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The Tip60 HAT-Complex is Recruited to Chromatin by the Transcription Factors E2F and Myc

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SUMMARY

The oncogenic transcription factors E2F and Myc bind to specific DNA sequences at distinct times during the G1 phase of the mammalian cell cycle, and regulate transcription of a plethora of target genes. E2F and Myc associate with TRRAP, a subunit of distinct macromolecular complexes that contain the histone acetyltransferases (HATs) Gcn5/PCAF or Tip60. During the course of my thesis I investigated several aspects of TRRAP-associated HAT-complexes. Firstly, I used chromatin immunoprecipitation (CHIP) to show that E2F recruits a TRRAP/Tip60 complex to its target genes. This was accompanied by localized changes in histone acetylation, centered to the E2F binding sites. Prevention of E2F-binding correlated with a failure to acetylate H4 on target promoters, and inability to enter the cell cycle upon mitogenic stimulation. Furthermore, the coactivator complex targeted most, if not all, promoters investigated in this study, establishing Tip60 recruitment and H4 acetylation as a common feature of E2F-dependent coactivation. Secondly, I demonstrated that Myc recruits HAT activity and associates with Tip60 and Gcn5/PCAF. Consistent with this, Tip60 was also recruited to Myc targets. Thirdly, at least two components of a Gcn5/PCAF-containing TRRAP complex were found associated with G1-specific cell cycle regulator Cyclin E. Moreover, the HAT activity of PCAF was susceptible to cell cycle arrest. Altogether, these results provide evidence that TRRAP-associated HAT-complexes play an important regulatory role during the mammalian cell cycle.

ZUSAMMENFASSUNG

Die onkogenischen Transkriptionsfaktoren E2F und Myc binden spezifische DNS-Sequenzen zu unterschiedlichen Zeitpunkten waehrend des Zellzyklus, und regulieren Transkription von vielen unterschiedlichen Genen. Sowohl E2F als auch Myc assoziieren mit TRRAP, einer Komponente von mindestens zwei verschiedenen macromolekularen Protein-Komplexen, welche die Histon-Acetyl-Transferasen (HAT) Gcn5/PCAF oder Tip60 enthalten. Waehrend meiner Dissertation studierte ich verschiedene Aspekte dieser TRRAP-assoziierten HAT-Komplexe. Zuerst verwendete ich Chromatin-Immunopraezipitation, um zu zeigen, dass ein TRRAP/Tip60 Komplex zu E2F-regulierten Genen rekrutiert wird. Dies war begleitet von lokalisierten Veraenderungen der Histon-Acetylierung, welche zu E2F Bindungssequenzen zentriert waren. Eine E2F-Mutante, welche das spezifische Binden von E2F zu Ziel-Genen blockierte, verhinderte Rekrutierung des TRRAP/Tip60-Komplexes, der Histon H4-Acetylierung, und des Wiedereintretens in einen neuen Zellzyklus nach Stimulierung mit Serum. Ausserdem beobachtete ich Histon H4-Acetylierung und Kofaktor-Rekrutierung bei allen untersuchten E2F Ziel-Genen, was diese Vorgaenge als klasisch fuer E2F Funktion etabliert. Des weiteren konnte ich zeigen, dass Myc HAT-Aktivitaet rekrutiert, und die beiden TRRAP assoziierten HAT-Enzyme Gcn5/PCAF und Tip60 binden kann. Schliesslich fand ich, dass mindestens zwei Komponenten eines Gcn5/PCAF Komplexes von dem Zellzyklus-Regulator Cyclin E gebunden werden, und dass die HAT-Aktivitaet von PCAF durch einer Blockierung des Zellzyklus beeintraechtigt wird. Zusammengenommen lassen diese Resultate vermuten, dass TRRAP und seine assoziierten HAT-Komplexe eine wichtige Rolle in der Regulierung des Zellzyklus von Saeugetier-Zellen spielen.

ABBREVIATIONS

aa	Amino acid
AcH	Acetyl-histone
achr	Acetylcholine receptor
APAF1	Assignment of apoptotic protease activating factor-1 protein
α-PT	Alpha Prothymosine
ARF	Alternative reading frame
ARP	Actin related protein
ATP	Adenosine Triphosphate
BAF	BRG1 associated factor
b	Bases
bp	Basepairs
BH2	Babe Hygromycin 2
bHLH	Basic Helix-Loop-Helix
BN2	Babe Neomycin
BP	Babe Puromycin
BrdU	Bromo-deoxyuridine
Brg1	Brahma-related gene 1 protein
Brm	Brahma
BSA	Bovine serum albumin
CAK	Cyclin/CDK activating kinase
CBP	CREB binding protein
CDC	Cell division cycle (protein)
CDK	Cyclin dependent kinase
cDNA	Complementary DNA
CHIP	Chromatin Immunoprecipitation
CKI	CDK inhibitor
CITED1	CBP/p300-interacting transactivator with Glutamate/Aspartate-rich C-terminal domain 1 protein
CMV	Cytomegalovirus
CNS	Central nervous system
CoA	Coenzyme A
con	Control
CREB	Cyclic AMP responsive element
ct	Cycle threshold
C-terminus, CT	Carboxy terminus
Cyc	Cyclin
DBD	DNA-binding-domain
DDB1	UV-DNA-damaged binding protein 1
DHFR	Dihdryofolate reductase
DMEM	Dulbecco's modified Eagle medium
d	Drosophila
DNA	Deoxyribonucleic acid
DNMT	DNA methyltransferase
DNApol	DNA polymerase
DP	Differentiation regulated transcription factor protein
DTT	Dithiothreitol

DUT	Deoxyuridine triphosphate nucleotidohydrolase protein
E2F	E2 factor
ECL	Enhanced chemoluminescence
E. coli	*Escherichia Coli*
EDTA	Ethylene diamine tetra-acetate
EST	Expressed sequence tag
FACS	Fluorescence activated cell sorting
FBS	Fetal bovine serum
FITC	Fluoresceine isocyanate
G1, G2	Gap phase 1, 2 (of the cell cycle)
Gar22	Growth arrest specific 22 protein
Gcn5	Glucose control negative 5 protein
GFP	Green fluorescent protein
GPAT	Glutamine PRPP amidotransferase
h	Human
HA	Hemagglutinin
HAT	Histone acetyltransferase
HBO1	HAT bound to ORC1
HDAC	Histone deacetylase
HMT	Histone methyltransferase
HRP	Horseraddish peroxidase
hrs	Hours
H. sapiens	*Homo sapiens*
HSP60	Heat shock protein of molecular weight 60kDa
IgG	Immunoglobulin G
INK	Inhibitor of kinase
IP	Immunoprecipitation
IPH	Immunoprecipitation Histone acetyltransferase assay
kDa	Kilodalton
KLH	Keyhole limpet hemocyanin
KO	Knockout
M	Mitosis
MCM	Minichromosome maintenance
MEF	Mouse embryonic fibroblast
min	Minutes
mRNA	Messenger RNA
MSH2	MutS homologue 2 protein
Mut	Mutant
NM23	Nucleoside diphosphate kinase gene
Non inf.	Not infected
N-terminus, NT	Amino terminus
NP40	Nonidet P-40 detergent
NPAT	Nuclear protein mapped to AT locus
NUC, Nuc	Nucleolin
OD	Optical density
4-OHT	4-Hydroxy tamoxifen (estrogen analog)
ORC1	Origin recognition complex protein 1
ORF	Open reading frame
pA	Protein A
PAGE	Polyacrylamide gel electrophoresis

PBS	Phosphate buffered saline
PCAF	p300/CBP associated factor
PcG	Polycomb group proteins
PCNA	Proliferating cell nuclear antigen
PCR	Polymerase chain reaction
pH	pondus hydrogenii
PI	Propidium iodide
PMSF	Phenymethylsulfonyl fluoride
PolA2	DNA Polymerase subunit A2
PVDF	Polyvinylidene fluoride
Rb	Retinoblastoma
RNA	Ribonucleic acid
RPM	Revolutions per minute
RRM1	Ribonucleotide reductase chain M1
RT	Room temperature
S	S-Phase of the cell cycle
SDS	Sodium dodecylsulfate
sec	seconds
Spt3	Suppressor of Ty 3 protein
TAD	transactivation domain
TAF	TBP associated factor
TBP	TATA binding protein
TFTC	TBP free TAFII-containing complex
TGF	Transforming growth factor
TK	Thymidine kinase
TKO	Triple knockout
TOP2A	Topoisomerase 2A protein
TRRAP	Transcription/transformation associated protein
Tip48/49	TBP interacting protein of molecular weight 48 kDa/ 49kDa
Tip60	Tat interacting protein of molecular weight 60kDa
TS	Thymidylate synthetase
U2OS	U2 osteosarcoma cell line
Ubi	Ubiquitin
UV	Ultraviolet radiation
W	Watt
WCL	Whole cell lysate
WT, wt	Wild type
V	Volt
y	Yeast, *Sacharomyces cerevisiae*

INTRODUCTION

1. Cancer Cells

The development of tumors in metazoans proceeds via the process of transformation. Accumulation of somatic mutations during the life-span of an individual organism, as well as inherited mutations, both contribute to transformation (Vogelstein and Kinzler 1993). Different tissues give rise to distinct tumors, but all cancer cells exhibit several common features: They avoid programmed cell death (apoptosis), grow and divide inappropriately, and (re-) acquire the capability to migrate and invade other tissues (Hanahan and Weinberg 2000). The genes affected by transforming mutations can be divided into two categories: Oncogenes, and tumor suppressors (Weinberg 1994). Oncogenes arise through mutations affecting their cellular counterparts, the proto-oncogenes. They act in a dominant manner, to induce cell division, or migration, for example. In contrast, loss of function of both alleles of tumor suppressor genes is required to contribute to transformation, as products of tumor suppressor genes signal to inhibit cellular growth and division, or detachment and migration. Transformation of cells is associated with changes of gene regulation (Leonhardt and Cardoso 2000; Baylin et al. 2001; Wade 2001), which results in altered signaling pathways, for instance deregulation of the cell division cycle (Sherr 1996). Thus, in order to understand the molecular mechanisms of transformation, we must know how these processes are controlled in both normal and transformed cells.

2. Chromatin and Transcriptional Regulation

2.1. Chromatin Structure

All eukaryotes package genomic DNA into chromosomes. These are comprised of chromatin, a highly ordered DNA-protein structure that allows for compact storage of the DNA in the limited space of the nucleus (Horn and Peterson 2002). The fundamental unit of chromatin is the nucleosome (Kornberg and Lorch 1999b), which consists of 146bp of genomic DNA tightly wrapped around a histone octamer (two copies of each core histone H2A, H2B, H3, and H4; Luger et al. 1997). Core histones are evolutionarily highly

conserved, positively charged proteins, which share two signature structural features (Hayes and Hansen 2001; Hansen 2002): A core "histone-fold" domain sufficient for histone-histone and histone-DNA interactions, an N-terminal tail, and a C-terminal tail. These tails protrude from the core, and contain sites for a plethora of covalent modifications (Figure 1; Goll and Bestor 2002).

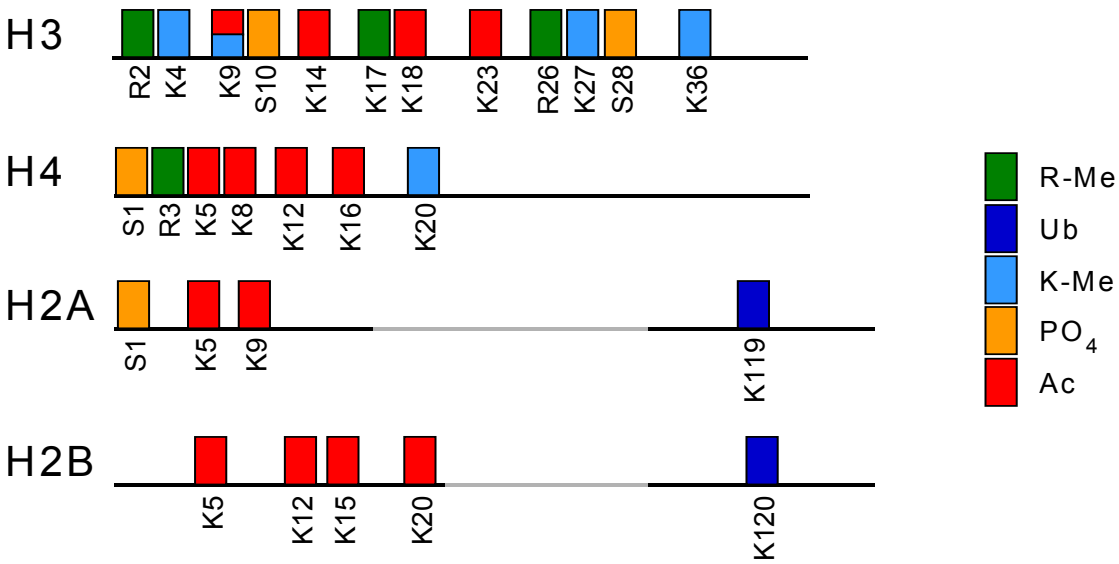

Figure 1: Histone Tail Modifications.
Schematic depicting covalent modifications of individual amino acid residues (S = Serine; K = Lysine; R = Arginine) in histone tails. Note that H3K9 can be either acetylated or methylated, but the two modifications are mutually exclusive. For further explanations, see text. Ac, Acetylation; Ub, Ubiquitination; R-Me, Arginine-Methylation; PO_4, Phosphorylation; and K-Me, Lysine-Methylation. Modified from Goll and Bestor, 2002.

In order to gain access to the DNA, the cellular transcription machinery must be able to modulate the structure of chromatin. Transcriptional activation correlates with relaxation of an inherently repressive chromatin structure, whereas repression is associated with condensation of chromatin. Two kinds of enzymatic activities that modulate chromatin structure have been identified: (a) chromatin-modifying-enzymes that covalently modify chromatin components (Workman and Kingston 1998), and (b) chromatin-remodeling-enzymes that non-covalently change the structure and/or the position of nucleosomes (Strahl and Allis 2000). Both classes of proteins occur *in vivo* as large multi-subunit protein complexes (Kornberg and Lorch 1999a). Chromatin-modifying-enzymes include histone acetyltransferases (HATs) and

deacetylases (HDACs), DNA methyltransferases (DNMTs), histone methyltransferases (HMTs), histone kinases, and histone ubiquitin-transferases. Recently, many of these activities have been associated with transcriptional regulation. In particular, HATs and HDACs have emerged as important co-regulators involved in a variety of transcriptional responses (Sterner and Berger 2000; Roth et al. 2001).

2.2. Histone Acetylation is Relevant for Transcriptional Regulation

It is now well established that regulation of histone acetylation is important for regulation of gene transcription (reviewed in Sterner and Berger 2000; Roth et al. 2001). In most cases, histone acetylation is linked to gene transactivation, while deacetylation is associated with transcriptional repression. A critical step in proving that acetylation can act to regulate transcription was the biochemical characterization of p55, the *Tetrahymena thermophila* orthologue of the known yeast transcriptional adaptor Gcn5p, as a HAT (Brownell et al. 1996). In metazoans, a regulatory role for histone acetylation in gene regulation was suggested by the characteristics of dosage compensation. For example, male fruit flies (*Drosophila melanogaster*) induce hyperacetylation of H4 lysine 16 (H4K16) throughout their single X-chromosome, which correlates with a two-fold increase in transcription (Lucchesi 1998; Pannuti and Lucchesi 2000). Similarly, in mammals, the transcriptionally inactive state of one of the two female X-chromosomes correlates with chromosome-wide hypoacetylation of both H3 and H4.

Precisely how acetylation regulates transcription is a matter of debate, but two, possibly overlapping models have emerged in the literature. The first model suggests that gene wide acetylation of histones, which reduces their net positive charges, weakens their interaction with the negatively charged DNA, thus creating a relaxed, more “open” chromatin structure. Deacetylation, in contrast, would cause stabilization of histone-DNA interactions, and induce a "closed" chromatin state. Consistent with this model, hypoacetylation of H3 throughout the coding regions of active genes correlates with transcriptional inhibition in yeast (Kristjuhan et al. 2002). In the alternative model, modification of histone tails creates a dynamic platform upon which the transcriptional machinery is assembled. Thus, different modifications may act

sequentially or in combination to create a “histone code”, which translates into initiation of promoter specific downstream events (Strahl and Allis 2000; Jenuwein and Allis 2001). This model is supported by a wealth of evidence; for example, methylated H3K9 specifically recruits HP1, which then induces heterochromatin formation, ultimately inducing gene silencing (Hall et al. 2002; Nielsen et al. 2002; Peters et al. 2002). The identification of domains that specifically interact with certain modified histones, such as the acetyl-histone binding bromodomain, also supports this hypothesis (Horn and Peterson 2001; Zeng and Zhou 2002). Ultimately, histone acetylation may regulate gene transcription via both kinds of mechanisms.

2.3. HATs and HDACs

HATs are enzymes that transfer acetyl-moieties onto the lysines of N-terminal histone tails (Sterner and Berger 2000; Roth et al. 2001). Both HATs and HDACs generally lack intrinsic DNA-binding activity. Instead, they are recruited to DNA by sequence specific transcription factors (TFs) to stimulate either transcriptional activation or repression. Therefore, HATs and HDACs play important roles in many transcriptional activation and repression pathways.

Mammalian HDACs can be subdivided into three classes (Fischle et al. 2001; Marks et al. 2001, Kuo et al. 1998). Class I HDACs (HDAC1-3, HDAC8) are similar to the yeast transcriptional repressor Rpd3p, and are subunits of at least two distinct corepressor complexes, the Sin3 complex and the NuRD complex. Class II HDACs (HDAC4-7, HDAC9-11) are related to yeast Hda1p, act as transcriptional repressors independent of Sin3 complexes, and have been implicated in various roles in cell differentiation and development. Class III HDACs (SIRT1-7) are related to yeast Sir2p, are biochemically and structurally distinct from Classes I and II, and appear to be involved in gene silencing and heterochromatin formation at centromeres and telomeres (Gasser and Cockell 2001).

HAT	Organisms containing the HAT	Known transcription-related functions	Specificity of recombinant enzyme *in vitro*	Known HAT-complexes, nucleosomal histone specificities *in vitro*
		GNAT family		
Hat1	Yeast to humans	None (histone-deposition-related HAT)	**H4**	Yeast HAT-B, HAT-A3 (no nucleosome acetylation)
Gcn5	Yeast to humans	Coactivator (adaptor)	**H3**/H4	Yeast ADA, SAGA (**H3**/H2B); human GCN5 complex, STAGA, TFTC (H3)
PCAF	Humans, mice	Coactivator	**H3**/H4	Human PCAF complex (**H3**/weak H4)
Elp3	Yeast	Transcript elongation	ND	Elongator, polymerase II holoenzyme (**H3**/weak H4)
Hpa2	Yeast	Unknown	**H3**/H4	
		MYST family		
Sas2	Yeast	Silencing	**H4**K16	
Sas3	Yeast	Silencing/ Transcription	**H3**/**H4**/H2A	NuA3 (**H3**)
Esa1	Yeast	Cell cycle progression	**H4**/H3/H2A	NuA4 (**H4**/H2A)
MOF	*Drosophila*	Dosage compensation	**H4**/H3/H2A	MSL complex (**H4**)
Tip60	Humans	HIV Tat interaction	**H4**/H3/H2A	Tip60 complex
MOZ	Humans	Leukemogenesis, upon chromosomal translocation	ND	
MORF	Humans	Unknown (strong homology to MOZ)	**H4**/H3/H2A	
HBO1	Humans	ORC interaction	H3/H4	HBO1 complex
		p300/CBP		
p300/CBP	Metazoans	Global coactivator	**H2A**/**H2B**/**H3**/**H4**	
		Nuclear Receptor Coactivators		
SRC-1	Humans, mice	Nuclear receptor coactivators (transcriptional response to hormone signals)	**H3**/H4	
ACTR	Humans, mice		**H3**/**H4**	
TIF2	Humans, mice		ND	
		General Transcriptional Factors		
$TAF_{II}250$	Various	TBP-associated factor	**H3**/H4	$TF_{II}D$
$TF_{III}C$				$TF_{III}C$ (**H2A**/**H3**/**H4**)
$TF_{III}C220$	Humans	RNA polymerase III transcription initiation	ND	
$TF_{III}C110$	Humans		ND	
$TF_{III}C90$	Humans		**H3**	

Table 1: Known and Putative HATs.
Known and putative HATs, their occurrence in distinct species, biological functions, substrate specificity towards histones (preferred substrate in bold), and associated complexes are listed. HATs are divided in five families. ND, not determined.

Like HDACs, HATs can be subdivided in several classes based on their sequence homologies, but all HATs share a highly conserved motif containing an acetyl-CoA binding site (Roth et al. 2001). Historically, HATs have been subdivided into type A (nuclear), and type B (cytoplasmic) HATs. The latter

are irrelevant for transcriptional regulation, and perform housekeeping roles in cells, functioning to acetylate newly synthesized histones (Brownell and Allis 1996). More precisely, HATs can be classified into several subfamilies based on their sequence homologies (Table 1): The GNAT family, the MYST family, $TAF_{II}250$, $TF_{III}C$, steroid receptor coactivators (SRC-1, ACTR, and Tif-2), and p300/CBP (for review, see Sterner and Berger 2000; Roth et al. 2001). Most HATs and HDACs are components of large multi-subunit protein complexes (Table 2). Interestingly, several HAT/HDAC-complexes have been described to interact with each other, and with other chromatin modifying complexes, and synergistic activation of transcription has been proposed (see chapter 2.7. pp.28).

2.3.1. HATs I: The GNAT Family

The GNAT (Gcn5-related N-Acetyltransferases) family of enzymes includes several non-histone acetyltransferases, including prokaryotic representatives (Neuwald and Landsman 1997). They have been grouped together on the basis of their sequence similarity in several homology regions and acetylation-related motifs. The family members that have been associated with transcription are yeast Gcn5p, mammalian PCAF and Gcn5 (yeast Gcn5p homologues), and Elp3p, a subunit of the yeast Elongator complex (Wittschieben et al. 2000; Kristjuhan et al. 2002).

Gcn5 is the most studied HAT to date. A variety of *in vitro* studies indicate that it acetylates preferably H3 and H2B (Kuo et al. 1996, Xu et al. 1998, Yang et al. 1996, Schiltz et al 1999, Grant 1999). Recent *in vivo* chromatin immunoprecipitation analysis (CHIP, see chapter 4.3; pp. 49) confirmed that the substrate specificity of yeast Gcn5p is directed towards H3 (K9, K14, K18, K23, and K27), and H2B (K11, and K16) (Suka et al. 2001). In addition to the HAT domain, Gcn5 proteins contain an adjacent Ada2 interaction domain, a C-terminal bromodomain that has been implicated in histone binding, and (only in higher eukaryotes) an N-terminal domain that appears to be critical for substrate recognition and protein-protein interactions. In yeast, Gcn5p acts as a transcriptional adaptor for several sets of target genes, including as *gal1*, *gal4*, and *his3* (Kuo et al. 1998; Wang et al. 1998). While Gcn5p itself is dispensable for growth, a composite mutation of Gcn5p and Sas3p

(apparently the other main H3 HAT in yeast, see chapter 2.3.2; pp. 20) causes terminal arrest of yeast cells in G2/M due to lack of H3-specific HAT activity (Howe et al. 2001). Humans and mice possess Gcn5 and PCAF, two highly similar homologues of yeast Gcn5p. However, they play distinct roles in mouse development (Table 2): While Gcn5$^{-/-}$-mice die during embryogenesis, PCAF$^{-/-}$-mice are viable and fertile (Xu et al. 1998; Yamauchi et al. 2000). Whether this results from biochemical differences between the two homologues, or due to differences in their developmental expression pattern, is unclear.

Gcn5 interacts with a variety of other transcription-associated proteins. In yeast, there are at least two distinct HAT-complexes containing Gcn5p as catalytic subunit, the SAGA and Ada complexes (Grant et al. 1997; Grant et al. 1998a; Grant et al. 1998c). No Ada-like complex has been described in higher metazoans, and no clear function has yet been attributed to the Ada complex. In contrast, the SAGA complex is critical for certain types of transcription both *in vitro* and *in vivo*. SAGA contains the Ada and Gcn proteins, that mediate activator interaction and nucleosome acetylation, the Spt proteins, that mediate TBP interaction, several TBP associated factors (TAFs), and the large protein Tra1p (Grant et al. 1998a; Saleh et al. 1998; Sterner et al. 1999). Mutation of different subunits affects transcription of distinct sets of target genes. In mammalian cells, three similar but distinct orthologues of the SAGA complex have been identified, the Gcn5 containing STAGA and TFTC complexes, and the PCAF containing PCAF complex (Table 3; Martinez et al. 1998; Ogryzko et al. 1998; Brand et al. 1999). They all exhibit subunit compositions remarkably similar to the SAGA complex. Both STAGA and TFTC have been linked to transcriptional activation (Hardy et al. 2002; Yanagisawa et al. 2002). However, the presence of spliceosome-associated proteins, as well as Damaged-DNA-Binding-Proteins, in both of these complexes suggests that they are also involved in chromatin modification in response to DNA-damage and/or mRNA processing (Brand et al. 2001; Martinez et al. 2001).

HAT	Genotype	Phenotype
PCAF	+/+	Normal
	+/-	Normal
	-/-	Normal
GCN5	+/+	Normal
	+/-	Normal
	-/-	Embryonic lethal (10.5 dpc) with loss of paraxial mesoderm and chordamesoderm due to increased apoptosis
PCAF & GCN5	+/- & +/-	Normal
	-/- & -/-	Embryonic lethal prior to 6.5 dpc
p300	+/+	Normal
	+/-	Decreased viability *in utero*
	-/-	Embryonic lethal (9-11 dpc), with decreased proliferation, heart defects, open neural tube
CBP	+/+	Normal
	+/-	Defective skeletal formation and hematopoietic differentiation; increased malignancies
	-/-	Embryonic lethal with characteristics similar to $p300^{-/-}$-mice
p300 & CBP	+/- & +/-	Embryonic lethal, similar to p300 or $CBP^{-/-}$-mice

Table 2: Comparison of Phenotypes of HAT-Deficient Mice.

2.3.2. HATs II: The MYST Family

MYST family HATs (named after the family's founding members MOZ, Ybf2p/Sas3p, Sas2p, Tip60; Borrow et al. 1996) share among them unique sequence features, most prominently the MYST-homology-domain, which includes an acetyl-CoA binding motif (Table 1). Most MYST family HATs also have a Zinc finger domain, the only exception being Esa1p (Sterner and Berger 2000; Roth et al. 2001).

MYST family HATs have been associated with a variety of functions. Yeast Sas2p is required to acetylate H4K16, establishing heterochromatin boundaries (Suka et al. 2002). Yeast Sas3p (and the associated NuA3 complex; Table 3) plays a role in silencing, transcriptional and/or replicational elongation, and genome wide acetylation (John et al. 2000; Howe et al. 2001; Reifsnyder et al. 1996, Takechi and Nakayama 1999). The gene encoding human MOZ is translocated to genes of other HATs (CBP or Tif2) in leukemia, and aberrant chromatin acetylation due to mistargeting of specific HAT

activities might ultimately lead to leukemogenesis (Borrow et al. 1996; Liang et al. 1998; Kitabayashi et al. 2001). MORF, a close homologue of MOZ, appears to function in both transcriptional repression and activation (Champagne et al. 1999; Panagopoulos et al. 2001; Pelletier et al. 2002). However, the physiological roles of MOZ and MORF remain unknown. Another human MYST HAT, HBO1, has been identified as a protein associated with the DNA replication initiation proteins MCM2 and Orc1, suggesting a role in DNA replication (Iizuka and Stillman 1999; Sharma et al. 2000; Burke et al. 2001). The best-characterized MYST proteins, however, are yeast Esa1p (Smith et al. 1998; Clarke et al. 1999), and its human homologue, Tip60 (Kamine et al. 1996).

Various *in vitro* studies indicate that Esa1 and Tip60 preferentially acetylate lysines in H4 and H2B (Yamamoto and Horikoshi 1997; Kimura and Horikoshi 1998). Consistent with this, Esa1p has recently been shown to target all lysines in the N-terminal tail of H4, except K16, plus H2A (K7), and H2B (K11 and K16) *in vivo* (Suka et al. 2001). Esa1p is essential for growth and viability in yeast. Interestingly, deletion of Esa1p causes terminal arrest in G2/M phase, similar to the composite *sas3/gcn5* mutation, suggesting that functional H3 and H4 HAT activities are essential for cell division in yeast. Esa1p is the catalytic subunit of a HAT-complex termed NuA4 (Table 3; Allard et al. 1999), which is involved in transcriptional regulation. Esa1p is targeted to a small subset of ribosomal protein (RP) promoters, depending on recruitment by the sequence specific transcription factors Rap1p or Abf1p (Galarneau et al. 2000; Eisen et al. 2001). Recruitment correlates with coordinate regulation of RP-genes in response to growth stimuli. In addition, the NuA4 complex is recruited to DNA double strand breaks *in vivo*, and appears to participate in non-homologous end-joining repair, and in a novel pathway of replication-coupled repair (Bird et al. 2002). Both pathways require Esa1p, suggesting that this HAT is also involved in chromatin-remodeling processes unrelated to transcriptional activation.

HAT-complex (species)	SAGA (yeast)	PCAF/STAGA/ TFTC (human)	ADA (yeast)	Tip60 (human)	NuA4 (yeast)
HAT	Gcn5p	Gcn5/PCAF	Gcn5p	Tip60	Esa1p
	Ada1p				
	Ada2p	hAda2	Ada2p		
	Ada3p	hAda3	Ada3p		
	Ada5p/Spt20p				
	Spt3p	hSpt3			
	Spt7p				
	Spt8p				
Tra1-like	Tra1p	TRRAP		TRRAP	Tra1p
H4-like	$TAF_{II}60$	PAF65α			
WD40 repeat containing	$TAF_{II}90$	PAF65ß			
H2B-like	$TAF_{II}61/68$	TAF 15/20			
	$TAF_{II}23/25$	TAF30			
H3-like	$TAF_{II}17/20$	TAF31			
	Sin4p				
			Ahc1p		
Actin				γ-actin	Act1p
ARP				BAF53	Act3p/Arp4p
				EPC	Epl1p
RuvB-like				Tip48/49	absent
				p400	
Chromodomain				MRG-X/ MRG15?	Eaf3p
				Ing1?	Yng2p

Table 3: Subunit Composition of Selected HAT-Complexes.
HAT-complex subunits of five HAT-complexes are listed in columns. Homologues appear in the same row. ARP, actin-related-protein.

The closest human homologue of Esa1p is Tip60. It was originally identified as a protein interacting with HIV Tat protein, suggesting a function in transcriptional regulation after infection with HIV. Indeed, Tip60 has been associated with transcriptional coactivation (Dechend et al. 1999; Cao and Sudhof 2001; Baek et al. 2002). NF-κB, Bcl-3, Jab1, and nuclear hormone receptors can all bind Tip60. Recently, a ternary complex consisting of Tip60, the Fe65 transcriptional adaptor, and the cytoplasmic tail of amyloid-β precursor protein (APP) has been identified. This complex is recruited to the *kai1* gene, and HAT activity of Tip60 was required for histone acetylation (Baek et al. 2002). However, another report suggests roles of Tip60 in repair of damaged DNA, and in apoptosis (Ikura et al. 2000). Consistent with this, Tip60 is normally degraded by the ubiquitin-proteasome pathway, but is stabilized following UV-irradiation (Legube et al. 2002).

Tip60 is the HAT of a multi-subunit protein complex in mammalian cells, the Tip60 complex (Table 3; Ikura et al. 2000). This complex also contains two ATP-dependent DNA helicases, Tip48 and Tip49 (Ikura et al. 2000; Fuchs et al. 2001). They are probably involved in transcriptional activation and repression in mammalian cells, and their *Drosophila* orthologues appear to play similar roles (Bauer et al. 2000). Other subunits of the Tip60 complex include γ-actin, the actin-related-protein (ARP) BAF53 (both of which are also components of other chromatin-remodeling-complexes; for review see Rando et al. 2000; Olave et al. 2002), and EPC, a human homologue of the *Drosophila* Enhancer-of-Polycomb protein, further suggesting a role in transcriptional regulation (Stankunas et al. 1998; Galarneau et al. 2000). Another subunit of the Tip60 complex is p400, a putative helicase (Fuchs et al. 2001). Interestingly, p400 was also purified as a component of the p400 complex, which is identical to the Tip60 complex, except that it does not contain Tip60 (but apparently does have HAT activity). Finally, the Tip60 complex contains TRRAP, a transcriptional regulatory protein also found in the human STAGA/TFTC and PCAF complexes (McMahon et al. 1998; Vassilev et al. 1998; Ikura et al. 2000). TRRAP is essential for mouse embryonic development (Herceg et al. 2001), and its homologue Tra1p is required for viability in yeast (Grant et al. 1998b). Tra1p is a component of the yeast HAT-complexes SAGA and NuA4 (Table 3). It is noteworthy, that TRRAP homologues are present in at least two distinct classes of HAT-complexes in both yeast and humans, and the fact that PCAF/STAGA/TFTC complexes are apparently analogous to SAGA suggests that the Tip60 complex may be analogous to NuA4. This possibility is supported by the fact that both catalytic subunits (Esa1p and Tip60) are MYST proteins. However, the human Tip60 complex differs from yeast NuA4 at least in containing the additional subunits Tip48, Tip49 and p400. Finally, the NuA4 complex contains the two additional subunits, Yng2p and Eaf3p, and while orthologues of both proteins have been identified in humans, it remains to be determined whether they are associated with the Tip60 complex (Loewith et al. 2000; Choy et al. 2001; Eisen et al. 2001; Nourani et al. 2001; Choy and Kron 2002).

2.3.3. HATs III: p300/CBP

The p300/CBP HATs are very large proteins present only in higher eukaryotes (for review see Goodman and Smolik 2000; Sterner and Berger 2000; Chan and La Thangue 2001; Roth et al. 2001). They play essential roles in balancing cell proliferation and differentiation. Both p300 and CBP are required for embryonic development in mice (Yao et al. 1998; Oike et al. 1999b; Kung et al. 2000). Their importance is further emphasized by gene-dosage-associated phenotypes in both humans and in mice (Table 2; Petrij et al. 1995; Giles et al. 1998; Oike et al. 1999a; Murata et al. 2001). Importantly, mutations in CBP or p300 have been found in tumors (Sobulo et al. 1997), and CBP has tumor suppressor activity in mice. In cultured cells, these two highly homologous proteins are, with few exceptions, functionally interchangeable, and thus are referred to as p300/CBP. They function as coactivators for a variety of transcription factors involved in proliferation or differentiation, such as nuclear hormone receptors, and differentiation specific transcription factors, e.g. GATA-1 (Boyes et al. 1998), EKLF (Zhang and Bieker 1998), and MyoD (Puri et al. 1997a; Puri et al. 1997b). Via MyoD, they are critical for muscle cell terminal differentiation *ex vivo* and *in vivo* (Puri et al. 1997a; Puri et al. 1997b). Furthermore, p300/CBP are essential for transformation by viral oncoproteins such as E1A (Arany et al. 1994; Eckner et al. 1994), and papilloma-virus E7 (Patel et al. 1999; Zimmermann et al. 1999). Consistent with this, there is evidence that they are required for the G1/S transition in cycling cells (Ait-Si-Ali et al. 1998; Ait-Si-Ali et al. 2000; Chan et al. 2001).

2.3.4. Acetylation of Non-Histone Substrates

Besides histones, many HATs can also acetylate other proteins. Substrates include non-histone chromatin proteins, such as the members of the high mobility group proteins (HMG; Wong et al. 1991). Moreover, many sequence-specific transcription factors, for example p53 (Gu and Roeder 1997, Liu et al. 1999), EKLF (Zhang and Bieker 1998), E2F1 (Martinez-Balbas et al. 2000; Marzio et al. 2000), HNF-4 (Soutoglou et al. 2000; Soutoglou et al. 2001), and dTCF (Waltzer and Bienz 1998), several cofactors, such as ACTR (Chen et al. 1999b), and general transcription factors (Imhof et al. 1997) can

be acetylated by one or more HATs (reviewed in Sterner and Berger 2000; Roth et al. 2001). Acetylation can alter their DNA-binding activities (E2F1, GATA-1, and p53), stabilize protein levels (E2F1), regulate nuclear localization (HNF-4), or affect protein-protein interactions (TCF and ACTR). Moreover, PCAF undergoes autoacetylation (Liu et al. 2000), which may facilitate intramolecular interactions and regulate HAT activity. Finally, proteins such as tubulin (reviewed in Polevoda and Sherman 2002; Palazzo et al. 2003) and two members of the importin-α protein family involved in nuclear import can also be acetylated (Bannister et al. 2000). Thus, while acetylation is apparently an important step in regulation of a plethora of transcriptional regulation pathways, it also regulates other cellular processes.

2.4. DNA and Histone Methylation

Many chromatin-modifying-activities other than HATs/HDACs have been identified. DNA methylation has been associated with epigenetic silencing and maintenance of genome integrity (Geiman and Robertson 2002, Robert, 2003). Aberrant DNA methylation contributes to carcinogenesis, as it results in alteration of gene expression (Rhee et al. 2002, Di Croce et al. 2002). For example, in colorectal cancer, the loci of *mlh1*, *mgmt*, and *cdkn2a* are hypermethylated, leading to downregulation of the corresponding gene products (Rocco and Sidransky 2001; Kondo et al. 2003). The enzymes catalyzing DNA methylation are the DNA methyltransferases (DNMTs). At least DNMT1, the predominant DNA methyltransferase in human cells, has been found to associate with transcriptional corepressors, notably with the tumor suppressor Rb (see chapter 3.1. pp. 29; Robertson et al. 2000).

Besides DNA, histone tails are also substrates for methylation, which can occur at both arginine and lysine residues (for review, see Zhang and Reinberg 2001). For example, methylation of H3K9 by lysine-specific histone methyltransferases (HMTs), such as SUV39H1, is a critical step in transcriptional silencing (Peters et al. 2001; Peters et al. 2002; Schotta et al. 2002). Methylated H3K9 recruits the HP1 protein, which induces heterochromatin formation (Lachner et al. 2001). Interestingly, SUV39H1 and HP1 can interact with HDACs, suggesting that histone deacetylation and

histone methylation cooperate to induce transcriptional silencing at chromosomal loci (Vaute et al. 2002). However, histone lysine methylation does not always coincide with repression or silencing. In yeast, active genes strictly correlate with trimethylated H3K4, whereas inactive genes exhibit both di- and trimethylated H3K4 (Santos-Rosa et al. 2002). Therefore, lysine methylation may be important for transactivation as well as repression, at least in yeast.

The enzymes catalyzing transfer of methyl-moieties to arginines are the protein arginine methyltransferases (PRMTs). Apparently, they are involved in both transactivation and repression (for reviews see McBride and Silver 2001; Stallcup 2001; Davie and Dent 2002; Kouzarides 2002). For example, PRMT1 and CARM1 can act as coactivators of hormone receptors following hormone treatment of human cells (Chen et al. 1999a; Koh et al. 2001; Ma et al. 2001; Xu et al. 2001b). In contrast, PRMT5 seems to be required for repression of the human *ccne1* gene (encoding Cyclin E), as it was recently purified as a subunit of a transcriptional repressor complex specific for the *ccne1* promoter (Fabbrizio et al. 2002). In summary, while DNA methylation is specific for gene silencing, histone methylation may result in gene repression or activation, depending on the cellular and promoter context.

2.5. Histone Phosphorylation

Histones are also substrates for kinase dependent phosphorylation (Berger 2002; Geiman and Robertson 2002). In human cells, phosphorylation of H3S10 occurs after treatment of cells with epidermal growth factor (EGF; Chadee et al. 1999; Sassone-Corsi et al. 1999). Moreover, recent CHIP data showed that EGF-activated genes indeed are both acetylated and phosphorylated at H3 (Cheung et al. 2000). Interestingly, kinetic analyses suggest that phosphorylation precedes acetylation. In fact, it may be an important determinant in HAT recruitment, as several HATs display preference for phosphorylated H3. The kinase responsible for histone phosphorylation appears to be Rsk2, which has been implicated in growth control (Sassone-Corsi et al. 1999; Merienne et al. 2001). In yeast, a H3S10 kinase complex has been purified that contains Snf1p as catalytic subunit (Lo et al. 2001). Snf1p and Gcn5p function in an obligate sequence to enhance

ino1 transcription by modifying histone H3S10 and K14, respectively (Lo et al. 2000). Thus, phosphorylation and acetylation are targeted to the same histone in a highly coordinated manner.

2.6. Chromatin-Remodeling-Complexes

All the enzymes described above covalently modify either DNA or DNA associated proteins. In contrast, ATP-dependent chromatin-remodeling-enzymes utilize the energy from ATP hydrolysis to induce conformational changes in chromatin (for reviews, see Guyon et al. 1999; Phelan et al. 1999; Becker and Horz 2002, Kingston and Narlikar 1999; Fry and Peterson 2002; Peterson 2002). By exposing or occluding specific DNA sequences, they determine whether DNA is accessible or not in the context of chromatin. Like HATs, many of the enzymatic subunits of chromatin-remodeling-complexes contain a variety of domains that are associated with transcriptional functions, for example bromodomains (in yeast Swi2p), chromodomains, and PhD fingers (both in human Mi-2). Based on sequence similarities in their catalytic ATPase domain, chromatin-remodeling-enzymes can be subdivided in three families: The Swi2/Snf2 family, the ISWI family, and the Mi-2 family (reviewed in Narlikar et al. 2002). Except for Mi-2, which has only been described in human cells, orthologues of the other two families have been identified in yeast, *Drosophila*, and humans, suggesting that some of these enzymes play highly conserved roles in chromatin-remodeling. While all three families are capable of inducing conformational changes in chromatin that alter DNA accessibility, there are critical differences in molecular mechanisms. For instance, histone tail removal negates remodeling by NURF, a complex containing *Drosophila* ISWI, but does not affect remodeling by Brg1, a human Swi2/Snf2 family protein (Langst et al. 1999; Langst and Becker 2001, Clapier et al. 2001). Thus, the precise mechanism of chromatin-remodeling employed by each family remains to be elucidated.

Like HATs, chromatin-remodeling-enzymes occur in cells as subunits of large multi-subunit complexes (Narlikar et al. 2002). In yeast, there are two complexes containing Swi2/Snf2-like subunits, the Swi/Snf complex, and the RSC complex. The former is conserved in humans as the so-called BAF complex (Wang et al. 1996a; Wang et al. 1996b; Zhao et al. 1998b). It

contains either of two catalytic subunits, Brg1 or hBrm, a variety of Brg/hBrm-associated-factors (BAFs), and γ-actin. The fact that γ-actin and BAF53 are common subunits of the BAF and Tip60 complexes suggests a general role for these proteins in chromatin-remodeling (Rando et al. 2000; Olave et al. 2002). The BAF complex interacts with, and is involved in, transcriptional activation and repression by several transcription factors, including NFI/CTF (Liu et al. 2001), the glucocorticoid receptor (GR; Fryer and Archer 1998), and Rb (Trouche et al. 1997; Strobeck et al. 2000). The latter interaction is noteworthy, since Brg1 and/or hBrm are necessary cofactors for Rb-dependent repression, and inhibition of proliferation. Cells lacking Brg1 or hBrm exhibit a transformed phenotype, and reintroduction of Brg1 induces cell cycle arrest and flat cell morphology, suggesting induction of senescence. Furthermore, Brg1 itself (Wong et al. 2000) and two other subunits of the BAF complex, BAF47/Ini1/Snf5 (Versteege et al. 1998; Klochendler-Yeivin et al. 2000; Roberts et al. 2002; Versteege et al. 2002) and BRCA1 (Bochar et al. 2000; Morrow and Gradishar 2002; Venkitaraman 2002), are tumor suppressors. In summary, the BAF complex appears to play an important role in transcriptional regulation and proliferation control in mammalian cells.

2.7. Combinatorial Function of Chromatin-Modifying and/or Remodeling-Complexes in Transcription

Studies in yeast have shown that chromatin-remodeling-complexes and HAT-complexes are functionally connected. For example, while neither SAGA subunit deletions, nor mutations of the Swi/Snf-complex-components are lethal, composite mutations of subunits of both complexes cause loss of viability (Roberts and Winston 1997). Consistent with this hypothesis, ordered recruitment of both kinds of complexes to the *HO* promoter by the transcription factor Swi5p has been demonstrated in yeast, revealing that Swi/Snf-complex-dependent chromatin-remodeling is a prerequisite for SAGA recruitment (Cosma et al. 1999; Cosma 2002). In contrast, on the *pho8* promoter SAGA recruitment appears to precede Swi/Snf-complex binding (Gregory et al. 1999). Thus, different promoters may exhibit distinct requirements for ordered action of various complexes. Additionally,

recruitment of SAGA to many genes involved in mitotic exit has been shown to require the Swi/Snf-complex, maybe due to the highly condensed metaphase chromosome structure (Krebs et al. 2000). In mammalian cells, E2F/Rb-dependent gene repression involves HDACs as well as the Brg1/hBrm, the homologues of the Swi2/Snf2-enzymes in yeast. In summary, transcriptional regulation often requires concerted function of several distinct complexes.

In addition, some HATs interact with each other *in vivo* (for example Chen et al. 1997). In fact, PCAF was first identified as a protein associated with p300/CBP (Yang et al. 1996). However, whether this interaction occurs within the context of the STAGA/TFTC/PCAF complexes, is not clear. During terminal differentiation of myocytes, the transcription factor MyoD recruits both p300 and PCAF (Puri et al 1997a; Puri et al. 1997b). However, while the HAT activity of PCAF appears to be required for acetylation of MyoD, the HAT activity of p300 is essential for gene specific expression in the cell fusion process, even though the specificity of these regulatory events is currently a matter of debate (Vaute et al. 2002). These data show that individual chromatin-remodeling-enzymes may perform specific functions even as parts of multi-subunit protein complexes. Furthermore, they may play roles in transcription that go beyond simple histone and factor acetylation, such as assembly of RNA polymerase or other rate limiting steps.

3. Cell Cycle Control in Mammalian Cells

3.1. The Cell Cycle

In order to reproduce, eukaryotic cells undergo the cell division cycle, or short, the cell cycle. This process can be divided into four phases (Figure 2): Two gap phases, G1 and G2, are interrupted by the synthesis phase (S), during which the DNA is replicated. Following G2 is mitosis (M), in which the nucleus and subsequently the cytoplasm divide to give rise to two daughter cells. G1 represents a regulatory switch, during which cells must decide whether to enter a new cell cycle, or whether to withdraw from the cycle and enter quiescence (G0), for example due to contact inhibition, serum

withdrawal, or TGF-β treatment (reviewed in Pardee 1989; Sherr 1994b; Sherr 1994a). The decision must be made before cells pass the restriction point (R), after which they are dedicated to completing the cycle even in absence of mitogenic factors (Zetterberg et al. 1995; Planas-Silva and Weinberg 1997; Blagosklonny and Pardee 2002). However, cell cycle progression can be halted or delayed in G1 and G2 through so-called checkpoint signaling cascades, which are activated, for example, by DNA-damage (reviewed in O'Connor 1997; Molinari 2000). During cellular transformation, deregulation of the cell cycle occurs due to accumulation of mutations in genes governing restriction point and checkpoint decisions (reviewed in Bartkova et al. 1997; Kaelin 1997; Molinari 2000). Thus, tumor cells commonly fail to withdraw from the cell cycle. In summary, whereas most normal cells in metazoans are not actively dividing (i.e. are in G0), tumor cells are able to cycle without restraint.

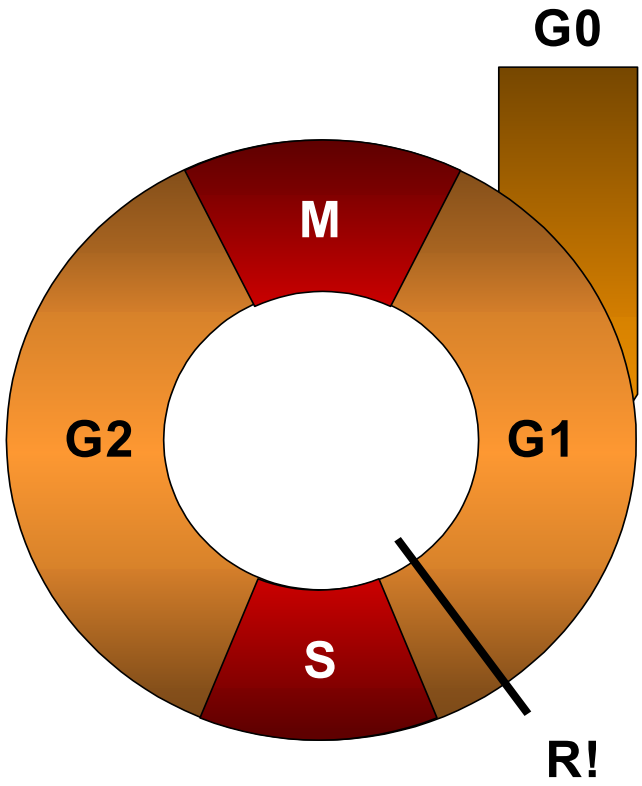

Figure 2: The Cell Cycle of Eukaryotes.
The eukaryotic cell cycle is divided in four phases: Two gap phases (G1 and G2) that are separated by the synthesis phase (S), during which the DNA is replicated. After G2, cells divide in mitosis (M), and enter a new G1. Cells can withdraw from the cycle, and arrest in quiescence (G0). Once cells are past the so-called restriction point (R) in late G1, they are dedicated to finishing the initiated cell cycle.

The G1 Phase of the cell cycle is regulated at a variety of levels (Figure 3). One critical player is the transcription factor E2F (reviewed in Nevins et al. 1997b; Johnson and Schneider-Broussard 1998; Nevins 1998; DeGregori 2002; Stevaux and Dyson 2002; Trimarchi and Lees 2002). E2F is an obligate heterodimer consisting of an E2F and a DP moiety. It regulates transcription of a variety of target genes (Ishida et al. 2001; Kel et al. 2001; Muller et al. 2001; Weinmann et al. 2001; Polager et al. 2002; Ren et al. 2002; Stanelle et al. 2002; Weinmann et al. 2002). These encode proteins involved in regulatory events before and during S-Phase (*ccna1, ccne1, ccne2, cdc2*, and *p107)*, and genes necessary for DNA metabolism (such as *dhfr, ts,* and *tk*) and replication (*mcm2-7*, *pol*α*2*, *cdc6*, and *orc1*). In addition, various other genes have recently been found to be regulated by E2F, suggesting roles for E2F in processes such as mitosis (for instance *bub1*), DNA repair (for example *pol*δ, *msh2*, and *brca1*), and apoptosis (several caspases, *p73*, *apaf1*, and *cdkn2a*). Interestingly, E2F may also participate directly in surveillance of replication, as it localizes to origins of replication in a ternary complex with Mre11 and Nbs1, proteins known to be crucial for genomic integrity (Maser et al. 2001). Such a complex may suppress replication after DNA-damage, thus preventing genomic instability.

In quiescence and during G1/S transition, E2F activity is negatively regulated by the three Retinoblastoma (Rb) family proteins, $p110^{Rb}$, p107, and p130 (Figure 3; reviewed in Nevins et al. 1997a; Harbour and Dean 2000b; Nevins 2001, Harbour and Dean 2000b; Classon and Harlow 2002, DeGregori 2002; Trimarchi and Lees 2002). All three bind E2F and these complexes repress activation of E2F target genes (see chapter 3.2.4. pp. 41). Repression of E2F targets is necessary for Rb-family proteins to exert their growth inhibitory effect.

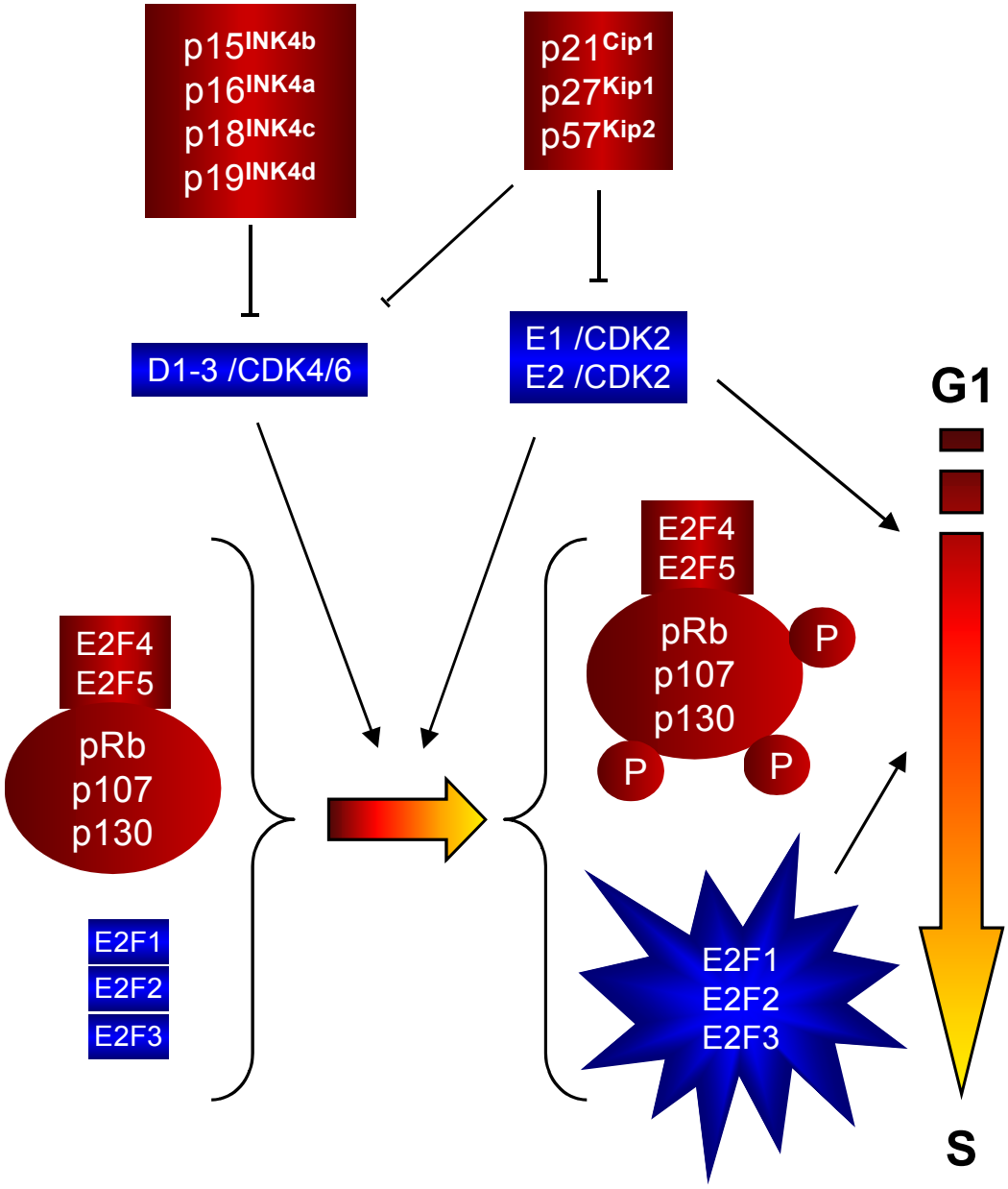

Figure 3: Regulation of the G1/S Transition in Mammalian Cells.
Regulation of the mammalian cell cycle in G1 converges on the E2F/Rb transcriptional network. For further details, see text. Growth promoting proteins are shown in blue, while growth inhibitory proteins are in red.

