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Development of temperature-sensitive mutants of the *Drosophila melanogaster* P-TEFb (Cyclin T/CDK9) heterodimer using yeast two-hybrid screening

Soyoun Kim [a,b,1,*], Irene M. Min [b,1], Shuo Ren [b], Alex Spector [a], Moonsoo M. Jin [c], John T. Lis [a,b,*]

[a] *Department of Molecular Biology and Genetics, Cornell University, Ithaca, NY 14853, USA*
[b] *Department of Biomedical Engineering, Dongguk University, Seoul 100-715, South Korea*
[c] *Department of Radiology, Weill Cornell Medical College, New York, NY 10065, USA*

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ABSTRACT

P-TEFb complex, a heterodimer of the kinase CDK9 and Cyclin T, is a critical factor that stimulates the process of transcription elongation. Here, we explored a fast and large-scale screening method to induce a temperature-dependent conditional disruption of the CDK9/Cyclin T interaction and developed an assay to validate their mutant phenotypes in a biological context. First, we used the yeast two-hybrid system to screen *Drosophila melanogaster* Cyclin T mutants at a large scale for temperature or cold sensitive (TS or CS) CDK9 interaction phenotypes. The isolated P-TEFb TS mutants were then expressed in *Drosophila* cells and were investigated for their effects on *Drosophila hsp70* transcriptional activity. Our results showed that these P-TEFb TS mutants had a reduced level of *hsp70* transcription at restrictive temperatures. A model structure of the Cyclin T and CDK9 complex suggested that the key TS mutations were found within the α2- and α3-helices at the interface of the complex, which may disrupt the binding of Cyclin T to CDK9 directly or indirectly by affecting the conformation of Cyclin T. The yeast two-hybrid-based screening strategy described here for isolating TS or CS interaction phenotypes can be directly applicable to other complexes in higher organisms. The use of TS or CS mutants will enable a 'real-time and reversible perturbation' restricted to specific protein–protein interactions, providing a mechanistic insight into the biological process mediated by a target complex.

1. Introduction

Recent studies indicate that much of the transcription regulation in higher eukaryotes occurs at the RNA polymerase II (Pol II) elongation step [1]. Pol II maturation into an elongationally competent complex at the promoter is accompanied by a biochemical modification at serine position 2 (Ser2) in the carboxyl-terminal domain (CTD) of Pol II [2]. The positive transcription elongation factor b (P-TEFb) is a serine/threonine kinase that phosphorylates the Pol II CTD and the pausing factors called negative elongation factor (NELF) and DRB sensitivity-inducing factor (DSIF) [3,4], allowing paused Pol II to progress into productive elongation.

P-TEFb is a heterodimer of the cyclin-dependent kinase 9 (CDK9) and Cyclin T in its active form [5]. In addition to its critical role in general cellular transcription, P-TEFb activity is specifically required for HIV-1 transcription [6]. TAT, a viral transactivator, recruits host P-TEFb at the 5′ end of the nascent transcript through the TAR RNA structure, and this interaction leads to the transcription of the full-length HIV genome. Therefore, P-TEFb may serve as a drug-susceptible target for transcriptional inhibition of HIV and suppression of many cellular genes. Small molecule compounds or mutant proteins that inhibit CDK9 kinase activity [7–10] and proteins or antibodies that block Cyclin T binding to CDK9 [8,11] have been developed as antagonists of P-TEFb activity. Usage of temporally controllable P-TEFb inhibitors may offer a better overview of the changes in cell physiology that result from P-TEFb disruption *in vivo* with fewer secondary effects arising from long-term treatment of cells with P-TEFb inhibitors.

