

Regulation and function of the ubiquitin ligase E6AP

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The most exciting phrase to hear in science, the one that heralds new discoveries, is not 'Eureka!' but 'That's funny...'

(Isaac Asimov)

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Abbreviations

AS	Angelman syndrome
ASD	Autism Spectrum Disorders
ATP	Adenosine triphosphate
β -Gal	beta-galactosidase
DDT	dithiothreitol
DNA	deoxyribonucleic acid
EDTA	ethylenediaminetetraacetic acid
ER	estrogen receptor
ERE	estrogen response element
FBS	fetal bovine serum
GST	Glutathion-S-transferase
HA-tag	Hemagglutinin-tag
HPV	human papillomavirus
IPTG	Isopropyl- β -D-thiogalactopyranosid
ivt	<i>in vitro</i> translated
kDa	kilo Dalton
ONPG	<i>o</i> -Nitrophenyl- β -D-galactopyranosid
OD	Optical density
PCR	Polymerase chain reaction
PBS	Phosphate-buffered saline
RNA	ribonucleic acid
RNAi	RNA interference
SHR	Steroid hormone receptor
shRNA	small hairpin RNA
SDS	Sodium dodecyl sulfate
TF	transcription factor
Ub	ubiquitin
UPS	Ubiquitin-Proteasome-System
wt	wild-type

Abstract

Post-translational modification of proteins by ubiquitin ("ubiquitination") determines proteolytic as well as non-proteolytic fates of the target proteins. It is catalyzed by the ubiquitination cascade, which is the sequential action of E1 ubiquitin-activating enzymes, E2 ubiquitin-conjugating enzymes and E3 ubiquitin-protein ligases. The substrate specificity relies on the E3 ligases, which covalently link ubiquitin to substrate proteins under formation of an isopeptide bond. A prominent member of the E3 ligase family is E6AP, which plays a key role in the development of distinct human diseases.

In cervical cancer, high-risk human papillomavirus (HPV) E6 proteins hijack E6AP to target the tumor suppressor p53 for ubiquitination and subsequent proteasomal degradation. In the absence of E6, p53 is not a substrate of E6AP. Besides, genetic evidence indicates that E6AP plays a crucial role in neurodevelopment. In particular, loss of E6AP expression is causally associated with the development of Angelman syndrome, while amplification of the E6AP gene causes autistic traits. Thus, level and/or activity of E6AP have to be tightly regulated; however, only little is known how this is achieved. Similarly, the physiological role of E6AP in HPV-negative cells is still largely unknown. To gain insights into the regulation of E6AP's E3 ligase activity and the function of E6AP within cells, *in vitro* and *in cellulo* studies were employed.

The first part of this thesis addressed the role of ubiquitin and HPV E6 proteins in E6AP-mediated catalysis *in vitro*. Previous studies revealed ubiquitin's "canonical" hydrophobic patch to play a crucial role in E6AP-mediated ubiquitination. However, once E6AP is bound to E6, this patch is not required for an efficient ubiquitination reaction anymore, suggesting that E6 does not only alter E6AP's substrate spectrum, but also acts as an allosteric activator of E6AP. To further clarify the role of ubiquitin in E6AP-mediated ubiquitination, studies were extended towards the "non-canonical" hydrophobic patch of ubiquitin. The obtained results clearly show that both patches of ubiquitin make a substantial contribution to E6AP-catalyzed isopeptide bond formation, but by different mechanisms. Eventually, experimental evidence indicates that E6 stimulates E6AP activity by enhancing its ability to catalyze isopeptide bond formation.

In the second part of this thesis, studies were focused on the role of E6AP in cell signaling pathways. Previously, we and others have reported that E6AP modulates estrogen receptor (ER) signaling – an effect that may not require E6AP's E3 ligase activity. Therefore, E6AP affects protein homeostasis not only at the post-translational, but also at the transcriptional level. However, in contrast to other reports, we could not observe a direct interaction between E6AP and ER. Hence, it was hypothesized that the effect of E6AP on ER might occur indirectly rather than directly. Indeed, experimental evidence suggests that E6AP affects the transactivation of ER by modulating protein kinase cascades, in particular the PI3K/Akt/GSK3 pathway, which in turn regulate ER activity by phosphorylation. Notably, the PI3K/Akt pathway regulates a variety of different signaling events and plays a crucial role in neurodevelopment. Thus, the pathology of the neurological disorders caused by altered E6AP expression may at least partially depend on E6AP's action on kinase-mediated signaling pathways.

Zusammenfassung

Die post-translationale Modifikation von Proteinen mit Ubiquitin ("Ubiquitinierung") hat proteolytische und nicht-proteolytische Folgen. Sie wird durch die Ubiquitinierungskaskade katalysiert, welche aus der sequentiellen Aktivität von E1 Ubiquitin-aktivierenden Enzymen, E2 Ubiquitin-konjugierenden Enzymen und E3 Ubiquitin-Protein Ligasen besteht. Dabei wird die Substratspezifität von den E3 Ligasen bestimmt, welche die kovalente Verknüpfung von Ubiquitin an Substratproteine unter Bildung einer Isopeptidbindung katalysieren. Eine wichtige E3 Ligase ist E6AP, welche eine Schlüsselrolle bei der Entstehung bestimmter humaner Erkrankungen spielt.

Bei Gebärmutterhalskrebs führt die Bindung der E6 Proteine humaner Papillomviren (HPV) an E6AP zur Ubiquitinierung und anschließendem proteasomalen Abbau von p53. In Abwesenheit von E6 ist p53 jedoch kein Substrat von E6AP. Außerdem zeigen genetische Befunde dass E6AP eine wichtige Rolle in der neuronalen Entwicklung spielt. So ist der Verlust der Expression von E6AP die kausale Ursache für die Entwicklung des Angelman Syndroms, während eine Amplifikation des E6AP-Gens autistische Merkmale erzeugt. Demnach müssen Level und/oder Aktivität von E6AP passend reguliert sein; wie dies erfolgt, ist aber bisher nur wenig verstanden. Ähnlich ist die Rolle von E6AP in HPV-negativen Zellen noch immer größtenteils ungeklärt. Um Einblicke in die Regulation der E3 Ligase Aktivität von E6AP und in die Funktion von E6AP in Zellen zu gewinnen, wurden Studien *in vitro* und *in cellulo* durchgeführt.

Der erste Teil dieser Dissertation behandelt den Einfluss von Ubiquitin und E6 Proteinen auf die E6AP-vermittelte Katalyse *in vitro*. Frühere Arbeiten zeigten, dass der "kanonische" hydrophobe Patch von Ubiquitin eine wichtige Rolle in der E6AP-vermittelten Ubiquitinierung spielt. Sobald E6AP an E6 gebunden ist, wird dieser Patch jedoch nicht mehr für eine effiziente Ubiquitinierungsreaktion benötigt. Demzufolge beeinflusst E6 vermutlich nicht nur die Substratspezifität von E6AP, sondern agiert auch als allosterischer Aktivator. Um den Beitrag von Ubiquitin zur E6AP-vermittelten Ubiquitinierung weiter zu untersuchen, wurden die Studien auf den "nicht-kanonischen" hydrophoben Patch von Ubiquitin erweitert. Die erhaltenen Ergebnisse zeigen deutlich, dass beide Patches von Ubiquitin einen wichtigen Beitrag zur Ausbildung der E6AP-katalysierten Isopeptidbindung leisten, wobei die zugrunde liegenden Mechanismen unterschiedlich sind. Zudem deuten die Ergebnisse auch an, dass E6 die Aktivität von E6AP durch Stimulation der Isopeptidbindungsbildung beeinflusst.

Im zweiten Teil dieser Arbeit konzentrierten sich die Studien auf die Rolle von E6AP in Signaltransduktionskaskaden. Wir und andere Arbeitsgruppen zeigten bereits, dass E6AP den Östrogen-Rezeptor (ER) beeinflusst – ein Effekt, der nicht die E3 Ligase Aktivität von E6AP benötigt. Demzufolge beeinflusst E6AP die Proteinhomöostase nicht nur auf der post-translationalen Ebene, sondern auch auf der Ebene der Transkription. Im Gegensatz zu anderen Berichten haben wir jedoch keine direkte Interaktion zwischen E6AP und dem ER beobachten können. Daher wurde vermutet, dass E6AP den ER eher indirekt beeinflusst. Tatsächlich weisen experimentelle Befunde darauf hin, dass E6AP den ER durch Modulation von Proteinkinase-

Kaskaden beeinflusst, insbesondere der PI3K/Akt/GSK3-Kaskade, welche wiederum die Aktivität des ER durch Phosphorylierung reguliert. Interessanterweise reguliert der PI3K/Akt-Signalweg vielfältige Signalvorgänge und spielt eine entscheidende Rolle in der neuronalen Entwicklung. Demzufolge könnte die Pathologie der neuronalen Krankheitsbilder, die durch eine veränderte E6AP-Expression verursacht werden, zumindest teilweise durch den Effekt von E6AP auf Signaltransduktionswege verursacht werden.

1. Introduction

Maintenance of proteostasis is crucial for keeping a cell or organism in a healthy state. However, the machineries that contribute to proteostasis must be adaptable to varying demands and hence a great diversity of mechanisms to do so has evolved. All steps from protein biogenesis to degradation have to be tightly controlled, which is achieved by a complex network of signaling events. In part, these signaling networks depend on the post-translational modification of proteins, thereby regulating interactions, subcellular localization and protein activity as well as stability. The latter can be achieved by post-translational modification of proteins by ubiquitin, a process that allows proteolysis in a substrate-specific manner (Ciechanover et al. 1984, Nandi et al. 2006).

1.1 The Ubiquitin-Proteasome-System

In 1953, it was found that intracellular protein degradation is an energy consuming reaction (Simpson 1953). However, it took almost 30 years to understand the underlying reason: the ubiquitin-proteasome system (UPS). It is based on covalent attachment of a small polypeptide, termed ubiquitin, to proteins that are designated for degradation by a large multisubunit protease, the proteasome (Hershko and Ciechanover 1992, Zwickl et al. 1999).

The process of ubiquitin attachment to a target protein (ubiquitination) involves the sequential action of distinct classes of enzymes: first, ubiquitin is activated by the E1 ubiquitin-activating enzyme in an ATP-consuming reaction. The E1 enzyme builds an ubiquitin-adenylate intermediate and subsequently forms a thioester bond between its catalytic cysteine residue and the C terminus of ubiquitin. Next, ubiquitin is transferred to an E2 ubiquitin-conjugating enzyme in a transthioylation reaction. Finally, an E3 ubiquitin-protein ligase is involved in the attachment of ubiquitin's C terminus to the ϵ -amino group of a target lysine residue under formation of an isopeptide bond (Hershko and Ciechanover 1992). Based on structure and catalytic mechanism, E3 ligases are divided into three different families: RING, RBR, and HECT ligases. In brief, RING E3 ligases serve mainly as an adapter between the E2 enzyme and the substrate (Deshaies and Joazeiro 2009, Metzger et al. 2013), while RBR (Smit and Sixma 2013, Spratt et al. 2014) and HECT E3 ligases (Rotin and Kumar 2009, Scheffner and Kumar 2014) accept ubiquitin from the E2 enzyme in a transthioylation reaction before catalyzing isopeptide bond formation (Figure 1). The reaction can be reversed by deubiquitinating enzymes, which cleave off ubiquitin from target proteins (Turcu-Reyes et al. 2009).

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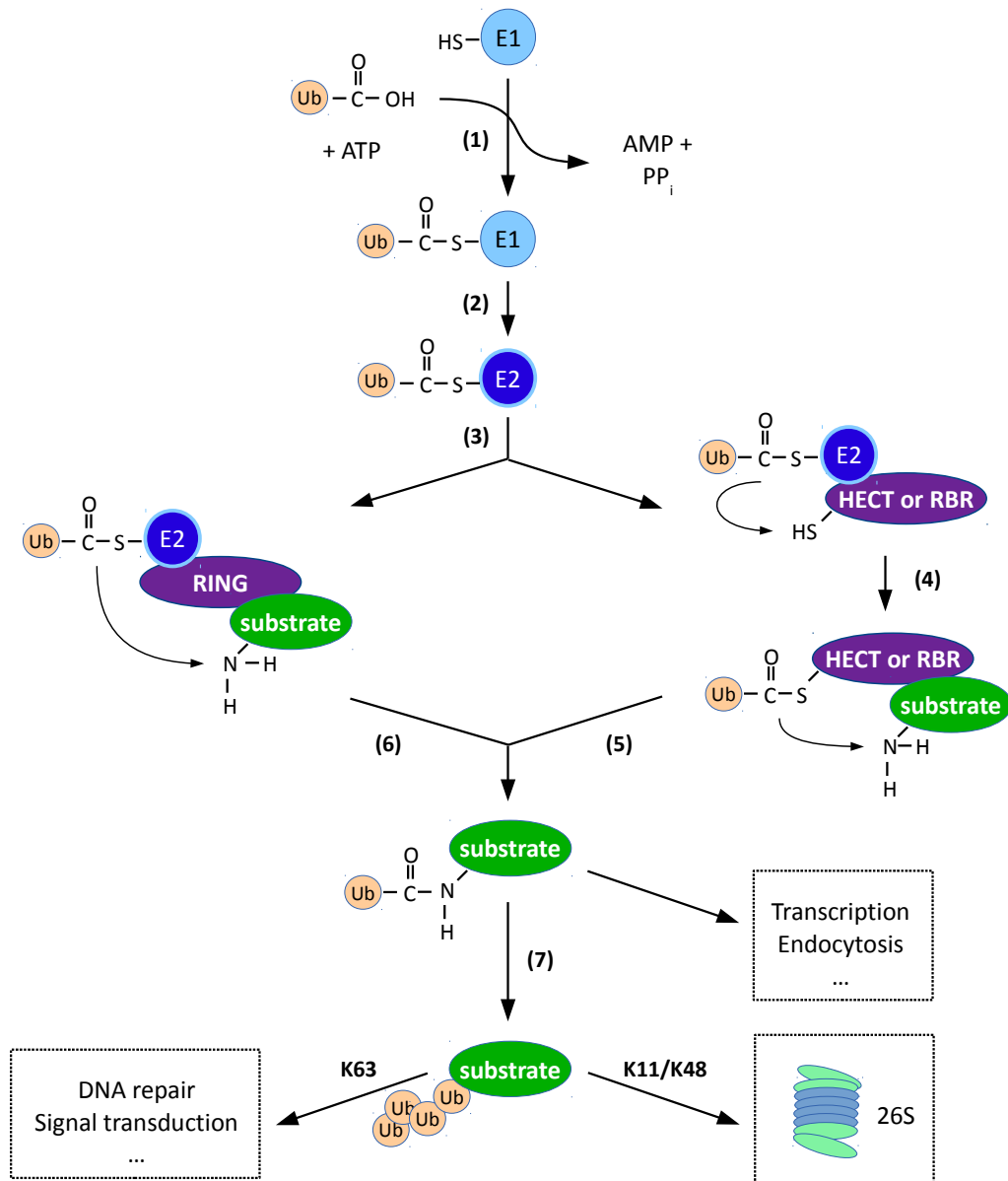


Figure 1: The ubiquitination cascade. (1) Ubiquitin (Ub) is activated by the E1 enzyme in an ATP-dependent reaction. The E1 enzyme forms a thioester between its catalytic cysteine and the C terminus of ubiquitin. (2) In a transthioylation reaction, ubiquitin is transferred to the active site cysteine of an E2 enzyme. (3) The E2 enzyme acts together with an E3 ligase in substrate ubiquitination. (4) In case of HECT and RBR ligases, ubiquitin forms a thioester with the catalytic cysteine of the E3 enzyme. (5) Subsequent isopeptide bond formation between a substrate lysine residue and ubiquitin is catalyzed by the E3 ligase. (6) RING ligases act as adapters for E2 and substrate. Isopeptide bond formation is catalyzed by the E2 enzyme. Monoubiquitination of substrate proteins can affect various cellular processes including gene expression and endocytosis. (7) In addition, one of ubiquitin's lysine residues (e.g. K63, K11, K48...) or the N terminus can be used to connect another ubiquitin, which results in the formation of ubiquitin chains. Depending on the linkage type, chains have different roles and outcomes (e.g., DNA repair, degradation by the 26S proteasome...).

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Quite early in the studies of the UPS it was noted that the modification of proteins by ubiquitin causes not only subsequent degradation but also modification of enzymatic functions (Ciechanover et al. 1984). Indeed, it was discovered that ubiquitination can occur in different modes: monoubiquitination is the attachment of single ubiquitin molecules to one or more lysine residues of the substrate. It is known to affect various cellular processes such as gene expression and endocytosis (Hicke 2001). In addition, ubiquitin itself can serve as acceptor for ubiquitin as it harbors seven lysine residues (K6, K11, K27, K29, K33, K48, K63), and also the α -amino group at the N terminus can be used for conjugation of another ubiquitin (Ye and Rape 2009). This so-called polyubiquitination may in some cases require a fourth class of enzymes (E4 ligases) for efficient chain formation (Hoppe 2005). Different chain types are linked to distinct effects: K48- and K11-linked ubiquitin chains serve as a signal for proteasomal degradation, whereas K63 chains are connected to non-proteolytic effects in various pathways including signal transduction and DNA repair (Ye and Rape 2009, Xu et al. 2009). These diverse effects are based on the different topologies of the different chain types and interactors which distinguish them. Hence the structure of ubiquitin (and ubiquitin chains) is a crucial determinant of recognition and specificity (Pickart and Fushman 2004).

1.2 Ubiquitin's structural features

Ubiquitin is a small polypeptide composed of 76 amino acids that is highly heat-stable and resistant to changes in pH as well as denaturing agents (Lenkinski et al. 1977). In 1987, Vijay-Kumar et al. solved the crystal structure of ubiquitin at 1.8 Å resolution. It revealed that ubiquitin is a compact globular protein, which is tightly hydrogen-bonded. It is composed of a β -sheet, a short 3_{10} helix and a 3.5-turn α -helix. The flexible C terminus is exposed to allow conjugation to primary amino groups (Vijay-Kumar et al. 1987; Figure 2).

In the past decades, it became more and more clear that distinct surface areas of ubiquitin play fundamental and distinguishable roles within cells. Residues around F4 play a crucial role in endocytosis, whereas a hydrophobic patch formed by L8, I44 and V70 is important for recognition by the proteasome (Beal et al. 1996, Sloper-Mould et al. 2001). However, for efficient binding to the proteasomal subunit S5a, it needs at least four ubiquitin molecules that are connected via K48 (Thrower et al. 2000). In addition, this so-called "canonical" hydrophobic patch is also the major interaction site for ubiquitin-binding domains of other proteins (reviewed in Dikic et al. 2009).

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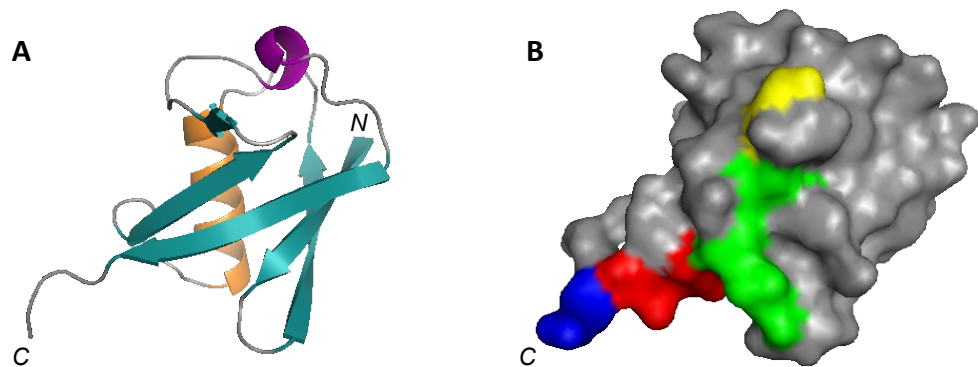


Figure 2: Crystal structure of ubiquitin. (A) The cartoon of the structure shows a five-stranded β -sheet (cyan), a short 3_{10} helix (magenta) and a 3.5-turn α -helix (orange). (B) Same as (A) except that ubiquitin's surface is shown. Ubiquitin is a compact globular protein with two exposed C-terminal glycine residues (blue). The adjacent residues I36, L71 and L73 form the "non-canonical" hydrophobic patch (red), which is important for ubiquitin-protein conjugate formation. The "canonical" hydrophobic patch (green) is composed of residues L8, I44 and V70 and serves as important platform for non-covalent interactions with ubiquitin-binding domains. For example, it mediates binding to the S5a subunit of the proteasome. However, to achieve efficient binding to the proteasome, it needs at least four ubiquitin molecules that are linked via K48 (yellow). - (PDB entry 1UBQ visualized with Pymol)

Another, so-called "non-canonical" hydrophobic patch is built by I36, L71 and L73. Since it is located quite close to ubiquitin's C-terminal tail, it is not surprising that it is important in ubiquitin-protein conjugate formation (Beal et al. 1996). For instance, in case of HECT ligases, the intact "non-canonical" patch is required for the E2-to-E3 ubiquitin transfer (Kamadurai et al. 2009). In addition, also RING-mediated ubiquitination depends on non-covalent contacts among ubiquitin's "non-canonical" patch, the E2 enzyme and the RING domain (Plechanovová et al. 2012).

Notably, recent findings expand the spectrum of ubiquitin signaling even further: ubiquitin itself is modified by phosphorylation. Furthermore, phosphorylated ubiquitin allosterically activates Parkin, an RBR E3 ligase (chapter 1.3.2) (Kane et al. 2014, Koyano et al. 2014). Hence, the mechanisms by which ubiquitin controls cellular processes may be even more versatile than initially expected as also free (non-conjugated) ubiquitin can affect protein function.

1.3 E3 ubiquitin-protein ligases

In humans, there are two known E1 ubiquitin-activating enzymes, around 40 E2 ubiquitin-conjugating enzymes and approximately 600-1000 putative E3 ubiquitin-protein ligases (Schulman and Harper 2009, Ye and Rape 2009, Li et al. 2008). The substrate specificity relies on the ligases. Depending on their structure and mechanism of action, E3 ligases can be grouped into three distinct families: RING E3s, RBR E3s and HECT E3s.

1.3.1 RING ligases

Amongst all E3 ubiquitin-protein ligases, the RING family displays the vast majority with about 600 putative members (Li et al. 2008). They serve mainly as scaffolds for E2 enzymes and substrate proteins, thereby assisting E2-mediated isopeptide bond formation.

RING E3s are characterized by a conserved structural motif, the so-called Really Interesting New Gene (RING) domain, which consists of two zinc atoms coordinated by a cysteine-rich cluster (Freemont et al. 1991). The RING domain serves as binding site for the E2 enzyme. Substrate binding is achieved through either binding to the RING ligase (e.g. Mdm2) or binding to other factors that assemble together with the RING ligase in a multi-subunit complex (e.g. SCF complexes) (summarized in Deshaies and Joazeiro 2009).

1.3.2 RBR ligases

In 1999, two groups independently described a new family of structurally related proteins characterized by two RING finger motifs, which are separated by a cysteine-rich cluster termed IBR (in-between-RING) or DRIL (double RING finger linked) (Morett and Bork 1999, Van Der Reijden et al. 1999). Nowadays, the term RING-in-between-RING (RBR) ligases is generally accepted. Amongst the 14 family members is Parkin (Marín et al. 2004), the protein connected to the development of parkinsonism (Kitada et al. 1998).

Parkin and other RBRs were found to possess E3 ubiquitin-protein ligase activity, but, in contrast to RING E3s, RBRs are responsible for catalyzing the isopeptide bond formation between ubiquitin and the target lysine residue (Wenzel et al. 2011). RBRs take over ubiquitin from the E2 enzyme that binds to one of the RING domains and form a thioester between ubiquitin and a cysteine residue of the other RING domain. As thioester formation is a feature of HECT E3 ligases, RBRs are thought of as RING/HECT hybrids (Spratt et al. 2014).

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1.3.3 HECT ligases

The third family of E3 ligases comprises about 30 members, which harbor a conserved C-terminal domain termed Homologous to E6AP Carboxy Terminus (HECT) domain (Rotin and Kumar 2009, Scheffner and Kumar 2014). The HECT domain has a bilobed structure, which provides binding to the ubiquitin-loaded E2 enzyme in the N lobe and harbors the catalytic site cysteine, which forms the thioester with ubiquitin, in the C lobe (Huang et al. 1999; Figure 3). N and C lobes are connected by a flexible linker, which enables the E2-to-E3 and E3-to-substrate ubiquitin transfer (Verdecia et al. 2003, Kamadurai et al. 2013).

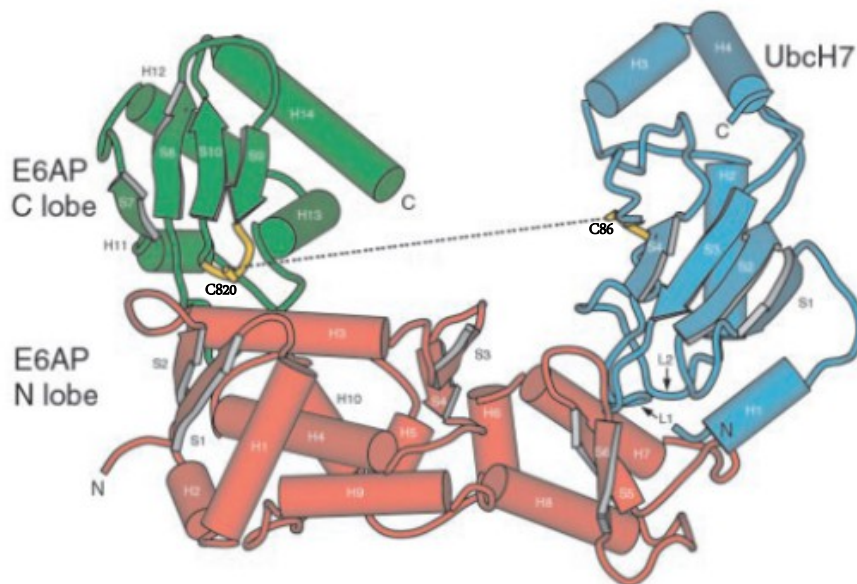


Figure 3: Crystal structure of the HECT domain of E6AP in complex with its cognate E2 enzyme Ubch7. The HECT domain has a bilobed structure consisting of N and C lobe. The N lobe (red) binds the E2 enzyme (Ubch7, blue), which can form the thioester with ubiquitin at its active site cysteine (C86, yellow). Subsequently, ubiquitin is transferred to E6AP's C lobe (green), which harbors the catalytic site cysteine of E6AP (C820, yellow). The distance between the two catalytic cysteine residues is rather big with approximately 40Å. Thus, the E2-to-E3 ubiquitin transfer depends on large conformational changes to bring both cysteines in closer proximity (modified from Huang et al. 1999).

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The N termini of HECT ligases provide surfaces for substrate binding and have regulatory functions (Kee and Huibregtse 2007). According to their N-terminal structural features, HECT ligases can be grouped into three subfamilies: NEDD4, HERC and Single HECT E3 ligases (Scheffner and Kumar 2014).

Neuronal precursor cell-expressed developmentally downregulated 4 (NEDD4) family members are characterized by a C2 phospholipid binding domain, which allows the association with the plasma membrane. This enables ubiquitination of membrane-bound substrates by NEDD4 E3s (Ingham et al. 2004). Besides, the interaction between the C2 and HECT domain can have an autoinhibitory effect on the E3 ligase activity (Wiesner et al. 2007). In addition, NEDD4 E3s harbor WW domains, which interact with PPxY motifs of substrate proteins (Ingham et al. 2004). However, in case of Smurf2, the WW domain interacts with the PPxY motif of Smad7, which activates Smurf2 by recruiting the E2 enzyme to the HECT domain (Ogunjimi et al. 2005).

The HECT and RCC1-like (HERC) subfamily of HECT ligases comprises 6 members (HERC1-HERC6), which have regulator of chromosome condensation 1 (RCC1)-like domains (RLDs) (Hochrainer et al. 2005). RCC1 is a protein that displays guanine nucleotide exchange factor (GEF) activity (Nishimoto 1999). However, this GEF activity has only been described for HERC1 but not for other HERC family members (Rosa et al. 1996).

HECT ligases that cannot be grouped into NEDD4 or HERC ligases are called Single HECT E3s. Members of this subfamily display a great diversity of structural features in their N-terminal parts. TRIP12 and HUWE1, for example, contain WWE domains, which are known poly(ADP-ribose) binding motifs (Scheffner and Kumar 2014). It is speculated that poly(ADP-ribosyl)ation of proteins primes them for ubiquitination by WWE domain-containing HECT ligases (Wang et al. 2011). For some Single HECT E3s, e.g. E6AP, no known N-terminal protein-protein interaction domains are annotated (Scheffner and Kumar 2014). Nonetheless, the N terminus of E6AP plays an important role in catalysis because it mediates the interaction with modulators and substrate recognition (Huibregtse et al. 1993, Kuhnle et al. 2011).

1.4 The HECT ligase E6AP

The E6-associated protein (E6AP) is a 100 kDa protein and the founding member of the HECT family of ubiquitin ligases (Huibregtse et al. 1995). It synthesizes K48-linked ubiquitin chains thus priming substrates for proteasomal degradation (Wang and Pickart 2005, Kim et al. 2007). E6AP is encoded by the *UBE3A* gene located at the long arm of chromosome 15 (15q11-13). The

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UBE3A gene contains 16 exons that, as a result of differential splicing, give rise to three E6AP isoforms. These isoforms differ in their very N terminus: compared to isoform 1, isoforms 2 and 3 are elongated by additional 20 and 23 amino acids, respectively (Yamamoto et al. 1997). The differences among these isoforms regarding tissue distribution or function are currently unknown.

