Self-association of adenovirus type 5 E1B-55 kDa as well as p53 is essential for their mutual interaction

PhD Thesis

in partial fulfilment of the requirements
for the degree “Doctor of Philosophy (PhD)”
in the Molecular Biology Program
at the Georg August University Göttingen,
Faculty of Biology

submitted by

Magdalena Morawska-Onyszczuk

born in
Pszczolki, Poland

2009

Affidavit

I hereby declare that this doctoral thesis has been written only by the undersigned and without any assistance from third parties.

Furthermore, I confirm that no sources have been used in the preparation of this thesis other than those indicated in the thesis itself.

Göttingen, 10 November 2009

Magdalena Morawska-Onyszczuk

"You're really not going to like it," observed Deep Thought.
"Tell us!"
"Alright," said Deep Thought. "The Answer to the Great Question..."
"Yes!.."
"Of Life, the Universe and Everything..." said Deep Thought.
"Yes!.."
"Is..." said Deep Thought, and paused.
"Yes!.."
"Is..."
"Yes!!!?.."
"Forty-two," said Deep Thought, with infinite majesty and calm.

"The Hitch Hikers Guide to Galaxy" by Douglas Adams (1952-2001)

List of publications:

Magdalena Morawska-Onyszczuk, Krystyna Bieńkowska-Szewczyk, Matthias Dobbelstein. Self-association of adenovirus type 5 E1B-55 kDa as well as p53 is essential for their mutual interaction. Accepted on 28.11.09 by Oncogene

TABLE OF CONTENTS

ACKNOWLEDGMENTS

This thesis would have not been possible without help, intellect and inspiration of many people that help me along the way.

The first people that I would like to acknowledge are my advisors Prof. Matthias Dobbelstein and Prof. Krystyna Bieńkowska-Szewczyk. I am truly grateful for giving me the chance to work on your teams. I appreciate the rare opportunity to work in two different laboratories in two different countries, at the same time. This has thought me not only to be flexible but also to be better organized to allow for this to work fluently. Although, at times I found this challenging, it was a true life changing experience. I am especially grateful to Prof. Dobbelstein for never giving up on me and being inspiring as a person and a scientist.

It would not be possible without the support from my husband Piotr, thank you for believing in me and allowing me to do this my way.

Elżbieta Brzuszkiewicz is a true friend whom I am very grateful not only for giving me a roof over my head in Goettingen but also for great discussions about science, life and universe.

I would like to express my gratitude to the members of my doctoral committee Prof. Frauke Melchior and Prof. Marcus Wahl for the inspiring discussions and support.

I am truly grateful to Dr. Steffen Burkhardt for his help and involvement.

The time at the laboratory would have not been the same without my labmates both in Goettingen and in Gdansk. I would like to acknowledge them for their help, discussions and sharing reagents. Special thanks to Irina Savelyeva, Muriel Lizé, Andrea Lipińska, Łukasz Rąbalski and Krzysztof Łepek ….you Rock!

In search of The Ultimate Question of Life, the Universe and Everything...
Magda

ABSTRACT

P53 is at the center of the control systems that mediate the response to DNA damage, leading to cell cycle arrest or apoptosis. Virus infection can cause a DNA damage response through virus replication intermediates, but many viruses have evolved mechanisms to interfere with this response. The adenovirus type 5 E1B-55 kDa oncoprotein has several known functions during the viral life cycle, but it is mostly known for its ability to bind and inactivate the tumor suppressor p53. Together with another viral protein, E4orf6, E1B-55 kDa joins an E3 ubiquitin ligase complex and marks p53 for proteasomal degradation. In the absence of viral binding partners, E1B-55 kDa forms cytoplasmic clusters and sequesters p53 to them. Interestingly, E1B-55 kDa and p53 are each capable of forming oligomers. We mapped the oligomerization domain of E1B-55 kDa to the central portion of the protein. Disturbing E1B-55 kDa self-association by point mutations at residues 285/286 or 307 not only impairs its intracellular localization to the cytoplasmic clusters, but in addition its association with p53. Strikingly, tetramerization of p53 is also required for efficient association with E1B-55 kDa. Moreover, two different E1B-55 kDa mutants defective for p53 binding but proficient for oligomerization can trans-complement each other for p53 relocalization. We propose that the homo-oligomerization of each component enables the efficient interaction between E1B-55 kDa and p53 through increased avidity.

ABBREVIATIONS

Ad – Adenovirus

APC- Adenomatous Polyposis of the Colon

ASPPs - Apoptosis Stimulating Protein of P53

ATM - Ataxia-Telangiectasia Mutated Kinase

ATR - ATM- and Rad3- related kinase

BRK - Baby Rat Kidney

BTB - Blot Transfer Buffer

CC - coiled-coil

DAPI - 4,6-diamidino-2-phenylindole

DNA - deoxyribonucleic acid

dNTP - Deoxyribonucleotide triphosphate

DMEM - Dulbecco's Modified Eagle's Medium

DTT – dithiothreitol

EGFP- Enhanced Green Fluorescence Protein

HA - hemagglutinin

HATs - Histone Acetyltransferases

HMW -High Molecular Weight

IP – immunoprecipitation

ITR - Inverted Terminal Repeat

kDa - kiloDalton

kB - Kilobasepair

LB - Luria Bertani

Mdm2- Murine Double Minute 2

MRN - Mre11/Rad50/Nbs1

mRNA - Messenger ribonucleic acid

mSin3A - Histone Deacetylase 1 Corepressor Complex

NES – Nuclear Export Signals

NHEJ - Non-homologous End-Joining

PBS - Phosphate Buffered Saline

PCNA - Proliferating Cell Nuclear Antigen

PTEN - Phosphatase and Tensin Homolog

RNP – Ribonucleoprotein

ROS - Reactive Oxygen Species

SDS - sodium dodecyl sulfate

SDS-PAGE – sodium dodecyl sulfate polyacrylamide gel electrophoresis

SV40 - Simian Vacuolizing Virus 40

TAD - Transcription Activation Domain

TEMED - tetramethylethylenediamine

VA - Virus-Associated

WB – Western Blot

LIST OF FIGURES

LIST OF TABLES

1. INTRODUCTION

1.1 The tumor suppressor p53

1.1.1 p53 functions

P53 is at the centre of the DNA damage sensory system. Its activation can induce different cell fates depending on input signals. The p53 protein is maintained at low levels in the cells because of its active degradation by the proteasome, mediated by the E3 ubiquitin ligase MDM2. In response to various forms of stress, like DNA strand breaks, ionizing radiation or UV induced damage, p53 is stabilized by protein–protein interactions and post-translational modifications that allow p53 to escape the degradation. Once stabilized, p53 integrates different signals and triggers gene expression to arrest the cell cycle or to induce apoptosis. Loss of p53 function leads to uncontrolled proliferation and promotes cancer development (Horn & Vousden, 2007; Olivier et al, 2009)-Fig. 1.

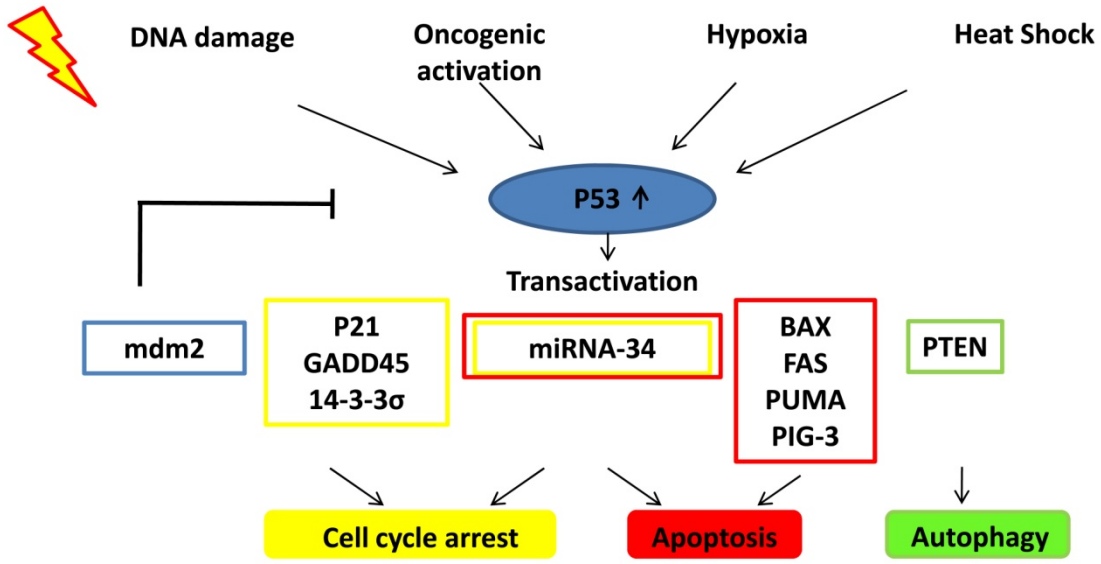

Figure 1. p53 signalling. In response to different stimuli p53 accumulates and binds to number of gene promoter inducing their expression and different cell faiths (modified from Stewart and Pietenpol, 2001).

1.1.1.1 Transcriptional activation

p53 is a transcription factor. It has a sequence-specific DNA-binding activity and the potential to induce the expression of a large number of genes. Between 500 and 1600 genes have been shown to contain p53-binding sequences and to respond to p53 (Cawley et al, 2004; Wei et al, 2006). p53 binds to the promoter regions and then recruits general transcription factors as well as histone acetyltransferases (HATs), such as CBP, p300 and

PCAF. These HATs acetylate lysine residues of histones, thereby increasing the accessibility of chromatin to the transcriptional apparatus (Liu et al, 1999). P53 binding can have both enhancing as well as repressing outcome. The differences in the sequence and spacing of the p53-binding sites, the overall levels and post-translational modifications of p53 and the presence or absence of transcriptional cofactors can all contribute to promoter selection and choice of response.

P53 can induce different cell fates. Therefore, pathways which induce cells to commit to either survival or cell death were investigated. Interestingly, it was found that p53 binds with high affinity to response elements found in cell cycle arrest gene promoters and with lower affinity to the response elements located in apoptosis related genes (Qian et al, 2002). Moreover, phosphorylation of p53 on serine 46 has been shown to contribute specifically to the activation of some apoptotic target genes, and mutation of this phosphorylation site reduces the ability of p53 to induce cell death but not proliferative arrest (Mayo et al, 2005; Oda et al, 2000b). Furthermore, acetylation of the C-terminus of p53 can affect its apoptotic activity (Chao et al, 2006; Knights et al, 2006). Alternatively, modifications may also function less directly, by regulating the interaction of p53 with co-activator proteins.

1.1.1.2 Cell cycle control

P53 targets genes that mediate cell cycle arrest and promote cell survival. Upon exposure to genotoxic stress, p53 induces p21. P21 inhibits cyclin-dependent kinases, resulting in pRB hypophosphorylation and subsequent G1/S arrest. Moreover, it binds to the Proliferating Cell Nuclear Antigen (PCNA) and prevents the elongation step in the DNA replication, blocking cells at the S-phase of the cell cycle (Stewart & Pietenpol, 2001). P53 can also control G2/M arrest via GADD45 and 14-3-3σ (Chan et al, 1999; Wang et al, 1999). Moreover, p53 can regulate the Adenomatous Polyposis of the Colon (APC) gene. APC is involved in the cell cycle control, chromosome segregation, mitotic spindle assembly, cell migration, cell adhesion and apoptosis. The phosphorylation status of p53 governs the transcription of APC (Jaiswal & Narayan, 2001). Interestingly, p53 can also protect cells from oxidative-stress-induced DNA damage and apoptosis by decreasing the levels of reactive oxygen species (ROS) in cells (Bensaad et al, 2006; Sablina et al, 2005).

1.1.1.3 Apoptosis

Apoptosis induction is an essential function of p53 as a tumor suppressor. It has been shown that p53 can affect both the extrinsic and the intrinsic pathway. P53 can activate FAS, DR4 and DR5 death receptors located at the plasma membrane, and so control the extrinsic apoptosis pathway (Liu et al, 2004; Muller et al, 1998; Sheikh et al, 1998). However, it seems mostly the intrinsic pathway that utilizes p53 induced responses. One of the first pro-apoptotic genes characterized was BAX. It is a Bcl-2 family member, p53 binds to its promoter and activates its expression. It induces apoptosis through cytochrome C release from the mitochondria and subsequent caspase pathway (Thornborrow & Manfredi, 1999). Similarly, p53 can induce other Bcl-2 family members that function upstream of BAX/BAK, to induce apoptosis following DNA damage. Among them are PUMA and NOXA, BH3 domain-only proteins (Han et al, 2007; Nakano & Vousden, 2001; Oda et al, 2000a; Yu et al, 2001).

P53 also induces apoptosis by promoting oxidative degradation of mitochondrial components through up-regulation of pro-oxidative genes, such as PIG-3 and PIG-6. PIG-3 encodes a protein homologous to NADPH-quinone oxidoreductase, an enzyme that generates reactive oxygen species (Polyak et al, 1997).

Interestingly, p53 can also directly activate apoptosis. It translocates to the mitochondria and triggers caspase activation, a function that is independent of its transactivating domain (Marchenko et al, 2000).

1.1.1.4 Other functions

Recently, many new unexpected roles of p53 emerged. P53 can regulate cellular autophagy through up-regulation of PTEN (Phosphatase and Tensin homolog). PTEN inhibits the mTOR pathway, which in turn increases autophagy (Feng et al, 2007). Cellular autophagy is a process involving lysosomal degradation of cytoplasmic organelles. Interestingly, it has recently been shown, that p53 regulates the transcription of some microRNAs. They are small non-coding RNAs which bind the 3'-UTRs of partially complementary mRNAs and inhibit their translation and destabilize them. P53 controls the miR-34 family, enhancing apoptosis, cell cycle arrest and senescence (Hermeking, 2009). Furthermore, p53 seems to play a role in many other pathways i.e., cell adhesion, protein catabolism, metabolism and ion transport.

1.1.2 p53 structure

1.1.2.1 Amino-terminal region

The primary structure of p53 can be separated into a few functional domains (Fig. 2). The N-terminal portion consists of a transcription activation domain (TAD) and an adjacent proline-rich domain. The TAD can be further subdivided into two smaller domains: TAD1 (residues 1-40) and TAD2 (residues 41-67). TAD1 is necessary to induce cell cycle arrest but not apoptosis. In contrast, TAD2 is required for p53-induced apoptosis (Harms & Chen, 2006). These two domains are major sites for post-translational modifications, which influence p53 interactions with regulatory proteins such as Murine Double Minute 2 (Mdm2) (Espinosa & Emerson, 2001; Gu et al, 1997). Similarly to TAD2, the proline-rich domain (residues 67-98) has been implicated in growth suppression and activation of apoptosis (Baptiste et al, 2002; Walker & Levine, 1996; Zhu et al, 2000).

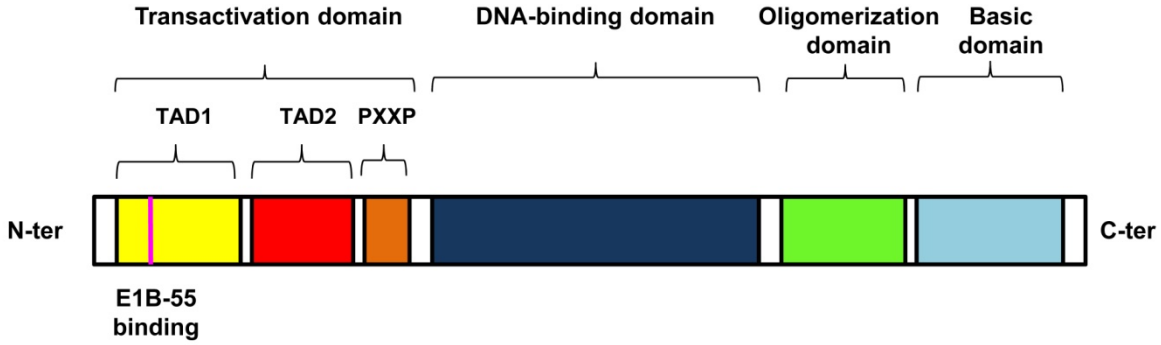

Figure 2. Linear schematic representation of the p53 domains (modified from Vousden and Prives, 2009).

1.1.2.2 DNA-binding region

Next to the transactivation domain is the DNA-binding domain (residues 98– 303). It serves mainly for DNA interactions. P53 recognizes the half-decamer DNA consensus sequences RRRC(A/T)(A/T)GYYY, where R stand for purine and Y for pyrimidine, which are separated by a 0-13 bp spacer. The spacer size affects the affinity of the p53 for target promoter sequence (el-Deiry et al, 1992; Funk et al, 1992; Tokino et al, 1994). Interestingly, the DNA-binding domain is also a site for interactions with the Apoptosis Stimulating Protein of P53 (ASPPs) and the p53 homologues p63 and p73 (Flores et al, 2002; Samuels-Lev et al, 2001).

1.1.2.3 Carboxy-terminal domain

The C-terminal portion of p53 contains the oligomerization domain and the basic domain. The oligomerization domain (residues 323–363) allows p53 to form tetramers. The p53 monomer is V-shaped, it comprises an alfa-helix and a beta-strand linked in a sharp turn. Two monomers associate via a beta-strand anti-parallel double sheet, the tetramer is formed by subsequent dimmers with helix-helix interactions (Fig. 3). The oligomers are held together largely by hydrophobic interactions (Chene, 2001; Harms & Chen, 2006). Interestingly, an additional basic domain can be found at the extreme C-terminus end (residues 363–393). It can both positively and negatively regulate p53. It binds DNA in a non-specific manner and increases DNA bending (Nagaich et al, 1999). This domain is also subjected to many post-translational modification and protein interactions (Appella & Anderson, 2001; Feng et al, 2005).

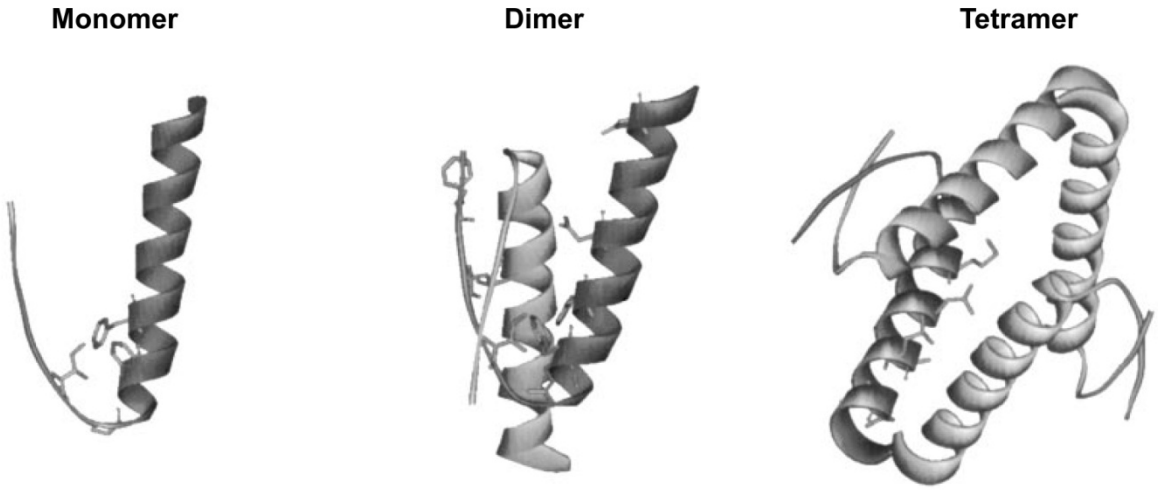

Figure 3. Structure of p53 oligomerization domain. Ribbon representation of a monomer (residues 326 ± 356); both chains of the dimer; four chains forming the tetramer (taken from Chene, 2001*).*

1.2.2.4 Oligomerization domain

P53 is only active in a tetrameric state, with regard to transcriptional activity and tumor suppression, as it recognizes and binds specific consensus sequences in a tetramerized form. Although p53 monomers can bind to the DNA and stimulate transcription under specific circumstances, the affinity of this binding is much weaker (Balagurumoorthy et al, 1995).

P53 tetramerization is also important for many protein-protein interactions. Some of the interactions map directly to the oligomerization domain: binding for casein kinase 2 (Gotz et al, 1999), or the Ca2+-dependnet protein kinase C (Delphin et al, 1997). Alternatively,

proteins bind efficiently only to tetrameric p53. The Mdm2 oncoprotein binds p53 and mediates its ubiquitination, marking p53 for degradation (Haupt et al, 1997; Kubbutat et al, 1997). Although the direct Mdm2/p53 interaction has been mapped to the N-terminal portion of p53 (Lin et al, 1994), the oligomerization domain of p53 enhances the interaction of the two proteins and p53 degradation. Only high Mdm2 concentrations allowed the degradation of monomeric p53 (Kubbutat et al, 1998). Many viral proteins also interact with p53, especially oncogenes from the family of small DNA tumor viruses. The large T-antigen protein from the simian vacuolizing virus (SV40) is forms oligomeric structures. Large T-antigen oligomerization is essential for the proper T-antigen/p53 interaction. Moreover, p53 oligomerization is required for the effective T-antigen/p53 binding (Montenarh et al, 1986; Tack et al, 1989). Interestingly, also the E2 oncogene from the human papilloma virus can bind to p53. The dimerization region of E2 is important for this interaction, and p53 oligomerization mutants are defective for the E2/p53 interaction (Massimi et al, 1999). The requirement for tetramerization of p53 in protein-protein interactions raises a possibility that these interactions often rely on the ability of both component to homo-oligomerize. Before this study was carried out, it was not clear if the oligomerization is required for the interaction of p53 with E1B-55 kDa, an adenoviral oncoprotein.

1.2 Adenovirus E1B-55 kDa oncogene product

1.2.1 Adenovirus

Adenoviruses constitute the Adenoviridae family of viruses, which traditionally is divided into two genera, Mastadenovirus and Aviadenovirus (Benko et al, 2000). Adenovirus (Ad) is a non-enveloped virus, with linear double stranded dsDNA genome and icosahedral particles architecture. The molecular weight of the whole virion has been estimated to be 175-185 x 10^6 daltons (Benko & Harrach, 2003).

The linear genome of an adenovirus contains two identical origins of DNA replication, each present in the terminal repeat (inverted terminal repeat: ITR). The adenovirus genes are subdivided into early genes (five transcript units: E1A, E1B, E2, E3, and E4), which are expressed before the onset of the viral DNA replication, intermediate genes (IX and IVa2), expressed at intermediate times in infection, and late genes (one late unit processed to generate five families of late mRNAs: L1-L5), which are transcribed after replication of the

adenovirus DNA has started. The chromosome carries also one or two (depending on the serotype) virus-associated (VA) genes (Flint, 1999).

Early region E1 contains genes involved in the cell transformation and in the regulation of transcription. E1A unit encodes three proteins that activate transcription and induce the host cell to enter the S phase of the cell cycle; E1B region encodes two proteins that block apoptosis; E2 encodes three proteins that function directly in the DNA replication; E3 encodes products that modulate the response of the host to infection; and the late family of mRNAs is concerned with the production and assembly of capsid components; E4 products mediate transcriptional regulation and mRNA transport and modulate DNA replication and apoptosis. Polypeptide IX is a minor structural component of the virion and polypeptide IVa2 appears to serve as a maturation protein during the adenovirus morphogenesis (Benko & Harrach, 2003; Branton, 1999).

1.2.2 E1B-55 kDa in adenovirus infection

1.2.2.1 Interaction with E4orf6

In adenovirus infected cells, E1B-55 kDa is mostly found together with another early gene product-E4orf6 (Sarnow et al, 1984). E1B-55 kDa requires E4orf6 for its nuclear localization (Goodrum et al, 1996). Moreover, these two proteins have been shown to accelerate the proteolytic degradation of p53. E1B-55 kDa/E4orf6 can be found together in high molecular mass complexes, which were shown to posses E3 ligase activity. They contain a Cullin family member –Cul5, elongins B and C, and the Rbx1 protein –RING-H2 finger protein. E4orf6 is required for the elongin C interaction, while E1B-55 kDa serves for the substrate recognition. To date, degradation of p53 (Harada et al, 2002; Querido et al, 2001a), the MRN complex (Stracker et al, 2002), and the DNA ligase IV (Baker et al, 2007) by this complex have been demonstrated (Fig. 4). One of the most interesting and yet not well understood functions of E1B-55 kDa is its importance in the late viral mRNA export and host mRNA inhibition. Together with E4orf6, it is essential for these processes. When either of the two proteins is missing, late protein synthesis and virus production are inefficient (Babiss et al, 1985; Halbert et al, 1985; Pilder et al, 1986; Weinberg & Ketner, 1986).

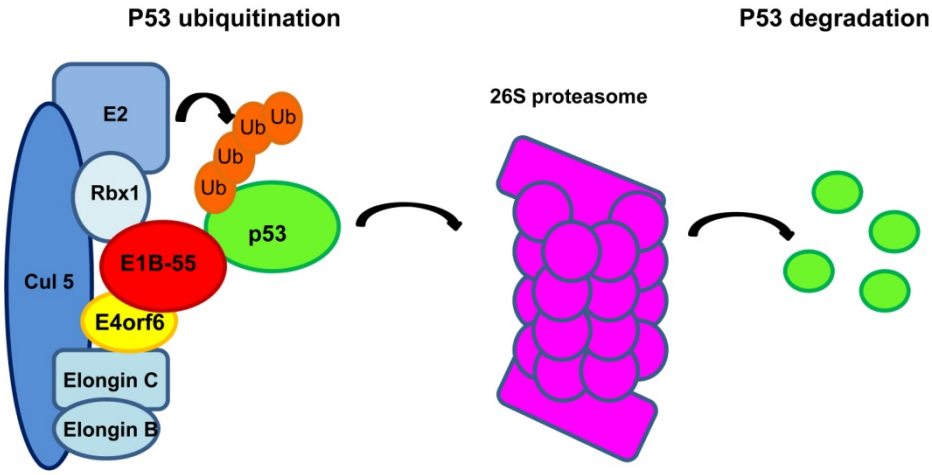

Figure 4. Schematic representation of E1B-55 kDa/E4orf6 E3 ligase complex activity on p53 (modified from Blackford and Grand, 2009).

1.2.2.2 Interaction with p53

E1B-55 kDa is expressed early during viral infection and has multiple functions in the virus life cycle. E1B-55 kDa importance first came into attention, when it was found that it can bind to p53 (Sarnow et al, 1982), neutralizes its activity and, as a result, blocks p53-dependent cell cycle arrest and apoptosis. E1B-55 kDa seems to utilize several mechanisms to accomplish this. E1B-55 kDa mostly localizes to cluster-shaped aggregates in the cytoplasm that were reported to represent aggresomes by some groups (Liu et al, 2005) but found distinct from aggresomes by others (Zhao & Liao, 2003). p53 is found in these clusters together with E1B-55 kDa (Blair Zajdel & Blair, 1988; Zantema et al, 1985). However, relocalization is not enough to inhibit p53 transactivating properties. Instead, E1B-55 kDa inhibits transcriptional activation of p53 through a direct interaction (Grand et al, 1995a). Several studies have shown that E1B-55 kDa carries a transcription repression domain that can be tethered to other proteins and in this way changes them from activators to repressors. This activity requires a cellular corepressor that has so far eluded identification (Martin & Berk, 1998). One of the candidates was the histone deacetylase 1 corepressor complex (mSin3A). It binds to E1B-55 kDa, however, it is not required for the p53 inhibition (Zhao et al, 2007). Furthermore, during the adenoviral infection, the p53 protein is degraded due to the E1B-55 kDa/E4orf6 induced ubiquitination and subsequent degradation (Harada et al, 2002; Querido et al, 2001a; Querido et al, 1997; Querido et al, 2001b; Roth et al, 1998; Steegenga et al, 1998).

