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Influence of EAPP on the DNA damage response of human osteosarcoma cells

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1 Abstract in English

Disruptions of the cell cycle regulation are often associated with cancerogenesis. The activity of E2F transcription factors plays an essential part of integrating cell cycle progression with transcription. E2F enhances the expression of genes which are indispensable for the regulation of cell cycle. The previously identified E2F-associated phospho- protein (EAPP) interacts with the E2F transcription factors, E2F-1 to E2F-3. It is supposed that high levels of EAPP play an important part in malignant transformations of cells by preventing cells from E2F-1 induced apoptosis. A RNAi-mediated knock down of EAPP results in cell death in U2OS cells.

EAPP is only required in low concentrations, but the concentration depends on cell conditions. Upon DNA damage, it increases and enhances the expression of p21, independently of p53, resulting in protecting cells from apoptosis via G1 arrest. EAPP can also negatively regulate the expression of the tumorsupressor $p14^{ARF}$. This can prevent cells from $p14^{ARF}$ regulated apoptosis and shows that EAPP might also be important for malignant transformations. Moreover, EAPP can reduce the efficacy of chemotherapeutics by inducing the MDR-1 promotor of p- glycoprotein, one of the most common regulator of multi drug resistance.

In this diploma thesis, U2OS cells were transfected with expression vectors for different truncations of EAPP to investigate specific binding fragments of EAPP that are essential for interactions with cell cycle related proteins. Double strand breaks were caused by treating cells with etoposide to investigate the role of EAPP during DNA damage. The influence of the endogenous and the different truncated versions of EAPP on the levels and activity of p-Akt S473, p21, p53 and Bax were examined. While p53 and p21 levels show similiar expression profiles like EAPP, Bax levels do not change at all. On the contrary, p-Akt S473 decreases during prolonged etoposide treatment when EAPP rises. This indicates an inverse relationship of these proteins. The effects on p-Akt S473 also variegate between the cell lines with different versions of EAPP.

2 Abstract in German

Zellzyklusstörungen werden oft mit der Entstehung von Krebs in Verbindung gebracht. Die Aktivität von Transkriptionsfaktoren der E2F Familie spielt eine wichtige Rolle im Verlauf des Zellzyklus. E2F verstärkt die Genexpression von Proteinen, die für die Regulation des Zellzyklus unverzichtbar sind. Das kürzlich identifizierte E2F-assozierte Phospho-Protein (EAPP) interagiert mit den Transkriptionsfaktoren E2F-1 bis E2F-3. Höhere EAPP Expression spielt vermutlich eine wichtige Rolle bei bösartigen Transformationen von Zellen, indem EAPP die Zellen vor der E2F-induzierten Apoptose schützt. In U2OS Zellen führte ein RNAi unterstützter Knockdown von EAPP zum Zelltod. In normalen Zellen ist EAPP nur in einer geringen Konzentration notwendig, aber die Konzentration ist vom Zustand der Zelle abhängig. DNA Schäden führen zu einem Anstieg von EAPP, welches die Expression von p21 induziert. Durch das Anhalten des Zellzyklus in G1, können Zellen vor Apoptose bewahrt werden. EAPP reguliert auch die Regulation des Tumorsuppressors $p14^{ARF}$, wodurch Zellen vom $p14^{ARF}$ regulierenden Zelltod geschützt sind. Dies zeigt, dass EAPP auch für bösartige Veränderungen in Zellen wichtig ist. Das p-Glycoprotein ist einer der wichtigsten Regulatoren der Multi-Drug-Resistence (MDR). EAPP kann auch die Wirksamkeit von Chemotherapeutika reduzieren, indem es den MDR-1 Promotor des p-Glycoproteins induziert.

In dieser Diplomarbeit wurden spezifische Bindungsfragmente von EAPP untersucht, die für mögliche Interaktionen mit wichtigen Proteinen des Zellzyklus wichtig sind. Dafür wurden U2OS Zellen mit Expressionsvektoren für diese spezifische Fragmente von EAPP transfekiert. Doppelstrangbrüche wurden durch Etoposid verursacht, um die Rolle von EAPP während eines DNA Schadens zu untersuchen. Der Einfluss von endogenem EAPP und der unterschiedlichen gekürzten Versionen von EAPP auf die Aktivität von p-Akt S473, p21, p53 und Bax wurden untersucht. Während p53 und p21 ein ähnliches Expressionsmuster wie EAPP gezeigt haben, veränderten sich die Proteinmengen bei BAX kaum. Andererseits, wird p-Akt S473 mit der Etoposid-Behandlung weniger, wenn EAPP steigt. Das könnte auf eine umgekehrte Beziehung der beiden Proteinen deuten. Die Effekte von p-Akt S473 variieren auch zwischen den einzelnen Zelllinien, die unterschiedliche Versionen von EAPP enthalten.

3 Abbreviations

ABC-	ATP-binding- cassette
APAF-1	apoptotic protease activating factor 1
ATM	ataxia telangiectasia mutant
ATR	rad3-related protein kinase
Bcl-2	B-cell lymphoma 2
CAK	CDK-activating kinase
cdk	cyclin dependent kinase
CHK-1/2	checkpoint- kinase 1/2
CTMP	carboxy-terminal modulator protein
DISC	death-inducing signaling complex
DP	DNA-binding protein
EAPP	E2F- associated phosphoprotein
Egr-1	Early growth response protein 1
f.l.	full length
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GPCR	G-protein coupled receptors
GSK	glycogen synthase kinase
mdm2	murine double minute 2
MDR	multi-drug- resistance
MRP	multi-drug-resistance-associated protein
NLS	nuclear localization signal
PDK-1/2	phosphoinositide-dependent kinase-1/2
PGP	p-glycoprotein
PH	pleckstrin-homology
PI3K	phosphoinsoitide-3-kinase
PIP2	phosphatidylinositol - biphosphate
PIP3	phosphatidylinositol- triphosphate
PKB	proteinkinase B
pRb	retinoblastoma protein
PTEN	phosphatase and Tensin homolog
RTK	receptor tyrosine kinases

sp-1/3	specificity protein 1/3
TNFR	tumor necrosis factor receptor
TRAIL-R1/2	TNF-related apoptosis inducing ligand receptor ½
wt	wild type

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5 Introduction

5.1 Cell cycle

The cell cycle is an essential mechanism for cell survial by passing a systematic progression. It describes the duplication of the cell's substance and the following division into two identical daughter cells.

Cell cycle can be subclassified into interphase and mitosis. The interphase comprises G1-, G2- and S- phase, seen in ***figure 1*****.** In this three phases cells grow constantly. In the first phase, G1- (gap) cell growth and synthesis of proteins for DNA-replication are important. In the following S-Phase (synthesis) DNA, RNA and further proteins are synthesized. In G2-Phase, the cells prepare themselves for mitosis by producing further proteins and RNA. During the mitotic phase (M-Phase) the DNA and the cytoplasm get split between the daughter cells. Cells have the opportunity to leave the cell cycle and to join the G0-Phase because of absence of nutritive substances. In contrast, resting cells from G0 can re-enter the cell cycle by stimulating with growth factos or nutritive substances.

5.1.1 Regulation of cell cycle

Checkpoints between the separat phases guarantee that enough cell materials are already produced and available to join the next stage. One of these checkpoints controlls the late G1-phase before cells enter S-Phase by checking excisiting DNA damages and cell size. Another checkpoint in G2- phase screens if DNA replication is completed successfully. The last checkpoint at the end of the M-Phase monitors if the cell is ready to disperse the duplicated chromosomes and to apportion them among the daughter cells (***figure 1***).

5.1.2 Cyclins and cyclin dependent kinases (cdk's)

The availability of growth factors determines the activity of these complexes and thus the decision between proliferation and cell cycle arrest. Through pausing the cell cycle, damage can be repaired or apoptosis can be initiated. Cyclins and cyclin dependent

kinases (cdk) are responsible for passing a checkpoint. (***figure 2***) Tumor cells often loose the scan of the checkpoints.

Several cyclins change their concentration throughout the cell cycle by a regulated reduction through the proteasom. Cdks are present during the whole cell cycle, contrary to cyclins. Normally a cyclin binds to a cyclin dependent kinase and the complex is regulated by reversible (de-) phosphorylations or specific inhibitorproteins. Cyclins can also regulate other cdks. By having inhibitory and activating domains, cdks can be regulated through other kinases (inactivate them) or phosphatases (activate them) [1].

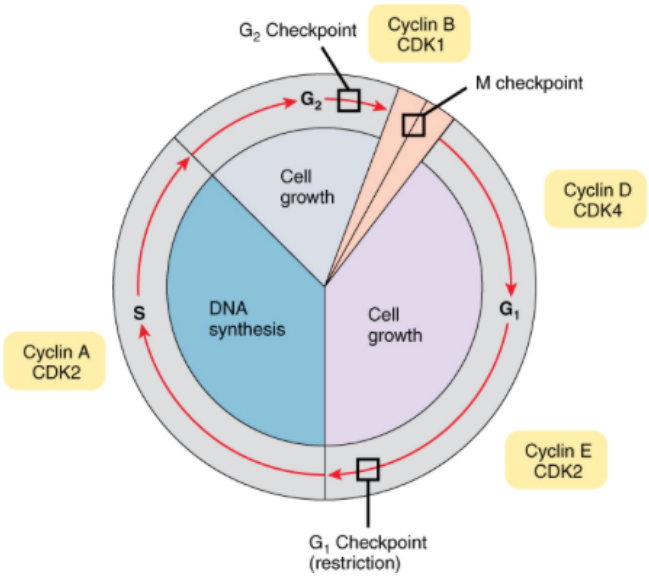

Figure 1: Organization of the cell cycle and its checkpoints.
Cell cycle contains of the interphase (G1-, S- and G2- phase) and mitosis (M-). G1 and G2 are primarily important for the cell growth and in S-phase cell synthesizes DNA. In the M- phase happens the separation of the doubled chromosomes. Three important checkpoints (G1, G2, M) secure the progression of cell cycle correctly.

Source:https://courses.lumenlearning.com/biology1/chapter/cell-cycle-with-cyclins-and-checkpoints/

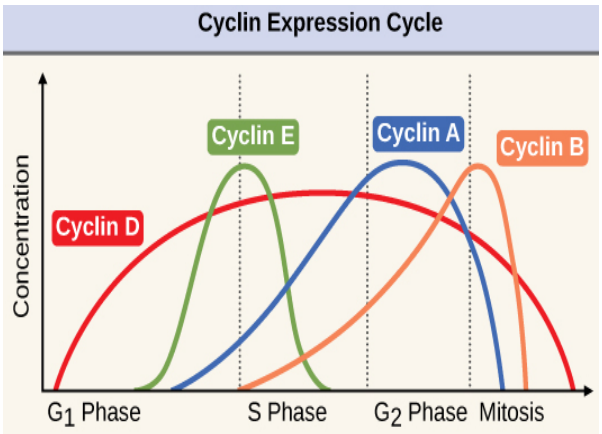

Figure 2: Variable expression of cyclins during cell cycle.
Different complexes of cyclin/cdks coordinate the cell cycle. Cyclin D/ cdk-4 and cyclin E/ cdk-2 complexes act in G1, cyclin A/ cdk-2 in S- and cyclin B/ cdk-1 in G2-phase (also figure 1).

Source:https://cnx.org/contents/abji7vNQ@6/Control-of-the-Cell-Cycle

5.2 Apoptosis

External factors, for example UV- or ionizing radiation, cytostatic drugs, hormons or absence of growth factors can lead to cell damage. If it is not repairable, the consequence is condensation of chromatin membran bledding and cell shrinking, which is characteristic for apoptosis.

Due to the fact that caspases are having proteolytic activity and under normal conditions lying dormant as inactive pro-caspase, they are eligible for initiating apoptosis as answer to cell damage. There are two main pathways, the intrinsic and the extrinsic that are responsible for apoptosis.

5.2.1 Extrinsic pathway

The extrinsic pathway is characterized by ligands, binding to the family of death receptors, including TNFR1, Fas, TRAIL-R1/2, that are a subfamily of tumor necrosis factor receptor (TNFR). If one of these is being activated the result is a platform called DISC, a clustering of the receptors on cytosolic membran site. This leads to mobilization of caspase 8 that initiate the executioner caspases caspase 3 and caspase 7. Otherwise it can go the indirect way by truncated Bid (t-Bid), the proteolysis form of Bid (***figure 3***), activating intrinsic apoptosis [2].

5.2.2 Intrinsic pathway

At the intrinsic way, p53 is being activated which leads to overexpression Bak and Bax that causes a release of cytochrome c from the mitochondria. The members of pro-apoptopic molecules (BH3- only proteins), Bax and Bak, and the anti-proapoptotic proteins (Bcl-2-like proteins) are regulated by the Bcl-2 protein family (Bid, Bim and Bad). In its neutralisated form Bax is bound to the anti-apoptotic factor Bcl-2. As the ratio of positive and negative apoptosis regulators is important for activation of Bax and Bak [3]. By oligomerizing through p53 or the extrinsic pathway, they make the mitochondrial membrane permeable which results in the release of cytchrome c from mitochondrial intermembrane space [4]. At the same time, APAF-1 is available as a

monomoer in the cytosol and inactive by binding to Desoxyadenosintriphosphat (dATP) or ATP. Together with cytochrome c and caspase 9, APAF-1 then represents the „apoptosome“ [5], that activates executioner caspases (caspase-3 and caspase-7) which causes cell death (***figure 3***) [4].

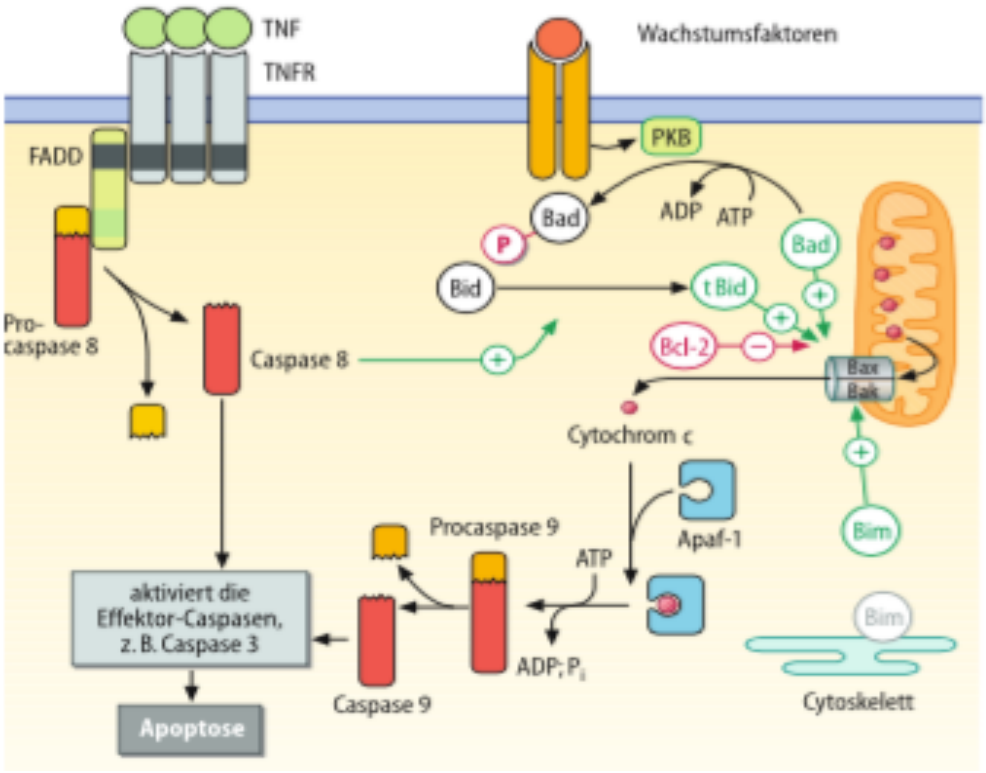

Figure 3: Apoptosis- extrinsic and intrinsic pathway. The extrinsic way is activated by TNFR/TNF and leads to initiate caspase 8, which can result in apoptosis by forcing executioner caspases or it can activate the intrinsic pathway alternatively. This way is characterized by a Bak/Bax overexpression resulting in the release of cytochrome, forming the apoptosome and activating executioner caspases.

