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DISSERTATION

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„EAPP: At the crossroad of apoptosis and p21-mediated cell cycle arrest“

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1. Abstract

1.1 Abstract (English)

Eukaryotic cells can be exposed to a huge number of extra- and intracellular factors causing a wide spectrum of different damages. It is essential for single as well as for multicellular organisms to counteract these impacts to maintain its integrity. Cells possess several checkpoints in the different cell cycle stages to respond to every defect in an appropriate manner. Mild forms of damage switch on cell cycle arrest and certain repair machineries, whereas severe damages can lead to programmed cell death. This so called apoptosis is an important step in multicellular organisms to prevent the malignant transformation of a cell which can result in an uncontrolled behavior and cancer.

In our laboratory a new interaction partner of the cell cycle regulator family E2F was discovered, namely E2F Associated Phosphoprotein (EAPP). In early studies we elucidated an involvement in the transcriptional regulation of the activator E2Fs at certain promoters. Several other proteins, necessary for the initiation of transcription, were found to interact with EAPP, like p53, the pocket protein family, or certain histone acetyl transferases. In the following work the impact of EAPP on the cell cycle and DNA damage checkpoints was studied. Overexpression of EAPP causes an arrest in the G1 cell cycle phase. It turned out that this effect is mediated by p21, a member of the Cip/Kip family of Cdk inhibitors. A detailed study of the interplay between EAPP and p21 revealed a regulation on the transcriptional level, independent of p53 one of the main inducers of p21 upon stress stimuli. Two Sp1/3 binding sites are responsible for this EAPP-dependent upregulation. Another protein where we could confirm an influence on the transcription by EAPP was Mdr1, a prominent ABC transporter involved in the multi drug resistance phenotype. The regulation of p21 had not only consequences in the normal cell cycle. Upon etoposide treatment, which induces DNA double strand breaks, the upregulation of p21 was dependent on the levels of EAPP. Overexpression of EAPP had an anti-apoptotic effect mediated, at least in some cases, by p21. On the other hand a knockdown of EAPP had severe consequences to the cells leading to massive apoptosis. We found several proteins being downregulated after an EAPP decrease. Growing cells with a 50% reduction of the

EAPP levels showed a quite normal behavior but upon DNA damage the apoptotic fraction greatly increases. Another protein involved in cell checkpoints which was influenced by EAPP was Chk2. The levels of the activating T68 phosphorylation of Chk2 increased upon an EAPP reduction and vice versa.

Taken together EAPP seems to play a prominent role in the decision between arrest and repair or apoptosis after cell damages due to the regulation of target genes on a transcriptional level. Cells with higher EAPP levels tend to avoid apoptosis which would explain the observed increase of EAPP in several transformed cancer cell lines.

1.2 Abstract (Deutsch)

Eukaryotische Zellen sind einer Vielzahl and extrazellulären Faktoren ausgesetzt welche ein grosses Spektrum an Schäden hervorrufen können. Es ist für einzellige wie auch für mehrzellige Organismen äußerst wichtig diesen Einflüssen entgegen zu wirken um die Integrität der Zelle zu bewahren. Sie besitzen verschiedene Checkpoints in unterschiedlichen Stadien des Zellzyklus, um in geeigneter Weise zu Antworten. Kleine Schäden verursachen einen Zellzyklusarrest und werden durch verschiedene Reparaturmechanismen beseitigt, wohingegen große Schäden zum programmierten Zelltod führen können. Diese sogenannte Apoptose ist ein sehr wichtiger Schritt in multizellulären Organismen um eine Zelltransformation zu verhindern, die zu einem unkontrollierten Verhalten und Krebs führen kann.

In unserem Labor wurde ein neuer Interaktionspartner der in die Zellzyklusregulation involvierten Transkriptionsfaktorfamilie E2F entdeckt, das E2F-Associated-Phosphoprotein (EAPP). In frühen Studien konnte ein Einfluss von EAPP auf die E2F abhängige Regulation bestimmter Promotoren nachgewiesen werden. Auch eine Interaktion mit anderen für die Initiation der Transkription wichtigen Proteine konnte nachgewiesen werden. Unter anderem p53, die Pocketprotein Familie, oder verschiedene Histonacetyltransferasen. In der nachstehenden Arbeit wurde der Einfluss von EAPP auf den Zellzyklus und durch DNA Schäden ausgelöste Checkpoints untersucht. Eine Überexpression von EAPP verursacht einen G1 Zellzyklusarrest. Es stellte sich heraus das dieser Effekt durch die Induktion von p21, einem Mitglied der Cdk inhibierenden Cip/Kip Proteinfamilie, verursacht wird. Eine genauere Analyse ergab eine Regulation durch EAPP auf transkriptioneller Ebene, unabhängig von p53, einem der Hauptaktivatoren von p21 speziell nach Zellschäden. Zwei Sp1/3 Bindungsstellen sind für diese EAPP abhängige Stimulierung notwendig. Ein anderes Protein bei dem wir einen Einfluss auf transkriptioneller Ebene durch EAPP nachweisen konnten war Mdr1, ein ABC Transporter, der eine wichtige Rolle bei der Resistenz von Zellen gegen unterschiedliche Drogen spielt.

Die Regulation von p21 hat nicht nur für den normalen Zellzyklus Konsequenzen. Seine Induzierung nach einer Behandlung mit Etoposide, welches DNA-Doppelstrangbrüche verursacht, ist EAPP abhängig. Eine EAPP Überexpression zeigte einen anti-apoptotischen Effekt, zumindest in einigen Fällen verursacht durch p21. Auf der anderen Seite führte ein EAPP Knockdown zu einem dramatischen

Anstieg an Apoptose. Mit reduziertem EAPP fanden wir einige Proteine in niedrigeren Mengen vor.
Bei normal wachsenden Zellen hatte eine Verminderung der EAPP Mengen auf 50% keine großen Auswirkungen, aber unter DNA schädigenden Bedingungen stieg der Anteil an apoptotischen Zellen verglichen mit den Kontrollzellen dramatisch an.
Ein weiteres wichtiges Checkpointprotein welches durch EAPP beeinflusst wird ist Chk2. Die Levels der aktivierenden T68 Phosphorylierung steigen bei einem EAPP Knockdown an und sind bei einer EAPP Überexpression vermindert.
Zusammenfassend scheint EAPP, durch seine Regulation verschiedener Proteine auf transkripionellem Niveau, eine wichtige Rolle in der Entscheidung zwischen Arrest und Reparatur oder Apoptose nach Zellschäden zu spielen. Zellen mit höheren Mengen EAPP tendieren dazu Apoptose zu vermeiden, was den EAPP Anstieg in einigen transformierten Krebszelllinien erklären würde.

2. Introduction

2.1. The cell cycle and its checkpoints

2.1.1 Regulation of the cell cycle

A crucial step in the life of a cell is the regulation and execution of the cell division. This is embedded in the so called cell cycle. In the standard model it can be separated into four different stages. G1-phase (gap 1), S-phase (synthesis), G2-phase (gap 2), which are summarized under the term interphase, and the M-phase (mitosis) where cells are divided. The G1-phase, between the end of mitosis and the beginning of a new DNA replication event, is also considered as the growth phase. Cells increase in size and have to decide, depending on intracellular and extracellular signals, whether they are ready for a new cell division or they stay in G1 for a longer period. This period can be extended and cells enter the so called G0-phase where they leave the cell cycle and remain quiescent, possibly indefinitely. Fully differentiated cells tend to stay in G0-phase. As an alternative to apoptosis cells can also enter G0 in response to for example severe DNA damage. This is called cellular senescence.

If cells enter S-phase they cross a point of no return. After the DNA is replicated cells enter G2 for final adaptations. They continue growing, prepare cell division, and check the DNA for damages before entering mitosis. (Tyson and Novak, 2008). During mitosis the nuclear membrane breaks down and the sister chromatids align at the metaphase plate. When all chromosomes are aligned a mitotic checkpoint is satisfied and they separate toward the opposite pole. In a process called cytokinesis the cell divides and a new G1-phase begins. (Glotzer, 2005; Nezi and Musacchio, 2009).

An important part in the regulation of the cell cycle is carried out by two classes of proteins, Cyclins and their interaction partners the Cyclin Dependent Kinases (Cdks), a family of serine/threonine kinases. Cyclins were discovered in the 80s in marine invertebrate embryos (Evans *et al.*, 1983). Their levels change during the cell cycle. If present they form heterodimers with their appropriate Cdks followed by the initiation through the Cdk-Activating-Kinase (CAK) complex, consisting of Cyclin H, Cdk7, and MAT1 (Fisher and Morgan, 1994). A further crucial step in their activation is the removal of inhibitory phosphate groups and the repression of Cyclin Dependent

Kinase Inhibitors (CKIs), which play a major role in checkpoints by arresting the cell cycle (Besson *et al.*, 2008).

In yeast we have a relatively simple model of regulation. There is one Cdk (Cdk1) which interacts with cell cycle stage specific Cyclins and therefore its activity oscillates and it is capable of inducing diverse cell cycle transitions (G1/S, S/G2, G2/M). In higher organisms we have a different picture. There are several phase specific homologues of the yeast Cdk1 which form heterodimers with Cyclins in complex combinations to specifically regulate only one cell cycle transition. Approximately 20 Cdk-related proteins and Cyclins are present. Due to extensive research a classical model of the cell cycle regulation was established. In the early G1 phase Cdk4/Cdk6 are activated by D-type Cyclins (D1, D2, D3) and phosphorylate the Retinoblastoma protein (pRb), resulting in the release of E2F transcription factors. E2F responsive genes are required for the G1/S transition, like Cyclin E (E1, E2) and Cyclin A (A1, A2). In the late G1 Cdk2 is activated through Cyclin E binding and dephosphorylation by Cdc25a. It further phosphorylates pRb, leading to additional E2F release and the passage through the G1/S restriction point. At the onset of S-phase Cyclin A is induced and interacts with Cdk2 and the heterodimer phosphorylates proteins necessary for DNA replication. To enter mitosis Cdk1/Cyclin A activity is necessary and finally Cdk1/Cyclin B (B1, B2) is responsible for completing it. Cdk1 is activated by switching off inhibitors like Wee1 or Myt1 and a dephosphorylation of crucial residues by Cdc25c. (Satyanarayana and Kaldis, 2009).

It seems that, in contrast to yeast, the function of each Cdk/Cyclin complex in higher organisms is restricted to a particular cell cycle stage. But in recent years more and more studies report a similar redundancy than in yeast with widespread compensations of different Cdk/Cyclin heterodimers. A triple knockout of the all D-type Cyclins for example showed a relatively normal proliferation behavior in the majority of tested cell lines. Only hematopoietic and myocardial cells had defects (Kozar *et al.*, 2004). A different situation revealed a triple knockout in mice which died around E16.5 (Kozar *et al.*, 2004). Single knockouts, even viable, displayed several cell type specific defects. Cyclin $D1^{-/-}$ had neurological abnormalities (Fantl *et al.*, 1995), Cyclin $D2^{-/-}$ showed defects in B-lymphocyte (Solvason *et al.*, 2000) and Cyclin $D3^{-/-}$ in T-lymphocyte development (Sicinska *et al.*, 2003). The reason for these cell type specific damages could be a lack of expression or a difference in the time point of expression of each of the D-type Cyclins in the particular cells. For example a Cyclin

$D3^{-/-}$ knockout defect could be compensated by expressing either Cyclin D2 (Carthon *et al.*, 2005) or Cyclin E1 (Geng *et al.*, 1999) from the cyclin D3 promoter. A similar picture appears with E-type Cyclins. Single knockout mice are viable and develop normally (Geng *et al.*, 2003) and the double knockout mouse dies at E11.5 (Parisi *et al.*, 2003). Contrary to this behave the A-type single knockouts. A Cyclin A1 knockout is viable (Liu *et al.*, 1998) but Cyclin $A2^{-/-}$ mice embryos die at E5.5 (Murphy *et al.*, 1997). Cyclin A2 is possibly necessary for early embryogenesis and can not be compensated. In line with this are the B-type knockouts. Cyclin $B1^{-/-}$ mice are embryonic lethal but Cyclin B2 knockout mice develop normal (Brandeis *et al.*, 1998). Cyclin A2 and Cyclin B1 appear to be the most nonredundant members of the Cyclin family.

An analogue pattern arises in Cdk knockdown studies. Mice with single knockouts of the interphase Cdks (Cdk2, Cdk4, and Cdk6) are viable and show similar minor defects than the knockouts of their interacting cyclins. $Cdk4^{-/-}$ (Rane *et al.*, 1999) and $Cdk6^{-/-}$ (Malumbres *et al.*, 2004) mice are viable but have a decreased size and defects in lymphocyte proliferation. MEFs (mouse embryonic fibroblasts) proliferate, despite an S phase entry delay of about 4h, quite normally (Tsutsui *et al.*, 1999). Cdk2 knockout mice are also viable but with a reduced body size. MEFs have again a delayed S-phase onset but behave normal (Ortega *et al.*, 2003). In contrast a Cdk1 deletion leads to embryonic lethality (Santamaria *et al.*, 2007). Interestingly embryos lacking all interphase Cdks develop till midgestation and MEFs derived from these embryos proliferate quite normal just with an extended cell cycle. Cdk1 binds to all Cyclins and is able to phosphorylate pRb and trigger S-phase induction in the absence of the other Cdks (Santamaria *et al.*, 2007). This longer description of knockout studies shall emphasize that even there is a broad variety of Cyclins and Cdks, which are necessary for the proper development of higher organisms, the basic cell cycle is still similar redundant than in yeast. In multicellular organisms a fine tuning of the cell cycle of different cell types has evolved, explaining the large number of Cyclins and Cdks in contrast to single cell eukaryotes.

Not only in the case of Cyclins and Cdks the basic model was challenged. Also the role of the activator E2Fs (E2F1, E2F2, and E2F3) in S-phase onset is not as simple as thought. In the classical model the activator E2Fs are released from pRb upon Cyclin/Cdk phosphorylation and trigger S-phase onset. Recent reports showed a different picture. In normal dividing murine embryonic stem cells E2F1-3 act as

transcriptional activators. But, as triple knockouts revealed, they are not necessary for cell division, instead they are required for cell survival. Interestingly in differentiated cells E2F1-3 stay in the complex with pRb and act as repressors for E2F target genes and hence support the exit from the cell cycle (Chong *et al.*, 2009).

These two examples, of the principle redundancy of Cdks and Cyclines and the additional and different functions of the E2F family, illustrate the complexity of the cell cycle regulation in higher organisms and how difficult it is to fit it into a simple model. This sometimes works in single cells but as soon as multicellular organisms are studied a further complexity arises due to the necessity to coordinate the development of different tissues and their sophisticated interplay.

A possibility to get an idea of the dynamics behind the cell cycle is to create a computational network model based on the experimental data. A nice model was created by Gerard and Goldbeter. It is centered on four modules (Cyclin D/Cdk4/6, Cyclin E/Cdk2, Cyclin A/Cdk2, and Cyclin B/Cdk1) and includes 44 variables. The model gives an idea how the regulatory structure results in a temporal self-organization and an orderly progression along cell cycle phases. It matches with many experimental data including the previously described phenomenon of the Cdk1 redundancy, or the observation that cell cycling can occur in the absence of pRb and growth factors (Gerard and Goldbeter, 2009).

A crucial point in regulating the cell cycle is the possibility to arrest cells as a consequence of damage. There exist several checkpoints during the cell cycle. First of all there is the so called restriction point at the G1/S transition. It indicates the time point when the cell cycle progression becomes independent of exogenous mitogenic stimuli. In the classical model this is the time point when pRb gets phosphorylated by growth factor activated Cyclin D/Cdk4/6 and the activating E2Fs are released. Additionally there is a number of Cdk inhibitors, like p21, p27, and p57 from the CIP/KIP family and p15 and p16 from the INK4A family, which can arrest cells in G1 upon different pathways. A broad variety of stimuli drives cells toward S-phase or keep them in G1. Cells check permanently their environment and intracellular conditions and damages, waiting for the right mixture of signals to proliferate. Interestingly the same mitogens can simultaneously activate Cyclin D and Cdk inhibitors, inducing proliferation and growth arrest signals at the same time. This is possibly to overcome short pulses of growth stimulating signals and only start the replication when the conditions are stable (Assoian and Yung, 2008).

The checkpoints during DNA replication fulfill different duties. One major point is the replication stop upon polymerase inhibition or nucleotide depletion (DNA replication checkpoint). A second checkpoint is activated by DNA damage (intra S-phase checkpoint). In contrast to a complete cell cycle arrest of the G1, G2 and M-phase checkpoints these S-phase checkpoints can only delay the progression. Double strand breaks for example lead to a phosphorylation and hence degradation of Cdc25a, via ATM and Chk2 activation, which can therefore not dephosphorylate and activate Cdk2, required for initiation of DNA synthesis (Costanzo *et al.*, 2000). This only delays the S-phase, an expected result considering the beforehand mentioned Cdk knockdown studies. The G2/M checkpoint senses a number of properties to ensure that the cell is ready for mitosis. Especially DNA damage is a major trigger of this checkpoint to prevent passing over defects to the daughter cells. Again this checkpoint results in the inactivation of Cdc25, in this case Cdc25c by Chk2, which in turn cannot dephosphorylate and activate Cdk1 provoking a G2 arrest (Peng *et al.*, 1997). Before entering mitosis there is also the antephase-checkpoint which prevents cell cycle progress in response to a range of stress agents (Chin and Yeong, 2009). Finally the Spindle Assembly Checkpoint (SAC) senses the correct alignment of the sister chromatids during the metaphase of mitosis. If this is fulfilled mitosis can be completed and cells divide through a process called cytokinesis (Dash and El-Deiry, 2004).

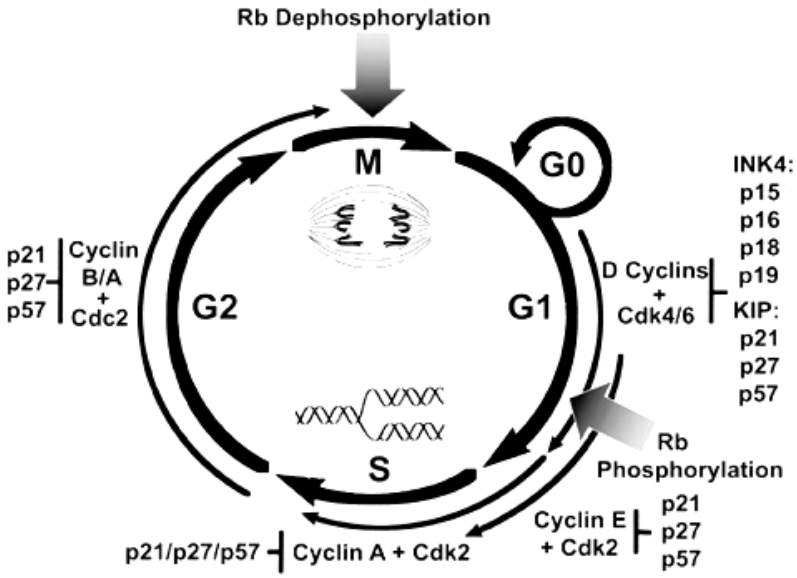


Fig 1: Diagram of the cell cycle, its regulating Cyclin/Cdk complexes, and their corresponding inhibitors (Donovan and Slingerland, 2000).

2.1.2 DNA damage - decision between arrest and apoptosis

A major checkpoint activator through out the whole cell cycle is DNA damage. In case of sustained damage cells have two principal possibilities. Either it is repairable, or the defects are too harmful to allow a proper cell behavior and they initiate a suicide process called apoptosis. This is a protective mechanism for the organism to prevent the transformation into cancer cells. DNA lesions can lead to altered functions and expression patterns of diverse proteins potentially resulting in an uncontrolled cell behavior. To give the repair machinery enough time, cells arrest in its actual cell cycle phase as long as lesions can be detected. It is comprehensible that this whole process plays a crucial role, especially in multi cellular organisms, to avoid unusual cell properties.

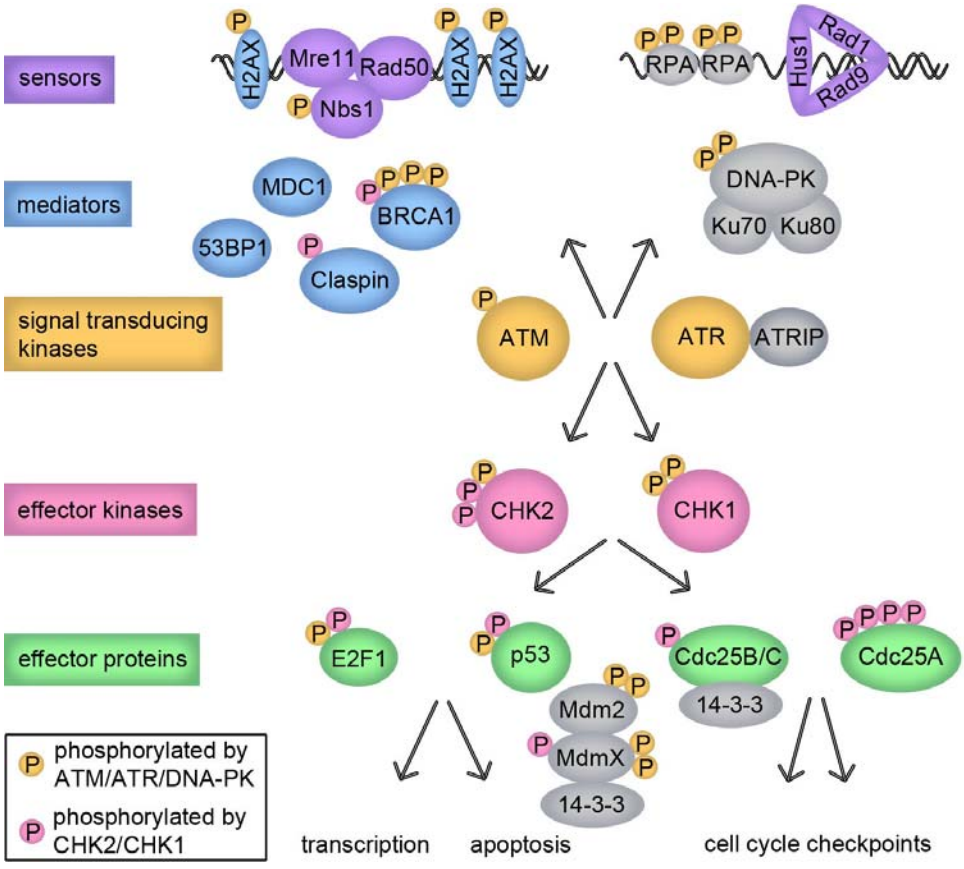


Fig. 2: Simplified summary of the DNA damage response. Double strand breaks are recognized by the MRN complex and single strand breaks by the 9-1-1 complex. The phosphorylations of ATM/ATR/DNA-PK are marked yellow whereas Chk1/2 phosphorylations are pink (Freeman and Monteiro, 2010).

There are different kinds of DNA damages with a diverse chemical nature, dispersed in the DNA hence it is an important question how checkpoint mechanism can recognize the presence of the lesions. Due to this diversity special factors are

necessary for recognition, processing, and repair but common to these processes is the activation of two protein kinases of the PIKK (Phosphatidylinositol 3-Kinase-related Kinase) family which are at the top of the checkpoint cascade, namely ATR and ATM. In the classical model ATM is mostly involved in double strand breaks whereas ATR responds to replication stress and different forms of DNA lesions where it overlaps with ATM. Additionally there exists the DNA-PK a kinase involved in the non-homologous end-joining double strand break repair pathway (Bartek and Lukas, 2007).

Double strand breaks (DSB) trigger the assembly of the MRN complex, comprising Mre11, Rad50, and Nbs1, which is involved in damage site recognition and is necessary for ATM recruitment (Uziel *et al.*, 2003). In undamaged cells ATM is inactivated by dimerization. For induction the ATM dimers are autophosphorylated and have to be converted into active monomers. There is still controversy how this initial activation of ATM/ATR is carried out. Recent reports support the idea that alterations of chromatin structure like short unwound regions exposing ssDNA are necessary to initiate this step (Bakkenist and Kastan, 2003). During G2/S phase active ATM, MRN, and CtIP/Sae2 are responsible for DSB end processing, promoting the formation of a 3'-ssDNA intermediate. This is a crucial step in the activation of ATR (Cortez *et al.*, 2001). Principally ATM is responsible for the initial rapid mediation of the damage whereas ATR is activated later and maintains the response (Tibbetts *et al.*, 1999). For the full activation ATR needs a ssDNA region and ATRIP, which mediates DNA binding, the 9-1-1 checkpoint clamp (comprising of Rad9, Hus1, and Rad1), and TopBP1 (Cimprich and Cortez, 2008). There is evidence from yeast that a minimal length of ssDNA, more than 10kb, is necessary to induce ATR, setting up a checkpoint threshold (Pellicioli *et al.*, 2001). But there are also contradictory results where no ssDNA intermediate is needed for checkpoint activation and that it rather serves as scaffold for other checkpoint proteins (Soutoglou and Misteli, 2008).

Not only for DSBs a single stranded intermediate state is suggested which activates the checkpoint. In non-cycling cells for example the photoproducts after low doses of UV light, mainly cyclobutane pyrimidine dimers and 6-4 pyrimidine-pyrimidone photoproducts, are repaired rapidly by the nucleotide excision repair (NER) without checkpoint activation. In contrast at higher UV doses the repair mechanisms become limiting hence further processing of the damaged DNA results in ssDNA which could be the main initiator of the ATR checkpoint (Marini *et al.*, 2006).

When the cells are not able to repair the DNA cycling cells can enter the S-phase with a damaged genome. During S-phase the replication stops in front of DNA lesions, producing ssDNA between the stalled polymerase and the uncoupled helicase, which induces the ATR checkpoint response (Sogo *et al.*, 2002).

An interesting connection seems to be between the translesion DNA synthesis (TLS) and the checkpoint response. If the cells can not repair all DNA lesions during S-phase there are also non-repair strategies known as DNA-damage tolerance pathways or post replication repair. In these cases replication is completed while the lesions can be removed at other cell cycle stages. Principally there are two possibilities. During the error-free pathway the undamaged sister chromatids serve in a recombination-like mechanism as a new temporary template for the replication machinery. The second way is error-prone. Special translesion DNA polymerases copy the damaged sites but induce, depending on the damage, more or less mistakes there. This so called translesion DNA synthesis (TLS) can be faithfully or mutagenic depending on the kind of polymerase and lesion (Friedberg, 2005). The key regulator in the decision between error-free and error-prone seems to be PCNA. Mono-ubiquitylation of PCNA leads to TLS (Hoege *et al.*, 2002) whereas poly-ubiquitylation and sumoylation induces the recombination-like error free pathway (Branzei *et al.*, 2008). One example of the interdependency between the TLS and checkpoint mechanism is the involvement of Chk1 in the TLS. It was shown that Chk1 inhibition, the first kinase activated by ATR, reduces the levels of mono-ubiquitylated PCNA and hence the amount of Pol κ associated PCNA (Bi *et al.*, 2006). Even there are also controversial results (Conde and San-Segundo, 2008) many data support this close connection between checkpoint and TLS (Kai and Wang, 2003). New evidence even seems to challenge the classical view of the DNA damage checkpoint and mutagenesis. In S. cervisiae loss of checkpoint activity generates a reduced mutation rate, possibly by lowering the amount of free dNTPs, which is normally induced upon DNA damage. TLS polymerases need high dNTP levels (10 times higher than replicative polymerases) and hence a reduction instead of an induction after DNA damage could impair this error-prone pathway (Chabes *et al.*, 2003; Shimizu *et al.*, 2002). It seems that in some cases the checkpoint activation surprisingly leads to more mutations instead of preventing them.

The activated apical checkpoint kinases ATM and ATR orchestrate diverse pathways responsible for DNA repair and cell cycle regulation. An initial step of ATM is the

remodeling of the chromatin via the massive phosphorylation of H2AX, a histone variant, providing an easier recruitment of checkpoint and repair factors to the damaged site (Burma *et al.*, 2001) or by phosphorylating KAP-1 leading to chromatin relaxation (Ziv *et al.*, 2006). An example is the recruitment of Mdc1 followed by several factors involved in repair including p53BP1, BRCA1, or Ubc13-Rnf8 (Cortez *et al.*, 1999; Stucki and Jackson, 2006; Wang and Elledge, 2007). Large complexes form at the damaged sites, comprising ATM, BRCA1, the MRE complex, the mismatch repair proteins MLH1, MSH2 and MSH6, the BLM helicase, and other repair proteins (Wang *et al.*, 2000b).

Additional to the repair ATM and ATR switch on several cell cycle checkpoints promoting the initiation of a cell cycle arrest or apoptosis, depending on the severity of the damage. Two of the key substrates in this process are Chk1 and Chk2. Even there is some crosstalk between ATM and Chk1 the standard cascade is ATR phosphorylates Chk1 and ATM Chk2. They have many overlapping substrates among the effector proteins (Bartek and Lukas, 2003). In contrast to an ATM or Chk2 knockdown is the ATR or Chk1 knockdown embryonic lethal (Shiloh, 2003). This is due to its additional involvement in the regular cell cycle, including the control of DNA replication (Sorensen *et al.*, 2004), or the induction of mitotic events on centrosomes (Kramer *et al.*, 2004).

Once activated Chk1 and Chk2 can arrest the cell cycle at different stages. Crucial targets for this are the members of the Cdc25 phosphatase family. As mentioned before they are necessary for the activation of several Cdks to promote a cell cycle stage transition. At the G1/S border and during S phase Cdc25a is phosphorylated upon DNA damage by Chk1/Chk2 to create a phosphodegron resulting in its ubiquitylation by the SCF-β-TRCP ubiquitin ligase and degradation and hence the inhibition of Cdk2 (Bartek *et al.*, 2004). The critical target for the G2 checkpoint is Cdc25c. It is phosphorylated by Chk1/Chk2 leading to an inhibitory sequestration by the 14-3-3 protein. Without Cdc25c the CyclinB/Cdk1 complex can not be activated and therefore entry into mitosis is blocked (Donzelli and Draetta, 2003). Another possibility for the damage checkpoint at the G2/M border is via Plk1. It is negatively regulated by ATM/ATR. Inhibition of Plk1 prevents the necessary degradation of Wee1 at G2/M. Wee1 is a kinase which, among other things, phosphorylates Cdk1 and therefore keeps its kinase activity low. For activation this phosphorylation has, as mentioned, to be removed by Cdc25c (Guardavaccaro and Pagano, 2006).

2.1.3 p53 and its role in checkpoint control

One of the most prominent targets of the DNA damage checkpoint is p53. ATM/ATR and Chk1/Chk2 stabilize p53 through a direct phosphorylation (Wahl and Carr, 2001) and the inhibition of Mdm2 an ubiquitin ligase responsible for p53 degradation (Maya *et al.*, 2001). The number of p53 target genes is huge. Depending on the activation context it can induce a broad variety of different pathways including cell cycle arrest, via p21 or 14-3-3, apoptosis, via Bax, Puma, or Noxa, senescence via Pai-1, autophagy via Dram, or it regulates the p53 pathway itself via Mdm2 (Vousden and Prives, 2009). Considering the number of p53 targets (up to now there are over 125 protein coding genes and noncoding RNAs known which are direct p53 targets) and their diverse functions a tight regulation is necessary (Riley *et al.*, 2008). p53 binding to the corresponding DNA recognition site is the first step in p53 mediated regulation. There exists a p53 response element (RE) where p53 binds as a tetramer. The functional binding sites vary in one or more base pairs compared to the originally defined response element (Gohler *et al.*, 2002). Most commonly they are located at different distances upstream from the transcription start site, although they can be situated close to it or even within intronic sequences (Riley *et al.*, 2008). The DNA topology around the RE and the sequence plays an important role in the binding affinity of p53. Recent studies in yeast revealed a 1000-fold difference in transactivation by p53 comparing weaker versus stronger binding sites. This difference seems to be largely depending on the central sequence element in the p53 response element (Inga *et al.*, 2002). In general cell cycle target genes contain more robust p53 binding sites than genes involved in apoptosis (Weinberg *et al.*, 2005). Due to this difference the nuclear concentration is a crucial factor in determining the target gene after stress (Szak *et al.*, 2001). Once bound p53 has several mechanisms to initiate the transcription machinery by attracting various cofactors to the promoter. It can recruit HATs, like p300/CBP, PCAF, GCN5, or TIP60, to open up the chromatin (Avantaggiati *et al.*, 1997; Candau *et al.*, 1997; Gevry *et al.*, 2007; Scolnick *et al.*, 1997). Paradoxically p300/CBP can activate p53 dependent transcription or, in a complex with Mdm2, triggers p53 degradation (Grossman *et al.*, 2003). p53 also assists in the recruitment of several components of the pre-initiation-complex like TBP (Farmer *et al.*, 1996; Seto *et al.*, 1992), TFIIA and TFIIH (Xing *et al.*, 2001), or TAF1 (Li *et al.*, 2007). Again considering the huge number of target genes it is not astonishing that similar levels of bound p53 can trigger various transcriptional

responses. Different mechanisms can be involved. First at some target promoters RNA Polymerase II is poised, lowering the activation barrier for these genes (Espinosa *et al.*, 2003). On the other hand cofactors can vary depending on the stimulus leading to a different outcome. An example is the recruitment of TFIIB and the p53 cofactor Cdk8 to the p21 promoter after DNA damage, but not after mild forms of stress, like UVC. This recruitment correlates well with the p21 RNA induction after the treatment (Donner *et al.*, 2007; Espinosa *et al.*, 2003). A Nutlin-3 treatment revealed the involvement of the chromatin architecture. It was shown that similar amounts of p53 bind to the 14-3-3 promoter after Nutlin-3 addition in both HCT116 and BV173 cells, but RNA Polymerase II was only recruited in the HCT116 cell. This is due to the high methylation status of the 14-3-3 promoter in BV173 (Paris *et al.*, 2008).

Interestingly p53 can also repress promoters. It can for example directly recruit corepressors like HDAC1 (Murphy *et al.*, 1999), or activate repressors indirectly as it is the case after p21 induction and hence a reduced pRb phosphorylation (Xiong *et al.*, 1993). Another possibility is the competition with a more potent transcription activator. After hypoxic stress p53 binds to the AFP promoter and displaces HNF3 which is a strong inducer of the gene (Lee *et al.*, 1999).

In the last years p53 posttranslational modifications were a major focus. p53 is acetylated, phosphorylated, ubiquitylated, and methylated mainly at the amino- and carboxy-termini, but also within the DNA binding domain. Different signaling pathways trigger different combinations of modifications and hence predispose p53 to selectively activate or repress certain target genes (Kruse and Gu, 2009). The precise combinatorial impact of different modifications at the same time is still not clear but a major goal for the future is to identify a kind of p53 modification “code”. Different single modification outcomes upon diverse stimuli are already well characterized. The phosphorylation on S46 after UV treatment for example leads to the specific induction of the pro-apoptotic AIP-1 gene (Oda *et al.*, 2000b). Several kinases like HIPK2 (D'Orazi *et al.*, 2002), AMPK (Okoshi *et al.*, 2008), PKC-δ (Yoshida *et al.*, 2006), or p38 (Perfettini *et al.*, 2005) are able to phosphorylate S46.

Upon genotoxic stress K120 or K164 in the DNA binding domain are acetylated by hMOF or TIP60, and p300/CBP, respectively. The K120 acetylation mediates the activation of Puma and Bax, two pro-apoptotic genes, whereas the K164 is indispensible for most p53 targets, like p21, Puma, or Pig3, except Mdm2 (Tang *et al.*, 2008). Different lysine residues at the carboxy terminal domain, like 305, 370, 372,

373, 381, or 382 by p300/CBP and 320 by PCAF can be acetylated and there are speculations if a different acetylation pattern differentially influences p53 target selection. p53 mutants support this idea. A K320Q acetylation mimicking mutant promotes cell cycle arrest due to a preference of p53 for its high-affinity binding sites like p21. In contrast a K373Q mutant favors p53 low-affinity binding sites, like in the Bax promoter, and triggers apoptosis. When K320 is acetylated it prevents the phosphorylation of residues necessary for nuclear retention, like S46 or S15, and it favors the binding to PCAF than to p300. In contrast K373 acetylation allows this phosphorylation and further stabilizes the interaction of p53 with HDAC1, p300, and SIRT1. These differences result in an increase of the number of repressed genes in the K373Q compared to the K320Q mutant, which seems to have a higher DNA binding affinity, and a distinct, partly contrasting, pattern of activated genes. To summarize, this acetylation difference results in an opposite outcome. Either, as in the case of a K320 acetylation cell cycle arrest and survival, or the commitment toward apoptosis after a K373 acetylation (Knights *et al.*, 2006). Interestingly Roy et al. could show that depending on the acetylation state p53 recruits totally different complexes to the target promoter (Roy and Tenniswood, 2007).

Furthermore the CTD lysines can also be methylated. A K382 methylation for example impedes the activation of strong p53 targets, like p21, whereas weaker targets are not affected (Shi *et al.*, 2007). Monomethylation of K370 results in inhibition of p53 whereas dimethylation increases p53 activity by enhancing the binding to its cofactor p53BP1 (Huang *et al.*, 2006; Huang *et al.*, 2007). Arginine methylation at R333, R335 and R337 in the tetramerization domain shifts transcription towards pro-apoptotic genes and reduces p21 induction (Jansson *et al.*, 2008). Also ubiquitylation plays a role. K320 ubiquitylation enhance the activation of cell cycle arrest targets like p21 and Cyclin G1 but has no effect on pro-apoptotic genes (Le Cam *et al.*, 2006). Taken together there is a huge complexity behind the posttranslational modification pattern of p53, facilitating an important mechanism for the p53 target selection upon different stimuli.

The next levels of regulation are p53 interaction partners which can alter response element recognition or the recruitment of transcriptional coactivators. p53 modifications also seem to play an important role in the selection of binding partners. Prominent examples are the ASPP family members. ASPP1 and ASPP2 bind to p53 and shift cells toward an apoptotic phenotype (Samuels-Lev *et al.*, 2001). In contrast

iASPP inhibits pro-apoptotic genes when bound to p53 (Bergamaschi *et al.*, 2006). It seems that the S46 phosphorylation is involved in the decision between these interaction partners. Pin1 recognizes this phosphorylation, binds to p53, resulting in the dissociation of iASPP and hence redirects the cells toward apoptosis (Mantovani *et al.*, 2007). Another example is YB1, which triggers the inhibition of Bax but not p21 when bound to p53 (Homer *et al.*, 2005). Or BRCA1, which binds to p53, leading to the induction of cell cycle arrest and repair genes (MacLachlan *et al.*, 2002). Furthermore it is also implicated in the stabilization of the S15 phosphorylation, resulting in a prolonged G1 arrest (Fabbro *et al.*, 2004). Examples for binding partners supporting an apoptotic response when interacting with p53 are the p52 subunit of NF-κB (Schumm *et al.*, 2006) or the p38 regulated p18/Hamlet (Cuadrado *et al.*, 2007; Lafarga *et al.*, 2007). Mdm2 influences p53 dependent transcription negatively. It binds to several p53 promoters, disrupts the HAT interaction with p53, recruits HDACs (Ito *et al.*, 2002) and induces the monoubiquitylation of histone H2B near the p53 response elements (Minsky and Oren, 2004).

