Functional characterization of the COOH-terminal kinase activity of the TBP-associated factor TAF1

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Für meine Großeltern.

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II. Abbreviations

°C	degree(s) Celsius
A	Ampere(s)
ATP	adenosinetriphosphate
bp	base pair(s)
BSA	bovine serum albumin
cm	centimeter(s)
CRC	chromatin-remodelling-complex
CTK	COOH-terminal kinase
Da	dalton(s)
DBD	doublebromodomain
DMSO	dimethylsulfoxide
DNA	desoxyribonucleic-acid
dNTP	deoxynucleoside triphosphate
ds	double-strand
DTT	dithiothreitol
E. coli	*Escherichia coli*
EDTA	ethylenediaminetetraacetic acid
et al.	and others
EtBr	ethidiumbromide
g	reciprocal centrifugal force (rcf)
GTF	general transcription factor
GTM	general transcription machinery
h	hour(s)
kb	kilobasepair(s) kilobase = 1000 bp
kDa	kilodalton(s)
l	liter
M	molarity
max	maximum
MCS	multiple cloning site
mg	milligram(s)
min	minute(s)
ml	milliliter(s)
mM	millimolarity
mm	millimeter(s)
ng	nanogram(s)
nm	nanometer(s)
NTK	NH_2-terminal kinase
OD	optical density
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
PIC	preinitiation-complex
RNA	ribonucleic-acid
rpm	rounds per minute
RT	room temperature
SDS	sodiumdodecylsulfate
sec	second(s)
ss	single-strand
TEMED	tetramethylendiamine
Tris	Trihydroxymethylamino-methane
U	unit, enzyme activity
UV	ultraviolet light
V	Volt(s)
w/v	weight per volume
µ	micro-
µg	microgram(s)

1. Introduction

The temporal and spatial control of gene expression is one of the most fundamental processes in biology. The transcription of genes constitutes an important medium for the regulation of gene expression. Although very basic principles of transcription are similar in prokaryotes and eukaryotes, eukaryotic transcription is distinguished by having multiple RNA polymerases and by highly more complicated control DNA sequences. The way how eukaryotic cells manage their genetic information with their much larger amounts of DNA that is packaged in a seemingly inaccessible structure called chromatin, requires regulatory machinery consisting of a conglomerate of protein factors, like the transcription factor TFIID. These factors function coordinately and in combinatorial fashion to switch the expression of genes on or off. In multicellular organisms, it is this selective expression of gene products in individual cell types that leads to the rich diversity of cells and tissues, which have evolved to perform highly specialized functions. The regulation of eukaryotic transcription of genes occurs on two levels: the level of the initiation of transcription at the DNA and the level of chromatin-dynamics (Lemon and Tjian 2000, Lee and Young 2000, Näär *et al*. 2001).

1.1 The complexity of transcription regulation

1.1.1 Regulatory DNA sequences

Eukaryotic genes consist of cis-regulatory regions (“enhancer” or “silencer”), the promoter and the protein-coding sequence. The core promoter is very compact and can contain at least one of three different sequence elements that contact the TBP-containing TFIID initiation complex (Smale and Kadonaga 2003): TATA box, initiator element (INR) and the downstream promoter element (DPE).

The TATA box is a DNA sequence found in the promoter region of many genes. It is the binding site for TBP (TATA-binding protein) of transcription factors like TFIID and is involved in the process of transcription by the RNA polymerase. Many genes lack a TATA box and use an INR and DPE instead. The INR is a conserved sequence element that encompasses the start site of transcription and can direct accurate transcription initiation in the absence of a TATA box (Smale

1997). It can also be represented in TATA-containing promoters where it leads to an increase in the strength of expression of the promoter (Lo and Smale 1996). The DPE was found only in TATA-less but INR-containing promoters and is responsible for the precise initiation of transcription through the INR (Burke and Kadonaga 1997).

The complexity of the eukaryotic promoter region seems to increase with the diversity of its correlating organism (Figure **1**): The regulatory DNAs of unicellular eukaryotes are often composed of short sequences, which are located immediately adjacent to the core promoter (Wyrick and Young 2002). These enhancer/silencer sequences are usually composed of two to three linked binding sites for one or two different sequence-specific transcription factors (de Bruin *et al.* 2001).

The regulatory region of higher eukaryotes likely contains several enhancers in 5' and 3' regulatory regions or even in introns, adding complexity to the level of transcription-regulation since silencers/enhancers present target sites for transcription factors.

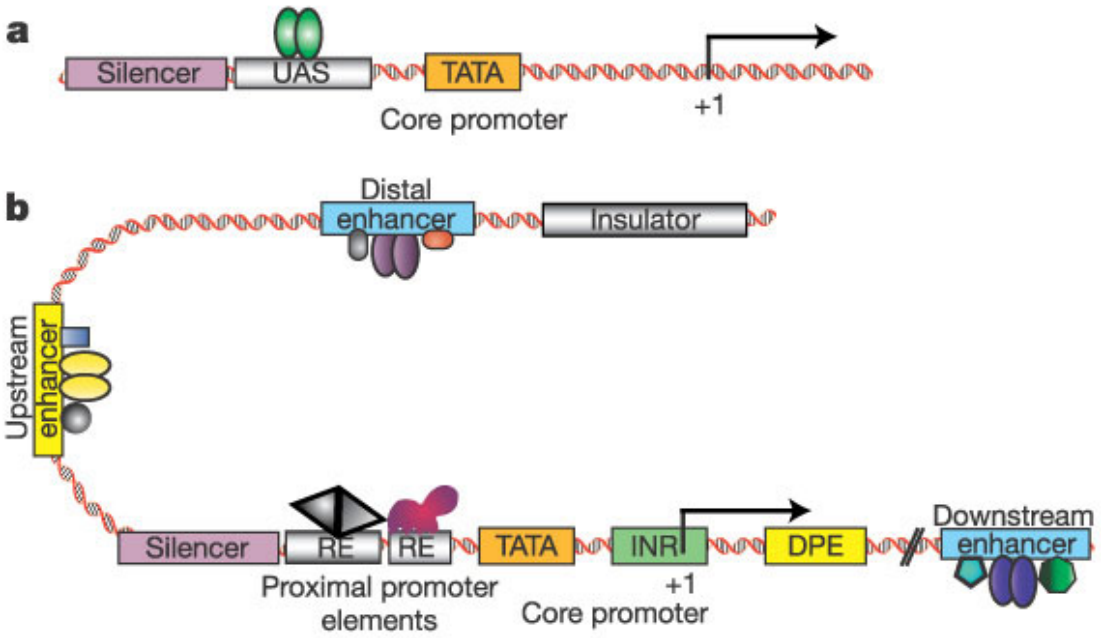

Figure 1. Comparison of a simple eukaryotic promoter and extensively diversified metazoan regulatory modules:
a Basic eukaryotic transcriptional promoter unit: a simple core promoter (TATA), upstream activator sequence (UAS) and silencer element spaced within 100-200 bp of the TATA box, typically for unicellular eukaryotes.
b Complex metazoan transcriptional control modules: Multiple clustered enhancer modules interspersed with silencer and insulator elements, located 10-50 kb up- or downstream of a composite core promoter containing TATA box (TATA), initiator sequences (INR) and downstream promoter elements (DPE) (Levine and Tjian 2003).

Examples among the known enhancers, for example in the upstream region of a gene, are the GC box, the CAAT box and the octamer element. The GC box is recognized by the transcription factor SP1 (Kadonaga *et al.* 1987, Pugh and Tjian 1990). The CAAT box is recognized by a variety of factors depending on the specific promoter, which it is part of. The octamer is also recognized by more than one factor, for example the ubiquitous transcription factor Oct-1 and the lymphoid specific Oct-2 (Lewin 1997).

Additional DNAs play an important role in enhancer function: They might serve as "tethering elements" (Figure **2**) that recruit distal enhancers to the core promoter and insulator DNA-sequences prevent gene-associated enhancers from inappropriately regulating neighboring genes (Calhoun *et al.* 2002, Burgess-Beusse *et al.* 2002).

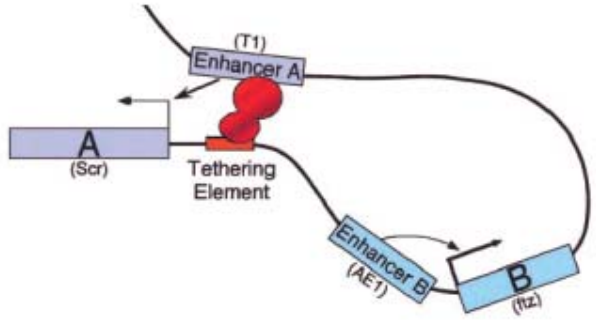

Figure 2. Function of a promoter proximal tethering element.
Given a promoter-close tethering sequence and the specific proteins, a loop in the DNA can form, bringing a distal enhancer (enhancer A) close to a proximal promoter A to activate transcription (Dorsett 1999).

1.1.2 Promoter recognition and the basal transcription apparatus

RNA polymerase II (Pol II) cannot initiate transcription itself, but is absolutely dependent on the auxiliary general transcription factors (GTFs) that bind to the specific regions of the gene (Roeder 1996, Woychik and Hampsey 2002). At least six GTFs (TFIIA, -B, -D, -E, -F and -H) are necessary to direct accurate and regulated initiation of transcription (Hampsey 1998, Hampsey and Reinberg 1999, Lemon and Tjian 2000, Lee and Young 2000, Näär *et al.* 2001).

Pol II, together with the GTFs, constitutes the basal transcription apparatus that is needed to initiate transcription of Pol II dependent genes and consists of more than 50 polypeptides that

must be assembled at promoter sequences upon activation of gene expression (Zawel and Reinberg 1993, Tjian and Maniatis 1994, Orphanides *et al.* 1996).

The current model for activation of transcription postulates, that in the first step transcription-factors bind specific DNA-sequences in the enhancer regions of their target genes (Figure **3**) following by the recruitment of one or more components of the basal transcription apparatus and/or cofactors (Ptashne and Gann 1997, Struhl 1996, Lemon and Tjian 2000, Näär *et al.* 2001). These interactions between enhancer-bound transcription-factors and components of the transcription apparatus probably stabilize the assembly of the apparatus to the promoter of the target gene and induce an increase in the rate of initiation of transcription (Tjian and Maniatis 1994, Roeder 1996, Ptashne and Gann 1997).

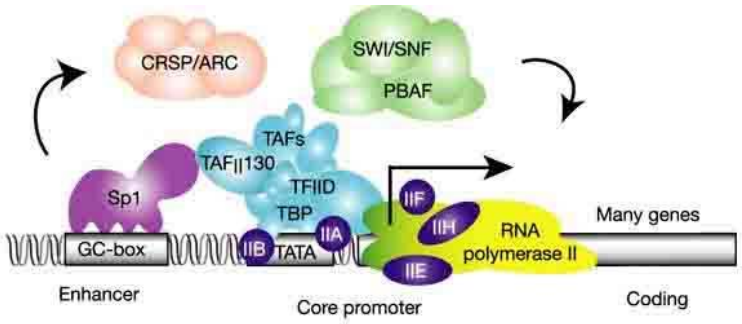

Figure 3. The transcription apparatus: a multi-subunit complex (Levine and Tjian 2003).

1.2 TFIID and TAFs

The GTF TFIID consists of TBP and, depending on the organism, 8-14 TBP-associated factors (TAFs) (Woychik and Hampsey 2002). Originally each TAF was named according to its relative weight (kD), but the names of the TAF-genes have been changed to reflect guidelines recently endorsed to avoid confusion (Tora 2002). An overview of the changes from old to new gene names for *S. cerevisiae, H. sapiens, D. melanogaster, C. elegans, and S. pombe* is shown in Figure **4**.

			C. elegans (ce)			
New name	*H. sapiens* (hs)	*D. melanogaster* (dm)	previous name	new name	*S. cerevisiae* (sc)	*S. pombe* (sp)
TAF1	$TAF_{II}250$	$TAF_{II}230$	*taf-1* (W04A8.7)	*taf-1*	Taf145/130	$TAF_{II}111$
TAF2	$TAF_{II}150$	$TAF_{II}150$	*taf-2* (Y37F11B.4)	*taf-2*	Taf150 or TSM1	(T38673)
TAF3	$TAF_{II}140$	$TAF_{II}155$ or BIP2	(C11G6.1)	*taf-3*	Taf47	
TAF4	$TAF_{II}130/135$	$TAF_{II}110$	*taf-5* (R119.6)	*taf-4*	Taf48 or MPT1	(T50183)
TAF4b	$TAF_{II}105$					
TAF5	$TAF_{II}100$	$TAF_{II}80$	*taf-4* (F30F8.8)	*taf-5*	Taf90	$TAF_{II}72$
TAF5b						$TAF_{II}73$
TAF5L	PAF65β	Cannonball				
TAF6	$TAF_{II}80$	$TAF_{II}60$	*taf-3.1* (W09B6.2)	*taf-6.1*	Taf60	(CAA20756)
TAF6L	PAF65α	(AAF52013)	*taf-3.2* (Y37E11AL.8)	*taf-6.2*		
TAF7	$TAF_{II}55$	(AAF54162)	*taf-8.1* (F54F7.1)	*taf-7.1*	Taf67	$TAF_{II}62$/PTR6
TAF7L	TAF2Q		*taf-8.2* (Y111B2A.16)	*taf-7.2*		
TAF8	(BAB71460)	Prodos	(ZK1320.12)	*taf-8*	Taf65	(T40895)
TAF9	$TAF_{II}32/31$	$TAF_{II}40$	*taf-10* (T12D8.7)	*taf-9*	Taf17	(S62536)
TAF9L	$TAF_{II}31$L (AAG09711)					
TAF10	$TAF_{II}30$	$TAF_{II}24$	*taf-11* (K03B4.3)	*taf-10*	Taf25	(T39928)
TAF10b		$TAF_{II}16$				
TAF11	$TAF_{II}28$	$TAF_{II}30\beta$	*taf-7.1* (F48D6.1)	*taf-11.1*	Taf40	(CAA93543)
TAF11L			*taf-7.2* (K10D3.3)	*taf-11.2*		
TAF12	$TAF_{II}20/15$	$TAF_{II}30\alpha$	*taf-9* (Y56A4.3)	*taf-12*	Taf61/68	(T37702)
TAF13	$TAF_{II}18$	(AAF53875)	*taf-6* (C14A4.10)	*taf-13*	Taf19 or FUN81	(CAA19300)
TAF14					Taf30	
TAF15	$TAF_{II}68$					
			B-TFIID			
BTAF1	$TAF_{II}170$/TAF-172	Hel89B	(F15D4.1)	*btaf-1*	Mot1	(T40642)

Figure 4. New Pol II TAF nomenclature including the corresponding known orthologs and paralogs (Tora 2002).

1.2.1 Function of TFIID in transcription

The inability of GTFs to access chromatin, DNA wrapped around core histones H2A, H2B, H3 and H4 in repeating subunits called nucleosomes, implies that a requisite step in transcription

initiation is the alteration of chromatin structure (Wolffe, 1998). TFIID, along with TFIIB, are the only components of the preinitiation complex (PIC) that can bind specifically to core promoters (Lagrange *et al.* 1998, Cosma *et al.* 1999, Krebs *et al.* 1999, Agalioti *et al.* 2000).
The binding of TFIID to a core promoter surrounding the transcription start site of a gene nucleates assembly of the PIC, which contains Pol II and the GTFs TFIIA, TFIIB, TFIIE, TFIIF and TFIIH (Orphanides *et al.* 1996; Hampsey 1998). The nucleating function of TFIID is thought to comprise several distinct activities, which are given to the TFIID complex by its TAF subunits (Wassarman and Sauer 2001). These are:

(1) Binding of TFIID to DNA sequence elements.
(2) The posttranslational modification of histones and GTFs.
(3) The recruitment to other GTFs via TAF-GTF interactions.
(4) The recruitment of TFIID to activators via TAF-activator interactions.
(5) Forming the scaffold for a stable TFIID complex via TAF-TAF and TAF-TBP interactions.

1.2.2 DNA binding of TFIID

Several TAFs contribute to the nucleating functions of TFIID by their structure. For example, *Drosophila* and human TAF12 are homologous to histone H2B and TAF6 and TAF9 are homologous to histone H4 and H3 respectively. In solution and in the crystalline state, the dTAF9/dTAF6 complex exists as a heterotetramer, resembling the $(H3/H4)^2$ heterotetrameric core of the histone octamer and suggesting that TFIID contains a histone octamer-like substructure and binds DNA in an octamer-like manner (Burley and Roeder 1996).
Early studies of Pol II transcription revealed, that TBP is sufficient for basal transcription *in vitro*, but that TFIID is required for the response to activators *in vivo*, indicating that TAFs play an important role in transcriptional activation (Näär *et al.* 2001). The binding of TFIID to the promoter constitutes a critical rate-limiting step of transcription (Orphanides *et al.* 1996, Hampsey and Reinberg 1997).
Activators directly bind TAFs, which is consistent with the idea that TAFs may function as conduits for the flow of transcriptional regulatory information between activators and the core transcriptional machinery. Numerous activators such as NTF-1, SP1, Bicoid, Hunchback and p53 cannot stimulate transcription *in vitro* unless their target TAFs are present in the TBP-TAF complex (Chen *et al.* 1994, Sauer *et al.* 1995, Thut *et al.* 1995).

In yeast, TAF-dependent promoters exhibit differential TAF-dependence, with some TAFs being required for activation while others are dispensable and TAF-dependence seems to be specified by core promoter elements (Green 2000). Human and *Drosophila* TAF subunits of TFIID have been identified as sequence-specific DNA binding proteins that recognize either the downstream promoter element (DPE) or the initiator (Smale 2001, Shen and Green 1997). Additionally, TAF-dependent transcription was found to correlate with promoter occupancy by TAFs, leading to the definition of two classes of promoters, TAF-independent and TAF-dependent promoters, to which TAFs are recruited with TBP, presumably as TFIID complex (Kuras *et al.* 2000, Li *et al.* 2000). Experiments with chimeric promoters using upstream activation sequences (UASs) and TAF-dependent and independent core-promoters showed that transcription from a TAF-dependent promoter but not TAF-independent promoter required both UAS and core promoter elements (Li *et al.* 2002).

TAFs mediate the activation of transcription of genes with TATA-less promoters and thus play a role in the TBP-independent activation of transcription by recruiting the TFIID complex to such core promoters (Chen and Hampsey 2002). TFIID might play a key regulatory role in the coordinate expression of the ribosomal protein gene regulon, where most ribosomal protein genes lack a canonical TATA element (Mencia *et al.* 2002). The suggestion that TAFs play a central role within the TBP-independent activation of transcription is supported by the identification of the TBP-less complex TFTC (TBP-free TAF-containing complex) in mammalian cells, which is similar to TFIID (Wieczorek *et al.* 1998).

TFIID was thought to be the only sequence-specific initiation factor recognising a Pol II promoter and thus to direct PIC assembly on all promoters. It has been widely believed that a PIC is composed of the same set of GTFs at every protein-coding gene promoter. However, recent findings show that initiation of transcription is also regulated at the level of PIC-formation at core promoters in form of distinct PICs with varying composition and with distinct promoter recognition factors (Müller and Tora 2004).

The observation that Pol II can initiate transcription using only a partial set of the GTFs has challenged the definition of GTFs (Parvin *et al.* 1994, Usheva and Shenk 1994). Additionally, database searches for paralogue genes encoding GTF subunits revealed that single-copy genes encode the components of TFIIB, -E, -F, -H and Pol II but TFIIA subunits which are not essential for transcription *in vitro,* are encoded by paralogue genes in *Drosophila* and humans (Aoyagi and Wassarman 2000).

TFIID might play a key regulatory role in the coordinate expression of the ribosomal protein gene regulon, where most ribosomal protein genes lack a canonical TATA element (Mencia *et al.*

2002). The suggestion that TAFs play a central role within the TBP-independent activation of transcription is supported by the identification of the TBP-less complex TFTC (TBP-free TAF-containing complex) in mammalian cells, which is similar to TFIID (Wieczorek *et al.* 1998). In contrast to the GTFs mentioned above, discoveries in the last years showed the TFIID complex in various compositions, with the rather diverse spectrum of TBP- and TAF-type factors allowing eukaryotic cells to form multiple promoter recognition complexes, differing in composition and function (see Figure **5**).

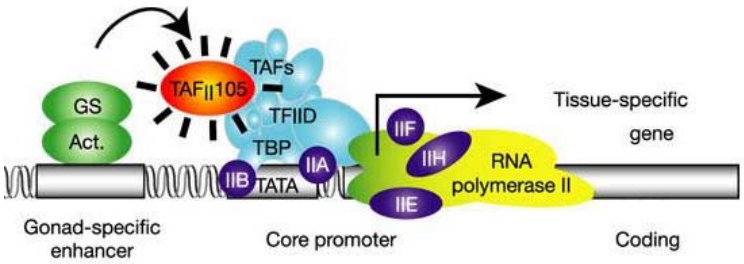

Figure 5. Metazoan gene-selective and tissue-specific TFIID-like complexes by using alternate TAFs and TRFs: An Ovarian-specific complex using TAF4b ($Taf_{II}105$) (Levine and Tjian 2003).

1.2.3 Different TFIID complexes

TAFs also have paralogue genes, which are often expressed in a cell type- or tissue-specific pattern. In yeast, two genes encode TAF5 homologues that are both present in the same TFIID complex (Mitsuzawa *et al.* 2001). *Drosophila melanogaster* has homologues of TAF4 (Nohitter, Nht) and TAF5 (Cannonball, Can), which are expressed only in testis and regulate the expression of specific target genes (Hiller *et al.* 2001). Additionally, *Drosophila* TAF10 and TAF10b have been shown to be differentially expressed during embryogenesis (Georgieva *et al.* 2000). In humans, TAF1L, a retroposed copy of TAF1 and TAF7L are specifically expressed during male germ-cell differentiation (Wang and Page 2002, Pointud *et al.* 2003). TAF1L lacks introns and evidently arose by retroposition of a processed TAF1 mRNA during primate evolution (Wang and Page 2002).

A tissue specific homologue of TAF4 in mammalians, TAF4b, is expressed in B-lymphocytes and in the granulosa cells of the ovary and testis (Dikstein *et al.* 1996b, Freiman *et al.* 2001, see Figure **5**). TAFs have also been described in complexes other than TFIID: yeast SAGA and *Drosophila* TFTC, human PCAF/GCN5 and STAGA complexes (Martinez 2002, Muratoglu *et al.*

2003). TFTC can replace TFIID or TBP both on TATA-containing and TATA-less promoters in *in vitro* transcription assays (Wieczorek *et al.* 1998, Brand *et al.* 1999).

1.2.4 TAF1 is essential for the nucleating function of TFIID

TAF1 interacts directly with TBP and other TAF subunits, is the scaffold for an assembly of the TFIID complex and a broadly acting regulator of transcription (Chen *et al.* 1994, Weinzierl *et al.* 1993, Verrijzer and Tjian 1996). Supporting the fact that TAF1 is an essential protein within TFIID is the observation that it is not possible to create mitotic and germ line TAF1-clones and null alleles of TAF1 are recessive larval lethal (Wassarman *et al.* 2000).

TAF1 engages in strong interactions with TBP, TAF12, TAF11, TAF6, TAF5, TAF4 and TAF2 (Chen *et al.* 1994). Immobilized TAF1 can serve as a scaffold for assembly of TFIID sub complexes and holo-TFIID from recombinant subunits, which suggests that assembly and integrity of TFIID are dependent on TAF1. Inactivation of TAF1 in yeast leads to degradation of other TFIID subunits and inactivation of all yeast TAFs tested to date results in degradation of other TAFs, which suggests that every TAF is necessary for the integrity of TFIID *in vivo* in yeast (Walker *et al.* 1996, Michel *et al.* 1998, Moqtaderi *et al.* 1998).

Defects in TAF1 result in the down regulation of key cell cycle proteins: TAF1 is identical to CCG1, a cell cycle regulatory protein found to be important for G1 cell cycle progression (Sekiguchi *et al.* 1991, Wassarman *et al.* 2000). The hamster ts13 cell line contains a single base pair substitution in the hamster gene encoding TAF1 (ts form of TAF1, glycine to aspartatic acid at position 690), which results in cell cycle arrest in G1 phase, followed by apoptosis of the cells at the restrictive temperature (Hayashida et al. 1994, Sekiguchi et al. 1995). Transcription from cyclin A and cyclin D promoters is reduced when the ts13 cells are shifted from permissive to the restrictive temperature, due to the defects in the transcription properties of TFIID containing the mutant form of TAF1 (Wang *et al.* 1997). Transient expression of human TAF1 in ts13 cells rescued the G1-specific cell cycle arrest (Wang and Tjian 1994). DNA microarray gene expression profiling in the ts13 cell line showed that the transcription of 18% of all protein-coding genes transcribed by Pol II is affected more than twofold at the non-permissive temperature (O'Brien and Tjian 2000).

TAF1 in yeast consists of two proteins (Figure **6**): yTAF1 contains the NH_2-terminal kinase domain (NTK) and bromodomain factor 1 and 2 (Bdf1, Bdf2) contain the COOH-terminal kinase domain (CTK, Matangkasombut *et al.* 2000). Both of them can interact with the TFIID-specific component yTAF7, which underlines that yTAF1 and Bdf1/2 represent the functional equivalent of TAF1 in higher eukaryotes. All TAF1 proteins contain a “high mobility group” (HMG) domain, called HMG-box that is involved in DNA-binding and protein-protein interactions. The HMG-box domain consists of three helices in an irregular array. HMG-box domains are found in one or multiple copies in "HMG motif proteins" which form a large, diverse protein family involved in the regulation of DNA-dependent processes such as transcription, DNA-replication, and DNA-repair. The HMG-box proteins are divided into three sub-families: HMGB, HMGN and the HMGA family. Each family has a characteristic functional sequence motif: the motif of the HMGB family is termed "HMG-box;" that of the HMGN family "nucleosomal binding domain" and that of the HMGA family "AT-hook." The functional motifs, which are characteristic of these canonical HMGs, are widespread among nuclear proteins in a variety of organisms.

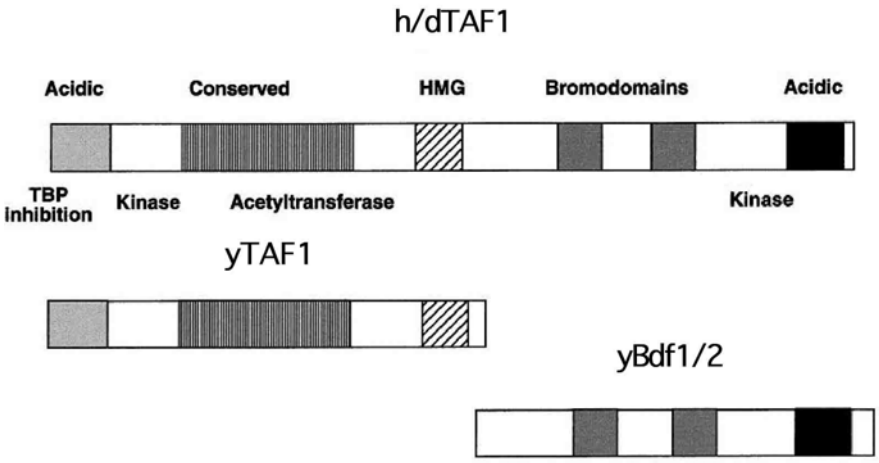

Figure 6. Bdf1 corresponds to the carboxy-terminal region of TAF1. Schematic alignment of yeast Taf1/Bdf1&2 and human/dTAF1 (Matangkasombut *et al.* 2000).

1.2.5 Activators regulate the function of TAF1

Enhancer-bound aactivator interactions increase promoter occupancy of TFIID (i.e. recruitment) and modulate TAF1 regulatory and enzymatic activities (i.e. regulation, Wassarman and Sauer 2001). Physical interactions between TAF1 and activators are a critical component of these mechanisms (Figure **7**). TAF1 binds activators like the HIV transactivator of viral transcription Tat (Weissman *et al.* 1998) or the viral activator protein ICP4 of the herpes simplex virus type 1 (Carrozza and DeLuca 1996), transcriptional repressors such as the retinoblastoma tumor suppressor protein RB (Siegert and Robbins 1999) and other proteins such as Cyclin D (Siegert et al. 2000).

Several lines of evidence support a model in which TAF1-activator interactions contribute to recruitment of TFIID to promoters:

1.) The activation domain of ICP4 binds TAF, indicating that TAF1-activator interactions contribute in recruiting TAF1 to promoters.

2.) Gene-specific promoter occupancy of yeast TAF1 is elevated in response to an activation signal and is reduced after removal of enhancer sites (Kuras *et al*. 2000, Li *et al*. 2000).

3.) Enhancers can confer TAF1 dependence on promoters that are normally TAF1 independent (Wang *et al*. 1997).

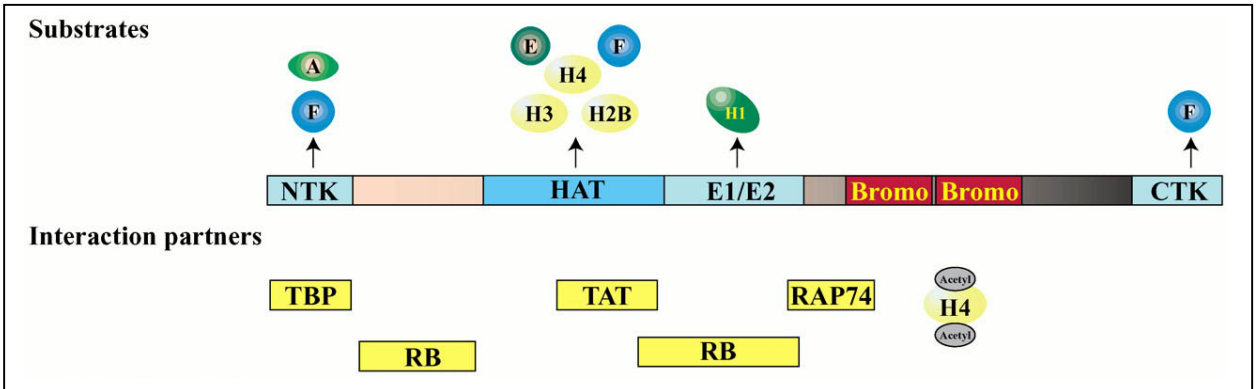

Figure 7. Schematic diagram of metazoan TAF1. Locations of enzymatic domains [NH_2-terminal kinase domain (NTK), COOH-terminal kinase domain (CTK), histone acetyltransferase domain (HAT), and ubiquitin-activating/conjugating domain (E1/E2)] and bromodomains (Bromo) are indicated within the linear TAF1 protein. Substrates for the enzymatic activities are indicated above the TAF1 protein, and interacting proteins are indicated below. Substrates and interacting partners are abbreviated as follows: TFIIA (A), TFIIE (E), TFIIF (F), TATAbinding protein (TBP), retinoblastoma protein (RB), HIV Tat (TAT) and TFIIFα (RAP74) (Wassarman and Sauer 2000).

Once recruited to a promoter, TAF1 could participate in PIC assembly by binding to the RAP74 subunit (TFIIFα) of TFIIF, as suggested by the finding that TAF1 mutants that fail to interact with RAP74 are unable to rescue the *ts13* cell cycle defect (Ruppert and Tjian 1995). The importance of TAF1 interactions with both activators and GTFs argues that TAF1 functions as a classically defined coactivator that bridges activators to PIC assembly.

Activators regulate transcription by modifying TAF1 activities to enhance formation or stability of the PIC on promoter-DNA. Binding of HIV Tat, an activator and repressor of viral gene transcription, to TAF1 inhibits TAF1 HAT activity (Weissman *et al.* 1998). Similarly, binding of RB to TAF1 inhibits TAF1 kinase activity (Siegert and Robbins 1999, Solow *et al*. 2001). In contrast, cyclin D1 suppresses the TAF1-kinase-inhibitory effect of RB (Siegert *et al*. 2000). This shows that regulation of TAF1 activities by interactions with activators, repressors and cell cycle regulators is an integral part to the process of transcriptional activation.

1.2.6 TAF1 plays an essential role in promoter recognition

It was shown that TAF1 contacts the INR, thus activating transcription on TATA-box- as well as TATA-less-promoters (Wu *et al.* 2001). A role for TAFs in recognizing the INR as start site for transcription was suggested by the finding that TFIID containing a TBP subunit that cannot bind to DNA can also not function on TATA-only promoters but can support transcription from TATA-less, INR-containing promoters (Martinez *et al*. 1994).

A minimal complex containing TBP and TAF1 directs basal but not activator-responsive transcription (Chen *et al*. 1994). A recombinant TBP-TAF1-TAF2 complex efficiently binds INR-containing promoters *in vitro* (Verrijzer *et al*. 1995). Moreover, a TAF1-TAF2 complex can support INR-mediated transcription and specifically binds sequences that match the INR consensus sequence from a pool of random sequence oligonucleotides (Verrijzer *et al*. 1995, Chalkley and Verrijzer 1999), indicating that TAF1, together with TAF2, mediates binding of TFIID to the INR.

1.2.7 TAF1 modifies GTFs and other proteins

TAF1 has intrinsic protein kinase activity (Dikstein *et al.* 1996a), histone acetyltransferase activity (Mizzen *et al.* 1996) and ubiquitin-activating and conjugating activity E1/E2 (Pham and Sauer 2000).

TAF1 is a bipartite kinase and contains terminal kinase domains at both ends of the protein (NTK and CTK, Figure **7**). Interestingly, the kinase domains do not show high similarities to other known kinases, suggesting that they might belong to a novel class (Dikstein *et al.* 1996a). The transcription regulator RB, which binds to the NTK-domain and thus inhibits its catalytic activity, regulates the activity of the NTK. However, transcription regulators EA1 and Cyclin D1 can suppress the RB-induced inhibition of the NTK (Shao *et al.* 1995, Siegert and Robbins 1999, Siegert *et al.* 2000).

The NTK phosphorylates the 74 kDa subunit (RAP74) of GTF TFIIF, the β-subunit of TFIIA or tumor suppressor p53 (Dikstein *et al.* 1996a, Solow *et al.* 2001, Li *et al.* 2004).

Dephosphorylation facilitates the ability of RAP74 to support transcription *in vivo*, thus implicating that phosphorylation of RAP74 by TAF1 may be an important mechanism for regulating transcription (Ruppert and Tjian 1995, Kitajima *et al.* 1994). RAP74 and TFIIA appear to represent reasonable candidate substrates for TAF1 kinase activity *in vivo*: endogenous RAP74 is hyperphosphorylated; dephosphorylation of RAP74 reduces its ability to support transcription elongation *in vitro* and phosphorylation of TFIIA stimulates TFIIA-TBP-TATA-element complex formation *in vitro* (Kitajima *et al.* 1994, Solow *et al.* 2001).

NTK-activity is required for TAF1-activity *in vivo*, because a recombinant TAF1 protein that lacks the NTK domain can not rescue the cell-cycle-arrest phenotype of ts13 cells, suggesting that the kinase activity is important for the progression through G1 (O'Brien and Tjian 1998, O'Brien and Tjian 2000).

Both human and *Drosophila* TAF1 contain a histone acetyl transferase (HAT) domain that acetylates not only histones H3 and H4, but also TFIIEβ, the smaller subunit of the GTF TFIIE, *in vitro* (Imhof *et al.* 1997). Additionally, cells expressing HAT-inactive TAF1 mutants arrest in the late G1-phase of the cell cycle, suggesting that TAF1 HAT-activity regulates the expression of genes involved in cell cycle progression (Dunphy *et al.* 2000).

TAF1 has also been demonstrated to mediate monoubiquitination of histone H1, a linker histone that binds to DNA between adjacent nucleosomes, via its E1/E2 domain (Pham and Sauer 2000).