The catalytic HECT domain is formed by the C-terminal ~350 amino acids with the catalytic site cysteine at position 820 (numbering according to isoform 1) (Huibregtse et al. 1995). The N terminus forms the platform for the interaction with human papillomavirus (HPV) E6 proteins (which gave E6AP its name) and HERC2, both of which modulate E6AP activity and/or function (Huibregtse et al. 1993, Kuhnle et al. 2011). In addition, E6AP contains three LXXLL motifs, which mediate protein-protein interactions and are found in many transcriptional regulators. One of these motifs mediates binding to HPV E6 (Figure 4) (El Hokayem and Nawaz 2014).

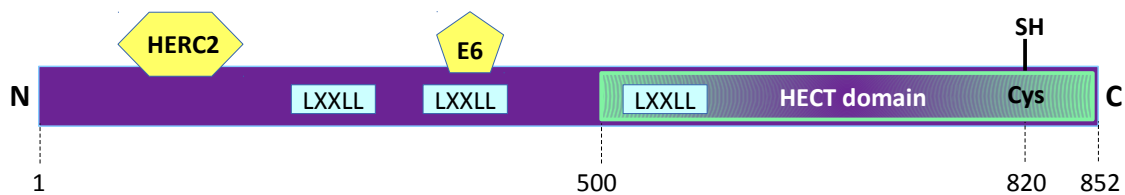


Figure 4: Schematic representation of E6AP's structure. Roughly, E6AP can be divided into an N-terminal part (amino acids 1-500), which provides binding to substrates and modulators of E3 ligase activity (HERC2 and HPV E6), and the C-terminal HECT domain with the catalytic site cysteine at position 820 (numbering according to isoform 1). Three LXXLL motifs, which are mediators of protein-protein interactions, are distributed over the protein.

Deregulation of E6AP activity is associated with distinct human diseases (Figure 5). Initially, E6AP was identified as the E3 ligase that is hijacked by the high-risk HPV E6 proteins to target the tumor suppressor p53 for ubiquitination and subsequent proteasomal degradation. This is a major step in cervical carcinogenesis (Scheffner et al. 1993).

Later, E6AP was found to play a substantial role in neurodevelopment as loss of E6AP expression is causally associated with the development of Angelman syndrome (AS) (Kishino et al. 1997, Matsuura et al. 1997). Additionally, increasing evidence connects improper E6AP expression or function also to other neurological disorders: Autism Spectrum Disorders (ASD) and Angelman-like syndromes (Miles 2011, Tan et al. 2014). The latter is implicated by the fact that loss of HERC2 protein causes a phenotype comparable to AS. Since HERC2 is a stimulator of E6AP, loss

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of HERC2 may cause a decreased activity of E6AP, which reflects the situation in AS (Kuhnle et al. 2011, Harlalka et al. 2013). However, in contrast to the role of E6AP in cervical cancer, the biochemical and cell biological mechanisms which make proper E6AP function indispensable for brain development are poorly understood.

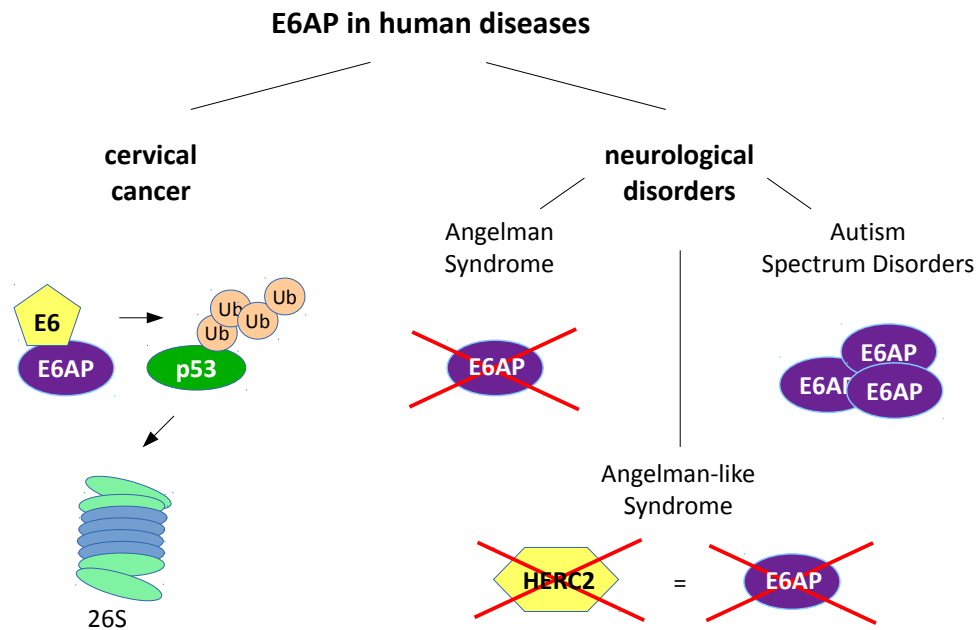


Figure 5: The role of E6AP in human diseases. In cervical cancer, binding of high-risk HPV E6 proteins to E6AP turns the tumor suppressor p53 into a substrate for E6AP-mediated ubiquitination. Subsequent proteasomal degradation of p53 by the 26S proteasome significantly contributes to malignancy. In addition, deregulation of E6AP activity is associated with neurological disorders. Loss of E6AP expression is causally associated with the development of Angelman syndrome while increased expression is found in patients with an autistic phenotype. Presumably, E6AP is also involved in the pathogenesis of a subset of Angelman-like syndromes. One example is indicated: HERC2 was identified as a stimulator of E6AP. Decreased E6AP activity due to loss of HERC2 expression may cause an Angelman-like phenotype in the HERC2 deficiency syndrome.

1.4.1 E6AP and cervical cancer

Cervical cancer is a malignant tumor of the lower part of the uterus, the cervix, and is one of the leading causes of cancer deaths in women worldwide (Ferlay et al. 2010). Predominantly it is caused by infection of the mucosa with small double-stranded DNA viruses, the HPVs (Walboomers et al. 1999). Notably, the discovery of a viral infection as a cause for cancer formation enabled the development of vaccines as cancer prophylaxis (Stanley 2007).

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About 40 mucosal HPV types are currently known to infect the anogenital tract, but not all of them have been identified in malignant tumors. Thus, they are grouped into high-risk HPVs (e.g. HPV16), which can cause cancer, and low-risk HPVs (e.g. HPV11), which are associated with the formation of benign lesions, commonly known as genital warts (Fernandes et al. 2013).

A common feature of malignant tumors is that at least one copy of the viral DNA is integrated into the genome of the host cell. This causes continuous expression of the two viral early genes E6 and E7. The products of these two genes, the E6 and E7 oncoproteins, cooperatively contribute to carcinogenesis by inactivating two important tumor suppressors: p53 and retinoblastoma protein (pRb), respectively (Narisawa-Saito and Kiyono 2007).

The pRb protein plays a substantial role in cell cycle control. In healthy cells, it hinders the G1-to-S transition by binding to the E2F family of transcription factors, which otherwise initiate the transcription of genes involved in DNA replication and cell cycle progression. Disruption of the growth-suppressive function of pRb is a key mechanism in cancer development (Nevins 2001). In HPV-positive cells, the E7 oncoprotein interferes with the interaction between pRb and E2F, which in turn stimulates cell division (McLaughlin-Drubin and Münger 2008).

P53 is an important transcription factor that, upon cellular stress, initiates cell cycle arrest, senescence or apoptosis. Lack of the functional p53 pathway causes genomic instability, which is a prerequisite for cancer development (Rinn and Huarte 2011). In cervical cancer, inactivation of p53 is caused by the HPV E6 oncoprotein (Scheffner et al. 1990). For that purpose, the E6 oncoprotein and the E3 ligase E6AP form a complex, in which two N-terminal Zinc binding domains of E6 engulf an α -helical LXXLL motif of E6AP (Zanier et al. 2013). The E6/E6AP complex acts as an E3 ligase towards p53, which leads to polyubiquitination and subsequent degradation of p53. Binding of p53 to E6 and E6AP requires initial complex formation of E6 with E6AP and accordingly, p53 is not a substrate of E6AP in the absence of E6 (Huibregtse et al. 1991, Scheffner et al. 1993, Ansari et al. 2012).

It is evident that the transforming potential of HPV E6 does not only depend on inactivation of p53 (Sedman et al. 1992). In fact, the ability of HPV E6 to interact with PDZ domain-containing proteins, e.g. Dlg, significantly contributes to hyperplasia *in vivo* and cell proliferation *in cellulo* (Nguyen et al. 2003, Choi et al. 2014). PDZ domains are common platforms for protein-protein interactions that influence cell polarity and signal transduction through scaffolding large protein complexes (Nourry et al. 2003). Binding of E6 to PDZ domains is accomplished via its C-terminal PDZ-binding motif and does not require complex formation with E6AP. However, the presence of E6AP can lead to polyubiquitination and degradation of E6-bound PDZ proteins (Kiyono et al.

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1997, Lee et al. 1997, Nakagawa and Huibregtse 2000).

Low-risk HPV types also express E6 proteins, e.g. HPV11 E6, which are able to bind to E6AP (Brimer et al. 2006, Kuballa et al. 2007). But, in contrast to high-risk HPVs, low-risk types do not lead to carcinogenesis, which is in part due to their inability to inactivate p53. In addition, the PDZ-binding motif of high-risk E6 is not present in the low-risk types (Ghittoni et al. 2010). Although the association of E6AP with low-risk HPV E6 is likely to be required for some pathogenic effects of low-risk HPVs, the exact role of this interaction remains obscure as there are no known ubiquitination targets for the low-risk HPV E6/E6AP complex (Brimer et al. 2006). However, the ability of high-risk HPV E6 proteins to reduce cellular E6AP levels is maintained in low-risk E6 proteins. Again, however, the physiological purpose of this is unknown (Kao et al. 2000, Brimer et al. 2006). Further studies are needed to clarify the role of E6AP in low-risk HPV infected cells.

In summary, many oncogenic effects of E6 depend on its association with E6AP and disruption of the E6/E6AP complex is a valuable strategy to treat cervical cancer. However, if and to what extent the physiological functions of E6AP are altered upon complex formation with E6 is unknown because the role of E6AP in HPV-negative cells is still unclear (Beaudenon and Huibregtse 2008, Matentzoglou and Scheffner 2008).

1.4.2 Role of E6AP in neurodevelopment

As E6AP was first described in the context of cervical cancer, much is known about the E6-dependent function of E6AP. However, genetic evidence shows that proper E6AP function is also important in HPV-negative cells. In particular, altered E6AP expression or function in neurons is associated with severe neurodevelopmental disorders, but the underlying biochemical mechanisms are poorly understood (Matentzoglou and Scheffner 2008, El Hokayem and Nawaz 2014).

Several E6-independent substrates of E6AP have been described, e.g. human homolog of yeast Rad23 (hHR23), which plays a role in DNA repair and acts as a shuttling factor for ubiquitinated proteins, and the RING E3 ligase Ring1b, which modulates gene expression by monoubiquitinating histone H2A (Kumar et al. 1999, Zaaroor-Regev et al. 2010). A possible contribution of these and other substrates to E6AP-associated neurological diseases has been proposed, but so far none of them is sufficient to explain the pathology of Angelman and Angelman-like syndromes as well as Autism Spectrum Disorders.

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1.4.2.1 E6AP and the Angelman syndrome

In 1965 the pediatrician Dr. Harry Angelman described patients with symptoms as speech impairment, jerky movements, seizures and a happy demeanor (Angelman 1965). This rare neurodevelopmental disorder, designated as the "Angelman syndrome (AS)", appears with an incidence of 1:15,000 to 1:40,000 (Mertz et al. 2013, Thomson et al. 2006). It turned out that the cause for AS is impaired expression of the *UBE3A* gene, which encodes E6AP (Kishino et al. 1997). In addition, it was noticed that E6AP is biallelically expressed in the majority of tissues, whereas the paternal allele is silenced in cells of the olfactory bulb and hippocampal as well as Purkinje neurons (Rougeulle et al. 1997, Albrecht et al. 1997). Hence, in these cells the maternal allele is the sole source for E6AP expression and in case of a disrupted maternal *UBE3A* gene no backup copy is available. Indeed, there are attempts to cure AS by unsilencing the paternal allele, which gave promising results in mouse models of AS (Huang et al. 2012, Meng et al. 2014).

Different mechanisms can lead to disruption of E6AP expression: in 70% of all AS patients the maternal *UBE3A* gene is deleted. Further 10% harbor mutations that lead to truncated or elongated protein products or amino acid exchanges. Notably, most of the identified single point mutations abrogate E6AP's E3 ligase activity (Cooper et al. 2004). Less than 10% of AS patients show imprinting defects or paternal uniparental disomy (Williams et al. 2010, Dagli et al. 2011). Of note, a defect of the *UBE3A* gene is not identified in the remaining 10% of AS patients. Often these patients do not exhibit all symptoms of AS and/or symptoms are not as severe. Thus, these patients are considered to have Angelman-like syndromes (Lossie et al. 2001, Tan et al. 2014).

1.4.2.2 Putative role of E6AP in Angelman-like syndromes

Individuals with Angelman-like (AS-like) syndromes share many phenotypic characteristics with AS patients. Symptoms in patients with AS-like syndromes are sometimes not as pronounced as in those with AS, and often not even all symptoms of AS are represented. This reflects the fact that the *UBE3A* gene is not affected. However, distinct genetic alterations affecting other genes and proteins have been identified in these patients (Tan et al. 2014). In some but not all cases, the indirect evidence suggests a possible link between E6AP and AS-like syndromes.

One interesting example is the *HERC2*-deficiency syndrome. Recently, patients with severe mental retardation were diagnosed to harbor a *HERC2* point mutation which leads to increased protein turnover rate and hence lower protein levels. Since *HERC2* was identified to stimulate

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E6AP's ligase activity, it has been proposed that loss of HERC2 protein results in a less active E6AP, which may cause the AS-like phenotype in these patients (Kuhnle et al. 2011, Harlalka et al. 2013).

Another example is Rett syndrome. It is caused by mutations of the *MECP2* gene located on chromosome Xq28 (Amir et al. 1999). The encoded Methyl CpG binding protein 2 (MECP2) affects gene expression by binding to methylated DNA (Nan et al. 1996). Two distinct findings indicate a connection between E6AP and Rett syndrome: Kim et. al suggest an involvement of E6AP in regulation of MECP2 activity (Kim et al. 2013). In contrast, two other groups showed that MECP2 activates the transcription of *UBE3A*. Therefore, loss of MECP2 function due to mutations would cause reduced E6AP levels, which might explain the similarities between Rett and Angelman syndromes (Samaco et al. 2004, Makedonski et al. 2005).

Furthermore, in the MECP2 duplication syndrome an additional copy of the *MECP2* gene is present in each cell. It is characterized by neuropsychiatric symptoms like depression and a broad autism phenotype (Ramocki et al. 2009). If in these patients E6AP levels are affected is not known. However, it is noteworthy that also *UBE3A* gene duplication is associated with the development of autistic traits (Schroer et al. 1998, Sebat et al. 2007). Hence, expression and activity of both, MECP2 and E6AP, plays a crucial role in neurodevelopment and has to be tightly regulated.

1.4.2.3 E6AP and Autism Spectrum Disorders

Autism Spectrum Disorders (ASD) represent a group of heterogenous neurodevelopmental disorders, which are characterized by impaired social interaction and repetitive behaviours (Miles 2011). With a prevalence of about 60/10,000, it is a rather abundant disorder that mainly affects males (Elsabbagh et al. 2012). The mechanisms that cause ASD are still a matter of debate, since more than 100 genes have been implicated in the development of autistic traits and additional environmental factors may also be involved. However, many genes that are involved in ASD play roles in translational regulation at the postsynaptic density (including scaffolding proteins and regulators of mTOR activity) and neuronal cell adhesion (Kelleher and Bear 2008, Noh et al. 2013, Rosti et al. 2014).

In approximately 20-25% of ASD patients, the genetic causes have been identified: chromosomal abnormalities (~5%), single-gene disorders (~5%) and copy number variants (CNVs) (10-20%). One of the most frequent CNVs is duplication of 15q11.2-15q11.3, the chromosomal region that

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contains the *UBE3A* gene (Miles 2011). In addition, it was shown that *UBE3A* duplication or triplication in mice result in an increased level of E6AP and autistic traits (Smith et al. 2011). Notably, these transgenic mice expressed an E6AP version with a C-terminal Flag tag, which is known to abrogate E6AP's E3 ligase activity (Salvat et al. 2004). Hence, E6AP may contribute to the observed phenotype by mechanisms other than ubiquitination. Strikingly, a cellular effect of E6AP that does not require its E3 ligase activity is modulation of the transcriptional activity of steroid hormone receptors.

1.4.3 E6AP as a modulator of steroid hormone signaling

As E6AP catalyzes the formation of K48-linked ubiquitin chains, many attempts to understand the physiological role of E6AP focused on the identification of its proteolytic targets. But in the past 15 years, emerging evidence suggests a role for E6AP in transcriptional regulation – an effect that may not depend on E6AP's E3 ligase function. In particular, it was shown that E6AP modulates steroid hormone receptor signaling (Nawaz et al. 1999).

1.4.3.1 Steroid hormone receptors (SHRs)

Steroid hormones (e.g. estradiol) are produced in endocrine glands and are spread over the body via the blood stream. Due to their lipophilic nature they can pass the cell membrane by diffusion. Their mechanism of action depends on specific binding to cellular factors: the steroid hormone receptors (SHRs), e.g. estrogen receptor alpha ($ER\alpha$). SHRs regulate a variety of cellular processes by modulating transcription as well as non-genomic pathways (Björnström and Sjöberg 2005). The generic mechanisms of estradiol and $ER\alpha$ signaling are summarized in Figure 6.

$ER\alpha$ is trapped in the cytoplasm, where it is stabilized through binding to heat shock proteins. Binding of the cognate steroid hormone estradiol induces receptor dimerization and release from the heat shock proteins. Ligand-activated $ER\alpha$ dimers translocate into the nucleus, where they bind to distinct DNA sequences to enhance or repress transcription (Figure 6A). Transcriptional activation is achieved through the recruitment of RNA polymerases and general transcription factors (TFs). Often this depends on coactivators that bridge between the sequence-specific SHRs and the general TFs. Most coactivators harbor LXXLL motifs which mediate the interaction with the SHRs. On the other hand, binding of corepressors to the SHRs

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can interfere with gene transcription by chromatin remodeling (Beato and Klug 2000).

Alternatively, activation of ER α can be achieved by phosphorylation through protein kinases that are activated by growth factor receptors (GF-Rs) (Figure 6B). Kinases that are known to phosphorylate and activate ER α are, amongst others, MAPK/Erk and Akt/PBK (Leeuw et al. 2011). The latter is implicated in the ligand-independent activation of ER α which may contribute to tamoxifen resistance in breast cancer treatment (Campbell et al. 2001).

Furthermore, ER α can modulate transcription by binding to other sequence-specific TFs (Figure 6C). In these cases ER α does not directly interact with DNA. A prominent example is the estradiol-dependent activation of AP-1 sites (Paech et al. 1997).

Last but not least, estradiol can affect protein kinase cascades in the cytoplasm. Consequently, estradiol influences not only gene expression but also non-genomic signaling pathways (Figure 6D). It is believed that these effects are mediated by membrane-associated ER α and/or the G protein-coupled receptor 30 (GPR30) (Pietras and Márquez-Garbán 2007, Maggiolini and Picard 2010).

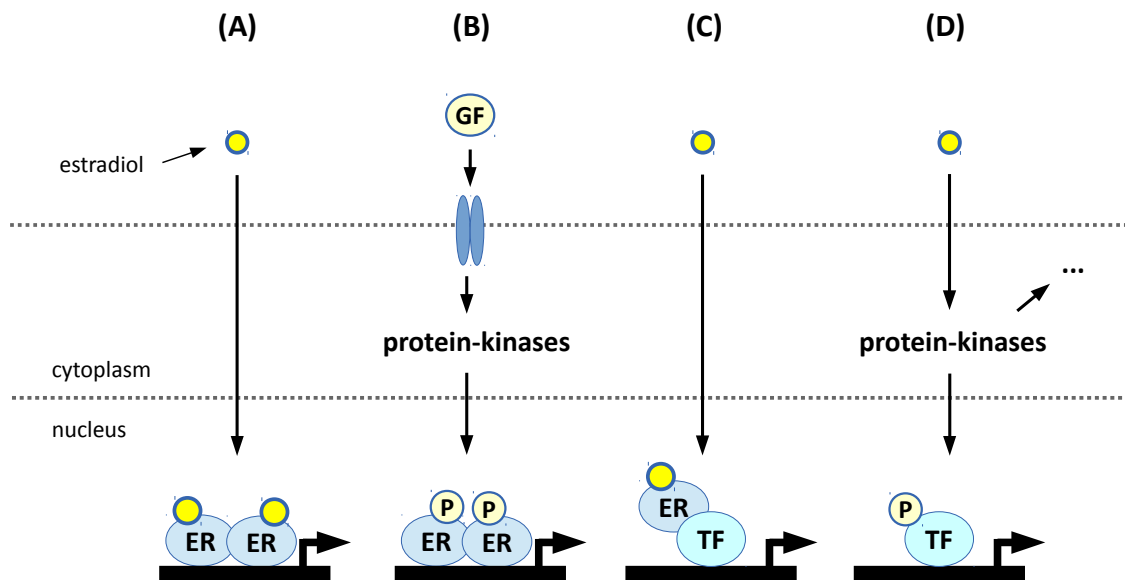


Figure 6: Mechanisms of estradiol and ER α signaling. (A) Estradiol diffuses into the cell, where it binds to the estrogen receptor (ER). Subsequent receptor dimerization enables DNA binding and transcriptional regulation. (B) Growth factors stimulate protein kinase cascades to activate the ER by phosphorylation. (C) Ligand-activated ER is able to regulate gene expression without direct binding to DNA. In these cases, other sequence-specific transcription factors (TFs) bridge between ER and DNA. (D) Estradiol activates protein kinases in the cytoplasm. The consequence is a great variety of different signaling events which can affect not only gene expression but also non-genomic pathways.

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1.4.3.2 Putative role of E6AP in SHR signaling

In 1999, Nawaz et al. identified E6AP as an interactor of SHRs in a yeast two-hybrid screen. The interaction of E6AP with the SHRs was hormone-dependent and caused a stimulation of the SHRs in transient transfection experiments. This stimulation was not abrogated by mutation of the catalytic cysteine of E6AP or deletion of the HECT domain, which suggested that the E3 ligase activity of E6AP was dispensable. However, a region that spans over all three LXXLL motifs of E6AP was required (Figure 4). In addition, they tested several point mutants of E6AP which originally have been found in AS patients. However, although these point mutations disrupted the E3 ligase activity of E6AP, they did not interfere with the co-activation function. Thus, Nawaz et al. concluded that E6AP has two independent functions in cells: as E3 ligase and SHR coactivator (Nawaz et al. 1999). In contrast, later findings suggested that E6AP targets SHRs for ubiquitination and degradation under certain conditions (Li et al. 2006). The proposed model encompasses the interaction of E6AP with ligand-activated SHRs, their binding to DNA, and subsequent ubiquitination and degradation of the SHRs (Reid et al. 2003, Ramamoorthy and Nawaz 2008). Later findings indicate that E6AP stimulates not only the genomic but also the non-genomic effects of steroid hormones (Khan et al. 2006, Srinivasan and Nawaz 2011). However, it took more than 10 years until the effect of endogenous E6AP on endogenous SHR target genes was tested. The starting point was the finding that AS mice display increased levels of the synaptic protein Arc (Greer et al. 2010). Arc plays a fundamental role in learning and memory by regulating receptor internalization in the post-synapse (Bramham et al. 2008). Hence, it was speculated that Arc significantly contributes to the pathology of AS. Indeed, later it turned out that normalization of Arc levels attenuates seizures (Mandel-Brehm et al. 2015). Initially, it was postulated that E6AP regulates Arc levels by targeting it for ubiquitination and subsequent proteasomal degradation. However, Kuhnle et al. provided evidence that E6AP is not an E3 ligase of Arc, but rather affects the estradiol-induced transcription of the *Arc* gene. Strikingly, in this particular case, E6AP acted as a repressor of *Arc* gene transcription. In addition, transient transfection experiments confirmed this observation not only with endogenous, but also with ectopic E6AP. However, the repressing effect of E6AP may be gene-specific as the estradiol-induced transcription of *CTSD* (another estradiol-regulated gene) required the presence of endogenous E6AP (Kuhnle et al. 2013).

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These findings lead to two important conclusions: (I) The pathogenesis of AS is at least partially dependent on the effect of E6AP on genomic steroid hormone action. It is very likely that the altered transcription of not only *Arc*, but also other genes contributes to disease development. (II) The mechanisms by which E6AP regulates SHR signaling might be more versatile than initially expected. E6AP acts not only as a coactivator, but also as a repressor of SHR signaling. However, the underlying biochemical and cell biological mechanisms are currently unknown.

2. Aims

It is evident that deregulation of E6AP activity is causally associated with the development of distinct human diseases. In cervical cancer, the interaction with high-risk HPV E6 proteins alters the substrate specificity of E6AP. This leads to E6AP-mediated ubiquitination and degradation of p53 as well as PDZ domain-containing proteins, which are not substrates of E6AP in the absence of E6. In addition, genetic evidence clearly shows that E6AP plays a crucial role in neurodevelopment. Impaired and increased expression of E6AP have been associated with the development of Angelman syndrome and with autistic traits, respectively. But so far the underlying biochemical mechanisms remain obscure. Hence, the aim of this thesis was to combine *in vitro* and *in cellulo* studies to provide insights into mechanisms that regulate E6AP activity and pathways that are regulated by E6AP.

Due to the role of E6AP in cervical carcinogenesis, it can be inferred that E6AP is a "modulatable" E3 ligase. Thus, the physiological role of E6AP may be specified by its interaction with other proteins. Recently, HERC2, an important player in neurodevelopment, was identified as a stimulator of E6AP's E3 ligase activity. However, whether the interaction with HERC2 alters the substrate spectrum of E6AP is currently unknown. Interestingly, in the course of those studies, it was found that ubiquitin's "canonical" hydrophobic patch plays a role in E6AP-catalyzed ubiquitination unless E6AP is bound to HERC2. Later, it was ascertained that the same holds true for HPV E6 proteins. Thus, modulators of E6AP not only affect the substrate specificity, but also act as allosteric activators by manipulating the interaction between ubiquitin and E6AP. To gain information about the underlying biochemical mechanisms, the first aim of this thesis was to clarify the contribution of ubiquitin and HPV E6 to E6AP-mediated catalysis.

On the other hand, there is accumulating evidence for a role of E6AP beyond the ubiquitin-proteasome system. In particular, E6AP affects transcription by manipulating steroid hormone receptor signaling. This effect may not require E6AP's catalytic activity, which suggests two distinct functions of E6AP within cells: E3 ligase and transcriptional modulator. Accordingly, E6AP can influence proteostasis by affecting not only protein stability, but also protein biogenesis, which may play a considerable role in the pathogenesis of the neurological disorders mentioned above. Thus, the second objective of this thesis was to unravel the mechanism by which E6AP affects steroid hormone signaling within cells.