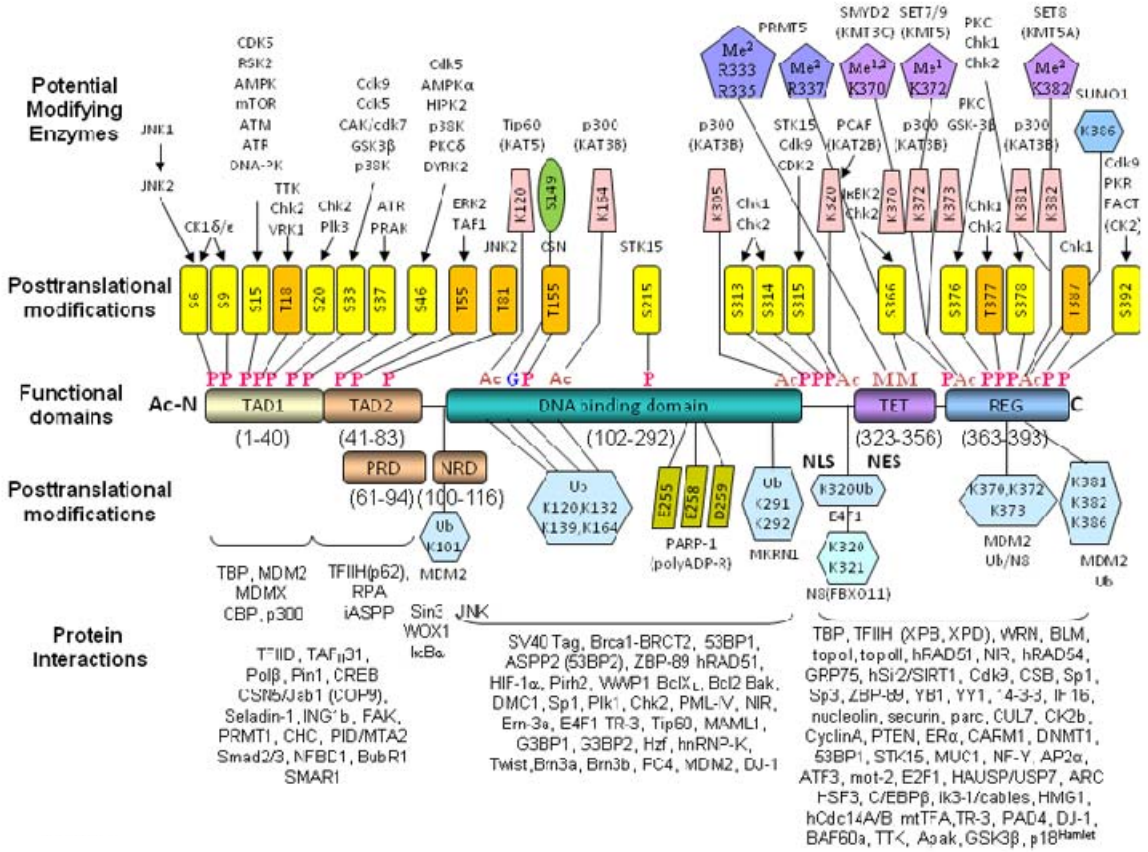


Fig 3: Diagram of the p53 functional domains, posttranslational modifications, and interaction partners (http://www.bnl.gov/biology/cellbio/human_p53.asp).

p53 isoforms are also involved in target selection. p53-β for example forms heterotetramers with wild-type p53 and promotes binding to the Bax but not to the p21 response element (Bourdon *et al.*, 2005). In contrast c-Abl phosphorylates p53 and hence stabilizes homotetramers favoring again the p21 and not the Bax RE (Wei *et al.*, 2005).

Finally the different dynamics of p53 levels upon different stimuli could provide another discriminating mechanism. Upon the induction of double strand breaks the first pulse of the p53 increase is the same independent of the concentration of the used drug. In contrast the first pulses due to an UV treatment are proportional to the dose in respect of amplitude and duration. On the other hand p53 levels are excitable upon double strand breaks, meaning that a second treatment further increases the levels of p53. Interestingly this is not the case after an UV triggered induction. These alterations in the dynamics of p53 amounts bring another level of specificity into the p53 dependent stress response (Batchelor *et al.*, 2011).

This more detailed description of p53 upon stress dependent activation should give an insight into the complex regulation of different outcomes via the same executing pathways. In the last years the old reductive paradigm of one stimulus resulting in the activation of one pathway gets more and more challenged. Lots of different factors are involved in the outcome by fine tuning prominent mediator proteins like p53, which can orchestrate different pathways depending on the quantity and quality of the stimulus. They “collect”, through for example modifications or different binding partners, various informations from the intra- and extra-cellular environment and induce appropriate mechanism when a certain threshold is reached. Cells are organized as scale free networks. A small number of proteins are highly connected and are in the centre of sub-networks. They are called hubs, whereas most proteins interact only with a few others (Albert *et al.*, 2000). Proteins like p53 represent hubs (Vogelstein *et al.*, 2000). This kind of organization has the big advantage that it is fault tolerant. Failures in the vast majority of the nodes can be tolerated hence they are less connected. But if hubs are affected the outcome can be severe. This is the disadvantage of scale free networks. A targeted damage to hubs can destabilize the network. Not for nothing are p53, and other important “hub” proteins, involved in many different cancer types. But in general the cost-benefit-ratio seemed to favor this kind of organization in nature as it is quite robust against random damages.

As mentioned, after DNA damage there are two possibilities. Either cell cycle arrest and repair or if the damage is to severe apoptosis is induced.

2.1.4 Apoptosis

Principally there are two signaling cascades that result in apoptosis, an extrinsic and an intrinsic pathway. The tumor necrosis factors (TNF) and their cognate receptors (TNFR) are gene superfamilies able to mediate the extrinsic pathway via a so called death domain. There exist more than 40 members of ligands and receptors executing diverse functions, mainly in the immune system, like cell death and survival, or cell differentiation. They are involved in host defense and inflammation as well as in autoimmune diseases (Ashkenazi, 2002). Members of the TNF superfamily can bind to more than one receptor, which would suggest a kind of redundancy within the family, but it seems that each receptor-ligand system has a unique function (Hehlgans and Pfeffer, 2005). Ligand and receptor can be transmembrane, cleaved from the cell surface, or expressed directly as soluble form. In all three cases they are still active and able to interact (Hehlgans and Pfeffer, 2005). Prominent TNF superfamily ligands are TNF, involved in inflammatory response to microbial infections, LT-, CD40L, LIGHT, RANKL, or BLYS/BAFF, which regulate diverse aspects of cellular or humoral immunity like lympohoid organ formation, stimulation or survival of T- and B-cells, or the activation of dentritic cells. Other examples are FASL and APO2L/TRAIL. They are involved in the apoptosis of peripheral lymphocytes and mediate apoptosis inducing activities of natural killer cells and cytotoxic lymphocytes against virus infected or oncogenically transformed cells (Ashkenazi, 2002).

The TNF receptors can be divided into three major groups. The first group contains a death domain in its cytoplasmic part. Examples are FAS (CD95), TNFR1, TRAIL-R1 (DR4), TRAIL-R2 (DR5), TRAIL-R4 (DcR2), TRAMP (DR3) or EDAR. If the receptors are activated adaptor proteins, like FAS-Associated Death Domain (FADD) and TNFR-Associated Death Domain (TRADD), are recruited to the death domains and induce the Caspase cascade followed by apoptosis. In the case of FADD a death inducing signaling complex (DISC) is recruited with FADD, FLIP and Procaspase 8 and 10 leading to the activation of Caspase 8 and 10 by self processing and releasing them to the cytoplasm (Kischkel *et al.*, 2000; Kischkel *et al.*, 2001). The effector Caspases 3, 6, and 7 are then cleaved and induced by Caspase 8 and 10 resulting in

the execution of apoptosis by carrying out its proteolytic activity in many cell compartements. This cleaving of proteins is responsible for most of the morphological changes of the cell during apoptosis. The blebbing of the cell is induced by the cleavage of the rho-kinase isoform ROCK-1 (Coleman *et al.*, 2001). Cleavage of proteins of the cytoskeleton and the Focal Adhesion Kinase (FAK) are responsible for the rounding up and substratum contact loss (Ndozangue-Touriguine *et al.*, 2008; Wen *et al.*, 1997). Cleavage of the Caspase activated DNAse Inhibitor ICAD results in the nuclear localization of the DNAse and the subsequent condensation and cleavage of the nuclear DNA (Nagata, 2000). Interestingly the 4 amino acid long Caspase cleavage motifs are highly conserved among widely divergent species like drosophila, xenopus and mammals (Aravind *et al.*, 1999; Walsh *et al.*, 2008).

The TNFR-Associated Death Domain pathway (TRADD) results in two different outcomes. When the receptor is kept membrane bound TRADD recruits RIP1 and TRAF2 resulting in the activation of the transcription factors NF-κB and AP1 leading to the protection from apoptosis. In contrast if the receptor with the bound ligand is internalized, TRADD recruits FADD and Procaspase 8 to form a DISC leading to a TNF-induced apoptosis (Hsu *et al.*, 1996; Krippner-Heidenreich *et al.*, 2002; Stanger *et al.*, 1995). Interestingly TNFR1 only signals cell death when at the same time protein synthesis is blocked (Hehlgans and Pfeffer, 2005).

The second group includes for example TNFRII, CD27, CD30, CD40, LTβR, OX40, BCMA, RANK, CAML, or TROY. They contain TNF-Receptor Associated Factor (TRAF)-Interacting Motifs (TIMs) in their cytoplasmic domain. If receptors are activated TRAF family members are recruited to the TIM and transduce the signal to various pathways like NF-κB, JNK, p38, ERK, and PI3K resulting in different outcomes, including proliferation, differentiation, and cell death, depending on background and combination. Up to now there are six TRAFs known (Hehlgans and Pfeffer, 2005).

The third group of receptors has no signaling motif at their cytoplasmic region. Instead they compete with other receptors for their corresponding ligands. Members are TRAIL-R3 (DcR1), DcR3, and OPG (Hehlgans and Pfeffer, 2005).

In the intrinsic pathway the Bcl2 family members are key transducers of the apoptotic signal. They share so called BH domains, which admit interactions among the family members. Bcl-2 itself has four domains. BH1, 2, and 4 form a hydrophobic groove whereas BH3 consists of an 8-12 amino acid long region that binds to this groove.

There are three kinds of family members. The first group includes the pro-survival members Bcl-2, Bcl-xL, Bcl-w, Mcl-1, and A1 which bind and therefore inhibit the anti-survival family members (Chipuk *et al.*, 2010). The second group contains the pro-apoptotic proteins Bax and Bak. They form homo-oligomers (both posses BH1-3 domains but not BH4) which can produce wide pores into the mitochondrial membrane resulting in apoptosis (Kuwana *et al.*, 2002; Wei *et al.*, 2000). The third group, the BH3-only proteins, includes Bad, Bik, Bid, Hrk, Bim, Noxa, Puma, and Bmf. They promote apoptosis but the exact mechanism is subject of recent controversy (Chipuk *et al.*, 2010).

In healthy cells Bax exists predominantly as a cytosolic momomer not pre-associated with Bcl-2 or other pro-survival members which are located at the intracellular membrane (Hsu *et al.*, 1997; Hsu and Youle, 1998; Lithgow *et al.*, 1994). Only small amounts of Bax are located at the mitochondrial membrane. The structure of Bax revealed that under normal conditions the BH3 domain is buried hence it can not interact with the pro-survival members (Suzuki *et al.*, 2000b). When apoptosis is induced Bax undergoes a conformational change, translocates to the mitochondrial membrane and forms oligomers leading to a pore in the membrane (Nechushtan *et al.*, 2001; Wolter *et al.*, 1997). Now the pro-survival members can bind to Bax (Hsu and Youle, 1997). There are several reports that post-translational modifications are involved in activation and translocation of Bax (Kim *et al.*, 2006a; Linseman *et al.*, 2004). In a recent paper they further showed that Bcl-xL constantly retranslocates Bax from the mitochondria to the cytosol to impede its activity (Edlich *et al.*, 2011).

In contrast Bak is constitutively localized on the mitochondrial membrane (Wei *et al.*, 2000). Under normal conditions it is at least partly associated with Mcl-1 and Bcl-xL but recent evidence again revealed a buried BH3 domain in healthy cells suggesting that most Bak is not associated with pro-survival members (Cuconati *et al.*, 2003; Moldoveanu *et al.*, 2006; Willis *et al.*, 2005).

Principally the pro-survival members show preference in their targets. Mcl-1 and Bcl-xL can bind to Bak and Bax, but Bcl-w and Bcl-2 only interact with Bax (Farrow *et al.*, 1995; Nijhawan *et al.*, 2003; Willis *et al.*, 2007).

At the moment there are two models how the interplay between the three sub-groups works. The first suggests two kinds of BH3-only proteins, activators and sensitizers. The activators, like Bim, Bid, or Puma can directly bind and induce Bax and Bak. The sensitizers, like Bad and Noxa, function only by displacing activators from the pro-

survival members, which, as mentioned, bind and inhibit all anti-survival family members (Kuwana *et al.*, 2005; Letai *et al.*, 2002).

But recent data showed that $Bim^{-/-}$ $Bid^{-/-}$ mice appear, in contrast to $Bax^{-/-}$ $Bak^{-/-}$ mice which die in utero, quite normal (Willis *et al.*, 2007). $Bim^{-/-}$ $Bid^{-/-}$ fibroblasts are sensitive to apoptotic stimuli like UV or etoposide (Willis *et al.*, 2007). Also $Bim^{-/-}$ $Puma^{-/-}$ thymocytes and fibroblasts or $Bim^{-/-}$ $Bid^{-/-}$ cells with significantly reduced Puma can activate apoptosis upon different treatment (Kim *et al.*, 2006b; Willis *et al.*, 2007). Bid can bind to Bax and Bak only when cleaved by Caspases into its active form tBid, which seems to be a downstram event after Bax/Bak activation (Li *et al.*, 1998; Luo *et al.*, 1998). In the second model the BH3-only proteins just neutralize the pro-survival proteins to prevent the inhibition of Bax and Bak (Fletcher and Huang, 2008). A recent work indicates a mixture of both models. They generated mice where the BH3 domain of Bim was replaced by that of Bad, Noxa, or Puma. The mutants bound the pro-survival members but could not interact with Bax anymore. Merino et al showed that apoptosis still could be induced but only at a minor level. The maximum apoptotic induction was gained with the wt-Bim. So they suggest that both mechanisms are involved, the inhibition of the interaction between pro-survival proteins and Bax and Bak, and a direct activation of Bax and Bak (Merino *et al.*, 2009). When in excess the survival members bind to the activated and membrane bound Bax and Bak and prevent the recruitment of additional Bax/Bak, therefore inhibiting apoptosis. Studies showed that membrane bound Bax recruits further Bax which can be blocked by the interaction with pro-survival proteins (Mikhailov *et al.*, 2001; Tan *et al.*, 2006).

If the apoptotic signals are too strong Bax and Bak form the mentioned pores into the mitochondrial membrane. Through this pores molecules can diverge from the intermembraneous space to the immediate peri-mitochondrial microenvironment. Among these proteins is Cytochrome c (Kuwana *et al.*, 2002). It triggers a conformational change of Apaf1, which hydrolysis bound ATP and forms a heptameric ring serving as a recruitment platform of the Procaspase 9 (Acehan *et al.*, 2002; Kim *et al.*, 2005; Yu *et al.*, 2005; Zou *et al.*, 1997). This complex is called apoptosome. The conformational change reveals the Caspase Recruitment Domain (CARD) on Apaf1, necessary for Procaspase 9 binding via a CARD-CARD interaction (Kim *et al.*, 2008a; Yuan *et al.*, 2010). There are two models how Caspase 9 is induced. In active form Caspases are dimers but, unlike the executing Caspases, Caspase 9 exists as a monomer under physiological conditions (Renatus *et al.*, 2001). One model supposes

that this CARD-CARD interaction is necessary for a conformational change in the Procaspase 9 which then can form dimers (Chao *et al.*, 2005). The second model, supported by most studies, argues that the apoptosome primarily increases the local concentration of Procaspase 9, facilitating the dimer formation (Boatright *et al.*, 2003). Once activated Caspase 9 can, like Caspase 8 and 10, induce the executing Caspases 3, 6, and 7 resulting in the above described outcomes of apoptosis (Riedl and Salvesen, 2007).

A broad range of proteins is involved in the regulation of the Bcl-2 family members. If the apoptotic signals are too strong and the pro-apoptotic family members reach a critical excess over the anti-apoptotic proteins Cytochrome c is released in sufficient amounts and the apoptotic outcome is inevitable (Fletcher and Huang, 2008).

The extrinsic apoptotic pathway is responsible for the recognition of external signals which force the cell to undergo apoptosis whereas the intrinsic pathway permanently senses the conditions in the cell. Every damage, e.g. DNA damage, oncogenic activation, or hypoxia, results in the upregulation of pro-apoptotic Bcl-2 family members. On the other hand anti-apoptotic signals induce the pro-survival family members (Wyllie, 2010).

Especially in the mammalian development the whole situation seems not to be as easy as described. Mutated mice with deficient apoptotic pathways show surprisingly low levels of developmental defects. There exist several alternative cell death mechanisms like necropotosis, death by cornification or shedding, entosis, or autophagy. In minor organisms, like yeast, an apoptosis like cell death is carried out in the absence of the canonical regulators like the Bcl-2 family or Caspases. Yuan and Kroemer for example describe and discuss in their review several of this alternative pathways (Yuan and Kroemer, 2010).

In the case of DNA damage p53 is a prominent mediator of the intrinsic pathway. Upon severe damage p53 is stabilized by ATM/ATR and Chk1/Chk2 and can induce apoptosis via several mechanisms. The most prominent way is through the direct transcriptional activation of Bcl-2 family members, including Bax, Noxa, and Puma (Mitry *et al.*, 1997; Nakano and Vousden, 2001; Oda *et al.*, 2000a). Also Apaf1 is a p53 target (Fortin *et al.*, 2001; Moroni *et al.*, 2001). On the other hand p53 represses also anti-apoptotic genes like Survivin (Hoffman *et al.*, 2002).

But there are additional more direct mechanisms. Stress stabilized cytoplasmic p53 physically interacts with Bcl-xL and Bcl-2. Nuclear p53 induces Puma transcription

which in turn binds to Bcl-xL and Bcl-2, liberating p53. The free cytosolic p53 supports Bax homo-oligomerisation followed by its translocation to the mitochondrial membrane. There is evidence that monoubiquitylated cytoplasmic p53 can directly translocate to mitochondria, where it again can interact with Bcl-xL and Bcl-2 inducing the oligomerization of Bax and Bak (Amaral *et al.*, 2010; Schuler *et al.*, 2000; Wolff *et al.*, 2008).

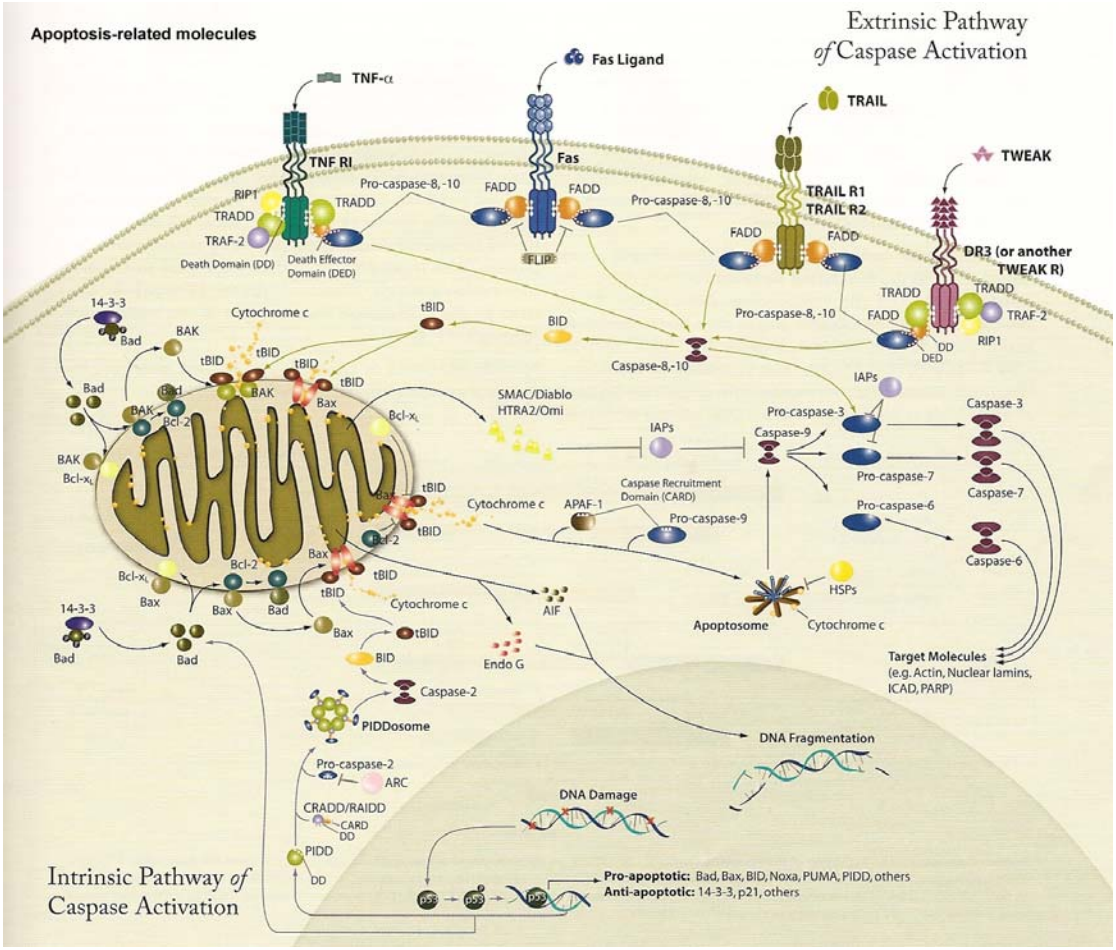


Fig. 4: Extrinsic and intrinsic pathways of Caspase activation (http://www.hixonparvo.info/model.htmlT)

Depending on its background p53 also acts on the extrinsic death receptor pathway. It stimulates Fas expression in the spleen, thymus, kidney and lung, promotes Fas trafficking to the cell surface, and activates DR5, the death domain containing receptor for TRAIL (Bennett *et al.*, 1998; Bouvard *et al.*, 2000; Wu *et al.*, 1997). DR5 was shown to be activated upon DNA damage (Wu *et al.*, 1997).

Beside p53 there are also direct mechanisms for the DNA damage response to interact with the apoptotic pathway. In the intra S-phase checkpoint ATM phosphorylation of Bid is necessary for a cell cycle arrest (Kamer *et al.*, 2005; Zinkel

et al., 2005). This would suppose a dual role for Bid, either pro-apoptotic or pro-survival depending on the background. But in other reports Bid is dispensable for the arrest (Kaufmann *et al.*, 2007). Further studies have to be done.

Another apoptotic regulator involved could be Apaf1. Knockout mice and cells showed defects in the S-phase checkpoint and it correlates with a defect in Chk1 activation, indicating a probable connection. There is no direct interaction between Apaf1 and Chk1 but possibly Apaf1 mediates the dephosphorylation of Chk1 (Zermati *et al.*, 2007).

Chk2 can induce apoptosis either directly by increasing the number of the promyelotic leukemia protein (PML) nuclear bodies, resulting in apoptosis (Stracker *et al.*, 2009), or indirectly by phosphorylating E2F1 on S364 leading to its stabilization and the expression of predominantly pro-apoptotic genes (Stiewe and Putzer, 2000).

Aven is a further example of the close interplay between the DNA damage signaling and the apoptotic pathway. It interacts with Bcl-xL and Apaf1 where it inhibits its Caspase 9 activating function (Chau *et al.*, 2000). Aven additionally binds to ATM and induces the ATM substrate phosphorylation and hence checkpoint activation. Aven depletion prevents ATM activation in human cells as well as in Xenopus egg extracts. Interestingly it is also a substrate of ATM, suggesting a positive feedback loop (Guo *et al.*, 2008).

Rad9, a member of the mentioned 9-1-1 checkpoint clamp, directly binds and inhibits Bcl-2 and Bcl-xL, supporting apoptosis (Komatsu *et al.*, 2000). A genetic loss of Hus1 induces Puma and Bim expression and further the release of Rad9 into the cytoplasm, increasing its pro-apoptotic features (Meyerkord *et al.*, 2008).

Interestingly during mitosis the apoptotic threshold is raised by an inhibitory phosphorylation of the T125 on Caspase 9 by the Cyclin B1/Cdk1 complex (Allan and Clarke, 2007). After all chromosomes are properly aligned at the metaphase plate and the Spindle Assembly Checkpoint (SAC) is satisfied, Cyclin B is degraded and the apoptotic threshold decreases again. This is possibly due to apoptotic stress signals created during mitosis. Upon prolonged mitosis cells undergo programmed cell death (Clarke and Allan, 2009). It is still not clear how the inhibitory effect on Caspase 9 is overcome, possibly by the increase of an upstream pro-apoptotic signal. Bcl-2, Bcl-xL, and Mcl-1 are all phosphorylated during mitosis and XIAP, a Caspase inhibitor, becomes degraded (Du *et al.*, 2005; Kobayashi *et al.*, 2007; Shi *et al.*, 2008). Another mechanism could be the observed slow degradation of Cyclin B during prolonged

mitosis which would lead to a constant increase in Caspase 9 activity while the upstream apoptotic signal stays constant (Clarke and Allan, 2009). When the Cyclin B degradation upon mitotic arrest was followed with a GFP tagged version in living cells a slow degradation leads to apoptosis whereas a fast degradation results in a mitotic exit (Tao *et al.*, 2005). Cells with high GFP-Cyclin B levels tended to apoptosis during mitosis in contrast to low levels (Gascoigne and Taylor, 2008). This is an example that the stress response is often not only genetically determined. Instead also a stochastic element constitutes the individual response.

2.1.5 p21 and its role in cell cycle checkpoints

p21 is one of the most prominent targets during DNA damage, especially for p53, to induce a cell cycle arrest. There are two big inhibitor classes impeding the activity of the cell cycle regulating Cdks and hence arresting the cells in different stages. One is the INK4 family comprising $p16^{INK4a}$, $p15^{INK4b}$, $p18^{INK4c}$, and $p19^{INK4d}$. They specifically inhibit Cdk4 and Cdk6. The other group is the CIP/KIP family, including $p21^{CIP1}$, $p27^{KIP1}$, and $p57^{KIP2}$, which inhibit various Cdks upon arrest stimuli (Sherr and Roberts, 1999). p21, a 164 amino acid protein, was identified as a complex component associated with Cyclin D1, a Cdk, and the Proliferating Cell Nuclear Antigen (PCNA) (Xiong *et al.*, 1992). It can bind and impede the activity of Cdk1, Cdk2, Cdk4, and Cdk6 paired with their associated Cyclins like A, B1, B2, D1 and D3 (Harper *et al.*, 1995). A Kinase Inhibitory Domain (KID) at the N-terminus is responsible for the Cylin/Cdk inhibition. In a recent paper it was shown that adaptive folding upon binding is responsible for the interaction with structurally distinct Cyclin/Cdk complexes (Wang *et al.*, 2011). Interestingly p21 and p27 are essential for Cyclin D/Cdk4/6 complex formation. At low concentration they promote the formation of active kinase complexes, whereas at higher concentrations they inhibit their activity. Primary mouse embryonic fibroblasts lacking p21 and p27 failed to build up Cyclin D/Cdk complexes and showed a reduced Cyclin D protein level. This effect could be reversed by restoring the p21 and p27 function. Often the loss of Cyclin D dependent kinase activity can be tolerated by cells (Cheng *et al.*, 1999). In endothelial cells Notch represses p21 which results in a cell cycle arrest due to reduced Cyclin D/Cdk4 complex formation and nuclear targeting (Noseda *et al.*, 2004). Interestingly in

mammalian epithelial cells Notch positively regulates p21 causing growth suppression (Rangarajan *et al.*, 2001).

Due to alternative promoters and splicing there are other p21 gene products. The most prominent one is p21B a 132 amino acid protein. Like p21 it is induced by p53 after DNA damage (from a p53 response element downstream the transcription start site) but unlike the full length protein it can trigger only apoptosis and not a cell cycle arrest (Nozell and Chen, 2002; Radhakrishnan *et al.*, 2006).

Originally p21 was discovered to arrest cells in G1 mainly by inhibiting the activity of Cyclin E/Cdk2 and Cyclin A/Cdk2, additionally to Cyclin D/Cdk4/6 (Brugarolas *et al.*, 1999). As expected p21 is accumulated in normal human fibroblasts which are arrested in G0 and a knock down leads to cell cycle entry and DNA synthesis (Nakanishi *et al.*, 1995). But its activity is by far not restricted to simply binding and inhibiting Cyclin/Cdk complexes. During S-phase PCNA is necessary for the formation of the DNA replication complex through its binding to the replicative Polymerase δ and the Replication Factor C (RFC). p21 is able to bind to PCNA and to compete with Polymerase binding hence blocking the replication by inhibiting this complex formation (Li *et al.*, 1994; Waga *et al.*, 1994). It depends on the levels of p21 how much PCNA is titrated out for an S-phase arrest. On the other hand this interaction induces the degradation of p21, which will be discussed later (Gottifredi *et al.*, 2004).

Additionally during S-phase p21 can directly associate with Polymerases δ (Li *et al.*, 2006). A mechanism to impede p21 activity at the S-phase entry is carried out by Myc through its activation of Cyclin D1 and D2. They in turn sequester p27 and p21, resulting in more active Cyclin E/Cdk2 complexes (Perez-Roger *et al.*, 1999).

Due to its inhibitory effect on S-phase a basal turnover of p21 exists at this cell cycle stage and a controversy arose if it is ubiquitin dependent or not. Beside the PCNA regulated degradation there are other mechanisms involved. Several cell cycle related E3 ligases target p21. SCF^{Skp2} and $CRL4^{CdT2}$ are responsible for degradation during S-phase facilitating a proper DNA replication, and APC/C^{Cdc20} targets p21 during prometaphase (Amador *et al.*, 2007; Bornstein *et al.*, 2003; Kim *et al.*, 2008b). Suppression of ubiquitylation and phosphorylation of p21 results in its stabilization (Bloom *et al.*, 2003; Coulombe *et al.*, 2004). In contrast there are reports suggesting an ubiquitin-independent mechanism for proteosomal turnover of p21 (Sheaff *et al.*, 2000) and p21 can directly interact with the 20S proteasome facilitating its degradation (Touitou *et al.*, 2001).

Also the G2 arrest is influenced by p21. Even it seems not to be necessary for G2 arrest induction it is essential for its sustaining. Beside its inhibitory effect on Cyclin B/Cdk1 through binding, a key regulator of G2/M transition, it additionally prevents its activation by retaining the complex in the nucleus, inhibiting the sequestration to the cytoplasm by 14-3-3σ where it is normally activated by Cdc25c (Chan *et al.*, 2000; Charrier-Savournin *et al.*, 2004). Additionally p21 downregulates Emi1, an inhibitor of the Anaphase Promoting Complex (APC) in G2 arrested cells, leading to APC activation and the degradation of key substrates like Cyclin A2 or B1. Therefore this G2 arrested cells cannot enter mitosis (Lee *et al.*, 2009a). The Cdc25 family, which dephosphorylates and hence activates Cyclin/Cdk complexes, competes with p21 for Cyclin binding and can therefore alleviate Cdk inhibition (Saha *et al.*, 1997).

In general p21 can influence gene regulation by several mechanisms. Clearly by inhibiting the Cdk activity but additionally it is also involved more directly by binding and inhibiting transcription factors like E2F1, STAT3, Myc, and p53 (Coqueret and Gascan, 2000; Delavaine and La Thangue, 1999; Kitaura *et al.*, 2000; Lohr *et al.*, 2003). Another mechanism is the hindrance of co-activator recruitment, like p300 or CBP, to the promoter, resulting in hypoacetylation and repression (Devgan *et al.*, 2005). But p21 can also activate genes by de-repressing p300-CREBBP (CREB binding protein) (Snowden *et al.*, 2000).

Due to its cell cycle arresting properties p21 plays a major role and is induced in the response to stress stimuli, like DNA damage, oxidative stress, cytokines and mitogens, tumor viruses, or anticancer agents. It triggers a cell cycle arrest and impairs actively and passively apoptosis, giving the cell time to repair their damages (Abbas and Dutta, 2009). Hence it is not surprising that a large number of different transcription factors are involved in its regulation. p53 is one of the most prominent transcriptional regulators of p21. It activates it upon various extrinsic and intrinsic signals, like DNA damage (Gartel and Tyner, 1999). Two p53 response elements (RE) are crucial for the activation (el-Deiry *et al.*, 1993). Additionally another p53 RE downstream of the transcription start site is responsible for p21B induction (Nozell and Chen, 2002; Radhakrishnan *et al.*, 2006). p63 and p73 the further two members of the p53 family can as well activate p21 and p21B through this response elements (Harms *et al.*, 2004).

Senescence, a permanent cell cycle arrest, caused for example by DNA damage is controlled by the p53-p21 and p16-pRb pathway (Jung *et al.*, 2010). p21 was identified

to be not only overexpressed in senescent cells, it could also induce premature senescence independently of p53 in normal and tumor cells (McConnell *et al.*, 1998; Noda *et al.*, 1994; Wang *et al.*, 1999). A p21 knockout in ATM null MEFs diminished the early onset of senescence (Shen *et al.*, 2005; Wang *et al.*, 1997b).

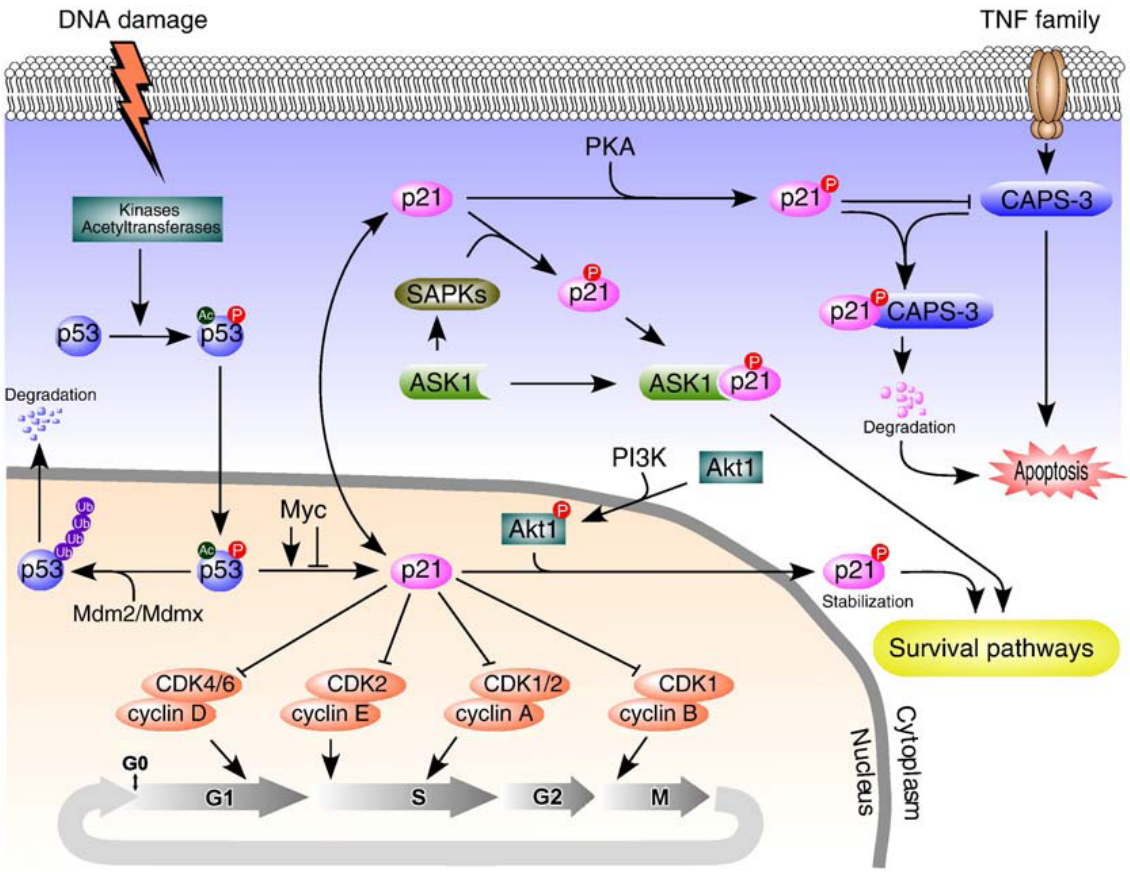


Fig. 5: Impact of p21 upon stress dependent activation (Jung *et al.*, 2010).

The role of p21 upon DNA damage concerning the repair processes is still a big controversy. It was long believed that p21 impairs repair due to the inhibition of PCNA, which localizes at the damaged sites and is required for Nuclear Excision Repair (NER), Base Excision Repair (BER), Mismatch Repair (MMR), and translesion DNA synthesis (Prives and Gottifredi, 2008). p21 inhibits for example the binding of PCNA to MLH1 and MLH2 which is required for MMR (Umar *et al.*, 1996). For long patch base excision repair PCNA stimulates the flap endonuclease 1 (FEN1), DNA ligase 1, and Polymerase δ, which is impeded by p21 binding (Tom *et al.*, 2001). But there are also reports showing a positive role for p21 in DNA repair. p21 null human fibroblasts are deficient in NER, which is set up downstream the recruitment of PCNA to the damaged sites (Shivji *et al.*, 1994; Stivala *et al.*, 2001). Several reports also showed an accumulation of p21, together with PCNA, at the damaged sites necessary for the

proper repair mechanism (Lee *et al.*, 2009b; Mocquet *et al.*, 2008; Perucca *et al.*, 2006). Another positive pathway is the inhibition of the p300-PCNA interaction during DNA repair by p21 leading to HAT activation which is suggested to provide chromatin accessibility to the Global Genome NER (GG-NER) machinery (Cazzalini *et al.*, 2008).
DNA damage does not always lead to an increase in p21 levels. It is not upregulated upon γ-radiation, hydroxyurea and aphidicolin and even down regulated after high UV treatment, whereas p53 levels increase in all cases (Soria and Gottifredi, 2010). This p21 regulation is due to an increase in protein degradation after the damage. The most prominent way is the PCNA binding dependent proteolytic degradation. When bound to chromatin associated PCNA the E3 ligase complex $CRL4^{CDT2}$ mediates the degradation of p21 (Abbas *et al.*, 2008; Nishitani *et al.*, 2008). Because it needs a chromatin bound PCNA, degradation happens only during S-phase and after DNA damage (Havens and Walter, 2009). p21 is not the only protein degraded after PCNA binding. Other candidates are Xic1, CDT1, Pol η, CK1 and E2F1 (Kim and Michael, 2008; Shibutani *et al.*, 2008). It seems that a special sequence of the PIP motif (PCNA interaction protein), responsible for PCNA and $CRL4^{CDT2}$ binding is necessary for degradation as not all proteins having a PIP are degraded (Havens and Walter, 2009).
A further mechanism for p21 turnover in S-phase, at least after DNA damage, was shown in a recent paper. Upon double strand breaks p53 is, via p21, responsible for a G1 and G2 arrest but cannot promote a S-phase arrest. This is initiated by the MRN complex and Cdc25. Ciznadija et al found out that the p53 response to ionizing radiation was the same in all cell cycle phases but an increase in p21 levels was only observed in G1 and G2. During S-phase p21 is degraded independent of the SCF^{Skp2}-E3 ligase but proteasome and Hdm2 dependent (Ciznadija *et al.*, 2011).
Interestingly most of the DNA damage inducing agents causing p21 degradation, like UV, MMS, or hydroxyurea, cause replication fork stalling (Soria *et al.*, 2006). This could be the signal to trigger p21 degradation, maybe also in normal S-phase cells. It is ATR dependent, which is activated after stalled replication, and the E3 ligase $CRL4^{CDT2}$ is associated to the replication forks after DNA damage (Abbas *et al.*, 2008; Lee *et al.*, 2007; Nishitani *et al.*, 2008). p21 degradation after UV is also necessary for efficient PCNA mono-ubiquitylation which is important for TLS (translesion DNA synthesis) induction (Soria *et al.*, 2006). p21 impairs the binding of the TLS-Polymerase η to PCNA (Soria *et al.*, 2008). At least for high levels of UV or MMS the p21 degradation could facilitate a proper TLS (Soria and Gottifredi, 2010). Hence p53

induction and PCNA dependent degradation of p21 are independent at low levels of UV or MMS, the degradation is possibly not strong enough to overcome inducing effects of p53 and p21 levels raise (Soria and Gottifredi, 2010; Soria *et al.*, 2006). In a recent paper Savio et al showed that p21 turnover in human fibroblasts was triggered mainly by the extent of treatment and not by the type of DNA damage or DNA repair pathway. They induced DNA damage which was repaired by NER and BER. Even for an efficient repair p21 removal was not required (Savio *et al.*, 2009). In general it seems logical that upon mild damage p21 is upregulated, inducing arrest. Following severe damage p21 is downregulated and cannot inhibit apoptosis (Cazzalini *et al.*, 2010).

