

# **Analysis of the HPV E6 “proteome”, “ubiquitome” and “interactome”**

Dissertation

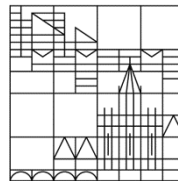
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## ABBREVIATIONS

aa	amino acid(s)
bp	base pairs
$\beta$ -Gal	$\beta$ -galactosidase
cDNA	coding DNA
CMV	cytomegalo virus
DMSO	dimethylsulfoxide
dNTP	deoxynucleoside triphosphate
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
E1	ubiquitin activating enzyme
E2	ubiquitin conjugating enzyme
E3	ubiquitin ligase
FACS	fluorescence activated cell sorting
FCS	fetal calf serum
GFP	green fluorescent protein
GST	glutathione-S-transferase
His-tag	6x Histidin-tag
IP	immunoprecipitation
IPTG	Isopropyl- $\beta$ -D-thiogalactopyranoside
HA	human influenza hemagglutinin
HPV	human papillomavirus
kDa	kilo Dalton
LB	Lucia Broth
Mdm	murine double minute
mRNA	messenger RNA
Ni-NTA	Nickel-nitrilotriacetic acid
OD	optical density
ONPG	o-nitrophenol- $\beta$ -galacto-pyranoside

ORF	open reading frame
PBS	phosphate buffered saline
PCR	polymerase chain reaction
puro <sup>r</sup>	puromycin resistance gene
rpm	revolutions per minute
RT	reverse transcription
SILAC	stable isotope labeling by amino acids in cell culture
TK	thymidine kinase
TUBEs	Tandem ubiquitin binding entities
Ubi	ubiquitin
wt	wild-type

## ABSTRACT

Human papillomaviruses (HPVs) are small double-stranded DNA viruses that infect cutaneous and mucosal epithelial cells. Mucosal HPVs can be divided in high risk and low risk types depending on their association with clinical lesions. Infection with high risk types can lead to the development of cervical cancer, whereas infection with low risk types induces the formation of benign genital tumors. E6 proteins of high risk HPVs contribute to the development of cervical cancer by utilizing the cellular ubiquitin ligase E6AP to target the tumor suppressor p53 and several other cellular proteins for degradation. In contrast, E6 proteins of low risk HPVs are only weakly oncogenic and only little is known about their biochemical and physiological properties.

In order to obtain further insights into the role of the high risk and low risk E6 proteins in the development of cervical cancer and benign tumors, in the first part of this thesis, the HPV E6 proteins were analyzed with respect to their influences on the “proteome” and “ubiquitome” of cells. In addition, interaction partners of HPV E6 were identified. To determine changes on proteomes upon expression of E6 proteins (“HPV E6 proteome”), we developed an inducible expression system that allows switching on and off E6 expression within cells. We used this system combined with quantitative mass spectrometry (stable isotope labeling of amino acids in cell culture, SILAC) and determined the effect of different E6 proteins on the protein expression pattern of a cell in general. For the identification of proteins that are ubiquitylated in an E6-dependent manner (“HPV E6 ubiquitome”), an *in cellulo* assay using tandem ubiquitin binding entities (TUBEs) as stabilization and isolation tool of ubiquitylated proteins was established. For the “interactome” studies (proteins that interact with E6), pulldown experiments with GST-tagged E6 and cell lysates from cells that do or do not express E6AP in combination with quantitative mass spectrometry (SILAC) were performed. With this approach, three subunits of the Anaphase Promoting Complex (APC/C) among others were identified as interaction partners of E6. APC/C is an E3 ligase whose major role is controlling the cell cycle. The interaction between APC/C and the HPV E6 proteins could be verified. However, further investigations are necessary to elucidate the physiological function of this interaction. In conclusion, this study contributes to the elucidation of the cellular pathways that are affected by both low risk and high risk E6 proteins.

In the second part of this thesis we showed that the E6 interacting protein LNX1 is able to bind to the tumor suppressor p53 and that it induces ubiquitylation and degradation of p53 in an E6-independent manner. p53 is a tightly regulated protein and has critical functions including regulation of cell death, senescence and proliferation. Our data show that an additional E3 ligase, LNX1 is involved in the regulation of p53.

## Zusammenfassung

Humane Papillomaviren (HPVs) sind kleine doppelsträngige DNA Viren, die kutane und muköse Epithelzellen infizieren. Muköse HPVs werden aufgrund ihres onkogenen Potenzials in Hoch-Risiko (*high risk*) und Niedrig-Risiko (*low risk*) -Typen unterteilt. Infektionen mit *high risk* HPVs können zur Entstehung von Zervixkarzinomen führen; die Folge einer Infektion mit *low risk* HPVs ist die Entstehung von gutartigen Tumoren. *High risk* E6 Proteine spielen bei der Entstehung von Zervixkarzinomen eine große Rolle: sie binden an die zelluläre E3-Ligase E6AP und induzieren dadurch den Abbau von p53 und weiteren zellulären Substraten. Im Gegensatz dazu besitzen die E6 Proteine der *low risk* HPV-Typen nur eine sehr geringe onkogene Wirkung. Über ihre biochemischen sowie physiologischen Funktionen ist bislang nur sehr wenig bekannt. Um neue Einblicke in die Rolle von *high risk* und *low risk* E6 Proteinen in der Entstehung von Zervixkarzinomen und gutartigen Tumoren zu gewinnen, wurden im ersten Teil dieser Arbeit E6 Proteine von *high risk* und *low risk* HPV-Typen hinsichtlich ihres Einflusses auf das "Proteom" und "Ubiquitom" untersucht. Weiterhin wurden HPV E6 Interaktionspartner identifiziert. Zur Untersuchung des Einflusses von E6 auf das "Proteom" einer Zelle wurde ein induzierbares Expressionssystem entwickelt, mit dem die Expression der E6 Proteine in Zellen an- und ausgeschaltet werden kann. In Kombination mit quantitativer Massenspektrometrie (SILAC, stable isotope labeling of amino acids in cell culture) wurden mit diesem System Effekte von verschiedenen E6 Proteinen auf die Expressionslevel der zellulären Proteine im Allgemeinen analysiert ("HPV E6 Proteom"). Um Proteine, die E6-abhängig ubiquitiniert werden ("HPV E6 Ubiquitom"), zu identifizieren, wurde mit Hilfe von TUBEs (*tandem ubiquitin binding entities*) ein *in cellulo* Assay etabliert, bei dem TUBEs als Stabilisierungs- und Isolierungstool für ubiquitinierte Proteine dienen. Zur Bestimmung neuer Interaktionspartner der E6 Proteine ("HPV E6 Interaktom") wurden Bindeassays mit GST-getagtem E6 Protein und Lysaten von Zellen, die entweder E6AP exprimieren, oder nicht, kombiniert mit quantitativer Massenspektrometrie (SILAC) durchgeführt. Im Zuge dieser Bindeassays konnte unter anderem drei Untereinheiten der E3 Ligase APC/C als Interaktionspartner für E6 identifiziert werden. APC/C ist eine E3 Ligase, die eine wichtige Rolle in der Zellzyklusregulation spielt. Die Interaktion von E6 mit APC/C konnte verifiziert werden, jedoch muss die physiologische Funktion dieser Interaktion noch weiter untersucht werden. Schlussfolgernd hat diese Arbeit zur Aufklärung der Funktionen der E6 Proteine von *high risk* und *low risk* HPV-Typen und deren Einfluss auf zelluläre Signalwege beigetragen.

Im zweiten Teil dieser Arbeit konnte gezeigt werden, dass die mit E6-interagierende E3 Ligase LNX1 in der Lage ist, den Tumorsuppressor p53 zu binden und dass LNX1 die

Ubiquitinierung und den Abbau von p53 induziert. Dies geschieht in einem E6-unabhängigen Mechanismus. p53 ist ein stark reguliertes Protein und spielt eine wichtige Rolle in verschiedenen zellulären Prozessen, wie Zelltod, Seneszenz und Proliferation. Unsere Daten zeigen, dass eine weitere E3 Ligase, LNX1, in der Regulation von p53 involviert ist.

# 1 INTRODUCTION

Controlled degradation of proteins is important for the regulation of many cellular processes like cell cycle progression, DNA damage response, regulation of immune response or quality control of proteins. In most cases degradation of proteins is mediated by the ubiquitin proteasome system (UPS). In an enzymatic cascade involving three different classes of enzymes, ubiquitin molecules are attached to substrates that are subsequently degraded by the proteasome (reviewed in Hershko and Ciechanover, 1998) (for details see 1.2). Viruses are known to manipulate the UPS for their own benefit. The main viral targets of UPS are deubiquitylation enzymes (DUBs) and E3 ubiquitin ligases, which are important for substrate specificity. There are viruses known that express their own E3 ubiquitin ligase or DUB. Others encode adaptor proteins which interact with cellular E3 ligases thereby changing their substrate specificity (Blanchette and Branton, 2009; Randow and Lehner, 2009). Examples for viral E3 ligases are the herpes simplex virus-1 protein ICP0 and Kaposi's sarcoma-associated herpesvirus proteins K3 and K5 (Boutell et al., 2002; Casey et al., 2010; Wang et al., 2008). A viral protein that acts as an adaptor for cellular E3 ligases is for example the human papillomavirus (HPV) E6 (reviewed in Moody and Laimins, 2010)(for details see 1.1).

## 1.1 HUMAN PAPILLOMAVIRUSES

Human papillomaviruses (HPVs) are small double-stranded DNA viruses which infect epithelial cells and cause a diverse range of epithelial lesions (reviewed in zur Hausen, 2002). So far, more than 100 different HPV types are known. Different HPV types are distinguished on the basis of DNA sequences in certain regions of their genome. A new type is defined if the sequence of the L1 protein's ORF (open reading frame) differs by more than 10 % from the closest known papillomavirus (Bernard et al., 2010; de Villiers et al., 2004). Depending on the tissue they infect, HPVs can be divided in mucosal and cutaneous HPVs. Furthermore, based on their association with clinical lesions the mucosal HPVs can be classified into high risk and low risk types (reviewed in zur Hausen, 2002). While infection with high risk types, for example HPV type 16 and 18, can lead to the development of cervical cancer, infection with low risk types such as HPV types 6 or 11 does not. Nevertheless, they are medically important since they cause benign genital tumors (reviewed in zur Hausen, 2002).

HPVs are non-enveloped viruses with an icosahedral capsid. Replication of the viral genome is dependent on the replication machinery of the infected cell and takes place in the nucleus of the host cell. The viral life cycle of HPVs is unique, as it is coupled to the differentiation program of the infected host cell, usually keratinocytes. Microwounds of the epithelium lead

to the exposure of cells in the basal layer thus making them susceptible to infections by HPVs (reviewed in Longworth and Laimins, 2004; Moody and Laimins, 2010). Viral entry is known to be a multistep process but so far, the exact mechanism is not completely understood. HPV entry is initiated by binding to heparan sulfated proteoglycans on the cell surface. After viral entry the production of approximately 20-100 extrachromosomal copies of the viral DNA per cell is initiated (Giroglou et al., 2001; Joyce et al., 1999). After basal cell division, viral genomes are portioned into daughter cells, of which one detaches from the basal layer, migrates toward the *stratum granulosum* and undergoes differentiation. In uninfected epithelium, differentiated cells exit cell cycle which leads to the loss of nuclei in suprabasal cells. In HPV-positive cells this is not the case due to the action of the viral E7 protein (for details see 1.1.5.1). Expression of E7 results in reentering of the cells in the cell cycle which leads to a characteristic retention of nuclei throughout all layers of infected epithelia (Cheng et al., 1995). In differentiated layers of the epithelium virion assembly takes place followed by the release of the viruses from the epithelial cells (reviewed in Conway and Meyers, 2009).

### **1.1.1 Role of HPV infection in cancer development**

Cervical cancer is according to the World Health Organization (WHO) the second most common cancer in women worldwide (web page, WHO). Nowadays it is accepted that development of cervical cancer is linked to infection with high risk HPV types. In 2008, Harald zur Hausen received the Nobel-prize in medicine for his research on this topic. In the early 1980s, his group isolated HPV DNA from cervical cancer biopsies for the first time (Boshart et al., 1984; Durst et al., 1983). Due to experimental research in the following years, the basic mechanism of the role of HPVs in cervical cancer development was elucidated and a direct role of HPV infection in cervical cancer development was shown. For example, expression of the viral E6 and E7 proteins was detected in cervical cancer cell lines and cancer biopsies (Schwarz et al., 1985). The integration of the viral DNA in the host genome was another important finding. Thereby the ORF encoding for the E2 protein, which regulates viral transcription, is disrupted. This leads to an uncontrolled expression of the E6 and E7 oncoproteins, which is believed to be one of the major causes of the development of cervical cancer after HPV infection (Durst et al., 1983) (for details see 1.1.5).

In 90 % of all cases of cervical cancer, HPV DNA of one of 15 different HPV types was found which are classified as high risk types. HPV type 16 and 18 represent the most frequent isolated virus types and are found in 50 % (type 16) and 20 % (type 18) of cervical lesions (Munoz et al., 2003; Smith et al., 2007). However, most infections do not lead to cancer formation. Instead, the viruses are cleared by the immune system. Only in a small percentage of cases infections persist, resulting in the development of low and high grade cervical

intraepithelial neoplasia (CIN). These may either regress or progress to an invasive cervical carcinoma (reviewed in Stanley, 2010). As HPVs are sexually transmitted, sexual behavior is one of the main risk factors for developing cervical cancer. Other risk factors include smoking, long term usage of oral contraceptives, age and impaired immune response (Almonte et al., 2008; Lenselink et al., 2008; Sellors et al., 2003; Shields et al., 2004).

Due to cytological screening programs ("pap smears") in developed countries the cervical cancer rate could be reduced (Humphries, 2012). In addition, two commercially available prophylactic HPV vaccines are available: a bivalent HPV 16 and 18 virus-like particle (VLP) vaccine from GlaxoSmithKline Biologicals (Cervarix™) and a quadrivalent HPV 6, 11, 16 and 18 VLP vaccine from MSD Merck (Gardasil™). As shown by a number of randomized clinical trials, these vaccines are effective in preventing HPV infections (Stanley, 2006). VLPs are made up of HPV L1 capsid proteins resulting in morphologically similar structure to the virus. After their injection antibodies are formed against VLPs (Kwak et al., 2011; Stanley, 2007). One disadvantage of these vaccines is that they only protect against infection by two of 15 high risk HPV types.

HPV infection is not only associated with the development of cervical cancer, it also plays a role in the development of other malignancies like anal cancer, penile cancer, vulvar and vaginal neoplasia and head and neck cancers (Parkin, 2006).

Cutaneous HPV types, mostly types 5 and 8, are associated with cancer development of the skin, in particular with the formation of non-melanoma skin cancer (NMSC) (reviewed in Akgul et al., 2006). First evidence was found in patients with a rare heritable disease, epidermodysplasia verruciformis (EV) (Majewski and Jablonska, 1995). These patients develop a large number of warts in early childhood. Some of them progress to skin cancer, especially in sun exposed epithelia sites. It was supposed that the malignant progression is due to a defect in immune surveillance (Orth, 1986). In addition, HPV induced skin cancer is found in patients with immune suppression upon organ transplantation (Boyle et al., 1984; Walder et al., 1971). Up to 80 % of NMSCs are HPV positive in these patients (de Jong-Tieben et al., 1995).

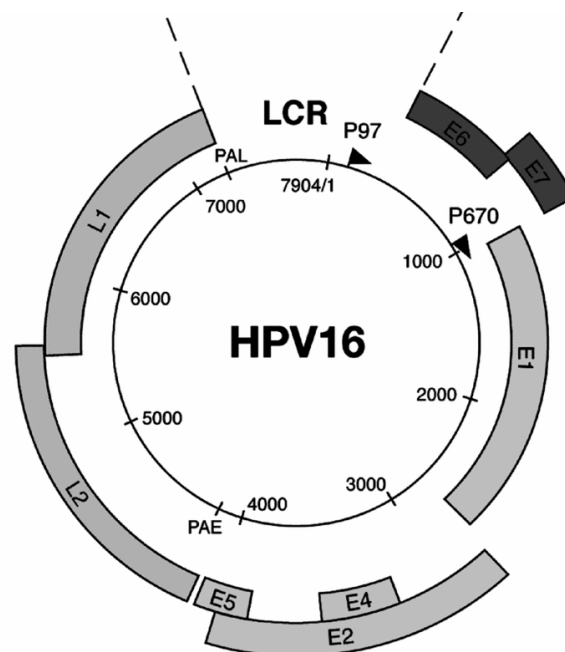
### **1.1.2 Low risk HPV related diseases**

Infection with low risk HPV types such as HPV type 6 and 11 only plays a marginal role in the progression to malignancies. Nonetheless these HPV types are medically interesting as they are linked to the development of benign tumors (Chan et al., 1995; Sinal and Woods, 2005). Infection with HPV 6 or 11 in the genital mucosa leads to the formation of genital warts (*condyloma acuminata*) which can grow to a so called Buschke-Lowenstein giant *condyloma* in rare instances. Genital warts are no life-threatening diseases, but they are a financial

burden on health systems (Hu and Goldie, 2008). HPV type 6 and 11 infection is also associated with the development of a disease called recurrent respiratory papillomatosis (RRP) which is characterized by the formation of benign squamous papillomas within the aerodigestive tract (reviewed in Derkay and Wiatrak, 2008). This disease is also not life-threatening, but it places a high burden on patients as repeated surgeries are necessary, also leading to high economic costs (Derkay, 1995).

### 1.1.3 HPV genome

The circular double-stranded genome of HPVs has a size of approximately 8 kbp and can be divided in three different regions: (i) a coding region containing the early genes (E1, E2, E4, E5, E6 and E7), (ii) a coding region containing the late genes (L1 and L2) and (iii) a non-coding region containing regulatory elements involved in viral replication and transcription (figure 1) (reviewed in Doorbar, 2006). The open reading frames of the genome are transcribed from one DNA strand and expressed from polycistronic mRNAs. The genome contains two promoters: the early one, which starts upstream of the E6 ORF and is called P97 in HPV type 16. The late one (P670 in HPV type 16) is activated after induction of productive replication (figure 1) (reviewed in Longworth and Laimins, 2004).



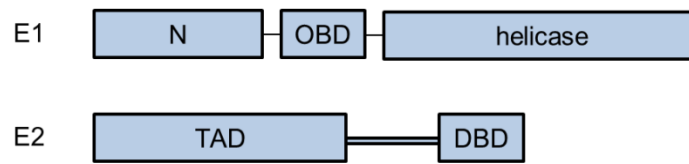
**Figure 1: Organization of the circular HPV 16 genome**

HPV 16 genome is shown as a black circle. It has a size of approximately 8 kbp and consists of six open reading frames (E1, E2, E4, E5, E6 and E7) which code for the early genes and two open reading frames (L1 and L2) which encode for the late genes. The early (P97) and late (P670) promoters are marked by arrows. The long control region (LCR) consists of regulatory elements for transcription and replication. PAE and PAL are the polyadenylation sites of early and late genes. Modified from (Doorbar, 2006).

It was shown that the HPV DNA is associated with cellular histones forming a chromatin-like complex (Howley, 1996). The first viral proteins expressed after infection are the E1 and E2 proteins which play an important role in viral replication (for details see 1.1.4). In addition, the E2 protein is a transcription factor that controls the viral transcription of the early promoter. Low E2 protein levels lead to an activation of the promoter, whereas high E2 protein levels inhibit transcription (Steger and Corbach, 1997). The role of the E4 protein in the viral life cycle is still not understood. However, it is known that it is expressed as a splice variant E1<sup>E4</sup>, where E4 is fused to the first 5 amino acids of E1. It is mainly found in the late stages of the viral life cycle in the upper layer of the epithelium (Doorbar et al., 1997). In addition, E1<sup>E4</sup> was shown to destabilize the cytokeratin network and to induce cell cycle arrest in G2 phase (Davy et al., 2002; Doorbar et al., 1991). E6 and E7 proteins are the main oncoproteins which contribute to transformation of the cell (for details see 1.1.5). The E5 protein is also an oncoprotein, but in contrast to E6 and E7, its role is still rather unclear. The E5 ORF is absent in a subset of HPV types suggesting that E5 is not essential for the viral life cycle. It was suggested that E5 is mainly expressed in differentiated epithelial cells during late phases of the viral life cycle (Fehrmann et al., 2003). L1 and L2 proteins are the capsid proteins which are expressed in differentiated cells. They form a capsid with icosahedral symmetry, consisting of 72 capsomeres which are built up of pentamers of L1 and several copies of L2 (Modis et al., 2002).

#### **1.1.4 HPV E1 and HPV E2 Proteins**

One of the major functions of E1 and E2 proteins is facilitating the viral genome replication (reviewed in Longworth and Laimins, 2004). However, viral replication is also dependent on the host replication machinery. The E1 protein has a size of about 68 kDa and ATPase as well as helicase activity. It can be divided in three main parts: the C-terminal helicase/ATPase domain, the central origin-binding domain (OBD) and an N-terminal domain (figure 2) (Amin et al., 2000; Titolo et al., 2003; Titolo et al., 2000). The E2 protein is a sequence-specific DNA binding protein which, beside its role in viral replication, is also a transcriptional regulator (reviewed in Hegde, 2002). It has a size of about 50 kDa and consists of an N-terminal transactivation domain (TAD) and a C-terminal DNA-binding domain (DBD), which are separated by a hinge region (figure 2). The function of this hinge region is so far poorly understood (reviewed in Hegde, 2002).



**Figure 2: Schematic map of the E1 and E2 proteins**

E1 consists of three main domains: an N-terminal domain (N), an origin binding domain (OBD) and a C-terminal ATPase/helicase domain (helicase). E2 has two domains: an N-terminal transactivation domain (TAD) and a C-terminal DNA binding domain (DBD). Both are linked by a hinge region (modified from Abbate et al., 2004).

E1 binds to AT-rich sequences in the origin of the viral genome via its OBD. This interaction is only weak and is enhanced through complex formation with E2. E2 binding sites (E2BS) are located on the viral DNA adjacent to the E1 recognition site (Frattini and Laimins, 1994a, b). The interaction between E1 and E2 is mediated by the TAD of E2 and the C-terminal helicase domain of E1 (Abbate et al., 2004; Yang et al., 1991; Yasugi et al., 1997). E1 and E2 proteins build a ternary prereplication complex consisting of a dimer of E1 and a dimer of E2. Following E1-E2 complex formation, additional E1 molecules are recruited to the DNA forming a double hexameric ring with helicase activity (Fouts et al., 1999; Lin et al., 2002). Around each DNA strand one hexamer is assembled. This process is ATP dependent, in particular the binding of E1 and E2 to the viral DNA and the induction of conformational changes of the E1-E2 complex that lead to the dissociation of E2 after the double hexameric E1 ring is formed (Titolo et al., 1999; White et al., 2001). Upon recruitment of several proteins including the DNA polymerase  $\alpha$  to the viral origin a full replication complex assembles resulting in replication of the viral genome (Masterson et al., 1998).

A truncated version of E1 consisting of OBD and helicase domain is sufficient to support viral replication *in vitro* (Amin et al., 2000; Sun et al., 1996). In contrast, this truncated version is inactive *in cellulo*, implicating that the N terminus has essential regulatory functions in this context. Indeed it could be shown that the N terminus of E1 regulates the nuclear-cytoplasmic shuttling of the protein (Deng et al., 2004; Fradet-Turcotte et al., 2010; Lin et al., 2000; Ma et al., 1999). In this region a CyclinE/A-Cdk2 binding motif (CBM), a bipartite nuclear localization signal (NLS) and a nuclear export signal (NES) are located.

Furthermore, three independent reports indicate that overexpression of E1 induces ATM-dependent DNA damage response and cell cycle arrest (Fradet-Turcotte et al., 2011; Reinson et al., 2012; Sakakibara et al., 2011). These studies suggest that overexpression of E1 results in S phase or in S and G2 phase arrests, which could favor viral replication.

In addition to its function during viral replication the E2 protein is a transcriptional regulator (reviewed in Hegde, 2002). Four E2BS are present in the long control region to which E2

binds with different affinities thereby regulating viral transcription (reviewed in Hegde, 2002). After infection, early gene transcription is regulated by the host transcription machinery via binding to the long control region upstream of the early promoter. As long as only low concentrations of E2 are present, this leads to an activation of early-gene expression. By binding to E2BSs which overlap with recognition sites of transcription factors E2 acts as transcriptional repressor when high E2 levels are reached (Demeret et al., 1997; Dostatni et al., 1991). By this mechanism, copy number of viral episomes is controlled in undifferentiated cells. Upon differentiation, a switch to the late promoter occurs which is not regulated by the E2 protein (Klumpp and Laimins, 1999). This results in an increased E1 and E2 expression leading to viral DNA amplification.

Infection with high risk HPV types can lead to an integration of the viral DNA in the host DNA (Durst et al., 1983). During this integration the ORFs of the E1 and E2 proteins are mostly disrupted, leading to an uncontrolled expression of proteins regulated by the early promoter, in particular of the E6 and E7 oncoproteins (Durst et al., 1983). Overexpression of E2 in HPV positive cancer cells with integrated HPV genomes, like HeLa cells, results in inhibition of E6 and E7 transcription. As a consequence, the cellular targets of E6 and E7, like p53 and pRB, accumulate (for details see 1.1.5) leading to cell cycle arrest and senescence (Dowhanick et al., 1995; Goodwin et al., 2000). Furthermore, high risk E2 proteins were shown to have an E6/E7 independent apoptotic effect via caspase 8 activation (Bermudez-Morales et al., 2009; Thierry and Demeret, 2008).

### **1.1.5 HPV oncoproteins**

It is commonly accepted that the oncogenic potential of HPVs is due to the action of the E6 and E7 oncoproteins (reviewed in Longworth and Laimins, 2004). With the help of transforming experiments, the oncogenic potential of HPV DNA of high risk types was confirmed. This was demonstrated for the first time by the transformation of NIH 3T3 cells by HPV 16 DNA (Tsunokawa et al., 1986; Yasumoto et al., 1986). A role of both E6 and E7 in transformation of primary cells was confirmed in rodent cells. HPV DNA constructs containing the open reading frames of E6 and E7 cotransfected with activated ras are able to transform primary baby rat kidney (BRK) cells (Matlashewski et al., 1987). The principal oncogene in this system is E7 (Phelps et al., 1988; Storey et al., 1988). While in these cells the transforming activity of E6 is less efficient compared to E7, in mouse cells it is comparable (Storey and Banks, 1993). These transforming assays show that both E6 and E7 can individually transform primary cells in a given context.

Immortalization of primary human keratinocytes can be achieved by the viral DNA from high risk HPV types (Durst et al., 1987; Kaur and McDougall, 1988; Schlegel et al., 1988). In this

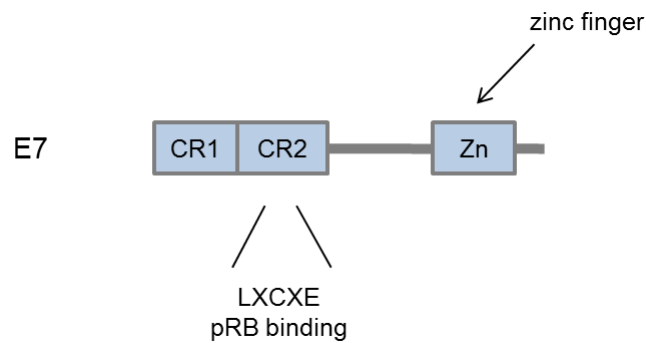
system, no coexpression of an oncogene like ras is necessary. The immortalization capacity is due to the expression of E6 and E7 oncoproteins, as constructs only expressing these proteins are able to immortalize primary human keratinocytes. However, expression of either E6 or E7 alone is not sufficient for immortalization of primary human keratinocytes (Barbosa and Schlegel, 1989; Hawley-Nelson et al., 1989). Immortalized cells are not tumorigenic per se. Only after passaging these cells over a long period of time they achieve this property (Hurlin et al., 1991). E6 and E7 expression contribute to genomic instability leading to additional mutations which favor cancer development (White et al., 1994). This could explain the long period of latency between infection and tumor formation.

The oncogenic potential of both E6 and E7 was also shown in transgenic mice expressing E6 and E7 driven by a Keratin14 promoter, which is activated in basal layers of keratinocytes. These mice develop tumors in skin and cervix (Arbeit et al., 1994; Lambert et al., 1993). Induction of cervical tumor formation in these mice is dependent on long-term estrogen exposure (Arbeit et al., 1996). E7 also seems to be the dominant oncogene in mice, as expression of E7 alone in combination with estrogen treatment leads to the formation of tumors of the reproductive tract. In contrast, expression of E6 results in cancer development only after estrogen treatment over a longer period of time. Coexpression of E6 and E7 under the same conditions causes larger tumors (Riley et al., 2003; Shai et al., 2007).

In the next sections, the contribution of E6 and E7 oncoproteins to cancer development is described in detail.

#### 1.1.5.1 HPV E7 Protein

HPV E7 proteins are composed of approximately 100 amino acids. In their N terminus they have regions of sequence similarity to a portion of conserved region 1 (CR1) and entire conserved region 2 (CR2) of adenovirus E1a proteins and related SV40 large tumor antigen (Figge et al., 1988; Phelps et al., 1988; Vousden and Jat, 1989). The C terminus of E7 contains two Cys-X-X-Cys motives (X represents any amino acid) building up a zinc finger motif, which functions as a dimerization domain (figure 3) (Barbosa et al., 1989; Clemens et al., 1995; Liu et al., 2006). NMR and crystal structures revealed that the N-terminal part of the protein is unstructured whereas the C-terminal part forms a tightly packed Zn-binding fold (Liu et al., 2006; Ohlenschlager et al., 2006).

**Figure 3: Schematic map of E7 protein**

In their N terminus, E7 proteins contain two regions of sequence similarity to conserved regions of adenovirus E1a and SV40 large tumor antigen: CR1 and CR2. Within the CR2 domain a LXCXE motif is located which facilitates binding to pRB. In addition, E7 proteins have a zinc finger motif at their C terminus (modified from McLaughlin-Drubin and Munger, 2009).

E7 proteins play a critical role in maintaining the viral life cycle. Differentiated epithelial cells do not perform DNA synthesis resulting in an environment which does not support HPV replication. Therefore, HPVs have to uncouple the process of cellular differentiation and proliferation. Infected cells remain competent to support DNA synthesis because of the action of the E7 proteins (Cheng et al., 1995). Low risk and high risk E7 proteins interact with pocket protein pRB via a highly conserved Leu-X-Cys-X-Glu (LXCXE) motif within the CR2 domain (figure 3) (Dyson et al., 1992; Munger et al., 1989). Pocket proteins are cell cycle regulators that modulate the activity of E2F transcription factors (reviewed in Dyson, 1998). The main function of E2F transcription factors is to regulate G1 exit and S phase progression. Cell cycle arrest is achieved in G1 phase by binding of pRB to E2F. In normal cells disruption of this pRB/E2F repressor complex is achieved by pRB phosphorylation in late G1. Free E2F is then able to act as transcriptional activator of genes necessary for S phase entry and progression (reviewed in Dyson, 1998).

High risk E7 proteins disrupt the pRB/E2F repressor complex by binding to pRB leading to an uncontrolled G1 exit and S phase entry (Huang et al., 1993; Wu et al., 1993). E7 of HPV type 16 recruits and reprograms a Cullin 2 based ubiquitin ligase complex which leads to proteasomal degradation of pRB (Boyer et al., 1996; Huh et al., 2007). The responsible E3 ligases for other E7 proteins have not been identified so far. Low risk E7 proteins bind more weakly to pRB compared to high risk E7 proteins (Gage et al., 1990; Munger et al., 1989). High risk E7 proteins also induce ubiquitylation of other pocket proteins like p130 and p107. Transforming capacity of E7 proteins is directly linked to degradation of pocket proteins, as E7 proteins with mutations in their LXCXE domain are not able to transform cells (Heck et al., 1992).

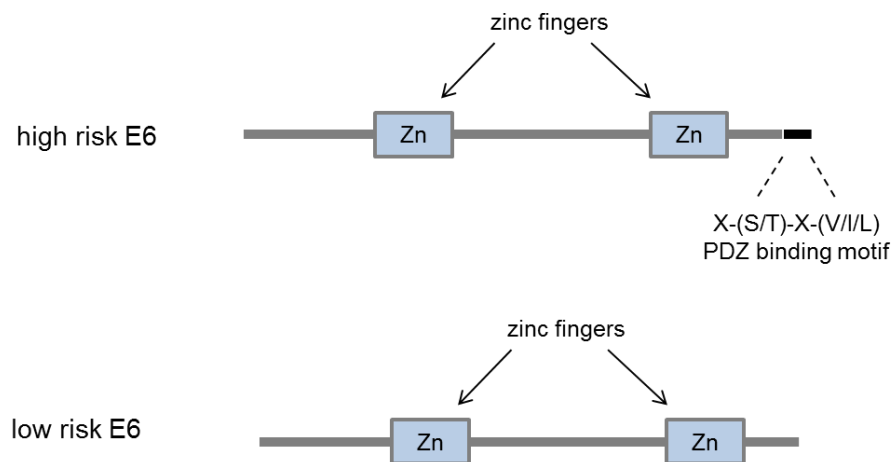
In addition to pRB binding, high risk E7 proteins accelerate G1/S transition by blocking the action of CDK inhibitors p21 and p27 (Jones et al., 1997a; Zerfass-Thome et al., 1996). In

contrast to high risk E7 proteins, the low risk ones bind p21 with reduced affinity. CDK2 activity is additionally regulated by the E7 proteins via direct or indirect binding to CDK2 and Cyclin subunits (He et al., 2003; Nguyen and Munger, 2008). Furthermore, E7 interacting proteins include transcription factors, cell cycle regulators and metabolic enzymes (reviewed in McLaughlin-Drubin and Munger, 2009).

In addition, high risk E7 proteins are associated with genomic instability. In particular E7 induces abnormal centrosome numbers and multipolar mitotic spindles (Duensing et al., 2000; Duensing and Munger, 2002).

#### 1.1.5.2 HPV E6 Protein

HPV E6 proteins consist of approximately 150 amino acids and contain two zinc finger domains each of them composed of two Cys-X-X-Cys motifs. In contrast to low risk E6 proteins, high risk E6 proteins possess a PDZ binding motif (X-(S/T)-X-(V/I/L)) at their very C terminus, which facilitates binding to PDZ domain-containing proteins (figure 4) (reviewed in Ghittoni et al., 2010).



#### Figure 4: Schematic map of E6 proteins

Low and high risk E6 proteins contain two zinc finger motifs. In addition, high risk E6 proteins have a C-terminal PDZ binding motive (modified from Ghittoni et al., 2010).

The best studied function of high risk HPV E6 proteins is the degradation of the tumor suppressor p53 (Scheffner et al., 1990). p53 is a transcription factor with critical regulatory functions in a cell including the induction of apoptosis. Simplified, p53 is activated in response to DNA damage, nucleotide depletion, hypoxia or other triggers, resulting in induction of genes involved in cell cycle regulation and apoptosis (reviewed in Efeyan and Serrano, 2007). p53 levels increase due to the action of E7 by binding to pRB and other cell

cycle regulators (see 1.1.5.1). As a consequence, these cells have an increased susceptibility to apoptosis (Jones et al., 1997b), which is counteracted by E6 via degradation of p53. E6 binds to p53 only in the presence of the cellular E3 ubiquitin ligase E6AP (E6 associated protein) forming a ternary complex (Huibregtse et al., 1991, 1993a). The E6/E6AP complex ubiquitylates p53, thereby leading to its proteasomal degradation (see 1.2), which contributes to the oncogenic potential of high risk HPVs. (Huibregtse et al., 1993b; Scheffner et al., 1993). In the absence of E6, E6AP is not involved in p53 degradation. As E6 mutants unable to induce degradation of p53 are competent to transform human mammary epithelial cells (HMEC) as efficiently as wild type E6, additional E6 functions must be necessary for their oncogenic potential (Liu et al., 1999).

Numerous cellular proteins have been reported to interact with high risk E6 proteins (reviewed in Pim and Banks, 2010) including PDZ domain-containing proteins (for details see 1.3) like hDlg, MAGI-1, -2, -3, and Scribble. The name PDZ derives from the first three proteins identified containing PDZ domains: PSD-95 (postsynaptic density-95 protein), DLG (*Drosophila* disc large protein) and ZO-1 (zonula occludens 1 protein) (reviewed in Harris and Lim, 2001). MAGUK proteins, including for example MAGI-1, -2, -3 and hDlg, are a group of PDZ domain-containing proteins which affect processes like cell polarity and maintenance of cell-to-cell interactions. Interaction of high risk E6 proteins with PDZ domain-containing proteins is mediated by the C-terminal PDZ binding motif of E6 (Kiyono et al., 1997). Importantly, this PDZ binding motif is not required for binding to E6AP or p53. However, high risk E6 proteins target PDZ domain-containing proteins like hDlg for proteasomal degradation in an E6AP dependent manner (Kuballa et al., 2007). The fact that the PDZ binding motif of E6 proteins is required for transformation in transgenic mice underlines its functional importance (Simonson et al., 2005).

Immortalization experiments revealed additional important functions of high risk E6 proteins, in particular the induction of telomerase activity (Gewin et al., 2004; Oh et al., 2001; Veldman et al., 2001; Veldman et al., 2003). Telomerase is only expressed in a small subset of normal cells (stem cells), however it is also expressed in tumor tissues and tumor derived cell lines (Kim et al., 1994b). E6 binds to hTERT (telomerase reverse transcriptase) promoter and induces its expression, which results in the activation of the telomerase complex. This activation is independent of the capability of E6 to degrade p53 and PDZ domain-containing proteins, but it is dependent on the binding of E6 to E6AP (Gewin et al., 2004; Kiyono et al., 1998; Liu et al., 2005). Two critical targets of E6/E6AP are known, namely Myc and NF $\kappa$ B, which act as transactivator and transrepressor of the hTERT promoter, respectively (Gewin et al., 2004; Katzenellenbogen et al., 2009; Liu et al., 2008). Furthermore, E6 can directly interact with the hTERT protein, which indicates that E6 may activate telomerase by two distinct mechanisms (Liu et al., 2009). However, the exact mechanisms how high risk E6

proteins activate telomerase are not completely understood so far.

Moreover, E6 proteins as well as E7 proteins induce genomic instability, precisely expression of high risk E6 proteins is associated with unaligned or lagging chromosomes (Duensing and Munger, 2002).

In contrast to high risk HPV E6 proteins, the functions of low risk HPV E6 proteins are only poorly characterized. Indeed they can interact with the cellular E3 ligase E6AP just like high risk E6 proteins (Brimer et al., 2007; Kuballa et al., 2007), but no targets for ubiquitylation are known so far. Low risk E6 proteins do not lead to the degradation of p53 (Scheffner et al., 1990) and as they do not contain a PDZ binding motif (figure 3), they presumably cannot interact and degrade PDZ domain-containing proteins, either (Brimer et al., 2007). Chimeric E6 proteins consisting of the N terminus of low risk E6 proteins and the very C terminus of high risk E6 proteins including the PDZ binding motif are able to bind and degrade PDZ domain-containing proteins, showing that the PDZ binding motif is sufficient to bind to these PDZ domain-containing proteins (Kuballa et al., 2007; Pim et al., 2002).

Some of the identified interaction partners and targets of high risk E6 proteins, e.g. Bak (Bcl-2 homologous antagonist/killer) have also been reported to be regulated by low risk E6 proteins (Kuhne and Banks, 1998; Thomas and Banks, 1999). However, in our experiments we neither could show binding of E6 to Bak nor E6-dependent ubiquitylation and degradation of Bak (Scheffner group, unpublished data).

As mentioned above, low risk and high risk E6 proteins bind to E6AP (Brimer et al., 2007; Kuballa et al., 2007). Both are stabilized by this complex formation, and it is believed that binding of E6AP to E6 protects it from ubiquitylation and proteasomal degradation (Tomaic et al., 2009; Weber, 2009). However, the responsible E3 ligase for E6 ubiquitylation remains unknown.

Another HECT E3 ligase, EDD (E3 ubiquitin ligase identified by differential display) was identified as an interaction partner of 18E6 (Tomaic et al., 2011). In the same study it was shown that EDD also interacts with low risk 11E6 and high risk 16E6 but with a lower affinity than 18E6. In addition, EDD interacts with E6AP in an E6 independent manner and thereby regulates the expression levels of E6AP, resulting in the modulation of the E3 ligase activity of the E6/E6AP complex (Tomaic et al., 2011).

Another published interaction partner for high risk and low risk E6 proteins is the tumor suppressor TIP60 (Tat-interaction protein 60 kDa) (Jha et al., 2010), a histone acetyltransferase which is involved in transcriptional regulation, check-point activation and p53-directed proapoptotic pathways (reviewed in Sapountzi et al., 2006). Both low risk and high risk E6 proteins destabilize TIP60 by proteasomal degradation in an E6AP independent manner. As TIP60 represses the early HPV promoter, the destabilization of TIP60 by E6 proteins leads to derepression of the promoter (Jha et al., 2010).

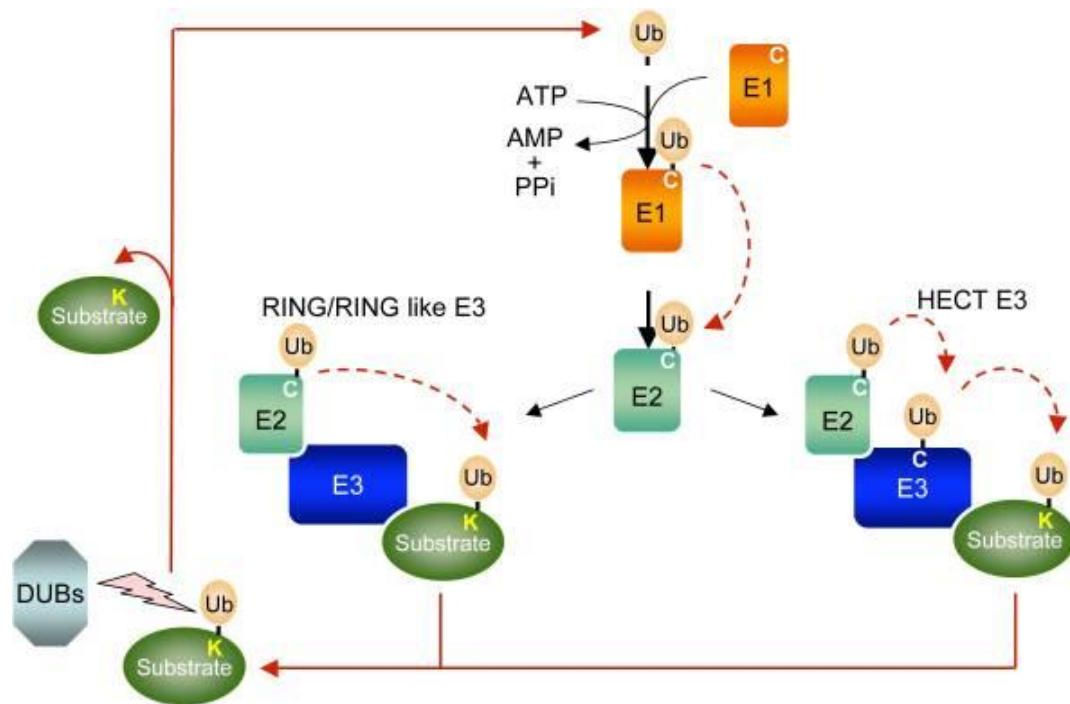
Finally, further investigations are necessary to elucidate different and common properties of high risk and low risk E6 proteins.

## 1.2 UBIQUITIN PROTEASOME SYSTEM (UPS)

Ubiquitin is a protein consisting of 76 amino acids which has a molecular mass of 8.6 kDa. It is a conserved protein which is ubiquitously expressed throughout all eukaryotic species and adopts a compact  $\beta$ -grasp fold with a flexible six-residue C-terminal tail (Goldstein et al., 1975; Vijay-Kumar et al., 1987). Ubiquitin can be attached to an  $\epsilon$ -amino group of a lysine residue of an acceptor protein or another ubiquitin molecule via its C terminus. This process termed ubiquitylation is one possible posttranslational modification of a protein. The covalent attachment of ubiquitin to its substrate is carried out by different classes of enzymes in a multistep mechanism (figure 5) (reviewed in Hershko and Ciechanover, 1998). In the first step, ubiquitin is activated by the ubiquitin activating enzyme E1. Upon hydrolysis of ATP a ubiquitin adenylate intermediate is formed, followed by the formation of a high energy thioesterbond between the active site cysteine of the E1 and the C-terminal glycine residue of ubiquitin (Haas et al., 1982). In the second step, the activated ubiquitin molecule is transferred to a ubiquitin conjugating enzyme (E2) thereby forming a thioesterbond with the active site cysteine of the E2. In the third step the activated ubiquitin is transferred in most cases to a lysine residue of a target protein forming an isopeptide bond. This step is catalyzed by an E3 ubiquitin ligase (E3) (reviewed in Hershko and Ciechanover, 1998; Pickart, 2001). Two different types of E3 ligases are known, RING (really interesting new gene)/RING like and HECT ligases (homologues to C terminus of E6AP) (figure 5; for details see 1.2.2).

Ubiquitylation is a hierarchical process. So far only two E1 enzymes (Groettrup et al., 2008; Jin et al., 2007; McGrath et al., 1991), around 40 E2 enzymes (Burroughs et al., 2008) and more than 600 E3 ligases are known in mammals (Li et al., 2008). As substrate specificity of the ubiquitylation process is mainly mediated by the E3 ligases, they form the largest group of enzymes.

Ubiquitin itself has seven lysine residues at positions 6, 11, 27, 29, 33, 48 and 63. All of them can be used to be attached to the C terminus of another ubiquitin molecule thereby forming ubiquitin chains with different linkage types. To elongate ubiquitin chains, in some cases a fourth enzyme termed E4 is required (Koegl et al., 1999). Ubiquitylation is a reversible process as ubiquitin molecules can be cleaved off by deubiquitylation enzymes (DUBs) which mainly exhibit cysteine protease activity (figure 5) (Nijman et al., 2005).



**Figure 5: Ubiquitin conjugation cascade**

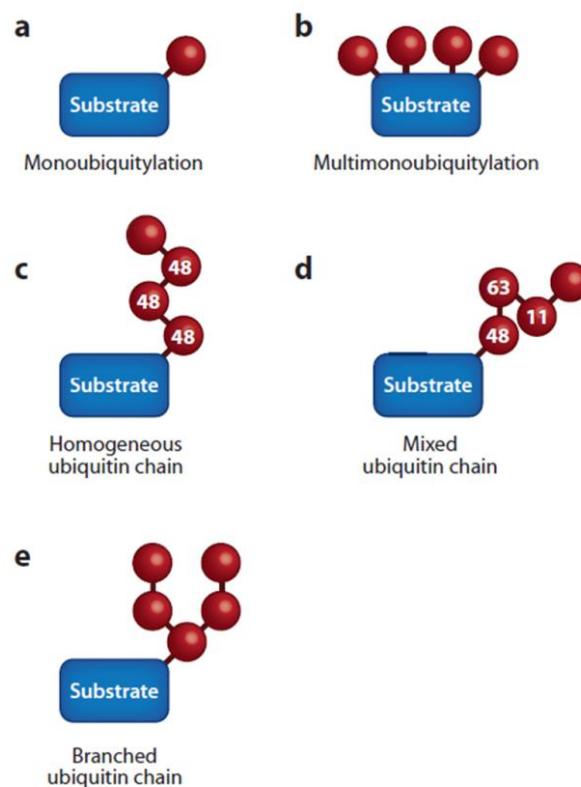
Three enzymes are necessary for the ubiquitylation process: ubiquitin is activated by the E1 enzyme and then transferred to the E2 enzyme. Substrate specificity is guaranteed by the E3 ligases. They bind substrate proteins and catalyze the transfer of the ubiquitin molecule to the lysine residue of the substrate protein. Two classes of E3 ligases are known RING/RING-like and HECT ligases. Ubiquitylation is a reversible process as deubiquitylation is performed by DUBs (Ravid and Hochstrasser, 2008).

Polyubiquitin chains display different cellular signals depending on the type of linkage. The best characterized ones are K11, K48 and K63 linked chains, the other forms of linkages are only poorly characterized so far. K48 linked chains are the most abundant linkage type and mark proteins for proteasomal degradation by the 26S proteasome, a multisubunit protease (reviewed in Pickart, 2000). For a long time it was believed that a chain length of at least four ubiquitin moieties attached to the substrate is necessary to be recognized by the proteasome (Thrower et al., 2000). However, recent reports show contradictory data implicating that monoubiquitylated and multimonomubiquitylated proteins exist which can also be recognized and degraded by the proteasome (Boutet et al., 2007; Kravtsova-Ivantsiv et al., 2009). The 26S proteasome is composed of two subcomplexes: the core 20S proteasome and the 19S regulatory particles (reviewed in Matyskiela and Martin, 2013). Ubiquitylated proteins are recognized by the 19S regulatory particle which in addition is responsible for their unfolding and translocation of to the 20S subcomplex, where proteolysis takes place. Furthermore, the 19S regulatory particle exhibits DUB activity preserving ubiquitin from degradation and resulting in free ubiquitin available for a new conjugation cycle (reviewed in Matyskiela and Martin, 2013; Sorokin et al., 2009). K11 linked chains are also known to serve as a proteolytic signal. The human E3 ubiquitin ligase APC/C, for example, which is known to target cell cycle

regulators for proteasomal degradation, forms K11 linked chains on its substrates (Jin et al., 2008).

