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M.Sc. Alicia Avenhaus
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E6AP Inhibition and Metformin Treatment: Two Novel Strategies to Block the Growth of HPV-Positive Cancer Cells?

Referees: Prof. Dr. Suat Özbek
Prof. Dr. Felix Hoppe-Seyler

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Summary

Oncogenic types of human papillomaviruses (HPVs) are major human carcinogens. The therapy of HPV-associated malignancies, such as cervical cancer, remains challenging and novel treatment options are urgently needed. This thesis aimed to gain a deeper understanding of the molecular mechanisms that sustain the proliferation of HPV-positive cancer cells and to identify new strategies to block their growth, thereby paving the way towards novel therapeutic approaches.

My studies uncovered that the cellular ubiquitin ligase E6-associated protein (E6AP), an interaction partner of the HPV E6 oncoprotein, is essential for maintaining the proliferation of HPV-positive cancer cells by exerting a previously unrecognized anti-senescent function. RNA interference (RNAi)-mediated E6AP silencing, unlike E6 silencing, led to a potent induction of cellular senescence, an irreversible growth arrest. Notably, the pro-senescent effect of E6AP downregulation was specifically observed in HPV-positive cancer cells. Proliferation studies and cell cycle analyses revealed that the cellular growth inhibition and senescence induction upon E6AP repression critically depend on the activation of the p53-p21 axis as well as on the pocket proteins retinoblastoma protein (pRb) and p130. Additionally, my results showed that Metformin, which is being discussed for repurposing in cancer therapy, can also inhibit the proliferation of HPV-positive cancer cells. However, this effect is reversible and, further, was found to be independent of Metformin's potential to downregulate the expression of the HPV *E6* and *E7* oncogenes. Moreover, my experiments demonstrated that Metformin exhibits complex functional interactions with chemotherapeutic agents in both HPV-positive and HPV-negative cancer cells. Whereas Metformin can protect against senescence induced by the pro-senescent drug Etoposide, it can enhance the pro-apoptotic effects of Cisplatin, particularly after pre-treatment with Metformin. My mechanistic studies further revealed functional key roles for p53 and a truncated form of the BH3-interacting domain death agonist (BID) protein, t-BID, in mediating the cooperative effects between Metformin and Cisplatin.

Collectively, my findings identify E6AP as a critical anti-senescent factor in HPV-positive cancer cells that is essential for their proliferation. E6AP might therefore serve as a new therapeutic target, with specificity for HPV-positive cancer cells. Moreover, my results uncover complex effects of Metformin on the phenotype of HPV-positive and HPV-negative cancer cells. Importantly, Metformin can substantially increase the anti-proliferative effects of Cisplatin, a drug that plays a key role in the treatment of HPV-positive cancers. This indicates that combinatorial treatments with Metformin and Cisplatin could potentially be of therapeutic benefit.

Zusammenfassung

Onkogene Typen humaner Papillomviren (HPV) sind wichtige humane Karzinogene. Die Therapie HPV-assoziiierter Tumorerkrankungen, wie etwa von Zervixkarzinomen, ist weiterhin herausfordernd, und es besteht dringender Bedarf an neuen Behandlungsoptionen. Ziel der vorliegenden Arbeit war es, ein tieferes Verständnis der molekularen Mechanismen zu gewinnen, die die Proliferation HPV-positiver Tumorzellen aufrechterhalten, und neue Strategien zu identifizieren, um ihr Wachstum zu blockieren.

Meine Untersuchungen zeigten, dass die zelluläre Ubiquitin-Ligase E6-assoziiertes Protein (E6AP), ein Interaktionspartner des HPV E6 Onkoproteins, eine essentielle Rolle für die Aufrechterhaltung der Proliferation HPV-positiver Krebszellen spielt, indem sie eine bislang unerkannte anti-seneszente Funktion ausübt. Im Gegensatz zu einer E6-Hemmung führte eine E6AP-Hemmung zur effizienten Induktion zellulärer Seneszenz, einem irreversiblen Wachstumsstopp. Bemerkenswerterweise war dieser pro-seneszente Effekt spezifisch in HPV-positiven Tumorzellen zu beobachten. Proliferationsstudien und Zellzyklusanalysen ergaben, dass die Wachstumshemmung und Seneszenzinduktion nach E6AP-Repression entscheidend von der Aktivierung der p53-p21 Achse sowie von den pRb und p130 Proteinen abhängen. Darüber hinaus zeigten meine Ergebnisse, dass Metformin, ein Antidiabetikum dessen Einsatz in der Krebstherapie diskutiert wird, ebenfalls die Proliferation HPV-positiver Tumorzellen hemmen kann. Dieser Effekt ist jedoch reversibel und zudem unabhängig von der Fähigkeit Metformins, die Expression der HPV *E6* und *E7* Onkogene zu hemmen. Des Weiteren ergaben meine Experimente, dass Metformin sowohl in HPV-positiven als auch in HPV-negativen Krebszellen komplexe funktionelle Wechselwirkungen mit Chemotherapeutika eingeht. Während Metformin vor der pro-seneszenten Wirkung von Etoposid schützen kann, kann es die pro-apoptotischen Effekte von Cisplatin verstärken – insbesondere nach einer Vorbehandlung mit Metformin. Mechanistische Untersuchungen deckten außerdem kritische funktionelle Rollen von p53 und einer trunkierten Form des BID-Proteins, t-BID, bei der Kooperation zwischen Metformin und Cisplatin auf.

Zusammenfassend identifizieren meine Befunde E6AP als wichtigen anti-seneszenten Faktor in HPV-positiven Tumorzellen, der essentiell für ihre Proliferation ist. E6AP könnte daher als neuer therapeutischer Angriffspunkt mit Spezifität für HPV-positive Tumorzellen dienen. Darüber hinaus zeigen meine Ergebnisse, dass die Effekte von Metformin auf den Phänotyp HPV-positiver und HPV-negativer Tumorzellen sehr komplex sind. Die Beobachtung, dass Metformin die antiproliferativen Effekte von Cisplatin erheblich verstärken kann, weist darauf hin, dass ihre kombinierte Anwendung potentiell von therapeutischem Nutzen sein könnte.

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Publications and Presentations

Publications

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Avenhaus A, Velimirović M, Bulkescher J, Scheffner M, Hoppe-Seyler F, Hoppe-Seyler K. E6AP is essential for the proliferation of HPV-positive cancer cells by preventing senescence. *PLOS Pathog*. 2025; 21(2):e1012914

Presentations

Avenhaus A, Velimirović M, Bulkescher J, Hoppe-Seyler F, Hoppe-Seyler K. Downregulation of the Cellular Protein E6AP leads to Efficient Senescence Induction in HPV-Positive Cancer Cells. Immunology, Infection and Cancer (IIC) Retreat. 7–9 October 2024. Bad Dürkheim, Germany. Poster presentation.

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CHAPTER 1

INTRODUCTION

1 Introduction

1.1 Human papillomaviruses

Papillomaviruses are a group of small, non-enveloped viruses, which infect mucosal or cutaneous epithelia of specific vertebrate host species, including humans.¹ They consist of a circular, double-stranded DNA (dsDNA) genome with a size of approximately 8000 base pairs and an icosahedric capsid, which is composed of the two viral structural proteins L1 and L2.² Infection with human papillomaviruses (HPVs) is often acquired through sexual activity and can remain asymptomatic or cause benign lesions, such as warts or papillomas.³ Harald zur Hausen pioneered the discovery of the association between HPV infection and cervical cancer.^{4,5} Today, several oncogenic (“high-risk”) HPV types (HPV16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, and 59) have been classified, which together cause approximately 4.5% of all human cancers.⁶ These include cervical, vulvar, vaginal, penile, anal, rectal, skin, and oropharyngeal cancers.^{3,7}

1.1.1 HPV genome structure and replication

The circular dsDNA genome of HPVs consists of one noncoding regulatory region and two protein-coding regions. The noncoding region, termed long control region (LCR), encompasses the viral origin of replication as well as promoter and enhancer elements, which contain binding sites for both cellular and viral transcription factors.^{8,9} The protein-coding regions are divided into early and late regions. The early region encompasses open reading frames (ORFs) encoding the highly conserved E1 and E2 proteins, which are involved in viral DNA replication, as well as the accessory proteins E4, E5, E6, and E7.¹⁰ Two ORFs in the late region encode the capsid proteins L1 and L2.¹⁰ HPV transcription is highly complex and is linked to the state of differentiation of the infected epithelial cells.¹¹ The use of multiple promoters, including the early (P_E) and late (P_L) viral promoters, together with different polyadenylation sites and alternative splicing, enables this complex pattern of transcription.¹²

The terminology of “early” and “late” proteins originates from their temporal expression during the infectious cycle.¹² In brief, HPVs, which have an epithelial tropism, infect dividing basal keratinocytes by entering through microlesions in the epithelium.³ The virus attaches to heparan sulfate proteoglycans (HSPGs) on the basement membrane, which facilitates conformational changes and enables cleavage of the L2 protein by furin proteases.^{13,14} After endocytosis, the viral genome, complexed with L2, enters the nucleus during cellular mitosis.^{15,16} Here, viral genome transcription takes place in association with promyelocytic leukemia (PML) nuclear bodies (NBs), leading to a persistent infection in the basal layer of the

epithelium.^{17,18} This requires the replication proteins E1, an ATP-dependent helicase, and E2, which together bind to the viral replication origin.¹⁹ Further, E2 ensures that the viral genome is retained in the nucleus during cell division by binding it to mitotic chromatin.²⁰ The early viral proteins E6 and E7 are expressed in the basal layers to drive cellular proliferation and expand the infected area by interfering with the activities of p53 and so-called “pocket proteins”, such as the retinoblastoma protein (pRb)²¹ – a mechanism that is also central to HPV-induced carcinogenesis. As infected basal cells differentiate and migrate into the suprabasal layer of the epithelium, E6 and E7 expression allows these normally non-dividing cells to re-enter the cell cycle.²² In addition, the E5 protein, which is only expressed by HPVs from the *Alphapapillomavirus* genus,²³ stimulates cellular proliferation and impairs keratinocyte differentiation.²⁴ At this stage, E1 and E2 expression is upregulated to promote vegetative genome amplification.²⁵ Eventually, increasing levels of E2 contribute to the downregulation of E6 and E7, thereby allowing further differentiation of the infected keratinocytes.¹² During late stages of the infection, the E4 protein is expressed in the uppermost epithelial layer to inhibit cellular proliferation and enhance the efficiency of genome amplification as well as the synthesis of the L1 and L2 capsid proteins.²⁶ L1 and L2 assemble with viral genomes to form virions in terminally differentiated keratinocytes, which are then shed from the epithelial surface (Figure 1).²⁷

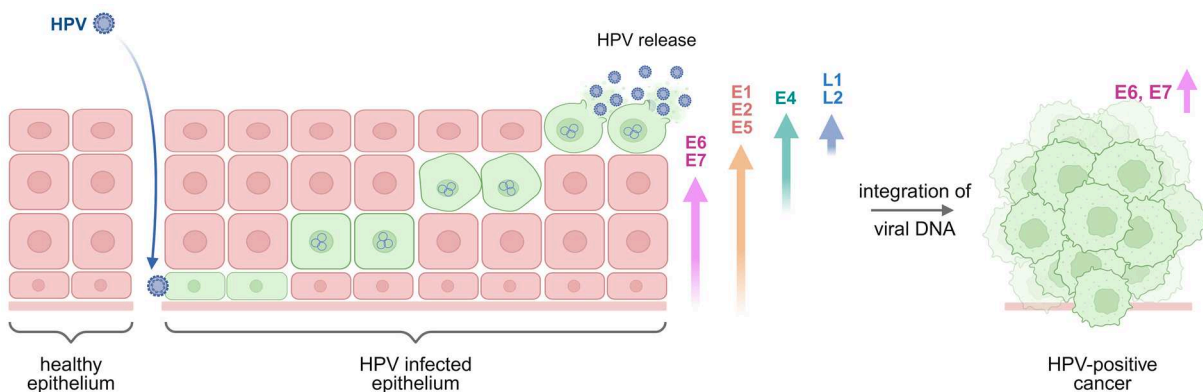


Figure 1. The life cycle of HPVs.

Left part: HPVs infect basal keratinocytes through microlesions in the epithelium. During differentiation, cells migrate into the uppermost epithelial layer, where virions are assembled and HPVs are released. Arrows indicate the expression of the viral E1, E2, E4, E5, E6, E7, L1, and L2 proteins in the respective layer of the epithelium. E6 and E7 expression facilitates the proliferation of HPV-positive cells (depicted in green). Right part: During cancer development, the viral DNA often integrates into the host chromosomes, which can lead to increased expression of the viral *E6/E7* oncogenes. Created with BioRender.com. The design was inspired by a Figure from Zhang et al., *Sig Transduct Target Ther* (2025).²⁸

1.1.2 HPV-mediated carcinogenesis

HPV-mediated carcinogenesis requires persistent infection with high-risk types of HPV, which often (in about 80%) – but not necessarily – leads to viral DNA integration into the host's genome.^{29,30} Integration events frequently lead to the loss of parts of the viral genome, such as the *E5* gene.³¹ A characteristic consequence of HPV integration is the dysregulation and overexpression of E6 and E7 (Figure 1, see also Chapter 1.2), for example, due to integration-induced disruption of *E2*, which results in decreased transcriptional repression of the E6/E7 promoter, or due to the expression of more stable, spliced viral-host *E6/E7* transcripts.^{29,32,33} As a consequence, cellular proliferation is enhanced and genomic instability increases, thereby promoting genetic changes contributing to the progression to invasive cancer.³⁴⁻³⁶

1.2 The viral *E6* and *E7* oncogenes

E6 and E7 play a central role in HPV-associated carcinogenesis. As a unique feature in high-risk HPVs, E6 and E7 are transcribed together as a polycistronic pre-mRNA that is regulated by a common promoter.^{3,37} When full-length E6 is expressed, the *E7* mRNA cannot be translated as there are only a few nucleotides between the E6 stop codon and the E7 start codon, making ribosomal re-initiation unlikely.³⁸ Therefore, E7 expression requires alternative RNA splicing of an intron within the E6 ORF, which results in a transcript that encodes E7 and a truncated E6 variant termed E6* (see Figure 42B,C in Chapter 4.1.2).^{38,39}

1.2.1 Molecular mechanisms of E6 and E7 activity

The E6 protein interacts with several cellular factors to enhance proliferation and to inhibit apoptosis. It is around 150 amino acids long and contains four zinc-binding CXXC motifs.⁴⁰ High-risk E6 oncoproteins also possess a PSD-95/DLG/ZO-1 (PDZ) binding motif (PBM), which enables binding and degradation of proteins that contain a PDZ domain and are involved in growth regulation.⁴¹ A key function of E6 is binding the cellular ubiquitin ligase E6-associated protein (E6AP) (described in detail in Chapter 1.3). While both high- and low-risk E6 proteins can interact with E6AP, only high-risk E6, in complex with E6AP, induces proteasome-dependent degradation of the tumor suppressor protein p53.⁴²⁻⁴⁴ Consequently, as p53 can act as a pro-apoptotic and pro-senescent factor,^{45,46} its degradation inhibits the induction of apoptosis and senescence. Furthermore, E6 has been reported to interfere with apoptosis by degrading the Fas-associated death domain (FADD) protein as well as Procaspase 8, thereby inhibiting the tumor necrosis factor-related apoptosis inducing ligand (TRAIL)-pathway.⁴⁷ Notably, E6-mediated p53 degradation not only inhibits apoptosis but also promotes cellular proliferation, for instance, by subsequent inhibition of the cell cycle regulator p21^{Waf1/Cip1}.⁴⁸

Moreover, E6 induces human telomerase reverse transcriptase (hTERT) expression, thereby increasing telomerase activity, which promotes cellular immortalization.^{49,50}

E7 cooperates with E6 in promoting cellular transformation. It is a protein with a length of about 100 amino acids and contains three conserved regions, including two CXXC motifs for zinc binding.⁵¹ High-risk E7 proteins bind to members of the pocket protein family, pRb, p130, and p107, and target them for proteasomal degradation.^{52,53} The pocket proteins are cell cycle regulators, which, in their active (hypophosphorylated) state, inhibit S phase entry (described in detail in Chapter 1.5.1).⁵⁴ Hypophosphorylated pRb forms active complexes with E2F family members (preferentially E2F1, E2F2, and E2F3) to repress transcription,^{55,56} a mechanism that is impaired by E7. Additionally, p130 and p107 function as part of the dimerization partner, Rb-like, E2F, and multi-vulval class B (DREAM) complex. The DREAM complex, which consists of either p130 or p107, E2F4 or E2F5, dimerization partner (DP), and the MuvB core complex (LIN9, LIN37, LIN52, LIN54, and RBBP4), transcriptionally represses genes involved in cell cycle progression.⁵⁷ The targeting of p130 and p107 by E7 leads to the disruption of the DREAM complex⁵⁸ and promotes Myb-MuvB (MMB) complex formation, which acts as a transcriptional activator of genes promoting cell cycle progression.⁵⁹ In this way, E7 overrides growth control and favors unscheduled cellular proliferation. This is further supported by E7's ability to interfere with p21^{Waf1/Cip1}- and p27^{Kip1}-mediated inhibition of Cyclin-dependent kinases (CDKs).^{60,61} As a result, CDK activity is increased and drives cell cycle progression by phosphorylating cell cycle regulators, including pRb, p130, and p107.⁵⁶ Moreover, E7 triggers the expression of CDK4/6 inhibitor p16^{INK4A}, which, under normal conditions, would lead to hypophosphorylation of pRb and cell cycle arrest.⁶²⁻⁶⁵ However, since E7 inactivates pRb, p16 is unable to induce cell cycle arrest in HPV-infected cells.^{64,66}

Altogether, the activities of the E7 protein induce uncontrolled cellular proliferation, which typically triggers p53-mediated cell death.⁶⁷ This is supported by the finding that p53 is stabilized in cells expressing E7.⁶⁸ Consequently, the cooperation of E7 with the E6 oncoprotein is essential for sustained proliferation, as E6 counteracts this response by mediating p53 degradation (Figure 2).⁶⁹

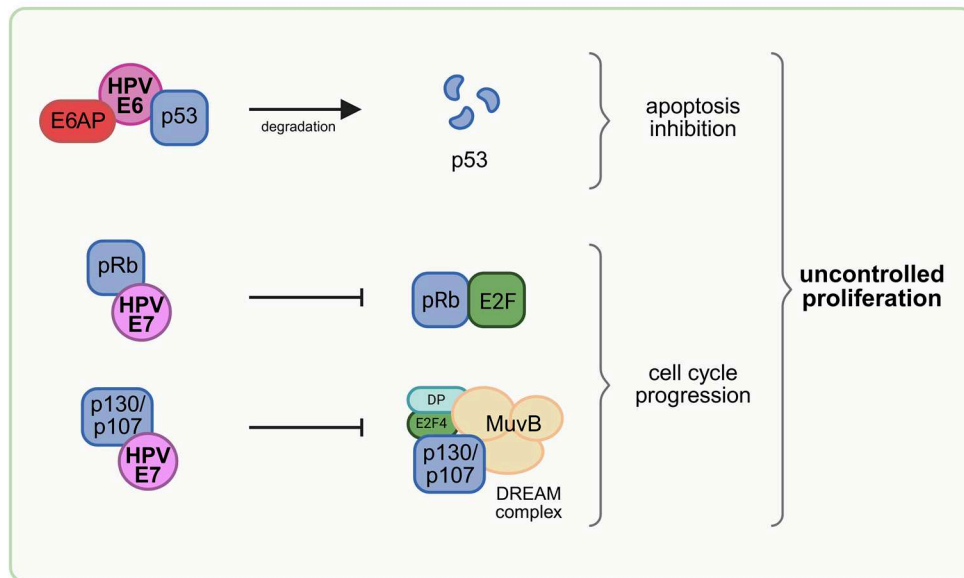


Figure 2. Key functions of the viral E6 and E7 oncoproteins in promoting uncontrolled proliferation of HPV-positive cancer cells.

HPV E6 interacts with the cellular ubiquitin ligase E6AP to mediate the proteasomal degradation of p53, thereby suppressing p53-mediated apoptosis. In parallel, HPV E7 targets the pocket proteins pRb, p107, and p130, which blocks the formation of the transcriptionally repressive pRb-E2F and DREAM complexes and thus drives cell cycle progression. Together, these anti-apoptotic and pro-proliferative functions of E6 and E7 enable the uncontrolled proliferation of HPV-positive cancer cells. Created with BioRender.com. Parts of the Figure have been adapted from Avenhaus et al., *PLOS Pathog* (2025).⁷⁰

1.2.2 E6 and E7 as therapeutic targets

The importance of E6 and E7 in HPV-mediated tumorigenesis is underlined by the fact that combined repression of these oncogenes efficiently induces rapid cellular senescence in HPV-positive cancer cells,⁷¹⁻⁷³ which is defined as an irreversible growth arrest.⁷⁴ Accordingly, continuous *E6/E7* oncogene expression is essential for the proliferation of these cells. Based on this critical dependency, E6 and E7 are viewed as ideal therapeutic targets in HPV-associated malignancies.⁶⁹ Although E6/E7-directed therapies have not yet been implemented in the clinic, several preclinical and clinical studies are investigating this approach.^{69,75}

For example, among the strategies being investigated, E6/E7-targeted immunotherapies have gained attention. Various types of therapeutic vaccines, which stimulate the immune system by delivering E6/E7 antigens, are currently in clinical trials.⁷⁶ Furthermore, adoptive T cell therapy with T cells reactive against HPV E6 or E7 is under clinical investigation.⁷⁷⁻⁷⁹

Beyond immunotherapy, several molecular tools and strategies have been developed to inhibit the expression or function of E6 and/or E7. For instance, E6 can be blocked by small-interfering RNAs (siRNAs), artificial microRNAs (miRNAs), or peptide aptamers.⁸⁰⁻⁸² Moreover, small-

molecule inhibitors such as Compound 12, as well as Myricetin and Spinacine, have been shown to block the activity of E6 in degrading p53.^{83,84} Combined repression of E6 and E7 can, for example, be achieved, by ectopic expression of E2 or by RNA interference (RNAi).^{71,73,85} Moreover, CRISPR/Cas9 genome editing has shown potential to disrupt *E6* and *E7* oncogene expression in HPV-positive cancer cells.^{86,87} In addition, a number of small molecule compounds have been shown in preclinical studies to inhibit the viral oncoproteins. For instance, human immunodeficiency virus (HIV)-1 protease inhibitors such as Saquinavir or Nelfinavir, the polyphenol Curcumin, the alkaloid Colchicine, the iron chelator Ciclopirox, or the anti-diabetic drug Metformin (described in detail in Chapter 1.6) can downregulate E6/E7 protein expression.⁸⁸⁻⁹² While the repression of E6/E7 can induce senescence,⁷¹⁻⁷³ inhibition of E6 alone has been shown in several studies to trigger apoptosis in HPV-positive cancer cells, although the reported levels of apoptosis are inconsistent.^{80-83,93-97} Furthermore, also the siRNA-mediated repression of E6AP, which together with E6 leads to the degradation of pro-apoptotic p53,⁴³ has been shown to potentially induce apoptosis in HPV-positive cancer cells.^{94,98-100}

1.3 The cellular ubiquitin ligase E6AP

Among the host cell factors targeted by HPV, cellular E6AP stands out for its essential role in mediating p53 degradation in HPV-positive cancer cells.^{42,43} E6AP, encoded by the *UBE3A* gene, is a cellular E3 ubiquitin ligase with a molecular weight of approximately 100 kDa.¹⁰¹ Together with cellular E1 and E2 enzymes, E3 ligases are components of a three-enzyme ubiquitination cascade that ultimately leads to the ubiquitination of specific target proteins.¹⁰² E6AP belongs to the homologous to the E6AP carboxyl terminus (HECT) E3 ligase family, a family that shares a HECT domain, which facilitates ubiquitin transfer from an E2 ligase to a substrate.¹⁰¹ Cellular ubiquitination substrates of E6AP are, for example, the DNA repair protein human homologue of yeast Rad23 (HHR23A), tumor suppressor PML, E3 ligase Ring1B, or β -catenin, an activator of Wnt/ β -catenin target gene transcription.¹⁰³⁻¹⁰⁷ In addition to its ubiquitin ligase activity, E6AP also functions as co-activator of steroid hormone receptor-linked transcriptional regulation.¹⁰⁸

In the context of HPV infections, the viral E6 protein hijacks E6AP. The interaction between E6AP and high-risk E6 is mediated by a leucine-rich LXXLL motif in E6AP and enables binding of p53 to a binding cleft in E6.¹⁰⁹ The formation of a ternary E6AP/E6/p53 complex then leads to polyubiquitination and proteasomal degradation of p53.¹¹⁰ Notably, in HPV-negative cells, p53 degradation is mediated by other E3 ligases such as murine double minute 2 (Mdm2),^{111,112} a pathway that is inactive in HPV-positive cancer cells.¹¹³ In addition to p53, also other proteins are targeted for degradation by the E6AP/E6 complex, such as human Scribble (hScrib), a

negative regulator of the cell cycle, Na⁺/H⁺ exchanger regulatory factor-1 (NHERF1), a negative regulator of the Wnt/β-catenin pathway, or NFX1-91, a negative transcriptional regulator of hTERT.^{44,114-116}

