Insights into the Tumor Suppressor p53:
Physiological Function and Proteolytic Regulation

Dissertation submitted for the degree of
Doctor of Natural Sciences

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Date of the oral examination: 22.03.2017

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Abbreviations

Units:
- m milli- \((10^{-3})\)
- μ micro- \((10^{-6})\)
- n nano- \((10^{-9})\)
- l liter
- g gram
- M molar
- sec second
- min minute
- bps base pairs
- cm centimeter
- kDa kilodalton

Hdm2 Human Mdm2
Hdm2_RING Isolated RING domain of Hdm2
HPV Human papilloma virus
IRES Internal ribosome entry site
Mdm2 Murine double minute 2
Ned8 Neural precursor cell-expressed developmentally down-regulated 8

p14ARF Human ARF
Puro\(^8\) Puromycin resistance gene
RING Really interesting new gene
RNA Ribonucleic acid
RNAi RNA interference
SDS-PAGE Sodium dodecyl sulfate polyacrylamide gel electrophoresis

Others:
- 16E6 HPV16 early protein 6
- 11E6 HPV11 early protein 6
- ARF Alternate reading frame
- BSA Bovine serum albumin
- Cas CRISPR-associated protein
- CRISPR Clustered regularly interspaced short palindromic repeats
- DMSO Dimethyl sulfoxide
- DNA Deoxyribonucleic acid
- E1 Ubiquitin-activating enzyme
- E2 Ubiquitin-conjugating enzyme
- E3 Ubiquitin ligase
- E6AP E6 associated protein
- GFP Green fluorescent protein
- GST Glutathione S-transferase
- p14ARF Human ARF
- Puro\(^8\) Puromycin resistance gene
- RING Really interesting new gene
- RNA Ribonucleic acid
- RNAi RNA interference
- SDS-PAGE Sodium dodecyl sulfate polyacrylamide gel electrophoresis
- sgRNA Single guide RNA
- SUMO Small ubiquitin-like modifier
Abstract

The tumor suppressor protein p53 is a stress-inducible transcription factor that regulates various cellular processes, including cell cycle arrest, senescence, apoptosis, DNA repair and energy metabolism. Owing to its growth-suppressive properties, p53 is maintained at a low steady-state level under normal cellular conditions via rapid proteasomal degradation. In most human cancers, p53 functions are inactivated by either mutation of the TP53 gene or several alternative mechanisms. In this work, we investigated the tumor suppressor p53 concerning its physiological function and proteolytic regulation.

Previous data in our laboratory indicated that HPV (human papillomavirus)-positive cancer cells cannot survive complete abrogation of p53 expression, implying that p53 may possess a pro-survival function. We accordingly intended to examine this phenomenon in HPV-negative cell lines. To monitor the bona fide effect of p53 deficiency on cell proliferation, we designed a bicistronic expression system whereby the antibiotic resistance of cells faithfully reflects the simultaneous RNA interference-mediated abrogation of p53 expression. However, this system failed to express the resistance marker protein at sufficient levels although knockdown of p53 expression was observed. Alternatively, via the CRISPR-Cas9 approach, we obtained cells harboring a knockout of the TP53 gene without significant impairment of cell growth, suggesting that expression of p53 proteins are not required for proliferation of the HPV-negative osteosarcoma cell line, U-2 OS.

Proteasome-mediated degradation of p53 relies on the covalent attachment of the small protein ubiquitin, termed ubiquitination, which is mainly catalyzed by the ubiquitin ligase Mdm2. This process is negatively regulated by the tumor suppressor protein ARF. In an attempt to delineate the underlying biochemical mechanism by which ARF inhibits Mdm2-mediated p53 ubiquitination, we performed a series of in vitro assays and corroborated that amino acids 2-14 of human ARF play a crucial role in the direct inhibition towards Mdm2 activity. Our data also imply a previously undescribed ARF binding site within the C-terminal domain of human Mdm2. According to the evidence obtained, we propose that ARF interferes with Mdm2-mediated p53 ubiquitination by blocking the ability of Mdm2 to facilitate ubiquitin transfer onto p53.

The development of cervical cancer is associated with p53 inactivation. HPV-related cervical cancers express wild-type p53 proteins, however, with basal levels attenuated by the expression of the HPV E6 oncoprotein. As currently available screening methods of cervical cancer suffer disadvantages of specificity or sensitivity, we also set out to develop a tool for clinical diagnosis that directly detects the expression of the HPV E6 oncoprotein. Although the developed fluorescence-based tool exhibits the potential to detect the HPV E6 oncoprotein, low specificity renders it unsuitable for reliable diagnosis of cervical cancer.
Zusammenfassung


Die Entwicklung des Zervixkarzinoms geht mit einer Inaktivierung von p53 einher. Durch HPV-verursachte Zervixkarzinome exprimieren ein Wildtyp-p53-Protein, das jedoch durch die Expression des HPV E6-Onkoproteins auf einem niedrigen Level gehalten wird. Da derzeit
verfügbare Screeningmethoden für Zervixkarzinome Nachteile entweder in der Spezifität oder in


der Sensitivität aufweisen, sollte in dieser Arbeit eine Methode für die klinische Diagnose


devontwickelt werden, die die Detektion des HPV E6-Onkoproteins auf einem direkten Weg


ermöglicht. Die Fluoreszenz-basierte Methode zeigte zwar das Potenzial, das HPV E6-Onkoprotein


tzdetektieren, eine geringe Spezifität macht sie in dieser Form für eine Diagnose von


Zervixkarzinomen allerdings unbrauchbar.
1 Introduction

Tumor suppressor genes prevent normal cells from tumor formation via regulation of various cellular pathways, including cell cycle, DNA damage repair, apoptosis and cell migration [1]. Inactivation or loss of these genes liberates cells from such growth-suppressive functions, leading to uncontrolled cell growth, which is one of the hallmarks of tumorigenesis.

The p53 tumor suppressor protein is a DNA sequence-specific transcriptional regulator that controls the expression of numerous genes in response to various cellular stresses. In 1979, p53 was first described in complexes with the viral oncoprotein SV40 T-antigen [2-4]. Because of its high levels in many tumor-derived cells but not normal cells, p53 was initially considered as a proto-oncoprotein (reviewed in [5]). A decade after its discovery, p53 was finally characterized as a tumor suppressor in 1989 [6-8]. Owing to its crucial role in tumor suppression, p53 has become one of the most intensively studied proteins in cancer research nowadays.

1.1 Physiological functions of p53

1.1.1 Structural composition of p53

The human p53 protein is encoded by the TP53 gene, which is located on the short arm of chromosome 17 (17p13.1) [9]. Full-length p53 consists of 393 amino acids and migrates with an apparent molecular mass of 53 kDa on SDS-PAGE. It is structurally characterized by several functional domains and regions (Figure 1).

Figure 1 Schematic structure of human p53 protein

The human p53 protein is composed of four structural and functional components, including the N-terminal transactivation domain followed by a proline-rich region (residues 1-92), the central DNA binding domain (residues 101-292), the C-terminal tetramerization domain (residues 326-356) and the regulatory region (residues 363-393).

The N-terminal region of p53 represents the transactivation domain (TAD), which is further subdivided into TADI (residues 1-42), TADII (residues 43-63) and a proline-rich region (residues 64-92) [10, 11] (in some cases, TADII is also referred to encompass residues 43-92 [12]). Moreover, the N-terminal region is intrinsically unstructured [13, 14], which facilitates the interaction of p53 with various proteins, including components of the transcription machinery, transcriptional
coactivators and regulators of p53 (reviewed in [15]).

Preceded by the N-terminal TAD, central residues 101-292 of p53 comprise the DNA binding domain (DBD, also called p53 core domain), which provides the basic scaffold for the DNA-binding interface. Six key residues in the DBD mediate the direct contact with DNA: Lys120, Ser241, Arg248, Arg273, Ala276, Cys277 and Arg280 [16, 17]. As DNA binding is essential for the transactivation function of p53, it is conceivable that mutations at these sites are associated with tumorigenesis (see below in 1.3.1). Furthermore, the consensus sequence for p53 binding, or the p53 response element, is widely found upstream of p53 target genes and is composed of two decamers that are separated by a spacer as follows: RRRCWGWYY…n…RRRCWWGWYY (where R is a purine, Y is a pyrimidine, W is an A or T and the spacer consists of 0-13 bases) [18]. The C-terminal 100 residues of p53 include the tetramerization domain (residues 326-356) and the regulatory region (residues 363-393). Through the tetramerization domain, p53 forms a tetrameric structure that refers to a “dimer of primary dimers”. Importantly, although the DBD directly contributes to the p53-DNA interface, the formation of a tetramer is another prerequisite for efficient DNA binding (reviewed in [19]). In addition, p53 tetramerization is also essential for its proteasomal degradation via Mdm2 [20, 21] (see below in 1.2). At the extreme C terminus, the regulatory region contains extensive sites for post-translational modifications, including acetylation, ubiquitination, phosphorylation, sumoylation, methylation and neddylation, which are relevant to the regulation of p53 functions and cellular protein levels (reviewed in [22]).

1.1.2 p53 functions as a transcription factor

The predominant physiological function of p53 is to regulate the expression of numerous genes, which are mostly involved in cellular pathways in response to stress signals. The sequence-specific binding of p53 to its response elements facilitates promoter opening and/or recruitment of the transcription machinery (reviewed in [23]), and thereby transcriptionally regulates cell cycle arrest, senescence, DNA repair, apoptosis or cellular energy metabolism.

1.1.2.1 Cell cycle arrest and senescence

It is commonly assumed that once cells suffer mild cellular stresses, p53 activates the transcription of the CDK (cyclin-dependent kinase) inhibitor $p21^{Cip1}$ gene (reviewed in [24]). Together with cyclin proteins, CDKs regulate the progression of cell cycle [25]. Through the interaction with cyclin-CDK complexes, $p21$ inhibits their kinase activity and blocks cell cycle progression, which allows cells to survive safely until the cellular damage has been resolved or the stress has been removed. In addition, activation of several p53 target genes such as 14-3-3 Sigma and GADD45 (growth arrest and DNA damage) also contribute to cell cycle arrest (reviewed in [24]). In case of irremovable cellular damage or high levels of sustained stress, p53 induces senescence, an irreversible cell cycle arrest, to prevent formation of malignant cells. Knockout of $p21^{Cip1}$ in
human fibroblasts extends their lifespan in culture, indicating this p53 target gene also plays a role in senescence [26]. Furthermore, PAI-1 (plasminogen-activator inhibitor 1) and YPEL3 (Yippee-like-3) have been recently identified as p53 target genes that induce senescence [27, 28].

1.1.2.2 Apoptosis
The ultimate weapon of p53 to prevent cancer development is the induction of programmed cell death, apoptosis. In the intrinsic pathway of apoptosis, members of the Bcl-2 family govern the release of cytochrome c from mitochondria and the activation of caspase-9, which promotes the apoptotic cascade and leads to the final destruction of the cell [29]. Many genes involved in this pathway are transcriptionally regulated by p53. First, p53 activates the expression of pro-apoptotic members in the Bcl-2 family such as BAX, BID, NOXA, and PUMA. In contrast, the expression of anti-apoptotic proteins, including BCL-2 and BCL-XL, are repressed by p53 (reviewed in [30]). Of note, p53 also enhances apoptosis in a transcription-independent manner. Via protein-protein interaction, p53 directly activates certain pro-apoptotic proteins and neutralizes the anti-apoptotic effects of BCL-2 and BCL-XL (reviewed in [31]).

1.1.2.3 DNA repair
Depending on the type of DNA damage, eukaryotic cells exert different DNA-repair processes, virtually all of which are positively regulated by p53 in both transcription-dependent and -independent manners (reviewed in [32, 33]). Moreover, p53 transcriptionally induces the expression of p53R2, a ribonucleotide reductase subunit, upon DNA damage [34]. The ribonucleotide reductase catalyzes the formation of deoxyribonucleotides from ribonucleotides and thus plays a crucial role in the regulation of DNA synthesis during cell division and DNA repair [35].

1.1.2.4 Cellular energy metabolism
Recent evidence has expanded p53 functions to the control of cellular energy metabolism. Through the action of AMP-activated protein kinase (AMPK), which senses reductions in the ATP:AMP ratio, p53 is activated by the cellular adversity of low energy [36]. Activated p53 then promotes a series of gene expression events, including transcription of the AMPK gene to negatively regulate the kinase mTOR (mammalian target of rapamycin) [37, 38]. As mTOR is the central node to coordinate cell growth via sensing nutrient availability and growth factor signaling [39], p53-mediated regulation of mTOR provides an alternative mechanism to prevent cell growth in response to metabolic stresses.

The role for p53 in response to metabolic stress is also consolidated by its ability to activate autophagy, a membrane trafficking process that mediates the delivery of cellular components to the lysosomal system for digestion [40]. Under starvation conditions, autophagy-mediated recycling of cellular components promotes the short-term survival of cells. As autophagy is
inhibited by mTOR [41], p53 indirectly activates autophagy via the negative regulation of mTOR signaling. Moreover, p53 also transcriptionally induces the expression of DRAM (damage-regulated autophagy modulator) to stimulate autophagy [42].

Virtually all cancer cells predominantly produce energy via a high rate of glycolysis followed by lactic acid fermentation in the cytosol instead of the oxidative phosphorylation in mitochondria, termed Warburg effect [43]. However, the intricate roles of p53 in the regulation of metabolic pathways appear to oppose to the Warburg effect. In general, p53 limits the conversion of glucose into pyruvate by blocking glucose uptake [44-46] and inhibiting the glycolytic flux [47, 48]. Furthermore, p53 enhances oxidative phosphorylation in mitochondria by transcriptional activation of SCO2 gene (synthesis of cytochrome c oxidase 2), which is a regulator of complex IV in the electron transport chain [49]. Additionally, the expression of subunit 1 of complex IV per se is also transcriptionally activated by p53 [50].

1.1.3 Isoforms of p53

In addition to the canonical full-length p53 protein (p53α), a number of p53 isoforms have been reported to be expressed by TP53 via different mechanisms, including alternative splicing of mRNA, alternative initiation of translation and/or alternative promoter usage (Figure 2, reviewed in [51]). TP53 comprises 11 exons (Figure 2 A). Complete excision of intron 9 generates the so-called α isoforms. In contrast, a partial retention of intron 9 via alternative splicing, also termed exon 9b or exon 9g, leads to the translation of the β and γ isoforms. Of note, the C-terminal region of α isoforms encoded by exon 10 and exon 11 is replaced by 10 and 15 new amino acids in β and γ isoforms, respectively, due to the presence of a stop codon in exon 9b and exon 9g (Figure 2 B).

Transcription of TP53 is driven by either the distal (P1) or the internal promoter (P2) (Figure 2 A). While the proximal promoter controls the expression of p53 (α, β, γ) and Δ40p53 (α, β, γ) variants, the internal promoter regulates the expression of Δ133p53 (α, β, γ) and Δ160p53 (α, β, γ) isoforms (Figure 2 B). Moreover, alternative initiation of translation determines the N-terminal variation of isoforms that are translated from the same transcript. In other words, this mechanism plays a decisive role in the expression of Δ40p53 (α, β, γ) and Δ160p53 (α, β, γ) isoforms.

1.1.3.1 p53β and p53γ

Owing to the truncation of the C-terminal tetramerization domain, the ability of p53β and p53γ to bind to DNA is impaired. In a p53-null cellular environment, transfection of p53β alone has no effect on p21 or Bax promoter transactivation. However, p53β and full-length p53 have been shown to form a protein complex, which enhances the transactivation activity of full-length p53 towards the Bax promoter and consequently apoptosis. Thus, p53β might function as a modulator to regulate the transcriptional activity of full-length p53 [52]. The physiological functions of p53γ remain poorly understood.
Alternative initiation of translation at codon AUG40 leads to the expression of Δ40p53 isoforms. This is mediated by the internal ribosome entry site (IRES) in the p53 mRNA upstream of AUG40, which recruits ribosomes and allows 5’ cap-independent internal initiation of translation [53]. Δ40p53α suppresses the transcriptional activity of full-length p53 and impairs p53-mediated growth suppression in a dominant-negative manner [54, 55]. In addition, Δ40p53α influences p53 ubiquitination and subcellular localization [55]. Compared to Δ40p53α, little is known about Δ40p53β and Δ40p53γ.

Figure 2 TP53 gene expresses 12 distinct p53 isoforms

(A) Composition of TP53 gene. TP53 comprises 11 exons (shown in boxes) and encodes at least 12 different isoforms via alternative splicing of mRNA, alternative initiation of translation and/or alternative promoter usage. Alternative splicing of mRNA in intron 9 (indicated by “^”) generates α, β and γ isoforms. Non-canonical exon 9b and exon 9g are shown as light and dark orange boxes, respectively. Alternative translation initiation sites (ATG) are indicated. The distal promoter (P1) and the internal promoter (P2) in intron 4 are denoted by arrows. Gray boxes indicate sequences of TP53 representing untranslated regions of p53 mRNA. Adapted from [51].

(B) Human p53 isoforms. p53α refers to full-length p53. TAD: transactivation domain; DBD: DNA binding domain; TD: tetramerization domain; CR: C-terminal regulatory region. Numbers represent the residue positions of full-length p53. The respective C-terminal amino acids encoded by exon 9b and exon 9g are indicated in the one-letter code.
1.1.3.3 Δ133p53 and Δ160p53 isoforms

Expression of Δ133p53 and Δ160p53 isoforms is controlled by the internal promoter in intron 4 of TP53. Translation of Δ133p53 and Δ160p53 is initiated at codon AUG133 and codon AUG160, respectively. Δ133p53α regulates the transcriptional activity of full-length p53, but in a target gene-dependent manner [56-58]. Of note, Δ133p53α was shown to stimulate angiogenesis, suggesting an active role of this isoform in tumorigenesis [59]. Unlike Δ133p53α, cellular roles of Δ133p53β, Δ133p53γ and also Δ160p53 isoforms remain unclear.

1.2 Regulation of p53 proteolysis via ubiquitin

Cells constitutively express full-length p53 to enable an instant response to cellular stresses. On the other hand, high expression levels of full-length p53 would activate undesired cell cycle arrest or even apoptosis in the absence of cellular insults. Thus, full-length p53 has to be maintained at a low steady-state level under normal cellular conditions, which is accomplished by post-translational modification with ubiquitin and subsequent proteasome-mediated degradation. This pattern of synthesis followed by immediate degradation might appear to be a futile and wasteful cycle for cells. However, it circumvents the time-consuming de novo protein synthesis starting at the DNA level and rapidly increases protein levels of full-length p53 by blocking degradation in response to cellular stresses. In the following, only full-length p53 is discussed.

1.2.1 Ubiquitin-proteasome system

1.2.1.1 Ubiquitination cascade

Ubiquitin is a globular protein of 76 amino acids, which is highly conserved among eukaryotes but not present in bacteria and archaea. The covalent attachment of ubiquitin to other proteins via its C terminus, termed ubiquitination, is an abundant post-translational modification involved in various cellular pathways. This process occurs sequentially in three steps, which are catalyzed by three different types of enzymes, respectively (Figure 3) [60, 61].

In the first step, the ubiquitin-activating enzyme (E1) activates ubiquitin in an ATP-consuming manner. This reaction starts with the formation of a ubiquitin-adenylate intermediate followed by the transfer of ubiquitin to the active cysteine residue of E1. This results in a high energy thioester linkage between the C-terminal carboxyl group of ubiquitin and the cysteine sulfhydryl group of E1. A ubiquitin-conjugating enzyme (E2) then accepts the activated ubiquitin from the E1 by forming a thioester linkage on its catalytic cysteine residue, termed “transthioesterification.” In the last step, ubiquitin is transferred from the E2 to the ε-amino group of a lysine residue on the substrate, forming an isopeptide bond, with the assistance of a ubiquitin ligase (E3).
In the ubiquitination cascade, E3 ligases account for the specific recognition of substrates. According to their structural and mechanistic features, E3 ligases can be divided into three classes: HECT, RING and RBR E3 ligases. HECT (homologous to E6AP carboxyl terminus) E3 ligases first accept ubiquitin from a cognate E2 enzyme by formation of a thioester linkage with ubiquitin through the catalytic cysteine residue in their conserved HECT domain and then catalyze the transfer of ubiquitin to the substrate. In contrast, RING E3 ligases serve as scaffolds that bring E2 and substrate into close proximity. RBR E3 ligases share common features with both HECT and RING E3 ligases. The RING1 domain facilitates thioester formation between ubiquitin and RING2. Ubiquitin is then transferred to the substrate. E3 ligases are denoted in green. Circles in dark green represent respective catalytic domains of each type E3 ligase. Ub: ubiquitin; E1: ubiquitin-activating enzyme; E2: ubiquitin-conjugating enzyme; E3: ubiquitin ligase; HECT: homologous to E6AP carboxyl terminus; RING: really interesting new gene; RBR: RING-in-between-RING.

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transfer of ubiquitin to the substrate [62]. In contrast, RING (really interesting new gene) E3 ligases contain a conserved E2 binding RING domain (or a RING-containing subunit in case of multi-subunit RING E3 complexes) and have been long considered as adaptors to assist in the E2-catalyzed isopeptide bond formation [62]. Notably, it has been recently reported that RING ligases allosterically facilitate the release of ubiquitin from the E2, suggesting their “enzymatic” relevance to ubiquitination in addition to substrate recognition [63]. Thirdly, RBR (RING-in-between-RING) E3 ligases are the most recently identified E3 class, which possess a highly conserved catalytic unit consisting of a RING1, an IBR (in-between RING) and a RING2 domain [64]. They share common features with both HECT and RING E3 ligases. The RING1 domain interacts with cognate ubiquitin-loaded E2s in a manner similar to RING E3 ligases, facilitating the HECT-like thioester formation between ubiquitin and RING2. Ubiquitin is then transferred from the RBR E3 to the substrate.

1.2.1.2 “Fates” of ubiquitinated proteins

The mode of ubiquitination determines the eventual fate of ubiquitinated proteins. Substrate proteins can be modified with a single ubiquitin molecule on one (mono-ubiquitination) or several lysine residues (multiple mono-ubiquitination). These types of ubiquitination regulate various cellular processes, including protein localization, DNA damage response, endocytosis, and transcriptional regulation, in a non-proteolytic manner (reviewed in [65]).

Moreover, ubiquitin chains comprising four or more ubiquitin molecules can be assembled and attached to a single lysine residue on the substrate (poly-ubiquitination). Ubiquitin contains seven lysine residues (K6, K11, K27, K29, K33, K48, and K63). All of these lysine residues can be used for isopeptide bond formation with the C-terminal carboxyl group of another ubiquitin molecule, resulting in the assembly of differently linked ubiquitin chains [66]. The pattern of lysine linkage within the ubiquitin chain contributes to the chain structure and the functional consequence. K11-linked ubiquitin chains target proteins for proteasome-mediated degradation and have been associated with cell cycle regulation [67] as well as endoplasmic reticulum-associated degradation (ERAD) [68], a protein quality control system of the ER. K48-linked chains serve as the canonical degradation signal that is recognized by the 26S proteasome (see below) and leads to proteolysis of the modified substrate [69, 70]. K63-linked ubiquitin chains are involved in cellular processes, such as DNA repair, intracellular signaling, and endocytosis, in a non-proteolytic manner [71-73]. The functional relevance of other ubiquitin chain types are only poorly understood.

1.2.1.3 Proteasome-mediated protein degradation

The 26S proteasome degrades 80-90% of intracellular proteins [74]. In eukaryotes, this multi-subunit protease complex consists of a 20S core particle and two 19S regulatory particles. The 20S core particle is a cylindrical chamber that exhibits caspase-like, trypsin-like and chymotrypsin-like proteolytic activities. The 19S regulatory particles are lid-like complexes that cover both sides of
the 20S core particle, and fulfill recognition of ubiquitinated substrates, removal of ubiquitin from
the substrate as well as substrate unfolding. In the process of proteasome-mediated protein
degradation, 19S regulatory particles first interact with the ubiquitinated substrate. Deubiquitinat-
ing enzymes (see below) and ATPase subunits of the regulatory particles then remove ubiquitin and unfold the substrate, respectively. Moreover, ATPase subunits also enhance
the opening of the gated 20S core particle, which facilitates the translocation of the substrate into
the 20S core particle. Eventually, protease subunits in the core particle accomplish the hydrolysis
of the peptide bonds of the substrate (reviewed in [75, 76]).

1.2.1.4 Deubiquitinating enzymes
Like other post-translational modifications such as phosphorylation, ubiquitination is also a
reversible process. Removal of ubiquitin from the substrate, termed deubiquitination, is catalyzed
by deubiquitinating enzymes (DUBs). Via this process, DUBs antagonize the functional
consequence of ubiquitination. Another function of DUBs is the activation of ubiquitin precursor
proteins. Within cells, ubiquitin is expressed as a fusion protein that consists either of multiple
copies of mono-ubiquitin “head-to-tail” linked by peptide bonds or of ubiquitin fused to the N
terminus of ribosomal subunits [77-79]. DUBs recognize the two C-terminal glycine residues of
ubiquitin and cleave the peptide bond between the C-terminal glycine of ubiquitin and the
following amino acid residue, resulting in free and mature ubiquitin. Moreover, DUBs are also
involved in other cellular functions such as ubiquitin recycling, proteasome-mediated degradation,
DNA repair and endocytosis (reviewed in [80]). Intriguingly, although bacteria do not possess
ubiquitin or a ubiquitin homolog with respect to sequence similarity, an E. coli protein, ElaD, has
been identified as a protease that exhibits efficient and specific DUB activity [81].

1.2.1.5 Ubiquitin-like proteins
Proteins that resemble ubiquitin in three-dimensional structure and the mode of attachment to
other proteins are defined as ubiquitin-like proteins (UBLs). In mammals, about 20 members have
been identified in the UBL family such as Nedd8 (neural precursor cell-expressed developmentally
down-regulated 8) and SUMO (small ubiquitin-related modifier). Similar to ubiquitination,
modifications by UBLs are accomplished by sequential enzyme cascades, but by using their
respective E1s, E2s and E3s. Furthermore, the respective modifications are also reversible by the
action of deconjugating enzymes. Within cells, UBLs are involved in the regulation of various
processes, including protein degradation, protein localization, autophagy, immune response and
development (reviewed in [82-84]).
1.2.2 Mdm2-mediated p53 ubiquitination

The human genome encodes two E1s, about 40 E2s and more than 500 putative E3 ligases [66]. This hierarchical order of enzymes provides not only a tight regulation of the ubiquitination reaction but also the specific recognition of a large substrates spectrum. Moreover, a defined E3 ligase can have several different substrates and vice versa. For instance, p53 has been described to be ubiquitinated by more than a dozen of different E3 ligases. These E3 ligases can catalyze different modes of p53 ubiquitination, namely mono- or poly-ubiquitination, leading to various regulatory consequences of p53, including proteasomal degradation and changes in cellular localization (reviewed in [85]).

1.2.2.1 Mdm2

Among all reported E3 ligases for p53, Mdm2 (murine double minute 2) is the principal endogenous regulator of p53 and tightly controls p53 levels [86-88]. Mdm2 is a member of the RING E3 ligase family and contains several structural domains (Figure 4). The N-terminal domain contributes to the interaction with the transactivation domain of p53 (Figure 1) [89]. The central acidic domain is regarded as a regulatory domain. Being intrinsically unstructured, this region serves as a flexible docking site of numerous proteins that regulate Mdm2 activity (reviewed in [90]), including the negative regulator ARF (see below in 1.2.3). In addition, the acidic domain also provides a second binding site for p53 by interaction with the DNA binding domain (Figure 1) [91]. The C-terminal RING domain represents the E2 binding site and is thus necessary for the transfer of ubiquitin from cognate E2s to p53. Moreover, dimerization of Mdm2 through the RING domain is a prerequisite for E3 activity [92, 93]. Mdm2 also contains nuclear localization and the nuclear export signals, which enable Mdm2 to shuttle between the cytoplasm and the nucleus to execute ubiquitination of nucleoplasmic p53. Finally, the acidic domain precedes the zinc binding domain of so far unknown function. Apart from p53, Mdm2 also ubiquitinates itself and other proteins such as MdmX and Numb [94-96]. Furthermore, Mdm2 is also known to catalyze the modification of p53 by the UBL Nedd8 [97].