3. Materials and Methods

3.1 Materials

3.1.1 Chemicals and Reagents

Amplifyer	GE Healthcare
Aprotinin/Leupeptin	Sigma Aldrich
Ammoniumperoxodisulfate	Roth
ATP	Sigma Aldrich
β -glycerophosphate	Sigma Aldrich
β -mercaptoethanol	Merck
BCA protein assay kit	Thermo Fisher
BSA, protease free	Sigma
CHIR99021 (GSK3 inhibitor)	Cayman Chemical
Coenzyme A	PJK
DTT	Roth
(ECL) Enhanced Chemiluminescence	Amersham
EDTA	Roth
Glutathione-S-Sepharose	GE Healthcare
glycine	Roth
L-cysteine	Merck
L-lysine monohydrochloride	Sigma Aldrich
LY294002 monohydrochloride (PI3K inhibitor)	Sigma Aldrich
Guanidinium hydrochloride	Roth
Imidazole	Roth
IPTG	Roth
Lipofectamine 2000	Invitrogen
Luciferin (D-)	PJK
MgCl ₂	ACROS organics
MgCO ₃	Roth
MgSO ₄	Roth
Midori Green	Biozym

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NaF	Sigma Aldrich
Na ₂ HPO ₄	Sigma Aldrich
NaH ₂ PO ₄	Merck
NaVO ₃	Sigma Aldrich
Ni-NTA Agarose	Qiagen
NP-40	MP Biomedicals
ONPG	Sigma-Aldrich
PBS	Gibco
PefaBloc	Boehringer Ingelheim
Q Sepharose Fast Flow	GE Healthcare
Rapamycin	Life Technologies
RotiBlock	Roth
Rotiphorese Gel 30	Roth
SDS	Roth
TEMED	Roth
Tricine	Roth
Triton-X 100	Roth
Tris (Trizma Base)	Sigma Aldrich
Tween-20	Roth
UO126 monoethanolate (MEK inhibitor)	Sigma Aldrich
Western Lightning ECL	Perkin Elmer

3.1.2 Buffers and Solutions

Laemmli loading buffer (2x)	125 mM Tris pH 6.8, 200 mM DTT, 4% SDS, 0.001 % Bromophenol blue
Laemmli running buffer (10x) (pH 8.4)	250 mM Tris, 2 M glycine, 1 % SDS
Stacking gel buffer	0.5M Tris pH 6.8, 0.4 % SDS, 0.001 % Bromophenol blue
Separating gel buffer	1.5M Tris pH 8.8, 0.4 % SDS
Transfer buffer (20x) (pH 8.3-8.6)	12.5 mM Tris, 0.1 M glycine
TNE-T (pH 7.5)	10 mM Tris, 2.5 mM EDTA, 50 mM NaCl, 0.1 % Tween-20

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Coomassie Blue staining solution	2 g/L Coomassie Brilliant Blue R250 in Destain solution
Destain solution	40 % methanol, 10 % acetic acid
Urea loading buffer (2x)	8 M Urea, 0.1 M Tris pH 6.8, 10 % Glycerol, 4 % SDS, 0.001 % Bromophenol blue
Stripping buffer for Western blots	6 M Guanidinium Hydrochloride, 20 mM Tris pH 7.5, 10 mM β -Mercaptoethanol, 0.2 % NP40
DNA loading buffer (10x)	60 % Saccharose, 0.25 M EDTA, 0.001 % Bromophenol blue
TAE buffer (50x)	2 M Tris, 950 mM acetic acid, 50 mM EDTA
TNN lysis buffer (pH 8)	0.1 M Tris, 0.1 M NaCl, 1% NP-40, 1 mM Pefabloc, 1 μ g/mL Aprotinin/Leupeptin, 1 mM DTT
Buffer Z (pH 7.0)	0.1 M NaH ₂ PO ₄ /Na ₂ HPO ₄ , 10 mM KCl, 1 mM MgSO ₄ , 50 mM β -Mercaptoethanol
ONPG (pH 7.0)	4 mg/mL in 0.1 M Na ₂ HPO ₄ /NaH ₂ PO ₄
Luria Broth medium (LB) (pH 7.5)	10 g/L NaCl, 5 g/L yeast extract, 0.5 g/L NaCl, 20 mM glucose
S1 (pH 8)	50 mM Tris, 10 mM EDTA, 100 μ g/mL RNaseA
S2	200 mM NaOH, 1 % SDS
S3 (pH 5.1)	2.8 M KAc
T25N50 (pH 7.5)	25 mM Tris-HCl, 50 mM NaCl
Luciferase substrate solution (pH 7.0-7.5)	470 μ M Luciferin, 530 μ M ATP, 270 μ M Coenzyme A, 200 μ M EDTA, 20 mM Tricine, 2.67 mM MgSO ₄ , 1.07 mM MgCO ₃ , 33.3 mM DTT
GST elution buffer (pH 8)	25 mM reduced Glutathion, 50 mM Tris-HCl

3.1.3 Bacterial strains

E.coli DH5 α

F- Φ 80*lacZ* Δ M15 Δ (*lacZYA-argF*) U169 *recA1 endA1 hsdR17* (rK-, mK+) λ -

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E.coli XL10-Gold

Tet^r Δ(*mcrA*)183 Δ(*mcrCB-hsdSMR-mrr*) 173 *endA1 supE44 thi 1 recA1 gyrA96 relA1 lac Hte* [F'*proAB lacI^q Z Δ M15 Tn10* (Tet^r) Amy Cam^r] (Stratagene)

E.coli BL21(DE3)

F⁻ *ompT gal dcm lon hsdS_B(r_B⁻ m_B⁻) λ(DE3 [lacI lacUV5-T7 gene 1 ind1 sam7 nin5])*

E.coli BL21(DE3) pLysS

F⁻ *ompT gal dcm lon hsdS_B(r_B⁻ m_B⁻) λ(DE3) pLysS(cm^R)*

3.1.4 Mammalian cell lines

H1299: non-small cell lung carcinoma;

H1299 E6APi: H1299 cells which were stably selected for RNAi-mediated knockdown of E6AP expression (Kuballa et al. 2007)

HEK293T: human embryonic kidney cells;

HEK293T E6APi: HEK293T cells which were stably selected for RNAi-mediated knockdown of E6AP expression (Kuhnle et al. 2013)

Cells were cultured in DMEM (Gibco) supplemented with 10 % FBS. H1299 E6APi and HEK293T E6APi were kept under antibiotics selection (to maintain the knockdown construct) with 4 μg/mL Puromycin (Gibco) and 100 μg/mL Hygromycin B (Invitrogen), respectively.

3.1.5 Antibodies

3.1.5.1 Primary antibodies

protein	name	source	dilution (buffer)	species
E6AP	anti-E6AP	AG Scheffner	1:5 (TNE-T)	mouse
His-tag	anti-6xHis-POX (HRP conjugated)	Sigma-Aldrich	1:5000 (TNE-T + 5 % BSA)	mouse
p53	DO-1	CalBiochem	1:1000 (TNE-T)	mouse

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HA-tag	HA 1.1	Covance	1:2500 (TNE-T)	mouse
β -Actin	anti-Actin	Sigma-Aldrich	1:7500 (TNE-T)	mouse
GSK3 α/β	anti-GSK3 α/β	invitrogen	1:1000 (TNE-T + 1 % BSA)	mouse
pGSK3 α/β (S21/9)	anti-pGSK3 α/β	Cell signaling	1:1000 (TNE-T + 5 % BSA)	rabbit
Akt	anti-Akt	Cell signaling	1:2000 (TNE-T + 5 % BSA)	rabbit
pAkt S473	anti-pAkt	Cell signaling	1:2000 (TNE-T + 5 % BSA)	rabbit
Erk1/2	anti-Erk1/2	Cell signaling	1:1000 (TNE-T + 5 % BSA)	rabbit
pErk1/2	anti-pErk1/2	Millipore	1:2000 (TNE-T + 5 % BSA)	rabbit

3.1.5.2 Secondary antibodies

name	source	dilution
HRP-coupled anti-mouse antibody	Dianova	1:20000
HRP-coupled anti-rabbit antibody	Dianova	1:20000

3.1.6 Primers

name	target	sequence	sites
FM43 5'ERa S118A	ER α	GCAGCTGGCGCCTTTCCTG	
FM44 3'ERa S118A	ER α	CAGGAAAGGCGCCAGCTGC	
FM45 5'ERa S167A	ER α	GAAAGATTGGCCGCTACCAATGAC	
FM46 3'ERa S167A	ER α	GTCATTGGTAGCGGCAATCTTTC	
FM47 5'ERa S104,106A	ER α	CCCCCACTCAACAGCGTGGCTCCGGCCC CGCTGATGCTACTGC	
FM48 3'ERa S104,106A	ER α	GCAGTAGCATCAGCGGGCCGGAGCCA CGCTGTTGAGTGGGGG	
FM65 5'ERa A118E	ER α	CGCCGCAGCTGGAGCCTTTCCTGCAG	

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FM66 3'ERa A118E	ER α	CTGCAGGAAAGGCTCCAGCTGCGGCG	
FM67 5'ERa A102'104'106E	ER α	GGGGGGTTTCCCCCACTCAACGAAGT GGAGCCGGAGCCGCTGATGCTACTGCA CCCGCC	
FM68 3'ERa A102'104'106E	ER α	GGCGGGTGCAGTAGCATCAGCGGCTCC GGCTCCACTTCGTTGAGTGGGGGAAA CCCCC	
FM57 5'Ub I36A	ubiquitin	GACAAGGAAGGCGCCCCTCCTGACCAG	
FM58 3'Ub I36A	ubiquitin	CTGGTCAGGAGGGGCGCCTTCCTTGTC	
FM59 5'Ub L71 73A	ubiquitin	GAAAGAGTCCACCCTGCACCTGGTCGC CCGTGCCAGAGGTGGT	
FM60 3'Ub L71 73A	ubiquitin	ACCCACCTCTGGCACGGGCGACCAGGT GCAGGGTGGACTCTTTC	
FM63 5'ERa S102A	ER α	GTTTCCCCCACTCAACGCCGTGGCTCC GGCCCCG	
FM64 3'ERa S102A	ER α	CGGGGCCGGAGCCACGGCGTTGAGTGG GGGAAAC	
FM84 5'E6APdC2	E6AP	CCATCACGTATGCCAAAGGATTTGGCT AACTGTAAAAGCTTAAG	
FM85 3'E6APdC2	E6AP	CTTAAGCTTTTACAGTTAGCCAAATCC TTTGGCATACTGATGG	
FM86 5'E6APdC4	E6AP	CACGTATGCCAAAGGATAAGGCATGCT GTAAAAGC	
FM87 3'E6APdC4	E6AP	GCTTTTACAGCATGCCTTATCCTTTGGC ATACGTG	
FM88 5'E6AP K847A	E6AP	CCATCACGTATGCCGCAGGATTTGGCAT GC	
FM89 3'E6AP K847A	E6AP	GCATGCCAAATCCTGCGGCATACGTGA TGG	
FM90 5'E6AP K847R	E6AP	CATCACGTATGCCAGAGGATTTGGCAT GC	
FM91 3'E6AP K847R	E6AP	GCATGCCAAATCCTCTGGCATACTGGA TG	

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FM120 5'HECTopt C820A	Codon-optimized HECT domain of E6AP	ACCAGCCATACCGCGTTTAATGTTCTG	
FM121 3'HECTopt C820A	Codon optimized HECT domain of E6AP	CAGAACATTAAACGCGGTATGGCTGGT	
5'E6AP(BamHI) (S. Kühnle)	E6AP	CGGGATCCATGAAGCGAGCAGCTG	BamHI
TB5_E6APdelta_C-lobe_rew (T. Barbic)	E6AP	GTAGTCGACTTAATTCCGGCTTCCACATATAA	Sall

3.1.7 Plasmids

Stock number	vector	insert	source
12	pRcCMV	β -Galactosidase	AG Scheffner
65 (S. K.)	pcDNA3	HA-Ring1b I53S	S. Kühnle
184 (S. K.)	pcDNA 3.1 (-) Hygro	empty	S. Kühnle
163 (S. K.)	pcDNA3	HA-ER α	S. Kühnle
154 (S. K.)	pGL2 basic	3xERE TATA Fluc	S. Kühnle
147 (S. K.)	pcDNA3	HA-E6AP wt RNAimut	S. Kühnle
146 (S. K.)	pcDNA3	HA-E6AP wt	S. Kühnle
227 (S. K.)	pcDNA3	HA-E6AP C820A	S. Kühnle
96 (S. K.)	pcDNA3	HA-E6AP C820A RNAi mut	S. Kühnle
ARF 46	pET3A	ubiquitin	A. Rojas-Fernandes
68	pcDNA3	HA-ER α S118A	mutagenesis PCR
69	pcDNA3	HA-ER α S167A	mutagenesis PCR
70	pcDNA3	HA-ER α S104A, S106A	mutagenesis PCR
74	pET3A	ubiquitin L71A, L73A	mutagenesis PCR
ARF 88	pET3A	ubiquitin L8A, I44A	A. Rojas-Fernandes

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86	pcDNA3	HA-ER α S102/104/106/118A	mutagenesis PCR
87	pcDNA3	HA-ER α S102/104/106A	mutagenesis PCR
88	pcDNA3	HA-ER α S118E	mutagenesis PCR
91	pcDNA3	HA-ER α S102/104/106/118E	mutagenesis PCR
92	pET3A	ubiquitin L71A, L73A, I36A	mutagenesis PCR
95	pcDNA3	HA-PTEN	H. Liang
103	TOP flash	β -Catenin reporter	AG Brunner
105	pcDNA3	HA-E6AP deltaC2 RNAimut	mutagenesis PCR
106	pcDNA3	HA-E6AP K847R RNAimut	mutagenesis PCR
107	pcDNA3	HA-E6AP deltaC4 RNAimut	mutagenesis PCR
108	pcDNA3	HA-E6AP K847A RNAimut	mutagenesis PCR
109	pcDNA3	HA-E6AP C820A deltaC2 RNAimut	mutagenesis PCR
110	pcDNA3	HA-E6AP C820A deltaC4 RNAimut	mutagenesis PCR
111	pcDNA3	HA-E6AP C820A K847A RNAimut	mutagenesis PCR
112	pcDNA3	HA-E6AP C820A K847R RNAimut	mutagenesis PCR
136	pGEX 2TK	GST-16E6	P. Kuballa
137	pGEX 2TK	GST-11E6	P. Kuballa
138	pGEX 2TK	GST-11C16	P. Kuballa
139	pGEX 2TK	GST-11E6 opt	E. Weber
142	pET15b	His-HECT E6AP	D. Schneider
143	pET15b	His-HECT E6AP C820A	mutagenesis PCR
147	pET15b	His-ubiquitin	subcloning (NdeI, BamHI)
148	pET15b	His-ubiquitin L8A, I44A	subcloning (NdeI, BamHI)

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149	pET15b	His-ubiquitin L71A, L73A	subcloning (NdeI, BamHI)
150	pET15b	His-ubiquitin L71A, L73A, I36A	subcloning (NdeI, BamHI)
ARF305	pGLO	AP-1 reporter	A. Rojas-Fernandes
ARF314	pGLO	CRE reporter	A. Rojas-Fernandes
61 (S. K.)	pcDNA3	HA-CaMKII	S. Kühnle
62 (S. K.)	pcDNA3	HA-CaMKII T305A, T306A (constitutive active)	S. Kühnle
179 (S. K.)	pcDNA3	HA-hHR23	S. Kühnle
TB6 (T. B.)	pcDNA3	HA-E6AP delta C-lobe	T. Barbic
196 (S.K.)	pcDNA3	HA-E6AP delta C6	S. Kühnle
1318 (P.K.)	pcDNA3	HA-rDlg	P. Kuballa
TB4	pcDNA3	HA-E6AP delta C6 C820A	T. Barbic

3.1.8 DNA and protein markers

-GeneRuler 1kb Plus DNA Ladder (Fermentas):

20000, 10000, 7000, 5000, 4000, 3000, 2000, 1500, 1000, 700, 500, 300, 200, 75 [bp]

- PageRuler Prestained Protein Ladder (Fermentas):

170, 130, 100, 70, 55, 40, 35, 25, 15, 10 [kDa]

- PageRuler Unstained Protein Ladder (Fermentas):

200, 150, 120, 100, 85, 70, 60, 50, 40, 30, 25, 20, 15, 10 [kDa]

3.2 Methods

3.2.1 PCR and restriction digests

Amplification of DNA fragments was performed with Phusion polymerase (Fermentas) according to manufacturer's instructions. For colony PCR, TAQ polymerase (AG Scheffner) was used together with Thermo Pol buffer (New England Biolabs). Mutagenesis PCR was carried out with Pfu turbo polymerase (Stratagene) according to manufacturer's instructions, and 10 μ L PCR product were digested with DpnI (New England Biolabs) before transformation into *E. coli* XL10 gold cells. Restriction enzymes (New England Biolabs) were incubated with DNA according to manufacturer's instructions.

3.2.2 Separation and extraction of DNA from agarose gels

Samples were supplemented with DNA-loading buffer and separated on 1% TAE agarose gels containing 0.1 % Midori green. DNA was visualized with UV light (254nm). Bands were excised and purified with the Nucleospin Extraction Kit (Macherey Nagel) according to manufacturer's instructions.

3.2.3 Ligation of DNA fragments

For ligation, DNA fragments were incubated with T4 DNA ligase (Fermentas) according to manufacturer's instructions.

3.2.4 Transformation

Ligation reactions, DpnI digested PCR reactions or purified plasmid DNA (100ng) were incubated with chemical competent *E.coli* cells (XL10 gold or DH5 α for DNA preparations, BL21 for protein expression) on ice for 30 minutes. Cells were subjected to heat shock for 90 seconds at 42°C. After cooling on ice for 10 minutes, cells were plated on LB agar plates or directly inoculated into 100 mL LB medium containing Ampicillin (100 μ g/mL) and, in case of protein expression in *E.coli* BL21 (DE3) pLysS, Chloramphenicol (34 μ g/mL). Cells were incubated over night at 37°C, liquid cultures were shaken at 180 rpm.

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3.2.5 Mini Preparation of plasmid DNA

2 mL of each *E.coli* culture were harvested by centrifugation at 16100 rcf. Pellets were resuspended in 200 μ L buffer S1. Cells were lysed by addition of 200 μ L buffer S2. After incubation for 3 minutes at room temperature, samples were supplemented with 200 μ L buffer S3 and incubated for 5 minutes on ice. Samples were centrifuged for 15 minutes at 16100 rcf and 4°C. Supernatants were mixed with 400 μ L isopropanol and centrifuged for 30 minutes at 16100 rcf and 4°C. After removal of the supernatants, pelleted DNA was washed with 600 μ L 70% ethanol and centrifuged for 15 minutes at 16100 rcf at room temperature. Supernatants were removed and the pellet was dried by vacuum. DNA was resuspended in 30 μ L water and DNA concentration was determined photometrically using the NanoDrop device (Implen). DNA was stored at -20°C.

3.2.6 Midi preparation of plasmid DNA

For preparation of larger amounts of DNA, 100-200 mL *E.coli* culture were inoculated and incubated over night at 37°C with gentle shaking (180 rpm). Cells were pelleted by centrifugation. Isolation of plasmid DNA was achieved with the PureYield Plasmid Midiprep System (Promega) according to manufacturer's instructions. DNA concentration was determined photometrically using NanoDrop. Purified plasmid DNA was stored at -20°C.

3.2.7 DNA sequencing

Sequencing reactions were performed by GATC (Köln).

3.2.8 Bacterial protein expression

Expression of untagged, GST-tagged or His-tagged proteins was performed by transformation of respective plasmids into *E.coli* BL21 cells (see 3.2.4). Liquid LB cultures were grown over night at 37°C under gentle shaking (180 rpm). Cultures were diluted to an OD_{600nm} of 0.1 with LB medium and further incubated. At an OD_{600nm} of 0.6-0.8, protein expression was induced by addition of 0.4 mM IPTG. Cultures were further incubated at 37°C for 5 hours or at 20°C over night. Cells were harvested by centrifugation (4221 rcf, 15 minutes) at 4°C. Pellets were stored at

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-80°C or directly used for protein purification. Bacterial cells were resuspended on ice in a lysis buffer containing PBS and 1% Triton-X 100, 1 mM DTT or 10 mM β -Mercaptoethanol (in case of His-tagged proteins), 1 μ g/mL Aprotinin/Leupeptin and 1 mg/mL Pefabloc. Cells were lysed by sonication using a Branson sonifier 250 (duty cycle 30-40, output control 3-4, 4 x 20 cycles). Extracts were cleared by centrifugation for 15 minutes at 10000 rpm and 4°C.

3.2.9 Affinity purification of GST-tagged proteins

Cleared supernatants (3.2.8) were incubated with Glutathione sepharose beads for 1-2 hours at 4°C. Beads were recovered by centrifugation for 5 minutes at 500 rcf and 4°C and washed 3 times with lysis buffer (see 3.2.8). Elution of GST-tagged proteins from the beads was achieved by incubating the beads 3-4x with 1 column volume of GST elution buffer (3.1.2). Elutions were analyzed by SDS-PAGE and Coomassie staining.

3.2.10 Affinity purification of His-tagged proteins

Cleared supernatants (3.2.8) were either incubated with Ni-NTA resin over night at 4°C or loaded onto HisTrap FF columns (GE Healthcare). After washing of the beads with lysis buffer (3.2.8), His-tagged proteins were eluted by increasing concentrations of Imidazole (5-500 mM) in 25 mM Tris-HCl (pH 7.5) and 300 mM NaCl. Elutions were analyzed by SDS-PAGE and Coomassie staining. Pooled fractions were dialyzed against T25N50.

3.2.11 Purification of bacterially expressed ubiquitin

Cleared supernatants (3.2.8) were boiled for 20-25 minutes at 70°C. Precipitates were removed by centrifugation for 15 minutes at 10000 rcf. Supernatants were analyzed by SDS-PAGE and Coomassie staining.

3.2.12 Preparation and detection of *in vitro* translated proteins

For preparation of *in vitro* translated proteins, the TNT coupled reticulocyte lysate kit (Promega) was used together with S^{35} -labeled methionine (Perkin Elmer) according to manufacturer's instructions. Proteins were separated by SDS-PAGE (3.2.13), and gels were fixed with Coomassie

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Destain solution and subsequently incubated with Amplifier (Amersham). Gels were dried by vacuum and applied to Imaging plates BASIII (Fuji). Detection was achieved using BAS Reader (Raytest).

3.2.13 SDS-PAGE

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to the protocol of Laemmli (Laemmli, U. K. 1970). To achieve an appropriate separation of proteins, separating gels were prepared with suitable polyacrylamide concentrations ranging from 7.5-17%. Stacking gels contained 5% polyacrylamide. Samples were boiled in Laemmli sample buffer for 5 minutes unless otherwise stated. Electrophoresis was carried out at a constant electric current of 50 mA unless otherwise stated. Detection of proteins was achieved by Coomassie staining (3.2.17), fluorography (3.2.12) or Western blot analysis (3.2.18).

3.2.14 *In vitro* ubiquitination assay

For *in vitro* ubiquitination, 1 μ L of rabbit reticulocyte lysate-translated 35 S-labeled substrate protein (E6AP, Ring1B-I53S, HHR23A) or 100 ng of recombinant substrate (baculo p53 or E6AP, bacterial HECT domain) were incubated with 50 ng of baculovirus-expressed E1, 50 ng of E2 enzyme (UbcH5b or UbcH7), 200 ng of baculovirus-expressed E6AP (where indicated), 20 μ g of ubiquitin or ubiquitin mutants (Ub LIA, Ub ILA) in the absence or presence of GST-16 E6 (200 ng) in 40- μ L volumes. In addition, reactions contained T25N50, 1 mM DTT, 2 mM ATP, and 4 mM MgCl₂. After incubation at 25°C for 2 hours, reactions were stopped by boiling in Laemmli sample buffer. Total reaction mixtures were electrophoresed in 8-15% SDS-polyacrylamide gels, and 35 S-labeled proteins were detected by fluorography (3.2.12), and recombinant proteins by Western blot analysis (3.2.18) or Coomassie staining (3.2.17).

3.2.15 *In vitro* thioester assays

For *in vitro* thioester assays, 50 ng of E1, 50 ng of UbcH7, UbcH5b or UbcH5b N77A, and 250 ng of E6AP or HECT domain of E6AP, where indicated, were incubated with 20 μ g of ubiquitin or the ubiquitin mutants (UbLIA, UbILA) for 1 minute at 30°C in 40 μ L volumes. In addition, reactions contained T25N50, 0.1 mM DTT, 4 mM ATP, and 10 mM MgCl₂. Reactions were terminated by

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incubating the mixtures for 15 min at 30°C in Urea loading buffer (to preserve ubiquitin thioester complexes) or by boiling the mixtures in Laemmli sample buffer (reducing conditions). Whole reaction mixtures were separated on 8-15% SDS-polyacrylamide gels (3.2.13) under a constant current of 30 mA and a maximum of 150 V. Gels were subjected to Western Blot analysis (3.2.18) using anti-E6AP or anti-His antibodies.

3.2.16 *In vitro* discharge assays

50 ng E2 enzymes (UbcH5b, UbcH5b N77A or UbcH7) were charged with 20 µg ubiquitin or the ubiquitin mutants by incubation together with 50 ng E1 and 20 µg ubiquitin version at 30°C for 1 minute. In addition, reactions contained T25N50, 0.1 mM DTT, 4 mM ATP, and 10 mM MgCl₂. To avoid further loading with ubiquitin, samples were supplemented with 50 mM EDTA before incubation in 50 mM cysteine or lysine (unless otherwise stated) for additional 20 minutes at 30°C. Reactions were stopped by addition of Urea loading buffer and further incubation at 30°C for 15 minutes. Whole reaction mixtures were separated on 8-15% SDS-polyacrylamide gels (3.2.13) under a constant current of 30 mA and a maximum of 150 V. Gels were subjected to Western Blot analysis (3.2.18) using an anti-His antibody.

3.2.17 Coomassie Blue and colloidal Coomassie staining

For staining of proteins separated by SDS-PAGE, gels were incubated in Coomassie Blue solution for 1 hour under gentle shaking. For destaining, gels were further incubated in Coomassie Destain solution. Staining with colloidal Coomassie was performed using RotiBlue (Roth) according to manufacturer's instructions.

3.2.18 Western blot analysis

Samples were subjected to SDS-PAGE as described (3.2.13) and transferred to PVDF membrane (Millipore) according to manufacturer's instructions. Transfer was performed in a wet transfer apparatus (BioRad) for 90 minutes at 60 V. Membranes were blocked in RotiBlock (Roth) for 1 hour at room temperature or overnight at 4°C. After washing the membranes with TNE-T (3x 5 minutes), they were incubated with primary antibody for 1 hour, washed 3x with TNE-T, and further incubated with the appropriate secondary antibody (except the used primary antibody was HRP-conjugated). Subsequently, membranes were washed 3x with TNE-T for 10 minutes.

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Detection of HRP-conjugates was performed with the Western Lightning ECL (Perkin Elmer) according to manufacturer's instructions in a LAS-3000 (Fujifilm).

3.2.19 Transient transfection experiments

Mammalian cell lines were prepared for transient transfection by seeding on 6-well culture dishes. 24 hours later, 70-90% confluent cells were transfected by lipofection using Lipofectamin 2000 (Invitrogen) according to manufacturer's instructions. Prior transfection, cells were supplemented with fresh media. Each well was transfected with 1000 ng ERE reporter, 50 ng pcDNA3 HA-ER α , 300 ng pRcCMV- β -Gal. Where indicated, 1.5 μ g pcDNA3 HA-E6AP, 0.5 μ g pcDNA3 HA-CaMKII or 1.5-3.0 μ g pcDNA3 HA-PTEN were cotransfected. Total DNA amounts in all samples were equalized by addition of empty vector (pcDNA3). If the effect of a kinase inhibitor was investigated, media was replaced by fresh media containing the inhibitor 6 hours after transfection. 24 hours after transfection, cells were harvested by scraping on ice in cold PBS and stored at -20°C or directly used for determination of protein concentration (3.2.24), and β -Gal (3.2.22) and luciferase (3.2.23) activities.

3.2.20 Large scale *in cellulo* luciferase assays in presence of kinase inhibitors

H1299 wt and E6APi cells were seeded onto 10-cm dishes 24 hours prior transfection. 70-90% confluent dishes were transfected by lipofection (Lipofectamin 2000, Invitrogen) according to manufacturer's instructions. Each dish was transfected with 6000 ng ERE reporter, 300 ng pcDNA3 HA-ER α , and 1800 ng pRcCMV- β Gal. 6 hours later, cells were detached by trypsinization, collected by centrifugation (10 minutes, 500 rcf) and resuspended in fresh media. Cells from one 10-cm dish were seeded onto 12 wells using 6-well plates. Upon seeding, treatment with the indicated inhibitor was started. Cells were harvested 16 hours later by scraping on ice in PBS, and stored at -20°C or directly analyzed.

3.2.21 Lysis of mammalian cells

Cell pellets were resuspended in 90 μ L (3.2.18) or 60 μ L (3.2.19) TNN lysis buffer, respectively, and incubated for 30 minutes on ice. Lysates were cleared by centrifugation for 30 minutes at 16100 rcf and 4°C. Supernatants were kept on ice until β -Gal (3.2.22) and luciferase (3.2.23) activities or protein concentrations (3.2.24) were determined.

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3.2.22 Determination of β -Gal activities in whole cell extracts

To adjust for transfection efficiencies, the activity of the co-expressed β -Galactosidase (β -Gal) was determined in cleared whole cell extracts (3.2.21) in duplicates. 5 μ L of lysate were mixed with 120 μ L Buffer Z and 5 μ L ONPG on a 96-well plate. Samples were prepared on ice, and reaction was initiated by incubation at 37°C for about 5 minutes. β -Gal activities were determined by measuring the absorbance at 405 nm in a micro-plate reader (Wallac 1420, Perkin Elmer).

3.2.23 Determination of luciferase activities in whole cell extracts

Cleared whole cell extracts (3.2.21) of cells transfected with the ERE reporter were transferred to a black 96-well plate (10 μ L lysate per well in duplicates). Plate was placed into a micro-plate reader (Wallac 1420, Perkin Elmer) which performed the assay automatically by pumping 100 μ L luciferase substrate into each well, brief mixing, 5 seconds incubation and measuring light emission for 10 seconds.

3.2.24 Determination of protein concentrations

Protein concentrations were determined by BCA assay (Thermo Fisher) according to manufacturer's instructions. Each sample was prepared in duplicates by mixing 5 μ L protein solution with 100 μ L working reagent. Absorbance was measured at 560 nm in a micro-plate reader (Wallac 1420, Perkin Elmer).

3.2.25 Sample preparation for phosphorylation-specific antibodies

Under normal culture conditions, mammalian cells were treated with 10 μ g/mL insulin (Sigma Aldrich) or 100 ng/mL Epidermal Growth Factor (EGF) (Sigma Aldrich) for 10 minutes. Cells were harvested on ice and either directly used for analysis or stored at -80°C after shock-freezing in liquid nitrogen. Pellets were lysed with TNN containing phosphatase inhibitors (5 mM NaF, 5 mM EDTA, 5 mM β -glycerophosphat, 2 mM NaVO₃) as described (3.2.21). Protein concentration was determined (3.2.24). Samples were boiled in Laemmli loading buffer and directly subjected to SDS-PAGE (3.2.13) and Western blot analysis (3.2.18) using phospho-specific antibodies.

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3.2.26 Identification of ubiquitination sites by mass spectrometry

For identification of the ubiquitination sites on baculo E6AP, an *in vitro* autoubiquitination assay was performed, as described (3.2.14), with UbcH5b as E2 enzyme. Samples were subjected to SDS-PAGE (3.2.13) and gels were stained with colloidal Coomassie (3.2.17). Slices used for analysis were excised with a scalpel and digested with Trypsin (Promega) according to the in-gel tryptic digestion protocol of the manufacturer. Peptides were recovered by ZipTip (Millipore) and eluted in 0.1% formic acid. Samples were analyzed by reversed phase liquid chromatography nanospray tandem mass spectrometry (LC-MS/MS) using an LTQ-Orbitrap mass spectrometer (Thermo Fisher) equipped with an Eksigent nano-HPLC. The dimensions of the reversed-phase LC column were 5 μm , 100 Å pore size C18 resin in a 75 μm 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 250 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 were searched against an appropriate protein database using Mascot (Matrix Science) with “Trypsin” enzyme cleavage, static cysteine alkylation by iodoacetamide, and methionine oxidation and di-glycine motifs at lysine residues as variable modifications. Data was analyzed by Proteome Discoverer (Thermo Fisher) and visualized by Xcalibur (Thermo Fisher).

