

# Regulatory Role of Transcription Factors TAF1 and TAF7 in Cyclin Gene Expression

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A dissertation

submitted in partial fulfillment of the  
requirements for the degree of

Doctor of Philosophy

University of Washington

2012

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Program Authorized to Offer Degree:

Molecular and Cellular Biology

## ABSTRACT

The largest transcription factor IID (TFIID) subunit, TBP associated factor 1 (TAF1) possesses protein kinase and histone acetyltransferase (HAT) activities. Both enzymatic activities are essential for transcription from a subset of genes and G1 progression in mammalian cells. TAF7, another TFIID subunit, binds TAF1 and inhibits TAF1 HAT activity. Here we present data demonstrating that disruption of the TAF1/TAF7 interaction within TFIID by protein phosphorylation leads to activation of TAF1 HAT activity and stimulation of cyclin D1 and cyclin A transcription. Overexpression and siRNA knockdown experiments confirmed that TAF7 functions as a transcriptional repressor at these promoters. Release of TAF7 from TFIID by TAF1 phosphorylation of TAF7 increased TAF1 HAT activity and elevated histone H3 acetylation levels at the cyclin D1 and cyclin A promoters. Serine-264 of TAF7 was identified as a substrate for TAF1 kinase activity. Using TAF7 S264A and S264D phospho-mutants, we determined that the phosphorylation state of TAF7 at S264 influences the levels of cyclin D1 and cyclin A transcription and promoter histone H3 acetylation. These studies have uncovered a novel function for the TFIID subunit TAF7 as a phosphorylation-dependent regulator of TAF1-catalyzed histone H3 acetylation at the cyclin D1 and cyclin A promoters.

## TABLE OF CONTENTS

LIST OF FIGURES .....	ii
INTRODUCTION .....	1
<b>CHAPTER 1:        PHOSPHORYLATION-DEPENDENT REGULATION OF                       TAF1 HAT ACTIVITY DURING THE CELL CYCLE.....</b>	<b>11</b>
INTRODUCTION.....	11
MATERIALS AND METHODS.....	12
RESULTS.....	17
CONCLUSIONS.....	24
<b>CHAPTER 2:        TAF1 PHOSPHORYLATION OF TAF7 SERINE-264 INCREASES                       HISTONE ACETYLATION AND TRANSCRIPTION LEVELS                       AT THE CYCLIN D1 PROMOTER.....</b>	<b>25</b>
INTRODUCTION.....	25
MATERIALS AND METHODS.....	26
RESULTS.....	29
CONCLUSIONS.....	34
<b>DISCUSSION.....</b>	<b>35</b>
<b>FIGURES.....</b>	<b>40</b>
<b>REFERENCES.....</b>	<b>66</b>

## LIST OF FIGURES

Figure Number	Page
1. Recognition of core promoter elements by TFIID and TFIIB	40
2. Pathways for preinitiation complex assembly	41
3. Structural organization of yeast TFIID	42
4. Schematic of TAF1	43
5. The stages of the cell cycle	44
6. TAF7 functions as a transcriptional repressor at a subset of promoters	45
7. TAF7 is a regulator of S-phase progression	46
8. Inverse correlation between TAF1 and TAF7 binding at the cyclin D1 and cyclin A promoters	47
9. TAF1 kinase activity opposes TAF7 binding at the cyclin D1 promoter	48
10. Schematic of TAF1 domains	49
11. Phosphorylated TAF7 shows decreased association with TAF1	50
12. TAF1 phosphorylates TAF7 within TFIID	51
13. TAF1 phosphorylation releases TAF7 from TFIID	52
14. Release of TAF7 from TFIID increases histone acetylation in vitro	53
15. TAF7 knockdown increases cyclin D1 and cyclin A promoter acetylation	54
16. TAF1 phosphorylates TAF7 in vitro	55
17. TAF1 kinase constructs	56
18. TAF1 NTK and CTK phosphorylate TAF7	57
19. Characterization of TAF1 NTK kinase activity	58
20. Mass spectrometry of TAF7 identifies S159 and S264 as phosphorylation sites	59

## **LIST OF FIGURES (cont.)**

21. S264A is a phosphosite for the TAF1 CTK	61
22. Phosphorylation of TAF7 at S264 disrupts TAF1 binding	62
23. TAF7 phosphorylated at S264 cannot incorporate into TFIID	63
24. TAF7 S264 phosphorylation stimulates cyclin D1 and cyclin A transcription	64
25. TAF7 S264A phosphorylation increases cyclin D1 and cyclin A promoter acetylation	65

## INTRODUCTION

### *Core promoter elements*

There are three eukaryotic RNA polymerases, RNA polymerases I, II, and III. Each RNA polymerase (RNA Pol) has unique biochemical properties and distinct nuclear localization. RNA Pol I is primarily involved in transcribing 18S and 28S ribosomal RNAs, while RNA Pol II transcribes mRNAs and RNA Pol III transcribes tRNAs and 5S rRNA. While these enzymes can transcribe a diverse set of DNA sequences, they require additional proteins to correctly specify the transcription start site. For RNA Pol II, a set of general transcription factors (GTFs) are required for transcription. These include transcription factors TFIIA, B, D, E, F and H (60).

Studies of eukaryotic promoters have thus far identified several core promoter elements, which are characteristic DNA sequences required for promoter function and for proper assembly and orientation of the transcription preinitiation complex (PIC) (Fig 1). The TATA box is an A/T-rich sequence located 25 to 30 nucleotides upstream of the transcription start site (TSS) in humans. It contains a consensus sequence that is recognized by the TATA-binding protein (TBP) subunit of the GTF TFIID complex (67). Another core promoter element, the initiator (Inr), surrounds the TSS. This element contains a pyrimidine-rich sequence that is capable of directing accurate transcription initiation alone or in conjunction with other core promoter elements. The TFIID subunits TAF1 and TAF2 are thought to mediate Inr recognition (7). The downstream promoter element (DPE) is located approximately 30 bp downstream of the TSS in many TATA-less promoters (5). This element is bound by TFIID subunits TAF6 and TAF9 (6).

There are two other downstream core promoter elements. The MTE (motif ten element) works in tandem with the Inr to enhance RNA Pol II mediated transcription (50). It can

functionally substitute for the TATA box and/or DPE as well as work synergistically with these elements to strengthen promoter activity (41). The presence of the downstream core element (DCE) seems to be mutually exclusive with the DPE as determined by analysis of database annotated human promoters (35). The TAF1 component of TFIID is able to contact the DCE, indicating that TFIID is also required for downstream promoter contacts (35).

The last two core promoter elements are recognized by the GTF TFIIB. The upstream TFIIB-recognition element (BRE<sup>u</sup>) is contacted by a helix-turn-helix (HTH) DNA-binding motif in TFIIB (33). This interaction helps to orient the directionality of the PIC. The downstream BRE (BRE<sup>d</sup>) is also bound by TFIIB through its recognition loop. These elements are found in both TATA-containing and TATA-less promoters, suggesting that TFIIB helps enhance TFIID binding to the core promoter through the addition of contact points (12).

The recruitment of PIC components to the core promoter remains an area of intense study and debate. There are currently two models of PIC assembly (Fig 2). In the sequential assembly model, the first step is TFIID recognition of the TATA box. This binding to the promoter is followed by TFIIA and B, which stabilize promoter-bound TFIID. Then Pol II and TFIIF are recruited to the complex, followed by TFIIIE and then TFIIH (3). An alternative model of PIC formation is known as the Pol II holoenzyme pathway. This model is based on the observations of several labs that showed Pol II could be purified in complex with several GTFs and chromatin remodeling complexes but did not co-purify with TFIID or TFIIA (53). Thus, the purification of a TFIID-deficient Pol II holoenzyme complex suggests that the DNA-bound TFIID may facilitate the entry of the Pol II holoenzyme to the promoter region. It is likely that both assembly pathways exist in vivo, as supporting evidence for both models has been reported in different regulatory systems (52).

### ***General transcription factor TFIID complex***

The TFIID complex was originally identified as a chromatographic fraction necessary to support site-specific transcription by Pol II in vitro (45). It was later determined that TFIID played a key role in binding to the TATA box (55). Eventually a single polypeptide possessing TATA box-binding activity was purified (4). However, recombinant TBP alone was not sufficient for transcription from TATA-containing promoters (57). Thus, it was proposed that additional proteins were required to work in conjunction with TBP to potentiate transcriptional activation. This hypothesis was supported by glycerol gradient sedimentation and immunoprecipitation analysis of partially purified TFIID, which indicated that TFIID was a multiprotein complex with a native size of ~750 kDa (16, 57).

Later studies determined that TFIID consists of TBP and 12-15 TBP-associated factors (TAFs) (72, 74). As discussed earlier, the TBP subunit contacts the TATA box, which allows TFIID to recognize TATA-containing promoters. TAF-Inr, TAF-DPE, and TAF-DCE interactions can also mediate TFIID binding to the core promoter. These additional TFIID-DNA contacts are particularly important at promoters lacking TATA sequences, as TAFs are required for transcription from TATA-less promoters (57). Characterization of TAFs revealed that many transcriptional activators interact directly with specific TAFs, including Sp1, YY1, USF, and adenovirus E1A (9, 16, 30, 31). These activator-TAF interactions imply that activators may function by recruiting TFIID to the promoter in order to nucleate PIC formation (75). Consistent with its role as a coactivator, only purified TFIID, but not TBP, supports activator-mediated transcription in partially purified cell-free transcription systems (16).

An initial analysis of the amino acid sequences of TAFs 6,9, and 12 revealed a striking similarity to the core H4, H3, and H2B histones, suggesting the existence of a histone octamer



structure within TFIID (18). Further studies identified histone fold domains within 9 of the TAFs, which create a set of 5 different heterodimers found within native TFIID complexes (62) (Fig 3). Recent EM studies have determined that both yeast and human TFIID consist of 3 lobes connected to form a clamp (23, 37, 38, 54). Immunolabeling of these EM structures determined that the core of the protein is composed of the histone fold TAF heterodimers (37). The C-terminus of TAF1 and TAF7 map together at the periphery of the complex, with the N-terminus of TAF1 found near TBP in the center of the clamp. The 3D architecture suggests that TFIID is composed of 2 subcomplexes. One complex contains TAF5 and the histone TAF heterodimers which form a stable core. The other complex consists of TAF1, TAF7, TBP and TAF2, which can be recruited to the core complex to form a complete TFIID (10).

Several TAF components are found in other complexes including TBP-free TAFII containing complex (TFTC) (77), Spt-Ada-Gcn5 acetyltransferase (SAGA) (22), and the polycomb repressive complex 1 (PRC1) (59). These TBP-lacking TAF-containing complexes are involved in diverse aspects of pol II-dependent transcription. Except for PRC1, which is primarily involved in gene silencing, the other TAF-containing complexes are implicated in activator-dependent transcription likely due to the histone acetyltransferase (HAT) activity inherent to each complex. However, much remains to be determined about the dynamics of promoter selection for each of these complexes and for TFIID.

### ***TAF1 enzymatic activity***

The TAF1 subunit of TFIID is an essential protein in yeast, flies, and hamster cell lines. The essential nature of TAF1 can be attributed to its broad requirement for RNA Pol II transcription. Knockout studies followed by microarray analysis in yeast and human cells

indicate that ~20% of genes are dependent on TAF1 for transcription (48, 63). Furthermore, inactivation of the yeast or hamster cell line TAF1 results in a G1 phase arrest (27, 63). Thus, it is not surprising that many of the genes identified as TAF1-dependent in the microarray studies are involved in growth control and cell cycle regulation (61, 76).

The TAF1 subunit of TFIID functions as a histone acetyltransferase (HAT), a protein kinase, and as a ubiquitin-activating and conjugating enzyme (Fig 4). The HAT domain of TAF1 maps to the central, most conserved portion of the protein. In vitro, *Drosophila* TAF1 acetylates free and nucleosomal histones (46). The preferred site of acetylation is histone H3 K14, however other lysines on histone H3 and H4 were acetylated. Human TAF1 acetylates TFIIE $\beta$  and TFIIF in vitro (32). Evidence for the importance of the TAF1 HAT domain comes from studies using the hamster ts13 mutant cell line. In this cell line, a temperature-sensitive missense mutation in the HAT domain of TAF1 causes a G1/S phase cell cycle arrest at the non-permissive temperature of 39.5°C (15, 27). The cell cycle defect could be rescued by transfection of full length TAF1, but not by transfection of TAF1 containing mutations in the HAT domain that disrupt HAT activity (15, 76). Thus, the TAF1 HAT domain is required for cell cycle progression.

TAF1 contains two independent serine/threonine kinase domains, one located in the N-terminus of the protein (NTK), and one located in the C-terminus (CTK) (14). Kinase activity has been demonstrated in vitro for yeast, *Drosophila*, and human proteins (14, 42, 68). Each domain is capable of autophosphorylation and transphosphorylation of substrates, including the TFIIF subunit RAP74, TFIIA, TAF7, p53, and histone H2B (14, 39, 42). TAF1 also possesses ubiquitin activating and conjugating activity (56). TAF1 monoubiquitination of histone H1 in vitro and in vivo promoted transcription of Dorsal genes in *Drosophila* embryos (56). TAF1

monoubiquitination also plays a role in muscle differentiation. In this system, the monoubiquitination of muscle differentiation inhibitor Pax3 by TAF1 promotes Pax3 degradation by the proteasome and leads to myogenic differentiation (2).

### ***Regulation of Histone Acetylation***

In eukaryotes, the packaging of DNA into chromatin interferes with DNA metabolic processes such as transcription, replication, and DNA repair. Chromatin structure and function can be affected by various post-translational modifications of the amino-terminal tails of nucleosomal histones, of which lysine acetylation is the best characterized. Acetylation is thought to increase DNA accessibility through the neutralization of the positive charge of lysine residues. This modification correlates largely with transcriptional activation, but is also involved in DNA replication, histone deposition, and DNA repair (70). Histone acetylation is catalyzed by histone acetyltransferases (HATs), whereas the reverse reaction is performed by histone deacetylases (HDACs). HATs and HDACs participate in the genome-wide turnover of acetyl groups on histones and some can modify other factors. These enzymes can be targeted to promoters through their sequence-specific interaction with transcription factors, thus regulating gene expression. Because of the importance of histone acetylation in chromatin function, HATs and HDACs have major roles in the control of cell fate and their misregulation is involved in the development of some human tumors (73). Consistent with the importance of HATs and HDACs, their function is tightly regulated in living cells and their activity can be modulated by signaling pathways.

Several modes of HAT regulation have been identified. As for all other proteins, an obvious way to regulate the activity of a HAT is to regulate the transcription or protein turnover

of that HAT. Transcription of the HAT Tip60, which is involved in DNA repair, is upregulated in response to DNA damage. After inactivation of the DNA damage pathway, the Tip60 protein is ubiquitinated by Mdm2 and targeted to the proteasome for degradation (36). Another way to regulate HAT activity is through post-translational modifications of the HAT or interactions with other proteins. The activity level of many HATs has been shown to be regulated through phosphorylation. For example, the HAT activity of CREB-binding protein (CBP) is stimulated upon phosphorylation by cyclin E/cyclin-dependent kinase 2 (1). The activity of CBP can also be stimulated through interaction with multiple sequence-specific transcription factors such as Sp1 and HNF1- $\alpha$  (8, 69).

### ***Cell Cycle***

Cell division consists of two consecutive processes, characterized by DNA replication followed by segregation of replicated chromosomes into two separate cells. Replication of DNA occurs during S phase, which is preceded by a gap called G1 during which the cell prepares for DNA synthesis. S phase is followed by another gap called G2 during which the cell prepares for mitosis, which is the process of nuclear division. Cells in G1 can enter a resting state called G0, which is the phase in which most differentiated cells in the body are found (Fig 5). All eukaryotes have evolved numerous mechanisms to minimize DNA damage. Cells monitor and maintain genomic integrity by means of a complex network of DNA repair pathways and cell cycle checkpoints, including ones at the G1/S phase transition and the G2/M phase transition (26). These cell cycle checkpoints are biochemical signaling pathways that sense DNA damage, resulting in activation of DNA repair and delay of cell cycle progression (78). During G1, cells require mitogenic signals to progress towards S-phase. Passage through the G1/S phase

checkpoint commits a cell to complete the cell cycle, as cells will continue through the cell cycle even if mitogenic stimulation is removed (64).

The transition from one cell cycle phase to another occurs in an orderly and directional fashion and is regulated by different proteins. One set of key regulatory proteins are the cyclin-dependent kinases (CDKs), which are a family of serine/threonine protein kinases that are activated at specific points of the cell cycle. When activated, CDKs induce downstream processes by phosphorylating their targets, the cyclin family of proteins (47). Different cyclins are required at different phases of the cell cycle. The three D type cyclins (cyclin D1, cyclin D2, cyclin D3) bind to CDK4 and CDK6 and are essential for G1 progression (65). Cyclin E associates with CDK2 to regulate progression from G1 into S phase (51). Cyclin A also interacts with CDK2 to promote S-phase progression (21). The expression of the cyclin family members rises and falls during the cell cycle and in this way they periodically activate their respective CDKs.

Because the G1/S checkpoint represents commitment to cell division and so many cancers have mutations in genes that control this checkpoint, understanding the transcriptional regulation of cyclins and CDKs may lead to new therapeutic targets for cancer. Cyclin D1, a growth factor sensor that integrates extracellular signals with the core cell cycle machinery, is frequently found overexpressed in human cancers and is often associated with a poor prognosis (24). Common mechanisms for cyclin D1 overexpression in cancer cells are gene amplification and gene rearrangements, causing abnormally elevated levels of transcript and protein (64). Such genomic aberrations are not a feature of all cancer cells that overexpress cyclin D1, suggesting the involvement of alternative transcriptional upregulation mechanisms.