1.4 Cervical cancer

Worldwide, about 690 000 new cancer cases yearly are attributable to infections with oncogenic HPVs.¹¹⁷ While HPV is the main driver of oropharyngeal cancers in high-income countries, cervical cancer remains the most prevalent HPV-associated malignancy in low-income countries and ranks fourth among the most common cancers in women worldwide.¹¹⁷⁻¹¹⁹ Nearly all cases of cervical carcinomas are caused by HPV, and the high-risk types 16 and 18 account for more than 70% of the cases.^{6,117}

The development of cervical cancer after an infection with HPV typically takes several decades and is preceded by pre-neoplastic lesions with increasing degrees of dysplasia, termed cervical intraepithelial neoplasia (CIN1-3).^{120,121} While CIN1 lesions often regress spontaneously due to immune clearance, CIN2 and CIN3 are more likely to progress to invasive cancer.^{121,122} Further, CIN2 and CIN3 are characterized by high expression levels of the widely investigated biomarker p16, which is used for diagnostic purposes.¹²³ Importantly, whereas a high-risk HPV infection alone appears to be essential, it is likely not sufficient to cause cervical cancer.¹²⁴ This is indicated by the long period between the initial infection and cancer formation (typically several decades) and the fact that only a small percentage of the infected females eventually develops cancer.⁵ Additional factors that are possibly involved include, for example, smoking, multiparity, HIV-infection, and long-term contraceptive use.¹²⁵⁻

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1.4.1 Prevention of cervical cancer

An effective strategy in reducing the incidence of cervical cancer and other HPV-related malignancies is the use of prophylactic HPV vaccines, available for over 15 years. These vaccines aim to induce HPV-specific neutralizing antibodies to prevent HPV infection.¹²⁹ They contain virus-like particles (VLPs) composed of L1, one of the two structural proteins contained in the HPV capsid.¹³⁰ Depending on the included VLPs, these vaccines protect against specific HPV types and are categorized by their valency. To date, 5 prophylactic HPV vaccines have been prequalified by the World Health Organization (WHO): the bivalent Cervarix, Cecolin, and Walrinvax against HPV16 and 18, the quadrivalent Gardasil against HPV16, 18, 6, and 11, and the ninevalent Gardasil9 against HPV16, 18, 31, 33, 45, 52, 58, 6, and 11.¹³¹ An additional quadrivalent HPV vaccine, Cervavac, is licensed in India.¹³² Since L1 VLP vaccines only

protect against specific HPV genotypes, current research is focusing on vaccines based on the minor capsid protein L2, which contains highly conserved epitopes and is thus capable of inducing cross-specific antibody responses.¹³³ Moreover, as current HPV vaccines do not have an effect on pre-existing infections, effort is also invested in developing vaccines that can act both prophylactically and therapeutically.^{134,135}

The efficacy of HPV vaccination is underscored by studies demonstrating a strong reduction in the prevalence of vaccine-covered HPV genotypes after vaccination.^{136,137} Initial studies also suggest a decreased incidence of cervical cancer in women eligible for HPV vaccination.¹³⁸⁻¹⁴¹ Current guidelines recommend vaccination for both girls and boys before the age of 15 and the WHO aims at a 90% HPV vaccination coverage in 2030 to eliminate cervical cancer.^{142,143} However, according to the WHO, global vaccination rates remain low, with only 31% of eligible girls globally receiving the first dose of the HPV vaccine as of 2024.¹⁴³ Specifically, many low- and middle-income countries have yet to establish national HPV vaccination programs, and challenges such as high vaccine prices, inadequate infrastructure, and cultural acceptance have to be overcome to ensure global access to HPV vaccines.^{144,145}

Another important pillar in the prevention of cervical cancer is screening, which includes, for example, the Papanicolaou (Pap) smear, a cytological test to identify cell abnormalities in the cervix, p16/Ki-67 dual staining of cytological samples, and high-risk HPV DNA-based tests.¹⁴⁶ In cases of positive results, these are typically followed by visual examination through colposcopy.¹⁴⁶

1.4.2 Therapeutic strategies in cervical cancer

The treatment of cervical cancer depends on the stage and progression of disease and typically includes surgery, chemotherapy (CT), and radiotherapy (RT). Surgery is primarily used in early-stage disease, while CT combined with RT is recommended for locally advanced, metastatic, and recurrent cervical cancer.^{147,148} The platinum-based drug Cisplatin is commonly used for CT, either as a single agent or, for increased efficiency, in combination with non-platinum-based chemotherapeutics such as Topotecan or Paclitaxel.¹⁴⁹ Some clinical studies also report positive effects of combining Cisplatin with the topoisomerase inhibitor Etoposide.¹⁵⁰⁻¹⁵²

1.4.2.1 Cisplatin: mechanisms of action

The primary target of Cisplatin is nuclear DNA, where it preferentially binds to nucleophilic N7-sites of purine bases, such as guanine, to form covalent adducts leading to intra- and interstrand crosslinks.^{153,154} These DNA lesions cause conformational changes that block

replication and transcription, leading to cell cycle arrest to allow DNA damage repair.¹⁵⁴ If the damage is extensive or cannot be repaired, the cell undergoes apoptotic cell death.^{153,155} Additionally, DNA damage signaling caused by low-dose Cisplatin has been shown to induce cellular senescence.¹⁵⁶⁻¹⁵⁸

The outcome of Cisplatin-induced DNA damage depends on the activated signaling pathways. For example, DNA damage upon Cisplatin treatment can activate the ataxia telangiectasia mutated (ATM)- and Rad3-related (ATR) kinase, which induces stabilization of p53.^{159,160} Activated p53 promotes cell cycle arrest, for example, by transactivating *p21* or *GADD45*.¹⁶¹ p53 also contributes to Cisplatin-mediated apoptosis by several mechanisms, such as counteracting anti-apoptotic B-cell lymphoma-extra-large (BCL-xL) or enhancing Fas death receptor expression.^{162,163} In line, lack of functional p53 can promote resistance towards Cisplatin-induced apoptosis.¹⁶³⁻¹⁶⁶ In HPV-positive cervical cancer cells, Cisplatin-mediated apoptosis depends on the activation of Caspase 8, which cleaves BH3-interacting domain death agonist (BID) into its active form truncated BID (t-BID), a mediator of the Fas apoptotic signaling pathway.^{167,168} Further studies suggest that mitogen-activated protein kinase (MAPK) pathways, including JNK, also contribute to Cisplatin-mediated apoptosis.^{169,170} In addition to nuclear DNA, Cisplatin can damage mitochondrial DNA and induce the generation of mitochondrial reactive oxygen species (ROS), thereby triggering apoptosis.¹⁷¹⁻¹⁷³

1.4.2.2 Targeted and immunotherapeutic approaches

While Cisplatin remains a key component of cervical cancer treatment, its limitations, including resistance and adverse effects, continue to pose significant challenges and have motivated the development of targeted therapies or immunotherapeutic strategies.^{149,174,175} For instance, the monoclonal antibody Bevacizumab, which neutralizes vascular endothelial growth factor (VEGF), a factor that plays a role in tumor growth by promoting angiogenesis, is used to treat cervical cancer.¹⁷⁶ A clinical study showed improved survival when CT was combined with Bevacizumab, leading to the approval of the combination of Cisplatin, Paclitaxel, and Bevacizumab for treatment of metastatic and recurrent cervical cancer by the US Food and Drug Administration (FDA) in 2014.¹⁷⁷⁻¹⁷⁹ Furthermore, the antibody-drug conjugate Tisotumab vedotin, which binds to the surface protein tissue factor (TF) and interferes with microtubules once internalized in the cell, is also approved for treatment of recurrent or metastatic disease.^{180,181} Due to recent advances in the development of immunotherapies, many clinical studies also investigate the potential of immunotherapeutics for the treatment of cervical cancer, and some have already been integrated into standard care.^{182,183} For example, immune checkpoint inhibitor Pembrolizumab, which targets the programmed death 1 (PD-1)/PD ligand-1 (PD-L1) checkpoint, was initially approved by the FDA in combination with CT to treat PD-L1-

positive, recurrent or metastatic cervical cancer.^{184,185} More recently, in 2024, Pembrolizumab received FDA approval also for use in combination with chemoradiotherapy for patients with high-risk locally advanced disease.^{186,187}

1.5 Cellular mechanisms of tumor suppression

Cancer develops through a complex, multi-step process during which cells accumulate genetic and epigenetic changes, thus acquiring characteristic capabilities.¹⁸⁸ These so-called hallmarks of cancer include, for example, evasion of growth suppressors, sustained proliferation, resistance to cell death, and replicative immortality.^{189,190} To avoid uncontrolled cellular proliferation and eliminate abnormal cells, organisms have evolved tumor suppression mechanisms such as the induction of apoptosis or senescence, and a tight regulation of the cell cycle, mechanisms which are often deregulated in cancer.¹⁹¹⁻¹⁹³ Importantly, these cellular countermeasures can not only prevent cancer development but can also be therapeutically exploited.¹⁹⁴

1.5.1 Cell cycle control

The cell cycle can be divided into the interphase and the mitotic phase. During the interphase, which comprises the G1, S, and G2 phases, the DNA is replicated, whereas during mitosis, two daughter cells are formed.¹⁹² Before re-entering the cell cycle, cells can remain in a non-proliferative state known as G0 or quiescence.¹⁹⁵ In response to mitogens or other stimuli, quiescent cells can enter the cell cycle.¹⁹⁶ Progression through the cell cycle phases is controlled by CDKs and their Cyclin partners. The transition from G0 to G1 is mediated by CDK4/6 binding Cyclin D, while CDK2 and Cyclin E drive the transition from G1 to the S phase and initiate DNA replication.^{197,198} CDK2-Cyclin A complexes mediate the progression through the S phase, and finally, CDK1 and Cyclin B facilitate the G2/M transition.^{197,198}

The expression of CDKs, Cyclins, and other cell cycle phase-specific genes is tightly regulated. The pRb-E2F and DREAM complexes repress transcription of cell cycle genes to maintain quiescence and restrict cell cycle entry.^{57,199} Specifically, hypophosphorylated pRb binds to the activating transcription factors E2F1, E2F2, and E2F3 to repress the transcription of G1/S genes via E2F binding sites in their promoters.^{56,200} In addition, hypophosphorylated p130 or p107 associate with the MuvB core, E2F4/5, and DP to form the DREAM complex, a process that depends on DYRK1A-mediated phosphorylation of LIN52.^{57,200,201} DREAM represses both G1/S genes by binding to E2F binding sites as well as G2/M genes by binding cell cycle genes homology region (CHR) promoter elements.^{200,202} In response to growth factors, Cyclin D leads to CDK4/6-mediated phosphorylation of pRb, p130, and p107.²⁰³ Consequently, the repression

of activator E2Fs by pRb is relieved, which results in increased expression of E2F-driven early cell cycle genes.²⁰³ Moreover, the DREAM complex is disrupted, leading to the association of the MuvB core with B-MYB and/or forkhead box protein M1 (FOXO1) during the S, G2, and M phases.^{204,205} The resulting MMB, FOXO1-MMB, or FOXO1-MuvB complexes activate the transcription of CHR-containing G2/M cell cycle genes to further promote cell cycle progression and proliferation.^{200,206} In line, disruption of these complexes, for example by depletion of B-MYB, acts anti-proliferative in various cell lines.²⁰⁷

To ensure genomic integrity, multiple cell cycle checkpoints monitor for DNA damage, replication errors, or spindle defects.²⁰⁸ These checkpoints delay or stop cell cycle progression to allow time for repair or resolution of stress signals.^{192,208}

1.5.2 Senescence

Cellular senescence is defined as an irreversible growth arrest in the G1 and G2 phases of the cell cycle.^{74,209} In contrast, quiescent cells are still capable of re-entering the cell cycle.¹⁹⁵ The foundation for the discovery of senescence was laid in 1961 by Paul Moorhead and Leonard Hayflick, who discovered that human fibroblasts do not proliferate indefinitely but stop dividing after a certain number of divisions while remaining viable.²¹⁰ This phenomenon, called replicative senescence, takes place during aging, primarily as a consequence of telomere shortening and the resulting activation of the DNA damage response (DDR).²¹¹ Senescence also plays a role during embryogenesis, where developmental senescence contributes to the specification of cell fates and tissue remodeling.²¹² In addition, pathological stressors can induce premature senescence.²¹³ Inactivation of tumor suppressor genes like *PTEN*, or activation of oncogenes such as *BRAF* or *Ras* can lead to oncogene-induced senescence.²¹⁴ Moreover, chemotherapeutic drugs, ionizing radiation, or oxidative stress can induce senescence by causing DNA damage.^{215,216} Importantly, while DNA damage and DDR signaling are often reported as the main cause of cellular senescence, senescence can also be induced in a DNA damage-independent manner.^{217,218}

The CDK inhibitor p21 is a main mediator of senescence-associated cell cycle arrest.²¹⁹ p21 is primarily activated by p53 in response to DNA damage and inhibits cell cycle progression during different cell cycle phases by inhibiting various Cyclin/CDK complexes, leading to the hypophosphorylation of pRb, p130, and p107.^{56,199} Consequently, the pRb-E2F and the DREAM repressor complexes are formed and can inhibit transcription of genes that are essential for both the G1/S and the G2/M transition, thereby promoting the induction of senescence.^{199,201}

Senescent cells undergo characteristic morphological and molecular alterations.²¹⁸ Interestingly, despite remaining metabolically active, these cells do not proliferate.²²⁰ Morphologically, senescent cells exhibit enlargement and flattening, as well as accumulation of cytoplasmic particles like Lipofuscin.^{220,221} Chromatin remodeling leads to the formation of senescence-associated heterochromatin foci (SAHF).²²² Additionally, senescent cells show enhanced lysosomal content and high expression of the lysosomal protein β -galactosidase, which underlies the senescence-associated β -galactosidase (SA- β -Gal) activity measurable at pH 6.0 and serves as a widely used senescence marker.^{223,224} Another defining feature is the senescence-associated secretory phenotype (SASP), which involves the release of many different factors, such as pro-inflammatory cytokines, chemokines, and growth factors, by senescent cells.²²⁵ The SASP reinforces senescence in surrounding cells in a paracrine manner and recruits immune cells to eliminate senescent cells.^{218,226}

Importantly, senescence has a dual role in tumors. On the one hand, it exerts tumor-suppressive effects by inducing an irreversible growth arrest.⁷⁴ On the other hand, accumulation of senescent cells and a persistent SASP can promote tumorigenesis by creating an immunosuppressive and growth-stimulatory environment.²²⁵⁻²²⁷ These potentially pro-tumorigenic capabilities of senescent cells are now recognized as contributing to the hallmarks of cancer.¹⁹⁰

1.5.3 Apoptosis

Apoptosis, a form of programmed cell death, is a process of cellular self-destruction following specific extracellular or intracellular signals.²²⁸ Apoptotic signaling can be initiated by two major pathways. The extrinsic (death receptor) pathway is activated by ligands binding to death receptors on the cellular surface, resulting in the activation of Caspase 8 and 10.²²⁹ The intrinsic (mitochondrial) pathway is triggered by intracellular perturbations, for example DNA damage, oxidative stress, or endoplasmic reticulum (ER) stress, ultimately inducing mitochondrial outer membrane permeabilization (MOMP) and Caspase 9 activation.²²⁸

The intrinsic apoptosis pathway is tightly controlled by proteins of the BCL-2 family, which comprises three subgroups: pro-survival proteins such as BCL-2, BCL-xL, BCL-W, and MCL-1, pro-apoptotic BH3-only proteins such as NOXA, PUMA, BID, BIM, and BAD, as well as the pro-apoptotic effector proteins BAX, BAK, and BOK.²³⁰ Intrinsic stress leads to an increase of the levels of BH3-only proteins.²³⁰ These proteins bind to and inhibit the pro-survival BCL-2 proteins, thereby releasing the apoptosis effectors BAX and BAK from inhibition.²³⁰ In addition, BH3-only proteins such as PUMA or the cleaved, active form of BID (t-BID) can directly activate BAX and BAK.^{231,232} Once activated, BAX and BAK oligomerize on the mitochondria, leading

to the formation of an apoptotic pore and Cytochrome c release.²³³ Cytochrome c, in turn, binds to apoptotic protease-activating factor-1 (APAF-1), facilitating the formation of the oligomeric apoptosome and subsequent Caspase 9 activation.²³⁴ Importantly, p53 is a central regulator of the BCL-2 family proteins and thereby plays a critical role in the induction of mitochondrial apoptosis. On the one hand, nuclear p53 induces the transcriptional activation of several BH3-only genes, (e.g., *NOXA*, *PUMA*) as well as *BAX* and *APAF-1*.^{235,236} On the other hand, cytoplasmic p53 can form complexes with BCL-2 family proteins, leading to their direct inhibition (anti-apoptotic BCL-2 and BCL-xL) or activation (pro-apoptotic BAX and BAK).²³⁶

The activation of the initiator Caspases 8, 9, and 10 at the end of both the intrinsic and extrinsic pathway leads to the activation of the downstream effector Caspases 3 and 7, which cleave various intracellular substrates.²³⁷ A well-known substrate represents the DNA repair enzyme poly(ADP-ribose) polymerase (PARP), whose cleavage product, together with those of caspases, is a commonly used marker for apoptosis.^{238,239} Apoptosis results in typical morphological changes like cell shrinking and membrane blebbing, as well as fragmentation of DNA.^{240,241} The latter can be visualized to detect apoptosis by terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assays.²⁴²

1.5.4 p53 and its role in tumor suppression

p53, a tumor suppressor protein that is encoded by the *TP53* gene, is often referred to as “guardian of the genome” due to its crucial role in maintaining DNA integrity.²⁴³ In most human cancers, p53 is either mutated or p53 signaling is impaired.²⁴⁴ p53 is activated in response to diverse stress signals, including DNA damage, oncogene activation, and replication stress.^{244,245} A key tumor-suppressive function of p53 lies in its potential to induce cell cycle arrest, thereby enabling DNA repair.^{56,246} If the damage is severe or irreparable, p53 can trigger senescence or apoptosis.^{45,46} Thus, p53 is a decisive factor in determining cell fate, and the outcome – temporary arrest, senescence, or apoptosis – likely depends on threshold-based mechanisms and p53 levels.²⁴⁷

p53 primarily acts as a transcription factor and regulates gene expression of a plethora of target genes.²⁴⁸ On the one hand, p53 directly activates gene expression by binding to p53 response elements typically located in the proximal promoter regions of its target genes.²⁴⁹ For instance, p53 activates *CDKN1A* (encoding p21), the pro-apoptotic genes *PUMA* and *BAX*, and genes involved in DNA repair, like *XPC* and *POLH*.²⁵⁰⁻²⁵⁴ On the other hand, p53 indirectly represses many cell cycle-related genes by inducing p21, thereby promoting the formation of the pRb-E2F and DREAM repressor complexes.²⁵⁵ Hence, as an essential part of the p53-p21-pRb and p53-p21-DREAM signaling pathways, p53 leads to cell cycle gene repression and

cell cycle arrest.⁵⁶ Besides regulating gene expression, p53 can also exert transcription-independent functions.²⁵⁶ For example, p53 directly interacts with BCL-2 family proteins in the cytoplasm, localizes at mitotic centrosomes to maintain their integrity, and binds to DNA damage sites, thereby recruiting repair factors.^{236,257,258}

Regulation of p53 at the protein level is mediated by several post-translational modifications (PTMs), including phosphorylation, acetylation, and ubiquitination.²⁵⁹ In response to DNA damage, p53 is phosphorylated by the ATM and ATR kinases at Ser15 residues, or by the ATM/ATR downstream effectors checkpoint kinase (CHK) 1 and CHK2 at Ser20 residues.²⁶⁰⁻²⁶³ These and other phosphorylation events can lead to p53 stabilization by inhibiting its interaction with Mdm2.^{264,265} Upon severe DNA damage, phosphorylation at Ser46 enhances apoptosis.²⁶⁶ Furthermore, phosphorylation at sites such as Ser15, Ser20, and Thr18 can promote the recruitment and binding of p300 to p53.²⁶⁷⁻²⁶⁹ p300 is one of the acetyltransferases mediating p53 acetylation, for example, at Lys373 or Lys382.²⁷⁰ These and other acetylation sites of p53 can also contribute to its transcriptional activity.²⁷¹

1.6 Metformin

Metformin (1,1-dimethylbiguanide hydrochloride) (Figure 3) is widely used in the treatment of type 2 diabetes mellitus (T2DM), a metabolic disorder characterized by the resistance to insulin and dysfunction of pancreatic β -cells.²⁷² The discovery of Metformin can be traced back to chemical analyses of the medicinal plant *Galega officinalis* (goat's rue).²⁷³ This plant was found to be rich in guanidine, an organic base that was shown in 1918 to lower blood glucose levels in rabbits.²⁷³ In 1922, Emil Werner and James Bell synthesized Metformin through condensing two guanidine molecules and eliminating ammoniac.²⁷⁴ About three decades later, Metformin, along with the related biguanides Phenformin and Buformin, began to be used clinically for the treatment of diabetes in Europe.²⁷³ However, due to a high risk of lactic acidosis, Phenformin and Buformin were taken off the market, while oral Metformin therapy was recognized as safe and well tolerated.^{273,275-277} Today, Metformin is considered the first-line pharmacological therapy for T2DM, and it is estimated that approximately 200 million people worldwide take the drug daily.²⁷⁸

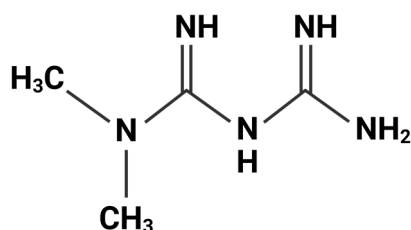


Figure 3. Chemical structure of the anti-diabetic drug Metformin.

Metformin is a biguanide compound that contains two linked guanidine molecules substituted with two methyl groups. Created with BioRender.com

The typical oral dose of Metformin ranges from 0.5 to 2.5 g per day.²⁷⁸ With an oral bioavailability of 40-60%, the steady-state plasma levels of Metformin achieved in T2DM patients are approximately 0.5-2.0 µg/mL (4-15 µM).^{278,279} Uptake from the intestinal lumen and subsequent distribution to key organs, including the liver and kidneys, is mediated by various transporters like organic cation transporter (OCT) 1, OCT2, and OCT3, plasma membrane monoamine transporter (PMAT), as well as multidrug and toxin extrusion (MATE) 1 and MATE2.²⁸⁰ Metformin is not metabolized, thus excreted unchanged by the kidneys, and has a relatively short plasma half-life of approximately five hours in individuals with normal renal function.^{279,281}

1.6.1 Molecular mechanisms of Metformin

Despite its clinical use for several decades, the complex molecular mechanisms underlying the therapeutic effects of Metformin remain incompletely understood. A major challenge in resolving these mechanisms arises from the uncertainty regarding which *in vitro* concentrations reflect therapeutically relevant levels achieved *in vivo*.²⁷⁸ Notably, many preclinical studies investigating the mechanisms of Metformin employ supra-pharmacological (millimolar) doses that are significantly higher than the concentrations achievable in patients.^{282,283}

The liver is one of the key target organs of Metformin, as the drug has frequently been reported to lower blood glucose levels by inhibiting hepatic gluconeogenesis.²⁸³⁻²⁸⁵ Various mechanisms have been proposed to explain this effect. One of the most-well established molecular actions of Metformin is the inhibition of complex I of the mitochondrial respiratory chain.^{278,286,287} By binding to its major inhibitory interaction site in complex I, the quinone-binding channel (Q-channel) that is exposed in the deactive state, Metformin prevents reactivation of the enzyme.^{288,289} As a result, oxidative phosphorylation (OXPHOS) is suppressed, leading to an increase in the AMP:ATP ratio in hepatocytes, which in turn activates AMP-activated protein kinase (AMPK).^{290,291} However, it is known today that Metformin-induced activation of AMPK and its upstream kinase liver kinase B1 (LKB1) is not required for the direct inhibition of hepatic glucose production.^{278,292,293} Instead, the inhibition of gluconeogenesis may be explained by the reduction in cellular energy levels and the increase in AMP, given that gluconeogenesis is an energy-intensive process.^{292,294} Still, while the inhibition of complex I by Metformin is widely accepted at the biochemical level, it is important to note that these effects may only occur at supra-pharmacological concentrations.^{278,295} An alternative proposed mechanism, which might be therapeutically more relevant, involves the Metformin-mediated inhibition of the mitochondrial glycerol-3-phosphate dehydrogenase, possibly as a downstream consequence of inhibition of complex IV of the mitochondrial OXPHOS system.²⁹⁶⁻²⁹⁸ This leads to an

enhanced cytosolic redox state, thereby reducing gluconeogenesis.^{296,297} However, this mechanism requires further investigation.