The role of p21 in apoptosis is another controversial story. There are reports claiming an induction or inhibition of it by p21. But the majority supports an anti-apoptotic property of p21. There are several mechanisms how p21 can protect against the programmed cell death. When localized in the cytoplasm it can bind and hence inhibit several proteins involved in apoptosis like the Procaspase 3, Caspase 8 and 10, the Stress-Activated Protein Kinases (SAPKs) and the Apoptosis Signal-Regulating Kinase 1 (ASK1) (Abbas and Dutta, 2009). Also transcriptional regulation is involved. p21 mediates the upregulation of anti-apoptotic secretion factors and additionally it suppresses pro-apoptotic genes by its ability to bind and inhibit E2F and Myc dependent transcription (Dotto, 2000). Additionally posttranslational modifications of p21 play an important role. PKA phosphorylation is for example necessary to bind and inhibit the Procaspase 3 in the cytoplasm (Suzuki *et al.*, 2000a). In general this cytoplasmatic localization seems to be a major criterion of the anti-apoptotic properties. Several kinases can phosphorylate p21 on T145 to promote its translocation to the cytoplasm by interrupting the PCNA binding, and on S146 which enhances stability and tumor cell survival. The most prominent one is Akt1 but also PKA, PKC, and Pim-1 trigger a phosphorylation on these sites (Jung *et al.*, 2010; Li *et al.*, 2002). Interestingly in two independent publications they found different outcomes after S146 phosphorylation depending on the isoform of the PKC. PKCζ phosphorylation on S146 decreases the half life of p21 whereas in another study PKCδ phosphorylation on the same residue enhances stability (Oh *et al.*, 2007; Scott *et al.*, 2002). S130 phosphorylation is another example where the type of kinase and possibly time point determines the outcome. During G1 and S-phase Cyclin E/Cdk2 phosphorylation on S130 decreases stability via ubiquitin dependent degradation

whereas JNK1 and p38 stabilize p21 through S130 phosphorylation (Bornstein *et al.*, 2003; Kim *et al.*, 2002a). An interesting modification related to the cell cycle control is the phosphorylation on T57 by Cdk2 or the Glycogen Synthase Kinase 3β (GSK3β), which increases the ability of p21 to interact with Cyclin B1/Cdk1 at the G2/M transition without inhibiting the complex, therefore promoting cell cycle progression (Dash and El-Deiry, 2005). In contrast to the cytoplasmatic localization the nuclear localization seems to favor apoptosis due to the degradation of p21 (Martinez *et al.*, 2002; Savio *et al.*, 2009). Another study showed that a p21 cleavage by Caspase 3 is important for a TGF-β induced apoptosis (Jung *et al.*, 2010).

Other reports attest p21 a direct positive role in apoptosis induction through the upregulation of Bax for example (Liu *et al.*, 2003). A critic to several studies presenting a pro-apoptotic effect of p21 is that they often only display that apoptosis concurred with p21 but not the dependency on it (Cazzalini *et al.*, 2010). Kraljeciv et al illustrated a relationship between the chemotherapeutic agent and the role of p21 in apoptosis. Human HEp-2 cells lacking p53 showed an induction of apoptosis through TNFRSF9 upregulation and Caspase 7 activation after p21 overexpression. Interestingly when they treated the same cells with two different chemotherapeutic agents they gained opposing results. p21 stimulation enhanced apoptosis when cells were treated with cisplatin, which induces DNA damage, and attenuates it when treated with methotrexate, which inhibits DNA synthesis by blocking the de novo synthesis of the nucleoside thymidine. The dual role of p21 was in a direct correlation with the Caspase 3 and 7 modulation (Kraljevic Pavelic *et al.*, 2008).

Additional to cell cycle and apoptosis regulation p21 promotes also cell differentiation (Besson *et al.*, 2008). It seems to be a barrier in reprogramming of iPS cells (Li *et al.*, 2009). Knockouts of p53 and p21 in MEFs increase the efficiency of the reprogramming step (Hong *et al.*, 2009; Kawamura *et al.*, 2009).

Taken all together p21 can be seen as a tumor suppressor and as an oncogene. In vivo data support both views. p21 promotes genomic stability in hematopoietic stem cells (Minucci *et al.*, 2002). The absence of p21 makes mice more susceptible to tumors (Martin-Caballero *et al.*, 2001; Topley *et al.*, 1999). Initial discoveries suggested p21 to be a major determinant of the tumor suppressor activity of p53 (el-Deiry *et al.*, 1993). Mice bearing a p53 mutant unable to induce apoptosis (p53R172P) exhibit longer survival than p53 null mice, but an additional p21 knockout shows accelerated tumorgenesis, aneuploidy, and chromosomal aberrations (Barboza *et al.*,

2006). A p21 dependent senescence in thymocytes protects them from chromosomal instability and cancer (Gartel, 2009). The loss of p21 in fumarylacetoacetate hydrolase deficient mice allows proliferation under DNA damage conditions, resulting in cancer development in liver and kidney (Willenbring *et al.*, 2008). MYCN is overexpressed in human neuroblastomas and additionally it modulates the suppression of p21. It is a target of the miRNA cluster 17-5p-92. An inhibitor of this miRNA reactivates p21 and Bim which abolishes the growth of therapy resistant neuroblastomas (Fontana *et al.*, 2008). These were a few examples of tumor suppressing properties of p21 in mouse models. Also for the oncogenic activity exist several studies. In p53 deficient or haploid mice loss of p21 leads to a significant extended survival (De la Cueva *et al.*, 2006). In ATM deficient mice a p21 knockout results in a delay in thymic lymphomagenesis (Wang *et al.*, 1997a). The development of oligodendrogliomas (ODG) is reduced upon loss of p21. After p21 transfer it accumulates in the nucleus of ODG tumor cells and induces the Cyclin D/Cdk4 complex formation, which contributes to an increase in proliferation and a reduction in apoptosis in the tumor cells (Liu *et al.*, 2007). Treatment of cancer cells with different drugs often upregulate p21, which in turn stimulates cellular senescence helping the cells to escape apoptosis triggered by the drugs (Gartel and Radhakrishnan, 2005).

This dual role of p21 in cancer formation makes it hard to target in a therapy. It also explains why p21 is regulated by a broad variety of proteins at the transcriptional and post transcriptional level. It has for sure one of the best studied promoters and still the list of regulators is constantly expanding. The most prominent one is p53 through its two response elements. Several co-factors can facilitate p53 dependent activation, like BRCA1 through p300 recruitment which activates and stabilizes p53 (Chai *et al.*, 1999; Lu and Arrick, 2000). The propyl isomerase Pin1 interacts with p53 in response to DNA damage, induces S33 and S46 phosphorylation resulting in p21 induction (Zacchi *et al.*, 2002). A similar mechanism is used by GADD34. It is induced by growth arrest and DNA damage and causes S15 phosphorylation of p53 enhancing p21 expression (Yagi *et al.*, 2003). The serine/threonine kinase LKB1 triggers a p21 dependent cell cycle arrest through p53 phosphorylation on S15 and S392 (Zeng and Berger, 2006). The Monocytic Leukemia Zinc-finger (MOZ) acetylates histones on the p21 promoter due to p53 binding (Rokudai *et al.*, 2009).

But there are several p53 independent mechanisms to induce the p21 promoter. Six Sp1/Sp3 binding sites are situated near the transcriptional start site and there are

various stress stimuli which promote a Sp1 dependent activation, like NGF, butyrate, phorbol myristate acetate (PMA), or TGF-β (Gartel and Tyner, 1999). Sp1 in association with p53 is also involved in DNA repair, cell growth, differentiation, and apoptosis by upregulating p21 (Koutsodontis *et al.*, 2001). Other proteins like pRb, the cGMP-dependent protein kinase (PDK), or Integrin β1 use Sp1 to induce the p21 promoter (Cen *et al.*, 2008; Decesse *et al.*, 2001; Fang *et al.*, 2007). Paradoxically there are also examples of Sp1 dependent repression of the promoter. In smooth muscle cells Sp1 represses p21 and disrupts therefore Cyclin D1/Cdk/p21 complexes, leading to proliferation inhibition and apoptosis induction. This indicates a cell type specific dependence in the regulation of p21 by Sp1 (Kavurma and Khachigian, 2003).
Other important regulators of p21 are HDACs, which repress its transcription. HDAC inhibitors can upregulate p21 either through enhancing directly the histone acetylation or through the Sp1/Sp3 binding sites where they cause HDAC release (Ocker and Schneider-Stock, 2007). HDAC1 for example interferes with the p21 activating p53 binding to Sp1. Genotoxic stress triggers an increase in p53 recruitment to the promoter causing a reduction in HDAC1 binding followed by hyperacetylation of the core promoter (Lagger *et al.*, 2003). The HDAC inhibitor TSA blocks this HDAC1 Sp1 binding, causing acetylation and transcriptional activation (Xiao *et al.*, 2000). It additionally mediates phosphorylation on S10 at histone 3 by MSK1 and the following acetylation on K14. 14-3-3 binds to this H3-S10ph-K14ac mark, which is necessary for the subsequent recruitment of RNA Polymerase II (Simboeck *et al.*, 2010).
SAHA another HDAC inhibitor was shown to increase Polymerase II loading to the promoter by decreasing the HDAC1 activity (Marks, 2004). Additionally it releases HDAC4 from the promoter which accelerates H3 and H4 acetylation (Mottet *et al.*, 2009; Richon *et al.*, 2000). A different mechanism utilizes the HDAC inhibitor Apicidin. It promotes the translocation of the Protein Kinase C (PKC) from the cytoplasm to the nucleus which induces Sp1 dependent p21 expression (Han *et al.*, 2001). The X-linked tumor suppressor FOXP3 activates p21 via inhibiting the binding of HDAC2 and 4 to the first intron, resulting in an acetylation increase (Liu *et al.*, 2009).
There are also various mechanisms to repress the p21 promoter. A prominent candidate is c-Myc. It suppresses transcription utilizing different ways. First it can sequester Sp1 from the promoter (Gartel *et al.*, 2001). It further binds and inhibits Miz1 an initiator sequence binding factor responsible for p21 induction during the hematopoietic cell differentiation (Cherrier *et al.*, 2009; Wu *et al.*, 2003). Myc also

represses p21 via the bHLH-LZ transcription factor AP4 (Jung *et al.*, 2008). In contrast Myc upregulates p21 through p19ARF which stabilizes p53 via Mdm2 inhibition (Felsher *et al.*, 2000). Other proteins repressing p21 are FBI-1 a proto oncogene and member of the transcription factor family POK. It competes with Sp1 and p53 for promoter binding (Choi *et al.*, 2009). CTIP2, a COUP-TF-interacting protein, is recruited to the Sp1 sites and inhibits the promoter epigenetically through the interaction with HDACs and lysine methyltransferases (Cherrier *et al.*, 2009). The homeodomain transcription factor CUT represses p21 via interacting with sequences overlapping the TATA box. (Coqueret *et al.*, 1998). ZNF76 and p53as, an alternative spliceform, inhibit p21 via TATA Box Binding Protein (TBP) interaction (Gartel, 2005; Wu *et al.*, 2002).

Further possibilities of regulation are influencing the transcriptional elongation. Chk1 can inhibit p21 by disassembling elongation factors like DSIF, CstF-64, or CpSF (Beckerman *et al.*, 2009). In contrast p53 activates p21 through recruitment of elongation factors such as DSIF, p-Tefb, and FACT, and the subsequent phosphorylation of the Polymerase II (Gomes *et al.*, 2006).

The next level of regulation happens at the mRNA level. RNA binding proteins, such as HuD or HuR, are translocated to the cytoplasm after UV or p16 accumulation, where they bind to the p21 mRNA and stabilize it (Al-Mohanna *et al.*, 2007; Joseph *et al.*, 1998; Wang *et al.*, 2000a). RBM38, also called RNPC1, stabilizes the p21 mRNA upon DNA damage induction (Shu *et al.*, 2006). PCBP1 and PCBP2 induce cell cycle arrest independent of p53 through p21 mRNA binding (Waggoner *et al.*, 2009). In contrast Msi-1 inhibits p21 translation upon mRNA binding (Battelli *et al.*, 2006).

Also miRNAs are involved in the regulation of p21. Examples are the miR-17-92, miR-106a-363, and miR-106b-25 clusters. Overexpression of these miRNAs cause proliferation and G1/S transition (Petrocca *et al.*, 2008a; Petrocca *et al.*, 2008b).

Taken together p21 seems to be one of the key players in the checkpoint response upon various stimuli, and hence cancer formation, by facilitating a cell cycle arrest and the inhibition of apoptosis to give the cells time to repair the damage. Not for nothing it is one of the best studied proteins, but due to the involvement in many different pathways and the great dependence on the cell background concerning the p21 way of action there is still a lot unclear.

2.2 Multi Drug Resistance

A serious problem in cancer therapy is the resistance of the target cells to various drugs used to kill them. In general this potential is called multi drug resistance. The multi refers to the fact that after a selection for resistances to a single drug, cells often show a cross resistance to other mechanistically and structurally unrelated drugs. There are three possibilities how a cell can evolve a resistance against certain agents.
The first mechanism is a reduced drug uptake. Water soluble drugs need a carrier to pass the membrane. These transporters normally facilitate the uptake of nutrients. Or they enter in an endocytosis dependent process. If this uptake mechanism is altered drugs can not accumulate in the cell anymore. Examples of drugs which are taken up actively are antifolates like methotrexate or the nucleotide analogues 5-fluorouracil and 8-azaguanine, or cisplatin (Shen *et al.*, 1998; Shen *et al.*, 2000).
The second mechanism includes all changes in cells affecting the ability of the drugs to kill the cells. Examples are alterations in the cell cycle, DNA repair, a changed drug metabolism through the cytochrome P450 superfamily, which are key enzymes in metabolizing xenobiotics, or the resistance due to a defect in the apoptosis induction (Lowe *et al.*, 1993; Schuetz *et al.*, 1996).
The third possibility involves ATP dependent efflux pumps. Hydrophobic drugs, like vinca alkaloids (vinblastine, vincristine), anthracyclines (doxorubicin, daunorubicin), the RNA transcription inhibitor actinomycin D or the microtubule stabilizing drug paclitaxel, which can easily diffuse into the cells, can be actively pumped out by ATP-Binding-Cassette (ABC) transporters. They show a broad drug specificity which allows them to facilitate the efflux of various xenobiotics (Gottesman *et al.*, 2002).
There are between 38 and 61 ABC genes in eukaryotic organisms, except plants, sequenced to date. Plants posses a greatly expanded number of efflux transporters. This is possibly due to a wider variety of toxins they have to deal with and the fact that they miss a filtering organ like the liver (Dean, 2009). The ABC proteins are grouped into 8 different subfamilies namely ABCA-ABCH. Humans have 48 ABC genes carrying out a broad variety of normal physiological functions (Dean and Annilo, 2005). The ABC transporters are classified according to their sequence in the nucleotide binding folds (NBFs), which are the ATP binding domains. A full transporter consists of two transmembrane (TM) domains with 6-12 membrane spanning alpha-helices and

two NBFs. Halftransporters contain only a single TM and a single NBF, therefore they need to form either homo- or heterodimer complexes (Dean, 2009).

In general ABC transporters gain their energy to efflux specific agents through binding or binding and hydrolyzing a pair of ATP molecules. A single ATP molecule binds parts of two NBFs causing a structural change which can be reversed by ATP hydrolysis and release. This conformational change enables the efflux pump to transport the drug to the other side of the membrane. It could be shown that not all transporters hydrolyse both ATP molecules (Kadaba *et al.*, 2008; Locher, 2004).

The ABC transporters are expressed in normal tissues transporting also numerous endogenous substrates. To their functions belongs the protection of the brain against toxins by the blood-brain-barrier and the blood-cerebrospinal-fluid barrier. The testicular tissue and the developing fetus are protected against various drugs through efflux pumps. The excretion of toxins from liver, gastrointestinal tract and kidney is also part of their function (Gottesman *et al.*, 2002).

A reason for the intensive research about ABC transporters is their involvement in cancer. Several transporters are upregulated in cancer cells, mainly ABCB1, ABCC1, and ABCG2 but also others seem to play a role in multi drug resistance (Szakacs *et al.*, 2006). In a screen with NCI60 cells 31 of 48 ABC transporters reduced the potency of anticancer drugs (Szakacs *et al.*, 2004). An example is ABCB5 which is suggested to be present in 2-20% of melanoma (Frank *et al.*, 2005). Immunodeficient mice which were injected with ABCB5 expressing cells could recapitulate the tumor (Schatton *et al.*, 2008). MRP2 could also be detected in cancer cell lines but its exact mechanism is still under study (Szakacs *et al.*, 2006).

The most prominent transporter is P-Glycoprotein (Pgp) a member of the ABCB subfamily. The gene names are ABCB1 or Mdr1 (Juliano and Ling, 1976; Ueda *et al.*, 1987). In over 50 percent of all drug resistant tumors Pgp was found to be expressed (Moitra *et al.*, 2011). It consists of 12 TM regions and two NBFs and has a broad spectrum of substrates. Unmodified neutral or positively charged hydrophobic drugs are the main targets. Examples are vinca alkaloids, anthracyclines, epipodophyllo-toxins and taxanes (Szakacs *et al.*, 2006). Normally Pgp is expressed at pharmacological barrier sites, like the blood-brain-barrier, where it prevents the uptake of drugs affecting the central nervous system, and in the transport epithelium of the kidney, liver, and the gastrointestinal tract. Additionally Pgp can be found in cells of the immune system and adult stem cells (Cordon-Cardo *et al.*, 1990; Kwan and

Brodie, 2005; Thiebaut *et al.*, 1989). Two other ABCB members confer the potential of drug resistance, ABCB11, a bile salt taransporter, and ABCB4 (Mdr3), a phosphatitylcholine flippase, both normally expressed in the liver (Gerloff *et al.*, 1998; van Helvoort *et al.*, 1996). As Pgp inhibitors the calcium channel blocker verapalmin and the immunosuppressant cyclosporine A are used (Gottesman *et al.*, 2002).

Another important ABC transporter is MRP1 (Multi drug Resistance associated Protein 1) a member of the ABCC subfamily. It has a similar structure than Pgp but additionally five membrane spanning N-terminal domains (Cole *et al.*, 1992). MRP1 recognizes neutral and anionic hydrophobic products and additionally transports organic anions and phase II metabolic products (Szakacs *et al.*, 2006). In general the ABCC subfamily, consisting of 12 members, is localized in the basolateral membranes. An exception is MRP2, which is expressed in the apical membrane of polarized cells including hepatocytes and enterocytes (Gottesman *et al.*, 2002). It plays a role in the protection against orally ingested drugs (Dietrich *et al.*, 2001).

MRP1 is expressed in many tumors and cancer cell lines suggesting a prominent role in the multi drug resistance. It shares many substrates with Pgp (Hipfner *et al.*, 1999; Szakacs *et al.*, 2004). Several other homologues of MRP1, but not all, have these additional N-terminal domains. They are putative targets in the treatment of drug resistance (Borst *et al.*, 2000).

ABCG2, also known as MXR or BCRP, is another transporter with a great potential in contributing resistance to drugs. It acts as a homodimer of two half-transporters and is a member of the ABCG subfamily (Honjo *et al.*, 2001). Studies revealed its expression in a variety of cell lines selected for anticancer drug resistance and the transported substrates span cytotoxic drugs, toxins, carcinogens found in food products and endogenous agents (Abbott, 2003).

Of great interest in the last years became the involvement of ABC transporters in stem cell biology. Many stem cells express high levels of specific transporters. Hematopoietic stem cells for example possess high levels of ABCG2 and/or ABCB1. During differentiation the expression is often turned off (Kim *et al.*, 2002b). Interestingly knockouts of ABC transporters, like ABCG2, ABCB1, or ABCC1 together or alone result in viable and fertile mice with normal stem cell development but they are more sensitive to drugs like vinblastine or mitoxantrone and the tissue distribution of substrate compounds differs, suggesting a role as a protector of the organism and stem cells from toxins (Jonker *et al.*, 2002; Schinkel *et al.*, 1994; Zhou *et al.*, 2002).

In the cancer stem cell model ABC transporters play a prominent role. Due to several reasons cancer stem cells are often resistant to chemotherapy. Among them are their quiescence, an enhanced DNA repair capacity, increased apoptosis threshold, and the expression of ABC transporters. These mechanisms provide the potential to survive and give rise to relapse at a later time point. Renal cell cancer for example, where Pgp is expressed in all cells, rarely responds to primary chemotherapy (Moitra *et al.*, 2011). Additional support for the cancer stem cell model and the ABC transporter involvement comes from the observation that many normal tissues, like bone marrow, gut lining, or hairs can recover after a chemotherapy due to their stem cells which seem to survive the treatment quite well (Dean, 2009).

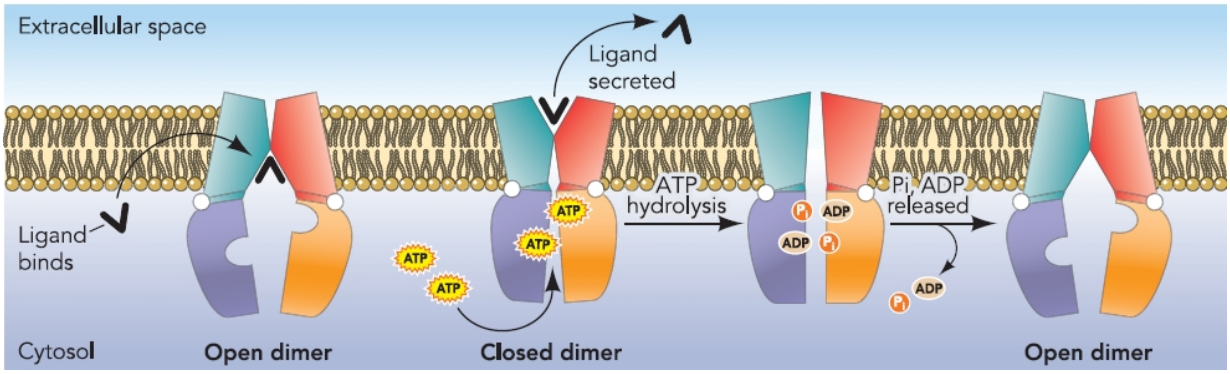


Fig 6: Mechanism of ABC transporters

2.3 The E2F Associated Phosphoprotein EAPP

As the E2F family of transcription factors is involved in a wide range of pathways, including cell cycle, DNA damage, or apoptosis, it is of great interest to find further associated partners. A yeast two hybrid screen was done in our laboratory to identify putative interaction candidates of E2F. The N-terminal domain of E2F1 (aa 1-125) was used as the bait, which contains a p45Skp2 (ubiquitin ligase), a Cyclin A/Cdk2, and a Sp1 binding domain, and the Nuclear Localization Sequence (NLS). One of the selected clones was a cDNA with a 99% homology to an uncharacterized human bone marrow protein called BM036. Because this 285 amino acid long protein was highly phosphorylated it was called E2F-Associated-Phosphoprotein (EAPP) (Novy *et al.*, 2005). Computer analysis showed a great homology between the human and the murine protein (86%) and revealed several putative phosphorylation and other modification sites. It contains a possible PEST motif (aa 2-34), a NLS (aa 120-131), a zinc finger motif (aa 159-216), and a coiled coil region (aa 226-254). GFP tagged EAPP was predominantly nuclear localized due to its NLS (Novy *et al.*, 2005).

GST pulldown and co-immunoprecipitation experiments revealed that the region between aa 175-240 seems to be necessary for binding the activator E2Fs 1-3. E2F4 does not interact with EAPP (Novy *et al.*, 2005). The same region is also responsible for the direct interaction with the pocket proteins pRb, p107, and p130, with p53, or the polyoma T-antigen (Novy, Rotheneder, Schwarzmayr, unpublished data). In an antibody array with cell cycle proteins further possible interaction partners were found like Cyclin D3, p19Skp1, Cul-1, Cyclin A, GAK, Rad52, Cdk6, Cdk1, Cdk2, and Stat3 (Rotheneder, unpublished data).

In pulldown experiments EAPP binds to the acetyl transferases PCAF and CBP. In a mammalian two-hybrid experiment also p300 interacted with EAPP (Ferner-Orthner, unpublished). Between the amino acids 120-175, including the NLS, an in vivo acetylation was detected (Ferner-Orthner, unpublished data).

The region between aa 175-180 seems to be important to form homodimers or homomultimers (Pohn, unpublished data).

In a human mRNA tissue blot EAPP mRNA was detected in all tested tissues, but in different amounts. Heart, placenta, skeletal muscle, and pancreas showed high EAPP levels, whereas in the brain, lung, and kidney the levels were lower (Novy *et al.*, 2005). Interestingly EAPP seems to be present at high levels in transformed cell lines

compared to the human diploid fibroblasts MRC5. SAOS-2 cells were the only exception of the tested cell lines (Novy *et al.*, 2005).

The promoter of EAPP possesses multiple binding sites for the Sp-family of transcription factors and an Egr1 binding site near the transcription start site. Egr1 (early growth response) is a transcription factor involved in the regulation of genes necessary for growth, proliferation, and differentiation (Thiel and Cibelli, 2002). Reporter gene assays where Sp1 and Egr1 were overexpressed increased the transcriptional activation of the EAPP promoter. In contrast Sp3 expression, which was shown to be able to repress Sp1 mediated transcriptional activation, repressed the promoter (Schwarzmayr *et al.*, 2008; Sjottem *et al.*, 1996).

In reporter gene assays the murine Thymidine Kinase (mTK) promoter, the p14ARF promoter, and two artificial ones, with either three functional or mutated E2F binding sites, were applied to elucidate the role of EAPP in the E2F dependent transcription. The artificial and the mTK promoter activity was further increased when EAPP was added to E2F1 in a dose dependent manner, but EAPP did not alter the E2F4 mediated transcriptional activity. The activity of the mutated promoter was not changed upon E2F1 and EAPP overexpression. In contrast EAPP repressed the E2F1 dependent activation of the p14ARF promoter (Novy *et al.*, 2005).

3. Outline of the work

The main focus in our laboratory is to elucidate the function of the newly discovered E2F-Associated-Phosphoprotein EAPP. The E2F family of transcription factors is a crucial regulator of the cell cycle. As EAPP interacts with the activator E2Fs and was able to influence the E2F dependent transcription a role in this process seemed to be a good starting point for further investigations. It turned out that also other proteins involved in the cell cycle are able to interact with EAPP, like the pocket protein family, or certain Cyclins and Cdks. Additionally levels of EAPP seem to decrease during mitosis. Therefore the question at the beginning of my dissertation was if EAPP is somehow involved in the regulation of the cell cycle and if this is the case which activities are carried out to regulate putative target proteins. Several drugs can be used which reversibly arrest cells at different cell cycle phases providing a useful tool to investigate this stages and the checkpoints leading to the arrest. Overexpression and knockdown studies of EAPP in combination with these drugs would give hints if EAPP really has an impact in these crucial processes. Several mechanisms could account for this, like a direct interaction of EAPP with target proteins, altering its activity due to sterical reasons or modifications. Another possibility would be the regulation of processes involved in the production of the protein, like transcription or translation. To reveal a potential mechanism various approaches can be carried out like western blot analysis, reverse transcriptase PCR, reporter gene assays, co-immunopresipitations, chromatin immunoprecipitations, diverse flow cytometrie applications, or immunofluorescence.

As EAPP is upregulated in several tested transformed cell lines it seems worth to study the role of EAPP in the cell, possibly finding another regulator involved in the transformation of normal cells to cancer.

4. Results

4.1 EAPP overexpression results in a G1 arrest

4.1.1 EAPP overexpression leads to a G1 arrest after different drug treatments

We had hints from immunofluorescence experiments that EAPP vanishes during mitosis. To further study this we used nocodazole, which induces a mitotic arrest, and released the cells afterwards. Nocodazole, or methyl[5-(2-thienyl-carbonyl)-1H-benzimidazol-2-yl]-carbamate, inhibits the polymerization of free tubulin molecules through binding an arginine residue of the ß-tubulin subunit (Jordan *et al.*, 1992). Hence the proper microtubule polymerization is impeded causing a mitotic arrest.

Surprisingly when we overexpressed EAPP in the osteosarcoma cell line U2OS not all cells showed the expected G2 DNA content, instead a G1 peak appeared in contrast to the control transfection (Andorfer,diploma thesis). To further investigate this we used other drugs with different impacts to find out if this effect is restricted to nocodazole. We tried colchicine, MMS, and etoposide. Colchicine acts similar than nocodazole, causing a mitotic arrest due to impeding the microtubule polymeriziation (Luduena *et al.*, 1992). Methyl-methanesulfonate (MMS) is an alkylating agent which adds methyl- or other alkylgroups onto guanine in the DNA (Sigma). This results in a fragmentation of the DNA when repair enzymes try to replace the alkylated bases. Etoposide is a Topoisomerase II inhibitor. Topoisomerases are ATP-dependent enzymes transiently breaking DNA to allow DNA strands pass through each other. This is necessary to solve topological problems, appearing after DNA replication, recombination, chromosome condensation and decondensation, or transcription. Topoisomerase II induces double strand breaks and etoposide can trap an intermediate state, called G-strand intermediate form, where the DNA is cut and it prevents the relegation and leads to an enzyme release leaving the DNA with a double strand break (Hande, 1998). Hence MMS and etoposide cause cell cycle arrests during S-phase and in G2.

The concentrations of the four drugs were tested in pre-experiments to find the proper amounts for U2OS. Cells were transfected with either a HA-EAPP or an empty expression vector using PEI. 24h after the transfections the drugs were added using

the appropriate concentration and 16h later cells were harvested and fixed in ethanol for FACS analysis (Fig 7).

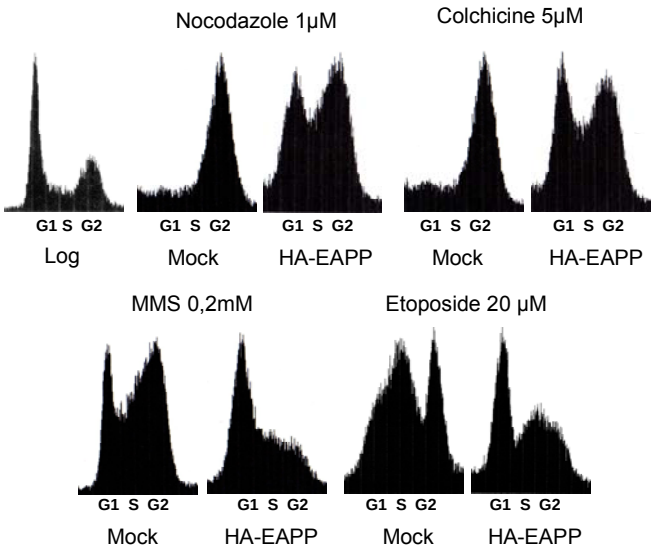


Fig 7: Drug treatment reveals an EAPP-induced G1 arrest. U2OS cells were transfected with either an empty or HA-EAPP expression vector and treated for 16h with nocodazole, colchicine, MMS, and etoposide. Cells were then harvested and fixed with ethanol for FACS analysis.

To test if this G1 induction is a phenomenon of only U2OS, which are p53 positive, we used other cell lines, namely T98G a glioblastoma cell line with a mutated p53, and SAOS an osteosarcoma cell line without p53. Both showed the same increase in the G1 proportion than U2OS upon EAPP overexpression and 16h nocodazole treatment (Fig 8).

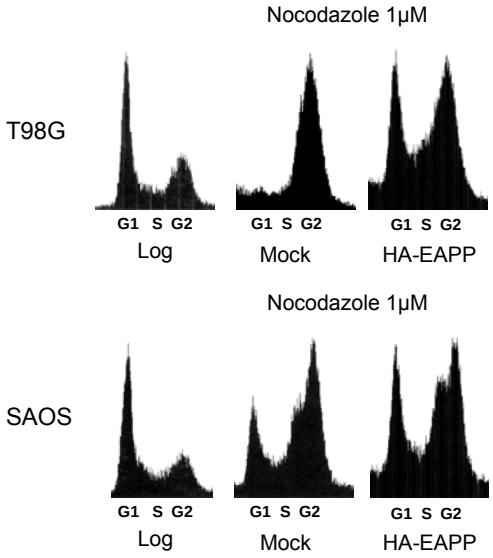


Fig 8: The EAPP triggered G1 arrest seems to be p53 independent. T98G and SAOS cells were transfected with either an empty or a HA-EAPP expression vector and treated for 16h with nocodazole. Cells were than harvested and fixed with ethanol for FACS analysis.

4.1.2 Long term nocodazole treatment does not lead to aneuploidy and apoptosis

If the cells would just not be able to arrest and would continue with the damaged microtubule and DNA, they finally would end up with a massive increase in aneuploidy and apoptosis. To examine this we applied the same procedure than before with the difference that the nocodazole was on the cells for 48 hours, enough time for two doublings (Fig 9).

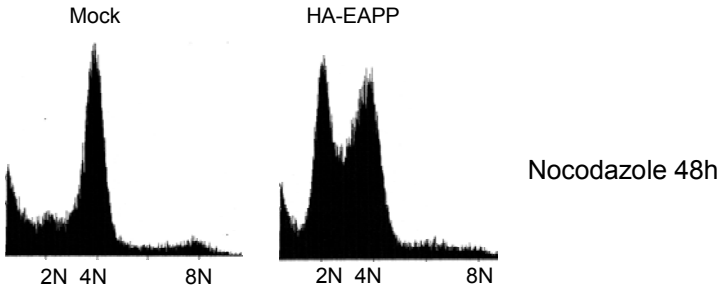


Fig 9: EAPP overexpressing cells do not show an increase in apoptosis and aneuploidy upon nocodazole treatment. U2OS cells were transfected with either an empty or HA-EAPP expression vector and treated for 48h with nocodazole. Cells were than harvested and fixed with ethanol for FACS analysis

A rough estimation about an apoptosis increase is done with the sub-G1 peak, which would rise because of the DNA fragmentation when apoptosis is initiated. If cells go through mitosis without proper segregation of their chromosomes an abnormal number would appear in the daughter cells, which is called aneuploidy. This can be seen in the DNA profile as cells would turn up with more than the doubled set of chromosomes and there should not be such a clear G1 peak.

Both, apoptosis and aneuploidy, do not seem to increase a lot after EAPP overexpression suggesting that the mechanism EAPP conveys is either established before the drugs act, this means that the cells arrest in G1 before entering the cell cycle giving the drugs no possibility to work, or they hinder the added agents in their proper way of action. Since we previously saw that EAPP overexpression causes an increase in S-phase in normal U2OS cells we concentrated on the second possibility (Novy, 2005). As we used different drugs it seemed unlikely that EAPP directly influences the way of action. More plausible would be a general mechanism to get rid of the drugs like the induction of ABC transporters. They would be able to pump them out of the cells.

4.2 Influence of EAPP on the ABC transporter ABCB1 (Mdr1)

4.2.1 EAPP can upregulate Mdr1 on a transcriptional level

To simply test a possible influence of EAPP on ABC transporters we used an antibody against the most common transporter, namely ABCB1 or Mdr1/Pgp, in T98G cells transiently overexpressing HA-EAPP (Fig 10).

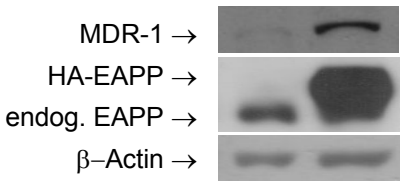


Fig 10: EAPP can upregulate Mdr1(Pgp). T98G cells were transfected with either an empty or HA-EAPP expression vector. Cells were harvested 48h later and whole cell extracts were made for Western analysis. 20µg cell extracts were loaded and the gained membrane was stained with the Mdr1, EAPP, and β-Actin antibody.

To further confirm this upregulation upon EAPP overexpression we applied luciferase reporter gene assays with a Mdr1-Luc vector (Fig 11,12).

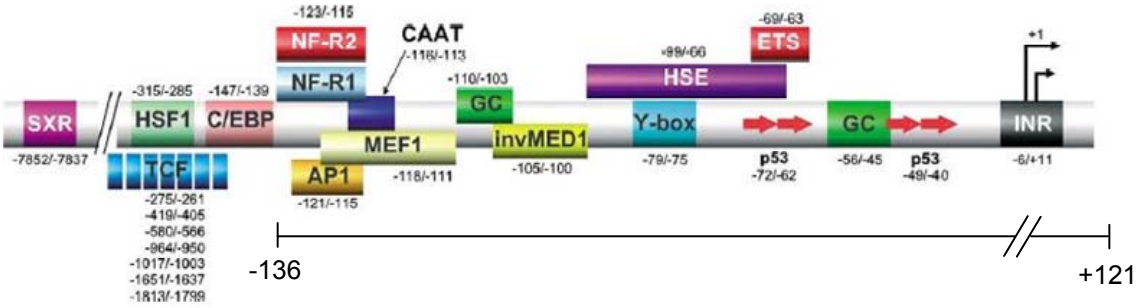


Fig 11: Diagram of the human Mdr1 promoter. The marked area between -136 and +121 was used for the reporter gene assay (Labialle *et al.*, 2002).

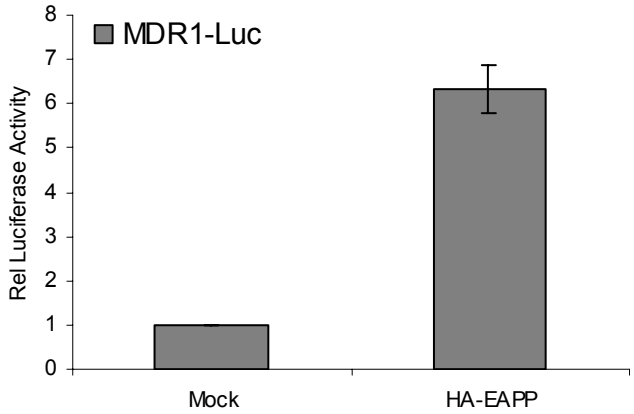


Fig 12 EAPP can upregulate Mdr1 on a transcriptional level. T98G cells were transfected with either an empty or HA-EAPP expression vector. Additionally the Mdr1-Luc and a β-Gal vector as transfection efficiency control were added. Cells were harvested 48h later and the luciferase reporter gene assay was applied. The bars show the average of three experiments.

The clear increase in Mdr promoter activity indicates a regulation on the transcriptional level. Using Western blot and luciferase assays we further investigated the induction of Mdr1 after EAPP overexpression and the addition of the different drugs used before (Fig 13,14).

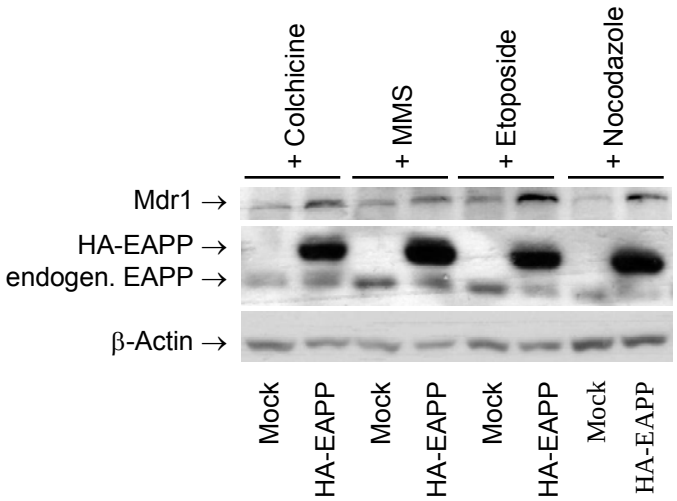


Fig 13: EAPP upregulates Mdr1(Pgp) differently upon different drug treatments. T98G cells were transfected with either an empty or HA-EAPP expression vector. The stated drugs were added after 24h for 16h. The cells were harvested and whole cell extracts were made for Western analysis. 20µg cell extracts were loaded and the membrane was stained with the Mdr1, EAPP, and β-Actin antibody.

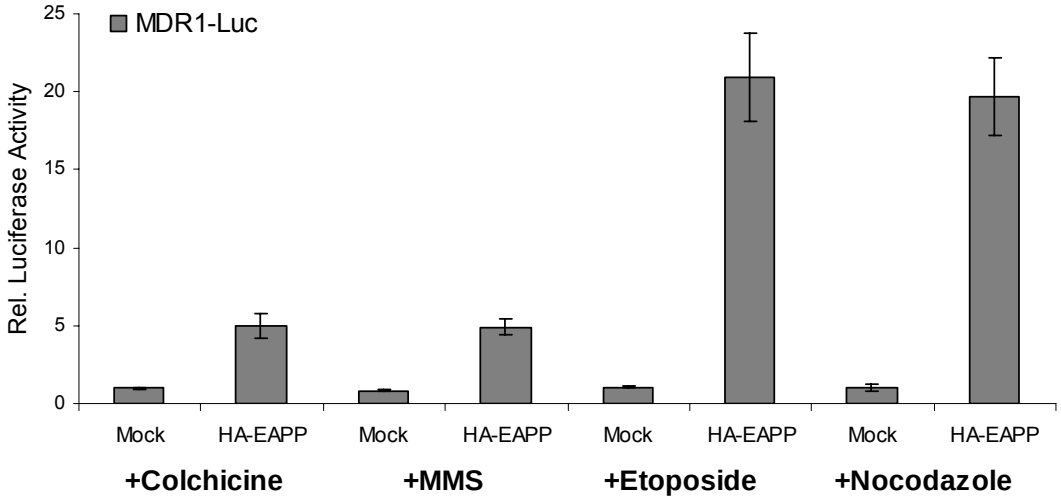


Fig 14: EAPP upregulates the Mdr1 promoter differently upon different drug treatments.T98G cells were transfected with either an empty or HA-EAPP expression vector. Additionally the Mdr1-Luc and a β-Gal vector as transfection efficiency control were added. After 24 hours cells were incubated with the stated drugs for 16h, harvested afterwards and the luciferase reporter gene assay was applied. The bars show the average of three experiments.

