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Protein-protein interactions and post-translational modifications of EAPP

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This thesis is dedicated to my wonderful parents, Nahid and Mohammad and to my dear brother, Bahador, for bringing hope to every day of my life, for supporting me in every step I take and for their belief in me.

I would be nothing without you.

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Contents

1. Abstract

An E2F associated phospho protein (EAPP) has been recently identified through yeast two-hybrid assay. EAPP consists of 285 amino acids, weighs about 40 KDa, is localized in the cell nucleus and interacts with a subgroup of E2F proteins. EAPP is involved in cell cycle regulation as it significantly stimulates the S-phase entry of the cell and disappears in M phase. EAPP can control E2F-mediated transcription and affect cell proliferation.

In this study the interactions and post-translational modifications of EAPP have been investigated. Different truncations of this protein were examined for activity and post-translational modifications, among that sumoylation and acetylation, to figure out the functional domains and regulation of EAPP. Besides, the stability of the protein and the domain affecting this character were also studied.

Treatment with TSA, an HDAC inhibitor, induced acetylation of EAPP at its N terminus, within amino acids 1-140.

Through FACS method it was shown that the cells expressing truncated EAPP 1-140, took much longer to re-enter the cell cycle after the release from Nocodazole which arrests the cells at M phase. It in turn shows the possibility of involvement of this truncation in inhibition of cyclin-dependent kinase (CDK) complexes which control the transitions between different phases of the cell cycle.

Utilizing Pulse Chase methods and treating the cells expressing full length or truncated versions of EAPP it was demonstrated that the amino-terminal part of EAPP confers acetylation-dependent stability to the protein.

1. Zusammenfassung

Das E2F assoziierte Phosphoprotein (EAPP) wurde kürzlich in einem Two-Hybrid-Versuch in Hefe identifiziert. Das ca. 40 kDa große EAPP besteht aus 285 Aminosäuren, ist im Zellkern lokalisiert und interagiert mit einer Untergruppe von E2F-Proteinen. EAPP stimuliert in der Zelle maßgeblich den Übergang von G1- zu S-Phase und verschwindet in der M-Phase. Somit spielt EAPP eine Rolle in der Zellzyklus-Regulation, kontrolliert aber auch E2F-vermittelte Transkription und beeinflusst die Zellteilung.

Thema dieser Arbeit ist EAPP und seine Interaktionen und Modifikationen nach der Translation. Hierzu wurden verschiedene Verkürzungen von EAPP auf Aktivität und Modifikationen wie Sumoylierung und Acetylierung untersucht, um mehr über die funktionellen Proteindomänen und ihre Regulation herauszufinden. Weiters wurden EAPP-Domänen hinsichtlich ihres Einflusses auf die Proteinstabilität untersucht.

Behandlung mit TSA (einem HDAC-Hemmstoff) induzierte Acetylierung am N-terminalen Teil der EAPP-Verkürzung 1-140. Mittels FACS-Analyse konnte gezeigt werden, dass Zellen, welche die EAPP-Verkürzung 1-140 exprimieren, sehr viel länger brauchen, um nach einer Behandlung mit Nocodazole (arretiert Zellen in M-Phase) eine neue Zellzyklusrunde zu starten. Diese Ergebnisse lassen darauf schließen, dass diese EAPP-Domäne an der Inhibierung von Cyclin-abhängigen Kinase (CDK)-Komplexen beteiligt ist, welche die Übergänge in die verschiedenen Zellzyklus-Phasen kontrollieren.

Mittels Pulse Chase-Versuchen und Cycloheximid-Behandlung von Zellen, welche die EAPP-Verkürzung 1-140 exprimieren, konnte gezeigt werden, dass EAPP an seinem N-terminalen Ende (Verkürzung 1-140) sehr viel stabiler ist. Die Quintessenz dieser Arbeit besagt, dass die Acetylierung von EAPP an seinem N-terminalen Ende seine Stabilität beeinflusst.

2. INTRODUCTION

2.1 The mammalian cell cycle

The cell cycle is a complex ordered set of events that control the cell division. The activation of two significant proteins; cyclins and cyclin dependent kinases (cdks) regulate the progression of the cell cycle. The cell cycle consists of four different phases, G1-, S-, G2-phase and Mitosis. The phosphorylation of numerous proteins caused by different sets of cdk- cyclin complexes leads to the entry of the cell into mitosis. There is an additional state of the cell, the G0-phase, which allows the cell to rest for a period of time, before going through the new cell cycle or terminal differentiation.

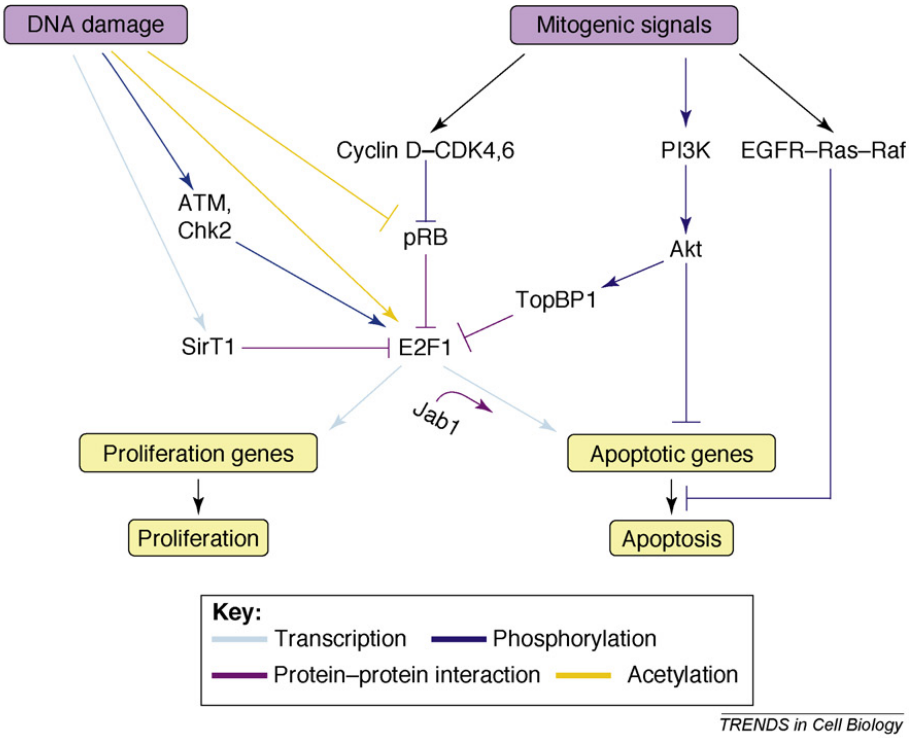
2.2 The E2F family of transcription factors

E2F play a crucial role in a wide range of biological processes, comprising DNA replication, mitosis, the mitotic checkpoint, DNA-damage checkpoints, DNA repair, differentiation, development and apoptosis (Polager et al., 2008). It can also function as transcriptional repressor or activator (De Gregori et al. 2006). The identification of E2F family of proteins was based on its role in advancing the G0 to S phase transition (Blais et al. 2007). E2F activity will be deregulated through many different mechanisms in the vast majority of human tumors. These comprise functional loss of the pocket protein family including the retinoblastoma protein (pRB); phosphorylation of pRB promoted by amplification of cyclin D; loss of p16 a cyclin-dependent kinase inhibitor that inhibits the phosphorylation of pRB (Polager et al., 2008). E2F transcription factors may thus play a significant role in regulating the transcription of different cellular processes far beyond the originally described cell cycle and proliferation (Rotheneder et al., 2007).

The E2F family comprises eight different genes (E2F1-8) in mammals, which code for nine distinct proteins (Polager et al., 2008 ; De Gregori et al., 2006). All members of this transcription factors contain a DNA-binding domain. E2F1–5 have a transactivation domain which enables the activation of gene expression. Additionally E2F1–6 comprise a dimerization domain which is necessary for their interaction with a member of the dimerization-partner family (DP1–DP4). This interaction enables them to bind DNA and function as transcriptional regulators. E2F1, E2F2 and E2F3a, are named “activator E2fs’” they are believed to function mostly in gene expression activation and they interact only with pRB. E2F3b, E2F4 and 5, on the other hand, are repressors and are believed to be capable of interacting with the other pocket proteins. The main role of E2F6–8 is repression of gene expression, they do not have any interaction with pocket proteins and are mostly regarded as the ‘repressor E2Fs’ (Polager et al., 2008; Alonso MM et al., 2008).

E2F-1, -2, and –3 can function as oncogene products (Xu etal., 1995), and E2F-1 is also known for its role as a tumor suppressor (Field et al., 1996; Yamasaki et al., 1996). This can be described by the capability of E2F-1 in inducing apoptosis, for instance by activating the expression of the tumor suppressor protein p14ARF (Bates et al., 1998).

E2F1 can either induce cell proliferation or cell death. Signal from DNA damage can lead E2F1 to apoptosis through p53-dependent and p53-independent pathways, (Rotheneder et al., 2007) whereas signal-transduction pathways, such as the phosphatidylinositol 3 kinase (PI3K)–protein kinase B (Akt) and epidermal-growth-factor receptor (EGFR)–ras pathways play a role in inhibition of E2F-induced apoptosis (Polager et al., 2008).



Upstream signals direct E2F1 to proliferation or apoptosis. Mitogenic signals and DNA damage activate different signaling pathways that determine whether E2F1 activity will induce transcription of proliferative or apoptotic genes. Mitogenic stimuli elevate cyclin-D levels, leading to repression of pRB activity via phosphorylation by cyclin D–CDK4 or cyclin D–CDK6 complexes. Subsequently, E2F1 is free to activate proliferative or apoptotic genes. When mitogenic signals also switch on the PI3K–Akt pathway or, in some situations EGFR–Ras–Raf signaling, the apoptotic activity of E2F1 is inhibited and it induces mainly proliferation. In response to DNA damage, signals direct E2F1 to activate its apoptotic target genes: pRB acetylation specifically releases E2F1, and not other E2Fs, to enable induction of apoptosis; E2F1 is phosphorylated by ATM and Chk2 and also acetylated these modifications direct it preferentially to its apoptotic target genes. In addition, binding of Jab1, an E2F pro-apoptotic co-factor, enhances the apoptotic activity of E2F1. Conversely, SirT1 is induced after DNA damage and it can inhibit the apoptotic functions of E2F1 (Polager et al. 2008).

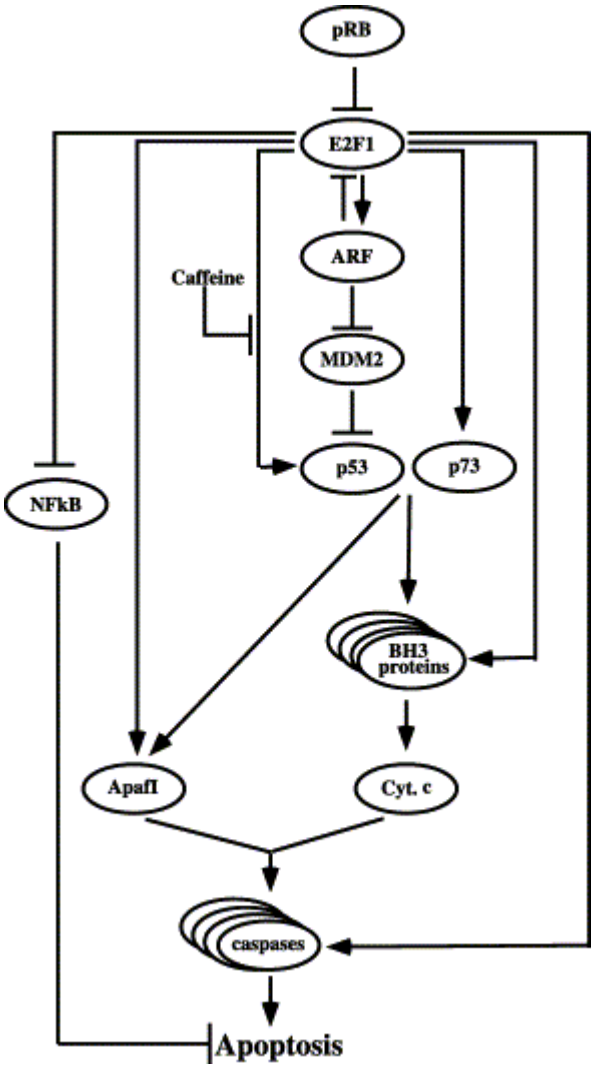
2.2.1 The role of p53 in E2F1-induced apoptosis

Accumulation of p53 is induced by ectopic expression of E2F1 (Kowalik et al., 1995; Hiebert et al., 1995) and direct transactivation of p14ARFtumor suppressor gene by E2F1 is one mechanism underlying this phenomenon (Bates et al 1998; Robertson et al., 1998). ARF has an interaction with the Mdm2 E3 ubiquitin ligase and inhibits the ability of Mdm2 to target p53 for ubiquitination and subsequent degradation (Weber et al., 1999). Therefore, Increase in ARF levels induced by E2F1, leads to activation and stabilization of p53 (Ginsberg et al., 2002).

Additionally, there are also ARF-independent functional links between E2F1 and p53 whereby E2F1 can increase p53 levels and p53-dependent apoptosis also in the absence of ARF. Recently a number of studies show that E2F1 over-expression or pRB inactivation leads to apoptosis that is inhibited by loss of p53 but not by loss of ARF (Russel et al., 2002; Tolbert et al., 2002; Tsai et al., 2002). E2F1 has an interaction with p53 via the cyclin A binding domain of E2F1 and this interaction increases the apoptotic activity of p53 (Hsieh et al., 2002). This function of E2F1 is shared by E2F2 and E2F3 (Ginsberg et al., 2002; Hsieh et al., 2002).

