ISAS - INTERNATIONAL SCHOOL
FOR ADVANCED STUDIES

NOVEL MECHANISM FOR THE REGULATION OF E2F-1 TRANSCRIPTION FACTOR BY INTERPLAY OF ACETYLATION AND UBIQUITINATION

Laura Galbiati

Thesis submitted for the degree of

Doctor Philosophiae

Supervisor:
Prof. Mauro Giacca

External examiner:
Dr. Kristian Helin

Academic Year 2002/2003

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SUPERIORE
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TRIESTE

to my father
who trusted me
the first and the most

to my mother
who loved me so much
to let me go...

The man and his safety must be the first preoccupation of any technological adventure.
Do never forget this when you're into your calculation, your equations and your experiments.

Albert Einstein

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1. INTRODUCTION

1.1 Transcription factor E2F

Cell cycle is a highly regulated process that is influenced by both positive and negative growth regulatory signals during the G1 phase. In particular, one of the most essential set of signals act by controlling the transcriptional activity of the cellular E2F factor, which links the activities of the cell cycle machinery with the transcription of genes whose products are required for S-phase entry and DNA synthesis. Activation of E2F is thus sufficient to irreversibly commit cells to undergo DNA replication (Sherr and Roberts, 1999). E2F activity has been shown to arise from concerted action of a family of proteins that have distinct biological properties.

1.1.1 E2F discovery and links to cancer

E2F was originally discovered as a cellular activity required for the early region 1A (E1A) transforming protein of adenovirus to mediate the transcriptional activation of the viral E2 promoter (Nevins, 1992). Subsequent studies showed that E2F controls transcription of cellular genes that are essential for cell division (Dyson, 1998), including cell cycle regulators and checkpoint genes in addition to enzymes involved in nucleotide biosynthesis and the regulatory components of the DNA replication machinery. E1A has been shown to posses the capability to stimulate cell-cycle entry by releasing transcriptionally active E2F molecules by sequestering pRB, a known E1A-associated protein that inhibits E2F activity (Bagchi et al., 1990) (see below).

In addition to adenovirus E1A, large T antigens of SV40 and polyoma viruses, and the E7 gene product of human papillomavirus also interact with pRB and dissociate E2F-pRB complexes (Chellappan et al., 1992). Thus activation of E2F appears to be a common mechanism by which these DNA viruses induce DNA replication in host cells to facilitate viral genome replication.

There is much indirect evidence to suggest that the activation of E2F transcription factors, via alterations in the E2F-RB pathway, is a key event in the development of most human cancers. Curiously, however, E2F which constitutes the central part of this pathway, is not frequent target of mutation in cancer, whereas pRB is absent or mutated in at least one-third of all human tumours, and molecules upstream pRB are frequent target for mutations and have a broader impact to cellular homeostasis.

pRB was the first tumour suppressor to be identified, the retinoblastoma gene was initially identified as a genetic locus associated with the development of an inherited eye tumour

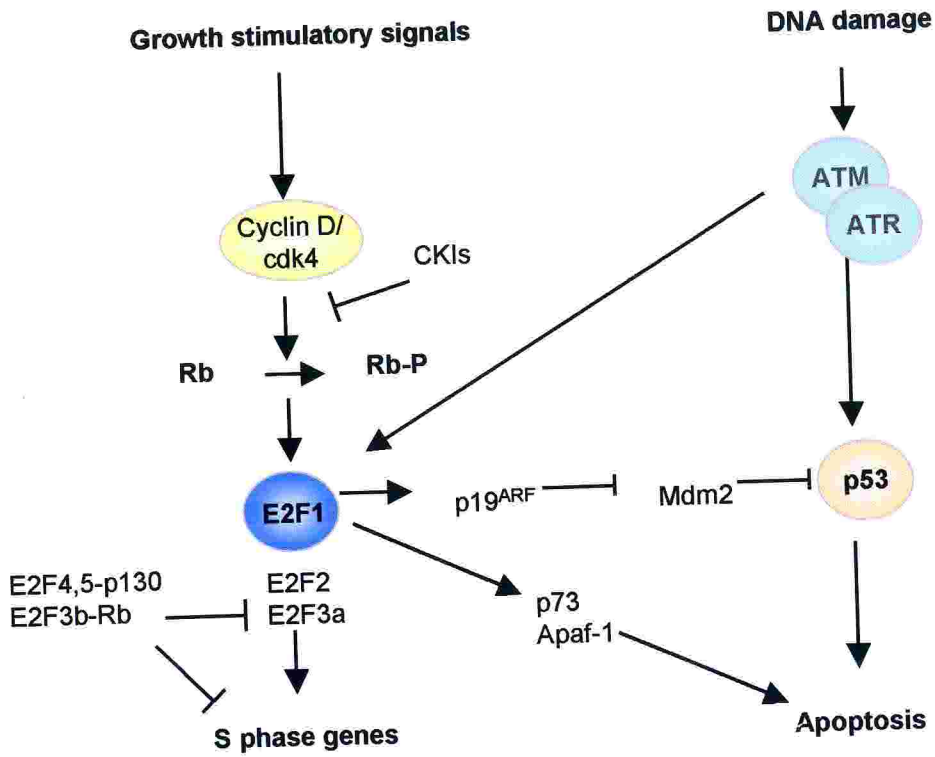

Figure 1.1
The pRB/E2F pathway in proliferation and apoptosis.

pRB is a central component of a signaling pathway that controls cell proliferation. D-type G1 cyclins, with their associated kinases Cdk4 and Cdk6, initiate the phosphorylation of pRB inactivating its capacity to interact with E2Fs. This phosphorylation allows the accumulation of E2F1,2 and 3 that activate the transcription of a large number of genes essential for replication an cell cycle progression. In addition pRB phosphorylation also disrupts complexes with E2F3b, E2F4 and E2F5 that function as transcriptional repressors of S phase genes as well as the genes encoding E2F1- 2 and 3 proteins. The Rb/E2F pathway has also been shown to integrate with pathways that control programmed cell death.
p53 plays a key role in cellular decision to either arrest the cell cycle, allowing the repair of damaged DNA, or to commit cell death. p53 accumulation is negatively regulated by MDM2, which targets it for ubiquitin-mediated proteasome degradation; MDM2 is in turn, negatively regulated by $p19^{ARF}$. E2F induces the expression of $p19^{ARF}$, thus directly connecting the RB/E2F pathway to p53 accumulation and apoptotic response. However E2F can also induce apoptosis in a p53 independent manner due to the activation of p73 or Apaf1. E2F is specifically induced following DNA damage via ATM and ATR protein kinases, which phosphorylate E2F and inhibit its degradation.

(Knudson, 1971) and E2F transcription factor activity was subsequentially identified as a key target for the growth suppressing action of the RB protein (Chellappan et al., 1991).

1.1.2 The RB/E2F pathway

It is well established that E2F regulates early cell cycle transitions, from G0 into G1 and from G1 to S phase. The transcriptional activity of E2F in G1 cells is regulated by the retinoblastoma tumour suppressor protein pRB. This protein is a member of the pocket protein family distinguished by the presence of evolutionary conserved pocket region which is necessary and sufficient for E2F binding, and able to modulate a number of functions leading to repression of transactivation, repression of apoptosis, protection from degradation and altered E2F-DNA specificity. pRB plays a pivotal role in the G1- to S-phase transition. By binding to the transcriptional activation domains of E2F, it maintains E2F in a transcriptionally inactive form; the ability of pRB to bind to E2F is regulated by its cell-cycle-dependent phosphorylation. pRB is hypophosphorylated during G0 and early G1, and this form binds and inhibits E2F.

Mitogenic growth factors induce the sequential activation of the cell-cycle dependent kinase complexes Cdk4/cdk6-cyclinD ad cdk2-cyclinE, which then phosphorylate pRB and cause it to release E2F. The resultant activation of E2F-responsive genes during G1 seems to commit the cells to initiate DNA replication.

Significantly, many growth-inhibitory signals, such as TGF-β and checkpoint pathways (including the p53/p21 pathway), mediate their effect by blocking the phosphorylation of pRB (DeGregori et al., 1995b; Mann and Jones, 1996; Schwarz et al., 1995). pRB is able to monitor and integrate both positive and negative growth signals determining whether or not the cell will divide (figure 1.1).

There is now considerable evidence that pRB regulates E2F-responsive genes through two distinct mechanisms that can refer to these as “inhibition of activation” and “active repression” (Trimarchi and Lees, 2002). The former involved binding of pRB to the transactivation domain of E2F, thereby blocking the ability of E2F to recruit the transcriptional machinery (Helin et al., 1993a; Helin et al., 1992). The pRB-E2F complex retains its ability to bind the promoters of E2F responsive genes and can thus recruit various factors that influence the chromatin structure of these genes and lead to active transcriptional repression (Zhang and Dean, 2001). These factors include histone deacetylase enzymes (HDACs) (Ait-Si-Ali et al., 1998; Brehm et al., 1998; Harbour and Dean, 2000), human brama related genes and its family member (hBRM) containing nucleosome remodelling activities (Zhang et al., 2000a), and the histone methytransferase SUV39H1 (Nielsen et al., 2001).

The recent finding that pRB-E2F complexes can also recruit a complex of SUV39H1 and heterochromatin protein 1 (HP1) to E2F responsive promoters (Bannister et al., 2001; Lachner et al., 2001) suggests that pRB can repress transcription at varying degrees, and that the repression could be a two-step process. First pRB-E2F complex could switch off actively transcribed genes by recruiting HDACs to deacetylate lysine 9 of histone H3. Subsequentially, the SUV39H1-HP1 complex could replace the HDACs allowing methylation of histone H3 on lysine 9 by SUV39H1 creating binding sites for HP1 leading to transcriptional silencing (Vandel et al., 2001) (figure 1.2).

pRB belongs to a family of proteins called pocket proteins including p107 and p130 that share many biological properties. Despite the existing similarities, the individual pocket proteins also have unique properties. First, they bind to different members of the E2F family *in vivo*, second these associations occur at distinct stages of cell cycle: whereas p130 is the predominant E2F regulator in G0, pRB binds E2F in both quiescent and actively dividing cells, and p107 is predominantly associated with E2F during the S phase. Distinct developmental requirements for these proteins, as well as partial functional redundancy between family members have been demonstrated. Unlike pRB, the other pocket proteins are not essential for embryonic development and low incidence of p107 and p130 mutation in human tumors indicate that they do not function as tumour suppressors (Mulligan and Jacks, 1998). This observations suggest that the closely related pRB family proteins affect cell cycle progression through distinct biochemical mechanisms and that their coordinated action may contribute to their diverse functions in various physiological settings (Classon et al., 2000).

Extensive networking exists between cellular pathways controlling proliferation and apoptosis. In the E2F/RB pathway (Dyson, 1998), p53 plays a key role in cellular decision to either arrest the cell cycle allowing the repair of damaged DNA, or to commit cell death. Its accumulation is negatively regulated by MDM2, which, in turn is negatively regulated by $p19^{ARF}$. E2F induces the expression of $p19^{ARF}$ thus directly connecting the E2F/RB pathway to p53 accumulation and the apoptotic response (DeGregori et al., 1997). However E2F-1 can also induce apoptosis in a p53 independent manner, which could be attributed, at least in part to the activation of p73 or other E2F-dependent factors (Nahle et al., 2002; Tolbert et al., 2002).

Further evidence for a unique role of E2F in an apoptotic response is seen from the observation that it is specifically induced following DNA damage. E2F-1 is phosphorylated by the DNA-

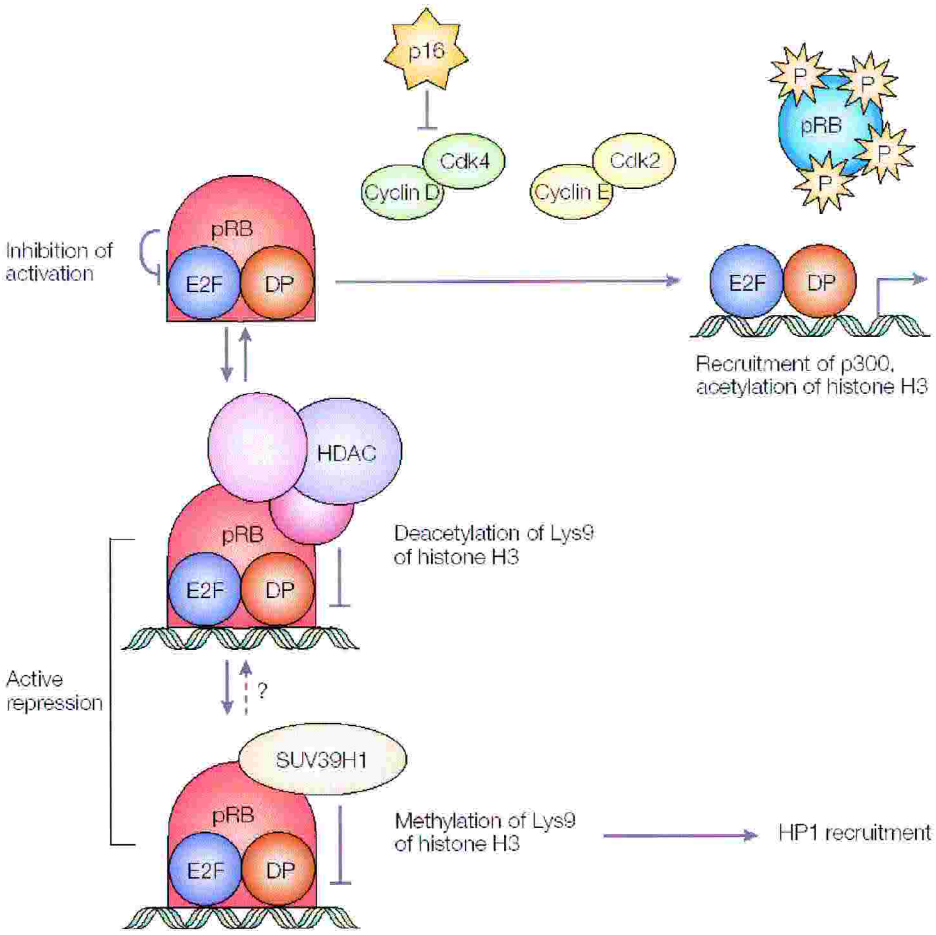

Figure 1.2
General mechanisms that control the repressive activity of E2F (from Trimarchi Nat. Rev. Mol. Cell. Biol. 2002)

The retinoblastoma protein binds to an E2F-DP complex in G0/G1 and this leads to repression of E2F-responsive genes through two possible mechanisms. First, pRB inhibits E2F from activating transcription by binding to its transactivation domain and preventing its interaction with the core transcriptional machinery. Second, the resulting complex binds to the promoters of E2F-responsive genes and enforces their 'active repression' trough the recruitment of either histone deacetylases (HDACs), which remove the acetyl group from lys9 of the histone H3 tail and thereby facilitate nucleosome packing , or SUV39H1, which methylates the same lysine residues to create binding sites for HP1, leading transcriptional silencing. Cell cycle entry is dependent on the sequential activation of the cell-cycle-dependent-kinases, Cdk4/6-cyclinD and Cdk2-cyclinE, which phosphorylate pRB and cause it to release E2F. The resultant activation of E2F-responsive genes seems to be at least partially dependent on the ability of E2F to recruit p300 leading to the acetylating of lys9 of histone H3. Several key component of the pRB regulatory pathway are known to be tumor suppressors (pRB, p16) or oncogenes (cyclinD, Cdk4).

damage-response kinases, ataxia-telangiectasia mutated (ATM) and ataxia-telangiectasia and Rad3-related (ATR), which leads to its stabilization (Lin et al., 2001) (figure 1.1).

1.1.3 The E2F family of transcription factors

Eight human genes have been so far identified as components of the E2F transcriptional activity (Dyson, 1998; Helin, 1998). On the basis of sequence homology and functional properties these genes have been divided into two distinct groups: the E2Fs (E2F1-E2F6) and the DPs (DP1 and DP2). They heterodimerize via interaction of the dimerization domains to give rise to functional E2F activity recognizing preferentially the same nucleotide sequence (TTTCCCGC).

E2F-1, E2F-2 and E2F-3 are potent transcriptional activators of E2F responsive genes (Helin et al., 1992). Overexpression of any of these proteins with functional DNA-binding and transcriptional activity is sufficient to induce quiescent cells to re-enter the cell cycle (Johnson et al., 1993).

E2F-1, -2 and -3 are specifically regulated by their association with pRB, but not with the related pocket proteins p107 or p130. Their release is triggered by the phosphorylation of pRB in late G1 and correlates closely with the activation of E2F-responsive genes.

The combined loss of these three E2F factors severely affects E2F target expression and is sufficient to completely block cellular proliferation (Wu et al., 2001) providing a direct genetic evidence for their essential role in cell cycle progression, proliferation and development and indicating that E2F-1, -2 and -3 could have overlapping roles in the induction of cell-cycle entry (Muller et al., 2001).

A second subclass of the E2F family includes E2F-4 and E2F-5. The sequences of these proteins diverge considerably from the other E2Fs; in particular, E2F-4 and E2F-5 lack most of the sequence that is amino-terminal to the DNA-binding domain. Consistent with this observation, E2F-4 and E2F-5 are regulated differently from E2F-1 -2, and -3 *in vivo*. First, significant levels of E2F-4 and E2F-5 are detected in quiescent (G0) cells are dispensable for cell cycle progression but necessary for pocket-protein mediated G1 arrest of cycling cells (Gaubatz et al., 2000), whereas E2F-1, E2F-2 and E2F-3 are primarily restricted to actively dividing cells. Second, the E2F subgroups bind to different pocket proteins *in vivo* (figure 1.3).

In contrast to the activating E2Fs, E2F-4 and E2F-5 are poor transcriptional activators and they are not able to drive quiescent cells to re-enter the cell cycle (Muller et al., 1997; Verona et al., 1997). The differential activity of the two E2F subgroups results from differences in their sub-cellular localization. Due to the presence of conserved amino acid terminal (NLS) E2F-1, E2F-2 and E2F-3 are induced to accumulate in the nucleus in G1 and downregulated as cell progress

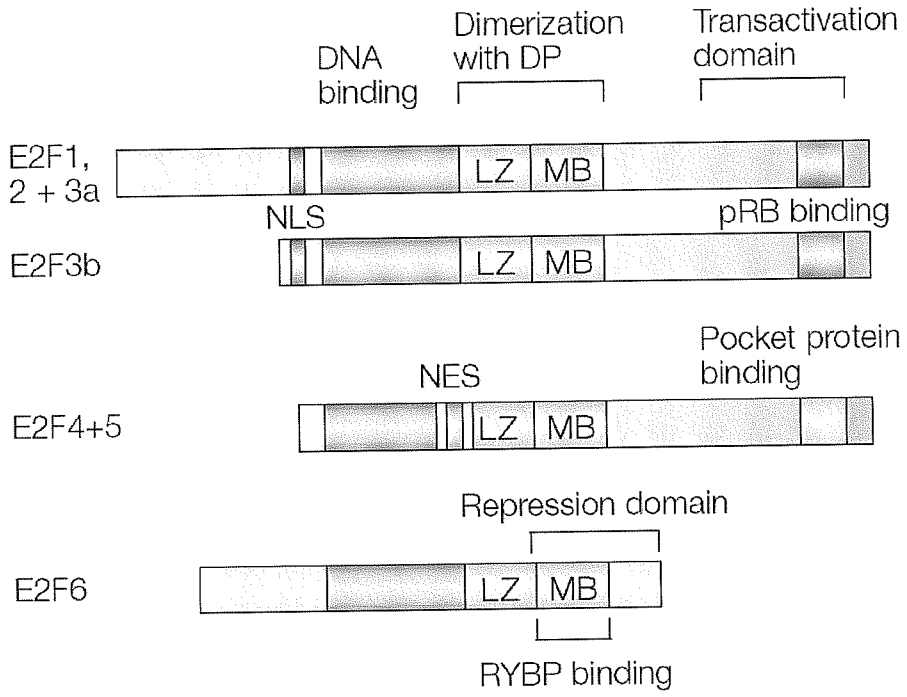

Figure 1.3
Structural comparison of the E2F family members
(from Trimarchi Nat. Rev. Mol. Cell. Biol. 2002)

All E2F family members share a domain structure with a core domain that mediate DNA binding or dimerization with DP, leucine zipper (LZ) and (MB) marked box. Sequences that are required for transcriptional activation and pocket protein binding are only present in E2F1-5. These E2Fs can be divided in two distinct subgroups - E2F1, E2F2, E2F3a+b, and E2F4 and E2F5 - on the basis of homology. Moreover the E2Fs from the first group share a canonical basic nuclear localization signal (NLS) that is absent in E2F4 and E2F5, which have a nuclear export signal (NES) instead. E2F6 diverges considerably from the other E2F family members: there is no almost no homology outside the core domain. The carboxy-terminal domain of E2F6 is required for recruitment of proteins that ensure the transcriptional repression.

trough the S phase, whereas E2F-4 and E2F-5, which lack defined nuclear localization signal (NLS) are predominantly cytoplasmic.

E2F-4 and E2F-5 are thought to be crucial for mediating the transcriptional repression of E2F responsive genes. The members of the DP class have been proposed to be responsible for translocating E2F members to the nucleus. An alternative mechanism for the nuclear translocation of E2F-4 and -5 has been ascribed to the pocket proteins p107 and p130, allowing an extra level of control in the activity of the E2F family, since nuclear translocation due to pocket protein will generate a negative effect on cell cycle progression, whereas DP-mediated nuclear accumulation will induce cell growth. Significant levels of E2F-4, p107 and p130 occupy the promoters of many E2F-responsive genes during these stages of the cell cycle. As cells progress through the cycle, the levels of E2F4–DP–p107/p130 complexes that are associated with promoters decline, and they seem to be replaced by the activating E2Fs (Takahashi et al., 2000; Wells et al., 2000).

However, it has been recently demonstrated that E2F-4 has two nuclear export signals (NES) and its cytoplasmic localization is dependent on the nuclear export factor CRM1 (Gaubatz et al., 2001). Nuclear import of E2F-5 only requires the first N-terminal 56 amino acid residues, and is not dependent on interaction with DP or pRB family proteins. E2F-5 is exported from the nucleus via CRM1-mediated transport, through a region corresponding to region that excludes the DNA- and the p130-binding domains. Thus, the subcellular distribution of E2F-5 is tightly regulated through multiple functional domains, which direct nucleocytoplasmic shuttling of this protein (Apostolova et al., 2002).

The sixth member of the family, E2F-6/EMA (E2F-modulating-activity) is the most recently identified and its biological properties are now beginning to be elucidated. It has an unusual organization in respect to the other family members: it has a truncated carboxyl-terminal region that cannot bind to pocket proteins, and also lacks transcription activation domains while DNA binding and dimerization domains are conserved and it has demonstrated to be an inhibitor of E2F dependent transcription (Cartwright et al., 1998; Trimarchi et al., 1998).

E2F-6 is a nuclear protein that contributes to gene silencing in a manner independent of retinoblastoma protein family members. The overriding view is that it directs transcriptional silencing by modifying chromatin. E2F-6 has been found in a multimeric protein complex that contains several distinct DNA binding activities such as Mga and Max that may allow its recruitment to the promoters of many different genes like Myc- and Brachyury. Moreover, the complex contains chromatin modifiers such as the novel identified histone methyltransferase

that modifies lysine 9 of histone H3, HP1γ, and Polycomb group (PcG) proteins. It is likely that E2F-6 silences gene expression in quiescent cells in vivo because E2F-6, Max, and HP1γ preferentially occupy sites in the promoters of cell cycle–regulated genes during G0. E2F-6 is replaced by other E2F family members including E2F-1 and E2F-4 as the cells move into G1. Moreover, the presence of distinct DNA binding activities in the E2F-6 complex may allow for the coordinated regulation of diverse target genes through a common long-term gene-silencing mechanism that depends on chromatin modification (La Thangue, 2002; Ogawa et al., 2002).

1.1.4 Regulation of E2F activity

The transcription factor E2F-1 plays a key role in regulating cell cycle progression. Accordingly, E2F-1 activity is itself tightly controlled by a series of transcriptional and post-transcriptional events (Dynlacht et al., 1994; Johnson et al., 1994). Regulation of E2F by **synthesis,** cell cycle-dependent activation of kinases and differential **subcellular localization**, have been reviewed extensively (Dyson, 1998).

E2F Modification by **phosphorylation** mediated by TFIIH plays a role in triggering its degradation during S phase (Vandel and Kouzarides, 1999).

Interaction of E2Fs with regulatory factors other than pocket- and DP-proteins are important for regulation of gene expression. Interaction with Sp1 appears also important for the regulation of several E2F target genes (Lin et al., 1996).

It has been reported that the p53 tumour suppressor protein makes functional interaction with both E2F-1 and DP-1 (O'Connor et al., 1995) causing inhibition of E2F dependent transcription, presumably due to competitive interference of p53 with the heterodimer formation. MDM2, the negative regulator of p53, seems to interact and regulate E2F activity (Martin et al., 1995). Other factors have been demonstrated to bind E2F proteins and augment its activity including CBP (Trouche and Kouzarides, 1996), TATA-binding protein (TBP) (Emili and Ingles, 1995), NF-κB (Kundu et al., 1997) and BRCA1 (Wang et al., 1997). It has recently reported that $p14^{ARF}$, which is an E2F target, forms a physical complex with E2F-1 and is capable of autonomously down-regulating E2F activity, which correlates with the inhibition of E2F-dependent apoptosis (Mason et al., 2002), interestingly it has also been demonstrated that ARF interaction might determine differential regulation of the E2F, DP and E2F/DP complex by altering the dynamics of their heterodimerization during progression from G1 to S (Datta et al., 2002).

The ability of RYBP (Ring1- and YY1-binding protein) to mediate an interaction between E2F-2 or E2F-3 and YY1 is an important component of Cdc6 activation and provides another example for proteins binding to E2F and influencing specificity of E2F function (Schlisio et al., 2002).

In order to stimulate transcription E2F proteins must reverse the pRB- imposed chromatin structure recruiting proteins capable of histone acetylation. Is reported that E2F stimulates transcription also by recruiting acetyltransferase activity and the essential cofactors GCN5 and TRRAPs (Lang et al., 2001).

E2F-1 was demonstrated to be **acetylated** both by P/CAF and p300/CBP (Martinez-Balbas et al., 2000; Marzio et al., 2000). These modifications were found to occur at three specific lysine residues close to the DNA binding domain, and have the effect of increasing DNA affinity *in vitro*. In this respect, it should be noted that E2F can also act as a repressor when complexed with pRB and that this interaction can also negatively regulate the FAT modification of E2F-1. Specifically, the HDAC1 associated with pRB is able to deacetylate E2F-1 demonstrating that the HDACs and HATs may act antagonistically on their non-histone substrates as they do on histones.

Regulation of E2F protein level can be mediated by the **ubiquitin-proteasome-dependent degradation** (see also paragraph below on ubiquitination). The only regulatory signal that has been clearly linked to the control of E2F stability is the binding with, and protection from degradation, by the retinoblastoma protein, both *in vitro* (Campanero and Flemington, 1997; Hateboer et al., 1996) and *in vivo* (Martelli and Livingston, 1999). A recent report suggests a model linking the control of E2F accumulation beyond G1/S phase to a specific interaction with ubiquitin protein ligase complex containing the $p45^{SKP2}$ F-box protein (Marti et al., 1999). *In vitro* studies demonstrate that multiple ROC-cullin ligases can specifically catalyze E2F-1 ubiquitination through a SKP1 and substrate phosphorylation-independent manner (Ohta and Xiong, 2001).