1.3 Chromatin

The fundamental structural unit of eukaryotic chromatin is the nucleosome. It consists of pairs of each of the core histones H2A, H2B, H3 and H4, thereby creating the histone octamer and a single molecule of the linker histone H1. The whole nucleosome spans about 180 base pairs (bp) of DNA. To gain access to the DNA itself, transcription-factors need to pass the organization of the DNA into the higher-structures of chromatin. Central mechanisms for regulating chromatin include reorganization as well as disassembly of the higher structures of the nucleosome (Narlikar *et al.* 2002).

1.3.1 The structure of chromatin

Eukaryotic genomic DNA is organized into repeating arrays of nucleosomes: 147 bp of DNA are wrapped 1.75 times around the histone octamer. The complex of a histone-octamer and the surrounding DNA is also termed “nucleosome-core-particle” (Hansen 2002).
Histone H1 and related "linker" histones bind to the extranucleosomal linker-DNA that separates core nucleosomes and H1-H1 interactions participate in the compaction of the 10 nm beads-on-a-string-structure into the 30 nm fiber (Thoma *et al.* 1979). Compared to core-histones, linker histones are evolutionary less conserved (Baxevanis *et al*. 1995, Lee and Young 2000, Horn and Peterson 2002).
The 30 nm fiber can coil to form a hollow tube (100 nm fiber), which finally forms the chromatin, which is further condensed into the compact chromosomes observed during mitosis (Hansen 2002, Horn and Peterson 2002). This complex compaction and structuring of DNA serves several functions: The overall negative charge of the DNA is neutralized by the positive charge of the histones and the large DNA fits into the small nucleus (Figure **8**).
Each core-histone consists of a globular domain, which intermediates histone/histone- and histone/DNA-interactions within the nucleosome and a flexible amino terminal region, called the NH_2-terminal tail (Arents *et al.* 1991, Luger *et al.* 1997a, Suto *et al*. 2000).

The current model of the nucleosome which is in part derived from high-resolution structures, states that the NH_2-terminal tails of all four histones protrude between the gyres of the DNA and the histone core binds the DNA backbone at 14 superhelical locations, resulting in more than 120 direct atomic histone-DNA interactions (Luger *et al.* 1997a, Khorasanizadeh 2004, Luger 2003). While the NH_2-terminal-tails are unstructured in the context of a single nucleosome, they take part in formation of higher-order chromatin organization by mediating interactions with nucleosomes or other chromatin proteins (Luger *et al.* 1997b, Hansen 2002) and the tails interact also with linker-DNA (Pruss and Wolffe 1993, Angelov *et al.* 2001).
Additionally, the flexible histone tails are targets for numerous covalent modifications (Strahl and Allis 2000, see below).
Histone H3 and H2A variants confer specialized function to nucleosomes. One prominent H3 variant is the “centromere protein A” (CENP-A) that participates in the assembly of specialized centromeric nucleosomes that are essential for centromere structure and function (Ahmad and Henikoff 2001). The histone H2A variant H2A.Z is important for silencing for a specific subset of genes. The crystal structure of a H2A.Z containing nucleosome core particles revealed only subtle changes on the accessible surface area of the nucleosome (Suto *et al.* 2000). However the role of histone variants is unclear and their structure and function remain unknown.

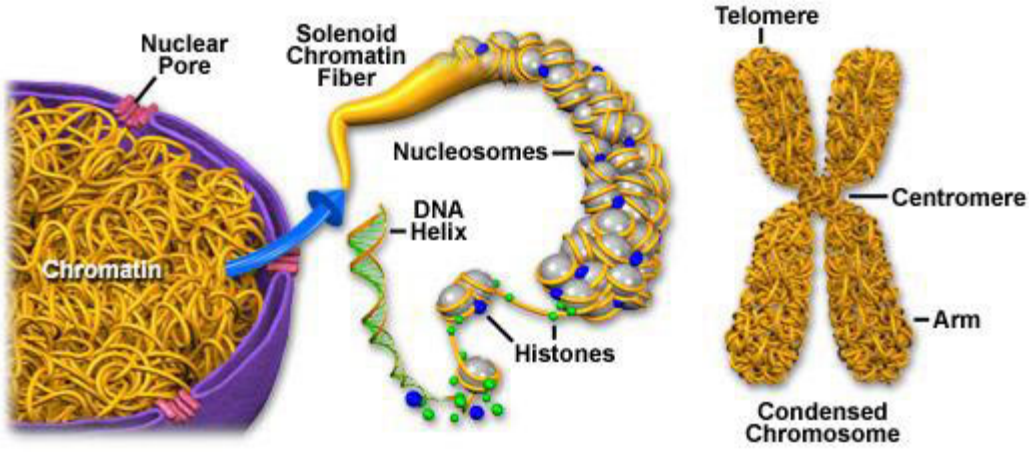

Figure 8. The structure of chromatin and the condensed chromosome.

1.3.2 The dynamic nucleosome

Earlier studies led to the proposal that the nucleosome is a monolithic, static assembly of DNA and protein. In contrast, newer studies provide evidence that chromatin is not static and describe

the model of the “dynamic nucleosome”: Nucleosomes were shown to have a dynamic equilibrium *in vitro* between a fully wrapped state and a set of partially unwrapped states, in which stretches of DNA transiently detach from the histone surface and then rewrap in a spontaneous and rapid fashion (Li *et al.* 2005).
Histone modifications and chromatin-remodelers facilitate changes in the structure of chromatin and DNA (Chakravarthy *et al.* 2005).
Chromatin remodeling ATPases, histone modifying enzymes as well as the underlying DNA sequence dynamically change chromatin structure, thus making the chromatin more or less accessible (Mellor 2005). Enzymes that act on chromatin themselves show dynamic interactions with the chromatin template and allow rapid changes to chromatin states (Karpova *et al.* 2004, Phair *et al.* 2004). Recent data *in vivo* suggest that within highly condensed mitotic chromosomes, both core histones and chromatin-associated factors are in dynamic equilibrium, which varies with the phases of mitosis (Chen *et al.* 2005). Additionally, replication-independent core histone replacement has been demonstrated and extensive nucleosome displacement and replacement occurs upon gene activation, particularly at highly active domains of the chromatin (Tagami *et al.* 2004, Schwabish and Struhl 2004, Schwartz and Ahmad 2005, Thiriet and Hayes 2005). This dissociative equilibrium is shifted in opposite directions, depending on the enzymes which modificate the state of the nucleosome.

1.4 Regulation of chromatin activity

Nucleosomes can restrict the binding of proteins to DNA. Among major classes of protein factors that regulate the dynamic structure of chromatin and therefore the accessibility of DNA to specific DNA binding factors, are chromatin remodeling complexes and enzymes that posttranslationally modify histones.
1.) ATP-dependent chromatin-remodeling complexes can “slide”, relocate or disassemble nucleosomes on DNA, in this way expose or occlude DNA sequences and thereby facilitate or inhibit the interaction of DNA-binding proteins with DNA (Becker and Horz 2002, Becker 2002).
2.) Specific enzymes can posttranslationally modify histones by adding or removing chemical moieties. Posttranslational histone modifications include acetylation, methylation, ubiquitination and phosphorylation, which may modulate the contacts between histones and DNA (Cheung *et al.* 2000a). Especially multiple covalent modifications of histone tails have been well

characterized and shown to play a global role in gene expression (Berger 2002, Jenuwein and Allis 2001).

1.4.1 ATP-dependent chromatin-remodeling complexes

ATP-dependent chromatin remodeling complexes (CRCs) are multi-protein complexes and use ATP-hydrolysis to overcome the considerable energy barrier for remodeling nucleosomes within chromatin (Becker and Horz 2002). The remodeling process requires ATP-binding to the conserved ATPase-subunit of these complexes, which show homology to each other within the yeast Snf2-ATPase (Narlikar *et al.* 2002).
Based on the identity of the catalytic ATPase-subunit, four families of CRCs have been identified so far, the SWI/SNF, ISWI, CHD (also called Mi-2 family) and INO80 family (Narlikar *et al.* 2002, Khorasanizadeh 2004).
The characterized chromatin-remodeling complexes have been implicated in at least three types of activities. These are DNA looping (Fan *et al.* 2003, Fazzio and Tsukiyama 2003), octamer sliding (Kassabov *et al.* 2003) and histone substitution (Mizuguchi *et al.* 2004).

1.4.2 Covalent modifications of histones: the “histone code”

A multitude of posttranslational histone modifications (Figure **9**) has been discovered, most of them attached to phylogenetically highly conserved amino acids in the histone tails, such as acetylation of lysine-residues, methylation of lysine- or arginine-residues and phosphorylation of serine- or threonine-residues. Additionally, histones can be ubiquitinated at their COOH-terminus (Zhang and Reinberg 2001, Khorasanizadeh 2004).
Recently, modifications of histones have been discovered within the structured globular domain (Zhang *et al*. 2003, Freitas *et al*. 2004). Several of these modifications occur near the nucleosome lateral surface, close to the DNA wrapped around the histones, indicating that these modifications may affect the interaction between the histone octamer and the DNA (Cosgrove *et al.* 2004). However, most of recent research efforts have focused on post-translational histone modifications occurring at the histone tails and have revealed that histone modifications play an essential role for chromosome function.

In context of the dynamic nucleosome, it appears that prior acetylation of histone tails is a prerequisite for nucleosome remodeling by CRCs *in vivo*, thus resulting in disassembly or repositioning of nucleosomes (Lomvardas and Thanos 2001, Reinke and Horz 2003, Nourani *et al.* 2004).

The correlation of specific histone modifications with the execution of specific biological events gave rise to the histone code hypothesis, which postulates that specific histone modifications determine chromosome function (Strahl and Allis 2000). Specifically modified amino acid residues are also referred to as “marks”, bearing information for the specific functional code of a histone and the packaged DNA.

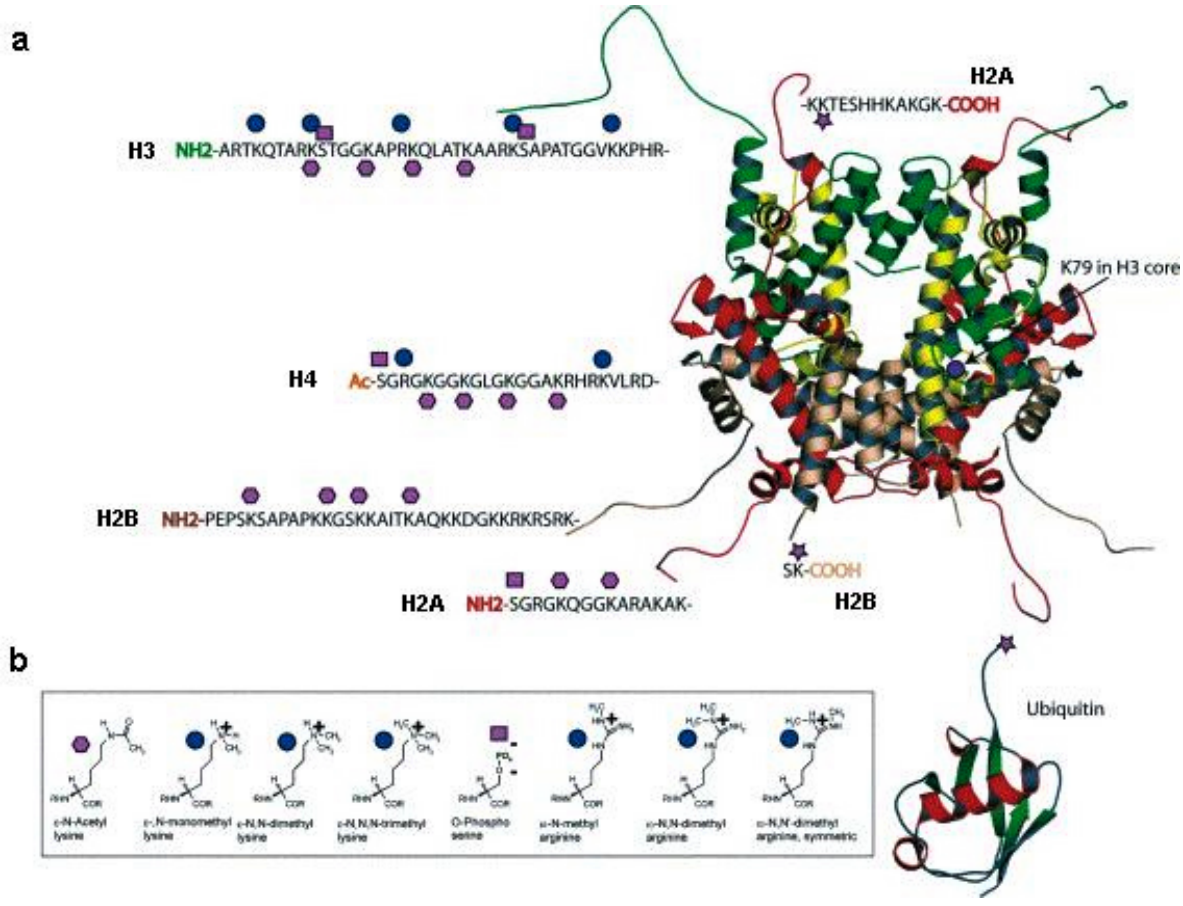

Figure 9. Posttranslational modifications on the core histones (human).
a The histone octamer portion of the nucleosome, the aminosequences of the NH_2- and COOH-terminal ends of single histones are shown, sites of modifications are marked.
b Chemical structure of the covalent modifications of the amino acids as shown in **a** (Khorasanizadeh 2004).

1.4.2.1 Acetylation

The reversible histone acetylation on lysine residues is so far the best characterized of all modifications (Grunstein 1997, Jenuwein and Allis 2001, Berger 2002, Kurdistani and Grunstein 2003). Hyperacetylation of lysine in the histone tails was proposed to be involved in activation of transcription over 40 years ago and has subsequently been strongly correlated with transcriptionally active genes (Allfrey *et al.* 1964) and hyperacetylated chromatin regions display a higher accessibility of their DNA (Hebbes *et al.* 1988 and 1994). The discovery that transcriptional regulators can have histone acetyltransferase- (HAT-) and histone deacetylase- (HDAC-) activity, provided evidence that acetylation is involved in transcription (Struhl 1998, Kornberg and Lorch 1999).

Besides transcription, histone acetylation contributes to other biological processes, for example, histone deposition: Histones H3 and H4 are brought to replicating chromatin in an acetylated state that becomes erased after replication (Turner and O'Neill 1995). Studies of the acetylation levels of bulk histones have shown that up to 13 of the 30 lysine-residues in the tail in a histone octamer are acetylated (Roth *et al.* 2001). Major acetylation sites are lysines 9, 14, 18 and 23 in histone H3 in most species (Thorne *et al.* 1990).

HATs are present in transcription factors and CRCs (Roth *et al.* 2001). HAT domains contain a central conserved core unit that is important for acetyl-coenzyme-A (acetyl-CoA) binding and a cleft, used for substrate recognition, which lies directly over the cofactor-binding pocket (Khorasanizadeh 2004). HATs reside within the context of large multisubunit complexes, separated into two main classes of histone acetyltransferases: Type A (nuclear) HATs and type B (cytoplasmic) HATs (Narlikar *et al.* 2002). Nuclear regulatory complexes contain mainly type A HATs, of which three families have been identified: the GNAT family, containing yeast GCN5 and human PCAF, the MYST family (containing yeast complex NuA4 with its HAT yESA1) and the P300/CBP family.

The different HAT-complexes have different but distinct histone specificities (Roth *et al.* 2001) even if they contain similar subunits: The HAT GCN5 is part of two complexes in yeast, Ada and SAGA, each with distinct biological functions (Marmorstein 2001a). GCN5 acetylates histones H3 and H4 *in vitro*, but in context with Ada or SAGA H3 and H2B *in vivo*.

Acetylation is reversed by HDACs, which fall into three main classes (Khochbin *et al.* 2001, Marmorstein 2001b). Those classes reside in different complexes: The class I HDAC family

contains for example the remodeling-complex NuRD with its subunit HDAC1 or the transcriptional corepressor Sin3 with its subunit HDAC2. Class II HDACs have yet to be purified. A prominent member of the class III HDACs is Sir2 that is involved in heterochromatin silencing at silent mating loci, telomeres and ribosomal DNA (Moazed 2001). The histone specificities of the class I HDAC family complexes are linked to transcriptional repression: the yeast homolog of HDAC1, Rpd3, deacetylates all sites except lysine 12 on histone H4, a site that is linked to heterochromatic silencing and whose acetylation by the *Drosophila* HAT dMOF is linked to activation of the male X chromosome (Roth *et al.* 2001, Suka *et al.* 2001). The fact that the NuRD complex includes the chromatin remodeling ATPase Mi-2 protein and HDAC activity is an example of functional cooperation between chromatin-remodelers and histone deacetylases (Tong *et al.* 1998, Wade *et al.* 1998, Zhang *et al.* 1998).
This interplay between HAT- and HDAC-complexes maintains the steady-state level of acetylation (Reid *et al.* 2000, Vogelauer *et al.* 2000).

1.4.2.2 Ubiquitination

The reversible covalent attachment of multiple copies of ubiquitin, a 79 amino acid polypeptide, or the ubiquitin-related SUMO (small ubiquitin-related modifier) is often associated with the degradation of proteins through the proteasome pathway (Varshavsky 1997). In contrast, histones are mono-ubiquitinated and transcriptionally active chromatin has been linked to ubiquitination:
Histone ubiquitination was first described for H2A, H2B and H3 and ubiquitinated H2A and H2B were detected in transcriptionally active chromatin (Nickel *et al.* 1989, Lee and Young 2000, Robzyk *et al.* 2000). Histone ubiquitination occurs at lysine residues at the COOH-tail and involves three enzymatic activities: ubiquitin activating- (E1), ubiquitin conjugating- (E2) and ubiquitin ligase-activity (E3) (Hershko and Ciechanover 1998). While histone ubiquitination has typically been attributed to positive control of transcription (Zhang *et al*. 2003), sumolation of histone H4 is involved in transcriptional repression (Shiio and Eisenmann 2003). Not only core-histones but also linker histones are the target for ubiquitination, TAF1 mono-ubiquitinates histone H1 in *Drosophila* embryos (Pham and Sauer 2000).

1.4.2.3 Methylation

Methylation occurs on lysine and arginine residues in histones and the ε-aminogroup of lysine residues can be mono-, di- or tri-methylated, while the addition of methyl-groups is correlated with an increase in the basicity of the lysine side chain. While acetylation is generally correlated with active transcription, histone methylation has been linked to both transcriptional activation and repression (Roth *et al.* 2001, Zhang and Reinberg 2001).

Histone methylation was believed to represent a stable modification with important functions in epigenetic gene control and for organizing chromatin domains and the enzymes that can add up to three methyl-groups to a single residue are called histone methyltransferases (HMTs). The domain containing the enzymatic activity responsible for lysine methylation of histone tails is called SET-domain, which consists of 130 amino acids folding into three discrete β-sheet regions flanked by α-helices (Khorasanizadeh 2004). The cofactor S-adenosyl-L-methionine (SAM) binds to a concave surface of the enzyme, providing methyl groups for modification. The histone tail inserts as a parallel strand between two strands of the SET-domain (Zhang and Reinberg 2001, Khorasanizadeh 2004). A HMT, which is untypical since it does not contain a SET-domain, is Dot1, which despite the lack of SET, still specifically methylates lysine 79 of histone H3 in the core domain. Dot1 methylation of lysine 79 in H3 mediates gene-silencing mechanisms in yeast (Min *et al.* 2003b, Park *et al.* 2002).

Methylation was long thought to be irreversible, but newer studies show evidence for a histone demethylase (LSD1) that reverts an activating methyl mark and is only compatible with mono- and dimethylation (Shi *et al.* 2004). Recent studies show that LSD1-demethylation works also on nucleosomes in combined action with other factors (Lee *et al.* 2005) and is involved in transcriptional events (Metzger *et al.* 2005). To date, histone arginine demethylases have not been identified.

1.4.2.4 Phosphorylation

Reversible histone phosphorylation contributes to transcription, chromosome condensation, DNA repair and apoptosis (Cheung *et al*. 2000a). Phosphorylation of specific amino acid residues alters the electrostatic charge of the modified and surrounding amino acids. Histone tails contain short stretches of basic amino acids, which are flanked by potential phosphorylation sites. These

"basic patches" may engage in histone-DNA interactions or other histones or chromatin-associated factors. Reversible phosphorylation represents a mechanism to disrupt the electrostatic interactions in order to unfold the nucleosomes (Cheung *et al.* 2000b). Studies of modulation of "charge patches" in the linker histone H1 via reversible phosphorylation showed that phosphorylation regulates transcription, presumably through altering higher-order chromatin structure (Dou and Gorovsky 2000).

H3 phosphorylation during gene expression was discovered in mammalian cells as an immediate early response to mitogenic stimulation, leading to transcription of c-Fos and c-Jun (Mahadevan *et al.* 1991). Kinases that phosphorylate H3 are Aurora-B/Ipl1, PKA, Rsk-2 and Msk-1, which tend to target serine/threonine sites that are surrounded by basic residues.

When mammalian cells are exposed to mitogen or stress, the time course of H3 phosphorylation corresponds to the transient expression of activated immediate early genes (Thomson *et al.* 1999a). The p42/p44 mitogen-activated protein (MAP) kinase pathway (also known as ERK pathway) as well as the stress-activated p38 pathway, can each induce histone H3 phosphorylation. Additionally, the ERK-activated Rsk-2 (ribosomal protein S6 kinase 2) kinase has been shown to be directly involved in H3 phosphorylation *in vivo* in studies with RSK-2-deficient cells, which were impaired in the transcriptional activation of c-fos gene and EGF-induced phosphorylation (De Cesare *et al.* 1998, Sassone-Corsi *et al.* 1999). Msk-1 (MAP- and Stress-activated kinase 1) is activated by ERK and p38 pathways and it is possible that both of these pathways can activate this kinase to induce H3 phosphorylation (Thomson *et al.* 1999b). These studies provide a direct link between signal transduction pathways and histone phosphorylation.

Nucleosomes containing phosphorylated and acetylated H3 are associated with EGF-activated genes, indicating that both modifications may cooperate in the transcription of these genes and several studies point out that H3 phosphorylation and acetylation are tightly coupled in response to EGF stimulation (Barratt *et al.* 1994, Cheung *et al.* 2000b, Clayton *et al.* 2000).

The probably best-studied histone-phosphorylation so far is at serine 10 in the NH_2-terminal tail of H3 (H3S10), which has been correlated with transcription activation, mitosis and chromosome condensation (Nowak and Corces 2004). The kinase Ipl1 in yeast, which is expressed in M-phase, phosphorylates H3S10 *in vivo* and genetic inactivation of *Ipl1* results in defects in chromosome segregation during mitosis (Francisco *et al.* 1994, Biggins *et al.* 1999) and complete loss of H3 phosphorylation during mitosis (Hsu *et al.* 2000). Phosphorylation of H3S10 is reversed by the protein phosphatase 1 (PP1) family and the yeast PP1 homolog Glc7, which acts also on H3S10 (Hsu *et al.* 2000).

Histone phosphorylation has been linked to cellular processes other than transcription, such as DNA damage response (Vidanes *et al.* 2005). Histone H2B becomes phosphorylated at serine 32 in apoptotic mammalian cells and the phosphorylation event coincides with nucleosomal DNA fragmentation (Ajiro 2000). The H2A variant of mammalian cells, H2A.X, is rapidly phosphorylated at its C-terminal serine 139 upon exposure to ionizing radiation or apoptosis-induced signals. In yeast, phosphorylation of the corresponding serine 129 has been found in association with DNA damage (Rogakou 1998, 1999 and 2000). Phosphorylation of histone H3 at serine 14 is uniquely associated with apoptotic chromatin in species ranging from frogs to humans (Cheung *et al.* 2003). Phosphorylation of nucleosome-associated proteins in areas of transcriptional active nucleosomes is mediated by the protein-kinase CK2 (casein kinase 2) in mammalian cells (Guo *et al.* 1998, Guo *et al.* 1999). A recent study suggests that phosphorylation of serine 1 of histone H4 in yeast requires CK2 and occurs within 1 kb of DNA double-strand breaks (DSBs). Though the mechanism how the phosphorylation might mediate DSB-repair is unclear, these are first indicators that there might be a histone-code for DNA-damage response (Cheung *et al.* 2005).

1.4.2.5 Functions of histone modifications

Histone modifications have different functions. They might change the charge of a specific area within the histone tail, resulting in a different electrostatic conformation. Another function is the change of binding sites for proteins that interact with histones. Specific histone modifications can increase the binding of specific proteins or inhibit binding to histones. Several histone modifications can work in a combinatorial fashion to have an effect, and this crosstalk between several possibly different kinds of modifications transfers specific characteristics to the modified region.

1.4.2.5.1 Histone modifications change the charge of histone tails

Acetylation and methylation result from addition of acetyl or methyl groups, usually to the basic amino acid lysine and these additions prevent positive charges from forming on the amino group. Phosphorylation adds a phosphate group, usually to the hydroxyl group of a serine or threonine residue, less frequently to a threonine residue. The phosphate group introduces negative

charges and has therefore a significant effect upon the electrostatic properties of the whole protein.

1.4.2.5.2 Histone modifications can recruit chromatin binding proteins

Histone modifications can function as target binding sites for chromatin binding proteins. Acetylation plays an essential role in transcriptional activation by recruitment of transcription factors:

An acetylated lysine residue has no longer a basic side chain, allowing it to be recognized by bromodomain modules which are conserved protein motifs of around 100 aminoacids and found in many chromatin-associated proteins. The bromodomain contacts the acetyl-carbonyl group of acetyl-lysine with a conserved asparagine residue and additional contacts to adjacent residues add specificity to the interaction (Owen *et al.* 2000, Jacobson *et al.* 2000). The central ATPase-subunits of various CRCs contain histone modification recognition motifs, which interact with acetylated/methylated histone tails (Langst and Becker 2004). Swi/Snf and Gcn5 bromodomains are required for the stable association of Swi/Snf with acetylated histone tails (Syntichaki *et al.* 2000, Hassan *et al.* 2002) and the tandem bromodomains of the yeast RSC nucleosome-remodeling complex recognize acetylated H3 Lysine 14 (Kasten *et al.* 2004). There is evidence, that the HAT Gcn5 sequentially acetylates a subset of lysines in histones H3 and H4, which leads to the ordered recruitment of bromodomain-containing transcription complexes (Agalioti *et al.* 2002). Acetylation of lysine 8 in histone H4 for example leads to recruitment of TFIID, whose bromodomains anchor it directly to nucleosomes at promoter regions (Khorasanizadeh 2004). TAF1 contains a double bromodomain (DBD), which enables TAF1 to bind to the acetylated N-terminal tail of histone H4. The DBD binds most tightly to H4 acetylated at lysine 5 and 12. This is consistent with the crystal structure of the DBD, which shows that the binding pockets for acetyllysine span a distance equivalent to seven residues (Jacobson *et al.* 2000). Therefore, the TAF1 bromodomains may target TFIID to chromatin-packaged promoters. Another example is the yeast SAGA complex, which interacts with acetylated nucleosomes through its bromodomain (Hassan *et al.* 2002).

Methylated lysine and arginine marks on histones can act as a recruitment signal or anchor for transcription factors:

The chromodomain conserved module, which is found in chromatin-associated proteins related to heterochromatin-protein HP1, binds to histone tails with methylated lysine in an affinity-

specific manner, being highest for tri-methyllysine and lowest for mono-methyllysine (Jacobs *et al.* 2001, Lachner *et al.* 2001, Bannister *et al.* 2001). Chromodomains are, like bromodomains, also found in several CRCs that also have HAT, SET or ATPase domains. Two distinct self-sustaining epigenetic silencing mechanisms are identified that are linked to the methylation of lysines 9 and 27 in H3. Methyllysine 9 plays a direct role in chromatin condensation and gene silencing. The chromodomain of the Polycomb protein specifically binds to H3 containing methyllysine 27 to mediate gene silencing during developmental stages (Fischle *et al.* 2003b, Min *et al.* 2003a). An example of epigenetic activation by lysine methylation involves the *Drosophila* Ash1 protein that methylates lysines 4 and 9 in histone H3 and lysine 20 in H4, creating a distinct signal for the recruitment of the epigenetic activator complex called Brahma to chromatin. Brahma binding inhibits HP1 association with a nucleosome and thus, Ash1 maintains a transcriptionally active state (Beisel *et al.* 2002). The human HMT Suv39H is a dominant modifier of heterochromatin-induced gene silencing. It methylates H3 at lysine 9 and contains both chromo- and SET-domains (Rea *et al.* 2000).

In the case of heterochromatin assembly, Suv39H methylates lysine-residue 9 of histone H3. The methylated lysine residue is then recognized and bound by the chromodomain protein HP1 (Bannister *et al.* 2001, Lachner *et al.* 2001). The Suv39H-HP1 methylation system may also mediate heterochromatin propagation.

In addition, studies show that H3S10 phosphorylation can also promote TBP recruitment through distinct promoter-specific mechanisms (Cheung *et al*. 2000a).

1.4.2.5.3 Crosstalk between different histone modifications

Several observations point out that specific histone modifications together play a role in transcription. For example, hyperacetylation of histone tails and H3 Lysine 4 methylation are both associated with transcriptionally active euchromatin, whereas hypoacetylation of H3 Lysine 9 is associated with heterochromatin (Noma *et al.* 2001).

Additionally, histone phosphorylation and methylation regulate histone acetylation in a combinatorial fashion (Berger 2001, Zhang and Reinberg 2001). For example phosphorylation of serine 10 in histone H3 enhances lysine 14 acetylation and these together promote transcription (Clements *et al.* 2003).

Besides the fact that multifunctional proteins like TAF1 show several modifying activities like HAT and ubiquitin-activating/conjugating (UBAC) activities, sequential ubiquitination and

deubiquitination can be concerted with lysine methylation and these events are important for transcription regulation (Henry *et al.* 2003, Daniel *et al.* 2004). For example, H2B ubiquitination at lysine 123 is important for the methylation of lysines 4 and 79 in histone H3 in yeast while Ubp8, a subunit of the SAGA complex, has been shown to remove the ubiquitin from the H2B lysine. A mutation that changes lysine at position 123 to an arginine perturbs silencing at the telomere and links ubiquitination to methylation of H3 and transcriptional silencing (Sun and Allis 2002).

A connection between methyllysine binding of chromodomains and acetylation has been discovered recently in yeast, which further underlines the cooperation between different chromatin modifying complexes in the nucleus (Pray-Grant *et al.* 2005).

Methylation of specific arginines in histone H3 and H4 correlate with acetylation and transcriptional activation (Zhang and Reinberg 2001). For example, the methylation of arginine 3 on histone H4 facilitates H4 acetylation and enhances transcription activation by nuclear hormone receptors (Wang *et al.* 2001).

Several transcription-associated HATs preferentially acetylate H3-S10P over the unmodified form as substrate (Cheung *et al.* 2000b, Lo *et al.* 2000). The genes INO1 and GAL1 are transcriptionally regulated in part by linked H3 modifications: Snf1-kinase-mediated phosphoserine 10 and GCN5-mediated acetyllysine 14 (Lo *et al.* 2001, Lo *et al.* 2005).

1.4.2.6 Histone code mechanisms: “binary switches” and “modification cassettes”

With the discovery of many histone modifications and their interplay in various organisms led to hypothetical models how the histone code might from a mechanism for the regulation of transcription. Most sites of post-translational modifications are extremely conserved and the high density and versatility observed on histones lead to the hypothesis that local clusters of modifications may be used in a combinatorial fashion to regulate chromosome structure and function. Possible models for the action of these clusters were described as “binary switches” and “modification cassettes” (Fischle *et al.* 2003a).

According to the binary switch model, modification by either phosphorylation or ubiquitination of a site adjacent or nearby to a methylation mark that engages an effector module, e.g. a CRC, could regulate binding to that factor.

It was shown that binding proteins of heterochromatic methylation marks have a fast mutual exchange rate with their environment and are not statically bound to their target sites, which

supports a kinetically controlled binding mechanism model (Festenstein et al. 2003, Cheutin et al. 2003). A binary switch has been proposed for the function of phosphoserines adjacent to methyllysines in histone tails in which the phosphorylation may work to neutralize the effect of the lysine methylation: especially in histone H3 serines 10 and 28 are phospho-marks flanking methyllysines, suggesting a possible link to the binary switch model in gene silencing (Fischle *et al.* 2003a).

Beyond binary switch mechanisms between two neighboring or nearby marks, linear strings of densely clustered histone modifications might act as discrete information units mediating signals. Short clusters of dense mark combinations could form defined "modification cassettes" with specific biological readouts that depend on their modification state. For example, a "cassette" of 3 marks could have 2^3 possible states if each mark can be modified independently. Such cassettes with dense assortment of modification marks have been described for histone H3 and H4 tails (Fischle *et al.* 2003a). They were are also found in minor histone variants as well as in the basic COOH-terminal region of the tumor suppressor p53, which is known to have similar crosstalk between different modification marks than histones: For example, phospho-marks facilitating consecutive acetylation and acetyl-marks possibly interfering with ubiquitination (Appella and Anderson 2000, Brooks and Gu 2003).

The NH_2-terminus of minor human H2A variants like H2A.1 and H2A.Z show conserved motifs which might be putative modification cassettes, where phosphorylation of serine 1 might interact with two other modifications, methylation of arginine 3 and acetylation of lysine 5. Interestingly, this motif appears also in the NH_2-terminal tail of human histone H4, where a second putative modification cassette might exist at an acid-labile phosphorylation site around histidine 18 (Fischle *et al.* 2003a, Fujitaki *et al.* 1981).

1.4.2.7 TAF1 modifies histones

TAF1 is a multifunctional enzyme and its many enzymatic activities also recognize histones as substrates:

TAF1 is a histone acetyltransferase and binds to acetylated histones: The HAT domain of TAF1 can acetylate NH_2-terminal-tail lysines in H3 *in vitro*, preferred there at lysine 14, H4 and H2A (Mizzen *et al.* 1996, Wassarman *et al.* 2000).

Additionally to its acetylating function, TAF1 is also an ubiquitin-activating/conjugating (ubac) enzyme: TAF1 has been demonstrated to mediate mono-ubiquitination of the linker histone H1 *in*

vitro. Mono-ubiquitination requires the sequential activity of ubiquitin-activating (E1) and ubiquitin-conjugating (E2) enzymes (Ciechanover *et al*. 2000). TAF1 contains both of these activities and it does not require an E3 ubiquitin-ligase to mono-ubiquitinate histone H1 *in vitro*: it links ubiquitin covalently via a thioester bond in an ATP-dependent manner (E1 activity) and it transfers activated ubiquitin to histone H1 via an isopeptide bond (E2 activity). The E1 and E2 activities of *Drosophila* and human TAF1 reside in the central region of the protein (figure 5, Pham and Sauer 2000, Wassarman and Sauer 2000). It is unclear how mono-ubiquitination of histone H1 by TAF1 mediates transcription, but inactivation of the TAF1 E1/E2 activity *in vivo* reduces the cellular level of mono-ubiquitinated histone H1 and the expression of genes targeted by the maternal activator Dorsal (Pham and Sauer 2000).

The COOH-terminal kinase domain of human TAF1 contains autokinase-activity (Dikstein *et al.* 1996). Interestingly, this CTK shows no apparent homology to other known protein kinases, including the NTK of TAF1. *Drosophila* TAF1 has a similar structure like its human counterpart and its NTK also autophosphorylates TAF1 and transphosphorylates RAP74. Previous studies advancing this work showed that hTAF1 and the CTK of dTAF1 are able to phosphorylate histone H2B, a new substrate for TAF1, *in vitro* (Kwoczynski 2002, Maile *et al.* 2004). Serine 33 (H2B-S33) is a conserved serine/threonine-phosphorylation site within H2B of several organisms (see Figure **10**).