Rb-family proteins in turn are negatively regulated by several Cyclin-Dependent-Kinases (CDKs). CDKs are serine/threonine kinases that govern the transitions between the distinct phases of the cell cycle (reviewed in Hengstschlager et al. 1999; Blagosklonny and Pardee 2002). Phosphorylation of Rb-family proteins by CDKs results in its dissociation from E2F, and from several corepressors (see chapter 3.2.4. pp. 41). In order to be active, CDKs must form heterodimers with a Cyclin subunit (reviewed in Hengstschlager et

al. 1999; Obaya and Sedivy 2002). mRNA and protein levels of distinct Cyclins are induced sequentially as cells exit from quiescence and progress through G1. At least some *ccn* and *cdk* genes (encoding the Cyclins and CDKs, respectively) are direct targets of E2F, creating a positive feedback regulation loop. During early G1, the principal kinases are CDK4 and CDK6, which bind D-type Cyclins (Cyclin D1, D2, and D3; reviewed in (Sherr 1993; Sherr 1994b; Hamel and Hanley-Hyde 1997; Planas-Silva and Weinberg 1997; Hengstschlager et al. 1999; Obaya and Sedivy 2002). Later, the predominant kinase is CDK2, bound by either Cyclin E1 or E2 (in late G1), or by Cyclin A (in S). While D-type Cyclins appear to strictly regulate Rb-family phosphorylation, Cyclin E1 is required for S-Phase entry even in the absence of functional Rb, suggesting that other substrates are rate limiting for Cyclin E1 induced proliferation (Ohtsubo et al. 1995; Geng et al. 1996; Alevizopoulos et al. 1997; Lukas et al. 1997; Alevizopoulos et al. 1998). These may include NPAT (which is involved in histone gene transcription during S-Phase; Zhao et al. 1998a; Ma et al. 2000; Zhao et al. 2000), several subunits of the BAF complex (Shanahan et al. 1999), the HAT p300/CBP (Perkins et al. 1997; Ait-Si-Ali et al. 1998; Ait-Si-Ali et al. 2000), and the CDK inhibitor $p27^{Kip1}$ (Sheaff et al. 1997; Vlach et al. 1997), all of which can be phosphorylated by Cyclin E/CDK2. During S-Phase CDK4/6 activities are low, but CDK2 activity is sustained, as E-type Cyclins are replaced by Cyclin A (reviewed in Yam et al. 2002). In mammalian cells, Cyclin A/CDK2 also regulates initiation of DNA replication, as it binds to, and colocalizes with, PCNA at the sites of DNA replication (Cardoso et al. 1993; Sobczak-Thepot et al. 1993; Fotedar et al. 1996). Furthermore, Cyclin A/CDK2 can bind E2F1, and it also phosphorylates DP1 in S/G2, resulting in loss of DNA-binding of E2F/DP heterodimers (see chapter 3.2.1. pp. 35; Krek et al. 1994; Krek et al. 1995). These events are presumably important in shutting down E2F-dependent transcription after S-Phase entry, preventing untimely DNA synthesis.

CDK inhibitors (CKIs) provide yet another level of regulation (Figure 3; reviewed in Ekholm and Reed 2000; Moller 2000; Blagosklonny and Pardee 2002; Ortega et al. 2002). CKIs can be divided into two classes, the INK family proteins ($p15^{INK4b}$, $p16^{INK4a}$, 18^{INK4c}, and $p19^{INK4d}$), and the Cip/Kip family ($p21^{Cip1}$, $p27^{Kip1}$ and $p57^{Kip2}$). While the INK proteins specifically inhibit

CDK4/6 by preventing their association with D-type Cyclins, Kip/Cip family proteins inhibit virtually all G1-specific Cyclin/CDK complexes. In contrast to INK family CKIs, Kip/Cip proteins do not prevent complex formation of active kinases, but directly bind to a Cyclin/CDK heterodimer to inhibit its catalytic activity. Interestingly, $p21^{Cip1}$ and $p27^{Kip1}$ can also act as assembly factors for D-type Cyclin/CDK complexes, and thus have somewhat antagonistic roles in cell cycle regulation (LaBaer et al. 1997). Nevertheless, overexpression of CKIs generally induces cell cycle arrest. Some CKIs appear to participate in regulation of specific functions, such as senescence ($p16^{INK4a}$; Haber 1997; Huschtscha and Reddel 1999; Sharpless and DePinho 1999; Bringold and Serrano 2000; Lundberg et al. 2000), TGFβ response ($p15^{INK4b}$ and $p21^{Cip1}$; Hannon and Beach 1994; Reynisdottir et al. 1995; Sandhu et al. 1997; Feng et al. 2000; Feng et al. 2002), and DNA-damage induced growth arrest (induction of $p21^{Cip1}$ through p53-dependent transactivation; for review see Boulaire et al. 2000; Bartek and Lukas 2001).

Many components of the network regulating S-Phase entry are targets of tumorigenic mutations. In fact, Rb exhibits features of a classical tumor suppressor in mice and humans, and deregulation of Rb/E2F-network proteins is a common event in tumorigenesis (reviewed in Weinberg 1991; Weinberg 1992; Sherr 1996; Nevins 2001; Classon and Harlow 2002; Sherr and McCormick 2002). For example, the gene encoding Cyclin D1 is often amplified in breast tumors, as well as some types of neck and head carcinomas (Donnellan and Chetty 1998; Steeg and Zhou 1998, Donnellan and Chetty 1998). While it is not clear whether *ccne1* itself is an oncogene, it is often overexpressed in breast cancer, and this correlates with poor prognosis (Donnellan and Chetty 1999). Low levels of $p27^{Kip1}$ also correlate with poor prognosis, and in one mouse knockout model $p27^{Kip1}$ behaves like a haplo-insufficient tumor suppressor (Fero et al. 1996; Fero et al. 1998; Donnellan and Chetty 1999; Moller 2000). $p16^{INK4a}$ is one of two tumor suppressors encoded by the *cdkn2a* locus (the other one being $p14^{ARF}$), which is frequently mutated or silenced in tumors (Sharpless and DePinho 1999; Serrano 2000; Sherr 2001; Ortega et al. 2002). In summary,

deregulation of the Rb/E2F pathway, and as a consequence, liberation of E2F activity, is probably an event necessary for tumorigenesis.

3.2. The Transcription Factor E2F: A Critical Regulator of S-Phase Entry

3.2.1. Biochemistry of E2F

The transcription factor E2F was originally identified as a cellular activity that is required for transcriptional activation of the adenoviral E2 gene promoter (reviewed in Nevins 1992). Like many transcription factors, E2F is a heterodimer of two different polypeptides, an E2F and a DP moiety, and dimerization is essential for DNA-binding and transcriptional regulation (reviewed in DeGregori 2002, Stevaux and Dyson 2002; Trimarchi and Lees 2002). *In vitro* binding assays revealed that all E2F/DP dimers preferentially bind the same DNA sequence, TTTSGCGCSAAA. However, while one CHIP study did not show any specific preference of distinct E2Fs for certain DNA sequences, other studies indicate, that individual E2Fs may indeed exhibit differential specificities for a given target gene (Takahashi et al. 2000; Wells et al. 2000). Thus, whether distinct E2F/DP heterodimers possess intrinsic ability to distinguish between target genes remains to be determined.

Until now, six *e2f* and two *dp* genes have been identified, giving rise to at least seven E2F proteins (E2F1, E2F2, E2F3a, E2F3b, E2F4, E2F5, and E2F6; Figure 4) and three DP proteins (DP1, DP2, and DP3; reviewed in (Dyson 1998; Helin 1998). The E2F proteins are commonly subdivided into three groups: “Activating” E2Fs (E2F1, E2F2, and E2F3a), “repressing” E2Fs (E2F4, and E2F5), and the structurally distinct E2F6, which is also associated with repression (reviewed in DeGregori 2002, Stevaux and Dyson 2002; Trimarchi and Lees 2002). The division into "activating" and "repressing" E2Fs is based on biological observations and is not fixed, as the domain containing transactivating capability overlaps with the domain responsible for repression (Figure 4). Furthermore, E2F1-3a may be involved in repression *in vivo*, while E2F4 and E2F5 can induce proliferation in some settings, again arguing in favor of some functional overlap between these two classes. The role of E2F3b is unclear, with roles consistent with both activation and repression (Leone et al. 2000; Wu et al. 2001).

Structurally, the E2F gene products are related polypeptides (Figure 4; reviewed in Trimarchi and Lees 2002). All E2F proteins contain a core domain consisting of a DNA-binding-domain (DBD), a marked-box (MB), and a leucine-zipper (LZ). This core is responsible for interaction with the DP proteins, and for DNA-binding (Bandara et al. 1993; Helin et al. 1993; Krek et al. 1993; Wu et al. 1995). In addition, all E2Fs except E2F6 possess a C-terminal transactivation domain (TAD)/Rb-family-binding domain. This domain mediates protein-protein interactions with both Rb-family proteins (Bagchi et al. 1991; Bandara et al. 1991; Chittenden et al. 1991; Ewen et al. 1991; Hannon et al. 1993; Li et al. 1993), and transcriptional coactivators, such as TRRAP (McMahon et al. 1998). Activating E2Fs (but not E2F3b) further possess an N-terminal domain crucial for the interaction with Cyclin A/CDK2 (Krek et al. 1994; Krek et al. 1995), and a nuclear localization signal (NLS) that is also present in E2F3b (Muller et al. 1997; Verona et al. 1997). In contrast, repressing E2Fs apparently contain a nuclear export signal (NES), and are subject to CRM1-dependent nuclear export (Gaubatz et al. 2001; Apostolova et al. 2002). All activating E2Fs can be acetylated by PCAF and/or p300/CBP, and three lysines critical for acetylation of E2F1 are conserved in E2F2 and E2F3, but not in repressing E2Fs or E2F6 (Martinez-Balbas et al. 2000; Marzio et al. 2000). Since it lacks the TAD/Rb-family-binding domain, E2F6 can not repress E2F targets via Rb-family proteins (Trimarchi et al. 1998). Instead, it recruits PcG-proteins to induce Rb-independent repression of E2F target genes (Trimarchi et al. 2001).

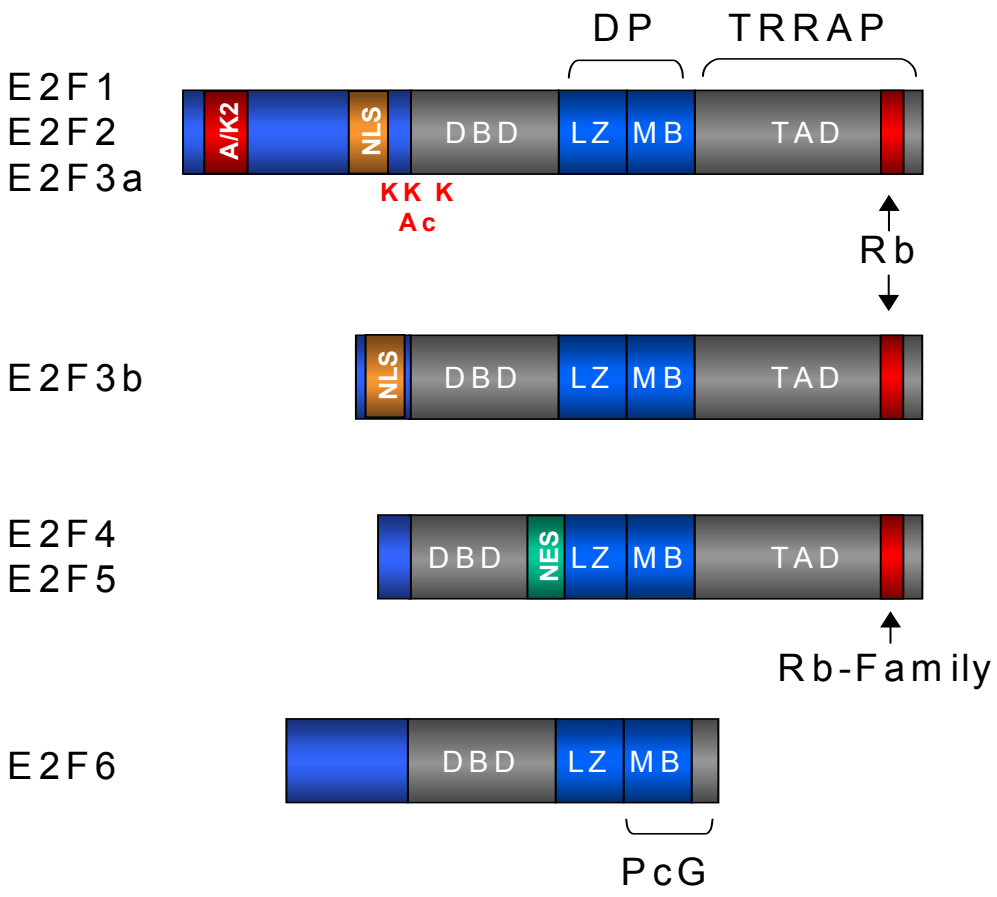

Figure 4: The E2F-family of Transcription Factors: Structural Features.
All E2F proteins possess a core domain that mediates DNA-binding and DP dimerization (encompassing the marked-box, MB, and the leucine-zipper, LZ sequence motifs). The TAD/Rb-family-binding domain (TAD) is only present in E2F1-5. The activating E2Fs (E2F1, -2, and -3a) also share a canonical nuclear localization signal (NLS), while the repressing E2Fs (E2F4 and -5) contain a nuclear export signal (NES). E2F6 lacks the TAD/Rb-family binding domain, and instead contains a C-terminal repression domain responsible for interaction with Polycomb-group (PcG) proteins.

3.2.2. Biological Activities of E2F

Activating E2Fs are capable of driving quiescent cells into S-Phase (and sometimes induce apoptosis), when overexpressed in mouse embryonic fibroblasts (MEFs) and transformed cells (Johnson et al. 1993; Lukas et al. 1996). Ectopic expression of E2F1 can also bypass G1 arrests imposed by expression of $p16^{INK4a}$, TGF-β, and DNA-damage (DeGregori et al. 1995; Mann and Jones 1996; Alevizopoulos et al. 1997). Overexpression of E2F1 can transform cell lines, or cooperate with oncogenic Ras to transform primary cells (Johnson et al. 1994; Singh et al. 1994; Xu et al. 1995). However, at least in some contexts, ectopic E2F1 can also induce senescence. Most

biological activities have been associated with the abilities of activating E2F to induce transcription of target genes required for entry and progression of S-Phase (reviewed in Lavia and Jansen-Durr 1999). Consistent with this, activating E2Fs are downregulated in quiescent cells, but accumulate as cells enter a new cycle (Slansky et al. 1993; Slansky and Farnham 1996; Leone et al. 1998). When bound by Rb, however, activating E2Fs loose the ability to activate transcription; instead, these complexes with Rb repress transcription (Helin et al. 1992; Flemington et al. 1993; Helin et al. 1993).

E2F4 also binds Rb, and seems to account for the majority of E2F activity in most cells (Beijersbergen et al. 1994). Furthermore, E2F4 and E2F5 specifically interact with p130 and p107, respectively, and these complexes accumulate in the nuclei of quiescent cells to repress transcription (Ikeda et al. 1996; Moberg et al. 1996), with E2F4/p130 complexes being predominant (Beijersbergen et al. 1994; Hijmans et al. 1995; Takahashi et al. 2000). In contrast to activating E2Fs, repressing E2Fs are expressed throughout the cell cycle, but are exported from the nucleus as cells progress into S-Phase (Lindeman et al. 1997; Verona et al. 1997; Gaubatz et al. 2001). Accordingly, E2F4 and E2F5 only possess weak S-Phase induction ability, but can induce S-Phase entry, when co-expressed with DP1 (Alevizopoulos et al. 1998).

Besides inducing proliferation, E2F1 can also promote apoptosis, probably through transactivation of genes such as *cdkn2a-arf* (Zhang et al. 1998; Parisi et al. 2002), *p73* (Lissy et al. 2000; Stiewe and Putzer 2000), *apaf1* (Moroni et al. 2001), several caspase genes (Nahle et al. 2002), and others (reviewed in Phillips and Vousden 2001; Ginsberg 2002). Both p53-dependent and p53-independent mechanisms contribute to E2F1-induced cell death (Qin et al. 1994; Shan and Lee 1994; Wu and Levine 1994; Hiebert et al. 1995; Hsieh et al. 1997; Pan et al. 1998). Consistent with this, protein levels of E2F1 are upregulated following DNA-damage, as is DNA-binding activity (Blattner et al. 1999; Hofferer et al. 1999; Lin et al. 2001). While these regulatory mechanisms are apparently unique to E2F1, both E2F1 and E2F3 contribute to apoptosis in developing mice (Phillips et al. 1997; Pan et al. 1998; Tsai et al. 1998; Phillips et al. 1999; Ziebold et al. 2001). Based on these data, a model has been suggested, whereby activating E2Fs contribute towards a pool of total activity, which induces proliferation when above a

certain threshold, and apoptosis, when total activity surpasses a second, higher threshold (Ziebold et al. 2001). Support for this model has been obtained from studies with mice harboring gene deletions for individual E2F genes (see next chapter).

3.2.3. Lessons from Mouse Models

All six individual E2F proteins have been inactivated in mice by targeted gene deletion. Unexpectedly, the observed phenotypes are often devoid of the general defects in proliferative processes that would be expected from mutations in major components of cell cycle regulation pathways. This has been attributed mostly to the redundancy of individual E2F proteins. Nevertheless, some important clues can be drawn from studies performed with mouse models and MEFs derived from these mice (reviewed in DeGregori 2002).

Mice lacking E2F1 show defects in T-lymphocyte maturation due to reduced apoptosis (Field et al. 1996). Additionally, hyperproliferation and tumor development can be observed in older mice, suggesting that E2F1 has growth inhibiting and tumor suppressor activities *in vivo* (Yamasaki et al. 1996). E2F2$^{-/-}$-mice also exhibit defects in T-cell maturation, but not due to diminished apoptosis (Murga et al. 2001). Instead, T-cells proliferate inappropriately, leading to an excess of self-reactive mature memory cells, and ultimately to autoimmune diseases. Thus, these two E2Fs cooperate to regulate the major components of immunological self-tolerance, at least in mice. E2F3 deletion results in partial lethality, as offspring arises only at a quarter of the expected frequency (Humbert et al. 2000b). MEFs from E2F3$^{-/-}$-mice exhibit decreased proliferation rates, and are delayed in entering S-Phase after exiting from quiescence. Induction of several E2F target genes is delayed, and weaker than in wild-type MEFs. While similar deregulation is absent from E2F1$^{-/-}$-MEFs, it is noteworthy that E2F1 overexpression can rescue the proliferation defects caused by deletion of E2F3. Another study showed that absence of all three activating E2Fs results in a total loss of proliferation, with MEFs arresting in all phases of the cell cycle (Wu et al. 2001). Somewhat contradictory to this, it was recently published that, while functional activating E2F is critically required for cell cycle entry from

quiescence, it is dispensable for exponential growth of MEFs (Rowland et al. 2002).

While MEFs lacking E2F4 exhibit no defects in proliferation or cell cycle control, E2F4$^{-/-}$-mice are smaller, and show several differentiation defects (Humbert et al. 2000a; Rempel et al. 2000; Fajas et al. 2002). Similarly, deletion of E2F5 results in no detectable proliferation defect, but mice die due to hydrocephalus resulting from impaired development of neuronal tissue (Lindeman et al. 1998). Thus, repressive E2Fs apparently have non-redundant functions in differentiation on mice. E2F4$^{-/-}$/E2F5$^{-/-}$-mice die during embryogenesis, while MEFs from these mice are not impaired in induction of quiescence, presumably due to compensatory mechanisms by other E2F proteins (Gaubatz et al. 2000). However, they fail to arrest in response to p16^{INK4a} overexpression, suggesting that repressive E2Fs are necessary for some, but not other growth inhibitory pathways. Finally, E2F6$^{-/-}$-mice are also viable, and MEFs from these mice show no defects in proliferation or induction of quiescence following serum withdrawal (Storre et al. 2002). However, mice lacking E2F6 exhibit homeotic transformation of the axial skeleton, similar to those found in mutations of Polycomb proteins, which fits well with the finding that E2F6 can associate with several PcG group proteins (Trimarchi et al. 2001).

The three members of the Rb-family have also been mutated in mice. Mice lacking one copy of the *rb1* gene are viable, but highly prone to tumor development. Deletion of both copies results in embryonic lethality due to excessive proliferation and apoptosis (Clarke et al. 1992; Jacks et al. 1992; Lee et al. 1992). Mice lacking p107 or p130 exhibit mouse-strain-dependent hyperplasia, cell cycle deregulation and apoptosis (p107$^{-/-}$), or embryonic lethality (p130$^{-/-}$), respectively (Cobrinik et al. 1996; Lee et al. 1996). Combined deletion of p107 and p130 results in death shortly after birth, again dependent on the mouse strain (LeCouter et al. 1998a; LeCouter et al. 1998b). Mutation of Rb and either p107 or p130 aggravates the defects observed in Rb$^{-/-}$-mice (Robanus-Maandag et al. 1998; Dannenberg et al. 2000). MEFs from mice lacking single Rb-family members display cell cycle defects, but still arrest in response to serum withdrawal. Composite p107$^{-/-}$/p130$^{-/-}$-MEFs fail to arrest in response to serum withdrawal and p16^{INK4a}

overexpression (Hurford et al. 1997). Finally, triple-knockout (TKO) MEFs completely fail to respond to any growth arrest signals, including DNA-damage and $p27^{Kip1}$ overexpression, and do not undergo senescence (Dannenberg et al. 2000; Sage et al. 2000). These data indicate that Rb-family members are critical for normal cell cycle regulation.

Interesting clues to the functions of individual E2F and Rb-family members stem from crosses of KO-mice heterozygous or homozygous for distinct E2F- and Rb-family proteins. For example, loss of E2F1 rescues, at least in part, from excessive proliferation and apoptosis found in $Rb^{-/-}$-mice (Tsai et al. 1998). Moreover, tumorigenesis in $Rb^{+/-}$-mice is reduced, when E2F1 is lost simultaneously (Pan et al. 1998). These data suggest growth-promoting roles for E2F1 *in vivo*, in addition to the growth inhibitory roles observed in $E2F1^{-/-}$-mice. Similarly, loss of E2F3 reduces excessive apoptosis and proliferation in $Rb^{-/-}$-mice, further proving the importance of E2F3 for proliferation (Ziebold et al. 2001). Interestingly, E2F4 loss also suppresses tumorigenesis, inappropriate gene expression, and proliferation in $Rb^{-/-}$-mice (Lee et al. 2002). Biochemical analyses suggest that this tumor suppression occurs via a novel mechanism: E2F4 loss allows p107 and p130 to regulate the normally Rb-regulated E2F1, -2, and -3. All these data argue in favor of a model, whereby Rb specifically regulates activating E2Fs, who contribute towards a pool of total E2F activity that must exceed a certain threshold to sustain proliferation. In contrast, E2F4 and -5 are regulated by p107 and p130, and are more critical for induction of quiescence. The fact that components of the Rb/E2F regulatory cascade are often mutated in tumors substantiates the relevance of this pathway in regulation of proliferation *in vivo*.

3.2.4. E2Fs and Rb-Family Proteins Modify Chromatin Structure

As described above, Rb and E2F proteins act to regulate transcription of E2F target genes. The molecular mechanisms underlying this regulation have only recently started to be understood. E2F-dependent transactivation may involve a variety of other transcription factors that have been shown to interact and/or cooperate with E2Fs, such as YY1 (Schlisio et al. 2002), and TBP (Hagemeier et al. 1993). Moreover, TRRAP and Gcn5/PCAF (Martinez-

Balbas et al. 2000; Marzio et al. 2000) as well as p300/CBP (Trouche et al. 1996; Trouche and Kouzarides 1996), also interact with E2F, making histone acetylation a likely participant in E2F-dependent transactivation.

Downregulation of transcription by E2F has been studied in-depth in the past few years (reviewed in Harbour and Dean 2000a). Importantly, inhibition of activation and active repression are biochemically distinct (Ross et al. 1999; Ross et al. 2001). The former appears to block interactions of E2F with activators, such as TBP (Hagemeier et al. 1993). The latter apparently involves several classes of chromatin-remodeling-enzymes. First, Rb, p107, and p130 all interact with Brg1/hBrm, the enzymatic subunits of the BAF complex (see above; Dunaief et al. 1994; Strober et al. 1996; Trouche et al. 1997). Growth arrest induced by Brg1/hBrm depends on functional Rb, and conversely, Brg1 is required for Rb to arrest some cell lines (Strobeck et al. 2000; Zhang et al. 2000). Thus, chromatin-remodeling by the BAF complex appears to be an important part of Rb-dependent repression of E2F targets in G0 and G1.

Rb also interacts with HDAC1, 2, and 3, and p107 and p130 bind HDACs as well (Brehm et al. 1998; Kuo and Allis 1998; Magnaghi-Jaulin et al. 1998). Recruitment of HDAC can reverse histone acetylation at E2F targets, and this correlates with transcriptional repression (Zhang et al. 1999; Zhang et al. 2000). Furthermore, the presence of repressive E2F4/p130/HDAC-complexes in quiescent cells correlates with hypoacetylated histone H3 and H4 on several E2F targets (Ferreira et al. 2001; Lai et al. 2001; Rayman et al. 2002). Thus, deacetylation of histones in E2F target gene promoters is an important role of Rb-family proteins.

Cyclin/CDK-dependent phosphorylation of Rb-family proteins affects their interactions with corepressors (Harbour et al. 1999). Specifically, Cyclin D/CDK4 activity disrupts the Rb/HDAC interaction in early G1, while Cyclin E/CDK2 inactivates Brg1/Rb complexes (Zhang et al. 2000). The interaction of Rb with HDACs depends on the LxCxE motif found in many Rb interacting proteins (Dahiya et al. 2000; Zhang et al. 2000). Even though it is unclear whether the LxCxE motif in HDACs is sufficient for Rb interaction, the binding of HDAC is apparently essential for Rb to exert its growth inhibitory functions. In contrast, the Brg1/Rb interaction occurs independently of the LxCxE motif,

which allows formation of a ternary Brg1/Rb/HDAC complexes. Therefore, the question arises of whether, or how, these two different machineries interact to induce Rb-dependent repression of E2F targets. Recent data suggest that Brg1 is required for Rb/HDAC dependent repression of the *ccne1* gene. In contrast, Rb/Brg1 is apparently sufficient to repress *cdc2* and *ccna1*, even after loss of HDAC from the complex in early G1. From these results a model has been suggested, whereby Cyclin D/CDK4 disrupts HDAC/Rb interaction, leading to increased expression of Cyclin E1, which then causes phosphorylation and inactivation of the remaining Rb/Brg1 complexes, leading to transcription of other E2F target genes, such as *cdc2*. Another level of regulation is provided by a distinct Rb-containing repressor complex, which contains the PcG-proteins HPC2, Ring1, and CtBP (Dahiya et al. 2001). This complex specifically represses *cdc2* and *ccna1*, but not *ccne1*, and apparently imposes cell cycle arrest in G2/M. In summary, individual target genes may exhibit different requirements for distinct corepressor and Cyclin/CDK complexes, and these requirements may ultimately lead to ordered, sequential gene expression of E2F targets (Harbour and Dean 2000a; Harbour and Dean 2000c).

Rb has also been shown to recruit a complex of SUV39H1 and HP1α to the *ccne1* promoter (Nielsen et al. 2001). Interestingly, these two proteins induce formation of transcriptionally silent heterochromatin (reviewed in Eissenberg and Elgin 2000; Dillon and Festenstein 2002; Peters et al. 2002). These data have led to a model, in which transcriptionally active targets are initially repressed by deacetylation, and then subjected to more permanent silencing by methylation of H3K9, which is followed by binding of HP1α and formation of heterochromatin. Another corepressor of the Rb/E2F is DNMT1, which co-purifies with Rb, E2F1, and HDAC1 (Robertson et al. 2000). These data suggest that DNA methylation is also important for silencing by Rb/E2F.

E2F6 is present in cells as part of at least two distinct multi-protein complexes. One contains two different HMTs, the Mga/Max dimer, and HP1γ (see chapter 3.3. pp. 44; Ogawa et al. 2002). CHIP analysis showed that this complex is present on four E2F targets in quiescent cells. The other complex contains several Polycomb-group proteins, including RYBP, Bmi1,

and Ring1 (Trimarchi et al. 2001), all of which have been associated with transcriptional repression. Therefore, E2F6 may repress transcription through Rb-independent chromatin-remodeling events at E2F targets.

3.2.5. DNA Tumor Viruses Target the Rb Pathway

Many DNA tumor viruses encode proteins that inactivate Rb-family proteins. These include adenovirus E1A, papilloma-virus E7, and SV40 large T antigen. For example, E1A contains the LxCxE motif found in many Rb binding proteins, and thus can disrupt interactions between Rb and corepressors, enabling derepression of E2F genes, and cell cycle entry (reviewed in Ben-Israel and Kleinberger 2002). Interestingly, while targeting of the Rb/E2F-pathway is essential for E1A to transform cells, E1A requires additional functions to overcome growth arrest (Alevizopoulos et al. 1998). It is noteworthy that the domain required for these biological activities allows association of E1A with TRRAP (Deleu et al. 2001). Furthermore, TRRAP and the TRRAP-containing p400 complex are targets necessary for transformation by E1A (Fuchs et al. 2001). The p400 complex is highly similar to the Tip60 complex and also contains TRRAP (but not Tip60). Finally, E1A also binds PCAF, and hence may target the other of the two TRRAP-containing HAT-complexes (Chakravarti et al. 1999; Hamamori et al. 1999a). Thus, it appears that TRRAP, and presumably TRRAP-associated HAT-complexes described above, are important targets of E1A in cellular transformation. In summary, E1A may liberate E2F activity by both relieving Rb-mediated repression, and by targeting cofactors of E2F.

3.3. The Myc/Max/Mad Network of Transcription Factors

The Myc-family of transcription factors is composed of three different genes, *L-myc*, *N-myc*, and *c-myc* (reviewed in Luscher 2001). While the former two are expressed in tissue or development specific fashion in mice and humans, Myc, the product of the *c-myc* gene, is ubiquitously expressed in proliferating cells. Myc is extremely unstable, and is subject to ubiquitin/proteasome-dependent degradation (Gross-Mesilaty et al. 1998; Salghetti et al. 1999; Sears et al. 1999). mRNA and protein are induced rapidly following mitogenic stimulation, and Myc levels and activity peak in

early G1 (reviewed in Kelly and Siebenlist 1986; Luscher and Eisenman 1990). Biological roles of Myc include induction of cell growth, proliferation, apoptosis, and suppression of terminal differentiation (reviewed in Cole and McMahon 1999; Dang et al. 1999; Lutterbach and Hann 1999; Eisenman 2000; Grandori et al. 2000). Furthermore, all members of the Myc-family are oncogenes, and deregulation of the *c-myc* gene is common in a variety of cancers, for example lymphomas and leukemias. Similarly, *N-myc* is commonly deregulated tumors such as neuroblastoma (reviewed in Boxer and Dang 2001; Hoffman et al. 2002; Lutz et al. 2002).

Myc is a member of the basic helix-loop-helix leucine-zipper (bHLH-LZ) family of transcription factors (reviewed in Grandori et al. 2000; Amati et al. 2001; Eisenman 2001; Luscher 2001). In addition to an N-terminal transactivation domain (TAD), it contains the C-terminal bHLH-LZ, which is responsible for DNA-binding and dimerization with its obligatory partner Max. Myc/Max dimers bind the E-box (CACGTG), as well as non-canonical sites, and activate transcription of a variety of target genes. These include genes involved in cell cycle regulation (for example *e2f1, ccnb1, ccnd2, ccne1, and cdk4*), apoptosis (several caspases, *apaf1*, *pig8*), ribosomal metabolism (Nucleolin, Nucleophosmin, and several ribosomal proteins), protein synthesis and folding (*eIF4F*, *eIF5A*, several HSP chaperones), basic metabolic pathways (*ldh*, and several enzymes of the glycolytic pathway), and others (Grandori et al. 1996; Coller et al. 2000; O'Hagan et al. 2000; Boon et al. 2001; Schuhmacher et al. 2001; Schuldiner and Benvenisty 2001; Menssen and Hermeking 2002; Schuldiner et al. 2002; Watson et al. 2002). Two conserved domains in the TAD of Myc, termed Myc-boxes 1 and 2 (Mb1 and Mb2) appear to be critical for transcriptional activation. They constitute interaction surfaces for a variety of proteins, including TRRAP (McMahon et al. 1998). It was speculated that Myc might induce histone acetylation of target genes, a hypothesis confirmed by our laboratory and others (Bouchard et al. 2001; Frank et al. 2001; Xu et al. 2001a). Another report demonstrated that Myc is capable of interacting with Snf5, a subunit of the BAF complex (Cheng et al. 1999). Furthermore, Myc associates with a variety of other known transcription factors and cofactors, such as p107, YY1, and Nmi1 (reviewed in Sakamuro and Prendergast 1999). Finally, at least *in vitro* Myc

can also bind TBP (Hateboer et al. 1993; McEwan et al. 1996), and might therefore directly recruit the core transcriptional machinery (Roy et al. 1993).

Interestingly, Myc can also repress transcription, through interaction with the Miz1 protein (Peukert et al. 1997; Schneider et al. 1997; Seoane et al. 2001; Staller et al. 2001). Miz1 activates transcription of target gene promoters containing an initiator (Inr) element. Myc binding to Miz1 leads to repression of this transactivation, presumably by inhibiting recruitment of coactivators such as p300. One of the key targets of Miz1 is the *cdkn2b* gene (encoding $p15^{INK4b}$), and suppression of *cdkn2b* transactivation is a key contribution of Myc-induced proliferation. Similarly, Myc represses Miz1-dependent activation of the *cip1/waf1* gene (encoding $p21^{Cip1}$) following DNA-damage (Seoane et al. 2002). In summary, Myc can activate and repress transcription, depending on the promoter context.

Opposing the transactivating and proliferative effects of Myc are Mad family members (reviewed in Eisenman 2000; Grandori et al. 2000; Baudino and Cleveland 2001; Luscher 2001). These include the Mad1, Mxi1 (Mad2), Mad3, and Mad4 proteins. Mad protein levels are upregulated following induction of differentiation. When overexpressed, Mad proteins inhibit proliferation, apoptosis, and transformation induced by Myc, and other factors. Like Myc, Mad is a bHLH-LZ protein, but contains an N-terminal Sin3 interaction domain (SID), instead of a TAD. As a consequence Mad/Max dimers repress target genes containing E-boxes by recruiting HDAC/Sin3 complexes to deacetylate promoters (see above; Ayer et al. 1995; Schreiber-Agus and DePinho 1998; Xu et al. 2001a). Another Myc related protein is Mnt, which is constitutively expressed, but whose role in regulation of transcription and proliferation is unclear (Hurlin et al. 1997; Meroni et al. 1997). Mga is yet another Myc-related transcription factor (Hurlin et al. 1999). It presumably acts in concert with E2F6 to repress transcription of target genes containing adjacent E2F-sites and E-boxes, and may induce cell cycle arrest (see above; Ogawa et al. 2002). In summary, switching from Mad/Max to Myc/Max is a key step in the induction of growth and proliferation in metazoans.

AIM OF THIS WORK

In recent years, the mechanisms of transcriptional repression by both E2F/Rb and Max/Mad have become understood in detail, in particular with the demonstration that HDACs act as corepressors of both pathways. However, the molecular pathways involved in activation by E2F and Myc still remain very much obscure. Recent data showed that chromatin-remodeling-complexes, in particular HATs, are involved in many transactivation pathways. Consistent with this, both E2F1 and Myc can associate with TRRAP, a subunit of two distinct HAT-complexes (the Tip60 complex, and STAGA/TFTC/PCAF complex). Nevertheless, at the onset of my thesis it was unclear whether this interaction had any functional consequences. Thus, I set out to analyze the biochemical and molecular roles of HATs, in particular TRRAP-associated complexes, in transactivation by E2F1 and Myc. Understanding how these two proteins regulate transcription will help to understand their biological functions, and ultimately their role in normal and transformed cells.

RESULTS

4. E2F Recruits the Tip60 Complex to Chromatin

4.1. Summary

TRRAP has been shown to associate with both E2F1 and c-Myc (McMahon et al. 1998). Importantly, it is required for cellular transformation induced by both c-Myc and E1A, the latter acting by inducing E2F activity. Since TRRAP is also a subunit of at least two different HAT-complexes, it was plausible that some of these complexes are involved in transactivation by E2F of Myc proteins.

In this chapter, I show that several subunits of the Tip60 complex, including TRRAP, are recruited to E2F targets following serum stimulation. E2F1 is sufficient to induce recruitment of these proteins, and acetylation of E2F target genes. Using a mutant E2F1, I demonstrate that functional activating E2F is required for association of the Tip60 complex with E2F targets, acetylation of histone H4, and entry of a new cell cycle. Moreover, H4 acetylation and Tip60 recruitment are common features of E2F targets.

4.2. Synchronization of T98G Cells

Selection of an appropriate cell system is crucial for studies of E2F, which is an intrinsic component of the cell cycle machinery. The glioblastoma cell line T98G has been successfully used to investigate E2F biology (for example Takahashi et al. 2000). These cells have retained growth arrest mechanisms characteristic of primary cells: They enter quiescence upon serum withdrawal, and re-enter the cell cycle when stimulated with serum. To achieve synchronous passage through the G1 and S-Phases, cells were allowed to grow to confluence, and then starved in serum free medium. After 48 hours, cells were passaged into fresh, serum containing medium. Cell cycle analysis was subsequently performed at different time-points by means of two-dimensional flow cytometry (Figure 5 A). Briefly, cells were labeled with 33μM BrdU for 30 minutes before harvesting at each time-point. Subsequently, nuclei were isolated, permeabilized, and stained with a monoclonal anti-BrdU antibody, as well as with propidium iodide (PI) dye, which stains DNA. Thus,

BrdU/PI double positive cells could be identified as S-Phase cells. T98G cells arrest in G0 when exposed to the serum deprivation/contact inhibition treatment (Figure 5 B). S-Phase commenced approximately 15 hours after release from quiescence, and peaked around 18 to 21 hours. Importantly, passage of the population through G1/S was highly synchronous, confirming the utility of T98G for E2F related cell cycle studies. A quantitation of the panels is shown in Figure 5 C.

4.3. Quantitative Chromatin Immunoprecipitation of E2F1: Binding of E2F1 to Targets

To study the function of different E2F proteins, I used chromatin immunoprecipitation (CHIP; Orlando et al. 1997). This technique allows analyzing the DNA-binding activities of different E2Fs in live cells at a given point in the cell cycle, and at multiple target genes. Here, I used a protocol for quantitative CHIP previously developed in our laboratory (Figure 6; Frank et al. 2001). Briefly, live cells were crosslinked with formaldehyde, and chromatin was subsequently sheared by sonication. Following SDS denaturation, protein-DNA complexes were immunoprecipitated with the antibody of choice (Figure 6 A). After extensive washes the crosslinks were reversed, the proteins digested, and the DNA purified by phenol/chloroform extraction and ethanol precipitation. Recovery of DNA was analyzed by real-time PCR. CHIP readout was expressed as the fraction of total input chromatin recovered in each immunoprecipitation (% total). Figure 6 B shows typical amplification curves obtained with primers specific for the E2F site in the *p107* promoter of an E2F1 CHIP from serum-stimulated T98G cells. To ensure accuracy of the technique, each CHIP was performed in triplicate within each experiment. Thus, single data points represent average and standard deviation of three independent IPs.

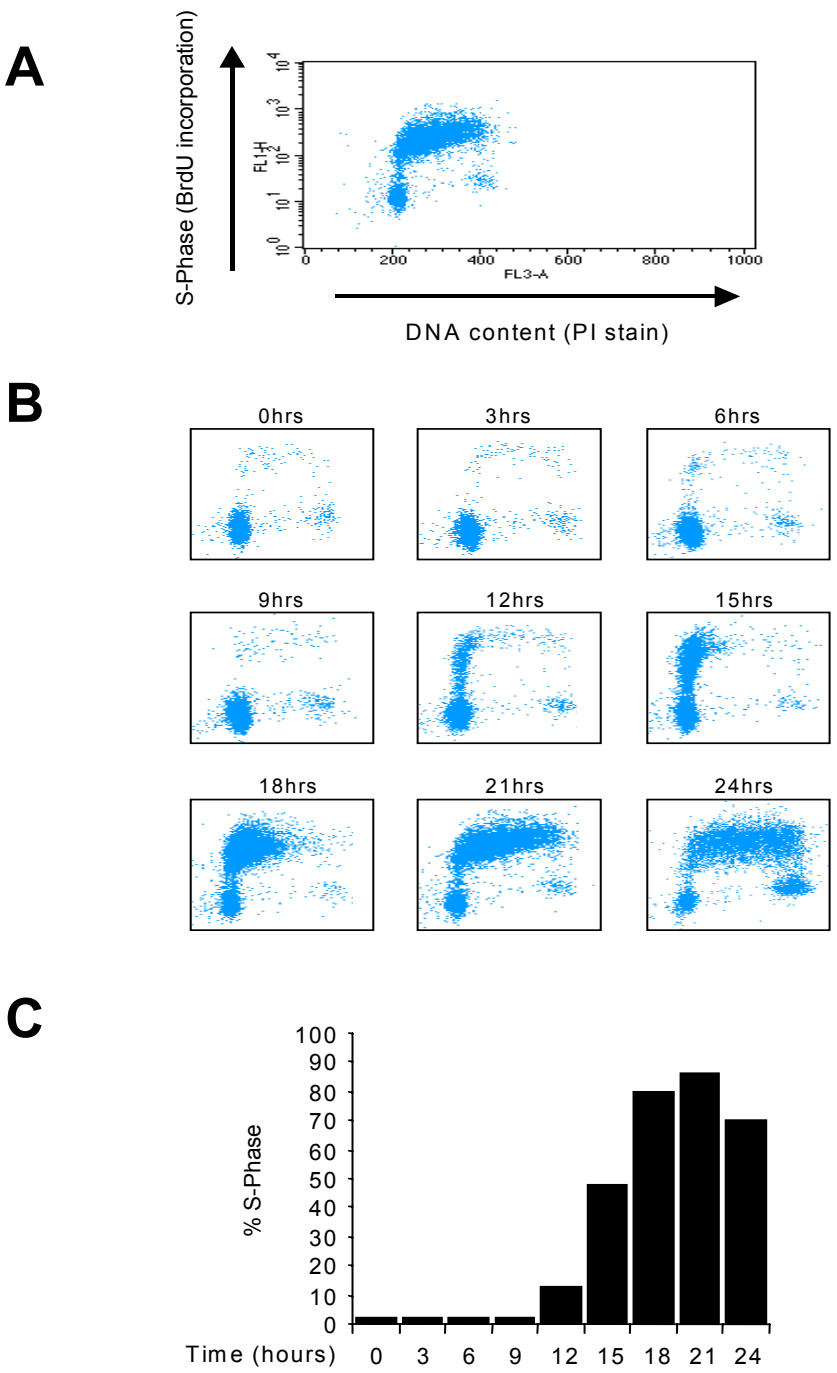

Figure 5: Cell Cycle Synchronization of T98G Cells.
T98G cells were rendered quiescent by serum starvation and contact inhibition, then stimulated with serum for the indicated lengths of time, and subsequently analyzed by two-dimensional flow cytometry. (A) Cells in S-Phase were detected as PI/BrdU double positive cells. (B) Cells synchronously pass through a new cell cycle after release from quiescence. (C) Quantitation of the same experiment is shown in. One of two independent experiments is shown.

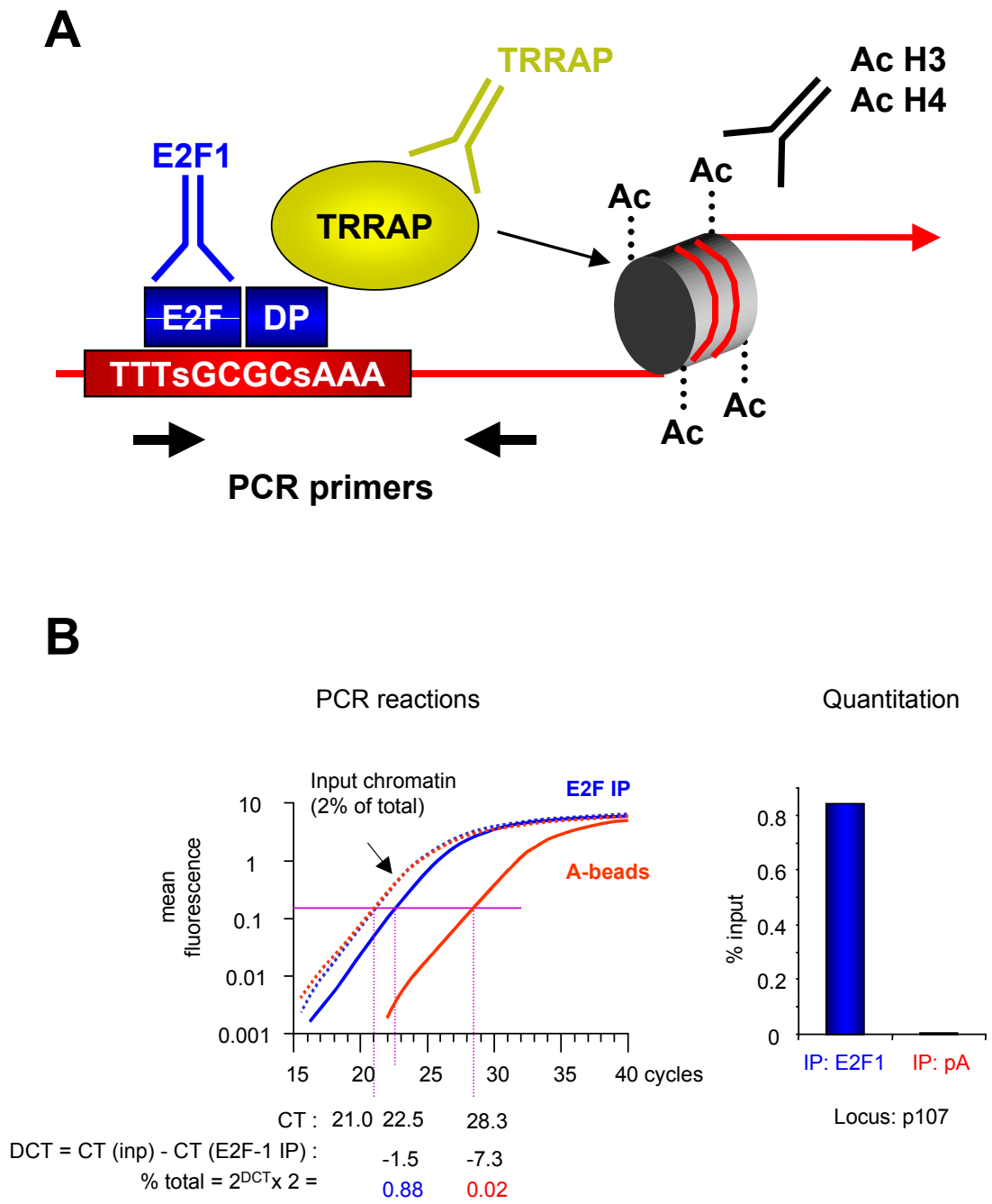

Figure 6: Quantitative Chromatin Immunoprecipitation (CHIP).
(A) Schematic representation of CHIP. IPs were performed with antibody of choice, washed, the DNA purified and analyzed with primers against the known DNA sequences. (B) Representative PCR amplification curves as obtained from the Taqman 5700 real-time PCR amplifier. In this example, primers specific for the E2F binding site in the *p107* promoter were used. Calculation of the amount of precipitated DNA relative to that present in total input chromatin is shown in the right panel. CT, cycle threshold, cycle number at which each reaction reaches a predetermined fluorescence threshold, set within the linear range of all reactions.

E2F proteins regulate a plethora of target genes, including many essential for S-Phase initiation and progression (see chapter 3.1. pp. 29). For a preliminary analysis, a subset of well-characterized target genes was chosen, consisting of *mcm3*, *mcm4*, *pcna*, and *p107*. I designed primers flanking known or putative consensus E2F binding sites in their promoters, as well as control primers for the *achr* promoter (that does not contain an E2F binding site). Binding of E2F1 to chromatin was analyzed in T98G cells that had been starved and released into a new cell cycle, as described above. E2F1 associated with all four targets, but not with the *achr* promoter (Figure 7 A). In every case, binding was minimal in quiescent cells (0 hours), peaked at 15 hours, and declined thereafter. This correlates well with the regulatory role of E2F1 in the G1/S transition. In order to further analyze the correlation of E2F1 binding and S-Phase entry, additional time-points were investigated by CHIP. Figure 7 B shows that maximal binding of E2F1 to the *mcm4* promoter immediately preceded S-Phase. To confirm the specificity of the CHIP assay, IPs were performed without antibodies. These nonimmune control CHIPs recovered very low levels of DNA, independently of the cell cycle stage (approximately 0.01% total for all investigated targets; Figure 7 C). Interestingly, E2F1 signals in quiescent cells were significantly higher than background (approximately 0.1% total), arguing that residual E2F1 is present on promoters in arrested cells. In conclusion, E2F1 is induced following serum stimulation of T98G cells and binds to E2F sites in the regulatory regions of four target genes.

Binding of transcription factors should be spatially limited to their consensus target sites. Thus, E2F-specific CHIP should exclusively recover DNA fragments containing E2F binding sites. To address this hypothesis, primers amplifying sequences upstream and downstream of E2F binding sites in each gene were designed (subsequently called walking primers). For convenience, only DNA from the 0- and 15-hour E2F1 CHIP was analyzed. Figure 7 D shows that recovery of amplicons upstream and downstream of the E2F sites strongly decreased as a function of distance from the E2F binding site. Thus, E2F1 localized to the predicted sites.