Regulation of gene expression in mammals through **methylation of cytosine residues at CpG** dinucleotides is involved in the development and progression of tumors. Many genes that are involved in the control of cell proliferation are regulated by members of the E2F family of transcription factors and some E2F DNA-binding sites are methylated in vivo. Methylation of E2F elements, derived from specific E2F dependent promoters, prevents or significantly inhibits the binding of E2F family members (Campanero et al., 2000).

Due to the complexity of E2F regulation and interaction between multiple regulatory mechanisms, loss of a single level of regulation (like mutation of pRB) therefore does not result in complete deregulation of E2F activity. This makes sense given the potency of E2F as a regulator of proliferation and apoptosis.

1.1.5 E2F transcriptional targets

The identification of E2F binding consensus site prompted the search for E2F-regulated genes. Most of the genes initially identified are related to cell cycle progression (Takahashi et al., 2000; Wells et al., 2000). Using a DNA microarray approach it has been recently possible to discover that E2Fs are also involved in transcriptional regulation of genes that encode components of the DNA damage checkpoint and repair pathways, as well as factors involved in chromatin assembly, condensation, chromosome segregation and the mitotic spindle checkpoint (Ishida et al., 2001; Ren et al., 2002; Wells et al., 2002).

Cell cycle regulatory genes are controlled by E2F through genetically distinct mechanisms: genes encoding essential enzymes in the nucleotide and DNA biosynthetic pathways are up-regulated in late G1, whereas genes ending regulators of cell cycle progression are repressed in G0 and activate at the G1/S transition.

E2F targets	**Ref.**
cell-cycle regulatory machinery	
E2F-1	(Johnson et al., 1994)
E2F-2	(Sears et al., 1997)
E2F-3	(Leone et al., 1998)
CycA	(Schulze et al., 1995)
CycB1	(Lukas et al., 1999)
CycE	(Ohtani et al., 1995)
Cdc2	(Dalton, 1992)
Cdc25A	(Chen and Prywes, 1999)
pRB	(Ohtani-Fujita et al., 1994)
p107	(Zhu et al., 1995)
p21	(Gartel et al., 1998),
p14AR	(Parisi et al., 2002)
c-myc	(Hiebert et al., 1989)
TGF-β	(Kim et al., 1994)
b-myb	(Lam et al., 1995)
BRCA1	(Wang et al., 2000)
Enzymatic machinery for DNA replication	
DHFR	(Blake and Azizkhan, 1989),
pol-α	(Pearson et al., 1991)
Tk	(Dou et al., 1994)
TS	(Dong et al., 2000)
RR2	(DeGregori et al., 1995a)
PCNA	(DeGregori et al., 1995a)
Regulatory machinery for DNA replication	
cdc6	(Yan et al., 1998)
MCM5 MCM6	(Ohtani et al., 1999)
MCM7	(Suzuki et al., 1998),
HsOrc1	(Ohtani et al., 1996)
RPA A2	(Kalma et al., 2001)

Table 1

Typical targets include those encoding cell cycle regulators that trigger S-phase entry, products involved in the assembly of pre-replication complex at origins of DNA replication and enzymes needed for the direct synthesis of DNA summarized in the table 1.

Recent findings link the process of **DNA repair, and recombination repair** in mammalian cells and suggest that their expression could be regulated through the common E2F factor (figure 1.4). The E2F-dependent DNA repair genes identified are involved in the full spectrum of repair process and include *msh1* and *msh2* (mismatch repair), *rpa3* (nucleotide excision repair), *ung* (base excision repair), *rad51* (homologous recombination), *rad54* and *DNA-PK* (non-homologous end-joining). E2F regulation of these genes at the G1/S transition is consistent with the view that DNA replication is an error-prone process, and that DNA repair is strictly linked to DNA synthesis.

The notion that E2F is simply a regulator of the G1/S transition is contradicted by evidence that E2F regulates the expression of several genes with **mitotic functions** including *cyclinB1*, *B2*, *Bub1* and *cdc2* involved in the progression through M-phase in addition to genes for cytokinesis (*Plk, PRC*), chromosome condensation (*SMC4*, *SMC2*), chromosome segregation (*securin* or *PTTG1*, *CENP-E*, *HEC1*), centrosome duplication (*RanBPM* (Meraldi et al., 1999), and mitotic spindle checkpoint (*CENP-E, Bub3, TTK Mad2*) whose misexpression contributes to genome instability.

E2F is known to downregulate the expression of anti-apoptotic factors, and several pro-apoptotic genes have been proposed to be induced by E2F-1, including *Apaf-1* (Moroni et al., 2001) and *caspase 3 and 7* (Muller et al., 2001). E2F-1 expression leads to stabilization of p53 through activation of the $p14^{ARF}$ gene, a component of the $p14^{ARF}$ MDM2 stabilization pathway (Bates et al., 1998; DeGregori et al., 1997; Mason et al., 2002).

p53 is induced in response to DNA damage and acts to enforce a cell cycle block in G1 phase (Zhou and Elledge, 2000). Its is now clear that the cellular response to DNA damage makes use of RB/E2Fpathway with a role for E2F as a specific inducer of **apoptosis** and p53 accumulation. DNA damage leads to specific induction of E2F-1 accumulation dependent on ATM kinase (Lin et al., 2001). *Chk1*, which is a kinase, activated by ATM that, was also identified as an E2F-dependent gene. Chk1 is required for the G2 DNA damage arrest **checkpoint**; interestingly pRB was required for Chk1 downregulation and resumption of G2 after damage (Gottifredi et al., 2001). The p53 homologue p73 is also E2F-1 inducible and can influence the rate of E2F-1 induced apoptosis (Irwin et al., 2000). Recent data demonstrate that ATM is transcriptionally

regulated by E2F-1 and suggest that ATM serves as a novel, ARF-independent functional link between the RB/E2F pathway and p53 (Berkovich and Ginsberg, 2003).

The observation that E2F can induce the expression of homeobox genes raises the possibility that E2F complexes may be also implicated in **development** and pattern formation. In particular several PcG genes have been identified, like *EZH2*, Embryonic Ectoderm Development protein (*EED*) and Homologue of Polyhomeotic (*EDR2/HPH2*).

E2F-dependent transcription factors that are involved in cell fate decision and **differentiation** include *HEY1*, associated with neurogenesis, *PTX1* craniofacial development, *MAF* (regulation of early differentiation), and *Sox9* (cartilage formation). These factors are exquisitely controlled, and in several cases it has been described that the over or misexpression of such genes leads to malformations and/or transformation.

Ran-binding protein1 (RanBP1) is a major regulator of the Ran GTPase and is encoded by an E2F-controlled gene (Di Fiore et al., 1999). RanBP1 protein controls a puzzling variety of cell cycle events, including checkpoint controls at the G1/S and G2/M transition and **nucleocytoplasmatic transport**.

The findings that the timing of RanBP1 transcriptional induction is coupled to that of genes required for S phase by the same E2F family of E2F activators and pocket proteins repressors, suggests that, in mammalian cells at least, RanBP1 might act as a sensor of the cell cycle phase, connecting the Ran cycle to the cell cycle (Lavia and Jansen-Durr, 1999).

1.1.6 Roles of E2F in initiation of DNA replication

The molecular mechanisms underlying the initiation of DNA replication in mammalian cells are still largely unknown. It remains difficult to identify discrete replication origins in the mammalian genome and thus the approach taken has been to focus on mammalian homologs of yeast genes that interact with origins and regulate DNA replication. The fact that homologs have been found for a large variety of genes strongly suggests conservation of basic regulatory mechanisms of DNA replication among yeast and other eukaryotes including mammals. Expression of most of the mammalian homologs has been shown to be regulated by E2F and it is likely that this is also the case for other as-yet-unidentified molecules. E2F could be a tool for identification of other participating players and there are many evidences that suggest E2F to have alternative non-transcriptional functions and to be directly involved on origins of DNA replication.

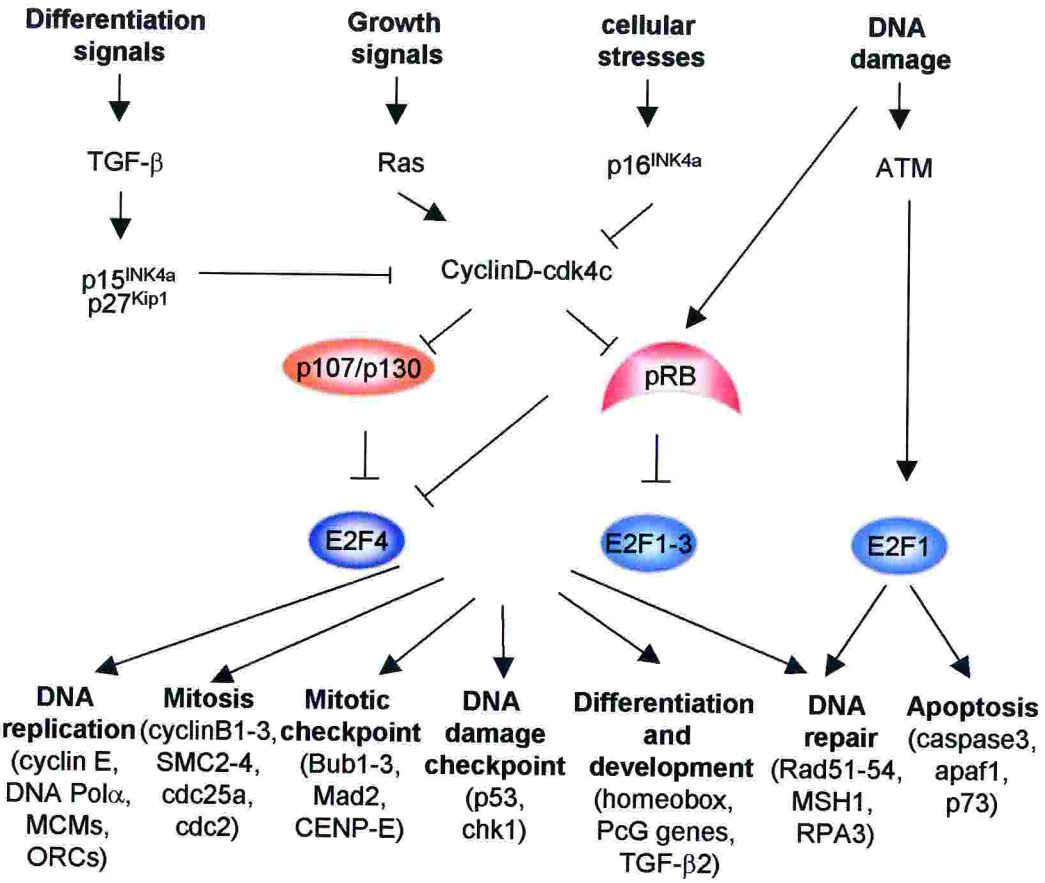

Figure 1.4
Transcriptional program of the E2F/pocket protein pathway

The E2F/RB pathway acts downstream of several important signaling cascades that trigger cell cycle arrest. Cellular senescence and TGF-β pathway induce cell cycle inhibitors such as p16, p27 and p15, which inactivate cyclinD-cdk4 and trigger pocket protein-dependent cell cycle arrest. Alternatively, the MAPK pathway induces cyclinD expression and leads to E2F transcriptional activation. DNA damage can induce two types of response depending on the context ad intensity of DNA damage: a cell cycle arrest requiring pRB or a proapoptotic response mediated through ATM and E2F1.
E2F functions beyond the G1/S transition of the cell cycle and its likely to regulate DNA replication, DNA repair, and DNA damage checkpoint genes. It also controls the transcription of genes that functions during mitosis, in mitotic checkpoints and in apoptosis. Finally, E2F induces genes that are involved in differentiation and development.

In mammalian cells E2F-4 and pRB co-localize in a limited number of discrete regions of the nucleus that represent the initial origins of DNA replication at foci that are sites of DNA synthesis early in S-phase (Lai et al., 2001).

During *Drosophila* oogenesis RBF and E2F-1 co-localize associated to replication proteins DmORC1 and 2 mediating developmental and cell cycle regulation of ORC1 (Royzman et al., 1999). Moreover E2F-1 and RBF form a complex with origin recognition complex proteins and are bound at origins of DNA replication sites, and indication that the E2F/RB pathway may directly function at origins of DNA replication to control S-phase entry (Bosco et al., 2001). In keeping with this conclusion, preliminary data from our laboratory indicate that a direct interaction exists between E2F-1 and ORC1 in human cells (unpublished data). The idea that pRB can block DNA synthesis is appealing, and may explain how it can function in a checkpoint pathway during S phase in mammal cells, at the time when E2F dependent transcription is not expected to be limiting.

Most intriguingly, E2F-1 has been also reported to associate with NBS1 and the MRE11 recombination/repair complex (Maser et al., 2001). The authors propose that E2F-1 is required to target the Mre11 complex near origins of DNA replication to suppress the firing of these origins when DNA is damaged.

1.2 Acetylation

Histones acetylation promoted by histone acetyl-transferases (HATs) plays an important role in coordinating gene expression, cell-cycle progression and differentiation. Component of the cell cycle regulatory apparatus are both regulated and bind directly to HATs. Moreover transcription factors have been identified as substrates for HATs. Several are the enzymes, acetylases and deacetylases that can regulate transcription by modifying the acetylation state of histones or transcription factors and some of them are present in multisubunit complexes. Acetylation of histonic or nonhistonic proteins is a reversible process, and the balance between acetylation and deacetylation has been demonstrated to be important in regulating gene expression and it is thus linked to the control of cell fate. As a consequence, hyperacetylation of normally silenced regions or deacetylation of normally actively transcribed regions can lead to various disorders, including developmental and proliferative diseases.

1.2.1 Chromatin modifying enzymes

Chromatin structure is known to have profound effects on gene expression in eukaryotic cells.

DNA in eukaryotes is typically packaged in repeating arrays of nucleosomes, in which 146 bp of DNA are wrapped around a histone octamer. Each octamer includes four histone proteins (H2A, H2B, H3 and H4). Chromatin structure is dynamically regulated by proteins that remodel chromatin in an ATP dependent manner through post-translational modifications including phosphorylation, ADP ribosylation, methylation, ubiquitination and acetylation.

Histone acetylation involves the transfer of an acetyl group from acetyl-CoA to the ε-amino group of lysine side chain within the substrate. The modification of the lysines groups of core histones by multiple post-translational modifications coincident with mitogenic signaling (Clayton et al., 2000) has led to a model in which the N-terminal tail of the core histone is considered to function as a signaling platform. Non-histone substrates of histone acetyl transferase FAT (factor acetyl transferase) might also function as signaling platforms in acetylation-phosphorylation cascades.

The acetylation reaction is both complex and specific since specific lysines are acetylated by specific HAT activities. How the post-translational modifications of histones activates gene expression remains unclear. The modification of lysine groups may disrupt electrostatic interactions between histones and DNA and increase accessibility of nuclear factors or co-activator complexes.

1.2.2 Histone acetyl-transferase families

HATs were historically classified as type A, located in the nucleus and known to acetylate nucleosomal histones within chromatin, and type B HATs, located in the cytoplasm with a housekeeping role consisting of acetylation of free histones in the cytoplasm. There are now six families of proteins known to exhibit a histone acetyltransferase activity (table 2).

The GNAT (Gcn5-related N-acetyltransferase) superfamily includes the best-characterized yeast Gcn5, originally discovered in the ciliate *Tetrahymena thermophila* (Brownell et al., 1996). In mammals, P/CAF (p300/CREB binding protein-associated factor) was identified on the basis of sequence homology to Gcn5 and was found to associate to the p300 co-activator protein (Yang et al., 1996).

The role of P/CAF in transcription has been investigated by multiple studies, and its requirement as a HAT and co-activator has been described for myogenesis and nuclear receptor-mediated, and growth factor-signaled activation among other processes. Although P/CAF was originally identified as a HAT, much recent work has focused on its acetylation of various non-histone transcription related protein as reported in the table 3.

Another group of evolutionary related proteins that are known or hypothesized to be HATs is the MYST family, named for its founding members: MOZ (monocytic leukaemia zinc finger protein), Ybf2/Sas3, Sas2 and Tip60. Additional members have more recently been identified including human HBO1 (histone acetyltransferase bound to ORC1) and MORF(MOZ-related factor). These proteins are grouped together on the basis of their close sequence similarities and their possession of a particular acetyltransferase homology region. Although containing regions similar in sequence, the members of the MYST family are involved in a wide range of regulatory functions in various organisms as reviewed in (Sterner and Berger, 2000).

After the discovery of histone acetylation by Gcn5 and P/CAF, the critical role of acetyl-transferases in transcriptional regulation was also demonstrated by the fact that the previously well-characterized co-activators of multicellular eukaryotes, p300 and its close homologue CBP (CREB binding protein), are themselves HATs (Bannister and Kouzarides, 1996; Ogryzko et al., 1996) and FATs. The interactions of p300/CBP (p300 and CBP are often referred as a single entity, since the two proteins are considered functional homolgs) with nuclear receptor co-activators, are examples of transcriptional regulatory complexes with multiple acetyltransferase activities. Overall p300/CBP is one of the most potent and versatile through the acetyl-transferases, consistent with its roles a global co-activator in higher eukaryotes. Like P/CAF,

p300/CBP is known to acetylate and regulate various transcription-related proteins other than the histones.

HAT proteins have also been directly implicated in transcriptional activation brought about by hormone signals. The HAT activities of human co-activators ACTR, SRC-1 and TIF2, which interact with nuclear hormone receptors, demonstrate the involvement of acetylation in yet another system of transcriptional regulation. The three proteins are a part of an evolutionary and functionally related HAT family and all three interact with p300/CBP.

Another direct connection between acetylation and activated transcription was demonstrated with the discovery that one of the TAFII (TATA-binding protein TBP- associated factor human TAFII250) subunits of the general transcription factor TFIID is itself a HAT (Mizzen et al., 1996). The HAT activity of TAFII250 suggests a model for the initiation of assembly of a transcriptional complex at chromatin-packaged promoters. As part of TFIID, TAFII250 may facilitate TBP binding directly by acetylating histones at the TATA box allowing formation of preinitiation complex.

HAT	**Histone modified**	**Reference**
GNAT superfamily		
hGCN5	H3, H4	(Brownell et al., 1996)
P/CAF	H3, H4	(Yang et al., 1996)
Hat1	H4	(Verreault et al., 1998)
MYST family		
Tip 60	H4, H3, H2A	(Kimura and Horikoshi, 1998)
MOZ	ND	(Borrow et al., 1996)
MORF	H4, H3, H2A	(Champagne et al., 1999)
HBO1	ND	(Iizuka and Stillman, 1999)
p300/CBP	H2A, H2B, H3, H4	(Bannister and Kouzarides, 1996)
Nuclear receptors co-activators		
SRC1	H3, H4	(Spencer et al., 1997)
ACTR	H3, H4	(Chen et al., 1997)
TAFII250	H3, H4	(Mizzen et al., 1996)
TAFIIIC	H3, H4, H2A	(Kundu et al., 1999)

Table 2

Evidence that histone acetylation is a general employed mechanism in transcription in supported by the fact also subunits of TFIIC, a general transcription factor in the RNA polymerase III basal machinery, were also recently identified as HATs (Kundu et al., 1999).

1.2.3 Acetylases in complexes

It is becoming increasingly apparent that acetyalses are mostly present within large nuclear complexes: several human protein complexes have been purified and characterized. Subunit identification has shown that some of these complexes are remarkably analogous to known yeast HAT complexes, and in each case an involvement in transcription is also suggested by subunits besides the HAT protein. As described above the TAFII250 is part of the well-characterized TATA-box binding protein (TBP)-containing, TFIID complex. Evidences from human and yeast cells indicated that P/CAF and GCN5 are also in large complexes (Struhl, 1998).

Interestingly, some of the proteins in the P/CAF complex have turned out to be TBP-associated factors (TAFs) that are also present in the TFIID complex.

1.2.4 Targets for acetylation

The acetyalses so far identified have the ability to modify histones in free solution but only a subset of them are able to acetylate histones in a nucleosomal structure. Although H3 and H4 are in general preferred substrates over H2A and H2B, acetylases such as p300 and CBP are able to modify all four. In addition, the specific lysine residues modified by each acetylase may differ, suggesting this difference may be an indicator of differences in function between acetylases (Davie, 1998).

It is also known that several HATs have a self-acetylating activity *in vitro*, including P/CAF, p300, Tip60 and MORF. However it is unknown whether these events have physiological relevance as self-regulation or not.

1.2.5 Acetylation and protein function

Some of the enzymes exhibiting histone acetyl-transferase activity are known to participate in transcriptional regulation by acetylating proteins other than the histones. FAT (factor acetyl transferase) activities have been demonstrated for P/CAF, p300/CBP and TAFII250, with transcription-related substrates ranging from activators and co-activators to basal transcription machinery factors and non-histone chromatin proteins. Acetylation has been demonstrated to affect either positively or negatively the activity in a numbers of activators involved in various cellular and developmental processes as summarized in table 3.

In the case of other DNA binding transcription factors (E2F-1 p53, EKLF and GATA-1) the acetylation site also falls directly adjacent to the DNA binding domain and acetylation results in

stimulation of DNA binding (Gu and Roeder, 1997; Martinez-Balbas et al., 2000; Marzio et al., 2000),

Substrates	Known function *in vivo*	Known FAT enzyme *in vitro*
Non histone Chromatin protein		
HMG1	Chromatin component	p300/CBP
HMG2	Chromatin component	ND
Yeast Sin1	Transcriptional regulator	Gcn5
HMG14	Nucleosome binding	p300/CBP
HMG17	Nucleosome binding	P/CAF
HMGI (Y)	Enhanceosome component	P/CAF, p300/CBP
Transcriptional activators		
p53	Tumour suppressor	P/CAF, p300/CBP
c-Myb	Proliferation, differentiation	p300/CBP, Gcn5
GATA-1	Blood cell differentiation	p300/CBP
Tal-1	Blood cell differentiation	P/CAF
EKLF	Globin gene expression	p300/CBP
MyoD	Muscle differentiation	P/CAF
E2F (1,2,3)	Cell cycle control	P/CAF
DTCF	Developmental regulation	P/CAF
HIV tat	HIV-1 transactivation	P/CAF
NF-κB (RelA subunit)	Inflammatory response	p300/CBP
Nuclear receptors co-activators		
ACTR	hormone signals	p300/CBP
SRC-1	transcriptional response	p300/CBP
TIF2		p300/CBP
General transcription factors		
TFIIE	General transcription machinery	P/CAF, p300/CBP, TAFII250
TFIIF	component	P/CAF, p300/CBP
TAF(I) 68	rRNA transcription component	P/CAF
Transcriptional repressors		
RB	Tumour suppressor	p300/CBP
MDM2	E3 ubiquitin ligase	p300/CBP
EVI1	Hematopoietic differentiation	P/CAF, p300/CBP
BCL6	Transcriptional repressor	p300/CBP
DNA metabolism control proteins		
Fen1	Chromatin remodeling	p300/CBP
TDG	DNA repair	p300/CBP
Other proteins		
Importin-α7, RcH1	Nuclear import	p300/CBP
α-tubulin	Microtubule component	ND
Adenovirus E1A	Cellular transformation	P/CAF, p300/CBP

Table 3 adapted form (Sterner and Berger, 2000)

(Boyes et al., 1998; Zhang and Bieker, 1998). Differently the lysines acetylates within the HMGI (Y) transcription factor or Fen-1 (Hasan et al., 2001b) fall within the DNA binding domain itself

and result in disruption of DNA binding. Besides affecting DNA binding, acetylation might also regulates protein-protein interactions either positively or negatively, and protein stability.

The growing list of protein modified by acetylation, including transcription factors like EVI1 (Chakraborty et al., 2001) and NF-κB RelA subunit (Chen et al., 2001), tumour suppressors as pRB (Chan et al., 2001) and MDM2 (Kawai et al., 2001), transcriptional repressors as BCL6 (Bereshchenko et al., 2002), transforming factors like E1A (Zhang et al., 2000b), co-activators, general transcriptional machinery components as TAF(I)68 (Muth et al., 2001) and nuclear import proteins, suggests that acetylation may function as a signaling mechanism which itself must be tightly regulated.

A new class of HAT substrates, which is represented by Fen1 (Hasan et al., 2001b) and TDG (Tini et al., 2002) that play a critical role in regulating DNA metabolic events, reveal a potential regulatory role for protein acetylation in maintaining genomic stability.

The binding of HATs to target substrates is transient and label; co-activator-substrate interaction can lead to transcriptional attenuation (Chen et al., 1999). The attenuation of co-activator signaling by acetylation illustrates the importance of feedback loops in controlling acetylation-signaling pathways. These findings raise the possibility that cancers may evade these normal mechanisms of attenuation leading to sustained proliferative signaling.

1.2.6 Histone deacetylases in cell proliferation and cancer

Transcription is controlled in part by the dynamic acetylation and deacetylation of histone proteins. The latter process is mediated by histone deacetylases (HDACs).

An increasing number of histone de-acetyalses have been characterized in higher eukaryotes. These proteins have been grouped in distinct families according to the similarity of their sequence to a yeast founding member (Gray and Ekstrom, 2001): HDAC1, 2, 3 and 8 are class I enzymes that share homology with yeast Rpd3, whereas HDAC4, 5, 6, 7, 9 (Zhou et al., 2001) and 10 (Guardiola and Yao, 2002) are class II enzymes that share homology with yeast HDA. Both classes share a common catalytic motif and they differentiate by unique N-terminal sequences found only in class II enzymes (Wade, 2001). Whereas some class II deacetylases are involved in regulating cell differentiation, in particular in muscle, some class I HDACs (1-3) participate in the control of the cell cycle progression by cooperating with the co-repressor pRB. HDAC11 is a novel and unique member of the histone deacetylase family, it contains conserved residues in the catalytic core regions shared by both class I and II mammalian HDAC enzymes and it may have distinct physiological roles from those of the known HDACs (Gao et al., 2002).

The classic paradigm for transcriptional regulation by histone deacetylases invokes recruitment to a gene regulatory region through a series of protein-protein interactions. Increased local concentration of deacetylase leads to a region of hypoacetylated chromatin that exerts a dominant repressive effect on transcription. The most characterized examples of regulation involve the class I HDACs. The class II HDACs are also thought to be recruited to distinct region of the genome by sequence specific DNA binding protein but they are subjected to an additional level of regulation. These enzymes actively shuttle between the nucleus and cytoplasm and their distribution appears to be under the control of cellular signaling pathways.

Inappropriate gene expression due to improper localization of histone deacetylase enzymes is known to be causal in some leukemias (Grignani et al., 1998; Lin et al., 1998).

The association of the deacetylase complex with the pRB tumour suppressor is another link to cancer. pRB mediates the induction of G1 arrest by repressing the activity of S-phase specific genes regulated by E2F. This repression is dependent on the association of pRB with the deacetyalase complex (Brehm and Kouzarides, 1999).

It has been also shown that in quiescent normal human cells HDAC complex colocalizes with both RB family members and E2F-4 in a limited number of discrete regions of the nucleus that in other studies have been shown to represent the initial origins of DNA replication following growth stimulation. These evidences suggest that RB family members, at least in part, drive exit from the cell cycle by recruitment of this HDAC complex to repress transcription from E2F-dependent promoters and possibly to alter chromatin structure at DNA origins (Lai et al., 2001).

1.3 p300/CBP

The CREB binding protein (CBP) and its homologue p300 are large nuclear molecules that coordinate and integrate multiple signal-dependent events with the transcription apparatus, allowing the appropriate level of gene activity to occur in response to diverse physiological cues that influence proliferation, differentiation and apoptosis, with chromatin remodeling. They are known to have very similar functions as extremely versatile co-activators.

The transcription regulating properties of p300 and CBP appear to be exerted through multiple mechanisms. They act as protein bridges connecting different sequence-specific transcription factors to the transcription apparatus. Providing a protein scaffold upon which to build a multicomponent transcriptional regulatory complex is likely to be an important feature of p300/CBP control.