Beyond its effects on hepatic gluconeogenesis, Metformin has also been reported to improve insulin sensitivity. For instance, Metformin may reduce lipid-induced resistance to insulin in the liver via lysosome-dependent AMPK activation.^{278,299,300} Additionally, Metformin could enhance insulin sensitivity by modulating immune cells.²⁹³ Importantly, Metformin also exerts pleiotropic effects on the gut, which could contribute to its therapeutic actions. These include increased intestinal utilization of circulating glucose, reduced intestinal glucose absorption, enhanced secretion of glucagon-like peptide 1 (GLP1), an incretin hormone that improves blood glucose homeostasis, and, as recently proposed, modulation of the gut microbiota composition.^{278,301-304}

1.6.2 Metformin as an anti-cancer agent

Over the past two decades, the repurposing potential of Metformin for cancer therapy has been intensely investigated and remains a subject of ongoing debate.³⁰⁵ In 2005, a retrospective clinical study first reported that Metformin use could reduce cancer incidence in T2DM patients.³⁰⁶ Subsequently, several epidemiological studies evaluated the association between Metformin intake and cancer risk. While some confirmed a reduction in cancer risk,³⁰⁷⁻³¹¹ others found no significant benefit.³¹²⁻³¹⁴ In parallel, numerous preclinical and clinical studies examined the potential anti-neoplastic effects of Metformin. A plethora of preclinical studies demonstrated that Metformin efficiently inhibits the proliferation of various cancer cell lines and reduces tumor growth in mouse models.^{276,305,315,316} Moreover, Metformin has been reported to act synergistic with CT and RT,³¹⁷⁻³²⁰ but other studies question these data.³²¹⁻³²⁷ Additionally, many clinical trials have explored the use of Metformin as an anti-cancer agent in both diabetic and non-diabetic patients, either as monotherapy or in combination with CT. While some of these trials reported beneficial effects,³²⁸⁻³³² many others found no improvement with Metformin treatment – whether used alone or in combination with CT.^{305,333-337} In rare cases, even decreased CT efficacy in the presence of Metformin has been observed.³³⁸

The proposed tumor-suppressive effects of Metformin are attributed to both direct and indirect mechanisms. The anti-proliferative effects observed *in vitro* point at direct mechanisms, likely linked to its inhibitory effects on complex I and subsequent activation of AMPK.^{305,316} Supporting this notion, overexpression of the Metformin-resistant NADH dehydrogenase NDI1 from *Saccharomyces cerevisiae*, which mimics complex I activity, abrogates the anti-proliferative effects of Metformin in cancer cells and xenograft models.^{305,339} Furthermore, numerous studies describe a range of tumor-suppressive mechanisms, most of which are

probably secondary to complex I inhibition.³⁰⁵ For instance, Metformin has been shown to inhibit mammalian target of Rapamycin complex 1 (mTORC1) signaling in an AMPK-dependent manner, reduce lipid synthesis, modulate the expression of miRNAs, and inhibit Wnt signaling in cancer cells.³⁴⁰⁻³⁴⁵ On the other hand, Metformin may also indirectly act anti-tumorigenic by lowering systemic glucose and insulin levels.^{305,346} This could decrease insulin receptor signaling and subsequent activation of the downstream growth-promoting PI3K/AKT/mTOR pathway.^{305,347} Additionally, Metformin has been proposed to modulate anti-cancer immunity, for example by altering cytokine production in immune cells or by enhancing the activity of natural killer cells.^{278,348,349}

Importantly, most studies investigating the anti-cancer effects have employed supra-pharmacological concentrations of Metformin. Thus, both the mechanisms underlying Metformin's anti-tumor activity as well as its interactions with CT remain incompletely understood and their relevance in the clinical setting is still uncertain.³⁰⁵

1.6.3 Metformin and cervical cancer

Evidence regarding the effects of Metformin use in cervical cancer remains inconclusive. While some studies suggest that Metformin may lower both the incidence^{350,351} and mortality³⁵² associated with cervical cancer in T2DM patients, others have not observed such benefits.^{353,354} In preclinical studies, Metformin has been shown to exert both anti-proliferative and pro-apoptotic effects in cervical cancer cell lines and several underlying mechanisms have been proposed.^{92,355} These include the reduction of AMPK O-GlcNAcylation, AMPK-mediated hyperacetylation of histone 3 lysine 9 (H3K9), and FOXM1 suppression.³⁵⁶⁻³⁵⁸ Additionally, Metformin has been reported to inhibit mTOR signaling in cervical cancer cells.⁹² Beyond its effects as a single agent, Metformin has also been shown to potentially enhance the anti-tumor activity of other compounds. Specifically, it enhances the effects of the oral mTOR inhibitor Everolimus and the HIV-1 protease inhibitor Nelfinavir in cervical cancer cells.^{359,360} Moreover, previous work from our group demonstrated that Metformin represses the expression of the viral *E6/E7* oncogenes in HPV-positive cervical cancer cells.⁹² Notably, however, despite downregulating *E6/E7*, Metformin does not induce senescence and was even found to counteract senescence triggered either by siRNA-mediated *E6/E7* silencing or by the chemotherapeutic agent Etoposide.⁹²

1.7 Research objectives

Inhibiting tumor cell proliferation is a central anti-cancer strategy. In HPV-positive cancer cells, the viral *E6* and *E7* oncogenes are the drivers of uncontrolled cell growth and could serve as important therapeutic targets. However, despite the development of effective strategies to inhibit *E6/E7 in vitro*, translation into the clinical setting remains challenging. My investigations aim to expand our knowledge on molecular strategies to interfere with the growth of HPV-positive cancer cells by addressing the following main research questions:

- (1) What role does the cellular ubiquitin ligase E6AP, which mediates p53 degradation together with E6, play for the proliferation of HPV-positive cancer cells? Which are the cellular fates resulting from E6AP inhibition in terms of their (i) proliferative behavior and cell cycle regulation, ii) expression of growth-regulatory factors, and iii) senescence and apoptosis regulation? Are these responses specific for HPV-positive cancer cells?
- (2) Does the phenotypic behavior of HPV-positive cancer cells upon E6AP, E6/E7, or E6 inhibition differ? Are there differences in the regulation of potential key downstream targets, such as p53, p21, pRb, and p130?
- (3) Does Metformin influence the phenotype of HPV-positive cancer cells, which is induced by E6AP or E6/E7 inhibition? Which mechanism may underlie its reported anti-senescent activity in HPV-positive cancer cells?
- (4) Why does Metformin impair the proliferation of HPV-positive cancer cells and inhibit viral *E6/E7* oncogene expression, but does not result in senescence, other than targeted E6/E7 repression by RNAi? Are these discrepant phenotypic responses linked to differences in global proteome composition or cell cycle regulation? Do the anti-proliferative effects of Metformin depend on its associated repression of E6/E7?
- (5) How effective are combination treatments of Metformin with standard chemotherapeutics? Does Metformin influence the anti-proliferative, pro-senescent, and pro-apoptotic effects of Etoposide or Cisplatin, and, in case, how does it affect potential downstream pathways involved? Are these responses conserved in HPV-negative cancer cells?

Overall, these studies aim to provide insights into the potential of E6AP to serve as a novel therapeutic target to specifically attack HPV-positive cancer cells. Furthermore, they seek to contribute to a better understanding of the phenotypic response of HPV-positive cancer cells to Metformin, which is currently under discussion for its possible use in cancer therapy.

CHAPTER 2

RESULTS

2 Results

2.1 Evaluation of the role of E6AP in the proliferation of HPV-positive cancer cells

It is well known that the growth of HPV-positive cancer cells depends on the expression of the viral *E6/E7* oncogenes, and inhibition of *E6/E7* induces rapid cellular senescence, an irreversible proliferative arrest.⁷¹⁻⁷⁴ Since the cellular ubiquitin ligase E6AP plays an important role in HPV-positive cancer cells by mediating, together with E6, the degradation of the growth-suppressive and pro-apoptotic protein p53,^{42,43,113} I aimed to investigate which cellular fates result from E6AP inhibition in these cells.

2.1.1 E6AP silencing induces senescence in HPV-positive cancer cells

In order to inhibit E6AP expression in HPV18-positive HeLa or HPV16-positive SiHa cervical cancer cells via RNAi, I employed three different siRNAs targeting the E6AP-encoding *UBE3A/E6AP* transcript (siE6AP-1, -2, -3). Cells were transfected and analyzed following the scheme in Figure 4A. RNA and immunoblot analyses showed a successful downregulation of *UBE3A/E6AP* transcript (Figure 4B) as well as E6AP protein levels (Figure 4C) in both HeLa and SiHa cells. In line with previous reports,^{100,361,362} E6AP knockdown was accompanied by a downregulation of E6 protein levels, while E7 levels remained unaffected. Furthermore, compared to cells transfected with control siRNA, inhibition of E6AP led to an expected increase in p53 and p21 protein levels,^{94,97,98,100,363} as well as an increase in post-translationally phosphorylated or acetylated p53 forms (P-p53 Ser15, P-p53 Ser20, and Ac-p53 Lys382) (Figure 4C). Strikingly, investigating the long-term effects of E6AP inhibition on the phenotype of HeLa and SiHa cells revealed efficient induction of cellular senescence and proliferation arrest in both cell lines. This was evidenced by a marked increase in the typical signs of senescence (morphological alterations, e.g. cytoplasmic extensions, cellular enlargement and flattening) and positive senescence-associated β -galactosidase (SA- β -Gal) staining²²³ (Figure 4D, upper panels for each cell line), as well as strongly impaired colony outgrowth in corresponding colony formation assays (CFAs) (Figure 4D, lower panels for each cell line). These data suggest that E6AP acts as a senescence-inhibiting factor in HPV-positive cancer cells.

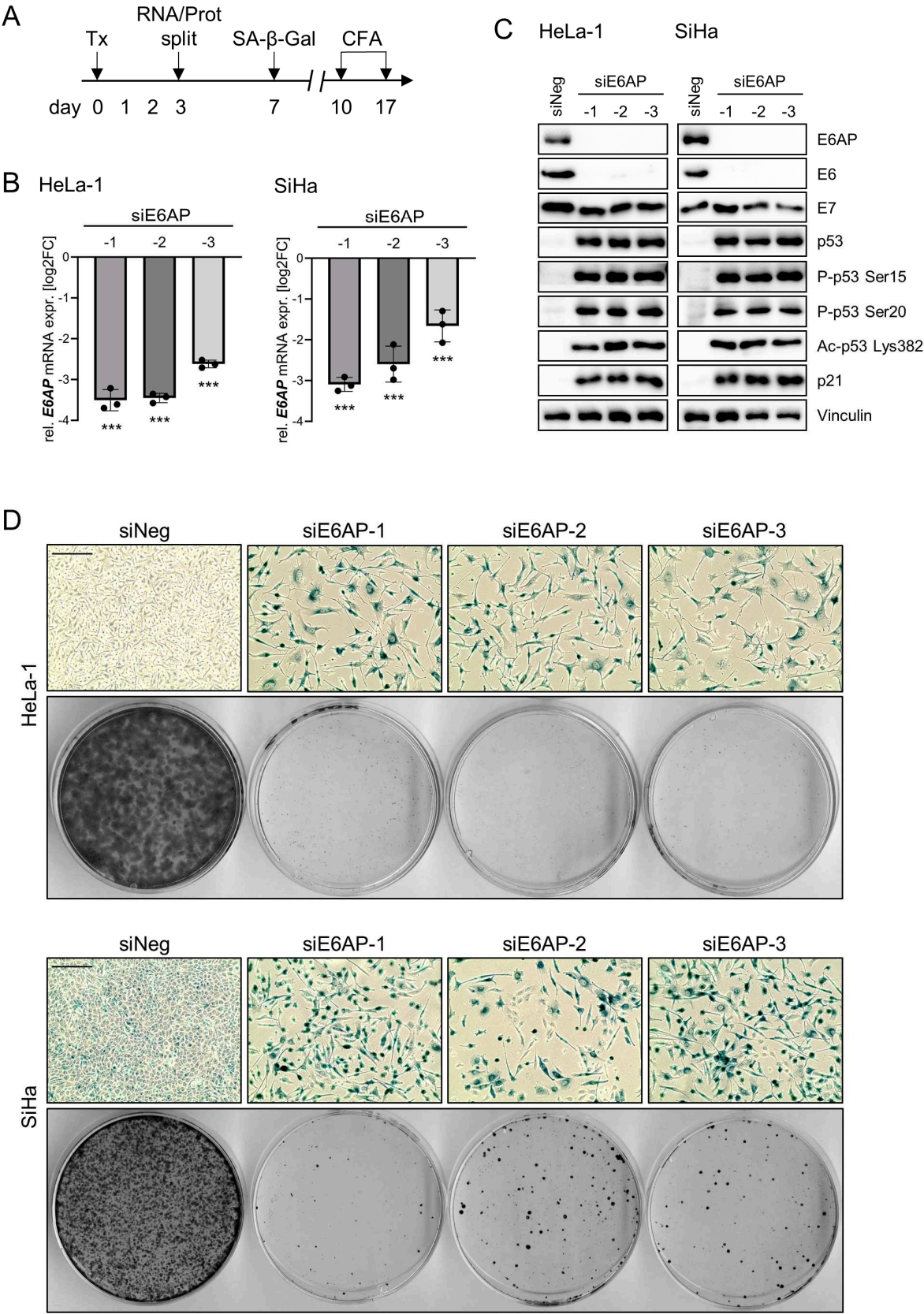


Figure 4. See Figure legend on the next page.

Figure 4. RNAi-mediated E6AP silencing induces senescence in HPV-positive cancer cells.

(A) Treatment scheme: HeLa-1 or SiHa cells were transfected (Tx) with three different E6AP-targeting siRNAs (siE6AP-1, -2, -3) or control siRNA (siNeg). Seventy-two hours post-Tx, cells were either harvested for RNA or protein (Prot) analyses or split and further cultivated for senescence assays (SA- β -Gal staining) or colony formation assays (CFA) after the indicated time period. (B) qRT-PCR analyses of *UBE3A/E6AP* mRNA levels. Shown are log₂-transformed fold changes (log₂FC) of mean expression with standard deviations ($n = 3$). Statistically significant differences between cells transfected with siE6AP-1, -2, or -3, and those transfected with siNeg (log₂FC = 0) were assessed using one-way ANOVA with Sidak's post hoc test for multiple comparisons. Comparisons between siNeg and each other condition are indicated. *** $p \leq 0.001$. rel., relative; expr., expression. (C) Immunoblot analyses of E6AP, E6, E7, p53, P-p53 Ser15, P-p53 Ser20, Ac-p53 Lys382, p21, and Vinculin protein levels. (D) Corresponding senescence assays (upper panels for each cell line; SA- β -Gal staining, blue; scale bar: 200 μ m) and CFAs (lower panels for each cell line). The Figure and Figure legend have been adapted from Avenhaus et al., *PLOS Pathog* (2025).⁷⁰

To confirm the results obtained from RNAi experiments with an independent method, I blocked E6AP expression in HeLa cells using CRISPR/Cas9 gene editing. HeLa cells were transfected with two distinct guide RNAs (gRNAs; gE6AP-1, -2) and subsequently treated following the scheme in Figure 5A. Compared to cells transfected with control plasmid LentiCRISPRv2, those transfected with E6AP-specific gRNAs exhibited an increased induction of cellular senescence (Figure 5B, upper panels) and an accompanying increase in the protein levels of p53, P-p53 Ser15, Ac-p53 Lys382, and p21 (Figure 5C, three left lanes). However, in addition to senescent cells, a substantial number of cells stained negative for the senescence marker SA- β -Gal and lacked typical features of senescent cells (Figure 5B, upper panels). Furthermore, both E6AP and E6 protein levels were only partially downregulated, if at all (Figure 5C, three left lanes), indicating insufficient CRISPR/Cas9-mediated silencing of E6AP. In order to remove the proliferating cell population, I co-treated the cells with Nocodazole, an inhibitor of microtubule polymerization that specifically targets proliferating cells.³⁶⁴ Upon Nocodazole treatment, cells transfected with control plasmid LentiCRISPRv2 were efficiently eliminated, whereas cells transfected with E6AP-targeting gRNAs showed efficient induction of cellular senescence (Figure 5B, lower panels). Moreover, E6AP and E6 protein levels were markedly decreased, while protein levels of total p53, post-translationally modified p53 forms, as well as p21 were strongly upregulated (Figure 5C, three right lanes). This result corroborates that downregulation of E6AP induces an irreversible growth arrest in HPV-positive cancer cells.

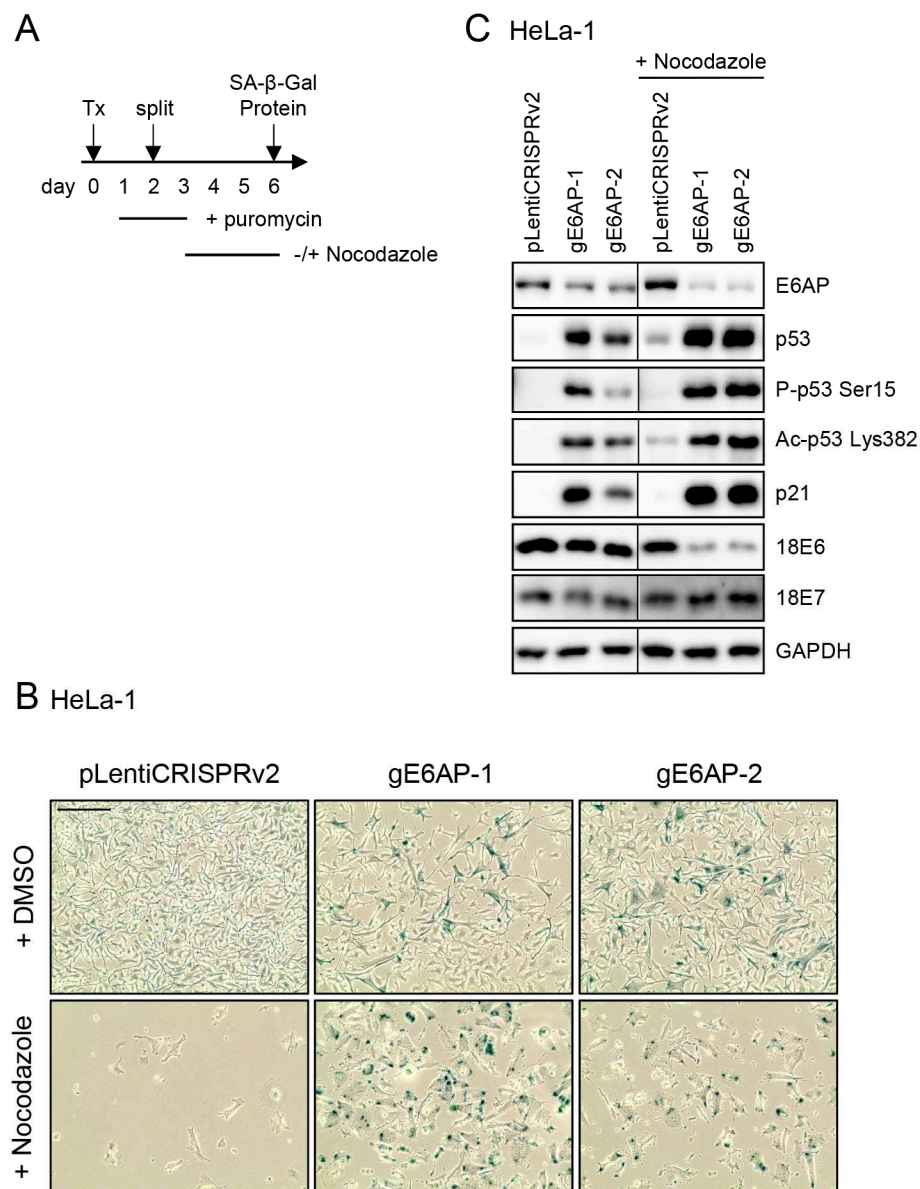


Figure 5. E6AP silencing via CRISPR/Cas9 genome editing leads to senescence induction in HeLa cells.

(A) Treatment scheme: HeLa-1 cells were transfected (Tx) with LentiCRISPRv2 expressing either gE6AP-1 or gE6AP-2 or with control plasmid LentiCRISPRv2. For selection of transfected cells, 1 μ g/mL puromycin was added into the cell culture medium 24 h post-Tx for 48 h. After 24 h of puromycin selection, cells were split and then cultured for another 24 h in the presence of puromycin. Subsequently, to eliminate proliferating cells, cells were treated with 1 μ g/mL Nocodazole, when indicated, and cultivated for another 72 h. DMSO served as solvent control. Six days post-Tx, cells were either harvested for protein analyses or investigated for senescence induction by senescence assays (SA- β -Gal staining). (B) Corresponding senescence assays (SA- β -Gal staining, blue; scale bar: 200 μ m). (C) Immunoblot analyses of E6AP, p53, P-p53 Ser15, Ac-p53 Lys382, p21, 18E6, 18E7, and GAPDH protein levels. Vertical lines between the lanes indicate where original images from the same blot and the same exposure time were cropped for clarity of presentation. The Figure and Figure legend have been adapted from Avenhaus et al., *PLOS Pathog* (2025).⁷⁰

In contrast to HPV-positive cancer cells, degradation of growth-suppressive p53 in HPV-negative cancer cells is not mediated by E6AP, but by other E3 ubiquitin ligases, such as Mdm2.^{111,112} To determine whether blocking E6AP also has pro-senescent effects in cells that do not rely on E6AP-mediated p53 degradation, I additionally investigated the long-term phenotypic effects of RNAi-mediated E6AP silencing in HPV-negative, wild-type p53 RKO and HCT116 colon cancer, as well as U2OS osteosarcoma cells. For this, I used an equimolar pool of the three E6AP-targeting siRNAs mentioned above (hereafter referred to as siE6AP). E6AP inhibition did not induce senescence in these cells (Figure 6A, upper panels for each cell line) and only marginally affected their colony formation capacity, if at all (Figure 6A, lower panels for each cell line). Further, while E6AP protein levels were efficiently downregulated, p53 and p21 levels were not or only slightly elevated (Figure 6B), consistent with the E6AP-independent mechanism of p53 degradation in these cells.^{111,112}

Overall, my findings indicate that E6AP has an anti-senescent function, which is specific for HPV-positive cancer cells.

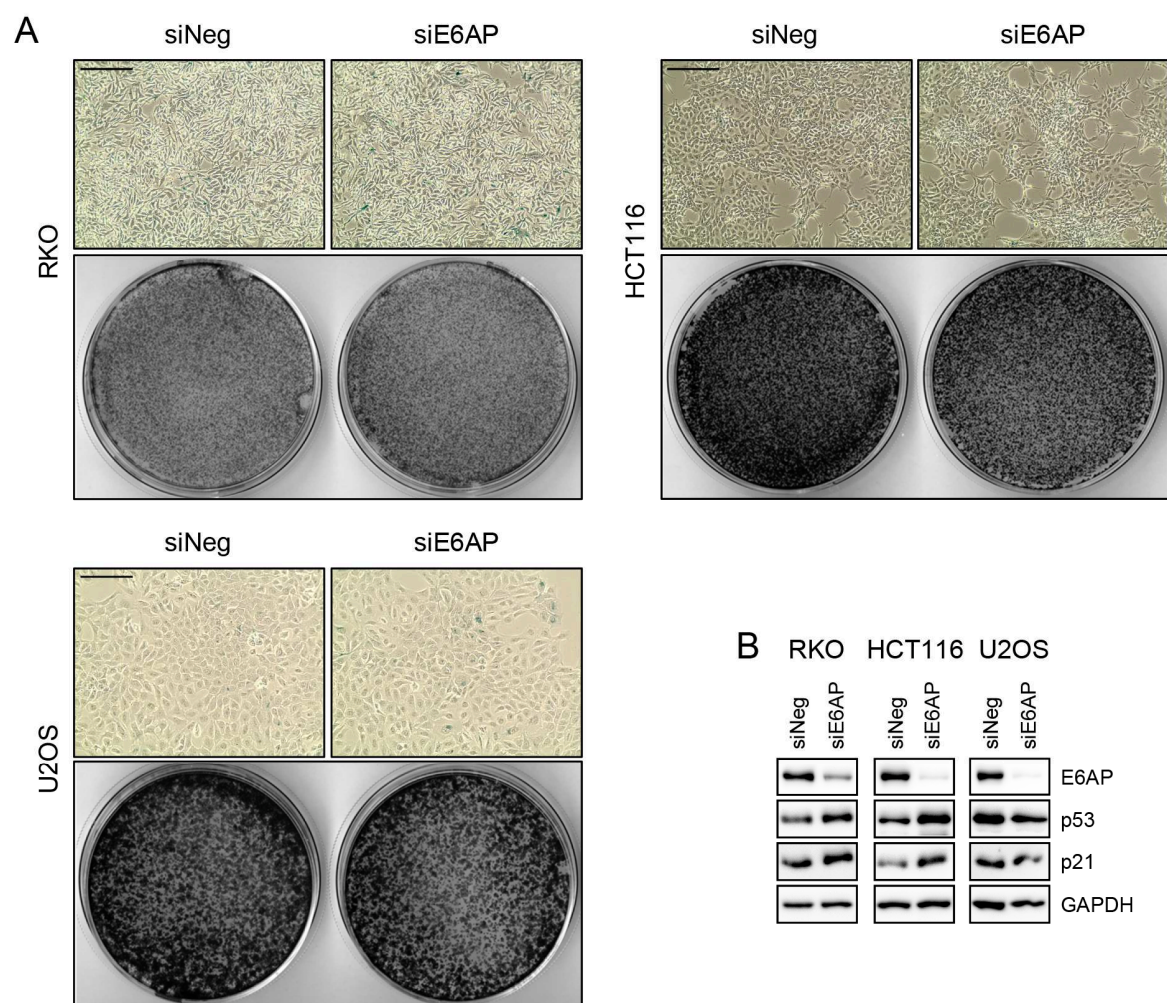


Figure 6. See Figure legend on the next page.