The Western blot and the luciferase assay show a similar pattern in the fold induction after EAPP overexpression and the addition of the appropriate drugs. It seems that the upregulation of Mdr1 by EAPP is at least partly dependent on the kind of applied agent. Even though the pathways switched on with nocodazole and colchicine should be more or less the same, there are possibly some differences which could lead to this result.

4.2.2 EAPP further upregulates the Mdr1 promoter after E2F1 overexpression

During reporter gene assay experiments it turned out that also E2F1 can upregulate the Mdr1 promoter. There is no hint in the literature but it is not implausible considering the huge number of promoters, possibly 20%, where E2F1 is present even without an E2F1 binding site (Bieda *et al.*, 2006). In luciferase reporter assays we examined a possible interplay between EAPP and E2F1 in regulating Mdr1 (Fig 15,16). We not only used the normal Mdr1 promoter, additionally we had a Mdr1-Luc vector with a mutated Y-box. The Y-box, or CCAAT-Box, is present in nearly one third of the eukaryotic housekeeping genes. It is situated at the proximal promoter and is known for binding general transcription factors which possess a specific binding site, like NF-Y, to enhance RNA polymerase II transcription (Fang *et al.*, 2004).

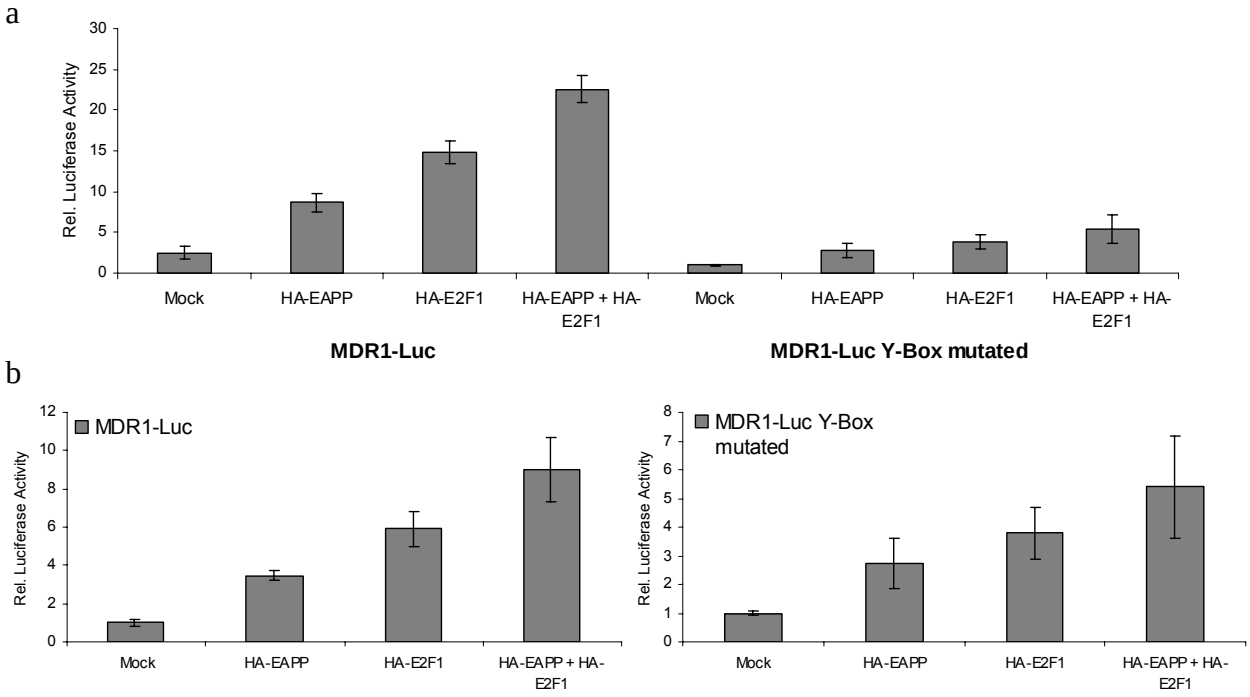


Fig 15: EAPP and E2F1 can upregulate the Mdr1 promoter even when the Y-Box is mutated. T98G cells were transfected with either an empty, HA-EAPP (1,5µg), or HA-E2F1 (0,5µg) expression vector. Additionally the Mdr1-Luc or the Mdr1-Luc Y-Box mutated and a β-Gal vector as transfection efficiency control were added. After 48 hours cells were harvested and the luciferase reporter gene assay was applied. The bars show the average of three experiments. (a) All experiments were put in one graph. (b) The Mdr1 and the Mdr1-Luc Y-box mutated were split and the control was set to one in both cases to compare the real increase in the promoter activity.

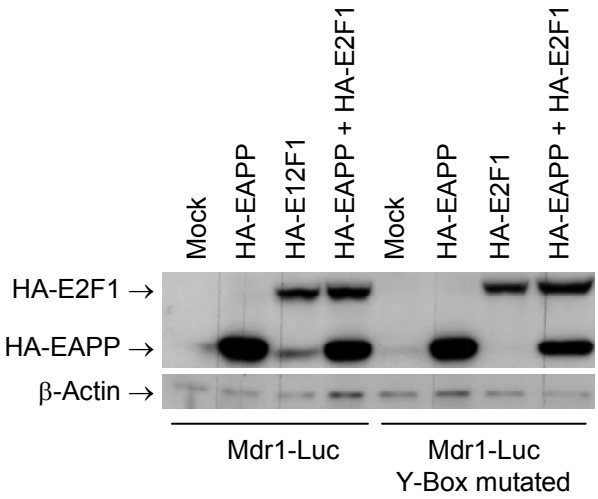


Fig 16: Control Western for the luciferase assay of Fig 15 to check if all transfected proteins are present. The blot was probed with an anti-HA and an anti-β-Actin antibody. The weak EAPP signal in the HA-E2F1 and Mock lane is possibly due to a contamination from the HA-EAPP+HA-E2F1 lane.

Both EAPP and E2F1 can upregulate the Mdr1 promoter in the presence of a Y-box mutation, indicating that their way of action does not depend on the CAATT-Box and their interaction partners. But the general expression goes significantly down when the Y-box is mutated pointing out its importance for the transcription of Mdr1. In both

cases a further upregulation of the promoter takes place when EAPP and E2F1 were transfected in combination. To get a hint if they work together or use different mechanism we did reporter gene assays with a pRb mutant, which can not be phosphorylated hence permanently binds E2F1 and inhibits it.

4.2.3 EAPP dependent Mdr1 upregulation seems to be independent of E2F1

In G1 resting cells E2F1 is bound by pRb, the most prominent member of the pocket protein family, and kept inactive. Upon growth stimuli pRb gets phosphorylated on several residues by Cyclin/Cdk complexes which trigger the release of E2F1, resulting in the activation of genes involved in cell cycle progression (Satyanarayana and Kaldis, 2009). A pRb mutant, where 15 out of 16 Cdk phosphorylation sites are altered to alanine and therefore is unable to be phosphorylated, would now bind constitutively to E2F1, leading to a permanent inhibition (Lents *et al.*, 2006). This pRb(∆cdk) can be transfected additionally to the pRb present in the cell hence it just targets all free E2F1 independently of the phosphorylation status of the endogenous pRb. We conducted luciferase assays transfecting various combinations of EAPP, E2F1, and pRb(∆cdk), together with the Mdr1-Luc vector (Fig 17). The question behind was if we can reduce the activation potential of E2F1 by this pRb mutant is than the EAPP dependent upregulation also affected.

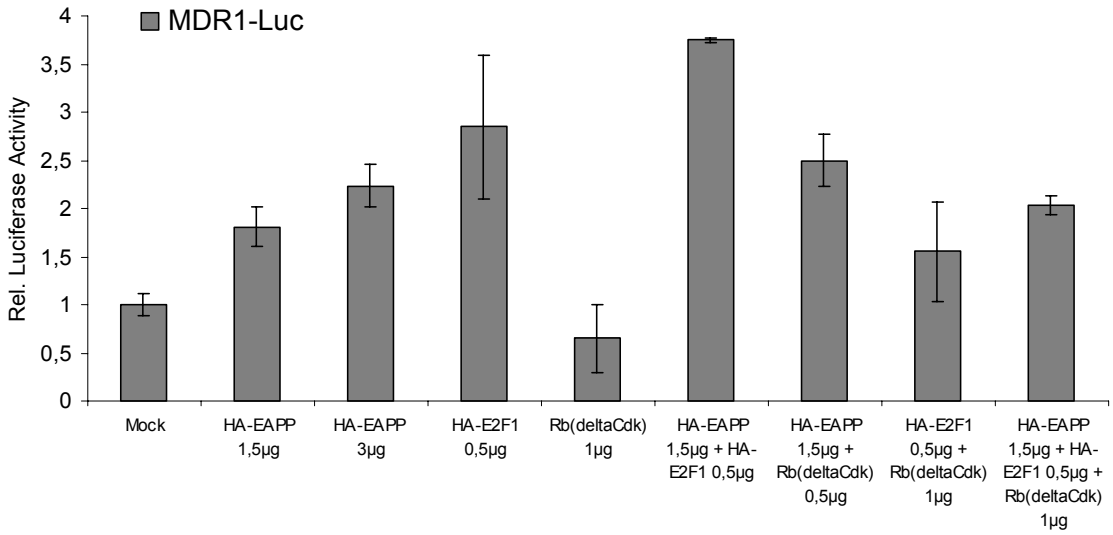


Fig 17: EAPP seems to regulate the Mdr1 promoter independently of E2F1. T98G cells were transfected with either an empty, HA-EAPP, HA-E2F1, or pRb(Δcdk) expression vector using the indicated amounts. Additionally the Mdr1-Luc and a β-Gal vector as transfection efficiency control were added. After 48 hours cells were harvested and the luciferase reporter gene assay was applied. The bars show the average of three experiments.

The luciferase reporter assays showed the expected downregulation of the promoter activity when pRb(Δcdk) was transfected. pRb(Δcdk) also reduces the E2F1 dependent upregulation. The levels do not go down as far as with pRb(Δcdk) alone, possibly due to an excess of the endogenous E2F1 taken together with the transfected one, as the amounts of transfected pRb(Δcdk) and HA-E2F1 seem to be quite similar (Fig 18). In contrast pRb(Δcdk) does not alter the EAPP dependent increase of the Mdr1 promoter activity and all three together reach a level similar to an EAPP induction alone. The rise of the Mdr1 promoter activity, when EAPP is transfected together with pRb(Δcdk), is quite high compared to EAPP alone. Possibly it up-regulates the promoter more in the absence of E2F1.

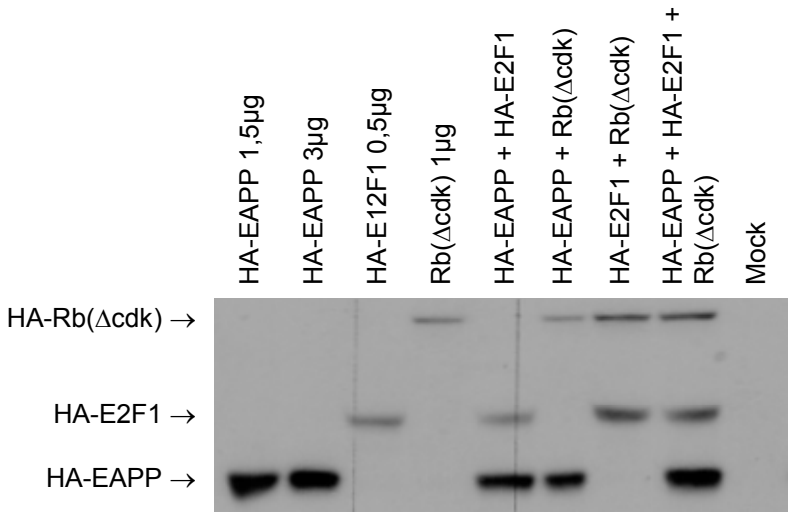


Fig 18: Control Western for the luciferase assay of Fig 17 to check if all transfected proteins are present. The blot was probed with an anti-HA antibody.

The next step was to examine if this upregulation has also in vivo consequences, resulting in an increase in the potential of pumping drugs out of the cell.

4.2.4 EAPP does not alter the efflux capacity of Mdr1

One of the most common P-glycoprotein substrates is rhodamine 123. It is pumped out of the cell by the ABC transporter and therefore used in functional assays to test the capacity of the multi drug resistance phenotype mediated by Pgp (Wuchter *et al.*, 2000). One big advantage of this dye is that it is fluorescent and its cell concentration can easily measured with flow cytometry. We used U2OS and T98G cells and transfected them with HA-EAPP and HA-E2F1. As control an empty expression vector was used. After 48h cells were harvested and incubated for one hour at 37°C with pre-tested amounts of rhodamine 123, washed and prepared for flow cytometry to measure the uptake of the dye. Unfortunately in both cell lines no difference in the dye concentration could be detected when either EAPP or E2F1 was overexpressed (Fig 19).

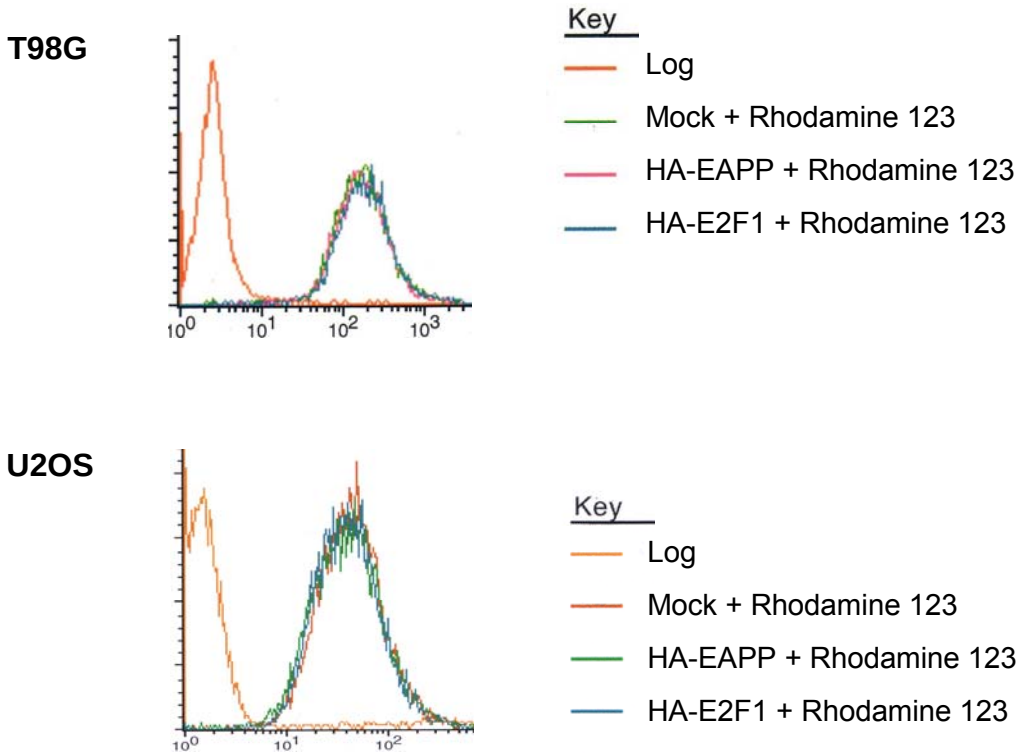


Fig 19: Neither in U2OS nor in T98G cells EAPP and E2F1 had any effect on the efflux capacity. The two indicated cell lines were transfected with either an empty, HA-EAPP or HA-E2F1 expression vector. After 48h cells were harvested incubated for one hour with rhodamine 123, washed two times and prepared for flow cytometry analysis of the intracellular dye concentration. A logarithmic growing population without dye addition was taken as a reference.

The expected pattern for an increase in Pgp function would be a kind of shoulder on the left side of the curve. About 20 percent of the cells are transfected hence only this part would show a raise in the drug efflux. In some experiments the whole curve was shifted a little bit (as can be seen in fig 20) but this has to be due to a general fluctuation between the cell populations as they were separated before the transfection.

To test if the method itself worked we used the Pgp inhibitor verapalmin. Because Mdr1 can be detected in the cells even without induction this inhibitor should reduce the capacity of the cells to pump the dye out. We again followed the procedure conducted for figure 19 with T98G. Before rhodamine was added mock, HA-EAPP, and HA-E2F1 transfected cells were split into two populations. One was incubated for one hour with rhodamine alone and the other with rhodamine and the Pgp inhibitor verapalmin. Afterwards cells were prepared for flow cytometry (Fig 20).

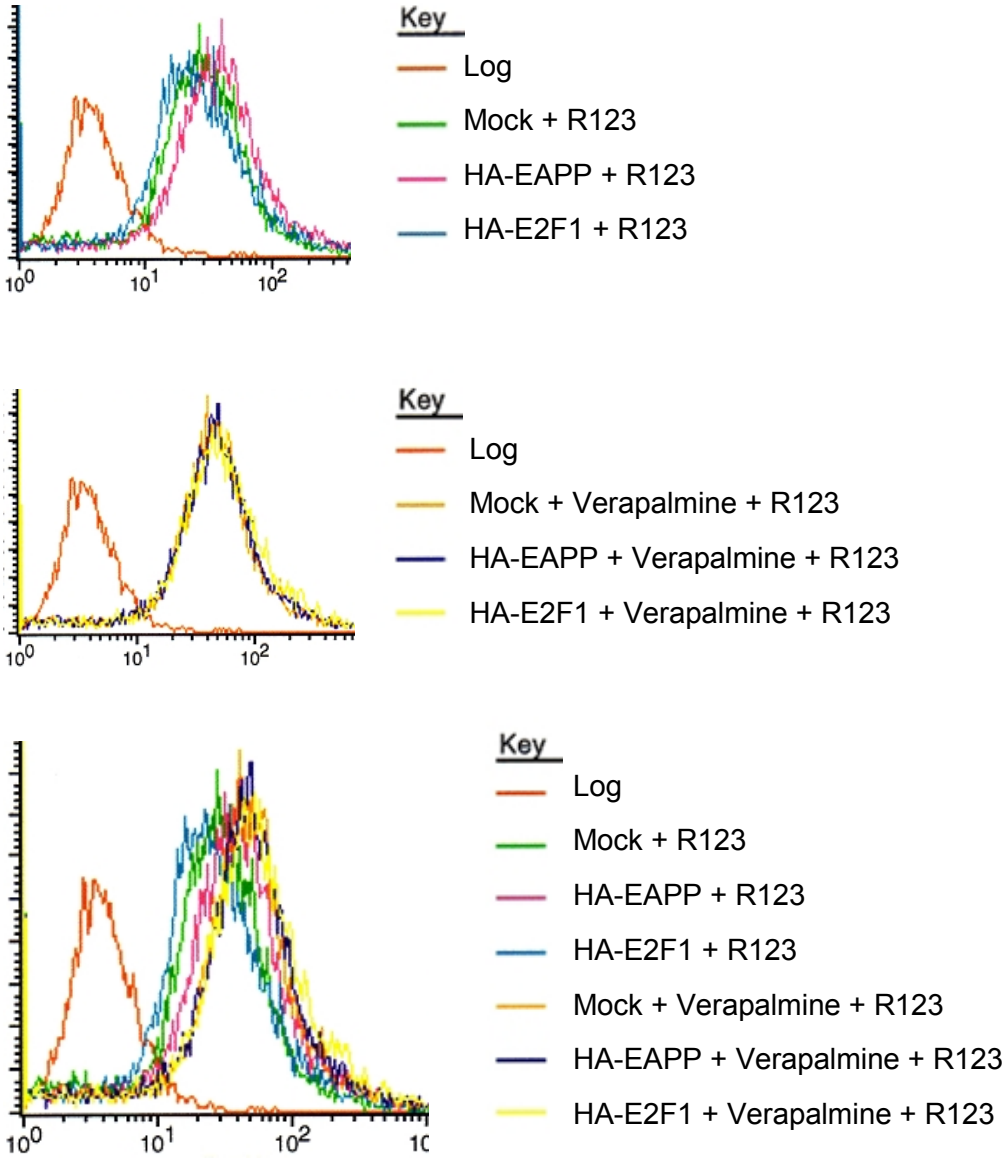


Fig 20: In T98G the Mdr1 inhibitor verapalmin results in an increase of the rhodamine 123 concentration. T98G cells were transfected with either an empty, HA-EAPP or HA-E2F1 expression vector. After 48h cells were harvested and each of the transfected cells split into two populations and either incubated for one hour with rhodamine 123 or with rhodamine and verapalmin. Afterwards they were washed two times and prepared for flow cytometry analysis of the dye concentration. A logarithmic growing population without dye addition was taken as a reference.

When verapalmin was added all transfected populations shifted, as expected, to a higher intracellular rhodamine concentration. This indicates that the experimental setup in general should work. The inhibition of Pgp results in a decrease of drug efflux. More Pgp should be able to pump more dye out of the cell and reducing the cell concentration. Unfortunately this we do not see when EAPP and E2F1 are overexpressed even we have an upregulation of Pgp in the Western analysis. Possibly this increase is not enough to get a shift in our experimental setup. We tried a final attempt with a pDsRed-EAPP fusion protein. As rhodamine 123 emits in the range of

GFP we needed a tag emitting at a higher wavelength like pDsRed. Because in the previous experiments we always compared two cell populations separated before transfection we could now additionally also compare the pDsRed positive and negative cells in one population where all cells are treated the same way. When analyzed with flow cytometry it is possible to distinguish between cells which are pDsRed positive, namely cells producing EAPP, and pDsRed negative, which have not taken up the pDsRed-EAPP expression vector. In this case, in contrast to the experiments before, the whole curve should shift as we compare 100% EAPP positive cells with control cells. Before we had only around 20% EAPP positive cells (Fig 21).

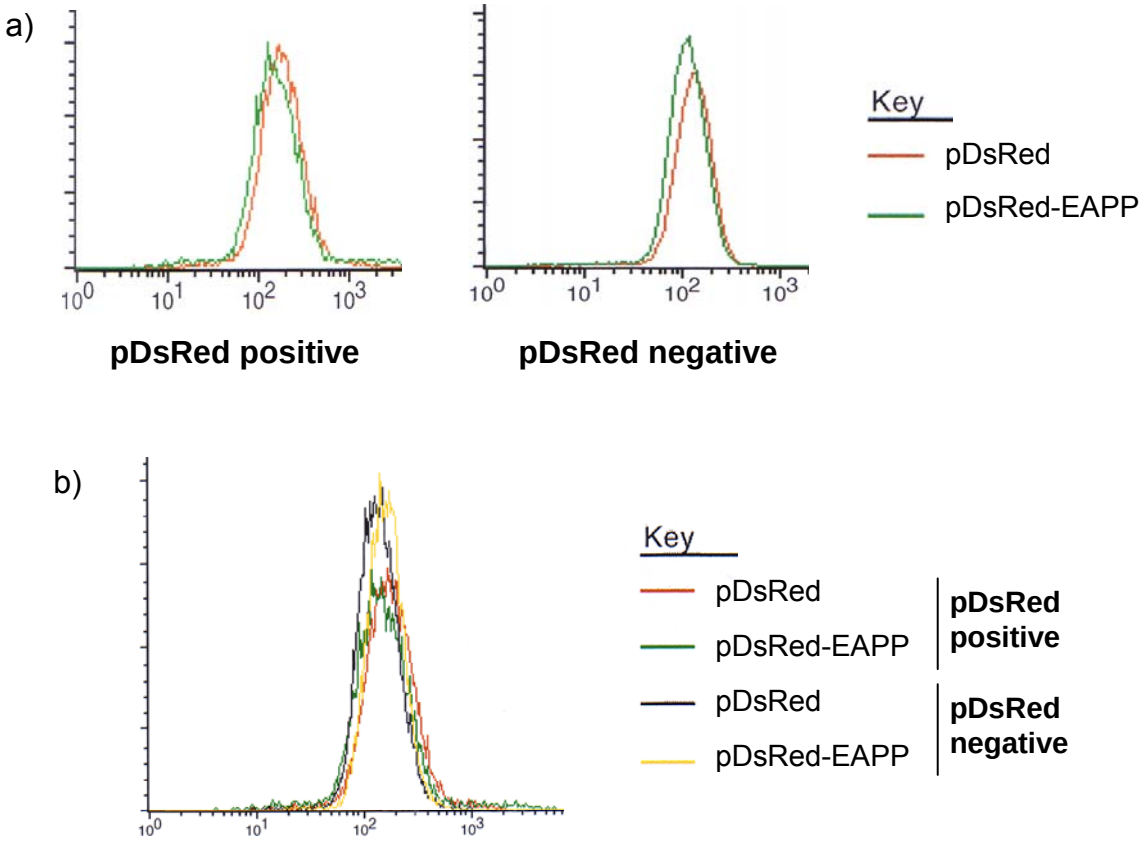


Fig 21: pDsRed-EAPP positive cells showed no significant increase in the efflux capacity due to Pgp upregulation. T98G cells were transfected with either a pDsRed alone or a pDsRed-EAPP expression vector. After 48h cells were harvested and incubated for one hour with rhodamine 123, washed two times and prepared for flow cytomentry. A logarithmic growing population without dye addition was taken as a reference (not shown). (a) Red fluorescent positive and negative pDsRed-empty and pDsRed-EAPP are compared in two separated graphs. (b) Both graphs from (a) are put together to see the difference between fluorescent and non fluorescent cells.

Even there is a slight shift to the left of pDsRed-EAPP positive cells compared to the pDsRed-empty positive cells indicating an increase in drug efflux, but as we got the similar pattern for the pDsRed negative cells this seems again to be due to a general effect of the different populations independently of the transfected protein.

We found an upregulation on a transcriptional level of the Mdr1 gene. Unfortunately the efflux experiments did not look very promising. Possibly the gained upregulation is not enough to see the effect in the used experimental setup. Nevertheless this indicates that ectopically expressed EAPP is not able to increase the efflux pumps in sufficient amounts to transport the delivered drugs out of the cells. To get a more or less normal cell cycle, explaining the G1 increase, most of the agents should be removed. Even low levels can arrest parts of the cells after 16 hours in other cell cycle stages. Only about 20-30 % of the cells are transfected, which resembles approximately the G1 fractions, suggesting that most of cells with an EAPP overexpression are in G1 and not in other stages. Taken together the upregulation of ABC transporters does not seem to be the reason for the changes in the DNA profile.

4.3 An EAPP dependent upregulation of p21 is responsible for the G1 arrest

4.3.1 EAPP overespression results in a G1 arrest even in unperturbed cells

In contrast to previous experiments where EAPP seemed to be able to drive cells into S-phase (Novy *et al.*, 2005) we found an increase in the G1 cell population in unperturbed cells. Because there was no drug treatment this was a further argument against the ABC transporter hypothesis. To further examine this G1 arrest upon EAPP overexpression we tried to measure the uptake of BrdU. Bromodeoxyuridine is a synthetic analogue of thymidine and becomes incorporated during DNA synthesis. If labeled with a fluorescent dye it is possible to measure the new uptake of BrdU which means that the cells start to replicate their DNA. If EAPP overexpressing cells would have a normal cell cycle and just pump the drugs out of the cells BrdU should be incorporated and cells should shift in flow cytometry analysis. If EAPP would arrest cells in G1 no BrdU would be taken up, therefore cells should not shift. Unfortunately the FITC dye, which was labeled to BrdU, is not a very strong fluorescent dye. We got not really conclusive results because it was difficult to say if there was a real shift or not (data not shown). As the results of the GFP tagged EAPP were quite promising we did not continue with the BrdU experiments.

To clarify these controversial data, if EAPP arrest or induce the cell cycle, we used a GFP tagged version of EAPP. The problem with the privious experiments was the low percentage of transfected cells. Only around 20% of the cells were transfected and this could lead to misleading results when analyzed together with the untransfected cells. Using a GFP tag it was now possible to gate and analyze just the GFP positive cells.

We tried two different approaches to elucidate the origin of this G1 peak. First we transfected U2OS cells with GFP or with GFP-EAPP. Cells were harvested and the DNA was stained with Hoechst 33258 and prepared for flow cytometry. We gated the GFP positive cells and compared the GFP and GFP-EAPP transfected cells in respect to either GFP negative, low GFP, and high GFP fluorescence (Fig 22).

In the GFP only cells there was no distinction between the different amounts of fluorescene intensity, also when compared to an untransfected control. In contrast the EAPP overexpressing cells showed an obvious increase of the G1 fraction versus the

other cell cycle stages. But there was no difference of the GFP-EAPP negative cells to the control and the GFP only cells.

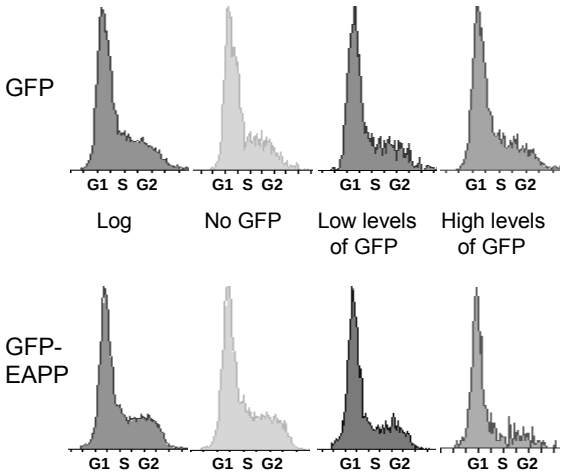


Fig 22: Upon GFP-EAPP overexpression the number of cells staying in the G1 cell cycle stage increases. U2OS cells were transfected with either GFP or GFP-EAPP expression vectors. 48h after transfection cells were harvested, stained with Hoechst 33258 and prepared for flow cytometry. Gates were set to get the fractions of non fluorescent cells and cells with low and high amounts of GFP signal.

It is unlikely that the effect is dependent on GFP as the HA tagged EAPP also leads to a G1 increase, but this can maybe seen only when treated with drugs as the effect is possibly to small for a constant and significant detection considering that only about 20 percent of the cells had an EAPP upregulation.

In the second experiment we transfected the same U2OS cell population with an empty pDsRed and a GFP-EAPP vector. Cells were harvested after 48 hours and the DNA was stained with Hoechst 33258. In a dot blot using the GFP channel against the pDsRed channel it is now possible to roughly distinguish cells having taken up both of them or only one. For these gated fractions the cell cycle distribution could now be determined (Fig 23).

Even the profiles are not the nicest it is clearly visible that cells harboring the GFP-EAPP vector showed an increase in the G1 cell cycle stage in contrast to the pDsRed positive cells. These two experiments indicate that EAPP is involved in the induction of a G1 arrest instead of driving cells into the S-phase.

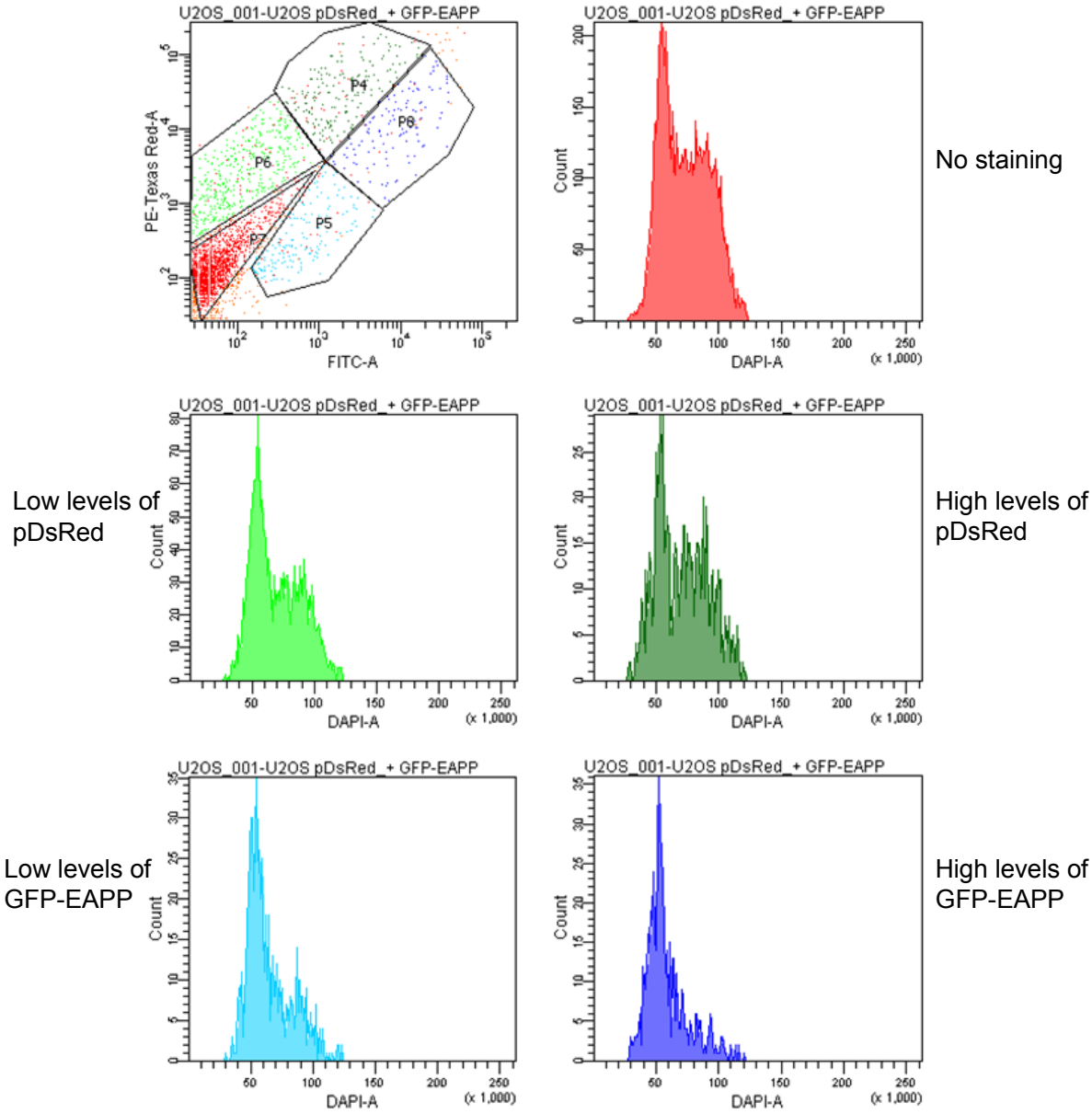


Fig 23: GFP-EAPP overexpressing cells increase the number of cells staying in the G1 cell cycle stage. U2OS cells were transfected with pDsRed and GFP-EAPP. 48h after transfection cells were harvested, stained with Hoechst 33258 and prepared for flow cytometry. Gates were set to get the fractions of non fluorescent cells and cells with low and high amounts of GFP and pDsRed signal.

4.3.2 EAPP levels and nuclear localization change upon a cell cycle arrest

Other experiments pointed in the same direction. U2OS cells were seeded out and grown to confluence. At the beginning, in the middle, and at the end of this period samples were taken and used for flow cytometry and Western analysis (Fig 24). Cells approaching a high density tend to slow down their cell cycle resulting in an increased G1 fraction as can be seen in the DNA profile. This arrest is at least partially

dependent on the upregulation of p21 (Perucca *et al.*, 2006). Simultaneously EAPP and p21 levels increased, in contrast to p53, in the highly dense cell population, suggesting an involvement in the cell cycle regulation of EAPP.

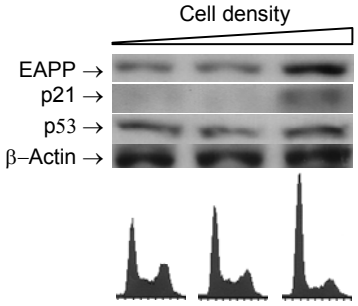


Fig 24: EAPP levels rise upon cell density increase. U2OS cells were grown to confluency and cells were harvested at the beginning, in the middle, and at the end and prepared for flow cytometry and Western analysis. To get the cell cycle profile U2OS were stained with Propidiumiodid. The Western blot was probed with the indicated antibodies.

T98G cells can be arrested upon serum depletion. We arrested and released them through addition of serum and harvested cells after indicated time points. Cell fractionation was done, separating cytoplasm and nucleus, and prepared for Western analysis (Fig 25). Interestingly EAPP seems to shift more to the nucleus upon a serum arrest and the pattern is reversed after the release. In immunofluoresence experiments EAPP can be mainly found in the nucleus but also a cytoplasmic fraction exists. It seems that either the import is increased or the export is reduced. In recent computer analysis of EAPP we found a nuclear export sequence. This again fits to the data suggesting that EAPP is able to trigger a G1 arrest.

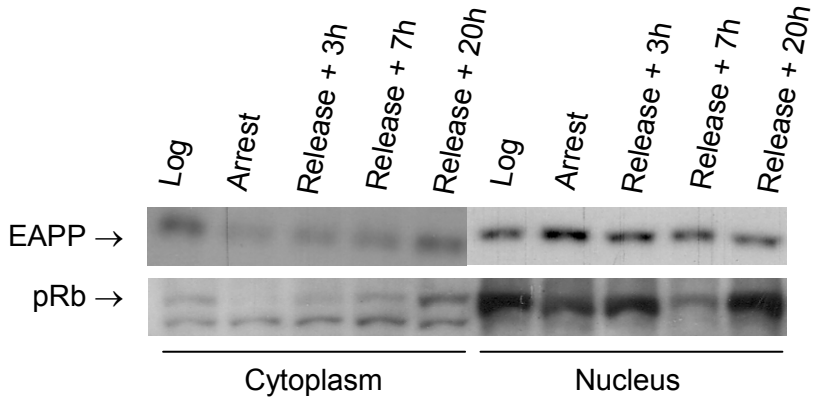


Fig 25: EAPP shifts to the nucleus upon serum arrest. T98G cells were serum arrested for 48 hour and released. Cells were harvested at the indicated time points and cytoplasm and nucleus were separated, prepared for Western analysis, and probed with the mentioned antibodies.

4.3.3 EAPP upregulates p21, causing a G1 arrest

To elucidate candidates responsible for this EAPP dependent G1 arrest we tested the most prominent cell cycle regulators able to block the progression to S-phase, like p21, p27, or p16. We found an upregulation of p21 upon an EAPP overexpression. To further investigate if this upregulation is required for the arrest we used a p21 knockdown vector in addition to the EAPP overexpression. U2OS cell were transfected with either a GFP or GFP-EAPP expression vector with or without a p21 mRNA targeting shRNA vector, followed by cell cycle analysis and Western blot (Fig 26).

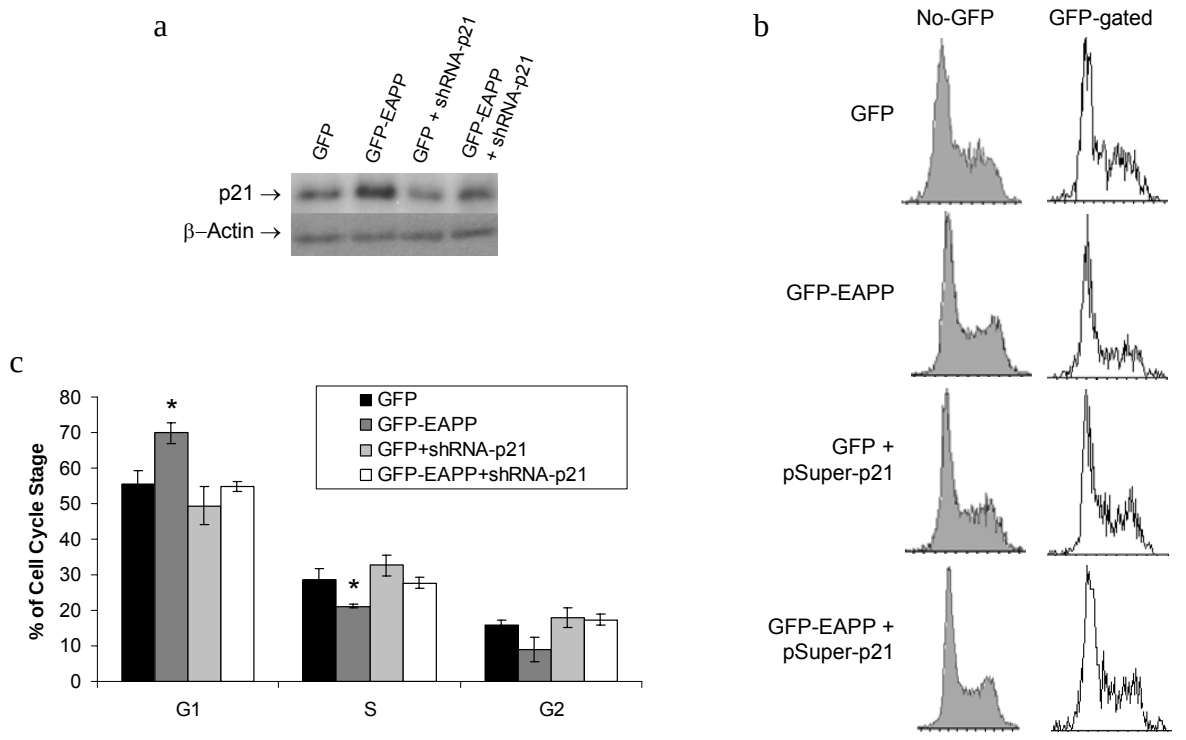


Fig 26: p21 is responsible for the EAPP triggered G1 arrest. U2OS cells were transfected with GFP alone or GFP-EAPP, with a scrambled shRNA or a p21 targeting shRNA. Cells were harvested after 48 hours and prepared for flow cytometry and Western analysis. (a) Western blot was probed with the indicated antibodies. (b) Cell cycle stage only for GFP positive cells were measured using Hoechst 33258. (c) Three independent experiments were quantified with FlowJo (Tree Star Inc, Ahland, OR, USA) and averaged. * P<0,05 (For P-value calculation see Data Analysis in Material and Methods)

Again the GFP tagged EAPP was used to gate just for the cells expressing the fusion protein, thus getting rid of the untransfected background. Even though the knockdown vector for p21 was not the best the results clearly show that an impeded upregulation of p21 prevents the EAPP triggered G1 arrest.