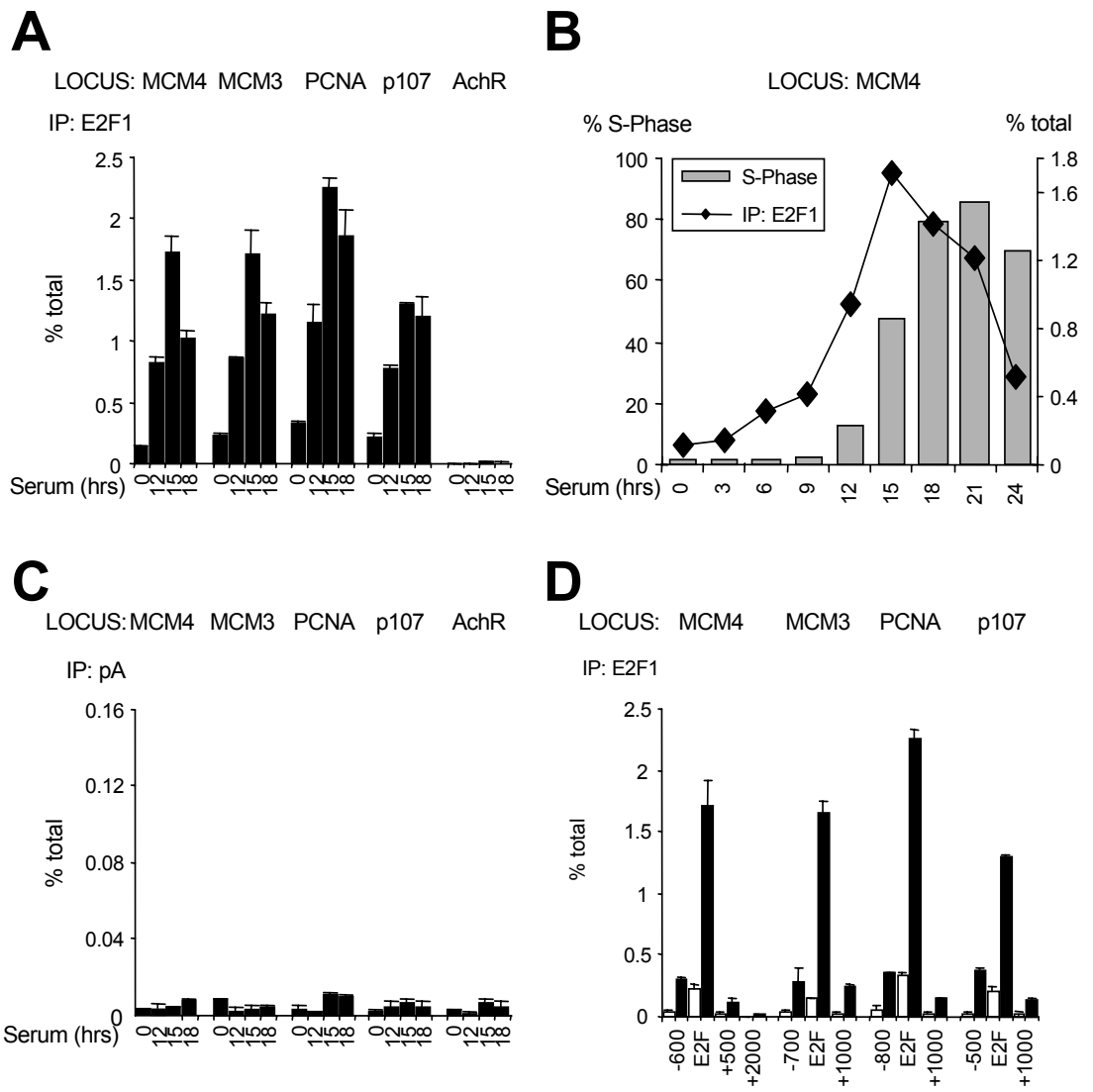

Figure 7: E2F1 Recruitment to Chromatin in T98G Cells.
(A) E2F1 binds to four target gene promoters in T98G cells stimulated with serum for the indicated time (in hours, on the x-axis). Binding is expressed as percentage of total input chromatin on the y-axis. No E2F1 is detected on the acetylcholine receptor. (B) Binding of E2F1 to the *mcm4* promoter was monitored, and every sample was also analyzed by two-dimensional flow cytometry. Binding of E2F1 (black line) peaks just prior to S-Phase entry (quantitated in grey bars). (C) Control CHIPs performed without antibody (only Protein A) fail to enrich for E2F specific targets at any time after serum stimulation. (D) Mapping of E2F1 binding along the different loci in quiescent cells (white bars), and cells at G1/S transition (15 hours; black bars). Numbers on the x-axis indicate the distance in bp from the E2F site. E2F = E2F binding site.

4.4. Binding Patterns of Other E2F-Family Members

Since several E2F proteins presumably have redundant functions (see chapter 3.2.1. and 3.2.2. pp. 35), it was important to address the binding patterns of other E2F-family members to chromatin during the serum

response of T98G cells. Thus, CHIP experiments were performed to investigate recruitment of E2F2, E2F3, and E2F4. Whereas E2F3 and E2F4 CHIPs yielded strong signals, recovery of DNA from E2F2 CHIPs was less efficient, a result that is consistent with previously published reports (Takahashi et al. 2000; Wells et al. 2000). This is possibly due to antibody inefficiency. Alternatively, E2F2 levels could be lower than E2F1 and E2F3 levels in T98G cells.

Both E2F2 and E2F3 exhibited similar, though not identical binding characteristics as those observed for E2F1 (Figures 8 A-B, compare with Figure 7 A). As seen for E2F1, E2F2 was virtually absent from target promoters in quiescent cells. After recruitment in G1 promoter binding peaked prior to S-Phase, and then decreased. E2F3, in contrast, was present in significant amounts on all promoters in quiescent cell populations. Even more DNA was recovered in E2F3 CHIPs from late G1 populations, although the increase was not as dramatic as for E2F1 or E2F2. It should be noted, however, that two differently spliced E2F3 proteins have been described, which the antibody used in this study does not distinguish. Thus, the recruitment of each of these would have to be assessed by isoform specific antibodies. In summary, activating E2Fs are mostly absent from target gene promoters in quiescent cells, but binding increased following serum stimulation.

CHIP with antibodies directed against E2F4 (a repressive E2F) showed occupancy at all four promoters in quiescent cells, but a weaker recovery from cells that were about to undergo S-Phase entry, as expected (Figure 8 C). Similarly p130, a Rb-family member was present on all targets in G0 cells but its signals are strongly reduced in G1/S-Phase cells (data not shown; see Figure 18 F). However, E2F4 was not completely absent from promoters in S-Phase. The remaining signal could originate from E2F4/p107 complexes, which have been previously described in cells undergoing DNA replication.

To verify localization patterns of E2F2, -3, and -4, the 0 and 15 hours samples were analyzed using the same walking primers as above. Again, binding of all the E2F proteins was limited to the E2F site as the signals amplified using up- or downstream amplicons were significantly weaker (Figures 8 D-F). In summary, the response of T98G cells to serum stimulation

exhibits a switch from repressive E2F4/p130 to activating E2F1, E2F2 and E2F3, which fits well with previously published reports (Takahashi et al. 2000; Wells et al. 2000).

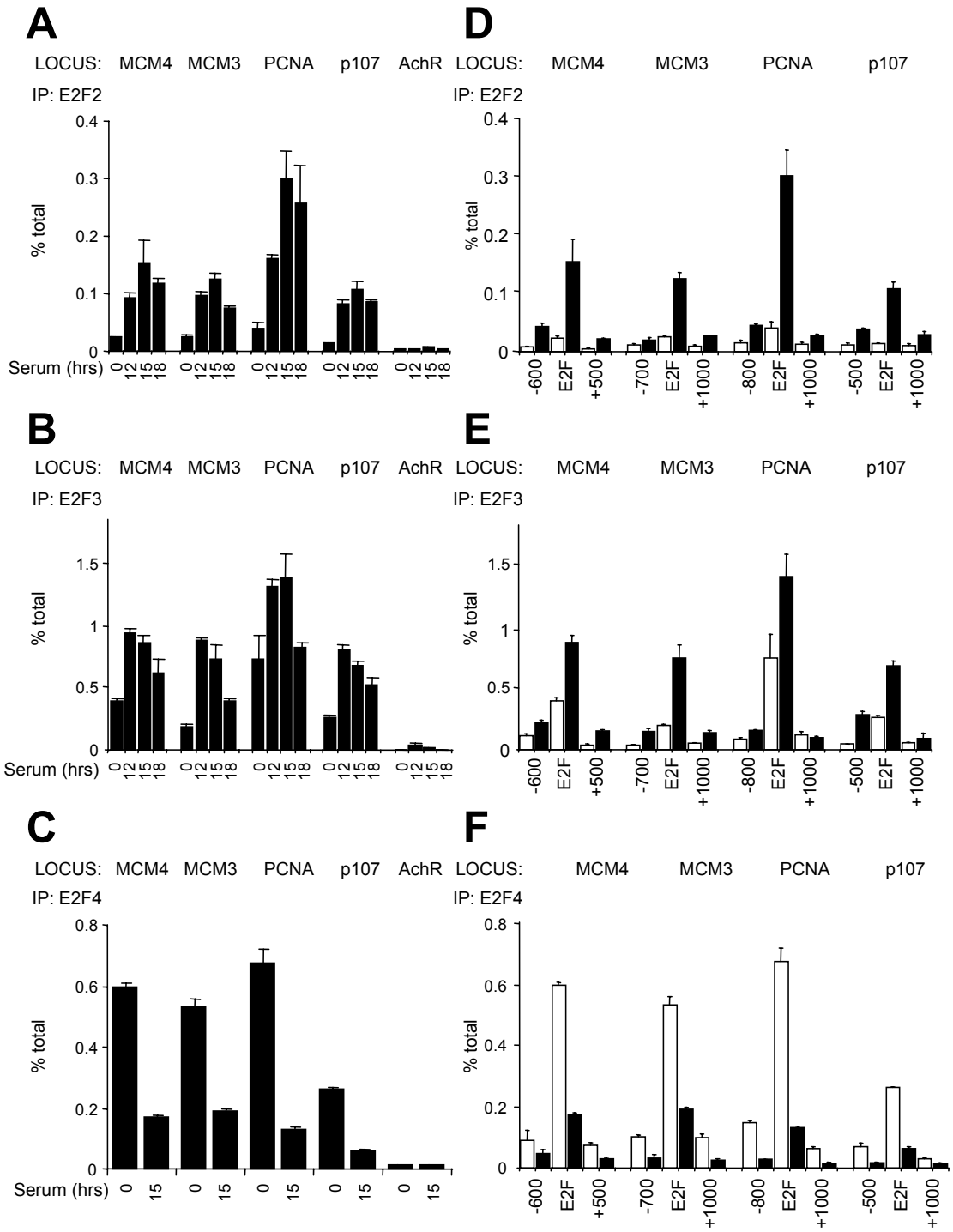

Figure 8: Specific Association of E2F-Family Members with E2F Targets.
CHIP was performed with antibodies for E2F2 (A, D), -3 (B, E), and -4 (C, F) in T98G cells. Temporal (A, B, C) and spatial (D, E, F) distributions of association with the indicated target loci are shown. Cell cycle populations and amplicons are identical to those used in Figure 7.

4.5. Acetylation of E2F Targets Temporally and Spatially Correlates with Recruitment of Activating E2F Proteins

Histone acetylation is regulated by many transcription factors, and both HATs and HDACs have been shown to interact with E2Fs (reviewed in Trimarchi and Lees 2002). Thus, I decided to verify if E2F targets undergo regulated histone acetylation at G1/S transition, when they are being actively transcribed. CHIP was performed with antibodies specifically recognizing acetylated H3 and H4. Both histones were hypoacetylated in quiescent T98G cells (Figure 9 A-B; 0hrs). As cells progressed through the cell cycle hyperacetylation occurred, and both H3 and H4 acetylation peaked at 12 to 15 hours. While this was a little earlier than maximal E2F1 binding, it should be noted that significant E2F1 (and E2F2 and E2F3) is bound at 12 hours, and this may be sufficient to induce acetylation. Furthermore, E2F3 binding peaked at 12 hours on all promoters and could contribute to acetylation. After 15 hours, acetylation decreased on all loci. Thus, the timing of maximal histone acetylation at E2F target promoters correlated well with the binding kinetics of activating E2F proteins.

If activating E2Fs were indeed responsible for histone acetylation, a spatial correlation should be expected as well. To verify this hypothesis, I used an extended set of walking primers. Interestingly, acetylation was more widespread, as most targets exhibited acetylation domains approximately 1.5 to 3kb wide (Figure 9 C-F). Nevertheless, acetylation of both histone H3 and H4 was centered to the E2F sites, which the exception of the *mcm4* gene, on which acetylation was strongest approximately 600bp upstream of the binding site. Another interesting case was the *pcna* promoter, which, while exhibiting most acetylation on the E2F site, also showed significant acetylation of upstream regions. This may be due to acetylation from other transcription factors. In summary, E2F1 binding appeared to induce hyperacetylation that reached the nucleosomes just adjacent to the E2F biding site. Since one nucleosome corresponds to approximately 146bp, acetylation probably covered not more than two or maximally three nucleosomes. Others have reported similar, narrow localization patterns of E2F-dependent responses (Morrison et al. 2002; Rayman et al. 2002). In conclusion, serum stimulation

of quiescent T98G cells induced acetylation of discrete domains of E2F target genes overlapping with and centered on the E2F binding site.

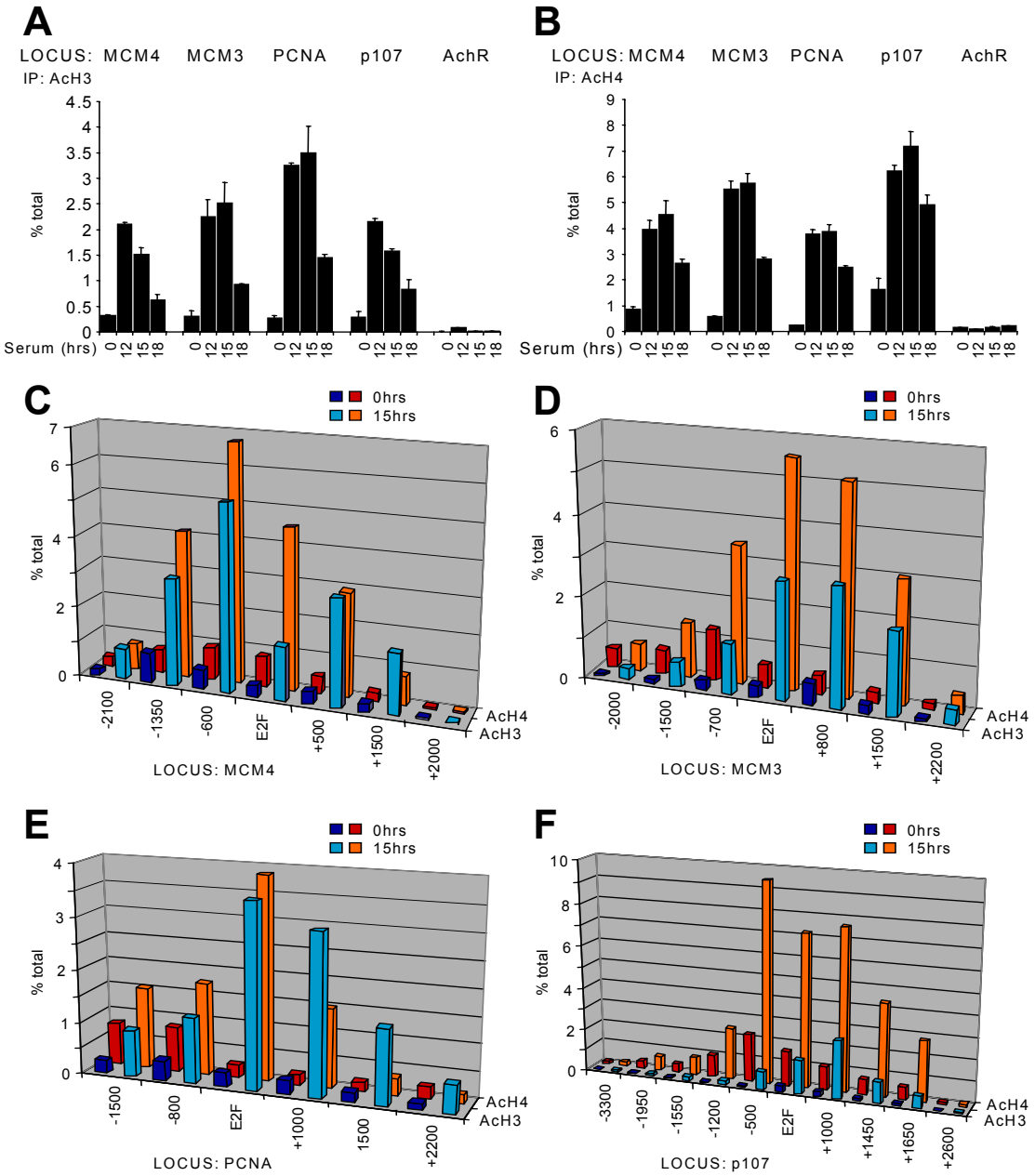

Figure 9: Induction of H3 and H4 Acetylation on E2F Targets in T98G Cells.
Acetylation of H3 and H4 at E2F targets was monitored by CHIP. Time-courses show that both H3 (A) and H4 (B) acetylation on all E2F targets is maximal prior to S-Phase (15 hours), whereas no acetylation is detected on the *achr*. Mapping of acetylation along the different loci using proximal and distal primer pairs revealed that acetylation of both H3 (dark blue: 0 hours; light blue 15 hours) and H4 (red: 0 hours; orange 15 hours) was centered to the E2F sites on *mcm4* (C), *mcm3* (D), *pcna* (E), and *p107* (F) promoters. Numbers on the x-axis indicate the distance in bp from the E2F site. E2F = E2F binding site.

4.6. The Tip60 Complex is Recruited to E2F Targets in T98G Cells

The simplest explanation for the above results is that activating E2Fs recruit HATs, which in turn acetylate nucleosomes in E2F target promoters. Supporting this hypothesis is the fact that E2F1 has been reported to bind TRRAP, which is a subunit of at least two distinct HAT-complexes. Furthermore, E2F has also been shown to bind several HATs, including the TRRAP-associated Gcn5 and PCAF (Martinez-Balbas et al. 2000; Marzio et al. 2000). Thus, I decided to investigate if subunits of HAT-complexes were recruited to E2F targets.

Antibodies to subunits of several HAT-complexes were purchased, or generated as described (see Materials and Methods, and Appendix A). TRRAP and Tip60 CHIP did enrich for E2F targets, so I analyzed recruitment of the Tip60 complex to E2F targets first.

CHIP was performed in T98G cells after serum stimulation. Both TRRAP and Tip60 were recruited to all investigated E2F targets, but not to the *achr* promoter (Figure 10 A-B). Kinetics of recruitment resembled the binding pattern of activating E2Fs: Maximal CHIP signals were obtained from populations just about to enter S-Phase, decreasing afterwards. While signals were weaker than those obtained for most E2Fs, they were reproducibly well above Protein A background (e.g. 0.1% total TRRAP on *mcm4* as opposed to 0.01% Protein A). Thus, even though the recovery of DNA was one order of magnitude lower than that obtained for E2F1 CHIP, it was significant.

To determine whether localization of the coactivators was identical to E2F1 localization, I ran the walking primers on TRRAP and Tip60 CHIPs (Figure 10 C-D). Both proteins exhibited a spatial distribution reminiscent of that detected for E2F1. Altogether, TRRAP and Tip60 were recruited to E2F targets in a spatial and temporal manner that resembled the binding pattern of activating activating E2Fs, suggesting that they are coactivators of E2F in a physiological setting.

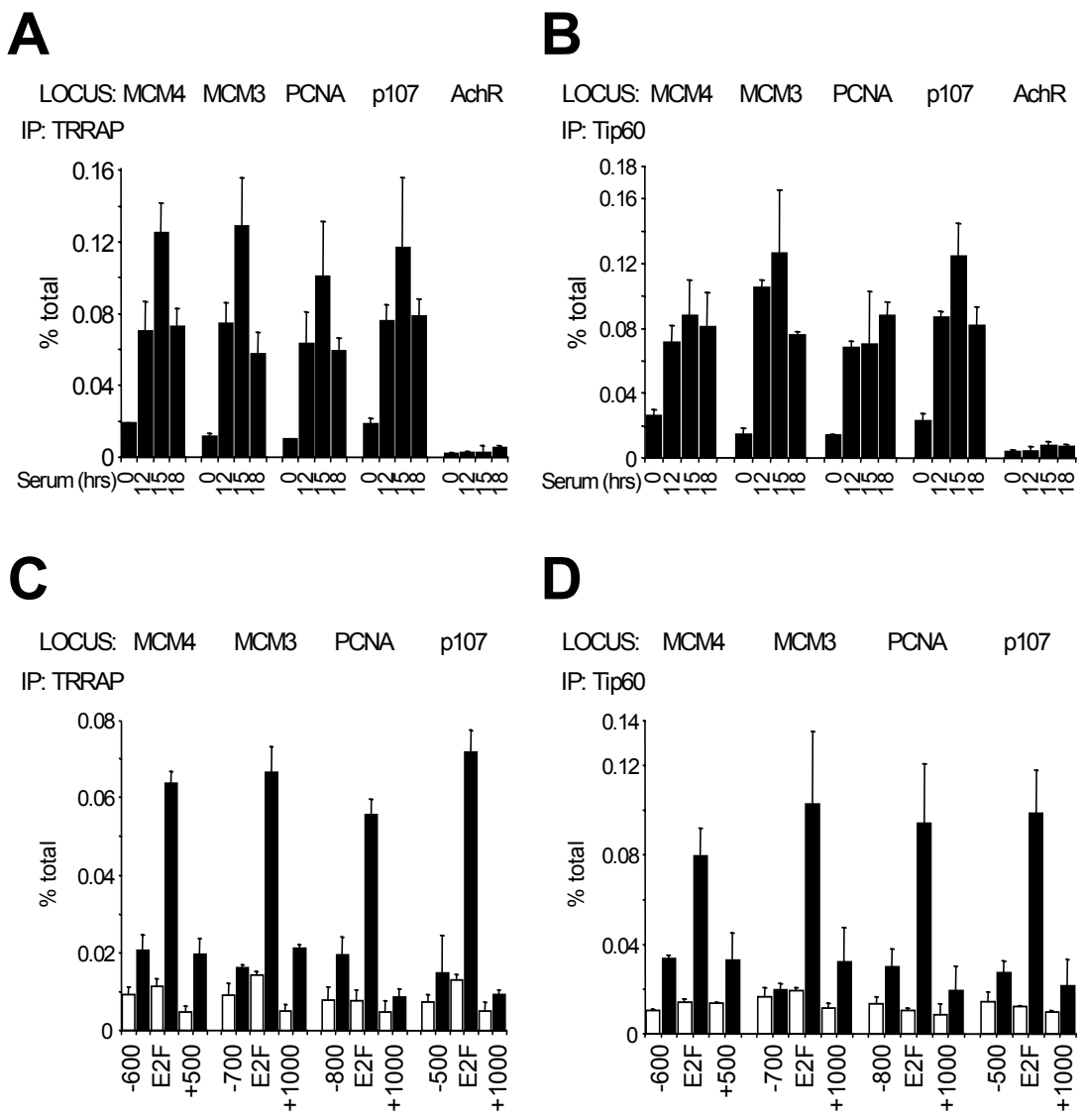

Figure 10: TRRAP and Tip60 are Recruited to E2F Targets in T98G Cells.
CHIP was performed with serum stimulated T98G cells using antibodies to TRRAP (A, C), and Tip60 (B, D). Recruitment of all proteins to E2F targets peaked just prior to S-Phase. Spatial distribution of binding was analyzed in (B) and (D) on chromatin from quiescent cells (white bars), and cells at G1/S transition (15 hours; black bars). Cell cycle populations and amplicons are identical to those used in Figure 7.

Several other subunits of the Tip60 complex have been identified (see introduction, Table 3), and of those, Tip49 has been shown to interact with E2F1 (Dugan et al. 2002). Thus, CHIPs were performed with Tip48, Tip49, and p400 antibodies. Recovery of DNA in p400 CHIPs, however, was extremely low, and no conclusive data were obtained in T98G cells (data not shown; but see Figure 16 F). Tip48 and 49 CHIP in contrast yielded strong signals. Both proteins bound to E2F targets. Induction was not as strong as

for TRRAP or Tip60, increasing only 2 to 3-fold over signals in quiescent cells (Figure 11 A-B). This was due to significant promoter binding by Tip48 and Tip49 in quiescent cells. This was probably E2F-independent recruitment, possibly by Pol_{II}, with which both proteins were recently shown to associate (M. Gstaiger, and W. Krek, unpublished results). However, regulated binding of Tip48 and Tip49 was localized to E2F consensus sites (Figure 11 C-D), as found for the other coactivators. One notable exception was *mcm4*, which additionally exhibited significant binding of both Tip48 and Tip49 in upstream regions. In summary, four subunits of the Tip60 complex are recruited specifically to E2F targets in T98G cells upon serum stimulation.

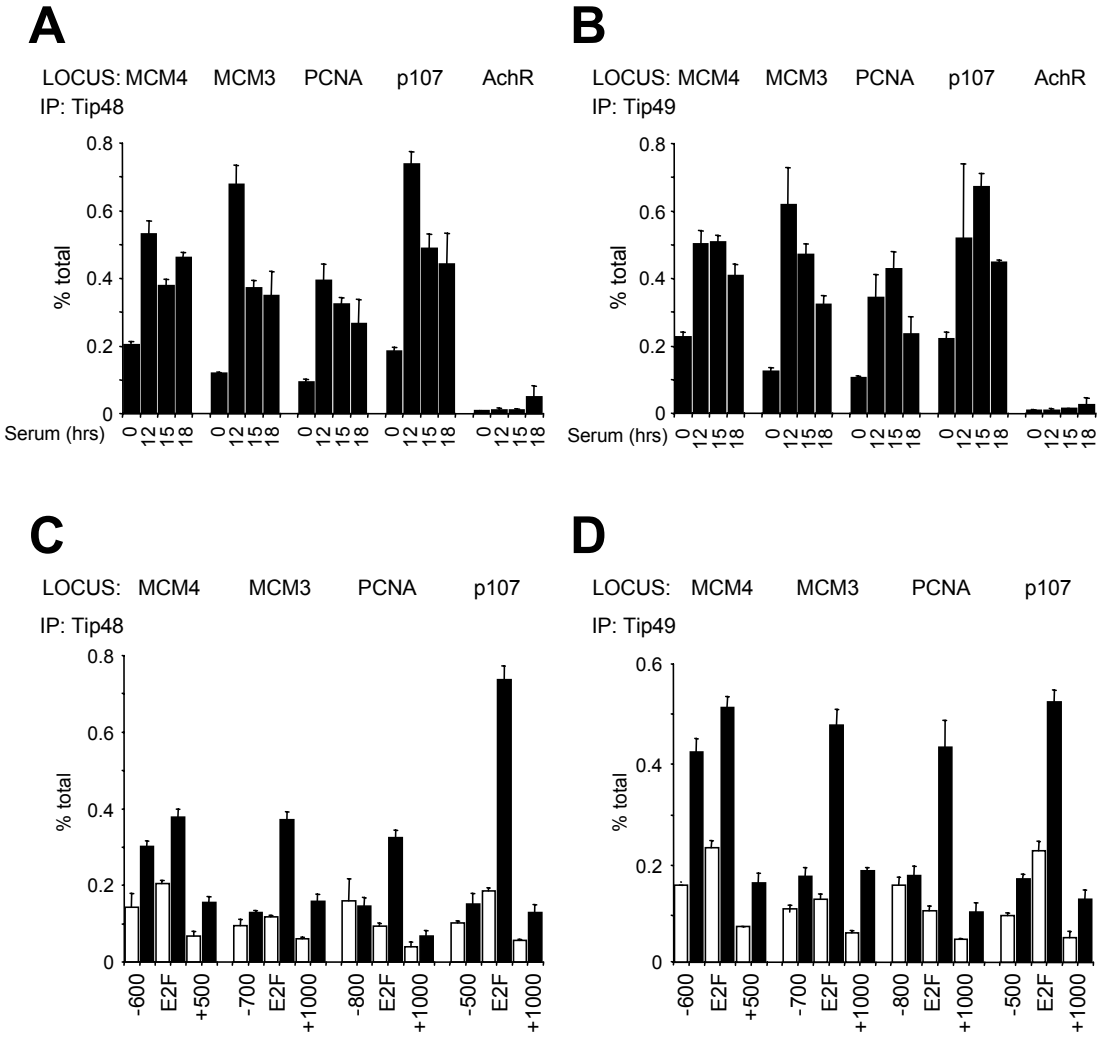

Figure 11: Tip48 and Tip49 are Recruited to E2F Targets in T98G Cells.
CHIP was performed with serum stimulated T98G cells using antibodies to Tip48 (A, C), and Tip49 (B, D). Recruitment of all proteins to E2F targets peaked before S-Phase. Spatial distribution of binding was analyzed in (B) and (D) on chromatin from quiescent cells (white bars), and cells at G1/S transition (15 hours; black bars). Cell cycle populations and amplicons are identical to those used in Figure 7.

Experiments performed with Gcn5/PCAF antibodies did not reproducibly yield significant enrichment (data not shown). Similarly, CHIP with antibodies recognizing p300 and CBP, proteins known to bind E2F1, did not reveal specific enrichment over background. Since negative CHIP data cannot be interpreted conclusively in the absence of a positive control, this does not exclude that these proteins function in transactivation of E2F targets. Further experiments will be required to address this question.

4.7. Tip60 Recruitment and H4 Acetylation are Features of Many E2F Targets

So far, I showed that the Tip60 complex is involved in E2F responses to serum stimulation of T98G cells. However, the subset of targets has been limited to four genes involved in G1/S-Phase transition. Recent studies have revealed that E2F proteins target hundreds of genes involved in several different cellular processes, including DNA replication, DNA repair, apoptosis, and others (see introduction). Therefore, I decided to conduct a survey of other E2F target genes. Primers against 30 known targets were designed and tested. Subsequently, 0 and 15 hours CHIP samples from T98G serum stimulation time-courses were analyzed. All promoters exhibited significant E2F1 binding, as was expected (Figure 12 A). H4 acetylation was also induced, but there appeared to be no direct correlation of signal strength of E2F1 binding and induction acetylation (Figure 12 B). Even though many genes exhibited significant Tip60 binding, some (such as *b-myb, polα2, rrm1,* and *p73*) showed weak recruitment that might not be sufficient to explain the strong induction of acetylation. *36B4* exhibited significant Tip60 recruitment and induced H4 acetylation, but probably independent of E2F1 binding, which was only weakly present on this promoter. In summary, while Tip60 was recruited to most E2F targets, other HATs probably also contribute towards total acetylation. A combination of CHIP experiments and molecular genetic approaches will be required to address this issue.

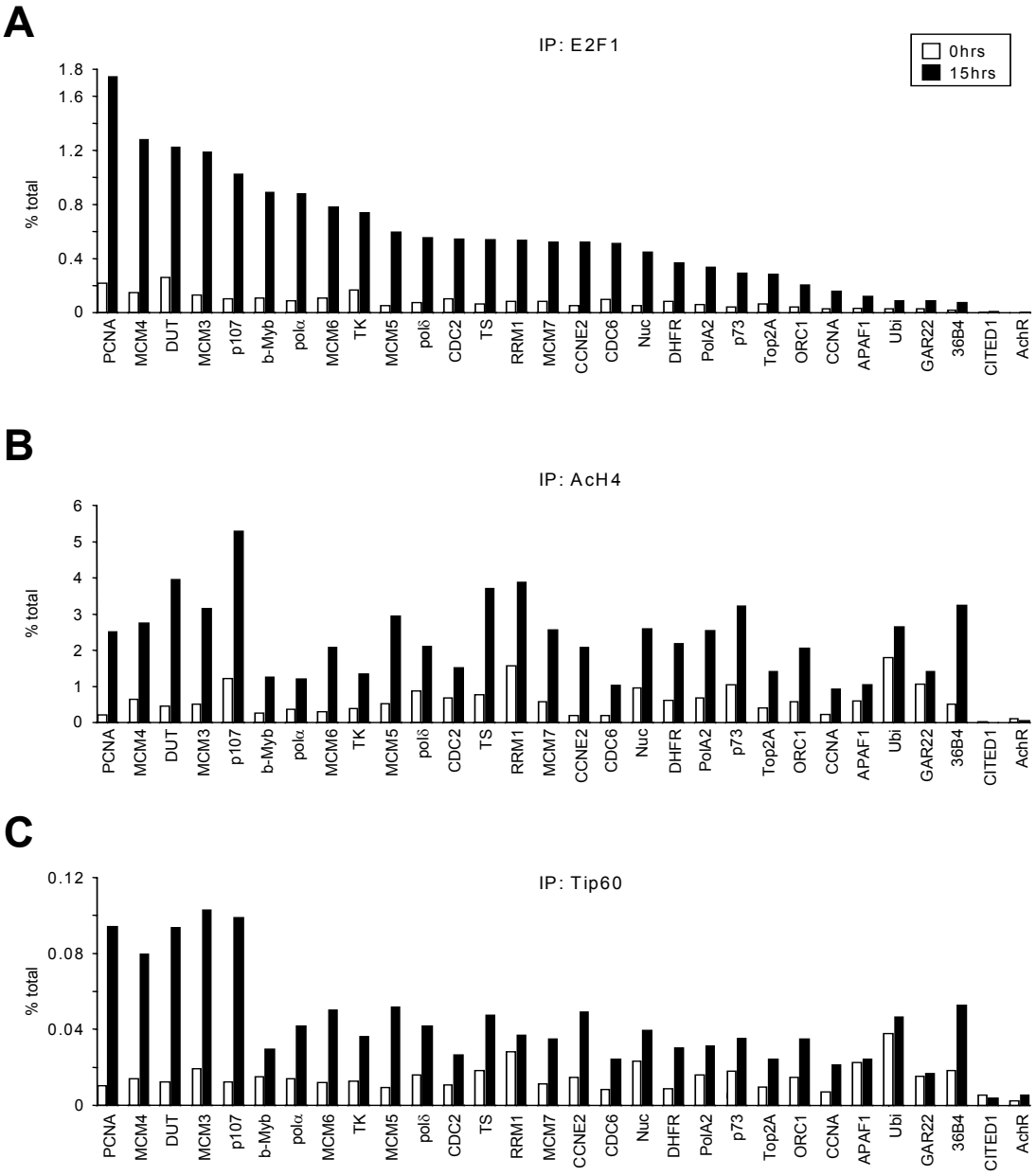

Figure 12: Tip60 Recruitment and H4 Acetylation is a Characteristic of Many E2F Targets.

E2F1 (A), AcH4 (B), and Tip60 (C) CHIP was performed with quiescent (white bars) and G1/S transition (15 hours; black bars) T98G cells. Primers for a selection of E2F targets were used. Loci are indicated below each panel, and are ordered for highest value of E2F1 binding along the X-axis.

4.8. ER-E2F1 is Sufficient to Induce Acetylation of E2F Targets

Having shown a correlation between histone acetylation and recruitment of activating E2F proteins, it was important to prove the role of activating E2Fs in this process. First, I addressed whether E2F1 is sufficient for induction of acetylation. In order to address this question, a human osteosarcoma cell line

expressing a chimeric ER-E2F1 protein was used (U2OS/ER-E2F1 cells; Vigo et al. 1999). This fusion of human E2F1 to the ligand-binding-domain of the estrogen-receptor (ER) is constitutively expressed, but only translocates from the cytoplasm to the nucleus when cells are treated with the estrogen analog 4-hydroxytamoxifen (4-OHT). Thus, it is possible to study rapid E2F1-dependent events in an otherwise unchanged cellular context.

Initially, I needed to verify if chimeric ER-E2F1 exhibited binding characteristics comparable to endogenous E2F1. Cells grown to confluence were starved for 96 hours in the absence of serum to eliminate endogenous E2F1. Then ER-E2F1 was activated by addition of 4-OHT for 0, 20, or 60 minutes, and cells were analyzed by CHIP. Judging from the amounts of DNA recovered in the CHIP, ER-E2F1 binding was at least as efficient as that of endogenous E2F1 during a serum response. ER-E2F1 rapidly associated with all four targets but not with the *achr* promoter (Figure 13 A). Moreover, localization of ER-E2F1 binding was the same as in serum stimulated T98G cells (Figure 13 B). As an additional control, U2OS cells without the transgene did not induce recruitment of E2F1 on any target upon stimulation with 4-OHT (Figure 13 C). Thus, 4-OHT rapidly induced localized binding of ER-E2F1 to its specific target genes, effectively mimicking the serum induced E2F1 response in T98G cells. A survey of four different clones of U2OS/ER-E2F1 revealed no differences in their responses to 4-OHT, confirming the reliability of this system (data not shown).

No H4 acetylation was induced in U2OS cells without the transgene (Figure 13 F), while binding of ER-E2F1 was accompanied by acetylation of both H3 and H4 on all targets, but not the *achr* (Figure 13 D-E). Initiation of acetylation in U2OS-ER-E2F1 cells was delayed, as it was barely detectable at 20 minutes. Possibly, assembly or activation of HAT-complexes is not as rapid as E2F1 binding. Interestingly, maximal acetylation of H4 was reduced when compared to levels reached in T98G cells (for example 3-4% total in U2OS/ER-E2F1 as opposed to 6-10% total in T98G). H3 acetylation was induced less dramatically than in the T98G system, due to higher initial acetylation levels in U2OS cells (compared to T98G cells). Spatial distributions of acetylation across the loci were very similar to those found in the T98G cells, with localization being centered to the E2F site at the *mcm3*

and the *p107* locus, and more widespread for *mcm4*, as seen in T98G cells (Figure 14 A-D). The *pcna* promoter exhibited a strong H3 and H4 acetylation upstream of the E2F binding site, which preceded 4-OHT treatment, and thus is presumably E2F-independent, results similar to those found in T98G cells. In summary, ER-E2F1 was sufficient to directly induce localized acetylation of both histone H3 and H4.

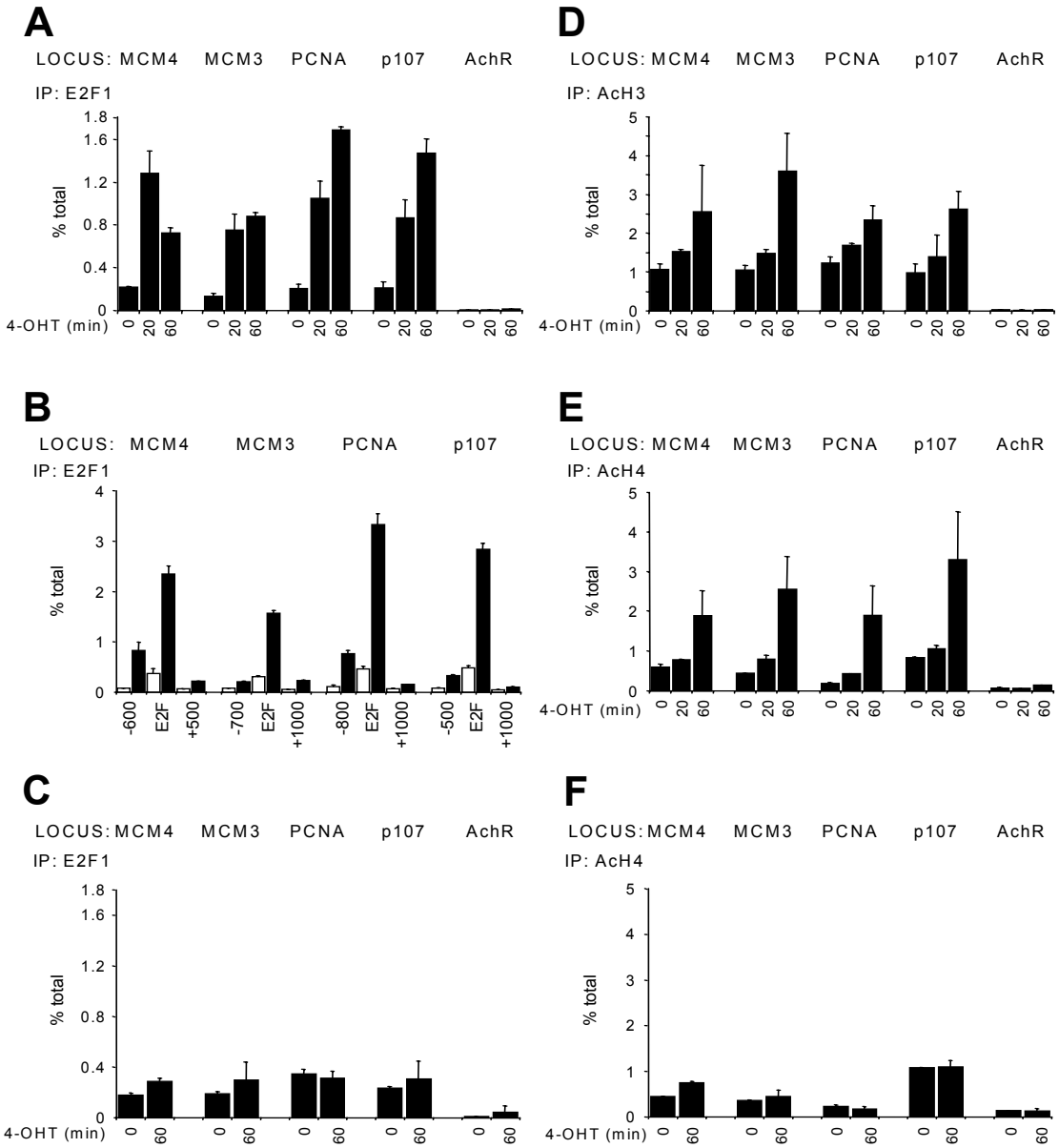

Figure 13: ER-E2F1 is Sufficient to Induce H3 and H4 Acetylation.
(A) Quiescent U2OS/ER-E2F1 cells were stimulated for 0, 20, and 60 minutes (on the x-axis) with 4-Hydroxytamoxifen (4-OHT), to induce nuclear localization of ectopic ER-E2F1. CHIP with antibodies specific for E2F1 showed rapid recruitment to E2F targets but not the control promoter (binding in % total on the y-axis). E2F1 recruitment was localized to E2F sites (B), and accompanied by acetylation of both H3 (D), and H4 (E). No E2F1 binding or H4 acetylation was detected when U2OS cells void of the transgene were stimulated with 4-OHT (C, F).

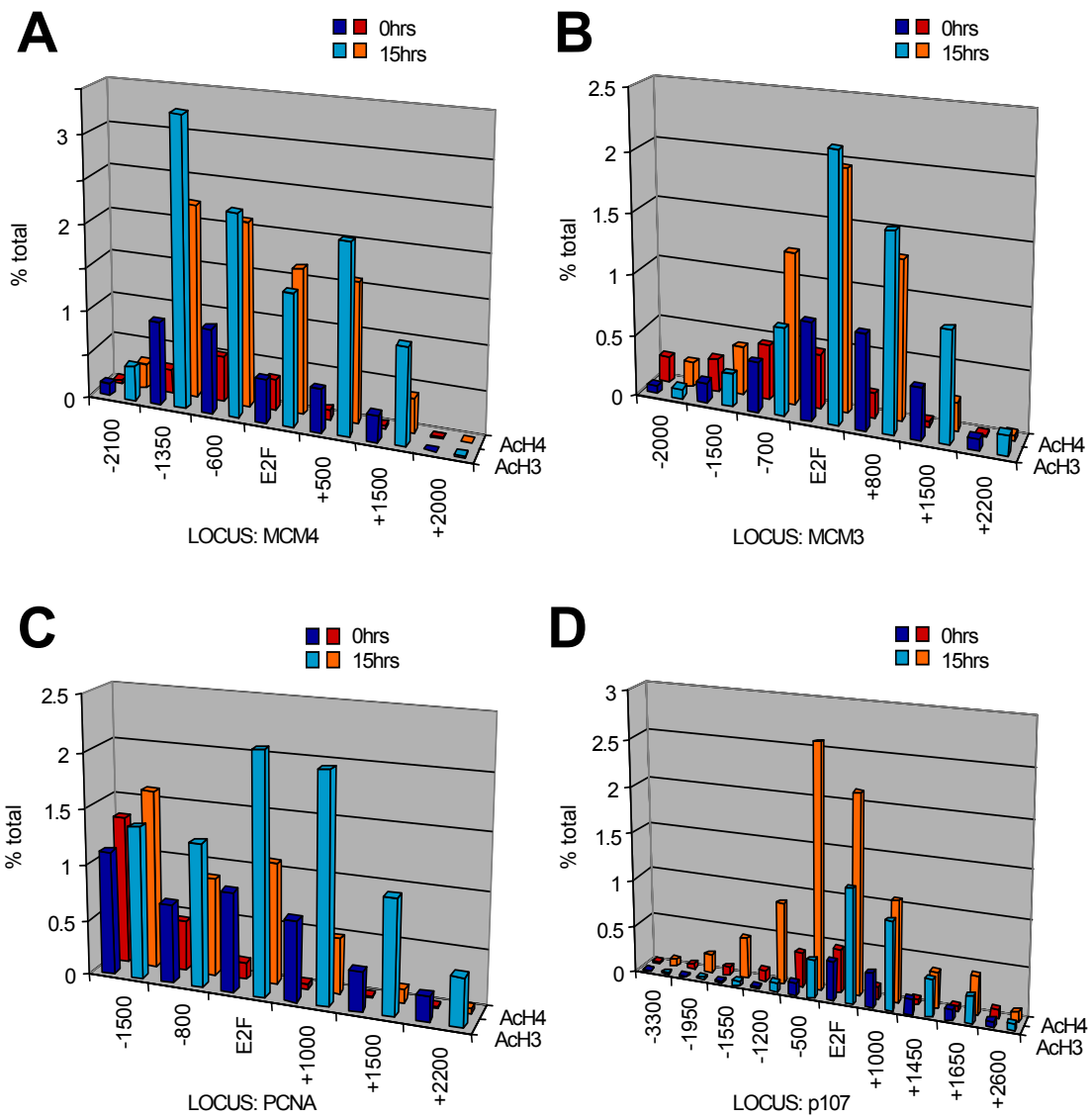

Figure 14: Acetylation of E2F Targets in U2OS/ER-E2F1 Cells is Localized to E2F Sites.

Spatial distribution of acetylation induced by ER-E2F1 was analyzed as above (Figure 9). Primers for *mcm4* (A), *mcm3* (B), *pcna* (C), and *p107* (D) promoters were run on AcH3 and AcH4 CHIPs from uninduced (dark blue and red bars, respectively) and induced cells (60 minutes stimulation, light blue and orange bars, respectively). In all cases acetylation is centered to the E2F sites.

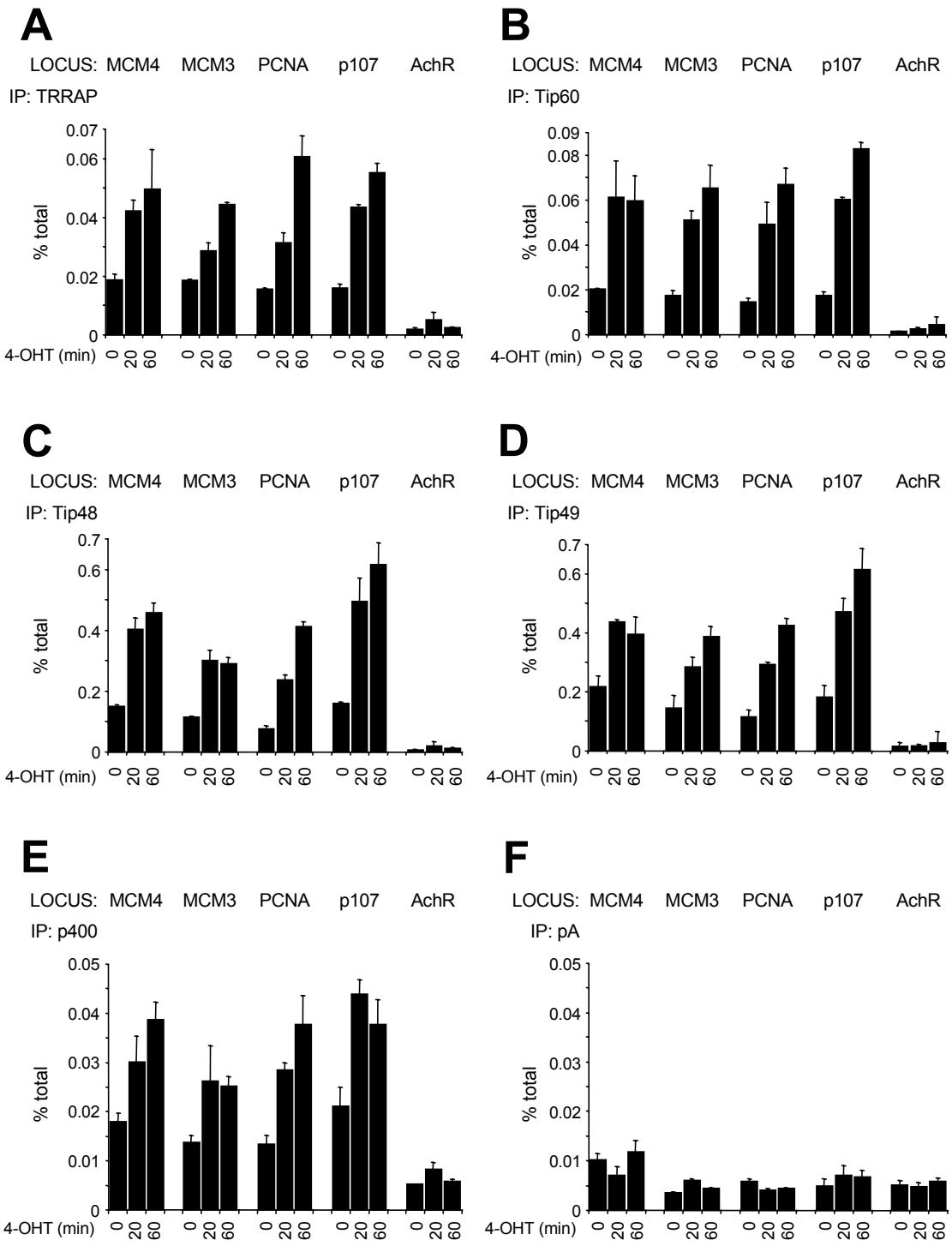

Figure 15: ER-E2F1 is Sufficient to Induce Recruitment of the Tip60 Complex.
U2OS/ER-E2F1 cells were stimulated as in Figure 13, and CHIP was performed with antibodies to TRRAP (A), Tip60 (B), Tip48 (C), Tip49 (D), and p400 (E). Control CHIP was performed without antibody (F). All five Tip60 complex subunits bound to E2F target promoters, while CHIP with Protein-A controls did not enrich for the same promoters.

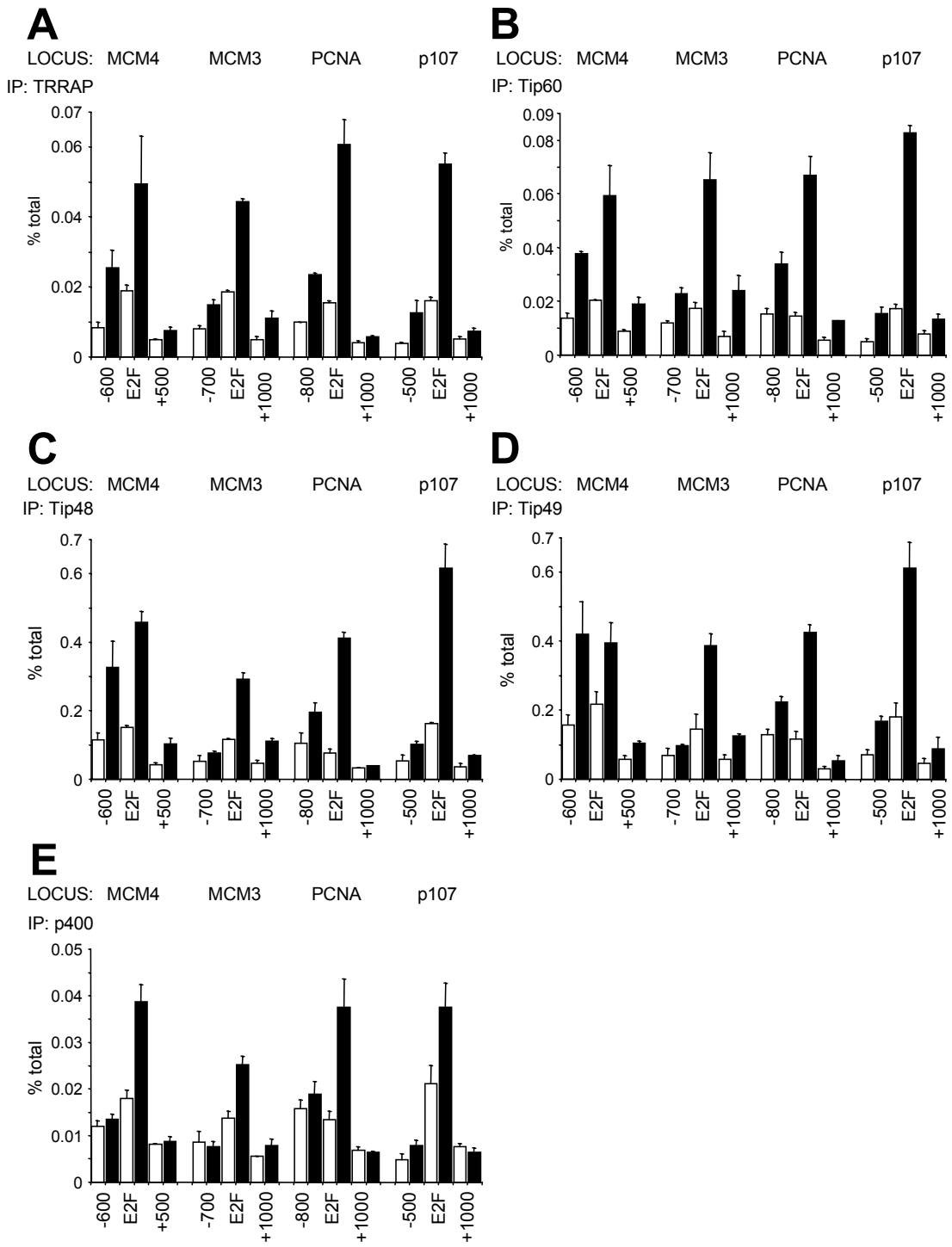

Figure 16: Tip60 Complex Subunits Localize to the E2F Sites.
Spatial distribution of Tip60 complex subunits was analyzed using the same primer pairs as described in Figure 7. TRRAP (A), Tip60 (B), Tip48 (C), Tip49 (D), and p400 (E) are all localized exclusively to the E2F binding sites. CHIP from unstimulated populations are represented in white bars, black bars represent populations from cells treated with 4-OHT for 60 minutes.

4.9. ER-E2F1 is Sufficient to Recruit Five Different Subunits of the Tip60 Complex

Since ER-E2F1 is sufficient to induce acetylation, it should also suffice to drive HAT-complex recruitment. In order to test this for the Tip60 complex, CHIP was performed with TRRAP, Tip60, Tip48, and Tip49 antibodies on U2OS/ER-E2F1 cells stimulated as described above. These experiments revealed that all proteins were rapidly recruited to E2F targets, paralleling the kinetics of E2F1 binding (Figure 15 A-D). p400 CHIP was not very efficient, but a low signal was reproducibly obtained, and was above signals from Protein-A control CHIPs (Figure 15 E, F). Again, kinetics of association with E2F targets resembled those of E2F1. If E2F1 were responsible for the recruitment of these five cofactors, localization would be expected to be limited to the E2F site. Using the same walking primers as in previous experiments showed that all proteins were recruited to the E2F sites, but not adjacent domains in chromatin, establishing the spatial correlation (Figure 16). In conclusion, E2F1 was sufficient to induce localized recruitment of five different subunits of the Tip60 HAT-complex, including Tip60, the catalytic subunit of the complex.