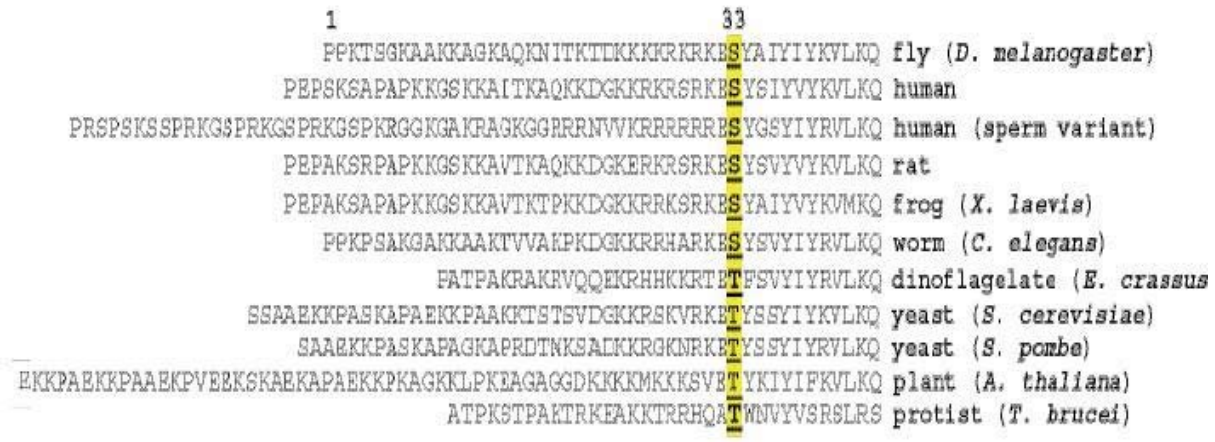

Figure 10. Serine/threonine at position 33 in the N-terminal tail of H2B is conserved among various organisms (Maile *et al.* 2004).

A target gene for TAF1 in Drosophila is the gene snail, which encodes a highly conserved, zinc-finger transcription factor that specifies mesodermal cell fate in a variety of organisms. *In situ*-hybridisation experiments showed that Snail expression is reduced in mutant TAF1 *Drosophila*

embryos containing a CTK-deletion of TAF1, leading to the hypothesis that the CTK may be important for the activation of transcription by TFIID *in vivo* (Kwoczynski 2002).

1.5 Specific aims of this study

During the last decade, the field of chromatin research proceeded in a fast pace. The discovery of the histone code and corresponding histone modifying enzymes added more complexity to the mechanisms of transcriptional regulation (Strahl and Allis 2000).

One of the histone modifying enzymes in the focus of research is TAF1, a subunit of the GTF TFIID and a multifunctional enzyme, whose activities contribute to transcription activation in multiple ways (Wassarman and Sauer 2001).

Previous studies revealed that the CTK of dTAF1 phosphorylates H2B-S33 *in vitro* and that the CTK is required for TFIID-dependent transcription activation (Kwoczynski 2002).

These results imply that CTK-mediated phosphorylation of H2B-S33 is involved in transcriptional regulation.

The main goal of this work was to uncover the role of the CTK-mediated phosphorylation of H2B-S33 in transcriptional activation. To achieve this goal, several tasks were assessed:

RNA interference was used to detect the CTK-mediated phosphorylation of H2B-S33 *in vivo.*

Another task was to characterize the kinase-activity as an integral part of the CTK of TAF1.

The identification and localization of the catalytic domain of the CTK and its specific kinase-motifs needed to be assessed with computational and experimental methods.

An important task was to correlate the transcriptional activation of target genes of TAF1 with the presence of H2B-S33P with *in vivo* assays.

The results of this work were intended to identify TAF1CTK as a major kinase of H2B-S33, to identify the catalytic motif in the CTK responsible for phosphorylation of H2BS33P and to reveal the importance of H2B-S33 phosphorylation by CTK at the promoter of TAF1 target genes for transcriptional activation in *Drosophila*.

2. Materials and Methods

2.1 Materials

2.1.1 Laboratory Equipment

ABI 7700 QPCR-Cycler	CIF: Core Instrumental Facility, UCR
Cell counting chamber	Neubauer
Centrifuge 5415D	Eppendorf
Centrifuge 5810R	Eppendorf
Centrifuge Biofuge pico	Heraeus
Chromatography columns	Pharmacia Biotech
Class II A2 Biological Safety Cabinet	ThermoForma
Computer, Hardware	iMac, 800 MHz
Computer, Software:	Microsoft Excel
	Microsoft Word
	Microsoft Powerpoint
	Mozilla Firefox Internet Browser
	Lasergene Navigator, DNAStar
	Adobe Photoshop 5.0
	DNA Strider 1.3
Custom DNA-gel system	ZMBH Heidelberg, Workshop
Eagle Eye II still video system	Stratagene
Freezer -20°C	Frigidaire
Freezer -80°C	ThermoForma
Fridge 4-10°C	Frigidaire
Gel Dryer Vacuum system	Fisher Scientific
Heatblock	Techne Dri-block DB-2A
Ice machine	Scotsman
Ice-bath	Neolab
Innova 4230 refrigerated incubator shaker	New Brunswick Scientific
Intensification cassettes for autoradiography	Suprema, Dr. Goos
LB-122 β-γ-detector	Berthold
Low Temperature Incubator	Fisher Scientific
Magnetic stirrer / heatplate	Fisher Scientific
Microscope	Nikon
Microwave oven	Sharp Carousel
Milli-Q Synthesis water purification system	Millipore
Mini Trans-Blot transfer cell	BioRad
Nutator	Clay Adams Brand
pH-meter (accumet® basic AB15)	Fisher Scientific

Pipettes (Pipetman: 2, 10, 20, 200, 1000 µl size)	Gilson
Electronic Pipettes	Brandt
Power supplies	Power Pac 300, BioRad
Printer HP Laserjet 4100	Hewlett Packard
Protean 3 minigel system	BioRad
PTC-100 Peltier Thermal Cycler	MJ Research
PTC-200 Peltier Thermal Gradient Cycler	MJ Research
Quarz Cuvette (1 cm, Z = 8.5)	Sarna Cells
QuixSep Micro Dialyzer dialysis chamber	Membrane Filtration Products Inc.
RC-5(B) refrigerated superspeed centrifuge	Sorvall
Rocker/shaker Roto Shake Genie	Scientific Industries Inc.
Scale	Fisher Scientific
Scale (analytical)	Fisher Scientific
Scanner	Epson
Scintillation Counter	Beckman
Shaker (horizontal)	GFL 3005
SmartSpec™Plus spectrophotometer	BioRad
Sonifier 450	Branson
Spinner flasks for cell culture	Wheaton, Bellco
Thermomixer	Eppendorf
Vortex Genie 2	Scientific Industries Inc.
Waterbath SUB 14	Grant
Zip-drive	Iomega

2.1.2 Consumables and Kits

10 cm cell culture dishes	Greiner
100 ml cell culture flask (with filter cap)	Greiner
15 cm cell culture dishes	Greiner
15 ml tubes	Fisher
50 ml tubes	Fisher
6-well plates for cell culture	Greiner
ECL-Plus Western Blotting Detection System	Amersham
Filter pipette tips (10, 20, 200, 1000 µl size)	Axygen
Filterpaper	Whatman
Parafilm “M”	American National Can
Perfectprep™ Gel Cleanup Kit	Eppendorf
Pipet tips (10, 20, 200, 1000 µl)	Axygen
QiagenMaxi Kit	Qiagen
QiagenMini Kit	Qiagen
Quick Ligation Kit	NEB
QuikChange™ Site-Directed Mutagenesis Kit	Stratagene
RNA Midiprep Kit	Qiagen
Scintillation fluid	Betamax
Serological pipettes	Falcon
Sterile filters (20 µm and 45 µm)	Millipore
Sterile filtration units	Nalgene
Streptavidine	BioRad
TOPO TA cloning kit	Invitrogen
X-OMAT™ autoradiography film	Kodak

2.1.3 Chemicals, Enzymes, Proteins and Molecular Weight Markers

Chemicals not listed were of p. A. quality and were purchased from the companies Sigma (St. Louis, MO), Fisher Scientific (Hampton, NH) and VWR (West Chester, PA).

Acrylamide (40% stock)	Fisher
Agarose (ultra pure)	VWR
APS (ammoniumpersulfate)	Merck
Baculovirus DNA	Baculo Gold, Pharmingen
BCIP (5bromo-4chloro-3indolyl-phosphate)	Boehringer Mannheim
Bromophenol Blue (Na-salt)	Sigma
Cellfectin	Invitrogen
Citric Acid	VWR/EMD
Coomassie® R250 Brilliant Blue	Serva
Deep Vent Polymerase	NEB
DTT	AppliChem GmbH
EDTA	VWR
Ethanol 95%	Biochemistry Dpt., UC Riverside
Ethidiumbromide	ICN Biomedicals Inc.
GeneRuler 1kb DNA-Ladder	MBI Fermentas
Glucose	MP Biomedicals
Glycerine (Glycerol)	VWR/EMD
Glycogen	J.T. Baker
Guanidine hydrochloride	VWR/EMD
Hepes (N-Cyclohexyl-2-aminoethanesulfonic acid)	Fisher
Histone-octameres	F. Sauer
Hotmaster Taq Polymerase	Eppendorf
Isopropanol (2-propanol)	VWR
L-Arginine free base	VWR/EMD
L-Glutamin/Penicillin/Streptomycin-Mix	Gemini Bioproducts
LB Broth, Miller	VWR
LB-Agar	VWR
Lysozyme	Roche
Methanol	VWR
NBT (nitro blue tetrazolium chloride)	Boehringer Mannheim
NONIDET® P40	Calbiochem
Nucleosomes	F. Sauer
Pipes (Piperazine-1,4-bis[2-ethanesulfonic acid])	Fisher
Pluronic® F-68	Gibco
PMSF	Roche
Ponceau-S protein staining solution	Serva
Potassiumtetrathionate	Pfaltz & Bauer Inc.
Protein marker, broad range	NEB
Protein marker, prestained	NEB
Proteinase K	Roche
Quick Ligase	NEB
Restriction enzymes and buffers	NEB

RNasin, ribonuclease inhibitor	Promega
RNaseA	Roche
ROX reference QPCR dye	Stratagene
Salmon testis DNA	Sigma
SDS (sodiumdodecylsulfate)	VWR
Shrimp alkaline phosphatase	Boehringer Mannheim
Sodiumchloride	VWR
sodiumdesoxycholate	Fisher
Skim milk powder	SACO Foods Inc.
SF900II serum-free insect cell media	Gibco
Sodiumhydroxide	VWR/EMD
Superscript RT	Invitrogen
SYBR-Green QPCR dye	Molecular Probes
TaKaRa ^{Ex}Taq Polymerase	TaKaRa
TEMED	Fisher
Tryptone	VWR/EMD
Tween®20	Fisher
Yeast extract	Gibco

2.1.4 Antibodies and Affinity Matrixes

Optitran reinforced NC membrane	Schleicher & Schüll
Westran PVDF-Membrane	Schleicher & Schüll
Immobilon-P (PVDF) Membrane	Millipore
Nitrocellulose membrane	Millipore
Anti-FLAG M2 affinity agarose	Sigma
Protein-A-Sepharose (PAS)	Amersham
Anti-H2B, rabbit	F. Sauer
Anti-phospho(H2BS33), rabbit	S. Kwoczynski / T. Maile
Anti-dTAF1M	D. Wassarman
Anti-dTAF1C	D. Wassarman
Anti-acetyl(H3K14), rabbit	Upstate
Anti-acetyl(H4), rabbit	Upstate
Anti-dimethyl(H3K9), rabbit	Upstate
Anti-phospho(H3S10/28P), rabbit	Upstate
Anti-rabbit-IgG-AP-conjugate	Sigma
Anti-rabbit-biotinylated	Amersham

2.1.5 Radioactive Nucleotides

Redivue adenosine-5'-[γ-^{32}P]-triphosphate, triethylammoniumsalt 10 mCi / ml (AA0018)	Amersham

2.1.6 Bacteria Stocks

XL-1 blue (Bullock *et al.* 1987); Stratagene

> recA1 end A1 gyrA46 thi-1 hsdR17 supE44 relA1 lac^- F' [$proAB^+$ $lacI^q$ lacZ∆M15 Tn10 (Tet^r)]

2.1.7 Insect Cells

Sf9 insect cells; Orbigen Inc.

> Cell line derived from pupal ovarian tissue of *Spodoptera frugiperda* (fall armyworm). Sf9 cells were used to isolate and propagate recombinant baculoviral stocks and to produce recombinant proteins.

Schneider S2 cells; Invitrogen

> Cell line derived from *Drosophila melanogaster* (fruit fly; wildtype-strain "Oregon R") dissociated, near hatching embryos. S2 cells were used to produce recombinant proteins and for XChIP experiments (Schneider 1972).

2.1.8 Oligonucleotides

Oligonucleotides (oligos) were produced by Sigma-Genosys in desalted purity grade. All sequences are shown in 5'-3' orientation.

2.1.8.1 Oligonucleotides for inserting point-mutations with PCR

Mutated codons are shown in bold letters.

5'-CTK-D1541A	GAGATGTTCCTCGAG**GCT**CTCAAGCAGATTGTGG
3'-CTK-D1541A	CCACAATCTGCTTGAG**AGC**CTCGAGGAACATCTC
5'-CTK-D1725A	GCACCAGAGTTT**GCT**GAAGCCTGGGGC
3'-CTK-D1725A	GCCCCAGGCTTC**AGC**AAACTCTGGTGC

2.1.8.2 Oligonucleotides for cloning

The positions of restriction sites are underlined and the specific enzyme is shown in brackets. Tags are shown in bold letters.

5'-TAF$_{II}$250-1500 (*Nde*I)	GGGCGCATATGCCATTCCTGTTCCCGGTAAG
3'-TAF$_{II}$250-Ende (*Xba*I)	CATGTCTAGACTAAAAAGTCCAGGCATAATCATTG
3'-CTKΔATP-A (*Sal*I)	ACGCGTCGACGTCGATGTAGTCATCTCCGG
5'-CTKΔATP-B1 (*Sal*I)	ACGCGTCGACCGCGGAAGGGGTAGGC
5'-CTKΔATP-B2 (*Sal*I)	GCGTCGACGAATCCATGGATGTGG
5'-Bdf1-FLAG (*Nco*I, *Nde*I)	CTAGCC**ATGGCTGACTACAAGGACGACGATGACAAGCAT**ATGACC GATATCACACCCGTACAGAACGATGTGGAT
3'-Bdf1-Stop (*Eco*RI, *Sma*I)	GCGTCCTCGAGGAATTCCCCGGGTCACTCTTCTTCATTTCG CTGCTAAC
5'-Bdf2-Start (*Nde*I)	GGAATTCCATATGTCTCGTACTAACATGGATACAAGAC
3'-Bdf2-Stop (*Xba*I)	CATGTCTAGATTAATCACTGTCACTGTCGCTACTATCAC

2.1.8.3 Oligonucleotides for RT-PCR-reactions

Name of oligo	5'-oligo (5'-3')	3'-oligo (5'-3')
actin5C	CGTTCTGGACTCCGGCGATGG	GTACTTGCGCTCTGGCGGGGC
cdc25-2 (string, stg)	GTGGATCTCGTCGTGCTCGCC	TGCTGGCGGTTCCGGGCGCTT

2.1.8.4 Oligonucleotides for XChIP-reactions (all in 5'-3' direction)

cdc25pro-5' (stg-promoter)	ATCATATGACTGCGGCCACTACC
cdc25pro-3' (stg-promoter)	CAGGATCATATGGACTCAGTTTTGGA

cdc25code-5’ (stg-coding)	ATTTCTTCGGCTTCAGACTCGCCAGC
cdc25code-3’ (stg-coding)	GCTTTTGGGTCACACGTCACGCAAG

2.1.8.5 Oligonucleotides for QPCR-reactions (5’-3’)

cdc25-SYBR-5’	TACGCGTCGCGGTATAAAAGCTCG
cdc25-SYBR-3’	ACCAATGTCCCGAGAAACGAGGAG
gtpro-SYBR-5’	TCCGGATTTTCGGTATAAATGCAG
gtpro-SYBR-3’	GACTGGTGATCTAGCTCTGATGTTGC

2.1.8.6 Oligonucleotides for sequencing (5’-3’)

pVL-fwd (vector forward)	ATAGTTGCTGATATCATGGAG
GST-Thr-fwd (tag fwd)	TCCAAAATCGGATCTGGTTC
T7 (vector forward)	TAATACGACTCACTATAGGG
T3 (vector revers)	AATTAACCCTCACTAAAGGG
dTAF1fwd487	GAGGCCAAGTTGACTAAGGAC
dTAF1fwd1024	GAGAACAGCGATCAGAAGC
dTAF1fwd1466	AGCAAAATGCTCAAGCAAAG
dTAF1fwd2001	CCTAAAGAAGTACTCCCACGG
dTAF1fwd2464	CGGTCGGTGAATTCAATATAC
dTAF1fwd2908	AAGCTTGACGACGAAGTAAAG
dTAF1fwd3446	GCAAGAATCTTGAGAACATGC
dTAF1fwd3898	CAGAACCAGAAGCTTCAGCC
dTAF1fwd4434	GTCCTCTATCCTGGAGATTATCC

dTAF1fwd4647 (250-CT-*)	CTGATCTACAATGGACCGC
dTAF1fwd5057 (250-1)	GAAGATACTTGAGTATGCCC
dTAF1fwd5585 (250-CT1-2)	CGAAGACGAGAACAATGCG
dTAF1fwd5884 (250-2)	TCGGCGAGCCCAGCAGC
Bdf1fwd305	AGAAGGAAGAAGGAGGACAAG
Bdf1fwd831	TGTAATTAGACGAGCCCAAAC
Bdf1fwd1277	ACTACGATTCCGATGAAGATTC
Bdf1fwd1723	TTAGATAATCACACCATCTTAACATTG
Bdf2fwd243	CGGTATCACTAACCTGAATTCTTC
Bdf2fwd713	CAATGCCACCAAGAGTTTTAC
Bdf2fwd1214	GCAATGAAGTCCATTCAATG
Bdf2fwd1643	CCGCAGATGAAATACTAACTTC

2.1.8.7 Linker-Oligonucleotides

Linker-oligonucleotides are pairs of long complementary nucleotides that were used for cloning new vectors with different multiple cloning sites (MCSs) or to introduce a variation in the NH_2-terminal tags of existing vectors used for proteinexpression.

LinkerA-5': *Not*I, ***Nco*I**, FLAG-tag, ***Nde*I**, *Spe*I, *Xba*I.	AAGGAAAAAAAGCGGCCG**CCATGG**CTGACTACAAGGACGACGATGACAAG**C ATATG**AGATCCACTAGTTCTAGACTAG
LinkerA-3' (complementary strand to LinkerA-5'):	CTAGTCTAGAACTAGTGGATCTCATATGCTTGTCATCGTCGTCCTTGTAGTCAGC CATGGCGGCCGCTTTTTTCCTT

LinkerB-5': ***Nco*I**, 10x HIS-tag, FLAG-tag, ***Nde*I**.	CATG**CCATGG**CTCATCATCATCATCATCATCATCATCACATGGCTGACTACA AGGACGACGATGACAAG**CATATG**GAATTCC
LinkerB-3':	GGAATTCCATATGCTTGTCATCGTCGTCCTTGTAGTCAGCCATGTGATGATGATG ATGATGATGATGATGATGAGCCATGGCATG

2.1.9 Plasmids

2.1.9.1 Cloning- and expressionvectors:

pBluescript II KS$^+$ (pBS, Stratagene)
Standard vector for cloning purposes (www.stratagene.com).

pTβSTOP (Jantzen *et al.* 1992)
Derivate of pT7βSal (Norman *et al.* 1988) that carries a multiple-cloning-site- (MCS)-insertion followed by three STOP-codons in three separate coding-frames.

pVL-FLAG (Näär and Tijan 1995)
Baculovirus-expressionvector and derivate of pAcSG2 (Pharmingen) containing an insertion for the sequence of the FLAG-epitope. This vector allows the production of recombinant baculoviruses to express NH_2-terminal-FLAG-tagged fusionproteins in *SF9*-cells.

pVL-GST-FLAG (Kwoczynski S., 2002)
Baculovirus-expressionvector, pVL-FLAG-derivate (Lichtsteiner and Tjian 1995) with an insertion of a 0.3 kbp GST-cDNA-fragment upstream of the sequence for the FLAG-epitope.

pBluescript-*Not*I/*Not*I-*dTAF$_{II}$250* (Kwoczynski 2002)
pBluescript KS$^+$ plasmid which contains the full length *dTAF$_{II}$250*-cDNA. The cDNA consists of three additional sequence-sections, which are not listed in the sequence of the NCBI (ID NM_057608.2). These sections are coded by an exon in the NTK and the additional exons 12a and 13a in the CTK.

pTβSTOP-*dTAF$_{II}$250-CT1500* (Kwoczynski 2002)
pTβSTOP-derivate, contains an insert of the C-terminal part of dTAF1 (CTK), starting at aminoacid 1496.

2.1.9.2 Constructed vectors for cloning and expression:

pBluescript-FLAG

Derivate of pBluescript II KS$^+$ with an insertion of the FLAG-tag sequence upstream of the MCS. This vector was constructed by inserting the annealed LinkerA-oligos (2.1.8.7) into pBluescript using the *Not*I/*Xba*I restriction sites.

pBluescript-His-FLAG

pBluescript KS$^+$ derivate with an insertion of the (10xHis)-FLAG-tag sequence upstream of the MCS. This vector was constructed by inserting the annealed LinkerB-oligos (2.1.8.7) into pBluescript-FLAG using the *Not*I/*Nde*I restriction sites.

2.1.9.3 Cloned constructs

pTβSTOP-*dTAF1CTK(D1541A)*

pTβSTOP-clone with insertion of the 1.9 kb CTK(D1541A)-PCR-fragment using *Nde*I/*BamH*I restriction sites. The PCR-fragment was amplified with the CTK-D1541A-primers (2.1.8.1.) following the protocol for inserting pointmutations into DNA (2.2.1.14.). Template-DNA was the vector pTβSTOP-*dTAF$_{II}$250-CT1500*.

pTβSTOP-*dTAF1CTK(M1537I-D1541A)*

pTβSTOP-clone with insertion of the 1.9 kb CTK(M1537I-D1541A)-PCR-fragment using *Nde*I/*BamH*I restriction sites. This PCR-fragment was amplified with the CTK-D1541A-primers following the protocol for inserting pointmutations into DNA. Template-DNA was the vector pTβSTOP-*dTAF$_{II}$250-CT1500*, but in this PCR, a second pointmutation (amino acid 1537 M -> I, codon ATG to ATA) was accidentally inserted, resulting in a double-pointmutation.

pTβSTOP-*dTAF1CTK(D1725A)*

pTβSTOP-clone with insertion of the 1.9 kb CTK(D1725A)-PCR-fragment using *Nde*I/*BamH*I restriction sites. The PCR-fragment was amplified with the CTK-D1725A-primers following the protocol for inserting pointmutations into DNA. Template-DNA was the vector pTβSTOP-*dTAF$_{II}$250-CT1500*.

pTβSTOP-*dTAF1CTKΔATP*

This pTβSTOP-construct carried the dTAF1CTK-insert lacking the putative ATP-binding domain. It was cloned in two steps: Fragment A was amplified in PCR with the primers 5'-TAF$_{II}$250-1500 / 3'-CTKΔATP-A and inserted into pTβSTOP using *Nde*I/*Sal*I. Fragment B was PCR-amplified with the primers 5'-CTKΔATP-B1 / 3'-TAF$_{II}$250-Ende and then inserted into the previously cloned pTβSTOP-*dTAF1CTK*A by using the restriction sites *Sal*I/*Xba*I. This resulted in a CTKΔATP construct which encodes amino acids 1496-1747 and 1780-2132 respectively, replacing the ATP-domain with a single aspartatic-acid residue (D).

pVL-GST-FLAG-*dTAF1CTK(D1541A)*

Expressionvector with the dTAF1CTK(D1541A)-insert cut out of pTβSTOP-*dTAF1CTK(D1541A)* via *Nde*I/*Sma*I and ligated into pVL-GST-FLAG.

pVL-GST-FLAG-*dTAF1CTK(M1537I-D1541A)*

Expressionvector with the dTAF1CTK(M1537I-D1541A)-insert cut out of pTβSTOP-*dTAF1CTK(M1537I-D1541A)* via *Nde*I/*Sma*I and ligated into pVL-GST-FLAG.

pVL-GST-FLAG-*dTAF1CTK(D1725A)*

Expressionvector with the dTAF1CTK(D1725A)-insert cut out of pTβSTOP-*dTAF1CTK(D1725A)* via *Nde*I/*Sma*I and ligated into pVL-GST-FLAG.

pVL-GST-FLAG-*dTAF1CTKΔATP*

Expressionvector with the 1.8 kb dTAF1CTKΔATP-insert cut out of pTβSTOP-*dTAF1CTKΔATP* via *Nde*I/*Xba*I and ligated into pVL-GST-FLAG.

pBluescript-FLAG-*Bdf1*

Derivate of pBluescript with a 2.1 kb Bdf1-PCR-fragment, amplified using the primers 5'-Bdf1-FLAG and 3'-Bdf1-Stop (2.1.8.1.) by following the protocol for PCR (2.2.1.2.1). The fragment was then cut with *Nco*I and *Eco*RI and ligated into a similar restriction-digested pBluescript-FLAG vector.

pBluescript-His-FLAG-*Bdf1*

Derivate of pBluescript-His-FLAG: This vector was constructed stepwise by insertion of fragments obtained from restriction-digests of pBluescript-FLAG-*Bdf1*:
Fragment Bdf1-A was created by restriction-digest of pBluescript-FLAG-*Bdf1* with *Nde*I and *Bam*HI. Fragment Bdf1-B was created by restriction-digest of pBluescript-FLAG-*Bdf1* with *Bam*HI and *Eco*RI. The Bdf1-A fragment was ligated into a similar restriction-digested pBluescript-His-FLAG plasmid, creating the plasmid pBluescript-His-FLAG-Bdf1-A. Fragment Bdf1-B was ligated into pBluescript-His-FLAG-Bdf1-A, which was cut with *Bam*HI and *Eco*RI, creating pBluescript-His-FLAG-*Bdf1*.

pBluescript-His-FLAG-*Bdf2*

Derivate of pBluescript-His-FLAG: A 2.0 kb PCR-fragment of Bdf2 was amplified using the primers 5'-Bdf2-Start and 3'-Bdf2-Stop, cut with the restriction enzymes *Nde*I and *Xba*I and ligated into a similar cut pBluescript-His-FLAG.

pVL-His-FLAG-*Bdf1*

Derivate of pVL-FLAG: pBluescript-His-FLAG-*Bdf1* was cut with the restriction enzymes *Nco*I and *Sma*I and the resulting His-FLAG-Bdf1-fragment was ligated into a similar cut pVL-FLAG vector.

pVL-His-FLAG-*Bdf2*

Derivate of pVL-FLAG: pBluescript-His-FLAG-*Bdf2* was cut with the restriction enzymes *Nco*I and *Xba*I and the resulting His-FLAG-Bdf2-fragment was ligated into a similar cut pVL-FLAG vector.

2.1.10 Baculoviruses for expression in *Sf9*-cellculture

Baculoviruses, which were created during this work to express proteins, are not additionally listed. The corresponding fusionproteins can be found in chapter three and are described by the constructed baculo-expressionvectors in chapter 2.1.9.3.

GST-FLAG-dTAF$_{II}$250CT1500 (S. Kwoczynski)
: Baculovirus for expression of the N-terminal GST-FLAG-tagged dTAF1CTK-fusionprotein.

2.1.11 Proteins

2.1.11.1 Histones and histone-peptides

Histone H2B: Purified *Drosophila* histone H2B, expressed in *E. coli* (F. Sauer).

Histones H2A, H2B, H3 and H4 from *Xenopus laevis*: Recombinant purified histones, expressed in *E. coli* (F. Sauer).

Histone-N-terminal-tail H2B-phosphoserine33-peptide (H2BT-S33P; Biosynthesis) for coupling to SulfoLink-columns, sequence: CKRKE(S-phospho)YAIY.

Unphosphorylated Histone-N-terminal-tail H2B-serine-33-peptide (H2BT; Biosynthesis) for coupling to SulfoLink-columns, sequence: CKRKESYAIY

Histone-N-terminal-tail H2B-peptide, sequence:
MPPKTSGKAAKKAGKAQKNITKTDKKKKRKRKES.

Histone-N-terminal-tail H3-peptide (H3T; S. Kwoczynski), sequence:
MARTKQTARKSTGGKAPRKQLATKAARKS.

Histone-N-terminal-tail H3-phosphoserine10/28-peptide (H3T-S10/28P; F. Sauer), sequence:
MARTKQTARK(S-phospho)TGGKAPRKQLATKAARK(S-phospho).

2.1.11.2 Fusionproteins

His- and GST-FLAG-tagged fusionproteins, cloned and expressed by S. Kwoczynski and F. Sauer. These proteins were used for transkinase-assays and the UV-crosslinking experiments (Maile *et al.* 2004, Kwoczynski 2002).

His-TAF$_{II}$250-CT1500 (dTAF1CTK)
: Renatured poly-His-tagged dTAF1CTK fusionprotein starting at amino acid 1496 of *Drosophila melanogaster* TAF1, S. Kwoczynski.

GST-FLAG-TAF$_{II}$250-CT1600 (dTAF1CTKΔ1600)
GST-FLAG-tagged dTAF1CTK fusionprotein starting at amino acid 1593 of *Drosophila melanogaster* TAF1, S. Kwoczynski.

GST-FLAG -TAF$_{II}$250-CT1800 (dTAF1CTKΔ1800)
GST-FLAG-tagged dTAF1CTK fusionprotein starting at amino acid 1797 of *Drosophila melanogaster* TAF1, S. Kwoczynski.

GST-FLAG -TAF$_{II}$250-CT1900 (dTAF1CTKΔ1900)
GST-FLAG-tagged dTAF1CTK fusionprotein starting at amino acid 1958 of *Drosophila melanogaster* TAF1, S. Kwoczynski.

GST-FLAG -TAF$_{II}$250-CT17-18 (dTAF1CTK17-18)
GST-FLAG-tagged dTAF1CTK fusionprotein, ranging from amino acid 1594 to 1802 of *Drosophila melanogaster* TAF1, S. Kwoczynski.

GST-FLAG -TAF$_{II}$250-CT18-19 (dTAF1CTK18-19)
GST-FLAG-tagged dTAF1CTK fusionprotein, ranging from amino acid 1797 to 1963 of *Drosophila melanogaster* TAF1, S. Kwoczynski.

GST-FLAG-dTAF1ΔCTK (dTAF1ΔCTK)
GST-FLAG-tagged dTAF1 full-length fusionprotein, but lacking the CTK due to site directed-mutagenesis using a primer pair, that introduces a stop codon behind amino acid 1728, F. Sauer.

2.1.11.3 Other proteins

TFIID (F. Sauer)
Purified *Drosophila* TFIID-complex (Maile *et al.* 2004).

Nucleosomes (F. Sauer)
Purified nucleosomes from 0-6 h old *Drosophila*embryos (Maile *et al.* 2004).

Octamers (F. Sauer)
Purified octamers from Schneider S2 cells (Maile *et al.* 2004).

2.1.12 Media, Buffers and Stock Solutions

All solutions were prepared in Milli-Q-filtered dH_2O unless otherwise mentioned below. Solutions which needed to be sterile were autoclaved or sterile-filtered depending on solution type. The pH of the solutions was adjusted with HCl and NaOH unless otherwise mentioned below.