2.2.2 E2F1 sensitizes cells to apoptotic stimuli

In a variety of cell types over-expression of E2F1 followed by treatment with ionizing radiation or chemotherapeutic drugs such as the topoisomerase II inhibitors, etoposide and adriamycin sensitizes cells to apoptosis (Nip et al., 1997; Pruschy et al., 1999). This increased level of E2F1 protein is due to protein stabilization which is mediated by an ATM-induced phosphorylation (Lin W.C. et al., 2001). Currently, the molecular mechanisms which underlie the capability of E2F1 to sensitize cells to apoptotic stimuli are not completely understood. However, it is significantly established that the pRB pathway is functionally inactivated in most human cancers leading to deregulation of E2F activity. Thus the increased sensitivity of tumor cells to radio- and chemotherapy might be due to the capability of E2F to induce apoptosis in reaction to genotoxic stress (Ginsberg et al., 2002).



Pathways of E2F1 induced apoptosis (Ginsberg et.al.2002).

2.3 E2F and the pocket protein family

The gene regulation of E2F results from a complex mechanism which enables the cell cycle to enter S phase and begin DNA replication only under approving conditions. For example, if genomic DNA sustained damages and requires repair, cell cycle will not proceed to S phase and in this way the accumulation of genetic defects within the cellular genome might be prevented. In this context, the regulators of E2F activity are the members of Rb family, which are capable of binding to E2F and preventing it from interacting with the promoter region of those genes which are crucial for S phase entry (Sun et al., 2007).

The pocket protein family consists of three members, pRb (pRb1/p105), p107 and p130 (pRb2). They are all nuclear proteins and operate mainly as regulators of the

cell cycle, although several studies presume that pocket proteins are also involved in development and differentiation (Lipinski and Jacks, 1999; Thomas et al., 2001).

The retinoblastoma susceptibility gene was identified as the first tumour suppressor gene. pRB, The product of this gene, is known as the negative regulator of cell proliferation. Active pRB has interactions with many nuclear proteins including numerous transcription factors and chromatin associated proteins (Morris et al., 2001; Goodrich et al., 2003 and 2006). The E2F transcription factors are the best-known targets of pRB (Bandara et al., 1991; Chellapan et al., 1991; Chittenden et al., 1991). Both pRB and pRB family members inhibit E2Fmediated activation and enhance E2F mediated repression. pRB family members are capable of repressing E2F dependent transcription and this mechanism is controlled by Cyclin -dependent kinases (CDKs) (van den Heuvel et al., 2008).

The three pocket proteins show similarities especially in the A and B pocket but there are also obvious distinctions. p107 and p130 have much closer similarity to each other than either to pRB. The expression pattern of pocket proteins varies, p107 is highly expressed in cycling cells and p130 is expressed at higher levels in cells that have exited the cell cycle (Classon et al., 2002; Mcpherson D., 2008).

In quiescent cells (G0) and cells in early G phase, pRb2/p130 is highly expressed (Cobrinik et al., 1993; Smith et al., 1996) it interacts mainly with E2F4 and to a less extent with E2F5 (Hijmans et al., 1995; Vairo et al., 1995; Sun et al., 2007). This complex first binds to the promoter regions of the genes required for S phase entry. pRb2/p130 and pRb/p107 mediate the transcriptional expression in G0 and early G1. In addition to that, there is another mechanism provided by pRB/p105, which represses the expression of genes inducing S phase entry. pRb/p105 is expressed at moderate and stable levels during the cell cycle in contrast to pRb2/p130 and pRb/ p107 (Buchkovich et al., 1989; Chen et al., 1989; Decaprio et al., 1989; Mihara et al., 1989).

Pocket proteins interfere with the E2F transactivtion domain and in this way can inhibit the E2F transactivation activity directly. They also build complexes with histone deacetylases, histone methyltransferases, histone demethylases, and other chromatin modulators, which act to confer a repressive chromatin state around E2F target genes (Mcpherson D., 2008). These chromatin-remodelling factors cause Histon deacetylation, which results in chromatin condensation, which is not permissive for transcriptional activity. Naturally this suppresses the expression of those genes that are needed for entering into S phase (Sun et al., 2007). It has been shown that pRB, besides controlling the transition from G1 to S phase, also regulates

ribosome biogenesis and in this way directly connects cell growth to cell proliferation. Genetic alteration involving the pathway leading to pRB inactivation, such as *RB1* mutation or deletion, *INK4a* mutation, deletion or gene silencing and cyclin D1 or Cdk4 overexpression, are observed regularely in human cancers (Sherr et al., 2002; Montanaro et al., 2008).

2.4 EAPP, a novel E2F binding protein

EAPP was verified as a novel E2F-binding protein by means of a yeast two-hybrid interaction system utilizing the N-terminal domain of E2F-1 as the bait. Due to the fact that this protein is highly phosphorylated, it was named EAPP (e2F-associated phosphoprotein). EAPP is localized in the cell nucleus and interacts with the E2F members 1-3, but not with E2F4. It was observed that EAPP is present during the cell cycle but vanishes during mitosis. An identical increase of cells in S phase was resulted when EAPP was over-expressed in U2OS cells, whereas knocking down of EAPP mediated by RNAi diminished the fraction of cells in S-phase. Coming to the conclusion, EAPP controls E2F-regulated transcription and stimulates cell proliferation (Novy et al, 2005).

On some promoters (e.g murine thymidine kinase promoter) that have just one E2F binding site but an Sp-1 binding site in addition (Ogris et al 1993) (Sp-1 also works synergistically with E2F) the activation by only E2F-1 alone was not that strong but the synergistic effect of E2F-1 and EAPP was much stronger than with the artificial E2F-promoter (Novy et al, 2005). As for p14 promoter it was shown that E2F-1 over-expression resulted in a strong activation, but contrary to all other examined E2F controlled promoters, a co-expression of EAPP caused a p14-repression, showing that EAPP can also be a transcriptional repressor (for p14 repression EAPP does not need to be mediated by E2F).

It was also examined if EAPP levels change during the cell cycle. Surprisingly the only phase of fluctuation was identified in mitotic cells, where EAPP disappeared in mitosis, showing that EAPP in this phase might interfere with the completion of the cell cycle. The mechanism of the EAPP degradation is not yet known. In M/G1 EAPP reappeared. In all other phases throughout the cell cycle EAPP levels remained rather constant (Novy et al, 2005).

EAPP might interfere with the degradation of E2F-1 via the proteasome pathway, as a slight increase of E2F-1 levels was found in cells transiently over-expressing EAPP

(Novy et al 2005).This could be a result of the repression of the p14 mediated E2F-1 degradation and/or enhanced E2F-1 promoter activity.

2.5 Histon acetylation

Intracellular and external mutagens attack DNA constantly. Sites of DNA damage need to be identified precisely so that the DNA repair machinery can be assembled for the proper location. The recognition of damaged sites, recruitment of repair factors and assembly of repair “factories” is modulated by posttranslational modifications(PTMs) These PTMs comprise acetylation, phosphorylation, methylation ubiquitination and sumoylation (Aimee N. Lake et al., 2007).

Histones are a family of nuclear proteins that have interactions with DNA. This interaction results in DNA being wrapped around a core of histone octamer in the nucleosome. Histon Acetyl Transferases (HATs) mediate the acetylation of histones on lysine residues, This important mechanism has a crucial role in regulation of chromatin remodelling and gene expression. Histone deacetylase (HDAC) inhibitors are the recent category of chemotherapeutic drugs which modulate gene expression by increasing histone acetylation , and therefore resulting in chromatin relaxation and modified gene expression. In preclinical studies, it has been demonstrated that HDAC inhibitors have strong anticancer activities (Rasheed at al. 2007). Hyperacetylation of histones leads to an open modification of chromatin structure and influences transcription of the gene through accessibility of DNA to the basal transcription initiation machinery (Ogryzko et al., 1996; Grünstein et al., 1997; Struhl et al., 1998 and Pons et al., 2009).

2.5.1 Chromatin modification in response to DNA double strand break

Chromatin makes a natural barrier against access to DNA throughout recombination, transcription and damage repair. Subsequent to DNA damage, chromatin structure is modified by (i) covalent histone modifications, (ii) ATP-dependent chromatin remodeling and (iii) incorporation of histone variants into nucleosomes (Vaquero et al., 2003; Shogren-Knaak et al., 2006).

One target of the ATM kinase subsequent to DNA damage is the histone H2A-variant H2AX (Redon et al., 2002). Histone H2AX is the major isoform in yeast and a minor H2A species in mammals. It is phosphorylated at the carboxy-terminal serine 139 in somatic cells in response to damage resulting from double strand breaks (DSBs) (Rogakou et al., 1998)

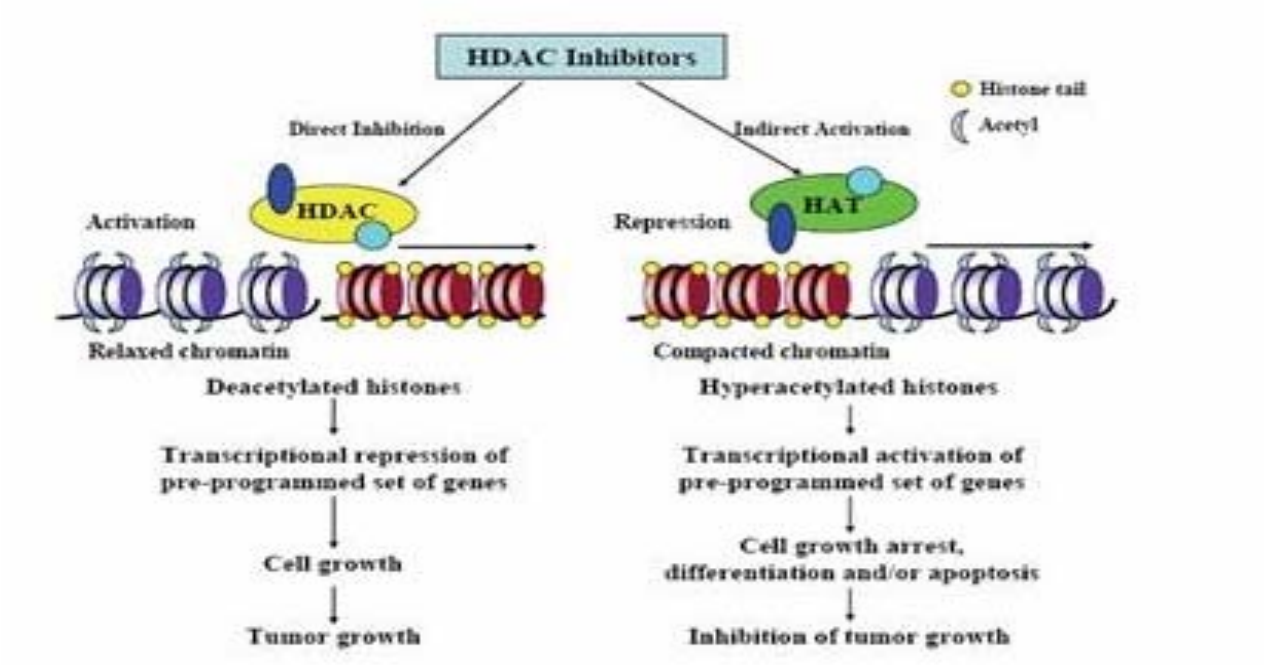
Within minutes of damage Phosphorylated H2AX (γ-H2AX) appears over large adjacent chromatin regions over tens of kilobase in yeast and up to 2 Mb in mammalian cells (Rogakou et al., 1999; Pandita et al., 2009).

2.5.2 The role of HDAC inhibitors

HDAC inhibitors, based on their structures, can be divided into four groups (Miller et al., 2003; Marks et al., 2004), comprising hydroximates, cyclic peptides, aliphatic acids and benzamides. The first discovered natural product belonging to hydroximates group was Trichostatin A (TSA) (Yoshida et al., 1990). Low concentration (nM) of TSA can result in cell differentiation and inhibit growth in tumors, with a tiny influence on normal cells (Marks et al., 2005). TSA causes cell cycle arrest or apoptosis by delaying the transition of G1/S phase (Mukhopadhyay et al., 2006). Despite the existence of strong evidence that TSA, as a common HDAC inhibitor, has a wide range of anti-cancer effects; it has not so far been used in the clinical trials, possibly because of its unrecognized potential side effects (Pan et al., 2007).

Histone deacetylase (HDAC) inhibitors have been employed over the last decade as an anti-proliferative strategy targeting solid or hematopoietic malignant disorders (Kelly et al., 2005).

Histone deacetylase (HDAC) inhibitors have been used as an anti-proliferative strategy targeting solid or hematopoietic malignant disorders over the last decade(Kelly et al., 2005; Glauben R. et al., 2009). They repress the pro-inflammatory cytokines which can lead to apoptosis (Glauben R. et al., 2006).



Molecular mechanism of HDAC inhibitors in anticancer effects. Transcriptional repression in chromatin with HDAC can lead to cell growth and tumor growth; transcriptional activation in chromatin with HAT can lead to cell growth arrest, differentiation and/or apoptosis and inhibition of tumor growth. HDAC inhibitor can directly inhibit HDAC and indirectly activate HAT (Bi et al., 2006).

There are many evidences which indicate that histone hypo-acetylation causes repression of tumor suppressor gene expression. Small molecular inhibitors of HDAC (HDACI) are very efficient in up-regulating the gene expression of tumor suppressor, resulting in tumor growth reduction and inducing programmed cell death (Liu et al., 2006).