1.2.2.3 Effect on the cellular DNA damage pathway

The adenovirus genome is present as a linear double stranded DNA, which is sensed as double-strand breaks by the host cells. Together with the replication intermediates, this activates the cellular DNA damage response and the non-homologous end-joining (NHEJ) process. The Mre11/Rad50/Nbs1 (MRN) complex is responsible for DNA double strand break repair. Its action leads to a covalent end joining of the viral genome, resulting in viral concatemerization. This, in turn, disturbs the viral replication and, later on, the efficient packaging of the viral genome into the capsid (Hearing et al, 1987; Rawlins et al, 1984). Degradation of the complex in an E1B-55 kDa/E4orf6 dependent manner avoids this, thereby supporting the viral life cycle (Stracker et al, 2002). Interestingly, the E1B-55 kDa/E4orf6 complex can target for degradation also other proteins important in downstream steps of NHEJ. The DNA ligase IV is necessary for the concatenation reaction itself. Adenovirus uses the ubiquitination machinery and the proteasomal degradation of DNA ligase IV to ensure that no concactamers are formed (Baker et al, 2007).

Furthermore, in response to DNA damage, ataxia-telangiectasia mutated kinase (ATM) and ATM- and Rad3- related kinase (ATR) can be activated. Indeed, in cells infected with E4orf6 deleted Ad, they are phosphorylated and they accumulate in the virus replication centres (Carson et al, 2003). This accumulation is dependent on the availability of NBS1, which in wild type infected cells is depredated, avoiding kinase activation (Lee & Paull, 2005).

Multiple ways, through which the E1B-55 kDa/E4orf6 complex can inactivate the DNA damage response, underlines the importance of E1B-55 kDa for the viral replication.

1.2.2.4 Effect on the virus and host mRNA

During its life cycle, adenovirus simultaneously stimulates the viral mRNA nuclear export and inhibits the host mRNA export (Beltz & Flint, 1979). These effects on mRNA depend on the E1B-55 kDa/E4orf6 complex formation and its ubiquitin ligase activity (Blanchette et al, 2008; Woo & Berk, 2007). Moreover, the protein complex can shuttle between the cytoplasm and the nucleus, using export signals (NES) which can be found both within the E4orf6 (Weigel & Dobbelstein, 2000) as well as in the E1B-55 kDa protein (Dosch et al, 2001; Kratzer et al, 2000). However, it does not seem to be the main platform for the viral mRNA

transport (Flint et al, 2005). Interestingly, the E1B-55 kDa protein has a homology with a family of RNA –binding proteins. Although E1B-55 kDa can bind RNA in a weak fashion in vitro, no specificity was observed for binding either the host cell or the viral mRNA (Horridge & Leppard, 1998). Mutants lacking E1B-55 kDa express a conditional phenotype. Elevating the temperature and infecting cells in the S phase of the cell cycle can rescue the late functions of the E1B-55 kDa (Goodrum & Ornelles, 1998; Goodrum & Ornelles, 1999; Harada & Berk, 1999). This suggests that cellular proteins are important to sustain these adenoviral needs. E1B-55 kDa together with E4orf6 could potentially affect these cellular partners' expression and or activation. Despite the intense research in this field, many aspects of E1B-55 kDa functioning still need elucidation and further investigation.

1.2.3 E1B-55 kDa in transformation and tumorigenesis

Adenoviruses can cause tumors in rodents. Although these cells do not produce viral progeny, portions of the viral genome are detected integrated into the chromosomes of the host cells. E1A alone can fully transform and immortalize primary rodent cells. However, this process is more efficient when the E1B-55 kDa protein is present in the cells (Levine, 2009). The role of E1B-55 kDa in transformation is not yet entirely clear. E1B-55kDa seems to be required, not only to block p53 this way counteracting apoptosis and the cell cycle arrest (Hutton et al, 2000; Teodoro & Branton, 1997; Yew & Berk, 1992). Recently, the importance of the E1B-55 kDa effect on the DNA double-strand break repair complex in the process of transformation was demonstrated (Hartl et al, 2008). Moreover, it was showed that the E1B-55 kDa domains required for the transformation are encoded within several patches of its primary sequence, including several clustered cysteine and histidine residues, some of which match the consensus for zinc fingers. In addition, two amino-acid substitutions (C454S/C456S) lead to substantially reduced E1B-55 kDa transforming activity (Hartl et al, 2008). Furthermore, it has been shown that the SUMO1 conjugation at the amino acid K104 is required for the efficient nuclear import of the viral protein in transformed baby rat kidney (BRK) cells (Endter et al, 2001).

1.2.4 E1B-55 kDa in tumor-specific virus replication

One of the most interesting E1B-55 kDa activities is the p53 inhibition. It was suggested that this function is connected to efficient viral growth. It was observed that for a ΔE1B-55 adenovirus mutant (ONYX015), replication was restricted in cells expressing wt p53, but efficient in cells with mutant or absent p53 (Bischoff et al, 1996). ONYX015 can replicate efficiently in tumour cells but not in primary cells. This led to the hypothesis that E1B-55 kDa deleted virus could potentially be used as an oncolytic therapeutic agent. This approach worked quite successfully in animals as well as later in patients, especially in trials to treat head and neck cancer (Dobbelstein, 2004). However, subsequent studies found that the mutant replication did not correlate with the p53 status but is rather based on the ability of E1B-55 kDa to influence the mRNA export/import at later stages of viral life cycle (Goodrum & Ornelles, 1998; Hall et al, 1998; Harada & Berk, 1999; Rothmann et al, 1998; Turnell et al, 1999). The success of the treatment with ONYX015 seems to depend on whether the specific tumour supports the late RNA export. As mentioned before, RNA export functions in ONYX015 infected cells can be rescued by a heat shock. In many tumours, pathways connected to heat shock proteins are deregulated, which may explain the replication selectivity of the ONYX015 virus. Better understanding of the E1B-55 kDa interaction with cell components might help developing more efficient strategies for cancer virotherapy.

1.2.5 E1B-55 kDa protein characteristics

1.2.5.1 Localization to cytoplasmic speckles

E1B-55 kDa mostly localizes to cluster-shaped bodies in the cytoplasm, both in virus infected and transformed cells. These seem to represent aggresomes (Liu et al, 2005). Aggresomes are cytoplasmic inclusion bodies that accumulate mis- or unfolded proteins, along with other proteins marked for degradation (Kopito, 2000). They are enriched in heat shock protein 70, vimentin and microtubules, and it was shown that E1B-55 kDa coprecipitates with them. Moreover, E1B-55 kDa seems to contribute to the formation of these cytoplasmic bodies (Liu et al, 2005). The E1B-55 kDa oncoprotein binds the proteins of interest and sequesters them in aggresome structures. p53, as well as MRN complexes and E4orf6, are relocalized to these structures together with E1B-55 kDa. Interestingly, also Cul5, member of the ubiquiting ligase complex, localizes to the same structures (Liu et al, 2005; Ornelles & Shenk, 1991;

Zantema et al, 1985). Enrichment of these proteins in aggresomes allows for an increased rate of polyubiquitination and subsequent degradation of E1B-55 kDa targets (Liu et al, 2005).

1.2.5.2 Post-translational modifications

Two different types of posttranslational modification have been attributed to Ad5 E1B-55 kDa until now. Ad5 E1B-55 kDa sequence analysis identified a sumoylation site at the position K104. SUMO-1 is a small ubiquitin-like modifier that can be conjugated to many cellular proteins though its carboxy-terminal glycine residue. It has been reported that SUMO-1 can modify the E1B-55 kDa lysine residue at position 104 and that this modification is required for the transforming functions of the oncoprotein (Endter et al, 2001). Moreover, it seems that SUMO-1 modification of E1B-55 kDa is needed for the CRM1-dependent nuclear export pathway (Kindsmuller et al, 2009).

Importantly, E1B-55 kDa is highly phosphorylated on serine and threonine residues (Malette et al, 1983; Teodoro & Branton, 1997). The phosphorylation of the carboxy-terminal serines 490 and 491 as well as threonine at position 495 have been shown to be essential in transforming and transcriptional repression activities of the E1B-55 kDa oncoprotein. While the double mutant S490/S491A still binds p53, it is no longer able to repress p53 transcriptional activation. Moreover, it seems that other E1B-55 kDa functions, like the late mRNA export, are not influenced (Teodoro & Branton, 1997). Interestingly, if all three phosphorylation sites are mutated, the E1B-55 kDa not only loses its aggresomal localization, but also is defective for the degradation of substrates (Schwartz et al, 2008).

1.2.5.3 E1B-55 kDa functional domains

Until now, not much structural data is available for E1B-55 kDa, although some functional domains of E1B-55 kDa have been characterised (Fig. 5). Early studies suggested that it is of nonglobular shape, with an elongated structure. Moreover, a purified protein can form dimers or even tetramers (Grand et al, 1995b; Martin & Berk, 1998). To better understand E1B-55 kDa and to try to narrow down residues responsible for different E1B-55 kDa functions, extensive mutagenesis was performed.

The E1B-55 kDa oncoprotein is a well-conserved protein across different adenovirus serotypes. The homology is least marked at the amino-terminal region. Interestingly, a nuclear export signal (NES) lies within the first 100 amino acids. It is well conserved within groups B, C, D and E, but not in A and F. This seems to correlate with the fact that the Ad12 E1B-55 kDa (group A) localizes predominantly to the nucleus, while the Ad5 E1B-55 kDa (group C) can be found in majority within the cytoplasmic clusters (Blackford & Grand, 2009). E1B-55 kDa has been shown to have a weak RNA-binding activity which maps to a region of the protein with the homology to a family of RNA-binding proteins (Horridge & Leppard, 1998). This domain within the central portion of E1B-55 kDa seems to be important for most of the E1B-55 kDa functions studied so far. Described insertions at positions H215, A262, R309, H326 create virus mutants which behave as E1B-55 null virus. It has been suggested that insertions at these positions disrupt the E1B-55 kDa structure and possibly its stability (Gonzalez & Flint, 2002; Liu et al, 2005).

Extensive mutational analysis of E1B-55, led to the discovery of separation-of-function mutants. E1B R240A cannot bind and relocalize p53, although it forms the cytoplasmic speckles. Furthermore, it did not induce degradation of p53, although the MRN complex and the DNA ligase IV degradation are intact. The interaction with the DNA ligase IV and the MRN complex occur in different E1B-55 kDa domains, although they are both important in the dsDNA damage pathway. Insertions at position H354 abrogate degradation of the MRN complex but not of DNA ligase IV (Schwartz et al, 2008; Shen et al, 2001). These results showed that E1B-55 kDa binding partners use different regions on E1B-55 kDa for the interactions.

As described above, the C-terminal region of E1B-55 kDa is also essential for its proper localization and functioning due to its phosphorylated residues.

Figure 5. Linear representation of E1B-55 kDa and its domains. Different motifs are indicated: nuclear export sequence (NES), homology to RNA-binding proteins domain (RNP), site of the SUMO-1 modification, three sites for phosphorylation (P) (modified from Blackford and Grand, 2009).

1.3 Aim of the study

Previously, we found that E1B-55 kDa forms SDS resistant protein adducts and that the central domain of E1B-55 kDa is necessary for their formation (Master thesis Morawska, 2007). The aim of my study was to investigate possible functional implications of these high molecular weight (HMW) complexes. In the course of the study, I discovered that E1B-55 kDa can form homo-oligomers in the cellular context. I investigated the role of the central portion of the protein in the oligomerization, and the requirement of E1B-55 kDa oligomerization for the protein proper functioning. The cellular localization of E1B-55 kDa oligomerization mutants was investigated, as well as, the interaction of E1B-55 kDa-mutants with E4orf6 and p53.

Interestingly, p53 is known to tetramerize. This process is essential for its proper functioning not only as a transcription factor but also in protein-protein interactions. Viral oncoproteins, like adenovirus 5 E1B-55 kDa, interact with p53 rising the possibility that these interactions rely on the ability of either component to homo-oligomerize.

2. MATERIALS AND METHODS

2.1 Materials

2.1.1 Antibodies

Table 4: Primary antibodies

Name	Antigen Origin	Clone	Dilution for WB	Dilution for IF	Company
Anti-E1B-55 kDa	mouse monoclonal	2A6	1:20	1:10	Home-made hybridoma supernatant
Anti-Myc tag	mouse monoclonal	4A6	1:1000	1:500	Upstate
Beta-actin	mouse monoclonal	AC-15	1:10000		Abcam
HA.11	mouse monoclonal	16B12	1:1000	1:500	Convance
Anti-HA tag	rabbit polyclonal	Y-11		1:50	Santa Cruz
GFP	mouse monoclonal		1:1000		Clontech
Anti-p53	mouse monoclonal	DO1	1:1000	1:500	Santa Cruz
Anti-p53	rabbit polyclonal	FL393	1:1000	1:500	Santa Cruz

Table 5: Secondary antibodies

Name	Dilution for WB	Company
Peroxidase-conjugated affiniPure F(ab')2 Fragment, donkey anti-mouse IgG (H+L)	1:10000	Jackson Immuno Research
Alexa Fluor 488 anti-mouse	1:500	Molecular Probes, Invitrogen
Alexa Fluor 488 anti-rabbit	1:500	Molecular Probes, Invitrogen
Alexa Fluor 596 anti-mouse	1:500	Molecular Probes, Invitrogen
Alexa Fluor 596 anti-rabbit	1:500	Molecular Probes, Invitrogen

2.1.2 Bacteria

Table 6: Bacterial strains

Name	Company
ElectroMAX™ DH10B™Electrocompetent cells	Invitrogen
SURE® Electro-competent cells	Stratagene

2.1.3 Chemicals

2-Mercaptoethanol	Roth
2-Propanol	Roth
Acetic Acid	Roth
Agar	Fluka
Agarose NEEO Ultra	Roth
Ammoniumpersulfat	Roth
Ampicillin anhydrous, 96,0-100,5%	Sigma-Aldrich
Bovine serum albumin (BSA)	New England Biolabs
Bromphenolblue	Sigma-Aldrich
Calciumchloride	Merck
Chloroform	Roth
Complete, EDTA-free	Roche
Disodium-hydrogen-phosphat	Merck
DMSO for Cell Culture	AppliChem
DTT (1,4-Dithiothreitol)	Roth
EDTA (Ethylendiaminetetraacetate)	Roth
Ethanol 99,9%	Merck
Formaldehyde	Roth
Glycerin	Roth
HCl	Roth
HEPES	Roth
Isopropanol, p.A.	Geyer

Magnesium chloride	Merck
Methanol	Roth
Milk powder	Naturaflor Töpfer
Natriumthiosulfate pentahydrate	Roth
Nonidet P40 substitute	Amersham
NaOH-pieces	Roth
PBS 10x	Invitrogen
PBS tablets	Invitrogen
Peptone	Casein Roth
pH-Solution 10,01	Roth
pH-Solution 4,01	Roth
pH-Solution 7,01	Roth
Ponceau S	Roth
Potassium chloride	Roth
Potassium-dihydrogen-phosphate	Roth
Rotiphorese Gel 30 (30% Acrylamid–Lösung)	Roth
Silver nitrate	Roth
SDS (Sodiumdodecylsulfat)	BioRad
Sodium acetate	Roth
Sodium azide	Applichem
Sodium carbonate	Roth
Sodium chloride	Roth
Sodium dihydrogenphosphate	Roth
Sodium hydrogencarbonate	Roth

TEMED (N,N,N´,N´-Tetramethylendiamin)	Roth
Tripton	Roth
Tris	Roth
Triton x-100	Applichem
Tween 20	AppliChem
Urea	Roth
Yeast Extract	Fluka

2.1.4 Consumable Materials

12-well Cell culture plates	Greiner
24-well Cell culture plates	Greiner
Cell culture flask (175 cm2, 75 cm2)	Greiner
4 well ChamberSlides	NUNC
Coverslips	Hounisen
Filter tips, Biosphere	Sarstedt
Gel-Blotting-Paper GB002 (Whatman-Papier)	Schleicher & Schuell
Gloves, „Safe Skin PFE“	Kimberly Clark
Nitrocellulose Protran Transfer Membrane BA83	Schleicher & Schuell
PCR-tubes (200 µl)	Sarstedt
Pipette tips „Tip One“	StarLab
Pipettes, sterile (5ml, 10ml und 25ml),	Sarstedt
Tube (1,5 ml/2 ml)	Sarstedt
Tube 15ml, 120x17mm, sterile	Sarstedt
Tube 50ml, 114x28mm, sterile	Sarstedt
X-ray Films (blue) RX 13x18 100Bl	Fuji, Ernst Christiansen

2.1.5 Eukaryotic cell lines

Cell line:

H1299 -The human lung adenocarcinoma, p53 null

Sustained in DMEM supplied with: 10 µg/ml Ciprofloxacin, 50 U/ml Penicillin, 50 µg/ml Streptomycin, 2 µg/ml Tetracycline, 10% FCS, 200 µM L-Glutamine.

2.1.6 Equipment

Biological Safety Cabinet „Hera Safe“	Heraeus Instruments
Counting Chamber (Neubauer)	Brand
Developing Machine „Optimax X-Ray Film Processor“	Protec Medizintechnik
Freezers –20° C	Liebherr
Freezers -80°C „Hera freeze“	Heraeus Instruments
Heating Block „Thermomixer comfort“	Eppendorf
Incubator (Cellculture) „Hera Cell 150“	Heraeus Instruments
Magnet stirrer „MR 3001“	Heidolph Instruments
Megafug 1.0R 4°C	Heraeus Instruments
Microscope „HBO 100“	Karl Zeiss
Microscope Hund Wetzlar „Wilovert LL“	Helmut Hund
Mini-centrifuge Model GMC-060	LMS Laboratory & Medical
pH-meter „CG 832“	Schott
Pipette-Aid ® portable XP”	Drummond
Pipettes „Research“ (2,5 µl, 20 µl, 200 µl und 1000 µl)	Eppendorf
Power Supply „Standard Power Pack P25T“	Whatman Biometra

Refrigerators 4°C „profi line“	Liebherr
SDS-PAGE-Chamber „MiniVE“	Amersham Biosciences
Shaker „Rocky“	Schütt Labortechnik
Spectrophotometer „NanoDrop ® ND-100“	Peqlab Biotechnologie
Table-centrifuge Type 5415R	Eppendorf
T-Personal (PCR-maschine)	Biometra
Vortex-mixer „Vortex Genie 2“	Scientific Industries
Weighing Machine	Sartorius
Western Blot Chamber for Semidry Blotting	Harnischmacher
X-Ray Cassettes 13x18cm	Rego X-Ray

2.1.7 Kits and Reagents

dNTP-Mix, 20mM	BioBudget
Fluorescent Mounting Medium	Dako Cytomation
GeneRuler 1kb DNA Ladder Mix	Fermentas
Lipofectamine™ 2000 Transfection Reagent	Invitrogen
PageRuler Prestained Protein Ladder	Fermentas
peqGOLD® Plasmid Miniprep Kit I (Classic Line)	Peqlab Biotechnologie
peqGOLD® Plasmid Miniprep Kit II (Classic Line)	Peqlab Biotechnologie
Invisorb Spin Plasmid Mini Two	Invitek
Protein A sepharose ™ CL-4B	GE Healthcare
Protein G sepharose	GE Healthcare
PfuUltra™ High-Fidelity DNA polymerase	Stratagene
QIAquick® PCR Purification Kit	Qiagen
SuperSignal West Dura Extended Duration	Pierce

SuperSignal West Femto Maximum Sensitivity	Pierce
Taq-Buffer $(NH4)_2SO_4$ and 25 mM $MgCl_2$	Fermentas
Taq-Polymerase	Fermentas

2.1.8 Plasmids

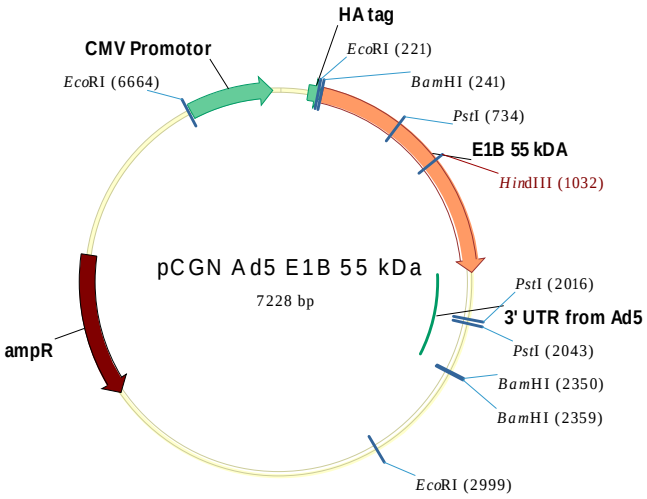

Figure 6. Plasmid maps of the pCGN E1B-55 kDa expressing vectors. Plasmid map of the Ad 5 E1B-55 kDa expressing vector pCGN Ad5 E1B-55 kDa. It is driven by the CMV promoter and contains an influenza flu tag (HA tag) at the amino-terminus.

Expression plasmids used:

- p53, p53 22/23 (Lin et al, 1994),
- HA-tagged Ad12 E1B-55 kDa (Wienzek et al, 2000),
- HA-tagged Ad5 E1B-55kDa and E4orf6 (Dobbelstein et al, 1997),
- P53 CC (Roth et al., 2000)
- p53-responsive firefly luciferase reporter plasmid -pBP100luc (Roth et al, 1998),
- p53DD and p53DDΔS - kind gifts of Moshe Oren,
- p53 LLL (L373, L380, L387) and p53 KEEK (K341, E344, E348, K355)- kind gifts of Karen Vousden,
- HA-tagged Ad5 E1B-55kDa Del 245-296, HA-tagged Ad5 E1B-55kDa Stop396 and MYC-tagged Ad5 E1B-55 kDa (Morawska Msc Thesis, 2007).

2.1.9 Oligonucleotides

Primers were synthesized at MWG Biotech, Ebersberg. Lyophilisates were solved in sterile water to give a 100 pmol/µl stock solution.

Table 4: Primers

Name and/or type of mutation	Sequence (5'- 3')
CMV	GGCGTG TACGGTGGGAGG TC
insE1B_rev	TCAAACGAGTTGGTGCTCAT
E1B R240A FnuDII	GTTATTATGAATGTCGCGTTTACTGGCCCC
E1B Δ250-255 KpnI	CCAATTTTAGCGGTACCAACCTTATCCTACACG
E1B Δ281-288 HhaI	CCTGGACCGATGTGCGCGTTTGGAAGGGGGTGG
E1B Δ296-310 BamHI	GGTGGTGTGTCGCACCTTGGGGATCCTGTCTG
E1B F307A BstUI	CAATTAAGAAATGCCTCGCGGAAAGGTGTACC
F264A no HindIII	CCTACACGGTGTAAGCGCCTATGGGTTTAA C
F285A BstUI	GGGGCTGTGCCGCGTACTGCTGCTGGAAGG
R281A NaeI	CCGATGTAAGGGTTGCCGGCTGTGCCTTTTACTGC
F251A no CfrI	CCCCAATTTTAGCGGTACGGTTGCCCTAGCCAATACCAACC
T255A no CfrI	GCGGTACGGTTTTCCTTGCCAATGCCAACCTTATCCTACACGG
Y286A HhaI	GGGGCTGCGCCTTTGCCTGCTGCTGGAAGGGG
E1B CCWK –AAAA PstI	GCTGTGCCTTTTACGCTGCAGCTGCTGGGGTGGTGTGTCG
E1B KKC AAA PstI	CCAAAAGCAGGGCTTCAATTGCTGCAGCCCTCTTTGAAAGGTG TACC
p53 Stop332 no PsuI	GAATATTTCACCCTTCAGTAGCGTGGGCGTGAG
p53 Stop310 MluI	CGAGCACTGCCCTAACGCGTCAGCTCCTCTCCC
p53rev	GGCGGGAGGTAGACTGACCC
GFP-E1B 244-310 for EcoRI GFP-E1B 244-310 rev BamHI	AAGGTTGAATTCCCCCAATTTTAGCG TACCGGATCCACACCTTTCAAAGAGG
HA-fragE1B for XbaI HA-fragE1B rev XbaI	AAGGTTTAGCGGCCGCAATTTTAGCG TACCGGATCCTCACCTTTCAAAGAGG
CC-Nterm-E1B for XbaI CC-Nterm-E1B rev XbaI	GTTGCGTCAGGGATCTAGAATGAAACAACT TAAACGAGCTCTGGATCTAGAGCGTTCGCC
CC-Middle-E1B for HindIII CC-Middle-E1Brev HindIII	CGAGATGTTCCGAAGCTTATCTAGAATGAAAC GCTCTGGATAAGCTTCGTTCGCCAACTAATTTC

2.1.10 Tissue Culture Materials

Ciprofloxacin (Ciprobay®)	Bayer
Dulbecco`s Modified Eagle Medium (DMEM)	GibcoBRL
Fetal calf serum, FCS	GibcoBRL
L-Glutamin	GibcoBRL
Penizillin/Streptomyzin	GibcoBRL
Tetracyclin	GibcoBRL
Trypsin/EDTA	GibcoBRL

2.1.11 Buffers and Solutions

Millipore water was used for preparation of all buffers and stock solutions.

Blocking solution: 10% FCS in PBS ++

10 x Blot Transfer Buffer (BTB): 58,2 g 48 mM Tris, 29,3 g 39 mM Glycine, 37,5 ml 10% SDS, add ddH_2O to 1L

1 x BTB (200ml): 20 ml 10 x BTB, 40 ml Methanol, 140 ml ddH_2O

Imunnoprecipitation buffer (IP-buffer):50 mM Tris-HCl, pH 7,5, 150 mM NaCl, 0,5 % NP-40

6 x Laemmli Buffer: 0,35 M Tris pH 6.8, 30% Glycerol (v/v), 10% SDS (w/v), 9,3% Dithiotreitol (DTT) (w/v), 0,02% Bromphenolblue (w/v)

2x Luria-Bertani Medium pH 7.0: 20g Trypton, 10g Yeast Extract, 10g NaCl, add H_2O until 1L,

add 15g agar per plate

Firefly Stock Buffer: 25mM Glycylglycine, 15mM K_2HPO_4, 4mM EGTA, pH 8.0

Renilla Stock Buffer: 1.1 M NaCl, 2.2mM Na_2EDTA, 0.22 M K_2HPO_4, pH 5.1

PBS (Phosphate buffered saline): 137 mM NaCl, 2,5 mM KCl, 8 mM Na2HPO4, 0,5 mM MgCl2, 1,47 mM KH2PO4, 0,9 mM CaCl2

PBS ++: 1x PBS with 10x Salt solution

PBS-T: 1x PBS with 0,1% Tween20.