2.1.12.1 Media

LB-media	1 %	peptone
	0,5 %	yeast extract
	0,5 %	NaCl
LB-agar-media	1.5 %	agar-agar in LB-media
SOB-media:	20 g	tryptone
	5 g	yeast extract
	0.5 g	NaCl
	2.5 mM	KCl
	pH 7.0	
	add dH_2O to a final volume of 1 l	
	autoclave	
	add 5 ml sterile 2 M $MgCl_2$ just before use	
serum-free *Sf9*-media:	1 l	SF900II serum-free media
	1%	Pluronic® F-68
	1%	L-Glutamin/Penicillin/Streptomycin-Mix

2.1.12.2 Buffers

AP-buffer:	100 mM	Tris-HCl, pH 8.0
	100 mM	NaCl
	50 mM	$MgCl_2$
10x PAGE-buffer:	2 M	Glycine
	250 mM	Tris-HCl pH 8.3
SDS-PAGE-runningbuffer	4.5 l	dH_2O
	500 ml	10x PAGE-buffer
	25 ml	20 % SDS
Transferbuffer I:	3.5 l	dH_2O
(proteins <150 kDa)	1 l	Methanol
	500 ml	10x PAGE-buffer
	2.5 ml	20 % SDS
Transferbuffer II:	50 mM	Tris-HCl pH 8.3
(proteins >150 kDa)	380 mM	Glycine
	0.1 %	SDS (w/v)
	20 %	Methanol

4x SDS-loadingbuffer:	125 mM	Tris-HCl pH 6.8
	10 %	β-Mercaptoethanol
	6 %	SDS
	20 %	Glycerol
	1 grain/10ml	Bromophenolblue
TBjap-buffer:	10 mM	Pipes
(Transformationbuffer japanese)	15 mM	$CaCl_2$
	250 mM	KCl
	set pH to 6.7 (KOH)	
	55 mM	$MnCl_2$
	filter-sterilization	
5x TBE-buffer :	54 g	Tris-HCl pH 8.0
	27.5 g	Boric acid
	20 ml	0.5 M EDTA
	add dH_2O (Milli-Q) to a total volume of 1 l	
TBST-buffer:	100 mM	Tris-HCl pH 8.0
	150 mM	NaCl
	0.1 %	Tween®20
TE-buffer:	10 mM	Tris-HCl, pH 8.0
	1 mM	EDTA
Plasmidprep-lysisbuffer:	7.8 ml	3.2 M LiCl
(LiCl-method)	1.3 ml	0.5 M EDTA pH 8.0
	0.4 ml	10% Triton X 100
	0.25 ml	2 M Tris pH 7.5
	0.25 ml	dH_2O
	250 µl	Lysozyme stock solution
DNA-loadingbuffer 10x:	50%	Glycerol
	0.1%	Orange-G
	49.9%	1x TBE-buffer
5x HEMG-buffer:	125 mM	Hepes pH 7.6
	62.5 mM	$MgCl_2$
	0.5 mM	EDTA
	50 %	Glycerol
0.5 – 1 M KCl-HEMG-buffer:	0.5 - 1 M	KCl
	0.2 M	PMSF
	1 %	NONIDET® P40
	20%	5x HEMG-buffer

His-lysisbuffer:	25 mM	Hepes pH 7.9
	1 M	NaCl
	10 %	Saccharose
	5 mM	Imidazol
	0.2 mM	PMSF
Buffer A:	25 mM	Hepes pH 7.9
	200 mM	NaCl
	10 %	Saccharose
	5 mM	Imidazol
	0.2 mM	PMSF
Buffer B:	25 mM	Hepes pH 7.9
	200 mM	NaCl
	10%	Saccharose
	500 mM	Imidazol
	0.2 mM	PMSF
Guanidinium-lysisbuffer:	6 M	Guanidinium
	25 mM	Hepes pH 8.0
	250 mM	NaCl
Buffer C1:	8 M	Urea
	25 mM	Hepes pH 8.0
	250 mM	NaCl
Buffer C2:	as C1 but with 50 mM imidazol instead	
Buffer D:	8 M	Urea
	25 mM	Hepes pH 8.0
	250 mM	NaCl
	1 M	Imidazol
Renaturationbuffer:	100 mM	Tris-HCl pH 7.5
	7.5 mM	EDTA
	100 mM	NaCl
	1 M	Arginin
	10 mM	DTT
	0.2 mM	PMSF
Kinasebuffer:	25 mM	Hepes pH 8.0
	12.5 mM	$MgCl_2$
	100 mM	KCl
	0.1 mM	EDTA
	0.1 %	NONIDET® P40
	0.2 mM	PMSF

Membrane-renaturationbuffer:	140 mM	NaCl
	10 mM	Tris-HCl pH 7.5
	2 mM	EDTA
	0.1 %	NONIDET® P40
	2 mM	DTT
Guanidine-denaturationbuffer:	7 M	Guanidine-HCl
	50 mM	Tris-HCl pH 8.3
	2 mM	EDTA
	50 mM	DTT (added fresh)
TBS (Tris buffered saline):	10 mM	Tris-HCl pH 7.5
	140 mM	NaCl
10x PBS:	1.4 M	NaCl
	27 mM	KCl
	100 mM	Na_2HPO_4
	18 mM	KH_2PO_4
	pH 7.3	
XChIP-lysisbuffer:	25 mM	Tris, pH 7.5
	140 mM	NaCl
	1 mM	EDTA
	1 %	Triton-X-100
	0.1 %	SDS
	filter-sterilization	
IP1:	similar as XChIP-lysisbuffer but with 500 mM NaCl instead	
IP2:	10 mM	Tris, pH 8.0
	250 mM	LiCl
	0.5 %	NONIDET® P40
	0.5 %	Sodiumdesoxycholate
	1 mM	EDTA
	filter-sterilization	

2.1.12.3 General stock solutions

Ethidiumbromide solution:	10 mg/ml	Ethidiumbromide in dH_2O
Ampicillin stock solution:	100 mg/ml	Ampicillin (Na-salt)
PMSF-stock solution:	100 mM	PMSF in dH_2O
Lysozyme stock solution:	10 mg/ml	Lysozyme in dH_2O
Proteinase-K solution:	20 mg/ml	Proteinase K in dH_2O

NBT-solution:	0.5 g	Nitro-blue-tetrazolium-chloride
	10 ml	70 % dimethylformamide (dH_2O)
BCIP-solution:	0.5 g	5bromo-4chloro-3indolylphosphate (disodium salt)
	10 ml	100 % dimethylformamide

2.1.12.4 Proteingel solutions

Coomassie-destaining-solution:	10 %	Acetic acid
	45 %	Methanol
	45 %	dH_2O
Coomassie-staining-solution:	0.25 %	Coomassie® R250 Brilliant Blue
	9.75 %	Acetic acid
	45 %	Methanol
	45 %	dH_2O
SDS-PAGE-resolving-gel:	25 %	4x resolving-gel buffer:
(in dH_2O)	6 – 18 %	Acrylamide (depending on gel concentration)
	0.1 %	TEMED
	0.6 %	APS
SDS-PAGE-stacking-gel:	25 %	4x stacking-gel buffer
(in dH_2O)	4 %	Acrylamide
	0.1 %	TEMED
	0.6 %	APS
4x resolving-gel buffer:	1.5 M	Tris-HCl pH 8.8
(lower tris buffer)	0.4 %	SDS
4x stacking-gel buffer:	0.5 M	Tris-HCl pH 6.8
(upper tris buffer)	0.4 %	SDS

2.1.12.5 Silverstaining-solutions

Fixative 1:	400 ml	Ethanol
	100 ml	Acetic acid
	add dH_2O (Milli-Q) to a total volume of 1 l	
Fixative 2:	300 ml	Ethanol
	2.5 g	Potassium tetrathionate
	41 g	Sodium acetate, anhydrous
	add dH_2O (Milli-Q) to a total volume of 1 l	

Silvernitrate-solution:	2 g	$AgNO_3$
	add dH_2O (Milli-Q) to a total volume of 1 l	
	add 250 µl 37 % formaldehyde just before use	
Developer-solution:	15 g	Potassium carbonate
	7.5 mg	Sodium thiosulfate
	add dH_2O (Milli-Q) to a total volume of 1 l	
	add 150 µl 37 % formaldehyde just before use	
Stop-solution:	50 g	Tris base
	20 ml	Acetic acid
	add dH_2O (Milli-Q) to a total volume of 1 l	

2.2 Methods

2.2.1 Analysis and Manipulation of Nucleic Acids

2.2.1.1 Photometric determination of DNA/RNA concentrations

Nucleic acids absorb light in the ultraviolet range. The absorption-maximum of DNA is at 260 nm, RNA and proteins (aromatic residues) at 280 nm respectively. To determine the concentration of DNA, the sample was tested for absorption at 260 nm in a spectrophotometer (2.1.1). To determine the degree of impurities due to RNA and proteins the absorption (optical density or OD) at 280 nm was also measured. The quotient of OD_{260}/OD_{280} is an indicator for the purity of DNA and lies within 1.8 to 2.0 for pure (column purification) solutions. For calculation of the DNA-concentration, following conventional unit was used: an OD_{260} of 1 resembles a concentration of 50 µg double-stranded DNA per ml.

2.2.1.2 Polymerase chain reaction (PCR)

2.2.1.2.1 PCR for cloning of cDNA-fragments

To amplify cDNAs out of template DNA-pools (Plasmidpreps, Libraries), primers containing specific restrictionenzyme cutting sites were created (2.1.8.2). The PCR reaction used 50-100 ng DNA template, 1-2 U DeepVent proofreading polymerase (NEB), 2 µl of 10x ThermoPol reaction buffer (NEB), 1 µl of 2.5 mM dNTP-Mix (TaKaRa) and 1 µM endconcentration of the corresponding 5'- and 3'-Primer. The reaction was set to a total volume of 20 µl (ad dH_2O). After initial denaturation of 95°C for 1.5 min. around 25 to 30 amplification cycles followed: melting temperature was set to 95°C (30 sec.), annealing around 5°C below the average melting temperature of the specific primerpair (45 sec.) and elongation was set to 75°C (1 min. per 1kb fragment length). The PCR ended with a final elongation at 75°C for 20 min. The amplified DNA was analyzed and purified afterwards via gelelectrophoresis on an agarose gel (2.2.1.6).

2.2.1.2.2 PCR for standard detection of specific DNA-sequences

To detect primer specific DNA-sequences in a sample by amplification, a PCR-reaction was set up as described above, but with ^{Ex}Taq-Polymerase (TaKaRa) using same amounts of ^{Ex}Taq-buffer. Denaturation temperature was set to 94°C, elongation to 72°C.

2.2.1.2.3 PCR for real-time quantitative detection of specific DNA-sequences (QPCR)

Detection of amplified cDNA in real-time was performed using an ABI 7700 light cycler. The reaction (20 µl total volume) used 8 µl of HotMasterMix (Eppendorf), 2 µl of template DNA, 1 µM primers, SYBR-Green (Molecular Probes) as corresponding dye (final concentration 1:10000)

and ROX (Stratagene) as reference dye (final concentration 1:300). 40 cycles were performed and temperature steps were set as described in 2.2.1.2.2.
The comparative threshold cycle (CT) method was used to compare the various amplification results obtained: The threshold cycle (CT) represents the fractional cycle number at which two or more different PCR reactions amplify DNA at the exact same ratio.

2.2.1.2.4 Reverse-Transcriptase-PCR (RT-PCR)

RNA, isolated with the midiprep kit from Qiagen, was measured on a spectrophotometer (Biorad) and used in a concentration of 10 µg per reaction. The RNA has been incubated at 65°C for 5 min and subsequently cooled down on ice. For the reverse transcriptase (RT) reaction 20 µl of 5x RT-buffer (Invitrogen), 10 µl 0.1 M DTT, 10 µl random hexamer primers (Boehringer Mannheim [200 ng/µl]), 10 µl of 10 mM dNTPs, 2.5 µl RNasin and 2 µl Superscript RT were added and the sample was set to a total volume of 100 µl (dH_2O). After incubation at 37°C for 1 h the sample was heated up to 95°C for 5 min and then cooled down on ice. The cDNA produced with this reaction could be stored at -20°C or immediately analyzed via PCR (2.2.1.2.2).

2.2.1.3 Annealing of single-strand DNA (ssDNA)

To create double-strand DNA (dsDNA) out of ssDNA strands like linker-oligonucleotides, 20 µg of each complementary oligonucleotide was added to a volume of 40 µl Milli-Q dH_2O containing 4 µl NEB-buffer 3 and mixed by inversion. The sample was heated up to 95°C for 5 min and then cooled down at room temperature. The annealed dsDNA was then frozen at -20°C or immediately digested with restrictionenzymes (2.2.1.4).

2.2.1.4 Digestion of DNA with restrictionendonucleases

The digestion of DNA with restrictionendonucleases was performed in a standard volume of 20 µl with sticky-end or blunt-end cutting restriction enzymes of bacterial origin. Single and double digest temperature, usage of BSA as a stabilizing agent and buffer settings were chosen following the recommendations of the manufacturer (NEB). 2-5 U (units) of enzyme were used for 2 h digest assays, overnight digests were performed using 5 U of enzyme. Digested DNA was either dephosphorylated (2.2.1.5) or directly analyzed and purified via gelelectrophoresis (2.2.1.6).

2.2.1.5 Dephosphorylation of linearised plasmids

To prevent intramolecular religation with the 3'-hydroxy-ends of digested linearised vector-DNA in following ligation reactions, the free 5'-phosphate-groups of the vectors were removed using shrimp alkaline phosphatase (SAP, Boehringer Mannheim) directly after digestion. 2.3 µl of 10x dephosphorylation buffer and 1 U of shrimp alkaline phosphatase were added to 20 µl digestion sample and incubated at 37°C for 1 h. The sample was then purified from contaminants using gelelectrophoresis (2.2.1.6).

2.2.1.6 Agarosegelelectrophoresis

Depending on the size of the expected DNA-fragments, gels were used within concentrations of 0.7 - 2% agarose. The specific amount of agarose was solubilised in 1x TBE by melting in a microwave and poured into a gel tray (custom DNA-gel system, 2.1.1) after addition of ethidiumbromide-solution (0.1 µl/ml of gel). 10x DNA-loadingbuffer has been added to the DNA samples in 1/10 concentration and the samples have been separated by electrophoresis. 0.1–1 µg DNA in 20-25 µl sample volume was separated, "Gene-Ruler™ 1 kb DNA ladder" (MBI) was used as DNA marker (0.1-1 µg). The gel was then analyzed and documented using the "Eagle Eye" UV-system (Stratagene).

2.2.1.7 Isolation of DNA-fragments out of agarose gels

DNA was extracted of agarose gels using the Perfectprep Gel Cleanup Kit from Eppendorf (2.1.2). The fragment of interest was cut out of the gel, melted in a binding buffer and then bound to the column matrix by means of centrifugation. Following a washing step, the purified DNA can be eluted using water or buffer.

2.2.1.8 Ligation of DNA-fragments

Ligation of DNA-fragments was done using the Quick Ligation Kit from NEB. Dephosphorylated vector-DNA and insert-DNA were combined in a volume of 10 µl, containing at least five molar excess of insert. 10 µl of 2x ligation buffer (NEB) was added and the sample was vortexed shortly. 1 µl of Quick T4 DNA ligase has been added, the sample was incubated for 5 min. at 25°C and then cooled down on ice. The ligated DNA could be stored at -20°C or directly transformed into *E.coli* cells.

2.2.1.9 Growing transformation-competent *E.coli* cells

The establishment of transformation-competent cells followed a modified method of Stratagene's Epicurian competent XL-1 blue (Inoue *et al.* 1990):
E. coli XL-1 blue cells (2.1.6) were grown over night in 5 ml SOB media. The culture was transferred afterwards into 250 ml SOB media and grown at 18°C for approximately 31 h until it reached an OD_{600} of 0.6. It was then incubated on ice for 10 min and centrifuged in a precooled Sorvall GS3 rotor for 10 min with 2500 g at 4°C. Meanwhile 2 ml of 100% DMSO was added to 100 ml of TBjap (2.1.12.2). After centrifugation, the supernatant has been discarded and the cell pellet has been resuspended in 80 ml TBjap buffer with DMSO. The suspension was incubated on ice for 10 min and centrifuged again. The supernatant has been discarded and the pellet was resuspended in 18.6 ml of precooled TBjap (without DMSO). After adding 1.4 ml of DMSO to the suspension (final concentration of 7%), it was incubated again on ice for 10 min. After incubation the cell suspension was dispensed into 200 µl aliquots in cryotubes which have been frozen immediately in liquid nitrogen and were stored at -80°C.

2.2.1.10 Transformation of competent *E.coli* cells

Frozen competent XL-1 blue cells were slowly thawed on ice. Pre-chilled DNA sample (25 ng ligation-mixture-DNA or 100 ng plasmid-DNA) was added to 100 µl of cells, the sample was mixed gently and incubated on ice for 30 min. After the following heat shock of 37°C for 1.5 - 2 min, the sample was chilled on ice again for 5 min. 900 µl of SOB or LB media was added and the solution was incubated at 37°C for 1 h. 100µl were spread on a 1:10 dilution LB-agar-plate containing 0.1 mg/ml ampicillin. The remaining 900 µl were centrifuged, the cell pellet was resuspended in 100 µl media and spread on a 1:1 plate. The plates were incubated at 37°C over night and checked for colonies the next day. Single colonies were picked with a pipet tip, transferred into a bacteria-culture vial with 5 ml LB-media containing 0.1 mg/ml ampicillin and were grown over night in an incubator-shaker.

2.2.1.11 Isolation of plasmid-DNA out of *E.coli* cells

2.2.1.11.1 Plasmid isolation with LiCl

1.5 ml out of the 5 ml overnight culture (2.2.1.9) were centrifuged (5 min, 1000g, Eppendorf centrifuge) and the resulting cell pellet was resuspended in 200 µl plasmidprep-lysisbuffer (2.1.12.2). After incubation for 5 min at room temperature, the sample was boiled at 100°C for 90 sec and immediately cooled on ice. Chromosomal DNA and denatured proteins were separated from the plasmid-DNA by centrifugation at 16000g for 8 min and the formed pellet was removed from the plasmid containing supernatant using a pipet tip. The plasmid-DNA was precipitated then by adding 0.7 volumes of isopropanol, incubated at room temperature for 10 min and centrifuged at 16000g for 15 min. The supernatant was discarded, the DNA-pellet washed with 70% ethanol and centrifuged again at 16000 g for 5 min. The supernatant was pipetted off carefully and the pellet was dried in the speedvac concentrator. After the sample was dry, the DNA got resuspended in 20 µl dH_2O.

2.2.1.11.2 Plasmid isolation with the QIAGEN Miniprep kit

To isolate plasmid-DNA with additional purification through a mini size column, the Qiagen miniprep kit was used. The isolation was performed following the protocol of the manufacturer. For each column DNA of 3 ml of the 5 ml overnight culture was used. The DNA has always been eluated from the column using 30 µl eluation buffer of the manufacturer.

2.2.1.11.3 Plasmid isolation with the QIAGEN Maxiprep kit

To isolate plasmid-DNA with additional purification through a maxi size column, Qiagens maxiprep kit was used. The 5 ml overnight culture was inoculated into a 250 ml LB-culture and again grown over night. The isolation was performed according to the protocol of the manufacturer, the plasmid-DNA was eluated in 100-500 µl TE-buffer.

2.2.1.12 Isolation of RNA out of S2 cells

For isolation of RNA out of Schneider S2 cells, the RNA midiprep kit from Qiagen was used. The experiment was done following the manufacturers protocol and the total amount of cells used per preparation was within the recommended range of 3–4x10^7 cells.

2.2.1.13 Phenole-chloroform extraction of DNA

To purify DNA-samples from hydrophobic impurities like lipids or proteins the samples were treated with phenole and ethanole-precipitated:
The total volume of the DNA-sample was set to 100 µl with TE-buffer (pH 8.0), 200 µl phenole was added and the sample was mixed (Vortex) and centrifuged at max speed for 1 min (Eppendorf microfuge). The upper aqueous phase was pipetted off into a new microcentrifuge tube and 100 µl TE were added to the remaining lower phenole phase to extract additional DNA. The two forming phases were mixed and centrifuged again and the aqueous phase was removed as before and combined with the first 100 µl of DNA-fraction. After adding 200 µl chloroform to the sample, it was mixed and centrifuged as before and the upper phase was pipetted into a new centrifuge tube.
To precipitate the DNA, 400 µl ice cold ethanol and 1 µl glycogen [20 mg/ml] was added to the sample which was then incubated for 30 min at -80°C. After incubation, the sample was centrifuged at max speed for 20 min, and the ethanol was discarded. The DNA-glycogen pellet was washed with 1 ml of 70% ethanol and the sample was centrifuged for another 20 min at max. speed. The ethanol was carefully pipetted off and the DNA was air-dried at room temperature. After the DNA was dry, the pellet was resuspended in 30 µl of dH_2O.

2.2.1.14 Insertion of point mutations in DNA

Point mutations were inserted into DNA using the QuikChange™Site-Directed Mutagenesis Kit protocol from Stratagene:
Site specific complementary primers, which both bind to the sequence to be mutated and carrying the desired pointmutations were used to amplify DNA from a template plasmid. The enzyme *Dpn*I was then used to digest the methylated non-mutant template DNA but not the unmethylated nicked circular strands that were amplified from it by the polymerase. The undigested DNA was then transformed into competent XL-1 blue cells, which repaired the nicks, forming mutated plasmid-DNA, to be analyzed by sequencing. The DNA of those clones which carried the specific pointmutated plasmid was then restriction-digested and re-cloned into a new plasmid to avoid complications due to unwanted mutations within the old vector during PCR.
The procedure was done following the settings in the protocol of the manufacturer, except that ^{Ex}Taq-Polymerase (TaKaRa, 5 U/µl) was used as polymerase and 5 µl of the amplified sample was added to 100 µl XL-1 blue bacteria suspension for transformation.

2.2.2 Analysis and Manipulation of Proteins

2.2.2.1 SDS-polyacrylamide-gelelectrophoresis (SDS-PAGE)

The separation of proteins according to their molecular weight was done via discontinual SDS-polyacrylamide-gelelectrophoresis following the method of Laemmli (Laemmli 1970):
Depending on the molecular weight of the separated proteins, matrixes of concentrations between 6 – 18% polymerised acrylamide were created:
Two glassplates of specific size were assembled, which were divided from each other by two vertical spacers of specific width (0.75 – 1.5 mm) on the sides and sealed on the bottom by another spacer (custom maxigel system) or a rubber mat (BioRad Protean minigel system). The resolving-gel (2.1.12.4) was then pipetted between the glassplates to polymerise at room temperature. Isopropanole was filled above the liquid resolving-gel solution before polymerisation to create a flat resolving-gel surface. After the gel was polymerised, the isopropanole was discarded, the gel surface was washed with MilliQ-dH_2O and the lower concentrated stacking-gel (2.1.12.4) was poured on top of the resolving-gel. Before the stacking-gel could polymerise, a comb of sample-specific length and width was stuck into the stacking-gel, creating slots for loading the proteins into the gel. Protein samples were boiled in SDS-loadingbuffer at 100°C for 5 min before they were loaded on the gel to disrupt the spacial structure (secondary, tertiary and quartiary structures) of the proteins within the β-mercaptoethanole-containing buffer and to anneal the SDS to create an overall negative charge according to the size of the protein for the electrophoretic separation in the gel.
The electrophoresis was started at 80 V (minigels) to 100 V (maxigels) to stack the proteins within the stacking-gel and set to 150 V (minigels) to 200 V (maxigels) once the proteins entered the resolving-gel.

2.2.2.2 Coomassie-staining of SDS-PAGE gels

To visualize electrophoretically separated proteins in a SDS-gel, the gels were incubated for 30 min to overnight in Coomassie-stainingsolution (2.1.12.4). Afterwards the gels were incubated with several washes of destaining-solution (2.1.12.4) until bands became visible. The gel was washed with dH_2O, documented and dried onto Whatman-paper with a vacuum-geldryer (2.1.1) at 80°C for 1-2 h.

2.2.2.3 Silverstaining of SDS-PAGE gels

Silverstaining allows a detection of proteins which is 10-100 x more sensitive than Coomassie staining:
The gels were incubated in fixative 1 (for all solutions used, see 2.1.12.5) for 1 h and then again 1 h in fixative 2. After fixation of the protein bands, the gels were washed in Milli-Q dH_2O 4x for 15 min. The gel was sensitized for 1 min in 0.1mM sodium thiosulfate solution and rinsed 3x for 20 sec in Milli-Q dH_2O. To stain the gel, it was incubated in silvernitrate-solution for at least 30 min and afterwards washed again in Milli-Q dH_2O for 1 min. The gel was placed into developer-solution for up to 30 min to visualize faint protein bands. When the gel was stained at the desired

intensity, the reaction was stopped placing the gel into stop-solution for 10 min. The gels were documented afterwards and stored in 1% acetic acid (Milli-Q dH2O) at 4°C or dried onto Whatman-paper as in 2.2.2.2.

2.2.2.4 Immunodetection of SDS-PAGE proteins (western-blot)

The power of the technique lies in the simultaneous detection of a specific protein by means of its antigenicity, and its molecular mass: proteins are first separated by mass in the SDS-PAGE, then specifically detected in the immunoassay step:
SDS-PAGE separated proteins can be transferred out of the gel onto a membrane in an electrical field without changing the separation pattern of the bands. Once on the membrane, protein bands can be immunologically assigned to specific proteins using specific antibodies in various immunodetection methods.

2.2.2.4.1 Western-blot and primary antibody

Transfer-membranes used for the western-blot analysis were either nitrocellulose- or PVDF-membranes. PVDF-membranes needed to be activated by a short incubation in methanol before the blotting procedure and were then, like the nitrocellulose membranes, equilibrated in transferbuffer. Two different transferbuffers (blottingbuffers) were used, transferbuffer II (Harlow 1999) for proteins above 150 kDa and transferbuffer I for smaller sized proteins.
Proteins were blotted using BioRad's Mini Trans-Blot transfer cell, at 300 mA (around 5 mA/cm^2 gel-surface). The blotting setup ("blotting sandwich", in blotting direction left to right) consisted of one fiber pad, three Whatman-filterpapers, SDS-PAGE gel, membrane, another three Whatman-filterpapers and the second fiber pad. If necessary, the blot was checked by staining the proteins on the membrane with Ponceau S and marking their position by piercing the membrane with a sterile needle. The membrane was incubated in blocking-solution (5% skim milk powder in TBST-buffer, 2.1.12.2) for 1 h at room temperature. The blocking-solution was discarded, the membrane was washed 3x for 10 min in TBST and incubated in the primary-antibody-TBST-dilution (set according to the antibody manufacturers protocol) for 1 h at room temperature or at 4°C over night. To remove remaining primary antibody, the membrane was washed again in TBST as before. The membrane was now ready to be incubated with the secondary antibody.

2.2.2.4.2 Immunodetection with alkaline phosphatase

In this type of immunodetection, the secondary antibody was coupled to the enzyme alkaline phosphatase (AP) and was diluted in TBST according to the manufacturers protocol. After incubation with the secondary-antibody-TBST-dilution at room temperature for 1 h, the membrane was washed again 3x for 10 min with TBST and then incubated in AP-buffer (2.1.12.2) with 4.5 µl/ml NBT and 3.5 µl/ml BCIP until the bands became visible and the reaction was stopped then by adding dH_2O.

2.2.2.4.3 Immunodetection with chemiluminescence (ECL-Plus-Kit)

The ECL-Plus-Kit (Amersham) uses biotinylated antibodies to detect proteins via chemiluminescence through the enzyme horse-raddish-peroxidase (HRP). After incubation with

the secondary-antibody-TBST-dilution and washing in TBST 3x for 10 min, the membrane was incubated in streptavidin-HRP solution (1:3000 in TBST) for 1 h at room temperature. The membrane was washed again in TBST as before and put in between two plastic foils with 1 ml of the ECL-solution (according to the manufacturers protocol). The fluorescence signals were detected using a Kodak X-Omat film in the darkroom within 5 min to 1 h.

2.2.3 Expression and Purification of recombinant Proteins out of *Sf9*-cells

For expression of the different tag-fusionproteins in *Spodoptera frugiperda* (*Sf9*) cells, the baculovirus-system was used:
pVL-baculovectors were cloned (2.1.9) and the specific viruses were created. For other proteins, certain baculoviruses already existed (F. Sauer).

2.2.3.1 Growing *Sf9*-cells in cell culture

Frozen *Sf9*-cells (-80°C) were quickly thawed at 37°C and transferred into a 100 ml cell-culture flask containing 20 ml serum-free *Sf9*-media (2.1.12.1). The cells were grown at 27°C, changing the media every two days until a stable adherent layer of young cells had been formed. The cells were then transferred into a spinner-flask and kept as suspensionculture, checking the amount of cells with a cell counting chamber (Neubauer) every two days. Cell numbers were kept at 1-2x10^6 cells per ml by repeated dilution of the culture with fresh media. To create backup-samples for growing new cell cultures, 1x10^6 cells per ml were slowly frozen to -80°C in fetal-bovine-serum containing 10% DMSO.

2.2.3.2 Baculovirus-transfection

2.2.3.2.1 Primary transfection

To create recombinant baculoviruses in the primary transfection, 10^6 *Sf9*-cells were pipetted in each cavity of a 6-well-plate and incubated at room temperature for 15-30 min to settle down and adhere to the plate. Mix A (175 ng Baculo Gold-DNA, 1.3-1.5 µg plasmid-DNA and 33µl serum-free *Sf9*-media) and Mix B (10µl Cellfectin reagent and 23 µl serum-free *Sf9*-media) were prepared seperately, then combined, mixed and incubated for 15 min at room temperature. After incubation, the *Sf9*-cells were washed 3x with 1 ml serum-free *Sf9*-media to remove old media and cell debris. 440 µl serum-free *Sf9*-media was added to mixture AB and the complete transfection-solution was added to the cells. The plate was sealed with parafilm to prevent dehydration and incubated on a rocker at low speed for 15 h at room temperature. The transfection-solution was pipetted off and the transfected cells were washed once with serum-free *Sf9*-media. 2 ml media was added to the cells which were sealed again and incubated at 27°C for 5 days. The cells were resuspended and the solution was centrifuged at 1000 rpm for 5 min at 4°C (Eppendorf microfuge). The supernatant was taken off and stored at -80°C or directly used for the amplification step.

2.2.3.2.2 Amplification and proteinexpression-test

The virus-concentration after primary transfection is usually low and there is no detectable proteinexpression at this stage. Therefore, the virus needs to be amplified in order to determine if the protein is expressed or not.
$7.5x10^6$ *Sf9*-cells were spread on a 10 cm cell culture dish and settled down for 15 min. The old culture media was replaced with 2.5 ml fresh *Sf9*-media and 1 ml of the primary transfection supernatant was added. The plate was incubated on a rocker for 1 h at room temperature and then 10 ml *Sf9*-media were added to the cells. The plate was incubated afterwards at 27°C for 5 days and the cells were resuspended and centrifuged as before in sterile 15ml tubes (Eppendorf centrifuge 5810R, rotor A-4-62). The supernatant was stored or used for a proteinexpressiontest. To test if the transfection was successful and protein is expressed, $7.5x10^6$ cells were spread on a 10 cm cell culture dish, settled down and washed as before. 0.5-1 ml of the amplification-supernatant was added and the solution was incubated for 1 h, slowly rocking. 10 ml media were added to the plate and the cells were incubated at 27°C for 2 days. After incubation, the media was removed, the cells were harvested and the protein purified for analysis with SDS-PAGE.

2.2.3.2.3 Reamplification and proteinexpression

After the transfected cells proved to express the protein, the virus was amplified again in order to be concentrated highly enough for a larger scale proteinexpression.
$12x10^6$ *Sf9*-cells were spread on a 15 cm cell culture dish and settled down for 15 min. The media was discarded and 5 ml fresh *Sf9*-media and 50 µl of the amplification supernatant was added. The plate was incubated on a rocker for 1 h at room temperature and 15 ml *Sf9*-media has been added to the cells. The cells were incubated afterwards at 27°C for 5 days and then resuspended and centrifuged as before in sterile 50 ml tubes. The supernatant was stored or used for proteinexpression.
To express the protein in a larger scale, amounts between 200 – 500ml of 10^6 cells/ml were added into a 4 l erlenmeyer flask. Between 1/50 – 1/100 volumes virus-reamplification-supernatant was added and the expression-culture incubated for 2-3 days at 27°C in a incubator-shaker (2.1.1) before the cells were harvested and protein was purified.

2.2.3.3 Purification of *Sf9*-expressed, FLAG-tagged proteins

2.2.3.3.1 Cell extracts of FLAG-protein-expressions

The protein expressing cells were harvested by decanting (shaker cultures) or resuspending with a pipet (culture plates) into appropriate centrifuge tubes. They were centrifuged at 4°C and 2000 rpm (Eppendorf centrifuge 5810R, rotor A-4-62) for 10 min, after this centrifugation step all other steps were done at 4°C. The cell pellet was resuspended in 1ml/plate or 40ml/erlenmeyer 0.5 M KCl-HEMG-buffer (2.1.12.2) and twice freezed in liquid nitrogen and thawed again to open the cells. The sample was sonified 2x for 15 sec and the cell debris was removed by centrifugation for 20 min (4°C) at 13000 rpm (Sorvall RC-5 superspeed centrifuge, SS34 rotor). The supernatant was subsequently passed through a 0.45 µm and a 0.20 µm filter. The filtrate was used directly for binding to FLAG-beads or was frozen in liquid nitrogen and stored in a low temperature freezer at -80°C.

2.2.3.3.2 Purification with FLAG-beads

Anti-FLAG M2 affinity beads (FLAG-beads) were washed once with 0.8 M KCl-HEMG-buffer and incubated with the cell extract filtrate (2.2.3.3.1.) over night on a nutator at 4°C. The sample was centrifuged for 5 min at 1000 rpm (4°C) and 3x washed with 1 M KCl-HEMG-buffer, carefully centrifuging the beads as before. The third wash with 1 M KCl-HEMG contained also 0.1% CHAPS as an additional cleaning step to remove other impurities bound to the FLAG-beads. After one additional washing step with 0.5 M KCl-HEMG-buffer, the sample was eluted off the beads by adding FLAG-peptide-solution [10mg/ml] in a relation of 1:1 to the beads-volume and incubating at 4°C on a nutator for at least 4 h. The sample was centrifuged as before and the eluate was pipetted into a new sterile tube and frozen in liquid nitrogen. The beads were stored at 4°C and used as eluation-control.

2.2.3.4 Purification of *Sf9*-expressed, HIS-tagged proteins under native conditions

Cells were harvested as described (2.2.3.3.1.), centrifuged at 2000 rpm for 10 min at 4°C (Eppendorf centrifuge 5810R, rotor A-4-62) and resuspended in 30 ml His-lysisbuffer (for all buffers see chapter 2.1.12.2) per 500 ml expression culture. The suspension was freezed in liquid nitrogen and thawed twice before sonification on ice 3x for 15 sec. After centrifugation at 20000rpm for 30 min at 4°C (SS34), the supernatant was passed through a 0.45 µm filter. The filtrate was incubated over night (4°C) with Ni^{2+}-beads (2.1.4) which have been equilibrated before in buffer A. The sample was loaded on a column and after the beads had settled down, the column was washed with buffer A until no proteins could be detected in the wash-flowthrough with the spectrophotometer. After washing, the bound proteins were eluted using a gradient of buffer B up to 100% over 10 ml. Proteins were collected in fractions of 0.5 ml, frozen in liquid nitrogen for storage at -80°C and analyzed on SDS-PAGE.

2.2.3.5 Denaturating/renaturating purification of *Sf9*-expressed, HIS-tagged proteins

2.2.3.5.1 Denaturation

Cells were harvested as described (2.2.3.3.1.), centrifuged at 2000 rpm for 10 min at 4°C (Eppendorf centrifuge 5810R, rotor A-4-62) and resuspended in 30 ml guanidinium-lysisbuffer (all buffers 2.1.12.2.) per 500 ml expression culture. The sample was incubated on a rocker for 1 h at room temperature, sonified on ice 3x for 15 sec, centrifuged at 20000 rpm for 30 min at 4°C (SS34) and the supernatant was passed through a 0.45 µm filter. To purify the His-epitope tagged proteins, the filtrate was incubated over night (4°C) with Ni^{2+}-beads which were equilibrated before in buffer C1. The sample was loaded on a column and after the beads had settled down, the column was washed with 15-20 column-volumes buffer C2 until no proteins could be detected in the washing-flowthrough with the spectrophotometer. After washing, the bound proteins were eluted using a gradient of buffer D up to 100% over 10 ml. Proteins were collected in fractions of 0.5 ml, frozen in liquid nitrogen for storage at -80°C and analyzed for purity on SDS-PAGE.
Pure fractions were afterwards renaturated with dialysis.

2.2.3.5.2 Renaturation

1 ml of protein-fraction from the denaturation was transferred into a dialysis chamber (2.1.1.) which was sealed with a dialysismembrane (Wheaton, MWCO 30 kDa) which was incubated stirring in 1 l renaturationbuffer for 16 h at 4°C. After the first incubation, the dialysate was incubated again under the same conditions in kinasebuffer. The concentration of the renatured proteins was analyzed via SDS-PAGE and the aliquots were frozen in liquid nitrogen and stored at -80°C.

2.2.4 *In vitro* Kinase Assays

2.2.4.1 Transkinase-assay

The transkinase-assay used in this work originates from an assay used for characterisation of the transkinase-activity of human TAF1 ($hTAF_{II}250$, Dikstein *et al*. 1996). In this assay was analyzed, if a protein is able to act as a kinase in transferring radioactive labeled phosphate to a specific substrate. ^{32}P-γ-ATP (2.1.5.) was used as donor for the phosphate group and substrates used were single histones and histone-peptides (2.1.11.1.).
Kinase-proteins, which were purified according to chapter 2.2.3.3, were incubated with the specific substrate and 0.25-1 µCi ^{32}P-γ-ATP for 25-60 min. at 30°C in kinasebuffer (2.1.12.2.) depending on the amount of kinase-proteins (100 - 400ng) used. The reactions were stopped by adding SDS-loadingbuffer (2.1.12.2.) and boiling of the samples at 100°C for 5 min. The samples were analyzed on a SDS-PAGE gel with coomassie staining (2.2.2.1. and 2.2.2.2.), the gel was dried and the phosphorylation was detected via autoradiography. The X-ray films (Kodak X-OMAT™) were exposed for 5 min or with weak signals over night in an intensification cassette (Suprema, Dr. Goos) at -80°C. Besides measuring the kinase activity on x-ray films, bands were also excised from the dried gel, put into scintillation vials containing scintillation fluid (2.1.2.) and incubated on a shaker for 1 h. After incubation, the radioactivity in each vial was measured on a scintillation counter (2.1.1.).

2.2.4.2 Transmembrane-autokinase-assay

This kinase-assay was used to detect autokinase activity of proteinkinases under repeatedly strong denaturing conditions involving western-blotting (Shackelford 1998). Purified kinase-proteins were first denatured in SDS-loadingbuffer containing 2 M urea and 2% SDS for 40 min before loading on a SDS-PAGE gel. The gel was blotted on an Immobilon-P-membrane (2.1.4.) and the bands were made visible on the membrane by staining with Ponceau-S. The membrane was rinsed afterwards with double-filtered (Milli-Q) water and was denatured again in guanidine-denaturationbuffer for 1 h at room temperature (for all buffers see chapter 2.1.12.2.). After this secondary denaturation-step, the membrane was rinsed 3x with TBS and incubated at 4°C over night in membrane-renaturationbuffer on a rocker at low speed. The membrane was then incubated for 1 h in 30 mM Tris-HCl pH 7.5 and incubated for 30 min in kinasebuffer containing 50 µCi of ^{32}P-γ-ATP. After that, the membrane was washed twice in standard 30 mM Tris-HCl (pH 7.5), then once in 30mM Tris-HCl with 0.1 % NONIDET® P40 and then again in standard 30 mM Tris-HCl. All washes were done for 10 min. After the Tris-HCl washing steps, the membrane was incubated for 10 min in 1 M KOH to remove ATP-background, rinsed with Milli-Q water, then with 10% acetic acid and again with Milli-Q water. An X-ray film (Kodak) was put on the

membrane for at least 3 days to detect the autophosphorylation-activity of the membrane-bound proteins in an intensification cassette at -80°C.