Figure 6. RNAi-mediated E6AP silencing does not induce senescence in HPV-negative cancer cells.

RKO, HCT116, or U2OS cells were transfected with siE6AP or control siRNA (siNeg). Cells were examined following the treatment scheme depicted in Figure 4A by (A) senescence assays (upper panels for each cell line; SA- β -Gal staining; scale bar: 200 μ m) and corresponding colony formation assays (lower panels for each cell line), and by (B) immunoblot analyses for E6AP, p53, p21, and GAPDH protein levels. The Figure and Figure legend have been adapted from Avenhaus et al., *PLOS Pathog* (2025).⁷⁰

2.1.2 The phenotypic responses resulting from E6AP, E6/E7, or E6 silencing in HPV-positive cancer cells exhibit pronounced differences

The previously unrecognized finding that E6AP silencing results in profound growth repression and efficient senescence induction in HPV-positive cancer cells suggests that E6AP inhibition may possess therapeutic potential. Therefore, I next compared the cellular response to E6AP silencing with that resulting from E6/E7 or E6 silencing, as E6 and E7 have been traditionally considered attractive therapeutic targets for inhibiting the growth of HPV-positive cancer cells.⁶⁹

2.1.2.1 Repression of E6AP triggers partial apoptosis, similar to E6 repression

Both E6AP and E6 are described as anti-apoptotic factors in HPV-positive cancer cells, since they promote the degradation of pro-apoptotic p53.^{45,46} Accordingly, their inhibition triggers apoptosis, however, the reported levels of apoptosis vary significantly between different studies.^{80-83,93-100} To test whether blocking either E6AP or E6 induces apoptosis in my experimental setup, I performed TUNEL analyses. The assays revealed an increase in apoptotic, TUNEL-positive HeLa and SiHa cells 96 hours after transfection with siE6AP or siE6, respectively (Figure 7A). Interestingly, the induction of apoptosis appeared to be strongly glucose-dependent, as the amount of TUNEL-positive cells decreased significantly when cells were cultivated in medium containing 25 mM glucose compared to cells cultivated in medium with 5.5 mM glucose. Since the increase in TUNEL-positive cells upon silencing of E6 in HeLa cells was not statistically significant, I further assessed apoptosis induction using two additional experimental approaches. First, immunoblot analyses revealed an increase of the apoptosis marker cleaved PARP upon E6AP or E6 knockdown (Figure 7B). Second, I analyzed the induction of cell death in live-cell imaging studies using the Incucyte® Cytotox Green reagent. Both E6AP and E6 silencing were accompanied by an increase in the number of Cytotox-positive HeLa cells over time (Figure 7C), corroborating further that E6AP or E6 silencing induces a pro-apoptotic response in HPV-positive cancer cells.

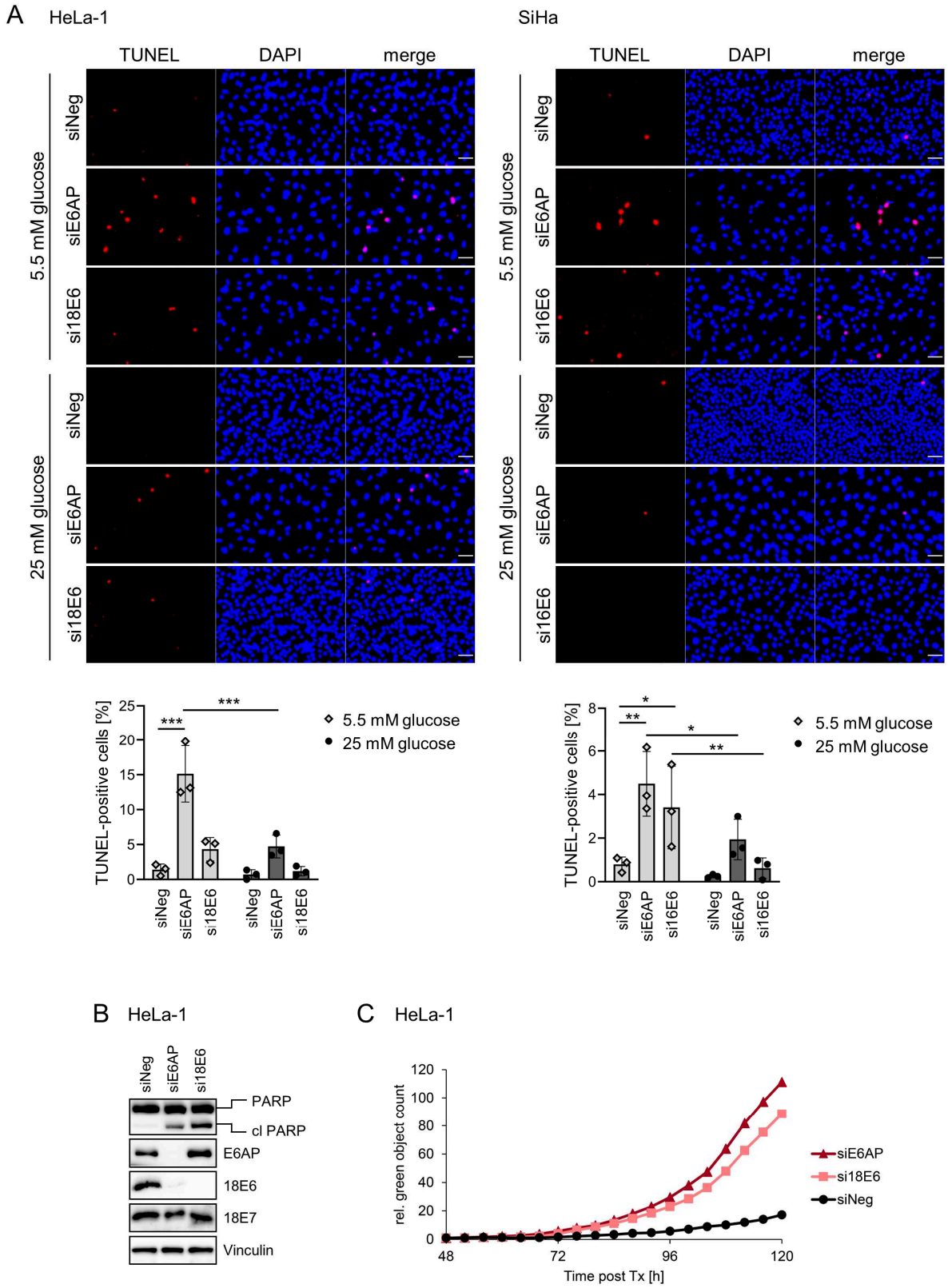


Figure 7. See Figure legend on the next page.

Figure 7. RNAi-mediated silencing of E6AP or E6 exerts pro-apoptotic effects in HPV-positive cancer cells.

(A) HeLa-1 or SiHa cells were transfected with 20 nM of siE6AP, si18E6 or si16E6, or control siRNA (siNeg), and cultivated under either 5.5 or 25 mM glucose in the cell culture medium for 96 h. Upper panels: TUNEL analyses of HeLa-1 or SiHa cells (scale bar: 50 μ m). Lower panels: Quantification of TUNEL-positive (apoptotic) cells relative to the number of DAPI-stained cells. Results are presented as mean percentages with standard deviations ($n = 15$ fields of view, each with ≥ 50 cells, from three independent experiments). Statistically significant differences were assessed using two-way ANOVA with Tukey's post hoc test for multiple comparisons. Only relevant comparisons are indicated. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$. (B) HeLa-1 cells were transfected with 20 nM of siE6AP, si18E6, or control siRNA (siNeg), cultivated under 5.5 mM glucose in the cell culture medium for 96 h, and examined by immunoblot for PARP, cleaved (cl) PARP, E6AP, 18E6, 18E7, and Vinculin protein levels. (C) HeLa-1-mKate2 cells were transfected with siE6AP, si18E6, or control siRNA (siNeg) and cultivated under 5.5 mM glucose in the cell culture medium. Twenty-four hours post-transfection, 50 nM Cytotox Green Dye was added. Cells were imaged by the Incucyte® S3 Live-Cell Analysis Instrument starting 24 h after the addition of Cytotox Green (48 h post-transfection). Dead cells (green objects) were quantified using the Incucyte® software package and are shown relative to the counts at 48 h post-transfection (set to 1). rel., relative; Tx, transfection. Subfigure (A) and the corresponding legend have been adapted from Avenhaus et al., *PLOS Pathog* (2025).⁷⁰

2.1.2.2 The long-term cellular fates resulting from E6AP or E6/E7 silencing differ from those induced by E6 silencing

The observation that E6AP silencing acts both pro-apoptotic and pro-senescent indicates that cells surviving the short-term pro-apoptotic stimulus of E6AP inhibition are ultimately blocked in their growth by inducing senescence. To compare this response to that of E6 and, also, E6/E7 inhibition, cells were monitored in comparative analyses over time following the scheme in Figure 4A. As described in Chapter 2.1.1, inhibition of E6AP led to efficient induction of senescence (Figure 8, upper panels for each cell line) and strongly impaired colony formation capacity (Figure 8, lower panels for each cell line) in HPV-positive HeLa, SiHa, and CaSki cells. In line with previous reports,⁷¹⁻⁷³ E6/E7 silencing also induced senescence and led to an equally efficient impairment of colony formation as E6AP inhibition in all three cell lines. Strikingly, the long-term effects of E6 silencing differed drastically, as only a small portion of cells showed signs of senescence, and colony outgrowth was only marginally reduced compared to cells transfected with control siRNA (Figure 8). This finding indicates that, while cells escaping siE6AP-mediated apoptosis induction undergo senescence, cells surviving E6 repression do not senesce, but continue to grow.

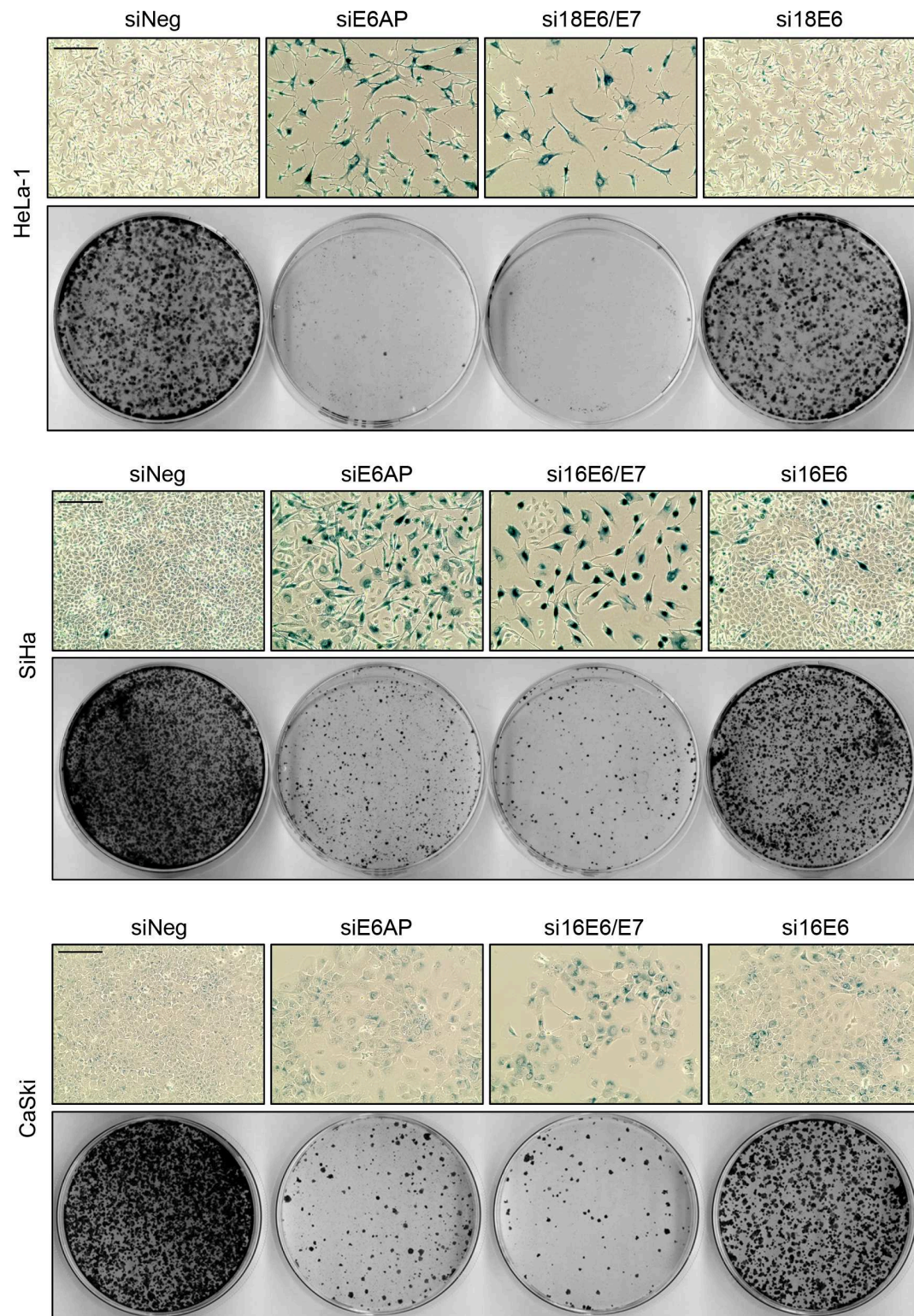


Figure 8. Silencing of E6AP, E6/E7, or E6 has differential effects on the senescence response in HPV-positive cancer cells.

HeLa-1, SiHa, or CaSki cells were transfected with siE6AP, si18E6/E7 or si16E6/E7, si18E6 or si16E6, or control siRNA (siNeg) and examined following the treatment scheme depicted in Figure 4A. Shown are corresponding senescence assays (upper panels for each cell line; SA-β-Gal staining, blue; scale bar: 200 μm) and colony formation assays (lower panels for each cell line). The Figure and Figure legend have been adapted from Avenhaus et al., *PLOS Pathog* (2025).⁷⁰

Interestingly, E6AP silencing, but not E6 silencing, induces senescence in HPV-positive cancer cells. However, E6 is concomitantly downregulated when E6AP is silenced by siRNAs or gRNAs, respectively (Figures 4C, 5C). To test whether siRNA-mediated E6 knockdown might not be efficient enough to fully suppress E6, which could possibly explain the lack of senescence induction, HeLa or SiHa cells were transfected either once or repeatedly with increasing concentrations of E6-targeting siRNAs following the scheme in Figure 9A.

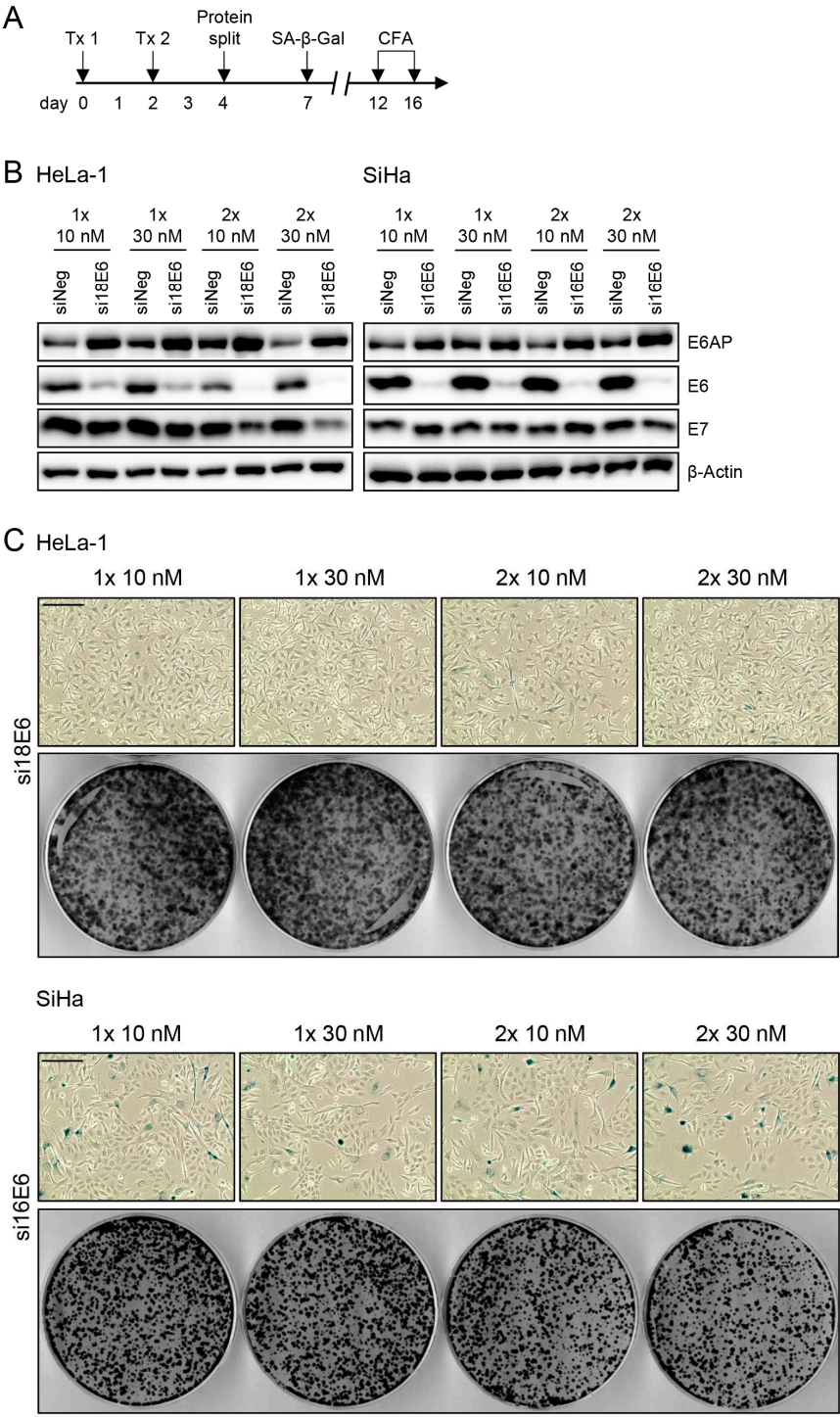


Figure 9. See Figure legend on the next page.

Figure 9. The senescence response of HPV-positive cancer cells is not enhanced by increased siE6 concentrations or repeated siE6 transfections.

(A) Treatment scheme: HeLa-1 or SiHa cells were transfected with either 10 or 30 nM si18E6 or si16E6 or control siRNA (siNeg). Forty-eight hours after the first transfection (Tx 1), half of the samples were transfected a second time with the same siRNAs (Tx 2). Ninety-six hours after Tx 1, cells were either harvested for protein analyses, or split and further cultivated for senescence assays (SA- β -Gal staining) or colony formation assays (CFAs) after the indicated time periods. (B) Immunoblot analyses of E6AP, E6, E7, and β -Actin protein levels following transfection with the indicated amounts of siRNAs, performed either once (1x) or twice (2x). (C) Corresponding senescence assays (upper panels for each cell line; SA- β -Gal staining, blue; scale bar: 200 μ m) and CFAs (lower panels for each cell line). The Figure and Figure legend have been adapted from Avenhaus et al., *PLOS Pathog* (2025).⁷⁰

Immunoblot analyses revealed an enhanced downregulation of E6 upon repeated transfections in HeLa cells, while no visibly stronger reduction in E6 levels was observed in SiHa cells (Figure 9B). E6AP and E7 levels were maintained in both cell lines, even after repeated transfections with higher siRNA concentrations. Moreover, enhanced E6 silencing was insufficient to appreciably induce senescence (Figure 9C, upper panels for each cell line) and impair colony outgrowth (Figure 9C, lower panels for each cell line) in both HeLa and SiHa cells. This suggests that RNAi-mediated repression of E6 alone is insufficient to induce pro-senescent.

In view of the proposed anti-senescent activity of E7,⁵¹ I further investigated whether E6AP inhibition might indirectly downregulate E7 levels, thereby contributing to the observed senescence upon E6AP inhibition. To this end, I conducted prolonged experiments in which cells were harvested 96 or 120 hours after transfection, and protein levels of E7 were assessed to compare the effects of E6AP or E6/E7 silencing. Notably, E7 levels remained unaffected by siE6AP in both HeLa and SiHa cells, even in prolonged experiments (Figure 10). This finding indicates that senescence induction following E6AP silencing does not depend on E7 downregulation.

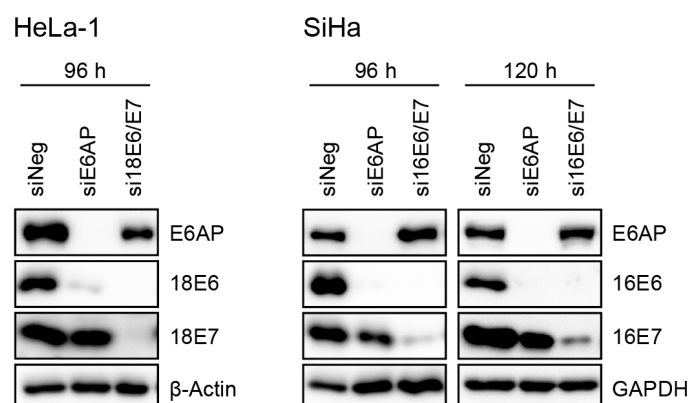


Figure 10. See Figure legend on the next page.

Figure 10. E6AP silencing does not lead to concomitant reduction in E7 levels in HPV-positive cancer cells.

HeLa-1 or SiHa cells were transfected with siE6AP, si18E6/E7 or si16E6/E7, or control siRNA (siNeg), cultivated for 96 h (HeLa-1, SiHa) or 120 h (SiHa), and examined by immunoblot for E6AP, E6, E7, and β -Actin (HeLa-1) or GAPDH (SiHa) protein levels.

2.1.2.3 The effects of E6AP, E6/E7, or E6 silencing on proliferation and cell cycle control vary substantially

The well-established senescence induction following E6/E7 repression has been described to be the result of an efficient arrest of HPV-positive cancer cells in the G1 phase of the cell cycle.^{207,365} To characterize the cell cycle regulation underlying the senescence induction upon E6AP repression, I next conducted comparative cell cycle and proliferation analyses. Proliferation curves acquired from live-cell imaging corroborated the results from senescence and colony formation assays, showing a strong impairment of proliferation upon E6/E7 repression and, with some delay, upon E6AP repression in HeLa, SiHa, and CaSki cells (Figure 11A). In contrast, E6 silencing caused an only transient proliferative slowdown after which the cells continued to proliferate.

Furthermore, cell cycle analyses revealed an efficient G1 arrest following E6/E7 silencing in all tested cell lines, as expected (Figure 11B, upper panels for each cell line). In contrast, despite potent inhibition of cellular proliferation (Figure 11A), E6AP silencing only marginally increased G1 populations in SiHa and CaSki cells, and led to an increase in S and G2/M phases in HeLa cells (Figure 11B, upper panels for each cell line). E6 inhibition induced a weak increase in G1 phases in SiHa and CaSki cells, while the cell cycle profile of HeLa cells remained largely unaffected. Notably, however, when cells were concomitantly treated with Nocodazole, which has been reported to induce efficient G2/M arrest in proliferating cells,³⁶⁴ both E6AP- and E6/E7-depleted cells were confirmed to be growth arrested. In specific, whereas Nocodazole-treated, control siRNA-transfected cells (which proliferate) showed a pronounced enrichment of cells in G2/M phases, these phases were only slightly increased by Nocodazole upon E6AP or E6/E7 inhibition, if at all (Figure 11B, lower panels for each cell line). E6 knockdown also counteracted the strong Nocodazole-induced G2/M arrest in SiHa and CaSki cells, whereas siE6-transfected HeLa cells showed a marked increase in G2/M populations, indicating that these cells were proliferating. This was consistent with the results obtained from proliferation curves, showing a longer proliferative delay upon E6 inhibition in SiHa and CaSki cells compared to HeLa cells (Figure 11A).