Ponceau S - Solution: 0,5 g Ponceau S, 1 ml Acetic acid, 100 ml ddH_2O

RIPA-Buffer: 0,1% Triton X-100 (v/v), 0,1% Desoxycholat (v/v), 0,1% SDS (w/v), 2 mM Tris/HCl, pH 8,5, 9 mM NaCl, 1 mM EDTA, 1,4% Trasylol (100000 KIE)

10x Salt solution: 1g $MgCl_2$, 1g $CaCl_2$, add H_2O up to 1L

50xTAE: 2 M Tris, pH 7.8, 0.25 M Sodium acetate, 50 mM EDTA

2.2 Methods

2.2.1 Cloning

2.2.1.1 Plasmid DNA isolation using Plasmid Miniprep Kit

Colonies of transformed bacteria grown on Petri dishes were picked with sterile pipette tips and transferred into 3 ml 2x LB medium or containing 100 µg/ml ampicillin. Bacteria were grown overnight at 37°C with agitation. 3 ml overnight bacterial culture was centrifuged for 5 min at max speed. The pellet was thoroughly re-suspended with 250 µl Solution I. The suspension was mixed with 250 µl Solution II in order to lyse the bacteria and inverted 4 times to mix and incubated for 2-5 min at RT. To neutralize mixture 350 µl of Solution III were added, mixed by inversion and immediately centrifuged 10 min at max speed. The supernatant was transferred into a column supplied with a collection tube and centrifuged at max speed for 1 min at RT. The flowthrough was discarded and a washing step was performed by adding on the column 500 µl Binding Buffer and centrifugation at max speed for 1 min at RT. In the next washing step, 750 µl ethanol containing Wash Buffer was added and centrifuged as before. To dry the membrane an additional centrifugation for 3 min at max speed was performed. Finally, DNA was eluted by adding 50 µl EB buffer (supplied with the kit), putting the column in a new 1,5 ml eppendorf tube and spinning down (1 min at max speed). Samples were stored at -20°C.

2.2.1.2 DNA quantification by NANO Drop spectrophotometer

To determine DNA yield and quality, absorption of the samples was measured at 260 nm and 280 nm. The DNA concentration is calculated as follows:

DNA conc. (μg/ml)= Absorption 260 x 50

2.2.1.3 Restriction digestion

DNA digestion was executed in conditions and in the presence of buffers recommended by producer (New England BioLabs or Fermentas).

About 0,5-1,5 μg of mini prep DNA was digested using 1 U of an enzyme for 1 hour at appropriate temperature (generally 37°C) in total reaction volume of 10 μl.
For digestion of backbone -2 μg of DNA was digested using 2 U of an enzyme for 2 hours at appropriate temperature (generally 37°C).

2.2.1.4 Agarose gel electrophoresis

1% agarose gels were made using agarose melted in electrophoresis buffer 1x TAE, supplied with ethidium bromide (from a solution stock of 10 mg/ml, to a final concentration of 0,5 μg/ml). DNA samples were diluted with 6x Loading Buffer (Fermentas) and loaded into the wells. Electrophoresis was performed by applying an electric field across the gel inducing the DNA migration. The gel was examined using a transilluminator at 254nm and a Polaroid picture was taken.

2.2.1.5 Ligation

Ligations were performed using 50-100 ng of vector with triple molar excess of insert. Reactions were performed in presence of ATP and 2 Units of T4 DNA ligase in a total volume of 20 μl for 2 hours at room temperature or overnight at 16°C. To inactivate the ligase and purify the reactions, QIAquick PCR purification Kit was used. Usually, 2 μl of reaction was used for bacterial transformation.

2.2.1.6 PCR reactions

The polymerase chain reaction (PCR) is a method for oligonucleotide primer-directed enzymatic amplification of a specific DNA sequence of interest. The PCR product was amplified from the 100ng DNA template using a thermostable DNA polymerase from *Thermus aquaticus* (Taq DNA polymerase) (reaction mix, Table 5) and using an automated thermal cycler to put the reaction through 25 cycles of denaturing, annealing of primers, and elongation (Table 6). Primer used for different reactions (Table 5). After amplification by PCR, the products were separated by 1% agarose gel electrophoresis and were directly visualized after staining with ethidium bromide.

Table 5: PCR reaction pipetting scheme

	concentration	for 1 reaction
Water, nuclease free		27,75 µl
10 x Taq buffer (NH4)2SO4	1x	5 µl
2mM dNTP mix	20 mM	0,5 µl
Primer rev	50 ng/µl	5 µl
Primer for	50 ng/µl	5 µl
TaqDNA-Polymerase	1.25 u/ 50 µl	0,25 µl
25 mM MgCl2	3 mM	6 µl
Template	50 ng/µl	1 µl

Table 6: PCR cycling conditions

	temperature	**time**	**Cycle**
heat up the lid to 99°C			
1	95°C	2min	
2	95°C	30sec	
3	55°C	30sec	
4	72°C	1 min	go to step 2: 25x
5	12°C	Pause	

Ready PCR products were stored at -20°C.

2.2.1.7 PCR purification (QIAGEN)

QIAquick PCR Purification Kit was used. 5 volumes of buffer PB were added to 1 volume of PCR sample and mixed. Sample was applied into a column supplied with a collection tube

and centrifuged at max speed for 1 min at RT. Flowthrough was discarded; column was washed with 750 µl of buffer PE and centrifuged as before. After that, column was put to a new 1.5 ml tube. To elute DNA 30 µl H_2O was added and samples were centrifuged for 1 min at max speed.

2.2.1.8 Site-directed mutagenesis

Site directed mutagenesis is a PCR based method to introduce desirable mutation into a plasmid DNA sequence using especially designed oligonucleotides caring the desired mutation. During the first cycle of primer –DNA binding, the mis-matches (mutation) are introduced into the plasmid template. After successive cycles, the amount of the mutagenized plasmid will grow exponentially. Moreover, to eliminate the template DNA, enzymatic digestion with DpnI restriction enzyme is performed as it cleaves only methylated DNA –the freshly amplified DNA is not methylated. In the first step, the PCR reaction was prepared (reaction mix, Table 7).

Table 7: Site-directed mutagenesis reaction pipetting scheme

reaction-mix	concentration	Volume
H_2O		32,5 µl
buffer	10x	5 µl
dNTP's	20mM	0,5 µl
Primer1	50 ng/µl	5 µl
Primer2	50 ng/µl	5 µl
DNA	50 ng	1 µl
Polymerase	2,5 U/µl	1 µl
Final volume		50 µl

Table 8: Site-directed mutagenesis cycling conditions

temperature		time	cycle
heat up the lid to 99°C			
1	95°C	2 min	
2	95°C	30 sec	
3	50°C	30 sec	
4	68°C	17 min	go to step 2: 19x
5	12°C	pause	

Primers (Table 4) and their complements were used. DNA was amplified with PfuUltra™ High-Fidelity DNA Polymerase, the cycling condition were as described in Table 8. In the next step, 2 µl of DpnI (Fermentas) restriction enzyme (10 U/µl) was added to each PCR tube and

incubated 2-4h at 37°C. Then the QIAquick PCR purification Kit was used to prepare DNA for bacterial transformation. To introduce mutated plasmid into bacteria, 2 µl of the purified plasmids were elctroporated into SURE® *E. coli*. Next day, 10 single colonies were picked up and analyzed by PCR with primers that can amplify the E1B-55 kDa insert (Table 4, CMV and insE1B_rev) or p53 (Table 4, CMV and p53rev). These PCR products were then digested with appropriate restriction enzyme for 2h at 37°C and analyzed by 1% agarose electrophoresis. For most of the mutations, an additional restriction site was created or restriction site was deleted, described in the name of the primer. To confirm presence of the desired mutation, sequencing of the insert was performed. All types of mutations together with primer sequences are described in Table 5.

2.2.1.9 Transformation of bacteria

DH10B or SURE® electrocompetent *E.coli or* ElectroMAX™ DH10B™ cells were thawed on ice, either 0,3 µl of plasmid DNA (0.1-100 ng) or 2 µl of mutagenesis reaction were mixed with 7 µl of cells, and transferred to the pre-cooled electroporation cuevette. The mixture was electroporated with a pulse at 1,7 kV , 200 Ω, 25 µF. Then cells were supplied with 200 µl 2x LB medium, added onto 2xLB/ampicillin (50mg/ml) pre-warmed plates and incubated overnight at 37°C. Next day, single small colonies were picked and inoculated in 3 or 10 ml LB medium supplied with ampicillin (100mg/ml) and incubated O.N at 37°C.

SURE *E.coli* cells were designed to facilitate cloning of DNA containing unstable inserts by removing genes involved in the rearrangement and deletion. E1B-55 kDa is preferentially recombined in bacterial cells, to ensure correct insert, SURE cells were always used for E1B-55 DNA purification and propagation.

2.2.2 Cell culture methods

2.2.2.1 Cultivation of mammalian cell lines

H1299 were maintained as monolayer cultures. The cells were grown on 75 cm^2 cell culture plates and chamber slides, or in 24 well plates, at 37°C, 5% CO_2 and 95% humidity in appropriate medium. Depending on the growth rate the cells were splitted 1 or 2 times per week: the medium was removed; cells were washed twice with sterile PBS (warmed up to

37°C) and incubated with trypsine/EDTA. After they detached fresh medium was added to stop the trypsinization reaction and to dilute the cells to desired density before placing them on a fresh dish.

2.2.2.2 Thawing mammalian cells

For thawing, a cryovial was kept at 37°C until the content was thawed. The cell suspension was transferred into a 15 ml falcon tube containing 10 ml growth medium. Cells were centrifuged at 800 x g for 10 min. The supernatant was removed, cells re-suspended in 25 ml fresh medium and placed in a cell culture dish in the incubator.

2.2.2.3 Cell transfection

The cultured cells were transfected with plasmid DNA using Lipofectamine 2000 reagent. On the day before transfection, cells were seeded according to culture plate size (Table 6). DMEM medium (without any addictives) was incubated with plasmid DNA or Lipofectamine 2000 (Table 9). After 5 min incubation at room temperature, both preparations were mixed and allowed for interaction for another 20 min. DNA-lipofectamine solution was applied on the cells (with the rich medium). The dishes were placed in the incubator, on the shaker for 15 min to allow equal distribution of transfection mix. After 4h the medium was changed.

Table 9: Transfection scheme

Plate type	Nr of seeded cells	DMEM	DNA	Lipofectamine
75 cm^2 flask	1 mln cells	1,5 ml	20 µg	10 µl
24 well plate	50 000 cells/well	50 µl	0,65 µg	2 µl
Chamber slides wells/plate	50 000 cells/well	50 µl	0,65 µg	2 µl
Chamber slides wells/plate	25 000 cells/well	25 µl	0,35 µg	1 µl

2.2.3 Biochemical methods

2.2.3.1 Cell harvesting and cell lysates preparation.

To prepare cell lysates, from cells seeded in 24 well plates, cells were scraped in the medium. Cell suspensions were centrifuged 3 min at 800 x g and the resulting cell pellet was

resuspended in 50 µl cold (4°C) RIPA buffer and 13 µl 6 x Laemmli-buffer. After vortexing, the solution was denatured by heating for 5 min at 95°C, unless stated otherwise. To decrease the viscosity of the samples, they were shaken 15 min at RT and then centrifuged 10 min at 16000 x g. Samples were run on the SDS polyacrylamide gels or stored at -20°C.

2.2.3.2 SDS-Polyacrylamide gel electrophoresis

We can separate proteins based on their mass by electrophoresis in a polyacrylamide gel under denaturing conditions. 10% separating gels were used and a 6% stacking gel (Table 10) to insure simultaneous entry of the proteins into the separating gel. SDS-PAGE was performed using 100V through the stacking gel and 150V through the separating gel. The negatively charged SDS-proteins complexes migrate in the direction of the anode at the bottom of the gel.

Table 10: Preparation of polyacrylamide gels

Gel name	**Reagents**	**Amount of reagents per 1 gel**
Stacking gel: 6%	dH_2O	3.4 ml
	30%Acrylamide-solution	0.85 ml
	1 M Tris-Buffer (pH 6,8)	0.625 ml
	10% SDS	50 µl
	10% APS	50 µl
	TEMED	5 µl
Separating gel: 10% gel	dH_2O	3,0 ml
	30% Acrylamide-Solution	2.5 ml
	1,5 M Tris-Buffer (pH 8,8)	1.9 ml
	10% SDS	75 µl
	10% APS	75 µl
	TEMED	5 µl

2.2.3.3 Western Blotting

To transfer the proteins from the gel onto a nitrocellulose membrane, a semi-dry electroblotting method was used. A ‘transfer sandwich’ was assembled: 2 pieces of Whatmann paper were soaked in 1 x BTB buffer and put on an anode plate. Then the

transfer membrane was placed on top of it, covered by the gel and another stack of soaked Whatmann paper. Extra care was taken not to trap air bubbles between gel and membrane. Everything was covered with the cathode plate and current was applied.

Transfer was performed at 14 V for 1h. The pre-stained molecular weight standards served as an indication of a successful transfer. Additionally to check loading accuracy, the membrane was stained with Ponceau S fixative dye solution.

2.2.3.4 Immunostaining

For immunostaining, the nitrocellulose membrane was firstly blocked in freshly prepared PBS-T containing 5% nonfat dry milk (blocking buffer) for 1h at RT with constant agitation. The primary antibody was diluted to the recommended concentration (for dilution see: Table 1) in blocking buffer and used for incubation of the membrane for 2 hour at room temperature or overnight at 4°C with agitation. The membrane was then washed three times with PBS-T, each time for 10 min. The donkey anti-mouse peroxidase-conjugated secondary antibody was diluted in PBS-T containing 5% nonfat dry milk, and added to the membrane (Table 2). After incubation at RT for 1h, membrane was washed once for 10 min and then 4 times 5 min with PBS-T. Finally the membranes were exposed to an enhanced chemiluminescence reaction (ECL-system): the membrane was incubated in a 1:1 mix of ECL solutions 1 and 2. The membrane was then covered in a plastic film and placed in a developing cassette. Specific bands were visualized by exposure the films to the X-ray for 1 s - 30 min.

2.2.3.5 Immunoprecipitation

H1299 cells seeded in 6-well dishes were transfected with plasmids as indicated in the figure legends. After 24 hours, the cells were harvested in 500 µl lysis buffer (50mM Tris, pH 7.5, 150mM NaCl, 0.5% NP-40) and mechanically disrupted with a syringe. The soluble fraction was precleared for 1h with 30 µl of a 50% slurry of Protein Sepharose CL-4B, followed by incubation with antibodies (0,5 µg) against the HA tag (HA.11, Covance) or p53 (FL393, Santa Cruz) for two hours. Protein-antibody complexes were pulled down for 30 min with 10 µl of a 50% slurry of Protein A sepharose (for HA.11) or Protein G sepharose (for FL-393), and

washed five times with 500 µl of the lysis buffer. Coprecipitated proteins were eluted from the sepharose by boiling with 30 µl of sample buffer (0,35 M Tris, pH 6.8, 30% Glycerol, 10% SDS, 9,3% Dithiothreitol (DTT), 0,02% Bromophenole Blue), resolved by sodium dodecyl sulfate-polyacrylamide electrophoresis (SDS-PAGE) and detected by immunoblot analysis.

2.2.3.6 Immunofluorescence.

H1299 cells were seeded onto plastic slides (Nunc) suitable for microscopy and transfected. After 24 hours, the cells were fixed with paraformaldehyde (4% in PBS for 20 min), permeabilized with Triton X-100 (0.2% in PBS, 25 min), and incubated with primary antibodies (Table 1). Primary mouse and rabbit antibodies were visualized by secondary antibodies coupled to AlexaFluor-596 or AlexaFluor-488 (Table 2). Prior to mounting with Dako fluorescent mounting medium, the cell nuclei were briefly stained with 4,6-diamidino-2-phenylindole (DAPI).

2.2.3.7 Luciferase assay.

H1299 cells were cotransfected with p53, E1B-55 kDa constructs and reporter plasmids as indicated in the figure legends. Luciferase assays (Firefly and Renilla luciferase) were performed using a self-made substrate mix as described (Dyer et al, 2000).

To prepare cell lysates, cells were scraped in the medium. Cell suspensions were centrifuged 3 min at 800 x g and the resulting cell pellet was resuspended in 50 µl of 1x Passive Lysis at RT. Then the cell lysates were shaken for 10 min at 1400 rpm in a thermoblock at the room temperature. Lysates were centrifuged at max speed for 5 min and the supernatant was collected. 20 µl of cell supernatants and 1x PLB were added to 96 well luciferase plate. 100 µl of Firefly luciferase buffer (Table 11) was added to each well and firefly luciferase was measured for 10 seconds. Then this signal was quenched by adding the Renilla buffer (Table 11), the renilla luciferase signal was then measured for 10 sec.

Table 11: Preparation of luciferase working buffers

Per 10 ml stock **Firefly Buffer**	200ul of 1M $MgSO_4$ 500ul of 100mM ATP pH 7.0 15ul of 1M DTT 120ul of 10mM CoA 1ml Luciferin
Per 10 ml stock **Renilla Buffer**	500ul of 10mg/mlBSA 12ul of 1.3M NaN_3 2,5ul of 6mM Coelenterazine

2.2.4 Cloning of recombinant proteins.

2.2.4.1 EGFP-fragE1B fusion protein.

To test if the E1B-55 kDa central domain can induce oligomerization on sits own, I created EGFP and E1B-55 kDa oligomerization domain fusion protein (Fig. 7). First I PCR amplified E1B-55 kDa fragment containing bases coding for amino acids 244-310, using primers containing enzyme adapters (Table 4). Then the PCR product was digested with EcoRI and BamHI restriction enzymes, and ligated within the C-terminus of EGFP digested backbone. ElectroMAX™ DH10B™ were transformed with the ligation. Single colonies were analyzed for presence of the fusion protein.

2.2.4.2 HA-fragE1B fusion protein.

To characterize behavior of E1B-55 kDa oligomerization domain, I created HA tag fusion with E1B-fragment. After PCR amplification, E1B fragment coding amino acids 245-310 was digested with XbaI enzyme and ligated with HA tag containing, also XbaI digested and dephosphorylated plasmid backbone (pCGN). ElectroMAX™ DH10B™ were transformed with the ligation. Single colonies were analyzed for presence of the fusion protein and correct orientation of the insert. To create HA-fragE1B Del 281-288, plasmid expressing E1BDel 281-288 was used as a template for the PCR reaction, otherwise the procedure was the same as for HA-fragE1B.

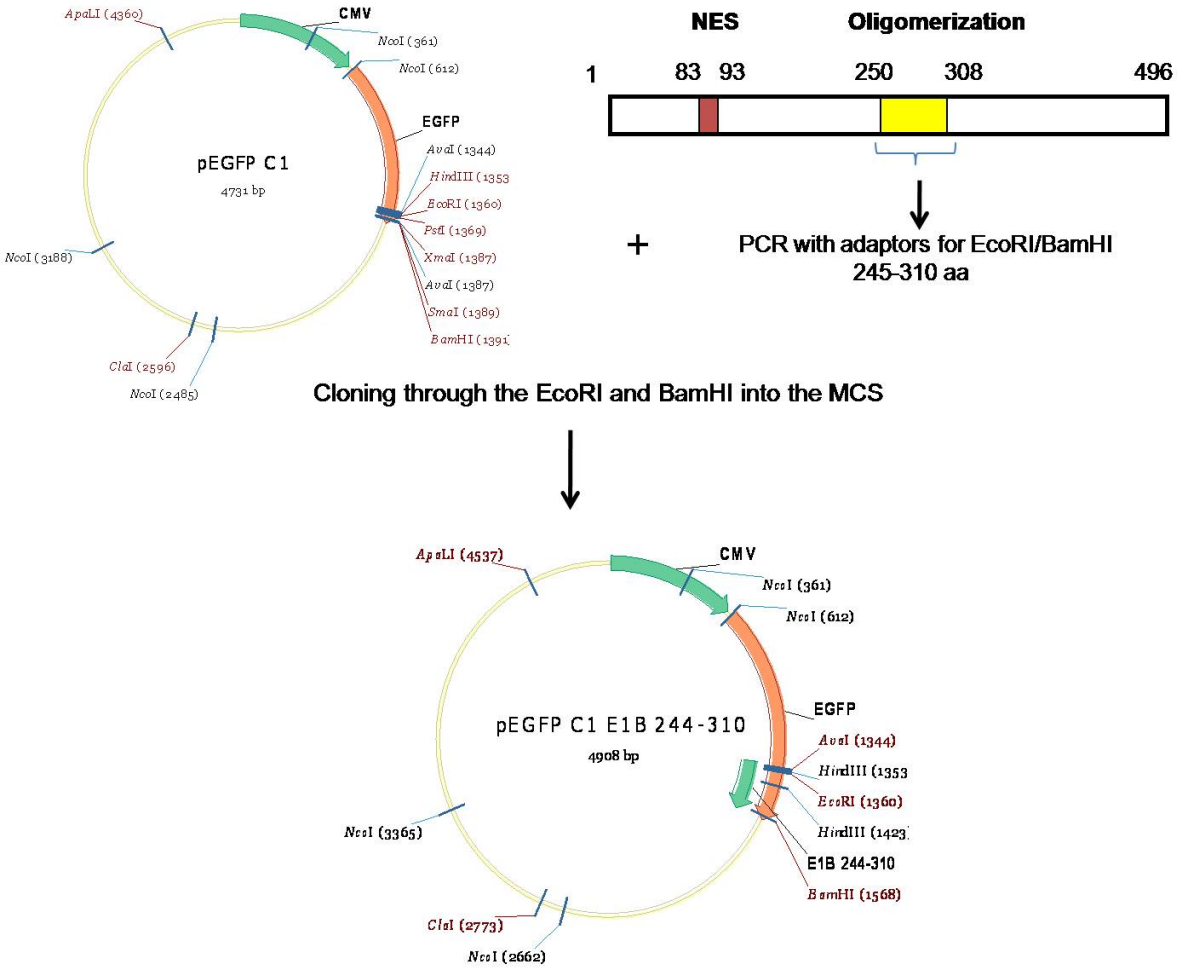

Figure 7. Schematic representation of cloning steps of EGFP-E1B 244-310 fusion protein.

2.2.4.3 Fusion of the yeast GCN4 oligomerization domain to E1B-55 kDa.

Fusion proteins between E1B-55 kDa and CC domain from yeast GCN4 gene were created; to test whether E1B-55 kDa oligomerization domain can be substituted with different oligomerization domain. After PCR amplification (primers Table 4), CC fragment was digested and ligated with E1B-55 kDa Del 281-288 or wt E1B-55 kDa digested backbones. CC domain was either cloned at the N-terminus (XbaI) of the E1B-55 kDa or at the middle portion of the protein (HindIII). SURE® Electro-competent cells were transformed with the ligations. Single colonies were analyzed for presence of the fusion protein and correct orientation of the insert.

3. RESULTS

In my Master Thesis I showed that E1B-55 kDa can form stable high molecular weight (HMW) species in SDS polyacrylamide gels. H1299 cells (p53 -/-) were transfected with doubly tagged HA/MYC E1B-55 kDa wild type protein. The HMW species were detected with all antibodies, either specific to the used tags (N-terminal HA tag vs. C-terminal MYC tag) or the E1B-55 kDa protein (2A6 –directs against an epitope lying within the first 180 aa of the protein)-Fig. 8. Therefore, these HMW species are specific to E1B-55 kDa. Mass spectrometry analysis of precipitated E1B-55 kDa, indeed detected the protein in the HMW complexes. The additional band present in MYC and 2A6 staining is probably a 49kDA E1B-55 kDa isoform. It is produced as a result of translation initiation at a downstream AUG codon of the E1B-55 kDa reading frame (Kindsmuller et al, 2009). Interestingly, site directed mutagenesis pointed at a central region of E1B-55 kDa as being necessary for the HMW complex formation. Deletion mutant 245-296 almost completely lost the aggregation properties. Here, I investigated the central portion of E1B-55 kDa, spanning residues 245-310, and its relevance for proper E1B functioning.

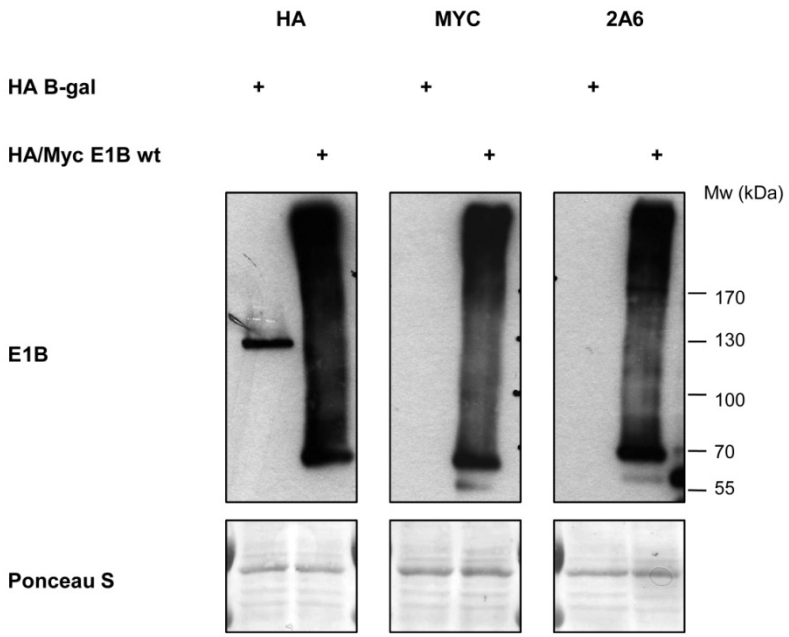

Figure 8. Formation of HMW complexes resistant to boiling in SDS. Lysates from H1299 cells transfected with the HA/MYC E1B-55 kDa construct (650ng) or HA-tagged beta-galactosidase (650ng), were analysed by immunoblotting and staining with anti-HA, anti-MYC antibodies.

3.1 Oligomerization of E1B-55 kDa

3.1.1 E1B-55 kDa exhibits a high molecular weight pattern which is connected to the denaturation status of the protein.

The E1B-55 kDa amino acid sequence shows a high percentage of hydrophobic residues as well as a few well conserved cysteine residues within the central portion. It has been previously observed that proteins with high portion of hydrophobic patches, especially membrane proteins, tent to aggregate in SDS while boiling (Sagne et al, 1996). To test whether different preparation temperatures can affect the HMW complexes, H1299 cells were transfected with wild type E1B-55 kDa or the Del 245-296 mutant, which served as a control. Cell lysates were re-suspended with Laemmli buffer and either boiled for 5 min or incubated at 57°C for 30 min.