To further confirm the regulation of p21 by EAPP we used overexpression and knockdown of EAPP to check corresponding p21 protein and mRNA levels (Fig 27). Especially the knockdown of EAPP dramatically reduces p21 protein and mRNA amounts.

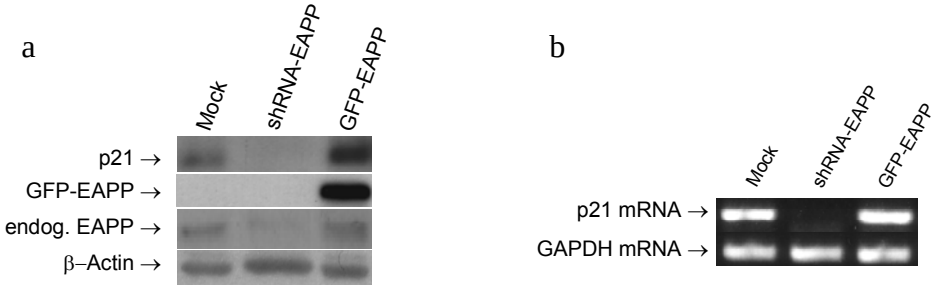


Fig 27: EAPP levels determine p21 protein and mRNA levels. U2OS cells were transfected with GFP-EAPP, a short hairpin RNA targeting the EAPP mRNA, and control vectors. After 48h cells were harvested and prepared for Western analysis and RT-PCR. (a) The Western blot was probed with indicated antibodies. (b) Reverse Transcriptase PCR was conducted using primers for p21 mRNA and GAPDH mRNA as a control and loaded onto a 2% agarose gel.

Knockdown vectors expressing shRNA often lead to artifacts due to pathways they activate independently of the target protein, for example the Toll Like Receptors (TLR). To rule out that the p21 downregulation is such an artifact we used different EAPP mRNA target sequences to test if they all show the same result (Fig 28). We additionally transfected GFP-EAPP and an EAPP knockdown vector at the same time in different concentrations to check if we get cells were the overexpression of EAPP together with the lowered endogenous EAPP resembles the control treated cells. As the same EAPP level should yield in the same p21 level we should be able to restore p21 amounts, what we indeed could do (data not shown).

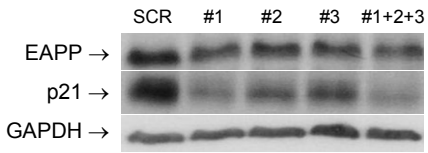


Fig 28: Different EAPP knockdown vectors can down regulate p21.Three different knockdown vectors expressing a shRNA against the EAPP mRNA were transfected, either alone or together, into U2OS. Additionally a scrambled shRNA was used as control. After 48 hours cells were harvested, prepared for Western analysis and immunostained with the indicated antibodies.

The p21 mRNA levels are affected by EAPP suggesting a transcriptional regulation. To examine this we performed reporter gene assays with the p21 wild type promoter, either elevating or reducing the levels of EAPP. As a further control we also checked a promoter which we know is unperturbed by EAPP (Fig 29).

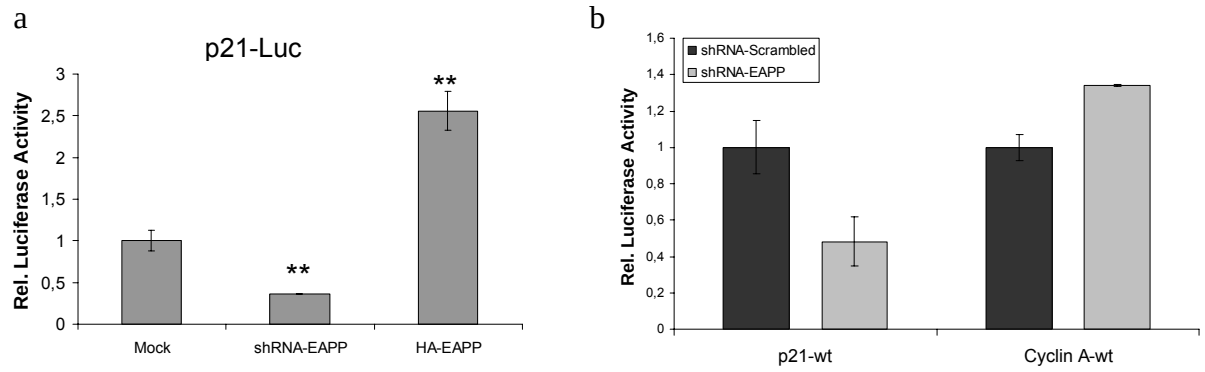


Fig 29: EAPP levels are responsible for the up and downregulation of the p21 promoter in reporter gene assays. (a) U2OS cells were transfected with either a control, a shRNA vector targeting the EAPP mRNA, and HA-EAPP in addition to a p21-Luc and a β-Gal vector as a transfection efficiency control. After 48h cells were prepared for luciferase reporter assays and measured. Transfection was checked with Western analysis (not shown) (b) U2OS cells were transfected with a control and an EAPP knockdown vector in addition to either a p21-Luc or Cyclin A-Luc vector. As a transfection efficiency control again the β-Gal vector was added. After 48h cell were prepared for luciferase reporter assays and measured. Transfection was checked with Western analysis (not shown). The assays were done in triplicate and data present the mean ± sd. * P<0,05; ** P<0,01

Upregulation of EAPP induces and downregulation inhibits the p21 promoter used for the reporter gene assay. In general these results indicate a transcriptional control. As an additional proof we did Chromatin Immunoprecipitations to check the occurrence of RNA Polymerase II at the exon 1 of the p21 gene (Fig 30). If the transcription is reduced, also less Polymerase should be found within the transcribed gene.

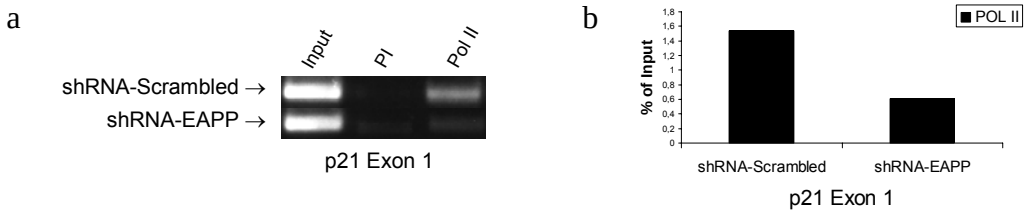


Fig 30: Polymerase II levels bound to the exon 1 of the p21 gene are reduced upon an EAPP knockdown. (a) U2OS cells were transfected with either a scrambled or an EAPP targeting shRNA vector. After 48 hours cells were harvested and a ChIP was performed using the indicated antibodies and primers and loaded onto a 2% agarose gel. (b) The agarose gel was quantified with ImageQuant with respect to the input.

As the level of Polymerase II at exon 1 of the p21 gene is reduced upon an EAPP knockdown the question was now how EAPP can regulate the promoter.

4.3.4 At least two Sp1 binding sites are necessary for the EAPP dependent p21 regulation

To get an idea of the regions involved in this process we used different truncated and mutated versions of the p21 promoter for reporter gene assays (Fig 31). The core promoter includes six Sp1/3 binding sites. Upstream of them are two p53 binding sites responsible for the p53 dependent upregulation of p21. The 5'-site (-2301) is bound more strongly by p53 than the 3'-site (-1394). Additional to the truncated versions of the p21 promoter (p21-BstX, p21-101) we used also promoters with mutations in the Sp1 binding sites (p21-101mt3, p21-101mt4, p21-Pstmt3) and a deletion mutant where the region between the p53 response elements and the first four Sp1 binding sites was removed (p21-Pst).

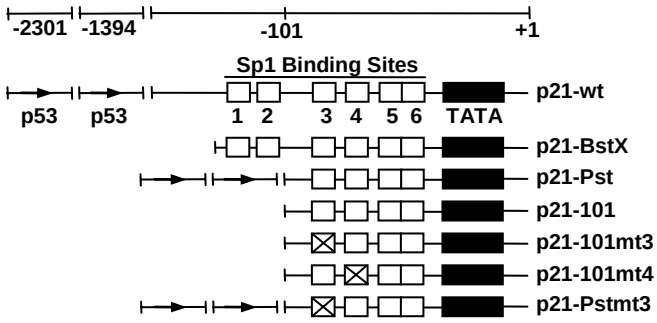


Fig 31: Diagram of the p21 promoter truncations. Used abbreviations are indicated on the right side. Crosses depict a mutation in the Sp1 binding site impeding the interaction.

The reporter gene assays were carried out again in U2OS cells, which were transfected with either a scrambled or an EAPP targeting shRNA together with the different p21 promoter constructs and a β-Gal vector for the transfection efficiency (Fig 32).

The reporter gene assays clearly pointed out that the proximal promoter including four of the six Sp1/3 binding sites can be downregulated by an EAPP knockdown. The importance of the Sp1/3 sites are indicated by the promoter truncations harboring mutated binding sites. When either the binding site number 3 or 4 are mutated the EAPP-dependent downregulation was abolished. They seem to be essential for this regulation. Even the p21-Pstmt3, including the two p53 binding elements, did not respond to the EAPP knockdown when the Sp1 site was inactive. The presence of the p53 response elements did not alter the influence of EAPP. The impact of the Sp1 binding sites 5 and 6 could not be checked as the promoter constructs harboring

mutations in these two sites had such a low activity that it was difficult to distinguish it from the background making any predictions impossible (data not shown).

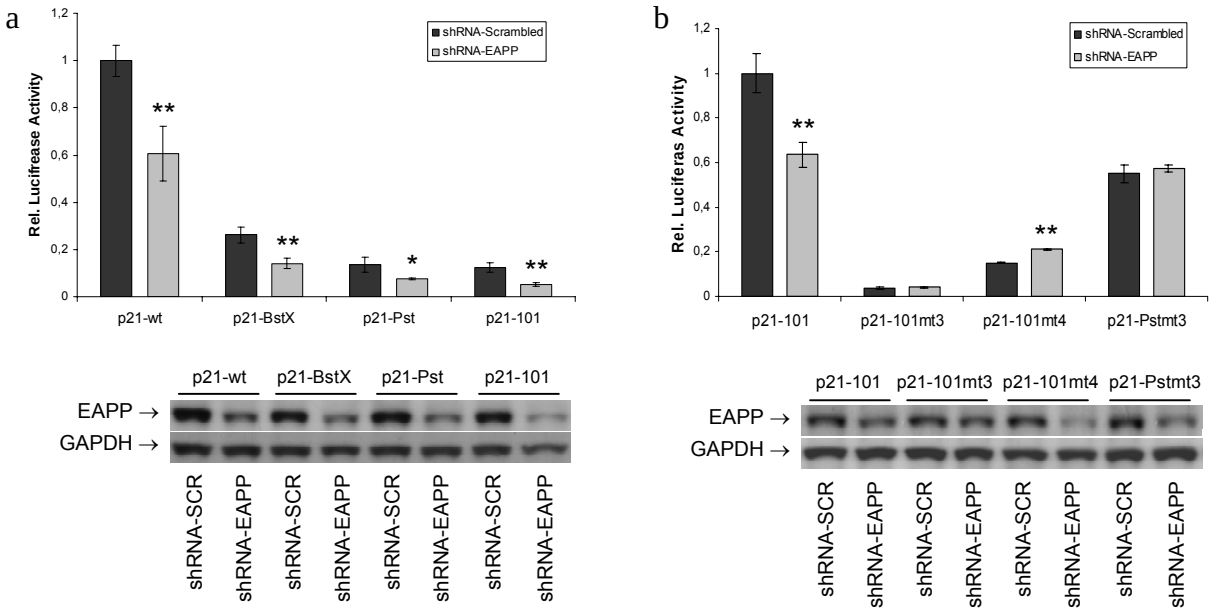


Fig 32: EAPP needs at least the Sp1 binding sites number 3 to 4 to mediate its p21 regulatory properties. (a,b) U2OS cells were transfected with either a scrambled or an EAPP mRNA targeting shRNA together with the indicated p21 promoter constructs and a β-Gal vector for the transfection efficieny. After 48 hours cells were harvested and prepared for the luciferase reporter assay and Western blot analysis for the loading control. As shorter promoters, especially without the p53 response elements, result in lower luciferase activity we split the blots using p21-101 as a reference, to make the differences for the less active constructs visible. The assays were done in triplicate and data present the mean ± sd. * P<0,05; ** P<0,01

4.3.5 H3 co-immunoprecipitation with EAPP

In a previous experiment we already got hints that EAPP might be associated with the chromatin. In a co-immunoprecipitation with the EAPP antibody histone 3 could be detected in the corresponding Western blot (Fig 33).

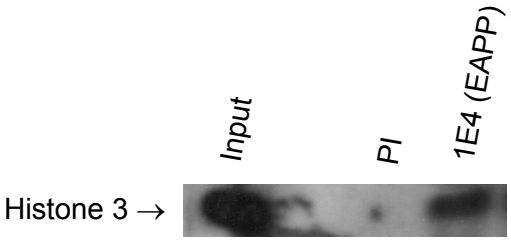


Fig 33: EAPP interacts with histone 3 in vivo. U2OS whole cell extracts were made and 400µg extracts were incubated for four hours either with an EAPP (1E4) antibody or a preimmuneserum of the EAPP antibody as control. After protein A incubation and washing a Western analysis was done using 20µg of the input. The blot was probed with a histone 3 antibody.

4.3.6 ChIP experiments reveal that EAPP binds around the TATA box

To figure out if EAPP binds to the p21 promoter we performed Chromatin IPs with a set of primers within the p21 promoter to target the site of EAPP association (Fig 34).

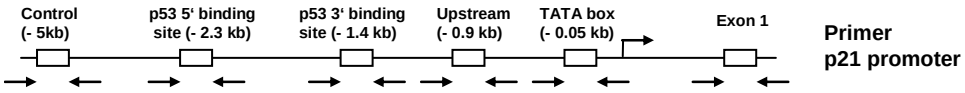


Fig 34: Map of the p21 promoter. The arrows indicate forward and reverse primer of the chosen region to be amplified for the ChIPs.

U2OS cells were transfected with a scrambled and an EAPP mRNA targeting shRNA and 48 hours after transfection cells were prepared for ChIP. Extracts were incubated with the EAPP antibody and the gained DNA was used for PCR with the above mentioned primer pairs of the p21 promoter (Fig 35). We concentrated our efforts on the knockdown of EAPP as the effect is better visible than with the overexpression.

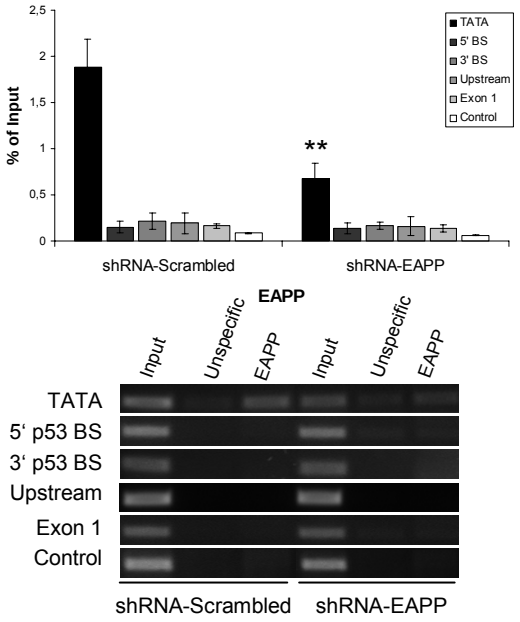


Fig 35: EAPP binds either direct or indirect to the region around the core p21 promoter. U2OS cells were transfected with a scrambled and an EAPP targeting shRNA. After 48 hours cells were harvested and ChIPs were performed with an EAPP antibody. The PCR was done with the indicated primers and the products were loaded onto a 2% agarose gel. As binding control a region far upstream of the promoter was used. The upper panel presents the mean ± sd of three independent experiments quantified with ImageQuant. The lower panel shows a representative experiment. *P<0,05; **P<0,01

The ChIPs supported the luciferase assay as they indicate that EAPP binds mainly either direct or indirect around the TATA box, as the gained DNA pieces after sonication are about 500 to 1000 bp.

4.3.7 An EAPP knockdown reduces several factors at the TATA box, but does not change acetylation and p53 binding

To further examine the effect on the p21 core promoter of an EAPP knockdown we continued ChIPs with different antibodies for proteins involved in the pre-initiation complex formation or with binding sites near the TATA box (Fig 36).

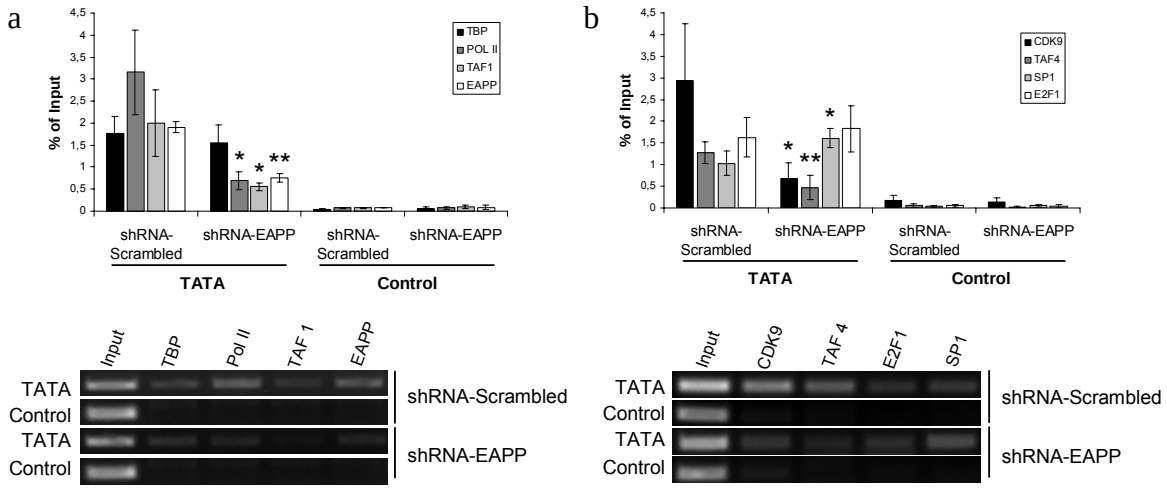


Fig 36: An EAPP knockdown seems to impede the pre-initiation complex formation. (a,b) U2OS cells were transfected with a scrambled and an EAPP targeting shRNA. After 48 hours cells were harvested and ChIPs were performed with the indicated antibodies. The PCR was done with the TATA and the control primer pair and the products were loaded onto a 2% agarose gel. The upper panel presents the mean ± sd of three independent experiments quantified with ImageQuant. The lower panel shows a representative experiment. *P<0,05; **P<0,01

Interestingly the reduction of EAPP significantly reduces the levels of proteins involved in the formation of the pre-initiation complex, like the RNA Polymerase II, TAF1 and TAF4, members of the TFIID complex, or Cdk9 involved in the elongation in association with pTEFb (Thomas and Chiang, 2006). Not affected is the TATA box binding Protein (TBP), or E2F1, which has several binding sites in the proximal p21 promoter and is also known to regulate p21 (Radhakrishnan *et al.*, 2004). In contrast Sp1 binding seems to rise a little bit upon EAPP knockdown.

As p53 is a prominent regulator of p21 it was worth to examine if there is a change in p53 promoter association upon EAPP protein decrease. According to the luciferase assays p53 should stay unperturbed. Again ChIP assays were carried out with a p53 antibody and a Polymerase II antibody for comparison (Fig 37). The ChIP results indicate no influence of EAPP for the promoter binding of p53 in contrast to the Polymerase II association. Interestingly p53 could be found at the TATA region but this interaction was reduced after an EAPP knockdown. In contrast Polymerase II

levels appear also in the 3'BS region and decreased upon EAPP reduction. There are reports that the whole promoter makes a loop as the p53 associated with the 3' response element can interact with the TATA box associated TAFs (Li *et al.*, 2007). With such a loop it would be possible to detect p53 and Polymerase II at other regions where they normally do not bind, as they will be pulled down because everything is cross linked.

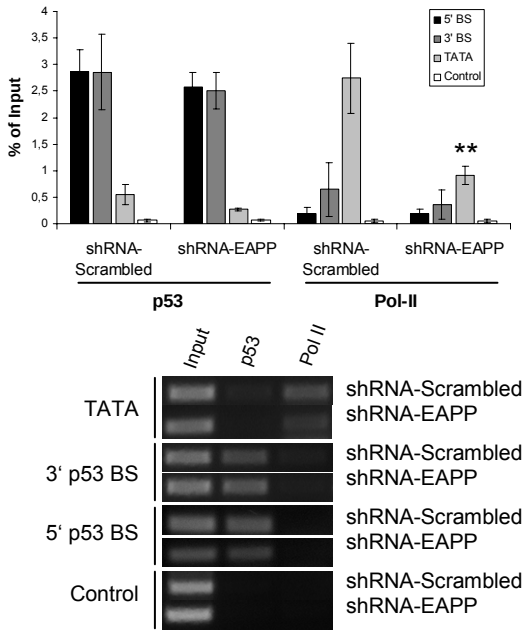


Fig 37: An EAPP knockdown does not alter the p53 association to the p21 promoter. U2OS cells were transfected with a scrambled and an EAPP targeting shRNA. After 48 hours cells were harvested and ChIPs were performed with the p53 and the Polymerase II antibodies. The PCR was done using the indicated primers and the products were loaded onto a 2% agarose gel. The upper panel presents the mean ± sd of three independent experiments quantified with ImageQuant. The lower panel shows a representative experiment. *P<0,05; **P<0,01

As DNA acetylation and phosphorylation are crucial steps in the regulation of genes we tested several modification marks using Chromatin IPs. The same procedure as before was carried out. We used antibodies against acetylated histone 3 and histone 4, against an acetylated K9 of histone 3 and a phosphorylated S10 of histone 3. An antibody targeting the c-terminus of histone 3 was used as a control to determine the amount of histones. We used a primer pair between the TATA box and the 3' p53 response element (Fig 38).

Neither acetylation nor the S10 phosphorylation of histone 3 showed any alteration upon an EAPP knockdown. The luciferase and ChIP experiments favor a regulation model where EAPP binds either direct or indirect to the Sp1 sites in the proximal promoter and alters the formation of the pre-initiation complex, resulting in more

expression when EAPP levels are increased and a reduction of p21 mRNA after a decrease of EAPP.

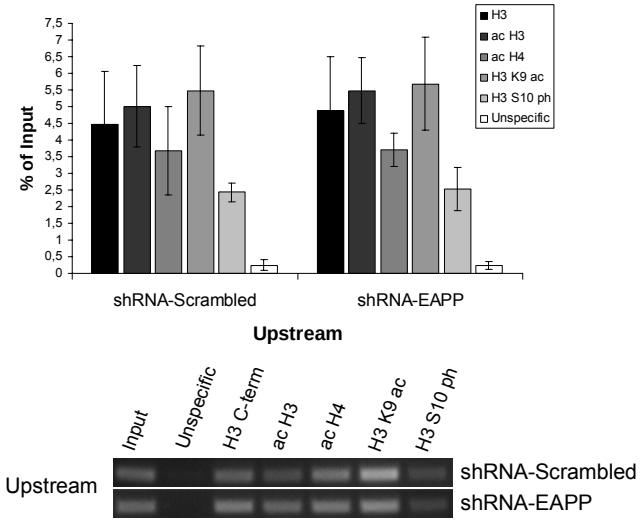


Fig 38: An EAPP knockdown does not alter the tested acetylation and phosphorylation of histone 3 and 4. U2OS cells were transfected with a scrambled and an EAPP targeting shRNA. After 48 hours cells were harvested and ChIPs were performed with the indicated antibodies. The PCR was done with primers against an upstream region and the products were loaded onto a 2% agarose gel. The upper panel presents the mean ± sd of three independent experiments quantified with ImageQuant. The lower panel shows a representative experiment. *P<0,05; **P<0,01

4.3.8 p53 and TSA do not influence the EAPP dependent regulation of p21

We additionally checked if p53 and/or acetylation are involved in the EAPP dependent p21 regulation. U2OS cells were transfected with a control, a shRNA vector targeting EAPP, and a GFP-EAPP expression vector. After 32 hours cells were either treated with etoposide, TSA, or not treated for 16 hours followed by whole cell extract preparation. Etoposide triggers DNA damage and TSA is a well established HDAC inhibitor. The corresponding Western blot was stained with the p21, EAPP, p53, and β-Actin antibody (Fig 39a). In T98G cells, which bear a mutated p53, EAPP was also knocked down and overexpressed and the cells were checked for p21 expression (Fig 39b).

The U2OS cells showed in all treatments a massive reduction of p21 levels upon an EAPP knockdown. For the overexpression of EAPP an effect on p21 was only detected in the untreated cells but not with etoposide and TSA. An explanation could be the extensive posttranscriptional regulation of p21 which would be independent of

EAPP. We later measured p21 mRNA levels where we got an upregulation (Fig 45). In contrast to p21 the amounts of p53 do not really change upon alterations in the levels of EAPP (Fig 39). Even when p53 is stimulated after etoposide treatment p21 is decreased with lowered EAPP. This indicates that EAPP acts downstream of p53 in the regulation of p21. The same is true when we checked T98G. The levels of EAPP correlate with the amounts of p21. TSA normally leads to an upregulation of p21 due to the inhibition of HDAC1 causing a promoter acetylation. Again the EAPP dependent downregulation of p21 is not impeded in the presence of TSA suggesting a mechanism more downstream in the transcriptional activation.

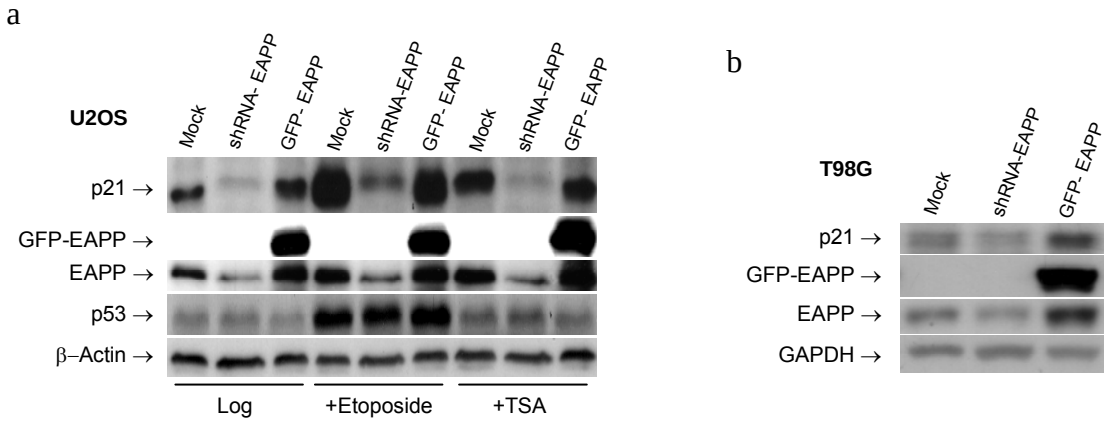


Fig 39: p53 and TSA do not influence the EAPP dependent regulation of p21. (a) U2OS cells were transfected with a control, a shRNA targeting EAPP, and a GFP-EAPP expression vector. After 32 hours cells were either treated with etoposide, TSA, or not treated for 16 hours followed by whole cell extract preparation and Western blot analysis using the indicated antibodies. (b) Same procedure was done as in (a) with T98G cells, except the treatment with etoposide and TSA.

As we had already hints that EAPP is upregulated upon DNA damage we further tried to find out more about the interplay between EAPP and p21 after treatment with damage inducing drugs.

4.4 Role of EAPP in DNA damage

A severe concern in cells is the appearance of damaged DNA. Several external impacts, like ionizing radiation, UV, or damage inducing substances like etoposide or MMS, can trigger harmful alterations of the DNA. The cell has different possibilities to react to these defects. If the injury is not to severe they go into a cell cycle arrest and try to repair it. Apart from that the induction of apoptosis or senescence are ways to prevent the cell getting dangerous for its environment due to an uncontrolled behavior. The key regulators in this process are well known proteins mutated in cancer cells like ATM, p53, or p21, which mediate the outcome of the cell damage. As severe DNA damage causes apoptosis drugs inducing this kind of defects are also prominent candidates for anti-cancer treatments.

In a Western analysis of U2OS cells treated with etoposide and harvested at several time points we observed not only the expected upregulation of p53, also EAPP levels increase (Fig 40).

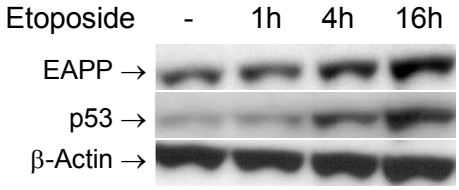


Fig 40: EAPP levels increase upon treatment with the DNA damage inducing drug etoposide. U2OS cells were treated with 20µM etoposide and harvested at the indicated time points. Western analysis was carried out and blots were probed with the EAPP, p53, and β-Actin antibody. Non treated cells served as control.

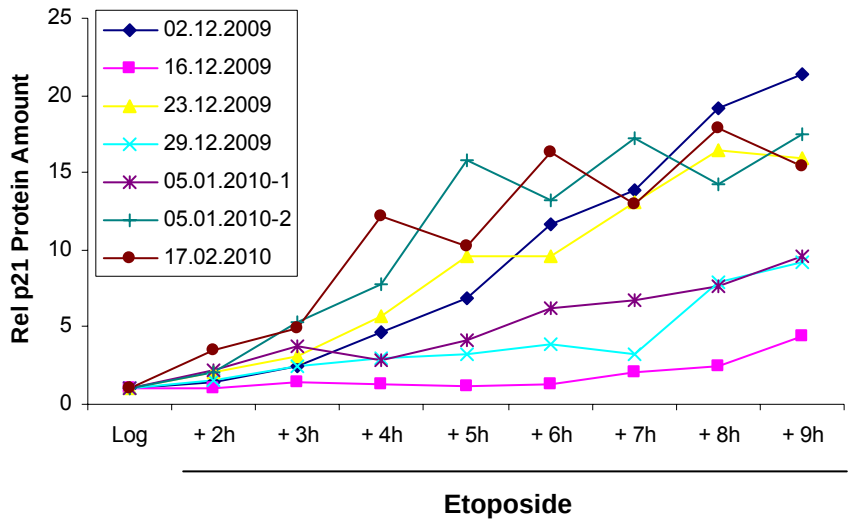
4.4.1 EAPP and p21 increase in parallel upon DNA damage

Not every DNA damage-triggering treatment induces p21, but with etoposide, causing DNA double strand breaks, a clear p21 upregulation could be observed. Interestingly if we applied several time the same etoposide concentration the pattern of the p21 increase was different. Possibly slight variations of the number of seeded cells might contribute to this. Both p21 and EAPP for example are quite sensitive to cell density.

We treated U2OS cells with etoposide and harvested the cells every hour for Western analysis. The blots were stained with the p21, the EAPP, and the β-Actin antibody as a loading control and quantified with ImageQuant using β-Actin as a reference.

Interestingly we got differences in the p21 induction even the etoposide concentration was the same. To further examine this we used two different start populations. One was not split for four days before seeding out (Fig 41a; 05.01.2010-2) and the other population was split every day (Fig 41a; 05.01.2010-1). With the different starting conditions we again got a different induction pattern. When the cells were not split for four days, resulting in a higher cell density, the p21 induction was much faster than in the cells split every day. Possibly when cells grow to confluency various factors are already near a threshold to induce p21 and this might facilitate a faster induction upon etoposide treatment. All results were put together in a diagram (Fig 41a). Two trends can be seen, either a slower increase of p21 or a faster one. We than put the curves of the faster increase together and the curves with the slower increase of p21 (Fig 41b).

a



b

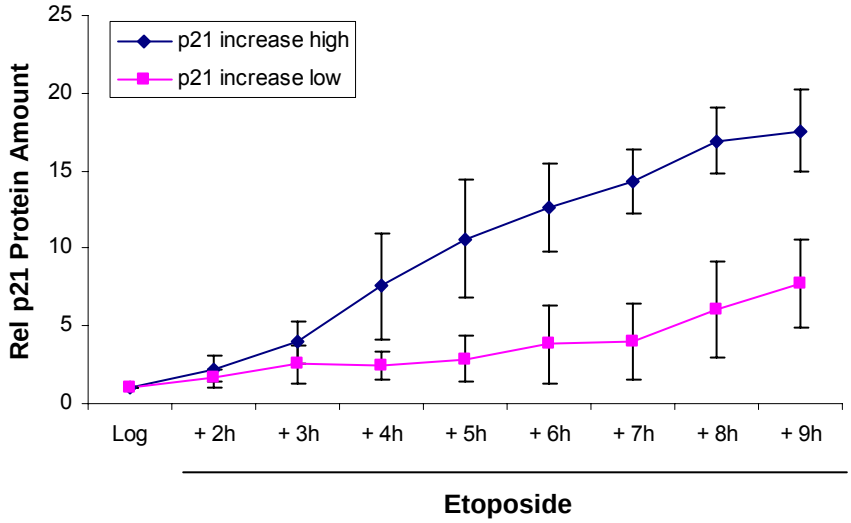


Fig 41: p21 induction upon etoposide treatment varies when initial cell population had slightly different properties. (a) U2OS cells were treated with etoposide, harvested after indicated time points and prepared for Western analysis. The blot was probed with a p21 and a β-Actin antibody as control. ImageQuant was used to quantify the results using β-Actin as a reference. (b) Curves of (a) with a faster and a slower p21 induction were put together and shown as mean ± sd.

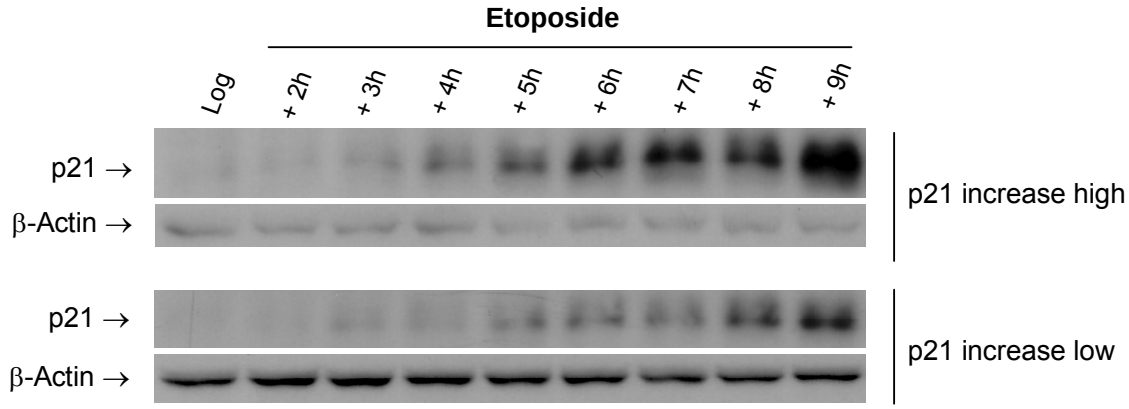
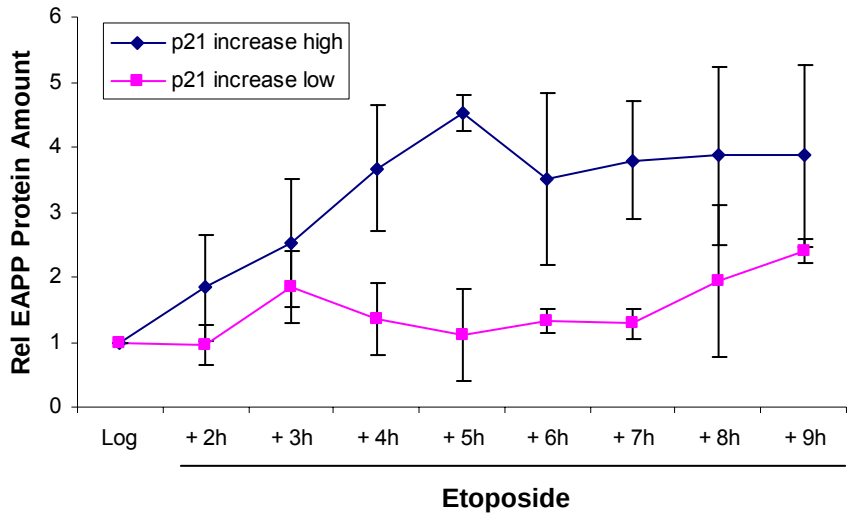


Fig 42: Representative Western blots for figure 41.

When we checked the EAPP increase we got a similar picture with different patterns of upregulation upon etoposide treatment. The interesting question was now is there a correlation between the p21 and the EAPP induction. To examine this we took the extracts, made Western analysis with an EAPP antibody, quantified the results with ImageQuant and examined the curves corresponding to high p21 increase and low p21 increase. We further included the p21 mRNA we had for some experiments (Fig 43).

The rough increase patterns of EAPP and p21 protein and mRNA upon DNA damage induction fit quite well, suggesting that EAPP could somehow be involved in the DNA damage checkpoint. It seemed worth to study if EAPP is necessary for the upregulation of p21 in the case of stress stimuli and which further part could EAPP play in the orchestration of the checkpoint response.

a



b

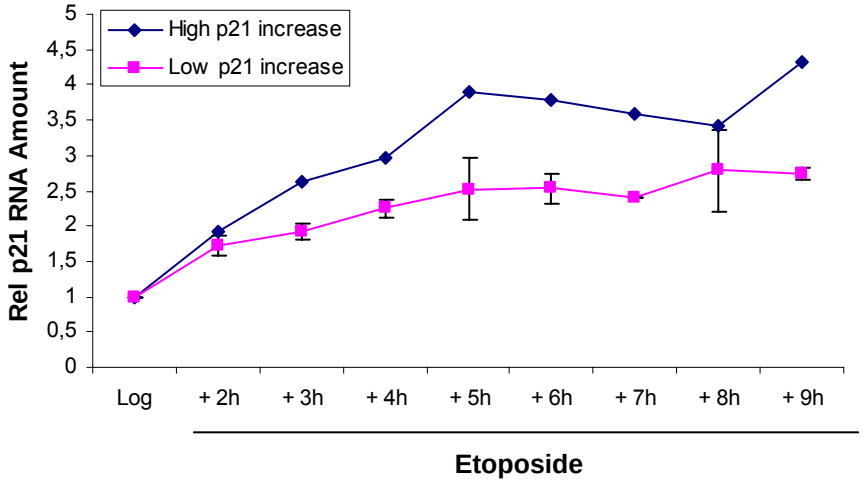


Fig 43: When the p21 induction upon etoposide treatment is high also the EAPP levels raise higher and vice versa. (a) Extracts of figure 41 were taken for Western analysis and probed with an EAPP and a β-Actin antibody as control. ImageQuant was used to quantify the results using β-Actin as a reference. Curves with a faster and a slower p21 induction in figure 41 were put together and shown as mean ± sd. (b) For one experiment with high p21 increase and two with low p21 increase of figure 41 mRNA was prepared and RT-PCR was done with p21 and GAPDH primers and loaded onto a 2% agarose gel. Quantification was done with ImageQuant using GAPDH as reference.