Figure 4 Schematic structure of human Mdm2 protein

Human Mdm2, or Hdm2, is composed of the N-terminal p53 binding domain (residues 18-101), the central acidic domain (residues 237-288) and the C-terminal RING domain (residues 436-482). Gray boxes represent the nuclear localization signal (residues 178-185), the nuclear export signal (residues 191-199) and the zinc binding domain (residues 289-331). The human ARF, or p14ARF, binding site (residues 210-244) is shown as a blue bar.
1.2.2.2 p53-Mdm2 feedback loop

Under normal cellular conditions, Mdm2 catalyzes p53 ubiquitination, thereby targeting it for proteasomal degradation. Importantly, mdm2 is also a target gene that is activated by p53. Hence, p53 and Mdm2 act in a negative feedback loop to maintain low levels of p53 [98]. When cells suffer cellular stresses, post-translational phosphorylation initiates the stabilization of p53. For instance, expression of various kinases such as ATM, ATR, DNA-PK, Chk1 and Chk2 are induced in response to DNA damage. These kinases phosphorylate p53 at Ser15 and Ser20 within the N-terminal transactivation domain [99-101] and thereby disrupt p53-Mdm2 interaction. Moreover, phosphorylation of Mdm2 by ATM at Ser395 within the RING domain impairs the E3 ligase activity of Mdm2 and prevents p53 ubiquitination [102]. A similar mechanism has also been described for metabolic stresses. As mentioned in 1.1.2.4, reduced nutrient or energy levels activate AMPK. This kinase then phosphorylates p53 at Ser15, leading to p53-Mdm2 disassociation and consequently, p53 accumulation [36]. After elimination of cellular stresses, surplus p53 activates mdm2 expression to restore p53 to basal levels.

1.2.3 ARF-Mdm2-p53 pathway

Besides phosphorylation mentioned above, Mdm2-mediated p53 ubiquitination is regulated at different layers. A prominent negative regulator of Mdm2 is the tumor suppressor ARF (alternative reading frame). ARF is encoded by INK4a/ARF gene locus (Figure 5), which resides on the short arm of chromosome 9 (9p21) in human [103]. In addition to ARF, this gene locus also encodes the structurally and functionally unrelated protein p16INK4a [104]. However, transcription of each gene is driven by different promoters. Furthermore, these two genes share the same exon 2 but use two distinct exon 1. The INK4a gene utilizes exons 1α, 2 and 3, whereas ARF uses exon 1β and an alternative reading frame of exon 2 for translation [104].

![Figure 5 INK4a/ARF gene locus](image)

The INK4a/ARF gene locus encodes two structurally and functionally unrelated proteins, p16INK4a and ARF, by using alternative reading frames of exon 2. The INK4a gene comprises exon 1α, exon 2 and exon 3, whereas ARF gene comprises exon 1β and exon2. Transcription of each gene is driven by respective promoters (denoted as green arrows). Gray boxes represent sequences corresponding to the untranslated regions of respective mRNAs. Blue lines represent the splice sites of each mRNA.
Both p16INK4a and ARF suppress tumorigenesis, though via different mechanisms. Being a well-known cell cycle inhibitor, p16INK4a directly inhibits the activity of cyclin D-dependent kinases CDK4 and CDK6, leading to G1/S cell cycle arrest [105]. In contrast, ARF interferes with Mdm2-mediated p53 ubiquitination. Mutation or overexpression of oncogenes, including Ras and Myc, induce hyper-proliferative signals, which transcriptionally activate ARF expression [106]. ARF binds to the central region of Mdm2 corresponding to the acidic domain (Figure 4) [107, 108]. Through the direct interaction with Mdm2, ARF attenuates Mdm2 activity towards p53 ubiquitination. As a consequence, p53 is stabilized and activates the expression of target genes involved in tumor suppression [109, 110]. Moreover, there are two, yet speculative mechanisms by which ARF activates p53. First, in vitro data indicate that ARF binds to Mdm2 and directly inhibits its E3 ligase activity [111]. On the other hand, in cellulo data imply that ARF separates Mdm2 and p53 spatially within cells by facilitating subcellular relocalization of Mdm2 [112].

ARF proteins have an unusual amino acid composition harboring about 20% arginine residues but little or no lysine (also see Figure 49 on page 91), which renders them highly basic with an isoelectric point greater than 12. Thus, it is speculated that ARF is normally unstructured and needs to be folded and neutralized at physiological pH with the assistance of other cellular molecules [113]. Probably because of this uncommon biochemical feature, ARF is highly insoluble when recombinantly expressed in bacteria, which accounts for the difficulties in studying this protein [114].

The N-terminal region of ARF encoded by exon 1β plays a crucial role in p53-associated ARF functions. It contributes to the interaction of ARF with Mdm2 and is required for ARF-dependent p53 stabilization. Conversely, deletion of the C-terminal segment encoded by exon 2 shows no effect on p53-associated ARF activity [107, 108, 115, 116]. Consistent with this, the earliest ARF ancestor found in chicken is exclusively encoded by exon 1β and remains capable of p53 activation [117], indicating that the N-terminal half of ARF is sufficient for p53-associated ARF function. It is also important to note that ARF has additionally p53-independent functions in the regulation of apoptosis, ribosome biogenesis (via nucleophosmin) and sumoylation (reviewed in [113]).
1.3 p53 and cancers

1.3.1 TP53 mutations in human cancers

Owing to its crucial role in the maintenance of genomic integrity, p53 has been dubbed the “guardian of the genome” [118]. Mutations of TP53 in the germline cause the cancer-prone predisposition known as Li-Fraumeni Syndrome (LFS), first described by Li and Fraumeni in 1969 [119, 120]. Mutant germline alleles of most tumor suppressor genes are typically associated with the susceptibility to a narrow range of cancer types. In contrast, LFS is usually accompanied with a wide variety of cancers occurring at a relatively early age [119]. Furthermore, the pattern of germline TP53 mutations in LFS patients is similar to that of somatic TP53 mutations found in p53-associated sporadic tumors, which includes hotspot residues [121] and a bias in favor of missense mutations [122] (see below).

Somatic mutations of TP53 are found in more than 50% of human sporadic cancers [123]. During tumor progression, mutations of most tumor suppressor genes result in loss of expression of the respective proteins or in expression of truncated forms [124]. However, cancer-associated mutations of TP53 are frequently single missense mutations (74%), where only a single base pair is substituted (Figure 6 A). In consequence, mutant p53 is expressed as a full-length form but with a single amino acid substitution [125].

Figure 6 Somatic mutations of TP53 in human cancers

(A) Different types of cancer-associated TP53 mutations. Missense mutations represent the majority of mutations found (74%). (B) Distribution of missense mutations along the amino acid sequence of p53. One-third of missense mutations occur at six hotspot residues. While mutations affecting DNA contact sites (R248 and R273) are denoted in green, conformational mutations (R175, G245, R249 and R282) are denoted as blue boxes. TAD: transactivation domain; DBD: DNA binding domain; TD: tetramerization domain; CR: C-terminal regulatory region. Data derived from the IARC TP53 Mutation Database version R13 (November 2008) [126]. Figure is adapted from [127].
Missense mutations of TP53 predominantly map to the DNA binding domain, impairing binding of p53 towards its response element (Figure 6 B) [123]. Moreover, about one-third of missense mutations occur at six hotspot residues, R175, G245, R248, R249, R273 and R282 [123, 128]. Mutations at the DNA contact sites (see 1.1.1), namely R248 and R273, directly eliminate the interaction between p53 and DNA without altering p53 conformation, termed DNA contact mutations [16, 129]. In contrast, conformational mutations (e.g. R175, G245, R249 and R282) disrupt DNA binding by destabilizing the tertiary structure of the p53 DNA binding domain [130].

Several mechanisms causally associate TP53 mutations with tumorigenesis. Firstly, the loss of wild-type p53 functions naturally increases the risk of genomic instability. However, mutations initially occur only in one allele of TP53, giving rise to heterozygosity, and a wild-type p53 protein is still expressed from the other allele. A former model suggested that the mutant p53 can antagonize wild-type p53 functions in a dominant-negative manner. As the DNA binding ability of p53 relies on the formation of tetramers, the hetero-tetramer consisting of mutant and wild-type p53 proteins interferes with p53-mediated transcriptional activation [131, 132]. However, recent data indicate that the proposed dominant-negative effect might not be sufficient to completely constrain wild-type p53. Importantly, initial mutation of one TP53 allele is usually followed by loss of heterozygosity during cancer progression, leading to inactivation of the remaining wild-type allele (reviewed in [127, 133]).

Another mechanism, termed gain-of-function, also contributes to cancer development. Many mutant p53 variants acquire new oncogenic functions not shared by the wild-type p53 protein. An important gain-of-function property of mutant p53 is the interaction with p63 and p73, members of the p53 protein family. Mutant p53 inactivates the tumor-suppressive activities of these two transcription factors and thereby promotes carcinogenesis [134, 135]. Furthermore, mutant p53 also interacts with other transcription factors and cellular proteins, causing oncogenic consequences (reviewed in [127]). Strikingly, mutant p53 also binds to DNA and transcriptionally regulates gene expression. Missense mutations within the DNA binding domain abolish the sequence-specific DNA binding ability towards the canonical p53 response element. However, mutant p53 appears to recognize and bind to another distinct response element and to activate genes involved in proliferation, anti-apoptosis and angiogenesis (reviewed in [136, 137]).

1.3.2 p53 and cervical cancer

As mentioned, mutations of TP53 highly correlate with cancer progression. However, maintenance of wild-type TP53 does not guarantee the prevention of carcinogenesis either. For instance, amplification of mdm2 gene [138] and certain single nucleotide polymorphisms found in the mdm2 promoter [139] result in overexpression of Mdm2, thereby increasing tumorigenic potential in the absence of TP53 mutations. In support of this notion, TP53 mutations and Mdm2 overexpression have been described as mutually exclusive events in sarcomagenesis [140].
Similarly, *TP53* is not mutated in the majority of cervical cancers. However, the growth-suppressive properties of wild-type p53 are inhibited via a different mechanism (see below). Cervical cancer is a carcinoma arising from the lower part of uterus, the cervix, and represents the fourth most common malignancy in women worldwide as estimated in 2012 [141]. Moreover, infection of human papillomaviruses (HPVs) is a necessary cause of cervical cancer, with an attributable fraction of more than 99%.

HPVs are small DNA viruses with a circular closed double-stranded genome of about 8,000 base pairs that infect squamous epithelia and cause various epithelial lesions. Over 120 types of HPVs have been identified and about 40 of them infect the anogenital tract [142]. According to their ability to cause malignant lesions, anogenital HPVs are roughly classified into high-risk and low-risk types. High-risk HPVs such as HPV16 and HPV18 are associated with cervical and other anogenital cancers. On the contrary, the infection of low risk HPVs, including HPV6 and HPV11, generally causes benign genital warts (reviewed in [143]).

The HPV genome encodes six early (E1, E2, E4, E5, E6 and E7) and two late proteins (L1 and L2) (reviewed in [144]). Among them, E6 and E7 act as oncoproteins that provide the primary transforming activity of high-risk HPVs. The high-risk E7 protein interacts with so called “pocket proteins”, comprising pRb (retinoblastoma protein), p107 and p130, thereby targeting them for degradation [145]. Other studies also suggested that high risk E7 protein disrupts the pRb-E2F complex by competing with E2F for pRb binding [146, 147]. E2F is a prominent transcription factor family that regulates the G1/S transition of cell cycle. By forming a complex with E2F proteins, pocket proteins repress cell cycle progression (reviewed in [148]). Thus, the inhibitory effect of high-risk E7 protein on pocket proteins activates cell proliferation, which facilitates not only DNA synthesis in host cells but also replication of HPV genome.

On the other hand, the uncontrolled cell proliferation induced by high-risk E7 triggers stabilization and activation of p53. To counteract p53-mediated cell cycle arrest (see 1.1.2.1), the other oncoprotein E6 interacts with the cellular protein E6AP (E6 associated protein), forming a functional E3 ligase complex and targeting p53 for ubiquitination as well as subsequent proteasomal degradation [149]. E6AP is encoded by the *UBE3A* gene, which is located on the long arm of human chromosome 15 (15q11-13) [150, 151] and expresses three E6AP isoforms [152]. E6AP represents the founding member of the HECT E3 ligase family [153] and was the first identified E3 ligase for p53 [154]. In addition, E6AP also catalyzes ubiquitination of other substrates [155], including itself [156], in both high-risk E6-dependent and -independent manners.

E6AP alone does not recognize p53. Therefore, high-risk E6 protein functions as an adaptor to bring E6AP and p53 into close proximity. In the high-risk E6 protein, two N-terminal zinc domains and a linker helix form a basic-hydrophobic pocket, which captures the α-helical LXXLL motif of E6AP (where L and X represent leucine and any residue, respectively) [157]. Subsequently, the
formation of the E6-E6AP complex leads to a conformational change of high-risk E6, structuring a p53 docking site [158].

Infection of high-risk HPVs is necessary but not sufficient for cervical carcinogenesis. Following infection, HPV genomes first exist as extrachromosomal elements, termed episomes [159]. In this state, the HPV E2 protein represses expression of the early genes [160], including E6 and E7, which are required for cancer progression. At this stage, HPV-infected cells are often cleared by the immune system of the host. The crucial step to initiate malignant progression of infected cells is the integration of at least one HPV genome into the host genome. Integration of the circular HPV DNA most often occurs within the E2 open reading frame, which abolishes the expression of the HPV E2 protein [161]. Ultimately, this results in deregulated expression of HPV E6 and E7 and thereby in uncontrolled proliferation of host cells.

1.4 CRISPR-Cas9 system for genome engineering

It is of considerable interest for researchers to site-specifically modify the genome at will. Recently, the advent of the CRISPR-Cas (clustered regularly interspaced short palindromic repeats-CRISPR-associated protein) system has greatly facilitated the advancement of genome engineering. This system was first described in bacteria and archaea as an adaptive immune system against viruses, which occurs in three stages: adaptation, expression and interference (Figure 7) (reviewed in [162]).

CRISPR-Cas immunity occurs in three stages. During the adaptation stage, adaptation Cas nuclease complex captures a fragment of the foreign DNA (green line) and integrates it into the CRISPR array between two direct repeats (red triangles) as a new spacer sequence (green hexagon). During the expression stage, the CRISPR array is transcribed and then processed into small CRISPR RNAs (crRNAs). Eventually, crRNA-guided Cas nuclease are responsible for the cleavage of foreign DNA at the specific sites complementary to the crRNA spacer sequence. Adapted from [162].
Once bacteria and archaea are infected, a fragment of the foreign DNA is first captured and integrated into the CRISPR array between two direct repeats as a new spacer sequence, which is executed by certain Cas nucleases exclusively involved in the adaptation stage. The spacer acquisition allows bacteria and archaea to memorize the infection of the respective virus and to transmit this information to the next generation. During the expression stage, the CRISPR array is transcribed and then processed into small CRISPR RNAs (crRNAs). Eventually, crRNAs guide Cas nucleases responsible for the interference stage, to cleave both strands of the foreign DNA specifically at sites that are complementary to the crRNA spacer sequence.

Three different types of the CRISPR-Cas system (I, II and III) are characterized by the molecular mechanism to achieve foreign DNA recognition and cleavage (reviewed in [163]). While type I and type III systems utilize a large complex of Cas proteins for crRNA-guided DNA cleavage, type II system needs only the Cas9 nuclease (Figure 8). Another property of type II system is the requirement of a trans-activating crRNA (tracrRNA). This noncoding RNA hybridizes with crRNA and has been reported not only to be necessary for processing the transcript of the CRISPR array but also to facilitate crRNA-guided DNA cleavage mediated by Cas9 [164]. In addition, the protospacer adjacent motif (PAM), a short sequence motif adjacent to the crRNA-targeted sequence on the foreign DNA, also plays an essential role in type I and type II systems. As the PAM sequence only exists on the foreign DNA and is not integrated into the CRISPR array, it determines the self-nonself discrimination of CRISPR-Cas-mediated DNA cleavage.

![Figure 8 Components of *Streptococcus pyogenes* type II CRISPR-Cas9 system](image)

In contrast to type I and type III systems, type II CRISPR-Cas system requires only one nuclease, Cas9 (light blue), to execute double-strand DNA cleavage of the target sequence (green). Additionally, tracrRNA (*trans*-activating crRNA, red) hybridizes with CRISPR RNA (crRNA, dark green), which facilitates crRNA-guided DNA cleavage mediated by Cas9. Moreover, PAM (protospacer adjacent motif, orange) determines the self-nonself discrimination of CRISPR-Cas-mediated DNA cleavage. For the application of genome engineering, the tracrRNA:crRNA hybrid has been engineered as a fusion version (gray dotted line), termed single guide RNA (sgRNA). Adapted from [165].

To date, many studies have accomplished genome engineering in eukaryotes using the system modified from CRISPR-Cas of *Streptococcus pyogenes*. This bacterium harbors a type II system. The *S. pyogenes* Cas9 protein, optimized by codon usage bias and acquisition of nuclear localization, has shown effective nuclease activity within eukaryotic cells [166, 167]. Moreover,
the tracrRNA:crRNA hybrid has also been engineered as a fusion version, termed single guide RNA (sgRNA), which remains functional to direct sequence-specific double-strand DNA cleavage [168].

The ability of the CRISPR-Cas9 system to recognize specific DNA sequences with subsequent cleavage of both DNA strands makes this system a powerful tool for genome engineering. In mammalian cells, double-strand DNA breaks are predominantly repaired by two different mechanisms: non-homologous end joining (NHEJ) and homology-directed repair (HDR) (Figure 9). As NHEJ repairs double-strand DNA breaks by blunt end ligation independently of sequence homology, it is an efficient pathway to protect genome integrity throughout the cell cycle. However, the error-prone mechanism of NHEJ frequently results in insertions or deletions of nucleotides (indels) at the break site [169]. In contrast, owing to the requirement of a homologous DNA template from the sister chromatid, HDR is confined to G2 phase of the cell cycle and executes accurate DNA repair [170]. For the application of CRISPR-Cas9-mediated genome engineering to mammalian cells, indels caused by NHEJ lead to frameshift mutations accompanied by premature stop codons, which enables the knockout of gene expression. Moreover, HDR can be induced by introduction of an additional donor DNA template that is homologous to the DNA sequence targeted by the sgRNA. Thus, genome editing can be achieved by the arrangement of the donor DNA with a desired sequence.

Figure 9 Biology of CRISPR-Cas9-mediated genome engineering

Double-strand DNA breaks caused by CRISPR-Cas9 system are repaired by HDR (homology-directed repair, left) or NHEJ (non-homologous end-joining, right) in mammalian cells. HDR requires a donor DNA template that is homologous to the DNA sequence targeted by sgRNA. By the arrangement of the donor DNA with the desired sequence, genome editing, including gene insertion or point mutagenesis, is achieved. In contrast, NHEJ repairs double-strand DNA breaks by blunt end ligation independently of sequence homology. Indels (insertions or deletions) caused by NHEJ lead to frameshift mutations accompanied by premature stop codons, which enables the knockout of gene expression.
1.5 Aims of this work

In response to oncogenic stresses, levels of the tumor suppressor p53 are upregulated, contributing to the activation of the growth-suppressive properties of p53. Under normal cellular conditions, however, p53 proteins levels are maintained low by Mdm2-mediated ubiquitination followed by proteasomal degradation. Mutations of the TP53 gene contribute to cancer progression. In many cases, cancers retaining wild-type TP53 are accompanied by inactivation of p53 at the protein level. In this work, we aimed at a characterization of the tumor suppressor p53 with respect to its function in tumor cells and proteolytic regulation.

Previous data in our laboratory indicated that it is a demanding task to establish p53-deficient cells using cell lines derived from HPV-positive tumors, which harbor wild-type TP53. Owing to the known tumor-suppressive features of p53, this was unexpected and implied that wild-type p53 has a novel pro-survival function in tumor cells. In the first project, it was thus attempted to address whether this phenomenon can also be observed in HPV-negative cell lines. For efficient knockdown of p53 expression, we envisioned to apply an RNA interference system that directly reflects the effect of p53 deficiency on cell proliferation. The anti-proliferative effects, if any, should also be reversed by ectopically expressed p53 protein in rescue experiments. Furthermore, with the advent of the CRISPR-Cas9 system, the data obtained should be corroborated by CRISPR-Cas9-mediated knockout of the TP53 gene.

Although it is generally accepted that ARF inhibits Mdm2-mediated p53 ubiquitination, thereby stabilizing p53, the underlying mechanisms remain controversial. Therefore, the second project was aimed at investigating the biochemical mechanism by which ARF affects Mdm2 activity. We intended to perform a series of in vitro ubiquitination assays in the presence of recombinant ARF protein. As the expression of ARF in bacteria is a challenge, it was first required to optimize the preparation scheme for bacterially expressed ARF protein. Moreover, as Mdm2 catalyzes not only ubiquitination but also neddylation of p53, effects of ARF on Mdm2-mediated p53 neddylation should also be inspected.

Additionally, we also intended to develop a novel tool for accurate early diagnosis of cervical cancer. An important step to initiate cervical carcinogenesis is the integration of the HPV genome into the host genome and the subsequent expression of the HPV E6 and E7 oncoproteins. Thus, an ideal diagnostic tool should exhibit high sensitivity towards the initiation of carcinogenesis rather than towards detection of HPV infection, which in many cases appears to be a transient event. Furthermore, as specimens obtained from patients contain numerous cellular molecules, this tool should also exhibit high specificity towards the chosen marker representing the initiation of carcinogenesis.
2 Material and Methods

2.1 Material

2.1.1 Chemicals

<table>
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<tr>
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<tbody>
<tr>
<td>Acetic acid</td>
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<td>APS (ammonium peroxydisulphate)</td>
<td>ROTH</td>
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<tr>
<td>Aprotinin</td>
<td>SIGMA-ALDRICH</td>
</tr>
<tr>
<td>ATP (adenosine triphosphate)</td>
<td>SIGMA-ALDRICH</td>
</tr>
<tr>
<td>Ampicillin</td>
<td>ROTH</td>
</tr>
<tr>
<td>Bromophenol blue</td>
<td>SERVA</td>
</tr>
<tr>
<td>BSA (bovine serum albumin)</td>
<td>ROTH</td>
</tr>
<tr>
<td>Chloramphenicol</td>
<td>ROTH</td>
</tr>
<tr>
<td>Coomassie Brilliant Blue R250</td>
<td>ROTH</td>
</tr>
<tr>
<td>Crystal violet</td>
<td>SERVA</td>
</tr>
<tr>
<td>Sodium deoxycholate</td>
<td>SIGMA-ALDRICH</td>
</tr>
<tr>
<td>DMSO (dimethyl sulfoxide)</td>
<td>SIGMA-ALDRICH</td>
</tr>
<tr>
<td>DTT (dithiothreitol)</td>
<td>ROTH</td>
</tr>
<tr>
<td>EDTA (ethylenediaminetetraacetic acid)</td>
<td>ROTH</td>
</tr>
<tr>
<td>Ethanol</td>
<td>ROTH</td>
</tr>
<tr>
<td>Formaldehyde solution</td>
<td>MERCK</td>
</tr>
<tr>
<td>Glycerol</td>
<td>ROTH</td>
</tr>
<tr>
<td>Glycine</td>
<td>ROTH</td>
</tr>
<tr>
<td>Guanidinium hydrochloride</td>
<td>ROTH</td>
</tr>
<tr>
<td>Isopropanol</td>
<td>SIGMA-ALDRICH</td>
</tr>
<tr>
<td>IGEPAL CA-630</td>
<td>MP Biochemicals</td>
</tr>
<tr>
<td>Imidazole</td>
<td>MERCK</td>
</tr>
<tr>
<td>Chemical</td>
<td>Supplier</td>
</tr>
<tr>
<td>---------------------------</td>
<td>---------------</td>
</tr>
<tr>
<td>IPTG (isopropyl-thio-β-D-galactopyranoside)</td>
<td>ROTH</td>
</tr>
<tr>
<td>KAc (potassium acetate)</td>
<td>MERCK</td>
</tr>
<tr>
<td>KCl</td>
<td>ROTH</td>
</tr>
<tr>
<td>L-glutathione reduced</td>
<td>SIGMA-ALDRICH</td>
</tr>
<tr>
<td>LB (lysogeny broth)</td>
<td>ROTH</td>
</tr>
<tr>
<td>Leupeptin</td>
<td>ROTH</td>
</tr>
<tr>
<td>Lysozyme</td>
<td>ROTH</td>
</tr>
<tr>
<td>β-Mercaptoethanol</td>
<td>MERCK</td>
</tr>
<tr>
<td>Methanol</td>
<td>SIGMA-ALDRICH</td>
</tr>
<tr>
<td>MgCl₂</td>
<td>ACROS ORGANICS</td>
</tr>
<tr>
<td>MgSO₄</td>
<td>ROTH</td>
</tr>
<tr>
<td>Milk powder</td>
<td>ROTH</td>
</tr>
<tr>
<td>NaCl</td>
<td>ROTH</td>
</tr>
<tr>
<td>NaH₂PO₄</td>
<td>MERCK</td>
</tr>
<tr>
<td>Na₂HPO₄</td>
<td>MERCK</td>
</tr>
<tr>
<td>NaOH</td>
<td>SIGMA-ALDRICH</td>
</tr>
<tr>
<td>ONPG (ortho-Nitrophenyl-β-galactoside)</td>
<td>SIGMA-ALDRICH</td>
</tr>
<tr>
<td>Orange G</td>
<td>ROTH</td>
</tr>
<tr>
<td>PBS (phosphate buffered saline)</td>
<td>gibco</td>
</tr>
<tr>
<td>Pefabloc</td>
<td>Boehringer Ingelheim</td>
</tr>
<tr>
<td>Ponceau S</td>
<td>ROTH</td>
</tr>
<tr>
<td>RNase A</td>
<td>SIGMA-ALDRICH</td>
</tr>
<tr>
<td>Rotiphorese Gel 30 (37.5 : 1) (acrylamide)</td>
<td>ROTH</td>
</tr>
<tr>
<td>SDS (sodium dodecyl sulfate)</td>
<td>ROTH</td>
</tr>
<tr>
<td>SOB (super optimal broth)</td>
<td>ROTH</td>
</tr>
<tr>
<td>D-(+)-Sucrose</td>
<td>ROTH</td>
</tr>
<tr>
<td>TEMED (tetramethylethylenediamine)</td>
<td>ROTH</td>
</tr>
<tr>
<td>Triton X-100</td>
<td>ROTH</td>
</tr>
<tr>
<td>Tris base</td>
<td>SIGMA-ALDRICH</td>
</tr>
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</table>
### Material and Methods

<table>
<thead>
<tr>
<th>Material</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tryptone/peptone</td>
<td>ROTH</td>
</tr>
<tr>
<td>Tween 20</td>
<td>ROTH</td>
</tr>
<tr>
<td>UltraPure Agarose</td>
<td>invitrogen</td>
</tr>
<tr>
<td>Yeast extract</td>
<td>ROTH</td>
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#### 2.1.2 Buffers and solutions

<table>
<thead>
<tr>
<th>Buffer</th>
<th>Composition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Buffer Z</td>
<td>100 mM Na₂HPO₄/NaH₂PO₄ (pH 7.0), 10 mM KCl, 1 mM MgSO₄, 50 mM β-Mercaptoethanol</td>
</tr>
<tr>
<td>Coomassie Blue staining solution</td>
<td>2 g/l Coomassie Brilliant Blue R250 in Coomassie destain solution</td>
</tr>
<tr>
<td>Coomassie destain solution</td>
<td>40% (v/v) methanol, 10% (v/v) acetic acid</td>
</tr>
<tr>
<td>Crystal violet solution</td>
<td>0.1% (w/v) crystal violet, 4% (v/v) formaldehyde in PBS</td>
</tr>
<tr>
<td>Elution buffer (GST-protein)</td>
<td>50 mM Tris-HCl (pH 8.0), 10 mM L-glutathione reduced</td>
</tr>
<tr>
<td>Elution buffer (His-protein)</td>
<td>25 mM Tris-HCl (pH 7.5), 50 mM NaCl, 300 mM imidazole</td>
</tr>
<tr>
<td>FACS (fluorescence-activated cell sorting) buffer</td>
<td>2% (v/v) FCS, 2 mM EDTA in PBS; 0.2 μm filtered</td>
</tr>
<tr>
<td>Fluorescence sample buffer</td>
<td>50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1mM DTT</td>
</tr>
<tr>
<td>Guanidinium hydrochloride lysis buffer</td>
<td>100 mM Na₂HPO₄/NaH₂PO₄ (pH 8.0), 6 M guanidinium-hydrochloride, 10 mM imidazole, 10 mM β-Mercaptoethanol</td>
</tr>
<tr>
<td>Laemmli resolving gel buffer</td>
<td>375 mM Tris-HCl (pH 8.8), 0.1% (w/v) SDS</td>
</tr>
<tr>
<td>Laemmli running buffer</td>
<td>25 mM Tris-HCl (pH 8.4), 200 mM glycine, 0.1% (w/v) SDS</td>
</tr>
<tr>
<td>Laemmli sample buffer</td>
<td>62.5 mM Tris-HCl (pH 6.8), 100 mM DTT, 10% (v/v) glycerol, 2% (w/v) SDS, 0.0005% (w/v) Bromophenol blue</td>
</tr>
</tbody>
</table>
### Material and Methods