They are potent histone acetyl-transferases, which link transcription to chromatin remodeling, mediate both positive or negative cross-talk between different signaling pathways, they participate in basic cellular functions, including DNA repair, cell growth, differentiation and apoptosis and play pivotal roles in embryonic development. p300/CBP can be under aberrant control in human disease particularly in cancer that may inactivate a p300/CBP tumour suppressor-like activity.

p300 and CBP were originally identified as proteins that bind to the adenoviral E1A and the cAMP-responsive-element binding protein (CREB) (Chrivia et al., 1993; Eckner et al., 1994). The p300/CBP genes are conserved in a variety of multicellular organisms from worms to humans. CBP is also closely related to p300: the proteins are 63% identical at the amino-acid level. Greater similarity is observed in specific regions, including the region encompassing the bromodomain, which is frequently found in mammalian HATs, the three cysteine-histidine rich domains (CH1, CH2 and CH3 which contains the E1A binding site), and the CREB binding site (KIX domain) The three CH regions are composed of four zinc fingers motifs which are TAZ1 in CH1, PHD in CH2 and ZZ and TAZ2 in CH3, an N-terminal nuclear receptor-interacting domain(RID) interacts with nuclear receptor activators. A bipartite nuclear localization signal (NLS-BP) resides at the middle part of p300/CBP and a glutamine/proline rich (QP) domain near the C-terminus associates with other co-activators and HAT proteins.

Both N-and the C-terminal regions of p300/CBP can activate transcription, and the HAT domain is located in the central region of the protein. It is associated with large conserved region spanning form the PHD domain to the ZZ motif (Yuan and Giordano, 2002).

Biochemical analysis of p300 revealed of distinct activation functions: (i) the CH3/E1A-binding region is thought to recruit transcription factors such as RNA polymerase II complex, TFIIB or P/CAF, (ii) the bromodomain, which may be important for the association of p300 with chromatin, (iii) the AT region, which catalyzes the acetylation of histones or other factors, and (iv) the SRC/p160-binding region in the C-terminus (figure 1.5).

This modular organization may allow p300/CBP to provide a scaffold for assembly of multicomponent transcription co-activator complexes, different activation functions are differentially required for the assembly of transcription initiation complexes (Kraus et al., 1999).

Although p300 and CBP share extensive homology, genetic and molecular analysis suggest that they perform not only overlapping but also unique functions. The complex phenotypes associated with a loss of p300/CBP function in humans and mice reflect the diverse roles of p300 and CBP in multiple developmental processes. It has been demonstrated that they have overlapping roles during embryonic development (Yao et al., 1998) but they perform unique tasks in certain physiological processes and they are not really interchangeable. It has been demonstrated that p300 and CBP are subjected to different regulation pathways: p300 protein is possibly degraded via the ubiquitin-dependent pathway during the retinoic acid (RA)-induced cell differentiation while CBP levels are constant through this process (Iwao et al., 1999).

It has also been reported that identical mutations in the p300 and CBP HAT domains impair HAT activity differently, providing the evidence for structural differences between p300 and CBP that may in part underlie a previously mentioned functional specialization of the two proteins (Bordoli et al., 2001).

1.3.1 Transcriptional control by p300/CBP

It is now widely accepted that p300/CBP proteins are versatile and perhaps rather general transcriptional integrators. Current evidences suggest that they act through a variety of mechanisms.

In the "bridging model", p300/CBP proteins connect sequence-specific transcription factors to the basal transcriptional machinery. Since p300/CBP proteins are involved in numerous signal transduction pathways, it has been proposed that a coordinated re-distribution of p300/CBP activity among different classes of factor in a signal dependent manner imparts specificity in transcriptional regulation.

In the "scaffold model", p300/CBP act as a protein scaffold for the assembly of multicomponent complexes that confer transcriptional activation. p300/CBP might nucleate the assembly of diverse cofactor proteins into multicomponent co-activator complexes. By providing a scaffold for

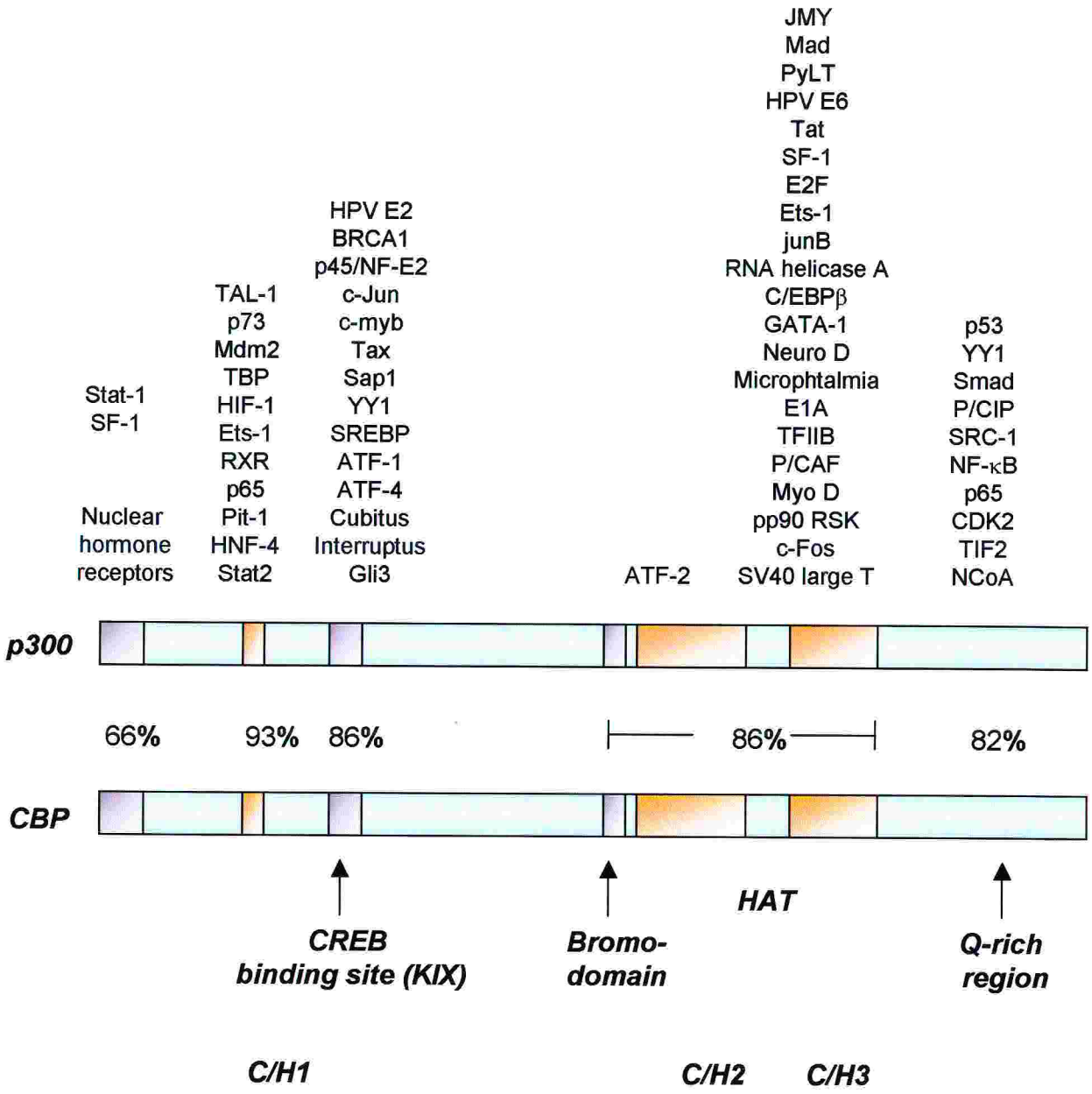

Figure 1.5
Structure of p300 and CBP, degree of homology and interacting factors

the assembly of transcription cofactors, it might increase the relative concentration of these factors in the local transcription environment and thereby facilitating protein-protein, protein-DNA interactions. Given a limited repertoire of activators, co-activators and cofactors available for responses to diverse regulatory cues, cells probably use cooperativity and transcriptional synergy so that a combination of a few ubiquitous signal- and tissue-specific activators can create a potentially very large number of regulatory complexes.

In the "HAT model", either the intrinsic HAT activity of p300/CBP or HATs assembled in multicomponent complexes target chromatin and/or transcription factors to facilitate a transcriptional response by weakening intranucleosomal interactions and destabilizing higher order of chromatin structure, by promoting transcription factors access to DNA in chromatin, or promoting the processivity of RNA polymerase through nucleosome array. More recently a functional interaction between p300/CBP and a family of proteins involved in nucleosome assembly (NAP nucleosome assembly proteins) has been documented (Shikama et al., 2000) demonstrating a direct connection between acetylation of nucleosomes to nucleosome remodeling in transcriptional regulation of E2F-1 and p53 target genes. Acetylation of transcription factors by p300/CBP can also regulate protein-protein interaction, and enhance DNA binding activity and consequently stimulate gene expression activity, as well as regulating DNA metabolic events inhibiting transcriptional activity and reducing its DNA binding capacity (Hasan et al., 2001b).

1.3.2 p300/CBP in cell growth regulation and development

It has become increasingly clear that p300/CBP proteins are versatile transcriptional co-activators that can influence different physiological processes, including cell growth, proliferation and cellular differentiation. They are likely to participate in DNA replication, tissue differentiation (such as skeletal myogenesis, adipogenesis and B cell differentiation), cell cycle checkpoints, cAMP nuclear signaling, response to hypoxia, cell adhesion control and the interplay between distinct signal transduction pathways, and they also seem to be involved in the mechanism that senses and controls the repair of damaged DNA as reviewed in (Goodman and Smolik, 2000).

Although the exact role of p300/CBP in regulating the behavior of a cell remains unknown, several lines of evidence suggest that p300/CBP is critical for the G1/S transition (Ait-Si-Ali et al., 2000) and hence for cell proliferation, thus standing in contrast to the widely accepted idea that p300/CBP is the product of a tumor suppressor gene.

Protein involved in cell fate control are generally turned on when a specific pathway is triggered in the cell and inactivated when cells are oriented toward other pathways through various pre or

posttranslational modifications. p300/CBP is an exception: it is essential for the balance between cell differentiation and cell proliferation. Although it is found in large complexes which include some of the components of the basal transcription machinery, it cannot be equated with a basal transcription factor, it is expressed only in multicellular organisms (Eckner, 1996), it is a target for transforming viral proteins such as E1A and SV40 T (Eckner et al., 1996) and, in mammalian cells, it is required only at certain stages of development. In particular p300 and CBP are crucial during embryogenesis in a dose dependent manner, either because they are not redundant in the process of embryogenesis due to functional or expression differences, or because total p300/CBP levels must be maintained for normal development.

They are also critical for muscle differentiation, in particular p300/CBP HAT activity is required for muscle cell terminal differentiation (Polesskaya et al., 2001).

A direct link between transcription, chromatin modifying activity and DNA repair by p300/CBP has been demonstrated for TDG (Thymine DNA Glycosylase) which, by forming a physical an functional complex, is able to stimulate CBP transcriptional activity, and reciprocally serves as a substrate for p300/CBP acetylation. Remarkably this acetylation triggers release of CBP from the complex and regulates the recruitment of repair endonucleases. These observations in addition to recent findings that p300 also associates with PCNA and Fen1, both required for DNA replication and repair, suggest a central role of p300/CBP in these processes (Hasan et al., 2001a; Hasan et al., 2001b). In this scenario p300/CBP-TDG may be recruited to promoter regions by transcription factors to first mediate repair of regulatory regions and subsequently to promote transcription; this would ensure that transcriptionally active genes are repaired prior to transcription. Hence, the transcriptional role of TDG may be linked to repair, which could be consistent with the association of numerous other factors with the CH3 domain. A direct regulatory role for acetylation in TDG-dependent DNA repair have been proposed, although histone hyperacetylation has been linked to enhanced DNA repair (Meijer and Smerdon, 1999).

1.3.3 Regulating p300/CBP activity

p300/CBP versatility and its involvement in opposing cellular processes raise the interesting issue of its regulation: indeed it has to be directed toward selected target genes during specific processes. Given the strong gene dosage effect observed. A simple competition between transcription factors for p300/CBP binding might participate in the regulation.

p300/CBP are nuclear phosphoproteins that, together with other factors known to be important regulators of the cell cycle and transcription such as p53, pRB, PML, and SUMO-1, reside in a

nuclear structures called the nuclear body (von Mikecz et al., 2000). The phosphorylation of p300/CBP appears to be under cell cycle control, during mitosis hyperphosphorylation mediated by cyclin-dependent-kinases has been observed, although relatively little is known about how phosphorylation affects p300/CBP function.

Another important question about p300/CBP function is whether HAT activity is controlled during the cell cycle. It has been reported that HAT activity peaks at the G1/S transition (Ait-Si-Ali et al., 1998). Furthermore, phosphorylation of p300/CBP by cyclinE-cdk2 in the C-terminal region of the protein appears to stimulate its HAT activity. p300/CBP HAT activity is regulated in cell cycle dependent manner suggesting a function for p300/CBP in the crucial G1/S step of the cell cycle. It is indeed essential for the activity of E2F, a transcription factor that controls the G1/S transition. Moreover p300/CBP HAT activity is required both for the G1/S transition and for E2F activity (Ait-Si-Ali et al., 2000).

1.3.4 p300/CBP and disease

The importance of p300/CBP in malignancy remains to be elucidated. Increasing evidences support the view that p300/CBP can be under aberrant control in tumor cells. Clinical lines of evidence suggest that p300/CBP are both implicated in tumorigenesis and mutations in the human CBP gene have been found in Rubinstein-Taybi syndrome (RTS), which is characterized by multiple developmental defects and mental retardation. Studies with CBP and p300 mouse mutants indicate that both proteins are required for normal development, and that there is an essential gene dosage-sensitive role for these transcriptional cofactors in embryogenesis, cell differentiation and proliferation. p300/CBP are thought to act as a tumor suppressor proteins firstly because RTS (Rubinstein-Taybi syndrome) patients have an increased predisposition to cancer (Petrij et al., 1995), secondly loss of p300/CBP function by bi-allelic inactivation allows uncontrolled cell growth and it is associated to gastric, hepatic, colon and breast malignancies (Muraoka et al., 1996 Suganuma, 2002 #299).

p300/CBP genes are involved in various chromosomal translocation events giving rise to specific hybrid proteins.

For example an MOZ-CBP fusion resulting from a translocation between *MOZ* (monocytic leukaemia zinc finger protein) and *CBP* was reported in AML (acute myeloid leukaemia) (Borrow et al., 1996). Although its function is not known, the *MOZ-CBP* gene product is considered to be a putative acetyl-transferase based on its homology to yeast silencing genes. A second translocation of the *CBP* gene to the *MLL* (mixed lineage leukaemia) gene occurs in chrornic myeloid leukaemia and myoelodysplastic syndrome (Sobulo et al., 1997), which arises as a

consequence of cancer therapies. In another case, a patient suffering from therapy-related AML was identified as having an in-frame fusion of *MLL* with *p300* (Ida et al., 1997).

These fusion proteins probably produce temporally aberrant and/or deregulated expression of proteins involved in differentiation or cell cycle control, resulting in neoplastic growth of myeloid cells. Presumably the fusion proteins can interact with the majority of p300/CBP targets, some of which, such as p53, and E2F, have been shown to play a regulatory role in haematopoietic cells. Furthermore, given the fact that p300/CBP MOZ and MLL are all associated with chromatin remodeling, altered pattern of chromatin acetylation seems likely to contribute to the oncogenic effects these fusion proteins exert. The fact that CBP is prone to translocations, inversions and deletions, suggests the presence of elements conferring genomic instability. The presence of an unstable genomic element might also explain why CBP seems to be more frequently target for chromosomal rearrangements than p300 (Giles et al., 1998). Recent findings suggest that alteration of putative tumour suppressor p300 and CBP may also deregulate TDG (thymine DNA glycosylase)-coupled repair and contribute to genomic instability commonly associated with cancer (Tini et al., 2002).

1.4 Ubiquitination

The conjugation of the conserved 76-residue polypeptide ubiquitin to other cellular proteins regulates a broad range of eukaryotic cell functions. The high efficiency and exquisite selectivity of the ubiquitination reaction reflect the properties of enzymes known as ubiquitin-protein ligases or E3s. An E3 recognizes its substrates based on the presence of a specific ubiquitination signal and catalyzes the formation of an isopeptide bond between a substrate (or ubiquitin) lysine residue and the C-terminus of ubiquitin. Although a great deal is known about the molecular basis of E3 specificity, much less is known about the molecular mechanisms of catalysis by E3s. Recent findings reveal that all known E3s utilize one of just two catalytic domains - a HACT domain or a RING finger domain - and crystal structures have provided the first detailed views of an active site of each type (Pickart, 2001a).

Substrates marked with a polymer of ubiquitins (a polyubiquitin chain) are selectively targeted to a multisubunit ATP-dependent protease known as the 26 proteasome, whereas certain substrates marked with one or a few ubiquitins are targeted for endocytosis, ultimately resulting in proteolysis in the lysosome. Ubiquitination regulates a host of critical cellular functions, frequently by mediating the selective degradation of master regulatory proteins. The progression of the cell cycle (Koepp et al., 1999), the induction of inflammatory response and antigen presentation (Rock and Goldberg, 1999) are just a few of the many processes regulated by ubiquitin/proteasome dependent proteolysis. Not surprisingly, deregulated ubiquitin dependent proteolysis has been implicated a causative factor in cancer and several inherited diseases.

1.4.1 The ubiquitin pathway

Ubiquitination usually results in the formation of a bond between the C-terminus of ubiquitin (G76) and the e-amino group of a substrate lysine residue. At least four distinct enzymes participate in polyubiquitination: ubiquitin-activating enzymes (E1), ubiquitn-conjugating enzymes (E2), ubiquitin ligase (E3), and ubiquitin multimerization proteins (E4) (Ciechanover et al., 2000a). These enzymes cooperate in a hierarchical manner to polyubiquitinate. In the first step, E1 enzymes activate a single ubiquitin moiety in an ATP-dependent reaction that requires Mg^{2+}. E1 enzymes form a thiol ester with the carboxyl group on G76, thereby activating the C-terminus of ubiquitin for nucleophilic attack. In the second step, E1 enzymes transfer the activated ubiquitin onto E2 enzymes that, like E1 enzymes, bind ubiquitin via a thioester bond. Next, ubiquitinated E2 enzymes associate with E3 enzymes. They are thought to define the substrate specificity of the ubiquitination reaction. Some E3 enzymes bind the substrate and

allow E2 to ubiquitinate the target protein. Other E3s serve as ubiquitin shuttle: they accept the ubiquitin from E2 enzymes via thioester bonds and transfer the ubiquitin onto the target protein. Finally, E4 enzymes bind to the ubiquitinated target protein and mediate polyubiquitination (figure 1.6).

Since ubiquitination has several possible consequences, the manner in which a covalent ubiquitin signal is interpreted must depend in some cases on additional factors, such as the subcellular localization of the substrate or the number and topology of the substrate-conjugated ubiquitins. Substrates destinated to proteasomes are usually conjugated to a polyubiquitin chain in which successive ubiquitins are linked by K48-G76 isopeptide bonds (Chau et al., 1989), whereas chains that are linked through K63-G76 bonds have been strongly implicated in non-proteolytic signals (Wang et al., 2001).

1.4.2 E3 ubiquitin ligases

The recognition of substrates for ubiquitination is governed by the presence and accessibility of primary sequence motifs in the substrate, known as ubiquitination signals, which are recognized by cognate E3s. Thus E3s are the central determinants of specificity in ubiquitination. The organization of the enzymatic conjugating cascade is hierarchical: there is one E1, a significant but limited number of E2s and a much larger number of E3s, each of which recognizes a set of substrates that share one or more ubiquitination signals. While E1 is an essential enzyme, redundancy for E3 ubiquitin ligases has been demonstrated.

The E3 reaction involves at least two distinct steps: E3 binding to the substrate via the ubiquitination signal, and covalent ligation of one or more ubiquitins to the substrate (figure 1.7).

Ubiquitination signals are frequently short regions of primary sequence. The first ubiquitination signal to be identified was the destruction box, found in mitotic cyclins, where the arginine and lysine residues are key determinants of specificity.

The known E3 enzymes are members of two protein families: HECT domain and RING E3s. The catalytic modules of the two families are unrelated in sequence and structure, nonetheless certain E2 are able to bind E3s from both families with the same efficiency.

The properties of the HECT domain where first revealed through studies on the conditional degradation of the p53 tumour suppressor. Biochemical studies showed that the recognition of p53 for virally induced ubiquitination depends on the HPV E6 gene product named E6-AP (E6-associated protein) that functions as a specific p53-specific E3 (Scheffner et al., 1993). The HECT domain (homologous to E6-AP carboxy terminus) is strictly conserved among different E3

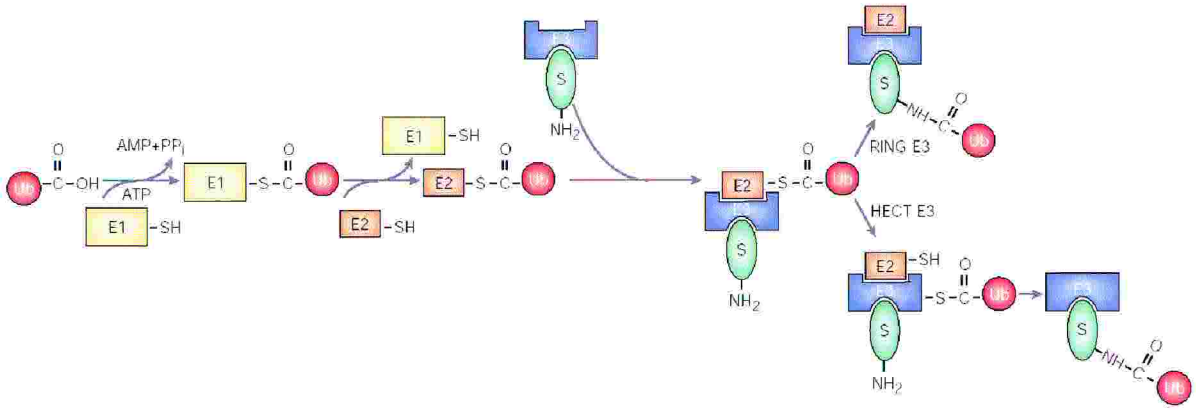

Figure 1.6
The ubiquitin pathway (from Weissman Nat. Rev. Mol. Cell. Biol. 2001).

Free ubiquitin is activated in ATP-dependent manner with the formation of a thiol-ester linkage between E1 and the C-terminus of ubiquitin. Ubiquitin is transferred to one of a number of different E2s, E2s associate with E3s, which might or might not have substrate already bound. For HECT domain E3s, ubiquitin is next transferred to the active-site cysteine of the HCT domain followed by transfer to substrate (S) as shown or to a substrate-bound multi ubiquitin chain. For RING E3s, current evidence indicates that ubiquitin might be transferred directly from the E2 to the substrate.

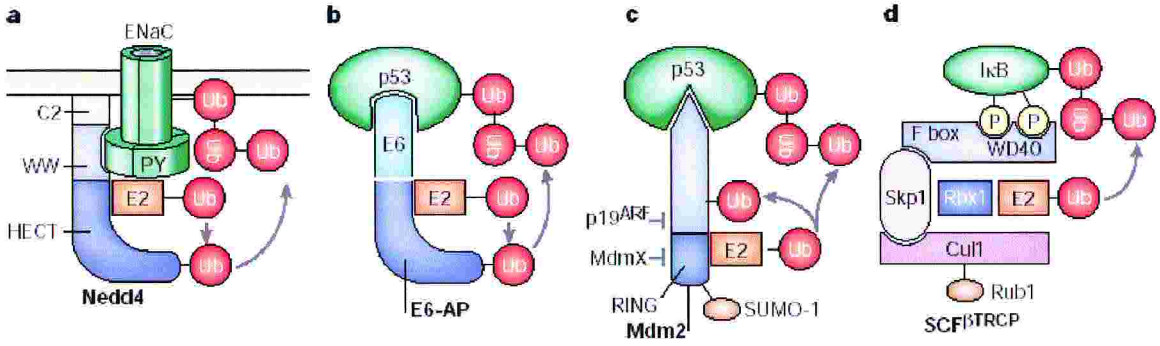

Figure 1.7
Representative E3-substrate interactions (from Weissman Nat. Rev. Mol. Cell. Biol. 2001).

A. Association of Nedd4 with the epithelial sodium channel at plasma membrane. **B.** Ternary complex of p53 with human papillomavirus E6 and E6-AP. **C.** MDM2 and p53 are substrates for modification by MDM2, p53 is substrate for ubiquitination both by MDM2 and E6-AP. **D.** $SCF^{\beta TRCP}$ as a prototypical cullin-containing E3.

ligases and compelling evidence indicates that HECT domain E3s uses a similar mechanism of covalent catalysis.

Until now, every known E3 without a HECT domain has a RING finger protein as one of its constituents. Although it is not known whether all RING finger protein are E3s, it is clear that there is al large family of RING E3s.

The RING finger motif (Really Interesting New Gene) displays a series of histidine and cysteine residues with a characteristic spacing that allows for the coordination of two zinc ions in a cross-brace structure. RING E3s come in two varieties: the most recently discovered one consists just in the RING finger protein. In a different type of RING E3s, the RING finger protein is one subunit of a multiprotein complex.

Three types of multisubunit E3s have been described in which a small RING finger protein is an essential component. The core components of these E3s are Skp1 and Cdc53, which assemble with different F-box protein into distinct protein complexes. The F-box protein subunits display selectivity in the recognition of potential ubiquitination targets. These units have been designed as SCFs protein ligase complexes (Skp1-cullin-F-box) (Feldman et al., 1997; Skowyra et al., 1997). Structural and functional homologs of Skp1 exist in evolutionary diverse organisms (Bai et al., 1996), likewise Cdc53 is a member of a large evolutionary conserved multigene family, referred to as the cullins (Kipreos et al., 1996). Finally the F-box, which is thought to mediate substrate binding to Skp1, in a conserved domain that is found in a large number of related family members may undergo combinatorial interactions to generate a large number of SCFs, each potentially with a unique cellular function (figure 1.8).

The $p19^{SKP1}$-CUL1-$p45^{SKP2}$ complex serves as E3 ubiquitin ligase in human cells recruiting specific substrates for ubiquitination to Cdc34/Ubc5 E2s. Interestingly, whereas $p19^{SKP1}$, CUL1 and Cdc34 are constant throughout the cell cycle (Lisztwan et al., 1998), the expression of $p45^{SKP2}$ is cell-cycle regulated and peaks in S phase (Marti et al., 1999). Genetic and biochemical data have implicated SCF complexes in a wide range of ubiquitination pathways, with a common feature being a requirement for substrate phosphorylation. This reflects the fact that many F-box proteins interact with substrates in a phosphorylation-dependent manner. Thus the timing of protein ubiquitination and destruction is controlled at the level of the interaction of the substrate with its specific SCF ubiquitin ligase, through the F-box component of this ligase complex.