K63 chains are the best characterized non-proteolytic linkages. They play a role in different pathways like DNA damage repair, endocytosis and NF- $\kappa$ B activation (Deng et al., 2000; Hicke and Riezman, 1996; Spence et al., 1995).

In addition to polyubiquitylation, other modes of ubiquitylation are known (figure 6). During monoubiquitylation only one ubiquitin molecule is attached to the substrate protein. Monoubiquitylated proteins play a role in DNA damage response and endocytosis (reviewed in Hicke, 2001). Upon multimonoubiquitylation, which is involved in receptor endocytosis, single ubiquitin molecules are linked to different lysine residues of the substrate protein (Haglund et al., 2003). Furthermore, branched and mixed ubiquitin chains exist, yet not much is known about their roles (figure 6). In most cases ubiquitin is conjugated to a lysine residue of a protein but it can also be attached to the N-terminal amino group (reviewed in Rieser et al., 2013). Moreover, it was shown that ubiquitylation can also take place at threonine, serine and cysteine residues of substrates (Cadwell and Coscoy, 2005; Shimizu et al., 2010).



**Figure 6: Modes of ubiquitylation**

Ubiquitin is attached to lysine residues of a substrate protein. In monoubiquitylation (a) one ubiquitin molecule is attached to a substrate molecule. During multimonoubiquitylation (b) multiple lysine residues become modified by ubiquitin. Ubiquitin exhibits seven lysine residues. All of them can be used to form ubiquitin chains. In homogeneous chains (c) always the same lysine is used for linkage (example is shown for K48 linked chains). In contrast, in mixed chains (d) different lysine residues are utilized to form chains. In addition branched chains exist (e) (modified from Komander and Rape, 2012).

### 1.2.1 Ubiquitin-like proteins (UBLs)

In addition to ubiquitin, ubiquitin-like proteins (UBLs) have been identified which all exhibit the typical  $\beta$ -grasp fold. All of them can be conjugated to substrates via an isopeptide bond in a similar manner as ubiquitin involving E1, E2 and mostly E3 enzymes. Each UBL features its own set of enzymes. Functions of modification with UBLs are diverse, including the regulation of localization and the stability of proteins, and the regulation of other cellular functions such as autophagy or inflammation (reviewed in Herrmann et al., 2007; Kerscher et al., 2006; Schulman and Harper, 2009). The best characterized UBLs are SUMO-1,-2,-3 (small ubiquitin related modifier-1,-2,-3) and NEDD8 (neuronal precursor cell-expressed developmentally downregulated 8), which shows the highest similarity to ubiquitin within the family of UBL proteins (Kumar et al., 1993). The exchange of only one amino acid (A72R in NEDD8 or R72A in ubiquitin) facilitates activation of this NEDD8 mutant by the ubiquitin E1 and vice versa (Whitby et al., 1998).

The best characterized NEDD8 substrates are Cullins that are scaffold proteins of multicomplex E3 ligases. Upon conjugation of NEDD8 to Cullins, conformational changes activate the Cullin RING ligase complex resulting in ubiquitylation of their substrates (Hori et al., 1999; Ohh et al., 2002).

### 1.2.2 E3 Ubiquitin Ligases

E3 Ubiquitin ligases represent the largest group of enzymes involved in the ubiquitylation cascade. They facilitate substrate specificity and together with the E2 enzymes direct the mode of ubiquitin linkage which is responsible for the fate of the ubiquitylated protein (reviewed in Passmore and Barford, 2004). Two different groups of E3 ligases are known: on the one hand HECT E3 ligases that exhibit a HECT domain with a catalytic cysteine residue and on the other hand RING and RING-like E3 ligases which have a RING or RING-like domain (reviewed in Metzger et al., 2012).

#### 1.2.2.1 RING E3 Ligases

Most ubiquitin E3 ligases are RING type enzymes. More than 600 potential RING E3s are encoded by the mammalian genome (Li et al., 2008). Most of them do not have intrinsic enzymatic activity, but serve as an adaptor protein to bring the ubiquitin loaded E2 and substrate proteins in close proximity. However, a subclass of RING E3 ligases known as RING-between-RINGs (RBRs) exist which have in addition to their RING domain two additional domains: an in between RING domain (IBR) and a domain named RING2 (reviewed

in Wenzel and Klevit, 2012). These E3 ligases combine features of RING and HECT E3 ligases and possess enzymatic activity.

E2 binding to RING E3 ligases is facilitated by their RING domain, a type of zinc finger domain, which is composed of specifically spaced cysteine and histidine residues coordinated by two zinc ions (Lorick et al., 1999). RING E3s can be classified in different groups depending on whether they function as monomers, dimers or multi-subunit complexes. Examples for homodimeric RING E3s are RNF4 and TRAF2 (Liew et al., 2010; Park et al., 1999), furthermore also heterodimeric ones exist (examples: Hdm2/HdmX, RING1b/BMI1 and BRCA1/BARD1). One of the two RING domains of the heterodimeric ligases often lacks ligase activity (HdmX, BMI1 and BARD1 respectively). It is believed that dimerization leads to a stimulation of the active RING domain (Brzovic et al., 2001; Linke et al., 2008). In addition to their RING domain, RING E3 ligases possess other domains which are involved in substrate binding. For RING E3s which are composed of multiple subunits such as Cullin RING ligases, these two domains are located on different subunits. Examples for multimeric E3 ligases are the SCF complex (SKP1-cullin-F-box protein) and the APC/C (anaphase promoting complex/cyclosome), both playing major roles in the regulation of the cell cycle (reviewed in Barford, 2011a; Petroski and Deshaies, 2005).

Moreover, RING-like E3 ligases are known, such as U-box proteins. Their U-Box domain is structurally similar to the RING domain, however it does not coordinate zinc ions (Hatakeyama et al., 2001).

#### 1.2.2.2 HECT E3 ligases

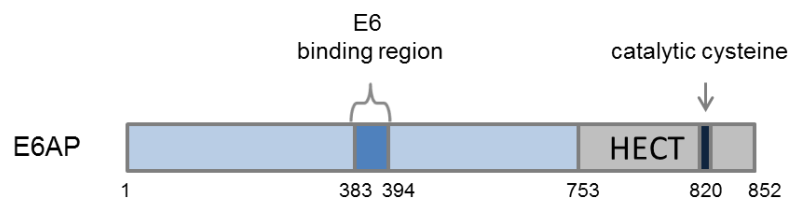
In mammals approximately 30 HECT E3 ligases exist and they are characterized by their C-terminal HECT (homologues to C terminus of E6AP) domain. This domain is named after the founding member of HECT E3 ligases, E6AP (E6 associated protein, see 1.2.2.3) (Huibregtse et al., 1995). In contrast to RING E3 ligases, HECT E3 ligases show direct catalytic activity during the transfer of ubiquitin to substrate proteins (see figure 5). The HECT domain is bi-lobed: the N-terminal lobe is responsible for the interaction with the ubiquitin loaded E2 protein, whereas the C-terminal lobe contains the active site cysteine residue that forms a thioester linkage with the C-terminal glycine residue of ubiquitin. Both lobes are connected by a flexible linker (Huang et al., 1999).

The C-terminal HECT domain of these ligases is conserved, while the N terminus of these proteins shows a high diversity and is responsible for substrate binding and therefore for substrate specificity.

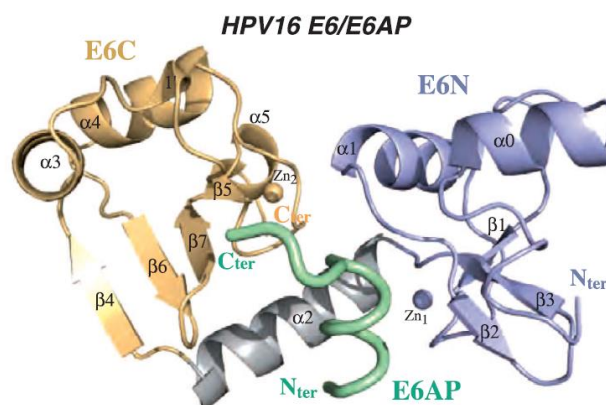
### 1.2.2.3 HECT E3 ligase E6AP

E6AP (E6 associated protein) (figure 7A) was discovered as an interacting protein of the human papillomavirus E6 protein and therefore its name derived (Huibregtse et al., 1991). It is encoded by the UBE3A gene and three isoforms of E6AP exist, resulting from alternative splicing. The isoforms only differ in their N termini (Kishino et al., 1997; Yamamoto et al., 1997). However, different roles of the isoforms are not known so far. Interaction with the human papillomavirus E6 protein causes a change in substrate specificity of E6AP. This is a “gain-of-function” mechanism as additional proteins are ubiquitylated by E6AP in the presence of E6. The best characterized E6/E6AP substrate is the tumor suppressor p53, but also other substrates are known like PDZ domain-containing proteins (for details see 1.1.5.2). E6 does not only modify E6AP substrate specificity, it also stimulates E6AP autoubiquitylation activity resulting in lower E6AP levels in the presence of E6 (Kao et al., 2000).

A



B



**Figure 7: Schematic map of E6AP and X-ray structure of E6/E6AP (LXXLL peptide) complex**

**A:** Schematic map of E6AP (isoform 1) is shown with its HECT domain and E6 binding region. Numbers indicate amino acid residues (amino acid and domains were annotated according to Uniprot/Swissprot database) **B:** Structure of 16E6 (F47R 4C/S) mutant and E6AP LXXLL peptide (amino acids 383-394, isoform 1) is shown. Blue pictures E6N (N-terminal zinc binding domain of E6), yellow E6C (C-terminal zinc binding domain of E6), grey linker helix of E6, green E6AP peptide. Helical E6AP peptide binds to a deep pocket formed by the two zinc finger motifs and the linker helix of E6 (Zanier et al., 2013).

Binding of E6 to E6AP is mediated by amino acids 383-394 of E6AP (isoform 1). In this region an LXXLL (where X represents any amino acid) motif exists, which is also present in other E6 binding partners (e.g. IFR-3 and MAML-1) and therefore probably represents a general binding motif for E6 proteins (Brimer et al., 2012; Ronco et al., 1998; Tan et al., 2012).

Recently the structure was solved of 16E6 and the LXXLL-containing region of E6AP (ELTLQELLGEER) (Zanier et al., 2013). As E6 undergoes self-oligomerization, a monomeric E6 mutant (F47R and 4 C residues were mutated to S) was used for crystallization. The structure reveals an  $\alpha$ -helical conformation of the E6AP peptide that is inserted in a deep pocket which is formed by the two zinc finger motifs and the linker helix of E6 (figure 7B) (Zanier et al., 2013).

In addition to its E6-dependent functions, which are believed to be one major trigger for the development of cervical cancer after HPV infection, E6AP is also involved in a neurological disease called Angelman Syndrome. E6AP is biallelically expressed in most types of cells. In special regions of the brain, however, in particular in Purkinje cells and CA-3 neurons of the hippocampus, it is only expressed by the maternal allele. Angelman Syndrome is caused by the loss of a functional maternal UBE3A gene (Rougeulle et al., 1997). The genetic mechanisms of the loss are diverse, comprising gene deletion, but also mutations in the maternal UBE3A gene (Fang et al., 1999). Angelman Syndrome patients suffer from severe developmental delays, seizures and jerky movements and they additionally show a happy demeanor (Williams et al., 1995; Williams et al., 2006). The molecular mechanism of how the loss of E6AP in the above mentioned regions of the brain results in the phenotype of Angelman Syndrome patients is so far only poorly understood. As E6AP is an ubiquitin E3 ligase one possible explanation could be that E6AP-dependent substrates accumulate in these cells and cause the symptoms. However, up to now only a few E6AP dependent substrates including HR23A, Ring1B and alpha-synuclein have been identified and none of them alone is responsible for the phenotype of Angelman Syndrome patients (Kumar et al., 1999; Mulherkar et al., 2009; Zaaroor-Regev et al., 2010). Furthermore, it is possible that E6AP has additional E3 ligase-independent functions which contribute to the phenotype of the patients. Indeed, it could be shown that E6AP can act as a transcriptional regulator (Kuhnle et al., 2013; Nawaz et al., 1999).

### **1.3 PDZ DOMAIN-CONTAINING PROTEINS**

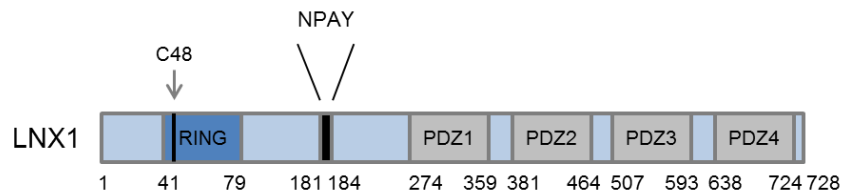
PDZ domain-containing proteins are named after the first identified members PSD95, DLG and ZO-1 and are found from bacteria to vertebrates (Ponting, 1997; Ponting et al., 1997). They are involved in many processes within a cell, including control of cell migration and invasion, cell proliferation, cell polarity, cell attachment and cell-cell contact, apoptosis and

immune cell recognition and signaling (reviewed in Subbaiah et al., 2011). PDZ domain-containing proteins can be classified in three families. The first family displays proteins, which contain no further protein domains apart from the PDZ domain. Proteins of the second family, the MAGUK proteins, (membrane associated guanylate kinases) have an SH3 and a guanylate kinase domain in addition to PDZ domains. Proteins of the third family contain protein-protein interaction domains (e.g. WW- or LRR-domains) in addition to PDZ domains. Examples for the third group are Scribble, LNX1 (ligand of numb protein X 1) and MUPP1 (multiple PDZ domain protein) (reviewed in Nourry et al., 2003).

PDZ domains are sites of protein-protein interaction. As PDZ domain-containing proteins with multiple PDZ domains exist, they can act as scaffolding molecules. PDZ domains consist of 80-90 amino acids and have certain defining structural elements: six  $\beta$ -sheets, one short and one long  $\alpha$ -helix (Doyle et al., 1996; Morais Cabral et al., 1996). PDZ domains interact with PDZ binding motifs which are short amino acid motifs found at the C terminus of proteins (Saras and Heldin, 1996). Examples for proteins with PDZ binding motifs are the high risk HPV E6 proteins (see 1.1.5.2), PTEN (phosphatase and tensin homologue deleted on chromosome 10) and HTVL-1 (human T cell leukemia virus 1) tax-1 protein (reviewed in Subbaiah et al., 2011). The specificity of PDZ domain - PDZ binding motif interaction is achieved by a variety of structural characteristics. PDZ domains are classified in four groups, dependent on the consensus sequence of the bound PDZ binding motif. However, a recent study showed that the situation is more complex and that 16 subclasses of PDZ binding motifs exist (Tonikian et al., 2008). On top of that, PDZ domain binding is often regulated by phosphorylation, since many PDZ binding motifs exhibit PKA (protein kinase A) recognition sites (Cohen et al., 1996).

### **1.3.1 RING E3 Ligase LNX (ligand of Numb protein X)**

LNX1 (ligand of Numb protein X 1) is a protein consisting of 728 amino acids which contains an N-terminal RING domain, four PDZ domains and an NPAY sequence motif (figure 8). It is the first PDZ domain-containing protein described that additionally exhibits a RING domain (Dho et al., 1998). The LNX family of proteins contains five members: in addition to the N-terminal RING domain, LNX1 and LNX2 have four PDZ domains, whereas LNX3 and LNX4 contain only two PDZ domains. Although LNX5 has no RING domain and only one PDZ domain, it shows sequence homology to LNX3 and LNX4 (Flynn et al., 2011).



**Figure 8: Schematic map of LNX1**

LNX1 is shown with its N-terminal RING domain, NPAY motif and four PDZ domains. Mutation of cysteine 48 to alanine within the RING domain leads to an inactive E3 ligase. Numbers indicate amino acid residues (amino acid and domains were annotated according to Uniprot/Swissprot database).

LNX1 was identified as interaction partner of Numb (Nie et al., 2002), which is a membrane-associated protein. Numb is asymmetrically localized in neuroblasts, and segregates to only one daughter cell after cell division and thereby functions as an intrinsic determinant of cell fate (Rhyu et al., 1994; Spana et al., 1995; Vervoort et al., 1997). Numb is a negative regulator of NOTCH and modulates neurogenesis by direct protein-protein interaction with NOTCH. The NOTCH signaling pathway is important for cell-cell communication and involves gene regulation mechanisms that control multiple cell differentiation processes (Guo et al., 1996). It was shown that LNX1 leads to the ubiquitylation and proteasomal degradation of Numb and thus enhances NOTCH signaling. Interaction between LNX1 and Numb is mediated by the NPAY sequence motif. However, for ubiquitylation of Numb, PDZ1 and the RING domain of LNX1 are also necessary (Nie et al., 2002). In addition to Numb, numerous additional interaction partners of LNX1 were described (Chen et al., 2005; Guo et al., 2012; Kansaku et al., 2006). Among all these identified interactors only PBK, Claudin and c-Src were shown to be ubiquitylation substrates of LNX1 (Guo et al., 2012; Takahashi et al., 2009; Weiss et al., 2007). Nonetheless, up to now the functional inter-relationships between LNX1 and its interactors are not well understood. Moreover, the high risk HPV E6 proteins were identified as binding partners of LNX1 (Weber, 2009). Interaction between these proteins is mediated by the C-terminal PDZ binding motif of E6 proteins and the PDZ1 domain of LNX1. The function of this interaction is so far unknown, as LNX1 is not a ubiquitylation substrate of the E6/E6AP complex in cells and it is also not the responsible E3 ligase for E6 ubiquitylation (Weber, 2009). Furthermore, no effect of E6 on LNX1 ligase activity was found (Schätzle, 2011). Moreover, Hek293 cells arrest in G1 phase of the cell cycle upon knockdown of LNX1 expression. The exact molecular mechanisms of this cell cycle arrest have not been elucidated so far (Zheng et al., 2011).

## 2 AIMS

High risk HPV E6 proteins play a major role in the induction of cervical cancer and are well studied. In contrast, low risk E6 proteins induce the formation of benign tumors and are only poorly characterized. As low risk and high risk E6 proteins share similarities on amino acid level (~ 50 % similarity) and binding to the cellular E3 ligase E6AP is a common feature among high risk and low risk E6 proteins, we hypothesized that they share some biochemical properties and interaction partners. Thus, the aim of the first part of this thesis was to identify common and different properties of the high risk and low risk HPV E6 proteins and to elucidate their impact on cellular pathways. In detail we aimed at:

1. developing an inducible expression system that allows to switch on and off E6 expression.
2. determining the effect of different E6 proteins on the protein expression pattern of a cell in general ("HPV E6 proteome").
3. identifying proteins, whose ubiquitylation status is altered by the different E6 proteins in the presence and absence of E6AP ("HPV E6 ubiquitome").
4. identifying proteins that interact with the different E6 proteins ("HPV E6 interactome")
5. functional characterizing the proteins which are altered by E6 with respect to their involvement in the formation of cervical cancer and benign tumors.

The aim of the second part of this thesis was the characterization of the E6 interaction partner LNX1. LNX1 is a RING E3 ligase that interacts with Numb and induces proteasomal degradation of it. Thereby LNX1 regulates Numb levels. Since Numb is a negative regulator of NOTCH, LNX1 enhances NOTCH signaling. The NOTCH signaling pathway is important for cell-cell communication and involves gene regulation mechanisms that control multiple cell differentiation processes.

To obtain more insights into LNX1 functions, this E3 ligase should be characterized with respect to E6-dependent and E6-independent functions.

## 3 MATERIAL AND METHODS

### 3.1 MATERIAL

#### 3.1.1 Buffers and solutions

β-Mercaptoethanol sample buffer (10 x)	7.15 M β-Mercaptoethanol, 40 % glycerol, 100 mM Tris-HCl, pH 7.5, orange G
Buffer Z (β-gal assay)	100 mM NaH <sub>2</sub> PO <sub>4</sub> , 10 mM KCl, 1 mM MgSO <sub>4</sub> , 50 mM β-Mercaptoethanol, pH 7.0
Coomassie Blue staining solution	2 g/l Coomassie Brilliant Blue R250 in Coomassie destain solution
Coomassie destain solution	40 % methanol, 10 % acetic acid
DNA loading buffer (10 x)	60 % Saccharose, 0.25 M EDTA, bromphenol-blue
Fixation solution (for FACS samples)	2 % (w/v) paraformaldehyde in PBS, pH 7.2
Guanidinium lysis buffer	100 mM Na <sub>2</sub> HPO <sub>4</sub> , 6 M Guanidinium-hydrochloride, 10 mM Imidazole, 10 mM β-Mercaptoethanol, pH 8.0
Laemmli loading buffer (2 x)	125 mM Tris-HCl, pH 6.8, 200 mM DTT, 20 % glycerol, 4 % SDS, 0.001 % bromphenol-blue
Laemmli loading buffer (5 x)	312.5 mM Tris-HCl, pH 6.8, 500 mM DTT, 50 % glycerol, 10 % SDS, 0.001 % bromphenol-blue
Laemmli running buffer (10 x)	250 mM Tris-HCl, pH 8.4, 2 M glycine, 1 % SDS
LB (Lucia Broth) medium	10 g/l NaCl, 5 g/l yeast extract, 10 g/l Bacto-tryptone, pH 7.5
ONPG	4 mg/ml 2-nitrophenyl β-D-galactopyranoside in 100 mM NaH <sub>2</sub> PO <sub>4</sub>
PBS	1.8 mM KH <sub>2</sub> PO <sub>4</sub> , 10.1 mM Na <sub>2</sub> HPO <sub>4</sub> , 137 mM NaCl, 2.7 mM KCl, pH 7.4
Propidium iodide solution (10 x)	5 mg Propidium iodide (PI) in 100 ml 38 mM sodium citrate, pH7.0
Propidium iodide stock solution	1 mg/ml Propidium iodide (PI), 2.5 mg/ml RNase A in PBS

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S1	50 mM Tris-HCl, pH 8, 10 mM EDTA, 100 µg/ml RNaseA
S2	200 mM NaOH, 1 % SDS
S3	2.8 KAc, pH 5.1
Separating gel buffer	1.5 M Tris-HCl, pH 8.8, 0.4 % SDS
SOC medium	20 g/l tryptone, 5 g/l yeast extract, 0.5 g/l NaCl, 20 mM glucose, pH 7
Stacking gel buffer	0.5 M Tris-HCl, pH 6.8, 0.4 % SDS
Stripping buffer for western blots	62.5 mM Tris-HCl, pH 6.8, 2 % SDS, 100 mM β-Mercaptoethanol
T <sub>25</sub> N <sub>50</sub>	25 mM Tris-HCl, 50 mM NaCl, pH 7.5
TAE-buffer (50 x)	2 M Tris-HCl, 950 mM acetic acid, 50 mM EDTA
TNE-T	10 mM Tris-HCl, pH 7.5, 2.5 mM EDTA, 50 mM NaCl, 0.1 % Tween
TNN	100 mM Tris-HCl, pH 8, 100 mM NaCl, 1 % NP-40
TNN lysis buffer	100 mM Tris-HCl, pH 8, 100 mM NaCl, 1 % NP-40, 1 mM Pefabloc, 1 µg/ml Aprotinin/Leupeptin, 1 mM DTT
Transfer buffer (20 x)	12.5 mM Tris-HCl, 100 mM glycine, pH 8.3
<i>Xenopus</i> CSF-XB	50 mM Sucrose, 10 mM K-Hepes, 0.5 mM EGTA, 0.5 mM MgCl <sub>2</sub> , pH 7.7 (KOH)
<i>Xenopus</i> Dejellying solution	2 % (w/v) cysteine in 1 x <i>Xenopus</i> XB salts, pH 7.8 (NaOH)
<i>Xenopus</i> fixing solution	60 % glycerol, 1 x MMR, 1 µg/ml Hoechst 33342, 10 % formaldehyde
<i>Xenopus</i> MMR (25 x)	125 mM Na-Hepes, 2.5 mM EDTA, 2.5 M NaCl, 50 mM KCl, 25 mM MgCl <sub>2</sub> , 50 mM CaCl <sub>2</sub> , pH 7.8 (NaOH)
<i>Xenopus</i> XB salts (20 x)	2M KCl, 20 mM MgCl <sub>2</sub> , 2 mM CaCl <sub>2</sub>

### 3.1.2 Chemicals

Amplifyer	GE Healthcare
Aprotinin/Leupeptin	Sigma
APS	Roth
ATP	Sigma
$\beta$ -Mercaptoethanol	Merck
BIO-RAD protein assay	Biorad
Cyclohexamide	Sigma
Doxycyclin hyclate	Sigma
DTT	Roth
ECL	Perkin Elmer
EDTA	Roth
Ethidiumbromide	Roth
Ganciclovir	InvivoGen
Geniticindisulfate (G418)	Roth
Glutathione Sepharose 4B	GE Healthcare
Gycine	Roth
Guanidinium Hydrochloride	Roth
HA-beads (monoclonal anti-HA agarose conjugate)	Sigma
Imidazole	USB
IPTG	Roth
Lipofectamine 2000	Invitrogen
MgCl <sub>2</sub>	ACROS organics
Milk powder	Roth
NaF	Sigma
Na <sub>2</sub> HPO <sub>4</sub>	Sigma
NaH <sub>2</sub> PO <sub>4</sub>	Merck
Na-o-vanadate	Sigma

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Ni-NTA-Agarose	Qiagen
Nocodazole	Sigma
NP-40	MP Biomedicals
ONPG	Sigma
PBS	Gibco
PefaBloc	Boehringer Ingelheim
Propidium iodide	Roth
Protein A-sepharose	GE Healthcare
Puromycin	Gibco
RNAse A	Roth
5 x Rotiphorese Gel 30	Roth
SDS	Roth
Sepharose CL-4B	GE Healthcare
TEMED	Roth
Thymidine	Sigma
Trizma Base (Tris)	Sigma
Turbofect	Fermentas
Tween-20	Roth

### 3.1.3 Bacterial strains

<i>E.coli</i> DH5 $\alpha$	M. Scheffner
<i>E.coli</i> XL10 Gold	M. Scheffner
<i>E.coli</i> BL21 (DE3)	M. Scheffner
<i>E.coli</i> Rosetta	Trenzyme GmbH
<i>E.coli</i> Rosetta/plys	Trenzyme GmbH
<i>E.coli</i> BL21/pRARE2	Trenzyme GmbH
<i>E.coli</i> GroEL/ES	Trenzyme GmbH
<i>E.coli</i> pArctic	Trenzyme GmbH

### 3.1.4 Mammalian cell lines

H1299: non-small cell lung carcinoma, p53 -/-	ATCC
H1299 E6APi: non-small cell lung carcinoma, p53 -/-, stable E6AP knockdown	M. Scheffner
Hek293T: human embryonal kidney cells	ATCC
U2OS: Osteosarcoma cells	ATCC

### 3.1.5 Cell culture material

#### 3.1.5.1 Cell culture media

DEMEM	Gibco
DEMEM R0K0 (not labeled Arg, Lys)	Dundee cell products
DEMEM R6K4 (Arg 6 $^{13}\text{C}_6^{14}\text{N}_4$ , Lys4 $^2\text{H}_4$ )	Dundee cell products
DEMEM R10K8 (Lys 8 ( $^{13}\text{C}_6^{15}\text{N}_2$ ), Arg 10 ( $^{13}\text{C}_6^{15}\text{N}_4$ ))	Dundee cell products
DEMEM without Arg and Lys	Silantes
Opti-MEM	Gibco
Foetal Calf Serum	Gibco
Foetal Calf Serum (dialyzed)	Dundee cell products

#### 3.1.5.2 Other material

Trypsin-EDTA	Gibco
Lys 0	Sigma
Arg 0	Sigma
Lys 8 ( $^{13}\text{C}_6^{15}\text{N}_2$ )	Silantes
Arg 10 ( $^{13}\text{C}_6^{15}\text{N}_4$ )	Silantes
Normocin	InvivoGen
Cellstar plates	Greiner

### 3.1.6 Antibodies

Primary antibodies:

protein	name	species	type	company/source	dilution
HA-tag	$\alpha$ HA 1.1	mouse	monoclonal	Covance	1:1000
Flag-tag	$\alpha$ Flag M2	mouse	monoclonal	Sigma	1:1000
E6AP	$\alpha$ E6AP	mouse	monoclonal	M. Scheffner	1:1000
p53	DO-1	mouse	monoclonal	Calbiochem	1:1000
Tubulin	$\alpha$ tubulin $\alpha$	mouse	monoclonal	Abcam	1:10000
hDlg	$\alpha$ hDlg	rabbit	polyclonal	Santa Cruz	1:1000
Cdc27	$\alpha$ Cdc27	rabbit	polyclonal	T.U. Mayer	1:1000
XeCdc20	$\alpha$ Cdc20 ( <i>Xenopus laevis</i> ), ab18217	mouse	monoclonal	Abcam	1:1000
hCdc20	$\alpha$ Cdc20 (human), ab26483	rabbit	polyclonal	Abcam	1:1000
ubiquitin	$\alpha$ ubiquitin, FK2	mouse	monoclonal	Enzo Life Science	1:1000
Xerp-1	$\alpha$ Xerp1 ( <i>Xenopus laevis</i> )	rabbit	polyclonal	T.U. Mayer	1:1000

Secondary antibodies

name	species	company	dilution
HRP-coupled a mouse	goat	Dianova	1:200000
HRP-coupled a rabbit	goat	Dianova	1:200000

### 3.1.7 Primers

name	sequence
5' BsmBI-HA	AACGTCTCTGATGTACCCATACGACGTCCCAGACTACGCT
3' AscI-16E6	AAGGCGCGCCTTACAGCTGGGTTTCTCTACGTG
3' AscI-11E6	AAGGCGCGCCTTAGGGTAACAAGTCTTCCATG
3' AscI-18E6	AAGGCGCGCCTTATACTTGTGTTTCTCTGCGTC
5' EcoRI HA	GGATTCATGTACCCATACGACGTCCCAGACTACGCT
3' XhoI HR23A Tubes with stopp	CACCTCGAGTTATTCCGGGGATCCACC
5' BglII HR23A Tube	ATCACCATCACAGATCTGGTGGAGGTG

<b>name</b>	<b>sequence</b>
5' BglII ubiquilin Tube	ATCACCATCACAGATCTGGAGGTGGAG
3' XhoI ubiquilin Tube with stopp	CACCTCGAGTTATTCCGGGGATCCTCC
3' XbaI cdc20	GCGTCTAGATCAGCGGATGCCTTG
5' BamHI cdc20	CAGGATCCATGGCACAGTTCGCGTTC
5' BglII HA	GCAGATCTATGGCGTACCCATACGAC
5' BamHI 18E1	GCGGATCCATGGCTGATCCAGAAG
5' BamHI 16E1	GAGGGATCCATGGCTGATCCTG
3' XhoI 16E1 stopp	GCGCTCGAGTTATAATGTGTTAGTATTTTG
3' XhoI 18E1 stopp	GCGCTCGAGTTATAGTGGTCTATGATTTTG
3' 16E1 106 S	CTTTTAATCTAGGACTAATATTATTGTCTAC
5' 16e1 106 S	GTAGACAATAATATTAGTCCTAGATTA AAAAG

### 3.1.8 DNA plasmids

#### 3.1.8.1 DNA Plasmids constructed and used in this thesis

<b>abbrev iation</b>	<b>vector name</b>	<b>insert</b>	<b>restriction sites</b>	<b>info</b>
MT27	pcDNA4 TO myc His B del BsaI			without BsaI in Amp resistance gene
MT28	pcDNA4 TO myc His B del BsaI puro-Ubi	puro-Ubi	ApaI/KpnI	subcloned from pExoIn
MT29	pcDNA4 TO myc His B del BsaI TK-puro-Ubi	TK	KpnI	subcloned from pAlli11TK
MT30	pcDNA4 TO myc His B del BsaI TK-puro-Ubi- HA-11E6	HA-11E6	Vector BsaI/AscI PCR BsmBI/AscI	PCR
MT31	pcDNA4 TO myc His B del BsaI TK-puro-Ubi- HA-16E6	HA-16E6	Vector BsaI/AscI PCR BsmBI/AscI	PCR
MT32	pcDNA4 TO myc His B del BsaI TK-puro-Ubi- HA-18E6	HA-18E6	Vector BsaI/AscI PCR BsmBI/AscI	PCR

<b>abbrev iation</b>	<b>vector name</b>	<b>insert</b>	<b>restriction sites</b>	<b>info</b>
MT33	pcDNA4 TO myc His B del BsaI TK-puro-Ubi- HA-11C18	HA-11C18	Vector BsaI/AscI PCR BsmBI/AscI	PCR
MT34	pTHE TK-puro-Ubi- HA-11E6	TK-puro-Ubi- HA-11E6	Blunt cloning in hpaI of pTHE	MT29
MT35	pTHE TK-puro-Ubi- HA-16E6	TK-puro-Ubi- HA-16E6	Blunt cloning in hpaI of pTHE	MT30
MT36	pTHE TK-puro-Ubi- HA-18E6	TK-puro-Ubi- HA-18E6	Blunt cloning in hpaI of pTHE	MT31
MT37	pTHE TK-puro-Ubi- HA-11C18	TK-puro-Ubi- HA-11C18	Blunt cloning in hpaI of pTHE	MT32
MT44	pSG5.0 His-NEDD8 A72R	NEDD8A72R	EcoRI/BamHI	PCR
MT45	pSG5.0 His-ubiquitin LIA	Ubiquitin LIA	EcoRI/BamHI	PCR
MT47	pcDNA3 HA-HR23A TUBEs	HR23A TUBES	Vector BamHI/XhoI PCR BglII/XhoI	PCR
MT48	pcDNA3 HA-Ubiquilin TUBEs	Ubiquilin TUBES	Vector BamHI/XhoI PCR BglII/XhoI	PCR
MT49	pcDNA3 HA-GST- HR23A TUBEs	GST-HR23A TUBES	Vector BamHI/XhoI PCR BglII/XhoI	PCR
MT50	pcDNA3 HA-GST- Ubiquilin TUBEs	GST-Ubiquilin TUBES	Vector BamHI/XhoI PCR BglII/XhoI	PCR
MT51	pcDNA3 Flag-HR23A TUBEs	HR23A TUBES	Vector BamHI/XhoI PCR BglII/XhoI	PCR
MT52	pcDNA3 Flag-Ubiquilin TUBES	Ubiquilin TUBES	Vector BamHI/XhoI PCR BglII/XhoI	PCR

<b>abbrev iation</b>	<b>vector name</b>	<b>insert</b>	<b>restriction sites</b>	<b>info</b>
MT53	pSG5.0 HisHA-HR23A TUBEs	HA-HR23A- TUBEs	EcoRI/XhoI	PCR
MT54	pSG5.0 HisHA- Ubiquilin TUBEs	HA-Ubiquilin TUBEs	EcoRI/XhoI	PCR
MT55	pcDNA3 Flag-11E6opt	11E6opt	BamHI/EcoRI	Subcloning from pcDNA3 HA-11E6opt
MT56	pcDNA3 HA-16E1 S106G	16E1 106S	BamHI/XhoI	PCR
MT57	pcDNA3 HA-16E1 without mutation	16E1		Mutagenesis PCR
MT59	pcDNA3 HA-16E2	16E2	BamHI/XhoI	Subcloning from pGex16E2
MT60	pcDNA3 HA-18E1	18E1	BamHI/XhoI	PCR
MT61	pcDNA3 HA-18E2	18E2	BamHI/XhoI	Subcloning from pGex18E2
MT62	pcDNA3 Flag-18E6	18E6	BamHI/EcoRI	Subcloning from pcDNA3 HA-18E6

### 3.1.8.2 Other DNA plasmids used in this thesis

<b>vector name</b>	<b>source</b>
pTHE	Addgene
pAlli11TK	Trenzyme
pExoIn	K. Matentzoglu
pcDNA3 HA-11E6	P. Kuballa
pcDNA3 HA-16E6	P. Kuballa
pcDNA3 HA-18E6	P. Kuballa
pcDNA3 HA-11C18	P. Kuballa
pcDNA3 HA-11E6opt	E. Weber
pcDNA3 HA-E6AP	K. Matentzoglu
pcDNA3 HA-Numb	E. Weber
pcDNA3 Flag-E6AP	K. Matentzoglu
pcDNA3 Flag-11C18	P. Kuballa

<b>vector name</b>	<b>source</b>
pcDNA3 Flag-16E6	P. Kuballa
pcDNA3 Flag-LNX1	E. Weber
pcDNA3 Flag-LNX1 C48A	E. Weber
pcDNA3 DHFR-HA-ubiquitin-p53	M. Scheffner
pRcCMV p53	M. Scheffner
pcDNA3 HA-eGFP	P. Kuballa
pcDNA3 eGFP	P. Kuballa
pcDNA3 eGFP 11E6	P. Kuballa
pcDNA3 eGFP 16E6	P. Kuballa
pcDNA3 3GFP 18E6	P. Kuballa
pcDNA3 Hismyc-ubiquitin	M. Scheffner
pCoc Mdm2	M. Scheffner
pGEX HR23A TUBEs	M. Rodriguez
pGEX Ubiquilin TUBEs	M. Rodriguez
pGEX 2TK 16E6	P. Kuballa
pGEX 2TK 18E6	P. Kuballa
pGEX 2TK 11C18	P. Kuballa
pGEX 2TK 11E6opt	E. Weber
pGEX 2TK E6AP	K. Matentzoglou
pGEX 2TK LNX1	E. Weber
pGEX 2TK Mdm2	M. Scheffner
pCS2 Flag <sub>3</sub> -Cdh1	O. Stemann
pCS2 Cdc20	T.U. Mayer
pCS2 Securin	T.U. Mayer
pGEX 18E1	M. Pawlita
pGEX 18E2	M. Pawlita
pGEX 16E1	M. Pawlita
pGEX 16E2	M. Pawlita
pSG5 EDD	Addgene

### 3.1.9 DNA- and protein marker

DNA marker:

- GeneRuler™ 1kb plus ladder (Fermentas)  
20000, 100000, 7000, 5000, 4000, 3000, 2000, 1500, 1000, 700, 400, 300, 200, 75 [bp]

Protein marker

- PageRuler™ Prestained Protein Ladder (Fermentas)  
170, 130, 95, 72, 55, 43, 34, 26, 17, 10 [kDa]
- PageRuler™ Unstained Protein Ladder (Fermentas)  
200, 150, 120, 100, 85, 70, 60, 50, 40, 30, 25, 20, 15, 10 [kDa]

### 3.1.10 Enzymes and reaction buffers

Taq Polymerase, selfmade (expressed in *E.coli*)

Phusion-Polymerase (Finnzyme)

Restriction digest enzymes (NEB)

Antarctic Phosphatase (NEB)

T4 DNA ligase (Fermentas)

## 3.2 METHODS

### 3.2.1 Preparation of plasmid DNA

#### 3.2.1.1 Mini preparation

For mini preparation 2 ml LB media was inoculated with a colony of a LB agar plate and grown over night at 37 °C. Cells were harvested by centrifugation. The plasmid DNA was isolated via alkalic lysis (Stephen et al., 1990).

#### 3.2.1.2 Midi preparation

For midi preparation 100 ml LB media was inoculated and bacteria were grown overnight. Plasmid DNA was isolated using Pure Yield™ Plasmid Midiprep System (Promega) according to the manufactures' instructions.

### 3.2.2 Determination of plasmid DNA concentration

Concentrations of plasmid DNA was estimated at a wavelength of  $\lambda=260$  nm with a nanophotometer (Implen).

### 3.2.3 Polymerase chain reaction (PCR)

For amplification of DNA fragments polymerase chain reactions were performed using phusion DNA polymerase (fermentas). The reaction was performed with 50 ng template DNA according to the manufactures instructions in a 50  $\mu$ l volume.

Screening PCR reactions on colonies were performed under similar conditions with self-prepared Taq polymerase and Thermo Pol buffer (fermentas) in a 20  $\mu$ l reaction volume.

### 3.2.4 Restriction digest

PCR products or plasmid DNA (2  $\mu$ g) were digested with the corresponding restriction enzymes and buffers (New England Biolabs) in a 20-60  $\mu$ l volume according to the manufactures instruction.

### 3.2.5 Ligation

Ligation was performed with T4 DNA ligase (Fermentas) in a 10  $\mu$ l reaction mixture containing 5 x rapid ligation buffer (Fermentas). The mixture was incubated for 15 minutes at room temperature followed by transformation in *E.coli* (3.2.8).

### 3.2.6 Agarose gel electrophoresis

Separation of DNA fragments was performed by agarose gel electrophoresis using 1 % TAE-agarosegels supplemented with 0.5  $\mu$ g/ml ethidium bromide. Agarose gels were loaded with DNA samples supplied with 10 x loading buffer and run at a constant voltage (100 V) for 30 minutes. Agarose gels were analyzed with a UV transilluminator and photographed using the LAS-3000 imaging system (Fujifilm).

### 3.2.7 Purification of DNA from agarose gels

After agarose gel electrophoresis (3.2.6) separated DNA fragementes were excised and DNA was purified using NucleoSpin extract II kit (Macherey Nagel).

### **3.2.8 Transformation of DNA in chemical competent *E.coli***

50 µl competent *E.coli* (XL10 Gold, DH5α or BL21 RIL) were incubated 30 minutes on ice with 1 µl plasmid DNA or 5 µl Ligation mixture (3.2.5) followed by a heat shock at 42 °C for 30 seconds. Afterwards transformation mixtures were incubated on ice for two minutes. Thereafter the mixtures were either plated on a LB agar plate with the corresponding antibiotics or were used to inoculate overnight cultures.

### **3.2.9 Preparation of electro competent *E.coli***

5 ml LB media was inoculated with *E.coli* (Rosetta, BL21, Origami or GroEL/ES) and grown to an OD of 0.4 to 0.6 at 37 °C. The mixture was cooled down on ice for 5-10 minutes. Afterwards the cultures were spinned down at 4600 rpm (heraeus multifuge) at 4 °C for 5 minutes. The pellet was washed with 2 ml ice cold water and spinned down at 4600 rpm (heraeus multifuge) for 5 minutes. Afterwards the pellet was resuspended in 2 ml 10 % Glycerol (ice cold) and spinned down at 4600 rpm (heraeus multifuge). The bacteria (pellet) were resuspended in 300-500 µl 10 % Glycerol. 50 µl were used for electroporation reactions (3.2.10).

### **3.2.10 Transformation of DNA in electro competent *E.coli***

50 µl of electro competent *E.coli* (3.2.9) were mixed with 1-2 µl DNA and filled in an electroporation cuvette (2 mm gap, peqlab) precooled on ice. Electroporation was performed with 2.5 kV in a multiporator (eppendorf).

### **3.2.11 Sequencing**

Sequencing was performed at GATC (Konstanz/Köln, Germany).

### 3.2.12 Cloning of pTHE-TK-puro<sup>r</sup>-ubiquitin-HA-E6

pTHE-TK-puro<sup>r</sup>-ubiquitin-HA-E6 vectors were cloned with a strategy that involved five steps:

1. The BsaI restriction site in pcDNA4TOmycHisB was removed
2. Sequence encoding for puro<sup>r</sup>-ubiquitin (source: pExoIn) was cloned into pcDNA4TOmycHisB(del BsaI) using KpnI and Apal restriction sites.
3. TK (source: pAlli11TK) was cloned into pcDNA4TOmycHisB(del BsaI)puro<sup>r</sup>-ubiquitin using KpnI restriction site
4. HA-E6 (source PCR) was cloned into pcDNA4TOmycHisB(del BsaI)TK-puro<sup>r</sup>-ubiquitin using BsaI/BsmBI and AscI restriction sites
5. TK-puro<sup>r</sup>-ubiquitin-HA-E6 was cloned in pTHE using blunt end cloning (HpaI restriction site on pTHE) resulting in pTHE-TK-puro<sup>r</sup>-ubiquitin-HA-E6

### 3.2.13 Protein expression in bacteria

Expression of GST-fusion proteins and His-tagged proteins was performed in *E.coli* BL21 (DE3) RIL if not indicated differently. Overnight cultures of LB Media with the respective antibiotics were inoculated. After 14 hours the culture was diluted 1:20 and grown to an OD of ~ 0.5 and induced with 0.5 mM IPTG (Isopropyl- $\beta$ -D-thiogalaktopyranoside, Roth). Proteins were expressed for 4 h at 37 °C. Afterwards bacteria cultures were spinned down (5000 rpm, 15 min, Sorvall centrifuge, SLA-3000 rotor) and bacteria pellets were either directly used for protein purification (3.2.17) or stored at -80 °C.

### 3.2.14 Optimization of expression of GST-fusion proteins

For optimization of GST protein expression, different *E.coli* strains were used: Rosetta, Rosetta/pLys, BL21, BL21/pRARE2, Arctic express and GroEL/ES. 2 ml cultures of LB media with the respective antibiotics were inoculated and grown overnight. Cultures were diluted to an OD of 0.05 and grown at 37 °C to an OD ~ 0.4. Half of the culture was grown at 37 °C and the other half was shifted to 20 °C. For the *E.coli* strain arctic express the cultures were shifted to 10 °C and for the strain GroEL/ES expression was performed at 30 °C. Cells were grown to OD ~ 0.5 and induced with either 0.5 or 0.05 mM IPTG. 0, 2, 4, and 20 hours after induction samples (0.5 OD) were taken. Bacteria pellets were resuspended in PBS 1 % triton and lysed by sonification. Samples of supernatant and pellets corresponding to an OD of 0.0625 were analyzed by SDS-PAGE (3.2.19) and Coomassie staining (3.2.20).

### 3.2.15 Optimized expression of GST-E6 proteins

Expression of GST-E6 proteins was performed in *E.coli* Rosetta/pLys. Overnight cultures were grown and diluted the next morning 1:20. Cells were grown to an OD ~ 0.4 and shifted to 20 °C and induced with 0.5 mM IPTG 30 minutes later. Bacteria were harvested by centrifugation (5000 rpm, SLA-3000 rotor) and bacteria pellets were either directly used for protein purification (3.2.17) or stored at -70 °C. Expression of GST-E1, GST-E2 and GST-LNX1 in *E.coli* BL21 RIL was also performed with this protocol.

### 3.2.16 Purification of His-tagged proteins

Bacteria pellets (3.2.13) were resuspended in a appropriate amount of PBS/1% Triton X100 and lysed on ice using sonification (Sonifier W-250 Branson, 3 x 20 pulses) followed by centrifugation (15000 rpm, 15 min, SS-34 or Fiberlite F21-8x50y rotor). Supernatents were transferred in new reaction tubes and Ni-NTA sepharose pre equilibrated in PBS/1% Triton X100 (1:1 Slurry) was added. Samples were incubated for 4 hours or overnight at 4 °C. Beads were collected by centrifugation (1000 rpm, 5 min, Eppendorf Centrifuge) and they were washed afterwards 3 x with PBS/1% Triton X100 and 3 additional x in T<sub>50</sub> (pH 8). Bound proteins were eluted 3 x with 250 mM imidazole in T<sub>50</sub> (pH 8).

### 3.2.17 Purification of GST-fusion proteins

Bacteria pellets (3.2.13 or 3.2.15) were resuspended in PBS/1% Triton X100 lysed on ice using sonification (Sonifier W-250 Branson, 3 x 20 pulses). Afterwards samples were centrifuged (15000 rpm, 15 min, SS-34 or Fiberlite F21-8x50y rotor) and supernatents were used for further purification. To 12.5 ml of supernatant 100 µl GSH-sepharose beads (GE Healthcare) pre equilibrated in PBS/1% Triton X100 (1:1 Slurry) were added. Samples were incubated 2-14 hours at 4 °C. Beads were collected by centrifugation (1000 rpm, 5 min, Eppendorf Centrifuge), and washed 3 times with PBS/1% Triton X100. Expression levels were analyzed by SDS-PAGE (3.2.19) and Coomassie staining (3.2.20). Purified GST Proteins were either used for GST coprecipitation assays (3.2.18) or eluted for *in vitro* ubiquitylation assays (3.2.25) or degradation assays in *Xenopus laevis* egg extracts (3.2.27). For elution, bound GST-fusion proteins to GSH-sepharose beads were first equilibrated in 50 mM Tris buffer and then eluted 3 x with 20 mM glutathione on ice. The volume of elution buffer was equal to the volume of beads.

### 3.2.18 GST coprecipitation assays (pulldown assays)

#### 3.2.18.1 GST coprecipitation assays with *in vitro* translated proteins

GST-fusion proteins bound to GSH-sepharose beads (3.2.17) were equilibrated in TNN. Similar protein amounts were used for each pulldown assay and bead amounts were equalized with empty GSH-sepharose beads. GST-proteins bound to GSH-sepharose beads were blocked with 5 % BSA in TNN for 30 minutes. Pulldown was performed with 5-10  $\mu$ l *in vitro* translation and adjusted to a volume of 300  $\mu$ l TNN lysis buffer. Samples were incubated for 4 hours or overnight at 4 °C and washed afterwards 3 x with TNN lysis buffer. Bound proteins were eluted with 2 x laemmli buffer at 95 °C for 5 minutes. Equal amounts of laemmli buffer to volume of beads were used. Samples were analyzed by SDS PAGE (3.2.19) and proteins were visualized by fluorography (3.2.22).