<table>
<thead>
<tr>
<th>Buffer / Solution</th>
<th>Composition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Laemmlie stacking gel buffer</td>
<td>125 mM Tris-HCl (pH 6.8), 0.1% (w/v) SDS</td>
</tr>
<tr>
<td>Lysis buffer (bacterial protein purification)</td>
<td>1% (v/v) Triton X-100, 1 μg/ml aprotinin/leupeptin, 1 mM pefabloc, 100 μg/ml lysozyme, 1 mM DTT in PBS</td>
</tr>
<tr>
<td>ONPG</td>
<td>4 mg/ml in 100mM Na₂HPO₄ (pH 7.0)</td>
</tr>
<tr>
<td>Orange G loading buffer</td>
<td>40 mg/ml sucrose, 0.2 mg/ml Orange G</td>
</tr>
<tr>
<td>PBS-T</td>
<td>1% (v/v) Triton X-100 in PBS</td>
</tr>
<tr>
<td>Ponceau S solution</td>
<td>0.2% (w/v) Ponceau S, 5% (v/v) acetic acid</td>
</tr>
<tr>
<td>RIPA lysis buffer</td>
<td>50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1 mM DTT, 1% (v/v) IGEPAL CA-630, 0.1% (w/v) SDS, 0.5% (w/v) sodium deoxycholate, 0.1 mM pefabloc, 1 μg/ml aprotinin/leupeptin</td>
</tr>
<tr>
<td>S1</td>
<td>50 mM Tris-HCl (pH 8.0), 10 mM EDTA, 100 μg/ml RNase A</td>
</tr>
<tr>
<td>S2</td>
<td>200 mM NaOH, 1% (w/v) SDS</td>
</tr>
<tr>
<td>S3</td>
<td>2.8 M KAc (pH 5.1)</td>
</tr>
<tr>
<td>SUMO protease buffer</td>
<td>50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1mM DTT, 5% (v/v) glycerol</td>
</tr>
<tr>
<td>T&lt;sub&gt;25&lt;/sub&gt;</td>
<td>25 mM Tris-HCl (pH 7.5)</td>
</tr>
<tr>
<td>T&lt;sub&gt;25&lt;/sub&gt;N&lt;sub&gt;50&lt;/sub&gt;</td>
<td>25 mM Tris-HCl (pH 7.5), 50 mM NaCl</td>
</tr>
<tr>
<td>T&lt;sub&gt;50&lt;/sub&gt;</td>
<td>50 mM Tris-HCl (pH 8.0 or 6.8)</td>
</tr>
<tr>
<td>TAE buffer</td>
<td>40 mM Tris-HCl, 0.11% (v/v) acetic acid, 1 mM EDTA</td>
</tr>
<tr>
<td>TBS-T</td>
<td>20 mM Tris-HCl (pH 7.6), 150 mM NaCl, 0.1% (v/v) Tween 20</td>
</tr>
<tr>
<td>TNE-T</td>
<td>50 mM Tris-HCl (pH 7.6), 50 mM NaCl, 2.5 mM EDTA, 0.1% (v/v) Tween 20</td>
</tr>
<tr>
<td>Transfer buffer</td>
<td>12.5 mM Tris-HCl (pH 8.3), 100 mM glycine</td>
</tr>
</tbody>
</table>


### 2.1.3 Bacterial culture media

<table>
<thead>
<tr>
<th>Medium</th>
<th>Composition</th>
</tr>
</thead>
<tbody>
<tr>
<td>LB (lysogeny broth)</td>
<td>1% (w/v) tryptone/peptone, 0.5% (w/v) yeast extract, 171.11 mM NaCl</td>
</tr>
<tr>
<td>SOB (super optimal broth)</td>
<td>2% (w/v) tryptone/peptone, 0.5% (w/v) yeast extract, 10 mM MgCl₂, 8.56 mM NaCl, 2.5 mM KCl</td>
</tr>
<tr>
<td>2YT</td>
<td>1.6% (w/v) tryptone/peptone, 1% (w/v) yeast extract, 85.56 mM NaCl</td>
</tr>
</tbody>
</table>

### 2.1.4 Bacterial strains

<table>
<thead>
<tr>
<th></th>
<th>Genotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>DH5α</td>
<td>F- endA1 glnV44 thi-1 recA1 relA1 gyrA96 deoR nupG purB20 ph80dlacZΔM15 Δ(lacZYA-argF)U169, hsdR17(rK mK⁺), λ⁻</td>
</tr>
<tr>
<td>BL21-CodonPlus (DE3)-RIL</td>
<td>E. coli B F’ ompT hsdS(rB mB) dcm+ Tet’ gal λ (DE3) endA Hte [argU ileY leuW Cam’]</td>
</tr>
</tbody>
</table>

### 2.1.5 Cell culture material

#### 2.1.5.1 Media and reagents

<table>
<thead>
<tr>
<th>Material</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>DMEM (Dulbecco's Modified Eagle Medium)</td>
<td>gibo</td>
</tr>
<tr>
<td>FCS (fetal calf serum)</td>
<td>Biochrom AG</td>
</tr>
<tr>
<td>Opti-MEM</td>
<td>gibo</td>
</tr>
<tr>
<td>Trypsin-EDTA (0.05%)</td>
<td>gibo</td>
</tr>
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</table>

#### 2.1.5.2 Antibiotics

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hygromycin B</td>
<td>InvivoGen</td>
</tr>
<tr>
<td>Neomycin (G418)</td>
<td>ROTH</td>
</tr>
<tr>
<td>Normocin</td>
<td>InvivoGen</td>
</tr>
</tbody>
</table>
2.1.6 Mammalian cell lines

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Tissue</th>
<th>Disease</th>
</tr>
</thead>
<tbody>
<tr>
<td>C-33A</td>
<td>Cervix</td>
<td>Carcinoma</td>
</tr>
<tr>
<td>H1299</td>
<td>Lung</td>
<td>Non-small cell lung carcinoma</td>
</tr>
<tr>
<td>HEK-293</td>
<td>Human embryonic kidney</td>
<td>-</td>
</tr>
<tr>
<td>HeLa</td>
<td>Cervix</td>
<td>Adenocarcinoma</td>
</tr>
<tr>
<td>MCF7</td>
<td>Breast</td>
<td>Adenocarcinoma</td>
</tr>
<tr>
<td>U-2 OS</td>
<td>Bone</td>
<td>Osteosarcoma</td>
</tr>
</tbody>
</table>

2.1.7 Antibodies

2.1.7.1 Primary antibodies

<table>
<thead>
<tr>
<th>Protein</th>
<th>Name</th>
<th>Species &amp; type</th>
<th>Supplier</th>
<th>Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-Actin</td>
<td>AC-15</td>
<td>mouse, monoclonal</td>
<td>SIGMA-ALDRICH</td>
<td>1:7,500</td>
</tr>
<tr>
<td>E6AP</td>
<td>-</td>
<td>rabbit, polyclonal</td>
<td>M. Scheffner</td>
<td>1:1,000</td>
</tr>
<tr>
<td>E6AP</td>
<td>3E5</td>
<td>mouse, monoclonal</td>
<td>SIGMA-ALDRICH</td>
<td>1:1,000</td>
</tr>
<tr>
<td>Flag-tag</td>
<td>M2</td>
<td>mouse, monoclonal</td>
<td>SIGMA-ALDRICH</td>
<td>1:1,000</td>
</tr>
<tr>
<td>p14ARF</td>
<td>ARF 4C6/4</td>
<td>mouse, monoclonal</td>
<td>abcam</td>
<td>1:5,000</td>
</tr>
<tr>
<td>p53</td>
<td>DO-1</td>
<td>mouse, monoclonal</td>
<td>CalBiochem</td>
<td>1:1,000</td>
</tr>
<tr>
<td>Tubulin</td>
<td>DM1A</td>
<td>mouse, monoclonal</td>
<td>abcam</td>
<td>1:5,000</td>
</tr>
<tr>
<td>Ubiquitin</td>
<td>P4G7</td>
<td>mouse, monoclonal</td>
<td>abcam</td>
<td>1:1,000</td>
</tr>
</tbody>
</table>

2.1.7.2 Secondary antibodies

<table>
<thead>
<tr>
<th>Name</th>
<th>Species</th>
<th>Supplier</th>
<th>Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>HRP-coupled α-mouse</td>
<td>goat</td>
<td>Dianova</td>
<td>1:30,000</td>
</tr>
<tr>
<td>HRP-coupled α-rabbit</td>
<td>goat</td>
<td>Dianova</td>
<td>1:30,000</td>
</tr>
</tbody>
</table>
### 2.1.8 Oligonucleotides

#### 2.1.8.1 Primers for PCR

<table>
<thead>
<tr>
<th>No.</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>HP38</td>
<td>CGGGGATCCATGGAGGAGCCGCAGTCAGATCC</td>
</tr>
<tr>
<td>HP49</td>
<td>CCGCTCGAGTCAGTCTGAGTCAGGGCCC</td>
</tr>
<tr>
<td>MT_31</td>
<td>GGCTCGAGTTAGGGAATAAGTTAGCAC</td>
</tr>
<tr>
<td>ToP_24</td>
<td>GCGGTCTCAGATGTTTCAAGGACCCACAG</td>
</tr>
<tr>
<td>ToP_27</td>
<td>GCGCTCGAGTTAGGGAATAAGTTAGCAC</td>
</tr>
<tr>
<td>ToP_30</td>
<td>AGGCGTCTCGGATGTAATACCAACATGTC</td>
</tr>
<tr>
<td>ToP_37</td>
<td>CCGGTCTCAGATGGAACACACACACACACCTCAAAGGAAGAG</td>
</tr>
<tr>
<td>ToP_38</td>
<td>CCGCCGTTACTGGAATTTCTCAGTCTGAGTCAGGGCCC</td>
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<tr>
<td>ToP_39</td>
<td>TCGGATCGATATCTCGGCGCATGGCGTACCACATGCAC</td>
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<tr>
<td>ToP_48</td>
<td>GCCGGATCCATGCTTGAATTTCTGAAATATAGTGTGTCAGG</td>
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<tr>
<td>ToP_49</td>
<td>AGGCTCGAGTCATTTAAGCGTCCGTTAAAATC</td>
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<td>ToP_50</td>
<td>GGAACAGCTTTTGAGTTGTGTGTTGTGCTGCTGCTG</td>
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<td>ToP_51</td>
<td>CAGGACAGGCACAAACACACACCTCAAAGCGTCTCC</td>
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<tr>
<td>ToP_52</td>
<td>GGGCGTCTCGGATGGAATATTCTCCATCCAGTGTGTTCTTTCT</td>
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<td>ToP_53</td>
<td>TTTGGCCGCGCATGGCGTACCACATACGACGTC</td>
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<tr>
<td>ToP_54</td>
<td>TTTGGGCGCGCCCTCAGTCTGAGTCGGCCCTCCTTTCTG</td>
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<tr>
<td>ToP_60</td>
<td>GAGCCTCGGATCATCTGCGCGACTGCGCCCTCCTCCG</td>
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<tr>
<td>ToP_68</td>
<td>AAGGTCTCAGAGAACGACAGATCGGACGACAGA</td>
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<td>ToP_69</td>
<td>ACTAGTGATTCCACTGAAATTTCTCAGGCGGCTCATAGGG</td>
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<td>ToP_70</td>
<td>TATGAGCCGGCCCTGAGTTGTGTTGCAACTGGG</td>
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<td>ToP_71</td>
<td>ACTAGTGATCCACTGAAATTTCTCAGGCGGAGTGTGTTATC</td>
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<td>ToP_72</td>
<td>TCGTAAAGTTGAAATTTGTAAGTTGAAATGGAGTTTAAATACA</td>
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<tr>
<td>ToP_73</td>
<td>ACTAGTGATCCACTGAAATTTCTCAGGCGGAGGAGG</td>
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<tr>
<td>ToP_74</td>
<td>CCTCTGGTGCAGATCCGTTGCGGGGTAGGCCGAGG</td>
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<tr>
<td>ToP_75</td>
<td>ACTAGTGATCCACTGCTCAGTCTGAGTCTGAGTCAGGGCCCTTCTGTC</td>
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<tr>
<td>ToP_95</td>
<td>AGCTTTCTCAGACATCATTTCTCCACC</td>
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### Material and Methods

#### 2.1.8.2 Oligonucleotides for annealing

<table>
<thead>
<tr>
<th>No.</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>ToA_9</td>
<td>TGGATATCTGCAGAATTACCGGTTATGCTATAAAAAACCTTAATAAC</td>
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<tr>
<td>ToA_10</td>
<td>GCCTCGAGTTACAGCTGGGTTTCTCTACGTGT</td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>No.</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>ToA_9</td>
<td>AAGCGGCCGCTGCTTGGACAGTGAGCGACCTCCACTCTACTTATTAGTGAAGCCACAGATG</td>
</tr>
<tr>
<td>ToA_10</td>
<td>AAGAAATTCTCCAGGCGATGAGCGCAGACTCCAGTGGTAATCTACTTACATCTGTGCTGCTTCACTAA</td>
</tr>
<tr>
<td>ToA_11</td>
<td>AAGCGGCCGCTGCTTGGACAGTGAGGAGGATGGTTTTGGGAGATGTATAGTGAAGCCACAGATG</td>
</tr>
<tr>
<td>ToA_12</td>
<td>AAGCGGCCGCTGCTTGGACAGTGAGGAGGATGGTTTTGGGAGATGTATACATCTGTGCTGCTTCACACTAC</td>
</tr>
<tr>
<td>ToA_13</td>
<td>AAGCGGCCGCTGCTTGGACAGTGAGGACACCTGGTTTTGGGAGATGTATAGTGAAGCCACAGATG</td>
</tr>
<tr>
<td>ToA_14</td>
<td>AAGAAATTCTCCAGGCGATGAGGATGAGGCTGCTTGGTTTTGGGAGATGTATACATCTGTGCTGCTTCACACTAC</td>
</tr>
<tr>
<td>ToA_15</td>
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</tr>
<tr>
<td>ToA_16</td>
<td>AAGCGGCCGCTGCTTGGACAGTGAGGACACCTGGTTTTGGGAGATGTATACATCTGTGCTGCTTCACACTAC</td>
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<tr>
<td>ToA_17</td>
<td>AAGCGGCCGCTGCTTGGACAGTGAGGACACCTGGTTTTGGGAGATGTATACATCTGTGCTGCTTCACACTAC</td>
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<tr>
<td>ToA_18</td>
<td>AAGCGGCCGCTGCTTGGACAGTGAGGACACCTGGTTTTGGGAGATGTATACATCTGTGCTGCTTCACACTAC</td>
</tr>
<tr>
<td>ToA_19</td>
<td>AAGCGGCCGCTGCTTGGACAGTGAGGACACCTGGTTTTGGGAGATGTATACATCTGTGCTGCTTCACACTAC</td>
</tr>
<tr>
<td>ToA_20</td>
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Material and Methods

2.1.9 Plasmids

2.1.9.1 Empty vectors used in this work

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<td>pcDNA3</td>
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Material and Methods

ToV-4  pGEX-SUMO  GE Healthcare/ this work
ToV-6  pExoIN  trezyme
ToV-8  pcDNA5/FRT/TO-FA  invitrogen/ T. U. Mayer
ToV-15  pIRES-GFP  Clontech/ this work
ToV-18  pRc/CMV  invitrogen
ToV-21  pSpCas9(BB)-2A-Puro  F. Zhang (Addgene plasmid # 48139) [171]
ToV-26  pPuroUb-SpCas9(BB)  F. Zhang (Addgene plasmid # 48139) [171]/ this work
ToV-29  pcDNA3puro  invitrogen/ this work
Tina_10 a)  pSpCas9(BsBs)-IRESneo  F. Zhang (Addgene plasmid # 48139) [171]/ this work
a) Construct was cloned by Tina Maxa under supervision.

2.1.9.2 Knockdown and knockout constructs cloned in this work

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### Material and Methods

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<td>5’-ToP_48 (BamHI); 3’-ToP_49 (XhoI)</td>
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2.1.9.3 Plasmids for protein expression constructed in this work

Tina_16 b) sg-mCherry sgRNA pSpCas9(BsBs)-IRESneo 5’-ToA_33 (Bsal); 5’-ToA_34 (Bsal)

Tina_20 b) sg-E6AP_iii sgRNA pSpCas9(BsBs)-IRESneo 5’-TM-28 (Bsal); 3’-TM-29 (Bsal)

a) and b) Constructs were cloned by Jasmin Taban and Tina Maxa, respectively, under supervision.
Material and Methods

a) Constructs were cloned by Jasmin Taban under supervision.

2.1.9.4 Other plasmids used in this work

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2.1.10 Synthetic peptides

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<td>ARF-Scrambled</td>
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2.1.11 Protein and DNA standards

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<td>GeneRuler 1kb Plus DNA Ladder</td>
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2.1.12 Software

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<td>Clone Manager 9</td>
<td>Analysis of DNA and protein sequences, design of cloning</td>
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<td>Clustal Omega [174] a)</td>
<td>Alignment of multiple amino acid sequences</td>
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<td>Image Studio Lite</td>
<td>Quantification of band intensity on SDS-PAGE</td>
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<td>NEBuilder Assembly Tool a)</td>
<td>Design of primers for Gibson Assembly</td>
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<td>PONDR VL-XT [175] a)</td>
<td>Prediction of naturally disordered regions</td>
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<td>PrimerX [176] a)</td>
<td>Design of primers for site-directed mutagenesis</td>
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<td>Spekwin32 [177]</td>
<td>Analysis of fluorescence spectrum</td>
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a) Online software
2.2 Methods

2.2.1 PCR and cloning

2.2.1.1 Polymerase chain reaction (PCR)
PCR was performed using Phusion High-Fidelity DNA Polymerase (New England Biolabs) in Phusion HF or GC buffer according to manufacturer’s instructions. Primers were synthesized by Integrated DNA Technologies.

2.2.1.2 Site-directed mutagenesis
To introduce point mutations into plasmid DNA, QuikChange Site-Directed Mutagenesis was performed using Pfu Turbo DNA Polymerase (Agilent) and complementary primers (designed with PrimerX [176]) containing the desired mutation in a 50 μl reaction volume according to manufacturer’s instructions. PCR products (25 μl) were then digested with DpnI (New England Biolabs) in the total volume of 50 μl supplemented with reaction buffer at 37°C for two hours. Digestion (10 μl) was then transformed into chemically competent DH5α E. coli cells.

2.2.1.3 Annealing of oligonucleotides and fill-in
To generate small DNA fragments that cannot be generated by PCR, fully or partially complementary oligonucleotides were annealed. Both oligonucleotides (5 μg) were combined in a total volume of 25 μl containing Phusion GC buffer. The mixed oligonucleotides were then annealed in a thermocycler under the following conditions. The reaction was first incubated at 94 °C for 5 min and then gradually cooled down to 70 °C with the rate of 1.2 °C per min. After incubation at 70 °C for 10 min, the reaction was cooled down to 20 °C with the rate of 1.2 °C per min.

Annealed oligonucleotides that are partially complementary to each other need to be filled-in to obtain the complete double-stranded DNA fragment. To do so, 25 μl annealed oligonucleotides was incubated at 72 °C for 30 min in the total volume of 50 μl containing 0.4 mM dNTPs (deoxynucleoside triphosphates), Phusion GC buffer and one unit Phusion High-Fidelity DNA Polymerase (New England Biolabs).

2.2.1.4 Restriction digest
All restriction enzymes were obtained from New England Biolabs. DNA (2 μg) was digest for 2 hours in a total volume of 45 μl containing the desired restriction enzymes under the conditions described by the manufacturer’s instructions.
2.2.1.5 Agarose electrophoresis
DNA fragments were separated via agarose electrophoresis. According to the size of DNA fragments, 0.8-2.0% (w/v) TAE agarose gels supplemented with 0.005% (v/v) Midori Green (Biozym) were prepared. Agarose gels were submerged in TAE buffer in a horizontal gel electrophoresis chamber and loaded with samples containing Orange G loading buffer. Electrophoresis was performed at 3 volt per cm of gel length. Separated DNA fragments were visualized using LAS-3000 Image Analyzer (Fujifilm).

2.2.1.6 Purification of DNA from agarose gels
After separation via agarose electrophoresis, desired DNA fragments were excised from the agarose gel under a UV transilluminator and purified using NucleoSpin Gel and PCR Clean-up kit (Macherey-Nagel) according to manufacturer’s instructions.

2.2.1.7 Ligation of DNA fragments
Purified vector and insert DNA fragments were mixed in a molar ratio of 1:3 and ligated using T4 DNA ligase (Thermo Scientific) in a total volume of 20 μl containing ligation buffer at 22 °C for 20 min. After incubation, the enzyme was inactivated at 65 °C for 10 min and the whole reaction was transformed into chemically competent *E. coli* DH5α cells (2.2.1.9).

2.2.1.8 Gibson Assembly
In addition to ligation, insert and vector were also alternatively assembled by Gibson Assembly [178]. Primers containing the overhang to generate the overlapping region upon PCR were designed with NEBuilder Assembly Tool (New England Biolabs) and PCR was performed as described in 2.2.1.1. Vector was linearized by restriction digest as described in 2.2.1.4. Vector (20 ng) and insert were assembled in a molar ratio of 1:2 using 2x HiFi DNA Assembly Master Mix (New England Biolabs) in a total volume of 4 μl. The reaction mixture was incubated at 50 °C for 20 min. After incubation, the whole reaction was transformed into chemically competent *E. coli* DH5α cells (2.2.1.9).

2.2.1.9 Transformation of DNA into chemically competent *E. coli*
Plasmid DNA (cloning reaction or 100 ng of purified DNA) was mixed with 80 μl corresponding chemically competent *E. coli* cells and incubated on ice for 30 min. After a heat shock at 42 °C for 90 sec, cells were incubated on ice for 10 min. Depending on the purpose of transformation, cells were treated as follows: (1) For transformation of purified plasmid DNA (large-scale DNA preparation or protein expression), cells were then inoculated into LB media or onto LB agar plates supplemented with appropriate antibiotic and incubated at 37 °C overnight. (2) For transformation of cloning reactions, cells were then incubated in 800 μl SOB media at 37 °C for 30 min with shaking at 500 rpm (Eppendorf Thermomixer compact). After centrifugation at 6,000 x
For 5 min, E. coli pellets were resuspended in 100 μl SOB media. Cell resuspension was then inoculated into LB media or onto LB agar plates containing the appropriate antibiotic and incubated at 37 °C overnight. (3) For any plasmid that contains kanamycin resistance gene, cells were always proceeded with the second procedure, regardless of the purpose of transformation, to allow the expression of kanamycin resistance gene.

2.2.1.10 Colony PCR
Colony PCR was performed to screen positive cloning colonies. An E. coli colony was picked from the LB agar plate and smeared into a PCR tube to serve as the DNA template. PCR was performed using self-made recombinant Taq polymerase in a total volume of 20 μl containing ThermoPol Reaction Buffer (New England Biolabs), Orange G loading buffer, 0.4 μM primers and 0.8 mM dNTPs. After reaction, PCR products were analyzed by agarose electrophoresis (2.2.1.5).

2.2.1.11 Small- and large-scale plasmid DNA preparation
Plasmid DNA preparation was performed using the alkaline lysis method [179]. For small-scale plasmid DNA purification, single E. coli colony was inoculated into 3 ml LB medium containing the appropriate antibiotic and incubated at 37 °C overnight. Subsequently, 1 ml culture was centrifuged at 16,100 x g, room temperature for 1 min and E. coli pellets were resuspended in 250 μl S1 buffer. Cell resuspension was then incubated with 250 μl S2 buffer at room temperature for 5 min. Afterwards, 300 μl S3 buffer was added, followed by 10-minute incubation on ice. After centrifugation at 16,100 x g, room temperature for 6 min, the supernatant was mixed with 600 μl isopropanol to allow the precipitation of the plasmid DNA and centrifuged at 16,100 x g, room temperature for 10 min. DNA pellets were then washed with 500 μl 70% ethanol and centrifuged at 16,100 x g, room temperature for 5 min. After aspiration of the supernatant, the DNA pellet was air-dried in Eppendorf Concentrator 5301 at room temperature. Eventually, the pellet was resuspended in 35 μl nuclease-free water and stored at -20 °C.

Large-scale plasmid DNA purification was performed using 200 ml overnight culture complemented with the appropriate antibiotics via PureYield Plasmid Midiprep System (Promega) according to manufacturer’s instructions. Purified plasmids were then stored at -20 °C.

2.2.1.12 Determination of DNA concentration
DNA concentration was measured using a NanoPhotometer (IMPLEN) according to manufacturer’s instructions.

2.2.1.13 DNA sequencing
DNA sequence was validated by GATC BIOTECH.
2.2.2 Preparation of recombinant proteins

2.2.2.1 In vitro translation

In vitro translation of proteins was performed using the TNT Coupled Reticulocyte Lysate System (Promega) according to manufacturer’s instructions. For visualization, proteins were labeled with $^{35}$S-methionine (Perkin Elmer).

2.2.2.2 Preparation of glycerol stocks

Glycerol stocks of the BL21 (DE3) RIL E. coli strain transformed with plasmids for protein expression were prepared by mixing 750 μl sterile 60% (v/v) glycerol and 750 μl overnight culture in cryovials and stored at -80 °C.

2.2.2.3 Conventional procedure for protein expression in E. coli

UbcH5b-His and GST-Ulp1 were recombinantly expressed by the E. coli strain BL21 (DE3) RIL using the following procedure. LB medium containing 50 μg/ml ampicillin and 34 μg/ml chloramphenicol was inoculated with transformed E. coli and cultivated at 37 °C overnight. The overnight culture was then diluted in fresh LB medium to the OD600 of 0.1 and grown at 37 °C. When the OD600 reached 1, protein expression was induced by 0.2 mM IPTG. The culture was then grown at 37 °C until OD600 reached 4. Cells were harvested by centrifugation at 4,000 x $g$, 4 °C for 20 min. E. coli pellets were either directly used for protein purification or stored at -80 °C.

2.2.2.4 Heat-shock protein expression in E. coli

All recombinant proteins not indicated in 2.2.2.3 were expressed by the E. coli strain BL21 (DE3) RIL using the heat-shock procedure. LB medium containing 50 μg/ml ampicillin and 34 μg/ml chloramphenicol was inoculated with transformed E. coli and cultivated at 37 °C overnight. On the next morning, the start culture was inoculated by diluting the overnight culture in fresh LB medium to the OD600 of 0.1. The start culture was then grown at 30 °C until the OD600 reached 0.6. E. coli cells were then harvested by centrifugation at 4,000 x $g$, 4 °C for 20 min and resuspended in the pre-warmed (42 °C) 2YT medium of the same volume as the start culture. The resuspension was cultivated at 42°C for 30 min and 1:4 diluted in pre-cooled (16 °C) 2YT medium. The culture was then grown at 16 °C until the OD600 reached 0.4-0.6. After induction of protein expression via 0.01 mM IPTG, the culture was grown at 16 °C until OD600 reached 10. Cells were harvested by centrifugation at 4,000 x $g$, 4 °C for 20 min. E. coli pellets were either directly used for protein purification or stored at -80 °C.
Material and Methods

2.2.2.5 Batch purification of GST-tagged proteins

After bacterial protein expression, the obtained cell pellet was resuspended in lysis buffer (lysis buffer: culture = 1:20, v/v) and sonicated by Branson Sonifier 250 (6x20 pulses, duty cycle: 40-50%, output control: 5). Cell debris was isolated by centrifugation at 27,000 \( x \) g, 4 °C for 20 min. The lysate was then transferred into a fresh tube and incubated at 4°C for 90 min under continuous rotation with the Glutathione Sepharose 4B (GE Healthcare; beads: culture = 1:1,000, v/v) equilibrated in PBS-T. After centrifugation at 500 \( x \) g, 4°C for 10 min, beads were washed three times with 15 ml PBS-T and twice with 15ml T50 (pH 8.0) by the incubation at 4°C for 5 min under the continuous rotation. Finally, GST-tagged proteins were eluted from the beads by incubation at 4°C for 30 min under continuous rolling using elution buffer containing 10 mM L-reduced glutathione (beads: elution buffer = 1:1, v/v).

