript during miRNA biogenesis [18].

In our first pulldown experiment involving DGCR8, Flag-tagged DGCR8 was pulled down by Flag-gel to find out whether the CTD peptides were pulled-down by DGCR8. In this experiment, CTD was pulled-down by DGCR8 in the absence of Abl kinase. Moreover, when active Abl kinase was present, such pulldown increased, suggesting that Abl kinase played a role in stabilizing the DGCR8-CTD interaction. In order to see whether Abl stabilized such interaction via its tyrosine phosphorylation

activity, Gleevec, a drug that inhibits Abl's kinase activity, was added to one of the samples. When Gleevec was added, the CTD pulldown decreased slightly, suggesting that the tyrosine phosphorylation by Abl had contributed to the CTD-DGCR8 interaction (figure 2b). An alternative pulldown experiment was conducted by pulling down the CTD peptides to find DGCR8. The results from this experiment also confirmed the DGCR8-CTD interaction (figure 2c). What these results did not provide was a definitive answer on whether the increase of DGCR8-CTD interaction was caused by the phosphorylation on the Tyr-1 position of the CTD.

The initial mass spectrometry assay was conducted by using specifically designed CTD peptides, which consisted of four YSPTSPS amino acid repeats. Since these peptides were biochemically made to be differentially phosphorylated on specific amino acid residues, they helped in revealing the specific phosphorylated condition of the CTD peptide involved in the binding. Using these synthetic peptides, DGCR8 was again confirmed to be interacting with unphospho-CTD, pY1-CTD, and pY1pS5-CTD (figure 3b and 3c). However, to repeat this result, or to simply have a positive pulldown proved to be difficult. Numerous attempts were done after the initial pulldown assay, but none succeeded. After thoroughly analyzing each trial, the most probable cause for the failures was the fact that these peptides are too volatile. Moreover, these 4-heptad repeat peptides were much smaller in size compared to the endogenous 52-heptad repeat CTD. It is not hard to imagine that landing an aircraft onto a massive runway would be much easier than to land the same aircraft onto an aircraft carrier. Same logic applies, since the CTD acts as a platform for proteins to bind to, the synthetic peptide, consisted of only 4-heptad

repeats, is a much more difficult platform for interaction, especially when compared to the endogenous form of CTD, which is consisted of 52-heptad repeats.

Since the in vitro binding assay based on synthetic CTD peptides was not working as well as we hoped, an alternative assay was required to confirm the DGCR8-CTD interaction. In order to explore the phosphorylated conditions of which the CTD can be pulled-down by DGCR8, we decided to utilize all the appropriate CTD-specific antibodies in our lab, including: Ser-5-, Ser-2-, phospho-, and unphospho-CTD-specific antibodies. Additionally, we also took a closer look at the protein motifs identified on DGCR8. The WW domain near the center of this protein is one of the least understood sites on DGCR8 in terms of its function. Together with a literature search that showed the WW domain on Yes-associated protein (YAP) is responsible for its interaction with CTD [25], we became interested in finding out if DGCR8 also interacts with CTD via its WW domain. To test the similarities between the two WW domains, and to confirm YAP WW domain's interaction with CTD, we included YAP in our CTD antibody assay.

As shown in figure 4, this experiment suggested that, interestingly, all three proteins, including: CID, YAP, and DGCR8 were pulled-down by CTD, but could be detected only by specific CTD antibodies. The CTD pulled-down by CID was detected by Ser-2 and Ser-5 specific antibodies; the CTD pulled-down by YAP was detected by unphospho-CTD specific antibody; and the CTD pulled-down by DGCR8 was detected by phospho-CTD specific antibody. This result successfully confirmed that YAP interacts with the unphosphorylated CTD, and that it interacts with neither the serine phosphorylated nor the tyrosine phosphorylated CTD. On the other hand, DGCR8

interacts with the phosphorylated CTD, but was detected by neither of the serine phosphorylated CTD antibodies. The result suggests that, while all three of these proteins are CTD-interacting proteins, the specific sites and phosphorylated conditions responsible for the CTD bindings are all different.

Based on the findings of this project, we can so far conclude that the interaction between CTD and the Microprocessor complex can be mediated by DGCR8. Furthermore, such interaction is enhanced by tyrosine phosphorylation on the first positions of each heptad CTD repeats. The results support the idea that, during RNA transcription, once DNA damage is detected, Abl kinase can be activated to induce Tyr-1 phosphorylation on CTD, and to enhance interaction with the active Microprocessor complex, which then leads to miRNA biogenesis. The finding of this project provided us an alternative perspective to look at the association between the RNA transcription machinery and miRNA Microprocessor complex. While the subject certainly requires more investigations, this project had hopefully shed some light on the less understood role of which the Tyr-1 phosphorylated CTD plays during RNA transcription.