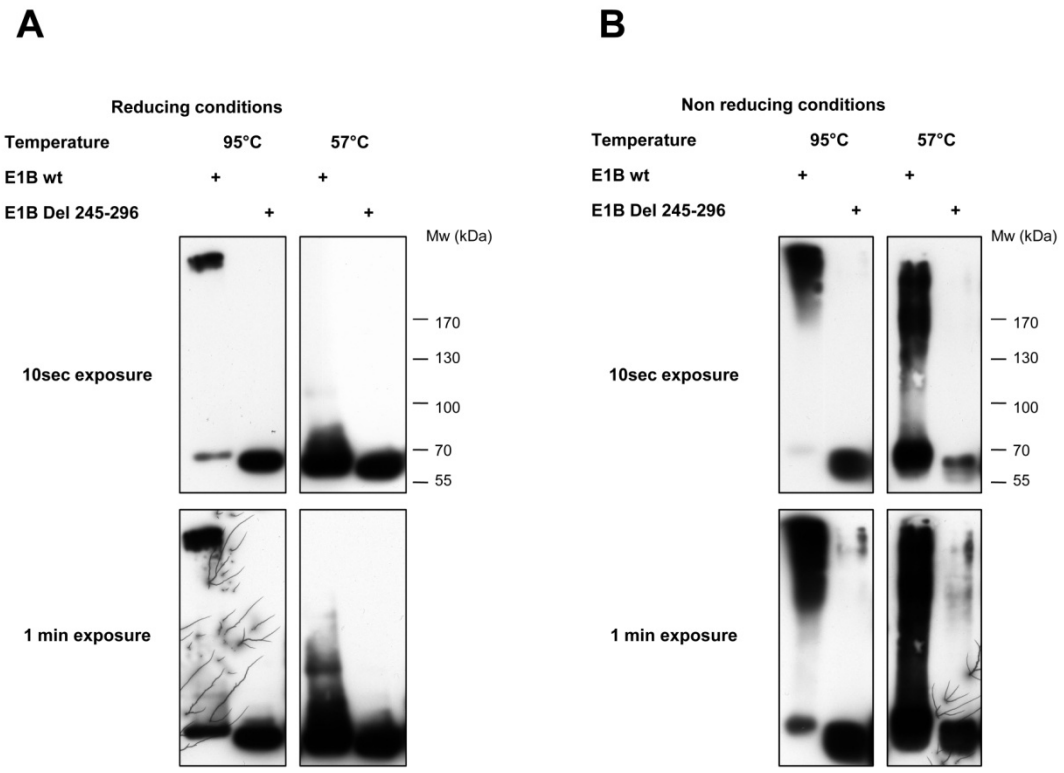

Figure 9. Formation of HMW complexes and denaturation status of the E1B-55 kDa protein. A) Lysates from H1299 cells transfected with the HA E1B-55 kDa construct (650ng) or HA Del 245-296 (650ng) were boiled for 5 min or incubated at 57°C for 30 min, and then analysed by immunoblotting and staining with anti-HA antibody; B) Lysates from H1299 cells transfected with the HA E1B-55 kDa construct (650ng) or HA Del 245-296 (650ng) were mixed with 6xLeammli buffer with or without betamercaptoethanol (reducing agent). Lysates were boiled for 5 min or incubated at 57°C for 30 min, and then immunoblotted and stained with anti-HA antibody.

After SDS-PAGE, immunoblots were stained with HA antibodies to detect E1B-55 kDa. Boiling of the wild type, but not the deleted mutant showed again additional HMW complexes, more prominent after longer exposure (Fig. 9A). However, incubation at 57°C, although it did not affect the banding pattern of the E1B-55 kDa deletion mutant, showed increased signals at lower molecular weights of the wt and loss of the HMW complexes (Fig. 9A). To analyse whether the sulphur bonds can also affect the presence of HMW complexes, additional transiently transfected H1299 cells were analysed. Interestingly, completely removing the reducing agent (beta-mercaptoethanol) from the loading buffer yielded highly "smeared" wt E1B-55 kDa bands which did not depend on the temperature of sample preparation. No HMW complexes were detected for the Del 245-296 (Fig. 9B). It seems that, at least in part, the HMW complexes are a reflection of incompletely reducing conditions. The fact that the HMW complexes seem to be the prevalent form of wt E1B-55 kDa in non-reducing conditions as well as the high hydrophobic amino acid content of the central portion of the protein suggest that this might be an oligomerization domain.

3.1.2 A central portion of E1B-55 kDa is necessary for its self-association.

It has been shown previously that purified E1B-55 kDa can form dimers (Martin & Berk, 1998). To test if dimerization can be detected in cells, I cotransfected H1299 cells (p53-/-) to synthesize differentially tagged E1B-55 kDa carrying the HA tag or the MYC tag. Cell lysates were then subjected to immunoprecipitation with an antibody to the HA tag. The immunoprecipitates were stained to detect HA and MYC tagged proteins. Self-association of wild type E1B-55 kDa was detected through coprecipitated MYC-E1B-55 kDa (Fig. 10A). To identify regions important for oligomerization, I first tested the large deletion mutant Del 245-296 and then smaller deletions that abolished the coprecipitation with wild type E1B-55 kDa (Fig. 10, A and B). These deletions cover a highly conserved specific region, amino acids 241-310, that has been previously characterized as having homology to Ribonucleoprotein (RNP) domains and weak unspecific RNA binding activity (Horridge & Leppard, 1998). From here on, I refer to this region as an oligomerization domain. E1B-55 kDa can self-associate in a cellular context, and this requires a conserved central portion of the protein.

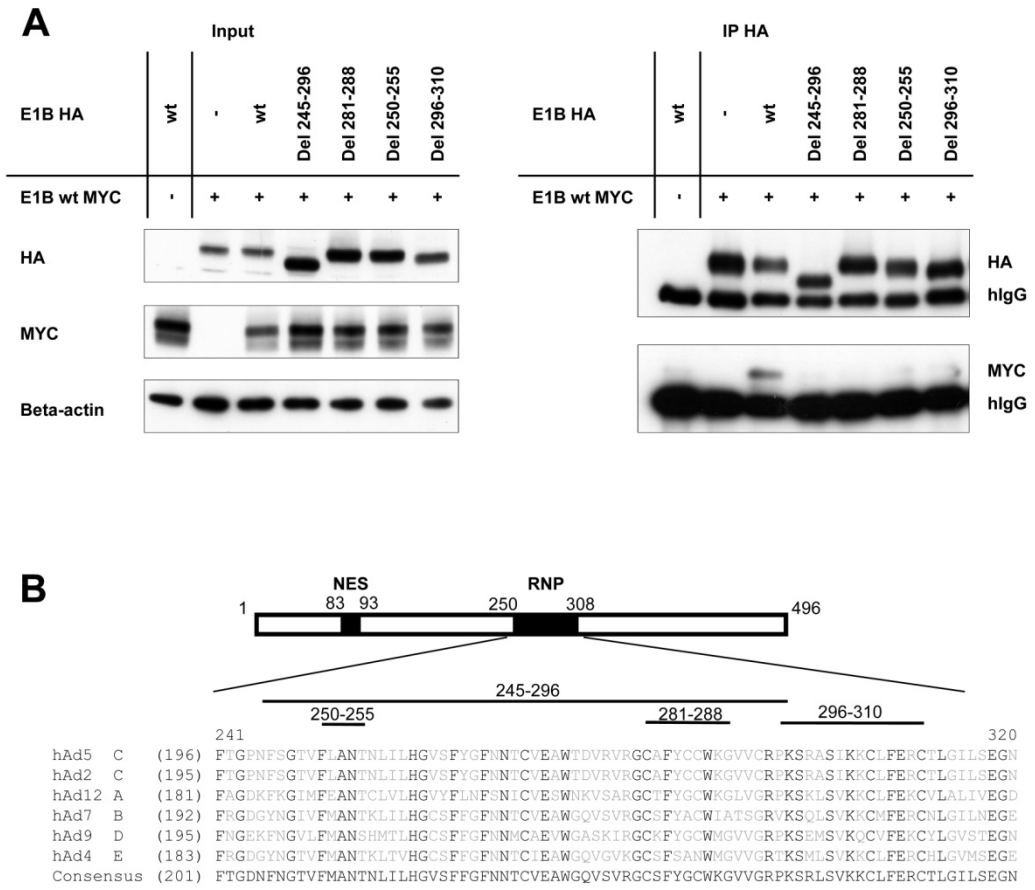

Figure 10. Self-association of E1B-55 kDa, and failure of deletion mutants to so. A) Lysates from H1299 cells cotransfected with expression constructs for differentially tagged E1B-55 kDa proteins (500ng HA-tagged E1B-55 kDa constructs and 1500ng MYC-tagged wild type E1B-55 kDa) were subjected to immunoprecipitation (IP) with the anti-HA antibody. 2% of the input lysates and approximately 20% of the immunoprecipitates were analyzed by immunoblotting and staining with anti-HA and anti-MYC antibodies. hIgG, heavy chain of immunoglobulin G. **B) Primary structure of E1B-55 kDa with putative functional regions.** Nuclear import signal (NLS) –position 83-93; putative ribonucleoprotein (RNP) motif homology region: position 250-308. Scheme modified from (Gonzalez & Flint, 2002). Protein sequence alignment between the Ad5 E1B-55 kDa protein and homologues from different adenovirus groups. Identical residues are marked in boldface. The deleted amino acids in the E1B-55 kDa mutants used here are indicated by bars above the alignment.

3.2 Functional characterization of E1B-55 kDa oligomerization mutants

3.2.1 E1B-55 kDa oligomerization segregates with proper localization.

Next, I analyzed how deletions in the central portion affect the intracellular localization of E1B-55 kDa. E1B-55 kDa can form characteristic cytoplasmic clusters (Blair Zajdel & Blair, 1988; Zantema et al, 1985). Immunofluorescence analysis of transfected H1299 cells showed that none of the E1B-55 kDa oligomerization mutants could form these clusters. Rather, these mutants of E1B-55 kDa were evenly distributed within the cytoplasm (Fig.

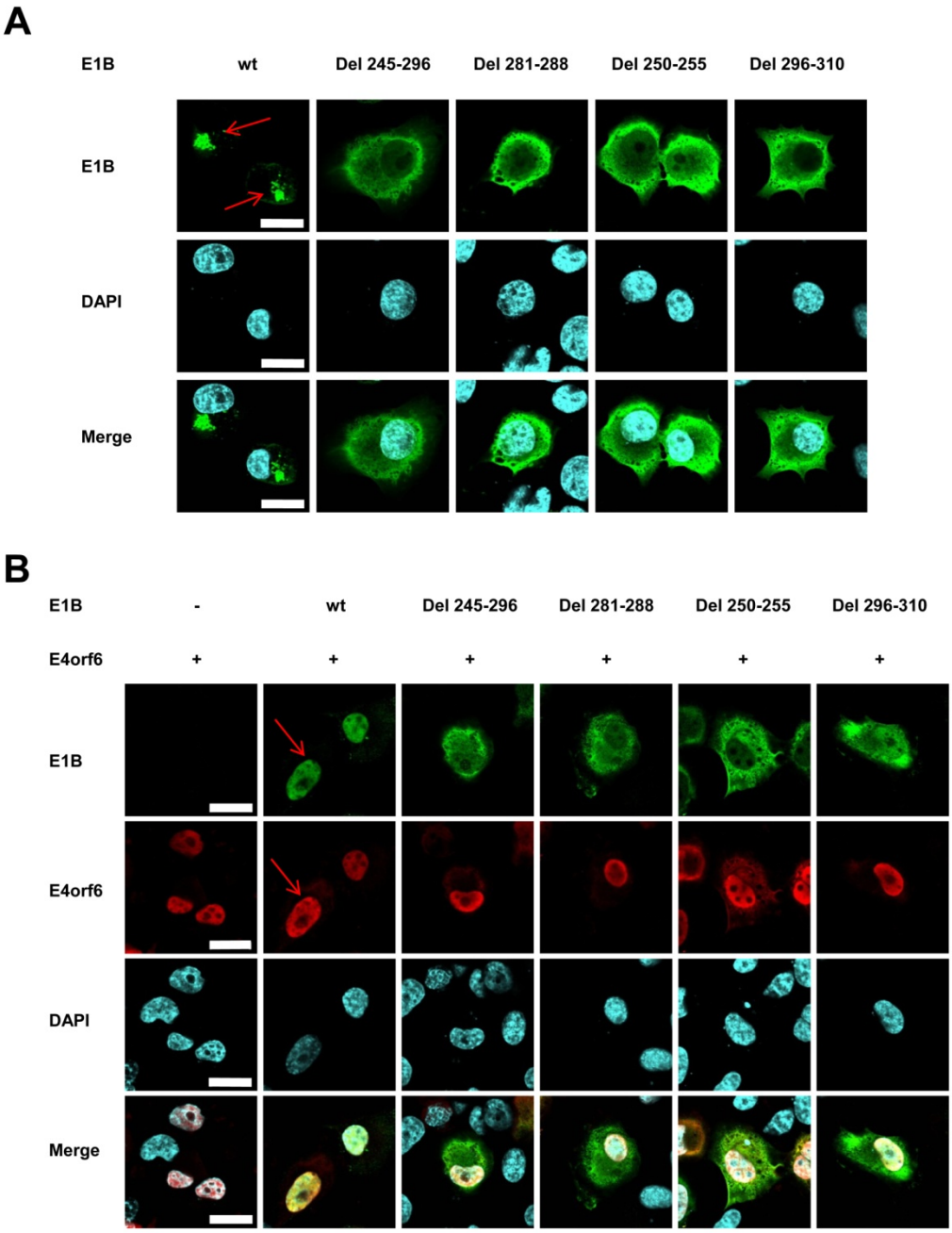

Figure 11. Intracellular localization of E1B-55 kDa oligomerization mutants. A) Formation of cytoplasmic clusters by E1B-55 kDa. H1299 cells were transfected with wild type E1B-55 kDa or the indicated E1B-55 kDa oligomerization mutants (650ng each). 24h after transfection, the cells were immunostained with the monoclonal antibody 2A6 specific for E1B-55 kDa, followed by a green-fluorescently labeled secondary antibody. The locations of the nuclei were visualized by 4'-6-diamidino-2-phenylindole (DAPI) staining. The sites of cytoplasmic clusters containing wild type E1B-55-kDa are marked by arrowheads. Bar=20µm. **B) Relocalization of E1B-55 kDa by E4orf6.** Expression plasmids for HA-tagged E1B-55-kDa (150ng) and E4orf6 (450ng) or 'empty' vector constructs were cotransfected into H1299 cells as indicated, followed by staining with a rabbit anti-HA antibody to detect E1B-55 kDa and the mouse monoclonal anti-E4orf6 antibody (RSA3). Corresponding secondary antibodies conjugated to green and red fluorescent dyes were used. The locations of the nuclei were visualized by DAPI staining. Bar=20µm.

11A). I then investigated the interaction of E1B-55 kDa with the E4orf6 protein. H1299 cells were cotransfected with E4orf6 and different E1B-55 kDa constructs.

Next day, the cells were fixed and immunostained for presence of both transfected proteins. Wild type E1B-55 kDa was relocalized to the nucleus by E4orf6, as reported previously (Goodrum et al, 1996). In contrast, all of the E1B-55 kDa deletion mutants remained mainly cytoplasmic despite the presence of E4orf6 (Fig. 11B). This suggests that E1B-55 kDa oligomerization is required for the efficient relocalization of E1B-55 kDa by E4orf6.

3.2.2 The central portion of E1B-55 kDa is required for interaction with p53.

To test whether deletions in the E1B-55 kDa oligomerization domain affect p53 binding, H1299 cells were cotransfected with wild type p53 and either wild type E1B-kDa or E1B-55 kDa oligomerization mutants. Immunostaining revealed that wild type E1B-55 kDa could efficiently relocalize p53 from the nucleus to the cytoplasm (Fig. 12A), as observed previously (Blair Zajdel & Blair, 1988; Zantema et al, 1985). In contrast, upon co-expression with E1B-55 kDa deletion mutants, p53 remained nuclear (Fig. 12A). Next, I assessed the inhibition of p53 activity by mutants of E1B-55 kDa in a luciferase reporter assay. H1299 cells were co-transfected to express p53 and E1B-55 kDa, together with a firefly luciferase plasmid containing a p53 inducible promoter, along with a plasmid that constitutively expressed renilla luciferase for normalization. As expected, wild type E1B-55 kDa could efficiently block p53-mediated transactivation (Fig. 12B). However, E1B-55 kDa oligomerization mutants almost completely lost this ability (Fig. 12B).

E1B-55 kDa not only inhibits p53 but, together with E4orf6, induces p53 ubiquitination and subsequent degradation (Querido et al, 1997; Steegenga et al, 1998) –Fig. 4. H1299 were transfected with p53, E4orf6 and E1B-55 kDa constructs. The levels of p53 were assessed by immunoblot analysis. As expected, p53 levels dropped upon co-expression with wild type E1B-55 kDa and E4orf6. However, the oligomerization mutants of E1B-55 kDa did not induce p53 degradation (Fig. 12C), in accordance with the lost colocalization with E4orf6 and p53. Hence, the oligomerization domain of E1B-55 kDa is needed for all aspects of antagonizing p53.

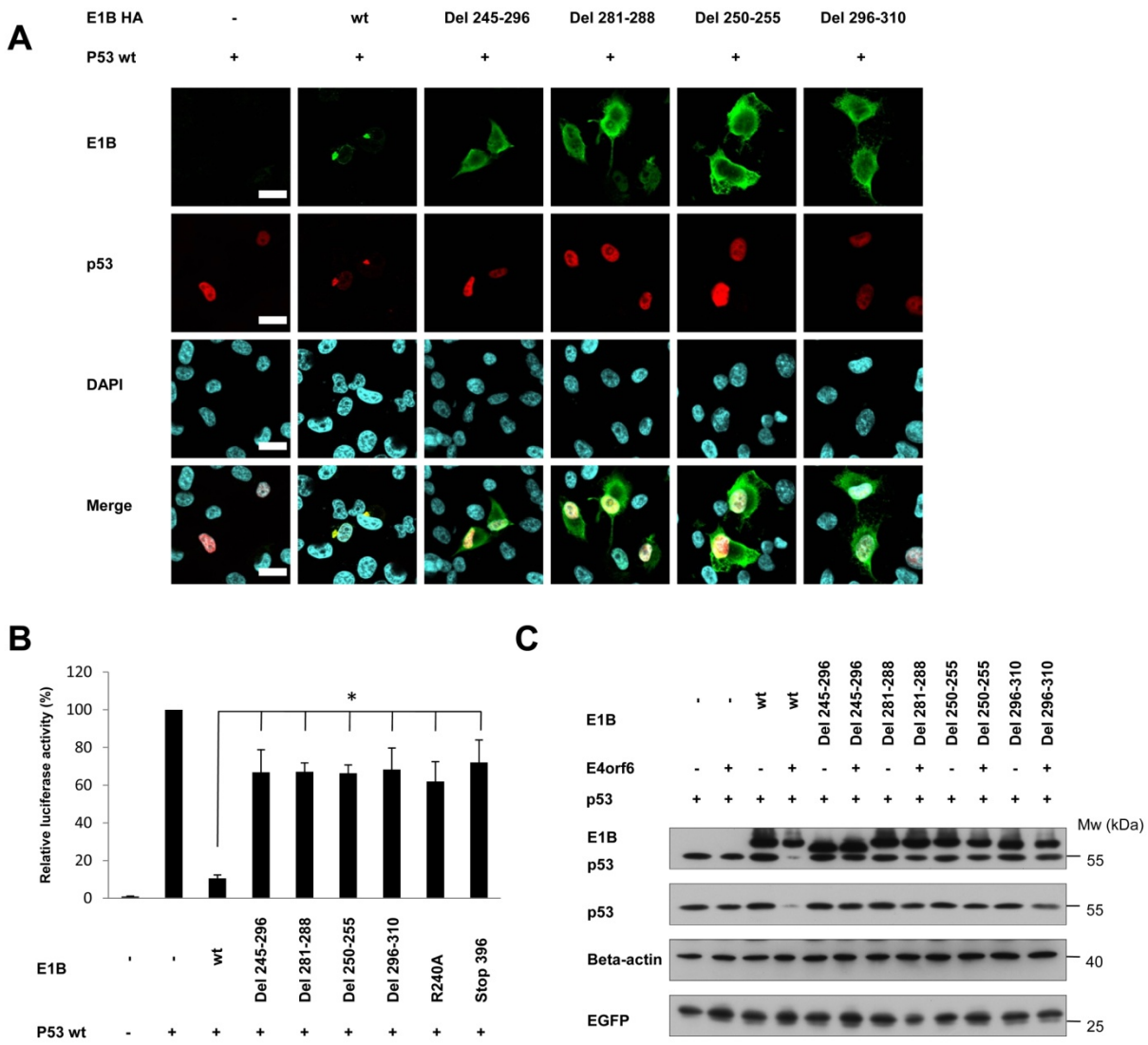

Figure 12. Interaction of p53 with E1B-55 kDa oligomerization mutants. A) Impact of E1B-55 kDa mutants on p53 localization. H1299 cells were cotransfected expression constructs for HA-tagged E1B-55 kDa (300ng) and wild type p53 (30ng), followed by immunostaining with a mouse monoclonal antibody to the HA-tag and a rabbit anti-p53 antibody (FL393). Nuclei were visualized by DAPI staining. Bar=20µm. **B) Impact of E1B-55 kDa mutants on p53 activity.** Expression plasmids for p53 (50ng), E1B-55 kDa mutants (500ng) or beta-galactosidase (500ng) were cotransfected along with 100ng of a p53 responsive firefly luciferase reporter plasmid, pBP100luc (Roth et al, 1998), and 50ng of a Tk promoter-driven renilla luciferase reporter plasmid into H1299 cells as indicated. Twenty four hours after transfection, both luciferase activities were determined. Firefly luciferase activities were divided by renilla luciferase activities and then normalized to the wild type p53 activity alone. The experiments were performed in triplicates, mean and standard deviations are indicated. P value *<0,01. **C) Ability of E1B-55 kDa mutants to mediate p53 degradation.** H1299 cells were transfected with plasmids to express p53 (50ng), E1B-55 kDa (100ng) and E4orf6 (450ng), or 'empty' vectors as indicated. Twenty four hours later the cells were harvested, followed by immunoblot analysis with the indicated antibodies. EGFP and beta-actin served as transfection and loading controls, respectively.

3.2.3 A carboxyterminal truncation mutant of E1B-55 kDa but not the central domain deletion mutants can be relocalized to cytoplasmic clusters by wild type E1B-55 kDa.

A carboxyterminal portion of E1B-55 kDa has been found necessary for its localization to cytoplasmic clusters (Schwartz et al, 2008; Teodoro & Branton, 1997). I used this as an independent means to observe intracellular self-association.

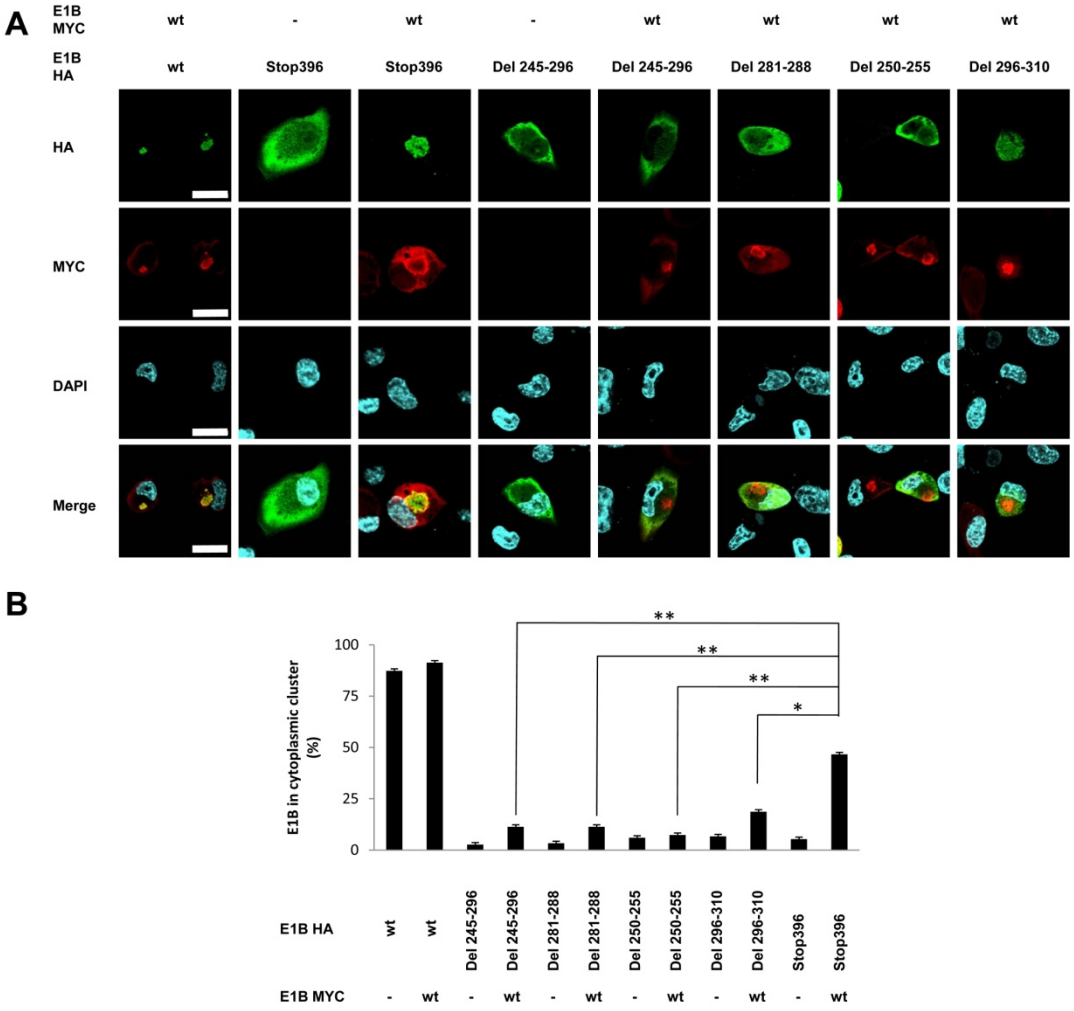

Figure 13. Relocalization of different E1B-55 kDa mutants by wild type E1B-55 kDa. A)Localization of HA-tagged E1B-55 kDa mutants in the presence of MYC-tagged wild type E1B-55 kDa. H1299 cells were cotransfected with expression plasmids caring wild type MYC-tagged E1B-55 kDa (400ng) and a panel of HA-tagged E1B-55 kDa mutants (200ng) as indicated, followed by staining with anti-HA and anti-MYC antibodies and DAPI. Bar=20µm. **B) Statistical evaluation of HA-E1B-55 kDa relocalization to the cytoplasmic clusters.** For each experiment three samples of 50 cells each were counted. Cells with HA-E1B-55 kDa in cytoplasmic clusters were counted as positive. The mean and standard deviation are indicated for each sample. Students T-test was performed to calculate the significance levels by which the indicated values are distinct. P value: * <0,02; ** <0,001, cf. Appendix.

A carboxyterminal truncation mutant with a stop codon at position 396 (E1B Stop396) was found diffusely distributed in the cytoplasm, as determined by immunofluorescence (Fig. 13, A and B). However, co-expressing it with differentially tagged wild type E1B-55 kDa led to the detection of both proteins in clusters (Fig. 13, A and B). This indicated that different E1B-55 kDa molecules associate with each other in cells. In contrast, the oligomerization deletion mutants remained cytoplasmic even after cotransfection with wild type E1B-55 kDa (Fig. 13, A and B).

I conclude that the central and the carboxyterminal regions of E1B-55 kDa are necessary for the formation of cytoplasmic clusters, but only the central domain seems essential for E1B-55 kDa oligomerization.

3.3 Characterization of the substitution mutants within E1B-55 kDa oligomerization domain.

3.3.1 Narrowing down the residues required for the E1B-55 kDa oligomerization.

In an attempt to identify specific amino acids responsible for E1B-55 kDa oligomerization by means of site directed mutagenesis I created series of alanine substitution mutants within the oligomerization domain. Cellular localization of E1B-55 kDa substitution mutants was analysed by H1299 cells transfection and then immunofluorescence analysis. All created mutants localized to cytoplasmic clusters similarly to wt E1B-55 kDa (Fig. 14A). Next, I tested their potency to inhibit p53 in a luciferase reporter assay. I co-transfected H1299 cells with different E1B-55 kDa constructs, p53 and luciferase constructs, as described earlier. Surprisingly, one of the single substitution mutants, Y286A, was not able to block p53 activity, while all the other mutants did (Fig. 14B). Tyrosine belongs to the group of aromatic amino acids. Although Y286A was only partially defective mutant, I decided to look at other aromatic residues within the oligomerization region and their contribution to oligomerization. Interestingly, phenylalanine residues within the 245-310 amino acid region are highly conserved across the human adenovirus E1B proteins (Fig. 10B). Interestingly, hydrophobic phenylalanine residues play an important role in p53 oligomerization (Chene, 2001).