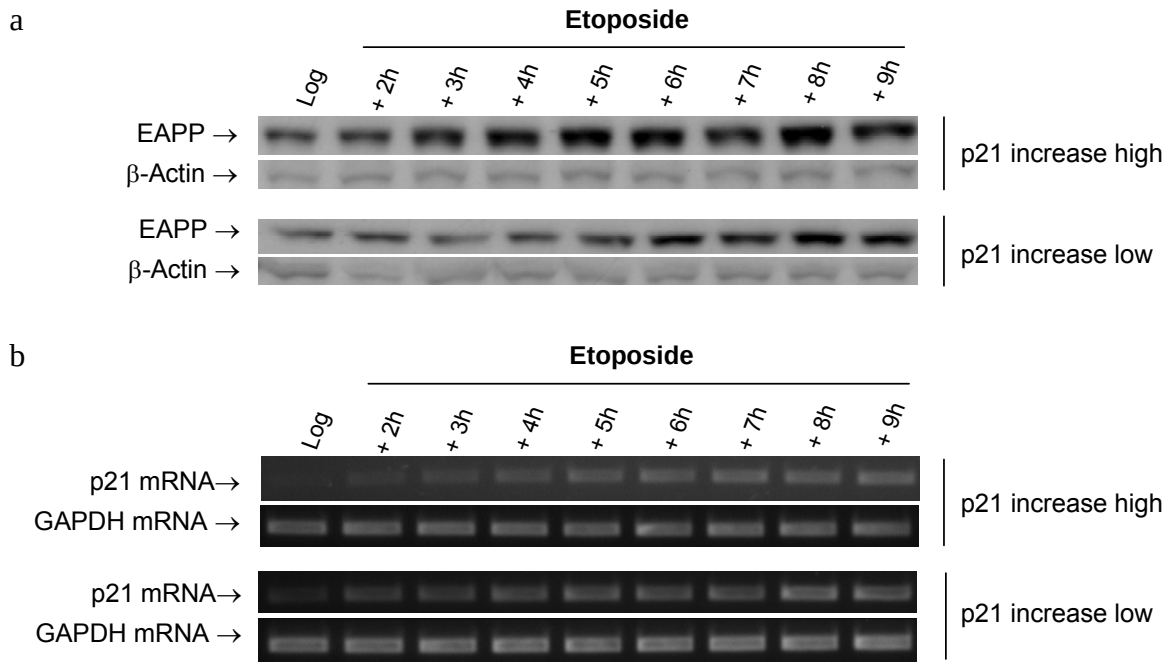


Fig 44: (a) Representative Western blots for figure 43a. (b) Representative RT-PCR for figure 43b.

4.4.2 EAPP is necessary for the p21 upregulation after DNA damage

To clarify the relationship between EAPP and p21 after DNA damage induction we first examined if an EAPP overexpression was able to further increase the levels of already stress-stimulated p21. Before we had done Western analysis and could not see any further change at the protein level (Fig 39). Possibly, because EAPP-induced transcriptional stimulation of p21 might be hidden by stress-induced posttranscriptional regulation of p21 (Soria and Gottifredi, 2010). We used U2OS and T98G cells, again to test if there is a p53 dependency of EAPP effects, treated them either with etoposide or not and prepared mRNA. We carried out RT-PCR with p21 and GAPDH primers, loaded the PCR on a 2% agarose gel and quantified the results with ImageQuant (Fig 45).

In both cases, untreated and etoposide treated, there is a clear upregulation of the p21 mRNA when EAPP was overexpressed. Also in the T98G cells the same pattern was observed. Only the general levels of p21 induction upon DNA damage were lower in T98G due to the mutation of p53.

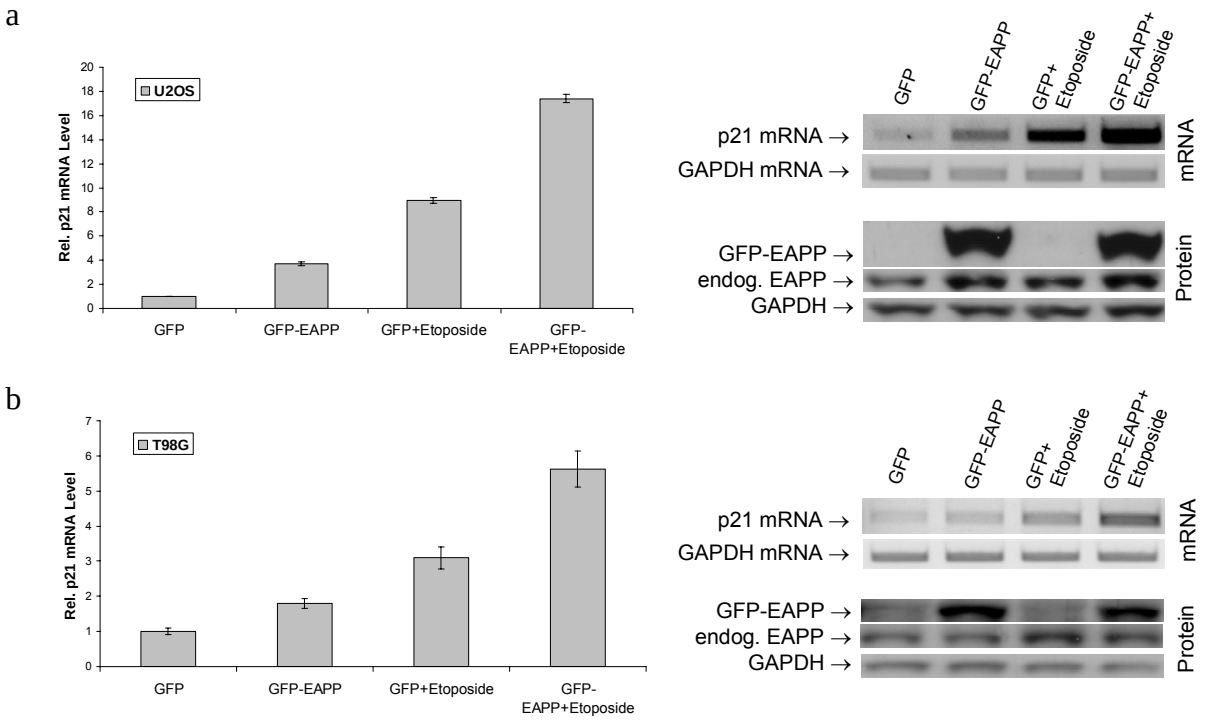


Fig 45: EAPP upregulates p21 mRNA even upon etoposide treatment in U2OS and T98G. (a) U2OS cells were transfected with GFP and GFP-EAPP and after 48 hours mRNA was prepared for RT-PCR. A p21 specific and as control a GAPDH specific primer pair was used. The PCR was loaded onto a 2% agarose gel, which was quantified with ImageQuant. The left diagram shows the mean ± sd of three PCRs. On the upper right side is one representative agarose gel and on the lower right side the corresponding Western blot as a transfection control. (b) Same procedure was done as in (a) with T98G instead of U2OS.

To confirm that EAPP is really necessary for the DNA damage triggered upregulation of p21 we knocked down EAPP in U2OS cells and treated them with etoposide, followed by harvesting them at several time points. We checked the EAPP protein levels and the p21 mRNA levels to detect a possible causal relationship (Fig 46).

We found a convincing similarity between the amount of EAPP and p21 mRNA. When EAPP is knocked down it is still upregulated in etoposide treated cells, but the overall levels are lower than in the control cells. We had the same picture for the p21 mRNA. It is upregulated upon DNA damage, but again the overall levels are lowered when EAPP is reduced, indicating a partial dependency on EAPP for its induction.

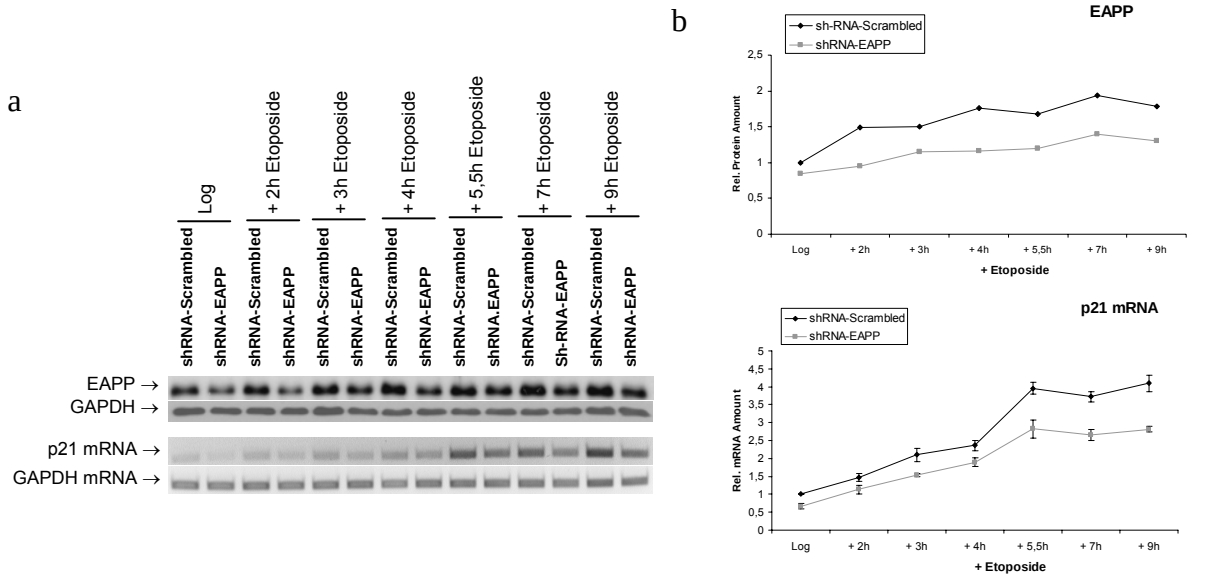
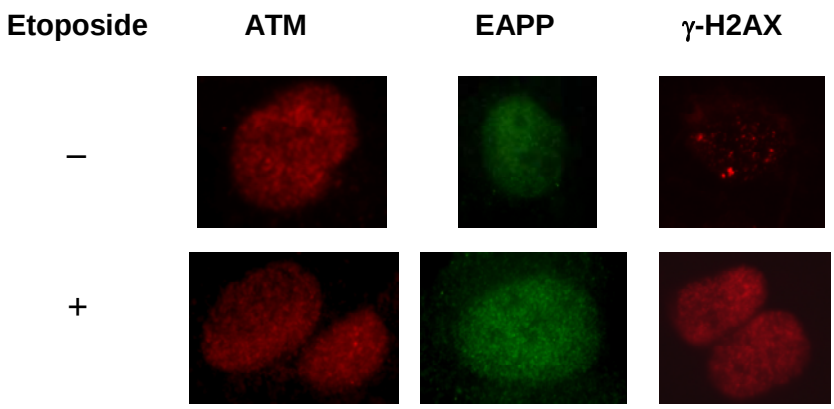


Fig 46: EAPP is necessary for the DNA damage dependent upregulation of p21. (a) U2OS cells were transfected with either a scrambled or an EAPP mRNA targeting shRNA. After 48 hours cells were treated with etoposide and harvested at indicated time points. Whole cell extracts and mRNA were prepared for Western analysis and RT-PCR respectively. For the Western analysis the blot was probed with an EAPP and GAPDH antibody. The RT-PCR was done with p21 mRNA and GAPDH mRNA primers. (b) Results from (a) were quantified with ImageQuant. The mRNA diagram is the mean ± sd of three PCRs.

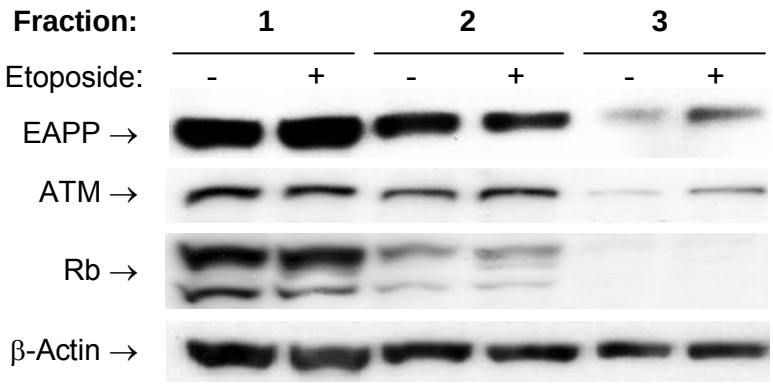
4.4.3 Levels of EAPP bound to chromatin increase after DNA damage

In former experiments we wanted to know if levels of EAPP bound to chromatin are altered when DNA damage is induced. This could be due to its recruitment to the damaged sites, as it happens with ATM for example which gets activated at these sites to fulfill its checkpoint mediating duties. In immunofluorescence experiments a difference between etoposide treated or untreated cells is difficult to see as EAPP and ATM are spread over the whole nucleus and the picture looks quite similar before and after damage induction. γ-H2AX in contrast, which is incorporated at broken DNA, can be found mainly at the damaged sites (Fig 47a). To circumvent this problem we used untreated or etoposide treated U2OS and prepared three fractions of extracts with increasing detergent amounts (NP40) and time. The longer cells are treated with NP-40 the more structures, as chromatin or membranes, are solved. If EAPP is bound mainly to chromatin upon DNA damage, like associated to the foci generated at the damaged sites, there should be an increase in the fraction obtained with higher NP40 and/or longer treatment when etoposide was added. As a positive control ATM was taken (Andegeko *et al.*, 2001). pRb was used as a negative control (Fig 47b).

a



b



Fraction: 1 0,2% NP40 for 5min
2 0,2% NP40 for further 5 min
3 0,5% NP40 for 45min

Fig 47: The association of EAPP with chromatin increases upon DNA damage induction. (a) Immunofluorescence was done with U2OS cells treated or not treated with etoposide. Cells were fixed with formaldehyde and stained with the indicated antibodies. Different cell populations were stained with the three antibodies just to compare minus and plus etoposide. (b) U2OS cells were either untreated or treated with etoposide and three fractions were taken using the indicated NP40 concentration and length of treatment. After every fraction cells were centrifuged, supernatant was collected and the pellet was further treated with the next fractionation buffer. The fractions were loaded onto a poly acrylamide gel and the blot was probed with the indicated antibodies.

The fractions 1 and 2 did not show any difference upon etoposide treatment but in fraction three an increase of EAPP and ATM is clearly achieved. In contrast, although the band is weak, the amounts of associated pRb did not change. This suggests that EAPP is either also recruited to the damaged foci as ATM or it is bound to transcriptional targets involved in the DNA damge checkpoint.

4.4.4 EAPP leads to reduced p53 nuclear accumulation before and after DNA damage

Another interesting observation was the fact that EAPP seems to be able to facilitate the nuclear export or can impede the import of p53 in either untreated or etoposide treated U2OS cells (Fig 48).

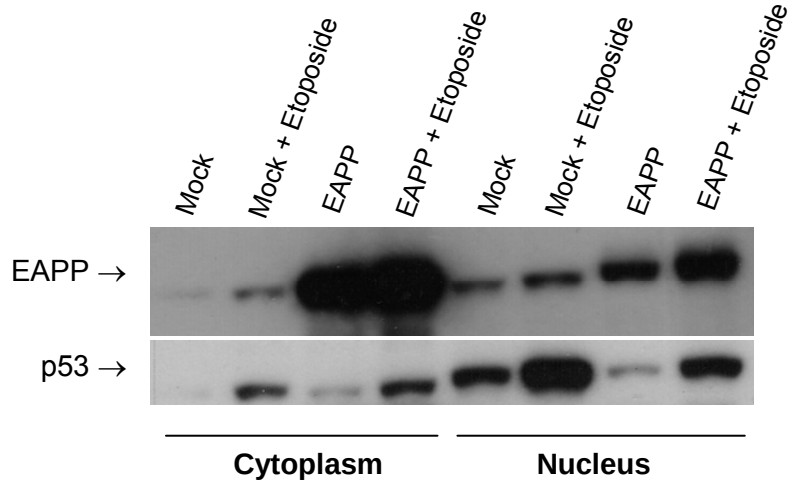


Fig 48: EAPP can alter the nuclear accumulation of p53 in untreated and etoposide treated cells. U2OS cells were transfected with a control or an untagged EAPP expression vector. After 32 hours cells were either not treated or treated with etoposide and fractionated to get cytoplasmic and nuclear extracts for Western blot analysis. An EAPP and p53 antibody was used.

Upon EAPP overexpression an increase of p53 in the cytoplasm and a decrease in the nucleus was obtained. In the case of DNA damage induction the overall levels of EAPP and p53 rose but the pattern was the same. Either EAPP is really involved in the spatial regulation of p53 or, as EAPP binds to p53 (Schwarzmayr, unpublished data), the massive amounts of EAPP in the cytoplasm after transfection just block the free p53 before it can enter the nucleus. But this is unlikely considering the huge number of proteins that migrate into and out of the nucleus.

4.4.5 A knockdown of EAPP prevents Chk2 dephosphorylation

A prominent target in the DNA damage response, especially after double strand breaks, is Chk2. It gets phosphorylated and hence activated by ATM and orchestrates several response pathways by phosphorylating its targets, among them p53. Since

EAPP can interfere with apoptosis (see below), it seemed reasonable to examine if EAPP has any influence on this crucial Chk2 phosphorylation. We took stable U2OS cell lines either overexpressing HA-EAPP or with decreased endogenous EAPP upon shRNA expression targeting the EAPP mRNA. We compared them to normal U2OS and treated them with etoposide. 16 hours after the addition of etoposide the medium was changed to release the cells from the damaging agent to further investigate the checkpoint recovery which is an important step. After the damaged cells are repaired the kinases like ATM and Chk2 have to be inactivated to switch off the checkpoint. We harvested the cells at the indicated timepoints, prepared them for Western analysis and probed the membrane with a phosphospecific Chk2 antibody targeting the crucial ATM-caused phosphorylation on T68 (Fig.49)

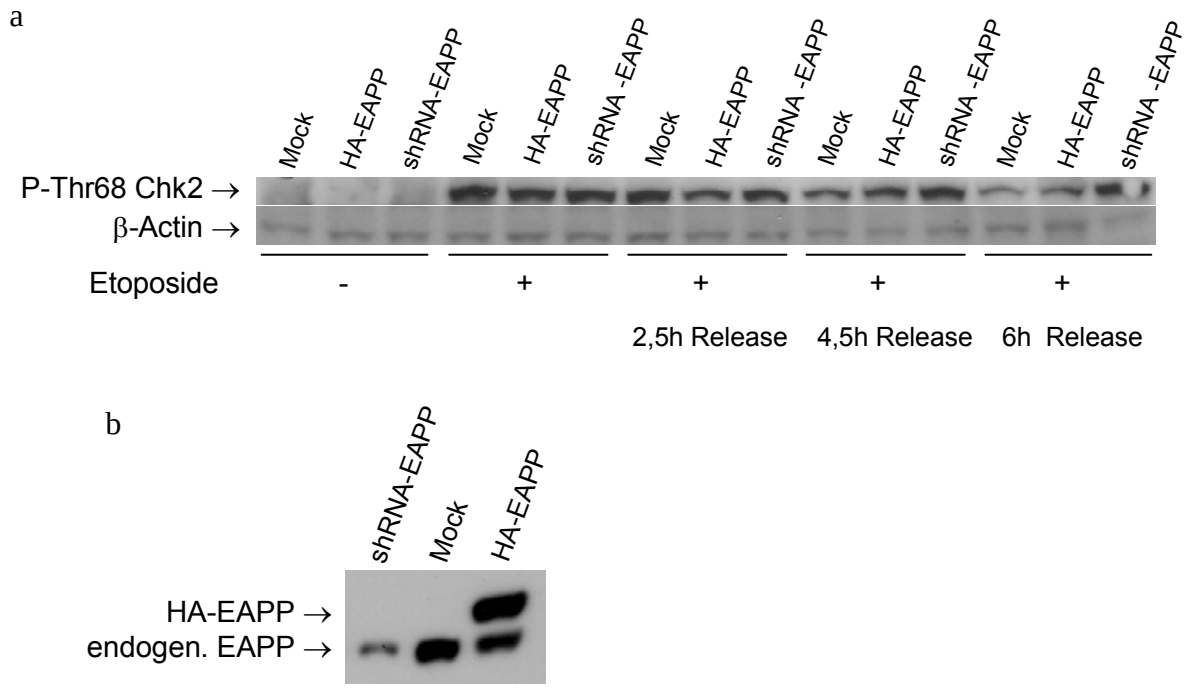


Fig 49: An EAPP knockdown prevents the dephosphorylation of Chk2 during checkpoint recovery. (a) Stable U2OS cell lines expressing HA-EAPP or a shRNA against the EAPP mRNA were taken together with normal U2SO cells and treated with etoposide. After 16 hours the medium was changed to release the cells, which were harvested at the indicated time points. Cells not treated with etoposide served as control. Western blot analysis was done and the blots were stained with a P-Thr68 specific Chk2 and an β-actin antibody. (b) EAPP expression of the stable U2OS cell lines compared to normal U2OS.

Interestingly when EAPP levels were reduced the Chk2 dephosphorylation after a release from etoposide, necessary for checkpoint recovery, was severely hampered. This could be either a direct mechanism through EAPP binding, or an indirect one by regulating phosphatases crucial for this step. We can not rule out either mechanism as we found a direct interaction of EAPP with phosphorylated Chk2 (Andorfer *et al.*,

2011) and also the downregulation of a phosphatase necessary for this step. In general PP1, PP2A, and Wip1 are involved in the dephosphorylation of Chk2 during checkpoint recovery. When we knocked down EAPP in U2OS cells we also found a decrease in Wip1, indicating that this could at least partly be responsible for the impeded Chk2 inactivation (Fig 50).

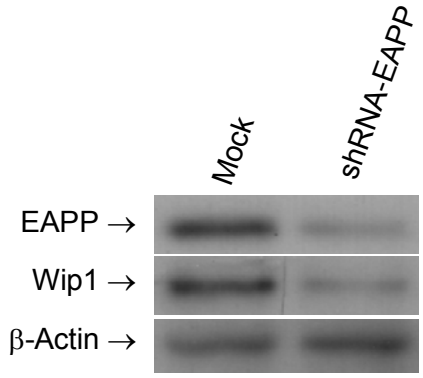


Fig 50: An EAPP knockdown leads to a Wip1 downregulation. U2OS cells were transfected with a scrambled and an EAPP mRNA targeting shRNA. After 48 hours cells were harvested, prepared for Western analyisis and probed with the indicated antibodies.

4.5 EAPP can prevent the induction of apoptosis

EAPP can arrest cells in G1 due to the upregulation of p21. An ongoing debate is the role of p21 in another crucial mechanism, namely the induction of apoptosis. Reports describing opposing activities exist, either p21 triggers apoptosis or prevents it. There seems to be a great dependence on the background which outcome is favored. Most of the studies support the prevention of the programmed cell death. First hints about the role of EAPP in this crucial process came from DNA profiles of EAPP-overexperssing cells after etoposide treatment.

4.5.1 EAPP overexpression reduces apoptosis levels after DNA damage

U2OS cells were transfected with GFP-EAPP, treated for 16 hours with etoposide and prepared for flow cytometry to measure the DNA. We observed an interesting fact when we compared GFP-EAPP positive with GFP-EAPP negative cells (Fig 51). A rough estimation of the apoptotic fraction can be done with the sub-G1 peak. When apoptosis is induced the DNA gets fragmented and apoptotic bodies are formed. This leads to deformed cells with a lower DNA content than normal G1 cells. As etoposide triggers double strand breaks cells induce apoptosis when the damage is too severe and the sub-G1 fraction increases. When EAPP was overexpressed in this etoposide treated cells they showed, additional to the expected decline of the S/G2 fraction at the expanse of G1, a reduction in the sub-G1 compared to cells with normal EAPP levels. This suggests a possible anti-apoptotic activity of EAPP.

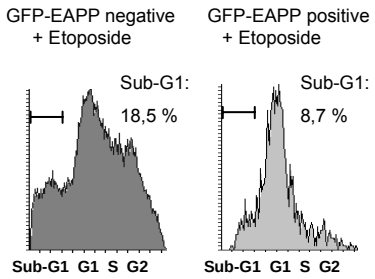


Fig 51: EAPP overexpression reduces the sub-G1 peak when cells were treated with etoposide. U2OS cells were transfected with GFP-EAPP and treated for 16 hours with etoposide. Cells were harvested, stained with Hoechst 33258 and prepared for flow cytometry. GFP positive and negative cells were gated to get the corresponding cell cycle distribution.

To further examine this we used a different way to determine the apoptotic fraction. Before cells fragment their DNA chromosomes condense which can be measured due to a brighter Hoechst 33258 staining. As apoptotic cells shrink they should appear in a dot blot displaying the size (forward scatter) and the Hoechst staining (Dapi channel) as a distinct population. We transfected U2OS cells with either GFP or GFP-EAPP, treated them with etoposide and harvested the cells after several time points (with the medium to collect also the dead cells for the proper rate between living and dead cells) to investigate if there is a change over time. The cells were stained with Hoechst 33258 and flow cytometry was carried out. GFP positive fractions were gated for smaller cells with a brighter Hoechst staining, as an indicator for apoptosis (Fig 52).

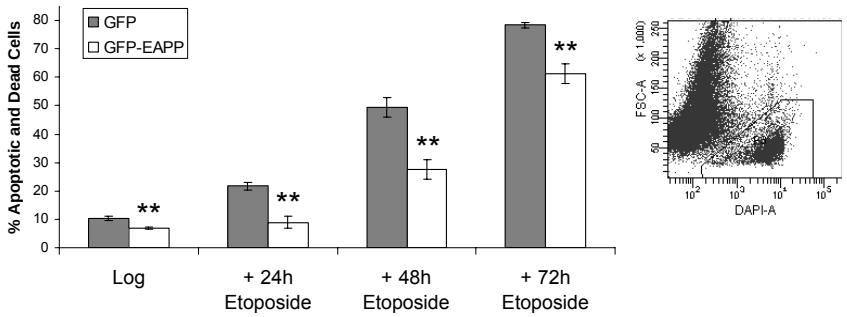


Fig 52: EAPP overexpression reduces apoptosis during an etoposide long term treatment. U2OS cells were transfected with either GFP or GFP-EAPP and treated with etoposide. After indicated time points cells were harvested (including the medium), stained with Hoechst 33258 and prepared for flow cytometry. GFP positive and apoptotic cells were gated and shown as the mean ± sd of three independent experiments. On the right panel a sample dot blot is shown with the gate for the GFP positive cells which are smaller (lower forward scatter) and the fluorescence is brighter in the DAPI channel as chromosomes condense during apoptosis.

The cells expressing more EAPP had reduced levels of apoptosis. The difference between the control and EAPP cells decreased the longer the cells were incubated

with etoposide suggesting that the damage is already so severe that the cells nevertheless switch on apoptosis.

But EAPP was not only able to reduce etoposide triggered cell death. E2F1 is a key regulator of the cell cycle but also induces apoptosis when overexpressed via the upregulation of pro-apoptotic proteins like p73. We transfected U2OS cells with either GFP, GFP-E2F1 plus HA-DP1, or GFP-E2F1 plus HA-DP1 plus GFP-EAPP. The cells were incubated with Draq5 for DNA staining and prepared for flow cytometry. The cell cycle distribution of the GFP positive cells was displayed and the transfection was controlled with a Western blot using the corresponding antibodies (Fig 53).

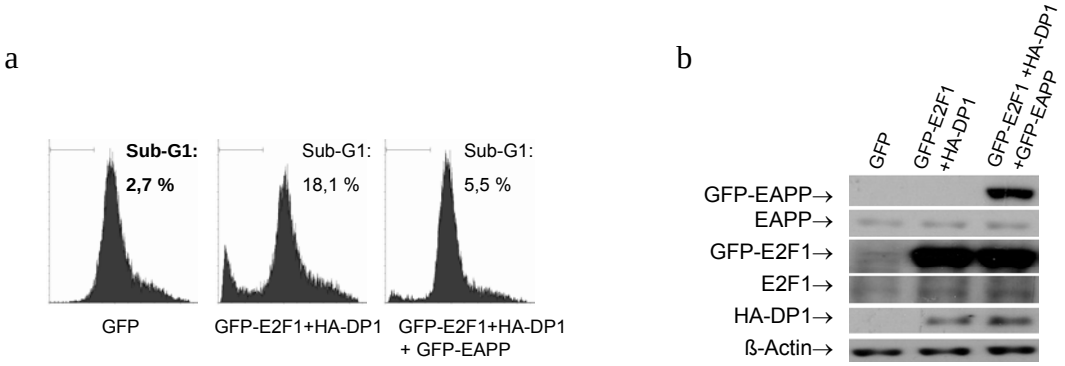


Fig 53: EAPP can reduce the sub-G1 peak triggered by E2F1 and DP1 overexpression. U2OS cells were transfected with GFP, GFP-E2F1 and HA-DP1, or GFP-E2F1, HA-DP1, and GFP-EAPP. After 48 hours cells were harvested, incubated with Draq5 for DNA staining and prepared for flow cytometry. (a) The GFP positive cells were gated to display their DNA distribution and the sub-G1 was calculated. (b) Western analysis was done and probed with the indicated antibodies as a transfection control.

As expected E2F1 was able to induce apoptosis when ectopically expressed, resulting in a sub-G1 increase. When EAPP was additionally overexpressed this sub-G1 fraction nearly disappeared, suggesting either a general anti-apoptotic mechanism conducted by EAPP or the hindrance of an E2F1 dependent activation.

4.5.2 A knockdown of p21 reduces the anti-apoptotic effect of EAPP

An overexpression of EAPP can protect cells from going into apoptosis (Fig 52). The question was now what happens when we simultaneously knock down p21. Can we reverse the effect or will it be unchanged? U2OS cells were transfected with GFP, or GFP-EAPP, and additionally with either a scrambled or a p21 mRNA targeting shRNA. Etoposide was applied and cells were harvested before and after 24, 48, and 72 hours of treatment and prepared for flow cytometry and RT-PCR. The levels of apoptosis

were, like in figure 52, measured by staining the cells with Hoechst 33258 and gating for GFP positive cells which are smaller in size and brighter in Hoechst staining. The RT-PCR was done to check the mRNA levels of p21 (Fig 54).

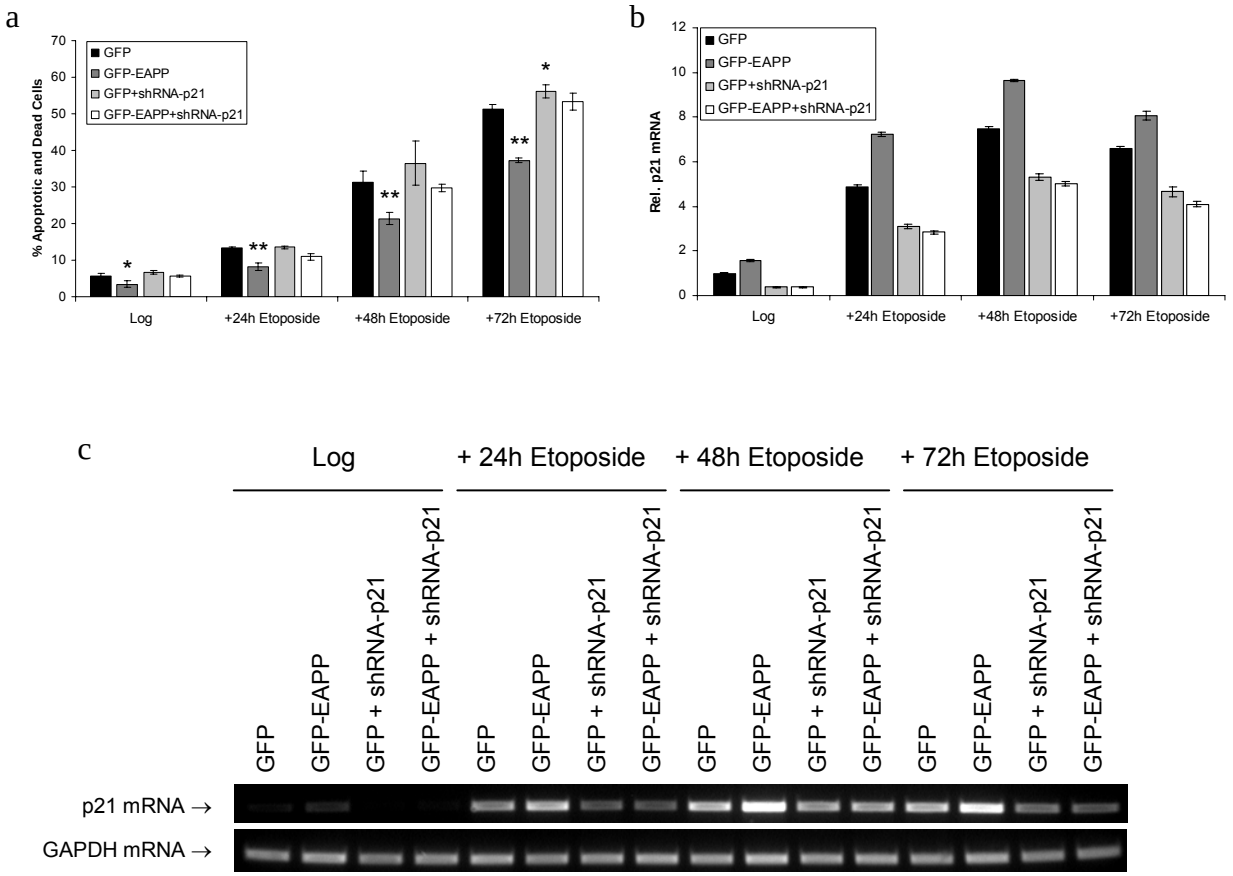


Fig 54: A p21 knockdown can abolish the EAPP triggered protective effect upon etoposide treatment. U2OS cells were transfected with GFP or GFP-EAPP in addition to either a scrambled or a p21 targeting shRNA. The cells were treated with etoposide and harvested at the indicated time points. (a) After Hoechst 33258 staining GFP positive cells with a smaller size and a brighter Hoechst staining were gated as a marker of apoptosis. The mean ± sd of three independent experiments is shown. (b) RT-PCR was done to determine the p21 mRNA levels. The mean ± sd of three PCRs is shown. (c) Representative agarose gel for the RT-PCR.

As expected overexpression of EAPP results in a reduced level of apoptosis upon etoposide treatment. When we knocked down p21 at the same time we could almost eliminate the anti-apoptotic effect of EAPP. This indicates that the protective properties of EAPP are at least partly mediated by p21. It correlates with the reported anti-apoptotic characteristics of p21.

4.5.3 A knockdown of EAPP induces apoptosis independent of p53

To test if vice versa a knockdown of EAPP was able to trigger apoptosis we took U2OS and T98G, knocked down EAPP and applied several methods to detect apoptosis. Additional to the sub-G1 we carried out Western analysis to check the levels of cleaved caspase 3, which is cut upon apoptosis induction. We also used a commercial apoptosis kit, which utilizes that during apoptosis the cytoplasmic membrane becomes slightly permeable. The green fluorescent dye Yo-Pro-1 can enter the cells in this stage whereas the red fluorescent dye propidium iodide (PI) is able to enter only already dead or at least late apoptotic cells where the membrane is more permeable. Using this two dyes it is now possible to distinguish normal cells (no staining), apoptotic cells (Yo-Pro-1 staining), and late apoptotic/dead cells (Yo-Pro-1 and PI staining). The T98G we again employed to check a p53 negative background (Fig 55).

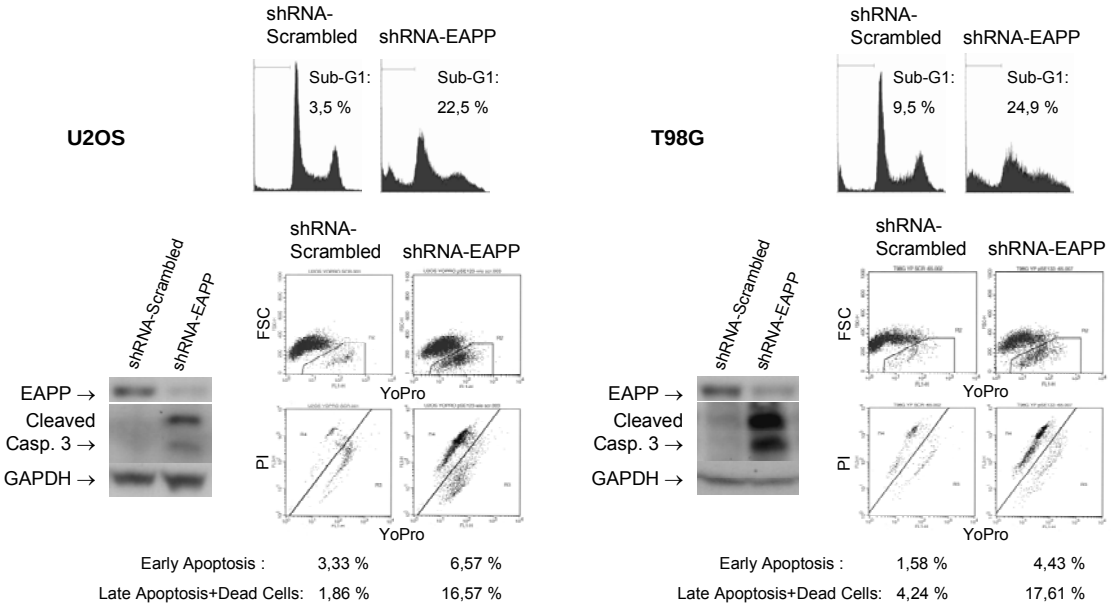


Fig 55: An EAPP knockdown induces apoptosis independently of p53. U2OS and T98G cells were transfected with either a scrambled or an EAPP mRNA targeting shRNA. After 48 hours cells were harvested and prepared for Western analysis and flow cytometry. The Western blot was stained with an EAPP, a cleaved caspase 3, and a GAPDH antibody. For flow cytometry the cells were either permeabilized and stained with PI to get the cell cylce distribution, or stained with Yo-Pro1 and PI to detect early and late apoptosis. First the Yo-Pro-1 signal was plotted against the size (forward scatter) and cells with a smaller size and Yo-Pro-1 positive were gated and splitted in a PI positive and negative fraction.

All different methods to detect apoptosis showed a significant increase upon a knockdown of EAPP. As U2OS and T98G showed the same results, the process seems to be independent of p53.

4.5.4 Reduced EAPP levels lead to a faster apoptosis induction upon long term treatment with moderate levels of etoposide

To induce apoptosis a really good knockdown of EAPP is necessary. If the EAPP levels are only moderately lowered the cells look quite normal. It was now interesting how cells would behave when we try to inhibit the EAPP upregulation upon etoposide treatment. To get a moderate knockdown we transfected U2OS cells with lower levels of the shRNA targeting the EAPP mRNA. We seeded equal amounts of transfected and non transfected cells either in normal growth medium or with the addition of low concentrations of etoposide. The cells were counted every day. We further carried out Western analysis and flow cytometry where we checked the cell cycle distribution and the amount of apoptosis using the Yo-Pro-1/PI kit (Fig 56).