## ***Eukaryotic Protein Kinases***

Protein kinases mediate most of the signal transduction in eukaryotic cells. By modification of substrate activity, protein kinases also control many other cellular processes, including metabolism, transcription, cell cycle progression, cytoskeletal rearrangement and cell movement, apoptosis, and differentiation. Mutations and dysregulation of protein kinases can cause human disease. Thus, agonists and antagonists of these enzymes could be used in disease therapies. Protein kinases are among the largest families of genes in eukaryotes and have been intensively studied. Most protein kinases belong to a single superfamily containing a eukaryotic protein kinase catalytic domain (43). However, about a dozen atypical protein kinase domains have been identified. These atypical protein kinase domains are found in proteins with reported biochemical activity, but which lack sequence similarity to the other eukaryotic protein kinases. Both the N-terminal kinase (NTK) and C-terminal kinase (CTK) of TAF1 are classified as atypical kinases.

In this dissertation, I present data demonstrating that the TAF7 subunit of TFIID is a phosphorylation-dependent transcriptional regulator, and show that altering the subunit composition of TFIID can have profound consequences on TFIID function and gene transcription. In Chapter 1, we demonstrate that TAF7 inhibits transcription from a subset of promoters dependent on TAF1 HAT activity. We identify TAF7 as a regulator of early S-phase progression and show differential recruitment of TAF1 and TAF7 at target promoters. We determine that release of TAF7 from the TFIID complex is required for TAF1 acetylation of histones in vitro and in vivo. This release of TAF7 from TFIID requires TAF1 phosphorylation of TAF7.

In Chapter 2, I characterize the mechanism for phosphorylation-dependent regulation of the TAF1 and TAF7 interaction and transcription of target genes. I identify the TAF1 site of phosphorylation on TAF7 at serine 264 and show that phosphorylation on serine 264 inhibits binding to TAF1 in vitro and promotes transcription and acetylation of histones at the promoters of TAF1-dependent target genes in vivo.

## **CHAPTER 1**

### **PHOSPHORYLATION-DEPENDENT REGULATION OF TAF1 HAT ACTIVITY DURING THE CELL CYCLE**

#### **INTRODUCTION**

In the ts13 mutant hamster cell line, a temperature-sensitive missense mutation in the HAT domain of TAF1 causes a G1/S phase cell cycle arrest at the non-permissive temperature of 39.5°C (15, 27). These cells exhibit transcriptional downregulation of cyclins A, D1, and E, but not c-fos or c-myc (61, 71, 76). Thus, TAF1 HAT inactivation does not induce a global defect in gene transcription but rather has an effect at only a subset of promoters. TAF1 also contains two independent serine/threonine kinase domains, one in the N-terminus (NTK) and one in the C-terminus (CTK) of the protein. Kinase activity has been demonstrated in vitro for human, yeast, and *Drosophila* TAF1 (14, 42, 44). Both domains are classified as atypical kinases and share little amino acid homology with each other; however the NTK and CTK domains both are capable of autophosphorylation and transphosphorylation of substrates such as the TFIID subunit TAF7 (20). Interestingly, transfection of TAF1 kinase mutants into ts13 cells failed to rescue the G1/S phase cell cycle arrest (49, 58). Thus, TAF1 HAT activity alone cannot promote G1/S phase progression, which suggests that the kinase activity of TAF1 also is required.

Recent work has suggested that TAF7, another TFIID subunit, may play a pivotal role in the regulation of TAF1 HAT activity. Gégonne et al identified TAF7 as a protein capable of directly binding to TAF1 and inhibiting TAF1 HAT activity in vitro (19). They also demonstrated that TAF1 can phosphorylate TAF7 in vitro (20). These observations led us to



propose a hypothesis in which TAF1 phosphorylation of TAF7 is necessary to release TAF7 from the TFIID complex. This would allow for activation of the TAF1 HAT domain and acetylation of promoter histones at target genes.

In this chapter, we present data in support of this hypothesis, in which TAF7, when associated with the TFIID complex and bound to TAF1, serves as a negative regulator of TAF1 HAT activity and cyclin D1 and cyclin A transcription. Overexpression of TAF7 in HeLa cells inhibited cyclin D1 and cyclin A transcription and caused the cells to accumulate in the early S phase of the cell cycle. By contrast, depletion of TAF7 from TFIID complexes by siRNA knockdown increased histone H3 acetylation at both cyclin promoters and stimulated cyclin D1 and cyclin A transcription.

## **MATERIALS AND METHODS**

### ***Tissue culture cell lines***

HeLa cells were grown in Dulbecco's modified Eagle medium (DMEM) (Gibco) supplemented with 10% fetal bovine serum (FBS) (HyClone), 2 mM L-glutamine, and penicillin/streptomycin in a humidified incubator containing 5% CO<sub>2</sub> at 37°C. Sf9 insect cells were propagated in Hink's TNM-FH insect medium containing 10% FBS, L-glutamine, and penicillin/streptomycin. Cultures were grown at 27°C in the absence of CO<sub>2</sub>.

### ***siRNA knockdown***

HeLa cells were seeded at  $3.3 \times 10^4$ /well in a 24-well plate and maintained for 16 hours in DMEM + 10% FBS without antibiotics. Cells were transfected with 50 nM siGENOME TAF7 siRNA (ThermoScientific) or non-targeting siRNA (Dharmacon) and 1 µl of

DharmaFECT 3 reagent (Dharmacon). After 72 hrs of incubation at 37°C in antibiotic-free DMEM + 10% FBS, cells were harvested for subsequent analysis.

### ***RNA isolation and qRT-PCR analysis***

RNA was harvested from cells using TRIZOL reagent according to the manufacturer's protocol (Gibco BRL). cDNA was synthesized from 1 µg of RNA using the iScript cDNA synthesis kit (Bio-Rad). One microliter of cDNA was amplified by quantitative PCR on a MX3000 Platform (Stratagene) using SsoFast EvaGreen Supermix (Bio-Rad) with primers for TAF7, CCND1, CCNE1, CCNA1, C-FOS, GAPDH, and ARBP.

### ***Transient Transfections***

HeLa cells were seeded at  $1 \times 10^5$  cells/well in a 24 well plate. Cells were transfected with 0.5 µg TAF7 expression plasmid using FuGENE HD transfection reagent (Roche) according to the manufacturer's protocol. Between 48-72 hours post-transfection, cells were harvested and subjected to RNA expression, protein expression or cell cycle analysis.

### ***HeLa nuclear extracts and immunoprecipitation of TFIID***

For preparation of nuclear extracts, HeLa cells were resuspended in Buffer A (10 mM HEPES, pH 7.9, 1.5 mM MgCl<sub>2</sub>, 10 mM KCl, 0.5 mM DTT) and incubated on ice for 15 min. Cells were lysed by pushing through a 25-gauge needle 5 times. The crude nuclear pellet was isolated by centrifugation for 20 sec at 12,000xg, resuspended in Buffer C (20 mM HEPES, pH 7.9, 25% (v/v) glycerol, 0.42 M NaCl, 1.5 mM MgCl<sub>2</sub>, 0.2 mM EDTA, 0.5 mM PMSF, 0.5 mM DTT), and incubated for 30 min at 4°C. After centrifugation for 5 min at 12,000xg, the

supernatant/nuclear extract was incubated with 0.5 µl anti-TBP polyclonal antibody (gift from Dr. R. Tjian) overnight at 4°C. Ten microliters of Protein A Sepharose CL-4B (GE Healthcare) was added, and the samples were nutated for 2 hrs at 4°C. The precipitated proteins were washed 5x with Buffer C and analyzed by western blot.

### ***Cell cycle analysis***

HeLa cells were resuspended at  $3 \times 10^6$  cells/ml and cold 70% ethanol was added dropwise while vortexing. Cells were fixed for 1 hr at 4°C, washed 2x with phosphate buffered saline (PBS), resuspended in PI staining buffer (1x PBS, 2% FBS, 50 µg/ml propidium iodide, 200 µg/ml RNase A, 0.1% Igepal) and incubated for 3 hr at 4°C. Data was collected on a FACScan machine using CellQuest Pro software (Becton, Dickinson and Company) and analyzed using FlowJo software (Tree Star, Inc).

### ***Cell synchronization***

HeLa cells were synchronized using a thymidine/nocodazole block protocol. Cells were incubated for 24 hrs in DMEM + 10% FBS containing 2 mM thymidine (USB). Thymidine was removed by washing once with PBS, and the cells were released from the block by incubation in DMEM + 10% FBS for 3 hours. Nocodazole was added to the media at 100 ng/ml for 12 hours to arrest the cells at G2/M. Cells were released into the cell cycle by washing with PBS and addition of fresh DMEM + 10% FBS. Cells were collected by centrifugation at different time points for subsequent analysis.

### **Chromatin immunoprecipitations**

Synchronized HeLa cells, seeded on 10-cm plates, were cross-linked by the addition of 400  $\mu$ l of 37% formaldehyde to 10 ml medium. After 15 min, cells were washed twice with PBS and lysed in 0.5 ml immunoprecipitation (IP) buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 5 mM EDTA, 1% Triton X-100, and 0.5% Nonidet P-40) containing protease inhibitors. The lysed cells were centrifuged and the nuclear pellet was collected and resuspended in 1.0 ml IP buffer and sonicated for 4 x 10 sec (Branson Sonifier 450, output 2, duty cycle 60). Half of the nuclear extract was incubated overnight at 4°C with either 1  $\mu$ l of anti-TAF1 (gift from Dr. X. Liu), anti-TBP (gift from Dr. R. Tjian), anti-histone H3 K9K14Ac (Upstate), anti-histone H3 K9Ac (Upstate) or anti-TAF7 (gift from Dr. I. Davidson) antibody. The other half of the nuclear extract was incubated with mouse IgG (Abcam). Ten microliters Protein A Sepharose CL-4B (GE Healthcare) was added, and the samples were nutated for 2 hr at 4°C. Immunoprecipitated complexes were washed five times with IP buffer and bound DNA/protein complexes were eluted with 2 x 250  $\mu$ l elution buffer (1% SDS, 0.1 M NaHCO<sub>3</sub>). Cross-links were reversed by the addition of 20  $\mu$ l 5 M NaCl and incubation overnight at 65°C. Samples were ethanol precipitated with glycogen as carrier and pellets were resuspended in 100  $\mu$ l proteinase K buffer (10 mM Tris-HCl, pH 7.8, 5 mM EDTA, 0.5% SDS) and digested with 200  $\mu$ g/ml proteinase K for 30 min at 50°C. DNA was purified by two phenol-chloroform extractions and one chloroform-isoamyl alcohol extraction, ethanol precipitated, and resuspended in 25  $\mu$ l TE (10 mM Tris-HCl, pH 8.0, 1 mM EDTA, pH 8.0). Twenty-five ng of purified ChIP DNA was amplified with SsoFast EvaGreen Supermix (Bio-Rad) using primers spanning the promoters of cyclin D1, cyclin A, cyclin E, c-fos, and GAPDH. qPCR reactions were performed on the MX3000 platform (Stratagene).

### **In vitro kinase assays**

TFIID complexes immunoprecipitated from synchronized HeLa cell nuclear extracts were immobilized on anti-TBP antibody bound to Protein A Sepharose. The immobilized proteins were incubated for 30 min at 30°C in 25 µl of phosphorylation buffer (25 mM HEPES, pH 7.9, 12.5 mM MgCl<sub>2</sub>, 100 mM KCl, 0.1 mM EDTA) with 1 µCi [ $\gamma$ -<sup>32</sup>P] ATP and 200 ng His-TAF7ΔN (aa 103-349) as the exogenous kinase substrate. Reaction products were subjected to SDS-PAGE, transferred to nitrocellulose, and the membrane exposed to film to detect phosphorylated His-TAF7ΔN. The same membrane was probed overnight at 4°C with anti-TAF1 antibody (gift from Dr. X. Liu), diluted 1:10,000 in PBST (PBS + 0.1% Tween-20), or anti-His antibody (Bethyl), diluted 1:5000 in PBST, to determine the total amount of total TAF1 and total His-TAF7ΔN in each reaction. The appropriate HRP-conjugated secondary antibody was used at 1:5000-1:10,000 dilution. For kinase reactions that did not involve the addition of recombinant His-TAF7ΔN as substrate, TFIID was immunoprecipitated from HeLa nuclear extracts that were fractionated on a phosphocellulose column as described (13). The 1.0 M KCl elution from the phosphocellulose column (P1.0) was incubated with anti-TBP antibody, Protein A Sepharose, and washed as described above. The immobilized proteins, equilibrated with the appropriate reaction buffer, were used in in vitro kinase assays. The identity of the phosphorylated TFIID subunits was determined by immunoblotting with different TAF antibodies. Anti-TAF7 antibody (a gift from Dr I. Davidson) was used at 1:5000 dilution and the anti-TAF5 antibody (mAb 6B1) at 1:2500 dilution.

### **Expression and purification of recombinant TAF1 and TAF7 proteins**

GST-tagged TAF1-CTK and GST-tagged TAF1-C.RAP were expressed and purified

from 15-cm plates of Sf9 cells infected with 1 ml of recombinant baculovirus as previously described (15). Proteins were affinity purified using glutathione-sepharose beads (GE Healthcare Biosciences). For expression of His-tagged TAF7 proteins, cDNA of TAF7 was PCR amplified and subcloned into the Bam HI and Sal I sites of pET28a. TAF7 expression constructs were transformed into BL21\* cells for protein expression. Starter cultures of 2 ml were diluted into 100 mL LB + 30 µg/ml kanamycin and cells were grown to an OD of 1.0 at 600 nm. TAF7 expression was induced by addition of 0.1 mM IPTG. After 16 hours, cells were harvested and lysed in 0.4 M HEMG buffer (400 mM KCl, 25 mM HEPES pH 7.9, 12.5 mM MgCl<sub>2</sub>, 0.5 mM EDTA, 0.1% NP-40, 10% glycerol). His-TAF7 was purified using Ni-NTA agarose (Qiagen) and competitively eluted from the beads with 200 mM imidazole.

### **In vitro histone acetyltransferase assay**

TFIID immunoprecipitated from P1.0 phosphocellulose fraction of HeLa nuclear extracts and immobilized on Protein A Sepharose was washed 2x with assay buffer (50 mM Tris-HCl, pH 8.0, 10% glycerol, 0.1 mM EDTA, 1 mM DTT, 0.2 mM PMSF), and resuspended in 30 µl assay buffer containing 300 ng histone H3 peptide (aa 1-20) and 0.25 µCi [<sup>3</sup>H]acetyl-coenzyme A (Amersham). Reactions were incubated for 60 min at 30°C, filtered through P81 paper, and washed with 50 mM sodium carbonate, pH 9.2. The amount of acetylated H3 peptide retained on the filter was measured by liquid scintillation counting.

## **RESULTS**

### ***TAF7 inhibits transcription at a subset of cell cycle genes***

The histone acetyltransferase (HAT) activity of TAF1, the largest subunit of TFIID, is required for transcription of cyclins D1, E and A as well as G1 to S phase progression (15, 27). It has been reported that TAF7, another TFIID subunit, binds to TAF1 and inhibits the HAT activity of TAF1 (19). These findings have led us to investigate whether TAF7 functions as an inhibitor of cyclin gene transcription. We performed siRNA knockdown experiments of TAF7 in HeLa cells and examined the effect of decreasing TAF7 protein levels on the transcriptional activity of different TAF1-dependent and independent genes by quantitative RT-PCR. After 72 hours of siRNA treatment, TAF7 mRNA (Fig. 6A) and total protein levels (data not shown) were reduced by 80-90% compared to cells treated with the non-targeting control siRNA. More importantly, the amount of TAF7 found incorporated into endogenous TFIID complexes also was reduced by ~50% compared to control treated cells (Fig 6B). We observed that loss of TAF7 resulted in a 40% increase in cyclin D1 and cyclin A transcript levels, but unexpectedly had no significant effect on the expression level of cyclin E, another TAF1-dependent cell cycle gene (Fig 6A). Knockdown of TAF7 also had no effect on c-fos and GAPDH expression levels (Fig 6A). These data suggest that TAF7 serves as an inhibitor of gene transcription, but only at a subset of RNA polymerase II promoters.

To gather additional evidence that TAF7 functions as a transcriptional repressor, we overexpressed TAF7 in HeLa cells with the expectation that increasing TAF7 protein levels will lead to a reduction in cyclin D1 gene transcription. Immunoprecipitation followed by western blotting revealed that the exogenously expressed TAP-tagged TAF7 was incorporated into endogenous TFIID complexes (Fig 6D). Consistent with our knockdown data, overexpression of TAF7 decreased cyclin D1 and A transcript levels by 50-75% but had no significant effect on cyclin E, c-fos, and GAPDH transcript levels (Fig 6C). These complementary loss and gain of

function studies strongly indicate that TAF7 is an inhibitor of gene transcription and that its repressor function displays promoter selectivity.

### ***TAF7 is a cell cycle regulator***

Cyclin D1 is required for progression through G1 into S phase (61). Our data suggest that TAF7 is a negative regulator of cyclin D1 expression, which prompted us to ask if altering TAF7 protein levels would change the dynamics of cell cycle progression. TAF7, as a YFP fusion protein, was overexpressed in HeLa cells, and the DNA content and cell cycle profile of YFP-positive and YFP-negative cells was determined using propidium iodide staining and flow cytometry. After 72 hours, we observed a significant increase in the percentage of YFP-positive, TAF7 overexpressing cells accumulating in early S-phase when compared to YFP-negative cells (Fig 7 A and B). This accumulation of cells in early S-phase coincided with a decrease in the number of YFP-positive cells in the subsequent G2 phase of the cell cycle (Fig 7C). Therefore, TAF7 can serve as a novel and unexpected regulator of progression through early S-phase.