At present, there is no evidence that association of E2F-1 with SKP2 requires phosphorylation of E2F-1, unlike other interactions between substrates and F-box proteins. Instead, the timing of

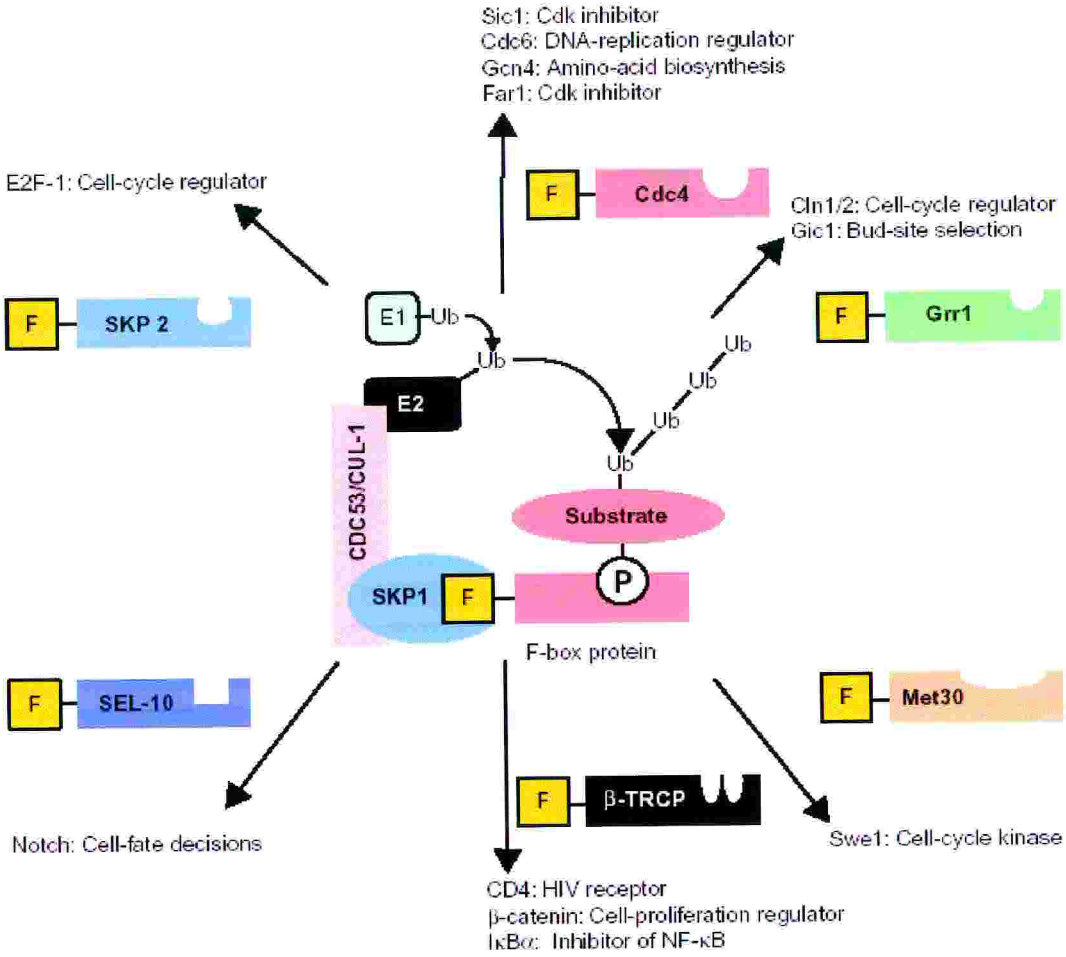

Figure 1.8
(from Harper Nat. Cell Biol. 1999)

F-box proteins are interchangeable adaptors that link a core ubiquitin ligase (SKP1-CDC53/CUL-1) with a wide array of substrates. The combination of SKP1and the F-box protein produces an SCF-type E3 complex. Shown in the center of the figure. Substrates destined to be degraded by the 26S proteasome are tagged with poyubiquitin chains by the action of E1, E2 and E3 enzymes. F-box proteins link the substrate to be labeled with polyubiquitin. These linkages frequently require that the substrate be phosphorylated. Around the periphery are shown many F-box proteins (marked with yellow boxes) and their substrates that have been so far identified. The F-box proteins all interact with SKP1 through their F-box and with one or more substrates, such as E2F-1 or Sic1, through other protein interacting motifs. In SKP2 and Grr1, C-terminal leucine -rich repeats interact with substrates, whereas CdC4, Met30, β-TrCP and Sel-10 contain WD40 repeats. Substrates of the SCF are often critical regulatory proteins whose level need to be altered rapidly for proper cellular function.

E2F-1 ubiquitination seems to depend on the timing of expression of SKP2 (Harper and Elledge, 1999; Marti et al., 1999).

F-box proteins are a versatile set of tools that can be used by the cell to perform its many tasks. A theme that has emerged is that F-box proteins can target multiple proteins that frequently bear no obvious relationship to one another.

1.4.3 Ubiquitination and degradation

Protein degradation plays an important role in a wide array of cellular processes. Eukaryotic cells have evolved two machineries to execute many of the controlled proteolytic events, the ubiquitination machinery and the proteasome. The ubiquitination machinery recognizes and tags specific proteins that are to be destroyed, whereas the proteasome degrades the ubiquitinated substrates. The collaborative action of these machineries is crucial for a variety of diverse processes including, development (DiAntonio et al., 2001; Hay et al., 1999; Karin and Ben-Neriah, 2000), apoptosis, signal transduction (Deng et al., 2000; Wang et al., 2001) and antigen presentation.

The role of ubiquitin in cell cycle progression is explained by the periodic ubiquitination of positive and negative regulators, leading to the appropriately timed degradation of these factors by proteasomes. Modulating the abundance of proteins such as cyclins is one means by which cells control their proliferation. Recent and ongoing work indicated that many important regulators of G1- and S-phases are targeted for ubiquitination and subsequent degradation by the 26S proteasome. The proteolysis of key proteins during these phases appears to be central for proper regulation of DNA replication ($p21^{Cip1}$, $p27^{Kip1}$, geminin, cyclin A, $p57^{Kip2}$, and $p19^{INK4d}$) and the maintenance of cellular homeostasis (E2F, cyclinE, cyclinD and Cdc6) (Yew, 2001). The inability of a cell to enter S-phase may ultimately lead to cell death or improper differentiation, while the disruption of normal cellular homeostasis and timely progression of cell cycle may eventually result in cellular transformation and tumorigenesis. Thus, not surprisingly, mutations in the genes encoding components of ubiquitin machinery may predispose individuals to cancer (Ciechanover et al., 2000b; Sakamoto, 2002).

The identification of a protein for ubiquitination involves a genetically encoded ubiquitination signal and/or a prior modification such as phosphorylation, or damage to the protein (Laney and Hochstrasser, 1999). Thus ubiquitination can best be thought of as a signal for localization of the protein to the proteasome and the regulation of the ubiquitination state of particular protein will

influence the half-life of that protein. An important unresolved question is how the ubiquitination machinery is functionally connected to the proteasome. Recent functional data suggest a role for hPLIC proteins in linking the ubiquitination machinery to the proteasome in vivo for the degradation of several proteins known to be ubiquitin-dependent substrates of the proteasome (Kleijnen et al., 2000). In addition other recent results provide genetic evidence that p300 plays a pivotal role in the regulation of MDM2-mediated p53 turnover by integrating the cellular ubiquitination and proteolytic processes (Zhu et al., 2001), as further discussed in the Discussion session.

The conjugation of ubiquitin (and ubiquitin-like) proteins is a reversible process; ubiquitination and deubiquitination pathways are combinatorial, and selectivity of proteolysis strongly depends on the exact combination of ubiquitinating and deubiquitinating enzymes present at any time. In addition to temporal control, it is likely that these modifications are also spatially regulated.

There are two classes of specific proteases acting on polymeric ubiquitin and cleaving at the C-terminal glycine of ubiquitin. These have been called deubiquitinating enzymes (DUB), and consist of the ubiquitin C-terminal Hydrolases (UCH) ad the ubiquitin specific processing proteases (UBP) (Wilkinson, 2000). In general the UCH isozymes are thought to be involved in processing ubiquitin-fusion proteins and are more active on ubiquitin with preference for substrates in which ubiquitins are fused to small peptides. UBP, on the other hand are generally larger and are thought to be responsable for removing ubiquitin from larger proteins and disassembling the polyubiquitin chains. At the proteasome, DUBs cleave multi-ubiquitin chains from residual peptides and shorten protein-bound multiubiquitin chains by removing the terminal ubiquityl group ensuring that highly ubiquitinated proteins remain associated to the proteasome. Another important function of DUBs is to prevent the accumulation of residual multi-ubiquitin chains at proteasomes and to be constitutively active in the removal of ubiquitin from substrates, as inhibition of proteasome function causes the accumulation of mostly non-ubiquitinated proteins (Weissman, 2001).

1.4.4 Regulation by ubiquitination

The way in which ubiquitin is linked to proteins has the potential to alter their fate. A single protein can be modified on one or more lysines with a single ubiquitin (monoubiquitination) (Hicke, 2001), with lysine-linked chains of ubiquitin (multi-ubiquitination) (Pickart, 2001b) or combinations of the two.

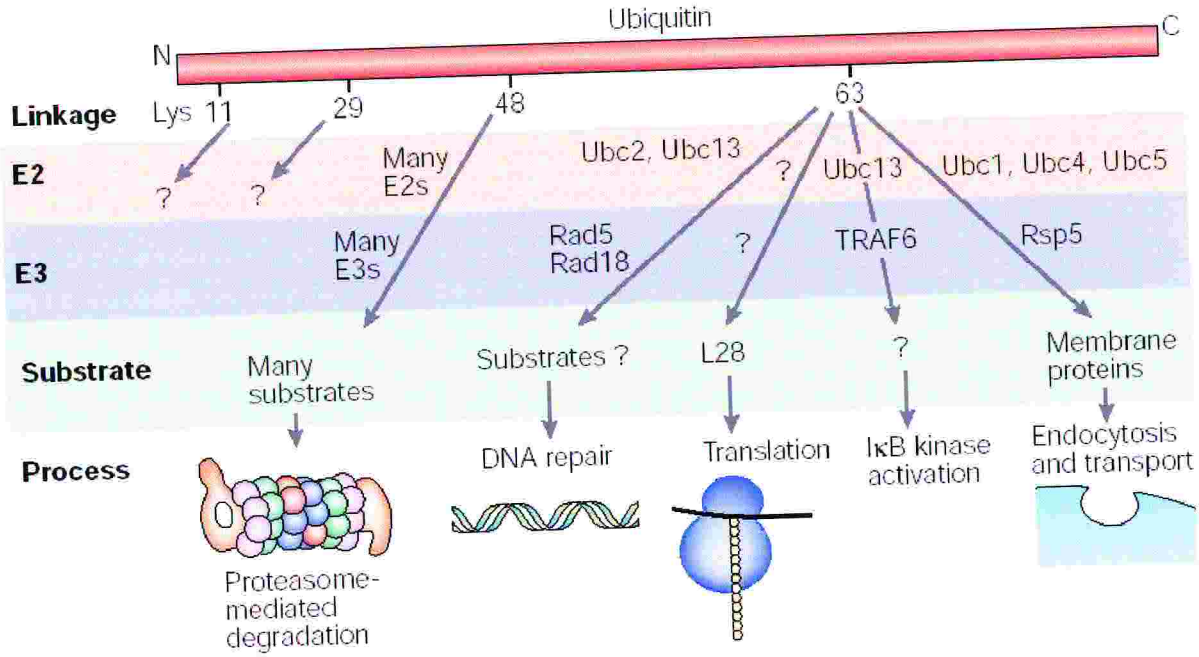

Figure 1.9
Different functions for different ubiquitin ligases (from Weissman Nat. Rev. Mol. Cell. Biol. 2001).

Ubiquitin has several lysine residues and in vivo, can form multi-ubiquitin chains linked through positions 11, 29, 48 and 63. The functions of Lys11- and Lys29-linked chains are unknown. Lys48-linked chains target proteins to the proteasome but might have other functions, and Lys63-linked chains have a range of fates.

While the attachment of K49-linked polyubiquitination chains targets proteins for degradation, polyubiquitin chains can also be synthesized with linkages between the C-terminus of ubiquitin and K6, K11, K29 and K63 on the adjacent ubiquitin. The precise role of these linkages is not known, although there is genetic evidence that K29-linkages are necessary for degradation of ubiquitin-fusion proteins and K63-linkages are vital in regulating post-replicational DNA repair (Hofmann and Pickart, 1999; Spence et al., 1995) and ubiquitination of cell surface receptors, but not required for degradation of short-lived proteins (Dubiel and Gordon, 1999). A recent and surprising observation is that a ribosomal subunit, L28, is a major substrate for modification with K63-linked chains, which enhances translation (Spence et al., 2000) (figure 1.9).

A variety of evidences suggesting that ubiquitin plays a direct role in transcriptional activation has emerged from several recent studies (Hoppe et al., 2000; Hoppe et al., 2001; Kaiser et al., 2000). Ubiquitin can be now considered as a key regulator of eukaryotic RNA synthesis, by mechanisms not only involving ubiquitin-dependent destruction of transcription factors by the proteasome (Blagosklonny, 2001; Desterro et al., 2000; Thomas and Tyers, 2000; Yu et al., 2000), but also involving other aspects of transcription.

A proteolysis-independent function for Cdc34/SCF (Kaiser et al., 2000) clearly indicate that ubiquitination of transcription factors can be utilized to directly regulate their activities. Moreover ubiquitylation not only regulates the same transcription factor by distinct degradation-dependent and -independent mechanisms, but exerts a higher level of regulation also controlling differential recruitment of a single transcription factor to distinct promoters, thereby diversifying transcriptional activator specificity (Kuras et al., 2002).

Based on the model of Salghetti et al. (Salghetti et al., 2001), binding of some transcription factors to promoters triggers their ubiquitination, which simultaneously activates them to initiate a round of transcription and tags them for subsequent destruction in a step that attenuates transcription. Although how recruitment of transcription factors to promoters might exactly trigger their ubiquitination is presently not known, several lines of evidence indicate that signals initiating ubiquitination of DNA-bound transcription factors could emanate directly from the Pol II general transcription machinery itself (Conaway et al., 2002).

It is currently unclear whether monoubiquitination of histones represents a marker for degradation. However, the presence of monoubiquitinated histones on chromatin and their low turnover ratio imply that modified histones remain attached to chromatin and that monoubiquitination of histones coincides with structural changes that accompany transcription or other DNA dependent events. It has recently been proposed a pathway leading to gene

regulation through concerted histone modifications on distinct histone tails by demonstrating regulation of H3 methylation and gene silencing by ubiquitination of histone H2B in yeast (Sun and Allis, 2002).

Most proteins have multiple lysine residues; in some cases specific lysines are targets for ubiquitination, whereas in others there is little specificity. Furthermore, there are now evidences that the N-terminal of the protein, rather than lysines, can serve as a ubiquitination site (Breitschopf et al., 1998).

The ubiquitin-proteasome system is responsible for the regulation and turnover of many short-lived proteins both in the cytoplasm and in the nucleus. Degradation can occur via two distinct pathways, an N-terminus dependent pathway and a lysine-dependent pathway. Different pathways are characterized by the site of initial ubiquitination of the protein, the N-terminus or an internal lysine, and can be differentially active in distinct cellular compartments. In the case of MyoD, for instance, the lysine-dependent pathway is the more active pathway within the cytoplasm while in the nucleus the two pathways are both active in protein degradation (Lingbeck et al., 2002).

2. MATERIALS AND METHODS

2.1 Vectors

The following plasmids where used in this study.

The expression vectors pCMVHAE2F-1 (Helin et al., 1993b) pCMVHAE2F-4 (Muller et al., 1997) containing the HA peptide sequence (YPYDVPDYA) from *influenzae* virus, as well as the mutant E2F pCMVE2F-1K(117/120/125)R (Marzio et al., 2000) which contains the three acetylatable lysines converted to arginines, in addition to vectors for *Escherichia coli* recombinant protein purification pGEX-E2F-1K(117/120/K125)R pGEX-E2F-1, have been kindly provided by K. Helin, European Institute of Oncology, Milan.

pCDNA3-p300 (Marzio et al., 1998) was constructed by cloning the cDNA of p300 (obtained from the expression plasmid pCMVβp300 a gift from D.M Livingston, Dana-Faber Cancer Institute, Boston) in pCDNA3 vector (Invitrogen, Carlsbad, CA).

The p300 acetylase-deficient point mutant DY (lysine 1399 converted to tyrosine), derived from a human tumor mutation, fails to induce p53 acetylation (Ito et al., 2001). The pCMVβp300DY-myc vector was generated by site direct mutagenesis and kindly provided by T.P.Yao (Duke University, Durham).

pGEXp300HAT plasmid (a gift from E. Verdin, Department of Medicine, University of California, San Francisco) was used for the production of recombinant p300 fragment, bearing histone acetyltransferase activity in *Escherichia coli*.

pCX-flag-P/CAF (Yang et al., 1996) was a gift from Y.Nakatani (National Institutes of Health, Bethesda, Maryland), provided by T. Kouzarides (Wellcome/CRC Institute and Department of Pathology, University of Cambridge, Cambridge).

The expression vector for tagged ubiquitin pMT107 (Treier et al., 1994) is an octameric ubiquitin precursor protein expressed from the CMV enhancer-promoter and containing a poly-adenylation signal from SV40. Each ubiquitin unit contains at its NH_2-terminus a His_6 tag. The precursor is expressed and efficiently processed by cellular ubiquitin-COOH-terminal hydrolases.

Plasmids pCS2$^+$cyclinE and pCS2$^+$p27 for expression of human cycilinE and p27 were kindly provided by B. Vogelstein (Johns Hopkins University School of Medicine, Baltimore).

pCMVRB expression vector was provided by Kaelin WG Jr. (Dana Faber Cancer Institute, Harvard Medical School, Boston).

Plasmid pHisRep68, a derivative of pET-16b (Novagen, Milwaukee, Wis.) used for the expression of N-terminally His-tagged Rep68, was obtained from M. Linden (Mt. Sinai School of Medicine, New York). pcDNA3-Rep68 and pcDNA3-Rep68N and pcDNA3-Rep68C were obtained by subcloning of Rep68 from phis-Rep68 into pcDNA3.

Expression plasmid for MDM2 protein pCMVMDM2 was a gift from C. Kühne and pCMVp53 was kindly provided by G.Del Sal.

2.2 Cell culture and transfection

Hek 293, 239T, U2OS, SAOS-2, HeLa and T98G Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) with glutamax (Life Technologies, Inc.) supplemented with 10% fetal bovine serum (Life Technologies, Inc.) and gentamicin (100 µg/ml) at 37 °C in a humidified 93% air, 7% CO_2 incubator. Transfections were performed by the standard calcium phosphate co-precipitation procedure or by effectene^{TM} (Qiagen) according to the instructions of the manufacturer.

Proteasome inhibition was achieved by 18 hours treatment with MG132 (1µM) and deacetylation inhibition by trichostatin A (TSA) (0.3mM). CRM1-mediated nuclear export inhibition was performed by a 18 hours Leptomicyn B (0.2nM) treatment.

2.3 Antibodies

For the production of polyclonal anti acetylated E2F-1, two rabbits were immunized with E2F-1 (114-128) peptide acetylated at positions 117,120 and 125 (produced in Protein Structure and Function laboratory in ICGEB, Trieste) after conjugation to keyhole limpet hemocyanin (Sigma) sera were collected, as well as the pre immune serum. For western blotting experiments, the antibodies where further subjected to affinity purification on a column harbouring the acetylated peptide (Immuno Pure® plus (A) igG Purification kit- Pierce).

The two antibodies were tested on recombinant GST-E2F-1 protein and GST-E2F-1 acetylated in vitro as described (Brownell and Allis, 1995) using recombinant p300HAT.

Mouse monoclonal E2F-1 (KH-95), rabbit polyclonal E2F-1 (C-20), tubulin (H -300), mouse monoclonal p53 (DO-1) and p27 (C-19) antibodies were provided by Santa Cruz Biotechnology (Santa Cruz, CA.), rat monoclonal HA high affinity (3F10) was purchased by Roche Diagnostics and the antibody anti-CyclinE ab-5 (CYE05) by NeoMarkers Inc. (Lab Vision corporation).

2.4 Immunoprecipitation and immunoblotting

Cleared cell lysates from transfected cells after sonication were incubated with the proper antibody overnight at 4°C. After incubation, 40 µl of a 50% suspension of protein-A-Sepharose CL-4B beads (Pharmacia) in RIPA 150 lysis buffer (50 mM Tris-HCl, pH 7.5; 150 mM NaCl; 1% NP-40; 1% deoxycholate; 0.1% SDS; 2mM EDTA) was added. After a 2-hr incubation at 4°C, beads were washed four times with 1 ml of RIPA buffer 150. Samples then were analysed by SDS page and Western blotting on a nitrocellulose membrane (Optitran Schleicher & Shuell). The antigen-antibody complexes were visualized using appropriate HRP-conjugated secondary antibodies (DAKO) and the enhanced chemiluminescence ECL detection system as recommended by the manufacturer (Amersham).

2.5 Recombinant proteins and GST pull down

Recombinant proteins including GST and GST-E2F-1, GST-E2F-1K(117/120/125)R GST-p300HAT (Ott et al., 1999) were expressed in bacteria and purified using glutathione-agarose affinity chromatography as previously described (Marzio et al., 1998). The TNT-coupled transcription-translation Reticulocyte Lysate System (Promega, Madison, WI) was used to produce [^{35}S] methionine-labelled proteins that were used in the pulldown assays. GST fusion proteins immobilized on agarose beads were washed and resuspended in NETN buffer (20 mM Tris-HCl, pH 7.5; 100 mM NaCl, 1 mM EDTA; 0.5% NP-40, 1 mM DTT, 1 mM phenilmethylsulphonyl fluoride) supplemented with 0.2 mg/ml ethidium bromide to impede the possible formation of aspecific interaction between residual DNA and proteins. [^{35}S] methionine-labelled proteins were added and incubated at 4°C on a rotating wheel. After 1 hour bound proteins were washed 5 times with 1 ml of NETN buffer. The GST-fusion proteins were analysed on 10% SDS-polyacrylamide gels for integrity and to normalize the amount of each protein. Dried gels were quantitated by Instant Imager (Packard Meriden, CT).

2.6 In vivo ubiquitination assay

Cells were cotransfected with His-tagged ubiquitin and different plasmids expressing target proteins for ubiquitination as indicated in the figure legends. Transfections were adjusted to the same content of transfected plasmid by addition of pCDNA3. Twenty-four hours after transfection cells were harvested and purification of His-ubiquitin-conjugates was performed as described in (Treier et al., 1994) and analysed by western blotting with antibodies anti-target proteins (figure 2.1).

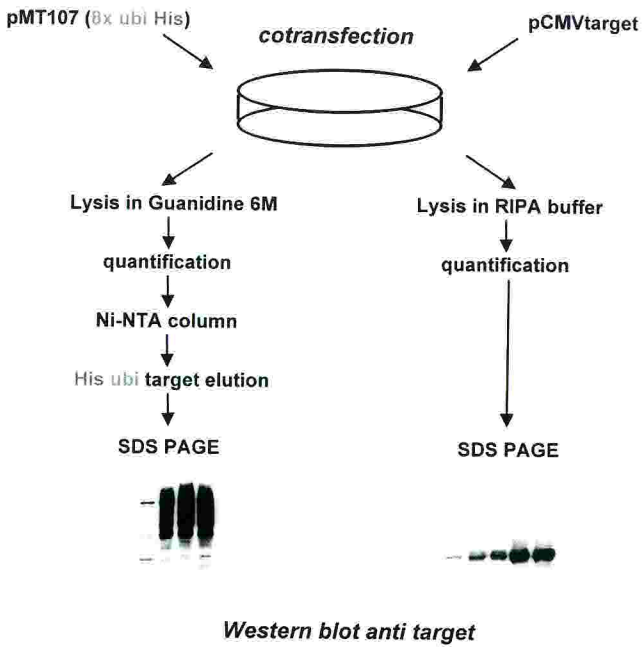

Figure 2.1 *In vivo* ubiquitination procedure

For purification of ubiquitin-protein conjugates: cells were lysed in 1.5 ml of 6M guanidinium-HCl, 0.1M $NaHPO_4/NaH_2PO_4$ (pH 8.0) plus 10mM imidazole per 100mm dish. The lysate was sonicated to reduce viscosity and mixed on a rotor with 0.2 ml (settled volume) of Ni^{2+}-NTA agarose (Qiagen) for 4 hours at 4 °C. The slurry was applied to a Bio-Rad Econo-Column. The column was successively washed with the following: 1ml of 6M guanidinium-HCl 0.1M $NaHPO_4/NaH_2PO_4$ (pH 8.0); 2ml of 6M guanidinium-HCl 0.1M $NaHPO_4/NaH_2PO_4$ (pH 5.8); 1ml of 6M guanidinium-HCl 0.1M $NaHPO_4/NaH_2PO_4$ (pH 8.0); 2ml of (6M guanidinium-HCl 0.1M $NaHPO_4/NaH_2PO_4$ [pH 8.0] protein buffer) 1:1; 2ml of (6M guanidinium-HCl 0.1M $NaHPO_4/NaH_2PO_4$ [pH 8.0] protein buffer) 1:3; 2ml of protein buffer; 2ml of protein buffer, 1ml of protein buffer plus 10mM imidazole. Elution was performed in 1ml protein buffer plus 500mM imidazole. Protein buffer is 50mM $NaHPO_4/NaH_2PO_4$ (pH 8.0), 100 mM KCl, 20% glycerol, and 0.2% NP-40. The eluate was TCA precipitated for further analysis.

Crude cell lysate was also performed in RIPA 150 buffer and defined amounts of lysate (standardized according to Bradford detection) were collected and boiled in Laemli sample buffer before being subjected to western blot analysis using the same antibodies.

2.7 Determination of protein half-life

293 cells were transfected using reagents and methods described above. 12 hours after transfection, cells where incubated for 1 hr in methionine-free DMEM medium and for an

additional hour with [^{35}S]-labelled methionine. Cells where then washed and incubated in DMEM supplemented with 300mg/ml methionine for the indicated time periods. The immunoprecipitated samples were separated by SDS-PAGE. The gels were dried and analysed by phosphoimage analysis.

2.8 Immunofluorescence staining and microscopy

Cells were transiently transfected by effectene^{TM} (Qiagen) transfection reagent or by the calcium phosphate method and observed for indirect immunofluorescence after 40h. Following paraformaldehyde fixation, cells were washed with 100 mM glycine and permeabilized with 0.1% Triton X-100 for 5 min. Primary antibodies against HA or E2F-1 (1/100) were incubated at 37 °C for 1 h in a humidified chamber in phosphate-buffered saline additioned with 1% bovine serum albumin and 0.1% Tween 20. FITC- or TRITC-conjugated secondary antibodies (Sigma) were diluted at 1/50 and incubated as described above. Nuclei were counterstained with 1 mg/ml Hoechst 33258 (Sigma). Slides were mounted in Vectashield (Vector) and observed with a Zeiss Axiovert X60 confocal microscope. Images were acquired with the LSM510 software.

3. RESULTS

3.1 Acetylation of E2F-1

The six members of the E2F family of transcription factors play a key role in the control of cell cycle progression by regulating the expression of genes involved in DNA replication and cell proliferation. E2F-1, -2, and -3 belong to a structural and functional subfamily distinct from those of the other E2F family members; this partition is also corroborated by their pattern of expression during the cell cycle and by their nuclear localization. In particular, E2F-1, -2, and -3, but not E2F-4, -5, and -6, associate with and are acetylated in vitro by p300 and CBP protein acetyl-transferases (Marzio et al., 2000) and by the p300/CBP-associated factor P/CAF (Martinez-Balbas et al., 2000).

The acetylation sites lie adjacent to the E2F-1 DNA-binding domain located in the N-terminal portion of the protein and involve lysine residues at positions 117, 120 and 125, which are highly conserved in E2F-1, 2 and 3. Acetylation of E2F-1 by p300/CBP in vitro and in vivo markedly increases its binding affinity for a consensus E2F DNA-binding site, which is paralleled by enhanced transactivation of an E2F-responsive promoter. Acetylation by P/CAF has three functional consequences on E2F-1 activity: increased DNA-binding ability, activation potential and protein half-life. These results suggest that acetylation stimulates the functions of the non-RB bound 'free' form of E2F-1. It has been also reported that the RB-associated histone deacetylase-1 can deacetylate E2F-1 indicating that reversible acetylation is a mechanism for regulation also of non-histone proteins.