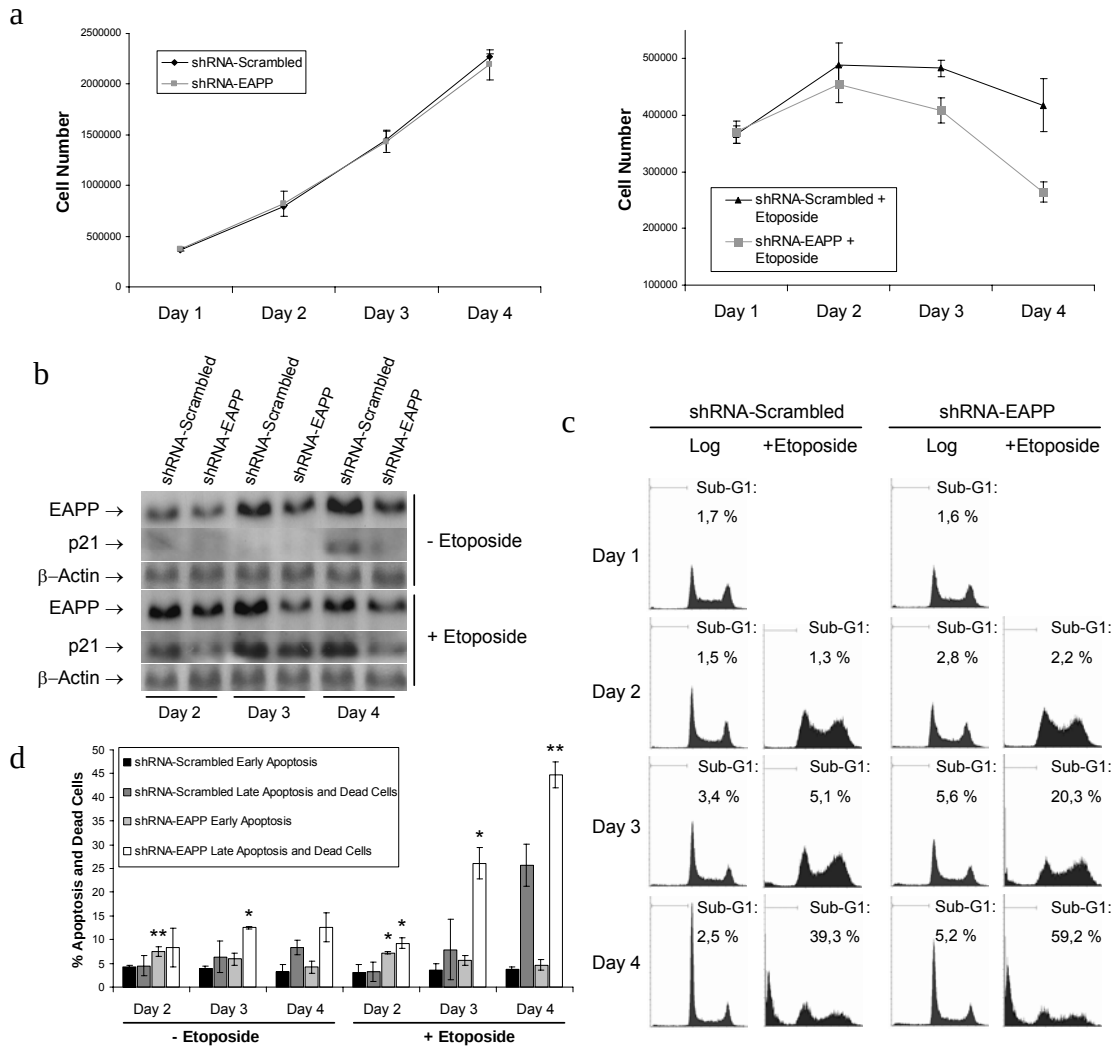


Fig 56: A moderate knockdown of EAPP, with only slight effects in normal growing cells, reduces viability in cells with low levels of etoposide. U2OS cells were transfected with a scrambled and a shRNA targeting the EAPP mRNA to gain a knockdown of about 50%. The cells were either untreated or treated with 5mM etoposide for three days. (a) Cells were counted with the Casy Counter every day and the mean ± sd of three independent experiments is shown. (b) To check the corresponsing protein

levels Western analysis was done with the indicated antibodies. (c) To get the cell cycle distribution cells were permeabilized, stained with PI and analyzed. The sub-G1 fraction was determined. (d) For apoptosis examination cells were stained with Yo-Pro-1/PI and analyzed by flow cytometry with the same gates as in figure 55. The mean ± sd of three independent experiments is shown.

In the untreated cells there was only a slight difference over time between the control and the EAPP knockdown cells. There was an increase in apoptosis when cell confluence rose, indicating a role of EAPP in this kind of stress response when the cells grow too dense. This fits to the observation that EAPP is upregulated when cells get confluent. And also under this condition an EAPP knockdown impedes the p21 upregulation. When the cells were treated with low levels of etoposide the moderate knockdown of EAPP had severe consequences. Apoptosis increased dramatically upon reduction of EAPP in comparison to the control cells. This can be seen in the Yo-Pro-1 data as well as in a sub-G1 increase. Also the numbers of cells decrease in the EAPP knockdown cells. As expected we also found p21 downregulated, raising the question how dependent is the protective function of EAPP on p21.

4.5.5 EAPP is also involved in the regulation of other genes

We found an influence of p21 according to the anti-apoptotic effects when EAPP is overexpressed. The situation for the knockdown of EAPP seems to be a little different. In general the overexpression has less effect on the levels of examined proteins than the knockdown. Possibly EAPP is crucial for the transcription of certain genes, but higher levels do not increase the expression of target genes dramatically as other co-factors are not elevated at the same time. Additionally a two-fold induction for example is often easier to tolerate than a more or less complete loss. After an EAPP knockdown p21 and Wip1 were not the only proteins we found to be downregulated. Other examples are p27 or Cdc25c (Fig 57). Further we found PCNA, Chk1, Mad2, Cyclin B, and Cdk1 downregulated upon an EAPP knockdown (Rotheneder unpublished data). So it seems that the apoptosis triggered by the loss of EAPP is not dependent on one protein, like p21, it is rather the result of the downregulation of a whole bunch of targets. With experiments where we tried to express p21 ectopically together with an EAPP knockdown vector we did not get really conclusive results but it did not seem that p21 was able to compensate the EAPP knockdown effect (data not shown).

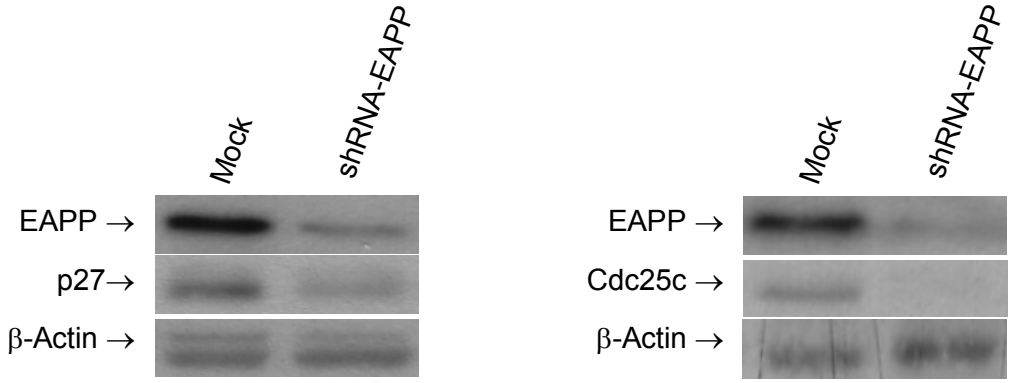


Fig 57: An EAPP knockdown leads to a decrease in p27 and Cdc25c levels. U2OS cells were transfected with either a scrambled or an EAPP mRNA targeting shRNA, harvested, prepared for Western analysis, and probed with the indicated antibodies.

When we overexpressed EAPP it was often difficult to see an upregulation of a target protein all the time. Possibly other factors are necessary which are present in different amounts depending on the cell property and also just on stochastic effects. When we checked the mRNA of a few proteins in the case of an ectopic expression of EAPP with and without etoposide treatment we found a slight upregulation of some independently of the treatment even when they are involved in the response to etoposide (Fig 58). This further suggests that EAPP is involved in the transcription of several genes in a quite general way depending on co-factors for the fine tuning.

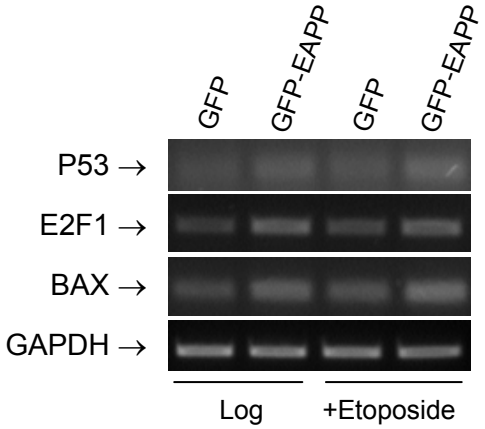


Fig 58: An EAPP overexpression leads to a slight upregulation of p53, E2F1, and Bax. U2OS cells were transfected with either GFP or GFP-EAPP, harvested, prepared for RT-PCR. The PCR was done with the indicated primers. GAPDH was used as control.

4.6 Confocal pictures of GFP-EAPP

To get additional information about the localization of EAPP in the nucleus we carried out confocal microscopy. We transfected U2OS with GFP-EAPP and fixed the cells for confocal microscopy. To get a three dimensional picture we performed Z-stacking making pictures of 16 slices through the cell (Fig 59).

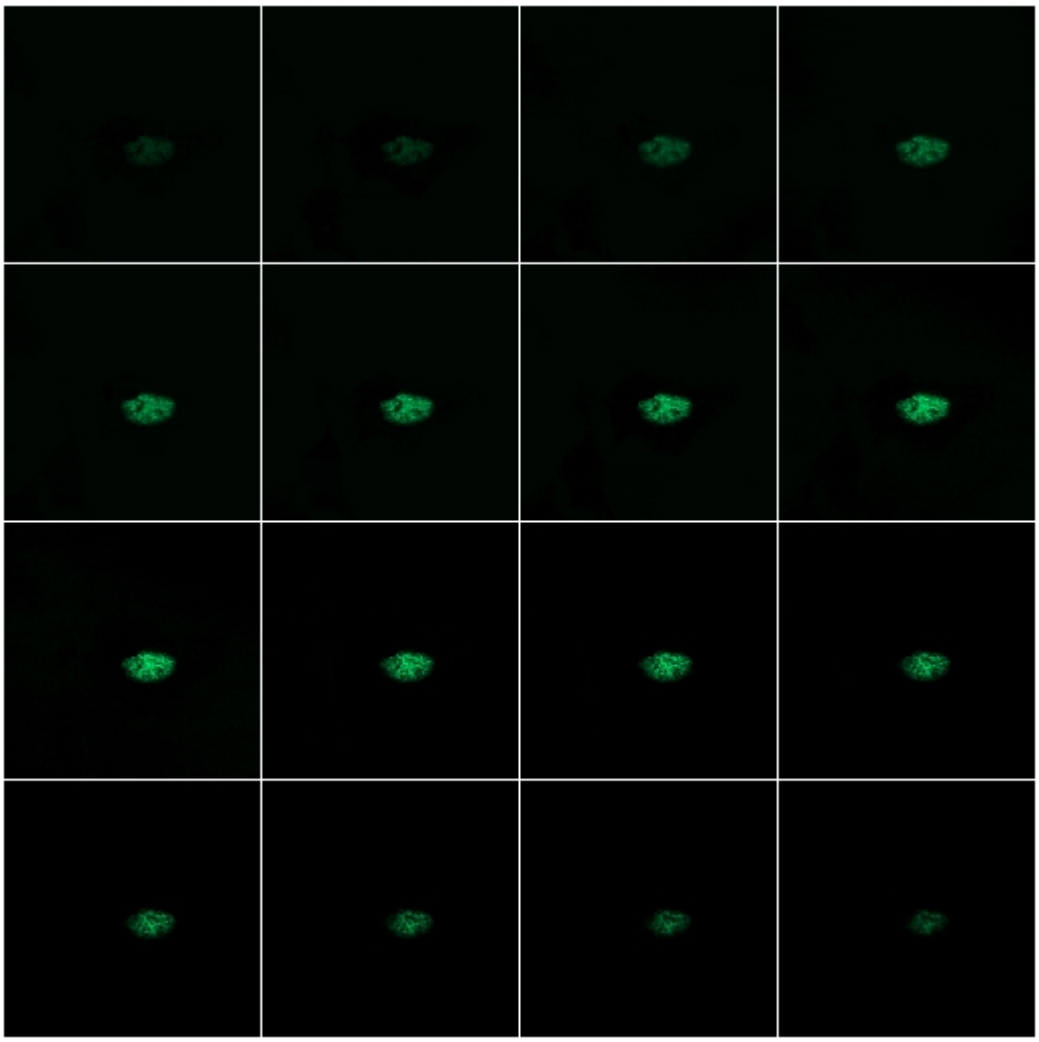


Fig 59: U2OS cells were transfected with GFP-EAPP, fixed and prepared for confocal microscopy. Z-stacking was carried out taking 16 slices through the cell.

It can be nicely seen that EAPP is spread over the whole nucleus except the nucleoli. For further examinations the endogenous EAPP should be stained and analysed.

5. Discussion

A crucial step in the live of cells is to sense the intracellular status and to coordinate it with extracellular signals. It is important that the cells are endowed with various different checkpoints to react on environmental and internal changes. Especially in multicellular organisms defects in one cell can be harmful for the whole organism. Hence it is necessary to provide mechanisms to deal with damages within the cell. Examples are delaying or arresting the cell cycle, giving the cells time to repair, or in the worst case to trigger apoptosis to prevent cells to get out of control. A huge part of research focuses on these checkpoints as a broad variety of diseases have their origin in defects in these sensing mechanisms, limiting the cells capability to switch on proper pathways in response to cell damaging stimuli. The application of different drugs causing damages recognized by certain checkpoints or the use of cell lines lacking proteins able to induce checkpoints or downstream targets of them, are widely used ways to study this response mechanisms. When we began to study the E2F1 Associated Phosphoprotein EAPP a possible involvement in the regulation of such sensing pathways turned out.

5.1 EAPP can trigger an increase in G1

As we had hints from immunofluorescence experiments that EAPP levels decrease during mitosis we tried to arrest cells in this cell cycle stage with the microtubule poison nocodazole. Surprisingly when we transfected cells with an EAPP expression vector, to study if the transcriptional control or degradation is responsible for this disappearance, these cells were not able to fully arrest in mitosis. Instead in the DNA profile a G1 peak appeared, representing roughly the percentage of transfected cells.
In general using drugs like nocodazole to arrest and synchronize cells during the cell cycle and determining this arrest afterwards using the DNA profile should be taken with a pinch of salt. Often it is not taken into account that the starting population is in different cell cycle stages, hence reach the checkpoint where they will stop at different time points. The problem is that the DNA amount and other cell properties like growth are not fully coupled. When cells are arrested with nocodazole they still grow in size, meaning that cells entering the arrest earlier have different cell properties as cells

entering the arrest later, even though they have the same DNA amount. This is also true for all other drugs. Cells which are in the triggered arrest for hours do not stand still and hence are different than cells entering the arrest right before harvesting the cells. All results gained after such a drug treatment triggered arrest should be regarded with suspicion. A method to get really synchronized cells is elutration where cells are separated due to their size (Cooper, 2003; Cooper *et al.*, 2006).

We additionally used other agents to investigate a possible restriction to nocodazole. But the same increase of the G1 fraction was also present with drugs arresting cells in S-phase upon DNA damage induction, or other tubuline poisons (Fig 7). The sub-G1 peak, an indicator of apoptosis due to DNA fragmentation, and the fraction of aneuploid cells, which possess abnormal numbers of chromosomes, did not increase. This suggests that EAPP is not able to overcome the arrest (Fig 9). It would be even implausible as the different drugs utilize different mechanisms at different cell cycle stages. The effect was also p53 independent as we got the same results with p53 negative or mutated cells (Fig 8).

In principle cells have three different mechanisms to deal with harmful drugs. Either they reduce the uptake, or they change the ability of the drug to act, or they pump the agents actively out of the cell. At least parts of the drugs we used freely diffuse into the cell, therefore the first mechanism can be ruled out. The applied drugs utilize all different mechanisms indicating that the way of action of EAPP has to be a general one like interfering the cell cycle. But in previous studies we found an induction of the cell cycle. Due to this observation we concentrated first on the third possibility, the active transport out of the cell by ABC transporters.

5.2 EAPP and E2F1 can regulate the Mdr1 gene

The use of ABC transporters to get rid of harmful drugs is an efficient way of the cells to prevent damage. Not for nothing efflux pumps account, upon other mechanisms, for the multi drug resistance of cancer cells. As Pgp is the most prominent pump it was the first candidate we tested and indeed we found an upregulation upon EAPP overexpression in Western analysis (Fig 10). Reporter gene assays indicated a transcriptional regulation of the promoter by EAPP (Fig 12). The varying levels of upregulation upon several drugs could be due to diverse pathways switched on, which

influence EAPP differently (Fig 13). Interestingly we found also E2F1 as a potential activator of Mdr1 in luciferase reporter assays, which was not described before (Fig 15). In a recent study they showed that E2F1 binds to about 10.000-11.000 genomic locations (Cao *et al.*, 2011). Most of them are situated in the core promoters and overlap to 83% with Polymerase II binding. Interestingly only 12% contain the classical E2F binding site, namely TTTSSCGC, whereas S is either G or C. To examine the question if E2F1 binds via other factors to the promoter or directly to another motif they used E2F1 mutations and repeated their Chip-seq experiments. The transactivation domain of E2F1 interacts with various transcription regulators like TBP, TFIIH, CBP/p300, TRAPP, Tip60, GCN5, the retinoblastoma family members, and ACTR/AIB1. But a deletion in this domain did not affect the binding pattern of E2F1. They next mutated the N-terminal domain, which is implicated in the binding of Sp1, Cyclin A, EAPP and other proteins. Sp1 would have been a potent mediator of the E2F1 binding as it is known to interact with GC rich sequences in the core promoter. But also this mutation did not alter E2F1 binding to the DNA. Even after a mutation in the marked box (MB), which is necessary for the E2F2 and E2F3 recruitment to the promoter and DP1 binding, the E2F1 interaction pattern was unaffected. Finally they used a known mutation of the DNA binding domain which only abolishes the DNA association but other functions, like pocket protein binding, are normal. This was the only alteration of the tested mutations that affected the interaction of E2F1 with the DNA, which was totally impeded, suggesting that E2F1 directly binds to the core promoter regions. A de novo motif analysis revealed a short CGCGC motif as common feature of about 70% of the E2F1 bound promoters (Cao *et al.*, 2011). Sp1 activates the Mdr1 promoter through a GC-rich region where possibly also E2F1 can bind and activate. The interplay of EAPP and E2F1 will be discussed later. Unfortunately in efflux experiments, where we tested the capacity of P-Glycoprotein, no change upon overexpression of EAPP or E2F1 could be observed. Possibly the increase is to less to detect a change in the drug transport in the carried out set up. Or a real stimulus like nocodazole or etoposide is required in addition.

5.3 EAPP regulates p21

As the overexpression of EAPP also triggered an increase in G1 of untreated logarithmic growing cells we further investigated the possibility of a cell cycle arrest (Fig 22). Due to the previous finding that EAPP overexpression increases the levels of S-phase we did not pay much attention to the feasibility of a G1 arrest at the beginning. Experiments measuring the uptake of BrDU to determine if cells are still growing or stick in G1 were not really conclusive (data not shown). In the old experiments we transfected cells with an EAPP expression vector and analyzed the cell cycle distribution of the whole cell population. Only about 20-30% were transfected. This could have led to wrong outcomes in some experiments as most of the assayed cells did not express ectopic levels of EAPP. To overcome this problem we now used a GFP tagged version of EAPP where we could gate just for the GFP positive cells. This allowed us to analyze the cell cycle distribution of only the EAPP overexpressing cells. We carried out two different approaches. In the first one we transfected U2OS cells with either GFP or GFP-EAPP and compared them with regard to low and high GFP amounts (Fig 22). To solve the problem of two different cell populations we transfected in the second attempt cells simultaneously with GFP-EAPP and an empty pDsRed vector, gated for GFP positive and pDsRed positive cells and checked their cell cycle distribution (Fig 23). In both cases EAPP clearly increases the levels of G1. When we looked at the most prominent putative targets which trigger a G1 arrest we found p21 upregulated upon EAPP overexpression and downregulated upon a knockdown of EAPP, at protein and mRNA level (Fig 27). The dependence on p21 for the cell cycle influence we could confirm with a p21 knockdown which strongly reduces the ability of EAPP to induce a G1 arrest (Fig 26). Additionally we got data fitting to the involvement of EAPP in a G1 arrest. Upon a cell density increase levels of EAPP rose in a similar pattern than p21 levels and the G1 cell cycle stage (Fig 24). It was shown that p21 is at least partially responsible for an arrest when cells approached confluency (Perucca, 2009). In serum starved T98G cells nearly all EAPP is in the nucleus, whereas upon release or in logarithmic cells also a cytoplasmic fraction is present (Fig 25).

Further experiments supported a possible transcriptional regulation of p21 by EAPP. In luciferase reporter assays EAPP modulated the p21 promoter activity. EAPP overexpression upregulates and a knockdown decreases p21 promoter activity (Fig

29). Reduced EAPP levels abate the amount of Polymerase II bound on exon 1 of the p21 gene, indicating a lower transcription rate (Fig 30). We used different p21 reporter constructs in luciferase assays which revealed a dependence of the regulation on at least two Sp1 binding sites in the core promoter (site 3 and 4). Additionally it seems to be independent of p53 (Fig 32). In Chromatin Immunoprecipitation experiments EAPP bound only to the core promoter and not upstream (Fig 35). An EAPP knockdown reduced the levels of proteins involved in the pre-initiation-complex, like Polymerase II, TAF1, TAF4, or the elongation factor Cdk9, at the transcriptional start site. The TATA-box Binding Protein (TBP) was unaffected as well as E2F1. Interestingly the promoter occupancy of Sp1 goes up with lower EAPP (Fig 36). Also the p53 levels on the promoter and different acetylation and phosphorylation marks were not altered (Fig 37, 38). The independency of p53 in the modulation of the p21 promoter was further shown with T98G cells, where p53 is mutated. When U2OS cells were treated with etoposide the p21 levels decreased upon an EAPP knockdown even though p53 is upregulated (Fig 39). An HDAC1 inhibition by TSA did not affect the EAPP dependent downregulation of p21. In the case of an EAPP upregulation upon etoposide and TSA treatment the protein levels of p21 did not show any difference compared to normal EAPP levels. This is possibly due to posttranscriptional regulation of p21 as the mRNA goes up in the presence of etoposide and EAPP overexpression in U2OS and T98G (Fig 45).

5.4 Transcriptional regulation of p21 and Mdr1 by EAPP

For the p21 promoter we have seen that at least two Sp1/3 sites are necessary for the regulation by EAPP. Interestingly in a recent paper it was shown that EAPP is able to bind directly to DNA. Furthermore it competes with Sp1 for its binding sites. But in contrast to the p21 promoter EAPP downregulates the Monoamine Oxidase B (MAO B) gene. MAO A and B degrade various neurotransmitters and are upregulated by glucocorticoids in a Sp1 dependent manner (Chen *et al.*, 2010). They found that EAPP interacts with the Sp1 binding sites at the core promoter and represses the promoter activity. Increasing EAPP levels in an EMSA decreased the Sp1 binding to the DNA. Sp1 alone greatly stimulates the promoter, but transfected together with EAPP this effect was diminished. When the cells were applied with dexamethasone, a synthetic

glucocorticoid hormone which induces MAO B, the levels of EAPP on the promoter dropped a bit whereas Sp1 levels increased about three-fold. In all experiments EAPP had no effect when the core promoter was not present. Taken together they suggested that EAPP competes with Sp1 for binding and represses the promoter when associated to the DNA (Chen *et al.*, 2010).

Inline with our data is the dependence on Sp1 sites in the action of EAPP. In Chromatin IPs we also observed an increase in bound Sp1 when EAPP is knocked down (Fig 36). This fits to the competition model they proposed.

We did not study the Mdr1 promoter in detail but in luciferase assays the activation upon EAPP overexpression seems to be independent of E2F1. A pRb mutant that constitutively binds E2F1, and thereby inhibits it, is not able to abolish the EAPP dependent upregulation. Quite the contrary it even seems to increase the promoter activity upon EAPP addition (Fig 17). The Mdr1 promoter is also regulated by Sp1 through a GC rich region between -61 to -43 (Cornwell and Smith, 1993). This could be the target for EAPP to act on the promoter but further studies should be done to elucidate this. Interestingly a prominent repressor of the Mdr1 gene is p53 through direct DNA binding to the HT site, which is a novel p53 binding site. The other p53 family members p63 and p73 can activate the Mdr1 gene through the APE site, an alternative p63/p73 element (Moitra *et al.*, 2011).

Contrary to the p21 and Mdr1 promoter the MAO B promoter is downregulated by EAPP. In luciferase reporter assays done with E2F1 target promoters EAPP could stimulate an artificial E2F-dependent promoter and the murine TK promoter but downregulated the p14ARF promoter (Novy *et al.*, 2005). It seems that other additional factors are important in the way of action of EAPP. Possibly it depends on the potential to upregulate better than Sp1 or not. Sp1 is not always a gene activator. In smooth muscle cells for example it even represses p21 on a transcriptional level (Kavurma and Khachigian, 2003).

In general the interplay between Sp1 and Sp3 is a good example how two proteins with the same binding sites can regulate genes differently depending on the promoter structure and cellular background. Sp1 and Sp3 are two transcription factors expressed in all mammalian cells and belong to the Specificity Protein/Krüppel-like Factor (SP/KLF) family, which has a highly conserved DNA binding domain (65%) and three Cys2His2-type zinc fingers. The similarity between Sp1 and Sp3 regarding their DNA binding domain is quite high (90%), hence they bind the same DNA sites with a

similar affinity (Suske *et al.*, 2005). But the binding pattern of Sp1 and Sp3 is different depending possibly on the surrounding context and the exact sequence of the site, which varies and therefore the affinity is changed.

Of great interest are the different isoforms, especially of Sp3. The two long forms of Sp3 are similar to Sp1 with the major difference that the inhibitory domain, which can repress the activation domain, is located in front of the DNA binding domain instead at the N-terminus. In contrast the two short isoforms of Sp3 lack the important first activation domain, suggesting that they are responsible for Sp3 mediated repression (Li and Davie, 2010).

In the human genome more than 12.000 promoters were found with putative Sp1/3 binding sites, involved in almost all cellular processes. It seems that a certain ratio between Sp1 and Sp3 is necessary for viability. Sp1 has three mechanisms to activate a gene. Either via a single Sp1 binding site, or synergistically by interaction of two or several Sp1 molecules bound to different Sp1 binding sites but without cooperative DNA binding, or through superactivation. This happens when a Sp1 molecule binds to a Sp1 site and a second one binds to the first one without DNA interaction. Sp3 can not activate synergistically as it has a different D-domain responsible for this kind of induction in Sp1. In promoters with more than one Sp1/3 site Sp3 is suggested to be a repressor as it can not activate synergistically resulting in a lower general activation compared to Sp1. In promoters with only one Sp1/3 site no difference between Sp1 and Sp3 transactivation appears (Wierstra, 2008). There are reports that Sp1 and Sp3 enhance certain promoters synergistically, like at the RAS Association Domain Family 1A (RASSF1A) gene (Lee *et al.*, 2009c). In other studies Sp1 and Sp3 where shown to independently regulate promoters, binding to distinct Sp1/Sp3 sites and hence being present in different complexes (Li and Davie, 2010). In general the exact sequence of Sp1/3 sites seems to be crucial for the outcome of the regulation. As mentioned Sp1 and Sp3 bind selectively to different sites in the same promoter and this may additionally decide if the bound Sp family member is an activator or repressor. In the Adenylate Cyclase gene promoter the distal Sp1/3 site enhances the activity whereas the middle Sp1/3 site represses activity (Rui *et al.*, 2008). In the regulation of the Monoamine Oxidase B (MAO B) promoter binding of Sp3 to the CACCC element represses the gene but when Sp3 binds to the proximal Sp1/3 site the gene is activated. Sp3 binds with an equal affinity to both sites (Ou *et al.*, 2004). Possibly the binding at different positions is responsible for the recruitment of different

factors to the Sp proteins. In general they can be seen as transcription platforms interacting with a huge number of different proteins involved in transcription, either activating or repressing. The old view of Sp1 acting as a transcriptional enhancer and Sp3 acting as repressor has been challenged in recent years. Sp1 interacts with many factors involved in the induction of genes like histone acetyl transferases as p300, ATP-dependent chromatin remodeling complexes like SWI/SNF, or transcription factors like E2F1, p53, or c-Jun. On the other hand also repressors associate with Sp1 as histone deacetylases like different HDACs, DNA methylases like DNMT1, or the pocket proteins p107 and pRb (Wierstra, 2008). An example of a gene where Sp1 is part of a repressor complex is the mentioned p21. In addition to the described downregulation in smooth muscle cells HDAC4 inhibits p21 via a Sp1 interaction and likely due to the recruitment of the HDAC4-HDAC3-N-CoR/SMRT complex. But Sp1 is also necessary for the basal p21 transcription as knockdown experiments of Sp1 showed (Wilson *et al.*, 2008). Also HDAC1 binds to Sp1 at the p21 promoter where it competes with p53 (Lagger *et al.*, 2003). In neuroblastoma cell lines TRKA and p75NTR promoters are repressed by a Sp1/Miz1/MYCN repression complex which recruits HDAC1 to the promoters. TRAK and p75NTR are membrane receptors activated by the nerve growth factor (NGF). The role of p75NTR is still controversial but TRAK is involved in survival, differentiation, and neuron development. Both are highly expressed in neuroblastoma. Interestingly a knockdown of Sp1 not only upregulates TRAK and p75NTR, also p21 was upregulated (Iraci *et al.*, 2010).

All members of the Sp family can interact with E2F1, which doesn not interfere with the Cyclin A binding to E2F1 (Rotheneder *et al.*, 1999). It was shown that certain genes are activated synergistically by Sp1 and E2F1. In the murine TK promoter a mutation of the Sp1/3 binding site attenuates also E2F1 association to the E2F binding site and vice versa. Possibly the cell cycle regulated E2F1 recruits Sp1 to the promoter to support the initiation of transcription (Karlseder *et al.*, 1996). Another example of the regulatory interplay between Sp1 and E2F1 is the Dehydrofolate Reductase (Dhfr) gene. Transcription of Dhfr in quiescent cells is low. Upon growth stimulation pRb gets phosphorylated and Dhfr is upregulated. After serum withdrawal pRb gets dephosphorylated and binds to Sp1-HDAC1 complexes, where HDAC1 possibly functions as a mediator, and to E2F1-3 associated with DP1 on the Dhfr core promoter establishing a repressive complex. Growth stimulation results in pRb dephosphorylation and its dissociation from Sp1 and E2F1-3. Activating cofactors can

bind, like p300 or CBP, and the Dhfr gene is transcribed. Interestingly HDAC1 was not replaced after cell cycle induction, it was bound constantly to Sp1. Possibly the dissociation of pRb is enough to impede HDAC1 activity of the Dhfr promoter (Chang *et al.*, 2001). E2F1 was shown to replace HDAC1 from Sp1 in certain promoters (Doetzlhofer *et al.*, 1999).

Posttranslational modifications play also an important role in the way of action of Sp1 and Sp3. They are phosphorylated, glycosylated, sumoylated, acetylated, ubiquitylated, and methylated, which alters protein level, the DNA-binding affinity, or the transactivation potential. Acetylated Sp3 for example is a transcriptional activator (Ammanamanchi *et al.*, 2003). The outcome of p300 dependent acetylation of Sp1 is regulated by the modified site. Acetylation in the DNA binding domain enhances its DNA interaction whereas acetylation of K703 prevents p300 association and hence reduces transcriptional activity (Wierstra, 2008). Sp1 is phosphorylated by at least nine kinases resulting in the activation of different target genes. ATM for example phosphorylates Sp1 upon DNA damage whereby Sp1 activates the IGF-IR (Insulin-like Growth Factor 1 Receptor) promoter. Phosphorylation by ERK2 induces the Sp1 dependent VEGF (Vascular Endothelial Growth Factor) transcription (Wierstra, 2008). Or a Cyclin A/Cdk complex phosphorylates Sp1 and therefore increases its DNA binding ability (Haidweger *et al.*, 2001). Another important modification is glycosylation. It can inhibit the transactivation domain hence leading to a promoter repression (Bouwman and Philipsen, 2002). But also activation is possible. The adipokine Resistin, which is involved in obesity and insulin resistance, can be repressed by PPARc dependent reduction of the Sp1 glycosylation (Chung *et al.*, 2006).

The broad variety of different modifications and binding partners is also reflected by the number and activity of the target genes. They are involved in nearly all cellular processes. Sp1 for example activates the D-type Cyclins, Cyclin E, Cdk2, E2F1, and c-Myc which leads to cell cycle progression. At the same time it induces the expression of the cell cycle inhibitors p15, p16, p18, p19, p21, and p27. Also for the induction of apoptosis the same picture exists. Sp1 target genes are pro- and anti-apoptotic factors. In many tumors Sp1 is upregulated, at least partly due to its interplay with the strong oncogene c-Myc. Sp1 knockdowns reduced tumor formation in several studies (Wierstra, 2008).

Taken together the outcome of Sp1 and Sp3 activity is highly context dependent with a huge number of cofactors involved in the regulation, whereas both Sp1 and Sp3 are able to repress and activate genes. There are some reports claiming that Sp1 is necessary for the basal transcription whereas Sp3 is responsible for the induced transcriptional activation (Li and Davie, 2010).

This longer summary of Sp1 and Sp3 should give an idea how complex transcriptional regulation can be and that one protein can be involved in quite contradictory events. It is reasonable that EAPP plays a similar role in the regulation of genes. First it seems to be able to bind and compete with Sp1 at the Sp1/3 binding sites. A knockdown prevents the assembly of the pre-initiation complex either direct or indirect. We further found an interaction of EAPP with other key players of gene regulation, like the transcription factors p53 and E2F1, the HATs p300 and PCAF, or the members of the pocket protein family pRb, p130, and p107 (Rotheneder, Schwarzmayr, Orthner, Novy unpublished data). As we have seen it for Sp1 they include activators, like p53 or the HATs, and repressor, like the pocket protein family. The outcome of the EAPP dependent gene regulation seems context dependent as it is able to induce gene expression, like p21 and Mdr1, or represses it, like the MAO B gene and p14ARF. Interestingly Sp3 activates p21 and Mdr1 and downregulates at least MAO B (Gartel *et al.*, 2000a; Hu *et al.*, 2000; Ou *et al.*, 2004). An overexpression of EAPP has often little impact on the levels of putative target genes, whereas the effects of a knockdown are much more severe. This indicates that other factors are involved, which possibly form complexes with EAPP.

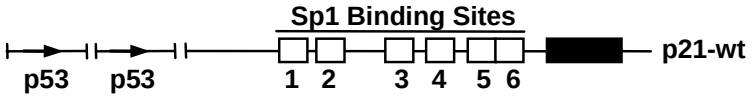


Fig 60: Diagram of the Sp1 and p53 binding sites in the p21 promoter

In the p21 promoter the Sp1/3 binding site number 3 seems to be necessary for the activation upon p53 upregulation or HDAC inhibition (Koutsodontis *et al.*, 2001; Lagger *et al.*, 2003). For EAPP dependent p21 regulation the Sp1/3 sites 3 and 4 are crucial. Maybe at the p21 promoter Sp1, Sp3 and EAPP activate the gene synergistically with different Sp1/3 binding site affinities. Concerning the MAO B promoter Sp1 and EAPP really compete for the same binding site whereas Sp1 is in this case the better activator, hence EAPP represses the promoter.At the p21 promoter Sp1 is possibly

more an activator when bound to the first two Sp1/3 sites and a repressor at the third Sp1/3 site as it was shown to interact with HDAC1 there. Only when HDAC1 is removed by p53 or HDAC inhibitors it activates even there. Sp1 and Sp3 bind to all six binding sites at the p21 promoter. In mutation studies of the Sp1/3 sites number 3 seems to be the most crucial one as its mutation reduces transcription to 10%. Interestingly in Drosophila SL2 cells, lacking Sp1/3, Sp1 and Sp3 can upregulate promoter constructs better when this third site is mutated. In contrast if the first binding site is either deleted or mutated the Sp1/3 dependent activation is nearly abolished (Koutsodontis *et al.*, 2002). This controversial data come possibly from the different cellular background. In Hep-G2 and Cos7 cells where the downregulation upon a site 3 mutation was shown a p53 site was present in the used luciferase reporter gene constructs. It is known that p53 needs this third site to activate the promoter (Koutsodontis *et al.*, 2001). If the p53 site is removed the activation goes as well to 10% indicating p53 as the major inducer of the gene. Mutating site 3 abolishes p53 dependent activation. Sp1 seems to more or less only facilitate a positive surrounding for p53, which needs Sp1 to activate the p21 promoter. p53 can even increase the levels of bound Sp1 (Wu *et al.*, 2011). Mutations in the sites 1, 5 and 6 do not really influence the activity whereas site 2 and 4 seem to be more involved. As we have heard In SL2 cells a mutation of site 3 causes a further activation of the promoter when Sp1 or Sp3 are expressed. It was shown that at this site Sp1 is associated with the repressor HDAC1, which is normally removed by p53 to activate the gene (Lagger *et al.*, 2003). When site 3 is mutated there is no Sp1/HDAC1 repressor complex anymore explaining the further upregulation in SL2. It seems that, in contrast to the tested mammalian cell lines, p53 does not interact with this site as a mutation has no effect. On the first Sp1 site Sp1 possibly interacts with activators hence it is necessary for the Sp1 dependent upregulation (independent of p53). Gartel et al showed that in Caco-2 cells site 1 and 2 are crucial for the Sp1/3 dependent upregulation of p21. Experiments in SL2 revealed that Sp1 binds to site 2 and Sp3 to site 1. In general Sp3 was a stronger activator than Sp1, which would fit to a Sp1/HDAC1 repressor complex (Gartel *et al.*, 2000a). Interestingly in HepG2 and Cos-7 a site 1 mutation was not really dramatic compared to a site 2 mutation. This would be in line with the model that Sp1 is crucial for the basal transcription and Sp3 for the further upregulation of genes. In contrast in SL2 cells site 1 seems to be quite important (Koutsodontis *et al.*, 2002).

EAPP could compete with this Sp1/HDAC1 repressor complex at site 3, recruiting activators to further upregulate the gene. Removing EAPP would lead to more Sp1/HDAC1 repressor complexes at this Sp1/3 site, explaining why an EAPP knockdown greatly reduces the p21 levels. But there has to be an additional mechanism as the knockdown of EAPP has quite severe consequences on the p21 levels, even in the presence of a p53 upregulation which competes with HDAC1 and should stimulate the promoter more than it can be seen in the Western analysis and RT-PCR experiments (Fig 39). As EAPP can interact also with p53 it is maybe in total a better activator when bound to p53 than Sp1/p53 at this site, possibly due to the recruitment of other activators. This would explain why EAPP can activate the promoter even further after etoposide treatment, as p53 is mainly responsible for the induction of p21 after DNA damage (Fig 45). It would be interesting to examine if p53 can activate the promoter via EAPP in a similar fashion than via Sp1. Contrary to most other multicellular organisms (including C. elegans), we did not find an EAPP homologue in Drosophila. The question is if an expression of EAPP in SL2 cells has similar effects than a Sp1/3 expression. In this cells site 3 seems not to be involved in a p53 dependent upregulation. This could be due to the lack of EAPP. It possibly does not only compete with Sp1 at this site it additionally could facilitate the interaction between p53 and Sp1. This would explain why the knockdown of EAPP is so severe as the promoter activity goes down to 10% without site 3 or the p53 binding sites. It also fits to our Chromatin IPs where p53 can not be found around the TATA box anymore after an EAPP knockdown, possibly due to the hindrance of the looping when p53 associates with Sp1. In our luciferase reporter assays the promoter activity goes dramatically further down when there are no p53 binding sites and site 3 is mutated. This is possibly due to the lack of site one and two in the used constructs (Fig 31).

Interestingly EAPP upregulates the Mdr1 promoter synergistically with E2F1, as both together further increase the activity. But when E2F1 is inhibited by a pRb mutant, unable to be phosphorylated, EAPP alone seems to upregulate the promoter more than without the E2F1 inhibition. As E2F1 interacts with Sp1 EAPP possibly binds better to the Sp1 site when E2F1 is not present and upregulates the promoter synergistically with itself. Experiments suggested that EAPP is able to form at least homodimers (Novy, unpublished data). Although the upregulation in the presence of this pRb mutant is less than with EAPP and E2F1 together it is more than with EAPP

alone. E2F1, as well as p53, compete with HDAC1 for Sp1 binding hence the repressor complex is changed into an activator complex. Maybe the affinity of Sp1 binding depends also on the associated cofactors and EAPP can compete better for binding when HDAC1 is bound to Sp1 rather than E2F1, assuming there is a Sp1/HDAC1 complex at the Mdr1 promoter. When E2F1 binds to its own target sequence and to Sp1 some stable loops may prevent an EAPP Sp1 competition. A mutation in the E2F1 binding site in the TK promoter reduces the Sp1 interaction with the DNA, indicating a cooperative binding (Karlseder *et al.*, 1996). Without E2F1 EAPP possibly does not need to compete with Sp1 and can further stimulate the promoter.

If EAPP competes with the Sp1/HDAC1 complex at the p21 promoter, there should be also changes in the acetlylation state. When EAPP is knocked down more Sp1-HDAC1 is bound to the promoter. Even HDAC1 represses the promoter in various ways than just altering the acetylation status, like preventing a p53 Sp1 interaction or the translocation of the Protein Kinase C to the nucleus (Han *et al.*, 2001; Lagger *et al.*, 2003). In Chromatin IPs we did not see any change in the acetylation status. We checked in detail only one region upstream of the core promoter and the sonication fragments were around 1000 bp. Possibly the fragment size is too high in respect to the tested site to really see acetylation changes around the core promoter. When Lagger et al showed the acetylation changes upon HDAC1 inhibition there was a massive change in the proximal region but not in the distal one. It would be worth to analyze the acetylation on the p21 promoter in more detail. On the other hand there are hints that also p300 levels on the promoter increase upon an EAPP knockdown (data not shown). Maybe the positive effect of additionall p300 and the negative effect of more Sp1/HDAC1 have an overall effect which is hard to resolve with Chromatin IPs. Additionally when EAPP is knocked down the HDAC1 inhibitor TSA did not alter the p21 decrease indicating that the function of HDAC1 is not necessary for the EAPP dependent downregulation of p21 (Fig 39). This could also explain why the acetylation state does not change. It suggests a role for EAPP more down stream, like the involvement in the assembly of the pre-initiation complex as Sp1. This would also fit to the severity of an EAPP knockdown. Sp1 recruits TBP and TFIID and stabilizes the assembly of TFIIB and TFIIE to the pre-initiation complex. (Wierstra, 2008). Possibly also E2F1 is involved in the recruitment of transcription factors to the start site. It binds to many core promoters and can interact with proteins involved in the pre-initiation

complex formation like TBP, TFIIH, CBP/p300 (Cao *et al.*, 2011). In general E2F1 seems to be, additionally to its classical role as a cell cycle regulator, a more global transcription factor, like Sp1, carrying out various functions depending on the different binding partners associated with it.