### ***TAF1 opposes TAF7 binding at the cyclin D1 and cyclin A promoters***

To elucidate the mechanism of TAF7's inhibitory function in gene transcription, the technique of chromatin immunoprecipitation (ChIP) was used to examine the localization of TAF1, TAF7, and TBP at the cyclin D1 and cyclin A promoter as cells progressed through the different stages of the cell cycle. HeLa cells were synchronized at early M phase using a thymidine/nocodazole block. At 4-hour intervals after removal of drug, DNA content was analyzed by propidium iodide staining and flow cytometry. We observed that under these conditions HeLa cells entered the G1 phase approximately 4 hours after drug removal.



Approximately 70% of the cells were in G1 at the 8 hour time point and progressed into S phase at 12 hours (Fig 8A). ChIP experiments with anti-TAF1 and anti-TBP antibodies revealed peaks of TAF1 and TBP recruitment to the cyclin D1 promoter during G1, when cyclin D1 is highly expressed in proliferating cells (Fig 8B, left panel). Peaks of TAF1 and TBP binding to the cyclin A promoter were observed at the G1/S phase boundary, consistent with the later time course of cyclin A induction during the cell cycle (Fig 8B, right panel). ChIP analysis of TAF7 promoter occupancy was performed using an anti-TAF7 antibody (19TA) kindly provided by Dr. I. Davidson. The Davidson group showed that 19TA was able to immunoprecipitate both free and TFIID bound TAF7 from cell lysates (34). We observed that at both the cyclin D1 and cyclin A promoters, high levels of TAF1 binding were associated with low levels of TAF7 (Fig 8C). The decrease in TAF7 binding could not be explained by the release of TFIID, as TBP remained bound to the cyclin D1 and cyclin A promoters under conditions of low TAF7 promoter occupancy. The inverse correlation between TAF1 and TAF7 binding suggests that these two TFIID subunits have opposing functions in the regulation of cyclin D1 and cyclin A transcription.

#### ***TAF1 kinase activity opposes TAF7 binding at the cyclin D1 promoter***

Protein phosphorylation by TAF1 kinase has been shown to disrupt TAF7 binding to TAF1 in vitro (20). We asked if the near-absence of TAF7 at the cyclin D1 promoter under conditions of high TAF1 occupancy could be mediated by TAF1 kinase activity. To address this question, TFIID was immunoprecipitated from HeLa nuclear extracts prepared from cells at different stages of the cell cycle. TAF1 kinase activity in the precipitated proteins was monitored by using purified recombinant TAF7 as exogenous substrate. We found that phosphorylation of

TAF7 was stimulated in early G1, approximately 4 hours after release from the nocodazole block, and remained elevated for approximately 4-6 hours (Fig 9A). When compared to the TAF7 occupancy profile shown in Fig 8C, high levels of TAF1 kinase activity coincided with low levels of TAF7 binding to the cyclin D1 promoter and vice versa (Fig 9B). These results are in agreement with our hypothesis that TAF1 phosphorylation of TAF7 regulates the association of TAF7 with TAF1 and thus with the TFIID complex.

### ***TAF1 phosphorylation of TAF7 disrupts their protein-protein interaction***

TAF1 possesses two independent serine/threonine kinase domains. Each of these domains is capable of autophosphorylation and transphosphorylation of substrates (14). It is unknown whether TAF1 autophosphorylation, transphosphorylation of TAF7, or both functions are necessary for the dissociation of TAF7 from TAF1 within the TFIID complex. Therefore, we examined the consequences of trans-phosphorylating TAF7 on TAF1 protein binding. Bacterially expressed and purified TAF7, with an N-terminal histidine-tag, (Fig 11A, His-TAF7) was pre-incubated under kinase conditions in the absence or presence of purified GST-tagged TAF1 C-terminal kinase domain (Fig 11A, GST-CTK). The GST-CTK fragment of TAF1 (Fig 10, CTK) lacks the interaction domain for TAF7 (RAPiD) but retains the ability to transphosphorylate TAF7. The mock- and pre-phosphorylated His-TAF7 were affinity purified away from the GST-CTK fragment using Ni-agarose beads. The isolated TAF7 proteins were subsequently incubated with a purified C-terminal TAF1 fragment that includes the TAF7 interaction domain, CTK domain and an N-terminal GST-tag (Fig 11A, GST-C.RAP and Fig 10, C.RAP) in the absence of any ATP. The amount of TAF7 that co-immunoprecipitated with the GST-C.RAP fragment was determined by immunoblotting. We observed that pre-

phosphorylated TAF7 exhibited a 50% reduction in C.RAP TAF1 binding when compared to the mock-phosphorylated protein (Fig 11B and C). These results demonstrate that trans-phosphorylation of TAF7 significantly lowers its binding affinity for the TAF1 protein.

### ***Phosphorylation and release of TAF7 activates the TAF1 HAT domain***

We have found that trans-phosphorylation of TAF7 can disrupt the interaction of TAF7 with TAF1. Thus, we wondered if TAF1 phosphorylation of TAF7 would be sufficient to stimulate release of TAF7 from the TFIID complex and lead to activation of TAF1 HAT activity. Intact TFIID complexes were immunoprecipitated from a partially fractionated HeLa cell nuclear extract enriched for TFIID, using an anti-TBP polyclonal antisera (72). Immunoprecipitated proteins were incubated with radiolabeled ATP under kinase reaction conditions to allow for protein phosphorylation. Reaction products were analyzed by autoradiography and immunoblotting (Fig 12, Kinase and IB). As previously shown, TAF1 was autophosphorylated in the context of TFIID; we also detected trans-phosphorylation of TAF7. In parallel reactions, the TFIID immobilized on sepharose beads was pre-incubated under kinase conditions in the absence or presence of 1 mM cold ATP to allow for protein phosphorylation, and the dissociation of TAF1, TBP and TAF7 from TFIID was subsequently examined. The amount of each TFIID subunit found in either the unbound supernatant (S) or associated with TFIID complexes (B) was determined by immunoblotting and quantified using ImageJ software (Fig 13A and B). We observed that incubation with ATP increased the amount of TAF7 found in the unbound supernatant by ~50%, representing TAF7 subunits no longer associated with the TFIID complex. This increase was accompanied by a corresponding decrease in the amount of TAF7 bound to TFIID (Fig 13B). By contrast, the amount of TAF1 and TBP detected in the unbound

supernatant remained unaffected by ATP. These findings further suggest that the association of TAF7 with the TFIID complex is regulated by protein phosphorylation.

The only component of TFIID shown to possess histone acetyltransferase activity is the TAF1 subunit, and histone H3 is a substrate for TAF1 HAT activity (46). We used these two pieces of information to investigate the role of TAF7 phosphorylation in regulating TAF1 HAT activity. TFIID, immunoprecipitated from phosphocellulose fractionated HeLa nuclear extracts, was preincubated with ATP to promote TAF7 phosphorylation and release from the TFIID complex. The level of histone acetyltransferase activity was subsequently measured using a histone H3 N-terminal peptide (aa 1-20) as substrate in an in vitro assay (Fig 14). The expectation is that release of TAF7, an inhibitor of TAF1 HAT activity, would lead to an increase in acetylation of the H3 peptide. We found that preincubation of TFIID with ATP (pre-phos TFIID) increased the level of H3 acetylation compared to preincubation in the absence of ATP (TFIID). Our protein binding and acetyltransferase assays suggest that dissociation of TAF7 from the TFIID complex via a phosphorylation-dependent mechanism stimulates TAF1 HAT activity.

To gather evidence that TAF7 functions as a regulator of TAF1 HAT activity in vivo, we used TAF7 siRNA knockdown to decrease the amount of TAF7 within TFIID complexes, and carried out ChIP experiments to examine the level of H3 acetylation at a number of different promoters. We previously reported that acetylation of histone H3-K9K14 by the TAF1 HAT domain is necessary for efficient cyclin D1 promoter function (15, 29). Treatment with TAF7 siRNA lead to an increase in H3-K9K14 acetylation levels at both the cyclin D1 and cyclin A promoters (Fig 15). Changes in H3 acetylation levels were not observed at the cyclin E, c-fos and GAPDH promoters (Fig 15), consistent with our earlier finding that the transcriptional

activity of these promoters is not subject to TAF7 repression (Fig 6C). These data suggest that TAF7 is a biologically significant negative regulator of TAF1 HAT activity in vivo.

## **CONCLUSIONS**

These studies have identified the TFIID subunit TAF7 as a novel regulator of cyclin D1 and cyclin A transcription. TAF7 inhibits transcription at the cyclin D1 and cyclin A promoters but has no effect on transcription of cyclin E, GAPDH, or c-fos. TAF7 also serves as a regulator of progression through the early S-phase of the cell cycle. We show that phosphorylation-dependent release of TAF7 from the TFIID complex is necessary for TAF1 acetylation of histones in vitro and in vivo. The phosphorylation and dissociation of TAF7 from TFIID complexes at the cyclin D1 and cyclin A promoters is most prevalent during the G1 phase of the cell cycle and potentially represents a novel downstream event of mitogenic signaling pathways that induce cyclin D1 and cyclin A transcription.

## **CHAPTER 2**

### **TAF1 PHOSPHORYLATION OF TAF7 SERINE-264 INCREASES HISTONE ACETYLATION AND TRANSCRIPTION LEVELS AT THE CYCLIN D1 PROMOTER**

#### **INTRODUCTION**

In Chapter 1, I presented data in support of a model that TAF7, when associated with the TFIID complex and bound to TAF1, serves as a negative regulator of TAF1 HAT activity and cyclin D1 and cyclin A transcription. Overexpression of TAF7 in HeLa cells inhibited cyclin D1 and cyclin A transcription and led to cells accumulating in the early S phase of the cell cycle. By contrast, depletion of TAF7 from TFIID complexes by siRNA knockdown increased histone H3 acetylation at both cyclin promoters and stimulated cyclin D1 and cyclin A transcription.

In Chapter 2, I expand on this model by identifying the mechanism for phosphorylation-dependent regulation of TAF1 and TAF7 at target genes. We show that TAF1 can phosphorylate TAF7 in vivo and in vitro. We identify the TAF1 site of phosphorylation on TAF7 at serine 264. We show that phosphorylation on TAF7 at serine 264 inhibits binding to TAF1 in vitro and promotes transcription of TAF1-dependent target genes in vivo. Expression of a TAF7 mutant, S264A, which is refractory to TAF1 phosphorylation, was even more effective at reducing H3 acetylation and transcription at target promoters than comparable levels of WT-TAF7.

#### **METHODS**

##### ***Tissue culture cell lines***

HeLa cells were grown in Dulbecco's modified Eagle medium (DMEM) (Gibco) supplemented with 10% fetal bovine serum (FBS) (HyClone), 2 mM L-glutamine, and penicillin/streptomycin in a humidified incubator containing 5% CO<sub>2</sub> at 37°C. Sf9 insect cells were propagated in Hink's TNM-FH insect medium containing 10% FBS, L-glutamine, and penicillin/streptomycin. Cultures were grown at 27°C in the absence of CO<sub>2</sub>.

### ***Expression and purification of recombinant TAF1 and TAF7 proteins***

Full-length and kinase construct HA-tagged TAF1s were expressed and purified from 15-cm plates of Sf9 cells infected with 1 ml of recombinant baculovirus as previously described (15). Proteins were affinity purified using an anti-HA antibody column. His-TAF7 S264A and S264D mutants were created using the QuikChange Site-Directed Mutagenesis Kit (Stratagene). TAF7 expression constructs were transformed into BL21\* cells for protein expression. Starter cultures of 2 ml were diluted into 100 mL LB + 30 µg/ml kanamycin and cells were grown to an OD of 1.0 at 600 nm. TAF7 expression was induced by addition of 0.1 mM IPTG. After 16 hours, cells were harvested and lysed in 0.4 M HEMG buffer (400 mM KCl, 25 mM HEPES pH 7.9, 12.5 mM MgCl<sub>2</sub>, 0.5 mM EDTA, 0.1% NP-40, 10% glycerol). His-TAF7 was purified using Ni-NTA agarose (Qiagen) and competitively eluted from the beads with 200 mM imidazole.

### ***In vitro kinase assays***

200ng of recombinantly expressed and purified HA-TAF1 and 200ng of MBP-TAF7 or His-TAF7 WT, S159A, or S264A were incubated for 30 min at 30°C in 25 µl of phosphorylation buffer (25 mM HEPES, pH 7.9, 12.5 mM MgCl<sub>2</sub>, 100 mM KCl, 0.1 mM EDTA) with 1 µCi [γ-

<sup>32</sup>P] ATP. Reaction products were subjected to SDS-PAGE, transferred to nitrocellulose, and the membrane exposed to film to detect phosphorylated TAF1 and TAF7.

### ***Mass spectrometry***

Excised coomassie-stained gel bands containing TAF7 were subjected to in-gel proteolytic digestion with trypsin as described (66). Following gel slice digestion, the digestion products were desalted using C18-micro ZipTips (Millipore) per the manufacturer's instructions and dried by vacuum centrifugation. The resulting peptide sample was resuspended in 14 uL of 0.1% formic acid and 5 uL was analyzed by LC/ESI MS with a 2D Nano-HPLC (Eksigent) coupled to a LTQ-OrbitrapXL (Thermo Scientific) mass spectrometer using an LC MS ion source configuration as described (40). The protein database search engine MASCOT (Matrix Science) version 1.01 was used to search a yeast protein database appended with the TAF7 sequence. Search parameters included variable phosphorylation modification on serine and threonine, and oxidation modification on methionine. Phosphopeptides identified with high confidence were manually validated by checking the mass deviation of the phosphopeptide identification (less than 5 ppm) and checking the thoroughness and quality of the fragmentation spectra.

### ***Transient Transfections***

HeLa cells were seeded at  $1 \times 10^5$  cells/well in a 24 well plate. Cells were transfected with 0.5 µg TAF7 expression plasmid using FuGENE HD transfection reagent (Roche) according to the manufacturer's protocol. Between 48-72 hours post-transfection, cells were harvested and subjected to RNA or protein expression analysis.



### ***HeLa nuclear extracts and immunoprecipitation of TFIID***

For preparation of nuclear extracts, HeLa cells were resuspended in Buffer A (10 mM HEPES, pH 7.9, 1.5 mM MgCl<sub>2</sub>, 10 mM KCl, 0.5 mM DTT) and incubated on ice for 15 min. Cells were lysed by pushing through a 25-gauge needle 5 times. The crude nuclear pellet was isolated by centrifugation for 20 sec at 12,000xg, resuspended in Buffer C (20 mM HEPES, pH 7.9, 25% (v/v) glycerol, 0.42 M NaCl, 1.5 mM MgCl<sub>2</sub>, 0.2 mM EDTA, 0.5 mM PMSF, 0.5 mM DTT), and incubated for 30 min at 4°C. After centrifugation for 5 min at 12,000xg, the supernatant/nuclear extract was incubated with 0.5 µl anti-TBP polyclonal antibody (gift from Dr. R. Tjian) overnight at 4°C. Ten microliters of Protein A Sepharose CL-4B (GE Healthcare) was added, and the samples were nutated for 2 hrs at 4°C. The precipitated proteins were washed 5x with Buffer C and analyzed by western blot.

### ***RNA isolation and qRT-PCR analysis***

RNA was harvested from cells using TRIZOL reagent according to the manufacturer's protocol (Gibco BRL). cDNA was synthesized from 1 µg of RNA using the iScript cDNA synthesis kit (Bio-Rad). One microliter of cDNA was amplified by quantitative PCR on a MX3000 Platform (Stratagene) using SsoFast EvaGreen Supermix (Bio-Rad) with primers for CCND1, CCNA1, C-FOS, and ARBP.

### ***Chromatin immunoprecipitations***

Chromatin immunoprecipitations were performed as in Chapter 1. Half of the nuclear extract was incubated overnight at 4°C with either 1µl of anti-histone H3 K9K14Ac (Upstate) or

anti-histone H3 K9Ac (Upstate). The other half of the nuclear extract was incubated with mouse IgG (Abcam). Twenty-five ng of purified ChIP DNA was amplified with SsoFast EvaGreen Supermix (Bio-Rad) using primers spanning the promoters of cyclin D1, cyclin A, or c-fos. qPCR reactions were performed on the MX3000 platform (Stratagene).

## RESULTS

### *TAF1 kinase domains phosphorylate TAF7 in vitro*

First, I began by confirming that I could observe TAF1 phosphorylation of TAF7 in vitro as had been previously shown (20). Recombinantly expressed and purified HA-TAF1 was used in an in vitro kinase assay with MBP-TAF7 as a substrate. As previously reported, TAF1 was capable of autophosphorylation in vitro (14, 20) (Fig 16). I also observed that increasing the amount of TAF1 in the reaction led to an increase in TAF1 transphosphorylation of TAF7 (Fig 16). Next, kinase assays were performed in the presence of 20uM apigenin. Previous reports have indicated that apigenin inhibits TAF1 kinase activity (39). TAF1 phosphorylation of TAF7 was inhibited by the addition of apigenin to the kinase assay reaction (Fig 16). This indicated that the observed phosphorylation events are due to TAF1 kinase activity and not a co-purifying protein.