Conditional mutants can provide a means for temporally dissecting molecular mechanisms *in vivo*. To achieve conditional regulation of cellular processes, reagents such as antibodies, small molecule compounds, RNAi [12], RNAia [13], and aptamers [13] have been developed. However, exogenously introduced inhibitors may have affinity for proteins other than their originally intended targets; it is often very difficult to rule out this possibility. In contrast, endogenous conditional mutants do not have this drawback,

Abbreviations: P-TEFb, positive transcription elongation factor b; CDK, cyclin-dependent kinase; TS, temperature sensitive; CS, cold sensitive; Gal4DBD, Gal4 DNA binding domain.

* Corresponding authors. Address: Department of Biomedical Engineering, Dongguk University, 3-26 Phil-Dong, Joong-Gu, Seoul 100-715, South Korea (S. Kim). Fax: +82 2 2290 1378 (S. Kim), fax: +1 607 255 6249 (J.T. Lis).

E-mail addresses: skim@dongguk.edu (S. Kim), johnlis@cornell.edu (J.T. Lis).

[1] These authors contributed equally to this work.

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since their effects are more likely to be limited to the gene product of interest.

Protein complexes maintain their functions by interacting with each other via specific domains. Conditional and temporal disruption of these interactions by TS mutations offers a novel means to examine the importance of specific interactions without having to account for the side effects caused by the loss of the entire protein, such as is found in a whole-protein knockout mutation. Here, a novel approach is proposed to rapidly generate TS and CS mutants of any targeted protein–protein interaction. We chose to generate conditional mutants of *Drosophila melanogaster* Cyclin T, a component of the P-TEFb complex, to disrupt its interaction with CDK9 in a controlled manner. Our methodology involved the yeast two-hybrid system, screening the mutants at different temperatures using CDK9 as 'bait', and using a library of the conserved Cyclin box portion of Cyclin T as 'prey'. Using this approach, several TS mutants of Cyclin T were identified in yeast, which were validated by a functional assay in *Drosophila* cells. To provide a structural basis for TS phenotypes of Cyclin T, a model structure of the Cyclin T and CDK9 complex was built, which revealed that the key mutations were localized to a region that is at the interface of the complex or that would alter the functional conformation of Cyclin T for its tight binding to CDK9.

2. Materials and methods

2.1. Plasmids and strains

The two-hybrid yeast strain PJ69-4A, in addition to the pGBDU and pGAD expression vectors, were kindly provided by Philip James [14]. To generate the CDK9 bait construct, the open reading frame of CDK9 was PCR amplified and cloned into the EcoRI and SalI sites of pGBDU-C2. For the Cyclin T box prey construct, the EcoRI-SalI fragment of pRMHA3-Cyclin T [15] was cloned into pGAD-C2. The Gal4 DBD-containing *Drosophila* expression vector pG was kindly provided by Carl Wu (NCI, Bethesda, MD). One SalI site in pG-Cyclin T [15] was modified, which was used as a positive control and for moving the yeast Cyclin box to pG. To generate pG-I 1–7, I 1–182 and III 6–14, NotI and SalI fragments of screened yeast Cyclin box in pGAD were moved to the NotI and SalI sites of modified pG-Cyclin T. *Hsp70-M* reporter construct, internal standard and Cyclin T/2xMut plasmids were gifts from Paul Mason [15].

2.2. Random mutagenesis using PCR

PCR reactions were carried out under the following conditions: 1 ng of DNA template, PCR buffer (Life Technologies), 0.2 mM of each dNTP, 0.5 mM of both primers, 2 mM MgCl2 and 2.5 U Taq DNA polymerase (Life Technologies) in 0.1 ml volume. Mutagenic PCR reactions were carried out with a reduced concentration of dATP (dGTP:dATP ratio of 5:1) in the presence of 0.5 mM MnCl2 as previously reported [16], but without addition of DMSO and β-mercaptoethanol. PCR reactions were performed at 94 °C for 1 min, 65 °C for 1 min and 72 °C for 1 min, for 30 cycles, followed by 7 min at 72 °C. PCR reaction yield was estimated by agarose gel electrophoresis. Sequences of PCR primers were as follows: Cyclin T Up 5′-GGCGAATTCAAAATGAGTCTCCTAGCCACGCCAATG-3′ and Cyclin T Down 5′-CTCGGTCGACTGGGGGATCTCCCATCG-3′.