4.10. ER-E2F1 Binds Targets only Weakly Bound in Serum Response

Transcription of some E2F target genes is presumably not fully induced after serum stimulation. For example, genes involved in apoptosis (*p73, apaf1*), or DNA repair (msh2, *pol*δ) may require additional stimuli to be strongly activated. Consistent with this, *p73* was bound only weakly by E2F1 and Tip60, and H4 acetylation was also lower (compared to, for example, *mcm3)*. However, when *p73* primers were run on CHIPs from an U2OS/ER-E2F1 experiment, it became apparent that the *p73* promoter is bound well by E2F1 and Tip60, even better than *mcm3*, therefore establishing it as bona fide E2F target (Figure 17 A). Acetylation of H4 and H3 was as strong as that observed on most targets in serum stimulated T98G cells (Figure 17 B-C). However, while also exhibiting increased E2F1 binding, no increase in histone acetylation was observed on other DNA-repair related E2F target genes (Figure 17 A-C). It may be that conditions, which induce transcription of these

genes, are necessary for increased recruitment of E2F1/Tip60 complexes, and enhanced histone acetylation.

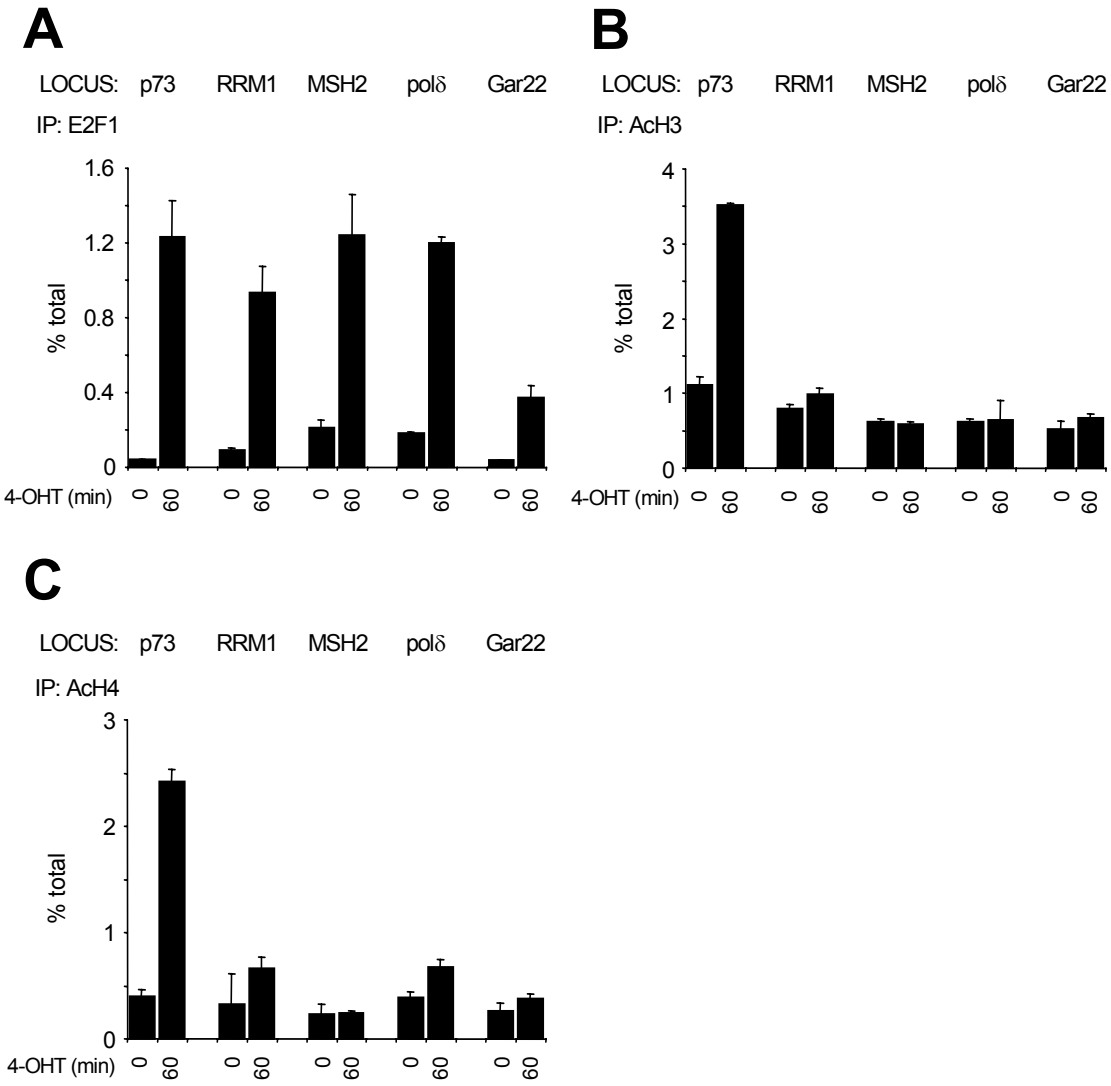

Figure 17: The *p73* promoter, but not other DNA-damage associated E2F-targets, is acetylated by ER-E2F1.
U2OS/ER-E2F1 cells were stimulated as in Figure 13, and CHIP was performed with antibodies to E2F1 (A), AcH3 (B), and AcH4 (c). All investigated promoters exhibit strong E2F1 binding, but only the *p73* target gene shows increased acetylation after 4-OHT stimulation.

4.11. Overexpression of a DNA-binding Deficient E2F1 Prevents Association of Endogenous E2F with Chromatin

Having shown that ER-E2F1 is sufficient for recruitment of the Tip60 complex, and for histone acetylation, I wished to investigate if activating E2F proteins are required for the recruitment of the Tip60 complex. In theory, this could be addressed by means of knockout mouse embryonic fibroblasts (KO-

MEFs) that lack single, or combinations of the activating E2Fs. However, it is easy to conceive that (a) all three individual E2F activators could potentially recruit coactivator complexes, and that (b) in case of absence of one or two of the E2Fs, the other E2F(s) could compensate for the loss, hampering studies designed to address the necessity of individual E2Fs. Therefore, I made use of adenoviruses expressing a dominant negative (DN) E2F.

Of the E2F mutants described in the literature, I decided to test the DNA-binding-deficient mutant E2F1-Eco132 (Johnson et al. 1993). Due to a single point mutation in its DNA-binding-domain, this mutant is unable to bind to E2F targets, but retains all protein-protein interaction surfaces, including the domain responsible for binding to the obligate dimerization partner DP. Previously, this mutant has not functioned as a DN mutant. However, when strongly overexpressed, one would expect that E2F1-Eco132 blocks any recruitment of functional E2F to promoters by titrating all DP away from endogenous E2F proteins.

To test this hypothesis, T98G cells were serum-stimulated to enter a new cell cycle and simultaneously infected with adenoviruses expressing GFP only (AdGFP, vector control), wild-type E2F1 (WT), and E2F1-Eco132 (Eco132). GFP expression (data not shown) and ectopic E2F1 expression (Figure 18 A) were comparable in all infected cells. E2F1 CHIP showed that infection with AdGFP did not affect the kinetics or localization of E2F1 recruitment to the *mcm4* promoter, when compared to non-infected T98G (Figure 18 A, compare with Figure 7 B; and data not shown). Overexpression of wild-type E2F1 caused premature binding to the *mcm4* promoter, whereas overexpression of E2F1-Eco132 prevented binding of E2F1 (Figure 18 A). Similar results were obtained for the *mcm3*, *pcna*, and *p107* promoters (Figure 18 B, and data not shown). Furthermore, CHIP with antibodies against E2F2 and E2F3 showed that both of them were also prevented from associating with target genes (Figures 18 C-D). E2F4 and p130 were present in quiescent cells, as expected (Figures 18 E-F). However, since both of these proteins are normally absent from G1/S transition cells, this was not influenced by expression of E2F1-Eco132. Thus, these experimental settings did not allow a formal assessment of whether the DN-E2F displaces repressive E2F. In summary, overexpressing E2F1-Eco132 efficiently prevented binding of

activating E2F complexes to target sites during the serum stimulation of T98G cells.

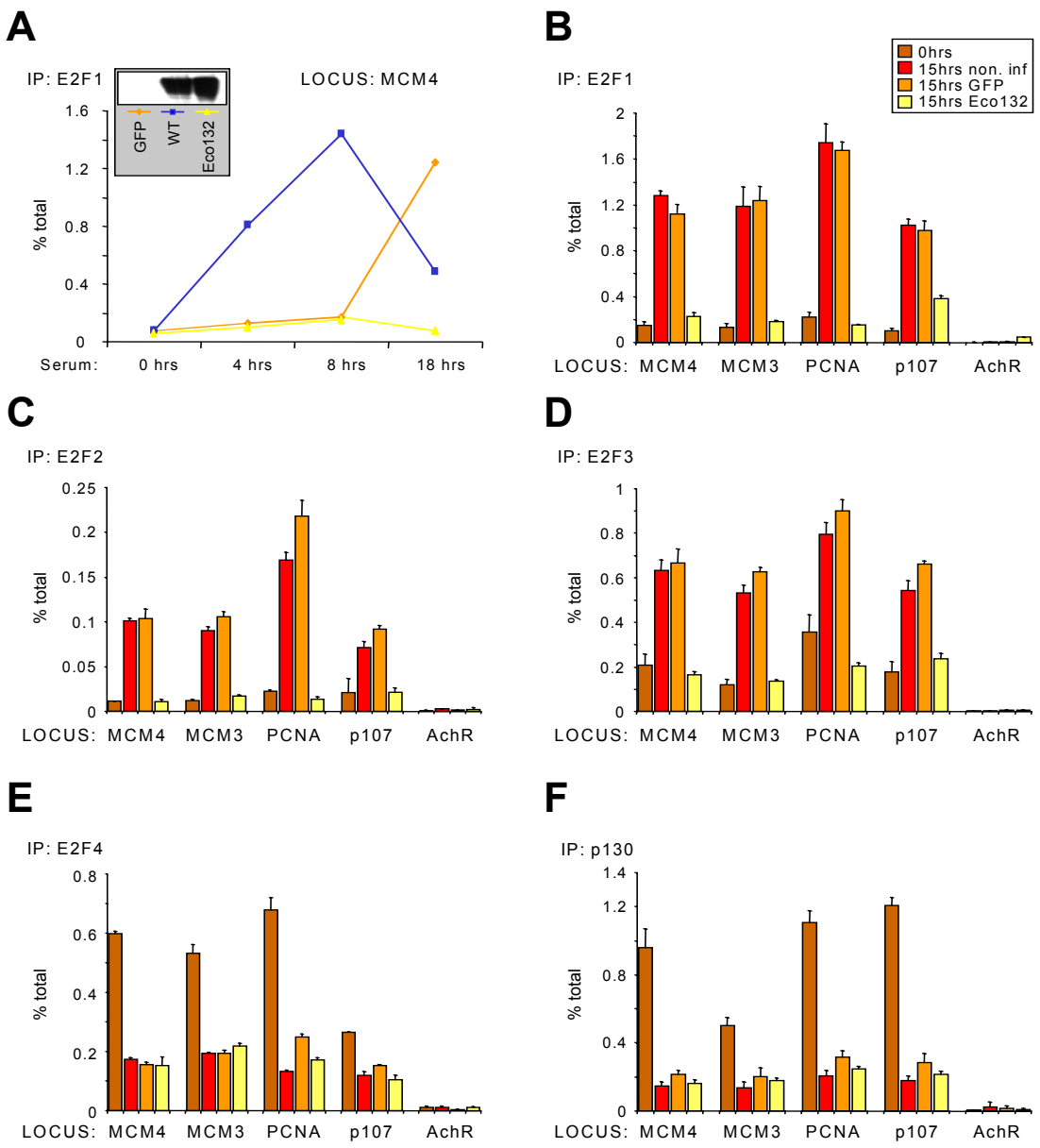

Figure 18: A DNA-Binding-Deficient Mutant E2F Inhibits Binding of E2F-family Members to E2F Targets when Expressed from Adenoviruses.

(A) T98G cells were starved as described. Upon releasing into a new cell cycle, they were infected with adenoviruses expressing GFP only (vector control, orange), wt E2F1 (blue), or the DNA-binding-deficient mutant E2F1-Eco132 (yellow). E2F1 CHIP was performed on cell populations at different points in the cell cycle, and showed that the WT E2F1 induced premature binding to the *mcm4* promoter, whereas E2F1-Eco132 prevented binding of any E2F1. Proteins were expressed at similar levels, as judged by Western blot (top left panel). Similarly, expression of E2F1-Eco132 (yellow bars), but not empty vector (orange bars), prevented induction of binding after quiescence (compare red bars to brown bars) of E2F1 (B), E2F2 (C), and E2F3 (D) to four target genes, as judged by CHIP. E2F4 (E), and p130 (F) were absent from promoters in all cell populations, except in quiescent cells. Signals from noninfected and control vector infected cells were comparable in all cases (compare red and orange bars).

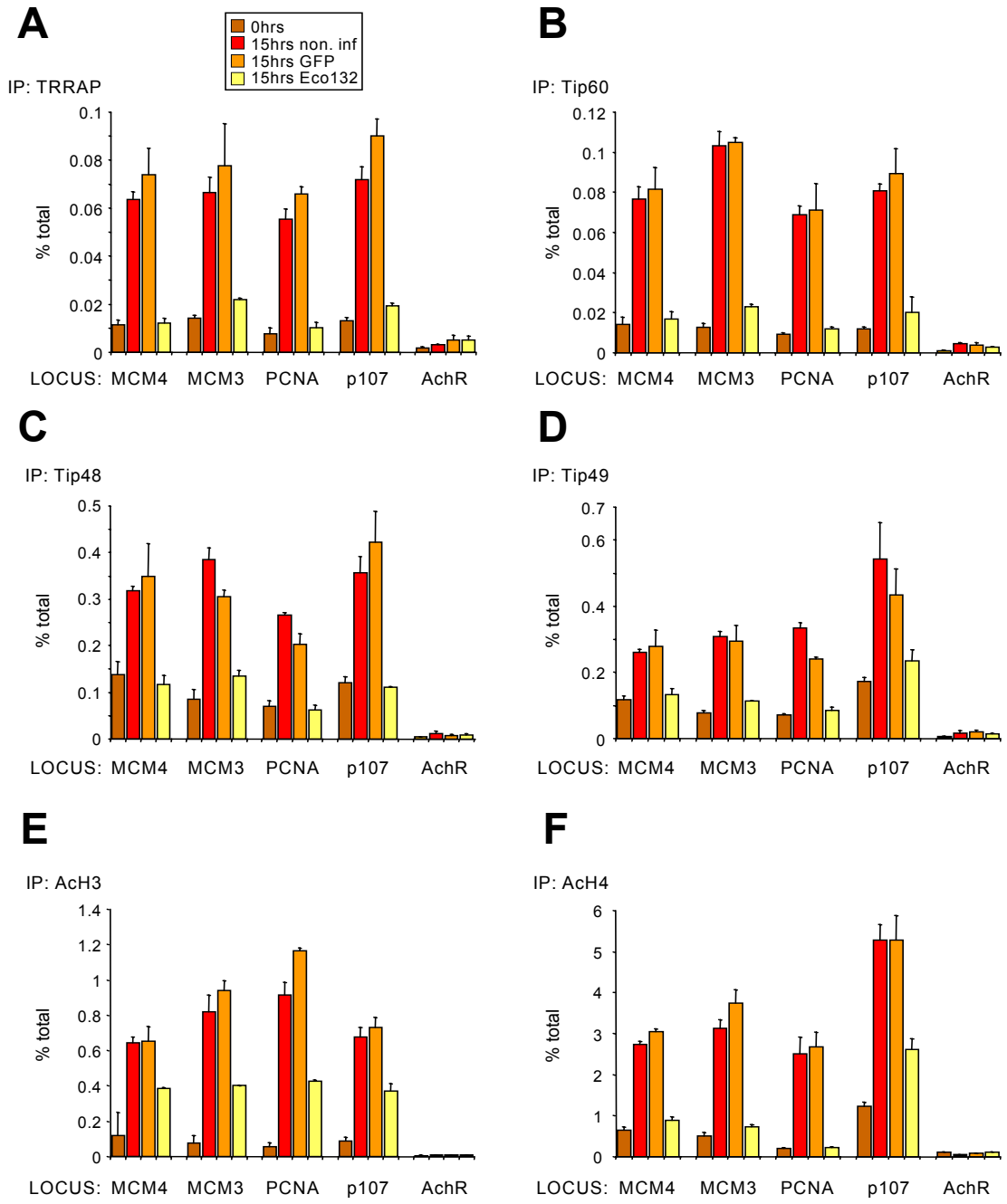

Figure 19: Activating E2F is Required for Recruitment of Tip60 Complex Subunits and Induction of H4 but not H3 Acetylation.

Cells were infected as in Figure 18, and CHIP was performed with antibodies to TRRAP (A), Tip60 (B), Tip48 (C), Tip49 (D), AcH3 (E), and AcH4 (F). Serum induction of cofactor recruitment (compare brown and red bars) is blocked in cells expressing E2F1-Eco132, but not upon expression of GFP only (compare orange and yellow bars). Acetylation of AcH3 is only reduced, whereas acetylation of H4 is totally blocked (E and F, compare orange and yellow bars).

4.12. Recruitment of the Tip60 Complex Requires Functional Activating E2F

The Tip60 complex was recruited to E2F target genes during serum stimulation of T98G cells (Figure 10), and ER-E2F1 is sufficient to drive this recruitment (Figure 14). In order to determine, whether activating E2F is required for this recruitment, I performed CHIP with antibodies against TRRAP, Tip60, Tip48, and Tip49 in cells infected with the DN-E2F as described above. Recruitment of all of these cofactors was blocked by expression of E2F1-Eco132, but not by infection with adenoviral vector, or in non-infected control cells (Figures 19 A-D). In summary, activating E2F is required for recruitment of the Tip60 complex to E2F target genes following serum stimulation of T98G cells.

4.13. Acetylation of H4 Depends upon Functional Activating E2F

Having shown that binding of activating E2F and coactivators is abolished in cells expressing E2F1-Eco132, I addressed, whether activating E2F is necessary for induction of acetylation. CHIP experiments showed that acetylation of H4 was reduced to levels similar to those found in quiescent cells, when E2F1-Eco132 was overexpressed, but not when cells only expressed GFP (Figure 19 F). Interestingly, some acetylation on the *p107* promoter remained detectable, which correlated with some residual E2F1 binding. This confirmed the strict correlation of E2F1 binding and H4 acetylation (Figure 18 B). In contrast to H4 acetylation, H3 reproducibly exhibited significant, but not full acetylation on all targets when E2F1-Eco132 was expressed (Figure 19 E). Thus, induction of H4 acetylation absolutely required binding of activating E2F, whereas H3 acetylation was only partially dependent upon it. Taken together, these data suggest that removal of repressive E2F complexes alone is not sufficient to induce H4 acetylation, although derepression may account for the partial increase in H3 acetylation.

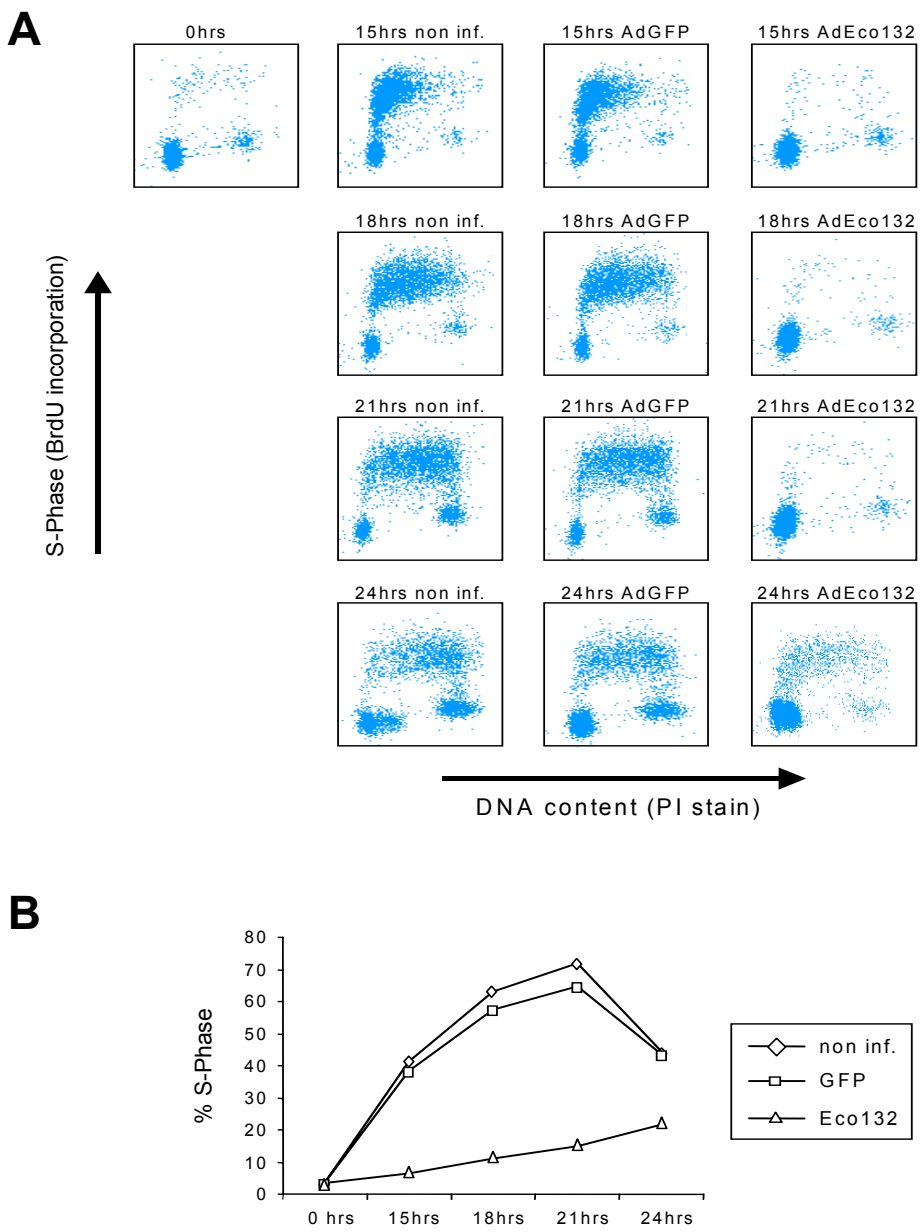

Figure 20: E2F Activity is Required for Cell Cycle Entry in T98G Cells.
Cells were infected as in Figure 18. At different times they were treated with 33μm BrdU for 30 minutes. (A) Analysis by two-dimensional flow cytometry showed that expression of mutant E2F1-Eco132 prevented S-Phase entry after cells were serum-stimulated whereas expressing of adenoviral vector only had no effect. Quantitation of the panels is shown in (B).

4.14. Binding of Activating E2F Proteins is Necessary for Cell Cycle Entry in T98G Cells

The results described show that activating E2F is required for Tip60 complex recruitment and histone acetylation of E2F targets. As a consequence, transcription and, ultimately, S-Phase entry should also depend on the presence of functional E2F activators. Thus, a mutant like E2F1-

Eco132 that removes all E2F protein from the cellular promoters should prevent cell cycle entry of T98G cells after serum stimulation. To test this hypothesis, two-dimensional flow cytometry was performed on T98G cells treated and infected as described. Expression of the E2F1-Eco132 mutant efficiently blocked S-Phase entry, whereas non-infected and AdGFP infected cells entered S-Phase efficiently (Figure 20). This implies that functional activating E2F is strictly required to drive quiescent T98G into S-Phase, whereas removal of repressive E2F4/p130 complexes alone is not sufficient. It has recently been published that activating, but not repressive E2Fs are dispensable in exponentially growing cells (Zhang et al. 1999; Rowland et al. 2002). In contrast, functional activator complexes were found to be required for cells entering a new cycle from quiescence. These results fit well with results described here.

4.15. Protein Levels of TRRAP and Tip60 do not vary throughout the Cell Cycle

Many proteins involved in cell cycle regulation exhibit tight regulation of protein levels, including activating E2Fs. Tip60 itself is regulated by ubiquitin/proteasome-dependent degradation (Legube et al. 2002), raising the possibility that its protein levels vary throughout the cell cycle. Thus, I decided to test if TRRAP and Tip60 levels change in T98G cells after serum stimulation. Cells were starved and allowed to enter a new cycle as before, and cell lysates were prepared at different time points. Proteins were separated by SDS-PAGE and analyzed by immunoblot with TRRAP and Tip60 antibodies. Neither TRRAP nor Tip60 levels varied considerably over an extended time-course (Figure 21). Similar results were obtained for Gcn5, HBO1, p300 and CBP (data not shown). Thus, it appears that levels of all these HATs are stable during transition from G0 to G1 and S-Phase. Furthermore, these data proved that the binding of Tip60 and TRRAP to E2F targets following serum stimulation was due to specific recruitment by activating E2F, and not a mere consequence of chromatin binding by the Tip60 complex after *de novo* protein synthesis following cell cycle entry.

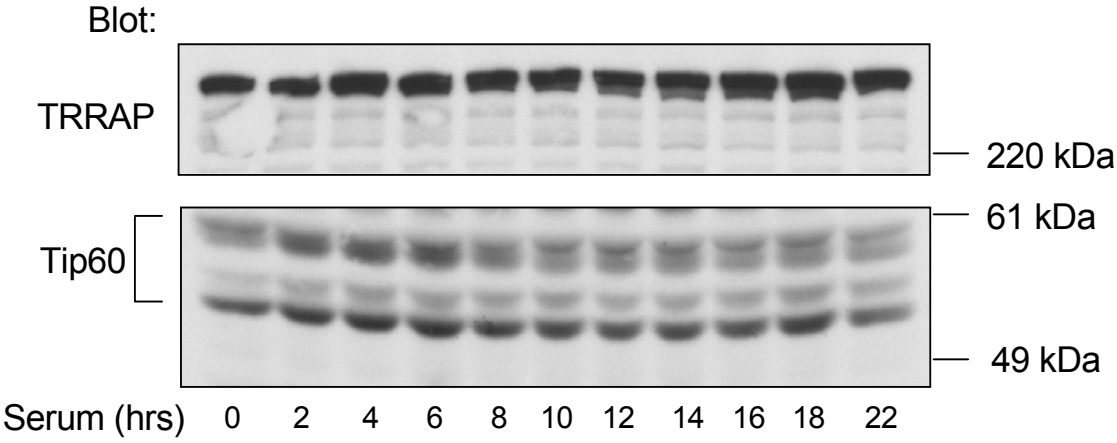

Figure 21: TRRAP and Tip60 Levels are Constant during G1/S-Phase Transition.

T98G cells were arrested and released into a new cycle as described. Cell lysates from different time points were analyzed by immunoblot with antibodies to TRRAP, and Tip60. Tip60 runs as a doublet, consistent with previously published results that two differently spliced proteins are present in cells. Protein levels do not vary during the cell cycle.

5. Myc Binds the HATs Gcn5 and Tip60

5.1. Summary

Besides E2F, TRRAP has been shown to bind Myc (McMahon et al. 1998). Thus, I decided to investigate if TRRAP/Tip60 complexes also play a role in Myc-dependent transactivation. Here, I show that Myc recruits HAT activity, and can bind the two TRRAP-associated HATs.

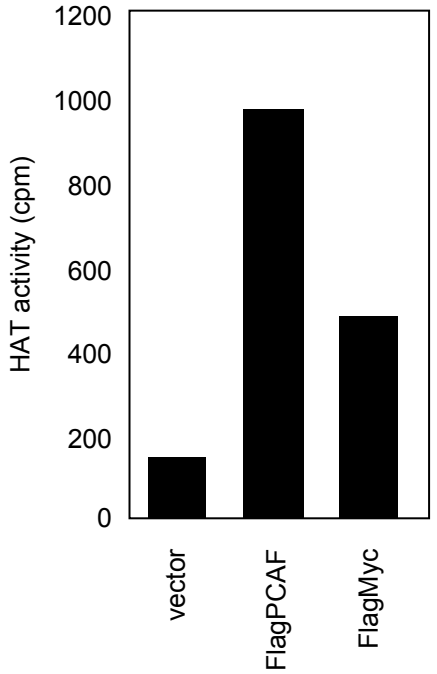

Figure 22: Transfected Myc Recruits HAT Activity.
293T cells were transiently transfected with vectors expressing the Flag-tag only, Flag-PCAF, or Flag-Myc. After 48 hours, cells were lysed, and IP-HAT assays were performed as described in Materials and Methods. Flag-PCAF and Flag-Myc both precipitate significant HAT activity, whereas no activity is recovered from cells only expressing the Flag-tag. Proteins were expressed at comparable levels (data not shown). One of two independent experiments is shown.

5.2. Myc Recruits HAT Activity when Overexpressed in 293T Cells

Myc has previously been published to bind TRRAP when expressed as a Flag-tagged protein in 293T cells. TRRAP is a subunit of at least two HAT-complexes in mammalian cells, suggesting that Flag-Myc can recruit HAT activity. To test this, 293T cells were transfected with vectors expressing Flag-Myc or Flag-PCAF (positive control). Flag-IPs were assayed for HAT activity

as described in Materials and Methods (chapter 8.2.9. p.111). These assays showed that Myc recruited a significant amount of HAT activity, nearly half as much as the potent HAT PCAF (Figure 22). Thus, Myc is capable of recruiting HAT activity in transient transfection assays.

5.3. Myc Binds to the SAGA Complex Components Gcn5 and Spt3

Since Gcn5 is a TRRAP-associated HAT, it could contribute to Myc-associated HAT activity. Therefore, transfection experiments were designed to find out whether Gcn5 associates with Myc. 293T cells were transiently transfected with vectors expressing Flag-tagged Myc, and un-tagged Gcn5. Lysates were immunoprecipitated with antibodies against the Flag-tag, and immunoblotted. Gcn5 was present in Flag IPs when cotransfected with Myc, but not in cells that expressed only Myc, or Gcn5 (Figure 23 A). Proteins were expressed at equal levels in all transfections.

In yeast, Gcn5p is part two distinct HAT-complexes composed of common and unique subunits (Grant et al. 1998). The SAGA complex is different from the other complex in that it contains the Spt class of proteins. The human complex STAGA is the orthologue of SAGA, and it contains the human counterpart of yeast Spt3p, hSpt3.

In order to find out whether Myc interacts with Gcn5 in the context of the STAGA complex, HA-tagged Myc was cotransfected with Flag-HA-tagged hSpt3, and lysates were immunoprecipitated with antibodies against Max, to test if the Myc/Max dimers can associate with hSpt3. When Myc was cotransfected, these IPs efficiently recovered transfected hSpt3 (Figure 23 B). In conclusion, Myc is capable of interacting with two different STAGA complex components in transient transfection experiments, suggesting that Myc recruits the STAGA complex to transactivate target genes. Similar results have been found by others (McMahon et al. 2000).

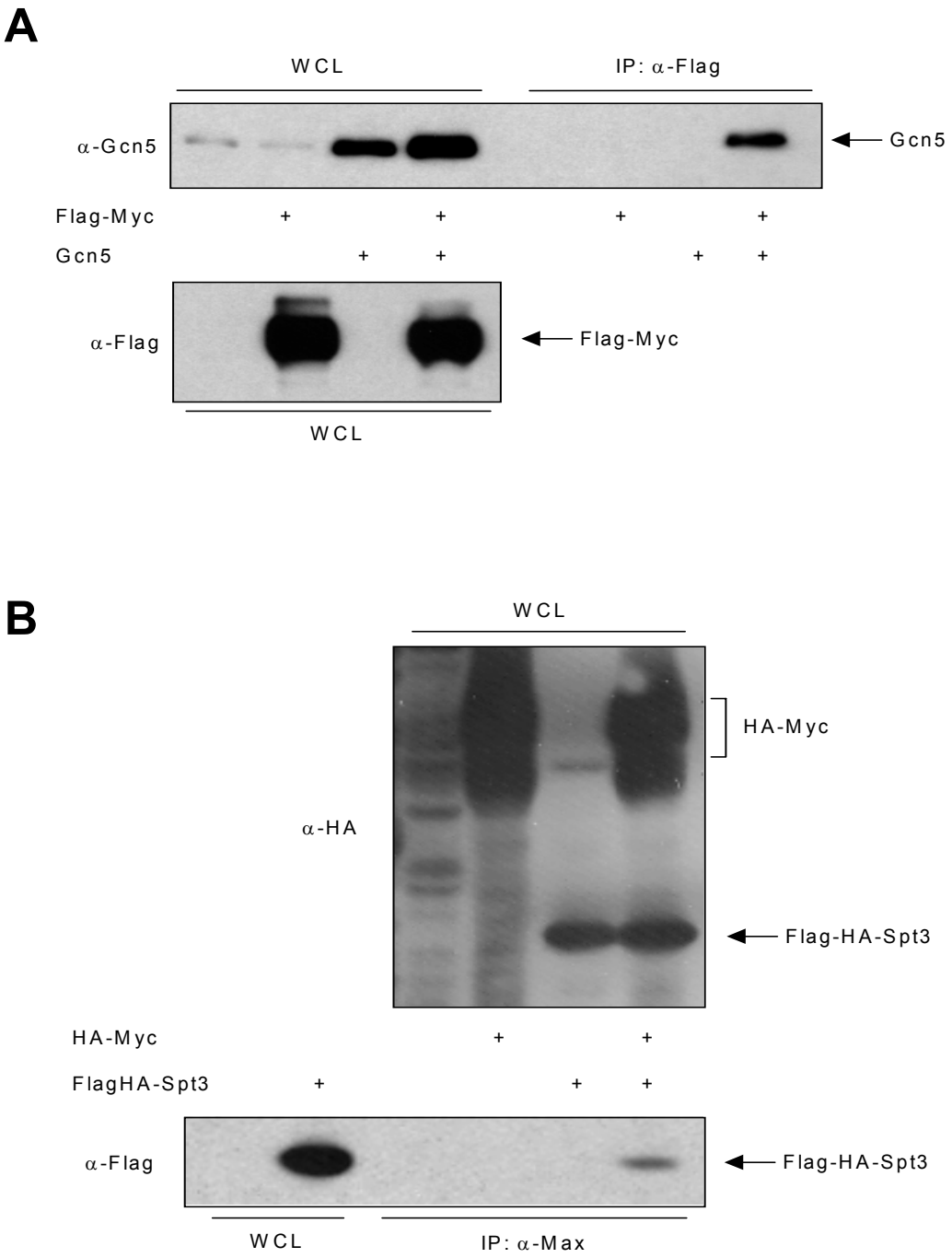

Figure 23: Myc Associates with STAGA Complex Components.
(A) Flag-Myc and un-tagged Gcn5 were transfected into 293T cells, alone, or in combination, as indicated. Cells were lysed after 48hrs, and lysates immunoprecipitated with Flag M2 monoclonal antibodies. IPs were immunoblotted for Gcn5, which was coprecipitated only when Myc and Gcn5 were cotransfected. Proteins were expressed at similar levels in all transfections. (B) HA-Myc and Flag-HA-Spt3 were transfected into 293T cells, alone or in combination. Cells were lysed after 48hrs, and lysates immunoprecipitated with Max2.1 antibodies. IPs were immunoblotted for Spt3, which was coprecipitated only when Myc and Spt3 were cotransfected. Proteins were expressed at similar levels in all transfections.

5.4. Myc and Tip60 Interact when Overexpressed in 293T Cells

The Tip60 complex also contains TRRAP, which raises the possibility that Myc can associate with Tip60 (Ikura et al. 2000). To verify, whether there is such an interaction, 293T cells were transfected with vectors expressing un-tagged Myc and Flag-HA-tagged Tip60, alone or in combination. Lysates were immunoprecipitated with antibodies against Tip60 or the Flag-tag, and IPs immunoblotted for coprecipitated Myc. Indeed, both Tip60 IPs contained significant amounts of Myc, whereas none was detected in control non-immune IPs (Figure 24 A). The interaction of Tip60 and Myc could also be detected when the lysates were immunoprecipitated with Myc antibodies, and immunoblotted for Tip60 (Figure 24 B).

As an additional control, I substituted the vector encoding Tip60 with an expression vector for HBO1 (Iizuka and Stillman 1999; Sharma et al. 2000; Burke et al. 2001). HBO1 is a Tip60 related protein, which has not been described to have a role in transcription. Consistent with this, no interaction with Myc was detected by immunoprecipitation-immunoblot experiments, arguing that the interaction is specific to Tip60 (Figure 24 C). In summary, Tip60 and Myc specifically interact in transfected cells, suggesting that a Tip60 containing HAT-complex plays a role in Myc function. Consistent with this, recent work in our lab demonstrated that TRRAP and Tip60 are recruited to chromatin by Myc (S. Frank, and B. Amati, unpublished results).

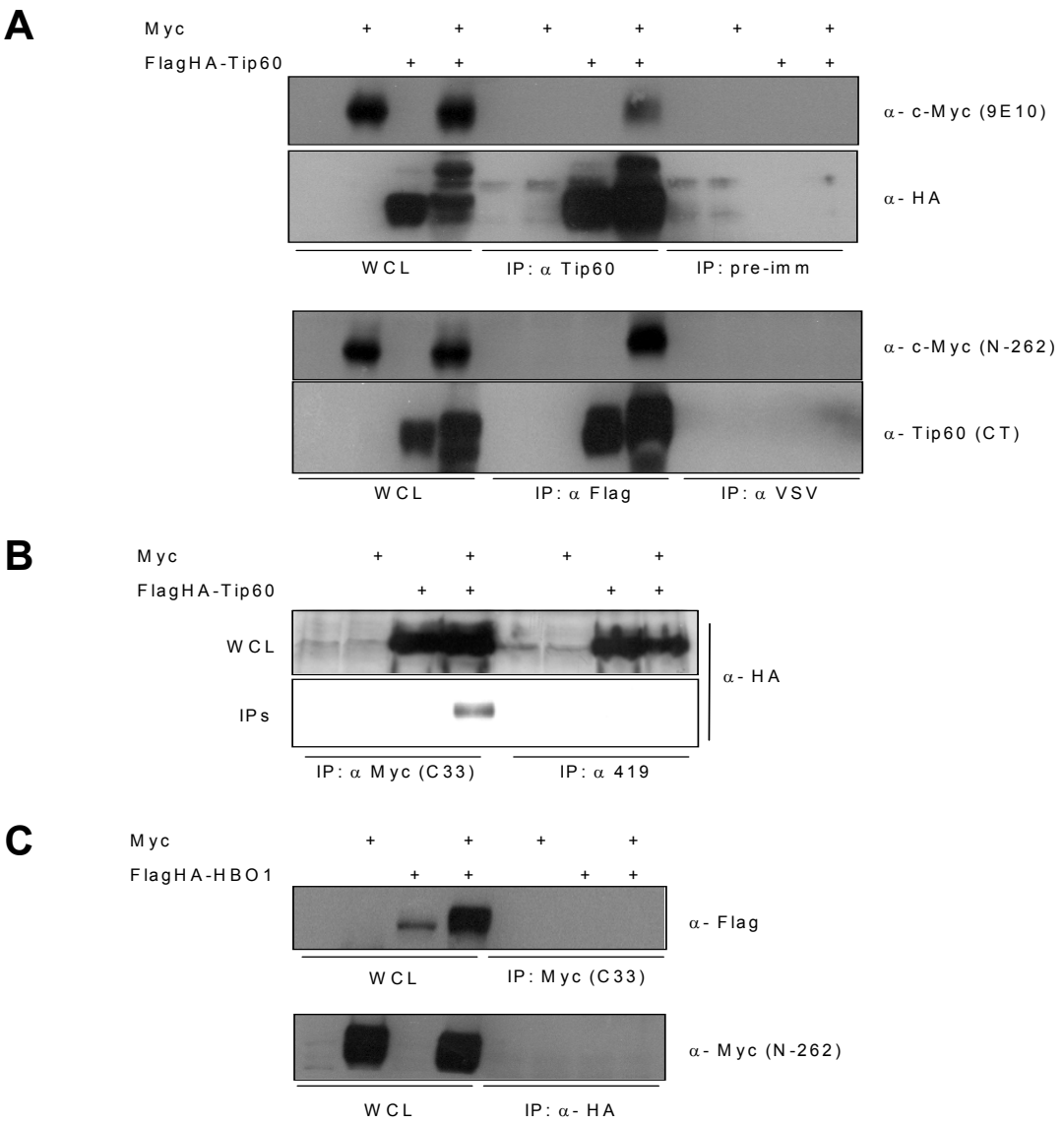

Figure 24: Myc Binds to Tip60, but not HBO1 in Transient Transfections.
(A) Flag-HA-Tip60 and un-tagged Myc were transiently transfected into 293T cells, alone or in combination. Cells were lysed after 48hrs and lysates immunoprecipitated with Flag M2 monoclonal antibodies (lower panel), or Tip60 polyclonal antibodies (upper panel). IPs were immunoblotted for Myc, which was coprecipitated only when Myc and Tip60 were cotransfected. No Myc was coprecipitated with VSV monoclonal isotype control antibody (lower panel), or preimmune Tip60 antisera (upper panel). Proteins were expressed at similar levels in all transfections. (B) Myc and Flag-HA-Tip60 were transfected into 293T cells, alone, or in combination. Cells were lysed after 48hrs, and lysates immunoprecipitated with Myc monoclonal antibodies, or 419 monoclonal isotype control antibodies. IPs were immunoblotted for Tip60, which was coprecipitated only when Myc and Tip60 were cotransfected (lower panel). Proteins were expressed at similar levels in all transfections (upper panel). (C) Myc and Flag-HA-HBO1 were transfected into 293T cells, alone, or in combination. Cells were lysed after 48hrs and lysates immunoprecipitated with Myc monoclonal antibodies (upper panel) or HA antibodies (lower panel). IPs were immunoblotted for HBO1 (Flag M2) or Myc. No interaction was detected. Proteins were expressed at similar levels in all transfections.

6. A Role for the STAGA Complex in the G1 Phase of the Cell Cycle

6.1. Summary

When mammalian cells are infected with adenovirus, the key function of the adenoviral oncoprotein E1A is to induce cell cycle progression (reviewed in Ben-Israel and Kleinberger 2002). It does so by bypassing the requirement for G1-specific Cyclin/CDK complexes, thus liberating E2F activity, and ultimately inducing E2F target genes. Interestingly, the E2F-binding HATs PCAF and p300 are targets of E1A (Reid et al. 1998; Chakravarti et al. 1999; Hamamori et al. 1999b). Furthermore, E1A associates with TRRAP, and the domain of E1A responsible for this interaction is required to overcome a $p27^{Kip1}$ imposed cell cycle arrest (Alevizopoulos et al. 1998; Deleu et al. 2001). The targeting of HATs by E1A suggests that in normal cells the activities of these HATs may be regulated by the cell cycle machinery, in particular by G1-specific Cyclin/CDK complexes.

Here, I show that Cyclin E and D are capable of interacting with the HATs Gcn5 and PCAF. Cyclin E also binds the STAGA complex subunit hSpt3. Finally, HAT activity of Flag-tagged PCAF is reduced in cells arrested with the CDK-inhibitors (CKI) $p16^{INK4a}$ or $p27^{Kip1}$.

6.2. Cyclins E and D Bind Gcn5 in Transient Transfections

If Cyclin/CDK complexes target the same set of substrates as E1A, an interaction between these cyclins and the HATs PCAF/Gcn5 would be expected. Thus, I tried to verify if Cyclins could interact with Gcn5 in transient transfections. 293T cells were cotransfected with vectors expressing HA-tagged Cyclin D, E1, or E2. Cells were lysed, and analyzed for protein expression by immunoblot. Cyclin E2 was expressed at lower levels, probably due to rapid degradation (Figure 25 A). Otherwise, protein levels were similar in all transfections (Figure 25 A). Lysates were then subjected to immunoprecipitation with Gcn5 antibodies (Figure 25 B) and HA or Cyclin E antibodies (Figure 25 C). An interaction of Gcn5 with Cyclin E was detected in cells coexpressing the two proteins. Furthermore, Gcn5 also associated with

Cyclin D1. Thus, it appears that Gcn5 is capable of interacting with two discrete, G1-specific Cyclins.

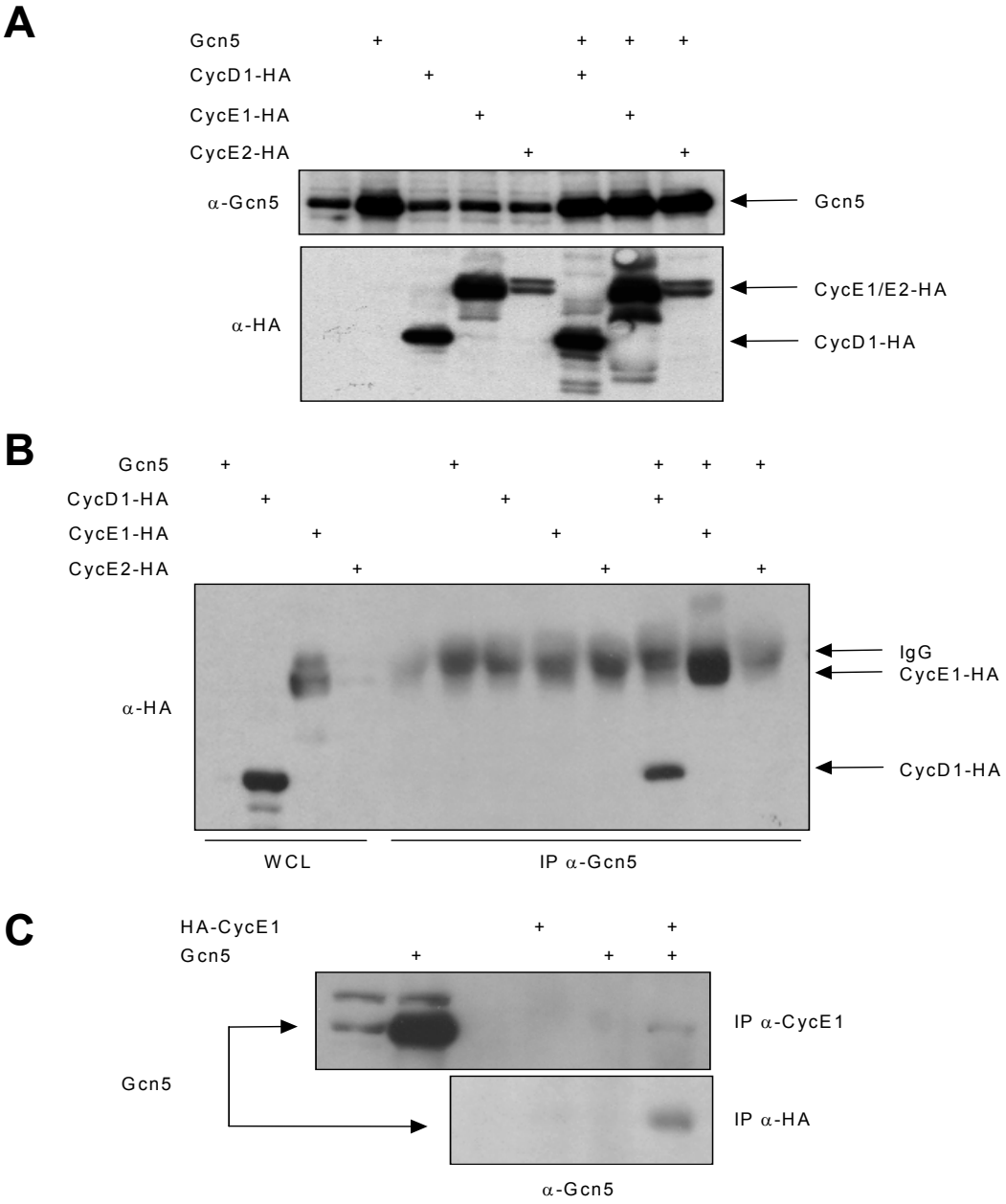

Figure 25: Cyclin E Interacts with Gcn5 when Overexpressed in Transient Transfections.

HA-tagged Cyclins were cotransfected with un-tagged Gcn5 in 293T cells. (A) Immunoblot of cell lysates with Gcn5 and HA antibodies revealed that all transfected proteins were expressed at equal levels in all transfections. (B) To detect interactions, lysates were immunoprecipitated with Gcn5 antibodies, and IPs immunoblotted for the presence of Cyclins using HA antibodies. Both Cyclin D1 and E were detected in Gcn5 immunoprecipitates. (C) Lysates were immunoprecipitated with Cyclin E antibody (HE172) or HA antibody (HA.11). Immunoblotting revealed presence of Gcn5 in Cyclin E precipitates. WCL = Whole cell lysate.

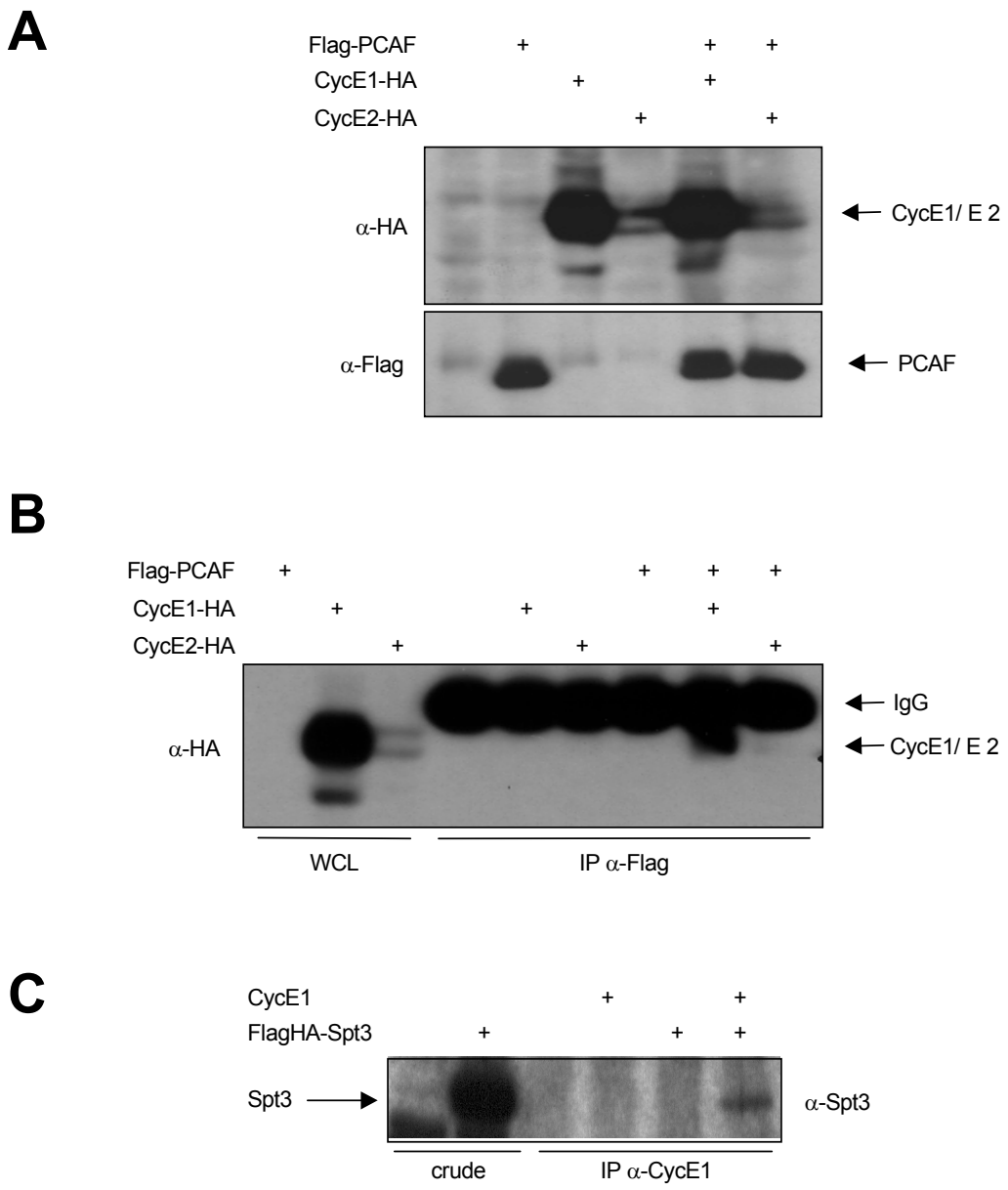

Figure 26: PCAF and hSpt3 are Capable of Interacting with Cyclin E when Cotransfected.

(A) HA-tagged Cyclins were cotransfected with Flag-PCAF into 293T cells. Lysates were immunoblotted for equal protein expression with Flag M2 and HA.11 monoclonal antibodies (upper panels). Flag-IPs were immunoblotted with HA-antibodies and revealed presence of coprecipitated Cyclin E (lower panel). (B) Flag-HA-tagged hSpt3 was coexpressed in 293T cells with un-tagged Cyclin E, and cell lysates were immunoprecipitated with Cyclin E monoclonal antibody HE172. Lysates were immunoblotted with Flag monoclonal antibody M2, which revealed presence of hSpt3 in the Cyclin E IPs.

6.3. Cyclin E Associates with PCAF and hSpt3 in Transient Transfections

Having shown that Gcn5 can bind to Cyclin E, I wanted to investigate, whether PCAF, a HAT closely related to Gcn5, can also bind Cyclin E. 293T cells were transiently transfected with expression vectors for HA-tagged

Cyclins E1 and E2, and Flag-tagged PCAF, alone or in combination. Lysates were analyzed for protein expression by immunoblot, showing equal expression in all cotransfections (Figure 26 A). Flag-IPs contained Cyclin E, indicating that PCAF is capable of binding it just as well as Gcn5 does (Figure 26 B).