4. Results

4.1 Ubiquitin's contribution to E6AP-mediated catalysis

It is known for almost 20 years that E6AP's level and activity have to be tightly controlled since its deregulation is associated with severe human diseases. Yet, there is still a lack of molecular explanations for the observed phenotypes in AS and ASD. Therefore, it is of the utmost interest to gain more insights into the mechanisms of E6AP-catalyzed ubiquitination and to identify factors that contribute to and/or control E6AP activity. Recently, an important modulator of E6AP's E3 ligase activity was identified which is, strikingly, ubiquitin itself (Kuhnle et al. 2011).

In the center of the current study were two hydrophobic surface patches of ubiquitin (Ub): the so-called "canonical" patch (leucine 8, isoleucine 44 and valine 70), which was shown previously to be important for E6AP-catalyzed ubiquitination, and the "non-canonical" patch (isoleucine 36, leucine 71 and 73). The respective Ub mutants Ub LIA (Ub L8A, I44A) and Ub ILA (Ub I36A, L71A, L73A) were bacterially expressed and analyzed in E6AP-catalyzed reactions.

4.1.1 Ubiquitin's hydrophobic patches affect E6AP-mediated substrate ubiquitination

To investigate the potential role of the hydrophobic patches of Ub, initially *in vitro* ubiquitination assays were performed using baculovirus-expressed E6AP (isoform 1) as E3 ligase, UbcH5b as E2 enzyme, and UBA1 as E1 enzyme (for reaction conditions, see 3.2.14). Experiments were conducted in the absence and presence of GST-tagged 16E6 as this is a well known interactor and modulator of E6AP (Figure 7A) (Scheffner et al. 1993, Kao et al. 2000). *In vitro* translated (ivt), radiolabeled Ring1b I53S and hHR23 served as 16E6-independent substrates of E6AP (Zaaroor-Regev et al. 2010, Kumar et al. 1999), while baculovirus-expressed p53 is exclusively a substrate of E6AP when complexed with 16E6 (Scheffner et al. 1993).

Compared to the reaction in presence of wild-type Ub (Ub wt), ubiquitination of both Ring1b I53S and hHR23 was strongly impaired when the hydrophobic patch mutants Ub LIA, Ub L71A/L73A and Ub ILA were used in the reaction. Furthermore, addition of 16E6 rescued the impairment caused by the LIA mutation and stimulated the reaction with Ub wt. Similarly, p53 was ubiquitinated in presence of 16E6 and Ub wt as well as Ub LIA. The same result was obtained in presence of a different E2 enzyme (UbcH7, Figure 7B). In contrast, the ubiquitination reaction in presence of Ub ILA was almost completely abolished under all conditions tested.

RESULTS

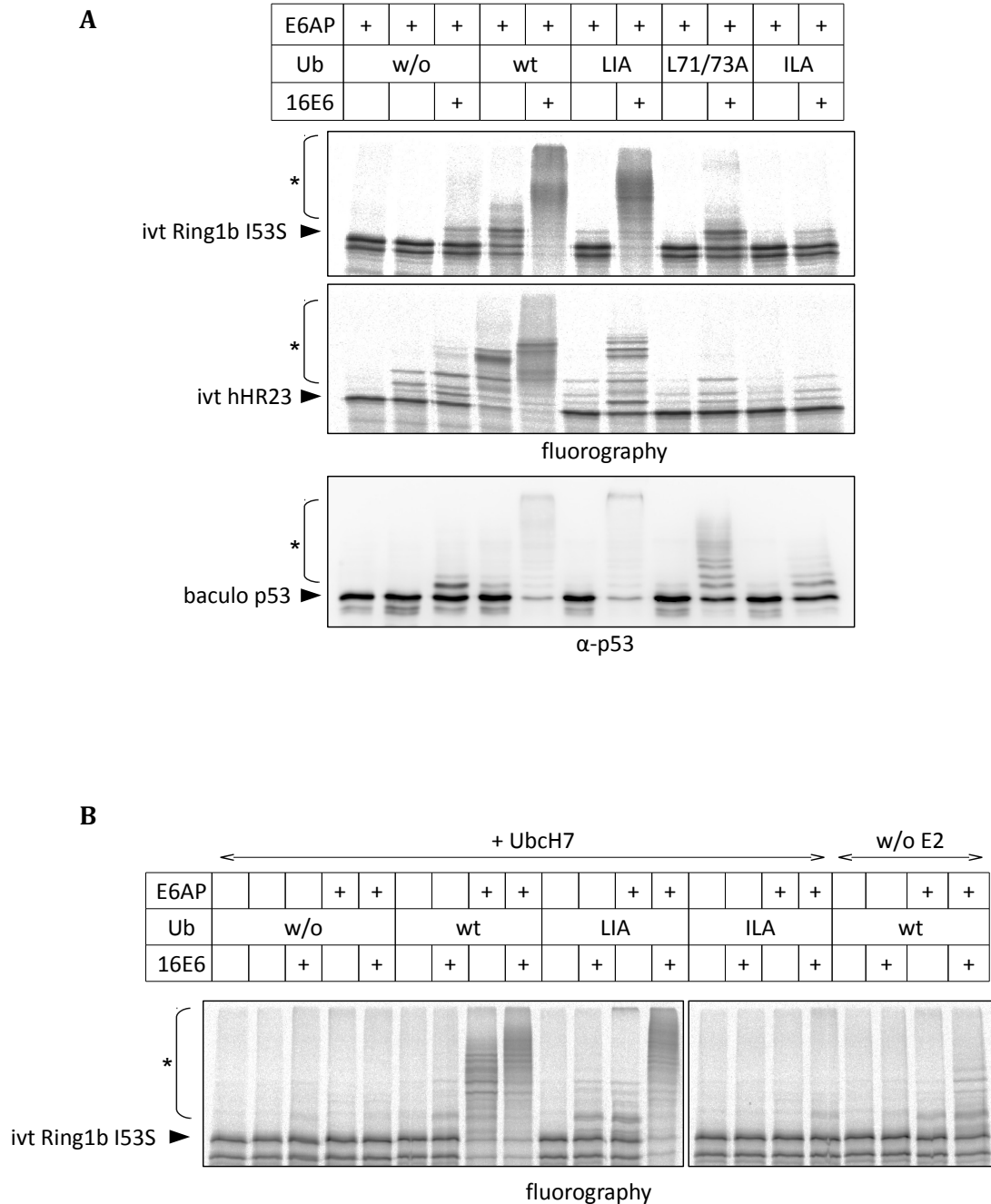


Figure 7: Intact hydrophobic patches of ubiquitin are indispensable for E6AP-mediated substrate ubiquitination. (A) *In vitro* ubiquitination assays with ivt Ring1b I53S, ivt hHR23 and baculo p53 were performed in presence of UBA1, UbcH5b, baculo E6AP and recombinant Ub versions as indicated (for reaction conditions see 3.2.14). Ubiquitination was almost abolished for Ub LIA and Ub ILA, but the defect with Ub LIA was rescued by addition of GST-16E6. - Ulf Gündisch, under supervision. (B) *In vitro* ubiquitination of ivt Ring1b I53S was performed in presence of UbcH7 as E2 enzyme. Additional controls indicate that the observed ubiquitination of Ring1b I53S with Ub wt and Ub LIA was dependent on the presence of baculo E6AP. - Nicole Richter-Müller, under supervision. The asterisks indicate ubiquitinated species.

RESULTS

4.1.2 Ubiquitin's hydrophobic patches influence E6AP auto-ubiquitination

Besides ubiquitination of other proteins, E6AP can serve as its own substrate. This process, termed auto-ubiquitination, occurs predominantly in the absence of other substrates (Nuber et al. 1998). Thus, to analyze the effect of the Ub patch mutants on auto-ubiquitination, baculovirus-expressed E6AP was incubated with UBA1, recombinant E2 enzymes, and different Ub versions as indicated. Reactions were also performed in the absence and presence of GST-tagged 16E6 (Figure 8).

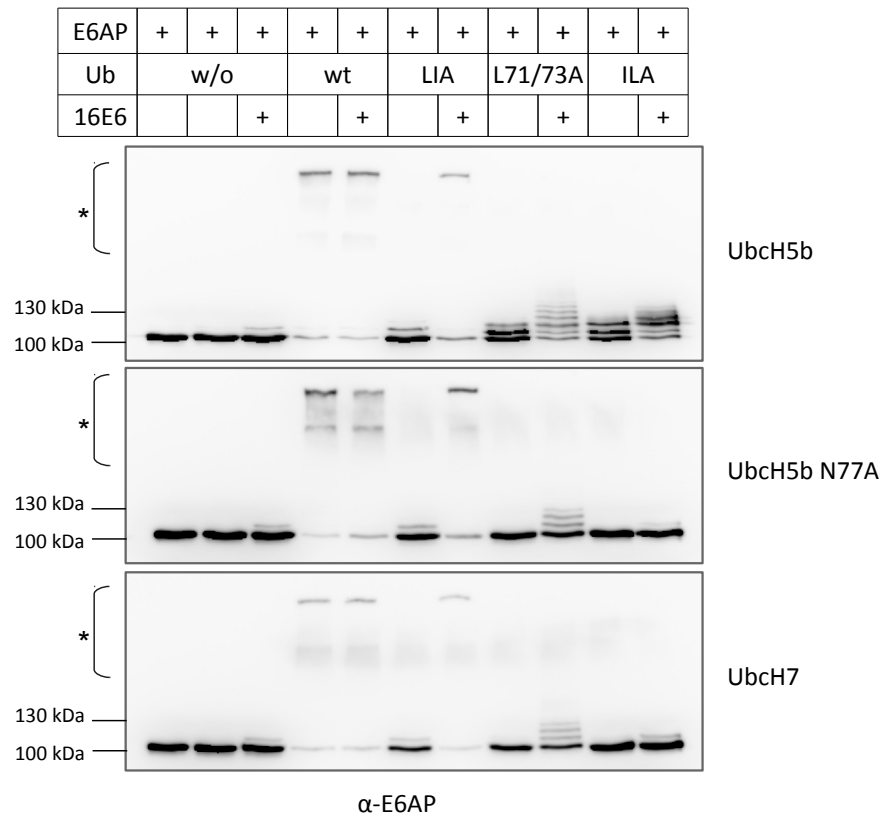


Figure 8: Mutation of ubiquitin's hydrophobic patches interferes with E6AP auto-ubiquitination. *In vitro* auto-ubiquitination assays with baculovirus-expressed E6AP were performed with different E2 enzymes (Ubch5b wt and N77A, Ubch7) and Ub versions under standard conditions (3.2.14). Samples were subjected to SDS-PAGE followed by Western blot analysis with an anti-E6AP antibody. Auto-ubiquitination was strongly impaired with Ub LIA and Ub ILA, yet addition of 16E6 efficiently rescued the deficit of Ub LIA. Remarkably, ubiquitination of E6AP with Ub ILA only took place in presence of Ubch5b, but not with its N77A mutant or Ubch7. The asterisks indicate ubiquitinated species.

RESULTS

As in the case of in E6AP-mediated substrate ubiquitination, the auto-ubiquitination reactions in presence of Ub LIA and Ub ILA were strongly impaired compared to the Ub wt control. Once again, the presence of 16E6 rescued the impairment of Ub LIA but not of Ub ILA. The different E2 enzymes used all led to the same result with one exception: strikingly, ubiquitination of E6AP with Ub ILA occurred in presence of UbcH5b, but was abolished if the UbcH5b mutant N77A (which is incapable to catalyze isopeptide bond formation, Wu et al. 2003) or UbcH7 were used. For further details on this unexpected finding, see 4.1.5.

4.1.3 Minor effects of ubiquitin's patch mutants on E1 and E2 enzymes

The above results demonstrated that Ub's hydrophobic patches play a fundamental role in E6AP-mediated ubiquitination. However, from these experiments it was not possible to specify the exact step of the reaction which is affected by these patches. One possibility would be that the respective Ub mutants are not efficiently transferred from the E1 enzyme to the E2 enzymes or that they cannot be released from the E2 enzymes. To analyze if and to what extent the patches affect loading and release from the E2 enzymes, thioester (3.2.15) and discharge assays (3.2.16) were performed. The formation of the Ub thioester conjugate should result in a mass shift of the E2 by 8,5 kDa compared to the uncharged E2 and the conjugate should be sensitive to treatment with DTT. Thioester formation of all tested E2s with Ub wt, Ub LIA and Ub ILA could be readily detected (Figure 9). Accordingly, activation of the Ub mutants by the E1 enzyme must also have occurred.

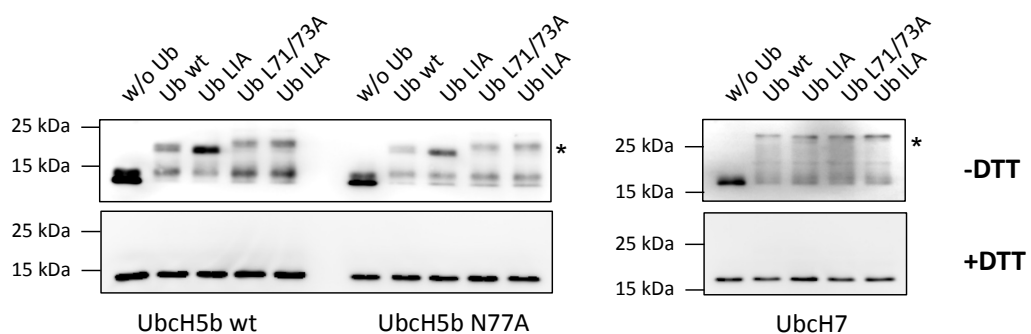


Figure 9: Ubiquitin's hydrophobic patches are not significantly affecting E1 and E2 enzyme activities. Thioester assays (3.2.15) proved the rapid thioester formation of UbcH5b wt, its N77A mutant and UbcH7 with the different Ub versions after incubation with UBA1 for 1 minute at 30°C. Samples were subjected to SDS-PAGE and Western blot analysis. Data was visualized by anti-His Western blot as the E2 enzymes harbor a C-terminal His-tag. The thioester-bonded Ub caused a mass shift of the E2 enzymes of approximately 8-9 kDa (upper panel) and was released upon addition of DTT (lower panel). The asterisks indicate the running positions of the Ub-loaded E2 enzymes.

RESULTS

In addition, release of the loaded Ub from the E2 enzymes should take place in presence of free cysteine and, in case of Ubch5b, free lysine. These so-called discharge assays reflect the ability of the respective E2 enzymes to transfer Ub to the active site cysteine of a HECT ligase under thioester formation or, in case of Ubch5b, the catalysis of isopeptide bond formation as it is performed together with RING ligases (Figure 10) (Pickart and Rose 1985, Wenzel et al. 2011). Prior to the incubation of the charged E2s with respective amino acids, samples were mixed with EDTA to prevent further activation of Ub by the E1 enzyme.

In case of Ubch5b, Ub wt was discharged in presence of cysteine and lysine. The ability to catalyze isopeptide bond formation is abolished in the Ubch5b N77A mutant, which therefore was solely able to transfer Ub to free cysteine (Wu et al. 2003). Ubch7 behaved, as already shown by Wenzel et al. (2011), like the Ubch5b N77A mutant. The results obtained with the hydrophobic patch mutants of Ub did not differ from those obtained with Ub wt.

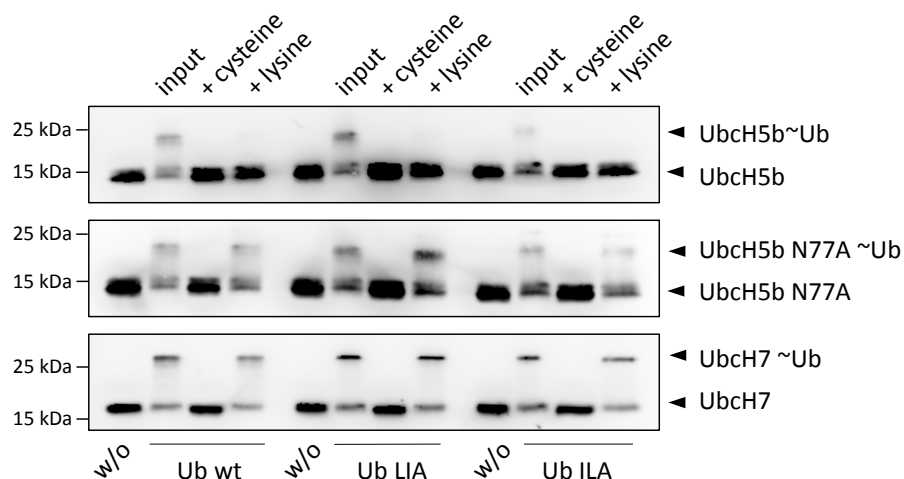


Figure 10: Discharge of hydrophobic patch Ub mutants from the E2 enzymes is not impaired. The different E2 enzymes were charged with the indicated Ub versions under thioester conditions. Subsequently, the charged E2s were incubated in presence of EDTA (50 mM) and cysteine or lysine (50 mM) for additional 20 minutes at 30°C. Samples were subjected to SDS-PAGE and Western blot analysis using an anti-His antibody (as all E2s harbor a C-terminal His-tag).

The reactivity of Ubch5b towards cysteine and lysine was analyzed in more detail. To do so, the discharge assay was repeated in presence of lower concentrations of the free amino acids (Figure 11). Discharge of Ub wt, Ub LIA and Ub ILA was readily achieved in presence of 0.5 mM cysteine, although the reaction was slightly more efficient with Ub ILA. In case of lysine, the

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discharge of Ub wt and Ub LIA required 50 mM free amino acid while discharge of Ub ILA took place already in presence of 5 mM lysine.

It was concluded that overall the Ub mutations did not lead to a significant impairment of catalysis on E1 and E2 enzyme level. These results highlighted that the observed loss of Ub ligation activity with Ub LIA and Ub ILA must be due to an effect on E6AP.

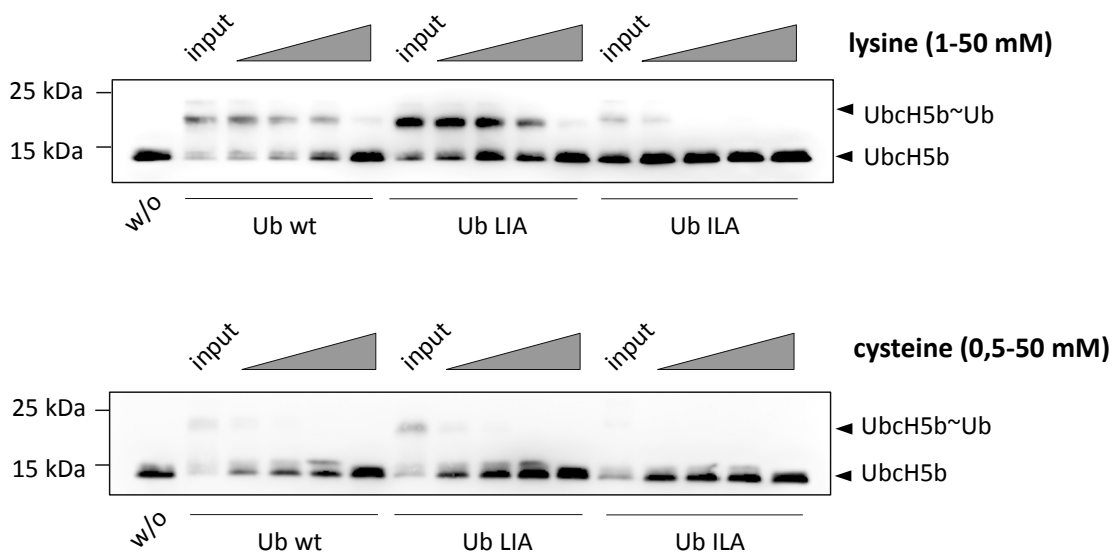


Figure 11: Discharge of Ub ILA from UbcH5b to lysine may be more efficient compared to Ub wt and Ub LIA. Incubation of ubiquitin-loaded UbcH5b in presence of increasing lysine concentrations (upper panel) showed a rapid release of Ub ILA already at a concentration of 5 mM, whereas the discharge of Ub wt and Ub LIA required 50 mM lysine. In contrast, incubation of the loaded E2 with different cysteine concentrations caused a rapid release of all Ub versions already at rather low concentrations (0.5 mM). Samples were subjected to SDS-PAGE and Western blot analysis using an anti-His antibody (as UbcH5b harbors a C-terminal His-tag).

4.1.4 Effect of ubiquitin's hydrophobic patches on HECT^{E6AP}- catalyzed reactions

In a simplistic view, E6AP can be divided into an N-terminal region that for example, provides binding sites for 16E6 (Huibregtse et al. 1993) and the Rcc1b domain of HERC2 (Kuhnle et al. 2011) and the C-terminal catalytic domain, termed HECT domain (HECT^{E6AP}) (Huibregtse et al. 1995). The HECT domain facilitates binding to the E2 enzyme within its N lobe and subsequent thioester formation with the C terminus of Ub at the active site cysteine of E6AP located in the C lobe (Figure 3) (Huang et al. 1999).

RESULTS

A possible contribution of ubiquitin's hydrophobic patches to thioester formation on HECT^{E6AP} was analyzed by incubating UBA1, UbcH7 and HECT^{E6AP} in presence of the different Ub versions under thioester conditions (for experimental conditions, see 3.2.15) (Figure 12). A mass shift of HECT^{E6AP} of approximately 9 kDa was detected for Ub wt, Ub LIA and Ub ILA. This shift was largely abolished in the presence of DTT, which proved that the respective band represents the HECT~Ub thioester conjugate. However, in case of Ub wt and Ub LIA small portions of the HECT~Ub conjugates remained stable in presence of DTT, which indicates that isopeptide bond formation must have occurred.

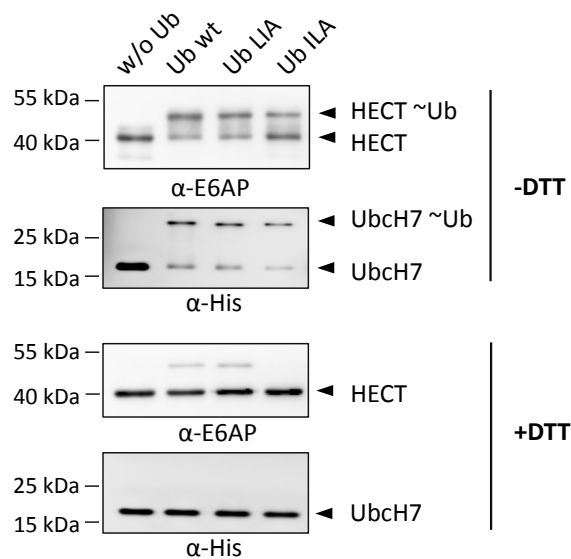


Figure 12: Ubiquitin's hydrophobic patches are not important for thioester formation with the catalytic HECT domain of E6AP. Under thioester conditions (3.2.15), UBA1, UbcH7 and HECT^{E6AP} were incubated with indicated Ub versions for 1 minute at 30°C. Reactions were stopped in the absence or presence of DTT and subjected to SDS-PAGE. The HECT domain and UbcH7 were visualized using antibodies against E6AP and His-tag (UbcH7-His), respectively. Thioester complex formation was readily detectable on UbcH7 and HECT^{E6AP}. Small portions of the HECT~Ub wt and HECT~Ub LIA conjugates were insensitive to treatment with DTT, which indicates the presence of an isopeptide bond between HECT^{E6AP} and Ub wt/LIA.

To demonstrate that the HECT domain of E6AP was indeed subjected to ubiquitination by the different Ub versions, the experiment was repeated under ubiquitination conditions (3.2.14) (Figure 13A). It turned out that HECT^{E6AP} auto-ubiquitination occurred in presence of Ub wt and Ub LIA. This auto-ubiquitination required the catalytic activity of HECT^{E6AP} as mutation of the catalytic cysteine of E6AP to an alanine (C820A) abolished it. HECT^{E6AP} auto-ubiquitination with Ub ILA was again very inefficient compared to Ub wt except when UbcH5b was used as E2 enzyme. However, in this case the ligase activity of HECT^{E6AP} was dispensable whereas the ability

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of UbcH5b to form isopeptide bonds between Ub and a target lysine residue seemed to be required. This is evident as the UbcH5b N77A mutant, which cannot catalyze isopeptide bond formation (Wu et al. 2003), did not lead to ubiquitination of HECT^{E6AP} with Ub ILA. Interestingly, recombinant ligase-active HECT^{E6AP} did not ubiquitinate *invitro* catalytically inactive HECT C820A, which indicates that this auto-ubiquitination occurs exclusively intramolecular (Figure 13B). This is of note as auto-ubiquitination of the full-length E6AP occurs mainly intermolecular (Nuber et al. 1998).

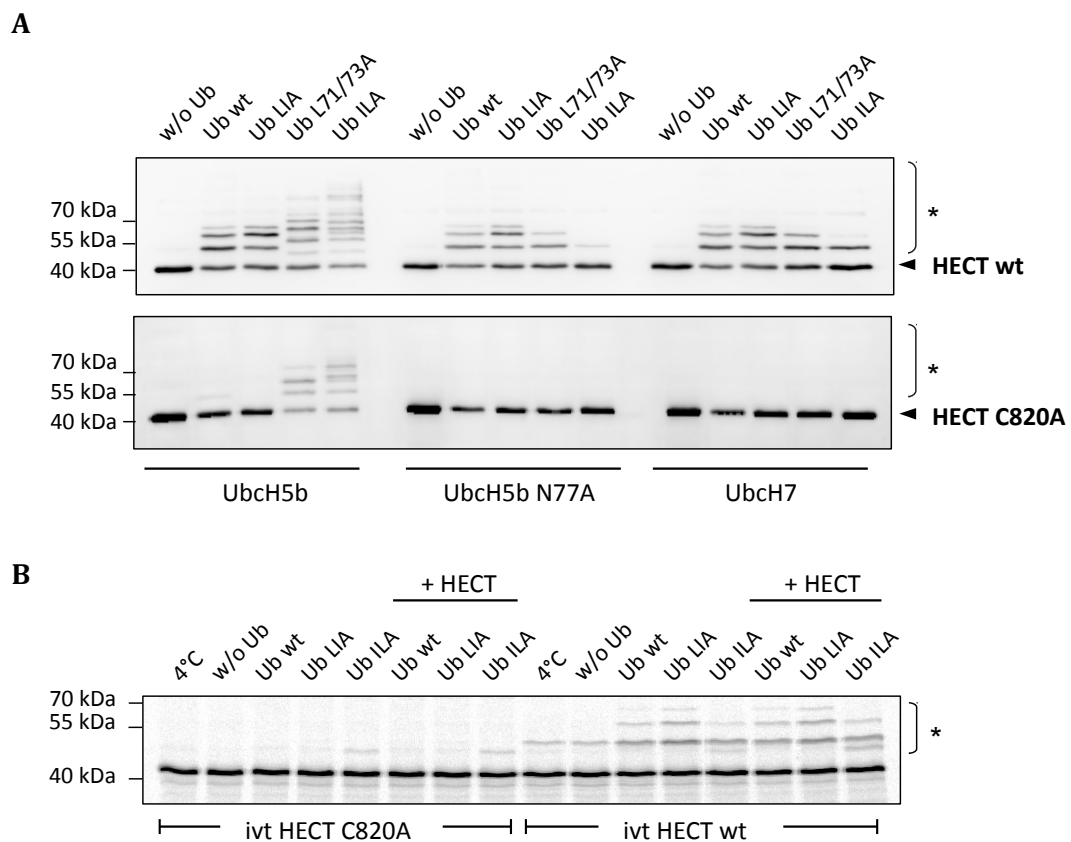


Figure 13: Ubiquitin's hydrophobic patches differentially affect HECT^{E6AP} auto-ubiquitination. A) Recombinant UBA1 and HECT^{E6AP} were incubated under ubiquitination conditions with different E2 enzymes and Ub versions as indicated. Samples were analyzed by SDS-PAGE and Western blot analysis with an anti-E6AP antibody. HECT^{E6AP} auto-ubiquitination with Ub wt and Ub LIA was catalyzed by the HECT domain as mutation of the catalytic cysteine to alanine (C820A) abolished the reaction. Auto-ubiquitination was rather inefficient with Ub ILA, except when UbcH5b served as E2 enzyme. However, in this case the reaction did not require the catalytic activity of the HECT domain. B) *Invitro* HECT or *invitro* catalytically inactive HECT C820A were incubated in presence of UBA1, UbcH7, recombinant HECT domain and Ub versions as indicated. Samples were analyzed by SDS-PAGE and fluorography. Addition of recombinant HECT domain did not lead to ubiquitination of *invitro* HECT domains. Thus, auto-ubiquitination takes place in an intramolecular fashion. The asterisks indicate ubiquitinated species.

RESULTS

4.1.5 Ubch5b attaches Ub ILA to a distinct lysine residue in E6AP's C lobe

As shown above, auto-ubiquitination of the isolated HECT domain of E6AP occurred with all tested Ub versions, although the reaction was strongly impaired with Ub ILA. However, when Ubch5b was the provided E2 enzyme, ubiquitination with Ub ILA was much more efficient. This reaction was not dependent on the presence of E6AP's catalytic cysteine residue (C820), but on the ability of Ubch5b to catalyze isopeptide bond formation. A similar result was obtained when *invit* full-length E6AP C820A was used as substrate (Figure 14). While in case of Ub wt and Ub LIA ubiquitination of E6AP C820A was largely dependent on the presence of ligase-active baculo E6AP, ubiquitination with Ub ILA was not.

