In compliance with the
Canadian Privacy Legislation
some supporting forms
may have been removed from
this dissertation.

While these forms may be included
in the document page count,
their removal does not represent
any loss of content from the dissertation.

Regulation of CAK activity of Cdk7 in *Drosophila melanogaster*

Jian Chen

Department of Biology

McGill University, Montreal

February 2003

A thesis submitted to McGill University in partial fulfillment of the requirements of the degree of Doctor of Philosophy (Ph.D)

Your file Votre référence
ISBN: 0-612-88437-6
Our file Notre référence
ISBN: 0-612-88437-6

Abstract

Cdk7 (Cyclin-dependent kinase 7) is conserved from yeast to human and involved in multiple functions. Cdk7 acts as a CAK (Cdk activating kinase) in a trimeric complex with Cyclin H and Mat1. The CAK activity is required for the full activation of the Cdks that directly regulate the cell cycle transitions. In addition, Cdk7 is the kinase subunit of TFIIH, the general transcription /DNA repair factor IIH. TFIIH is required for the general transcription of messenger RNAs by RNA polymerase II and for the transcription-coupled nucleotide excision repair functions. As in other systems, *Drosophila* Cdk7 has multiple functions. In order to understand how different functions of Cdk7 are regulated, I performed genetic screens to identify the regulators or downstream factors of multiple functions of Cdk7. Several candidate dominant suppressors and enhancers were identified in these screens. One strong suppressor of *cdk7*, *xpd*, encodes another subunit of TFIIH. The genetic suppression by *xpd* attracted me to further characterize the biological significance of this interaction. I showed that Xpd does have a novel function in regulating CAK activity of *cdk7*, it down-regulates mitotic CAK activity. Furthermore, I found that Xpd protein levels are cell cycle dependent, being down-regulated at the beginning of the mitosis. Based on these data, I propose a model that mitotic down-regulation of Xpd results in increased CAK activity, positively regulating mitotic progression. Simultaneously, this down-regulation can be expected to contribute to the mechanisms of mitotic silencing of basal transcription.

Résumé

Cdk7 (Cyclin-dependent kinase 7) est conservée de la levure jusqu'à l'homme et est impliquée dans plusieurs processus. Cdk7 joue le rôle d'une CAK (Cdk activating kinase) dans un complexe trimérique avec Cycline H et Mat1. L'activité CAK est essentielle pour l'activation complète des Cdks qui contrôlent directement les transitions du cycle cellulaire. De plus, Cdk7 est la sous-unité kinase de TFIIH, qui est le principal facteur IIH de transcription et de réparation de l'ADN. TFIIH est nécessaire à la transcription des ARN messagers par l'ARN polymérase II ainsi qu'à certaines fonctions de réparation de l'ADN. Comme dans d'autres systèmes, la Cdk7 de *Drosophila* participe à de multiples fonctions. Afin de comprendre comment les diverses fonctions de Cdk7 sont regulées, j'ai conduit des criblâges génetiques pour identifier les régulateurs ou facteurs en aval des multiples fonctions de Cdk7. Plusieurs candidats pour des suppresseurs et renforceurs dominants furent identifiés dans ces criblâges. Un fort suppresseur de *cdk7,xpd* , code pour une autre sous-unité de TFIIH. La suppression génétique de *xpd* m'a poussée à caractériser plus en profondeur la signification biologique de cette intéraction. J'ai prouvé que Xpd éxécute une nouvelle fonction dans la régulation de l'activité CAK de *cdk7*, elle régule négativement l'activité mitotique de CAK. En outre, j'ai constaté que les niveaux de la protéine Xpd dépendent du cycle cellulaire, cette dernière étant régulée négativement au début de la mitose. En me basant sur ces données, je propose un modèle où la régulation négative de Xpd a pour conséquence une activité CAK accrue, ainsi régulant positivement la progression mitotique. Simultanément, on peut s'attendre à ce que cette régulation négative participe aux mécanismes qui rendent la transcription basale silencieuse pendant la mitose.

Acknowledgments

First of all, I would like to thank my supervisor, Beat Suter for his invaluable support throughout the research, for his scientific supervision and advises, his encouragement and his financial support. Thank you for bringing me into a fantastic research area.

Special thanks to Stéphane Larochelle for leaving me an excellent project, for teaching me molecular biology, for valuable advise and discussion, for inspiring me throughout the research. Thanks to Andrew Swan for his fruitful discussions in fly genetics and immunostaining tips. Thanks to Judit Pandur for technical help with fly work and for organizing great parties. Thanks to Sayena Azarbar and Lydia Trvalec for their work on the *xpd* mutation screen. Thanks to Mathieu Miron, Oona Johnston and Mirlene Lindor for helping with *Drosophila* cell cultures. Thanks to Matthieu Cavey and Nisrine Masrouha for French translating of this thesis abstract. Thanks to Beili Hu for microinjection. Thanks to Long Yang and Nianqun Liu for discussions on ideas and techniques. Thanks to Yanru Li and Kate Levesque for yummy fly food. Thanks to Malcolm Whiteway, Richard Roy and Siegfried Hekimi for sitting on my supervisory committee. Thanks to Weihua Li for Lunch company and technical discussion, to Sirine Hijal for healthy jokes and to Chantel Pare for harmless food. Thanks to previous and present members in Suter lab, Judit Pandur, Thuy Nguyen, Nisrine Masrouha, Matthieu Cavey, Li Zhang, Beili Hu for helping to make these years enjoyable. Thanks to members of the Lasko, Roy and Bussey lab for help, advise and ideas. Finally, thanks to Bloomington Stock Center for the deficiency kit and other lines.

Contributions to Original Knowledge

Chapter 2 reports general characterization of Cdk7 in *Drosophila*, including Cdk7 isoforms in cell cycle phases, immunostaining pattern of Cdk7 and Cdk7's involvement in transcription.
All of the result presented in this chapter are the results of the efforts of the candidate.
Some data in this chapter has been published in:
Larochelle, S., Chen, J., Knights, R., Pandur, J., Morcillo, P., Erdjument-Bromage, H., Tempst, P., Suter, B., and Fisher, R. P. 2001. T-loop phosphorylation stabilizes the CDK7-cyclin H-MAT1 complex in vivo and regulates its CTD kinase activity. *Embo J* **20**, 3749-59.

Chapter 3 describes the genetic screens I performed to identify dominant suppressors and enhancers of *cdk7*. Several candidate suppression region and enhance region were isolated. All of the result presented in this chapter are the results of the efforts of the candidate with the following exceptions: Judit Pandur helped to collect male flies for the primary screen.

Chapter 4 reports that *Drosophila* Xpd negatively regulates the mitotic CAK activity of Cdk7. One strong suppressor from the genetic screen was mapped to *xpd,* another TFIIH component. I showed that Xpd is down-regulated before metaphase. Down-regulation of Xpd appears to contribute to the up-regulation of mitotic CAK activity and to positively regulate mitotic progression. Simultaneously, this down-regulation can be expected to be a major mechanism of mitotic silencing of basal transcription.

All of the result presented in this chapter are the results of the efforts of the candidate with the following exception: Xiaoming Li generated the antibody against Xpd.
The candidate was responsible for carrying out the experiements, obtaining and interpreting the results reported in this chapter.

Chapter 4 has been submitted as:
A novel function for Xpd/Ercc2 in regulating CAK activity and mitotic progression.
Jian Chen, Stéphane Larochelle, Xiaoming Li and Beat Suter.
The paper was written by the candidate with help from B.Suter.

Acknowledgements for materials provided are included in the Marterials and Methods in Chapter 4.

Chapter 5 reports the strategy for generating and identifing the *xpd* mutations. Sayena Azarbar and Lydia Trvalec carried out the experiements under the supervision of Beat Suter and the candidate. Sayena Azarbar performed the EMS mutagenesis and primary screen for the *xpd* mutations using 2606 Df(2R)Pu-D17 large deficiency. Lydia continued the work by further mapping the mutations using smaller deficiencies. Appendix I describes the deficiency kit from the Bloomington Stock Center used for the primary genetic screen for dominant enhancers and suppressors

Appendix I provides the deficiency kit from the Bloomington Stock Center used for the primary genetic screen for dominant enhancers and suppressors

Appendix II lists the raw data from the primary screen for dominant suppressors and enhancer of $cdk7^{ts1}$

Appendix III lists the candidate dominant suppressors and enhancers of $cdk7^{ts1}$ with their genotypes and breakpoints.

Appendix IV T-loop phosphorylation stabilizes the CDK7-cyclin H-MAT1 complex in vivo and regulates its CTD kinase activity. *Embo J* **20**, 3749-59.

Table of Contents

List of Figures

Chapter 2

Chapter 3

Chapter 4

Chapter 5

Chapter 1

General Introduction

TFIIH is composed of nine subunits, the CAK complex Cdk7, Cyclin H, MAT1, the two helicase Xpb and Xpd, and p34, p44, p52, p62 (Egly, 2001; Frit et al., 1999). The TFIIH holo-enzyme and its sub-complexes are thought to function in three different cellular events: cell cycle control, regulation of transcription and DNA repair (Egly, 2001; Frit et al., 1999). Therefore, I will introduce the regulation of these three functions in the following.

I. Cell cycle regulation

The cell cycle is a highly ordered, precisely controlled progression which undergoes two important major transitions, G1/S and G2/M, and ends up with the duplication of a cell. Cdks (Cyclin-dependent-kinase) are key regulators of these two cell cycle transitions (Morgan, 1997; Nigg, 1995). The integration of the internal and external signals leads to their successive activation and inactivation, which in turn drives the cell cycle. In higher eukaryotes, different Cdks with different cyclins act as enzyme complexes to regulate the cell cycle. Cdk2 with S phase cyclins regulate the G1/S transition and S phase progression, and Cdc2/B-type Cyclins play a role in mitosis; Cdk4,6/CyclinDs are involved in the exit from quiescence and the passage from G1 to S. In *Drosophila*, DmCdc2/CyclinB and DmCdc2/CyclinA function in mitosis (Knoblich and Lehner, 1993; Lehner and O'Farrell, 1990; Stern et al., 1993) and the DmCdk2/CyclinE is required for entry into and progression through S phase (Knoblich et al., 1994; Richardson et al., 1993; Sauer and Lehner, 1995). In yeast, a single Cdk, (Cdc2 in *S. pombe* and CDC28 in *S. cerevisiae*) in conjunction with phase specific cyclins are used for the regulation of the cell cycle (Morgan, 1997; Nigg, 1995).

To ensure perfect timing of Cdk activity and precise coordination of cell cycle events, a very elaborate mechanism exists that can precisely regulate different Cdk activities during the cell cycle. This regulation can be through selective proteolysis of cyclins, Cdk inhibitors and several phosphorylation events (Morgan, 1995).

Proteolysis pathway

Cdks require a Cyclin as partner to be active and each Cdk has its specific cyclin partner. Cyclins undergo periodic synthesis and ubiquitin-dependent proteolysis during the cell cycle, thus providing periodic activation and inactivation of Cdk activity.

The ubiquitin-proteasome pathway is a multi-step process that targets the substrate to be degraded by the proteasome with the help of ubiquitins. Ubiquitin is first activated by a ubiquitin-activating enzyme called E1 and subsequently transferred to E2, the ubiquitin-conjugating enzymes or UBC. Then, ubiquitin is transferred from the E2 enzymes to substrates and this step requires ubiquitin-protein ligase E3. Now, a polyubiquitin chain can be formed by conjugation of more ubiquitin molecules to those already attached to the substrate. Finally, the polyubiquitin chain is recognized by the 26S proteasome, a multisubunit protease specific for multiubiquitinated substrates (Baumeister et al., 1998; Coux et al., 1996; Zachariae and Nasmyth, 1999). The proteasome is active throughout the cell cycle, suggesting that cyclin ubiquitination rather than its degradation is cell cycle regulated (Mahaffey et al., 1993).

Cells usually contain a single, conserved E1 enzyme and a family of related E2 enzymes, while E3 enzymes appear to be structurally diverse and range from single proteins to large multi-subunit complexes. APC/C, SCF and VBC are members of complex E3 enzymes (Zachariae and Nasmyth, 1999). E3 are thought to determine which proteins are ubiquitinated and degraded (Zachariae and Nasmyth, 1999). APC/C is shown to function at multiple steps during the cell cycle, APC/C targets and destroys B-type Cyclins, thus inactivating mitotic CDKs. Such inactivation of Cdks is essential for cell cycle progression. At the end of mitosis, Cdk1 inactivation is essential for disassembly of the mitotic spindle, chromosome decondensation, cytokinesis, reformation of the nuclear envelope, reactivation of transcription and rebuilding the Golgi apparatus (Gallant and Nigg, 1992; Gottesfeld and Forbes, 1997; Holloway et al., 1993; Luca et al., 1991; Murray and Kirschner, 1989; Sigrist et al., 1995b; Surana et al., 1993). Degradation of mitotic Cyclins appears to be a conserved mechanism for Cdk1 inactivation. APC/C also destroys anaphase inhibitors such as Pds1 and Cut2, and promotes sister chromatid separation at the metaphase-to-anaphase transition.

APC is under complex control by phosphorylation and binding of one of two WD 40 repeat proteins: Cdc20 and Cdh1/Hct1 in yeast, Fizzy and Fizzy-related in *Drosophila*, or p55cdc/hCdc20 and hCdh1 in humans (Morgan, 1999). These WD 40 proteins are evolutionarily conserved and act as both substrate recognition and activating modules for APC (Pfleger et al., 2001a). In *Drosophila, fizzy* encodes a protein composed of seven WD repeats and is required for degradation of Cyclin A and B during mitosis (Dawson et al.,

1995; Sigrist et al., 1995a). Fizzy-related, a related WD protein, is expressed later in development when cell cycles contain a G1 phase (Sigrist and Lehner, 1997). In these somatic cycles, APC/C-dependent degradation during mitosis depends on Fizzy, whereas the maintenance of the APC activity during G1 requires Fizzy-related.

In budding yeast, the Fizzy homolog Cdc20 mediates degradation of the anaphase inhibitor Pds1, S phase B-type cyclin Clb5 and the mitotic cyclin Clb3 at the metaphase-to-anaphase transition (Visintin et al., 1997a; Shirayama et al., 1999; Alexandru et al.,1999). During anaphase, the Fizzy-related homolog Cdh1/Hct1 is required for degradation of another set of substrates including the mitotic cyclin Clb2, the polo kinase Cdc5, and the spindle protein Ase1 (Charles et al., 1998; Schwab et al., 1997; Shirayama et al., 1998; Visintin et al., 1997b). In yeast and human cells, levels of Cdc20 protein rise and fall as cells enter and exit mitosis, with the result that Cdc20 is bound to the APC/C only during M phase (and possibly during late G2). In contrast, the level of Cdh1 remains constant during the cell cycle, but it only binds the APC/C during G1. For the rest of the cell cycle, the association of Cdh1 with the APC/C is inhibited due to its phosphorylation by Cdk1 (Zachariae et al., 1998).

The APC/C appears to possess other activator proteins besides Cdc20 and Cdh1. Budding yeast contain a third Cdh1/Cdc20-related gene called AMA1, which is expressed and spliced only during meiosis. Fission yeast contain a family of at least five related WD proteins, including a Cdc20 homolog (Slp1) and a Cdh1 homolog (Srw1/Ste9) (Kitamura et al., 1998; Kominami et al., 1998; Matsumoto, 1997; Yamaguchi et al., 1997). How the WD coactivators promote ubiquitination is not clear.

The APC is regulated by the spindle checkpoint. Spindle checkpoint will be activated to delay anaphase if the chromosomes do not attach well to both spindle poles (Li and Nicklas, 1995; Rieder et al., 1995; Rieder et al., 1994). APC-Cdc20 can be inactivated by the checkpoint through a signal transduction pathway composed of the Mad and Bub proteins (Hardwick, 1998; Taylor, 1999). This prevents the degradation of securin and consequent chromosome segregation until all the chromosomes are attached to both spindle poles (Alexandru et al., 1999; Yamamoto et al., 1996). Cdh1-APC is regulated at two levels, by phosphorylation dependent dissociation of Cdh1 from APC and by Mad2l2 (Mad2-related protein) inhibition of APC-Cdh1 activity (Pfleger et al., 2001b).

Cyclin proteolysis in cell cycle regulation

In all systems studied thus far, Cyclin A degradation is shown to precede cyclin B degradation and this order of destruction of mitotic Cyclins seems to be conserved (Lehner and O'Farrell, 1990; Luca and Ruderman, 1989; Minshull et al., 1990; Pines and Hunter, 1990; Whitfield et al., 1990). This sequential destruction of the different Cyclins controls and coordinates late mitotic progression (Parry and O'Farrell, 2001). In *Drosophila*, Cyclin A is degraded prior to the metaphase/anaphase transition; Cyclin B is degraded at the metaphase/anaphase transition; and Cyclin B3 is degraded during anaphase (Huang and Raff, 1999; Jacobs et al., 1998; Lehner and O'Farrell, 1990; Maldonado-Codina and Glover, 1992; Sigrist et al., 1995a; Whitfield et al., 1990). The expression of stable versions of each of the mitotic Cyclins shows that they block the mitosis at successive stages (Sigrist et al., 1995a). Stable Cyclin A blocked the metaphase/anaphase transition, stable Cyclin B blocked anaphase spindle pole separation and cytokinesis. Stable Cyclin B3 blocked cells in a late anaphase configuration except that deep cytokinetic furrows developed (Parry and O'Farrell, 2001).

Both Cyclins A and B1 are degraded in mitosis by ubiquitin-mediated proteolysis (Glotzer et al., 1991; Hershko et al., 1994; King et al., 1995; Sudakin et al., 1995). Cyclin B1 and the anaphase inhibitor securin are substrates of Cdc20-APC and have well-defined destruction boxes (D box) composed of the sequence R-X-X-L-X-X-X-X-N. These D-boxes are essential for the Cdc20-APC mediated degradation (Yamano et al., 1998). Recently, it was shown that Cdh1-APC requires a KEN box, composed of K-E-N, as a general targeting signal. Cdc20 is itself a Cdh1-APC substrate and it contains a KEN box (Pfleger and Kirschner, 2000). Cyclin A has a D-box, however it is shown that D box sequences are not essential for mitotic destruction of Cyclin A in *Drosophila* (Kaspar et al., 2001). Destruction of Cyclin A is mediated by at least three different elements. These elements are a KEN box, a D box and an un-characterized sequence (Kaspar et al., 2001). *Xenopus* oocyte extracts seem to be exceptional as in this system both Cyclin A and B1 have a D-box that is required for their degradation (Glotzer et al., 1991; King et al., 1996; Kobayashi et al., 1992; Lorca et al., 1998).

Cyclin A levels begin to decrease in early prometaphase in HeLa and the non-transformed PtK1 cells (den Elzen and Pines, 2001). Destruction of Cyclin A is mediated by APC (Geley et al., 2001). However, in animal cells, evidence indicates that during prometaphase the spindle checkpoint keeps Cdc20 inactive until all the chromosomes have attached to both poles of the spindle (Dobles et al., 2000; Fang et al., 1998; Gorbsky et al., 1998; Li et al., 1997). While these contradictory results question the role of APC in Cyclin A degradation, genetic data and immunodepletion results clearly established the role of Cdc20 in this process. Cdc20 is required for Cyclin A degradation in *Drosophila*, *Xenopus* extracts and human cells because mutations in the Cdc20 homologue, *fizzy*, or anti-Fizzy/ Cdc20 antibodies, stabilize Cyclin A (Dawson et al., 1995; Geley et al., 2001; Lorca et al., 1998; Sigrist et al., 1995a). It therefore seems that Cyclin A is a better substrate for APC/C than securin and Cyclin B, and Cyclin A may be degraded at low Cdc20 activity (Geley et al., 2001).

CAK in cell cycle

Cdks are also regulated by positive and negative phosphorylation. Among these regulatory systems, CAK (Cdk Activating Kinase) is a pivotal positive regulator of Cdks (Harper and Elledge, 1998; Nigg, 1996). Most Cdks require the phosphorylation of a threonine residue located in the T-loop to achieve full kinase activity (Desai et al., 1992; Gould et al., 1991; Solomon et al., 1992). This threonine residue is conserved in all Cdks that function in cell cycle regulation (Nigg, 1995). The enzyme responsible for this phosphorylation is therefore termed Cdk-activating-kinase or CAK. The crystal structure of Cdk2-CyclinA showed that this threonine phosphorylation induces a conformation change that is important for substrate binding (Jeffrey et al., 1995; Russo et al., 1996). Interestingly, CAK itself is a Cdk/Cyclin complex, composed of Cdk7 and its partner CyclinH (Fesquet et al., 1993; Fisher and Morgan, 1994; Makela et al., 1994; Poon et al., 1993; Solomon et al., 1993).

Initial characterization of Cak activity was done with *Xenopus* egg extracts (Solomon et al., 1992) and mammalian cell extracts (Desai et al., 1992). CAK complexes were then purified from various systems and were found to be composed of Cdk7, Cyclin H and Mat1 (Devault et al., 1995; Fesquet et al., 1993; Fisher et al., 1995; Fisher and Morgan, 1994;

Labbe et al., 1994; Makela et al., 1994; Poon et al., 1993; Solomon et al., 1993; Tassan et al., 1995). Cdk7 was shown to be required for CAK activity *in vivo* in *Drosophila melanogaster* (Larochelle et al., 1998). By using conditional *cdk7* mutants, *cdk7* is demonstrated to be essential for CAK activity *in vivo* and it is required for the activation of both Cdc2/Cyclin A and Cdc2/Cyclin B, and for cell division. Recently, it is shown in *C.elegans* embryos that Loss of *cdk7* activity leads to cell cycle arrest in M phase and interphase (Wallenfang and Seydoux, 2002).

The CAK complex containing Cdk7 appears to constitute the major Cak activity in the cell because Cak activity fractionates as a single main peak after many chromatographic steps in *Xenopus* and mammalian cell extracts (Solomon et al., 1993; Fisher and Morgan, 1994) and immunodepletion and genetic inactivation of Cdk7 eliminates most detectable CAK activity (Larochelle et al., 1998; Poon et al., 1993; Solomon et al., 1993).

Recently, one report showed that CAK is required for human Herpersvirus 8 to re-enter the cell cycle (Child and Mann, 2001). In this report, viral DNA replication is generally dependent on circumventing host cell cycle control to force S phase entry in an otherwise quiescent cell. K cyclin encoded by human Herpersvirus 8 has high sequence similarity with D-type cyclin and functions like Cyclin E (Child and Mann, 2001). It has been shown that viral K Cyclin/Cdk6 requires CAK activity to be fully active to mediate S phase entry (Child and Mann, 2001).

Bulk CAK activity and levels of Cdk7 do not fluctuate during the cell cycle (Brown et al., 1994; Poon et al., 1994; Tassan et al., 1994). This argues against a role for CAK in cell cycle regulation. However, for a polypeptide that is required for multiple unrelated functions in cell cycle regulation and transcription, regulation is not restricted to the control of its level or total activity, but regulation may occur by differential recruitment of the Cdk7 from the large pool.

G1/S transition

G1 Cyclin/Cdk complexes, Cyclin D/Cdk4, Cyclin D/Cdk6 and Cyclin E/Cdk2, are the major regulators of G1/S transition. They all phosphorylate retinoblastoma protein RB during G1 progression to release the E2F transcription factors (Ewen et al., 1993; Kato and Sherr, 1993; Lundberg and Weinberg, 1998). Since only the hypophosphorylated form of

RB binds E2F, RB phosphorylation in G1 releases transcriptionally active E2F-1 (Mittnacht, 1998). E2F can then activate the transcription of genes required for entering S phase. E2F-1-binding sites are present in the promoters of several cell cycle regulators, such as *cdc2, cyclin E, cyclin A* and *E2F-1* itself (Dalton, 1992; Geng et al., 1996; Hsiao et al., 1994; Johnson et al., 1994; Ohtani et al., 1995; Schulze et al., 1995). In addition, they were found in the promoters of some proto-oncogenes involved in cell proliferation, including *c-myb, B-myb, c-myc* and *N-myc* (Amati et al., 1998; Hiebert et al., 1991; Hiebert et al., 1989; Lam and Watson, 1993; Mudryj et al., 1990).

Regulation of E2F occurs at different levels. E2F-1 is transcriptionally induced in late G1 and targeted to degradation in S phase (Hsiao et al., 1994; Johnson et al., 1993; Slansky et al., 1993). The tumor suppressor proteins p53 and Rb inhibit the transcriptional activity of E2F-1 (Brehm et al., 1998; Hagemeier et al., 1993; Hiebert et al., 1992; Luo et al., 1998; Magnaghi-Jaulin et al., 1998; O'Connor et al., 1995), whereas the onco-protein MDM2 increases its activity (Martin et al., 1995). Phosphorylation of the E2F-1 and DP-1 subunits by Cyclin A/Cdk2 inhibits the DNA-binding activity of the dimer (Dynlacht et al., 1994; Kitagawa et al., 1995; Krek et al., 1994; Xu et al., 1994). The activity of the E2Fs is also regulated by degradation via ubiquitin-dependent proteolysis *in vivo,* and targeting for degradation requires the E2F-1 activation domain. E2F-1 is stabilized by its interaction with Rb (Campanero and Flemington, 1997; Hateboer et al., 1996; Hofmann and Livingston, 1996).

Regulation of G1 Cdks occurs at different levels. Just like all other Cdks, G1 Cdks can be regulated through regulating the expression and degradation of Cyclins. In addition, the activity of Cdk4 and Cdk6 is negatively regulated by CKIs. The CKI INK4 family includes $p16^{INK4A}$, $p15^{INK4B}$, $p18^{INK4C}$ and $p19^{INK4D}$. These polypeptides have significant similarity in their primary structures and they all bind and inactivate Cdk4 and Cdk6 (Guan et al., 1994; Hannon and Beach, 1994; Serrano et al., 1993). Binding of the INK4 family inhibitors prevents Cdk4 and Cdk6 from phosphorylating Rb.

G2/M transition

Cdk1/Cdc2, master regulator of mitosis entry and progression

Mitosis is subdivided into four phases: prophase, metaphase, anaphase and telophase. In eukaryotic cell cycle, Cdc2 (Cdk1) is the major regulator of the G2-M transition and mitotic progression. The activity of Cdc2/Cyclin peaks at mitosis. Activated Cdc2/Cyclin complexes then phosphorylate numerous substrates, such as nuclear lamins, kinesin-related motors and other microtubule-binding proteins, condensins and Golgi Matrix components. These events are important for nuclear envelope break down, centrosome separation, spindle assembly, chromosome condensation and Golgi fragmentation, respectively (Nigg, 2001). Cdc2-Cyclin complexes also contribute to mitosis exit by activating APC that destructs Cyclin. With Cyclin destruction, Cdc2 is inactivated, allowing cells to exit mitosis. Cdc2 inactivation also allows the reformation of pre-initiation complexes at origins of replication, thereby licensing cellular chromosome for the next round of replication.

Regulation of Cdc2 activity occurs at multiple levels. First, Cdc2 is activated by Cyclin B binding. Cyclin B undergoes cell cycle dependent degradation and synthesis. Second, Cdc2/Cyclin B is subjected to active nuclear import and export during the cell cycle. During G2, Cdc2/Cyclin B is exported to the cytoplasm by the nuclear export protein Crm1, and at the G2/M transition it is translocated to the nucleus to phosphorylate its targets and to initiate mitosis (Hagting et al., 1998; Yang et al., 1998). Third, Cdc2 is activated by CAK through phosphorylation at Thr161 in its T-loop (Nigg, 1996). At last, Cdc2-Cyclin B is inhibited by phosphorylation at two negative regulatory sites, Thr14 and Tyr15. Phosphorylation of these two sites is catalyzed by two protein kinases Wee1 and Myt1 (Nigg, 2001). Cdc2-Cyclin B is activated by dephosphorylation of these two sites through phosphatase Cdc25 (King et al., 1994).

As a key regulator of mitosis, Cdc2 is the major target for the G2/M DNA damage checkpoint (Abraham, 2001). DNA damage prevents Cdc2 activation through multiple pathways. First, DNA damage seems to affect nuclear translocation of Cyclin B, sequestering Cyclin B/Cdc2 in the cytoplasm (Chan et al., 1999; Jin et al., 1998; Toyoshima et al., 1998). Second, It was shown that phosphorylation of Cdc2 on Thr161 was blocked by

p21 (Smits et al., 2000). The Cdk inhibitor p21 accumulates upon DNA damage and this is induced by the tumor suppressor p53 (el-Deiry et al., 1993; Harper et al., 1993). p21 was initially identified as inhibitor of G1 Cdks but recently it was shown to also play a crucial role in G2 DNA damage checkpoint (Bunz et al., 1998; Harper et al., 1993; Xiong et al., 1993). Third, DNA damage prevents the dephosphorylation of Thr14 and Tyr15 through Chk1 (Walworth et al., 1993; Walworth and Bernards, 1996). Chk1 was shown to phosphorylates Cdc25C and cause Cdc25C to bind to the 14-3-3 proteins (Peng et al., 1997; Sanchez et al., 1997). This binding prevents Cdc25C from translocating to the nucleus and activating Cdc2 (Lopez-Girona et al., 1999).

II. Transcription by RNA polymerase II

General transcription and TFIIH in transcription

In eukaryotes, RNA polymerase II (pol II) is responsible for synthesis of messenger RNAs. The best characterized budding yeast pol II is composed of 12 different polypeptides with a total mass of about 0.5 megadaltons (MD). All subunits of the human pol II are highly conserved with their yeast counterparts. At least 10 mammalian pol II genes can be substituted for their yeast counterparts and nine of them are also conserved among the three eukaryotic RNA polymerases I, II, and III. Pol II can unwind the DNA double helix, polymerize RNA, and proofread the nascent transcript on its own. With general transcription factors and other cofactors, Pol II can assemble larger initiation and elongation complexes, it is capable of promoter recognition and it responds to regulatory signals (Cramer et al., 2000). Transcription initiation involves a succession of events including the formation of a stable preinitiation complex, the ATP-dependent activation of the complex, promoter opening, first phosphodiester bond formation, and promoter escape. (Conaway and Conaway, 1993; Roeder, 1996). Transcription initiation by pol II is a multistep process that requires as a minimum the five general initiation factors TFIIB, TFIID, TFIIE, TFIIF and TFIIH (Dvir et al., 2001). First, the TATA binding protein [TBP] or the TBP-containing multiprotein complex TFIID bind sequence-specifically to the promoter to establish a nucleoprotein recognition site for pol II on the DNA. Second, TFIIB binds to both polymerase and TBP to form the TFIID/IIB/Pol II intermediate complex. Third, TFIIF binds to and strongly stabilizes the TFIID/IIB/Pol II complex, and it recruits TFIIE and TFIIH into the complex. After formation of the fully assembled preinitiation complex, a DNA

helicase activity associated with TFIIH catalyzes ATP-dependent unwinding of the DNA template at the transcriptional start site to form the open complex (Conaway and Conaway, 1993; Roeder, 1996).

TFIIH in general transcription

Among all the polypeptides necessary to initiate the transcription reaction, enzymatic activities have been defined for only three: Xpb, Xpd, and Cdk7, all of which are subunits of TFIIH [Roy, 1994 #124; Schaeffer, 1994 #460; Serizawa, 1995 #127; Schaeffer et al, 1993, (Shiekhattar et al., 1995). For the transcription initiation, the Xpb helicase plays a dominant role, whereas the Xpd helicase plays a minor role. The CAK subcomplex of TFIIH improves the efficiency of initiation, but this involves only the structural contributions of CAK rather than enzymatic activity (Bradsher et al., 2000). TFIIH functions in transcription initiation as well as in the promoter escape reaction. For the latter action, the Xpb helicase plays an enzymatic role in the movement of the polymerase, whereas the Xpd helicase activity appears to be dispensable although its physical presence plays an important role (Bradsher et al., 2000). Another study showed that TFIIH requires distinct regions of the DNA template, 23-39 and 39-50 bp downstream from the transcriptional start site in order to function in transcription initiation and promoter escape (Spangler et al., 2001).

CTD phosphorylation

The largest subunit of RNA Pol II contains an essential CTD (Carboxy-terminal domain) composed of tandem repeats of a heptapeptide with the consensus sequence Tyr-Ser-Pro-Thr-Ser-Pro-Ser (Bregman et al., 2000). The CTD is phosphorylated *in vivo* mostly at Ser2 and Ser5 but also at Thr4 and Tyr1 (Dahmus, 1995). In yeast cells, replacing either Ser2 or Ser 5 with Ala causes lethality (West and Corden, 1995). CTD phosphorylation plays a predominant role in transcription regulation and post-transcriptional RNA processing. During the initiation stage of transcription, a hypophosphorylated CTD form (RNA PIIa) is associated with the pre-initiation complex. Subsequently, CTD gets hyperphosphorylated (RNA PIIo) and it then assembles into a transcription elongation complex. At this time the other proteins that are involved in pre-mRNA processing (e.g., 5'

capping, polyadenylation, and splicing) bind to the CTD. After synthesis of a complete pre-mRNA transcript, the CTD must be dephosphorylated to regenerate the initiation competent form (IIa). Therefore, CTD kinases and CTD phosphatases are required for the dynamic activity of RNA Pol II.

Cdk7 (Cyclin H), Cdk8 (Cyclin C) and Cdk9 (Cyclin T) are three major CTD kinases Other possible CTD kinases include Cdc2, CTDK-1 in budding yeast, casein kinase II(CKII), and c-abl (Bregman et al., 2000; Majello and Napolitano, 2001). It was shown that the kinase activity of Cdk7 is required for CTD phosphorylation and for promoter clearence (Adamczewski et al., 1996; Akoulitchev et al., 1995; Cismowski et al., 1995; Dahmus, 1995; Dahmus, 1996; Feaver et al., 1994; Laybourn and Dahmus, 1989; Laybourn and Dahmus, 1990; Makela et al., 1995; Roy et al., 1994; Serizawa et al., 1995; Shiekhattar et al., 1995; Valay et al., 1995). Consistent with this, it was shown in *Drosophila* that the elongating RNA polymerase II has either a partially phosphorylated or hyper-phosphorylated CTD, and the pausing polymerase contains an unphosphorylated CTD (O'Brien et al., 1994). Strong evidence for Cdk7 function in transcription comes from the unicellular *Saccharomyces cerevisiae*. Kin28/Ccl1, the yeast protein with the best sequence similarity to metazoan Cdk7/CyclinH, is an active CTD kinase *in vivo* and *in vitro* (Cismowski et al., 1995; Feaver et al., 1994; Valay et al., 1995). By using a temperature sensitive allele of *kin28*, It was shown that loss of *kin28* function leads to significant decrease in mRNA synthesis (Cismowski et al., 1995; Valay et al., 1995).

CTD phosphatase activity has been identified in the FCP1 phosphatase (Majello and Napolitano, 2001). Human FCP1 is able to dephosphorylate the CTD *in vitro* (Cho et al., 1999). The yeast Fcp1 gene is essential for viability and for CTD dephosphosrylation *in vivo* (Kobor et al., 2000). Moreover, FCP1 phosphatase activity is required for the dissociation of the capping enzymes from the elongation complex (Schroeder et al., 2000).

CTD phosphorylation is a regulatory step for RNA Pol II transcription. RNAP II transcriptional activity is modulated during the cell cycle and this is altered by cell cycle dependent changes in CTD phosphorylation (Bregman et al., 2000). Tat stimulates HIV-1 transcription elongation by recruitment of the CTD kinases Cdk9 and Cdk7 to the HIV-1 promoter (Cujec et al., 1997; Zhou et al., 2000). The human immunodeficiency virus (HIV) encodes the transcriptional transactivator Tat, which binds to the transactivation response

(TAR) RNA stem-loop in the viral long terminal repeat (LTR) and increases rates of elongation rather than initiation of transcription by RNA polymerase II (Pol II) (Cujec et al., 1997). Interestingly, substrate specificity of CTD kinase is altered in the presence of the Tat. Cdk9 targets Ser2 in the absence of Tat but targets both Ser2 and Ser5 in the presence of Tat. On the other hand, Cdk7 phosphorylates Ser 5 of the CTD in the presence or absence of Tat (Zhou et al., 2000).

III. DNA repair

DNA repair pathways and genes

Various environmental agents, such as ultraviolet (UV) light from the sun, cigarette smoke, or incompletely defined dietary factors, can induce DNA damage and affect the structure and integrity of DNA molecules. Some DNA damage can arise spontaneously through intrinsic instability of chemical bonds in DNA. To keep the genome stability, DNA repair enzymes continuously monitor chromosomes and correct damaged nucleotide residues.

Among the DNA repair pathways that operate in response to the presence of damaged bases in the DNA, three biochemical pathways result in the excision of damaged or inappropriate bases. These are called base excision repair (BER), mismatch repair (MMR) and nucleotide excision repair (NER) (Friedberg, 2001; Wood et al., 2001). NER incorporates the excision of damaged bases as part of an oligonucleotide fragment (~25-30 bp), in contrast to the BER and MMR processes, in which damaged or mismatched bases are excised as a free base or single nucleotide.

NER and the function of TFIIH in NER

NER removes a wide variety of lesions, including UV-induced lesions, bulky chemical adducts and some forms of oxidative damage (de Boer and Hoeijmakers, 2000). In *E.coli*, three proteins UvrA, UvrB, and UvrC can locate a lesion and incise on either side of it to remove the damaged DNA segment. Eukaryotes use a more elaborate system to perform NER, involving the action of at least 30 proteins in a "cut-and paste" like mechanism (de Boer and Hoeijmakers, 2000). NER consists of several steps (de Boer and

Hoeijmakers, 2000; de Laat et al., 1999): 1) *Sensing of damaged DNA*. XPC/HHR23B was recently shown to be a DNA-damage sensor and the repair-recruitment factor (Sugasawa et al., 1998). XPE and XPA were also proposed to play a role in this step since they have high affinity for injured DNA (Jones and Wood, 1993; Keeney et al., 1993). 2) *Unwinding the DNA double helix around the injury*. This step requires the TFIIH complex, may be facilitated by RPA and may depend on ATP (Evans, 1997; Wood, 1997). Two subunits of TFIIH, XPB and XPD, contain ATPase dependent 3'-5' and 5'-3' helicase activity, respectively (Schaeffer et al., 1994; Schaeffer et al., 1993), and direct unwinding of the DNA helix (Evans, 1997). 3) *Dual incision of 24-32 nucleotides containing the lesion.* This requires XPG and ERCC1/ XPF, which cut the damaged strand at the 3' and 5' side, respectively (O'Donovan et al., 1994; Sijbers et al., 1996). In this step, RPA and TFIIH seem to help to position XPG (and XPA) at the damaged DNA (de Laat et al., 1998; Iyer et al., 1996). 4) *Filling the resulting gap by DNA repair synthesis.* For this step *in vitro* data shows that efficient repair synthesis requires RPA, RFC, PCNA and DNA polymerase δ and ε (Shivji et al., 1995). 5*) Ligating the newly synthesized strand to the pre-existed one*. DNA ligase I was shown to be a likely enzyme for this step (Barnes et al., 1992).

Two modes of NER can be distinguished: repair of lesions over the entire genome, referred to as global genome NER (GG-NER), and repair of transcription-blocking lesions present in transcribed DNA strands, hence called transcription-coupled NER (TC-NER) (de Laat et al., 1999). The process described above is the process of GG-NER. TC-NER has essentially the same process except for the initial damage recognization step. In the TC-NER, the stalled RNA polymerase II complex itself seems to be the DNA damage signal to attract the NER machinery (Donahue et al., 1994; Mu and Sancar, 1997). XPC/HHR23B seems to be the only known NER factor that is dispensable for TC-NER (Venema et al., 1991).

Core-TFIIH and Xpd are required for the DNA repair process (Araujo et al., 2000; Frit et al., 1999; Winkler et al., 2000). However, The requirement for Cak is not clear, it is shown that Cak is not required for *in vitro* NER (Svejstrup et al., 1996) and *in vitro* core-TFIIH has higher dual incision activity than holo-TFIIH (Araujo et al., 2000).

NER defect syndromes

The consequences of a defect in one of the NER proteins result in one or a combination of the three rare recessive syndromes: Xeroderma pigmentosum (XP), Cockayne Syndrome (CS) and Trichothiodystrophy (TTD) (de Boer and Hoeijmakers, 2000). Seven complementation groups within the NER-deficient class of XP patients are identified and designated XP-A to XP-G. Two complementation groups for CS, CS-A and CS-B, and three for TTD groups, XP-B, XP-D and TTD-A are identified. In addition, combined XP and CS (XP/CS) syndromes exist and they are assigned to three complementation groups: XP-B, XP-D and XP-G (de Boer and Hoeijmakers, 2000).

XP is characterized by hyperpigmentation of the skin under sun exposure and cutaneous abnormalities. In addition, it is associated with 1,000-2,000 fold increased risk to develop skin cancer, mainly basal cell carcinomas, squamous cell carcinomas and melanomas (de Boer and Hoeijmakers, 2000). Some XP patients also display neurological abnormalities caused by primary neuronal degeneration. CS and TTD patients display sun sensitivity but have no predisposition to skin cancer. CS and TTD also displays severe clinically distinct developmental retardation and neurological degeneration (Lehmann, 2001).

IV. Other substrates or interacting factors of TFIIH

Besides Cdks and CTD, a large variety of substrates of TFIIH/CAK were identified *in vitro*. They are mainly transcription regulators that function in various pathways. It appears that the activity of a transactivator can be activated through phosphorylation by TFIIH associated Cdk7. TFIIH-associated Cdk7 efficiently phosphorylates the nuclear receptor RARα (Retinoic Acid Receptor) on Ser77, and this phosphorylation is critical for the transactivation by RARα (Rochette-Egly et al., 1997; Rochette-Egly et al., 1995). Similarly, TFIIH associated Cdk7 phosphorylates ERα (Estrogen receptor) on its Ser118 and this phosphorylation is required for efficient transactivation by ERα (Chen et al., 2000). Collectively, TFIIH was shown to phosphorylate the POU (DNA binding) domain of Oct factors (Octamer binding transcription

factors), transcription factor TBP, TFIIE(p56) and TFIIF(RAP74) (Inamoto et al., 1997; Rossignol et al., 1997; Roy et al., 1994; Yankulov and Bentley, 1997).

TFIIH and E2F

Pearson and Greenblatt (1997) pointed to the connection between TFIIH and E2F. TFIIH was shown to bind to E2F and this binding stimulates the transactivation of E2F. Interestingly, Rb competes with TFIIH to bind to E2F at its activation domain (Pearson and Greenblatt, 1997). Another group showed that E2F-1 is phosphorylated by the TFIIH kinase *in vitro* and this phosphorylation may trigger its rapid degradation (Vandel and Kouzarides, 1999). E2F-1 is phosphorylated by TFIIH kinase *in vitro* at two sites in its activation domain, Ser 403 and Ser433. TFIIH was found associate specifically in S phase with E2F-1 (AD) *in vivo* when E2F is degraded rapidly. This interaction is restricted to S phase because during the G1 phase, RB associates with E2F and this prevents the binding of TFIIH (Dyson, 1998; Helin, 1998; Vandel and Kouzarides, 1999).

Cdk7 and p16 ^{INK4A}

INK4 family inhibitors prevent Cdk4 and Cdk6 from phosphorylating Rb. However, only mutations in $p16^{INK4A}$ have been found to correlate with human tumors. This suggests that the ability to inhibit pRb kinase activity may not be the sole determinant of the tumor suppressor activity of $p16^{INK4A}$ and that tumor suppressor function of $p16^{INK4A}$ is at least partially mediated by a different pathway (Kamb et al., 1994; Koh et al., 1995; Lukas et al., 1995; Monzon et al., 1998; Nobori et al., 1994; Yarbrough et al., 1999). Recent experiments have revealed an additional function of $p16^{INK4A}$. $p16^{INK4A}$ (but not $p15^{INK4B}$) specifically inhibits phosphorylation of the CTD by TFIIH and it is suggested that this inhibition contributes to the ability of $p16^{INK4A}$ to induce cell cycle arrest (Nishiwaki et al., 2000; Serizawa, 1998). In Hela cells, $p16^{INK4A}$ and Cdk7 coimmunoprecipitate and co-localize with each other, indicating their physical interaction (Nishiwaki et al., 2000). It is suggested that $p16^{INK4A}$ is in the transcription complex and that it regulates the cell cycle not only via the inhibition of pRb phosphorylation but also at the transcription level via the inhibition of CTD phosphorylation (Nishiwaki et al., 2000).