The interaction data presented above indicate that Cyclin E binds to a Gcn5/PCAF containing HAT-complex. If this were the STAGA complex, an interaction with hSpt3 would also be expected. Thus, 293T cells were transfected with plasmids encoding a Flag-HA-tagged hSpt3 and un-tagged Cyclin E. Lysates were immunoprecipitated with the Cyclin E monoclonal antibody HE172, and immunoblotted for the presence of hSpt3. Only when both proteins were coexpressed, such an interaction was detected (Figure 26 C). In conclusion, Cyclin E is capable of interacting with at least two components of the STAGA complex in transient transfection assays.

6.4. Cell Cycle Arrest by $p16^{INK4a}$ and $p27^{Kip1}$ Reduces the HAT Activity of Transduced PCAF

The fact that Cyclins bind to a Gcn5/PCAF suggests that they may regulate the activity of a Gcn5/PCAF HAT-complex, for example by CDK-dependent phosphorylation. To address this possibility, I used a Rat1 cell line stably transduced with a retrovirus expressing Flag-tagged PCAF (Rat1/FlagPCAF). Exponentially growing cells were superinfected with retroviruses expressing either the $p16^{INK4a}$ or $p27^{Kip1}$ CKI, or empty vector (BP). After infection, cells were grown to confluence, and then passaged into media containing Puromycin for selection purpose. After two days, all cells of an uninfected control population not expressing the resistance gene had died. Cells expressing the CKIs were growth arrested, as determined by colony formation assay (data not shown). Cells were harvested, and lysates analyzed by immunoblot and HAT assay. Whereas PCAF protein levels were not reduced in cells expressing either of the CKIs, HAT activity dropped to background levels (Figure 27). This suggests that HAT activity be regulated in response to growth inhibitory stimuli. However, whether modulation of HAT activity is a cause, or a consequence of growth arrest, remains to be determined.

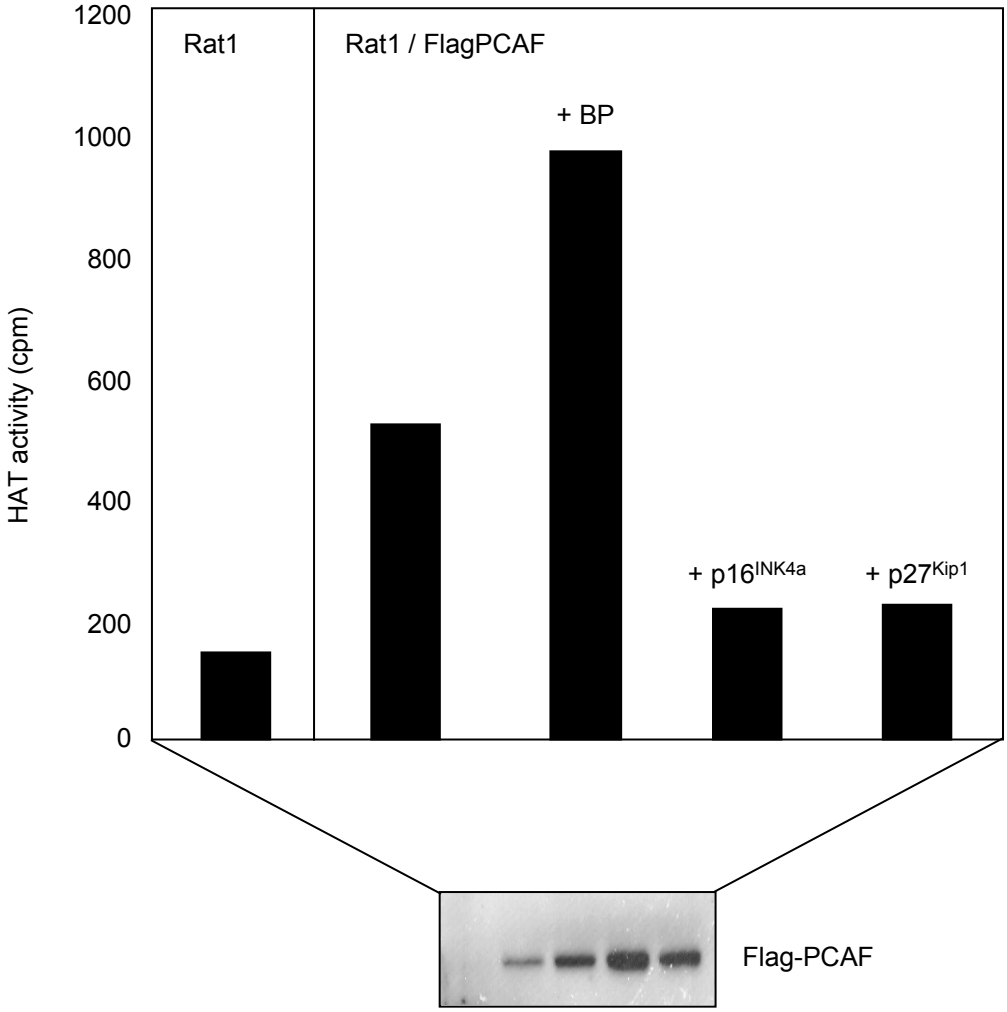

Figure 27: Cell Cycle Arrest by CKIs Reduces PCAF Associated HAT Activity.
Rat1 cells were transduced with a retrovirus expressing FlagPCAF. For HAT assays, exponentially growing populations were transduced with either BP retrovirus (empty vector control), or vectors expressing either $p16^{INK4a}$ or $p27^{Kip1}$. After two days of selection with Puromycin, the stably transduced cells were harvested, and IP-HAT assays were performed as described in Materials and Methods (upper panel). For expression analysis, cell lysates were immunoblotted with M2 Flag monoclonal antibody (lower panel). HAT activity in arrested cells was significantly reduced, compared to control infected cells. One of three independent experiments is shown.

DISCUSSION

7. TRRAP-Associated HAT-Complexes are Involved in E2F and Myc Transactivation and in Cell Cycle Control

7.1. Analysis of Binding Patterns of E2F Proteins

During my thesis, I analyzed the role of TRRAP-associated HAT-complexes in E2F function. To this aim, I initially investigated a number of genomic loci for their association with different E2F proteins during the cell cycle using quantitative CHIP. I found that in quiescent T98G cells E2F4/p130 complexes are predominant on the promoters of E2F targets. As cells progressed through G1, activating E2Fs (E2F1, -2, and -3a) replaced E2F4. Their binding peaked just before to S-Phase, then dropped. Presumably Cyclin A/CDK2, which is activated in S-Phase, phosphorylates all activating E2Fs to release them from their targets, as was shown for E2F1. These results fit well with previously published reports (Takahashi et al. 2000; Wells et al. 2000; Rayman et al. 2002), and establish T98G cells as a system useful for analysis of cofactor recruitment and histone acetylation by E2F.

Others and we detected robust signals for both E2F1 and E2F3, while E2F2 CHIP resulted in only weak specific enrichment, suggesting that E2F2 may not be highly expressed in these cells (Takahashi et al. 2000; Wells et al. 2000). Alternatively, the antibody used in these studies might not be very efficient. However, in exponentially growing, but not in synchronized MEFs, significant E2F2 and E2F3 signals have been obtained by CHIP (Wells et al 2000). Thus, E2F2 may be more important in cycling cells than in the first cycle after quiescence, as has been previously proposed for E2F3. Alternatively, individual E2Fs may play distinct roles in different cell types, as has been suggested from results obtained with mouse models (see chapter 3.2.3; pp. 39). Another interesting result was that, while promoter occupancy by E2F1 and -2 in quiescence was low, E2F3 was present in significant amounts. As noted above, the antibody used in this study does not distinguish between the E2F3a and E2F3b isoforms. Nevertheless, the data suggest that E2F3 can play a role in transcriptional repression in G0.

Using an artificial system expressing hormone inducible E2F1 (U2OS-ER-E2F; Vigo et al. 1999), it was possible to analyze E2F1 binding to target genes that were not strongly bound after mitogenic stimulation. Indeed, some of those were efficiently bound by ER-E2F1, for example *p73*. Presumably, additional signals are required to achieve enhanced binding of endogenous E2F1 to these promoters. In this context it is noteworthy, that E2F1, but not E2F2 or E2F3, exhibits protein stabilization and enhanced DNA-binding after DNA-damage (Lin et al. 2001). Such conditions may favor binding of E2F1 to the promoters of *p73* and other DNA-damage related genes like *msh2, polδ, and apaf1*, but this hypothesis remains to be tested.

Ectopic ER-E2F1 bound to targets at least well as endogenous E2F1. Furthermore, when nuclear localization of ER-E2F1 was induced, or when E2F1 was overexpressed from an adenoviral vector, association with E2F binding sites was immediate. Thus, E2F1 is fully capable of binding to its targets as soon as it is expressed, or imported into the nucleus, without the need of additional signals (for example serum) that modulate DNA-binding. This suggests that E2F1 is able to displace E2F4/p130 complexes, which presumably are occupying E2F targets in quiescent U2OS/ER-E2F1 cells. The reason for this might be a higher specific affinity of E2F1 (compared to E2F4) for its binding sites.

E2F sites are commonly located within promoters, close to the transcriptional start site. Many promoters also possess binding sites for other transcription factors, and these may contribute to transcriptional regulation. For instance, a complex of p107, a repressive E2F, and Smad2/3 has recently been found to specifically repress transcription of the *c-myc* gene after TGF-β treatment (Chen et al. 2002). While not recruited via E2F sites, the entity of an E2F and a Rb-family protein may recruit corepressors to the Smad-sites in the *c-myc* gene, just as for "regular" E2F target genes. Another possible co-regulator is YY1, which has recently been shown to interact with E2F2 and E2F3, and cause synergistic activation of the *cdc6* promoter, even though the nature of this synergy has not been determined (Schlisio et al. 2002). Similarly, binding sites for Sp1 occur in many E2F target genes (*cdkn2a, cdc6*, and *tk*), and combinatorial coactivation has been suggested (for

example Chang et al. 2001; Huang et al. 2001; Blais et al. 2002; Parisi et al. 2002; Nichols et al. 2003). Moreover, at least some E2F target genes (for instance *nuc*, *mcm4*, *ccne2*) also contain Myc binding sites, and Myc binding can induce acetylation of promoters in early G1, at least on *nuc* (Greasley et al. 2000; Fernandez et al. 2002). Transcriptional activation upon mitogenic stimulation might thus be a complex response achieved by simultaneous, or sequential, action of several transcription factors. Interestingly, a chromatin-modifying-complex containing E2F6/DP1 and Mga/Max, antagonists of the E2F and Myc activators, has been shown to bind several E2F targets in quiescent primary fibroblasts (Ogawa et al. 2002). Thus, promoters containing both E-boxes and E2F sites might be subject to coregulation by repressive and activating branches of two independent transcription factor families. Lastly, it is important to note that at least some of the E2F targets involved in DNA-damage are also transactivated by p53 (for example *p73, apaf1;* reviewed in Xu and Raafat el-Gewely 2001). Therefore, transcriptional regulation of these genes in response to DNA-damage might also depend on combined actions of at least two distinct transcription factors.

7.2. Activating E2F is Required for Cell Cycle Entry in T98G Cells

The role of activating versus repressing E2F in the cell cycle has been a focus of debate (see introduction). Depending on the cellular context, overexpression of activating E2F proteins can cause S-Phase induction, overcome artificially imposed G1 arrest, or induce apoptosis. E2F4 and E2F5 are also capable of inducing S-Phase when overexpressed, raising the possibility that these studies do not necessarily reflect the role of endogenous E2Fs. Nevertheless, it is clear that at least E2F3 plays an important role in G1 progression as E2F3$^{-/-}$-MEFs are delayed in S-Phase entry following mitogenic stimulation, and gene expression of specific targets is reduced. Consistent with this E2F1 and E2F3 loss also reduce excess proliferation in Rb$^{-/-}$-mice. Furthermore, the combined mutation of E2F3 with E2F1, or E2F2, or both leads to progressive reduction in cell cycle entry, such that TKO-MEFs do not detectably enter the cell cycle, and fail to proliferate. However, in these MEFs repressive E2F may become dominant, so that lack of proliferation may not be due to absent activators, but enhanced inhibitory E2F. Therefore, the

necessity of activating E2F for proliferation and cell cycle entry remains formally unproven.

In this study, I used a mutant E2F1 that is deficient in DNA-binding, but retains the capacity to interact with DP1 (E2F1-Eco132). Expression from an adenoviral vector resulted in levels far above endogenous E2F1 protein. The ectopic E2F1-Eco132 presumably titrates all DP protein away from endogenously expressed E2F, effectively acting as a dominant negative E2F. Indeed, as analyzed by CHIP, no E2F (including E2F4) was detected on promoters after infecting T98G cells with Ad-E2F1-Eco132. In these cells, BrdU incorporation was strongly delayed. Thus, even though repressive E2F complexes were absent from promoters, cells expressing Ad-E2F1-Eco132 are unable to efficiently enter S-Phase. These experiments suggest that activating E2F is strictly required for cell cycle entry in T98G cells. Consistent with my results, it has recently been published that activating E2F is dispensable for continuous proliferation, but required for mitogen stimulated induction of S-Phase (Rowland et al. 2002; Zhang et al. 1999). Possibly, activating E2F is specifically required to induced transcription of proteins that are downregulated in quiescence, but are expressed in cycling cells, due to the action of alternative transcription factors. Alternatively, in quiescent cells, E2F target genes may exist in a chromatin conformation that requires recruitment of cofactors for remodeling and transcription. Accordingly, in cycling cells the role of activating E2F may be different, maybe effecting timing, or levels of target gene transcription. Altogether, studies with a DN-E2F1 confirm the importance of activating E2F for S-Phase induction from quiescence following mitogenic stimulation.

7.3. E2F Induces Histone Acetylation in a Temporally and Spatially Restricted Fashion

In many eukaryotes histone acetylation is a hallmark of transcriptional activation. Indeed, several E2F targets have previously been found to be acetylated in T98G cells (Takahashi et al. 2000). Here, I significantly expanded these studies. The data show that acetylation is not limited to a subset of E2F target genes involved in G1/S transition. Instead, all 25 investigated target genes exhibited induction of H4 acetylation in serum

stimulated T98G cells, including DNA-damage/apoptosis related target genes like *p73*, *pol*δ, and *msh2*. Thus, it appears that histone acetylation is a general feature of E2F target gene activation. Interestingly, there are strong differences in total acetylation on individual targets. Possibly, acetylation on each locus depends on events other than just the simple recruitment of one transcription factor to a target promoter at a given time.

Furthermore, I analyzed in-depth the histone acetylation patterns on four well-characterized E2F targets (*mcm3*, *mcm4*, *pcna*, and *p107*). Interestingly, acetylation of both H3 and H4 was transient, increasing through G1, peaking at G1/S transition, and then decreasing. Thus, acetylation paralleled the binding of activating E2Fs. The fact that acetylation was transient suggests that a deacetylase activity must be recruited once cells are in S-Phase. Consistent with this, complexes of E2F4 and p107 have been described in S-Phase cells, and these may recruit HDACs to E2F target genes during DNA replication. Alternatively, global and non-targeted deacetylation may restore histone acetylation to basal levels following induction, as has recently been suggested (Vogelauer et al. 2000; Katan-Khaykovich and Struhl 2002).

In addition to the temporal pattern of acetylation, its spatial distribution was also analyzed. Using amplicons that reach over several kb of these target promoters, I found that, unlike E2F binding, acetylation was more widespread. In fact, while being centered to the E2F sites, it extended over the whole promoter. However, acetylation did not spread far into the coding region, as little regulated acetylation was observed beyond two kb downstream of the transcriptional start site. This suggests that activating E2F remodels chromatin locally. Rather than increasing access to a large chromatin domain, the observed acetylation may represent a signal-like modification. This fits with the “histone code” model, where distinct modifications in the tails of histones can act sequentially or in combination, to create a code that is read by other proteins to bring about downstream events. Therefore, while histone acetylation of E2F target promoters may be important in transcriptional activation, it may not constitute the ultimate or total signal generated by E2F activators. Furthermore, it is important to note that the acetyl-histone antibodies used in this study recognize acetylation on any of the lysines in H3

or H4 tails. While total acetylation increased from G0 to G1 to S, acetylation of individual lysines may not follow the same temporal or spatial acetylation pattern. Recently, lysine specific CHIP grade antibodies against both H3 and H4 have been described (Suka et al. 2001). Making use of these reagents would allow determining the exact acetylation patterns on individual E2F target genes. Possibly, different HATs and/or HDACs contribute acetylation/deacetylation of different lysines on E2F targets. In summary, while induction of histone acetylation is likely to be an important part of E2F function, the exact “histone code” remains to be determined.

7.4. E2F is Sufficient for Induction of Histone Acetylation

Using the ER-E2F1 chimera, I showed that induced binding of ectopic E2F1 is followed by histone acetylation. Compared to the acetylation induced by serum, however, maximal levels of acetylation were lower, suggesting that additional signaling is required to achieve full acetylation, for example serum. Remarkably, acetylation induced by ER-E2F1 was delayed: E2F1 and cofactor recruitment significantly increased after only 20 minutes of treatment, whereas acetylation was not yet strongly induced above initial levels. Presumably assembly of HAT-complexes is followed by another step necessary for histone acetylation. Interestingly *p73*, a target promoter that was only weakly bound by E2F1 in serum stimulation, but well in the ER-E2F1 experiments, exhibited strong H3 and H4 acetylation. Possibly, a serum response is not sufficient to induce full activation of the *p73* gene, whose transcription is induced in responses to apoptotic stimuli. Other target genes (for example *msh2*, or *pol*δ) were not acetylated in ER-E2F1 experiments, even though E2F1 binding was induced, suggesting a requirement for additional signaling events. Nevertheless, these experiments show that E2F1 is sufficient to induce significant changes in acetylation on most E2F targets.

7.5. Activating E2F is Required for Full H4 but not H3 Acetylation

Time-course analysis in T98G cells showed that H4 acetylation closely correlated with binding of activating E2Fs, suggesting that they are responsible for this modification. Interestingly, for H3 the correlation was less

strict, as acetylation appeared to peak a little before maximal binding of E2F activators. Using the DN-E2F proved that inhibition of activator recruitment correlates with block of H4 acetylation. In contrast, only a partial block of H3 acetylation was observed. Thus H4, but not H3 acetylation appears to strictly depend on recruitment of activating E2F. Nevertheless, in the context of the DN-E2F, H3 did not become fully acetylated, indicating that E2F-dependent HAT recruitment contributes to full H3 acetylation. In cells infected with DN-E2F, removal of G0-specific E2F4/p130/HDAC complexes may be followed by binding of H3 HATs, which cause the observed partial H3 acetylation. For instance, Gcn5 may bind promoters in E2F-independent fashion, as has been suggested in yeast (Vogelauer et al. 2000). Alternatively, distinct HATs may contribute to full H3 acetylation by E2F-dependent and independent recruitment.

The differences between H3 and H4 acetylation are compelling, and suggest that H3 and H4 exhibit different susceptibility to HDAC removal. Interestingly, in quiescent $p107^{-/-}/p130^{-/-}$-KO MEFs, H3 acetylation of the *e2f1* gene was strongly increased (in comparison to WT MEFs), whereas only a modest increase in H4 acetylation was detected (Rayman et al. 2002). This fits with my findings that repressive E2F is predominantly required to maintain hypoacetylated H3. However, in these MEFs the absence of p107 and p130 also induced a moderate change in H4 acetylation on the E2F1 locus, a result not reproduced by expression of the dominant negative E2F mutant. This could result from untimely recruitment of activating E2F to the promoter in quiescent cells, recruiting an H4 HAT. In summary, it appears that H4 acetylation is strictly dependent on functional activating E2F, whereas removal of repressive complexes is sufficient to result in partial, but not full H3 acetylation.

7.6. Activating E2F Recruits the Tip60 Complex

The centerpiece of this study is the result that the Tip60 complex is involved in the transcriptional response of activating E2F. Four lines of evidence indicate that this is the case: First, four subunits of the Tip60 complex were recruited to E2F target promoters after serum stimulation of T98G cells. Importantly, the kinetics of TRRAP and Tip60 recruitment

paralleled association of activating E2F with target promoters. Second, whereas histone acetylation was more widespread, localization of all four subunits was strictly limited to the E2F sites in all tested promoters. Third, an ER-E2F1 chimera was sufficient to induce rapid binding of five different Tip60 complex components to E2F targets. Again, both timing and spatial distribution mimicked the E2F1 binding pattern. Fourth, when DN-E2F was overexpressed, no Tip60 complex components were recruited to promoters, proving a necessity of activating E2F in the process. How individual components of the Tip60 complex, and its HAT activity, contribute towards E2F-dependent transactivation, remains to be elucidated. Nevertheless, these results strongly imply an important role for the Tip60 complex in chromatin-remodeling at E2F target genes.

Recently, it has been published that, when overexpressed, not only E2F1, but also E2F4 interacts with TRRAP (Lang et al. 2001). Therefore, one would assume that TRRAP would be recruited to chromatin in quiescent cells, when E2F4 is predominant on all investigated E2F targets. However, my CHIP experiments failed to detect TRRAP and Tip60 on E2F targets in quiescent cells. Thus, no temporal correlation between E2F4 and TRRAP/Tip60 binding could be established, suggesting that the observed interaction may be an artifact of overexpression. Alternatively, TRRAP may be recruited by E2F4 in conditions not investigated in this study, (for instance differentiation specific events), or such a complex may be target gene specific.

While it is clear that Tip60 and E2F associate with each other on chromatin, I failed to show a physical association between Tip60 and E2F1 in lysates (data not shown). It is possible that this interaction can only occur on native chromatin. However, three subunits of the Tip60 complex (TRRAP, p400, and Tip49) have been published to bind E2F1 (McMahon et al. 1998; Fuchs et al. 2001; Dugan et al. 2002). It is also noteworthy that a complex highly similar to the Tip60 complex has recently been purified from mammalian cells (the p400 complex; Fuchs et al. 2001). The only difference between the two is the absence of Tip60 in the p400 complex, raising the possibility that Tip60 is a substoichiometric component of this complex. Interestingly, the p400 complex was identified as a critical target of the adenoviral oncoprotein E1A, whose main function is induction of cell cycle

entry by liberating E2F activity. Thus, based on my studies, targeting of the Tip60 complex by E1A may provide an additional avenue by which this oncoprotein regulates E2F-dependent transcription (Figure 28).

CHIP with antibodies to other HATs did not yield any reproducible and significant result. Previously, others have also failed to CHIP p300/CBP and Gcn5/PCAF on E2F targets (Rayman et al. 2002). Nevertheless, both classes of HATs have been shown to interact with activating E2Fs in biochemical assays (Marzio et al 2000; Martinez-Balbas 2000). Possibly, antibodies are not able to interact with these HATs on E2F targets in the context of chromatin, even though all these proteins have been successfully detected by CHIP at other loci. It will be important to determine their role in E2F-dependent transactivation. p300, CBP, Gcn5, and PCAF possess intrinsic, H3-specific HAT activity, but CBP and PCAF have also been published to acetylate E2F itself. Thus, distinct HATs might be responsible for histone and E2F acetylation. Furthermore, p300/CBP possess dual activity towards H3 and H4, and hence might contribute to H4, as well as H3 acetylation on E2F targets.

The fact that E2F itself can be acetylated is interesting. Both p300/CBP, and PCAF (but not Gcn5) were found to acetylate E2F1, E2F2, and E2F3 (but not E2F4, E2F5, or E2F6). Target lysines lie just adjacent to the DNA-binding-domain, and acetylation leads to prolonged protein half-life, enhanced DNA-binding, and increased transactivation potential (Martinez-Balbas et al. 2000; Marzio et al. 2000). A mutant E2F1 having the three acetylation-targeted lysines substituted by alanines has been described. It would be interesting to see how such a protein effects cofactor recruitment, and/or histone acetylation. Such questions could be addressed using the adenoviral system described in this study, or using retroviruses. Similarly, it would be useful to analyze other E2F mutants in these systems, such as the mutant lacking the Cyclin A/CDK2 interaction domain, which should prevent downregulation of E2F's DNA-binding in S-Phase (Krek et al. 1995).

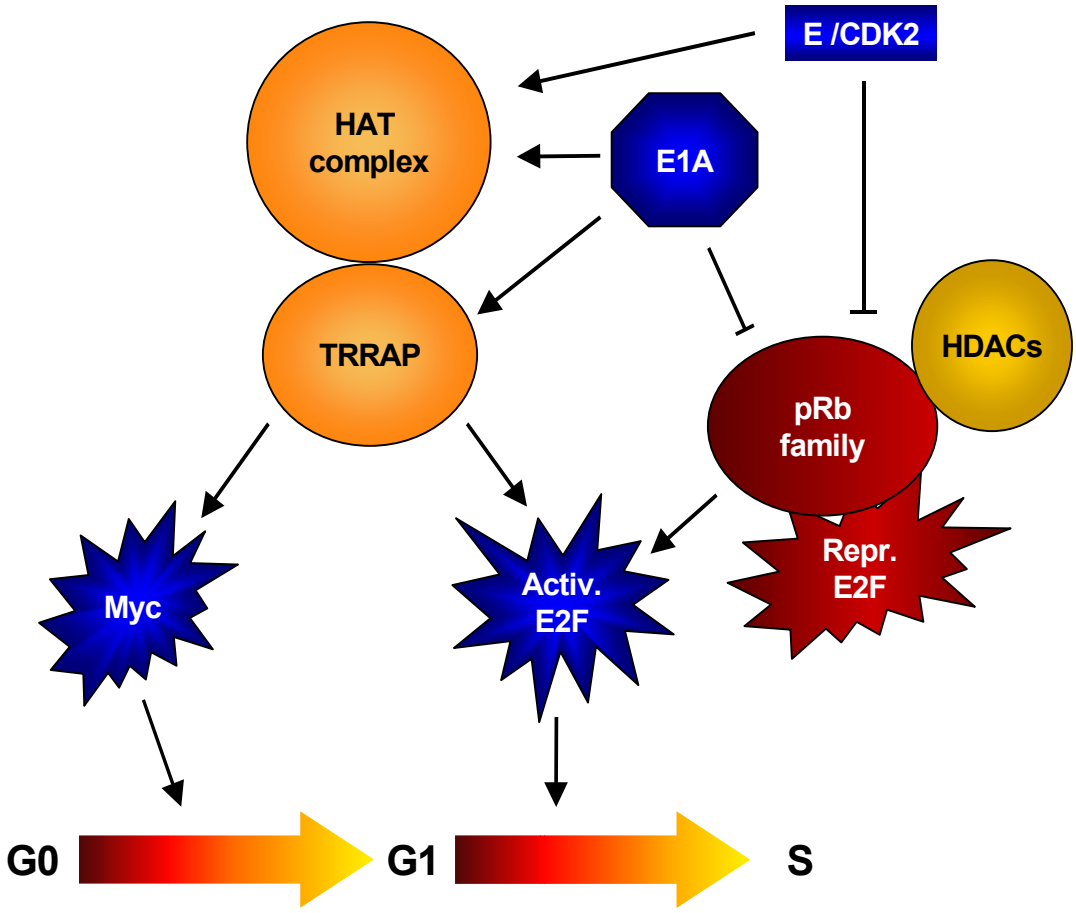

Figure 28: A Model for a Role of TRRAP and its HAT-Complexes in the Regulation of Growth and Proliferation.
The E2F/Rb and Myc/Max/Mad networks of transcription factors regulate growth and proliferation. Both recruit TRRAP and its associated HATs. These proteins are also targets of E1A, and/or components of the cell cycle regulatory network. Thus, TRRAP or its associated proteins may play an important role in tumor development.

Consistent with a role of E2F acetylation, Rb-associated HDACs are capable of deacetylating E2F1. Interestingly, Rb itself can also be acetylated by p300 (Chan et al. 2001). In this case, acetylation inhibits phosphorylation by Cyclin/CDK complexes, thus stabilizing Rb-repression. Taken together, these results suggest that p300 can contribute to both S-Phase progression (by acetylation of activating E2Fs, and possibly by coactivating E2F transcription), and S-Phase inhibition (by maintaining repressive Rb/E2F complexes). In summary, whether the differential effects of p300 in G1/S transition result in signals promoting or inhibiting the cell cycle, remains to be determined.

7.7. Do E2F Targets Require Additional Chromatin-Remodeling Before Transactivation?

HAT-complexes represent only one type of chromatin modifying enzymes involved in transcriptional responses. E2F proteins have been found to interact with a variety of other chromatin-remodeling-complexes, for example the Swi2/Snf2-family proteins Brg1/hBrm, and also with DNA methyltransferases (DNMT1), histone deacetylases (HDACs1 and 2), histone methyltransferases (SuVAR39H1), and Polycomb-group proteins (PcGs, such as Bmi1, Ring1, HPC2). However, HDACs, HMTs, and PcG proteins are all primarily involved in repression and/or silencing. In contrast, Swi/Snf-chromatin-remodeling-complexes can coactivate transcription by several transcription factors. Thus, it would be interesting to test whether chromatin-remodeling participates in E2F coactivation, prior or subsequent to G1 specific histone acetylation events. Cell lines lacking, or harboring mutations in, some of the subunits of these complexes have been described, and present a potential tool to study such events (Zhang et al. 2000; Dahiya et al. 2001).

7.8. The Tip60 Complex is Involved in the Transcriptional Activity of Myc

Myc recruits TRRAP, and induces H4 acetylation in early G1 following mitogenic stimulation, suggesting that TRRAP-associated HAT-complexes are involved in Myc-dependent chromatin-modifying events (McMahon et al 1998; McMahon et al. 2000; Bouchard et al. 2001; Frank et al. 2001). Consistent with this, I showed that Myc interacts with Tip60 in transient transfections. Tip60 was also recruited to several Myc targets, as judged by CHIP (S. Frank and B. Amati, unpublished data). Additionally, like E2F, Myc recruited at least four other subunits of the Tip60 complex to chromatin, TRRAP, p400, Tip48, and Tip49 (S. Frank, T. Parisi, and B. Amati, unpublished data). Previously, it has been proposed that Myc interacts with Tip48/49 and TRRAP within different complexes (Nikiforov et al. 2002). However, this contrasts with the observation that Myc can interact with these proteins in the context of the purified p400 complex, suggesting that Myc recruits a TRRAP/Tip60/p400 complex to chromatin (Fuchs et al. 2001). In any case, this does not rule out the possibility that other, TRRAP or Tip48/49 containing HAT-complexes are also recruited by Myc. In fact, I showed that Myc bound two STAGA complex

components in transient transfections. Myc can also induce H3 acetylation on several promoters in a variety of cell lines (P. Fernandez, S. Frank, and B. Amati, unpublished data). In summary, Myc probably recruits two classes of TRRAP-associated HATs, Tip60 and GCN5/PCAF, to mediate acetylation of H4 and H3, respectively. Alternatively, unidentified HATs may also contribute to total histone acetylation in a Myc-response.

7.9. Are TRRAP Containing HAT-Complexes Involved in G1/S Transition?

The finding that E2F and Myc both recruit a TRRAP/Tip60/p400 complex is striking, and establishes this complex as a part of the machinery that controls the cell cycle. Interestingly, adenoviral E1A protein can bind the p400 complex through a short domain in its N-terminus. Chimeric proteins in which this domain was fused to the Myc DNA-binding-domain mimic Myc activity by binding TRRAP, inducing histone acetylation, activating hTERT expression, and promoting cellular transformation (S. Frank and B. Amati, unpublished results; Deleu et al. 2001; Nikiforov et al. 2002). Thus, the TRRAP/p400-binding motif of E1A functionally substitutes for the Myc N-terminus, strengthening the notion that recruitment of these co-factors is crucial for Myc activity. In the context of full-length E1A, the TRRAP/p400-binding domain is required for maximal transforming activity, and for bypassing cell cycle arrest imposed by the CDK2 inhibitor $p27^{Kip1}$ (Alevizopoulos et al 1998; Deleu et al. 2001). These data indicate that regulation of TRRAP/p400 complexes is an important role of E1A. In addition, several groups also reported a modulation of PCAF's HAT activity by E1A, thus implying the other major TRRAP-containing HAT-complex as an E1A-target (Chakravarti et al. 1999; Hamamori et al. 1999a; Reid et al. 1998). However, if HAT activity is actually enhanced or inhibited by E1A remains controversial. In fact, it might depend on the cellular context, since PCAF is involved in a broad variety of coactivation events. Nevertheless, all these observations suggest that, like other cellular targets of E1A, TRRAP complexes are key regulators of cellular growth and proliferation.

E1A substitutes for Cyclin/CDKs function after adenoviral infection. For example, E1A binds and inactivates Rb-family proteins, usually a critical function of Cyclin/CDK complexes. Similarly, E1A stimulates the HAT activity

of p300, also a target of Cyclin E (Ait-Si-Ali et al. 1998). Interestingly, the timing of this stimulation (G1/S transition) fits with a possible role in E2F-dependent transcription. Here, I show that Cyclin E is capable of binding two STAGA complex components, again suggesting that a TRRAP-associated HAT-complex is regulated during G1/S transition. Consistent with this, the HAT activity of PCAF was downregulated upon cell cycle arrest by CKIs. Overall, it appears likely that different HAT-complexes are playing important roles in regulation of G1-specific transcriptional events, and are in turn controlled by G1-specific Cyclin/CDKs. This regulation highlights a second potential pathway essential for cell cycle control that runs in parallel to the Cyclin/CDK/Rb pathway in mammalian cells (Figure 28).

7.10. A Possible Role for TRRAP-Associated HATs in DNA Repair and Apoptosis

In yeast, Esa1p-dependent H4 acetylation is required for double strand break repair (Bird et al. 2002), and in human cells the Tip60 complex is involved in apoptosis following γ-irradiation (Ikura et al. 2000). Tip60 levels increase after DNA-damage by UV irradiation, presumably because ubiquitin/proteasome-dependent degradation of Tip60 is downregulated (Legube et al. 2002). In contrast, Tip60 levels remained constant during the G0/G1 and G1/S-Phase transitions in T98G cells. However, it is possible that the protein is differentially regulated in response to serum and to DNA-damage. Interestingly, DNA-damage also induces stabilization of E2F1. This may represent a distinct upregulation of E2F1/Tip60, which is not normally activated in cells responding to mitogenic stimuli, but specifically after DNA-damage.

Similar to the Tip60 complex, Gcn5-containing HAT-complexes may also play a role in DNA-damage responses, as the human STAGA complex contains the UV-damaged-DNA-binding-protein 1 (DDB1), which is involved in nucleotide excision repair (Brand et al. 2001; Martinez et al. 2001). The STAGA related TFTC-Complex preferentially binds UV-irradiated DNA, and UV-damaged DNA inhibits TFTC-mediated Pol_{II}-transcription. Moreover, TFTC is recruited in parallel with the nucleotide-excision-repair-protein XP-A

to UV-damaged DNA. Finally, the DNA-binding subunit of DNA-dependent protein kinase, which is involved in DNA-damage signaling, interacts with and phosphorylates Gcn5, downregulating its HAT activity (Barlev et al. 1998). In all those cases, the role of the HAT-complexes may be both transcription-related and unrelated. For example, they might remodel chromatin structure to allow easier access for the repair machinery to sites of damaged DNA.

Another place where HATs may be involved in DNA repair is via p53, a regulator of transcription in response to DNA-damage. p53 induces transcription of various target genes, inducing either cell cycle arrest, or apoptosis (for review, see Vousden 2002). Several coactivators of p53 have been identified, including TRRAP and Gcn5 (Barlev et al. 2001). In yeast, p53 is capable of interacting with the NuA4 complex, predicting an interaction with the Tip60 complex in mammalian cells (Nourani et al. 2001). Consistent with this, preliminary analysis indicates that Tip60 is indeed recruited to p53 targets following DNA-damage (D. Donjerkovic and B. Amati, unpublished results). Therefore, the Tip60 and STAGA complexes may be involved in several distinct functions after DNA-damage has been inflicted.

7.11. TRRAP-Associated HATs and Tumorigenesis

Results from this study and from other recent publications indicate that TRRAP and its associated HATs are involved in a variety of cellular processes. Interestingly, E2F, Myc, and p53 are transcription factors commonly deregulated in human tumors. Thus, TRRAP, and/or Gcn5/PCAF and Tip60 may be targets of pathways deregulated in tumorigenesis. In fact, PCAF has previously been suggested to be a tumor suppressor (Schiltz and Nakatani 2000), and cell lines with mutations in the PCAF gene have recently been reported (Ozdag et al. 2002). The oncoprotein E1A apparently targets both PCAF/Gcn5 containing complexes and the TRRAP/p400 complex, results that strengthen the notion that TRRAP containing complexes are involved in tumorigenesis (Figure 28). Furthermore, the HATs p300/CBP behave as haplo-insufficient tumor suppressors in mice (Petrij et al. 1995; Yao et al. 1998; Oike et al. 1999b), and mutations of p300 have been found in human tumors (Muraoka et al. 1996; Gayther et al. 2000; Bryan et al. 2002). Heterozygosity for CBP, occurring in human Rubinstein-Taybi-Syndrome, also

predisposes for tumor development (Petrij et al. 1995; Giles et al. 1998; Oike et al. 1999a; Murata et al. 2001). Similarly, the gene encoding MOZ, another HAT, is a target of translocations that can lead to leukemogenesis (Giles et al. 1997; Borrow et al. 1996). Finally, the BAF complex components Ini1/Snf5 and Brg1 are tumor suppressors of the Rb/E2F-pathway, proving that mutations of other chromatin-remodeling-complexes can occur in cancer development (reviewed in Phillips and Vousden 2000; Klochendler-Yeivin and Yaniv 2001).

CONCLUSIONS AND PERSPECTIVES

E2F-family proteins are critical regulators of S-Phase entry, and quiescence. Hence, they govern cellular proliferation in metazoans by regulating transcription of hundreds of target genes, whose individual functions and contributions towards proliferation are just emerging in recent years. Importantly, while the mechanisms of transcriptional repression by E2F also have begun to be understood, how E2F transactivates its targets still remains an unresolved mystery.

Tip60 is involved chromatin-remodeling by E2F. An important clue towards elucidating these molecular mechanisms came from the identification of TRRAP, a subunit of chromatin-modifying HAT-complexes, as a protein interacting with E2F. Later, it was shown that E2F targets are deacetylated (by a complex of HDAC1/mSin3B) in G0, but acetylated at G1/S transition. Here, I expanded these studies by showing that E2F is required and sufficient for induction of histone acetylation on E2F targets. It does so by recruiting at least one of the two TRRAP containing HAT-complexes, the Tip60 complex. To my knowledge, this is the first time that a chromatin-modifying-enzyme has been experimentally implicated in E2F-dependent acetylation. However, while these results shed light on events governed by E2F, they raise even more questions.

How about additional enzymatic activities? First, it will be critical to determine whether the Tip60 complex is indeed required for E2F-dependent induction of transcription. Experiments to address these issues are currently underway. Furthermore, while explaining H4 acetylation, the Tip60 complex is unable to acetylate H3. Thus, other HAT-complexes must also be present at E2F targets. Moreover, other chromatin modifying and/or remodeling enzymes may combine with HATs to create a chromatin environment that allows for efficient transcription of E2F target genes. It will be important to determine the identity of such cofactors in future experiments.

What is the role of Tip60 *in vivo*? HAT-complexes are probably involved in regulatory processes other than just transcriptional regulation. Thus, while it will be important to find out what other transcription factors depend on Tip60

to induce transcription, other chromatin based processes should also be investigated. In this context, it is noteworthy that the Tip60 participates in DNA-damage response, and apoptosis. The human STAGA/TFTC/PCAF complexes may also participate in various processes, as suggested by the presence of splicing-associated and DNA-damage-associated proteins within these complexes. Similarly, TRRAP may have functions other than just being a subunit of HAT-complexes. The identification of the remaining subunits of the Tip60 complex, alternate complexes, and mechanisms regulating these complexes will help us understand such functions.

TRRAP and Tip60 – potential tumor suppressors? The work presented here shows that Tip60 and TRRAP participate in the E2F/Rb, as well as the Myc/Max/Mad transcriptional regulatory pathways. Both of these are key elements of a network that governs growth and proliferation, and tumorigenesis. Thus, it is tempting to speculate that Tip60 and/or TRRAP may themselves play important roles in the processes that lead to tumor formation in mammalian organisms. Other chromatin-remodeling subunits, such as Ini1/Snf5 and Brg1, cofactors of E2F/Rb-dependent repression, are indeed known tumor suppressors. Moreover, PCAF, another TRRAP-associated HAT, has been suggested to be a tumor suppressor, and p300/CBP, a possible coactivator of E2F, does exhibit tumor suppressor characteristics. In addition, TRRAP and Gcn5 interact with the tumor suppressor p53. Finally, the adenoviral oncoprotein E1A targets several of these HATs, and other chromatin-remodeling-complexes. Taken together, these data suggest that regulation of chromatin structure is critical for normal cellular behavior, and that mis-regulation of this process can ultimately contribute to cellular transformation. Thus, understanding how chromatin-remodeling and modification cooperate to control transcription, may help us elucidate mechanisms causing transformation, and ultimately provide clues how to fight cancer. Consistent with these ideas, it has been suggested that the enzymes affecting the acetylation status of chromatin should be targets in cancer therapies (reviewed in Marks et al. 2001; Johnstone 2002; Kelly et al. 2002). Indeed, small inhibitory molecules targeting HDACs are active *in vitro* and *in vivo*, causing cancer cell growth arrest, differentiation and/or apoptosis. Several HDAC inhibitors are currently in clinical trials as anticancer agents,

and some have shown activity against cancers. In the light of these results, it may well be worth expanding these searches for molecules inhibiting HATs, as well as other chromatin modifying enzymes.

MATERIALS AND METHODS

8.1. Cell Culture Techniques

8.1.1. Mammalian Cell Culture

Adherent mammalian cells (T98G, U2OS/ER-E2F1, U2OS, WS1, 293T, Bosc23, and Rat1 cells) were cultured in DMEM (Biowhittaker 12-064F) supplied with 10% Fetal Bovine Serum (FBS; Gemini Bioproducts 00-106) and antibiotics (Penicillin 10000 u/ml, and Streptomycin10mg/ml). Cells were incubated at 37°C in a 10% CO_2 atmosphere saturated with water. Cells were rendered quiescent by growing to confluence followed by incubation for two days in serum-free medium. To induce cell cycle entry, cells were harvested by trypsinization, and reseeded onto plates containing DMEM/10%FCS.

8.1.2. Transient Transfection of Bosc23 or 293T Cells

Cells at 60 to 80% confluence were transiently transfected with 2 to 20 μg of total plasmid according to standard calcium phosphate transfection protocols, or using Fugene 6 Lipofect transfection reagent (Roche) according to the manufacturer's protocol.

8.1.3 Generation of High Titer Retroviral Supernatants and Infection Protocol

Generation of high titer retroviruses was performed as described in previous protocols (Morgenstern and Land 1990; Vlach et al. 1996). Briefly, the gene of interest was cloned into the polylinker of pBabe retroviral vectors. Plasmids were transfected into Bosc23 or Phoenix cells (60% confluent 10cm dishes) in the presence of 25μM Chloroquine using transient transfection methods. After 12 hours the medium was aspirated, and replaced by 5ml of fresh medium. The retroviral supernatant was harvested after 48hrs, filtered to remove cell debris, and aliquots stored at -70°C. For infection, an aliquot was thawed at 37°C, added to 5ml of growth medium containing 4-8 μg/ml polybrene. This infection cocktail was vortexed and added to subconfluent, growing cells. The next day, the cells were passaged and selected for at least two days with the appropriate drug (Puromycin 2.5μg/ml, G418 1mg/ml, Phleomycin 50μg/ml, and Hygromycin B 150μg/ml).

8.1.4. Generation of Adenoviruses

The cDNAs expressing human WT E2F1 or E2F1-Eco132 were cloned into the polylinker of pAdTrackCMV, followed by recombination in *E. coli*, viral production and amplification in 293T cells, and purification of recombinant adenovirus as described previously (He et al. 1998).

8.1.5. High Efficiency Infection of T98G Cells with Adenovirus

In order to achieve highly efficient adenoviral infection, I used a modification of a previously described protocol (Fasbender et al. 1997; Frank et al. 2001). Briefly, an infection cocktail of adenovirus ($0.5x10^9$ particles/confluent plate of T98G cells), 5 to 10ml of serum free DMEM, and 5 to 10μl of Superfect transfection reagent (QIAGEN) was mixed by vortexing and incubated at RT for 10min. Trypsinized cells were resuspended directly in the mixture, and plated into fresh, serum containing DMEM. Infection efficiency was verified by scoring for GFP positive cells by direct fluorescence in a Zeiss Axiovert S100 AttoArc 2 HBO 100W fluorescent microscope (adenoviral vectors contain a separate mRNA encoding GFP). Typically, after 12 hours of infection 100% of the cells exhibited GFP expression.

8.1.6. BrdU/PI FACS Analysis

Cells were treated with 10μg/ml (approx. 33μM) BrdU for the desired time (pulse labeling 30min) by directly adding 33.3μl of 10mM BrdU aqueous stock solution per 10mls of DMEM. Cells were harvested by trypsinization, washed once in PBS, and resuspended in 200μl PBS. The suspension was added slowly to 5ml ice cold 70% v/v ethanol while vortexing (fixation step). Cells were then stored on ice for at least 30min. For FACS, fixed cells were pelleted at 1200rpm for 10min at 4^oC. The supernatant was aspirated; the cells were resuspended in 0.5ml PBS, and transferred to Eppendorf tubes. Cells were spun in a tabletop centrifuge at 2000rpm at RT for 5min, and the supernatant was aspirated. Pellets were resuspended in 0.5ml 3M HCl, and incubated for 20min at RT. 0.5ml of PBS was added and cells pelleted by centrifugation. Then cells were resuspended in 100mM sodium borate ($Na_2B_4O_7$) pH 8.5, and incubated at RT for 3min. 0.5ml PBS containing 2.5% v/v FBS (block) was

added, and cells pelleted were by centrifugation. Cells were resuspended in 0.5ml block, and incubated at RT for 15min. After pelleting cells by centrifugation, they were directly resuspended in 20 to 50μl of FITC-conjugated anti-BrdU antibody (Becton Dickinson #7583), and incubated on ice in the dark for 45min. 0.5ml of block was added and after centrifugation pellets were resuspended in 0.5ml of block, containing 20μl of PI (stock solution of 250μg/ml is 25x) and 20μl of RNase A (stock solution of 10mg/ml is 400x), and transferred to FACS tubes. After incubating at 37°C for 5 to 30min cells were stored on ice and analyzed in a FACScalibur (Becton Dickinson).

8.2. Biochemical Techniques

8.2.1. List of Antibodies

E2F1 sc-193 (C-20) and KH20&KH95 (Upstate Biotech 05-379); E2F2 sc-633 (C-20); E2F3 sc-878 (C-18); E2F4 sc-1082 (A-20); E2F5 sc-999 (E-19); p130 sc-317 (C-20); c-Myc sc-764 (N-262); c-Myc sc-42 (C-33), sc-40 (9E10), sc-788 (C-19), or sc-41 (C-8); Max sc-765 (C-124), or Max2.1; Pol_{II} sc-899 (N-20); Acetyl-Histone H3 Upstate Biotech 06-599; Acetyl-Histone H4 Upstate Biotech 06-866; Tip60 affinity purified CLHF, CLGT, and RLPV (see 8.2.2; p. 111); TRRAP CT affinity purified (K. Alevizopoulos); Gcn5 N-terminal (S. Roth); Spt3 #623 (E. Martinez); Flag M2 (Sigma); HA HA.11 (Babco Ascites); Tip48 and Tip49 affinity purified (T. Parisi); p400 affinity purified F20/21 (M. Fuchs, H.M. Chan, D.M. Livingston, unpublished); Cyclin E HE12, HE172 (E. Lees); Cyclin E2 8E6 (E. Lees); NRS and 419 controls (D. Parry).

8.2.2. Peptides for Tip60, Gcn5, PCAF, and HBO1 Antibodies

Peptides for antibodies were chosen according to some criteria from published human protein sequences. The following peptides were synthesized by Research Genetics:

Tip60 RLPV peptide: CRLPVLRRNQDNEDEWPLAE (aa 11-29)

Tip60 CIEL peptide: CIELGRHRLKPW (aa 234-245)

Tip60 CLGT peptide: CLGTDEDSQDSSDGIPSAPRM (aa 192-212)

Tip60 CLHF peptide: CLHFTPKDWSKRGKW (aa 499-512)

Gcn5 CT peptide: CASALEKFFYFKLKEGGLIDK (aa 817-837)

PCAF CT peptide: CANILEKFFFSKIKEAGLIDK (aa 783-813)

HBO1 CT peptide: KRSNSNKTMDPSCLKWTPPKGT (aa 589-611)

8.2.3. PIERCE KLH Kit Coupling Protocol

For conjugation, peptides were dissolved in 0.2 to 0.5ml of conjugation buffer (83mM sodium phosphate buffer pH 7.2, 100mM EDTA, 900mM NaCl, 0.02% sodium azide). The peptide must be in molar excess of the KLH carrier, thus up to 2mg peptide per 2mg KLH carrier protein was used. 2mg of KLH carrier protein were suspended in 0.2ml H_2O without vortexing or heating. The peptide and the carrier solutions were mixed immediately, and incubated for two hours at RT. If a precipitate formed, the reaction mix was briefly centrifuged (the precipitate was later added to the pooled, desalted fractions). The conjugate was purified by Gel-Filtration to remove EDTA. The conjugate containing fractions were filter sterilized, and frozen to -20°C before being sent for rabbit immunization. Pocono Rabbit Farm generated immune sera according to standard protocols.

8.2.4. PIERCE Sulfolink Kit Protocol: Coupling of Peptides to Sulfolink Resin

For coupling, a column was packed with 4ml 50% v/v Sulfolink beads, and equilibrated with 6 volumes of coupling buffer (50mM Tris/HCl, 5mM sodium EDTA pH 8.5), then drained. The peptide was dissolved in sample preparation buffer (100mM sodium phosphate buffer, 1mM sodium EDTA pH 6.0), and added to the beads. The column was filled up with coupling buffer, and the mix was incubated on a shaker at RT for 15min. After settling the beads for 30min, the buffer was drained and the column washed with 3 volumes of coupling buffer. The OD_{280} of the wash was monitored to determine the efficiency of peptide binding. Then, nonspecific binding sites of the resin were blocked for 15 minutes at RT by incubating on a shaker in 1ml of blocking solution (80mg L-cysteine to 10ml coupling buffer, which creates a 50mM cysteine solution) per column. After settling the beads for 30min, the buffer was drained and the column washed with 2 to 4 volumes of washing buffer (1M NaCl) and 2 times with 4 volumes of PBS. Columns were stored in PBS containing 0.05% sodium azide at 4°C.

8.2.5. Affinity Purification of Crude Antisera

10ml of crude rabbit serum were diluted in 40ml PBS containing 10% glycerol. The peptide affinity column to was brought to RT, and washed with 5 volumes of PBS containing 10% glycerol to equilibrate. Then the serum mix was added to the beads, and incubated overnight on a shaker at $4^{o}C$. The column was subsequently washed with PBS to eliminate nonspecific proteins (efficiency of wash checked by monitoring OD_{280}), then washed once with 10mM sodium phosphate buffer pH 6.8. Bound antibody was eluted with 4 volumes of elution buffer (100mM L-glycine pH 2.5-3) by collecting 1ml fractions (100μl of 1M sodium phosphate pH8.0 was added to each tube before elution to neutralize the factions). The OD_{280} of the elution was monitored, and the elution repeated if necessary. Then, the column was eluted again with 4ml of 100mM triethylamine pH11.5 (100μl of 1M sodium phosphate pH8.0 was added to each tube before elution to neutralize the factions). Fractions containing protein were pooled, and dialyzed against PBS overnight at $4^{o}C$. Dialyzed antibody was concentrated in a Centricon 30. To determine purity of the antibody, samples were separated by SDS-PAGE and stained with Coomassie Brilliant Blue. To restore the purification column for further use, it was washed with 8 volumes of PBS to remove any protein. Columns were stored in 2ml degassed PBS containing 0.05% sodium azide.

8.2.6. Coupling Antibodies to Protein-A Beads

2mg purified antibody were bound to 1ml Protein-A beads (50% w/v) by incubating overnight at $4^{o}C$ on a shaker. The beads were collected by spinning at 3000g for 10min (the supernatant was also retained, as it may contain residual antibody), and washed twice with 10 volumes sodium borate (50-200mM, pH > 8.3). For coupling, the beads were resuspended in 10ml sodium borate buffer containing 20mM DMP. The solution was incubated at RT for 30min while rocking. The reaction was stopped by washing in 10 volumes of 200mM ethanolamine pH 8.0. To completely block the reaction, the beads were incubated in 10 volumes of 200mM ethanolamine pH 8.0 for 2hrs. The beads were then washed in PBS, and resuspended in PBS/0.1% sodium azide.

8.2.7. Generation of Protein Extracts and Immunoprecipitation (IP)

Cultured cells were washed once in PBS and harvested by scrapping with a rubber policeman. Suspensions were briefly centrifuged in a tabletop centrifuge and the supernatant aspirated. Pellets were lysed on ice in IPH buffer (50mM Tris/HCl pH8.0, 150mM NaCl, 5mM EDTA, 0.5%v/v NP40) containing protease inhibitors (Roche Complete protease inhibitor cocktail), phosphatase inhibitors (20mM sodium fluoride and 40μM sodium orthovanadate), and deacetylase inhibitors (5mM sodium butyrate). After incubation for 20 to 60min cells were centrifuged for 10min at $4^{o}C$ at 14000 RPM. Protein concentration was determined by PIERCE BCA protein assay kit. For immunoblotting, small amounts were boiled in sample buffer, and separated by SDS PAGE. For IPs, antibodies against the desired proteins and 30μl of a 50% protein-A (or protein-G) slurry were added to the extract, and the mix was incubated at $4^{o}C$ on a shaker for 2 to 6 hrs. Cells were pelleted by centrifugation and washed 3 to 4 times with IPH buffer. The pellets were boiled in SDS-PAGE sample buffer, and precipitated proteins separated by SDS-PAGE.