2.2.2.6 Batch purification of His-tagged proteins

Bacterial lysate containing the desired His-tagged proteins was prepared as described in 2.2.2.5. The lysate was incubated at 4°C for four hours under the continuous rotation with 10 mM imidazole and Ni-NTA-Agarose (Qiagen; beads: culture = 1:5,000, v/v) equilibrated in PBS-T. After centrifugation at 500 \( x \) g, 4°C for 10 min, beads were washed three times with 15 ml PBS-T containing 20 mM imidazole and twice with 15ml T25N50 (pH 7.5), 20 mM imidazole by incubation at 4°C for 5 min under continuous rotation. Elution was performed by incubation at 4°C for 30 min under continuous rolling with the elution buffer containing 300 mM imidazole (beads: elution buffer = 1:1, v/v).

2.2.2.7 Purification of tag-free proteins via on-beads cleavage

For preparation of tag-free proteins, protein of interest (POI) was first recombinantly expressed by the E. coli strain BL21 (DE3) RIL as a GST-SUMO fusion protein (see 3.2.2). Meanwhile, GST-tagged SUMO-specific proteases, Ulp1 (Ubl-specific protease 1) was individually expressed. Both proteins were then isolated by Glutathione Sepharose 4B (GE Healthcare) as described in 2.2.2.5, followed by three washes with 15 ml PBS-T by the incubation at 4°C for 5 min under continuous rotation. Subsequently, both bead batches were individually transferred onto chromatography columns and washed with 25 ml SUMO protease buffer.

To allow the cleavage between SUMO and POI, two bead batches bound respectively with GST-SUMO-POI and GST-Ulp1 were combined in a 2:1 ratio (v/v) and incubated at 4°C overnight. After centrifugation at 500 \( x \) g, 4°C for 10 min, the supernatant, which contains the tag-free POI, was collected and stored at -80°C.
Material and Methods

2.2.3 In vitro assays

2.2.3.1 Hdm2-mediated ubiquitination and neddylation
For Hdm2-mediated in vitro ubiquitination assays, 0.8 μl in vitro translated \(^{35}\)S-labeled substrate was incubated with 100 ng ubiquitin-activating E1 (baculovirus-expressed Uba1), 500 ng ubiquitin-conjugating E2 (bacterially expressed UbcH5b-His) and either 10 μg ubiquitin (SIGMA-ALDRICH) or 10 μg Nedd8 (bacterially expressed) in a total volume of 40 μl. Depending on the reaction, either 0.1 μM GST-Hdm2, 0.05 μM GST-Hdm2_RING (GST-tagged isolate RING domain of Hdm2), 0.33 μM Hdm2 or 0.43 μM Hdm2_RING was used as ubiquitin ligase E3 (all bacterially expressed). Additionally, each reaction contained 1 mM DTT, 2 mM ATP and 2 mM MgCl\(_2\) and was buffered by Tris at pH 7.5. After incubation at 25°C for 20 min, the reaction was stopped by the addition of 5x Laemmli sample buffer and analyzed by SDS-PAGE (2.2.5.2) and fluorography (2.2.5.4).

2.2.3.2 E6-E6AP-mediated p53 ubiquitination assay was performed under the same conditions as described above in 2.2.3.1, except that the ubiquitin ligase E3 was replaced by bacterially expressed tag-free 16E6 in the presence (in 3.2) or absence (in 3.3) of 0.8 nM baculovirus-expressed E6AP.

2.2.3.3 E6AP auto-ubiquitination
For E6AP in vitro auto-ubiquitination assay, 0.8 μl in vitro translated \(^{35}\)S-labeled E6AP was incubated with 0.2 μM bacterially expressed tag-free 16E6 in the presence of ubiquitin-activating E1, ubiquitin-conjugating E2, ubiquitin, DTT, ATP and MgCl\(_2\) under the conditions as described in 2.2.3.1.

2.2.3.4 Free ubiquitin chain formation assay
Free ubiquitin chain formation was performed by incubating 0.43 μM Hdm2_RING with ubiquitin-activating E1, ubiquitin-conjugating E2, ubiquitin, DTT, ATP and MgCl\(_2\) under the conditions as described in 2.2.3.1. After incubation, the reaction was stopped by the addition of 5x Laemmli sample buffer and analyzed by SDS-PAGE (2.2.5.2) and Western blot (2.2.5.5) by using the monoclonal ubiquitin antibody P4G7.

2.2.3.5 Measurement of fluorescence spectra
To prepare the samples for measurement, 3.3 μM compound B049 was diluted in the fluorescence sample buffer to a total volume of 1.5 ml and pipetted into a quartz cuvette. Four identical replicates were prepared. The samples were then placed into the luminescence spectrometer LS 50 (Perkin-Elmer) to measure the emission spectrum. After recording the emission intensity at
521 nanometers, 125 μl fluorescence sample buffer (serves as the background sample) or proteins (10 μM 16E6, 11E6 or BSA) were spiked into individual cuvettes followed by the second measurement of emission spectrum. The same procedure was repeated for the third measurement. Subsequently, 250 and 500 μl fluorescence sample buffer or proteins were spiked into individual cuvettes for the 4th and the 5th measurement, respectively.

2.2.4 In cellulo experiments

2.2.4.1 Maintenance of mammalian cells
Adherent mammalian cells were cultivated at 37°C, 95% humidity and 5% CO₂ in the Hareus CO₂ Incubator BBD 6220 (Thermo Scientific) on 10 cm dishes. All parental cell lines were cultured in DMEM supplemented with 10% FCS (v/v), 50 μg/ml Normocin and penicillin/streptomycin (1:100, v/v).

For passaging cells, the medium was aspirated. Cells were then washed with PBS and treated with 0.05% Trypsin-EDTA. After incubation at 37°C for 3-5 min, depending on the cell line, detached cells were collected with supplemented DMEM to stop the trypsin reaction. Cells were then resuspended and distributed according to experimental requirements.

2.2.4.2 Transfection of mammalian cells
Six to eight hours before transfection, cells were seeded at a density corresponding to 90% confluency at the time of transfection. For in cellulo ubiquitination and neddylation assays (see 2.2.4.8), H1299 cells were seeded onto 6 cm dishes and plasmid DNA in a total amount of 5.5 μg was transfected. For other experiments, cells were seeded onto six-well plates and plasmid DNA in a total amount of 2.5 μg was transfected. Transfection was performed by using Lipofectamine 2000 Reagent (invitrogen) according to the manufacturer’s instructions in a ratio of DNA: Lipofectamine = 1:2 (μg: μl). Cells were harvested or applied with other treatments 24-48 hours post-transfection depending on the cell line.

2.2.4.3 Generation of stable Flp-In expression cell line
The Flp-In System (invitrogen) allows integration of the ectopic gene of interest at a specific genomic location via homologous recombination, which avoids that the random integration disrupts the endogenous gene expression [180]. To generate a stable Flp-In cell line that ectopically expresses wild-type p53, the Flp-In host cell line (HeLa Flp, from Thomas U. Mayer’s group, University of Konstanz) was seeded onto 6 cm dishes. After six to eight hours, cells were co-transfected with 0.5 μg Flp-In plasmid DNA carrying the coding sequence of wild-type p53, and 5 μg expression construct of the Flp recombinase. After 24 hours, transfected cells were selected by 314 μg/ml hygromycin.
Material and Methods

2.2.4.4 Isolation of clonal cell lines by sorting
To generate single cell clones, cells were sorted onto 96-well plates by FACSria Illu II (BD Biosciences). For each cell line, 1 x 10^6 cells were resuspended in 1 ml FACS buffer and filtered (100 μm). Well plates were prepared with the medium supplemented with the appropriate antibiotic prior to sorting. Single cell clones were cultured until being confluent on 10 cm dishes and harvested for further analyses.

2.2.4.5 Cryopreservation of mammalian cells
Cells (90% confluent) on 10 cm dishes were trypsinized and collected by centrifugation at 500 x g for 5 min. The cell pellet was resuspended in 1.5 ml FCS containing 10% DMSO (v/v) and transferred into a cryogenic storage vial. These vials were then placed into Mr. Frosty cryo-freezing container (Nalgene) and were stored at -80°C. After one day, cryovials were stored in the vapor phase of liquid nitrogen.

2.2.4.6 Lysis of mammalian cells
Harvested mammalian cells were lysed in a volume dependent on cell number using RIPA lysis buffer. After incubation at 4°C, 1,000 rpm (VXR basic IKA Vibrax Shaker) for 30 min, samples were centrifuged at 16,000 x g for 30 min and the lysate was transferred into a fresh tube for protein analyses.

2.2.4.7 Crystal violet cell staining
To visualize cell colonies, cells on 6 cm dishes were washed twice with ice-cold PBS. Afterwards, cells were fixed and stained with the crystal violet solution by incubating for 20 min. Stained cells were washed with abundant tap water. Cells were then dried at room temperature and scanned with an office scanner.

2.2.4.8 In cellulo p53 ubiquitination and neddylation assays
H1299 cells on 6 cm dishes were co-transfected with expression constructs for p53 (0.1 μg), Mdm2 (1 μg) and β-galactosidase (0.5 μg). For in cellulo ubiquitination and neddylation assays, His-ubiquitin (2 μg) and His-Nedd8 (2 μg) expression constructs were co-transfected, respectively. DNA amount was taken up to 5.5 μg by using pcDNA3 empty vector. Transfection was performed as described in 2.2.4.2. Post-transfection (24 hours), cells were harvested and divided into two parts. While one-sixth total cells was analyzed by β-galactosidase assay (see 2.2.4.9), five-sixths cells were subjected to the isolation of ubiquitinated or neddylated proteins.

Cells were lysed by resuspension in 1 ml guanidinium hydrochloride lysis buffer under denaturing conditions. The cell lysate was then incubated with 50 μl Sepharose CL-4B (GE Healthcare) at 4°C for one hour under continuous rotation to remove proteins that unspecifically bind to the beads. After centrifugation at 16,100 x g, 4°C for 10 min, the supernatant was transferred to a fresh tube.
The pre-cleared lysate was then mixed with 50 μl Ni-NTA (Qiagen) and incubated at 4°C overnight under continuous rotation.

Subsequently, Ni-NTA beads were centrifuged at 500 x g, 4°C for 10 min. Beads were washed twice with 1 ml guanidium hydrochloride lysis buffer. For renaturation, beads were washed twice with 1 ml 1:4 mixture between guanidinium hydrochloride lysis buffer and T50 (pH 6.8), 20mM imidazole, followed by four times washing with 1 ml T50 (pH 6.8), 20mM imidazole. His-tagged proteins were then eluted by using Laemmli sample buffer supplemented with 200 mM imidazole. Samples were normalized with transfection efficiency and analyzed by SDS-PAGE (2.2.5.2) and Western blot (2.2.5.5) via the monoclonal p53 antibody DO-1.

2.2.4.9 β-galactosidase assay

To determine the transfection efficiency of in cellulo ubiquitination and neddylation assays, expression constructs for β-galactosidase were co-transfected. The DNA amount of β-galactosidase construct displayed one-tenth total DNA amount. After harvest, the cell lysate was prepared as described in 2.2.4.6 by using 100 μl RIPA lysis buffer. The lysate (10 μl) was then incubated with 100 μl Buffer Z and 10 μl ONPG in a 96-well plate. Each sample was prepared in duplicates. For the blank reference, 10 μl RIPA lysis buffer, instead of lysate, was used. The reaction was incubated at 37°C until an adequate yellow color appeared. The absorbance was measured at 405 nanometers in a micro-plate reader (VICTOR³ Multilabel Reader, Perkin Elmer).

2.2.5 Protein analysis

2.2.5.1 BCA assay

To determine the protein concentration of the cell lysate, the bicinchoninic acid (BCA) assay was performed using the Pierce BCA Protein Assay Kit (Thermo Scientific) according to manufacturer’s instructions. Each sample was prepared in duplicates by mixing 10 μl lysate dilution and 100 μl working reagent. After incubation at 37°C for 30 min and cooling down at room temperature for 5 min, the absorbance was measured at 560 nanometers by the VICTOR³ Multilabel Reader (Perkin Elmer).

2.2.5.2 SDS-PAGE

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to the protocol of Laemmli [181]. According to the size of the analyzed protein, 8%, 10% or 12.5% final concentration of acrylamide in separating gels were used. Prior to loading onto the gel, protein samples were incubated with Laemmli sample buffer at 95°C for 10 min under reducing conditions. Electrophoresis was carried out at a constant current of 40 mA. Proteins were then visualized by either Coomassie Blue staining (2.2.5.3) or Western blot (2.2.5.5). Radio-labeled proteins were detected via fluorography (2.2.5.4).
2.2.5.3 Coomassie Blue staining
After SDS-PAGE, gels were stained with Coomassie Blue for 15 min at room temperature and destained for 30 min by the destain solution.

2.2.5.4 Fluorography
For the detection of $^{35}$S-labeled proteins, gels were fixed for 20 min in the destain solution. Afterwards, the gel was incubated for 20 min in Autoradiography Enhancer (Perkin Elmer). After drying at 80°C for two hours, gels were subjected to an Imaging Plate BAS-MS 2040 (Fujifilm) and signals were detected by the BIO-IMAGING ANALYZER IPR-1000 (Fujifilm).

2.2.5.5 Western blot
After SDS-PAGE, gels were equilibrated with the transfer buffer. The PVDF (polyvinylidene difluoride) membrane (Millipore) was first activated in methanol and consecutively washed in transfer buffer. Western blotting was performed at 60 V, room temperature for 90 min in a wet Trans-Blot Electrophoretic Transfer Cell (BIO-RAD). Subsequently, the membrane was incubated in 5% milk (w/v) in TNE-T at 4°C overnight, followed by two washes with TNE-T at room temperature for 10 min. Incubation with the primary antibody was performed at room temperature for one hour. After three washes with TNE-T, the blot membrane was incubated at room temperature for one hour with the secondary antibody coupled to horseradish peroxidase, again followed by three washes with TNE-T. The blot was then developed using Western Lightning Plus-ECL (Perkin Elmer) in the LUMINESCENT IMAGE ANALYZER LAS-3000 (Fujifilm).

2.2.5.6 Dot blot
To perform the dot blot, proteins were directly spotted onto the nitrocellulose membrane (Whatman). After air-drying at room temperature, the blot was stained with Ponceau S solution for the direct protein detection or was analyzed by other approaches.

2.2.5.7 Detection of fluorescence on dot blot
The blot spotted with proteins was first blocked via incubation with 5% BSA (w/v) in TBS-T at room temperature for one hour, followed by two washes with TBS-T at room temperature for 10 min. The blot was then incubated with 10 μM compound B049 in TBS-T at room temperature for 30 min. After three washes with TBS-T at room temperature for 10 min, the fluorescence was detected at 473 nanometers by the FLUORESCENT IMAGE ANALYZER FLA-5000 (Fujifilm).
3 Results

3.1 Modulation of TP53 expression by RNAi and CRISPR-Cas9

The tumor suppressor p53 is well-known for its ability to induce cell cycle arrest, senescence and apoptosis in cells that have acquired oncogenic potential. Furthermore, p53 functions have been expanded beyond the prevention of tumor initiation: By affecting energy metabolism, p53 restrains the proliferation of tumor cells [182], which underpins the role of p53 in tumor suppression. Therefore, it is conceivable that cancer cells prefer loss of functional p53.

Intriguingly, previous work in our laboratory indicated that the complete loss of p53 expression by RNA interference (RNAi) appears to be a difficult task in HPV (human papillomavirus)-positive cells, which express wild-type p53 [183]. The introduction of an RNAi construct against p53 mRNA into the HPV18-positive Hela cells did reduce p53 protein levels in the mixed population of transfected cells. To corroborate the rigorous abrogation of p53 expression, single cell clones with stable knockdown of p53 expression were established. Of note, from hundreds of clones tested, only two showed a complete loss of p53 expression under normal growth conditions. When treated with the anticancer drug actinomycin D, which triggers accumulation of p53 proteins [184], minute amounts of p53 can be detected in the two clones. Moreover, a similar phenomenon was observed with the HPV16-positive SiHa cell line and none of established single clones exhibited full depletion of p53. Accordingly, it appears that in HPV-positive cells, p53 protein levels can be reduced but still need to be maintained at a certain level, suggesting an unexpected, potential pro-survival function of wild-type p53. In this work, we intended to address whether or not p53 may have a similar pro-survival role in HPV-negative cell lines by investigating the effect of RNAi-mediated p53 abrogation on cell proliferation. In addition, we also exploited the recently burgeoning CRISPR-Cas9 approach to knockout the TP53 gene.

3.1.1 Knockdown of p53 expression by bicistronic shRNAmir-IRES system

RNAi has become a widely used approach for knockdown of gene expression via degradation of mRNA. Nowadays, researchers implement RNAi by introducing either small interfering RNA (siRNA) or a construct encoding an artificial short hairpin RNA (shRNA) into cells. Although synthetic siRNAs instantly trigger degradation of the complementary mRNA in the cytosol upon penetrating cells, their short lifespan of about 48 hours limits the duration of action [185]. In contrast, vector-based expression of shRNA allows sustained downregulation of gene expression. After introduction into cells, the vector is transported into the nucleus. Transcribed shRNA is then processed by the endogenous micro RNA (miRNA) biogenesis machinery into siRNA [186]. Moreover, once the vector is integrated into genomic DNA, constitutive expression of shRNA enables the generation of cell lines possessing stable gene silencing.
To monitor the introduction of shRNA constructs into cells, DNA sequences encoding transfection markers such as reporters or antibiotic resistance proteins are often co-transfected. The expression of shRNA relied originally on the precise transcription driven by RNA polymerase III (pol III) [187], whereas transcription of messenger RNA (mRNA) of proteins is mediated by RNA polymerase II (pol II). As a consequence, the expression of shRNA and the transfection marker were driven by distinct promoters and thus differed from each other. In contrast to the original shRNA system, the transcription of endogenous miRNA is driven by RNA pol II [188]. Embedding the shRNA sequence into the context of the human endogenous miR-30 precursor miRNA (pre-miRNA), termed “shRNAmir”, is able to be expressed under the control of the CMV promoter, an RNA pol II promoter, and has been shown to effectively reduce the levels of mRNA containing the complementary target site [189].

Here, we designed a bicistronic shRNAmir-IRES system, where shRNAmir and the puromycin resistance gene (Puro\textsuperscript{R}) were combined into the same expression cassette and linked by an IRES (internal ribosome entry site) element (Figure 10). The transcription of this cassette results in only one transcript containing both components. The stem-loop structure of shRNAmir is released from the transcript and further processed into siRNA through the miRNA biogenesis pathway. Meanwhile, the liberated IRES-Puro\textsuperscript{R} mRNA is exported into the cytosol. Upon recruitment of ribosomes to the IRES element, puromycin-N-acetyltransferase is translated. Therefore, this system enables the simultaneous expression of siRNA and a resistance marker against an antibiotic, in our case puromycin. As shRNAmir and the resistance marker are expressed from the same transcript, any cell that is resistant to the puromycin selection, by definition, produces siRNA.

**Figure 10 Bicistronic shRNAmir‐IRES system**

The cistron contains shRNAmir, an IRES element and puromycin resistance gene (Puro\textsuperscript{R}). After transcription driven by the CMV promoter, the stem-loop structure of shRNAmir (pri-miRNA) is processed into pre-miRNA by the endogenous miRNA biogenesis machinery. Pre-miRNA is then exported into the cytosol and further processed into siRNA. Similarly, the IRES-Puro\textsuperscript{R} mRNA is exported into the cytosol and translated with the assistance of the IRES sequence to recruit ribosomes, generating puromycin-N-acetyltransferase. CMV: cytomegalovirus; IRES: internal ribosome entry site; pri-miRNA: primary micro RNA; pre-miRNA: precursor micro RNA; siRNA: small interfering RNA.
We designed four shRNAmir sequences targeting different sites of p53 mRNA (Table 1 and Figure 11). While p53-i_II and III target the cDNA region of p53 encoding the DNA binding domain (DBD), p53-i_I and IV are complementary to the sequences within the 5’ and 3’ untranslated region (UTR), respectively.

<table>
<thead>
<tr>
<th>RNAi</th>
<th>Target sequence</th>
</tr>
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<tbody>
<tr>
<td>p53-i_I</td>
<td>(5’UTR) AAAAGUCUAGAGCCACCGU</td>
</tr>
<tr>
<td>p53-i_II</td>
<td>(948i) CACUACAACUACAGUGUA</td>
</tr>
<tr>
<td>p53-i_III</td>
<td>(1026i) GACUCCAGUGGUAACUCAC</td>
</tr>
<tr>
<td>p53-i_IV</td>
<td>(3’UTR) AGGGAGUUUUGGGAGAUGU</td>
</tr>
</tbody>
</table>

Table 1 Target sequences of p53 mRNA knockdown constructs

Four different shRNAmir sequences for p53 mRNA knockdown were designed. Target sites of respective shRNAamirs are shown in Figure 11.

Figure 11 Target sites of knockdown constructs on p53 mRNA

Target sites of each designed shRNAmir are denoted as red bars. UTRs and the open reading frame of full-length p53 mRNA are shown as gray and blue boxes, respectively. Coding sequences of p53 main functional domains are shown as solid light blue boxes. While p53-i_II and III target the region encoding the DBD, p53-i_I and IV target 5’ and 3’ UTR, respectively. UTR: untranslated region; TAD: transactivation domain; DBD: DNA binding domain; TD: tetramerization domain; CR: C-terminal regulatory region. Numbers represent the residue positions of the full-length human p53 protein.

To evaluate the knockdown efficiency, osteosarcoma U-2 OS cells, which express wild-type p53, were transiently transfected with designed p53 knockdown constructs. In addition, the empty vector pIRESpuro and a “non-silencing” RNAi (non-i) construct, which does not target any known human transcript, were also transfected to serve as controls. Cell lysates were prepared 48 hours post-transfection and the same protein amount of each sample was subjected to SDS-PAGE. Knockdown efficiency was determined by Western blot analysis using an antibody against p53. As shown in Figure 12, transfection of the p53-i constructs I, II and IV resulted in a decrease in p53 levels, whereas p53-i_III did not significantly change p53 protein levels as compared to vector and non-i controls. It is noteworthy that knockdown efficiency of p53-i_I with about 20% was reproducibly observed and that p53-i_II and IV consistently showed higher knockdown efficiency in other independent experiments (data not shown). Thus, except for p53-i_III, all p53-i constructs exhibited the ability to knockdown p53 expression in U-2 OS cells. Furthermore, the rather low knockdown efficiency is likely due to the low transfection efficiency of at best 40% in U-2 OS.
Results

To investigate whether knockdown of p53 expression affects cell proliferation, we performed a colony formation assay. HPV-negative U-2 OS cells were transfected with pIRESpuro empty vector, the non-i construct or p53 knockdown constructs. Transfected cells were then selected by treatment with the antibiotic puromycin and surviving cell colonies were visualized by crystal violet staining. As shown in Figure 13, cells transfected with the empty vector or non-i construct showed similar efficiency in colony formation after puromycin selection, indicating that expression of shRNAmir per se does not significantly influence cell viability. In contrast, cells transfected with p53-i constructs I, II or IV severely suffered from puromycin treatment. Of note, although here cells transfected with p53-i_III exhibited less colony formation as compared to vector and non-i controls, colony numbers obtained with p53-i_III were reproducibly comparable to those of the non-i control in other independent experiments (data not shown).

As shown above (Figure 12), however, p53-i_III did not induce a knockdown of p53 expression, indicating that cell viability correlates with the preservation of p53 protein expression within cells. Additionally, surviving cells from p53-i_III experiments were also analyzed by Western blot and did not show any knockdown of p53 expression (data not shown). In conclusion, the data obtained indicate that abrogation of p53 expression might constrain proliferation of U-2 OS cells.

Figure 12 Knockdown efficiency of p53-i constructs in U-2 OS cells
U-2 OS cells were transfected with either pIRESpuro empty vector or indicated shRNAmir-IRESpuro constructs. Cells were harvested and lysed 48 hours post-transfection. The total protein amount of cell lysates was determined by BCA assay (2.2.5.1). Each cell lysate (100 μg) was analyzed by SDS-PAGE and Western blot using the monoclonal p53 antibody DO-1. For the loading control, 10 μg of each cell lysate were analyzed using the monoclonal tubulin antibody DM1A. Band intensity of p53 was quantified as indicated at the bottom. Running positions of molecular mass standards (kDa) are indicated on the left.

3.1.1.1 Cells transfected with p53-i constructs do not survive puromycin selection
To investigate whether knockdown of p53 expression affects cell proliferation, we performed a colony formation assay. HPV-negative U-2 OS cells were transfected with pIRESpuro empty vector, the non-i construct or p53 knockdown constructs. Transfected cells were then selected by treatment with the antibiotic puromycin and surviving cell colonies were visualized by crystal violet staining. As shown in Figure 13, cells transfected with the empty vector or non-i construct showed similar efficiency in colony formation after puromycin selection, indicating that expression of shRNAmir per se does not significantly influence cell viability. In contrast, cells transfected with p53-i constructs I, II or IV severely suffered from puromycin treatment. Of note, although here cells transfected with p53-i_III exhibited less colony formation as compared to vector and non-i controls, colony numbers obtained with p53-i_III were reproducibly comparable to those of the non-i control in other independent experiments (data not shown).

Figure 13 U-2 OS cells transfected with p53-i_I, II and IV constructs fail to survive puromycin selection
U-2 OS cells were transfected with either pIRESpuro empty vector or indicated shRNAmir-IRESpuro constructs. After 48 hours, cells were treated with 3 μg/ml puromycin for 14 days. Formation of cell colonies was visualized by crystal violet staining (2.2.4.7).

As shown above (Figure 12), however, p53-i_III did not induce a knockdown of p53 expression, indicating that cell viability correlates with the preservation of p53 protein expression within cells. Additionally, surviving cells from p53-i_III experiments were also analyzed by Western blot and did not show any knockdown of p53 expression (data not shown). In conclusion, the data obtained indicate that abrogation of p53 expression might constrain proliferation of U-2 OS cells.
To substantiate the notion that the cytotoxicity observed above was specifically caused by knockdown of p53 expression, the colony formation assay was performed in the p53-null non-small cell lung carcinoma cell line, H1299. Knockdown constructs that induced cytotoxicity in U-2 OS cells, namely p53-i_I, II and IV, were transfected into H1299 cells followed by puromycin treatment (Figure 14). Transfection of neither non-i control nor p53-i constructs caused significant cell death. Slightly inferior colony formation was observed in cells transfected with p53-i_II, implying a mild off-target effect of this construct on cell viability. In conclusion, the overall high viability observed in H1299 cells upon puromycin treatment suggested that the cytotoxicity observed in U-2 OS cells is probably a result of RNAi-mediated knockdown of p53 expression.

We further examined the generality of the p53 knockdown-mediated cytotoxicity in several cell lines (summarized in Table 2). Regardless of p53 status (wild-type or mutant p53), knockdown of p53 expression exhibited striking cytotoxicity in all tested cancer cell lines, including C-33A, HeLa and MCF-7. Moreover, transfection of p53-i constructs also led to cell death of immortalized HEK-293 cells, which are not cancerous. Taken together, p53 might be required for cell proliferation in cancer as well as non-cancer cells. Moreover, this novel pro-survival function of p53 appears to be a common feature of wild-type and mutant p53 proteins.