Cdk7 and p53

Activation of p21 by p53 in response to DNA damage causes cells to arrest in G1 (Harper et al., 1993; Pietenpol et al., 1994), allowing some time for repair of damaged DNA or for triggering apoptosis if DNA repair fails (Attardi et al., 1996). p53 is subjected to various post-translational modification like e.g. phosphorylation, glycosylation and acetylation (Hecker et al., 1996; Knippschild et al., 1996; Lohrum and Scheidtmann, 1996; Meek, 1994). *In vitro* studies showed that Cdk7/CyclinH is able to phosphorylate p53 in a manner that is strongly stimulated by Mat1, and this phosphorylation of p53 enhances its specific DNA-binding activity (Ko et al., 1997; Lu et al., 1997). On the other hand, p53 down-regulates CAK activity, resulting in significant down-regulation of phosphorylation of Cdk2 and CTD. This down-regulation is observed in p21-/- cells upon irradiation, indicating the involvement of a CAK dependent and CKI-independent mechanism in p53 triggered growth arrest (Schneider et al., 1998).

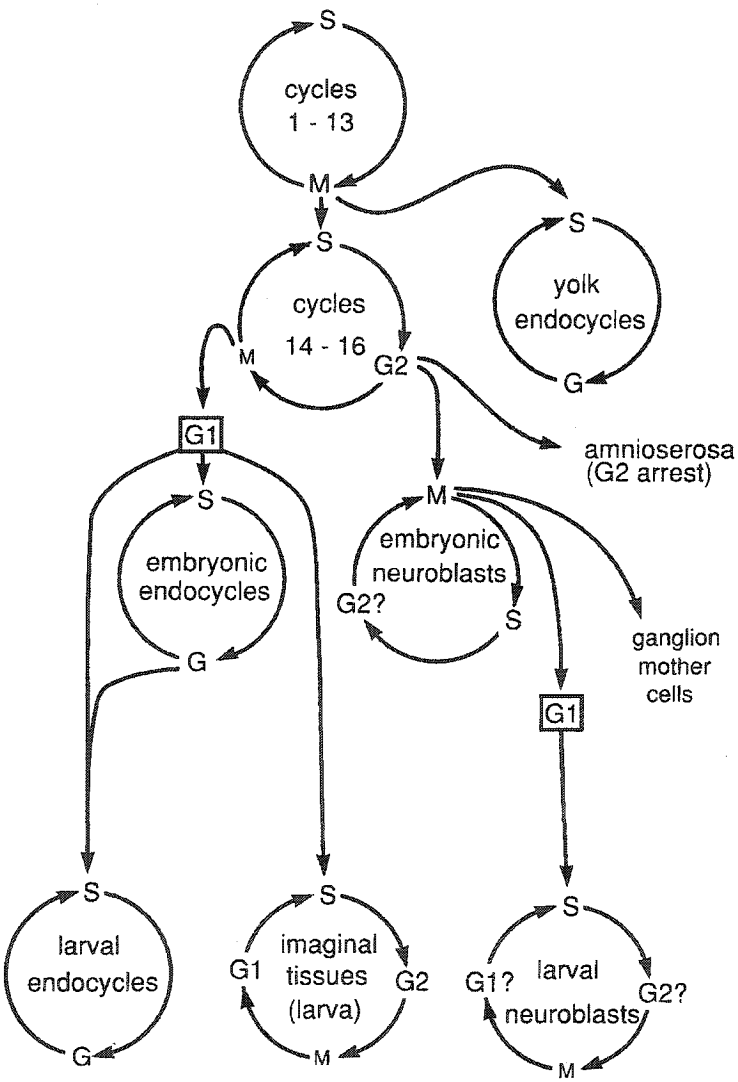
It is found that Xpb/Ercc3, Xpd/Ercc2 and p62 are capable to interact with p53 *in vitro* and it has been demonstrated that p53 potentially modulates TFIIH-associated NER activity by inhibiting the intrinsic DNA helicase activity of Xpd and Xpb (Wang et al., 1996).

TFIIH and *c-myc* expression

Liu *et al* (2002) reported that TFIIH mutations found in xeroderma pigmentosum block transcription activation by the FUSE binding protein (FBP), a regulator of *c-myc* expression, and repression by the FBP interacting repressor (FIR) (Liu et al., 2001). FBP activates transcription by interacting with TFIIH and stimulating initiation and promoter escape, whereas FIR represses transcription by interacting with TFIIH and inhibiting promoter escape by pol II. These findings provide direct evidence that activators and repressors can regulate pol II transcription at both initiation and promoter escape by controlling the activity of TFIIH.

V. Overview of the *Drosophila* system

Drosophila embryos undergo 17 rounds of mitotic cycles before they reach the larval stage (see graph below, adopted from (Foe, 1993)). The first 13 cycles are nearly synchronized syncytial cycles and the nuclear divisions occur very rapidly in a common cytoplasm. These 13 cycles are driven by maternal factors that have only the two phases S and M.
Starting in cycle 14, the cell divisions are controlled by zygotic factors and a G2 phase is introduced before the mitotic phase 14. During this interphase 14, the cell cycle slows down and



the embryos cellularize. Also starting with mitotic Cycle14, cell divisions become asynchronous. Most embryonic cells enter an extended G1 phase after Cycle 16 (Foe, 1993). At the larval stage,

the animals mainly grow and do not have mitotic divisions except for the imaginal disc cells which use all four cell cycle phases G1/S/G2/M and will give rise to the adult body. Most larval tissues, such as salivary glands, undergo endoreplication without cell division, giving rise to polyploid larval cells (Cohen, 1993). One advantage for studying the function of Cdk7 in cell cycle and transcription regulation in *Drosophila* is that various tissues have different versions of the cell cycle. Different requirements can therefore be analyzed in an individual animal.

Chapter 2

Drosophila Cdk7 and its role in transcription

I. Introduction

Although *in vitro* CTD kinase activity of vertebrate Cdk7 is well demonstrated, *in vivo* evidence was missing (when this project was initiated). Only recently, it was shown in *C.elegans* embryos that loss of *cdk7* activity reduces CTD phosphorylation and mRNA transcription (Wallenfang and Seydoux, 2002). Previous studies showed that expression of a dominant negative Cdk7 delays the onset of transcription in *Drosophila* embryos (Leclerc et al., 2000). However, with the cdk7 temperature sensitive allele $cdk7^{ts1}$, transcriptional defects were not detected (Larochelle et al., 1998).

To find out whether *Dmcdk7* is involved in transcription and to further understand the *in vivo* functions of *Drosophila* Cdk7, I analyzed the expression and the localization pattern of Cdk7 in endoreplicating cells. These cells undergo DNA replication without cell division, therefore their development is independent of mitosis. Moreover, I performed *in vitro* experiment to test whether *cdk7* is involved in expression of heat shock proteins.

II. Results

Cdk7 in endoreplicating and mitotic tissues

Two isoforms of Cdk7 can be resolved from wild type flies and the fast migrating isoform is the T-loop phosphorylated Cdk7 (Figure 1). The ratio of the two isoforms of Cdk7 varies in different tissues (Fig 1A) while the amount shows no obvious change (data not shown). It appears that in tissue containing endoreplicating cells (G/S), such as whole larvae and salivary glands, The ratio of phosphorylated to unphosphoylated Cdk7 is relatively high. In mitotic cells from imaginal discs (G1/S/G2/M), the two isoforms of Cdk7 are almost evenly represented.

Cdk7 expression in different cell cycle phases

I analyzed Cdk7 levels and T-loop phosphorylation during the cell cycle in early embryonic cells (cycle 10-13). Figure 1B shows that Cdk7 levels and phosphorylation do not vary significantly during different cell cycle phases, interphase, prophase, metaphase, anaphase and telophase. This is consistent with previous work showing that bulk CAK activity and Cdk7

levels do not vary during the cell cycle (Brown et al., 1994; Poon et al., 1994; Tassan et al., 1994).

Germline Cdk7 localizes to nuclei of nurse cells and oocytes

Drosophila nurse cells undergo endoreplication and they are very active in transcription. They synthesize large amounts of various transcripts and proteins, which are transported to the oocyte where they are sorted until they are used to support the development of early stage embryos (Spradling, 1993). Since the physiological function of a protein is often reflected by its localization pattern, I analyzed the localization of Cdk7 in nurse cells. Immunostainings show that germline Cdk7 protein is predominantly found in the nuclei of nurse cells and oocytes (Figure 2). This nuclear localization of Cdk7 is consistent with previous reports and with its role in transcription (Tassan et al., 1994). Interestingly, some dots stained by anti-Cdk7 antibody were repeatedly found arround the nuclei (Figure 2). We have not identified these dots, but evidence suggests that these dots could be Cajal or coiled bodies. Cajal bodies (CBs) are small nuclear organelles that contain the three eukaryotic RNA polymerases and a variety of factors involved in transcription and processing of all types of RNAs. It is suggested that pol I, pol II, and pol III transcription and processing complexes are pre-assembled in the CBs before transport to the sites of transcription on the chromosomes and in the nucleoli (Gall, 2000; Gall, 2001). Cdk7 was previously reported to localize within Cajal bodies (Grande et al., 1997; Jordan et al., 1997). It is worth to point out that many interacting factors or substrates of Cdk7 were found in Cajal bodies, including TBP, TFIIF and CTD of RNA polymerase II (Gall et al., 1999; Schul et al., 1998). Ser-5 phosphorylated CTD was also found in Cajal bodies in amphibian oocytes and Ser-5 is the target site of Cdk7 (Morgan et al., 2000). These data suggest that Cajal bodies are sites of assembly or modification of the polymerase subunits and transcription machinery of the nucleus.

Another Cdk, Cdk2 is shown to accumulate within Cajal bodies. This accumulation is cell cycle-dependent and depends on Cyclin E (Liu et al., 2000). Cdk2 is localized to Cajal bodies at the G1/S transition and /or early S phase, coinciding with the expression of Cyclin E. During the G2 phase, Cdk2 redistributes from Cajal bodies to the nucleoplasm and this correlates with the disappearance of Cyclin E. It is suggested that it is in Cajal bodies where Cdk2/Cyclin E gets activated and where it activates its diverse substrates (Liu et al., 2000). In late S phase, Cdk2

associates with Cyclin A and is incorporated into DNA replication forks (Cardoso et al., 1993). Unlike Cyclin E, Cyclin A is not found in Cajal bodies (Liu et al., 2000).

cdk7 loss-of-function flies have defects in endoreplication tissues

To address the question of whether Cdk7 is involved in transcription *in vivo*, I characterized the phenotype of *cdk7* loss-of-function mutations and compared it with that of the *cdc2* loss-of-function mutations to find out if Cdk7 has functions other than activating Cdc2. If Cdk7 only functions through the Cdc2 pathway, the phenotype of the *cdk7* loss-of-function mutation should be a subset of the *cdc2* loss-of-function phenotype. If novel phenotypes specific to *cdk7* loss-of-function are found, they would point to additional functions of *cdk7*.

The salivary gland cells of fly larvae go through endocycles during which DNA replicates but without intervening mitoses and they do not require *cdc2* activity. Consistent with this notion, the same experiment done with the *cdc2* mutant (Stern et al., 1993) showed that the salivary glands of 3rd instar larvae from wild type and *cdc2* mutants are indistinguishable in both the intensity of the DNA staining and in total size.

DNA staining of intact salivary glands in wild type and $cdk7^{null}$ flies showed that the intensity of DNA staining in mutant flies (Figure 3B and 3B1-3) is the same as in wild type (Figure 3A and 3A1-3) and total number of salivary gland cells is also similar to wild type. However, the size of the salivary glands in *cdk7* loss-of-function flies (Figure 3B) is only about one-fourth or less than that of wild type flies (Figure 3A). This smaller size is due to reduced cytoplasm but not due to nuclear differences such as different DNA contents. Taken together, these observations strongly suggest that *in vivo* Cdk7 is not only involved in Cdc2 activation but has at least one other function. This additional function may include transcriptional regulation.

cdk7 loss-of-function flies have defects in synthesis of heat shock proteins

To study whether *cdk7* loss-of-function flies have defects in gene expression, I used the heat shock system. Under the stress of heat shock, most preexisting transcription and protein synthesis events are shut off, while some heat shock proteins rapidly accumulate (Arrigo and Tanguay, 1991; Lindquist, 1980; Petersen and Lindquist, 1988; Yost and Lindquist, 1986). Because of its stringent control and its inducibility, heat shock response has been an useful system to study transcription regulation. I tried to find out if the isolated tissue from the *cdk7*

loss-of-function mutant has the ability to express specific genes. In heat shock experiment, I compared the ability to synthesize heat shock proteins in wild type flies and *cdk7* loss-of-function flies. Figure 4 shows that small Heat shock proteins are not detectable in $cdk7^{null}$ larvae upon heat shock and Hsp83 is not induced above non-heat shock levels either.

Cdk7 T-loop phosphorylation varies depending on different temperatures

Interestingly, Cdk7 T-loop phosphorylation increases upon temperature up-shift (Figure 5). I did two types of experiments. In one experiment, I gave a 30 min heat shock (37°C) to a collection of embryos (two to four hour old). A higher ratio of T-loop phosphorylated Cdk7 versus unphosphorylated Cdk7 was seen in heat shocked embryos (Figure 5A, upper panel). As a control, I looked at the phosphorylation level of the CTD of the largest subunit of RNA polymerase II. I also recovered a high level of hyper-phosphorylated RNA polymerase II (IIo) in my heat shock experiments [Figure 5A, lower panel]. This is consistent with result in in mammalian systems. It was shown in mammalian systems that during heat shock the overall phosphorylation of RNA polymerase II increases (Dubois et al., 1994; Dubois et al., 1997), while in unstressed cells, the ratio of hypo- (IIa) to hyper-phosphorylated (IIo) RNA polymerase II is about 1:1, in heat-shocked cells more hyper-phosphorylated RNA polymerase II is recovered (Dubois et al., 1997).

In order to confirm the effect on Cdk7 phosphorylation by temperature up-shift, I raised the adults at different temperatures (18°C, 25°C, 29°C), let them lay eggs and harvested them after four hours at the respective temperature. Similarly, in parallel to increasing temperature, the ratio of phosphorylated to unphosphorylated Cdk7 rises (Figure 5B). Because embryonic development is faster at high temperature than at low temperature, the 29°C collection was composed of older embryos than the collection at 18°C. However, the increase of Cdk7 phosphorylation is not likely to be due to the older age of the embryos collected at high temperature, because the T-loop phosphorylation of Cdk7 does not increase with embryonic age (Larochelle et al., 2001).

Heat shock protein synthesis is delayed in *cdk7*^{S164AT170A} mutants

Drosophila Cdk7, as its human counterpart, is phosphorylated at two sites within its T-loop, Ser164 and Thr170. Since Cdk7 T-loop phosphorylation increases upon temperature up-shift and under heat shock, I asked whether this phosphorylation is essential for the heat shock response. To answer this, I analyzed heat shock protein synthesis in *cdk7*^{S164AT170A} mutants which have both Ser164 and Thr170 sites mutated to alanine (Larochelle et al., 2001). Figure 6 shows that upon heat shock small heat shock proteins are not efficiently synthesized in *cdk7*^{S164AT170A} mutants compared to wild type animals. However, the amount of small heat shock protein in the mutants is increasing upon prolonged heat shock time (after 120 min). It thus appears that *cdk7*^{S164AT170A} mutants require longer time to synthesize the heat shock proteins, indicating that their synthesis is delayed. This observation is consistent to the temperature sensitive phenotype of *cdk7*^{S164AT170A}. Adults of these *cdk7*^{S164AT170A} mutant are viable at 18°C but die after 3 days at 29°C. Furthermore, *cdk7*^{S164AT170A} mutant larvae do not survive after a 120-min heat shock.

III. Discussion

Cdk7 and transcription

The fact that *cdk7* loss-of-function flies have defects in salivary gland cells, which grow independent of *cdc2* activity, strongly suggests that Cdk7 participates in cell cycle independent functions. I also found that heat shock proteins are not inducible in *cdk7* loss-of-function flies, indicating that Cdk7 is involved in synthesis of heat shock proteins, at least under our experimental condition. Because heat shock response is mainly controlled at the level of transcription, these data strongly suggested that *cdk7* has a role in transcription. Furthermore, the *cdk7* T-loop *cdk7*^{S164AT170A} causes delays synthesis of heat shock proteins. Together with the finding that T-loop phosphorylation occurs at high temperatures, this raises the question whether Cdk7 T-loop phosphorylation is required for the synthesis of heat shock proteins. Supporting this view, T-loop phosphorylation of Cdk7 stabilizes the Cdk7/Cyclin H/ MAT1 complex and greatly stimulates the activity of this complex towards the CTD of RNA polymerase II (Larochelle et al., 2001) (see Appendix IV).. Thus, T-loop mutation in flies is likely unable to support the CTD

phosphorylation that normally occurs after heat shock stress, resulting in a defect in heat shock response.

IV. Materials and Methods

Western blot

For Western blots with syncytial embryos (cycle 10 to 13), the embryos were treated as described by (Edgar et al., 1994). Briefly, the embryos were stained with DNA dye Hoechst 33258 and embryos at different cell cycle phases were sorted by their DNA pattern and handpicked under the microscope. Antibodies against Cdk7 were used at 1:20 dilution.

For analyzing heat shock proteins in $cdk7^{null}$ (Figure 4), wild type and $cdk7^{null}$ third-instar larvae were heat shocked at 37°C for 30, 60 and 90 minutes, and protein extracts corresponding to 1/4 of a larva were loaded on each SDS-PAGE lane. The antibodies against sHsp and Hsp83 were used at 1:100 dilution.

For Cdk7 phosphorylation at high temperatures (Figure 5A), wild type 2-4 hour embryos were collected and half of them were subjected to heat shock for 30 minutes at 37°C, the other half was kept at room temperature as control. For the Western blot shown in Figure 5B, wild type flies were continuously raised and 0-4 hour embryos were collected at 18°C, 25°C and 29°C.

For analyzing the heat shock protein synthesis in $cdk7^{S164A/T170A}$ (Figure 6), wild type and $cdk7^{S164A/T170A}$ third-instar larvae were heat shocked at 37°C for 30, 60, 90, 120 and 150 minutes. Protein extracts corresponding to 1/4 of a larva were loaded on each lane. The antibody against α-Tubulin was used at 1:500 dilution.

Immunostaining and DNA staining

Immunostaining of ovaries was performed as previously discribed (Suter and Steward, 1991). Cdk7 antibody was used at 1:2 dilution. Pre-absorbed Oregon Green conjugated anti-mouse antibodies (Molecular Probes) were used as secondary antibodies at 1:2000 dilution. Confocal images were taken on the Leica,TCS SP2 confocal laser scanning microscope.

For DNA staining of salivary glands, Hoechst 33258 was used at 1ug/ml during a one hour incubation at room temperature. The images were taken with the Zeiss Axioplan microscope.

Figure 1. Cdk7 expression in different tissues and cell cycle phases

A) Western blot of Cdk7 from the total protein extracts of different tissues.
salivary gland * : There are few cells in the anterior region of the salivary gland that are mitotic stem cells. In these salivary gland samples, the stem cells were completely removed.

B) Western blot of Cdk7 from the extracts of young embryos at different cell cycle phases.
pCdk7: T-loop phosphorylated isoform of Cdk7.

Figure 1. Cdk7 in different tissues and cell cycle phases

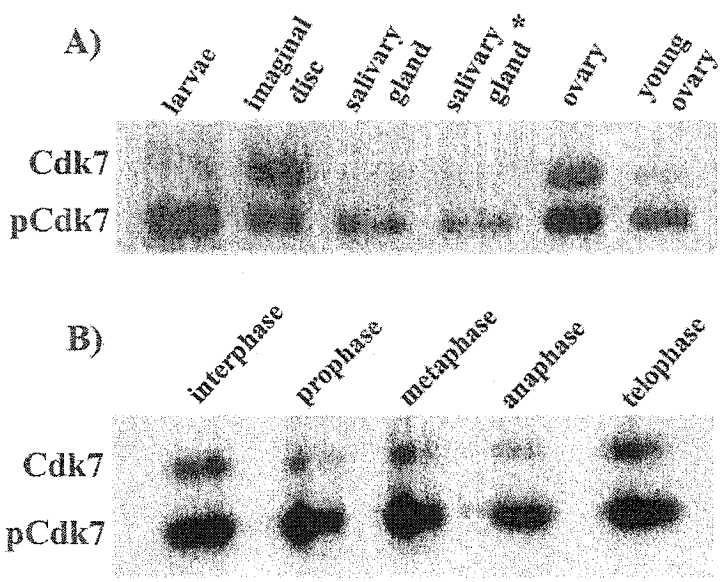


Figure 2. Cdk7 localizes to the nuclei of oocytes and nurse cells.

Confocal images of Cdk7 immunostaining of egg chambers. Cdk7 is predominantly localized to nuclei of oocytes and nurse cells. A) A stage 10 egg chamber is composed of one oocyte (its nucleus is marked with a *) and 15 nurse cells (only 8 are seen in this section). Number 1 and 2 indicate two nurse cell nuclei. B) and C) show magnified confocal sections of nurse cell nuclei 1 and 2, they are taken at different levels. Arrows indicate the Cajal body-like dots with Cdk7 staining.

Immunostaining of Cdk7 in egg chamber

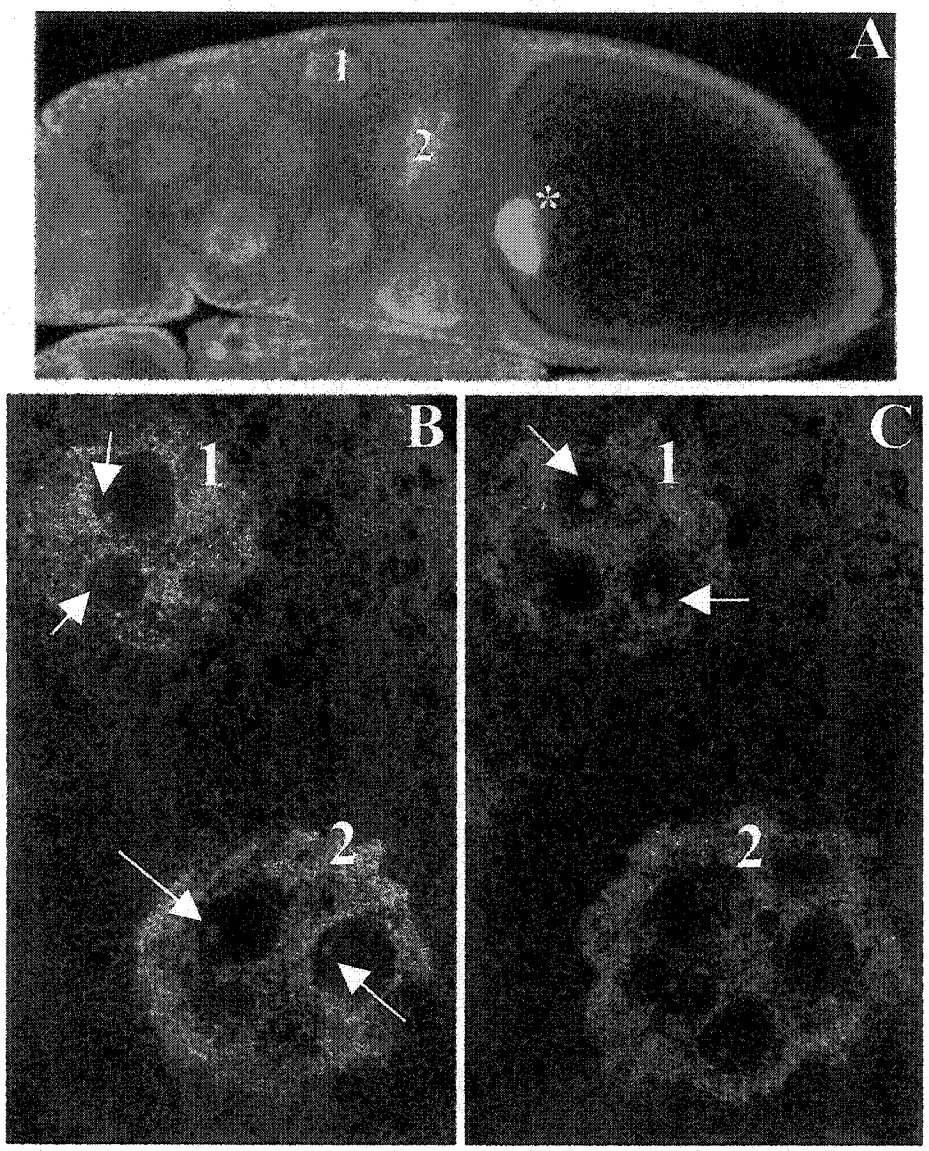


Figure 3. DNA staining of salivary glands from wt and *cdk7* ^{null} flies

DNA staining (Hoechst 33258) of the salivary glands from 3rd instar of wild type and *cdk7*^{null} larvae. A and A1-3 show salivary glands of wild type larvae and A1-3 are different magnified portions of wild type salivary glands. B and B1-3 are salivary glands from *cdk7* ^{null} and B1-3 are different magnified portions of the mutant salivary glands. The corresponding regions of the salivary gland from wt and *cdk7* ^{null} are shown side by side.

DNA staining of salivary glands from wt and *cdk7*^{null} larvae

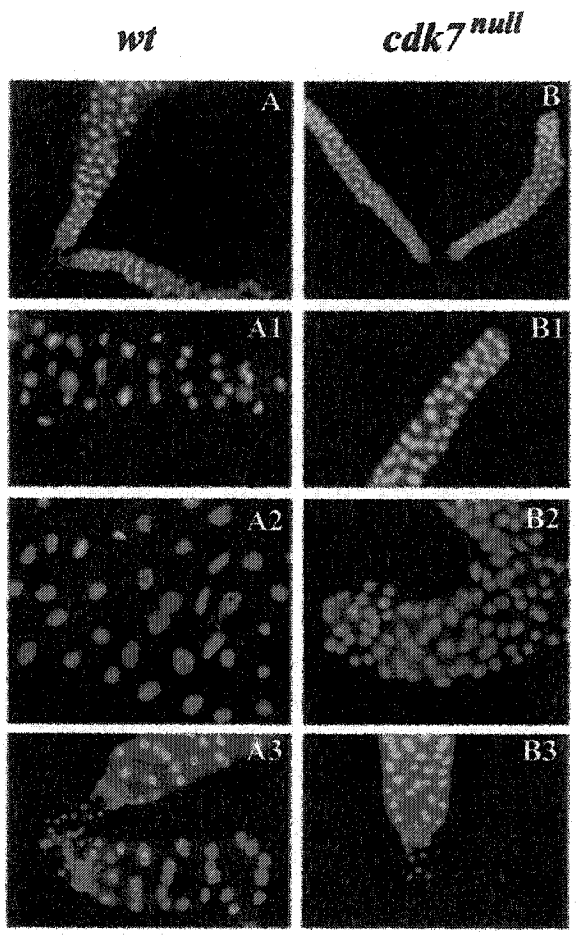


Figure 4 *cdk7*^{null} have defects in synthesis of heat shock proteins

A) western blot with antibodies against the small Hsps and Hsp83. B) total protein stained by Ponceau red as loading control. W: wild type; M: *cdk7*^{null}.

Heat shock proteins are not induced in *cdk7* loss-of-function flies

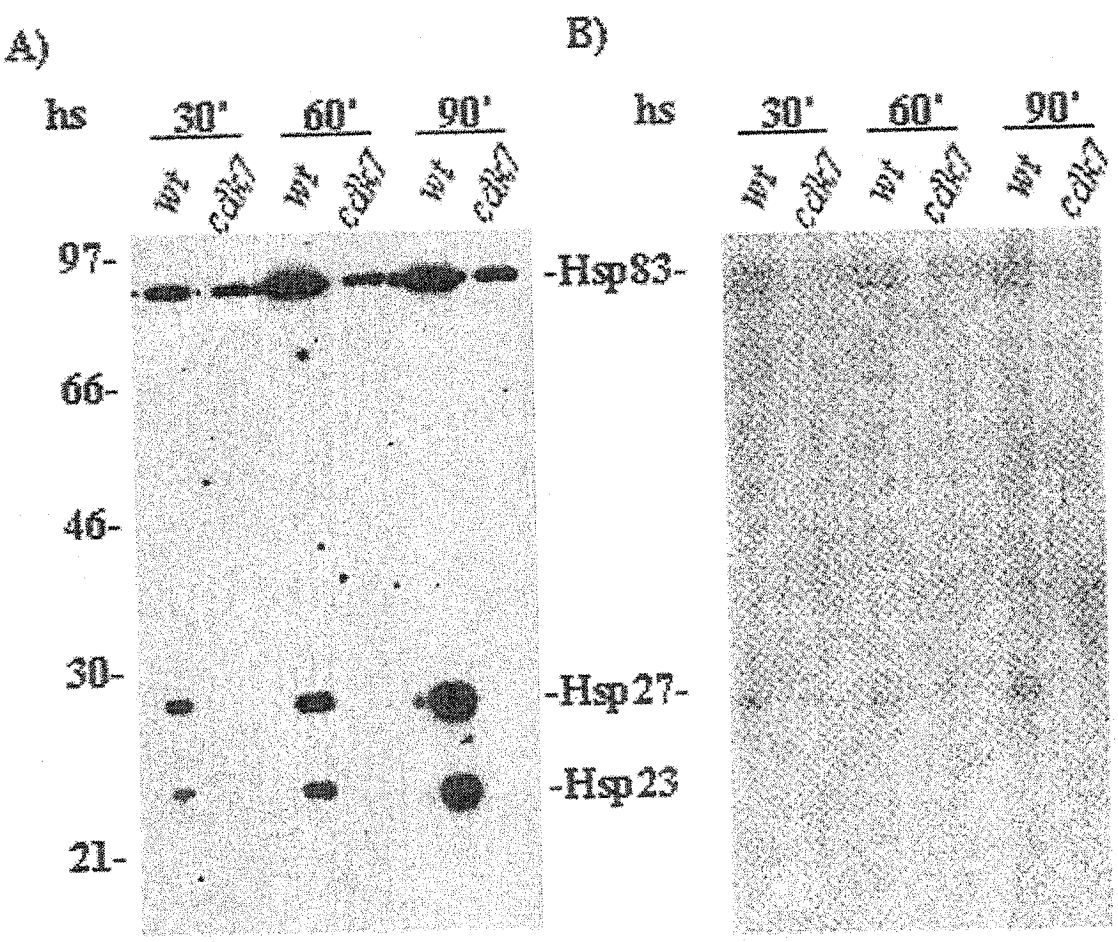


Figure 5. Cdk7 phosphorylation at high temperatures

A) Western blot of Cdk7 and the largest subunit of Pol II in embryos after heat shock. B) Western blot of Cdk7 from embryos kept at 18°C, 25°C and 29°C.

Figure 5. Cdk7 phosphorylation at high temperature

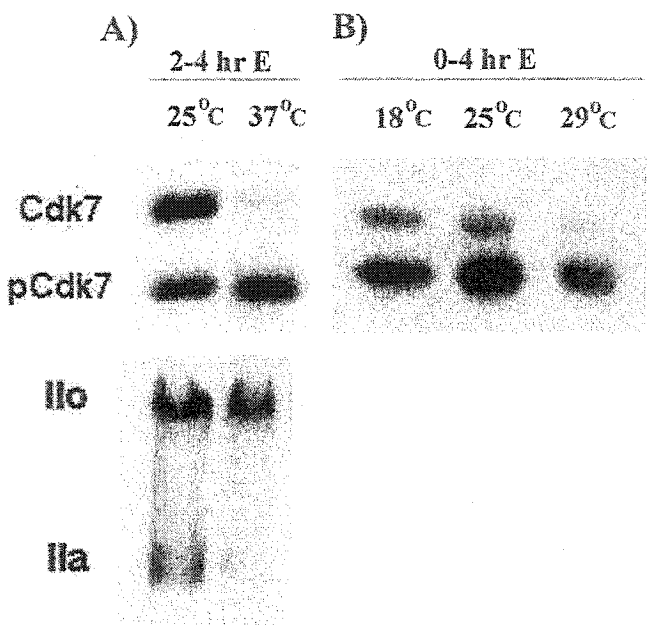
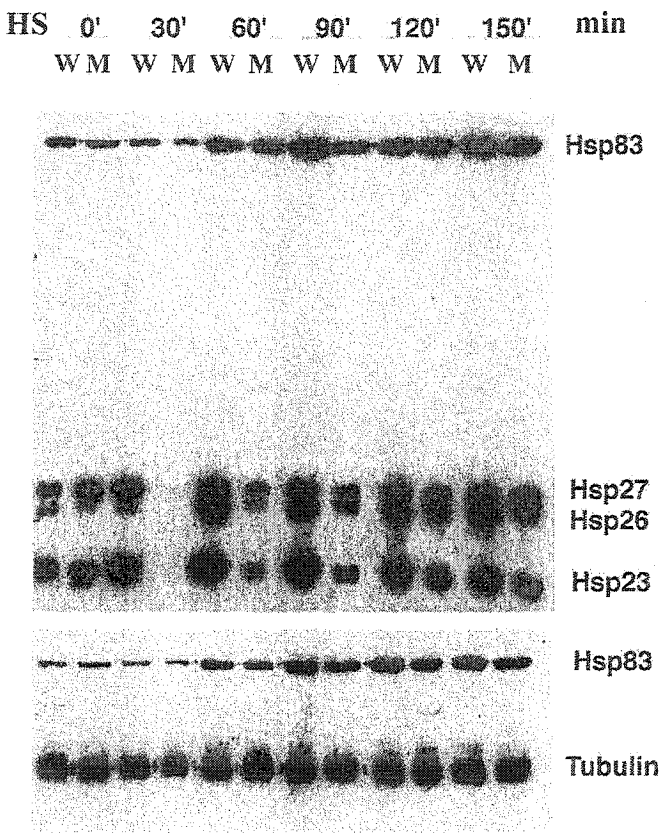


Figure 6. Heat shock protein synthesis in *cdk7*$^{S164A/T170A}$

The immunoblot was probed with antibodies against the small Hsps and Hsp83 for the upper blot. It was then reprobed with antibodies against Hsp83 and α-tubulin. α-tubulin serves as loading control. W: wild type; M: *cdk7*$^{S164A/T170A}$.

Figure 6. Heat shock protein synthesis in *cdk7*$^{S164A/T170A}$ flies



W: wild type M: *cdk7*$^{S164A/T170A}$

Chapter 3

Genetic screen for dominant *enhancers* and *suppressors* of *Dmcdk7*^{ts1}

I. Introduction

Drosophila Cdk7 acts as a Cak at least toward Cdc2 (Larochelle et al., 1998) and has a role in transcription, as suggested by dominant negative mutants (Leclerc et al., 2000) and my analysis of the null mutant (Chapter II). I am interested in the question whether and how different functions of Cdk7 are regulated *in vivo*. To address this question, I tried to identify factors that interact with Cdk7 in an attempt to find regulators of Cdk7's multiple functions. To identify genes involved in directing Cdk7 activity towards transcription or cell cycle function, I performed a screen for dominant suppressors and enhancers of a hypomorphic *cdk7* mutant ($cdk7^{ts1}$) that has only a cell cycle but no transcriptional defect.

Our $cdk7^{ts1}$ mutants are 100% viable at the permissive temperature (18°C) and non-viable at the restrictive temperature (27°C) (Larochelle et al., 1998). By growing the flies at intermediate temperatures we create a sensitized background in which reduction in the dose of *cdk7*-interacting genes could affect the viability of the $cdk7^{ts1}$ mutants. At the semi-permissive temperature of 22-25°C, a significant proportion of $cdk7^{ts1}$ mutants are viable, and we predict that reducing the dose of positive regulators of the cell cycle function of *cdk7* may reduce the viability of the mutant flies. At the restrictive temperature, $cdk7^{ts1}$ mutants are nonviable, and we predict that reducing the dose of dosage sensitive negative cell cycle regulators may increase the viability of the mutant flies.

II. Results

The genetic screens for dominant suppressors and enhancers were carried out by crossing the flies from the deficiency kit (a collection of deficiencies covering 80% of the fly genome provided by the Bloomington Stock Center, see Appendix 1) into the $cdk7^{ts1}$ mutants and testing for viability of the offspring at selected temperatures (Figure 1A). For the primary screen, a total of 109 stocks were used. Appendix II documents raw data from the primary screen, 103 deficiency stocks were tested at 25°C and 75 were tested at 23°C. 16 Candidate suppressors and 22 candidate enhancers are listed with their genotypes and breakpoints in Appendix III.

Thirteen candidate suppressors were selected for a large scale secondary screen. For 10 of them suppression of *cdk7* could be confirmed (Table 1). Amongst them, two strong suppressors, #3467 *[Df (2R) AA21]* and *#2606 [Df (2R) Pu-D17]*, have overlapping deletions and the cytological region 57B-57D is absent in both of them, suggesting that a suppressor gene maps to this interval (Table 1). To narrow down the suppressor gene, flies containing smaller deletions, #3469 *Df(2R)PK1*, #1916 *Df(2R)PI13* and #1510 *Df(2R)exu1*, were then tested for suppression. This allowed me to map the suppressor to 57C05-57D09 (Table 1b, Figure 1B). In this region, 10 complementation groups had been genetically characterized before. The ten point mutations in the 57C5-57D9 region are *l(2) 57Cc, l(2) 57Cd, anon-57C1, tud, l(2)57Ce, l(2) 57Da, l(2) 57Db, l(2) 57Dd, l(2) 57De, l(2) 57Dc*. Representative alleles of these were tested but showed no suppression. 5 other genes have been characterized at the molecular level but no mutation has been identified in them. In this latter group, the gene encoding Xpd is a likely candidate. Xpd/ERCC2 is known to physically associate with Cdk7 in TFIIH (Drapkin et al., 1996; Reardon et al., 1996; Schaeffer et al., 1994). I further mapped this dominant suppressor to *xpd* and I focused on the characterization of the biological significance of the negative genetic interaction between *cdk7* and *xpd* (see next chapter for detail).

For the enhancer screen, the 22 candidate enhancers are listed in Appendix III. From them, 13 stocks were selected for the large scale secondary screen, and for nine of them, their enhancing interaction with *cdk7* could be confirmed (Table 2). Further work is required to map them to individual genes.

Figure 1. Scheme of the genetic screen for suppressors of *cdk7^{ts1}* and cytological mapping of one suppressor region.

A) Virgin females of *cdk7^{ts1}* flies were crossed to the 109 male strains heterozygous for second chromosome deletions (shown as *Df*/+) or third chromosomes deletions (not shown). In the F1 generation, females (either *cdk7^{null}*/+; *Df*/+; *P[cdk7^{ts1}]*/+ or *cdk7^{null}*/+; *CyO*/+; *P[cdk7^{ts1}]*/+) are all heterozygous for the endogenous *cdk7^{+}* gene (on the 1st or X chromosome) and serve as positive control for the viability. All F1 males are *cdk7^{null}* for endogenous *cdk7* and have one *cdk7^{ts1}* transgene (on 3rd chromosome) as the only source of Cdk7, they are temperature sensitive. Half of the males inherit the *Df* chromosome (*cdk7^{null}*/Y; *Df*/+; *P[cdk7^{ts1}]*/+) and the other half the balancer chromosome (*cdk7^{null}*/Y; *CyO*/+; *P[cdk7^{ts1}]*/+). The appearance of these flies shows the survival of *cdk7^{ts1}* flies under the chosen condition and is therefore the internal standard. B) Suppression of *cdk7^{ts1}* was noticed initially in two strains *Df*(2R)AA21 and *Df*(2r)Pu-D17. The flies with the smaller deletion *Df*(2R)PK1, *Df(2R)PI13* and *Df(2R)exu1* were used to narrow down the suppression region. Suppressions was found with *Df(2R)PK1* and *Df(2R)PI13* but not with *Df(2R)exu1*. Therefore the suppression region is within the overlapping region of *Df(2R)PK1* and *Df(2R)PI13*, corresponding to 57C05-57D09.

A)

$$\frac{cdk7^{null}}{cdk7^{null}} \; ; \; \frac{+}{+} \; ; \; \frac{P[w^+,\ cdk7^{ts1}]}{P[w^+,\ cdk7^{ts1}]} \otimes \frac{+}{\neg} \; ; \; \frac{Df}{CyO} \; ; \; \frac{+}{+}$$

F1 :

♀

$$\frac{cdk7^{null}}{+} \; ; \; \frac{Df}{+} \; ; \; \frac{P[w^+,\ cdk7^{ts1}]}{+} \qquad \frac{cdk7^{null}}{+} \; ; \; \frac{CyO}{+} \; ; \; \frac{P[w^+,\ cdk7^{ts1}]}{+}$$

Both female classes are viable and serve as positive internal control

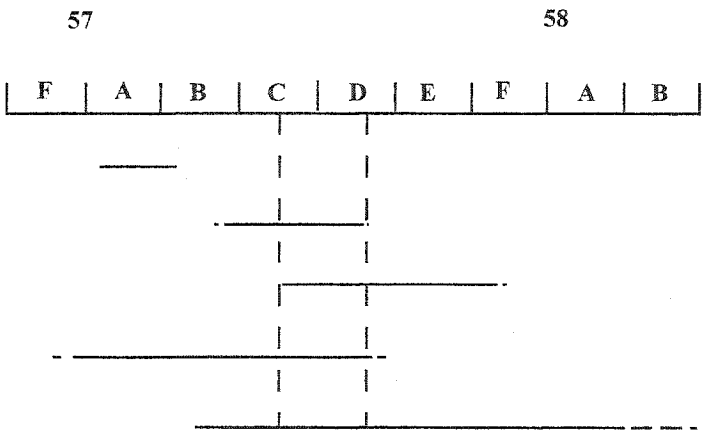
♂

$$\frac{cdk7^{null}}{\neg} \; ; \; \frac{Df}{+} \; ; \; \frac{P[w^+,\ cdk7^{ts1}]}{+} \qquad \frac{cdk7^{null}}{\neg} \; ; \; \frac{CyO}{+} \; ; \; \frac{P[w^+,\ cdk7^{ts1}]}{+}$$

viable at "restrictive" temperature
if Df acts as suppressor

lethal at restrictive temperature
as negative control

B)



Deficiency	Suppression
Df(2R)exu1	–
Df(2R)PI13	+
Df(2R)PK1	+
Df(2R)AA21	+
Df(2R)Pu-D17	+

Table 1. Secondary screen for dominant suppressors of *cdk7*^{ts1}

Table 1a) Secondary screen for dominant suppressors of *cdk7*

Stock No. region deleted	female		male		comments
	balancer	deficiency	balancer	deficiency	
3346	64	78	1	1	
038A01;039D03-E01	59	44	0	0	
	31	110	19	92	
	32	78	8	60	suppressor
757	57	91	0	0	
055E02-04; 056C01-11	51	53	0	0	
	62	53	1	2	
	91	74	49	68	
343	22	33	0	0	
036E06-F01; 036F07-09	18	14	0	0	
	61	65	33	49	
	43	90	32	75	
* 3467	119	132	4	34	suppressor
056F09-17;057D11-12	143	148	0	46	suppressor
	59	50	5	39	suppressor
	53	61	36	43	
1990.2	79	86	0	1	
083C01-02; 083D04-05	103	92	0	4	suppressor
084A04-05; 084B01-02	21	26	7	23	suppressor
	4	10	4	8	
3369	45	45	1	0	
100C;100D	31	20	0	0	
	28	34	0	12	suppressor
	30	60	16	31	
3127	73	83	0	0	
077B-C;077F-78A	72	88	0	0	
	17	18	0	0	
* 2606	110	95	0	11	suppressor
057B04;058B	97	104	0	2	suppressor
	14	23	0	0	
759	68	52	1	0	
102E02;102E10	42	44	0	0	
	45	46	7	15	suppressor
3000	66	90	0	0	
076A03;076B02	57	78	0	5	suppressor
	44	35	5	21	suppressor
2585	114	79	0	0	
095A05-07;095D06-11	72	61	0	0	
	50	54	1	7	suppressor
	36	52	26	26	
3157	66	52	1	1	
060E06-08;060F01-02	89	82	0	1	
	62	44	2	15	suppressor
	76	54	50	48	

556	92	71	0	0	
030C01-02;030F, 030B09-10	133	133	1	1	
	44	59	36	41	

Different colours indicate different temperatures at which flies were tested.
Red, blue, green and purple indicate that flies were kept at 27, 25, 23, 21-22 degrees.
* the deficiencies of the two strong suppressors 3467 *[Df(2R) AA21]* and #2606 *[Df (2R) Pu-D17]* overlap in the 57B-57D region.