As discussed earlier, TAF1 contains two independent serine/threonine kinase domains, one in the N-terminus of the protein and one in the C-terminus. To determine which TAF1 kinase domain was capable of phosphorylating TAF7, we created a series of HA-tagged TAF1 kinase constructs (Fig 17). These include the minimal NTK and CTK domains (NTK and CTK) and two longer constructs which include a kinase domain and the RAPiD domain, the TAF7 binding site on TAF1 (N.RAP and C.RAP). These constructs were expressed and purified using

the baculovirus expression system in Sf9 cells. Purified proteins were used in an in vitro kinase assay with radiolabeled ATP and full-length TAF7 as substrate. Both autophosphorylation and transphosphorylation of TAF7 were examined. Each kinase construct was capable of autophosphorylation, as has been previously reported (14) (Fig 18), similar to the full length protein (Fig 17). Furthermore, both kinase domains of TAF1 could trans-phosphorylate TAF7 (Fig 18). We also observed that TAF1 binding to TAF7 is not required for phosphorylation as TAF7 was phosphorylated in the absence of the RAPID domain (NTK and CTK). Next, we performed these kinase assays in the presence of 20uM apigenin. After treatment with apigenin, we observed almost non-existent levels of TAF1 autophosphorylation and transphosphorylation of TAF7 (Fig 18). These results further indicated that the observed phosphorylation events are due to TAF1 kinase activity and not a co-purified kinase.

### ***Kinetic analysis of the TAF1 NTK***

Kinetic studies of the TAF1 N-terminal kinase were performed using baculovirus expressed and purified HA-TAF1 NTK. A mock purification from uninfected Sf9 cells was performed in parallel as a negative control. First, kinase assays were performed with saturating levels of full length His-TAF7 and [ $\gamma$ - $^{32}$ P] ATP as substrates. Samples were removed from the reaction at the indicated time points and reaction products were analyzed by SDS-PAGE and silver staining. Bands corresponding to TAF7 were excised from the gel and analyzed by Cerenkov counting for  $^{32}$ P incorporation. Over time, we observed that TAF1 shows a linear rate of phosphorylation of TAF7 (Fig 19A).

Next, I performed kinase assays with increasing TAF7 concentration while keeping TAF1 concentration the same. TAF7 concentration ranged from 0-250 nM. For these

experiments, we observed a biphasic response curve of TAF7 phosphorylation with a plateau around 25nM when the kinase and substrate are in 1:1 ratio (Fig 19B). This curve shape suggests that TAF7 has at least two sites of TAF1 kinase activity, with one site preferred when TAF7 concentration is limiting.

### ***TAF7 phosphosite identification***

In order to identify potential TAF1 phosphosites on TAF7, we decided to overexpress tagged TAF7 in HEK293 cells, purify the TAF7 using immunoprecipitation and SDS-PAGE, and then analyze TAF7 by mass spectrometry. TAF7 was subcloned from the bacterial 6x His-TAF7 expression vector into a mammalian tandem-affinity purification (TAP) tag vector. Our TAP-TAF7 expression vector (a gift of R. Moon) contains a strepavidin and hemagglutinin tag separated by a TEV cleavage site. TAP-TAF7 constructs were transfected into HEK293 cells using Lipofectamine Plus reagent. After 48 hours, cells were harvested and lysed. TAP-TAF7 was pulled down using strepavidin beads and competitively eluted by the addition of biotin to the buffer. Elutions were separated by SDS-PAGE and proteins visualized by Coomassie staining. Bands corresponding to TAF7 were excised and analyzed by mass spectrometry by Phil Gafken at FHCRC and Jeff Ranish at ISB. Two phosphosites were identified on TAF7, one at serine-159 and one at serine-264 (Fig 20 and Table 1).

### ***TAF1 phosphorylates TAF7 on serine-264 to regulate protein binding of TFIID subunits***

To determine if TAF1 is the kinase responsible for these post-translational modifications, serine to alanine point mutants of TAF7 at residues 159 and 264 were created, expressed in bacteria, and used as substrates for TAF1 in in vitro kinase assays. While the S159A mutant was

phosphorylated at levels similar to wild type TAF7, S264A exhibited a large reduction in total phosphorylation levels, suggesting that this amino acid is a substrate for TAF1 kinase activity (Fig 21). Next, we engineered a phosphomimetic serine to aspartic acid mutation at S-264 (S264D) to test if phosphorylation of TAF7 S-264 is sufficient to disrupt TAF1 binding. Comparable to what was observed for pre-phosphorylated TAF7 (Fig 11E), the phosphomimetic S264D showed a 50% reduction in TAF1 C.RAP binding compared to WT-TAF7 and the S264A mutant, which cannot be phosphorylated on serine 264 (Fig 22A and B). These data suggest that TAF1 phosphorylation of TAF7, and more specifically at S-264, can disrupt their protein-protein interaction.

To demonstrate that phosphorylation of S-264 is biologically relevant, we examined the effect of this post-translational modification on the efficiency of TAF7 incorporation into endogenous TFIID complexes in vivo. For these studies, YFP-tagged WT or S264D TAF7 was expressed in HeLa cells, and the amount of exogenously expressed TAF7 present in endogenously assembled TFIID complexes was determined. We found that when expressed at comparable levels, the phosphomimetic S264D TAF7 was incorporated less efficiently into TFIID complexes than its WT counterpart (Fig 23). No change in the levels of TAF1 and TBP found in TFIID was observed with expression of the phosphomimetic mutant. These studies demonstrate that the phosphorylation state of TAF7 on S-264 can dictate the subunit composition of TFIID in cells.

### ***Regulation of gene expression and histone H3 acetylation by TAF7 serine-264***

#### ***phosphorylation***

Next, we wanted to explore the consequences of overexpressing the S264A and S264D TAF7 mutants on cyclin D1 and cyclin A transcription. Following transfection of the control, WT-TAF7, S264A or S264D expression plasmid into HeLa cells (Fig 24A), cyclin D1, cyclin A and c-fos transcript levels were measured by qRT-PCR. The S264A mutant, which should be refractory to regulation by TAF1 phosphorylation, was even more effective at repressing cyclin D1 and cyclin A transcription than WT-TAF7 (Fig 24B and C). To our surprise, the S264D mutant actually had a stimulatory effect on cyclin D1 and cyclin A transcription and appeared to be functioning as a dominant negative mutant (Fig 24B and C). One possible explanation for this unexpected increase in mRNA expression is that the S264D mutant competes with the endogenous WT-TAF7 for assembly into TFIID complexes. Due to the reduced ability of S264D to remain associated with TFIID, the outcome is an increase in TFIID complexes lacking a TAF7 subunit, leading to cyclin D1 and cyclin A expression above normal levels. Another possibility is that S264D is binding to a negative regulator and sequestering it away from the transcription machinery since it cannot efficiently associate with TFIID. As expected, neither WT-TAF7 nor the TAF7 mutants had any effect on the transcription levels of the c-fos gene (Fig 24D). These data further support the hypothesis that the inhibitory function of TAF7 is regulated by serine-264 phosphorylation.

According to our model, the inability of TAF1 to phosphorylate the S264A mutant should produce TFIID complexes in which TAF7 cannot be dissociated by phosphorylation, thus inhibiting TAF1 HAT activity and reducing histone H3 acetylation at target promoters. We used ChIP to investigate the levels of histone H3 acetylation at the cyclin D1, cyclin A and c-fos promoters following expression of WT, S264A and S264D TAF7 in HeLa cells (Fig 25A). In agreement with our gene expression results, promoter-selective inhibition of H3 acetylation was

observed with WT-TAF7 expression at both cyclin loci. S264A TAF7 was the most effective at reducing H3 acetylation levels at these loci while having no effect at the c-fos promoter (Fig 25B,C and D). Once again, the S264D mutant was an ineffective repressor of H3 acetylation in comparison to WT-TAF7 and the S264A mutant. These ChIP results establish a connection between phosphorylation of TAF7, histone H3 acetylation and gene regulation at the cyclin D1 and cyclin A promoters.

## CONCLUSIONS

In this chapter, we have demonstrated that TAF1 can phosphorylate TAF7 in vitro and in vivo. TAF7 is a substrate for both the TAF1 N-terminal and C-terminal kinases. We identified serine-264 as a TAF1 phosphorylation site on TAF7. TAF1 phosphorylation of TAF7 on serine-264 disrupted TAF7 binding to TAF1 and TFIID. Release of TAF7 from the TFIID complex via a phosphorylation dependent mechanism activated TAF1 HAT activity and H3 histone acetylation at the cyclin D1 and cyclin A promoters. Expression of a TAF7 mutant, S264A, which cannot be phosphorylated by TAF1, was even more effective at reducing H3 acetylation and transcription at target promoters than comparable levels of WT-TAF7. These studies have uncovered a novel function for the TAF7 subunit of TFIID, as a phosphorylation-dependent transcriptional regulator, and demonstrate that altering the subunit composition of TFIID can have profound consequences on TFIID function and gene transcription.

## DISCUSSION

These studies have identified the TFIID subunit TAF7 as a novel regulator of cyclin D1 and cyclin A transcription. We propose that activation of cyclin D1 and cyclin A transcription can occur via a phosphorylation-dependent mechanism in which TAF7 is phosphorylated by the TAF1 subunit of TFIID. This phosphorylation event results in release of TAF7 from the TFIID complex, stimulation of TAF1 HAT activity, acetylation of core promoter histones and increased transcription. The phosphorylation and dissociation of TAF7 from TFIID complexes at the cyclin D1 and cyclin A promoters is most prevalent during the G1 phase of the cell cycle and potentially represents a novel downstream event of mitogenic signaling pathways that induce cyclin D1 and cyclin A transcription.

Acetylation of histones is well-established as an important mechanism for activating gene transcription. Much effort has been placed on the characterization of histone acetyltransferases (HATs), enzymes that catalyze these post-translational modifications. HATs can be regulated through a variety of molecular mechanisms. Auto-acetylation within the activation loop of p300 and phosphorylation of CREB binding protein (CBP) stimulate their rates of histone acetylation (1, 25). CBP HAT activity also can be stimulated by its interaction with select transcriptional activators (8, 25). We have discovered that TAF1 HAT activity is regulated by an inhibitory protein interaction that is disrupted by protein phosphorylation. Intriguingly, the protein kinase responsible resides in TAF1, the same polypeptide that possesses the HAT domain subject to regulation. To our knowledge, TAF1 is the first example of a polypeptide that possesses two catalytic activities that are functionally connected. Whether this mode of regulation takes place within a single molecule in cis or between two TAF1 proteins in trans remains to be determined.



ts13 cells are conditional mutants that arrest in the late G1 phase of the cell cycle when shifted to the nonpermissive temperature of 39.5°C. Characterization of these mutant cells established that the G1/S phase cell cycle arrest is due to a missense mutation in the TAF1 HAT domain that abrogates catalytic activity (15, 27). Expression of WT-TAF1 rescued the ts13 cell cycle arrest while expression of HAT deficient mutants was ineffective (15). Unexpectedly, the introduction of TAF1 constructs containing disruptive kinase domain mutations also failed to complement the ts13 mutant phenotype (49). These data indicate that both TAF1 HAT and kinase activities are required for normal cell cycle progression. Our studies have extended these findings by placing both enzymatic activities of TAF1 in the same signaling pathway and adding to our molecular understanding of their function during G1 to S phase progression in mammalian cells.

We and others have previously reported that the HAT activity of TAF1 is required for efficient transcription from a subset of protein encoding genes (48, 58, 63, 76). TAF7 is a negative regulator of TAF1 HAT activity and inhibits transcription from only a subset of genes (19). Based on these results, we anticipated that transcription levels of genes driven by promoters dependent on TAF1 HAT activity also would be subject to inhibitory actions by TAF7. Unexpectedly, elevating TAF7 levels only inhibited transcription of the TAF1 dependent cyclin A and D1 genes and had no effect on cyclin E transcription, another gene that requires TAF1 HAT activity for efficient promoter activity. These data indicate that although the subset of genes dependent on TAF1 HAT activity overlaps genes regulated by TAF7, these two subpopulations are not identical. One possible explanation is that the inhibitory effects of TAF7 can be bypassed by the recruitment of another enzyme that catalyzes the histone modifications necessary for efficient gene transcription. Further studies will be necessary to identify additional

TAF1-dependent genes resistant to the inhibitory effects of TAF7 and to determine the specific set of factors present at each of these promoters that accounts for their differential regulation.

Overexpression of TAF7 was sufficient to inhibit cyclin D1 and cyclin A mRNA expression levels (Fig 6C). A question that comes to mind is “How does TAF7 overexpression mechanistically work in the cell to repress gene transcription?” One hypothesis is that excess levels of TAF7 protein would change the dynamics of TFIID assembly and create more complexes containing TAF7. This shift in TFIID composition would increase the number of DNA-bound TAF7-containing complexes at the cyclin D1 and cyclin A promoters, thereby inhibiting transcription of these genes.

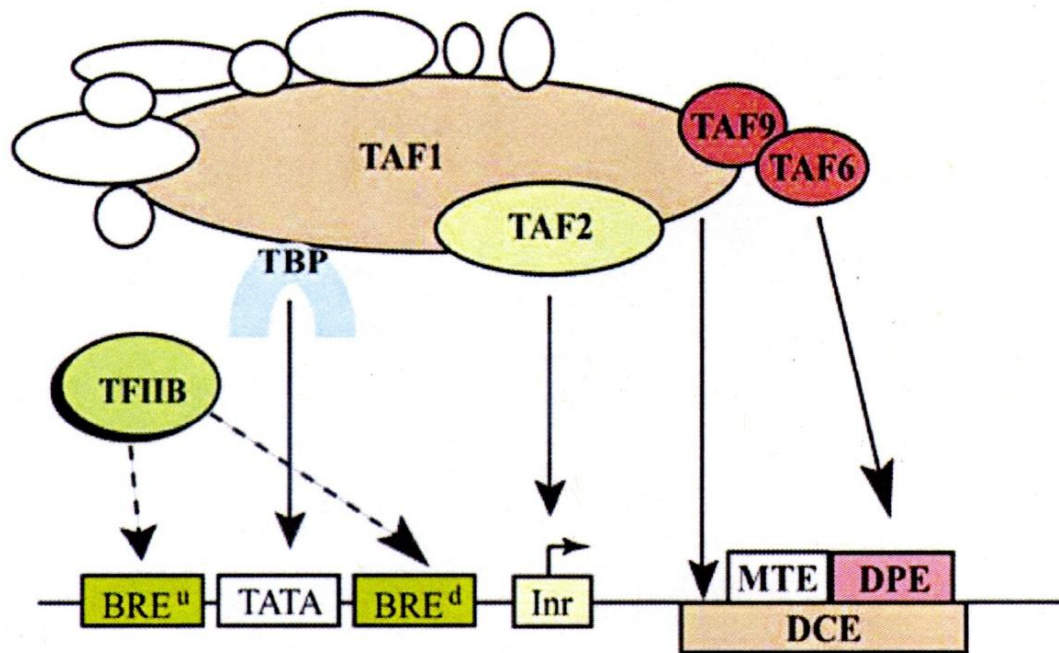
When first isolated, it was generally thought that the subunit composition of a functional TFIID complex was invariant and contained a defined set of TAFs conserved from yeast to humans. Here we present data suggesting that TAF7 dissociates from the TFIID complex following phosphorylation by the TAF1 kinase, as cells transition through the G1 phase of the cell cycle (Figure 9B and 13A). Immuno-electron microscopy performed with a TAF7 antibody mapped TAF7 at the periphery of the TFIID complex, near the TAF1 HAT domain (37). This peripheral localization suggests that TAF7 could easily enter and exit the TFIID complex. Our findings are consistent with recent studies that have demonstrated TFIID is variable and dynamic in both its subunit composition and overall structure. Several groups have identified TAF variants that incorporate into the TFIID complex at different stages of development or in different cell types (17, 28). The disruption and replacement of the canonical TFIID with a novel complex composed of the TBP related factor 3 (TRF3) and TAF3 also has been reported in myoblasts undergoing differentiation into myotubes (11). In addition, TAF subunits originally identified in TFIID can be found in other transcription regulatory complexes (77). Therefore,

regulating the composition of canonical and non-canonical TFIID complexes represents an important mechanism for controlling gene transcription.

Loss of the TAF2 subunit of TFIID relative to the other subunits has been repeatedly observed during the purification of TFIID from yeast and human cells, suggesting that the TAF2 subunit readily dissociates from the complex (10). Subsequent structural analysis of yeast TFIID by cryo-electron microscopy and electron tomography confirmed the existence of two subpopulations, TFIID complexes containing the Taf2 subunit and those lacking Taf2 (54). In the absence of Taf2, significant reorganization of different domains was observed suggesting that TFIID demonstrates significant plasticity and is capable of varying its overall structure. However, the presence of Taf2 appeared to stabilize one of four abundant states identified for TFIID. The ts13 single missense mutation in TAF1, which disrupts the ability of TAF1 to acetylate histones, is thought to shift the TAF1 protein to an inactive state for HAT activity under nonpermissive conditions. These results have led us to hypothesize that TAF7 binds to TAF1 and locks the TFIID complex into a conformation in which the structure of the TAF1 HAT domain is no longer favorable for catalytic activity. To test this hypothesis, our lab has established a collaboration to determine the structure of a TAF1-TAF7 dimer using X-ray crystallography.

In summary, we discovered a novel role for TAF7 as a negative regulator of cyclin D1 transcription and cell cycle progression. These studies have determined that activation of cyclin D1 and cyclin A transcription requires a previously uncharacterized phosphorylation dependent switch in the composition of TFIID. Phosphorylation catalyzed by the TAF1 kinase leads to dissociation of the TAF7 subunit from TFIID and activation of TAF1 HAT. There is a growing body of evidence that TFIID is a highly dynamic molecule, and we have demonstrated that

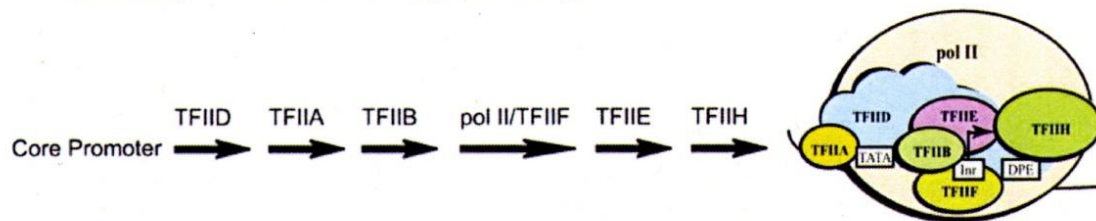
altering its subunit composition can have profound consequences on TFIID function and gene transcription.