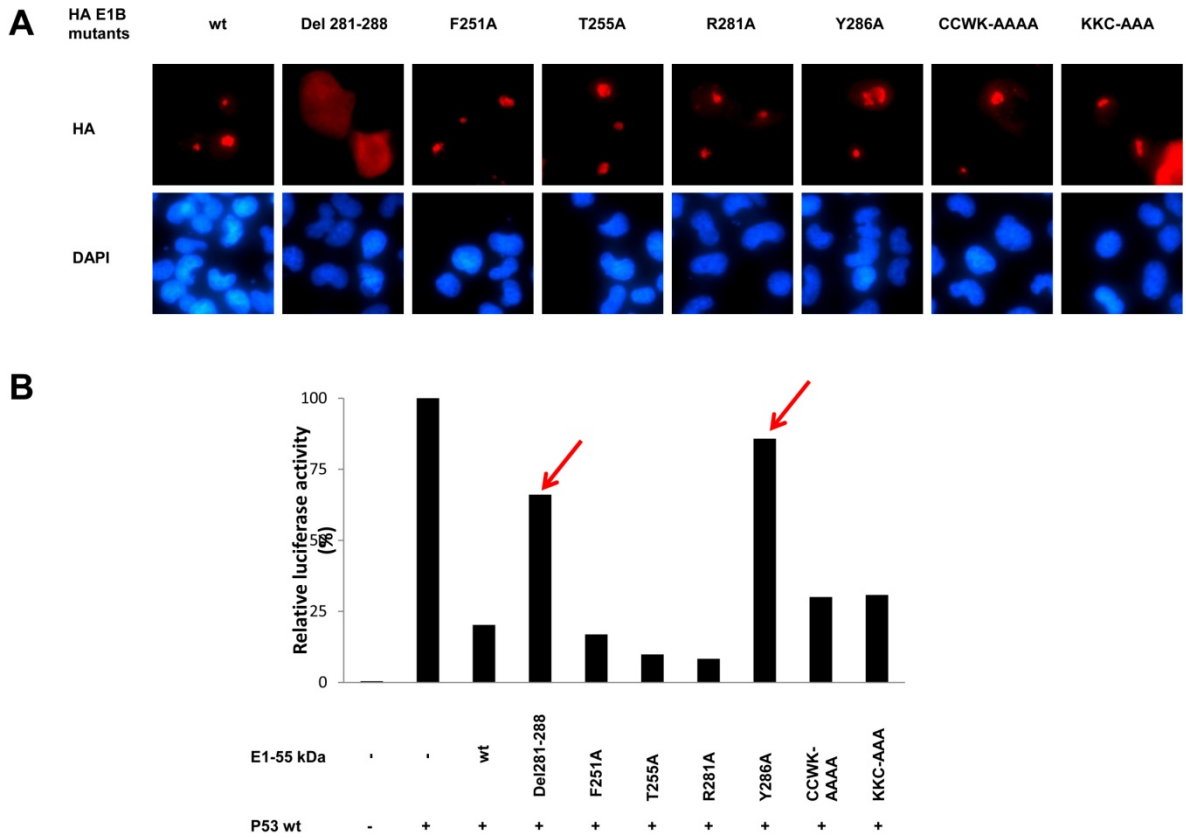

Figure 14. Characteristics of the substitution mutants. A) Localization of HA-tagged E1B-55 kDa substitution mutants. H1299 cells were cotransfected with a panel of HA-tagged E1B-55 kDa mutants (650ng) as indicated, followed by staining with anti-HA and DAPI. Visualized by non-confocal fluorescence microscopy. B) The impact of the E1B-55 kDa substitution mutants on p53 activity was assessed as in Fig. 12B.

3.3.2 The E1B-55 kDa mutants F307A and FY285AA largely fail to self-associate

Three phenylalanine residues within the conserved region 245-310 of E1B-55 kDa were mutated to alanines. Localization of the phenylalanine mutants was analyzed by H1299 cells transfection and immunofluorescence staining.. Mutants E1B F264A and E1B F285A still localized in discrete clusters, albeit with reduced efficiency in the case of E1B F285A (Fig. 15, A-C). However, the substitution of phenylalanine 307 abolished the mutant's ability to form cytoplasmic clusters (Fig. 15A).

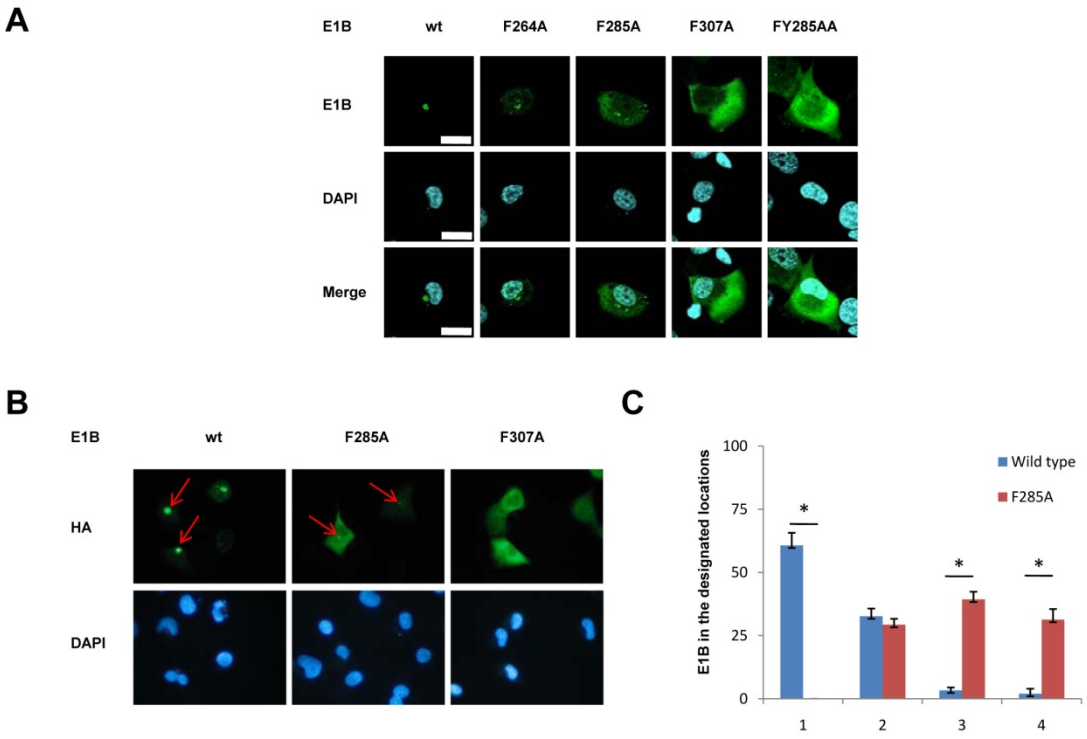

Figure 15. Intracellular localization of phenylalanine substitution mutants. A) Formation of cytoplasmic clusters by indicated E1B-55 kDa mutants was assessed as in Fig. 11A. Bar=20µm. A lower magnification of the samples from Fig. 15A is shown, as seen by non-confocal fluorescence microscopy. A) Statistical evaluation of E1B-55 kDa localization in wt or F285A transfected H1299 cells. For each experiment three samples of 50 cells each were counted. Four different categories for E1B localization were defined: 1-E1B exclusively in discrete cytoplasmic clusters; 2-E1B in cytoplasmic clusters and diffusely in the cytoplasm, but with the signal from the cluster brighter than in any other area of the cytoplasm; 3-E1B in cytoplasmic clusters still visible, but mostly in a diffuse cytoplasmic pattern, with the signal in at least some of these areas as bright as in the clusters; 4-E1B diffusely in the cytoplasm, with no visible clusters. The mean and standard deviation are indicated for each sample. Student's T-test was performed to calculate the significance levels by that the indicated values are distinct. * p<0,002.

Next to F285, E1B-55 kDa contains the aromatic residue, Y286, whose substitution to alanine also created partially defective mutant. This raised the possibility that these two residues might functionally substitute for each other, resulting in partial but not complete functional impairment of E1B F285A. In agreement with this assumption, the double mutant FY285AA completely lost the ability to form cytoplasmic clusters (Fig. 15A).

Next, I investigated the association of these mutants with E4orf6. Mutants F264A and F285A could be relocalized by E4orf6 (Fig. 16), in contrast F307A was defective for this interaction (Fig. 16). Interestingly, E1B FY285YY still associated with E4orf6 to some extent (Fig. 16), arguing that it did not simply loose all known functions of E1B-55 kDa.

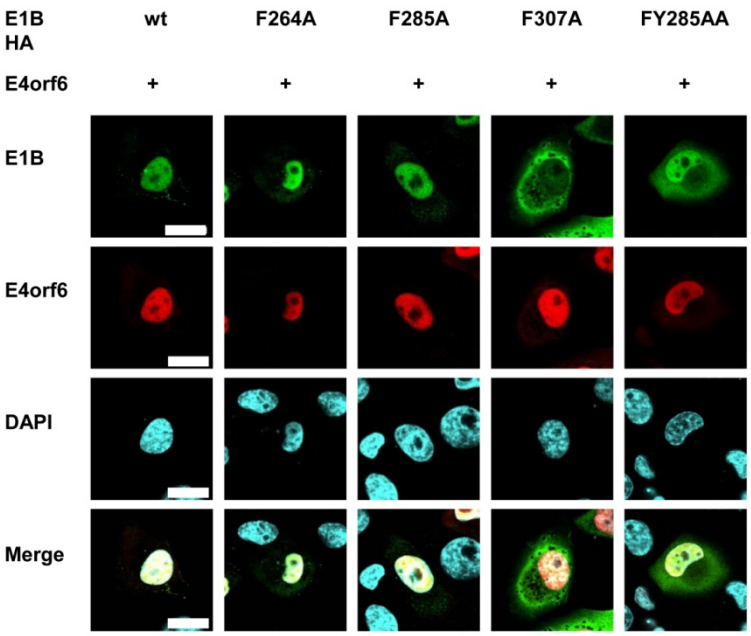

Figure 16. Relocalization of single amino acid substitution mutants of E1B-55 kDa by E4orf6 was assessed as in Fig. 11B. Bar=20µm.

To confirm the failure of the E1B-55 kDa point mutants FY285AA and F307A to self-associate, I co-expressed them with differentially tagged wild type E1B-55 kDa, followed by immunostaining. Whereas E1B F264A and F285A strongly associated with wildtype E1B-55 kDa, much less of this colocalization was seen for the E1B mutants FY285AA and F307A (Fig. 17, A and B).

To test if these mutations could abolish the ability of E1B-55 kDa to bind itself, I performed a co-immunoprecipitation. I cotransfected H1299 cells to synthesize differentially tagged E1B-55 kDa constructs carrying the HA or the MYC tags. Cell lysates were then subjected to immunoprecipitation with an antibody to the HA tag. The immunoprecipitates were stained to detect HA and MYC tagged proteins. Self-association of differentially tagged wild type E1B-55 kDa was used as a control. Wild type E1B-55 kDa could efficiently self-associate (Fig. 17C). F307A and FY285AA mutations largely abolished the ability to interact both with the wt E1B-55 kDa as well as with MYC-tagged versions of E1B-55 kDa proteins with corresponding mutations (Fig. 17C).

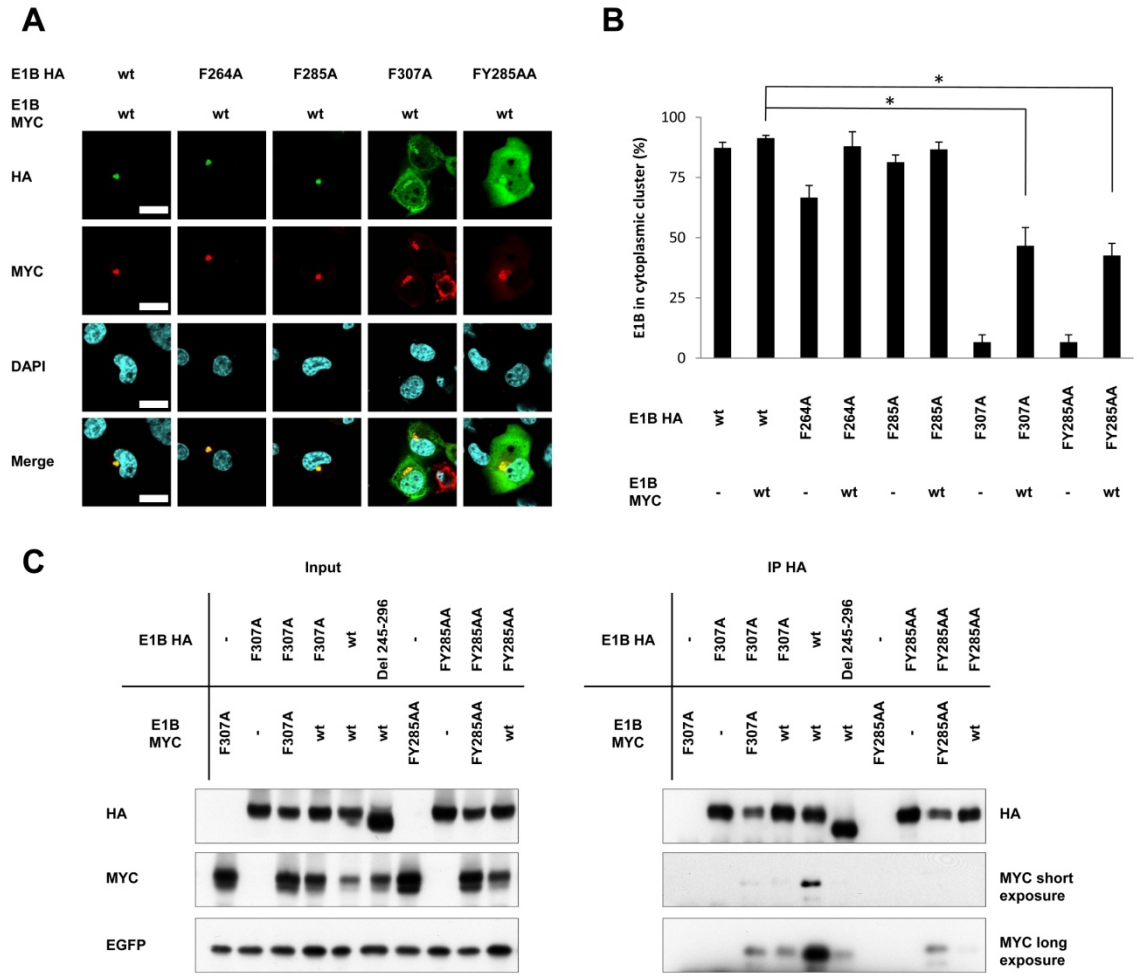

Figure 17. Self-association of substitution mutants of E1B-55 kDa. A) Relocalization of HA-tagged E1B-55 kDa mutants by MYC-tagged wild type E1B-55 kDa constructs was assessed as in Fig. 13A. Bar, 20µm. Note that all cells that showed fluorescence in clusters are summarized here, even if an additional signal was seen in a diffusely cytoplasmic pattern. B) Statistical evaluation of the experiment B in analogy to the Fig. 13B. P value: *<0,005. C) Direct interaction of F307A and FY285AA with wild type E1B-55 or with itself was assessed by immunoprecipitation as described in Fig. 10A.

3.3.3 The E1B-55 kDa mutants F307A and FY285AA inefficiently antagonize p53.

Next, I determined the ability of E1B-55 kDa substitution mutants to associate with and inhibit p53. The E1B-55 kDa mutant F264A was still capable to move p53 to the cytoplasm, as determined by immunofluorescence analysis (Fig. 18A). In contrast, E1B F285A did so only to a lesser extent, and the mutants F307A and FY285AA did not detectably relocalize p53. In accordance, E1B F264A still reduced the transcriptional activity of p53 in reporter assays, but the mutants F285A, FY285AA, and F307A were all impaired in this ability (Fig. 18B). All point mutants of E1B-55 kDa that fail to self-associate are also defective in their ability to associate with p53 and to reduce

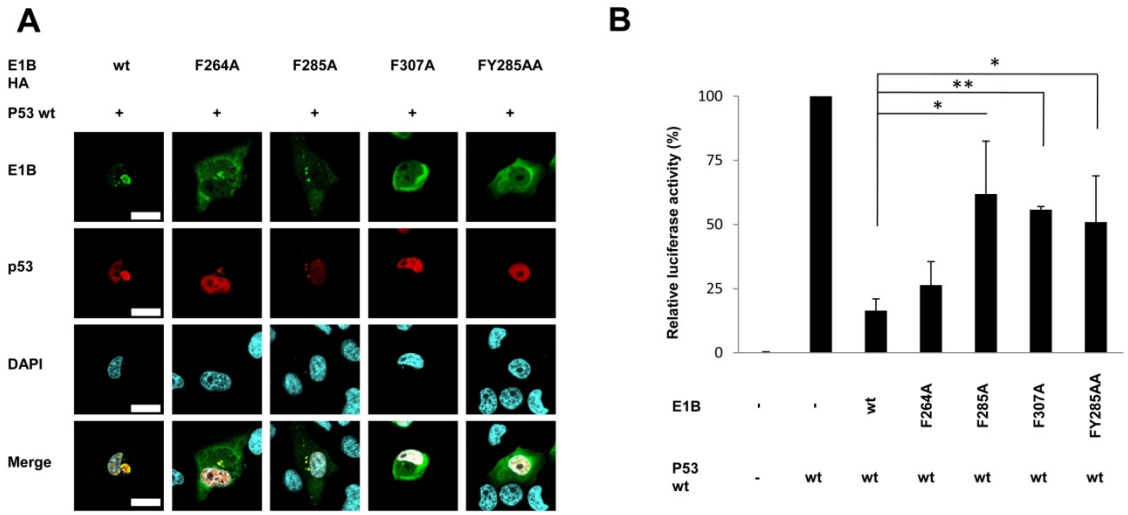

Figure 18. Interaction of substitution mutants of E1B-55 kDa with p53. A) The impact of single amino acid substitution mutants of E1B-55 kDa on p53 localization was assesses as in Fig. 12A. Bar, 20µm. B) The impact of single amino acid substitution mutants of E1B-55 kDa mutants on p53 activity was assessed as in Fig. 12B. P values: *<0,05; **<0,005.

transactivation by p53. This is at least compatible with the assumption that the self-association of E1B-55 kDa might represent a prerequisite for p53 binding.

3.4 E1B-55 kDa oligomerization allows for partial trans-complementation regarding the relocalization of p53.

The point mutant R240A of E1B-55 kDa cannot bind p53 but can still interact with E4org6 and form cytoplasmic clusters (Shen et al, 2001). The E1B-55 kDa mutant Stop396 also fails to antagonize p53 (Fig. 12B), perhaps as a result of its failure to form cytoplasmic clusters. I therefore hypothesized that E1B R240A, despite its inability to bind p53 on its own, might restore the p53 association of E1B Stop396. Immunofluorescence analysis of H1299 transfected cells, showed that the E1B R240A mutant can indeed form cytoplasmic clusters and relocalize E1B Stop396 to a similar extent as wild type E1B-55 kDa (Fig. 19, A and B). While neither E1B Stop396 nor E1B R240A alone were capable of relocalizing p53 to the cytoplasm, their coexpression allowed the detection of p53 in cytoplasmic clusters (Fig. 19, C and D). Oligomerization thus allows for trans-complementation and partially restores the ability of two defective mutants to associate with p53.

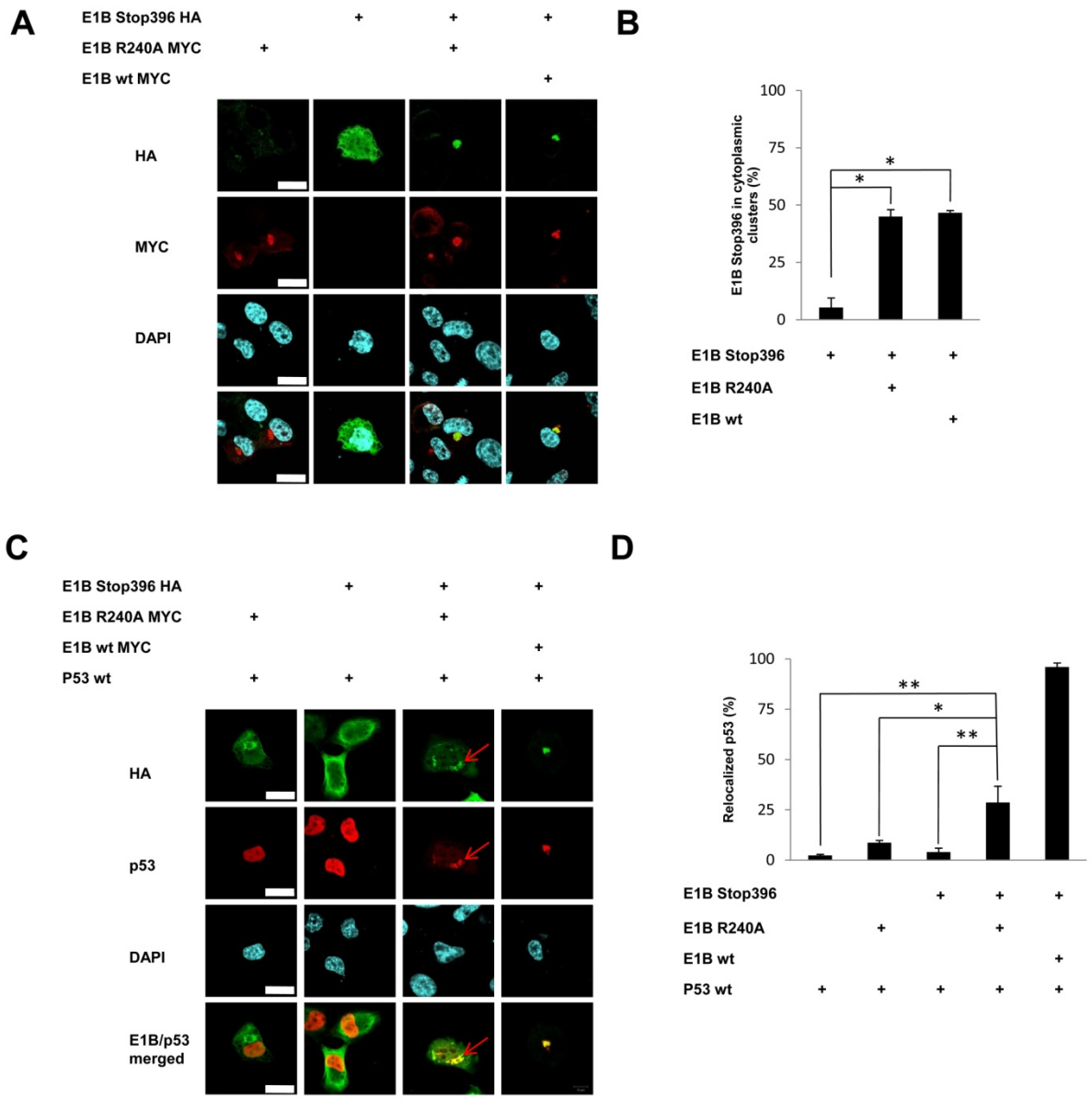

Figure 19. p53 relocalization by transcomplementation of E1B-55 kDa mutants. A) Colocalization of E1B-55 kDa mutants. H1299 cells were transfected with HA–tagged E1B Stop396 (200ng) and MYC-tagged E1B R240A (400ng), or MYC-tagged wild type E1B-55 kDa as indicated. The cells were stained with anti-HA and anti-MYC antibodies, as well as DAPI. Bar, 20µm. B) Atatistical evaluation of HA-tagged Stop396 relocalized to the cytoplasmic clusters. The percentage of cells with E1B Stop396 in cytoplasmic clusters was determined as in Fig. 3B. P values: *<0,0005. C) P53 relocalization by combining E1B-55 kDa mutants. H1299 cells were transfected with wild type p53 (50ng), HA–tagged Stop396 (200ng) and MYC-tagged R240A (400ng) or MYC-tagged wild type E1B-55 kDa as indicated, followed by staining with anti-HA and anti-p53 (FL393) antibodies and DAPI. The sites of cytoplasmic clusters containing both Stop396 and p53 are marked by arrowheads. Bar, 20µm. D) Statistical evaluation of p53 relocalized to the cytoplasmic clusters. The percentage of cells with p53 found in cytoplasmic clusters was determined as in Fig. 3B. P values: *<0,015; **<0,025

3.5 Further characterization of E1B-55 kDa oligomerization domain

3.5.1 E1B-55 kDa fragment spanning amino acids 245-310 fused to EGFP can form cytoplasmic clusters and HMW complexes.

Mutational analysis defined a region within E1B-55 kDa that is required for self-association. This raises the question whether this region is also sufficient to form oligomers. 244-310 amino acid fragment of E1B-55 kDa was fused to the C-terminus of EGFP protein, yielding an EGFP-fragE1B construct.

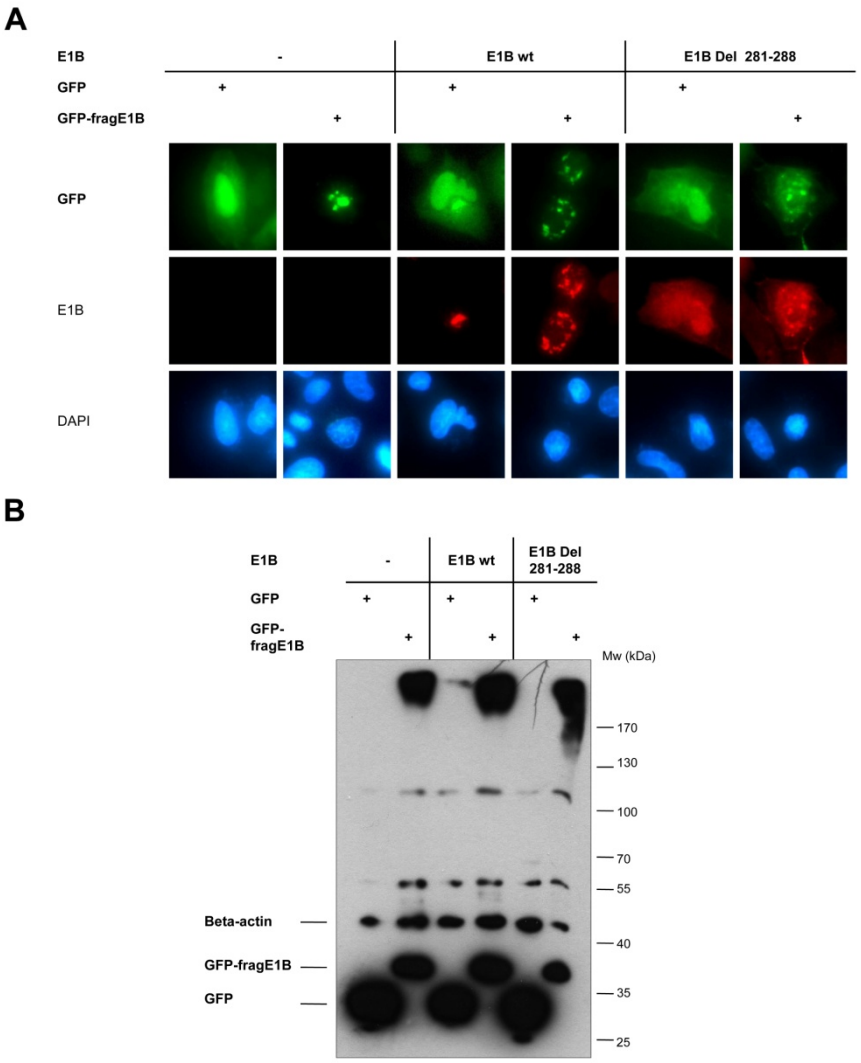

Figure 20. Characterization of EGFP-fragE1B fusion protein. A) Localization of EGFP-fragE1B. H1299 cells were transfected with EGFP-fragE1B (200ng) and HA E1B-55 kDa wt or Del 281-288 (200ng) as indicated. The cells were stained with anti-HA antibody, as well as DAPI. B) Expression pattern of EGFP-fragE1B. Lysates from H1299 cells transfected with the EGFP-fragE1B (650ng) or HA E1B-55 kDa wt or Del 281-288 (200ng) as indicated, were analysed by immunoblotting and staining with anti-EGFP antibody.