Table 1b. Fine mapping of S*uppressor* of *cdk7in the* 57B-57D region

Stock No. Deficiency	female		male		comments
	Balancer	Deficiency	Balancer	Deficiency	
3467 *Df(2R) AA21*	73	69	23	80	suppressor
3469 *Df(2R)PK1*	41	47	20	43	suppressor
1510 *Df(2R)exu1*	136	118	98	82	non
1916 *Df(2R)PI13*	125	135	70	110	suppressor
2606 *Df (2R) Pu-D17*	28	62	22	104	suppressor

Four stocks with smaller deletions were tested, two of them, 3469 *Df(2R)PK1* and 1916 *Df(2R)PI13* showed suppression.

Table 2. Secondary screen for dominant enhancers of *cdk7*^{ts1}

Table 2. Secondary screen for enhancers of *cdk7*

Stock No. region deleted	female		male		comments
	Balancer	Deficiency	Balancer	Deficiency	
3573	170	188	4	0	enhancer
023C;023E03-06	89	101	17	2	enhancer
	90	111	91	57	enhancer
190	108	79	2	0	enhancer
047D03;048B02	73	71	39	31	
	96	102	77	38	enhancer
167	133	108	0	0	
038A06-B01;040A04-B01	69	57	1	0	
	96	48	61	33	
2892	68	29	1	0	
029C01-02;030C08-09	72	67	44	9	enhancer
	81	55	25	47	
1642	249	248	1	0	
049A-B;049D-E	66	74	26	17	
	68	76	33	47	
2583	220	185	0	0	
035F-036A;036D	64	77	1	0	
	63	38	61	48	
3343	92	86	0	0	
070A02-03;070A05-06	57	46	24	4	enhancer
	61	63	46	38	
3591	131	129	2	0	
044F10;045D09-E01	51	41	20	4	enhancer
	31	39	30	24	
442	150	124	0	0	
049C01-04;050C23-D02	46	48	40	15	enhancer
	49	37	30	23	
3520	97	94	1	0	
052F05-09;052F10-53A01	46	42	0	0	
	55	42	49	34	
2362	88	127	0	0	
095E08-F01;095F15	20	26	2	0	
	40	37	35	15	enhancer
1702	143	57	0	0	
046C;047A01	81	32	33	7	
	105	50	52	27	
693	137	136	2	0	*
024C02-08;025C08-09	46	42	36	0	*
	65	56	56	0	*

Different colours indicate different temperatures at which flies were tested.
blue, green and purple indicates that flies were kept at 25, 23, 21-22 dergee.
* contain a duplication of 024D04; 025F02 on the X chromosome; the deficiency causes dominant lethality in males that do not have the duplication

Chapter 4

A novel function for Xpd/Ercc2 in regulating CAK activity and mitotic progression

I. Abstract

Cyclin dependent kinase 7 (Cdk7) performs multiple distinct cellular functions. As the kinase subunit of general transcription factor IIH (TFIIH), Cdk7 acts in basal transcription. In addition, Cdk7 acts independent of TFIIH as a catalytic subunit of the Cdk-activating-kinase (CAK) to phosphorylate and activate other cell cycle regulating Cdks. Here we show that *Drosophila* Xpd, another TFIIH component, negatively regulates the mitotic CAK activity of Cdk7. Reduction of Xpd facilitates Cdk7's mitotic activity, while excess Xpd titrates CAK activity, resulting in reduced Cdk1/Cdc2 T-loop phosphorylation, mitotic defects and lethality. At the beginning of mitosis, when Cdc2, the cell cycle target of Cdk7, needs to be active, the negative regulator Xpd is down-regulated. Down-regulation of Xpd thus appears to contribute to the up-regulation of mitotic CAK activity and to positively regulate mitotic progression. Simultaneously, this down-regulation can be expected to be a major mechanism of mitotic silencing of basal transcription.

II. Introduction

TFIIH is composed of nine subunits, the CAK complex Cdk7, Cyclin H, MAT1, the two helicases Xpb and Xpd, and p34, p44, p52, p62 (Adamczewski et al., 1996; Frit et al., 1999; Roy et al., 1994; Serizawa et al., 1995; Shiekhattar et al., 1995). All TFIIH components are conserved from yeast to human (Frit et al., 1999). TFIIH phosphorylates the CTD (Carboxy-Terminal-Domain) of the largest subunit of RNA polymerase II (Akoulitchev et al., 1995; Makela et al., 1995)a step thought to be essential for transition of the RNA polymerase II holoenzyme from transcription initiation to elongation (Dahmus, 1995; Dahmus, 1996; Laybourn and Dahmus, 1989; Laybourn and Dahmus, 1990). Consistent with this idea, it was shown in *Drosophila* that the elongating RNA polymerase II has a hyper-phosphorylated CTD, and the pausing polymerase contains an unphosphorylated CTD (O'Brien et al., 1994). Strong evidence for Cdk7 function in transcription comes from *Saccharomyces cerevisiae* where Kin28/Ccl1, the yeast ortholog of Cdk7/CyclinH, is an active CTD kinase (Cismowski et al., 1995; Feaver et al., 1994; Valay et al., 1995). Such a transcriptional function was also found for *Drosophila cdk7* (Leclerc et al., 2000) (Chapter II).

Cdk7 is also found in a smaller complex, together with Cyclin H and Mat1. This free CAK complex can phosphorylate and activate other Cdks (Cyclin dependent kinases), the key regulators of the cell cycle. The successive activation and inactivation of these Cdks are the force that drives the cell cycle forward (Morgan, 1997; Nigg, 1995). The activities of Cdks are under precise regulation by multiple mechanisms such as positive and negative phosphorylation and binding of regulatory proteins like Cyclins and Cdk inhibitors (Morgan, 1995; Morgan, 1997). Most Cdks require the phosphorylation of a conserved threonine residue located in the T-loop to achieve full kinase activity (Desai et al., 1992; Gould et al., 1991; Nigg, 1995; Solomon et al., 1992). And *Drosophila* Cdk7 is essential for the T-loop phosphorylation of Cdc2(Cdk1) and for its activity (Larochelle et al., 1998).

Because Cdk7 is involved in such diverse functions as cell cycle control and transcription, it has long been postulated that Cdk7 may represent a critical link between the cell cycle and transcriptional programs (Fisher and Morgan, 1996; Frit et al., 1999; Nigg, 1995). *Drosophila* is a good system to address the question of whether and how these two functions of Cdk7 are regulated *in vivo* in a multicellular organism. To identify genes involved in directing Cdk7 activity towards transcription or cell cycle function, we performed a screen for dominant suppressors and enhancers of a hypomorphic *cdk7* mutant that has only a cell cycle but no transcriptional defect. In this report we describe that lack of one copy of *xpd/Ercc2* (Xeroderma Pigmentosum disorder group D/ Excision repair complementing group C) leads to a strong suppression of the hypomorphic *cdk7* cell cycle defect. To understand the biological significance of this genetic interaction, we studied Xpd in further detail and found that Xpd is specifically down-regulated during mitosis. In contrast, high Xpd expression negatively regulates the CAK activity of Cdk7, and prevents entry into and progression through mitosis.

III. Result

xpd acts as a dominant suppressor of *cdk7^{ts1}* cell cycle defects

The *cdk7^{ts1}* mutant shows cell cycle but no transcription defects (Larochelle et al., 1998). *cdk7^{ts1}* flies are fully viable at the permissive temperature (18°C) but exhibit complete lethality at the restrictive temperature (27°C). Between 25°C and 26°C a small proportion of the *cdk7^{ts1}* flies survive. Conditions under which the mutant phenotype is partially penetrate provide a sensitive environment to identify genetic interactions. We therefore used these conditions to scan the second and third chromosomes for regions capable of suppressing the temperature sensitive lethality of *cdk7^{ts1}* mutants. The screen was carried out by crossing *cdk7^{ts1}* flies to a collection of 144 deficiency strains that are heterozygous for a deletion on the second or third chromosome (the entire genetic screen will be described elsewhere). In the F1 generation, the viability of the two male *cdk7^{ts1}* classes from the same cross is directly compared. A significant increase in population size of the deficiency class (*Df/+;* flies that have a *cdk7^{ts1}* and the heterozygous deficiency) over the Balancer control class (*CyO/+;* flies that only have a *cdk7^{ts1}* and no deficiency) indicates the presence of a dominant suppressor. In this way, we identified a strong suppressor and, using smaller deficiencies, mapped it to cytological region 57C5-57D9 on the second chromosome (Figure 1a). Mutant alleles are available for ten complementation groups in this region, but none of them was able to dominantly suppress *cdk7^{ts1}*.

However, one gene in this interval, *xpd*, is an excellent candidate for a *cdk7* interactor. *xpd*, Xeroderma Pigmentosum disorder group D gene, encodes an ATPase / DNA helicase that functions as part of TFIIH in transcription and DNA repair (Lehmann, 2001). *In vitro* biochemical data showed that Xpd can be found in a complex with Cdk7 and may anchor Cak into TFIIH (Coin et al., 1999; Drapkin et al., 1996; Reardon et al., 1996; Rossignol et al., 1997; Roy et al., 1994; Schaeffer et al., 1994). In the absence of *xpd* point mutations we could not directly test whether *xpd* acts as a suppressor of *cdk7^{ts1}*. We therefore tested whether suppression of *cdk7^{ts1}* by the *xpd* deletion can be rescued by adding back transgenic *xpd*. As *xpd* rescue construct we used a *xpd* cDNA driven by a *hsp70* promoter (*hsp-xpd*) (Reynaud et al., 1999). Flies carrying the *cdk7^{ts1}* allele are crossed to flies that contain both, *Df(2R)Pu-D17* (*xpd* deficiency) and the transgene *hsp-xpd*. The viability of the different genotypes resulting from this

cross was noted and compared to the expected number (Figure 1b). Under the assay conditions used for this experiment, $cdk7^{ts1}$ flies have only 16.4% viability. The viability of $cdk7^{ts1}$ increases to 70.3% in $cdk7^{ts1}$ flies lacking one copy of the *xpd* region and this increased viability is reverted to 19.4% in heterozygous deficiency flies that, in addition, express *xpd* from the transgene. Because $cdk7^{ts1}$ suppression by the deletion can be rescued by adding back *xpd*, we conclude that reduction of *xpd* causes the suppression of $cdk7^{ts1}$.

To find out whether this suppression is specific for *xpd* or whether it could be caused by reduction of any other TFIIH component, we also tested whether reduction in genetic dose of *xpb*, *p62*, *p34*, or *Mat1* can suppress $cdk7^{ts1}$. None of these genes acted as a suppressor of $cdk7^{ts1}$ (data not shown). To find out whether reduction of *xpd* specifically suppresses the cell cycle function of *cdk7*, we tested whether a heterozygous deletion of *xpd* also suppresses another temperature sensitive allele ($cdk7^{S164A/T170A}$) that does not show a clear cell cycle defect (Larochelle et al., 2001). Deletion of *xpd* does not increase the viability of $cdk7^{S164A/T170A}$ flies in an analogous assay. We therefore conclude that reduction of *xpd* specifically suppresses the cell cycle defect of $cdk7^{ts1}$, indicating that reduced levels of *xpd* facilitate cell cycle progression. Under these experimental conditions, *xpd* therefore seems to negatively interfere with Cdk7's cell cycle function.

Down-regulation of Xpd at the beginning of mitosis

While Xpd is known to function in TFIIH for basal transcription and DNA repair (Lehmann, 2001) no cell cycle function had been ascribed to it yet. To evaluate Xpd's potential cell cycle role, we studied Xpd expression during the cell cycle.

Drosophila embryos undergo 17 rounds of mitotic cycles before they reach the larval stage. The first 13 cycles are synchronized syncytial cycles and the nuclear divisions occur very rapidly in a common cytoplasm. These 13 cycles are driven by maternal factors that have only the two phases S and M. Starting in cycle 14, the cell divisions are controlled by zygotic factors and a G2 phase is introduced before the mitotic phase 14. During this interphase 14, the cell cycle slows down and the embryos cellularize. Also starting with mitotic Cycle14, cell divisions become asynchronous. Groups of cells, termed mitotic domains, enter mitosis in sequence and the domains are named by numbers indicating the order in which they initiate mitoses [Foe, 1993

#391]. Within each domain, cells enter mitosis in close synchrony, starting near the center of the domain and progressing concentrically outward to its borders. The cells of one domain share the same cell cycle length but cell cycle length varies between different domains (Foe, 1993). Because of this, the embryos at Cycle 14 or later embryonic cycles contain a mixture of cells in different cell cycle phases, making the system well suited to study in situ the expression pattern of proteins during different cell cycle phases.

On *Drosophila* embryos the anti-Xpd antibody produces a highly cell cycle dependent staining pattern (Figure 2a-f). *Drosophila* embryos undergo 17 rounds of mitotic cycles before reaching the larval stage. The first 13 cycles are synchronized syncytial cycles that do not require transcription and are composed of only two phases; S phase and mitosis. Starting with mitotic Cycle 14, cell divisions become asynchronous. Figures 2a-c show asynchronic cells of a wild type cycle 14 embryo. In interphase and prophase cells, strong Xpd signal is found. In cells at metaphase, anaphase and telophase, the signal drops to almost background levels (Figure 2a-d). A Similar pattern was observed in Cycle 15 embryos (data not shown). In Cycle 13 embryos, Xpd signal is significantly lower in metaphase cells than in interphase cells (Figures 2e,f). As a control, we performed immunostainings with antibody against Xpb/Hay, we did not see the cell cycle dependent staining pattern (data not shown).

To find out whether the drop in Xpd signal is caused by down-regulation of Xpd, Schneider cells were synchronized at G2/M and aliquots of the cells were collected at indicated time points post-release from the block (Figure 2g). Starting at 120 minutes, the percentage of cells in G1 increases at the expense of G2/M cells, indicating that G2/M arrested cells have started to progress through mitosis. Also at 120 minutes Xpd levels start to drop. At 240 minutes, Xpd has reached its lowest levels, coinciding with the rapid down-regulation of Cyclin B and Cyclin A. Together with the embryonic in situ staining data, the observed down-regulation of Xpd in the tissue culture cells indicates that Xpd polypeptide is down-regulated during prometaphase. To our knowledge, this is the first report of a cell cycle dependent regulation of Xpd or any subunit of TFIIH.

Elevated Xpd levels reduce CAK activity and Cdc2 T-loop phosphorylation

Genetically *xpd* acts as a repressor of the cell cycle activity of *cdk7*. Since Xpd and Cdk7 are in the same TFIIH complex, one simple hypothesis would be that Xpd titrates Cdk7 and prevents it from acting as a CAK. The fact that Xpd is down-regulated at mitosis, when Cdk7 is required for its mitotic CAK function, raises the question whether Xpd down-regulation is required to release Cdk7 for its mitotic CAK activity. To test this possibility and to understand the physiological function of Xpd down-regulation, we over-expressed Xpd in wild type flies.

First, we analyzed the *in vitro* CAK activity from embryos with or without *xpd* over-expression. We induce *xpd* over-expression in 2-4 hour embryos by heat shocking embryos containing two copies of the *hsp-xpd* transgene for 30 minutes at 36°C. After a recovery time of forty-five-minutes at room temperature, CAK activity of embryonic extracts was measured using the *in vitro* kinase assay described previously (Larochelle et al., 1998). Without heat shock, flies containing the *hsp-xpd* transgene show CAK activity levels almost as high as wild type flies lacking the transgene (Figure 3a). However, after heat shock induction, embryonic extracts from the *xpd* over-expressing embryos had significantly less CAK activity than the heat shocked wild type flies without this *hsp-xpd* transgene (Figure 3b). Because the difference in CAK activity between the two lines only becomes pronounced after heat shock induction, CAK activity is not reduced due to disruption of an endogenous gene at the insertion site of the transgene. We therefore conclude that CAK activity is reduced by the induction of Xpd.

To verify that CAK activity is reduced, we analyzed the phosphorylation of Cdc2 on threonine 161 (T161). *Drosophila* Cdc2 polypeptide can be resolved into three to four isoforms with the fastest migrating isoform 1 being the T161 phosphorylated Cdc2 (Figure 3c) (Edgar et al., 1994). After the induction of Xpd overexpression and a subsequent 30-min recovery period, isoform 1 is not detectable in either wild type flies or flies containing the *hsp-xpd* transgene. However, after a 60-minute recovery period, wild type embryos have significant levels of the T161 phosphorylated isoform, comparable to non-heat-shocked control wild type flies at the same stage. In flies containing the *hsp-xpd* transgene, on the other hand, the T161 phosphorylated isoform is still undetectable after 60 min. of recovery. This indicates that the embryos that over-express Xpd are not able to phosphorylate Cdc2 at T161 as efficiently as wild type embryos, and we therefore expect these embryos to display mitotic defects.

Xpd over-expression reduces viability

In 2-3hr old wild type embryos, the heat treatment caused only 10% of the embryos to show a lethal phenotype 90 minutes into the recovery period. However, upon *xpd* over-expression, 50% of the embryos show lethality (Figure 4a) as judged by large areas of aggregation, irregular mitotic figures and degenerating cells (data not shown). The embryonic lethality is comparable to the overall lethality during the entire life, indicating that pulse over-expression of *xpd* in the embryo has only a rapid effect on embryonic viability and no long term effects caused by, for instance, general transcriptional problems. This lethality was not observed with control transgenic lines that either contain *hsp-xpb/hay* or a *hsp-xpd* construct that lacks the N-terminal CAK-interaction domain (*hsp-xpdC)*, indicating that elevated Xpd levels reduce fly viability (Figure 4a) (Sandrock and Egly, 2001).

We also analyzed the effects of different levels of Xpd on viability. A single cross yielded four classes of F1 siblings, each with a different *xpd* dose, raised under identical conditions (Figure 4b). Upon daily induction of *hsp-xpd* on top of wild type *xpd* level, the viability of transgenic *hsp-xpd* lines (*CyO/+; +/hsp-xpd)* is reduced to 14.3% (36/251) of wild type. This reduced viability can be partially restored to 40% (100/251) by removing one copy of endogenous *xpd* (*Df (xpd)/+; +/hsp-xpd*). These results confirm that over-expression of *xpd* reduces fly viability. In addition, the viability of heterozygous deficiency flies containing only one endogenous copy of *xpd* (*Df(xpd)/+; +/PrDr*) is reduced to about half the level of wild type (57%, 143/251) under our experimental condition with heat shock stress. This suggests that the cellular levels of Xpd are critical for the cell.

***Xpd* over-expression causes mitotic defects**

To test whether the lethality resulting from Xpd over-expression could be due to reduction of mitotic CAK and Cdc2 activity. We analyzed the cellular phenotype of embryos expressing elevated levels of Xpd and found cell cycle arrests in these embryos. At 45 minutes into the recovery period, transgenic and wild type embryos restarted their cell cycle progression and *xpd* over-expressing embryos have not started to die yet (Figure 4a). To visualize *cdk1* activity, we stained embryos at this stage with the antibody against phospho-Histone H3 (PH3). PH3 serves as a reporter of local Cdk1 activity in syncytial and cellularized embryos (Su et al., 1998).

In wild type embryos (Figure 5a and 5b), cells in each mitotic domain enter mitoses in a slightly asynchronic manner, starting from the center and continuing to the outside of each domain (Foe, 1993). In domain 1 and 4, telophase, metaphase and prophase figures are seen concentrically in this order from the center towards the outside (magnified for domain 1 in 5a1 and 5b1). In the center cells, the mitoses start earlier and finish earlier as seen by the appearance and loss of PH3 signal. Monitoring the same mitotic domain in embryos at later developmental stages would reveal that more peripheral cells have entered mitosis and are PH3 positive. In addition, more central cells have finished mitosis and have become PH3 negative. This progression can be seen by comparing the two wild type embryos shown in Figure 5a and b. The one in Figure 5b is slightly (a few minutes) older than the one in Figure 5a, and a wider central area devoid of PH3 staining is seen in this older embryo (arrows in Figure 5a1, 5b1). Therefore, within one mitotic domain, cells are normally not completely synchronized and if the same mitotic phase is found in many additional cells, this would be indicative of a cell cycle arrest.

In mitotic domain 1 of embryos from *xpd*-overexpressing lines, there are many more metaphase figures (arrowheads in Figure 5c1) than in the same domain of wild-type embryos (Figure 5a1 & 5b1), indicating that cells undergo metaphase arrests. Similar metaphase arrests could be seen in domain 1 and 4 of the *xpd* over-expressing embryo (compare Figure 5d to Figure 5b). In addition to metaphase arrests we observe prophase arrests. In domain 4 of *xpd* overexpressing embryos, many more cells are in prophase (Figure 5c4) compared to the same domain in wild type embryos (Figure 5a and 5b). Besides these mitotic arrests, we also observed G2/M arrests. Various metaphase and prophase figures are seen in domain 5 and 6 in wild type embryos (Figure 5a and 5b). However, in *xpd* overexpressing embryos (Figure 5c), prophase-like condensation of PH3 signal is seen in very few cells in domain 5 and almost no PH3 signal is seen in domain 6 (Figure 5c). This indicates that cells in these domains are unable to enter M phase, probably arresting at the G2/M boundary. Similar arrests are also seen in domain 5 and 6 of the other *xpd* over-expressing embryo shown in Figure 5d. The lack of PH3 signal in domain 5 and 6 in both *xpd* overexpressing embryos is not due to the younger age because they have a comparable or wider mitotic domain 1 and 4 than wild type embryos shown in Figure a and b. The fact that morphogenesis progresses normally also shows that under our experimental conditions the over-expression of *xpd* does not block all basal transcription. Furthermore, Cyclin A and Cyclin B levels are normal as assessed by Western blot analysis (data not shown),

indicating that the transcription of Cyclin A and Cyclin B are not affected and the cell cycle arrests in the *xpd* over-expressing embryos are not due to the lower levels of mitotic Cyclins.

Xpd over-expression in cycle 13 embryos prevents cell division

Cycle 14 has a cell cycle composed of three phases S, G2 and M whereas Cycle 13 consists only of an S and M phase. Because we observe local down-regulation of Xpd also in Cycle 12 and 13 (not shown), we wanted to find out whether *xpd* over-expression causes similar cell cycle arrests in a different cell cycle. Wild type embryos and *hsp-xpd* transgenic embryos were heat shocked in Cycle 13 (to induce *xpd* over-expression) and then aged to Cycle 14. At this time they were fixed and stained with anti α-tubulin antibodies to detect their mitotic spindles. Figure 5e and 5f show one wild type and one Xpd-overexpressing embryo, respectively. Both embryos have cells in metaphase 14 and are at the same morphogenetic stage. Xpd-overexpressing embryos (Figure 5f) contain fewer cells than wild type embryos (Figure 5e). In the wild type, 22 cells could be counted along the cephalic furrow and 23 from the anterior of the head to the cephalic furrow. In *xpd* overexpressing embryos, however, only 16 and 15 cells are counted along the corresponding lines. Thus, the wild type embryo has about twice as many cells as the *xpd* overexpressing embryo. One reason for this may be that *xpd* overexpression causes the cells to permanently arrest in cycle 13. This does not seem to be the case because we see mitotic figures appearing in the *xpd* overexpressing embryos (Figure 5f). Another possibility is that half of the cells die as a consequence of *xpd* overexpression. This also does not seem to be the case because we do not find evidence for cell death in these embryos. Instead, the cells in these embryos are larger. This points to a third explanation, that *xpd* over expression in cycle 13 causes these cells to skip mitotic phase 13 and to continue with cycle 14. Consistent with this interpretation, mitotic cells appear in regions of the embryos that correspond to cycle 14 mitotic domains (Figure 5f). The fact that we do not observe a cell cycle arrest at cycle 13 may have to do with the lack of an appropriate cell cycle checkpoint in this syncytial cycle.

Xpd over-expression disrupts sub-cellular localization of Cdk7

To study how Xpd affects Cdk7 activity, we analyzed the subcellular localization of Cdk7 during the cell cycle. Cdk7 is predominantly localized found in the nucleus and we have observed this pattern also in fly ovaries (Chapter II). However, in embryos, the Cdk7 localization pattern is different. Figure 6a shows a wild type embryo in Cycle 14. In its mitotic domain 1, the cells are in different mitotic phases. During meta- ana- and telophase, the Cdk7 signal is abundant throughout the cytoplasm and 'nuclear' plasm. Outside the mitotic domains the cells are in interphase. We compared the Cdk7 and Xpd expression pattern with or without Xpd over-expression. In control embryos that have wild type levels of *xpd*(Non-HS), strong Xpd staining is seen in the nucleus in prophase, but not metaphase cells (Figure 6b, left two panels, also Figure 2c). During this period Cdk7 signal becomes uniformly distributed (Figure 6b, left two panels). However, in *xpd* over-expressing embryos, the staining patterns of Cdk7 and Xpd are altered and signals co-localize in the different cell cycle phases (Figure 6b, right two panels).
In these embryos, metaphase-like figures are seen in some cells, indicating that these cells were able to start mitotic DNA condensation. However, the uniform mitotic staining of Cdk7 is never seen (compare the Cdk7 staining of the second and fourth panel). Second, in these embryos the drop in overall Xpd signal is not seen in prophase (compare Xpd signal in the two left panels to those in the two right panels). Third, ectopicly localized Cdk7 and Xpd seem to colocalize in the *xpd* over-expressing embryos. We conclude that the over-expression of Xpd alters the overall distribution of Xpd and causes the mislocalization of Cdk7.

Reduction of Xpd levels results in elevated CAK activity and higher proliferation

We next analyzed whether reduced Xpd levels affect CAK activity and cell proliferation using RNA interference in *Drosophila* S2 cells (Clemens et al., 2000). As a control, we also performed RNAi with dsRNA of *xpb/hay* and *cdk7*. Two to four days after the dsRNA incubation, Xpd and Cdk7 are depleted almost completely, while Xpb/Hay levels varied more between experiments and were only reduced to 5-30% (Figure 7a). On the fourth day after dsRNA incubation, significant differences between *xpd* RNAi and the untreated control are seen when comparing CAK activity and cell proliferation. Averaged data are shown in Figures 4c and 4d. *xpd* RNAi treatment causes an increase in CAK activity to 125% (normalized to total protein levels, Figure 7c). In contrast, *cdk7* RNAi reduced CAK activity to 42% and *xpb/hay* RNAi to

82% (Figure 7c). Therefore, Xpd also negatively regulates CAK activity of Cdk7 in S2 cells. In parallel to the increase in CAK activity on day four, *xpd* RNAi treatment also causes an increase in cell numbers to 139% of the control, while *cdk7* and *xpb/hay* RNAi show lower cell numbers (76% and 69% of the control, respectively; Figures 7b, d).

IV. Discussion

Xpd, a fine tuner between mitosis and transcription?

Although T-loop phosphorylation of Cdks appears to be an essential step in their *in vitro* activation (Morgan, 1997; Nigg, 1995) no evidence was found that would indicate that phosphorylation by CAK is regulated or has a regulatory role *in vivo*. Indeed, early attempts to uncover fluctuations in the level of CAK activity during the cell cycle have failed (Brown et al., 1994; Matsuoka et al., 1994; Poon et al., 1994; Tassan et al., 1994). However in *Drosophila*, fluctuations in Cdk1 activity during embryonic cycles 8-13 correlate both with Cyclin degradation and T-loop phosphorylation (Edgar et al., 1994). Beginning in interphase 9 there is an apparent reduction in the level of activating (Thr-161) phosphorylation, this fluctuation then gradually increases so that by the interphase of cycle 13 the Thr-161 phosphorylated form of Cdk1 is undetectable. Here we observe that Xpd is down-regulated at mitosis, and that the over-expression of Xpd results in delayed or blocked entry into mitosis during embryonic cycles 13 and 14. Xpd over-expression also results in a reduction of total CAK activity and Cdc2 T-loop phosphorylation that can be measured in embryonic extracts. Similarly, we show that reducing the dose of Xpd can rescue the lethality of a *cdk7* allele that is specifically defective in its mitotic cell cycle function. The possible mechanism of action could be that high concentration of Xpd favors incorporation of CAK into the TFIIH complexes, thereby reducing the amount available to activate Cdk1. In this way, the cell cycle dependent regulation of Xpd levels could cause cell cycle dependent alteration of mitotic CAK activity.

In eukaryotic cells, mitosis is accompanied by a global repression of transcription. The direct inactivation of components of the transcription machinery accounts for the majority (≥90%) of the transcription repression observed *in vitro* (Gottesfeld and Forbes, 1997). The targets of mitotic inactivation include all three nuclear RNA polymerases and some transcription factors (Gottesfeld et al., 1994; Heix et al., 1998; Leresche et al., 1996; Segil et al., 1996; White

et al., 1995). For the mitotic repression of basal transcription of RNA polymerase II, TFIIH was shown to be the major target, and TFIIH associated CTD kinase activity and transcription activity are specifically repressed at mitosis (Akoulitchev and Reinberg, 1998; Long et al., 1998). Here we show that Xpd down-regulation occurs during prophase, coinciding with general transcription silencing (Gottesfeld and Forbes, 1997). The timing of this down-regulation also coincides with the rise of Cdc2 activity. Because Xpd mediates the interaction between CAK and core TFIIH, the down-regulation of Xpd is expected to cause the dissociation of the CAK complex from holo-TFIIH, thereby causing TFIIH to lose its CTD kinase activity (Figure 8). This in turn would contribute to the inhibition of transcription observed during mitosis. In our model, the abundance of Xpd acts as a critical regulator of both cell cycle and basal transcription by determining the distribution of Cdk7 between its CAK and CTD kinase forms. Although it remains to be determined if this mechanism is ubiquitous in eukaryotic cells, or is limited to certain types of cell cycles, we provide the first tangible evidence for such a coupling between the cell cycle and the regulation of basal transcription through Xpd and Cdk7.

V. Materials and Methods

Fly strains, culture conditions and crosses

Drosophila was cultured on standard corn meal food at room temperature if not specified otherwise. The temperature sensitive alleles are grown at 18°C. $cdk7^{null}$ is *w Df(1)JB254* $P[w^{+}snf^{+},dhd^{+}]$. The $cdk7^{ts1}$ flies are $cdk7^{null}$ / $cdk7^{null}$; +/+; $P[w^{+}\ cdk7^{P140S}]$/ $P[w^{+}\ cdk7^{P140S}]$ (Larochelle et al., 1998). $cdk7^{S164A/T170A}$ was described elsewhere (Larochelle et al., 2001). The five strains used for testing suppression of $cdk7^{ts1}$ are *Df(2R)AA21, Df(2R)Pu-D17, Df(2R)PK1, Df(2R)PI13* and *Df(2R)exu1*. Two 3rd chromosomal *hsp-xpd* strains were obtained from Mario Zurita (Reynaud et al., 1999). The ten point mutations in the 57C5-57D9 region that showed no suppression are *l(2) 57Cc, l(2) 57Cd, anon-57C1, tud, l(2)57Ce, l(2) 57Da, l(2) 57Db, l(2) 57Dd, l(2) 57De, l(2) 57Dc.*

To screen for dominant suppressors of *cdk7* (Figure 1a) and to map the suppressors, $cdk7^{ts1}$ virgins were crossed to balanced deficiency males. Eggs were laid at room temperature for four to eight hours and then transferred to the incubator where they were reared between 25°C

and 26°C. The two classes of F1 male flies were scored and their frequency compared. Suppressors were identified as containing more male flies with the deficiency chromosome (*cdk7^{null}/Y; Df/+; P[w^{+}cdk7^{ts1}] /+* than the siblings without the deficiency chromosome (*cdk7^{null}/Y; CyO/+; P[w^{+}cdk7^{ts1}] /+*).

To test if *xpd* is the suppressor of *cdk7^{ts1}* (Figure 1b), *cdk7^{null} / FM7C; +/+; P[w^{+}cdk7^{P140S}]/ P[w^{+}cdk7^{P140S}]* females were crossed to *w/Y; Df(xpd)/ CyO; P[w^{+} hsp-xpd] /PrDr* males. Eggs were collected in bottles for 4-8 hours and grown at room temperature. To induce *xpd* over-expression, the bottles were kept in an incubator that cycled between 25°C (22hrs) and 37°C (1hr and 1hr slope time) until adult flies enclosed. To test whether over-expression of *xpd* causes lethality (Figure 4b), either *Df(2R)AA21/CyO; +/+* or *Df(2R)PI13/CyO; +/+* females were crossed to *+/+; P[w^{+} hsp-xpd] /PrDr* males. *xpd* over-expression was induced as above.

Cell culture, synchronization and flow cytometry

Drosophila S2 cell lines were cultured at 27°C in Schneider's *Drosophila* medium (Gibco 11720-034), 10% FBS (26140-079), 50µg/ml penicillin-streptomycin (15140-122), 1mM L-glutamin (25030-081). Cells were first synchronized at G1/S with double Thymidine block (2mM, two 16-hour treatments with a 10-hour interval) followed by a G2/M block (16 hours) with 0.5µg/ml nocodazole (Sigma). For flow cytometry analysis, cells were washed once with 1xPBS, fixed in 75% ice cold ethanol for 15 minutes and washed once with PBS. After 30 min RNase treatment (0.8mg/ml; 37°C), they were stained with propidium iodide (Molecular probes; 5µg/ml, 15 minutes) and analyzed by FACS scan (Becton Dickinson). Results were analyzed with the ModfitLT program.

xpdC transgenic lines

The *hsp-xpdC* construct has a deletion of the 5' region from codon 6-441, which corresponds to codons 1-430 of its human counterpart. To generate the *hsp-xpdC* construct, the 3' part of the *xpd* cDNA was amplified by PCR using sense primer 5' ggaattcc TAAATGAAAGTACT CCTTCCCATTTTGCACTTTA and antisense primer 5' ggaattcc GCAGAGCACCTTCCACATCT. The resulting PCR fragment has EcoRI sites at both ends that allowed insertion into the EcoRI site of the *hsp70SCS* vector to clone it behind the hsp70 promoter. The *hsp70-xpdC* fusion gene was then transferred into pCaSpeR vector using Not1 and Kpn1, and transgenic lines were established.

Embryo collection, Xpd induction and CAK assay

Flies were allowed to pre-lay for 1 hour before egg collections. For CAK assays, two to four hour old embryos were collected, heat shocked for 30 min and allowed to recover for 45 min. Embryos used for CAK assays or immuno-staining were dechorionated with 50% bleach, frozen in dry ice and stored at -70°C. For immuno-staining with anti-PH3 antibody, embryos at S or G2 14 were heat shocked for 30 min. and harvested 45 min. thereafter. Based on Western blot results from embryos collected after a 30 min heat shock at 36°C, Xpd levels are 36% higher than in the wild type.

For induction of *xpd* over-expression in Cycle 13, 0-30 min old eggs were collected and aged for 110 min. to reach Cycle 13. They were then heat shocked for 30 min. at 36°C in a water bath, allowed to recover at room temperature for 100 minutes (to reach M14), and fixed.

To determine the lethal period after *xpd* induction, 2-3 hr old embryos were collected and given a 30 min. heat shock at 36°C. Every 30 min, an aliquot was harvested and fixed with methanol. A portion of the eggs was aged to the adult stage.

Antibodies and immunostaining

To generate antibodies against Xpd, the cDNA clone (LD11051) was digested with Spl I and Pml I and the resulting 2.1 kb fragment was subcloned into pSL1180. A BamH1 fragment containing most of the *xpd* cDNA was inserted into the expression vector pET3b and expressed in the BL21(DE3) E.coli strain. A 88KD polypeptide was purified using the Bio-RAD Prep Cell system. This purified protein was injected into rabbits to produce polyclonal antiserum, which

was affinity purified with bacterially expressed Xpd coupled to CNBr-activated Sepharose 4B beads (Amersham Pharmacia Biotech, 17-0430-01). A 1:500 dilution of this antibody was used for immuno-staining and a 1:2000 dilution was used for the Western blots. The antibody against Cdk7 (Larochelle et al., 1998) was used at 1:20 and 1:2 dilution for western blot and immunostaining, respectively. Anti-PSTAIR was used at 1:2000 for Cdc2 Western blots. Cdc2 isoforms were resolved as reported (Edgar et al., 1994). Anti PH3 (Upstate) and α-tubulin (Sigma DM1a) were used at 1:200 and 1:500 dilution, respectively. Anti Xpb/ERCC3/Haywire was obtained from M. Fuller (Mounkes et al., 1992) and used at 1:1000. Protein extraction from embryos has been described previously (Larochelle et al., 1998). To extract proteins from Schneider cells, $1.5x10^6$ cells were pelleted and resuspended in RIPA buffer, followed by sonication and boiling in SDS sample buffer.

For immunostainings, embryos were fixed either in 95% methanol, 5% 0.25M EGTA (for anti-α-tubulin staining) or in 4% paraformaldehyde (for anti-Xpd, Cdk7 and PH3 staining). Embryos were then rinsed in cold 95% methanol / 5% 0.25M EGTA and gradually rehydrated in PBST. Blocking and primary antibody staining was described previously (Suter and Steward, 1991). Secondary antibodies (Oregon Green or Texas Red conjugated anti-mouse or rabbit antibodies; Molecular Probes) were pre-absorbed and used at 1/500 to 1/2000. If DNA staining was needed, embryos were also incubated one hour with 1ug/ml Hoechst 33258 or 15 minute with 2uM To-Pro 3 (Molecular Probes). The embryos were observed under the microscope (Axioplan). Confocal images were taken on a confocal laser scanning microscope (Leica,TCS SP2). A Z stack of 12-15 serial images taken at 0.3μm intervals contained all of the Xpd signal. Such stacks were used to quantify the Xpd signal using the Leica software for measuring and analysis of pixel intensity. 15-30 cells were quantified for each cell cycle phase.

RNA interference in S2 cells

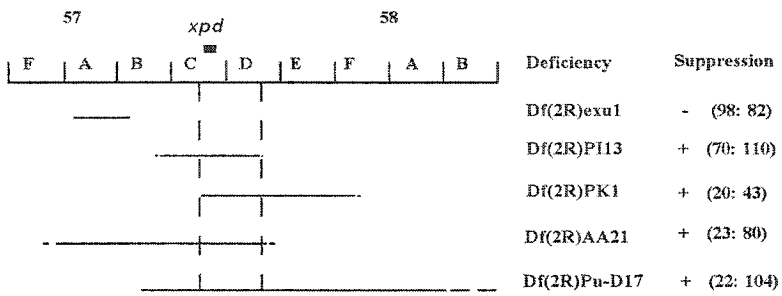
RNAi experiment were performed essentially as (Clemens et al., 2000). PCR products were amplified from the cDNA clones, *xpd* in pBS, *xpb* in pNB40, *cdk7* in pBS. The primers used in PCR reactions contain a T7 RNA polymerase promotor sequence at 5' end (5'-TAATACGACTCACTATAGGGAGG-3'), followed by sequences specific for the targeted genes. The gene-specific sequences are as follow: cdk7 sense 5'-TGACAA-AACGGAACGCTATG, cdk7 anti-sense 5'-GCCGGTTTGTTAGCGAAATA; hay sense 5'-

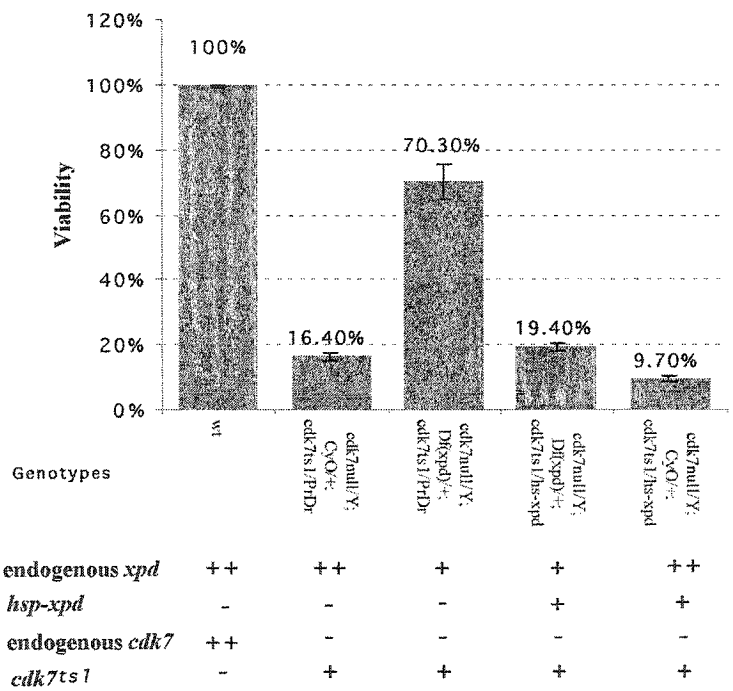
GGTCAAGCTGGTCTTGAAGC, hay anti sense 5'-AGTAGTGTGGCCGTC-AATCC ; xpd sense 5'-GACGGCCTGTTGG-TGTACTT, xpd anti-sense CACCTCGG-TTAGGACATCGT.

VI. Acknowledgments to reagents and equipment

I am grateful to Mario Zurita for *hsp-xpd* transgenic flies and to Margaret Fuller for anti Haywire antibody and to Trudi Schupbach, William J. Mackay, and Ben-Zion Shilo for fly strains. I thank Robert Tanguay for providing S2 and KC cells. I would like to thank Adrian Tsang for use of the confocal microscope and Aleks Spurmanis for technical assistance. Thanks to Paul Lasko and Richard Roy for use of the Axioplan microscope and the Phosphor-Imager.

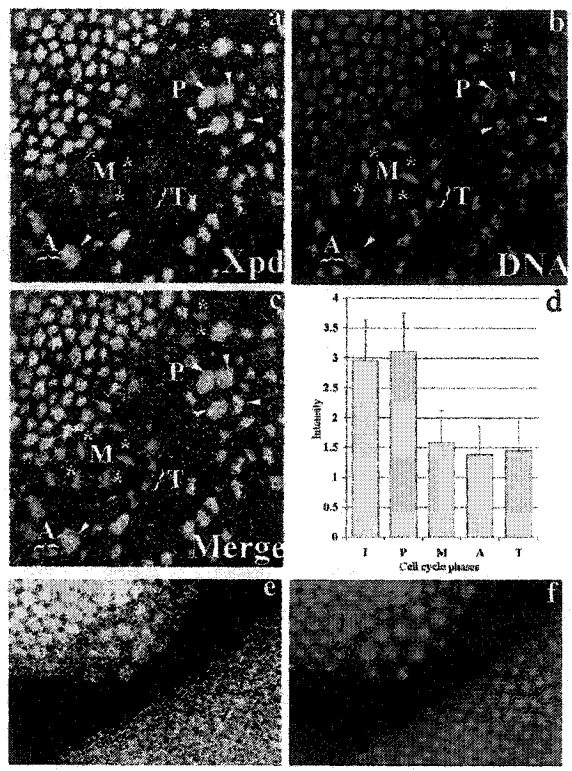
Figure 1. *xpd* is a dominant suppressor of *cdk7* ^{ts1}. **a,** "+" or "-" indicates whether suppression was or wasn't observed. Shown in brackets is the number of balanced control flies and the number of deficiency flies (separated by a colon). *Df*: deficiency chromosome; 2R: second chromosome, right arm. 57 and 58 designates the chromosomal regions that are subdivided into smaller regions (A to F). Solid lines represent the deleted region in each deficiency chromosome. Horizontal dashed lines indicate uncertainty in breakpoint location; vertical dashed lines delineate the suppression region. **b,** The total number of wild type females obtained in these crosses was 1318. The expected number of flies for each male genotype is one eighth of this, 165. *Df(xpd)* is *Df (2R)Pu-D17*; *hsp-xpd* is a *xpd* transgene under *hsp70* promoter; *PrDr*: dominant markers for third chromosome. each "+" refers to one copy of endogenous *xpd, cdk7* or transgene *hsp-xpd* or *cdk7*^{ts1}.





endogenous *xpd*	++	++	+	+	++
hsp-xpd	-	-	-	+	+
endogenous *cdk7*	++	-	-	-	-
cdk7ts1	-	+	+	+	+

Figure 2 Xpd levels are cell cycle dependent. **a-c,** Confocal images of Cycle 14 embryos stained with antibodies against Xpd (**a**) and the DNA dye To-Pro-3 (**b**). The merged picture is shown in **c**. M and "*" mark metaphase figures, P and arrowheads prophase figures, A and T with "}"anaphase and telophase figures, respectively. **d**, Quantification of the cellular Xpd signal obtained from stacks of confocal images. I, P, M, A, and T refer to interphase, prophase, metaphase, anaphase and telophase, respectively. Because background fluorescence was not subtracted, the actual difference in Xpd signal levels are higher. **e** and **f** show confocal images of Cycle 13 embryos stained with antibodies against Xpd (**e**) and the DNA dye To-Pro-3 (**f**). Weaker Xpd signal coincides with condensed chromosomes staining. **g**, Western blot analysis of Xpd protein levels in S2 cells at indicated time points post-release from nocodazole block. Numbers in upper panels indicate the percentage of cells in specific cell cycle phases. Xpd and Cdk1 signals were quantified by Storm PhosphorImager (Molecular Dynamics). Cdk1 was used as the internal standard. Normalized Xpd levels (quant.) are shown for each time point with the highest value set at 1.0.



g

t[min]=	Time after release from the nocodazole block 0	60	120	180	240	300
G2/M	46.6	53.2	52.7	39.8	25.7	11.5
G1	14.8	16.1	20.9	30.5	38.7	44.6
S	38.7	30.7	26.5	29.7	35.6	43.9

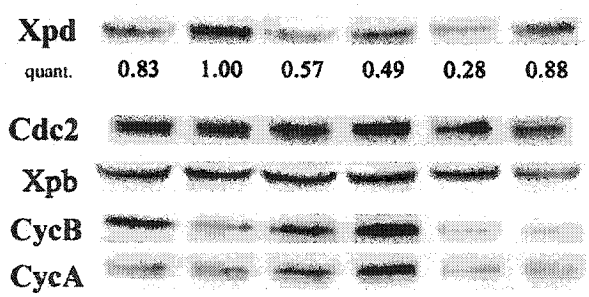


Figure 3 CAK activity and Cdc2 T-loop phosphorylation are reduced in embryos that over-express *xpd*. **a,** CAK levels without *xpd* induction. **b,** Upon heat induction in embryos, CAK activity is significantly reduced in the *hsp-xpd* transgenic line. The experiments were repeated four times with consistent results. The autoradiographic signal of Histone H1 (^{32}P-H1) shown at the bottom was quantified with a PhosphorImager and the integrated pixel intensity is shown on the y axis. **c,** T161 phosphorylated Cdc2 (isoform 1) is still reduced in *hsp-xpd* embryos after a 60 minute recovery. Four isoforms of Cdc2 are indicated by numbers 1 to 4 on the right side. According to Edgar et al (1994), isoform 1: T161 phosphorylated Cdc2; 2: unphosphorylated Cdc2; 3: Y15 phosphorylated Cdc2, 4: T14, Y15 phosphorylated Cdc2.