Source: Löffler- Biochemie und Pathobiochemie, 8. Auflage 2007

5.2.3 The Bcl2- family

About 20 members are classified to the B-cell lymphoma 2 family (Bcl2), divided in three interacting groups and generally in two functional groups (pro- or antiapoptotic) (***figure 5).*** Every member has at least one of four important Bcl-2 Homology (BH1- BH4) domains but all members are able to form homo- or heterdimeric complexes with other members [6].

The first group, including Bcl-2 and Bcl-x, are important for cell survival and differ themselves in localization and function. By having three domains (BH1, BH2, BH3), the second group (for example Bax) is also called as the „multi- BH domain group“ and seems to be an important part of apoptosis initiation. The last group only consists of a BH3 interaction domain which probably is important for antagonizing the pro-survival group (***figure 4***) and initiating apoptosis [7,8].

The relation of pro- and apoptotic members determines how cells manage a pro-apoptotic signal. Interacting with organelles by their hydrphobic C- terminal is mostly essential for the function of the Bcl-2 family members [4].

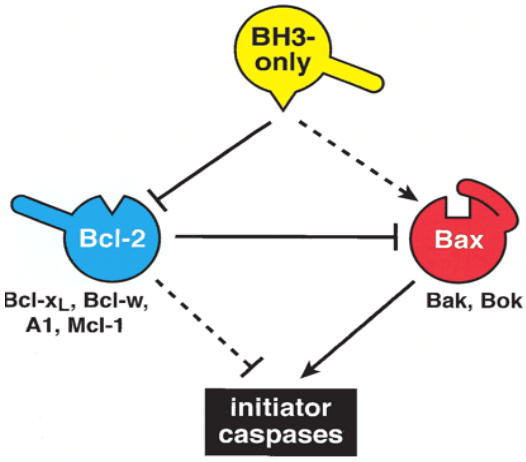

Figure 4: Bcl-2 subfamilies and their interactions. The BH3- only subf. suppresses the pro-survial Bcl-2 subf. and can act pro-apoptotic via the Bax-subf. indirectly. The Bax- subf. can induce initiator caspases indirectly and is negatively regulated by the Bcl-2 subfamily.

Source: http://genesdev.cshlp.org/content/17/20/2481/F1.expansion.html

subfamily	examples	apoptosis related orientation
Bcl-2	Bcl-2, Bcl-x_L	anti-apoptotic
multi-BH domain	Bax, Bak	pro- apoptotic
BH3- only	Bad, Bim, Bik, Bid	pro- apoptotic

Figure 5: The subfamilies of Bcl-2 and their apoptosis related orientatation

5.2.3.1 Bax and its activation by Bcl2- family

DNA damage-induced activation of p53 can induce the pro-apoptotic proteins Bax and Bak. In normal cells, Bax occurs predominantly as a monomer in the cytoplasm. Apoptosis induces an interaction between Bcl-2 proteins, which are integrated to the

mitochondrial membrane, and cytosolic BH-3 only proteins. Conformational changes of Bax enable its translocation to the outer mitochondrial membrane where Bax oligomerizes homodimerically. By inserting Bax into the outer mitochondrial membrane, the permeabilization of the membrane for apoptogenic proteins, also cytochrome c, increases (***figure 6***). Together with Apaf-1 and procaspase 9, cytochrome c then represents the „apoptosome" [4].

BH3- only proteins have two faces. On the one side, they inhibit anti-apoptotic proteins, but on the other side, BH3- only proteins trigger the activity of Bax (Bak as well) [3,8]. Latter is possible by t-Bid and Bad (***figure 3***) [9]. The activation of Bax is still not clearly understood. In the „direct activation model" BH3-only proteins (Bim, t-Bid) bind Bax (or Bak) primarily. Contrarily, the „indirect activation model" suggests that Bax is induced by inhibiting the anti-apoptotic Bcl-2 subfamily (by BH3-only proteins) [10].

Recent studies suppose that the anti-apoptotic effects of Bcl-2 and Bcl-x_L on Bax might depends on Bax's initiate state of activation, for example conformation, post-translational modification, localization [11,12].

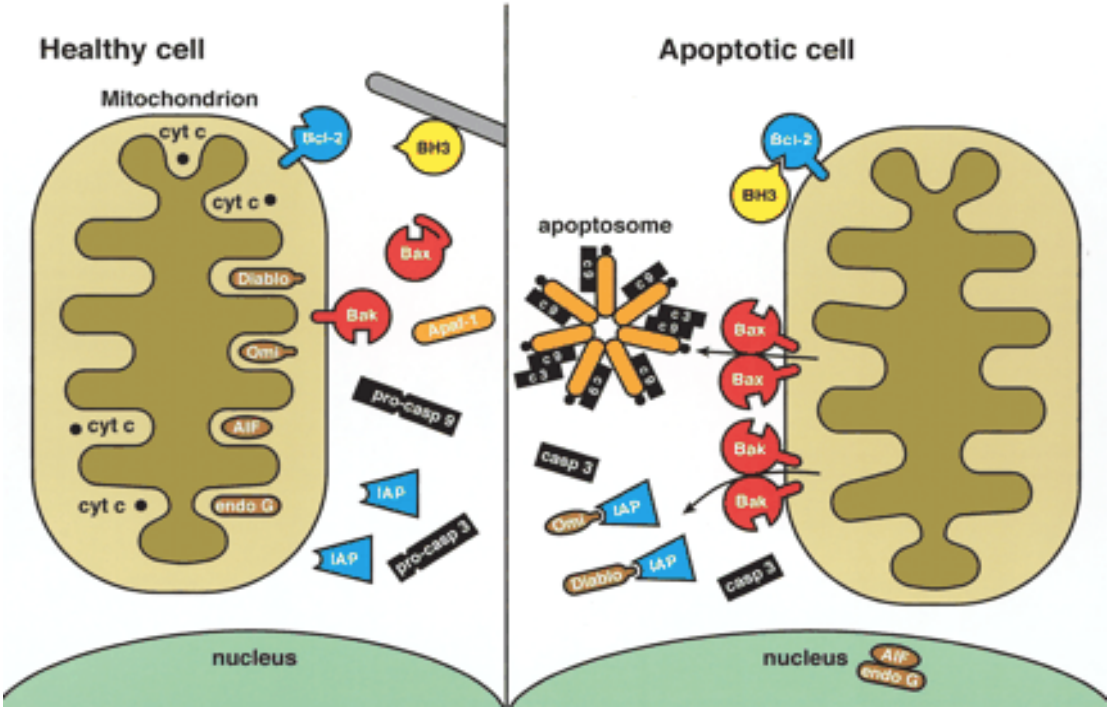

Figure 6: Outer mitochondrial membrane in healthy cell and apoptotic cell. During apoptosis Bcl-2 associates with BH3-only proteins and this complex stabilizes the oligomerization of Bax proteins on the outer mitochondrial membrane. Released cytochrome c associates with Apaf-1 and procaspase 9 and together they represent the „apoptosom".
Source: http://genesdev.cshlp.org/content/17/20/2481/F2.expansion.html

5.3 The E2F transcription family

E2F plays an important role in cell cycle progress and expression of genes which are essential for passing G1-/ S- phase. E2F acts variably by cyclical interactions with regulator proteins, pocket proteins, cyclins or cdk's.

The E2F family comprises 8 E2F genes which are classified into 4 subgroupes by conseridering their functions, sequence homology or how they become activated. One subgroup, E2F-1, E2F-2 and E2F-3a, are known as transciptional activators with a N-terminal domain comprising a nuclear localization signal and providing a template for interactions with other proteins. This group only binds to pRb and is mainly present at the passage G1-/ S- phase.

The retinoblastoma protein (Rb), p107 and p130 belong to the pocket proteins. Unphosphorylated pocket proteins can repress the activity of E2F by interacting directly with it (most of G1- phase). At the end of G1, cyclin/ cdk complexes phosphorylate the pocket proteins, resulting in an inactivation of them [13].

The second subgroup contains E2F-3b, E2F-4 and E2F-5 and are known as pocket protein- dependent repressors. The third group consists only E2F-6 and the last group of E2F-7a, E2F-7b and E2F-8 (***figure 7***) [14].

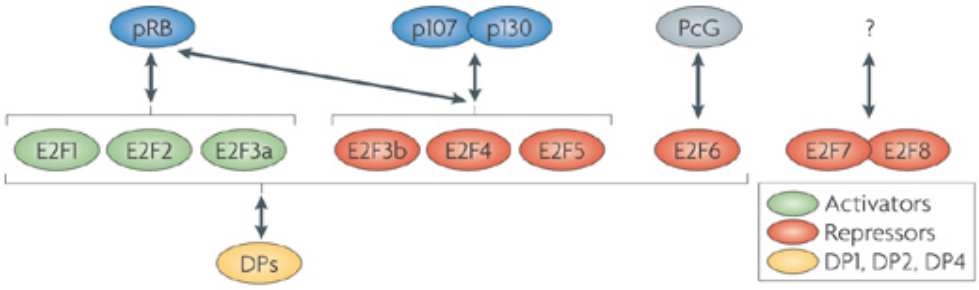

Figure 7: The 4 Subgroups of E2F and their interactions with other proteins. The first group (E2F-1 to E2F-3a) is known as activators by binding to pRb. The second group, E2F-3b to E2F5 interact with p107 and p130. This group is together with the third group and the fourth group classified into repressors.

Source: http://www.nature.com/nrm/journal/v9/n9/box/nrm2469_BX1.html

5.3.1 Structure of E2F subtypes

All E2F proteins have a DNA binding domain and with the exception of E2F7- E2F8 also a dimerization domain. E2F-1 to E2F-6 bind DNA by forming a heterodimeric- complex with a DP-protein (DNA-binding protein) (***figure 7***). On the contrary, E2F-7 to E2F-8 can bind the DNA directly without DPs [15]. DPs stabilize the DNA binding of E2F and they are together with pocket proteins important for the entry of E2F-4 and E2F-5 into the nucleus [16].

By having a C-terminal transactivation domain (the transactivation domain is primarily for interactions with the basal transcription apparatus) for binding to pRb, E2F-1 to E2F-5 also can link to the pocket proteins. PRb, p107 and p130 are the main regulators of E2F activity [17]. E2F-6 has no pocket binding domain and transactivation domain, but it acts as a repressor of the E2F- dependent transcription.

The association of E2F and DP looses its DNA binding activity by phosphorylation through cyclin/ cdk complexes. This is an important requirement for the cell cycle progression. E2F can be secured from proteasom pathway degradation by hiding the C-terminal sequences through pocket proteins. Because of splitting from pRb, E2F is unprotected and then ready for ubiquitin-dependent degradation [18].

5.3.2 E2F in the cell cycle

The E2F family plays an important role in gene expression during the cell cycle. E2F proteins can be regulated by cyclins, cdk's, pocket proteins or prosttranslational modifications which change the DNA-binding activity or stability of E2F. The respective activity level of an E2F protein is defined by it's interactions with other proteins. It seems that the subunit E2F is responsible for the regulatory activity of the heterodimeric complexe of E2F/DP and DP is important for DNA binding primarily [19].

5.3.3 E2F1-3a

E2F-1 to E2F-3a act as transcriptional activators when pRb dissociates, but they are also repressors (by pRb). Contrary to E2F-4 and E2F-5, they have their own nuclear

localization signal (NLS). Consequently, E2F-4 and E2F-5 are only repressors because they are stably located in the nucleus in the pocket protein bound state.

E2F-1 to E2F-3a have the highest potential of interactions by having a cyclin A/cdk-2 binding-, pocket protein -, DNA binding and dimerization domain. The interaction with the cyclin A/ cdk-2 complex results from the protein's N-termini. By forming a complex with E2F1-3/ DP during S-phase, cyclin A/ cdk-2 phosphorylate this association. Consequential the complex separates from DNA and the cell cycle makes progress. E2F1-3 is unprotected by pRb in S-/ G2- phase, as a result, these E2F proteins are degraded by the proteasom [20]. E2F-1 to E2F-3a also have the ability to interact with transcription factors of the Sp-1 family or EAPP. While E2F/ Sp1 interactions cause contributive effects (activation or repression of transcription), EAPP suppresses the $p14^{ARF}$ promotor which is activated by E2F1. EAPP induces important genes in the cell cycle like E2F [21].

5.3.4 E2F3b-5

E2F-3b to E2F-5 are graduated as repressors by binding pRb (E2F-3b), other pocket proteins (E2F-4, E2F-5) or cofactors. E2F-4 and E2F-5 have no NLS, but the associations of E2F4-5/pocket proteins translocate to nucleus where they are active. Inserting a NLS into E2F-4,5 make them independent of pocket proteins and they act as activators of transcription [22].

By phosphorylating the pocket proteins through cyclin/cdk complexes and causing a conformational change, pocket proteins can dissociate from E2F. Consequently, the dissociation seems to make the DNA- binding unstable. E2F4-5 goes back to cytoplasma, firstly in G0-/ G1-phase and then in late G1-phase through cyclin E/cdk-2 [20].

5.3.5 pRb and E2F

The cyclin D/ cdk-4 complex is required to pass G1- phase and to enter S- phase. After being phosphorylated by CAK, the complex migrates to the nucleus. There, E2F is inactivated because it binds to unphosphorylated retinoblastom protein (pRb) [23]. An active cyclin D/ cdk-4 complex is important for phosphorylating pRb which dissociates

from E2F. This process also requires the complexes cyclin E / cdk-2 and cyclin A/ cdk, resulting in the release of E2F (***figure 8***).

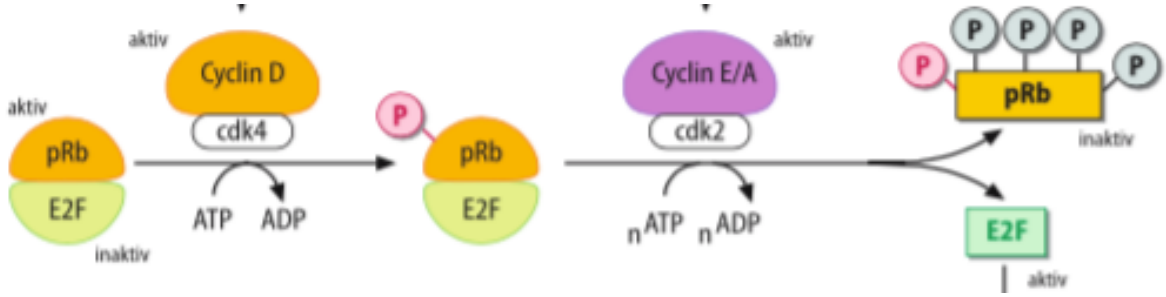

Figure 8: pRb and E2F interaction. E2F is inactive by binding pRb to it. The complexes cyclin D/ cdk4 phosphorylates pRb resulting in a conformational change. With cyclin E(A)/ cdk2 is the dissociation of pRb from E2F possible and E2F is now active.

Source: Löffler- Biochemie und Pathobiochemie, 8. Auflage 2007

E2F regulates the expression of enzymes which are important for DNA synthesis and is consequently required for the passage of G1- phase to S- phase. The pRb inhibits E2F transcriptional activity via a direct interaction with E2F. Cell growth triggers the phosphorylation of pRb. On the contrary, the cell growth decreases when an activated inhibitor of cdks (for example p21) prevents the phosphorylation of pRb [1].

Tumorsuppressors, such as pRb, prevent damaged cells from uncontrolled cell proliferation. Maligne transformation of cells and furthermore carcinogenisis are probably caused by mutations of tumorsuppressors.

5.4 EAPP and its function

The E2F-Associated-Phosphoprotein (EAPP) is involved in regulating the cell cycle. It is present in several tissues in variable amounts. The name is given due to its strong phosphorylation and its interaction with E2F. Analysis showed that there several amino acids, especially serine but threonine and tyrosin as well, are phosphorylated. Human EAPP consists of 285 amino acids. With an expected molecular weight of about 35 kDa, Western analyses show that HA-tagged EAPP migrates like a 45 kDa protein.Posttranscriptional modifications might at least partly explain this discrepancy [17].