2.3. Yeast two-hybrid screening

Yeast screening was performed as described in [14] with some modifications. We first introduced CDK9 bait construct pGBDU-C2 into yeast strain PJ69-4A. In order to introduce the randomly mutagenized Cyclin box sequences by homologous recombination, both the NotI/ClaI digested fragment of the prey construct pGAD-C2 and the randomly mutagenized Cyclin box cDNA were introduced into PJ69-4A. After transformation using the high efficiency LiAc method [14], cells were incubated for 6 h in YPD media for recovery from transformation and homologous recombination. Transformants were obtained on medium lacking uracil, leucine, and adenine at 25 °C after 3–4 days of growth. Resulting transformants were transferred to four plates by replica plating. Replica plates were incubated at four different temperatures, 18 °C, 25 °C, 30 °C and 37 °C. Colonies that could not grow at specific temperatures were selected and re-streaked on media lacking uracil, leucine and histidine with 1.5 mM 3-aminotriazole at four different temperatures. These confirmed colonies were then plated on 5-FOA media to evict bait constructs containing the URA marker. These colonies, containing only Cyclin T mutants (not CDK9), were then tested for autoactivation by mutant Cyclin T alone. Finally, these screened mutants were grown in liquid media for 2 days and plasmid DNA was purified (EZ yeast plasmid minipreparation kit, Geno Technology). These individual plasmids were then transformed into PJ69-4A containing CDK9 bait for retesting the temperature dependent growth.

2.4. Liquid growth test

Single colonies from each individual transformant were inoculated and grown for 2 days in liquid minimal media lacking uracil and leucine. 5 μl of liquid cultures were spotted onto medium lacking uracil, leucine and adenine. For spotting, fully-grown liquid cultures were serially diluted by 1/10.

2.5. DNA sequencing

DNA plasmids were prepared with the EZ yeast plasmid minipreparation kit (Geno Technology) from yeast culture grown for 2 days at 30 °C in liquid media lacking uracil and leucine. Each yeast-purified plasmid was electro-transformed into DH5α *Escherichia coli*. Plasmids were prepared (Miniprep Kit, Qiagen) from overnight cultures of *E. coli* and sequenced by the Cornell University Life Sciences Core Laboratories Center with the Gal4 AD primer (Clontech).

2.6. Cell transfection

Drosophila Kc cell transfection was performed as previously described [15]. Before the copper induction step, each triplicate of transfected Kc cells was preincubated at three different temperatures, 18 °C, 25 °C or 30 °C. After 1.5 h of preincubation, $CuSO_4$ was added to a final concentration of 1.4 mM and cells were incubated for 18 h at 18 °C, 25 °C or 30 °C.

2.7. Primer extension assay

From 2 ml of copper-induced Kc cells, total RNA was prepared with the RNeasy kit (Qiagen) according to the manufacturer's instructions except that RNA was eluted once with 100 μl water. Primer labeling and primer extension assays were performed as previously described [15]. The sequence of the *malE* primer used in this assay is 5′-CGGTCGTCAGACTGTCGATGAAGC-3′.

2.8. Structural model of the Cyclin T/CDK9 complex

Drosophila Cyclin T (AAC73052), a region of Trp45 to Pro240, has a sequence homology of 65% with human Cyclin T2. The structure of the *Drosophila* Cyclin T was modeled after human Cyclin T2 (PDB Code: 2IVX) using the Modeller program (Andrej Sali, UCSF) with default parameters. The Cyclin T model structure was