3.1.1 Production of anti-acetylated E2F-1 specific antibodies

In order to further verify the *in vivo* acetylation of E2F-1, we raises specific rabbit polyclonal antibodies for acetylated E2F-1.

In collaboration with the Protein Structure and Function laboratory in ICGEB (Trieste), we obtained two 15-amino acid peptides corresponding to E2F-1 (114-128), either acetylated or not acetylated at positions 117, 120 and 125 (Figure 3.1 panel A). After coupling to KHL (Keyhole Limpet Hemocyanin) and rabbit immunization, sera were collected and the specificity of the antibodies was tested by western blotting against increasing amounts of both synthetic peptides as substrates in a dot-blot experiments. As shown in Figure 3.1 panel B, the serum raised against the acetylated E2F-1 peptide showed markedly increased recognition specificity for the

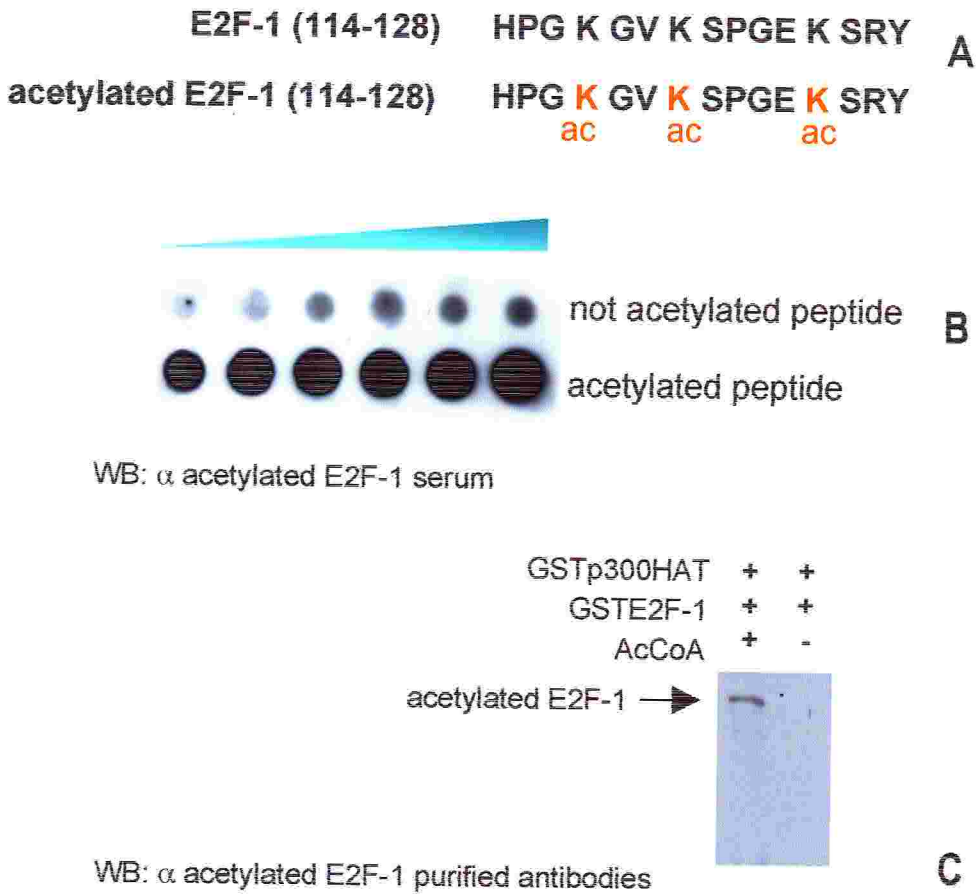

Figure 3.1
Production of anti-acetylated E2F-1 antibodies

A. Synthetic acetylated and not acetylated E2F-1 (114-128) peptides containing lysines 117, 120 and 125 used for rabbit immunization.
B. The specificity of sera from injected rabbits for the acetylated peptide was tested by dot blot: increasing amounts of acetylated and non-acetylated peptide were blotted with serum form rabbits immunized with acetylated peptide showing specificity for the acetylated form.
C. Purified anti-acetylated E2F-1 antibodies tested against *in vitro* acetylated recombinant protein showing the specificity only for the acetylated form of E2F-1. The experiment was performed by incubating, referring to the first lane, recombinant GST-E2F-1 fusion protein, GST-p300(HAT) fusion protein and acetylCoA, and without acetylCoA in the second lane.

acetylated peptide as compared to non acetylated one. Subsequently, antisera were affinity-purified on columns bearing the corresponding (acetylated or not acetylated) peptide.

Purified anti-acetylated E2F-1 antibodies were then tested by western blotting against an *in vitro* acetylated recombinant E2F-1. The experiment was performed by incubating a recombinant GST-E2F-1 fusion protein with a recombinant protein encompassing the HAT domain of p300 (GST-p300HAT), in the presence or absence of acetyl-CoA. In these conditions, E2F-1 gets readily acetylated (Marzio et al., 2000). As shown in Figure 3.1 panel C, the antibody raised against the acetylated E2F-1 peptide recognized recombinant E2F-1 only if incubated with p300HAT and acetyl-CoA.

3.1.2 Detection of *in vivo* acetylated E2F-1

The antibody raised against acetylated E2F-1 was then used to detect *in vivo* acetylated E2F-1. For this purpose, we transfected either 293 or 293T cells with E2F-1 and P/CAF, followed by immunoprecipitation of E2F-1 using the antibody raised against the acetylated peptide and by visualization of immunoprecipitated E2F-1 by western blotting with the anti-E2F-1 monoclonal antibody (KH-95). To increase the amount of acetylated E2F-1, cells were treated with trichostatin A (TSA), an inhibitor of de-acetylates. The results of these experiments are shown in Figure 3.2. Acetylated E2F-1 was readily detected in both 293 and 293T cells transfected with P/CAF and exogenous E2F-1, and treated with TSA (lanes 5 and 10). In 293 cells, omission of any of these three treatments resulted in negative results. In 293T cells (which express the SV40 T-antigen, which amplifies transfected plasmid copy number), acetylated transfected E2F-1 was also detected without overexpression of P/CAF and treatment with TSA (lane 3). In both 293 and 293T cells, however, we always failed to visualize the endogenous acetylated protein.

Analogous results have been also obtained using p300 instead of P/CAF or by using the anti-E2F-1 antibody (sc KH-95) for immunoprecipitation followed by visualization with the antibody against acetylated E2F-1 (data not shown).

These data clearly indicate that E2F-1 can be found as an acetylated protein also inside the cells. By taking advantage of the developed antibody, another member of the laboratory is currently investigating whether E2F-1 acetylation might occur in a specific phase of the cell cycle.

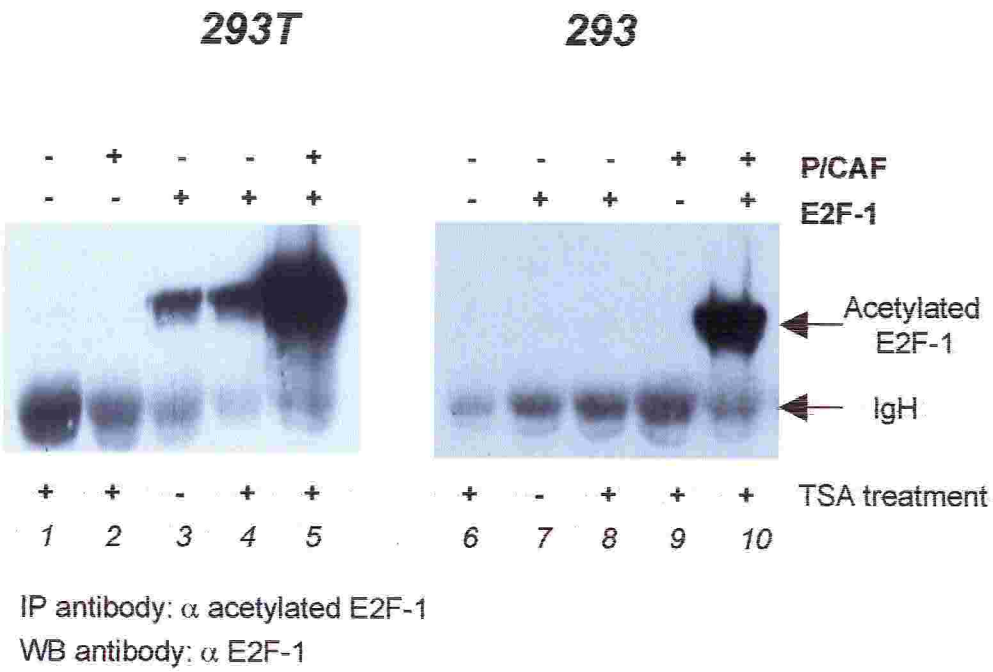

Figure 3.2
Acetylated E2F-1 *in vivo*

Whole cell lysates prepared from 293T and 293 cells either transfected with E2F-1 (lanes 3,4,5,7,8, and 10) or not (lanes 1,2,6,and 9) and with P/CAF (lanes 2,5,9 and 10), treated or untreated (lanes 3 and 7) with trychostatin A (TSA) were immunoprecipitated using the specific rabbit polyclonal antibody we raised against acetylated E2F-1, and immunoprecipitates were analyzed by SDS PAGE and western blot using a mouse monoclonal anti-E2F-1 (KH95). The position of acetylated E2F-1 and igH (immunoglobulin heavy chain) are indicated.

3.2 Ubiquitination of E2F-1

Several aspects of eukaryotic cell-cycle regulation rely on the selective and temporally controlled elimination of key regulatory proteins through the ubiquitin/proteasome pathway. Among these proteins, activity of E2F-1 must be tightly regulated in a cell-cycle-dependent manner to enable gene expression programs to be closely coupled with cell-cycle progression. E2F-1 is known to be ubiquitinated and degraded by the proteasome in a manner dependent upon its dissociation from pRB, p107, or p130 (Campanero and Flemington, 1997; Hateboer et al., 1996; Hoffman et al., 1996).

In vivo, E2F-1 is physiologically ubiquitinated and degraded by the proteasome pathway during the S-to-G2-phase transition through its interaction with $p45^{SKP2}$, which is the cell cycle-regulated component of the ubiquitin protein ligase SCF^{SKP2} (Marti et al., 1999). The timing of E2F-1 degradation implies that it is degraded after it has fulfilled its essential function at the G1-to-S phase transition. Degradation might be important for resetting conditions for the next cell cycle.

In vitro, ubiquitination of E2F-1 is mediated by Ubc5 E2 and by CUL1-ROC1 E3 ligase. In contrast to substrates of the SKP1-Cullin1-F-box (SCF) complexes, E2F-1 is ubiquitinated in vitro without requirement of phosphorylation, moreover it has been demonstrated that multiple ROC-Cullin ligases can specifically catalyze E2F-1 ubiquitination through a novel SKP1-independent mechanism (Ohta and Xiong, 2001).

Acetylation and ubiquitination are both post-translational modifications that affect protein-protein interaction, protein function and protein stability, and both occur on lysines. Several evidences suggest that a functional interplay exists between these two modifications. Among these are the findings that histone acetylase TAFII250 also exhibits an ubiquitin ligase activity (Pham and Sauer, 2000), and that HDACs regulate protein ubiquitination (Seigneurin-Berny et al., 2001).

Therefore we set out to investigate a relationship a correlation might exist between ubiquitination and acetylation of E2F-1 and whether the acetyl-transferases that bind and acetylate the protein might have an influence on the ubiquitination process as well.

3.2.1 P/CAF and p300 increase E2F-1 ubiquitination

For the study of ubiquitination we exploited an *in vivo* ubiquitination assay based on cell co-transfection with a plasmid for the target protein together with a plasmid encoding for his-tagged ubiquitin. After transfection, cell lysates are processed through a nickel column, which binds poly-histidines, and histidin-tagged ubiquitinated target proteins are eluted and detected by western blotting.

This assay was performed by transfecting an E2F-1 expression vector, a plasmid expressing histidin-tagged ubiquitin, and a plasmid expressing either p300 or P/CAF in the relative ratio of 2:1:1.5. The results of these experiments are shown in Figure 3.3. Upon transfection of p300 or P/CAF, E2F-1 ubiquitination resulted impressively increase, with the generation of high molecular weight E2F-ubiquitin conjugates (lanes 4,5,8 and 9).

A similar result was obtained in all the different cell lines we tested, which included HEK 293, 293T, U2OS, HeLa and T98G (figure 3.3) indicating that this event is not cell-type specific.

These results indicate that p300 and P/CAF, either directly or indirectly enhance the extent of E2F-1 ubiquitination *in vivo*, with an effect that is functionally analogous to that of known ubiquitin ligases. To further corroborate this notion, the effects of other known ubiquitin ligases were tested by the same assay. These included SKP2, which is known to be the specific ubiquitin ligase for E2F-1, the E3 ubiquitin ligase MDM2 and another unrelated protein as a negative control: AMF-1/Gps2 (Peng et al., 2000).

In addition to E2F-1 we also examined E2F-4 as a target protein for ubiquitination upon overexpression of the same ubiquitin ligases. The results of these experiments are shown in Figure 3.4a. We confirmed that both p300 and P/CAF strongly augmented E2F-1 poly-ubiquitination, detectable for the presence of a smear in the corresponding lanes, at an extent that is similar to SKP2. In contrast, overexpression of MDM2 mostly promoted E2F-1 monoubiquitination, since high molecular weight E2F1-ubiquitin conjugates are not visible in the corresponding lane as in the other cases.

In contrast to E2F-1, increase of ubiquitination was not detected for E2F-4, which lacks the N-terminal segment of E2F-1 that is involved in SCF^{SKP2}- and p300-binding and that contains the acetylation site. Surprisingly, P/CAF increased ubiquitination of both E2F-1 and E2F-4 (Figure 3.4b) suggesting different mechanisms exhibited by P/CAF and p300 in the regulation of this process.

3.2.2 p300 determines accumulation of E2F-1 ubiquitin-conjugates similar to inhibition of the proteasome

The results shown above indicate that overexpression of p300 and of P/CAF increase the intracellular concentration of ubiquitinated E2F-1. One possibility is that this increased ubiquitination might parallel increased degradation of the protein. Therefore, we decided to visualize the total levels of E2F-1 to whole cell lysates, in addition to selection of its ubiquitinated version. As shown in Figure 3.5, we surprisingly observed that the amount of total E2F-1 was indeed increased in the lysates. This increase, as well as the remarkable accumulation of E2F-1

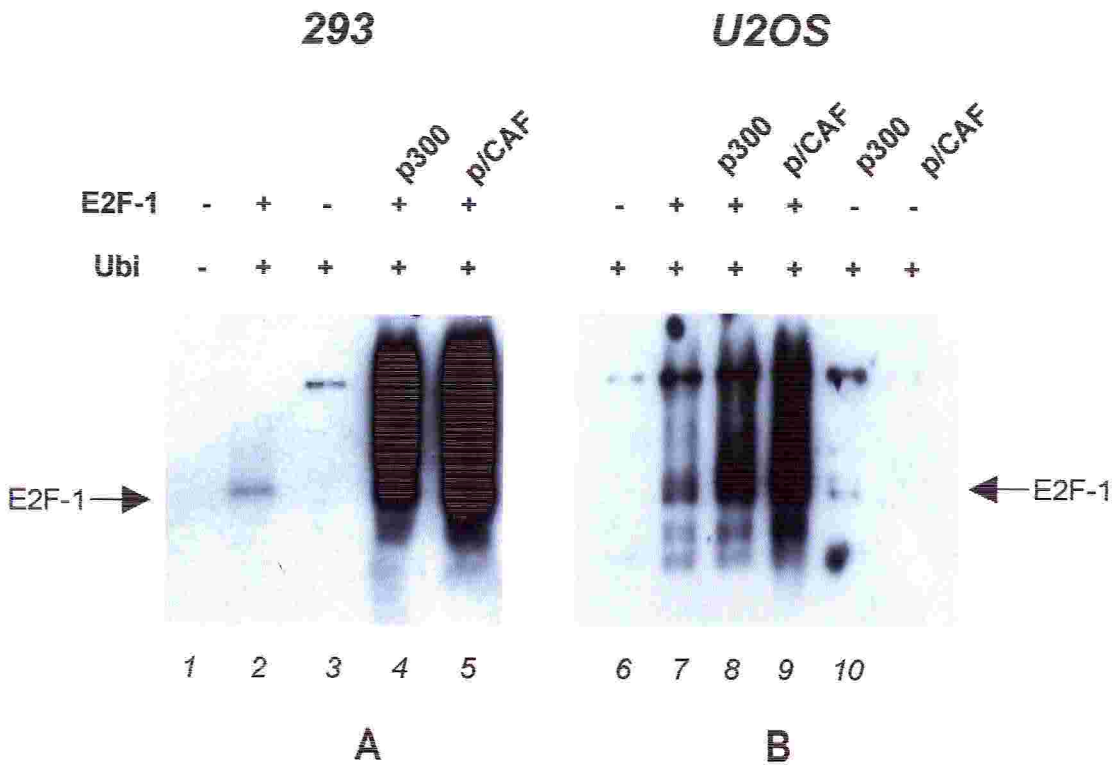

Figure 3.3
P/CAF and p300 increase E2F-1 ubiquitination *in vivo*

E2F-1 is physiologically ubiquitinated and degraded by the proteasome pathway. Here we show that overexpression of p300/CBP and P/CAF determine a marked increase of the E2F-1 ubiquitinated form *in vivo*.
Increase of E2F-1 ubiquitination occurs in multiple cell types, including HEK 293 (**A**), U2OS (**B**), and HeLa cells (not shown).

poly-ubiquitin conjugates, was comparable to that obtained by cell treatment with MG132, an inhibitor of proteasome degradation (shown in lanes 4 and 5). These results raise the unexpected possibility that ubiquitination of E2F-1 by p300 might be a signal leading to increased stability of the protein, instead than increased degradation. In these experiments, no additive effect of p300 and MG132 is detectable. This might suggest that the two effects are neither additive nor cooperative; alternatively – and most plausibly - the experiment was performed at saturating conditions thus impeding to compare small differences in E2F levels.

The N-terminal domain of E2F-1 is not the only element of E2F-1 primary structure that contributes to its degradation. Another such element appears to reside within the C terminus of the transcription factor, close to the RB-binding site, and unphosphorylated pRB, when produced with E2F-1, can protect E2F-1 from degradation (Campanero and Flemington, 1997) (Hateboer et al., 1996) (Hoffman et al., 1996). Such regulation may augment the stability of RB–E2F-1–repressor complexes and may contribute to proper control of exit from G1 phase. Therefore we wished to test whether co-expression of pRB in our system might regulate the extent accumulation of E2F-1 poly-ubiquitin conjugates. As shown in Figure 3.5b, however, no effect could be detected after transfection of pRB, thus ruling out an involvement of this protein in the observed regulation of E2F-1 ubiquitination by p300.

3.2.3 Increase of E2F-1 ubiquitination is specific and depends upon binding to p300

p300 has been shown to specifically bind E2F-1 both *in vitro* and *in vivo* (Marzio et al., 2000). Binding occurs between the N-terminal domain of E2F-1 and both the N-terminal and HAT domains of p300 (R. Mendoza-Maldonado, data not shown). A representative GST pulldown experiment between recombinant E2F-1 and *in vitro* translated p300 is shown in Figure 3.6 panel A. The same panel also shows the effect of incubation of the binding reaction with increasing amounts of the 68 amino acid form of the adeno-associated virus (AAV) Rep protein, which has been shown to disrupt co-activation by p300 (Marcello et al., 2000). In the presence of this protein, binding between p300 and E2F-1 is progressively diminished (from 14% to 4% of input radiolabeled p300). Given this specific effect of Rep, we decided to use this protein as a probe to test whether disruption of E2F-1-p300 interaction inside the cells might result in modification of the effects of p300 on ubiquitination of E2F-1. Figure 3.6 panel B shows the result obtained by the co-transfection of 293 cells with E2F-1 and p300 either in the presence or absence of transfected Rep68. In the absence of Rep, p300 promotes accumulation of ubiquitin-conjugated E2F-1; in its presence, this effect is markedly reduced. The inhibitory activity of Rep on p300 co-activation has been ascribed to the C-terminus of the molecule (Marcello et al.,

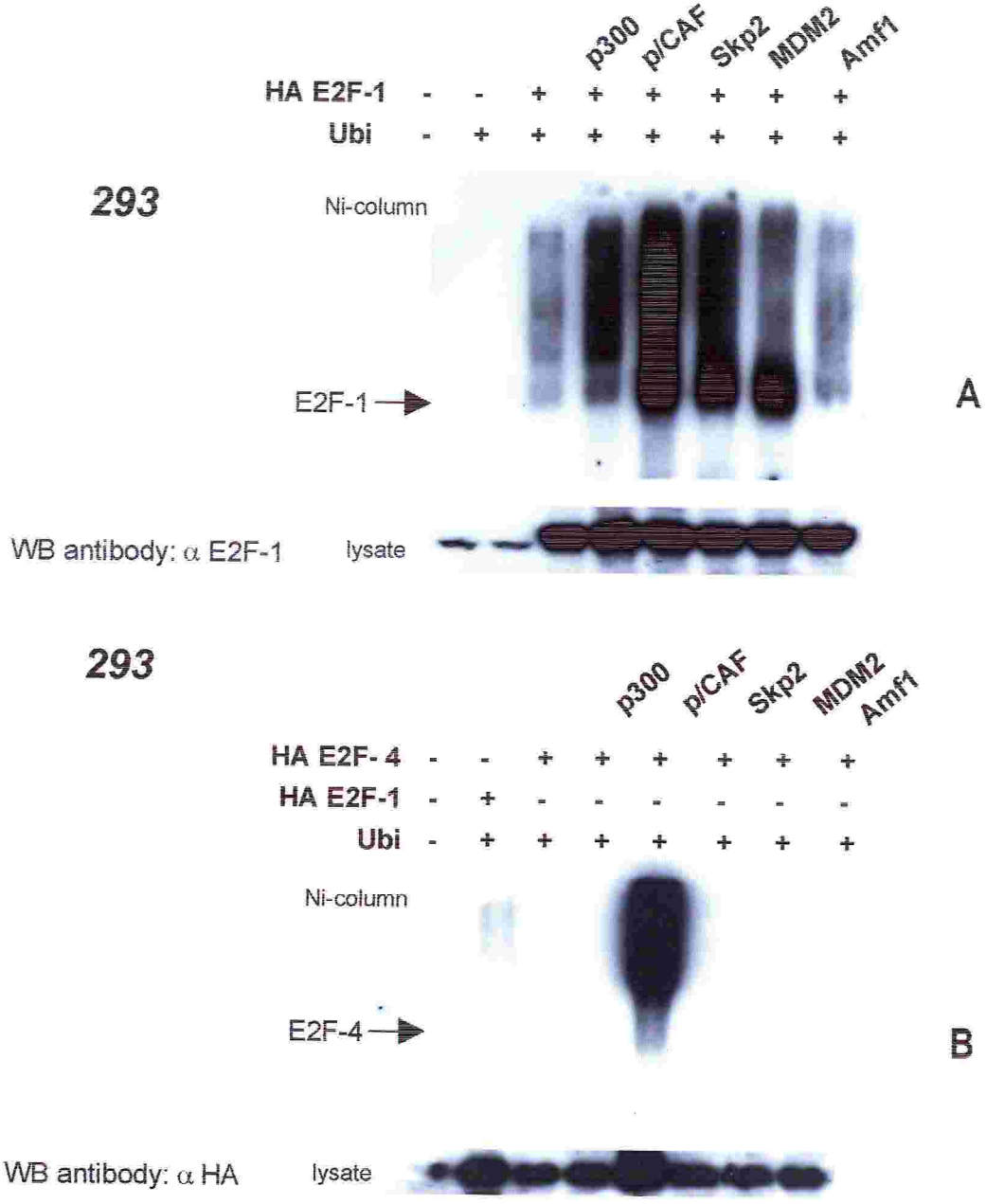

Figure 3.4
p300 increase E2F-1 but not E2F-4 ubiquitination *in vivo*

We compared E2F-1 and E2F-4 as targets for ubiquitination upon overexpression of different ubiquitin ligases, including Skp2, p300, p/CAF and MDM2.
Ubiquitination of E2F-1 was increased after expression of Skp2, p300 and P/CAF; this effect is not present for E2F-4, which lacks the p300-binding domain and is not acetylated by p300. Surprisingly, P/CAF increases ubiquitination of both E2F-1 and E2F-4.

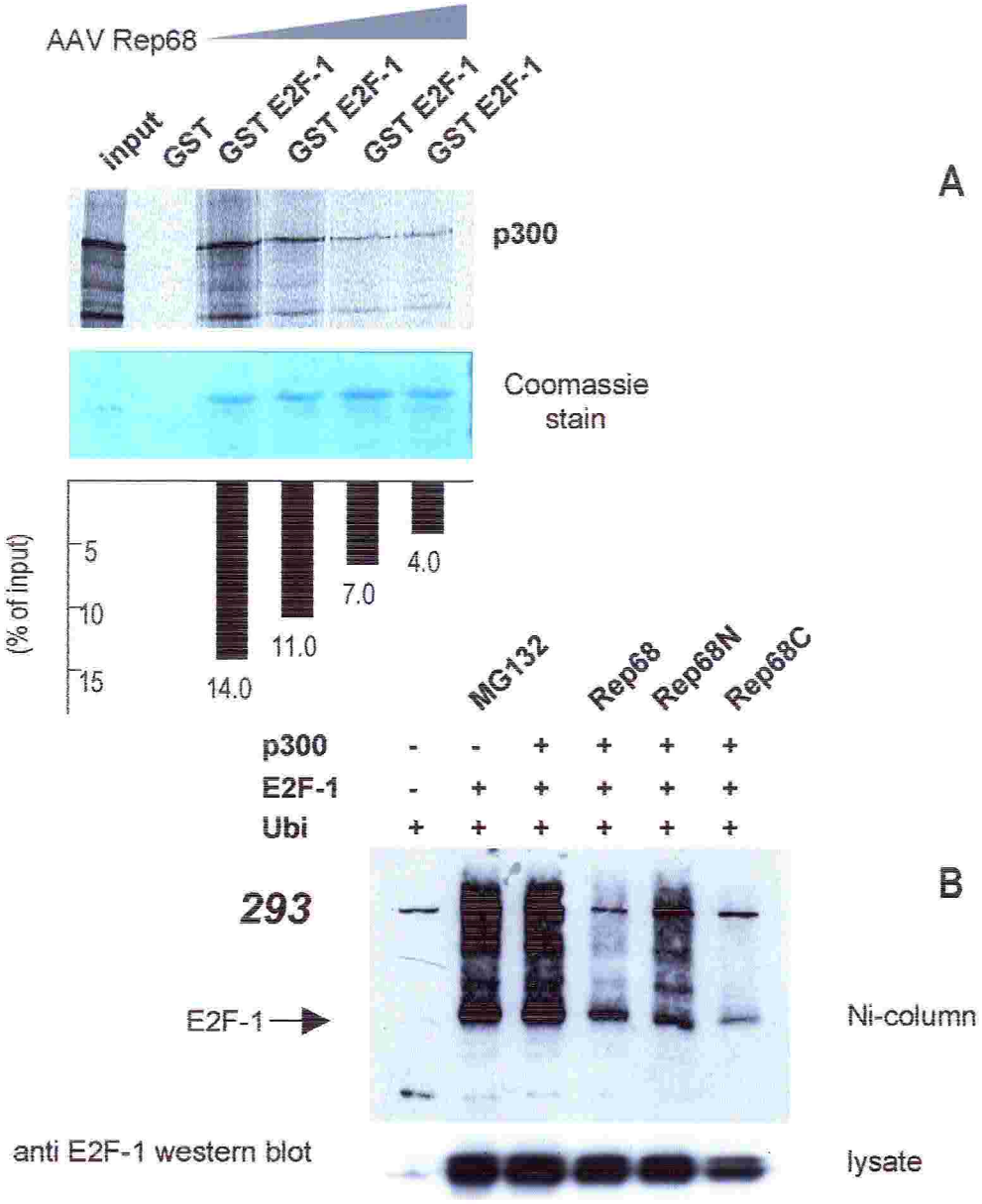

Figure 3.6
p300-mediated E2F-1 ubiquitination is dependent on the direct interaction between the two proteins.