Analysis showed that the EAPP gene has a promotor with binding sites for the specificity proteins (Sp) and the transcription factor early growth response 1 (Egr-1). By binding to GC-rich regions, Sp1 is important to induce the expression some proteins, for example the thymidine kinase. On the contrary, Sp3 is described as a repressor of transcription when it is sumoylated (a posttranscriptional modifikation). The EAPP promotor has four closely spaced binding sites and one for Egr-1. The EAPP gene comprises a TATA-less promotor (consequently rich in GC-residues) where Sp-1 and Egr-1 stimulate its expression, whereas Sp-3 acts as a repressor of transcription.

The activity of Sp family variegates by its posttranscriptional modifications, for example phosphorylation, O- glycolisation or acetylation. The EAPP promotor is induced in dose dependent manner by Sp-1 and Egr-1, but treating cells with both does not stimulate EAPP additionally. The activity of the EAPP promotor is nearly completly blocked by Sp-3 overexpression. The affinity of the repressor Sp-3 to the EAPP promotor is more dominant than Sp-1 and Egr-1 to it [21].

5.4.1 Role of EAPP in multi-drug-resistance

EAPP plays also an important part in resistance to anti-cancer drugs and moreover in the efficacy of chemotherapeutics. P-glycoprotein (PGP) and the multi-drug-resistance-associated protein (MRP) are the most common regulators of multi drug resistance (MDR). As members of the ABC transporters (ATP-binding cassette), they prevent cells

from xenobiotics by enhancing the removal of drugs out of the cells. PGP can be induced through EAPP by stimulating the MDR-1 promotor or, independently of EAPP, by E2F-1. It seems that E2F-1 interacts with Sp-1 to activate certain GC-boxes of the promotors. Unphosphorylated pRb represses and disturbes the activity of E2F-1 while EAPP competes with Sp-1 for binding site of the MDR-1 promotors [23].

5.4.2 EAPP in cell cycle

As mostly located in the nucleus, EAPP plays a substantial role in cell proliferation and apoptosis, interacting with the E2F-1, -2 and 3. During the cell cycle the EAPP levels stay rather unaltered, but EAPP dissapears in M- phase. This seems to be important for the completion of the cell cycle. The quick comeback indicates that EAPP is necessary to stimulate G1-phase and E2F transcription activity in S-phase. The concentration of EAPP is depending on cells condition, inasmuch as under normal terms, EAPP is only required in low concentrations, while under stress, for instance due to DNA damage, EAPP is significantly higher expressed. Induced EAPP stimulates p21 independently of p53 by enhancing p21's promotor activity. Increased p21 level results in prevention of apoptosis by arresting cells in G1-phase. On the contrary, low EAPP levels enhance apoptosis [24,25].

5.5 Cell proliferation

5.5.1 Activation of p53 and its role in cell cycle

DNA damage can be caused by chemical reagents, UV- or X-rays, spontaneous mutations or free radicals (produced during metabolic processes). Cells react to DNA damage by activating checkpoints [26]. DNA damage results in the activation of ataxia telangiectasia mutant (ATM) or rad3-related protein kinase (ATR). They can enhance their signal strength by phosphorylating checkpoint-kinase 1 (CHK-1) and checkpoint kinase 2 (CHK-2) (***figure 9***).

When double- strand breaks occur, ATM phosphorylates p53 directly on Ser15 and indirectly on Ser20 by checkpoint kinase 2 (CHK-2). Single strand breaks (e.g. during replication stress) result in the activation of ATR. Then checkpoint kinase 1 (CHK-1) is phosphorylated (and active) by ATR and finally p53 is actived by phosphorylating through CHK-1 [27].

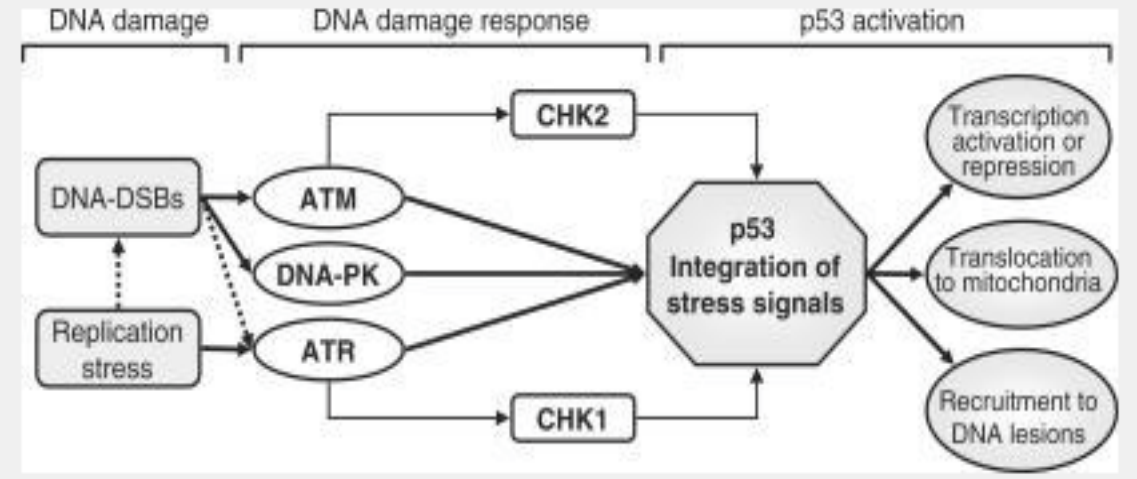

Figure 9: Response of cells to DNA damage. Double strand breaks activate ATM which phosphorylates p53 directly or indirectly by CHK-2. During replication stress single strand breaks occur and ATR phosphorylates p53 directly or indirectly by CHK-1.

Source: https://openi.nlm.nih.gov/detailedresult.php?img=PMC2190721_gkm744f1&req=4

p53 regulates the transcription by binding the promotor of the cdk-inhibitor p21. Thus the expression of p21 increases resulting in cell cycle arrest by inhibiting cyclinD/ cdk-4,

cyclin E/ cdk-2 and cyclin A/ cdk-2. This happens during oxidative stress or DNA damage. Now cells have time to repair the DNA or p53 initiates apoptosis [28].

If p53 doesn't modulate the expression of p21 correctly, cells are able to enter S- phase despite damage. p53 plays a critical role in carcinogenesis by protecting cells from malignant transformation [17,29].

5.5.2 Relationship between mdm2 and p53

The presence of p53 depends on the activity of murine double minute 2 (mdm2). Thus, mdm2 is one of the most important proteins that regulates p53 negatively. In unstressed cells, p53 is removed continously to keep p53 levels low.

As an E3 ubiquitin ligase, mdm2 enhances proteasomal degradation of p53 by catalyzing polyubiquitination. Mdm2 recognizes the N-terminal transactivation domain of p53. The mdm2/ p53 complex in the nucleus translocate to the cytoplasm where p53 degradation starts, but it can be degraded in the nucleus as well.

DNA damage causes phosphorylation of p53 by different ways (for example ATM, ATR, CHK-1, CHK-2). This reduces the affinity of mdm2 to p53 and prevents p53 from proteasomal degradation (***figure 10***). ATM could also phosphorylate mdm2 on Serin 395 what prevents p53 also from degradation or export out of the nucleus. Moreover phosphorylated mdm2 enhances the p53 expression after genotoxic stress [30].

On the other side, p53 is positive regulator of mdm2 by inducing the expression of mdm2 as a transcription factor. This makes sense after DNA repair to remove excessive p53 quickly [31]. Blocking the degradation of p53 is also possible through binding mdm2 by the tumor supressor $p14^{ARF}$ [32]. $P14^{ARF}$ binds mdm2 directly without disturbing p53-mdm2 interactions [33].

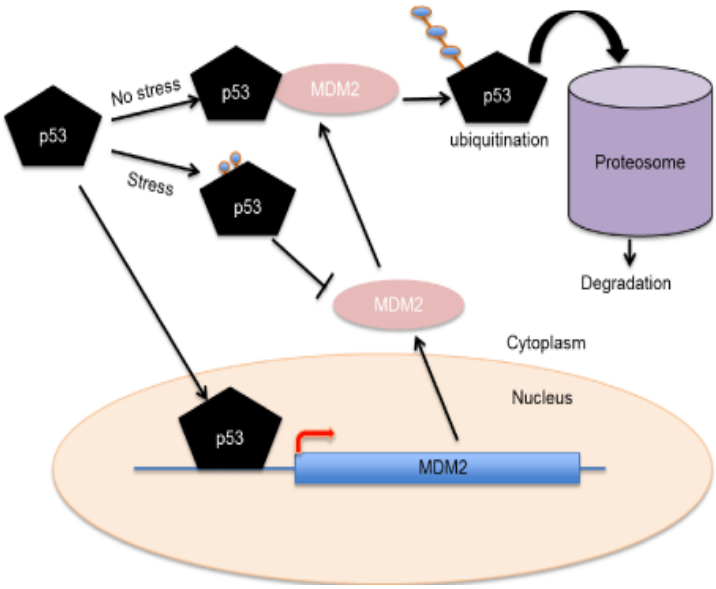

Figure 10: Activation and degradation of p53/mdm2.
In stressless cells, mdm2 forces the ubiquitination of p53, resulting in the degradation it. When cellular stress (for example DNA damage) occurs, p53 is phosphorylated and prevented from the proteasom. Phosphorylated Mdm2 by ATM is also possible. Figure does not show that mdm2 is also in the nucleus.

Source: http://austinpublishinggroup.com/blood-disorders /fulltext/ blood-disorders-v2-id1028.php

5.5.3 E2F and EAPP in cellcycle and apoptosis

The N-termini domain of E2F enables interactions between E2F1-3 and EAPP. With the loss of this domain, E2F-4 does not bind to EAPP. Bindings seem to be regulated by the C-terminal part of EAPP [17].

E2F-1 is also known as a tumor supressor by inducing apoptosis through activating the tumor supressor protein $p14^{ARF}$ and p73 [17]. EAPP negatively regulates the expression of $p14^{ARF}$ independently of E2F. This may save cells from $p14^{ARF}$ regulated apoptosis, when p53 is inactivated. With this model, EAPP can stimulate E2F- dependent transcription in S-phase and act as controller of the repressor $p14^{ARF}$, which could be important for malignant transformation. On the contrary, low p53 levels induce $p14^{ARF}$, resulting in p53-independent apoptosis [21].

Cells were EAPP is overexpressed arrest in G1 Phase and tend to avoid E2F-1 induced apoptosis which could explain its increased levels in cancer cell lines and its possible involvement in transformation of malignant cells. EAPP seems to disturb finishing of the cell cycle. This could explain the absence of EAPP in mitosis. Finally, a RNAi- mediated knock down of EAPP demonstrates that EAPP is essential for cell cycle progression [17].

5.5.4 Function of p21

In normal cells, p21 has its function as a cell cycle inhibitor, leading to cell arrest during G1-Phase until DNA damage is successfully repaired or apoptosis has been induced. P53 regulates the higher acitivity of p21 mainly, when DNA damage or oxidative stress occurs [17].

But p21 has two faces, it has positive effects on cells by preventing from malignant transformations. So it could act as keeper of cancerogenesis by arresting the cell cycle. Contrarily, p21 induces gene expression via promotor elements and transcriptional factors what can result in a higher cell cycle activity [15]. Moreover, an overexpression of p21 and deficit of p53 can direct in malignant transformation [34]. Knockouts of p21 show that p21 is the main mediator of p53- induced cell cycle arrest [29].

5.5.5 EAPP and p21

Smaller fractions of apoptotic cells were found in EAPP overexpressing cells, while knockout of EAPP results in higher fractions of apoptotic cells. In recent studies we found out that anti-apoptotic effect of EAPP is diminished in p21-lowered cells. Therefore we assume that the antiapoptic activity of EAPP is mediated by p21. This effect seems to be independent of p53s' task. EAPP competes with Sp-1 for the same binding site of p21's promotor [17]. Cells with high p21 levels by EAPP are prevented from apoptosis, but lower EAPP levels enhance cell death [25].

5.6 PI3K/PKB- pathway

The phosphoinsoitide-3-kinase (PI3K)/ proteinkinase B (PKB) pathway plays an important role in cell growth, cell proliferation, apoptosis by using insulin signaling for protein synthesis and regulating processes. It seems to be also important in human malignancies what marks the PI3K/PKB pathway as an exciting target in cancer therapy.

By having a membrane-associated phosphatidic acid group, phosphatidylinositol phosphates also consist of a glycerol fraction for binding to a phosphorylated inositol head group in cytosol. Signals from receptor tyrosine kinases (RTKs) and G-protein coupled receptors (GPCRs) are transferred by the PI3Ks.

The phosphoinositide-3-kinases can be divided into several groups, whereas class 1 is able to phosphorylate the 3'- hydroxyl group of the inositol ring of phosphatidylinositol-4, 5-biphosphate (PI- 4, 5- P2) to form phosphatidylinositol-3,4,5-trisphosphate (PIP_3) [35]. As a phosphatase, PTEN regenerates PIP_2 by dephosphorylating PIP_3.

If a RTK is being activated in the plasma membrane, P13K starts modeling a complex, consisting of a regulatory (p85) and a catalytically component (p110). In normal cells, activity of PI3K, including regulation of cell proliferation, apoptosis, and other cellular processes, is being restricted. Two forms of P13K exist, class IA and IB with their subunits, while only class IA is discussed to be involved in ontogenesis processes. PI3Ks main function consists in synthesizing second messenger, such as PIP3 in the membrane that activates AKT by interacting with the pleckstrin-homology (PH) domaine (***figure 8***).

5.6.1 The proteinkinase B / Akt

The AKT family consists of 3 different serin/threoninekinases, AKT1, AKT2 and AKT3, that differ in structure and function. Regulation of proliferation, cell growth and survival are major functions of proteinkinase B.

5.6.2 Role of Akt in apoptosis

AKT affects apoptosis, a main mechanism to control exessive cell proliferation in normal cells. It can act anti-apoptotic by preventing cells from PTEN- mediated apoptosis. Akt operates as a kinase, influencing other proteins, such as caspase-9 or pro apoptotic BAD. Both, caspase-9 and BAD, are negatively regulated by Akt-catalyzed phosphorylation. Consequently, the activity of BAD is reduced and this seems to prevent apoptosis [36].

Due to phosphorylation of mdm2 at Ser166 and Ser186 by AKT, mdm2 transloctates from the cytoplasm into the nucleus. There it downregulates p53 by an increased binding of the tumor supressor. Consequently, p53 can decrease by exporting p53/mdm2 complexes from the nucleus and the following degradation by the proteasom. Thus, AKT can prevent cells from p53- dependent apoptosis. This mechanism could demonstrate possible targets for the efficacy of cancer therapies in question p53 [37].

5.6.3 Activation of Akt and its action in cell proliferation

Two mechanism for entire activation of AKT are required: First, through interaction of its N-terminal PH-domaine with PIP3, AKT is being translocated to the inside of the membrane. This effect can be blocked by carboxy-terminal modulator protein (CTMP) that stops phosphorylation of AKT at the membrane. An unknown kinase is able to phosphorylate CTMP and consequently dissolutes CTMP from AKT again. The second mechanism, that consists of phosphorylation of Thr308 by PDK1 and Ser473 by PDK-2 is then mandatory for reaching maximum effects of AKT (***figure 11***).

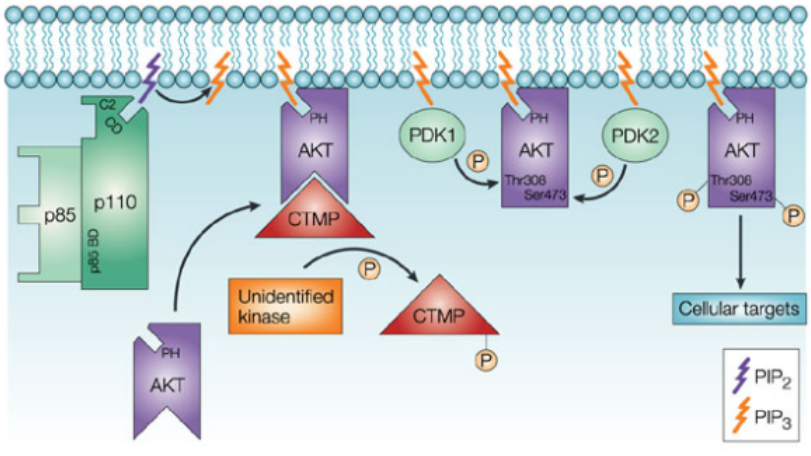

Figure 11: Activation of Akt.
PIP3 phosphorylates AKT via the PH- domaine.
By an unknown kinase phosphorylated CTMP can suppress this interaction. PDK1 and PDK2 trigger the effects of AKT.