Table 2 Transfection of p53-i constructs leads to cytotoxicity in various p53-positive cell lines

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Origin</th>
<th>p53 status</th>
</tr>
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<tbody>
<tr>
<td>C-33A</td>
<td>cervical carcinoma</td>
<td>mutant p53 (R273C)</td>
</tr>
<tr>
<td>HEK-293</td>
<td>human embryonic kidney</td>
<td>wild-type, inactivated</td>
</tr>
<tr>
<td>HeLa a)</td>
<td>cervical adenocarcinoma</td>
<td>wild-type</td>
</tr>
<tr>
<td>MCF-7</td>
<td>breast adenocarcinoma</td>
<td>wild-type</td>
</tr>
</tbody>
</table>

Figure 14 Transfection of p53-i constructs does not cause cytotoxicity in H1299 cells

H1299 cells were transfected with indicated shRNAmir-IRESpuro constructs. After 24 hours, cells were treated with 4 μg/ml puromycin for 14 days. Formation of cell colonies was visualized by crystal violet staining (2.2.4.7). The experiment was performed by Jasmin Taban under supervision.

Table 2 Transfection of p53-i constructs leads to cytotoxicity in various p53-positive cell lines

Indicated cell lines were transfected with shRNAmir-IRESpuro constructs of non-i, p53-i_I, II or IV. After treatment with puromycin, formation of cell colonies was visualized by crystal violet staining (2.2.4.7). All cell lines exhibited the similar result to that shown in Figure 13. Experiments were performed by Jasmin Taban under supervision.

a) The experiment was performed using HeLa Fp stable cell line, a kind gift from Thomas U. Mayer (University of Konstanz).
As RNAi approaches are often accompanied by off-target effects, results obtained from RNAi experiments need to be validated by rescue experiments using RNAi-resistant versions of the target mRNA. We established a series of stable cell lines that ectopically express different p53 variants (summarized in Table 3). The stable cell lines were then transfected with p53-i constructs to examine their proliferation in the context of loss of endogenous p53. However, none of the ectopically expressed full-length p53 proteins (wild-type, R273H, R273C) was able to rescue cell proliferation from the cytotoxic effect caused by knockdown of endogenous p53 expression.

<table>
<thead>
<tr>
<th>Ectopic p53</th>
<th>Host cell line</th>
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<td>U-2 OS</td>
<td>wild-type</td>
<td>Mutant and wild-type p53 proteins share the same potential pro-survival function.</td>
</tr>
<tr>
<td>p53 R273H</td>
<td>C-33A</td>
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<td>HeLa</td>
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<td>Loss of endogenous p53 function is supposed to be rescued by ectopic expression of the same version of p53 protein.</td>
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<tr>
<td>p53β R273C</td>
<td>C-33A</td>
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<td>p53 isoforms are involved in the pro-survival function.</td>
</tr>
<tr>
<td>p53γ R273C</td>
<td>C-33A</td>
<td>p53 R273C</td>
<td>p53 isoforms are involved in the pro-survival function.</td>
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<tr>
<td>TP53 R273C</td>
<td>U-2 OS</td>
<td>wild-type</td>
<td>More than one of the p53 isoforms are required for cell proliferation. Mutant and wild-type p53 proteins share the same potential pro-survival function.</td>
</tr>
</tbody>
</table>

Table 3 Ectopic p53 proteins fail to rescue cells from p53-i constructs-induced cytotoxicity

Stable cell lines ectopically expressing different p53 variants were established. The rationale why a given p53 variant may rescue cell proliferation is listed. Stable cell lines were transfected with shRNAmir-IRESpuro constructs of non-i, p53-i_1, II or IV. After treatment with puromycin, formation of cell colonies was visualized by crystal violet staining (2.2.4.7). a) Experiments were performed by Jasmin Taban under supervision. b) Stable cell line was established using Flp-In system with HeLa Flp cells (2.2.4.3), a kind gift from Thomas U. Mayer (University of Konstanz). All p53 expression constructs were designed such that they are not RNAi-resistant to p53-i_II. However, they all lack the UTR sequences of p53 mRNA and thus cannot be targeted by p53-i_1 and IV.
As none of the full-length p53 proteins was able to rescue cell proliferation, we assumed that other p53 isoforms are involved in the potential pro-survival function. TP53 has been described to express at least 12 different isoforms (Figure 2) [51] and all of them are targeted by our p53-i constructs. We first examined the most abundantly expressed isoforms p53β and p53γ but both isoforms also failed to restore cell viability.

We then speculated that more than one of the p53 isoforms are required for cell proliferation. Since there was no information as to which isoform may be indispensable for cell survival, the most straightforward strategy to ascertain our argument was the ectopic expression of the whole TP53 gene (i.e. including introns and exons). Thus, we set out to generate a TP53 sequence starting from exon 2 up to the stop codon of full-length p53 in exon 11 (Figure 15 A, top). Exon 1 and the rest of exon 11 encode the 5’ and the 3’ UTR of p53 mRNAs, respectively, and are not involved in alternative expression of different p53 isoforms. Therefore, mRNAs transcribed from this TP53 construct are resistant to p53 i_I and IV. Moreover, given that the overexpression of full-length wild-type p53 induces apoptosis in transfected cells, the TP53 construct was designed based on the p53 R273C mutant in C-33A cells. The designed TP53 R273C sequence contains about 7,000 nucleotides. To perform the polymerase chain reaction (PCR) with high fidelity, amplification of the desired TP53 sequence from C-33A genomic DNA was divided into four fragments (Figure 15 A, bottom). PCR products of each fragment were in turn assembled into the expression vector pIRESneo via Gibson assembly (Figure 15 B) [178].

Figure 15 Cloning of TP53 R273C construct

(A) Schematic TP53 sequence in the TP53 R273C construct. Top: designed TP53 sequence encompasses from the exon 2 to the exon 11. Note that the region after the stop codon of full-length p53 in the exon 11 (of 1207 bps) is not shown and was not applied to the cloning. Bottom: Four fragments designed for PCR. Fragment I: 1764 bps, II: 1652 bps, III: 2491 bps, IV: 1107 bps. Arrows represent designed primers for PCR. Red and blue overhangs are required for Gibson assembly and indicate the sequence overlapping the vector and the adjacent fragment, respectively. (B) Via Gibson assembly (2.2.1.8), PCR product of fragment I was assembled with pIRESneo vector that was linearized by restriction enzymes NotI and EcoRI. The constructed plasmid was then linearized again by EcoRI digest, and PCR product of fragment II, Fragment III as well as IV were in turn cloned into the vector via the same strategy. After the insertion of fragment IV, the EcoRI site was destroyed.
Expression of the $TP53$ R273C construct was first inspected in the p53 null H1299 cell line. Transfection of the $TP53$ R273C construct led to the expression of a species that migrates at the same position as full-length p53 on SDS-PAGE, indicating that in general, the $TP53$ R273C construct is capable of appropriate mRNA splicing resulting in the expression of full-length p53 R273C. Other isoforms were not detected by Western blot analysis, which might be due to low expression levels (data not shown).

The $TP53$ R273C construct was transfected into U-2 OS cells to establish a stable cell line, which was then transfected with p53-i constructs followed by puromycin selection to perform colony formation assays (Figure 16). However, the $TP53$ R273C stable cell line behaved the same as the parental cell line as well as a control cell line that harbors only the empty vector pIRESneo. This suggested that even the ectopic expression of p53 isoforms cannot rescue the cytotoxic effects of the p53-i constructs used.

![Figure 16 TP53 R273C fails to rescue proliferation of U-2 OS cells transfected with p53-i constructs](image)

During this work, Fellmann et al identified an optimized shRNAmir form, termed miR-E, which strongly increases knockdown efficiency [190]. The miR-E shares the same core stem-loop structure with shRNAmir but possesses additionally modified 5’ and 3’ flanking regions of the stem-loop (Figure 17 A). We applied miR-E to knockdown p53 expression. As the shRNAmir form of p53-i_III showed neither effective knockdown of p53 expression (Figure 12) nor cytotoxicity towards U-2 OS cells (Figure 13), we first cloned p53-i_III in the miR-E form by inserting sequences of 5’ and 3’ flank region into the existing p53-i_III shRNAmir construct. For positive and negative controls, miR-E of p53-i_II and non-i were also constructed. The generated miR-E constructs were
transiently transfected into U-2 OS cells to evaluate the knockdown efficiency. As shown in Figure 17 B, the miR-E form of p53-i_II did not show a significant difference in knockdown efficiency as compared to the shRNAmir form. On the other hand, while shRNAmir of p53-i_III was consistently incompetent to reduce p53 levels, miR-E of p53-i_III resulted in a 30% knockdown of p53 expression. This result verified miR-E as an improvement of the shRNAmir system regarding knockdown efficiency.

A colony formation assay was then performed in U-2 OS cells using miR-E constructs to examine the p53-i_III-mediated cytotoxicity. Under this condition, not only p53-i_II but also p53-i_III led to cell death under puromycin treatment (Figure 18). Strikingly, while shRNAmir-non-i did not exhibit a cytotoxic effect on U-2 OS (Figure 13), transfection of miR-E-non-i construct unexpectedly disabled U-2 OS to grow in the presence of puromycin. Since the cytotoxic effect caused by knockdown of p53 expression could also not be rescued by ectopic expression of p53, this observation raised concerns as to whether the observed p53-i-associated cytotoxicity is indeed dependent on abrogation of p53 expression.

Figure 17 miR-E enhances the knockdown efficiency of p53-i_III

(A) Schematic comparison between shRNAmir and miR-E. shRNAmir and miR-E share the same core stem-loop structure and miR-E possesses additionally modified 5’ and 3’ flanking region of the stem-loop (denoted in yellow). (B) miR-E-p53-i_III exhibits superior knockdown efficiency to shRNAmir-p53-i_III does. U-2 OS cells were transfected with either pIRESpuro empty vector or indicated knockdown constructs. Cells were harvested and lysed 48 hours post-transfection. The total protein amount of cell lysates was determined by BCA assay (2.2.5.1). Each cell lysate (70 μg) was analyzed by SDS-PAGE and Western blot using the monoclonal p53 antibody DO-1. For the loading control, 10 μg of each cell lysate was analyzed using the monoclonal tubulin antibody DM1A. Band intensity of p53 was quantified as indicated at the bottom. Running positions of molecular mass standards (kDa) are indicated on the left.

A colony formation assay was then performed in U-2 OS cells using miR-E constructs to examine the p53-i_III-mediated cytotoxicity. Under this condition, not only p53-i_II but also p53-i_III led to cell death under puromycin treatment (Figure 18). Strikingly, while shRNAmir-non-i did not exhibit a cytotoxic effect on U-2 OS (Figure 13), transfection of miR-E-non-i construct unexpectedly disabled U-2 OS to grow in the presence of puromycin. Since the cytotoxic effect caused by knockdown of p53 expression could also not be rescued by ectopic expression of p53, this observation raised concerns as to whether the observed p53-i-associated cytotoxicity is indeed dependent on abrogation of p53 expression.

Figure 18 U-2 OS cells transfected with miR-E-IRESpuro constructs do not survive puromycin treatment

U-2 OS cells were transfected with either pIRESpuro empty vector or indicated miR-E-IRESpuro constructs. After 48 hours, cells were treated with 3 μg/ml puromycin for 14 days. Formation of cell colonies was visualized by crystal violet staining (2.2.4.7).
According to Fellmann et al, the miR-E system increases knockdown efficiency by boosting the processing of shRNAmir into siRNA [190]. This may suggest that the enhanced siRNA processing somehow impedes cell proliferation in the presence of puromycin. To understand the underlying mechanism, we constructed a pcDNA3puro vector, where the neomycin resistance gene was replaced by the puromycin resistance gene (\textit{Puro} \textsuperscript{R}). The miR-E-non-i construct was then co-transfected with either pcDNA3 or pcDNA3puro into U-2 OS cells to perform colony formation assays. After puromycin treatment, cells co-transfected with pcDNA3puro significantly formed more colonies than those co-transfected with the original pcDNA3 vector did (Figure 19 A, left). As the presence of an additional \textit{Puro} \textsuperscript{R}-containing construct was capable of rescuing cell viability, this result indicated that expression of \textit{Puro} \textsuperscript{R} in the miR-E-IRESpuro construct was somehow inhibited. Furthermore, viability of cells transfected with miR-E-p53-i_II and III was also restored by co-transfection of pcDNA3puro (Figure 19 A), supporting the notions that abolished or reduced expression of \textit{Puro} \textsuperscript{R} in the miR-E-IRESpuro system is a common consequence and that the cytotoxicity was not caused by knockdown of p53 expression.

We subsequently examined the cytotoxic effect of the three shRNAmir-IRESpuro p53-i constructs that led to cytotoxicity in p53-positive cell lines, namely p53-i_\textit{I}, II and IV (see 3.1.1.1). Strikingly, the cell proliferation was also rescued by co-transfection of pcDNA3puro construct in U-2 OS cells (Figure 19 B), which further corroborated that the cytotoxicity observed above was not dependent on RNAi-mediated abrogation of p53 expression but the lack of efficient expression of the puromycin resistance marker.
It was then addressed whether the expression of any gene preceded by the IRES element is also reduced in the shRNAmir-IRES system. We replaced the \textit{Puro} in the shRNAmir-IRESpuro system with the coding sequence of GFP (green fluorescent protein). Constructs were transiently transfected into U-2 OS cells to determine GFP expression. Consistent with the colony formation assay (Figure 13), cells transfected with shRNAmir-IRES-GFP construct in the context of p53-i_II showed only about 10% GFP expression as compared to those transfected with shRNAmir-IRES-GFP-non-i or the pIRES-GFP vector (determined by flow cytometry, data not shown).

Furthermore, the experiment was also performed in H1299 cells and similar results were obtained: Cells transfected with shRNAmir-IRES-GFP constructs of p53-i_II and IV expressed lower levels of GFP than those transfected with shRNAmir-IRES-GFP-non-i or the pIRES-GFP vector control did (Figure 20). Although transfection of shRNAmir-IRESpuro-p53-i constructs did not cause significant cell death in H1299 upon puromycin treatment (Figure 14), data obtained from GFP expression indicated that the inefficient expression of transfection markers may be a general phenomenon in the shRNAmir-IRES system.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure20.png}
\caption{shRNAmir-IRES-GFP-p53-i constructs exhibit reduced GFP expression}
\end{figure}

\textbf{H1299 cells were transfected with either pIRES-GFP empty vector or shRNAmir-IRES-GFP constructs. After 24 hours, expression of GFP was determined by fluorescence microscopy. Bright-field images are not shown, but cells were 100% confluent in all samples.}

\subsection*{3.1.2 Impact of p53 knockdown on U-2 OS proliferation}

The incompetence of the shRNAmir-IRES system to efficiently express the puromycin resistance marker motivated us to switch to the co-transfection of RNAi and \textit{Puro} constructs. As the miR-E system indeed enhanced knockdown efficiency (Figure 17 B), all four p53-i sequences (Table 1) were cloned as the miR-E form into a pIRES vector, where \textit{Puro} was removed. We first validated the ability of our RNAi constructs to knockdown p53 expression. To do so, we established a H1299 cell line (H1299_p53), which stably expresses the p53 mutant R273C from a cDNA containing not only the open reading frame but also the 5’ and 3’ UTRs of p53 mRNA (Figure 11). The miR-E-p53-i constructs were then co-transfected with pcDNA3puro (see 3.1.1.3) into H1299_p53. After transient selection with puromycin for three days, transfected cells were analyzed for expression levels of p53 protein by Western blot (Figure 21). Co-transfection of miR-E-p53-i_II, III and IV constructs led to significant knockdown of p53 expression, whereas miR-E-p53-i_I construct did not cause detectable reduction in p53 levels.
We then co-transfected the miR-E-p53-i constructs with pcDNA3puro vector into U-2 OS cells to investigate the impact of p53 expression knockdown on cell proliferation. After treatment with puromycin, cells transfected with miR-E-p53-i_I, II and III constructs showed an efficiency in colony formation similar to that of cells transfected with the empty vector (Figure 22 A). Colony numbers were less in the miR-E-p53-i_IV sample than in other miR-E-p53-i samples. As compared to non-i control, however, transfection of p53-i_IV construct did not show obvious cytotoxicity. In addition,
cells surviving puromycin selection were also harvested and analyzed for p53 levels by Western blot (Figure 22 B). While miR-E-p53-i_II and III led to about 50% knockdown of p53 expression, miR-E-p53-i_I and IV did not cause a significant reduction in p53 levels. Since p53 was still expressed at considerable levels, we were not able to draw a definite conclusion as to whether p53 knockdown is cytotoxic to U2OS cells or not.

### 3.1.3 Application of CRISPR-Cas9

As the RNAi approach could not provide conclusive evidence whether p53 is required for the proliferation of HPV-negative cells, we looked for an alternative system to regulate gene expression. Recently, the CRISPR-Cas9 (clustered regularly interspaced short palindromic repeats-CRISPR-associated protein 9) technology has become a mature and widely used tool for genome manipulation [165]. Thus, we made use of this system to address the effects of TP53 knockout on cell proliferation.

#### 3.1.3.1 Knockout of UBE3A expression via CRISPR-Cas9 system

In our laboratory, the applicability of CRISPR-Cas9 system to knockout gene expression within human cells was first examined with the UBE3A gene, which encodes the E6 associated protein (E6AP). We designed three different single guide RNAs (sgRNAs) targeting UBE3A using the online software CHOPCHOP [173]. The first two sgRNAs (sg-E6AP_i and ii) are complementary to a sequence within exon 3 and the third sgRNA (sg-E6AP_iii) targets a sequence within exon 4 of UBE3A (Figure 23 A). As negative control, a sgRNA targeting the coding sequence of “non-endogenous” fluorescent protein mCherry (sg-mCherry) was used. The sgRNAs were cloned downstream of the U6 promoter in a dual expression system where the U6 promoter drives the expression of sgRNA, and the CMV promoter drives the simultaneous expression of a modified form of S. pyogenes Cas9 as well as the neomycin resistance marker via an IRES element (Figure 23 B).

![Figure 23 Implementation of CRSPR-Cas9-mediated UBE3A knockout](image)  
(A) Target sites of E6AP sgRNAs on UBE3A. Target sites of each designed sgRNA are denoted as blue bars. Numbers represent the residue positions of the E6AP protein (isoform 1).  
(B) UBE3A knockout construct. The U6 promoter drives the expression of sgRNA. Moreover, simultaneous expression of Cas9 and NeoR via the IRES element is under the control of CMV promoter. Both expression cassettes are part of the same vector. Cas9: modified S. pyogenes Cas9 protein; CMV: cytomegalovirus; IRES: internal ribosome entry site; NeoR: neomycin resistance gene; sgRNA: single guide RNA.
The various sgRNA constructs were transfected into H1299 cells followed by neomycin treatment to establish stable cell lines. Cells of the mixed population were then harvested and expression levels of endogenous E6AP were analyzed by Western blot. The cell line H1299 K3, which harbors a stable knockdown of E6AP expression via RNAi [191], was included as control (Figure 24). The knockout constructs sg-E6AP_i and sg-E6AP_iii resulted in reduced E6AP expression, whereas H1299 parental cells and cells transfected with sg-E6AP_ii construct exhibited comparable E6AP levels. Together, our data revealed the ability of sg-E6AP_i and sg-E6AP_iii to guide CRISPR-Cas9-mediated knockout of UBE3A.

Western blot analysis of the stable cell lines did not show a complete abrogation of E6AP expression in the mixed population. To ascertain whether CRISPR-Cas9 is capable of 100% knockout of UBE3A (i.e. both alleles), cells of the mixed population of the sg-E6AP_iii stable cell line were sorted onto 96-well plates in a ratio of one cell per well, resulting in 107 single clones. However, only 13 clones survived during culturing. Expression levels of E6AP in the single clones were examined by Western blot analysis (Figure 25). In six single clones, E6AP protein was not expressed at detectable levels. Thus, our data validated the ability of CRISPR-Cas9 to execute the effective knockout of UBE3A. Furthermore, the other seven UBE3A knockout-negative single clones did not express the Cas9 protein, indicating the correlation between Cas9 expression and UBE3A knockout. The UBE3A gene in clone 1F2, 3A3 and 3D1 was analyzed by Sanger sequencing, which showed the presence premature stop codons in the vicinity of the CRISPR-Cas9-associated cleavage site (data not shown). This result verified UBE3A knockout at the genome level.
Next, we applied the CRISPR-Cas9 system to knockout TP53. We designed two sgRNAs (sg-p53_ii and iii) that are complementary to the region correspond to p53-i_II target site (Figure 26 A and Table 4). In addition, an sgRNA (sg-p53_i) was designed to target the 5’ region of the p53 open reading frame to avoid the expression of C-terminally truncated forms of p53. For the expression of Cas9, we tested the ubiquitin-puro fusion system (Figure 26 B) [192] as an alternative to the IRESneo_Cas9 construct (Figure 23 B). In this system, the CMV promoter drives the transcription of a cDNA sequence encoding a fusion protein consisting of the puromycin resistance marker, ubiquitin and a modified form of S. pyogenes Cas9. The transcript is translated into a single polypeptide, which is then cleaved by endogenous deubiquitinating enzymes (DUBs) at the C-terminal end of ubiquitin. As a result, the ubiquitin-tagged puromycin resistance marker and tag-free Cas9 protein are simultaneously expressed in a 1:1 ratio.

### Table 4 Target sequences of TP53 sgRNAs

Three different sgRNAs for TP53 knockout were designed. Target sequences of sg-p53_ii and iii partially overlap the region correspond to the target site of p53-i_II (left), which is denoted in red. Target sites of respective sgRNAs are shown in Figure 26 A.
We first examined cell growth of U-2 OS cells transfected with TP53 knockout constructs by performing a colony formation assay. However, this did not yield any conclusive results (Figure 27 A). Although cells transfected with sg-p53_ii construct grew slowly, a comparable growth pattern was also observed in cells transfected with empty vector or sg-mCherry constructs. In contrast, transfection of sg-p53_i and iii constructs showed even superior colony formation. Cells surviving the puromycin treatment were also analyzed by Western blot using an antibody against p53 (Figure 27 B). While sg-p53_i and ii resulted in about 80% and 60% knockout efficiency, respectively, sg-p53_iii showed about 40% reduction of p53 levels. These results indicated that the different growth patterns of U-2 OS cells shown in Figure 27 A are not related to the actual p53 protein levels within cells. Moreover, a p53 species with a lower molecular mass than full-length p53 was observed in cells transfected with sg-p53_ii and iii constructs (Δ p53 in Figure 27 B), suggesting the formation of a premature stop codon and thus, the expression of a truncated form of p53.
The lack of correlation between p53 expression levels and cell proliferation raised a question of whether cells that have obtained knockout of TP53 expression can still proliferate normally and maintain the knockout over the time of growth. To address this, we first repeated the experiment shown in Figure 27 A and reproducible colony formation for each construct was observed upon puromycin treatment (data not shown). Subsequently, identical numbers of surviving cells from each sample were seeded onto 6 cm dishes, termed as passage 0. To monitor any changes of transfected cells in proliferation, untransfected parental cells were also seeded. After five passages in puromycin-free cell culture media, all samples obtained similar colony numbers (Figure 28 A). Moreover, cells at passage 0 and 5 were also harvested and analyzed by Western blot (Figure 28 B). In cells transfected with sg-p53_i and ii constructs, no significant difference of p53 levels between passage 0 and 5 was observed, indicating that U-2 OS cells maintain a TP53 knockout over time and that depletion of p53 protein does not affect cell proliferation of U-2 OS (detailed in 4.1.1.2).

Figure 27 Proliferation of U-2 OS cells does not correlate with p53 expression levels

(A) Effect of TP53 knockout on U-2 OS proliferation. U-2 OS cells were transfected with either Puro-Ubi-Cas9 empty vector, mCherry or TP53 knockout constructs. After 48 hours, cells were treated with 3 μg/ml puromycin for 14 days. Formation of cell colonies was visualized by crystal violet staining (2.2.4.7). (B) Surviving cells in (A) were harvested and lysed. The total protein amount of cell lysates was determined by BCA assay (2.2.5.1). Each cell lysate (120 μg) was analyzed by SDS-PAGE and Western blot using the monoclonal p53 antibody DO-1. For Cas9 and loading control, 20 μg of each cell lysate was analyzed using the monoclonal Flag antibody M2 and the monoclonal tubulin antibody DM1A, respectively. Band intensity of p53 was quantified as indicated at the bottom. Running positions of molecular mass standards (kDa) are indicated on the left.
Figure 28 U-2 OS cells maintain TP53 knockout over several passages

(A) Effect of TP53 knockout on U-2 OS proliferation. U-2 OS cells were transfected with either Puro-Ub-Cas9 empty vector, mCherry or TP53 knockout constructs. After 48 hours, cells were treated with 3 μg/ml puromycin. Same numbers of surviving cells as well as untransfected parental cells were seeded onto 6 cm dishes and termed as passage 0. After five passages, cells were visualized by crystal violet staining (2.2.4.7). (B) Cells in (A) were harvested and lysed. The total protein amount of cell lysates was determined by BCA assay (2.2.5.1). Each cell lysate (120 μg) was analyzed by SDS-PAGE and Western blot using the monoclonal p53 antibody DO-1. For Cas9 and loading control, 20 μg of each cell lysate was analyzed using the monoclonal Flag antibody M2 and the monoclonal tubulin antibody DM1A, respectively. Band intensity of p53 was quantified as indicated at the bottom. Running positions of molecular mass standards (kDa) are indicated on the left. (A) was performed by Daniela Eichbichler under supervision.
Results

3.2 Inhibitory effect of p14ARF on Hdm2-mediated p53 ubiquitination

Owing to its growth-suppressive functions, the tumor suppressor p53 needs to be maintained at low levels under normal growth conditions. This is mainly achieved by the E3 ligase Mdm2 (murine double minute 2), which catalyzes ubiquitination of p53, thereby targeting p53 for proteasomal degradation [22]. However, once cells receive stress signals induced by mutated or overexpressed oncogenes, the ARF promoter is activated [193]. The expressed ARF protein attenuates Mdm2 activity towards p53 ubiquitination, which then leads to the accumulation of p53 in response to oncogenic stress.

The mechanisms by which ARF interferes with the Mdm2-p53 interaction remain controversial. First, it was previously reported that ARF interacts with Mdm2 and sequesters it in the nucleolus. As p53 is mostly localized in the nucleoplasm, nucleolar localization of Mdm2 leads to spatial separation from p53 and thus inhibition of p53 ubiquitination [112]. On the other hand, in vitro data from another group showed that ARF directly inhibits the ubiquitin ligase activity of Mdm2 towards both p53 ubiquitination and Mdm2 auto-ubiquitination [111], implying that the subnuclear localization does not play the central role in the ARF-Mdm2-p53 connection. In agreement with this, more recent in cellulo work also corroborated that the co-localization of ARF and Mdm2 in the nucleolus is not essential for attenuating p53 degradation, although it may enhance stabilization of p53 [194]. Taken together, the ARF-Mdm2 interaction apparently suffices for the direct inhibition of Mdm2-mediated ubiquitination. In this work, we accordingly attempted to delineate the biochemical mechanisms underlying the effect of ARF on the Mdm2-mediated p53 ubiquitination.

3.2.1 GST-p14ARF inhibits GST-Hdm2-mediated p53 ubiquitination in vitro

In order to enable the detection of the putative inhibitory effect of ARF on Mdm2-mediated p53 ubiquitination, the in vitro ubiquitination reaction has to be tightly controlled insofar as ubiquitination of p53 should not be too efficient to be able to monitor the inhibitory effect of ARF. Thus, conditions including E3 protein amount, incubation time and temperature of the reaction were optimized. By doing so, E3 protein amounts indicated in each experiment and incubation at 25 °C for 20 min were determined as optimal. Compared to incubation at 30 °C for 120 min, which is the standard in vitro ubiquitination condition for Mdm2 in our laboratory [195], ubiquitination under the optimized condition is less efficient. However, we were still able to readily detect ubiquitination (see below).

We first employed bacterially expressed GST (glutathione S-transferase)-tagged human Mdm2 (Hdm2) and human ARF (p14ARF) to address the p14ARF effect on Hdm2-mediated p53 ubiquitination. In vitro translated and $^{35}$S-labeled p53 was incubated with ubiquitin, the ubiquitin-activating enzyme Uba1, the ubiquitin-conjugating enzyme UbcH5b, GST-Hdm2 and increasing
amounts of GST-p14ARF under the conditions indicated above. After incubation, samples were subjected to SDS-PAGE followed by fluorography (Figure 29, left). The addition of GST-Hdm2 led to the covalent attachment of ubiquitin to p53, resulting in a shift of molecular mass on SDS-PAGE (3rd lane from the left in Figure 29), which indicated ubiquitination of p53. Moreover, this shift in molecular mass was reduced in the presence of increasing amounts of GST-p14ARF with simultaneous increase in intensity of the band representing non-modified p53, suggesting inhibition of p53 ubiquitination. Thus, consistent with published data [111], GST-p14ARF inhibited GST-Hdm2-mediated p53 ubiquitination in vitro.