So far the exact protein motif responsible for DGCR8 binding with CTD has not been confirmed. In future experiments, we can utilize the DGCR8 mutant constructs acquired from Dr. Narry Kim's lab to confirm whether the WW domain on DGCR8 interacts with CTD. We have acquired five DGCR8 mutant constructs from Dr. Kim's lab, including: wild-type DGCR8 (1-773), WW domain and dsRBD1/2 containing DGCR8 (276-773), dsRBD1/2 containing DGCR8 (484-773), dsRBD1/2 containing and Drosha interacting site truncated DGCR8 (484-738), and a dsRBD1/2 truncated and WW

domain containing DGCR8 (1-483). By employing these DGCR8 mutant constructs on previously described pulldown assays, we can hopefully gain more insights in the binding between DGCR8 and the Tyr-1 phosphorylated CTD.

IV:

Materials and Methods

Cell culture and transfection. 293T cells were maintained in DMEM medium supplemented with puromycin and 10% FBS. Cells were cultured overnight at 37 degree C to a confluency of 80-85%, and then transiently transfected with expression plasmid (10 μg), using GeneTran reagent following the manufacturer's instructions.

Protein extraction and Western blotting. Protein extracts were harvested and sonicated twice in cold lysis buffer (20 mM Tris [pH 8.0], 100 mM NaCl, 1 mM EDTA, 1% NP-40, 10% Glycerol, 1 mM dithiothreitol [DTT], and 1 mM PMSF). After centrifugation at 14,000 r.p.m at 4 degree C for 15 minutes, the supernatant was isolated and labeled as whole-cell-lysate (WCL). Protein lysates were separated on 4%-12% Tris-Glycine gels (Invitrogen) or 8% SDS-poly-acrylamide gels, and transferred to Nitrocellulose membrane. Membranes were blocked by 5% milk or 5% BSA (milk blocking for mouse antibodies and BSA blocking for rabbit antibodies) for 30 minutes before antibodies were added before they were incubated for three hours to overnight.

In vitro protein binding assay by antibody-conjugated beads. Antibody-conjugated beads were prepared by incubating the desired pulldown antibody with protein A/G beads (Thermo Scientific) for 3 hours. For Flag-tagged proteins, the conjugated beads were replaced, and applied directly by FLAG Affinity Gels (Sigma). Antibody-conjugated beads were then aliquoted to the mixture of extracted proteins and CHAPS IP buffer (0.1% CHAPS, 50 mM HEPES, 150 mM KCl, 10% Glycerol, 1 mM PMSF, 10 mM NaOV, 50 mM NaF, 10 mM beta-Glycerolphosphate, and10 mM 4NaPP). The mixtures

were then rotated in 4 degree C for 5-8 hours before the beads were washed 3 times with 5% TBST, and eluted with SDS sample buffer. Resulting fractions were left in room temperature for 10 minutes before they were boiled for another five minutes. Finally, the fractions were loaded to prepared gels for Western blotting analysis.

In vitro protein binding assay by differentially phosphorylated synthetic CTD peptides. extracted proteins were added to titrated synthetic Streptavidin-tagged CTD peptides and rotated in 4 degree C for 1 hour. Then the mixture was added to Streptavidin agarose beads (Thermo Scientific) prepared in CHAPS IP buffer (0.1% CHAPS, 50 mM HEPES, 150 mM KCl, 10% Glycerol, 1 mM PMSF, 10 mM NaOV, 50 mM NaF, 10 mM beta-Glycerolphosphate, and10 mM 4NaPP). The mixture were then rotated in 4 degree C for another hour before the beads were washed 3 times with 5% TBST, and eluted with SDS sample buffer. Resulting fractions were left in room temperature for 10 minutes before they were boiled for another five minutes. Finally, the fractions were loaded to prepared gels for Western blotting analysis

References

1. Friedberg, E. C. DNA Damage and repair. *Nature*, 2003. **421**: 436-440.

2. Wang, J. Y. and Cho, S. K. Coordination of repair, checkpoint, and cell death responses to DNA damage. *Adv Protein Chem*, 2004. **69**: 101-35.

3. Roos, W. P. and Kaina, B. DNA damaged-induced apoptosis: from specific lesions to the DNA damage response and apoptosis. *Cancer Lett*, 2012. doi: 10.1016/j.canlet. 2012.01.007

4. Wang, J. Y. Ledley, F. Goff, S. Lee, R. Groner, Y. Baltimore, D. The mouse c-abl locus: molecular cloning and characterization. *Cell*, 1984. **36**: 349-56.

5. Wang, J. Y. Regulation of cell death by the Abl tyrosine kinase. *Oncogene*, 2000. **19**: 5643-50.

6. Baskaran, R. Chiang, G. C. and Wang, J. Y. Identification of a binding site in c-Abl tyrosine kinase for the C-terminal repeated domain of RNA polymerase II. *Mol Cell Biol*, 1996. **16**: 3361-9.

7. Baskaran, R. Dahmus, M. E. and Wang, J. Y. Tyrosine phosphorylation of mammalian RNA polymerase II carboxyl-terminal domain. *Proc Natl Acad Sci U S A,* 1993. **90**: 11167-71.