Source:https://www.researchgate .net/publication/11282035_The_Phosphatidylinositol_3_Kinase_AKT_Pathway_in_human_cancer

AKT can also play a role in cell cycle by regulating reduction of cyclin D1, that is important for switching from G1- to S-stage. GSK3beta is being phosphorylated by AKT that causes its inactivation, and as consequence the depletion of Cyclin D1 is being reduced and accumulates [36].

6 Material

6.1 Media and Dishes

- DMEM high glucose with 10% FCS, 1% Penicillin/ Streptomycin and 1% Glutamax
- Ø 100 mm plate, 10 ml media

6.2 Cell lines

Only U2OS wildtype (human osteosarcoma cell line) and truncations of them were used for analysis. Mutants expressed additional to the endogoneus EAPP a specific domain of EAPP.

- U2OS wild type
- U2OS HA-EAPP full length
- U2OS HA-EAPP 1-120
- U2OS HA-EAPP 1-160
- U2OS HA-EAPP 1-180
- U2OS HA-EAPP 10-240
- U2OS HA-EAPP 55-c
- U2OS HA-EAPP 95-c
- U2OS HA-EAPP pm (serin and thyrosin amino acids were replaced by alanin)
- U2OS HA- EAPP ps (continous decreased amount of protein by siRNA)
- U2OS mEAPP (J) (knockout of endogoneus EAPP and integration of murine EAPP cDNA)

6.3 Antibodies

- BAX: (2D2) sc-20067 mouse monoclonal Antibody- Santa Cruz
- CDK-1: Cdc2 p34 (17) sc-54 mouse monoclonal IgG2a 200 μg/ml- Santa Cruz

- EAPP- Antibody: generated by 1E4 cells
- GAPDH: mouse monoclonal from E.Ogris
- p-AKT (S473): D9E XP rabbit monoclonal Antibody #4060 Cell Signaling
- p21: (Waf1/Cip1) mouse antibody Ig2a #2946 Cell Signaling
- p53: (DO1) sc-126 monoclonal IgG2a 200 μg/ml- Santa Cruz
- YB1: rabbit polyclonal Antibody, produced by Franz Wohlrab Lab

6.4 Treatments

- Etoposid- treatment: final concentration 0,005 mM

6.5 Equipment

- X-Ray Film developer Curix 60 AGFA
- FUJI Medical X-Ray Film 100 NIF 18x24
- Trans-Blot Turbo Transfer System from Biorad
- Trans- Blot Turbo Mini Nitrocellulose Transfer Packs
- Thermomixer 5436 from Eppendorf
- Centriguge 5471R from Eppendorf
- Microcentrifuge FugeOne two I
- Mixer Rotary Latinco Typ LD76
- Incubator WTB Binder (Articl.nr. 9040-0005)
- Victorr V 1420 multilabel counter
- NanoDrop

6.6 Further Materials/ Solutions

Polyacrylamid gel

A) separation gel	8 %	10 %	12 %	14 %
acrylamid	1,92 ml	2,4 ml	2,64 ml	3,36 ml
d H2O	2,98 ml	2,5 ml	2,36 ml	1,54 ml
Tris ph 8,7	3 ml	3 ml	3 ml	3 ml
SDS 10%	40 µl	40 µl	40 µl	40 µl
TEMED	8 µl	8 µl	8 µl	8 µl
APS	40 µl	40 µl	40 µl	40 µl

B) stacking gel	1x	2x
acrylamid	600 µl	1,2 ml
d H2O	2,86 ml	5, 72 ml
Tris ph 6,8	500 µl	1 ml
SDS 10%	20 µl	40 µl
TEMED	4 µl	8 µl
APS	20 µl	40 µl

APS 20%

0,2 g APS
1ml dH2O

Blocking Solution

300 ml (1x) TBS-T
15 g milk powder
200 µl azid

Bradford reagent

BIORAD Protein Assay Dye Reagent
Concentrate was diluted 1:5 with dH2O

ECL (selfmade)	0,1 M Tris (pH= 8,5) p-Coumaric acid 3-Aminophtalhydrazide (luminol) 3% hydrogen peroxide solution
ECL (BIORAD)	Clarity Western ECL
Hunt- Buffer	20 mM Tris-HCl (pH= 8,0) 100 mM NaCl 1 mM EDTA 0,5 % NP-40 1 mM DTT 1 mM PMSF 50 mM NaF
Hunt- Mix	0,01 % complete mini 0,01 % Na-Vo4 0,01 % Butyrat 0,002 % PMSF remain Hunt- Buffer
Marker Antibody	DNA- marker Lambda HindIII
Milk 0,5 % for secondary antibody	0,5 % milk powder remain (1x) TBS-T
Ponceau red	
Protein marker	Protein marker Precision Plus Protein Standards all blue from BIORAD
(10x) Running Buffer	60,5 g Tris 288 g Glycin

	20 g SDS filled up to 2L with dH2O
(1x) Running Buffer	200 ml (10x) Running Buffer 1800 ml dH2O
Sample Buffer	100 mM Tris (pH= 6,8) 20 % Glycerol 10 % beta- Mercaptoethanol 2 % SDS 0,01 % Brom- Ethanolblue
(10x) TBS	121,2 g Tris 175,2 g NaCl ad 1800 ml dH2O adjusted ph- value to 7,5 and filled up to 2L with dH2O
(5x) Transfer Buffer	from BIORAD
(1x) Transfer Buffer	200 ml (5x) Transfer Buffer 600 ml dH2O 200 ml ETOH

7 Methods

7.1 Cell Culture

All cells were cultivated in DMEM high glucose with 10% FCS, 1% Penicillin/ Streptomycin and 1% Glutamax at 37°, 90% rH and 7,5 % CO_2 . The medium was removed, the cells were washed with PBS and trypsinized with 5-10 drops for splitting.

The same procedure was used for freezing cells. Therefore the cells were resupendend in 1ml medium and transported to a cryotube which was filled up with 1/10 DMSO. After cooling down on ice, the cells were frozen at -80°. For defrosting, the cells were hold in the water bath until nearly complete thawed. Then they were transferred to a cell culture dish with heated medium by resupending the remaining ice cube of cells.

7.2 Generation of different cell lines with additional variable versions of EAPP

Former studies showed a total knockout of EAPP resulted in death of human U2OS cells. Thus EAPP plays an important role in cell cycle. EAPP interacts with different proteins involved in the cell cycle. To investigate which functional domains of EAPP are necessary for interactions with other proteins, several truncated versions of EAPP were generated. In earlier experiments, additional to the essential endogoneus EAPP of U2OS cells, particular segments of EAPP were transfected (***figure 12***).

1: U2OS wild type
2: U2OS HA-EAPP full length
3: U2OS HA-EAPP 1-120
4: U2OS HA-EAPP 1-160
5: U2OS HA-EAPP 1-180
6: U2OS HA-EAPP 10-240
7: U2OS HA-EAPP 55-c
8: U2OS HA-EAPP 95-c
9: U2OS EAPP pm
10: U2OS ps EAPP
11: U2OS mEAPP (J)
marker

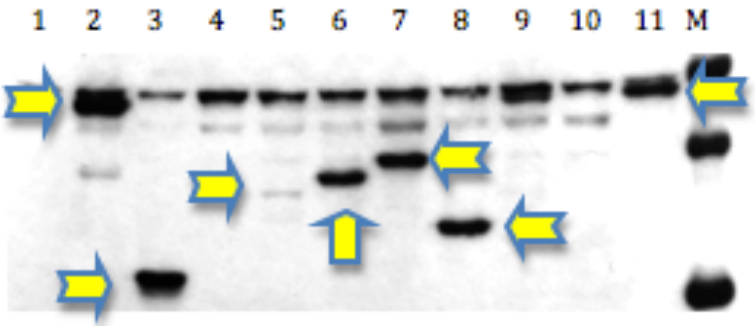

Figure 12: truncations of EAPP in Western blot analysis. All truncations were HA-tagged, proved with anti-HA-antibody with Western Blot researches. HA- tags (yellow arrows) from 1-160 and 95-c weren't visible in the short exposure.

7.3 Transfection and generation of U2OS cells expressing EAPP truncations

After cloning various truncations of EAPP cDNA in an expression vector, the U2OS cells were transfected with the vector who carried the specific sequence of EAPP. The transfections were performed according to the PEI method. Cells were selected with geniticin (G418) until cells do not die any more. After making a dilution (1:20.000-50.000) of the selected cells, single clones were isolated and cultured in new dishes. The truncations of EAPP stably expressed the respective domain of EAPP.

7.3.1 Transfection via PEI Method

For transfections 1ml of a DNA-mix was added to the cells. The corresponding DNA amount was filled up with HBS to 0,5 ml. This solution was mixed with 0,5 ml containing 2 times µl the amount of µg DNA in HBS. After vortexing and incubating 10 minutes at room temperature, the cells were treated with the mix.

7.4 Generation of U2OS EAPP pm cells

U2OS cells were transfected and selected for the expression of HA-EAPPpm, a mutant were all serines and threoinines known to become phosphorylated had been replaced by alanines.

U2OS EAPP pm:

1	MNRLPDDYDPYAVEEPADEEPALAAAEDEVDVLLHGAPDQKRKLIRECLAGEAEAAAEDE	60
61	FEKEMEAELNATMKTMEDKLSSLGTGSSSGNGKVATAPTRYYDDIYFDADAEDEDRAVQV	120
121	KKKKKKQHKIPTNDELLYDPEKDNRDQAWVDAQRRGYHGLGPQRSREQQPVPNSDAVLN	180
181	CPACMTTLCLDCQRHESYKTQYRAMFVMNCSINKEEVLRYKASENRKKRRVHKKMRSNQE	240
241	DAAEKAETDVEEIYHPVMCTECSTEVAVYDKDEVFHFFNVLASHS	285

U2OS wt:

1	MNRLPDDYDPYAVEEPSDEEPALSSSEDEVDVLLHGTPDQKRKLIRECLTGESESSSEDE	60
61	FEKEMEAELNSTMKTMEDKLSSLGTGSSSGNGKVATAPTRYYDDIYFDSDSEDEDRAVQV	120
121	KKKKKKQHKIPTNDELLYDPEKDNRDQAWVDAQRRGYHGLGPQRSREQQPVPNSDAVLN	180
181	CPACMTTLCLDCQRHESYKTQYRAMFVMNCSINKEEVLRYKASENRKKRRVHKKMRSNQE	240
241	DAAEKAETDVEEIYHPVMCTECSTEVAVYDKDEVFHFFNVLASHS	285

7.5 Protein Extraction

The medium was removed and 1 ml PBS was given to the cells. The cells should be stored on ice. After scraping the cells off, they were collected, centrifuged and washed with 1 ml PBS. The volume of the pellet was rated, resupended with the tenfold amount of its volume with Hunt- Mix and frozen at -80°C. After thawing, the cells were centrifugated with the centrifuge 5471R (Eppendorf) for 10min at 2°C. Storing the cells on ice was also important, when the supernatants were transferred to new Eppendorf tubes. The finished extracts were stored at -80°C.

7.5.1 Protein Concentration Measure via UV/VIS- Spectroscopy

To determine the concentration of the protein, 1 µl was sampled and given to 1 ml (room temperature heated) Bradford reagent. After vortexing and incubating 5 minutes at room temperature, the protein concentration was measured at wavelenght λ = 595 nm.

To use a specific amount of the proteins, this formula was used:

$$\frac{\left(\frac{1000}{Abs.}\right)}{70} = ug/ul$$

7.6 Western Blot

7.6.1 Preparation and Run

Usually an amount of 20 µg protein was mixed with 10 µl sample buffer and shaked on a Eppendorf Thermomixer 4 minutes at 95°. If the volume of the probe was higher than 15 µl, the Eppendorf vessels were opened during the shake. After cooling down on ice, the

probes were spinned down in a microzentrifuge for 5 seconds and stored at -20° until using them.

The extracts were loaded on a polyacrylamid gel with 3 µl BIORAD protein marker and run on 110 Volt until the running front leaked out.

7.6.2 Blotting

After removing the stacking gel and assembling the transfer sandwich the blotting procedure was started. By using the Trans-Blot Turbo Transfer System (BIORAD), only semi dry electrophoresis apparatus was excercised at 1,3A ; 25V ; for 10 minutes.

7.6.3 Checking the protein loading

The nitrocelluloses with the transferred proteins were washed with water to remove the transfer buffer to enable a coloring of the protein loads with Ponceau red. To decolorize the blots again, 1x TBS-T was used.

7.6.4 Immunodetection and analysis

The blots were blocked with 5 % milk blocking solution for at least 30 minutes. After adding the primary antibodyies, the blots were stored at 4° slow-moving. For phospho-specific- antibodies the blots were washed with 1x TBS-T before adding the primary antibodies.

7.6.4.1 Editing and representation of the blots

The immunostaining of the blots were reworked with the program „ImageJ“. Brightness and contrast were adjusted. For removing the backgrounds, the results were converted into peaks by choosing the respective area of the immunostainings. The areas under curve (AUC) of the investigated proteins were normalized to the highest one of each run. The same was done for GAPDH, as comparison. At last, each line was divided by the respective GAPDH level. Graphs were generated with the outcomes by „Excel“.

7.7 Assembly of antibodies

7.7.1 primary antibodies

normal primary antibody	phospho-specific antibody	EAPP – antibody (mouse)
1/10 5% blocking solution 9/10 (1x) TBS-T 1/1000 azid 1: x-000 antibody	1/5 5% BSA 4/5 (1x) TBS-T 1/1000 azid 1: x-000 antibody	1/10 5% Blocking Solution 26/30 (1x) TBS-T 1/30 1E4- antibody

7.7.2 Secondary antibodies

anti- mouse antibody (1:10.000)	anti- rabbit antibody (1:30.000)
3 µl anti- mouse antibody and 1 µl anti-marker antibody were mixed in 30 ml 0,5% milk for blots with 8- 15 slots	1 µl anti- rabbit antibody was mixed in 30 ml 0,5% milk for blots with 8-15 slots

The following day the blots were washed 3 times with 1x TBS-T for each time 10 minutes. After incubating the blots with secondary antibodies for at least 60 minutes, they were washed with TBS-T 3 times again. To assay the blots with X-Ray films a selfmade ECL detection solution or ECL from BIORAD was used. Therefore the blots were incubated 30 seconds with ECL and shorty after analysed with Curix 60 AGFA developer.

7.8 Treatments

7.8.1 Etoposide- treatment

To show how EAPP responds to a DNA damage signal, the cells were treated with etoposide with a final concentration of 0,005 mM, visible below in ***figure 13***. After

treating them with etoposide 16 hour, the medium was removed, the cells were washed with 1x PBS and fresh culture medium was added.

						+ Etoposide 16h				
untreated	**+ Eto 2h**	**+ Eto 4h**	**+ Eto 8h**	**+ Eto 16h**		**MC 2h**	**MC 4h**	**MC 8h**	**MC 24h**	**MC 48h**

Figure 13: Process of etoposide treatments

8 Aim of the project

EAPP plays an important role in the cell cycle. Depending on the cell type, complete loss of EAPP can be lethal. Its overexpression results in elevated p21 levels, a subsequent cell cycle arrest, and heigthened resistance against apoptosis. EAPP interacts with a number of proteins among them the phosphorylated and thus active form of Chk2 This protein is involved in the response to DNA double strand breaks. Aim of this project was to investigate the functional domains of EAPP by using different truncations and versions of EAPP in addition to the endogenous EAPP, in U2OS cells. The consequences of their expression on the levels and the activity of selected cell cycle- and apoptosis regulators should be examined under normal and DNA damage conditions. Following DNA damage induction, the expression of the endogenous EAPP and the different version of EAPP were to be compared with important cell cycle and apoptosis- related proteins by Western blotting and immunostaining. The truncations of EAPP can be very useful for the determination of important domains of EAPP and their possible influence on other proteins.