As the isolated RING domain of Hdm2 (Hdm2_RING in the following) is sufficient to ubiquitinate p53 in vitro (personal communication in our laboratory), the effect of p14ARF on Hdm2_RING-mediated p53 ubiquitination was also evaluated. Importantly, the p14ARF binding site has been identified within the central region of Hdm2 encompassing amino acid 210 to 244 (Figure 4) [107]. Although the isolated RING domain lacks the known p14ARF binding site, GST-p14ARF unexpectedly inhibited Hdm2_RING-mediated p53 ubiquitination (Figure 29, right).

In addition to ubiquitination, Hdm2 catalyzes neddylation of p53 within cells [97]. We accordingly attempted to investigate the effect of p14ARF on Hdm2-mediated p53 neddylation. Although in vitro neddylation of p53 by Hdm2 has not yet been achieved via the E1 and E2 of neddylation cascade, Hdm2 is capable of p53 neddylation by using enzymes of the ubiquitin system in vitro (unpublished observation of our laboratory). Therefore, the in vitro neddylation assay of p53 was performed under in vitro ubiquitination conditions with the exception that ubiquitin was replaced by Nedd8. As shown in Figure 30 A, p53 was modified by ubiquitin and Nedd8 with similar efficiency. Furthermore, GST-p14ARF exhibited a comparable inhibitory effect on both p53 ubiquitination and neddylation, indicating that the p14ARF effect is not limited to ubiquitination.
When Hdm2_RING was used as E3 ligase, we again observed the unexpected inhibitory effect (Figure 30 B). Notably, GST protein alone did not inhibit p53 ubiquitination, suggesting that the inhibition was a specific effect of p14ARF (data not shown). Previously, a direct interaction between ARF and p53 was reported via in vitro co-precipitation assays [196]. Thus, our data may indicate that p14ARF inhibits p53 ubiquitination at the substrate level, namely through interaction with p53 rather than with Hdm2.

**Figure 30 GST-p14ARF inhibits both Hdm2-mediated ubiquitination and neddylation of p53**

In vitro translated 35S-labeled p53 was incubated with either (A) GST-Hdm2 or (B) GST-Hdm2_RING and with increasing amounts of GST-p14ARF under conditions described in 2.2.3.1. All GST fusion proteins were bacterially expressed. Reaction products were analyzed by SDS-PAGE followed by fluorography. Running positions of unmodified and modified forms of p53 are denoted by an arrow and an asterisk, respectively. Running positions of molecular mass standards (kDa) are indicated on the left. Experiments were performed by Julia Mader under supervision.

### 3.2.2 On-beads cleavage for processing recombinant SUMO fusion proteins

The specificity of GST-p14ARF to inhibit GST-Hdm2_RING-mediated p53 ubiquitination needed to be further corroborated. As GST is known to dimerize [197], it is of great importance to exclude that the observed inhibitory effect of GST-p14ARF on GST-Hdm2_RING was indirectly caused by GST-GST interaction. To validate this, we took advantage of the SUMO (small ubiquitin-like modifier) fusion technology [198] to prepare tag-free recombinant proteins (Figure 31). In this system, the coding sequence of the protein of interest (POI) is fused downstream of the SUMO cDNA on an expression vector. After expression as a fusion protein, the SUMO tag can be removed.
by the ubiquitin-like-specific protease 1 (Ulp1), resulting in tag-free POI. Conventionally, a His-tag is introduced at the N terminus of SUMO in order to allow protein purification via Ni\(^{2+}\)-affinity chromatography. Subsequently, the purified His-SUMO fusion protein is incubated with recombinant Ulp1. As the obtained protein preparation also contains Ulp1 and His-SUMO, POI needs to be isolated by a further purification step such as size exclusion or ion exchange chromatography, which leads to laborious procedures of protein purification.

**Figure 31 SUMO fusion expression system**

The expression vector encodes a polypeptide consisting of SUMO fused to the N terminus of the POI. Upon translation, the SUMO fusion protein is cleaved by the SUMO protease Ulp1, resulting in tag-free POI. The SUMO protein can also be N-terminally tagged to allow the protein purification via affinity chromatography. POI: protein of interest; SUMO: small ubiquitin-like modifier; Ulp1: ubiquitin-like-specific protease 1.

To simplify the purification procedure, the “on-beads cleavage” was performed. We fused GST to the N terminus of SUMO. As illustrated in Figure 32 A, GST-Ulp1 and GST-SUMO-Hdm2 are separately expressed in *E.coli*. After lysis, both GST-tagged proteins are isolated via batch affinity purification using glutathione-Sepharose. Subsequently, the two bead batches harboring GST-Ulp1 and GST-SUMO-Hdm2, respectively, were combined to allow cleavage. After centrifugation, GST-Ulp1 and GST-SUMO, which are bound to glutathione-Sepharose, are in the pellet, whereas the tag-free Hdm2 is retained in the supernatant.

As shown in Figure 32 B, the beads mixture prior to the GST-Ulp1-mediated cleavage contained species representing GST-Ulp1 and GST-SUMO-Hdm2. After cleavage, however, the band of GST-SUMO-Hdm2 disappeared and an additional band representing GST-SUMO emerged. The tag-free Hdm2 was exclusively observed in the supernatant after centrifugation. Moreover, the tag-free Hdm2 appeared purer than GST-Hdm2 as less background bands were observed. Apart from Hdm2, we also achieved the preparation of Hdm2\_RING and the HPV 16E6 protein using this approach, and all these proteins exhibited activity with respect to ubiquitination (see below). Taken together, our data demonstrate the applicability of the developed on-beads cleavage procedure for purification of GST-SUMO fusion proteins.
3.2.3 The N-terminal p14ARF peptide inhibits p53 ubiquitination in vitro

Having successfully prepared tag-free Hdm2 and Hdm2_RING, we confirmed the activity of both proteins towards p53 ubiquitination in vitro (Figure 33). However, when the GST-SUMO fusion approach was used to prepare p14ARF, we failed to obtain satisfactory protein yield and purity. Expression of GST-p14ARF resulted in higher protein yield but the purity was also not optimal (data not shown). Although tag-free Hdm2_RING is in principle sufficient to determine whether or not the inhibitory effect observed above was due to GST-GST dimerization, we considered that the inhibitory effect may be caused by contaminating proteins, which are co-purified with GST-p14ARF. To exclude this possibility, we made use of a synthetic peptide consisting of the N-terminal 20 amino acids of p14ARF (peptide 3 or “pep3” in the following), which has been shown to be capable of inhibiting Hdm2-mediated p53 ubiquitination in vitro [107].
3.2.3.1 Pep3 inhibits Hmd2- and Hdm2_RING-mediated ubiquitination of p53

The ability of pep3 to inhibit p53 ubiquitination was first verified in the in vitro p53 ubiquitination assay, where tag-free Hdm2 and Hdm2_RING were used as E3 ligases. Required amounts of these two enzymes were beforehand tested so that ubiquitination of p53 by both E3 ligases is similarly efficient (data not shown).

As shown in Figure 33, pep3 was able to inhibit p53 ubiquitination catalyzed by both Hdm2 and Hdm2_RING. Whereas 0.5 μM pep3 was already sufficient to inhibit Hdm2-mediated p53 ubiquitination (note the increase in unmodified p53), the decrease in Hdm2_RING-mediated p53 ubiquitination was only observed at higher concentration of pep3. Notably, 2.5 μM and 10 μM pep3 led to similar inhibition towards Hdm2- and Hdm2_RING-mediated p53 ubiquitination, respectively, suggesting that the inhibitory efficiency of pep3 exhibits about fourfold difference between Hdm2 and Hdm2_RING. Furthermore, pep3 was reconstituted in DMSO. Controls in the presence of DMSO only (4th and 9th lane from the left) did not detectably change p53 ubiquitination efficiency, indicating that p53 ubiquitination was specifically inhibited by pep3. Overall, these results revealed that p14ARF indeed inhibits Hdm2_RING-mediated p53 ubiquitination in vitro but less efficiently than it does Hdm2-mediated p53 ubiquitination.

![Figure 33 Pep3 inhibits both Hdm2- and Hdm2_RING-mediated p53 ubiquitination in vitro](image)

In vitro translated 35S-labeled p53 was incubated with either 0.33 μM Hdm2 or 0.43 μM Hdm2_RING and with increasing amounts of pep3 under conditions described in 2.2.3.1. Hdm2 and Hdm2_RING were bacterially expressed as tag-free proteins. Reaction products were analyzed by SDS-PAGE followed by fluorography. Ubiquitination of p53 mediated by Hdm2 and Hdm2_RING were adjusted in order to have the similar reaction efficiency. Running positions of unmodified and ubiquitinated forms of p53 are denoted by an arrow and an asterisk, respectively. Running positions of molecular mass standards (kDa) are indicated on the left.
Results

3.2.3.2 Pep3 inhibits E6-E6AP-mediated p53 ubiquitination

We hypothesized above that p14ARF might inhibit p53 ubiquitination at substrate level (see 3.2.1), which was supported by the observation that pep3 also inhibits Hdm2_RING-mediated ubiquitination (Figure 33). If this holds true, pep3 may also inhibit p53 ubiquitination catalyzed by other E3 ligases. p53 is also a known substrate of the HECT E3 ligase E6AP in complex with HPV E6 oncoproteins [154]. Thus, in vitro ubiquitination of p53 was performed using either Hdm2 or E6-E6AP as E3 ligase to further examine the pep3 effect on p53 ubiquitination.

Reaction efficiencies of Hdm2- and E6-E6AP-mediated p53 ubiquitination were first normalized by adjusting protein amounts of E6-E6AP (data not shown). Subsequently, increasing amounts of pep3 were added to the p53 ubiquitination assay and showed reproducible inhibitory effects on Hdm2-mediated p53 ubiquitination (Figure 34). In case of E6-E6AP, the inhibition of p53 ubiquitination was detectable only in the presence of the highest concentration of pep3, which is similar to the observation for Hdm2_RING-mediated p53 ubiquitination (Figure 33). This suggested that in both cases of Hdm2_RING and E6-E6AP, pep3 might interfere with p53 ubiquitination through the same mechanism, possibly by binding to p53. However, additional experiments would be required to substantiate this possibility.

Figure 34 Pep3 inhibits E6-E6AP-mediated ubiquitination of p53 in vitro

In vitro translated $^{35}$S-labeled p53 was incubated with either 0.33 μM Hdm2 or 16E6-E6AP complex (0.2 μM 16E6 and 0.8 nM E6AP) and with increasing amounts of pep3 under conditions described in 2.2.3.1 and 2.2.3.2, respectively. Hdm2 and 16E6 were bacterially expressed as tag-free proteins. E6AP was baculovirus-expressed. Reaction products were then analyzed by SDS-PAGE followed by fluorography. Running positions of unmodified and ubiquitinated forms of p53 are denoted by an arrow and an asterisk, respectively. Running positions of molecular mass standards (kDa) are indicated on the left.
3.2.4 Pep3 does not inhibit auto-ubiquitination of E6AP in vitro

Based on data from our and other laboratories, the first 20 amino acids of p14ARF are indeed sufficient to inhibit Hdm2-mediated p53 ubiquitination. However, the observation that pep3 inhibits Hdm2_RING- and E6-E6AP-mediated p53 ubiquitination with similar efficiency raised concerns about the specificity of this effect. To our knowledge, it has not been investigated whether pep3 specifically acts on Hdm2-mediated ubiquitination or interferes with ubiquitination in general. To further study pep3 effects on E6-E6AP-mediated ubiquitination, an E6AP auto-ubiquitination assay was performed [156]. In vitro translated E6AP was incubated with bacterially expressed E6 protein of HPV type 16 (16E6) under in vitro ubiquitination conditions, as 16E6 is known to enhance E6AP-mediated ubiquitination [199]. As shown in Figure 35, 16E6 significantly enhanced E6AP auto-ubiquitination. With increasing pep3 concentrations, a decrease in E6AP ubiquitination, if at all, was hardly detectable, which ruled out that pep3 acts directly on E6AP. In addition, this result provided clear evidence that pep3 does not work at the upstream steps of the ubiquitination cascade, namely E1 and/or E2. However, the exact mechanism by which p14ARF inhibits Hdm2-mediated p53 ubiquitination remained to be determined.

3.2.5 Pep3 inhibits Hmd2_RING-mediated ubiquitination of HdmX and Numb in vitro

As Hdm2 targets various proteins for ubiquitination, we then asked whether p14ARF also inhibits Hdm2-mediated ubiquitination of other substrates. We first tested the pep3 effect on ubiquitination of human MdmX (HdmX), another well-known substrate of Hdm2 [95]. The in vitro ubiquitination assay showed that pep3 also inhibits both Hdm2- and Hdm2_RING-mediated HdmX ubiquitination (Figure 36 A). This result confirmed the ability of p14ARF to inhibit Hdm2_RING-mediated ubiquitination although a significant inhibition of Hdm2_RING-mediated HdmX ubiquitination was only observed in the presence 10 μM pep3, like in case of p53 (Figure 33).
We also investigated another substrate of Hdm2, Numb [96]. However, under our in vitro ubiquitination conditions, full-length Hdm2 failed to efficiently ubiquitinate Numb (data not shown). We consequently used only Hdm2_RING as E3 ligase for Numb in the in vitro ubiquitination assay. Intriguingly, pep3 also inhibited Hdm2_RING-mediated Numb ubiquitination (Figure 36 B) with an efficiency similar to that observed above for HdmX and p53. Taken together, the data obtained imply that p14ARF does not act at substrate level but directly affects Hdm2_RING. Accordingly, the observed inhibition of E6-E6AP-mediated p53 ubiquitination by pep3 (Figure 34) might be caused by a different mechanism.

**Figure 36** Pep3 inhibits Hdm2_RING-mediated ubiquitination of HdmX and Numb in vitro

In vitro ubiquitination of (A) HdmX and (B) Numb. In vitro translated 35S-labeled HdmX and Numb were incubated with either 0.33 μM Hdm2 or 0.43 μM Hdm2_RING and with increasing amounts of pep3 under conditions described in 2.2.3.1. Hdm2 and Hdm2_RING were bacterially expressed as tag-free proteins. Reaction products were analyzed by SDS-PAGE followed by fluorography. Running positions of unmodified and ubiquitinated forms of HdmX or Numb are denoted by an arrow and an asterisk, respectively. Running positions of molecular mass standards (kDa) are indicated on the left.
3.2.6 Pep3 directly inhibits Hdm2_RING activity

In addition to catalyzing substrate ubiquitination, the isolated Hdm2 RING domain is also known to stimulate free ubiquitin chain formation in the absence of ubiquitination substrates [195]. Thus, to corroborate the direct effect of pep3 on Hdm2_RING activity, we investigated the effect of pep3 on Hdm2_RING-mediated ubiquitin chain formation. Ubiquitin was incubated with E1 (Uba1), E2 (UbcH5b), Hdm2_RING and increasing amounts of pep3 under in vitro ubiquitination conditions. Samples were subsequently subjected to SDS-PAGE and analyzed by Western blot using an antibody against ubiquitin (Figure 37). Addition of Hdm2_RING resulted in a shift of molecular mass of ubiquitin on SDS-PAGE, indicating free ubiquitin chain formation (3rd lane from the left in Figure 37). Furthermore, 2.5 μM pep3 already significantly inhibited ubiquitin chain formation. As we have shown above that pep3 does not interfere with the loading of ubiquitin onto E1 and E2 (see 3.2.4), this result not only ascertained the direct effect of p14ARF on Hdm2_RING activity but also implied a novel p14ARF binding site within the C-terminal RING domain of Hdm2.

Figure 37 Pep3 inhibits Hdm2_RING-mediated in vitro ubiquitin chain formation

Bacterially expressed tag-free Hdm2_RING (0.43 μM) was incubated with increasing amounts of pep3 under conditions described in 2.2.3.4. Note that conditions used here is the same as Hdm2_RING-mediated in vitro ubiquitination assay except for the absence of in vitro translated substrate. Reaction products were then analyzed by SDS-PAGE and Western blot using the monoclonal ubiquitin antibody P4G7. Running positions of mono-ubiquitin and conjugated ubiquitin chains are denoted by an arrow and an asterisk, respectively. Running positions of molecular mass standards (kDa) are indicated on the left.
3.2.7 Electrostatic effect of pep3 on ubiquitination

ARF proteins have an unusual amino acid composition. Human p14ARF consists of 132 amino acids, among which are 25 (19%) positively charged arginine residues but no lysine. These arginine residues are distributed throughout the entire protein. Pep3 possesses six arginine residues, which leads to a highly positive charge of this peptide at physiological pH. Thus, it is possible that the phenomena observed above were the result of potentially unspecific electrostatic effects. To evaluate this possibility, we studied the ability of peptides, with the same amino acids composition as pep3 but with either the reverse or scrambled sequence (Figure 38 A), to inhibit ubiquitination.

When Hdm2 was utilized as E3 ligase in the in vitro p53 ubiquitination assay, the reverse peptide surprisingly inhibited p53 ubiquitination with the efficiency similar to that of pep3 (Figure 38 B). In contrast, the inhibitory effect of the scrambled peptide was less significant as compared to pep3 and the reverse peptide. Although it also completely inhibited p53 ubiquitination at 10 μM, 2.5 μM scrambled peptides led to an inhibition of p53 ubiquitination similar to that caused by 0.5 μM of pep3 or the reverse peptide. Thus, the precise sequence arrangement apparently plays a certain role in pep3’s function. However, the similarity in inhibition shared by pep3 and the reverse peptide needed to be further examined. Additionally, the weak inhibitory ability of the scrambled peptide might represent the electrostatic effect, suggesting that the overall charge of pep3 also partially contributes to inhibition of p53 ubiquitination.

Figure 38 Reverse and scrambled p14ARF peptides inhibit Hdm2-mediated p53 ubiquitination
(A) Sequences of p14ARF peptides. Pep3 represents the first 20 amino acids of p14ARF [107]. Reverse and scrambled peptides consist of the same amino acids as pep3 but with different sequence arrangement. Arginine residues are denoted in red. (B) Effects of p14ARF peptides on Hdm2-mediated p53 ubiquitination in vitro. In vitro translated 35S-labeled p53 was incubated with 0.33 μM Hdm2 and with increasing amounts of p14ARF peptides under conditions described in 2.2.3.1. Hdm2 was bacterially expressed as a tag-free protein. Reaction products were analyzed by SDS-PAGE followed by fluorography. Running positions of unmodified and ubiquitinated forms of p53 are denoted by an arrow and an asterisk, respectively. Running positions of molecular mass standards (kDa) are indicated on the left.
Further examination into the effect of the reverse and scrambled peptides on Hdm2_RING activity showed that pep3 and the reverse peptide inhibited Hdm2_RING-mediated p53 ubiquitination with comparable efficiency, whereas the scrambled peptide showed lower efficiency (Figure 39 A). Moreover, a similar result was obtained for Hdm2_RING-mediated free ubiquitin chain formation (Figure 39 B). Taken together, these results indicated that pep3 inhibits Hdm2_RING-mediated ubiquitination, at least to a certain extent, in a specific manner rather than only via potential electrostatic effects.

Figure 39 Scrambled peptide inhibits Hdm2_RING activity less effectively than pep3 and reverse peptide do
Scrambled peptide inhibits Hdm2_RING-mediated (A) p53 ubiquitination and (B) free ubiquitin chain formation in vitro, but less efficiently than pep3 and reverse peptide do. In (A), in vitro translated [35S]-labeled p53 was incubated with 0.43 μM Hdm2_RING and increasing amounts of p14ARF peptides under conditions described in 2.2.3.1. Hdm2_RING was bacterially expressed as a tag-free protein. Reaction products were then analyzed by SDS-PAGE followed by fluorography. Running positions of unmodified and ubiquitinated forms of p53 are denoted by an arrow and an asterisk, respectively. In (B), Hdm2_RING (0.43 μM) was incubated with increasing amounts of p14ARF peptides under conditions described in 2.2.3.4. Reaction products were analyzed by SDS-PAGE and Western blot using the monoclonal ubiquitin antibody P4G7. Running positions of mono-ubiquitin and conjugated ubiquitin chains are denoted by an arrow and an asterisk, respectively. Running positions of molecular mass standards (kDa) are indicated on the left.
We also tested the reverse and scrambled peptides in E6-E6AP-mediated ubiquitination assays. Consistent with the data above, the reversed peptide behaved like pep3 in slightly inhibiting E6-E6AP-mediated p53 ubiquitination (Figure 40 A) and in exhibiting no inhibitory effect on E6AP auto-ubiquitination (Figure 40 B). Notably, the scrambled peptide inhibited neither E6-E6AP-mediated p53 ubiquitination nor E6AP auto-ubiquitination.

**Figure 40** Scrambled peptide does not inhibit 16E6-E6AP activity in vitro

The scrambled peptide inhibits neither (A) 16E6-E6AP-mediated p53 ubiquitination nor (B) E6AP auto-ubiquitination. In (A), in vitro translated 35S-labeled p53 was incubated with 16E6-E6AP complex (0.2 μM 16E6 and 0.8 nM E6AP) and increasing amounts of p14ARF peptides under conditions described in 2.2.3.2. 16E6 was bacterially expressed as a tag-free protein. E6AP was baculovirus-expressed. In (B), in vitro translated 35S-labeled E6AP was incubated with 0.2 μM 16E6 and with increasing amounts of p14ARF peptides under conditions described in 2.2.3.3. Reaction products were analyzed by SDS-PAGE followed by fluorography. Running positions of molecular mass standards (kDa) are indicated on the left. Running positions of unmodified and ubiquitinated forms of (A) p53 as well as (B) E6AP are denoted by an arrow and an asterisk, respectively.
3.2.8 Effect of p14ARF on p53 ubiquitination and neddylation in cellulo

In addition to in vitro assays, we were also interested in determining the effect of p14ARF on p53 ubiquitination within cells. Here we utilized mouse Mdm2 instead of human Hdm2 as E3 ligase since according to our experience, the construct encoding Mdm2 leads to stronger p53 ubiquitination in cellulo than the one encoding Hdm2 does. Because of 80% similarity of Mdm2 and Hdm2 at the amino acid sequence level, results obtained with Mdm2 can likely be extrapolated to p14ARF’s effect on Hdm2. As Mdm2 also catalyzes neddylation of p53 within cells [97], we investigated effects of p14ARF on both p53 ubiquitination and neddylation. To do so, the non-small cell lung carcinoma cell line H1299 (p53 null) was co-transfected with expression vectors encoding p53, Mdm2, β-galactosidase and either His-ubiquitin or His-Nedd8. In addition, increasing amounts of p14ARF expression vector were also included in the transfection. Cells were harvested 24 hours post-transfection and lysed. Ubiquitinated or neddylated proteins were isolated by Ni²⁺-affinity chromatography. The transfection efficiency of each sample was determined by β-galactosidase activity and normalized. Samples were then subjected to SDS-PAGE and analyzed by Western blot using an antibody against p53.

In Figure 41 A, significant amounts of modified p53 were only observed in the presence of both Mdm2 and His-ubiquitin/His-Nedd8, indicating that ubiquitination and neddylation are caused by overexpression of Mdm2. Co-transfection of p14ARF encoding plasmids led to a decrease in both modifications, suggesting that p14ARF also inhibits Mdm2 activity in cellulo. Furthermore, inhibition of p14ARF towards p53 ubiquitination was superior to that towards p53 neddylation. This could be explained by the notion that in the absence of p14ARF, p53 neddylation was more efficient than p53 ubiquitination, and thus higher amounts of p14ARF may be required to observe an inhibitory effect. Of note, low amounts of p14ARF (0.5 μg) appeared to slightly enhance p53 neddylation, while higher amounts of p14ARF (1 and 2 μg) resulted in inhibition of p53 neddylation. To ascertain this phenomenon, even lower amounts of p14ARF plasmids were transfected in the in cellulo p53 neddylation assay (Figure 41 B). Indeed, p53 neddylation was enhanced with increasing amounts of p14ARF plasmid transfected. However, when 2 μg of p14ARF plasmids were co-transfected, p53 neddylation was significantly decreased. Thus, p14ARF might exhibit a dual regulatory effect on p53 neddylation within cells: At low protein levels, p14ARF enhances p53 neddylation. In contrast, when p14ARF levels reach a certain threshold, it starts to interfere with p53 neddylation.
Figure 41 Effects of p14ARF on p53 ubiquitination and neddylation in cellulo
(A) p14ARF inhibits both Mdm2-mediated p53 ubiquitination and neddylation in cellulo. (B) Low levels of p14ARF slightly enhance Mdm2-mediated p53 neddylation in cellulo. H1299 cells were co-transfected with expression constructs for p53, Mdm2, β-galactosidase, His-ubiquitin or His-Nedd8, and p14ARF. Cells were harvested and lysed 24 hours post-transfection. Ubiquitinated or neddylated forms of p53 were isolated by Ni²⁺-affinity chromatography as described in 2.2.4.8. Samples were analyzed by SDS-PAGE and Western blot using the monoclonal p53 antibody DO-1. Transfection efficiency was determined by monitoring β-galactosidase activity (2.2.4.9). p53 input (unmodified form) and ubiquitinated or neddylated forms of p53 are denoted by an arrow and an asterisk, respectively. Running positions of molecular mass standards (kDa) are indicated on the left. (A) was performed by Nicole Richter-Müller.
Overexpression of p14ARF has been reported in many cancer cells possessing mutations in TP53, including H1299 [200]. If the dual effect of p14ARF on p53 neddylation is physiologically relevant, a reduction in basal levels of p14ARF in H1299 would conceivably further attenuate p53 neddylation. To study this possibility, we first established a H1299 p14ARF knockdown (p14ARF-i) cell line. As compared to the control cell line with non-silencing RNAi (non-i), a knockdown efficiency of more than 70% was achieved (Figure 42 A). We subsequently performed in cellulo p53 neddylation assays with H1299_non-i and H1299_p14ARF-i cell lines. However, there was no significant difference in p53 neddylation between non-i and p14ARF-i cell lines in the presence of 0.5 μg Mdm2 plasmids (Figure 42 B). Moreover, when 0.1 μg of Mdm2 plasmids were co-transfected, p14ARF-i cells even showed slightly higher p53 neddylation than non-i cells did, which argues against the possibility that low levels of p14ARF enhance p53 neddylation.

Figure 42 Knockdown of endogenous p14ARF expression in H1299 cells did not attenuate p53 neddylation
(A) Knockdown of p14ARF expression in H1299. H1299 cells were transfected with shRNAmir-IRESpuro constructs (see 3.1.1) of either non-i or p14ARF-i. After establishment of stable cell lines by treatment with 4 μg/ml puromycin, cells of the mixed population were harvested. Cells were lysed and the total protein amount of cell lysates was determined by BCA assay (2.2.5.1). Each cell lysate (250 μg) was analyzed by SDS-PAGE and Western blot using the monoclonal p14ARF antibody ARF 4C6/4. Band intensity of p14ARF was quantified as indicated at the bottom.

(B) Mdm2-mediated p53 neddylation within H1299_non-i and _p14ARF-i stable cell lines. The mixed population of stable cell lines in (A) were co-transfected with expression constructs for p53, Mdm2, β-galactosidase and His-Nedd8. Cells were harvested and lysed 24 hours post-transfection. Neddylated form of p53 was isolated by Ni²⁺-affinity chromatography as described in 2.2.4.8. Samples were analyzed by SDS-PAGE and Western blot using the monoclonal p53 antibody DO-1. Transfection efficiency was determined by monitoring β-galactosidase activity (2.2.4.9). p53 input (unmodified form) and neddylated form of p53 are denoted by an arrow and an asterisk, respectively. Running positions of molecular mass standards (kDa) are indicated on the left.
3.3 Fluorescence-based approach for early diagnosis of cervical cancer

The tumor suppressor p53 has been dubbed the “guardian of the genome” [118]. As genome instability is one of the underlying causes of cancer, it comes as no surprise that more than 50% of human cancers have been associated with mutations in TP53 [127]. Even in cancers retaining wild-type TP53, the functions of p53 proteins are often inactivated by alternative mechanisms. For instance, almost all cervical cancers are linked to infection with high-risk human papillomaviruses (HPVs), and all of these cancers appear to retain wild-type TP53. Instead, the viral oncoprotein E6 interacts with endogenous E6AP in the host cells, forming a functional E3 ligase complex and targeting p53 for ubiquitination as well as proteasomal degradation [149].