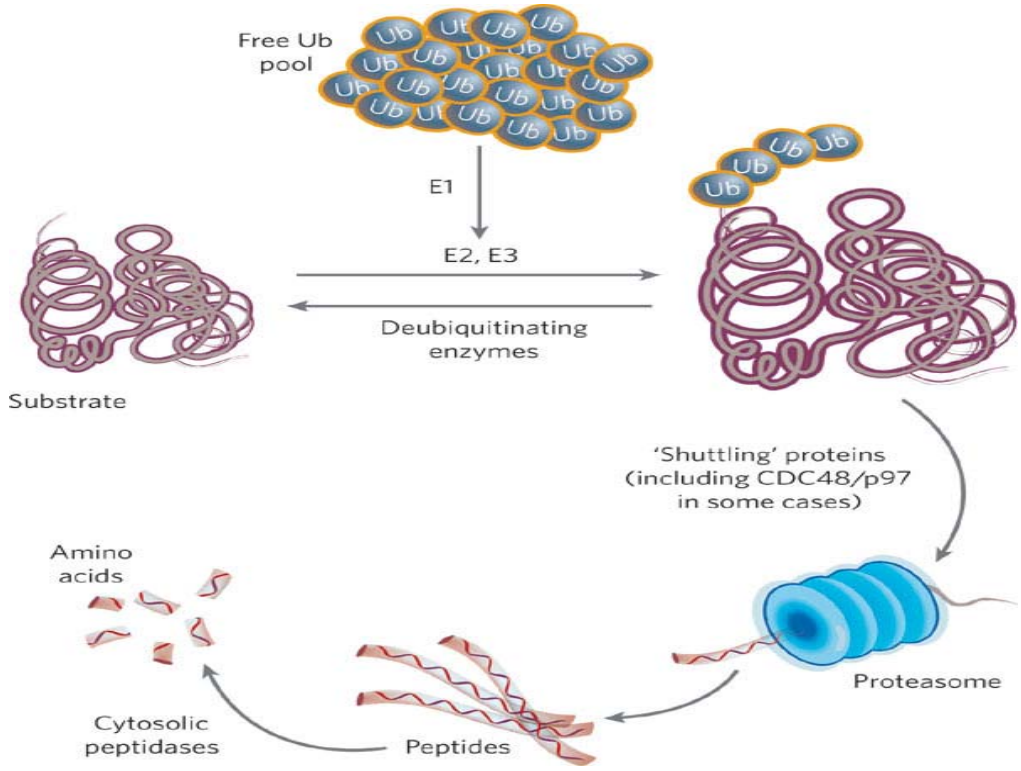
Repressing HDAC activity and preventing the deacetylation of histone may result in hyperacetylation of histone, then unfolding the chromatin and promoting transcription factors to bind to DNA and in this manner, genes which are inhibited can be expressed (Brown et al., 2002). HDAC enzymes eliminate the acetyl group from the histone (hypoacetylation), thereby decline the space between the nucleosome and the DNA wrapped around it, reducing transcription factor access and inducing transcriptional repression (De Ruijter et al., 2003; Bi et al., 2006).

2.6 Protein stability

Intracellular proteins are turning over extensively, this process is specific, and the stability of many proteins is controlled induvidually and can change under various conditions. Proteolysis of cellular proteins is a temporally regulated and tightly controlled process which plays major roles in broad array of basic pathways. Among these processes are cell cycle, development, differentiation, regulation of transcription, antigen presentation, signal transduction, receptor-mediated endocytosis, quality control, and regulation of different metabolic pathways. The stability of all cellulare proteins can be influenced by alteration in pathophysiological conditions, such as starvation or re-supplementation of nutrients (Aaron Ciechanover, 2007).

2.6.1 Protein degradation

In eukaryotic cells the ubiquitin–proteasome and autophagy–lysosome pathways are the two main routes of protein and organelle clearance. Short-lived nuclear and cytosolic proteins are mainly degraded by proteasomes which are barrel-shaped multiprotein complexes (Ciechanover, A. 2006; David C. Rubinsztein 2006).



Schematic diagram of the ubiquitin–proteasome system. Before they are targeted for proteasomal degradation, most proteins are covalently modified with ubiquitin (Ub). Typically, three enzyme types are involved in this process — ubiquitin-activating (E1), ubiquitin-conjugating (E2) and ubiquitin ligase (E3) enzymes. Proteins tagged with chains of four or more ubiquitins are shuttled to the the proteasome by various proteins such as CDC48/p97. In the proteasome, proteins are reduced to peptides, which are then released into the cytosol and further broken down by peptidases (David C. Rubinsztein 2006).

Most proteins are targeted for proteasomal degradation after being covalently moderated with ubiquitin, which is conjugated through its carboxy terminus, usually to lysine residues. Three different kinds of enzymes involve in this conjugation: E1 is an ubiquitin activating enzyme. Hydrolyses ATP and makes a thioester-linked conjugate between itself and ubiquitin.; E2 which is an ubiquitin-conjugating enzyme forms a similar thioester intermediate with ubiquitin receiving it from E1; and E3 as ubiquitin ligase transfers the ubiquitin to the substrate binding both E2 and the substrate (Richly H. et al., 2005; Weihl et al., 2006; Rubinsztein DC. 2006).

Transcription can be influenced by Ubiquitin conjugation through different mechanisms (Muratani et al., 2003). Many transcription factors are ubiquitinated and degraded by the proteasome. In fact, in many cases,. , transcriptional activation domains and signals for Ubiquitin conjugation directly overlap. Ubiquitination and proteolysis of repressors even may stimulate transcriptional activity and reset a promoter for further rounds of transcription (Lipford et al., 2005; H.Lecker et al. 2006).

Aims of the project

Considering the fact that EAPP interacts with different proteins playing important roles in the cell cycle (Ludwig Schwartzmayer unpublished data), different EAPP truncations should be examined for interactions and post-translational modifications to identify the regulatory domains of this protein.

Recognition of the sumoylation and acetylation sites of EAPP was planned based on the unpublished data of Peter Andorfer demonstrating the fact of post-translational modifications of EAPP.

Identification of the protein domain playing a role in the stability of EAPP was also among the questions to be answered.

3. RESULTS

3.1. Generation of U2OS cells stably expressing EAPP truncations

Considering the fact that EAPP interacts with different proteins playing important roles in the cell cycle (Ludwig Schwartzmayer unpublished data), this experiment was done with the aim of revealing the binding site of interacting proteins with EAPP.

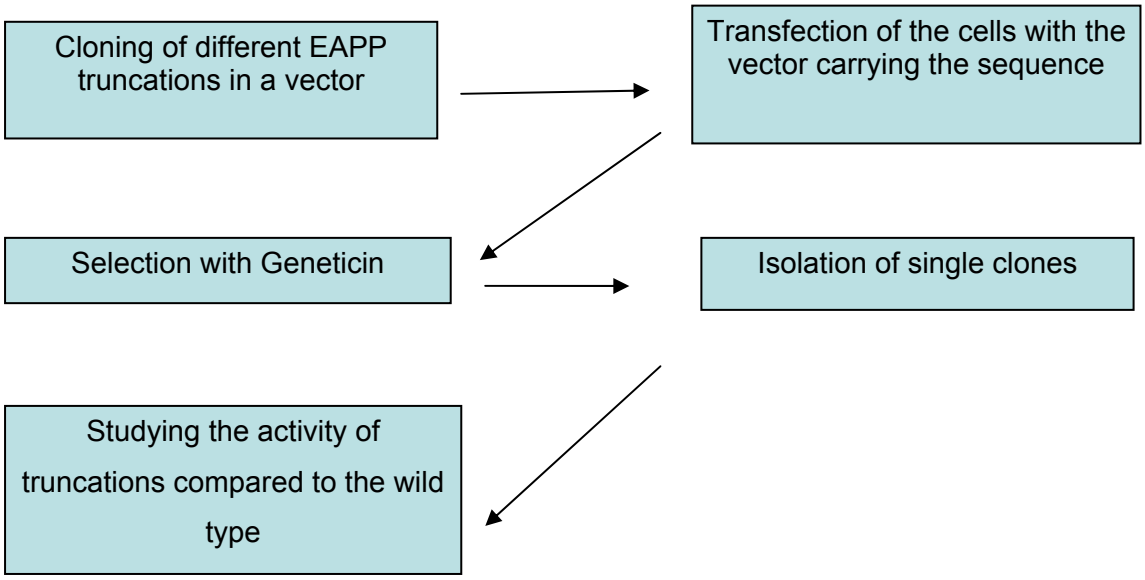
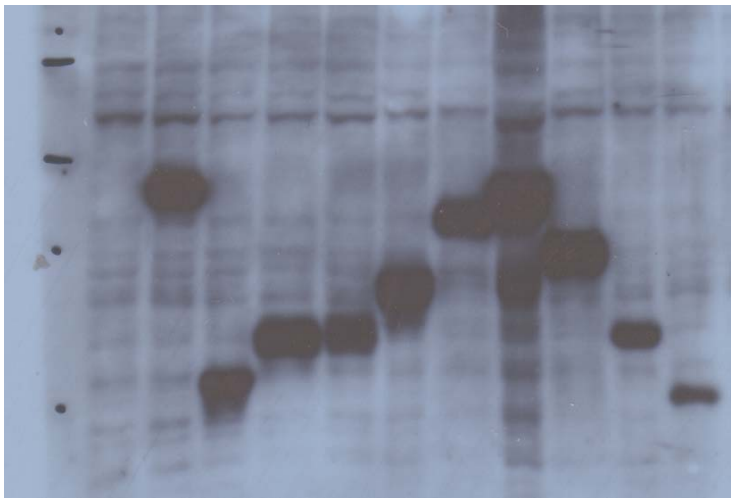


Fig.3.1.1. Different EAPP truncations were cloned in a vector. Then the U2OS cells were transfected with the vector carrying the sequence. The single clones were isolated after selection with geneticin and western blotting was performed with the cell lysate to study the activity of the truncations.

M 1 2 3 4 5 6 7 8 9 10 11

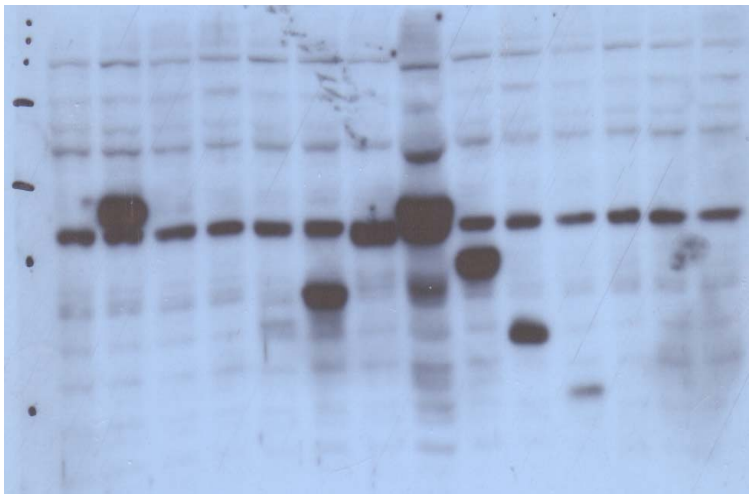


1. U2OS
2. HA-EAPP
3. 1-120
4. 1-140
5. 1-160
6. 1-180
7. 1-240
8. 1-266
9. 55-C
10 .95-C
11. 135-C

Fig.3.1.2: Western blot with α HA, the stably expressed levels of different EAPP truncations (as shown in the box) were studied in this figure. All the truncations have a HA tag and the expression could be followed by anti HA antibody in western blot. U2OS were loaded as a negative control.

All the EAPP truncations were stably expressed, confirming the expected weight and size.

M 1 2 3 4 5 6 7 8 9 10 11 12 13 14



1. U2OS
2. HA-EAPP
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4. 1-140
5. 1-160
6. 1-180
7. 1-240
8. 1-266
9. 55-C
10 .95-C
11. 135-C
12.175-C
13. 195-C
14. 215-C

Fig.3.1.3. 1E4 western blot, a western blot was performed with anti EAPP by EAPP truncations as shown in the box to study the expression of endogenous EAPP.

As it can be seen, 1E4 antibody recognized the truncations 1-180, 55-C, 95-C and 135-C indicating that the recognition site of 1E4 is between135-180.

The protein amounts were higher in truncations 1-180, 55-C and 95-C compared to endogenous EAPP.

3.2 Effect of drugs influencing cell cycle on EAPP truncations

The fact of post translational modification and among that, putative sumoylation of EAPP (Peter Andorfer unpublished data), brought up the idea of finding these sumoylation sites on EAPP. The U2OS cells, stably expressing different EAPP truncations, were treated with Iodoacetamide which is the inhibitor of isopeptidases that remove sumo moieties from the protein.

They were also treated with TSA which is a histon deacetylase inhibitor and also with Etoposide, which inhibits topoisomerase II and leads to DNA double strand break. With this experiment the question was whether the truncations are differentially affected by different drugs.

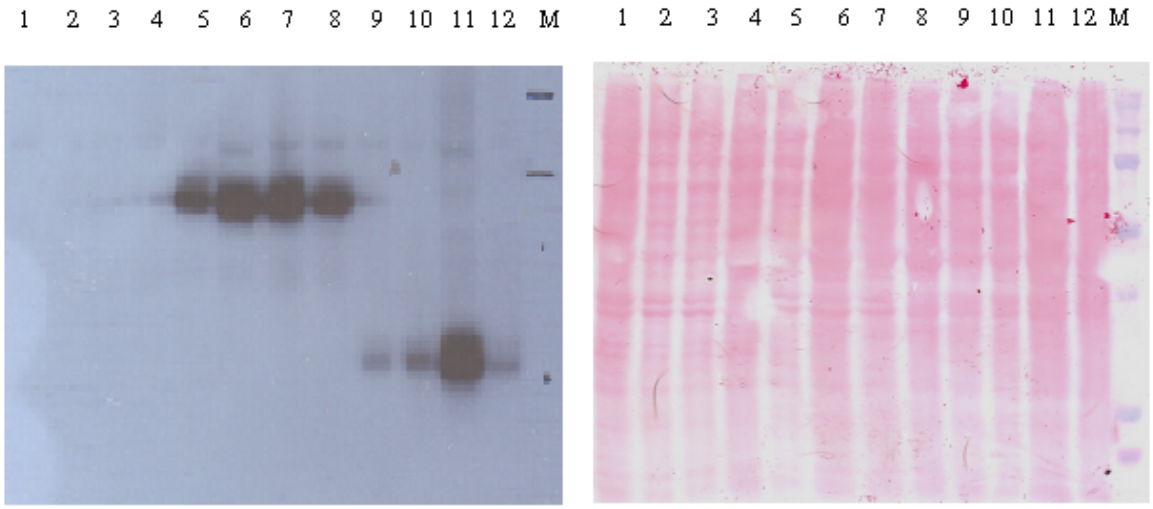


Fig.3.2.1 α-HA Western blot, a western blot was performed with anti HA, by EAPP truncations tagged with HA and treated with different drugs, as shown in the box, to examine if they are differentially affected by these drugs. On the right, the ponceau stain belonging to this blot is shown.