8.2.8. Immunoblotting

SDS PAGE was carried out according to standard procedures. Proteins were transferred to PVDF membranes at 25V for at least 2 hours. Membranes were incubated in blocking solution (PBS, 0.1% Tween-20, 5% w/v milk powder) for at least 1 hour, followed by incubation in blocking solution containing the appropriate antibody (see above) for at least 1 hour at RT. After washing 4 times for 5min at RT with PBS containing 0.1% Tween-20, membranes were incubated in blocking solution containing the appropriate, HRP-conjugated secondary antibody for at least 30min. After washing 4 times for 15min at RT with PBS containing 0.1% Tween-20, and rinsing twice quickly with PBS, reactive proteins were detected by enhanced chemoluminescence (ECL; Amersham) according to the manufacturer's protocol.

8.2.9. Immunoprecipitation-Histone-Acetyltransferase (IP-HAT) Assay

IP-HAT assays were performed similarly to previously published protocols (Brownell and Allis 1995; Brownell et al. 1996). Cell culture and Immunoprecipitation was performed as described above, except that the IPs were incubated overnight. After the last wash of the IP, the supernatant was totally aspirated and the pelleted beads were resuspended in 30μl of reaction buffer (50mM Tris/HCl pH 8.0, 10% glycerol, 1mM DTT, 1mM PMSF, 0.1mM EDTA) containing 25μg histones (Boehringer Mannheim histone mix; stock solution: 5mg/ml histones in 1mM HEPES pH 7.5, 100mM PMSF), 1mM sodium butyrate, and 50nCi ^{3}H or ^{14}C labeled Acteyl-CoA (Amersham; e.g. 0.2 to 0.4μl of 2-10Ci/mmol). The reaction mix was incubated at 30°C for 10 to 60min. Then the mix was spotted onto Whatman P81 phospho-cellulose squares (2x2 cm) and allowed to air dry. Dry filters were washed 3 times for 10min at RT with 200mM sodium bicarbonate pH 9.2 to remove unincorporated radioactive Acetyl-CoA. The filters were then placed in scintillation vials, and 2mls of scintillation fluid was added. After incubating the vials at RT for 10min, the amount of incorporated radioactivity was determined using a liquid scintillation counter. Alternatively the reaction mixtures were added to 30μl of 2x SDS-PAGE loading buffer and proteins separated by SDS-PAGE. Gels were dried on a Stacked Gel Dryer SGD300 (SAVANT) for 1 hour at 80°C, and exposed to Kodak Biomax Films at -70°C for at least one day.

8.2.10. CHIP Assay

CHIP was performed according to previous protocols (Orlando et al. 1997; Frank et al. 2001). Formaldehyde was added directly to cultured cells (approx. $5x10^{6}$–$1x10^{7}$ cells per CHIP, i.e. six to eight confluent plates of T98G cells per time-out for four CHIP triplicates) to a final concentration of 1% to fix at RT for 10min. Fixation was stopped by addition of glycine to final concentration of 0.125M, and cells incubated for 5 min. Then plates were rinsed twice with TBS at RT. TBS was aspirated completely, and cells harvested in SDS buffer (100mM NaCl, 50mM Tris/HCL, pH8.1, 5mM EDTA ph 8.0, 0.2% sodium azide, 0.5% SDS) containing protease inhibitors. Cells were pelleted by

spinning in tabletop centrifuge for 10min at 2000RPM, and resuspended in 4ml of ice-cold IP Buffer for sonication (IP buffer: 1 volume SDS Buffer + 0.5 volume Triton Dilution Buffer. Triton Dilution Buffer: 100mM Tris/HCl pH 8.6, 100mM NaCl, 5mM EDTA, 0.2% sodium azide, 5.0% Triton X-100). Samples were sonicated to an average length of 500-1000bp (e.g. pulse for 20 seconds at power setting 3 and 100% duty cycle of Branson Sonifier 450, big probe), and the volume adjusted with IP buffer to 1ml/IP. Lysates were precleared with 30μl of Protein-A beads (50% v/v in TE containing 0.2mg/ml sonicated salmon sperm DNA and 0.5mg/ml lipid-free BSA) for 30min at 14000RPM. Then, 20μl lysate were removed to be used as total. The primary antibody was added, and the mix incubated overnight at $4^{o}C$ on a rotating wheel. After centrifugation for 20min at 14000RPM at $4^{o}C$, the supernatant was added to 30μl of 50% Protein-A beads, and incubated on a rotating wheel for two hours at $4^{o}C$. Pellets were washed 3 times in 1ml Mixed Micelle Wash Buffer (15ml 5M NaCl, 10ml 1M Tris/HCl, pH 8.1, 5ml 0.5M EDTA, pH 8.0, 40ml 65% w/v sucrose, 1ml 10% sodium azide, 25ml 20% Triton X-100, 10ml 10% SDS, H_20 to 500ml) 2 times in 1ml Buffer 500 (0.1% (w/v) deoxycholic acid, 1mM EDTA, 50mM HEPES, pH 7.5, 500mM NaCl, 1% (v/v) Triton X-100, 0.2% sodium azide), 2 times in 1ml LiCl/detergent buffer (0.5% (w/v) deoxycholic acid (sodium salt), 1mM EDTA, 250mM LiCl, 0.5% (v/v) NP-40, 10mM Tris/HCl, pH 8.0, 0.2% sodium azide) and once in 1ml Tris/EDTA pH 7.4. 250μl of a 1% SDS, 0.1M $NaHCO_3$ solution was added to both the pellets and the totals, and samples incubated overnight at $65^{0}C$ to elute immune complexes, and to reverse the crosslinks. After a brief centrifugation, the elute was removed and added to 250 μl of proteinase K solution (1.5μl 20mg/ml glycogen in sterile H_20, 5μl 20mg/ml proteinase K stock, 244.5μl TE buffer, pH 7.6), and incubated at $37^{o}C$ for 2 hours. 50 μl of 4 M LiCl was added, then 500μl phenol/chloroform/iso-amyl-alcohohl. Tubes were shaken vigorously for 90 seconds and phases separated for 10min at 14000RPM at RT. The upper phase was added to 1ml of 100% ethanol, mixed, and incubated at $-20^{o}C$ for 15 min. The DNA was precipitated by microcentrifuging for 30min at $4^{o}C$ at 14000RPM, and the pellets washed with 1ml 70% ethanol at $4^{o}C$ for 5min at

14000RPM. The supernatant was aspirated, the sample air-dried for 10min, and resuspended in 300µl H_20.

8.3. Molecular Biology Techniques

8.3.1. Subcloning

Subcloning of cDNAs into mammalian expression, retroviral, and adenoviral vectors was as described (Sambrook et al. 1989), using QIAGEN plasmid miniprep (#12161), maxiprep (#12163), and QIAquick gel extraction (#28704) kits according to the manufacturer's protocols.

8.3.2. Taqman Real Time PCR Analysis

For analysis of DNA samples by real time PCR, a reaction mix of 6µl DNA, 4µl primer mix (H_2O containing 0.33µl of 45µM primer stock per primer) and 10µl of SYBR Green PCR Master mix (Applied Biosystems #4309155) was analyzed in a Perkin Elmer Gene Amp 5700 Sequence Detector for 40 cycles (15sec 94°C, 15sec, 55°C, 1min 72°C).

8.3.3. Primer Design and List of Primers

Primers against DNA of E2F targets for both CHIP and mRNA analysis was performed by using computer assisted primer design (PrimerExpress 2.1).

Gene	Position	Acc. Num.	AP number	Sequence 5'-3' (upper primer 1st line, lower primer second line)	Comment	Reference
e2f1	E2F site				OK	Primers from Takahashi et al. 2000
cdc2	E2F site				good	
cdc6	E2F site				no good	
cdc25a	E2F site				no good	
b-myb	E2F site				no good	
p107	E2F site				great	
ccna1	E2F site				good	
actin					no good	
mcm4	E2F site	NT023806	2755/56	GCGGTTTGGGAGCGCTA GACGACGCTCGCGGACT	good	Leone et al. 1998; Kel et al. 2001
	E2F site		2757/58	GCCTACTTCTGGTTTACGCACG CGCCACTGCGCATCG		
	E2F site		2759/60	CCGAGCGAGGCCTACTTCT GGACAGTGCCGCTTCTTTCA	great	
	E2F site		2771/72	ccaccacctcccgtccttaa aatcacagcggcgctcgtac		
mcm5	E2F site	AB003469	2761/62	GAAGGAGGCGAGGTCATGC AGCGATTGGACCGTTCTGAG	OK	Leone et al. 1998; Kel et al. 2001

	E2F site		2763/64	GCCAAATTGTTCCGCACAC TGATTGGCTGCAAAGTGCAT	great	
	E2F site		2773/74	tccttcccagccagaagttt tcccactagcctcacctctg	no good	
dhfr	E2F site	K01612	2787/88	GTCCTCCCGCTGCTGTCAT GGACACAGCGACGATGCA		Kel et al. 2001; Fry et al. 1999; reviewed in Slansky and Farnham 1996
	E2F site		2789/90	GCCTCGCCTGCACAAATAG CAGAACGCGCGGTCAAGT	great	
e2f1	E2F site	U47675	2791/92	GGCGGCTCGTGGCTCT GCCGCTGCCTGCAAAG		Takahashi et al. 2000; Kel et al. 2001
	E2F site		2793/94	GGCGGCTCGTGGCTCT GAGGGCTCGATCCCGCT		
b-myb	E2F site	X82032	2795/96	CGCTTGGCGGGAGATAGA TCCTCGCTCGCAGGAACT	great	Lam and Watson 1993; Kel et al. 2001
	E2F site		2797/98	AGTTCCTGCGAGCGAGG TCGAAGGCGTCAGCGTG	good	
cdc2	E2F site	X66172	2799/00	GGACGACACTCTCCCGACTG GCGCCAACTGAGTGCGA	great	Dalton 1992; Tommasi and Pfeifer 1995
	E2F site		2801/02	CTGGAGGAGAGCGCTTGCT GGGTGGCTAGAGCGCGA		
cdc6	E2F site	AJ009560	2803/04	GTGACTACAGCCAATCAGAATCGA AATCCGAATGGCCACAGC	great	Hateboer et al. 1998; Yan et al. 1998
	E2F site		2805/06	CGCTGTGCAGTTTGTTCAGG CACGCAGCCTCTCGGACT	great	
mcm6	E2F site	D89335	2871/72	GCGCCGTTCATTGGTCAG AATCGTGACACAGGAGCTGGA	good	Leone et al. 1998
	E2F site		2873/74	CGAAATCTCCAGCTCCTGTGT TTCCGACCTGCACGGC	OK	
mcm3	E2F site	AL034343	2921/22	TCTTTGGCAGCGGGCAT CGCAGCTCCACATCGTCC	OK	Leone et al. 1998
	E2F site		2923/24	GCGGGAAGAGTTCGGAAGTT GTGGAGGTTCCCAGGATGACT		
orc1	E2F site	U40152	2939/40	GCGATTGGCGCGAAGTT CAGGACCAAAGCGTGTGTCTC	great	Ohtani et al. 1996; Kel et al. 2001
	E2F site		2941/42	CGCGATTGGCGCGA GCCAGGACCAAAGCGTGT		
nuc	E2F site	M60858	2943/44	GCTGCCCAAGCCTACGG TGGAATCGGGCGGTCTC	good	Kel et al. 2001
	E2F site		2945/46	CGGAGACCGCCCGATT TCGTGGGACTGGCGTTTT	good	
polα	E2F site	M64481	2947/48	GCCCGACCGCTTCTTTCT TTGGCGCCCTGTGATGA	great	Pearson et al. 1991; Kel et al. 2001
	E2F site		2949/50	GGCACGTCAGTGGCCTTC GAAAGCCAATCAGCGGCTC		
pcna	E2F site	J04718	2951/52	CTGGCTGCTGCGCGA CACCACCCGCTTTGTGACT	good	DeGregori et al. 1995; Kel et al. 2001
	E2F site		2953/54	TTTCGCGCCAAAGTCACA GAACAGTGTTCCTGGCACTGC		
tk	E2F site	X15509	2955/56	ACGTCCATCGCCCTGATTT GAATCCGGGACGTGCG	good	Dou et al. 1994; Kel et al. 2001
	E2F site		2957/58	GCACGTCCCGGATTCCTC GCTGACCTGGCGGGAGAT	good	
mcm7	E2F site	AB004270	1179/80	CCGCCTTGTCCGATTGG	good	Leone et al. 1998

				AAATTGGCGCGAAACGTC		
	E2F site		1181/82	GGCTAGCGGGAGGTGAAGA	good	
				CCGGCCAACCGAAATTG		
dut	E2F site	AF018429	3089/0	CTGCGGCGACGCTCAT	good	Ren et al. 2002
				AGAAGGCGAGCGAGGAGAC		
	E2F site		3091/92	GCTCGCCTTCTGGCTCTG		
				AAATGGCGGGTGTCTCTTCA		
rrm1	E2F site	AF107045	3093/94	AGCAGGAGAGCAGCATTTCC	good	DeGregori et al 1995
				CATGGGCCCCATTGGAT		
	E2F site		3095/96	TGGGAGAGGCGTAGTCTTCTG		
				TGTAGCCGCCATGTTGAGTCT		
top2a	E2F site	AF071738	3097/98	CAAGCTTTCCGCACGAGAA	good	Polager et al. 2002; Ren et al. 2002
				GGAATCTGGCCAATGAGAAGG		
	E2F site		3099/100	TCCCTGTCAATCTCTCCGCTAT		
				GACTAGAGAGGCTTCAAAAGGCAC		
polα2	E2F site	XM006519	3101/02	CACTGCACAAACCATTTGGC	good	Polager et al. 2002; Ren et al. 2002
				CGGTGGCAGAACTGAGTGG		
	E2F site		3103/04	GCAGGACGTTCTCACCAGGA		
				TGGAGGGAGCAGAAATCTCG		
ung	E2F site	X89398	3105/06	CAATTGCTGACCGCCACA		Polager et al. 2002; Ren et al. 2002
				TCATCCTGGAGCTGAGGAGG		
	E2F site		3107/08	GGCGCGATCAAAGCTCAC		
				GAGGATCGCTTGAGGCCAG		
msh2	E2F site		3109/10	AGCTCTACTAAGGATGCGCGTC		Polager et al. 2002; Ren et al. 2002
				CTTTAGCTACTGCGCATGCCT		
	E2F site		3111/12	GACTCCCACCCACCGAAAC	OK	
				CACGGCGACCACACCC		
polδ	E2F site	L38719	3113/14	GCGGGAAACGCTGTTTGA		Ren et al. 2002
				TCCCTACCAGGCCCACTG		
	E2F site		3115/16	GGCCACTTCGGAAGCTGAG	OK	
				CAAGTGCGGGCAAGGC		
gar22	E2F site	3164077	3117/18	CGGTCCGTCCTCTGCAGTT	OK	Weinmann et al. 2002
				GGACGCGGGAGGGTTAAG		
	E2F site		3119/20	TTATTGGAGCTCTGAGCCTGG		
				GGCGGCAGCCATGCTA		
rcql2	E2F site	L36140	3121/22	GACCGCAGGAAAACGTGG		Weinmann et al. 2002
				TCGACCGCGCCTTCTC		
	E2F site		3123/24	TTCCCGGGTTTCCTAAGACTC		
				GGTGTGGCACAGAACGGAG		
ts	E2F site	D00596	3125/26	CCGGGTTTCCTAAGACTCTCAG		DeGregori et al. 1995
				GGTGTGGCACAGAACGGAG		
	E2F site		3127/28	CTGTGCTGCTGGCTTAGAGAAG		
				GGCTGGGAAGGACTGCG		
mcm4	-600	NT023806	3129/30	ACGCATTCCTGCCCCA		
				AGGAGACCTTGTCCGCTGC		
	-600		3131/32	CTGCTGCTCAGGACGCATT	OK	
				CCTGGCCGGTCATCAACT		
	-400		3133/34	GGCTACTTGGTGTTGGACTTGG		
				TCGCTTGTTTTATCTGCCTCTG		
	-400		3135/36	GGCCTAGGTCAAGAGTTCCAAGT		
				GACTGCAGGCGGGCATC		
	-200		3137/38	AAGTGAGAAATGCATCTGTAATGTCCT		
				CTAGGGAGCGTGTGCTTCTTTATC		
	-300		3139/40	AGTCCCAACACTTTAGGAAGCTGA		
				GTCTCGAACTCCTGCGATCC		
	+200		3141/42	TCTGGGTCTCGCGGTTTG		
				GCTTGCCGACGACGCT		

	+200		3143/44	TCTGGGTCTCGCGGTTTG GCCGACGACGCTCGC		
	+500		3145/46	ACGTAGAGGCGAGGATTCCAC CAGGTCCACTCCAGGCGA	good	
	+500		3147/48	TCAGCTATCCCTCTTGACTTTGATG GGTTCCCTCTACCCGAGAGC		
	+1000		3149/50	ACAACTCATCTCTTACCCACAGGAA ACAGCCATGTCAAAAGTTGGAA		
	+1500		3151/52	GCATCTATCGAGCTGTGCCTATT TAGACAGACTTCACATTACTCACTCTTGG	bad	
	+2000		3153/54	GGCCTCACTGCGTACGTAATG CAGGACCAGCTGCCTTGTCT	good	
	+2000		3155/56	GGATCATCTGTCAGCTCAATGC TTCCACTGAGACTCAATGGGATT		
	+3000		3157/58	GGGAAGACCGTGCGCTT ACCCATGTGGCGTGCAG	good	
	+3000		3159/60	ACTCGGGAGGTTGAGGCAG AGTGCAGTGGCGCGATCT		
ubi	TATA	X04803	3161/62	CTTTCAGCGGCGCACG GTTGCGTCACTTATCACCCCTC		
	TATA		3163/64	GAGACGGCGTCTACGTGAGG AGCTCACCCAACAAAGCCAA	good	
36B4	prom	17475111	3165/66	TGAGCTCCCTGTCTCTCCTCA CGTCAGGGATTGCCACG	good	
	prom		3167/68	GCGATTGCGCGTGTCC AGGAGAGACAGGGAGCTCAGG		
achr	prom	Y00508	3169/70	CAACCAAAGCCCATGTCCTC AGGCACGCTACAGGGCTTC		
	prom		3171/72	CCTTCATTGGGATCACCACG AGGAGATGAGTACCAGCAGGTTG	good	
cited1	E2F site	NM004143	3203/04	TTCCCGCCAATTTATCCAACT ACGTTGTTGGCATTTCAGAGC	good	Muller et al. 2001
	E2F site		3205/06	GATGTCAAGGGTGGCACCTC CATCTCTTGGTTGGCATCCTC		
ccne2	E2F site	NM057735	3207/08	ATATATTGGGTTGGCGCCG CCAGACCAGCTACCGCTCG	good	A. Beck, N. Lauper, and B. Amati, unpublished
	E2F site		3209/10	CAGCTGAGCCGAGCGGTA TCTTTCAGGTGTATAAAACCTCGC		
p107	E2F site	AL136172	3237/38	TGGATGACAACACGTCCCG AACATCCCTTCAGGCCCC		
	E2F site		3239/40	TGGATGACAACACGTCCCG CACGGCCCCCGACTTC	good	
	-500		3241/42	CCACTCACTGGGCACTAGGAC AAAGTAGCCCCTGGACGAAGA	good	
	-300		3243/44	GAGGCCACCTCGCAGTAGG CTGGTTAGGCTCTTGGGTTTTG		
	500		3245/46	AAGCTCAGGAAAGGAGCGG GGTAGAGGGAAGTGTGGTCAGG	good	
	500		3247/48	CTAGGTCCCGCGCCG GTAGAGGGAAGTGTGGTCAGGAAG		
	1000		3249/50	TCAGCCCTACGTTTCCTCCTC TGATGCAAAGCATCAGGACG		
	1000		3251/52	AATGAGTATTAGACTGCTTTCCAGGTG AAGACAACCTTCTGAGCCTTGC		
mcm3	-700	AL034343	3295/96	TTTCCAAAATTTGTATAACACACGATG GTTTACCCTGCCCCCTAGAGA	good	
	-400		3297/98	GAATTTGGTCGTTGCATCGG TTTTTGCAGGGTTTTTCAAGC		

	-450		3299/00	CAGCTTGAAAAACCCTGCAAA		
				GCGTTCTGGGAGTTGTAGTGTTCT		
	-200		3301/02	TCAGTGAGTGAGGCGGAGG		
				ACGTACCATCCGGGATTCTCT		
	+350		3303/04	TCCAGCCCCAATCTTGTAACTT		
				ATGGGAACCTGTCGCCTTTT		
	+500		3305/06	GCGTTGCAGAGTGCTCAGATT		
				TTTTCCTCCAGGTAAAACGTCAGT		
	+1000		3307/08	GGATTTTATGTGCAACCCAAGAC	good	
				GATGGTTTTTTCAACATTCTATGGC		
pcna	-800	J04718	3309/10	CTCCACATATGCCCGGACTT	good	
				CCTCTTTGACTCCTGAACCCG		
	-900		3311/12	TGCAGCAGCTGTACTCTCTTCAG		
				GCCTACCGGGAGGAAAAGC		
	-400		3313/14	AGGCCTGCTGGGATATTAGCTC	good	
				GAGTCCATGCTCTGCAGGTTTAC		
	-500		3315/16	GCTCCTGAAGCCGAAACTAGC		
				GCTCCTGAAGCCGAAACTAGC		
	+400		3317/18	AATGGCATCCTCCAGCAGTC		
				AGGCCGTCTCAGAACTGGTG		
	+450		3319/20	CCTTCTAACCGCGTTCGAAATAC	good	
				TTAAAATCAACGCCGTCTGCT		
	+900		3321/22	GTCTTGGCTCTGTTGCCCAG		
				AGAGAGCTATATGGCACTACTGCACT		
	+1000		3323/24	CTCAAGCAGTCCTCTTGCCTTAGT	good	
				GGCCCGTAGTCCTTCCTAGC		
dut	-1000	AF018429	3333/34	CAGAGCAAACAAGAAGAGCGAA		
				GCCGCCTCTGGCTGC		
	-650		3335/36	CTCTGCTACCATTTCCTTACGTCTCT		
				CGCGTTTTGCATCGCTG		
	-600		3337/38	TGCTACCATTTCCTTACGTCTCTG		
				CTCGCGCGTTTTGCATC		
	+650		3339/40	GCAGACTGCCTGTATCTACCACA		
				ACAGTAAAACGTGCTGTAGAGATGAAA		
	+650		3341/42	ATTTGAGAAGCATCTGAAAACGAA		
				AAAGTGTGGTAGATACAGGCAGTCTG		
p73	-3500	AF235000	3343/44	GATTTAAGCACCCTTCTGCCAC	good	Stiewe and Putzer 2001
				TGGCTGGGATTTGAGGTCTT		
	-1750		3345/46	TGTGCCGCAGGGCTTTA		
				AAATCCCTGGTCGGAAGAAAC		
	#2		3347/48	TCAAGGAGAGAACGAATTTGCC	good	
				TCAAGGAGAGAACGAATTTGCC		
	#2		3349/50	ATCACCCGCGCGCAT		
				CCAGACCCGCACGATTCTT		
	#2/#3		3351/52	CGTGCGCCCAGCAAAC		
				CCGGCTGACTCGCACATC		
	#2/#3		3353/54	GCGGTGCCGGCCTT		
				AGACGAATCCCATGCAGAGG		
	#3		3355/56	AGAGGCTCTCCGCGGG		
				AAGCAGGGCTTGCCACC		
	#3		3357/58	GATCCTCTGCCGGGCG		
				CAGGGCTTGCCACCCAC		
	#7/#8		3359/60	ATCGGCCCCTGGGACTT		
				CTCCTCGCAGGCTAGACTCTG		
	#7/#8		3361/62	GAGTTGGATCGGCCCCTG		
				GCTAGACTCTGGCCGCTCC		
	#9		3363/64	GTGCAGGTGGAAATCGCC		
				TCCTCAGGGAAGCTGAGGC		
	#7		3369/70	GGGACGAGCTAGTGGACAAGG	good	

				CCGATCCAACTCCGAGGG		
	#9 +600		3371/72	CCTTTGCTCAGCTTGTATTTTGG	good	
				TACATGGGCTTATCTCCTAGCGT		
	#9 +600		3373/74	CAGCTTGTATTTTGGCCTGGA		
				TGTGCATACTACATGGGCTTATCTC		
	#9 +1kb		3375/76	AGCTACTCGGGAGGCTGAGG		
				CCTCTCCATCCCAGGTTCAAG		
	#9 +1kb		3377/78	ATGGTGAAACCCAGTCTCTACTTGA		
				TGCCACCATGCCCAGC		
cdkn2a-arf	site #2	AF082338	3417/18	GGGTGGGAAGATGGTGGTG		Kel et al. 2001
				CCACTTTCCCGCCCTGT		
	site #2		3419/20	GGTGGGAAGATGGTGGTGG		
				CCTCTCCCTCCCGCCTAC		
	site #1		3421/22	CCTCCTGATTGGCGGATAGA		
				GGAAGAAAGGAAAGCGAGGTC		
	site #1		3423/24	ATAGAGCAATGAGATGACCTCGCT		
				TTTAAAAATGAAAAAGGAAGAAAGGAA		
	site #1		3425/26	GTTCAAAACTGACATTCAGCCTCC		
				AGGTCATCTCATTGCTCTATCCG		
	-1500		3427/28	CGTGGCATAGGTGCAGAGG		
				GCTCTATGGGAACCAGATCCTTT		
	-1000		3429/30	CGGCTCACGCTGTGGTTC		
				GCGCTTGGGCCTAGTGG		
	-3000		3431/32	AATTCCCCTGCATACTGCAACT		
				ACTGCCGCTGCCCAATAC		
apaf1	E2F site	AB070829	3433/34	GACTGCTCCGCTGTCCAGA		Moroni et al. 2001
				TCACGTCCACTCGCTACCTCT		
	E2F site		3435/36	TCCGGCGGGATTTGACT		
				GCTACCTCTTCTTCTCCGCCTC		
	p53 site		3437/38	TCTGGAGACCCTAGGACGACA		
				CACGTCCTGCTCCCCCT		
	p53 site		3439/40	CGACTTCTTCCGGCTCTTCA		
				TGCCCTGGGCTTGTCGT		
p107	-1200	AL136172	3491/92	ACTGGGATTACAGGCAAAGCA	good	
				CTGTCTTCGTTCTTTTGTTGTTCC		
	-1100		3492/93	GGGCCCAGACACAGAATCTTC		
				TGGGAAACTTCTAGAGGGTATTTGG		
	-2000		3495/96	CATGCTTTGGGAGAACTCTGAA		
				TCTATGATTAGCCTCCCTGTTGTG		
	-1950		3497/98	CACACAATGGGATCAAAATAGAAACA	good	
				TTTTGAGCCAGATACACATGAAGG		
	-1600		3499/00	CTGCCTCAGCCTCCCAAAG		
				GGTTGCAGTGGCTCACACCT		
	-1550		3501/02	TACAGGTGTGAGCCACTGCAAC	good	
				CTGAATAGAAAATGAAGTATGGAAGGC		
	-2650		3503/04	GGTGGCGTGTGCCTGTAGT		
				AATTCTCCTTCCTCAGCCCC		
	-2500		3505/06	AGATCTTGCCACTGCACTCCA		
				TGAGACAGAGTCTCACTCTGTCACC		
	-3300		3507/08	GGAGCTTGGCTTAACCCTGA	good	
				TTTGAAAATGTTCTAGTGACTCCCC		
	-3100		3509/10	CCGGGCACAGTGGTTCAC		
				GCCTCGGCCTCCCAAA		
	+1450		3511/12	AGCATAAGCCCAGTGTAGACAGAA	good	
				TGCTACATGAAATACACTCCATCACA		
	+1650		3513/14	ATGTGTGCCTATATATGAAGGAGTAGTAC CT	good	

				CCATTGTCCTTTACCCAGTCTATCC	
	+2200		3515/16	CGGTGGCTCACACCTGTAATC	bad
				TCTGCCCACCTCAGCCTC	
	+2250		3517/18	ACATCTTCAATGTTTGCTCTACTTATTTAT TT	
				GGCCAAGACCCTGTTTCTAAGG	
	+2500		3519/20	GGCCTGGAATGCAGTGGT	
				CAGGAACATCACTTGAGCACAGA	
	+2600		3521/22	GATGTTCCTGCTTCAGCCTCC	OK
				TGGGCAACATAGTCAGATACTATCTCC	
pcna	-2000	J04718	3571/72	GGCCTGTAATCCCAGCACTTT	bad
				TCTATTTATATGTCTGACTCCACAGCAG	
	-2000		3573/74	ATTGAAAATTTCTGGCTGGGC	
				CAAAGTGCTGGGATTACAGGC	
	-1500		3575/76	TGTGATGCTATGTTTTAAAGAGGTACTGA	OK
				AATTTTATGCCACGTACATCTTTTTATC	
	-1500		3577/78	TGACTCAGCTGTATTACTTCAAGGAGTT	OK
				ACCTTTGAGCACTTAGGCAATTTT	
	+1500		3579/80	TCATGATTTCTGTGCCATACTCTAATG	OK
				TCCTCCAGGGATAAAGTAAGAGAACA	
	+1500		3581/82	TTTGAGTGGAGTTTGTTAAGAAATTACG	
				TTCTGATTACTTTAAAATATGACTTGCGT	
	+2200		3583/84	ATCAAGTCCACACCGGCTCT	OK
				TTATACGCCTAACTTCCCAATCCT	
	+2400		3585/86	TTTAAAGGTGGAGAAGCAGGCT	OK
				TTTGGCTAAATTGTCACTTAAGGTTG	
mcm3	-1500	AL034343	3587/88	TTGGCTAAGGATCGATTTATCTGC	OK
				ACTGTATGTTTTGTGCCATCCTAGTG	
	-2000		358990	GCAACAACCTGATTGATTTTGG	OK
				CATGAAATCTTTAAGCAGAGGAAATG	
	-2200		3591/92	TGGAAAGAAAATTGAAATGGAGG	OK
				CAGTTTCCATACTTGGGTCACATTC	
	+1500		3593/94	GGCTGTTTTGCAGGAAGACC	OK
				GATCAGCTCCCGAACTTTGC	
	+1400		3595/96	TACCGGCTGATTGTCAATGTG	OK
				GTTAGCCCTCTTCTCGTTTTTCC	
	+2000		3597/98	GGACTCTTACCTCCTGCTTCCTC	
				GACAATGCCCTCCACACAGA	
	+2200		3599/00	TACGTGGTAAAACTTGGAGGGC	OK
				TTCATCATTCTACTAATACATGTGATTACTCAA	
mcm4	-1350	NT023806	3601/02	TTCTCAAAGTAGGAACGGCAAAT	
				GCCCGGCCAAGAGAAAGTAT	
	-1300		3603/04	CTGGTGGGATTAGGCGAGATT	OK
				GCCCGGCCAAGAGAAAGT	
	-2200		3605/06	ACTTCCCCTGGAATCTCTCAAGT	
				ACCTTCCATTTATGCCATATCTCC	
	-2100		3607/08	TTGAAACCCTGAGAAGAGAAAGTAATC	OK
				TCAAACAAAAAACTAACATTGCGTG	
	+1400		3609/10	AGTATGCAGTCTCCTGAAACCATCT	
				GAGTTACCCACATGATACCCGC	
	+1500		3611/12	TGTGTAGGCAGCAGCAGAAGA	OK
				TCTGTTCCCCAGATCACAAGTTT	

Table 4: List of Primers Used in this Study.

All primers tested in this study are listed. Distances are in bp, from the E2F site. Primers that work in CHIP are indicated. For all E2F targets, references are listed.

APPENDIX A: Generation and Characterization of Gcn5, HBO1, PCAF, Tip60, and TRRAP Antibodies

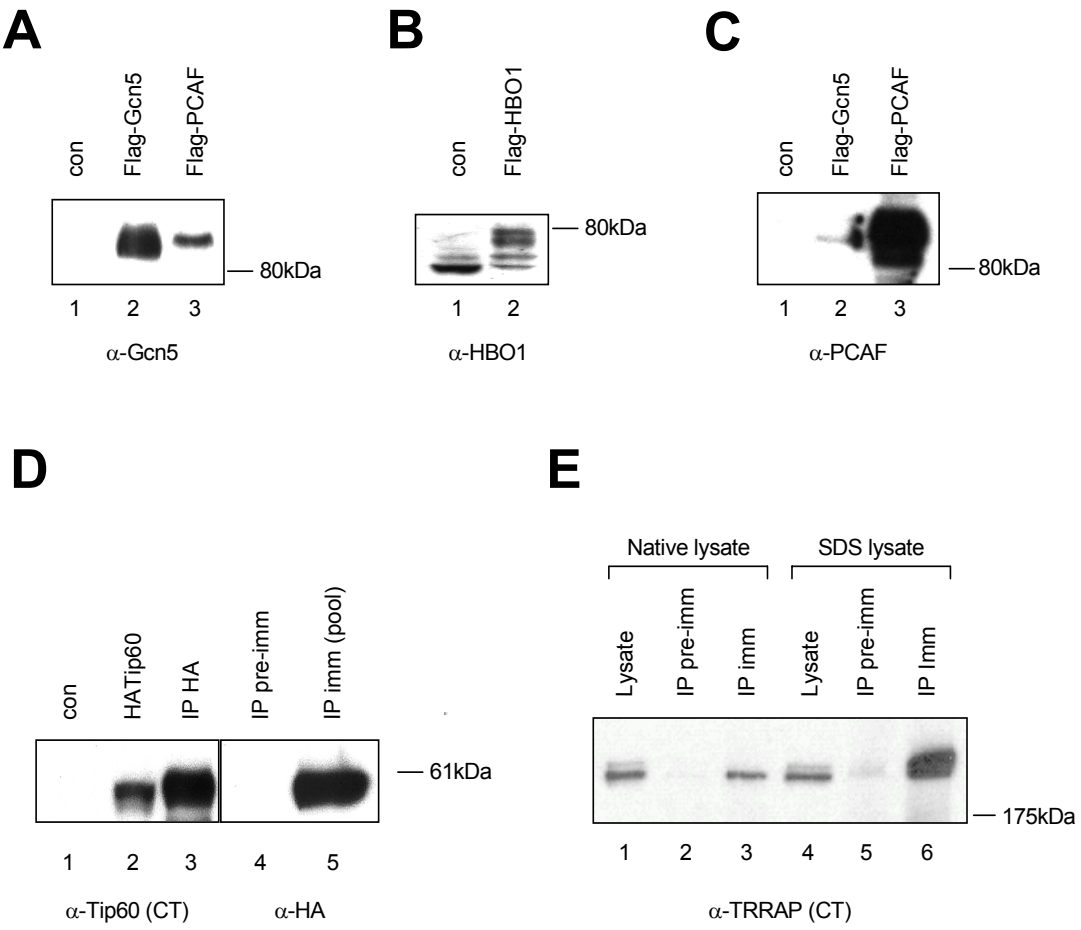

Figure 29: Characterization of Polyclonal Antibodies.
Rabbit polyclonal antibodies to Gcn5, HBO1, PCAF, Tip60, and TRRAP were affinity purified as described in Materials and Methods. Then, they were used to immunoblot lysates from 293T cells transiently transfected with expression vectors for the immunogenic protein, Gcn5 (A), HBO1 (B), PCAF (C), and Tip60 (D); for analysis of TRRAP antisera, untransfected cells were used. All antibodies recognized a single band corresponding to the transfected protein. Gcn5 and PCAF antisera also crossreacted weakly with the related PCAF, and Gcn5 proteins (A, C). Tip60 (a pool of all three peptide antibodies) and TRRAP antisera specifically immunoprecipitated proteins under SDS lysis conditions used in CHIP assay (D, E). For further information, see text.

In order to investigate the recruitment of HAT-complex subunits by CHIP, rabbit polyclonal peptide antisera were raised against TRRAP, Gcn5, HBO1, Tip60, and PCAF (see Materials and Methods). Crude bleeds were affinity purified against the immunogenic peptides. For rapid characterization the purified antibodies were used to immunoblot lysates from 293T cells

transfected with expression vectors encoding the respective HAT (for TRRAP untransfected 293T cells were used). Both Gcn5 (Figure 29 A, lane 2) and PCAF (Figure 29 C, lane 3) antisera recognized single bands corresponding to the transfected HATs. However, antisera also crossreacted weakly with the other HAT, respectively, probably due to their high homology (Figures 29 A, lane 3, and C, lane 2). Nevertheless, crossreactions with unrelated HATs were not observed, even when the proteins were strongly overexpressed (for example, adenovirally expressed Tip60 was not revealed by Gcn5 blot; data not shown). HBO1 antisera detected a doublet in nontransfected cells, and a doublet of higher molecular weight in transfected cells, consistent with the higher increased weight of tagged, transfected Flag-HA-HBO1 (Figure 29 B, compare lanes 1 and 2). Tip60 antisera detected a single band in cells transfected with Flag-HA-Tip60 (Figure 29 D, lane 2). Finally, TRRAP antisera detected a single band of high molecular weight, correlating with the large size of TRRAP (434kDa; Figure 29 E, lanes 1 and 4).

In addition, the TRRAP and Tip60 antisera were characterized for their ability to IP transfected (Tip60) or endogenous (TRRAP) proteins. Tip60 was immunoprecipitated under denaturing conditions (0.5% SDS) by a pool of all three individual peptide antisera, but not by the pre-immune control sera (Figure 29 D, compare lanes 4 and 5). The IP was as efficient as an IP performed with monoclonal antibodies against the Flag-tag of the transfected Tip60 (Figure 29 D, compare lanes 5 and 3). Similarly, endogenous TRRAP was efficiently immunoprecipitated under denaturing conditions by immune, but not pre-immune sera (Figure 28 E, compare lanes 6 and 5). In contrast, IPs were less efficient under native conditions (Figure 29 D, compare lanes 6 and 3), even though both lysates contained a similar amount of TRRAP (Figure 29 D, compare lanes 1 and 4).

APPENDIX B: Analysis of E2F-Target Binding by Distinct E2F Proteins, and by p130.

E2F proteins bind to promoter DNA with the consensus sequence TTTsGCGCsAAA. When analyzed by *in vitro* electro-mobility-shift assays (EMSA), E2F proteins do not exhibit target sequence specificity, i.e. E2F target sequences are bound equally well by all species of E2F proteins (reviewed in Trimarchi and Lees 2002). However, a recent study has suggested that different target genes exhibit distinct binding by various E2F proteins (Wells et al. 2000). Similarly, it is believed that individual E2F polypeptides may perform unique functions (reviewed in DeGregori 2002). For example, E2F1 may have a specific role in DNA repair and/or induction of apoptosis after DNA damage (Blattner et al. 1999; Hofferer et al. 1999; Lin et al. 2001). Consistent with this, some E2F target genes, such as APAF-1, are apparently induced specifically by E2F1, but not other E2F species (Moroni et al. 2001).

During my investigations of the role of the Tip60 complex in E2F function I generated a collection of primers specific for approximately 25 bona fide E2F target genes. This allowed to analyze binding of individual E2Fs to these targets, by using CHIP experiments described before (see chapter 4.3. and 4.4., pp. 50. Figure 30 A-C shows that all "activating" E2Fs exhibit similar binding patterns, i.e. targets with a lot of bound E2F1 also bound a lot of E2F2 and E2F3. In contrast, there was no correlation between the binding of activating E2Fs and E2F4 (compare Figure 30 A-C with Figure 30 D). Similarly, there was no correlation between binding of E2F1 and p130 (Figure 30 E-G), which is as expected, as E2F1 presumably acts mostly as an activator in T98G cells. However, there also was only a weak correlation of p130 binding and E2F4 binding (Figure 30 H-I). Possibly, p107 is predominant on targets with less bound p130.

It is particularly noteworthy that DNA damage associated genes, such as p73, polδ, and Top2a, are bound by all activating E2Fs, rather than just E2F1, suggesting that they may be targeted by all of them in the serum response of T98G cells. Nevertheless, this does not exclude a specific role for E2F1 in

induction of these targets after DNA damage. In summary, these results suggest that the activating E2F proteins exhibit similar target gene specificities, at least in T98G cells.

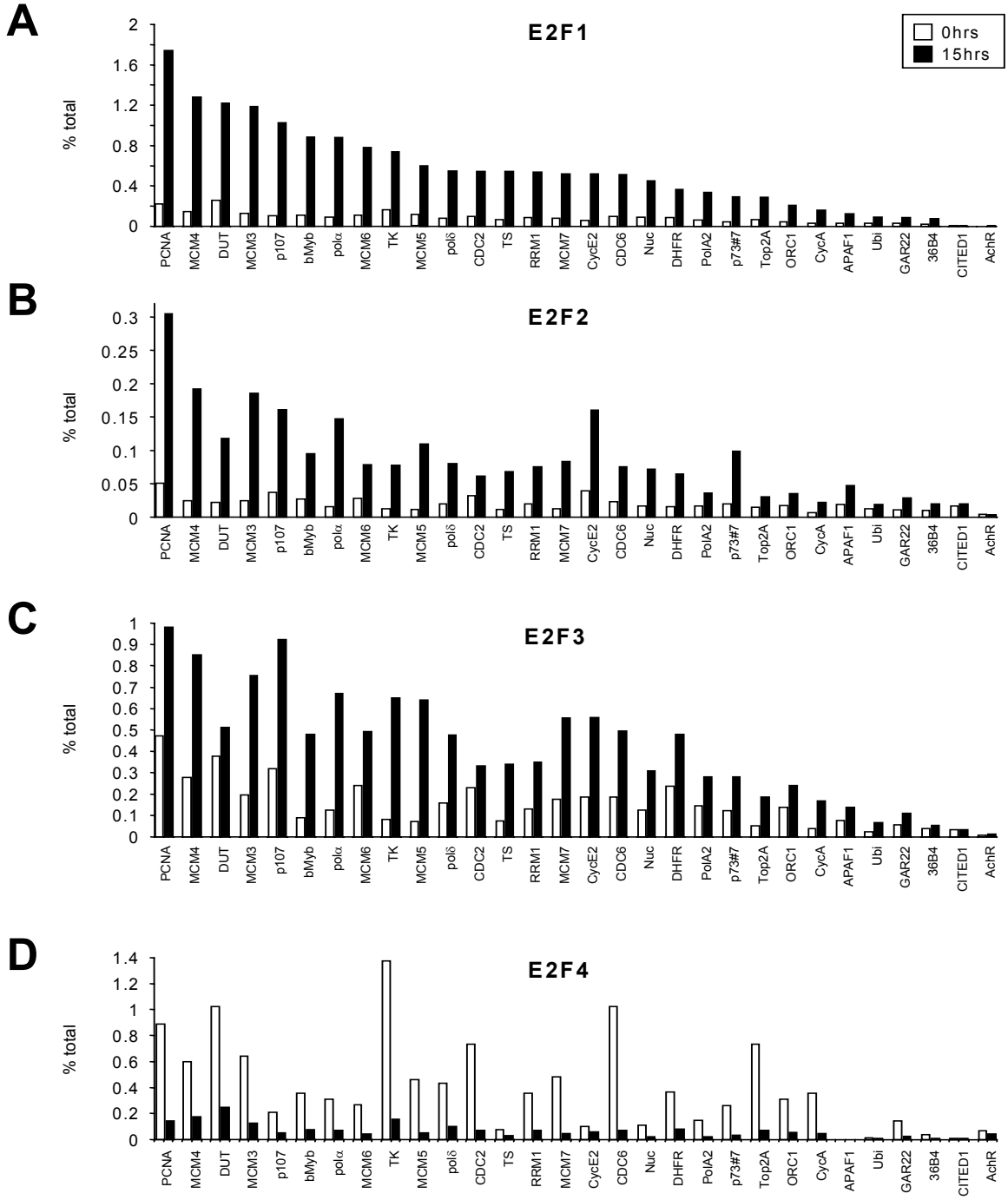

Figure 30: Analysis of Binding Patterns of Individual E2F Proteins, and p130 in T98G Cells.

CHIP with antibodies specific for individual E2F proteins and p130 was performed as described in Figures 7 and 8. Cell populations were identical those used in Figure 7. Primers for E2F target genes are described in Materials and Methods, chapter 8.3.3.

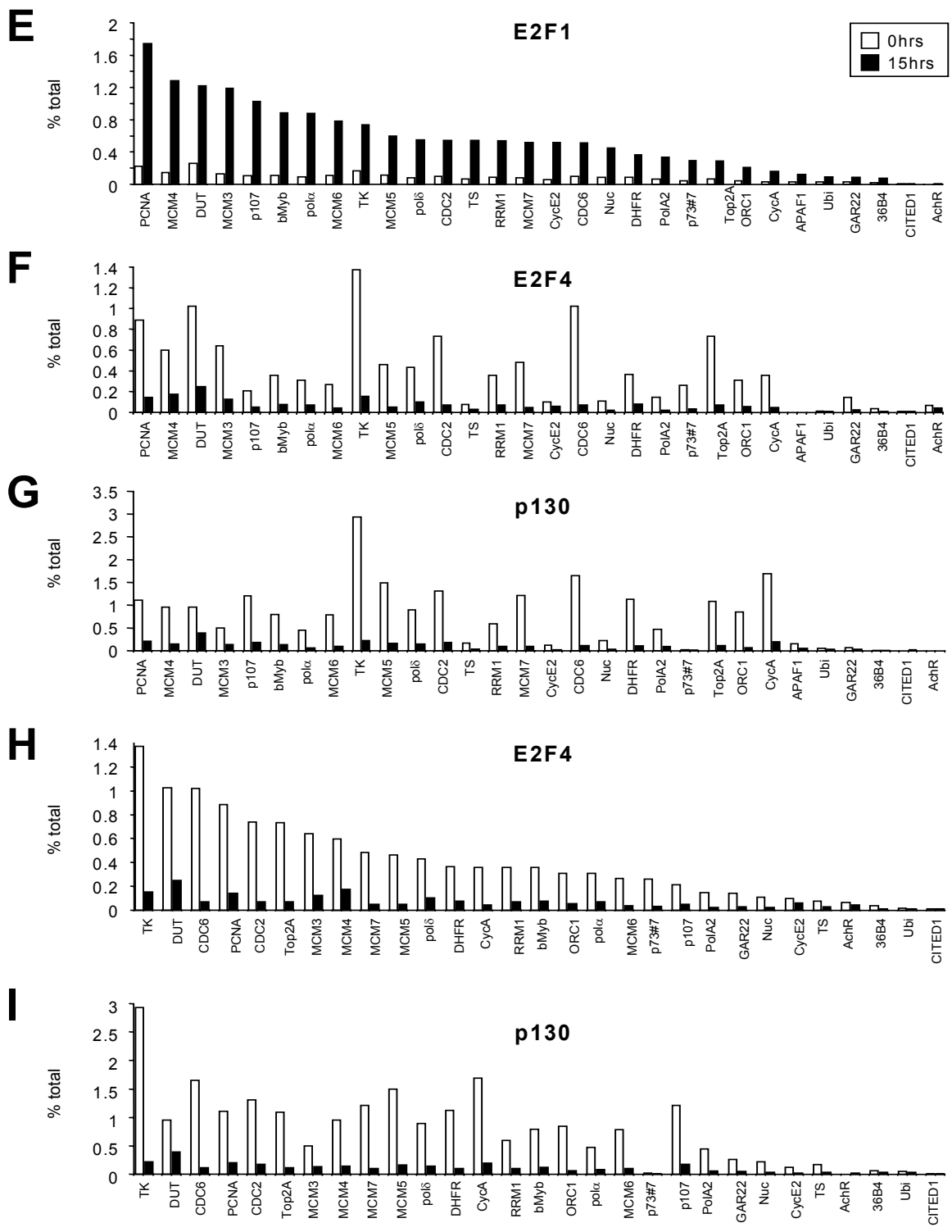

Figure 30: Analysis of Binding Patterns of Individual E2F Proteins, and p130, in T98G Cells (continued).

REFERENCES

Ait-Si-Ali, S., A. Polesskaya, S. Filleur, R. Ferreira, A. Duquet, P. Robin, A. Vervish, D. Trouche, F. Cabon, and A. Harel-Bellan. 2000. CBP/p300 histone acetyl-transferase activity is important for the G1/S transition. *Oncogene* **19**: 2430-7.

Ait-Si-Ali, S., S. Ramirez, F.X. Barre, F. Dkhissi, L. Magnaghi-Jaulin, J.A. Girault, P. Robin, M. Knibiehler, L.L. Pritchard, B. Ducommun, D. Trouche, and A. Harel-Bellan. 1998. Histone acetyltransferase activity of CBP is controlled by cyclin-dependent kinases and oncoprotein E1A. *Nature* **396**: 184-186.

Alevizopoulos, K., B. Catarin, J. Vlach, and B. Amati. 1998. A novel function of adenovirus E1A is required to overcome growth arrest by the CDK2 inhibitor p27(Kip1). *EMBO J* **17**: 5987-97.

Alevizopoulos, K., J. Vlach, S. Hennecke, and B. Amati. 1997. Cyclin E and c-Myc promote cell proliferation in the presence of $p16^{INK4a}$ and of hypophosphorylated Retinoblastoma-family proteins. *EMBO J* **16**: 5322-5333.

Allard, S., R.T. Utley, J. Savard, A. Clarke, P. Grant, C.J. Brandl, L. Pillus, J.L. Workman, and J. Cote. 1999. NuA4, an essential transcription adaptor/histone H4 acetyltransferase complex containing Esa1p and the ATM-related cofactor Tra1p. *Embo J* **18**: 5108-5119.

Amati, B., S.R. Frank, D. Donjerkovic, and S. Taubert. 2001. Function of the c-Myc oncoprotein in chromatin-remodeling and transcription. *Biochim Biophys Acta* **1471**: M135-M145.

Apostolova, M.D., I.A. Ivanova, C. Dagnino, S.J. D'Souza, and L. Dagnino. 2002. Active nuclear import and export pathways regulate E2F-5 subcellular localization. *J Biol Chem* **277**: 34471-9.

Arany, Z., W.R. Sellers, D.M. Livingston, and R. Eckner. 1994. E1A-associated p300 and CREB-associated CBP belong to a conserved family of coactivators [letter]. *Cell* **77**: 799-800.

Ayer, D.E., Q.A. Lawrence, and R.N. Eisenman. 1995. Mad-Max transcriptional repression is mediated by ternary complex formation with mammalian homologs of yeast repressor Sin3. *Cell* **80**: 767-76.

Baek, S.H., K.A. Ohgi, D.W. Rose, E.H. Koo, C.K. Glass, and M.G. Rosenfeld. 2002. Exchange of N-CoR corepressor and Tip60 coactivator complexes links gene expression by NF-kappaB and beta-amyloid precursor protein. *Cell* **110**: 55-67.

Bagchi, S., R. Weinmann, and P. Raychaudhuri. 1991. The retinoblastoma protein copurifies with E2F-I, an E1A-regulated inhibitor of the transcription factor E2F. *Cell* **65**: 1063-72.

Bandara, L., V.M. Buck, M. Zamanian, L.H. Johnston, and, and N. LaThangue. 1993. Functional synergy between DP-1 and E2F-1 in the cell cycle regulating transcription factor DRTF1/E2F. *EMBO J* **12**: 4317-4324.

Bandara, L.R., J.P. Adamczewski, T. Hunt, and N.B. La Thangue. 1991. Cyclin A and the retinoblastoma gene product complex with a common transcription factor. *Nature* **352**: 249-51.

Bannister, A.J., E.A. Miska, D. Gorlich, and T. Kouzarides. 2000. Acetylation of importin-alpha nuclear import factors by CBP/p300. *Curr Biol* **10**: 467-70.

Barlev, N.A., L. Liu, N.H. Chehab, K. Mansfield, K.G. Harris, T.D. Halazonetis, and S.L. Berger. 2001. Acetylation of p53 activates transcription through recruitment of coactivators/histone acetyltransferases. *Mol Cell* **8**: 1243-54.

Barlev, N.A., V. Poltoratsky, T. Owen-Hughes, C. Ying, L. Liu, J.L. Workman, and S.L. Berger. 1998. Repression of GCN5 histone acetyltransferase activity via bromodomain-mediated binding and phosphorylation by the Ku-DNA-dependent protein kinase complex. *Mol Cell Biol* **18**: 1349-58.

Bartek, J. and J. Lukas. 2001. Pathways governing G1/S transition and their response to DNA damage. *FEBS Lett* **490**: 117-22.

Bartkova, J., J. Lukas, and J. Bartek. 1997. Aberrations of the G1- and G1/S-regulating genes in human cancer. *Prog Cell Cycle Res* **3**: 211-20.

Baudino, T.A. and J.L. Cleveland. 2001. The Max network gone mad. *Mol Cell Biol* **21**: 691-702.

Bauer, A., S. Chauvet, O. Huber, F. Usseglio, U. Rothbacher, D. Aragnol, R. Kemler, and J. Pradel. 2000. Pontin52 and reptin52 function as antagonistic regulators of beta-catenin signalling activity. *Embo J* **19**: 6121-30.

Baylin, S.B., M. Esteller, M.R. Rountree, K.E. Bachman, K. Schuebel, and J.G. Herman. 2001. Aberrant patterns of DNA methylation, chromatin formation and gene expression in cancer. *Hum Mol Genet* **10**: 687-92.

Becker, P.B. and W. Horz. 2002. ATP-dependent nucleosome remodeling. *Annu Rev Biochem* **71**: 247-73.