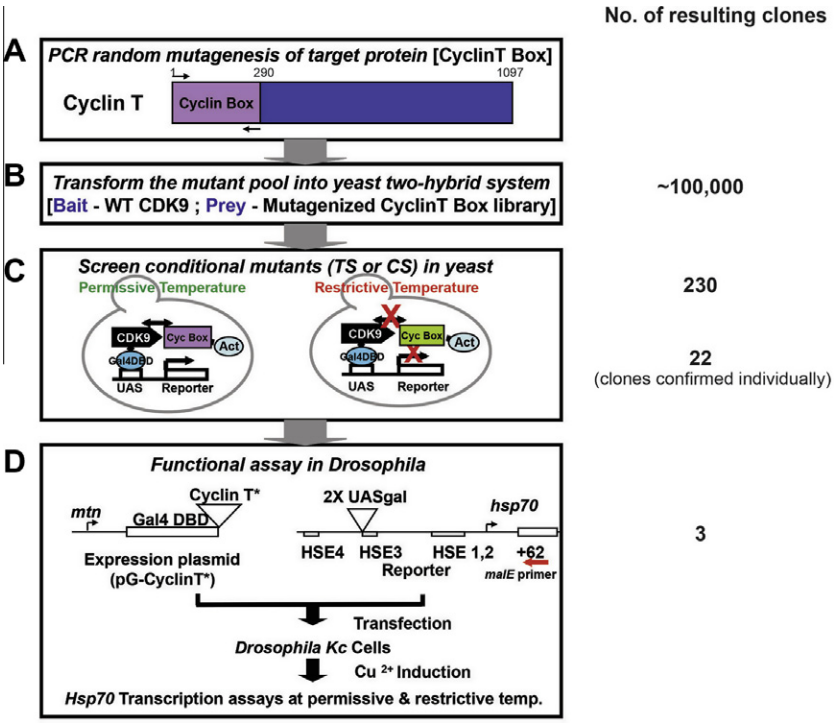

Fig. 1. Scheme for the discovery of conditional mutants of P-TEFb. (A–C) Conditionally interacting mutants of P-TEFb were screened on a large scale using the yeast two-hybrid system, where CDK9 fused to the GAL4 DNA binding domain (Gal4DBD) fragment acted as the 'bait' protein, and the wild type or mutant 'Cyclin Box' fused to the GAL4 activation domain (Act) fragment served as the 'prey' protein. The interaction between 'prey' and 'bait' leads to the expression of reporter (β-galactosidase) and auxotrophic markers (ADE2 for the growth in media lacking adenine) in the strain PJ69-4A. (D) The yeast-screened Cyclin T mutants were tested for their abilities to activate *hsp70* transcription in *Drosophila*. The wild type Cyclin T or TS Cyclin T mutants (Cyclin T*) fused to the Gal4DBD protein (the expression controlled by the metallothionein (*mtn*) promoter) were tested for their abilities to bind to a pair of GAL4-binding sites (2X UASgal) and activate *hsp70* transcription in the reporter plasmid *hsp70*-M. The *hsp70*-M has four heat shock elements (HSE). Bacterial malE gene was inserted in the reporter hsp70 (−194/+62), and the primer (small arrow) located at the *malE* allowed primer extension analysis to quantify the levels of *hsp70* transcripts. The number of resulting clones in each step is shown at right.

superimposed onto the human Cyclin A (PDB Code: 1VYW) with a root mean square deviation of 1.3 Å. The model structure of the *Drosophila* CDK9 was constructed using human CDK2 (1VYW) as a template.