1. U2OS WT
2. U2OS+etoposide
3. U2OS+TSA
4. U2OS+iodoacetamide
5. HA EAPP normal
6. HA EAPP+etoposide
7. HA EAPP+TSA
8. HA EAPP+iodoacetamide
9. 1-120 normal
10. 1-120+etoposide
11. 1-120+TSA
12. 1-120+iodoacetamide

Comparing the signal strength with the protein amount shown in ponceau stain, EAPP 1-120 showed higher levels of protein after treatment with TSA.

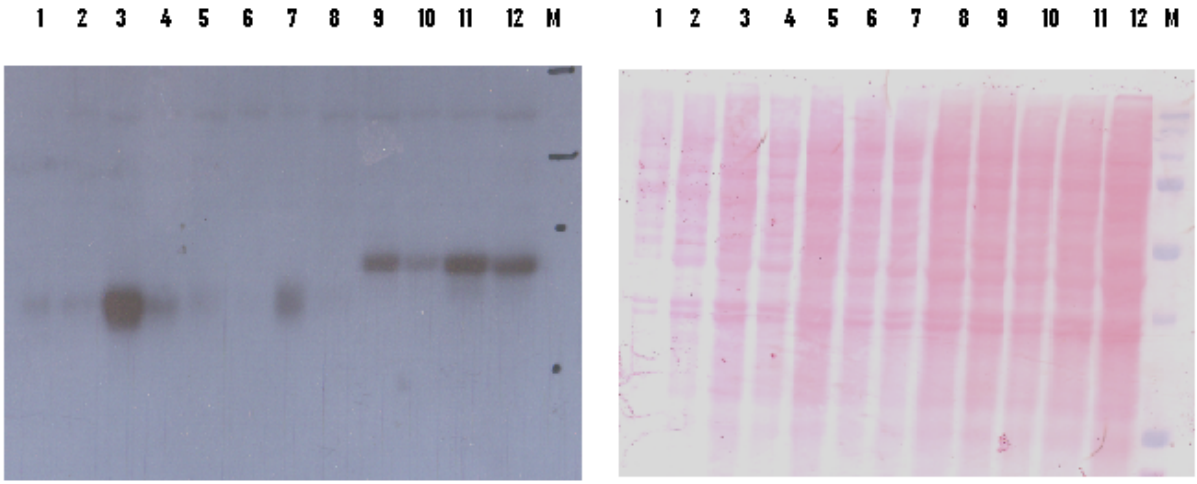


Fig.3.2.2 α-HA Western blot a western blot was performed with anti HA, by EAPP truncations tagged with HA and treated with different drugs, as shown in the box, to examine if they are differentially affected by these drugs. On the right, the ponceau stain belonging to this blot is shown.

1. 1-140 normal
2. 1-140+etoposide
3. 1-140+TSA
4. 1-140+iodoacetamide
5. 1-160 normal
6. 1-160+etoposide
7. 1-160+TSA
8. 1-160+iodoacetamide
9. 1-180 normal
10. 1-180+etoposide
11. 1-180+TSA
12. 1-180+iodoacetamide

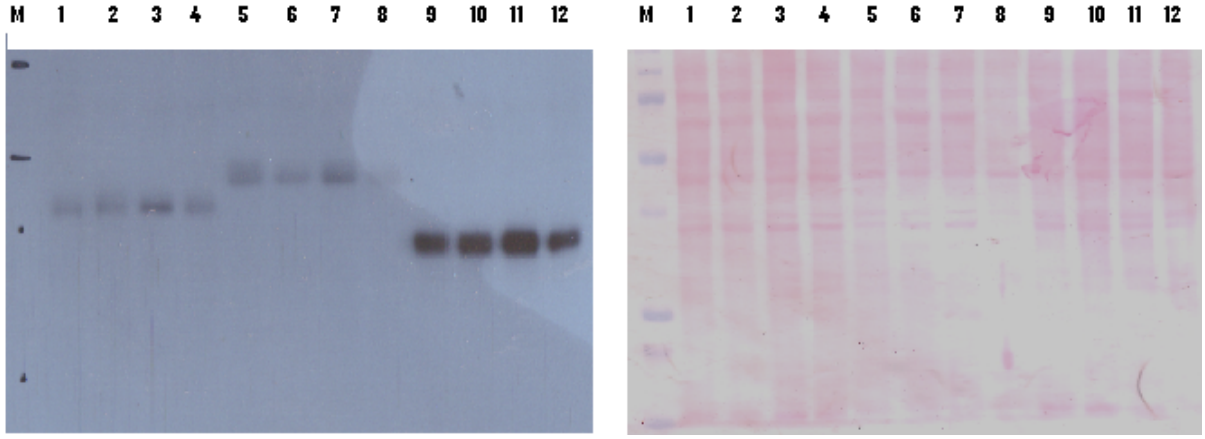


Fig.3.2.3 α-HA Western blot a western blot was performed with anti HA, by EAPP truncations tagged with HA and treated with different drugs, as shown in the box, to examine if they are differentially affected by these drugs. On the right, the ponceau stain belonging to this blot is shown.

1. 1-240 normal
2. 1-240+etoposide
3. 1-240+TSA
4. 1-240+iodoacetamide
5. 1-266 normal
6. 1-266+etoposide
7. 1-266+TSA
8. 1-266+iodoacetamide
9. 55-C normal
10. 55-C+etoposide
11. 55-C+TSA
12. 55-C+iodoacetamide

Comparing the results of this part, higher protein amounts of some truncations (1-120, 1-140, 1-160) were distinguished after treatment with TSA but this effect was not observed with other truncations.
This difference in protein amount can either depend on levels of protein translation and mRNA transcrioption or might depend on stability of the protein at this part.

3.3 Inhibition of gene expression in translational level

Comparing the results of part 3.2, higher protein amounts of some truncations (1-120, 1-140, 1-160) were seen after treatment with TSA. To examine if this difference depends on protein translation, the cells expressing full length and truncated EAPP, were treated with Cycloheximide which is an inhibitor of protein biosynthesis in eukaryotic cells.
Increased levels of protein amount can result from either higher expression or higher stability of the protein.
This experiment was performed to examine the main cause of increased level of protein.
If the induction by TSA occurs after translation then no expression was expected after treatment with cycloheximide. If the protein amount is maintained by TSA in the presence of cycloheximid, it indicates that TSA increases protein stability.

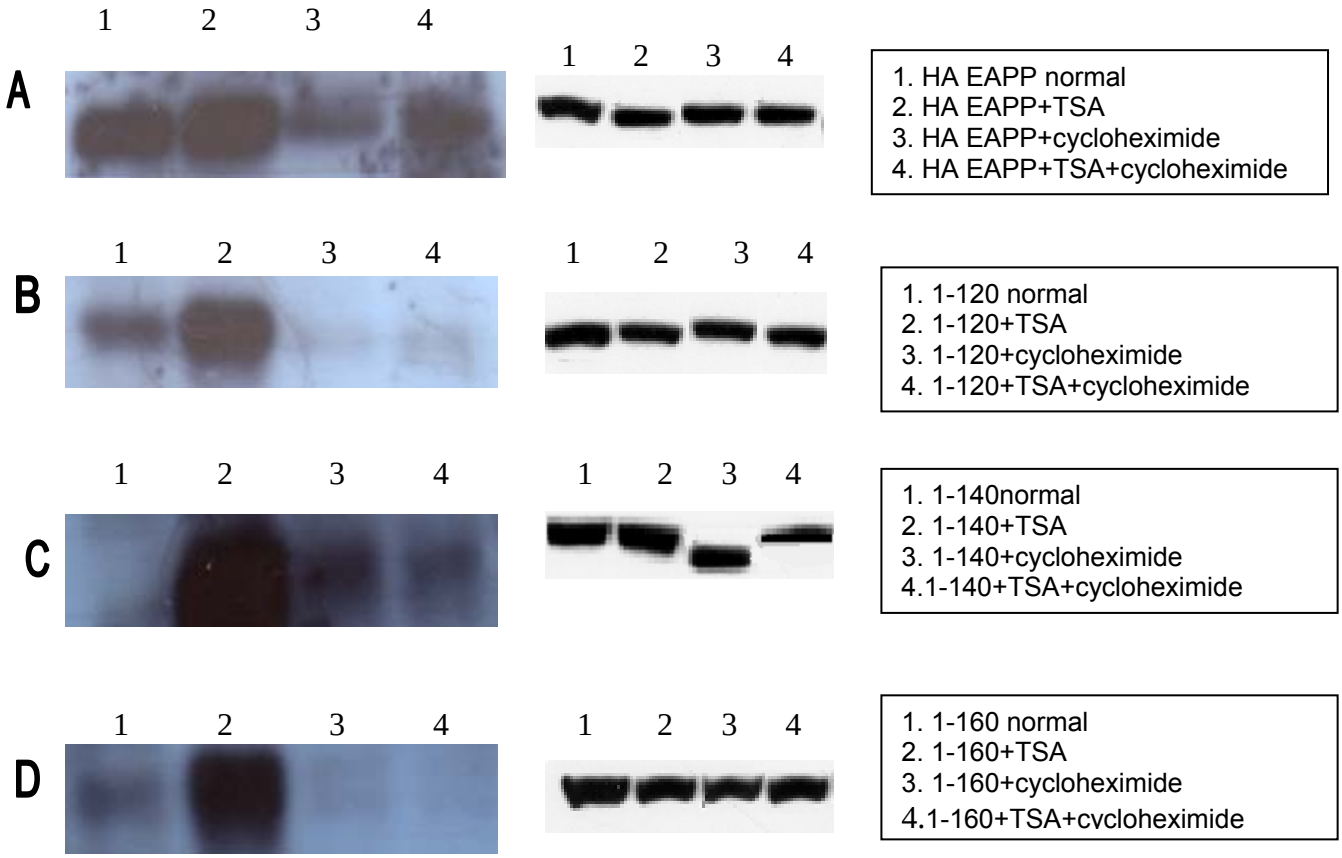


Fig.3.3.1, α-HA Western blot Fig.3.3.2, α βActin Western blot

Fig.3.3.1 A,B,C & D; western blot was performed with anti HA, by full length and truncated EAPP tagged with HA and treated with cycloheximide and TSA, as shown in the boxes.

Fig.3.3.2 A,B,C & D; western blot with anti beta Actin, as loading control.

As it can be seen in (Fig.3.3.1 B and D) the protein amount of EAPP1-120 and EAPP 1-160 were both decreased after treatment with Cycloheximide; Treatment with TSA in the presence of Cycloheximid even did not affect the maintaining of protein amount.

Full length EAPP together with EAPP1-140 (Fig.3.3.1 A and C) showed less decrease in protein amount as they were treated with Cycloheximid and surprisingly even higher levels of protein amount were observed when they were treated with both Cycloheximide and TSA which in turn can indicate that TSA-induced acetylation, accompanied with increased protein amount, can increase the protein stability.

3.4 Cell cycle analysis with flow cytometry

To examine the effects of EAPP on cell cycle, a flow cytometry was performed on U2OS cells with over expressed EAPP, Knocked down EAPP and the cells expressing truncation of EAPP, 1-140. The cells were pre-treated with Nocodazole to be arrested at G2/M phase and were then released by different time intervals.