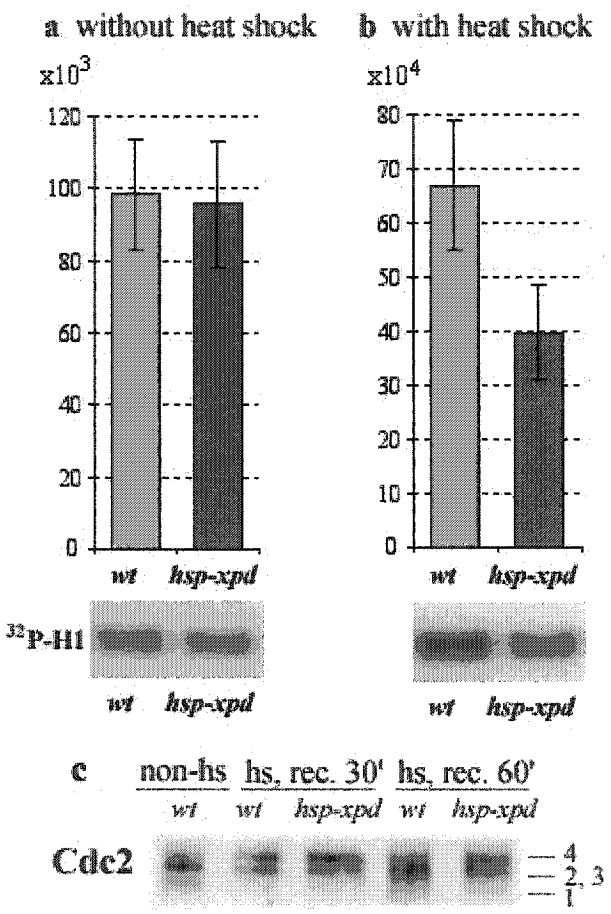

a without heat shock
b with heat shock
x10^3
x10^4
120
100
80
60
40
20
0
80
70
60
50
40
30
20
10
0
wt
hsp-xpd
32P-H1
c
non-hs
hs, rec. 30'
hs, rec. 60'
wt
wt
hsp-xpd
wt
hsp-xpd
Cdc2
4
2, 3
1

Figure 4 Xpd over-expression results in lethality. **a,** Over-expression of *xpd* at the embryonic stage results in embryonic lethality. Embryos were stained with α-tubulin antibodies and Hoechst 33258. A strong drop in viability was seen 90 minutes (but not 60 min.) after the end of induction of *hsp-xpd.* The same heat shock procedure caused only slight reduction of viability in wild type embryos. According to previous reports, heat shock causes interphase arrest of nuclei in both syncytial (Foe and Alberts, 1985) and cellularized embryos (Maldonado-Codina et al., 1993). Most embryos show mitotic spindles and condensed DNA 30 minutes after heat shock treatment, indicating that most embryos have recovered from heat shock and re-entered mitosis at this time. This recovery time is close to the one described by others (Maldonado-Codina et al., 1993). **b,** Viability of flies with different *xpd* levels. Four genotypes with different *xpd* levels were analysed. *CyO/+; +/PrDr* flies have wild type *xpd* levels and their viability serves as control (100%). The *xpd* dosage corresponding to each genotype is shown at the bottom, each "+" refers to one copy of *xpd* or *hsp-xpd* transgene.

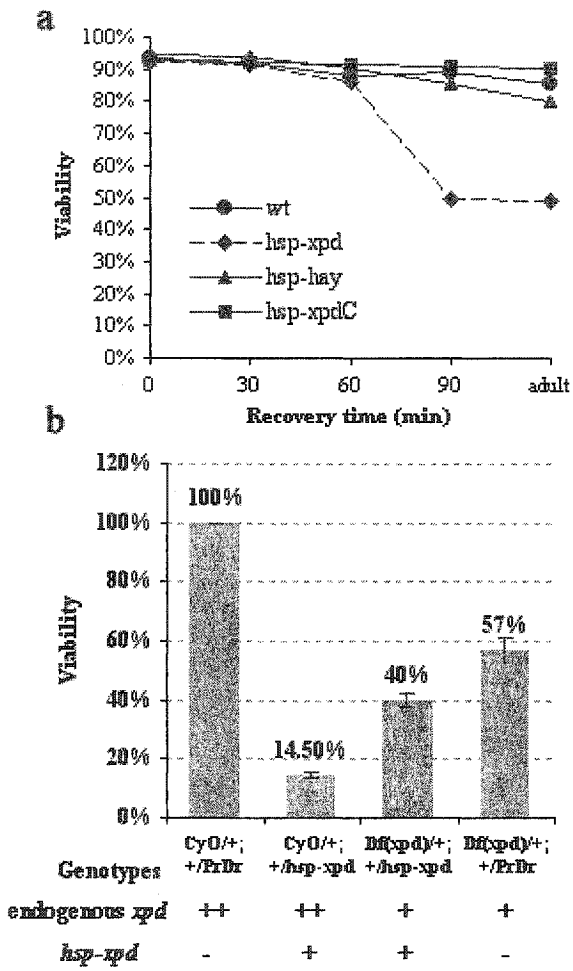

a
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
Viability
wt
hsp-xpd
hsp-hay
hsp-xpdC
0
30
60
90
adult
Recovery time (min)
b
120%
100%
80%
60%
40%
20%
0%
Viability
100%
14.50%
40%
57%
CyO/+; +/PrDr
CyO/+; +/hsp-xpd
Df(xpd)/+; +/hsp-xpd
Df(xpd)/+; +/PrDr
Genotypes
endogenous xpd
hsp-xpd

Figure 5 Over-expression of *xpd* causes cell cycle arrest in Cycle 14 embryos and fewer cell divisions in Cycle 13 embryos. **a-d** show α-PH3 staining of Cycle 14 embryos. The two wild type embryos (**a** and **b**) and the two *hsp-xpd* embryos (**c** and **d**) are at about the same gastrulation stage in mitotic Cycle 14. **a** and **c** are ventral views, **b** and **d** are ventrolateral views of the embryos. Numbers 1,4,5,6 indicate respective mitotic domains. Images in **a1, b1** and **c1** are magnified domain 1 (boxed region) of embryos in **a**, **b** and **c**, respectively. **c4** is the magnified domain 4 of the embryo in C. "( )" with letter p, m and t in B1 indicate prophase, metaphase and telophase figures, respectively. Arrows point to the central region of the mitotic domains, which contain cells that have exited mitosis and have lost the PH3 signals. Arrowheads in **c1** point to metaphase figures.

e and **f** show α-tubulin staining of Cycle 14 embryos (lateral view). Mitotic spindles (arrows) can be seen in both embryos, indicating that they are in mitosis. Wild type embryos (**e**) and embryos from a *hsp-xpd* transgenic line (**f**) undergo gastrulation and are at about the same developmental stage. "*" indicates the progress of germ band extension. Two arrowheads indicate the position of the cephalic furrow. The white doted lines parallel and perpendicular to the cephalic furrow aid counting the cells along the a-p and d-v axis.

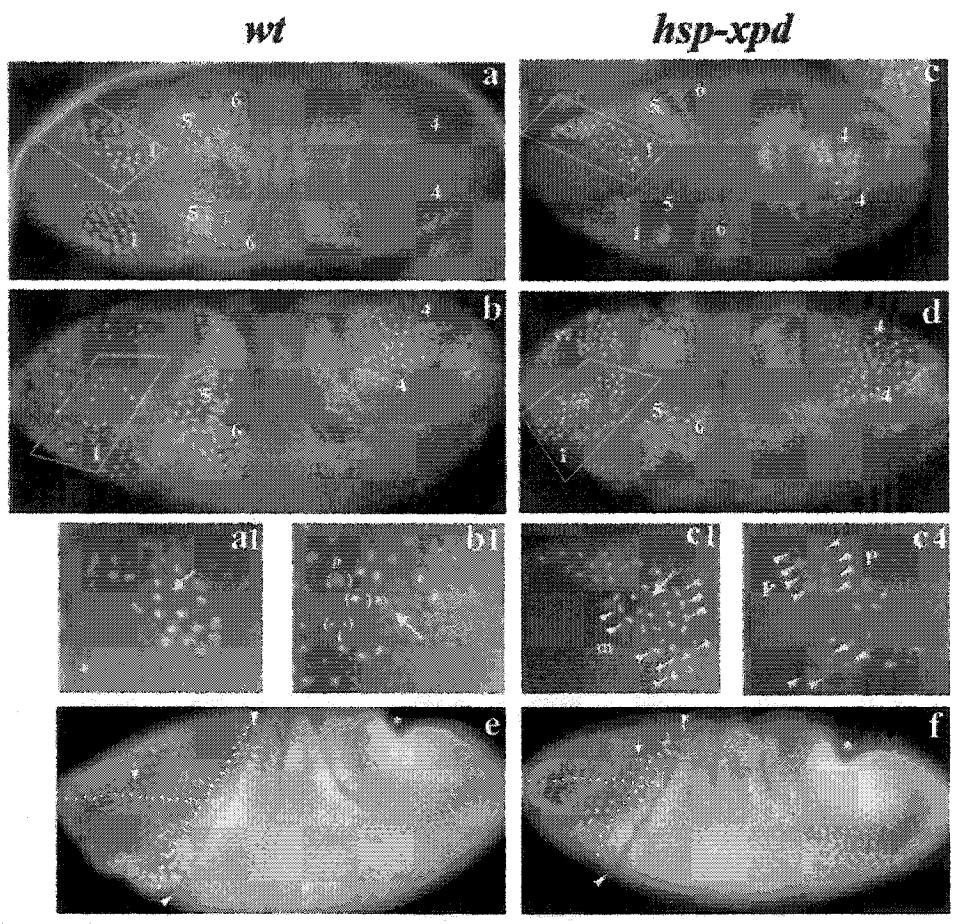

wt
hsp-xpd
a
b
c
d
a1
b1
c1
c4
e
f

Figure 6 Xpd over-expression results in mislocalization of Xpd and Cdk7. **a,** Embryonic Cdk7 distribution is cell cycle dependent. The confocal image from a mitotic Cycle 14 embryo (M14) shows Cdk7 (green) and DNA (red). **b,** Xpd over-expression disrupts the normal pattern of both Xpd and Cdk7 and causes ectopic co-localization of Xpd and Cdk7. The confocal images from M14 embryos show DNA in blue, Xpd signal in red and Cdk7 staining in green. The cells shown in the two panels on the left have wild type levels of *xpd* (Non-HS), while the cells in the two panels on the right have been heat shocked to over-express *hsp-xpd* (HS). M and "*" refer to metaphase figures; P and arrowheads refer to prophase figures; A and T with "}" stands for anaphase and telophase figures, respectively.

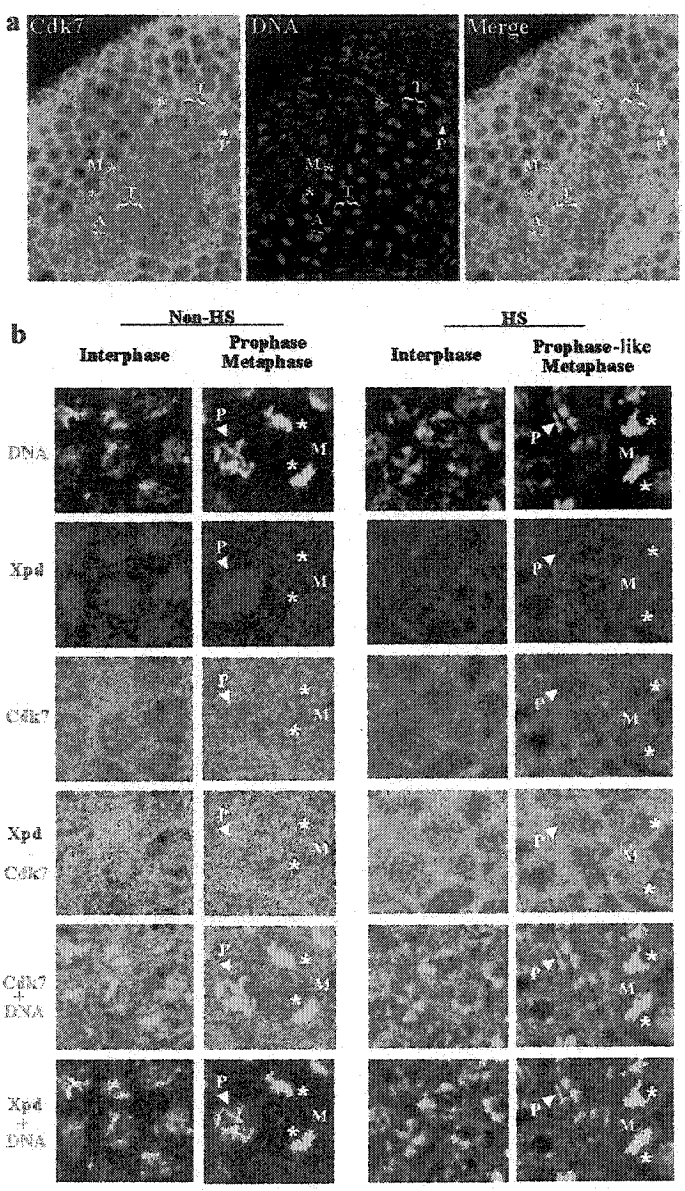

a
Cdk7
DNA
Merge
b
Non-HS
HS
Interphase
Prophase Metaphase
Interphase
Prophase-like Metaphase
DNA
Xpd
Cdk7
Xpd Cdk7
Cdk7 + DNA
Xpd + DNA

Figure 7 RNA interference in S2 cells. non: control cells with same treatment as other samples except that no dsRNA was added. **a,** Western blots show protein levels of Cdk7, Xpb/Hay and Xpd in S2 cell extracts after RNAi. CR: Cross reactivity band as additional control. Results from the second day, third day and fourth day are shown. **b,** Total cell numbers of S2 cells with *xpd*, *cdk7*, *xpb/hay* RNAi as well as non-dsRNA control were shown for every day after dsRNA incubation. **c,** Average CAK activity at the fourth day after RNAi. non-dsRNA control was set as 100%. **d,** Average cell numbers of RNAi samples. non-dsRNA control was set as 100%. Both CAK activity and cell proliferation vary strongly in response to *xpb/hay* RNAi treatment. This may be due to the incomplete and variable knockdown of Xpb/Hay. In addition, *xpb/hay* RNAi reduces not only Xpb/Hay levels but also Xpd levels (Figure 7a), complicating the interpretation of these results. In contrast, *xpd* and *cdk7* RNAi affect only their respective polypeptide levels.

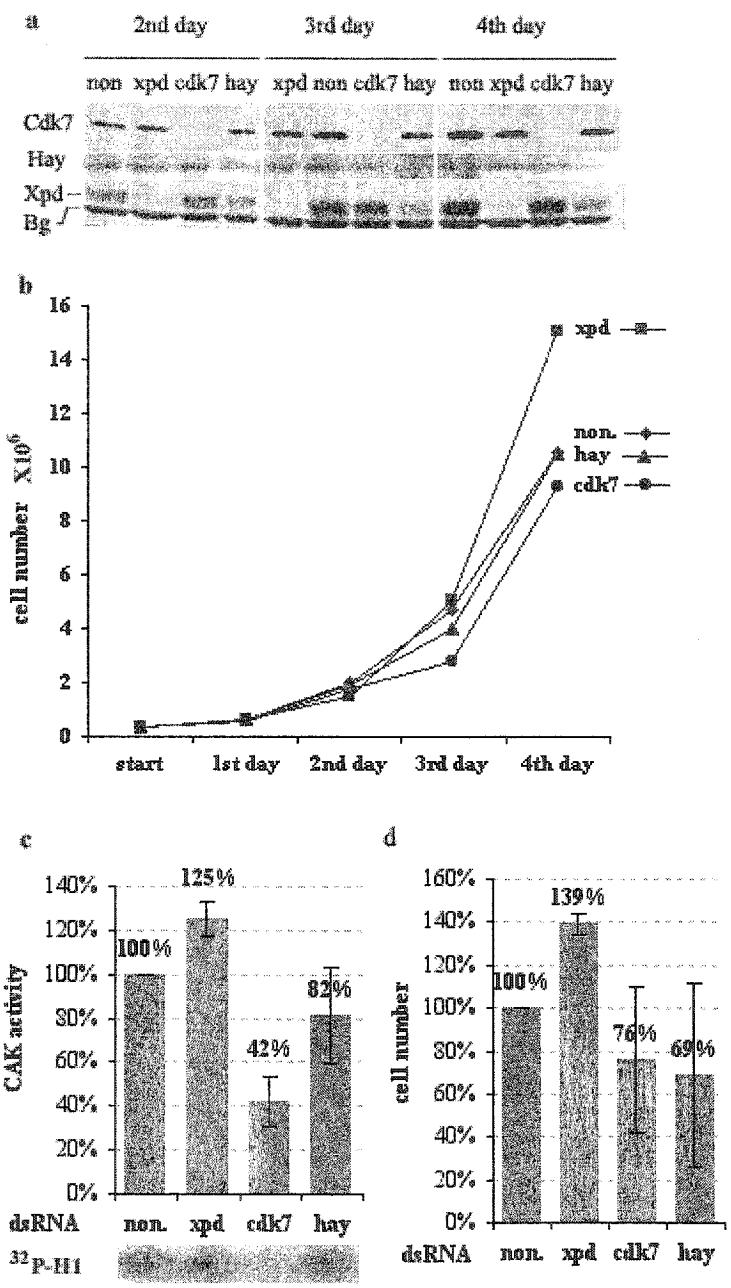
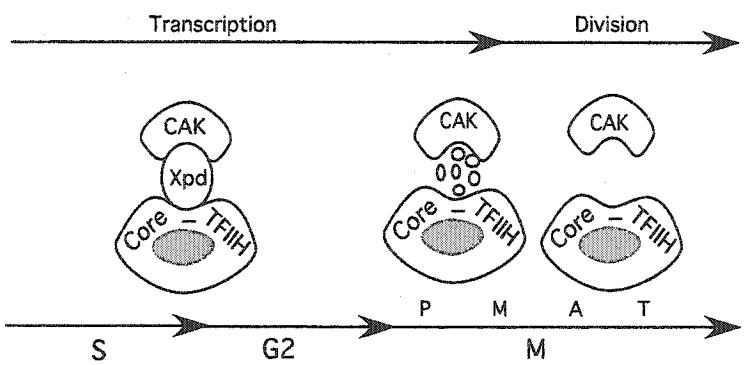

a
2nd day
3rd day
4th day
non xpd cdk7 hay xpd non cdk7 hay non xpd cdk7 hay
Cdk7
Hay
Xpd
Bg
b
cell number X10^6
xpd
non.
hay
cdk7
start
1st day
2nd day
3rd day
4th day
c
CAK activity
125%
100%
82%
42%
dsRNA non. xpd cdk7 hay
32P-H1
d
cell number
139%
100%
76%
69%
dsRNA non. xpd cdk7 hay

Figure 8 Model: dual role for down-regulation of Xpd during mitotic prophase. Transcription and mitosis are two mutually exclusive events during the cell cycle. The down-regulation of Xpd during mitotic prophase would directly inactivate RNA polymerase II transcription by dissociating TFIIH. Simultaneously, this Xpd down-regulation would free the CAK complex and permit it to perform its CAK function. S, G2 and M refer to the three cell cycle phases; P, M, A, T refer to the four mitotic phases: prophase, metaphase, anaphase and telophase, respectively.


Transcription
Division
CAK
Xpd
Core – TFIIH
CAK
Core – TFIIH
CAK
Core – TFIIH
P M A T
S
G2
M

Chapter 5

xpd mutagenesis

I. Introduction

Both Xpd and Xpb are helicase subunits in TFIIH and mutations in *xpd* and *xpb* result in similar syndromes, XP, TTD and XP/CS. Xpb plays an essential enzymatic role in transcription and this function is responsible for the severity of the phenotypes and the fact that a very limited number (3) of patients has been identified (Guzder et al., 1994). Other clinical features found in Xpb patients, such as UV sensitivity and high skin cancer susceptibility, are attributed to a defect in DNA repair (Evans, 1997; Lehmann, 2001).

However, the complex human syndromes caused by *xpd* mutations can not fully be explained by defects in transcription and NER. While Xpb plays an essential enzymatic role in basal transcription, the enzymatic activity of Xpd is dispensable for this action. Mutations in either its ATP binding site or in one of the helicase motifs do not alter basal transcription and do not result in lethality (Sung et al., 1988; Tirode et al., 1999; Winkler et al., 2000). In contrast, the deletion of *xpd* in the mouse leads to pre-implantation lethality (de Boer et al., 1998). These data suggest that transcription requires the physical presence of Xpd rather than its helicase activity. The essential function of Xpd in cellular viability is consistent with the notion that only subtle Xpd mutations are found in XP, TTD and XP/CS patients. In contrast to the limited numbers of patients identified with *xpb* mutation, numerous *xpd* patients are registered over the world and cell lines form *xpd* patients have been established and intensively studied. Figure 1 shows the mutations in the Xpd protein found in TTD, XP, and XP/CS patients (Botta et al., 1998). One can see that mutations at specific sites are syndrome specific but mutations in neighboring codons can result in different syndromes.

Researchers attempted to find other defects than transcription and NER in *xpd* mutations to explain the syndromes varying from neurological abnormalities to growth retardation and cancer. Recently, the Egly group showed that *xpd* mutations of the XP and TTD type prevent phosphorylation of RARα by Cdk7 kinase and thus cause defects in hormone-mediated transcription activation of their target genes (Keriel et al., 2002). *in vivo* phosphorylation of RAR is carried out only by TFIIH even though free CAK is able to phosphorylate RAR *in vitro* (Rochette-Egly et al., 1997). Because Xpd acts as a linker between CAK and core-TFIIH, *xpd* mutations that weaken the connection between Xpd and core-TFIIH reduce TFIIH-associated

kinase activity towards RAR. This finding helps to explain some clinical features of Xpd deficient patients such as growth defects, developmental abnormalities or sterility.

To develop a model for studying *xpd* function in a genetically tractable system, we attempted to generate *xpd* mutations in *Drosophila melanogaster*. These tools should allow us to analyze its *in vivo* function in NER, transcriptional regulation and cell cycle control.

II. Results

xpd EMS mutagenesis in fly

xpd is conserved form yeast to human. *xpd* homologues have been studied in different systems, *rad 3* in budding yeast, *rad15* in fission yeast, mouse *Ercc2*, human *xpd* (Lehmann, 2001). *Drosophila xpd* shows a high degree of homology with its human counterpart, with 73% identity in the predicted amino acid sequence, and this identity increases to almost 100% in the nucleotide-binding box and the proposed DNA-DNA and DNA-RNA helicase domain (Reynaud et al., 1999).

We assume that flies that are lacking both copies of genomic *xpd* will be lethal. To generate *xpd* mutations in *Drosophila*, we randomly induced point mutations and screened for the mutations that caused lethality over a *xpd* deficiency. (Figure 2). From this screen, we obtained 157 mutation strains that were lethal over the large *xpd* deficiency *Df(2R)Pu-D17*. We are now in the process of mapping these mutations with two smaller *xpd* deficiencies. Finally, in order to identify *xpd* mutations, we will try to rescue them with a *hsp-xpd* transgene (Figure 3). It is possible that some weak *xpd* alleles over smaller *xpd* deficiencies are not 100% lethal. In this case, I will be able to use western blot to analyze Xpd protein level in these viable escapers and find out if they are lacking Xpd or have less Xpd. In addition, I can also sequence their *xpd* gene to find out if they have mutations in the ORF of *xpd* gene.

III. Materials and Methods

EMS mutagenesis and *xpd* mutation screen

To make the sugar-EMS solution, add 260 µl EMS (Methanosulfonic acid ethyl ester, Sigma M 0880) with a 1 ml syringe to 100 ml 1% Sucrose solution in a glass beaker (500 ml). Put 1 Kimwipe into a disposable plastic fly vial, add 2 ml of sugar-EMS solution and push the paper down to the bottom. Transfer 50 males into each vial and keep them over night in the hood. The next day, transfer males on normal food and let them recover over night, then mate them to about 50 females per vial. Point mutations in germ cells of these F1 male flies were induced by the EMS mutagene and different sperm cells are expected to contain distinct mutations. Therefore, each individual of their progeny F2 would bear different mutations. 7,000 F2 males were singled out to cross to the *xpd* deficiency (heterozygous for the *xpd* deficiency). In the F3 generation we expect two classes of the flies that contain EMS mutations. One class has the EMS mutation over the *xpd* deficiency chromosome, the second class has the EMS mutation over the balancer chromosome. If the flies of the first class are lethal, this mutation was selected as potential *xpd* mutation. Their siblings, the second class that has the EMS mutation over the balancer, were recovered to establish a fly stock of this mutation.

Figure 1. Adopted from Bott et al (Botta et al., 1998). Alterations of the human XPD protein in TTD, XP, and XP/CS patients.

The diagram shows the XPD protein, with the helicase domains (blackened boxes in bar). The amino acid changes resulting from the mutations associated with the different pathological phenotypes are shown boxed, with the change shown as either white on black (for TTD) or black on white (for XP [dotted border] and for XP/CS [unbroken border]). The deletions are indicated by unbroken, dashed and dotted arrows. Numbers "1" and "2" after the patient code denote the different alleles.

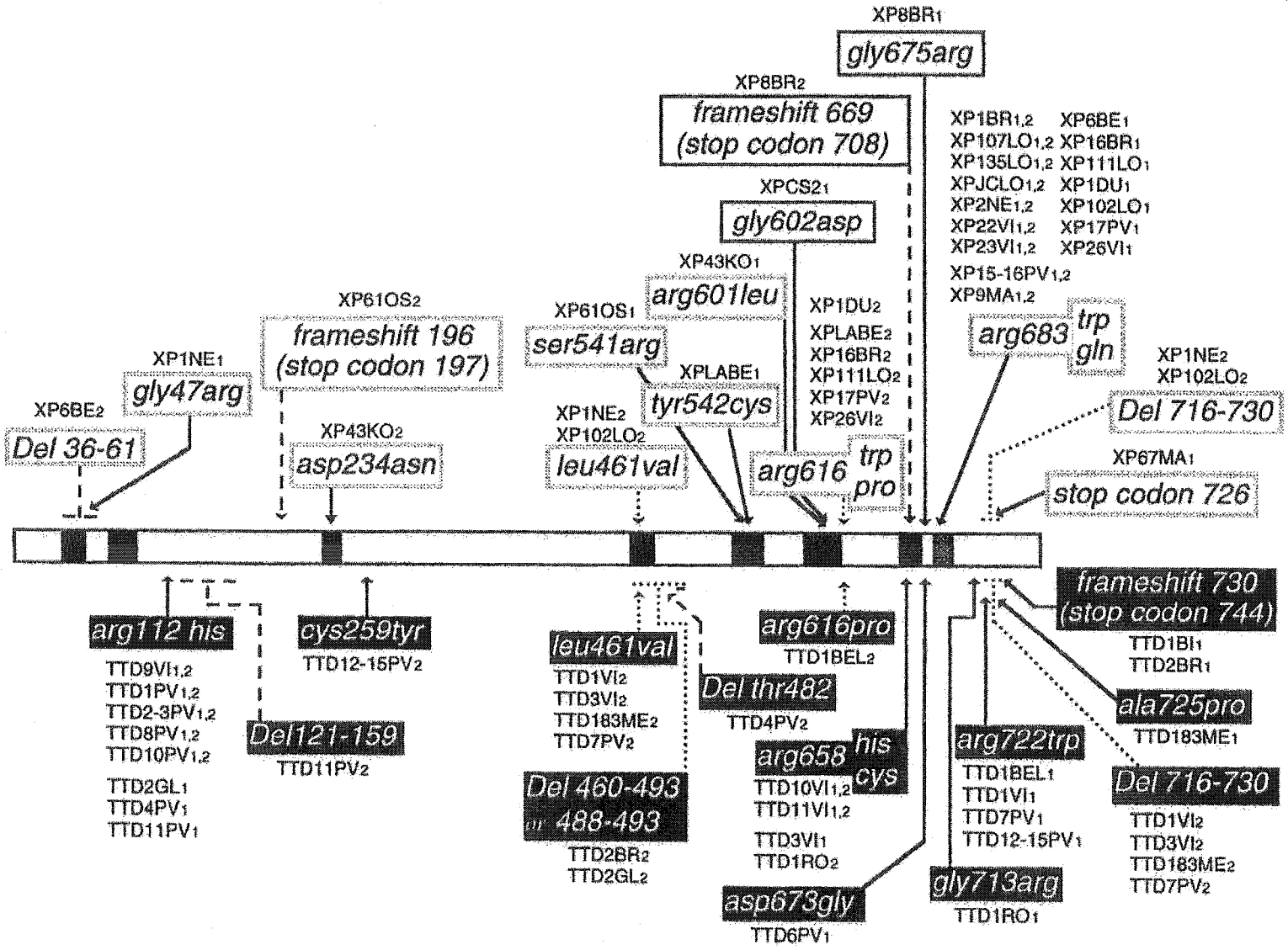

XP8BR1
gly675arg
XP8BR2
frameshift 669
(stop codon 708)
XPCS21
gly602asp
XP43KO1
arg601leu
XP61OS2
frameshift 196
(stop codon 197)
XP61OS1
ser541arg
XP1NE1
gly47arg
XP6BE2
Del 36-61
XP43KO2
asp234asn
XPLABE1
tyr542cys
XP1NE2
XP102LO2
leu461val
XP1DU2
XPLABE2
XP16BR2
XP111LO2
XP17PV2
XP26VI2
arg616 trp pro
XP1BR1,2 XP6BE1
XP107LO1,2 XP16BR1
XP135LO1,2 XP111LO1
XPJCLO1,2 XP1DU1
XP2NE1,2 XP102LO1
XP22VI1,2 XP17PV1
XP23VI1,2 XP26VI1
XP15-16PV1,2
XP9MA1,2
arg683 trp gln
XP1NE2
XP102LO2
Del 716-730
XP67MA1
stop codon 726
arg112 his
TTD9VI1,2
TTD1PV1,2
TTD2-3PV1,2
TTD8PV1,2
TTD10PV1,2
TTD2GL1
TTD4PV1
TTD11PV1
Del121-159
TTD11PV2
cys259tyr
TTD12-15PV2
leu461val
TTD1VI2
TTD3VI2
TTD183ME2
TTD7PV2
Del 460-493
or 488-493
TTD2BR2
TTD2GL2
arg616pro
TTD1BEL2
Del thr482
TTD4PV2
arg658 his cys
TTD10VI1,2
TTD11VI1,2
TTD3VI1
TTD1RO2
asp673gly
TTD6PV1
frameshift 730
(stop codon 744)
TTD1BI1
TTD2BR1
ala725pro
TTD183ME1
arg722trp
TTD1BEL1
TTD1VI1
TTD7PV1
TTD12-15PV1
gly713arg
TTD1RO1
Del 716-730
TTD1VI2
TTD3VI2
TTD183ME2
TTD7PV2

Figure 2. Genetic scheme for EMS mutagenesis

Males fed with EMS were from two strains 1) A204: *b pr cn wx bw; 2)* AP203: *b pr cn sca.* A204 *b pr cn wx bw* was used as example for this genetic scheme. Following EMS mutagenesis, the males were mated to *bTft/CyO bw* virgin females. The *bTft/b pr cn wx bw** males resulting from this cross were then mated to *xpd* deficiency virgins *Df(xpd), cn bw/ SM1*. In the progeny, the absence of *Df(xpd), cn bw / b pr cn wx bw** indicates that the EMS point mutation is lethal over the *xpd* deficiency. In cases where this is observed, their siblings *b pr cn wx bw*/SM1* were recovered to establish a stock.

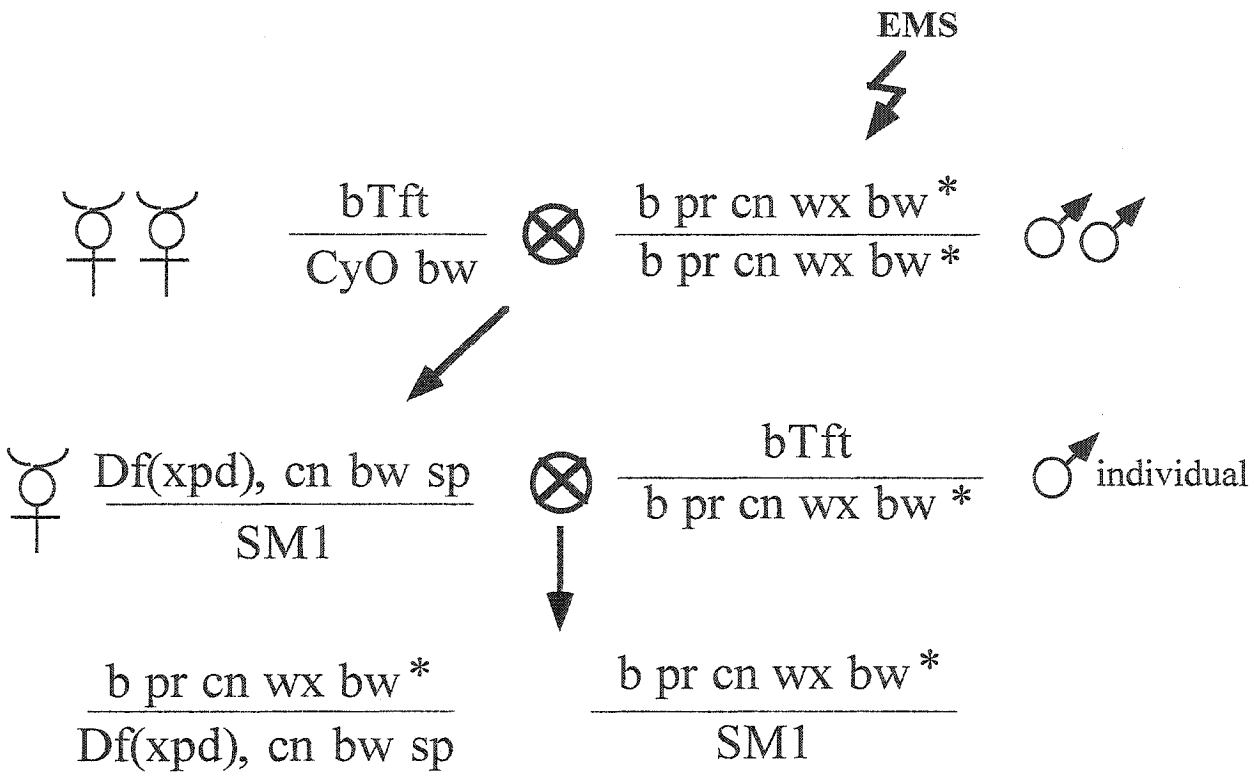

EMS
bTft / CyO bw
b pr cn wx bw* / b pr cn wx bw*
Df(xpd), cn bw sp / SM1
bTft / b pr cn wx bw *
individual
b pr cn wx bw* / Df(xpd), cn bw sp
b pr cn wx bw* / SM1

Figure 3. Rescue the EMS mutations with an *hsp-xpd* transgene

Amongst the siblings of the F3 generation, two classes of flies that contain the point mutations over the *xpd* deficiency chromosome are compared. One class of flies has the *hsp-xpd* transgene (*b pr cn wx bw*/ Df(xpd)cn bw* sp^1*; hsp-xpd/+)*, but the other not (*b pr cn wx bw*/Df(xpd)cn bw* sp^1*; PrDr/+)*. If the mutation is within the *xpd* region, then the *xpd* transgene will rescue the first class. If the mutation is not in *xpd* region, then neither class will be rescued.

♀♀ $\frac{w}{w}$; $\frac{+}{SM1\ (Cy\ cn^2\ sp^2)}$; $\frac{P[w^+\ hsp\text{-}xpd]}{TM3,Sb}$ X $\frac{w}{\neg}$; $\frac{Df(xpd)\ cn\ bw\ sp^1}{CyO}$; $\frac{+}{PrDr}$ ♂♂

↓

♀♀ $\frac{w}{w}$; $\frac{Df(xpd)\ cn\ bw\ sp^1}{SM1\ (Cy\ cn^2\ sp^2)}$; $\frac{P[w^+\ hsp\text{-}xpd]}{PrDr}$ X $\frac{+}{\neg}$; $\frac{b\ pr\ cn\ wx\ bw^*}{SM1\ (Cy\ cn^2\ sp^2)}$; $\frac{+}{+}$ ♂♂

↓ (with heat shock)

$\frac{w}{\neg}$; $\frac{b\ pr\ cn\ wx\ bw^*}{Df(xpd)\ cn\ bw\ sp^1}$; $\frac{P[w^+\ hsp\text{-}xpd]}{+}$	If non-Cy, non-PrDr, reddish eye flies viable, recues
$\frac{w}{\neg}$; $\frac{b\ pr\ cn\ wx\ bw^*}{Df(xpd)\ cn\ bw\ sp^1}$; $\frac{PrDr}{+}$	lethal
$\frac{w}{\neg}$; $\frac{SM1\ (Cy\ cn^2\ sp^2)}{Df(xpd)\ cn\ bw\ sp^1}$; $\frac{PrDr\ or\ P[w^+\ hsp\text{-}xpd]}{+}$	viable
$\frac{w}{\neg}$; $\frac{b\ pr\ cn\ wx\ bw^*}{SM1\ (Cy\ cn^2\ sp^2)}$; $\frac{PrDr\ or\ P[w^+\ hsp\text{-}xpd]}{+}$	viable

Chapter 6

General discussion

I. Xpd in the regulation of CAK activity

TFIIH can be found in a small number of sub-complexes, core-TFIIH with the five subunits p34, p44, p52, p62 and Xpb, and the CAK sub-complex with Cdk7, Cyclin H and Mat1. TFIIH and its sub-complexes are involved in the regulation of transcription, DNA repair and cell cycle control (Egly, 2001; Frit et al., 1999). Free CAK and TFIIH associated CAK display different substrate specificity. TFIIF (rap 74), TFIIE (p56) and E2F are phosphorylated by TFIIH but not by CAK. Histone H1 and CTD are better substrates for TFIIH than for CAK, but Cdk2 is better phosphorylated by CAK than by TFIIH (Rossignol et al., 1997; Vandel and Kouzarides, 1999; Yankulov and Bentley, 1997). It appears that the multiple functions of Cdk7 may be regulated by complex formation.

The molecular structure of human TFIIH shows that Xpd is located close to the CAK complex (Schultz et al., 2000). Structural and biochemical data indicates that Xpd links the CAK complex to the core -TFIIH (Drapkin et al., 1996; Reardon et al., 1996; Schultz et al., 2000). The N-terminal domain of Xpd is required for interaction between Xpd and the CAK complex and the C-terminus of Xpd facilitates or enhances this interaction (Sandrock and Egly, 2001). It further appears that Xpd associates with core TFIIH through p44 and with CAK through Mat1, and that this interaction helps CAK integrate into core-TFIIH (Busso et al., 2000; Coin et al., 1998; Feaver et al., 2000; Schultz et al., 2000). Therefore, the fact that Xpd has an organizing role in TFIIH assembly and that it links the CAK complex to core TFIIH makes Xpd an excellent candidate for a regulator of TFIIH complex composition and function.

Our data strongly support the idea that the multiple functions of Cdk7 could be regulated by complex formation and that this regulation through Xpd is cell cycle dependent. This provides the first evidence that TFIIH is a link between cell cycle and transcription.

II. What mechanism mediates the Xpd down-regulation during prometaphase?

Evidence that a protein degradation pathway influences the organization and stability of TFIIH has started to build up very recently. A four hybrid screen using the CAK components Cdk7, Cyclin H and Mat1 as bait identified three subunits of the proteasome machinery (Sandrock and Egly, 2001). In addition, more indirect evidence comes from the finding that

strongly reduced levels of TFIIH cause the TTD phenotype in the TTD-A group patients (Stefanini et al., 1993; Vermeulen et al., 2000). It was therefore suggested that the TTD-A mutations affect an unknown factor required either to stabilize TFIIH or to protect TFIIH from degradation (Bergmann and Egly, 2001; Vermeulen et al., 2000). Consistent with this interpretation, this TTD phenotype can be rescued by injecting TFIIH. This rescue is only transient, further suggesting that the TFIIH complexes are either inactivated or degraded (Vermeulen et al., 2000). Our results now show directly that wild type cells down-regulate Xpd at the beginning of mitosis very abruptly. This provides evidence that Xpd may be regulated through a protein degradation pathway. The timing of this degradation further suggests that it has a biological significance for either cell cycle progression and/or for transcription silencing during mitosis.

I have shown that Xpd is down-regulated during prometaphase, coinciding with Cyclin A degradation. The mechanism of its down-regulation remains to be clarified. The first interesting question is whether Xpd down-regulation is through ubiquitin-mediated proteolysis. This could be addressed by analyzing the down-regulation of Xpd in the presence of inhibitors of the ubiquitin system. If Xpd does require ubiqutin to be down-regulated, it will be interesting to find out whether it requires APC and fizzy/fizzy-related? From the sequence analysis, Xpd does not have a typical D-box (R-X-X-L-X-X-X-X-N) as Cyclin B1 or a KEN box as Cyclin A. But Xpd has a few putative D-boxes (R-X-X-L) as Cyclin A does. Perhaps Xpd requires multiple signals to mediate its degradation, similar to Cyclin A. The requirement for *fizzy* or *fizzy-related* should be answered by analyzing Xpd levels in *fizzy* or *fizzy-related* mutants.

III. High cancer risk in XP type Xpd patients

Mutations in *xpd* can result in three clinical distinct, autosomal recessive disorders: Xeroderma pigmentosum (XP), Trichothiodystrophy (TTD) and occasionally Cockayne Syndrome that is seen only in combination with XP (XP/CS) (Lehmann, 2001). XP is characterized by hyperpigmentation of the skin under sun exposure and cutaneous abnormalities. In addition, it is associated with 1,000-2,000 fold increased risk to develop skin cancer (Lehmann, 2001). Some XP patients also display neurological abnormalities caused by primary

neuronal degeneration. Some TTD patients display sun sensitivity but have no predisposition to skin cancer and all of them display severe developmental retardation and neurological degeneration (de Boer and Hoeijmakers, 2000; Lehmann, 2001).