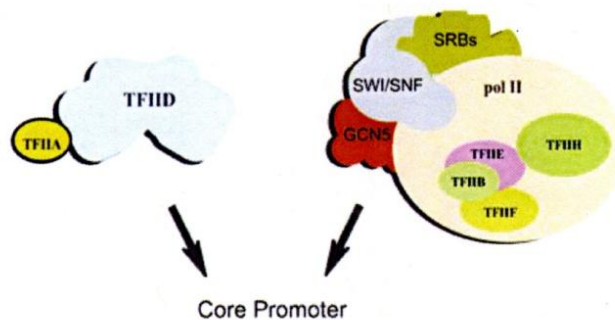
**Figure 1 Recognition of core promoter elements by TFIID and TFIIB**

Depiction of the interactions that occur between TFIID and TFIIB with the seven core promoter elements discussed in this dissertation. Adapted from Thomas, MC and Chaing, C-M 2006.

## A

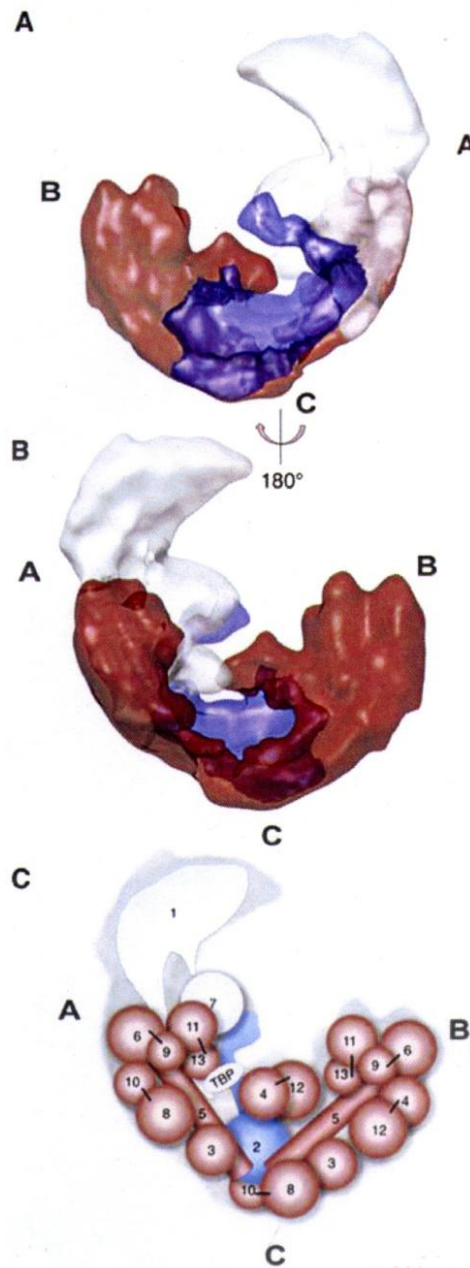


## B



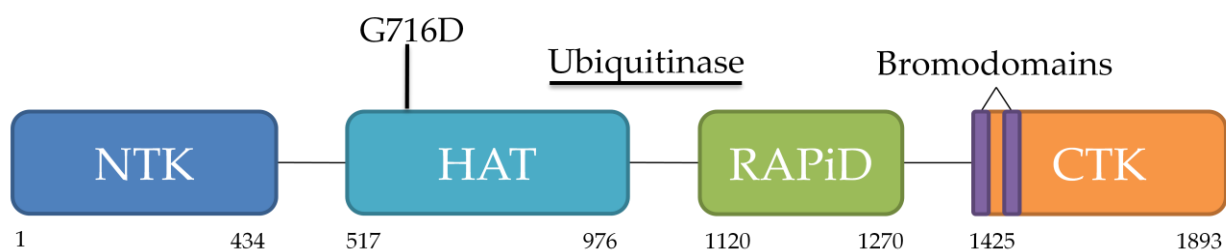
### Figure 2 Pathways for preinitiation complex assembly

Preinitiation complex (PIC) formation may occur by stepwise recruitment of the general transcription machinery (A, the sequential assembly pathway) or by recruitment of preassembled pol II holoenzyme and TFIID complexes as depicted in the two-component pathway (B). Adapted from Thomas, MC and Chiang, C-M 2006.



### Figure 3 Structural organization of yeast TFIID

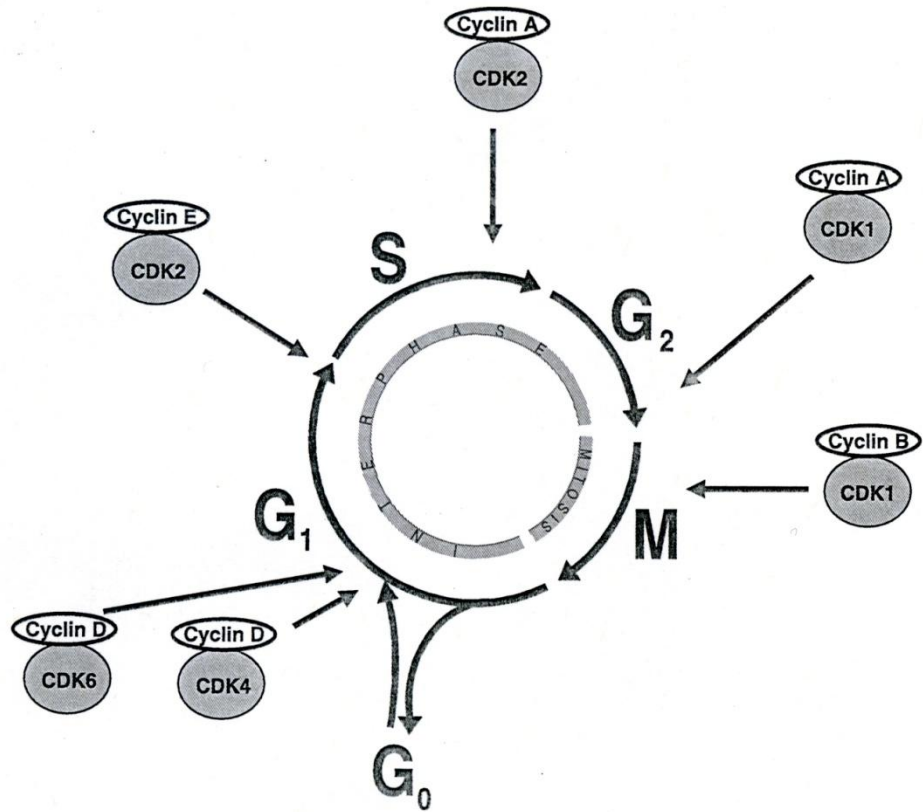
(A and B) Two opposite views of the TFIID complex showing the location of TAF1, TAF7 and TBP (white), TAF2 (blue) and a quasi symmetric core module (red). (C) Schematic representation of TAF localization within TFIID. The approximate positions of the subunits are derived from antibody-labelling experiments. The size of the spheres is proportional to the molecular mass of the proteins. Black lines represent documented protein-protein interactions of histone fold domain containing TAFs. Adapted from Cler, E et al 2009.



#### Figure 4 Schematic of TAF1

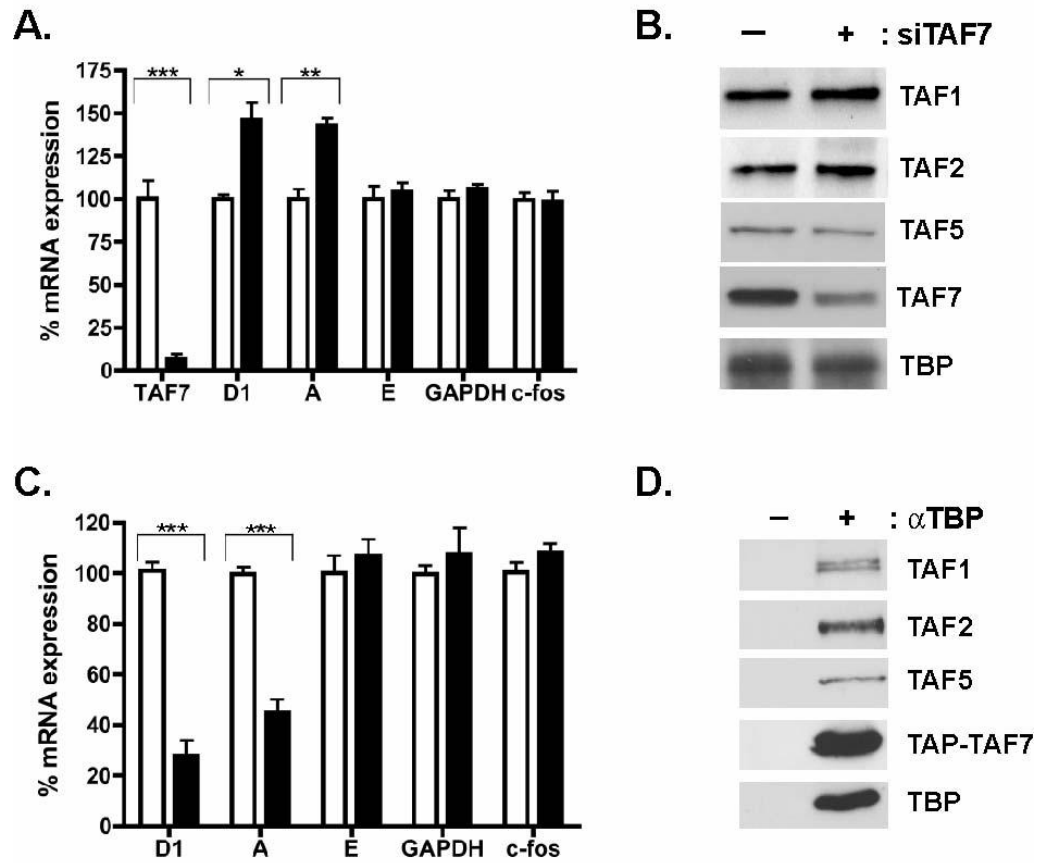
Diagram of the largest TFIID subunit TAF1. There are two kinase domains, one in the N-terminus (NTK) and one in the C-terminus (CTK). The histone acetyltransferase (HAT) domain is found in the central, most conserved part of the protein. Ubiquitinase activity also maps to the center of the protein. The RAP74 interaction domain (RAPiD) can bind the TFIIF subunit RAP74 and the TFIID subunit TAF7. TAF1 also possess two bromodomains capable of binding acetylated lysine residues. These are located within the CTK domain. The location of the human equivalent residue of the ts13 point mutation is also indicated (G716D).





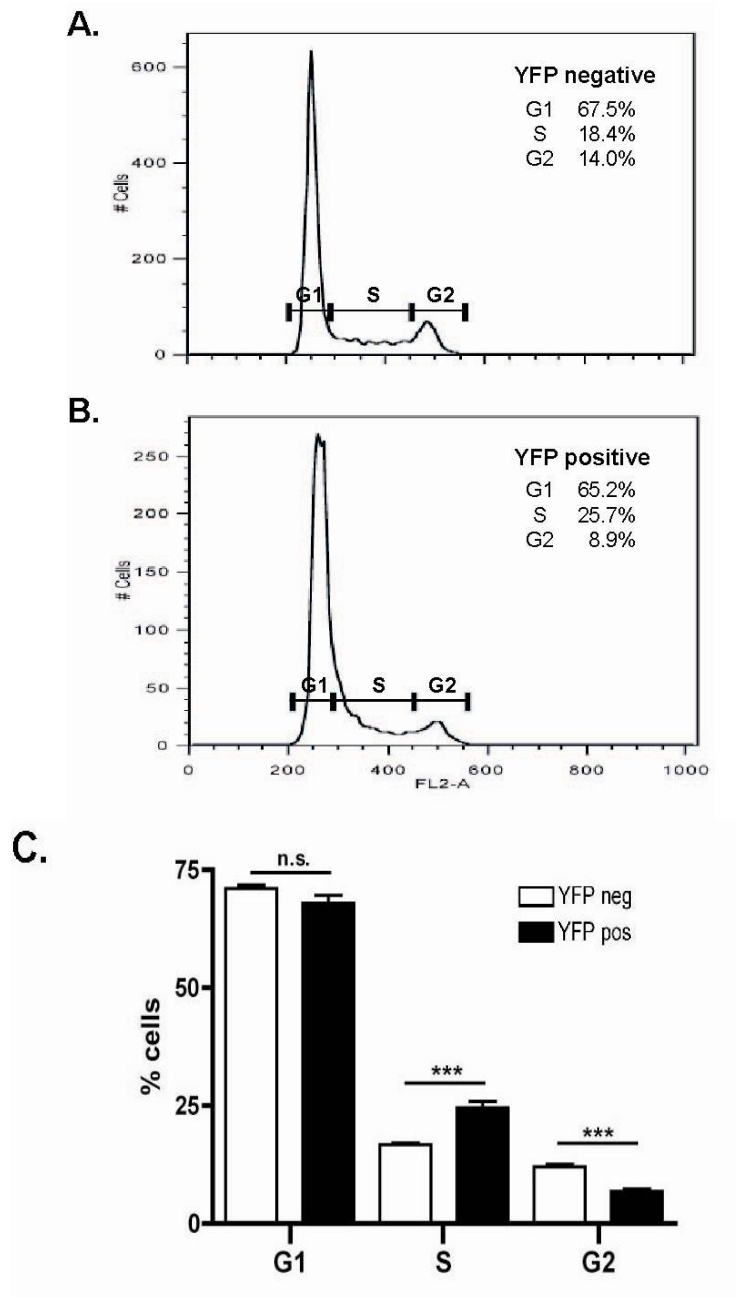
**Figure 5 The stages of the cell cycle**

The site of activity of regulatory CDK/cyclin complexes is also indicated. Adapted from Vermeulen, K, Van Bockstaele, DR and Berneman, ZN 2003.



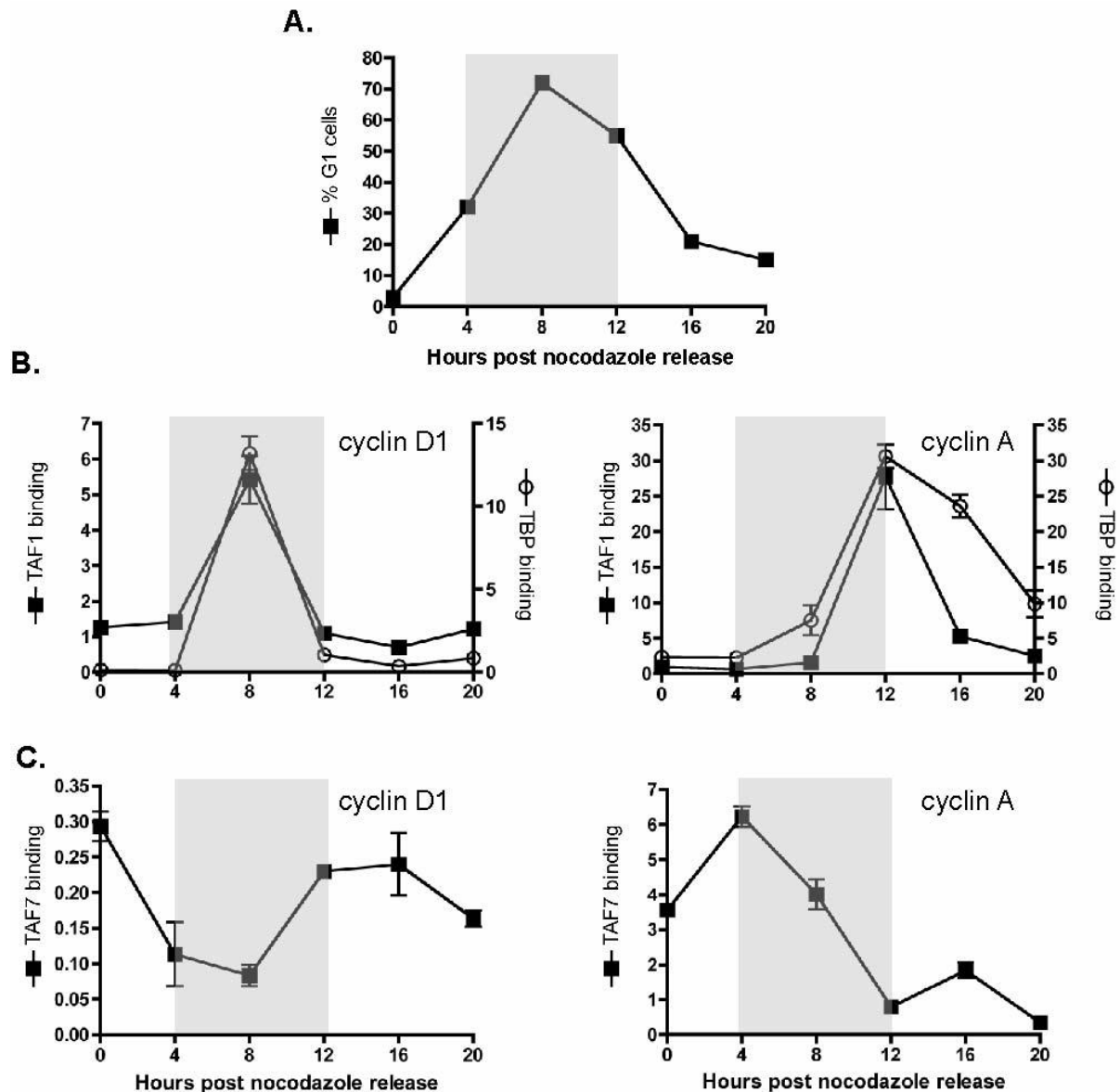
**Figure 6 TAF7 functions as a transcriptional repressor at a subset of promoters**

HeLa cells were treated with 50 nM control (white bars) or TAF7 (black bars) siRNA for 72 hours. Total RNA was collected and transcript levels for TAF7, cyclin D1, cyclin A, cyclin E, GAPDH, and c-fos were determined by qRT-PCR. Average of 3 independent experiments, each in triplicate, is provided. \* $p < 0.05$ , \*\* $p < 0.005$ , \*\*\* $p < 0.001$  (B) HeLa cells were treated with siRNA as described in A. TFIID complexes were immunoprecipitated using an anti-TBP antibody. Precipitated proteins were separated on SDS-polyacrylamide, and the indicated TFIID subunits were detected by immunoblotting. (C) HeLa cells were transfected with pGLUE empty vector (white bars) or TAP-TAF7 expression plasmid (black bars). Forty-eight hours post-transfection, total RNA was collected and analyzed by qRT-PCR as in A. Results are averaged from 3 independent experiments, each in triplicate. \*\*\* $p < 0.001$  (D) HeLa cells were transfected with TAP-TAF7 expression plasmid. After 48 hours, immunoprecipitations were carried out using an anti-TBP antibody. Precipitated proteins were analyzed by SDS-PAGE and immunoblotted for the indicated TFIID subunits.