3. Results and discussion

3.1. Strategy for generating conditionally active P-TEFb using the yeast two-hybrid system

We developed a novel strategy to screen conditional mutants of *Drosophila* proteins. For this, we took advantage of the yeast two-hybrid system [14]. We hypothesized that an interaction that is conditionally disrupted by mutations in yeast would also be conditionally disrupted at the same temperature in *Drosophila*. The yeast two-hybrid system 'converts' protein–protein interactions into the activation of reporters, and the ease of screening a large number of colonies makes this system an efficient screening tool. Furthermore, the interaction between prey and bait in our yeast two-hybrid system leads to the expression of a reporter (*β-galactosidase*) and an auxotrophic marker (ADE2 for the growth in media lacking adenine). Prior to the construction and screening of the library, we first confirmed that the expression of wild type Cyclin T and CDK9 as a prey-bait pair allowed the activation of *β-galactosidase* and growth of cells on media lacking auxotrophic markers (data not shown). Alternative to the use of whole *Drosophila* Cyclin T, which is a large protein (118 kDa) consisting of 1097 amino acids, we have chosen to focus on a region called 'Cyclin box', a domain in Cyclin T that provides major molecular contacts with CDK9 (Fig. 1A). The Cyclin box is highly conserved among different species and among other Cyclin members that form complexes with the CDK9 kinase subunit *in vitro* [5,17]. The expression of wild type Cyclin box was also confirmed to be as active as full-length Cyclin T in forming a complex with CDK9, judging from a comparable cell growth (Fig. 2D).

The overall strategy for generating conditionally interacting mutants of P-TEFb using the yeast two-hybrid system is as follows

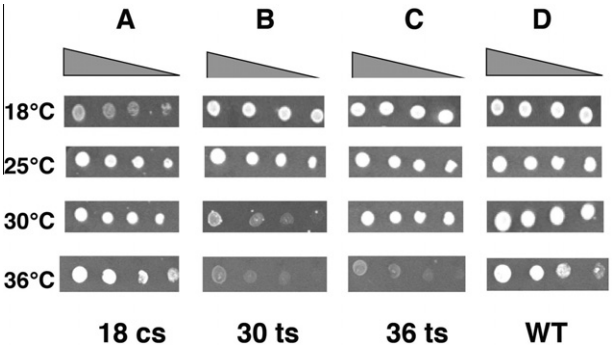

Fig. 2. Yeast growth with conditional P-TEFb mutants at different temperatures. Yeast colonies that showed temperature-dependent phenotypes were retested individually by recovering plasmids harboring the Cyclin box and reintroducing them into fresh yeast cells along with the CDK9 bait plasmid. Yeast growths were assessed at four different temperatures: 18 °C, 25 °C, 30 °C, and 36 °C. (A) CS mutant 18cs did not grow at or below 18 °C. (B) TS mutant 30ts did not grow at or over 30 °C. (C) 36ts Did not grow at or over 36 °C. (D) The yeast clone that was transformed with the wild type Cyclin box 'prey' plasmid and the CDK9 'bait' vector survived at all temperatures tested. Every panel indicates 1/10 of serial dilution, from left to right, of fully-grown liquid culture.

(Fig. 1). First, a mutant library of the Cyclin box as a 'prey' vector containing the GAL4 activation domain (Act) [16] were introduced into yeast strain PJ69-4A, which is transformed with a 'bait' vector containing the CDK9 gene fused to the GAL4 DNA binding domain (Gal4DBD) (Fig. 1B). The strain PJ69-4A contains the GAL4-inducible promoters regulating the *lacZ* reporter gene and ADE2 auxotrophic marker gene [14]. Therefore, when the 'bait' and 'prey' protein factors interact, GAL4 activates the *β-galactosidase* reporter and allows growth of cells on media lacking the auxotrophic marker. Approximately 100,000 colonies were screened at four different temperatures: 18 °C, 25 °C, 30 °C, and 36 °C. Out of these, the growth of 230 colonies differed significantly at different temperatures; phenotypes were retested by recovering plasmids harboring the Cyclin box and reintroducing them into fresh yeast cells along with the CDK9 bait plasmid. Finally, we identified 22 clones that survived the retest: the clones that were unable to grow at or below 18 °C were designated as 18cs (selected clones are shown in Fig. 2A), at or over 30 °C as 30ts (Fig. 2B), and at or over 36 °C as 36ts (Fig. 2C). Compared to these clones, yeast clones expressing the wild type Cyclin box/CDK9 grew well at all temperatures tested (Fig. 2D).