The biochemical activity of XPD is to unwind DNA in the 5'-3' direction and it seems to be doing this both, during nucleotide excision repair (NER) and during transcription initiation. During transcription, Xpd participates in opening up the DNA at the promoter site, thereby facilitating access to the transcriptional start site for the RNA polymerase II transcription machinery (Holstege et al., 1996; Tirode et al., 1999). However, while *xpd* is essential for this process, its helicase activity is dispensable for transcription and is only required for DNA repair (Bradsher et al., 2000; Coin et al., 1999; Tirode et al., 1999; Winkler et al., 2000).

Despite the knowledge of Xpd's dual role in NER and transcription, Xpd syndromes are not fully understood. The sequence analysis of *xpd* alleles showed that *xpd* mutations are syndrome specific, wherein the alteration of a given residue causes either the XP or the TTD phenotype (Botta et al., 1998; Takayama et al., 1996; Takayama et al., 1995; Taylor et al., 1997). However, because *xpd* mutations changing even neighboring residues can result in the different phenotypes, no disease specific domain could be identified (Botta et al., 1998; de Boer and Hoeijmakers, 2000; Lehmann, 2001). Two research studies addressed the question how mutations in one *xpd* gene can give such different clinical phenotypes. One study suggests a potential link between *xpd* and *xpb* mutations and deregulation of *c-myc* expression (Liu et al., 2001). The second showed that Xpd mutations, both XP and TTD type, prevent phosphorylation of RARα by Cdk7 kinase and thus cause defects in hormone-mediated transcription activation (Keriel et al., 2002).

A question that still remained unclear is why the repair-deficient photosensitive TTD phenotype is not associated with cancer while XP mutations are. It was proposed that XP mutations may interfere with the helicase activity and that this would mainly disrupt NER. TTD mutations, on the other hand, were thought to cause subtle defects in interaction between Xpd and other components of TFIIH or transcription factors. The TTD mutations were therefore expected to have their primary defect in transcription (Berneburg and Lehmann, 2001; Coin et al., 1999; Hoeijmakers et al., 1996). However, both XP and TTD cell lines show defects in transcription and in DNA repair. Furthermore, the severity of these defects is in a similar range in XP and TTD cell lines, arguing that neither qualitative nor quantitative differences in NER and transcription

account for the clinical differences (Berneburg et al., 2000; Botta et al., 1998; Coin et al., 1999; de Boer and Hoeijmakers, 2000). Therefore, symptoms in XP type and TTD type *xpd* patients cannot be fully explained by NER defects and subtle defects in transcription, suggesting that other defects might contribute to the phenotypes.

Our finding that *xpd* negatively regulates the CAK activity of Cdk7 points to a new mechanism that may explain the high cancer risk found only in XP and not in TTD type *xpd* patients. XP mutations may alter Xpd such that it fails to repress CAK. The molecular basis of this could be that the XP mutations interfere with CAK complex binding to core-TFIIH or with CAK stability in holo-TFIIH, thereby leading to excessive accumulation of free CAK. This excess CAK may then interfere with normal entry into or progression through mitosis and this may increase the risk of developing cancer in the NER defective cell. Consistent with this model, a previous study had established a tentative link between elevated levels of Cdk7 and cancer. Comparing tumor cell lines to their normal cell counterparts, this work showed that the tumor cells have 1.4 to 3 fold elevated levels of Cdk7 (Bartkova et al., 1996).

Other evidence supporting this model comes from the study of the TTD-A phenotype caused by highly reduced levels of TFIIH. The study showed that CAK components are still anchored to TFIIH of TTD-A cells and that the Xpd-CAK interaction is not disrupted in TTD-A patients (Vermeulen et al., 2000). In contrast, the XP type *xpd* mutation R683W weakens CAK anchoring on core-TFIIH (Keriel et al., 2002) and the associated tumors did not respond to retinoid treatment (Keriel et al., 2002; Kraemer et al., 1988). There is further support for this model from a direct measurement of the interaction capacity between different mutant forms of Xpd and the CAK complex (Sandrock and Egly, 2001). In a yeast four-hybrid assay, one of each mutant *xpd*, TTD (R112H), XP (D234N) and XP/CS (G602D), was used as bait. The interaction between wild type and mutant forms of Xpd and CAK was then measured with a reporter gene. Compared to wild type Xpd, the TTD mutation had only little effect, and the Xpd-CAK interaction capacity was approx. 90% of wild type. XP and XP/CS mutations, on the other hand, caused a much stronger reduction in interaction capacity. The XP form of Xpd reduces the interaction capacity between Xpd and CAK to about 55% and the XP/CS to approx. 60%. In this experiment there is a good correlation between reduced Xpd–CAK interaction and the cancer risk observed in XP patients. However, because of the limited sample size in the above experiment,

further work is required to systematically test whether increased CAK activity is a signature of the XP syndrome.

IV. Cdk7 and Alzheimer's disease

Alzheimer disease (AD) is characterized by the degeneration of selected neuronal populations in the hippocampus and other cortical brain regions (Smith, 1998). Neurofibrillary tangles are typical pathological signs observed in Alzheimers disease and these structures were identified as a highly insoluble form of the microtubule-binding protein tau (Haass and Mandelkow, 1999; Mandelkow and Mandelkow, 1998). The insolubility of tau is due to its hyperphosphorylation. A number of protein kinases phosphorylate tau *in vitro*, but the most physiologically relevant ones appear to be GSK-3 (Tau protein kinase I), CDK5/p25 (Tau protein kinase II), cAMP-dependent protein kinase (PKA) and casein kinase 1 (CK1) (Haass and Mandelkow, 1999; Imahori and Uchida, 1997; Mandelkow and Mandelkow, 1998).

CDK5 was initially identified on the basis of its sequence similarity with Cdk1 (Meyerson et al., 1992), later as tau protein kinase II (Kobayashi et al., 1993; Paudel et al., 1993; Tang and Wang, 1996). In brains of AD patients, Cdk5 has increased activity and is found preferentially in neurofibrillary tangles [Lee, 1999 #719; (Leost et al., 2000; Yamaguchi et al., 1996). This suggests that Cdk5 may be involved in the cytoskeletal abnormalities and neuronal death observed in AD. *Drosophila* homologues of Cdk5 have been identified and they show 78% identity in the kinase domain with their human counterpart (Sauer et al., 1996). *Drosophila* Cdk5 is also expressed in neuronal system and it functions in axon patterning in the embryos (Connell-Crowley et al., 2000).

Recent findings suggest that the mechanism of abnormal neuronal death might be due to cell apoptosis triggered by de-regulation of the cell cycle control. Normal neuron cells are terminally differentiated and maintain their quiescent status. In contrast, in neuron cells of AD patients, up-regulation of the cell cycle regulators was found. The affected cell cycle proteins include Cdks (Cdc2, Cdk2, Cdk4), Cyclins (CyclinD, CyclinB Cyclin E) and CKIs (p16 and p21) (Arendt et al., 1996; McShea et al., 1997; Nagy et al., 1997; Smith and Lippa, 1995; Vincent et al., 1997). It thus seems that these cells attempt to re-enter into the

cell cycle. Because these cells can not undergo an entire division cycle, cell cycle entry might trigger apoptosis and result in neurodegeneration.

Cdk7 is a major positive regulator of other Cdks and its expression in neuron cells from AD patients was analyzed. Immunostaining results showed that Cdk7 is significantly elevated in susceptible neurons of early onset AD patients compared with age-matched controls (Zhu et al., 2000). With respect to the progression of the disease, the elevation of Cdk7 in AD patients seems to be an early event in AD pathogenesis. This study established a tentative link between Cdk7 up-regulation and early onset of Alzhermer's disease. Another known substrate of Cdk7, RNAP II-LS, also changes in phoshorylation status and subcellular localization in Alzhermer's disease brains (Bregman et al., 2000). Since Cdks and RNAP II-LS are the potential targets of Cdk7 and because Cdk7 itself is up-regulated in AD patients, Cdk7 may play a causative role in AD pathogenesis.

References

- Abraham, R. T. 2001. Cell cycle checkpoint signaling through the ATM and ATR kinases. *Genes & Development* **15**, 2177-96.
- Adamczewski, J. P., Rossignol, M., Tassan, J. P., Nigg, E. A., Moncollin, V., and Egly, J. M. 1996. MAT1, cdk7 and cyclin H form a kinase complex which is UV light-sensitive upon association with TFIIH. *Embo J* **15**, 1877-84.
- Akoulitchev, S., Makela, T. P., Weinberg, R. A., and Reinberg, D. 1995. Requirement for TFIIH kinase activity in transcription by RNA polymerase II. *Nature* **377**, 557-60.
- Akoulitchev, S., and Reinberg, D. 1998. The molecular mechanism of mitotic inhibition of TFIIH is mediated by phosphorylation of CDK7. *Genes Dev* **12**, 3541-50.
- Alexandru, G., Zachariae, W., Schleiffer, A., and Nasmyth, K. 1999. Sister chromatid separation and chromosome re-duplication are regulated by different mechanisms in response to spindle damage. *Embo J* **18**, 2707-21.
- Amati, B., Alevizopoulos, K., and Vlach, J. 1998. Myc and the cell cycle. *Front Biosci* **3**, D250-68.
- Araujo, S. J., Tirode, F., Coin, F., Pospiech, H., Syvaoja, J. E., Stucki, M., Hubscher, U., Egly, J. M., and Wood, R. D. 2000. Nucleotide excision repair of DNA with recombinant human proteins: definition of the minimal set of factors, active forms of TFIIH, and modulation by CAK. *Genes Dev* **14**, 349-59.
- Arendt, T., Rodel, L., Gartner, U., and Holzer, M. 1996. Expression of the cyclin-dependent kinase inhibitor p16 in Alzheimer's disease. *Neuroreport* **7**, 3047-9.
- Arrigo, A. P., and Tanguay, R. M. 1991. Expression of heat shock proteins during development in Drosophila. *Results Probl Cell Differ* **17**, 106-19.
- Attardi, L. D., Lowe, S. W., Brugarolas, J., and Jacks, T. 1996. Transcriptional activation by p53, but not induction of the p21 gene, is essential for oncogene-mediated apoptosis. *Embo J* **15**, 3693-701.
- Barnes, D. E., Tomkinson, A. E., Lehmann, A. R., Webster, A. D., and Lindahl, T. 1992. Mutations in the DNA ligase I gene of an individual with immunodeficiencies and cellular hypersensitivity to DNA-damaging agents. *Cell* **69**, 495-503.

- Bartkova, J., Zemanova, M., and Bartek, J. 1996. Expression of CDK7/CAK in normal and tumor cells of diverse histogenesis, cell-cycle position and differentiation. *Int J Cancer* **66**, 732-7.
- Baumeister, W., Walz, J., Zuhl, F., and Seemuller, E. 1998. The proteasome: paradigm of a self-compartmentalizing protease. *Cell* **92**, 367-80.
- Bergmann, E., and Egly, J. M. 2001. Trichothiodystrophy, a transcription syndrome. *Trends Genet* **17**, 279-86.
- Berneburg, M., Clingen, P. H., Harcourt, S. A., Lowe, J. E., Taylor, E. M., Green, M. H., Krutmann, J., Arlett, C. F., and Lehmann, A. R. 2000. The cancer-free phenotype in trichothiodystrophy is unrelated to its repair defect. *Cancer Res* **60**, 431-8.
- Berneburg, M., and Lehmann, A. R. 2001. Xeroderma pigmentosum and related disorders: defects in DNA repair and transcription. *Adv Genet* **43**, 71-102.
- Botta, E., Nardo, T., Broughton, B. C., Marinoni, S., Lehmann, A. R., and Stefanini, M. 1998. Analysis of mutations in the XPD gene in Italian patients with trichothiodystrophy: site of mutation correlates with repair deficiency, but gene dosage appears to determine clinical severity. *Am J Hum Genet* **63**, 1036-48.
- Bradsher, J., Coin, F., and Egly, J. M. 2000. Distinct roles for the helicases of TFIIH in transcript initiation and promoter escape. *J Biol Chem* **275**, 2532-8.
- Bregman, D. B., Pestell, R. G., and Kidd, V. J. 2000. Cell cycle regulation and RNA polymerase II. *Front Biosci* **5**, D244-57.
- Brehm, A., Miska, E. A., McCance, D. J., Reid, J. L., Bannister, A. J., and Kouzarides, T. 1998. Retinoblastoma protein recruits histone deacetylase to repress transcription. *Nature* **391**, 597-601.
- Brown, A. J., Jones, T., and Shuttleworth, J. 1994. Expression and activity of p40MO15, the catalytic subunit of cdk- activating kinase, during Xenopus oogenesis and embryogenesis. *Mol Biol Cell* **5**, 921-32.
- Bunz, F., Dutriaux, A., Lengauer, C., Waldman, T., Zhou, S., Brown, J. P., Sedivy, J. M., Kinzler, K. W., and Vogelstein, B. 1998. Requirement for p53 and p21 to sustain G2 arrest after DNA damage. *Science* **282**, 1497-501.

- Busso, D., Keriel, A., Sandrock, B., Poterszman, A., Gileadi, O., and Egly, J. M. 2000. Distinct regions of MAT1 regulate cdk7 kinase and TFIIH transcription activities. *J Biol Chem* **275**, 22815-23.
- Campanero, M. R., and Flemington, E. K. 1997. Regulation of E2F through ubiquitin-proteasome-dependent degradation: stabilization by the pRB tumor suppressor protein. *Proc Natl Acad Sci U S A* **94**, 2221-6.
- Cardoso, M. C., Leonhardt, H., and Nadal-Ginard, B. 1993. Reversal of terminal differentiation and control of DNA replication: cyclin A and Cdk2 specifically localize at subnuclear sites of DNA replication. *Cell* **74**, 979-92.
- Chan, T. A., Hermeking, H., Lengauer, C., Kinzler, K. W., and Vogelstein, B. 1999. 14-3-3Sigma is required to prevent mitotic catastrophe after DNA damage. *Nature* **401**, 616-20.
- Charles, J. F., Jaspersen, S. L., Tinker-Kulberg, R. L., Hwang, L., Szidon, A., and Morgan, D. O. 1998. The Polo-related kinase Cdc5 activates and is destroyed by the mitotic cyclin destruction machinery in S. cerevisiae. *Curr Biol* **8**, 497-507.
- Chen, D., Riedl, T., Washbrook, E., Pace, P. E., Coombes, R. C., Egly, J. M., and Ali, S. 2000. Activation of estrogen receptor alpha by S118 phosphorylation involves a ligand-dependent interaction with TFIIH and participation of CDK7. *Mol Cell* **6**, 127-37.
- Child, E. S., and Mann, D. J. 2001. Novel properties of the cyclin encoded by Human Herpesvirus 8 that facilitate exit from quiescence. *Oncogene* **20**, 3311-22.
- Cho, H., Kim, T. K., Mancebo, H., Lane, W. S., Flores, O., and Reinberg, D. 1999. A protein phosphatase functions to recycle RNA polymerase II. *Genes Dev* **13**, 1540-52.
- Cismowski, M. J., Laff, G. M., Solomon, M. J., and Reed, S. I. 1995. KIN28 encodes a C-terminal domain kinase that controls mRNA transcription in Saccharomyces cerevisiae but lacks cyclin-dependent kinase-activating kinase (CAK) activity. *Mol Cell Biol* **15**, 2983-92.
- Clemens, J. C., Worby, C. A., Simonson-Leff, N., Muda, M., Maehama, T., Hemmings, B. A., and Dixon, J. E. 2000. Use of double-stranded RNA interference in Drosophila cell lines to dissect signal transduction pathways. *Proc Natl Acad Sci U S A* **97**, 6499-503.

- Cohen, S. M. 1993. Imaginal disc development. In *The development of Drosophila melanogaster*747-842.
- Coin, F., Bergmann, E., Tremeau-Bravard, A., and Egly, J. M. 1999. Mutations in XPB and XPD helicases found in xeroderma pigmentosum patients impair the transcription function of TFIIH. *Embo J* **18**, 1357-66.
- Coin, F., Marinoni, J. C., and Egly, J. M. 1998. Mutations in XPD helicase prevent its interaction and regulation by p44, another subunit of TFIIH, resulting in Xeroderma pigmentosum (XP) and trichothiodystrophy (TTD) phenotypes. *Pathol Biol (Paris)* **46**, 679-80.
- Conaway, R. C., and Conaway, J. W. 1993. General initiation factors for RNA polymerase II. *Annu Rev Biochem* **62**, 161-90.
- Connell-Crowley, L., Le Gall, M., Vo, D. J., and Giniger, E. 2000. The cyclin-dependent kinase Cdk5 controls multiple aspects of axon patterning in vivo. *Curr Biol* **10**, 599-602.
- Coux, O., Tanaka, K., and Goldberg, A. L. 1996. Structure and functions of the 20S and 26S proteasomes. *Annu Rev Biochem* **65**, 801-47.
- Cramer, P., Bushnell, D. A., Fu, J., Gnatt, A. L., Maier-Davis, B., Thompson, N. E., Burgess, R. R., Edwards, A. M., David, P. R., and Kornberg, R. D. 2000. Architecture of RNA polymerase II and implications for the transcription mechanism. *Science* **288**, 640-9.
- Cujec, T. P., Okamoto, H., Fujinaga, K., Meyer, J., Chamberlin, H., Morgan, D. O., and Peterlin, B. M. 1997. The HIV transactivator TAT binds to the CDK-activating kinase and activates the phosphorylation of the carboxy-terminal domain of RNA polymerase II. *Genes Dev* **11**, 2645-57.
- Dahmus, M. E. 1995. Phosphorylation of the C-terminal domain of RNA polymerase II. *Biochim Biophys Acta* **1261**, 171-82.
- Dahmus, M. E. 1996. Reversible phosphorylation of the C-terminal domain of RNA polymerase II. *J Biol Chem* **271**, 19009-12.
- Dalton, S. 1992. Cell cycle regulation of the human cdc2 gene. *Embo J* **11**, 1797-804.
- Dawson, I. A., Roth, S., and Artavanis-Tsakonas, S. 1995. The Drosophila cell cycle gene fizzy is required for normal degradation of cyclins A and B during mitosis and has homology to the CDC20 gene of Saccharomyces cerevisiae. *J Cell Biol* **129**, 725-37.

- de Boer, J., Donker, I., de Wit, J., Hoeijmakers, J. H., and Weeda, G. 1998. Disruption of the mouse xeroderma pigmentosum group D DNA repair/basal transcription gene results in preimplantation lethality. *Cancer Res* **58**, 89-94.
- de Boer, J., and Hoeijmakers, J. H. 2000. Nucleotide excision repair and human syndromes. *Carcinogenesis* **21**, 453-60.
- de Laat, W. L., Appeldoorn, E., Sugasawa, K., Weterings, E., Jaspers, N. G., and Hoeijmakers, J. H. 1998. DNA-binding polarity of human replication protein A positions nucleases in nucleotide excision repair. *Genes Dev* **12**, 2598-609.
- de Laat, W. L., Jaspers, N. G., and Hoeijmakers, J. H. 1999. Molecular mechanism of nucleotide excision repair. *Genes Dev* **13**, 768-85.
- den Elzen, N., and Pines, J. 2001. Cyclin A is destroyed in prometaphase and can delay chromosome alignment and anaphase. *J Cell Biol* **153**, 121-36.
- Desai, D., Gu, Y., and Morgan, D. O. 1992. Activation of human cyclin-dependent kinases in vitro. *Mol Biol Cell* **3**, 571-82.
- Devault, A., Martinez, A. M., Fesquet, D., Labbe, J. C., Morin, N., Tassan, J. P., Nigg, E. A., Cavadore, J. C., and Doree, M. 1995. MAT1 ('menage a trois') a new RING finger protein subunit stabilizing cyclin H-cdk7 complexes in starfish and Xenopus CAK. *Embo J* **14**, 5027-36.
- Dobles, M., Liberal, V., Scott, M. L., Benezra, R., and Sorger, P. K. 2000. Chromosome missegregation and apoptosis in mice lacking the mitotic checkpoint protein Mad2. *Cell* **101**, 635-45.
- Donahue, B. A., Yin, S., Taylor, J. S., Reines, D., and Hanawalt, P. C. 1994. Transcript cleavage by RNA polymerase II arrested by a cyclobutane pyrimidine dimer in the DNA template. *Proc Natl Acad Sci U S A* **91**, 8502-6.
- Drapkin, R., Le Roy, G., Cho, H., Akoulitchev, S., and Reinberg, D. 1996. Human cyclin-dependent kinase-activating kinase exists in three distinct complexes. *Proc Natl Acad Sci U S A* **93**, 6488-93.
- Dubois, M. F., Bellier, S., Seo, S. J., and Bensaude, O. 1994. Phosphorylation of the RNA polymerase II largest subunit during heat shock and inhibition of transcription in HeLa cells. *J Cell Physiol* **158**, 417-26.

- Dubois, M. F., Vincent, M., Vigneron, M., Adamczewski, J., Egly, J. M., and Bensaude, O. 1997. Heat-shock inactivation of the TFIIH-associated kinase and change in the phosphorylation sites on the C-terminal domain of RNA polymerase II. *Nucleic Acids Res* **25**, 694-700.
- Dvir, A., Conaway, J. W., and Conaway, R. C. 2001. Mechanism of transcription initiation and promoter escape by RNA polymerase II. *Curr Opin Genet Dev* **11**, 209-14.
- Dynlacht, B. D., Flores, O., Lees, J. A., and Harlow, E. 1994. Differential regulation of E2F transactivation by cyclin/cdk2 complexes. *Genes Dev* **8**, 1772-86.
- Dyson, N. 1998. The regulation of E2F by pRB-family proteins. *Genes Dev* **12**, 2245-62.
- Edgar, B. A., Sprenger, F., Duronio, R. J., Leopold, P., and O'Farrell, P. H. 1994. Distinct molecular mechanism regulate cell cycle timing at successive stages of Drosophila embryogenesis. *Genes Dev* **8**, 440-52.
- Egly, J. M. 2001. The 14th Datta Lecture. TFIIH: from transcription to clinic. *FEBS Lett* **498**, 124-8.
- el-Deiry, W. S., Tokino, T., Velculescu, V. E., Levy, D. B., Parsons, R., Trent, J. M., Lin, D., Mercer, W. E., Kinzler, K. W., and Vogelstein, B. 1993. WAF1, a potential mediator of p53 tumor suppression. *Cell* **75**, 817-25.
- Evans, H. J. 1997. Historical perspectives on the development of the in vitro micronucleus test: a personal view. *Mutat Res* **392**, 5-10.
- Ewen, M. E., Sluss, H. K., Sherr, C. J., Matsushime, H., Kato, J., and Livingston, D. M. 1993. Functional interactions of the retinoblastoma protein with mammalian D- type cyclins. *Cell* **73**, 487-97.
- Fang, G., Yu, H., and Kirschner, M. W. 1998. The checkpoint protein MAD2 and the mitotic regulator CDC20 form a ternary complex with the anaphase-promoting complex to control anaphase initiation. *Genes Dev* **12**, 1871-83.
- Feaver, W. J., Huang, W., Gileadi, O., Myers, L., Gustafsson, C. M., Kornberg, R. D., and Friedberg, E. C. 2000. Subunit interactions in yeast transcription/repair factor TFIIH. Requirement for Tfb3 subunit in nucleotide excision repair. *J Biol Chem* **275**, 5941-6.

- Feaver, W. J., Svejstrup, J. Q., Henry, N. L., and Kornberg, R. D. 1994. Relationship of CDK-activating kinase and RNA polymerase II CTD kinase TFIIH/TFIIK. *Cell* **79**, 1103-9.
- Fesquet, D., Labbe, J. C., Derancourt, J., Capony, J. P., Galas, S., Girard, F., Lorca, T., Shuttleworth, J., Doree, M., and Cavadore, J. C. 1993. The MO15 gene encodes the catalytic subunit of a protein kinase that activates cdc2 and other cyclin-dependent kinases (CDKs) through phosphorylation of Thr161 and its homologues. *Embo J* **12**, 3111-21.
- Fisher, R. P., Jin, P., Chamberlin, H. M., and Morgan, D. O. 1995. Alternative mechanisms of CAK assembly require an assembly factor or an activating kinase. *Cell* **83**, 47-57.
- Fisher, R. P., and Morgan, D. O. 1994. A novel cyclin associates with MO15/CDK7 to form the CDK-activating kinase. *Cell* **78**, 713-24.
- Fisher, R. P., and Morgan, D. O. 1996. CAK in TFIIH: crucial connection or confounding coincidence? *Biochim Biophys Acta* **1288**, O7-10.
- Foe, V. E., and Alberts, B. M. 1985. Reversible chromosome condensation induced in Drosophila embryos by anoxia: visualization of interphase nuclear organization. *J Cell Biol* **100**, 1623-36.
- Foe, V. E., Odekk, G.M., and Edgar, B.A. 1993. Mitosis and morphogenesis in the Drosophila Embryo: Point and Counterpoint. In *The development of Drosophila melanogaster*149-300.
- Friedberg, E. C. 2001. How nucleitide excision repair protects against cancer. *Nature reviews* **1**, 22-33.
- Frit, P., Bergmann, E., and Egly, J. M. 1999. Transcription factor IIH: a key player in the cellular response to DNA damage. *Biochimie* **81**, 27-38.
- Gall, J. G. 2000. Cajal bodies: the first 100 years. *Annu Rev Cell Dev Biol* **16**, 273-300.
- Gall, J. G. 2001. A role for Cajal bodies in assembly of the nuclear transcription machinery. *FEBS Lett* **498**, 164-7.
- Gall, J. G., Bellini, M., Wu, Z., and Murphy, C. 1999. Assembly of the nuclear transcription and processing machinery: Cajal bodies (coiled bodies) and transcriptosomes. *Mol Biol Cell* **10**, 4385-402.

- Gallant, P., and Nigg, E. A. 1992. Cyclin B2 undergoes cell cycle-dependent nuclear translocation and, when expressed as a non-destructible mutant, causes mitotic arrest in HeLa cells. *J Cell Biol* **117**, 213-24.
- Geley, S., Kramer, E., Gieffers, C., Gannon, J., Peters, J. M., and Hunt, T. 2001. Anaphase-promoting complex/cyclosome-dependent proteolysis of human cyclin A starts at the beginning of mitosis and is not subject to the spindle assembly checkpoint. *J Cell Biol* **153**, 137-48.
- Geng, Y., Eaton, E. N., Picon, M., Roberts, J. M., Lundberg, A. S., Gifford, A., Sardet, C., and Weinberg, R. A. 1996. Regulation of cyclin E transcription by E2Fs and retinoblastoma protein. *Oncogene* **12**, 1173-80.
- Glotzer, M., Murray, A. W., and Kirschner, M. W. 1991. Cyclin is degraded by the ubiquitin pathway. *Nature* **349**, 132-8.
- Gorbsky, G. J., Chen, R. H., and Murray, A. W. 1998. Microinjection of antibody to Mad2 protein into mammalian cells in mitosis induces premature anaphase. *J Cell Biol* **141**, 1193-205.
- Gottesfeld, J. M., and Forbes, D. J. 1997. Mitotic repression of the transcriptional machinery. *Trends Biochem Sci* **22**, 197-202.
- Gottesfeld, J. M., Wolf, V. J., Dang, T., Forbes, D. J., and Hartl, P. 1994. Mitotic repression of RNA polymerase III transcription in vitro mediated by phosphorylation of a TFIIIB component. *Science* **263**, 81-4.
- Gould, K. L., Moreno, S., Owen, D. J., Sazer, S., and Nurse, P. 1991. Phosphorylation at Thr167 is required for Schizosaccharomyces pombe p34cdc2 function. *Embo J* **10**, 3297-309.
- Grande, M. A., van der Kraan, I., de Jong, L., and van Driel, R. 1997. Nuclear distribution of transcription factors in relation to sites of transcription and RNA polymerase II. *J Cell Sci* **110**, 1781-91.
- Guan, K. L., Jenkins, C. W., Li, Y., Nichols, M. A., Wu, X., O'Keefe, C. L., Matera, A. G., and Xiong, Y. 1994. Growth suppression by p18, a p16INK4/MTS1- and p14INK4B/MTS2-related CDK6 inhibitor, correlates with wild-type pRb function. *Genes Dev* **8**, 2939-52.

- Guzder, S. N., Sung, P., Bailly, V., Prakash, L., and Prakash, S. 1994. RAD25 is a DNA helicase required for DNA repair and RNA polymerase II transcription. *Nature* **369**, 578-81.
- Haass, C., and Mandelkow, E. 1999. Proteolysis by presenilins and the renaissance of tau. *Trends in Cell Biology* **9**, 241-4.
- Hagemeier, C., Cook, A., and Kouzarides, T. 1993. The retinoblastoma protein binds E2F residues required for activation in vivo and TBP binding in vitro. *Nucleic Acids Res* **21**, 4998-5004.
- Hagting, A., Karlsson, C., Clute, P., Jackman, M., and Pines, J. 1998. MPF localization is controlled by nuclear export. *Embo J* **17**, 4127-38.
- Hannon, G. J., and Beach, D. 1994. p15INK4B is a potential effector of TGF-beta-induced cell cycle arrest. *Nature* **371**, 257-61.
- Hardwick, K. G. 1998. The spindle checkpoint. *Trends Genet* **14**, 1-4.
- Harper, J. W., Adami, G. R., Wei, N., Keyomarsi, K., and Elledge, S. J. 1993. The p21 Cdk-interacting protein Cip1 is a potent inhibitor of G1 cyclin- dependent kinases. *Cell* **75**, 805-16.
- Harper, J. W., and Elledge, S. J. 1998. The role of Cdk7 in CAK function, a retro-retrospective. *Genes Dev* **12**, 285-9.
- Hateboer, G., Kerkhoven, R. M., Shvarts, A., Bernards, R., and Beijersbergen, R. L. 1996. Degradation of E2F by the ubiquitin-proteasome pathway: regulation by retinoblastoma family proteins and adenovirus transforming proteins. *Genes Dev* **10**, 2960-70.
- Hecker, D., Page, G., Lohrum, M., Weiland, S., and Scheidtmann, K. H. 1996. Complex regulation of the DNA-binding activity of p53 by phosphorylation: differential effects of individual phosphorylation sites on the interaction with different binding motifs. *Oncogene* **12**, 953-61.
- Heix, J., Vente, A., Voit, R., Budde, A., Michaelidis, T. M., and Grummt, I. 1998. Mitotic silencing of human rRNA synthesis: inactivation of the promoter selectivity factor SL1 by cdc2/cyclin B-mediated phosphorylation. *Embo J* **17**, 7373-81.
- Helin, K. 1998. Regulation of cell proliferation by the E2F transcription factors. *Curr Opin Genet Dev* **8**, 28-35.

- Hershko, A., Ganoth, D., Sudakin, V., Dahan, A., Cohen, L. H., Luca, F. C., Ruderman, J. V., and Eytan, E. 1994. Components of a system that ligates cyclin to ubiquitin and their regulation by the protein kinase cdc2. *J Biol Chem* **269**, 4940-6.
- Hiebert, S. W., Blake, M., Azizkhan, J., and Nevins, J. R. 1991. Role of E2F transcription factor in E1A-mediated trans activation of cellular genes. *J Virol* **65**, 3547-52.
- Hiebert, S. W., Chellappan, S. P., Horowitz, J. M., and Nevins, J. R. 1992. The interaction of RB with E2F coincides with an inhibition of the transcriptional activity of E2F. *Genes Dev* **6**, 177-85.
- Hiebert, S. W., Lipp, M., and Nevins, J. R. 1989. E1A-dependent trans-activation of the human MYC promoter is mediated by the E2F factor. *Proc Natl Acad Sci U S A* **86**, 3594-8.
- Hoeijmakers, J. H., Egly, J. M., and Vermeulen, W. 1996. TFIIH: a key component in multiple DNA transactions. *Curr Opin Genet Dev* **6**, 26-33.
- Hofmann, F., and Livingston, D. M. 1996. Differential effects of cdk2 and cdk3 on the control of pRb and E2F function during G1 exit. *Genes Dev* **10**, 851-61.
- Holloway, S. L., Glotzer, M., King, R. W., and Murray, A. W. 1993. Anaphase is initiated by proteolysis rather than by the inactivation of maturation-promoting factor. *Cell* **73**, 1393-402.
- Holstege, F. C., van der Vliet, P. C., and Timmers, H. T. 1996. Opening of an RNA polymerase II promoter occurs in two distinct steps and requires the basal transcription factors IIE and IIH. *Embo J* **15**, 1666-77.
- Hsiao, K. M., McMahon, S. L., and Farnham, P. J. 1994. Multiple DNA elements are required for the growth regulation of the mouse E2F1 promoter. *Genes Dev* **8**, 1526-37.
- Huang, J., and Raff, J. W. 1999. The disappearance of cyclin B at the end of mitosis is regulated spatially in Drosophila cells. *Embo J* **18**, 2184-95.
- Imahori, K., and Uchida, T. 1997. Physiology and pathology of tau protein kinases in relation to Alzheimer's disease. *J Biochem (Tokyo)* **121**, 179-88.
- Inamoto, S., Segil, N., Pan, Z. Q., Kimura, M., and Roeder, R. G. 1997. The cyclin-dependent kinase-activating kinase (CAK) assembly factor, MAT1, targets and enhances

CAK activity on the POU domains of octamer transcription factors. *J Biol Chem* **272**, 29852-8.

- Iyer, N., Reagan, M. S., Wu, K. J., Canagarajah, B., and Friedberg, E. C. 1996. Interactions involving the human RNA polymerase II transcription/nucleotide excision repair complex TFIIH, the nucleotide excision repair protein XPG, and Cockayne syndrome group B (CSB) protein. *Biochemistry* **35**, 2157-67.
- Jacobs, H. W., Knoblich, J. A., and Lehner, C. F. 1998. Drosophila Cyclin B3 is required for female fertility and is dispensable for mitosis like Cyclin B. *Genes Dev* **12**, 3741-51.
- Jeffrey, P. D., Russo, A. A., Polyak, K., Gibbs, E., Hurwitz, J., Massague, J., and Pavletich, N. P. 1995. Mechanism of CDK activation revealed by the structure of a cyclinA-CDK2 complex [see comments]. *Nature* **376**, 313-20.
- Jin, P., Hardy, S., and Morgan, D. O. 1998. Nuclear localization of cyclin B1 controls mitotic entry after DNA damage. *J Cell Biol* **141**, 875-85.
- Johnson, D. G., Ohtani, K., and Nevins, J. R. 1994. Autoregulatory control of E2F1 expression in response to positive and negative regulators of cell cycle progression. *Genes Dev* **8**, 1514-25.
- Johnson, D. G., Schwarz, J. K., Cress, W. D., and Nevins, J. R. 1993. Expression of transcription factor E2F1 induces quiescent cells to enter S phase. *Nature* **365**, 349-52.
- Jones, C. J., and Wood, R. D. 1993. Preferential binding of the xeroderma pigmentosum group A complementing protein to damaged DNA. *Biochemistry* **32**, 12096-104.
- Jordan, P., Cunha, C., and Carmo-Fonseca, M. 1997. The cdk7-cyclin H-MAT1 complex associated with TFIIH is localized in coiled bodies. *Mol Biol Cell* **8**, 1207-17.
- Kamb, A., Shattuck-Eidens, D., Eeles, R., Liu, Q., Gruis, N. A., Ding, W., Hussey, C., Tran, T., Miki, Y., Weaver-Feldhaus, J., and et al. 1994. Analysis of the p16 gene (CDKN2) as a candidate for the chromosome 9p melanoma susceptibility locus. *Nat Genet* **8**, 23-6.
- Kaspar, M., Dienemann, A., Schulze, C., and Sprenger, F. 2001. Mitotic degradation of cyclin A is mediated by multiple and novel destruction signals. *Curr Biol* **11**, 685-90.
- Kato, J. Y., and Sherr, C. J. 1993. Inhibition of granulocyte differentiation by G1 cyclins D2 and D3 but not D1. *Proc Natl Acad Sci U S A* **90**, 11513-7.

- Keeney, S., Chang, G. J., and Linn, S. 1993. Characterization of a human DNA damage binding protein implicated in xeroderma pigmentosum E. *J Biol Chem* **268**, 21293-300.
- Keriel, A., Stary, A., Sarasin, A., Rochette-Egly, C., and Egly, J. M. 2002. XPD mutations prevent TFIIH-dependent transactivation by nuclear receptors and phosphorylation of RARalpha. *Cell* **109**, 125-35.
- King, R. W., Glotzer, M., and Kirschner, M. W. 1996. Mutagenic analysis of the destruction signal of mitotic cyclins and structural characterization of ubiquitinated intermediates. *Mol Biol Cell* **7**, 1343-57.
- King, R. W., Jackson, P. K., and Kirschner, M. W. 1994. Mitosis in transition. *Cell* **79**, 563-71.
- King, R. W., Peters, J. M., Tugendreich, S., Rolfe, M., Hieter, P., and Kirschner, M. W. 1995. A 20S complex containing CDC27 and CDC16 catalyzes the mitosis-specific conjugation of ubiquitin to cyclin B. *Cell* **81**, 279-88.
- Kitagawa, M., Higashi, H., Suzuki-Takahashi, I., Segawa, K., Hanks, S. K., Taya, Y., Nishimura, S., and Okuyama, A. 1995. Phosphorylation of E2F-1 by cyclin A-cdk2. *Oncogene* **10**, 229-36.
- Kitamura, K., Maekawa, H., and Shimoda, C. 1998. Fission yeast Ste9, a homolog of Hct1/Cdh1 and Fizzy-related, is a novel negative regulator of cell cycle progression during G1-phase. *Mol Biol Cell* **9**, 1065-80.
- Knippschild, U., Milne, D., Campbell, L., and Meek, D. 1996. p53 N-terminus-targeted protein kinase activity is stimulated in response to wild type p53 and DNA damage. *Oncogene* **13**, 1387-93.
- Knoblich, J. A., and Lehner, C. F. 1993. Synergistic action of Drosophila cyclins A and B during the G2-M transition. *Embo J* **12**, 65-74.
- Knoblich, J. A., Sauer, K., Jones, L., Richardson, H., Saint, R., and Lehner, C. F. 1994. Cyclin E controls S phase progression and its down-regulation during Drosophila embryogenesis is required for the arrest of cell proliferation. *Cell* **77**, 107-20.
- Ko, L. J., Shieh, S. Y., Chen, X., Jayaraman, L., Tamai, K., Taya, Y., Prives, C., and Pan, Z. Q. 1997. p53 is phosphorylated by CDK7-cyclin H in a p36MAT1-dependent manner. *Mol Cell Biol* **17**, 7220-9.

- Kobayashi, H., Stewart, E., Poon, R., Adamczewski, J. P., Gannon, J., and Hunt, T. 1992. Identification of the domains in cyclin A required for binding to, and activation of, p34cdc2 and p32cdk2 protein kinase subunits. *Mol Biol Cell* **3**, 1279-94.
- Kobayashi, S., Ishiguro, K., Omori, A., Takamatsu, M., Arioka, M., Imahori, K., and Uchida, T. 1993. A cdc2-related kinase PSSALRE/cdk5 is homologous with the 30 kDa subunit of tau protein kinase II, a proline-directed protein kinase associated with microtubule. *FEBS Lett* **335**, 171-5.
- Kobor, M. S., Simon, L. D., Omichinski, J., Zhong, G., Archambault, J., and Greenblatt, J. 2000. A motif shared by TFIIF and TFIIB mediates their interaction with the RNA polymerase II carboxy-terminal domain phosphatase Fcp1p in Saccharomyces cerevisiae. *Mol Cell Biol* **20**, 7438-49.
- Koh, J., Enders, G. H., Dynlacht, B. D., and Harlow, E. 1995. Tumour-derived p16 alleles encoding proteins defective in cell-cycle inhibition. *Nature* **375**, 506-10.
- Kominami, K., Seth-Smith, H., and Toda, T. 1998. Apc10 and Ste9/Srw1, two regulators of the APC-cyclosome, as well as the CDK inhibitor Rum1 are required for G1 cell-cycle arrest in fission yeast. *Embo J* **17**, 5388-99.
- Kraemer, K. H., DiGiovanna, J. J., Moshell, A. N., Tarone, R. E., and Peck, G. L. 1988. Prevention of skin cancer in xeroderma pigmentosum with the use of oral isotretinoin. *N Engl J Med* **318**, 1633-7.
- Krek, W., Ewen, M. E., Shirodkar, S., Arany, Z., Kaelin, W. G., Jr., and Livingston, D. M. 1994. Negative regulation of the growth-promoting transcription factor E2F-1 by a stably bound cyclin A-dependent protein kinase. *Cell* **78**, 161-72.
- Labbe, J. C., Martinez, A. M., Fesquet, D., Capony, J. P., Darbon, J. M., Derancourt, J., Devault, A., Morin, N., Cavadore, J. C., and Doree, M. 1994. p40MO15 associates with a p36 subunit and requires both nuclear translocation and Thr176 phosphorylation to generate cdk-activating kinase activity in Xenopus oocytes. *Embo J* **13**, 5155-64.
- Lam, E. W., and Watson, R. J. 1993. An E2F-binding site mediates cell-cycle regulated repression of mouse B- myb transcription. *Embo J* **12**, 2705-13.
- Larochelle, S., Chen, J., Knights, R., Pandur, J., Morcillo, P., Erdjument-Bromage, H., Tempst, P., Suter, B., and Fisher, R. P. 2001. T-loop phosphorylation stabilizes the

CDK7-cyclin H-MAT1 complex in vivo and regulates its CTD kinase activity. *Embo J* **20**, 3749-59.

- Larochelle, S., Pandur, J., Fisher, R. P., Salz, H. K., and Suter, B. 1998. Cdk7 is essential for mitosis and for in vivo Cdk-activating kinase activity. *Genes Dev* **12**, 370-81.
- Laybourn, P. J., and Dahmus, M. E. 1989. Transcription-dependent structural changes in the C-terminal domain of mammalian RNA polymerase subunit IIa/o. *J Biol Chem* **264**, 6693-8.
- Laybourn, P. J., and Dahmus, M. E. 1990. Phosphorylation of RNA polymerase IIA occurs subsequent to interaction with the promoter and before the initiation of transcription. *J Biol Chem* **265**, 13165-73.
- Leclerc, V., Raisin, S., and Leopold, P. 2000. Dominant-negative mutants reveal a role for the Cdk7 kinase at the mid- blastula transition in Drosophila embryos. *Embo J* **19**, 1567-75.
- Lehmann, A. R. 2001. The xeroderma pigmentosum group D (XPD) gene: one gene, two functions, three diseases. *Genes Dev* **15**, 15-23.
- Lehner, C. F., and O'Farrell, P. H. 1990. The roles of Drosophila cyclins A and B in mitotic control. *Cell* **61**, 535-47.
- Leost, M., Schultz, C., Link, A., Wu, Y. Z., Biernat, J., Mandelkow, E. M., Bibb, J. A., Snyder, G. L., Greengard, P., Zaharevitz, D. W., *et al.* 2000. Paullones are potent inhibitors of glycogen synthase kinase-3beta and cyclin-dependent kinase 5/p25. *Eur J Biochem* **267**, 5983-94.
- Leresche, A., Wolf, V. J., and Gottesfeld, J. M. 1996. Repression of RNA polymerase II and III transcription during M phase of the cell cycle. *Exp Cell Res* **229**, 282-8.
- Li, X., and Nicklas, R. B. 1995. Mitotic forces control a cell-cycle checkpoint. *Nature* **373**, 630-2.
- Li, Y., Gorbea, C., Mahaffey, D., Rechsteiner, M., and Benezra, R. 1997. MAD2 associates with the cyclosome/anaphase-promoting complex and inhibits its activity. *Proc Natl Acad Sci U S A* **94**, 12431-6.
- Lindquist, S. 1980. Varying patterns of protein synthesis in Drosophila during heat shock: implications for regulation. *Dev Biol* **77**, 463-79.