A. E2F-1 binds to p300 in GST-pulldown assay and the interaction is disrupted by increasing amounts of AAV rep68 protein. A coomassie stain to verify that equal amounts of recombinant GST-E2F-1 have been incubated with equal amounts of p300, and a diagram with the rate of E2F/p300 binding are also reported.
B. Disruption of interaction results in decrease of ubiquitination. In *in vivo* ubiquitination assay when AAV rep68 is cotransfected together with E2F1 and p300, ubiquitination is compromised. More precisely C-terminal rep68 appears to play a direct role exhibiting the strongest effect on p300-mediated E2F-1 ubiquitination.

2000). Therefore, we tested the effects on p300-induced E2F-1 ubiquitination of transfection of either the N-terminal or the C-terminal fragments of Rep68. Consistent with the expected results, we found that Rep68C inhibits p300-mediated E2F-1 ubiquitination similar to the full-length protein, while Rep68N had no apparent effect.

Altogether these results suggest that binding of E2F-1 to p300 is required to mediate the effects of the latter protein on E2F-1 ubiquitination.

The above conclusion would predict that p300 expression would have no effect on ubiquitination of E2F-4, an E2F family member that does not bind p300 (Marzio et al., 2000). The results of an *in vivo* ubiquitination assay in which we co-transfected E2F-4 together with p300 are shown in Figure 3.7. These results clearly indicate that, in contrast to E2F-1, E2F-4 ubiquitination is unaffected by p300.

Biochemical studies revealed that ubiquitination of E2F-1 is promoted by binding to SCF^{SKP2}, an F-box protein that is functions as an E3 receptor component of the SCF ubiquitin ligase complex (Harper and Elledge, 1999). The same protein also determines specific ubiquitination and proteasome-mediated degradation of several other cellular targets, including cyclin E and the cyclin kinase inhibitor $p27^{kip1}$ (Nakayama et al., 2000). In order to understand whether the effect of p300 might extend to these other cellular targets, we transfected 293 cells with plasmids expressing histidin-tagged ubiquitin, each one of these targets, and p300 in the molar ratio 2:1:1.5. Ubiquitin-conjugates recovered from total cell lysates at 24 hours after transfection were analyzed by Western blotting using antibodies against cyclin E and p27. As clearly evident in Figure 3.7, and conversely to E2F-1, cyclin E and p27 ubiquitination resulted not affected by p300 overxpression. However, when the same cells were treated with the proteasome inhibitor MG132, marked accumulation of poly-ubiquitinated conjugates and of total protein amount was evident for both proteins, in agreement with the fact that they are physiologically regulated via the proteasome pathway.

These results indicate that the p300-mediated accumulation of ubiquitinated E2F-1 is a novel mechanism for post-translational regulation of E2F-1 that does not extend to other targets of SKP2 ubiquitination. However, having performed all the experiments in a $SKP2^{+/+}$ background, we cannot formally exclude that p300-mediated E2F-1 ubiquitination might still depend on SKP2.

3.2.4 No acetylation, no ubiquitination

It is of considerable interest that both acetylation and ubiquitination are post-translational modifications occurring on lysine residues, which eventually result in different consequences. This consideration might suggest that the two modifications could compete for the same

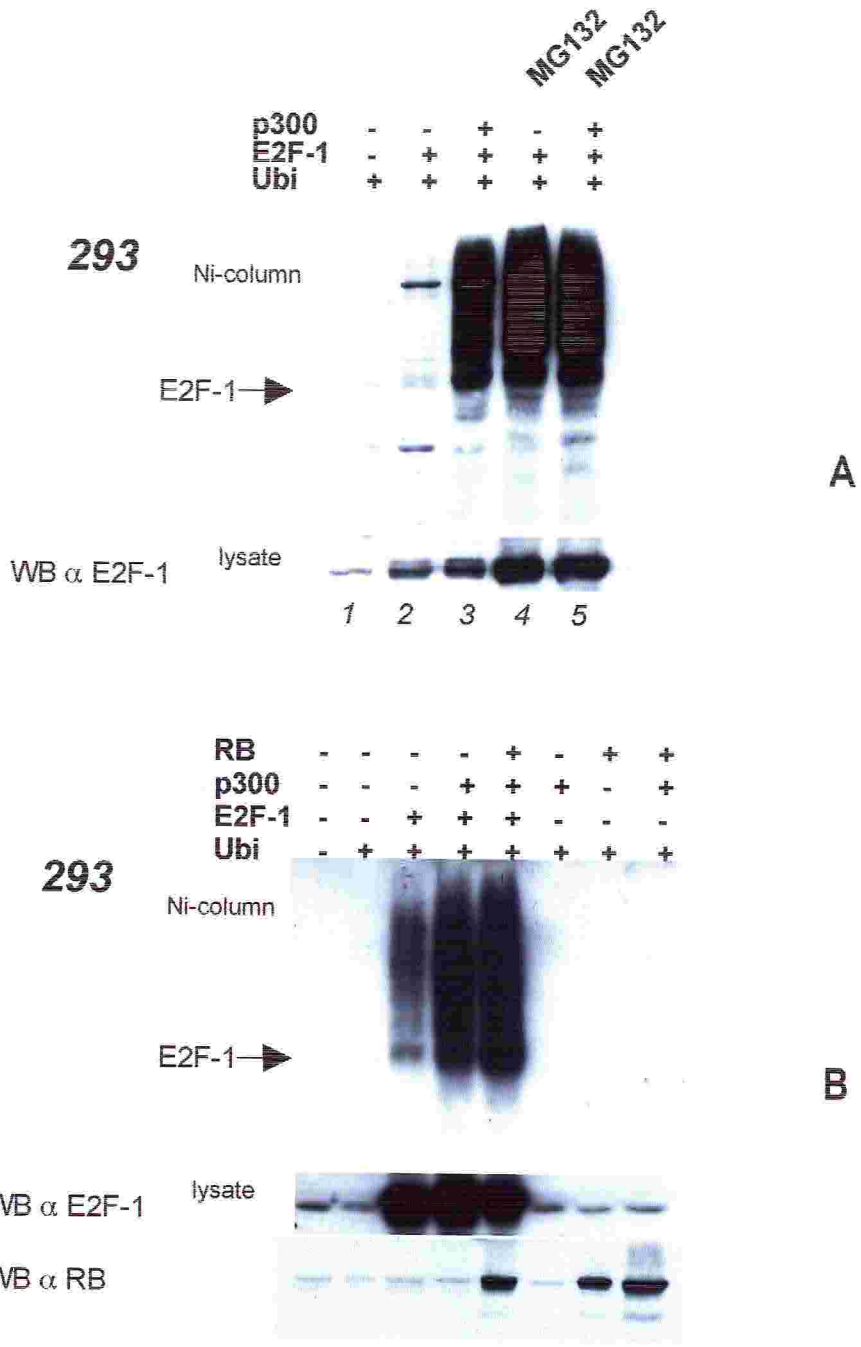

Figure 3.5
p300 stabilizes E2F-1 ubiquitinated isoforms

A. E2F-1 exhibits the same ubiquitination pattern in the presence of the proteasome inhibitor MG132 or upon p300 overexpression suggesting that p300 might have a role in the stabilization of ubiquitinated E2F-1.
B. p300-mediated E2F-1 ubiquitination is not affected by RB overexpression.

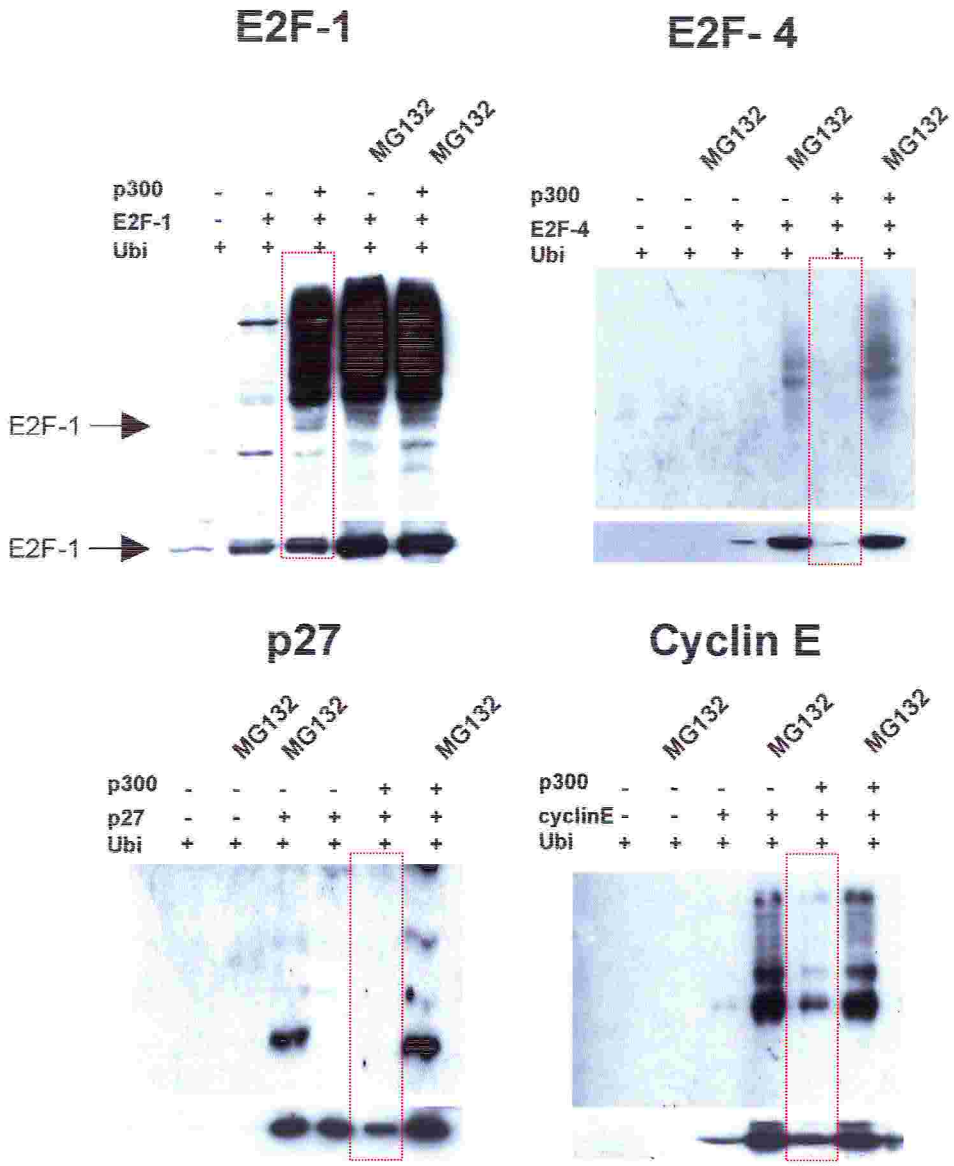

Figure 3.7
p300-mediated E2F-1 ubiquitination is unique for E2F-1 and alternative to skp2.

Overexpression of p300 increases ubiquitination of E2F-1. Ubiquitination of E2F-4, a member of the E2F family that is not able to bind p300/CBP, is unaffected. Thus, p300 apparently acts as a novel ubiquitin ligase for E2F-1. This ubiquitin-ligase activity is alternative to $p45^{skp2}$, since ubiquitination of p27 and cyclin E, two other substrates for $p45^{skp2}$, is unaffected by overexpression of p300. The red dotted boxes indicate those lanes in which both p300 and the respective factors were transfected to allow easy comparison of the results.

substrate, thus representing a novel mechanism to regulate protein stability. Indeed, recent observations indicate that this is the case for p53 and Smad7. In these two proteins, the same lysine residues can be actually either acetylated by p300 or P/CAF or ubiquitinated; if acetylated, ubiquitination is prevented (Gronroos et al., 2002; Ito et al., 2002; Li et al., 2002). As far as E2F-1 is concerned, experiments performed in the T. Kouzarides laboratory have indicated that expression of P/CAF increases the overall levels of E2F-1, and that this increase was dependent on the HAT activity of P/CAF and on the integrity of the acetylated lysine residues of E2F-1 (Martinez-Balbas et al., 2000).

Given the above considerations, if was of utmost importance to determine whether competition between acetylation and ubiquitination for modification of common lysine residues in response to P/CAF or p300 expression was also a mechanism for regulation of E2F-1 stability. To address this issue, we performed the *in vivo* ubiquitination assay taking advantage of the availability of both a mutant of p300 that is impaired in its enzymatic activity and of a mutant of E2F-1 which is no longer acetylated since it bears substitution of K at positions 117, 120, and 125 with R (a kind gift of K. Helin, Milan). The results of these experiments are shown in Figure 3.8. As expected, overexpression of p300 and P/CAF increased ubiquitination of wt E2F-1 but, interestingly, it did not affected modification of the E2F-1 triple mutant. This result indicates a possible role for acetylation in determining the extent of ubiquitination of the factor. This possibility is further reinforced by the observation that transfection of the p300 mutant p300DY, which bears a single point mutation in its acetyl-transferase domain (Ito et al., 2001) did not increase the extent of ubiquitination of E2F-1. The specificity of this result was also proven by an experiment in which the effects of p300, P/CAF and the inactive p300 mutant p300DY were tested on the ubiquitination of transfected p53. To prevent interference due to endogenous p53, this experiment was performed in the SAOS-2 cell line. As shown in figure 3.9a, in contrast to what observed for E2F-1, all the three proteins failed to increase p53 ubiquitination.

Altogether these data indicate that, for E2F-1, a novel mechanism for the regulation of protein stability might occur, which involves both acetylation and ubiquitination coordinated in a non competitive fashion, and in which acetylation acts as a signal for subsequent ubiquitination.

To further reinforce the evidence that E2F-1 acetylation plays a key role in the extent of its ubiquitination, we also investigated ubiquitination after inhibition of cellular deacetylases, in order to increase the overall acetylation of the intracellular environment. For this purpose, 293 cells, either transfected or not transfected with E2F-1, were treated with the histone deacetylase

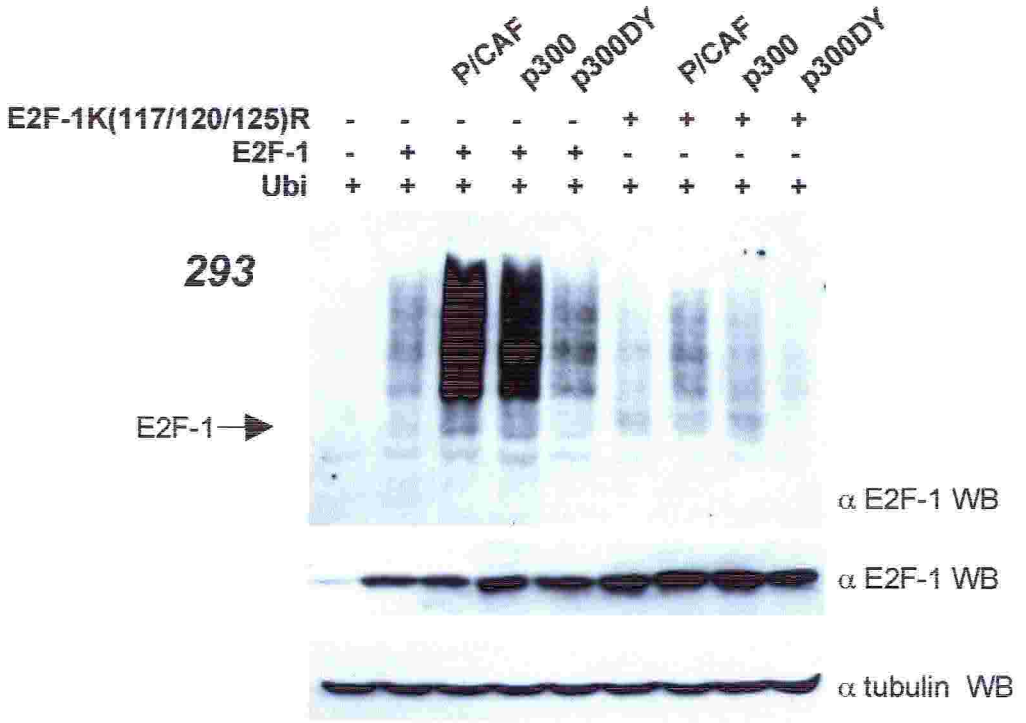

Figure 3.8a
E2F-1 ubiquitination by p300 depends on its acetyl-transferase activity

Overexpression of p300 and P/CAF increases ubiquitination of wt E2F-1 but not of E2F-1 mutated at the three acetylatable lysines at positions 117, 120, and 125. These results indicate a possible role for acetylation in determining the extent of ubiquitination of the factor. This possibility is further reinforced by the observation that transfection of a mutant from a p300 bearing a single point mutation in its acetyl-transferase domain (p300DY) does not increase the extent of ubiquitination of E2F-1.

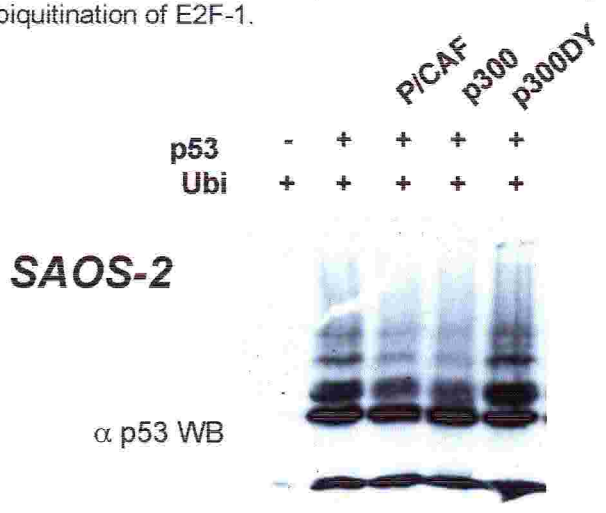

Figure 3.8b
p53 ubiquitination by p300 is unaffected

Overexpression of p300 and P/CAF, in contrast to E2F, does not increase ubiquitination of p53. These results indicate that p53 and E2F are subjected to different mechanisms of regulation.

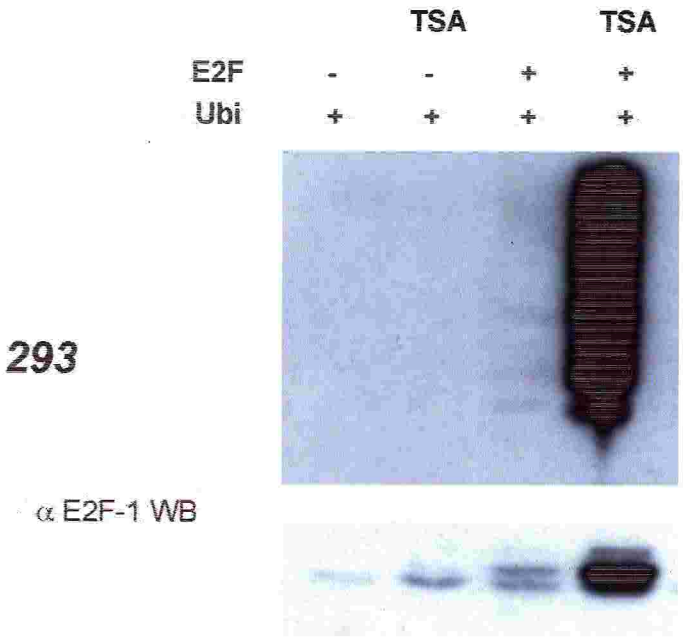

Figure 3.9
Hyperacetylation leads to E2F-1 hyperubiquitination

E2F-1 ubiquitination is markedly increased upon treatment with histone deacetylase inhibitor TSA, suggesting that the more E2F-1 is acetylated, the more is prone to be ubiquitinated. By promoting acetylation, TSA treatment leads to increased total protein amount.

inhibitor Trychostatin A (TSA), then processed through the *in vivo* ubiquitination assay, and E2F-1 ubiquitination patterns from treated and untreated cells were compared.

Inhibition of deacetylation after TSA treatment led to a strong increase of E2F-1 poly-ubiquitination (Figure 3.9). Moreover this effect appeared to be dependent on the dose of TSA used (data not shown), suggesting that the increase in ubiquitination parallels the increase in acetylation of the protein. TSA treatment also gave rise to an increase of total E2F-1 levels, as detected by Western blot analysis of whole cell lysates using an antibody anti-E2F-1, either in transfected or untransfected cells, a results which is consistent with the fact that E2F-1 acetylation enhances protein stability (Martinez-Balbas et al., 2000).

3.2.5 p300 promotes stabilization of E2F-1

The results so far reported suggest that a link is likely to exist between acetylation of E2F-1 by p300 and the increase in the cellular concentration of its ubiquitinated forms. One key question to be answered is obviously whether this markedly increased ubiquitination also impacts on the stability of the factor, given the rapid degradation of ubiquitinated proteins by the proteasome.

To address this issue, we the levels of ectopically expressed E2F-1 protein were monitored in a pulse-chase experiment following immunoprecipitation at different time points; the factor was expressed either in the presence or absence of transfected p300. Figure 3.10 shows that, overexpression of p300 clearly increases stability of E2F-1. This effect is most likely due to acetylation of the factor by p300, since stability of mutant K(117/120/125)R, which still binds to p300 but is neither acetylated nor ubiquitinated by p300, is insensitive to p300 overexpression.

These results indicate that acetylation of E2F-1 by p300 significantly increases protein stability, a result which is in agreement with previously published data that reported stabilization of E2F-1 by overexpression of the P/CAF acetyl-transferase (Martinez-Balbas et al., 2000). Since both P/CAF and p300 overexpression also determines remarkable ubiquitination of the factor, a likely possibility exists that stability of the protein might be consequent to the latter modification.

3.3 Subcellular localization of E2F-1

Several different mechanisms are required to elicit the correct transcriptional responses following intra- or extracellular stimuli during the cell cycle; each family of transcription factors has evolved its own specific mechanism for activation. One of these mechanisms occurs through regulation of nucleo-cytoplasmatic shuttling. The involvement of cytoplasmic and

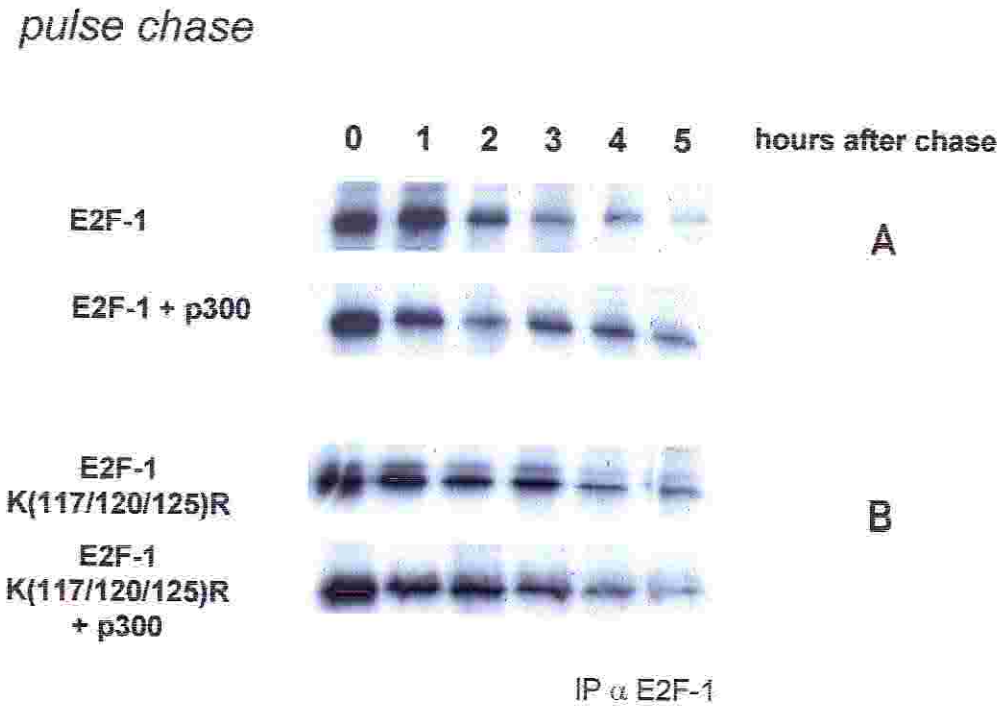

Figure 3.10

A.To determine the protein half-life of E2F-1 in the presence or absence of p300, 293 cells were transfected with 2 μg of pCMVE2F-1 HA in the absence or presence of 1.5 μg of pCMVβp300. At 24 hours after medium change, cells were pulse-labelled for 1 hour with [^{35}S] methionine. Chase was performed in medium with 10-fold excess of cold methionine and cysteine for the time periods indicated. cells were lysed and immunoprecipitations were performed with the anti-E2F-1 (KH-95) antibody. Immunoprecipitates were separated by SDS-PAGE, blotted and autoradiographed. The intensity of ^{35}S-labelled proteins was misured by phosphoimage analysis.
B.The effect of the half-life of E2F-1K(117/120/125)R were assayed under the same conditions as described in (A) therefore 2 μg of pCMVE2F-1K(117/120/125)R were transfected with or without p300 in 293 cells.

nuclear accessory molecules, in addition to membrane transport components, is essential for this process. Given these considerations, we set out to understand whether the regulation of E2F-1 by acetylation and ubiquitination might impact on the subcellular localization of this factor.

3.3.1 Overexpression of p300 affects E2F-1 localization

Subcellular localization of E2F-1 after overexpression of p300 and P/CAF acetyl-transferases was studied by immunofluorescence. Human epithelial HeLa cells were cultured on glass chamber slides and transiently transfected with expression vectors encoding for HA-tagged E2F-1, p300 and P/CAF. After 40 hours, cells were fixed and processed for indirect immunofluorescence microscopy as described in Materials and Methods, using an anti-HA primary antibody and a TRITC-conjugated secondary antibody.

According to established data about the subcellular distribution of E2F family members, ectopically expressed E2F-1 in cycling cells was mostly detectable in the nucleus, with only limited staining of the cytoplasm (Figure 3.11 panel A; >80% of the cells). Only <20% of the cells showed predominant cytoplasmic localization with exclusion of the nucleus (C). The picture in the left side of panel A shows a representative cell with N localization.

In contrast, overexpression of p/CAF or p300 determined clear re-localization of a considerable portion of E2F-1. As shown in Figure 3.11 panel B, E2F-1 was predominantly located in the cytoplasm and excluded from the nucleus in ~65% of the cells transfected with P/CAF, with a peculiar accumulation in the perinuclear region (a representative cell is shown in the picture on the left side of panel B); less than 35% of the cells showed E2F-1 fluorescence in the nucleus or both in the nucleus and the cytoplasm (N). Overexpression of p300 had a similar effect on E2F-1 subcellular localization, with ~60% of the cells showing cytoplasmic localization with evident accumulation around the nuclear membrane and exclusion of the nuclei. All these experiments were conducted in triplicate samples by evaluating E2F subcellular localization in at least 400 cells per sample.