#### 3.2.18.2 GST coprecipitation assays with *Xenopus laevis* egg extract

GST-fusion proteins bound to GSH-sepharose beads were equilibrated in CSF-XB buffer. Pulldown experiments were performed with 10  $\mu$ l beads and 100  $\mu$ l of either meiotic (CSF) or interphase *Xenopus laevis* egg extract (3.2.26). By addition of Ca<sup>2+</sup> (0.6 mM) and Cyclohexamide (0.35 mM) to CSF for 1 hour at 20 °C interphase extract were received. Samples were incubated for 2 hours at 4 °C and washed afterwards 3 x with CSF-XB buffer. Bound proteins were eluted with equal amounts of 2 x laemmli buffer. Electrophoresis on SDS-PAGE (3.2.19) was performed and separated proteins were transferred to PVDF membrane (western blot analysis 3.2.21).

#### 3.2.18.3 GST coprecipitation assays with cell lysate

GST-fusion proteins bound to GSH-sepharose beads were equilibrated in TNN buffer. 30  $\mu$ l 1:1 Slurry of GST-proteins were incubated with 800  $\mu$ l cell lysate (cells from 1 x 15 cm plate were lysed in 1 ml TNN lysis buffer) for 4 hours or overnight at 4 °C. Samples were washed 3 x with TNN lysisbuffer and bound proteins were eluted with 30  $\mu$ l 2 x laemmli buffer. Proteins were separated by electrophoresis on SDS-PAGE (3.2.19) followed by transfer to PVDF membrane (western blot analysis 3.2.21).

#### 3.2.18.4 GST coprecipitation assays with cell lysate - SILAC Experiment

12 x 15 cm plates H1299 cells were labeled with light, 12 x 15 cm plates H1299 cells were labeled with heavy and 12 x 15 cm plates H1299 E6APi cells were labeled with medium amino acids (dundee cell products) for 8 days. Cells were lysed in 12 ml TNN lysis buffer for 1

hour at 4 °C on an overhead shaker. Afterwards lysates were centrifuged (1/2 hour, 20000 rpm, SS-34 rotor) and supernatants were used for coprecipitation experiments. 3 ml of supernatant were incubated with 100 µl 1:1 Slurry GST-proteins (GST or GST-11C18) for 5 hours at 4 °C. Beads were washed 3 x with TNN lysis buffer and 5 µl 1:1 Slurry were taken for western blot analysis. The remaining beads of the respective GST or GST-11C18 coprecipitations with lysates of light, medium and heavy labeled cells were pooled. Proteins were eluted from beads 4 x with 150 µl T<sub>25</sub>N<sub>1000</sub> buffer following protein precipitation with Chloroform/Ethanol (Wessel Flüge protocol, (Wessel and Flugge, 1984)). Precipitated proteins were dissolved in 54 µl 2 % SDS and 6 µl 10 x β-mercaptoethanol sample buffer was added. Samples were boiled and separated by a gradient (5-20 %) electrophoresis on SDS-PAGE (3.2.19). Separated proteins were stained by colloidal Coomassie. Each lane was cut in 20 slices and analyzed by mass spectrometry (see 3.2.41, 3.2.42 and 3.2.43).

### **3.2.19 SDS-polyacrylamide gel electrophoresis (SDS-PAGE)**

Separation of proteins by electrophoresis was performed according to Laemmli (Laemmli, 1970). The amount of acrylamide in the separating gel (370 mM Tris-HCl, 0.1 % SDS, pH 8) varied from 5 % to 20 % depending on the molecular mass of the proteins separated. The stacking gel (125 mM Tris-HCl, 0.1 % SDS, pH 6.8) had always a percentage of 5 % acrylamide. Laemmli sample buffer was added to protein samples and boiled at 100 °C for 2-5 minutes. Electrophoresis was performed in Laemmli running buffer at constant current (50 mA). Detection of proteins was conducted by coomassie blue staining (2.2.20), immunodetection (western blot analysis 2.2.21) or radiolabeled proteins were visualized by fluorography (2.2.22).

### **3.2.20 Coomassie blue staining**

Separated proteins on SDS-gels were stained for 20 minutes with Coomassie Blue staining solution followed by destaining with destain solution. SDS-gels for mass spectrometry analysis were stained by colloidal Coomassie (Roth) and destained with a 25 % methanol solution.

### **3.2.21 Western blot analysis**

Transfer of proteins from SDS-gels on PVDF-membranes (Roth) was performed in a blotting chamber (BioRad) in 1 x transfer buffer (12.5 mM Tris-HCl, 100mM Glycine, pH 8.3) at a constant voltage (60 V) for 90 minutes. For immunodetection of the transferred proteins, membranes were first blocked for 1 hour at room temperature or overnight at 4 °C in

blocking buffer (5 % milk powder (Roth) in TNE-T). The following steps were all performed at room temperature. After 3 washing steps (TNE-T) followed the incubation with the respective primary antibody (see 3.1.6) for 1 hour. Afterwards, membranes were washed 3 x with TNE-T and they were incubated with the horse radish peroxidase coupled secondary antibody. Subsequently membranes were washed again with TNE-T to remove unbound secondary antibodies and proteins were detected by an ECL- (enhanced chemiluminescence) reaction on the FUJI LAS imaging system.

### **3.2.22 Fluorography**

For visualization of <sup>35</sup>S methionine labeled proteins, SDS-gels were first incubated in destain solution for 20 minutes followed by incubation in Amplifyer (Amersham) for 20 minutes. Afterwards, gels were dried at 80 °C under vacuum conditions. Proteins of the dried gels were visualized by an Imaging Plate BAS-IIIIs (Fuji) in BAS Cassette 2 1040 (Fujifilm). Exposition was dependent on the intensity of the signals and was performed for 1 to 72 hours. Images were taken by BASReader (Raytest).

### **3.2.23 Determination of protein concentration**

Determination of protein concentrations of cell lysates was performed with Bradford reagents (BioRad or Roth) according to the manufactures' instructions. Measurements were conducted at a wavelength of 595 nm and for blank sample TNN lysis buffer was added.

### **3.2.24 *in vitro* translation**

*In vitro* translation of proteins was performed with rabbit reticulocyte lysate (RRL) or wheat germ extract (WG) with the TNT-*in vitro* translation kit according to the manufactures' instructions. Proteins were radioactively labeled by <sup>35</sup>S labeled methionine (Perkin Elmer).

### **3.2.25 *in vitro* ubiquitylation assay**

*In vitro* ubiquitylation assays were performed in a reaction volume of 40 µl. E1 and E6AP were expressed in the baculovirus system in insect cells. E2 enzyme (UbcH5b), GST E6 proteins, His-16E6 and GST-LNX1 were expressed in *E.coli*. For ubiquitylation assays 1-2 µl of *in vitro* translation, 20 µg ubiquitin (Sigma), 50 ng E1 and 50 ng E2 and different amounts of E3 ligases (GST-LNX1 or E6/E6AP) were mixed. In addition each reaction contained 2 mM ATP, 2 mM MgCl<sub>2</sub>, 1 mM DTT and were filled up to 40 µl with T<sub>25</sub>N<sub>50</sub> (pH 7.5). Samples were incubated at 25 °C (E6/E6AP) or 30 °C (GST-LNX) for 120 minutes followed by addition of 5 x

laemmli sample buffer and separation on SDS-PAGE (3.3.19). Labeled proteins were visualized by fluorography (3.2.22).

### 3.2.26 Preparation of *Xenopus laevis* egg extract (CSF)

Frogs were injected with 50 units PMSG (Pregnant mare's serum gonadotropin) 3 to 7 days prior extract preparation. In the evening before extract preparation, frogs were induced to ovulate by injection with HCG (human chorionic gonadotropin) (500 units). Next morning eggs were washed with 1 x MMR and activated eggs were sorted out. Afterwards eggs were dejellied with dejelling solution (2 % w/v cysteine in 1 x XB salts, pH 7.8 adjusted with NaOH) for 7 minutes followed by washing in CSF XB buffer (1 x XB salts, 50 mM Sucrose, 10 mM K-Hepes, 0.5 mM EGTA, 0.5 mM MgCl<sub>2</sub>) to remove cysteine. 8 µl Cytochalasin B (0.1 mg/ml) and 1 ml of CSF XB buffer were pre-filled in centrifugation tubes before eggs were added. Eggs were centrifuged for 1 minute at 1000 rpm followed by an additional minute at 2000 rpm (Sorvall HB-6 rotor). Excessive buffer was removed and eggs were lysed through centrifugation at 10000 rpm for 10 minutes (Sorvall HB-6 rotor). The yellowish middle layer consist the cytoplasmic fraction and was removed with a 1 ml syringe and 18 G needle. Extract was kept on ice and cytochalasin B (0.1 mg/ml) was added (1:1000 dilution). Thereafter extracts were tested: 2 x 20 µl extract were supplemented with sperm nuclei. In one sample release to interphase of extract was performed through addition of Ca<sup>2+</sup> (0.6 mM). Samples were incubated for 45 minutes at 20 °C and morphology of DNA was analyzed by Hoechst staining. 2 µl of extract were mixed with 3 µl of *Xenopus* fixing solution followed by squashing under a cover slip. Samples were analyzed by microscopy (Zeiss, Axio Imager M1). Extracts were used for coprecipitation (3.2.18.2) or degradation (3.2.27) assays when the sample without Ca<sup>2+</sup> showed chromosomal DNA and the released interphase sample (+Ca<sup>2+</sup>) exhibited nuclei.

### 3.2.27 Degradation assay in *Xenopus laevis* egg extract

*In vitro* translated Securin was added to *Xenopus laevis* egg extract (CSF, 3.2.28) (1:20 dilution). In addition GST proteins were added and each sample was divided in 2 parts. One was released by addition of Ca<sup>2+</sup>. Samples were incubated at 20 °C and at 0, 5, 10, 20, 40, 60 and 90 minutes 3 µl of each sample were removed and mixed with 27 µl 2 x laemmli sample buffer and boiled. Samples were analyzed by SDS-PAGE (3.2.19) and fluorography (3.2.21).

### **3.2.28 Cell culture of mammalian cells**

Mammalian tumor cell lines (Hek293T, U2OS, H1299) were cultured on cellstar plates (Greiner) in DEMEM (Gibco) and 10 % FCS (Gibco) in an incubator at 37 °C and 5 % CO<sub>2</sub>.

### **3.2.29 Synchronization of mammalian cells**

To arrest cells at G1/S boundary of the cell cycle they were treated for 18 hours with 2 mM thymidine (sigma). To obtain arrested cells at G2/M boundary, cells were first treated with 2 mM thymidine (sigma) for 18 hours. Afterwards thymidine was removed by washing the cells 3 x with 37 °C warm PBS. After addition of fresh DEMEM (10 % FCS) cells were released for 6 hours followed by addition of nocodazole (100 ng/ml) (sigma) to the media for 12 hours. Cells were harvested and used for FACS analysis (3.2.38) or coprecipitation assays (3.2.37).

### **3.2.30 Transfection of mammalian cells**

Mammalian cells were transfected by lipofection. H1299 and U2OS cells were transfected with Lipofectamine2000 (Invitrogen) and Hek293T cells with Turbofect (Fermentas) according to the manufactures instructions. 24 hours after transfection cells were harvested or subjected for stable selection. For FACS analysis cells were fixed 48 hours after transfection (see 3.2.38).

### **3.2.31 Harvesting of cells**

Media of the cells was discarded and cells were washed 3 x with cold PBS. Thereafter cells were harvested on ice with a cell scraper in an appropriate volume of PBS. Cells were transferred in a reaction tube and centrifuged at 3000 rpm for 3 minutes at 4 °C. (ependorf centrifuge) Cell pellets were either directly used for cell lysis (3.2.32) or stored at -80 °C.

### **3.2.32 Cell lysis**

The amount of lysis buffer was adjusted to the number of cells. Lysis was performed in TNN buffer supplemented with 100 μM DTT and protease inhibitors Aprotin/Leupeptin and Pefabloc (TNN lysis buffer). For His-ubiquitylation assays, pulldown samples were lysed in Guanidium-hydrochloride lysis buffer. Cells were resuspended with the respective lysisbuffer and incubated on ice for 30 minutes followed by centrifugation at 13200 rpm for 30 minutes

(Eppendorf centrifuge). Lysates were transferred in fresh reaction tubes and cell pellets were discarded.

### **3.2.33 Determination of transfection efficiency**

For the determination of transfection efficiency a vector encoding  $\beta$ -galactosidase was cotransfected. After cell lysis  $\beta$ -galactosidase activity was measured: 5  $\mu$ l lysate was added to 120  $\mu$ l Z buffer and 5  $\mu$ l ONPG (o-nitrophenol- $\beta$ -galacto-pyranoside) as substrate. Samples were incubated at 37 °C until they became a yellow color. Afterwards absorbance was measured at a wavelength of 405 nm. To determine the blank value the addition of the corresponding lysis buffer was used.

### **3.2.34 Degradation assay in mammalian cells**

We cotransfected DNAs encoding a potential substrate protein (e.g.:p53) and an E3 ligase (e.g.: Mdm2) in H1299 cells to test the ability whether the E3 ligase is able to degrade this protein. Afterwards cells were harvested, lysed in TNN lysis buffer and adjusted to transfection efficiencies (3.2.33). Proteins were separated by SDS-PAGE (3.2.19) and analyzed by western blot analysis (3.2.21).

### **3.2.35 Degradation assay using DHFR-HA-ubiquitin-p53**

The DHFR-HA-ubiquitin fusion system was used to determine whether p53 levels were affected by LNX1. H1299 cells were cotransfected with DHFR-HA-ubiquitin-p53 and the respective E3 ligase (Mdm2 or LNX1). DHFR-HA-ubiquitin is expressed as a polyprotein and cleaved in cells by ubiquitin specific proteases in DHFR-HA-ubiquitin and p53 resulting in same amounts of both proteins. DHFR-HA-ubiquitin is a very stable protein. Thus, a clear result about p53 degradation can be drawn by comparing p53 levels with those of DHFR-HA-ubiquitin. Cells were lysed with TNN lysis buffer and samples were adjusted to transfection efficiencies (3.2.33). Proteins were separated by SDS-PAGE (3.2.19) and analyzed by western blot analysis (3.2.21). p53 and DHFR-HA-ubiquitin levels were quantified (ImageJ). p53 levels were normalized according DHR-HA-ubiquitin levels.

### **3.2.36 His-ubiquitylation assay**

For His-ubiquitylation assays 1/3 of each sample were lysed with an appropriate amount of TNN lysisbuffer (60-100  $\mu$ l). Samples were adjusted to transfection efficiency (see 3.2.33) and

separated by SDS-PAGE (3.2.19) following western blot analysis (3.2.21). 2/3 of each sample were lysed in 0.5 ml Guanidin-HCl lysisbuffer (see 3.1.1) followed by a preclear of lysates with 200 µl 1:1 slurry sepharose for 1 hour. Samples were centrifuged for 5 minutes at 13200 rpm (Eppendorf centrifuge) and supernatants were transferred in reaction tubes. Subsequently 100 µl 1:1 slurry Ni-NTA agarose beads (Qiagen) was added to each sample and binding of His-ubiquitin to Ni-NTA was performed for 4 hours or overnight at 4 °C. Beads were washed 2 x with GuHCl-buffer followed by 2 x washing steps with 1:5 mixture of GuHCl buffer and 50 mM Tris, 20 mM Imidazole, pH 6.8. Thereafter beads were washed 4 x with 50 mM Tris, 20 mM Imidazole, pH 6.8. Bound proteins were eluted from beads by addition of a 1:1 mixture 100 µl 5 x Laemmli sample buffer and 0.5 mM Imidazole following boiling. Samples were separated on SDS-PAGE (3.2.19) followed by western blot analysis and proteins (3.2.21).

### **3.2.37 Immunoprecipitation Assays**

#### **3.2.37.1 Coimmunoprecipitation Assays**

Cells were lysed in 500 µl TNN lysis buffer for Coimmunoprecipitation assay. Afterwards, samples were adjusted to transfection efficiency and filled up with TNN lysis buffer to 300 µl total sample volume. 30 µl of each samples were taken as input samples and supplemented with 5 x Laemmli buffer. To the remaining 270 µl lysate 35 µl 1:1 slurry Protein A sepharose were added and incubated for 1 hour as preclear. Samples were centrifuged and lysate was transferred to new reaction tubes and supplemented with 1 µl Flag or HA antibody. Samples were incubated at 4 °C for 1 hour followed by addition of 50 µl 1:1 slurry of Protein A beads. After incubation for 4 hours or overnight at 4 °C beads were washed 3 x with TNN lysis buffer and bound proteins were eluted by 2 x sample buffer and boiling. Samples were analyzed by SDS-PAGE (3.2.19) and western blot analysis (3.2.21).

#### **3.2.37.2 Immunoprecipitation of HA-TUBEs**

Cells (6 cm plates) were lysed in 100 µl TNN lysis buffer. Samples were adjusted according to transfection efficiency (3.2.33) and filled up to a total volume of 300 µl and supplemented with 10 µl 1:1 slurry HA-beads (Sigma). Samples were incubated at 4 °C for 4 hours or overnight followed by 3 x washing steps with TNN lysis buffer. Bound proteins were eluted by addition of 2 x Laemmli buffer and boiling. Samples were analysed by SDS-PAGE (3.2.19) and western blot analysis (3.2.21).

HA-TUBEs IP for mass spectrometry analysis was performed with 1 x 15 cm plate for each sample as outlined above. Volumes of TNN lysis buffer and HA-beads were adjusted to the higher number of cells. Samples were analysed on a gradient (5-15 %) SDS-PAGE (3.2.19)

followed by colloidal coomassie staining (3.2.20). Each lane was cut in 20 slices and analyzed by mass spectrometry (see 3.2.41 and 3.2.42).

### **3.2.38 Flowcytometry (FACS)**

#### **3.2.38.1 DNA content analysis with PI staining**

5 x 10<sup>6</sup> cells were harvested by trypsination and washed 2 x with PBS. Cells were resuspended in 300 µl PBS 1 % FCS and 900 µl ice cold EtOH was added dropwise during vortexing to fix them. Cells were stored at 4 °C overnight or up to 1 week. Fixed cells were washed twice with PBS (3000 rpm, 5 min, Eppendorf Centrifuge) and afterwards resuspended in 500 µl PBS. 50 µl of 10 x PI solution (3.1.1), 50 µl of RNase A solution (10 mg/ml) and 6 µl of 400 µg/ml PI (aqueous) solution was added to the cell suspension. Samples were incubated at 37 °C for 30 minutes. Cells were afterwards stored on ice and were analyzed on Becton Dickinson FACS Calibur and Cellquest Pro software. 10000 events were measured and data were evaluated with Cyflogic software.

#### **3.2.38.2 DNA content analysis with PI staining in combination with measurement of fluorescent protein expression**

To determine DNA content in combination with eGFP measurement cells were fixed with 1 % formaldehyde, permeabilized with 70 % EtOH and then stained with PI. In detail 2 x 10<sup>6</sup> cells were harvested by trypsination and washed 2 x with PBS. Cells were resuspended in 500 µl cold PBS followed by the addition of 500 µl of cold fixation solution (3.1.1). Samples were incubated for 1 hour at 4 °C. Afterwards cells were washed 1 x with PBS and 1 ml ice cold 70 % EtOH was added dropwise to the cell pellet during vortexing. Cells were incubated overnight or up to 1 week at 4 °C. Cells were centrifuged (5 min, 300 g, Heraeus Multifuge) and cell pellets were resuspended in 1 ml PI working solution (1:25 dilution of PI stock solution (3.1.1)) and incubated for 30 minutes at 37 °C. Samples were analyzed on Becton Dickinson FACS Calibur with Cellquest Pro software. 25000 events of GFP positive cells were measured. Data evaluation was performed with Cyflogic software.

### **3.2.39 Labeling of proteins of cell lines with SILAC media**

Proteins of H1299 and H1299 E6APi cell lines were test labeled for 10 days with SILAC media (R0K0, R6K4, R10K8) (Dundee cell products). After 3, 5, 7, 8, 9 and 10 days samples were taken and cells were lysed in TNN lysis buffer and analyzed by mass spectrometry for

incorporation of labeled amino acids. After 7 days more than 95 % of all proteins were labeled with heavy amino acids.

Proteins of U2OS cells were test labeled for 10 days with SILAC media (light R0K0, heavy R10K8) (Silantes). Samples of cells were taken after 3, 5, 7, 8, 9 and 10 days lysed in TNN lysis buffer and analyzed by mass spectrometry for incorporation of labeled amino acids. More than 95 % of all proteins were labeled after 7 days.

### **3.2.40 SILAC-Proteome approach**

Proteins of U2OS 18E6 and U2OS 11C18 cell lines were both labeled with light (R0K0) and heavy (R10K8) amino acids for 8 days. Cells which were labeled with heavy amino acids were induced with doxycycline to express the corresponding E6 proteins 2 days before harvesting. Cells were lysed in TNN lysis buffer and protein concentrations were determined by Bradford assay. Same protein amounts of light and heavy labeled cell lysates (100 µg) were mixed and 5 x Laemmli sample buffer was added. Samples were boiled and separated on a 5-15 % gradient SDS-PAGE. The gel was stained with Colloidal Coomassie (3.2.20) and each lane was cut in 20 slices and analyzed by mass spectrometry (3.2.41, 3.2.42 and 3.2.43) (in collaboration with Proteomics Facility, Konstanz).

### **3.2.41 In-gel trypsin digestion of proteins and peptide extraction**

Excised bands were in gel trypsin digested prior analysis by LC-MS/MS. Gel pieces were incubated in 1 mM DTT in 50 mM  $\text{NH}_4\text{HCO}_3$  for 1 hour at 56 °C. DTT solution was afterwards removed. Alkylation was performed with 5 mM Iodacetamide in 50 mM  $\text{NH}_4\text{HCO}_3$  for 1 hour at room temperature. Gel pieces were afterwards washed with MilliQ-water following an additional washing step with 50 mM  $\text{NH}_4\text{HCO}_3$ . Gel pieces were dehydrated using 3/2 acetonitrile/MilliQ-water. This step was repeated until gel pieces were colorless. After the solution was discarded pure acetonitrile was added and samples were incubated for 10 minutes and air dried to complete dryness. Afterwards gel pieces were reswelled on ice for 45 minutes in cold buffer containing trypsin (10 ng/µl) and 50mM  $\text{NH}_4\text{HCO}_3$ . Solution was discarded and gel pieces were incubated in 50mM  $\text{NH}_4\text{HCO}_3$  without trypsin over night at 37 °C. Supernatants were collected and peptides were extracted by adding 3/2 acetonitrile/0.1% TFA in MilliQ-water for 1 hour at room temperature. Elutions were afterwards collected and gel pieces were covered with acetonitrile for 15 minutes. Collected washes and elutions were purified using ZipTip. Bound peptides were eluted in 2 µl acetonitrile /0.1%TFA (8:2) and diluted with 0.1 % formic acid to a final volume of 20 µl (in collaboration with Proteomics Facility, University of Konstanz).

### 3.2.42 LC-MS/MS

All samples were analyzed by reversed phase liquid chromatography nanospray tandem mass spectrometry (LC-MS/MS) using an LTQ-Orbitrap mass spectrometer (Thermo Fisher) and an Eksigent nano-HPLC. The dimensions of the reversed-phase LC column were 5  $\mu\text{m}$ , 100  $\text{\AA}$  pore size C18 resin in a 75  $\mu\text{m}$  i.d.  $\times$  10 cm long piece of fused silica capillary (Acclaim PepMap100, Thermo Scientific). After sample injection, the column was washed for 5 min with 95 % mobile phase A (0.1 % formic acid) and 5 % mobile phase B (0.1 % formic acid in acetonitrile), and peptides were eluted using a linear gradient of 5% mobile phase B to 40 % mobile phase B in 65 min, then to 80 % B in an additional 5 min, at 300 nl/min. The LTQ-Orbitrap mass spectrometer was operated in a data dependent mode in which each full MS scan (30 000 resolving power) was followed by five MS/MS scans where the five most abundant molecular ions were dynamically selected and fragmented by collision-induced dissociation (CID) using a normalized collision energy of 35% in the LTQ ion trap. Dynamic exclusion was allowed. Tandem mass spectra (ubiquitome approach) were searched against the Swissprot human protein database using Mascot (Matrix Science) with “Trypsin/P” enzyme cleavage, static cysteine alkylation by iodoacetamide and variable methionine oxidation (in collaboration with Proteomics Facility, University of Konstanz).

### 3.2.43 Data analysis of mass Spectrometry data with Maxquant and Perseus

Tandem mass spectra (3.2.42) (proteome and interactome approach) were processed for quantification with Maxquant software and data evaluation was performed with Perseus software (Version 1.3.0.4).

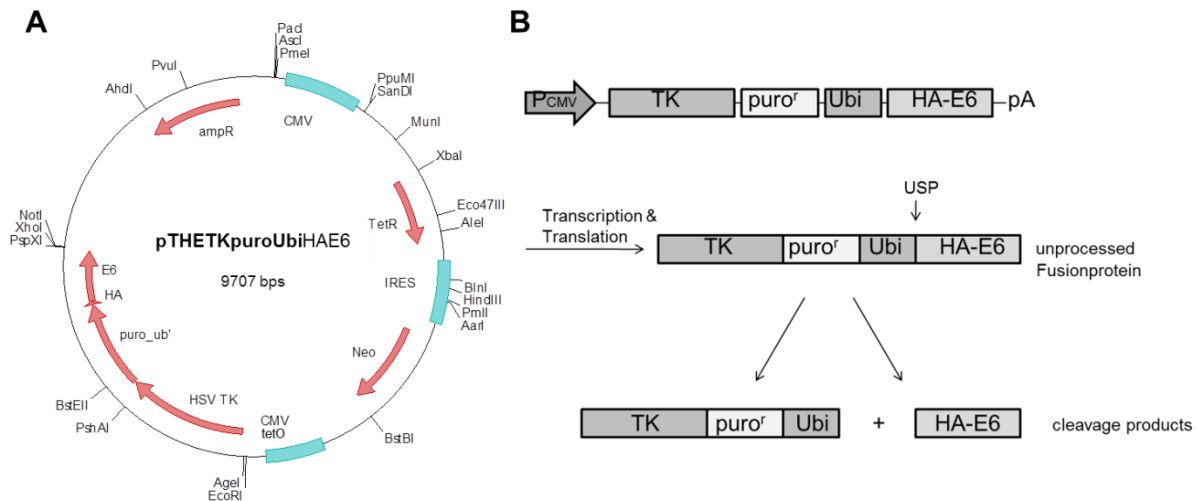
## 4 RESULTS

In contrast to high risk HPV E6 proteins, low risk HPV E6 proteins are only poorly characterized. However, high risk and low risk E6 proteins show similarity on amino acid level and presumably they have similar functions during the viral life cycle. In addition, both proteins bind to the E3 ligase E6AP assuming that they share some E6AP-dependent ubiquitylation substrates (Brimer et al., 2007; Kuballa et al., 2007). In order to obtain better insight into common and different functions of high risk and low risk HPV E6 proteins we have characterized them by a mass spectrometric-driven approach. First we analyzed the “HPV E6 proteome” (proteins whose expression is up- or down- regulated in the presence of E6), second the “HPV E6 ubiquitome” (proteins that are ubiquitylated in an E6-dependent manner) and third the “HPV E6 interactome” (proteins that interact with E6).

### 4.1 ESTABLISHMENT AND CHARACTERIZATION OF STABLE CELL LINES, INDUCIBLY EXPRESSING THE E6 PROTEINS

As our intention was to analyze the HPV E6 proteins by quantitative mass spectrometry with respect to their “proteome”, “ubiquitome” and “interactome”, especially for the proteome approach, it is necessary to work with defined cellular systems. To do so, we established stable cell lines which inducibly express HA-tagged E6 proteins (HA-11E6, HA-16E6, HA-18E6 and the chimeric HA-11C18, which consists of the 140 N-terminal amino acids of the low risk 11E6 and 17 C-terminal amino acids of the high risk 18E6 protein including the PDZ binding motif). For this purpose, we used the already described ubiquitin-fusion system combined with an inducible system, which is regulated via the Tet repressor. Varshavsky and colleagues showed that a fusion protein consisting of ubiquitin fused to the N terminus of another protein is site-specifically cleaved by ubiquitin specific proteases (USP) into the free proteins (Varshavsky, 2005). The ubiquitin-fusion protein system makes use of this phenomenon by fusing a resistance marker protein (in this case, puromycin N-acetyltransferase or, in brief, puor<sup>r</sup>) to the N terminus of ubiquitin, which in turn is fused to the N terminus of a protein of interest, in our case the HA-E6 proteins (Matentzoglou and Scheffner, 2009). We developed a new system, where the puor<sup>r</sup>-ubiquitin-HA-E6 fusion protein is further expanded by fusion of the herpes simplex virus-derived thymidine kinase (TK) to the N terminus of puor<sup>r</sup> (figure 9). The resulting polyprotein is processed by ubiquitin specific proteases (USP) into the HA-E6 protein and the TK-puor<sup>r</sup>-Ubi (figure 9B). The predicted advantages of the system are: (i) the resistance marker (puor<sup>r</sup>) and HA-E6 are expressed as a fusion protein and thus, by definition, any cell that is resistant to puromycin

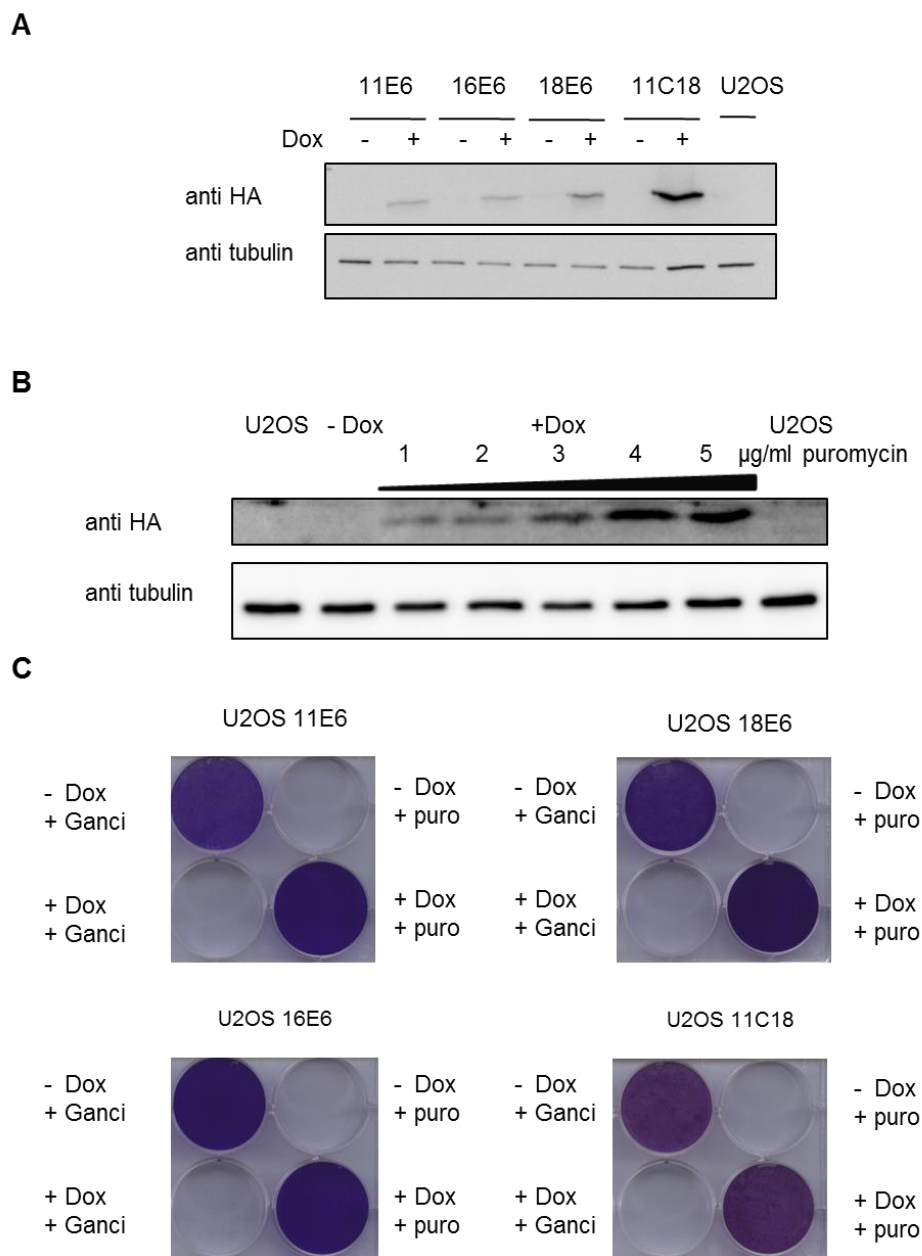
expresses also the HA-E6 protein, and (ii) the tightness of the system is guaranteed by TK in combination with ganciclovir. If TK is expressed, it converts ganciclovir into cytotoxic products. Thus, cells in the uninduced state (i.e. in the absence of doxycycline) can be treated with ganciclovir and all cells expressing the polyprotein will be eliminated (i.e. "leaky" cells), while cells that do not express the polyprotein will survive.



**Figure 9: Inducible TK-puro<sup>r</sup>-Ubi fusion system**

**A:** Vector map of the pTHE vector (Addgene) containing a Tet-On system for inducible expression and cDNA encoding the TK-puro<sup>r</sup>-Ubi-HA-E6 protein. **B:** TK-puro<sup>r</sup>-Ubi fusion system: the vector encodes a fusion protein consisting of the thymidine kinase (TK) fused to the N terminus of puromycin N-acetyltransferase (puro<sup>r</sup>) fused to the N terminus of ubiquitin (Ubi), which itself is fused to the N terminus of HA-E6. The fusion protein is expressed as one polyprotein and afterwards it is cleaved by ubiquitin specific proteases (USP) after the C-terminal glycine of ubiquitin into HA-E6 and TK-puro<sup>r</sup>-Ubi (modified from Matentzoglou and Scheffner, 2009).

With our newly developed vector system we established stable U2OS cell lines by transfection of the respective expression vectors and subsequent selection. First, cells were selected using G418 to ensure that cells contain the respective expression vector, and second, cells were induced with doxycycline followed by selection with puromycin to guarantee that all cells express the TK-puro<sup>r</sup>-Ubi-HA-E6 polyprotein. Upon these selection steps, stable inducible cell lines for the different E6 proteins were established ("U2OS 11E6", "U2OS 16E6", "U2OS 18E6" and "U2OS 11C18"). Western blot analysis of induced and uninduced cells proofed inducible expression of the HA-tagged E6 proteins (figure 10A), which could be enhanced by selection with increasing amounts of puromycin (figure 10B, data shown for U2OS 11C18).



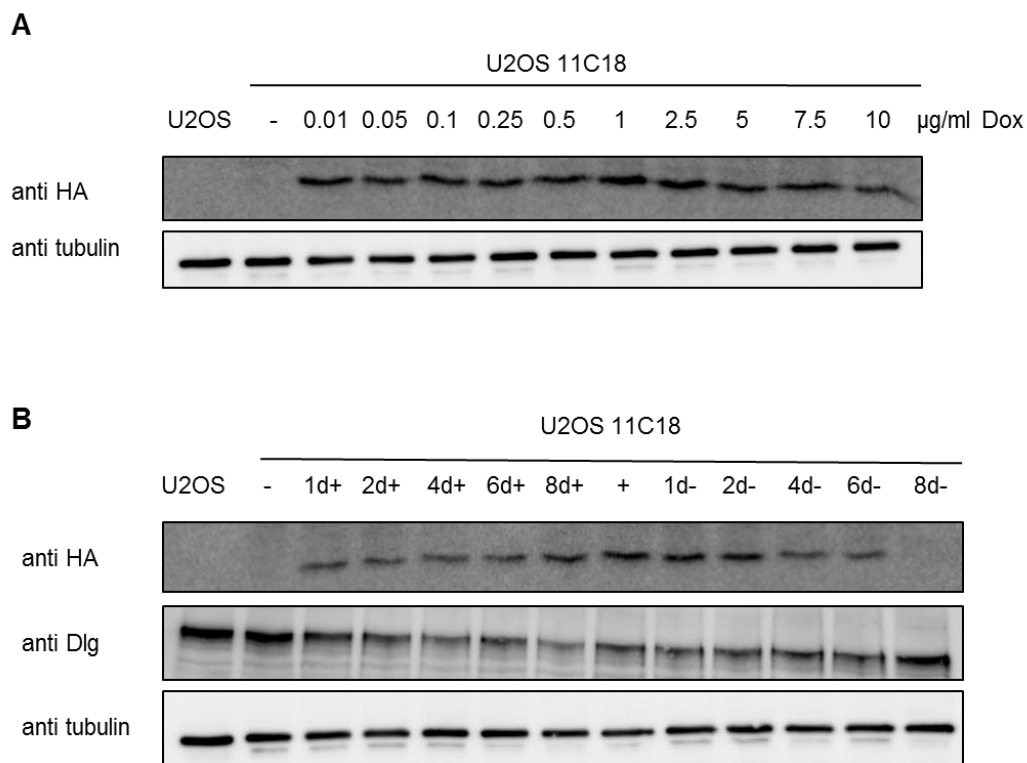
**Figure 10: U2OS E6 cell lines inducibly express HA-E6 proteins and respond to puromycin and ganciclovir selection**

**A:** U2OS 11E6, U2OS 16E6, U2OS 18E6 and U2OS 11C18 cell lines were induced for 2 days with doxycycline (Dox) (1 mg/ml) followed by puromycin selection (5 µg/ml). Cells were lysed under native conditions and protein levels were detected by western blot analysis using indicated antibodies. Expression of tubulin served as loading control. **B:** U2OS 11C18 cells were induced for 2 days with doxycycline (Dox) (1 mg/ml) followed by puromycin selection for 24 hours with indicated amounts of puromycin. Cell lysates were analyzed for protein levels by western blot analysis using indicated antibodies. Expression of tubulin served as loading control. **C:** Puromycin and ganciclovir treatment: induced and uninduced U2OS 11E6, U2OS 16E6, U2OS 18E6 and U2OS 11C18 cells were treated with puromycin (puro) (5 µg/ml) or with ganciclovir (Ganci) (0.015 mM). 12 days after selection cells were stained with crystal-violet to visualize viable cells.

Furthermore, we characterized the U2OS E6 cell lines with respect to their selection markers. For this purpose we analyzed induced and uninduced cells treated with puromycin or

ganciclovir over a time period of twelve days. Upon treatment, cells were stained with crystal violet to visualize viable cells. As expected, most of the induced cells survived in the presence of puromycin, whereas uninduced cells died after two days of puromycin treatment. Furthermore, ganciclovir selection showed that in the absence of doxycycline for U2OS 16E6 and U2OS 11C18, cell growth was not affected, whereas most of the induced cells died. For U2OS 11E6 and U2OS 18E6, fewer uninduced cells survived after ganciclovir treatment. This was likely due to “leaky” expression of the polyprotein (figure 10C). However, repeated treatment (3 times) of these cell lines with ganciclovir did not result in cell lines without “leaky” expression of the polyprotein (data not shown). In all following experiments, cells which were not selected with ganciclovir were used. In conclusion, these results show that the vector system behaves as expected with respect to puromycin and ganciclovir treatment. However, elimination of “leaky” cells with ganciclovir was not successful as expected.

In addition, cell lines were characterized with respect of inducibility (figure 11, data shown for U2OS 11C18). Therefore, U2OS 11C18 cells were induced with different amounts of doxycycline (0.01-10 mg/ml). The highest amount of HA-11C18 protein was achieved by induction with 1 mg/ml doxycycline, but also with the other concentrations good expression levels were obtained (figure 11A). Moreover, a time course experiment with U2OS 11C18 cells was performed, which revealed that optimal expression was observed one to two days after induction (Figure 11B). In all future experiments, U2OS E6 cells were induced with 1 mg/ml doxycycline over a time period of two days. Finally, cells were induced with doxycycline and two days upon induction, doxycycline was removed to analyze the stability of the expressed protein. This experiment showed that up to six days after removal of doxycycline (6d-), HA-11C18 levels were only slightly affected, while there was a significant decrease in HA-11C18 levels eight days after removal (8d-) (Figure 11B). Additionally, degradation of the PDZ domain-containing protein hDlg was shown in the presence of HA-11C18 (Figure 11B), demonstrating that the expressed HA-11C18 protein is functional. However, degradation of p53 by HA-16E6 and HA-18E6 in U2OS 16E6 and U2OS 18E6, respectively, was not observed (data not shown, for details see 5.1). In conclusion, we established defined systems for the analysis of the functions of E6 proteins by mass spectrometry and the subsequent characterization of the potential functions of E6.



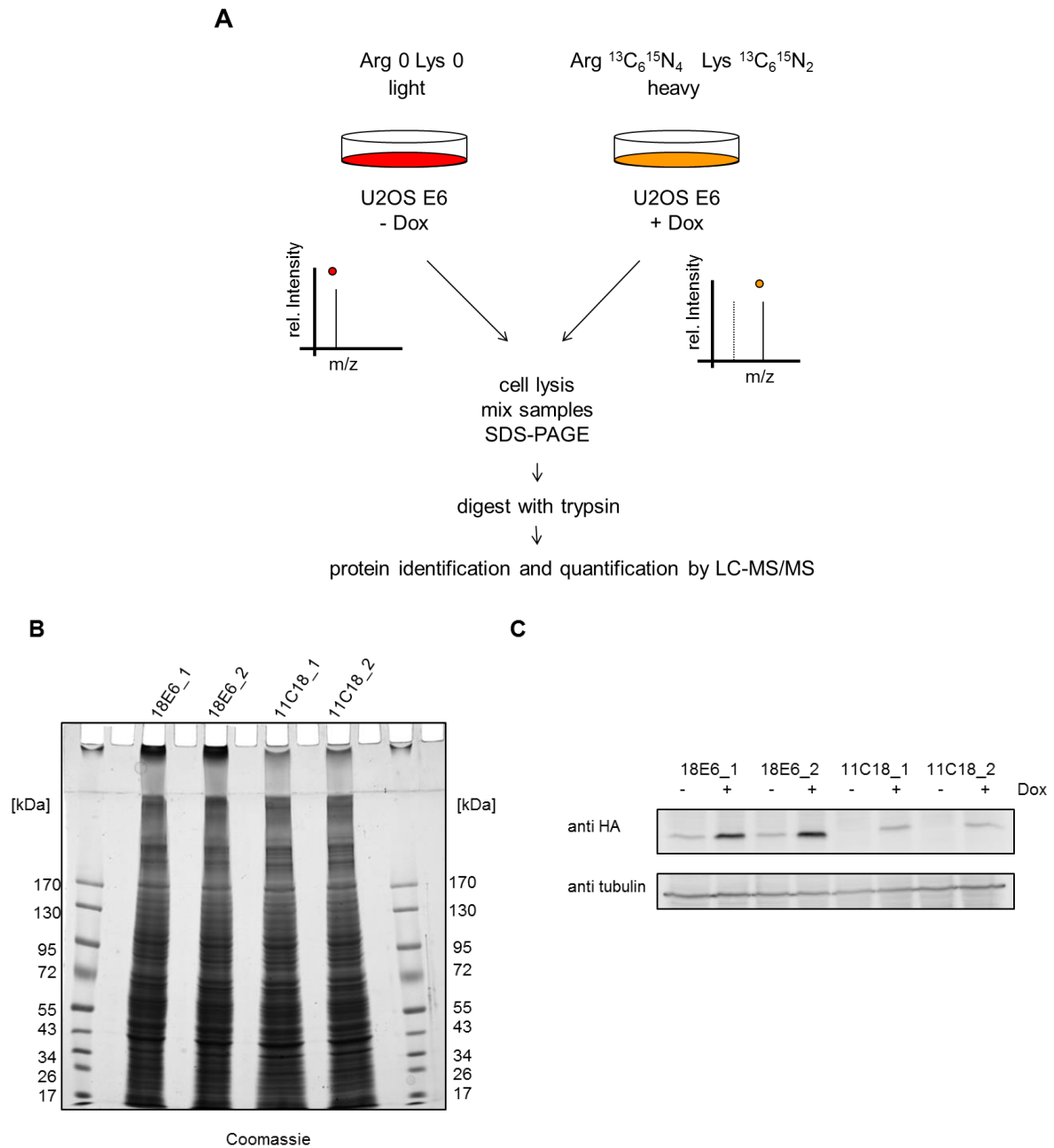
**Figure 11: Characterization of inducibility of U2OS E6 cell lines**

**A:** Doxycycline titration: U2OS 11C18 cells were induced for 2 days with different amounts of doxycycline (Dox) (0.01-10 mg/ml, 48 hours). Cells were harvested and lysed. Protein levels were detected by western blot analysis using indicated antibodies. Expression levels of tubulin served as loading control. **B:** Time course experiment: U2OS 11C18 cells were treated with doxycycline (Dox) (1 mg/ml) up to 7 days. In addition, cells were treated for two days with doxycycline which was removed afterwards. Cells were harvested at different time points and lysed. Protein levels were analyzed by western blot analysis using indicated antibodies. Expression levels of tubulin served as loading control.

## 4.2 ANALYSIS OF “HPV E6 PROTEOME”

To identify and quantify proteome changes in the presence of the E6 proteins, we used our established stable inducible cell lines (see 4.1) and performed SILAC (stable isotope labeling by amino acids in cell culture) experiments combined with LC-MS/MS analysis. SILAC is a powerful approach in (MS)-based proteomics (Ong, 2012). With this method, two or three different cell lines can be compared and analysed by mass spectrometry in a quantitative manner. To do so, essential natural (“light”) amino acids (arginine, lysine) are replaced in the growth media by different stable isotope containing variants (“heavy”). During the growth of the cells, the heavy amino acids are incorporated into newly synthesized proteins (“labeled cells”). Thus, two or three different cell lines can be compared using different labels (scheme of SILAC proteome experiment is outlined in figure 12A). Due to analysis by LC-MS/MS of cell lysates of different labeled cells, heavy and light peptide pairs (SILAC pairs) can be detected and be distinguished by a defined mass difference. Thus, through comparing signal

intensities from light and heavy samples quantitative comparison of relative abundances is possible (Ong and Mann, 2006).



**Figure 12: SILAC experiment: U2OS 18E6 and U2OS 11C18**

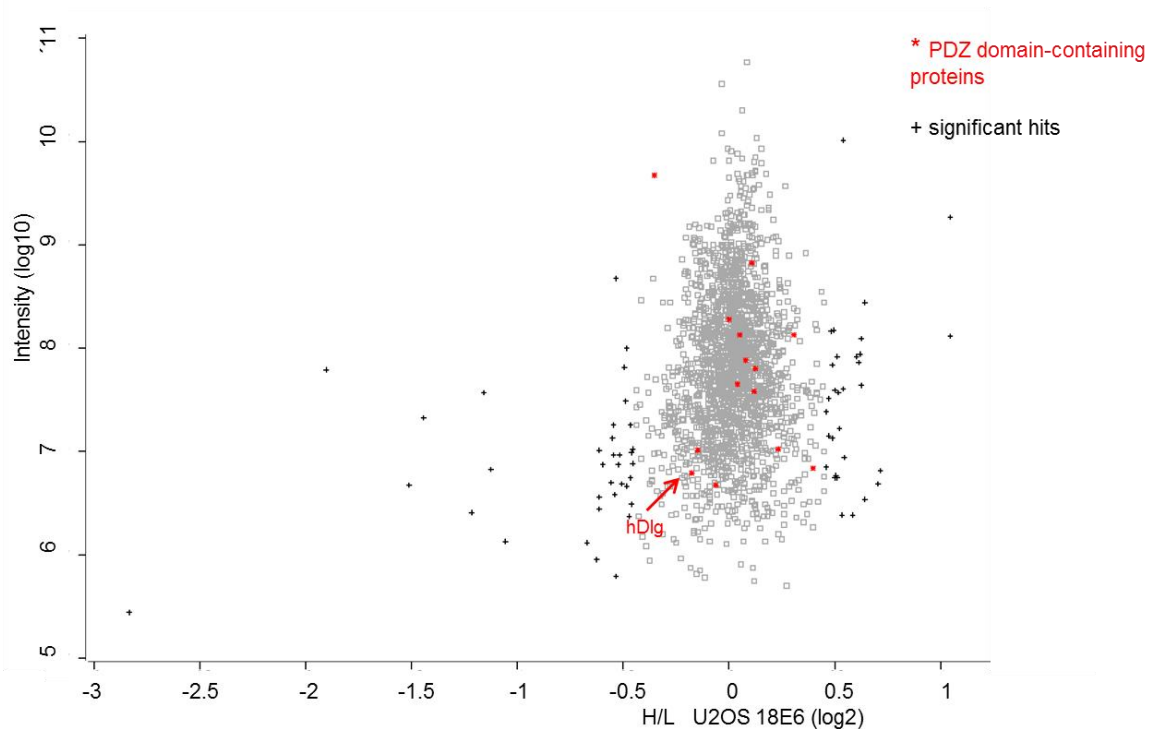
**A:** Scheme of SILAC experiment: U2OS 18E6 and U2OS 11C18 cell lines were both labeled with light and heavy amino acids for 8 days. Two days before harvesting, heavy labeled cells were induced to express HA-18E6 or HA-11C18 respectively (+Dox). Cells were lysed and light and heavy labeled cells were pooled and separated by SDS-PAGE with subsequent Coomassie staining. SDS-gel was sliced and proteins were in-gel digested with trypsin and subjected to LC-MS/MS (Proteomics Facility, University of Konstanz). SILAC peptide pairs can be distinguished by a defined mass shift and this allows quantification of relative abundances of these peptides (ratios). The experiment was performed in duplicate. **B:** Coomassie-stained SDS-PAGE of samples for mass spectrometric analysis. **C:** Samples of cell lysates were analyzed by SDS-PAGE and subsequent western blot analysis with anti-HA and anti-tubulin antibodies. Tubulin levels served as loading control.