3.2. Sequence analysis of TS Cyclin box mutants

From the yeast clones that exhibited TS growth (30ts and 36ts), the prey plasmids were extracted and sequenced, which identified three unique clones designated as I 1–7 (30ts), I 1–182 (30ts), and III 6–14 (36ts) (Fig. 3A). Within the Cyclin box across different species, there is a highly conserved pair of amino acids within the α3-helix (Lys126 and Glu129 in *Drosophila* Cyclin box); it was previously shown that the mutation of both K126L and E129K (2xMut) totally abolished the ability of Cyclin T to form

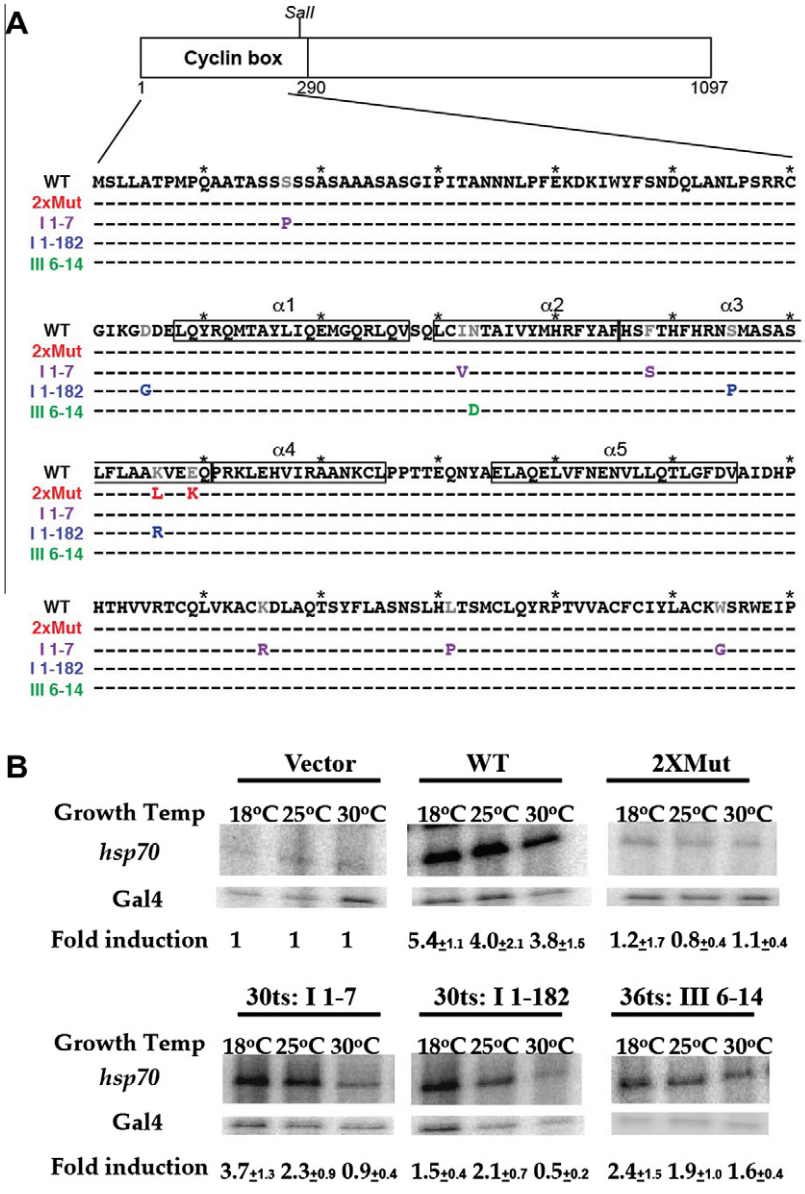

Fig. 3. Mutations in temperature dependent Cyclin T mutants and effects on P-TEFb activity. (A) Sequence comparison of selected TS *Drosophila* Cyclin T mutants, wild type Cyclin T, and 2xMut. Altered amino acids in each mutant are noted with colored letters. α-Helices are indicated according to the notations by Peng et. al. [5]. Every 10th residue is marked with an asterisk. (B) Primer extension assay to measure transcriptional activity of an *hsp70* reporter by conditional mutants of P-TEFb in *Drosophila* Kc cells. The *hsp70* (−194/+62) reporter is fused to the bacterial *malE* gene for primer extension. Gal4 transcripts were used as expression controls, and used to normalize *hsp70* activation levels. All experiments were repeated at least 3 times. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this book.)

an active complex with CDK9 [18]. We found that one of the three clones (I 1–182) contained a mutation in one of the two conserved positions (K126), while the other two clones contained mutations in the α2-or α3-helices that create the molecular contacts with CDK9 (refer to Fig. 4).

3.3. TS mutants screened in yeast recapitulate the same phenotype in Drosophila cells