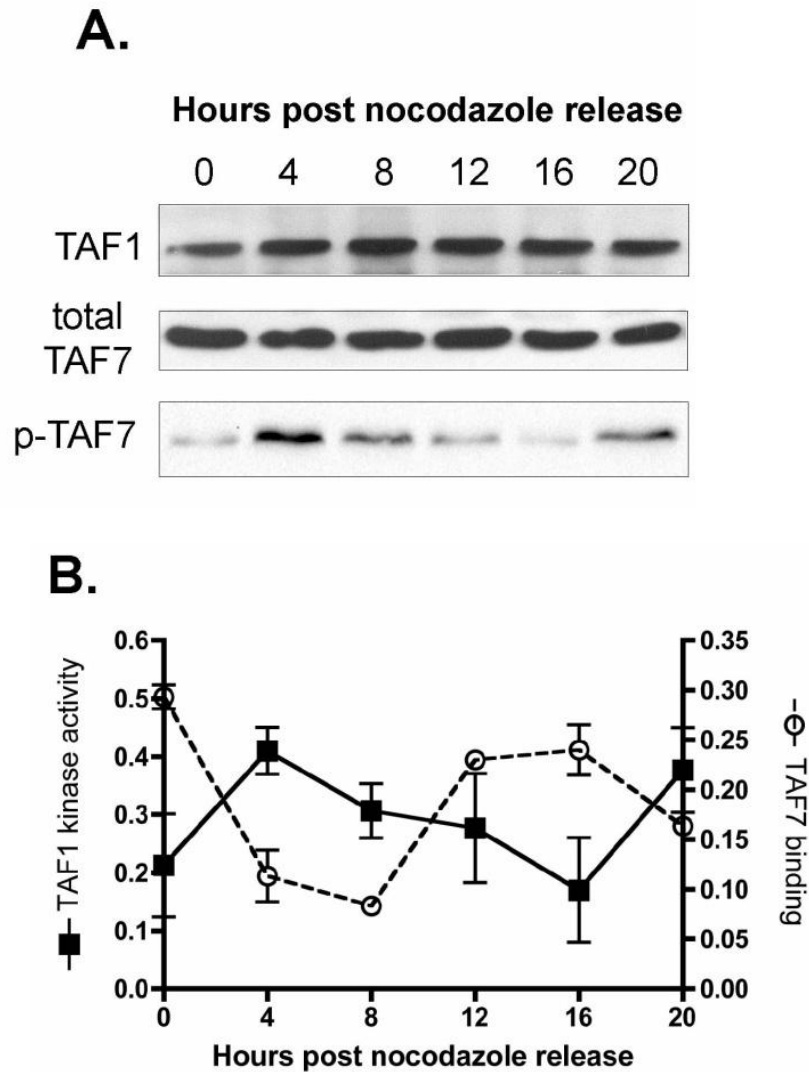
### Figure 7 TAF7 is a regulator of S-phase progression

HeLa cells were transiently transfected with YFP-TAF7 expression plasmid. After 72 hours, cells were fixed and stained with propidium iodide. YFP fluorescence and DNA content were determined by flow cytometry. The cell cycle profiles of YFP negative (A) and YFP positive (B) cells from one representative experiment are provided. Percentage of cells in each cell cycle phase, as defined by the indicated gates, is provided. (C). Cell cycle distribution of YFP negative (white bars) and YFP positive (black bars) from 11 independent transfections is shown. \*\*\*  $p < 0.001$ , n.s. = not significant.



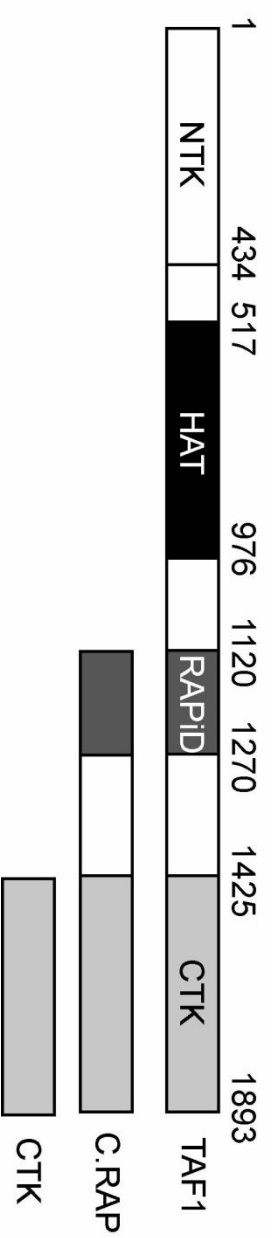
**Figure 8 Inverse correlation between TAF1 and TAF7 binding at the cyclin D1 and cyclin A promoters**

(A) HeLa cells were synchronized by thymidine/nocodazole block and collected at the indicated times after drug removal. Percent of G1 cells was determined by propidium iodide staining and flow cytometry. (B) Chromatin immunoprecipitation experiments were performed using anti-TAF1 (closed squares) or anti-TBP (open circles) antibodies and samples analyzed by qPCR using primers spanning the cyclin D1 (left panel) and cyclin A (right panel) promoters. Y-values represent the average percent input detected from one representative experiment carried out in triplicate. Time period when cells are predominantly in G1 is indicated by the shaded grey box. (C) ChIP experiments using anti-TAF7 antibody were carried out and analyzed as described in B.



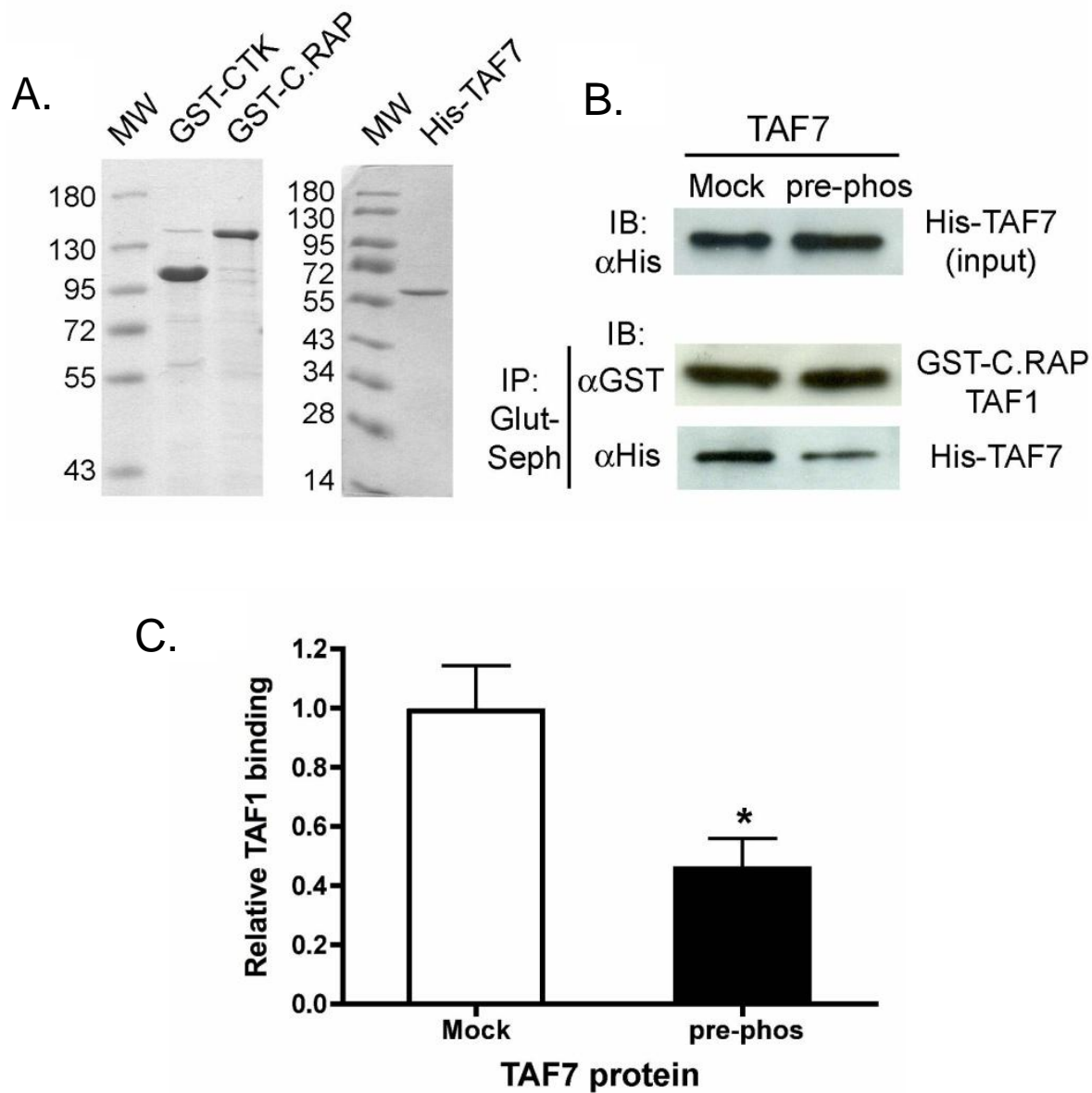
### Figure 9 TAF1 kinase activity opposes TAF7 binding at the cyclin D1 promoter

(A) HeLa cells were synchronized using a thymidine/nocodazole block and collected at the indicated time points after drug removal. For TAF1 kinase activity, HeLa nuclear extracts were prepared and immunoprecipitated with anti-TBP sepharose beads. Bound proteins were added to in vitro kinase assays using recombinant His-TAF7 as substrate. Phosphorylated TAF7 was detected by autoradiography (p-TAF7). Total TAF1 and His-TAF7 protein levels were monitored by western blotting. (B) Phosphorylated TAF7 in kinase assays was quantified using ImageJ software. TAF1 kinase activity is expressed as intensity of TAF7 phosphorylation corrected for total TAF1 protein (closed squares). Kinase activity was compared to TAF7 ChIP data presented in Fig 8C.



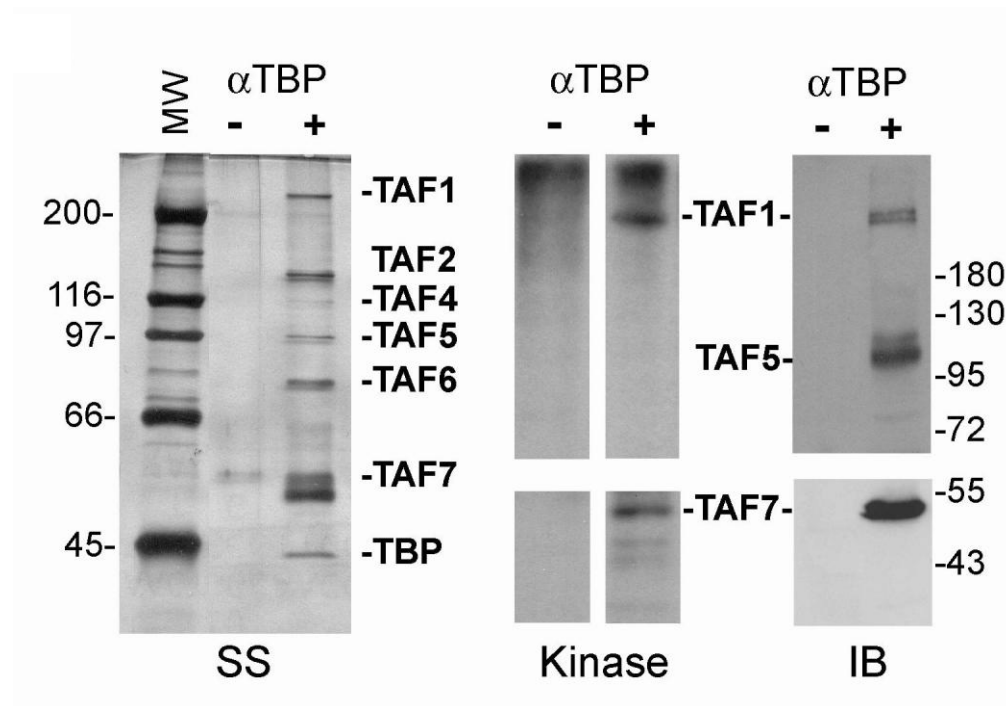
**Figure 10 Schematic of TAF1 domains**

A diagram of full length TAF1 and TAF1 fragments used in Figure 11 is shown. Domains indicated are NTK: N-terminal kinase, HAT: histone acetyltransferase domain, RAPiD: TAF7 interaction domain, CTK: C-terminal kinase.



**Figure 11 Phosphorylated TAF7 shows decreased association with TAF1**

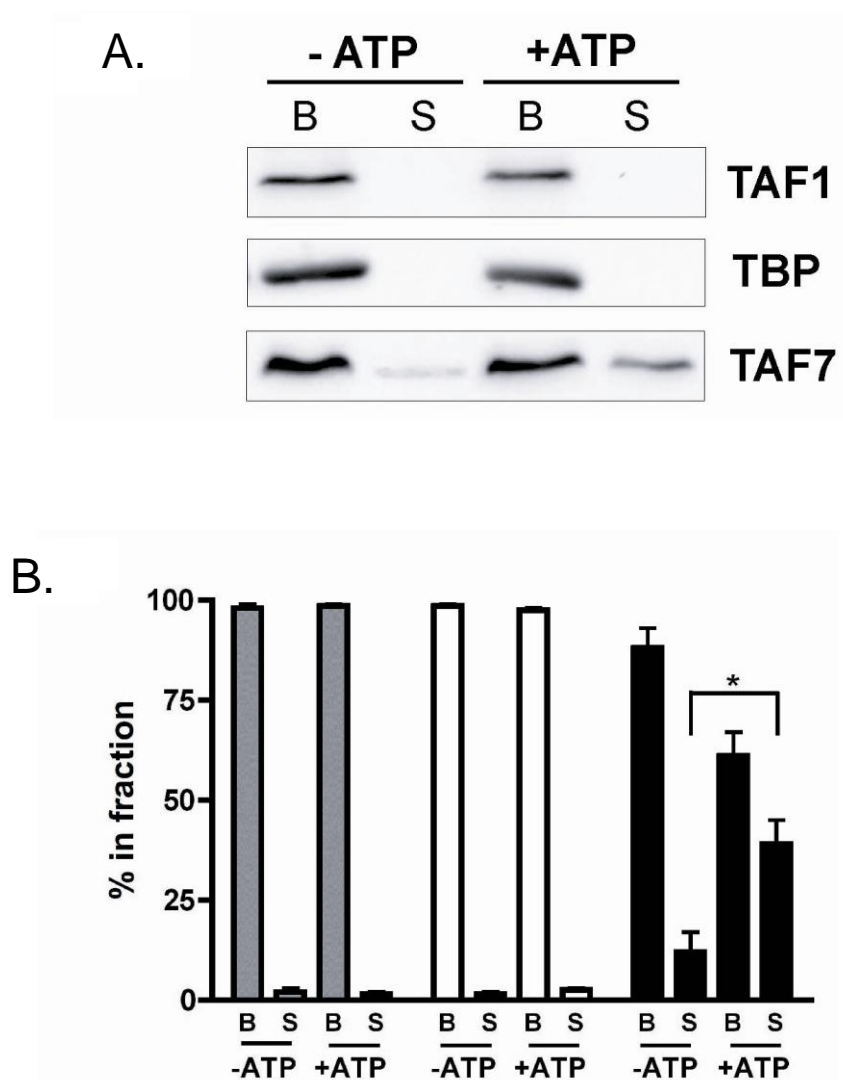
(A) Purified baculovirus expressed TAF1 fragments, GST-CTK and GST-C.RAP, and bacterially expressed His-TAF7 were visualized by Coomassie blue staining. (B) Purified His-TAF7 pre-incubated with buffer (Mock) or TAF1 CTK (pre-phos) was isolated using Ni-agarose beads (input) and incubated with C-terminal TAF1 fragment (GST-C.RAP). Proteins co-precipitating with GST-C.RAP (IP:Glut-Seph) were detected by immunoblotting with the indicated antibody. (C) TAF1 binding was quantified using ImageJ software. \* $p < 0.05$ ,  $n = 5$ .