Since not much is known about low risk E6 proteins, we decided to investigate the effect of 11C18 on the proteome. This chimeric protein was chosen as it exhibits the C-terminal PDZ binding motif, leading to binding and degradation of PDZ domain-containing proteins (Kiyono et al., 1997; Kuballa et al., 2007), which was supposed to serve as positive control for our experiments. In addition, we elucidated changes in the proteome upon expression of the high risk 18E6 protein.

The scheme of our SILAC experiment is outlined in figure 12A: uninduced cells were incubated with media containing light amino acids (“L”) and induced cells with media containing heavy amino acids (“H”) over a time period of eight days to allow incorporation of labeled amino acids in the whole proteome (“labeled cells”). Afterwards, cells were harvested and lysed under native conditions. Lysates of light and heavy labeled cells were combined, the proteins were separated by SDS-PAGE and subsequently stained by Coomassie (figure 12B). Each lane was cut into 20 slices and analyzed by LC-MS/MS (in collaboration with Proteomics Facility, University of Konstanz).

Induction of HA-E6 protein expression by doxycycline was confirmed for U2OS 18E6 and U2OS 11C18 cell lines by western blot analysis using anti-HA antibody (figure 12C). For U2OS 18E6 cell line weak expression of HA-18E6 was detectable due to “leaky” cells even in the absence of induction.

By LC-MS/MS, a total of 2071 proteins were detected for U2OS 18E6, and 2160 proteins were detected for U2OS 11C18. 2015 and 1935 proteins were quantified, respectively. Of these proteins, levels of 31 proteins were significantly up- (positive values of  $\log_2$  heavy/light (H/L) ratios) and levels of 35 proteins down-regulated (negative values of  $\log_2$  H/L ratios) in the presence of HA-18E6 according to significance A (Significance A was calculated by Perseus software). The experiment was performed as duplicate and data were combined by an experimental design (using Maxquant software). However, data from samples whose ratios varied significantly between both experiments were excluded, finally leading to 27 proteins whose expression levels were up- and 32 proteins whose expression levels were down-regulated in the presence of HA-18E6 (figure 13, table S1).

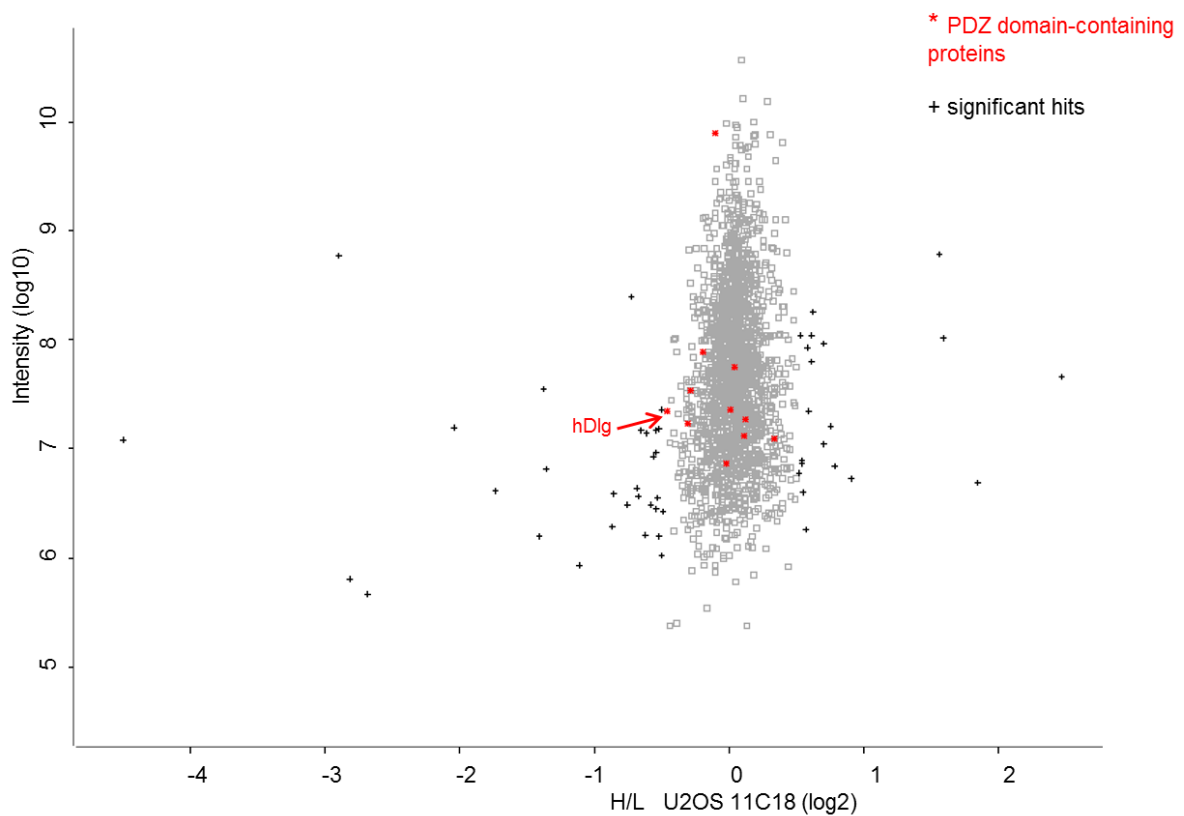


**Figure 13: Proteome wide quantification of U2OS 18E6**

Signal intensities ( $\log_{10}$ ) of all quantified proteins are shown as a function of their fold change ( $\log_2$ ). Negative values of H/L ratios ( $\log_2$ ) represent proteins whose levels are down-regulated in the presence of HA-18E6. Positive values of H/L ratios ( $\log_2$ ) represent proteins whose levels are up-regulated in the presence of HA-18E6. Significant altered proteins (black crosses) were evaluated by significance A (perseus software). Red stars mark PDZ domain-containing proteins.

After consideration of distinct variation between the two experiments, following results ensued for the U2OS 11C18 cell line: levels of 19 proteins were up- and of 27 down-regulated in the presence of HA-11C18 according to significance A (figure 14, table S2).

Although we identified PDZ domain-containing proteins by LC-MS/MS (figure 13 and 14, red stars) including hDlg, which is a known substrate of E6/E6AP (Kuballa et al., 2007), none of them were significantly altered in the presence of HA-18E6 or HA-11C18. Expression levels of six proteins were significantly altered by both HA-18E6 and HA-11C18: levels of three proteins were down-regulated and levels of two proteins were up-regulated in the presence of both E6 proteins, while the protein expression levels of Sorting nexin-1 was differently altered by HA-18E6 and HA-11C18 (table 1).



**Figure 14: Proteome wide quantification of U2OS 11C18**

Signal intensities ( $\log_{10}$ ) of all quantified proteins are shown as a function of their fold change ( $\log_2$ ). Negative values of H/L ratios ( $\log_2$ ) represent proteins whose levels are down-regulated in the presence of HA-11C18. Positive values of H/L ratios ( $\log_2$ ) represent proteins whose levels are up-regulated in the presence of HA-11C18. Significant regulated proteins (black crosses) were evaluated by significance A (perseus software). Red stars mark PDZ domain-containing proteins.

**Table 1: Proteins whose levels are significantly altered in presence of HA-11C18 and HA-18E6**

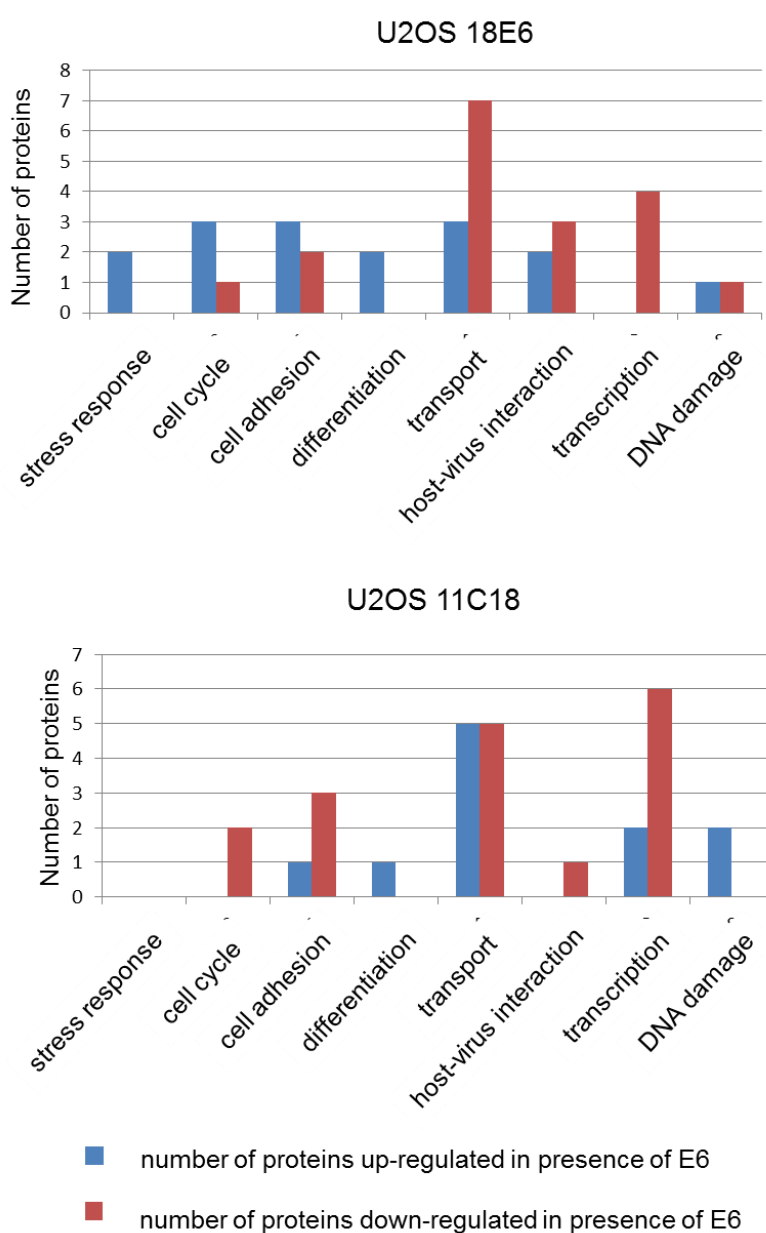
SILAC experiment combined with LC-MS/MS of U2OS 18E6 and U2OS 11C18 cells identified potential proteins whose levels are altered. Table includes only proteins which were altered by HA-18E6 and HA-11C18. Negative H/L ratios ( $\log_2$ ) represent proteins whose levels are down-regulated in the presence of HA-E6, whereas positive H/L ratios (H/L) represent proteins whose levels are up-regulated in the presence of HA-E6.

Protein	H/L 11C18	H/L 18E6
Desmoplakin	-2.68223	-1.16278
ALL1-fused gene from chromosome 4p12 protein	-0.69108	-0.535164
PAI1 RNA-binding protein 1	-0.549388	-0.484348
Sorting nexin-1	0.532866	-0.483682
MYH-1c	0.543397	0.485118
Myosin I beta	0.604641	0.615228

For further analysis of proteomics data, biological processes were assigned to affected proteins according to the UniProtKB/Swiss-Prot data base (table S1 and S2). If at least two

proteins whose expression is either by HA-18E6 or HA-11C18 altered were found to be involved in a biological process, these processes (stress response, cell cycle, cell adhesion, differentiation, transport, host-virus interaction, transcription and DNA damage) were considered and data were summarized (figure 15).

Of all biological processes found transport was most frequently represented. Levels of different proteins involved in transport were either up- or down-regulated in the presence of HA-E6. In contrast, levels of proteins playing a role in transcription were mainly down-regulated in the presence of E6. For all other biological processes no clear pattern was detectable.



**Figure 15: SILAC proteome approach - biological processes**

Biological processes were assigned to proteins whose levels were significantly affected by E6 proteins (see table S1 and S2). Schematic overview shows proteins whose levels were affected (at least 2 proteins) by 18E6 (upper panel) or 11C18 (lower panel) and their involvement in biological processes.

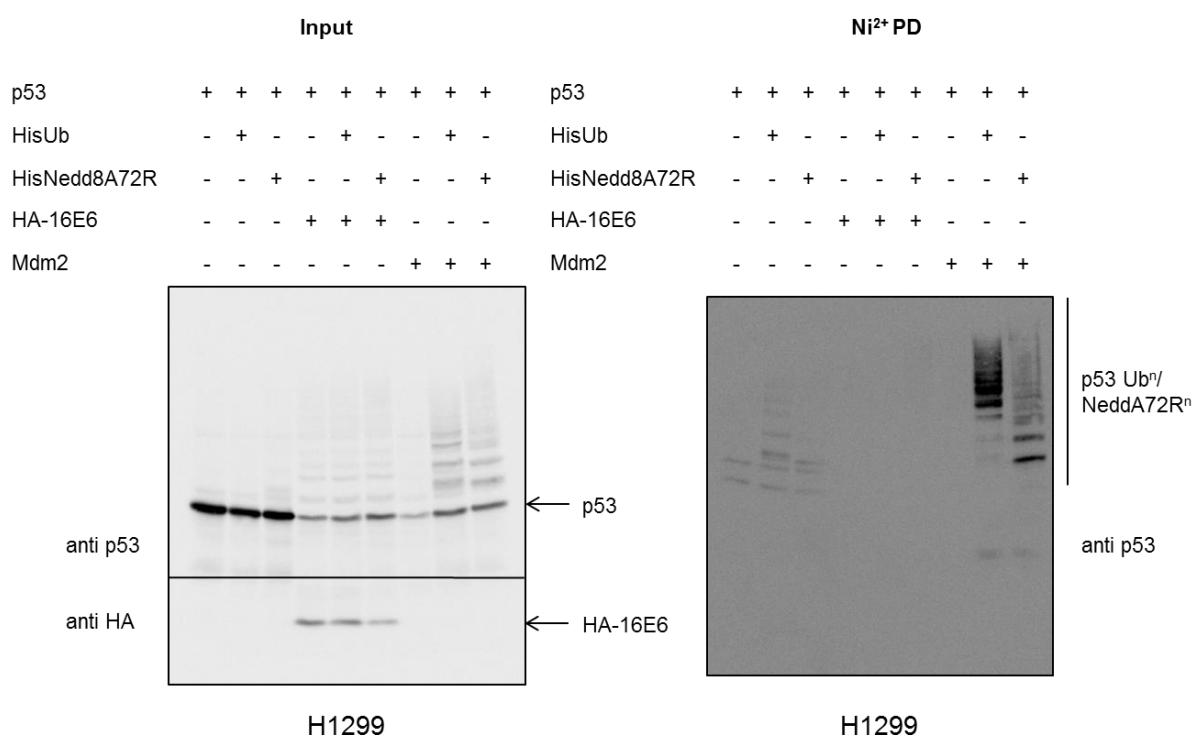
### 4.3 ANALYSIS OF “HPV E6 UBIQUITOME”

With the “proteome” approach we do not obtain any information about how levels of target proteins are altered by the E6 proteins which could be due to direct or indirect regulation on protein or mRNA levels. Thus, we additionally analyzed the “HPV E6 ubiquitome”. With the “ubiquitome” approach we aimed at identifying potential substrates that are ubiquitylated in the presence of the E6 proteins. Although both low risk and high risk E6 proteins interact with the cellular E3 ligase E6AP (Brimer et al., 2007; Kuballa et al., 2007), no common ubiquitylation substrates are known so far. However, we propose that they should share at least some ubiquitylation substrates as E6 proteins have presumably similar roles during the viral life cycle. Prior to the analysis of the “HPV E6 ubiquitome”, the setup of an *in cellulo* ubiquitylation assay was necessary, as in previous studies E6 mediated ubiquitylation of known substrates could not be shown in a cellular system (Camus et al., 2003; Scheffner group, unpublished data).

#### 4.3.1 E6 mediated ubiquitylation of p53 cannot be shown with His-ubiquitin Assay

For the identification of novel ubiquitylation substrates it is necessary to show that under the assay conditions used known substrates are ubiquitylated. In previous studies E6 mediated ubiquitylation of p53 and other known substrates could only be demonstrated *in vitro* but not *in cellulo* (Kuballa et al., 2007). To display ubiquitylation of a substrate within cells, usually a so called His-ubiquitin assay is performed. For this assay, cells are transfected with expression vectors encoding for His-tagged ubiquitin (His-ubiquitin) which is conjugated to substrates in a similar manner as endogenous ubiquitin. Cells are lysed under denaturing conditions to avoid deubiquitylation and afterwards ubiquitylated proteins are isolated by Ni-NTA chromatography. Input samples are lysed under native conditions. However, His-ubiquitin assays performed with H1299 cells cotransfected with expression vectors encoding His-ubiquitin (HisUb), p53 and HA-16E6 showed only p53 degradation in input samples but no ubiquitylated p53 in pulldown samples in western blot analysis (figure 16). Furthermore, in input samples higher migrating bands in samples with E6 were observed (figure 16 left panel), which could be ubiquitylated forms of p53. However, we were not able to isolate these modified versions of p53 by Ni<sup>2+</sup> chromatography (figure 16, right panel) In contrast, in His-ubiquitin assays with cells coexpressing His-ubiquitin, p53 and Mdm2, another E3 ligase for p53, p53 degradation in input samples and p53 ubiquitylation in pulldown samples was observed (figure 16). In conclusion, the His-ubiquitin assay is not suitable to show E6 mediated ubiquitylation of p53. As the lack of detection of ubiquitylated p53 may be due to rapid proteasomal degradation of ubiquitylated p53, we additionally

performed this assay with His-tagged Nedd8A72R. This Nedd8 mutant can be utilized by enzymes of the ubiquitin machinery (Whitby et al., 1998) and we assumed that it cannot be recognized by the 26S proteasome. However, also with this mutant we were not able to show E6 mediated p53 neddylation (figure 16). In addition, we analyzed E6 mediated p53 ubiquitylation by His-ubiquitin assays in Hek293T cells using endogenous and overexpressed p53, but also in this cellular system we were not able to detect ubiquitylated p53 species (data not shown). Moreover, titration experiments in H1299 cells with different amounts of His-ubiquitin and E6 did not lead to detection of p53 ubiquitylation (data not shown).



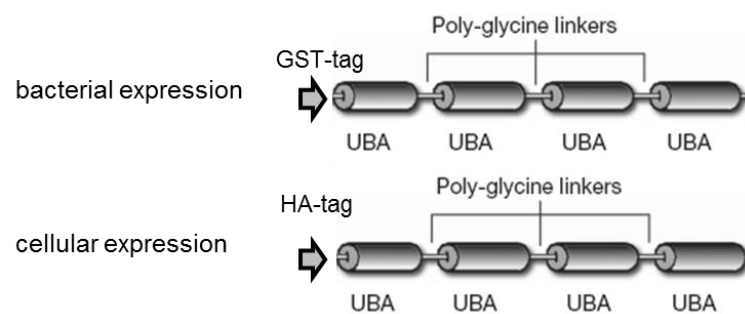
**Figure 16: E6 mediated ubiquitylation of p53 cannot be shown with His-ubiquitin assay**

H1299 cells were cotransfected with expression vectors encoding p53 and His-ubiquitin (HisUb) or His-Nedd8A72R (HisNedd8A72R) and HA-16E6 or Mdm2 as indicated. Input samples were lysed under native conditions with TNN lysis buffer and samples were adjusted to transfection efficiency. Pulldown samples were lysed under denaturing conditions and ubiquitylated/neddylated proteins were purified by Ni-NTA pulldown (Ni<sup>2+</sup>PD). Input and PD samples were analyzed by SDS-PAGE and subsequent western blot analysis using anti-p53 (DO-1) and anti-HA antibodies. p53 Ub<sup>n</sup>/NeddA72R<sup>n</sup> – with ubiquitin or NEDD8A72R modified p53.

#### 4.3.2 Ubiquitylated p53 can be stabilized by coexpression of tandem ubiquitin binding entities (TUBEs)

As already mentioned, one possible explanation why we were not able to detect any ubiquitylated p53 species could be that degradation of E6-dependent substrates by the 26S proteasome is very efficient and thus ubiquitylated species do not accumulate. However, inhibition of the proteasome by MG132 did not lead to stabilization of ubiquitylated p53 in previous studies either (Camus et al., 2003; Scheffner group, unpublished data).

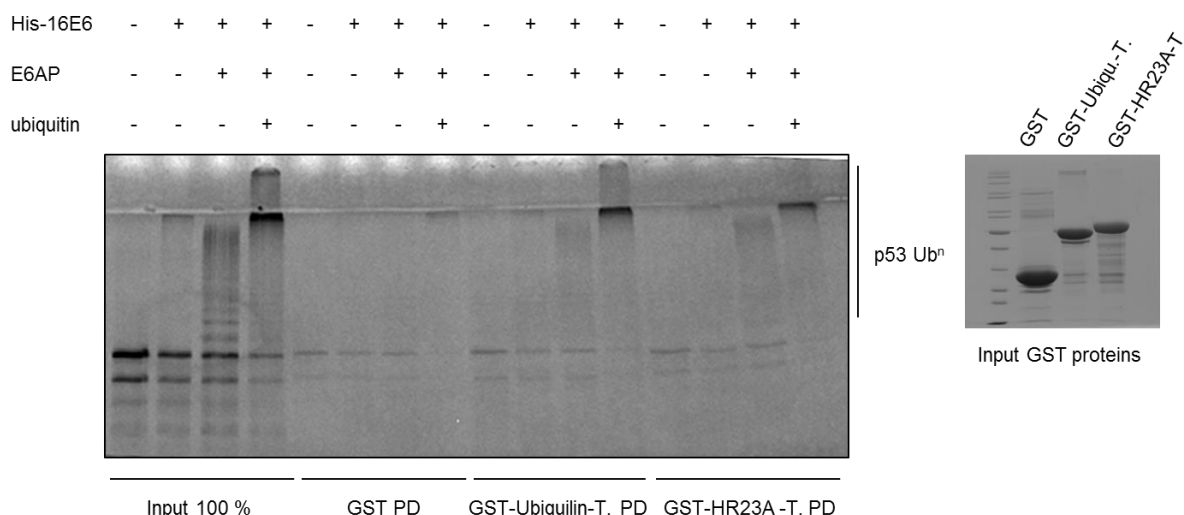
Consequently, we decided to stabilize ubiquitylated proteins by coexpression of tandem ubiquitin binding entities (TUBEs) (Hjerpe et al., 2009). TUBEs consist of 4 UBA domains in a row connected by a flexible linker (figure 17) and each UBA domain is able to bind to one ubiquitin molecule. Binding of TUBEs to ubiquitin chains protects them from deubiquitylation enzymes (DUBs) and proteasomal degradation (Hjerpe et al., 2009). TUBEs were originally established by the Rodriguez group who performed cell lysis in the presence of bacterially expressed GST-TUBEs to avoid deubiquitylation. In extension of their work, we aimed to stabilize ubiquitylated proteins by expression of TUBEs directly within cells. Thus, in our studies we used bacterially expressed GST-TUBEs for *in vitro* studies and HA-TUBEs for expression within cells. Furthermore, two different TUBEs were utilized, built up of four UBA domains derived from either HR23A or Ubiquilin (Hjerpe et al., 2009).



**Figure 17: Schematic map of Tandem Ubiquitin Binding Entities**

Tandem Ubiquitin Binding Entities (TUBEs) consist of 4 UBA domains connected by a flexible poly-glycine linker. Each UBA domain binds to one ubiquitin molecule and therefore TUBEs interact with ubiquitin chains and protect them from deubiquitylation and proteasomal degradation. In our studies we used bacterially expressed GST-tagged TUBEs for *in vitro* experiments and TUBEs with a HA-tag for expression within cells (modified from Hjerpe et al., 2009).

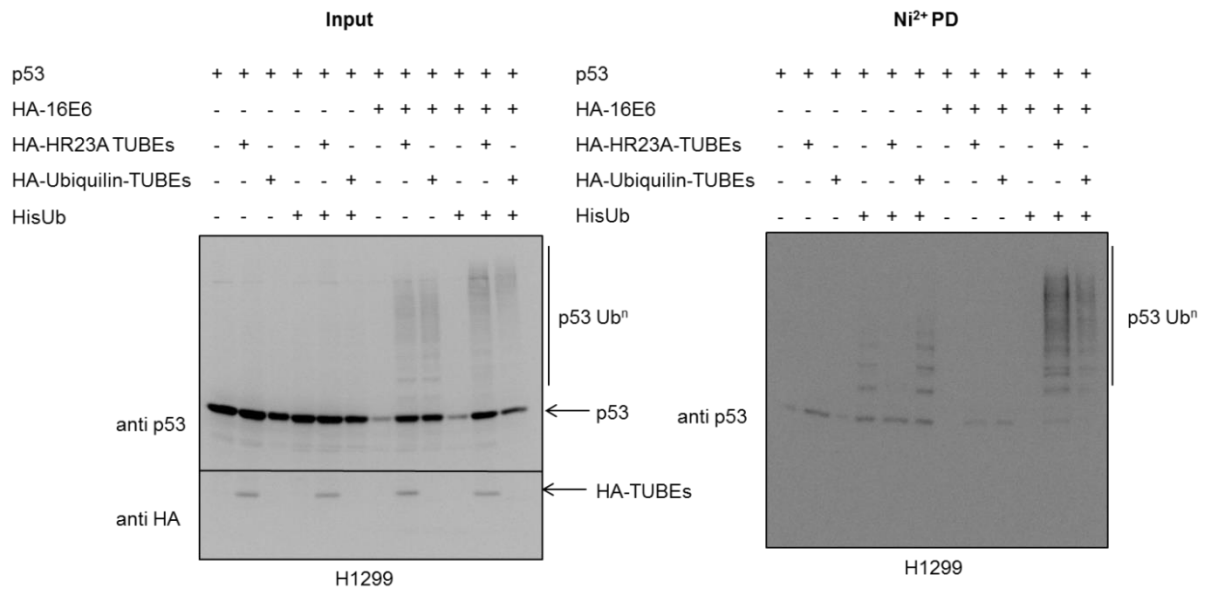
First, we investigated if bacterially expressed and purified GST-TUBEs are able to bind *in vitro* ubiquitylated p53. To do so, we performed an *in vitro* ubiquitylation assay of *in vitro* translated p53 in the presence of His-16E6, E6AP and ubiquitin. One fourth of each assay was used as an input sample and respectively one fourth was used for pulldown experiments with GST, GST-HR23A-TUBEs or GST-Ubiquilin-TUBEs. As expected, p53 was ubiquitylated in an E6/E6AP-dependent manner shown by the appearance of a high molecular weight smear and the disappearance of unmodified p53 (figure 18, input samples). GST-HR23A-TUBEs and GST-Ubiquilin-TUBEs bound efficiently to ubiquitylated p53. In contrast, only a weak background binding was detected in pulldown samples with GST protein (figure 18, pulldown (PD) samples). From this experiment, we concluded that both types of GST-TUBEs are able to bind ubiquitylated p53.



**Figure 18: GST-TUBEs bind to *in vitro* ubiquitylated p53**

Ubiquitylation assay with *in vitro* translated p53 was performed for 2 hours at 25 °C. Samples were divided in 4 parts. One part was used as input samples and with the other parts pull-down (PD) experiments with bacterially expressed GST, GST-Ubiquilin TUBEs (GST-Ubiquilin T.) or GST-HR23A TUBEs (GST-HR23A T.) was conducted. Input and PD samples were analyzed by SDS-PAGE followed by fluorography (left panel). Coomassie stained gel shows input levels of GST and GST-fusion proteins (right panel). p53 Ub<sup>n</sup> – with ubiquitin modified p53.

In the next step we wanted to analyze if TUBEs expressed in cells can stabilize ubiquitylated p53. Therefore, H1299 cells were cotransfected with expression vectors encoding p53, HA-16E6, His-ubiquitin (HisUb) and HA-HR23A-TUBEs or HA-Ubiquilin-TUBEs, and a His-ubiquitin assay was performed. Western blot analysis revealed that p53 was degraded in the presence of HA-16E6, however coexpression of HA-TUBEs stabilized p53 (figure 19, Input samples, left panel). Moreover, a slight high molecular weight smear was detected in the presence of HA-16E6 and HA-TUBEs independent of the expression of His-ubiquitin (figure 19, input samples, left panel). As indicated by the high molecular weight smear, ubiquitylated p53 was pulled down by Ni-NTA agarose dependent on expression of His-ubiquitin, HA-16E6 and HA-TUBEs (figure 19, PD samples, right panel). In contrast without HA-16E6, no or only a weak ubiquitylation of p53 in the presence of HA-TUBEs was detected. In conclusion, coexpression of HA-TUBEs not only stabilizes ubiquitylated p53, but also the unmodified form.

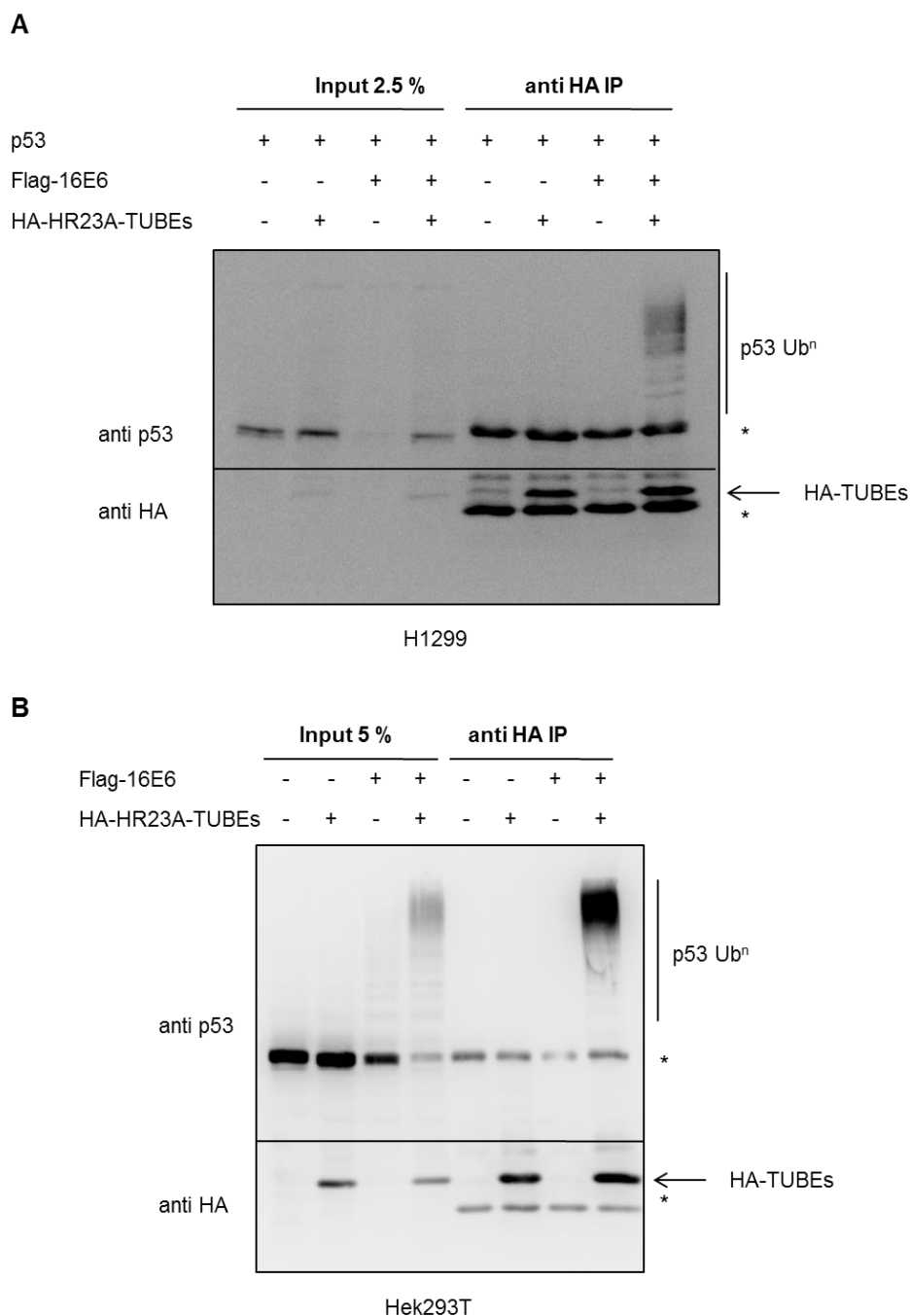


**Figure 19: Ubiquitylated p53 is stabilized by coexpression of HA-TUBEs**

H1299 cells were transfected as indicated with the respective expression constructs. Input samples were lysed under native conditions and equalized for transfection efficiency. Pulldown (Ni<sup>2+</sup>PD) samples were lysed under denaturing conditions and PD was performed with Ni-NTA agarose to bind ubiquitylated proteins. Input and PD samples were analyzed by SDS-PAGE followed by western blot analysis using anti-HA and anti-p53 (DO-1) antibodies as indicated. p53 Ub<sup>n</sup> – with ubiquitin modified p53.

### 4.3.3 Ubiquitylated p53 can be isolated by TUBEs

Western blot analysis of input samples of the p53 His-ubiquitin assay (figure 19, left panel) revealed a high molecular weight smear in the presence of HA-16E6 and HA-TUBEs. This high molecular weight smear may represent p53 modified with endogenous ubiquitin, since it appeared independently of His-ubiquitin overexpression. Thus, endogenous ubiquitin may be sufficient to monitor E6 mediated p53 ubiquitylation. Isolation of ubiquitylated proteins by bacterial expressed GST-TUBEs has been shown before (Hjerpe et al., 2009) and we could demonstrate that they bind to *in vitro* ubiquitylated p53 (figure 18). Therefore, in the next step we wanted to determine if ubiquitylated p53 can be isolated by TUBEs when coexpressed within cells. To do so, we used HA-TUBEs (HA-HR23A-TUBEs) to allow isolation of ubiquitylated proteins by immunoprecipitation using an anti-HA antibody (anti HA-IP). To investigate if ubiquitylated p53 can be detected in this assay, we cotransfected H1299 cells with expression vectors encoding p53, Flag-16E6, and HA-TUBEs as indicated (figure 20). p53 levels decreased in the presence of Flag-16E6 and p53 was stabilized upon coexpression of HA-TUBEs (figure 20A, input samples). HA-TUBEs and bound ubiquitylated p53 were efficiently isolated by immunoprecipitation using anti-HA antibody (figure 20A, IP samples). Also in this case, p53 ubiquitylation was E6 dependent, because no ubiquitylated forms of p53 were detectable without E6.



**Figure 20: Ubiquitylated p53 binds to HA-TUBEs and can be isolated by anti HA-IP**

**A:** H1299 cells were transfected with expression vectors encoding p53, Flag-16E6 and HA-TUBEs as indicated. Cells were lysed under native conditions and 2.5 % of each sample was used as input. Anti HA-IPs were conducted with the remaining samples and anti HA-beads. **B:** Expression vectors for Flag-16E6 and HA-TUBEs were transfected in Hek293T cells. Cells were lysed under native conditions. 5 % of each sample was used as input and with the remaining parts an anti HA-IP was performed. Input and IP samples of A and B were analyzed by SDS-PAGE, followed by western blot analysis using anti-HA and anti-p53 (DO-1) antibodies. p53 Ub<sup>n</sup> – with ubiquitin modified p53. Asterisks mark heavy and light chain of HA antibody.

Note that the heavy chain of the anti-HA antibody has nearly the same molecular mass as p53, so we were unable to conclude anything about background binding of unmodified p53 to anti-HA antibody coupled to beads.

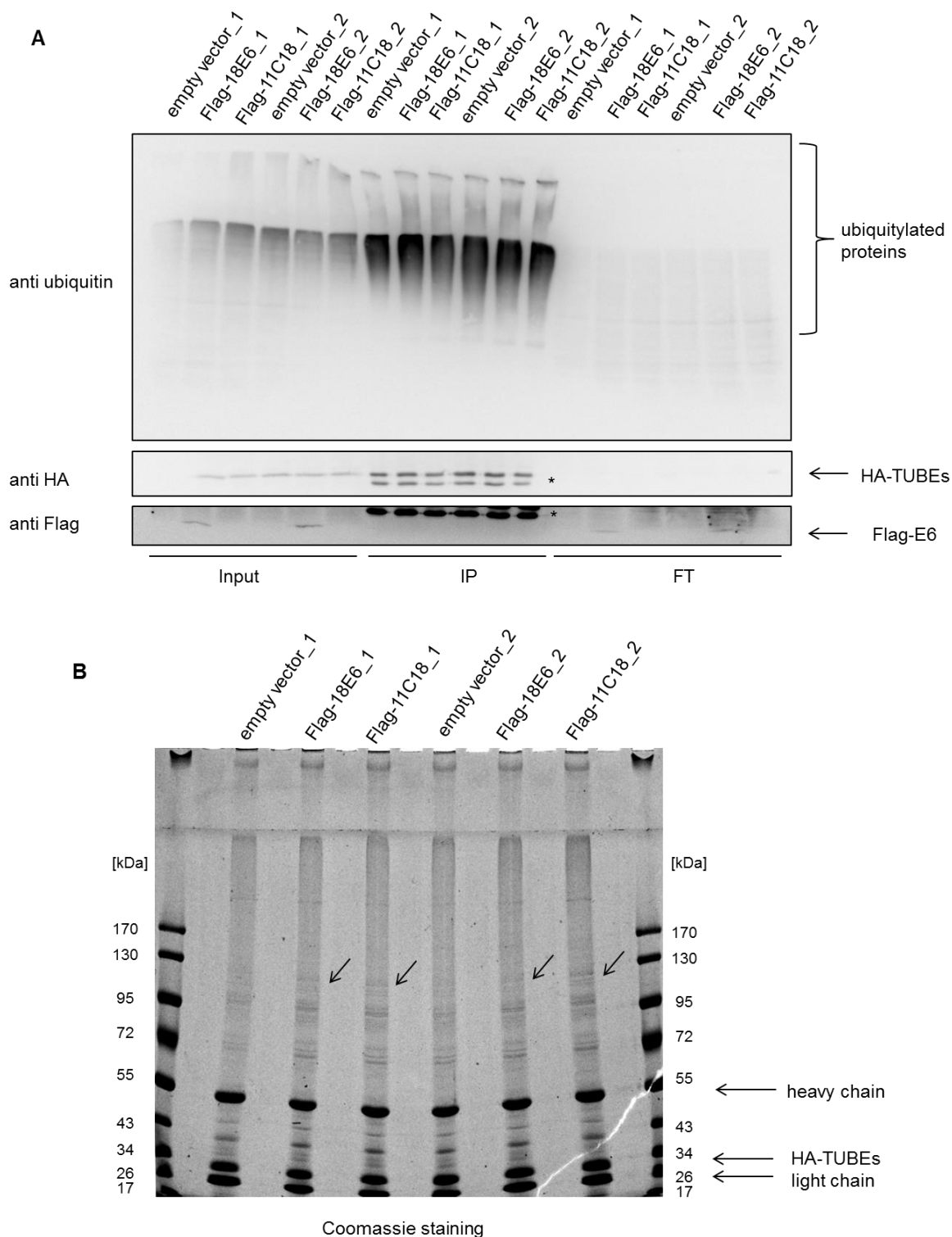
In addition, we tested if our newly developed HA-TUBEs assay also works in another cell line. We analyzed E6 mediated ubiquitylation of endogenous p53 in Hek293T cells. Expression vectors encoding Flag-16E6 and HA-TUBEs were transfected. Because of very high levels of endogenous p53 in Hek293T, the threefold amount of Flag-16E6 expression vector was transfected in comparison to the assay in H1299 cells, in order to detect a decrease in p53 levels (figure 20B, input samples). Also in Hek293T cells ubiquitylated endogenous p53 could be efficiently stabilized and precipitated by HA-TUBEs using anti-HA antibody (figure 20B, IP samples).

In conclusion, we established a straightforward assay to isolate ubiquitylated p53 via HA-TUBEs by an anti HA-IP. However, in contrast to E6 mediated ubiquitylation of p53, we could not detect ubiquitylated forms of other E6 dependent substrates, e.g. hDlg with this assay (data not shown, for details see 5.3.1).

#### **4.3.4 Identification of ubiquitylation substrates of E6**

Our final aim was to identify ubiquitylation substrates of 18E6 and the chimeric 11C18 E6. As all previous experiments to setup the method for identification of ubiquitylation substrates were conducted in the presence of 16E6, we repeated the HA-TUBEs assay for p53 ubiquitylation with Flag-18E6 and Flag-11C18. As expected, p53 ubiquitylation was detected in the presence of Flag-18E6, but not of Flag-11C18 (data not shown).

For identification of E6 dependent substrates by mass spectrometric analysis, the HA-TUBEs assay was performed with a higher amount of cells (1 x 15 cm plate H1299 for each sample) and in duplicate. Upon coexpression of HA-TUBEs and Flag-18E6 or Flag-11C18 HA-TUBES were isolated. Binding of HA-TUBEs to anti-HA-beads and coimmunoprecipitation of ubiquitylated proteins were confirmed by western blot analysis of an aliquot with anti-ubiquitin, anti-Flag and anti-HA antibodies respectively (figure 21A). As HA-TUBEs were enriched in IP samples and not detectable in the flow-through samples (FT), the IP was proofed to be efficient. Furthermore, similar results were obtained for ubiquitylated proteins showing their efficient binding to HA-TUBEs (figure 21A). Moreover, the presence of Flag-18E6 in input and flow-through samples was detected, in contrast to Flag-11C18 which could not be found. The remaining IP samples were subjected to SDS-PAGE, Coomassie staining and subsequent analysis by LC-MS/MS (in collaboration with Proteomics Facility, University of Konstanz).



**Figure 21: Identification ubiquitylation substrates of E6 by HA-TUBEs assay**

H1299 cells were transfected with HA-HR23A-TUBEs and Flag-18E6 or Flag-11C18 as indicated. Cells were lysed under native conditions. Immunoprecipitation of HA-TUBEs was performed using anti HA-beads. **A:** Efficiency of immunoprecipitation of HA-TUBEs and coprecipitated ubiquitylated proteins were analyzed by SDS-PAGE and westernblot analysis using anti-HA, anti-Flag and anti-ubiquitin antibodies. Asterisks mark heavy and light chains of anti-HA antibody. **B:** Remaining samples were separated by SDS-PAGE and stained by Coomassie. Gel was cut in 20 slices and analyzed by LC-MS/MS (Proteomics Facility, University of Konstanz). Arrows within gel mark additionally bands in samples with Flag-18E6 or Flag-11C18.

The stained SDS-gel showed a high molecular weight smear in all lanes. In addition, light and heavy chains of anti-HA antibody were detectable as well as a band with the size of about 26 kDa representing HA-TUBEs. Compared to control samples, additional bands were visible with a size of about 120 kDa in samples with Flag-18E6 or Flag-11C18 (figure 21B, additional bands are marked by arrows).

Proteins were digested with trypsin prior to LC-MS/MS analysis leading in case of ubiquitylated proteins in branched peptides with di-glycine modifications on lysine residues which were linked to ubiquitin (see 5.3.2, figure 41). Ubiquitin peptides including those with di-glycine modified lysines corresponding to modifications at position 11, 48 and 63 were identified by LC-MS/MS analysis, confirming that we were able to isolate ubiquitin chains linked to each other via these lysine residues. Additional proteins were identified as potential ubiquitylation substrates. However, an attached di-glycine motif was not detected. From this result we cannot deduce if the isolated proteins were modified by ubiquitin, or if they were unspecifically bound to HA-beads. Moreover, most of the proteins were present also in the absence of E6 (table S3). In conclusion, the isolation and identification of E6-dependent ubiquitylation substrates by TUBEs needs further optimization (see 5.3.2).

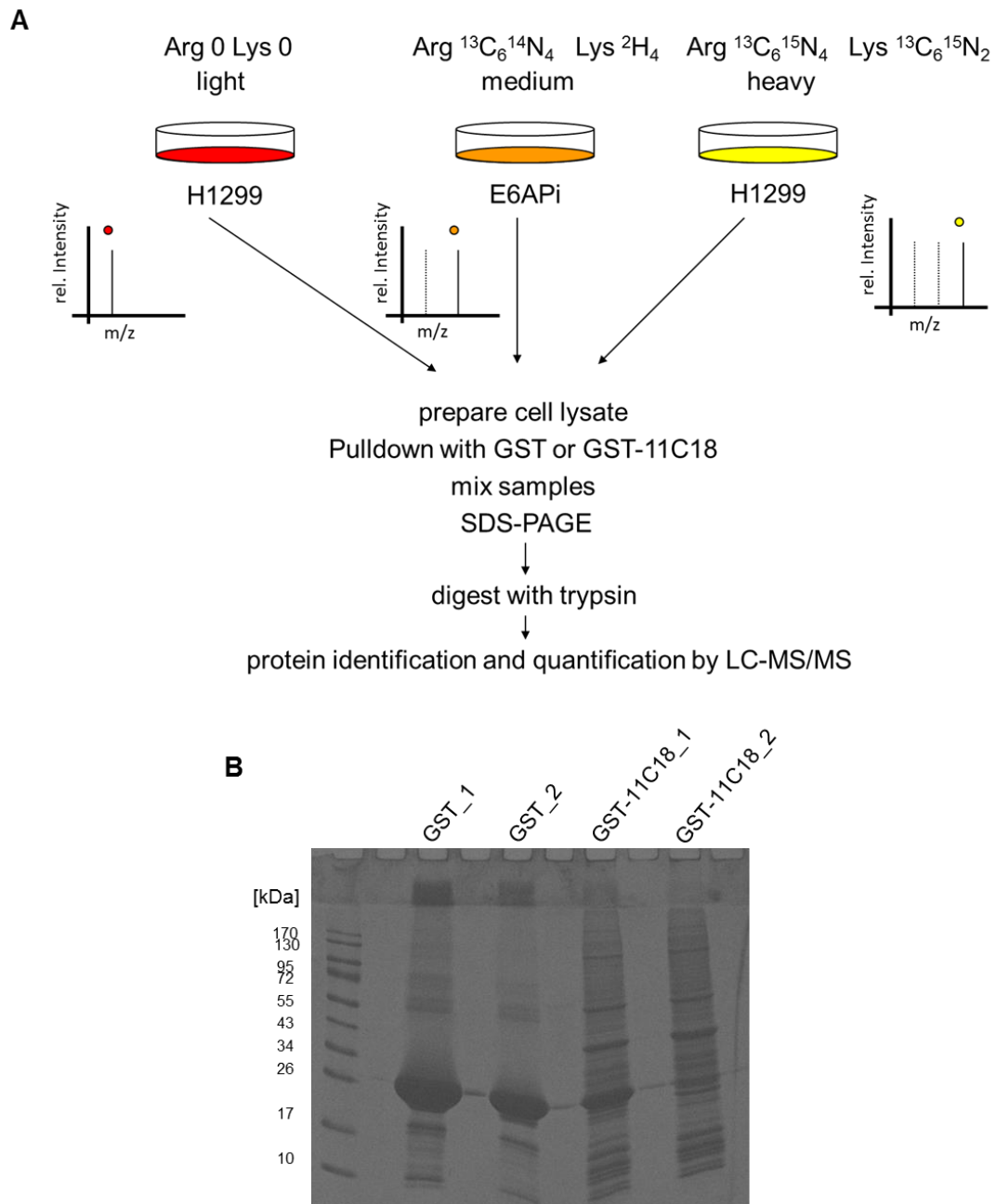
#### **4.4 ANALYSIS OF “HPV E6 INTERACTOME”**

In addition to the elucidation of the “HPV E6 proteome” and “ubiquitome” we aimed to identify interaction partners of E6 (“HPV E6 interactome”). As we were mainly interested in the low risk E6 protein 11E6, we chose to identify interaction partners of the chimeric 11C18 protein. This chimeric protein has the C terminus of the high risk 18E6 protein and therefore can interact with PDZ domain-containing proteins (Kiyono et al., 1997; Kuballa et al., 2007), which served as positive control for our binding assays.

##### **4.4.1 Identification of interaction partners of GST-11C18 via a SILAC based approach**

To identify interaction partners of GST-11C18 we performed GST pulldown assays with bacterially expressed GST-11C18 protein and cell lysate derived from H1299 cells. In addition, we wanted to elucidate which proteins bind to GST-11C18 in an E6AP-dependent manner. To do so, we combined pulldown experiments with a SILAC approach (see 4.2). In detail, pulldown experiments were either conducted with GST-11C18 protein and cell lysate of H1299 cells (light (“L”) or heavy (“H”) labeled) or with cell lysate of H1299 E6APi (K3; (Kuballa et al., 2007)) cells which exhibit a stable knockdown of E6AP expression (medium (“M”) labeled). As a control, the same experiment was performed with GST protein to

determine background binding. The schematic overview of the SILAC approach combined with pulldown experiments is outlined in figure 22A.