- Liu, J., Akoulitchev, S., Weber, A., Ge, H., Chuikov, S., Libutti, D., Wang, X. W., Conaway, J. W., Harris, C. C., Conaway, R. C., *et al.* 2001. Defective interplay of activators and repressors with TFIH in xeroderma pigmentosum. *Cell* **104**, 353-63.
- Liu, J., Hebert, M. D., Ye, Y., Templeton, D. J., Kung, H., and Matera, A. G. 2000. Cell cycle-dependent localization of the CDK2-cyclin E complex in Cajal (coiled) bodies. *J Cell Sci* **113**, 1543-52.
- Lohrum, M., and Scheidtmann, K. H. 1996. Differential effects of phosphorylation of rat p53 on transactivation of promoters derived from different p53 responsive genes. *Oncogene* **13**, 2527-39.
- Long, J. J., Leresche, A., Kriwacki, R. W., and Gottesfeld, J. M. 1998. Repression of TFIIH transcriptional activity and TFIIH-associated cdk7 kinase activity at mitosis. *Mol Cell Biol* **18**, 1467-76.
- Lopez-Girona, A., Furnari, B., Mondesert, O., and Russell, P. 1999. Nuclear localization of Cdc25 is regulated by DNA damage and a 14-3-3 protein. *Nature* **397**, 172-5.
- Lorca, T., Castro, A., Martinez, A. M., Vigneron, S., Morin, N., Sigrist, S., Lehner, C., Doree, M., and Labbe, J. C. 1998. Fizzy is required for activation of the APC/cyclosome in Xenopus egg extracts. *Embo J* **17**, 3565-75.
- Lu, H., Fisher, R. P., Bailey, P., and Levine, A. J. 1997. The CDK7-cycH-p36 complex of transcription factor IIH phosphorylates p53, enhancing its sequence-specific DNA binding activity in vitro. *Mol Cell Biol* **17**, 5923-34.
- Luca, F. C., and Ruderman, J. V. 1989. Control of programmed cyclin destruction in a cell-free system. *J Cell Biol* **109**, 1895-909.
- Luca, F. C., Shibuya, E. K., Dohrmann, C. E., and Ruderman, J. V. 1991. Both cyclin A delta 60 and B delta 97 are stable and arrest cells in M- phase, but only cyclin B delta 97 turns on cyclin destruction. *Embo J* **10**, 4311-20.
- Lukas, J., Parry, D., Aagaard, L., Mann, D. J., Bartkova, J., Strauss, M., Peters, G., and Bartek, J. 1995. Retinoblastoma-protein-dependent cell-cycle inhibition by the tumour suppressor p16. *Nature* **375**, 503-6.
- Lundberg, A. S., and Weinberg, R. A. 1998. Functional inactivation of the retinoblastoma protein requires sequential modification by at least two distinct cyclin-cdk complexes. *Mol Cell Biol* **18**, 753-61.

- Luo, R. X., Postigo, A. A., and Dean, D. C. 1998. Rb interacts with histone deacetylase to repress transcription. *Cell* **92**, 463-73.
- Magnaghi-Jaulin, L., Groisman, R., Naguibneva, I., Robin, P., Lorain, S., Le Villain, J. P., Troalen, F., Trouche, D., and Harel-Bellan, A. 1998. Retinoblastoma protein represses transcription by recruiting a histone deacetylase. *Nature* **391**, 601-5.
- Mahaffey, D., Yoo, Y., and Rechsteiner, M. 1993. Ubiquitin metabolism in cycling Xenopus egg extracts. *J Biol Chem* **268**, 21205-11.
- Majello, B., and Napolitano, G. 2001. Control of RNA polymerase II activity by dedicated CTD kinases and phosphatases. *Front Biosci* **6**, D1358-68.
- Makela, T. P., Parvin, J. D., Kim, J., Huber, L. J., Sharp, P. A., and Weinberg, R. A. 1995. A kinase-deficient transcription factor TFIIH is functional in basal and activated transcription. *Proc Natl Acad Sci U S A* **92**, 5174-8.
- Makela, T. P., Tassan, J. P., Nigg, E. A., Frutiger, S., Hughes, G. J., and Weinberg, R. A. 1994. A cyclin associated with the CDK-activating kinase MO15. *Nature* **371**, 254-7.
- Maldonado-Codina, G., and Glover, D. M. 1992. Cyclins A and B associate with chromatin and the polar regions of spindles, respectively, and do not undergo complete degradation at anaphase in syncytial Drosophila embryos. *J Cell Biol* **116**, 967-76.
- Maldonado-Codina, G., Llamazares, S., and Glover, D. M. 1993. Heat shock results in cell cycle delay and synchronisation of mitotic domains in cellularised Drosophila melanogaster embryos. *J Cell Sci* **105**, 711-20.
- Mandelkow, E., and Mandelkow, E. 1998. Tau in Alzheimer's disease. *Trends in Cell Biology* **8**, 425-7.
- Martin, K., Trouche, D., Hagemeier, C., Sorensen, T. S., La Thangue, N. B., and Kouzarides, T. 1995. Stimulation of E2F1/DP1 transcriptional activity by MDM2 oncoprotein. *Nature* **375**, 691-4.
- Matsumoto, T. 1997. A fission yeast homolog of CDC20/p55CDC/Fizzy is required for recovery from DNA damage and genetically interacts with p34cdc2. *Mol Cell Biol* **17**, 742-50.
- Matsuoka, M., Kato, J. Y., Fisher, R. P., Morgan, D. O., and Sherr, C. J. 1994. Activation of cyclin-dependent kinase 4 (cdk4) by mouse MO15-associated kinase. *Mol Cell Biol* **14**, 7265-75.

- McShea, A., Harris, P. L., Webster, K. R., Wahl, A. F., and Smith, M. A. 1997. Abnormal expression of the cell cycle regulators P16 and CDK4 in Alzheimer's disease. *Am J Pathol* **150**, 1933-9.
- Meek, D. W. 1994. Post-translational modification of p53. *Semin Cancer Biol* **5**, 203-10.
- Meyerson, M., Enders, G. H., Wu, C. L., Su, L. K., Gorka, C., Nelson, C., Harlow, E., and Tsai, L. H. 1992. A family of human cdc2-related protein kinases. *Embo J* **11**, 2909-17.
- Minshull, J., Golsteyn, R., Hill, C. S., and Hunt, T. 1990. The A- and B-type cyclin associated cdc2 kinases in Xenopus turn on and off at different times in the cell cycle. *Embo J* **9**, 2865-75.
- Mittnacht, S. 1998. Control of pRB phosphorylation. *Curr Opin Genet Dev* **8**, 21-7.
- Monzon, J., Liu, L., Brill, H., Goldstein, A. M., Tucker, M. A., From, L., McLaughlin, J., Hogg, D., and Lassam, N. J. 1998. CDKN2A mutations in multiple primary melanomas. *N Engl J Med* **338**, 879-87.
- Morgan, D. O. 1995. Principles of CDK regulation. *Nature* **374**, 131-4.
- Morgan, D. O. 1997. Cyclin-dependent kinases: engines, clocks, and microprocessors. *Annu Rev Cell Dev Biol* **13**, 261-91.
- Morgan, D. O. 1999. Regulation of the APC and the exit from mitosis. *Nat Cell Biol* **1**, E47-53.
- Morgan, G. T., Doyle, O., Murphy, C., and Gall, J. G. 2000. RNA polymerase II in Cajal bodies of amphibian oocytes. *J Struct Biol* **129**, 258-68.
- Mounkes, L. C., Jones, R. S., Liang, B. C., Gelbart, W., and Fuller, M. T. 1992. A Drosophila model for xeroderma pigmentosum and Cockayne's syndrome: haywire encodes the fly homolog of ERCC3, a human excision repair gene. *Cell* **71**, 925-37.
- Mu, D., and Sancar, A. 1997. Model for XPC-independent transcription-coupled repair of pyrimidine dimers in humans. *J Biol Chem* **272**, 7570-3.
- Mudryj, M., Hiebert, S. W., and Nevins, J. R. 1990. A role for the adenovirus inducible E2F transcription factor in a proliferation dependent signal transduction pathway. *Embo J* **9**, 2179-84.
- Murray, A. W., and Kirschner, M. W. 1989. Cyclin synthesis drives the early embryonic cell cycle. *Nature* **339**, 275-80.

- Nagy, Z., Esiri, M. M., and Smith, A. D. 1997. Expression of cell division markers in the hippocampus in Alzheimer's disease and other neurodegenerative conditions. *Acta Neuropathol (Berl)* **93**, 294-300.
- Nigg, E. A. 1995. Cyclin-dependent protein kinases: key regulators of the eukaryotic cell cycle. *Bioessays* **17**, 471-80.
- Nigg, E. A. 1996. Cyclin-dependent kinase 7: at the cross-roads of transcription, DNA repair and cell cycle control? *Curr Opin Cell Biol* **8**, 312-7.
- Nigg, E. A. 2001. Mitotic kinases as regulators of cell division and its checkpoints. *Nat Rev Mol Cell Biol* **2**, 21-32.
- Nishiwaki, E., Turner, S. L., Harju, S., Miyazaki, S., Kashiwagi, M., Koh, J., and Serizawa, H. 2000. Regulation of CDK7-carboxyl-terminal domain kinase activity by the tumor suppressor p16(INK4A) contributes to cell cycle regulation. *Mol Cell Biol* **20**, 7726-34.
- Nobori, T., Miura, K., Wu, D. J., Lois, A., Takabayashi, K., and Carson, D. A. 1994. Deletions of the cyclin-dependent kinase-4 inhibitor gene in multiple human cancers. *Nature* **368**, 753-6.
- O'Brien, T., Hardin, S., Greenleaf, A., and Lis, J. T. 1994. Phosphorylation of RNA polymerase II C-terminal domain and transcriptional elongation. *Nature* **370**, 75-7.
- O'Connor, D. J., Lam, E. W., Griffin, S., Zhong, S., Leighton, L. C., Burbidge, S. A., and Lu, X. 1995. Physical and functional interactions between p53 and cell cycle co-operating transcription factors, E2F1 and DP1. *Embo J* **14**, 6184-92.
- O'Donovan, A., Davies, A. A., Moggs, J. G., West, S. C., and Wood, R. D. 1994. XPG endonuclease makes the 3' incision in human DNA nucleotide excision repair. *Nature* **371**, 432-5.
- Ohtani, K., DeGregori, J., and Nevins, J. R. 1995. Regulation of the cyclin E gene by transcription factor E2F1. *Proc Natl Acad Sci U S A* **92**, 12146-50.
- Parry, D. H., and O'Farrell, P. H. 2001. The schedule of destruction of three mitotic cyclins can dictate the timing of events during exit from mitosis. *Curr Biol* **11**, 671-83.
- Paudel, H. K., Lew, J., Ali, Z., and Wang, J. H. 1993. Brain proline-directed protein kinase phosphorylates tau on sites that are abnormally phosphorylated in tau associated with Alzheimer's paired helical filaments. *J Biol Chem* **268**, 23512-8.

- Pearson, A., and Greenblatt, J. 1997. Modular organization of the E2F1 activation domain and its interaction with general transcription factors TBP and TFIIH. *Oncogene* **15**, 2643-58.
- Peng, C. Y., Graves, P. R., Thoma, R. S., Wu, Z., Shaw, A. S., and Piwnica-Worms, H. 1997. Mitotic and G2 checkpoint control: regulation of 14-3-3 protein binding by phosphorylation of Cdc25C on serine-216. *Science* **277**, 1501-5.
- Petersen, R., and Lindquist, S. 1988. The Drosophila hsp70 message is rapidly degraded at normal temperatures and stabilized by heat shock. *Gene* **72**, 161-8.
- Pfleger, C. M., and Kirschner, M. W. 2000. The KEN box: an APC recognition signal distinct from the D box targeted by Cdh1. *Genes Dev* **14**, 655-65.
- Pfleger, C. M., Lee, E., and Kirschner, M. W. 2001a. Substrate recognition by the Cdc20 and Cdh1 components of the anaphase- promoting complex. *Genes Dev* **15**, 2396-407.
- Pfleger, C. M., Salic, A., Lee, E., and Kirschner, M. W. 2001b. Inhibition of Cdh1-APC by the MAD2-related protein MAD2L2: a novel mechanism for regulating Cdh1. *Genes Dev* **15**, 1759-64.
- Pietenpol, J. A., Tokino, T., Thiagalingam, S., el-Deiry, W. S., Kinzler, K. W., and Vogelstein, B. 1994. Sequence-specific transcriptional activation is essential for growth suppression by p53. *Proc Natl Acad Sci U S A* **91**, 1998-2002.
- Pines, J., and Hunter, T. 1990. Human cyclin A is adenovirus E1A-associated protein p60 and behaves differently from cyclin B. *Nature* **346**, 760-3.
- Poon, R. Y., Yamashita, K., Adamczewski, J. P., Hunt, T., and Shuttleworth, J. 1993. The cdc2-related protein p40MO15 is the catalytic subunit of a protein kinase that can activate p33cdk2 and p34cdc2. *Embo J* **12**, 3123-32.
- Poon, R. Y., Yamashita, K., Howell, M., Ershler, M. A., Belyavsky, A., and Hunt, T. 1994. Cell cycle regulation of the p34cdc2/p33cdk2-activating kinase p40MO15. *J Cell Sci* **107**, 2789-99.
- Reardon, J. T., Ge, H., Gibbs, E., Sancar, A., Hurwitz, J., and Pan, Z. Q. 1996. Isolation and characterization of two human transcription factor IIH (TFIIH)-related complexes: ERCC2/CAK and TFIIH [published erratum appears in Proc Natl Acad Sci U S A 1996 Sep 17;93(19):10538]. *Proc Natl Acad Sci U S A* **93**, 6482-7.

- Reynaud, E., Lomeli, H., Vazquez, M., and Zurita, M. 1999. The Drosophila melanogaster homologue of the Xeroderma pigmentosum D gene product is located in euchromatic regions and has a dynamic response to UV light-induced lesions in polytene chromosomes. *Mol Biol Cell* **10**, 1191-203.
- Richardson, H. E., O'Keefe, L. V., Reed, S. I., and Saint, R. 1993. A Drosophila G1-specific cyclin E homolog exhibits different modes of expression during embryogenesis. *Development* **119**, 673-90.
- Rieder, C. L., Cole, R. W., Khodjakov, A., and Sluder, G. 1995. The checkpoint delaying anaphase in response to chromosome monoorientation is mediated by an inhibitory signal produced by unattached kinetochores. *J Cell Biol* **130**, 941-8.
- Rieder, C. L., Schultz, A., Cole, R., and Sluder, G. 1994. Anaphase onset in vertebrate somatic cells is controlled by a checkpoint that monitors sister kinetochore attachment to the spindle. *J Cell Biol* **127**, 1301-10.
- Rochette-Egly, C., Adam, S., Rossignol, M., Egly, J. M., and Chambon, P. 1997. Stimulation of RAR alpha activation function AF-1 through binding to the general transcription factor TFIIH and phosphorylation by CDK7. *Cell* **90**, 97-107.
- Rochette-Egly, C., Oulad-Abdelghani, M., Staub, A., Pfister, V., Scheuer, I., Chambon, P., and Gaub, M. P. 1995. Phosphorylation of the retinoic acid receptor-alpha by protein kinase A. *Mol Endocrinol* **9**, 860-71.
- Roeder, R. G. 1996. The role of general initiation factors in transcription by RNA polymerase II. *Trends Biochem Sci* **21**, 327-35.
- Rossignol, M., Kolb-Cheynel, I., and Egly, J. M. 1997. Substrate specificity of the cdk-activating kinase (CAK) is altered upon association with TFIIH. *Embo J* **16**, 1628-37.
- Roy, R., Adamczewski, J. P., Seroz, T., Vermeulen, W., Tassan, J. P., Schaeffer, L., Nigg, E. A., Hoeijmakers, J. H., and Egly, J. M. 1994. The MO15 cell cycle kinase is associated with the TFIIH transcription- DNA repair factor. *Cell* **79**, 1093-101.
- Russo, A. A., Jeffrey, P. D., and Pavletich, N. P. 1996. Structural basis of cyclin-dependent kinase activation by phosphorylation. *Nat Struct Biol* **3**, 696-700.
- Sanchez, Y., Wong, C., Thoma, R. S., Richman, R., Wu, Z., Piwnica-Worms, H., and Elledge, S. J. 1997. Conservation of the Chk1 checkpoint pathway in mammals: linkage of DNA damage to Cdk regulation through Cdc25. *Science* **277**, 1497-501.

- Sandrock, B., and Egly, J. M. 2001. A yeast four-hybrid system identifies Cdk-activating kinase as a regulator of the XPD helicase, a subunit of transcription factor IIH. *J Biol Chem* **276**, 35328-33.
- Sauer, K., and Lehner, C. F. 1995. The role of cyclin E in the regulation of entry into S phase. *Prog Cell Cycle Res* **1**, 125-39.
- Sauer, K., Weigmann, K., Sigrist, S., and Lehner, C. F. 1996. Novel members of the cdc2-related kinase family in Drosophila: cdk4/6, cdk5, PFTAIRE, and PITSLRE kinase. *Mol Biol Cell* **7**, 1759-69.
- Schaeffer, L., Moncollin, V., Roy, R., Staub, A., Mezzina, M., Sarasin, A., Weeda, G., Hoeijmakers, J. H., and Egly, J. M. 1994. The ERCC2/DNA repair protein is associated with the class II BTF2/TFIIH transcription factor. *Embo J* **13**, 2388-92.
- Schaeffer, L., Roy, R., Humbert, S., Moncollin, V., Vermeulen, W., Hoeijmakers, J. H., Chambon, P., and Egly, J. M. 1993. DNA repair helicase: a component of BTF2 (TFIIH) basic transcription factor. *Science* **260**, 58-63.
- Schneider, E., Montenarh, M., and Wagner, P. 1998. Regulation of CAK kinase activity by p53. *Oncogene* **17**, 2733-41.
- Schroeder, S. C., Schwer, B., Shuman, S., and Bentley, D. 2000. Dynamic association of capping enzymes with transcribing RNA polymerase II. *Genes Dev* **14**, 2435-40.
- Schul, W., van Driel, R., and de Jong, L. 1998. Coiled bodies and U2 snRNA genes adjacent to coiled bodies are enriched in factors required for snRNA transcription. *Mol Biol Cell* **9**, 1025-36.
- Schultz, P., Fribourg, S., Poterszman, A., Mallouh, V., Moras, D., and Egly, J. M. 2000. Molecular structure of human TFIIH. *Cell* **102**, 599-607.
- Schulze, A., Zerfass, K., Spitkovsky, D., Middendorp, S., Berges, J., Helin, K., Jansen-Durr, P., and Henglein, B. 1995. Cell cycle regulation of the cyclin A gene promoter is mediated by a variant E2F site. *Proc Natl Acad Sci U S A* **92**, 11264-8.
- Schwab, M., Lutum, A. S., and Seufert, W. 1997. Yeast Hct1 is a regulator of Clb2 cyclin proteolysis. *Cell* **90**, 683-93.
- Segil, N., Guermah, M., Hoffmann, A., Roeder, R. G., and Heintz, N. 1996. Mitotic regulation of TFIID: inhibition of activator-dependent transcription and changes in subcellular localization. *Genes Dev* **10**, 2389-400.

- Serizawa, H. 1998. Cyclin-dependent kinase inhibitor p16INK4A inhibits phosphorylation of RNA polymerase II by general transcription factor TFIIH. *J Biol Chem* **273**, 5427-30.
- Serizawa, H., Makela, T. P., Conaway, J. W., Conaway, R. C., Weinberg, R. A., and Young, R. A. 1995. Association of Cdk-activating kinase subunits with transcription factor TFIIH. *Nature* **374**, 280-2.
- Serrano, M., Hannon, G. J., and Beach, D. 1993. A new regulatory motif in cell-cycle control causing specific inhibition of cyclin D/CDK4. *Nature* **366**, 704-7.
- Shiekhattar, R., Mermelstein, F., Fisher, R. P., Drapkin, R., Dynlacht, B., Wessling, H. C., Morgan, D. O., and Reinberg, D. 1995. Cdk-activating kinase complex is a component of human transcription factor TFIIH. *Nature* **374**, 283-7.
- Shirayama, M., Zachariae, W., Ciosk, R., and Nasmyth, K. 1998. The Polo-like kinase Cdc5p and the WD-repeat protein Cdc20p/fizzy are regulators and substrates of the anaphase promoting complex in Saccharomyces cerevisiae. *Embo J* **17**, 1336-49.
- Shivji, M. K., Podust, V. N., Hubscher, U., and Wood, R. D. 1995. Nucleotide excision repair DNA synthesis by DNA polymerase epsilon in the presence of PCNA, RFC, and RPA. *Biochemistry* **34**, 5011-7.
- Sigrist, S., Jacobs, H., Stratmann, R., and Lehner, C. F. 1995a. Exit from mitosis is regulated by Drosophila fizzy and the sequential destruction of cyclins A, B and B3. *Embo J* **14**, 4827-38.
- Sigrist, S., Ried, G., and Lehner, C. F. 1995b. Dmcdc2 kinase is required for both meiotic divisions during Drosophila spermatogenesis and is activated by the Twine/cdc25 phosphatase. *Mech Dev* **53**, 247-60.
- Sigrist, S. J., and Lehner, C. F. 1997. Drosophila fizzy-related down-regulates mitotic cyclins and is required for cell proliferation arrest and entry into endocycles. *Cell* **90**, 671-81.
- Sijbers, A. M., de Laat, W. L., Ariza, R. R., Biggerstaff, M., Wei, Y. F., Moggs, J. G., Carter, K. C., Shell, B. K., Evans, E., de Jong, M. C., *et al.* 1996. Xeroderma pigmentosum group F caused by a defect in a structure- specific DNA repair endonuclease. *Cell* **86**, 811-22.

- Slansky, J. E., Li, Y., Kaelin, W. G., and Farnham, P. J. 1993. A protein synthesis-dependent increase in E2F1 mRNA correlates with growth regulation of the dihydrofolate reductase promoter. *Mol Cell Biol* **13**, 1610-8.
- Smith, M. A. 1998. Alzheimer disease. *Int Rev Neurobiol* **42**, 1-54.
- Smith, T. W., and Lippa, C. F. 1995. Ki-67 immunoreactivity in Alzheimer's disease and other neurodegenerative disorders. *J Neuropathol Exp Neurol* **54**, 297-303.
- Smits, V. A., Klompmaker, R., Vallenius, T., Rijksen, G., Makela, T. P., and Medema, R. H. 2000. p21 inhibits thr161 phosphorylation of cdc2 to enforce the G2 DNA damage checkpoint. *J Biol Chem* **275**, 30638-43.
- Solomon, M. J., Harper, J. W., and Shuttleworth, J. 1993. CAK, the p34cdc2 activating kinase, contains a protein identical or closely related to p40MO15. *Embo J* **12**, 3133-42.
- Solomon, M. J., Lee, T., and Kirschner, M. W. 1992. Role of phosphorylation in p34cdc2 activation: identification of an activating kinase. *Molecular Biology of the Cell* **3**, 13-17.
- Spangler, L., Wang, X., Conaway, J. W., Conaway, R. C., and Dvir, A. 2001. TFIIH action in transcription initiation and promoter escape requires distinct regions of downstream promoter DNA. *Proc Natl Acad Sci U S A* **98**, 5544-9.
- Spradling, A. C. 1993. Developmental genetics of oogenesis. In *The development of Drosophila melanogaster* 1-70.
- Stefanini, M., Vermeulen, W., Weeda, G., Giliani, S., Nardo, T., Mezzina, M., Sarasin, A., Harper, J. I., Arlett, C. F., Hoeijmakers, J. H., and et al. 1993. A new nucleotide-excision-repair gene associated with the disorder trichothiodystrophy. *Am J Hum Genet* **53**, 817-21.
- Stern, B., Ried, G., Clegg, N. J., Grigliatti, T. A., and Lehner, C. F. 1993. Genetic analysis of the Drosophila cdc2 homolog. *Development* **117**, 219-32.
- Sudakin, V., Ganoth, D., Dahan, A., Heller, H., Hershko, J., Luca, F. C., Ruderman, J. V., and Hershko, A. 1995. The cyclosome, a large complex containing cyclin-selective ubiquitin ligase activity, targets cyclins for destruction at the end of mitosis. *Mol Biol Cell* **6**, 185-97.
- Sugasawa, K., Ng, J. M., Masutani, C., Iwai, S., van der Spek, P. J., Eker, A. P., Hanaoka, F., Bootsma, D., and Hoeijmakers, J. H. 1998. Xeroderma pigmentosum group

C protein complex is the initiator of global genome nucleotide excision repair. *Mol Cell* **2**, 223-32.

- Sung, P., Higgins, D., Prakash, L., and Prakash, S. 1988. Mutation of lysine-48 to arginine in the yeast RAD3 protein abolishes its ATPase and DNA helicase activities but not the ability to bind ATP. *Embo J* **7**, 3263-9.
- Surana, U., Amon, A., Dowzer, C., McGrew, J., Byers, B., and Nasmyth, K. 1993. Destruction of the CDC28/CLB mitotic kinase is not required for the metaphase to anaphase transition in budding yeast. *Embo J* **12**, 1969-78.
- Suter, B., and Steward, R. 1991. Requirement for phosphorylation and localization of the Bicaudal-D protein in Drosophila oocyte differentiation. *Cell* **67**, 917-26.
- Svejstrup, J. Q., Vichi, P., and Egly, J. M. 1996. The multiple roles of transcription/repair factor TFIIH. *Trends Biochem Sci* **21**, 346-50.
- Takayama, K., Salazar, E. P., Broughton, B. C., Lehmann, A. R., Sarasin, A., Thompson, L. H., and Weber, C. A. 1996. Defects in the DNA repair and transcription gene ERCC2(XPD) in trichothiodystrophy. *Am J Hum Genet* **58**, 263-70.
- Takayama, K., Salazar, E. P., Lehmann, A., Stefanini, M., Thompson, L. H., and Weber, C. A. 1995. Defects in the DNA repair and transcription gene ERCC2 in the cancer-prone disorder xeroderma pigmentosum group D. *Cancer Res* **55**, 5656-63.
- Tang, D., and Wang, J. H. 1996. Cyclin-dependent kinase 5 (Cdk5) and neuron-specific Cdk5 activators. *Prog Cell Cycle Res* **2**, 205-16.
- Tassan, J. P., Jaquenoud, M., Fry, A. M., Frutiger, S., Hughes, G. J., and Nigg, E. A. 1995. In vitro assembly of a functional human CDK7-cyclin H complex requires MAT1, a novel 36 kDa RING finger protein. *Embo J* **14**, 5608-17.
- Tassan, J. P., Schultz, S. J., Bartek, J., and Nigg, E. A. 1994. Cell cycle analysis of the activity, subcellular localization, and subunit composition of human CAK (CDK-activating kinase). *J Cell Biol* **127**, 467-78.
- Taylor, E. M., Broughton, B. C., Botta, E., Stefanini, M., Sarasin, A., Jaspers, N. G., Fawcett, H., Harcourt, S. A., Arlett, C. F., and Lehmann, A. R. 1997. Xeroderma pigmentosum and trichothiodystrophy are associated with different mutations in the XPD (ERCC2) repair/transcription gene. *Proc Natl Acad Sci U S A* **94**, 8658-63.

- Taylor, S. S. 1999. Chromosome segregation: dual control ensures fidelity. *Curr Biol* **9**, R562-4.
- Tirode, F., Busso, D., Coin, F., and Egly, J. M. 1999. Reconstitution of the transcription factor TFIIH: assignment of functions for the three enzymatic subunits, XPB, XPD, and cdk7. *Mol Cell* **3**, 87-95.
- Toyoshima, F., Moriguchi, T., Wada, A., Fukuda, M., and Nishida, E. 1998. Nuclear export of cyclin B1 and its possible role in the DNA damage- induced G2 checkpoint. *Embo J* **17**, 2728-35.
- Valay, J. G., Simon, M., Dubois, M. F., Bensaude, O., Facca, C., and Faye, G. 1995. The KIN28 gene is required both for RNA polymerase II mediated transcription and phosphorylation of the Rpb1p CTD. *J Mol Biol* **249**, 535-44.
- Vandel, L., and Kouzarides, T. 1999. Residues phosphorylated by TFIIH are required for E2F-1 degradation during S-phase. *Embo J* **18**, 4280-91.
- Venema, J., van Hoffen, A., Karcagi, V., Natarajan, A. T., van Zeeland, A. A., and Mullenders, L. H. 1991. Xeroderma pigmentosum complementation group C cells remove pyrimidine dimers selectively from the transcribed strand of active genes. *Mol Cell Biol* **11**, 4128-34.
- Vermeulen, W., Bergmann, E., Auriol, J., Rademakers, S., Frit, P., Appeldoorn, E., Hoeijmakers, J. H., and Egly, J. M. 2000. Sublimiting concentration of TFIIH transcription/DNA repair factor causes TTD-A trichothiodystrophy disorder. *Nat Genet* **26**, 307-13.
- Vincent, I., Jicha, G., Rosado, M., and Dickson, D. W. 1997. Aberrant expression of mitotic cdc2/cyclin B1 kinase in degenerating neurons of Alzheimer's disease brain. *J Neurosci* **17**, 3588-98.
- Visintin, R., Prinz, S., and Amon, A. 1997a. CDC20 and CDH1: a family of substrate-specific activators of APC- dependent proteolysis. *Science* **278**, 460-3.
- Visintin, R., Prinz, S., and Amon, A. 1997b. CDC20 and CDH1: a family of substrate-specific activators of APC- dependent proteolysis. *Science* **278**, 460-3.
- Wallenfang, M. R., and Seydoux, G. 2002. cdk-7 Is required for mRNA transcription and cell cycle progression in Caenorhabditis elegans embryos. *Proc Natl Acad Sci U S A* **99**, 5527-32.

- Walworth, N., Davey, S., and Beach, D. 1993. Fission yeast chk1 protein kinase links the rad checkpoint pathway to cdc2. *Nature* **363**, 368-71.
- Walworth, N. C., and Bernards, R. 1996. rad-dependent response of the chk1-encoded protein kinase at the DNA damage checkpoint. *Science* **271**, 353-6.
- Wang, X. W., Vermeulen, W., Coursen, J. D., Gibson, M., Lupold, S. E., Forrester, K., Xu, G., Elmore, L., Yeh, H., Hoeijmakers, J. H., and Harris, C. C. 1996. The XPB and XPD DNA helicases are components of the p53-mediated apoptosis pathway. *Genes Dev* **10**, 1219-32.
- West, M. L., and Corden, J. L. 1995. Construction and analysis of yeast RNA polymerase II CTD deletion and substitution mutations. *Genetics* **140**, 1223-33.
- White, R. J., Gottlieb, T. M., Downes, C. S., and Jackson, S. P. 1995. Mitotic regulation of a TATA-binding-protein-containing complex. *Mol Cell Biol* **15**, 1983-92.
- Whitfield, W. G., Gonzalez, C., Maldonado-Codina, G., and Glover, D. M. 1990. The A- and B-type cyclins of Drosophila are accumulated and destroyed in temporally distinct events that define separable phases of the G2-M transition. *Embo J* **9**, 2563-72.
- Winkler, G. S., Araujo, S. J., Fiedler, U., Vermeulen, W., Coin, F., Egly, J. M., Hoeijmakers, J. H., Wood, R. D., Timmers, H. T., and Weeda, G. 2000. TFIIH with inactive XPD helicase functions in transcription initiation but is defective in DNA repair. *J Biol Chem* **275**, 4258-66.
- Wood, R. D. 1997. Nucleotide excision repair in mammalian cells. *J Biol Chem* **272**, 23465-8.
- Wood, R. D., Mitchell, M., Sgouros, J., and Lindahl, T. 2001. Human DNA repair genes. *Science* **291**, 1284-9.
- Xiong, Y., Hannon, G. J., Zhang, H., Casso, D., Kobayashi, R., and Beach, D. 1993. p21 is a universal inhibitor of cyclin kinases. *Nature* **366**, 701-4.
- Xu, M., Sheppard, K. A., Peng, C. Y., Yee, A. S., and Piwnica-Worms, H. 1994. Cyclin A/CDK2 binds directly to E2F-1 and inhibits the DNA-binding activity of E2F-1/DP-1 by phosphorylation. *Mol Cell Biol* **14**, 8420-31.
- Yamaguchi, H., Ishiguro, K., Uchida, T., Takashima, A., Lemere, C. A., and Imahori, K. 1996. Preferential labeling of Alzheimer neurofibrillary tangles with antisera for tau

protein kinase (TPK) I/glycogen synthase kinase-3 beta and cyclin-dependent kinase 5, a component of TPK II. *Acta Neuropathol (Berl)* **92**, 232-41.

- Yamaguchi, S., Murakami, H., and Okayama, H. 1997. A WD repeat protein controls the cell cycle and differentiation by negatively regulating Cdc2/B-type cyclin complexes. *Mol Biol Cell* **8**, 2475-86.
- Yamamoto, A., Guacci, V., and Koshland, D. 1996. Pds1p is required for faithful execution of anaphase in the yeast, Saccharomyces cerevisiae. *J Cell Biol* **133**, 85-97.
- Yamano, H., Tsurumi, C., Gannon, J., and Hunt, T. 1998. The role of the destruction box and its neighbouring lysine residues in cyclin B for anaphase ubiquitin-dependent proteolysis in fission yeast: defining the D-box receptor. *Embo J* **17**, 5670-8.
- Yang, J., Bardes, E. S., Moore, J. D., Brennan, J., Powers, M. A., and Kornbluth, S. 1998. Control of cyclin B1 localization through regulated binding of the nuclear export factor CRM1. *Genes Dev* **12**, 2131-43.
- Yankulov, K. Y., and Bentley, D. L. 1997. Regulation of CDK7 substrate specificity by MAT1 and TFIIH. *Embo J* **16**, 1638-46.
- Yarbrough, W. G., Buckmire, R. A., Bessho, M., and Liu, E. T. 1999. Biologic and biochemical analyses of p16(INK4a) mutations from primary tumors. *J Natl Cancer Inst* **91**, 1569-74.
- Yost, H. J., and Lindquist, S. 1986. RNA splicing is interrupted by heat shock and is rescued by heat shock protein synthesis. *Cell* **45**, 185-93.
- Zachariae, W., and Nasmyth, K. 1999. Whose end is destruction: cell division and the anaphase-promoting complex. *Genes Dev* **13**, 2039-58.
- Zachariae, W., Schwab, M., Nasmyth, K., and Seufert, W. 1998. Control of cyclin ubiquitination by CDK-regulated binding of Hct1 to the anaphase promoting complex. *Science* **282**, 1721-4.
- Zhou, M., Halanski, M. A., Radonovich, M. F., Kashanchi, F., Peng, J., Price, D. H., and Brady, J. N. 2000. Tat modifies the activity of CDK9 to phosphorylate serine 5 of the RNA polymerase II carboxyl-terminal domain during human immunodeficiency virus type 1 transcription. *Mol Cell Biol* **20**, 5077-86.

- Zhu, X., Rottkamp, C. A., Raina, A. K., Brewer, G. J., Ghanbari, H. A., Boux, H., and Smith, M. A. 2000. Neuronal CDK7 in hippocampus is related to aging and Alzheimer disease. *Neurobiol Aging* **21**, 807-13.

Appendix I

Deficiency kit (1998 Version)

from Bloomington stock center

Bloomington Deficiency Kit stocks listed by deficiency breakpoints

Please do not specify individual stock numbers when ordering deficiency kits. Request kits as DK1 (the X chromosome), DK2 (chromosome 2), DK3 (chromosome 3), or DK4 (chromosome 4). You may order the kit for an autosomal arm by requesting DK2L, DK2R, etc.

Stock #	Genotype	Breakpoints
1329	Df(1)BA1, w[*]/FM7; Dp(1;2)E1, y[+]/+	001A01;002A
1546	T(1;3)sc[J4], sc[J4]/C(1)DX, y[1] f[1]; Dp(1;f)z9 ! = Df(1)sc-J4	001B;003A03 (Df in T(1;3))
936	Df(1)64c18, g[1] sd[1]/Dp(1;2;Y)w[+]/C(1)DX, y[1] w[1] f[1]	002E01-02;003C02
935	Df(1)JC19/FM7c	002F06;003C05
729	Df(1)N-8/FM7c	003C02-03;003E03-04
939	In(1)dm75e19/FM7c ! = Df(1)dm75e19	003C11-003E04
940	Df(1)A113/C(1)DX, y[1] w[1] f[1]; Dp(1;2)w[+]64b/+	003D06-E01;004F05
944	Df(1)JC70/FM7c, sn[+]	004C15-16;005A01-02
945	Df(1)C149/FM6	005A08-09;005C05-06
946	Df(1)N73/FM6	005C02;005D05-06
1665	Df(1)5D, y[1]/FM6 ! ry[506] floating	005D01-02;005E
579	Df(1)JF5, f[1] car[1]/FM7	005E03-05;005E08
1925	In(1)G4e[L]H24i[R], f[1]/FM7 ! = Df(1)G4e[L]H24i[R]	005E03-08;006B
3196	Df(1)Sxl-bt, y[1]/Binsinscy	006E02;007A06
948	Df(1)ct-J4, In(1)dl-49, f[1]/C(1)DX, y[1] w[1] f[1]; Dp(1;3)sn[13a1]/Ki[1]	007A02-03;007C01
3221	Df(1)ct4b1, y[1]/Binsn	007B02-04;007C03-04
949	Df(1)C128/FM6	007D01;007D05-06
950	Df(1)RA2/FM7c	007D10;008A04-05
951	Df(1)KA14/FM7c	007F01-02;008C06
3651	Df(1)lz-90b24, y[2] w[a]/FM7c	008B05-06;008D08-09 or 008D01-02;008E01-02
952	Df(1)C52/FM6	008E;009C-D
954	Df(1)v-L15/FM6	009B01-02;010A01-02
3560	Df(1)v-N48, f[*]/Dp(1;Y)B[S-]v[+]y[+]/C(1)DX, y[1] f[1]	009F;010C03-05
957	Df(1)KA7/C(1)DX, y[1] w[1] f[1]; Dp(1;2)v[+]65b/+	010A09;010F06-07
959	Df(1)HA85/FM7c	010C01-02;011A01-02
962	Df(1)N105/FM6	010F07;011D01
964	Df(1)JA26/FM7c	011A01;011D-E
967	Df(1)C246/FM6	011D-E;012A01-02
966	Df(1)N12, ras[1] v[1]/FM6	011D01-02;011F01-02
727	Df(1)g, f[1] B[1]/In(1)AM	012A;012E or 011F10;012F01
998	Df(1)RK2/FM7a	012D02-E01;013A02-05
1039	Df(1)RK4/FM7c/Dp(1;Y)y[+], y[1]	012F05-06;013A09-B01
3347	Df(1)sd72b/FM7	013F01;014B01
125	Df(1)4b18, y[1] cv[1] v[1] f[1] car[1]/In(1)sc[S1L]sc[8R], In(1)S, w[a] B[1]	014B08;014C01
993	Df(1)r-D1, v[1] f[1]/C(1)DX, y[1] w[1] f[1]; Dp(1;4)r[+]*/+	014C02-04;015B02-C01
723	Df(1)B/In(1)sc[7], In(1)AM, ptg[4] car[1]	016A02;016A06
970	Df(1)N19/FM6	017A01;018A02
971	Df(1)JA27/FM7c	018A05;018D
972	Df(1)HF396/FM7c	018E01-02;020
977	Df(1)DCB1-35b/FM6/Dp(1;Y)y[+]mal[106]	019F01-02;020E-F
3714	Df(1)A209/FM7a	020A;020F
3520	w[a] N[fa-g]; Df(2R)Jp8, w[+]/CyO	052F05-09;052F10-53A01
3638	Df(2L)net-PMF/SM6a	021A01;021B07-08
3548	Df(2L)al/In(2L)Cy, Cy[1]	021B08-C01;021C08-D01
3084	Df(2L)ast2/SM1	021D01-02;022B02-03
3133	Df(2L)dp-79b, dp[DA] cn[1]/In(2LR)bw[V1], b[1] ! does not delete dp	022A02-03;022D05-E01
90	Df(2L)C144, dpp[d-ho] ed[1]/In(2LR)Gla, Gla[1] Bc[1] Egfr[E1]	023A01-02;023C03-05
97	Df(2L)JS32, dpp[d-ho]/SM6a	023C03-05;023D01-02
3573	In(2LR)DTD16[L]DTD42[R], bw[1] sp[1]/CyO ! = Df(2L)23C;23E3-6	023C;023E03-06
712	Df(2L)ed1, al[1] b[1]/SM5	024A03-04;024D03-04
593	Df(2L)sc19-8/SM6b?, Cy[1] Roi[1]; Dp(2;1)B19, y[1], ed[1] dp[o2] cl[1]	024C02-08;025C08-09
627	Df(2L)sc19-5/SM6b?, Cy[1] Roi[1]; Dp(2;1)B19, Df(1)y-ac, sc[1] pn[1]: ed[1] dp[o2] cl[1]	025A04-05;025D05-07
781	Df(2L)cl-h3/SM6b?, Cy[1] Roi[1]	025D02-04;026B02-05
490	In(1)w[m4]; Df(2L)E110/CyO	025F03-026A01;026D03-11
3571	Df(2L)Dwee-delta5/Dp(?;2)bw[D], S[1] wg[Sp-1] Ms(2)M[1] bw[D]/CyO	027A;028A
1357	Df(2L)J-H/SM5 ! = Df(2L)J136-H52	027C02-09;028B03-04
3077	Df(2L)spd, al[1] dp[ov1]/CyO	027D-E;028C