9 Results

9.1 Effects of etoposide on endogenous EAPP and ectopic EAPP (and truncations thereof)

In normal cells, EAPP is only required in low concentrations. Following DNA damage, EAPP increases and stimulates the expression of p21, independently of p53. To demonstrate the effects of endogenous EAPP and the additional truncations of EAPP during DNA damage, the cells were treated with a final concentration 0,005mM of etoposide.

Endogenous EAPP seems to be rather increased in most of the cell lines due to etoposide, but the 55-c, 1-180 and 10-240 HA-EAPP mutants differ sligthly from the rest. In wildtype cells, an increase of EAPP is visible until 16 hours etoposide treatment (***figure 14, B***), but after medium change, the cells are rather unchanged at high levels (***figure 14, A***). There, higher EAPP levels may be caused by the fresh medium which induces the whole protein expression generally.

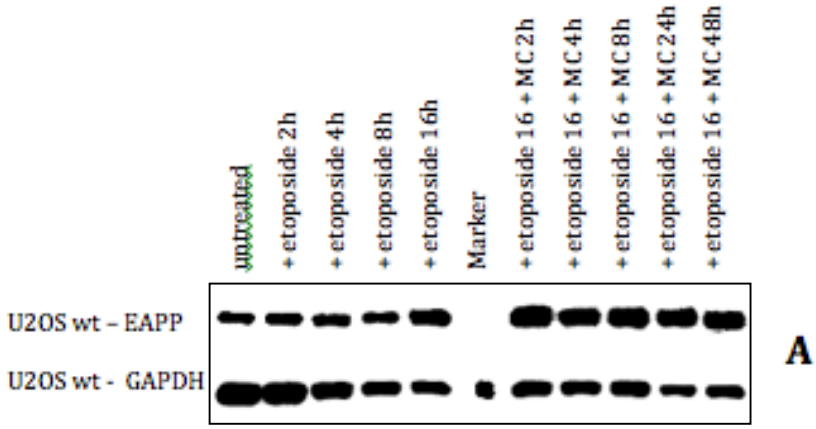

Figure 14 A: Effects on EAPP in wild type cells by etoposide treatment
Wester blot analysis and immunodectection of EAPP and GAPDH. EAPP increases with etoposid treatment until 16 hours. After media change, EAPP stays rather unchanged.

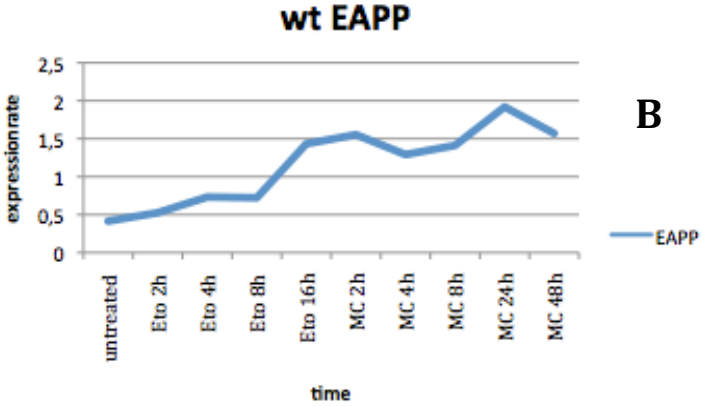

Figure 14 B: Influence on EAPP in U2OS wild type cells by DNA damage (graph)
Western blot analysis and immunostaining of EAPP in wt cells are compared with GAPDH by transforming the results via ImageJ.

In 55-c (***figure 15, C***) and 1-180 HA-EAPP cells (***figure 15, B***), endogenous EAPP and the truncations of EAPP are unchanged until medium change for 8 hours. There we can see an increase of the truncated EAPP in both cell lines which disappears 24 hours after medium change again.

In contrast to this, in 10-240 cells, both EAPPs stay constant with etoposide until medium change, with the exception of the endogenous EAPP. It seems to increase only after 16 hours of etoposide treatment. An enormous induction of the 10-240 truncation of EAPP is visible after the removal of etoposide until 8 hours medium change. In contrast to this, the endogenous EAPP stays unchanged until 8 hours fresh medium. Notable, at 24 hours medium change, the endogenous is higher expressed than before, respecting the GAPDH level. On the other hand, the expression of the 10-240 EAPP reflects the expression of GAPDH at that time (***figure 15, A***).

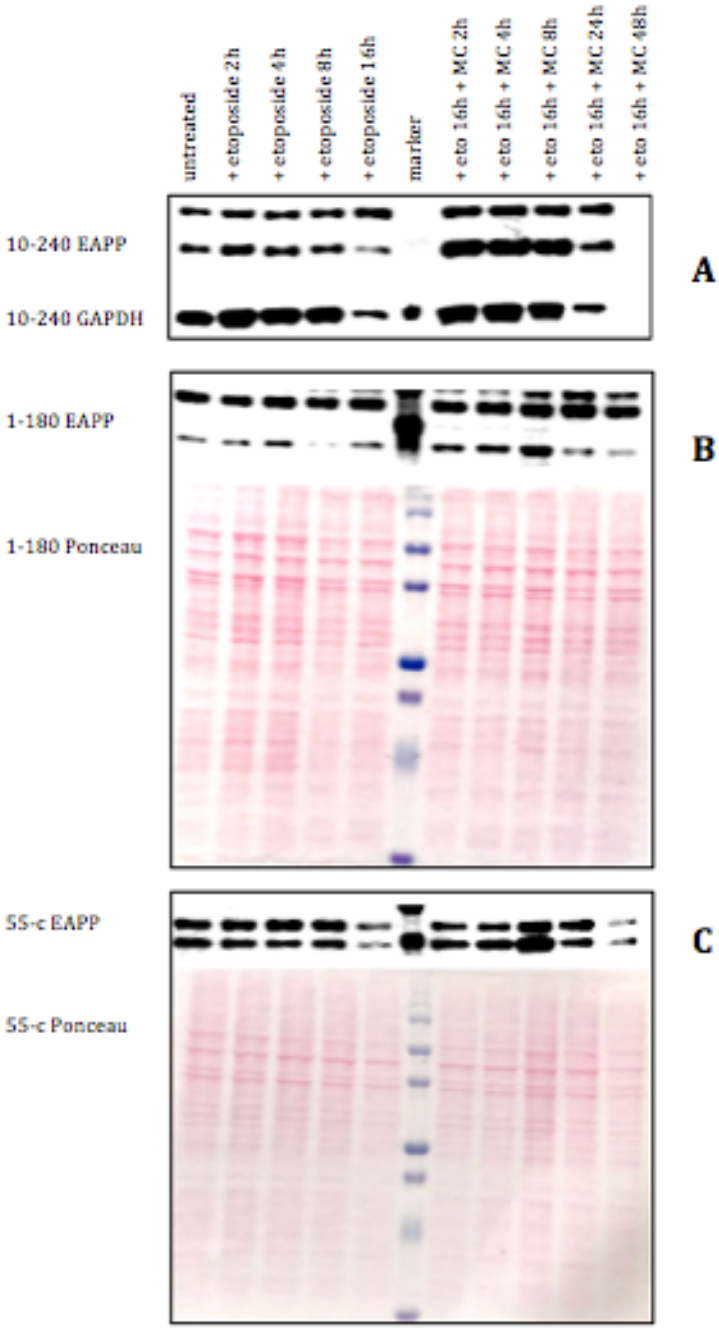

Figure 15: Western blot analysis and immunodetection of different truncations of EAPP.
A, B, C show in the first line the endogenous EAPP and below the respective truncation of EAPP. Effects on EAPP are compared with GAPDH in A, B and C with their Ponceau. The effects on the truncations of EAPP differ in effects on endogenous EAPP.

9.2 Different effects on p- Akt S473

In U2OS wild type cells, phosphorylated Akt S-473 rather goes down after 8 hours etoposide treatment. After replacing the medium for 2 hours, an increase of p-Akt and a following decrease after 4 hours are observed (***figure 16, A***). Comparing the p-Akt expression with EAPP levels suggests that p-Akt decreases after 8 hours etoposide and EAPP rises. After medium change, EAPP is clearly higher expressed than p-Akt. Both proteins are induced by etoposide after 16 hours and decrease after 24 hours medium change (***figure 17***). Cells expressing HA-EAPP 10-240 in addition to endogenous EAPP, show similiar effects on p-Akt S473 due to etoposide, compared with GAPDH (***figure 16, B***). But after medium change, the induced p-Akt levels stay unchanged and reflect the respective GAPDH levels.

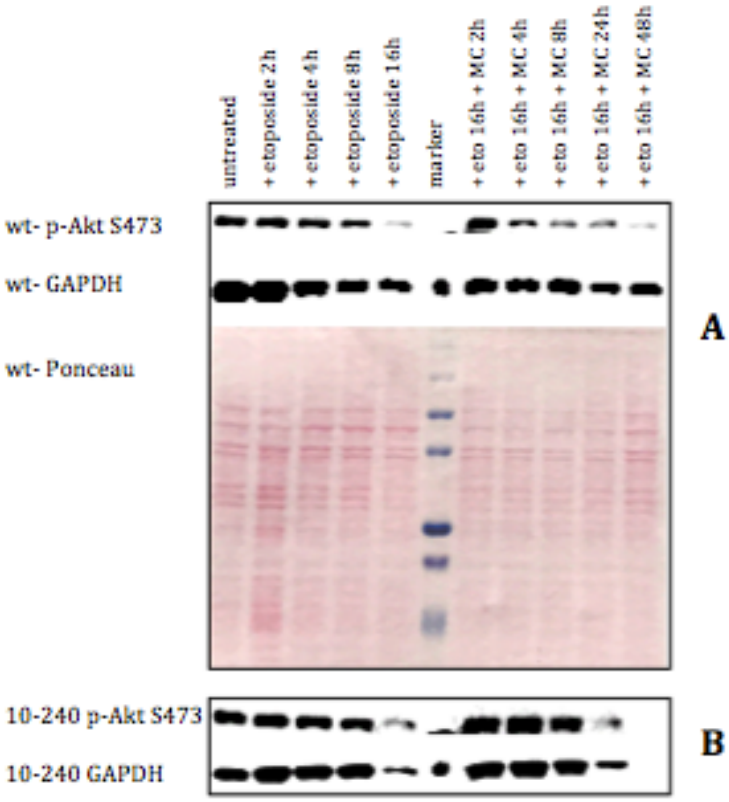

Figure 16: Effects on p-Akt S473 in wild type cells and cells with 10-240 truncation of EAPP
Western blot analysis and immunodetection of p-Akt in wt cells (A) and in U2OS HA-EAPP 10-240 (B). MC 48h is missing in B.

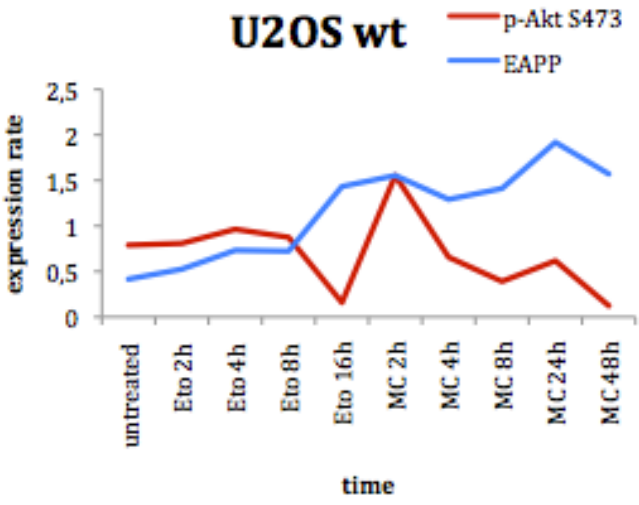

Figure 17: EAPP and p-Akt S473 levels in wild type cells
The diagramm shows the expression of EAPP (red) and p-Akt S473 (blue), normalized to their respective GAPDH levels.

On the contrary, in cells expressing HA-EAPP 1-180 in addition, p-Akt does not go down by etoposide, but the removal of etoposide by fresh medium tends to result in an increase of phosphorylated Akt S473 (***figure 18, A***). U2OS ps EAPP (expressing lower amounts of endogenous EAPP) and cells expressing HA-EAPP 55-c in addition, show Akt constant phosphorylation upon etoposide treatment. Moreover, different p-Akt levels in 55-c cells result from the discrepancy of the respective GAPDH levels (***figure 18, B+C***). HA-EAPP 1-120 expressing cells tend to result in a decrease of p-Akt until 8 hours etoposide. At 8 hours and 16 hours, an induction of p-Akt is visible. After the removal of etoposide, p-Akt S473 is induced after 2 hours. Followed by lower levels and ending in an increase between 24 hours and 48 hours again, can be seen in ***figure 18, D***.

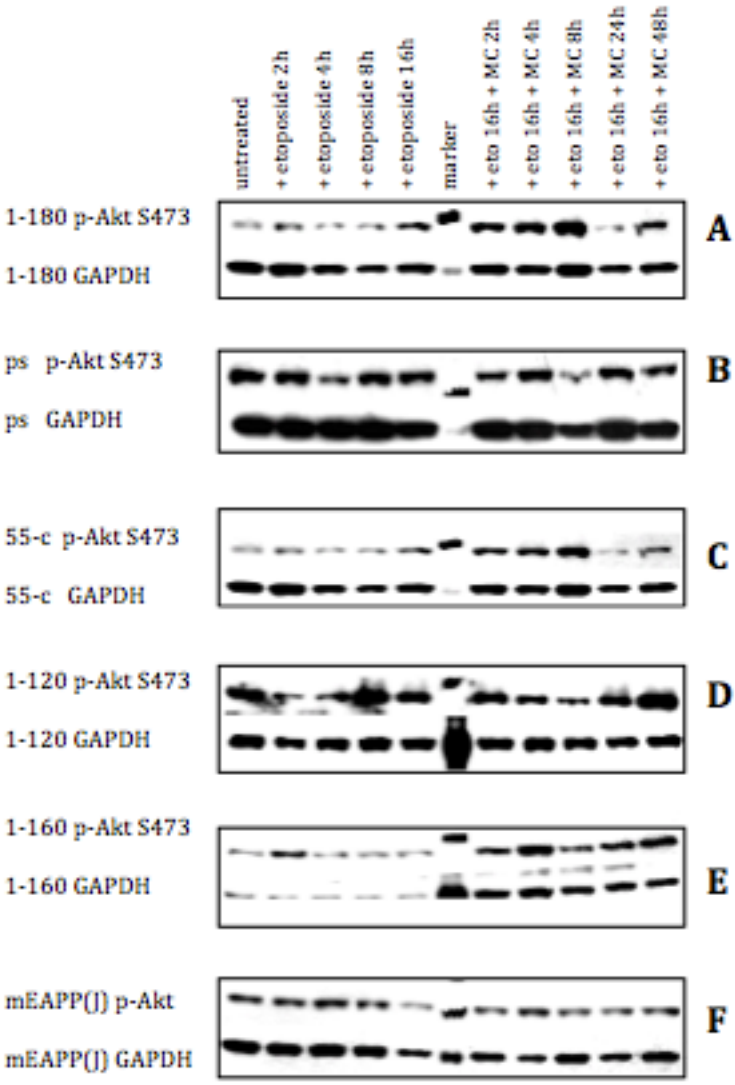

Figure 18: effects on p-Akt S473 in cells with different EAPP truncations.
All blots (C-H) show the p-Akt S473 levels in the first line in comparison with GAPDH below.Etoposide treatment seems to have different effects on phosphorylated S473 Akt.

In contrast to EAPP 1-120, cells with the truncation of EAPP 1-160 are slightly induced after 2 hours etoposide and remain then on low levels again. By adding new medium, an increase of p-Akt at 4 hours is visible which reduces gradually and rises at 48 hours again (***figure 18, E***) In cells with murine EAPP, p-Akt seems to stay unchanged, but new medium tends to result in an increase of it at 4 hours (***figure 18, F***).

9.3 Role of EAPP in the activation of p53

EAPP has been shown to interact with p53 (Rotheneder unpublished results). Therefore it is conceivable that EAPP influences the dynamics of p53 activation following DNA damage. Etoposide inhibits the DNA synthesis by forming a complex with topoisomerase II and DNA, resulting mostly in double strand breaks of the DNA. To indicate effects on EAPP by DNA damage induced activation of p53, the cells with different versions of EAPP were treated with etoposide for specific times.