2.2.5 Crosslinked Chromatin Immunoprecipitation (XChIP)

Protein-A-Sepharose (PAS) was blocked with XChIP-lysisbuffer, BSA [1mg/ml, Roth] and salmon testis DNA [1mg/ml] for 2-3 h and then washed 3x with XChIP-lysisbuffer.
Schneider S2 cells were added into an erlenmeyer flask at a concentration of around $400x10^6$ cells. Formaldehyde (crosslinker) was added to a final concentration of 1.8% and incubated 15 min on a shaker at room temperature. Glycine was added to a final concentration of 125 mM and incubated 5 min at room temperature. The cells were centrifuged at 1000 rpm (Eppendorf centrifuge) and the pellet was washed in precooled 1x PBS. The sample was then centrifuged again at 1000rpm and the pellet was resuspended in XChIP-lysisbuffer (3 ml per 200 ml of cell pellet). The suspension was frozen and thawed twice in liquid nitrogen, sonified (6x for 30 sec) to an average fragment length of 700 basepairs, afterwards centrifuged at 15000 rpm for 15 min and the supernatant (x-linked-chromatin) has been taken off with a filterpipet-tip. 25 µl blocked PAS was added to each 1000 µl of the supernatant, incubated for 1.5 h at 4°C and then centrifuged at 1000 rpm for 1 min. These pre-cleaned supernatants were pooled again and, at same amounts of chromatin, incubated with the specific antibodies (around 4 µg or 1:100 dilution, depending on antibody, except control sample) at 4°C over night.
25µl of blocked PAS was added to the samples and incubated at 4°C for 3 h. The samples were centrifuged at 4000 rpm for 1 min. and washed 4x in XChIP-lysisbuffer, 4x in IP1, 4x in IP2 and 4x in TE (pH 8.0). The PAS-complexes were resuspended in 100 µl TE, RNaseA was added (final conc. 50 µg/ml) and incubated at 37°C for 30 min. SDS was added to a concentration of 0.5% and Proteinase-K was added to a concentration of 0.5mg/ml. The samples were then incubated over night at 37°C.
In a second incubation step to reverse the cross-links, the samples were heated to 65°C for 6 h. The DNA was cleaned up with phenol-chloroform-extraction (2.2.1.13) and analyzed with PCR.

2.2.6 UV-crosslinking experiments

Crosslinking experiments using ultraviolet (UV)-light were used to determine the exact position of the ATP-binding domain in the kinase-proteins. Proteins were combined with [0.5 µCi] ^{32}P-γ-ATP in 1x Kinase-buffer (2.1.12.2.). The samples were immediately exposed to 9600 joules of UV-radiation in a crosslinking-chamber (2.1.1) for 10 min at room temperature. A secondary sample for each tested protein was incubated in normal daylight conditions as a negative control. The samples were then subject to SDS-PAGE (2.2.2.1.) with subsequent drying of the gel followed by analysis on X-ray film as described before.

2.2.7 Purification of polyclonal H2B-phosphoserine-33-antibodies

To purifiy the α-H2BS33P-antibody out of serum from immunized rabbits (S. Kwoczynski), two purification steps were performed. The first step implied the removal of antibodies unspecific to phosphoserine-33-H2B, the second implied the concentration of phosphoserine-33-H2B specific antibodies.

To separate H2B-specific from H2B-phospho-S33-specific antibodies, two affinity-columns were generated: 1 mg H2B-phosphoserine-33-peptide (2.1.11.1.) was coupled to 1 ml “SulfoLink”-Matrix (Pierce) via its reactive SH-group of the N-terminal cysteine, which was added to the peptide sequence. The second column was created in the same way but using unphosphorylated H2B-peptide (2.1.11.1.) instead. The coupling of both peptides was done following the protocol of the manufacturer, but all buffers used contained protease and phosphatase inhibitors at manufacturer-recommended concentrations (Roche). The rabbit-serum was run three times over the H2B-peptide column to remove the phosphoserine-unspecific antibodies from the flowthrough-serum. The flowthrough was then run again three times on the phosphoserine-H2B-peptide-column to concentrate the remaining α-H2BS33P-antibody which was eluted from the matrix by adding 100 mM glycine-buffer at pH 2.7. Fractions of 1 ml were collected, immediately neutralized with 100 µl 1M Tris-HCl at pH 7.5 and analyzed for the antibody on a SDS-PAGE gel.

2.2.8 RNAi

Plasmids used to produce TAF1-dsRNA were generated by PCR amplifying basepairs 3873-4879 from a TAF1-cDNA and cloning the product in both orientations into the pCRII-TOPO cloning vector using the TOPO TA cloning kit (Invitrogen).
RNAi was carried out as described in Pile *et al*. 2002: Briefly, 2 x 10^6 cells were plated into a 60-mm-diameter dish. After 1 h, FBS-containing medium was removed and replaced with 2 ml of serum-free medium. Approximately 40 µg of dsRNA was added per dish and mixed by swirling. After 30 min, 4 ml of medium containing 10% FBS, 100 U of penicillin per ml, and 100 µg of streptomycin per ml was added. Cells were assayed at a specified day following addition of dsRNA. To determine the growth curves of RNAi cells, cells were mixed and counted each day following the addition of dsRNA for a total of 4 days.

3. Results

This chapter addresses the question whether the phosphorylation of H2B at serine 33 (H2BS33) is truly intrinsic to dTAF1CTK and if this kinase-activity can be correlated with transcriptional activation of specific target genes.

Histone H2B has been reported in previous studies to be phosphorylated in apoptotic mammalian cells (Ajiro 2000). Interestingly, the dTAF1 protein used in this work and in initial preceding studies has been discovered in apoptotic cells and bears three additional exons. The first one resides in the NTK, adding three more amino acids at position 113. The other two exons, named 12a and 13a are located in the CTK, ranging from position 1809 to 1841 (for exon 12a) and from 1907 to 1937 (exon 13a). Exon 12a adds an additional AT-hook motif to the CTK of dTAF1, which bears the first one at amino acids 1792-1801 (Kwoczynski 2002).

The protein kinase-activity of TAF1 appears to be highly substrate specific, as it is unable to phosphorylate a variety of common phosphoacceptor proteins. Moreover, none of the other eight subunits of TFIID that are in contact with TAF1 appear to be targeted for phosphorylation by TAF1. Interestingly, in addition to RAP74, TAF1 is able to weakly phosphorylate the large subunits of TFIIA and TFIIE but not other components of the basal machinery. These findings are consistent with the notion that the phosphorylation of RAP74, and possibly TFIIA and TFIIE, by TAF1 might represent highly specific and potentially important steps during transcriptional regulation (Dikstein *et al.* 1996a). A big question regarding the specificity of the kinase-activity was, if there are other physiologically relevant substrates for the TAF1 kinase.

It has been argued that the TAF1-mediated activation of transcription is connected to enzymatic modifications of histones, which leads to the hypothesis that TAF1-kinase-dependent gene-expression gets activated due to TAF1-mediated phosphorylation of specific components of the chromatin, e.g. histones (Mizzen *et al.* 1996, Imhof *et al.* 1997, Wassarman *et al.* 2000). That implies that TAF1 has the ability to specifically modify not only GTMs but also histones posttranslational.

Earlier studies have characterized TAF1 as a bipartite kinase containing a NTK- and a CTK-activity (Dikstein et al. 1996a). The NTK phosphorylates RAP74, a part of the TFIIF complex but a substrate for the CTK has not been discovered.

In studies preceding this work, using similar kinase-assays *in vitro*, histone H2BS33 was found to be a substrate of the CTK of TAF1, underlining the hypothesis that TAF1 might activate transcription via histone-modification (Kwoczynski 2002).
The discovery of H2B as CTK-activity substrate, which is phosphorylated at serine 33 posed new questions whether the observed kinase-activity is truly intrinsic to the CTK and if so, which kinase-motifs responsible for the phosphorylation were located.
In summary, this work examines and characterizes the c-terminal kinase-activity of TAF1 closer and investigates the functional properties of TAF1 kinase-activity in transcription with several *in vitro* and *in vivo* experiments:
Experiments underlining the observed kinase-activity of dTAF1CTK as an intrinsic part of CTK were conducted. The positions and true natures of the unknown kinase-motifs were investigated *in silico* by computational analysis of the CTK-sequence. Putative kinase-motifs were modified via insertion of point mutations and/or deletions and subsequent investigation of the mutant-proteins *in vitro* by using similar kinase-assays as done in the previous studies (Dikstein *et al.* 1996, Kwoczynski 2002). An important part of this work was to investigate whether H2BS33 is phosphorylated *in vivo* and if this specific histone modification can be linked to transcriptional activation. To address this issue, various immunodetection-experiments have been conducted, using a specific antibody targeted against phosphorylated H2BS33.

3.1 Kinase-activity is intrinsic to the CTK of dTAF1

The first step in characterizing the kinase activity of the CTK was to rule out the possibility that the observed kinase-activity may be due to a CTK-associated proteinkinase, which might have been co-purified with the expressed CTK protein. To investigate whether the kinase-activity of the CTK of TAF1 is the result of intrinsic kinase activity rather than an associated kinase activity, a transmembrane-kinase assay (2.2.4.2.) was conducted with CTK and bovine-serum-albumin (BSA) as a negative control. During this assay, the proteins are denatured via SDS-polyacrylamid gelelectrophoresis (SDS-PAGE) and blotted on a membrane. The membrane-bound proteins are subsequently denatured again, then renatured and finally tested for autokinase activity. The repeated denaturing steps in this experiment and the correlated unfolding of the CTK should result in the removal of associated proteins such as co-purified kinases:

His-tagged *Drosophila* TAF1CTK (2.1.11.2.) and BSA were denatured with SDS/urea-buffer for 40 min. The denatured proteins were separated by SDS-PAGE, electrophoretically transferred onto PVDF membrane and the position and amount of transferred dTAF1CTK and BSA on the membrane was determined by staining with Ponceau-S. The membrane-bound proteins were subsequently denatured, washed, renatured, incubated with radiolabeled ATP and exposed to an X-ray film (Kodak).

A clear signal of radiolabeled dTAF1CTK was detected in the assay. In contrast, radiolabeled BSA was not detected and this although 10 fold more BSA was used in the assay than CTK (Figure **11**). Due to the repeated denaturing and washing steps used in this experiment, it is highly unlikely that another kinase, initially co-purified with dTAF1CTK, is still bound to dTAF1CTK and therefore responsible for the observed phosphorylation of dTAF1CTK. This result indicates that dTAF1CTK has an intrinsic kinase-activity in *Drosophila* and auto-phosphorylation is not mediated by another dTAF1CTK-associated kinase.

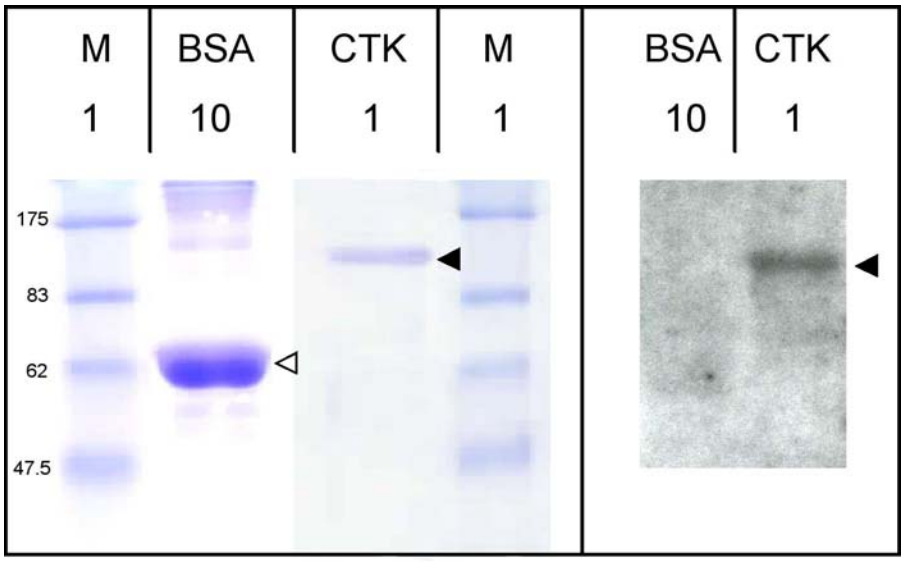

Figure 11. dTAF1-Membrane-autokinaseassay, the size of the protein marker bands are indicated on the left.
Left box: Coomassie-stained gels showing dTAF1CTK (CTK) and bovine-serum-albumin (BSA) with the loaded amounts in µg.
Right box: Autokinaseassay on denatured / renatured membrane-bound BSA (white arrowhead) and dTAF1CTK (black arrowhead). M = prestained protein marker (NEB).

3.2 Identification of the essential kinasedomains of dTAF1

Proteinkinases are divided in two classes of kinases depending on their amino acid substrate: the protein-serine/threonine kinases and the protein-tyrosine kinases (Hanks and Hunter 1995). These enzymes use the y-phosphate of ATP (or GTP) to generate phosphate monoesters using protein alcohol groups (on serine and threonine residues) and/or protein phenolic groups (on tyrosine residues) as phosphate acceptors. The kinase-activity of protein kinases lies within their conserved catalytic domain. Eukaryotic protein kinases share a conserved catalytic core common to both serine/threonine and tyrosine protein kinases.

Protein kinases contain a number of conserved subdomains, also referred to as motifs, in their catalytic domain of which two are essential for kinase function: the ATP-binding motif and the amino acid specific (serine/threonine or tyrosine) catalytic motif (Hanks *et al.* 1988). The ATP-binding motif is usually located NH_2-terminal to the amino acid specific motif (Figure **12**).

This chapter addresses the search for putative kinase motifs in the CTK by computational means *in silico* and by experimental means *in vitro*.

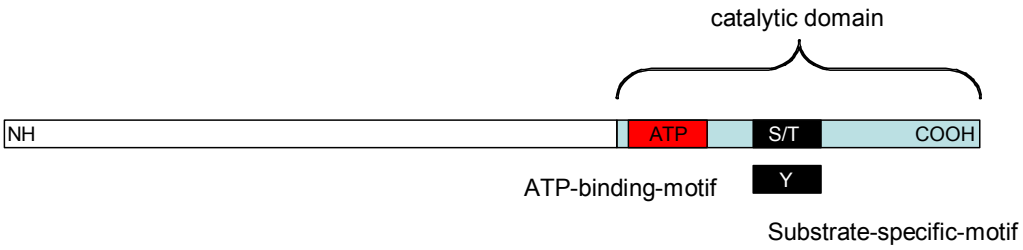

Figure 12. Typical structure of a proteinkinase. The catalytic motif of 250-300 aminoacids is located COOH-terminal with the ATP-binding motif (red) at the NH_2-terminal part of the catalytic domain followed by the substrate specific motif for serine/threonine (S/T) or tyrosine (Y, black).

3.2.1 Computational identification and characterization of the kinase motifs

To identify putative ATP-binding and amino acid specific motifs, the amino acid sequence of dTAF1CTK was examined *in silico* for homologies to sequences of known kinase motifs:

These two types of motifs are defined by three signature patterns shown in Figure **13** (PROSITE database: documentation PDOC 00100, http://ca.expasy.org/prosite/PDOC00100), which can be used for computational screening of protein sequences to detect the presence of putative kinase-motifs.

In the majority of protein kinases the ATP-binding motif is located in the NH_2-terminal extremity of the catalytic domain. The motif consists of a glycine-rich stretch of residues (basic sequence-pattern GxGxx[SGA]) in the vicinity of a conserved lysine-residue (K) that has been shown to be involved in ATP-binding. The amino acid specific kinase-motif (either serine/threonine- or tyrosine-specific), which is located in the central part of the catalytic domain, contains a conserved aspartic-acid-residue, which is essential for the catalytic activity of the kinases and a conserved lysine- and asparagine-residue (Knighton *et al.* 1991).

To identify motifs in dTAF1CTK, two signature patterns were used to detect the amino acid specific kinase-specific motifs: one pattern specific for serine/threonine-kinases (Ser/Thr) and

[LIV] - G - {P} - G - {P} - [FYWMGSTNH] - [SGA] - {PW} - [LIVCAT] - {PD} - x - [GSTACLIVMFY] - x(5,18) - [LIVMFYWCSTAR] - [AIVP] - [LIVMFAGCKR] - K (K binds ATP)

Protein-kinase ATP-binding region pattern: Detects the majority of known protein kinases but it fails to find especially viral kinases which are quite divergent in this region and are completely missed by this pattern (Prosite-pattern PS00107).

[LIVMFYC] - x - [HY] - x - D - [LIVMFY] - K - x(2) - N - [LIVMFYCT](3)
(D is an active-site residue)

Serine/threonine-kinase active-site pattern: Detects most serine/threonine specific protein kinases with 10 exceptions (half of them viral kinases) and also Epstein-Barr virus BGLF4 and Drosophila ninaC which have respectively serine and arginine instead of the conserved lysine, which are detected by the Tyrosine-pattern (Prosite-pattern PS00108).

[LIVMFYC] - {A} - [HY] - x - D - [LIVMFY] - [RSTAC] - {D} - x - N - [LIVMFYC](3)
(D is an active site residue)

Tyrosine-kinase active-site pattern: Detects all tyrosine specific protein kinases with the exception of human ERBB3 and mouse blk (Prosite-pattern PS00109).

Figure 13. Overview of the patterns used for identification of kinase-specific and ATP-binding motifs: each aminoacid (AA) is separated by a hyphon (-), several possible allowed AAs at one location are shown in square parentheses, forbidden AAs are shown in curly parentheses. The number of repetitions of AAs is shown in standard parentheses. An x stands for any AA that is possible at that location.

the other for tyrosine-kinases (Tyr). If an analyzed protein includes both of the two protein-kinase signatures covering the sequence of the same motif, the probability of it being a protein-kinase is close to 100% (http://ca.expasy.org/prosite/PDOC00100).

In the study preceding this work, the sequence of dTAF1CTK was examined for the kinase motifs described above (Kwoczynski 2002). As shown in Figure **14**, a tandem ATP-motif (amino acids 1747 to 1780) was detected within the CTK of dTAF1 by computational search with the PROSITE patterns using the protein-pattern search program of the internet-application "Network protein sequence analysis" NPS@ (NPSA) at the Pôle Bioinformatique Lyonnais ("PBIL" in Lyon, France: http://pbil.univ-lyon1.fr).

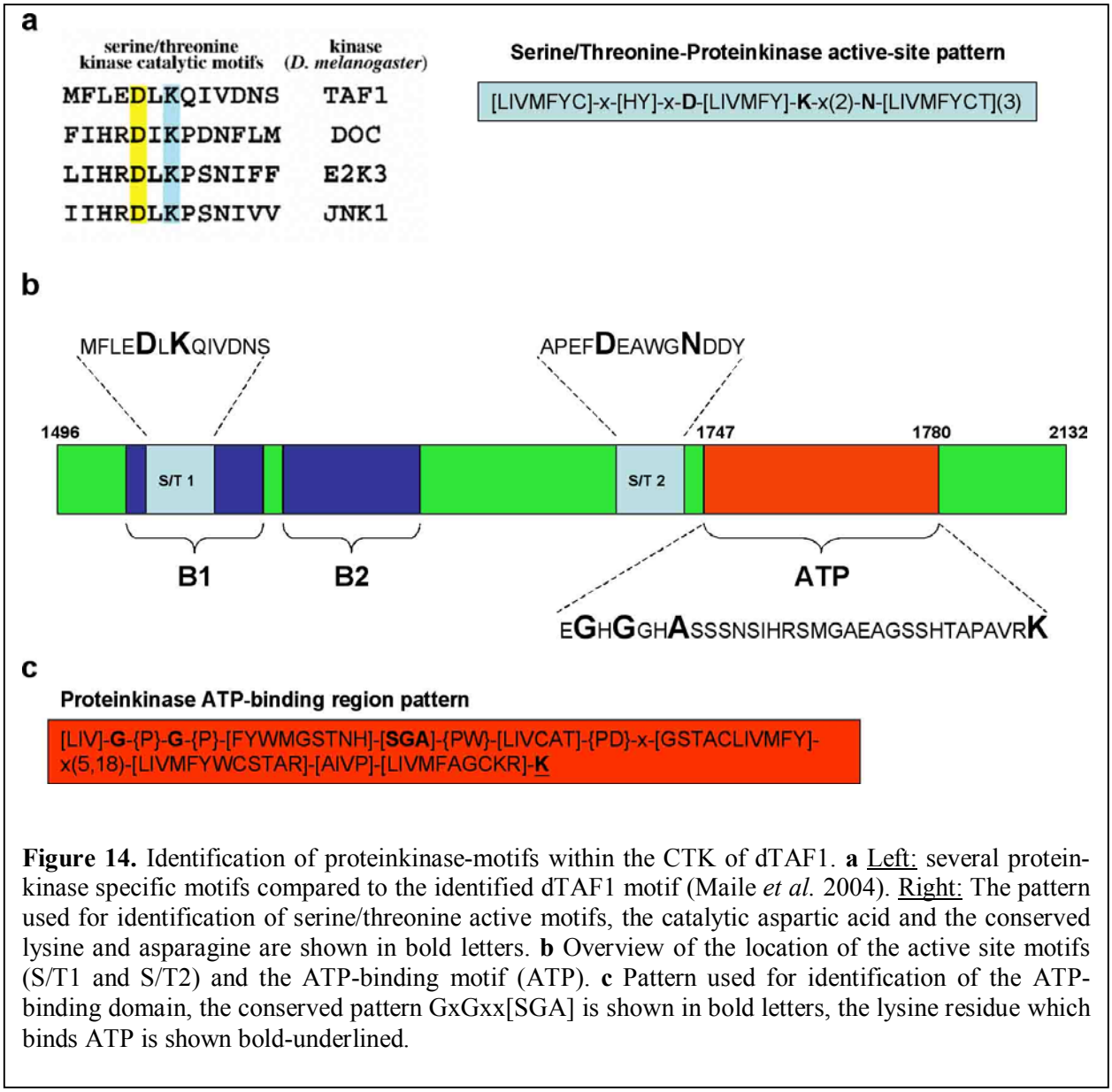

Figure 14. Identification of proteinkinase-motifs within the CTK of dTAF1. **a** Left: several protein-kinase specific motifs compared to the identified dTAF1 motif (Maile *et al.* 2004). Right: The pattern used for identification of serine/threonine active motifs, the catalytic aspartic acid and the conserved lysine and asparagine are shown in bold letters. **b** Overview of the location of the active site motifs (S/T1 and S/T2) and the ATP-binding motif (ATP). **c** Pattern used for identification of the ATP-binding domain, the conserved pattern GxGxx[SGA] is shown in bold letters, the lysine residue which binds ATP is shown bold-underlined.

However, although the tandem ATP-motif showed high homology to the prosite-pattern used (84-90% respectively), the program at PBIL was unable to detect any amino acid specific motifs since it can not detect kinase motifs that show less homology to the patterns than 50%.

A manual approach to detect putative amino acid specific motifs resulted in the discovery of two putative serine/threonine-specific motifs. Interestingly, one of the putative serine/threonine-specific motifs (S/T-1, amino acids 1537 to 1549) resides within the first bromodomain (B1) of the DBD, whereas the second amino acid specific motif (S/T-2, amino acids 1721 to 1733) was found closely NH_2-terminal to the ATP-binding motif (Kwoczynski 2002).

A second computational approach detecting the amino acid specific motifs was done in this work using both available patterns for the amino acid specific motifs: While the pattern for serine/threonine-specific kinase motifs repeatedly did not show any motifs (homology >50% for Ser/Thr), the pattern for tyrosine-specific (Tyr) kinase motifs recognized sequences overlapping the previously manually detected sequences. With a low pattern-homology of 50% the sequence for S/T-1 was detected. S/T-2 was detected also, but with a higher homology of 65% to the tyrosine specific pattern.

However, only the motif S/T-1 contained the conserved aspartatic-acid residue and the lysine residue both of which are essential for S/T-specific kinase activity (Figure **14a**).

Additionally, the detected overlapping tandem ATP-binding motif is located COOH-terminal of the detected amino acid specific motifs, which is contrary to the NH_2-terminal location of this motif as it is observed in other discovered protein-kinases.

3.2.2 Functional characterization of kinase-motifs in dTAF1CTK

To investigate whether the *in silico* identified specific kinase-motifs are responsible for the CTK-activity of dTAF1, mutant dTAF1CTK-derivates containing mutations or deletions of kinase motifs were generated.

To address the role of the S/T-motifs, proteins containing point mutations which exchange single amino acid converting the essential aspartatic-acid- to a catalytic inactive alanine-residue at the putative catalytic-active amino acid position 1541 (dTAF1CTK[D1541A]) of the first serine/threonine-specific motif S/T-1. For the second amino acid specific motif S/T-2, a protein

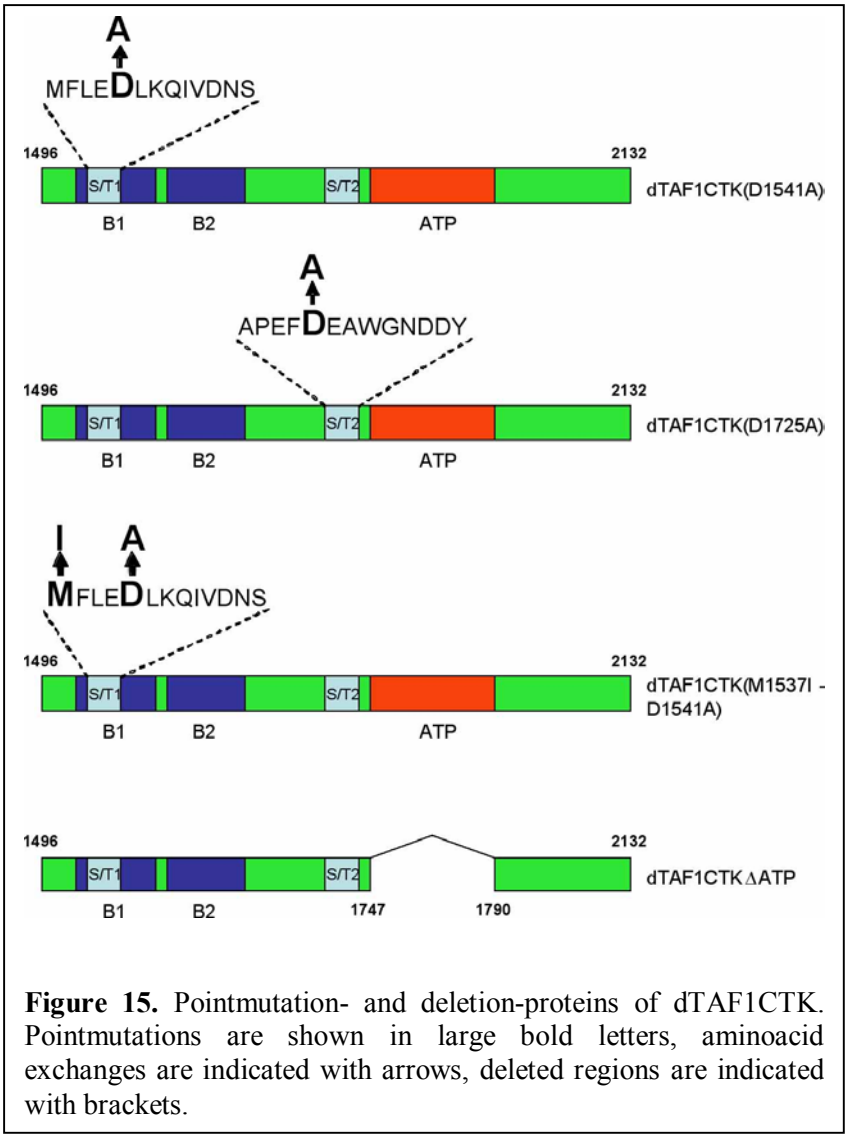

Figure 15. Pointmutation- and deletion-proteins of dTAF1CTK. Pointmutations are shown in large bold letters, aminoacid exchanges are indicated with arrows, deleted regions are indicated with brackets.

was created that contained a similar point-mutation at amino acid 1725 (dTAF1CTK[D1725A]). Another protein was created that carried a double-pointmutation in positions 1537 and 1541 of the motif S/T-1. This protein contained an additional amino acid exchange of the methionine residue at position 1537 to an isoleucine residue (dTAF1CTK[M1537I/D1541A]).

To address the role of the detected ATP-binding motif, a deletion-mutant (dTAF1CTKΔATP) was created that lacks the entire ATP-binding motif (Figure **15**).

Mutant dTAF1CTK-proteins were expressed in Sf9-cells as His-FLAG-fusionproteins and immunoaffinity-purified using FLAG-beads (2.2.3.3, Figure **16**):

The purified recombinant mutant proteins were subjected to *in vitro* kinase-assays to detect phosphorylation activity. Additionally to the created point mutation- and deletion-containing dTAF1CTK-proteins several other proteins were also investigated in the assays:

Besides unmodified wildtype dTAF1CTK, purified TFIID and unmodified full-length wildtype

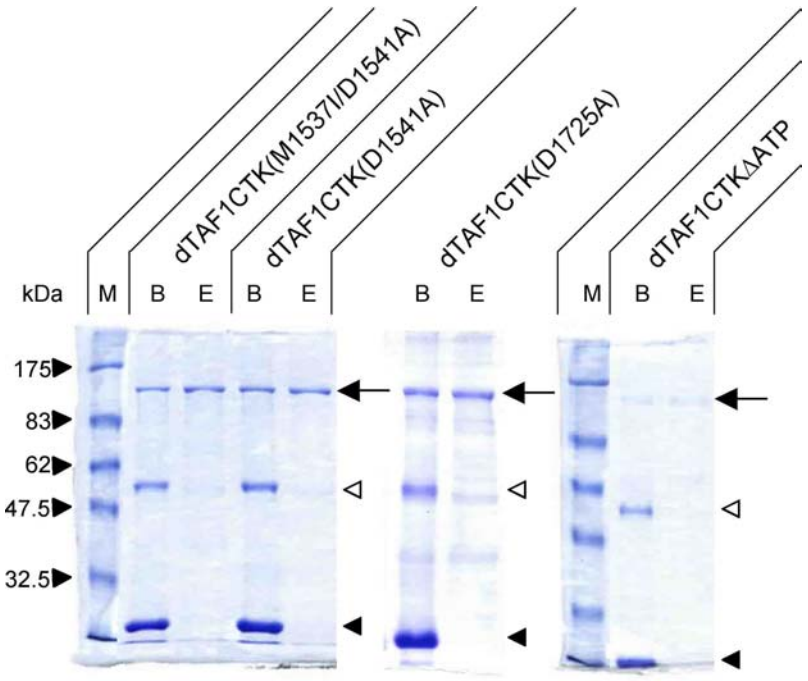

Figure 16. Coomassie-stained gels of expressed and FLAG-purified GST-FLAG-fusionproteins. M = prestained protein marker, S/T-dbl = dTAF1CTK(M1537I/D1541A), S/T-1 = dTAF1CTK(D1541A), S/T-2 = dTAF1CTK(D1725A), ΔATP = dTAF1CTKΔATP. B = 10 µl of washed, FLAG-bead-bound protein. E = 15 µl of protein, eluted with FLAG-peptide. Black arrow = fusionproteins, white arrowhead = heavy FLAG-antibody chain, black arrowhead = light FLAG-antibody chain. The size of the marker bands is indicated on the left.

dTAF1-protein (dTAF1) an additional full-length protein bearing a point mutation resulting in a stop codon at position 1728, thus lacking the CTK-domain (dTAF1ΔCTK), was subjected to kinase-assays. Additionally to this COOH-terminal-deleted dTAF1 protein an NH_2-terminal-deleted dTAF1CTK protein was used in the assay (dTAF1CTKΔ1600), which started at amino acid 1593 and thus lacked the B1 region and S/T-1 motif.

Kinase reactions were programmed with 1 μg recombinant histone H2B and contained 400 ng endogenous TFIID or 250 ng recombinant TAF1 derivatives and 0.25 μCi γ-^{32}P-ATP. The reactions were performed in 20 μl kinase buffer (see 2.1.12.2.) for 25 min at 25°C and the proteins were analyzed by SDS-PAGE using 15% SDS-polyacrylamide-gels. To compare the protein amount present in the reactions, the intensity of protein bands was detected by coomassie-blue staining. After staining, gels were dried on filter-paper (Whatman) and the kinase activity was analysed after exposure of an X-ray film (Kodak, 2.2.4.1.) to the dried gels.

The observed signal of both auto- and transphosphorylation of H2B was strongest for the TFIID complex, followed by wildtype dTAF1 and dTAF1CTK. Compared to the TFIID complex and the two wild type proteins, all mutant proteins showed reduced auto- and transkinase-activity (Figure **17**).

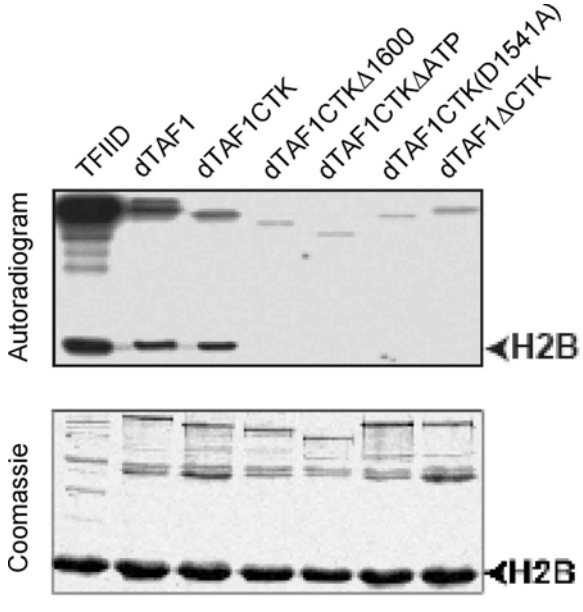

Figure 17. Autoradiogram (top) of *in vitro* kinase assays with recombinant H2B and TFIID, dTAF1 and dTAF1 derivatives.

dTAF1ΔCTK showed no transkinase-activity compared to TFIID and the unmodified wild type proteins. The auto- and transkinase-activities of dTAF1CTK(D1541A) and the double-mutant dTAF1CTK(M1537I/D1541A) were significantly reduced, indicating that the serine/threonine-specific motif S/T-1 is responsible for the serine-specific kinase-activity of dTAF1CTK (see Figures **17** and **18**).

This result is underlined by the observed reduced kinase signal of dTAF1CTKΔ1600, showing that without the complete S/T-1 motif, there is still a small amount of autokinase activity, but no detectable transkinase activity (Figure **17**).

The mutation of S/T-2 in dTAF1CTK(D1725A) resulted in a partial reduction of CTK-activity, suggesting that S/T-1 dominates over S/T-2.

Deleting the ATP-binding motif in dTAF1CTKΔATP leads to a significant decrease in kinase-activity comparable to dTAF1CTK(D1541A), indicating that the computational detected ATP-binding motif is important for kinase function of dTAF1CTK (figures **17** and **18**).