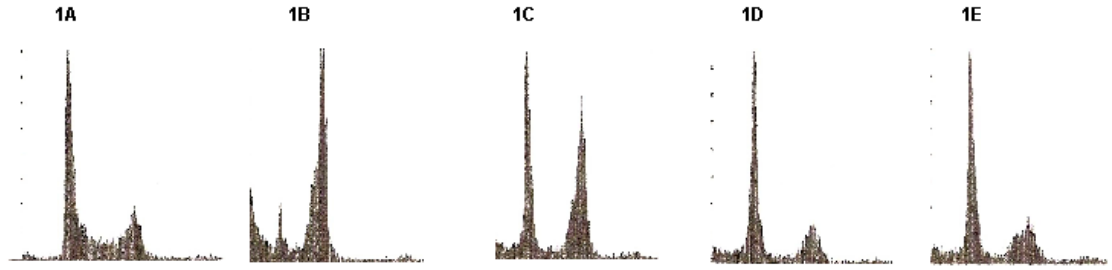


Fig.3.4.1.A U2OS cells log; 1B U2OS cells with nocodazole; 1C U2OS cells released after 3h;1D U2OS cells released after 6h; 1E U2OS cells released after 9h

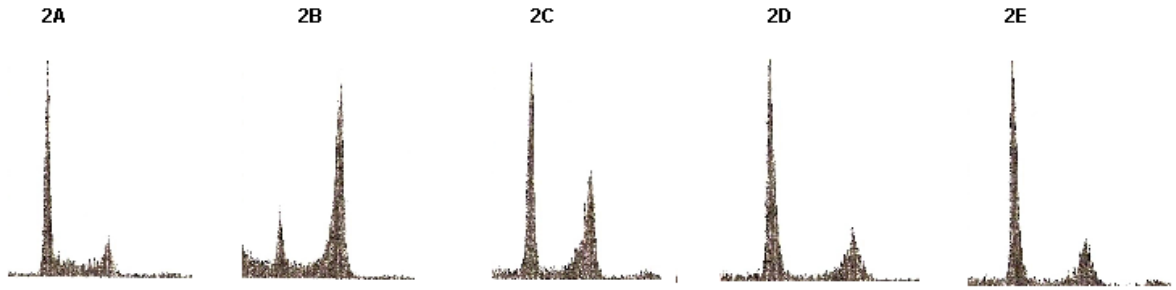


Fig. 3.4.2.A HA-EAPP log; 2B HA-EAPP with nocodazole; 2C HA-EAPP released after 3h; 2D HA-EAPP released after 6h; 2E HA-EAPP released after 9h

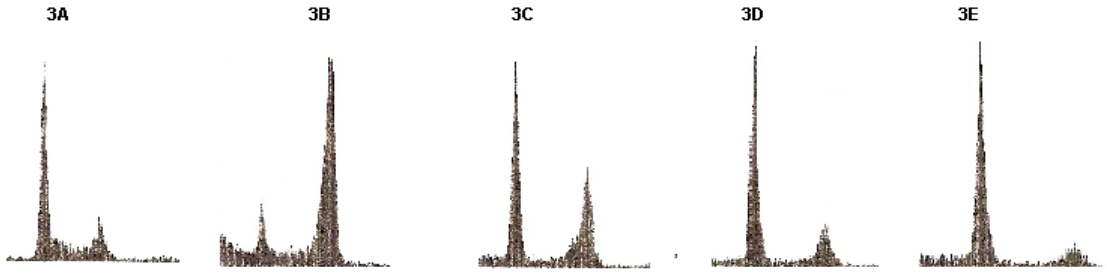


Fig.3.4.3A P-Super EAPP log; 3B P-Super EAPP with nocodazole; 3C P-Super EAPP released after 3h; 3D P-Super EAPP released after 6h; 3E P-Super EAPP released after 9h

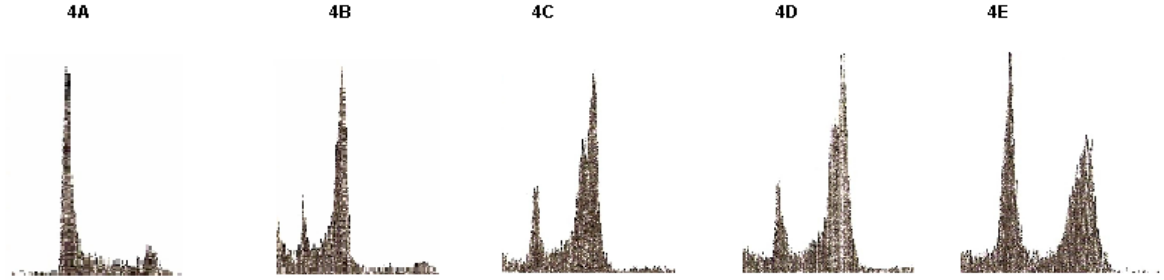


Fig.3.4.4A 1-140 log; 4B 1-140 with nocodazole; 4C 1-140 released after 3h; 4D 1-140 released after 6h; 4E 1-140 released after 9h

Varying the amounts of full length EAPP within the range of our experiments had no effect on cell cycle because no difference was observed in cell cycle profile by Fig.3.4.2 which shows the effects of over expressed EAPP and Fig.3.4.3 which shows the effects of knocked down EAPP. Controversially the truncated EAPP, 1-140, showed that the cells come out from M phase, slower than full length EAPP after release.

3.5. Effect of EAPP 1-140 on cell cycle

Flow cytometry was performed with the cells expressing EAPP 1-140 because the TSA effect was more pronounced by this truncation. The cells were pre-treated with nocodazole to arrest them in G2/M phase. Then they were treated once with and once without TSA by different time intervals after release. This experiment was done to examine if this truncation does affect the cell cycle in M phase and to see if this effect changes after release when they are treated with TSA.

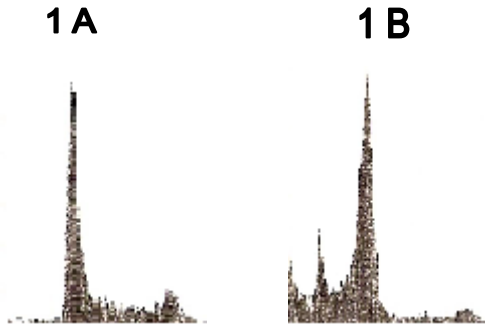


Fig.3.5.1A; 1-140 Log
Fig.3.5.1.B; 1-140 with nocodazole treatment overnight

Fig.3.5.2.A; 1-140 w/o TSA 1.5h after release
Fig.3.5.2B; 1-140 with TSA 1.5h after release

Fig.3.5.3.A; 1-140 w/o TSA 3h after release
Fig.3.5.3B; 1-140 with TSA 3h after release

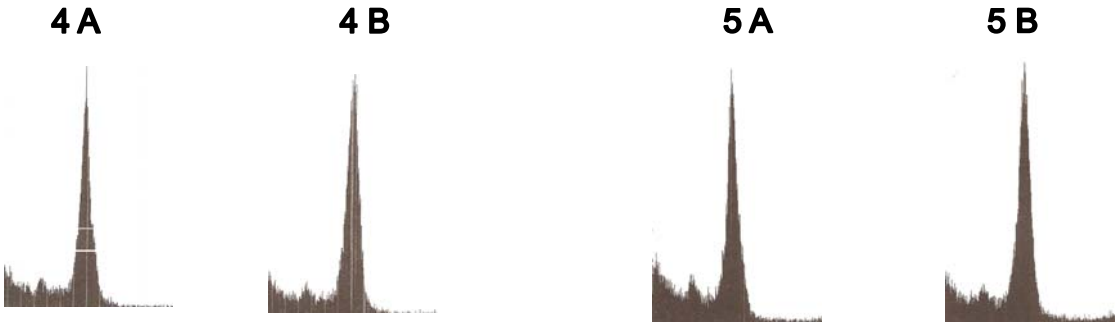


Fig.3.5.4.A; 1-140 w/o TSA 4.5h after release
Fig.3.5.4B; 1-140 with TSA 4.5h after release

Fig.3.5.5.A; 1-140 w/o TSA 6h after release
Fig.3.5.5B; 1-140 with TSA 6h after release

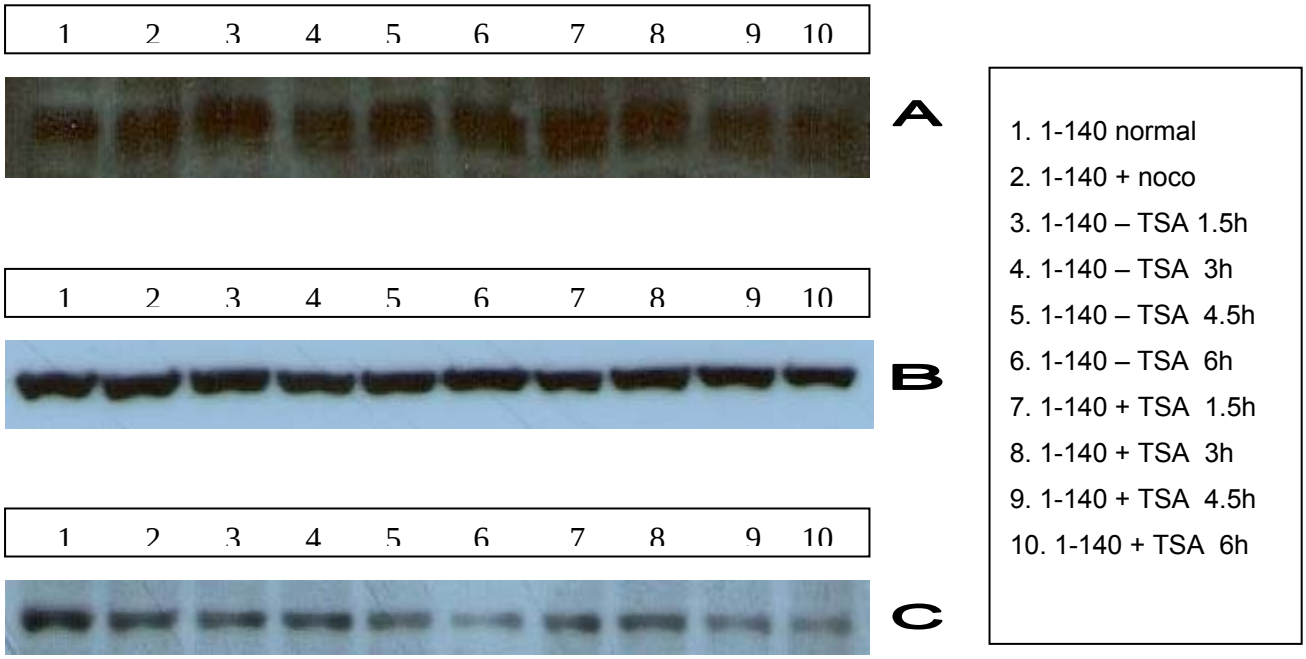


Fig 3.5.6 A, αHA Western blot; Western blot was performed with each of the FACS samples to check the levels of protein amount. They were tagged with HA and the loading map is shown in the box.
Fig.3.5.6B, α β Actin Western blot; western blot with anti beta actin as loading control.
Fig.3.5.6 C, 1E4 (α EAPP); western blot with anti EAPP to check the levels of endogenous EAPP

Generally the truncation EAPP 1-140 took much longer to re-enter the cell cycle after the release from Nocodazole.

With a small difference, the cells without TSA treatment, shifted to S phase more than the TSA treated cells, in the first 1.5h and the last 6h after release.

Comparing the western blot analyses, the cells with and without TSA treatment showed the same protein amount after release (Fig.3.5.6A) but regarding the endogenous EAPP (Fig 3.5.6C) the cells showed less protein, the longer the intervals were after release and no strong difference was observed between the cells with and without TSA treatment.

3.6. Protein stability and protein degradation

After obtaining no adequate results to realize the reason for increased amount of expression by some TSA treated EAPP truncations (1-120, 1-140 and 1-160), it was thought that maybe the acetylation of EAPP has some effect on protein stability and not necessarily the protein biosynthesis results in increased levels of expression. Therefore the next step was to check the protein stability with “pulse chase” method. The truncation 1-140 by which the effects of treatment with TSA was more pronounced, together with other truncation of EAPP which covers the C terminal of the protein, 55-C and also the full length HA-EAPP were put through this method. Each of the samples was treated with and without TSA. Stability of all forms was evaluated by labelling for 30 min with [^{35}S]methionine/cysteine-containing medium and a subsequent chase with medium containing non-radioactive methionine and cysteine.(Klenk et. al. 2006) The time intervals for harvesting were 5min, 1h and 3h after “chase” phase.

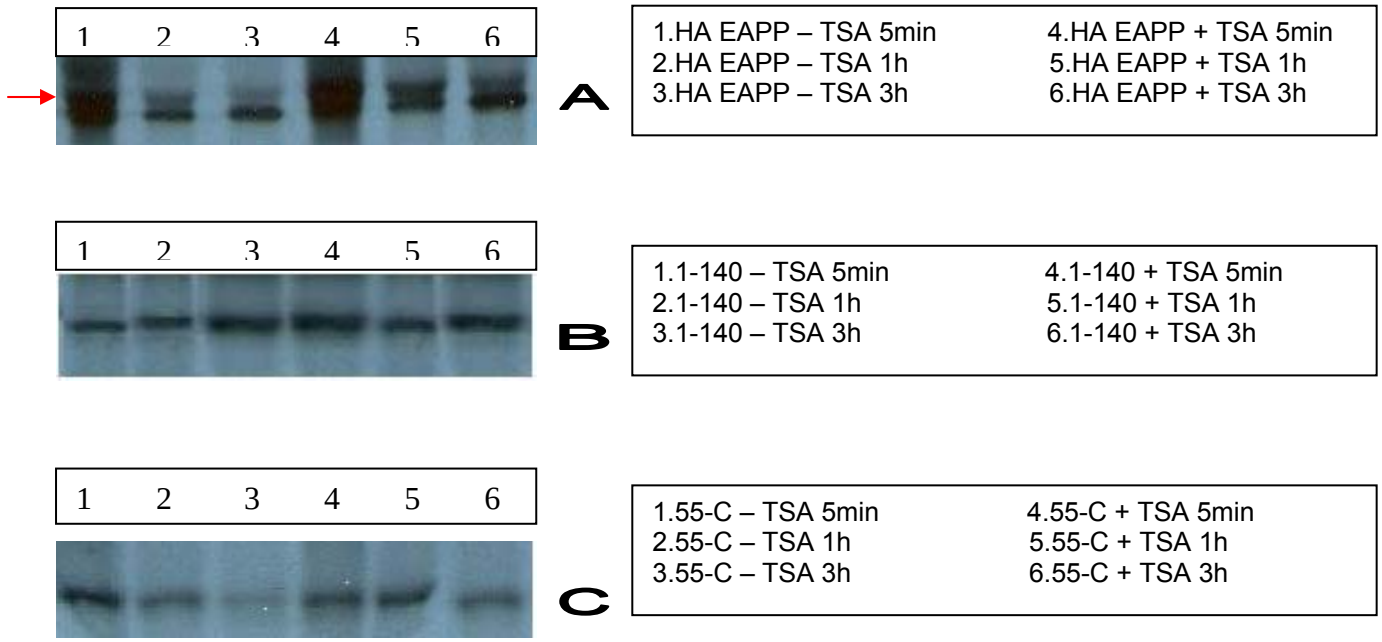


Fig.3.6.1 A,B & C; full length EAPP and truncated EAPP treated with and without TSA, harvested by different time intervals of 5 min, 1h and 3h after chase phase. Western blot was performed with αHA. The loading map is shown in the box.