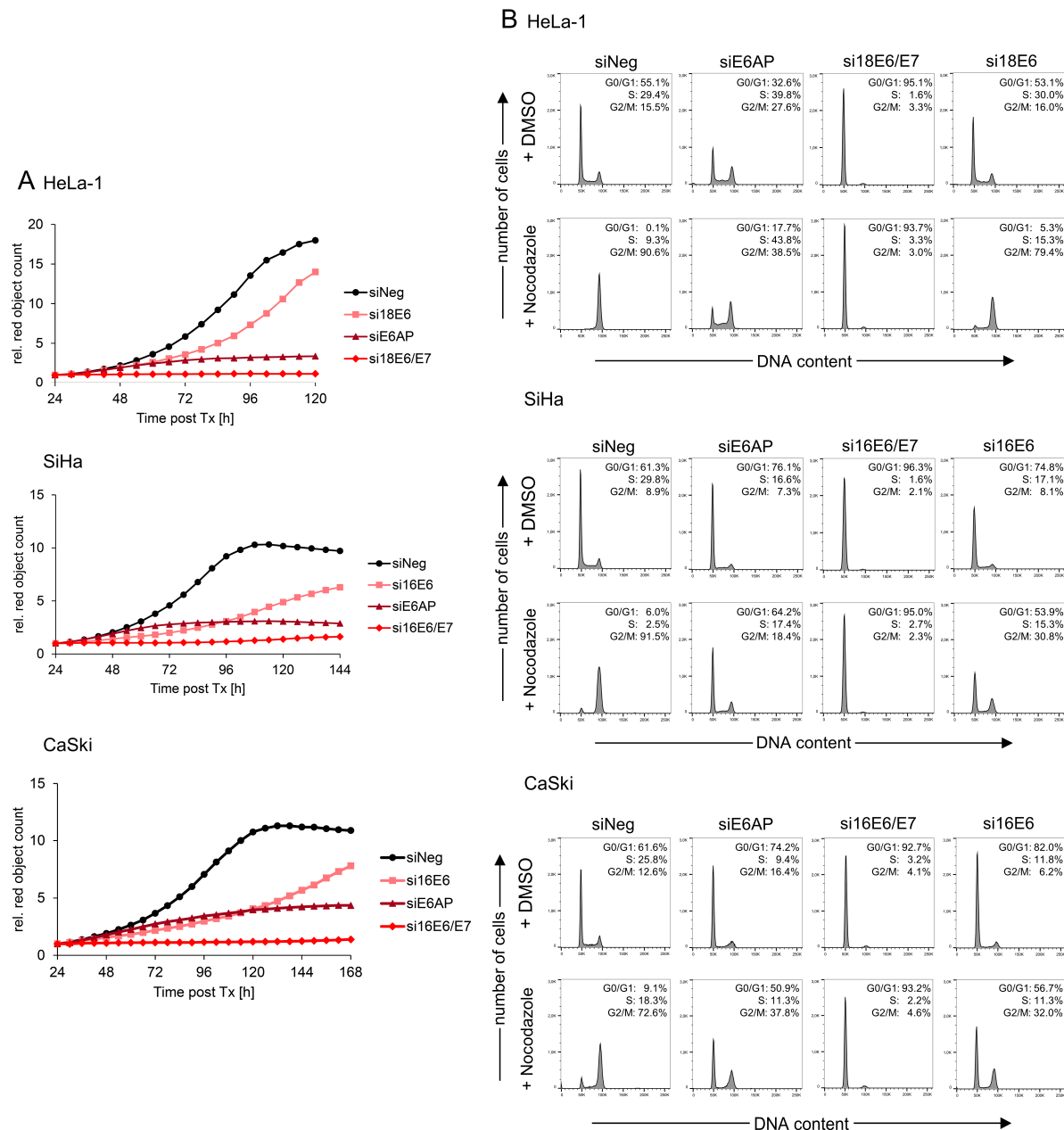


Figure 11. Differential effects of silencing E6AP, E6/E7, or E6 on the proliferation and cell cycle profiles of HPV-positive cancer cells.

(A) HeLa-1-mKate2, SiHa-mKate2, or CaSki-mKate2 cells were transfected with siE6AP, si18E6/E7 or si16E6/E7, si18E6 or si16E6, or control siRNA (siNeg) and imaged by the Incucyte® S3 Live-Cell Analysis Instrument for up to 168 h post-transfection. Cell counts (red objects) were quantified using the Incucyte® software package and are shown relative to the counts at 24 h post-transfection (set to 1). rel., relative; Tx, transfection. (B) HeLa-1, SiHa, or CaSki cells were transfected with siE6AP, si18E6/E7 or si16E6/E7, si18E6 or si16E6, or control siRNA (siNeg) and cultivated for 72 h. Nocodazole at a final concentration of 0.1 $\mu\text{g}/\text{mL}$ or DMSO (solvent control) was added for the last 16 h (HeLa-1) or 24 h (SiHa, CaSki) of cultivation. Cell cycle profiles and quantifications of the percentages of cell populations in the individual cell cycle phases are shown. The Figure and Figure legend have been adapted from Avenhaus et al., *PLOS Pathog* (2025).⁷⁰

Since cell cycle progression is characterized by a tightly regulated expression of genes that coordinate the transition through the cell cycle phases,²⁰⁰ I additionally analyzed the expression of a spectrum of cell cycle regulators, which either peak during G1/S or G2/M transition,⁵⁸ upon E6AP, E6/E7, or E6 inhibition, respectively. The efficient G1 arrest resulting from E6/E7 inhibition (Figure 11B) was accompanied by a pronounced downregulation of B-MYB, FOXM1, E2F1, Cyclin A, Cyclin B1, CDC2, CDK2, and CKS1 protein levels in both HeLa and SiHa cells (Figure 12A), as expected.⁵⁸ In strong contrast, inhibition of either E6AP or E6 had no effect on the levels of these proteins. This differential regulation was also reflected at the transcript level, where mRNA expression of *MYBL2* (coding for B-MYB), *FOXM1*, *CCNA2* (coding for Cyclin A2), and *E2F1* was significantly reduced in E6/E7-depleted HeLa and SiHa cells, while it was only marginally downregulated upon E6AP or E6 inhibition, if at all (Figure 12B).

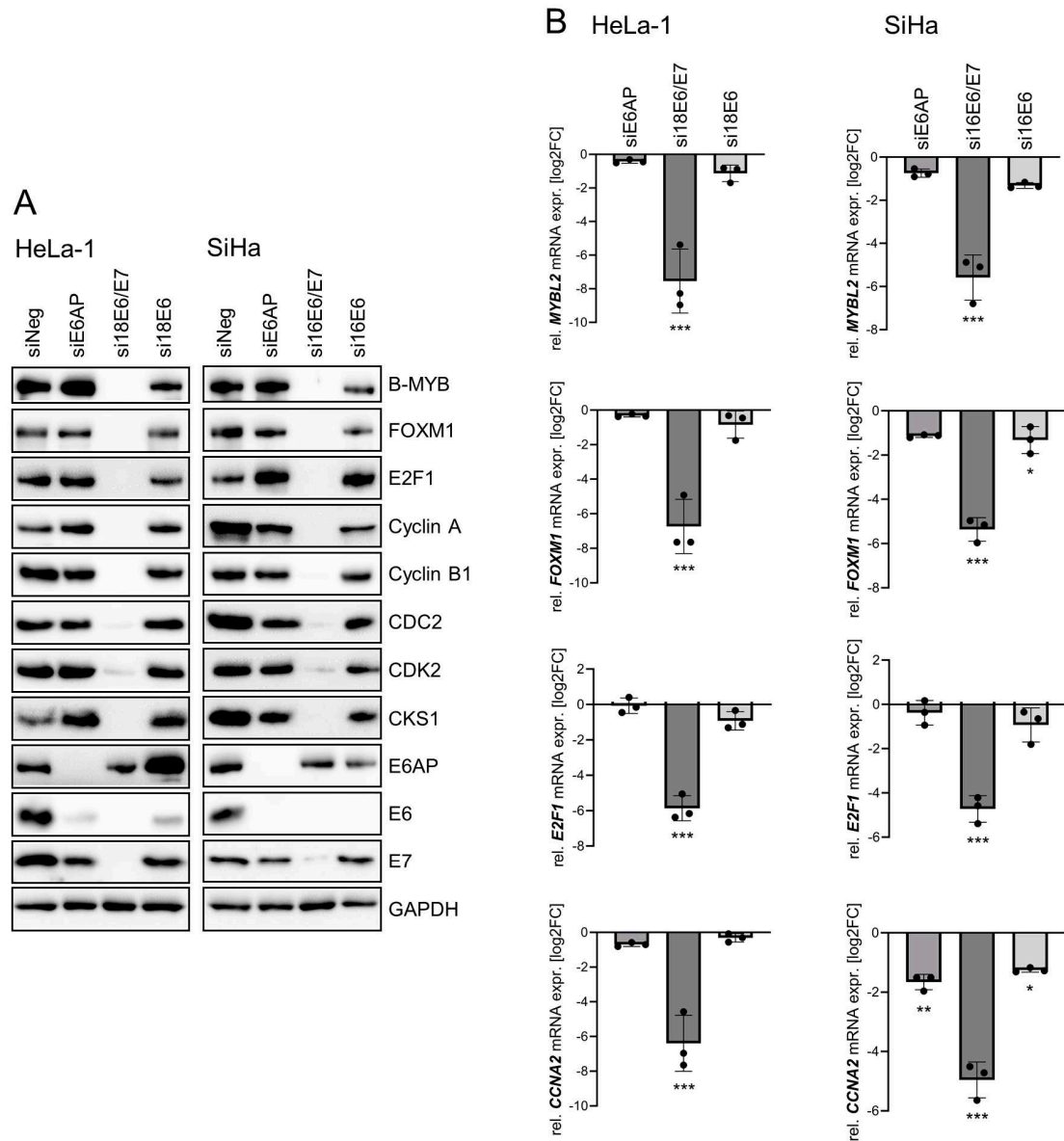


Figure 12. See Figure legend on the next page.

Figure 12. Differential expression of cell cycle regulators upon silencing of E6AP, E6/E7, or E6 in HPV-positive cancer cells.

HeLa-1 or SiHa cells were transfected with siE6AP, si18E6/E7 or si16E6/E7, si18E6 or si16E6, or control siRNA (siNeg) and cultivated for 72 h. (A) Immunoblot analyses of B-MYB, FOXM1, E2F1, Cyclin A, Cyclin B1, CDC2, CDK2, CKS1, E6AP, E6, E7, and GAPDH protein levels. (B) Corresponding qRT-PCR analyses of *MYBL2* (coding for B-MYB), *FOXM1*, *E2F1*, and *CCNA2* (coding for Cyclin A2) mRNA levels. Shown are log₂-transformed fold changes (log₂FC) of mean expression with standard deviations ($n = 3$). Statistically significant differences between cells transfected with siE6AP, siE6/E7, or siE6, and those transfected with siNeg (log₂FC = 0) were assessed using one-way ANOVA with Sidak's post hoc test for multiple comparisons. Comparisons between siNeg and each other condition are indicated when statistically significant. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$. rel., relative; expr., expression. The Figure and Figure legend have been adapted from Avenhaus et al., *PLOS Pathog* (2025).⁷⁰

Taken together, these findings suggest that cells continue to proliferate upon E6 silencing, whereas both E6AP and E6/E7 silencing efficiently impair cellular proliferation and induce senescence. However, the underlying cell cycle regulation differs significantly. E6/E7 silencing leads to a G1 arrest, while E6AP inhibition induces senescence without a marked enrichment of the cells in a particular cell cycle phase. This is further supported by distinct expression profiles of cell cycle regulators, which align with the respective cell cycle profiles.

2.1.3 Activation of the p53-p21 axis is critical for senescence induction upon E6AP silencing

Having characterized the underlying cell cycle regulation, I next aimed to gain insights into the mechanisms responsible for senescence induction upon E6AP inhibition. Importantly, I found that senescence following E6AP silencing is specifically induced in HPV-positive cancer cells, which rely on E6AP-mediated p53 degradation,⁴³ but not in HPV-negative cancer cells, where p53 is regulated independently of E6AP.^{111,112} Based on this observation, and since both p53 and its downstream transcriptional target p21^{56,250} are well-established mediators of senescence under various biological conditions,⁷⁴ I hypothesized that the p53-p21 axis might contribute to this phenotype.

2.1.3.1 p53 and p21 induction is stronger following E6AP silencing than following E6/E7 or E6 silencing

To first investigate in more detail how p53 and p21 levels are regulated in response to E6AP inhibition, I compared the activation of the p53-p21 axis following E6AP, E6/E7, or E6 inhibition – three conditions that all prevent p53 degradation in HPV-positive cancer cells due to the loss of E6AP and/or E6. Interestingly, the induction of p53 and p21 protein levels was more pronounced upon E6AP inhibition compared to E6/E7 or E6 inhibition in both HeLa and SiHa

cells (Figure 13A). Moreover, post-translationally modified forms of p53, P-p53 Ser15, P-p53 Ser20, and Ac-p53 Lys382, were considerably more strongly upregulated in E6AP-depleted cells compared to the other conditions. This was further accompanied by a more pronounced increase in the transcript levels of *CDKN1A* (encoding p21) in E6AP-depleted HeLa and SiHa cells, compared to either E6/E7- or E6-depleted cells, which was at least partially statistically significant (Figure 13B).

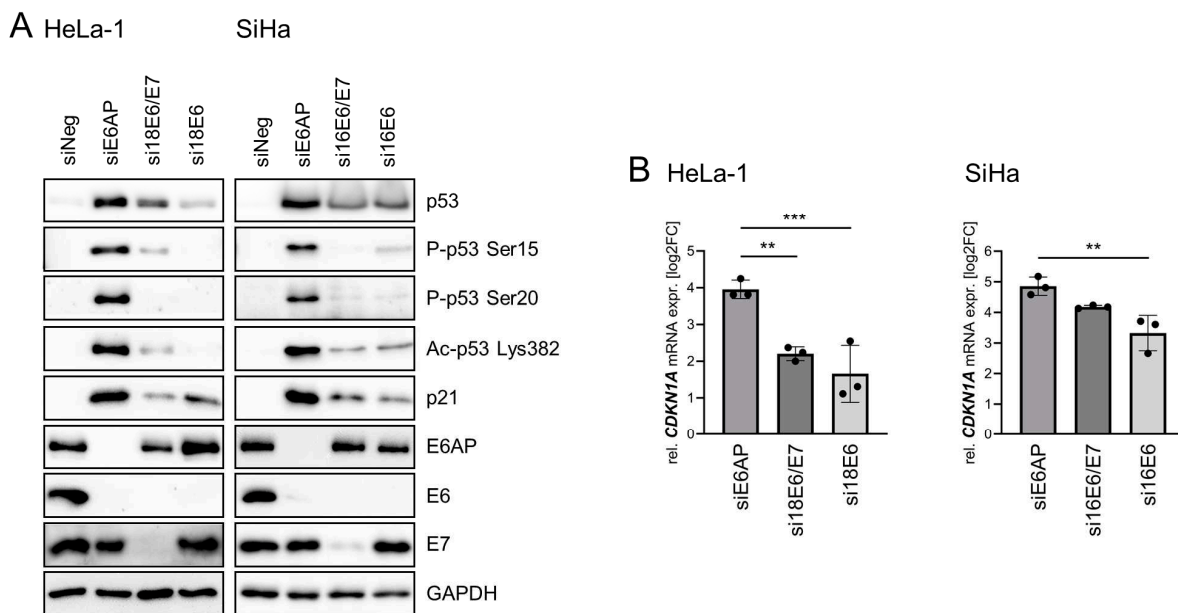


Figure 13. p53 and p21 induction upon E6AP silencing is stronger than upon E6/E7 or E6 silencing in HPV-positive cancer cells.

HeLa-1 or SiHa cells were transfected with siE6AP, si18E6/E7 or si16E6/E7, si18E6 or si16E6, or control siRNA (siNeg) and cultivated for 72 h. (A) Immunoblot analyses of p53, P-p53 Ser15, P-p53 Ser20, Ac-p53 Lys382, p21, E6AP, E6, E7, and GAPDH protein levels. (B) Corresponding qRT-PCR analyses of *CDKN1A* (coding for p21) mRNA levels. Shown are log₂-transformed fold changes (log₂FC) of mean expression with standard deviations ($n = 3$). Statistically significant differences between cells transfected with siE6AP, siE6/E7, or siE6, and those transfected with siNeg (log₂FC = 0) were assessed using one-way ANOVA with Sidak's post hoc test for multiple comparisons. Only relevant comparisons are indicated. ** $p \leq 0.01$, *** $p \leq 0.001$. rel., relative; expr., expression. The Figure and Figure legend have been adapted from Avenhaus et al., *PLOS Pathog* (2025).⁷⁰

2.1.3.2 Senescence induction and proliferative arrest following E6AP silencing strongly depend on p53 and p21 activation

To next assess the functional role of p53 and p21 in growth arrest and senescence induction upon E6AP inhibition in comparison to E6/E7 or E6 inhibition, co-transfection experiments were performed in which either p53 or p21 were concomitantly silenced.

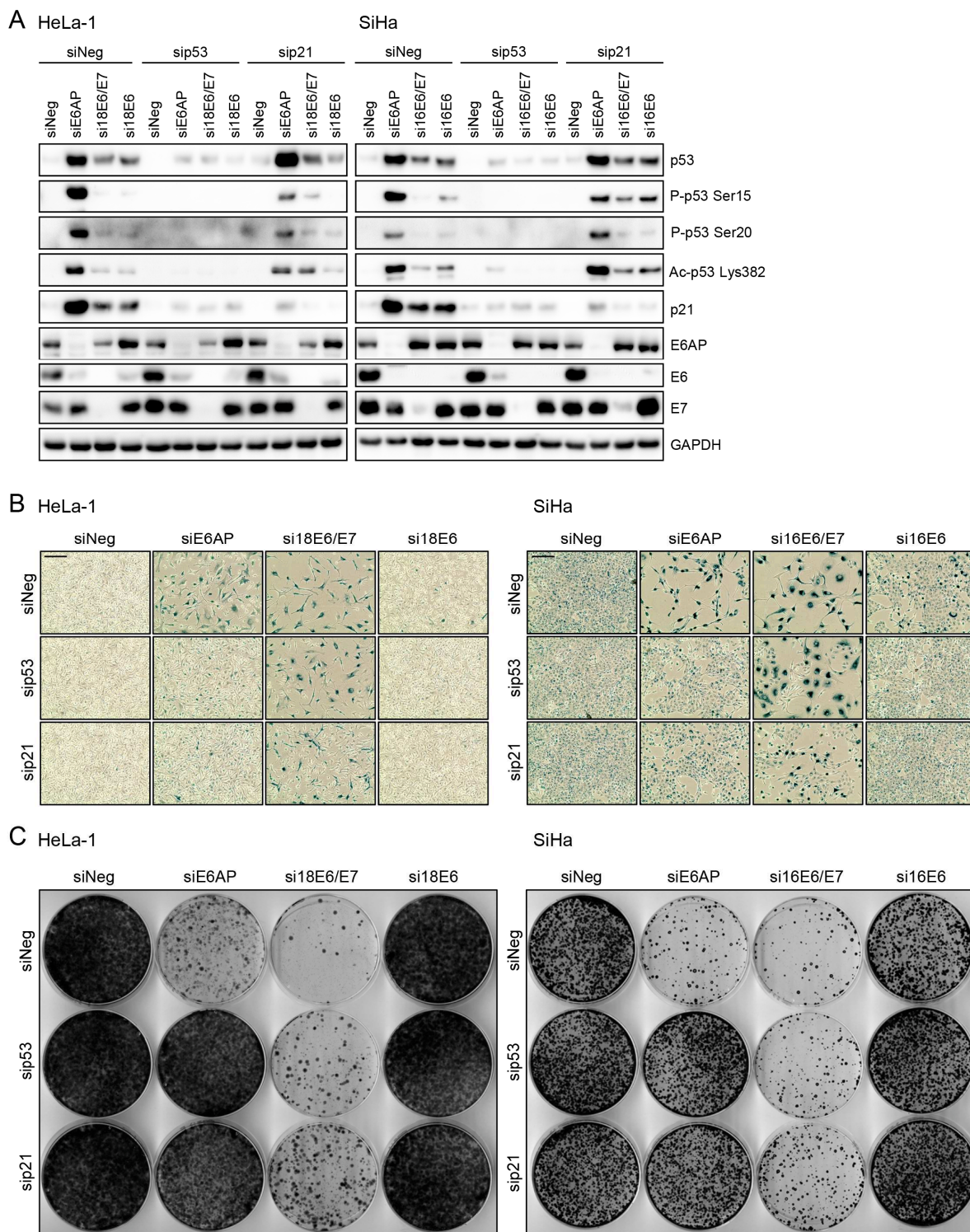


Figure 14. See Figure legend on the next page.

Figure 14. p53 and p21 are critical for senescence induction upon E6AP silencing in HPV-positive cancer cells.

HeLa-1 or SiHa cells were transfected with siE6AP, si18E6/E7 or si16E6/E7, si18E6 or si16E6, or control siRNA (siNeg), alone or in combination with sip53 or sip21, as indicated. Cells were examined following the treatment scheme depicted in Figure 4A by (A) immunoblot for p53, P-p53 Ser15, P-p53 Ser20, Ac-p53 Lys382, p21, E6AP, E6, E7, and GAPDH protein levels, (B) senescence assays (SA- β -Gal staining, blue; scale bar: 200 μ m), and (C) corresponding colony formation assays. The Figure and Figure legend have been adapted from Avenhaus et al., *PLOS Pathog* (2025).⁷⁰

Immunoblot analyses showed efficient downregulation of the respective target proteins, as well as downregulation of p21 and post-translationally modified forms of p53 upon p53 knockdown in both HeLa and SiHa cells (Figure 14A). Strikingly, while p53 or p21 silencing alone did not affect the phenotype of the cells, senescence induction following E6AP silencing was efficiently blocked upon concurrent downregulation of either p53 or p21 in both HeLa and SiHa cells (Figure 14B), indicating a strong dependency of siE6AP-induced senescence on both p53 and p21 induction. This dependency was further supported by corresponding CFAs, showing efficient colony outgrowth upon concomitant silencing of E6AP and p53 or p21 (Figure 14C). In contrast, co-silencing of E6/E7 and p21 resulted in the emergence of few non-senescent cells (Figure 14B) and moderately enhanced colony formation in both HeLa and SiHa cells (Figure 14C), whereas co-silencing of p53 had minor to no effects. Moreover, the phenotype of E6-depleted cells was largely unaffected by concomitant inhibition of p53 or p21 (Figure 14B,C).

In agreement with the finding that senescence induction upon E6AP inhibition depends on p53 and p21, their depletion also efficiently counteracted siE6AP-induced growth arrest, as evidenced in proliferation assays of HeLa, SiHa, and CaSki cells (Figure 15A). This was further confirmed by cell cycle analyses, in which concomitant inhibition of E6AP and p53 or p21 restored a cell cycle distribution comparable to that of control siRNA-transfected HeLa cells (Figure 15B, upper panels for HeLa cells). Moreover, it restored the ability of both HeLa and SiHa cells to induce an efficient G2/M cell cycle arrest following Nocodazole treatment, indicating active proliferation of cells in which E6AP and p53 or p21 are concomitantly silenced (Figure 15B, lower panels for each cell line).