Early recognition of abnormal cell growth in tissues may prevent progression to invasive lesions. Abnormalities in cytology in the cervix is generally detected via the Papanicolaou smear test (Pap test), which allows diagnosis of cervical dysplasia at an early stage. However, this method lacks a quantitative readout and completely relies on visual monitoring of cytological abnormalities by experienced pathologists. Moreover, Pap tests detect cancerous tissues only in about 70% of cases and this low sensitivity needs to be compensated by regular repetition of the test [201]. In contrast to Pap tests, PCR-based genotyping of HPV DNA in cervical specimen has 90-95% sensitivity [202]. However, infection of high-risk HPVs is essential but not sufficient for cervical carcinogenesis. Statistical studies showed that 90% of cervical infections are cleared by the immune system within two years [203, 204]. Thus, a general screening program using HPV genotyping would identify too many patients who will not develop cervical cancer. In this project, we therefore aimed to develop a sensitive and specific technique to reliably diagnose cervical cancer at an early stage.

3.3.1 Fluorophore-conjugated peptide 11 inhibits E6-E6AP-mediated p53 ubiquitination

HPV E6 proteins recognize leucine (L)-rich LXXLL motifs in interacting proteins (reviewed in [205]). In 2009, Dymalla et al. identified another E6-binding motif consisting of the cysteine (C)-rich sequence CXChXCh (Figure 43) [206]. A peptide containing this motif (peptide 11 or pep11 in the following) showed slightly higher binding affinity than that containing the LXXLL motif. Notably, pep11 strictly interacts with the E6 oncoprotein of HPV type 16 (16E6). As pep11 is highly hydrophobic and thus barely soluble in aqueous solution, a series of mutants of pep11 were made to improve the solubility. The binding affinity to 16E6 of a solubility-improved variant, termed pep11** (Figure 43), was five times greater than that of the peptide containing the LXXLL motif [206]. Thus, pep11** possesses the potential for a sensitive tool to detect 16E6 proteins within cells.
Recently, one of our collaborators conjugated a fluorophore to a peptide to synthesize a fluorescent compound. Interestingly, when this compound was incubated with a given binding protein of the peptide, a change in emission intensity of the fluorophore was observed (unpublished data). We accordingly came up with the idea to conjugate a fluorophore to pep11 or pep11**. If the observation of our collaborator is a general phenomenon, we should detect a change in fluorescent property of the synthetic compound in the presence of 16E6. Moreover, expression of HPV E6 oncoproteins is a representative marker for the initiation of cervical carcinogenesis [159]. Thus, after incubation of the conjugated compound with a specimen derived from a patient, the change in the emission intensity could serve as a quantitative readout for the detection of 16E6 proteins. In other words, through the sensitive and specific interaction between pep11 variants and 16E6, we envisioned a precise early diagnosis of cervical cancer induced by HPV 16.

Figure 43 Sequences of pep11 and pep11**
Pep11 contains the novel 16E6 binding motif CXChXCh. Pep11** was modified from pep11 to improve solubility via replacement of several hydrophobic residues and addition of hydrophilic KEKE residues at N terminus (shaded in gray) [206]. Consensual cysteine and hydrophobic residues in the novel motif are denoted in red and blue, respectively. h: hydrophobic amino acid; X: any amino acid.

Figure 44 Pep11 and pep11** inhibit 16E6-E6AP-mediated p53 ubiquitination
In vitro translated 35S-labeled p53 was incubated with 30 nM 16E6 and increasing amounts of pep11 or pep11** under conditions described in 2.2.3.2. 16E6 was bacterially expressed as a tag-free protein. No recombinant E6AP protein was added in reactions as the reticulocyte lysate used for in vitro translation contains trace amounts of E6AP. Reaction products were analyzed by SDS-PAGE followed by fluorography. Running positions of unmodified and ubiquitinated forms of p53 are denoted by an arrow and an asterisk, respectively. Running positions of molecular mass standards (kDa) are indicated on the left.

We first intended to corroborate the capability of pep11 and pep11** to interact with 16E6 when conjugated with fluorophores. In addition to interaction with 16E6, pep11 is also known to inhibit 16E6-E6AP-mediated ubiquitination by competing with E6AP for 16E6 binding [206]. Consistently,
our data also showed that pep11 and pep11** inhibit 16E6-E6AP-mediated p53 ubiquitination (Figure 44). This indicated that the in vitro p53 ubiquitination assay is a suitable approach to test whether synthesized compounds retain the ability to bind to 16E6.

Various fluorophores were conjugated to either pep11 or pep11** by our collaboration partner. Then, the ability of each synthesized compound to inhibit 16E6-E6AP-mediated p53 ubiquitination was examined (exemplified in Figure 45). Furthermore, the inhibitory capacity of each compound was compared with that of its respective non-modified peptide (summarized in Table 5). To sum up, pep11** compounds generally showed a greater inhibitory effect on p53 ubiquitination than pep11 compounds did, probably owing to higher solubility. Except for B038, all conjugated compounds appeared to retain the property of pep11/pep11** to interact with 16E6.

![Figure 45 Most conjugated compounds retain the ability to inhibit 16E6-E6AP-mediated p53 ubiquitination](image)

In vitro translated 35S-labeled p53 was incubated with 30 nM 16E6 and increasing amounts of pep11, pep11**, or conjugated compounds under conditions described in 2.2.3.2. 16E6 was bacterially expressed as a tag-free protein. No recombinant E6AP protein was added in reactions as the reticulocyte lysate used for in vitro translation contains trace amounts of E6AP. Reaction products were analyzed by SDS-PAGE followed by fluorography. Running positions of unmodified and ubiquitinated forms of p53 are denoted by an arrow and an asterisk, respectively. Running positions of molecular mass standards (kDa) are indicated on the left. The inhibitory ability of each conjugated compound is listed in Table 5. For instance, as B049 and pep11** inhibited p53 ubiquitination with the similar efficiency, the inhibitory ability was indicated as “+++. Moreover, as compared to pep11, the inhibition of B043 towards p53 ubiquitination was impaired, but was observed at the highest concentration. Therefore, the inhibitory ability was indicated as “+”.

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We subsequently investigated whether 16E6 affects the photophysical properties of the conjugated compounds. As compound B049, which consists of Alexa Fluor 488 and pep11**, exhibited not only significant inhibition towards p53 ubiquitination (Figure 45) but also high solubility in aqueous solution (data not shown), we determined the emission spectrum of this compound in the presence of recombinant 16E6. To do so, the emission spectrum of 3.3 μM B049 was first measured in a volume of 1.5 ml. Subsequently, 16E6 was stepwise spiked into the sample to monitor the change in the emission spectrum. It is noteworthy that the addition of 16E6 did not shift the emission wavelength (data not shown). As background control, another sample was measured using the very same procedure but via the addition of sample buffer rather than 16E6.

For the evaluation, emission intensity values of the background sample were subtracted from those of the 16E6 sample to exclude quenching effects caused by sample dilution. Normalized values were then plotted in Figure 46.

The emission intensity of B049 rose with the addition of 16E6 (orange line in Figure 46) suggesting that 16E6 dequenched B049. As mentioned, pep11** interacts exclusively with 16E6 [206]. To ensure that the observed phenomenon specifically resulted from the interaction between pep11** and 16E6, effects of 11E6 (E6 of HPV type 11) and BSA on B049 emission intensity were also examined (gray and blue line in Figure 46, respectively). Strikingly, both 11E6 and BSA also

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Table 5 Inhibitory ability of conjugated compounds towards 16E6-E6AP-mediated p53 ubiquitination

The inhibitory ability of each conjugated compound was compared with that of its respective non-modified peptide (pep11 or pep11**). +++: Inhibition towards p53 ubiquitination was as good as the non-modified peptide; ++: Inhibition was slightly impaired, but comparable to the non-modified peptide; +: Inhibition was impaired, but inhibition was observed at the highest concentration; -: no inhibition. a) “F”: specified fluorophores generated by our collaboration partner; numbers represent the excitation wavelength of each fluorophore. b) Note that pep11 inhibits p53 ubiquitination less efficiently than pep11** (Figure 44).

3.3.2 Proteins lead to dequenching of the compound B049

We subsequently investigated whether 16E6 affects the photophysical properties of the conjugated compounds. As compound B049, which consists of Alexa Fluor 488 and pep11**, exhibited not only significant inhibition towards p53 ubiquitination (Figure 45) but also high solubility in aqueous solution (data not shown), we determined the emission spectrum of this compound in the presence of recombinant 16E6. To do so, the emission spectrum of 3.3 μM B049 was first measured in a volume of 1.5 ml. Subsequently, 16E6 was stepwise spiked into the sample to monitor the change in the emission spectrum. It is noteworthy that the addition of 16E6 did not shift the emission wavelength (data not shown). As background control, another sample was measured using the very same procedure but via the addition of sample buffer rather than 16E6. For the evaluation, emission intensity values of the background sample were subtracted from those of the 16E6 sample to exclude quenching effects caused by sample dilution. Normalized values were then plotted in Figure 46.

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increased the emission intensity of B049, though with lower efficiency. This result indicated that 16E6 is potentially capable of altering the photophysical properties of Alexa Fluor 488 but at least to a certain extent, in an unspecific manner.

The specificity of the interaction between 16E6 and compound B049 was further inspected by dot blot analysis. 16E6 and Hdm2_RING (see 3.2) proteins were spotted onto nitrocellulose membrane, which was then incubated with B049 to allow interaction. After washing out unbound B049, the blot was analyzed by fluorescence spectroscopy (Figure 47, left). Fluorescence of Alexa Fluor 488 was detected in the area spotted with both 16E6 and Hdm2_RING proteins. Consistent with above data, this result suggested unspecific interaction between B049 and proteins, rendering specific detection of 16E6 by designed fluorescence-based approach impossible.

Each protein (1 μg) was spotted onto nitrocellulose membrane. After blocking the non-specific area by BSA, the blot was incubated with B049. The blot was then analyzed by the fluorescence spectroscopy (left) (2.2.5.7). A replicate blot was stained by Ponceau S (right).
4 Discussion

4.1 Characterization of p53 function via RNAi and CRISPR-Cas9

The tumor suppressor p53 predominantly functions as a transcription factor that activates the expression of genes involved in various cellular pathways. By doing so, p53 prevents normal cells from embarking on tumorigenesis. Interestingly, previous attempts in our laboratory to abrogate p53 expression in HPV-positive cancer cell lines via RNAi showed that despite a detectable reduction in p53 levels, complete abrogation of p53 proteins seemed to be cytotoxic [183]. Because of this putative pro-survival function of p53 in HPV-positive cells, we aimed to address whether p53 also exhibits a similar effect on the proliferation of HPV-negative cells. We utilized both RNAi and CRISPR-Cas9 techniques to abolish p53 expression in the HPV-negative cell line U-2 OS. Although knockdown of p53 expression did not provide conclusive evidence, knockout of TP53 via CRISPR-Cas9 system indicated that p53 is not required for cell proliferation in U-2 OS. In addition, experiences obtained from RNAi and CRISPR-Cas9 experiments also extended our technical knowledge of these two approaches.

4.1.1 p53 is not required for proliferation of HPV-negative U-2OS cells

4.1.1.1 Knockdown of p53 expression via RNAi

The underlying question of this project was whether p53 expression could be completely abrogated within HPV-negative cells without affecting cell proliferation or not. When transfected with shRNAmir-p53-i_I, II or IV constructs that executed an efficient knockdown of p53 expression (Figure 12), cell lines harboring endogenous p53, regardless of wild-type or mutant, showed severe cytotoxic effect upon treatment with puromycin (Figure 13 and Table 2). Furthermore, transfection of these p53-i constructs into p53-null H1299 cells did not cause significant cytotoxicity (Figure 14), which appeared to serve as an ideal control to corroborate the specificity of cytotoxic effects caused by knockdown of p53 expression. Along with our misjudgment of the applicability of the shRNAmir-IRESpuro system (see below in 4.1.3.1), these data misled us to believe that HPV-negative cell lines are not able to survive a complete abrogation of p53 expression.

Hence, we further examined the cytotoxicity caused by knockdown of p53 expression via rescue experiments (Table 3). The mutation of tumor suppressor genes frequently results in the expression of truncated protein forms with loss-of-function or in complete depletion of gene expression. In contrast, TP53 typically harbors missense mutations in tumors [127], which results in the expression of the full-length protein and suggests a selective advantage of p53 expression over p53 deficiency. Thus, we first hypothesized that the potential pro-survival function of p53 is
shared by wild-type and mutant p53. That is to say, in addition to the generally accepted gain-of-function feature, mutant p53 proteins might maintain a so far unknown function of wild-type p53 to keep cancer cells proliferative. On this basis, we ectopically and stably expressed p53 R273H, which is the most common and studied p53 mutant found in cancers [127], in U-2 OS cells. However, this stable cell line was not able to rescue cell proliferation when transfected with p53-i constructs (Table 3). Furthermore, neither p53 R273C nor wild-type p53 rescued cells from the cytotoxicity caused by knockdown of p53 expression in C-33A cells (which express endogenous p53 R273C) and in HeLa cells (which express endogenous wild-type p53), respectively. In addition, as TP53 expresses various isoforms of p53 (Figure 2) [51], we expressed p53 isoforms by different means, which also did not result in rescue of cell proliferation. Finally, co-transfection of a DNA plasmid expressing Puro\(^6\) (pcDNA3puro) rescued cell growth of U-2 OS (Figure 19), indicating that our belief in the necessity of p53 for cell proliferation is presumably based on a shortcoming of the shRNAmir-IRES system used.

After realizing that the shRNAmir-IRES is not suitable for our purpose, we used a conventional strategy to introduce RNAi constructs into cells. We first placed both miR-E-p53-i and Puro\(^6\) on the same expression vector but each was preceded by an individual promoter. However, we could not accomplish satisfactory knockdown efficiency, which was probably due to the use of a weak promoter for miR-E-p53-i (data not shown). Consequently, we co-transfected two separate constructs, miR-E-p53-i and Puro\(^6\) (pcDNA3puro). To minimize the possibility that cells surviving antibiotic selection do not express siRNA, a DNA amount ratio of 20:1 between miR-E-p53-i construct and pcDNA3puro was used. When miR-E-p53-i constructs were co-transfected into U-2 OS cells, no significant cytotoxicity effect was observed upon puromycin treatment (Figure 22 A). Moreover, cells surviving from colony formation assay were analyzed by Western blot, which showed no correlation between p53 levels and colony formation manner (Figure 22). Thus, we concluded that the shRNAmir-IRES system causes insufficient expression of the puromycin resistance marker (see below in 4.1.3.1) and that knockdown of p53 expression via RNAi does not provide conclusive evidence concerning the proposed pro-survival function of p53 in HPV-negative cancer cells.

### 4.1.1.2 Knockout of gene expression via CRISPR-Cas9

In addition to RNAi, we applied the CRISPR-Cas9 system to knockout gene expression. We first examined the applicability of CRISPR-Cas9 by studying knockout of UBE3A, which encodes the E3 ligase E6AP. Among three different designed sgRNAs, sg-E6AP_i and iii efficiently reduced E6AP levels in H1299 cells (Figure 24). Moreover, upon single clone selection, we obtained six clones harboring full depletion of E6AP expression according to Western blot analysis (Figure 25). Clone 1F2, 3A3 and 3D1 were further analyzed by sequencing a region of UBE3A that encompasses the targeting site of sg-E6AP_iii. The result showed that all of them exhibited either insertion or
deletion of nucleotides at the CRISPR-Cas9-associated cleavage site, leading to premature stop codons (data not shown, performed by Tina Maxa under supervision). Ultimately, in these three clones, ectopically expressed p53 proteins were not degraded when 16E6 was co-expressed (data not shown, performed by Tina Maxa under supervision), which on a functional basis substantiated the loss of E6AP in H1299 cells (note that E6AP is required for 16E6 to target p53 for degradation). In conclusion, by using the CRISPR-Cas9 system, we indeed accomplished the knockout of UBE3A expression.

We subsequently exploited the CRISPR-Cas9 system to address the effect of TP53 knockout on U-2 OS proliferation. When p53 knockout constructs were transfected into U-2 OS cells, none of the knockout constructs led to a decrease in colony formation compared to control constructs (Figure 27 A), and the efficiency in colony formation did not correlate with p53 expression levels (Figure 27 B). We then asked whether cells harboring a TP53 knockout have a growth disadvantage that can be monitored over time. After culturing for five passages, no decrease in colony formation was observed in cells harboring TP53 knockout (Figure 28 A) and p53 levels remained low except for cells transfected with the sg-p53_iii construct (Figure 28 B).

CRISPR-Cas9 modulates gene expression at the DNA level. As diploid cells usually possess two alleles of each gene, a decrease in protein levels of greater than 50% in a mixed cell population in principle indicates that certain cells have received full knockout of both alleles. Although DNA amplification occurs frequently in cancer cells, an amplification of the TP53 locus at 17p13.1, at least to our knowledge, has not been reported for U-2 OS (reviewed in [207]). Furthermore, U-2 OS cells transfected with sg-p53_i and ii constructs still exhibited about 70% knockdown efficiency in p53 expression levels after five passages (Figure 28 B), indicating that some cells in the mixed population harbor a complete knockout of TP53. Taken together, as the complete abrogation of p53 expression does not affect proliferation of those p53 null cells, our data obtained from CRISPR-Cas9-mediated TP53 knockout implied that p53 does not play a relevant role in cell proliferation in HPV-negative cells. However, this notion may rely on an oversimplified mathematical calculation. For a robust conclusion, the genuinely complete knockout of TP53 would need to be verified at the genome level.

As previous data of HPV-positive cells were acquired from RNAi experiments, it would also be interesting to corroborate the observation via the CRISPR-Cas9 system. We accordingly performed CRISPR-Cas9-mediated TP53 knockout in the HPV-positive HeLa cell line. Transfection of TP53 knockout constructs did not constrain cell proliferation and the Western blot analysis did not show a detectable reduction in p53 levels either (data not shown), implying the failure in TP53 knockout within HeLa cells. Thus, the experimental condition needs to be further optimized.
4.1.2 Effective knockdown of protein expression via shRNAmir and miR-E

4.1.2.1 Implementation of RNAi under the control of RNA pol II promoter

In order to execute a knockdown of p53 expression, we first used the modified pre-miRNA of human endogenous miR-30 [189], termed shRNAmir, under the control of the RNA pol II promoter CMV (Figure 10). Within animal cells, naturally occurring miRNAs are partially complementary to the 3' UTR of target mRNAs and fulfill RNA interference by translational repression or mRNA destabilization (reviewed in [208]). In contrast, siRNA processed from shRNAmir is perfectly complementary to the desired sequence of a target mRNA, and directly induces mRNA cleavage (reviewed in [209]). These differences render shRNAmir-processed siRNA more capable of specific target recognition and effective gene silencing.

When transiently transfected into U-2 OS cells, shRNAmir-p53-i_I, II and IV constructs indeed led to a detectable reduction in p53 levels (Figure 12). Although these constructs exhibited rather low knockdown efficiency (about 20-40%), this might be due to the “hard-to-transfect” feature of U-2 OS cells. According to our experience, transfection of a GFP construct into U-2 OS cells can reach at best 40% transfection efficiency under optimized transfection conditions (determined by flow cytometry, data not shown). Accordingly, the knockdown efficiency of shRNAmir will be expectedly greater, if transfection of U-2 OS could be further improved.

In principle, shRNA and shRNAmir regulate gene expression via the same mechanism. However, the efficient expression of shRNA relies on the precise transcription driven by RNA pol III promoters, including U6 [210, 211] and H1 [187]. Contrary to RNA pol II promoters, RNA pol III promoters usually lack tissue-specific and inducible regulation of gene expression. Although inducible systems for shRNA expression have been recently developed [212], the tight suppression of shRNA transcription in the non-induced state remains a major concern. Thus, the successful knockdown of p53 expression via shRNAmir under the control of CMV promoter also enables a more flexible choice among well-established expression systems with RNA pol II promoters.

4.1.2.2 miR-E structure enhances knockdown efficiency

In addition to shRNAmir, we also employed its advanced version miR-E (Figure 17 A), which enhances knockdown efficiency by boosting processing of shRNAmir into siRNA [190]. In agreement with reported data [190], miR-E-p53-i_III showed superior knockdown of p53 expression in U-2 OS cells as compared to shRNAmir-p53-i_III (Figure 17 B). In fact, the target sequence of p53-i_III was first published in the context of shRNA structure [187], which has been successfully used for knockdown of p53 expression in many studies. Thus, the inferior knockdown ability of shRNAmir-p53-i_III is presumably attributable to inefficient processing rather than poor target recognition. This notion is also supported by the observation that transfection of shRNAmir-IRESpuro-p53-i_III construct does not lead to cell death under puromycin treatment (Figure 13).
Since we concluded that in regard of the bicistronic shRNAmir-IRESpuro construct, the processing of shRNAmir interferes with efficient expression of the transfection marker (see below in 4.1.3.1), our observation reflects the inefficient siRNA processing and thus the intact expression of puromycin resistance marker.

The miR-E structure did not significantly enhance the knockdown efficiency of p53-i_II (Figure 17 B). Our interpretation is that shRNAmir-p53-i_II is already highly efficiently processed, suggesting that the target sequence of p53-i_II, for an elusive reason, also possesses the potency to facilitate siRNA processing. Taken together, our data demonstrate that the processing of shRNAmir into siRNA crucially determines the knockdown efficiency of RNAi, and that both basal stem design (i.e. the modified flanking regions in miR-E) and target sequence influence the processing efficiency.

4.1.3.3 p53-i_1 is ineffective to knockdown p53 expression
U-2 OS cells transfected with the shRNAmir-IRESpuro-p53-i_1 construct failed to survive puromycin selection (Figure 13), suggesting the effective processing of shRNAmir-p53-i_1. However, when transiently transfected into U-2 OS cells, this construct reproducibly resulted in only 20% knockdown of p53 expression (Figure 12 and not shown data). Furthermore, miR-E-p53-i_1 did not show any ability to knockdown p53 expression (Figure 21), which implies that factors other than siRNA processing can affect knockdown efficiency.

The target sequence of p53-i_1 is localized within the 5’ UTR of p53 mRNA. This region is known to form a complex secondary structure [213], which might hinder target recognition of p53-i_1. Furthermore, expression levels of p53-i_1 might also be associated with its low knockdown efficiency. One should note that in Figure 12, p53-i constructs targeted the endogenous p53 mRNA in U-2 OS. In Figure 21, however, p53 mRNA is transcribed from ectopic p53 cDNA in the H1299_p53 stable cell line. As the stable cell line was established by random integration of plasmid DNA into the genome, we cannot exclude the possibility that multiple copies of p53 cDNA were inserted into the genomic DNA, which led to higher levels of p53 cDNA transcripts. In support of poor target recognition, only relatively high levels of p53-i_1 in U-2 OS cells are capable of a rather mild elimination of p53 expression. Although this speculation will need to be corroborated by direct comparison between shRNAmir- and miR-E-p53-i_1 under the same cellular conditions, it indicates the importance to select the proper target site of RNAi.

4.1.3 shRNAmir-IREs system: You cannot have your cake and eat it too
The combination of vector-based RNAi with antibiotic selection allows to select for cells that indeed harbor the RNAi construct. This is usually achieved by the introduction of two expression vectors that contain the resistant gene and the RNAi sequence, respectively, into cells. Alternatively, both elements can be encoded on the same expression vector but their expression is individually driven by two different promoters. Both approaches are confronted with the
disadvantage that only some of the selected cells may exhibit a knockdown in expression of the gene of interest since the RNAi element may not be expressed in all cells harboring the resistant gene.

4.1.3.1 siRNA processing attenuates expression of transfection markers

To ensure the simultaneous expression of siRNA and resistance marker, we designed the shRNAmir-IRESpuro system (Figure 10). In the colony formation assay, U-2 OS cells individually transfected with pIRESpuro empty vector and shRNAmir-IRESpuro-non-i construct showed comparable cell proliferation upon puromycin treatment (Figure 13). This result led to the misinterpretation that the shRNAmir-IRESpuro system is indeed capable of simultaneous and efficient expression of both siRNA and resistance marker. However, when the miR-E form of non-i was used, U-2 OS cells failed to survive puromycin treatment (Figure 18). As mentioned, the miR-E system increases knockdown efficiency by boosting the processing of shRNAmir into siRNA [190]. This observation suggested that the enhanced siRNA processing somehow impedes cell proliferation upon puromycin treatment. To obtain insight into the mechanism underlying this phenomenon, pIRES-GFP empty vector or shRNAmir-IRES-GFP constructs were transfected into U-2 OS and H1299 cells (not shown data and Figure 20, respectively) and GFP expression levels were determined. This showed that lower levels of GFP were observed when shRNAmir-IRES-GFP-p53 i_II or IV were transfected, implying that in the shRNAmir-IRES system, effective siRNA processing attenuates expression of transfection markers. Indeed, co-transfection of a DNA plasmid containing Puro rescued the cell growth of U-2 OS (Figure 19), indicating that the siRNA processing results in inefficient expression of the puromycin resistance marker.

It seems highly unlikely that our siRNAs also target the mRNAs of the respective transfection markers. First, sequence alignment of siRNA targets does not show high sequence identity with coding sequences of the puromycin resistance marker or GFP. Similarly, it is also rather impossible that our different siRNA sequences all recognize mRNAs of puromycin resistance marker and GFP, even in an unspecific manner. However, transcription of shRNAmir results in pri-miRNA, which is further processed into pre-miRNA by excising the flanking region of the stem-loop structure (Figure 10). As Puro is located downstream of shRNAmir in the designed system, the liberated Puro mRNA lacks a 5’ cap, which is involved in nuclear export and protection of mRNAs [214]. Hence, the Puro mRNA might be rapidly degraded by abundant RNA nucleases within the nucleus and/or fail to be exported into the cytosol.

The bicistronic shRNAmir-IRES system is not a novel concept. It has been reported that a construct containing the tandem arrangement of GFP coding sequence and shRNAmir (Figure 48) is capable of simultaneous expression of siRNA and GFP [215, 216]. A similar design, but in the context of murine miR-155, has also been launched on the market (BLOCK-iT RNAi Kit, invitrogen). However, when we inspect the published data, they are similar to our results. Although the knockdown of
the target gene expression was successful, cells containing the knockdown constructs mostly showed lower GFP expression levels in comparison to control cells harboring the empty vector (i.e. lacking shRNAmir) [215, 216]. If our argument concerning the inefficient expression of transfection marker in the shRNAmir-IRES system is true, the set-up of GFP-shRNAmir system may be confronted with a similar shortcoming: The processing of shRNAmir results in the GFP mRNA that lacks 3’ poly-A tail. As poly-A tail is involved in numerous regulatory processes of mRNA, including cellular localization, stability and translation [217], the absence of 3’ UTR attenuates expression levels of GFP. Taken together, this caveat of bicistronic RNAi systems needs to be considered when performing respective experiments.

Figure 48 Tandem expression of GFP and shRNAmir
This system has been shown to simultaneously express siRNA and GFP [215, 216] but unlike our shRNAmir-IRES design (Figure 10), possesses the GFP coding sequence upstream of shRNAmir.

4.1.3.2 H1299 exhibits high resistance to puromycin
H1299 cells transfected with shRNAmir-IRESpuro-p53-i constructs remained viable under puromycin treatment (Figure 14), which may contradict the proposed model for the incompetence of the shRNAmir-IRES system to generate sufficient amounts of the puromycin resistance marker. H1299 cells may have an advantage over other cell lines with respect to puromycin resistance. According to the antibiotic kill curve, although 1 μg/ml puromycin was already sufficient to kill untransfected cells, H1299 cells transfected with a Puro<sup>a</sup> construct could survive under the treatment of up to 8 μg/ml puromycin. By comparison, transfected U-2 OS cells were viable at best with 4 μg/ml puromycin (data not shown). Furthermore, H1299 cells transfected with shRNAmir-IRES-GFP-p53-i constructs expressed low but detectable levels of GFP (Figure 20). Thus, the level of puromycin resistance marker expressed from shRNAmir-IRESpuro-p53-i constructs in H1299 cells may still suffice for cell survival upon puromycin selection. Importantly, the shRNAmir-IRESpuro system was successfully applied for the establishment of H1299 cells that harbor stable knockdown of p14ARF expression (H1299_p14ARF-i, Figure 42 A), and no severe cytotoxicity was observed. Taken together, we speculate that like other cell lines, H1299 cells also experienced an attenuated expression of puromycin resistance marker from the shRNAmir-IRES system. However, owing to the high tolerance of H1299 towards puromycin, transfected H1299 cells are still able to survive puromycin treatment.
4.2 Biochemical insights into ARF-Mdm2-p53 pathway

It is commonly accepted that ARF interferes with Mdm2-mediated ubiquitination of p53 and thereby stabilizes p53. However, the underlying mechanism remains controversial. In vitro data showing that ARF inhibits Mdm2 auto-ubiquitination as well as p53 ubiquitination have implied a direct effect of ARF on Mdm2 activity [111]. In this work, we confirmed this concept by performing a series of in vitro experiments. Additionally, our data provide deeper insight into the biochemical mechanism by which ARF inhibits Mdm2 activity.