First, I analysed the localization of the fusion protein. H1299 cells were cotransfected with EGFP-fragE1B and wt E1B-55 kDa or Del 281-288 to investigate if they could interact with the fusion protein through the oligomerization domain. Immunofluorescence indicated that the EGFP-fragE1B can be found in small cytoplasmic bodies, in contrast to the EGFP alone (Fig. 20A). Moreover, wt E1B-55 kDa was found to colocalize with the EGFP-fragE1B (Fig. 20A). However, EGFP alone was also relocalized by wt E1B-55 kDa into the cytoplasmic speckles (Fig. 20A), albeit less efficiently. This suggests the presence of another site of interaction between E1B-55 kDa and EGFP, independently of the presence of the E1B-55 kDa fragment. The fact that the oligomerization deficient Del 281-288 mutant can be relocalized to cytoplasmic speckles by the EGFP-fragE1B further supports this view. Next, I investigated banding pattern of expressed EGFP-fragE1B by western blotting. EGFP alone was detected as a single band (Fig. 20B). Interestingly, EGFP-fragE1B formed HMW complexes, similarly to the wt E1B-55 kDa (Fig. 20B). These were not increased by co-expression with the wt or mutant E1B-55 kDa (Fig. 20B).

These results suggest that the EGFP fusion partner contributes to protein aggregation and therefore is unsuitable to study E1B-55 kDa oligomerization domain properties.

3.5.2 The 245-310 region of E1B-55 kDa is sufficient to form oligomers when synthesized as a protein fragment.

The EGFP-fragE1B fusion protein studies raised the question whether the E1B-55 kDa oligomerization region is also sufficient to form oligomers when synthesized as a protein fragment without the EGFP fusion partner. I chose to fuse the E1B oligomerization domain to a HA tag. It is a small tag that, in contrast to the EGFP, should not be able to bind E1B-55 kDa on its own. Again 245-310 amino acid fragment of E1B-55 kDa was fused to the C-terminus of HA, creating HA-fragE1B fusion. Additionally, a mutant lacking E1B amino acids 281-288 was created, as described above. HA-tagged betagalactosidase was used as a control. Upon transfection of H1299 cells with HA fusion mutants, I did indeed see big cytoplasmic cluster forming within the periphery of the nucleus (Fig. 21A). However, HA-fragE1B Del281-288 was also present in a big cytoplasmic cluster (Fig 21A). Moreover, analysis of immunoblotted

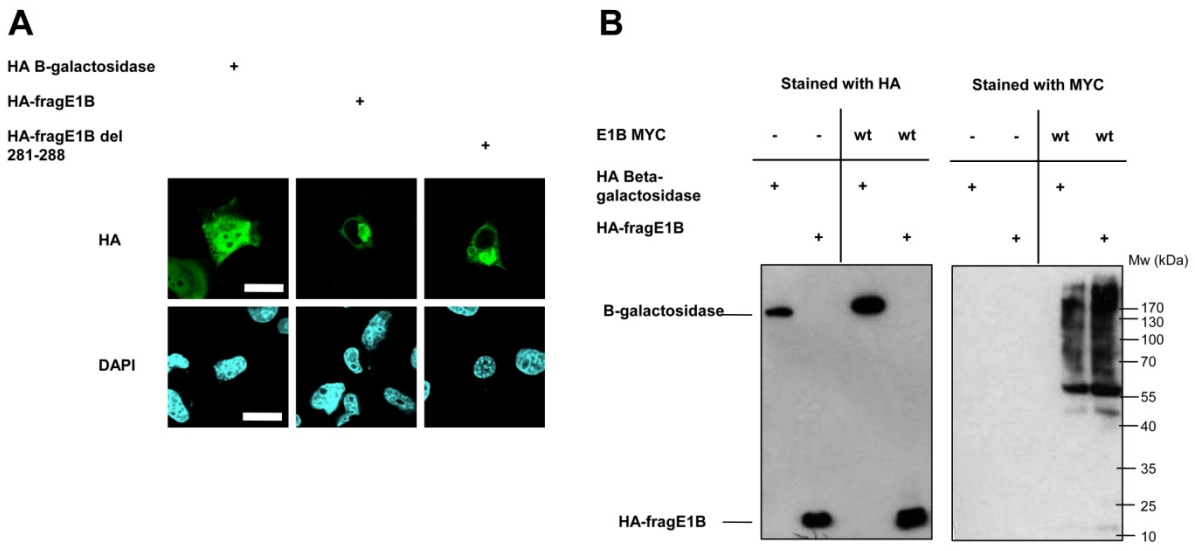

Figure 21. Characterization of HA-fragE1B fusion protein. A) Formation of cytoplasmic clusters by indicated HA-fragE1B constructs was assessed as in Fig. 11A. Bar=20µm. B) Expression pattern of HA-fragE1B. H1299 cells were transfected with the HA-fragE1B (200ng) or in combination with MYC E1B-55 kDa wt (200ng), as indicated. HA-tagged betagalactosidase was used as a control. Lysates were analysed by immunoblotting and staining with anti-HA and anti-MYC antibodies.

proteins revealed that HA-fragE1B is expressed, but I detected only a single band and no HMW complex, in contrast to the EGFP-fragE1B (Fig. 21B). It is therefore seems that the self-association of E1B-55 kDa fragment shows different characteristics compared to whole E1B-55 kDa.

3.5.3 Fusion of the yeast GCN4 oligomerization domain to a E1B-55 kDa oligomerization mutant does not restore its oligomerization.

It has been previously shown, that the p53 oligomerization domain can be replaced by the coiled-coil (CC) domain of the yeast GCN4 protein that mediates oligomerization (Ellenberger et al, 1992; Hu et al, 1990), yielding a functional p53 protein (Roth et al, 2000). To investigate, whether the fusion of a different oligomerization domain to E1B Del 245-296 could restore its proper localization and functions, I created E1B-55 kDa fusion proteins. CC domain was cloned to the N-terminus or within the central region of the E1B-55 kDa protein. Immunofluorescence of H1299 transfected cells revealed, that fusion with the CC domain not only did not restore a proper function of E1B Del 245-296, but also disturbed a proper localization of the wild type E1B-55 kDa (Fig. 22A). Moreover, p53 inhibition in the luciferase reporter assay was not restored (Fig. 22B).

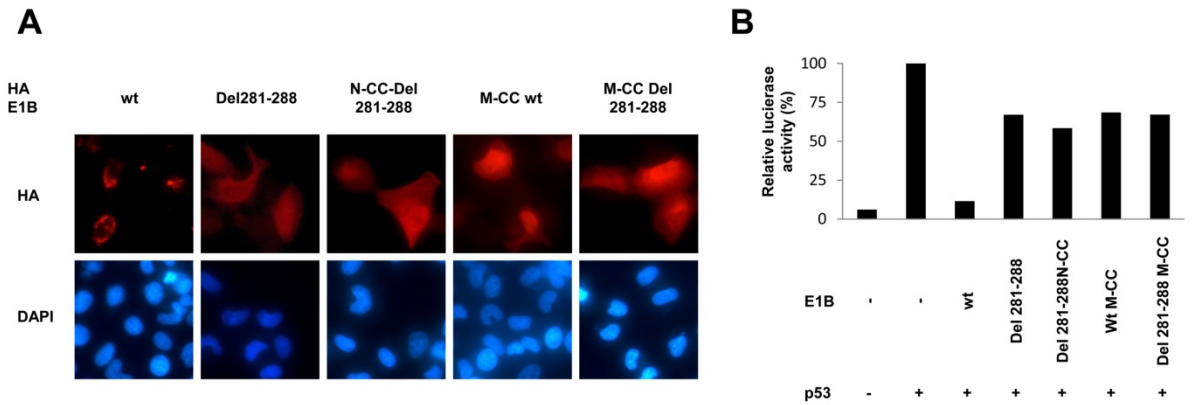

Figure 22. Characterization of CC-E1B fusion constructs. A) Formation of cytoplasmic clusters by indicated CC-E1B fusion mutants was assessed as in Fig. 11A. B) The impact of the CC-E1B-55 fusion constructs on p53 activity was assessed as in Fig. 13B.

3.6 The interaction of E1B-55 kDa and p53 requires p53 oligomerization.

3.6.1 Monomeric p53 cannot be relocalized by wild type E1B-55 kDa.

The fact that p53 forms a tetramer raised the question whether the oligomerization of p53 would also contribute to the interaction of E1B-55 kDa and p53. To answer this, I employed a truncated, oligomerization deficient mutant of p53 carrying a stop codon at position 332. Moreover, specific point mutations within the tetramerization domain of p53 were analyzed. H1299 cells were co-transfected to synthesize wild type E1B-55 kDa and p53 mutants, followed by immunofluorescence analysis. As expected, wild type p53 was efficiently relocalized by E1B-55 kDa from the nucleus to the cytoplasmic clusters (Fig. 23A). In contrast, p53 with mutations at the residues 22/23 remained nuclear (Fig. 23A), in agreement with its defective interaction with E1B-55 kDa (Lin et al, 1994). Interestingly, p53 Stop332 and the monomeric p53 mutant KEEK also remained mostly nuclear, and only a small portion could be detected within the cytoplasmic clusters of E1B-55 kDa (Fig. 23A), as confirmed by quantification of the cells that contained cytoplasmic p53 (Fig. 23B). In contrast, a p53 mutant that forms dimers but not tetramers, p53 LLL, was almost completely relocalized to the cytoplasmic clusters by E1B-55 kDa (Fig. 23, A and B). I also used a dominant inhibitor of p53 oligomerization termed p53DD, a polypeptide that largely consists of the p53 oligomerization domain. It competitively induces the formation of inactive hetero-oligomers with p53, eliminating p53 homotetramers (Shaulian et al, 1992).

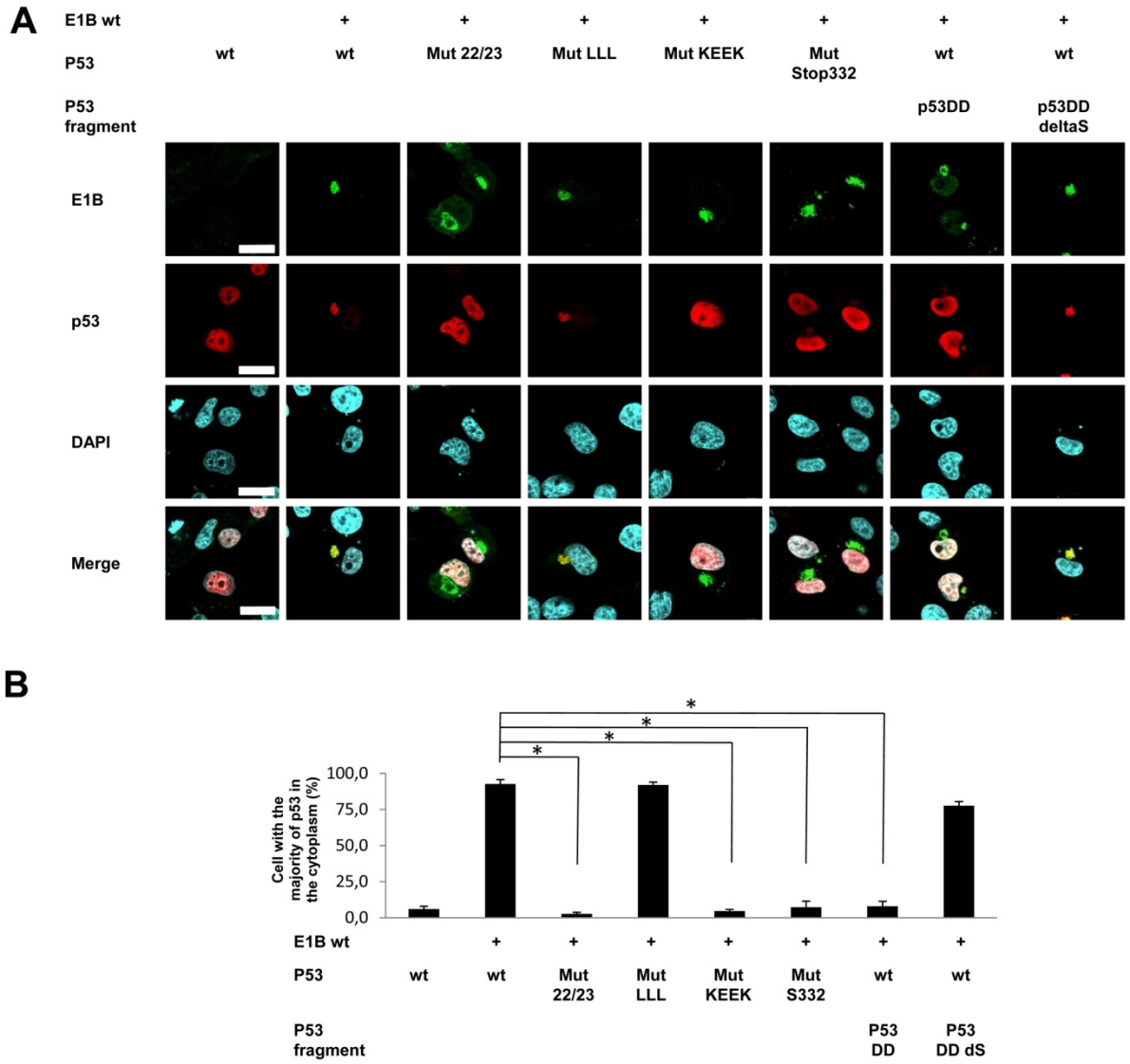

Figure 23. Interaction of wild type E1B-55 kDa with oligomerization-deficient p53. A) H1299 cells were cotransfected with plasmid encoding HA-tagged wild type E1B-55 kDa (400ng) and wild type p53 (50ng), p53 Stop332 (50ng), p53 L22G T23S (50ng), and the oligomerization competitor p53DD or its inactive mutant p53DDdeltaS (200ng) as indicated. After twenty four hours, the cells were fixed and stained with rabbit HA Y-11 antibody specific for E1B-55 kDa, and the mouse monoclonal anti-p53 antibody (DO1). The rabbit FL393 antibody was used to visualize p53 L22G T23S mutant, since it is not recognized by DO1. The locations of the nuclei were visualized by DAPI. Bar=20µm. **B) Statistical evaluation of p53 relocalization to the cytoplasm.** For each experiment, three samples of 50 cells each were counted by a person who was unaware of the identity of the samples. Cells with the majority of the p53's signal in the cytoplasm were counted as positive. The mean and standard deviation of the each sample are indicated for in each case. P values: *<0,00005.

When I coexpressed wild type p53 with wild type E1B-55 kDa and p53DD, p53 mainly stayed nuclear. However, when the inactive control construct p53DDdeltaS was used, p53 relocalization was not disturbed (Fig. 23, A and B).

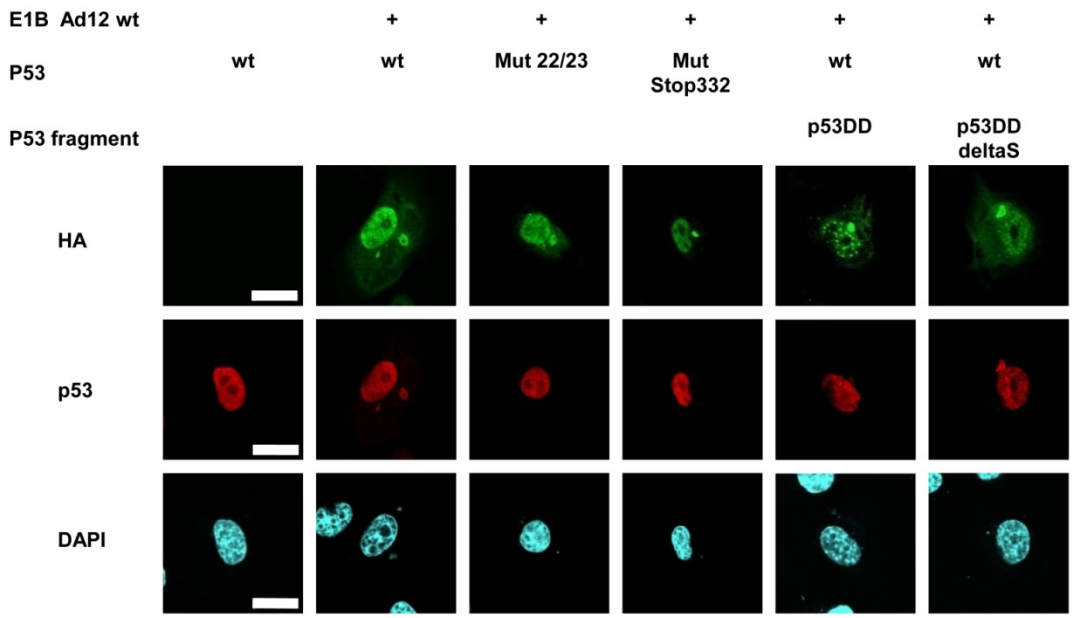

Figure 24. Interaction of wild type Ad12 E1B-55 kDa with p53 oligomerization-deficient mutant was assessed as in Fig. 24A. Bar, 20µm.

Interestingly, the adenovirus type 12 E1B-55 kDa protein also relocalized wild type p53 but not p53 Stop332 (Fig. 24). Earlier studies had reported that p53 relocalization by Ad12 E1B-55 kDa does not depend on the oligomerization status of p53 (Zhao & Liao, 2003). However, in those studies, GFP-tagged Ad12-E1B was used. GFP fusion seemed to localize Ad12 E1B-55 kDa mainly to the cytoplasmic speckles, while I detected it mainly in the nucleus, perhaps representing the reason for the discrepancy.

3.6.2 Monomeric p53 can only weakly bind to E1B-55 kDa.

These results demonstrate that monomeric p53 retains only weak ability to associate with E1B-55 kDa. To further confirm this result, I performed coimmunoprecipitations of p53 and E1B-55 kDa. H1299 cells were co-transfected to synthesize wild type E1B-55 kDa, as well as mutantof p53. Cell lysates were subjected to precipitations with an antibody against p53, followed by immunoblot and staining of p53 and E1B-55 kDa. Analysis of the soluble and insoluble input fractions showed that mutant E1B-55 kDa was more soluble than the wild type protein, apparently reflecting a reduced solubility of the cytoplasmic cluster formed by the latter (Fig. 25A). Upon coexpression with wild type E1B-55 kDa, p53 was found less soluble, perhaps through its association with the cytoplasmic clusters. Wild type E1B-55 kDa

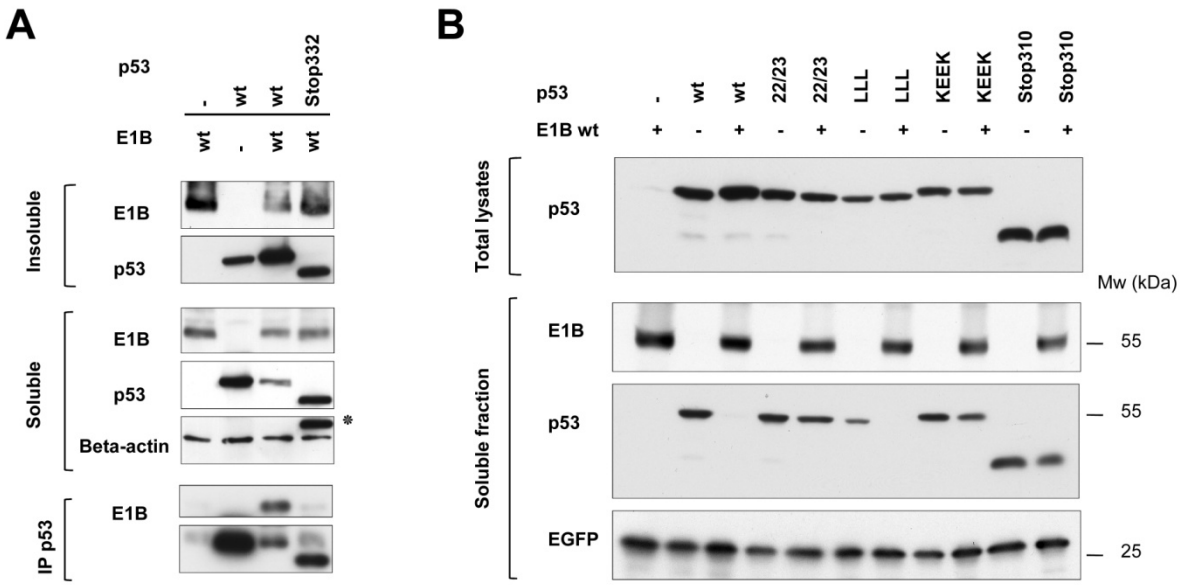

Figure 25. Impact of p53 oligomerization on its interaction with E1B-55 kDa. A) Detection of p53/E1B-55 kDa interaction by co-immunoprecipitation. H1299 cells were transfected with 100ng of a p53 expression constructs and 2ug of E1B-55 kDa expression plasmids as indicated. After twenty four hours the cell lysates were subjected to immunoprecipitation with the FL393 antibody against p53. 2% of the soluble input, 15% of insoluble input, and 20% of the immunoprecipitate were analyzed by immunoblotting with anti-HA and DO1 (anti-p53) antibodies (the later antibody directly coupled to horse reddish peroxidase) Beta-actin staining served as loading control. P53 and beta-actin staining were performed consecutively on the same blot. Therefore p53 Stop332 can be observed in the beta-actin panel indicated by an asterisk. Note the different solubility of wild type E1B-55 kDa and wild type p53 when they were coexpressed, in comparison with the E1B-55 kDa mutants. B) Detection of p53/E1B-55 kDa interaction by changed p53 solubility. H1299 cells were transfected with 50ng of a p53 expression constructs and 500ng of E1B-55 kDa expression plasmid as indicated. After twenty four hours the cell lysates were prepared by incubation with lysis buffer (as described for IP) for 1h on ice. Subsequently, soluble lysates were analyzed by immunoblotting with anti-HA and Fl393 (anti-p53) antibodies. EGFP staining served as transfection control.

was co-precipitated with wild type p53 but not with p53 Stop332 or control (Fig. 25A). The differences in p53 solubility induced by E1B-55 kDa allowed me to use the solubility assay as separate means to look at the interaction of different p53 mutanst with E1B-55 kDa. Wild type p53 as well as dimeric p53 LLL were rendered less soluble upon addition of E1B-55 kDa (Fig. 25B). Monomeric p53 mutants (KEEK and Stop310), and control p53 22/23, stay in majority in soluble fraction, even if E1B-55 kDa is present (Fig. 25B). Thus, p53 solubility seems to decrease through association with E1B-55 kDa. I suggest that both p53 and E1B-55 kDa need to oligomerize for efficient mutual interaction, and that this is due to increased

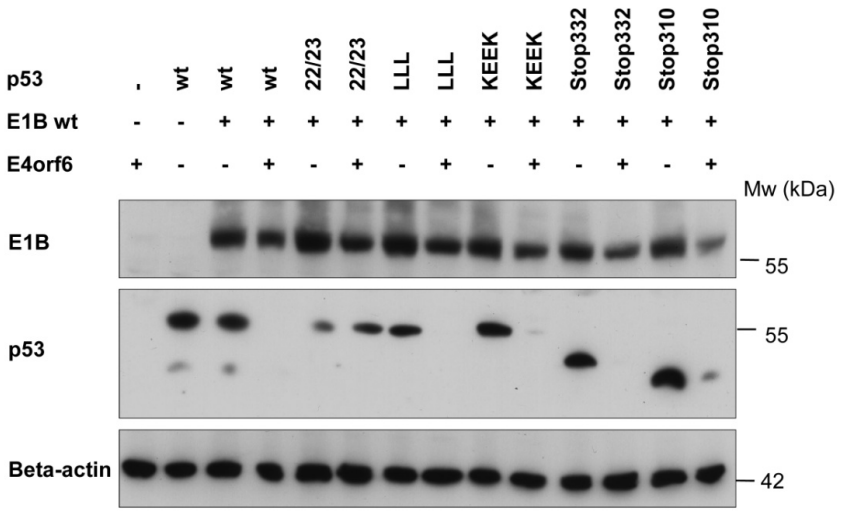

Figure 26. Impact of p53 oligomerization on its degradation by E1B-55/E4orf6 complex. Ability of p53 oligomerization mutants to be degradated upon coexpression with E1B-55 kDa and E4orf6. H1299 cells were transfected with plasmids to express p53 (50ng), E1B-55 kDa (150ng) and E4orf6 (450ng), or 'empty' vectors as indicated. Twenty four hours later the cells were harvested, followed by immunoblot analysis with the indicated antibodies. EGFP and beta-actin served as transfection and loading controls, respectively.

avidity, i.e. complex stabilization though multiple interactions, as known for the interaction between antigens and bi- or polyvalent antibodies.Curiously, p53 does not require its oligomerization domain for degradation when both E1B-55 kDa and E4orf6 are present in the same cell (Fig. 26), as described previously (Querido et al, 2001b). This may be due to the ability of E1B-55 kDa (Sarnow et al, 1982) as well as E4orf6 (Dobner et al, 1996) to interact directly with different regions on p53, possibly giving rise to a trimeric complex that is again held together through multiple interactions but without the need for p53 oligomerization.

4. DISCUSSION

4.1 E1B-55 kDa evolved to inhibit p53-induced cell cycle arrest and apoptosis

The large E1B proteins of adenoviruses are prototype p53 antagonists; in fact, p53 and its importance in cancer were first discovered through its interaction with DNA tumor virus proteins (Berk, 2005; Levine, 2009). Adenovirus belongs to the family of small DNA tumor viruses, which need host cells to enter the S-phase of the cell cycle to complete their life cycle. For their replication they need the host replication machinery: synthesis precursors, DNA polymerase, deoxynucleotides etc. To accomplish this they developed proteins which bind the pRB protein and release E2F to accumulate and induce cells to enter the S-phase (Levine, 2008). Adenovirus uses its first early gene, E1A for this interaction. However, host cells have developed many mechanisms to sense virus entry as well changes in the balance of the cell cycle. At the centre of the defence mechanism, lays p53. It accumulates as a result of increased free levels of E2F, or other stress signals. Viral genome and its replication intermediates are sensed as DNA damage. P53 integrates all these signals and after it is induced, it activates expression of target genes, like p21, which can induce G1 or G2 arrest, cell senesce or even apoptosis (as described in introduction). Thus the control of p53 is essential for adenovirus life cycle. In fact it developed a 'double safe' mechanism for p53 inactivation. E1A not only can bind to pRB it has also been shown to be an inhibitor of p53 transcription (Somasundaram & El-Deiry, 1997). However, the main p53 inhibitor is E1B-55 kDa. E1B-55 kDa developed different ways to eliminate p53 and allow for stable virus growth and replication. It not only binds p53 and blocks it transcription domain, but also it relocalizes p53 from the nucleus to the cytoplasm and together with E4orf6 induces p53 ubiquitination and subsequent degradation (Berk, 2005; Levine, 2009).