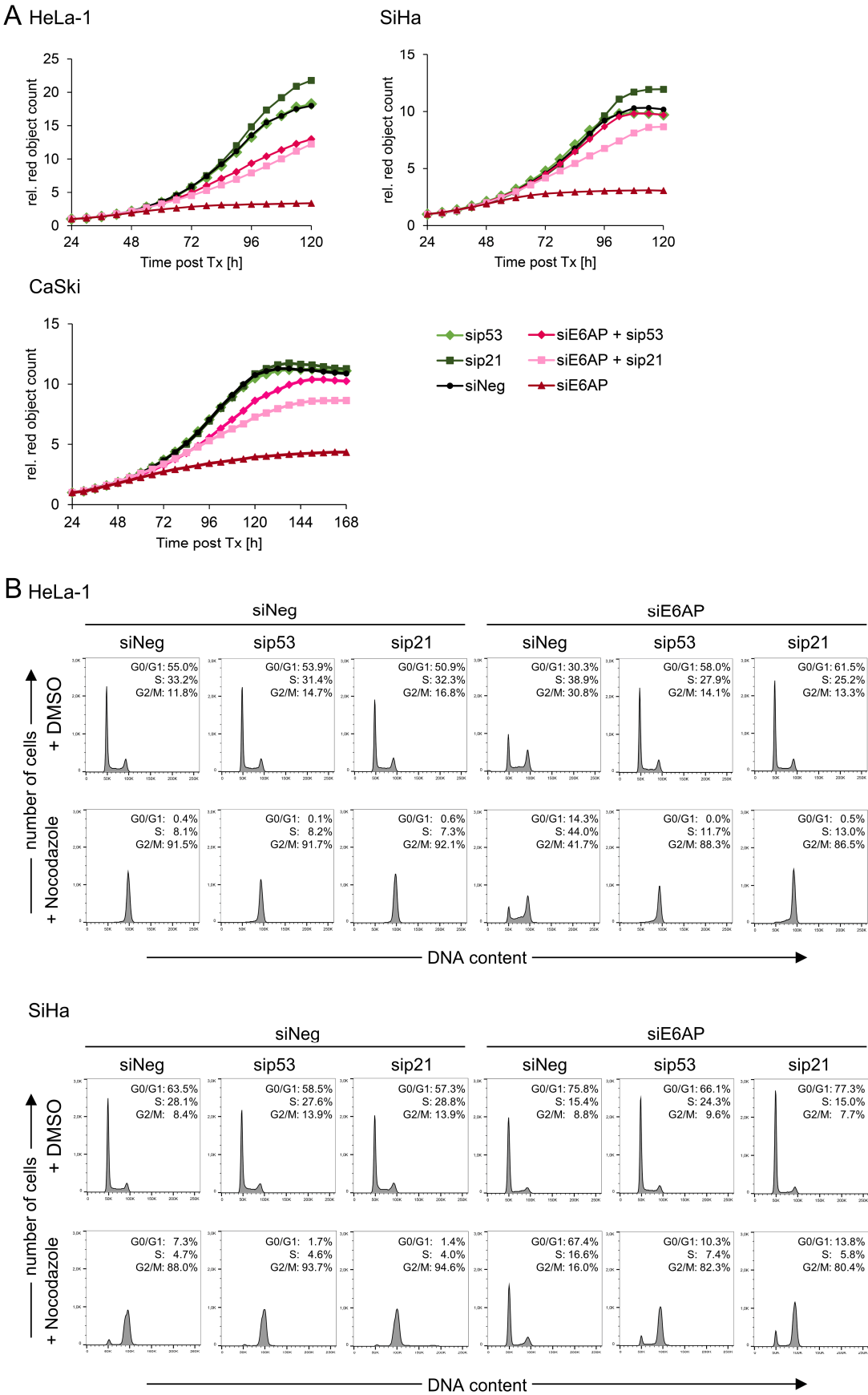


Figure 15. See Figure legend on the next page.

Figure 15. p53 and p21 are critical for proliferation arrest upon E6AP silencing in HPV-positive cancer cells.

(A) HeLa-1-mKate2, SiHa-mKate2, or CaSki-mKate2 cells were transfected with siE6AP or control siRNA (siNeg), either alone or in combination with sip53 or sip21, as indicated, and imaged by the Incucyte® S3 Live-Cell Analysis Instrument for up to 168 h post-transfection. Cell counts (red objects) were quantified using the Incucyte® software package and are shown relative to the counts at 24 h post-transfection (set to 1). rel., relative; Tx, transfection. (B) HeLa-1 or SiHa cells were transfected with siE6AP or control siRNA (siNeg), either alone or in combination with sip53 or sip21, as indicated, and cultivated for 72 h. Nocodazole at a final concentration of 0.1 µg/mL or DMSO (solvent control) was added for the last 16 h (HeLa-1) or 24 h (SiHa) of cultivation. Cell cycle profiles and quantifications of the percentages of cell populations in the individual cell cycle phases are shown. The Figure and Figure legend have been adapted from Avenhaus et al., *PLOS Pathog* (2025).⁷⁰

2.1.3.3 E6AP repression fails to induce senescence in “p53 null” cells

To further validate the dependency of siE6AP- and siE6/E7-induced senescence on p53 activation, I used two HeLa cell clones in which p53 is stably silenced by RNAi (“p53 null” cells: HeLa A1 and HeLa C2)⁹⁸ and compared their phenotypic response to E6AP or E6/E7 silencing to those of their parental HeLa cells.

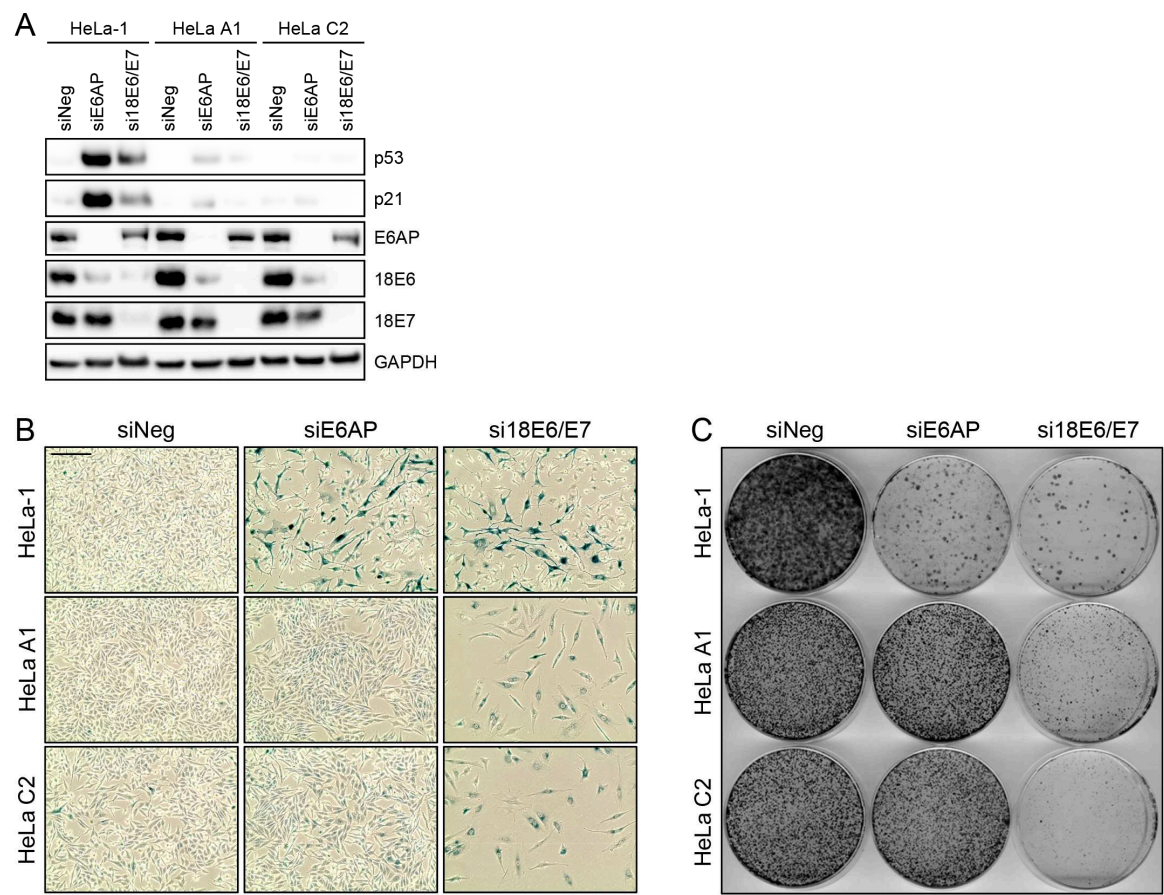


Figure 16. See Figure legend on the next page.

Figure 16. Silencing of E6AP does not induce senescence in “p53 null” HeLa cells.

HeLa-1 or HeLa “p53 null” cells (HeLa A1, HeLa C2) were transfected with siE6AP, si18E6/E7, or control siRNA (siNeg). Cells were examined following the treatment scheme depicted in Figure 4A by (A) immunoblot for p53, p21, E6AP, 18E6, 18E7, and GAPDH protein levels, (B) senescence assays (SA- β -Gal staining, blue; scale bar: 200 μ m), and (C) corresponding colony formation assays. The Figure and Figure legend have been adapted from Avenhaus et al., *PLOS Pathog* (2025).⁷⁰

While inhibition of E6AP or E6/E7 led to increased protein levels of p53 and p21 in the parental HeLa cells, p53 and p21 levels remained largely unaffected in the “p53 null” derivatives (Figure 16A). Furthermore, E6/E7 silencing induced senescence (Figure 16B) and impaired colony formation (Figure 16C) in both parental and “p53 null” cells. In strong contrast, senescence and reduced colony outgrowth following E6AP inhibition were only observed in parental, but not in “p53 null” HeLa cells (Figure 16B,C).

Altogether, these experiments reveal substantial differences in the functional significance of the p53-p21 axis for the pro-senescent activities of either E6AP or E6/E7 silencing. While p53 and p21 play a critical role in senescence and growth arrest upon E6AP knockdown, they are dispensable for the pro-senescent response triggered by E6/E7 silencing.

2.1.4 pRb and p130 are critical for senescence induction upon both E6AP and E6/E7 silencing

A difference between downregulation of E6AP or E6 on the one hand and E6/E7 on the other hand is the sustained expression of E7 in the first two conditions. Whereas the central target of E6AP and E6 in HPV-positive cancer cells is p53, critical targets of E7 include the pocket proteins pRb and p130.^{52,53} These proteins negatively control the cell cycle in their active, hypophosphorylated forms by repressing genes involved in cell cycle progression.^{54,56,57,200-202} E7 mediates the inactivation of hypophosphorylated pocket proteins, which results in the prevention of cell cycle arrest and senescence.⁵⁸ Thus, I hypothesized that the presence or absence of E7 – and resulting differences in pocket protein activity – could underlie the differential senescence responses and mechanistic distinctions observed following repression of E6AP, E6/E7, or E6.

2.1.4.1 E6AP, E6/E7, or E6 silencing reduces phosphorylated pRb levels to different extents

In order to evaluate potential differences in pocket protein activities, I first compared expression levels of pRb, phosphorylated pRb (P-pRb; inactive), and p130 upon siRNA-mediated silencing of E6AP, E6/E7, or E6 in SiHa cells. Consistent with the ability of E7 to induce the degradation

of hypophosphorylated pocket proteins,^{52,53,58} E6/E7 silencing led to an upregulation of total pRb and p130 protein levels, as well as a strong downregulation of P-pRb (Figure 17A), indicating pocket protein activity.⁵⁴ In contrast, and consistent with sustained E7 expression, levels of total pRb and p130 were maintained or even moderately decreased upon both E6AP and E6 inhibition. Surprisingly, however, the levels of P-pRb were also reduced, especially upon E6AP silencing. Notably, even upon repeated transfections or increased siRNA concentrations, P-pRb levels were largely maintained following E6 silencing (Figure 17B), indicating that E6 repression alone, in contrast to E6AP repression, does not fully inhibit pRb phosphorylation.

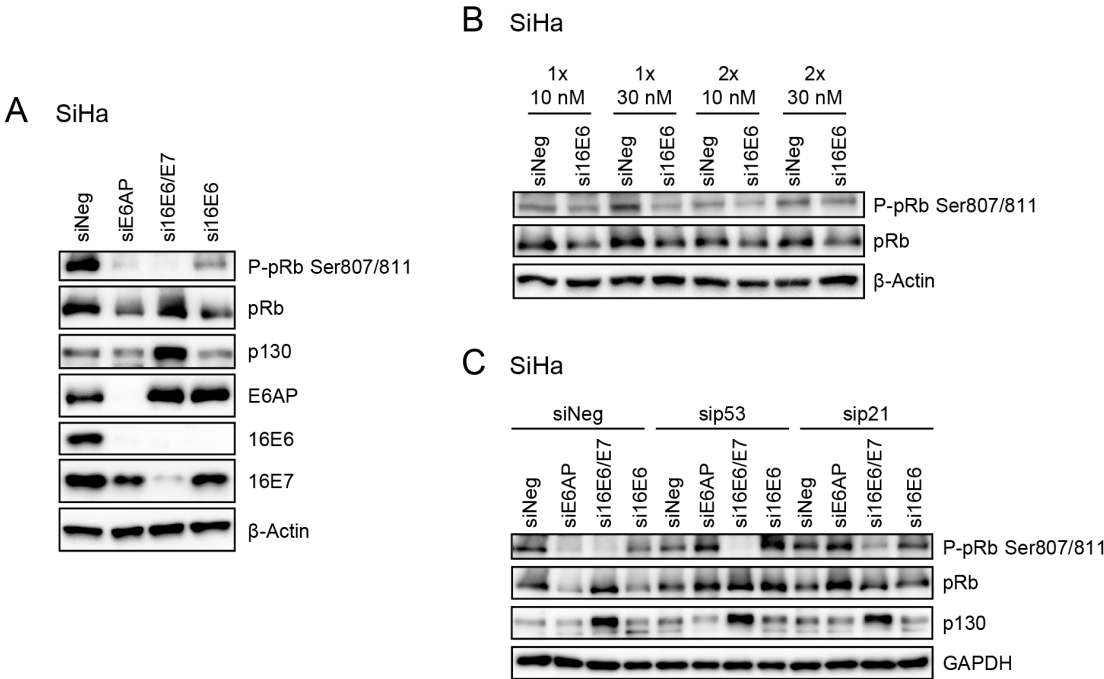


Figure 17. E6AP, E6/E7, or E6 silencing reduces the levels of phosphorylated pRb to varying degrees in HPV-positive cancer cells.

(A) SiHa cells were transfected with siE6AP, si16E6/E7, si16E6, or control siRNA (siNeg), cultivated for 72 h, and examined by immunoblot for P-pRb Ser807/811, pRb, p130, E6AP, 16E6, 16E7, and β -Actin protein levels. (B) SiHa cells were transfected with either 10 or 30 nM si16E6 or control siRNA (siNeg), either once (1x) or twice (2x), following the treatment scheme depicted in Figure 9A, and examined by immunoblot for P-pRb Ser807/811, pRb, and β -Actin protein levels. (C) SiHa cells were transfected with siE6AP, si16E6/E7, si16E6, or control siRNA (siNeg), alone or in combination with sip53 or sip21, as indicated, cultivated for 72 h, and examined by immunoblot for P-pRb Ser807/811, pRb, p130, and GAPDH protein levels. The Figure and Figure legend have been adapted from Avenhaus et al., *PLOS Pathog* (2025).⁷⁰

Interestingly, inhibition of either p53 or p21 – both of which are required for senescence induction upon E6AP knockdown (Figure 14) – effectively reverted P-pRb downregulation following siRNA-mediated E6AP inhibition in SiHa cells (Figure 17C). In contrast, siE6/E7-induced downregulation of P-pRb was only marginally rescued by concomitant inhibition of p21, and remained largely unaffected upon simultaneous knockdown of p53. This may indicate that inhibition of siE6AP-induced senescence by p53 or p21 depletion is linked to the phosphorylation of pRb and suggests a connection between the p53-p21 axis and pocket protein activity.

2.1.4.2 Senescence induction and proliferative arrest upon both E6AP and E6/E7 repression strongly depend on pRb and p130

To explore whether pocket protein activity is functionally relevant to senescence induction upon E6AP or E6/E7 inhibition, HeLa or SiHa cells were transfected with either siE6AP or siE6/E7, and p130, pRb, or both were concomitantly silenced. Immunoblot analyses confirmed efficient knockdown of the respective proteins (Figure 18A). Strikingly, both siE6AP- and siE6/E7-induced senescence were, at least partially, reversible by concomitant inhibition of p130 and/or pRb, as evidenced by the emergence of non-senescent cells next to senescent cells staining positive for SA- β -Gal in both cell lines (Figure 18B). This effect was accompanied by enhanced colony outgrowth in CFAs, particularly in HeLa cells (Figure 18C). In SiHa cells, the effect was less pronounced, which may be due to the transient nature of siRNA transfections that can cause phenotypic effects to diminish at later time points. Another interesting observation in this context was that knockdown of either pRb alone or pRb and p130 in combination not only led to an inhibition of siE6/E7-induced senescence (Figure 18B,C), but also counteracted the siE6/E7-mediated downregulation of the cell cycle regulators E2F1, Cyclin A, Cyclin B1, CDC2, CDK2, and CKS1 in both HeLa and SiHa cells (Figure 18A). This supports the concept that siE6/E7-mediated activation of pocket proteins leads to the downregulation of these cell cycle regulators.

In conclusion, my data demonstrate that senescence and proliferation arrest induced by E6AP or E6/E7 inhibition are strongly dependent on pRb and p130 activities. This is particularly noteworthy since E7 is still expressed upon E6AP knockdown. It therefore can be hypothesized that E7 is not able to fully block the pocket proteins under this condition. Further, this indicates that, although p53 and p21 contribute differently, both the siE6AP- and siE6/E7-induced senescence cascades ultimately converge and share the same downstream pathway.

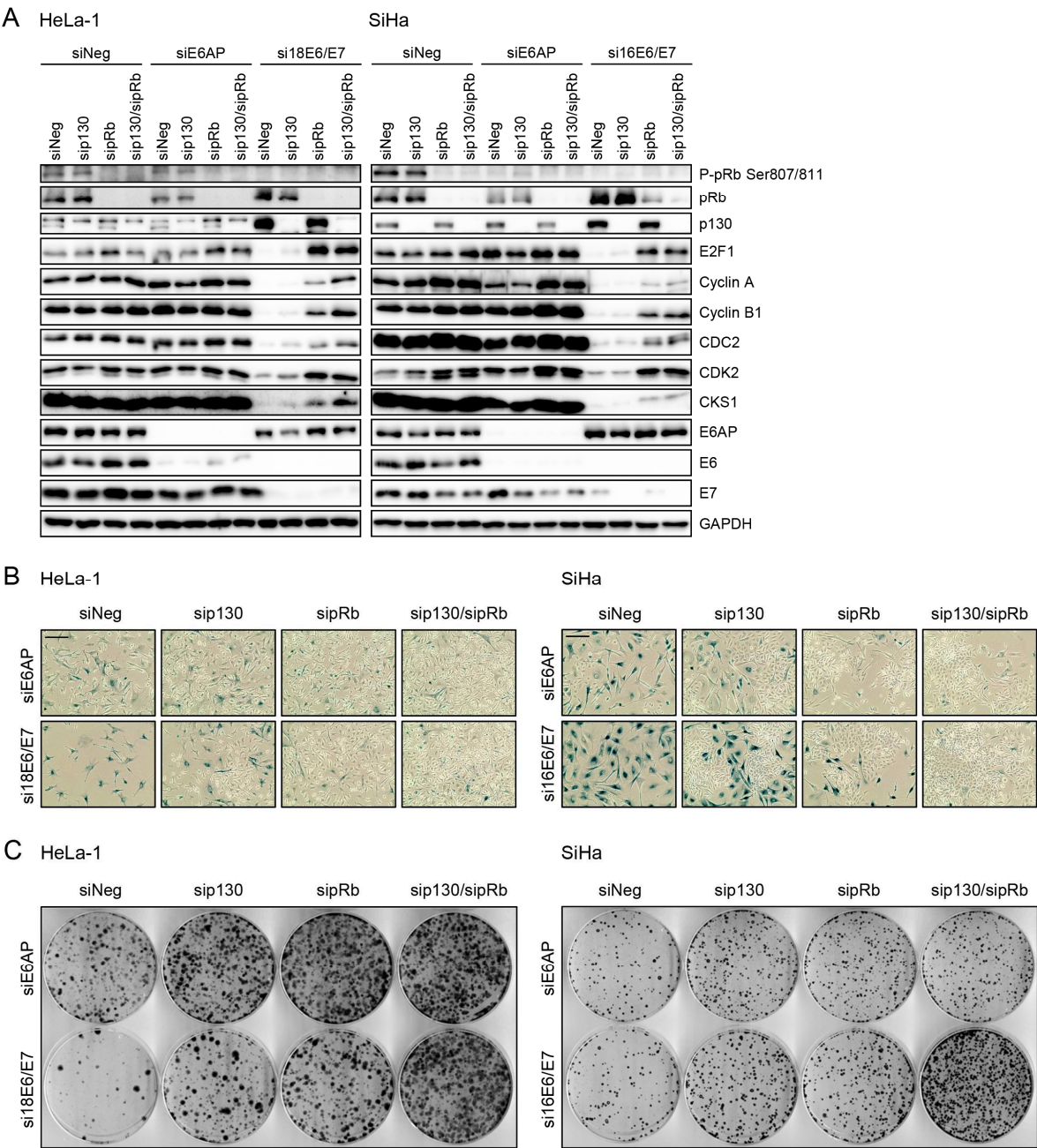


Figure 18. pRb and p130 are critical for senescence induction upon both E6AP and E6/E7 silencing in HPV-positive cancer cells.

HeLa-1 or SiHa cells were transfected with siE6AP, si18E6/E7 or si16E6/E7, si18E6 or si16E6, or control siRNA (siNeg), alone or in combination with sip130, sipRb, or a combination of both (sip130/sipRb), as indicated. Cells were examined following the treatment scheme depicted in Figure 4A by (A) immunoblot for P-pRb Ser807/811, pRb, p130, E2F1, Cyclin A, Cyclin B1, CDC2, CDK2, CKS1, E6AP, E6, E7, and GAPDH protein levels, (B) senescence assays (SA-β-Gal staining, blue; scale bar: 200 μm), and (C) corresponding colony formation assays. Subfigures (A) and (B) and their corresponding legends have been adapted from Avenhaus et al., *PLOS Pathog* (2025).⁷⁰

2.1.5 Metformin counteracts the senescence induction following both E6AP and E6/E7 silencing

Collectively, the findings referenced above have uncovered that E6AP inhibition leads to efficient senescence induction and may have potential to serve as a novel therapeutic strategy to block the growth of HPV-positive cancer cells. Importantly, it has been previously shown that the anti-diabetic drug Metformin, which is discussed to have anti-tumorigenic potential,³⁰⁵ protects HPV-positive cancer cells from siE6/E7-induced senescence.⁹² To further compare the characteristics of senescence induced by E6AP or E6/E7 inhibition, and to gain insights into the phenotypic effects of Metformin in HPV-positive cancer cells, I tested whether Metformin can also counteract senescence induced by E6AP silencing.

Cells were treated according to the scheme shown in Figure 19A. In line with earlier results presented in Figure 8, blocking of either E6AP or E6/E7 efficiently induced senescence, as indicated by the positive staining for SA- β -Gal and the typical senescent morphology of two different HeLa strains (HeLa-1, -2), which differ in their sensitivity towards Metformin, as well as SiHa cells (Figure 19B, left panels for each cell line). Treatment with Metformin reduced overall cell numbers in control siRNA-transfected cells, consistent with its anti-proliferative potential.⁹² Interestingly, Metformin treatment not only strongly reduced the number of SA- β -Gal-positive cells upon E6/E7 silencing, as previously reported,⁹² but also upon E6AP silencing (Figure 19B, right panels for each cell line). In view of the central role of p53 and p21 and/or pRb and p130 in senescence induction upon siRNA-mediated E6AP or E6/E7 inhibition, I investigated the expression of these proteins in corresponding immunoblot analyses. Strikingly, Metformin treatment strongly impaired the siE6AP- and siE6/E7-induced upregulation of p53, Ac-p53 Lys382, and p21 protein levels in both HeLa and SiHa cells (Figure 19C). In addition, Metformin also counteracted the siE6/E7-induced upregulation of pRb and p130 in both cell lines, particularly after 72 hours of treatment. This suggests that Metformin might prevent senescence by downregulating factors that are critically involved in senescence induction following E6AP or E6/E7 inhibition.