These results indicate that subcellular localization of E2F-1 is remarkably influenced by the levels of P/CAF or p300 acetyl-transferases. To find out whether this event is a specific property of HeLa cells, we performed the same experiments in U2OS cells. E2F-1 was again detected in the cytoplasm also in the majority of these cells (data not shown; see also Figure 3.15), also ruling out any influence of the human papillomavirus oncoproteins expressed in HeLa cells in this process.

We next focused our attention to the effects of p300 and P/CAF on E2F-4. In contrast to E2F-1, -2 and –3, which have a consensus NLS and are predominantly nuclear, E2F-4 has the intrinsic

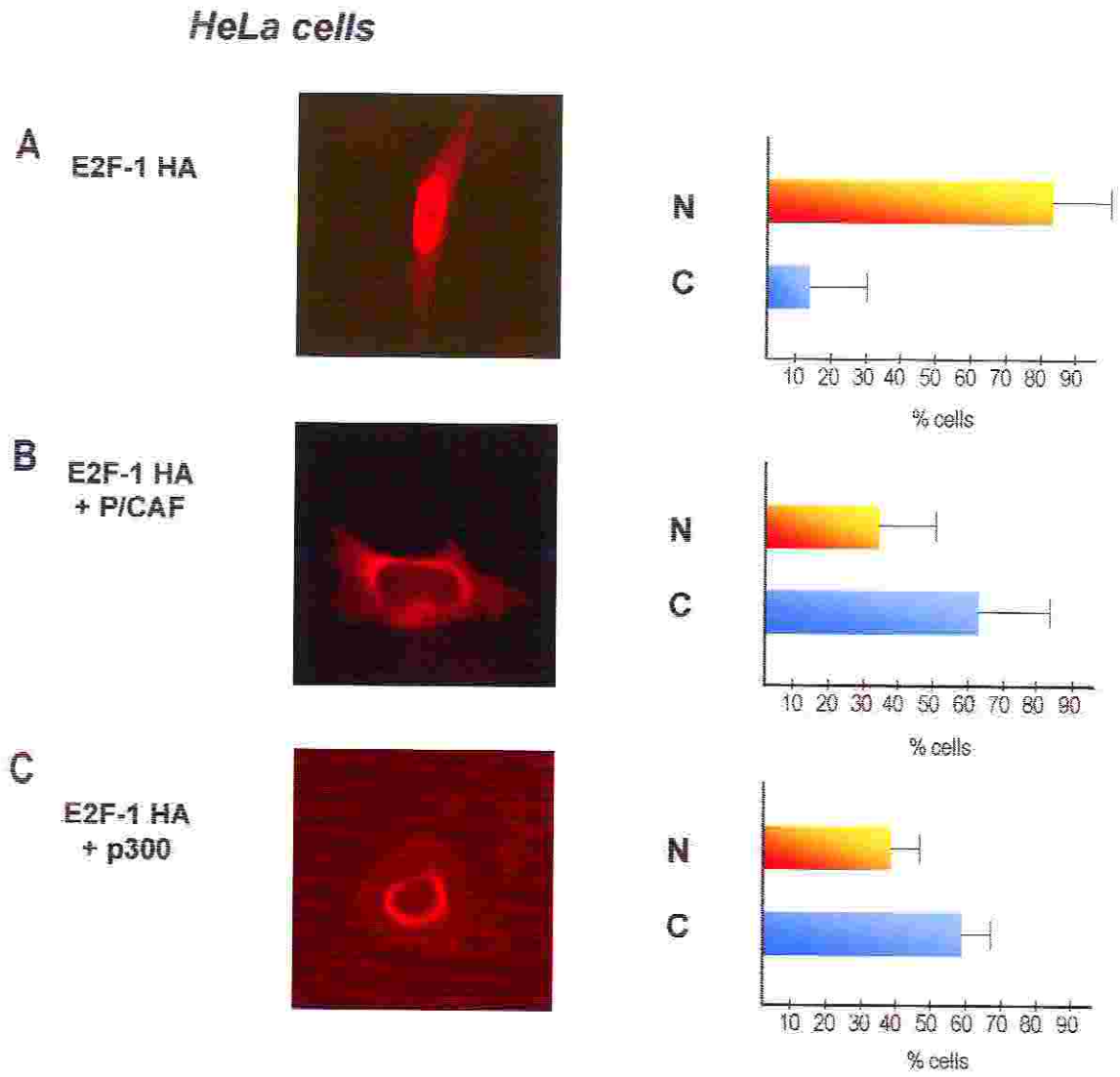

Figure 3.11
p/CAF and p300 affect E2F-1subcellular localization

Asynchronously growing HeLa cells were transiently transfected with expression vectors encoding for the proteins indicated on the side of each panel. After 20 hours, overproduced E2F-1 protein was detected in paraformaldehyde-fixed cells by indirect immunofluorescence using an anti-HA primary antibody and an anti-rat TRITC-conjugated secondary antibody. Nuclei were revealed by Hoechst staining (not shown). Overexpression of P/CAF or p300 acetyltransferases leads to E2F-1 cytoplasmic localization. Histograms on the right side of the micrographs indicate the percentage of cells where E2F-1 localization has been detected primarily in the nucleus (N) or in the cytoplasm (C). The results were calculated from experiments conducted in triplicate samples and by evaluating E2F subcellular localization in 400 cells/sample.

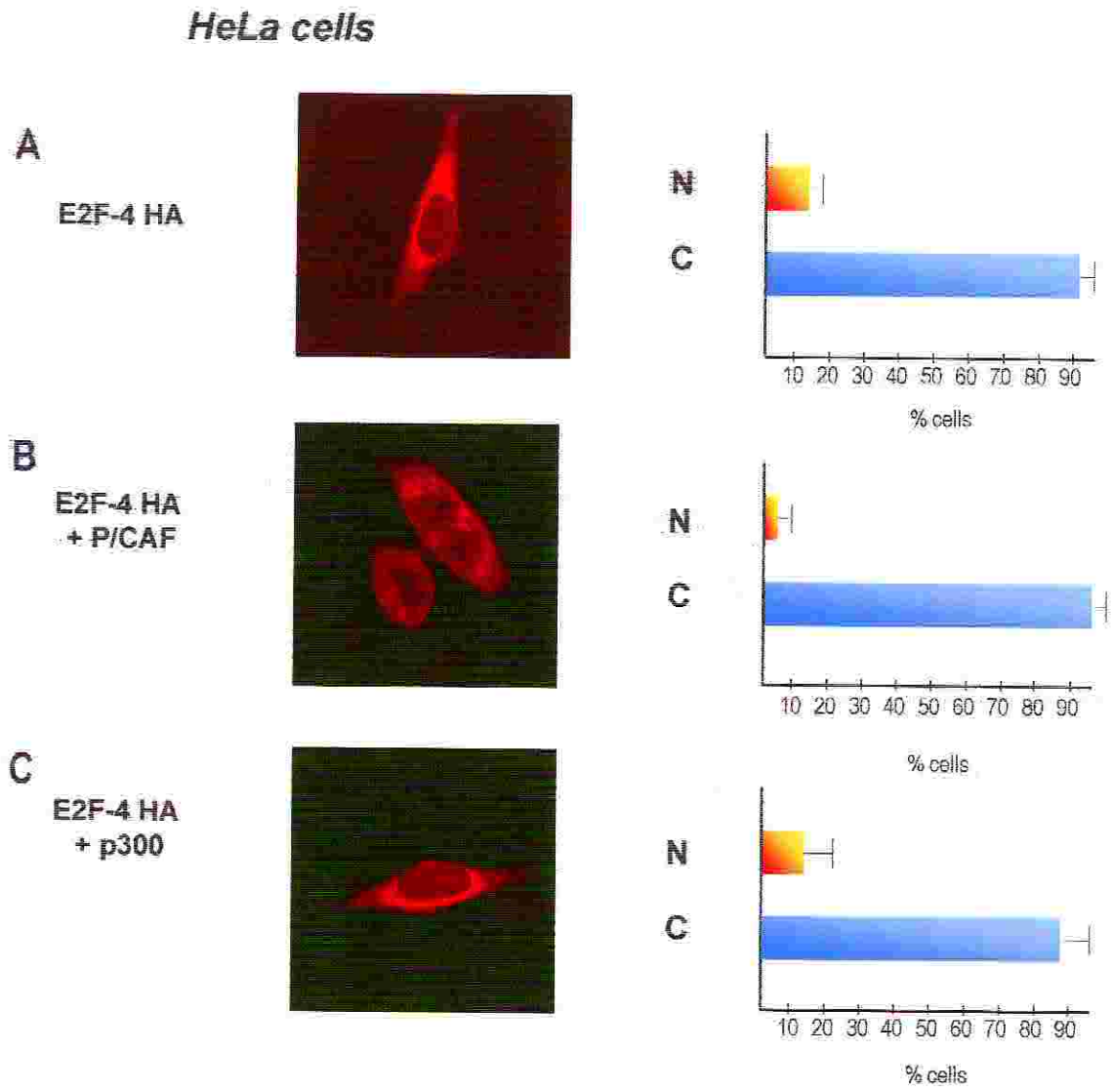

Figure 3.12
E2F-4 localization is not affected by p300 and P/CAF

Exogenously expressed E2F-4 localizes in the cytoplasm. Asynchronous HeLa cells were transfected with CMV expression vector encoding HA-tagged E2F-4 and immunofluorescence was detected as described in materials and methods with and anti-HA antibody. Expression of P/CAF or p300 does not promote any change in E2F-4 subcellular distribution. Histograms on the right side of the micrographs indicate the percentage of cells where E2F-4 localization has been detected primarily in the nucleus (N) or in the cytoplasm (C). The results were calculated from experiments conducted in triplicate samples and by evaluating E2F subcellular localization in 400 cells/sample.

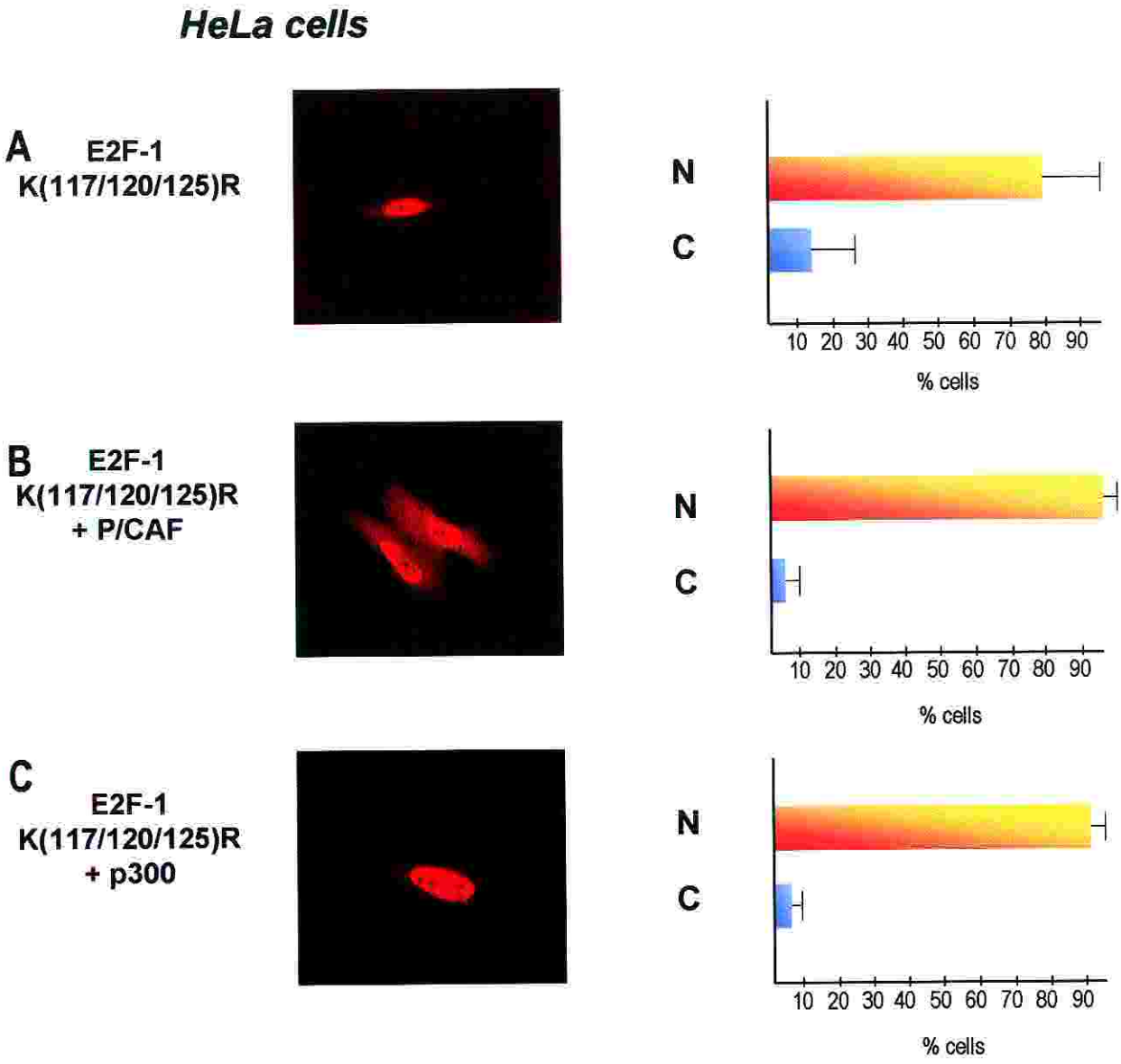

Figure 3.13
No effects of P/CAF and p300 on the localization of E2F-1 mutant

HeLa cells were transiently transfected with expression plasmids encoding for an E2F-1 mutated in the lysines (117, 120, 125) target for acetylation, in the presence or absence of p300 or P/CAF overexpressed as indicated beside each panel. E2F mutant was detected by indirect immunofluorescence using anti-E2F-1 antibody and TRITC-conjugated anti-mouse secondary antibody. Cytoplasmic localization of E2F-1 mutant is not affected by overexpression of both acetyl-transferase activities. Histograms on the right side of the micrographs indicate the percentage of cells where E2F-1K(117/120/125)R mutant localization has been detected in the nucleus (N), or the cytoplasm (C).The results were calculated from experiments conducted in triplicate samples and by evaluating E2F subcellular localization in 400 cells/sample.

property to shuttle between the cytoplasm and the nucleus in a cell cycle dependent manner (Lindeman et al., 1997; Muller et al., 1997; Verona et al., 1997). Specifically, endogenous E2F-4 is predominantly nuclear in G0 and progressively moves to cytoplasm as cells approach S phase; passive nuclear accumulation follows interaction with pocket proteins. As expected, in asynchronous cells E2F-4 was predominantly detected in the cytoplasm; expression of p300 and P/CAF did not affect E2F-4 subcellular distribution (Figure 3.12).

These results are consistent with the fact that E2F-4 lacks the N-terminal domain necessary for p300 interaction and acetylation (and thus ubiquitination), and further reinforce the notion that the change in subcellular localization of E2F-1 might be a consequence of its acetylation, and probably subsequent ubiquitination. To better address this issue we performed another set of experiments aimed at analyzing the localization of the E2F-1 mutant at lysines 117,120 and 125 in response to p300 and P/CAF overexpression. As previously demonstrated, this mutant is not subjected to modification by acetylation, and subsequent p300-dependent ubiquitination is severely compromised. This mutant was found predominantly localized in the nucleus (>80%), with very limited cytoplasmic fluorescence (a representative cell is shown in Figure 3.13 panel A). Transfection of P/CAF (panel B) or p300 (panel C) did not significantly change the pattern of subcellular distribution of the E2F-1 K(117/120/125)R mutant.

As a final indication that acetylation of E2F-1 by p300 is involved in determining its subcellular localization, we also tested the effect of the p300DY mutant, bearing a point mutation that abolishes HAT activity. As shown in Figure 3.14, overexpression of this mutant had no effect neither on wild type E2F-1 nor on the E2F-1 K(117/120/125)R mutant. Altogether these data strongly support the notion that acetylation of E2F-1 plays a key role in the subcellular localization of the factor.

Finally, as an additional indication of a specific role of p300 in the determination of the subcellular localization of E2F-1, we also tested co-localization of the two factors in U2OS cells by confocal microscopy. Asynchronous U2OS cells were transfected with expression vector encoding for E2F-1 or E2F-1 together with p300 or p300DY and analyzed by immunofluorescence. As expected, p300 transfection determined re-distribution of E2F-1 localization to the cytoplasm, with peculiar accumulation in the perinuclear region. Surprisingly, p300 was also localized to the same perinuclear site in these experiments (Figure 3.15 panel B). The normal localization of p300 is diffuse nuclear, with co-localization with PML in nuclear bodies in some cells - (Marcello et al., 2001) and Figure 3.15 panel D-, not dissimilar from the one which is detectable for the p300DY mutant, shown in Figure 3.14 panel C.

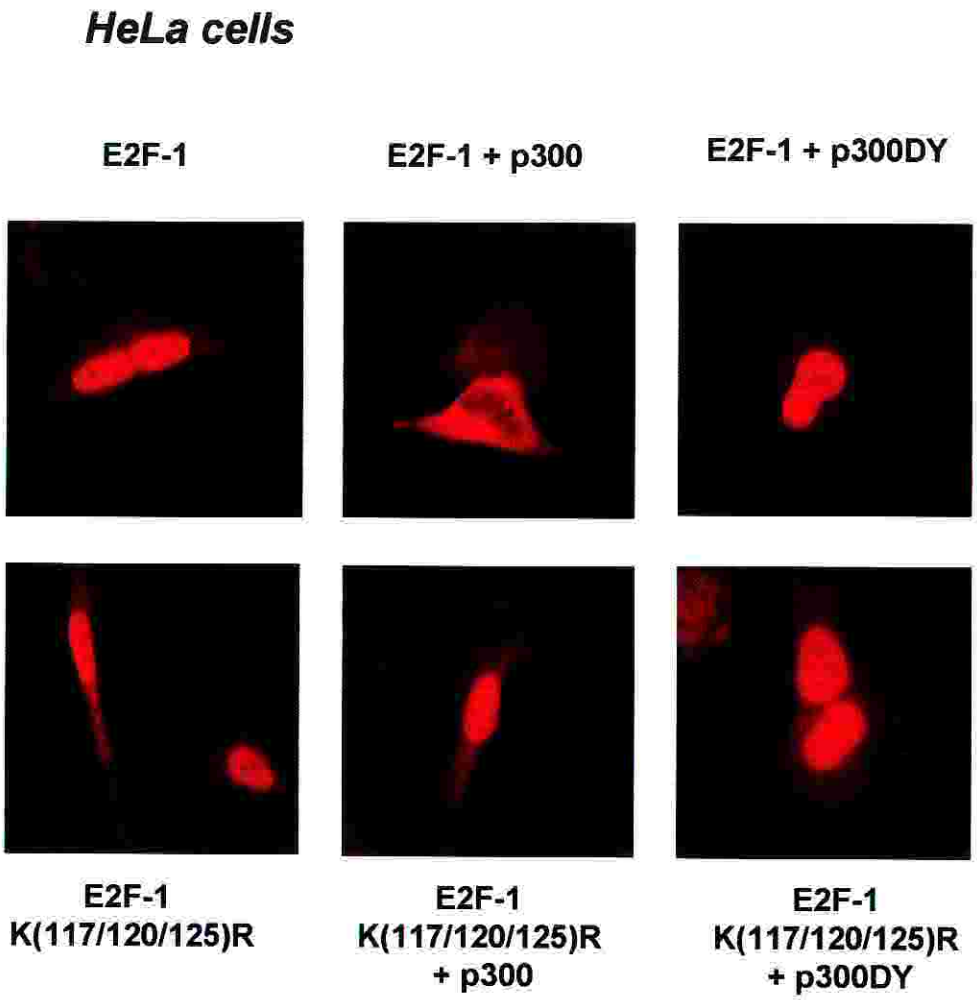

Figure 3.14
Integrity of p300 HAT activity is indispensable for nuclear export

Upon p300 transfection the majority of asynchronous HeLa cells E2F-1, but not E2F1 mutant which lacks acetylatable lysines, localizes in the cytoplasm suggesting a role for the acetylation in the control of protein export. This hypothesis is enforced by the evidence that p300DY mutant bearing a point mutation that abolishes HAT activity, does not affect nuclear export. E2F-1 and E2F-1K(117/120/125)R mutant were detected by indirect immunofluorescence using anti-E2F-1 antibody and TRITC-conjugated anti-mouse secondary antibody as already described.

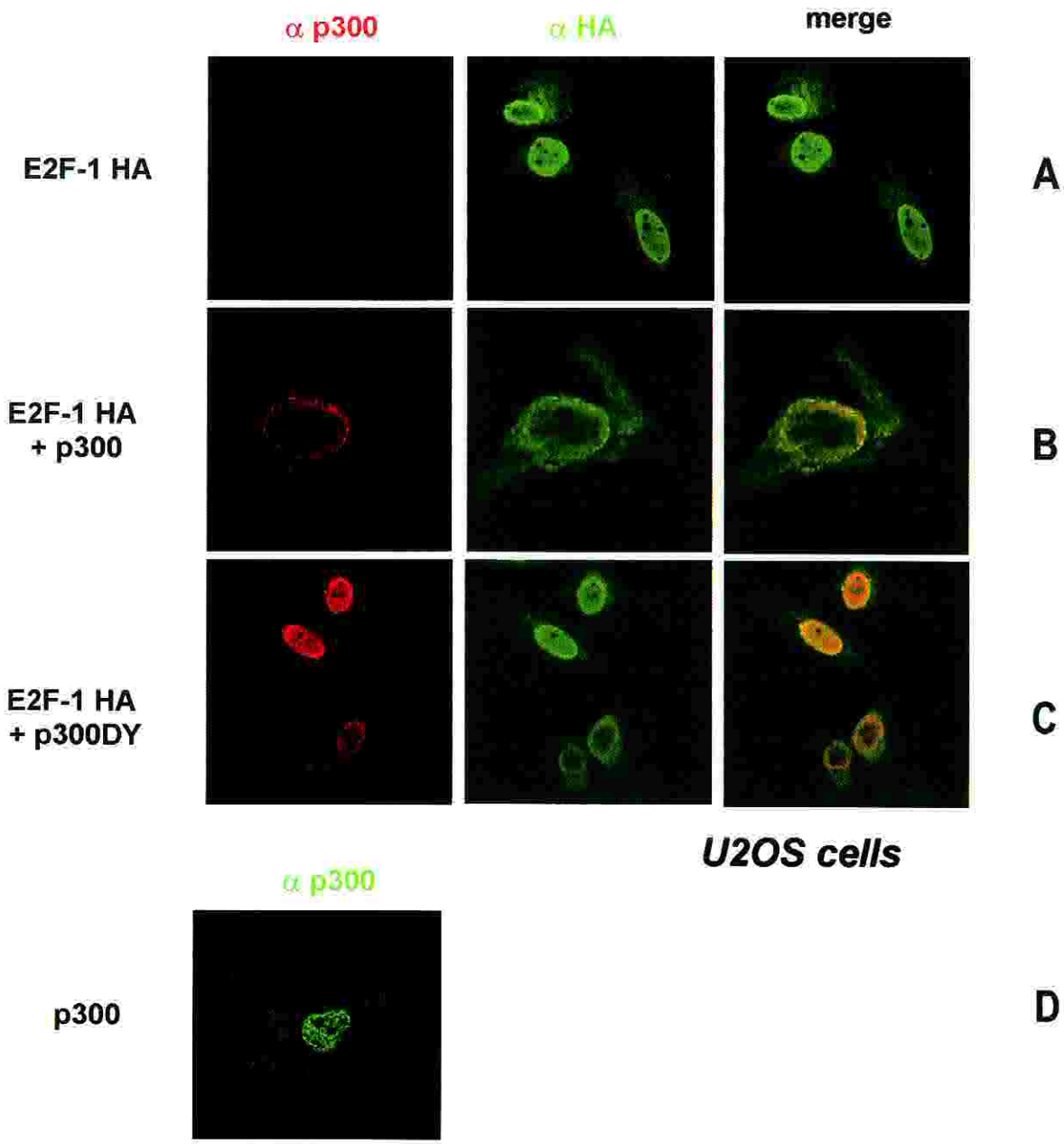

Figure 3.15
E2F-1 and p300 co-localization in U2OS cells

U2OS cells were transfected with expression plasmid encoding for HA tagged-E2F-1, p300, and acetylation deficient p300DY and analyzed by immunofluorescence with anti-HA and anti p300 primary antibodies and FITC- and TRITC-conjugated secondary antibodies. E2F and p300 appear to localize out of the nucleus but are also present in evident aggregates around nuclear membrane, while E2F-1 and p300DY co-localize diffusely in the nucleus, meaning that functional significance might involve acetylation in the regulation of E2F activity.

3.3.2 p300 determines nuclear export of E2F-1 through a CRM1-dependent pathway

In search of experimental evidence that E2F-1 is actively exported from the nucleus upon p300 expression, we utilized Leptomycin B, a specific inhibitor of nuclear export. Exportin or CRM1, has been identified as a receptor that is responsible for nuclear export of proteins that contain specific nuclear sequences (NES) (Fukuda et al., 1997) (Fornerod et al., 1997). Leptomycin B binds to CRM1 and inhibits its interaction with NES-containing proteins (Kudo et al., 1999).

Consistent with our previous studies, ectopically expressed E2F-1 was detected in the nucleus in asynchronous cells and in the cytoplasm when co-transfected with p300. However, after incubation with Leptomycin B, E2F-1 was detected in the nuclei of most cells, either with or without transfection with p300 (figure 3.16). Consistently, E2F-1 K(117/120/125)R was found nuclear either with or without LMB treatment and with or without p300 expression). From these evidences we can conclude that p300-mediated E2F-1 nuclear export is CRM-1 dependent.

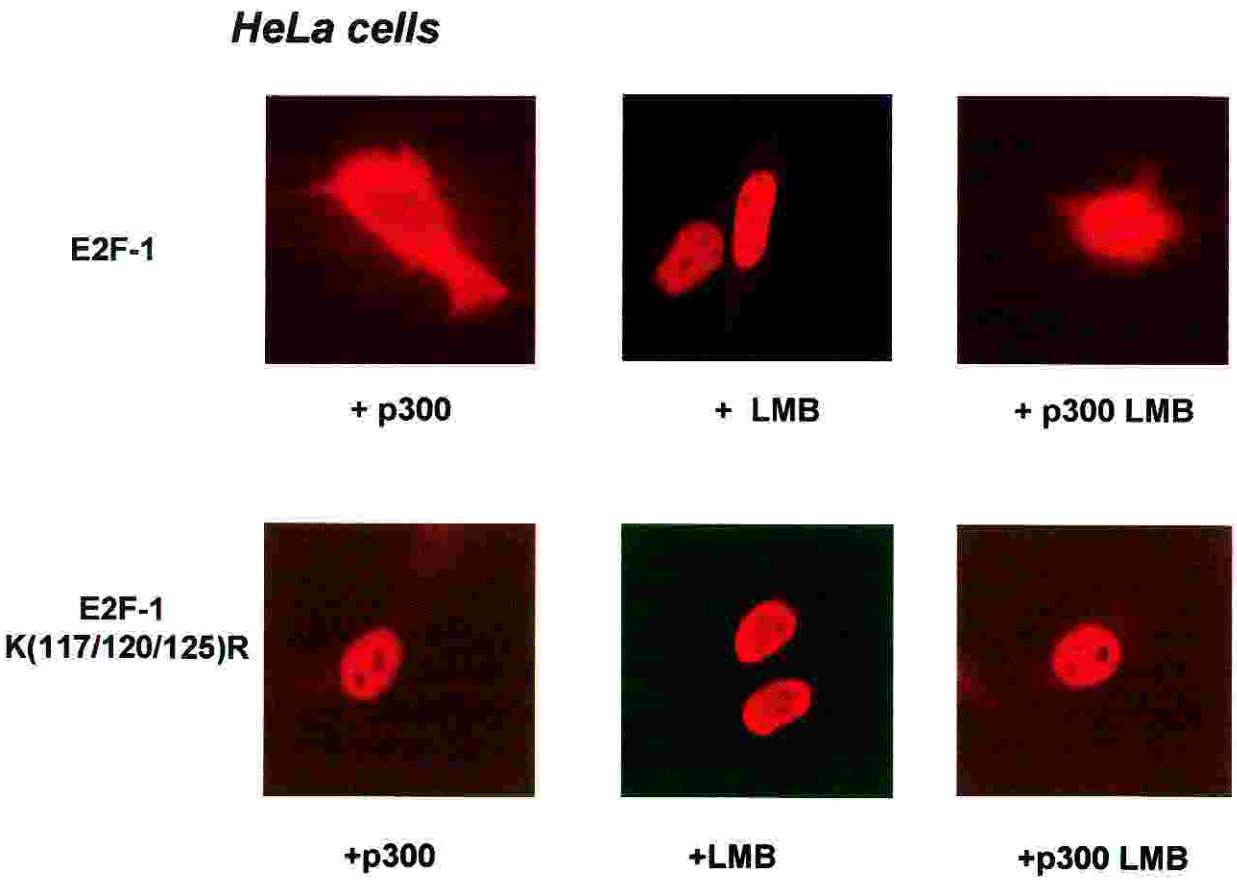

Figure 3.16
Leptomycin B inhibits p300 mediated E2F-1 nuclear export

HeLa cells were transfected as indicated, treated with 0.2nM Leptomycin B (LMB) for 18 hours and then analyzed by indirect immunofluorescence as described in Materials and Methods by staining with an anti-HA primary antibody and anit-rat TRITC-conjugated secondary antibody. Nuclei were counterstained with Hoechst. E2F-1 is nuclear in the majority of asynchronously growing cells and cytoplasmic when p300 is cotransfected. In LMB-treated cells, transfected or not transfected with p300, E2F-1 is no longer exported from the nucleus.