U2OS wild type cells show already an induction of p53 after 2 hours etoposide treatment, clearly seen at 16 hours etoposide (***figure 19, A***). In contrast to wild type cells, DNA damage increases p53 after 4 hours and p53 remains at high levels in 10-240 cells (***figure 19, B***). In both cell lines, p53 levels are after 16 hours etoposide treatment at their maximums and stay rather unchanged after medium change for 24 hours. P53 in wt cells decreases at 48 hours again (***figure 19, A***).

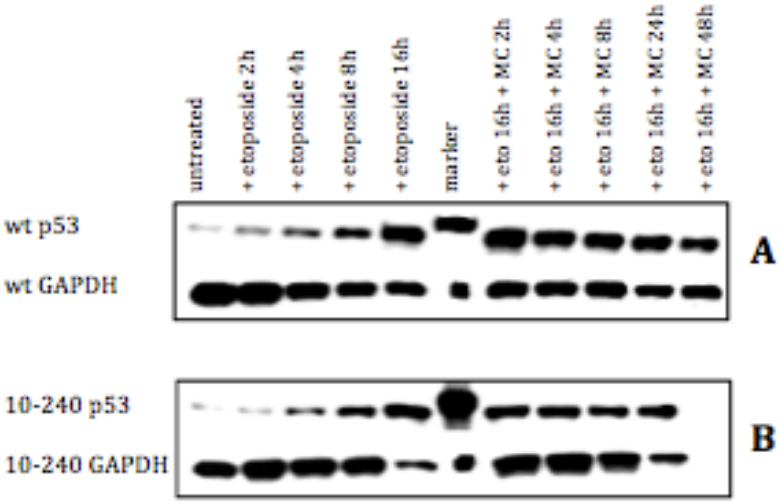

Figure 19: p53 increaes by DNA damage in wt and 10-240 cells.
A shows p53 levels U2OS wild type cells and B U2OS HA-EAPP 10-240 cells by using Western blot analysis and immunodetection.

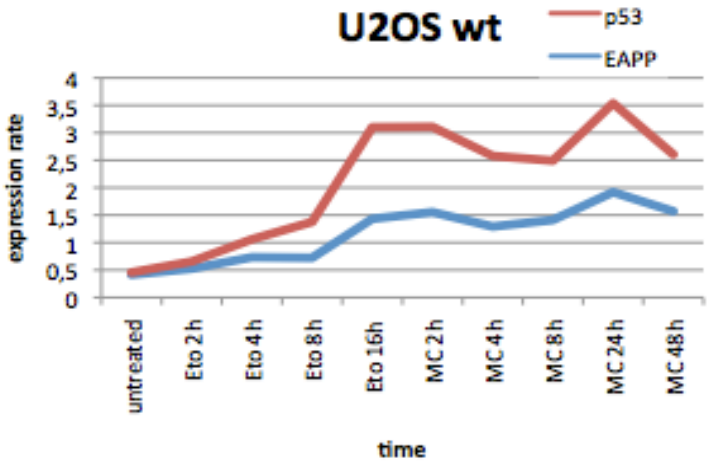

Figure 20: EAPP and p53 levels in wild type cells after DNA damage
Western blot analysis and immunostaining of EAPP (red) and p53 (blue) show a similiar expression profile due to etoposide.

Western blot analysis and immunostaining of EAPP and p53 show a similiar

expression profile following etoposide treatment in wild type cells. The respective graph suggests that p53 needs a long time to go back to beginning levels (***figure 20***).

In cells expressing HA-EAPP 1-180, p53 increases firstly after 4 hours etoposide and it has its maximum between 8 hours and 16 hours. After the removal of etoposide, p53 levels go down slightly. At 24 hours after medium change, p53 rises again and decreases at 48 hours (***figure 21, A***). Similar p53 levels are in U2OS ps EAPP visible. Adding fresh culture medium does not affect the expression of p53 (***figure 21, B***).

In cells expressing HA-EAPP full-length, p53 becomes induced by etoposide after 2 hours. An increase of p53 after 16 hours treatment is clearly visible. After the removal of etoposide, p53 stays rather unchanged. It seems that fresh culture medium does not alter the expression of p53 in cells with HA-EAPP full length (***figure 21, C***). P53 levels are higher at the begin of medium change in HA-EAPP full length cells than in ps cells (***figure 21, B+C***).

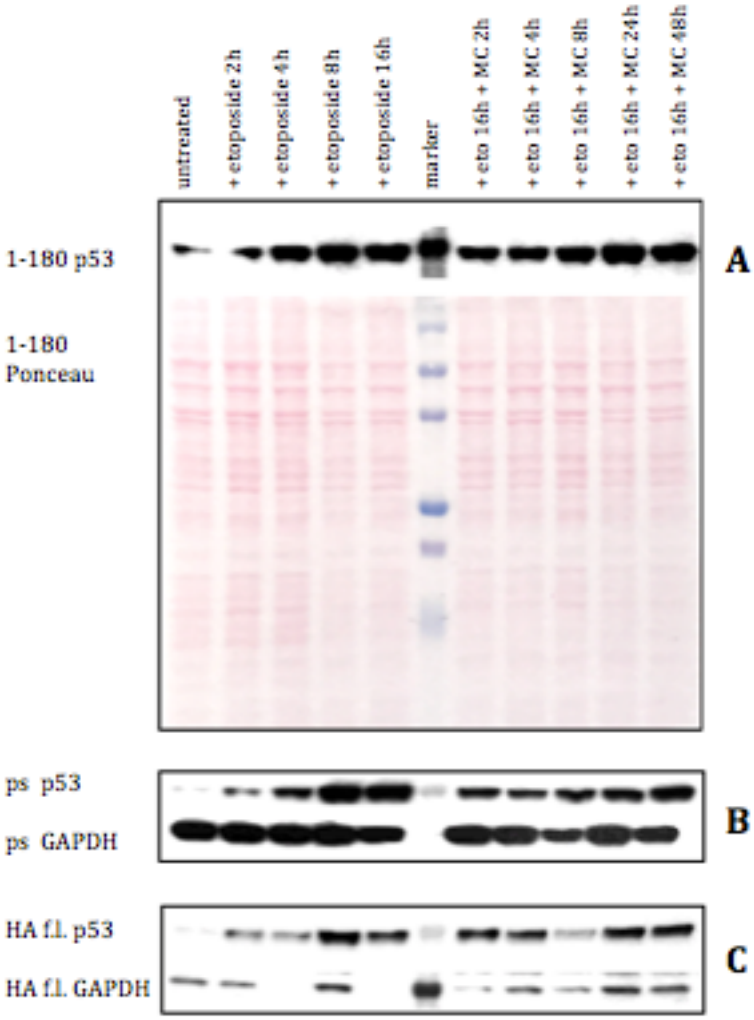

Figure 21: Induction of p53 in 1-180,EAPP f.l. and ps EAPP cells
Western blot analysis and immunodetection show different increases of p53 in 1-180 (A), ps EAPP (B) and HA-EAPP full length (C) cells.

In cells with the truncation of EAPP 1-120, p53 is highest after 8 hours etoposide treatment and after the removal of etoposide, p53 is constantly expressed (***figure 22, A***). U2OS EAPP pm and 95-c cells show an increase of p53 until 16 hours by etoposide. In pm cells, p53 remains almost at high levels after the removal of etoposide (***figure 22, B+C***). On the contrary, fresh media seems to decrease p53 firstly, but then p53 levels rise again in truncated EAPP 95-c cells (***figure 22, C***).

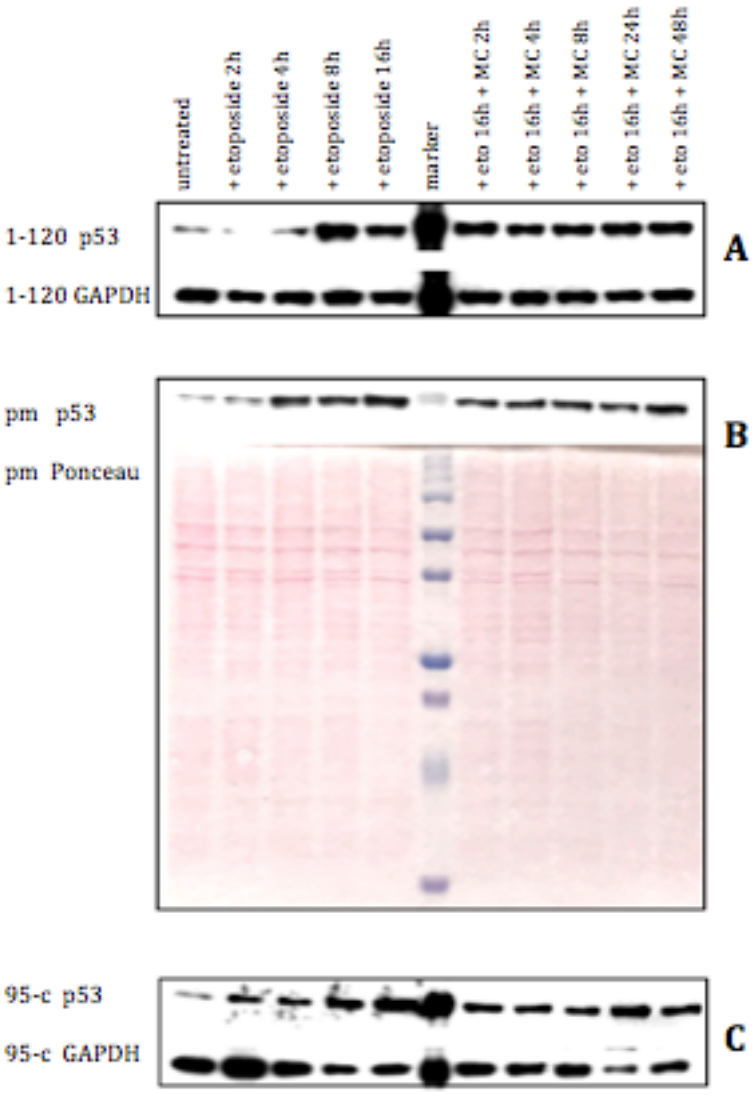

Figure 22: Western blot analysis of p53 in U2OS cells with different versions of EAPP.
1-120 (A), EAPPpm (B), 95-c (C); Blots show effects on p53 after treating cells with 10 µl etoposide by immunostaining.

9.4 Induction of p21 by DNA damage

Higher EAPP levels can prevent cells from apoptosis by p21 induction [25]. Cells expressing different versions of EAPP were treated with etoposide to investigate possible consequences of their expression on p21 activation.

U2OS wt cells and the cells expressing the truncations of EAPP 1-180, 10-240, 55-c cells show similiar effects on p21 after etoposide addition. p21 increases after 8 hours etoposide and stays constant until medium change, with the exception of wild type and 10-240 cells, where p21 is further induced (***figure 23, A+C***). p21 seems to be stronger induced by etoposide in wt and 10-240 cells than in 55-c and 1-180 cells (***figure 23 A-D***). After the removal of etoposide by adding fresh culture medium, p21 stays constant at high levels in all four cell lines. p21 levels in cells expressing only murine HA-EAPP (but no endogenous one) are rather high in untreated cells already and only modestly increase upon etoposide addition (***figure 23, E***).

p21 and EAPP show a similiar expression profile after DNA damage induction, with the exception that p21

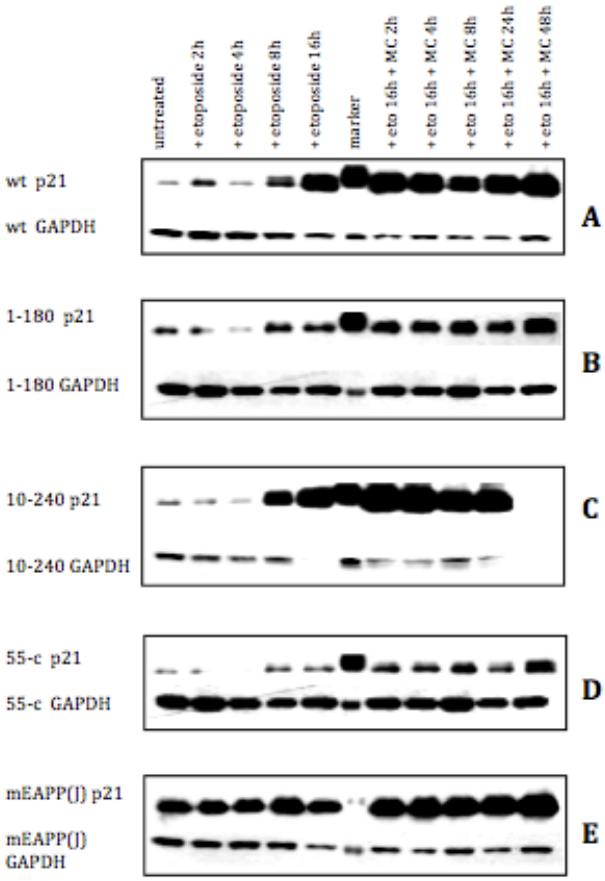

Figure 23: Western blot analysis and immunostaining of p21 in cells with different versions of EAPP.
In wild type cells (A), 1-180 (B), 10-240 (C) and 55-c cells (D), p21 increases after 8 hours by etopiside and its expression stays rather high. Murine EAPP (E) shows similiar effects on p21.

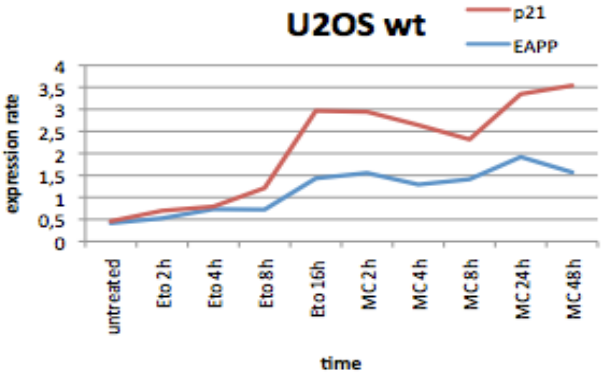

Figure 24: EAPP and p21 levels in U2OS wild type cells after DNA damage
Western blot analysis and immunostaining are transformed into a graph by ImageJ. EAPP (red) and p21 (blue) show a similiar graph.

decreases and EAPP rises after 4 hours and 48 hours medium change (***figure 24***).

In cells with the truncation of EAPP 1-120, an induction of p21 is already visible after 2 hours. After changing the medium, the expression of p21 remains at high levels (***figure 25, A***). Cells with the truncation of EAPP 1-160 show a similiar induction of p21. After the removal of etoposide, p21 levels are unaltered high (***figure 25, B***). Same effects on p21 can be observed in 95-cells with etoposide, but with the removal of etoposide, p21 seems to be stable until 4 hours. Then it decreases and rises again at 24 hours (***figure 25, C***). In pm cells, an increase of p21 is slightly visible after 8 hours, and after medium change p21 rises till 4 hours and goes down again after 8 hours already. In contrast to the other cell lines, pm cells do not show a strong induction of p21 at 16 hours by etoposide (***figure 25, D***).