As it is shown in Fig. 3.6.1C, it seems that the C terminus of the protein (55-C) is less stable than the N terminal, where 1-140 belongs. Generally the protein had shown a longer half life when it was treated with TSA indicating that acetylation regulates its stability. Amazingly the truncation 1-140 showed the most stability.

4. DISCUSSION

4.1. U2OS cells stably expressing EAPP truncations

4.1.1 Western blot analysis

The production of cells, expressing different EAPP truncations, as it was explained in chapter 3.1, worked properly and the results shown Fig.3.1.2 did confirm our anticipation, considering the weight and size of each truncation.
As it can be seen in Fig.3.1.3, the antibody 1E4 recognizes EAPP at the truncations 1-180, 55-C, 95-C and 135-C confirming that the recognition site of 1E4 should be between135-180.

4.2 Post translational modification of EAPP

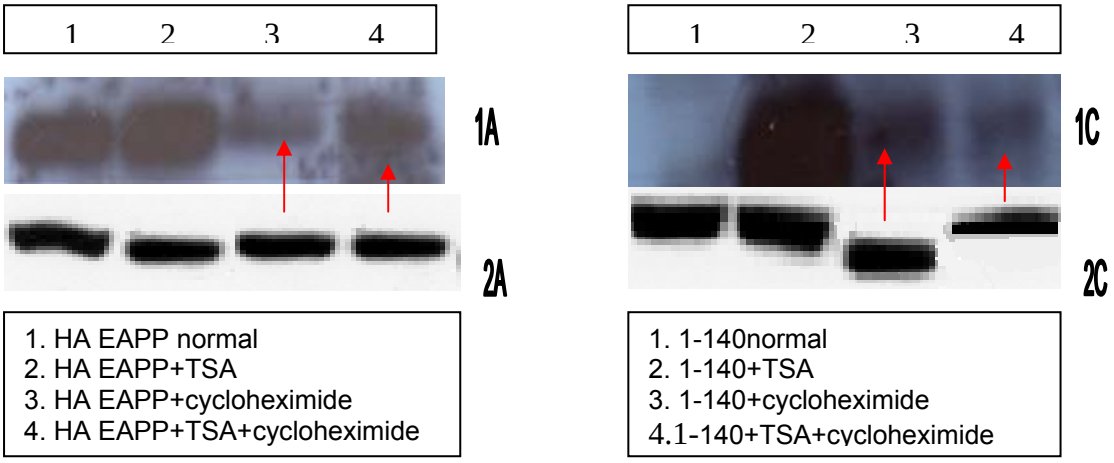
As it can be seen in figures of part 3.2, some of TSA treated EAPP truncations showed higher levels of expression. Trichostatin A (TSA) is a well-characterized histone deacetylase (HDAC) inhibitor. TSA is known to modify the balance between histone acetyltransferase and HDAC activities that induce histone hyperacetylation and regulate gene expression (Sung-Hye Kim et al. 2010). HDACs class I and class II, are TSA-sensitive and form a multiprotein repressor complex to remove the acetyl group from lysine residues of histones (Yang XJ et al. 2007). Recently, the effect of TSA in acetylation/ deacetylation of nonhistone proteins has been demonstrated as a diverse regulatory event, including ubiquitination/proteasomal degradation. (Sung-Hye Kim et al. 2010).
Regarding Fig.3.2.1 and 3.2.2, two truncations, 1-120 and 1-140 appeared to have increased level of expression in comparison to other truncations. It can somehow depend on the clonal effect and that the inserts do not always integrate in the same site of DNA. The problem can be solved by Flip in system by which the insert and the host DNA have two flanking sites on a definite part, which could be recognized by integration.

On the other hand it can also depend on amount of protein translation or may rely on protein stability which were examined in the subsequent experiments.

4.3 Inhibition of gene expression in translational level

Treatment with Cycloheximide inhibits the protein biosynthesis in the cell. Referring to Fig.3.3.1, effects of treatment was observed with EAPP 1-120 and 1-160 but surprisingly not with EAPP 1-140 and full length EAPP, although it was supposed to maintain no expression, if the induction by TSA occurs after translation.

On the other hand, the protein amount of EAPP 1-140 and full length EAPP in the presence of Cycloheximide was maintained by addition of TSA, observing higher amount of protein. Therefore it can be concluded that Trichostatin A increases acetylation and protein stability at this part of EAPP.



1A and 1C; western blot was performed with anti HA, by full length EAPP and 1-140, tagged with HA and treated with cycloheximide and TSA, as shown in the boxes.
2A and 2C; western blot with anti beta Actin, as loading control.

With truncation EAPP 1-140, TSA effect appeared to depend on stability of the protein at this part but still could also be influenced by transcription.

4.5 Effect of EAPP on cell cycle profile

4.5.1 FACS Analysis

It was proved that there are more cells in S-phase if EAPP is over-expressed. On the other hand, EAPP knock down by RNAi, reduces the fraction of cells in S-phase dramatically (Novy et al., 2005). Another result suggests that cells overexpressing EAPP abrogate nocodazole -induced arrest (Peter Andorfer, unpublished data).

In the DNA profile analyses in FACS experiments, however there was no difference shown between cells that had an EAPP knockdown and cells with an EAPP overexpression. This in turn could depend on the density of the cells, as it is indirectly proportional to the cell number in S-phase. The denser they are, the fewer cells are in S phase.
Contradicting P.Andorfer's result, the cells overexpressing EAPP, do undergo Nocodazole arrest.

U2OS cells expressing EAPP 1-140 took much longer than control cells or cells over-expressing full length EAPP to re-enter the cell cycle after the release from Nocodazole, It may be due to a clonal effect or may be exposure to stress through treatment with Nocodazole, has been highlighted in this part of protein or maybe 1-140 has interplay with mitotic control proteins which undergo depletion after arrest.

4.6 Effect of 1-140 on cell cycle

4.6.1 FACS analysis

Generally the truncation EAPP 1-140 took much longer to re-enter the cell cycle after the release from Nocodazole.
With a small difference, the cells without TSA treatment, shifted to S phase more than the TSA treated cells, in the first 1.5h and the last 6h after release.
This truncation of EAPP might have some effects on inhibition of cyclin-dependent kinase (CDK) complexes. CDK complexes regulate transitions between different phases of the cell cycle. (Bey-Dih Chang et al, 2000). Consequences of CDK inhibition include dephosphorylation of pRb and downregulation of a large group of E2F-dependent genes that are involved in DNA replication and cell cycle progression (Nevins, 1998).

Considering the DNA profile of TSA treated cells, acetylation of this truncation seems to have stronger effects on inhibition of mitotic control proteins.

4.6.2 Western blot analysis

Considering the western blot analyses with anti HA in Fig.3.5.6A, treatment with TSA in different time intervals after release, didn't affect the protein amount of EAPP.
Interestingly the amount of endogenous EAPP was descending over the time after release. Regarding the endogenous EAPP levels, a tiny difference was recognized with TSA treated cells.

4.7 Protein stability and degradation

4.7.1 Analysis with pulse chase

It seems that EAPP proteins with a deleted N-terminus (55-C) is less stable than the part containing this part (1-140). Generally the protein had shown more stability as it was treated with TSA compared to without TSA.
This result indicates the TSA affected protein stability at the N terminus. Referring to chapter.3.3, the protein level of 1-140 was sustained by TSA in the presence of Cycloheximide, indicating that TSA increases the protein stability of this truncation.
It seems that Trichostatin A enhances acetylation as well as the stability of the protein but the precise mechanism remains unclear (Sung-Hye Kim et al. 2010).
Amazingly the truncation 1-140 showed the most stability in comparison to full length EAPP and 55-C.

5. Material and Methods

5.1 Cell lines:

U2OS (ATCC: HTB-96): Human osteosarcoma cell line. The cells were grown in DMEM with 10% FCS and AB.
T98G (ATCC: CRL 1690): Human glioblastoma cell line. The cells were grown in DMEM with 10% FCS and AB.

5.2 Vectors:

pCIneo-HA-MCS: Expression vector with a Hemaglutinin-tag and a multiple cloning site
pSuper: RNAi vector based on the pBlueScript-KS vector with ampicilline resistance

5.3 Special chemicals, antibodies and solutions

ECL detection solution
Reagents:
p-Coumaric acid Sigma
3-Aminophtalhydrazide (Luminol) Fluka
Hydrogen Peroxide 30% Aldrich
Nitrocellulose Transfer Membrane Protran
PEI 25000 Aldrich
Protein-Assay Dye Reagent BIO-RAD Protein Assay
Silica Sigma
X-Ray Films Fuji Medical X-Ray Film
Antibodies:
α-EAPP polyclonal Mouse polyclonal
α-EAPP [1E4] Mouse monoclonal
α-EAPP [4A6] Mouse monoclonal
α-HA [16B12] Babco
α-β-Actin

αP53 Santa Cruz

5.4 Solutions

40% Acrylamid (100ml H_2O):	38 g Acrylamid 2 g NN´Methylenbisacrylamid add one spoon DOWEX-Ionenaustauscherharz. Store at 4°C in the dark
Antibiotics:	1000 x Ampicillin (50 mg / ml) 1000 x Kanamycin (50 mg / ml)
Blocking Solution for primary ab´s:	1 x PBS 0,1 % Tween Sodium Azide *(20 % NaN_3 = 20000 stock)* 3 % Milkpowder
Blocking Solution for secondary ab´s	1 x PBS 0,1% Thinerazol 0,5% Milkpowder
Coomassie blue:	0,25 % (w/v) Coomassie R 10 % (v/v) Aceticacid 50 % (v/v) Methanol Filtration
Destaining Solution:	10% Acetic acid 30% MeOH 60% distilled H_2O
DNA-Loading dye:	0,25 % Bromphenolblau (25 mg) 0,25 % Xylen-Cyanin (25 mg) 30 % v/v Glyzerin x ml H_2O to 10 ml
1000x Ethidium bromide:	5 mg Ethidium bromide / ml H_2O
Hunt extraction buffer	20 mM TRIS/HCL pH=8,0 100 mM NaCl

	1 mM EDTA 0,5 % NP-40 1 mM DTT 1 mM PMSF
Laemmli sample buffer:	100 mM TRIS-HCl pH=6,8 20 % Glycerol 0,01 % Bromphenolblue 10 % ß-Mercaptoethanol 5 % SDS
10 x PBS:	80 g / l NaCl 2 g / l KCl 2 g / l Kaliumdihydrogenphosphate 11,5 g / l Disodiumhydrogenphosphate pH= 7,4
Ponceau:	0,2 % w/v Ponceau S in 3 % Trichloracetic acid
10x Running buffer:	192mM Glycin 25mM TRIS 0,1 % SDS
Stripping buffer:	100mM 2-Mercaptoethanol 2 % (w/v) SDS 62,5mM TRIS-HCl pH 6,7
1x TE:	10mM TRIS/Cl 1mM EDTA pH= 8.0
5 x Transfer-buffer (2000 ml):	125mM TRIS.....30,29 g 960mM Glycin...144,13 g For 1 x Buffer add 20 % v/v Methanol prior of use.
UltraNewWash:	50mM NaCl 10mM TRIS/HCl pH=7,5 25mM EDTA 50 % v/v Ethanol

5.5 SDS-PAGE Minigel

	6 %	8 %	10 %	12 %	14 %	Stacking
40 % Acrylamid	600 µl	800 µl	1 ml	1,2 ml	1,4 ml	250 µl
ddH2O	1,85 ml	1,65 ml	1,45 ml	1,25 ml	1,05 ml	1,48 ml
1 M TRIS-Cl, pH= 8,7	1,5 ml	1,5 ml	1,5 ml	1,5 ml	1,5 ml	250 µl
20 % SDS	20 µl	20 µl	20 µl	20 µl	20 µl	10 µl
TEMED	4 µl	4 µl	4 µl	4 µl	4 µl	2 µl
10 % APS	20 µl	20 µl	20 µl	20 µl	20 µl	10 µl

6. EXPERIMENTAL METHODS

6.1 Bacterial Cultures

6.1.1 Luria Broth (LB)

1% Trypton, 1% NaCl, 0,5% Yeast-extract, (1,5% Agar-Agar for plates)
LB has to be sterilized by autoclavation

6.1.2 For Agar Dishes

1% Trypton
1% NaCl
0,5% Yeast extract
1,5% Agar
The Antibiotics (Ampiciline/Tetracycline) were supplied after cooling down the autoclaved agar to 50°C
Pour out the solution into petri-dishes

6.1.3 Storage of bacteria

0,9 ml of an overnight culture were added to the same volume of freezing buffer and stored at –80°C
2x Freezing buffer:
12.6g/l K_2HPO_4
3.6g/l KH_2PO_4
0.9g/l Na-Citrat
1.8g/l $(NH_4)_2SO_4$
0.18g/l $MgSO_4 \cdot 7H_2O$
88.0g/l Glycerol

6.1.4 Transformation of competent E.coli

Mix DNA and an aliquot of competent cells.

Keep the mixture 20min on ice

Heat shock the cells 2 min at 42°C.

Add 400µl LB and keep the cells on 37°C for 30min.

Plate the suspension on antibiotic-plates.

6.2 Mammalian cells

6.2.1 Media for mammalian cell

DMEM (10 Litre):

„Dulbeco´s modified Eagles´s Medium“ [Gibco / BRL # 52100 /039] powder solved in 5 Litre Aqua. dest. Addition of 30g $NaHCO_3$. Addition of 5 Litre aqua. dest. Sterilfiltration [Satorius P plus]. Immediately before use ad 10 % FCS (foetal calf serum) or 10 % CS (Calf serum) and AB or AB+G.

100 x antibiotic-stock (AB):

6g/l Penicillin G, Potassium salt, 10g/l Streptomycinsulfate. Antibiotics are solved in 1x PBS and sterile filtrated [0,2µm].

50 x AB+G in 1x PBS:

5,8 g / l Glutamat, 6 g/l Penicillin G, Pothassium salt, 10 g/l Streptomycinsulfat.