4. DISCUSSION

The E2F family of transcription factors plays a key role in the regulation of the timely expression of genes essential for both DNA replication as well as for surveillance of genomic integrity. Activity of E2F family members is tightly regulated during cell cycle progression by several molecular mechanisms, which include control of gene expression, binding to inhibitory proteins, post-translational modification, and regulation of protein turn-over, in order to match protein supply and availability to the cellular request. Besides normal regulation of cell cycle progression, tight regulation is also essential for cellular responses to external stimuli, DNA damage or other kind of cellular stresses, as well as during development (for recent reports, see Ishida et al., 2001; Lin et al., 2001; Muller et al., 2001; Ren et al., 2002; Wu et al., 2001).

In this work we present evidence that one of the mechanisms that regulate E2F-1 activity involves proteins acetylation by the cellular histone- and factor-acetyltransferases p300/CBP and P/CAF *in vitro* and *in vivo*, and that acetylation of E2F-1 by these enzymes: i) is paralleled by poly-ubiquitination of the protein; ii) increases protein stability; iii) determines a remarkable change in its subcellular localization.

Acetylation of E2F-1 leads to its ubiquitination

Lysine residues in transcription factors as well as other regulatory proteins are the targets of different covalent modifications, including sumoylation, ubiquitination, acetylation, and methylation. Each individual lysine residue can only be conjugated by a single modification at a time; however, multiple lysine residues of a protein may be modified simultaneously by two different modifications. Therefore, lysine residues may undergo sequential or cascades of covalent modifications, where modification of an individual residue may influence the modification of a neighbouring residue (Freiman and Tjian, 2003).

The cellular p300 and CBP transcriptional co-activators play an integral role in various cellular events, including cell proliferation, differentiation, and development by interacting with a variety of cellular proteins as extensively reviewed in: (Goodman and Smolik, 2000). In addition, p300/CBP possess histone and factor acetyl-transferase activity that originates either from intrinsic activity and/or from the associated protein, P/CAF, responsible for modification by acetylation of a number of histonic and nonhistonic targets. Curiously, CBP/p300 appears to be capable of contributing to opposite cellular processes since both co-activators participate in various tumor-suppressor pathways as well in the activities of several oncogenes. Consistent with this notion, CBP and p300 have been shown to promote apoptosis and cell proliferation –

two opposite effects that appear to be highly context-dependent -, as well as ubiquitination (Fukuchi et al., 2001). For example, p300 plays a dual role in the regulation of p53: by binding and acetylating p53 it increases its stability, while through its interaction with MDM2 it augments the ability of this protein to target p53 for degradation (Kawai et al., 2001).

Earlier work performed in this laboratory and in the laboratory of T. Kouzarides has provided evidence that E2F-1 is a target for binding and acetylation by p300/CBP and P/CAF (Martinez-Balbas et al., 2000; Marzio et al., 2000). In the work presented in this thesis, using antibodies specifically raised against acetylated E2F-1, we could verify, by immunoprecipitation, the actual existence of acetylated E2F-1 *in vivo* upon transfection either p300/CBP or P/CAF. Unexpectedly, we also observed that overexpression of p300/CBP or P/CAF is paralleled by a marked increase in the accumulation of ubiquitin-conjugated E2F-1 inside the cells. This increase was again dependent on factor acetylation, since it required both the presence of the E2F-1 lysines that are the targets for the acetylation and the integrity of enzymatic activity of p300. This effect appears independent of regulation E2F regulation by RB.

The available evidence about E2F-1 indicates that ubiquitin-modification of the factor regulates its proteolysis and thus controls its turnover in physiological conditions (Campanero and Flemington, 1997; Hateboer et al., 1996; Martelli and Livingston, 1999). The timing of E2F-1 ubiquitination apparently depends on the timing of expression of the $p14^{SKP2}$ F-box protein, acting as an E3 ubiquitin ligase for the factor (Marti et al., 1999). During the S-phase, E2F-1 becomes phosphorylated by cyclin A–CDK2 (Krek et al., 1994) or by other kinases including TFIIH (Vandel and Kouzarides, 1999) and detaches from DNA (Krek et al., 1995). It is thus conceivable that SKP2 interacts with E2F-1 after detachment from DNA and determines its ubiquitination and degradation. However, at present no evidence exists that association of E2F-1 with SKP2 actually requires phosphorylation of E2F-1, unlike other interactions between F-box proteins and their cognate substrates. In addition, SKP2 is itself cell-cycle-regulated at the transcriptional level, being controlled by E2F-1. Thus, E2F-1 might also have a hand in its own destruction by regulating transcription of its ubiquitin ligase, much as mitotic cyclins set up their own destruction by activating the anaphase-promoting complex. The N-terminal segment of E2F-1 that is required for binding to SKP2 – as well as for binding to p300 and acetylation -, is absent in E2F-4. This difference essentially contributes to explain the biological differences between the two proteins in terms of stability and turnover.

By taking advantage on an *in vivo* assay for purification of ubiquitin-conjugates, here we provide evidence that overexpression of either p300/CBP or P/CAF results in the accumulation of polyubiquitin-tagged E2F-1 in the same manner as detected by overexpression of SKP2. The

effect of the two acetyl-transferases is most likely directed toward E2F and not toward the E2F-ubiquitin ligase, since i) it does not affect other targets of SKP2, including p27 and cyclin, E and ii) it requires acetylation of the substrate, since the mutated p300DY effector and the mutated E2F-1 K(117,120,125)R target are both ineffective. Which is the ubiquitin ligase that mediates these effects of p300/CBP and P/CAF? The above considerations exclude a direct effect of the two acetyl-transferases on SKP2. However, they are still consistent with the possibility that SKP2 might be involved in the ubiquitination process of acetylated E2F-1. Experiments performed in SKP2 knock out cells will definitely clarify this issue. In this respect however, it should be considered that another ubiquitin ligase certainly exists in mammalian cells that mediates ubiquitination and proteasome degradation of E2F-1, since mouse SKP2 -/- cells are viable and show normal regulation of E2F-1 turnover (Nakayama et al., 2000). Consistent with this possibility, also p27 undergoes two different mechanisms of regulation by degradation, which are either SKP2-dependent or independent (Hara et al., 2001), and E2F-1 itself has been shown to be the target of multiple distinct E3 ligases (SCF-type or ROC-cullin ligases) *in vitro* (Ohta and Xiong, 2001).

Might p300/CBP and P/CAF exhibit themselves E3 ubiquitin ligase activity for E2F-1? This intriguing observation is indirectly supported by analogous speculations reported for p53. Similar to what observed for E2F-1, p300 has been reported to co-activate and acetylate p53 in DNA damaged cells but to promote ubiquitin-proteasome degradation of p53 in unstressed cells, through the formation of a complex with the MDM2 ubiquitin ligase (E3) (Grossman et al., 1998). Results from Grossman et al. (oral presentation at "Cancer Genetics & Tumor Suppressor Genes" CSHL meeting, August 2002) suggest that p300 itself might be an ubiquitin ligase for p53 ubiquitination. E3 activity was localized to a central domain of p300 without homology to other known E3 motifs. More precisely, in this context, MDM2 E3 activity towards p53 is limited to the addition of single ubiquitin moieties to multiple lysines, suggesting that an additional factor (E4) is required to achieve p53 poly-ubiquitination. p300 may thus serve as an E4 enzyme for p53, cooperating with MDM2 E3 function to generate poly-ubiquitinated p53 intermediates required for proteasomal turnover.

The HAT domains of CBP and p300 are characterized by the presence of a highly conserved putative plant homeodomain (PHD) (C4HC3) type zinc finger, which is part of the functionally uncharacterized cysteine- histidine-rich region 2 (CH2). This region conforms to the PHD type zinc finger consensus (Kalkhoven et al., 2002). Thus the PHD finger forms an integral part of the enzymatic core of the HAT domain of CBP but it is dispensable for p300 HAT activity, providing evidence for structural differences between p300 and CBP that may in part underlie a previously reported functional specialization of the two proteins (Bordoli et al., 2001; Yuan et al., 1999).

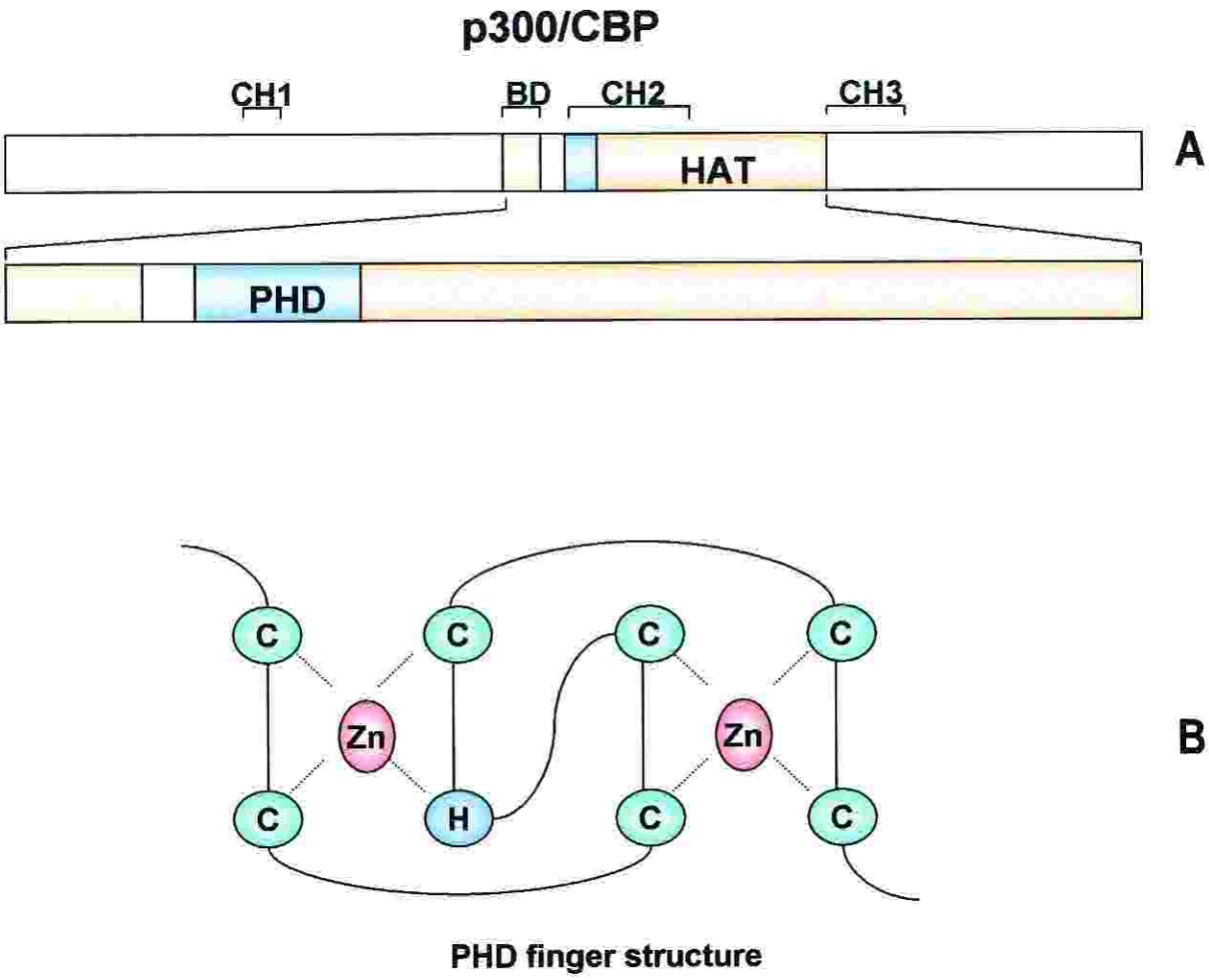

Figure 4.1
A conserved PHD finger in the acetyl-transferase domain of p300/CBP

A. Schematic representation of p300/CBP showing the position of the bromodomain (BD), the acetyl-transferase domain (HAT) the PHD finger, and the CH1 through -3.
B. Putative structure of the PHD finger. The zinc-coordinating cysteines (C) and histidine (H) and zinc atoms (Zn) are indicated.

PHD domains constitute a widely distributed subfamily of zinc fingers whose biochemical functions have been unclear until now. Recently, several PHD-containing viral proteins have been identified that promote immune evasion by downregulating proteins that govern immune recognition (Boname and Stevenson, 2001; Coscoy et al., 2001; Lorenzo et al., 2002; Mansouri et al., 2003). Different evidence indicates that these viral regulators lead to ubiquitination of their targets by functioning as E3 ubiquitin ligases - an activity that requires integrity of the PHD motif. These are the first examples linking the PHD domain to E3 activity, but the recent discovery of PHD-dependent E3 activity in the cellular kinase MEKK1 (Lu et al., 2002) and the close structural relation of PHD domains to RING fingers hint that many other PHD proteins might share this activity (Coscoy and Ganem, 2003). Thus, it is conceivable that p300/CBP dependent E2F-1 ubiquitination might be directly mediated by an E3 ubiquitin ligase activity featured in its PHD finger. In this context, it is also worth mentioning that another acetylase, the TAFII250 subunit of the RNA polymerase II general initiation factor TFIID, also has ubiquitin ligase function. At variance with canonical ubiquitination enzymes, this factor exhibits both E1 ubiquitin activating, and E2 ubiquitin conjugating activities (Pham and Sauer, 2000; Wassarman and Sauer, 2001).

Increased stability of acetylated E2F-1

Since stability of proteins in the cells is importantly regulated by the rate of its post-translation modification by ubiquitination followed by proteasome-mediated degradation, we reasonably expected that the observed effects of p300 and P/CAF on E2F-1 ubiquitination might lead to increased protein degradation and more rapid turnover. In contrast and to our surprise, we observed that overexpression of the two acetyl-transferases determined a significant increase in E2F-1 stability. This increase is most likely dependent on acetylation of the factor, since the half-life of a mutant E2F-1 which cannot be longer acetylated is insensitive to p300 overexpression, even if this protein is still capable of binding to p300 indistinguishable from the wild type (Marzio et al., 2000). These results, together with analogous observations already published for P/CAF (Martinez-Balbas et al., 2000), indicate that a clear link exists between the regulation of protein stability and its acetylation.

When evaluating the effects of p300/CBP and P/CAF overexpression on one side on ubiquitination and on the other side on stability it should be considered that the two experiments probably look at different fractions of E2F-1 inside the cells. As a matter of fact, the *in vivo* ubiquitination assay is based on the selection of the portion of E2F-1, which is tagged with his-ubiquitin and is selected on the metal column. This fraction clearly represents a minority of the

expressed E2F; otherwise poly-ubiquitination would be visible in western blotting of total cell lysates. In contrast, analysis of E2F-1 stability measures the average half-life of the whole E2F-1 pool inside the cells. Thus, the extrapolation that the poly-ubiquitinated form of E2F-1 is also the form, which is more stable, must be taken with prudence.

Notwithstanding the above note of caution, it is clear that a strong relationship exists between E2F-1 acetylation, poly-ubiquitination and the control of its stability. In this respect, E2F-1 is not the only factor in which these three events have been found intertwined.

Regulation of protein stability by acetylation can be achieved by different mechanisms. The most intuitive one is competition between acetylation and ubiquitination for the same lysine substrates, as is the case for Smad7. Here, acetylation by p300 or mutation of the acetylated lysine residues, stabilizes and protects the protein from TGFβ-induced degradation mediated by the ubiquitin ligase Smurf1 (Gronroos et al., 2002).

As already mentioned, p53 can be also acetylated by both p300 (Gu and Roeder, 1997) and P/CAF (Liu et al., 1999; Sakaguchi et al., 1998) and, also in this case, acetylation plays a positive role in the accumulation of the protein as a response to cellular stress. p53 is normally unstable and degraded by the ubiquitin proteasome pathway (Prives and Hall, 1999). Ubiquitination and degradation of p53 is dependent on MDM2 (Haupt et al., 1997; Kubbutat et al., 1997), a RING-type E3 ubiquitin ligase that can recruit and stimulate E2 conjugating enzyme (Honda and Yasuda, 2000). Recent data suggest that p300 may operate in concert with MDM2 to affect the normal turnover of p53 in unstressed cycling cells (Grossman, 2001; Grossman et al., 1998). Thus p300 may help to control the switch in p53 stability that occurs after DNA damage or oncogene activation by integrating acetylation, ubiquitination and proteolysis (Zhu et al., 2001). Recent evidence also indicates that the cellular MDM2 protein inhibits the intrinsic HAT activity of p300/CBP (Ito et al., 2001; Kobet et al., 2000) and P/CAF (Jin et al., 2002), and, interestingly, that acetylation of p53 inhibits its ubiquitination by MDM2 (Li et al., 2002) furthermore p53 deacetylation mediated by MDM2-HDAC1 is required for its degradation (Ito et al., 2002), suggesting a mechanism of competition between acetylation and ubiquitination for the control of protein stability.

The link between control of acetylation and ubiquitination has recently received further support also from the identification of the components of the murine HDAC6 complex (Seigneurin-Berny et al., 2001). A number of the proteins in this complex appear involved in the ubiquitin-signalling pathway; in addition, active deacetylation of p53 by HDAC1 and Sir2 plays a role in the regulation of p53-dependent processes, including transcription, cell cycle regulation, and apoptosis (Luo et al., 2001; Luo et al., 2000; Vaziri et al., 2001).

Finally, positive regulation of protein stability by acetylation has been also recently described for E2F-1. In prostate carcinoma cells, it has been observed that acetylated E2F-1 dissociates from the SKP2 component of its E3 ubiquitin ligase (Farhana et al., 2002). Clearly, this mechanism is different from the one described in this thesis, since it would imply that increased stability of the factor correlates with diminished ubiquitination, opposite to what observed by overexpression of p300/CBP and P/CAF. In contrast, in our experiment even broad inhibition of de-acetylases by cell treatment with TSA resulted in a remarkable accumulation of E2F-1 ubiquitin conjugates.

Acetylation of E2F-1 controls its subcellular localization

In search of a possible mechanism accounting from increased stability of E2F-1 upon its ubiquitination, we also analyzed subcellular protein localization. The results obtained provided evidence that that p300 and P/CAF are also involved in E2F shuttling from the nucleus to the cytoplasm. Cytoplasmic translocation of E2F-1 was again dependent on acetylation, since it required integrity of HAT domain of p300 and availability of lysines on the target. A conceivable model that could account for these results is that acetylation acts as a signal both for the export of the protein from the nucleus to the cytoplasm, where ubiquitination might take place.

The possibility that p300/CBP might have a role in subcellular localization of target proteins also receives support from recent findings. p300/CBP have been described to mediate ubiquitination of Smad3 by facilitating its interaction with the ROC1-SCF^{Fbw1a} E3 ubiquitin ligase and to trigger its nuclear export that finally results in proteasome-dependent degradation (Fukuchi et al., 2001). In addition, interaction of transcriptional repressor EVI1 with CBP and P/CAF has been shown to result in reversible acetylation and in co-localization in nuclear speckles (Chakraborty et al., 2001).

Although we found that p300 mediated nuclear export of E2F-1 is CRM1 dependent, simple overexpression of CRM1 has been demonstrated not to affect E2F-1 nuclear localization, possibly suggesting that E2F-1 export is determined by acetylation and not limited by CRM1 concentration. Among the E2F family members, nuclear export appears to be a specific property of E2F-4 (Gaubatz et al., 2001) and E2F-5 (Apostolova et al., 2002) but not of E2F-1. Thus, it might be conceived either that acetylation unmasks a functional NES that does not conform to the canonical NES consensus or that acetylation favours interaction with another unrecognized protein that in turn mediates nuclear export. It is known, for instance, that MDM2 contributes to CRM1-dependent nuclear export of p53 depending on a series of ubiquitination-induced conformational changes in the p53 molecule that lead to the activation of its NES (Appella and Anderson, 2001; Geyer et al., 2000; Gu et al., 2001). Although both export and degradation of

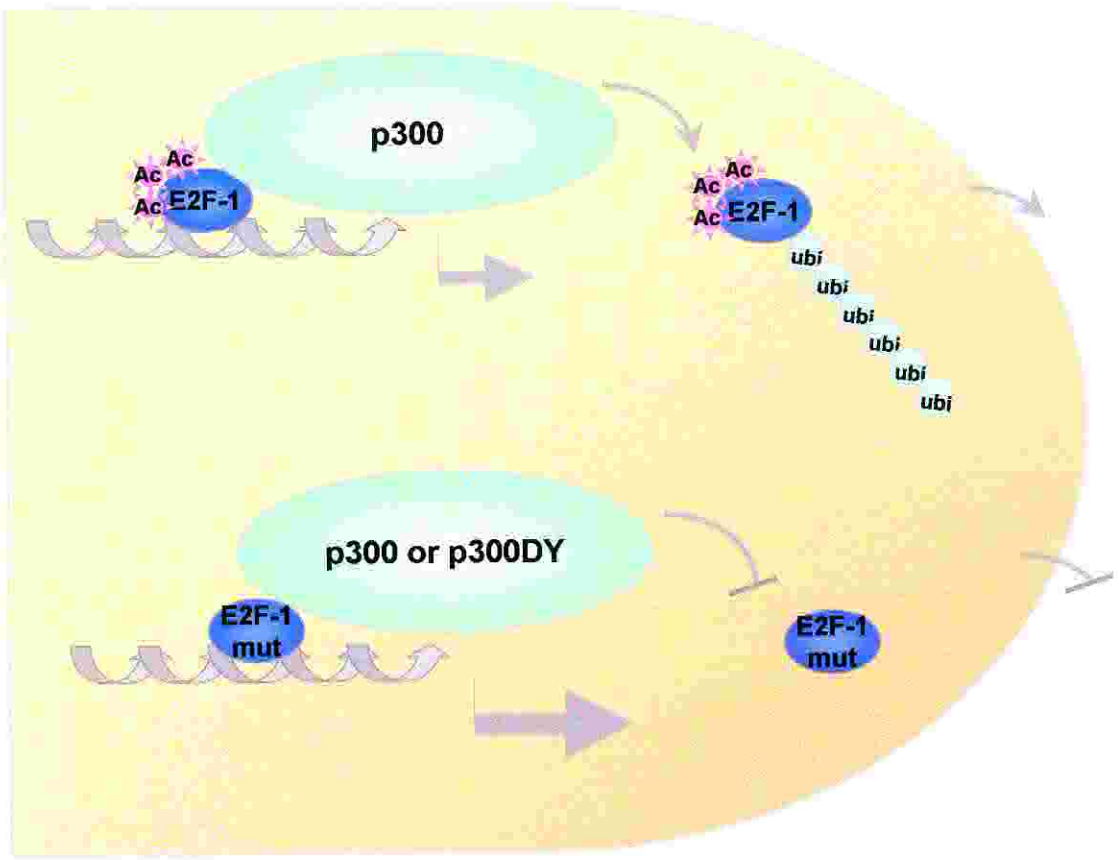

Figure 4.2
Possible model for E2F regulation by acetylation and ubiquitination

The ability of E2F-1 transcription factor to bind p300 coactivator correlates to E2F-1 acetylation at three specific lysines at position 117,120 and 125 that enhances affinity for DNA. p300 also promotes ubiquitination of E2F-1 and availability of acetylated lysines has been shown to be a signal for this modification and for CRM1-mediated nuclear export.
Lysine mutated version of E2F-1 maintains its ability to bind DNA and p300 (or p300 HAT deficient p300DY) but does not undergo neither acetylation nor ubiquitination and is retained in the nucleus. Due to the accumulation in the nucleus E2F mutant and is able to activate E2F responsive promoters five-fold more than the wild type.

p53 are mediated by MDM2, these activities are not absolutely dependent on each other (Lohrum et al., 2001). It has also been reported that p53 nuclear export can be a consequence of ubiquitination, but is not a necessary condition for ubiquitination and degradation (Yu et al., 2000). In this respect, our experiments cannot determine whether E2F ubiquitination occurs in the nucleus or in the cytoplasm and cannot distinguish whether acetylation, ubiquitination or both are the primary responsible for nuclear export.

Ubiquitination and proteasome-dependent degradation of proteins can occur both in the nucleus and in the cytoplasm. In the case of MyoD, for example, lysine-dependent degradation is more active in the cytoplasm, while in the nucleus operates both lysine-dependent and N-terminus dependent degradation (Lingbeck et al., 2002). The degradation of p27 in the nucleus during subsequent phases of the cell cycle appears to be regulated by SCF^{SKP2} ubiquitin ligase, which targets p27 for ubiquitination. In contrast p27 appears to be translocated from the nucleus in a CRM1 dependent manner and ubiquitinated in the cytoplasm at the G0 G1 transition by a yet unidentified ubiquitin ligase that functions independently of SKP2 suggesting that a poly-ubiquitination activity in the cytoplasm contributes to the early phase of p27 degradation, thereby promoting cell cycle progression from G0 to G1 (Hara et al., 2001; Ishida et al., 2002).

Taken all the above considerations together, the results obtained suggest a novel model for E2F-1 regulation by acetylation and ubiquitination. E2F-1 binds to p300/CBP, and binding is required for specific acetylation at three specific lysine residues at positions 117, 120 and 125; acetylation of these lysines correlates with both ubiquitination of E2F-1 and export of the factor to the cytoplasm. Ubiquitination by p300 is specific for E2F-1 and unrelated to the SKP2 pathway.

A central role for the p300 co-activator has been established in coordinating the interplay between the pathways of control mediated by E2F and p53, supporting the idea that p300 functions as a negative regulator of cell cycle progression and that p300 levels are instrumental in influencing whether cell cycle progression, G1 arrest or apoptosis occur. Regulation of E2F-1 stability and availability in the nucleus might be important for these effects to be exerted. Thus, it will be of interest to observe regulation of E2F-1 ubiquitination and subcellular localization in cells exposed to stressing conditions.

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