1541	y[1] w[1] N[spl-1]; Df(3L)66C-G28/TM3	066B08-09;066C09-10
3024	Df(3L)h-i22, Ki[1] roe[1] p[p]/TM3	066D10-11;066E01-02
1688	Df(3L)Rdl-2/TM3, Sb[1]	066F05;066F05
2479	Df(3L)29A6, ri[1] p[p]/TM3	066F05;067B01
997	Df(3L)AC1, roe[1] p[p]/TM3, Sb[1]	067A02;067D07-13 or 067A05;067D09-13
89	y[1?]; Df(3L)lxd6/TM3, Sb[1] Ser[1]	067E01-02;068C01-02
2547	Df(3L)vin2, ru[1] h[1] gl[2] e[4] ca[1]/TM3	067F02-03;068D06
2611	Df(3L)vin5, ru[1] h[1] gl[2] e[4] ca[1]/TM3, Sb[1] Ser[1]	068A02-03;069A01-03
2612	Df(3L)vin7, ru[1] h[1] gl[2] e[4] ca[1]/TM3, Sb[1] Ser[1]	068C08-11;069B04-05
4366	In(3LR)C190[L]Ubx[42TR], Ubx[-]/sti[1]	069F03-04;070C03-04 + 089;089 (small df somewhere in 89)
3124	Df(3L)fz-GF3b, P{w[+tAR] ry[+t7.2AR]=wA[R]}66E/TM6B	070C01-02;070D04-05
3126	Df(3L)fz-M21, th[1] st[1]/TM6	070D02-03;071E04-05
2992	Df(3L)BK10, ru[1] Ly[1] red[1] cv-c[1] Sb[sbd-1] sr[1] e[1]/TM3	071C;071F
3640	Df(3L)brm11/TM6C, cu[1] Sb[1] ca[1]	071F01-04;072D01-10
2993	Df(3L)st-f13, Ki[1] roe[1] p[p]/TM6B	072C01-D01;073A03-04
2998	Df(3L)81k19/TM6B	073A03;074F
2608	Df(3L)W10, ru[1] h[1] Sb[sbd-2]/TM6B ! = see comment	075A06-07;075C01-02
2990	Df(3L)Cat, ri[1] Sb[sbd-1] e[*]/TM3, Ser[1]	075B08;075F01
3000	Df(3L)VW3/TM3	076A03;076B02
3617	Df(3L)kto2/TM6B	076B01-02;076D05
2052	Df(3L)rdgC-co2, th[1] st[1] in[1] ri[1] p[p]/TM6C, Sb[1] cu[1] Tb[1]	077A01;077D01
3127	Df(3L)ri-79c/TM3	077B-C;077F-78A
4429	Df(3L)ME107, mwh[1] red[1] e[1]/TM1, red[*]	077F03;078C08-09
3627	Df(3L)31A/Dp(3;3)C126, st[1] cp[1] in[1] ri[1] p[p]	078A;078E, 078D;079B
4430	Df(3L)Pc-2q, ry[506]/TM2	078C05-06;078E03-079A01
4370	Df(3L)Delta1AK, ru[1] h[1] ry[506] sr[1] e[s] ca[1]/TM3, ry[RK] Sb[1] Ser[1]	079F;080A
3688	Dp(3;1)2-2, w[1118]/?; Df(3R)2-2/TM3	081F;082F10-11
339	w[1118]; Df(3R)6-7/TM3, Sb[1]	082D03-08;082F
1990	Df(3R)Tpl10, Tp(3;3)Dfd[rv1], ri[1] p[p]/TM3	083C01-02;084B01-02
1884	Df(3R)Scr, p[p] e[s]/TM3	084A01-02;084B01-02
1842	Df(3R)Antp17/TM3 ! = Antp[N+R17]	084B01-02;084D11-12 or A06,D14
1968	Df(3R)p712, red[1] e[1]/TM3	084D04-06;085B06
1962	Df(3R)p-XT103, ru[1] st[1] e[1] ca[1]/TM3	085A02;085C01-02
1931	Df(3R)by10, red[1] e[1]/TM3	085D08-12;085E07-F01
1893	Df(3R)by62, red[1] e[1]/TM1	085D11-14;085F16
3128	Df(3R)M-Kx1/TM3, Sb[1] Ser[1]	086C01;087B01-05
3003	Df(3R)T-32, (ri[1]) cu[1] sr[1] e[s]/MRS ! = TE32	086E02-04;087C06-07
3007	Df(3R)ry615/TM3, Sb[1] Ser[1]	087B11-13;087E08-11
1534	Tp(3;Y)ry506-85C/MKRS	087D01-02;088E05-06;Y
383	Df(3R)ea, ri[1] p[p]/TM3, Ser[1]	088E07-13;089A01
1467	Df(3R)P115, e[11]/TM1; Dp(3;1)P115/+ ! = Tp(3;1)P115	089B07-08;089E07-08;020
4431	Df(3R)DG2/TM2	089E01-089F04;091B01-B02
3071	Df(3R)C4, p[*]/Dp(3;3)P5, Sb[1]	089E;090A
3011	Df(3R)Cha7/TM6B?	090F01-F04;091F05
3012	Df(3R)Dl-BX12, ss[1] e[4] ro[1]/TM6B	091F01-02;092D03-06
3340	Df(3R)e-R1, Ki[1]/TM3	093B03-05;093D02-04
2425	Df(3R)e-N19/TM2	093B;094
2586	Df(3R)23D1, ry[506]/TM3, Sb[1]/mus309[Horka] e[1]	093F;094F
2585	cn[1]; Df(3R)mbc-R1, ry[506]/TM3, Sb[1] ry[*]	095A05-07;095D06-11
4432	Df(3R)crb-F89-4, st[1] e[1]/TM3, Ser[1]	095D07-D11;095F15
2363	Df(3R)crb87-5, st[1] e[1]/TM3, Ser[1]	095F07;096A17-18
1972	In(3R)Ubx[7LL]ats[R], asp[1] ats[1] p[p]/TM6B; y[1]/Dp(1;Y)y[+] ! = Df(3R)XS, Dp(3R)XS	096A01-07;096A21-25
2366	Df(3R)XTA1, th[1] st[1] ri[1] roe[1] p[1]/Dp(3;3)M95A[+]13, st[1] e[1]	096B;096D
1910	Df(3R)Tl-P, e[1] ca[1]/TM3, Ser[1]	097A;098A01-02
823	Df(3R)D605/TM3, Sb[1] Ser[1]	097E03;098A05
430	w[1118]; Df(3R)3450/TM6B	098E03;099A06-08
669	w[*]; Df(3R)Dr-rv1, ry[506]/TM3, ry[RK] Sb[1] Ser[1]	099A01-02;099B06-11
3547	Df(3R)L127/TM6; Dp(3;1)B152	099B05-06;099E04-F01
3546	Ts(YLt;3Lt)B81, P{ry[+t7.2]=RP49}F2-80A e[1]/TM3; Dp(3;1)67A ! = Df(3R)B81	099C08;100F05
3369	Df(3R)awd-KRB, ca[1]/TM3, y[+] Sb[1] e[1] Ser[1]	100C;100D
1011	Df(3R)faf-BP/TM6B	100D;100F05
1785	C(4)RM, ci[1] ey[R]	
758	Df(4)M101-62f/Dp(2;4)ey[D], Alp[eyD]: ey[D]	101E;102B10-17
759	Df(4)G/ci[D] ! see comment	102E02;102E10

140	Df(1)w67c23, y[1]; Df(2L)Trf-C6R31/CyO	028DE (within)
179	In(1)w[m4h], y[1]; Df(2L)TE29Aa-11/CyO	028E04-07;029B02-C01
2892	Df(2L)N22-14/In(2LR)O, Cy[1] dp[lvl] pr[1] cn[2]	029C01-02;030C08-09
556	Df(2L)s1402/CyO	030C01-02;030F
1045	Df(2L)Mdh, cn[1]/Dp(2;2)Mdh3, cn[1] ! see comment	030D-30F;031F
1469	Df(2L)J39/In(2L)Cy; Dp(2;Y)cb50, Dp(1;Y)B[S]Yy[+]/C(1)RM ! = J-der-39	031C-D;032D-E
3079	Df(2L)Prl/CyO	032F01-03;033F01-02
3344	Df(2L)prd1.7, b[1] Adh[n2] pr[1] cn[1] sca[1]/CyO-vKa, P{ry[+t*]=elav-lacZ.H}YH2	033B02-03;034A01-02
3138	Df(2L)b87e25/In(2L)NS	034B12-C01;035B10-C01
3588	Df(2L)TE35BC-24, b[1] pr[1] pk[1] cn[1] sp[1]/CyO	035B04-06;035F01-07
1491	Df(2L)r10, cn[1]/CyO ! ry floating?	035E01-02;036A06-07
2583	Df(2L)cact-255rv64/CyO; ry[506]	035F-036A;036D
3180	Df(2L)H20, b[1] pr[1] cn[1] sca[1]/CyO ! = Df(2L)dl2034	036A08-09;036E01-02
420	Df(2L)TW137, cn[1] bw[1]/CyO, Dp(2;2)M(2)m[+]	036C02-04;037B09-C01
3189	Df(2L)TW50, cn[1]/CyO, Dp(2;2)M(2)m[+]	036E04-F01;038A06-07
3346	Df(2L)TW84/CyO	037F05-38A01;039D03-E01
167	Df(2L)TW161, cn[1] bw[1]/CyO ! weak stock	038A06-B01;040A04-B01
739	Df(2R)M41A4/SM1	041A
749	In(2R)bw[VDe2L]Cy[R]/In(2LR)Gla, Gla[1]	041A-B;042A02-03
1006	Df(2R)nap1/In(2LR)Gla, Dp(2;2)BG, Gla[1]	041D02-E01;042B01-03
1007	Df(2R)nap9/In(2LR)Gla, Dp(2;2)BG, Gla[1]	042A01-02;042E06-F01
1888	Df(2R)ST1, Adh[n5] pr[1] cn[*]/CyO	042B03-05;043E15-18
1930	In(2R)pk78s/CyO? ! = Df(2R)pk78s	042C01-07;043F05-08 or 042B;042C max or 42F;43F+
3368	Df(2R)cn9/SM6b?, Cy[1] Roi[1] <P> ! poor or no Cy expression	042E;044C
198	w[118]; Df(2R)H3C1/CyO	043F;044D
201	w[118]; Df(2R)H3E1/CyO	044D;044F12
3591	w[1]; Df(2R)Np5, In(2LR)w45-32n, cn[1]/CyO	044F10;045D09-E01
1743	w[1118]; Df(2R)B5, px[1] sp[1]/CyO, Adh[nB]	046A;046C
1702	Df(2R)X1/CyO, Adh[nB]	046C;047A01
596	Df(2R)Stan2, b[1] pr[1] P{ry[+t7.2]=neoFRT}42D/CyO	046F01-02;047D01-02
520	Df(2R)E3363/CyO-CR2, P{ry[+t7.2]=sevRas1.V12}*	047A;047F
190	Df(2R)en-A/CyO	047D03;048B02-05
1145	Df(2R)en30/SM5; Dp(1;Ybb[-])B[S]	048A03-04;048C06-08
1642	Df(2R)vg135/CyO, S[*] bw[1]	049A-B;049D-E
754	Df(2R)vg-C/SM5	049A04-13;049E07-F01
442	Df(2R)CX1, b[1] pr[1]/SM1	049C01-04;050C23-D02
1896	Df(2R)trix/CyO?	051A01-02;051B06
1150	w[1]/Dp(1;Y)y[+]; Df(2R)knSA3, Tp(1;2)TE21F22A/CyO	051B05-11;051F05-13
3518	w[a] N[fa-g]; Df(2R)Jp1/CyO	051C03;052F05-09
3064	Df(2R)Pcl7B/CyO	054E08-F01;055B09-C01
3120	Df(2R)Pcl11B, al[1] dp[ov1] b[1] pr[1]/CyO-vKa, P{ry[+t*]=elav-lacZ.H}YH2	054F06-55A01;055C01-03
1547	Df(2R)PC4/CyO	055A;055F
757	y[*] w[*]/Dp(1;Y)y[+]; Df(2R)P34/CyO	055E02-04;056C01-11
543	Df(2R)017/SM1	056F05;056F015
3467	Df(2R)AA21, c[1] px[1] sp[1]/SM1	056F09-17;057D11-12
2606	Df(2R)Pu-D17, cn[1] bw[1] sp[1]/SM1	057B04;058B
283	Dp(1;Y)y[+]/y[1]; Df(2R)X58-7, pr[1] cn[1]/CyO, bw[1]	058A01-02;058E04-10
282	Dp(1;Y)y[+]/y[1]; Df(2R)X58-12/SM5	058D01-02;059A
3909	w[*]; Df(2R)59AD/SM1	059A01-03;059D01-04
1682	Df(2R)or-BR6, cn[1] bw[1] sp[1]/In(2LR)lt[G16L]bw[V32gR]	059D05-10;060B03-08
2604	Df(2R)Px2/SM5	060C05-06;060D09-10
2471	Df(2R)M60E/In(2LR)bw[V32g]	060E02-03;060E11-12
3157	Df(2R)ES1, b[1] pr[1] cn[1] wx[wxt] Kr[If-1]/SM1	060E06-08;060F01-02
2577	Df(3L)emc-E12/TM6B	061A;061D03
439	Df(3L)Ar14-8, red[1]/TM2, emc[2] p[p] Ubx[130] e[s] ! = Df(3L)emc5	061C05-08;062A08
600	Df(3L)Aprt-1, ru[1] h[1]/TM3, Sb[1] Ser[1]	062A10-B01;062D02-05
2400	Df(3L)R-G7, ve[1]/TM6B, Tb[+]	062B08-09;062F02-05
3650	Df(3L)M21, ri[1] p[p]/In(3LR)T33[L]f19[R] ! see comment	062F;063D
3649	Df(3L)HR119/TM6B	063C06;063E
3687	w[1118]; Df(3L)GN50/TM8, l(3)DTS4[1] th[1] st[1] Sb[1] e[1]	063E01-02;064B17
3686	Df(3L)GN24/TM8, l(3)DTS4[1] th[1] st[1] Sb[1] e[1]	063F04-07;064C13-15
3096	Df(3L)ZN47, ry[506]/TM3	064C;065C
4393	Df(3L)XDI98/TM6B	065A02;065E01
1420	Df(3L)pbl-X1/TM6B ! w[*] floating	065F03;066B10

Appendix II

Primary genetic screen for dominant suppressors and enhancers of *cdk7*

Different colors indicate different temperatures under which the tests were carried out. Red and blue refer to 25 and 23 degree, respectively. Balancer and deficiency stand for the flies contain balancer chromosomes and deficiency chromosomes, respectively. Dp stands for the duplication. "*s*"refers to suppressor and "*e*" stands for enhancer. "?" indicates the uncertain interaction.

Primary Screen for dominant suppressors and enhancers of Cdk7

	female		male		
Stock No.	balancer	deficiency	balancer	deficiency	comments
343	50	42	4	10	*s*
	24	30	3	4	
1469	37	13	0	1	
490	31	23	0	0	
3781	19	16	1	0	
	2	3	0	0	
3079	39	35	1	0	
3571	26	23	0	0	
	3	3	0	0	
3084	25	9	1	1	
1357	23	53	1	1	
	20	12	0	3	*s*
3187	0	37	0	2	
167	54	52	11	6	*e*
	62	39	10	0	*e*
3346	45	54	17	49	*s*
	53	52	5	9	
3588	35	31	0	0	
	44	36	3	2	
1006	23	22	24	9	*Dp in balancer*
	13	7	7	0	*Dp in balancer*
420	46	55	4	4	
	6	20	0	1	
3189	29	49	6	14	
	12	20	0	2	
3180	52	34	21	5	*e*
	33	29	12	1	*e*
2583	46	47	3	0	*e*
	26	23	7	1	*e*
781	46	32	2	0	
	11	26	2	1	
2892	52	44	0	0	
	30	28	6	0	*e*
627	34	34	0	0	
693	53	62	0	0	
	24	24	18	5	*e?*
712	36	38	0	0	
	2	26	1	13	

Stock No.	female		male		comments
	balancer	deficiency	balancer	deficiency	
3573	29	30	0	0	
	28	33	18	2	*e*
97	21	14	0	0	
179	42	63	1	0	
	26	28	16	9	
140	40	39	0	0	
1491	55	40	1	0	
	21	28	3	5	
3077	40	43	0	0	
	15	8	7	9	
3076	15	42	6	7	
759	15	29	5	20	*s*
3157	62	50	9	19	*s*
4430	18	13	6	1	*e*
1547	22	19	0	0	
757	33	42	0	0	
	29	17	3	16	*s*
543	23	21	1	1	
1150	21	16	1	1	
	14	16	4	5	
442	39	23	0	0	
	18	20	11	4	*e*
283	25	26	4	3	
	19	12	0	2	*s*
2606	42	38	0	7	*s*
	18	28	3	16	*s*
3467	54	43	7	14	*s*
	15	32	0	7	*s*
754	25	21	0	0	
1642	43	35	0	0	
	9	11	5	0	*e*
3120	27	26	11	2	*e*
	21	10	3	1	
3520	20	118	7	3	
	13	7	6	1	*e*
1145	30	31	0	0	
	10	10	1	2	
190	33	47	0	0	
	20	13	15	6	*e*
3632	30	26	7	0	*e*

Stock No.	female		male		comments
	balancer	deficiency	balancer	deficiency	
3078	5	67	3	8	
3518	24	25	1	1	
	9	7	2	0	
3129	39	36	3	3	
1473	45	49	0	0	
	11	11	2	4	
1545	28	8	0	0	
520	16	22	2	0	*e*
	23	20	3	0	*e*
596	24	23	2	0	*e*
3591	12	3	0	0	
	28	22	12	4	*e*
201	29	25	0	0	
	42	49	11	5	*e*
198	39	31	0	0	
	9	14	10	13	
1930	9	8	0	0	
	16	22	6	1	*e*
1007	9	17	0	0	
	17	20	15	2	*Dp in balancer*
556	29	27	0	5	*S*
	32	30	0	1	
1420	12	51	1	0	
3340	28	29	9	13	
3687	15	48	0	3	
	4	10	0	0	
2363	5	11	5	3	
	57	62	6	7	
2362	10	8	0	0	
	24	29	12	2	*e*
3010	19	24	0	0	
	18	18	6	7	
1011	8	5	0	0	
2586	5	12	0	1	
1416	19	25	2	2	
2585	19	21	1	1	
	27	31	9	18	*s*
4432	5	6	0	0	
	7	18	2	0	
2426	6	34	0	0	
	14	25	2	5	

Stock No.	female		male		comments
	balancer	deficiency	balancer	deficiency	
3369	8	5	0	0	
	15	7	0	9	*s*
1972	8	8	0	0	
3079	4	6	0	0	
1990.1	21	12	0	0	
1990.2	46	61	0	2	*s*
	48	68	11	30	*s*
3343	37	28	0	0	
	21	19	15	3	*e*
1910	5	6	0	0	
	17	7	2	1	
430	1	3	0	0	
4366	18	1	0	1	
3683	20	0	0	1	
	30	2	3	12	
3343	34	38	0	0	
758	18	13	1	1	
	32	26	7	8	
383	27	229	0	0	
	1	5	0	0	
3071	27	33	0	0	
3007	13	19	0	0	
	15	12	0	1	
1541	41	43	0	0	
3128	46	41	0	0	
	23	28	3	0	*e*
1534	13	48	0	0	
	21	1	1	11	*s ?*
3640	26	16	0	0	
	7	6	0	0	
3024	32	39	0	0	
	25	6	3	3	
2993	14	24	0	0	
	2	3	2	5	
997	25	18	0	0	
1702	41	13	25	3	*e*
339	7	14	0	0	
	4	12	2	13	
3127	24	25	0	0	
	8	11	0	7	*s*
2547	4	20	0	0	

Stock No.	female		male		comments
	balancer	deficiency	balancer	deficiency	
3632	1	5	0	0	
3686	26	8	0	0	
147	10	6	2	5	*s*
1968	14	9	0	0	
	14	6	0	0	
2052	33	46	0	0	
2612	16	20	0	0	
3071	4	3	0	0	
600	8	29	0	0	
3000	25	21	0	1	*s*
	5	1	0	5	*s*
4393	10	12	1	0	

Appendix III

Candidate *suppressors* and *enhancers* of *cdk7* from the primary screen

Candidate *suppressors* and *enhancers* of *cdk7*from the primary screen are listed with their detailed genotypes and breakpoints. Notice that the two candidate suppressors 2606 and 3467 have overlapping deletion (indicated by *). Among candidate *enhancers*, four pairs of *enhancers* have overlapping deletions (indicated by *), they are 2583 and 3180, 3632 and 167, 3591 and 201, 596 and 520. **Please note that fly database is being updated, the breakpoints of the deficiencies can be mapped more precisely from the recent data.**

candidate *enhancers*

Stock No.	genotype	chromosome	breakpoints/Insertion
3573	In(2LR)DTD16LDTD42R, bw1 sp1/CyO	2	023C;023E03-06
693	Df(2L)sc19-8/SM6b; Dp(2;1)B19, y1, ed1 dpo2 cl1	2	024C02-08;025C08-09, 024D04;025F02;009B-C
2892	Df(2L)N22-14/CyO	2	029C01-02;030C08-09
*2583	Df(2L)cact-255rv64, cactchif64/CyO; ry506	2;3	035F-036A;036D
*3180	Df(2L)H20, b1 pr1 cn1 sca1/CyO	2	036A08-09;036E01-02
*3632	Df(2L)pr-A14, cn1 bw1/SM5	2	037D02-07;039A04-07
*167	Df(2L)TW161, cn1 bw1/CyO	2	038A06-B01;040A04-B01
1930	Df(2R)pk78s/CyO	2	042F;043F08; 059F05-08 (Df + In)
*3591	w1; Df(2R)Np5, In(2LR)w45-32n, cn1/CyO	1;2	044F10;045D09-E01, 031B;045D09-E01
*201	w118; Df(2R)H3E1/CyO	1;2	044D01-04;044F12
1702	Df(2R)X1, Mef2X1/CyO, AdhnB	2	046C;047A01
*596	Df(2R)stan2,b1 pr1 P{ry+t7.2=neoFRT}42D/ CyO	2	046F01-02;047D01-02
*520	Df(2R)E3363/CyO, P{ry+t7.2=sevRas1.V12}FK1	2	047A;047F
190	Df(2R)en-A/CyO	2	047D03;048B02
1642	Df(2R)vg135, nompAvg135/CyO, S bw1	2	049A-B;049D-E, 047F04-048A;049A-B
442	Df(2R)CX1, b1 pr1/SM1	2	049C01-04;050C23-D02
3520	wa Nfa-g; Df(2R)Jp8, w+/CyO	1;2	052F05-09;052F10-53A01
3120	Df(2R)Pcl11B, al1 dpov1 b1 pr1/ CyO, P{ry+t=elav-lacZ.H}YH2	2	054F06-55A01;055C01-03
3343	Df(3L)Ly, mwh1 Ly1/TM1, jv	3	070A02-03;070A05-06
4430	Df(3L)Pc-2q, ry506/TM2	3	078C05-06;078E03-079A01
3128	Df(3R)M-Kx1/TM3, Sb1 Ser1	3	086C01;087B01-05
2362	Df(3R)crb87-4, st1 e1/TM3, Ser1	3	095E08-F01;095F15

candidate *suppressors*

Stock No.	genotype	chromosome	breakpoints/Insertion
1357	Df(2L)J-H/SM5	2	027C02-09;028B03-04
556	w; Df(2L)s1402, P{w+mC=lacW}s1402/CyO	1;2	030C01-02;030F, 030B09-10
343	Df(2L)M36F-S6/SM5	2	036E06-F01; 036F07-09
3346	Df(2L)TW84, l(2)74i1, Tft1 LarTW84/CyO	2	038A01;039D03-E01
757	y1 w/Dp(1;Y)y+; Df(2R)P34/CyO	1;Y;2	055E02-04; 056C01-11
*3467	Df(2R)AA21, c1 px1 sp1/SM1	2	056F09-17;057D11-12
*2606	Df(2R)Pu-D17, cn1 bw1 sp1/SM1	2	057B04;058B
283	Dp(1;Y)y+/y1; Df(2R)X58-7, pr1 cn1/CyO, bw1	1;Y;2	058B01-02;058E01-04
3157	Df(2R)ES1, b1 pr1 cn1 wxwxt/SM1	2	060E06-08;060F01-02
3000	Df(3L)VW3/TM3	3	076A03;076B02
3127	Df(3L)ri-79c/TM3, Sb1	3	077B-C;077F-78A
1990	Df(3R)Tpl10, Dp(3;3)Dfdrv1, kniri-1 Dfdrv1 pp Doa10/TM3, Sb1	3	083C01-02;084B01-02, 083D04-05;084A04-05;098F01-02
1534	Tp(3;Y)ry506-85C/MKRS	3	087D01-02;088E05-06;Y
2585	cn1; Df(3R)mbc-R1, ry506/TM3, ry Sb1 Ser1	2;3	095A05-07;095D06-11
3369	y; Df(3R)awd-KRB, e ca1/TM3, Sb1 Ser1	3	100C;100D
759	Df(4)G/In(4)ciD, ciD panciD	4	102E02;102E10

Appendix IV

T-loop phosphorylation stabilizes the CDK7-cyclin H-MAT1 complex in vivo and regulates its CTD kinase activity. ***Embo J*** **20, 3749-59.**

The EMBO Journal Vol. 20 No. 14 pp. 3749–3759, 2001

T-loop phosphorylation stabilizes the CDK7–cyclin H–MAT1 complex *in vivo* and regulates its CTD kinase activity

Stéphane Larochelle[1,2], Jian Chen[2], Ronald Knights[1], Judit Pandur[2], Patrick Morcillo[1], Hediye Erdjument-Bromage[3], Paul Tempst[3], Beat Suter[2] and Robert P.Fisher[1,4]

[1]Cell Biology Program and [3]Molecular Biology Program, Memorial Sloan-Kettering Cancer Center, 1275 York Avenue, New York, NY 10021, USA and [2]Department of Biology, McGill University, 1205 Dr Penfield Avenue, Montreal, PQ, Canada H3A 1B1

[4]Corresponding author
e-mail: r-fisher@ski.mskcc.org

Cyclin-dependent kinase (CDK)7–cyclin H, the CDK-activating kinase (CAK) and TFIIH-associated kinase in metazoans can be activated *in vitro* through T-loop phosphorylation or binding to the RING finger protein MAT1. Although the two mechanisms can operate independently, we show that in a physiological setting, MAT1 binding and T-loop phosphorylation cooperate to stabilize the CAK complex of *Drosophila*. CDK7 forms a stable complex with cyclin H and MAT1 *in vivo* only when phosphorylated on either one of two residues (Ser164 or Thr170) in its T-loop. Mutation of both phosphorylation sites causes temperature-dependent dissociation of CDK7 complexes and lethality. Furthermore, phosphorylation of Thr170 greatly stimulates the activity of the CDK7–cyclin H–MAT1 complex towards the C-terminal domain of RNA polymerase II without significantly affecting activity towards CDK2. Remarkably, the substrate-specific increase in activity caused by T-loop phosphorylation is due entirely to accelerated enzyme turnover. Thus phosphorylation on Thr170 could provide a mechanism to augment CTD phosphorylation by TFIIH-associated CDK7, and thereby regulate transcription.

Keywords: CAK/cell cycle/cyclin-dependent kinase/*Drosophila*/TFIIH

Introduction

Two events are essential for the activation of the cyclin-dependent kinases (CDKs) that drive the cell cycle: binding to a cyclin partner and phosphorylation on the activation segment or T-loop by a CDK-activating kinase (CAK) (Morgan, 1995). CDK7 was first purified from metazoan sources as the catalytic subunit of CAK, and later shown to be required for CAK activity *in vivo* in *Drosophila melanogaster* (Harper and Elledge, 1998; Larochelle *et al.*, 1998). CDK7 also plays a central role in the regulation of transcription as the kinase subunit of the general transcription factor IIH (TFIIH). In that context, CDK7 phosphorylates the C-terminal domain (CTD) of RNA polymerase II (RNA pol II) to facilitate promoter clearance (Dahmus, 1996). The dual role of CDK7 has not been universally conserved, however, because the budding yeast *Saccharomyces cerevisiae* maintains distinct enzymes for the two functions (Kaldis, 1999).

To form a stable binary complex with its activating partner, cyclin H, *in vitro*, CDK7 must be phosphorylated on a conserved threonine (Thr170) in its own T-loop (Fisher *et al.*, 1995; Martinez *et al.*, 1997). CDK7 has an additional phosphorylated serine (Ser164) within the T-loop, but it is not required for binding cyclin H or for activating CDK7 complexes *in vitro* (Fisher *et al.*, 1995). Remarkably, the requirement for T-loop phosphorylation can be bypassed *in vitro* altogether by the association of CDK7 and cyclin H with the RING finger protein, MAT1 (Devault *et al.*, 1995; Fisher *et al.*, 1995; Tassan *et al.*, 1995; Martinez *et al.*, 1997; Garrett *et al.*, 2001).

Although bulk CAK activity and levels of CDK7, cyclin H and MAT1 proteins do not appear to fluctuate during the cell cycle (Brown *et al.*, 1994; Poon *et al.*, 1994; Tassan *et al.*, 1994), CDK7 could be regulated by differential association with other proteins, or by other post-translational modifications. For example, it has been reported that TFIIH-bound CDK7 phosphorylates the CTD more efficiently than it does CDK2 (Rossignol *et al.*, 1997). In addition, TFIIH binding appears to confer sensitivity to UV irradiation on CDK7 activity *in vivo* (Adamczewski *et al.*, 1996). Within the trimeric complex, MAT1 has been proposed to increase the activity of CDK7 towards the CTD at the expense of CAK activity (Yankulov and Bentley, 1997). Finally, TFIIH-associated kinase activity appears to decrease at mitosis (Long *et al.*, 1998), and a recent study suggested that changes in the levels of Ser164 phosphorylation are responsible for that repression (Akoulitchev and Reinberg, 1998).

To address the functional significance of CDK7 T-loop phosphorylation *in vivo*, we have combined genetics in *Drosophila* with biochemical analysis of purified mammalian components. *Drosophila* CDK7 is phosphorylated on two sites, Ser164 and Thr170, within the T-loop, as is its mammalian counterpart. These phosphorylations are important determinants of CDK7–cyclin H–MAT1 complex stability; the trimeric CAK complex dissociates *in vivo* and *in vitro* in the absence of T-loop phosphorylation. *In vitro*, Thr170 phosphorylation specifically stimulates the CTD kinase activity of the CDK7 trimeric complex by increasing enzyme turnover ~20-fold, but has little effect on CAK activity. Ser164 phosphorylation by itself, in contrast, has only minimal stimulatory effects on both CAK and CTD kinase activities.

Therefore, T-loop phosphorylation and binding to MAT1 are cooperating rather than alternative modes of

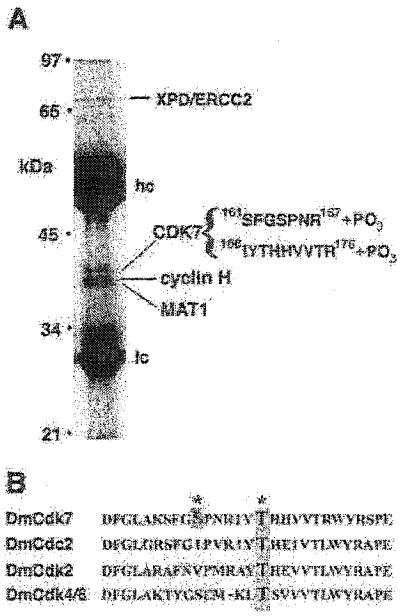


Fig. 1. DmCAK contains cyclin H, MAT1 and XPD. (**A**) Immunoprecipitations were carried out on 0–16 h embryonic extracts, and the isolated proteins were subjected to mass spectrometry. The identities of the four major proteins present in the immunoprecipitates are indicated on the right. Complete *Drosophila* cyclin H (AF024618) and MAT1 (AF071227) sequences can be obtained from GenBank. DmXPD has been described previously (Reynaud *et al.*, 1999). (**B**) T-loop sequences and phosphorylation sites of *Drosophila* CDKs. In addition to the conserved threonine at position 170, Ser164 within the T-loop of CDK7 is also a target of phosphorylation.

CDK7 activation *in vivo*, with modifications at Ser164 and Thr170 playing redundant roles in the stabilization of the trimeric CAK complex. The phosphorylation state of TFIIH-bound CDK7, moreover, could be an important determinant of CTD phosphorylation rates during the transcription cycle.

Results

Drosophila CAK complexes

The only component of CAK described to date in *Drosophila* is the catalytic subunit, CDK7 (Larochelle *et al.*, 1998). We identified in the database *Drosophila* genes coding for proteins homologous to the known partners of vertebrate CDK7, cyclin H and MAT1, and isolated corresponding cDNAs from an embryonic library. The putative *Drosophila* cyclin H is 42% identical to human cyclin H, and the candidate *Drosophila* MAT1 protein shares 52% amino acid identity with human MAT1 (not shown). To determine the composition of physiological *Drosophila* CAK complexes, we immunoprecipitated CDK7 from embryonic extracts and identified the associated proteins by mass spectrometry of tryptic peptide fragments. We confirmed that CDK7 complexes contain the products of the *cycH* and *MAT1* cDNAs we identified (Figure 1A). Therefore, *Drosophila* CAK, like its vertebrate counterpart, contains the three subunits: CDK7, cyclin H and MAT1. A fraction of CDK7 is also bound to XPD (Figure 1A), which is found along with CAK in TFIIH. A quaternary complex composed of CDK7, cyclin H, MAT1 and XPD has also been described in mammalian cell extracts (Drapkin *et al.*, 1996; Reardon *et al.*, 1996; Rossignol *et al.*, 1997).

Mass spectrometric analysis of the *Drosophila* CAK peptides (Supplementary data are available at *The EMBO Journal* Online) indicated that both Ser164 and Thr170 are phosphorylated *in vivo*, as are the corresponding residues in vertebrate CDK7 (Labbé *et al.*, 1994). CDK7 is the only member of the CDK family with two documented phosphorylations within the T-loop (Figure 1B).

Table I. Temperature-sensitive phenotypes associated with mutations of CDK7 phosphorylation sites

Mutation	Rescue $cdk7^{null}$		
	18°C	25°C	29°C
S180A	yes	yes	yes
S164A	yes	yes	yes
T170A	yes	yes	no[a]
S164A/T170A	yes	no[a]	no[b]

[a]Die as pharate adults.
[b]Die as embryos.

Abrogation of CDK7 phosphorylation in vivo

We mutated Ser164 and Thr170, individually (S164A, T170A) and in combination (S164A/T170A), to alanine. A third mutation, Ser180 to alanine (S180A), was a control. Ser180 is part of the conserved WYR(A/S)PE motif of protein kinases and is an alanine in most other CDKs, including mammalian CDK7. The activity of $CDK7^{S180A}$ is identical to that of wild-type CDK7 (not shown).

We assessed the ability of a given allele of *cdk7* to rescue the lethality associated with the $cdk7^{null}$ mutation by crossing males carrying the mutant transgene on the third chromosome (*yw*/Y; +/+; $Pw^{+}[cdk7^{mutant}]$/+) to balanced $cdk7^{null}$ females ($cdk7^{null}$/*FM7c*; +/+; +/+). The presence of any males carrying the $cdk7^{null}$ chromosome in the progeny from this cross indicates that the transgene rescued the lack of *cdk7*. All mutations tested were able to rescue the lethality of the null mutation at 18°C (Table I). Although relative viability varied somewhat among individual transgenic lines, stocks of each line could be established and maintained at 18°C ($cdk7^{null}$/$cdk7^{null}$; +/+; Pw^{+} $[cdk7^{mutant}]$/$Pw^{+}[cdk7^{mutant}]$). Thus, CDK7 T-loop phosphorylation is not absolutely essential *in vivo*. However, the $cdk7^{S164A/T170A}$ double mutant transgene was unable to rescue viability at 25°C, and the T170A transgene, when present as a single copy, could only rescue viability consistently at temperatures below 29°C (Table I).

Our results are in contrast to a recent report suggesting that the T170A mutation causes CDK7 to behave in a dominant-negative fashion (Leclerc *et al.*, 2000). The fact that $CDK7^{T170A}$ expressed at or near endogenous levels in a $cdk7^{null}$ background can fully rescue viability (Table I) argues that the dominant effects observed by Leclerc *et al.* were probably secondary to overexpression. Our data also indicate that $CDK7^{T170A}$ is less active than wild-type CDK7 towards at least one substrate (see below), possibly explaining why $CDK7^{T170A}$ failed to rescue viability of the

null mutation when expressed at levels much lower than that of the endogenous protein (Leclerc *et al.*, 2000). We therefore conclude that $cdk7^{T170A}$ behaves genetically as a weak loss-of-function, rather than a dominant-negative, mutation at expression levels near that of wild-type *cdk7*.

In contrast to the effects of the previously described conditional allele of *cdk7* ($cdk7^{P140S}$; Larochelle *et al.*, 1998), the temperature sensitivity of the $cdk7^{S164A/T170A}$ allele is expressed almost immediately upon transfer to the restrictive temperature, resulting in a rapid arrest of egg laying by adults, and in embryonic lethality at 29°C. Furthermore, the $cdk7^{S164A/T170A}$ adult flies die after 48–72 h at 29°C, also in contrast to the $cdk7^{P140S}$ mutant, in which viability at high temperatures was not compromised after animals reached adulthood (Larochelle *et al.*, 1998). S164A/T170A larvae, moreover, do not survive a 60 min heat shock at 37°C, probably due to a failure to induce a normal heat-shock response. This suggests a more complete loss of CDK7 activity *in vivo* upon temperature shift when the T-loop cannot be phosphorylated.

The steady-state levels of CDK7 T-loop phosphorylation change during development

The various CDK7 phospho-isoforms observed in ovaries of mutant animals are shown in Figure 2A. Phosphorylation of CDK7 on the T-loop increases electrophoretic mobility, as has been observed for other CDKs. We can resolve at least three phospho-isoforms under optimal conditions. In wild-type (or S180A) adults, the fastest migrating, doubly phosphorylated isoform predominates, but we also observe significant amounts of the slowest migrating, unphosphorylated form. In the S164A mutant animals, the doubly phosphorylated form disappears, and an isoform with intermediate electrophoretic mobility, presumably representing CDK7 singly phosphorylated on Thr170, appears.

We asked whether the T-loop phosphorylation state of CDK7 changes in a number of physiological contexts. During embryonic development, the distribution of CDK7 between a predominant, doubly phosphorylated form and a minor, unphosphorylated form appears to be relatively constant (Figure 2B). Likewise, CDK7 isoforms do not fluctuate appreciably in early embryos fractionated into interphase (I), prophase (P), metaphase (M), anaphase (A) and telophase (T) populations (Figure 2C). In contrast, we observe variations when we compare different developmental stages and different tissues (Figure 2D). In third instar larvae (L3), the unphosphorylated isoform is virtually absent. Instead, we see a doublet probably corresponding to doubly and singly phosphorylated CDK7, with the singly, presumably Thr170-phosphorylated, form usually predominating. In contrast, the unphosphorylated form is a major one in imaginal disc, and is also abundant in ovaries. All of the extracts for the analysis shown in Figure 2D were prepared under denaturing conditions (boiling in SDS sample buffer); extraction under non-denaturing conditions solubilized little or no unphosphorylated CDK7 (Figure 2E), precluding meaningful comparison of CDK7-associated kinase activity in different tissues. Although we do not yet understand their physiological significance, these tissue-specific differences suggest that CDK7 T-loop phosphorylation *in vivo* could modulate kinase activity in response to developmental or environmental signals.

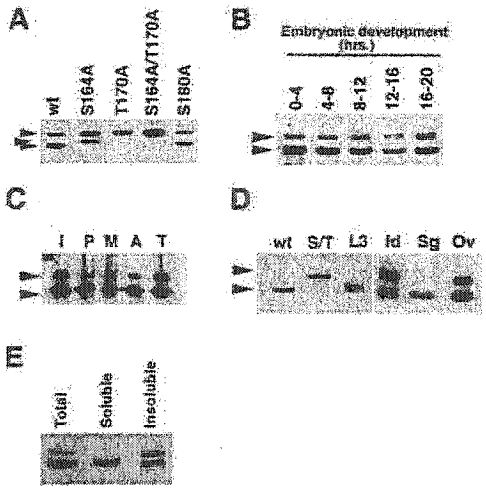


Fig. 2. Phospho-isoforms of *Drosophila* CDK7 detected by electrophoretic mobility shift. (A) CDK7 protein from ovaries of wild-type and mutant animals is shown. Dual T-loop phosphorylation (bottom arrowhead) results in a large increase in mobility compared with the unphosphorylated isoform (top arrowhead). Phosphorylation on Thr170 alone in the S164A mutant causes an intermediate shift (middle arrow). In this analysis, we could not distinguish the form singly phosphorylated on Ser164 from the completely unphosphorylated form. (B) The relative abundance of CDK7 protein and its state of T-loop phosphorylation vary little during embryonic development. (C) The state of CDK7 T-loop phosphorylation does not change appreciably during the embryonic cell cycles. Embryos were selected individually (embryonic cycle 9–13) after methanol fixation and staining for DNA, and identified by microscopic inspection as being in interphase (I), prophase (P) metaphase (M), anaphase (A) or telophase (T). (D) The state of CDK7 T-loop phosphorylation varies among different tissues: ovaries from wild-type (wt) and the double-mutant (S/T) animals, third instar larvae (L3), imaginal discs (Id), salivary glands (Sg) and ovaries (Ov). Note that the difference in isoform representation between the first and last lanes, which both contain extracts of wild-type ovaries, reflects differences in the age of the animals. We typically see more unphosphorylated CDK7 in more mature ovaries. Proteins were extracted by directly boiling the tissues in SDS sample buffer. (E) The unphosphorylated form of CDK7 exhibits low solubility. Embryos were homogenized in SDS sample buffer (Total) or HoB buffer. Following centrifugation, the supernatant (soluble) and pellet (insoluble) were boiled in SDS sample buffer. The unphosphorylated form of CDK7 is extracted efficiently only in the presence of SDS (lanes 1 and 3).

T-loop phosphorylation protects trimeric CAK from thermal inactivation in vitro

To understand the temperature-sensitive phenotype in *cdk7* mutant animals, we tested whether the mutant proteins could be inactivated by a temperature shift *in vitro*. We measured the activity of CDK7 immunoprecipitated from embryos or adult flies raised at 18°C towards both CDK2 and CTD after incubation at either room temperature or 33°C. Remarkably, mutation of Thr170 to alanine differentially affected activity towards the two different substrates, revealing a previously unsuspected role for this residue in determining substrate specificity (see also Figure 7). In addition, both the CAK and CTD kinase activities of all T-loop mutant forms of CDK7 were reduced after a short incubation at 33°C

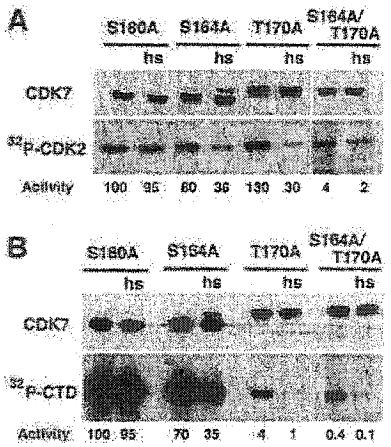


Fig. 3. T-loop mutant forms of *Drosophila* CDK7 are temperature sensitive *in vitro*. CDK7 was immunoprecipitated from the various mutants; the immunoprecipitates were divided in half and either left on ice or incubated at 33°C for 30 min (hs) prior to kinase assays with either $CDK2^{D145N}$–cyclin A (**A**) or GST–CTD (**B**) as substrates. Note that the exposure times for experiments with $CDK7^{S164A/T170A}$ were longer than for the wild-type and single mutants. For both substrates, incorporation was quantified by PhosphorImager and expressed as a percentage of incorporation by wild-type CDK7 not subjected to heat shock (defined as 100%). Measured values are indicated below each lane in (A) and (B). Top panel, anti-CDK7 immunoblot; bottom panel, ^{32}P incorporation into substrates.

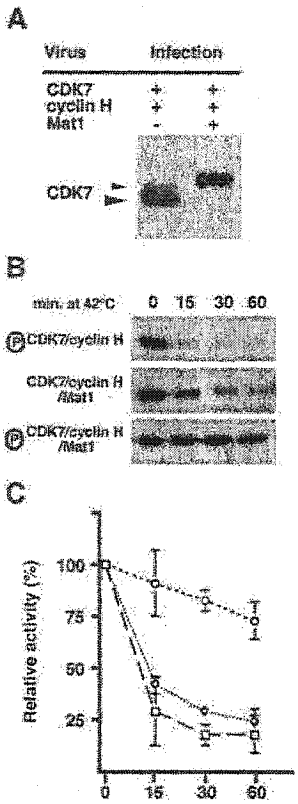


Fig. 4. T-loop phosphorylation protects mammalian CDK7 from thermal inactivation. (**A**) Anti-CDK7 immunoblots of recombinant CAK purified from Sf9 cells infected with baculoviruses encoding the mammalian CAK subunits. Double infections with CDK7 and cyclin H viruses produce CDK7 protein that is doubly phosphorylated on the T-loop (left: large arrowhead). The small arrowhead indicates a less abundant, probably singly phosphorylated CDK7 isoform. Triple infections with CDK7, cyclin H and MAT1 result in the isolation of CDK7 protein that is entirely unphosphorylated (right). (**B**) Time course of thermal inactivation of the different forms of CAK *in vitro*. We incubated the three complexes at 42°C for the indicated periods of time before we measured activity towards a CDK2–cyclin A substrate. (**C**) Relative thermal stability of the CAK dimer, unphosphorylated trimer and phosphorylated trimer quantified on the basis of triplicate experiments (represented by B). The phosphorylated trimer (circle) is resistant to heat inactivation compared with either the unphosphorylated trimer (diamond) or the phosphorylated dimer (square).

in vitro (Figure 3A and B). Interestingly, the activity associated with CDK7 in the S164A/T170A mutant is <5% (CAK) or 1% (CTD kinase) that of wild-type CDK7 (Figure 3), although the animals are viable. Thus, wild-type CDK7 activity vastly exceeds the level required to sustain its essential function or, alternatively, compensatory mechanisms can act to rescue a drastic drop in CAK and CTD kinase activity.

T-loop phosphorylation appears to protect CDK7 from thermal inactivation. It remained possible, however, that increased thermal lability was due to disruption of protein conformation because alanine cannot substitute adequately for unphosphorylated serine or threonine. To address this possibility, we compared the activity and stability of phosphorylated and unphosphorylated forms of trimeric wild-type CDK7, by using mammalian CDK7, cyclin H and MAT1 proteins expressed with baculoviruses. When we co-infect insect cells with CDK7 and cyclin H viruses only, the dimeric complex that we purify contains CDK7 that is almost quantitatively phosphorylated on both Ser164 and Thr170 (Figure 4A). In contrast, co-infection with CDK7, cyclin H and MAT1 baculoviruses results in a trimeric complex containing completely unphosphorylated CDK7 (Figure 4A). We compared the thermal stability of the three forms of CDK7—phosphorylated dimer, unphosphorylated trimer and phosphorylated trimer—by incubating the different complexes for various times at 42°C before assaying their activity (Figure 4B and C). Both dimer and unphosphorylated trimer rapidly lost activity at 42°C, whereas the phosphorylated trimer, reconstituted by addition of pure MAT1 to the dimer, was resistant to thermal inactivation. We therefore conclude that T-loop phosphorylation is an important contributor to the thermal stability of physiological CDK7 complexes, even when they contain MAT1.