AB+G are solved in 1x PBS and sterile filtrated [0,2µm].

Trypsin/EDTA Solution:

500mg / 1000ml Trypsin

0,2 w/v% NaEDTA

solved in H_2O and sterile filtrated [0,2µm].

6.2.2 Storage of mammalian cells

Mix cells in media + 10 % v/v DMSO + 50 % v/v FCS. Freeze 2 hours at –20, then at – 80°C up to a view weeks, then in N_2.

6.2.3 Propagation and splitting of cells

Attached cells were grown in petri-dishes at 37°C in special chambers with waterstream saturated atmosphere containing 7,5% CO_2. For splitting confluent cells the petri-dish had to be washed once with warm 1xPBS and incubated with about 12 drops T/E for 1-10 min. The floating cells were now resuspended in complete DMEM medium to stop the activity of T/E and were diluted to the preferred density.

6-wells, 3 ml media per well (10% FCS or 10% CS)
Ø 100 mm plate, 10 ml media (10% FCS or 10% CS)
Ø 140 mm plate, 20 ml media (10% FCS or 10% CS)

6.2.4 Transfection of mammalian cells (PEI –method)

Ø 100 mm plate, 10 ml media (10 % FCS). Change media two hours prior to transfection and 24 hours after transfection.
Transfection mix:
x µg DNA in H_2O
ad 200µl 150mM HBS (steril)
Add 200µl 150mM NaCl with PEI working solution (ratio DNA(µg) : PEI(µl) ~ 1:2,5)
Vortex 10 sec and let mixture rest for 10 min
Add to cells

6-Wells	1-5 µg DNA and 200 µl HBS
Ø 100 mm plate	10-20 µg DNA and 400 µl HBS
Ø 140 mm plate	20-30 µg DNA and 1000 µl HBS

2x HBS:
280 mM NaCl
50 mM HEPES
1,5 mM Na2HPO4
PEI working solution:
42.2g PEI 25000 in 42.2g H_2O
Mix well for 4-5h

Use 87µl of this solution and add 100ml H_2O
Adjust pH to 7.0 with HCl
Steril fitration, aliquote and store at 4°C

6.2.5 Cell-extracts of mammalian cells

Wash cells two times with 1x PBS.
Resuspend the cell pellet in 5x volume HUNT-extraction buffer or 3x volume WCE-extraction buffer.
Freeze-thaw 2-3 times in liquid N_2
Centrifuge 10min with 14000rpm
Store supernatant at -80°C

HUNT-Extraction buffer:
20 mM TRIS / HCl pH=8,0
100 mM NaCl
1 mM EDTA
0,5 % NP-40
1mM DTT
1mM PMSF

6.2.6 Protein Quantification (Bradford)

For the quantification of proteins in extracts the method of Bradford was used. Therefore 1ml Bio-Rad Solution (1:5) was incubated with 3, 5 and 10 µl of the concentration standard BSA (1µg/µl) and 1-10µl of the sample extracts for 5-10 min. The specific extinction was analysed in a spectrophotometer at 595nm. With the known BSA concentrations a standard curve can be obtained and the protein concentration of the cell extracts can be determined by comparing the O_Ds.

6.3 Methods for DNA

6.3.1 Plasmid DNA preparation

6.3.1.1 over night culture

Bacteria, picked directly from the petri dishes or taken from frozen cultures, were incubated in 2-6 ml LB-media with antibiotics and shaked over night at 37°C under aerobe conditions. These cultures can be used either for mini- or maxipreps, for protein expression experiments, of freezing.

6.3.1.2 Small scale plasmid DNA preparation with silica milk (Miniprep)

Harvest cells by centrifugating of bacteria culture in eppendorf tube 5min 14000rpm; RT
Remove supernatant and resuspend pellet in 200µl Solution I plus 2µl of RNaseA (10mg/ml), 10min on ice
Add 400µl of Solution II and shake, 10min on ice
Add 300µl of Solution III and shake, 10min on ice
Add 20-30µl of silica milk and incubate on a shaker for 10-20 min; 37°C, 900 rpm.
Wash the silica-pellet 2 x with Ultra New Wash.
Resuspend the pellet in 20-50 µl H_2O and heat the probe 5 min 55°C.
Transfer the supernatant into a fresh Eppendorf-tube.

Silika milk:
Wash silica two times with H_2O
Prepare a 50/50 Solution of silica and H_2O
Store at 4°C in the dark

Solution 1: 50mM Glucose
25mM TRIS-HCl pH=8
10mM EDTA
Solution 2: 0,2 N NaOH

1 % SDS

Solution 3: 3 M KOAc

5 M Acetic acid

6.3.1.3 Large-scale plasmid DNA preparation:

Harvest cells of a 500 ml bacteria-culture by centrifugation (5 min, 6000 rpm, 4°C).
Resuspend the pellet in 20 ml cold Solution 2. Add a small aliquot Lysozym and shake .
Add 40 ml Solution 2 and wait 10min, RT.
Add 20 ml Solution 3 and wait 10min, RT.
Centrifugation (20 min, 8000 rpm, 4°C). Filter the supernatant through a fine paper layer into 50ml isopropanol.
Centrifugation (20 min, 10000 rpm, 4°C).
Drain the pellet and resuspend in 5 ml TE. Transfer the solution into a clean SS34-beaker and add 5 ml 5M LiCl.
Centrifugation (20 min, 10000 rpm, 4°C). Transfer the supernatant into a clean SS34-beaker and add 10 ml isopropanol.
Centrifugation (20 min, 10000 rpm, 4°C). Drain the pellet and resuspend in 500 µl TE.
Add 20 µl RNaseA (10 mg / ml), 30 min, RT.
Add 500 µl 2 x PN and shake. Put the mixture 10min on ice.
Centrifugation (10 min, 14000 rpm, 4°C). Resuspend the pellet in 400 µl TE.
Add 10 µl RNaseA (10 mg / ml), 30 min, RT.
2 x extraction with 400µl phenol / chloroform / isoamylalkohol. 1 x Extraction with 400µl chloroform.
Precipitate the supernatant with 100µl 10M NH4OAc and 800 µl cold 96 % ethanol.
Centrifugation (10 min, 14000 rpm, 4°C). Wash the pellet 2 x with 1ml 70 % ethanol.
Dry pellet in speed-vac
Resuspend the dry pellet in 400 µl TE.

2 x PN: 30 % PEG 6000

1,5 M NaCl

Lithiumchlorid: 5M LiCl

Ammoniumacetat: 10M NH4OAc

TE : 10mM TRIS-HCl pH=8

1mM EDTA pH=8

6.3.2 DNA Purification

6.3.2.1 DNA isolation from agarosegel with silica-milk:

Melt agarose-DNA slices in 3x volume of 6M NaJ at 55°C, 600 rpm.
Add 10-15µl silica suspension and incubate on a shaker for 10-20 min; 30°C, 600 rpm.
Wash the silica-pellet 2 x with Ultra New Wash.
Resuspend the pellet in 30 µl TE and heat the probe 5 min 55°C.
Transfer the supernatant into a fresh Eppendorf-tube.

6.3.3 DNA Quantification

To estimate the amount of DNA, separated DNA samples in the agarose gel were compared to the DNA markers of known concentrations under UV.
For precise quantification y µl of DNA-solution (in TE) were diluted with water to 1 ml and measured in a photometer: µg/µl = OD_{260} x 50 x y (used µl of DNA)
If OD_{260} us between 1,8 and 2,0 the DNA is clean, but less than 1,8 indicates for impurities in the solution

6.4 Special methods

6.4.1 Immunoblot analysis

Samples:
Mix 10-20 µg cell extract with an equal volume of Laemmli sample buffer and boil for 3-5 minutes at 95°C
Unused samples may be stored at –20°C

Electrophoresis:
Load 2-3 µl protein marker per well and the samples
Electrophorese in 1x Running buffer: 125mV

Transfer to membrane:
Transfer proteins from the gel to a nitrocellulose membrane
Use an electroblotting apparatus

Blotting:
250mA for 2,5 hours at 4°C
Use ice for cooling the transfer buffer

Immunostaining:
Block non-specific binding by incubating the membrane in a blocking solution for 30 min at RT
Incubate the blocker membrane for 1, 5 hours with primary antibody-milk
Wash three times for 10 min in PBST
Incubate the membrane for 1 hour with secondary antibody
Wash three times for 10 min in PBST

Detection of bound antibodies:
Incubate the membrane for 1 min with ECL detection solution

6.4.2 Fluorescence activated cell sorter (FACS) -Analysis

Preparation of cells for FACS:
Wash cells twice with PBS. Add Trypsin/EDTA. Incubate at 37°C.
Stop the reaction by addition of 1ml media + 10% FCS.
Centrifugate for 5min at 1000rpm, discard the supernatant.
Add 1ml PBS and centrifugate (1000 rpm, 5min).
Resuspend the pellet in 1mlPBS.
Pour the necessary amount of suspension into 5ml cold 85% ethanol and store at –20°C.

Staining of cells for FACS:

Centrifugation of fixed cells (1000 rpm, 2min, 4°C). Remove supernatant and resuspend the
pellet in 100-200µl 0,05% Pepsin (Sigma # P-7000). Add 1-2 ml staining solution (3min, RT).
Store at 4°C and use the preparation for FACS-analysis in between the next two hours.
Staining solution:
100mM TRIS-HCl pH= 7,5
2mM MgCl2
0,1 % Triton-X 100
2µg / ml DAPI (4,6-Diamidino-2-phenylindolhydrochlorid; Merck # 25653)
15µg / ml Sulforhodamin 101 (Sigma #. S-7635)

6.4.3 Nocodazole arrest and release

Grow cells up to 80% confluency
Add nocodazole and remove medium after 16h through centrifugation
Wash cell pellet once with 1xPBS
Resuspend cell pellet in new medium and add to the cells
To harvest the cells, use the cell scraper

6.4.4 Metabolic Labelling of Proteins and Immunoprecipitation (palse chase method)

Cells were starved for 30 min in 1 ml of methionine/cysteine^and FCS free medium, then metabolically labeled for 30 min with 125 µCi/ml of ^{35}S-methionin in methionine/cysteine-free medium (pulse), washed free of unbound radioactive amino acids, and incubated in prewarmed complete medium (chase). At the indicated time points, the cells were disrupted in ice-cold hunt lysis buffer and immunoprecipitated with anti-HA antibodies. Proteins were subjected to SDS-PAGE.

7. References

Alexandre Blais and Brian D Dynlacht (2007) E2F-associated chromatin modifiers and cell cycle control. A 2007 Dec;19(6):658-62. Epub 2007 Nov 19.

Alonso MM, Alemany R, Fueyo J, Gomez-Manzano (2008) CE2F1 in gliomas: a paradigm of oncogene addiction. Cancer Lett. 2008 May 18; 263(2):157-63. Epub 2008 Mar 10.

Bandara, L. R. & La Thangue, N. B. (1991): Adenovirus E1a prevents the retinoblastoma gene product from complexing with a cellular transcription factor. Nature 351, 494–497 (1991).

Bates, S., Phillips, A. C., Clark, P. A., Stott, F., Peters, G., Ludwig, R. L., and Vousden, K. H. (1998): p14ARF links the tumour suppressors RB and p53. Nature 395, 124–125.

Bi G, Jiang G. (2006): The molecular mechanism of HDAC inhibitors in anticancer effects. Cell Mol Immunol. 2006 Aug; 3(4):285-90.

Bracken, A.P. et al. (2004): E2F target genes: unraveling the biology.Trends Biochem. Sci. 29, 409–417.

Brown R, Strathdee G. (2002): Epigenomics and epigenetic therapy of cancer. Trends Mol Med. 2002; 8:43-48.

Buchkovich K, Duffy LA, Harlow E. (1989): The retinoblastoma protein is phosphorylated during specific phases of the cell-cycle. Cell 58:1097–1105.

Buchkovich K, Duffy LA, Harlow E. (1989): The retinoblastoma protein is phosphorylated during specific phases of the cell-cycle. Cell 58:1097–1105.

Cam, H. et al. (2004): A common set of gene regulatory networks links metabolism and growth inhibition. Mol. Cell 16, 399–411.

Chen PL, Scully P, Shew JY, Wang JYJ, Lee WH. (1989): Phosphorylation of the retinoblastoma gene-product is modulated during the cell-cycle and cellular-differentiation. Cell 58:1193.

Chen PL, Scully P, Shew JY, Wang JYJ, Lee WH. (1989): Phosphorylation of the retinoblastoma gene-product is modulated during the cell-cycle and cellular-differentiation. Cell 58:1193.

Chellappan, S. P., Hiebert, S., Mudryj, M., Horowitz, J. M. & Nevins, J. R. (1991): The E2F transcription factor is a cellular target for the RB protein. Cell 65, 1053–1061.

Chittenden, T., Livingston, D. M. & Kaelin, W. G. Jr. (1991): The T/E1A-binding domain of the retinoblastoma product can interact selectively with a sequence-specific DNAbinding protein. Cell 65, 1073–1082.

Ciechanover A. (2007): Intracellular protein degradation from a vague idea through the lysosome and the ubiquitin-proteasome system and on to human diseases and drug targeting. Ann N Y Acad Sci. 2007 Nov;1116:1-28.

Ciechanover, A. (2006): The ubiquitin proteolytic system: from a vague idea, through basic mechanisms, and onto human diseases and drug targeting. Neurology 66, S7–S19.

Classon M, Harlow E (2002): The retinoblastoma tumour suppressor in development and cancer. Nat Rev Cancer 2002, 2: 910-917.

Cobrinik D. (2005): Pocket proteins and cell cycle control. Oncogene 24: 2796–2809.

Decaprio JA, Ludlow JW, Lynch D, Furukawa Y, Griffin J, Piwnicaworms H, Huang CM, Livingston DM. (1989): The product of the retinoblastoma susceptibility gene has properties of a cell-cycle regulatory element. Cell 58: 1085 –1095.