4.2.1 p14ARF inhibits E3 ligase activity of Hdm2 directly and specifically

Our first results showed that GST-p14ARF inhibits GST-Hdm2-mediated p53 ubiquitination in vitro (Figure 29), which is consistent with reported in vitro data [107, 111]. By using tag-free Hdm2 prepared via the GST-SUMO fusion system (Figure 32) and pep3, consisting of the first 20 amino acids of p14ARF, we excluded that the inhibitory effect is caused by an unspecific link via GST-GST interaction (Figure 33). Owing to the absence of cellular organelles in the in vitro reaction, our results support the notion that the subcellular separation of Hdm2 and p53 is not necessary for stabilization of p53. Instead, p14ARF is capable of directly inhibiting Hdm2 activity.

To date, most p53-related ARF studies have exclusively focused on the ARF-Mdm2-p53 axis. We expanded this not only to other known substrates of Mdm2, but also to another E3 ligase of p53, E6-E6AP. The fact that pep3 also inhibits ubiquitination of other Hdm2 substrates, HdmX and Numb (Figure 36), validated the inhibitory effect of p14ARF on Hdm2 activity and helped us to delineate the biochemical mechanism of the ARF effect on Mdm2-mediated p53 ubiquitination (see 4.2.3.1). Furthermore, the inability of p14ARF to impede E6AP auto-ubiquitination (Figure 35) indicated that p14ARF interferes with neither E6-E6AP activity nor the activation and conjugation of ubiquitin by E1 and E2, respectively. Although pep3 slightly inhibited 16E6-E6AP-mediated p53 ubiquitination (Figure 34), this might result from an alternative mechanism rather than direct interference with E6-E6AP activity (see 4.2.4).

4.2.2 Relevance of p14ARF N-terminal amino acids 2-14

Amino acid sequence alignment of ARF proteins derived from mammalian species or even chicken indicates a conserved region encompassing approximately the N-terminal 30 residues of human p14ARF (Figure 49 A). Considering the overall dissimilarity of ARF protein across species, this conserved region might be relevant to ARF functions and thus is selectively maintained during evolution. Additionally, we analyzed the predicted ordered and disordered regions of ARF proteins in different species (Figure 49 B). Strikingly, a conserved protein topology is only found within about the N-terminal 15 residues of ARF proteins. Furthermore, several studies have demonstrated that the very N-terminal region of p14ARF plays a crucial role in the interaction with Hdm2 or in inhibition of Hdm2-facilitated p53 degradation [107, 108, 115, 116].
Figure 49 Amino acid sequence analysis of ARF orthologs derived from different species

(A) ARF orthologs share high conservation within the first 30 amino acids. Sequence alignment was conducted using Clustal Omega [174]. Percentage of arginine residues in each ARF protein is shown. Consensus symbols are denoted at the bottom. Asterisk (*): identical residues; colon (:): conservation of residues with strongly similar properties; period (·): conservation of residues with weakly similar properties.

(B) The first 15 residues of ARF proteins share the conserved protein topology. Folding potential was predicted using PONDR VL-XT analysis [175]. Human: Homo sapiens; Mouse: Mus musculus; Rat: Rattus norvegicus; Hamster: Mesocricetus auratus; Pig: Sus scrofa; Opossum: Monodelphis domestica; Chicken: Gallus gallus.
Discussion

Our data that pep3 suffices to inhibit Hdm2 activity support the functional relevance of the N-terminal region of p14ARF. As arginine residues represent 30% of the sequence in this peptide, we made use of a peptide with the reverse amino acid sequence of pep3 to analyze potential electrostatic effects. Notably, pep3 and the reverse peptide inhibited Hdm2 and Hdm2_RING activity with similar efficiencies (Figure 38 and Figure 39), suggesting that the two peptides share similar features. Indeed, when aligning these two peptides, we found a region of high similarity ranging from residues 2 to 14 of pep3 (Figure 50 A, top). Moreover, the pattern of this consensus sequence is conserved among ARF proteins from mammals (Figure 50 B). It has been reported that residues 2 to 14 of p14ARF are not only necessary for Hdm2 binding but also partially contribute to the nucleolar localization of p14ARF [108]. Thus, the unexpected inhibition of Hdm2-mediated ubiquitination caused by the reverse peptide coincidentally consolidates the importance of residues 2 to 14 in p14ARF regarding Hdm2 regulation. Of note, the corresponding region in mouse p19ARF is not required for nucleolar localization of p19ARF, but plays a role in the interaction with mouse Mdm2 [108]. Along with our data, the function of the conserved region encompassing residues 2 to 14 would appear to be the direct inhibition of Hdm2 activity via specific interaction rather than the sequestration of Hdm2 in the nucleolus. Moreover, the threonine at position eight is the only conserved residue within this region with exception of arginine residues. It would therefore be interesting to investigate whether phosphorylation of this threonine residue alters the regulation of ARF-Mdm2-p53 pathway.

Figure 50 Amino acid sequence alignment of p14ARF peptides
(A) Pep3 shares high similarity from residues 2 to 14 with the reverse peptide but not with the scrambled peptide. (B) The consensus sequence shared by pep3 and the reverse peptide is also found in ARF proteins derived from different species. Sequence alignment was conducted using Clustal Omega [174]. Consensus symbols are denoted at the bottom. Asterisk (*): identical residues; colon (:): conservation of residues with strongly similar properties; period (·): conservation of residues with weakly similar properties. For (B), residues 2 to 14 of p14ARF are indicated in the red box. The threonine residue in the consensus region is denoted in green. Identical residues or residues with similar hydrophobicity are shown at the bottom. The difference in consensus between ARF proteins and p14ARF peptides is denoted in blue. h: hydrophobic amino acid; X: any amino acid.
In addition to the reverse peptide, the peptide with the scrambled sequence of pep3 also inhibited Hdm2 and Hdm2_RING activity but with lower efficiency (Figure 38 and Figure 39). The sequence alignment of pep3 and the scrambled peptide shows little in common (Figure 50 A, bottom), suggesting that the scrambled peptide-associated reduction in ubiquitination reflects presumably unspecific electrostatic effects resulting from the abundant arginine residues. However, this notion does not contradict the specificity of pep3 and the reverse peptide, as the inhibitory efficiency of these two peptides is four to five times greater than that of the scramble peptide (Figure 38 and Figure 39). Thus, only 20-25% of the inhibitory efficiency were probably contributed by electrostatic effects.

4.2.3 Association between p14ARF and Hdm2 RING domain

4.2.3.1 Inadequacy of the substrate level hypothesis
The p14ARF binding site has been localized within the Hdm2 central region encompassing amino acid 210 to 244 (Figure 4) [107]. Thus, at the initial stage of this project, the most surprising result was that GST-p14ARF inhibits p53 ubiquitination catalyzed by GST-Hdm2_RING (Figure 29). This observation was further corroborated by exploiting tag-free Hdm2_RING and pep3 in the in vitro p53 ubiquitination assay (Figure 33). Lacking direct evidence that p14ARF interacts with the C-terminal RING domain of Hdm2, we first proposed that p14ARF inhibits Hdm2-mediated p53 ubiquitination at the substrate level by binding to p53. Indeed, a direct interaction between ARF and p53 has been observed by baculovirus-mediated co-expression of both proteins in insect cells [196], although this interaction has not yet been demonstrated within mammalian cells.

To address this hypothesis, we determined whether p14ARF also inhibits ubiquitination of other Hdm2 substrates. Indeed, pep3 also inhibits Hdm2_RING-mediated ubiquitination of HdmX and Numb (Figure 36). Whether there is a direct interaction between HdmX and p14ARF remains controversial. Different groups have reported opposite data concerning the potential p14ARF-HdmX interaction [218, 219]. Regarding Numb, to our knowledge, an interaction between Numb and p14ARF has not been reported so far. In any case, it seems unlikely that p14ARF interferes with the ubiquitination of these different substrates by acting at the substrate level rather than at the Hdm2 level.
4.2.3.2 p14ARF directly inhibits Hdm2_RING activity

If p14ARF does not inhibit Hdm2-mediated ubiquitination at the substrate level, the obvious interpretation of our results would be the direct association of p14ARF with the RING domain of Hdm2. To support this possibility, we performed ubiquitin chain formation assays under the same conditions as Hdm2_RING-mediated in vitro ubiquitination of substrates. The observation that pep3 also inhibited Hdm2_RING-mediated ubiquitin chain formation (Figure 37) disproved the substrate level hypothesis and indicated that p14ARF directly inhibits the "enzymatic" activity of Hdm2_RING.

The specificity of the pep3 effect on Hdm2_RING activity was further substantiated. Compared to Hdm2-mediated p53 ubiquitination, pep3 inhibited Hdm2_RING-mediated p53 ubiquitination with a lower efficiency of 25% (Figure 33). As discussed above, 20-25% of the inhibition ability of pep3 might be attributed to electrostatic effects (see 4.2.2). This raised the concern whether the inhibition of Hdm2_RING-mediated p53 ubiquitination by pep3 was exclusively caused by potential electrostatic effects. However, the scrambled peptide inhibited both Hdm2_RING-mediated p53 ubiquitination and free ubiquitin chain formation less efficiently than pep3 and the reverse peptide did (Figure 39), which indicated that inhibition of Hdm2_RING-mediated ubiquitination does not only rely on electrostatic interactions. Moreover, as full-length Hdm2 additionally possesses the known p14ARF binding site in the central region, the superior inhibitory effect of pep3 on Hdm2-mediated ubiquitination can readily be explained by higher binding affinity of full-length Hdm2 for pep3.

We observed that 5 μM pep3 was capable of full inhibition of Hdm2_RING-mediated free ubiquitin chain formation (Figure 37), whereas 10 μM pep3 only partially constrained Hdm2_RING-mediated p53 ubiquitination (Figure 33). As mentioned, the only difference of the reaction between the ubiquitin chain formation assay and the in vitro ubiquitination assay was the presence of the ubiquitination substrate, which was in vitro translated using reticulocyte lysate. Accordingly, the different inhibitory efficiency of pep3 might be caused by components present in the reticulocyte lysate. However, when the ubiquitin chain formation assay was performed in the presence of reticulocyte lysate, a similar inhibitory efficiency of pep3 to that shown in Figure 37 was observed (data not shown, performed by Oliver Hartmann under supervision). Thus, it will need to be further investigated whether pep3 inhibits substrate ubiquitination and free ubiquitin chain formation via different mechanisms or pep3 has a distinct affinity to interact with Hdm2_RING in the presence of substrates.

Although we have proven that pep3 does not influence 16E6-E6AP activity (Figure 35), the effect of pep3 on other RING E3 ligases should also be examined in the future. Especially, whether pep3 also impedes the activity of the isolated RING domain of other RING E3 ligases has to be elucidated. It is known that both the full-length form and the isolated RING domain of E3 ligase RLIM [220]
are able to execute auto-ubiquitination in vitro (unpublished data from our laboratory). Thus, the investigation of potential pep3 effects on RLIM and RLIM_RING auto-ubiquitination will help us to validate the specificity of the p14ARF effect on Hdm2_RING activity.

4.2.3.3 Putative interaction between p14ARF and the RING domain of Hdm2
The direct inhibition of Hdm2_RING activity by pep3 also suggests the existence of a novel interaction site for p14ARF within the Hdm2 RING domain. As direct interaction between p14ARF and the isolated Hdm2 RING domain has never been addressed so far, it will be of interest to confirm the putative interaction by respective binding assays. At the time of writing this thesis, preliminary data from an ongoing project indeed indicate a direct interaction between pep3 and Hdm2_RING (data not shown, performed by Oliver Hartmann under supervision). If reproducible, this result will underpin that p14ARF inhibits Hdm2_RING activity via direct interaction.

4.2.3.4 p14ARF may constrains ubiquitin transfer from E2 to the substrate
Unlike HECT E3 ligases, which genuinely exhibit enzymatic activity to catalyze ubiquitin transfer from E3 to the substrate, RING E3 ligases have long been considered as inert adaptor proteins between E2s and substrates. However, more recent data suggest that RING E3 ligases in fact act as allostERIC activators of E2s. By the preferential interaction with ubiquitin-loaded E2, dimerized RING domains bring the thioester bond between E2 and ubiquitin in an even more reactive state, which facilitates ubiquitin transfer onto the substrate [63]. Moreover, this model has also been empirically proven for the Hdm2 RING domain [221].

As our data showed that p14ARF directly inhibits the activity of isolated Hdm2 RING domain, we propose a potential biochemical mechanism of the ARF effect on Mdm2-mediated ubiquitination of p53: Through direct interaction with Hdm2 RING domain, p14ARF interferes with the release of ubiquitin from E2 (Figure 51). To ascertain this model, we need to perform a ubiquitin discharge assay [222, 223] in the presence of pep3, where ubiquitin is first loaded onto E2 proteins via thioester bond formation followed by the addition of Hdm2_RING to activate the release of ubiquitin. Despite being capable of thioester bond formation between ubiquitin and E2 proteins, we still failed to achieve an Hdm2_RING-mediated ubiquitin discharge. The experimental conditions of the ubiquitin discharge assay will thus need to be further optimized. If the proposed model proves to be correct, to obtain complete insight into the mechanism of ARF inhibition, it will need to be determined whether p14ARF interferes with ubiquitin release by abolishing RING-RING dimerization, which is required for the E3 ligase activity of Hdm2 [92, 93], or by interference with the ability of the RING-RING dimer to release ubiquitin from the E2.
4.2.4 ARF’s role in E6-E6AP-mediated p53 ubiquitination

A debatable result was that pep3 and the reverse peptide, but not the scrambled peptide, also inhibited 16E6-E6AP-mediated p53 ubiquitination (Figure 34 and Figure 40 A). Although this observation was in line with our initial substrate level hypothesis, the inadequacy of this notion for Hdm2 (see 4.2.3.1) made us reconsider the role of ARF in E6-E6AP-mediated p53 ubiquitination. An unspecific electrostatic effect does also not seem to be a satisfactory interpretation. As we have concluded above that the ability of the scrambled peptide to inhibit ubiquitination is a consequence of electrostatic interactions (see 4.2.2), all of our p14ARF peptides should inhibit 16E6-E6AP-mediated p53 ubiquitination with equal efficiency, if it were an unspecific effect. Furthermore, overexpression of p14ARF inhibited 16E6-E6AP-mediated p53 degradation also within cells (data not shown), where the charge of p14ARF is presumably buffered or neutralized by forming complexes with other cellular molecules [113].

We accordingly attempted to elucidate the impact of p14ARF on E6-E6AP activity. As mentioned, E6AP belongs to the HECT class of E3 ligases. In fact, in addition to the inhibition towards the RING ligase Hdm2, ARF is known to restrain the activity of HectH9 (also called ARF-binding protein 1, ARF-BP1), a HECT E3 ligase catalyzing the ubiquitination of a number of proteins including p53 [224, 225]. However, the observation that p14ARF peptides did not inhibit 16E6-facilitated E6AP auto-ubiquitination (Figure 40 B) indicates that p14ARF does not influence E6-E6AP activity per se. Moreover, we observed that pep3 does not inhibit E6-E6AP-mediated ubiquitination of the tumor suppressor hDlg (human homologue of the Drosophila discs large) (data not shown, performed by Julia Mader under supervision), another E6-dependent substrate of E6AP [191]. Thus, the p14ARF-mediated inhibitory effect on E6-E6AP is apparently specific to p53 ubiquitination.

While being beneficial but not necessary for E6AP auto-ubiquitination, high-risk E6 is indispensable to both hDlg and p53 ubiquitination. Serving as a platform, high-risk E6 mediates the interaction of E6AP with hDlg and p53 but via different mechanisms. Dlg, which is a PDZ domain containing protein, interacts with the C-terminal PDZ binding motif of high-risk E6 [226].
On the other hand, the PDZ binding motif is not required for the high-risk E6-mediated interaction between p53 and E6AP [227]. Instead, E6AP renders the conformation of high-risk E6 competent for the interaction with p53 by structuring a p53-binding cleft on high-risk E6 [158]. As p14ARF does not seem to disrupt the formation of functional 16E6-E6AP complex, we speculate that p14ARF interferes with the interaction between high-risk E6 and p53. It is hypothesized that high-risk E6 remains able to interact with p53, when p53 complexes with DNA or other proteins [158]. Thus, p14ARF may bind to high-risk E6 and somehow perturb the p53-binding interface, which will of course need to be proven by a series of interaction assays. Moreover, in contrast to the lack of reported evidence regarding the correlation between p14ARF and high-risk E6, ARF has been shown to inhibit high-risk E7-induced pRb degradation and DNA replication [228]. Ultimately, further investigation of the influence of p14ARF on HPV-related carcinogenesis may be meaningful to the prevention or the treatment for cervical cancer.

4.2.5 Effect of p14ARF on p53 neddylation

In addition to in vitro assays, the inhibitory effect of p14ARF on p53 ubiquitination was confirmed in in cellulo assays (Figure 41 A). Moreover, we also examined the effect of p14ARF on p53 neddylation within cells. While high levels of p14ARF inhibited Mdm2-mediated neddylation of p53, low levels of p14ARF appeared to slightly enhance p53 neddylation (Figure 41 A). In an attempt to corroborate this phenomenon, we observed that p53 neddylation indeed increased with the amount of transfected p14ARF constructs within a certain range (Figure 41 B). This result alluded to opposite regulatory effects of p14ARF on Mdm2-mediated p53 neddylation that vary with different expression levels of p14ARF. Therefore, a greater reduction in p53 neddylation was rationally expected when endogenous p14ARF expression is abrogated by RNAi. However, despite a knockdown efficiency of greater than 70% (Figure 42 A), no significant decrease in p53 neddylation was observed in H1299_p14ARF-i stable cell lines (Figure 42 B), which rendered the notion of the dual regulatory effect untenable.

Another explanation of our data would be that Mdm2-mediated p53 ubiquitination is more sensitive to p14ARF than neddylation. In the absence of ectopic p14ARF, p53 ubiquitination was inferior to p53 neddylation (Figure 41 A, also personal communication in our laboratory), suggesting that basal levels of p14ARF inhibit preferentially Mdm2-mediated p53 ubiquitination rather than neddylation. In the in cellulo p53 neddylation assay, ectopically expressed p14ARF presumably inhibited modification of p53 by endogenous ubiquitin, thereby resulting in increased p53 levels (Figure 41 B, note the increase in levels of unmodified p53). Since more p53 was available in the presence of ectopically expressed p14ARF, p53 neddylation was apparently increased (Figure 41 B, 0.05-1 μg of p14ARF). However, when p14ARF levels reached a certain threshold, p14ARF started to negatively influence Mdm2-mediated neddylation (Figure 41 B, 2 μg of p14ARF). In the non-i stable cell line, p53 neddylation in the presence of low Mdm2 levels was
less efficient as compared to high Mdm2 levels, which was not observed in the p14ARF-i stable cell line (Figure 42 B). This may be attributable to the endogenous levels of p14ARF present in the non-i stable cell line, which, relative to the low Mdm2 levels, already exceeded the speculative threshold to execute inhibition of p53 neddylation. Along this line, since the expression of endogenous p14ARF was decreased in the p14ARF-i stable cell line, p53 was neddylated by low and high levels of Mdm2 with similar efficiency. In any case, Mdm2-mediated p53 ubiquitination is generally inferior to p53 neddylation in H1299 parental cells. Thus, comparison between Mdm2-mediated p53 ubiquitination and neddylation in p14ARF-i stable cells will be helpful to further characterize the different inhibitory efficiency of p14ARF towards these two post-translational modifications of p53.

According to our in vitro experiments, GST-p14ARF inhibited p53 ubiquitination and neddylation with similar efficiencies (Figure 30). However, one should note that our assays were performed by using ubiquitin E1 and E2 since efficient Hdm2-mediated p53 neddylation via Nedd8 E1 and E2 has so far not been achieved in vitro. Such an in vitro p53 neddylation assay (i.e. with Nedd8 enzymes) will eventually allow to delineate the p14ARF effect on p53 neddylation. It was recently reported that phosphorylation of Hdm2 on Tyr281 and Tyr302 by the tyrosine protein kinase Src enhances the affinity of Hdm2 for the Nedd8 E2 Ubc12, and thereby switches the activity of Hdm2 from ubiquitination to neddylation [229]. Thus, application of Hdm2 phosphorylated at Tyr281 and Tyr302 might provide a viable solution to the establishment of an Hdm2-mediated p53 in vitro neddylation assay.

4.3 GST-SUMO fusion system for the preparation of recombinant proteins

Expression and purification of recombinant proteins have become an invaluable technique for biochemical studies. However, expression of mammalian proteins in E. coli often encounters problems including misfolding and aggregation of foreign proteins, which leads to low solubility and low yield. In this work, we made use of a GST-SUMO fusion system for the preparation of tag-free recombinant Hdm2, Hdm2_RING and 16E6 proteins (see 3.2, 3.3, and Figure 32 B). The overall satisfactory protein yield and functionality support published data that the SUMO-fusion technology enhances the solubility of expressed proteins in E. coli (reviewed in [198]). Although p14ARF was not successfully expressed via the same procedure, this might be due to its unusual amino acid composition and biochemical properties.

Another advantage of the SUMO fusion system is the efficient removal of the SUMO tag by the SUMO protease Ulp1 (Figure 31), which specifically cleaves the peptide bond directly after the last residue of SUMO. By using cloning strategies like Golden Gate cloning [230] or Gibson assembly
[178], where the restriction site between coding sequences of SUMO and POI can be eliminated, no additional amino acids will remain at the N terminus of the native POI after the cleavage via Ulp1.

In this work, we successfully simplified the purification procedure of the SUMO fusion system by performing “on-beads cleavage” (Figure 32). As on-beads GST-Ulp1 directly liberates the tag-free POI into the solution, our method saves not only the time required for the elution of tagged proteins from chromatography beads, but also the cost of eluents. Furthermore, the one-step removal of the GST-SUMO tag avoids the multi-step purification for separation of GST-SUMO and the POI that are present in the same solution. Although we have not tested the applicability of this approach in the context of a His-tag, it is supposed to be as feasible as the GST-tag version. If this is the case, researchers that already possess expression constructs for His-SUMO-tagged POI can simply turn to the on-beads cleavage procedure without any additional efforts and costs.

### 4.4 Fluorescence-based detection of 16E6 protein

In addition to the primary prevention of HPV infection via vaccination, the cytology-based Pap test and PCR-based HPV genome-typing comprise a secondary strategy against development of cervical cancer. However, both methods suffer from disadvantages of diagnostic accuracy. We accordingly attempted to develop a novel tool that is capable of both sensitive and specific diagnosis of HPV-infected cells that are on the way to cancer at an early stage. Our approach is based on the conjugation of a fluorophore to a peptide (pep11 or pep11**, Figure 43) that is known to bind HPV type 16E6 proteins with high affinity [206]. In the presence of 16E6 protein, we expected a photophysical change of the conjugated fluorophore. Since expression of HPV E6 oncoproteins is a representative marker for the initiation of cervical carcinogenesis [159], the quantitative fluorescence measurement would serve as an approach for 16E6 detection, thereby diagnosing cervical cancer induced by HPV 16 at an initial stage.

For our purpose, it is of great importance that pep11 and pep11** retain their ability to interact with 16E6 when conjugated with a fluorophore. We corroborated the interaction by performing in vitro p53 ubiquitination assays using 16E6-E6AP as E3 ligase since pep11 and pep11** are known to compete with E6AP for 16E6 binding and thereby inhibit 16E6-E6AP-mediated ubiquitination [206]. This alternative approach allowed us to circumvent the restrictions of conventional interaction assays, such as the requirement of massive amounts of synthesized compounds or special instruments. Except for B038, all synthesized compounds retained the ability to inhibit 16E6-E6AP-mediated ubiquitination (Figure 45 and Table 5). Because of the inavailability of most unconjugated fluorophores, we were unable to examine the effect of
fluorophores per se on p53 ubiquitination. However, the pep11** conjugated compound B048 showed higher inhibitory efficiency as compared to the respective analog containing pep11, B038, which exhibited no inhibition towards p53 ubiquitination (Table 5). This indicated that, at least for this fluorophore (F 500), inhibition of p53 ubiquitination was not caused by the fluorophore itself. Taken together, results obtained from 16E6-E6AP-mediated in vitro p53 ubiquitination assays provided us with indirect but reliable evidence that the conjugated compounds are capable of interaction with the 16E6 protein.

When we measured emission spectra, 16E6 proteins indeed dequenched compound B049. However, this change in the photophysical properties of Alexa Fluor 488 was not specific to 16E6 since 11E6 as well as BSA also showed similar effects, though with lower efficiency (Figure 46). As pep11 and pep11** only bind to 16E6, the dequenching effect caused by 11E6 and BSA is probably due to an unspecific interaction of proteins with the hydrophobic fluorophore. Supporting this notion, compound B049 also bound to the isolated RING domain of Hdm2 that was immobilized on a blot membrane (Figure 47). Moreover, when compound B046, which is conjugate of FITC (fluorescein isothiocyanate) and pep11** (Table 5), was incubated with 16E6 or BSA, no significant change in emission intensity was observed. FITC alone, however, showed dramatic quenching effects in the presence of 16E6 or BSA (data not shown). Other synthesized compounds were not tested owing to their low solubility. In conclusion, our data demonstrate the unspecific and unpredictable effects of proteins on the emission intensity of fluorophores and point to the unsuitability of this set-up to detect 16E6 proteins with high specificity.
5 References


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Acknowledgment

First and foremost I would like to express my sincerest gratitude to my advisor Prof. Martin Scheffner for providing me the opportunity to work on such fascinating and challenging projects. Martin has given me not only great freedom but also the overwhelming support. I really enjoyed the unforgettable time working in this lab.

My immense appreciation is also extended to Prof. Thomas U. Mayer for being a member of my thesis committee and for the support of many experimental materials as well as the help from his lab members.

I am also grateful to Prof. Florian Stengel for being the oral examiner in my examination committee. My special gratitude goes to our technicians: Nicole Richer‐Müller and Silke Büstorf. Their enthusiasm and a strong sense of responsibility not only facilitates the progress of my work but also ensures the proper day-to-day operation of our laboratory.

I am much obliged to Dr. Ashit Rao, Dr. Dana Becker, Dr. Elisabeth Stürner, Dr. Franziska Mortensen and Dr. Simone Kühnle for the critical revision of this thesis. An additional thank you to Dr. Dana Becker for the translation of the abstract into German.

Without the contribution from my colleagues, this dissertation would have been impossible:

For my first project, I thank Jasmin Taban for her endless patience to perform a series of laborious cytotoxicity and rescue experiments. I also thank Daniela Eichbichler for her short but valuable assistance. Furthermore, I am grateful to Dr. Julia Häfner from Mayer’s group for demonstrating us the Flp-In system.

I thank Julia Mader and Oliver Hartmann for their brilliant contribution to set up conditions of various in vitro assays in the second project.

The third project was accomplished by a great team consisting of experts from various fields. Dr. Paul Jansen was in charge of the synthesis of fluorescence compounds. In addition, I am thankful to Prof. Dominik Wöll and his lab member Dr. Maren Dill, as well as Dr. Martin Winterhalder from Zumbusch’s group for helping the measurement of fluorescence spectra and for the fruitful discussion. I also want to especially thank Dr. Konstantin Matentzoglu for managing the proposal of this project, which was funded by EuroTransBio.

I also thank Tina Maxa. With the assistance of Dr. Naowras Al-Obaidi from Mayer’s group, we established the CRISPR-Cas9 system in Scheffner’s group together.

Last but not least, I thank all members in Scheffner’s and Stengel’s groups for the excellent working atmosphere. I will always miss the happy time spending with you guys.