TBP binding is not changed upon an EAPP knockdown but the levels of the TFIID members TAF1 and TAF4 on the promoter decrease, as well as the RNA-Polymerase II itself. The levels of p53, which can be found at the TATA box, decreased upon an EAPP knockdown but not p53 bound to its response elements, indicating that the crucial looping of p53 to the TATA box can not take place possibly due to a hindered complex formation around the transcription start site (Fig 37, (Li *et al.*, 2007)). If EAPP is necessary for the assembly of the pre-initiation complex, which is present as a poised complex in the case of p21, it would explain why an EAPP knockdown nearly abolishes the level of p21, whereas an overexpression has lower effects due to the missing recruitment of other factors which are not present in excess or cannot bind the promoter for sterical reasons.

Taken together there are several mechanisms EAPP could apply to regulate target genes. As we can see for other proteins like p53 or E2F1 different ways can facilitate transcription at the same time. In the case of the p21 promoter EAPP could simultaneously compete for Sp1/3 binding sites, stabilize the p53 Sp1 binding, and assist the pre-initiation complex formation.

It would be worth to carry out an expression screen for an EAPP knockdown to get an idea of the number of target genes. Possibly EAPP acts in a similar way than Sp1 as a kind of platform, recruiting factors that either activate or repress the transcription of the target gene.

5.5 Consequences of the p21 regulation by EAPP in the case of DNA damage

We had already hints that EAPP is upregulated upon DNA damage induction (Fig 40). It was now interesting to examine if the regulation of p21 by EAPP has also consequences there. p21 is involved in various DNA damage responses to arrest the cells giving time to repair their DNA.

Etoposide induces double strand breaks and was reported to increase p21 levels. In a time course up to nine hours after the addition of etoposide we checked the

upregulation of p21. Interestingly we got different induction pictures with either a higher or a lower increase. This is possibly due to slightly different cell properties at the beginning. In one series we compared cells split at the day before with cells grown for a few days without splitting. Interestingly in the cells not split the induction was faster than in the split cells (Fig 41; 5.1.2010-1 were split and 5.1.2010-2 were not split). Maybe the potential for the p21 induction was already raised due to a higher cell density. When cells grew to confluence various positive regulators could be already near a threshold to induce p21, which facilitates a faster induction upon etoposide treatment. After 24 hours both reached a similar level (data not shown). This further indicates that the speed of induction depends on the background of the cells. When we combined the p21 induction of different experiments into two groups, with either a higher or lower increase, and compared it with the levels of EAPP we got a similar pattern. A steep rise of p21 coincides with a stronger EAPP increase compared to the lower p21 induction where also the EAPP upregulation was low (Fig 43). As expected the p21 mRNA behaved in the same way (Fig 43). To further confirm the interplay between EAPP and p21 during DNA damage we overexpressed EAPP and checked the p21 mRNA levels in normal and etoposide treated U2OS and T98G. In all cases we got an upregulation of the mRNA upon additional EAPP, indicating that EAPP is able to further induce the already increased p21 levels after DNA damage in cells with and without a functional p53 (Fig 45). The interesting question was now what happens with the p21 rise when cells possessing lower EAPP levels are treated with etoposide. We performed a time course in U2OS which revealed a clear correlation between the EAPP amount and the level of p21 mRNA induction (Fig 46). These experiments fit to the crucial role EAPP seems to play in the regulation of p21 in normal and stressed cells.

Cell fractionations with rising detergent concentrations and treatment time revealed an increase of EAPP in the presence of etoposide in the last fraction which partly includes also chromatin bound proteins (Fig 47). This could be due to the recruitment to the damaged foci of EAPP similar than ATM which shows the same pattern. Interestingly EAPP, even if it has no effect on the protein level of p53 (Fig 39) seems to influence the location of it. In untreated and etoposide treated cells an overexpression of EAPP leads to a p53 shift to the cytoplasm (Fig 48).

To investigate a possible further impact of EAPP in the DNA damage response we investigated its interplay with prominent checkpoint proteins like ATM and Chk2. We

found an influence of EAPP on the phosphorylation state of Chk2. After double strand breaks ATM is activated and phosphorylates Chk2 at T68 which results in its homodimerization and trans-phosphorylation at T383 and T387 leading to the full activation of Chk2 (Ahn *et al.*, 2000). As a consequence of this, Chk2 modulates several proteins involved in checkpoint control. Examples are the stabilization of p53 or the inactivation of Cdc25c resulting in cell cycle arrest, DNA repair or apoptosis. Interestingly an EAPP knockdown induced the T68 phosphorylation even without damaged DNA whereas an overexpression of EAPP leads to a reduction of the phosphorylation after etoposide treatment (Rotheneder, (Andorfer *et al.*, 2011)). Additionally EAPP seems to bind preferentially to phosphorylated Chk2 (Schwarzmayr, (Andorfer *et al.*, 2011)). To analyze checkpoint induction and subsequent recovery we treated cells with etoposide for 16 hours, removed it by washing the cells and harvested them at several time points. We compared stable cell lines with lower, normal, and increased EAPP levels. After six hours of release from etoposide it is clearly visible that in cells with lower EAPP levels the T68 phosphorylation of Chk2 is not removed which further indicates an important role for EAPP in Chk2 regulation. There are principally two possibilities how EAPP could facilitate this effect. Either it hinders the phosphorylation or it induces the dephosphorylation. Several phosphatases, namely PP2A, PP1, and Wip1 are responsible for the removal of the phosphate group (Freeman and Monteiro, 2010). As EAPP seems to bind preferentially to phosphorylated Chk2 the second way is the more plausible. In our suggested model EAPP interacts with the modified Chk2 and recruits the phosphatases, resulting in the dephosphorylation and the dissociation of the complex. Also in normal healthy cells Chk2 is phosphorylated constantly but a continuous dephosphorylation compensates this effect. In an EAPP knockdown this dephosphorylation does not take place anymore leading to a permanent accumulation of the Chk2 phosphorylation. On the other hand EAPP overexpression increases the dephosphorylation step reducing the overall phosphorylation (Fig 49, (Andorfer *et al.*, 2011)).

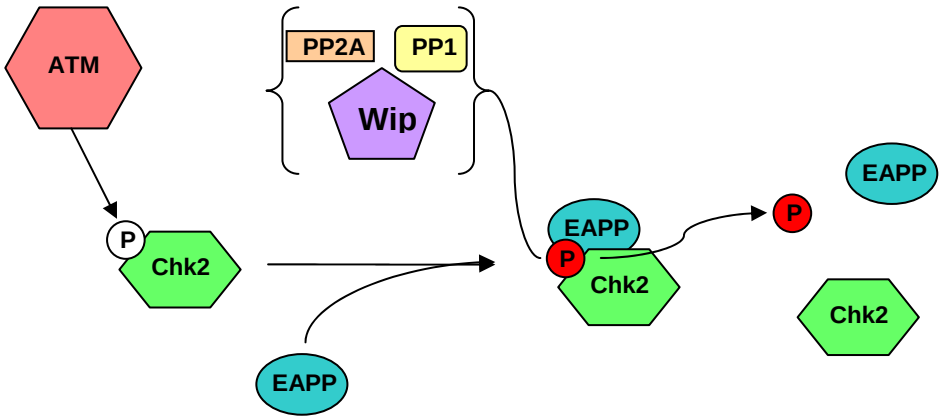


Fig 61: Proposed model of the interaction between EAPP and Chk2 in respect of the phosphorylation state of Chk2.

Additionally a knockdown of EAPP reduces the levels of Wip1 which maybe further contributes to the observed effect (Fig 50). This could happen in two ways. First less Wip1 leads probably to a decrease in the dephosphorylation of Chk2 and second Wip1 was also reported to modulate the ATM activity. It dephosphorylates ATM at S1981, a crucial site for monomerization and activation. This has the same purpose as the Chk2 dephosphorylation namely to deactivate ATM for checkpoint recovery. Loss of Wip1 results in ATM activation and hence further Chk2 phosphorylation (Shreeram *et al.*, 2006).

Taken together EAPP seems to impede the activation of Chk2, providing a mechanism for the recovery from the checkpoint. The DNA damage triggered increase of EAPP is slower than the phosphorylation of Chk2 which gives Chk2 the time to activate its targets before levels of active Chk2 are reduced again. But the increase of EAPP is early enough to be present in sufficient amounts for the activation of p21. Interestingly p21 is also responsible for the downregulation of Chk1 which supports our own observations that an overexpression of EAPP results in a reduction of Chk1 ((Gottifredi *et al.*, 2001), Rotheneder, unpublished data). These feedback loops provide a mechanism to switch of the checkpoint response by proteins which are in turn activated by the checkpoint. Since this activation needs time the checkpoint proteins can modulate their targets first. If the damage is too severe these early response proteins are activated constitutively counteracting this deactivation and at some point certain target proteins will reach a threshold to induce apoptosis. EAPP seems to be involved in such a feedback loop by activating p21 and by facilitating the deactivation of Chk2.

As we have seen overexpression of EAPP triggered a shift of the p53 protein equilibrium to the cytoplasm. This suggests either a direct mechanism of EAPP on the p53 localization, as EAPP interacts with p53 (Schwarzmayr, unpublished data) or via another target than p21. ATM inhibition for example leads to more p53 in the cytoplasm (Pang *et al.*, 2011). We have hints that EAPP interacts with ATM and possibly it can also trigger its dephosphorylation via Wip1 or directly like Chk2 which would lead to its deactivation, but this are only speculations. Interestingly p21 null cells were also reported to cause a cytoplasmic p53 accumulation (Pang *et al.*, 2011)

5.6 EAPP and its influence on apoptosis

When we overexpressed EAPP in the presence of etoposide we got the described increase in the G1 fraction. When we did the same with the GFP tagged EAPP and gated the fractions afterwards we observed not only a G1 increase but the sub-G1 amount decreased dramatically (Fig 51). In an etoposide time course cells with higher EAPP levels showed a significant reduction in apoptosis compared to normal U2OS (Fig 52). In line with this result was an earlier observation that overexpression of EAPP could abrogate the E2F1 mediated apoptosis. Ectopically expressed E2F1 was shown to induce apoptosis via several mechanisms. Either p53 dependent due to the upregulation of p14ARF which blocks the p53 inhibitor Mdm2, or p53 independent via activation of pro-apoptotic genes like p73, Apaf1, several Caspases, or pro-apoptotic BH3-only proteins like Bim, Puma, or Noxa. Additionally E2F1 inhibits proteins involved in survival pathways like NF-κB or Mcl-1 (Wu *et al.*, 2009). How EAPP is able to abolish the E2F1 induced apoptosis and if it is due to the general anti-apoptotic effect or specific for E2F1 we do not know but it would be a further hint why EAPP is overexpressed in several tested cancer cell lines. In normal cells the interplay between pro-survival and pro-apoptotic properties of E2F1 has to be regulated tightly to enable a proper cell behavior. Studies have shown that in cancer cells the pro-apoptotic activities of E2F1 are often disabled due to certain alterations in the downstream pathways (Wu *et al.*, 2009). As EAPP restrains this pro-apoptotic property of E2F1 it would provide a mechanism for cancer cells to avoid the E2F1 triggered cell death. EAPP was shown to support E2F1 dependent transcription (Novy *et al.*, 2005). It would be worth to examine if EAPP is able to specifically inhibit only

the pro-apoptotic activities of E2F1, like the activation of p14ARF, and in contrast enhancing the survival related E2F1 targets. In the case of cell cycle induction it counteracts E2F1 due to the activation of p21 but E2F1 is additionally involved in other survival functions like DNA repair which would provide targets for EAPP. (Chen *et al.*, 2011). E2F1 is reported to regulate p21 as well, but this seems to be more at the basal level (Gartel *et al.*, 2000b).

To investigate the influence of p21 in the pro-apoptotic properties of EAPP in the case of DNA damage induction we treated cells with etoposide and performed a time course comparing cells with elevated EAPP and reduced p21 levels in different combinations (Fig 54). Most reports suggest an anti-apoptotic effect mediated by p21. It can modulate several proteins involved in the induction of apoptosis. Cytoplasmic p21 is able to inhibit a number of pro-apoptotic proteins like Procaspase 3, Caspase 8 and 10 or the kinases SAPK and ASK1. Additionally it mediates the upregulation of anti-apoptotic genes and suppresses the transcription of pro-apoptotic genes by binding and inhibiting E2F and Myc (Abbas and Dutta, 2009; Dotto, 2000). A knockdown of p21 greatly reduced the ability of EAPP to protect cells of the DNA damage triggered apoptosis suggesting a crucial function of p21 in this mechanism. As the apoptosis levels upon p21 downregulation are still a bit lower in the presence of more EAPP additional anti-apoptotic effects of EAPP could be involved but the major target to prevent cell death seems to be p21 (Fig 54). A further mechanism could be the inhibition of Chk2 phosphorylation and hence activation by EAPP (Fig 49, (Andorfer *et al.*, 2011)). Chk2 is activated upon DNA damage and can influence apoptosis p53 dependent and independent. On the one hand it facilitates p53 stabilization via the phosphorylation on S20. There are hints that the modification of p53 by Chk2 preferentially triggers the induction of pro-apoptotic genes whereas the phosphorylation by ATM on S15 leads to cell cycle arrest (Hirao *et al.*, 2002). Chk2 also phosphorylates E2F1 on S364 which enhances E2F1 stability and possibly alters its promoter specificity leading to the expression of pro-apoptotic genes (Stiewe and Putzer, 2000). This stabilization establishes the connection between the DNA damage response and the E2F1 pathway. Additionally it was shown that Chk2 induced by IR interacts with and phosphorylates the Promyelotic Leukemia protein (PML) resulting in an increase in the number of PML nuclear bodies. This in turn induces apoptosis (Stracker *et al.*, 2009). In the case of the cell survival functions of EAPP in logarithmic

cells (Fig 53) Chk2 seems not to be the crucial target of EAPP as the active form is present only at low levels in undamaged cells.

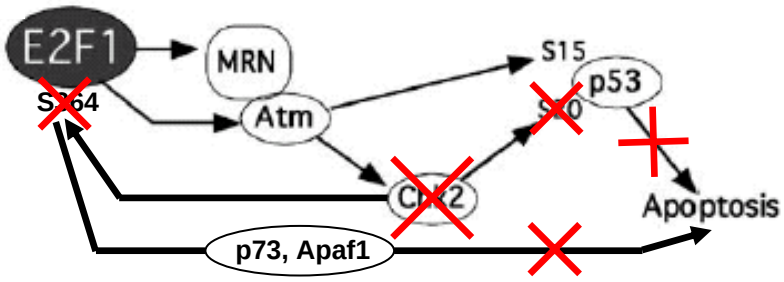


Fig 62: Inhibition of Chk2 activation attenuates its activation and its stabilizing phosphorylations on E2F1 and p53 reducing the ability to induce apoptosis.

In contrast a knockdown of EAPP induced apoptosis in U2OS, which have a wild type p53, and T98G, which have a mutated p53, suggesting a p53 independent way of action (Fig 55). In both cases the EAPP levels have to be reduced greatly to result in cell death. A moderate knockdown of about 50% has no obvious effect on the cells. But when we treated these cells with etoposide they induced apoptosis earlier as the control cells with normal EAPP levels (Fig 56). This further suggests a crucial role of EAPP in the decision between life and death upon certain stress stimuli. Interestingly in growing cells p21 was upregulated upon increased cell density in the case of normal EAPP levels but not when EAPP was reduced (Fig 56). Also the G1 fraction in the cell cycle distribution seems to be reduced in the EAPP knockdown cells, supporting the importance of EAPP in the general regulation of p21 (Fig 56).

The mechanism how reduced EAPP levels shift the damaged cells towards apoptosis still has to be examined. The most plausible target is again p21. Reduction of p21 levels favors the induction of apoptosis upon stress. In general low levels of DNA damage result in the stabilization of p21 whereas severe defects trigger the proteolysis of p21 allowing cells to undergo a programmed cell death (Cazzalini *et al.*, 2010). We tried to add p21 in addition to an EAPP knockdown under DNA damage inducing conditions but did not get fully conclusive results. Another candidate could be the above mentioned Chk2. Downregulation of EAPP results in a massive increase in Chk2 phosphorylation. This in turn could be responsible for an increase of E2F1, the PML bodies, and p53 phosphorylation followed by the induction of apoptosis. In the case of p53 we do not see any alterations in the S20 phosphorylation and protein stability (data not shown). But there are several reports which claim that the role of

Chk2 in the stabilization and hence activation of p53 upon DNA damage is not as crucial as previously described (Ahn *et al.*, 2004). Purified Chk2 from tumor cell lines harboring DNA damages was not able to phosphorylate p53 but it could phosphorylate Cdc25C. Additionally knockdowns of Chk1 and Chk2 in several tumor cell lines did not alter p53 S20 phosphorylation and stabilization (Ahn *et al.*, 2003). Maybe other proteins can compensate the loss of Chk2.

Assigning the EAPP-dependent apoptosis when knocked down to really low levels to one mediator protein seems to be difficult. Low EAPP not only results in decreased p21 or Wip1 levels. We found several other proteins being influenced like p27 or Cdc25c (Fig 57). Other targets were PCNA, Chk1, Mad2, Cyclin B, and Cdk1 (Rotheneder, unpublished data). This suggests a severe impact on the cell regulation at several levels. The moderate knockdown of EAPP seems to provide enough EAPP for the normal cell functions. Only upon stress EAPP would need to be upregulated to a certain level to provide the transcription of target genes involved in the pro-survival checkpoint response. As this is impeded cells tend to undergo apoptosis. Upregulation of EAPP often shows only a minor effect on putative target genes which were downregulated upon a knockdown. Other factors seem to be necessary for the upregulation. They are, depending on the cell background, not always present in additional amounts when only EAPP is overexpressed. In some cases we found a slight upregulation of the mRNA levels like p53, E2F1 or even the proapoptotic Bax (Fig 58). This increase of Bax fits to the reports that p21 can induce Bax directly (Liu *et al.*, 2003).

EAPP could be involved in the transcription in a more general way, like Sp1 or E2F1. On some targets it has more influence, like on p21, and on other targets it is more dependent on co-factors but still necessary to maintain the transcription. It would be worth to perform an expression array to get a hint how many proteins show an unusual behavior when EAPP is knocked down.

In the case of p21 we propose the following model: In normal cells EAPP is required for the transcription of p21. Upon stress it is upregulated which is necessary for the further induction of p21. When EAPP is present in higher amounts, like in several cancer cells, p21 levels are higher, which favors a G1 arrest and additional anti-apoptotic activities crucial for cancer cells. This effect is possibly not restricted to the upregulation of p21 as the Chk2 modulation could also play a prominent role. When

EAPP levels are reduced p21 and several other proteins are decreased, resulting in apoptosis especially when the cells are stressed (Fig 63).

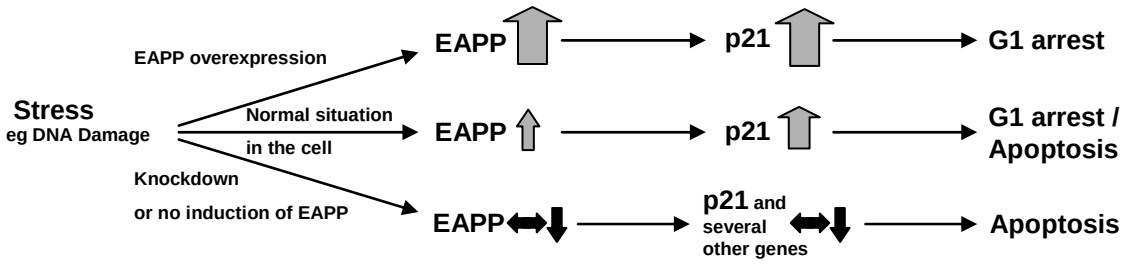


Fig 63: Proposed model of the influence of EAPP upon certain stress stimuli

Taken together EAPP seems to be crucial in maintaining the integrity of the cells particularly when stressed and response checkpoints have to be switched on. This would explain why EAPP is present at higher levels in several tested cancer cell lines. A complete knockdown seems to be lethal, which is also supported by the fact that we were not able to produce a stable cell line with a knockdown greater than 50%. Further studies have to be done to shed light on the exact mechanism of EAPP and the pathways in which it is involved.

6. Material and Methods

6.1 Materials

6.1.1 Cell lines

Cell line	ATCC No	Description
U2OS	HTB-96	Human osteosarcoma cell line
T98G	CRL-1690	Human glioblastoma cell line

6.1.2 Vectors

Vector	Reference
pCIneo-HA-MCS	(Novy *et al.*, 2005)
pCIneo-HA-HA-EAPP	(Novy *et al.*, 2005)
pCIneo-HA-HA-E2F1	(Novy *et al.*, 2005)
pCIneo-HA-DP1	
pCIneo-HA-pRb(Δcdk)	(Lukas *et al.*, 1999)
pSuper-Scrambled	(Andorfer and Rotheneder, 2011)
pSuper-p21	(Andorfer and Rotheneder, 2011)
pSuper-EAPP #1	(Novy *et al.*, 2005)
pSuper-EAPP #2	(Andorfer and Rotheneder, 2011)
pSuper-EAPP #3	(Andorfer and Rotheneder, 2011)
pEGFP-C1	(Novy *et al.*, 2005)
pEGFP-EAPP	(Novy *et al.*, 2005)
pEGFP-E2F1	(Andorfer and Rotheneder, 2011)
pDsRed-C1	BD #632466
pDsRed-EAPP	Novy, unpublished data
pDsRed-E2F1	Novy, unpublished data
β-galactosidase	(Novy *et al.*, 2005)
p21-wt-Luc	(Andorfer and Rotheneder, 2011)
p21-BstX-Luc	(Andorfer and Rotheneder, 2011)
p21-Pst-Luc	(Andorfer and Rotheneder, 2011)
p21-101-Luc	(Andorfer and Rotheneder, 2011)
p21-101mt3-Luc	(Andorfer and Rotheneder, 2011)
p21-101mt4-Luc	(Andorfer and Rotheneder, 2011)
p21-Pstmt3-Luc	(Andorfer and Rotheneder, 2011)
Mdr1-Luc	(Okamoto *et al.*, 2000)
Mdr1(Y-Box mutated)-Luc	(Okamoto *et al.*, 2000)

6.1.3 Primers

	Annealing Temperature	Sequence
RT PCR		
p21 mRNA	58°C	Fwd: CCGTGAGCGATGGAACTTC Rev: GAGACTAAGGCAGAAGATGTAGAG
p53 mRNA	58°C	Fwd: GCCCCCAGGGAGCACTA Rev: GGGAGAGGAGCTGGTGTTG
E2F1 mRNA	58°C	Fwd: ACCAGGGTTTCCAGAGATGC Rev: CACCACACAGACTCCTTCCC
GAPDH mRNA	58°C	Fwd: TCTTCTTTTGCGTCGCCAG Rev: AGCCCCAGCCTTCTCCA
Bax mRNA	58°C	Fwd: TGGAGCTGCAGAGGATGATTG Rev: GAAGTTGCCGTCAGAAAACATG
CHIP p21 promoter		
Control	59°C	Fwd: GGAGTCCTGTTTGCTTCTGG Rev: CTTTGGCCACACTGAGGAAT
p53 5' binding site	59°C	Fwd: GTGGCTCTGATTGGCTTTCTG Rev: CTGAAAACAGGCAGCCCAAG
p53 3' binding site	59°C	Fwd: GCAGAGGAGAAAGAAGCCTG Rev: CATCTGAACAGAAATCCCACTG
Upstream	59°C	Fwd: GAGGCAAAAGTCCTGTGTTC Rev: GGTTGCAGCAGCTTTGTTGG
TATA box	59°C	Fwd: CTCTCCAATTCCCTCCTTCC Rev: AGAAGCACCTGGAGCACCTA
Exon 1	59°C	Fwd: CCTAATCCGCCCACAGGAAGC Rev: CAGCACTCTTAGGAACCTCTC

6.1.4 Antibodies

Antibody	Company/Reference
β-Actin	Sigma # A5316
Acetyl-Histone 3	Upstate # 06-599
Acetyl-Histone 4	Upstate # 06-866
ATM	Santa Cruz # sc-23921
Cdk9	Santa Cruz # sc-8338
Cdc25c	Santa Cruz # sc-13138
Chk2 T68	Santa Cruz # sc-16297-R
Cleaved Caspase 3	Cell Signaling # 9664S
E2F1	Santa Cruz # sc-251
EAPP	(Novy *et al.*, 2005)
GAPDH	Chemicon # MAB374
HA (16B12)	Abcam # ab72479
Histone 3 C-terminus	Abcam # ab1791
Histone 3 Acetyl K9	Upstate # 06-942
Histone 3 Phosph. S10	Upstate # 05-598

Mdr1	Santa Cruz # sc-8313
p21	Santa Cruz # sc-817 / # sc-56335
p27	Santa Cruz # sc-776
p53	Santa Cruz # sc-126
Pol II	Santa Cruz # sc-899
pRb	Santa Cruz # sc-50
Sp1	Santa Cruz # sc-59
TAF1	Santa Cruz # sc-735
TAF4	Santa Cruz # sc-736
TBP	Santa Cruz # sc-273
Wip1	Santa Cruz # sc-20712

6.1.5 Special Chemicals

Chemical	Company	Description
Colchicine	Sigma	Microtubule poison
Etoposide	Sigma	Topoisomerase II inhibitor
MMS	Sigma	DNA alkylating agent
Nocodazole	Sigma	Microtubule poison

6.1.6 Common solutions:

40% Acrylamid (100ml H2O):	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 Chloramphenicol (30 mg / ml) 1000 x Kanamycin (50 mg / ml) 1000 x Tetracyclin (30 mg / ml)
Blocking Solution:	1 x PBS 0,1 % Tween Nariumazid (20 % NaN3 = 20000 stock) 3 % Milkpowder
Salmon Sperm DNA:	1g 20min sonicated salmonsperm-DNA in 100 ml TE. Heat the suspension before use (95°C) and put immediately on ice.
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
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
10x TAE:	400mM TRIS/Acetat 20mM EDTA pH= 8.0
1 x TBE:	0,9 M TRIS-HCl, pH 8,3 0,9 M Borat 25mM EDTA
1x TE:	10mM TRIS/Cl 1mM EDTA pH= 8.0
5 x Transfer-buffer (2000 ml):	125mM TRIS 960mM Glycin 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

6.2 Bacterial Cultures

6.2.1 Luria Broth (LB)

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

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

6.2.2 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 $(NH4)2\ SO_4$
0.18g/l $MgSO_4$ .7 H_2O
88.0g/l Glycerol

6.2.3 $CaCl_2$ competent E.coli

Inoculate 2,5 ml overnight culture in 500 ml LB until OD600nm = 0,2.
Centrifugation (5000 rpm, 10 min).

Resuspend the pellet in 150 ml sterile, cold 100mM $CaCl_2$; keep 20 min on ice!
Centrifugation (5000 rpm, 10 min).
Resuspend the pellet in 5 ml sterile, cold 100mM $CaCl_2$ + 1/5 volume autocl. glycerol (87 %).
Prepare 100 µl aliquots and store at -80°C.
Competent E.coli after Nishimura
(Nishimura et al., 1990)
Inoculate 50 ml medium A with 0,5 ml over night culture
Grow to a mid logarithmic phase
Keep cells on ice for 10 min, then fuge at 1500g, 10min, 4°C
Resuspend pellet gently in 0,5ml precooled medium A
Add 2,5ml of storage solution B and mix well without vortexing
Divide in aliquots of 100µl, store at -80°C

Medium A:
5ml 1M $MgSO_4$
10 ml 10% glucose
100 ml 5x LB
H_2O ad 500ml, autoclav.

Storage solution B:
Supplement LB-broth with:
36% glycerin
12% PEG 7500
12 mM $MgSO_4$ 7 H_2O
sterilize by filtration

6.2.4 Transformation of competent E.coli

Mix DNA and an aliquot of competent cells.
Keep the mixture 20min on ice
Heat shock the cells 2min at 42°C.
Add 300µl LB and keep the cells on 37°C for 30min.
Plate the suspension on antibiotic-plates.

6.3 Methods for DNA

6.3.1 Plasmid DNA preparation

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, or freezing.

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

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 NH_4OAc 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 NH_4OAc

6.3.2 DNA purification (with Phenol-Chloroform-Isoamylalcohol)

Phenol was equilibrated with 0,5M Tris/HCl (pH = 7,5) followed by equilibration with 0,1 M Tris/HCl (pH=7,5) and subsequently with 0,001M Tris/HCl (pH=7,5). Afterwards a 25:24:1 mixture of phenol:chloroform:isoamylalcohol (PCI) and a 1:1 mixture of chloroform:isoamylalcohol (CI) was prepared, covered with 0,01 M Tris/HCl (pH=7,5) and stored at 4°C under light protection.
100 µl of DNA in TE were vortexed with 100 µl PCI, centrifuged for 1 min at full speed and the (upper) water-phase was vortexed with 100 µl CI and centrifuged again for 1 min. Afterwards the watery phase was transferred to a new eppendorf tube.

6.3.3 DNA precipitation

For DNA precipitation of the watery phase EtOH (2-3 times the volume) was added in the presence of 0,3 M CH_3CO_2Na at pH 5,2 (1/10 of the volume). Very small amounts were precipitated in the presence of glycogen (20 mg/ml). After half an hour or overnight at –20°C the precipitate was centrifuged for 10 min at 15000 rpm, washed once with 70% EtOH, and the pellet dissolved in TE.

6.3.4 Agarose gel

Corresponding amounts of agarose was mixed with either 130 ml (big gel) or 50 ml (small gel) of a TAE Buffer and heated in the microwave till all the agarose was dissolved. The solution was cooled down (eg under the water pipe) to ~50°C and Ethidiumbromide was added (3µl for a small gel, 7µl for a big gel; Stock 10mg/ml) and pored into a tray.

6.3.5 DNA isolation from agarose gel 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.

6.3.6 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 = OD260 x 50 x y (used µl of DNA)
If OD260 is between 1,8 and 2,0 the DNA is clean, but less than 1,8 indicates for impurities in the solution

6.4 Mammalian Cells

6.4.1 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 H2O and sterile filtrated [0,2µm].

6.4.2 Storage of mammalian cells

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

6.4.3 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 twice with warm 1xPBS and incubated with about 12 drops Trypsin/EDTA 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, 2 ml media per well (10% FCS or 10% CS)
Ø 60 mm plate, 3 ml media (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)

6.4.4 Transfection of mammalian cells (PEI-method)

Transfection mix (for eg 100mm):
x µg DNA in H_2O
ad 200µl HBS (steril)
Add 200µl HBS 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
Change media 24 hours after transfection

Plate	Size	Cells for transfection	Cells/cm²	DNA amount	HBS
6-Well	1,7cm 9,08 cm^2	~ 170.000	18722/cm2	1-4 µg	2x100 µl
60mm	2,6cm 21,24 cm^2	~ 400.000	18832/cm2	5-10 µg	2x200 µl
100mm	4,2cm 55,42 cm^2	~ 1.000.000	18044/cm2	10-20 µg	2x400 µl
140mm	6,75cm 143,13 cm^2	~ 2.700.000	18864/cm2	20-30 µg	2x600 µl

1x HBS:
150 mM NaCl
20 mM HEPES
0,75 mM Na_2HPO_4

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

6.4.5 Whole 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 N2
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

6.4.6 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 analyzed 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 ODs.

6.5 Special Methods

6.5.1 Western immunoblot analysis with SDS - Polyacrylamide Gels

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

	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

Electrophoresis:

Load 2-3 µl protein marker per well and the samples onto a polyacrylamide gel

Electrophorese in 1x Running buffer: 120mV

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 or 40mA o/n.

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 (or o/n at 4°C)

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 (HRP coupled)
Wash three times for 10 min in PBST

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

6.5.2 RNA Isolation with Trizol

This was done according to the Invitrogen Protocol for the Trizol Reagent

6.5.3 Reverse Transcriptase cDNA synthesis and PCR

RevertAid™ First Strand cDNA Synthesis Kit (#K1622) was used.
For the corresponding PCR the Maxima® Hot Start Green PCR Master Mix (2X) was used. 2 µl of the gained cDNA was taken, mixed with 1µl of the indicated primers (diluted 1/10) and the master mix and filled up to 25µl. Annealing temperatures are shown in the primer section.

PCR Protocol
95°C 4 min |
95°C 45 sec | not more than 30 cycles
x °C 1 min |
72 °C 45 sec
72 °C 4 min

x…annealing temperature

6.5.4 Cell-cycle analysis

The cell-cycle distribution was measured with DAPI (Merck, Darmstadt, Germany, Figures 7,8,9), PI (Sigma, Figures 24,55,56), Hoechst 33258 (Invitrogen, Figures 22,23,26,51), or Draq5 (Biostatus Limited, Leicestershire, UK; Figure 53).Cells were

trypsinized, washed once with phosphate-buffered saline and around 5.105 cells were taken for analysis. For Hoechst 33258 and Draq5, which stain viable cells, appropriate concentrations of the dyes were used and incubated for20 min on 4 1C in the dark (Draq5 10 mM, Hoechst 33258 5 mg/ml). For DAPI and PI cells were fixed in 85% ethanol and incubated at least 30 min on ice. PI was used in a final concentration of 50 mg/ml and 100 mg/ml RNAse Typ 1-A wasadded and incubated for 30 min at room temperature in the dark. DAPI concentration was 2 ng/ml and fixed cells were incubated for 20 min in the dark at 4 1C. To analyze the cell cycle distribution different devices were used. DAPI staining was measured with a Pas-III from Partec (Muenster, Germany), PI and Draq5 with a FACS-Calibur (BD Bioscience, San Jose, CA, USA), and Hoechst 33258 with a FACS-Aria (BD Bioscience). Hoechst 33258 and Draq5 allowed an additional staining with and gating for GFP. With the FACS-Calibur and FACS-Aria cell debris and doublets were excluded (Andorfer and Rotheneder, 2011).

6.5.5 Apoptosis and cell count

Cells were counted with a CASY Cell Counter TTC (Schärfe System, Reutlingen, Germany). To detect apoptosis different assays were carried out. For all procedures cells were trypsinized, washed once with phosphate-buffered saline and around $5x10^5$ cells were taken for measurement. The same procedure as described in the cell cycle analysis part was applied for sub-G1 analysis. Beforehand a threshold was set to discriminate against the cell debris. For the Vybrant Apoptosis Assay Kit (Invitrogen) cells were stained with PI and Yo-Pro EAPP: At the crossroad of apoptosis and arrest according to the protocol from Invitrogen. Yo-Pro enters only into early apoptotic cells, whereas PI stains all permeabilized cells. Again a threshold was set to exclude cell debris. Necrotic cells have either a higher forward scatter signal due to cell swelling or disappear in the cell debris upon bursting. In contrast, apoptotic cells have a smaller forward scatter signal before they give origin to many small apoptotic bodies, which generally end up as debris. Yo-Pro-positive cells with a smaller forward scatter were gated and separated into PI-positive cells (late apoptosis or already dead cells) and PI-negative cells (early apoptosis). Hoechst 33258 brightly stains the condensed chromatin of apoptotic cells, hence, the fluorescence intensity increases. A threshold excluding the cell debris was set and GFP-positive cells were gated and percentage of cells with higher Hoechst 33258 values and smaller forward scatter values were

measured. All flowcytometry experiments were done with a FACS-Calibur or a FACS-Aria from BB Bioscience (Andorfer and Rotheneder, 2011).

6.5.6 Chromatin Immunoprecipitations (ChIP)

CHIP's were done as described in Hauser *et al.* (Hauser *et al.*, 2002).

6.5.7 Immunoprecipitation

Whole cell extracts were prepared and 500µg incubated over night at 4°C with the corresponding antibody. Protein A or Protein G beads were added for one hour and beads were washed afterwards 4-5 times with the Hunt buffer, boiled in a Laemmli buffer and loaded onto a PA gel.

ProtA- or ProtG-Agarosebeads for Immuneprecipitation:

Mix 0.15g of ProtA- or ProtG-agarose beads with 1,2ml coating buffer. Incubate 30min at RT, wash 3x with H2O. For storage add NaN3.

Coating buffer: 50mM TRIS pH=8
0.02% NaN_3
10% (w/v) BSA

6.5.8 Luciferase reporter gene assay

Wash cells 2x with 1x PBS.
Add 300µl luciferase-assay buffer.10 min on ice.
Freeze in liquid nitrogen two times
Centrifugation (5 min, 14000 rpm, 4°C).
Use 200µl of the supernatant for measurement.
Right before measurement add 16µl Luciferin, 4µl ATP (stock 0,1 M), and 1µl DTT (stock 1M)

Luciferase-assay buffer:
25mM Tricine
0,5mM EDTA
0,54 mM Na-Tripolyphosphate
16,3 mM MgSO4.7H_2O
0,1 % Triton X-100
H2O to 1L; pH 7,8

Luciferin:
Dissolve 10mg in 35,6 ml H2O. Adjust pH to 7,5 with 0,25M Na_2CO_3. Store dark at -20°C.

β-Galactosidase-assay
For each sample mix the following ingredients.

30 µl Protein-extract
3 µl 100 x Mg-Lösung
66 µl ONPG
201 µl Sodiumphosphate pH =7,5

Vortex the samples and put on 37°C until a slight yellow color appears. Stop the reaction with 500µl 1M Na2CO3. Centrifuge the samples and transfer the supernatant into fresh Eppendorf-tubes. Measurement at 420nm (linear range: OD420nm = 0,2 - 0,8).

100 x Mg-Solution:
0,1M $MgCl_2$
4,5M β-Mercaptoethanol

1 x ONPG:
4mg / ml o-nitrophenyl--D-Galactopyranosid in 0,1M Potassiumphosphat pH= 7,8

0,1 M Sodium phosphate buffer pH=7,5:
41ml 0,2 M Na_2HPO_4 2 H_2O

9ml 0,2 M NaH_2PO_4 2 H_2O
50ml H_2O

6.5.9 Efflux Measurement

Cells were trypsinized and washed once in PBS. 2 µl Rhodamine123 (0,8 mg/ml) was added and incubated for one hour at 37°C. Afterwards cells were washed once with PBS and analyzed with the FACS Calibur.

6.5.10 Cell Fractionation

Harvest about one million cells. Spin down for 15 sec and remove supernatant. Resuspend cells in 200 µl cold buffer A by gentle pipetting. Swell cells on ice for 15 min. Add 12,5 µl of 10 % NP-40. Vortex tube for 10 sec (or just rotate it). Spin tube for 30 sec. Collect supernatant as cytosolic fraction.
The nuclear pellet is washed three times with 300 µl buffer A . Add 50 µl buffer C. Vortex for 15 min in the cold room (or freeze three times in liquid N2). Spin 5 min at 15.000 rpm

Buffer A:
10 mM HEPES pH 7,9
10 mM KCl
0,1 mM EDTA
0,1 mM EGTA
1 mM DTT
Inhibitor cocktail

Buffer C:
4 M NaCl
1 mM EDTA
1 mM EGTA
1 mM DTT
Inhibitor cocktail

6.5.11 Immunfluorescence

Grow cells on cover slips in a 6-well plate
Wash cells twice with PBS
Cover cells with 1ml fresh 3% Formaldehyde in PBS
After 10 min wash twice with PBS
Cover the cells with 1ml 0,1% Triton-X in PBS
After 10 min wash twice with PBS
Pipette antibody solution directly onto the cover slip
Store the cover slips over night at 4°C in a wet chamber
Wash the cover slips three times with PBS
Cover the cells with secondary antibody solution and keep store the probes in the dark for 1h at 4°C
Wash the cover slips twice with PBS
Wash the cover slips once with PBS + 1μg/ml DAPI or Hoechst for 10 min
Wash once with PBS
Dry slide
Mount with Dako Fluorescent Mounting Medium

6.5.12 Data analysis

Data in all experiments are represented as mean±s.d. Statistical analysis was carried out using unpaired t-test. The P-values of <0.05 were considered to be statistically significant. *$P<0.05$; **$P<0.01$

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