**Figure 12 TAF1 phosphorylates TAF7 within TFIID**

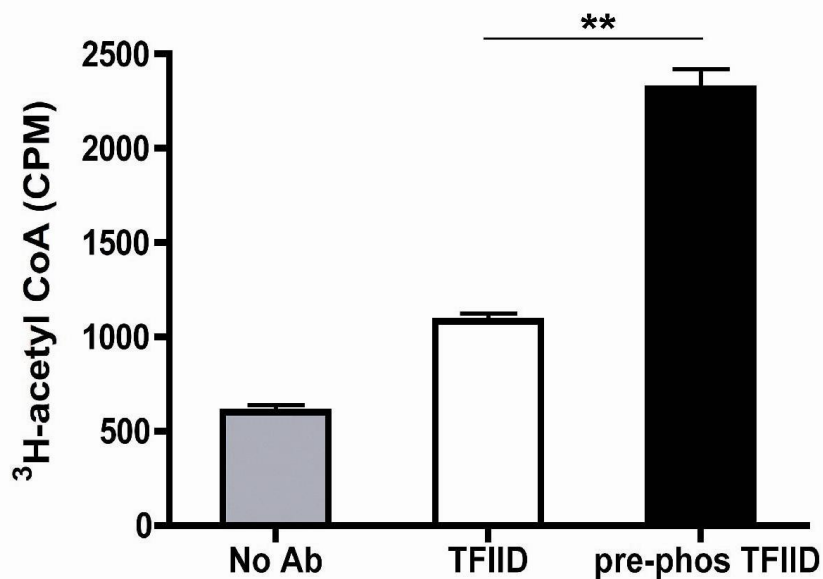
TFIID was immunoprecipitated from fractionated HeLa nuclear extracts (enriched for TFIID) with an anti-TBP antibody and visualized by silver staining (SS). Proteins precipitated in the absence of antibody also are shown. In vitro kinase assays were performed with the precipitated proteins, and reaction products separated on SDS-polyacrylamide, transferred to nitrocellulose, visualized by autoradiography (Kinase) and subjected to immunoblotting (IB) for the indicated proteins. The position of molecular weight standards is shown.





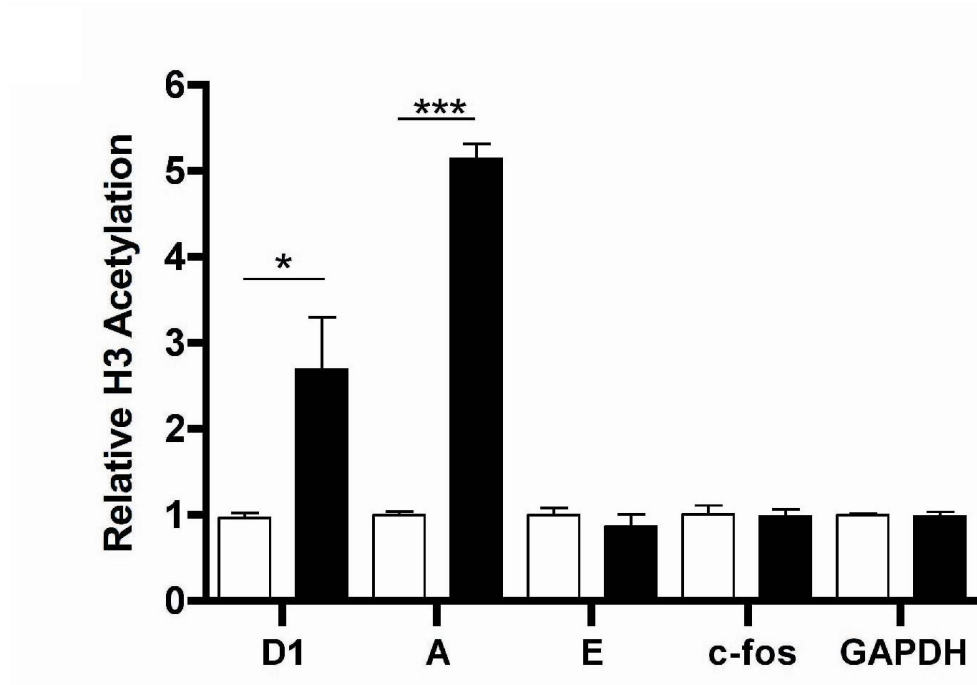
### Figure 13 TAF1 phosphorylation releases TAF7 from TFIID

(A) TFIID was immunoprecipitated as described in Figure 12. In vitro kinase assays using cold ATP were carried out with TFIID immobilized on anti-TBP sepharose beads. The supernatant (S) and bound (B) fractions were collected and the amount of TAF1, TBP and TAF7 present was determined by immunoblotting. (B) Signal intensities were quantified using ImageJ software, and the percent total for each protein in the bound and supernatant fractions was calculated. Gray bars represent TAF1 bands, white represent TBP, and black represent TAF7. \* $p < 0.05$ ,  $n = 3$ .



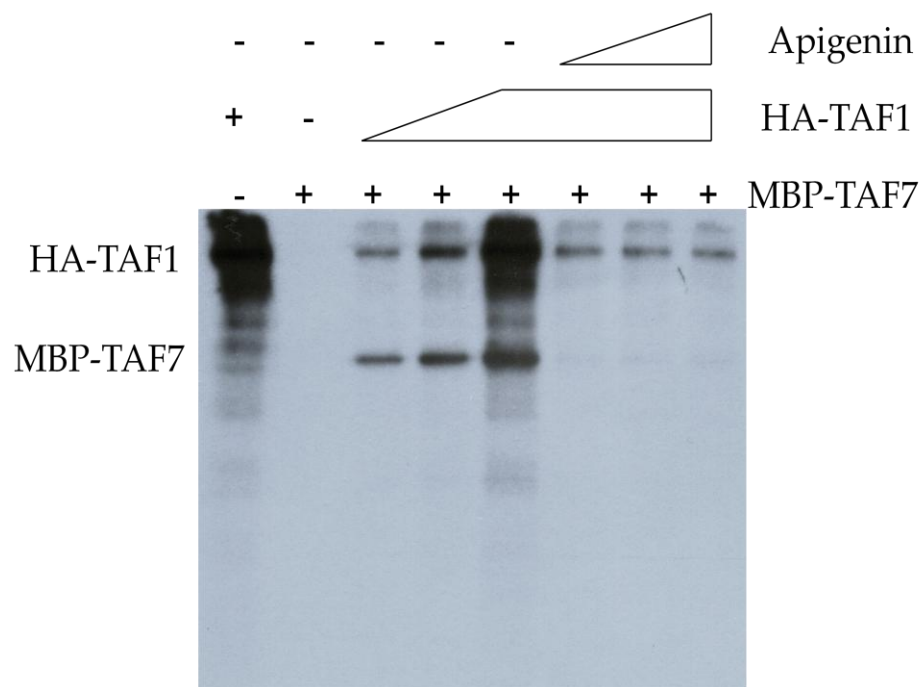
**Figure 14 Release of TAF7 from TFIID increases histone acetylation in vitro**

Immunoprecipitated TFIID was incubated in the absence (TFIID) or presence (pre-phos TFIID) of cold ATP prior to assaying for HAT activity. <sup>3</sup>H-acetyl-CoA incorporation into a human histone H3 peptide (aa 1-20) was measured by liquid scintillation. Results from one representative experiment are shown. \*\*p < 0.005, n = 3



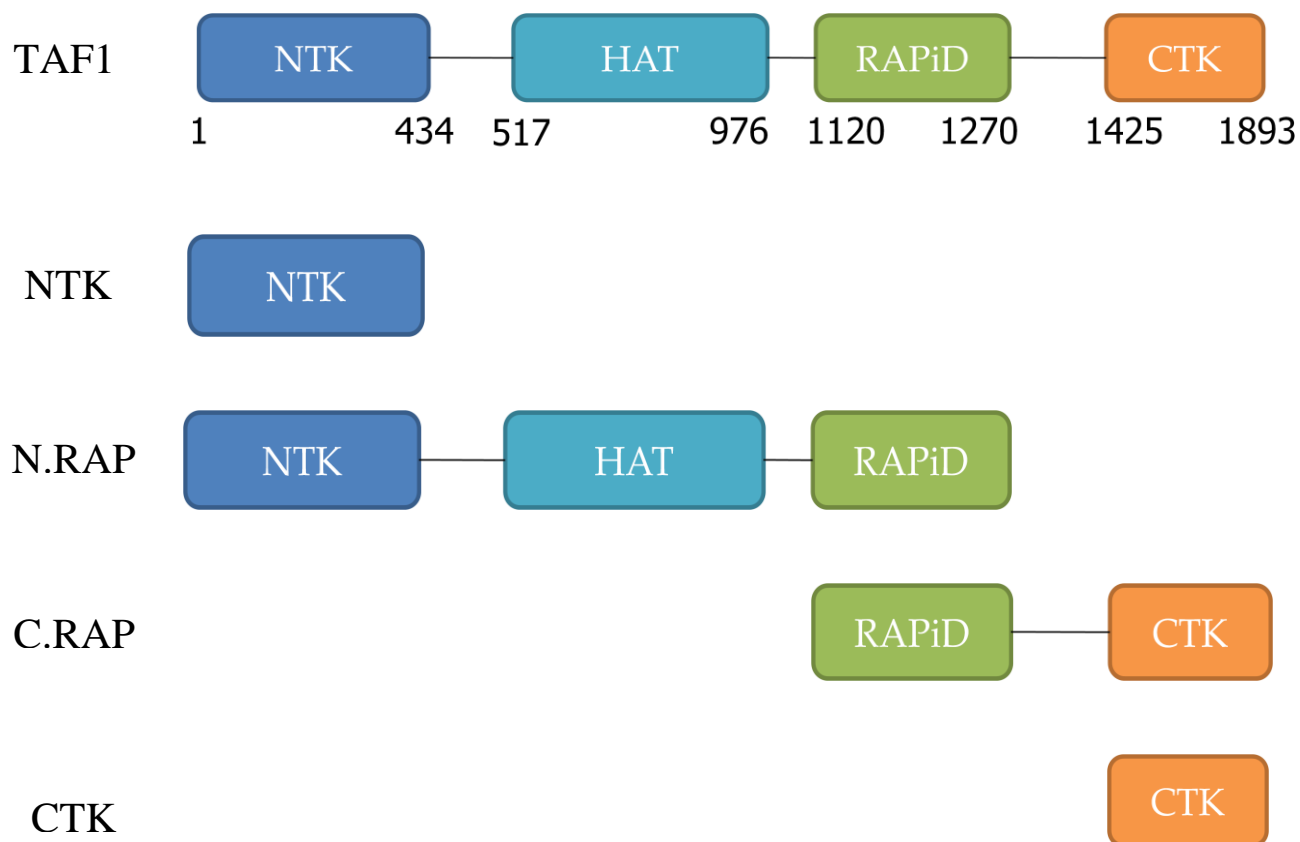
**Figure 15 TAF7 knockdown increases cyclin D1 and cyclin A promoter acetylation**

HeLa cells were treated with 50 nM control (white bars) or TAF7 (black bars) siRNA for 72 hours. ChIPs were performed using anti-histone H3 K9K14Ac antibody and analyzed by qPCR at the indicated promoters. Y-axis represents fold-change in H3 K9K14Ac relative to control-treated cells. \* $p < 0.05$ , \*\*\* $p < 0.001$ ,  $n = 3$ .



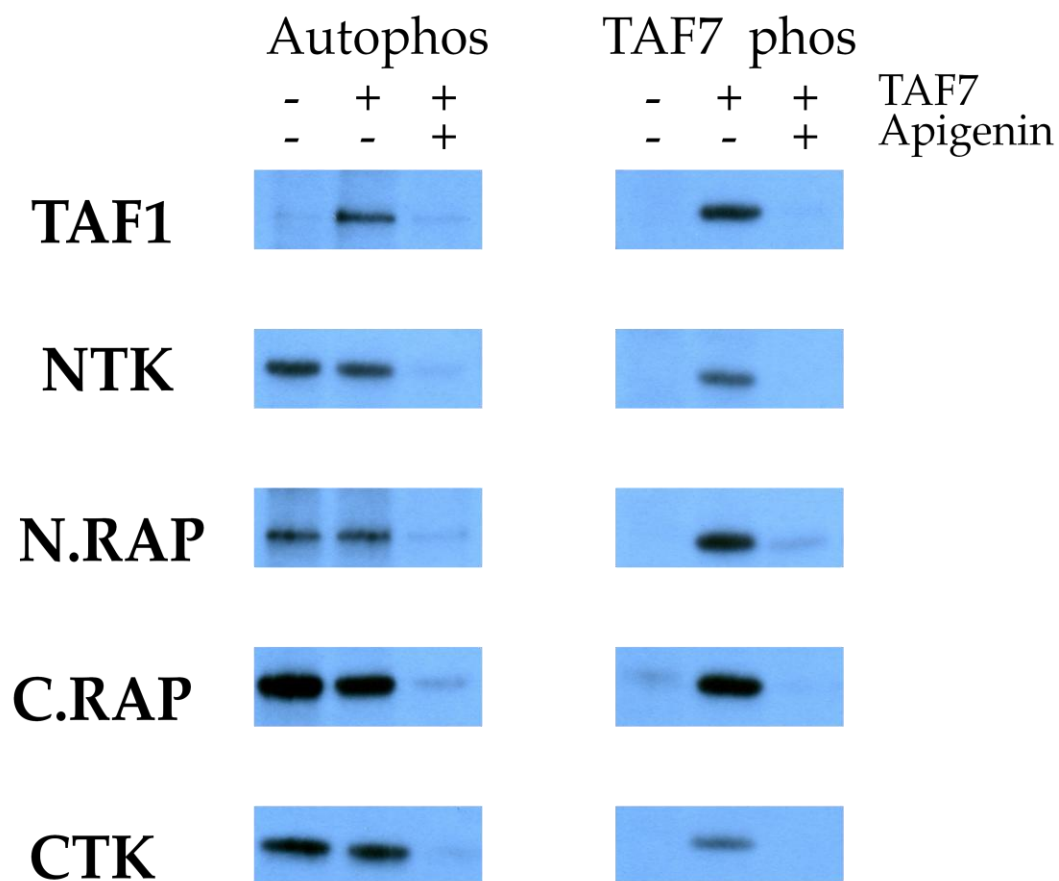
**Figure 16 TAF1 phosphorylates TAF7 in vitro**

In vitro kinase assay with immunopurified TAF1 and TAF7 proteins. Reactions were performed in the absence or presence of increasing concentrations of a TAF1 kinase inhibitor apigenin (20  $\mu$ M). Phosphorylated proteins were visualized by autoradiography.



**Figure 17 TAF1 kinase constructs**

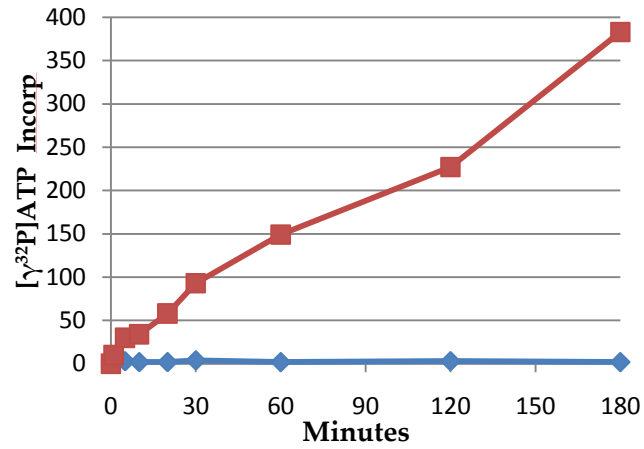
A schematic of the baculovirus-expressed HA-tagged full length TAF1 and TAF1 fragments used in Figures 16 and 18 is shown. Domains indicated are NTK: N-terminal kinase, HAT: histone acetyltransferase domain, RAPiD: TAF7 interaction domain, CTK: C-terminal kinase.



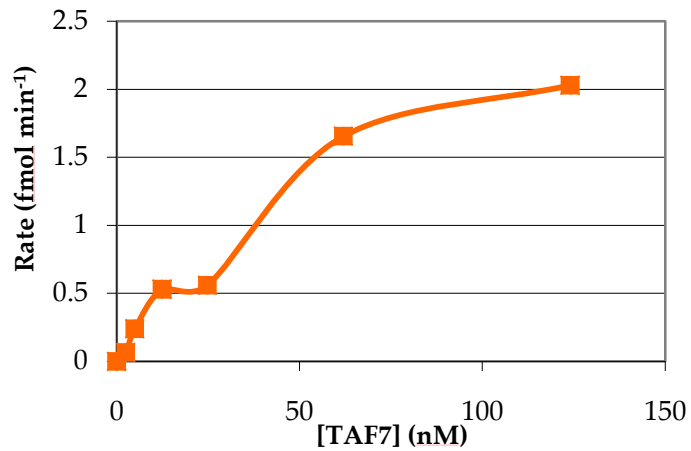
**Figure 18 TAF1 NTK and CTK phosphorylate TAF7**

Baculovirus-expressed HA-TAF1 kinase constructs were immunopurified from Sf9 cell lysates. Purified proteins were used in an in vitro kinase assay to demonstrate autophosphorylation activity and transphosphorylation of TAF7 in the presence or absence of the TAF1 kinase inhibitor apigenin (20uM). The domains of TAF1 shown are: NTK = N-terminal kinase, HAT = histone acetyltransferase, RAPiD = RAP74 interaction domain, CTK = C-terminal kinase.

A.



B.



**Figure 19 Characterization of TAF1 NTK kinase activity**

(A) Time course of TAF7 phosphorylation comparing proteins immunopurified from NTK infected (red) or uninfected Sf9 cells (blue). (B) Biphasic kinetics for 25 nM of TAF1 NTK with a saturating level of [ $\gamma^{32}\text{P}$ ]ATP (75  $\mu\text{M}$ ) and increasing concentrations of WT TAF7.