Beijersbergen, R.L., R.M. Kerkhoven, L. Zhu, L. Carlee, P.M. Voorhoeve, and R. Bernards. 1994. E2F-4, a new member of the E2F gene family, has oncogenic activity and associates with p107 in vivo. *Genes Dev* **8**: 2680-2690.

Ben-Israel, H. and T. Kleinberger. 2002. Adenovirus and cell cycle control. *Front Biosci* **7**: d1369-95.

Berger, S.L. 2002. Histone modifications in transcriptional regulation. *Curr Opin Genet Dev* **12**: 142-8.

Bird, A.W., D.Y. Yu, M.G. Pray-Grant, Q. Qiu, K.E. Harmon, P.C. Megee, P.A. Grant, M.M. Smith, and M.F. Christman. 2002. Acetylation of histone H4 by Esa1 is required for DNA double-strand break repair. *Nature* **419**: 411-415.

Blagosklonny, M.V. and A.B. Pardee. 2002. The restriction point of the cell cycle. *Cell Cycle* **1**: 103-10.

Blais, A., D. Monte, F. Pouliot, and C. Labrie. 2002. Regulation of the human Cyclin-dependent kinase inhibitor p18INK4c by the transcription factors E2F1 and Sp1. *J Biol Chem* **277**: 31679-93.

Blattner, C., A. Sparks, and D. Lane. 1999. Transcription factor E2F-1 is upregulated in response to DNA damage in a manner analogous to that of p53. *Mol Cell Biol* **19**: 3704-13.

Bochar, D.A., L. Wang, H. Beniya, A. Kinev, Y. Xue, W.S. Lane, W. Wang, F. Kashanchi, and R. Shiekhattar. 2000. BRCA1 is associated with a human SWI/SNF-related complex: linking chromatin-remodeling to breast cancer. *Cell* **102**: 257-65.

Boon, K., H.N. Caron, R. van Asperen, L. Valentijn, M.C. Hermus, P. van Sluis, I. Roobeek, I. Weis, P.A. Voute, M. Schwab, and R. Versteeg. 2001. N-myc enhances the expression of a large set of genes functioning in ribosome biogenesis and protein synthesis. *Embo J* **20**: 1383-1393.

Borrow, J., V.P. Stanton, Jr., J.M. Andresen, R. Becher, F.G. Behm, R.S. Chaganti, C.I. Civin, C. Disteche, I. Dube, A.M. Frischauf, D. Horsman, F. Mitelman, S. Volinia, A.E. Watmore, and D.E. Housman. 1996. The translocation t(8;16)(p11;p13) of acute myeloid leukaemia fuses a putative acetyltransferase to the CREB-binding protein. *Nat Genet* **14**: 33-41.

Bouchard, C., O. Dittrich, A. Kiermaier, K. Dohmann, A. Menkel, M. Eilers, and B. Lüscher. 2001. Regulation of Cyclin D2 gene expression by the Myc/Max/Mad network: Myc-dependent TRRAP recruitment and histone acetylation at the Cyclin D2 promoter. *Genes Dev* **15**: 2042-2047.

Boulaire, J., A. Fotedar, and R. Fotedar. 2000. The functions of the cdk-Cyclin kinase inhibitor p21WAF1. *Pathol Biol (Paris)* **48**: 190-202.

Boxer, L.M. and C.V. Dang. 2001. Translocations involving c-myc and c-myc function. *Oncogene* **20**: 5595-610.

Boyes, J., P. Byfield, Y. Nakatani, and V. Ogryzko. 1998. Regulation of activity of the transcription factor GATA-1 by acetylation. *Nature* **396**: 594-8.

Brand, M., J.G. Moggs, M. Oulad-Abdelghani, F. Lejeune, F.J. Dilworth, J. Stevenin, G. Almouzni, and L. Tora. 2001. UV-damaged DNA-binding protein in the TFTC complex links DNA damage recognition to nucleosome acetylation. *Embo J* **20**: 3187-96.

Brand, M., K. Yamamoto, A. Staub, and L. Tora. 1999. Identification of TATA-binding protein-free TAFII-containing complex subunits suggests a role in nucleosome acetylation and signal transduction. *J Biol Chem* **274**: 18285-18289.

Brehm, A., E.A. Miska, D.J. McCance, J.L. Reid, A.J. Bannister, and T. Kouzarides. 1998. Retinoblastoma protein recruits histone deacetylase to repress transcription. *Nature* **391**: 597-601.

Bringold, F. and M. Serrano. 2000. Tumor suppressors and oncogenes in cellular senescence. *Exp Gerontol* **35**: 317-29.

Brownell, J.E. and C.D. Allis. 1995. An activity gel assay detects a single, catalytically active histone acetyltransferase subunit in Tetrahymena macronuclei. *Proc Natl Acad Sci U S A* **92**: 6364-8.

Brownell, J.E. and C.D. Allis. 1996. Special HATs for special occasions: linking histone acetylation to chromatin assembly and gene activation. *Curr Opin Genet Dev* **6**: 176-84.

Brownell, J.E., J. Zhou, T. Ranalli, R. Kobayashi, D.G. Edmondson, S.Y. Roth, and C.D. Allis. 1996. Tetrahymena histone acetyltransferase A: a homolog to yeast Gcn5p linking histone acetylation to gene activation. *Cell* **84**: 843-51.

Bryan, E.J., V.J. Jokubaitis, N.L. Chamberlain, S.W. Baxter, E. Dawson, D.Y. Choong, and I.G. Campbell. 2002. Mutation analysis of EP300 in colon, breast and ovarian carcinomas. *Int J Cancer* **102**: 137-41.

Burke, T.W., J.G. Cook, M. Asano, and J.R. Nevins. 2001. Replication factors MCM2 and ORC1 interact with the histone acetyltransferase HBO1. *J Biol Chem* **276**: 15397-408.

Cao, X. and T.C. Sudhof. 2001. A transcriptionally active complex of APP with Fe65 and histone acetyltransferase Tip60. *Science* **293**: 115-120.

Cardoso, M.C., H. Leonhardt, and B. Nadal-Ginard. 1993. Reversal of terminal differentiation and control of DNA replication: Cyclin A and Cdk2 specifically localize at subnuclear sites of DNA replication. *Cell* **74**: 979-92.

Chadee, D.N., M.J. Hendzel, C.P. Tylipski, C.D. Allis, D.P. Bazett-Jones, J.A. Wright, and J.R. Davie. 1999. Increased Ser-10 phosphorylation of histone H3 in mitogen-stimulated and oncogene-transformed mouse fibroblasts. *J Biol Chem* **274**: 24914-20.

Chakravarti, D., V. Ogryzko, H.-Y. Kao, A. Nash, H. Chen, Y. Nakatani, and R.M. Evans. 1999. A viral mechanism for inhibition of p300 and PCAF acetyltransferase activity. *Cell* **96**: 393-403.

Champagne, N., N.R. Bertos, N. Pelletier, A.H. Wang, M. Vezmar, Y. Yang, H.H. Heng, and X.J. Yang. 1999. Identification of a human histone acetyltransferase related to monocytic leukemia zinc finger protein. *J Biol Chem* **274**: 28528-36.

Chan, H.M., M. Krstic-Demonacos, L. Smith, C. Demonacos, and N.B. La Thangue. 2001. Acetylation control of the retinoblastoma tumour-suppressor protein. *Nat Cell Biol* **3**: 667-74.

Chan, H.M. and N.B. La Thangue. 2001. p300/CBP proteins: HATs for transcriptional bridges and scaffolds. *J Cell Sci* **114**: 2363-73.

Chang, Y.C., S. Illenye, and N.H. Heintz. 2001. Cooperation of E2F-p130 and Sp1-pRb complexes in repression of the Chinese hamster dhfr gene. *Mol Cell Biol* **21**: 1121-31.

Chen, C.R., Y. Kang, P.M. Siegel, and J. Massague. 2002. E2F4/5 and p107 as Smad cofactors linking the TGFbeta receptor to c-myc repression. *Cell* **110**: 19-32.

Chen, D., H. Ma, H. Hong, S.S. Koh, S.M. Huang, B.T. Schurter, D.W. Aswad, and M.R. Stallcup. 1999a. Regulation of transcription by a protein methyltransferase. *Science* **284**: 2174-7.

Chen, H., R.J. Lin, R.L. Schiltz, D. Chakravarti, A. Nash, L. Nagy, M.L. Privalsky, Y. Nakatani, and R.M. Evans. 1997. Nuclear receptor coactivator ACTR is a novel histone acetyltransferase and forms a multimeric activation complex with P/CAF and CBP/p300. *Cell* **90**: 569-80.

Chen, H., R.J. Lin, W. Xie, D. Wilpitz, and R.M. Evans. 1999b. Regulation of hormone-induced histone hyperacetylation and gene activation via acetylation of an acetylase. *Cell* **98**: 675-686.

Cheng, S.W., K.P. Davies, E. Yung, R.J. Beltran, J. Yu, and G.V. Kalpana. 1999. c-MYC interacts with INI1/hSNF5 and requires the SWI/SNF complex for transactivation function. *Nat Genet* **22**: 102-105.

Cheung, P., K.G. Tanner, W.L. Cheung, P. Sassone-Corsi, J.M. Denu, and C.D. Allis. 2000. Synergistic coupling of histone H3 phosphorylation and acetylation in response to epidermal growth factor stimulation. *Mol Cell* **5**: 905-15.

Chittenden, T., D.M. Livingston, and W.G. Kaelin, Jr. 1991. The T/E1A-binding domain of the retinoblastoma product can interact selectively with a sequence-specific DNA-binding protein. *Cell* **65**: 1073-82.

Choy, J.S. and S.J. Kron. 2002. NuA4 subunit Yng2 function in intra-S-phase DNA damage response. *Mol Cell Biol* **22**: 8215-25.

Choy, J.S., B.T. Tobe, J.H. Huh, and S.J. Kron. 2001. Yng2p-dependent NuA4 histone H4 acetylation activity is required for mitotic and meiotic progression. *J Biol Chem* **276**: 43653-62.

Clapier, C. R., Langst, G., Corona, D. F., Becker, P. B., Nightingale, K. P. 2001. Critical role for the histone H4 N terminus in nucleosome remodeling by ISWI. *Mol Cell Biol* **21**: 875-83.

Clarke, A.R., E.R. Maandag, M. vanRoon, N.M.T. vanderLugt, M. vanderValk, M.L. Hooper, A. Berns, and H. teRiele. 1992. Requirement for a functional Rb-1 gene in murine development. *Nature* **359**: 328-330.

Clarke, A.S., J.E. Lowell, S.J. Jacobson, and L. Pillus. 1999. Esa1p is an essential histone acetyltransferase required for cell cycle progression. *Mol Cell Biol* **19**: 2515-26.

Classon, M. and E. Harlow. 2002. The retinoblastoma tumour suppressor in development and cancer. *Nat Rev Cancer* **2**: 910-7.

Cobrinik, D., M.-H. Lee, G. Hannon, G. Mulligan, R.T. Bronson, N. Dyson, E. Harlow, D. Beach, R.A. Weinberg, and T. Jacks. 1996. Shared role of the pRB-related p130 and p107 proteins in limb development. *Genes Dev* **10**: 1633-1644.

Cole, M.D. and S.B. McMahon. 1999. The Myc oncoprotein: a critical evaluation of transactivation and target gene regulation. *Oncogene* **18**: 2916-2924.

Coller, H.A., C. Grandori, P. Tamayo, T. Colbert, E.S. Lander, R.N. Eisenman, and T.R. Golub. 2000. Expression analysis with oligonucleotide microarrays reveals that MYC regulates genes involved in growth, cell cycle, signaling, and adhesion. *Proc Natl Acad Sci U S A* **97**: 3260-3265.

Cosma, M.P. 2002. Ordered Recruitment: Gene-Specific Mechanism of Transcription Activation. *Mol Cell* **10**: 227-236.

Cosma, M.P., T. Tanaka, and K. Nasmyth. 1999. Ordered recruitment of transcription and chromatin-remodeling factors to a cell cycle- and developmentally regulated promoter. *Cell* **97**: 299-311.

Dahiya, A., M.R. Gavin, R.X. Luo, and D.C. Dean. 2000. Role of the LXCXE binding site in Rb function. *Mol Cell Biol* **20**: 6799-805.

Dahiya, A., S. Wong, S. Gonzalo, M. Gavin, and D.C. Dean. 2001. Linking the Rb and Polycomb pathways. *Mol Cell* **8**: 557-69.

Dalton, S. 1992. Cell cycle regulation of the human cdc2 gene. *Embo J* **11**: 1797-804.

Dang, C.V., L.M. Resar, E. Emison, S. Kim, Q. Li, J.E. Prescott, D. Wonsey, and K. Zeller. 1999. Function of the c-Myc oncogenic transcription factor. *Exp Cell Res* **253**: 63-77.

Dannenberg, J.H., A. van Rossum, L. Schuijff, and H. te Riele. 2000. Ablation of the retinoblastoma gene family deregulates G(1) control causing immortalization and increased cell turnover under growth-restricting conditions. *Genes Dev* **14**: 3051-64.

Davie, J.K. and S.Y. Dent. 2002. Transcriptional control: an activating role for arginine methylation. *Curr Biol* **12**: R59-61.

Dechend, R., F. Hirano, K. Lehmann, V. Heissmeyer, S. Ansieau, F.G. Wulczyn, C. Scheidereit, and A. Leutz. 1999. The Bcl-3 oncoprotein acts as a bridging factor between NF-kappaB/Rel and nuclear co-regulators. *Oncogene* **18**: 3316-23.

DeGregori, J. 2002. The genetics of the E2F family of transcription factors: shared functions and unique roles. *Biochim Biophys Acta* **1602**: 131-50.

DeGregori, J., T. Kowalik, and J.R. Nevins. 1995. Cellular targets for activation by the E2F1 transcription factor include DNA synthesis- and G1/S-regulatory genes. *Mol Cell Biol* **15**: 4215-4224.

Deleu, L., S. Shellard, K. Alevizopoulos, B. Amati, and H. Land. 2001. Recruitment of TRRAP required for oncogenic transformation by E1A. *Oncogene* **20**: 8270-8275.

Di Croce, L, Raker, V.A, Corsaro, M, Fazi, F, Fanelli, M, Faretta, M, Fuks, F, Lo Coco, F, Kouzarides, T,Nervi, C,Minucci, S,Pelicci, P.G. 2002. Methyltransferase recruitment and DNA hypermethylation of target promoters by an oncogenic transcription factor. Science **296**: 1079-1082.

Dillon, N. and R. Festenstein. 2002. Unravelling heterochromatin: competition between positive and negative factors regulates accessibility. *Trends Genet* **18**: 252-8.

Donnellan, R. and R. Chetty. 1998. Cyclin D1 and human neoplasia. *Mol Pathol* **51**: 1-7.

Donnellan, R. and R. Chetty. 1999. Cyclin E in human cancers. *Faseb J* **13**: 773-80.

Dou, Q.P., S. Zhao, A.H. Levin, J. Wang, K. Helin, and A.B. Pardee. 1994. G1/S-regulated E2F-containing protein complexes bind to the mouse thymidine kinase gene promoter. *J Biol Chem* **269**: 1306-13.

Dugan, K.A., M.A. Wood, and M.D. Cole. 2002. TIP49, but not TRRAP, modulates c-Myc and E2F1 dependent apoptosis. *Oncogene* **21**: 5835-43.

Dunaief, J.L., B.E. Strober, S. Guha, P.A. Khavari, K. Alin, J. Luban, M. Begemann, G.R. Crabtree, and S.P. Goff. 1994. The retinoblastoma protein and BRG1 form a complex and cooperate to induce cell cycle arrest. *Cell* **79**: 119-30.

Dyson, N. 1998. The regulation of E2F by pRB-family proteins. *Genes Dev* **12**: 2245-2262.

Eckner, R., Z. Arany, M. Ewen, W. Sellers, and D.M. Livingston. 1994. The adenovirus E1A-associated 300-kD protein exhibits properties of a transcriptional coactivator and belongs to an evolutionarily conserved family. *Cold Spring Harb Symp Quant Biol* **59**: 85-95.

Eisen, A., R.T. Utley, A. Nourani, S. Allard, P. Schmidt, W.S. Lane, J.C. Lucchesi, and J. Cote. 2001. The yeast NuA4 and Drosophila MSL complexes contain homologous subunits important for transcription regulation. *J Biol Chem* **276**: 3484-91.

Eisenman, R.N. 2000. The Max network: coordinated transcriptional regulation of cell growth and proliferation. *Harvey Lect* **96**: 1-32.

Eisenman, R.N. 2001. Deconstructing myc. *Genes Dev* **15**: 2023-2030.

Eissenberg, J.C. and S.C. Elgin. 2000. The HP1 protein family: getting a grip on chromatin. *Curr Opin Genet Dev* **10**: 204-10.

Ekholm, S.V. and S.I. Reed. 2000. Regulation of G(1) Cyclin-dependent kinases in the mammalian cell cycle. *Curr Opin Cell Biol* **12**: 676-84.

Ewen, M.E., Y.G. Xing, J.B. Lawrence, and D.M. Livingston. 1991. Molecular cloning, chromosomal mapping, and expression of the cDNA for p107, a retinoblastoma gene product-related protein. *Cell* **66**: 1155-1164.

Fabbrizio, E., S. El Messaoudi, J. Polanowska, C. Paul, J.R. Cook, J.H. Lee, V. Negre, M. Rousset, S. Pestka, A. Le Cam, and C. Sardet. 2002. Negative regulation of transcription by the type II arginine methyltransferase PRMT5. *EMBO Rep* **3**: 641-5.

Fajas, L., R.L. Landsberg, Y. Huss-Garcia, C. Sardet, J.A. Lees, and J. Auwerx. 2002. E2Fs regulate adipocyte differentiation. *Dev Cell* **3**: 39-49.

Fasbender, A., J. Zabner, M. Chillon, T.O. Moninger, A.P. Puga, B.L. Davidson, and M.J. Welsh. 1997. Complexes of adenovirus with polycationic polymers and cationic lipids increase the efficiency of gene transfer in vitro and in vivo. *J Biol Chem* **272**: 6479-6489.

Feng, X.H., Y.Y. Liang, M. Liang, W. Zhai, and X. Lin. 2002. Direct interaction of c-Myc with Smad2 and Smad3 to inhibit TGF-beta-mediated induction of the CDK inhibitor p15(INK4B). *Mol Cell* **9**: 133-43.

Feng, X.H., X. Lin, and R. Derynck. 2000. Smad2, Smad3 and Smad4 cooperate with Sp1 to induce p15(INK4B) transcription in response to TGF-beta. *Embo J* **19**: 5178-5193.

Fernandez, P., D. Donjerkovic, S.R. Frank, M. Schroeder, L. Wang, S. Liu, J. Greene, and B. Amati. 2002. Genomic targets of the c-Myc protein. *manuscript in preparation*.

Fero, M.L., E. Randel, K.E. Gurley, J.M. Roberts, and C.J. Kemp. 1998. The murine gene p27Kip1 is haplo-insufficient for tumour suppression. *Nature* **396**: 177-80.

Fero, M.L., M. Rivkin, M. Tasch, P. Porter, C.E. Carow, E. Firpo, K. Polyak, L.H. Tsai, V. Broudy, R.M. Perlmutter, K. Kaushansky, and J.M. Roberts. 1996. A syndrome of multiorgan hyperplasia with features of gigantism, tumorigenesis, and female sterility in p27(kip1)-deficient mice. *Cell* **85**: 733-744.

Ferreira, R., I. Naguibneva, M. Mathieu, S. Ait-Si-Ali, P. Robin, L.L. Pritchard, and A. Harel-Bellan. 2001. Cell cycle-dependent recruitment of HDAC-1 correlates with deacetylation of histone H4 on an Rb-E2F target promoter. *EMBO Rep* **2**: 794-9.

Field, S.J., F.Y. Tsai, F. Kuo, A.M. Zubiaga, W.G. Kaelin, D.M. Livingston, S.H. Orkin, and M.E. Greenberg. 1996. E2F-1 functions in mice to promote apoptosis and suppress proliferation. *Cell* **85**: 549-561.

Fischle, W., V. Kiermer, F. Dequiedt, and E. Verdin. 2001. The emerging role of class II histone deacetylases. *Biochem Cell Biol* **79**: 337-48.

Flemington, E.K., S.H. Speck, and W.G. Kaelin, Jr. 1993. E2F-1-mediated transactivation is inhibited by complex formation with the retinoblastoma susceptibility gene product. *Proc Natl Acad Sci U S A* **90**: 6914-8.

Fotedar, A., D. Cannella, P. Fitzgerald, T. Rousselle, S. Gupta, M. Doree, and R. Fotedar. 1996. Role for Cyclin A-dependent kinase in DNA replication in human S phase cell extracts. *J Biol Chem* **271**: 31627-37.

Frank, S.R., M. Schroeder, P. Fernandez, S. Taubert, and B. Amati. 2001. Binding of c-Myc to chromatin mediates mitogen-induced acetylation of histone H4 and gene activation. *Genes Dev* **15**: 2069-2082.

Fry, C.J., Pearson, A., Malinowski, E., Bartley, S. M., Greenblatt, J., Farnham, P. J. 1999. Activation of the murine dihydrofolate reductase promoter by E2F1. A requirement for CBP recruitment. *J Biol Chem* **274**: 15883-91.

Fry, C.J. and C.L. Peterson. 2002. Transcription. Unlocking the gates to gene expression. *Science* **295**: 1847-8.

Fryer, C.J. and T.K. Archer. 1998. Chromatin remodelling by the glucocorticoid receptor requires the BRG1 complex. *Nature* **393**: 88-91.

Fuchs, M., J. Gerber, R. Drapkin, S. Sif, T. Ikura, V. Ogryzko, W.S. Lane, Y. Nakatani, and D.M. Livingston. 2001. The p400 complex is an essential E1A transformation target. *Cell* **106**: 297-307.

Galarneau, L., A. Nourani, A.A. Boudreault, Y. Zhang, L. Heliot, S. Allard, J. Savard, W.S. Lane, D.J. Stillman, and J. Cote. 2000. Multiple links between the NuA4 histone acetyltransferase complex and epigenetic control of transcription. *Mol Cell* **5**: 927-37.

Gasser, S.M. and M.M. Cockell. 2001. The molecular biology of the SIR proteins. *Gene* **279**: 1-16.

Gaubatz, S., J.A. Lees, G.J. Lindeman, and D.M. Livingston. 2001. E2F4 is exported from the nucleus in a CRM1-dependent manner. *Mol Cell Biol* **21**: 1384-92.

Gaubatz, S., G.J. Lindeman, S. Ishida, L. Jakoi, J.R. Nevins, D.M. Livingston, and R.E. Rempel. 2000. E2F4 and E2F5 play an essential role in pocket-protein-mediated G1 control. *Mol Cell* **6**: 729-35.

Gayther, S.A., S.J. Batley, L. Linger, A. Bannister, K. Thorpe, S.F. Chin, Y. Daigo, P. Russell, A. Wilson, H.M. Sowter, J.D. Delhanty, B.A. Ponder, T. Kouzarides, and C. Caldas. 2000. Mutations truncating the EP300 acetylase in human cancers. *Nat Genet* **24**: 300-3.

Geiman, T.M. and K.D. Robertson. 2002. Chromatin-remodeling, histone modifications, and DNA methylation-how does it all fit together? *J Cell Biochem* **87**: 117-25.

Geng, Y., E.N. Eaton, M. Picon, J.M. Roberts, A.S. Lundberg, A. Gifford, C. Sardet, and R.A. Weinberg. 1996. Regulation of Cyclin E transcription by E2Fs and retinoblastoma protein. *Oncogene* **12**: 1173-1180.

Giles, R.H., D.J. Peters, and M.H. Breuning. 1998. Conjunction dysfunction: CBP/p300 in human disease. *Trends Genet* **14**: 178-83.

Giles R.H., Dauwerse J.G., Higgins C., Petrij F., Wessels J.W., Beverstock G.C., Dohner H., Jotterand-Bellomo M., Falkenburg J.H., Slater R.M., van Ommen G.J., Hagemeijer A., van der Reijden B.A., Breuning M.H. Detection of CBP rearrangements in acute myelogenous leukemia with t(8;16). *Leukemia*. 1997 **11**:2087-96.

Ginsberg, D. 2002. E2F1 pathways to apoptosis. *FEBS Lett* **529**: 122-5.

Goll, M.G. and T.H. Bestor. 2002. Histone modification and replacement in chromatin activation. *Genes Dev* **16**: 1739-42.

Goodman, R.H. and S. Smolik. 2000. CBP/p300 in cell growth, transformation, and development. *Genes Dev* **14**: 1553-77.

Grandori, C., S.M. Cowley, L.P. James, and R.N. Eisenman. 2000. The Myc/Max/Mad Network and the Transcriptional Control of Cell Behavior. *Annu Rev Cell Dev Biol* **16**: 653-699.

Grandori, C., J. Mac, F. Siebelt, D.E. Ayer, and R.N. Eisenman. 1996. Myc-Max heterodimers activate a DEAD box gene and interact with multiple E box-related sites in vivo. *Embo J* **15**: 4344-4357.

Grant, P.A., L. Duggan, J. Cote, S.M. Roberts, J.E. Brownell, R. Candau, R. Ohba, T. Owen-Hughes, C.D. Allis, F. Winston, S.L. Berger, and J.L. Workman. 1997. Yeast Gcn5 functions in two multisubunit complexes to acetylate nucleosomal histones: characterization of an Ada complex and the SAGA (Spt/Ada) complex. *Genes Dev* **11**: 1640-1650.

Grant, P.A., D. Schieltz, M.G. Pray-Grant, D.J. Steger, J.C. Reese, J.R. Yates, 3rd, and J.L. Workman. 1998a. A subset of TAF(II)s are integral components of the SAGA complex required for nucleosome acetylation and transcriptional stimulation. *Cell* **94**: 45-53.

Grant, P.A., D. Schieltz, M.G. Pray-Grant, J.R. Yates, 3rd, and J.L. Workman. 1998b. The ATM-related cofactor Tra1 is a component of the purified SAGA complex. *Mol Cell* **2**: 863-867.

Grant, P.A., D.E. Sterner, L.J. Duggan, J.L. Workman, and S.L. Berger. 1998c. The SAGA unfolds: convergence of transcription regulators in chromatin-modifying complexes. *Trends Cell Biol* **8**: 193-197.

Grant, P.A., Berger, S. L., Workman, J. L. Identification and analysis of native nucleosomal histone acetyltransferase complexes. *Methods Mol Biol* **119**: 311-7.

Greasley, P.J., C. Bonnard, and B. Amati. 2000. Myc induces the nucleolin and BN51 genes: possible implications in ribosome biogenesis. *Nucleic Acids Res* **28**: 446-453.

Gregory, P.D., A. Schmid, M. Zavari, M. Munsterkotter, and W. Horz. 1999. Chromatin remodelling at the PHO8 promoter requires SWI-SNF and SAGA at a step subsequent to activator binding. *Embo J* **18**: 6407-14.

Gross-Mesilaty, S., E. Reinstein, B. Bercovich, K.E. Tobias, A.L. Schwartz, C. Kahana, and A. Ciechanover. 1998. Basal and human papillomavirus E6 oncoprotein-induced degradation of Myc proteins by the ubiquitin pathway. *Proc Natl Acad Sci U S A* **95**: 8058-8063.

Gu, W. and R.G. Roeder. 1997. Activation of p53 sequence-specific DNA-binding by acetylation of the p53 C-terminal domain. *Cell* **90**: 595-606.

Guyon, J.R., G.J. Narlikar, S. Sif, and R.E. Kingston. 1999. Stable remodeling of tailless nucleosomes by the human SWI-SNF complex. *Mol Cell Biol* **19**: 2088-97.

Haber, D.A. 1997. Splicing into senescence: the curious case of p16 and p19ARF. *Cell* **91**: 555-8.

Hagemeier, C., A. Cook, and T. Kouzarides. 1993. The retinoblastoma protein binds E2F residues required for activation in vivo and TBP binding in vitro. *Nucleic Acids Res* **21**: 4998-5004.

Hall, I.M., G.D. Shankaranarayana, K. Noma, N. Ayoub, A. Cohen, and S.I. Grewal. 2002. Establishment and maintenance of a heterochromatin domain. *Science* **297**: 2232-7.

Hamamori, Y., V. Sartorelli, V. Ogryzko, P.L. Puri, H.Y. Wu, J.Y. Wang, Y. Nakatani, and L. Kedes. 1999a. Regulation of histone acetyltransferases p300 and PCAF by the bHLH protein twist and adenoviral oncoprotein E1A. *Cell* **96**: 405-13.

Hamamori, Y., V. Sartorelli, V. Ogryzko, P.L. Puri, H.-Y. Wu, J.Y.J. Wang, Y. Nakatani, and L. Kedes. 1999b. Regulation of acetyltransferases p300 and PCAF by the HLH protein twist and adenoviral oncoprotein E1A. *Cell* **96**: 405-413.

Hamel, P.A. and J. Hanley-Hyde. 1997. G1 Cyclins and control of the cell division cycle in normal and transformed cells. *Cancer Invest* **15**: 143-52.

Hanahan, D. and R.A. Weinberg. 2000. The hallmarks of cancer. *Cell* **100**: 57-70.

Hannon, G.J. and D. Beach. 1994. p15 INK4B is a potential effector of TGFß-induced growth arrest. *Nature* **371**: 257-261.

Hannon, G.J., D. Demetrick, and D. Beach. 1993. Isolation of the Rb-related p130 through its interaction with CDK2 and Cyclins. *Genes Dev* **7**: 2378-2391.

Hansen, J.C. 2002. Conformational dynamics of the chromatin fiber in solution: determinants, mechanisms, and functions. *Annu Rev Biophys Biomol Struct* **31**: 361-92.

Harbour, J., R. Luo, A. Dei Santi, A. Postigo, and D. Dean. 1999. Cdk Phosphorylation Triggers Sequential Intramolecular Interactions that Progressively Block Rb Functions as Cells Move through G1. *Cell* **98**: 859–869.

Harbour, J.W. and D.C. Dean. 2000a. Chromatin-remodeling and Rb activity. *Curr Opin Cell Biol* **12**: 685-689.

Harbour, J.W. and D.C. Dean. 2000b. Rb function in cell-cycle regulation and apoptosis. *Nat Cell Biol* **2**: E65-7.

Harbour, J.W. and D.C. Dean. 2000c. The Rb/E2F pathway: expanding roles and emerging paradigms. *Genes Dev* **14**: 2393-2409.

Hardy, S., M. Brand, G. Mittler, J. Yanagisawa, S. Kato, M. Meisterernst, and L. Tora. 2002. TATA-binding protein-free TAF-containing complex (TFTC) and p300 are both required for efficient transcriptional activation. *J Biol Chem* **277**: 32875-82.

Hateboer, G., H.T. Timmers, A.K. Rustgi, M. Billaud, L.J. van't Veer, and R. Bernards. 1993. TATA-binding protein and the retinoblastoma gene product bind to overlapping epitopes on c-Myc and adenovirus E1A protein. *Proc Natl Acad Sci U S A* **90**: 8489-93.

Hateboer, G., A. Wobst, B.O. Petersen, L. Le Cam, E. Vigo, C. Sardet, and K. Helin. 1998. Cell cycle-regulated expression of mammalian CDC6 is dependent on E2F. *Mol Cell Biol* **18**: 6679-97.

Hayes, J.J. and J.C. Hansen. 2001. Nucleosomes and the chromatin fiber. *Curr Opin Genet Dev* **11**: 124-9.
He, T.C., S. Zhou, L.T. da Costa, J. Yu, K.W. Kinzler, and B. Vogelstein. 1998. A simplified system for generating recombinant adenoviruses. *Proc Natl Acad Sci U S A* **95**: 2509-2514.
Helin, K. 1998. Regulation of cell proliferation by the E2F transcription factors. *Curr Opin Genet Dev* **8**: 28-35.
Helin, K., J.A. Lees, M. Vidal, N. Dyson, E. Harlow, and A. Fattaey. 1992. A cDNA encoding a pRB-binding protein with properties of the transcription factor E2F. *Cell* **70**: 337-350.
Helin, K., C.L. Wu, A.R. Fattaey, J.A. Lees, B.D. Dynlacht, C. Ngwu, and E. Harlow. 1993. Heterodimerization of the transcription factors E2F-1 and DP-1 leads to cooperative trans-activation. *Genes Dev* **7**: 1850-1861.
Hengstschlager, M., K. Braun, T. Soucek, A. Miloloza, and E. Hengstschlager-Ottnad. 1999. Cyclin-dependent kinases at the G1-S transition of the mammalian cell cycle. *Mutat Res* **436**: 1-9.
Herceg, Z., W. Hulla, D. Gell, C. Cuenin, M. Lleonart, S. Jackson, and Z.Q. Wang. 2001. Disruption of Trrap causes early embryonic lethality and defects in cell cycle progression. *Nat Genet* **29**: 206-211.
Hiebert, S.W., G. Packham, D.K. Strom, R. Haffner, M. Oren, G. Zambetti, and J.L. Cleveland. 1995. E2F-1:DP-1 induces p53 and overrides survival factors to trigger apoptosis. *Mol Cell Biol* **15**: 6864-6874.
Hijmans, E.M., P.M. Voorhoeve, R.L. Beijersbergen, L.j. van't Veer, and R. Bernards. 1995. E2F-5, a new E2F family member that interacts with p130 in vivo. *Mol Cell Biol* **15**: 3082-3089.
Hofferer, M., C. Wirbelauer, B. Humar, and W. Krek. 1999. Increased levels of E2F-1-dependent DNA-binding activity after UV- or gamma-irradiation. *Nucleic Acids Res* **27**: 491-5.
Hoffman, B., A. Amanullah, M. Shafarenko, and D.A. Liebermann. 2002. The proto-oncogene c-myc in hematopoietic development and leukemogenesis. *Oncogene* **21**: 3414-21.
Horn, P.J. and C.L. Peterson. 2001. The bromodomain: a regulator of ATP-dependent chromatin-remodeling? *Front Biosci* **6**: D1019-23.
Horn, P.J. and C.L. Peterson. 2002. Molecular biology. Chromatin higher order folding--wrapping up transcription. *Science* **297**: 1824-7.
Howe, L., D. Auston, P. Grant, S. John, R.G. Cook, J.L. Workman, and L. Pillus. 2001. Histone H3 specific acetyltransferases are essential for cell cycle progression. *Genes Dev* **15**: 3144-54.
Hsieh, J.K., S. Fredersdorf, T. Kouzarides, K. Martin, and X. Lu. 1997. E2F1-induced apoptosis requires DNA-binding but not transactivation and is inhibited by the retinoblastoma protein through direct interaction. *Genes Dev* **11**: 1840-52.
Huang, D., M. Jokela, J. Tuusa, S. Skog, K. Poikonen, and J.E. Syvaoja. 2001. E2F mediates induction of the Sp1-controlled promoter of the human DNA polymerase epsilon B-subunit gene POLE2. *Nucleic Acids Res* **29**: 2810-21.
Humbert, P.O., C. Rogers, S. Ganiatsas, R.L. Landsberg, J.M. Trimarchi, S. Dandapani, C. Brugnara, S. Erdman, M. Schrenzel, R.T. Bronson, and J.A. Lees. 2000a. E2F4 is essential for normal erythrocyte maturation and neonatal viability. *Mol Cell* **6**: 281-91.
Humbert, P.O., R. Verona, J.M. Trimarchi, C. Rogers, S. Dandapani, and J.A. Lees. 2000b. E2f3 is critical for normal cellular proliferation. *Genes Dev* **14**: 690-703.
Hurford, R.J., D. Cobrinik, M. Lee, and N. Dyson. 1997. pRB and p107/p130 are required for the regulated expression of different sets of E2F responsive genes. *Genes Dev* **11**: 1447-1463.
Hurlin, P., C. Quéva, and R. Eisenman. 1997. Mnt, a novel Max-interacting protein is coexpressed with Myc in proliferating cells and mediates repression at Myc binding sites. *Genes Dev* **11**: 44-58.
Hurlin, P.J., E. Steingrimsson, N.G. Copeland, N.A. Jenkins, and R.N. Eisenman. 1999. Mga, a dual-specificity transcription factor that interacts with Max and contains a T-domain DNA-binding motif [published erratum appears in EMBO J 2000 Jul 17;19(14):3841]. *Embo J* **18**: 7019-28.
Huschtscha, L.I. and R.R. Reddel. 1999. p16(INK4a) and the control of cellular proliferative life span. *Carcinogenesis* **20**: 921-6.

Iizuka, M. and B. Stillman. 1999. Histone acetyltransferase HBO1 interacts with the ORC1 subunit of the human initiator protein. *J Biol Chem* **274**: 23027-34.
Ikeda, M.A., L. Jakoi, and J.R. Nevins. 1996. A unique role for the Rb protein in controlling E2F accumulation during cell growth and differentiation. *Proc Natl Acad Sci U S A* **93**: 3215-20.
Ikura, T., V.V. Ogryzko, M. Grigoriev, R. Groisman, J. Wang, M. Horikoshi, R. Scully, J. Qin, and Y. Nakatani. 2000. Involvement of the TIP60 histone acetylase complex in DNA repair and apoptosis. *Cell* **102**: 463-473.
Imhof, A., X.J. Yang, V.V. Ogryzko, Y. Nakatani, A.P. Wolffe, and H. Ge. 1997. Acetylation of general transcription factors by histone acetyltransferases. *Curr Biol* **7**: 689-92.
Ishida, S., E. Huang, H. Zuzan, R. Spang, G. Leone, M. West, and J.R. Nevins. 2001. Role for E2F in control of both DNA replication and mitotic functions as revealed from DNA microarray analysis. *Mol Cell Biol* **21**: 4684-99.
Jacks, T., A. Fazeli, E.M. Schmitt, R.T. Bronson, M.A. Goodell, and R.A. Weinberg. 1992. Effects of an Rb mutation in the mouse [see comments]. *Nature* **359**: 295-300.
Jenuwein, T. and C.D. Allis. 2001. Translating the histone code. *Science* **293**: 1074-80.
John, S., L. Howe, S.T. Tafrov, P.A. Grant, R. Sternglanz, and J.L. Workman. 2000. The something about silencing protein, Sas3, is the catalytic subunit of NuA3, a yTAF(II)30-containing HAT-complex that interacts with the Spt16 subunit of the yeast CP (Cdc68/Pob3)-FACT complex. *Genes Dev* **14**: 1196-208.
Johnson, D.G., W.G. Cress, L. Jakoi, and J.R. Nevins. 1994. Oncogenic capacity of the E2F-1 gene. *Proc Natl Acad Sci U.S.A.* **91**: 12823-12827.
Johnson, D.G. and R. Schneider-Broussard. 1998. Role of E2F in cell cycle control and cancer. *Front Biosci* **3**: d447-8.
Johnson, D.G., J.K. Schwartz, W.D. Cress, and J.R. Nevins. 1993. Expression of transcription factor E2F-1 induces quiescent cells to enter S phase. *Nature* **365**: 349-352.
Johnstone R.W. Histone-deacetylase inhibitors: novel drugs for the treatment of cancer. *Nat Rev Drug Discov*. 2002 **1**:287-99
Kaelin, W.G., Jr. 1997. Alterations in G1/S cell-cycle control contributing to carcinogenesis. *Ann N Y Acad Sci* **833**: 29-33.
Kamine, J., B. Elangovan, T. Subramanian, D. Coleman, and G. Chinnadurai. 1996. Identification of a cellular protein that specifically interacts with the essential cysteine region of the HIV-1 Tat transactivator. *Virology* **216**: 357-66.
Katan-Khaykovich Y, Struhl K. 2002. Dynamics of global histone acetylation and deacetylation in vivo: rapid restoration of normal histone acetylation status upon removal of activators and repressors. *Genes Dev*. **16**: 743-52.
Kel, A.E., O.V. Kel-Margoulis, P.J. Farnham, S.M. Bartley, E. Wingender, and M.Q. Zhang. 2001. Computer-assisted identification of cell cycle-related genes: new targets for E2F transcription factors. *J Mol Biol* **309**: 99-120.
Kelly, K. and U. Siebenlist. 1986. The regulation and expression of c-myc in normal and malignant cells. *Annu Rev Immunol* **4**: 317-338.
Kelly W.K., O'Connor O.A., Marks P.A. Histone deacetylase inhibitors: from target to clinical trials. *Expert Opin Investig Drugs*. 2002 **11**:1695-713.
Kingston, R. E., Narlikar, G. J. ATP-dependent remodeling and acetylation as regulators of chromatin fluidity. 1999. *Genes Dev* **13**: 2339-2352.
Kimura, A. and M. Horikoshi. 1998. Tip60 acetylates six lysines of a specific class in core histones in vitro. *Genes Cells* **3**: 789-800.
Kitabayashi, I., Y. Aikawa, L.A. Nguyen, A. Yokoyama, and M. Ohki. 2001. Activation of AML1-mediated transcription by MOZ and inhibition by the MOZ-CBP fusion protein. *Embo J* **20**: 7184-96.
Klochendler-Yeivin, A., L. Fiette, J. Barra, C. Muchardt, C. Babinet, and M. Yaniv. 2000. The murine SNF5/INI1 chromatin-remodeling factor is essential for embryonic development and tumor suppression. *EMBO Rep* **1**: 500-6.
Klochendler-Yeivin, A. and M. Yaniv. 2001. Chromatin modifiers and tumor suppression. *Biochim Biophys Acta* **1551**: M1-10.
Koh, S.S., D. Chen, Y.H. Lee, and M.R. Stallcup. 2001. Synergistic enhancement of nuclear receptor function by p160 coactivators and two coactivators with protein methyltransferase activities. *J Biol Chem* **276**: 1089-98.
Kondo, Y., L. Shen, and J.P. Issa. 2003. Critical role of histone methylation in tumor suppressor gene silencing in colorectal cancer. *Mol Cell Biol* **23**: 206-15.

Kornberg, R.D. and Y. Lorch. 1999a. Chromatin-modifying and -remodeling complexes. *Curr Opin Genet Dev* **9**: 148-51.
Kornberg, R.D. and Y. Lorch. 1999b. Twenty-five years of the nucleosome, fundamental particle of the eukaryote chromosome. *Cell* **98**: 285-94.
Kouzarides, T. 2002. Histone methylation in transcriptional control. *Curr Opin Genet Dev* **12**: 198-209.
Krebs, J.E., C.J. Fry, M.L. Samuels, and C.L. Peterson. 2000. Global role for chromatin-remodeling-enzymes in mitotic gene expression. *Cell* **102**: 587-598.
Krek, W., M.E. Ewen, S. Shirodkar, Z. Arany, W.G. Kaelin, Jr., and D.M. Livingston. 1994. Negative regulation of the growth-promoting transcription factor E2F-1 by a stably bound Cyclin A-dependent protein kinase. *Cell* **78**: 161-72.
Krek, W., D.M. Livingston, and S. Shirodkar. 1993. Binding to DNA and the retinoblastoma gene product promoted by complex formation of different E2F family members. *Science* **262**: 1557-1560.
Krek, W., G. Xu, and D.M. Livingston. 1995. Cyclin A-kinase regulation of E2F-1 DNA-binding function underlies suppression of an S phase checkpoint. *Cell* **83**: 1149-1158.
Kristjuhan, A., J. Walker, N. Suka, M. Grunstein, D. Roberts, B.R. Cairns, and J.Q. Svejstrup. 2002. Transcriptional inhibition of genes with severe histone h3 hypoacetylation in the coding region. *Mol Cell* **10**: 925-33.
Kung, A.L., V.I. Rebel, R.T. Bronson, L.E. Ch'ng, C.A. Sieff, D.M. Livingston, and T.P. Yao. 2000. Gene dose-dependent control of hematopoiesis and hematologic tumor suppression by CBP. *Genes Dev* **14**: 272-7.
Kuo, M.H. and C.D. Allis. 1998. Roles of histone acetyltransferases and deacetylases in gene regulation. *Bioessays* **20**: 615-26.
Kuo, M.H., J.E. Brownell, R.E. Sobel, T.A. Ranalli, R.G. Cook, D.G. Edmondson, S.Y. Roth, and C.D. Allis. 1996. Transcription-linked acetylation by Gcn5p of histones H3 and H4 at specific lysines. *Nature* **383**: 269-72.
Kuo, M.H., J. Zhou, P. Jambeck, M.E. Churchill, and C.D. Allis. 1998. Histone acetyltransferase activity of yeast Gcn5p is required for the activation of target genes in vivo. *Genes Dev* **12**: 627-39.
LaBaer, J., M.D. Garrett, L.F. Stevenson, J.M. Slingerland, C. Sandhu, H.S. Chou, A. Fattaey, and E. Harlow. 1997. New functional activities for the p21 family of CDK inhibitors. *Genes Dev* **11**: 847-862.
Lachner, M., D. O'Carroll, S. Rea, K. Mechtler, and T. Jenuwein. 2001. Methylation of histone H3 lysine 9 creates a binding site for HP1 proteins. *Nature* **410**: 116-20.
Lai, A., B.K. Kennedy, D.A. Barbie, N.R. Bertos, X.J. Yang, M.C. Theberge, S.C. Tsai, E. Seto, Y. Zhang, A. Kuzmichev, W.S. Lane, D. Reinberg, E. Harlow, and P.E. Branton. 2001. RBP1 recruits the mSIN3-histone deacetylase complex to the pocket of retinoblastoma tumor suppressor family proteins found in limited discrete regions of the nucleus at growth arrest. *Mol Cell Biol* **21**: 2918-32.
Lam, E.W. and R.J. Watson. 1993. An E2F-binding site mediates cell-cycle regulated repression of mouse B-myb transcription. *EMBO J* **12**: 2705-2713.
Lang, S.E., S.B. McMahon, M.D. Cole, and P. Hearing. 2001. E2F transcriptional activation requires TRRAP and GCN5 cofactors. *J Biol Chem* **276**: 32627-34.
Langst, G. and P.B. Becker. 2001. Nucleosome mobilization and positioning by ISWI-containing chromatin-remodeling factors. *J Cell Sci* **114**: 2561-8.
Langst, G., E.J. Bonte, D.F. Corona, and P.B. Becker. 1999. Nucleosome movement by CHRAC and ISWI without disruption or trans-displacement of the histone octamer. *Cell* **97**: 843-52.
Lavia, P. and P. Jansen-Durr. 1999. E2F target genes and cell-cycle checkpoint control. *Bioessays* **21**: 221-30.
LeCouter, J.E., B. Kablar, W.R. Hardy, C. Ying, L.A. Megeney, L.L. May, and M.A. Rudnicki. 1998a. Strain-dependent myeloid hyperplasia, growth deficiency, and accelerated cell cycle in mice lacking the Rb-related p107 gene. *Mol Cell Biol* **18**: 7455-65.
LeCouter, J.E., B. Kablar, P.F. Whyte, C. Ying, and M.A. Rudnicki. 1998b. Strain-dependent embryonic lethality in mice lacking the retinoblastoma-related p130 gene. *Development* **125**: 4669-79.
Lee, E.Y., H. Cam, U. Ziebold, J.B. Rayman, J.A. Lees, and B.D. Dynlacht. 2002. E2F4 loss suppresses tumorigenesis in Rb mutant mice. *Cancer Cell* **2**: 463-72.

Lee, E.Y.-H.P., C.-Y. Chang, N. Hu, Y.-C.J. Wang, C.-C. Lai, K. Herrup, W.-H. Lee, and A. Bradley. 1992. Mice deficient for Rb are nonviable and show defects in neurogenesis and haematopoiesis. *Nature* **359**: 288-294.

Lee, M.-H., B.O. Williams, G. Mulligan, S. Mukai, R.T. Bronson, N. Dyson, E. Harlow, and T. Jacks. 1996. Targeted disruption of p107: functional overlap between p107 and Rb. *Genes Dev* **10**: 1621-1632.

Legube, G., L.K. Linares, C. Lemercier, M. Scheffner, S. Khochbin, and D. Trouche. 2002. Tip60 is targeted to proteasome-mediated degradation by Mdm2 and accumulates after UV irradiation. *Embo J* **21**: 1704-12.

Leone, G., J. DeGregori, Z. Yan, L. Jakoi, S. Ishida, R.S. Williams, and J.R. Nevins. 1998. E2F3 activity is regulated during the cell cycle and is required for the induction of S phase. *Genes Dev* **12**: 2120-2130.

Leone, G., F. Nuckolls, S. Ishida, M. Adams, R. Sears, L. Jakoi, A. Miron, and J.R. Nevins. 2000. Identification of a novel E2F3 product suggests a mechanism for determining specificity of repression by Rb proteins. *Mol Cell Biol* **20**: 3626-32.

Leonhardt, H. and M.C. Cardoso. 2000. DNA methylation, nuclear structure, gene expression and cancer. *J Cell Biochem Suppl* **Suppl**: 78-83.

Li, Y., C. Graham, S. Lacy, A.M. Duncan, and P. Whyte. 1993. The adenovirus E1A-associated 130-kD protein is encoded by a member of the retinoblastoma gene family and physically interacts with Cyclins A and E. *Genes Dev* **7**: 2366-2377.

Liang, J., L. Prouty, B.J. Williams, M.A. Dayton, and K.L. Blanchard. 1998. Acute mixed lineage leukemia with an inv(8)(p11q13) resulting in fusion of the genes for MOZ and TIF2. *Blood* **92**: 2118-22.

Lin, W.C., F.T. Lin, and J.R. Nevins. 2001. Selective induction of E2F1 in response to DNA damage, mediated by ATM-dependent phosphorylation. *Genes Dev* **15**: 1833-44.

Lindeman, G.J., L. Dagnino, S. Gaubatz, Y. Xu, R.T. Bronson, H.B. Warren, and D.M. Livingston. 1998. A specific, nonproliferative role for E2F-5 in choroid plexus function revealed by gene targeting. *Genes Dev* **12**: 1092-8.

Lindeman, G.J., S. Gaubatz, D.M. Livingston, and D. Ginsberg. 1997. The subcellular localization of E2F-4 is cell-cycle dependent. *Proc Natl Acad Sci U S A* **94**: 5095-5100.

Lissy, N.A., P.K. Davis, M. Irwin, W.G. Kaelin, and S.F. Dowdy. 2000. A common E2F-1 and p73 pathway mediates cell death induced by TCR activation. *Nature* **407**: 642-5.

Liu, L., Scolnick, D. M., Trievel, R. C., Zhang, H. B., Marmorstein, R., Halazonetis, T. D., Berger, S. L. p53 sites acetylated in vitro by PCAF and p300 are acetylated in vivo in response to DNA damage. *Mol Cell Biol* **19**: 1202-1209.

Liu, R., H. Liu, X. Chen, M. Kirby, P.O. Brown, and K. Zhao. 2001. Regulation of CSF1 promoter by the SWI/SNF-like BAF complex. *Cell* **106**: 309-18.

Liu, Y., A.L. Colosimo, X.J. Yang, and D. Liao. 2000. Adenovirus E1B 55-kilodalton oncoprotein inhibits p53 acetylation by PCAF. *Mol Cell Biol* **20**: 5540-53.

Lo, W.S., L. Duggan, N.C. Tolga, Emre, R. Belotserkovskya, W.S. Lane, R. Shiekhattar, and S.L. Berger. 2001. Snf1--a histone kinase that works in concert with the histone acetyltransferase Gcn5 to regulate transcription. *Science* **293**: 1142-6.

Lo, W.S., R.C. Trievel, J.R. Rojas, L. Duggan, J.Y. Hsu, C.D. Allis, R. Marmorstein, and S.L. Berger. 2000. Phosphorylation of serine 10 in histone H3 is functionally linked in vitro and in vivo to Gcn5-mediated acetylation at lysine 14. *Mol Cell* **5**: 917-26.

Loewith, R., M. Meijer, S.P. Lees-Miller, K. Riabowol, and D. Young. 2000. Three yeast proteins related to the human candidate tumor suppressor p33(ING1) are associated with histone acetyltransferase activities. *Mol Cell Biol* **20**: 3807-16.

Lucchesi, J.C. 1998. Dosage compensation in flies and worms: the ups and downs of X-chromosome regulation. *Curr Opin Genet Dev* **8**: 179-84.

Luger, K., A.W. Mader, R.K. Richmond, D.F. Sargent, and T.J. Richmond. 1997. Crystal structure of the nucleosome core particle at 2.8 A resolution. *Nature* **389**: 251-260.

Lukas, J., T. Herzinger, K. Hansen, M. Moroni, D. Resnitzky, K. Helin, S. Reed, and J. Bartek. 1997. Cyclin E-induced S phase without activation of the pRb/E2F pathway. *Genes Dev* **11**: 1479-1492.

Lukas, J., B. Otzen Petersen, K. Holm, J. Bartek, and K. Helin. 1996. Deregulated expression of E2F family members induces S-phase entry and overcomes p16INK4A-mediated growth suppression. *Mol Cell Biol* **16**: 1047-1057.