DeGregori, J. and Johnson, D.G. (2006): Distinct and overlapping roles for E2F family members in transcription, proliferation and apoptosis. Curr. Mol. Med. 6, 739–748.

De Ruijter AJ, van Gennip AH, Caron HN, Kemp S, van Kuilenburg AB. (2003): Histone deacetylases (HDACs): Characterization of the classical HDAC family. Biochem J. 2003; 370:737-749.

Dimova, D.K. and Dyson, N.J. (2005): The E2F transcriptional network: old acquaintances with new faces. Oncogene 24, 2810–2826.

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

Glauben, R., Batra, A., Stroh, T., Erben, U., Fedke, I., Lehr, H. A., Leoni, F., Mascagni, P., Dinarello, C. A., Siegmund, B. (2008) Histone deacetylases: novel targets for prevention of colitis-associated cancer in mice. Gut 57,613-622.

Glauben R, Sonnenberg E, Zeitz M, Siegmund B. (2009): HDAC inhibitors in models of inflammation-related tumorigenesis. Cancer Lett. 2009 Aug 8;280(2):154-9. Epub 2008 Dec 19.

Goodrich, D. W. (2003): How the other half lives, the aminoterminal domain of the retinoblastoma tumor suppressor protein. J. Cell Physiol. 197, 169–180.

Goodrich, D. W. (2006): The retinoblastoma tumor-suppressor gene, the exception that proves the rule. Oncogene 25, 5233–5243.

Grunstein M. (1997): Histone acetylation in chromatin structure and transcription. Nature 1997; 389:349–352.

Hijmans EM, Voorhoeve PM, Beijersbergen RL, Vantveer LJ, Bernards R. (1995): E2f-5, a new E2f family member that interacts with P130 in-vivo. Mol Cell Biol 15:3082– 3089.

Hiebert, S.W., Packham, G., Strom, D.K., Ha‹ner, R., Oren, M., Zambetti, G. and Cleveland, J.L. (1995): E2FI: DP-1 induced p53 and overrides survival factors to trigger apoptosis. Mol. Cell Biol. 15, 6864^6874.

Hsieh, J.K., Yap, D., O'Connor, D.J., Fogal, V., Fallis, L., Chan, F., Zhong, S. and Lu, X. (2002): Novel function of the Cyclin A binding site of E2F in regulating p53-induced apoptosis in response to DNA damage. Mol. Cell. Biol. 22, 78^93.

Kim SH, Kang HJ, Na H, Lee MO. (2010): Trichostatin A enhances acetylation as well as protein stability of ERalpha through induction of p300 protein. Breast Cancer Res. 2010;12(2):R22. Epub 2010 Apr 13.

Kowalik, T.F., DeGregori, J., Schwarz, J.K. and Nevins, J.R. (1995): E2F1 overexpression in quiescent fibroblasts leads to induction of cellular DNA synthesis and apoptosis. J. Virol. 69, 2491^2500.

Lake AN, Bedford MT (2007): Protein methylation and DNA repair. Mutat Res. 2007 May 1;618(1-2):91-101. Epub 2007 Jan 21.

Lipford JR, Smith GT, Chi Y, Deshaies RJ. (2005): A putative stimulatory role for activator turnover in gene expression. Nature 438: 113–116.

Liu T, Kuljaca S, Tee A, Marshall GM. (2006): Histone deacetylase inhibitors: multifunctional anticancer agents. Cancer Treat Rev. 2006; 32:157-165.

Lin, W.C., Lin, F.T. and Nevins, J.R. (2001): Selective induction of E2F1 in response to DNA damage, mediated by ATM-dependent phosphorylation. Genes Dev.15, 1833^1844.

Lipinski, M. M., and Jacks, T. (1999): The retinoblastoma gene family in differentiation and development. Oncogene 18, 7873-7882.

Lorenzo Montanaro, Davide Trere´ ,and Massimo Derenzini (2008): Nucleolus, Ribosomes, and Cancer. The American Journal of Pathology, Vol. 173, No. 2.

Macpherson D. (2008): Insights from mouse models into human retinoblastoma. Cell Div. 2008 May 19;3:9.

Marks PA, Dokmanovic M. Histone deacetylase inhibitors (2005): discovery and development as anticancer agents. Expert Opin Investig Drugs. 2005;14:1497-1511.

Marks PA, Richon VM, Miller T, Kelly WK. (2004): Histone deacetylase inhibitors. Adv Cancer Res. 2004; 91:137-168.

Mihara K, Cao XR, Yen A, Chandler S, Driscoll B, Murphree AL, Tang A, Fung YKT. (1989): Cell-cycle dependent regulation of phosphorylation of the human retinoblastoma gene-product. Science 246:1300–1303.

Miller TA, Witter DJ, Belvedere S. (2003): Histone deacetylase inhibitors. J Med Chem. 2003; 46:5097-5116.

Morris, E. J. & Dyson, N. J. (2001): Retinoblastoma protein partners. Adv. Cancer Res. 82, 1–54.

Muratani M, Tansey WP (2003): How the ubiquitin-proteasome system controls transcription. Nat Rev Mol Cell Biol 4: 192–201.

Mukhopadhyay NK, Weisberg E, Gilchrist D, Bueno R, Sugarbaker DJ, Jaklitsch MT. (2006): Effectiveness of trichostatin A as a potential candidate for anticancer therapy in non-small-cell lung cancer. Ann Thorac Surg. 2006;81:1034-1042.

Nevins JR. (1998): Toward an understanding of the functional complexity of the E2F and retinoblastoma families. Cell Growth Differ. 1998 Aug; 9(8):585-93.

Nip, J., Strom, D.K., Fee, B.E., Zambetti, G., Cleveland, J.L. and Hiebert, S.W. (1997): E2F-1 cooperates with topoisomerase II inhibition and DNA damage to selectively augment p53-independent apoptosis. Mol. Cell. Biol. 17, 1049^1056.

Novy M, Pohn R, Andorfer P, Novy-Weiland T, Galos B, Schwarzmayr L, Rotheneder H.(2005): EAPP, a novel E2F binding protein that modulates E2F-dependent transcription. Mol Biol Cell. 2005 May;16(5):2181-90. Epub 2005 Feb 16.

Ogris, E., H. Rotheneder, et al. (1993). "A binding site for transcription factor E2F is a target for trans activation of murine thymidine kinase by polyomavirus large T antigen and plays an important role in growth regulation of the gene." J Virol **67**(4): 1765-71

Ogryzko VV, Schiltz RL, Russanova V, Howard BH, Nakatani Y.(1996): The transcriptional coactivators p300 and CBP are histone acetyltransferases. Cell 1996; 87: 953–959.

Pan LN, Lu J, Huang B. (2007): HDAC inhibitors: a potential new category of anti-tumor agents. Cell Mol Immunol. 2007 Oct; 4(5):337-43.

Pandita TK, Richardson C. (2009): Chromatin remodeling finds its place in the DNA double-strand break response. Nucleic Acids Res. 2009 Apr;37(5):1363-77. Epub 2009 Jan 12.

Polager S, Ginsberg D. (2008): E2F - at the crossroads of life and death. Trends Cell Biol. 2008 Nov;18(11):528-35. Epub 2008 Sep 18.

Pons D, de Vries FR, van den Elsen PJ, Heijmans BT, Quax PH, Jukema JW. (2009): Epigenetic histone acetylation modifiers in vascular remodelling: new targets for therapy in cardiovascular disease. Eur Heart J. 2009 Feb;30(3):266-77. Epub 2009 Jan 15.

Pruschy, M., Wirbelauer, C., Glanzmann, C., Bodis, S. and Krek, W. (1999): E2F-1 has properties of a radiosensitizer and its regulation by cyclin A kinase is required for cell survival of fibrosarcoma cells lacking p53 Cell Growth Differ. 10, 141^146.

Rasheed WK, Johnstone RW, Prince HM. (2007): Histone deacetylase inhibitors in cancer therapy. Expert Opin Investig Drugs. 2007 May;16(5):659-78.

Redon C, Pilch D, Rogakou E, Sedelnikova O, Newrock K, Bonner W. (2002): Histone H2A variants H2AX and H2AZ. Curr. Opin. Genet. Dev. 2002;12:162–169.

Ren, B. et al. (2002): E2F integrates cell cycle progression with DNA repair, replication, and G2/M checkpoints. Genes Dev. 16, 245–256

Richly, H. et al. (2005) A series of ubiquitin binding factors connects CDC48/p97 to substratemultiubiquitylation and proteasomal targeting. Cell 120, 73–84.

Robertson, K.D. and Jones, P.A. (1998): The human ARF cell cycle regulatory gene promoter is a CpG island which can be silenced by DNA methylation and down-regulated by wild-type p53. Mol. Cell Biol. 18, 6457^6473.

Russell, J.L., Powers, J.T., Rounbehler, R.J., Rogers, P.M., Conti, C.J. and Johnson, D.G. (2002): ARF Differentially Modulates Apoptosis Induced by E2F1 and Myc Mol. Cell Biol. 22, 1360^1368.

Rogakou EP, Pilch DR, Orr AH, Ivanova VS, Bonner WM.(1998): DNA double-stranded breaks induce histone H2AX phosphorylation on serine 139. J. Biol. Chem. 1998;273:5858–5868.

Rogakou EP, Boon C, Redon C, Bonner WM. (1999): Megabase chromatin domains involved in DNA double-strand breaks in vivo. J. Cell Biol. 1999;146:905–916.

Rotheneder, H., Schwarzmayr, L., Andorfer, P., Sarr, A. (2007). Transcription Factors of the E2F Family and DNA Damage control. *Dynamic Cell Biology* 1(1): 48-59. issn: 1749-0561

Rubinsztein DC. (2006): The roles of intracellular protein-degradation pathways in neurodegeneration. Nature. 2006 Oct 19;443(7113):780-6.

Sherr, C.J. and McCormick, F. (2002): The RB and p53 pathways in cancer. Cancer Cell 2, 103–112.

Sherr CJ, McCormick F (2002): The RB and p53 pathways in cancer. Cancer Cell 2002, 2:103–112.

Shogren-Knaak M, Ishii H, Sun JM, Pazin MJ, Davie JR, Peterson CL. (2006): Histone H4-K16 acetylation controls chromatin structure and protein interactions. Science. 2006;311:844–847.

Smith EJ, Leone G, DeGregori J, Jakoi L, Nevins JR. (1996): The accumulation of an E2F-p130 transcriptional repressor distinguishes a G(0) cell state from a G(1) cell state. Mol Cell Biol 16:6965–6976.

Struhl K. (1998): Histone acetylation and transcriptional regulatory mechanisms. Genes Dev 1998;12:599–606.

Sun A, Bagella L, Tutton S, Romano G, Giordano A. (2007): From G0 to S phase: a view of the roles played by the retinoblastoma (Rb) family members in the Rb-E2F pathway. J Cell Biochem. 2007 Dec 15;102(6):1400-4.

Sun A, Bagella L, Tutton S, Romano G, Giordano A. (2007): From G0 to S phase: a view of the roles played by the retinoblastoma (Rb) family members in the Rb-E2F pathway. J Cell Biochem. 2007 Dec 15;102(6):1400-4.

Thomas, D. M., Carty, S. A., Piscopo, D. M., Lee, J. S., Wang, W. F., Forrester, W. C., and Hinds, P. W. (2001): The retinoblastoma protein acts as a transcriptional coactivator required for osteogenic differentiation. Mol Cell 8, 303-316.

Tolbert, D., Lu, X., Yin, C., Tantama, M. and Van Dyke, T. (2002): p19ARF Is Dispensable for Oncogenic Stress-Induced p53-Mediated Apoptosis and Tumor Suppression in Vivo Mol. Cell Biol. 22, 370^377.

Tsai, K.Y., MacPherson, D., Rubinson, D.A., Crowley, D. and Jacks, T. (2002): ARF is not required for apoptosis in Rb mutant mouse embryos Curr. Biol. 12, 159^163.

Vairo G, Livingston DM, Ginsberg D. (1995): Functional interaction between E2f-4 and P130—Evidence for distinct mechanisms underlying growth suppression by different retinoblastoma protein family members. Genes Dev 9:869–881.

Van den Heuvel S, Dyson NJ. (2008): Conserved functions of the pRB and E2F families. Nat Rev Mol Cell Biol. 2008 Sep; 9(9):713-24.

Vaquero A, Loyola A, Reinberg D. (2003): The constantly changing face of chromatin. Sci. Aging Knowledge Environ. 2003;2003:RE4.

Weihl, C. C., Dalal, S., Pestronk, A. & Hanson, P. I. (2006): Inclusion body myopathy-associated mutations in p97/VCP impair endoplasmic reticulum-associated degradation. Hum. Mol. Genet. 15, 189–199 (2006).

Weber, J.D., Taylor, L.J., Roussel, M.F., Sherr, C.J. and Bar- Sagi, D. (1999): Nucleolar Arf sequesters Mdm2 and activates p53. Nat. Cell Biol. 1, 20^26.

W.K. Kelly, P.A. Marks. (2005): Drug insight: histone deacetylase inhibitors–development of the new targeted anticancer agent suberoylanilide Hydroxamic acid, Nat. Clin. Pract. Oncol. 2 (2005) 150–157.

Xu, G., Livingston, D. M., and Krek, W. (1995): Multiple members of the E2F transcription factor family are the products of oncogenes. Proc. Natl. Acad. Sci. USA 92, 1357–1361.

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

Yang XJ, Seto E. (2007): HATs and HDACs: from structure, function and regulation to novel strategies for therapy and prevention. Oncogene 2007, 26:5310-5318.

Yoshida M, Kijima M, Akita M, Beppu T. (1990) Potent and specific inhibition of mammalian histone deacetylase both in vivo and in vitro by trichostatin A. J Biol Chem. 1990;265:17174-17179.

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