To test whether the *Drosophila* Cyclin T mutants screened in yeast also show the same temperature-dependent phenotypes in *Drosophila*, we used the artificial recruitment system in *Drosophila* Kc cells using the pG vector containing a Gal4 DNA binding domain (Gal4DBD) and an *hsp70*-M reporter vector containing GAL4-binding event (UASgal) reporters (Fig. 1D). The pG expression vector contained the copper-inducible metallothionein (*mtn*) promoter driving the expression of Gal4DBD alone or Gal4DBD with either wild type (WT) or Cyclin T mutants (2xMut, I 1–7, I 1–182, and III 6–14; Fig. 3B). The *hsp70–M* reporter plasmid was inserted with a pair of GAL4-binding sites (2x UASgal) upstream of the *hsp70* reporter gene. Bacterial gene malE was fused downstream of *hsp70* to distinguish Gal4DBD-driven expression from endogenous *hsp70*. After transfection of Kc cells with both plasmids, total RNA was analyzed for *hsp70* reporter activity by primer extension with reverse transcriptase and primers within the malE gene. Previously, the same system was used to demonstrate that the recruitment of wild type Cyclin T can activate transcription of an *hsp70* reporter gene [15].

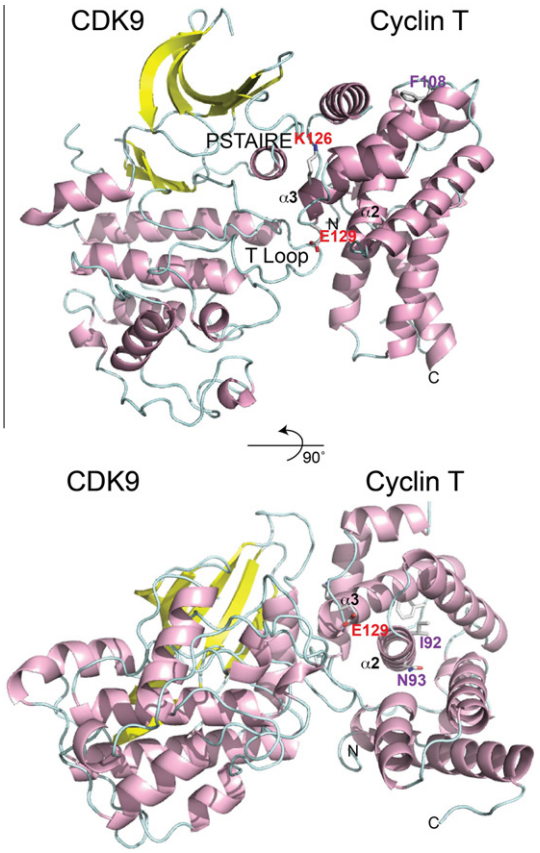

Fig. 4. Structural model and key TS amino acid mutations in the Cyclin T and CDK9 complex. The complex structure of Cyclin T and CDK9 was drawn in cartoon diagram using PyMOL. Key mutations are identified, with the wild-type residues drawn in stick models. Selected α-helices, and N- and C-termini of Cyclin T are labeled. The two residues that were mutated in 2xMut (K126L and E129K) are shown in stick models. The mutations I92V and N93D found in mutants I 1–7 and III 6–14 are located in the α3-helix of Cyclin T.