CDK7 T-loop phosphorylation is required for efficient assembly of the CAK trimer

In vertebrates, CDK7 exists in two major complexes that can be separated by gel filtration: a >600 kDa complex corresponding to TFIIH; and an ~100 kDa heterotrimeric complex comprising CDK7, cyclin H and MAT1, which migrates aberrantly with an apparent size of ~240 kDa (Devault *et al.*, 1995; Fisher *et al.*, 1995). We fractionated *Drosophila* embryonic extracts to determine the apparent size of the CDK7-containing complexes (Figure 5A). As controls, we analyzed mammalian CDK7–cyclin H dimer and CDK7–cyclin H–MAT1 trimer on the same column

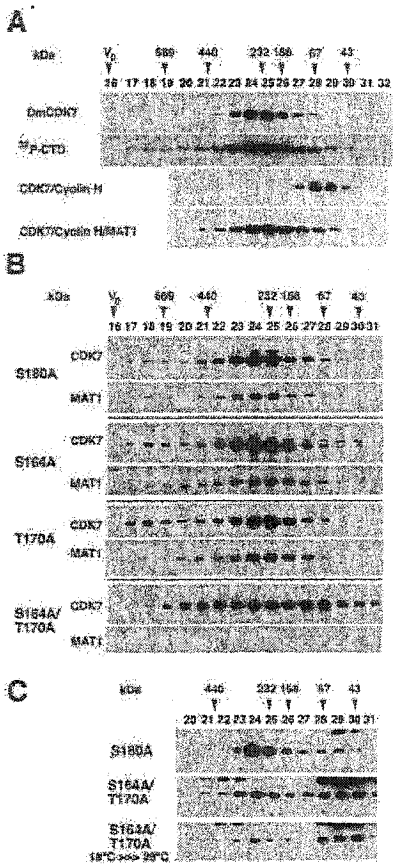


Fig. 5. T-loop phosphorylation is required for the formation of a stable CDK7–cyclin H–MAT1 complex. (**A**) Embryonic extracts were fractionated on a Superdex 200 gel filtration column; each fraction was subjected to immunoprecipitation with anti-CDK7 antibodies and analyzed by immunoblot or assayed for phosphorylation of GST–CTD *in vitro*. Pure mammalian dimeric and trimeric CAK complexes were used as size standards in chromatography, and detected by immunoblotting with anti-CDK7 antibodies for the dimer and with anti-MAT1 antibodies for the trimer. (**B**) Size distribution of CDK7 protein in the various T-loop mutants. Embryonic extracts from the indicated mutants were fractionated as in (A); the fractions were immunoprecipitated with anti-CDK7 antibodies and subjected to SDS–PAGE followed by immunoblotting for CDK7 and MAT1 proteins. Due to the fragility of the complex in the total absence of CDK7 T-loop phosphorylation, no MAT1 could be detected in immunoprecipitates from the S164A/T170A mutant. (**C**) Decreased thermal stability of unphosphorylated CDK7-containing complexes. Embryonic extracts were fractionated as in (A) and analyzed directly (without immunoprecipitation) by SDS–PAGE and immunoblotting with anti-CDK7 antibodies. When S164A/T170A embryos are shifted from 18 to 29°C, the recovery of the complex is diminished further (bottom panel).

(Figure 5A). Most endogenous *Drosophila* CDK7 chromatographs with the same apparent size as the mammalian trimer. Therefore, soluble CDK7 in embryos is predominantly in the form of free CAK trimer, most of which is phosphorylated on the T-loop. This is consistent with the apparently stoichiometric amounts of CDK7, cyclin H and MAT1 we typically recover in anti-CDK7 immunoprecipitates from embryonic extracts (Figure 1A). We cannot exclude, however, the presence of minor CDK7 complexes lacking MAT1 or containing other subunits, such as XPD, which might not affect chromatographic behavior. After chromatography, we immunoprecipitated the fractions with an anti-CDK7 antibody, and measured kinase activity towards a recombinant CTD substrate (Figure 5A). We consistently observed a minor peak of both immunoreactivity (Figure 5B) and CTD kinase activity (Figure 5A) in fraction 18, which probably corresponds to TFIIH. Thus *Drosophila* CDK7 forms most or all of the same complexes as does vertebrate CDK7.

We next looked at the size distribution of the CDK7 proteins with T-loop mutations (Figure 5B). When we analyzed extracts from either $cdk7^{S164A}$ or $cdk7^{T170A}$ embryos, the majority of CDK7 remained in fractions corresponding to the trimeric form. In both cases, however, detectable amounts of CDK7 protein appeared in the smaller size fractions, possibly corresponding to free CDK7 monomer. Interestingly, the unphosphorylated CDK7 isoform was enriched in the monomer-sized fractions of the S164A lysate (Figure 5B, fractions 29 and 30). In $cdk7^{S164A/T170A}$ lysates, the redistribution of CDK7 protein to low molecular weight forms was even more pronounced (Figure 5B), indicating a defect in complex formation when CDK7 cannot be phosphorylated. Consistent with this interpretation, we could detect little or no MAT1 in immunoprecipitates of fractions of the S164A/T170A lysate, although it was detected readily in wild-type and both single mutants (Figure 5B). Because the $cdk7^{S164A/T170A}$ mutation caused lethality at high temperature and altered the distribution of CDK7 between different complexes, we asked whether the basis for temperature sensitivity might be an impaired ability to interact with cyclin H and MAT1. Indeed, after $cdk7^{S164A/T170A}$ embryos were shifted from 18 to 29°C, CDK7 complexes dissociated almost completely (Figure 5C). This correlated well with the inactivation of mutant CDK7 complexes *in vitro* (Figure 3), suggesting that the basis for thermal instability in the absence of T-loop phosphorylation is due, at least in part, to decreased affinity of CDK7 for its positive regulators, cyclin H and MAT1.

Disappearance of the trimer did not correlate perfectly with the amount of apparent monomer recovered (Figure 5C). In fact, we also reproducibly observed increased immunoreactivity in higher molecular weight fractions and in fractions intermediate in size between trimers and monomers (Figure 5B), possibly representing aggregated or misfolded mutant CDK7 protein, partially dissociated complexes or disruption of higher order complexes not extracted efficiently from wild-type embryos. To confirm the release of CDK7 protein from active complexes, we measured the relative amounts of dissociated CDK7 in the various mutants by immunoprecipitation with antibodies that distinguish between free and complexed CDK7. Monoclonal antibody (mAb) 4A7 recognizes an epitope that is inaccessible when CDK7 is in a functional complex, but is fully accessible when complexes are dissociated *in vitro* (Figure 6A). Although mAb 4A7 precipitated very little CDK7 from wild-type, S164A and T170A homogenates, it readily precipitated CDK7 protein from the S164A/T170A lysates (Figure 6B, right). mAb 20H5, which recognizes an epitope available in both the complex and the monomeric forms,

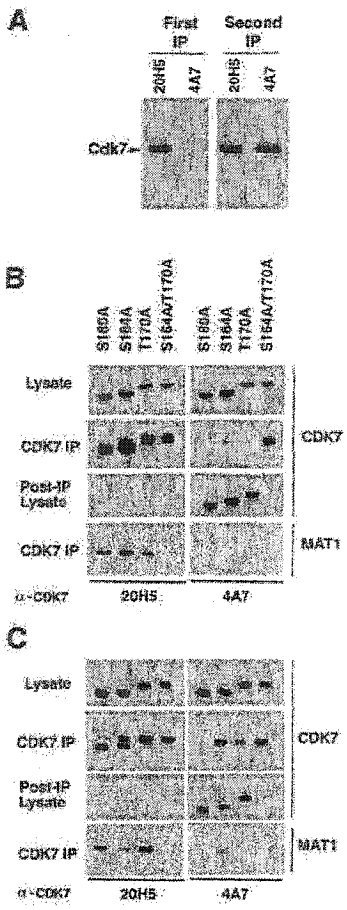


Fig. 6. Dephosphorylation of the T-loop destabilizes CDK7 complexes. (A) Anti-Cdk7 4A7 is specific for the monomeric form of CDK7, whereas the 20H5 antibody recognizes both complex and monomeric forms of CDK7. Antibody 20H5 can precipitate Cdk7 from an embryonic lysate whereas 4A7 cannot (Left). Following immunoprecipitation with 20H5 and denaturation by boiling in SDS, 4A7 and 20H5 precipitate Cdk7 with equal efficiency (right). (B) The 4A7 antibody immunoprecipitates large amounts of protein only from the S164A/T170A mutant, and a small amount from the S164A mutant, whereas the 20H5 antibody precipitates similar amounts from all samples. Top panel, anti-CDK7 immunolot of total lysates prior to immunoprecipitation; second panel, anti-CDK7 immunoblot of immunoprecipitates; third panel, anti-CDK7 immunoblot of post-immunoprecipitation supernatants; bottom panel, anti-MAT1 immunoblot on the immunoprecipitated material. (C) Similar to (B) after 15 h incubation at 4°C. Detectable amounts of CDK7 can be immunoprecipitated from all samples with the 4A7 antibody. Both single mutants (S164A and T170A) dissociate to a much greater extent than does the wild-type (S180A) control. In each case, however, only the slow migrating (unphosphorylated) form can be precipitated, indicating that dephosphorylation of the T-loop is required to destabilize complexes. Dephosphorylation of the S164A protein correlates with the loss of MAT1 from the complex (bottom panel).

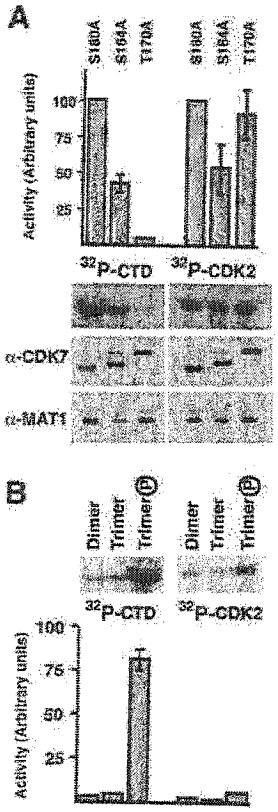


Fig. 7. T-loop phosphorylation of CDK7 regulates activity in a substrate-dependent manner. (A) CDK7 was immunoprecipitated from S180A, S164A and T170A mutants. Each sample was divided in half and tested for kinase activity towards either GST–CTD or CDK2–cyclin A (top panel). Incorporation was quantified and expressed as a percentage of incorporation by the wild-type (S180A) control immunoprecipitate. The T170A mutation results in a loss of activity that is specific to the CTD substrate. Both wild-type and T170A immunoprecipitates contain similar amounts of MAT1 protein, indicating that the substrate-specific effect is caused by the lack of phosphorylation rather than by loss of MAT1 from the complex (bottom panel). Some of the loss of activity by the S164A mutant with both substrates probably stems from the fact that S164A immunoprecipitates contain significant amounts of completely dephosphorylated (monomeric) CDK7, which is paralleled by a reduced amount of MAT1 in S164A immunoprecipitates as compared with both wild-type and T170A. (B) The purified mammalian phosphorylated trimer exhibits increased activity (25- to 50-fold) towards the CTD substrate when compared with either phosphorylated dimer or unphosphorylated trimer, but the effect of phosphorylation on CAK activity is modest (~2-fold).

precipitated similar amounts of CDK7 proteins from all samples (Figure 6B, left). These data indicate that the $CDK7^{S164A/T170A}$ protein dissociated from cyclin H and MAT1 even with minimal handling of the samples *in vitro*, and is probably dissociated extensively *in vivo*.

After several hours of incubation at 4°C, $CDK7^{S164A}$ and $CDK7^{T170A}$ proteins can also be immunoprecipitated readily with mAb 4A7 (Figure 6C, right), consistent with spontaneous dissociation of the complex in crude extracts. Only a barely detectable amount of wild-type (S180A) CDK7 can be precipitated under similar conditions. Interestingly, only the slow migrating (unphosphorylated) isoform of CDK7 can be precipitated from the wild-type and S164A lysates with mAb 4A7 (Figure 6C), arguing that the inability to form a stable complex is strictly a function of phosphorylation, not of misfolding caused by serine/threonine to alanine substitutions. We conclude that T-loop phosphorylation of CDK7 is required to form a stable CDK7–cyclin H–MAT1 complex *in vitro* and *in vivo*. Phosphorylation of either Ser164 or Thr170

Table II. Apparent kinetic parameters of the diferent CAK complexes with the CTD and CDK2–cyclin A substrates

Parameter	CTD			$CDK2^{D145N}$–cyclin A		
	Dimer	Trimer	P-Trimer	Dimer	Trimer	P-Trimer
K_m (μM)	7.5 ± 2.5	3.8 ± 1.6	4.3 ± 1.4	0.26 ± 0.08	0.39 ± 0.07	0.23 ± 0.05
V_{max} (pmol P_i/min)	1.6 ± 0.3	1.6 ± 0.2	26.6 ± 3.3	0.094 ± 0.010	0.054 ± 0.010	0.19 ± 0.02
k_{cat} (/s)	0.23	0.22	3.69	0.013	0.008	0.026
k_{cat}/K_m	0.03	0.06	0.86	0.04	0.02	0.11

is sufficient to stabilize the complex, because only unphosphorylated CDK7 can be precipitated by the monomer-specific 4A7 antibody. Indeed, the CAK complexes from either *cdk7*^{T170A} or *cdk7*^{S164A} mutant animals initially are relatively stable (Figure 6B), but decay upon prolonged incubation at 4°C as shown by the loss of MAT1 in parallel with dephosphorylation of the T-loop of CDK7 (Figure 6C).

T-loop phosphorylation stimulates kinase activity of trimeric CDK7 in a substrate-specific manner

In the absence of heat treatment, we observed little difference between wild-type *Drosophila* CDK7 and the single phosphorylation site mutants in activity towards a CDK2 substrate (Figure 3A). However, the T170A mutant protein had dramatically reduced activity towards CTD, compared with wild-type (Figure 3B). To measure the relative effects of Ser164 and Thr170 phosphorylation on CDK7 activity towards CDK2 and the CTD, anti-CDK7 immunoprecipitates were divided in half and assayed with both substrates. The immunoprecipitations were done under conditions that minimized CDK7 complex dissociation *in vitro* (similar to Figure 6B). The $CDK7^{S164A}$ protein is ~50% as active as wild-type CDK7 with either substrate (Figure 7A), possibly because it is more prone than wild-type and T170A proteins to dephosphorylation and consequent destabilization of the complex. Indeed, the $CDK7^{S164A}$ immunoprecipitate shows a reduced amount of MAT1 relative to the wild-type, which correlates with the presence of completely unphosphorylated CDK7 (Figure 7A). The $CDK7^{T170A}$ protein, in contrast, is nearly identical to wild-type CDK7 in activity towards CDK2, but only 4% as active towards the CTD (Figure 7A). Moreover, complexes containing $CDK7^{T170A}$ remain intact throughout the immunoprecipitation and the kinase assays, as judged by the stable association of MAT1 (Figure 7A). Thus, phosphorylation of Thr170 stimulates CTD kinase activity ~25-fold under these assay conditions without significantly affecting CAK activity. To confirm these observations, we carried out similar experiments with purified wild-type mammalian components in both the phosphorylated and unphosphorylated states (Figure 7B). The phosphorylated trimer is ~20-fold more active towards the GST–CTD substrate, but only slighly more active towards CDK2. Although the relative contributions of phosphorylations on Ser164 and Thr170 can only be measured in *Drosophila*, the results with either the fly or mammalian enzymes are in remarkably good agreement: phosphorylation of trimeric CDK7 on Thr170 specifically and dramatically (~20-fold) stimulates activity towards the CTD without significantly affecting CAK activity. Phosphorylation on Ser164, in contrast, causes only a modest stimulation of activity without substrate preference.

Phosphorylation of the CDK7 T-loop could boost CTD kinase activity in either of two ways: an increase in catalytic efficiency specific to the Tyr-Ser-Pro-Thr-Ser-Pro-Ser target sequence of the CTD, or a change in the enzymatic mechanism from a distributive to a processive one. Because the full-length CTD has 52 potential sites of phosphorylation by CDK7, an increase in processivity could well account for the apparent increase in activity when CDK7 is doubly phosphorylated on the T-loop. To test this idea, we compared the activity of CDK7 immunoprecipitated from wild-type, S164A and T170A animals towards a CTD peptide containing only two heptad repeats. The results obtained with the peptide substrate were identical to those obtained with the full-length CTD (data not shown), suggesting an increased catalytic efficiency rather than processivity. In addition, kinetic analysis of the three mammalian CAK complexes (phosphorylated dimer, unphosphorylated trimer and doubly phosphorylated trimer) shows that the substrate-specific increase in catalytic efficiency is due entirely to accelerated enzyme turnover (Table II). Thr170 phosphorylation could therefore be a key step in regulating the rate of CTD phosphorylation by TFIIH during transcription.

A defect in CTD hyperphosphorylation after heat shock in cdk7^{T170A} *mutant larvae*

The effect of Thr170 phosphorylation on the kinetics of CTD kinase activity *in vitro* suggests that its abrogation might compromise normal RNA pol II phosphorylation *in vivo*. The fact that *cdk7*^{T170A} flies are viable under most conditions tested, however, argues against a constitutive, global disruption of CTD phosphorylation. It is therefore not surprising that we saw no major difference in the steady-state levels of CTD phosphorylation, on either Ser2 or Ser5, among the *cdk7* mutants raised at different temperatures (Figure 8; data not shown). In contrast, when we subjected larvae of the different mutant backgrounds to a brief heat shock, phosphorylation of the CTD reproducibly increased above basal levels in both the wild-type and S164A mutant, whereas the response was blunted in the T170A mutant animals (Figure 8). Although the mechanisms underlying these fluctuations are likely to be complex, our data suggest that a kinetically defective, $CDK7^{T170A}$ mutant enzyme is unable to support the increased rate of CTD phosphorylation that normally occurs after an acute thermal stress.

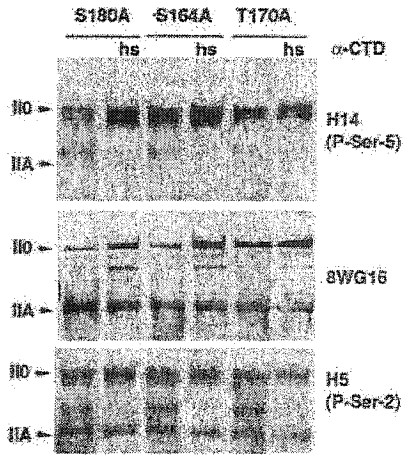


Fig. 8. RNA polymerase II phosphorylation in CDK7 mutants. Third instar wandering larvae were homogenized directly in SDS sample buffer either prior to, or after a 30 min heat shock at 37°C (hs). Each sample was immunoblotted with antibodies to the CTD of the large subunit of RNA pol II: 8WG16, H5 (specific for phosphorylated Ser2 of the CTD heptad repeat) and H14 (specific for phosphorylated Ser5 of the CTD heptad repeat). Although the 8WG16 antibody preferentially recognizes unphosphorylated repeats (enriched in form IIA), it cross-reacts to some extent with form IIO that is not completely phosphorylated.

Discussion

T-loop phosphorylation: a stabilizer of the CDK7–cyclin H–MAT1 trimeric complex

In the best studied cases of CDKs that drive the cell cycle, T-loop phosphorylation is essential for physiological function (Morgan, 1997). Although that requirement has been demonstrated for Cdc2 in fission yeast (Gould *et al.*, 1991), Cdc28 in budding yeast (Lim *et al.*, 1996; Cross and Levine, 1998) and Cdc2 in *Drosophila* (Larochelle *et al.*, 1998), its basis can only be inferred from structural studies of the human CDK2–cyclin A complex (De Bondt *et al.*, 1993; Jeffrey *et al.*, 1995; Russo *et al.*, 1996). Phosphorylation of Thr160 in the T-loop of CDK2 stimulated the rate of catalysis ~300-fold, but also strengthened the association between the catalytic subunit and the cyclin (Russo *et al.*, 1996). T-loop phosphorylation is also required to stabilize complexes of CDC2 and cyclin A (Ducommun *et al.*, 1991; Desai *et al.*, 1995; Larochelle *et al.*, 1998). Here we demonstrate that a major role *in vivo* for T-loop phosphorylation of CDK7 is the stabilization of the predominant physiological form of the kinase: the CDK7–cyclin H–MAT1 trimer.

Our results suggest that the two mechanisms for CDK7 complex stabilization and activation—MAT1 addition and T-loop phosphorylation—which can operate independently *in vitro*, actually cooperate under physiological conditions to maintain complex integrity. With prolonged exposure to elevated temperature, dissociation to monomeric subunits occurs *in vivo* when CDK7 is dephosphorylated, even in the presence of MAT1.

Might there be physiological situations in which the trimeric CAK complex needs to be destabilized, for example to facilitate subunit rearrangements or redistribution of CAK between free and TFIIH-bound forms? Interestingly, we have observed spontaneous dephosphorylation, and dissociation of *Drosophila* CDK7 in *vitro*, either after prolonged incubation on ice or when immunoprecipitated complexes are subjected to a brief heat treatment. In the latter case, we may infer the presence, in complex with CDK7, of a phosphatase capable of dephosphorylating Thr170. Under both conditions, $CDK7^{S164A}$ appears especially susceptible to dephosphorylation and dissociation, whereas the doubly phosphorylated, wild-type form appears largely resistant. Thus the dual phosphorylation might be part of a regulatory circuit controlling CDK7 function at the level of complex stability.

CDK7 T-loop phosphorylation: separate controls for cell cycle and transcription?

Since its discovery as a component of both CAK and TFIIH in metazoans, CDK7 has been studied as a possible link between the cell cycle and transcriptional machinery. Those investigations have uncovered several potential regulatory mechanisms, but no clear evidence for their usefulness *in vivo*. T-loop phosphorylation is an example of such a mechanism in search of a biological context. Our studies have uncovered two important functions of CDK7 phosphorylation: stable complex assembly and modulation of CTD kinase activity. Whereas neither function is absolutely essential, impairment of either may cause temperature-sensitive loss of viability.

The ability to bypass T-loop phosphorylation of CDK7 in *vitro* (Devault *et al.*, 1995; Fisher *et al.*, 1995; Tassan *et al.*, 1995) appeared to obviate its requirement *in vivo*. Kin28, the budding yeast ortholog of CDK7, is phosphorylated uniquely on Thr162 by Cak1, which also activates Cdc28 (Espinoza *et al.*, 1998; Kimmelman *et al.*, 1999). Although phosphorylation stimulates the activity of the Kin28 complex towards the CTD (Espinoza *et al.*, 1998; Kimmelman *et al.*, 1999), strains containing Kin28 lacking Thr162 are viable, with no obvious transcriptional defects or thermal sensitivity. The $kin28^{T162A}$ allele is, however, synthetically lethal with mutations in the gene encoding Tfb3, the budding yeast ortholog of MAT1 (Kimmelman *et al.*, 1999). Thus T-loop phosphorylation and the RING subunit reinforce each other in yeast as they do in metazoans, but either one may suffice to ensure adequate kinase function. Both appear to be necessary in *Drosophila*, which die even at moderate temperatures when T-loop phosphorylation is blocked, despite having a full complement of wild-type MAT1.

No CDK7-activating kinase has been identified conclusively in a metazoan species, but candidates have emerged from studies of CDK activation *in vitro* as well as in yeast. Vertebrate CDC2 and CDK2 activate CDK7 in *vitro* (Fisher *et al.*, 1995; Martinez *et al.*, 1997), and are able to phosphorylate both Ser164 and Thr170 with equal efficiency (Garrett *et al.*, 2001). Budding yeast Cak1 and fission yeast Csk1, both monomeric kinases distantly related to CDKs, activate the CDK7 orthologs, Kin28 and Mcs6, respectively, *in vivo* (Molz and Beach, 1993; Espinoza *et al.*, 1998; Hermand *et al.*, 1998; Kimmelman *et al.*, 1999; Lee *et al.*, 1999). Thus, the T-loop of CDK7 may be a critical regulatory target, possibly helping to coordinate patterns of gene expression with cell cycle progression.

T-loop phosphorylation: a regulator of CTD phosphorylation and transcription rates?

It has been reported that the addition of MAT1 to the CDK7–cyclin H complex alters its substrate specificity, favoring CTD phosphorylation at the expense of CAK activity (Yankulov and Bentley, 1997). The phosphorylation state of the CDK7 catalytic subunit was not determined and the effects of MAT1 addition were also quite modest: an ~4-fold increase in the CTD:CDK2 phosphorylation ratio (Yankulov and Bentley, 1997). In contrast, we consistently observe an ~20-fold stimulation of the CTD kinase activity of trimeric CDK7–cyclin H–MAT1 when Thr170 is phosphorylated, with no loss (or gain) of CAK activity, under conditions where neither substrate is in limiting concentration. MAT1 is required for this effect; the phosphorylated dimeric complex is no more active than the unphosphorylated trimer. Indeed, the modest lowering of the K_m for CTD when MAT1 joins the complex (Table II) could explain the apparent stimulation observed previously (Yankulov and Bentley, 1997). We suggest, however, that MAT1 merely serves to facilitate substrate-specific stimulation by Thr170 phosphorylation, and that cycles of phosphorylation and dephosphorylation of the T-loop are more likely to regulate the function of CDK7 *in vivo* than are association and dissociation of MAT1.

The CTD of RNA pol II undergoes a cycle of phosphorylation and dephosphorylation during the process of transcription. RNA pol II with a hypophosphorylated CTD initiates transcription, the CTD becomes phosphorylated as the enzyme proceeds from initiation to elongation and, finally, the CTD is dephosphorylated as it completes the transcription cycle (Dahmus, 1996). Phosphorylation of Thr170 uniquely regulates the activity of CDK7 towards the CTD. The mechanism is direct acceleration of the catalytic rate of the enzyme, and so would provide a way to increase CTD phosphorylation rates and thereby favor promotor clearance, perhaps in opposition to dephosphorylation by a CTD phosphatase (Cho *et al.*, 1999). Whether this modulation is critical to regulation of gene expression has yet to be tested thoroughly. However, our studies raise the intriguing possibility that a kinase cascade or network regulates transcription through changes in the state of CDK7 T-loop phosphorylation. The failure to observe any changes in the steady-state levels of CTD phosphorylation in our *cdk7* mutants may reflect the complex network of kinases and phosphatases that act in concert on the CTD. Regulation of CDK7 T-loop phosphorylation may be critical, however, when rapid changes in gene expression are induced, for example by heat shock.

The dual function of metazoan CDK7 in control of cell cycle and transcription programs remains a puzzle. Although the notion that CDK7 coordinates gene expression with cell division in some fashion is intriguing, it has received little experimental support, and so the question of why two seemingly disparate functions are combined in one enzyme is still unanswered. We have more insight into how CDK7 can phosphorylate both the T-loops of CDKs and the CTD of RNA pol II, despite the complete lack of sequence homology between its two physiological substrates, by adopting different strategies for substrate recognition (Garrett *et al.*, 2001). Moreover, in this report, we have demonstrated how the CTD kinase activity of CDK7 can be regulated, by Thr170 phosphorylation, independently of CAK activity. Strikingly, Thr170 phosphorylation of trimeric CDK7 enables the enzyme to catalyze CTD phosphorylation at ~100 times the maximal rate for CDK2 phosphorylation (Table II). Because the CTD contains many (~52) target sites for CDK7-mediated phosphorylation, whereas CDK2 contains only one, this rate enhancement could allow the major physiological form of CDK7, the phosphorylated trimer, to catalyze CDK activation and CTD hyperphosphorylation at very similar rates.

Materials and methods

Drosophila stocks and growth conditions

Unless otherwise noted, adult flies and embryos were kept at 18°C. The different mutant stocks were obtained by crossing balanced *cdk7^{null}* females [*wDf(1)JB254, Pw$^+$(snf$^+$,dhd$^+$)/FM7c*; +/+; +/+] (Larochelle *et al.*, 1998) to the different lines carrying the *cdk7* mutant transgene on the third chromosome. Stocks were: [*wDf(1)JB254, Pw$^+$(snf$^+$,dhd$^+$)/wDf(1)JB254, Pw$^+$(snf$^+$,dhd$^+$); Pw$^+$(cdk7^m)/Pw$^+$ (cdk7^m)*; +/+] or [*wDf(1)JB254, Pw$^+$(snf$^+$,dhd$^+$)/wDf(1)JB254, Pw$^+$(snf$^+$,dhd$^+$); +/+; Pw$^+$(cdk7^m)/Pw$^+$ (cdk7^m)*]. Embryos were collected at the indicated temperatures on apple juice–agarose plates, dechorionated by treatment with 50% bleach, washed thoroughly, flash frozen in liquid nitrogen and stored dry at −80°C until use.

Size exclusion chromatography

Embryos were homogenized on ice in HoB [25 mM HEPES pH 7.4, 150 mM NaCl, 20 mM NaF, 20 mM β-glycerophosphate, 1 mM EDTA, 1 mM dithiothreitol (DTT), 0.1% Triton X-100] + protease inhibitors. We typically used 250 mg dry weight of embryos per ml of buffer, resulting in a total protein concentration of ~15 mg/ml after 30 min centrifugation at 100 000 *g*. We loaded 0.2–0.5 ml over a Superdex 200 10/30 HR gel filtration column (Amersham Pharmacia Biotech) equilibrated with 25 mM HEPES pH 7.4, 150 mM NaCl, 1 mM EDTA, 0.5 mM DTT, 10% glycerol. Fractions (0.5 ml) were collected and 10 µl of each was used for immunoblots. For immunoprecipitations, Triton X-100 was added to each fraction to a final concentration of 0.1%.

Antibodies

Monoclonal antibodies specific for *Drosophila* CDK7 were raised against the full-length protein as described previously (Larochelle *et al.*, 1998). The monoclonal antibody detecting the N-terminal part of MAT1 (1G6) was a kind gift of Jean-Marc Egly. Antibodies directed against the CTD of RNA pol II (8WG16, H5 and H14) were obtained from Covance, Inc.

Electrophoresis and immunoblotting

In order to separate the various phospho-isoforms of CDK7 reliably, SDS–PAGE was carried out with piperazine di-acrylamide instead of bis-acrylamide as the cross-linker (Kumagai and Dunphy, 1995), and the pH of the resolving gel was increased to 9.2. Separation of the different CDK7 phospho-isoforms increased with distance migrated through the gel, and may not be obvious in shorter runs. Immunoblots to detect *Drosophila* CDK7 were carried out with a mixture of monoclonal antibodies (4A7, 4D12 and 20H5) in the form of hybridoma culture supernatant at a dilution of 1:20–1:50 (~0.5 µg/ml). Affinity-purified polyclonal antibodies against human CDK7 and MAT1 were used at 1:1000 (~0.2 µg/ml). For pol II immunoblots, 10 third instar wandering larvae of each genotype were either collected at 25°C or heat shocked in a 1.5 ml microtube immersed in a 37°C water bath for 30 min. The samples were frozen in liquid nitrogen, and later homogenized directly in SDS sample buffer.

Immunoprecipitation and kinase assays

For measurements of activity in CDK7 immunoprecipitates (IPs), embryos or adult flies were homogenized in HoB as described above. For each IP, 150 µl of mAb supernatant (4A7, 4D12 or 20H5) and 15 µl of protein G–agarose were mixed with ~2 mg of total protein for 2 h at 4°C. The IPs were washed three times with HoB and three times with kinase buffer (25 mM HEPES pH 7.4, 150 mM NaCl, 10 mM $MgCl_2$, 0.5 mM DTT) and tested for kinase activity towards 7 µg of recombinant

GST–CTD, or 2 μg of catalytically inactive CDK2^{D145N}–cyclin A complex, in a volume of 30 μl of kinase buffer + 0.1 mM ATP + 2.5 μCi of [γ-^{32}P]ATP. Thermal inactivation experiments for both immunoprecipitated *Drosophila* CDK7 and pure mammalian CDK7 complexes were carried out in kinase buffer. Mammalian CDK7–cyclin H and CDK7–cyclin H–MAT1 complexes were purified as described previously (Fisher, 1997) from insect Sf9 cells infected doubly or triply, respectively, with the appropriate recombinant baculoviruses. Briefly, lysates were subjected to sequential chromatography with HiTrap Q, ATP-agarose and Superose 12 columns. The phosphorylated trimeric complex was generated by mixing the purified dimer (phosphorylated *in vivo* during infection) with purified MAT1 in an equimolar ratio. The mixture was incubated on ice for 1 h prior to kinase assays. Assays with purified proteins were carried out using 2–50 ng of enzyme in a final volume of 20–50 μl.

Measurements of kinetic parameters

Kinase assays with the purified mammalian complexes were carried out using a final CAK concentration of 6 nM in a volume of 20 μl for CTD and 10 μl for CDK2 kinase assays. Each assay was carried out in triplicate in 25 mM HEPES pH 7.4, 150 mM NaCl, 10 mM $MgCl_2$, 0.5 mM DTT, 200 μM ATP, 5 μCi of [γ-^{32}P]ATP for 5 min at 23°C. The concentrations of substrate were: for the CTD, 0.35, 0.875, 1.70, 3.40, 8.75, 14 and 17.5 μM; and for CDK2^{D145N}–cyclin A, 0.05, 0.125, 0.25, 0.5; 1.25 and 2.5 μM. The reactions were stopped by adding two volumes of 2× sample buffer and boiling. Samples were separated by SDS–PAGE and incorporation was quantified by scanning dried gels with a STORM 840 phosphorimager using the ImageQuant software. Incorporation was determined using various dilutions of [γ-^{32}P]ATP spotted on paper as calibration standards. Apparent K_m and V_{max} values were calculated by fitting the data to the Michaelis–Menten equation using the GraphPad Prism software for Macintosh.

Supplementary data

Supplementary data for this paper are available at *The EMBO Journal* Online.

Acknowledgements

We thank Leah Bernstein for technical assistance, Arpi Nazarian and Anita Grewal for expert help with mass spectrometric analysis, Jean-Marc Egly for MAT1 antibody, Andrew Koff, Karen Lee and Stewart Shuman for critical reading of the manuscript, Kathryn Anderson and Mary Baylies for access to *Drosophila* rearing facilities, and Sabino Guzman for preparing fly food. S.L. is especially grateful to Julia Saiz, Karen Lee, Bill Barton, Jack Liao, Christophe Rachez, Andrew Swan and Reiko Cyr for stimulating discussions and support during the course of this work. This work is supported by grants to R.P.F. from the NIH and the American Cancer Society, and to B.S. from the National Cancer Institute of Canada with funds from the Canadian Cancer Society. Also supported by NCI Cancer Center grant P30 CA08748. J.C. was the recipient of a studentship from the Fonds pour la formation de chercheurs et l'aide à la recherche (FCAR Quebec). S.L. is a research fellow of the National Cancer Institute of Canada, supported with funds provided by the Terry Fox run.

References

Adamczewski,J.P., Rossignol,M., Tassan,J.-P., Nigg,E.A., Moncollin,V. and Egly,J.-M. (1996) MAT1, cdk7 and cyclin H form a kinase complex which is UV light-sensitive upon association with TFIIH. *EMBO J.*, **15**, 1877–1884.

Akoulitchev,S. and Reinberg,D. (1998) The molecular mechanism of mitotic inhibition of TFIIH is mediated by phosphorylation of CDK7. *Genes Dev.*, **12**, 3541–3550.

Brown,A.J., Jones,T. and Shuttleworth,J. (1994) Expression and activity of p40^{MO15}, the catalytic subunit of cdk-activating kinase, during *Xenopus* oogenesis and embryogenesis. *Mol. Biol. Cell*, **5**, 921–932.

Cho,H., Kim,T.K., Mancebo,H., Lane,W.S., Flores,O. and Reinberg,D. (1999) A protein phosphatase functions to recycle RNA polymerase II. *Genes Dev.*, **13**, 1540–1552.

Cross,F.R. and Levine,K. (1998) Molecular evolution allows bypass of the requirement for activation loop phosphorylation of the Cdc28 cyclin-dependent kinase. *Mol. Cell. Biol.*, **18**, 2923–2931.

Dahmus,M.E. (1996) Reversible phosphorylation of the C-terminal domain of RNA polymerase II. *J. Biol. Chem.*, **271**, 19009–19012.

De Bondt,H.L., Rosenblatt,J., Jancarik,J., Jones,H.D., Morgan,D.O. and Kim,S.-H. (1993) Crystal structure of cyclin-dependent kinase 2. *Nature*, **363**, 595–602.

Desai,D., Wessling,H.C., Fisher,R.P. and Morgan,D.O. (1995) The effect of phosphorylation by CAK on cyclin binding by CDC2 and CDK2. *Mol. Cell. Biol.*, **15**, 345–350.

Devault,A., Martinez,A.-M., Fesquet,D., Labbé,J.-C., Morin,N., Cavadore,J.-C. and Dorée,M. (1995) MAT1 ('ménage à trois') a new RING finger protein subunit stabilizing cyclin H–cdk7 complexes in starfish and *Xenopus* CAK. *EMBO J.*, **14**, 5027–5036.

Drapkin,R., Le Roy,G., Cho,H., Akoulitchev,S. and Reinberg,D. (1996) Human cyclin-dependent kinase-activating kinase exists in three distinct complexes. *Proc. Natl Acad. Sci. USA*, **93**, 6488–6493.

Ducommun,B., Brambilla,P., Felix,M.-A., Franza,B.R., Karsenti,E. and Draetta,G. (1991) cdc2 phosphorylation is required for its interaction with cyclin. *EMBO J.*, **10**, 3311–3319.

Espinoza,F.H.E., Farrell,A., Nourse,J.L., Chamberlin,H.M., Gileadi,O. and Morgan,D.O. (1998) Cak1 is required for Kin28 phosphorylation and activation *in vivo*. *Mol. Cell. Biol.*, **18**, 6365–6373.

Fisher,R.P. (1997) Reconstitution of mammalian CDK-activating kinase. *Methods Enzymol.*, **283**, 256–270.

Fisher,R.P., Jin,P., Chamberlin,H.M. and Morgan,D.O. (1995) Alternative mechanisms of CAK assembly require an assembly factor or an activating kinase. *Cell*, **83**, 47–57.

Garrett,S., Barton,B.A., Knights,R., Jin,P., Morgan,D.O. and Fisher,R.P. (2001) Reciprocal activation by cyclin-dependent kinases 2 and 7 is directed by substrate specificity determinants outside the T-loop. *Mol. Cell. Biol.*, **21**, 88–99.

Gould,K.L., Moreno,S., Owen,D.J., Sazer,S. and Nurse,P. (1991) Phosphorylation at Thr167 is required for *Schizosaccharomyces pombe* p34^{cdc2} function. *EMBO J.*, **10**, 3297–3309.

Harper,J.W. and Elledge,S.J. (1998) The role of Cdk7 in CAK function, a retro-retrospective. *Genes Dev.*, **12**, 285–289.

Hermand,D., Pihlak,A., Westerling,T., Damagnez,V., Vandenhaute,J., Cottarel,G. and Mäkelä,T.P. (1998) Fission yeast Csk1 is a CAK-activating kinase (CAKAK). *EMBO J.*, **17**, 7230–7238.

Jeffrey,P.D., Russo,A.A., Polyak,K., Gibbs,E., Hurwitz,J., Massagué,J. and Pavletich,N.P. (1995) Mechanism of CDK activation revealed by the structure of a cyclin A–CDK2 complex. *Nature*, **376**, 313–320.

Kaldis,P. (1999) The cdk-activating kinase (CAK): from yeast to mammals. *Cell Mol. Life Sci.*, **55**, 284–296.

Kimmelman,J., Kaldis,P., Hengartner,C.J., Laff,G.M., Koh,S.S., Young, R.A. and Solomon,M.J. (1999) Activating phosphorylation of the kin28p subunit of yeast TFIIH by cak1p. *Mol. Cell. Biol.*, **19**, 4774–4787.

Kumagai,A. and Dunphy,W.G. (1995) Control of the Cdc2/cyclin B complex in *Xenopus* egg extracts arrested at a G_2/M checkpoint with DNA synthesis inhibitors. *Mol. Biol. Cell*, **6**, 199–213.

Labbé,J.-C. *et al.* (1994) p40^{MO15} associates with a p36 subunit and requires both nuclear translocation and Thr176 phosphorylation to generate cdk-activating kinase activity in *Xenopus* oocytes. *EMBO J.*, **13**, 5155–5164.

Larochelle,S., Pandur,J., Fisher,R.P., Salz,H.K. and Suter,B. (1998) Cdk7 is essential for mitosis and for *in vivo* Cdk-activating kinase activity. *Genes Dev.*, **12**, 370–381.

Leclerc,V., Raisin,S. and Léopold,P. (2000) Dominant-negative mutants reveal a role for the Cdk7 kinase at the mid-blastula transition in *Drosophila* embryos. *EMBO J.*, **19**, 1567–1575.

Lee,K.M., Saiz,J.E., Barton,W.A. and Fisher,R.P. (1999) Cdc2 activation in fission yeast depends on Mcs6 and Csk1, two partially redundant Cdk-activating kinases CAKs). *Curr. Biol.*, **9**, 441–444.

Lim,H.H., Loy,C.J., Zaman,S. and Surana,U. (1996) Dephosphorylation of threonine 169 of Cdc28 is not required for exit from mitosis but may be necessary for Start in *Saccharomyces cerevisiae*. *Mol. Cell. Biol.*, **16**, 4573–4583.

Long,J.J., Leresche,A., Kriwacki,R.W. and Gottesfeld,J.M. (1998) Repression of TFIIH transcriptional activity and TFIIH-associated cdk7 kinase activity at mitosis. *Mol. Cell. Biol.*, **18**, 1467–1476.

Martinez,A.-M., Afshar,M., Martin,F., Cavadore,J.-C., Labbé,J.-C. and Dorée,M. (1997) Dual phosphorylation of the T-loop in cdk7: its role in controlling cyclin H binding and CAK activity. *EMBO J.*, **16**, 343–354.

Molz,L. and Beach,D. (1993) Characterization of the fission yeast *mcs2* cyclin and its associated protein kinase activity. *EMBO J.*, **12**, 1723–1732.

Morgan,D.O. (1995) Principles of CDK regulation. *Nature*, **374**, 131–134.

Morgan,D.O. (1997) Cyclin-dependent kinases: engines, clocks and microprocessors. *Annu. Rev. Cell Dev. Biol.*, **13**, 261–291.

Poon,R.Y.C., Yamashita,K., Howell,M., Ershler,M.A., Belyavsky,A. and Hunt,T. (1994) Cell cycle regulation of the $p34^{cdc2}/p33^{cdk2}$-activating kinase $p40^{MO15}$. *J. Cell Sci.*, **107**, 2789–2799.

Reardon,J.T., Ge,H., Gibbs,E., Sancar,A., Hurwitz,J. and Pan,Z.-Q. (1996) Isolation and characterization of two human transcription factor IIH (TFIIH)-related complexes: ERCC2/CAK and TFIIH*. *Proc. Natl Acad. Sci. USA*, **93**, 6482–6487.

Reynaud,E., Lomelí,H., Vázquez,M. and Zurita,M. (1999) The *Drosophila melanogaster* homologue of the xeroderma pigmentosum D gene product is located in euchromatic regions and has a dynamic response to UV light-induced lesions in polytene chromosomes. *Mol. Biol. Cell*, **10**, 1191–1203.

Rossignol,M., Kolb-Cheynel,I. and Egly,J.-M. (1997) Substrate specificity of the cdk-activating kinase (CAK) is altered upon association with TFIIH. *EMBO J.*, **16**, 1628–1637.

Russo,A.A., Jeffrey,P.D. and Pavletich,N.P. (1996) Structural basis of cyclin-dependent kinase activation by phosphorylation. *Nature Struct. Biol.*, **3**, 696–700.

Tassan,J.-P., Schultz,S.J., Bartek,J. and Nigg,E.A. (1994) Cell cycle analysis of the activity, subcellular localization and subunit composition of human CAK (CDK-activating kinase). *J. Cell Biol.*, **127**, 467–478.

Tassan,J.-P., Jaquenod,M., Fry,A.M., Frutiger,S., Hughes,G. and Nigg,E.A. (1995) *In vitro* assembly of a functional human cdk7/cyclin H complex requires MAT1, a novel 36 kDa RING finger protein. *EMBO J.*, **14**, 5608–5617.

Yankulov,K.Y. and Bentley,D.L. (1997) Regulation of CDK7 substrate specificity by MAT1 and TFIIH. *EMBO J.*, **16**, 1638–1646.

Received December 1, 2000; revised April 30, 2001; accepted May 25, 2001