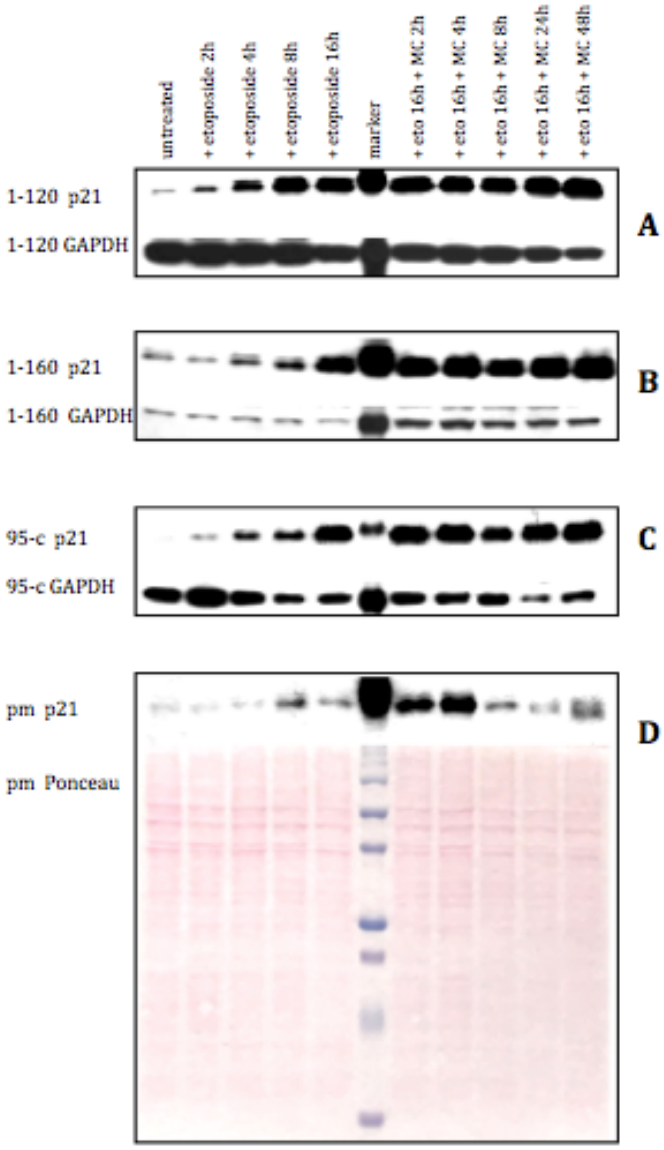

Figure 25: p21 levels of U2OS cells with variable versions of EAPP

Wester blot analysis and immunostaining show increased p21 expression in 1-120 (A), 1-160 (B) and 95-c (C) cells by etoposide. P21 in cells with EAPPpm (D) were compared with the ponceau of pm cells.

9.5 Unaltered Bax levels by DNA damage-induced activation of p53

Following DNA damage, p53 can induce the pro-apoptotic protein Bax. Therefore, the expression of Bax in cell lines with different versions of EAPP were checked. DNA damage was caused by etoposide with a final concentration of 0,005 mM. An increased expression of Bax in relation to high p53 levels was to be expected (effects on p53 seen in 8.3.) Interestingly, Bax does not increase by etoposide in most cells lines. Some effects on Bax are visible, but only in a slight form.

In U2OS wild type cells, inducing DNA damage by etoposide does not affect the expression of Bax. Levels of Bax correspond to the protein loading of the blot, with the exception at 48 hours medium change, where an induction of Bax is visible (***figure 26, A***). Cells with HA-EAPP full length also do not show an impact on Bax, respecting the discrepancy of the ponceau coloring (***figure 26, B***).

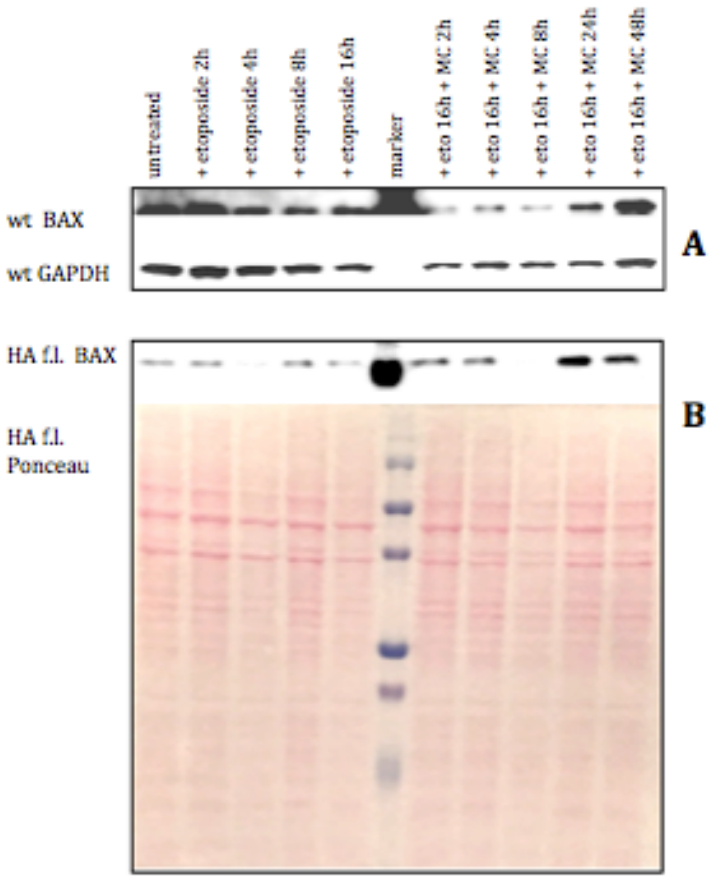

Figure 26: Effects on Bax in wild type and cells with HA-EAPP full length

Western blot analysis and immunostaining show no impact on Bax by DNA damage in wild type cells (A) and in cells with HA-EAPP full length (B).

On the contrary, cells with the truncation of EAPP 1-120 show a slight induction of Bax after 16 hours etoposide. After the removal of etoposide by fresh culture medium, the Bax protein stays at high levels constantly (***figure 27, A***). The expression of Bax in cells with the truncation of EAPP 1-180 reflects the protein amounts in the ponceau staining. There, a small induction of Bax after medium change is visible, which may occurs by the fresh medium itself (***figure 27, B***). In cells with 95-c EAPP, Bax does not show any induction by etoposide, respecting the protein loading, seen in ***figure 28 C***.

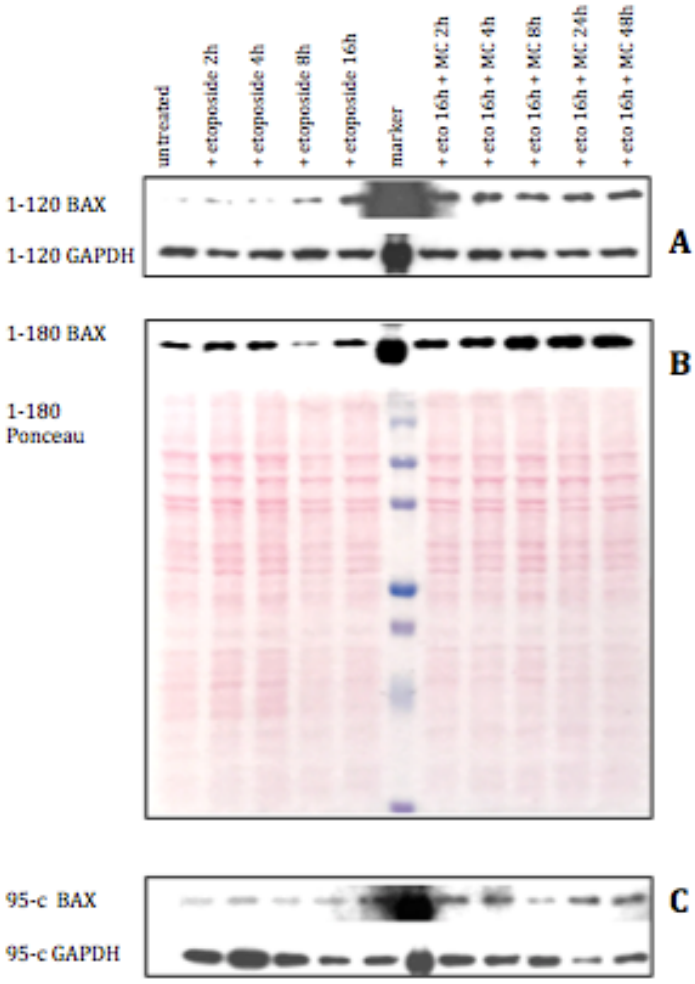

Figure 27: Effects on Bax in U2OS 1-120, 1-180 and 95-c EAPP cells.
In 1-120 cells (A) is a slight induction of Bax at 16 hours etoposide visible. 1-180 cells (B) and 95-c (C) stay rather unchanged, respecting ponceau and GAPDH.

In U2OS cells with EAPPpm, etoposide does not show any impact on Bax levels and Bax is also barely visible (***figure 28, A***). If this effect depends on the cell line itself or on the exposure of immunostaining, has to be checked in a further experiment.

Cells with psEAPP also do not show an induction of Bax due to etoposide treatment. Moreover, it seems that the Bax protein rather goes down during treatment. After adding fresh medium, Bax levels stay unchanged low at the begin. Respecting the ponceau staining, Bax rises after 24 hours medium change and after 48 hours a further increase of Bax is visible (***figure 28, B***).

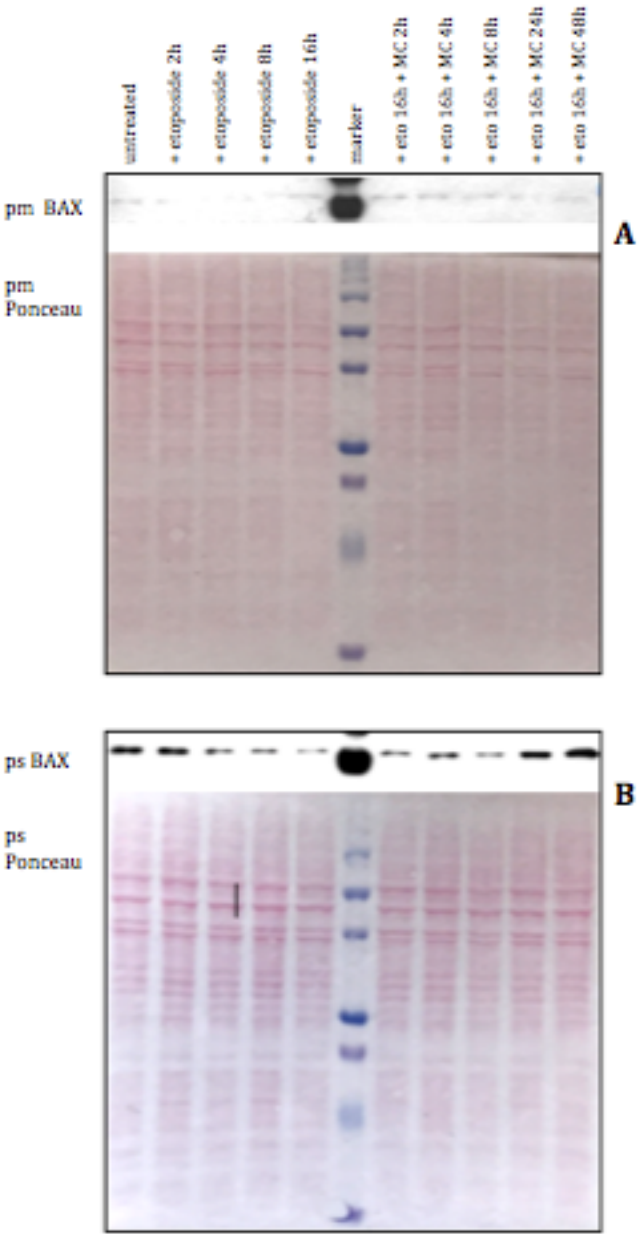

Figure 28: Bax levels in U2OS EAPP pm and U2OS psEAPP cells after DNA damage

Western blot analysis and immunostaining show unchanged Bax levels in U2OS EAPPpm cells (A) and wavering protein levels in U2OS psEAPP cells (B). Both are compared with their respective ponceau staining.

9.6 Effects on cells at 8 hours etoposide treatment

After 8 hours, most cells with different version of EAPP show a clearly impact of etoposide. To compare the proteins of choice between the cell lines, the respective extracts of the proteins were plotted together at the same time of etoposide treatment.

In U2OS wild type cells and cells with the truncation of EAPP 10-240, levels of p-Akt S473 are higher than in other cell lines with different truncations of EAPP. Moreover, cells expressing HA-EAPP 1-120, 1-160 or 1-180 in addition, show only low levels of p-Akt despite their GAPDH levels. In cells with HA-EAPP full length in addition, p-Akt is not visible because the p-Akt level corresponds to its low protein loading. In cells with the truncation of EAPP 55-c and 95-c, p-Akt is also not visible (in 55-c only barely seen) despite its acceptable protein loadings. p-Akt S473 is also not visible in pm cells despite its high GAPDH level. In ps cells which express lower amounts of endogenous EAPP, p-Akt S473 is clearly seen after etoposide treatment. Similiar to ps cells, in cells expressing only murine HA-EAPP, the levels of p-Akt S473 are also high, respecting the GAPDH level (***figure 29, A***).

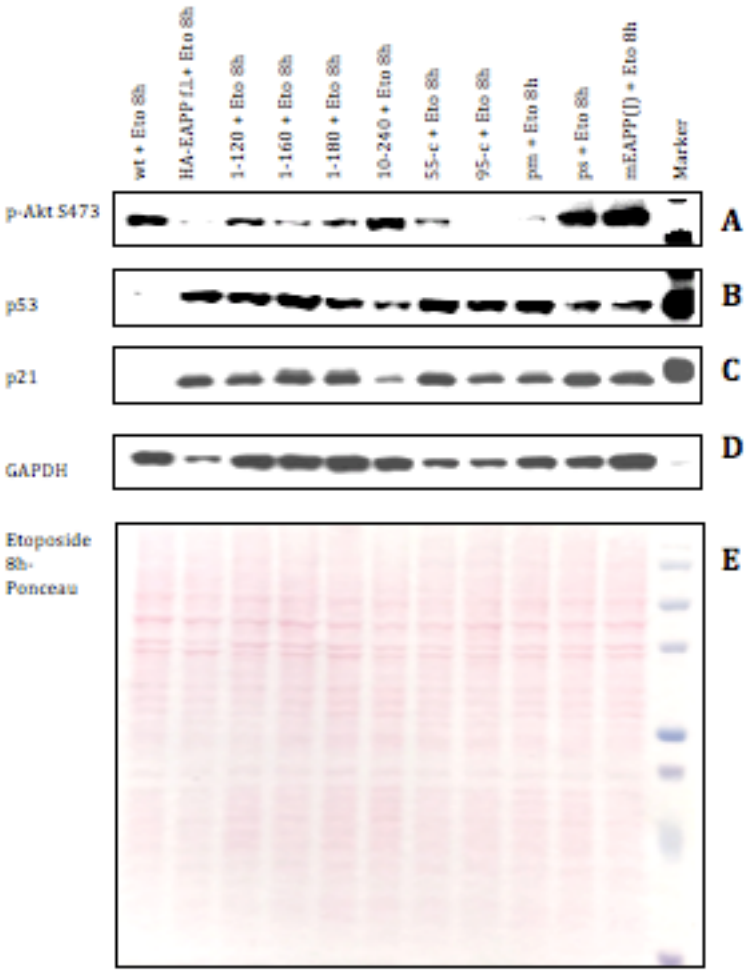

Figure 29: p-Akt S473, p53 and p21 levels at 8 hours etoposide treatment
All investigated cell lines seem to differ themselves in the expression of p-Akt S473 (A) and p53 (B). P21 (C) is rather equal induced in all cells, with the exception of wild type cells. GAPDH (D) and the ponceau stainining (E) represent the protein loadings of the different cell lines.

The expression of p53 is not visible in wild type cells despite the high level of GAPDH. However, p53 in wild type cells also increases by etoposide (***8.3., Figure 19, A***), but the intensity of the induction seems to be lesser than in other cells. Simliar to wild type cells, p53 is barely visible in cells expressing HA-EAPP 10-240. In cells with HA-EAPP full length or the truncation of EAPP 55-c in addition, the strong expression of p53 is clearly visible, respecting the low protein loading and ponceau staining. In pm cells and cells with the truncation of EAPP 1-120 and 1-160, p53 reflects the expression of its respective GAPDH level. Despite a good protein loading, p53 is only weak expressed in cells with only murine EAPP (***figure 29, B***).

p21 is in cells rather equally expressed and it mostly reflects the expression of its GAPDH level. But U2OS wild type cells differ from the rest. There, p21 is not visible respecting the high protein amount of GAPDH (***figure 29, C***). However, p21 is also induced by etoposide (seen in ***8.4., figure 23, A***) after 8 hours in wild type cells. In cells with the truncation of EAPP 10-240, p21 is also hardly visible, respecting the protein loading. In cells expressing HA-EAPP full length in addition, p21 is clearly expressed, respecting the protein amount of GAPDH (***figure 29, C***).