8. Baskaran, R. Escobar, S. R. and Wang, J. Y. Nuclear c-Abl is a COOH-terminal repeated domain (CTD) kinase specific for the mammalian RNA polymerase II: possible role in transcription elongation. *Cell Growth Differ*. 1999, **10**: 387-96.

9. Buratowski, S. The CTD code. *Nat Struct Biol*, 2003. **10**: 679-80.

10. West, M. L. and Corden, J. L. Construction and analysis of yeast RNA polymerase II CTD deletion and substitution mutations. *Genetics*, 1995. **140**:1223-33.

11. Bentley, D. L. Rules of engagement: co-transcriptional recruitment of pre-meRNA processing factors. *Curr Opin Cell Biol*, 2005. **17**: 251-6.

12. Proudfott, N. J. Furger, A. and Dye, M. J. Integrating mRNA processing with transcription. *Cell*, 2002. **108**: 501-12.

13. Hirose, Y. and Manley, J. L. RNA polymerase II and the integration of nuclear events. *Gene Dev,* 2000. **14**: 1415-29.

14. Bartel, D. P. MicroRNAs: genomics, biogenesis, mechanism, and function. *Cell*, 2004. **116**: 281-97.

15. Gregory, R. I. Yan, K. P. Amuthan, G. Chendrimada, T. Doratotaj, B. Cooch, N. and Shiekhattar, R. The Microprocessor complex mediates the genesis of microRNAs. *Nature,* 2004. **432**: 235-40.

16. Han, J. Lee, Y. Yeom, K. H. Nam, J. W. Heo, I. Rhee, J. K. Sohn, S. Y. Cho, Y. Zhang, B. T. and Kim, V. N. Molecular basis for the recognition of primary microRNAs by the Drosha-DGCR8 complex. *Cell*. 2006. **125**: 887-901.

17. Kim, V. N. Han, J. and Siomi, M. C. Biogenesis of small RNAs in animals. *Nat Rev Mol Cell Biol*, 2009. **10**: 126-39.

18. Han, J. Lee, Y. Yeom, K. H. Kim, Y. K. Jin, H. and Kim, N. V. The Drosha-DGCR8 complex in primary microRNA processing. *Genes Dev*, 2004. **18**: (3016-27).

19. Yeom, K. Lee, Y. Han, J. H. Suh, M. R. and Kim, N. V. Characterization of DGCR8/ Pasha, the essential cofactor for Drosha in primary miRNA processing. *Nucleic Acids Res*, 2006. **16**: 4622-9.

20. Senturia, R. Faller, M. Yin, S. Loo, J. A. Cascio, D. Sawaya, M. R. Hwang, D. Clubb, R. T. and Guo, F. Structure of the dimerization domain of DiGeorge Critical Region 8. *Protein Science,* 2010. **19**: 1354-65.

21. Angers, S. Thorpe, C. J. Biechele, T. L. Goldenberg, S. J. Zheng, N. MacCoss, M. J. and Moon, R. T. The KLHL12-Cullin-3 ubiquitin ligase negatively regulates the Wnt-ß-catenin pathway by targeting Dishevelled for degradation. *Nat Cell Biol,* 2006. **8**: 348-57.

22. Morlando, M. Ballarino, M. Gromak, N. Pagano, F. Bozzoni, I. and Proudfoot, N. J. Primary microRNA transcripts are processed co-transcriptionally. *Nat Struct Mol Biol*, 2008. **9**: 902-9.

23. Buchdunger, E. Zimmermann, J. Mett, H. Meyer, T. Mueller, M. Druker, B. J. and Lydon, N. B. Inhibition of the Abl protein-tyrosine kinase in vitro and in vivo by a 2-phenylaminopyrimidine derivative. *Cancer Res*, 1996. **1**: 100-4.

24. Pires, J. R. Taha-Nejad, F. Toepert, F. Ast, Thomas, Hoffmueller, U. Schneider-Mergener, J. Kuehne, R. Macias, M. J. and Oschkinat, H. Solution L30K in Complex with the Peptides GTPPPPYTVG, N-(n-octyl)-GPPPY and PLPPY and the application of peptide libraries reveal a minimal binding epitope. *J Mol Biol,* 2001. **314**: 1147-56.

25. Macias, M. Hyvoenen, M. Baraldi, E. Schultz, J. Sudol, M. Saraste, M. and Oschkinat, H. Structure of the WW domain of a kinase-associated protein complexed with a proline-rich peptide. *Nature*, 1996. **382**: 646-9.

26. Gavva, N. R. Gavva, R. Ermekova, K. Sudol, M. and Shen, C. K. Interaction of WW domains with hematopoietic transcription factor p45/NF-E2 RNA polymerase II. *J Biol Chem,* 1997. **39**: 24105-8.

27. Bienkiewicz, E. A. Woody Y. M. and Woody, R. W. Conformation of the RNA polymerase II C-Terminal Domain: circular dichroism of long and short fragments. *J Mol Biol*, 2000. **297**: 119-33.