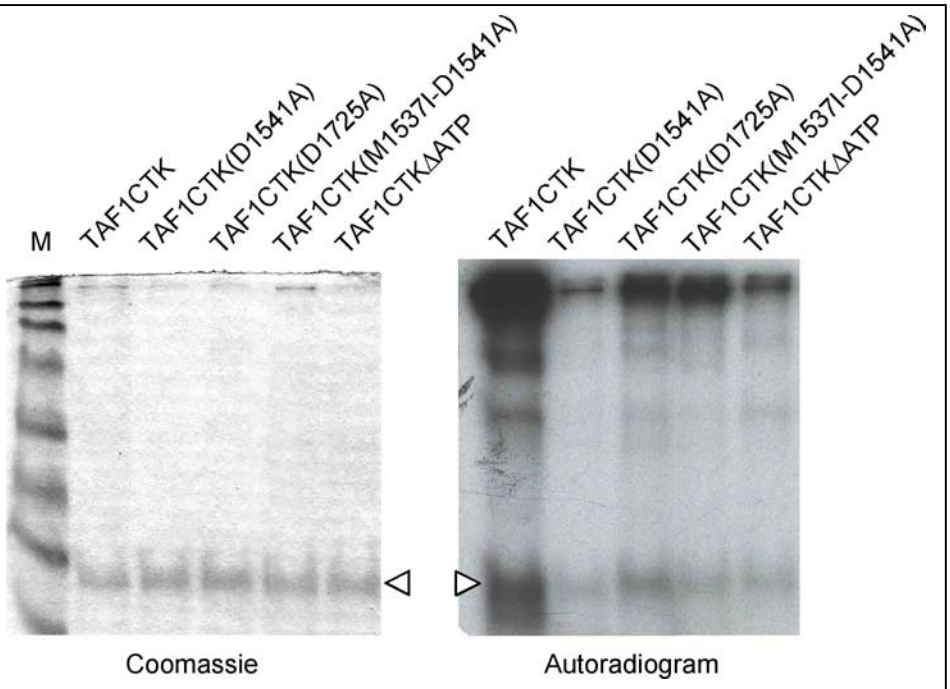

Figure 18. Transkinase-activity of *Drosophila* TAF1-pointmutation- and deletion-proteins.
Left: 15% coomassie-stained SDS-polyacrylamide-gel.
Right: autoradiogram, 24 h exposure of film to gel on left side. M = Marker, white arrowhead = Histone H2B.

3.2.3 UV-crosslinking experiments

To determine the position of the ATP-binding motif independently of transkinase-assays, crosslinking experiments, using ultraviolet- light (UV-light) as crosslinking agent, were performed. By exposing several deletion-constructs to UV-radiation, which was intended to crosslink γ^{32}P-ATP with the ATP-binding motif, an identification of an ATP-binding domain in dTAF1 by binding of the radiolabeled ATP was expected.

Various NH_2- and COOH-terminal truncated dTAF1CTK-proteins, consisting of different regions within the CTK (Figure **19a**), had been generated (Kwoczynski 2002, see chapter 2.1.11.2.).

Three proteins used in the UV-crosslinking experiments were truncated at the NH_2-terminal end: Besides dTAF1CTKΔ1600 (see above), these proteins were dTAF1CTKΔ1800, starting at amino acid 1797 thus lacking any motifs except the two exons 12a and 13a and dTAF1CTKΔ1900, starting at amino acid 1958 which lacked any identified motifs.

Two proteins were truncated at the NH_2- and COOH-terminal ends: dTAF1CTK17-18 consisted of the amino acid specific motif S/T-2 and the ATP-binding motif, ranging from amino acid 1594 to 1802. Ranging from amino acid 1797 to 1963, dTAF1CTK18-19 contained only exons 12a and 13a.

Proteins were combined with radioactive γ-labeled ATP in kinase buffer and treated with UV-light in a crosslinking chamber (2.2.6). A second set of corresponding samples was assembled in the same way but exposed to standard daylight conditions as control. After exposure to UV-light, the proteins were separated by SDS-PAGE. The positions of the truncated proteins were detected by coomassie-blue staining, the stained gels were dried and radiolabeled ATP, if bound by the specific protein, was detected by autoradiography.

As shown in Figure **19b**, dTAF1CTKΔ1900, which lacks any identified motifs, did not bind ATP indicating that no ATP-binding motifs are located in this fragment.

An unexpected result of this experiment was the observation that the untreated control samples showed as much radioactive signal as their corresponding UV-treated samples. This indicates that the binding of ATP to the specific motifs is strong enough to withstand the denaturing effects of SDS-PAGE and/or that the truncated proteins still have auto-phosphorylating kinase activity.

Interestingly, signals were detected on the proteins containing only the exons and no other identified motifs. The intensity of the signals detected at dTAF1CTKΔ1800 and dTAF1CTK18-19 is comparable to each other, indicating that one or both exons also bind ATP.

However, dTAF1CTKΔ1600 that also contains the two exons plus the ATP-binding motif, the S/T2 motif and the second bromodomain shows a comparably stronger background, indicating CTK-activity. Additionally, dTAF1CTK17-18, containing the S/T-2 and the ATP-binding motif, which are only 20 amino acids apart from each other, shows the strongest signal compared to the other samples.

In conclusion, these results show that even without the first kinase-domain, the motifs within dTAF1 still contain some ability to bind ATP strongly enough without the need of a UV-light as a crosslinking agent. Both proteins that contain the detected ATP-binding motif, dTAF1CTKΔ1600 and dTAF1CTK17-18, show the strongest detected signals compared to the other truncated

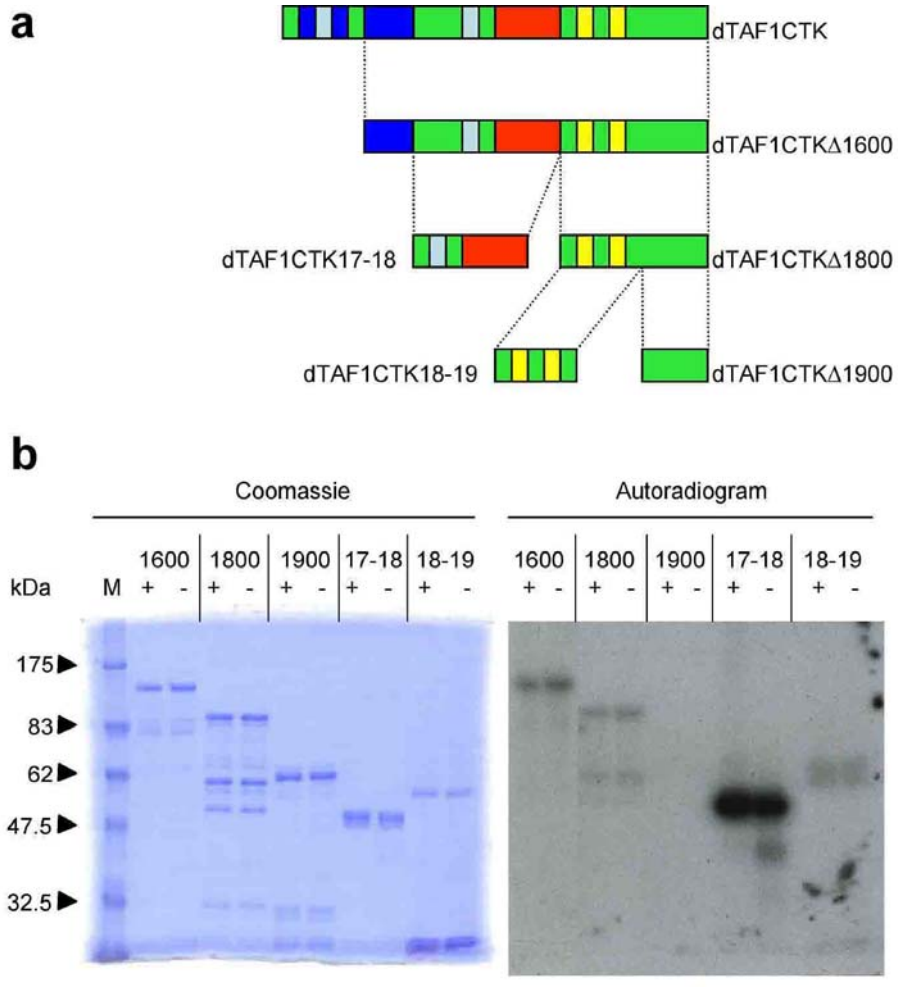

Figure 19. UV-X-link experiment with various dTAF1 deletion constructs. **a** Schematic representation of dTAF1-derivates: dark-blue = bromodomain, light-blue = S/T-motifs, red = ATP-motif, yellow = exon 12a and 13a. Shown are d TAF1-derivatives lacking the first kinase motif (dTAF1CTKΔ1600), containing only S/T-2 and the ATP-motif (dTAF1CTK17-18), only the c-terminal part (dTAF1CTKΔ1800), only the exons (dTAF1CTK18-19) or the C-terminal part without exons (dTAF1CTKΔ1900). **b** Coomassie-stained gel (left) and corresponding autoradiogram (right) of an UV-crosslink of γ-^{32}P-ATP to the constructs shown above, incubated with the radiolabeled ATP under UV-radiation (+) or daylight (-) for 10 min. M = prestained protein marker (size indicated on the left side), 1600 = dTAF1CTKΔ1600, 1800 = dTAF1CTKΔ1800, 1900 = dTAF1CTKΔ1900, 17-18 = dTAF1CTK17-18, 18-19 = dTAF1CTK18-19.

proteins. This also indicates that the S/T-2 motif and the ATP-binding motif together have kinase activity, thus forming a basic catalytic domain of a proteinkinase. An intriguing observation is that dTAF1CTKΔ1600, which contains the additional NH_2-terminal regions missing in dTAF1CTK17-18, shows a weaker signal compared to the smaller dTAF1CTK17-18. This indicates that the additional regions, including B2, might have an inhibitory effect on the kinase activity of the ATP-binding motif and S/T-2.

The ATP-binding motif might also be longer than previously expected and might encompass the region of the additional two exons. The fact that the motif-lacking dTAF1CTK1900 shows no detectable signal underlines this theory.

Interestingly, a region ranging from amino acids 1791 to 1821 with weak homology to the ATP-binding motif pattern (71%) was detected in an earlier study (Kwoczynski 2002). This region also contains two AT-hooks, which are characteristic motifs for DNA-binding proteins. AT-hooks can bind to AT-rich DNA-sequences via adenosine-binding. This ability might be responsible for the observation that the proteins dTAF1CTKΔ1800 and dTAF1CTK18-19, containing only this identified motif, can still bind ATP and that the binding is strong enough to result in a signal even after exposure of the proteins to SDS-PAGE (Figure **20**).

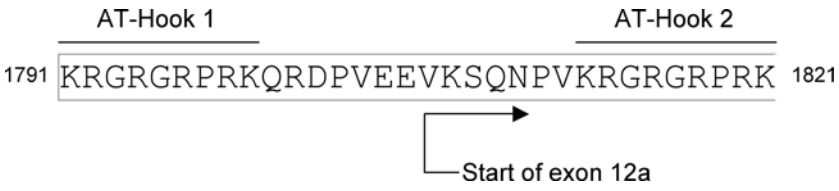

Figure 20. AT-Hooks in dTAF1. The start of exon 12a is indicated at position 1806. Both AT-Hook regions (KRGRGRPRK) are marked overlined.

3.2.4 Determination of the turnover-rate of dTAF1CTK-kinase activity

An interesting question was whether the kinase activity of the CTK of dTAF1 shows any differences in regard to the full-length dTAF1-protein or in the case of association with other proteins as TFIID complex. To determine the potency of the dTAF1CTK-kinase, we determined the turnover-rate of the kinase activity of TFIID, dTAF1 and dTAF1CTK correlated to recombinant *Drosophila* H2B and nucleosomes as substrates.

All enzymes and substrates were subjected to kinase assays and the turnover values for phosphorylation of H2B-S33 were determined by measuring the incorporation of radiolabeled phosphate groups into H2B in the presence of 3 μg TAF1, TFIID, or CTK-derivatives and 3 μg H2B or 12 μg nucleosomes. The reaction products of *in vitro* kinase assays were separated by SDS-PAGE, stained with coomassie-blue dye and the gels were afterwards dried on filter paper. The bands of radioactive H2B, nucleosomes and controls were excised from the dried gel and incorporation of radioactivity was determined by scintillation counting (2.2.4.1.).

All samples measured, reached a kinetic plateau in kinase activity within 5 to 15 minutes. The time point when the measured values reached this plateau was used to calculate the turnover values specific for each enzyme/complex with the specific substrate as shown in Figure **21**. The calculated turnover values are:

TFIID (nucleosomes: 3.3/sec; H2B: 2.6/sec); dTAF1: (nucleosomes: 2.5/sec; H2B: 10.5/sec); CTK: (nucleosomes: 4.4/sec; H2B: 2.6/sec).

The full-length dTAF1-protein showed the highest kinase activity on H2B as substrate in comparison to the TFIID complex or dTAF1CTK, while TFIID showed slightly higher kinase activity than dTAF1CTK. This indicates that within the TFIID complex, the kinase activity of dTAF1 might be inhibited due to other TAFs binding to dTAF1 and that the NH_2-terminal dTAF1 regions, which are missing in dTAF1CTK, are also contributing to the overall kinase-activity of the CTK.

In contrast, when nucleosomes were used as substrate, dTAF1CTK showed about double as much kinase activity compared to dTAF1 and TFIID, which kinase activities were measured at about the same intensities. This result indicates that the smaller size of dTAF1CTK, which lacks the 1495 NH_2-terminal amino acids of dTAF1, might be of sterical advantage for dTAF1CTK to access H2B as substrate within the nucleosome structure.

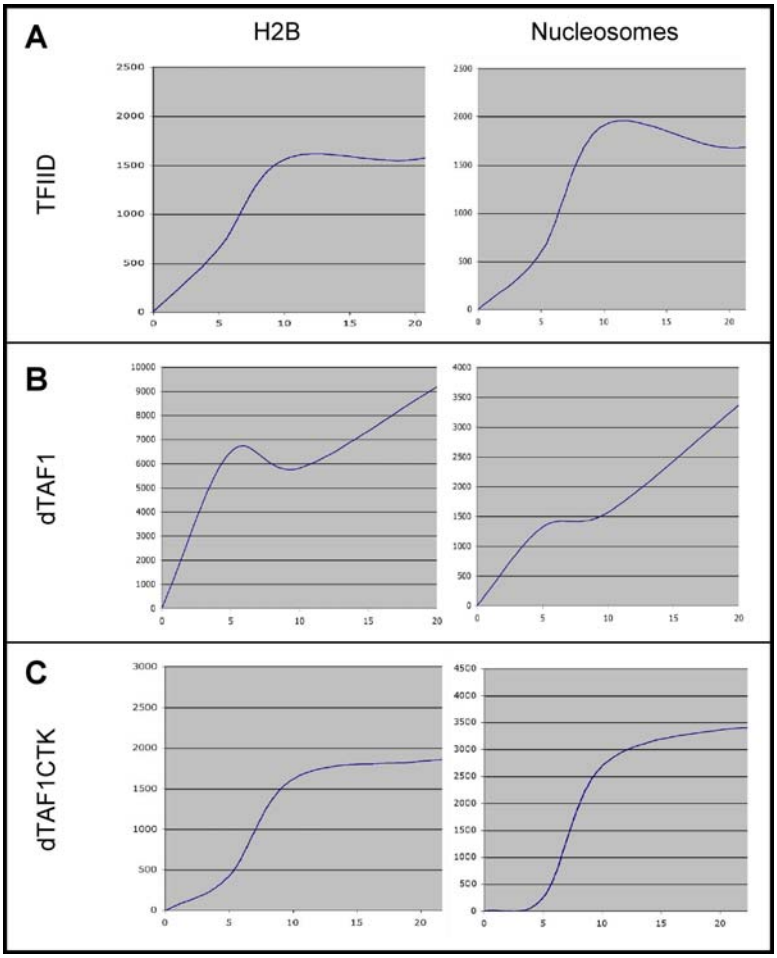

Figure 21. Kinase-activity of the TFIID-complex (A), dTAF1 (B) and dTAF1CTK (C) over time with recombinant H2B and nucleosomes as substrate. The x-axis shows the amount of incorporated radiolabeled phospho-groups measured with scintillator-counts and the y-axis shows the time in minutes.

3.3 Immunodetection of phosphorylated H2B in vivo

The results shown in the previous chapters show that dTAF1CTK has intrinsic kinase activity. To prove *in vivo* kinase activity of dTAF1CTK, it is essential to show that dTAF1 phosphorylates histone H2B *in vivo*. This chapter addresses the issue by various immunodetection experiments, which were conducted on nucleosomes, octamers and specific H2B derivates.
For these experiments, an antibody was needed that specifically recognizes the phosphorylated NH_2-terminal tail of H2B, differing in only one additional phosphate group at serine-residue 33 from the unmodified histone. A study preceding this work showed that dTAF1CTK phosphorylates H2BS33 and established an antibody recognizing phosphorylated H2BS33. However, this study did not detect phosphorylated histone H2B *in vivo* (Kwoczynski 2002).

3.3.1 Purification of the H2BS33P-antibody

To confirm the specificity of the antibody against serine 33 phosphorylated H2B (anti-H2BS33P), antibody was purified over sulfolink columns as described in chapter 2.2.7.
The two NH_2-terminal peptides of H2B, which were used to purify the antibody, were separated on two similar SDS-PAGE gels. One peptide contained phosphorylated S33 whereas the serine-residue of other peptide was unmodified (2.1.11.1). One SDS-PAGE gel was silver-stained to visualize the peptide-bands. The peptides on the other SDS-PAGE gel were blotted on a PVDF membrane, probed with anti-H2BS33P and the signals on the western-blot were detected with alkaline phosphatase (2.2.2.4.2). Purified antibody in a 1:100 dilution was able to detect as little as 10 ng of the phosphorylated H2B-peptide (not detectable on silver-stained gels), but did not detect the unphosphorylated H2B peptide (Figure **22**). Besides the peptide signals, no other signals were detected on the membrane.
The NH_2-terminal tails of histones H2B and H3 have slight structural similarities. For example, the H3 tail can be phosphorylated at serine-residues 10 and 28. To detect if anti-H2BS33P shows a possible cross-reaction with histone H3 in a phosphorylated or unmodified state, several NH_2-terminal tail peptides of H3 were probed with anti-H2BS33P.
Nucleosomes, histone H2B and NH_2-terminal tail peptides of H2B (H2BT), phosphorylated H2B (H2BT-S33P), unmodified H3 (H3T) and double-phosphorylated H3 at serine-residues 10 and 28

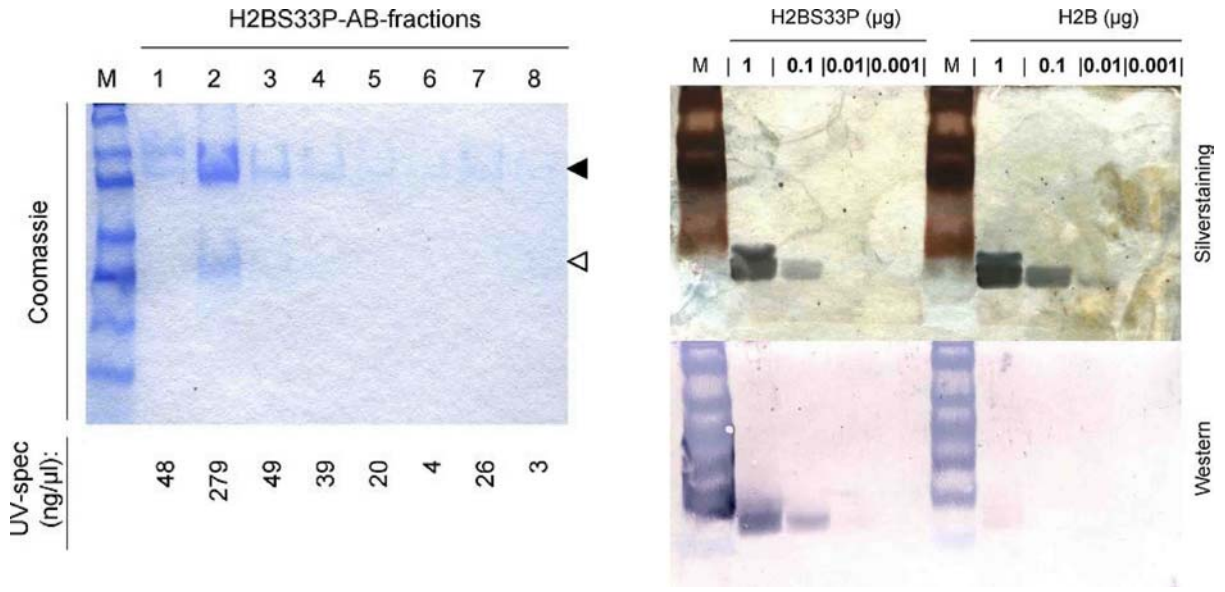

Figure 22. Purification of the H2BS33P-antibody over sulfolink columns.
Left: Eluated fractions containing antibody after purification over both sulfolink-columns. Top: Coomassie-stained SDS-PAGE gel (15%) of purified antibody fractions 1-8. Black arrowhead = heavy antibody chain at around 62 kDa; white arrowhead = light antibody chain at 27 kDa; M = prestained protein marker (NEB). Bottom: Concentration of corresponding fractions estimated with UV-spectrophotometer in ng/µl.
Right: The column-purified H2BS33P-antibody is specific for serine 33 phosphorylated H2B N-terminal tail peptide. Top: Silver-stained SDS-PAGE gel (18%) of phosphorylated peptide and standard H2B tail peptide. Amounts of phosphorylated and unmodified peptide are given in µg. Bottom: Western-blot on similar gel as on top, purified H2BS33P-antibody (fraction 2) used in 1:100 dilution with secondary antibody coupled to AP (1:3000 dilution). M = prestained protein marker (NEB). H2BS33P = serine 33 phosphorylated N-terminal-tail H2B peptide, H2B = N-terminal-tail unmodified peptide.

(H3T-S10/28P, amino acids 1 to 32) were separated on two SDS-PAGE gels. One gel was stained with coomassie-blue while the proteins on the other gel were blotted on a PVDF membrane and probed with anti-H2BS33P (Figure **23**). The antibody-signals on the western-blot were detected by chemiluminescence using the ECL-Plus-Kit of Amersham (2.2.2.4.3).

Anti-H2BS33P did not recognize an unmodified H3-tail peptide (H3T) or the double-phosphorylated H3 peptide (H3T-S10/28P), containing phosphorylated serine 10 and serine 28 (2.1.11.1.).

These results indicate that anti-H2BS33P specifically recognizes H2BS33P.

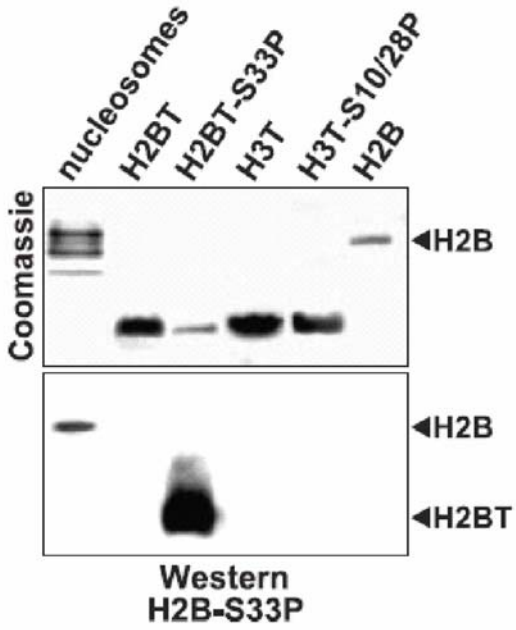

Figure 23. H2B serine 33 is phosphorylated in *Drosophila*:
Top: Coomassie-stained SDS-PAGE gel of purified nucleosomes, H2B-tail peptide (H2BT), serine 33 phosphorylated H2B-tail peptide (H2BT-S33P), H3-tail peptide (H3T), serine 10/28 doublephosphorylated H3-tail peptide (H3T-S10/28P) and recombinant histone H2B. Bottom: Corresponding western-blot with H2BS33P-antibody (1:1000 dilution, ECL-detection).

3.3.2 *Drosophila* H2B serine 33 is phosphorylated *in vivo*

To determine if H2BS33 is phosphorylated in *Drosophila*, purified *Drosophila* nucleosomes and recombinant H2B were probed on western blots with anti-H2BS33P.

The antibody recognized a protein in nucleosomes with a molecular weight similar to that of H2B, providing evidence that H2BS33 is a target for phosphorylation *in vivo* (Figure **23**).

Since phosphorylated H2BS33 was now detected in *Drosophila*, the next step was to investigate if the kinase activity of dTAF1 can be linked to this specific histone modification.

To examine whether dTAF1 phosphorylates H2BS33 *in vivo*, RNA interference (RNAi) experiments were conducted, targeting the expression of dTAF1. In short, RNAi involves post-

transcriptional gene silencing initiated by the introduction of double-stranded RNA (dsRNA) as a tool to knock out expression of specific genes in a variety of organisms. The dsRNAs direct the creation of small interfering RNAs (siRNAs), which target RNA-degrading enzymes to destroy transcripts complementary to the siRNAs (Gura 2000).

Schneider-S2 cells were treated with a 1006 bp long dTAF1-dsRNA fragment (2.2.8), thus reducing the levels of expressed dTAF1 in these cells (dTAF1-RNAi). Endogenous histone octamers were purified from the dTAF1-RNAi treated and untreated (mock) cells (source: F. Sauer) and equal amounts were separated by SDS-PAGE and examined by western-blot analysis using anti-H2BS33P (Figure **24**). Phosphorylation of H2BS33 was reduced in octamers originating from TAF1-RNAi cells compared to mock-RNAi cells. These results indicate that TAF1 is a major kinase for H2BS33 *in vivo*.

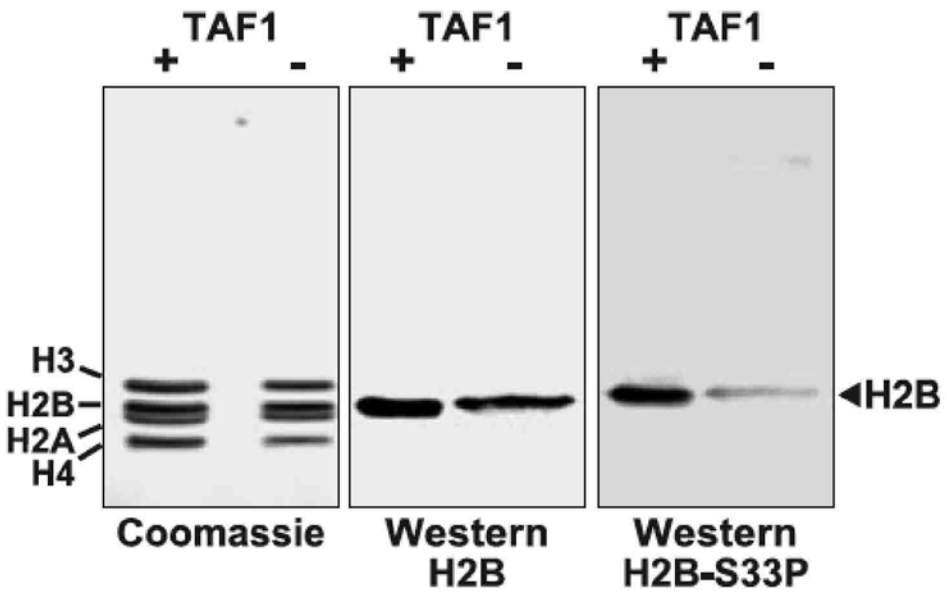

Figure 24: Coomassie blue–stained gel (left) and corresponding Western blots (middle and right) of histone octamers purified from mock- (+) or TAF1- (–) RNAi treated S2 cells.
The same Western blot was first probed with the H2B-S33P antibody (right), then stripped off the first antibody and subsequently reprobed with an antibody against unmodified H2B to present a loading control. Positions of the histones are indicated on the left, the position of H2B on the corresponding westerns is indicated on the right side.

3.4 Role of TAF1 phosphorylation of H2BS33 in transcriptional activation

Flow cytometry analysis of dTAF1-RNAi treated cells revealed that loss of dTAF1 results in cell cycle arrest in G2/M-phase (figure **25**), which suggests that dTAF1 regulates the expression of genes involved in G2/M-phase cell cycle progression (Maile *et al.* 2004).

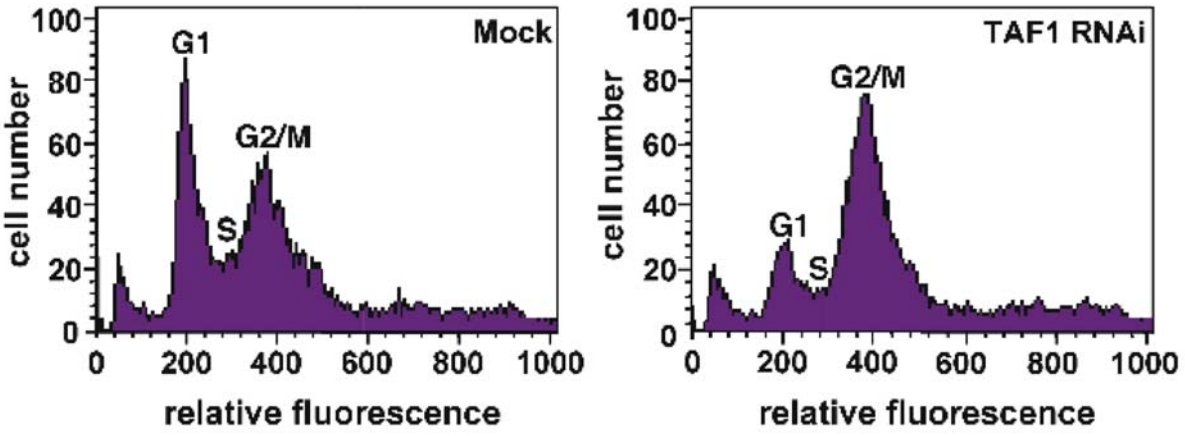

Figure 25. Knockdown of TAF1 causes G2/M phase cell cycle arrest in *Drosophila* cells. RNAi-untreated (mock, left) and TAF1-RNAi treated (right) S2 cells were analyzed by flow cytometry. Phases of the cell cycle (G1, S, and G2/M) are indicated. On average, for asynchronously growing mock-RNAi cells 40% of the cells were in G2/M while for TAF1-RNAi cells 83% of the cells were in G2/M.

This leads to the question what genes might be affected in their expression due to the loss of TAF1 mediated transcription.

One of the key cell cycle regulators of is *cdc2*, which regulates G2/M-phase (Figure **26**). The gene encodes a protein (CDK1 in humans) that is a member of a family called cyclin dependent kinases (CDKs). The activation of CDK is dependent on reversible phosphorylation. Cyclins are cell cycle proteins formed and degraded periodically during the cell cycle. The cyclins bind to the activated CDK molecules, thereby regulating CDK activity and selecting the proteins to be phosphorylated. For example, to proceed from G2 to M phase, a complex of cdc2 and cdc13 (cyclinB) is essential. The kinase Wee phosphorylates cdc2, thus inactivating it, whereas its

counterpart, the phosphatase cdc25 (*Drosophila* string, *stg*) activates cdc2 by dephosphorylation (Figure **27**).

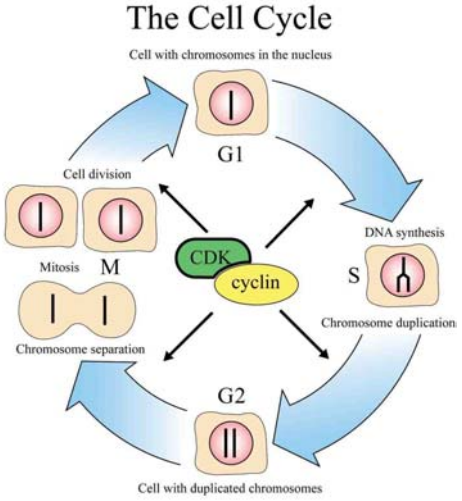

Figure 26. The eukaryotic cell cycle.

The intriguing question, if dTAF1 can be correlated to the transcription activation of specific genes, was investigated in this chapter. By use of reverse transcriptase PCR (RT-PCR), the transcription level of genes of interest in dTAF1-RNAi and RNAi-untreated (mock-RNAi) cells was monitored. To examine the modification status of histones associated to the promoters of genes that showed reduced transcription levels in cells, cross-linked chromatin immuno-precipitation (XChIP) experiments were conducted.

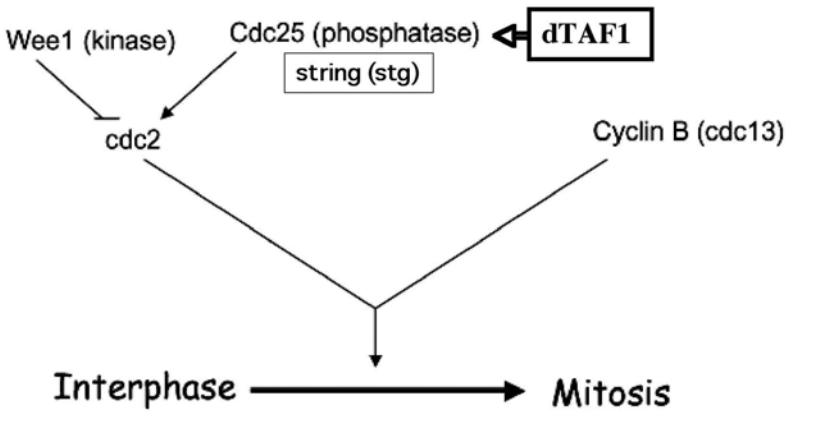

Figure 27. TAF1 might mediate cell cycle progression by mediating the transcription of essential cell cycle proteins like cdc25 (*Drosophila string*).

3.4.1 Transcription of genes in S2 cells

To investigate whether TAF1 regulates the transcription of G2/M-phase specific genes, the transcriptional status of specific genes was compared in mock-RNAi and dTAF1-RNAi treated Schneider S2 cells by using RT-PCR (2.2.1.2.4).

RNA was isolated from mock-RNAi and dTAF1-RNAi treated cells, reverse transcribed and specific primers were used to detect the presence of cell cycle specific gene transcripts in the cDNA pools. The cDNA pools were standardized by monitoring the transcription of actin5C in mock and RNAi-treated cells.

Interestingly, the transcription of the cell-cycle gene *string* (*stg*), the homolog of yeast cdc25 in *Drosophila*, was significantly reduced in dTAF1-RNAi cells compared to mock-RNAi cells (Figure **28**). In contrast, transcription of actin5C was not reduced, indicating that the observed differences reflect changes in the transcription levels rather than in the cDNA amount.

In summary, this indicates that dTAF1 regulates cdc25 transcription.

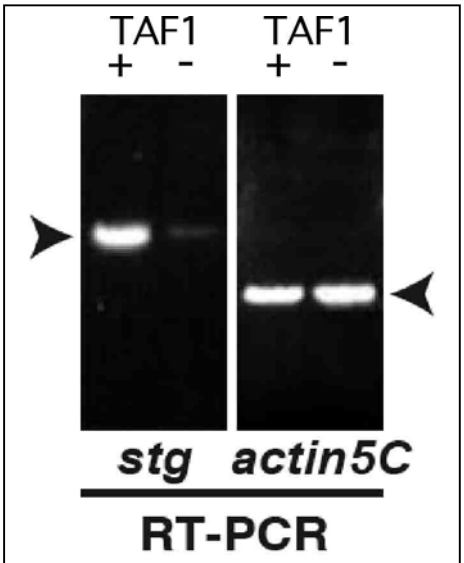

Figure 28. Ethidium-bromide stained agarose gels showing RT-PCR products for *stg* (left) and *actin5C* (right) transcripts in mock (+) and TAF1 (-) RNAi treated S2 cells.