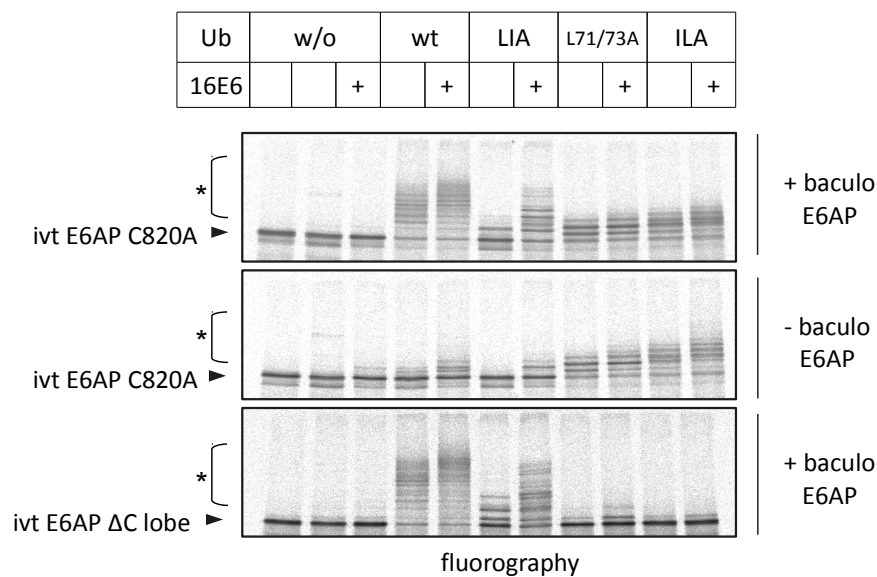


Figure 14: Ubiquitination of E6AP with Ub ILA does not require E6AP's ligase activity. *In vitro* ubiquitination of *invit* catalytically inactive E6AP versions (C820A and Δ C lobe) was performed with Ubch5b as E2 enzyme and Ub versions as indicated. Auto-ubiquitination with Ub wt and Ub LIA, but not Ub ILA, was largely dependent on the presence of ligase-active baculo E6AP. Interestingly, ubiquitination with Ub ILA was abrogated when the C lobe of E6AP was deleted.

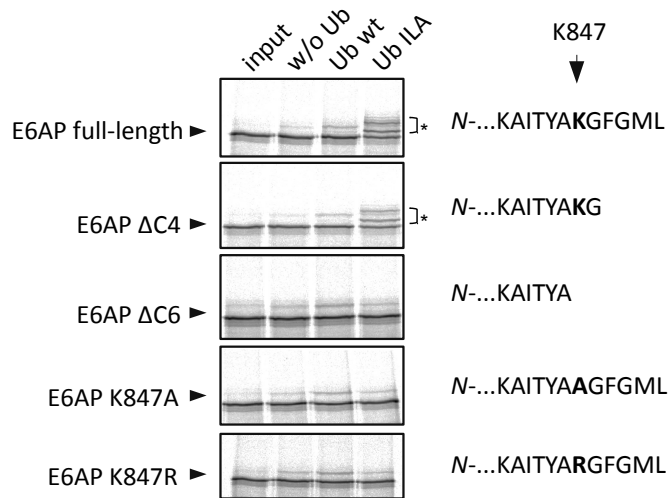
RESULTS

Furthermore, the stimulating effect of 16E6 was only detectable for those reactions that were catalyzed by baculovirus-expressed E6AP. However, the mild effects of 16E6 in the absence of baculovirus-expressed E6AP may be derived from the E6AP present in the reticulocyte lysate which was used to prepare the ivt proteins (Huibregtse et al. 1991). The lack of any effect of 16E6 in the presence of Ub ILA supports the notion that these ubiquitination reactions were not catalyzed by E6AP but by UbcH5b. Interestingly, ubiquitination with Ub ILA was abolished when the catalytically inactive E6AP Δ C lobe mutant was used as ivt substrate. This mutant is still able to bind the E2 enzyme, but lacks the C lobe which harbors the catalytic cysteine residue.

Based on the above findings, it was hypothesized that UbcH5b catalyzed the attachment of Ub ILA to a lysine residue within E6AP's C lobe. Indeed, mutational analysis revealed that E6AP's very C-terminal lysine K847 was targeted by UbcH5b with Ub ILA, but not with Ub wt (Figure 15A). This result was also confirmed by mass spectrometric analysis of the reaction products obtained by incubating baculovirus-expressed E6AP with UbcH5b and Ub ILA. The tryptic peptide corresponding to E6AP's C terminus harbored a di-glycine motif at K847 that originated from the attached Ub ILA (Figure 15B).

RESULTS

A



B

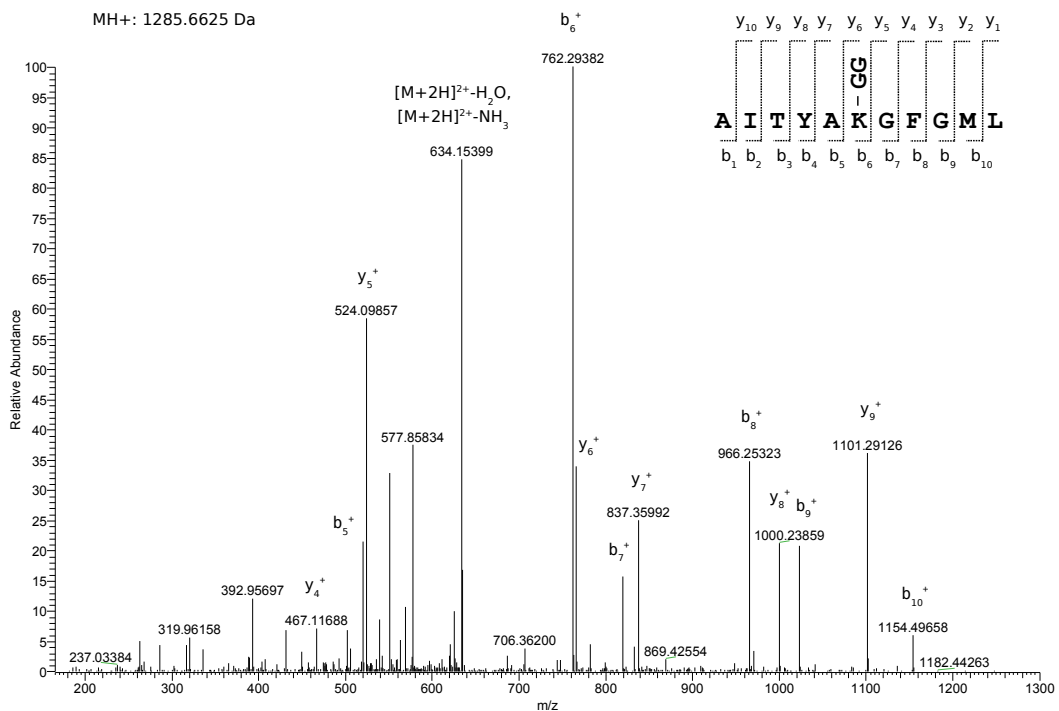


Figure 15: The UbcH5b catalyzed ubiquitination of E6AP with Ub ILA is mapped to lysine 847. A) The *in vitro* ubiquitination assay with indicated *in vitro* E6AP versions and Ub variants was performed in presence of UbcH5b. All E6AP mutants lacked catalytic activity (C820A) so that the observed covalent attachment of Ub ILA is assigned to the activity of the E2 enzyme. The corresponding C-terminal amino acid sequences of the E6AP mutants are shown with lysine 847 highlighted in bold. The asterisks indicate ubiquitinated species. Loss of lysine 847 abolished the attachment of Ub ILA to E6AP. B) Mass spectrometric analysis of recombinant E6AP incubated with UbcH5b and Ub ILA confirmed that E6AP's K847 is targeted for ubiquitination because a tryptic peptide harboring a GG-tag at K847 of E6AP was identified.

RESULTS

4.1.6 Dissecting the role of ubiquitin's patches on full-length E6AP

The data obtained so far suggested that the patch mutants Ub LIA and Ub ILA were not impaired in forming thioester complexes with the isolated HECT domain of E6AP. In order to prove that this holds true also for the full-length protein, thioester assays were performed with baculovirus-expressed E6AP (Figure 16). As E6AP is a comparably large protein (~ 100 kDa), the mass shift caused by charging of E6AP with Ub is rather small (~ 8.5 kDa). Therefore, the Ub versions used in these assays harbored an N-terminal His-tag which was used for detection by Western blot analysis. First, it was proven that the tag did not alter the results obtained with the HECT domain (data not shown). Subsequently, the His-tagged Ub versions were incubated with UBA1 and UbcH7 in the absence and presence of E6AP.

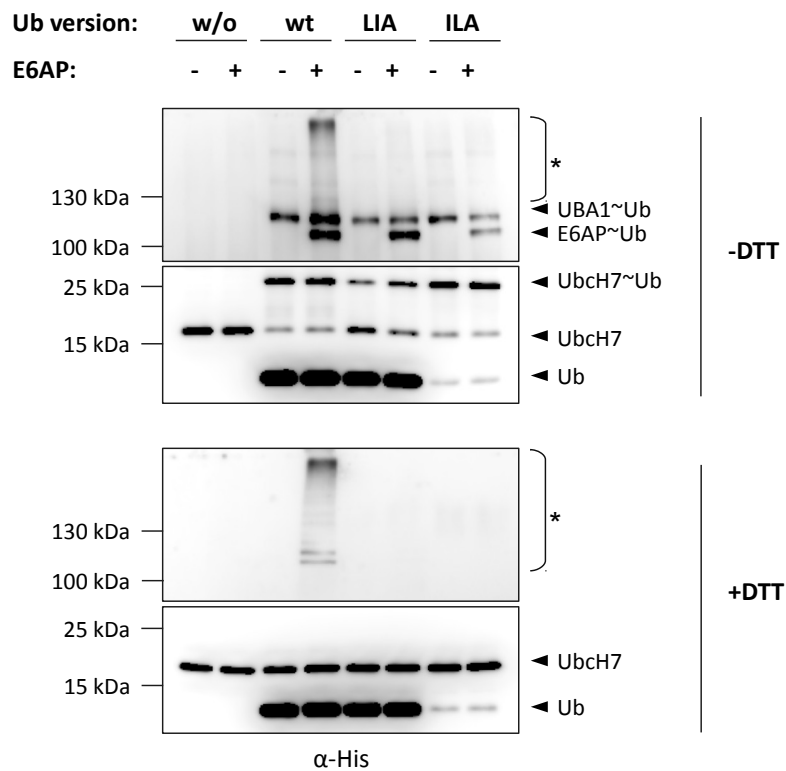


Figure 16: Efficient thioester formation of full-length E6AP with Ub wt, Ub LIA and Ub ILA. The thioester assay was performed with His-tagged Ub versions to allow the detection of the E6AP~Ub thioester conjugate via an anti-His antibody. Incubation of UBA1, UbcH7 and E6AP, where indicated, caused a mass shift of Ub to molecular masses corresponding to the UBA1~Ub and E6AP~Ub conjugates. Addition of DTT disrupted the thioester but, in case of Ub wt and in presence of E6AP, a high molecular mass smear remained stable. This smear represents polyubiquitinated forms of E6AP (indicated by the asterisks). The low levels of detectable unconjugated His-Ub ILA are a result of defective transfer to the Western blot membrane.

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A mass shift of each Ub version to molecular masses corresponding to the UBA1~Ub and E6AP~Ub thioester intermediate could be readily observed. Thus, thioester formation of the full-length E6AP with Ub wt, Ub LIA and Ub ILA was as efficient as with the isolated HECT domain. In case of Ub wt and in presence of E6AP a high molecular weight smear remained stable in presence of DTT. This corresponds to Ub chains which were formed by E6AP on itself.

4.1.7 Role of E6AP's N terminus in catalysis

The previous data indicate that the two examined hydrophobic patches of Ub do not significantly contribute to the thioester formation on E1, E2 and E6AP but rather play a role in the formation of isopeptide bonds catalyzed by E6AP. Additionally, there were different effects of the patch mutants observed on full-length E6AP and the isolated HECT domain. While ubiquitination with Ub ILA was impaired with HECT^{E6AP} as well as full-length E6AP, Ub LIA functioned very well in HECT^{E6AP} auto-ubiquitination but not with the full-length protein. Therefore, the role of ubiquitin's "canonical" patch, which is mutated in Ub LIA, must be somehow related to the N terminus of E6AP.

Hence, an important aspect in gaining information about ubiquitin's contribution to E6AP-catalyzed ubiquitination was to analyze the contribution of E6AP's N terminus to the reaction. For this purpose, *in vitro* auto-ubiquitination experiments were performed in parallel with full-length E6AP and HECT^{E6AP} together with His-tagged Ub versions. Western blot analyses of the reaction products were performed using anti-His and anti-E6AP antibodies (Figure 17). The most important difference between HECT^{E6AP} and full-length E6AP was that the ability to form long Ub chains was restricted to the full-length protein. Hence, a putative defect of Ub LIA in chain elongation could have been hypothesized. However, it was noticed that also in case of Ub LIA long chains appeared after incubation with full-length E6AP, though to a small extent. Thus, it was concluded that Ub LIA did not just cause a drastic failure in E6AP-mediated Ub chain formation, but rather affected isopeptide bond formation in general.

RESULTS

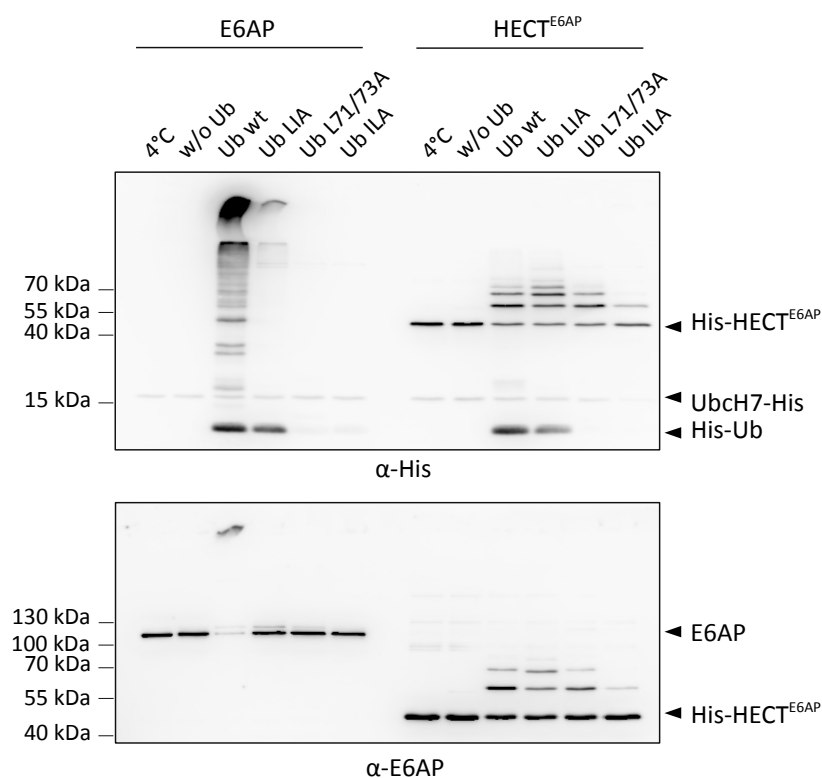


Figure 17: Comparison of auto-ubiquitination reactions catalyzed by full-length E6AP and HECT^{E6AP}. Recombinant E6AP or HECT^{E6AP} were incubated with UBA1, UbcH7 and indicated His-tagged Ub versions. Samples were analyzed by SDS-PAGE and anti-His (upper panel) or anti-E6AP (lower panel) Western blot analysis. Incubation of full-length E6AP with Ub wt resulted in a complete loss of unmodified E6AP and the formation of high-molecular mass Ub chains. The reactions of full-length E6AP with Ub LIA or Ub ILA were strongly impaired. In contrast, HECT^{E6AP} did not catalyze long Ub chains, but the auto-ubiquitination reaction with Ub LIA was as efficient as with Ub wt.

4.1.8 HPV16 E6 stimulates E6AP-catalyzed isopeptide bond formation

As the defect of Ub LIA was restricted to the full-length E6AP-catalyzed isopeptide bond formation, it was assumed that the effect of 16E6 on E6AP arose by stimulation of isopeptide bond formation with substrate proteins and not by stimulation of Ub thioester formation of E6AP. In order to prove this, baculovirus-expressed E6AP was charged with His-Ub LIA under thioester conditions and subsequently incubated with or without GST-16E6 (Figure 18). Western blot analysis with an anti-His antibody enabled the detection of the UBA1~Ub LIA and E6AP~Ub LIA conjugates as shown before. To distinguish between thioester and isopeptide bonds, samples were treated with or without DTT prior to SDS-PAGE analysis.

RESULTS

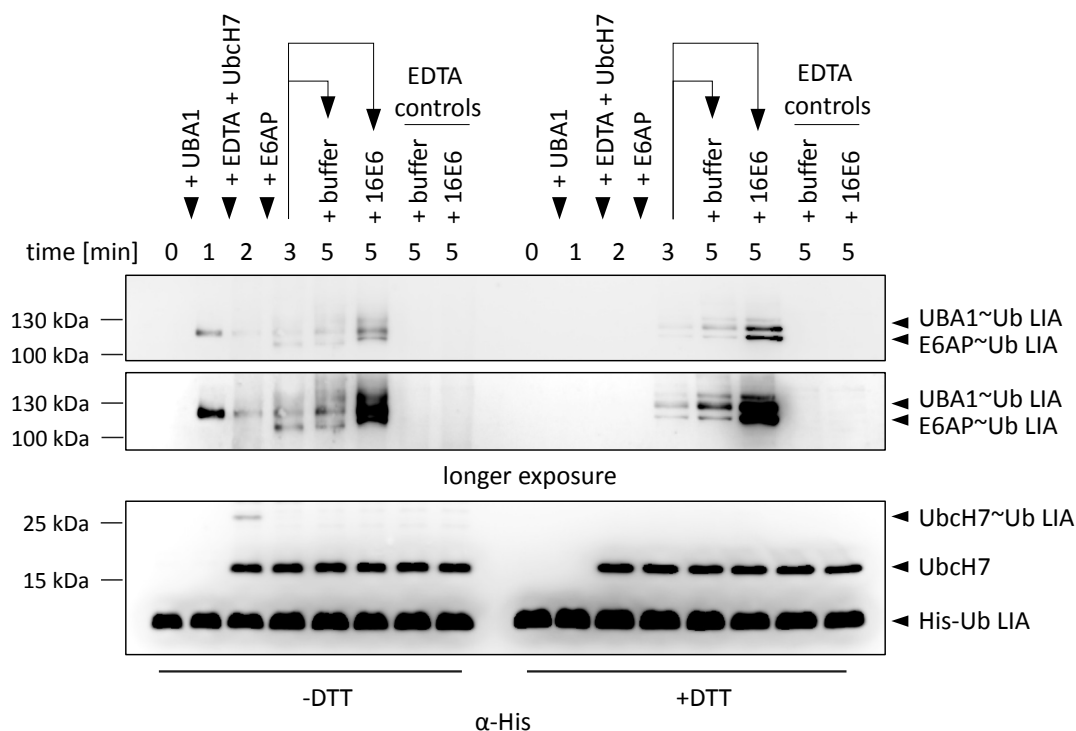


Figure 18: The oncoprotein 16E6 stimulates E6AP-mediated isopeptide bond formation. To distinguish the effect of 16E6 on the thioester and the isopeptide bond formation ability of E6AP, E6AP was first charged with His-tagged Ub LIA and then further incubated in absence and presence of 16E6. To do so, UBA1 was charged with Ub LIA under thioester conditions. To avoid further activation of Ub LIA, 50 mM EDTA was added. Additional controls, where EDTA was supplied before charging of UBA1 occurred, show that the amount of EDTA completely blocked any reaction. Subsequently, Ub LIA was released from UBA1 to UbCh7. Afterwards, E6AP was added to the reaction and incubated for further two minutes. Thioester conjugated UbCh7 was not present anymore and thus any further reaction depended on the E6AP~Ub LIA thioester. Further incubation was performed in absence and presence of 16E6. Samples were subjected to SDS-PAGE in the presence or absence of a reducing agent (DTT). Ub LIA was visualized by anti-His Western blot. Incubation of the E6AP~Ub LIA thioester complex with 16E6 increased the presence of the DTT-insensitive E6AP-Ub LIA complex. Thus, 16E6 appears to support E6AP-mediated isopeptide bond formation.

For Ub charging of E6AP, first UBA1 was incubated with Ub LIA. Afterwards, the addition of EDTA prevented further activation of Ub LIA so that the E1 was entirely discharged by addition of UbCh7. Incubation of the loaded E2 enzyme with E6AP resulted in complete loss of the UbCh7~Ub LIA conjugate and the E6AP~Ub LIA thioester complex appeared. This was now the starting point for further incubation with or without 16E6. In absence of 16E6, only a small portion of the E6AP~Ub LIA conjugate remained stable after treatment with DTT. This corresponded to the weak ability of E6AP to perform auto-ubiquitination with Ub LIA. However, addition of 16E6 drastically increased the DTT-insensitive amount of E6AP-Ub LIA conjugate. As the presence of EDTA in the reaction hindered further activation of Ub LIA, the E6AP~Ub LIA

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thioester acted as the sole source of conjugatable Ub LIA. Therefore, any effect of 16E6 on thioester formation of E6AP with Ub LIA was excluded. Consequently, 16E6 appears to stimulate the E6AP-catalyzed transfer of Ub LIA from the active site cysteine of E6AP to a lysine residue within E6AP.

4.1.9 Low-risk HPV E6 proteins also affect E6AP's E3 ligase activity

Besides the high-risk HPV16 E6 protein, E6 proteins from low-risk HPVs (e.g. HPV11 E6) also interact with E6AP's N terminus (Brimer et al. 2006, Kuballa et al. 2007). In order to investigate whether the effect on E6AP-mediated catalysis is restricted to high-risk E6 proteins, *in vitro* ubiquitination experiments were executed in presence of low-risk E6 versions. In addition to 16E6 and 11E6, the chimeric versions 16C11 (N terminus of 16E6, C terminus of 11E6) and 11C16 were used (Figure 19).

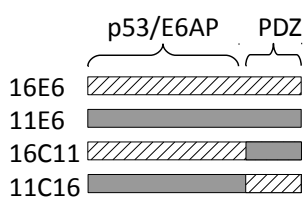


Figure 19: Schematic illustration of chimeric E6 proteins used in this study. High-risk HPV16 E6 harbors a C-terminal PDZ-binding motif that is not required for the interaction with p53 and E6AP but for interaction with PDZ domain-containing proteins such as Dlg. Low-risk HPV11 E6 binds E6AP but not p53 or PDZ domain-containing proteins. The chimeric 16C11 protein lost its ability to bind Dlg, while 11C16 is enabled to bind Dlg via the PDZ-binding motif of 16E6 (Brimer et al. 2006, Kuballa et al. 2007).

All E6 proteins tested caused the formation of a high molecular mass smear in the presence of E6AP and Ub LIA (Figure 20A). Switching the C-terminal parts of the E6 proteins did not appear to affect the results, which indicates that the C-terminal PDZ-binding motif of 16E6 (Lee et al. 1997, Kiyono et al. 1997) does not play a role in modulating E6AP activity. However, while 16E6 and 16C11 caused the loss of non-modified E6AP, for low-risk 11E6 and 11C16 a decrease of non-modified E6AP could not be observed (Figure 20B). Hence, the 11E6/E6AP complex probably catalyzed the formation of free Ub LIA chains.

RESULTS

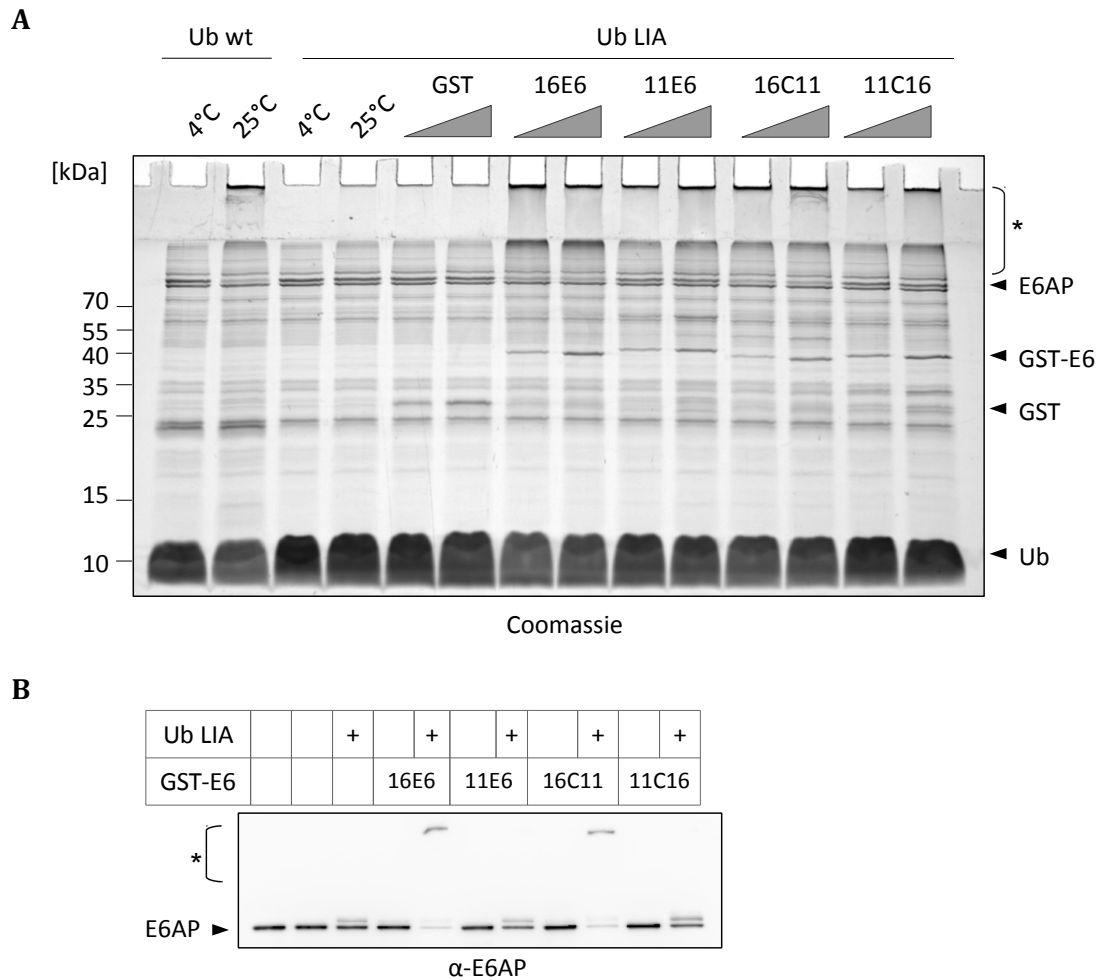


Figure 20: Interaction of E6AP with low-risk 11E6 rescues the defective usage of Ub LIA. A) The E6AP auto-ubiquitination assay was performed in the absence or presence of indicated Ub versions and E6 proteins. Samples were subjected to SDS-PAGE and visualized by Coomassie staining. Incubation of E6AP with Ub LIA did not lead to the formation of a high molecular mass smear unless the indicated GST-tagged E6 proteins were present. The C-terminal PDZ-binding motif of 16E6 was not required for this reaction since the chimeric 16C11 and 11C16 proteins behaved like the 16E6 and 11E6 proteins, respectively. B) E6AP auto-ubiquitination assay was performed with Ub LIA and E6 proteins as indicated. Samples were subjected to SDS-PAGE and Western blot analysis with an anti-E6AP antibody. In contrast to high-risk 16E6, low-risk 11E6 did not induce auto-ubiquitination of E6AP with Ub LIA. Switching the C-terminal portions of the E6 proteins did not alter the result. The asterisks indicate ubiquitinated species.

To prove that 11E6 can indeed stimulate E6AP activity, it was hypothesized that in presence of a substrate bound to the 11E6/E6AP complex, ubiquitination of this substrate would occur in the presence of Ub LIA. Indeed, ivt rDlg, which binds to the C-terminal PDZ-binding motif of 16E6 and thus also to the chimeric 11C16 protein (Brimer et al. 2006, Kuballa et al. 2007), was modified with Ub LIA by the 11C16/E6AP complex as it was also the case for the 16E6/E6AP complex (Figure 21).

RESULTS

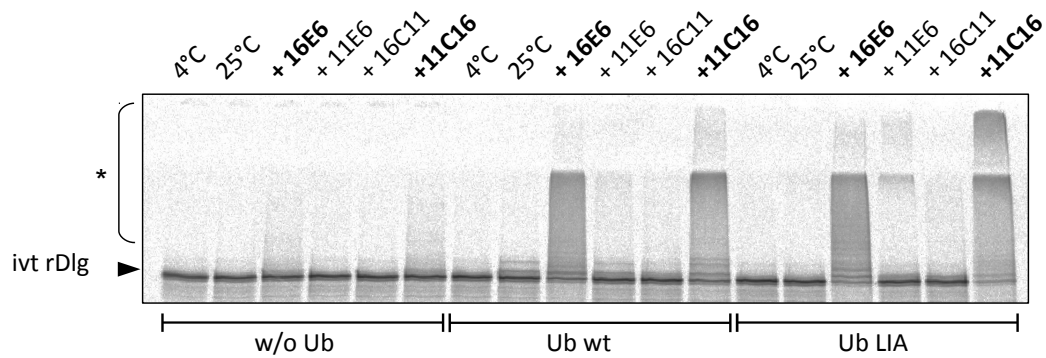


Figure 21: Ivt rDlg is a target for the 16E6/E6AP and 11C16/E6AP complexes with both, Ub wt and Ub LIA. Ivt rDlg was incubated with UBA1, UbcH7, baculo E6AP and indicated Ub versions. Samples were subjected to SDS-PAGE and visualized by fluorography. The C-terminal PDZ-binding motif of 16E6 and 11C16 mediated binding to rDlg and was necessary to induce efficient ubiquitination. Mutation of ubiquitin's "canonical" patch (Ub LIA) did not abrogate the reactions, which supports the notion that also low-risk 11E6 rescues the deficit of E6AP to function with Ub LIA. The asterisk indicates ubiquitinated species.