The results reported here indicate that the interference of E1B-55 kDa with p53 function is not merely determined by a simple bimolecular interaction. Instead, both partners need to self-associate in order to interact efficiently with each other. This principle may pertain to a large variety of binding partners that associate with E1B-55 kDa and/or with p53.

4.2 Avidity enables the interaction between E1B-55 kDa and p53.

Multiple interactions within a higher-order complex of molecules can strongly enhance the stability of the complex as a whole, in a synergistic fashion. This phenomenon has first been described for antibodies: Since immunoglobulin M antibodies contain ten undistinguishable antigen binding sites (paratopes), the interaction with their epitopes is far stronger than the interaction observed between single Fab fragments and antigens, as long as the antigen is presented in aggregates with multiple epitopes of the same kind. This phenomenon has since then been designated by the term “avidity”, to distinguish the combined stability of such multiple interactions from the affinity of a single bond (Dissanayake et al, 1977). My results strongly suggest that the interaction between E1B-55 kDa and p53 requires a similar avidity phenomenon. Here again, the self association of both partners is required for efficient interaction with each other.

p53 requires the ability to dimerize but does not need to form tetramers for its association with E1B-55 kDa. A simple avidity model would predict that tetramerization of p53 would further increase its association with E1B-55 kDa when compared to p53 dimers. The most straight-forward explanation for the seeming discrepancy consists in the assumption that E1B-55 kDa predominantly forms dimers, leaving only two binding sites for a p53 oligomer. In agreement, dimers were observed when analyzing baculovirus-expressed E1B-55 kDa by gel filtration (Martin & Berk, 1998). However, the large E1B protein from adenovirus type 12 was observed at molecular weights compatible with tetramers or even higher oligomers in a previous study (Grand et al, 1995b). If transferable to the Ad2/Ad5 situation, this suggests that E1B-55 kDa complexes could provide at least four p53 binding sites at a time. Both studies were using in vitro assays, whereas my experiments support the view that E1B-55 kDa also self-associates in mammalian cells. However, it should be taken into account that a four-fold interaction between p53 and E1B-55 kDa would require considerable flexibility of both tetramers. In the case of immunoglobulin M, this flexibility is brought about by the adoption of a “polyp”-like shape that allows the simultaneous binding of several paratopes to aggregated epitopes (Davis & Shulman, 1989). p53 and/or E1B-55 kDa cannot automatically be expected to provide the same degree of structural flexibility, possibly allowing only two p53 molecules within one tetramer to interact with an E1B-55 kDa oligomer. Interestingly, it has been observed that tetramers can be formed from pre-

assembled dimers, as in case of p53 (Chene, 2001). Thus, it is also possible that if E1B-55 kDa forms indeed tetramers, they could be assembled through anti-parallel alignment of the dimers, creating two binding site for p53 interaction.

4.3 Self-association of E1B-55 kDa co-segregates with the formation of cytoplasmic clusters.

For all the mutations within the central region of E1B-55 kDa analyzed here, their ability to self-associate coincided with the accumulation in cytoplasmic clusters. Mutants with a deletion in the carboxyterminal region of E1B-55 kDa also failed to form such clusters but did associate with wild type E1B- 55kDa in these structures. This argues that self-association is a prerequisite for the accumulation of E1B-55 kDa in this interesting intracellular location, while the highly phosphorylated carboxy terminus probably serves as an interaction site for agressome forming proteins. Some reports have provided evidence that E1B-55 kDa accumulates in a structure termed “aggresomes”, characterized by high concentrations of vimentin (Liu et al, 2005). It seems possible that E1B-55 kDa not only associates with pre-formed intracellular structures but actively shapes them or even leads to their formation in the first place. If E1B-55 kDa assumes such a role as a structure-defining element, its ability to self-associate could represent a key mechanism for doing so. High-order multimers could be formed by the aggregation of smaller-order complexes of E1B-55 kDa. In agreement with such a scenario, high molecular weight aggregates that contain E1B-55 kDa (and possibly additional components) have been observed in a previous study (Grand et al, 1995b). Moreover, I have consistently observed high molecular weight aggregates of E1B-55 kDa. Their presence seems to depend on the cysteine bridges formation and can be affected by the denaturation of the proteins. These HMW complexes co-segregate with presence of the oligomerization domain. Although the biochemical nature of these aggregates is unclear at this point, they might reflect cluster formation in vivo. This is also supported by the notion that E1B-55 kDa relocates p53 to a less soluble fraction of cell lysates. We suppose that the accumulation of E1B-55 kDa and p53 in cytoplasmic clusters is associated with multiple protein aggregations and hence decreased solubility.

4.4 Structure predictions of the E1B-55 kDa oligomerization domain

Mutational analysis defined a region within E1B-55 kDa that is required for self-association and proper E1B-55 kDa functioning (Table 12).

Table 12: Summary of the characteristics determined for the E1B-55 kDa mutants under study.

E1B	Wild type	Del 245-296	Del 250-255	Del 281-288	Del 296-310	Stop 396	F264A	F285A	F307A	FY285 AA	R240A
Formation of cytoplasmic clusters	+++	-	-	-	-	-	++	+	-	-	++
P53 inactivation	+++	-	-	-	-	-	+++	-	-	-	-
P53 degradation	+++	-	-	-	-	-	+++	+++	+	+	-
E4orf6 relocalization	+++	-	-	-	-	N/A	+++	++	-	++	+++
Coimmunoprecipitation with E1B-55 kDa wt	+++	+	+	+	+	+	N/A	N/A	+	+	N/A
Colocalization with E1B-55 kDa wt	+++	-	-	+/-	+	+++	+++	+++	+	+	+++

This raises the question whether this region is also sufficient to form oligomers when synthesized as a protein fragment. Indeed the E1B-55 kDa residues 245-310 fused to either EGFP or HA peptide produced cytoplasmic clusters. However, the response of such fragments to point mutations differs from that of full-length E1B-55 kDa. It is therefore difficult to assess whether the self-association of E1B-55 kDa fragments maintains the same characteristics as whole E1B-55 kDa. It seems that the complex conformation of E1B-55 kDa does not allow its division into separate functional domains.

Unfortunately, we do not know much about the E1B-55 kDa folding. It is interesting to speculate on possible E1B-55 kDa structure. A typical oligomerization domain from GCN4 gene or p53, consists of hydrophobic heptat repeats which form a coiled-coil (CC) domain. A monomer forms a beta-strand linked to an alfa-helix by a single glycine. The V shaped monomers interact through their beta-strand forming an anti-parallel double-stranded sheet. The tetramerization is triggered though interaction of the helixes (Fig. 3).

E1B-55 kDa sequence was analysed by meta-server on the genesilico.pl platform. It is a gateway to various third-party programs/servers for protein structure prediction, which has been developed in the Prof. Janusz Bujnicki laboratory in IIMCB, Warsaw, Poland. No coiled-coiled domains have been detected throughout the E1B-55 kDa amino acid sequence,

Table 13: E1B-55 kDa oligomerization domain secondary structure perditions.

	1.....10.....20.....30.....40.....50.....60.....70.....
	FTGPNFSGTVFLANTNLILHGVSFYGFNNTCVEAWTDVRVRGCAFYCCWKGVVCRPKSRASIKKCLFERCTLGILSEG
sspro4	--------EEEE---EEEEE--EEE----EEEEE----EE---EEEEEEE-E------HHHHHHHHHHHHEEEEE---
cdm	--------EEEE-----------------------EEE---HH--------------EEEHHHHHH--EEE----
psipred	--------EEEE----EEEEEEEEE----HHEEE---EEEEEEEEEEEEEE-------EEEE----HH--EEEEEE--
fdm	----------HHH----------------------EEE---HH-----------------HHHHHHHHH-----
jnet	--------EEEEE--EEEEEEEEEE----EEEEEE--EEEEEEEEEEEEEEEE----EEEEEEEEEEEEEEEEE---
porter	-------EEEEEE--EEEEEEEEEEE---EEEEEEE--EE--EEEEEEEEEEE----EEEEEEEEEEEEEEEEE--
sable	--------EEEEE--EEEEEEEEEE-----EEEE---EEEEEEEEEEEEEEEE----EEEEEEEEHEEEEEEEEE--
prof	-------EEEEEE---EEEEEEEEE----EEEEE---EE----EEEEEE--EE------EEEEEEEEEEEEEEEE--
gor	--------EEEE---EEEE----------EEEE----------EEEE-EEEE------EEEEEEEEE---EEE----
Consensus	--------EEEE---EEEEEEEEEE----EEEEE---EEE---EEEEEEEEE------EEEEEEE-EE--EEEEE---
	E –beta-strand; H-alfa-helix

suggesting different oligomerization mechanism compared to p53. Moreover, the CC domain is usually found on the amino—terminal or carboxy–terminal parts of the proteins. In case of E1B-55 kDa it is the central domain, which is responsible for the oligomerization. Thus, it is not surprising that the fusion of the CC domain either to the N-terminus or in the middle of the E1B-55 kDa oligomerization mutant did not restore its functions.

Secondary structure perditions suggest that this region consists mainly of beta-strands, alfa-helixes were rarely predicted (Table 13). Although usually it is the helixes that are described as oligomerizing units, in fact beta-strand are capable of this action too.

Irrespectively of the oligomerization motifs, it often is the hydrophobic amino acids that are necessary for stabilization of the oligomers. E1B-55 kDa oligomerization domain contains many well conserved hydrophobic residues. It is especially rich in the phenylalanine residues. The phenylalanine 307 is especially important in the oligomerization process as substitution of this amino acid to alanine disturbs both the oligomerization as well as E1B-55 kDa proper functioning.

Despite my efforts, I did not manage to obtain probable model of E1B-55 kDa. It is especially hard for 'in vitro' modelling, as it is a viral protein with a very low homology to cellular genes, therefore not suitable for homology-based tertiary structure predictions. 'De novo' platform simulations, without the homology constrains, also did not provide reliable predictions (Selvita Protein Modelling Platform 2.0).

A possible clue to E1B-55 kDa structure comes from the studies of Horridge and Leppard. They showed that the central domain of E1B-55 kDa bears homology to RNP binding proteins (Horridge & Leppard, 1998). Interestingly, the same conserved residues are important for both E1B-55 kDa oligomerization and for RNP protein functions. RNP structures are well characterized. Most important for the interaction are two beta-strands which form an anti-parallel b-sheet, which serves as the interaction plane (Kenan et al, 1991). In E1B-55 kDa they would correspond to amino acids 250-255 and 281-288, which are necessary for E1B oligomerization. Although RNP proteins posses many different function, oligomerization with this domain was not yet characterized. It would be of considerable interest to investigate if the RNP fold allows except for protein-protein and protein-RNA or DNA interactions also for oligomerization. Moreover, at this point we cannot exclude that RNA is involved in the E1B-E1B interaction. I have showed before that at least the HMW complexes formation does not seem to depend on the presence of either RNA or DNA (Morawska Master Thesis, 2007). However, not direct biochemical studies were performed.

To investigate any protein structure there is a need for high amount of pure protein. Previous attempts to solve the E1B-55 kDa structure were complicated by the fact that it cannot be produced in bacteria. Even the E1B-55 kDa DNA sequence seems to be toxic and recombined away by normal bacteria strains. Only special *E. coli* lacking recombinase genes can be used for E1B-55 kDa containing plasmid propagation. Although E1B-55 kDa has been produced from baculovirus infected insects cells, most of the produced E1B-55 kDa is insoluble due to aggregation –cytoplasmic clusters (Martin & Berk, 1998). Oligomerization mutants of E1B-55 kDa did not form aggresomes anymore and were in majority found in the soluble fraction of the cellular lysates. Baculoviruses expressing 307 or 285/286 E1B-55 kDa mutants could become a voluble tool not only to further biochemically characterization of E1B-55 kDa oligomerization but also high scale purification and for further structural studies.

4.5 Interactions of E1B-55 kDa with partners other than p53 may also depend on self-association.

Besides p53, E1B-55 kDa is known to associate with a variety of viral and cellular proteins. Most notably, the association of E1B-55 kDa with E4orf6 co-segregates with E1B-55 kDa self-association in the mutational analysis presented here. This could either indicate that E1B-55 kDa needs to form oligomers in order to associate with E4orf6. Alternatively, however, the region of E1B-55 kDa that binds E4orf6 might overlap with the region forming oligomers. Without being able to decide between these two possibilities, we favour the idea that E1B-55 kDa oligomers associate with E4orf6 more efficiently than monomers; even a discrete point mutation at residue 307 fails to form cytoplasmic clusters and to associate with E4orf6 in the nucleus, while similar mutants are not affected in either ability (Fig. S5). Again, this points out the co-segregation of homo- and hetero-oligomerization. At present though, it is not known whether E4orf6 alone is capable of forming oligomers. Moreover, the interaction between E1B-55 kDa and E4orf6 is further complicated by the need for RUNX1 (Marshall et al, 2008). The E4orf6 protein, rather than E1B-55 kDa, is the principal binding partner for cullin 5 and the ubiquitin ligase complex (Harada et al, 2002; Querido et al, 2001a), making it an even more intriguing question whether and how E4orf6 forms higher-order complexes with itself or in association with E1B-55 kDa.

Another viral E1B-55 kDa association partner is E4orf3 (Konig et al, 1999; Leppard & Everett, 1999). This protein, although highly insoluble and therefore difficult to study, forms large aggregates in the nucleus and cytoplasm (Araujo et al, 2005), suggesting that it might self-associate. E4orf3 could hence provide another example of increased association with E1B-55 kDa through avidity.

Concerning cellular proteins, and on top of p53, E1B-55 kDa associates with mediators of the DNA damage response and DNA repair. Namely, the MRN complex (Stracker et al, 2002) and the associated DNA ligase IV are subject to degradation by E1B-55 kDa and E4orf6. Interestingly, the MRN complex can form higher oligomers to achieve the association of DNA ends (de Jager et al, 2001). It is therefore conceivable that the association with the MRN complex is also based on E1B-55 kDa self-association and the resulting increase in avidity.

Other association partners of E1B-55 kDa, like DNA ligase IV (Baker et al, 2007), Integrin alpha 3 (Dallaire et al, 2009) or the single-stranded DNA-binding protein 2 (Fleisig et al, 2007) are not (yet) known to oligomerize but may possibly form similar complexes.

4.6 p53-oligomerization may increase its avidity to associate with many binding partners.

p53 is known to associate with a large variety of cellular proteins, and our study on E1B-55 kDa raises the question whether the ability of p53 to form tetramers contributes to at least some of these associations.

In the case of viral proteins, it is noteworthy that the SV40 large T antigen, a known p53 binding oncoprotein, forms hexamers. It would be tempting to speculate that, again, this would provide a basis for efficient interaction with a p53 tetramer. However, the crystal structure of the complex rather suggests that T antigen disrupts the ability of p53 to form oligomers through massive conformational changes (Lilyestrom et al, 2006). Apparently, the affinity of T antigen to p53 is sufficient to bind p53 monomers without the need for higher order complex formation.

In contrast, the principal cellular p53 antagonist, Mdm2, has been found to form oligomers either with itself or with its paralogue MdmX/Mdm4 (Singh et al, 2007). Moreover, the oligomerization of p53 is required for its degradation through Mdm2 (Kubbutat et al, 1998; Maki, 1999). Together, these findings strongly suggest that the interaction of Mdm2 and p53, or at least the subsequent ubiquitin ligation, are fortified by an avidity effect analogous to the E1B 55 kDa-p53-interaction.

Many additional interaction partners of p53 have not been assessed as to their capability to self-associate. This includes histone acetyltransferases, kinases, and numerous other direct or indirect modifiers of its activity (Bode & Dong, 2004). A majority of these modifiers is found in large protein complexes. This raises the intriguing possibility that the stabilization of associations between p53 and its partners through avidity effects is a general phenomenon that reaches far beyond the interaction with adenovirus E1B-55 kDa.

5. APPENDIX

Table 14: P values (levels of significance, as determined by Student's T-Test) for the statistical comparisons throughout the thesis

Figure	First Value	Second Value	P value
3B	Del245-296/wt	Stop396/wt	0,000241897
3B	Del281-288/wt	Stop396/wt	0,000315023
3B	Del281-288/wt	Stop396/wt	0,000158614
3B	Del296-310/wt	Stop396/wt	0,014277023
4B	Wt	Del245-296	0,00660396
4B	Wt	Del281-288	0,000343019
4B	Wt	Del250-255	0,000266309
4B	Wt	Del296-310	0,005671853
4B	Wt	R240A	0,005428277
4B	Wt	Stop396	0,004009248
5D	Wt	F264A	0,219245776
5D	Wt	F285A	0,052123465
5D	Wt	F307A	0,004138994
5D	Wt	FY285AA	0,001197456
6B	Wt/wt	F264A	0,096203193
6B	Wt/wt	F285A	0,028171378
6B	Wt/wt	F307A	0,001320027
6B	Wt/wt	FY285AA	0,036303128
7B	Wt	22/23	3,79904E-05
7B	Wt	LLL	0,385012819
7B	Wt	KEEK	4,0238E-05
7B	Wt	Stop332	9,4791E-06
7B	Wt	p53DD	3,39592E-06
7B	Wt	p53DDdeltaS	0,001758674
8B	Stop396	Stop396/R240A	0,000403211
8B	Stop396	Stop396/wt	0,000132611
8D	p53	Stop396/R240A	0,014731918
8D	Stop396	Stop396/R240A	0,023910472
8D	R240A	Stop396/R240A	0,014059254
S2B	Wt, c1	F285A, c1	0,001143271
S2B	Wt, c3	F285A, c3	0,000394321
S2B	Wt, c4	F285A, c4	0,000970944

6. REFERENCES

Appella E, Anderson CW (2001) Post-translational modifications and activation of p53 by genotoxic stresses. *Eur J Biochem* **268**(10)**:** 2764-2772

Araujo FD, Stracker TH, Carson CT, Lee DV, Weitzman MD (2005) Adenovirus type 5 E4orf3 protein targets the Mre11 complex to cytoplasmic aggresomes. *J Virol* **79**(17)**:** 11382-11391

Babiss LE, Ginsberg HS, Darnell JE, Jr. (1985) Adenovirus E1B proteins are required for accumulation of late viral mRNA and for effects on cellular mRNA translation and transport. *Mol Cell Biol* **5**(10)**:** 2552-2558

Baker A, Rohleder KJ, Hanakahi LA, Ketner G (2007) Adenovirus E4 34k and E1b 55k oncoproteins target host DNA ligase IV for proteasomal degradation. *J Virol* **81**(13)**:** 7034-7040

Balagurumoorthy P, Sakamoto H, Lewis MS, Zambrano N, Clore GM, Gronenborn AM, Appella E, Harrington RE (1995) Four p53 DNA-binding domain peptides bind natural p53-response elements and bend the DNA. *Proc Natl Acad Sci U S A* **92**(19)**:** 8591-8595

Baptiste N, Friedlander P, Chen X, Prives C (2002) The proline-rich domain of p53 is required for cooperation with anti-neoplastic agents to promote apoptosis of tumor cells. *Oncogene* **21**(1)**:** 9-21

Beltz GA, Flint SJ (1979) Inhibition of HeLa cell protein synthesis during adenovirus infection. Restriction of cellular messenger RNA sequences to the nucleus. *J Mol Biol* **131**(2)**:** 353-373

Benko M, Harrach B (2003) Molecular evolution of adenoviruses. . In *Adenoviruses:Model and Vectors in Virus Host Interactions:Virion and Structure, Viral Replication, Host Cell Interactions*, Doerfler W, Bohm P (eds). Berlin: Springer

Benko M, Harrach B, Russell WC (2000) Family Adenoviridae. In *Virus Taxonomy,Seventh Report of the International Committee on Taxonomy of Viruses*, M. H. V. van Regenmortel, C. M. Fauquet, D. H. L. Bishop, E. B. Carstens, M. K. Estes, S. M. Lemon, J. Maniloff, M. A. Mayo, D. J. McGeoch, C. R. Pringle, Wickner RB (eds), pp 227–238. San Diego: Academic Press

Bensaad K, Tsuruta A, Selak MA, Vidal MN, Nakano K, Bartrons R, Gottlieb E, Vousden KH (2006) TIGAR, a p53-inducible regulator of glycolysis and apoptosis. *Cell* **126**(1)**:** 107-120

Berk AJ (2005) Recent lessons in gene expression, cell cycle control, and cell biology from adenovirus. *Oncogene* **24**(52)**:** 7673-7685

Bischoff JR, Kirn DH, Williams A, Heise C, Horn S, Muna M, Ng L, Nye JA, Sampson-Johannes A, Fattaey A, McCormick F (1996) An adenovirus mutant that replicates selectively in p53-deficient human tumor cells. *Science* **274**(5286)**:** 373-376

Blackford AN, Grand RJ (2009) Adenovirus E1B 55-kilodalton protein: multiple roles in viral infection and cell transformation. *J Virol* **83**(9)**:** 4000-4012

Blair Zajdel ME, Blair GE (1988) The intracellular distribution of the transformation-associated protein p53 in adenovirus-transformed rodent cells. *Oncogene* **2**(6)**:** 579-584

Blanchette P, Kindsmuller K, Groitl P, Dallaire F, Speiseder T, Branton PE, Dobner T (2008) Control of mRNA export by adenovirus E4orf6 and E1B55K proteins during productive infection requires E4orf6 ubiquitin ligase activity. *J Virol* **82**(6)**:** 2642-2651

Bode AM, Dong Z (2004) Post-translational modification of p53 in tumorigenesis. *Nat Rev Cancer* **4**(10)**:** 793-805

Branton P (1999) Early Gene Expression. In *Adenoviruses: Basic Biology to Gene Therapy*, Seth P (ed), 5. Texas: R.G. Landes Company Austin

Carson CT, Schwartz RA, Stracker TH, Lilley CE, Lee DV, Weitzman MD (2003) The Mre11 complex is required for ATM activation and the G2/M checkpoint. *EMBO J* **22**(24)**:** 6610-6620

Cawley S, Bekiranov S, Ng HH, Kapranov P, Sekinger EA, Kampa D, Piccolboni A, Sementchenko V, Cheng J, Williams AJ, Wheeler R, Wong B, Drenkow J, Yamanaka M, Patel S, Brubaker S, Tammana H, Helt G, Struhl K, Gingeras TR (2004) Unbiased mapping of transcription factor binding sites along human chromosomes 21 and 22 points to widespread regulation of noncoding RNAs. *Cell* **116**(4)**:** 499-509

Chan TA, Hermeking H, Lengauer C, Kinzler KW, Vogelstein B (1999) 14-3-3Sigma is required to prevent mitotic catastrophe after DNA damage. *Nature* **401**(6753)**:** 616-620

Chao C, Wu Z, Mazur SJ, Borges H, Rossi M, Lin T, Wang JY, Anderson CW, Appella E, Xu Y (2006) Acetylation of mouse p53 at lysine 317 negatively regulates p53 apoptotic activities after DNA damage. *Mol Cell Biol* **26**(18)**:** 6859-6869

Chene P (2001) The role of tetramerization in p53 function. *Oncogene* **20**(21)**:** 2611-2617

Dallaire F, Blanchette P, Groitl P, Dobner T, Branton PE (2009) Identification of integrin alpha3 as a new substrate of the adenovirus E4orf6/E1B 55-kilodalton E3 ubiquitin ligase complex. *J Virol* **83**(11)**:** 5329-5338

Davis AC, Shulman MJ (1989) IgM--molecular requirements for its assembly and function. *Immunol Today* **10**(4)**:** 118-122; 127-118

de Jager M, van Noort J, van Gent DC, Dekker C, Kanaar R, Wyman C (2001) Human Rad50/Mre11 is a flexible complex that can tether DNA ends. *Mol Cell* **8**(5)**:** 1129-1135

Delphin C, Huang KP, Scotto C, Chapel A, Vincon M, Chambaz E, Garin J, Baudier J (1997) The in vitro phosphorylation of p53 by calcium-dependent protein kinase C--characterization of a protein-kinase-C-binding site on p53. *Eur J Biochem* **245**(3)**:** 684-692

Dissanayake S, Hay FC, Roitt IM (1977) The binding constants of IgM rheumatoid factors and their univalent fragments for native and aggregated human IgG. *Immunology* **32**(3)**:** 309-318

Dobbelstein M (2004) Replicating adenoviruses in cancer therapy. *Curr Top Microbiol Immunol* **273:** 291-334

Dobbelstein M, Roth J, Kimberly WT, Levine AJ, Shenk T (1997) Nuclear export of the E1B 55-kDa and E4 34-kDa adenoviral oncoproteins mediated by a rev-like signal sequence. *EMBO J* **16**(14)**:** 4276-4284

Dobner T, Horikoshi N, Rubenwolf S, Shenk T (1996) Blockage by adenovirus E4orf6 of transcriptional activation by the p53 tumor suppressor. *Science* **272**(5267)**:** 1470-1473

Dosch T, Horn F, Schneider G, Kratzer F, Dobner T, Hauber J, Stauber RH (2001) The adenovirus type 5 E1B-55K oncoprotein actively shuttles in virus-infected cells, whereas transport of E4orf6 is mediated by a CRM1-independent mechanism. *J Virol* **75**(12)**:** 5677-5683

Dyer BW, Ferrer FA, Klinedinst DK, Rodriguez R (2000) A noncommercial dual luciferase enzyme assay system for reporter gene analysis. *Anal Biochem* **282**(1)**:** 158-161

el-Deiry WS, Kern SE, Pietenpol JA, Kinzler KW, Vogelstein B (1992) Definition of a consensus binding site for p53. *Nat Genet* **1**(1)**:** 45-49

Ellenberger TE, Brandl CJ, Struhl K, Harrison SC (1992) The GCN4 basic region leucine zipper binds DNA as a dimer of uninterrupted alpha helices: crystal structure of the protein-DNA complex. *Cell* **71**(7)**:** 1223-1237

Endter C, Kzhyshkowska J, Stauber R, Dobner T (2001) SUMO-1 modification required for transformation by adenovirus type 5 early region 1B 55-kDa oncoprotein. *Proc Natl Acad Sci U S A* **98**(20)**:** 11312-11317

Espinosa JM, Emerson BM (2001) Transcriptional regulation by p53 through intrinsic DNA/chromatin binding and site-directed cofactor recruitment. *Mol Cell* **8**(1)**:** 57-69

Feng L, Lin T, Uranishi H, Gu W, Xu Y (2005) Functional analysis of the roles of posttranslational modifications at the p53 C terminus in regulating p53 stability and activity. *Mol Cell Biol* **25**(13)**:** 5389-5395