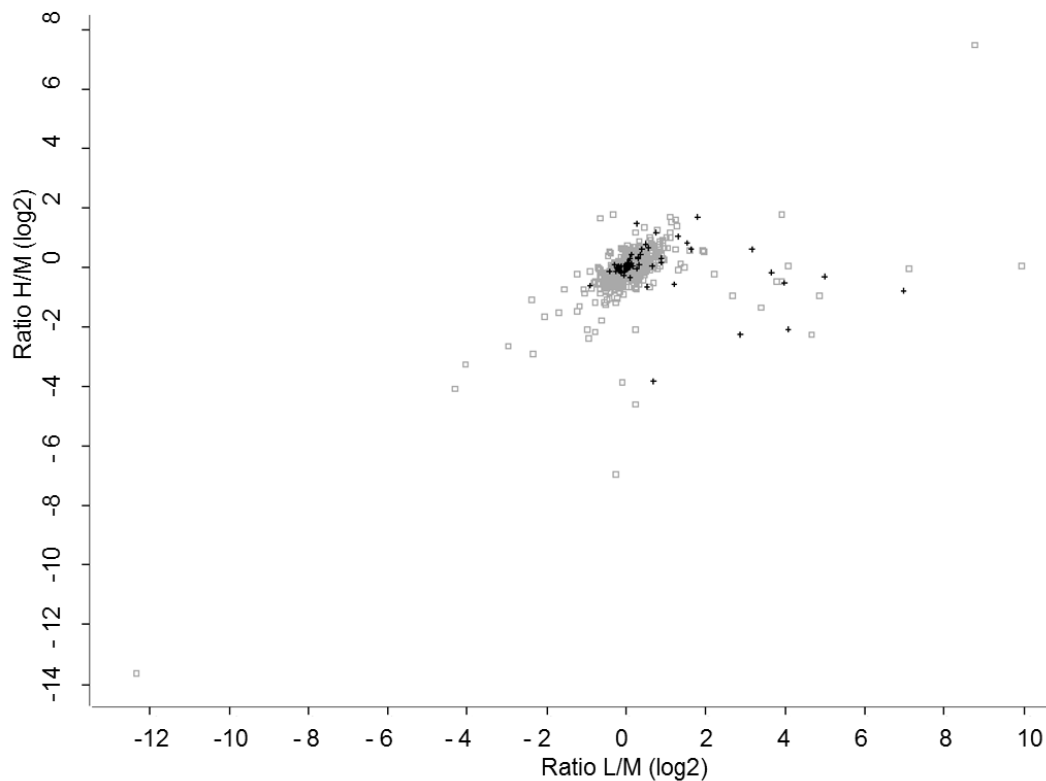
**Figure 22: Scheme of SILAC experiment combined with GST pulldown experiment**

**A:** The scheme of SILAC experiment: to identify E6-binding proteins, parental H1299 cells were labeled with light and heavy amino acids, respectively, and H1299 E6APi cells, which have a stable knockdown of E6AP expression (E6APi), with medium amino acids. Upon labeling, cells were lysed and pulldown (PD) experiments were performed with bacterially expressed GST or GST-11C18 protein. Subsequently, samples were combined and proteins separated by SDS-PAGE and stained by Coomassie. The gel was sliced and proteins were in-gel digested with trypsin and subjected to LC-MS/MS (Proteomics Facility, University of Konstanz). **B:** Coomassie stained SDS-gel of SILAC experiment.

After performing pulldown assays of GST and GST-11C18 protein with cell lysates, samples of the three GST-11C18 or GST pulldown assay respectively were combined and analyzed by

SDS-PAGE and Coomassie staining (figure 22B) and subsequent analysis by LC-MS/MS (in collaboration with Proteomics Facility, University of Konstanz). The GST pulldown was performed in duplicate and GST-11C18 pulldown in triplicate (figure 22B, data not shown). While in GST-11C18 pulldown samples bands at the whole ranges of molecular masses were visible in Coomassie stained gel (figure 22B), for GST pulldowns fewer bands were detectable. A strong band in GST pulldown samples with a molecular mass of about 25 kDa was visible, which corresponds to the size of GST protein (figure 22B).

Proteins identified by LC-MS/MS analysis were further analyzed with respect to E6AP-dependent binding. Data of all experiments were combined and ratios L/M and H/M (log<sub>2</sub> values) were blotted against each other (figure 23). Black crosses mark proteins which were found in both, control GST and GST-11C18 pulldown assays and therefore represent background proteins.



**Figure 23: Data analysis of GST-11C18 pulldown**

H/M and L/M ratios (log<sub>2</sub>) were blotted against each other showing that most proteins bind to GST-11C18 in an E6AP independent manner. Black crosses mark background proteins which were also found in GST pulldown assays.

As H1299 cells were labeled with light and heavy amino acids and H1299 E6APi cells with medium amino acids, L/M and H/M ratios should reveal the same values. Thus, we could use these ratios as internal control for our experiment (see 5.4.1). In addition, the values of the H/L ratios (log<sub>2</sub>) should have the value 0. Most of the proteins bound to GST-11C18 in an

E6AP independent manner (main population with a ratio (log<sub>2</sub>) of about 0; figure 23). Proteins with a negative value in H/M and L/M ratios (log<sub>2</sub>) are proteins which bound to GST-11C18 in the presence of E6AP more efficiently, and proteins with a positive value (log<sub>2</sub>) bound to GST-11C18 in E6AP knockdown cells more efficiently. After consideration of the H/L ratios only a few proteins were shown to bind significantly (according to significance A, perseus software) to GST-11C18 in an E6AP-dependent manner (table 2), including Paxillin a known interaction partner of E6 (Tong and Howley, 1997). Seven proteins were bound in the presence of E6AP more efficiently. Moreover, one protein bound to GST-11C18 only in E6AP knockdown cells. However, for all of these proteins only one to two peptides were identified. So these data seem not to be reliable ones.

**Table 2: Significant hits of SILAC experiment combined with GST-11C18 pulldown**

Proteins identified by LC-MS/MS which bound significantly to GST-11C18 in an E6AP-dependent manner. Positive values of L/M and H/M ratios (log<sub>2</sub>) display proteins that bind to GST-11C18 more efficiently in the absence of E6AP. Negative values of L/M and H/M ratios (log<sub>2</sub>) display proteins that bind to GST-11C18 more efficiently in the presence of E6AP. NaN (not a number).

ratio L/M (log <sub>2</sub> )	ratio H/L (log <sub>2</sub> )	ratio H/M (log <sub>2</sub> )	gene name	protein name	peptides
3.89985	0.0737312	1.75677	JUN	Transcription factor AP-1	2
-1.24793	-0.432552	-1.49196	CUL7	Cullin-7	1
-1.72317	0.412619	-1.55937	MBOAT7	Lysophospholipid acyltransferase 7	1
-2.07861	0.487178	-1.66433	PSMC3	26S protease regulatory subunit 6A	1
-2.35727	NaN	-2.92807	PXN	Paxillin	1
-4.06901	0.586116	-3.25512	MRPL53	39S ribosomal protein L53, mitochondrial	1
-4.32474	NaN	-4.11548	ATP4A	Potassium-transporting ATPase alpha chain 1	1
-12.3312	NaN	-13.6189	ATP13A4	Probable cation-transporting ATPase 13A4	1

In total, more than 880 proteins were identified by LC-MS/MS which bound to GST-11C18 in pulldown assays including PDZ-domain containing proteins (see 54.1, table 4; e.g. hScribb, a known binding partner of E6 (Nakagawa and Huibregtse, 2000)).

However, only around 380 proteins could be confirmed in all three GST-11C18 binding assays (table S4). Moreover, nine of the potential interaction partners are known to play a role in the ubiquitin proteasome pathway (according to annotated KEGG pathways, table 3). Among these proteins, three subunits of the anaphase promoting complex (APC/C) were found. In addition, the HECT-E3 ligase UBR5 was identified as a potential interaction partner, which is a reported binding protein of the E6 proteins (Tomaic et al., 2011).

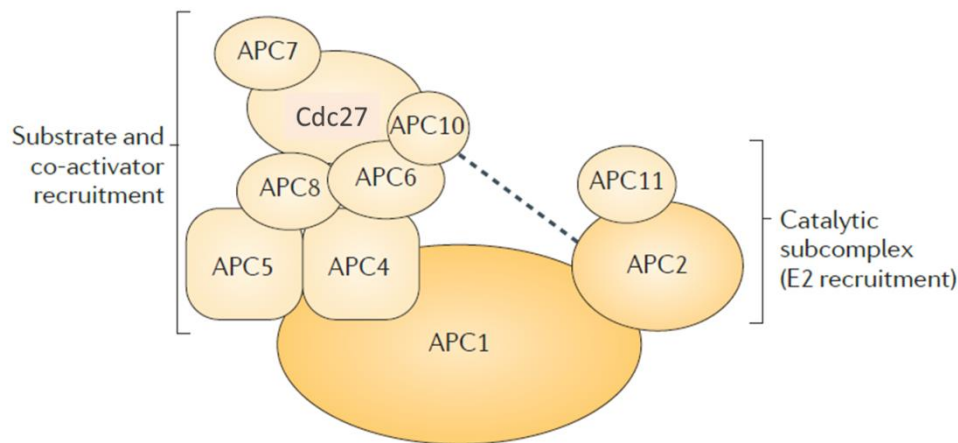
**Table 3: Proteins of ubiquitin proteasome pathway identified as potential interaction partners of GST-11C18**

Proteins identified as potential interaction partners of GST-11C18 by LC-MS/MS. Displayed are only proteins which play a role in the ubiquitin proteasome pathway.

gene name	protein name	number of peptides		
		PD 1	PD2	PD3
TRIP12	E3 ubiquitin-protein ligase TRIP12	7	3	7
ANAPC1	Anaphase-promoting complex subunit 1	1	1	2
ANAPC5	Anaphase-promoting complex subunit 5	2	3	-
CDC27	Cell division cycle protein 27 homolog	4	4	-
CUL7	Cullin-7	1	-	-
UBR5	E3 ubiquitin-protein ligase UBR5	4	1	1
KLHL9	Kelch-like protein 9	1	-	1
PRPF19	Pre-mRNA-processing factor 19	5	8	11
SKP1	S-phase kinase-associated protein 1	2	-	3

#### 4.5 CHARACTERIZATION OF THE INTERACTION OF THE HPV E6 PROTEINS WITH APC/C

With the interactome approach, three subunits of the anaphase-promoting complex (APC/C), namely APC1, APC5 and Cdc27 could be identified as potential interaction partners of the chimeric GST-11C18 protein (see 4.4). APC/C is a RING E3 ligase composed of at least 13 subunits, which form two subcomplexes connected by the scaffolding subunit APC1 (reviewed in Pines, 2011; Zhang et al., 2013). One subcomplex consists of the catalytic core formed by APC11 (RING protein) and APC2 (a Cullin like protein), and is involved in the recruitment of the E2 enzyme. The second subcomplex is important for substrate recognition and coactivator binding (figure 24) (reviewed in Pines, 2011). APC/C regulates cell division and the timing of re-entry into S phase by targeting key cell cycle regulators like cyclins for proteasomal degradation (reviewed in Peters, 2006). Two coactivators (Cdc20 and Cdh1) of APC/C are known which interact only transiently with the complex. Cdc20 acts as a coactivator of APC/C during early mitosis until telophase, whereas Cdh1 acts as a coactivator in telophase and G1 phase. Cdc20 binds to APC/C already in G2 phase (reviewed in Manchado et al., 2010). However, in this stage of the cell cycle the SAC (spindle assembly checkpoint) is active. Checkpoint proteins bind to Cdc20, thereby leading to inhibition of APC/C<sup>Cdc20</sup>. After all chromosomes have been aligned correctly to the mitotic spindle, the SAC is satisfied, APC/C<sup>Cdc20</sup> is activated and the cell progresses through mitosis, leading to cell division (reviewed in Manchado et al., 2010; Peters, 2006).



**Figure 24: Schematic composition of APC/C**

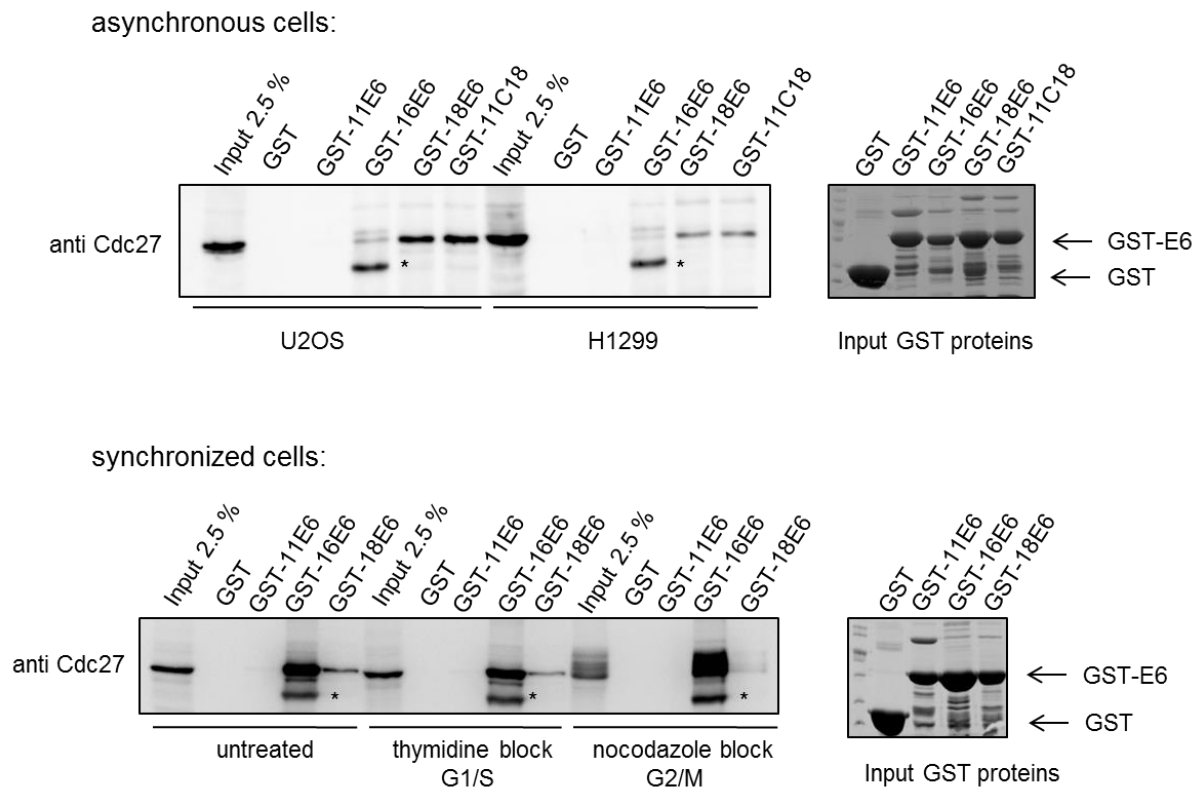
APC/C is composed of two subcomplexes which are linked by the scaffold protein APC1. One subcomplex made up of APC11, a RING protein, and APC2, a Cullin like protein, has catalytic activity and is involved in recruitment of the E2 ubiquitin enzyme. The second subcomplex is responsible for substrate and coactivator recruitment (modified from Pines, 2011).

Deregulation of APC/C leads to a defective DNA damage checkpoint in G2 phase, loss of control of genome replication, aberrant M phase exit or premature G1/S transition (reviewed in Mo et al., 2012). As viruses need to create an intracellular environment which supports viral replication, manipulation of APC/C seems to be an attractive target to achieve this task. Indeed several viral proteins are known to inhibit (e.g. adenovirus E1A, chicken anemia virus (CAV) apoptin) or activate (e.g. adenovirus E4orf4, human T cell lymphotropic virus type 1 (HTLV-1) Tax) APC/C. The mechanisms how these viral proteins achieve inhibition or activation of APC/C are diverse (reviewed in Mo et al., 2012). Thus, after the identification of APC/C as a potential interaction partner of the chimeric 11C18 protein, we aimed to verify this interaction and obtain insights into its potential function.

#### 4.5.1 HPV E6 proteins with the exception of 11E6 bind to APC/C in pulldown experiments

To confirm the interaction of 11C18 with APC/C, pulldown experiments with bacterially expressed GST-11C18 and cell lysate of H1299 or U2OS cells were performed. To determine if binding to APC/C is a common feature of the E6 proteins, low risk GST-11E6 and high risk GST-16E6 and GST-18E6 were included in the assay. Binding of APC/C was monitored with a specific antibody detecting the APC/C subunit Cdc27 as shown in figure 25 (upper panel), an interaction between APC/C and high risk GST-16E6, GST-18E6 and the chimeric GST-11C18 could be detected. However, no binding to GST-11E6 was observed, suggesting that the PDZ binding motif within the C terminus of high risk proteins is necessary for the interaction.

To obtain first hints about the function of the interaction of E6 with APC/C, pulldown experiments with extracts derived from synchronized cells were performed. This showed that high risk GST-16E6 and GST-18E6 bind to APC/C in a cell cycle independent manner (figure 25, lower panel).



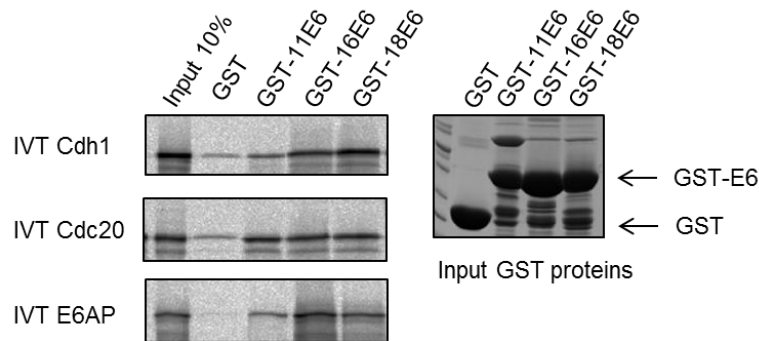
**Figure 25: GST-E6 proteins with the exception of GST-11E6 bind to APC/C**

Bacterially expressed GST, GST-11E6, GST-16E6, GST-18E6 and GST-11C18 were incubated with cell lysate derived from asynchronously growing H1299 or U2OS (upper panel) or synchronized U2OS (lower panel) cells. Binding of APC/C was analyzed by SDS-PAGE with subsequent western blot analysis using anti-Cdc27 antibody (left panel). Asterisks mark an unspecific band recognized by anti-Cdc27 antibody. Coomassie stained gels show input levels of GST proteins (right panels).

#### 4.5.2 HPV E6 proteins bind to *in vitro* translated APC/C coactivators Cdh1 and Cd20

Two coactivators are known to bind to and activate APC/C. Cdc20 activates APC/C in early mitosis and in late mitosis Cdh1 takes over. APC/C Cdh1 is also active during G1-phase (reviewed in Pines, 2011). As high risk E6 proteins appear to bind APC/C cell cycle independent manner, we wanted to analyze if the E6 proteins are able to interact with the coactivators of APC/C directly. To do so, we performed pulldown experiments with bacterially expressed GST-E6 proteins and *in vitro* translated Cdc20, Cdh1 or E6AP. The latter served as positive control. Surprisingly, binding of all GST-E6 proteins including the low risk

GST-11E6 to both coactivators was observed (figure 26). Furthermore, binding of all GST-E6 proteins to E6AP was detected as expected. However, binding of Cdc20 and Cdh1 to other GST-fusion proteins including GST-16E7 and GST-Mdm2Ring was also observed (see 4.5.10, figure 36). Since the latter proteins were meant to serve as negative controls we cannot exclude that the observed binding of GST-E6 proteins to the coactivators is due to an unspecific interaction.



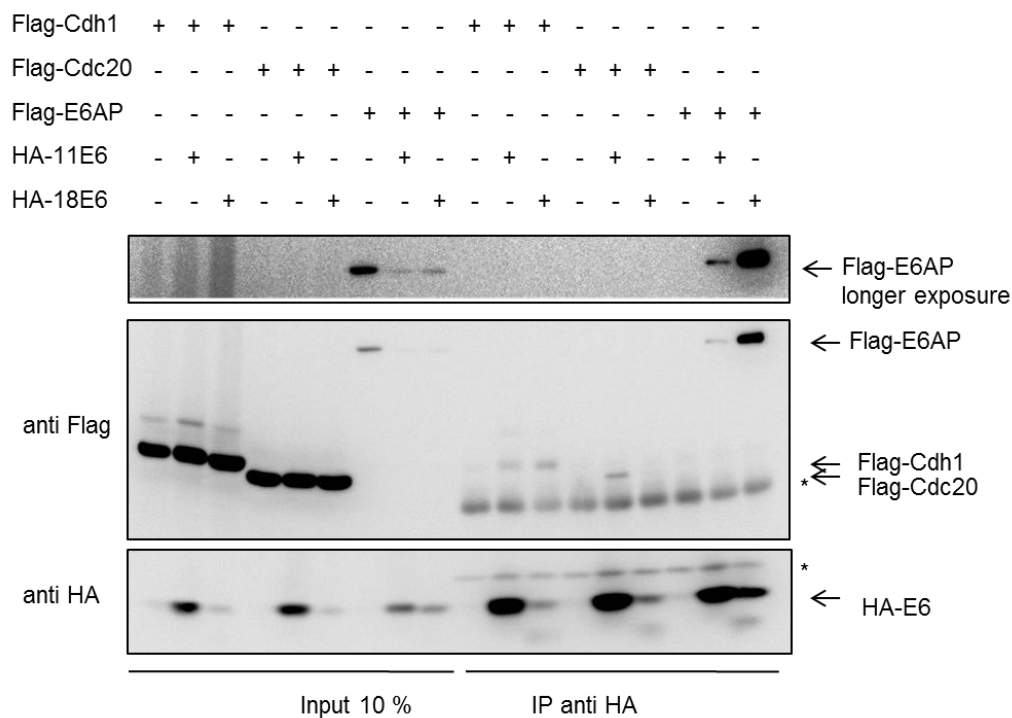
**Figure 26: GST-E6 proteins bind to *in vitro* translated APC/C coactivators Cdh1 and Cdc20**

Bacterially expressed GST, GST-11E6, GST-16E6 and GST-18E6 were incubated with *in vitro* translated Cdh1, Cdc20 or E6AP, whereas E6AP served as positive control. Binding of *in vitro* translated (IVT) proteins to GST-E6 proteins were analyzed by SDS-PAGE and subsequent fluorography (left panel). Coomassie stained gel shows input levels of GST proteins (right panel).

#### 4.5.3 HPV E6 proteins bind to APC/C coactivators Cdh1 and Cdc20 in coimmunoprecipitation experiments

Since binding assays with *in vitro* translated coactivators of APC/C and GST-E6 proteins did not reveal clear results, we went on to study whether the E6 proteins and coactivators bind to each other in overexpression experiments within cells. To do so, coimmunoprecipitation experiments with overexpressed proteins were conducted in Hek293T cells. In detail HA-11E6 or HA-18E6 were coexpressed together with either Flag-Cdh1, Flag-Cdc20 or Flag-E6AP in Hek293T cells. Flag-E6AP was used as positive control. For immunoprecipitation (IP) anti-HA antibody coupled to protein A sepharose was utilized. Western blot analysis of input samples revealed that all overexpressed proteins were expressed. However, the amount of HA-11E6 was higher than the amount of HA-18E6, presumably because a codon optimized version was used. Furthermore, Flag-E6AP levels decreased in the presence of both HA-11E6 and HA-18E6, which was probably due to the stimulating effect of E6 on E6AP autoubiquitylation (Kao et al., 2000). Both HA-E6 proteins were immunoprecipitated using anti-HA antibody and both coactivators were coprecipitated with HA-11E6 (figure 27). For HA-18E6, only Flag-Cdh1 but not Flag-Cdc20 was bound (figure 27). However, binding of HA-18E6 to Flag-Cdc20 could be detected in other binding experiments (data not shown).

Binding of Flag-E6AP to both HA-E6 proteins was detected as expected with binding of HA-18E6 stronger than to HA-11E6.

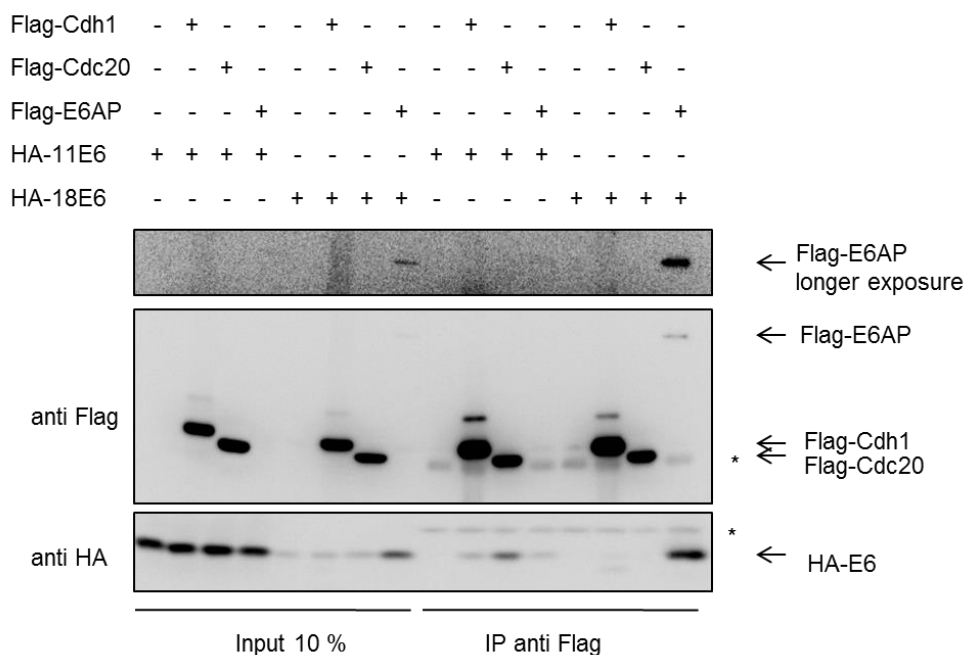


#### Figure 27: Coimmunoprecipitation assays of HA-E6 proteins and Flag-coactivators

Hek293T cells were transfected with expression constructs for HA-11E6 or HA-18E6 and Flag-Cdh1, Flag-Cdc20 or Flag-E6AP as indicated. Coimmunoprecipitation was performed using anti-HA antibody bound to protein A sepharose. Input (10 %) and IP samples were analyzed by SDS-PAGE followed by western blot analysis using anti-HA and anti-Flag antibodies. Asterisks mark heavy and light chains of anti-HA antibody.

In addition, reciprocal coimmunoprecipitation experiments were performed using an anti-Flag antibody. Input samples showed that all proteins were expressed and Flag-Cdh1, Flag-Cdc20 and Flag-E6AP were efficiently immunoprecipitated (figure 28). However, expression levels of Flag-E6AP were very low in the presence of HA-11E6. In Flag-Cdh1 samples, an additional higher migrating band was visible which also bound to the anti-Flag antibody indicating that it is a modified version of Flag-Cdh1. Binding of HA-11E6 to Flag-Cdh1 and Flag-Cdc20 was observed. However, also in this experiment HA-18E6 was able to bind only to Flag-Cdh1 and not to Flag-Cdc20. Binding of Flag-E6AP to HA-11E6 and HA-18E6 was detected as expected (figure 28).

From coimmunoprecipitation experiments it appears that the low risk 11E6 binds to the APC/C coactivators. However, if this interaction occurs in a direct manner cannot be deduced. In addition, binding of high risk 18E6 to Cdh1 was observed, while the results for binding to Cdc20 are not clear as binding could not be reproducibly demonstrated.



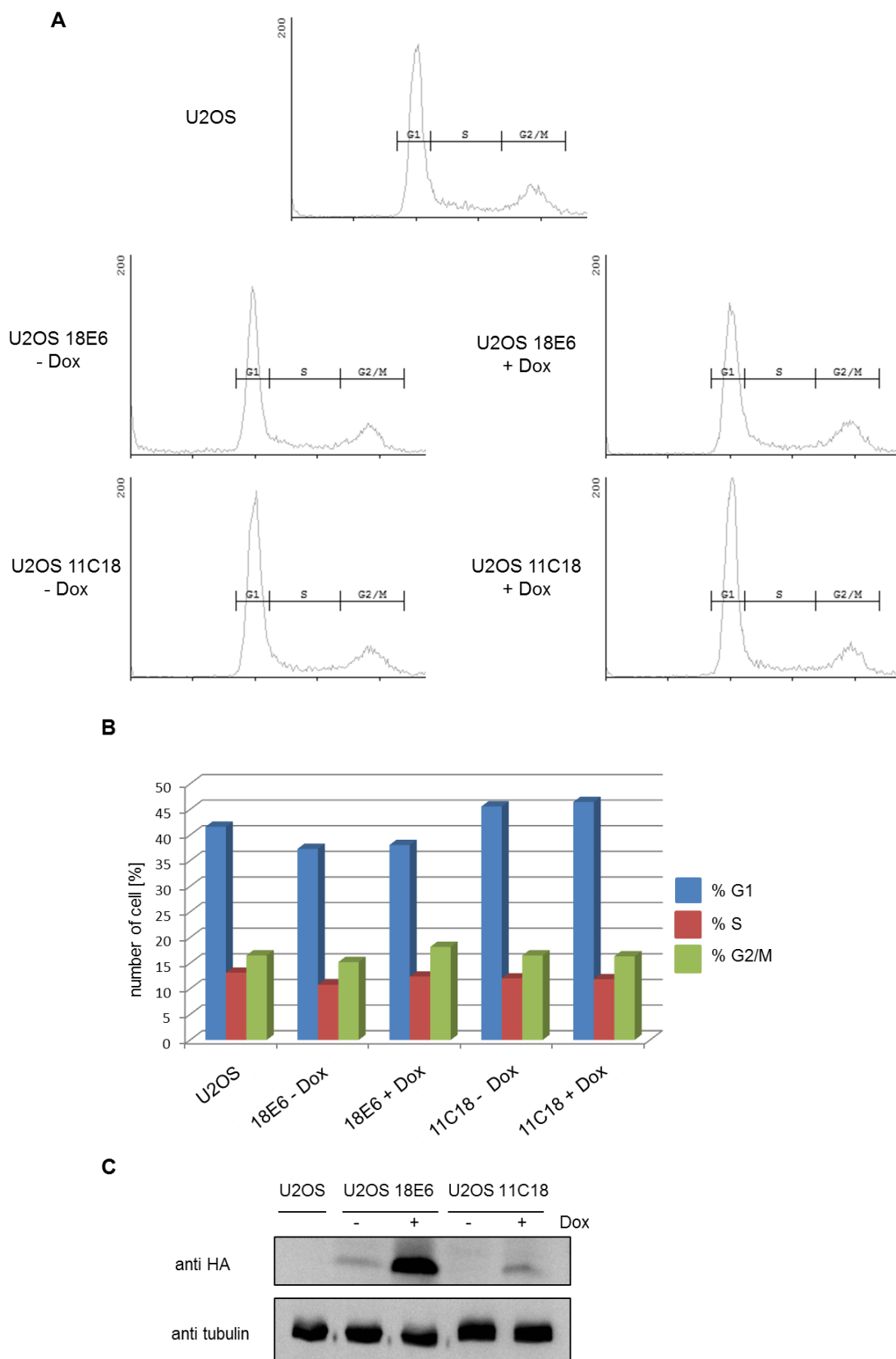
**Figure 28: Coimmunoprecipitation assays of HA-E6 proteins and Flag-coactivators**

Hek293T cells were transfected with Flag-Cdh1, Flag-Cdc20 or Flag-E6AP and HA-11E6 or HA-18E6 as indicated. Coimmunoprecipitation was performed using anti-Flag antibody bound to protein A sepharose. Input (10 %) and IP samples were analyzed by SDS-PAGE followed by western blot analysis using anti-HA and anti-Flag antibodies. Asterisks mark heavy and light chains of anti-Flag antibody

#### 4.5.4 Cdc20 and Cdh1 are no ubiquitylation substrates of E6/E6AP complex

One possible function of the interaction of the E6 proteins with APC/C could be that a subunit of APC/C or one of its coactivators is a substrate of the E6/E6AP complex. To examine this possibility, we performed *in vitro* ubiquitylation assays using *in vitro* translated Cdh1 or Cdc20 as substrates. In addition *in vitro* translated p53 served as positive control. In Cdh1 and Cdc20 ubiquitylation assay weak higher migrating bands in the presence of E6/E6AP which could represent ubiquitylated forms of Cdh1/Cdc20 were detected (figure 29). However, based on our experience we can detect weak ubiquitylation of any substrate in the presence of E6/E6AP using *in vitro* ubiquitylation assays. Thus, we assume that neither Cdh1 nor Cdc20 were specifically ubiquitylated in an E6/E6AP dependent manner (figure 29). In contrast, efficient p53 ubiquitylation was shown in the presence of E6 and E6AP.





**Figure 30: Cell cycle analysis of U2OS E6 cell lines by DNA staining and flow cytometry**

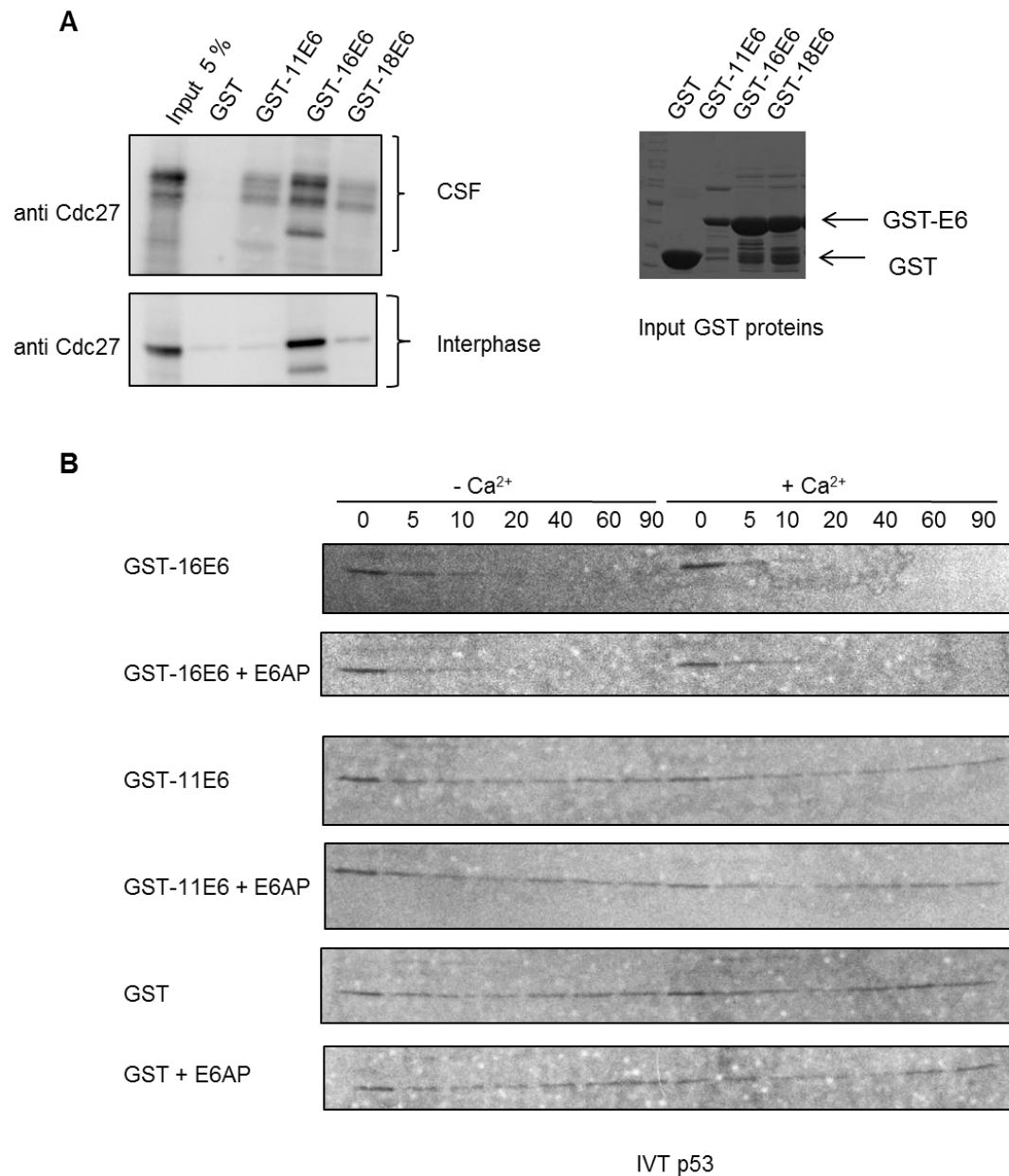
U2OS cells inducibly expressing the indicated E6 proteins were fixed with ethanol and DNA was stained with propidium iodide (PI). (-Dox) represents not induced cells and (+Dox) induced cells. Fixed cells were analyzed by flow cytometry. **A:** Cell cycle profile of each sample is shown in a histogram. **B:** Schematic representation of the cell cycle distributions. **C:** Aliquots of harvested cells were lysed and lysates were analyzed for protein expression by SDS-PAGE and western blot using anti-HA and anti-tubulin antibodies. Tubulin levels served as loading control.

#### 4.5.6 HPV E6 proteins do not inhibit or activate APC/C in *Xenopus laevis* egg extracts

As tumor cells per se have a deregulated cell cycle, possible effects of E6 on the E3 ligase activity of APC/C may not be detectable in U2OS cells. Therefore, we switched the system to *Xenopus laevis* egg extracts. CSF (cytostatic factor) *Xenopus laevis* egg extract is arrested in metaphase of meiosis II due to inhibition of APC/C by phosphorylated Xerp1. Addition of calcium ions ( $\text{Ca}^{2+}$ ) mimics fertilization and thereby leads to degradation of Xerp1 (Rauh et al., 2005). This results in activation of APC/C and release of the extract into interphase. As these extracts are an “open system” it is possible to analyze the influence of proteins or other components on APC/C activity in a simple manner (Hannak and Heald, 2006).

Before analyzing the effect of bacterially expressed GST-E6 proteins on APC/C activity in this system, we investigated if they also bind to *Xenopus laevis* APC/C. Pulldown experiments with GST-11E6, GST-16E6 or GST-18E6 meiotic (CSF) extracts showed that all of them were able to bind to APC/C. However, binding to interphase APC/C was only detected for GST-16E6 and GST-18E6 but not for GST-11E6 (figure 31A).

In the next step, we tested if GST-16E6 is active in *Xenopus laevis* egg extracts and as a consequence is able to induce degradation of *in vitro* translated p53 added to extracts. For E6-induced degradation of p53 E6AP is required. Thus, we first attempted to determine if E6AP is present in *Xenopus laevis* egg extracts. In a western blot analysis of *Xenopus laevis* egg extract E6AP was not detected. However, the anti-E6AP antibody used was prepared against the human E6AP HECT domain and therefore it may not recognize *Xenopus laevis* E6AP (data not shown). Therefore, for the degradation assay, p53 was *in vitro* translated in wheat germ extract and not in reticulocyte lysate, as the latter contains E6AP. Subsequent, p53 degradation assays in meiotic and released extracts showed that addition of high risk GST-16E6 protein induced the degradation of p53 within 5 to 10 minutes independent of the cell cycle status. Supplement of baculovirus expressed E6AP to the extracts did not have an additional effect on the degradation of p53 (figure 31B, upper two panels). Moreover, low risk GST-11E6 protein and GST alone did not induce the degradation of p53 as expected (figure 31B, panel 3-6). From these experiments we concluded that *Xenopus laevis* egg extracts contain active E6AP and that it can form an active E3 ligase complex with GST-16E6 to degrade p53 and that GST-16E6 is functional in *Xenopus laevis* egg extracts.

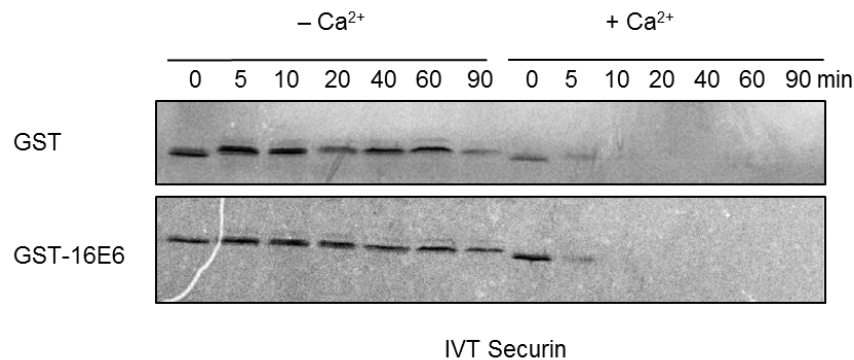


**Figure 31: GST-E6 proteins are functional in *Xenopus laevis* egg extracts and bind to *Xenopus laevis* APC/C**

**A:** Pulldown experiments were performed with bacterially expressed GST, GST-11E6, GST-16E6 and GST-18E6. Coomassie stained gel shows input levels of GST proteins (right panel). GST-fusion proteins were incubated with *Xenopus laevis* egg extracts (CSF or interphase extract). Binding of APC/C was analyzed by SDS-PAGE with subsequent western blot analysis using anti-Cdc27 antibody (left panel) (Nadine Dreser, under supervision). **B:** *Xenopus laevis* egg extract (CSF) was supplemented with *in vitro* translated p53 and GST-fusion proteins and baculovirus expressed E6AP as indicated. Degradation of p53 was analyzed in CSF extract (-Ca<sup>2+</sup>) and in CSF extracts directly after the addition of Ca<sup>2+</sup> (+Ca<sup>2+</sup>). Samples were taken after 0, 5, 10, 20, 40, 60 and 90 minutes and analyzed by SDS-PAGE and subsequent fluorography.

After verifying that GST-16E6 is active in *Xenopus laevis* egg extracts, we analyzed its effect on APC/C activity. To do so, we supplemented extracts with *in vitro* translated Securin, a known substrate of APC/C (Zou et al., 1999), to monitor its E3 ligase activity. In meiotic extracts (CSF), which contain inactive APC/C, Securin was stable whereas after addition of Ca<sup>2+</sup> APC/C

was activated leading to degradation of Securin after 5-10 minutes. Addition of GST-16E6 to extracts did not show any inhibition or activation of APC/C as Securin remained stable in meiotic CSF extract and was also degraded 5-10 minutes after release of extract into interphase (figure 32). In conclusion, E6 proteins do not have an effect on APC/C mediated ubiquitylation of Securin in *Xenopus laevis* extracts (see 5.5.2).

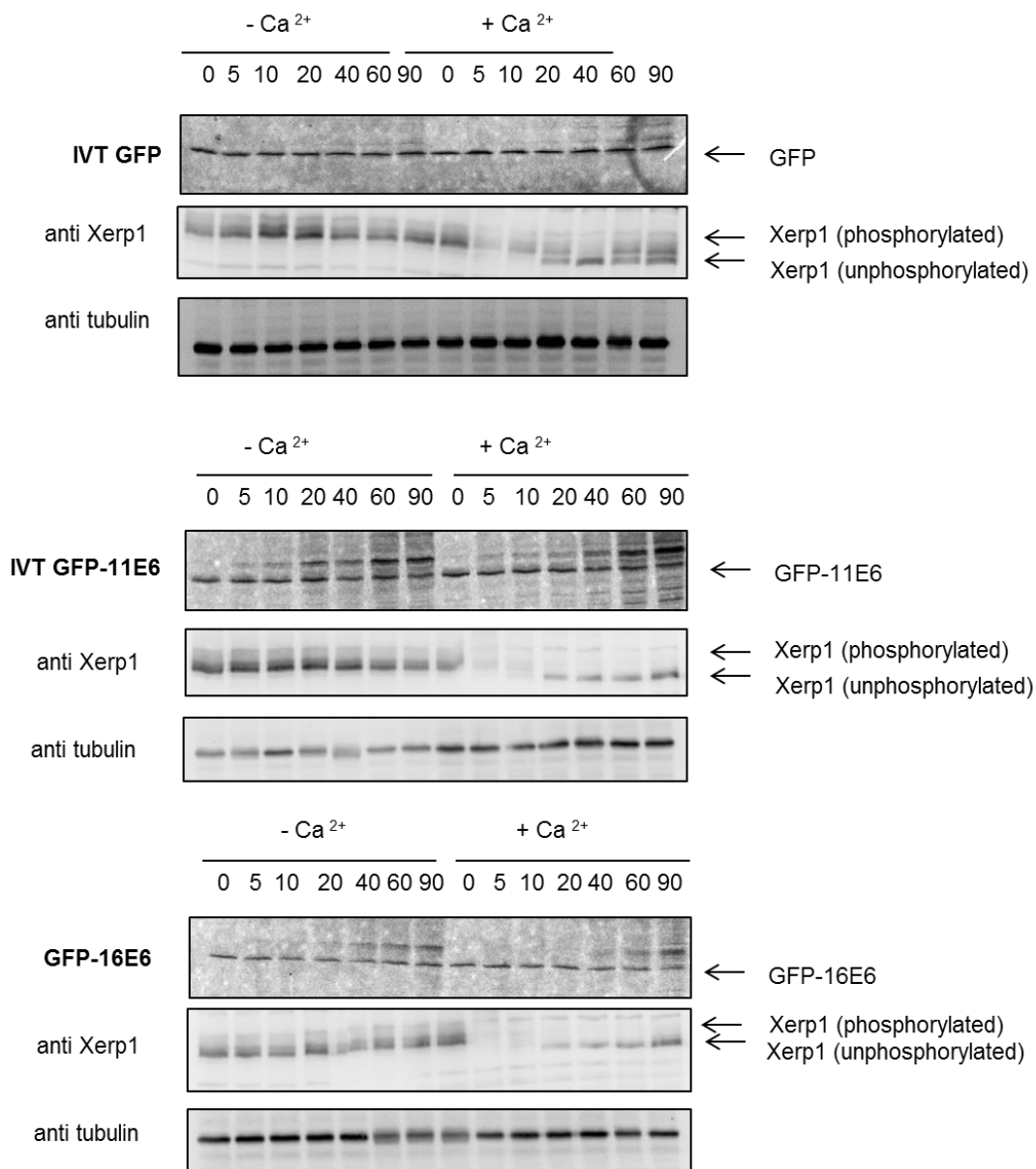


**Figure 32: GST-16E6 does not inhibit or activate APC/C in *Xenopus laevis* egg extract**

*Xenopus laevis* egg extract (CSF) was supplemented with *in vitro* translated Securin and bacterially expressed GST or GST-16E6 protein as indicated. Degradation of Securin was analyzed with CSF extract (-Ca<sup>2+</sup>) and with CSF extract directly after the addition of Ca<sup>2+</sup> (+Ca<sup>2+</sup>). Samples were taken after 0, 5, 10, 20, 40, 60, and 90 minutes and analyzed by SDS-PAGE and subsequent fluorography.

#### 4.5.7 APC/C does not ubiquitylate HPV E6 proteins

E6 proteins are substrates of a so far unknown E3 ligase (Kehmeier et al., 2002; Stewart et al., 2004). As APC/C interacts with the E6 proteins, another potential function of this interaction could be that APC/C is an E3 ligase for E6. To analyze this hypothesis we performed E6 degradation assays in *Xenopus laevis* egg extracts. We used *in vitro* translated GFP-E6 fusion proteins for this assay, as E6 proteins themselves only exhibit one or two methionines and therefore cannot be efficiently radiolabeled. For degradation assay, meiotic extracts were supplemented with GFP, GFP-11E6, GFP-16E6 (figure 33) or GFP-18E6 (data not shown) and part of the extracts were released by Ca<sup>2+</sup> into interphase which results in activation of APC/C. The degradation assay showed that all GFP fusion proteins were stable after the addition of CSF and released extract (figure 33, data not shown). Xerp1 was degraded within 5 minutes upon release of extracts showing that APC/C was activated. Furthermore, 20 minutes after release unphosphorylated newly synthesized Xerp1 was detectable as expected (figure 33). For all GFP-E6 proteins tested a higher migrating band was detected in the degradation assays. However, this band was probably due to the incorporation of free radiolabeled methionine into a newly synthesized protein in the extract. This band was also visible when using GFP in the assay (data not show). From these degradation assays in *Xenopus laevis* egg extract we conclude that APC/C is not an E3 ligase for E6 ubiquitylation.