3.4.2 H2BS33P is correlated to transcription at the *stg* promoter

To assess whether TAF1-mediated phosphorylation is involved in activation of *stg* transcription, XChIP-experiments were chosen as a means to examine the phosphorylation status of the *stg*-promoter in mock- and dTAF1-RNAi cells.

XChIP-assays were performed as described in chapter 2.2.5: *In vivo* cross-linked chromatin was isolated from mock- or RNAi-treated cells. The *in vivo* cross-linked chromatin was sheared to an average fragment length of 700 bp and was immunoprecipitated with the following antibodies: H2B-S33P, dimethyl-H3K9 and an additional non-antibody sample as control. Chromatin-antibody complexes were purified by Protein-A sepharose affinity-chromatography, incubated with RNase and Proteinase K to remove RNA and proteins respectively, and incubated at 65°C for 6 h to reverse the cross-link. The precipitated DNA was purified (2.2.1.13) and used as a template for PCR. Specific PCR-primer pairs (2.1.8.4) were used to detect the *string* promoter region from position [-333] to [+157] (relative to the start of transcription at position +1) in DNA pools.

PCR products were analyzed by gel electrophoresis using ethidium bromide containing agarose gels and detected by UV-illumination. Phosphorylation of H2B serine 33 was detected at the *stg* promoter in mock-treated cells. In contrast, H2BS33 phosphorylation was strongly reduced in dTAF1RNAi cells (Figure **29**).

TAF1 contains a histone acetyltransferase (HAT) domain that acetylates histone H3 lysine 14 (H3-K14) and unidentified lysine-residues in histone H4 *in vitro* (Mizzen *et al*. 1996). To assess the role of dTAF1 mediated histone actylation towards transcription of *stg*, XChIP assays with antibodies to acetylated H3-K4 and H4 (2.1.4) were performed. Interestingly, acetylated H3-K14 and H4 were detected at the transcriptionally active *stg* promoter in mock-RNAi cells but not at the inactive *stg* promoter in TAF1-RNAi cells (Figure **30a**).

In conclusion, these results indicate that there is a correlation between TAF1-mediated phosphorylation of H2B-S33 and acetylation of H3 and H4 to potentiate transcriptional activation in *Drosophila* cells.

To distinguish whether the loss of H2B-S33 phosphorylation at the *stg* promoter is due directly to the loss of dTAF1 due to RNAi or indirectly due to G2/M-phase arrest, XChIP assays were performed on S2 cells, which were arrested in G2/M-phase by RNAi of the SIN3 transcriptional corepressor. Although *stg* transcription is repressed in SIN3-RNAi cells (Pile *et al*. 2002), yet the

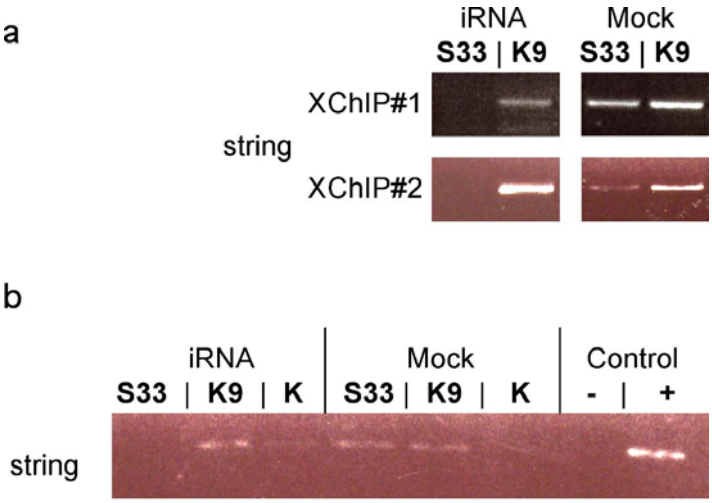

Figure 29. Photographs of ethidiumbromide-stained agarose-gels showing XChIP-experiments with antibodies against H2B-phosphoserine 33 and H3-lysine 9 in TAF1-iRNA (iRNA) and mock treated S2-cells.
a. XChIP-PCRs using a primerpair for the cdc25/string-promoterregion (string).
b. XChIP-PCR using primerpairs for the string promoter region. S33 = H2BS33P-antibody, K9 = H3K9-dimethyl-antibody, K = antibody-negative IP control, minus (-) = PCR-negative control, plus (+) = PCR-positive control (genomic-DNA template).

stg promoter remains associated with H2B-S33P and dTAF1, indicating that loss of H2B-S33 phosphorylation in dTAF1 RNAi cells is because of elimination of dTAF1 rather than G2/M-phase cell cycle arrest (Figure **30b**).

To confirm these results, Realtime-PCR was performed. The *stg* promoter was detected in DNA-pools, using Realtime-PCR-primer pairs (2.1.8.5) for generating a short amplicon of 100 bp in the presence of SYBR-green. The comparative threshold cycle (C^T) method was used to compare the presence of the *stg* promoter in immunoprecipitated DNA pools derived from dTAF1- or mock-RNAi cells. The data shown in Figure **30c** represents the relative amount of precipitated *stg* promoter of mock-RNAi treated cells compared to dTAF1-RNAi or SIN3-RNAi treated cells, supporting the results which were obtained from the XChIP experiments.

In summary, these results indicate that the loss of phosphorylation of H2BS33 is not due to indirect effects of cell cycle arrest in G2/M-phase and that the transcription of *stg* is not only correlated with dTAF1-mediated phosphorylation of H2BS33 but also with acetylation of H3 and H4.

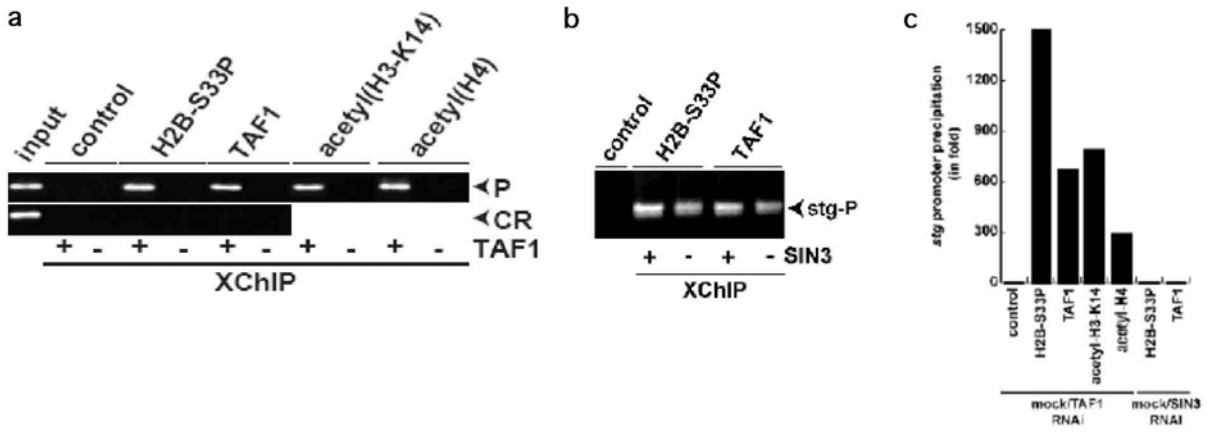

Figure 30. XChIP-experiment and corresponding Realtime-PCR.
a Photographs of ethidium bromide-stained agarose gels showing PCR products for the *stg* promoter (P) or coding region (CR) in mock (+) and TAF1 (-) RNAi S2 cells. In vivo cross-linked chromatin was immunoprecipitated with the indicated antibodies or rabbit preimmune serum (control). Input represents the amount of *stg* promoter present in 0.1% of the chromatin used for XChIP.
b Phosphorylation of H2B-S33 at the *stg*-promoter in SIN3-RNAi cells. Photograph of an ethidiumbromide-stained agarose gel. PCR products are representing a 500bp fragment containing the *stg*-promoter in mock (+) and SIN3 (-) RNAi treated S2 cells. control = rabbit preimmune serum.
c Schematic representation of Realtime-PCR experiments with the XChIP-precipitated DNA from **a** and **b** detecting the *string* promoter.

3.4.3 H2BS33 is phosphorylated at the *gt* promoter

Both maternal as well as zygotic genes control the process of segmentation in Drosophila. Members of the gap class of segmentation genes play a key role in this process by interpreting maternal information and controlling the expression of pair-rule and homeotic genes. The *Drosophila* gap gene *giant (gt)* expresses a short-range transcriptional repressor, which refines the expression pattern of other gap and pair-rule genes in the *Drosophila* blastoderm-embryo (Rivera-Pomar and Jäckle 1996). *Gt* is transcribed in two domains along the anterior-posterior axis of blastoderm-stage embryos: Transcription of the posterior *gt*-transcription-domain (pgt) is activated by the maternal activator Caudal (Cad), whereas transcription of the anterior *gt*-transcription-domain (agt) is Cad-independent (Rivera-Pomar *et al.* 1996).

To investigate the role of TAF1-mediated phosphorylation of H2BS33 during fly development, a recessive lethal dTAF1-allele, $TAF1^{CTK}$, was used which contains a nonsense mutation at amino acid 1728 that truncates the CTK downstream of the DBD (Maile *et al*. 2004). The corresponding protein (dTAF1ΔCTK) is expressed in *Drosophila* but presumably does not have CTK activity,

since it does not phosphorylate H2B *in vitro* (see Figure **17**). *In situ* hybridization was used to monitor the transcription of *gt* in embryos, which were homozygous mutant for *TAF1*^{CTK} and heterozygous mutant for *cad* (Rivera-Pomar and Jäckle 1996, Maile *et al.* 2004). The hybridization experiments indicated that in this genetic background, the transcription of *giant* was reduced. Relative to controls (*cad*/+ or *TAF1*^{CTK}), pgt but not agt transcription was reduced in *cad*/+;*TAF1*^{CTK} embryos (Figure **31a**).

To examine whether dTAF1-mediated phosphorylation of H2B-S33 contributes to pgt transcription, the histone modification status at the *gt*-promoter was investigated by XChIP-analysis.

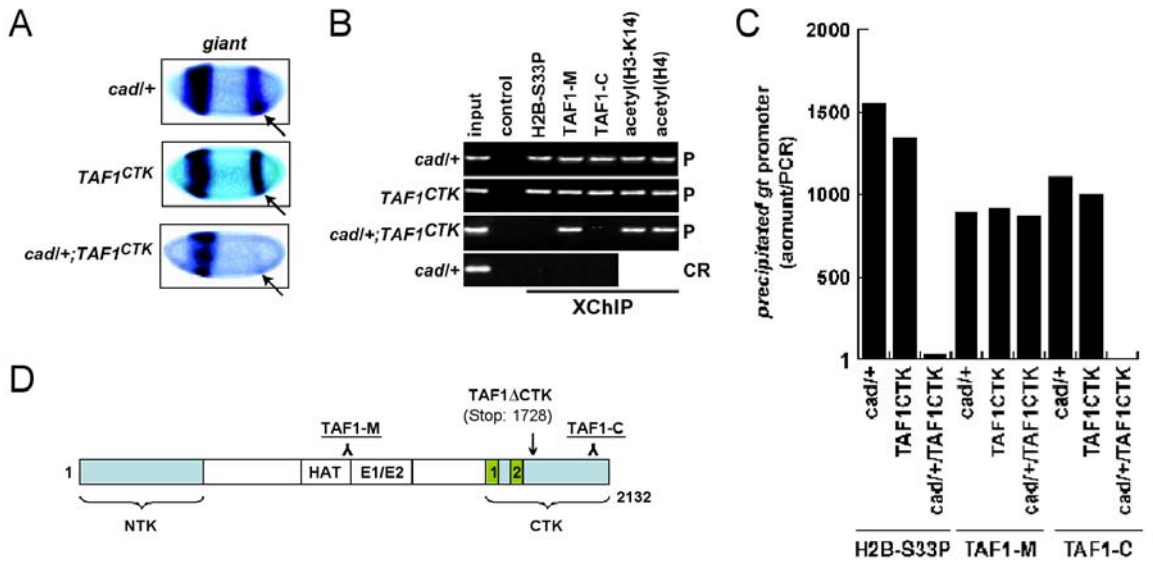

Figure 31.

A *Gt*-transcription in heterozygous mutant *cad* (top, *cad*/+), homozygous mutant *TAF1ΔCTK* (middle, *TAF1*^{CTK}), or heterozygous mutant *cad* and homozygous mutant *TAF1ΔCTK* (bottom, *cad*/+;*TAF1*^{CTK}) blastoderm-stage *Drosophila* embryos. *Gt*-transcription was detected by *in situ* hybridization with *gt* anti-sense RNA. Anterior is to the left and dorsal is up. The positions of the posterior *gt*-transcription-domains are indicated by arrows.

B Photographs of ethidiumbromide-stained agarose-gels showing PCR products for the *gt* promoter (P) or coding region (CR) in embryos described in A. In vivo cross-linked chromatin was isolated from the posterior halves of embryos and immunoprecipitated with the indicated antibodies or rabbit preimmune serum (control). Input represents the amount of *gt* promoter present in 2% of the chromatin used for XChIP.

C Schematic representation of the relative amounts of precipitated *gt* promoter in DNA pools generated by XChIP using the indicated antibodies. Chromatin was prepared from embryos laid by *cad*/+ females expressing wild type TAF1 or TAF1ΔCTK or homozygous mutant *TAF1*^{CTK} embryos.

D Schematic representation of TAF1 and the epitopes recognized by TAF1 antibodies TAF1-M and TAF1-C. The position of the premature stop-codon mutation in *TAF1ΔCTK* is indicated.

Cross-linked chromatin was isolated from the posterior halves of *cad*/+;*TAF1*^{CTK}- and control-embryos and immunoprecipitated with antibodies against H2B-S33P, acetylated histones, or dTAF1. PCR detected H2BS33P at the transcriptionally active *gt* promoter in control embryos (*cad*/+ or *TAF1*^{CTK}), but not at the transcriptionally silent promoter in *cad*/+;*TAF1*^{CTK}-embryos (Figure **31b**). To monitor the binding of dTAF1 to the promoter, two different antibodies (TAF1-M and TAF1-C) were used, which recognize the middle domain (TAF1-M) or the CTK of TAF1 (TAF1-C), respectively (Figure **31d**). The *gt*-promoter was precipitated by both antibodies from control embryos indicating that dTAF1CTK and maternally contributed wild-type TAF1 are present at the *gt*-promoter in the pgt (Figure **31b**).

Interestingly, although the TAF1-M antibody precipitated the *gt* promoter from *cad*/+;*TAF1*^{CTK}-embryos, TAF1-C did not. This result suggests that TAF1ΔCTK is preferentially recruited to the *gt*-promoter in the pgt (Figure **31b**), suggesting that TAF1ΔCTK, which is recognized by TAF-M, is present at a higher concentration in *cad*/+;*TAF1*^{CTK}-embryos than maternal contributed dTAF1.

This result is supported by the presence of TAF1-mediated histone acetylation at the transcriptionally silent *gt*-promoter, since TAF1ΔCTK still contains HAT activity and a functional DBD (Figure **31d**). Thus, TAF1-mediated phosphorylation of H2B-S33 contributes to transcriptional activation during *Drosophila* embryogenesis.

To confirm these results, Realtime-PCR was used to detect the *gt* promoter in the same immunoprecipitated DNA pools that were used to generate the results shown in Figure **31b**.

Realtime-PCR assays were programmed with primers (2.1.8.5) detecting the *gt* promoter in the presence of SYBR-green to generate a short amplicon of about 100 nucleotides to compare the presence of the *gt* promoter in immunoprecipitated DNA pools derived from precipitation with H2B-S33P, TAF1-M, or TAF1-C antibodies. Figure **31c** shows the relative amounts of precipitated *gt* promoter in the DNA pools generated by XChIP using the indicated antibodies. As shown with the XChIP assays before, the *gt* promoter could not be detected by Realtime-PCR in the DNA pools of *cad*/+;*TAF1*^{CTK}-embryos.

Summarizing these results, H2BS33 is phosphorylated at the *gt* promoter and this modification status can be correlated with transcription activation of *gt* in *Drosophila*. The finding that loss of H2B-S33P from the promoter results in reduced transcription, despite the presence of TAF1-mediated histone acetylation indicates that dTAF1 CTK- and HAT-activities can cooperate in transcriptional activation of some genes. Thus, TAF1-mediated phosphorylation of H2B-S33 contributes to transcriptional activation during *Drosophila*embryogenesis.

3.5 TAF1CTK homologs in yeast

The sequence of yeast TAF1 does not contain a CTK, which instead is formed by the two proteins Bdf1 and Bdf2 (Matangkasombut *et al.* 2000). Both proteins contain a DBD and the acidic COOH-terminal regions, similarly to dTAFCTK (see Figure **6**). In intriguing question was if these proteins also contain kinase motifs and if kinase activity towards histones as substrates can be detected. In this chapter, Bdf1 and Bdf2 were screened for the presence of kinase motifs and kinase assays were used to detect specific kinase activity towards the core histones H2A, H2B, H3 and H4.

3.5.1 Saccharomyces cerevisiae Bdf1 and Bdf2 contain putative kinase motifs

Using the PROSITE patterns shown in Figure **13**, the amino acid sequences of Bdf1 and Bdf2 were screened for the presence of ATP-binding and amino acid specific motifs with the internet application PATTINPROT.

This computational analysis of Bdf1 and Bdf2 revealed that these proteins share a putative conserved serine/threonine-specific motif in the second bromodomain (B2) of the DBD, which shows homology to the specific PROSITE pattern used to identify S/T-motifs in dTAF1. This motif ranges from amino acids 353-365 in Bdf1 and 357-369 in Bdf2. The calculated homology of the detected motifs towards the PROSITE patterns was 52% for Ser/Thr and 69% for Tyr in Bdf1 and Bdf2. However, this S/T-motif does not contain the conserved lysine at position seven within the 13 amino acid motif sequence (Figure **32**).

Additionally, Bdf2 contains an NH_2-terminal ATP-binding motif (amino acids 44-77) showing 83% homology to the pattern, which is missing in Bdf1. Although Bdf1 has glycine-rich areas in the NH_2-terminal part of the protein sequence, the conserved sequence GxGxx[SGA] missed at least one of the conserved amino acids and thus, a conserved ATP-binding motif could not be detected.

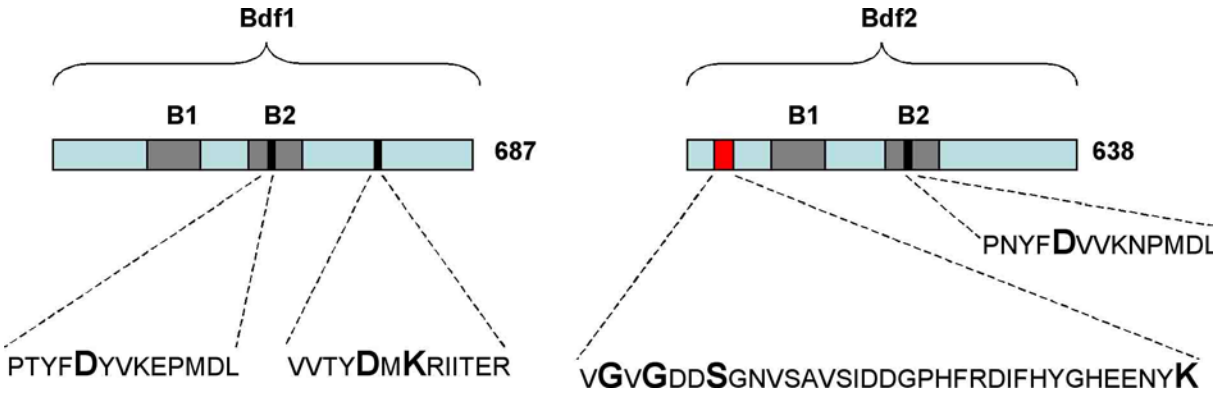

Figure 32. Kinase motifs found in yeast Bdf1 and Bdf2 by computational search.
Grey areas = regions of the bromodomains 1 and 2 (B1, B2). Kinase-specific motifs are shown in black and ATP-motifs in red boxes. Conserved and catalytic active residues in the amino-acid sequence are shown highlighted (graphical motif-size shown in scale to overall length of protein).

Instead, Bdf1 contains a second S/T-motif besides the motif in B2, ranging from amino acids 528-540. Interestingly, a conserved lysine at position seven is present in this motif but the conserved asparagine-residue at position 10 could not be found in any of the detected S/T-motifs. The Bdf1 S/T-motif shows weak homology compared to the pattern from the PROSITE database (Ser/Thr 53%, Tyr 50%), which is comparable to the homology of S/T-1 in dTAF1CTK.
The presence of an NH_2-terminal ATP-binding-motif followed by an S/T-motif in Bdf2 might form a functional catalytic domain as found in other typical proteinkinases.
Thus, Bdf2 appears like a mirror image of dTAF1CTK that contains the kinase-motif in the first bromodomain and a C-terminal ATP-binding motif (Figures **12** and **32**).

3.5.2 Bdf1 and Bdf2 phosphorylate H3 *in vitro*

To investigate, whether Bdf1 and Bdf2 have kinase activity, FLAG-epitope tagged fusionproteins of Bdf1 and -2 were expressed and purified from Sf9 cells (Figure **33**). The recombinant Bdf1 and -2 proteins were subjected to *in vitro* kinase assays to detect kinase activity.

Kinase-assays were programmed with 1 µg of recombinant *X. laevis* H2A, H2B, H3 or H4 respectively and 400 ng of Bdf1 or Bdf2. Bdf1 and Bdf2 have autokinase-activity and phosphorylate H3 (Figure **34**). This shows that both Bdf-proteins are histone kinases.

Bdf2 seems to have a stronger transkinase-activity towards H3 when compared to the transkinase-signal obtained with Bdf1. Additionally, the autokinase-activity seems to decrease in the presence of H3, indicating that H3 as substrate competes with the autokinase-activity of Bdf1 and Bdf2.

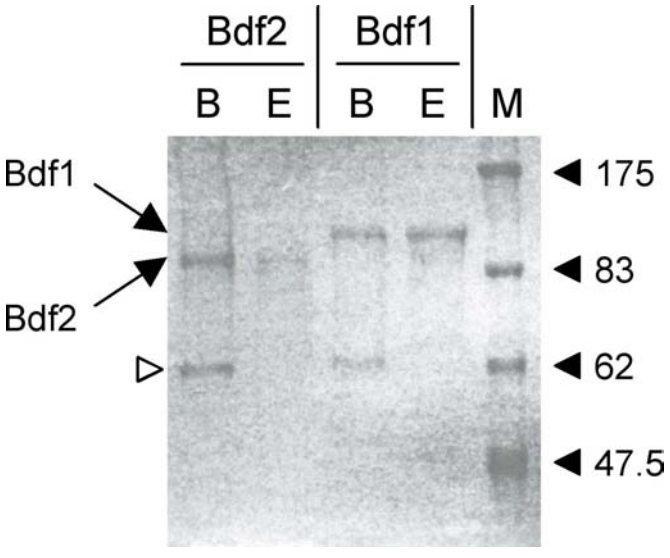

Figure 33. Sf9-expressed Bdf1&2 fusionproteins. B = beads, E = eluate, black arrows show the bands of the fusionproteins, white arrowhead = heavy antibody chain. M = prestained protein marker (NEB), molecular weights (in kDa) are indicated on the right.

This result indicates that Bdf1 and Bdf2 have histonekinase-activity. The higher activity of Bdf2 might be correlated to the more conserved ATP-binding motif. However, since Bdf1 shows kinase activity too, a not very high conserved ATP-binding motif might be located within the glycine-rich areas in the NH_2-terminal part of the protein.

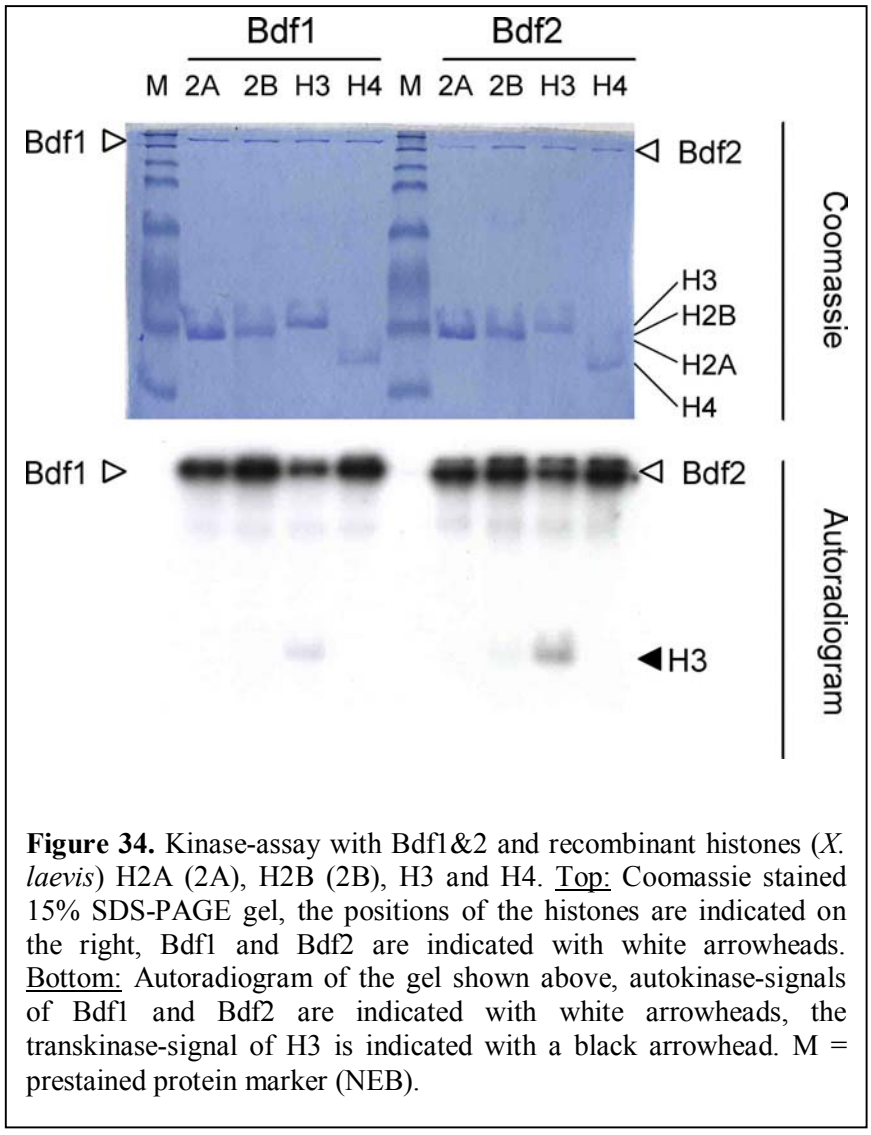

Figure 34. Kinase-assay with Bdf1&2 and recombinant histones (*X. laevis*) H2A (2A), H2B (2B), H3 and H4. Top: Coomassie stained 15% SDS-PAGE gel, the positions of the histones are indicated on the right, Bdf1 and Bdf2 are indicated with white arrowheads. Bottom: Autoradiogram of the gel shown above, autokinase-signals of Bdf1 and Bdf2 are indicated with white arrowheads, the transkinase-signal of H3 is indicated with a black arrowhead. M = prestained protein marker (NEB).

4. Discussion

The coactivator TAF1 plays an essential role in the regulation of essential biological processes by activating for example the transcription of cell cycle, cell-growth- and developmental factors (Wang *et al.* 1997, Walker *et al.* 1997, O'Brien and Tjian 2000, Pham and Sauer 2000). TAF1 activates transcription of target genes by initiating the assembly and activity of the PIC at the promoter and through enzymatic modifications of components of the general transcription machinery (GTM) and histones, resulting in the transfer of activation-signals to the GTM (Dikstein *et al.* 1996a, O'Brien and Tjian 1998, Solow *et al.* 1999; 2001).
The HAT-activity of TAF1 can acetylate histones H3 and H4 while the E1/E2-activities mono-ubiquitinate histone H1. In contrast, the kinase activity of the NTK targets components of the GTM (Dikstein *et al.* 1996a, O'Brien and Tjian 1998, Wassarman *et al.* 2000).
A previous study discovered that the CTK of dTAF1 phosphorylates H2BS33 and is essential for TAF1 activity *in vivo* (Kwoczynski 2002). However, the kinase motifs of the CTK remained unknown. Furthermore, it remained mysterious whether phosphorylated H2BS33 existed *in vivo* and if this modification is involved in transcriptional activation of specific TAF1-target genes.
This work functionally characterizes the CTK of dTAF1 as an atypical serine-kinase and uncovered the characteristic kinase motifs, the serine/threonine-specific motif and the ATP-binding motif in the CTK. The results provided evidence that TAF1 is a major kinase of H2BS33 *in vivo*. Genetic and RNAi assays revealed that TAF1 plays an essential role in the transcription of cell cycle and developmental genes. Therefore, the kinase activity can be linked to TAF1-mediated transcriptional activation.

4.1 dTAF1 is an atypical histone-kinase

The most important difference of TAF1 compared to other kinases is the fact that TAF1 contains with NTK and CTK two distinct kinase-domains.
However, the kinase activity of human TAF1 (hTAF1) is under dispute. Recently, Sawa *et al*. described that the phosphorylation of the CTK of hTAF1 is not mediated by CTK autokinase activity but rather by the TAF1-associated protein kinase CK2, responsible for phosphorylation of

the yeast TAF1CTK-homolog Bdf1 (Dikstein *et al.* 1996a, O'Brien and Tjian 1998, Sawa *et al.* 2004). This result is supported by the fact that *E. coli*-expressed TAF1 shows no autophosphorylation activity, probably due to the fact that highly structured molecules of the size of TAF1 get differently folded or lack eukaryotic posttranslational modifications. To verify that dTAF1 has intrinsic autokinase activity, His-tagged dTAF1CTK, which has been denatured and subsequently renatured, was subjected in this study to an additional denaturing/renaturing step by a transmembrane-autokinase-assay. The results revealed that, contrary to the findings by Sawa *et al*., dTAF1CTK contained autokinase activity, suggesting that there is no additional kinase involved in the autophosphorylation of dTAF1CTK (Figure **11**). This data demonstrates that dTAF1CTK has intrinsic kinase activity.

4.1.1 dTAF1CTK differs in kinase motif alignment from other proteinkinases

While dTAF1 shares common structural characteristics with typical protein kinases in its NTK, the CTK differs from the typical alignment in several points.

A characteristic attribute of serine/threonine- and tyrosine-specific kinases is a COOH-terminal localised catalytic domain consisting of around 250-300 amino acids, which can be separated into eleven motifs, also called subdomains (subdomains I-XI) with partially highly conserved sequences (Hanks *et al.* 1988, Hunter and Hanks 1995). These sequences have been identified by analyzing protein sequences of a selection of 65 different serine/threonine- and tyrosine-specific kinases, whose biological function was unknown (Hunter and Hanks 1995). Further studies showed that only a few motifs were relevant for the catalytic activity of these kinases and that they could be summarized into two essential types of kinase motifs as shown in the PROSITE documentation PDOC00100: the ATP-binding motif and the substrate-specific-binding motif (Figures **12** and **13**). Both of these kinase-motifs were detected by computational sequence comparison in dTAF1CTK, but except for the ATP-binding motif, homologies of the TAF1 S/T-motifs to other serine/threonine-specific motifs of protein kinases were very low, indicating that dTAF1 might belong to a novel class of protein kinases. Supporting this hypothesis is the fact that a number of protein kinases have been described whose catalytic domains do not share the same attributes as eukaryotic protein kinases (Hunter and Hanks 1995). However, the majority of known serine/threonine- and tyrosine-kinases contain the conserved ATP-binding motif (Ryazanov *et al.* 1997). As detected by the experiments in this and previous studies, both NTK and CTK contain the conserved ATP-binding motif.

Interestingly, while the NTK of dTAF1 shows a typical alignment of kinase motifs forming the catalytic domain, the CTK shows an untypical catalytic domain: The NTK contains a common serine/threonine-kinase-specific alignment, involving a NH_2-terminal localised ATP-binding motif followed by a conserved serine-threonine-specific motif within the catalytic domain (Kwoczynski 2002). In contrast, the motifs identified in the CTK show a vice-versa assembly (Figure **14**), thus mirroring the standard pattern of subdomains as found in the NTK.

Except for the double-bromodomain of hTAF1, no crystal structure of TAF1 exists to date (Jacobson *et al*. 2000). It requires further examination of the three-dimensional structure to determine whether a mirrored assembly of two terminal kinases has advantages in terms of substrate-recognition, ATP-binding or general stability of the kinase-structure. The TFIID-complex was documented to have a horseshoe-like three-dimensional structure (Andel *et al.* 1999). Since TAF1 builds the main structural scaffold of the TFIID-complex, it is imaginable that TAF1 also might form a horseshoe-like substructure, maybe with two parallel-aligned kinase-domains at the ends of the complex (Figure **35**).

So far, both kinases have been shown to have autokinase activity and specifically recognize and phosphorylate different substrates: NTK binds and phosphorylates RAP74 (Ruppert and Tjian 1995, Dikstein *et al.* 1996a) whereas CTK phosphorylates H2BS33 (Maile *et al.* 2004). However, the efficient transphosphorylation of RAP74 seems to require the combined action of both

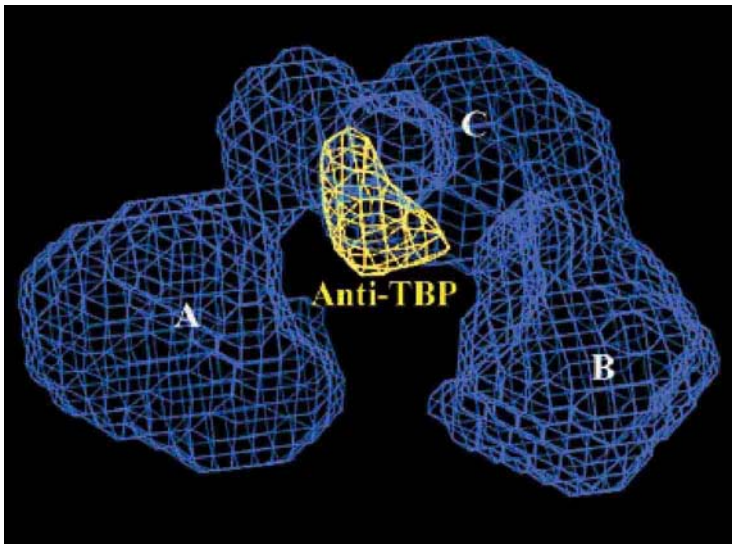

Figure 35. The 3D-structure of the TFIID-complex. The blue mesh corresponds to the holo-TFIID, with the A, B, and C lobes indicated. The yellow mesh shows the density difference between the holo-TFIID and TFIID that is bound to the TBP antibody (Andel *et al.* 1999).

functional kinase domains (Dikstein *et al.* 1996a), in which, hypothetically, a specific parallel assembly of the two kinase domains at the terminal ends of TAF1 might be of functional significance, bringing both kinase domains into a close spatial alignment.

4.1.2 Bromodomain 1 of dTAF1CTK contains the amino acid specific motif

The performed kinase experiments identified the substrate-specific motif of the CTK catalytic domain. The amino acid specific motif of a total length of 13 amino acids is characterized by three highly conserved amino acids: a catalytic aspartic-acid-residue at position five, a conserved asparagine-residue at position ten and a substrate specific amino acid at position seven which is, in case of typical Ser/Thr-kinases, a lysine-residue (see also Figure **13**).
dTAF1CTK contains such a motif with the two conserved amino acids apartatic-acid and lysine in its first bromodomain B1. The aspartic-acid of the amino acid specific motif is presumed to act as a base to remove a proton from the protein substrate hydroxyl group (Figure **36**), although the catalytic mechanism is not definitively established.