To sum up, it seems to be that there are differences in the expression of p-Akt S473, p53 and p21 between the investigated cells, compared with GAPDH (***figure 29, D***) and ponceau staining (***figure 29, E***). The protein loading was rather equal with the exception of cells with HA-EAPP f.l., 55-c and 95-c EAPP (***figure 29, D***).

10 Discussion

The regulation of the cell cycle is one of uttermost importance to prevent cells from malignant transformations. By delaying or arresting the cell cycle, cells have time to repair the DNA damage or when the damage is irreversible, they can initiate apoptosis to secure cells from uncontrolled growth.

The E2F transcription factor family is important for the cell progress and gene expression during the whole cell cycle The E2F-Associated Phosphoprotein (EAPP) interacts with the activators of the E2F family, E2F-1 to E2F-3, thereby influencing E2F dependent transcription. EAPP also stimulates the expression of p21, leading to an arrest in G1. While high EAPP levels secure cells from apoptosis via p21, low levels of EAPP enhance the cell death. Moreover, when EAPP is overexpressed and cells arrest in G1, cells tend to avoid E2F-1 induced apoptosis. EAPP is often elevated in human cancer cells and it is supposed to play an important role in the regulation of the cell cycle and in managing DNA damage.

Certain DNA damage-inducing drugs can be used to investigate the regulation of cell cycle. In our experiments, cells were treated with etoposide that inhibits the DNA synthesis, resulting mostly in double strand breaks of the DNA. Besides wt U2OS, we used U2OS cells stably expressing different truncations or mutations of EAPP. Western blot analysis and immunostaining should uncover specific domains of EAPP that are important for interactions with cell cycle and apoptosis -involved proteins. Moreover, the different versions of EAPP and their influences on other proteins in cell cycle regulation were investigated. The results show different effects on the truncated versions of EAPP and also on p53, p21, p-Akt S473, Bax. This indicates that EAPP may need to form various complexes with other proteins for being involved in several pathways.

In normal cells, EAPP is only required in low concentrations but following DNA damage, EAPP increases and stimulates the expression of p21, independently of p53. An induction of the endogenous EAPP can be seen at 16 hours etoposide treatment in all cell lines (*seen in **9.1***.). The truncations of HA-EAPP 10-240 and 1-180 (***figure 15, A+B***),

do not show a similar increased expression at that time. On the contrary, the truncation of HA-EAPP 55-c is rather equally expressed as the endogenous EAPP (***figure 15, C***). Taken the results of the effects on EAPP together, either the whole EAPP protein seems to be important for an clear induction of it or certain part of it. Specific binding domains for the activation of EAPP by DNA damage may be incomplete or important pieces of the domains are missing.

p53 can be controlled by different post-translational modifications (for example ubiquitylation, phosphorylation, acetylation,..). Following DNA damage, the acetylation of p53 is important for its response to DNA damage and also facilitates associations with other proteins. Because of an interaction of EAPP with p53 (Rotheneder unpublished data), EAPP may influences the dynamics of p53 activation following DNA damage. An induction of p53 is visible in every cell line, but the induction period of p53 seems to differ from wild type cells, in cells expressing a truncation of HA-EAPP in addition. In cells expressing HA-EAPP 10-240 in addition, the induction of p53 seems to be delayed in time (***figure 19, B***), in contrast to wild type cells. However, the expression of p53 after medium change is almost at high levels.

The p53 degradation by the proteasom seems to take a long time (***figure 20***). One possible reason is that the acetylation and other modifications of p53 may block its ubiquitylation. Among these modifications are phoshorylations of p53 by ATM, ATR, CHK-1 or CHK-2, which might prevent p53 from its proteasomal degradation. It is conceivable that higher expressed EAPP after medium change may interacts with p53. Only a specific part of the EAPP protein seems to be responsible for interactions with p53. Following the last thesis, ps EAPP cells express lower amounts of endogenous EAPP, but p53 is as similiar expressed as in U2OS wild type cells (***figure 21, B***). Moreover, in U2OS pm cells (where all serines and threonines of EAPP known to become phosphorylated had been replaced by alanines) p53 levels correspond with their GAPDH levels (***figure 22, B***). Further experiments have to be performed to understand the impact on p53 by EAPP.

Following DNA damage, p21 arrests the cell cycle in G1 and this can prevent cells from malignant transformations. The concentration of EAPP is depending on cells condition. EAPP is significantly higher expressed after DNA damage. By enhancing p21's promotor

activity, induced EAPP stimulates the expression of p21, independently of p53. Thus, higher EAPP levels can prevent apoptosis by p21 induction. In cells with different versions of EAPP in addition, a clear increase of p21 till 16 hours etoposide treatment is visible. By adding fresh culture medium, p21 remains at high levels in all investigated cells. In U2OS pm cells, p21 is only slightly induced by etoposide. Some serines and threonines of EAPP, which become phosphorylated and are important for the stimulation of p21, are replaced by alanines. This might explain the low expression of p21 at 8 hours and 16 hours after etoposide treatment (***figure 25, D***).

Following DNA damage induced activation, p53 can induce the pro-apoptotic protein Bax. An induction of Bax in relation to high p53 levels was to be expected, but only slight effects on Bax occured (***figure 26-28***). p53 and BH3- only proteins (Bim, t-bid) can activate Bax directly. But Bax is also induced indirectly by inhibiting the anti-apoptotic Bcl-2 subfamily (this happens by BH3- only proteins). The stimulation of Bax may occur by another pathway, because the activation of p53 is clearly seen in 9.5.. Studies suppose that the inactivation of Bax by the anti-apoptotic Bcl-2 and Bcl-xL depends on Bax's conformation, post-translational modifications or localization. Moreover, it is conceivable that these factors are also important for the activation of Bax. Akt can also act anti- apoptotic by a negative regulation of BAD. If EAPP influences the activation of Bax, it would might act indirectly via other proteins (for example p53).

Via the phosphorylation of caspase-9, BAD or mdm2 (affects p53 activity), Akt can prevent cells from p53- dependent apoptosis. The effects on phosphorylated Akt S473 are various. On the one hand, in wild type cells, p-Akt decreases when EAPP rises following DNA damage (***figure 16, A***). Cells expressing lower amounts of endogenous EAPP (ps cells) show a constant high expression of p-Akt S473 upon etoposide treatment. It is conceivable that there is an opposite impact/regulation of both proteins. This is maybe possible by the strong phosphorylation of EAPP (directly) or by disturbing the cell cycle progression (indirectly, EAPP stimulates p21, resulting in an arrest of the cell cycle). On the other hand, in cells with the truncations of EAPP 1-180, 55-c and 1-120, p-Akt S473 rather does not decrease by etoposide treatment (***figure 18, A+C+D***). Maybe the truncations of EAPP in addition to the endogenous EAPP disturb the reduced expression of p-Akt (directly or indirectly). To see clear a clear influence of EAPP on p-

Akt S473, further experiments are required, for example immunoprecipitation or protein pull-down.

The respective protein extracts of the different cells were analysed together at the same time (8 hours) of etoposide treatment (***figure 29***). There are great differences in the expression-intensity of the investigated proteins p-Akt S474, p53 and p21. One possibility is that the truncations of HA-EAPP in addition to endogenous EAPP, affect the expression of these proteins. EAPP seems to form dimers or even higher order oligomers (Rotheneder unpublished results). The role of these higher order structures is unclear. Depending on their ability to take part in this process, truncations of EAPP might thus either stimulate or inhibit certain activites in the cell. Perhaps, some effects by EAPP did not occure because of the abscence of important domains in the truncations of HA-EAPP. The possible conformations/allostery and 3D- structure of EAPP are not fully understood.

It should also be kept in mind, that we used U2OS cells. They are immortalized tumor cells (we can split them indefinitely) for biomedical researches. Post- translational modifications can differ from normal (healthy) human cells. Moreover, single clones were isolated and cultured. The truncations of EAPP stably expressed the respective domain of EAPP in single clones and the expressions can variegate from single clone to single clone. The expression of the truncation of EAPP depends on the copy number and the location of the integration.

EAPP is involved in the regulation of the cell cycle. DNA damage can induce cell cycle arrest, senescence or apoptosis. A dysfunction of these mechanisms is often associated with malignant transformations of cells and can play an important role in canceriogenesis. The fact that EAPP is necessary for the survival of a cell, makes it interesting for further researches. To investigate the role of EAPP and its specific domains for possible interactions, further experiments (immunoprecipitation, protein pull-down) have to be performed.

11 References

1. Löffler. Biochemie und Pathobiochemie Auflage 8. Springer 2006, DOI:
2. Green DR, Llambi F. Cell Death Signaling. Cold Spring Harb Perspect Biol 2015; 7:
3. Willis SN, Adams JM. Life in the balance: how BH3-only proteins induce apoptosis. Curr Opin Cell Biol 2005; 17: 617-625
4. Adams JM. Ways of dying: multiple pathways to apoptosis. Genes Dev 2003; 17: 2481-2495
5. Bratton SB, Salvesen GS. Regulation of the Apaf-1-caspase-9 apoptosome. J Cell Sci 2010; 123: 3209-3214
6. Yin XM, Oltvai ZN, Korsmeyer SJ. BH1 and BH2 domains of Bcl-2 are required for inhibition of apoptosis and heterodimerization with Bax. Nature 1994; 369: 321-323
7. Liu X, Dai S, Zhu Y et al. The structure of a Bcl-xL/Bim fragment complex: implications for Bim function. Immunity 2003; 19: 341-352
8. Martinou JC, Youle RJ. Mitochondria in apoptosis: Bcl-2 family members and mitochondrial dynamics. Dev Cell 2011; 21: 92-101
9. Zaltsman Y, Shachnai L, Yivgi-Ohana N et al. MTCH2/MIMP is a major facilitator of tBID recruitment to mitochondria. Nat Cell Biol 2010; 12: 553-562
10. Tait SW, Green DR. Mitochondria and cell death: outer membrane permeabilization and beyond. Nat Rev Mol Cell Biol 2010; 11: 621-632
11. Renault TT, Teijido O, Antonsson B et al. Regulation of Bax mitochondrial localization by Bcl-2 and Bcl-x(L): keep your friends close but your enemies closer. Int J Biochem Cell Biol 2013; 45: 64-67
12. Moreau C, Cartron PF, Hunt A et al. Minimal BH3 peptides promote cell death by antagonizing anti-apoptotic proteins. J Biol Chem 2003; 278: 19426-19435
13. Ferreira R, Magnaghi-Jaulin L, Robin P et al. The three members of the pocket proteins family share the ability to repress E2F activity through recruitment of a histone deacetylase. Proc Natl Acad Sci U S A 1998; 95: 10493-10498
14. Gaubatz S, Lees JA, Lindeman GJ et al. E2F4 is exported from the nucleus in a CRM1-dependent manner. Mol Cell Biol 2001; 21: 1384-1392
15. de Bruin A, Maiti B, Jakoi L et al. Identification and characterization of E2F7, a novel mammalian E2F family member capable of blocking cellular proliferation. J Biol Chem 2003; 278: 42041-42049
16. Girling R, Partridge JF, Bandara LR et al. A new component of the transcription factor DRTF1/E2F. Nature 1993; 365: 468
17. Novy M, Pohn R, Andorfer P et al. EAPP, a novel E2F binding protein that modulates E2F-dependent transcription. Mol Biol Cell 2005; 16: 2181-2190
18. Hateboer G, Kerkhoven RM, Shvarts A et al. Degradation of E2F by the ubiquitin-proteasome pathway: regulation by retinoblastoma family proteins and adenovirus transforming proteins. Genes Dev 1996; 10: 2960-2970
19. Vairo G, Livingston DM, Ginsberg D. Functional interaction between E2F-4 and p130: evidence for distinct mechanisms underlying growth suppression by different retinoblastoma protein family members. Genes Dev 1995; 9: 869-881
20. Dynlacht BD, Flores O, Lees JA et al. Differential regulation of E2F transactivation by cyclin/cdk2 complexes. Genes Dev 1994; 8: 1772-1786

21. Rotheneder H, Geymayer S, Haidweger E. Transcription factors of the Sp1 family: interaction with E2F and regulation of the murine thymidine kinase promoter. J Mol Biol 1999; 293: 1005-1015
22. Muller H, Moroni MC, Vigo E et al. Induction of S-phase entry by E2F transcription factors depends on their nuclear localization. Mol Cell Biol 1997; 17: 5508-5520
23. Andorfer P, Rotheneder H. Regulation of the MDR1 promoter by E2F1 and EAPP. FEBS Lett 2013; 587: 1504-1509
24. Georgakilas AG, Martin OA, Bonner WM. p21: A Two-Faced Genome Guardian. Trends Mol Med 2017, DOI: 10.1016/j.molmed.2017.02.001:
25. Andorfer P, Schwarzmayr L, Rotheneder H. EAPP modulates the activity of p21 and Chk2. Cell Cycle 2011; 10: 2077-2082
26. Su TT. Cellular responses to DNA damage: one signal, multiple choices. Annu Rev Genet 2006; 40: 187-208
27. Matt S, Hofmann TG. The DNA damage-induced cell death response: a roadmap to kill cancer cells. Cell Mol Life Sci 2016; 73: 2829-2850
28. Riley T, Sontag E, Chen P et al. Transcriptional control of human p53-regulated genes. Nat Rev Mol Cell Biol 2008; 9: 402-412
29. Fischer M, Grossmann P, Padi M et al. Integration of TP53, DREAM, MMB-FOXM1 and RB-E2F target gene analyses identifies cell cycle gene regulatory networks. Nucleic Acids Res 2016; 44: 6070-6086
30. Hu W, Feng Z, Levine AJ. The Regulation of Multiple p53 Stress Responses is Mediated through MDM2. Genes Cancer 2012; 3: 199-208
31. Shi D, Gu W. Dual Roles of MDM2 in the Regulation of p53: Ubiquitination Dependent and Ubiquitination Independent Mechanisms of MDM2 Repression of p53 Activity. Genes Cancer 2012; 3: 240-248
32. Midgley CA, Desterro JM, Saville MK et al. An N-terminal p14ARF peptide blocks Mdm2-dependent ubiquitination in vitro and can activate p53 in vivo. Oncogene 2000; 19: 2312-2323
33. Ashcroft M, Taya Y, Vousden KH. Stress signals utilize multiple pathways to stabilize p53. Mol Cell Biol 2000; 20: 3224-3233
34. Horn HF, Vousden KH. Coping with stress: multiple ways to activate p53. Oncogene 2007; 26: 1306-1316
35. Lien EC, Dibble CC, Toker A. PI3K signaling in cancer: beyond AKT. Curr Opin Cell Biol 2017; 45: 62-71
36. Vivanco I, Sawyers CL. The phosphatidylinositol 3-Kinase AKT pathway in human cancer. Nat Rev Cancer 2002; 2: 489-501
37. Mayo LD, Donner DB. A phosphatidylinositol 3-kinase/Akt pathway promotes translocation of Mdm2 from the cytoplasm to the nucleus. Proc Natl Acad Sci U S A 2001; 98: 11598-11603

Figures

Figure 1: https://courses.lumenlearning.com/biology1/chapter/cell-cycle-with-cyclins-and-checkpoints/

Figure 2: https://cnx.org/contents/abji7vNQ@6/Control-of-the-Cell-Cycle

Figure 3: Löffler- Biochemie und Pathobiochemie, 8. Auflage 2007

Figure 4: http://genesdev.cshlp.org/content/17/20/2481/F1.expansion.html

Figure 6: http://genesdev.cshlp.org/content/17/20/2481/F2.expansion.html

Figure 7: http://www.nature.com/nrm/journal/v9/n9/box/nrm2469_BX1.html

Figure 8: Löffler- Biochemie und Pathobiochemie, 8. Auflage 2007

Figure 9: https://openi.nlm.nih.gov/detailedresult.php?img=PMC2190721_gkm744f1&req=4

Figure 10: http://austinpublishinggroup.com/blood-disorders /fulltext/ blooddisorders-v2-id1028.php

Figure 11: https://www.researchgate.net/publication/11282035_The_Phosphatidylinositol_3_Kinase_AKT_Pathway_in_human_cancer

12 Statutory declaration

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