Lundberg, A.S., W.C. Hahn, P. Gupta, and R.A. Weinberg. 2000. Genes involved in senescence and immortalization. *Curr Opin Cell Biol* **12**: 705-9.
Luscher, B. 2001. Function and regulation of the transcription factors of the Myc/Max/Mad network. *Gene* **277**: 1-14.
Luscher, B. and R.N. Eisenman. 1990. New light on Myc and Myb. Part I. Myc. *Genes Dev* **4**: 2025-2035.
Lutterbach, B. and S.R. Hann. 1999. c-Myc transactivation domain-associated kinases: questionable role for map kinases in c-Myc phosphorylation. *J Cell Biochem* **72**: 483-491.
Lutz, W., J. Leon, and M. Eilers. 2002. Contributions of Myc to tumorigenesis. *Biochim Biophys Acta* **1602**: 61-71.
Ma, H., C.T. Baumann, H. Li, B.D. Strahl, R. Rice, M.A. Jelinek, D.W. Aswad, C.D. Allis, G.L. Hager, and M.R. Stallcup. 2001. Hormone-dependent, CARM1-directed, arginine-specific methylation of histone H3 on a steroid-regulated promoter. *Curr Biol* **11**: 1981-5.
Ma, T., B.A. Van Tine, Y. Wei, M.D. Garrett, D. Nelson, P.D. Adams, J. Wang, J. Qin, L.T. Chow, and J.W. Harper. 2000. Cell cycle-regulated phosphorylation of p220(NPAT) by Cyclin E/Cdk2 in Cajal bodies promotes histone gene transcription. *Genes Dev* **14**: 2298-313.
Magnaghi-Jaulin, L., R. Groisman, I. Naguibneva, P. Robin, S. Lorain, J.P. Le Villain, F. Troalen, D. Trouche, and A. Harel-Bellan. 1998. Retinoblastoma protein represses transcription by recruiting a histone deacetylase. *Nature* **391**: 601-605.
Mann, D.J. and N.C. Jones. 1996. E2F-1 but not E2F-4 can overcome p16-induced G1 cell-cycle arrest. *Curr Biol* **6**: 474-483.
Marks, P., R.A. Rifkind, V.M. Richon, R. Breslow, T. Miller, and W.K. Kelly. 2001. Histone deacetylases and cancer: causes and therapies. *Nat Rev Cancer* **1**: 194-202.
Martinez, E., T.K. Kundu, J. Fu, and R.G. Roeder. 1998. A human SPT3-TAFII31-GCN5-L acetylase complex distinct from transcription factor IID. *J Biol Chem* **273**: 23781-23785.
Martinez, E., V.B. Palhan, A. Tjernberg, E.S. Lymar, A.M. Gamper, T.K. Kundu, B.T. Chait, and R.G. Roeder. 2001. Human STAGA complex is a chromatin-acetylating transcription coactivator that interacts with pre-mRNA splicing and DNA damage-binding factors in vivo. *Mol Cell Biol* **21**: 6782-95.
Martinez-Balbas, M.A., U.M. Bauer, S.J. Nielsen, A. Brehm, and T. Kouzarides. 2000. Regulation of E2F1 activity by acetylation. *Embo J* **19**: 662-71.
Marzio, G., C. Wagener, M.I. Gutierrez, P. Cartwright, K. Helin, and M. Giacca. 2000. E2F family members are differentially regulated by reversible acetylation. *J Biol Chem* **275**: 10887-92.
Maser, R.S., O.K. Mirzoeva, J. Wells, H. Olivares, B.R. Williams, R.A. Zinkel, P.J. Farnham, and J.H. Petrini. 2001. Mre11 complex and DNA replication: linkage to E2F and sites of DNA synthesis. *Mol Cell Biol* **21**: 6006-16.
McBride, A.E. and P.A. Silver. 2001. State of the arg: protein methylation at arginine comes of age. *Cell* **106**: 5-8.
McEwan, I.J., K. Dahlman-Wright, J. Ford, and A.P. Wright. 1996. Functional interaction of the c-Myc transactivation domain with the TATA binding protein: evidence for an induced fit model of transactivation domain folding. *Biochemistry* **35**: 9584-93.
McMahon, S.B., H.A. Van Buskirk, K.A. Dugan, T.D. Copeland, and M.D. Cole. 1998. The novel ATM-related protein TRRAP is an essential cofactor for the c- Myc and E2F oncoproteins. *Cell* **94**: 363-374.
McMahon, S. B., Wood, M. A., Cole, M. D. The essential cofactor TRRAP recruits the histone acetyltransferase hGCN5 to c-Myc. *Mol Cell Biol* **20**: 556-562.
Menssen, A. and H. Hermeking. 2002. Characterization of the c-MYC-regulated transcriptome by SAGE: identification and analysis of c-MYC target genes. *Proc Natl Acad Sci U S A* **99**: 6274-6279.
Merienne, K., S. Pannetier, A. Harel-Bellan, and P. Sassone-Corsi. 2001. Mitogen-regulated RSK2-CBP interaction controls their kinase and acetylase activities. *Mol Cell Biol* **21**: 7089-96.
Meroni, G., A. Reymond, M. Alcalay, G. Borsani, A. Tanigami, R. Tonlorenzi, C.L. Nigro, S. Messali, M. Zollo, D.H. Ledbetter, R. Brent, A. Ballabio, and R. Carrozzo. 1997. Rox, a novel bHLHZip protein expressed in quiescent cells that heterodimerizes with Max,

binds a non-canonical E box and acts as a transcriptional repressor. *Embo J* **16**: 2892-2906.
Moberg, K., M.A. Starz, and J.A. Lees. 1996. E2F-4 switches from p130 to p107 and pRB in response to cell cycle reentry. *Mol Cell Biol* **16**: 1436-49.
Molinari, M. 2000. Cell cycle checkpoints and their inactivation in human cancer. *Cell Prolif* **33**: 261-74.
Moller, M.B. 2000. P27 in cell cycle control and cancer. *Leuk Lymphoma* **39**: 19-27.
Morgenstern, J.P. and H. Land. 1990. Advanced mammalian gene transfer: high titre retroviral vectors with multiple drug selection markers and a complementary helper-free packaging cell line. *Nuc Acids Res* **18**: 3587-3596.
Moroni, M.C., E.S. Hickman, E.L. Denchi, G. Caprara, E. Colli, F. Cecconi, H. Muller, and K. Helin. 2001. Apaf-1 is a transcriptional target for E2F and p53. *Nat Cell Biol* **3**: 552-8.
Morrison, A.J., C. Sardet, and R.E. Herrera. 2002. Retinoblastoma protein transcriptional repression through histone deacetylation of a single nucleosome. *Mol Cell Biol* **22**: 856-65.
Morrow, M. and W. Gradishar. 2002. Breast cancer. *Bmj* **324**: 410-4.
Muller, H., A.P. Bracken, R. Vernell, M.C. Moroni, F. Christians, E. Grassilli, E. Prosperini, E. Vigo, J.D. Oliner, and K. Helin. 2001. E2Fs regulate the expression of genes involved in differentiation, development, proliferation, and apoptosis. *Genes Dev* **15**: 267-285.
Muller, H., M.C. Moroni, E. Vigo, B.O. Petersen, J. Bartek, and K. Helin. 1997. Induction of S-phase entry by E2F transcription factors depends on their nuclear localization. *Mol Cell Biol* **17**: 5508-20.
Muraoka, M., M. Konishi, R. Kikuchi-Yanoshita, K. Tanaka, N. Shitara, J.M. Chong, T. Iwama, and M. Miyaki. 1996. p300 gene alterations in colorectal and gastric carcinomas. *Oncogene* **12**: 1565-9.
Murata, T., R. Kurokawa, A. Krones, K. Tatsumi, M. Ishii, T. Taki, M. Masuno, H. Ohashi, M. Yanagisawa, M.G. Rosenfeld, C.K. Glass, and Y. Hayashi. 2001. Defect of histone acetyltransferase activity of the nuclear transcriptional coactivator CBP in Rubinstein-Taybi syndrome. *Hum Mol Genet* **10**: 1071-6.
Murga, M., O. Fernandez-Capetillo, S.J. Field, B. Moreno, L.R. Borlado, Y. Fujiwara, D. Balomenos, A. Vicario, A.C. Carrera, S.H. Orkin, M.E. Greenberg, and A.M. Zubiaga. 2001. Mutation of E2F2 in mice causes enhanced T lymphocyte proliferation, leading to the development of autoimmunity. *Immunity* **15**: 959-70.
Nahle, Z., J. Polakoff, R.V. Davuluri, M.E. McCurrach, M.D. Jacobson, M. Narita, M.Q. Zhang, Y. Lazebnik, D. Bar-Sagi, and S.W. Lowe. 2002. Direct coupling of the cell cycle and cell death machinery by E2F. *Nat Cell Biol* **4**: 859-64.
Narlikar, G.J., H.Y. Fan, and R.E. Kingston. 2002. Cooperation between complexes that regulate chromatin structure and transcription. *Cell* **108**: 475-87.
Neuwald, A.F. and D. Landsman. 1997. GCN5-related histone N-acetyltransferases belong to a diverse superfamily that includes the yeast SPT10 protein. *Trends Biochem Sci* **22**: 154-5.
Nevins, J.R. 1992. E2F: a link between the Rb tumor suppressor protein and viral oncoproteins. *Science* **258**: 424-429.
Nevins, J.R. 1998. Toward an understanding of the functional complexity of the E2F and retinoblastoma families. *Cell Growth Differ* **9**: 585-93.
Nevins, J.R. 2001. The Rb/E2F pathway and cancer. *Hum Mol Genet* **10**: 699-703.
Nevins, J.R., J. DeGregori, L. Jakoi, and G. Leone. 1997a. Functional analysis of E2F transcription factor. *Methods Enzymol* **283**: 205-19.
Nevins, J.R., G. Leone, J. DeGregori, and L. Jakoi. 1997b. Role of the Rb/E2F pathway in cell growth control. *J Cell Physiol* **173**: 233-6.
Nichols, A.F., T. Itoh, F. Zolezzi, S. Hutsell, and S. Linn. 2003. Basal transcriptional regulation of human damage-specific DNA-binding protein genes DDB1 and DDB2 by Sp1, E2F, N-myc and NF1 elements. *Nucleic Acids Res* **31**: 562-9.
Nielsen, P.R., D. Nietlispach, H.R. Mott, J. Callaghan, A. Bannister, T. Kouzarides, A.G. Murzin, N.V. Murzina, and E.D. Laue. 2002. Structure of the HP1 chromodomain bound to histone H3 methylated at lysine 9. *Nature* **416**: 103-7.
Nielsen, S.J., R. Schneider, U.M. Bauer, A.J. Bannister, A. Morrison, D. O'Carroll, R. Firestein, M. Cleary, T. Jenuwein, R.E. Herrera, and T. Kouzarides. 2001. Rb targets histone H3 methylation and HP1 to promoters. *Nature* **412**: 561-5.

Nikiforov, M.A., S. Chandriani, J. Park, I. Kotenko, D. Matheos, A. Johnsson, S.B. McMahon, and M.D. Cole. 2002. TRRAP-dependent and TRRAP-independent transcriptional activation by Myc-family oncoproteins. *Mol Cell Biol* **22**: 5054-5063.

Nourani, A., Y. Doyon, R.T. Utley, S. Allard, W.S. Lane, and J. Cote. 2001. Role of an ING1 growth regulator in transcriptional activation and targeted histone acetylation by the NuA4 complex. *Mol Cell Biol* **21**: 7629-40.

Obaya, A.J. and J.M. Sedivy. 2002. Regulation of Cyclin-Cdk activity in mammalian cells. *Cell Mol Life Sci* **59**: 126-42.

O'Connor, P.M. 1997. Mammalian G1 and G2 phase checkpoints. *Cancer Surv* **29**: 151-82.

Ogawa, H., K. Ishiguro, S. Gaubatz, D.M. Livingston, and Y. Nakatani. 2002. A complex with chromatin modifiers that occupies E2F- and Myc-responsive genes in G0 cells. *Science* **296**: 1132-6.

Ogryzko, V.V., T. Kotani, X. Zhang, R.L. Schlitz, T. Howard, X.J. Yang, B.H. Howard, J. Qin, and Y. Nakatani. 1998. Histone-like TAFs within the PCAF histone acetylase complex. *Cell* **94**: 35-44.

O'Hagan, R.C., N. Schreiber-Agus, K. Chen, G. David, J.A. Engelman, R. Schwab, L. Alland, C. Thomson, D.R. Ronning, J.C. Sacchettini, P. Meltzer, and R.A. DePinho. 2000. Gene-target recognition among members of the myc superfamily and implications for oncogenesis. *Nat Genet* **24**: 113-119.

Ohtani, K., J. DeGregori, G. Leone, D.R. Herendeen, T.J. Kelly, and J.R. Nevins. 1996. Expression of the HsOrc1 gene, a human ORC1 homolog, is regulated by cell proliferation via the E2F transcription factor. *Mol Cell Biol* **16**: 6977-84.

Ohtsubo, M., A.M. Theodoras, J. Schumacher, J.M. Roberts, and M. Pagano. 1995. Human Cyclin E, a nuclear protein essential for the G1-to-S phase transition. *Mol Cell Biol* **15**: 2612-2624.

Oike, Y., A. Hata, T. Mamiya, T. Kaname, Y. Noda, M. Suzuki, H. Yasue, T. Nabeshima, K. Araki, and K. Yamamura. 1999a. Truncated CBP protein leads to classical Rubinstein-Taybi syndrome phenotypes in mice: implications for a dominant-negative mechanism. *Hum Mol Genet* **8**: 387-96.

Oike, Y., N. Takakura, A. Hata, T. Kaname, M. Akizuki, Y. Yamaguchi, H. Yasue, K. Araki, K. Yamamura, and T. Suda. 1999b. Mice homozygous for a truncated form of CREB-binding protein exhibit defects in hematopoiesis and vasculo-angiogenesis. *Blood* **93**: 2771-9.

Olave, I.A., S.L. Reck-Peterson, and G.R. Crabtree. 2002. Nuclear actin and actin-related proteins in chromatin-remodeling. *Annu Rev Biochem* **71**: 755-81.

Orlando, V., H. Strutt, and R. Paro. 1997. Analysis of chromatin structure by in vivo formaldehyde cross-linking. *Methods* **11**: 205-14.

Ortega, S., M. Malumbres, and M. Barbacid. 2002. Cyclin D-dependent kinases, INK4 inhibitors and cancer. *Biochim Biophys Acta* **1602**: 73-87.

Ozdag, H., S.J. Batley, A. Forsti, N.G. Iyer, Y. Daigo, J. Boutell, M.J. Arends, B.A. Ponder, T. Kouzarides, and C. Caldas. 2002. Mutation analysis of CBP and PCAF reveals rare inactivating mutations in cancer cell lines but not in primary tumours. *Br J Cancer* **87**: 1162-5.

Palazzo, A., B. Ackerman, and G.G. Gundersen. 2003. Cell biology (Communication arising): Tubulin acetylation and cell motility. *Nature* **421**: 230.

Pan, H., C. Yin, N.J. Dyson, E. Harlow, L. Yamasaki, and T. Van Dyke. 1998. Key roles for E2F1 in signaling p53-dependent apoptosis and in cell division within developing tumors. *Mol Cell* **2**: 283-92.

Panagopoulos, I., T. Fioretos, M. Isaksson, U. Samuelsson, R. Billstrom, B. Strombeck, F. Mitelman, and B. Johansson. 2001. Fusion of the MORF and CBP genes in acute myeloid leukemia with the t(10;16)(q22;p13). *Hum Mol Genet* **10**: 395-404.

Pannuti, A. and J.C. Lucchesi. 2000. ReCycling to remodel: evolution of dosage-compensation complexes. *Curr Opin Genet Dev* **10**: 644-50.

Pardee, A.B. 1989. G1 events and regulation of cell proliferation. *Science* **246**: 603-608.

Parisi, T., A. Pollice, A. Di Cristofano, V. Calabro, and G. La Mantia. 2002. Transcriptional regulation of the human tumor suppressor p14(ARF) by E2F1, E2F2, E2F3, and Sp1-like factors. *Biochem Biophys Res Commun* **291**: 1138-45.

Patel, D., S.M. Huang, L.A. Baglia, and D.J. McCance. 1999. The E6 protein of human papillomavirus type 16 binds to and inhibits co-activation by CBP and p300. *Embo J* **18**: 5061-72.

Pearson, B.E., H.P. Nasheuer, and T.S. Wang. 1991. Human DNA polymerase alpha gene: sequences controlling expression in cycling and serum-stimulated cells. *Mol Cell Biol* **11**: 2081-95.

Pelletier, N., N. Champagne, S. Stifani, and X.J. Yang. 2002. MOZ and MORF histone acetyltransferases interact with the Runt-domain transcription factor Runx2. *Oncogene* **21**: 2729-40.

Perkins, N.D., L.K. Felzien, J.C. Betts, K. Leung, D.H. Beach, and G.J. Nabel. 1997. Regulation of NF-kappaB by Cyclin-dependent kinases associated with the p300 coactivator. *Science* **275**: 523-7.

Peters, A.H., J.E. Mermoud, D. O'Carroll, M. Pagani, D. Schweizer, N. Brockdorff, and T. Jenuwein. 2002. Histone H3 lysine 9 methylation is an epigenetic imprint of facultative heterochromatin. *Nat Genet* **30**: 77-80.

Peters, A.H., D. O'Carroll, H. Scherthan, K. Mechtler, S. Sauer, C. Schofer, K. Weipoltshammer, M. Pagani, M. Lachner, A. Kohlmaier, S. Opravil, M. Doyle, M. Sibilia, and T. Jenuwein. 2001. Loss of the Suv39h histone methyltransferases impairs mammalian heterochromatin and genome stability. *Cell* **107**: 323-37.

Peterson, C.L. 2002. Chromatin-remodeling: nucleosomes bulging at the seams. *Curr Biol* **12**: R245-7.

Petrij, F., R.H. Giles, H.G. Dauwerse, J.J. Saris, R.C. Hennekam, M. Masuno, N. Tommerup, G.J. van Ommen, R.H. Goodman, D.J. Peters, and et al. 1995. Rubinstein-Taybi syndrome caused by mutations in the transcriptional co-activator CBP. *Nature* **376**: 348-51.

Peukert, K., P. Staller, A. Schneider, G. Carmichael, F. Hanel, and M. Eilers. 1997. An alternative pathway for gene regulation by Myc. *Embo J* **16**: 5672-5686.

Phelan, M.L., S. Sif, G.J. Narlikar, and R.E. Kingston. 1999. Reconstitution of a core chromatin-remodeling-complex from SWI/SNF subunits. *Mol Cell* **3**: 247-53.

Phillips, A.C., S. Bates, K.M. Ryan, K. Helin, and K.H. Vousden. 1997. Induction of DNA synthesis and apoptosis are separable functions of E2F-1. *Genes Dev* **11**: 1853-63.

Phillips, A.C., M.K. Ernst, S. Bates, N.R. Rice, and K.H. Vousden. 1999. E2F-1 potentiates cell death by blocking antiapoptotic signaling pathways. *Mol Cell* **4**: 771-81.

Phillips, A.C. and K.H. Vousden. 2000. Acetyltransferases and tumour suppression. *Breast Cancer Res* **2**: 244-6.

Phillips, A.C. and K.H. Vousden. 2001. E2F-1 induced apoptosis. *Apoptosis* **6**: 173-82.

Planas-Silva, M.D. and R.A. Weinberg. 1997. The restriction point and control of cell proliferation. *Curr Op in Cell Biol* **9**: 768-772.

Polager, S., Y. Kalma, E. Berkovich, and D. Ginsberg. 2002. E2Fs up-regulate expression of genes involved in DNA replication, DNA repair and mitosis. *Oncogene* **21**: 437-46.

Polevoda, B. and F. Sherman. 2002. The diversity of acetylated proteins. *Genome Biol* **3**: reviews0006.

Puri, P.L., M.L. Avantaggiati, C. Balsano, N. Sang, A. Graessmann, A. Giordano, and M. Levrero. 1997a. p300 is required for MyoD-dependent cell cycle arrest and muscle-specific gene transcription. *Embo J* **16**: 369-83.

Puri, P.L., V. Sartorelli, X.J. Yang, Y. Hamamori, V.V. Ogryzko, B.H. Howard, L. Kedes, J.Y. Wang, A. Graessmann, Y. Nakatani, and M. Levrero. 1997b. Differential roles of p300 and PCAF acetyltransferases in muscle differentiation. *Mol Cell* **1**: 35-45.

Qin, X.-Q., D.M. Livingston, W.G. Kaelin, and P.D. Adams. 1994. Deregulated transcription factor E2F-1 expression leads to S-phase entry and p53-mediated apoptosis. *Proc Natl Acad Sci U S A* **91**: 10918-10922.

Rando, O.J., K. Zhao, and G.R. Crabtree. 2000. Searching for a function for nuclear actin. *Trends Cell Biol* **10**: 92-7.

Rayman, J.B., Y. Takahashi, V.B. Indjeian, J.H. Dannenberg, S. Catchpole, R.J. Watson, H. te Riele, and B.D. Dynlacht. 2002. E2F mediates cell cycle-dependent transcriptional repression in vivo by recruitment of an HDAC1/mSin3B corepressor complex. *Genes Dev* **16**: 933-47.

Reid, J.L., A.J. Bannister, P. Zegerman, M.A. Martinez-Balbas, and T. Kouzarides. 1998. E1A directly binds and regulates the P/CAF acetyltransferase. *Embo J* **17**: 4469-4477.

Reifsnyder, C., J. Lowell, A. Clarke, and L. Pillus. 1996. Yeast SAS silencing genes and human genes associated with AML and HIV-1 Tat interactions are homologous with acetyltransferases. *Nat Genet* **14**: 42-9.

Rempel, R.E., M.T. Saenz-Robles, R. Storms, S. Morham, S. Ishida, A. Engel, L. Jakoi, M.F. Melhem, J.M. Pipas, C. Smith, and J.R. Nevins. 2000. Loss of E2F4 activity leads to abnormal development of multiple cellular lineages. *Mol Cell* **6**: 293-306.

Ren, B., H. Cam, Y. Takahashi, T. Volkert, J. Terragni, R.A. Young, and B.D. Dynlacht. 2002. E2F integrates cell cycle progression with DNA repair, replication, and G(2)/M checkpoints. *Genes Dev* **16**: 245-256.

Reynisdottir, I., K. Polyak, A. Iavarone, and J. Massagué. 1995. Kip/Cip and INK4 Cdk inhibitors cooperate to induce cell cycle arrest in response to TGF-beta. *Genes Dev* **9**: 1831-1845.

Rhee, I., K.E. Bachman, B.H. Park, K.W. Jair, R.W. Yen, K.E. Schuebel, H. Cui, A.P. Feinberg, C. Lengauer, K.W. Kinzler, S.B. Baylin, and B. Vogelstein. 2002. DNMT1 and DNMT3b cooperate to silence genes in human cancer cells. *Nature* **416**: 552-6.

Robanus-Maandag, E., M. Dekker, M. van der Valk, M.L. Carrozza, J.C. Jeanny, J.H. Dannenberg, A. Berns, and H. te Riele. 1998. p107 is a suppressor of retinoblastoma development in pRb-deficient mice. *Genes Dev* **12**: 1599-609.

Robert, M. F., Morin, S., Beaulieu, N., Gauthier, F., Chute, I. C., Barsalou, A., MacLeod, A. R. DNMT1 is required to maintain CpG methylation and aberrant gene silencing in human cancer cells. *Nat Genet* **33**: 61-5.

Roberts, C.W., M.M. Leroux, M.D. Fleming, and S.H. Orkin. 2002. Highly penetrant, rapid tumorigenesis through conditional inversion of the tumor suppressor gene Snf5. *Cancer Cell* **2**: 415-25.

Roberts, S.M. and F. Winston. 1997. Essential functional interactions of SAGA, a Saccharomyces cerevisiae complex of Spt, Ada, and Gcn5 proteins, with the Snf/Swi and Srb/mediator complexes. *Genetics* **147**: 451-465.

Robertson, K.D., S. Ait-Si-Ali, T. Yokochi, P.A. Wade, P.L. Jones, and A.P. Wolffe. 2000. DNMT1 forms a complex with Rb, E2F1 and HDAC1 and represses transcription from E2F-responsive promoters. *Nat Genet* **25**: 338-42.

Rocco, J.W. and D. Sidransky. 2001. p16(MTS-1/CDKN2/INK4a) in cancer progression. *Exp Cell Res* **264**: 42-55.

Ross, J.F., X. Liu, and B.D. Dynlacht. 1999. Mechanism of transcriptional repression of E2F by the retinoblastoma tumor suppressor protein. *Mol Cell* **3**: 195-205.

Ross, J.F., A. Naar, H. Cam, R. Gregory, and B.D. Dynlacht. 2001. Active repression and E2F inhibition by pRB are biochemically distinguishable. *Genes Dev* **15**: 392-7.

Roth, S.Y., J.M. Denu, and C.D. Allis. 2001. Histone acetyltransferases. *Annu Rev Biochem* **70**: 81-120.

Rowland, B.D., S.G. Denissov, S. Douma, H.G. Stunnenberg, R. Bernards, and D.S. Peeper. 2002. E2F transcriptional repressor complexes are critical downstream targets of p19(ARF)/p53-induced proliferative arrest. *Cancer Cell* **2**: 55-65.

Roy, A.L., C. Carruthers, T. Gutjahr, and R.G. Roeder. 1993. Direct role for Myc in transcription initiation mediated by interactions with TFII-I. *Nature* **365**: 359-361.

Sage, J., G.J. Mulligan, L.D. Attardi, A. Miller, S. Chen, B. Williams, E. Theodorou, and T. Jacks. 2000. Targeted disruption of the three Rb-related genes leads to loss of G(1) control and immortalization. *Genes Dev* **14**: 3037-50.

Sakamuro, D. and G.C. Prendergast. 1999. New Myc-interacting proteins: a second Myc network emerges. *Oncogene* **18**: 2942-2954.

Saleh, A., D. Schieltz, N. Ting, S.B. McMahon, D.W. Litchfield, J.R. Yates, 3rd, S.P. Lees-Miller, M.D. Cole, and C.J. Brandl. 1998. Tra1p is a component of the yeast Ada.Spt transcriptional regulatory complexes. *J Biol Chem* **273**: 26559-26565.

Salghetti, S.E., S.Y. Kim, and W.P. Tansey. 1999. Destruction of Myc by ubiquitin-mediated proteolysis: cancer-associated and transforming mutations stabilize Myc. *Embo J* **18**: 717-726.

Sambrook, J., E.F. Fritsch, and M. T. 1989. Molecular Cloning.

Sandhu, C., J. Garbe, N. Bhattacharya, J. Daksis, C.H. Pan, P. Yaswen, J. Koh, J.M. Slingerland, and M.R. Stampfer. 1997. Transforming growth factor beta stabilizes p15INK4B protein, increases p15INK4B-cdk4 complexes, and inhibits Cyclin D1-cdk4 association in human mammary epithelial cells. *Mol Cell Biol* **17**: 2458-2467.

Santos-Rosa, H., R. Schneider, A.J. Bannister, J. Sherriff, B.E. Bernstein, N.C. Emre, S.L. Schreiber, J. Mellor, and T. Kouzarides. 2002. Active genes are tri-methylated at K4 of histone H3. *Nature* **419**: 407-11.

Sassone-Corsi, P., C.A. Mizzen, P. Cheung, C. Crosio, L. Monaco, S. Jacquot, A. Hanauer, and C.D. Allis. 1999. Requirement of Rsk-2 for epidermal growth factor-activated phosphorylation of histone H3. *Science* **285**: 886-91.

Schiltz, R. L., Mizzen, C. A., Vassilev, A., Cook, R. G., Allis, C. D., Nakatani, Y. Overlapping but distinct patterns of histone acetylation by the human coactivators p300 and PCAF within nucleosomal. *J Biol Chem* **274**: 1189-92.

Schiltz, R. L. and Y. Nakatani. 2000. The PCAF acetylase complex as a potential tumor suppressor. *Biochim Biophys Acta* **1470**: M37-53.

Schlisio, S., T. Halperin, M. Vidal, and J.R. Nevins. 2002. Interaction of YY1 with E2Fs, mediated by RYBP, provides a mechanism for specificity of E2F function. *Embo J* **21**: 5775-86.

Schneider, A., K. Peukert, M. Eilers, and F. Hanel. 1997. Association of Myc with the zinc-finger protein Miz-1 defines a novel pathway for gene regulation by Myc. *Curr Top Microbiol Immunol* **224**: 137-46.

Schotta, G., A. Ebert, V. Krauss, A. Fischer, J. Hoffmann, S. Rea, T. Jenuwein, R. Dorn, and G. Reuter. 2002. Central role of Drosophila SU(VAR)3-9 in histone H3-K9 methylation and heterochromatic gene silencing. *Embo J* **21**: 1121-31.

Schreiber-Agus, N. and R.A. DePinho. 1998. Repression by the Mad(Mxi1)-Sin3 complex. *Bioessays* **20**: 808-818.

Schuhmacher, M., F. Kohlhuber, M. Holzel, C. Kaiser, H. Burtscher, M. Jarsch, G.W. Bornkamm, G. Laux, A. Polack, U.H. Weidle, and D. Eick. 2001. The transcriptional program of a human B cell line in response to Myc. *Nucleic Acids Res* **29**: 397-406.

Schuldiner, O. and N. Benvenisty. 2001. A DNA microarray screen for genes involved in c-MYC and N-MYC oncogenesis in human tumors. *Oncogene* **20**: 4984-4994.

Schuldiner, O., S. Shor, and N. Benvenisty. 2002. A computerized database-scan to identify c-MYC targets. *Gene* **292**: 91-99.

Sears, R., G. Leone, J. DeGregori, and J.R. Nevins. 1999. Ras enhances Myc protein stability. *Mol Cell* **3**: 169-79.

Seoane, J., H.V. Le, and J. Massague. 2002. Myc suppression of the p21(Cip1) Cdk inhibitor influences the outcome of the p53 response to DNA damage. *Nature* **419**: 729-34.

Seoane, J., C. Pouponnot, P. Staller, M. Schader, M. Eilers, and J. Massagué. 2001. TGFß influences Myc, Miz-1 and Smad to control the CDK inhibitor p15INK4b. *Nat Cell Biol* **3**: 400-408.

Serrano, M. 2000. The INK4a/ARF locus in murine tumorigenesis. *Carcinogenesis* **21**: 865-9.

Shan, B. and H.-W. Lee. 1994. Deregulated expression of E2F-1 induces S-phase entry and leads to apoptosis. *Mol Cell Biol* **14**: 8166-8173.

Shanahan, F., W. Seghezzi, D. Parry, D. Mahony, and E. Lees. 1999. Cyclin E associates with BAF155 and BRG1, components of the mammalian SWI-SNF complex, and alters the ability of BRG1 To induce growth arrest. *Mol Cell Biol* **19**: 1460-1469.

Sharma, M., M. Zarnegar, X. Li, B. Lim, and Z. Sun. 2000. Androgen receptor interacts with a novel MYST protein, HBO1. *J Biol Chem* **275**: 35200-8.

Sharpless, N.E. and R.A. DePinho. 1999. The INK4A/ARF locus and its two gene products. *Curr Opin Genet Dev* **9**: 22-30.

Sheaff, R.J., M. Groudine, M. Gordon, J.M. Roberts, and B.E. Clurman. 1997. Cyclin E-CDK2 is a regulator of p27Kip1. *Genes Dev* **11**: 1464-1478.

Sherr, C.J. 1993. Mammalian G1 Cyclins. *Cell* **73**: 1059-1065.

Sherr, C.J. 1994a. G1 phase progression: Cycling on cue. *Cell* **79**: 551-555.

Sherr, C.J. 1994b. Growth factor-regulated G1 Cyclins. [Review]. *Stem Cells (Dayt)* **1**: 47-55.

Sherr, C.J. 1996. Cancer cell cycles. *Science* **274**: 1672-1677.

Sherr, C.J. 2001. The INK4a/ARF network in tumour suppression. *Nat Rev Mol Cell Biol* **2**: 731-7.

Sherr, C.J. and F. McCormick. 2002. The RB and p53 pathways in cancer. *Cancer Cell* **2**: 103-12.

Singh, P., S.H. Wong, and W. Hong. 1994. Overexpression of E2F-1 in rat fibroblasts leads to neoplastic transformation. *EMBO J* **13**: 3329-3338.

Slansky, J.E. and P.J. Farnham. 1996. Transcriptional regulation of the dihydrofolate reductase gene. *Bioessays* **18**: 55-62.

Slansky, J.E., Y. Li, W.G. Kaelin, and P.J. Farnham. 1993. A protein synthesis-dependent increase in E2F1 mRNA correlates with growth regulation of the dihydrofolate reductase promoter. *Mol Cell Biol* **13**: 1610-8.

Smith, E.R., A. Eisen, W. Gu, M. Sattah, A. Pannuti, J. Zhou, R.G. Cook, J.C. Lucchesi, and C.D. Allis. 1998. ESA1 is a histone acetyltransferase that is essential for growth in yeast. *Proc Natl Acad Sci U S A* **95**: 3561-3565.

Sobczak-Thepot, J., F. Harper, Y. Florentin, F. Zindy, C. Brechot, and E. Puvion. 1993. Localization of Cyclin A at the sites of cellular DNA replication. *Exp Cell Res* **206**: 43-8.

Sobulo, O.M., J. Borrow, R. Tomek, S. Reshmi, A. Harden, B. Schlegelberger, D. Housman, N.A. Doggett, J.D. Rowley, and N.J. Zeleznik-Le. 1997. MLL is fused to CBP, a histone acetyltransferase, in therapy-related acute myeloid leukemia with a t(11;16)(q23;p13.3). *Proc Natl Acad Sci U S A* **94**: 8732-7.

Soutoglou, E., N. Katrakili, and I. Talianidis. 2000. Acetylation regulates transcription factor activity at multiple levels. *Mol Cell* **5**: 745-51.

Soutoglou, E., B. Viollet, M. Vaxillaire, M. Yaniv, M. Pontoglio, and I. Talianidis. 2001. Transcription factor-dependent regulation of CBP and P/CAF histone acetyltransferase activity. *Embo J* **20**: 1984-92.

Stallcup, M.R. 2001. Role of protein methylation in chromatin-remodeling and transcriptional regulation. *Oncogene* **20**: 3014-20.

Staller, P., K. Peukert, A. Kiermaier, J. Seoane, J. Lukas, H. Karsunky, T. Möröy, J. Bartek, J. Massagué, F. Hänel, and M. Eilers. 2001. Repression of p15INK4b expression by Myc through association with Miz-1. *Nat Cell Biol* **3**: 392-399.

Stanelle, J., T. Stiewe, C.C. Theseling, M. Peter, and B.M. Putzer. 2002. Gene expression changes in response to E2F1 activation. *Nucleic Acids Res* **30**: 1859-67.

Stankunas, K., J. Berger, C. Ruse, D.A. Sinclair, F. Randazzo, and H.W. Brock. 1998. The enhancer of Polycomb gene of Drosophila encodes a chromatin protein conserved in yeast and mammals. *Development* **125**: 4055-66.

Steeg, P.S. and Q. Zhou. 1998. Cyclins and breast cancer. *Breast Cancer Res Treat* **52**: 17-28.

Sterner, D.E. and S.L. Berger. 2000. Acetylation of histones and transcription-related factors. *Microbiol Mol Biol Rev* **64**: 435-459.

Sterner, D.E., P.A. Grant, S.M. Roberts, L.J. Duggan, R. Belotserkovskaya, L.A. Pacella, F. Winston, J.L. Workman, and S.L. Berger. 1999. Functional organization of the yeast SAGA complex: distinct components involved in structural integrity, nucleosome acetylation, and TATA-binding protein interaction. *Mol Cell Biol* **19**: 86-98.

Stevaux, O. and N.J. Dyson. 2002. A revised picture of the E2F transcriptional network and RB function. *Curr Opin Cell Biol* **14**: 684-91.

Stiewe, T. and B.M. Putzer. 2000. Role of the p53-homologue p73 in E2F1-induced apoptosis. *Nat Genet* **26**: 464-9.

Storre, J., H.P. Elsasser, M. Fuchs, D. Ullmann, D.M. Livingston, and S. Gaubatz. 2002. Homeotic transformations of the axial skeleton that accompany a targeted deletion of E2f6. *EMBO Rep* **3**: 695-700.

Strahl, B.D. and C.D. Allis. 2000. The language of covalent histone modifications. *Nature* **403**: 41-5.

Strobeck, M.W., K.E. Knudsen, A.F. Fribourg, M.F. DeCristofaro, B.E. Weissman, A.N. Imbalzano, and E.S. Knudsen. 2000. BRG-1 is required for RB-mediated cell cycle arrest. *Proc Natl Acad Sci U S A* **97**: 7748-53.

Strober, B.E., J.L. Dunaief, Guha, and S.P. Goff. 1996. Functional interactions between the hBRM/hBRG1 transcriptional activators and the pRB family of proteins. *Mol Cell Biol* **16**: 1576-83.

Suka, N., K. Luo, and M. Grunstein. 2002. Sir2p and Sas2p opposingly regulate acetylation of yeast histone H4 lysine16 and spreading of heterochromatin. *Nat Genet* **32**: 378-83.

Suka, N., Y. Suka, A.A. Carmen, J. Wu, and M. Grunstein. 2001. Highly specific antibodies determine histone acetylation site usage in yeast heterochromatin and euchromatin. *Mol Cell* **8**: 473-479.

Takahashi, Y., J.B. Rayman, and B.D. Dynlacht. 2000. Analysis of promoter binding by the E2F and pRB families in vivo: distinct E2F proteins mediate activation and repression. *Genes Dev* **14**: 804-16.

Takechi, S. and T. Nakayama. 1999. Sas3 is a histone acetyltransferase and requires a zinc finger motif. *Biochem Biophys Res Commun* **266**: 405-10.

Tommasi, S. and G.P. Pfeifer. 1995. In vivo structure of the human cdc2 promoter: release of a p130-E2F-4 complex from sequences immediately upstream of the transcription initiation site coincides with induction of cdc2 expression. *Mol Cell Biol* **15**: 6901-13.

Trimarchi, J.M., B. Fairchild, R. Verona, K. Moberg, N. Andon, and J.A. Lees. 1998. E2F-6, a member of the E2F family that can behave as a transcriptional repressor. *Proc Natl Acad Sci U S A* **95**: 2850-2855.

Trimarchi, J.M., B. Fairchild, J. Wen, and J.A. Lees. 2001. The E2F6 transcription factor is a component of the mammalian Bmi1-containing Polycomb complex. *Proc Natl Acad Sci U S A* **98**: 1519-24.

Trimarchi, J.M. and J.A. Lees. 2002. Sibling rivalry in the E2F family. *Nat Rev Mol Cell Biol* **3**: 11-20.

Trouche, D., A. Cook, and T. Kouzarides. 1996. The CBP co-activator stimulates E2F1/DP1 activity. *Nucleic Acids Res* **24**: 4139-45.

Trouche, D. and T. Kouzarides. 1996. E2F1 and E1A(12S) have a homologous activation domain regulated by RB and CBP. *Proc Natl Acad Sci U S A* **93**: 1439-1442.

Trouche, D., C. Le Chalony, C. Muchardt, M. Yaniv, and T. Kouzarides. 1997. RB and hbrm cooperate to repress the activation functions of E2F1. *Proc Natl Acad Sci U S A* **94**: 11268-73.

Tsai, K.Y., Y. Hu, K.F. Macleod, D. Crowley, L. Yamasaki, and T. Jacks. 1998. Mutation of E2f-1 suppresses apoptosis and inappropriate S phase entry and extends survival of Rb-deficient mouse embryos. *Mol Cell* **2**: 293-304.

Vassilev, A., J. Yamauchi, T. Kotani, C. Prives, M.L. Avantaggiati, J. Qin, and Y. Nakatani. 1998. The 400 kDa subunit of the PCAF histone acetylase complex belongs to the ATM superfamily. *Mol Cell* **2**: 869-875.

Vaute, O., E. Nicolas, L. Vandel, and D. Trouche. 2002. Functional and physical interaction between the histone methyl transferase Suv39H1 and histone deacetylases. *Nucleic Acids Res* **30**: 475-81.

Venkitaraman, A.R. 2002. Cancer susceptibility and the functions of BRCA1 and BRCA2. *Cell* **108**: 171-82.

Verona, R., K. Moberg, S. Estes, M. Starz, J.P. Vernon, and J.A. Lees. 1997. E2F activity is regulated by cell cycle-dependent changes in subcellular localization. *Mol Cell Biol* **17**: 7268-82.

Versteege, I., S. Medjkane, D. Rouillard, and O. Delattre. 2002. A key role of the hSNF5/INI1 tumour suppressor in the control of the G1-S transition of the cell cycle. *Oncogene* **21**: 6403-12.

Versteege, I., N. Sevenet, J. Lange, M.F. Rousseau-Merck, P. Ambros, R. Handgretinger, A. Aurias, and O. Delattre. 1998. Truncating mutations of hSNF5/INI1 in aggressive paediatric cancer. *Nature* **394**: 203-206.

Vigo, E., H. Muller, E. Prosperini, G. Hateboer, P. Cartwright, M.C. Moroni, and K. Helin. 1999. CDC25A phosphatase is a target of E2F and is required for efficient E2F-induced S phase. *Mol Cell Biol* **19**: 6379-95.

Vlach, J., S. Hennecke, K. Alevizopoulos, D. Conti, and B. Amati. 1996. Growth arrest by the Cyclin-dependent kinase inhibitor $p27^{Kip1}$ is abrogated by c-Myc. *EMBO J.* **15**: 6595-6604.

Vlach, J., S. Hennecke, and B. Amati. 1997. Phosphorylation-dependent degradation of the Cyclin-dependent kinase inhibitor $p27^{Kip1}$. *EMBO J* **16**: 5334-5344.

Vogelauer, M., J. Wu, N. Suka, and M. Grunstein. 2000. Global histone acetylation and deacetylation in yeast. *Nature* **408**: 495-498.

Vogelstein, B. and K.W. Kinzler. 1993. The multistep nature of cancer. *Trends Genet* **9**: 138-141.

Vousden KH. Activation of the p53 tumor suppressor protein. *Biochim Biophys Acta*. 2002 **1602**: 47-59.

Wade, P.A. 2001. Transcriptional control at regulatory checkpoints by histone deacetylases: molecular connections between cancer and chromatin. *Hum Mol Genet* **10**: 693-8.

Waltzer, L. and M. Bienz. 1998. Drosophila CBP represses the transcription factor TCF to antagonize Wingless signalling. *Nature* **395**: 521-525.

Wang, L., L. Liu, and S.L. Berger. 1998. Critical residues for histone acetylation by Gcn5, functioning in Ada and SAGA complexes, are also required for transcriptional function in vivo. *Genes Dev* **12**: 640-53.

Wang, W., J. Cote, Y. Xue, S. Zhou, P.A. Khavari, S.R. Biggar, C. Muchardt, G.V. Kalpana, S.P. Goff, M. Yaniv, J.L. Workman, and G.R. Crabtree. 1996a. Purification and biochemical heterogeneity of the mammalian SWI-SNF complex. *Embo J* **15**: 5370-5382.

Wang, W., Y. Xue, S. Zhou, A. Kuo, B.R. Cairns, and G.R. Crabtree. 1996b. Diversity and specialization of mammalian SWI/SNF complexes. *Genes Dev* **10**: 2117-2130.

Watson, J.D., S.K. Oster, M. Shago, F. Khosravi, and L.Z. Penn. 2002. Identifying genes regulated in a Myc-dependent manner. *J Biol Chem* **in press**.

Weinberg, R.A. 1991. Tumor suppressor genes. [Review]. *Science* **254**: 1138-1146.

Weinberg, R.A. 1992. The retinoblastoma gene and gene product. [Review]. *Cancer Surv* **12**: 43-57.

Weinberg, R.A. 1994. Oncogenes and tumor suppressor genes. [Review]. *Ca Cancer J Clin* **44**: 160-170.

Weinmann, A.S., S.M. Bartley, T. Zhang, M.Q. Zhang, and P.J. Farnham. 2001. Use of chromatin immunoprecipitation to clone novel E2F target promoters. *Mol Cell Biol* **21**: 6820-32.

Weinmann, A.S., P.S. Yan, M.J. Oberley, T.H. Huang, and P.J. Farnham. 2002. Isolating human transcription factor targets by coupling chromatin immunoprecipitation and CpG island microarray analysis. *Genes Dev* **16**: 235-244.

Wells, J., K.E. Boyd, C.J. Fry, S.M. Bartley, and P.J. Farnham. 2000. Target gene specificity of E2F and pocket-protein family members in living cells. *Mol Cell Biol* **20**: 5797-807.

Wittschieben, B.O., J. Fellows, W. Du, D.J. Stillman, and J.Q. Svejstrup. 2000. Overlapping roles for the histone acetyltransferase activities of SAGA and elongator in vivo. *Embo J* **19**: 3060-8.

Wong, A., F. Shanahan, Y. Chen, L. Lian, P. Ha, K. Hendricks, S. Ghaffari, D. Iliev, B. Penn, A. Woodland, R. Smith, G. Salada, A. Carillo, Laity K, J. Gupte, B. Swedlund, S. Tavtigian, D. Teng, and E. Lees. 2000. BRG1, a component of the SWI-SNF complex, is mutated in multiple human tumor cell lines. *Cancer Res* **60**: in press.

Wong, L.C., D.J. Sharpe, and S.S. Wong. 1991. High-mobility group and other nonhistone substrates for nuclear histone N-acetyltransferase. *Biochem Genet* **29**: 461-75.

Workman, J.L. and R.E. Kingston. 1998. Alteration of nucleosome structure as a mechanism of transcriptional regulation. *Annu Rev Biochem* **67**: 545-79.

Wu, C.L., L.R. Zukerberg, C. Ngwu, E. Harlow, and J.A. Lees. 1995. In vivo association of E2F and DP family proteins. *Mol Cell Biol* **15**: 2536-2546.

Wu, L., C. Timmers, B. Maiti, H.I. Saavedra, L. Sang, G.T. Chong, F. Nuckolls, P. Giangrande, F.A. Wright, S.J. Field, M.E. Greenberg, S. Orkin, J.R. Nevins, M.L. Robinson, and G. Leone. 2001. The E2F1-3 transcription factors are essential for cellular proliferation. *Nature* **414**: 457-62.

Wu, X. and A.J. Levine. 1994. p53 and E2F-1 cooperate to mediate apoptosis. *Proc Natl Acad Sci U S A* **91**: 3602-3606.

Xu, D., N. Popov, M. Hou, Q. Wang, M. Bjorkholm, A. Gruber, A.R. Menkel, and M. Henriksson. 2001a. Switch from Myc/Max to Mad1/Max binding and decrease in histone acetylation at the telomerase reverse transcriptase promoter during differentiation of HL60 cells. *Proc Natl Acad Sci U S A* **98**: 3826-3831.

Xu, G., D.M. Livingston, and W. Krek. 1995. Multiple members of the E2F transcription factor family are the products of oncogenes. *Proc Natl Acad Sci U S A* **92**: 1357-1361.

Xu, H. and M. Raafat el-Gewely. 2001. P53-responsive genes and the potential for cancer diagnostics and therapeutics development. *Biotechnol Annu Rev* **7**: 131-64.

Xu, W., H. Chen, K. Du, H. Asahara, M. Tini, B.M. Emerson, M. Montminy, and R.M. Evans. 2001b. A transcriptional switch mediated by cofactor methylation. *Science* **294**: 2507-11.

Xu, W., D.G. Edmondson, and S.Y. Roth. 1998. Mammalian GCN5 and P/CAF acetyltransferases have homologous amino-terminal domains important for recognition of nucleosomal substrates. *Mol Cell Biol* **18**: 5659-5669.

Yam, C.H., T.K. Fung, and R.Y. Poon. 2002. Cyclin A in cell cycle control and cancer. *Cell Mol Life Sci* **59**: 1317-26.

Yamamoto, T. and M. Horikoshi. 1997. Novel substrate specificity of the histone acetyltransferase activity of HIV-1-Tat interactive protein Tip60. *J Biol Chem* **272**: 30595-8.

Yamasaki, L., T. Jacks, R. Bronson, E. Goillot, E. Harlow, and N.J. Dyson. 1996. Tumor induction and tissue atrophy in mice lacking E2F-1. *Cell* **85**: 537-548.

Yamauchi, T., J. Yamauchi, T. Kuwata, T. Tamura, T. Yamashita, N. Bae, H. Westphal, K. Ozato, and Y. Nakatani. 2000. Distinct but overlapping roles of histone acetylase PCAF and of the closely related PCAF-B/GCN5 in mouse embryogenesis [In Process Citation]. *Proc Natl Acad Sci U S A* **97**: 11303-6.

Yan, Z., J. DeGregori, R. Shohet, G. Leone, B. Stillman, J.R. Nevins, and R.S. Williams. 1998. Cdc6 is regulated by E2F and is essential for DNA replication in mammalian cells. *Proc Natl Acad Sci U S A* **95**: 3603-8.

Yanagisawa, J., H. Kitagawa, M. Yanagida, O. Wada, S. Ogawa, M. Nakagomi, H. Oishi, Y. Yamamoto, H. Nagasawa, S.B. McMahon, M.D. Cole, L. Tora, N. Takahashi, and S. Kato. 2002. Nuclear receptor function requires a TFTC-type histone acetyl transferase complex. *Mol Cell* **9**: 553-62.

Yang, X.J., V.V. Ogryzko, J. Nishikawa, B.H. Howard, and Y. Nakatani. 1996. A p300/CBP-associated factor that competes with the adenoviral oncoprotein E1A. *Nature* **382**: 319-324.

Yao, T.P., S.P. Oh, M. Fuchs, N.D. Zhou, L.E. Ch'ng, D. Newsome, R.T. Bronson, E. Li, D.M. Livingston, and R. Eckner. 1998. Gene dosage-dependent embryonic development and proliferation defects in mice lacking the transcriptional integrator p300. *Cell* **93**: 361-72.

Zeng, L. and M.M. Zhou. 2002. Bromodomain: an acetyl-lysine binding domain. *FEBS Lett* **513**: 124-8.

Zetterberg, A., O. Larsson, and K.G. Wiman. 1995. What is the restriction point? *Curr Opin Cell Biol* **7**: 835-842.

Zhang, H.S., M. Gavin, A. Dahiya, A.A. Postigo, D. Ma, R.X. Luo, J.W. Harbour, and D.C. Dean. 2000. Exit from G1 and S phase of the cell cycle is regulated by repressor complexes containing HDAC-Rb-hSWI/SNF and Rb-hSWI/SNF. *Cell* **101**: 79-89.

Zhang, H.S., A.A. Postigo, and D.C. Dean. 1999. Active transcriptional repression by the Rb-E2F complex mediates G1 arrest triggered by p16INK4a, TGFbeta, and contact inhibition. *Cell* **97**: 53-61.

Zhang, W. and J.J. Bieker. 1998. Acetylation and modulation of erythroid Kruppel-like factor (EKLF) activity by interaction with histone acetyltransferases. *Proc Natl Acad Sci U S A* **95**: 9855-9860.

Zhang, Y. and D. Reinberg. 2001. Transcription regulation by histone methylation: interplay between different covalent modifications of the core histone tails. *Genes Dev* **15**: 2343-60.

Zhang, Y., Y. Xiong, and W.G. Yarbrough. 1998. ARF promotes MDM2 degradation and stabilizes p53: ARF-INK4a locus deletion impairs both the Rb and p53 tumor suppression pathways. *Cell* **92**: 725-734.

Zhao, J., B.K. Kennedy, B.D. Lawrence, D.A. Barbie, A.G. Matera, J.A. Fletcher, and E. Harlow. 2000. NPAT links Cyclin E-Cdk2 to the regulation of replication-dependent histone gene transcription. *Genes Dev* **14**: 2283-97.

Zhao, J.Y., B. Dynlacht, T. Imai, T. Hori, and E. Harlow. 1998a. Expression of NPAT, a novel substrate of Cyclin E-Cdk2, promotes S-phase entry. *Genes Dev* **12**: 456-461.

Zhao, K., W. Wang, O.J. Rando, Y. Xue, K. Swiderek, A. Kuo, and G.R. Crabtree. 1998b. Rapid and phosphoinositol-dependent binding of the SWI/SNF-like BAF complex to chromatin after T lymphocyte receptor signaling. *Cell* **95**: 625-36.

Ziebold, U., T. Reza, A. Caron, and J.A. Lees. 2001. E2F3 contributes both to the inappropriate proliferation and to the apoptosis arising in Rb mutant embryos. *Genes Dev* **15**: 386-91.

Zimmermann, H., R. Degenkolbe, H.U. Bernard, and M.J. O'Connor. 1999. The human papillomavirus type 16 E6 oncoprotein can down-regulate p53 activity by targeting the transcriptional coactivator CBP/p300. *J Virol* **73**: 6209-19.

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DECLARATION FOR THE FACULTY

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Palo Alto, February 10th 2003

To whom it may concern:

Subject: Thesis presented to the University of Fribourg, in order to obtain the title of a *Doctor Rerum Naturalium*

Ladies and Gentleman:

I hereby certify that I have written my thesis "The Tip60 HAT-complex is recruited to Chromatin by E2F and Myc" on my own and based on my own work performed without any illegitimate help.

Sincerely

Stefan Taubert

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PUBLICATIONS

Taubert S., Frank S.R., Parisi T., Helin K., and Amati B. (2002). The Tip60 complex is a coactivator of E2F transactivation *in vivo*. Manuscript in preparation.

Frank S.R., Parisi T., Taubert S., and Amati B. (2002). Myc recruits the histone acetyltransferase Tip60 to chromatin. Submitted

Frank S.R., Schroeder M., Fernandez P., Taubert S., Amati B. (2001). Binding of c-Myc to chromatin mediates mitogen-induced acetylation of histone H4 and gene activation. Genes Dev. 15(16): 2069-82.

Amati B., Frank S.R., Donjerkovic D., Taubert S. (2001). Function of the c-Myc oncoprotein in chromatin-remodeling and transcription. BBA 1471(3): M 135-45.

Susstrunk U., Pidoux J., Taubert S., Ullmann A., Thompson C.J. (1998) Pleiotropic effects of cAMP on germination, antibiotic biosynthesis and morphological development in *Streptomyces coelicolor*. Mol. Microbiol. 30(1): 33-46.

MEETINGS

1) 01/2001: Cancer and the Cell Cycle, Lausanne, Switzerland; Poster Presentation
2) 05/2000: The Cell Cycle, Cold Spring Harbor; Oral Presentation
3) 10/1999: Chromatin; Domains and Dynamics; Villars-sur-Ollon, Switzerland
4) 08/1999: EMBO Workshop "Molecular Medicine of the Gut", Arolla, Switzerland
5) 01/1999: Cancer and the Cell Cycle, Lausanne, Switzerland