$H_3N^+$ MSKSKDDAPH ELESQFILRL PPEYASTVRR AVQSGHVNK DRLTIELHPD  
 GRHGIVRVDR VPLASKLVDL PCVMESLKI DKKTFYKTAD ICQMLVSTVD  
 GDLYPPVEEP VASTDPKASK KKDKDKEKKEF IWNHGITLPL KNVRKRRFRK  
 TAKKKYIESP DVEKEVKRLL STDAEAVSTR WEIIAEDETK EAENQGLDIS  
 SPGMSGHRQG HDSLEHDELK EIFNDLSSSS EDEDETQHQD EEDINIIDTE  
 EDLERQLQDK LNESDEQHQE NEGTLQLVMG IQKQIDNMKG KLQETQDRAK  
 RQEDLIMKVE NLALKNRFQA VLDELKQKED REKEQLSSLQ EELESLEK-COOH

**Figure 20 Mass spectrometry of TAF7 identifies S159 and S264 as phosphorylation sites**

Baculovirus-expressed GST-TAF7 was immunopurified from Sf9 cell lysates, electrophoresed and Coomassie stained. The TAF7 band was excised and subjected to in-gel tryptic digestion. Digested peptides were analyzed by electrospray ionization followed by neutral-loss on an Orbitrap instrument. Detected peptides are shown in red and identified serine phosphosites are shown in blue.

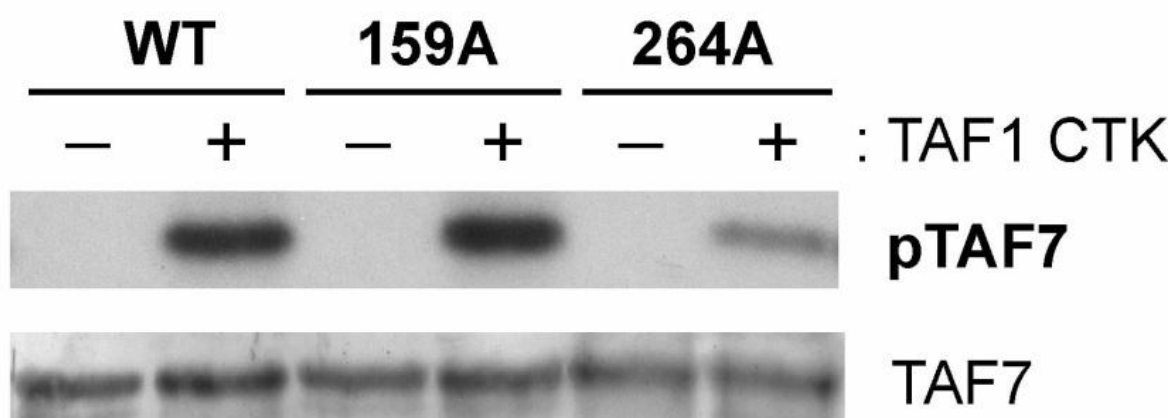


**Table 1: TAF7 phosphopeptides identified by mass spectrometry**

Phosphopeptide *	Position **	p-value
YIEpSPDVEK	S159	0.920
YIEpSPDVEKEVKR	S159	
LNEpSDEQHQENEGTNQLVMGIQK	S264	0.996
QLQDKLNEpSDEQHQENEGTNQLVMGIQK	S264	0.991

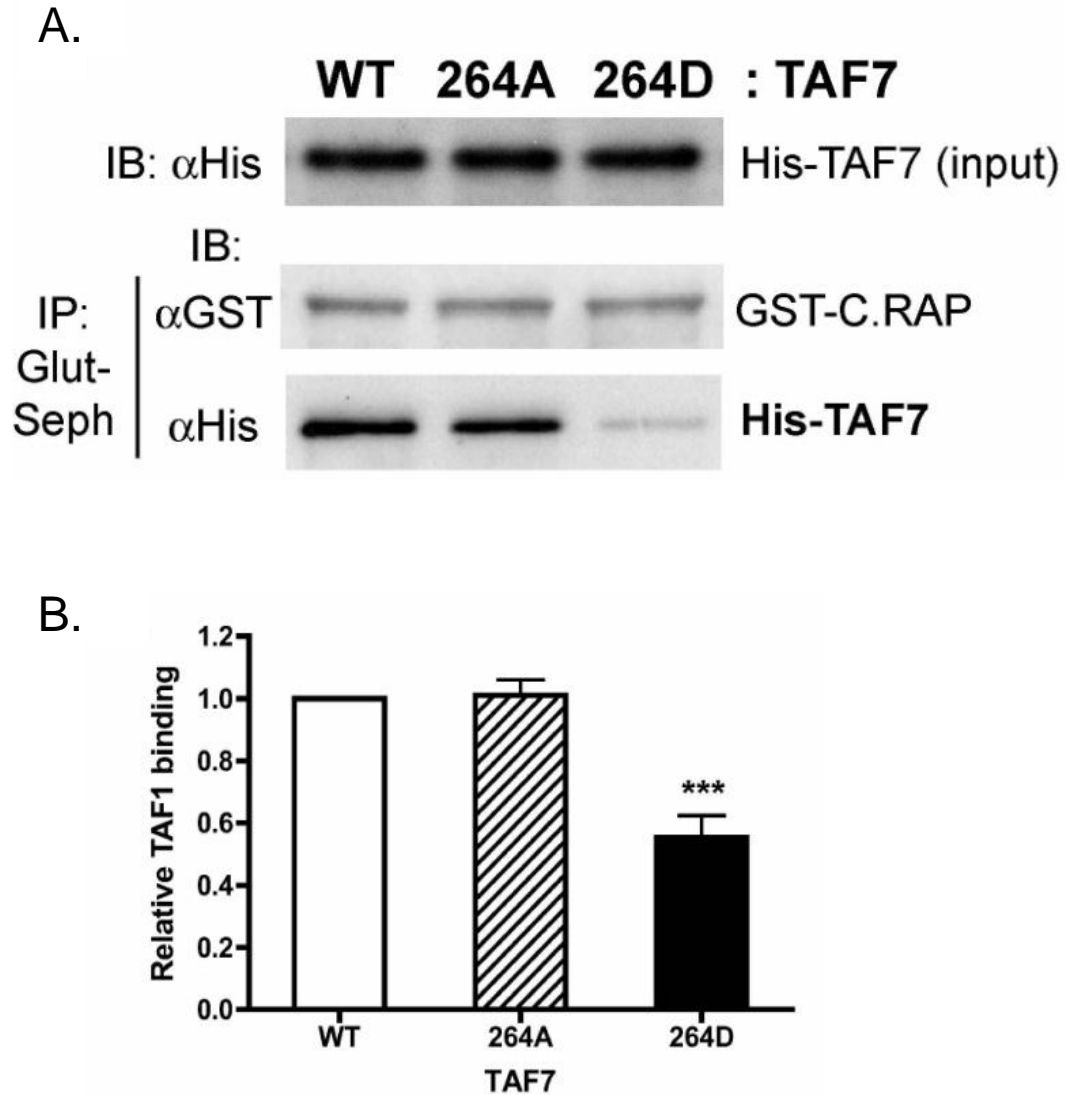
\* Phosphorylated amino acid is preceded with 'p'

\*\* Position of phosphorylated amino acid in TAF7 protein



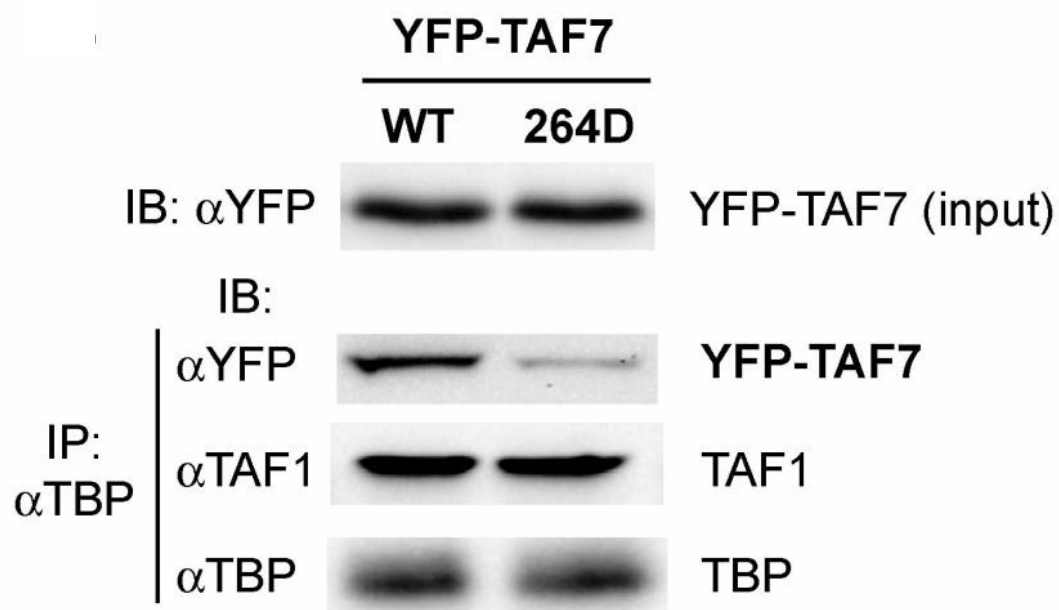
**Figure 21 S264 is a phosphosite for the TAF1 CTK**

His-tagged WT and mutant TAF7 proteins were expressed in bacteria, affinity purified, and used as substrates for TAF1 CTK. Phosphorylated TAF7 was detected by autoradiography (pTAF7). Equal loading of TAF7 substrates was confirmed by silver staining (TAF7).



**Figure 22 Phosphorylation of TAF7 at S264 disrupts TAF1 binding**

(A) The indicated His-tagged WT and mutant TAF7s (input) were incubated with GST-C.RAP TAF1. Pulldowns with glutathione-sepharose were performed, and precipitated proteins were subjected to SDS-PAGE and immunoblotting. (B) TAF1 binding was quantified as described in Fig 11C. \*\*\* $p < 0.001$ ,  $n = 4$ .



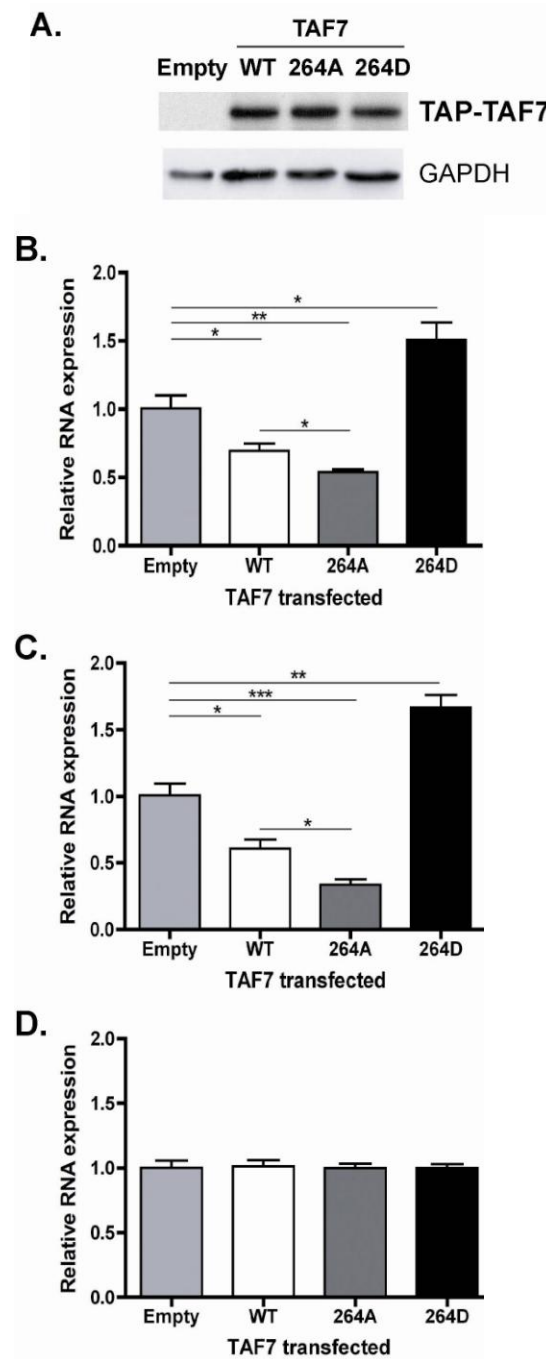
**Figure 23 TAF7 phosphorylated at S264 cannot incorporate into TFIID**

YFP-tagged WT-TAF7 or S264D mutant was transiently expressed in HeLa cells for 48 hours. Nuclear extracts were prepared and total levels of YFP-tagged WT and 264D TAF7 were determined by immunoblotting (input) using anti-YFP antibody. TFIID was immunoprecipitated using an anti-TBP antibody and the indicated subunits were detected by immunoblotting.

Cyclin D1

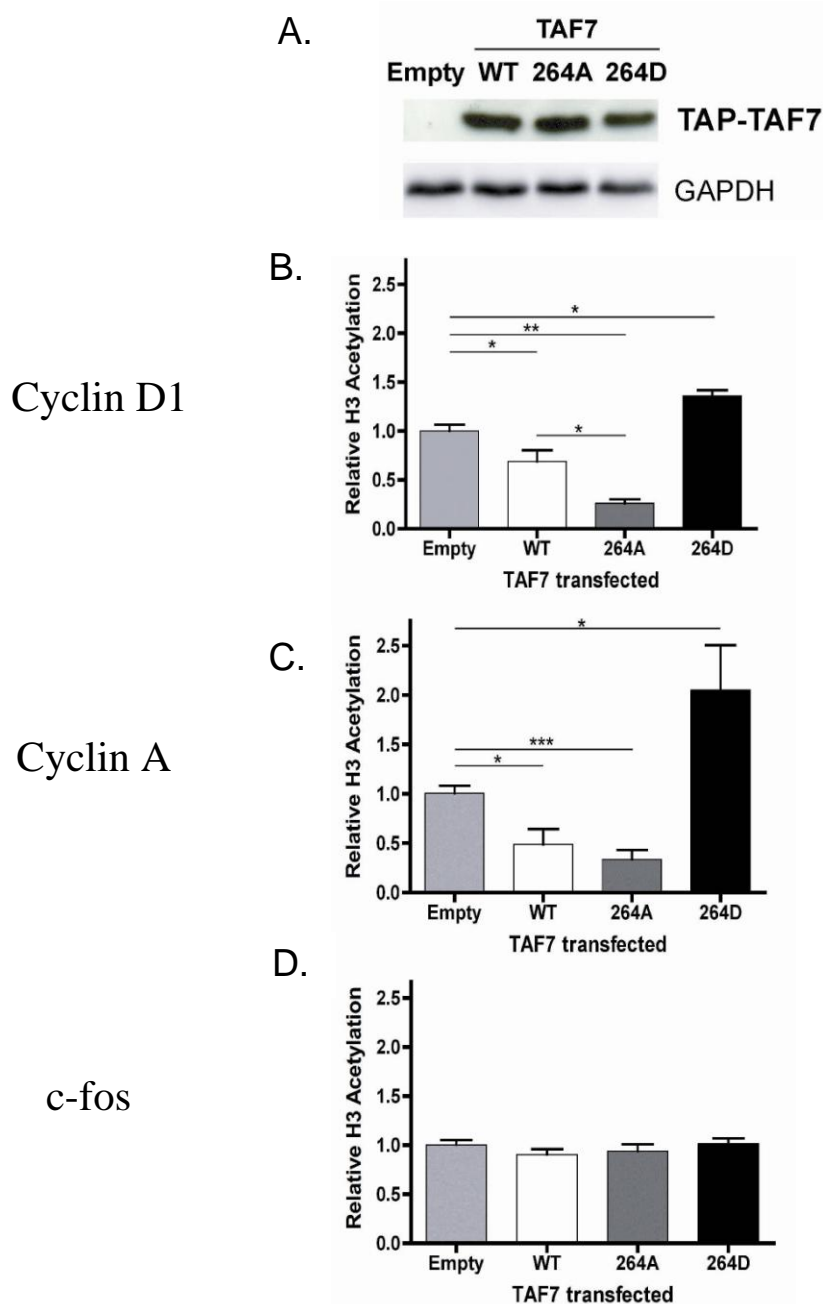
Cyclin A

c-fos



**Figure 24 TAF7 S264 phosphorylation stimulates cyclin D1 and cyclin A transcription**

HeLa cells were transfected with TAP-tag control (Empty), TAP-TAF7 (WT), TAP-264A or TAP-264D plasmid. After 48 hours, whole cell extracts were prepared and expression levels of TAF7 proteins were determined by immunoblotting using an anti-HA antibody. GAPDH protein levels were measured to ensure equal protein loading. For RNA expression, total RNA was collected and transcript levels for cyclin D1 (B), cyclin A (C), and c-fos (D) were measured by qRT-PCR. Y-axis represents mRNA levels relative to cells transfected with the empty control plasmid. n = 3.



**Figure 25 TAF7 S264 phosphorylation increases cyclin D1 and cyclin A promoter acetylation**

(A) HeLa cells were transfected with TAP-tag control (Empty), TAP-TAF7 (WT), TAP-264A or TAP-264D plasmid. After 48 hours, whole cell extracts were prepared and expression levels of TAF7 proteins were determined by immunoblotting using an anti-HA antibody. GAPDH protein levels were measured to ensure equal protein loading. (B,C,D) In parallel transfections, H3 K9 acetylation levels at the indicated promoters were determined by ChIP using anti-histone H3 K9Ac antibody and qPCR. Y-axis indicates H3 acetylation levels relative to cells transfected with the empty control plasmid for each promoter.  $n = 3$ . \* $p < 0.05$ , \*\* $p < 0.005$ , \*\*\* $p < 0.001$ .

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