**Figure 33: *In vitro* translated GFP-E6 proteins are not degraded by *Xenopus laevis* egg extracts**

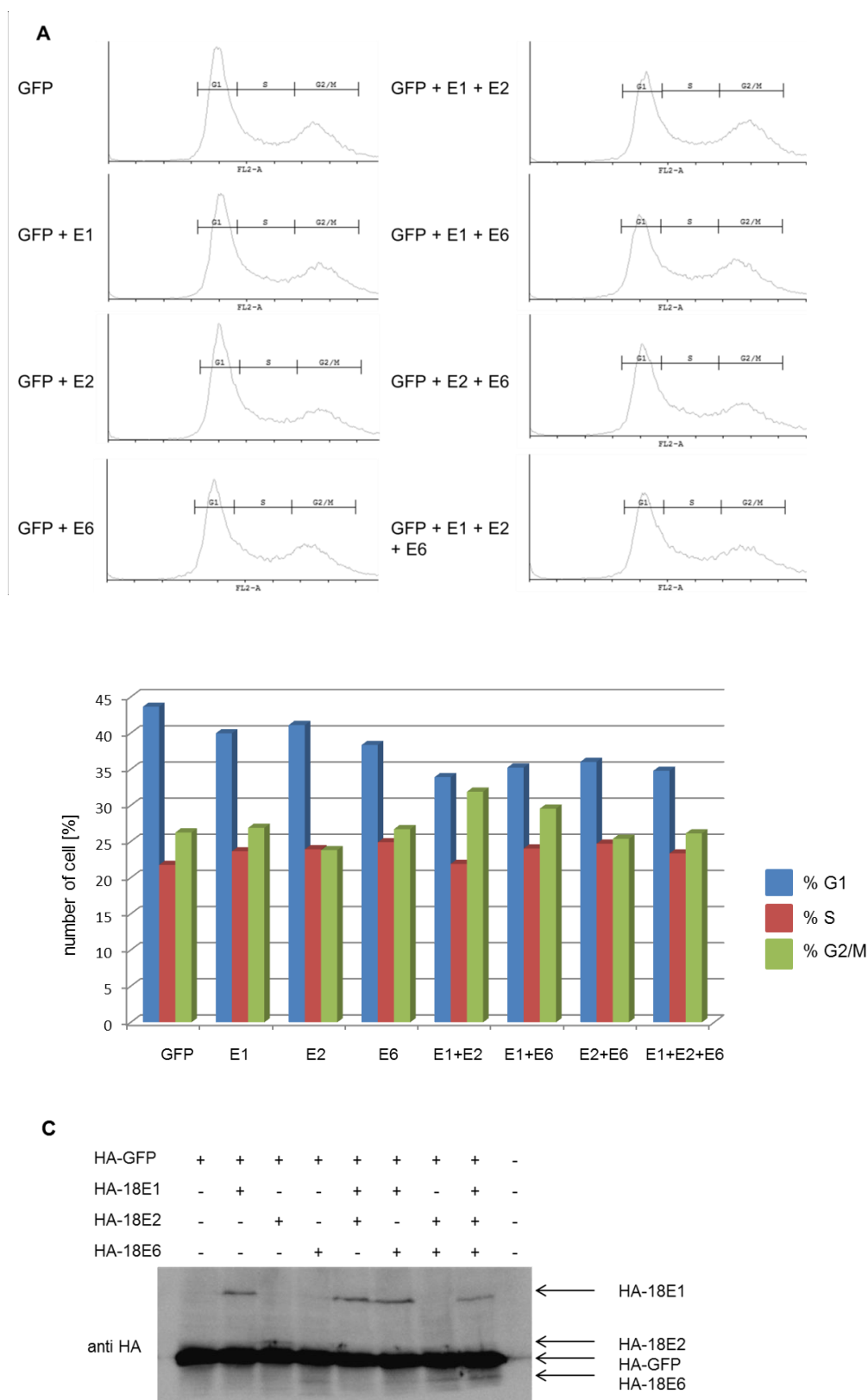
*Xenopus laevis* egg extract (CSF) was supplemented with *in vitro* translated GFP or GFP-E6 proteins as indicated. Degradation of GFP-fusion proteins was analyzed in CSF extract ( $-Ca^{2+}$ ) or directly after the addition of  $Ca^{2+}$  to CSF extract ( $+Ca^{2+}$ ). Samples were taken after 0, 5, 10, 20, 40, 60 and 90 minutes and analyzed by SDS-PAGE following fluorography and western blot analysis using anti-Xerp1 and anti-tubulin antibodies. Protein levels of tubulin served as loading control.

#### 4.5.8 Analysis of HPV E1, E2 and E6 protein expression on cell cycle progression

In the previous experiments, we did not observe any effect of the E6 proteins on APC/C activity or cell cycle progression. One possible explanation for these results could be that effects are only detectable in the presence of other HPV proteins. Along this line, HPV E1 and E2 proteins play a role in viral replication (see 1.1.4) and it is believed that cell cycle arrest in the G2/M phase favors viral replication (reviewed in Davy and Doorbar, 2007). During this

arrest, the replication machinery is available for viral replication as it is not blocked by replication of cellular DNA. Furthermore, it was reported that the expression of HPV E1 and HPV E2 proteins induces cell cycle arrests. Expression of high risk E2 proteins lead to a G2/M arrest in Saos cells (Bellanger et al., 2005) and two reports showed different results for the effect of the expression of E1 on cell cycle progression. C33A cells were arrested in S phase after expression of E1, whereas expression of E1 in U2OS cells lead to an arrest of the cells in S phase and G2/M phase, but only in the presence of the HPV E2 protein (Fradet-Turcotte et al., 2011; Reinson et al., 2012).

Thus, based on the above, we hypothesized that one HPV protein (e.g. E1, E2) is necessary to arrest cells, for example in G2/M phase and that another HPV protein (e.g. E6) is needed to overcome this arrest leading to continuation of cell cycle progression which is needed for the HPV life cycle (see 1.1). Another possibility would be that more than one HPV protein is necessary to arrest cells. To analyze these hypotheses we performed cell cycle analysis by DNA staining and subsequent flow cytometry of H1299 cells transfected with expression vectors encoding HA-18E1, HA-18E2, HA-18E6, combinations of them and HA-GFP as control (figure 34). Western blot analysis revealed that HA-GFP, HA-18E1, HA-18E6 and HA-18E2 were expressed. However, HA-E2 levels were very low (figure 34 C). HA-GFP positive cells were used for cell cycle analysis and expression of a single HPV protein did not show a significant influence on cell cycle distributions compared to HA-GFP control. However, coexpression of two HPV proteins, no matter which ones, lead to less cells in G1 phase. But not in all cases higher amounts of cells in S and G2/M phase were detected (figure 34). Furthermore, expression of all three proteins did not show additional effects. In addition, possible effects of HPV proteins on cell cycle progression were studied in a different cell line, U2OS cells. Cell cycle analysis with this cell line revealed similar results as obtained in H1299 cells (data not shown). In conclusion we showed that coexpression of different HPV proteins in H1299 and U2OS cells leads to effects on cell cycle progression. However, the elucidation of the exact mechanisms underlying these effects needs further investigations.

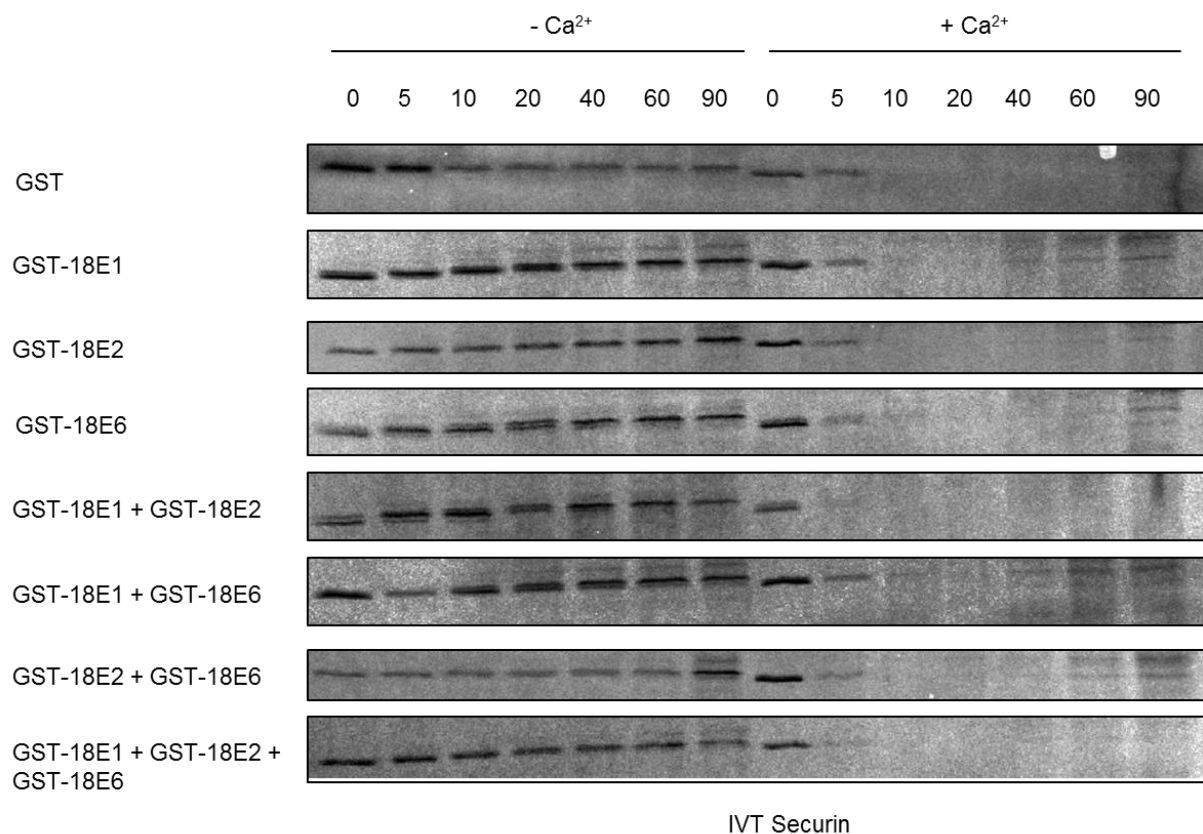


**Figure 34: Cell cycle analysis upon expression of E1, E2 and E6 proteins in H1299 cells**

H1299 cells were transfected as indicated. HA-GFP transfected cells served as control. Cells were harvested and fixed with formaldehyde and were subsequently permeabilized with 70 % ethanol. DNA was stained with propidium iodide (PI) and DNA content of cells was analyzed by flow cytometry. Only HA-GFP positive cells were analyzed for cell cycle distribution. **A:** Cell cycle profile of each sample is shown in a histogram. **B:** Schematic representation of the cell cycle distributions. **C:** Aliquots of harvested cells were lysed and lysates were analyzed for protein expression by SDS-PAGE and western blot analysis using anti-HA antibody.

#### 4.5.9 HPV E1, E2 and E6 proteins do not activate or inhibit APC/C in *Xenopus laevis* egg extracts

Cell cycle analysis suggested that coexpression of HPV proteins affects cell cycle progression. However, it remained unclear if these effects are due to binding of E6 to APC/C. Furthermore, also binding of E2 proteins to APC/C coactivators was described before (Bellanger et al., 2005). To elucidate if any of the HPV proteins has an effect on APC/C E3 ligase activity, we performed a Securin degradation assay in *Xenopus laevis* egg extracts (Hannak and Heald, 2006). None of the HPV proteins tested was able to activate APC/C in this assay as Securin remained stable in CSF extract (-Ca<sup>2+</sup>) where APC/C is normally inactive. Furthermore, Securin was degraded 5 minutes after releasing the extract by Ca<sup>2+</sup> into interphase upon APC/C activation. The presence of any of the HPV proteins did not show an inhibitory effect on Securin degradation, as in these samples Securin was also degraded after 5 minutes. Moreover, combinations of the HPV proteins also did not show any effects (figure 35).

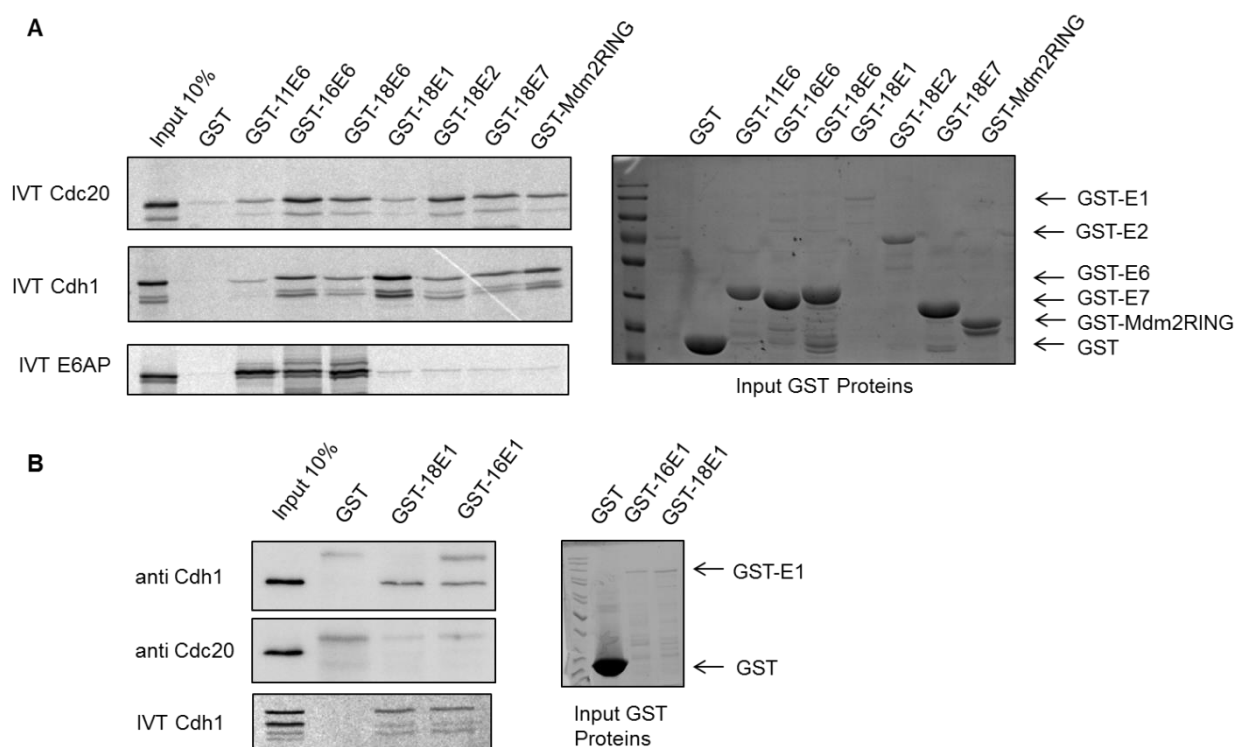


**Figure 35: E1, E2 und E6 proteins do not have an influence on APC/C activity in *Xenopus laevis* egg extracts**

*Xenopus laevis* egg extract (CSF) was supplemented with *in vitro* translated Securin and bacterially expressed GST or GST-fusion proteins as indicated. Levels of Securin were analyzed by degradation assays in CSF extract (-Ca<sup>2+</sup>) and in CSF extract directly after the addition of Ca<sup>2+</sup> (+ Ca<sup>2+</sup>). Samples were taken after 0, 5, 10, 20, 40, 60, and 90 minutes and analyzed by SDS-PAGE and subsequent fluorography.

#### 4.5.10 HPV E1 proteins bind to Cdh1 but not to Cdc20

As mentioned before (3.5.8), binding of high risk HPV E2 proteins to APC/C coactivators Cdh1 and Cdc20 was described before (Bellanger et al., 2005). To reproduce these data we performed binding assays with GST-18E2 and *in vitro* translated Cdh1 and Cdc20. In addition, we also performed binding assays with GST-E6 proteins as positive controls and GST-18E7 and GST-Mdm2RING as negative controls. As nearly all proteins, including the negative controls with the exception of GST, bound to Cdc20 and Cdh1, these assays appeared to be very unspecific (figure 36A, upper and middle panel). In contrast, binding of E6AP was only detected to the GST-E6 proteins as expected (figure 36A, lower panel).



**Figure 36: GST-E1 proteins bind to Cdh1 but not to Cdc20**

**A:** GST pull-down assays were performed with the GST-fusion proteins indicated and *in vitro* translated Cdc20, Cdh1 or E6AP. Bound proteins were analyzed by SDS-PAGE and subsequent fluorography (left panel). Coomassie staining of gel shows input levels of GST proteins used for pull-down experiments (right panel). GST-fusion proteins are marked by arrows.

**B:** GST pull-down assays were performed with bacterially expressed GST and GST-E1 proteins and recombinant in baculovirus system expressed His-Cdh1, His-XeCdc20 or *in vitro* translated Cdh1. Bound proteins were analyzed by SDS-PAGE and subsequent western blot analysis for binding to recombinant proteins and fluorography for radiolabeled proteins (left panel). Expression of GST proteins was confirmed by SDS-PAGE and Coomassie staining (right panel).

Furthermore, we investigated if GST-18E1 is also able to bind to APC/C coactivators. Surprisingly, GST-18E1 was the only protein which seemed to bind to Cdh1 but not to Cdc20. To verify binding of GST-E1 proteins to Cdh1 we performed binding assays with baculovirus expressed His-Cdh1 (human) and His-XeCdc20 (*Xenopus laevis* Cdc20 and human Cdc20

display 75 % identity). Indeed, binding of GST-16E1 and GST-18E1 to baculovirus expressed His-Cdh1 as well as to *in vitro* translated Cdh1 was detected. Whereas binding of GST-E1 proteins to baculovirus expressed XcCdc20 was not detected (figure 36B).

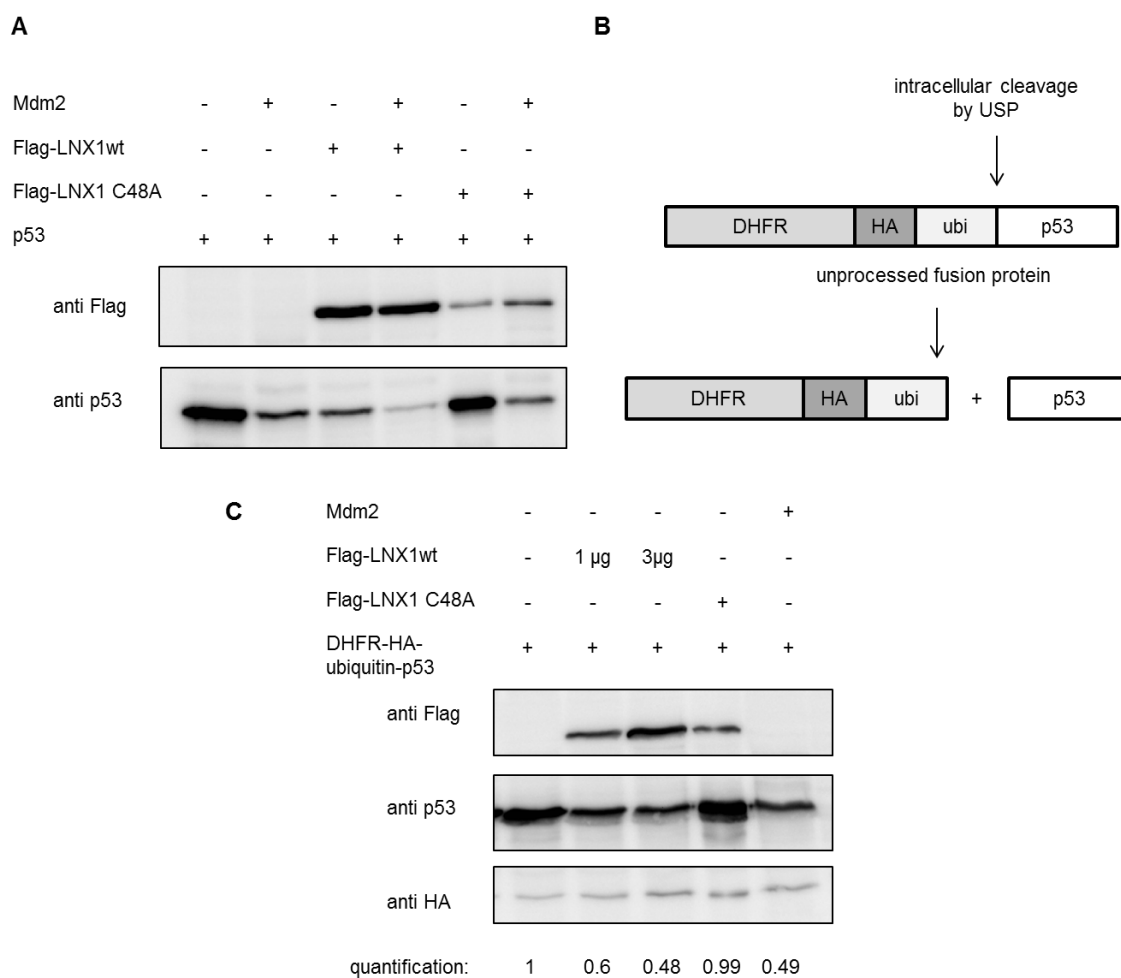
In conclusion, our results indicate that GST-16E1 and GST-18E1 bind to Cdh1 but not to Cdc20. To elucidate the function of the interaction of the E1 proteins and Cdh1, further investigations are necessary. For all other GST-fusion proteins, no conclusion about binding to APC/C coactivators can be drawn.

## 4.6 UBIQUITYLATION OF P53 BY THE E3 LIGASE LNX1

### 4.6.1 LNX1 induces reduction of p53 levels *in cellulo*

LNX1 is a member of the LNX family of proteins which contain a RING domain and PDZ domains (for details see 1.3.1). LNX1 was identified as interaction partner of Numb and it was shown that LNX1 catalyzes ubiquitylation of Numb and targets it for proteasomal degradation and thereby regulating the NOTCH pathway (Nie et al., 2002). Multiple other interaction partners of LNX1 have been identified including the high risk HPV E6 proteins (Weber, 2009). However, we were not able to elucidate the function of this potential interaction, since LNX1 does not appear to be a ubiquitylation substrate of the E6/E6AP complex within cells and is not an E3 ligase for E6 ubiquitylation (Weber, 2009). Furthermore, no effect of E6 on LNX1 ligase activity was observed (Schätzle 2011, under supervision). When we used Flag-LNX1 in a p53 degradation assay in H1299 cells as control, we observed that levels of p53 are decreased in the presence of Flag-LNX1 (data not shown). The tumor suppressor protein p53 has critical functions including regulation of cell death, senescence and proliferation (reviewed in Efeyan and Serrano, 2007). p53 is tightly regulated by post-translational modifications including ubiquitylation and subsequent proteasomal degradation.

Thus, the obtained result pointed to an unexpected role of LNX1 in the degradation of p53. To further address this issue, we tested whether the RING E3 ligase activity is needed to observe the decrease in p53 levels. Therefore, we analyzed p53 protein levels in the presence of wild-type Flag-LNX1 or Flag-LNX1 C48A, which has a mutation in the RING domain and is inactive as an E3 ligase. Indeed, coexpression of LNX1 leads to reduced p53 levels and reduction of p53 levels by LNX1 is dependent on LNX1 E3 ligase activity (figure 37A). Moreover, we investigated the effect of coexpression of Mdm2 and wild-type Flag-LNX1 or Flag-LNX1 C48A on p53 levels. Coexpression of wild type Flag-LNX1 and Mdm2 lead to a further reduction of p53 levels compared to samples with only one E3 ligase overexpressed, whereas coexpression of Flag-LNX1 C48A and Mdm2 did not show an additive effect on p53 degradation (figure 37A).



### Figure 37: p53 levels are reduced in the presence of LNX1

**A:** p53 degradation assay was performed in H1299 cells. Cells were transfected with the respective expression vectors for p53, Mdm2, Flag-LNX1wt and Flag-LNX1 C48A. Cells were lysed under native conditions and equalized for transfection efficiencies. Lysates were analyzed by SDS-PAGE, followed by western blot analysis using anti-Flag and anti-p53 (DO-1) antibodies (Nicole Richter-Müller, under supervision). **B:** Schematic presentation of DHFR-HA-ubiquitin-p53 system. (DHFR: dihydro folat dehydrogenase). **C:** Degradation assay was performed in H1299 cells. Expression vectors coding for the respective proteins were transfected in H1299 cells. DHFR-HA-ubiquitin-p53 is expressed as one polyprotein and cleaved by ubiquitin specific proteases into p53 and DHFR-HA-ubiquitin. Cells were lysed under native conditions equalized for transfection efficiencies and analyzed by SDS-PAGE, followed by western blot analysis using anti-Flag, anti-HA and anti-p53 (DO-1) antibodies. Levels of p53 and DHFR-HA-ubiquitin were quantified (ImageJ) and p53 levels were adjusted to DHFR-HA-ubiquitin signals (Dolde, 2013, under supervision).

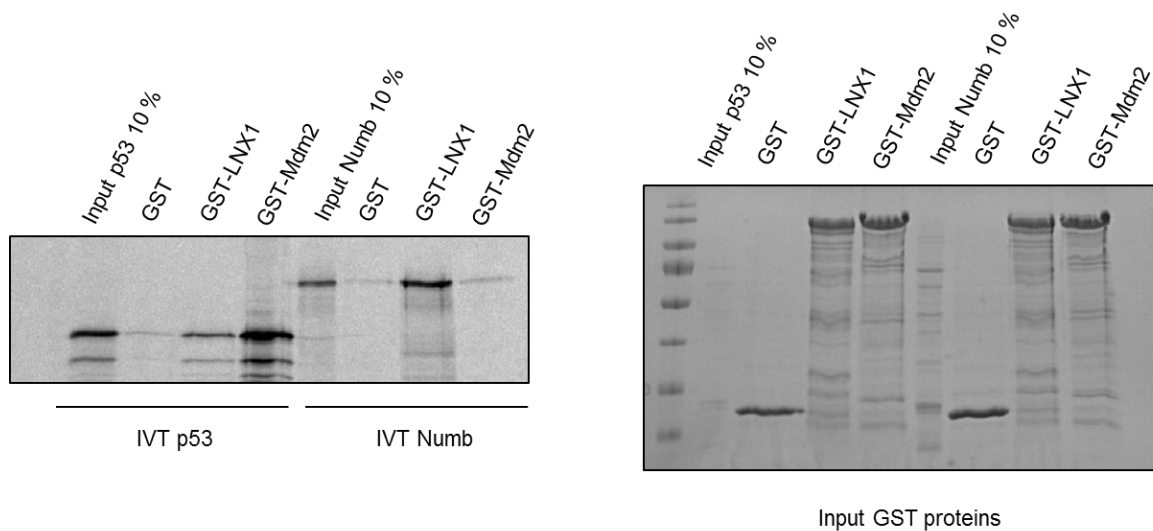
The above results indicate that the E3 ligase activity of LNX1 is necessary for reduction of p53 levels and that coexpression of Mdm2 and Flag-LNX1 has an additive effect on p53 levels.

To exclude that these results are due to differences in transfection efficiencies and to show that the decrease occurs on the posttranscriptional level, we repeated the degradation assay by transfecting a construct encoding DHFR-HA-ubiquitin-p53 (figure 37B), which is expressed as one polyprotein. This polyprotein is cotranslationally cleaved by ubiquitin

specific proteases into p53 and DHFR-HA-ubiquitin resulting in same amounts of both proteins (figure 37B and C). Hence, a clear conclusion about p53 degradation within cells can be drawn by determining the ratios between DHFR-HA-ubiquitin and p53. Note that DHFR-HA-ubiquitin is a rather stable protein (Varshavsky, 2005); quantification of p53 levels showed a dose-dependent reduction of p53 levels by Flag-LNX1 dependent on its E3 ligase activity (figure 37C).

#### 4.6.2 GST-LNX1 binds to p53 *in vitro*

As we observed reduction of p53 levels by Flag-LNX1 in cells dependent on its E3 ligase activity we speculated that this is due to ubiquitylation of p53 by LNX1 and its subsequent degradation by the 26S proteasome. If this is the case, both proteins should interact with each other. Therefore, we performed an *in vitro* binding assay using bacterially expressed GST, GST-LNX1 or GST-Mdm2 and *in vitro* translated p53. We used as positive control *in vitro* translated Numb, which is a known interaction partner of LNX1 and Mdm2 (Nie et al., 2002; Yogosawa et al., 2003). This assay revealed that GST-LNX1 is able to interact with p53 (figure 38). However, GST-Mdm2 binds stronger to p53 than GST-LNX1. In contrast, binding of Numb to GST-LNX1 was more efficient than to GST-Mdm2 (figure 38).

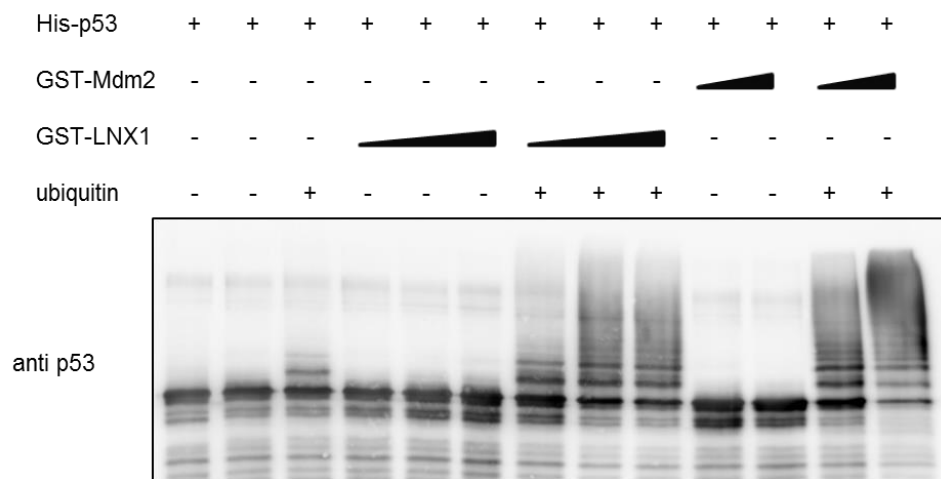


#### Figure 38: GST-LNX1 binds to p53 *in vitro*

GST pull-down assays were performed with GST-fusion proteins and *in vitro* translated p53 and Numb. Bound proteins were detected by SDS-PAGE and subsequent fluorography (left panel). Coomassie staining shows input levels of GST proteins used for pull-down experiments (right panel) (Nicole Richter-Müller, under supervision).

### 4.6.3 p53 is a ubiquitylation substrate of LNX1

Since we could show that p53 and LNX1 bind to each other, we next investigated whether LNX1 is able to ubiquitylate p53 *in vitro*. To do so, we performed an *in vitro* ubiquitylation assay using baculovirus expressed His-p53 as substrate. GST-Mdm2 was used as positive control. As expected, we detected ubiquitylation of p53 by GST-Mdm2 shown by the reduction of levels of unmodified p53 and the appearance of a high molecular weight smear in samples with GST-Mdm2 and ubiquitin (figure 39). Moreover, similar effects were obtained in the presence of GST-LNX1 and ubiquitin showing that GST-LNX1 is able to ubiquitylate p53 *in vitro*. Furthermore, ubiquitylation of p53 by LNX1 occurs in a dose-dependent manner (figure 39).



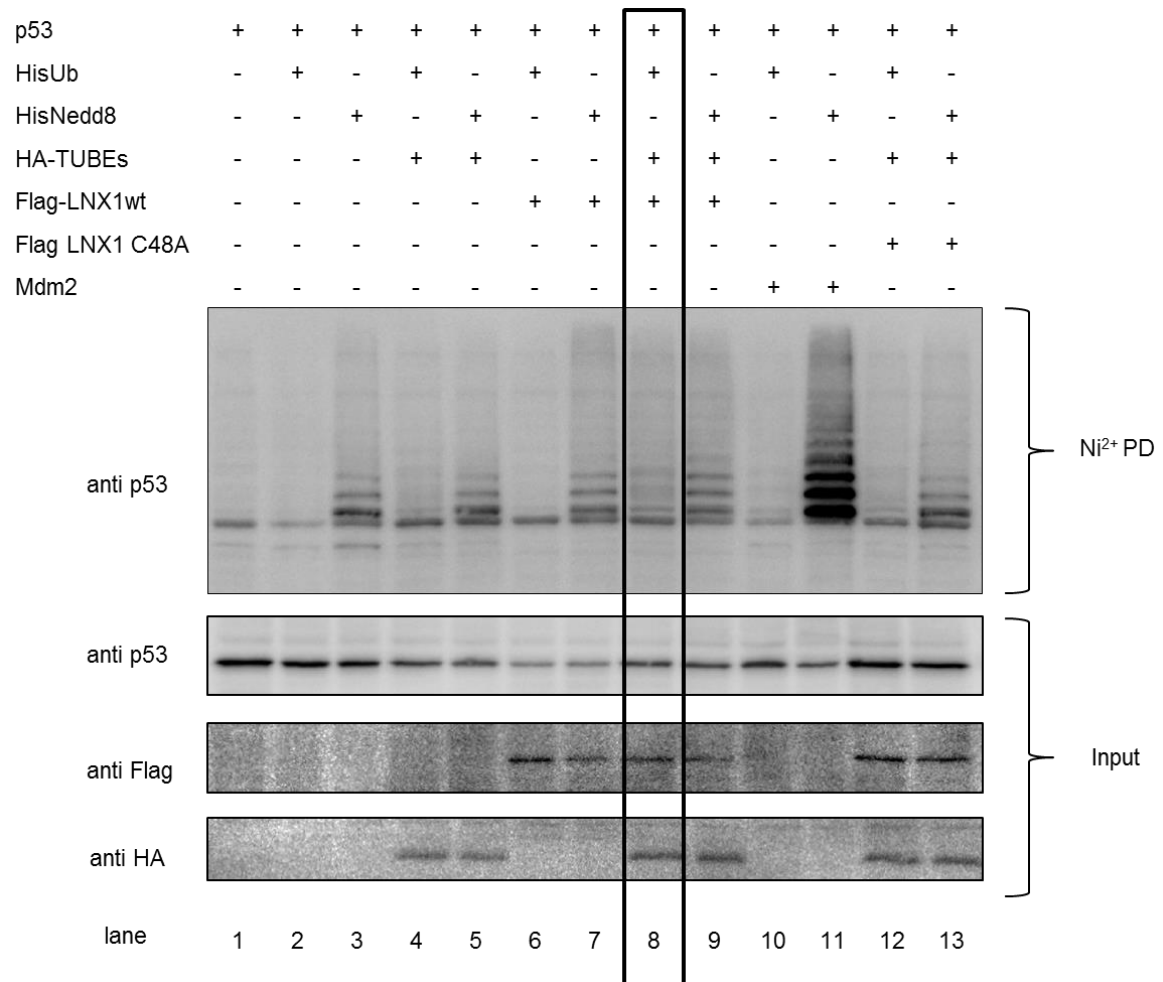
#### Figure 39: GST-LNX1 ubiquitylates p53 *in vitro*

*In vitro* ubiquitylation assay was performed with baculovirus expressed His-p53 as substrate and GST-LNX1 or GST-Mdm2 as E3 ligase. Samples were analyzed by SDS-PAGE and subsequent western blot analysis using anti-p53 antibody (DO-1) (Dolde, 2013, under supervision).

In the next step, we investigated whether LNX1 is able to ubiquitylate p53 *in cellulo*. In addition we analyzed if LNX1 is able to neddylation p53 *in cellulo*, as from our experience *in cellulo* neddylation assays are more efficient than ubiquitylation assays (Scheffner group, unpublished data). To do so, we performed a His-ubiquitin/NEDD8 assay in the presence and absence of HA-HR23A-TUBEs (see figure 17) in H1299 cells (figure 40). TUBEs serve as stabilization tool for ubiquitylated proteins (for details see 4.3.2)

In contrast to overexpression of Mdm2, where an enhanced neddylation of p53 was observed (figure 40, comparing lane 3 and 11), overexpression of Flag-LNX1 did not have a significant effect on p53 neddylation (figure 40, comparing lane 3 and 7). Moreover, expression of HA-TUBEs did not have an effect on p53 neddylation. Ubiquitylation of p53 by Flag-LNX1 could not be detected in the absence of HA-TUBEs even though lower p53 levels were found in the input samples (figure 40, lane 6). However, ubiquitylation of p53 by Mdm2 which

should have served as positive control could also not be shown in this assay (figure 40, lane 10). In the presence of HA-TUBEs a weak high molecular weight smear was detected dependent on the expression of Flag-LNX1 with an intact RING domain (figure 40, comparing lane 4, 8, 12). This result may indicate that LNX1 is able to ubiquitylate p53 *in cellulo*.



**Figure 40: Analysis of ubiquitylation and neddylation of p53 within cells**

H1299 cells were transfected with the respective expression constructs. Input samples were lysed under native conditions and equalized for transfection efficiencies. Pulldown samples (Ni<sup>2+</sup>PD) were lysed under denaturing conditions and PD was performed with Ni-NTA agarose to bind ubiquitylated or neddylated proteins. Input and PD samples were analyzed by SDS-PAGE followed by western blot analysis using anti-HA, anti-Flag and anti-p53 (DO-1) antibodies as indicated (Nicole Richter-Müller, under supervision).

## 5 DISCUSSION

### 5.1 ESTABLISHMENT AND CHARACTERIZATION OF STABLE CELL LINES, INDUCIBLY EXPRESSING THE E6 PROTEINS

For the elucidation of the HPV E6 “proteome”, “ubiquitome” and “interactome” by a mass spectrometry driven approach it is necessary to work with defined cellular systems. As overexpression of the HPV E6 proteins is toxic (Scheffner group, unpublished data), we combined the already described puromycin<sup>r</sup>-Ubi fusion system (Matentzoglou and Scheffner, 2009) with a TetOn system (figure 9). The generated inducible puromycin<sup>r</sup>-Ubi fusion system is well suited for an establishment of cell lines that express a protein of interest in a tightly regulated manner. With this system, the protein of interest is only expressed in the presence of the respective inducing agent, which allows propagation of cells in the absence of induction. Therefore, it can be used for the characterization of proteins, whose expression has toxic effects. Here, we combined this advantage with the puromycin<sup>r</sup>-Ubi fusion system. The principle of this system is that in cells the resistance markers (in our case, puromycin<sup>r</sup> and TK) are expressed as a polyprotein with the E6 protein, which is posttranslationally processed to the respective free proteins (figure 9). With conventional inducible expression systems, single cell clones need to be established to avoid the presence of “leaky” cells in a mixed population. Because of TK expression in our system, “leaky” cells should be readily eradicated by ganciclovir treatment, while cells that express the protein of interest in the presence of inducing agent survive. However, in our experiments this elimination of “leaky” cells could not be fully accomplished (figure 10C). Furthermore, as shown previously (Matentzoglou and Scheffner, 2009), the puromycin<sup>r</sup>-Ubi fusion system allows homogenous expression of a protein of interest in a mixed cell population and, thus, time-consuming selection of clonal cell lines is not required. Altogether, our expression vector provides a potential powerful tool for rapid selection of cell lines that express a protein of interest, in our case the HA-E6 proteins, in a regulated manner.

Using this system we established U2OS cell lines which inducibly express the HA-11E6, HA-16E6, HA-18E6 or the chimeric HA-11C18 protein (figure 10A). These cell lines behaved as expected according to puromycin and ganciclovir treatment (figure 10). Moreover, we showed that induction of HA-11C18 expression with 0.01-10 mg/ml doxycycline revealed similar protein levels of HA-E6 (figure 11A). Levels of HA-11C18 protein were analyzed by a time course experiment showing that HA-11C18 levels did not significantly decrease until day eight after removal of doxycycline (figure 11B). So far, it remains unclear whether the change in protein levels is due to the half-life of the E6 protein, of the mRNA or a combination of both. To investigate this issue, the analysis of TK-puromycin<sup>r</sup>-Ubi-HA-E6 mRNA levels by qPCR

would be required. However, similar results were obtained by a time course experiment using Hek293 cells stably expressing the same polyprotein TK-puro<sup>r</sup>-Ubi-HA-11C18 construct (data not shown). In this case, we analyzed mRNA levels of cells by qPCR and observed that TK-puro<sup>r</sup>-Ubi-HA-11C18 mRNA was still present after six days upon removal of doxycycline but it was significantly decreased after eight days upon removal (data not shown) indicating that at least in this cell line, the change in protein levels correlates with mRNA levels. We were able to show, that in U2OS 11C18 cells hDlg - a known substrate of the chimeric E6 protein (Pim et al., 2002) - is degraded upon induction of HA-11C18 expression. This result indicates that the expressed HA-11C18 protein is functional (figure 11B). However, degradation of p53 by HA-16E6 or HA-18E6 in U2OS E6 cell lines could not be shown (data not shown). U2OS cells contain wild-type p53 and Hdm2, targeting p53 for degradation (Florenes et al., 1994) which could explain why we were not able to detect E6 mediated degradation of p53 as it is already a short-lived protein.

To gain further insight into E6 functions, it would be worthwhile to establish stable H1299 or HaCat cells inducibly expressing the E6 proteins using this new expression system.

HaCat cells are transformed keratinocytes (Boukamp et al., 1988) and are therefore interesting cells to analyze as keratinocytes are the natural target of HPVs. H1299 is a tumor cell line which does not express p53, so with this cell line we could analyze the effect of E6 proteins in a p53 null background. However, we were not able to establish these stable cell lines with our newly developed expression system which may be due to promoter silencing of the CMV promoter, a common phenomenon. As already the first selection using G418 for cells which contain the expression vector failed is another possibility that “leaky” expression of the toxic E6 protein leads to cell death. In addition the transfection efficiencies of HaCat cells are very low. Due to the lack of inducible HaCat and H1299 cell lines we restricted our analyses of changes upon E6 expression on the proteome to the U2OS E6 cell lines (see 4.2 and 5.2).

## 5.2 ANALYSIS OF “HPV E6 PROTEOME”

High risk and low risk HPV E6 proteins share identity on amino acid level (~ 50 %) and presumably have similar roles in the HPV life cycle. Thus, our hypothesis is that they share some common properties. To identify common and different functions of the high risk and low risk HPV E6 proteins we elucidated changes of the proteome in the presence of different E6 proteins (“HPV E6 proteome”). We used a mass spectrometry driven approach (SILAC) and analyzed which proteins were altered after induction of HA-E6 expression in the established U2OS E6 cell lines (see 4.1) (scheme is outlined in figure 12A). We analyzed the effect of the high risk HA-18E6 protein and of the chimeric HA-11C18 protein on the proteome. The chimeric 11C18 protein was chosen instead of the low risk 11E6 protein, as it contains the

PDZ binding motif which is responsible for binding of PDZ domain-containing proteins and therefore should serve as positive control for our approach.

For both cell lines (U2OS 18E6, U2OS 11C18) we detected around 2100 proteins by LC-MS/MS for each experiment. As expected protein levels of most of these proteins were not altered by the E6 proteins, as can be seen by the population of H/L ratios 0 (log2) (figure 13 and 14). In the presence of HA-18E6 levels of 31 proteins were up- and of 35 proteins were down-regulated whereas in the presence of HA-11C18 levels of 19 proteins were up- and of 27 proteins were down-regulated (table S1 and S2). In these proteome approaches, we identified PDZ domain-containing proteins (14 for U2OS 18E6 and 11 for U2OS 11C18, figure 13 and 14, red stars) including hDlg, a known substrate of the E6/E6AP complex (Kuballa et al., 2007). However, levels of none of the PDZ domain-containing proteins were significantly altered by the respective E6 protein. For U2OS 18E6 cells this could be due to “leaky” expression of HA-18E6 in the absence of doxycycline (figure 12B), assuming that low amounts of HA-18E6 are sufficient to degrade hDlg. One would not necessarily expect an additional effect upon induction of HA-18E6 expression. For U2OS 11C18 cells “leaky” expression of HA-11C18 was not detected (figure 12B), but in these cells ratios for hDlg were on the border to significant values (figure 14). Moreover, degradation of hDlg was shown for U2OS 11C18 but also for U2OS 18E6 by western blot analysis (figure 11B, data not shown). We did not identify MAGI-1, although this protein is believed to be the strongest binding partner and most relevant substrate of E6 proteins among PDZ domain-containing proteins (Kranjec and Banks, 2011; Thomas et al., 2001; Zhang et al., 2007), maybe due to low expression levels in U2OS cells. As PDZ domain-containing proteins, like hDlg and MAGI-1 should have served as positive control in our experiments, the data suggest that we were not able to identify all proteins whose levels were altered by HA-18E6 or HA-11C18, respectively. One aim of these proteome studies was to identify common targets of the low risk and high risk E6 proteins. However, the expression levels of only six proteins were identified to be altered by both HA-18E6 and HA-11C18 (table 1). Low risk and high risk E6 proteins both interact with the cellular E3 ligase E6AP (Brimer et al., 2007; Kuballa et al., 2007) and we hypothesized that they share some ubiquitylation substrates. As E6AP catalyzes the assembly of K48 linked chains on its substrates (Kim et al., 2007; Wang and Pickart, 2005), respectively modified proteins are marked for subsequent proteasomal degradation which should result in decreased protein levels in the presence of E6. Among the proteins whose levels are altered by HA-18E6 and HA-11C18, levels of three proteins (desmoplakin, ALL1-fused gene from chromosome 4p12, PAL1 RNA-binding protein 1) were down-regulated (table 1). As we only compared protein but not mRNA levels, it is not clear whether lower protein levels of the proteins in the presence of HA-E6 are due to ubiquitylation by E6/E6AP and subsequent

proteasomal degradation. It could be also the case that E6 regulates these protein levels by indirect regulation at the protein level, or due to regulation at the mRNA level.

Among these three proteins desmoplakin is the most interesting one as it is a component of desmosomes which are intercellular junctions found in different tissues including epithelia (reviewed in Garrod and Chidgey, 2008). Thus, desmosomes play a role in adhesion between cells and may be therefore an attractive target for E6 proteins. Since the natural target of HPVs are keratinocytes and the viral life cycle is linked to the differentiation program of the keratinocytes (reviewed in Doorbar, 2006), the disruption of desmosomes and cell-cell adhesions by E6 could favor the viral life cycle. Desmoplakin was also isolated by HA-TUBEs in our “ubiquitome” approach in the presence of Flag-11C18 (see 4.3.3 and table S3) but not in the presence of Flag-18E6. However, desmoplakin was not found to be modified by a di-glycine motif (see 4.3.3). Thus, it is not clear if desmoplakin was ubiquitylated prior to the affinity purification. In our “interactome” approach we did not observe specific binding of desmoplakin to GST-11C18 assuming that E6 proteins regulate desmoplakin levels in an indirect manner.

PAL1 RNA binding protein 1 was also identified in the “interactome” approach. All other proteins whose expression levels were up- or down-regulated by both HA-11C18 and HA-18E6 were not found to be ubiquitylated in the presence of E6 or bound to GST-11C18 in our “ubiquitome” and “interactome” approaches (see 3.3 and 3.4). Thus, further investigations are necessary to verify an effect of E6 on these proteins. Western blot analyses of U2OS E6 cell lines with specific antibodies should reveal whether levels of these proteins including desmoplakin are indeed altered in the presence of HA-E6. Moreover, it should be investigated if this takes place in an E6AP-dependent manner. Finally, further investigations are necessary to elucidate the functions and mechanisms of these E6-dependent changes in protein levels.

For a more detailed analysis of the proteomes of both U2OS E6 cell lines we grouped the proteins altered by HA-E6 according to their involvement in different biological processes (stress response, cell cycle, cell adhesion, differentiation, transport, host-virus interaction, transcription and DNA damage) (figure 15). However, no clear pattern could be observed with the exception of transcription for which proteins whose levels were down-regulated were found. However, no protein was found whose levels are altered by both E6 proteins (table S1 and S2).

Future experiments should also include proteome analysis of the U2OS 11E6 and U2OS 16E6 cell lines to compare whose protein levels are altered by the high risk 16E6 and 18E6 proteins. By comparing the proteome data of U2OS 11E6 and U2OS 11C18 we would be able to identify proteins whose levels are altered due to the presence of the PDZ binding motif. In addition, it would be worthwhile to analyze proteome changes upon expression of E6

proteins in transformed keratinocytes (HaCat cells), as keratinocytes are the natural target cells of HPVs. However, we were not yet able to establish stable inducible cell lines using HaCat cells (see 5.1). Moreover, it would be interesting to identify changes in the proteome triggered by E6 expression using primary keratinocytes.

In conclusion, we identified changes of the proteome in U2OS 18E6 and U2OS 11C18 cell lines after induction of HA-E6 expression. Further investigations using additional E6 proteins and additional cell lines are necessary to obtain more insights in the pathways and the specific proteins affected by E6. Moreover, we have to increase the sensitivity of the mass spectrometric analysis to identify a higher number of proteins.

### **5.3 ANALYSIS OF “HPV E6 UBIQUITOME”**

Low risk and high risk E6 proteins bind to the cellular ubiquitin E3 ligase E6AP (Brimer et al., 2007; Kuballa et al., 2007), so it seems very likely that the respective E6/E6AP complexes share some ubiquitylation substrates. Up to now cellular ubiquitylation of p53 by E6/E6AP could not be shown (Camus et al., 2003), which should serve as positive control. Thus, the establishment of a new cellular ubiquitylation assay was necessary to identify E6-dependent substrates. However, until this thesis, E6/E6AP-mediated ubiquitylation of p53, the best characterized substrate of E6/E6AP could not be observed in cells in transfection experiments (Camus et al., 2003; Scheffner group, unpublished data). Thus, to identify ubiquitylation substrates of E6 it was first necessary to establish a robust ubiquitylation assay for E6.

#### **5.3.1 Establishment of a ubiquitylation assay using TUBEs**

For the identification of ubiquitylation substrates of the E6/E6AP complex, it is advantageous to use an experimental setup where a positive control is available. However, using standard cellular ubiquitylation assays (His-ubiquitin assay), E6/E6AP mediated ubiquitylation of p53 or of other known substrates could not be shown (figure 16). We tested various conditions, cell lines, ubiquitin and Nedd8 mutants (figure 16, data not shown), but we were not able to detect p53 ubiquitylation/neddylolation by E6/E6AP under any condition although we consistently observed degradation of unmodified p53 in the presence of E6 (figure 16, data not shown). One explanation for this phenomenon could be that ubiquitylated p53 is degraded by the 26S proteasome very rapidly. However, it is known that the usage of proteasome inhibitors also does not lead to an accumulation of ubiquitylated p53 species. But proteasome inhibitors have also not an effect on levels of the unmodified form of p53 (Camus et al., 2003; Scheffner group, unpublished data). These results indicate that also

deubiquitylation plays a role. So we decided to stabilize ubiquitylated p53 using TUBEs (tandem repeated ubiquitin binding entities) (Hjerpe et al., 2009).