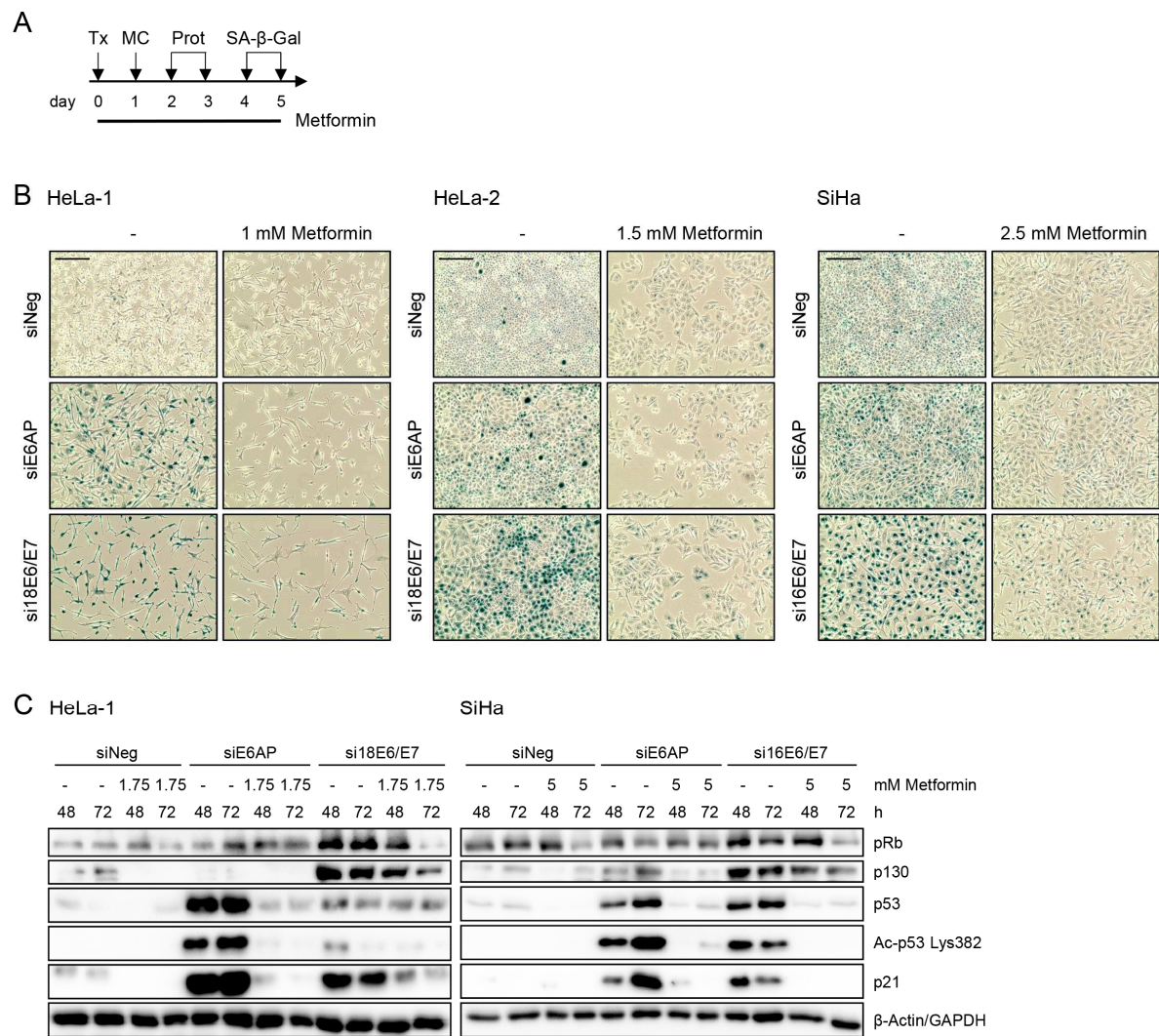


Figure 19. Metformin treatment counteracts the pro-senescent effects of both E6AP and E6/E7 silencing in HPV-positive cancer cells.

(A) Treatment scheme: HeLa-1, HeLa-2, or SiHa cells were forward transfected (Tx) with siE6AP, si18E6/E7 or si16E6/E7, or control siRNA (siNeg) and simultaneously treated with the indicated concentrations of Metformin. After 48 or 72 h of Metformin treatment, HeLa-1 and SiHa cells were harvested for protein (Prot) analyses. After 96 or 120 h of Metformin treatment, HeLa-1, HeLa-2, and SiHa cells were fixed for senescence assays (SA-β-Gal staining). MC, medium change. (B) Corresponding senescence assays (SA-β-Gal staining, blue; scale bar: 200 μm). (C) Immunoblot analyses of pRb, p130, p53, Ac-p53 Lys382, p21, and β-Actin (HeLa-1) or GAPDH (SiHa) protein levels. Subfigures (A) and (C) and their corresponding legends have been adapted from Avenhaus et al., *J Med Virol* (2025).³⁶⁶

Metformin is known to be a potent inhibitor of mTORC1 signaling,^{92,340-342} which can play a role in senescence induction.^{367,368} In line, mTOR inhibition has been shown to counteract siE6/E7-induced senescence.³⁶⁹ Hence, I aimed to determine whether the anti-senescent effects of Metformin could depend on its ability to block mTOR signaling rather than its effects on the

p53-p21 axis or the pocket proteins. To this end, I compared the effects of Metformin with those of mTORC1 inhibitor Rapamycin and mTORC1/2 inhibitor KU-0063794 (Figure 20).

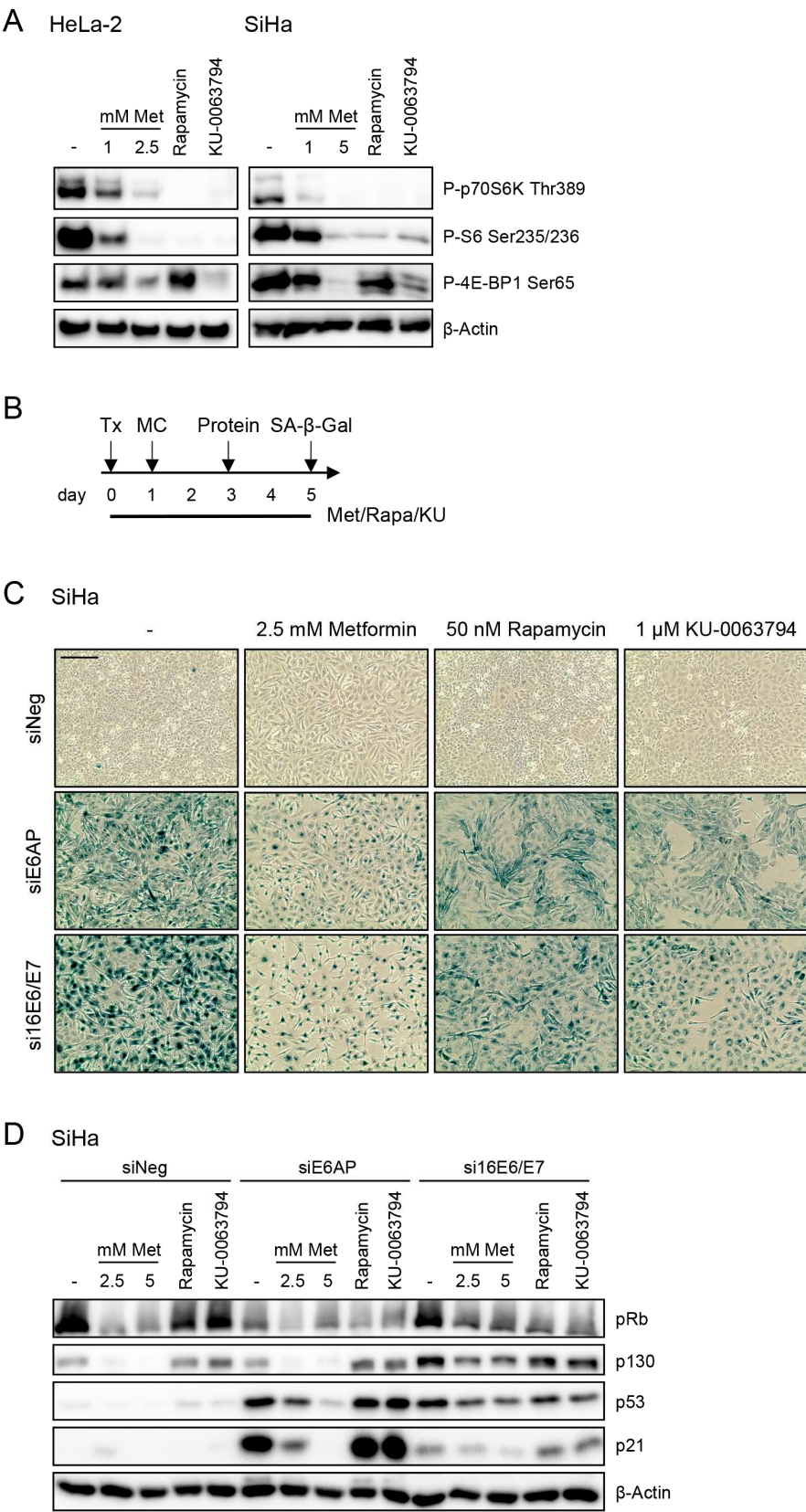


Figure 20. See Figure legend on the next page.

Figure 20. Metformin treatment inhibits mTOR signaling in HPV-positive cancer cells and counteracts the pro-senescent effects of E6AP or E6/E7 silencing more efficiently than chemical mTOR inhibitors.

(A) HeLa-2 or SiHa cells were treated with the indicated concentrations of Metformin (Met), 50 nM Rapamycin, 1 μ M KU-0063794, or DMSO (- , solvent control) for 24 h and examined by immunoblot for P-p70S6K Thr389, P-S6 Ser235/236, P-4E-BP1 Ser65, and β -Actin protein levels. (B) Treatment scheme for Subfigures (C) and (D): SiHa cells were forward transfected (Tx) with siE6AP, si16E6/E7, or control siRNA (siNeg) and simultaneously treated with 2.5 mM Metformin (Met), 50 nM Rapamycin (Rapa), 1 μ M KU-0063794 (KU), or DMSO (- , solvent control). After 72 h of treatment, cells were harvested for protein analyses. After 120 h of treatment, cells were fixed for senescence assays (SA- β -Gal staining). MC, medium change. (C) Corresponding senescence assays (SA- β -Gal staining, blue; scale bar: 200 μ m). (D) Immunoblot analyses of pRb, p130, p53, p21, and β -Actin protein levels.

While 1 mM Metformin only marginally affected mTOR signaling, higher doses of Metformin (2.5 mM [HeLa] or 5 mM [SiHa]) were confirmed to inhibit mTOR, as evidenced by downregulated phosphorylation levels of the mTOR substrates p70-S6 kinase (p70S6K), S6 ribosomal protein (S6), and eukaryotic initiation factor 4E-binding protein 1 (4E-BP1) in both HeLa and SiHa cells (Figure 20A). Similarly, these substrates were downregulated after treatment with Rapamycin or KU-0063794 (with the exception that Rapamycin did not inhibit phosphorylation of 4E-BP1)³⁶⁹ in both cell lines. Despite effective mTOR inhibition, Rapamycin and KU-0063794 only marginally reduced signs of senescence induced by either E6AP or E6/E7 inhibition in SiHa cells (Figure 20B,C). Moreover, unlike Metformin, Rapamycin and KU-0063794 did not counteract p53 and p21 induction following E6AP inhibition nor p130 induction upon E6/E7 inhibition in SiHa cells, as observed in corresponding immunoblot analyses (Figure 20D). In contrast, both inhibitors, like Metformin, counteracted the pRb increase following E6/E7 knockdown. Notably, however, Metformin had stronger inhibitory effects on basal pRb and p130 levels compared to Rapamycin and KU-0063794.

Taken together, these findings show that Metformin counteracts senescence induction upon both E6AP and E6/E7 inhibition. The anti-senescent effects of Metformin appear to be more efficient than the effects of the mTOR inhibitors Rapamycin and KU-0063794, which could result from Metformin's capacity to not only inhibit mTOR signaling, but also target the p53-p21 axis as well as pRb and p130.

Importantly, these observations raise questions about the biological and therapeutic implications of the anti-senescent activity of Metformin. However, although the senescence-suppressive effects might appear counterproductive in the context of tumor suppression, Metformin remains a candidate for anti-cancer therapy, particularly due to its known ability to repress viral *E6/E7* oncogene expression and inhibit tumor cell proliferation.^{92,370-372} To investigate this contradiction and explore its therapeutic potential further, I next conducted a systematic investigation of the effects of Metformin on HPV-positive cancer cells.

2.2 Evaluation of Metformin and combination therapies as strategies to inhibit HPV-positive cancer cell proliferation

The anti-senescent activity of Metformin, along with its ability to counteract p53 and p21 induction, is particularly interesting given the previous finding from our group that Metformin itself downregulates the expression of the viral *E6/E7* oncogenes at both the transcript and protein level.⁹² This is usually a strong pro-senescent trigger and leads to p53 and p21 upregulation in HPV-positive cancer cells.^{71,369,373} To better understand this paradox and to evaluate how Metformin affects the proliferation of HPV-positive cancer cells, I aimed to characterize the effects of Metformin on the phenotype of these cells in more detail by comparing it to those of RNAi-mediated *E6/E7* downregulation.

2.2.1 The effects of *E6/E7* inhibition using RNAi or Metformin treatment differ drastically

First, I compared the effects of siRNA-mediated *E6/E7* downregulation – which efficiently induces senescence – or Metformin-mediated *E6/E7* repression – which does not induce senescence but a proliferative stop that can be reversed upon drug removal⁹² – on central *E6/E7* targets involved in cell cycle control and senescence. In line with previous results,⁹² *E6* and *E7* protein levels were efficiently downregulated by si*E6/E7*, and also following Metformin treatment in a dose-dependent manner in both HeLa and SiHa cells (Figure 21A). As expected,^{207,369,373} *E6/E7* repression upon siRNA-mediated *E6/E7* silencing was accompanied by an upregulation of the *E6/E7* key targets p53, p21, pRb, and p130, as well as downregulation of P-pRb in both cell lines (Figure 21A, left panels for each cell line). Moreover, Cyclin D1 levels, which are typically increased in senescent cells,³⁷⁴ were elevated. Surprisingly, Metformin-induced downregulation of *E6/E7* did not induce the anticipated increase in these targets, but was even accompanied by a downregulation of p53, p21, P-pRb, p130, and Cyclin D1 protein levels in both cell lines (Figure 21A, right panels for each cell line). Of note, while total pRb levels remained largely unaffected in this experimental setup (24 hours of Metformin treatment), they decreased upon longer treatment with Metformin in SiHa cells (Figures 19C, 20D).

Among the factors analyzed, the reduction of p53 and p21 levels upon Metformin treatment stood out, particularly given their key role in coordinating cell cycle arrest and senescence.^{56,201} To investigate whether this regulation occurs at the transcriptional or post-transcriptional level, *TP53* mRNA expression was examined and found to be unaffected by Metformin treatment in both HeLa and SiHa cells (Figure 21B). Moreover, I expanded my analyses to HPV-negative cancer cells to determine whether the unexpected downregulation of p53 and p21 could be independent of HPV status. Notably, basal p53 and p21 levels were also dose-dependently

downregulated after Metformin treatment in U2OS osteosarcoma cells (Figure 21C). Metformin further not only counteracted p53 and p21 induction triggered by E6AP or E6/E7 inhibition in HPV-positive cancer cells (Figures 19C, 20D), but also counteracted p53 and p21 upregulation upon inhibition of Mdm2-mediated p53 degradation using Nutlin-3³⁷⁵ in HPV-negative U2OS as well as HCT116 colon cancer cells (Figure 21D). This identifies a strong suppressive effect of Metformin on p53 and p21 protein levels, which appears to be HPV-independent.

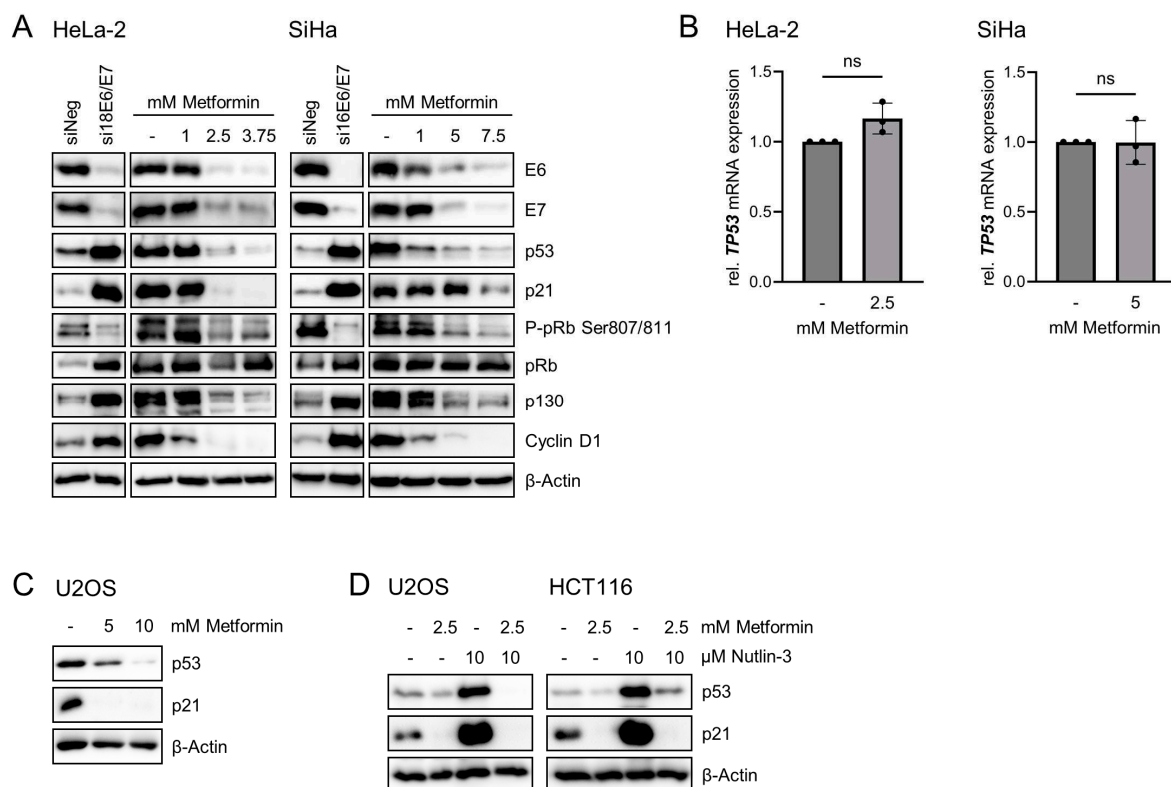


Figure 21. Metformin- or siRNA-induced downregulation of E6/E7 exhibits pronounced differences on key E6/E7 targets in HPV-positive cancer cells.

(A) HeLa-2 or SiHa cells were either transfected with si18E6/E7 or si16E6/E7, or control siRNA (siNeg) and cultivated for 72 h, or treated with the indicated concentrations of Metformin for 24 h and examined by immunoblot for E6, E7, p53, p21, P-pRb Ser807/811, pRb, p130, Cyclin D1, and β-Actin protein levels. (B) HeLa-2 or SiHa cells were treated with the indicated concentrations of Metformin for 24 h. qRT-PCR analyses of *TP53* mRNA levels were conducted and are shown as mean relative expression with standard deviations ($n = 3$), with the untreated control set to 1. Statistically significant differences were assessed using a two-sided unpaired *t*-test. ns, not significant. rel., relative. (C) U2OS cells were treated with the indicated concentrations of Metformin for 24 h and examined by immunoblot for p53, p21, and β-Actin protein levels. (D) U2OS or HCT116 cells were treated with 2.5 mM Metformin for 48 h. Nutlin-3 at a final concentration of 10 μM or DMSO (-, solvent control) was added for the last 24 h of treatment. Cells were examined by immunoblot for p53, p21, and β-Actin protein levels. The Figure and Figure legend have been adapted from Avenhaus et al., *J Med Virol* (2025).³⁶⁶

In view of the differential regulation of key E6/E7 targets, the effects of either siRNA-mediated or Metformin-induced E6/E7 downregulation were further investigated using mass-spectrometry (MS)-based proteomics. In collaboration with Dr. Bianca J. Kuhn and Prof. Dr. Jeroen Krijgsveld (DKFZ, Heidelberg), I analyzed global proteomic changes of 4649 detected proteins in SiHa cells in which E6/E7 expression was inhibited by RNAi. These changes were compared to published proteome data (5929 detected proteins) from SiHa cells in which E6/E7 expression was repressed by treatment with Metformin for 24 hours.^{92,376}

Significantly enriched pathways upon siRNA-mediated E6/E7 knockdown were identified using Gene Set Enrichment Analysis (GSEA)^{377,378} and the C2 database.^{379,380} Notably, a pronounced negative enrichment was observed for the FISCHER_DREAM_TARGETS²⁰² gene set, which comprises cell cycle-related genes regulated by the DREAM complex (Figure 22A, left panel). This indicates high activity of the cell cycle suppressive p53-p21-DREAM pathway, as the DREAM complex – containing the pocket protein p130 – acts as a transcriptional repressor.^{56,57,201,202} Similarly, E6/E7 silencing by RNAi led to a significant negative enrichment of the FISCHER_G1_S_CELL_CYCLE²⁰² gene set (Figure 22B, left panel), which consists of genes that promote the G1/S transition. In contrast, and despite efficient E6/E7 downregulation, Metformin treatment did not induce a significant negative enrichment of these gene sets (Figure 22A,B, right panels). This differential regulation was further visualized in heatmaps showing a stronger downregulation of leading-edge genes (proteins) from the FISCHER_DREAM_TARGETS gene set, which contribute most to the enrichment result³⁷⁸ (Figure 22C, left panel), as well as more pronounced negative enrichment of genes (proteins) in the FISCHER_G1_S_CELL_CYCLE gene set (Figure 22C, right panel) following siRNA-mediated E6/E7 silencing compared to E6/E7 downregulation after Metformin treatment. Of note, only subsets of genes of the respective gene sets were detected in each proteome data set. Among these subsets, the majority of detected genes (proteins) overlapped between the Metformin and the siE6/E7 data set, while some genes were unique to either condition (Supplementary Figure 1). However, the distribution of these unique genes was largely similar to that of the overlapping genes (not shown), indicating that they did not strongly affect the overall results derived from GSEA.

To validate the proteomics data, I performed immunoblot analyses for a selection of proteins also detected in the proteome screens. In line with the proteome analyses (Supplementary Figure 2) as well as earlier results presented in Figure 12A, the DREAM and/or pRb targets B-MYB, FOXM1, E2F1, Cyclin A, Cyclin B1, Cyclin B2, CDC2, CDK2, CKS1, RRM1, RRM2, PCNA, and PLK1 were efficiently downregulated following E6/E7 repression by RNAi, but unaffected or only modestly decreased after treatment with increasing doses of Metformin in both HeLa and SiHa cells (Figure 22D).

In conclusion, siRNA-mediated E6/E7 inhibition induces senescence and efficiently downregulates proteins critical for cell cycle progression, likely through activation of the p53-p21-pRb and p53-p21-DREAM pathways. Conversely, Metformin treatment – which induces growth arrest but not senescence – has only limited effects on these cell cycle regulators and differentially affects E6/E7 key targets, despite also downregulating E6/E7 expression.

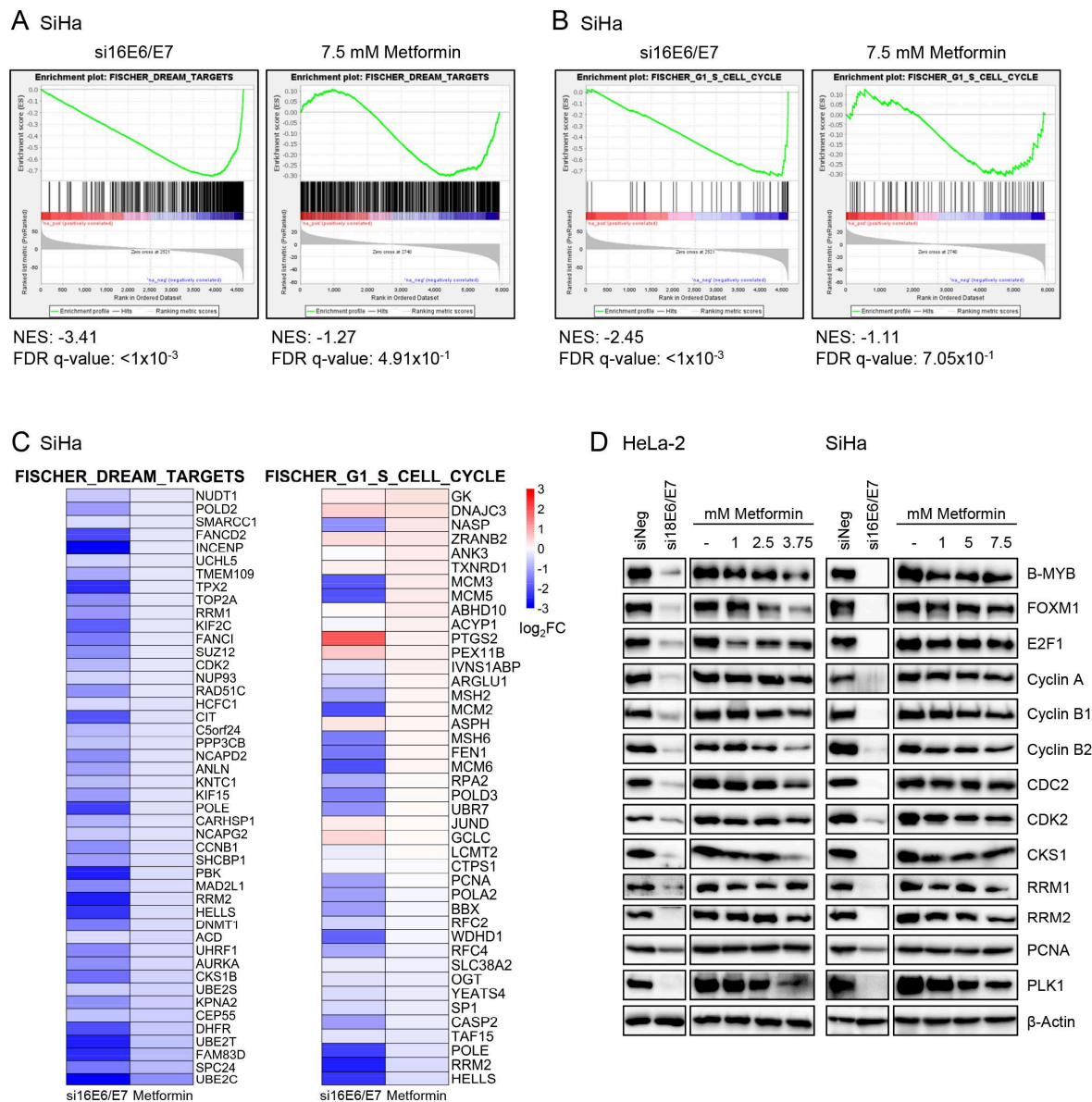


Figure 22. Metformin- or siRNA-induced downregulation of E6/E7 exhibits pronounced differences on cell cycle regulators in HPV-positive cancer cells.