Feng Z, Hu W, de Stanchina E, Teresky AK, Jin S, Lowe S, Levine AJ (2007) The regulation of AMPK beta1, TSC2, and PTEN expression by p53: stress, cell and tissue specificity, and the role of these gene products in modulating the IGF-1-AKT-mTOR pathways. *Cancer Res* **67**(7)**:** 3043-3053

Fleisig HB, Orazio NI, Liang H, Tyler AF, Adams HP, Weitzman MD, Nagarajan L (2007) Adenoviral E1B55K oncoprotein sequesters candidate leukemia suppressor sequence-specific single-stranded DNA-binding protein 2 into aggresomes. *Oncogene* **26**(33)**:** 4797-4805

Flint J (1999) Organization of the Adenoviral Genome. In *Adenoviruses: Basic Biology to Gene Therapy*, Seth P (ed), 3. Texas: R.G. Landes Company Austin

Flint SJ, Huang W, Goodhouse J, Kyin S (2005) A peptide inhibitor of exportin1 blocks shuttling of the adenoviral E1B 55 kDa protein but not export of viral late mRNAs. *Virology* **337**(1)**:** 7-17

Flores ER, Tsai KY, Crowley D, Sengupta S, Yang A, McKeon F, Jacks T (2002) p63 and p73 are required for p53-dependent apoptosis in response to DNA damage. *Nature* **416**(6880)**:** 560-564

Funk WD, Pak DT, Karas RH, Wright WE, Shay JW (1992) A transcriptionally active DNA-binding site for human p53 protein complexes. *Mol Cell Biol* **12**(6)**:** 2866-2871

Gonzalez RA, Flint SJ (2002) Effects of mutations in the adenoviral E1B 55-kilodalton protein coding sequence on viral late mRNA metabolism. *J Virol* **76**(9)**:** 4507-4519

Goodrum FD, Ornelles DA (1998) p53 status does not determine outcome of E1B 55-kilodalton mutant adenovirus lytic infection. *J Virol* **72**(12)**:** 9479-9490

Goodrum FD, Ornelles DA (1999) Roles for the E4 orf6, orf3, and E1B 55-kilodalton proteins in cell cycle-independent adenovirus replication. *J Virol* **73**(9)**:** 7474-7488

Goodrum FD, Shenk T, Ornelles DA (1996) Adenovirus early region 4 34-kilodalton protein directs the nuclear localization of the early region 1B 55-kilodalton protein in primate cells. *J Virol* **70**(9)**:** 6323-6335

Gotz C, Scholtes P, Prowald A, Schuster N, Nastainczyk W, Montenarh M (1999) Protein kinase CK2 interacts with a multi-protein binding domain of p53. *Mol Cell Biochem* **191**(1-2)**:** 111-120

Grand RJ, Lecane PS, Owen D, Grant ML, Roberts S, Levine AJ, Gallimore PH (1995a) The high levels of p53 present in adenovirus early region 1-transformed human cells do not cause up-regulation of MDM2 expression. *Virology* **210**(2)**:** 323-334

Grand RJ, Mustoe T, Roberts S, Gallimore PH (1995b) The quaternary structure of the adenovirus 12 early region 1B 54K protein. *Virology* **207**(1)**:** 255-259

Gu W, Shi XL, Roeder RG (1997) Synergistic activation of transcription by CBP and p53. *Nature* **387**(6635)**:** 819-823

Halbert DN, Cutt JR, Shenk T (1985) Adenovirus early region 4 encodes functions required for efficient DNA replication, late gene expression, and host cell shutoff. *J Virol* **56**(1)**:** 250-257

Hall AR, Dix BR, O'Carroll SJ, Braithwaite AW (1998) p53-dependent cell death/apoptosis is required for a productive adenovirus infection. *Nat Med* **4**(9)**:** 1068-1072

Han J, Goldstein LA, Hou W, Rabinowich H (2007) Functional linkage between NOXA and Bim in mitochondrial apoptotic events. *J Biol Chem* **282**(22)**:** 16223-16231

Harada JN, Berk AJ (1999) p53-Independent and -dependent requirements for E1B-55K in adenovirus type 5 replication. *J Virol* **73**(7)**:** 5333-5344

Harada JN, Shevchenko A, Pallas DC, Berk AJ (2002) Analysis of the adenovirus E1B-55K-anchored proteome reveals its link to ubiquitination machinery. *J Virol* **76**(18)**:** 9194-9206

Harms KL, Chen X (2006) The functional domains in p53 family proteins exhibit both common and distinct properties. *Cell Death Differ* **13**(6)**:** 890-897

Hartl B, Zeller T, Blanchette P, Kremmer E, Dobner T (2008) Adenovirus type 5 early region 1B 55-kDa oncoprotein can promote cell transformation by a mechanism independent from blocking p53-activated transcription. *Oncogene* **27**(26)**:** 3673-3684

Haupt Y, Maya R, Kazaz A, Oren M (1997) Mdm2 promotes the rapid degradation of p53. *Nature* **387**(6630)**:** 296-299

Hearing P, Samulski RJ, Wishart WL, Shenk T (1987) Identification of a repeated sequence element required for efficient encapsidation of the adenovirus type 5 chromosome. *J Virol* **61**(8)**:** 2555-2558

Hermeking H (2009) MiR-34a and p53. *Cell Cycle* **8**(9)**:** 1308

Horn HF, Vousden KH (2007) Coping with stress: multiple ways to activate p53. *Oncogene* **26**(9)**:** 1306-1316

Horridge JJ, Leppard KN (1998) RNA-binding activity of the E1B 55-kilodalton protein from human adenovirus type 5. *J Virol* **72**(11)**:** 9374-9379

Hu JC, O'Shea EK, Kim PS, Sauer RT (1990) Sequence requirements for coiled-coils: analysis with lambda repressor-GCN4 leucine zipper fusions. *Science* **250**(4986)**:** 1400-1403

Hutton FG, Turnell AS, Gallimore PH, Grand RJ (2000) Consequences of disruption of the interaction between p53 and the larger adenovirus early region 1B protein in adenovirus E1 transformed human cells. *Oncogene* **19**(3)**:** 452-462

Jaiswal AS, Narayan S (2001) p53-dependent transcriptional regulation of the APC promoter in colon cancer cells treated with DNA alkylating agents. *J Biol Chem* **276**(21)**:** 18193-18199

Kenan DJ, Query CC, Keene JD (1991) RNA recognition: towards identifying determinants of specificity. *Trends Biochem Sci* **16**(6)**:** 214-220

Kindsmuller K, Schreiner S, Leinenkugel F, Groitl P, Kremmer E, Dobner T (2009) A 49 kDa isoform of the adenovirus type 5 early region 1B 55K protein is sufficient to support virus replication. *J Virol*

Knights CD, Catania J, Di Giovanni S, Muratoglu S, Perez R, Swartzbeck A, Quong AA, Zhang X, Beerman T, Pestell RG, Avantaggiati ML (2006) Distinct p53 acetylation cassettes differentially influence gene-expression patterns and cell fate. *J Cell Biol* **173**(4)**:** 533-544

Konig C, Roth J, Dobbelstein M (1999) Adenovirus type 5 E4orf3 protein relieves p53 inhibition by E1B-55-kilodalton protein. *J Virol* **73**(3)**:** 2253-2262

Kopito RR (2000) Aggresomes, inclusion bodies and protein aggregation. *Trends Cell Biol* **10**(12)**:** 524-530

Kratzer F, Rosorius O, Heger P, Hirschmann N, Dobner T, Hauber J, Stauber RH (2000) The adenovirus type 5 E1B-55K oncoprotein is a highly active shuttle protein and shuttling is independent of E4orf6, p53 and Mdm2. *Oncogene* **19**(7)**:** 850-857

Kubbutat MH, Jones SN, Vousden KH (1997) Regulation of p53 stability by Mdm2. *Nature* **387**(6630)**:** 299-303

Kubbutat MH, Ludwig RL, Ashcroft M, Vousden KH (1998) Regulation of Mdm2-directed degradation by the C terminus of p53. *Mol Cell Biol* **18**(10)**:** 5690-5698

Lee JH, Paull TT (2005) ATM activation by DNA double-strand breaks through the Mre11-Rad50-Nbs1 complex. *Science* **308**(5721)**:** 551-554

Leppard KN, Everett RD (1999) The adenovirus type 5 E1b 55K and E4 Orf3 proteins associate in infected cells and affect ND10 components. *J Gen Virol* **80 (Pt 4):** 997-1008

Levine AJ (2009) The common mechanisms of transformation by the small DNA tumor viruses: The inactivation of tumor suppressor gene products: p53. *Virology* **384**(2)**:** 285-293

Lilyestrom W, Klein MG, Zhang R, Joachimiak A, Chen XS (2006) Crystal structure of SV40 large T-antigen bound to p53: interplay between a viral oncoprotein and a cellular tumor suppressor. *Genes Dev* **20**(17)**:** 2373-2382

Lin J, Chen J, Elenbaas B, Levine AJ (1994) Several hydrophobic amino acids in the p53 amino-terminal domain are required for transcriptional activation, binding to mdm-2 and the adenovirus 5 E1B 55-kD protein. *Genes Dev* **8**(10)**:** 1235-1246

Liu L, Scolnick DM, Trievel RC, Zhang HB, Marmorstein R, Halazonetis TD, Berger SL (1999) p53 sites acetylated in vitro by PCAF and p300 are acetylated in vivo in response to DNA damage. *Mol Cell Biol* **19**(2)**:** 1202-1209

Liu X, Yue P, Khuri FR, Sun SY (2004) p53 upregulates death receptor 4 expression through an intronic p53 binding site. *Cancer Res* **64**(15)**:** 5078-5083

Liu Y, Shevchenko A, Berk AJ (2005) Adenovirus exploits the cellular aggresome response to accelerate inactivation of the MRN complex. *J Virol* **79**(22)**:** 14004-14016

Maki CG (1999) Oligomerization is required for p53 to be efficiently ubiquitinated by MDM2. *J Biol Chem* **274**(23)**:** 16531-16535

Malette P, Yee SP, Branton PE (1983) Studies on the phosphorylation of the 58000 dalton early region 1B protein of human adenovirus type 5. *J Gen Virol* **64**(Pt 5)**:** 1069-1078

Marchenko ND, Zaika A, Moll UM (2000) Death signal-induced localization of p53 protein to mitochondria. A potential role in apoptotic signaling. *J Biol Chem* **275**(21)**:** 16202-16212

Marshall LJ, Moore AC, Ohki M, Kitabayashi I, Patterson D, Ornelles DA (2008) RUNX1 permits E4orf6-directed nuclear localization of the adenovirus E1B-55K protein and associates with centers of viral DNA and RNA synthesis. *J Virol* **82**(13)**:** 6395-6408

Martin ME, Berk AJ (1998) Adenovirus E1B 55K represses p53 activation in vitro. *J Virol* **72**(4)**:** 3146-3154

Massimi P, Pim D, Bertoli C, Bouvard V, Banks L (1999) Interaction between the HPV-16 E2 transcriptional activator and p53. *Oncogene* **18**(54)**:** 7748-7754

Mayo LD, Seo YR, Jackson MW, Smith ML, Rivera Guzman J, Korgaonkar CK, Donner DB (2005) Phosphorylation of human p53 at serine 46 determines promoter selection and whether apoptosis is attenuated or amplified. *J Biol Chem* **280**(28)**:** 25953-25959

Montenarh M, Vesco C, Kemmerling G, Muller D, Henning R (1986) Regions of SV40 large T antigen necessary for oligomerization and complex formation with the cellular oncoprotein p53. *FEBS Lett* **204**(1)**:** 51-55

Muller M, Scaffidi CA, Galle PR, Stremmel W, Krammer PH (1998) The role of p53 and the CD95 (APO-1/Fas) death system in chemotherapy-induced apoptosis. *Eur Cytokine Netw* **9**(4)**:** 685-686

Nagaich AK, Zhurkin VB, Durell SR, Jernigan RL, Appella E, Harrington RE (1999) p53-induced DNA bending and twisting: p53 tetramer binds on the outer side of a DNA loop and increases DNA twisting. *Proc Natl Acad Sci U S A* **96**(5)**:** 1875-1880

Nakano K, Vousden KH (2001) PUMA, a novel proapoptotic gene, is induced by p53. *Mol Cell* **7**(3)**:** 683-694

Oda E, Ohki R, Murasawa H, Nemoto J, Shibue T, Yamashita T, Tokino T, Taniguchi T, Tanaka N (2000a) Noxa, a BH3-only member of the Bcl-2 family and candidate mediator of p53-induced apoptosis. *Science* **288**(5468)**:** 1053-1058

Oda K, Arakawa H, Tanaka T, Matsuda K, Tanikawa C, Mori T, Nishimori H, Tamai K, Tokino T, Nakamura Y, Taya Y (2000b) p53AIP1, a potential mediator of p53-dependent apoptosis, and its regulation by Ser-46-phosphorylated p53. *Cell* **102**(6)**:** 849-862

Olivier M, Petitjean A, Marcel V, Petre A, Mounawar M, Plymoth A, de Fromentel CC, Hainaut P (2009) Recent advances in p53 research: an interdisciplinary perspective. *Cancer Gene Ther* **16**(1)**:** 1-12

Ornelles DA, Shenk T (1991) Localization of the adenovirus early region 1B 55-kilodalton protein during lytic infection: association with nuclear viral inclusions requires the early region 4 34-kilodalton protein. *J Virol* **65**(1)**:** 424-429

Pilder S, Moore M, Logan J, Shenk T (1986) The adenovirus E1B-55K transforming polypeptide modulates transport or cytoplasmic stabilization of viral and host cell mRNAs. *Mol Cell Biol* **6**(2)**:** 470-476

Polyak K, Xia Y, Zweier JL, Kinzler KW, Vogelstein B (1997) A model for p53-induced apoptosis. *Nature* **389**(6648)**:** 300-305

Qian H, Wang T, Naumovski L, Lopez CD, Brachmann RK (2002) Groups of p53 target genes involved in specific p53 downstream effects cluster into different classes of DNA binding sites. *Oncogene* **21**(51)**:** 7901-7911

Querido E, Blanchette P, Yan Q, Kamura T, Morrison M, Boivin D, Kaelin WG, Conaway RC, Conaway JW, Branton PE (2001a) Degradation of p53 by adenovirus E4orf6 and E1B55K proteins occurs via a novel mechanism involving a Cullin-containing complex. *Genes Dev* **15**(23)**:** 3104-3117

Querido E, Marcellus RC, Lai A, Charbonneau R, Teodoro JG, Ketner G, Branton PE (1997) Regulation of p53 levels by the E1B 55-kilodalton protein and E4orf6 in adenovirus-infected cells. *J Virol* **71**(5)**:** 3788-3798

Querido E, Morrison MR, Chu-Pham-Dang H, Thirlwell SW, Boivin D, Branton PE (2001b) Identification of three functions of the adenovirus e4orf6 protein that mediate p53 degradation by the E4orf6-E1B55K complex. *J Virol* **75**(2)**:** 699-709

Rawlins DR, Rosenfeld PJ, Wides RJ, Challberg MD, Kelly TJ, Jr. (1984) Structure and function of the adenovirus origin of replication. *Cell* **37**(1)**:** 309-319

Roth J, Koch P, Contente A, Dobbelstein M (2000) Tumor-derived mutations within the DNA-binding domain of p53 that phenotypically resemble the deletion of the proline-rich domain. *Oncogene* **19**(14)**:** 1834-1842

Roth J, Konig C, Wienzek S, Weigel S, Ristea S, Dobbelstein M (1998) Inactivation of p53 but not p73 by adenovirus type 5 E1B 55-kilodalton and E4 34-kilodalton oncoproteins. *J Virol* **72**(11)**:** 8510-8516

Rothmann T, Hengstermann A, Whitaker NJ, Scheffner M, zur Hausen H (1998) Replication of ONYX-015, a potential anticancer adenovirus, is independent of p53 status in tumor cells. *J Virol* **72**(12)**:** 9470-9478

Sablina AA, Budanov AV, Ilyinskaya GV, Agapova LS, Kravchenko JE, Chumakov PM (2005) The antioxidant function of the p53 tumor suppressor. *Nat Med* **11**(12)**:** 1306-1313

Sagne C, Isambert MF, Henry JP, Gasnier B (1996) SDS-resistant aggregation of membrane proteins: application to the purification of the vesicular monoamine transporter. *Biochem J* **316 (Pt 3):** 825-831

Samuels-Lev Y, O'Connor DJ, Bergamaschi D, Trigiante G, Hsieh JK, Zhong S, Campargue I, Naumovski L, Crook T, Lu X (2001) ASPP proteins specifically stimulate the apoptotic function of p53. *Mol Cell* **8**(4)**:** 781-794

Sarnow P, Hearing P, Anderson CW, Halbert DN, Shenk T, Levine AJ (1984) Adenovirus early region 1B 58,000-dalton tumor antigen is physically associated with an early region 4 25,000-dalton protein in productively infected cells. *J Virol* **49**(3)**:** 692-700

Sarnow P, Ho YS, Williams J, Levine AJ (1982) Adenovirus E1b-58kd tumor antigen and SV40 large tumor antigen are physically associated with the same 54 kd cellular protein in transformed cells. *Cell* **28**(2)**:** 387-394

Schwartz RA, Lakdawala SS, Eshleman HD, Russell MR, Carson CT, Weitzman MD (2008) Distinct requirements of adenovirus E1b55K protein for degradation of cellular substrates. *J Virol* **82**(18)**:** 9043-9055

Shaulian E, Zauberman A, Ginsberg D, Oren M (1992) Identification of a minimal transforming domain of p53: negative dominance through abrogation of sequence-specific DNA binding. *Mol Cell Biol* **12**(12)**:** 5581-5592

Sheikh MS, Burns TF, Huang Y, Wu GS, Amundson S, Brooks KS, Fornace AJ, Jr., el-Deiry WS (1998) p53-dependent and -independent regulation of the death receptor KILLER/DR5 gene expression in response to genotoxic stress and tumor necrosis factor alpha. *Cancer Res* **58**(8)**:** 1593-1598

Shen Y, Kitzes G, Nye JA, Fattaey A, Hermiston T (2001) Analyses of single-amino-acid substitution mutants of adenovirus type 5 E1B-55K protein. *J Virol* **75**(9)**:** 4297-4307

Singh RK, Iyappan S, Scheffner M (2007) Hetero-oligomerization with MdmX rescues the ubiquitin/Nedd8 ligase activity of RING finger mutants of Mdm2. *J Biol Chem* **282**(15)**:** 10901-10907

Somasundaram K, El-Deiry WS (1997) Inhibition of p53-mediated transactivation and cell cycle arrest by E1A through its p300/CBP-interacting region. *Oncogene* **14**(9)**:** 1047-1057

Steegenga WT, Riteco N, Jochemsen AG, Fallaux FJ, Bos JL (1998) The large E1B protein together with the E4orf6 protein target p53 for active degradation in adenovirus infected cells. *Oncogene* **16**(3)**:** 349-357

Stewart ZA, Pietenpol JA (2001) p53 Signaling and cell cycle checkpoints. *Chem Res Toxicol* **14**(3)**:** 243-263

Stracker TH, Carson CT, Weitzman MD (2002) Adenovirus oncoproteins inactivate the Mre11-Rad50-NBS1 DNA repair complex. *Nature* **418**(6895)**:** 348-352

Tack LC, Cartwright CA, Wright JH, Eckhart W, Peden KW, Srinivasan A, Pipas JM (1989) Properties of a simian virus 40 mutant T antigen substituted in the hydrophobic region: defective ATPase and oligomerization activities and altered phosphorylation accompany an inability to complex with cellular p53. *J Virol* **63**(8)**:** 3362-3367

Teodoro JG, Branton PE (1997) Regulation of p53-dependent apoptosis, transcriptional repression, and cell transformation by phosphorylation of the 55-kilodalton E1B protein of human adenovirus type 5. *J Virol* **71**(5)**:** 3620-3627

Thornborrow EC, Manfredi JJ (1999) One mechanism for cell type-specific regulation of the bax promoter by the tumor suppressor p53 is dictated by the p53 response element. *J Biol Chem* **274**(47)**:** 33747-33756

Tokino T, Thiagalingam S, el-Deiry WS, Waldman T, Kinzler KW, Vogelstein B (1994) p53 tagged sites from human genomic DNA. *Hum Mol Genet* **3**(9)**:** 1537-1542

Turnell AS, Grand RJ, Gallimore PH (1999) The replicative capacities of large E1B-null group A and group C adenoviruses are independent of host cell p53 status. *J Virol* **73**(3)**:** 2074-2083

Walker KK, Levine AJ (1996) Identification of a novel p53 functional domain that is necessary for efficient growth suppression. *Proc Natl Acad Sci U S A* **93**(26)**:** 15335-15340

Wang XW, Zhan Q, Coursen JD, Khan MA, Kontny HU, Yu L, Hollander MC, O'Connor PM, Fornace AJ, Jr., Harris CC (1999) GADD45 induction of a G2/M cell cycle checkpoint. *Proc Natl Acad Sci U S A* **96**(7)**:** 3706-3711

Wei CL, Wu Q, Vega VB, Chiu KP, Ng P, Zhang T, Shahab A, Yong HC, Fu Y, Weng Z, Liu J, Zhao XD, Chew JL, Lee YL, Kuznetsov VA, Sung WK, Miller LD, Lim B, Liu ET, Yu Q, Ng HH, Ruan Y (2006) A global map of p53 transcription-factor binding sites in the human genome. *Cell* **124**(1)**:** 207-219

Weigel S, Dobbelstein M (2000) The nuclear export signal within the E4orf6 protein of adenovirus type 5 supports virus replication and cytoplasmic accumulation of viral mRNA. *J Virol* **74**(2)**:** 764-772

Weinberg DH, Ketner G (1986) Adenoviral early region 4 is required for efficient viral DNA replication and for late gene expression. *J Virol* **57**(3)**:** 833-838

Wienzek S, Roth J, Dobbelstein M (2000) E1B 55-kilodalton oncoproteins of adenovirus types 5 and 12 inactivate and relocalize p53, but not p51 or p73, and cooperate with E4orf6 proteins to destabilize p53. *J Virol* **74**(1)**:** 193-202

Woo JL, Berk AJ (2007) Adenovirus ubiquitin-protein ligase stimulates viral late mRNA nuclear export. *J Virol* **81**(2)**:** 575-587

Yew PR, Berk AJ (1992) Inhibition of p53 transactivation required for transformation by adenovirus early 1B protein. *Nature* **357**(6373)**:** 82-85

Yu J, Zhang L, Hwang PM, Kinzler KW, Vogelstein B (2001) PUMA induces the rapid apoptosis of colorectal cancer cells. *Mol Cell* **7**(3)**:** 673-682

Zantema A, Schrier PI, Davis-Olivier A, van Laar T, Vaessen RT, van der EA (1985) Adenovirus serotype determines association and localization of the large E1B tumor antigen with cellular tumor antigen p53 in transformed cells. *Mol Cell Biol* **5**(11)**:** 3084-3091

Zhao LY, Liao D (2003) Sequestration of p53 in the cytoplasm by adenovirus type 12 E1B 55-kilodalton oncoprotein is required for inhibition of p53-mediated apoptosis. *J Virol* **77**(24)**:** 13171-13181

Zhao LY, Santiago A, Liu J, Liao D (2007) Repression of p53-mediated transcription by adenovirus E1B 55-kDa does not require corepressor mSin3A and histone deacetylases. *J Biol Chem* **282**(10)**:** 7001-7010

Zhu J, Zhang S, Jiang J, Chen X (2000) Definition of the p53 functional domains necessary for inducing apoptosis. *J Biol Chem* **275**(51)**:** 39927-39934

CURRICULUM VITAE

Magdalena Morawska-Onyszczuk

Contact details **Dabrowskiego 7/8A, Sopot, 81-754, Poland**
Tel: 0048 504633862
Tel: 0049 1636852270
mmoraws@gwdg.de

Personal Information

Date of Birth: 27.06.1983
Place of Birth: Pszczolki (Poland)
Citizenship: polish

Education:

Expected date to finish PhD by January 2010

- 2007 PhD in the Department of Molecular Oncology, University Goettingen, Germany, in the lab of Prof. Matthias Dobbelstein
with a collaboration with Department of Molecular Virology, Intercollegiate Faculty of Biotechnology, Gdansk University and Medical University of Gdansk, Poland, in the lab of Prof. Krystyna Bienkowska-Szewczyk

2007 - 2005 MSc at International Master's/PhD Molecular Biology Program, International Max Planck Research School. University Goettingen, Germany (highly selective 20/400 applications accepted)

2005 - 2002 BSc at International Program First Level Degree: "Job Creation Oriented Biotechnology", University Perugia, Italy

Professional Experience:

- 04/2007 Provisional PhD thesis title: Self-association of adenovirus type 5 E1B-55 kDa”,

03/2007-9/2006 Master thesis: “Molecular interactions of the adenovirus E1B-55 kDa oncoprotein”. Department of Molecular Oncology, University Goettingen, Germany, Grade: A.

06/2005-01/2005 Bachelor thesis: "Isolation of novel chimpanzee adenovirus and development of vector transfer system". IRBM Merck Research Laboratories, Rome, Italy, Grade: A.

08/2004-06/2004 Short Research project: "Parvoviruses: role of infection with adeno-associated virus (AAV) of human heart tissue, addressing a possible role of this virus in cardiomyopathies". Department of Applied Tumor Virology, German Cancer Research Centre (DKFZ), Heidelberg, Germany

08/2003-06/2003 Short Research project "The lambda pR promoter activity in *Escherichia coli* cgtA mutant". Institute of Biochemistry and Biophysics of Polish Academy of Science, Gdansk, Poland

Techniques:

- Cloning;
- PCR;
- Site-directed mutagenesis
- Real timer PCR
- Cell culture and transfection (adherent cells and liquid culture)
- SDS-PAGE
- Silver staining
- Western Blot
- Immunostaining
- Immunoprecipitation
- Immunofluorescence
- Luciferase assay
- Adenovirus growth and purification
- Infection of eukaryotic cells

Additional Courses:

- Introduction to animal experiments
- Applications of the Tandem Affinity Purification tag
- Posttranslational modification with ubiquitin-related protein SUMO
- Intercultural Communication Workshop
- Poznan Summer School of Bioinformatics 2009

Awards and Honours:

- 2005 - 2006 Stipend International Max Planck Research School
- 2003 - 2005 Scholarship for academic excellence, Dept. of Biotechnology, Gdansk, Poland
- 09/2004 - 12/2004 Socrates/Erasmus Scholarship

Additional qualifications:

- Microsoft Office,
- Cambridge Young Enterprise International Examination, 2001, grade A
- Fluent in English,
- Conversation Level German and Italian,

Hobbies:

- Work in Student Radio,
- Art,
- Theatre.

Career goals:

I want to become an independent researcher and a group leader.

References:

Prof. Dr. med. Matthias Dobbelstein
Department of Molecular Oncology
Ernst Caspari Haus
Göttingen Center of Molecular Biosciences, GZMB
Faculty of Medicine, University of Göttingen
Justus von Liebig Weg 11, Room 3.234
D-37077 Göttingen
Germany
phone: 0049 551 39 13840
fax: 0049 551 39 13713
e-mail: mdobbel@gwdg.de

Prof. Krystyna Bienkowska-Szewczyk
Department of Molecular Virology
Intercollegiate Faculty of Biotechnology
University Gdansk and Medical University Gdansk
Kładki 24
80-822 Gdańsk
Poland
phone: 0048 58 5236381
e-mail: krysia@biotech.ug.gda.pl