4.2 Effect of E6AP on cell signaling and transcription

E6AP is a member of the HECT family of ubiquitin-protein ligases and is known to synthesize K48-linked ubiquitin chains (Huibregtse et al. 1995, Kim et al. 2007). It is therefore taken for granted that it modulates protein stability by post-translational modification of substrate proteins, thereby targeting them for proteasomal degradation (Chau et al. 1989, Gregori et al. 1990, Deveraux et al. 1994). However, increasing evidence indicates that E6AP regulates protein synthesis also on the level of transcription – an effect that presumably contributes to some pathological symptoms observed in AS and ASD (reviewed in El Hokayem and Nawaz 2014). To understand the reasons that make an appropriate E6AP level so important for proper neurodevelopment, it is therefore mandatory to also investigate E6AP's function in gene expression as well.

4.2.1 E6AP modulates steroid hormone receptor (SHR) signaling

Any gene expression depends on binding of transcription factors to the promoter-enhancer region located upstream of the gene. Besides the so-called general transcription factors, which are largely indispensable for initiating transcription, there is a huge variety of transcriptional modulators which either enhance or slow down transcription (Latchman 2013). Prominent examples of transcriptional activators are steroid hormone receptors (SHRs), which, classically upon hormone binding, are capable of interacting with particular DNA sequences in the enhancer region of a gene, thereby initiating transcription (reviewed in Mangelsdorf et al. 1995, Björnström and Sjöberg 2005).

To analyze the effect of E6AP on SHR signaling, transient luciferase reporter assays were carried out in H1299 cells that either express endogenous E6AP (H1299 wt) or harbor a stable knockdown of E6AP expression (H1299 E6APi) (Kuballa et al. 2007). These reporter assays are based on the binding of a certain SHR (e.g. estrogen receptor) to its specific target sequence (e.g. ERE – estrogen response element) in the enhancer region of the reporter construct that drives transcription of a luciferase gene. Thus, the amount of expressed luciferase protein, which is determined in whole cell extracts, correlates with the activity of the transactivator (for further details on the experimental set-up see 3.2.19). Experiments were focused on the effect of E6AP on estrogen receptor alpha (ER α). As H1299 cells do not express a transcriptionally active ER α (Zhang et al. 2008), it had to be co-expressed. However, treatment with the steroid hormone

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estradiol was not necessary to induce ER α -mediated transcription because the cell culture media contains hormones (derived from the FBS) and phenolred, which acts as an ER α agonist (Berthois et al. 1986).

The RNAi-mediated loss of endogenous E6AP expression led to an increased luciferase activity which was antagonized by co-expression of ectopic HA-tagged E6AP (Figure 22). Thus, an off-target effect of the shRNA used for downregulation of expression of endogenous E6AP was unlikely. The same result was obtained in HEK293T cells (data not shown), indicating that, at least under the conditions used, E6AP acts as a repressor of the transactivation function of ER α . Interestingly, this effect did not depend on the catalytic activity of the overexpressed E6AP, because mutation of its catalytic cysteine residue at position 820 to an alanine (C820A) did not abrogate the repression. Therefore, E6AP did not act as a repressor of SHR signaling by ubiquitinating the receptor thus priming it for degradation. Accordingly, the expression level of ectopic HA-tagged ER α was not altered upon E6AP co-expression.

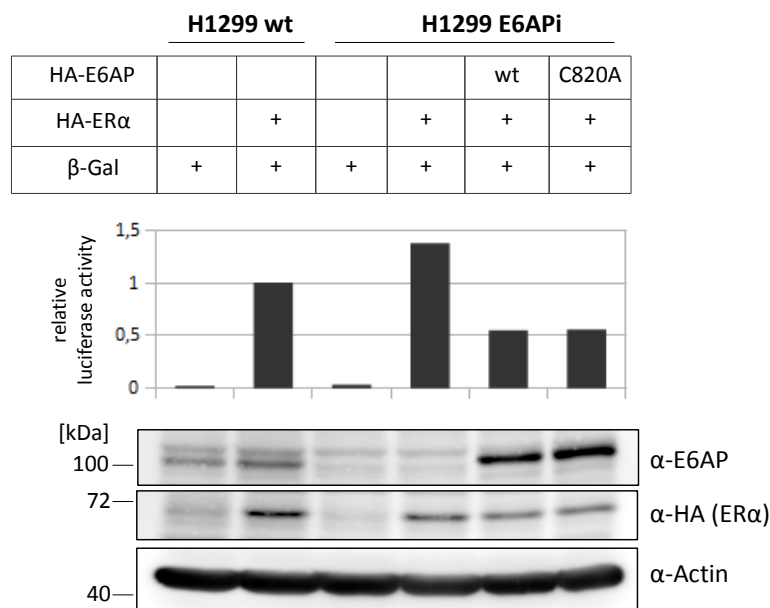


Figure 22: Expression of endogenous and ectopic E6AP reduce ER α mediated transcription in transient luciferase reporter assays. H1299 cells were transfected with the ERE-reporter, β -Gal and HA-tagged constructs (HA-ER α and HA-E6AP versions – wt and catalytically inactive C820A mutant) where indicated (for experimental set-up, see 3.2.19). Transfection efficiencies were determined by β -Gal assay (3.2.22) and detected luciferase activities (3.2.23) are expressed as the ratio of luciferase per β -Gal activities. Lysates were adjusted for transfection efficiencies and subjected to SDS-PAGE and Western blot analysis with anti-HA, anti-E6AP and anti-Actin antibodies (lower part). Note that loss of endogenous E6AP expression (H1299 E6APi) led to increased luciferase values which were decreased again via ectopic E6AP expression. The experiment shown is a representative result out of three independent experiments.

RESULTS

4.2.2 E6AP affects SHR signaling via a PI3K-dependent pathway

As the repressing effect of E6AP on ER α did not depend on its ligase activity, it seemed unlikely that E6AP caused ubiquitination and subsequent proteasomal degradation of the receptor. Thus, it was speculated that the underlying mechanism might also not depend on binding of E6AP to the receptors (which indeed could never be observed in *in vitro* or *in cellulo* experiments – data not shown). A mechanism that affects the transcriptional activity of SHRs is the post-translational modification by phosphorylation (reviewed in Leeuw et al. 2011, Björnström and Sjöberg 2005).

Based on extensive literature research, two kinase cascades came into focus: the MAPK/Erk and PI3K/Akt pathways. In a simplified view, the MAPK/Erk pathway is best known for modulating the activities of transcription factors (Yoon and Seger 2006) and the PI3K/Akt pathway for controlling translational initiation by mTORC1 (Mamane et al. 2006). Both pathways are known to (I) play pivotal roles in neurodevelopment (Thomas and Haganir 2004, Acebes and Morales 2012) and (II) regulate SHR signaling (Leeuw et al. 2011, Björnström and Sjöberg 2005) – two features that are also true for E6AP (El Hokayem and Nawaz 2014). To investigate if deregulation of these kinase cascades leads to the observed increase in SHR signaling in H1299 E6APi cells, transient luciferase reporter assays were conducted in presence of specific kinase inhibitors (Figure 23, for experimental set-up, see 3.2.20).

Inhibition of the MAPK/Erk pathway by UO126 led to a concentration-dependent increase of ER α transactivation in H1299 wt and E6APi cells. This increase occurred in both cell lines to a similar extent, and, thus, presumably independent of endogenous E6AP expression levels. Western blot analysis confirmed that Erk phosphorylation (and thus activity) was completely blocked by UO126 (data not shown). This result was unexpected, because according to Thomas et al. (2008) Erk appears to phosphorylate and stimulate ER α . If this had been the case in the current experimental setting, Erk inhibition should have caused a decrease in ER α activity. However, later findings allowed a conclusive interpretation of this observation (chapter 4.2.4).

In contrast, inhibition of PI3K by LY294002 reduced ER α transactivation. This decrease was much more drastic in H1299 E6APi compared to H1299 wt cells, which resulted in comparable luciferase activities in both cell lines when the highest concentration of LY294002 was applied. A higher concentration of the inhibitor did not result in a further decrease of ER α activity (data not shown). The functionality of the inhibitor was confirmed by Western blot analysis. Akt/PKB is a direct target of PI3K and is phosphorylated when PI3K is active (Datta et al. 1999). Accordingly, PI3K inhibition led to a loss of phosphorylated Akt. The same result was obtained with a different pair of cell lines (HEK293T wt and E6APi, data not shown). Thus, it was concluded that E6AP regulates SHR activity in a PI3K-dependent manner.

RESULTS

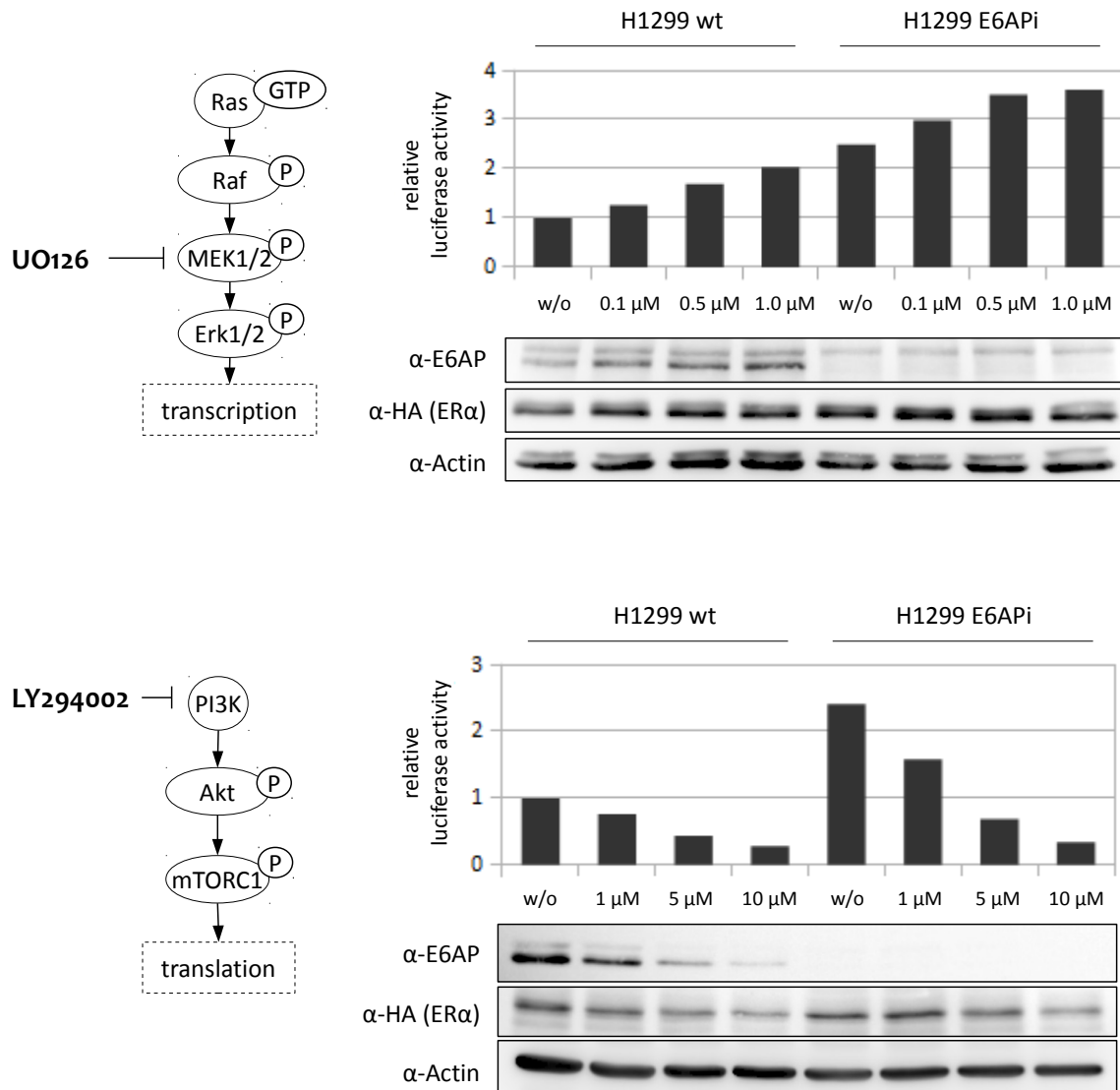


Figure 23: Inhibition of the MAPK/Erk and PI3K/Akt pathways has opposing effects on ER α activity. H1299 wt and E6APi cells were transfected with the ERE reporter, HA-tagged ER α and β -Gal (for experimental set-up, see 3.2.20). To test the impact of MAPK/Erk and PI3K/Akt pathways on ER α transactivation, cells were cultured in the presence of the MAPK/Erk inhibitor UO126 (upper panel) or the PI3K inhibitor LY294002 (lower panel) using the indicated concentrations. Luciferase activities were determined and are expressed relative to the β -Gal values. Lysates were adjusted for transfection efficiency and subjected to Western blot analysis with anti-HA, anti-E6AP and anti-Actin antibodies. The observed increase (UO126) or decrease (LY294002) of E6AP expression is largely an artefact since samples were not adjusted for protein amount prior SDS-PAGE and Western blot analysis. The schemes on the left side depict the pathways that were analyzed. While inhibition of MAPK/Erk caused increased luciferase activities, treatment with the PI3K inhibitor decreased them. Furthermore, block of PI3K activity abrogated the effect of endogenous E6AP on ER α . The results shown are representative for results obtained from at least three independent experiments.

RESULTS

4.2.3 The PI3K target GSK3 regulates ER α through phosphorylation

As loss of PI3K activity drastically reduced ER α transactivation, it was hypothesized that one of its downstream targets mediates the effect of PI3K on the SHR. It is well established that many of PI3K's effects are based on its activation of Akt/PBK, which in turn leads to the activation of mTORC1 (and its downstream target S6K1) and repression of GSK3 (Datta et al. 1999, Hay and Sonenberg 2004, Cross et al. 1995). While mTORC1 activity is crucial for initiating protein translation and thus plays a role in cell proliferation (Hay and Sonenberg 2004, Mamane et al. 2006), GSK3 is usually active in resting cells thereby inhibiting a number of transcription factors (Woodgett 2001). Akt, GSK3, and S6K1 were found to stimulate ER α by phosphorylation, although there is also evidence for an Akt-independent effect of PI3K on ER α (Campbell et al. 2001, Medunjanin et al. 2005, Yamnik et al. 2009). To unravel the mechanism by which PI3K modulates ER α in H1299 cells, transient luciferase reporter assays were conducted in the presence of the mTORC1 inhibitor rapamycin and the GSK3 inhibitor CHIR99021 (Figure 24). While inhibition of mTORC1 did not show any effect, inhibition of GSK3 led to a decrease in luciferase activity. This decrease was again much more prominent in H1299 E6APi compared to wt cells which resulted in similar luciferase activities with the highest concentration of the inhibitor. Thus, GSK3 activity is (I) involved in stimulation of ER α transactivation and (II) might be the mediator of E6AP's effect on SHR signaling.

RESULTS

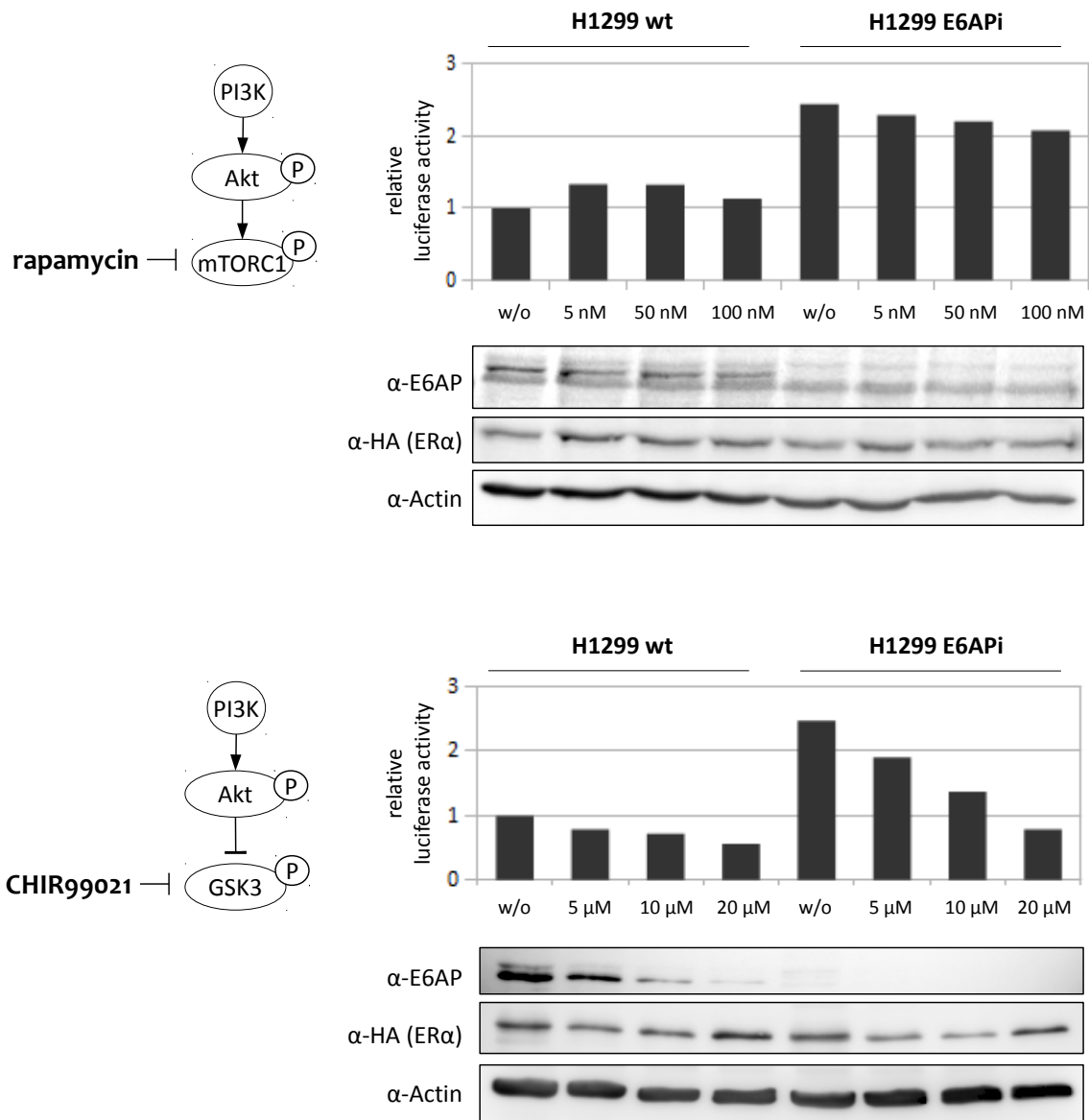


Figure 24: The PI3K/Akt target GSK3, but not mTORC1, contributes to ER α activity in H1299 cells. H1299 wt and E6APi cells were transfected with the ERE reporter, HA-tagged ER α and β -Gal. Cells were incubated with the mTORC1 inhibitor rapamycin (upper panel) or the GSK3 inhibitor CHIR99021 (lower panel) as indicated (for experimental set-up, see 3.2.20). Luciferase activities were determined and are expressed relative to the β -Gal values. Lysates were adjusted for transfection efficiency and subjected to Western blot analysis with anti-HA, anti-E6AP and anti-Actin antibodies. The observed decrease of E6AP expression after incubation of H1299 wt cells with CHIR99021 is an artefact, since samples were not adjusted for protein amount prior to SDS-PAGE and Western blot analysis. The schemes on the left side illustrate the connection of the PI3K/Akt pathway. Inhibition of GSK3 led to a remarkable reduction of luciferase activities, while loss of mTORC1 activity showed no effect. The results shown with the GSK3 inhibitor are representative for results obtained in at least three independent experiments.

RESULTS

Medunjanin et al. have reported that GSK3 regulates the activity of ER α by phosphorylation of several N-terminal serine residues (S102/104/106 and S118). The authors showed that mutation of these residues to alanine decreased ER α activity in MCF-7 cells, which supports their conclusion that ER α activity is positively regulated by the GSK3-mediated phosphorylation (Medunjanin et al. 2005). To confirm that the same holds true in H1299 cells, several phospho-mutant ER α 's were generated and tested in transient luciferase reporter assays (for experimental set-up, see 3.2.19). The results clearly showed that replacing the GSK3-targeted serines of ER α by alanine (to avoid phosphorylation) decreased the luciferase activities drastically (Figure 25).

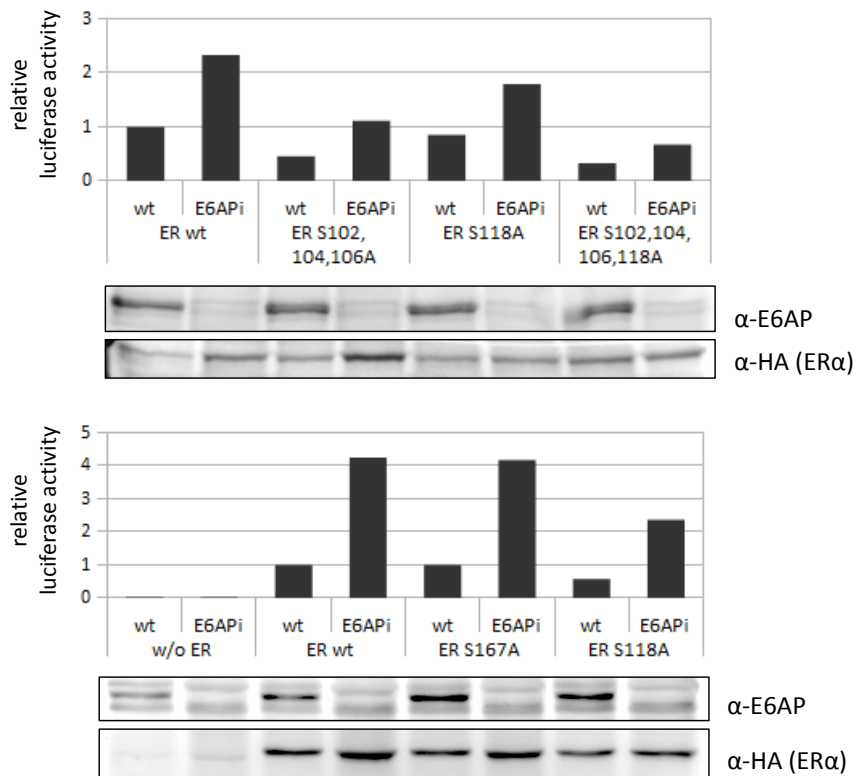


Figure 25: Replacement of S102/104/106 and/or S118, but not S167, of ER α by alanine reduces ER α transactivation. H1299 wt and E6APi cells were transfected with the ERE reporter, HA-tagged ER α versions as indicated and β -Gal (for experimental set-up, see 3.2.19). Luciferase activities were determined and are expressed relative to the β -Gal values. Lysates were adjusted for transfection efficiency and subjected to Western blot analysis with anti-HA, anti-E6AP and anti-Actin antibodies. S102/104/106 and S118 of ER α are targeted by GSK3 for phosphorylation. Replacement of these residues by alanine reduced ER α transactivation which corresponds to the effect of the GSK3 inhibitor on ER α wt. S167 is a target for Akt and S6K1, and replacement by alanine had no impact on transactivation. Two independent experiments gave similar results.

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Accordingly, replacing these serines by glutamic acid, which mimics phosphorylation, increased luciferase activities (data not shown). This supports the finding that the transcriptional activity of ER α is modulated by the GSK3-mediated phosphorylation of these residues. In addition, mutation of S167 (targeted by Akt (Campbell et al. 2001) or S6K1 (Yamnik et al. 2009)) to alanine had no effect on ER α transactivation.

4.2.4 Effect of other GSK3 modulators on ER α transactivation

The data obtained so far indicate that E6AP might act as a repressor of ER α signaling by interfering with the GSK3-mediated stimulatory phosphorylation of ER α . Thus, also other inhibitors of GSK3 should cause decreased ER α activities in transient luciferase reporter assays. One of such negative regulators is Erk (Ding et al. 2005), and indeed it was already shown that inhibition of Erk (thus activation of GSK3) caused increased luciferase activities (Figure 23). Another GSK3 repressor is CamKII (Song et al. 2010), and indeed overexpression of CaMKII wt or CaMKII T305/306A (constitutive active mutant; Elgersma et al. 2002) resulted in lowered ER α transactivation (Figure 26). This finding is of particular interest as it was shown in a mouse model of AS that expression of the constitutive active CaMKII T305V/T306A mutant overcomes some severe phenotypes caused by knockout of the E6AP gene (van Woerden et al. 2007).

RESULTS

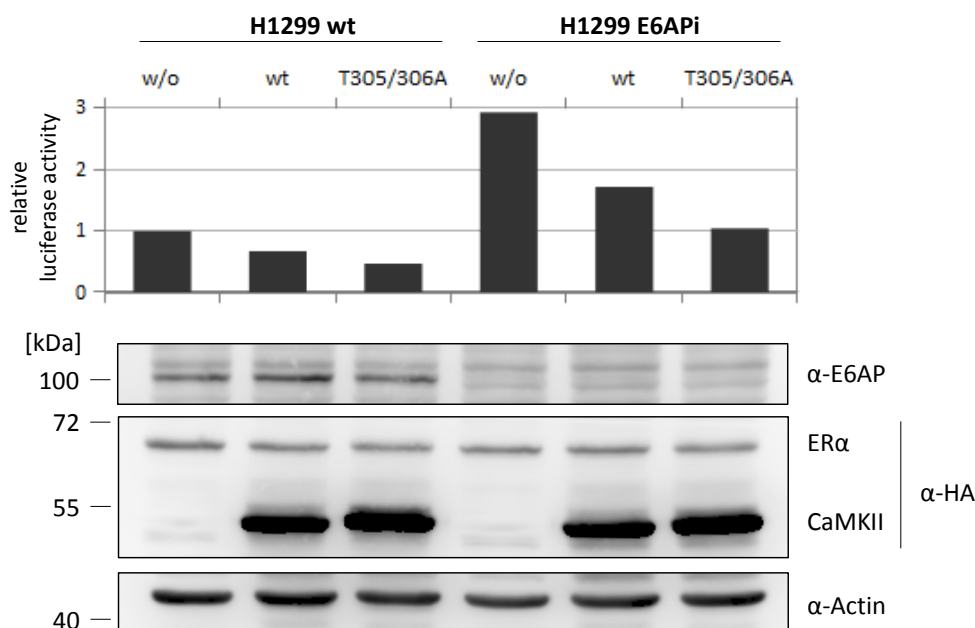


Figure 26: CaMKII, a negative regulator of GSK3, interferes with ER α transactivation in H1299 cells. H1299 wt and E6APi cells were transfected with the ERE reporter, HA-tagged ER α and β -Gal. HA-tagged CaMKII wt or T305/306A (constitutive active mutant) was co-expressed as indicated. Luciferase activities were determined and are expressed relative to the β -Gal values. Lysates were adjusted for transfection efficiency and subjected to Western blot analysis with anti-HA, anti-E6AP and anti-Actin antibodies. Expression of CaMKII caused decreased ER α activity, most likely due to the repression of GSK3 activity. Two independent experiments gave similar results.

GSK3 can also be repressed through phosphorylation by Akt/PKB (Cross et al. 1995). Activation of Akt/PKB is induced by stimulation of PI3K activity with growth factors like insulin and epidermal growth factor (EGF) (Datta et al. 1999). However, treatment of H1299 cells with insulin did not show any effect on ER α transactivation, and the presence of EGF caused only a mild repression (Figure 27).

RESULTS

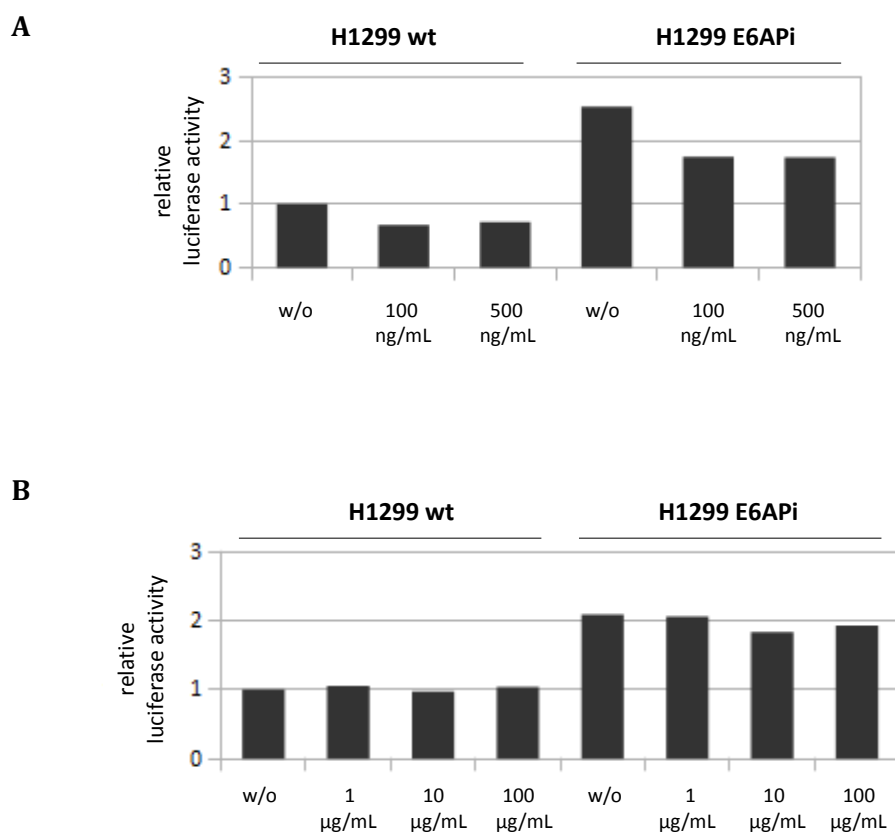


Figure 27: EGF (A) or insulin (B) do not affect ER α transactivation in H1299 cells. H1299 wt and E6APi cells were transfected with the ERE reporter, HA-tagged ER α and β -Gal. Cells were incubated with EGF (A) or insulin (B) with indicated concentrations (for experimental set-up, see 3.2.20). Luciferase activities were determined and expressed relative to the β -Gal values. Treatment with EGF and insulin did not severely affect ER α transactivation. In both cases, two independent experiments gave similar results.