TUBEs consist of four UBA domains derived of either HR23A or Ubiquilin which are connected by a flexible linker (figure 17). They were originally established by the Rodriguez group to isolate ubiquitylated proteins (Hjerpe et al., 2009). Hjerpe et al. showed that the addition of bacterially expressed GST-TUBEs during cell lysis protects ubiquitylated proteins from DUBs and proteasomal degradation. Thus, we hypothesized that ectopic expression of TUBEs should also stabilize ubiquitylated proteins within cells. To test this, we used different tagged versions of TUBEs: HA-TUBEs, His-HA-TUBEs and HA-GST-TUBEs. First we investigated whether coexpression of HA-TUBEs or HA-GST-TUBEs stabilizes ubiquitylated p53 species using His-ubiquitin assays. Indeed, in the presence of HA-TUBEs ubiquitylation of p53 by E6/E6AP could be shown (figure 19). For p53 ubiquitylation mediated by Mdm2 we detected ubiquitylated p53 even in the absence of TUBEs, but in the presence of HA-TUBEs we were able to detect higher amounts of ubiquitylated p53 (data not shown). These data indicate that TUBEs bind to ubiquitylated proteins within cells and protect them from deubiquitylation and/or proteasomal degradation. In the presence of HA-GST-TUBEs stabilization of ubiquitylated p53 in the presence of E6 or Mdm2 could not be detected (data not shown), maybe due to steric hindrance of the larger HA-GST-tag. However, results of *in vitro* experiments using GST-tagged TUBEs argues against this possibility as GST-TUBEs are able to bind to *in vitro* ubiquitylated p53 (figure 18).

Since we detected a high molecular weight smear in the input samples where no His-ubiquitin was expressed (figure 18, left panel) we speculated that in the presence of TUBEs endogenous ubiquitin is sufficient to detect ubiquitylated p53 and that TUBEs could be used to isolate these. So we decided to simplify our assay and expressed His-HA-TUBEs in H1299 cells to isolate ubiquitylated proteins by Ni-NTA chromatography under native conditions. Indeed, we were able to detect ubiquitylated p53 in samples where His-HA-TUBEs, p53 and Flag-16E6 were coexpressed (data not shown). These data provide evidence that TUBEs can be useful to isolate ubiquitylated proteins not only *in vitro* but also directly from cells. However, we also detected unmodified p53 due to unspecific binding to Ni-NTA-agarose (data not shown) which is frequently observed when using Ni-NTA-agarose as affinity matrix. Thus, we decided to use differently tagged TUBEs (HA-TUBEs) to isolate ubiquitylated proteins via immunoprecipitation using an anti-HA-antibody. With this approach we could efficiently purify HA-TUBEs and ubiquitylated proteins bound to the TUBEs including ubiquitylated p53 (figure 20, 21A).

Since in the presence of TUBEs, we could monitor E6 mediated ubiquitylation of p53 in cells, we also tried to show ubiquitylation of other known E6/E6AP substrates like hDlg and MAGI-1 using coexpression of HA-TUBEs in His-ubiquitin assays and isolation of

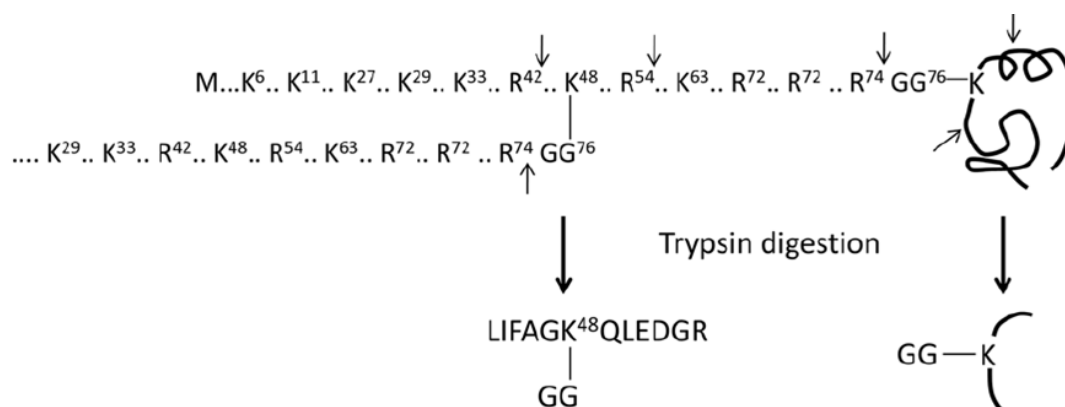
ubiquitylated proteins by HA-TUBEs using anti-HA antibody. However, in these cases we could not detect ubiquitylated species of the proteins. Since both proteins have a relatively high molecular mass (130 kDa and 150 kDa) and ubiquitylated species exhibit even higher molecular masses, the conditions used for transferring such proteins by western blot may have been not optimal. In other words, the failure to detect ubiquitylated hDlg and MAGI-1 may be explained by incomplete transfer of the ubiquitylated hDlg and MAGI-1. In fact, in these and the assays using p53 as substrate, we observed an accumulation of unmodified p53, hDlg and MAGI-1 in the presence of TUBEs (figure 19, data not shown) indicating that the presence of TUBEs interfered with degradation of ubiquitylated forms of hDlg and MAGI-1. Thus, in the future *in cellulo* assays using His-hDlg or His-MAGI-1, and TUBEs allowing the purification of His-hDlg or His-MAGI-1 and their ubiquitylated forms respectively under denaturing conditions by Ni-NTA chromatography should be performed. Subsequent analysis of bound proteins by mass spectrometry could verify that ubiquitylated forms of hDlg and MAGI-1 exist.

In conclusion, we found conditions to show that ubiquitylation of p53 by E6/E6AP occurs within cells which has not been shown before and can serve as positive control for the identification of ubiquitylation substrates of E6/E6AP. Moreover, we established a very simple assay to isolate ubiquitylated proteins that has several advantages compared to existing methods. Most of these methods make use of overexpression of a tagged version of ubiquitin. HA-ubiquitin and isolation of ubiquitylated proteins via an immunoprecipitation using HA-antibody or His-ubiquitin and purification by Ni-NTA chromatography are often used (Callis and Ling, 2005). Advantage of the latter is that isolation of ubiquitylated proteins is possible under denaturing conditions, where DUBs are inactive and therefore deubiquitylation is blocked. However, as overexpressed ubiquitin is used in both cases, the observed ubiquitylation pattern may not reflect the endogenous situation (reviewed in Hjerpe and Rodriguez, 2008). In contrast in our system, proteins modified with endogenous ubiquitin can be isolated by ectopic expression of TUBEs. A further advantage of this system is that proteins, which are very efficiently ubiquitylated and subsequently degraded by the 26S proteasome are stabilized within cells and, thus, can be detected and isolated. To determine if our new approach can be applied in general further investigations with additional E3 ligases and their cognate substrates should be performed.

### 5.3.2 Identification of ubiquitylation substrates of E6

After the establishment of an *in cellulo* ubiquitylation assay, we aimed to identify ubiquitylation substrates of E6 ("HPV E6 ubiquitome"). To do so, we isolated ubiquitylated proteins in the presence of Flag-18E6 or Flag-11C18 using HA-TUBEs and subsequently

analyzed these by LC-MS/MS (figure 21). In all samples (without E6, in the presence of Flag-18E6 and in the presence of Flag-11C18) we identified up to 129 proteins which copurified with HA-TUBEs (table S3). Prior to LC-MS/MS analysis, proteins bound were digested by trypsin, which in case of ubiquitylated proteins results in branched peptides containing a di-glycine motif (GG) on lysine residues which were linked to ubiquitin (figure 41).



**Figure 41: Trypsin digestion of ubiquitylated proteins generates branched peptides containing a di-glycine motif**

Schematic overview: trypsin digest of ubiquitylated proteins leads to peptides with a di-glycine motif (GG) on ubiquitylated sites of modified proteins and ubiquitin itself (Na and Peng, 2012).

Ubiquitin derived peptides modified with a di-glycine motif on lysine residues 11, 48 and 63 were found, corresponding to chain formation at these residues. This result verified nicely that in principle our approach to isolate polyubiquitylated proteins by TUBEs worked. However, for all other identified proteins no peptides modified with a di-glycine motif could be observed by LC-MS/MS analysis. This could be due to low sequence coverage of the identified proteins (i.e. peptides modified by di-glycine motifs are not detected even though they are present). Another possibility is that these peptides were too small to be detected by LC-MS/MS analysis or that the proteins identified bound unspecifically to HA-beads. In any case, most of the proteins were also identified in the absence of E6, indicating that even if the proteins identified were ubiquitylated prior purification, this happened very likely not in an E6-dependent manner. One problem during ubiquitome analyses by LC-MS/MS is that the amount of ubiquitin peptides in the samples is very high which impedes the identification of other proteins. As we were interested in proteins modified with ubiquitin chains, gel pieces of the upper part of the SDS-gel corresponding to proteins with a molecular mass of 98 kDa and higher were of interest to us (figure 21). However, in these samples mainly ubiquitin was detected, and also some E3 ligases like EDD and RNF213 (table S3). This is not unexpected as E3 ligase are known to autoubiquitylate themselves (de Bie and Ciechanover, 2011). Moreover, proteins, like cytoskeletal and structural proteins (e.g. tubulin, actin), were also

found in this part of the gel which are known as unspecific binders (Trinkle-Mulcahy et al., 2008). Thus, it seems that many of the identified proteins bind unspecific to HA-beads. Furthermore, all proteins identified in this upper part of the gel bound also in the absence of E6 including the area where additional bands in the presence of Flag-18E6 and Flag-11C18 were detected (figure 21). Thus, further optimizations of this method are necessary to reliably identify ubiquitylation substrates of E6. One additional reason why we did not detect ubiquitylated proteins could be that the amount of cell lysate was not sufficient. In future experiments up-scaling of the assay should be considered. Moreover, after tryptic digest of the proteins, a second purification step using an antibody that recognizes di-glycine modified peptides could be performed. This technique has already been applied in other ubiquitome studies (Udeshi et al., 2012; Udeshi et al., 2013). However, also in this case there will be a high amount of ubiquitin peptides in the samples. To avoid this, one should cleave ubiquitin from proteins prior LC-MS/MS analysis. However, ubiquitin chains bound by TUBES are protected from deubiquitylation by DUBs (Hjerpe et al., 2009) making this approach difficult. Another possibility to identify ubiquitylation substrates of E6 is to switch to an *in vitro* ubiquitylation assay using recombinantly expressed and purified E1, E2, E3 (E6AP) and E6 and cell lysates, which contain the so far unidentified substrates. In such an assay, TUBEs and/or a tagged ubiquitin version could be used to allow the isolation of ubiquitylated proteins. For example biotinylated ubiquitin could be used with subsequent isolation of ubiquitylated proteins by binding to avidin/streptavidin. This has the advantage that the biotin-avidin/streptavidin interaction is a very strong non-covalent binding (Gitlin et al., 1987). Thus, the usage of stringent washing conditions to reduce background binding would be possible. Therefore, it may be worthwhile to establish such a ubiquitylation assay for the identification of novel ubiquitylation substrates of E6.

## 5.4 ANALYSIS OF “HPV E6 INTERACTOME”

### 5.4.1 Identification of potential interaction partners of the chimeric 11C18 protein

To gain insights into the functions of low risk E6 proteins, we attempted to identify potential interaction partners of the chimeric 11C18 protein (“HPV E6 interactome”) in addition to the “proteome” and “ubiquitome” approaches (see 4.2 and 4.3). For the interaction study we used the chimeric 11C18 instead of the low risk 11E6 since it has the advantage that PDZ domain-containing proteins can serve as positive control. Indeed a number of PDZ-domain containing proteins were pulled down by 11C18 (table 4) and hScribb was among them, which is a known binding partner of E6 (Nakagawa and Huibregtse, 2000), demonstrating the applicability of our approach.

**Table 4: PDZ-domain containing proteins identified as potential interaction partners of GST-11C18**

Proteins identified as potential interaction partners of GST-11C18 by LC-MS/MS. Displayed are only proteins which contain PDZ domains.

gene name	protein name	number of peptides		
		PD 1	PD 2	PD 3
MLLT4	Afadin	20	9	3
TJP1	Tight junction protein ZO-1	18	11	27
Lin7C	Lin-7 homolog C	1	-	4
MPP6	MAGUK p55 subfamily member 6	8	5	7
MPP5	MAGUK p55 subfamily member 5	3	2	-
ERBB2IP	Protein Lap2	1	-	1
SCRIB	Protein scribble homolog	1	-	6
SNX27	Sorting nexin-27	2	2	2
SYNJ2BP	Synaptojanin-2-binding protein	3	2	2
TAX1BP3	Tax1-binding protein 3	2	2	1
SNTB2	Beta-2-syntrophin	1	1	-
PTPN4	Tyrosine-protein phosphatase non-receptor type 4	-	1	1

In addition, we identified several potential interaction partners of the chimeric 11C18 protein. Taken the three pulldown experiments together, we identified more than 880 proteins (figure 23) as potential interaction partners of GST-11C18, but only less than the half of these interaction partners were identified in all three experiments (table S4) showing a high variation between the different experiments.

Furthermore, we were interested in proteins that bind in an E6AP-dependent manner to 11C18, because it is known that low risk E6 proteins, as well as high risk E6 proteins, are able to interact with E6AP assuming that both types of E6-E6AP complexes share at least some ubiquitylation substrates (Brimer et al., 2007; Kuballa et al., 2007). However, in combined GST-11C18 pulldown/SILAC experiments we only identified a few possible E6AP-dependent binding partners (table 2). Although we performed the assay with an internal control, the determined ratios varied a lot: H1299 cells were labeled with light (L) and heavy (H) amino acids which should have resulted in values of H/L ratios of 1 (log2 value 0). In addition H1299 E6APi cells were labeled with medium (M) amino acids and therefore H/M and L/M ratios should have had the same values. However, for most of the identified proteins this was not the case. This finding may be due to the outline of the experiment: pulldown experiments with GST-11C18 and cell lysate of H1299 (light and heavy labeled) or H1299 E6APi (medium labeled) were performed separately and samples were combined at a late time point of the experiment. This implicates a high error rate during washing and elution steps. Nonetheless, seven proteins were identified that bind to GST-11C18 only in the presence of E6AP and one protein that binds only in the absence of E6AP (table 2). However, only one or two peptides were identified of these proteins. Thus, additional approaches are necessary to verify these

data. Moreover, one of the potential interaction partners is a mitochondrial protein and E6 is normally not located in this compartment (E6 was reported to localize to the nucleus or cytosol (Choulier et al., 2002; Kim et al., 1994a; Leverrier et al., 2007)). In conclusion we identified a few potential E6AP-dependent binding partners of GST-11C18 (table 2). However, further validation of these data using other approaches like coimmunoprecipitations is necessary.

Moreover, we identified several proteins that play a role in the ubiquitin proteasome pathway (table 3) as potential binding partners of 11C18. Among these, several E3 ligases were present but not E6AP. This was presumably due to the fact that bound proteins were eluted by high salt concentrations and under these conditions E6AP seems to remain bound to GST-11C18 (data not shown).

Two HECT-E3 ligases UBR5 (ubiquitin protein ligase E3 component n-recognin 5) and TRIP12 (Thyroid receptor-interacting protein 12) were found as potential binding partner. UBR5, also known as EDD (E3 Identified by Differential Display), has recently been reported to bind to E6 (Tomaic et al., 2011). Accordingly, we were able to confirm binding of *in vitro* translated EDD to E6 using GST-E6 pulldown assays (data not shown). Moreover, it was reported that EDD binds to E6AP in an E6-independent manner thereby regulating E6AP expression. However, using *in vitro* pulldown experiments, we were not able to reveal binding of GST-E6AP to EDD (data not shown).

EDD and TRIP12 were reported to be involved in the DNA-damage response (Gudjonsson et al., 2012). Both proteins control another E3 ligase, RNF168 which is involved in histone ubiquitylation after induction of DNA double strand breaks. Histones modified with a K63-linked ubiquitin chain serve as interacting platform for chromatin-associated repair and signaling factors (e.g. 53BP1) (reviewed in Bekker-Jensen and Mailand, 2011). Knockdown of EDD, TRIP12 or double knockdown of both proteins in U2OS cells leads to accumulation of RNF168 resulting in uncontrolled histone ubiquitylation with the consequence of excessive transcriptional silencing. This was shown by formation of enlarged G1 nuclear bodies (stained with anti 53BP1 antibody) after knockdown of EDD or TRIP12 (Gudjonsson et al., 2012). Intriguingly, enlarged G1 nuclear bodies were also described in advanced HPV positive cervical cancers (Matsuda et al., 2011). As E6 proteins can bind to EDD, one possible explanation for the enlarged G1 nuclear bodies in cervical cancer cells could be that E6 leads to proteasomal degradation of EDD resulting in elevated amounts of RNF168. *In vitro* ubiquitylation assays with EDD as potential substrate and E6/E6AP complex as E3 ligase showed only an inefficient ubiquitylation of EDD (data not shown). Furthermore, we investigated if induction of E6 expression in U2OS 16E6 cells (see 4.1) lead to enlarged G1 nuclear body formation (in collaboration with Bioimaging Center, University of Konstanz). However, no effect of E6 on nuclear body size was found (data not shown). Consequently,

these results do not provide strong evidence for the involvement of E6 in the formation of enlarged G1 nuclear bodies by reduction of EDD levels. Furthermore, although we identified EDD and TRIP12 in our “proteome” approach (see 4.2), the levels of these E3 ligases were not significantly changed upon induction of expression of HA-18E6 or HA-11C18. However, in the inducible U2OS E6 cell lines E6 levels are very low and in future experiments the G1 nuclear body size should be analyzed after transient expression of E6. In addition, the interaction of E6 with TRIP12 needs to be confirmed as E6 may regulate G1 nuclear body size via this E3 ligase and not via EDD. Another possibility for enlarged G1 nuclear bodies in cervical cancer tissues could be due to E7 expression as both E6 and E7 are usually expressed in these tissues (Durst et al., 1983).

In addition to EDD and TRIP12, we identified several other proteins involved in the ubiquitin proteasome pathway as potential interaction partners of GST-11C18 (table 3). However, for Cullin-7, Kelch-like protein 9, and S-phase kinase-associated protein 1 only one to three peptides for each protein were identified and even though we identified up to 11 peptides of the pre-mRNA factor 19 these potential interactions were not investigated further in this thesis for reasons of time.

## **5.5 CHARACTERIZATION OF THE INTERACTION OF THE HPV E6 PROTEINS WITH APC/C**

### **5.5.1 HPV E6 proteins bind to APC/C**

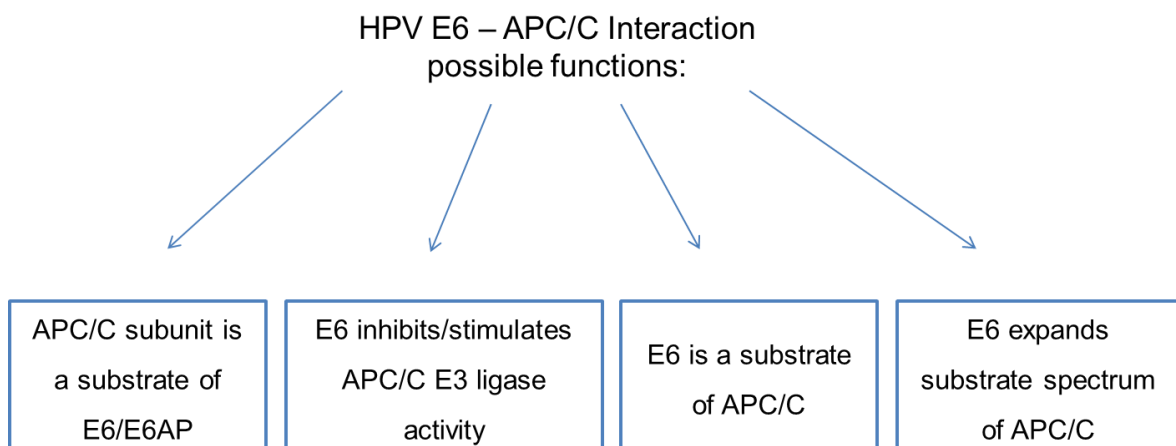
Three subunits of the multimeric E3 ligase APC/C namely APC1, APC5 and Cdc27 (table 3) were identified as potential interaction partners of the GST-11C18 protein (see 4.4). The best understood function of the multicomponent E3 ligase APC/C is regulation of the cell cycle by targeting key cell cycle regulators like cyclins for proteasomal degradation (reviewed in Pines, 2011). Some viral proteins are known to interact and manipulate APC/C for the benefit of the virus (reviewed in Mo et al., 2012). Thus, APC/C could also be an attractive target for HPV E6 proteins. We were able to confirm binding of GST-11C18 to APC/C in GST pulldown experiments using cell lysates of H1299 and U2OS cells (figure 25). Moreover, we showed that binding to APC/C is a common feature of the E6 proteins (figure 25, 26, 27 and 28).

As pulldown and coimmunoprecipitation assays were performed with cell lysates, binding can occur in a direct or indirect manner. However, further *in vitro* experiments with individual APC/C subunits are difficult to perform since recombinant expression and purification of single APC/C subunits in *E.coli* or insect cells (baculovirus system) and subsequent purification is problematic. The individual subunits are highly unstable presumably because of incorrect folding in the absence of the other proteins of the complex (Zhang et al., 2010).

Exceptions are the coactivators of APC/C (Cdh1 and Cdc20). Binding of all GST-E6 proteins to *in vitro* translated or baculovirus expressed Cdh1 or Cdc20 could be observed (figure 26, data not shown). However, these interactions seemed to be rather unspecific, because several other GST-fusion proteins that were supposed to serve as negative controls bound to Cdh1 and Cdc20 as well (figure 37A, data not shown). Thus, conclusions about the binding mode could not be drawn and further investigations are necessary to elucidate if E6 proteins bind to APC/C in an indirect or direct manner. If the latter is the case, the question arises which subunit(s) of APC/C are responsible for binding. As very recently recombinant expression of catalytically active APC/C complex was described direct binding to E6 proteins could be tested in the future using *in vitro* binding assays (Zhang et al., 2013). Moreover, binding assays with cell lysates derived of cells with knockdown of different subunits of the APC/C and could reveal insights in the mode of binding.

### 5.5.2 Functional characterization of the interaction of the E6 proteins and APC/C

Next we aimed to elucidate possible functions of the interaction between the E6 proteins and APC/C (Figure 42). For example, an APC/C subunit could serve as a substrate for the E6/E6AP complex. Another possibility is that E6 inhibits or stimulates the E3 ligase activity of APC/C or that E6 is a substrate of the APC/C. Finally, E6 could modulate the APC/C E3 ligase activity with respect to substrate recognition, leading to additional substrates degraded by APC/C in the presence of E6. This would be a similar mechanism compared to manipulation of E6AP by E6 proteins. However, it is very difficult to address the last possibility experimentally, as these additional substrates would need to be identified. Consequently, this potential function of the interaction of E6 with APC/C was not further analyzed.



**Figure 42: Possible functions of the interaction of HPV E6 and APC/C**  
Scheme shows possible functions of the interaction of HPV E6 with APC/C.

(i) *A subunit of APC/C is a ubiquitylation substrate* – firstly *in vitro* ubiquitylation assays with APC/C coactivators as potential substrates revealed no evidence for this hypothesis (figure 29). Secondly, we did not find protein levels of APC subunits (APC1, APC2, APC4 and APC7) to be altered in the presence of HA-18E6 or HA-11C18 in our proteome approach (see 4.2). These findings rather argue against an E6-dependent proteasomal degradation of APC/C subunits. However, it is believed that E6 can recruit a so far unknown E3 ligase (i.e. other than E6AP) for substrate ubiquitylation (Massimi et al., 2008) which could be also the case for an APC/C subunit.

(ii) *E6 affects E3 ligase activity of APC/C* - binding of E6 to APC/C may modulate the APC/C E3 ligase activity which should result in altered cell cycle progression. In more detail, inhibition of APC/C leads to an arrest of cells in G2/M or G1 phase of the cell cycle, while a stimulation or activation of APC/C activity should lead to premature exit of mitosis or G1 phase. However, cell cycle analysis by DNA staining and flow cytometry of inducible U2OS E6 cell lines did not reveal any effect on cell cycle progression upon expression of HA-18E6 or HA-11C18 (figure 30). Similar results were obtained upon transient expression of HA-18E6 in H1299 (figure 34) and U2OS (data not shown) cells. In addition, we analyzed U2OS 18E6 and U2OS 11C18 cells by time-laps-microscopy and determined the time from nuclear envelope breakdown to cytokinesis (data not shown). Again, we did not observe any differences in the presence of E6 suggesting that E6 does not have an effect on mitotic progression. However, cell cycle and time-laps-microscopy analyses were only performed with tumor derived cell lines which per se have abnormal cell cycle progression. Thus, in future experiments the effect of E6 expression on cell cycle progression in primary cells (e.g. human foreskin keratinocytes) should be analyzed. In fact, in such cells mitotic defects upon expression of the high risk 16E6 protein were described (Duensing et al., 2000; Duensing and Munger, 2002). Thus, it should also be tested whether these mitotic defects are due to binding of E6 to APC/C.

To analyze the effect of E6 proteins on E3 ligase activity of APC/C we switched to *Xenopus laevis* egg extracts (CSF). Using this system, we did not observe any effect of GST-16E6 or GST-18E6 on APC/C E3 ligase activity towards its substrates Securin and Cyclin B2 (figure 32 and 35, data not shown) although both proteins are able to bind *Xenopus laevis* APC/C (figure 31A). Moreover, GST-16E6 is functional in this extracts as it leads to the degradation of *in vitro* translated p53 which was added to the extract (figure 31B). However, as manipulation of APC/C could happen not only in a catalytically but also in a stoichiometric mechanism, the amounts of E6 proteins added to the extracts could have been too low to detect effects. Moreover, as *Xenopus laevis* egg extracts exclusively are assumed to contain APC/C<sup>Cdc20</sup> and not APC/C<sup>Cdh1</sup>, we cannot rule out that E6 has an effect on APC/C<sup>Cdh1</sup> activity. This issue could be addressed by degradation assays with an APC/C<sup>Cdh1</sup> dependent substrate and cell extracts of

G1 arrested cells which contain active APC/C<sup>Cdh1</sup>. Alternatively, *in vitro* ubiquitylation assays with APC/C immunoprecipitated from cells as E3 ligase could be performed (Rape and Kirschner, 2004; Williamson et al., 2009).

(iii) *E6 is a substrate of APC/C* - it is known that E6 proteins are ubiquitylated by an up to now unknown E3 ligase (Kehmeier et al., 2002; Stewart et al., 2004). Most APC/C substrates contain a degradation motif such as a D box (RXXL) or KEN box (reviewed in Barford, 2011b). E6 proteins do not comprise such a degradation motif and we have shown that GFP-E6 proteins are stable in *Xenopus laevis* egg extracts harboring active APC/C<sup>Cdc20</sup> (figure 33). Moreover, E6 ubiquitylation and levels within cells are not affected upon overexpression of Cdh1 or Cdc20 (data not shown), as shown for other APC/C substrates (Ichim et al., 2013). From these results, we conclude that APC/C is not an E3 ligase for E6.

Thus, further studies are necessary to identify the E3 ligase for E6. Potential candidates could be the E3 ligases identified in the interactome approach (table 3).

### 5.5.3 Functional characterization of the interaction of E6 proteins and APC/C in the presence of HPV E1 and E2 proteins

Since we were not able to obtain insights into the potential function of the interaction of E6 with APC/C in systems where only the E6 protein was present (see 4.5.4-4.5.7), we speculated that effects of this interaction are only detectable in the presence of other HPV proteins. HPV E1 and E2 proteins play an essential role in viral replication (see 1.1.4) and it was shown that HPV replication cannot only occur during S phase but also during G2 phase of the cell cycle (Reinson et al., 2012). So, our hypothesis was that one HPV protein (e.g. E1) is necessary to block cells in G2/M phase and another HPV protein (e.g. E6) is responsible for the cell to overcome this arrest and that modulation of APC/C activity plays a role in this process. This could favor the HPV life cycle, which is unique as it is linked to the differentiation program of the host cell, the keratinocyte (see 1.1). Furthermore, the possibility exists that more than one HPV protein is necessary to arrest cells. Cell cycle analyses of H1299 and U2OS cells transfected with E1, E2 or E6 proteins and coexpression of these proteins showed that coexpression of two HPV proteins, no matter which ones, leads to a modulation of cell cycle progression in so far as a decrease in cells in G1 phase was observed (figure 34, data not shown). However, an accumulation of cells in G2/M and S phase was not always detected. Eventually, analysis of progression of the cells throughout the complete cell cycle after cells were blocked at different cell cycle stages will be necessary to elucidate which stage is affected by expression of the HPV proteins.

Although it was reported that high risk E2 expression induces G2/M cell cycle arrest (Bellanger et al., 2005), we did not detect an effect on cell cycle progression upon expression

of HA-18E2 (figure 34, data not shown). This discrepancy in data could be due to the fact that cell cycle analyses were performed in different cell lines. We used in our studies H1299 and U2OS cells, whereas Bellanger et al. observed cell cycle arrests in Saos-2 and HeLa cells. Furthermore, different expression levels and the usage of differently tagged E2s could have resulted in these different findings (we used HA-tagged E2s, Bellanger et al. used GFP-E2s). Moreover, it is interesting to note that Bellanger et al. observed a G2/M cell cycle arrest in HeLa cells upon E2 expression, because these cells are HPV positive and express the HPV E6 and E7 proteins.

Expression of HPV E1 proteins in U2OS and C33A cells was described to arrest cells in S phase and G2/M phase via response to DNA damage checkpoint activation (Fradet-Turcotte et al., 2011; Reinson et al., 2012). However, in our experiments we did not detect an alteration of cell cycle progression after expression of HA-18E1. Fradet-Trucotte et al. analyzed the effect of various E1 proteins on cell cycle progression including 31E1 and 16E1 but not 18E1. This fact and that they used C33A cells could explain why we obtained different data. Reinson et al. worked with the same cellular system as we did (U2OS cells) and they did not observe considerable changes in the cell cycle profile upon expression of 18E1 or 18E2 alone in comparison to the mock control which is consistent with our data. Furthermore, our results are in line with the published data that coexpression of E1 and E2, results in a decreased fraction of cells in G1 phase (figure 34, data not shown). However, Reinson et al. observed a significant accumulation of cells in S and G2 phase after coexpression of E1 and E2 which we only observed in H1299 cells (figure 34) but not in U2OS cells (data not shown). In contrast to our experiments in which we cotransfected GFP to analyze only transfected cells, Reinson et al. achieved a transfection efficiency of 80-90 % which may have resulted in different data. In addition, our cell cycle analyses were performed only once and repetition experiments are necessary to draw conclusions about the effect of HPV proteins on cell cycle progression. In any case, from such cell cycle analyses we cannot conclude if the observed effects are indeed due to manipulation of APC/C by the HPV proteins.

To further analyze whether HPV proteins affect APC/C E3 ligase activity we used the *Xenopus laevis* egg extract system. Regardless of which GST tagged HPV protein we added to *Xenopus laevis* egg extracts we did not observe an effect on APC/C E3 ligase activity as its substrate Securin was degraded in a similar manner as in the GST control (figure 35). Addition of a combination of GST tagged HPV proteins to extracts did also not show an effect on APC/C E3 ligase activity (figure 35). However, as we were limited in the amount of GST proteins, which we can add to the *Xenopus laevis* egg extracts, because *Xenopus laevis* egg extracts can be diluted maximally 1:10 (Hannak and Heald, 2006), the amount of HPV proteins might have been too low to considerably affect APC/C activity. In future experiments, mRNAs coding for the different HPV proteins could be added to *Xenopus laevis* egg extracts, as in these extracts

translation of mRNAs occurs, which could lead to higher protein levels of the HPV proteins. An advantage of this approach is that untagged versions of the HPV proteins or at least proteins with a smaller epitope tag (e.g. HA) could be used as maybe the large GST-tag of the bacterially expressed HPV proteins could interfere with their functionality. However, at least for the E6 – p53 interaction (figure 26) and degradation of p53 in the *Xenopus laevis* egg extract system this is not the case (figure 31).

Furthermore, it should be considered to extend the analysis of the cell cycle and effects on APC/C E3 ligase activity to the HPV E7 protein. E7 proteins drive cells into S phase by the interaction with pRB pocket proteins (for details see 1.1.5.1). Thus maybe E7 needs to be present to detect effects of the other HPV proteins on cell cycle progression. Finally, HPV infection is restricted to keratinocytes, tumor cell lines and *Xenopus laevis* egg extracts may be the wrong systems to study the effects of HPV proteins on cell cycle and APC/C E3 ligase activity. Thus, primary cells or possibly HaCat cells (transformed keratinocytes) should be used to further investigate whether HPV proteins affect cell cycle progression and APC/C E3 ligase activity.

#### 5.5.4 HPV E1 proteins bind to Cdh1 but not to Cdc20

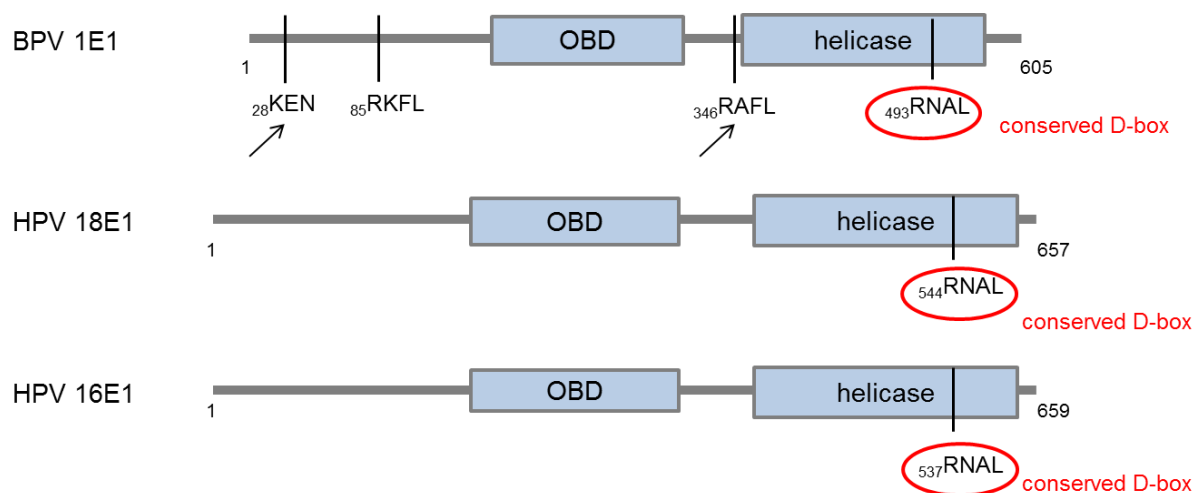
Binding of high risk E2 proteins to APC/C coactivators Cdc20 and Cdh1 was reported already in 2005 (Bellanger et al., 2005). However, we did not arrive at a clear-cut conclusion in this aspect, since *in vitro* binding assays with either *in vitro* translated or baculovirus expressed Cdh1/Cdc20 appeared to be rather unspecific (i.e. nearly every protein fused to GST we tested bound to them) (figure 36A, data not shown). Of note, Bellanger et al. performed their binding assays in a similar manner as we did. They showed that GST-E2 binds via its TAD (transactivation domain) to *in vitro* translated Cdh1 and Cdc20. As negative control they used GST-E2 $\Delta$ TAD, where they could not observe binding. However, binding was not verified using additional methods like coimmunoprecipitations. We also analyzed in our *in vitro* binding assays if GST-18E1 is able to interact with Cdh1 and Cdc20. Interestingly, it was the only GST-fusion protein which we tested that bound to *in vitro* translated Cdh1 but not to Cdc20 (figure 36 A, left panel) even though the protein amount of GST-18E1 used for the assay was lower than the amount of the others (figure 37 A, right panel). Moreover, binding to Cdh1 was also shown for GST-16E1 (figure 36 B) and a direct interaction between GST-16E1 and GST-18E1 and Cdh1 was verified using baculovirus expressed Cdh1. Next, coimmunoprecipitation assays with cell lysates should be performed to verify the interactions under more physiological conditions.

As mentioned above, Reinson et al. reported that coexpression of E1 and E2 induces an G2/M phase arrest (Reinson et al., 2012). Moreover, it is known that APC/C<sup>Cdh1</sup> is activated upon

DNA damage in G2 phase leading to cell cycle arrest (Bassermann et al., 2008) raising the question if the observed G2/M phase arrest by E1/E2 is mediated via APC/C<sup>Cdh1</sup>.

So far we did not obtain any evidence that E1 affects APC/C activity even in the presence of E2 using the *Xenopus laevis* egg extract system (figure 35). However, E1 and E2 are expressed at rather low levels which may not be sufficient to observe any effect. Moreover, *Xenopus laevis* extracts do not contain Cdh1. Thus, effect of E1 on APC/C activity should be repeated using cell extracts that contain active APC/C<sup>Cdh1</sup>.

For bovine papillomavirus (BPV) 1E1 it was described that it is a target for proteasomal degradation by APC/C (Mechali et al., 2004). Mechali et al. showed that this is only the case when E1 is replicative active and they assume that it is a mechanism to control E1 levels and thereby viral replication. This could be a mechanism to maintain a low copy number of the viral genome during latent infection (Mechali et al., 2004). Since HPV 18E1/HPV 16E1 and BPV 1E1 show similarity on amino acid level (39 % identity) and have the same functions during viral replication it should be considered that HPV 18E1 and HPV 16E1 are substrates of APC/C. BPV1 E1 consists three D-box motives and one KEN-box and Mechali et. al identified the KEN box and one D-box as degradation motifs (figure 43, marked by arrows). However, these two motifs are not conserved among HPV 16E1 and HPV 18E1, but one of the two additional D-box motives is also present in HPV 16E1 and HPV 18E1 and located in the helicase domain (figure 43). Mutation of this conserved D-box in BPV1 E1 resulted in a replicative inactive form of E1 (Mechali et al., 2004).



**Figure 43: Degradation motifs (D-boxes and KEN-boxes) of E1 proteins**

In BPV 1E1 three D-boxes (RXXL) and one KEN-box are present and the KEN box and <sup>346</sup>RAFL D-box were identified as the degradation motives responsible for APC/C mediated degradation (marked by arrow) (Mechali et al., 2004). The D-box present in the helicase domain is conserved among BPV1 E1, HPV 18E1 and HPV 16E1 whereas the KEN box and the other D-boxes are not conserved. Sequence alignments to evaluate conserved degradation motifs were performed with Uniprot/Swissprot Blast. Numbers indicate amino acid residues. OBD: origin binding domain.

Thus, this D-box mutant could not be analyzed with respect to APC/C mediated degradation of E1, as only the replicative active E1 protein is targeted for degradation. Future experiments should clarify if degradation of E1 proteins by APC/C is a common mechanism to control papillomavirus E1 protein levels. Furthermore, the involvement of the conserved D-box motif should be analyzed.

## 5.6 UBIQUITYLATION OF P53 BY THE E3 LIGASE LNX1

LNX1 is a RING E3 ligase that contains four PDZ domains and interacts with high risk E6 proteins mediated by the PDZ binding motif of E6 and the PDZ 1 domain of LNX1 (Weber, 2009). However, the function of this interaction is so far unknown. Using LNX1 as a control in p53 degradation assays in H1299 cells, we surprisingly found that LNX1 expression results in reduction of p53 levels (data not shown). As indicated (1.4.6) p53 is tightly regulated by post-translational modifications including ubiquitylation and subsequent proteasomal degradation. So far, Mdm2 (Mdm2 in mouse, Hdm2 in human) is the best characterized E3 ligase for p53 ubiquitylation and is believed to be the prime factor for regulation of p53 protein levels (Ringshausen et al., 2006). However, p53 can still be degraded in Mdm2-deficient mice and multiple other E3 ligases were identified to play a role in regulation of p53 (Camus et al., 2003).

Since an involvement of LNX1 in p53 ubiquitylation and degradation has not been described before, we verified that p53 levels are reduced in the presence of LNX1 and showed in addition that this is dependent on the E3 ligase activity of LNX1 (figure 37A). To do so, we used the DHFR-HA-ubiquitin-p53 system (figure 37B) and quantification of p53 levels showed that LNX1 mediates degradation of p53 with an apparent efficiency similar to Mdm2 (figure 37C). However, since Mdm2 levels cannot be detected by western blot analysis, it is not possible to compare expression levels of Flag-LNX1 and Mdm2 and thereby efficiencies. Moreover, coexpression of both E3 ligases showed an additional effect on p53 degradation indicating that both E3 ligases degrade p53 independent of one other (figure 37A).

Reduction of p53 levels by LNX1 is dependent on its E3 ligase activity (figure 37), suggesting that LNX1 induced ubiquitylation of p53 leading to subsequent degradation. Indeed, we could show that LNX1 ubiquitylates p53 *in vitro* (figure 39). However, ubiquitylation assay in H1299 cells did not reveal a clear result concerning ubiquitylation of p53 by LNX1. A slight high molecular weight smear in the presence of HA-TUBEs, which stabilize ubiquitylated proteins, was detected when LNX1 was coexpressed. This effect was reproducibly seen and dependent on E3 ligase activity of LNX1 (figure 40, data not shown) suggesting that LNX1 ubiquitylates p53 *in cellulo*. Another hint that LNX1 ubiquitylates p53 *in cellulo* is that unmodified p53 levels are stabilized in input samples in the presence of LNX1 and TUBEs

(figure 40), which was also detected for E6 mediated ubiquitylation of p53 (see figure 19). However, further studies with for example proteasome inhibitors are necessary to show that reduction of p53 levels by LNX1 is indeed mediated by ubiquitylation and proteasomal degradation. Moreover, since high risk E6 proteins also interact with LNX1 (Weber, 2009), it should be investigated if E6 has an effect on LNX1 mediated p53 ubiquitylation/degradation. Though, it should be noted that E6 does not have an effect on LNX1 auto-ubiquitylation and Numb ubiquitylation (Schätzle, 2011, under supervision).

The family of LNX proteins consists of five members (for details see 1.3.1) and among these proteins LNX1 and LNX2 share an identical domain structure and are 50 % identical on amino acid level (Flynn et al., 2011). Moreover, it was shown that LNX2 also functions as an E3 ligase for Numb which is the best characterized substrate of LNX1 (Rice et al., 2001) assuming that they share some common functions. Thus, it would be worthwhile to examine if LNX2 is also able to ubiquitylate and degrade p53.

It was reported that Mdm2 can bind and ubiquitylate Numb (Yogosawa et al., 2003), a known LNX1/2 substrate (Nie et al., 2002; Rice et al., 2001). Moreover, a role of Numb in the stabilization and activation of p53 was shown in an Mdm2-dependent manner (Colaluca et al., 2008). However, the actual mechanism of Numb-induced activation of p53 is so far not understood. One could envisage at least two mechanisms, by which LNX1 regulates p53 levels: direct regulation by ubiquitylation and indirect regulation by controlling Numb protein levels. Future studies should analyze if the observed degradation of p53 by LNX1 is dependent on Numb and Mdm2. *In vitro*, we have shown that LNX1 alone is able to bind and ubiquitylate p53 indicating that Numb and Mdm2 are not involved in this mechanism. Nonetheless, degradation assays in Mdm2 and Numb knockdown cells should be performed to elucidate if this is also the case within cells.

All *in cellulo* experiments were performed with ectopically expressed proteins. Thus, it will be important to determine if endogenous p53 and LNX1 are able to interact with each other. In addition, the regions on both proteins which are responsible for binding should be identified to obtain first insights in potential functions of this interaction. Moreover, experiments using the RNA interference technology to down-regulate (knock down) endogenous LNX1 expression levels should be performed to investigate if p53 is stabilized and activated under these conditions. In fact, knockdown of LNX1 in Hek293 cells was shown to arrest cells in G0/G1 phase (Zheng et al., 2011). A whole-genome expression microarray analysis of LNX1 knockdown and control cells indicated that several pathways including p53 are involved in this cell cycle arrest and several p53 target genes were found to be activated, for example Noxa, XRCC5, STAG1 and VCAN (Zheng et al., 2011). However, this study was performed with Hek293 cells, which are human embryonic kidney cells transformed by adenovirus with the consequence that p53 should be inactive in these cells (Grand et al., 1995). Thus, it seems

very unlikely that the observed G0/G1 phase arrest in these cells is due to p53 stabilization and activation. Knockdown of LNX1 and its effect on the p53 signaling pathway should be performed in cell lines which contain active wild-type p53 as it is the case in U2OS cells.

In conclusion, we identified LNX1 as a new E3 ligase that may play a role in p53 ubiquitylation. Further investigations are necessary to elucidate the complete mechanism and function of LNX1 mediated ubiquitylation of p53.