With the TS Cyclin T mutants, we then tested the ability of the mutant P-TEFb to activate transcription of *hsp70* at different temperatures (18 °C, 25 °C, and 30 °C) using a primer extension assay (Fig. 3B). We avoided conditions (e.g., 36 °C) that may activate heat shock elements in the reporter construct by endogenous heat shock responses. Two 30ts mutants, I 1–7 and I 1–182, displayed dramatic (4–8-fold) reductions in *hsp70* transcription levels at 30 °C relative to wild type, while the levels of reduction at 18 °C and 25 °C were less pronounced (less than 2-fold) (Fig. 3B). Consistent with the phenotype of 36ts mutants and their ability to grow normally at 30 °C (Fig. 2C), 36ts III 6–14 displayed comparable levels of *hsp70* transcription across three different temperatures. While 30ts mutants severely compromised *hsp70* transcription only at 30 °C, the 2xMut of Cyclin T showed background levels of *hsp70* transcription at all temperatures tested. Our results therefore demonstrated that the phenotypes of TS mutants (30ts and 36ts), discovered by the TS growth of yeast in the yeast two-hybrid screen, were faithfully reproduced in *Drosophila* cells based on TS P-TEFb interaction and downstream transcriptional activity.

3.4. Structural basis of the TS mutants of Cyclin T

Given the model structure of the Cyclin T/CDK9 complex (Fig. 4), all the TS mutants contained one or two mutations between the α2- and α3-helices (Fig. 3A), which interact with the CDK2-equivalent PSTAIRE and T-loop in CDK9 [19]. In Cyclin T, Glu-129 is in the vicinity of two arginines in the PSTAIRE (Arg-97) and T-loop (Arg-204) of CDK9, and the mutation to Lys found in the 2xMut may create an electrostatic repulsion likely to disrupt the complex. The 2xMut and I 1–182 mutants contained mutations in Lys-126 in Cyclin T, which is in direct contact with the residues in the CDK9 PSTAIRE loop. Therefore, introducing a hydrophobic residue Leu (K126L) in the 2xMut or a bulky residue Arg (K126R) in the I 1–182 will reduce a favorable electrostatic attraction of Lys126 with neighboring acidic residues from CDK9. In Cyclin T, the α2-helix is positioned in the middle of the structure and surrounded by neighboring α-helices. Therefore, the mutations found in the I 1–7 and III 6–14 mutants, I92V and N93D, will likely to induce an overall conformational change of Cyclin T and affect the complex formation. Furthermore, the mutations in a region with direct contact with the CDK9 in the 2xMut and I 1–182 led to complete or more severe disruptions of P-TEFb activity relative to those that occurred in a buried region in the Cyclin T, as in I 1–7 and III 6–14 (Figs. 3B and 4).

In this study, we demonstrated that conditional mutants for temporally disrupting protein–protein interactions could be isolated using the yeast two-hybrid system. This screening platform is fast, efficient, and can be performed on a large scale. Conditional mutants isolated in yeast can then be readily validated in cells or in the higher organisms to which the target molecules belong, such as the assay used herein to test temperature-dependent disruption of transcriptional activation by P-TEFb.

The ability to quickly isolate temporally controllable and reversible phenotypes, without a prior knowledge of sequence homology or structural information, will be a valuable tool for mechanistic studies of signaling pathways. This technique could accelerate current efforts to build and confirm protein–protein interaction networks on a genome-wide scale.

Acknowledgments

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