As this result was unexpected, it was speculated that H1299 cells may not respond to insulin or EGF, e.g. due to lack of the respective endogenous receptors. However, Western blot analysis revealed that upon stimulation of H1299 cells with insulin and EGF, the activation of Akt/PKB is observed, but no effect on GSK3 phosphorylation and therefore activity could be detected (Figure 28). This may explain the lack of any effect of insulin and EGF treatment on ER α transactivation in H1299 cells. The mild effect of EGF can also be caused by an unrelated mechanism as EGF affects not only the PI3K/Akt pathway, but also many other signaling cascades (Oda et al. 2005, Yarden and Sliwkowski 2001).

RESULTS

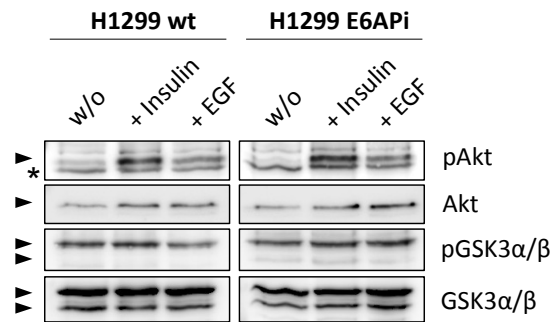


Figure 28: Stimulation of H1299 cells with EGF and insulin causes activation of Akt but no inhibition of GSK3. H1299 wt and E6APi cells were treated with 10 $\mu\text{g}/\text{mL}$ insulin or 100 ng/mL EGF (for experimental set-up, see 3.2.25). Lysates were adjusted for protein amount and subjected to Western blot analysis using antibodies directed against pAkt^{Ser473}, Akt, pGSK3 α/β ^{S21/9}, and GSK3 α/β . Both, insulin and EGF, caused a rapid phosphorylation of Akt (and thus activation) in both cell lines. Active Akt can inhibit GSK3 by phosphorylation. However, insulin and EGF did not cause a detectable change on GSK3 phosphorylation in two out of two experiments. Note that in the absence of insulin and EGF the phosphorylation of Akt in H1299 wt cells is higher than in E6APi cells. The asterisk indicates an unrelated background band.

An alternative way to modulate Akt activity is overexpression of PTEN. PTEN is the phosphatase that converts the PI3K product PI(3,4,5)P3 back into PI(4,5)P2 and thus prevents many downstream signaling events, including activation of Akt, that are usually triggered by PI3K activity (Downes et al. 2001, Datta et al. 1999). As Akt negatively regulates GSK3 (Cross et al. 1995), Akt inhibition should cause increased GSK3 activity and increased ER α transactivation. However, overexpression of PTEN caused decreased ER α transactivation (Figure 29). This supports the previous data obtained with the PI3K inhibitor LY294002 (Figure 23). Thus, the transcriptional activity of ER α is dependent not only on GSK3, but also on PI3K activity. Consequently, the effect of E6AP on SHR signaling might be mediated by a PI3K-dependent pathway that does not only include GSK3-mediated phosphorylation of ER α .

RESULTS

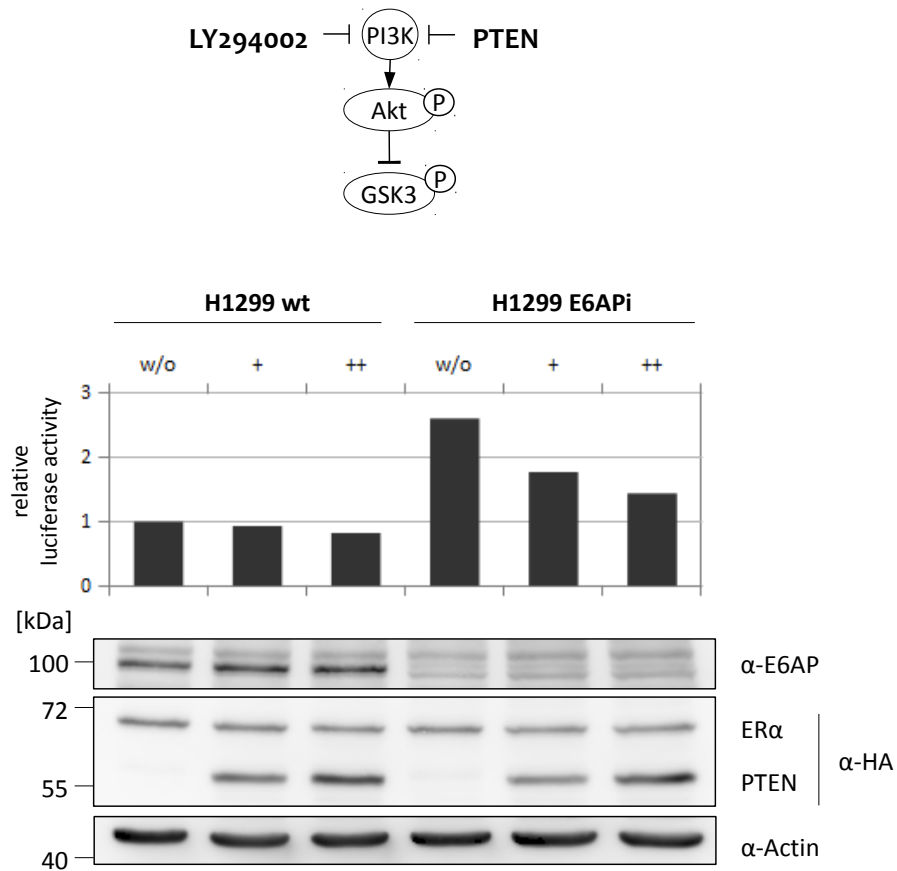


Figure 29: PTEN, a counteractor of PI3K/Akt signaling, interferes with ER α transactivation in H1299 cells. H1299 wt and E6APi cells were transfected with the ERE reporter, HA-tagged ER α and β -Gal. HA-tagged PTEN was co-expressed as indicated (+ = 1500ng, ++ = 3000ng). Luciferase activities were determined and are expressed relative to the β -Gal values. Lysates were adjusted for transfection efficiency and subjected to Western blot analysis with anti-HA, anti-E6AP and anti-Actin antibodies. In two out of two experiments the expression of PTEN caused decreased ER α activities, which fits to the result obtained with the PI3K inhibitor LY294002 (Figure 23). The scheme illustrates that both, PTEN and LY294002, inactivate PI3K-mediated signaling.

4.2.5 Effect of E6AP on other GSK3 targets

It appears that the activity of SHRs, and in particular ER α , in H1299 cells is connected to the kinase activity of GSK3. Thus, it was hypothesized that E6AP might act as a repressor of GSK3 leading to the decreased activity of ER α when E6AP is expressed. To investigate this hypothesis, the effect of E6AP on other GSK3 targets, namely CREB, AP-1 and β -Catenin, was investigated (reviewed in Sutherland 2011).

Phosphorylation of CREB by GSK3 is dependent on initial phosphorylation of CREB by PKA and supposed to stimulate its transcriptional activity (Fiol et al. 1994, Bullock and Habener 1998).

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However, there are contradictory results indicating that GSK3 can repress CREB DNA binding ability (Grimes and Jope 2001, Tullai et al. 2007), and it is still a matter of debate how these opposing observations can be reconciled. AP-1 is a dimeric transcription factor composed of Jun and Fos proteins, and phosphorylation of Jun by GSK3 interferes with its transactivation function (Karin et al. 1997, Nikolakaki et al. 1993, Tullai et al. 2011).

In contrast to CREB and AP-1, β -Catenin does not directly interact with DNA, but is a coactivator for the TCF/LEF family of transcription factors. GSK3-mediated phosphorylation of β -Catenin renders it inactive in the cytoplasm. Inhibition of GSK3 releases β -Catenin which translocates into the nucleus and activates transcription (reviewed in Waltzer and Bienz 1999).

In all cases, the respective luciferase reporter constructs were used to estimate the activities of CREB, AP-1 and beta-catenin/TCF upon co-expression of E6AP or treatment with the GSK3 inhibitor CHIR99021 in H1299 E6APi cells. Treatment with the GSK3 inhibitor resulted in the activation of CREB, AP-1 and β -Catenin as indicated by increased luciferase activities, although the effect on AP-1 is comparably mild. In contrast, overexpression of E6AP caused decreased luciferase values, most prominent in case of AP-1. Thus, E6AP may not be a negative regulator of GSK3 in all cases.

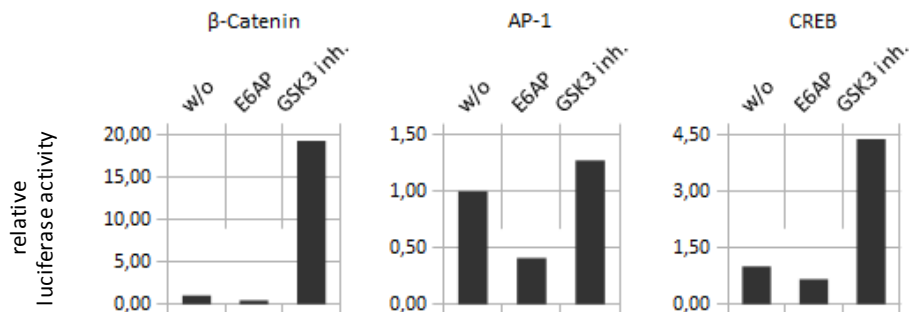


Figure 30: Effect of E6AP and CHIR99021 (GSK3 inhibitor) on other GSK3 targets. H1299 E6APi cells were transfected with the reporter constructs for β -Catenin/TCF, AP-1 or CREB and either co-transfected with E6AP or treated with the GSK3 inhibitor CHIR99021 (20 μ M). Luciferase activities were determined and are expressed relative to the protein amount of the whole cell extracts. Inhibition of GSK3 by CHIR99021 significantly activated β -Catenin and CREB, while overexpression of E6AP repressed AP-1. Thus, E6AP did not act as a negative regulator of GSK3 in these cases. In all cases two independent experiments gave similar results.

Correspondingly, it is assumed that different pools of GSK3 act on different target proteins. These targets are affected by differing stimuli and do not necessarily depend on each other (Sutherland 2011). Furthermore, the analyzed transcription factors are certainly not only regulated by GSK3 and therefore the effect of E6AP, especially on AP-1, may depend on other regulators.

5. Discussion

5.1 Modulation of E6AP activity by interactors and ubiquitin

E6AP is a prominent member of the E3 ubiquitin ligase family and known to play a substantial role in different human diseases. Genetic evidence clearly indicates that an appropriate E6AP level is indispensable for proper brain development and function. Nonetheless, there is still a lack of molecular explanations for the observed pathology of AS (loss of E6AP expression) and ASD (higher E6AP expression). However, E6AP's role in cervical cancer supports the idea of E6AP being a "modulatable" E3 ligase, because in this disease binding of HPV E6 proteins alters the substrate specificity of the ligase.

Recently, HERC2, a giant HECT ligase that is also important in neurodevelopment, was identified to interact with E6AP. Upon binding, E6AP's ligase activity was stimulated *in vitro* and *in cellulo*. If and to what extent the substrate spectrum of E6AP is also affected remains unclear so far. However, in the course of those studies it was also found that the so-called "canonical" hydrophobic patch of ubiquitin (L8, I44, V70) is indispensable for proper E6AP function. Interestingly, this patch is less relevant for E6AP-mediated ubiquitination as long as binding to HERC2 occurs (Kuhnle et al. 2011). Further studies revealed that the same holds true for the HPV E6/E6AP complex (S. Kuhnle, unpublished results).

Therefore, a common mode of action of E6AP modulators might be the manipulation of the interaction between E6AP and ubiquitin (Ub). To gain more insights into the role of Ub in E6AP-mediated catalysis, studies were extended towards another, so-called "non-canonical" hydrophobic patch that is formed by I36, L71 and L73 of Ub. E6AP-mediated ubiquitination was again dramatically impaired, but this time no rescue was achieved in presence of an HPV E6 protein (e.g. Figure 7). This was the starting point to survey for the mechanisms that make both hydrophobic patches essential in E6AP-mediated catalysis.

5.1.1 The effects of ubiquitin's hydrophobic patches are not substrate-dependent

In order to investigate which proteins involved in the ubiquitination reaction are affected by ubiquitin's hydrophobic patches, several *in vitro* ubiquitination assays were performed using different substrate proteins. This was necessary to determine if parameters like substrate binding efficiencies and the biochemical environment of the target lysine residue contribute to the inability of E6AP to use the hydrophobic patch Ub mutants (note that in previous studies, only E6AP auto-ubiquitination was studied).

DISCUSSION

A number of potential substrates for E6AP-mediated ubiquitination have been reported so far, though only few have been confirmed under *in vitro* conditions. Amongst them are Ring1b (Zaaroor-Regev et al. 2010) and hHR23 (Kumar et al. 1999), which therefore were suitable to address potential substrate-specific effects (Figure 7). Furthermore, some analyzed substrates are not targeted by E6AP alone but only in complex with high-risk HPV E6 proteins (e.g. HPV16 E6). The most prominent one is the tumor suppressor p53 (Figure 7) (Scheffner et al. 1993), but ubiquitination of the PDZ domain-containing protein Dlg also depends on binding of E6 to E6AP (Figure 21) (Kiyono et al. 1997, Lee et al. 1997, Nakagawa and Huibregtse 2000). Last but not least E6AP auto-ubiquitination (Nuber et al. 1998) was examined in the absence of other substrates (Figures 8 and 20).

In all cases ubiquitination reactions were strongly impaired compared to the Ub wt control, when the "canonical" patch mutant Ub LIA (L8A, I44A) and the "non-canonical" patch mutant Ub ILA (I36A, L71/73A) were employed. However, the activity towards Ub LIA was restored by addition of HPV16 E6 and also by low-risk HPV11 E6. Interestingly, stimulation by HPV16 E6 was also observed with Ub wt, which concurs with earlier reports showing stimulation of E6AP auto-ubiquitination by 16E6 (Kao et al. 2000). These results suggest that the effects of Ub LIA and Ub ILA are not dependent on the target substrate but rather on the E1-E2-E3 enzyme cascade that catalyzes the ubiquitination reaction.

5.1.2 Minor roles of ubiquitin's patches in E1 and E2 enzyme functions

Attachment of ubiquitin to substrate proteins is mediated by the sequential action of E1, E2 and E3 enzymes (Figure 1). The initial step is activation of ubiquitin by the E1 enzyme under ATP hydrolysis, resulting in the E1~Ub thioester conjugate. Subsequently, ubiquitin is transferred to the active site cysteine of an E2 enzyme in a transthiolation reaction (Hershko and Ciechanover 1992). Any difficulties in these first steps of the ubiquitination cascade would also affect the final attachment of ubiquitin to a substrate protein. Hence, it had to be analyzed if and how ubiquitin's hydrophobic patches influence E1 and E2 enzyme function. This was of particular interest for interpreting the results obtained with Ub ILA as under none of the tested conditions an efficient ubiquitination reaction was detectable.

In all reactions UBA1 served as the ubiquitin-activating enzyme. UbcH5b and UbcH7 served as E2 enzymes as they are known to function together with E6AP (Scheffner et al. 1993, Nuber et al. 1996). Both E2 enzymes yielded the same results in E6AP-mediated substrate ubiquitination

DISCUSSION

with Ub LIA and Ub ILA (Figure 7). Thioester assays proved the efficient charging of UBA1 and both E2 enzymes with all tested Ub versions (Figure 9). Subsequently, the release of the Ub versions from the E2 enzymes to free cysteine or lysine was analyzed, because this mimics the transfer to the catalytic site cysteine of HECT ligases (e.g. E6AP) or a target lysine residue (as it is necessary in RING E3-mediated ubiquitination), respectively (Figure 10).

As both E2 enzymes are able to work with HECT ligases, the release of all Ub versions took place in presence of cysteine. Furthermore, as Ubch5b is also a cognate E2 for RING ligases, the discharge was also achieved with free lysine (as also shown by Wenzel et al. 2011). As expected, this property is abolished in the Ubch5b N77A mutant because asparagine 77, which is part of the so-called "HPNI-motif", appears to stabilize the oxyanion that is formed during formation of the isopeptide bond with a primary amino group. Hence, Ubch5b N77A is still able to work with E6AP, but not with RING ligases (Wu et al. 2003, Plechanovová et al. 2012).

The release of each Ub version from Ubch7, Ubch5b and Ubch5b N77A to free cysteine or lysine (Ubch5b) was not severely affected by both hydrophobic patch mutants of Ub. However, it has to be noted that the conjugates formed by Ubch5b wt and N77A with Ub ILA seemed to be less stable compared to the conjugates with Ub wt. In addition, a more efficient release of Ub ILA from Ubch5b wt to free lysine was observed already at rather low concentrations of the amino acid (Figure 11).

From the crystal structures of Ubch5b and the Ubch5b~Ub conjugate, a large interaction of Ubch5b with the loaded ubiquitin cannot be observed. Additionally, the conformational changes that take place on Ubch5b and Ub upon loading are marginal (Sakata et al. 2010). Nevertheless, mutation of Ub's "non-canonical" patch may cause a groove in the proximity of the thioester bond. Thus, it can be speculated that the accessibility of the Ubch5b~Ub ILA thioester bond for water or free amino acid side chains is increased and causes a higher susceptibility for hydrolysis and discharge, respectively.

In other words, the "non-canonical" patch of ubiquitin appears to act as a selectivity filter for Ubch5b. Its mutation to Ub ILA interferes with the mechanism that allows Ubch5b to release the loaded Ub in a site-specific manner. This would also explain why Ubch5b mediated the transfer of Ub ILA to the very C-terminal lysine residue K847 (= -6K) of E6AP, which was not observed with Ub wt, because the intact "non-canonical" patch hinders this Ubch5b-catalyzed "off-target" reaction (Figure 15). In case of Ubch7 this selectivity filter is not needed because it does not react with primary amino groups anyway.

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If and how E6AP contributes to this selectivity can only be speculated because a crystal structure for UbcH5b~Ub in complex with E6AP is not available, but as UbcH5b-mediated ubiquitination of E6AP with Ub ILA takes place in the flexible C terminus of E6AP (Figure 3), a contribution through a non-covalent interaction between E6AP and UbcH5b and/or the loaded Ub appears to be possible. Notably, a similar interaction was shown to be essential for the E2-to-E3 Ub transfer for UbcH5b and NEDD4L. The authors showed that ubiquitin's "non-canonical" patch (I36 and L71/73), which they partially mutated to either L71A or I36D, was essential for thioester formation on the HECT domains of NEDD4L, Rsp5 and Itch. However, this was not the case for E6AP (Kamadurai et al. 2009). This emphasizes the remarkable differences between HECT family members.

5.1.3 Ubiquitin's patches contribute to E6AP-mediated catalysis

As no severe effects of Ub's patches on the functions of E1 and E2 enzymes were detectable, their contribution to the ubiquitination reaction must originate from the E3 enzyme level. In case of HECT ligases like E6AP, the E3 enzyme takes over ubiquitin from the E2 enzyme under thioester formation (Rotin and Kumar 2009, Scheffner and Kumar 2014). This step is performed by the HECT domain, which harbors the binding site for the E2 enzyme and the active site cysteine used for the thioester formation with ubiquitin (Kumar et al. 1997, Huang et al. 1999). The results obtained in this thesis clearly showed that both hydrophobic patches do not significantly contribute to the ability of E6AP to form thioesters with Ub and that this is the case not only for the isolated HECT domain but also for the full-length protein (Figures 12 and 16). Thus, the E2-to-E3 Ub transfer does not depend on Ub's "canonical" and "non-canonical" patches. This is in good agreement with the aforementioned data that shows no contribution of Ub's "non-canonical" patch to thioester formation on E6AP but on other HECT ligases (Kamadurai et al. 2009). In conclusion, the data strongly indicate that Ub's patches assist in E6AP-catalyzed isopeptide bond formation.

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5.1.4 Mechanistic insights into isopeptide bond formation: role of ubiquitin's "non-canonical" patch

For catalyzing the attachment of Ub to the ϵ -amino group of a target lysine residue several requirements have to be met. Firstly, the thioester-bound Ub in E6AP's C lobe has to come in close proximity to the substrate. Secondly, the ϵ -amino group of the targeted lysine has to be deprotonated to enable its nucleophilic attack at the carbon of the thioester bond. However, due to the high pK of primary amines of about 10 to 11, they are under physiological conditions predominantly protonated (Nelson and Cox 2009). Thus, the enzyme has to create an appropriate environment which facilitates removal of the proton and stabilizes the unprotonated state. Thirdly, the nucleophilic attack of the amino group results in the formation of an oxyanion as a transition state. Such high-energy intermediates are stabilized by enzymes through hydrogen bonds, which in turn lowers the activation energy and favors product formation (Nelson and Cox 2009).

In order to assign which of these steps are influenced by Ub's "canonical" and "non-canonical" patches, comparison of the results obtained with the catalytic HECT domain of E6AP and the full-length protein is helpful (Figure 17). In both cases, auto-ubiquitination required the intact "non-canonical" patch, which highlights its fundamental role in E6AP-mediated catalysis. Mutation to Ub ILA might result in a lowered hydrophobicity in close proximity to the catalytic center, which may be necessary for stabilizing a deprotonated amino group. As no crystal structure of the ubiquitin-loaded HECT^{E6AP} is available, it can also be speculated that this patch makes important non-covalent contacts with certain residues on the HECT domain, thereby inducing a more active conformation. However, there is no evidence for a non-covalent interaction between the HECT domain of E6AP and Ub, like it was found for Nedd4 HECT family members (French et al. 2009, Kim et al. 2011, Maspero et al. 2011, Ogunjimi et al. 2010). In those cases, the non-covalent interaction with Ub takes place in the N lobe and requires Ub's "canonical" patch. This interaction facilitates binding to ubiquitinated substrates and is predominantly important in Ub chain formation.

Ub's "non-canonical" patch is not the only factor that is required for isopeptide bond but not thioester formation: an E6AP lacking the conserved phenylalanine residue F849 (numbering according to isoform 1) located four amino acids from the very C terminus (-4F) is still efficiently charged with Ub, but substrate ubiquitination is impaired (Salvat et al. 2004). Recently, a putative role of this residue in substrate ubiquitination was suggested. Crystal structure analysis

DISCUSSION

indicates that upon loading of the HECT domain of Rsp5 with Ub, the flexible C terminus of the C lobe winds around the C terminus of Ub, thereby making contacts with the N lobe. Disturbing these interactions for example by mutating the -4F hinders the E3-to-substrate Ub transfer probably because the HECT domain is not stabilized in its active conformation (Kamadurai et al. 2013). In addition, mutation of the adjacent -6K of E6AP to an arginine (K847R) dramatically increased the ligase activity, while mutation to alanine (K847A) showed a mild negative effect (data not shown). This highlights again the important role of the C terminus in catalysis. In addition, it has to be noted that indeed the very C-terminal amino acids of at least some HECT ligases determine the type of Ub chain that is formed. Hence, the C lobe does not only provide the catalytic residue for Ub thioester formation, but is also required for isopeptide bond formation (Kim and Huibregtse 2009, Maspero et al. 2013).

Taken together a model can be suggested in which Ub and E6AP's C terminus collectively contribute to isopeptide bond formation (Figure 31): while Ub's "non-canonical" patch assists in retaining the deprotonated ϵ -amino group of the targeted lysine, E6AP's -6K stabilizes the oxyanion intermediate (a mechanism that might be strengthened by replacing the -6K by arginine: the guanidino group of arginine has a higher pK than the ϵ -amino group of lysine and thus is to a larger extent protonated and, accordingly, positively charged under physiological conditions; Nelson and Cox 2009). The -4F ensures that these processes take place by interacting with the N lobe, thereby properly positioning the amino acids that are involved in catalysis.

However, to validate this hypothesis further experiments are needed. The most valuable insight would certainly be provided by a crystal structure of the Ub-loaded HECT^{E6AP} as this would visualize how Ub is interacting with E6AP and which conformational changes take place on E6AP upon loading. Possibly this would also allow identification of those basic and hydrophobic amino acid residues necessary for initial target-lysine deprotonation. Similarly, a mutational analysis could be executed in a way that the putative interacting amino acid side chains of E6AP and Ub are altered or swapped. However, without crystal structure the interpretation of these data would still be rather speculative and error-prone.

DISCUSSION

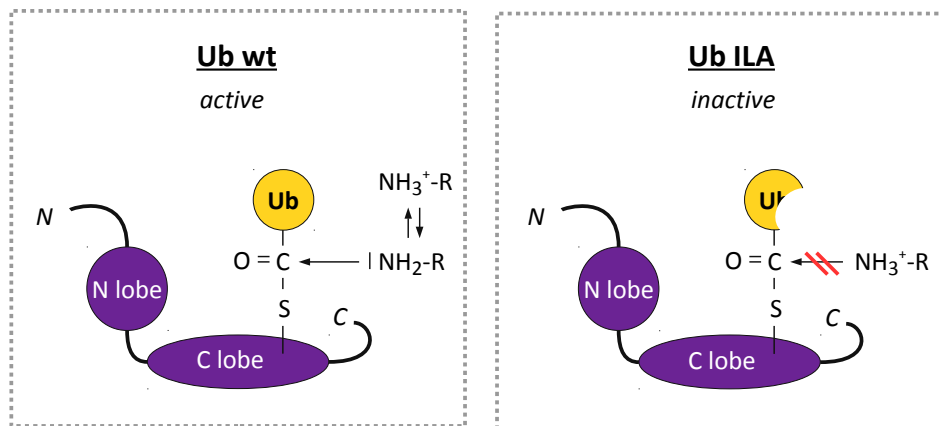


Figure 31: Putative role of ubiquitin's "non-canonical" patch in E6AP-mediated isopeptide bond formation. The amino group of a target lysine residue has to be deprotonated (NH₂R) to enable the nucleophilic attack at the carbonyl carbon of the thioester bond that is formed between the HECT domain of E6AP (purple) and ubiquitin (Ub, yellow). The intact "non-canonical" patch traps the deprotonated amino group (Ub wt, left side). Mutation of the patch (Ub ILA, right side) decreases the hydrophobicity at the catalytic center and the targeted amino group is not stabilized in its deprotonated state. The reaction with the protonated form is not possible.

5.1.5 Role of ubiquitin's "canonical" hydrophobic patch for the activity of full-length E6AP: E6AP vs. HECT^{E6AP}

As the "non-canonical" patch of Ub appears to fulfill a very basic role in E6AP-mediated catalysis, effectors like 16E6 that bind outside the HECT domain did not influence its function. However, this was not the case for the "canonical" patch. While its mutation to Ub LIA did not affect the auto-ubiquitination reaction of HECT^{E6AP}, a significant impairment of the isopeptide bond formation ability of full-length E6AP was observed (Figure 17). Furthermore, binding of 16E6 to E6AP, which occurs in the N terminus, eliminated the defect and the "canonical" patch did not appear to play a major role in catalysis anymore (Figures 7, 8 and 14). This effect is not restricted to high-risk HPV16 E6 as also low-risk 11E6 (Figures 20 and 21) and the Rcc1b domain of HERC (Kuhnle et al. 2011) stimulated E6AP activity by binding to E6AP's N terminus. Hence, these interactors do not only alter E6AP's substrate spectrum (as it is well documented for 16E6) but also act as allosteric activators of E6AP's catalytic activity. Thus, the N terminus of E6AP does not only facilitate substrate binding, but also plays a fundamental role in the formation of isopeptide bonds. This is further supported by the finding that point mutations which are mapped to the N terminus of E6AP, e.g. S349P, play a causative role in the development

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of AS (Malzac et al. 1998). Biochemical analysis revealed that the E6AP S349P mutant is still able to form a thioester complex with ubiquitin, but subsequent substrate ubiquitination is impaired (Cooper et al. 2004).

In order to understand this observation, it has to be postulated that a non-covalent interaction between E6AP's N terminus and the Ub-loaded HECT domain takes place. This interaction is (I) to a certain extent mediated by Ub's "canonical" patch and (II) modified by interactors that bind to the N terminus (Figure 32). However, neither in size exclusion chromatography nor coprecipitation experiments a non-covalent binding of the N terminus or the full-length ligase to Ub or the HECT domain was observed. In addition, a functional full-length E6AP could not be reconstituted by providing the recombinant N terminus to the catalytic HECT domain (data not shown). This indicates that the respective affinities are probably very low; however, as usually these parts are present on one polypeptide chain, high affinities are dispensable.