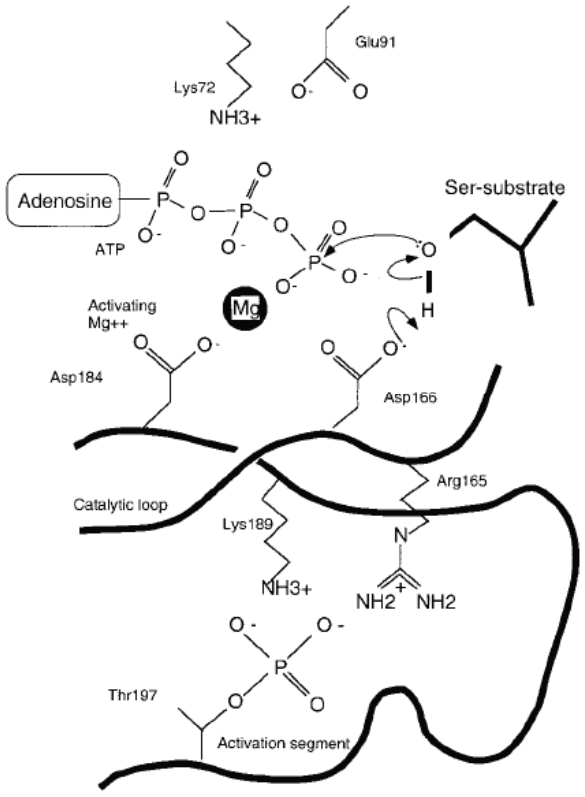

Figure 36: An example of the roles of conserved kinase motif amino acid residues important for binding of ATP shown for the Ser/Thr-kinase cAPK:
The correct alignment of the ATP with respect to the catalytic residues appears crucial for catalysis. The adenine is located in a hydrophobic pocket, and the ribose is stabilized by hydrogen bonds. The phosphates of ATP are aligned for catalysis by interactions with the main chain nitrogens of a NH_2-terminal glycine-rich loop, by interactions with Lys-72 (which is localized by a salt bridge to Glu-91), and by interactions with Mg^{2+} ions. An activatory Mg^{2+} binds to the β- and γ- phosphates of ATP and to Asp-184. The catalytic aspartate, Asp-166, which is invariant in all kinases, is located in the COOH-terminal lobe in a loop, termed the catalytic loop. The aspartate is presumed to act as a base to remove a proton from the protein substrate hydroxyl group, although the catalytic mechanism is not definitively established. The resulting alcoholate or phenolate ion is positioned to attack the γ-phosphate of ATP (Johnson *et al.* 1996).

The resulting alcoholate or phenolate ion is positioned to attack the γ-phosphate of ATP. All amino acid specific motifs detected in dTAF1CTK contain these catalytic residues.
The results in this work show that the discovered motif S/T-1, which is mainly responsible for transkinase-activity of the CTK is located in the DBD. Although no kinase-specific motif has been identified in the DBD of hTAF1CTK by the computational approach, the DBDs of human and *Drosophila* TAF1 are highly conserved, indicating that their three-dimensional structure might be similar. A comparison of the homologous dTAF1 and hTAF1 DBD-sequences with the crystal structure of the DBD of human TAF1 (Jacobson *et al.* 2000) suggests that the position of the *Drosophila* kinase-specific motif might lie on the lateral surface of the DBD within one of the four helices in the helix-bundles of bromodomain 1 and does not overlap with the acetylated lysine-binding surface which consists of two deep pockets at the bottom surface of the two helix-bundles of the DBD.
These findings underline the theory that the kinase-specific motif might be an independent functional unit of the DBD.

4.1.3 dTAF1CTK contains a secondary amino acid specific motif

A secondary S/T-specific kinase motif was detected in dTAF1CTK, termed S/T-2. Point mutations that exchange the catalytic active aspartic-acid residue to an alanine in S/T-1 revealed that this motif dominates over S/T-2. However, point mutations in S/T-2 resulted in reduction of kinase activity, indicating that this motif is also involved in kinase activity.
Interestingly, UV-crosslink experiments intended to locate the ATP-binding motif involving proteins lacking S/T-1 revealed that the proteins dTAF1CTKΔ1600 and especially dTAF1CTK17-18 show stronger ATP-binding than all other tested proteins. Both proteins contain the highly conserved ATP-binding domain found together with the amino acid specific motif S/T-2.
The protein dTAF1CTK17-18 showed the strongest ATP-binding compared to all other tested deletion-proteins. This might indicate that this protein could have a higher affinity to ATP by combining the ATP-binding capabilities of the conserved lysine in the ATP-motif and the aspartic-acid plus the asparagine residue of the S/T2-motif. More likely however is the fact that the protein, consisting mainly of both kinase motifs, folds into a functional catalytic domain that confers auto-kinase activity to the protein.
Comparably, dTAF1CTKΔ1600, which still contains the secondary bromodomain and thus folds structurally different from dTAF1CTK17-18 might form a more inhibitory structure, thus resulting

in less autokinase activity than dTAF1CTK17-18 but more activity than all other proteins used in the crosslinking experiment.
These results are intriguing in two ways since they suggest that S/T-2 and the ATP-binding motif actually form a catalytic core domain capable of autophosphorylation without additional NH_2- and COOH-terminal located motifs and indicate that additional sequences have a regulatory function on this catalytic domain as shown by the protein dTAF1CTKΔ1600. Thus, the observation that the motif S/T-1 dominates over S/T-2 may be the result of inhibitory effects by additional sequences located NH_2-terminal to S/T-2 like, for example, the structure of the DBD.
This result assists the hypothesis that multifunctional proteins indeed show different preferential activities depending to their folding in tertiary structure, which results in specific conformations. Depending on the conformation, specific domains like the catalytic domain might be spatially more or less available to binding partners such as substrates like histone proteins and phosphodonors like ATP-molecules.

4.1.4 dTAF1CTK contains a conserved ATP-binding motif

An ATP-binding motif in dTAF1CTK shows high homology (90%) to the PROSITE-pattern used in the computational search (Figure **13**), containing the catalytic ATP-binding lysine and each conserved residue within the G-{P}-G-{P}-x-[SGA] pattern. This motif is well conserved and existent in even the most atypical kinases like for example *C. elegans'* eEF-2K, which does not even show the conserved lysine in the ATP-binding motif and instead contains two cysteines that take over the catalytic activity of the missing lysine (Ryazanov *et al.* 1997, Maru and Witte 1991).
Transkinase-assays with CTK proteins lacking the ATP-binding-motif (Figure **15**) indicate that the motif is essential for kinase activity (Figures **17** and **18**).
UV-crosslinking experiments were conducted to confirm that the identified ATP-binding motif binds ATP since additional possible ATP-binding domains were found in the computational search (Figure **20**). Several deletion-proteins were used to find the exact location of the ATP-motif responsible for binding the ATP-molecule and subsequently, radiolabeled ATP was crosslinked via UV-light to the specific constructs (Figure **19**).
Interestingly, all of the proteins that showed ATP-binding on autoradiography by treatment with UV-radiation also showed ATP-binding in similar strength when incubated under standard

conditions without being exposed to UV-radiation. This result suggests that these proteins still have autokinase activity.

An intriguing observation is that the proteins containing exon 12a (dTAF1CTKΔ1800 and dTAF1CTK18-19), which contain AT-hooks, bind ATP while the protein containing only the acidic COOH-terminus (dTAF1CTKΔ1900), which contains no ATP-binding motifs, did not. AT-hooks are part of HMG proteins and bind to adenine-thymine rich DNA-sequences (Reeves and Nissen 1990, Geierstanger *et al.* 1994). Interestingly, the AT-hook of exon 12a is part of the secondary ATP-binding motif discovered as part of the tandem ATP-domain in an earlier study (Kwoczynski 2002). This secondary domain (Figure **20**) contains two AT-hooks and the UV-crosslinking results suggest that the AT-hooks might contribute to the capability of this region to bind ATP. It might also be possible that the ATP-binding motif of dTAF1CTK consists of two closely overlapping functional ATP-binding motifs that form a functional ATP-binding surface. This double-motif might be necessary to secure kinase activity of TAF1 when binding partners of dTAF1 such as other TAFs sterically block parts of the complete ATP-binding motif. This theory is supported by the function of dTAF1 as flexible scaffold protein of TFIID, which inclines that dTAF1 might exist in different conformations depending on the conformation of TFIID, but still needs to maintain specific functions like ATP-binding active regardless of its changing conformational state.

4.2 Other bromodomain kinase proteins

Three-dimensional structural analysis of several protein kinases revealed, that the catalytic domain is composed of two loops, a short NH_2-terminal loop that forms a twisted β-sheet structure and represents the ATP-binding motif and a bigger loop located COOH-terminal to the ATP-binding motif, which mostly consists of α-helices that bind the substrate (Johnson *et al.* 1996, Ryazanov *et al.* 1997). This alignment of the motifs, with the conserved GxGxx[SGA] sequence of the ATP-binding motif localized at the very NH_2-terminal end of the catalytic domain, followed by the amino acid specific motif, is typical for the majority of the discovered protein kinases so far (Hardie and Hanks 1995, Taylor *et al.* 1992, Hanks and Hunter 1995). The short loop contains the conserved GxGxx[SGA]-sequence of the ATP-binding motif and interacts directly with the phosphates of the ATP-molecule (Hardie and Hanks 1995, Taylor *et al.* 1992, Johnson *et al.* 1996) whereas the conserved lysine, localised up to 20 amino acids COOH-

terminal of the GxGxx[SGA]-sequence, takes part at the binding of the β- and γ-phosphates of the ATP-molecule (Hanks and Hunter 1995, Johnson *et al.* 1996).
Bromodomains consist of a bundle of α-helices (Jacobson *et al*. 2000) and it might be possible that one or both bromodomains in dTAF1 could form a similar structure like the substrate recognizing, α-helices-containing loop of protein kinases.

4.2.1 The BET family

Members of the *fsh*/RING3 (BET) family of DBD proteins have kinase activity, suggesting that TAF1 might be a member of a kinase family whose catalytic motif resides within the DBD (Zheng and Zhou 2002, Florence and Faller 2001). By a computational approach involving kinase-specific search patterns in various bromodomains, several bromodomain-kinase proteins of the BET-family of proteins have been identified that contain kinase-motifs with higher homology to the motifs found in dTAF1.
The BET proteins are a novel class of transcriptional regulators whose members can be found in animals, plants and fungi. Founding members are *Drosophila* Fs(1)h, human RING3 and yeast Bdf1. BET proteins are distinguished by an N-terminal single bromodomain or DBD and an ET domain. As predicted by the presence of the (double-) bromodomain, these proteins have been found to be associated with chromatin. Additionally, the poorly characterized ET domain functions as a protein-protein interaction motif and may be part of a serine-kinase activity (Florence and Faller 2001), which underlines the possibility of the dTAF1-bromodomain being an essential part of a chromatin-associated kinase activity and points towards a possible interplay between the acetylation of histones, possibly the binding to those residues by a bromodomain and subsequent kinase-activity.
In an additional computational approach conducted in this work, kinase-motifs were detected in both bromodomains of *drosophila* fs(1)h. The motif in the first bromodomain shows high homology to the typical kinase pattern (58%-59%), indicating that fs(1) seems to be a member of the typical serine/threonine-kinases. However, the kinase-motif found in the second bromodomain shows very high homology to the one found in ring3, which does not contain a highly homologous kinase-motif in bromodomain 1 like the one found in fs(1)h (see Figure **37**). Interestingly, several transcripts of *fs(1)h* exist, of which isoform B with a length of 2038 amino acids (compared to 1110 amino acids of isoform A) has additional sequences between both bromodomains, which encode for transmembrane-regions. These genes have generally been

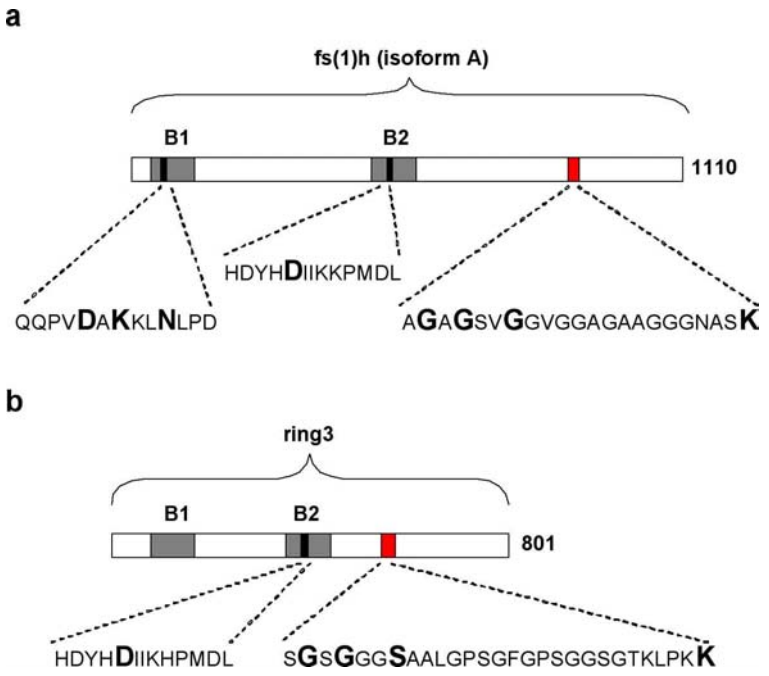

Figure 37. Motifs found in the bromodomain-kinases fs(1)h (isoform A) in *Drosophila* (**a**) and its human homolog ring3 (**b**). grey = bromodomains 1 and 2. Kinase-specific motifs are shown as black, ATP-motifs as red boxes. Conserved and catalytic active residues in the amino-acid sequence are shown highlighted (all graphical motif-sizes are shown in scale to overall length of the proteins).

shown to code for membrane proteins (Haynes *et al.* 1989), thus pointing towards different specifities of the two kinase-motifs in the two distinct bromodomains, maybe depending on separated spatial compartments within the cell.

All putative kinase-motifs found in bromodomain 2 (B2) are very homologous in fs(1)h, ring3, Bdf1 and 2 (Figures **32** and **37**). They all lack the conserved lysine (at position seven) as well as the conserved asparagine (at position ten) within their 13 amino acid pattern and thus show homology to the kinase-domain described in human TAF1 by Tjian and O'Brien, although this kinase-motif is not located within a bromodomain (Tjian and O'Brien 1998). It might be that this group of kinase-motifs defines a novel class of proteinkinases, specific for phosphorylating substrates within the nucleus such as nucleosomes or other chromatin-binding proteins like transcription factors.

The human homolog to *Drosophila* fsh, ring3 (Brd2), has been shown to be highly phosphorylated in response to mitogens (Denis and Green 1996). Like hTAF1, this protein has been proposed to have autokinase-activity despite limited sequence similarity to known kinases.

Another mammalian BET family member, Brd4, has been implicated in cell cycle regulation of chromosome structure (Dey *et al.* 2003), which suggests a connection between signal transduction and cell cycle pathways to chromatin and gene expression.
Therefore, bromodomains with different specificity and maybe additional functions besides acetyllysine-binding add to the complexity of the histone code.

4.2.2 Yeast Bdf1 and Bdf2

Bdf1 and Bdf2 share the conserved S/T-kinase-motif in bromodomain 2 (Figure **32**), but only Bdf2 carries a typical ATP-binding motif. Bdf1 instead contains an additional amino acid specific kinase motif which shows similarity to S/T-1 in dTAF1CTK, but is located COOH-terminal to the DBD.
The kinase assays performed in this work documented that both Bdf-proteins contain histone-specific kinase activity, suggesting that the BET family contains specific histone-protein kinases related to dTAF1CTK.
Genetic analyses show that while each gene alone is not essential, disruption of both *bdf1* and *bdf2* is lethal in yeast (Matangkasombut *et al.* 2000). This implies that besides the essential nature of the bromodomain to bind to acetylated lysines, there might be other essential functions located in bromodomain 2, such as histone kinase activity.
An intriguing question is whether there are known interactions of Bdf-proteins with other histone modifications. Yeast Bdf1 is known to associate with TFIID and binds histone H4 with preference for multiply acetylated forms, whereas, in contrast, Bdf2 shows no preference for acetylated forms. Additionally, point mutations within the bromodomain, that block Bdf1-binding to histones, disrupt transcription and reduce Bdf1 association with chromatin *in vivo* (Matangkasombut and Buratowski 2003). The same study shows that deletion of Bdf1 but not Bdf2 is lethal when combined with a mutant allele of ESA1, a histone H4 acetyltransferase, or with nonacetylatable histone H4 variants.
Interestingly, the study shows also that there may be some interactions between Bdf1 and histone H3. While a deletion of the H4 tail is lethal in combination with a deletion of Bdf1, a deletion of the H3 tail is not lethal, but this double mutant shows a synthetic slow growth phenotype. This suggests that both histone tails may play a role in the function of the Bdfs *in vivo*, with H4 being particularly important.

This difference between Bdf1 and Bdf2 in the preference of binding to acetylated H4 and a possible role of H3 correlates with the observation in this study that Bdf1 and Bdf2 phosphorylate H3. It also provides an explanation the observed differences in kinase activity, with Bdf1 revealing a weaker kinase activity than Bdf2. It is possible that Bdf1 needs acetylated H4 to unfold a stronger kinase activity compared to Bdf2 or that the kinase activity of Bdf1 is restricted to a specific serine-/threonine- or tyrosine-residue within H3 while the kinase activity of Bdf2 might target several residues. An intriguing fact is that these results may suggest a possible interplay between acetylation and histone phosphorylation by the CTK-homologous Bdf-proteins in yeast.

4.3 Phosphorylation of H2B is essential for transcriptional activation

One general question of this work was how the coactivator dTAF1 functions *in vivo* as histone kinase and especially how the documented kinase activity contributes to activation of transcription.

It is known that histone phosphorylation can activate transcription, but most of these events were restricted to phosphorylation of histone H3 (Cheung *et al.* 2000a). Specific phosphorylation of H2B was shown before, but only in context of apoptotic mammalian cells (Ajiro 2000) and this phosphorylation event coincides with nucleosomal DNA fragmentation. The phosphorylation of mammalian H2B is occurring at serine 32 by an unidentified kinase and is probably dependent on caspase-induced signaling pathways. Compared to *Drosophila* H2B, mammalian H2B contains serines at position 32 and 36 (Figure **10**).

Are there any potential links between apoptotic pathways and histone modifications? A connection might exist since the dTAF1-protein used in this work was initially identified as a splice-variant of dTAF1, which was expressed in *Drosophila* embryo cells after induction of apoptosis (Kwoczynski 2002).

4.3.1 TAF1 phosphorylates serine 33 in the NH_2-terminal H2B tail *in vivo*

The experiments described in chapter 2 show that TAF1 phosphorylates H2BS33 *in vivo* (Maile *et al.* 2004). Interestingly, serine 33 is the only evolutionary conserved position of serines or threonines in the NH_2-terminus of metazoan H2Bs (Figure **10**). The crystal structure of the

nucleosome from *Xenopus laevis* reveals that the respective serine-residue links the H2B NH_2-terminal tail, which binds DNA, to the histone-fold domain (Luger et al. 1997a, Luger and Richmond 1998). It might be possible that this exchange of the hydroxyl group on serine 33 with a bulkier negatively charged phosphate group might drastically affect H2B-tail interactions with DNA. This is particularly important because the H2B-tail is supposed to regulate nucleosome mobility. For example, deletion of the H2B-tail bypasses the requirement for the SWI/SNF nucleosome-remodeling complex in yeast (Recht and Osley 1999) and the NH_2-terminal tail shows to be also critical for maintaining the position of histone octamers in *in vitro* sliding assays (Hamiche *et al.* 2001).

These results display a model in which the TAF1-mediated phosphorylation of H2BS33 changes the overall conformation of the H2B NH_2-terminal tail, which may result in the disruption of DNA-histone interactions, leading to local decondensation of chromatin. This decondensation might be a trigger for chromatin remodeling and the formation of a chromatin structure that facilitates the assembly of other GTFs at a given promoter, a function that is primarily attributed to TFIID (Lemon and Tjian 2000, Orphanides and Reinberg 2002, Dikstein *et al.* 1996a).

4.3.2 The role of TAF1-phosphorylated H2BS33 for cell cycle regulation

Experiments show that cell cycle arrest of TAF1RNAi cells correlates with reduced transcription of *string*.

The protein phosphatase String is predominantly expressed during G2-phase and activates the cell cycle by dephosphorylating Cdc2 (Lee *et al.* 1992, Figure **38**). Therefore, the loss of *stg* from S2 cells by treatment with Stg-RNAi causes G2-M arrest (Pile *et al.* 2002), indicating that TAF1 may regulate G2/M-phase progression by activating the transcription of *stg*.

XChIP experiments revealed that activation of *stg* coincides with TAF1-mediated phosphorylation of H2BS33. Similarly, transcription of *string* is repressed in SIN3-RNAi treated cells (Pile *et al.* 2002). However the results obtained in this work show that the *stg*-promoter remains associated with H2BS33P and dTAF1. This indicates that loss of phosphorylation of H2B serine 33 in TAF1RNAi-treated cells is directly due to the elimination of TAF1 rather than arrest in G2/M-phase (Figure **30**).

In addition to the kinase-activity, TAF1 has a histone acetyltransferase activity that acetylates H3 lysine 14 and unidentified lysine residues in histone H4 in vitro (Mizzen *et al.* 1996). Analysis by XChIP detected acetylated histone H3 lysine 14 and histone H4 at the transcriptionally active

stg-promoter in mock-treated RNAi cells but not at the inactive *stg*-promoter in TAF1RNAi-treated cells (Figure **30**).

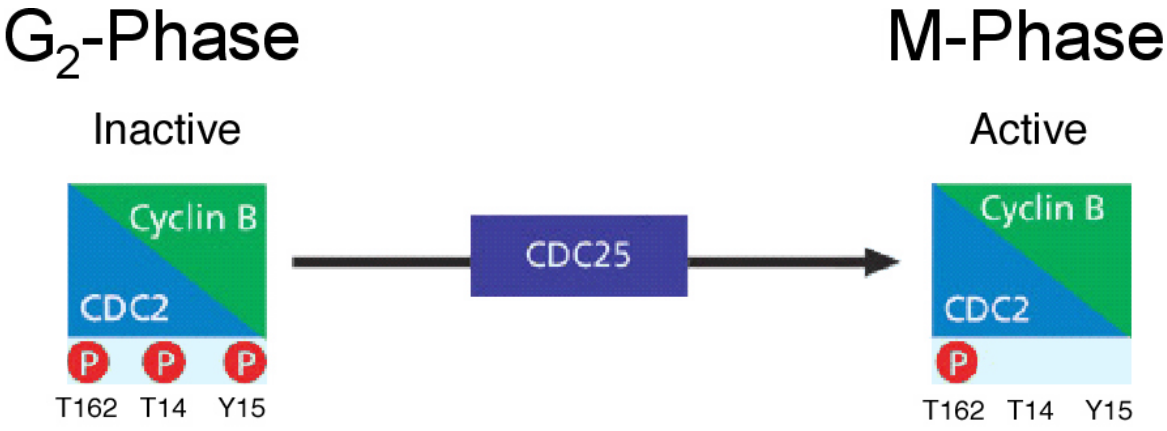

Figure 38. The transition from G2 phase to mitosis is triggered by the Cdc25-mediated activation (dephosphorylation) of the cyclinB/cdc2 complex. CyclinB/cdc2 is activated by phosphorylation of threonine 162 and the dephosphorylation of threonine 14 - tyrosine 15. The complex is kept in an inactive state due to the phosphorylation of threonine 15, which is catalyzed by the kinase Wee1. Cyclin B/cdc2 activation is triggered when Cdc25, a phospatase, dephosphorylates threonine 15.

4.3.3 The role of TAF1-acetylation in *stg* transcription

These results indicate that in case of *stg*-activation, several histone modifications might play a combinatory role at specific promoters and they suggest that the dTAF1-mediated phosphorylation of H2BS33 is essential for transcriptional activation of *string*/*cdc25* and thus cell cycle progression. Furthermore, phosphorylation of H2BS33 and acetylation of H3 and H4 seem to be correlated with transcriptional activation in *Drosophila*, underlining that TAF1-CTK- and TAF1-HAT-activities may cooperate in transcriptional activation of some genes.

Other components of TFIID also showed to have influence over the cell cycle in various organisms. Depletion of yeast TAF5, human TAF2 or a twofold reduction in TBP from chicken was shown to result in arrest in the G2/M-phase (Apone *et al.* 1996, Martin *et al.* 1999, Um *et al.* 2001). Besides, as shown in this work with TAF1, also TBP is regulating expression of *stg*/*cdc25*, thus providing additional support for the results in this study, indicating that the H2B-S33 kinase activity of TAF1 occurs in the context of TFIID (Um *et al.* 2001). Another interesting fact is that the depletion of yeast TAF1, which does not possess a CTK, and the inactivation of

the HAT-activity of TAF1 induce G1-phase arrest because of a reduced transcription of B- and D-type cyclins, respectively (Apone *et al.* 1996, Dunphy *et al.* 2000). That means that the loss of all TAF1 activities causes G2/M-phase arrest whereas the loss of TAF1 HAT-activity causes arrest in G1-phase, which suggests a more gene-specific requirement for the CTK- and HAT-activities of TAF1.

4.3.4 The role of TAF1-phosphorylation of H2BS33 in *Drosophila* development

It was shown by XChIP-PCR-analysis, that phosphorylation of H2BS33 by CTK is essential for transcription of the gene *giant*.

Additionally, acetylation was found at the transcriptionally silent *gt*-promoter since the HAT-activity of the TAF1CTK-mutants was not disabled, which suggests that acetylation occurs independently of the phosphorylation of H2BS33 (Maile *et al.* 2004). Additionally, missing ubiquitination at the *gt* promoter is correlating with missing phosphorylation (F. Sauer, personal correspondence).

These results point towards the theory that TAF1-mediated phosphorylation of H2BS33 is a dominant factor in the transcription of *giant*. It is possible that prior acetylation of histones at the *gt* promoter introduces phosphorylation of H2BS33 due to better binding of the kinase motif carrying DBD to the multiple-acetylated target region. Phosphorylation then changes the structure of the histone tail in a way to allow better binding of TAF1 or other transcription factors to the promoter.

giant (gt) is a gap gene that codes for a transcriptional repressor, which is located in the nucleus. In keeping with its developmental group, lack of *giant* expression produces gaps in both anterior and posterior structures, specifically the labial and labral head structures and abdominal segments A5 through A7. Giant helps to define the domains of expression for the pair-rule genes *even-skipped, paired* and *fushi tarazu* and it also delimits the anterior boundary of early *antennapedia* expression. While giant is expressed in an anterior and a posterior stripe in early fly development, for example, *GT* RNA is first detected quite early in development during nuclear cycle 12, the expression pattern becomes increasingly complex over time (Eldon and Pirotta 1991, Kraut and Levine 1991). This complex expression pattern during development is likely linked to an equal complex regulation of transcription. Therefore it is possible that besides TAF1-mediated phosphorylation, other histone modifications might interfere with the transcriptional

regulation of *giant* in different developmental stages. During the last years, it has become clear that there is interplay between core histone modifications. For example, the choice of methylation of H3 lysine 9 could be directed by phosphorylation of H3 serine 10: In *Saccharomyces cerevisiae*, Snf1 and GCN5 appear to work synergistically to mediate gene activation by phosphorylating H3 serine 10 and acetylating H3 lysine 14, respectively (Lo *et al.* 2001). There might be even other TAF1-mediated histone modifications that are correlated with kinase-activity: TAF1 has been shown to contain histone-specific ubiquitin-activating/conjugating (E1/E2) activity for histone H1 (Pham and Sauer 2000). An intriguing cross-talk linking ubiquitination of H2B to methylation of H3 was shown also in yeast: Genetic experiments demonstrated a link between ubiquitination of H2B by Rad6/Ubc2 and the methylation of H3 on lysine 4 in budding yeast (Sun and Allis 2002). Recently, a novel histone-kinase, NHK-1 (nucleosomal histone kinase-1), was discovered to phosphorylate H2A threonine 119 specifically in context of the nucleosome during mitosis in the *Drosophila* embryo (Aihara *et al.* 2004). Mammalian H2A lysine 119, which is next to the putative unique phosphorylation site in mammal H2A threonine 120, is well conserved and known to be modified by mono ubiquitination (Olson *et al.* 1976, Goldknopf *et al.* 1977), suggesting a possible interplay between both modification sites. With the gene *giant*, this study shows that the kinase activity of dTAF1 might also be involved in the transcriptional activation of developmental genes. It might be interesting to conduct further ChIP experiments to get more insight into the connections of dTAF1 histone kinase activity and the role of the functional interplay between histone modifications in the context of the genome.

5. Summary

Activation of eukaryotic transcription involves an orchestrated interplay between transcription factors and the general RNA polymerase II (Pol II) transcription machinery (GTM), which consists of Pol II and general transcription factors (GTFs).

The GTF TFIID consists of the TATA-box binding protein (TBP) and several TBP-associated factors (TAFs). The binding of TFIID to promoters can nucleate transcription.

TAF1 is the largest subunit of TFIID and plays a central role within the nucleating function of TFIID in transcription. TAF1 mediates the binding of TFIID to promoters and interacts with enhancer-bound transcription factors and several GTFs. Additionally, TAF1 contains four enzymatic activities that are essential for viability of eukaryotes and mediate posttranslational modification of GTFs and histones. TAF1 is a bipartite protein kinase and contains an NH_2-terminal kinase domain (NTK) and a COOH-terminal kinase domain (CTK). A previous study demonstrated that the CTK phosphorylates serine-residue 33 in histone H2B (H2BS33). However, the role of TAF1-mediated phosphorylation in transcription regulation remained unknown.

In this study, the functional importance of H2BS33 phosphorylation (H2BS33P) by TAF1 was investigated by using a combination of biochemical and *in vivo* assays.

In vitro kinase assays uncovered the two essential kinase motifs in TAF1CTK, the ATP-binding motif and the serine/threonine-specific catalytic motif, and indicate that the TAF1 CTK has intrinsic kinase-activity. Western blot analysis using an antibody to H2BS33P revealed that H2BS33 is phosphorylated in *Drosophila*. RNA-interference (RNAi) assays, designed to attenuate TAF1 expression (TAF1RNAi), revealed that TAF1 is a major kinase for H2BS33 in *Drosophila* Schneider cells.

Flow-cytometry analysis of TAF1RNAi cells indicated that loss of TAF1 expression results in cell cycle arrest in G2/M-phase. Screening the transcription of cell cycle genes in TAF1RNAi cells by using reverse-transcriptase-PCR demonstrated that the transcription of the cell cycle gene *string* (*stg*) is reduced in the absence of TAF1.

Chromatin immunoprecipitation assays (XChIP) indicate that H2BS33P is detectable at the transcriptionally active *stg* promoter but not at the silent *stg* promoter in TAF1RNAi cells. These results demonstrate that phosphorylation of H2BS33 is involved in *stg* transcription.

XChIP-assays using chromatin prepared from Drosophila embryos, which express a mutant

TAF1 lacking the CTK, revealed that CTK-mediated phosphorylation of H2BS33 plays an essential role in the activation of transcription of the Drosophila segmentation gene *giant*.
In vitro kinase assays demonstrate that Bdf1 and Bdf2, the yeast homologues of the TAF1CTK, phosphorylate histones suggesting that the kinase activity of the TAF1CTK is phylogenetically conserved. The results of this work demonstrate that TAF1CTK is a major histone kinase of H2BS33 and that TAF1-mediated phosphorylation of H2BS33 plays an essential role in the transcription events during cell cycle progression and development.

6. Zusammenfassung

Die Aktivierung eukaryotischer Transkription umfaßt eine geordnete Interaktion zwischen Transkriptionsfaktoren und der generellen RNA Polymerase II (Pol II) Transkriptionsmaschinerie (GTM), welche aus Pol II und generellen Transkriptions-Faktoren (GTFs) gebildet wird.

Der GTF TFIID besteht aus dem TATA-Box-bindenden Protein (TBP) und mehreren TBP-assoziierten Faktoren (TAFs). Die Bindung von TFIID an Promotoren kann Transkription nukleieren. TAF1 ist die größte Untereinheit von TFIID und spielt eine zentrale Rolle bei der nukleierenden Funktion von TFIID in Transkription. TAF1 vermittelt die Bindung von TFIID an Promotoren und interagiert mit Enhancer-gebundenen Transkriptionsfaktoren sowie mehreren GTFs. Desweiteren enthält TAF1 vier enzymatische Aktivitäten, welche essentiell für die Entwicklung von Eukaryoten sind und posttranslationelle Modifikationen von GTFs und Histonen vermitteln. TAF1 ist eine zweiteilige Proteinkinase und enthält eine NH_2-terminale Kinasedomäne (NTK) sowie eine COOH-terminale Kinasedomäne (CTK). Eine frühere Studie zeigte daß die CTK Serin 33 in Histon H2B (H2BS33) phosphorylieren kann. Welche Rolle die TAF1-vermittelte Phosphorylierung in der Transkription spielt, blieb jedoch unbeantwortet.

In dieser Studie wurde die funktionelle Bedeutung der Phosphorylierung von H2BS33 (H2BS33P) durch TAF1 mittels Kombination von biochemischen Assays und Experimenten *in vivo* untersucht.

Mit Hilfe von *in vitro* Kinase-Assays wurden die zwei essentiellen Kinasemotive in TAF1CTK, das ATP-bindende Motiv und das Serin/Threonin-spezifische katalytische Motiv, aufgedeckt und gezeigt, daß TAF1CTK intrinsische Kinaseaktivität besitzt. Western-Blot-Analysen mit Antikörpern gegen H2BS33P zeigen, daß H2BS33 in Drosophila phosphoryliert ist. RNA-Interferenz Experimente (RNAi), welche eine Abschwächung der Expression von TAF1 bewirkten, zeigen, daß TAF1 eine bedeutende Kinase für H2BS33 in *Drosophila* Schneider-Zellen darstellt.

Durchflußzytometrische Analysen von TAF1RNAi-Zellen ergaben, daß die Reduzierung der Expression von TAF1 einen Zellzyklusarrest in G2/M-Phase bewirkt. Ein Screening der Transkription von Zellzyklusgenen in TAF1RNAi-Zellen zeigte, daß die Transkription des Zellzyklusgens *string* (*stg*) in der Abwesenheit von TAF1 reduziert ist.

Chromatin Immunopräzipitierungsassays (XChIP) detektierten H2BS33P am transkriptionell aktiven *stg* promoter, jedoch nicht am transkriptionell inaktiven *stg* promoter in TAF1RNAi-

Zellen. Diese Ergebnisse demonstrieren, daß die Phosphorylierung von H2BS33 an der Transkription von *stg* beteiligt ist.

XChIP-Assays mit Chromatin aus *Drosophila* Embryos, welche eine TAF1-Mutante ohne CTK exprimieren, deckten außerdem auf, daß die CTK-vermittelte Phosphorylierung von H2BS33 eine essentielle Rolle bei der Transkriptionsaktivierung des *Drosophila* Segmentierungsgens *giant* spielt.

In vivo Kinase-Assays zeigen daß Bdf1 und Bdf2, Homologe von TAF1CTK in Hefe, Histone phosphorylieren und legen nahe, daß die Kinaseaktivität von TAF1CTK phylogenetisch konserviert ist.

Die Ergebnisse dieser Arbeit charakterisieren TAF1CTK als eine bedeutende Histonkinase von H2BS33 und zeigen daß die TAF1-vermittelte Phosphorylierung von H2BS33 eine bedeutende Rolle in der Transkription zellzyklus- und entwicklungsspezifischer Gene spielt.

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8. Publications

Parts of this study have been published in:

Maile T., Kwoczynski S., Katzenberger R. J., Wassarman D. A., Sauer F.:
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