## 6 SUPPLEMENTARY DATA

**Table S1: “HPV E6 proteome” - proteins with significantly altered expression levels in the presence of HA-18E6 protein, identified by LC-MS/MS**

Protein names	gene names	ratio H/L (log2)	peptides	sequ. cov. [%]	biological processes
Heat shock 70 kDa protein 1A/1B	HSPA1A	1.04096	19	38.5	stress response
highly similar to HEAT SHOCK 70 kDa PROTEIN 1	DAAP-21F2.8-002	1.03991	16	32.4	stress response
Cytospin-A	SPECC1L	0.714136	3	3.4	cell cycle, cell division
Neural cell adhesion molecule L1	L1CAM	0.703455	3	3	cell adhesion, differentiation, neurogenesis
Prolyl 4-hydroxylase subunit alpha-2	P4HA2	0.640714	12	35.5	N/A
Laminin subunit gamma-2	LAMC2	0.639417	2	1.8	cell adhesion, differentiation, neurogenesis
Procollagen-lysine,2-oxoglutarate 5-dioxygenase 2	PLOD2	0.621524	12	20.8	N/A
Replication protein A 14 kDa subunit	RPA3	0.621337	2	29.8	DNA damage, DNA recombination, DNA repair, DNA replicaton
Myosin I beta	MYO1C	0.615228	9	11.9	protein transport, transport, translocation, mRNA transport
E3-keto-steroid reductase	HSD17B7	0.598651	2	8.5	lipid biosynthesis, lipid metabolism, steorid biosynthesis
Elongation factor Tu GTP-binding domain-containing protein 1	EFTUD1	0.579759	4	2.9	protein biosynthesis, ribosome biogenesis
Palladin	PALLD	0.540027	3	2.3	cytoskeleton organization
Calretinin	CALB2	0.537843	4	23.2	N/A
Vimentin	VIM	0.534659	33	68.2	host-virus interaction
GDH/6PGL endoplasmic bifunctional protein	H6PD	0.529371	2	2.3	carbohydrate metabolism, glucose metabolism
Pogo transposable element with ZNF domain	POGZ	0.518233	2	1.2	cell cycle, cell division
Endothelial protein C receptor	PROCR	0.511468	3	13.1	blood coagulation, hemostasis
NADH dehydrogenase [ubiquinone] complex I, assembly factor 7	NDUFAF7	0.510456	2	6.6	N/A
Plastin-3	PLS3	0.504875	2	3.7	N/A
DnaJ homolog subfamily A member 1	DNAJA1	0.49375	3	9.6	Cochaperone of Hsc70
Keratin, type I cytoskeletal 18	KRT18	0.49211	8	26.7	cell cycle, host virus interaction
MYH-1c	MYO1B	0.485118	7	6.3	Motorprotein

Table S1 continued:

protein names	gene names	ratio H/L (log2)	peptides	sequ. cov. [%]	biological processes
ADP-ribosylation factor 5;ADP-ribosylation factor 5	ARF5	0.484087	5	30.6	ER-Golgi transport, protein transport, transport
Peptidyl-prolyl cis-trans isomerase C	PPIC	0.477884	3	12.7	N/A
Collagen alpha-2(IV) chain	COL4A2	0.470198	2	1.4	angiogenesis
14-3-3 protein eta	YWHA	0.466966	6	28.5	N/A
Chloride intracellular channel protein 4	CLIC4	0.455123	3	19.8	ion transport, transport
Laminin subunit beta-3	LAMB3	0.453965	5	6.1	cell adhesion
Nucleobindin-1	NUCB1	-0.456564	2	5.2	N/A
La-related protein 1	LARP1	-0.456702	6	6.5	N/A
Coiled-coil-helix-coiled-coil-helix domain-containing protein 3, mitochondrial;	CHCHD3	-0.464047	2	12.9	transcription, transcription regulation
Golgi to ER traffic protein 4 homolog	GET4	-0.466098	2	7.9	transport
Kinesin-like protein KIF2C	KIF2C	-0.468352	4	8	cell cycle, cell division, chromosome partition, mitosis
NES protein	NES	-0.471589	17	34.5	N/A
Nuclear pore complex protein Nup153	NUP153	-0.475976	2	2.3	host-virus interaction, protein transport, translocation, transport, viral penetration into nucleus, virus entry into host cell, mRNA transport
Sorting nexin-1	SNX1	-0.484348	3	6.8	protein transport, transport
Protein phosphatase methylesterase 1	PPME1	-0.493865	2	5.4	demethylation of proteins
E3 ubiquitin-protein ligase CHIP	STUB1	-0.497079	2	7.6	DNA damage, DNA repair, Ubl conjugation pathway
Nucleolar RNA helicase 2	DDX21	-0.506804	2	4.8	RNA helicase, RNA foldase
Importin subunit alpha-3	KPNA3	-0.518618	3	8.3	Host-virus interaction, protein transport, transport, viral penetration into nucleus, virus entry into host cell, mRNA transport
Adenosine 3-phospho 5-phosphosulfate transporter 1	SLC35B2	-0.52772	2	5.8	N/A
Protein furry homolog-like	FRYL	-0.535164	4	1.2	transcription, transcription regulation
Importin-8	IPO8	-0.54103	2	1.9	protein transport, transport
2,5-phosphodiesterase 12	PDE12	-0.547784	3	6.2	N/A

**Table S1 continued:**

protein names	gene names	ratio H/L (log2)	peptides	sequ. cov. [%]	biological processes
Dimethyladenosine transferase 1, mitochondrial	TFB1M	-0.550655	2	6.6	transcription, transcription regulation, rRNA processing
NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 2	NDUFA2	-0.555672	2	31.3	electron transport, respiratory chain, transport
Peptidyl-prolyl cis-trans isomerase-like 4	PPIL4	-0.602438	2	6.1	folding of proteins
ATP-dependent RNA helicase DDX54	DDX54	-0.614205	2	1.9	transcription, transcription regulation
SPARC	SPARC	-0.615707	2	9.9	cell growth
Acylamino-acid-releasing enzyme	APEH	-0.617698	2	3.1	N/A
Endoplasmic reticulum metalloproteinase 1	ERMP1	-0.629523	2	2.5	N/A
NADH-ubiquinone oxidoreductase chain 5	MT-ND5	-0.675858	2	4.6	electron transport, respiratory chain, transport
Desmoplakin	DSP	-1.05871	3	0.9	cell-cell adhesion
Zyxin	ZYX	-1.12727	2	6.8	cell adhesion, host-virus interaction
Protein S100-A10	S100A10	-1.16278	3	40.2	N/A
N-acetylneuraminic acid cytidyltransferase	CMAS	-1.22092	2	6.2	N-acetylneuraminic acid metabolic process
Iporin	RUSC2	-1.44886	2	1.4	N/A
Heterogeneous nuclear ribonucleoprotein L	HNRNPL	-1.51655	6	20.2	mRNA splicing
POTE ankyrin domain family member 1	POTE1	-1.90358	5	5.9	N/A
Receptor tyrosine-protein kinase erbB-2	ERBB2	-2.83681	2	2.2	transcription, transcription regulation

**Table S2: "HPV E6 proteome" - proteins with significantly altered expression levels in the presence of HA-11C18 protein, identified by LC-MS/MS**

protein name	gene name	ratio H/L (log2)	peptides	sequ. cov. [%]	biological processes
Glycine N-acyltransferase	GLYAT	2.4551	2	6.4	detoxification
Syntaxin-7	STX7	1.83329	2	9.2	alternative splicing
VIP36-like protein	LMAN2L	1.57773	1	3.1	N/A
Adenylyl cyclase-associated protein	CAP1	1.54987	3	15.7	cell polarity
Isobutyryl-CoA dehydrogenase, mitochondrial	ACAD8	0.899485	1	3.1	Branched-chain amino acid catabolism, transcription, transcription regulation
Interleukin 18	IL18	0.777788	3	13.5	Cytokine

Table S2 continued:

protein name	gene name	ratio H/L (log2)	peptides	sequ. cov. [%]	biological processes
Solute carrier family 2, facilitated glucose transporter member 14	SLC2A14	0.74992	2	2.8	differentiation, spermatogenesis, sugar transport, transport
Phospholipase A-2-activating protein	PLAA	0.696261	2	4.3	involved in the maintenance of ubiquitin levels
Annexin A6	ANX6	0.690819	8	15.8	calcium ion transport
Acidic leucine-rich nuclear phosphoprotein 32 family member A	ANP32A	0.611833	4	28.1	N/A
Superoxide dismutase [Cu-Zn]	SOD1	0.605874	2	44.8	destroys radicals
Myosin I beta	MYO1C	0.604641	13	15.1	protein transport, transport, translocation, mRNA transport
Protein CutA	CUTA	0.581689	2	16.2	N/A
Replication protein A 14 kDa subunit	RPA3	0.568908	2	29.8	DNA damage, DNA recombination, DNA repair, DNA replication
CDP-diacylglycerol--inositol3-phosphatidyltransferase	CDIPT	0.561497	1	4.6	lipid biosynthesis, lipid metabolism, phospholipid biosynthesis, phospholipid metabolism
MYH-1c	MYO1B	0.543397	2	1.9	Motorprotein
Golgi-specific brefeldin A-resistance guanine nucleotide exchange factor 1	GBF1	0.536053	3	1.9	COPI coating of vesicle, Guanine-nucleotide releasing factor
Sorting nexin-1	SNX1	0.532866	2	7.4	protein transport, transport
Histidine triad nucleotide-binding protein 1	HINT1	0.521553	3	40.5	apoptosis, transcription, transcription regulation
DNA replication complex GINS protein PSF3	GINS3	0.515309	1	3.1	DNA replication
RNA polymerase-associated protein LEO1	LEO1	-0.493236	1	2	transcription, transcription regulation, Wnt signaling pathway
Antigen KI-67	MKI67	-0.504305	1	0.8	cell cycle
Methylmalonyl-CoA isomerase	MUT	-0.509553	1	1.5	degradation of amino acids, odd chain fatty acids and cholesterol
YLP motif-containing protein 1	YLPM1	-0.530156	2	1.6	transcription, transcription regulation
Ancient ubiquitous protein 1	AUP1	-0.53621	1	3.8	Ubl conjugation pathway

Table S2 continued:

protein name	gene name	ratio H/L (log2)	peptides	sequ. cov. [%]	biological processes
Ras-related protein Rab-32	RAB32	-0.549325	2	9.8	protein transport
Plasminogen activator inhibitor 1 RNA-binding protein	SERBP1	-0.549388	1	4.7	regulation of mRNA stability
Kinase D-interacting substrate of 220 kDa	KIDINS220	-0.551099	2	1.2	MAPKinase signaling
Uncharacterized protein C7orf50	C7orf50	-0.569693	1	6.2	N/A
Protein PRRC2A	PRRC2A	-0.590549	1	1.1	regulation of pre mRNA splicing
OCIA domain-containing protein 1	OCIAD1	-0.623176	1	7.2	N/A
E3 ubiquitin-protein ligase TRIM33	TRIM33	-0.62431	2	1.7	transcription, transcription regulation, Ubl conjugation pathway
ADP/ATP translocase 1	SLC25A4	-0.654694	8	29.9	Host-virus interaction, transport
Probable RNA-binding protein 19	RBM19	-0.678049	2	2.4	N/A
Protein furry homolog-like	FRYL	-0.69108	3	0.9	transcription, transcription regulation
DAZ-associated protein 1	DAZAP1	-0.762803	1	5.2	differentiation, spermatogenesis
Melanoma-associated antigen B2	MAGEB2	-0.865726	1	3.8	Ubl conjugation pathway
Kinesin-like protein KIF23	KIF23	-0.874825	1	1.7	cell cycle, cell division, mitosis
DNA-directed RNA polymerase I subunit RPA34	CD3EAP	-1.11422	1	3.5	transcription
cDNA FLJ60028, highly similar to Sodium/potassium-transporting ATPase alpha-2 chain (EC 3.6.3.9)	N/A	-1.35775	2	16	ion transport
Metaxin-2	MTX2	-1.38605	2	12.2	protein transport, transport
Exocyst complex component 5	EXOC5	-1.41361	2	4.5	exocytosis, protein transport, transport
Eukaryotic translation initiation factor 2 subunit 2	EIF2S2	-1.73423	2	7.8	proteinbiosynthesis

**Table S2 continued:**

protein name	gene name	ratio H/L (log2)	peptides	sequ. cov. [%]	biological processes
Apolipoprotein B-100	APOB	-2.04174	2	0.5	lipid metabolism, lipid transport, steroid metabolism, sterol metabolism, transport
Desmoplakin	DSP	-2.68223	2	0.5	cell-cell adhesion
Prolactin-inducible protein	PIP	-2.82296	1	6.8	N/A
Endoplasmic reticulum mannosyl-oligosaccharide 1,2-alpha-mannosidase	MAN1B1	-2.89486	1	1.8	glycoprotein quality control
Lysozyme C	LYZ	-4.49691	2	16.2	defense response to bacterium

**Table S3: "HPV E6 ubiquitome" - potential E6 dependent ubiquitylated proteins isolated by TUBEs**

protein name	gene name	number of peptides					
		empty 1	empty 2	Flag-18E6_1	Flag-18E6_2	Flag-11C18_1	Flag-11C18_2
116 kDa U5 small nuclear ribonucleoprotein component	EFTUD2	-	3	-	3	-	4
26S protease regulatory subunit 4	PSMC1	-	-	-	3	-	2
26S protease regulatory subunit 7	PSMC2	-	-	-	-	-	2
40S ribosomal protein S12	RPS12	-	-	-	-	2	-
40S ribosomal protein S15a	RPS15A	-	2	-	2	1	-
40S ribosomal protein S16	RPS16	3	4	-	2	1	2
40S ribosomal protein S19	RPS19	-	-	-	3	-	-
40S ribosomal protein S2	RPS2	-	-	-	-	4	-
40S ribosomal protein S24	RPS24	2	-	-	2	-	-

**Table S3 continued:**

protein name	gene name	number of peptides					
		empty 1	empty 2	Flag-18E6_1	Flag-18E6_2	Flag-11C18_1	Flag-11C18_2
40S ribosomal protein S3a	RPS3A	-	-	2	-	3	-
60 kDa heat shock protein, mitochondrial	HSPD1	3	2	-	7	2	7
60S acidic ribosomal protein P0	RPLP0	4	6	-	3	3	4
60S ribosomal protein L11	RPL11	-	2	-	2	2	2
60S ribosomal protein L12	RPL12	2	-	-	-	2	2
60S ribosomal protein L13	RPL13	-	2	-	3	2	-
60S ribosomal protein L22	RPL22	-	-	-	-	-	2
60S ribosomal protein L23	RPL23	3	3	2	-	3	-
60S ribosomal protein L23	RPL23	-	-	-	-	-	3
60S ribosomal protein L24	RPL24	-	2	-	-	-	-
60S ribosomal protein L27a	RPL27A	-	-	-	-	2	-
60S ribosomal protein L6	RPL6	-	-	-	-	-	2
60S ribosomal protein L7a	RPL7A	-	-	-	-	2	-
6-phosphofructokinase type C	PFKP	2	-	-	-	-	-
78 kDa glucose-regulated protein	HSPA5	4	11	-	7	11	8
Actin, cytoplasmic 1	ACTB	6	8	8	8	7	10
Aladin	AAAS	-	-	-	-	-	2
Anaphase-promoting complex subunit 1	ANAPC1	-	-	-	-	-	2
Arginine--tRNA ligase	RARS	-	-	-	2	-	-

**Table S3 continued:**

protein name	gene name	number of peptides					
		empty 1	empty 2	Flag-18E6_1	Flag-18E6_2	Flag-11C18_1	Flag-11C18_2
Aspartate--tRNA ligase, cytoplasmic	DARS	2	-	-	-	-	-
ATP synthase subunit alpha	ATP5A1	2	10	-	9	-	12
ATP synthase subunit f, mitochondrial	ATP5J2	2	2	2	2	2	2
ATP synthase subunit O, mitochondrial	ATP5O	-	-	-	-	-	2
ATPase family AAA domain-containing protein 3A	ATAD3A	4	4	-	4	3	5
ATPase family AAA domain-containing protein 3B	ATAD3B	-	-	-	2	-	-
ATPase WRNIP1	WRNIP1	2	4	-	3	4	5
ATP-dependent RNA helicase A	DHX9	3	6	2	7	2	7
ATP-dependent RNA helicase DDX3X	DDX3X	-	3	6	6	9	8
ATP-dependent RNA helicase DDX3Y	DDX3Y	3	-	-	-	-	-
ATP-dependent zinc metalloprotease YME1L1	YME1L1	-	-	-	-	-	2
BTB/POZ domain-containing adapter for CUL3-mediated RhoA degradation protein 3	KCTD10	-	2	-	-	3	-
CAD protein	CAD	-	4	-	2	-	4
Calcium-binding mitochondrial carrier protein Aralar1	SLC25A12	-	-	-	-	-	4
Calcium-binding mitochondrial carrier protein Aralar2	SLC25A13	-	3	3	4	2	6

**Table S3 continued:**

protein name	gene name	number of peptides					
		empty 1	empty 2	Flag-18E6_1	Flag-18E6_2	Flag-11C18_1	Flag-11C18_2
Cathepsin D	CTSD	3	-	-	-	-	-
Cell division cycle protein 23 homolog	CDC23	-	-	-	-	-	3
Clathrin heavy chain 1	CLTC	-	2	2	2	-	6
CTP synthase 1	CTPS1	-	-	-	-	-	2
Cullin-3	CUL3	2	-	-	-	-	-
Dermcidin	DCD	-	2	-	-	3	-
Desmoplakin	DSP	-	-	-	-	3	4
DNA damage-binding protein 1	DDB1	-	5	-	4	-	-
DNA replication licensing factor MCM7	MCM7	3	4	3	4	2	4
DNA-dependent protein kinase catalytic subunit	PRKDC	4	3	-	7	8	12
DNA-directed RNA polymerase II subunit RPB1	POLR2A	-	2	-	2	-	6
DNA-directed RNA polymerase II subunit RPB2	POLR2B	-	4	-	4	-	5
DNA-directed RNA polymerase II subunit RPB3	POLR2C	-	-	-	-	-	2
DnaJ homolog subfamily C member 10	DNAJC10	2	-	-	-	-	-
Dolichyl-diphosphooligosaccharide --protein glycosyltransferase 48 kDa subunit	DDOST	3	5	2	4	3	4

**Table S3 continued:**

protein name	gene name	number of peptides					
		empty 1	empty 2	Flag-18E6_1	Flag-18E6_2	Flag-11C18_1	Flag-11C18_2
Dolichyl-diphosphooligosaccharide--protein glycosyltransferase subunit 1	RPN1	2	4	2	5	6	5
E3 ubiquitin-protein ligase RNF213	RNF213	2	4	-	3	3	8
E3 ubiquitin-protein ligase UBR5	UBR5	8	9	7	6	9	22
Elongation factor 1-alpha 1	EEF1A1	-	-	-	-	2	2
Elongation factor 1-gamma	EEF1G	-	-	-	-	-	2
Elongation factor Tu, mitochondrial	TUFM	5	5	-	5	6	7
Endoplasmic reticulum chaperone	HSP90B1	2	-	-	-	-	-
Erlin-1	ERLIN1	-	3	-	-	2	4
Eukaryotic initiation factor 4A-I	EIF4A1	-	-	-	-	-	2
Fatty acid synthase	FASN	2	2	-	-	-	-
F-box only protein 28	FBXO28	-	2	-	-	2	2
General transcription factor II-I	GTF2I	-	2	-	-	-	-
Gigaxonin	GAN	2	-	-	4	5	4
Glyceraldehyde-3-phosphate dehydrogenase	GAPDH	2	-	-	-	3	3
Golgi to ER traffic protein 4 homolog	GET4	2	4	3	3	3	-
Guanine nucleotide-binding protein subunit beta-2-like	GNB2L1	-	-	-	-	3	-
Heat shock 70 kDa protein 1A/1B	HSPA1A	6	7	8	8	6	2

**Table S3 continued:**

protein name	gene name	number of peptides					
		empty 1	empty 2	Flag-18E6_1	Flag-18E6_2	Flag-11C18_1	Flag-11C18_2
Heat shock cognate 71 kDa protein	HSPA8	12	15	-	14	14	8
Heat shock protein HSP 90-beta	HSP90AB1	4	4	-	-	2	14
Heterogeneous nuclear ribonucleoprotein A1	HNRNPA1	-	-	4	-	-	5
Heterogeneous nuclear ribonucleoprotein A1-like 2	HNRNPA1 L2	-	2	-	-	2	-
Heterogeneous nuclear ribonucleoprotein D0	HNRNPD	-	-	-	2	-	4
Heterogeneous nuclear ribonucleoprotein D-like	HNRPDL	-	-	-	-	-	2
Heterogeneous nuclear ribonucleoprotein F	HNRNPF	2	2	3	3	4	4
Heterogeneous nuclear ribonucleoprotein H	HNRNPH1	-	-	-	-	-	2
Heterogeneous nuclear ribonucleoprotein H3	HNRNPH3	2	3	3	3	3	3
Heterogeneous nuclear ribonucleoprotein K	HNRNPK	-	-	-	-	-	2
Heterogeneous nuclear ribonucleoprotein M	HNRNPM	5	6	-	5	8	5
Heterogeneous nuclear ribonucleoprotein Q	SYNCRIP	-	-	3	2	-	3
Heterogeneous nuclear ribonucleoprotein R	HNRNPR	-	-	-	-	3	4
Heterogeneous nuclear ribonucleoprotein U	HNRNPU	2	2	-	-	2	4
Heterogeneous nuclear ribonucleoprotein U-like protein 1	HRNPUL1	17	16	8	18	19	21
Heterogeneous nuclear ribonucleoproteins A2/B1	HNRNPA2 B1	-	2	-	-	-	3

**Table S3 continued:**

protein name	gene name	number of peptides					
		empty 1	empty 2	Flag-18E6_1	Flag-18E6_2	Flag-11C18_1	Flag-11C18_2
Histone H2A type 1-H	HIST1H2A H	2	2	2	2	2	2
Histone H4	HIST1H4A	-	3	2	2	2	-
Importin subunit beta-1	KPNB1	-	-	-	-	2	3
Insulin-like growth factor 2 mRNA-binding protein 1	IGF2BP1	2	-	-	2	3	-
Interleukin enhancer-binding factor 2	ILF2	3	3	2	4	4	4
Interleukin enhancer-binding factor 3	ILF3	2	-	-	-	-	5
Isoleucine--tRNA ligase, cytoplasmic	IARS	-	-	-	2	-	3
Junction plakoglobin	JUP	-	-	-	-	-	5
Kelch-like protein 12	KLHL12	-	-	-	-	-	2
Large proline-rich protein BAG6	BAG6	5	10	5	12	10	2
L-lactate dehydrogenase B chain	LDHB	-	-	-	-	2	6
Long-chain-fatty-acid--CoA ligase 3	ACSL3	-	2	-	-	-	-
Lysine--tRNA ligase	KARS	-	2	-	-	-	-
Lysozyme C	LYZ	2	-	-	-	-	-
Major vault protein	MVP	8	10	7	3	7	17
Medium-chain specific acyl-CoA dehydrogenase, mitochondrial	ACADM	2	2	-	-	-	2
Mitochondrial import inner membrane translocase subunit TIM50	TIMM50	-	3	-	3	3	3
mRNA cap guanine-N7 methyltransferase	RNMT	2	-	-	-	-	2
Myb-binding protein 1A	MYBBP1A	2	-	-	-	3	3

**Table S3 continued:**

protein name	gene name	number of peptides					
		empty 1	empty 2	Flag-18E6_1	Flag-18E6_2	Flag-11C18_1	Flag-11C18_2
Myoferlin	MYOF	-	-	-	-	-	2
Myosin-9	MYH9	9	4	-	7	7	21
NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 9, mitochondrial	NDUFA9	-	3	-	-	-	-
Nuclear protein localization protein 4	NPLOC4	-	-	-	2	-	3
Nucleolar protein 56	NOP56	-	-	-	-	2	-
Nucleolar RNA helicase 2	DDX21	2	2	2	3	2	4
Nucleolin	NCL	-	9	2	2	7	8
Nucleophosmin	NPM1	-	2	-	2	3	2
Phosphate carrier protein, mitochondrial	SLC25A3	-	-	-	-	2	-
Plectin	PLEC	2	-	-	4	-	4
Poly(U)-binding-splicing factor PUF60	PUF60	2	-	-	-	-	3
Polyadenylate-binding protein 1	PABPC1	4	5	4	2	6	8
Polyadenylate-binding protein 4	PABPC4	-	-	-	-	6	6
Polyubiquitin-C	UBC	6	6	7	6	5	8
Prelamin-A/C OS=Homo sapiens	LMNA	-	-	-	-	2	-
Pre-mRNA-processing-splicing factor 8	PRPF8	-	-	-	-	2	-
Probable arginine--tRNA ligase, mitochondrial	RARS2	-	-	-	-	-	2
Probable ATP-dependent RNA helicase DDX17	DDX17	-	-	-	-	-	4
Probable ATP-dependent RNA helicase DDX41	DDX41	2	-	-	4	4	5
Probable ATP-dependent RNA helicase DDX5	DDX5	7	5	4	10	8	11

**Table S3 continued:**

protein name	gene name	number of peptides					
		empty 1	empty 2	Flag-18E6_1	Flag-18E6_2	Flag-11C18_1	Flag-11C18_2
Prohibitin-2	PHB2	2	3	-	2	3	3
Protein TFG	TFG	3	4	-	5	4	4
Putative ATP-dependent RNA helicase DHX30	DHX30	2	6	-	7	5	8
Putative pre-mRNA-splicing factor ATP-dependent RNA helicase DHX15	DHX15	2	-	-	2	3	3
Putative pre-mRNA-splicing factor ATP-dependent RNA helicase DHX16	DHX16	-	-	-	-	-	2
Putative ribosomal RNA methyltransferase NOP2	NOP2	-	-	-	-	-	2
Pyruvate kinase isozymes M1/M2	PKM	-	-	-	5	-	4
Ribosomal L1 domain-containing protein 1	RSL1D1	-	-	-	2	-	-
RNA-binding protein FUS	FUS	3	3	-	4	2	5
rRNA 2'-O-methyltransferase fibrillarin	FBL	-	-	-	-	2	-
RuvB-like 1	RUVBL1	-	-	-	-	-	3
Serine hydroxymethyltransferase, mitochondrial	SHMT2	-	-	-	2	-	-

**Table S4: “HPV E6 interactome” - GST-11C18 interaction partners identified in all three pulldown experiments**

protein names	gene names	number of peptides		
		PD1	PD2	PD3
DNA-dependent protein kinase catalytic subunit	PRKDC	41	12	3
ATP-dependent RNA helicase A	DDX9	31	23	37
Putative ATP-dependent RNA helicase DHX30	DDX30	28	16	30
Pre-mRNA-processing-splicing factor 8	PRPF8	27	11	40
Plectin-1	PLEC1	26	13	7
Myb-binding protein 1A	MYBBP1A	20	17	37
Afadin	MLLT4	20	9	3
Heterogeneous nuclear ribonucleoprotein U	HNRNPU	20	11	19
116 kDa U5 small nuclear ribonucleoprotein component	SNRP116	19	12	22
ATP-dependent DNA helicase 2 subunit 1	XRCC6	19	15	23
Ras GTPase-activating-like protein IQGAP1	IQGAP1	18	8	12
Probable ATP-dependent RNA helicase DDX5	DDX5	18	16	27
Probable ATP-dependent RNA helicase DDX17	DDX17	18	13	17
Splicing factor 3B subunit 3	SF3B3	18	13	18
Tight junction protein ZO-1	ZO1	18	11	27
Nucleolar RNA helicase 2	DDX21	17	12	34
Pre-mRNA-splicing factor SF3b 155 kDa subunit	SF3B1	17	12	18
5'-3' exoribonuclease 2	XRN2	17	9	6
ATP-dependent RNA helicase DDX3X	DDX3X	17	14	17
Superkiller viralicidic activity 2-like 2	SKIV2L2	17	11	11
Polyadenylate-binding protein 1	PAB1	16	11	16
Myosin-9	MYH9	16	14	58
Dolichyl-diphosphooligosaccharide--protein glycosyltransferase subunit 1	RPN1	16	6	8
ATP-dependent RNA helicase DDX1	DDX1	16	17	18
DNA-directed RNA polymerase, mitochondrial	POLRMT	16	10	7
Pentatricopeptide repeat-containing protein 3, mitochondrial	PTCD3	16	10	15
highly similar to Poly(A)-binding protein 1		16	11	16
Heterogeneous nuclear ribonucleoprotein Q	HNRPQ	14	13	21
Trifunctional enzyme subunit beta, mitochondrial	HADHB	14	10	12
Ubiquitin carboxyl-terminal hydrolase 7	USP7	13	9	4
Tubulin beta chain	TUBB	13	12	15
La-related protein 1	LARP1	13	8	10
Ribosome-binding protein 1	RRBP1	13	6	16

**Table S4 continued:**

protein names	gene names	number of peptides		
		PD1	PD2	PD3
Caseinolytic peptidase B protein homolog	CLPB	13	10	8
Syntaxin-binding protein 3	STXBP3	13	8	8
DnaJ homolog subfamily C member 13	DNAJC13	13	6	31
Leucine-rich repeat-containing protein 59	LRRC59	13	11	6
U4/U6.U5 tri-snRNP-associated protein 1	SART1	12	5	10
Pre-rRNA-processing protein TSR1 homolog	TSR1	12	3	8
Putative uncharacterized protein MATR3	MATR3	11	6	9
Tubulin beta-4 chain	TUBB4	11	10	10
Probable ATP-dependent RNA helicase DDX47	DDX47	11	9	13
Heterogeneous nuclear ribonucleoprotein M	HNRNPM	11	6	20
Chromodomain-helicase-DNA-binding protein 3	CHD3	11	4	2
ATP synthase subunit alpha, mitochondrial	ATP5A1	11	13	11
HNRPR protein	HNRPR	11	7	18
cDNA FLJ58806, highly similar to DNA-directed RNA polymerase, mitochondrial (EC 2.7.7.6)		11	8	4
Exosome component 10	EXOSC10	10	9	12
28S ribosomal protein S22, mitochondrial	MRPS22	10	16	12
Tubulin beta-2A chain	TUBB2A	10	12	15
GTP-binding protein GUF1 homolog	GUF1	10	5	4
Cell division cycle 5-like protein	CDC5L	10	7	14
Interleukin enhancer-binding factor 2	ILF2	9	8	9
Squamous cell carcinoma antigen recognized by T-cells 3	SART3	9	7	10
Myosin-Ic	MYO1C	9	6	17
Splicing factor 3 subunit 1	SF3A1	9	5	2
Splicing factor, arginine/serine-rich 1	SFRS1	9	6	10
Pre-mRNA-splicing factor SF3b 145 kDa subunit	SF3B2	9	3	10
Ribonucleases P/MRP protein subunit POP1	POP1	9	5	11
N-acetyltransferase 10	NAT10	9	3	17
Transcription intermediary factor 1-beta	TRIM28	9	4	4
Protein phosphatase 1G	PPM1G	8	4	8
Protein tyrosine phosphatase-like protein PTPLAD1	PTPLAD1	8	3	2
28S ribosomal protein S9, mitochondrial	MRPS9	8	8	9
Casein kinase II subunit alpha	CSNK2A1	8	8	9
Nucleosome assembly protein 1-like 1	NAP1L1	8	7	10
ATP-dependent RNA helicase DDX50	DDX50	8	5	16
RNA-binding protein 39	RBM39	8	4	11

**Table S4 continued:**

protein names	gene names	number of peptides		
		PD1	PD2	PD3
40S ribosomal protein S4	RPS4X	8	4	16
Putative rRNA methyltransferase 3	FTSJ3	8	4	13
Nucleophosmin	NPM1	8	8	12
MAGUK p55 subfamily member 6	MPP6	8	5	7
Nucleolar GTP-binding protein 1	GTPBP4	8	6	10
Heterogeneous nuclear ribonucleoproteins A2/B1	HNRNPA2B1	8	11	11
mRNA cap guanine-N7 methyltransferase;mRNA (guanine-N(7))-methyltransferase	RNMT	8	6	7
DNA topoisomerase 1;DNA topoisomerase I	TOP1	8	7	18
UPF0027 protein C22orf28	C22orf28	8	9	11
Insulin-like growth factor 2 mRNA-binding protein 3	IGF2BP3	8	2	7
39S ribosomal protein L12, mitochondrial	MRPL12	7	2	6
UPF0568 protein C14orf166	C14orf166	7	3	8
Calcium-binding mitochondrial carrier protein Aralar2	SLC25A13	7	2	5
ATP synthase subunit O, mitochondrial	ATP5O	7	1	8
60S acidic ribosomal protein P0	RPLP0	7	13	14
Insulin-like growth factor 2 mRNA-binding protein 1	IGF2BP1	7	6	11
Ribosomal L1 domain-containing protein 1	RSL1D1	7	3	17
Ras GTPase-activating protein-binding protein 1	G3BP1	7	6	6
28S ribosomal protein S25, mitochondrial	MRPS25	7	2	9
40S ribosomal protein S2	RPS2	7	8	10
Ribosomal protein L23a	RPL23A	7	3	9
39S ribosomal protein L15, mitochondrial	MRPL15	7	5	10
Heterogeneous nuclear ribonucleoprotein D0	HNRNPD	7	6	9
60S ribosomal protein L9	RPL9	7	4	10
Probable E3 ubiquitin-protein ligase TRIP12	TRIP12	7	2	7
RRP12-like protein	RRP12	7	4	12
Putative uncharacterized protein FAM98B	FAM98B	7	6	9
40S ribosomal protein S15a	RPS15A	7	7	9
40S ribosomal protein S16	RPS16	7	4	9
40S ribosomal protein S17	RPS17	7	4	8
AP-2 complex subunit alpha-1	AP2A1	7	4	5
DNA damage-binding protein 1	DDB1	7	5	4
Aspartyl/asparaginyl beta-hydroxylase	ASPH	7	5	3
ATPase family AAA domain-containing protein 3A	ATAD3A	7	4	18
Large subunit GTPase 1 homolog	LSG1	7	5	8

**Table S4 continued:**

protein names	gene names	number of peptides		
		PD1	PD2	PD3
tRNA (cytosine-5-)-methyltransferase NSUN2	NSUN2	7	3	1
THO complex subunit 4	THOC4	7	7	9
40S ribosomal protein S3a	RPS3A	7	6	19
Poly(A) RNA polymerase, mitochondrial	MTPAP	7	7	9
Serine hydroxymethyltransferase	SHMT2	6	4	3
Probable ATP-dependent RNA helicase DDX49	DDX49	6	3	5
Large proline-rich protein BAT2	BAT2	6	1	1
Heterogeneous nuclear ribonucleoprotein U-like protein 1	HNRNPUL1	6	7	14
Serine/threonine-protein kinase PRPF4 homolog	PRPF4B	6	2	10
AP-2 complex subunit alpha-2	AP2A2	6	6	4
Casein kinase II subunit alpha'	CSNK2A2	6	3	5
28S ribosomal protein S35, mitochondrial	MRPS35	6	4	7
Death domain-associated protein 6	DAXX	6	3	2
ATP-dependent RNA helicase DDX55	DDX55	6	3	6
40S ribosomal protein S13	RPS13	6	1	8
WD40 repeat-containing protein SMU1	SMU1	6	8	12
Heterogeneous nuclear ribonucleoprotein A/B	HNRNPAB	6	6	3
Pre-mRNA-processing factor 40 homolog A	PRPF40A	6	3	5
Replication factor C subunit 1	RFC1	6	5	16
Uncharacterized protein C4orf14	C4orf14	6	9	12
Ribosomal RNA processing protein 1 homolog A	RRP1	6	7	5
Guanine nucleotide-binding protein subunit beta-2-like 1	GNB2L1	6	11	16
Caprin-1	CAPRIN1	6	4	4
Double-stranded RNA-binding protein Staufen homolog 1	STAU1	5	3	6
Guanine nucleotide-binding protein-like 3	GNL3	5	1	10
60S ribosomal protein L4	RPL4	5	6	15
Pre-mRNA-processing factor 19	PRPF19	5	8	11
39S ribosomal protein L44	MRPL44	5	1	6
down-regulator of HLA II (IK)	IK	5	6	3
Heterogeneous nuclear ribonucleoprotein A0	HNRNPA0	5	3	5
Fragile X mental retardation syndrome-related protein 1	FXR1	5	3	8
28S ribosomal protein S28	MRPS28	5	2	6
Cleavage and polyadenylation specificity factor subunit 1	CPSF1	5	3	6
40S ribosomal protein S14	RPS14	5	3	8
Reticulocalbin-2	RCN2	5	4	7
60S ribosomal protein L7	RPL7	5	2	13

**Table S4 continued:**

protein names	gene names	number of peptides		
		PD1	PD2	PD3
Splicing factor U2AF 65 kDa subunit	U2AF2	5	6	7
28S ribosomal protein S23, mitochondrial	MRPS23	5	3	9
Putative methyltransferase NSUN5	NSUN5	5	3	3
U4/U6 small nuclear ribonucleoprotein Prp4	PRPF4	5	1	8
MKI67 FHA domain-interacting nucleolar phosphoprotein	MKI67IP	5	5	8
KRR1 small subunit processome component homolog	KRR1	5	2	8
Protein FAM98A	FAM98A	5	4	6
40S ribosomal protein S19	RPS19	5	4	7
Probable ATP-dependent RNA helicase DDX27	DDX27	5	4	9
28S ribosomal protein S31, mitochondrial	MRPS31	5	5	8
Putative uncharacterized protein NOP2	NOP2	5	5	21
ATP-dependent RNA helicase DDX18	DDX18	5	2	10
ATP synthase subunit beta, mitochondrial	ATP5B	5	8	3
Heat shock 70 kDa protein 1	HSPA1A	5	4	7
Protein LYRIC	MTDH	5	4	6
Putative pre-mRNA-splicing factor ATP-dependent RNA helicase DHX15	DDX15	5	3	13
60S ribosomal protein L3	RPL3	5	4	8
Zinc finger CCHC domain-containing protein 8	ZCCHC8	5	5	2
28S ribosomal protein S17, mitochondrial	MRPS17	5	2	5
39S ribosomal protein L38, mitochondrial	MRPL38	5	3	4
39S ribosomal protein L11, mitochondrial	MRPL11	4	1	8
Pre-mRNA 3'-end-processing factor FIP1	FIP1L1	4	2	6
60S acidic ribosomal protein P1	RPLP1	4	4	4
60S acidic ribosomal protein P2	RPLP2	4	4	4
40S ribosomal protein S25	RPS25	4	2	4
40S ribosomal protein S7	RPS7	4	2	8
Heterogeneous nuclear ribonucleoprotein H3	HNRNPH3	4	4	2
Ribosome biogenesis regulatory protein homolog	RRS1	4	3	8
Nucleolar GTP-binding protein 2	GNL2	4	5	3
Cell growth-regulating nucleolar protein	LYAR	4	4	14
40S ribosomal protein S6	RPS6	4	6	7
28S ribosomal protein S18b, mitochondrial	MRPS18B	4	3	5
SPATS2-like protein	SPATS2L	4	1	5
60S ribosomal protein L12	RPL12	4	2	6
E3 ubiquitin-protein ligase UBR5	UBR5	4	1	1

**Table S4 continued:**

protein names	gene names	number of peptides		
		PD1	PD2	PD3
Procollagen-lysine,2-oxoglutarate 5-dioxygenase 1	PLOD1	4	3	2
Probable ATP-dependent RNA helicase DHX36	DHX36	4	1	1
Ribophorin II	RPN2	4	5	3
Small nuclear ribonucleoprotein E	SNRPE	4	4	4
Splicing factor 3A subunit 3	SF3A3	4	1	4
Nuclease-sensitive element-binding protein 1	YBX1	4	2	6
Mitochondrial ribonuclease P protein 1	MRPP1	4	6	4
28S ribosomal protein S5, mitochondrial;S5mt	MRPS5	4	5	6
Pentatricopeptide repeat-containing protein 1	PTCD1	4	4	1
Insulin-like growth factor 2 mRNA-binding protein 2	IGF2BP2	4	3	8
40S ribosomal protein S8	RPS8	4	5	8
40S ribosomal protein S9	RPS9	4	3	7
RNA-binding protein FUS	FUS	4	3	6
ADP/ATP translocase 3	SLC25A6	4	4	4
Dimethyladenosine transferase 1, mitochondrial	TFB1M	4	7	6
Signal recognition particle 14 kDa protein	SRP14	4	1	5
Intron-binding protein aquarius	AQR	4	2	5
60S ribosomal protein L7a	RPL7A	4	8	10
Protein kinase C alpha type	PRKCA	4	5	3
60S ribosomal protein L17	RPL17	4	1	9
Heterogeneous nuclear ribonucleoprotein A3	HNRNPA3	4	5	9
La-related protein 5	LARP5	4	4	6
Serine/threonine-protein phosphatase PP1-gamma catalytic subunit	PPP1CC	3	7	4
Probable ATP-dependent RNA helicase DDX23	DDX23	3	3	10
KH domain-containing, RNA-binding, signal transduction-associated protein 1	KHDRBS1	3	3	2
Ras GTPase-activating protein-binding protein 2	G3BP2	3	3	3
Pre-mRNA-splicing factor SRP20	SFRS3	3	3	3
Pre-mRNA-splicing factor SRp30C	SFRS9	3	3	8
Pre-mRNA-splicing factor SRP40	SFRS5	3	2	2
Exosome complex exonuclease RRP4	EXOSC2	3	4	8
Pre-mRNA-splicing factor SF3b 49 kDa subunit	SF3B4	3	3	3
Protein SDA1 homolog	SDAD1	3	2	5
Cell division cycle 2-related protein kinase 7	CRKRS	3	1	5
RNA-binding protein 42	RBM42	3	3	5

**Table S4 continued:**

protein names	gene names	number of peptides		
		PD1	PD2	PD3
Transcriptional activator protein Pur-alpha	PURA	3	2	1
Cell division cycle 2-like protein kinase 1	CDC2L1	3	1	2
Histone H2A	H2AX	3	3	5
Serine/threonine protein phosphatase	PPP1CA	3	7	4
Heterogeneous nuclear ribonucleoprotein L	HNRNPL	3	2	4
Syntaxin-4	STX4	3	5	4
Nuclear RNA export factor 1	NXF1	3	4	9
Heterogeneous nuclear ribonucleoprotein D-like	HNRPDL	3	6	7
ATP-dependent RNA helicase DDX54	DDX54	3	3	13
DnaJ homolog subfamily C member 9	DNAJC9	3	1	4
39S ribosomal protein L37, mitochondrial	MRPL37	3	3	6
Pre-mRNA-splicing factor SYF1	XAB2	3	3	1
Putative ATP-dependent RNA helicase DHX57	DHX57	3	2	1
MAGUK p55 subfamily member 5	MPP5	3	2	2
Polymerase I and transcript release factor	PTRF	3	1	2
60S ribosomal protein L18	RPL18	3	3	5
Histone H2A type 2-A	HIST2H2AA	3	3	5
3'-5' exoribonuclease 1	ERI1	3	2	2
Histone H1.5	HIST1H1B	3	3	4
Neuropathy target esterase	PNPLA6	3	4	3
Serine/threonine-protein phosphatase PP1-beta catalytic subunit	PPP1CB	3	6	5
60S ribosomal protein L22	RPL22	3	3	7
39S ribosomal protein L23, mitochondrial	MRPL23	3	2	3
La-related protein 7	LARP7	3	1	6
DNA replication licensing factor MCM7	MCM7	3	3	2
Splicing factor, arginine/serine-rich 10	SFRS10	3	2	3
Probable ATP-dependent RNA helicase DDX56	DDX56	3	1	4
Pre-mRNA-processing factor 6	PRPF6	3	2	11
ATPase family AAA domain-containing protein 3B	ATAD3B	3	2	10
Sorting nexin-27	SNX27	3	2	2
Mannosyl-oligosaccharide glucosidase	MOGS	3	2	1
39S ribosomal protein L50, mitochondrial	MRPL50	3	1	2
60S ribosomal protein L6	RPL6	3	4	8
RNA methyltransferase-like protein 1	RNMTL1	3	3	9
Brix domain-containing protein 1	BXDC1	3	2	2

**Table S4 continued:**

protein names	gene names	number of peptides		
		PD1	PD2	PD3
Nucleolar protein 56	NOP56	3	1	15
60S ribosomal protein L35	RPL35	3	1	3
General transcription factor 3C polypeptide 1	GTF3C1	3	2	2
Inactive ubiquitin-specific peptidase 39	USP39	3	2	10
Cyclin-K	CCNK	3	3	2
Polymerase delta-interacting protein 3	POLDIP3	3	2	1
Heterogeneous nuclear ribonucleoprotein U-like protein 2	HNRNPUL2	3	1	6
Growth arrest and DNA-damage-inducible proteins-interacting protein 1	PRG6	3	1	7
Peptidyl-prolyl cis-trans isomerase-like 4	PPIL4	3	2	4
ATP-dependent RNA helicase DDX39	DDX39	3	2	2
Probable rRNA-processing protein EBP2	EBNA1BP2	3	2	5
60S ribosomal protein L5	RPL5	2	3	5
AFG3-like protein 2	AFG3L2	2	3	1
60S ribosomal protein L14	RPL14	2	2	3
Splicing factor, arginine/serine-rich 7	SFRS7	2	2	4
18S rRNA dimethylase	DIMT1L	2	3	4
Tax1-binding protein 3;Tax interaction protein 1;Glutaminase-interacting protein	TAX1BP3	2	1	1
Splicing factor U2AF 35 kDa subunit	U2AF1	2	1	2
Polyadenylate-binding protein 2	PAB2	2	2	2
Pre-mRNA-splicing factor 3	PRPF3	2	2	5
CD2 antigen cytoplasmic tail-binding protein 2	CD2BP2	2	2	3
28S ribosomal protein S7, mitochondrial	MRPS7	2	3	9
28S ribosomal protein S26, mitochondrial3	MRPS26	2	2	3
ATP-dependent RNA helicase DDX24	DDX24	2	1	6
Nicotinamide mononucleotide adenylyltransferase 1	NMNAT1	2	1	7
Phosphatidylserine synthase 1	PTDSS1	2	1	2
Transformer-2 protein homolog alpha	TRA2A	2	2	1
Exosome complex exonuclease RRP42	EXOSC7	2	2	3
Lysyl-tRNA synthetase	KARS	2	1	3
Replication factor C subunit 4	RFC4	2	5	2
Translocon-associated protein subunit delta	SSR4	2	1	1
Histone H1x	H1FX	2	2	5
AP-2 complex subunit mu-1	AP2M1	2	2	3
39S ribosomal protein L4, mitochondrial	MRPL4	2	2	3

**Table S4 continued:**

protein names	gene names	number of peptides		
		PD1	PD2	PD3
ESF1 homolog	ESF1	2	1	2
Putative RNA-binding protein 3	RBM3	2	1	2
rRNA 2'-O-methyltransferase fibrillarin	FBL	2	3	10
Nucleoplasmin-3	NPM3	2	1	3
Prohibitin-2;B-cell receptor-associated protein BAP37	PHB2	2	3	4
Glutamate-rich WD repeat-containing protein 1	GRWD1	2	1	1
Cytochrome c1, heme protein, mitochondrial	CYC1	2	2	1
Exosome complex exonuclease RRP45	EXOSC9	2	2	4
DNA-directed RNA polymerase II subunit RPB1	POLR2A	2	2	5
Ribosomal RNA processing protein 1 homolog B	RRP1B	2	1	9
Nucleolar protein 16	NOP16	2	2	4
Zinc finger protein 622	ZNF622	2	1	1
Heterogeneous nuclear ribonucleoprotein G	HNRPG	2	2	3
CDKN2AIP N-terminal-like protein	CDKN2AIPNL	2	2	1
Protein SET	SET	2	2	2
39S ribosomal protein L39, mitochondrial	MRPL39	2	4	3
Splicing factor U2AF 26 kDa subunit	U2AF1L4	2	1	2
U3 small nucleolar RNA-associated protein 14 homolog A	UTP14A	2	2	1
TCOF1 protein	TCOF1	2	1	1
GTP-binding protein 10	GTPBP10	2	2	2
DnaJ homolog subfamily A member 3, mitochondrial	DNAJA3	2	3	6
Protein phosphatase 1 regulatory subunit 12A	PPP1R12A	2	2	2
Succinate dehydrogenase [ubiquinone] flavoprotein subunit, mitochondrial	SDHA	2	1	1
39S ribosomal protein L41, mitochondrial	MRPL41	2	1	3
Probable ATP-dependent RNA helicase DHX37	DDX37	2	2	1
60S ribosomal protein L27	RPL27	2	2	5
60S ribosomal protein L30	RPL30	2	3	4
Eukaryotic translation initiation factor 2 subunit 1	EIF2S1	2	3	4
DNA-directed RNA polymerase I subunit RPA49	POLR1E	2	1	5
Serine/threonine-protein kinase SRPK1	SRPK1	2	5	2
Dolichyl-diphosphooligosaccharide--protein glycosyltransferase 48 kDa subunit	DDOST	2	2	2
U2 small nuclear ribonucleoprotein A'	SNRPA1	2	2	5
Eukaryotic translation initiation factor 2 subunit 3	EIF2S3	2	1	6
Synaptojanin-2-binding protein	SYNJ2BP	2	2	2

**Table S4 continued:**

protein names	gene names	number of peptides		
		PD1	PD2	PD3
ELAV-like protein 1	ELAVL1	2	3	2
N-acylneuraminate cytidyltransferase	CMAS	2	1	2
28S ribosomal protein S6, mitochondrial	MRPS6	2	2	1
AP-2 complex subunit beta-1	AP2B1	2	5	5
Zinc finger CCCH-type antiviral protein 1	ZC3HAV1	2	1	2
TRM1-like protein	TRM1L	2	2	1
ATP synthase subunit gamma, mitochondrial	ATP5C1	2	2	4
Coiled-coil domain-containing protein 137	CCDC137	2	1	3
NF-X1-type zinc finger protein NFXL1	NFXL1	2	1	1
40S ribosomal protein S15	RPS15	2	1	2
39S ribosomal protein L1, mitochondrial	MRPL1	2	1	6
p21-activated protein kinase-interacting protein 1	PAK1IP1	2	2	4
Probable RNA-binding protein 19	RBM19	1	1	4
Transducin beta-like protein 2	TBL2	1	1	4
Importin subunit alpha-21	KPNA2	1	1	3
Pyruvate dehydrogenase E1 component subunit beta, mitochondrial	PDHB	1	2	1
SPTLC1 protein	SPTLC1	1	2	1
Vesicle-associated membrane protein-associated protein A	VAPA	1	1	2
FUS-interacting serine-arginine-rich protein 1	FUSIP1	1	2	2
Importin subunit alpha-4	KPNA4	1	1	1
28S ribosomal protein S21, mitochondrial	MRPS21	1	1	2
General transcription factor 3C polypeptide 3	GTF3C3	1	2	1
Small nuclear ribonucleoprotein G-like protein	SNRPG	1	2	3
Replication factor C subunit 2	RFC2	1	2	1
Small nuclear ribonucleoprotein Sm D3	SNRPD3	1	2	3
Eukaryotic translation initiation factor 2 subunit 2	EIF2S2	1	3	6
40S ribosomal protein S11	RPS11	1	1	5
Probable histidyl-tRNA synthetase, mitochondrial	HARS2	1	1	1
Transcriptional repressor CTCF	CTCF	1	1	2
Uncharacterized protein C7orf50	C7orf50	1	2	4
Nucleolar complex protein 4 homolog	NOC4L	1	2	3
Pre-mRNA branch site protein p14	SF3B14	1	2	2
28S ribosomal protein S16, mitochondrial	MRPS16	1	2	3
Anaphase-promoting complex subunit 1	ANAPC1	1	1	2

**Table S4 continued:**

protein names	gene names	number of peptides		
		PD1	PD2	PD3
Histone H2A-Bbd type 2/3	H2AFB2	1	2	3
Formin-like protein 2	FMNL2	1	2	2
WD repeat-containing protein 82	WDR82	1	1	1
39S ribosomal protein L10, mitochondrial	MRPL10	1	1	1
Junctophilin-1	JPH1	1	1	1
40S ribosomal protein S29	RPS29	1	1	1
39S ribosomal protein L45, mitochondrial	MRPL45	1	2	2
Heterogeneous nuclear ribonucleoproteins C1/C2	HNRNPC	1	4	4
NADH dehydrogenase [ubiquinone] 1 beta subcomplex subunit 4	NDUFB4	1	2	1
Kinesin-like protein KIF23	KIF23	1	2	3
Serine beta-lactamase-like protein LACTB, mitochondrial	LACTB	1	1	2
Putative uncharacterized protein CBARA1	CBARA1	1	1	1
Translocon-associated protein subunit alpha	SSR1	1	2	2
Small nuclear ribonucleoprotein Sm D1	SNRNP1	1	2	2
Phosphorylated adapter RNA export protein	PHAX;	1	1	1
Protein NipSnap homolog 1	NIPSNAP1	1	1	3
60S ribosomal protein L13a	RPL13A	1	1	7
Histone-binding protein RBBP4	RBBP4	1	1	1
G patch domain-containing protein 4	GPATCH4	1	1	3
Myelin expression factor 2	MYEF2	1	1	7
Ribosomal protein S27	RPS27	1	1	2
PAI1 RNA-binding protein 1	SERBP1	1	2	1
60S ribosomal protein L37a	RPL37A	1	1	1
60S ribosomal protein L13	RPL13	1	3	7
Target of EGR1 protein 1	TOE1	1	2	2
Exosome complex exonuclease RRP43	EXOSC8	1	1	3
39S ribosomal protein L51, mitochondrial	MRPL51	1	1	1
EF-hand domain-containing family member A1	EFHA1	1	1	1
Heterochromatin protein 1-binding protein 3	HP1BP3	1	2	8
Tumor necrosis factor receptor superfamily member 6B	TNFRSF6B	1	1	1
Putative uncharacterized protein ZFR	ZFR	1	1	3

## 7 LITERATURE

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