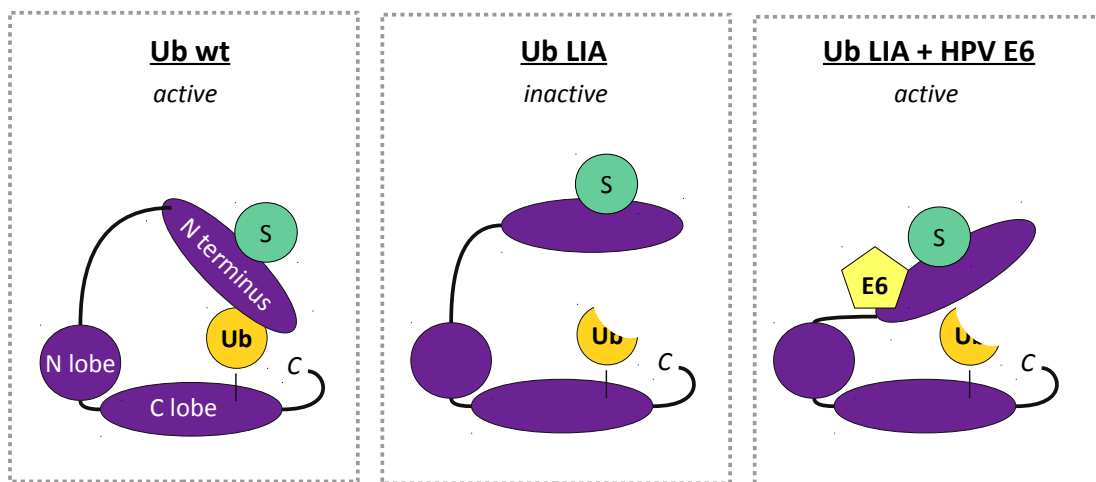


Figure 32: E6AP-mediated substrate ubiquitination may depend on non-covalent contacts between its N terminus and ubiquitin's "canonical" patch. To allow transfer of the thioester-conjugated ubiquitin (Ub, yellow) from the C lobe to a substrate (S, green) which is bound to the N terminus, the C lobe may have to come in closer proximity to the N terminus of E6AP (purple). This active conformation (Ub wt, left side) is stabilized by non-covalent binding of ubiquitin to the N terminus. Since the "canonical" patch of ubiquitin mediates this interaction, its mutation to Ub LIA (middle part) abolishes substrate ubiquitination. The interaction of E6AP with HPV E6 proteins (E6, yellow) causes an active conformation that does not require ubiquitin's "canonical" patch anymore (right side).

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Still it was necessary to analyze the role of E6AP's N terminus in catalysis to understand the role of Ub's "canonical" patch in more detail. The most striking difference was the unique ability of full-length E6AP to rapidly form long Ub chains on itself, which fits to previously published data (Wang and Pickart 2005). Therefore, it was speculated that Ub's "canonical" patch assists in E6AP-mediated Ub chain elongation. This hypothesis was also supported by the fact that this patch is located adjacent to Ub's K48, which is used by E6AP to form Ub chains (Figure 2) (Kim et al. 2007). However, experimental evidence indicated that not only chain formation, but rather any isopeptide bond formation is dependent on the intact "canonical" patch. Along this line, long chains were also formed with Ub LIA, though the reaction was much less efficient than the one with Ub wt (Figure 17).

Furthermore, the N terminus is required for substrate binding as for example, the HECT domain alone does not bind to and ubiquitinate the substrate Ring1b (data not shown). Consequently, the N-terminally bound substrate and the Ub-loaded HECT domain must come in close proximity to enable isopeptide bond formation with a target lysine residue. It can be speculated that in the absence of the intact patch, this "active conformation" is not stabilized due to a disturbed interaction between E6AP's N terminus and the thioester-conjugated Ub in the HECT domain. Effectors like 16E6 may cause a fully active conformation of E6AP that does not depend on the "canonical" patch anymore.

Last but not least there was another difference between the isolated HECT domain and full-length E6AP, which was the mechanism of auto-ubiquitination: while ubiquitin transfer on HECT^{E6AP} occurred exclusively intramolecular, the full-length protein catalyzed – at least in part – an intermolecular attachment (compare Figures 13B and 14). This argues that under the conditions used E6AP forms an oligo- or multimer in solution while the HECT domain alone does not. This is supported by the finding that mutation of HECT's F727 did not alter its auto-ubiquitination ability (data not shown). This phenylalanine is, according to the crystal structure of HECT^{E6AP}, most important for mediating the contact between two HECT domains. However, the authors indicate that this might be an artefact caused by the high protein concentrations needed for crystallization (Huang et al. 1999). In fact, mutation of F727 on either full-length E6AP or HECT alone does not alter the efficiency of the ubiquitination reactions which are catalyzed by E6AP concentrations in the nanomolar range (data not shown).

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If and to what extent Ub's surface patches act in E6AP multimerization is still a matter of debate. However, it can only be speculated if E6AP multimerization is indeed important for ubiquitination of substrates other than E6AP itself and how the multimer is constituted because neither size exclusion chromatography nor coprecipitation experiments revealed any non-covalent interaction between E6AP molecules (data not shown). Recently, data was published, showing that E6AP multimerization is necessary for its activity. In addition, the authors claim that binding of HPV16 E6 increases this multimerization thereby increasing the E3 ligase activity (Ronchi et al. 2014). However, the latter observation contradicts previous findings that E6 leads to *cis*-auto-ubiquitination of E6AP (intramolecular Ub transfer), which would actually indicate that E6 interferes with multimerization (Kao et al. 2000; unpublished observation).

It has to be mentioned that although binding of HERC2 to E6AP also stimulates E6AP's ligase activity and rescues the impairment with Ub LIA, it does not lead to the *cis*-auto-ubiquitination of E6AP as observed with E6 (Kuhnle et al. 2011). In any case, it cannot be excluded that, in the absence of modulators, the "canonical" patch is required for E6AP multimerization and therefore activity, but in the presence of modulators it is not. For example, it seems possible that there is a non-covalent interaction of E6AP's N terminus with the Ub-loaded HECT domain but this interaction does not take place on one polypeptide but rather intermolecular.

To achieve a comprehensive understanding of the mechanism by which Ub's "canonical" patch acts in catalysis, it will be necessary to elucidate the catalytic mechanism of E6AP-mediated isopeptide bond formation in detail. However, this appears to be a difficult task since so far several attempts for crystallization of the full-length protein failed. Furthermore, as E6AP is a comparably large protein (~ 100 kDa), a mutagenesis analysis in the absence of structural data to identify N-terminal residues that are required for the E3 ligase activity would be rather labor-intensive. Nevertheless, a good starting point may be to generate N-terminal truncation versions of E6AP. Investigation of their auto-ubiquitination activity with Ub LIA as well as chain formation ability should then be compared to the full-length E6AP as well as HECT^{E6AP}. Perhaps in this way, the putative non-covalent binding site between Ub-loaded HECT domain and the N terminus could be delineated. To further confirm an interaction, co-immunoprecipitation and size exclusion chromatography experiments may not be suitable as affinities might be rather low. Hence, more sensitive techniques have to be used. Weak interactions (up to K_d values in the millimolar range) can be identified by detection of hydrogen-deuterium exchange reactions via nuclear magnetic resonance- or mass spectrometry-based approaches (Fielding 2007, Wales and Engen 2006). Furthermore, labeling of Ub with a fluorophore and detection of its altered

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fluorescence polarization upon incubation with E6AP fragments may be a suitable method (Lea and Simeonov 2011). Weak and transient interactions can also be stabilized by crosslinking agents (Kluger and Alagic 2004), which might enable the detection of inter- as well as intramolecular interactions between E6AP and Ub. Another possibility is to subject a mixture of e.g. E6AP and Ub to limited tryptic digestion. Trypsin is a protease that cleaves behind lysine and arginine residues. If a cleavage site is not accessible due to the conformation of a protein or shielding by an interactor, the pattern of tryptic peptides is altered (see e.g. Helmich-de Jong et al. 1987).

Finally, the results obtained offer a valuable strategy to identify further modulators of E6AP activity. As E6AP can only inefficiently utilize Ub LIA for ubiquitination, the stimulating effect of an interactor should be more readily detected in the presence of Ub LIA than in the presence of Ub wt. Without any doubt, the search for additional E6AP modulators will be an important step in understanding the role of E6AP within cells.

5.2 Effect of E6AP on cell signaling and transcription

E6AP is a prominent member of the E3 ubiquitin-protein ligase family and contributes to maintaining a functional proteome by targeting proteins for proteasomal degradation. Thus, to understand the physiological role of E6AP a number of studies have focused on the identification of its proteolytic targets, but so far, none of them has been successful in explaining the pathology of AS and ASD (reviewed in Matentzoglou and Scheffner 2008, El Hokayem and Nawaz 2014). Besides E6AP's role in regulation of protein stability by ubiquitination, accumulating evidence indicates that E6AP also affects gene transcription, and thus protein synthesis. Strikingly in this case, the E3 ligase activity of E6AP may be dispensable, which suggests an unexpected role for E6AP in proteostasis (Nawaz et al. 1999, Ramamoorthy and Nawaz 2008).

5.2.1 E6AP affects steroid hormone receptor signaling

We and others have reported that E6AP affects the activity of steroid hormone receptors (SHRs) in transient luciferase reporter assays and on endogenous target genes (Nawaz et al. 1999, Khan et al. 2006, Kuhnle et al. 2013). However, the underlying mechanism is still a matter of debate. In contrast to other reports (Nawaz et al. 1999, Reid et al. 2003, Li et al. 2006, Gao et al. 2005, Khan et al. 2006), we could not neither detect direct binding of E6AP to SHRs nor observe an effect of E6AP on the protein levels of ectopically expressed SHRs (Kuhnle et al. 2013). Another pronounced difference to previous reports is that, under the conditions used in our lab, E6AP acts as a repressor in SHR signaling (Figure 22). Furthermore, as the E3 ligase activity of E6AP is dispensable for its action on the SHRs, at least in overexpression experiments (Nawaz et al. 1999), it seems likely that E6AP is not the E3 ligase which ubiquitinates and degrades SHRs. Thus, we speculated that E6AP affects receptor activity by a rather indirect mechanism.

The activity of SHRs is modulated by a number of different events: binding to their ligand, interaction with cofactors, and post-translational modifications such as phosphorylation (reviewed in Björnström and Sjöberg 2005). The latter came into focus after a survey of the literature which revealed the importance of certain protein kinase cascades (MAPK- and PI3K-dependent pathways) in neurodevelopment (Thomas and Haganir 2004, Acebes and Morales 2012). As they also modulate the activity of SHRs (Leeuw et al. 2011, Björnström and Sjöberg 2005), we hypothesized that E6AP alters SHR signaling by affecting these kinase pathways.

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5.2.2 E6AP modulates ER α via a PI3K-dependent mechanism

To address this possibility, the transcriptional activity of ectopically expressed ER α in H1299 wt and E6APi cells (which harbor a stable knockdown of E6AP expression by RNAi) was studied in transient luciferase reporter assays, in the absence and presence of specific kinase inhibitors. It was postulated that if E6AP affects SHRs via modulation of the MAPK/Erk or PI3K pathways, treatment with respective kinase inhibitors should cause a differential response in H1299 wt compared to E6APi cells.

According to several publications the activity of MEK and its downstream target Erk are in some cases crucial for ER α activity (Kato et al. 1995, Atanaskova et al. 2002). However, in H1299 cells, inhibition of MEK by U0126 caused an increase in ER α transactivation that was not affected by the presence of endogenous E6AP (Figure 23). Subsequently, evidence was obtained that this effect was probably caused by a downstream target of Erk, which upon Erk inhibition became more active and stimulated ER α activity (chapter 4.2.4).

In contrast, inhibition of PI3K by LY294002 caused a decrease of ER α transactivation in a concentration-dependent manner (Figure 23). This decrease was significantly more prominent in E6APi cells than in wt cells. Accordingly, this led to similar luciferase activity values and thus ER α activities in both cell lines when PI3K was completely inhibited. In other words, when PI3K activity is blocked, an effect of E6AP on SHR signaling can no longer be observed. Thus, the effect of endogenous E6AP on the ER α is dependent on PI3K activity and therefore, E6AP might act on ER α by modulation of a PI3K-dependent pathway.

In fact, there is already some direct and indirect evidence from the literature that E6AP affects the PI3K/Akt/mTOR pathway in a positive way, although there is a debate about the underlying mechanism. Zheng et al. claimed that E6AP targets TSC2, a repressor of mTOR, for ubiquitination and degradation. Furthermore, E6AP-mediated TSC2 degradation was stimulated upon binding of the HPV E6 oncoprotein to E6AP (Zheng et al. 2008). In contrast, Spangle et al. showed that TSC2 levels are not affected by E6 expression. According to their data, E6/E6AP might stimulate mTOR through activation of Akt, although the exact mechanism remained unknown (Spangle and Munger 2010, Spangle et al. 2012). At the same time, Srinivasan and Nawaz claimed that E6AP acts as a stimulator of Akt by affecting non-genomic steroid hormone action and by modulating the levels of RhoA (a negative regulator of Akt) (Srinivasan and Nawaz 2011).

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The mechanisms by which the PI3K/Akt pathway modulate the transcriptional activity of ER α are well documented. Phosphorylation of ER α at S167 stimulates its transcriptional activity and is attributed to Akt (upstream of mTORC1) or S6K1 (downstream of mTORC1) activity (Campbell et al. 2001, Yamnik et al. 2009). In addition, phosphorylation at S102/104 and S118 is associated with ER α stabilization and transcriptional activation and mediated by GSK3, a direct target of Akt. However, additional phosphorylation of S106 (by a yet unknown priming kinase) might be necessary for subsequent GSK3-mediated phosphorylation (Medunjanin et al. 2005, Cross et al. 1995). Finally, PI3K can also affect ER α in an Akt-independent manner, although the exact mechanism is unknown (Campbell et al. 2001).

To obtain insight into the mechanism by which PI3K regulates ER α activity in H1299 cells, the effect of inhibition of the PI3K/Akt downstream targets mTORC1 and GSK3 was studied (Figure 24). Treatment with rapamycin, an mTORC1 inhibitor, did not show any effect on ER α transactivation. Thus, S6K1-mediated S167 phosphorylation does not appear contribute to ER α activity in H1299 cells. In contrast, inhibition of GSK3 with CHIR99021 decreased ER α activity, which again led to similar luciferase values in H1299 wt and E6APi cells. Thus, GSK3 may contribute to the effect of E6AP on ER α activity and furthermore, E6AP might act as a negative regulator of GSK3.

These findings were further supported by phospho-mutants of ER α . While mutation of S167 to alanine did not cause any difference in ER α transactivation compared to ER α wt, mutation of S102/104/106 and/or S118 reduced ER α transactivation, which is in agreement with the presumed stimulatory role of GSK3-mediated phosphorylation of these residues (Figure 25). The finding that GSK3 stimulates ER α activity could even explain the previous observation that inhibition of Erk, which is a negative regulator of GSK3, leads to a stimulatory effect in luciferase reporter assays (Figure 23).

The indication that E6AP might affect ER α signaling by repression of GSK3 activity is highly interesting with respect to results obtained in a mouse model of AS. In 2003, Weeber et al. reported that loss of E6AP expression causes reduced CaMKII activity (Weeber et al. 2003). CaMKII acts as a molecular switch in calcium signaling and its persistent activity plays a fundamental role in memory and learning (reviewed in Lisman et al. 2002). Four years later, van Woerden et al. published that the introduction of a constitutive active CaMKII T305V/T306A in AS mice reversed some of the severe defects caused by loss of E6AP expression (van Woerden et al. 2007). So far, it remains elusive how E6AP expression affects CaMKII activity. Nevertheless, it has to be noted that CaMKII is a repressor of GSK3 (Song et al. 2010). Therefore, if loss of E6AP

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expression does indeed cause a higher GSK3 activity, expression of a GSK3 repressor (e.g. CaMKII) can be postulated to rescue some of the cognitive deficits in AS mice. In agreement with this hypothesis, co-expression of CaMKII wt or the constitutive active CaMKII T305/306A mutant reversed the higher ER α activity in H1299 E6APi cells (Figure 26).

5.2.3 E6AP is not in general a negative regulator of GSK3

In view of the data discussed above, E6AP may act as a repressor of GSK3 by activating Akt. Indeed, Western blot analysis reproducibly showed a higher level of phosphorylated Akt and hence increased Akt activity in H1299 wt cells compared to E6APi cells. However, an altered inhibitory phosphorylation status of GSK3 (GSK3 α at S21 and GSK3 β at S9), which is supposed to be mediated by Akt (Cross et al. 1995), was not detectable (Figure 28). In addition, the effect of ectopic E6AP expression on other GSK3 targets was analyzed. The transcriptional activities of AP-1, CREB, and β -Catenin are usually negatively regulated by GSK3 (Sutherland 2011, Nikolakaki et al. 1993, Grimes and Jope 2001, Waltzer and Bienz 1999). Thus, if E6AP acts as a general repressor of GSK3, E6AP overexpression should lead to the stimulation of transactivation mediated by AP-1, CREB and β -Catenin. However, E6AP showed either no or even repressing effects (Figure 30).

This discrepancy is also reflected by the effect of the PI3K inhibitors LY294002 and PTEN (Figures 23 and 29, respectively). Upon treatment with LY294002 or co-expression of PTEN, Akt is inhibited. Thus, a higher activity of GSK3 and ER α should result. But, as mentioned before, the opposite was actually observed. As several publications show a connection between PI3K and MAPK/Erk pathways (reviewed in Mendoza et al. 2011), it was hypothesized that inhibition of PI3K causes an activation of Erk. However, Western blot analysis revealed no difference in Erk phosphorylation status and activity upon PI3K inhibition (data not shown).

It should be noted that the action of GSK3 on a substrate often depends on prior phosphorylation of the substrate by a priming kinase (Sutherland 2011). Thus, it can be speculated that the effect of E6AP on ER α may not, or not only, be mediated at the level of GSK3, but also by another, yet unknown, priming kinase, which may depend on PI3K activity.

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Taken together, the data obtained point to a rather complex mechanism that underlies (A) the effect of the PI3K/Akt/GSK3 pathway on ER α transactivation and (B) the role of E6AP in ER α signaling. At this stage, it is almost impossible to draw a conclusive model that fits all observations. However, the majority of the present findings together with the data published by others strongly suggest that E6AP negatively regulates ER α activity by activating PI3K-dependent pathways (summarized in Figure 33).

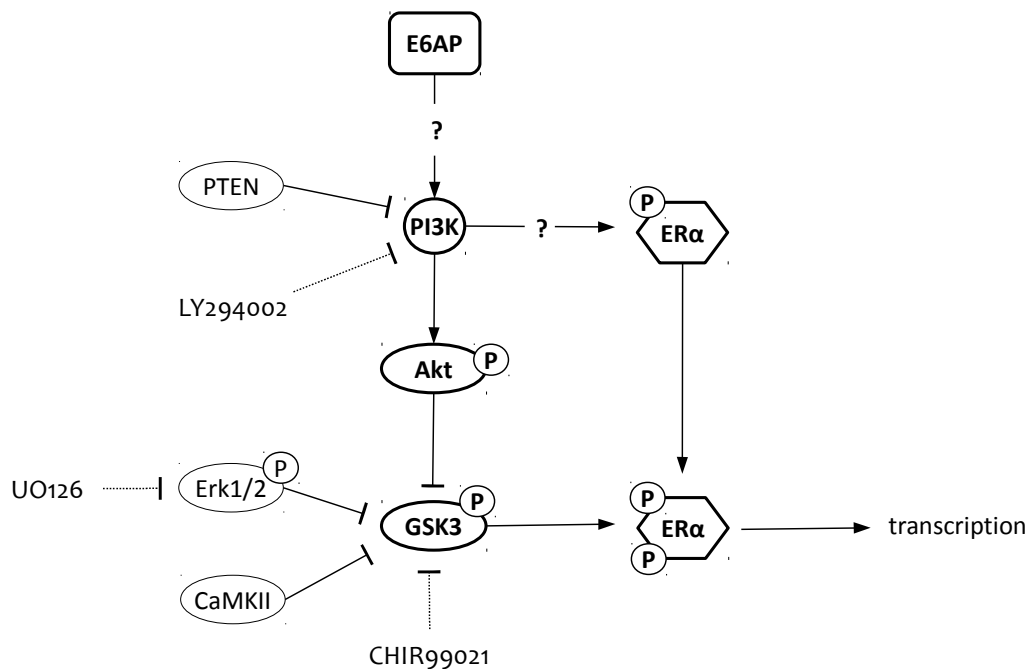


Figure 33: Schematic representation of the mechanism that may underlie the effect of E6AP on ER α transactivation. ER α transactivation is to a large extent dependent on GSK3 activity. Consequently, inhibitors of GSK3 (Erk, CaMKII) reduce the activity of ER α . E6AP acts as a repressor of GSK3 by activating PI3K/Akt. In addition, ER α activity depends on a PI3K-mediated mechanism, which does not act via Akt. It is speculated that PI3K causes a priming phosphorylation of ER α which is a prerequisite for further GSK3-mediated phosphorylation of ER α .

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5.2.4 Concluding remarks and future perspectives

The basic idea at the beginning of this project was to bring several pieces of a puzzle together. Loss of E6AP expression is causally associated with the development of AS (Kishino et al. 1997). However, AS is a comparably rare monogenic disorder with an incidence of approximately 1:25000 to 1:40000 (Mertz et al. 2013, Thomson et al. 2006). Consequently, only a few groups are studying the role of E6AP in neurodevelopment. However, in the past years, accumulating evidence associates E6AP overexpression (due to gene amplification) with ASD (Glessner et al. 2009, Hogart et al. 2010, Urraca et al. 2013). In contrast to AS, ASD is a very complex polygenic disorder with a ~hundredfold higher prevalence than AS (prevalence of ASD: 1:100, Kogan et al. 2009, Levy et al. 2009, Rosti et al. 2014). Thus, there is a huge interest in unraveling the mechanisms causing ASD, and the sheer number of studies (approximately 10-20 publications in PubMed every day) allows to generalize common findings. Amongst these generally accepted observations is that (A) the up-regulation of the Akt/mTOR pathway and (B) the deregulation of MAPK pathways are common and disease-causing events in ASD (Kelleher and Bear 2008, Noh et al. 2013). As E6AP plays a role in AS and ASD, it was speculated that E6AP modulates these protein kinase cascades and that this is a major step in pathogenesis. In a very simplistic view, proteins/pathways that are known to cause ASD (high E6AP expression, high mTOR activity) might be reciprocally affected in AS (low E6AP expression, low mTOR activity). Indeed, there is evidence to support this idea (Khan et al. 2006, Zheng et al. 2008, Srinivasan and Nawaz 2011), and to indicate that the same principle can be applied to HPV-induced carcinogenesis. Here, the HPV E6 oncoprotein might also cause a stimulation of the Akt/mTOR pathway through binding to E6AP (Spangle and Munger 2010, Spangle et al. 2012). Strikingly, we also observed a stimulation of the effect of E6AP on ER α activity upon co-expression of HPV E6 oncoproteins (data not shown).

Currently it can only be speculated that upon knockdown of E6AP expression, reduced activity of Akt also affects the activity of its downstream target GSK3; but without any doubt, GSK3 plays a pivotal role in neurodevelopment (Hur and Zhou 2010) and hence might be involved in the development of AS. However, in this study no effect of E6AP on the inhibitory S21/S9 phosphorylation of GSK3 α/β , which is mediated by Akt (Cross et al. 1995), was observed. This may be due to the remarkable diversity of mechanisms that regulate GSK3 (e.g., kinases other than Akt, subcellular localization, other phosphorylation sites on GSK3, protein complex formation, substrate phosphorylation by a priming kinase – reviewed in Jope et al. 2006) and

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hence the effect of E6AP may become blurred. Most notably it has to be considered that the effect of E6AP on GSK3 may be highly substrate-specific and hence an effect on total GSK3 activity in general may not occur under any condition.

With respect to the effect of E6AP on ER α signaling, the findings made in this work are in some disagreement with published data. While Nawaz et al. (1999) refer to E6AP as an activator of ER α signaling, in our hands E6AP acted as a repressor. The difference in these findings might now be explained by the complex action of E6AP on protein kinase signaling networks, which certainly depends on a number of factors like cell lines and culture conditions.

Nevertheless, it should be noted that the current findings are also indirectly supported by studies carried out with SH-SY5Y cells. Greer et al. found that in AS mice, Arc protein levels are increased, suggesting that Arc may be a substrate of E6AP (Greer et al. 2010). Later it was discovered that this increase in protein levels may be due to estradiol-induced Arc transcription rather than stabilization of the Arc protein – again positioning E6AP as a transcriptional repressor (Kuhnle et al. 2013). Moreover, exactly this estradiol-induced Arc transcription was reported to depend on intact MAPK- and PI3K-dependent pathways (Chamniansawat and Chongthammakun 2009). However, it remains to be clarified to what extent GSK3 contributes to Arc transcription in SH-SY5Y cells.

Therefore, further studies to understand the role of E6AP in ER α signaling should not deal with the steroid receptor itself but rather take a closer look at protein kinase signaling pathways. These may be the actual targets of E6AP and may account for many observations that the experimenter will make when studying the effect of E6AP in a cellular context. However, this will not be a simple task, and data obtained will have to be interpreted with caution because: (A) PI3K is a key regulator of both translation and transcription, so its modulation causes quite a number of different downstream events; (B) PI3K as well as MAPK pathways are often deregulated in cancer cells and contribute to carcinogenesis (e.g. H1299 cells contain mutated NRAS which may cause a constitutive active MAPK/Erk pathway - Sunaga et al. 2011); (C) Methods available to study the receptors that trigger PI3K and MAPK *in vitro* or *in cellulo* are limited, biased, and cost intensive. If further work relies on Western blot analysis with phospho-specific antibodies, one has to be aware of the fact that under normal culture conditions possible effects may be too small to be detected. More drastic conditions (serum or hormone starvation) may need to be applied to lower the "baseline" of all signaling events.

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It remains to be determined how E6AP causes the stimulation of Akt/mTOR. Regarding this issue, the data generated in this work do not allow a decisive conclusion. However, attention should be drawn on the **non-genomic action of steroid hormones** (Figure 34A). This action is based on the activation of protein kinase signaling cascades by membrane-associated steroid receptors or GPR30 (Losel et al. 2003, Pietras and Márquez-Garbán 2007, Maggiolini and Picard 2010). According to Zhang et al. (2008), the non-genomic signaling also takes place in H1299 cells. This cell line, which was mainly used in this thesis, expresses a form of ER β that does not drive transcription from estrogen response elements but presumably causes rapid activation of MAPK and PI3K pathways after estradiol treatment. It can be speculated that E6AP affects this non-genomic signaling leading to the observed activation of PI3K/Akt, as it has already been suggested by Srinivasan and Nawaz (2011). Interestingly, co-expression of ectopic ER α with an androgen-responsive reporter caused a significant increase of transcription of the reporter construct in H1299 cells, which may depend on the non-genomic activation of the endogenous androgen receptor (data not shown).

Another attractive possibility is the involvement of **RhoA as a mediator of PI3K/Akt modulation** (Figure 34B). RhoA is known to stimulate PTEN activity thus counteracting the PI3K/Akt pathway (Li et al. 2005). However, there are also other mechanisms implicated in the cross-talk between RhoA and PI3K pathways, and some even refer to RhoA being an activator of Akt (Reuveny et al. 2004, Del Re, Dominic P. et al. 2008). Khan et al., as well as Srinivasan and Nawaz, have reported that loss of E6AP expression is accompanied by increased RhoA levels and activity. They speculate that E6AP activates Akt by decreasing RhoA levels. How E6AP affects RhoA is unknown (Khan et al. 2006, Srinivasan and Nawaz 2011); but interestingly, RhoA repressed androgen receptor-mediated transcription (Khan et al. 2006), and similarly inhibition of RhoA resulted in stimulation of ER α -mediated transcription in H1299 cells (data not shown). Finally, it should to be noted that some reports indicate a connection between **E6AP and the oxidative stress response** (Figure 34C). In 2010, Nasu et al. found that E6AP expression causes reduction of Prx1 levels. Prx1 is involved in clearance of reactive oxygen species (ROS), and hence loss of E6AP reduced ROS accumulation (Nasu et al. 2010, Wolyniec et al. 2012). Accordingly, the presence of E6AP may increase the levels of ROS, and ROS are known to stimulate the PI3K/Akt pathway (reviewed in Martindale and Holbrook 2002). However, this may be a rather bold hypothesis. In any case, further studies regarding the role of E6AP in cell signaling will bring more pieces of the puzzle together.

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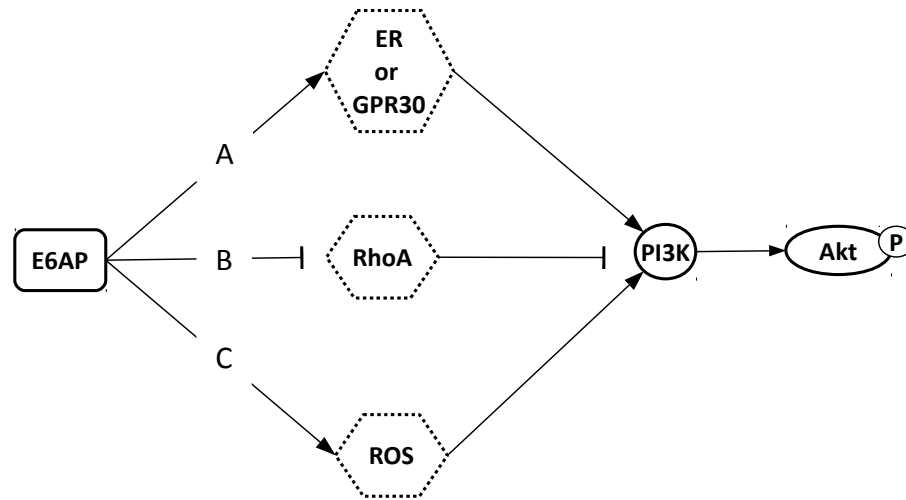


Figure 34: Hypothetical mechanisms by which E6AP may activate the PI3K/Akt pathway. (A) E6AP might stimulate the non-genomic action of steroid hormones. Subsequent activation of membrane-bound ER or GPR30 causes activation of PI3K. (B) E6AP may negatively regulate RhoA. Reduced RhoA activity causes an increase of PI3K-mediated signaling. (C) Expression of E6AP may cause accumulation of reactive oxygen species (ROS). ROS stimulate the activity of PI3K.

6. References

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7. Acknowledgements

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