(A-C) SiHa cells were transfected with si16E6/E7 or control siRNA (siCtrl1) and cultivated for 72 h. Global proteomic changes were analyzed by mass spectrometry (preparation of the samples was performed by Claudia Lohrey, proteome analyses were performed by Dr. Bianca J. Kuhn). Proteome profiles of SiHa cells in which E6/E7 was silenced by RNAi were compared to published proteomic data from SiHa cells treated with 7.5 mM Metformin or DMSO for 24 h^{92,376} using gene set enrichment analysis (GSEA). Signed log₁₀ *p* values of the proteome data sets siE6/E7 vs. siCtrl1 (*n* = 3) or Metformin vs. DMSO (*n* = 3) were used as input for pre-ranked GSEA with 1000 permutations using the C2 collection. NES, normalized enrichment score; FDR, false discovery rate.

Figure 22. (continued) (A) Enrichment plots for the gene set FISCHER_DREAM_TARGETS.²⁰² (B) Enrichment plots for the gene set FISCHER_G1_S_CELL_CYCLE.²⁰² (C) Corresponding heatmaps showing average log₂ fold change (log₂FC) values of leading-edge genes (proteins) from the FISCHER_DREAM_TARGETS gene set (left panel) or average log₂FC values of genes (proteins) from the FISCHER_G1_S_CELL_CYCLE gene set (right panel) detected in both data sets. Expression changes are coded by color: red, upregulation; blue, downregulation. (D) HeLa-2 or SiHa cells were either transfected with si18E6/E7 or si16E6/E7, or control siRNA (siNeg) and cultivated for 72 h, or treated with the indicated concentrations of Metformin for 24 h and examined by immunoblot for B-MYB, FOXM1, E2F1, Cyclin A, Cyclin B1, Cyclin B2, CDC2, CDK2, CKS1, RRM1, RRM2, PLK1, and β -Actin protein levels. Subfigures (A), (B), and (D) and their corresponding legends have been adapted from Avenhaus et al., *J Med Virol* (2025).³⁶⁶

2.2.2 Metformin can inhibit cellular proliferation independently of E6/E7 suppression

Since siRNA-mediated E6/E7 inhibition and Metformin treatment differentially affect cell cycle-promoting factors (Figure 22), I next examined whether these differences translate into distinct effects on cell cycle profiles.

As described above, E6/E7 inhibition via RNAi leads to an efficient induction of G1 arrest in HPV-positive HeLa and SiHa cells (Figure 11B). Interestingly, although Metformin downregulates E6 and E7 at higher doses (Figure 21A), Metformin treatment did not trigger a G1 arrest but rather moderately increased G2/M populations in both HeLa and SiHa cells (Figure 23A, upper panels for each cell line). Given previous reports about the anti-proliferative potential of Metformin,^{92,370-372} I additionally employed co-treatment with microtubule polymerization inhibitor Nocodazole to further assess the proliferative capacity of Metformin-treated cells. Nocodazole induced a pronounced increase in G2/M phases in proliferating control cells (Figure 23A, lower panels for each cell line). In contrast, the cell cycle profile of Metformin-treated cells remained largely unaffected upon Nocodazole co-treatment. This indicates a strong proliferative arrest of Metformin-treated cells. Consistently, proliferation curves obtained from live-cell imaging showed a strong reduction in proliferation upon Metformin treatment in SiHa cells (Figure 23B).

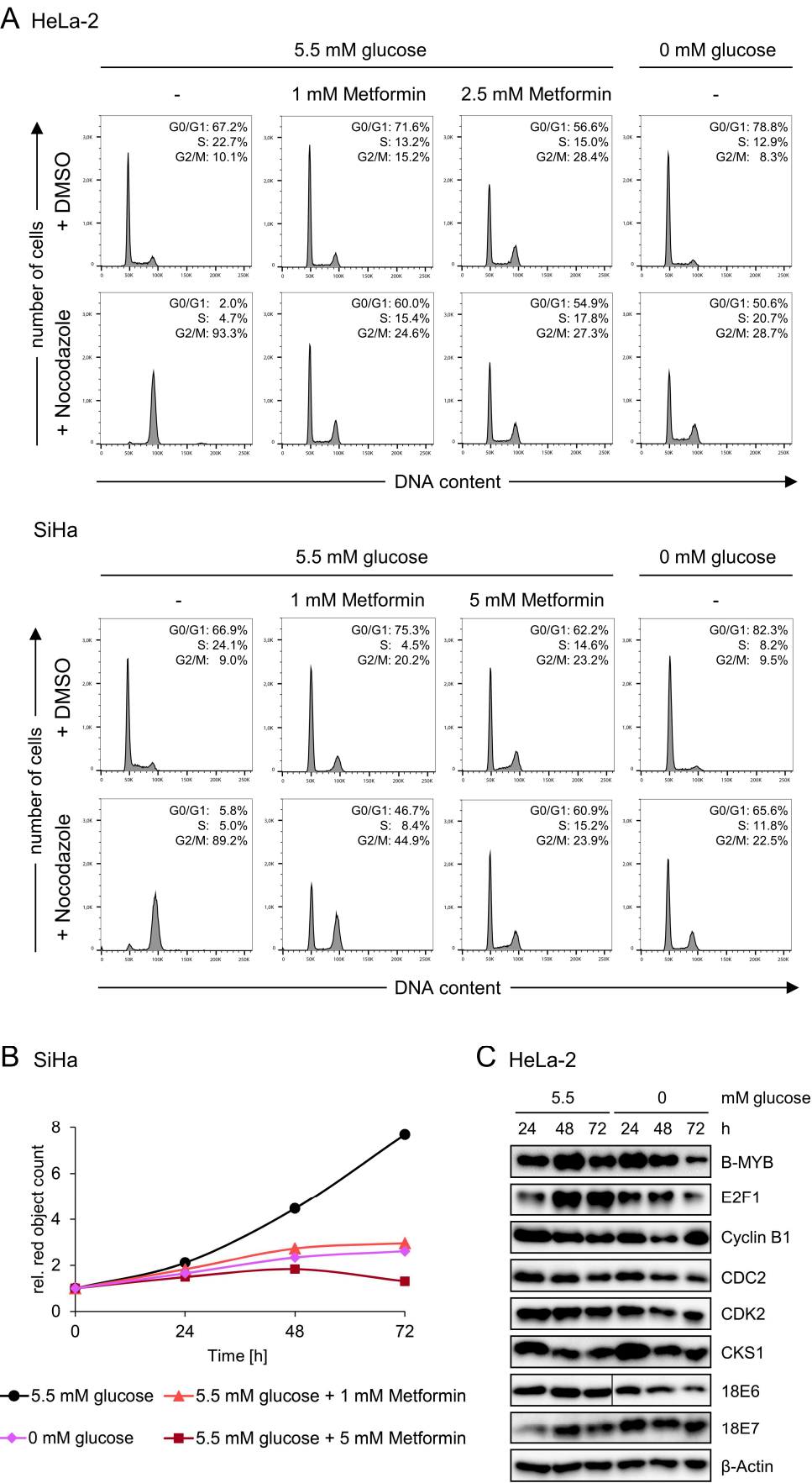


Figure 23. See Figure legend on the next page.

Figure 23. Metformin treatment blocks the proliferation of HPV-positive cancer cells without inducing a pronounced accumulation of cells in a specific cell cycle phase.

(A) HeLa-2 or SiHa cells were treated with the indicated concentrations of Metformin or cultivated in medium containing no glucose (0 mM) for 48 h. Nocodazole at a final concentration of 0.1 $\mu\text{g/mL}$ or DMSO (solvent control) was added for the last 24 h of treatment. Cell cycle profiles and quantifications of the percentages of cell populations in the individual cell cycle phases are shown. (B) SiHa-mKate2 cells were treated 24 h after seeding (time point 0) with the indicated concentrations of Metformin or cultivated in medium containing no glucose (0 mM) for 72 h. Cells were imaged by the Incucyte[®] S3 Live-Cell Analysis Instrument. Cell counts (red objects) were quantified using the Incucyte[®] software package and are shown relative to the counts at time point 0 (set to 1). rel, relative. (C) HeLa-2 cells were cultivated under either 0 or 5.5 mM glucose in the cell culture medium for up to 72 h and examined by immunoblot for B-MYB, E2F1, Cyclin B1, CDC2, CDK2, CKS1, 18E6, 18E7, and β -Actin protein levels. Vertical lines between the lanes indicate where original images from the same blot and the same exposure time were cropped for clarity of presentation. Subfigures (A) and (B) and their corresponding legends have been adapted from Avenhaus et al., *J Med Virol* (2025).³⁶⁶

Importantly, the Metformin-induced growth arrest observed in cell cycle analyses (Figure 23A) and proliferation assays (Figure 23B) was already evident upon treatment with 1 mM Metformin, a dose that is insufficient to downregulate E6 and E7 (Figure 21A). This could indicate that the anti-proliferative effect of Metformin is independent of its suppressive effects on the viral oncogenes.

To explore whether other growth inhibitory conditions can impair the proliferation of HPV-positive cancer cells without suppressing E6/E7 levels, I compared the effects of Metformin to those of glucose deprivation (0 mM), which is reported to inhibit cellular proliferation³⁸¹⁻³⁸⁴ but did not affect E6/E7 expression in HeLa cells (Figure 23C). Glucose deprivation induced an increase of cells in the G1 phase of the cell cycle in both HeLa and SiHa cells (Figure 23A, upper right panels for each cell line) which was, however, not as robust as the G1 arrest observed upon E6/E7 silencing by RNAi (Figure 11B). Moreover, similar to Metformin treatment, glucose-starved cells were efficiently growth arrested despite sustained E6/E7 expression, as evidenced by co-treatment with Nocodazole that failed to induce efficient G2/M arrest in both cell lines (Figure 23A, lower right panels for each cell line), as well as proliferation curves showing strongly impaired proliferation of SiHa cells (Figure 23B). In addition, glucose withdrawal from the medium, similar to Metformin treatment (Figure 22D), had only minor effects on the expression of cell cycle-promoting factors such as B-MYB, E2F1, Cyclin B1, CDC2, CDK2, and CKS1 in HeLa cells (Figure 23C).

Collectively, my data reveal that, unlike siRNA-mediated E6/E7 silencing, Metformin-induced growth arrest is not associated with an arrest in a specific phase of the cell cycle. Moreover, my findings show that both treatment with Metformin and glucose deprivation can inhibit cell cycle progression and cellular growth in HPV-positive cancer cells despite continued E6/E7 expression. Overall, this indicates that Metformin can inhibit HPV-positive cancer cell proliferation independently of its ability to suppress the viral oncogenes.

2.2.3 Cell cycle arrest upon Metformin treatment protects cells from CT-induced senescence

It has been previously shown by our group that Metformin-treated cells not only evade siE6/E7-induced senescence (Figure 19B), but are also protected from senescence induced by the chemotherapeutic drug Etoposide.⁹² This drug causes DNA damage in the S phase of the cell cycle by inhibiting nuclear topoisomerase II, thereby leading to an accumulation of S phase populations as well as G2/M arrest.³⁸⁵⁻³⁸⁷ Since Metformin induces growth arrest in different cell cycle phases, I hypothesized that this arrest could protect cells from Etoposide-induced DNA damage occurring in S phase.

2.2.3.1 Metformin treatment or glucose deprivation prevents Etoposide-induced cell cycle perturbations and senescence in HPV-positive cancer cells

Cell cycle analyses of HeLa or SiHa cells treated with Etoposide, as outlined in the scheme in Figure 24A, revealed an accumulation of cells in the S phase as well as increased G2/M populations in both cell lines, as expected (Figure 24B). Metformin treatment alone led to only minor alterations of cell cycle profiles. Remarkably, also co-treatment with Metformin and Etoposide resulted in largely unchanged cell cycle profiles with only slight increases in S phases compared to the corresponding controls, if at all. Interestingly, a similar effect was observed upon glucose deprivation (Figure 24B, right panels for each cell line), suggesting that cell cycle arrest induced by either Metformin treatment or glucose deprivation prevents DNA damage as well as resulting S phase accumulation and G2/M arrest following treatment with Etoposide.

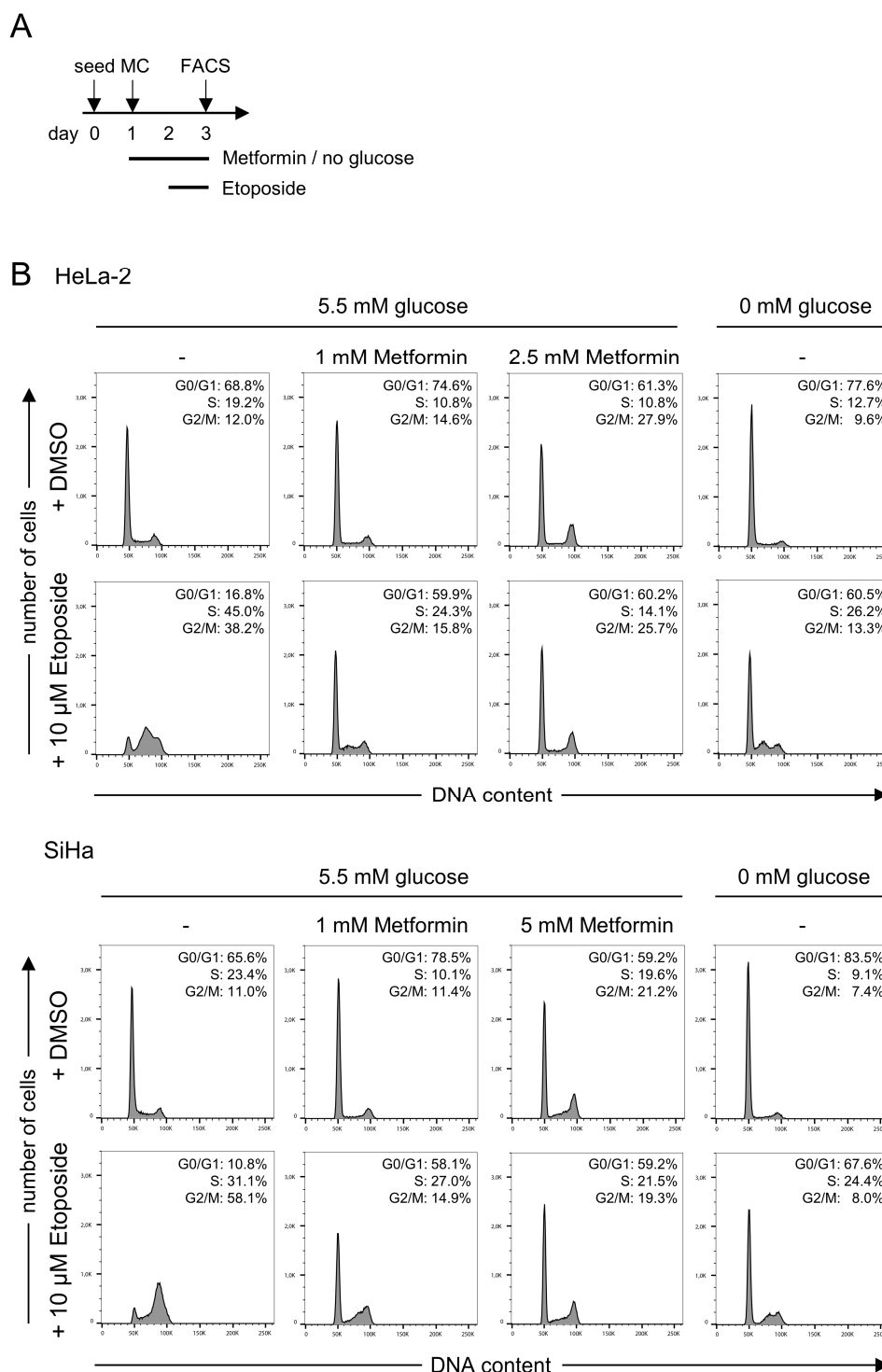


Figure 24. Metformin treatment or glucose deprivation counteracts Etoposide-induced cell cycle perturbations in HPV-positive cancer cells.

(A) Treatment scheme: HeLa-2 or SiHa cells were treated with the indicated concentrations of Metformin or cultivated in medium containing no glucose (0 mM) for 48 h. Etoposide at a final concentration of 10 μ M or DMSO (solvent control) was added for the last 24 h of treatment. Subsequently, cells were harvested for flow cytometry analyses (FACS). MC, medium change. (B) Corresponding cell cycle profiles and quantifications of the percentages of cell populations in the individual cell cycle phases are shown. The Figure and Figure legend have been adapted from Avenhaus et al., *J Med Virol* (2025).³⁶⁶

It thus seems likely that the absence of G2/M arrest upon co-treatment with Metformin and Etoposide underlies the ability of Metformin-treated cells to evade Etoposide-induced senescence. Accordingly, when analyzing the phenotypic effects over time following the treatment scheme in Figure 25A, I found that both Metformin-treated and glucose-deprived HeLa and SiHa cells were protected from Etoposide-induced senescence.

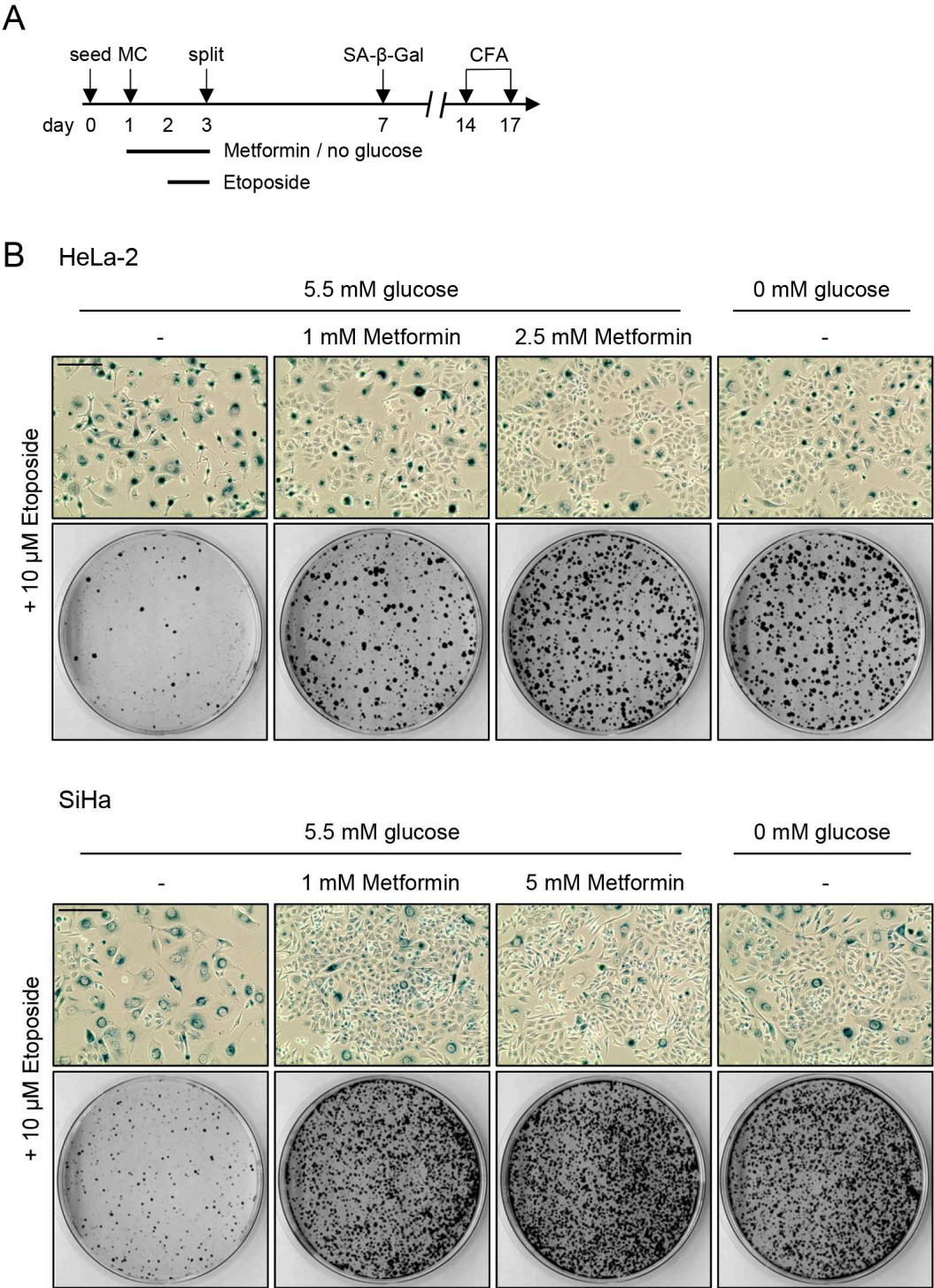


Figure 25. See Figure legend on the next page.

Figure 25. Metformin treatment or glucose deprivation counteracts the pro-senescent effects of Etoposide in HPV-positive cancer cells.

(A) Treatment scheme: HeLa-2 or SiHa cells were treated with the indicated concentrations of Metformin or cultivated in medium containing no glucose (0 mM) for 48 h. Etoposide at a final concentration of 10 μ M was added for the last 24 h of treatment. Subsequently, cells were split and further cultivated in drug-free medium for senescence assays (SA- β -Gal staining) or colony formation assays (CFAs) after the indicated time periods. MC, medium change. (B) Corresponding senescence assays (upper panels for each cell line; SA- β -Gal staining, blue; scale bar: 200 μ m) and CFAs (lower panels for each cell line). The Figure and Figure legend have been adapted from Avenhaus et al., *J Med Virol* (2025).³⁶⁶

In specific, Etoposide *per se* efficiently induced cellular senescence (Figure 25B, upper left panels for each cell line), which was accompanied by a lack of colony outgrowth in CFAs (Figure 25B, lower left panels for each cell line) in both cell lines. In contrast, cells either pre-treated with Metformin or cultivated in medium lacking glucose prior to Etoposide treatment displayed a substantial emergence of non-senescent cells as well as increased colony formation capacity upon drug removal (Figure 25B).

2.2.3.2 Metformin treatment protects HPV-negative cancer cells from Etoposide-induced senescence

Since both treatment with 1 mM Metformin and glucose deprivation did not affect the expression of E6 and E7 (Figures 21A, 23C), but induced cell cycle arrest (Figure 23A,B) and prevented senescence induction upon Etoposide co-treatment (Figure 25), I hypothesized that these effects are HPV-independent and could also apply to HPV-negative cancer cells. Therefore, I further analyzed the effects of Metformin and Etoposide co-treatment in MCF-7 breast cancer or U2OS osteosarcoma cells. Metformin monotreatment only marginally affected the cell cycle profiles of both MCF-7 and U2OS cells (Figure 26A). Etoposide monotreatment led to an increase in G2/M phases in both cell lines whereas, in contrast to HPV-positive cells, an accumulation of S phases was not observed. Importantly, however, similar to the results obtained in HPV-positive cancer cells, co-treatment with Metformin and Etoposide had only mild effects on the cell cycle profiles of both MCF-7 and U2OS cells, indicating a protective effect of Metformin against the cell cycle perturbations caused by Etoposide. Consistent with these findings, senescence assays (Figure 26B, upper panels for each cell line) and CFAs (Figure 26B, lower panels for each cell line) revealed efficient induction of senescence in both cell lines upon Etoposide monotreatment, whereas a pronounced increase in non-senescent cells and colony formation capacity was observed upon concomitant treatment with Metformin.

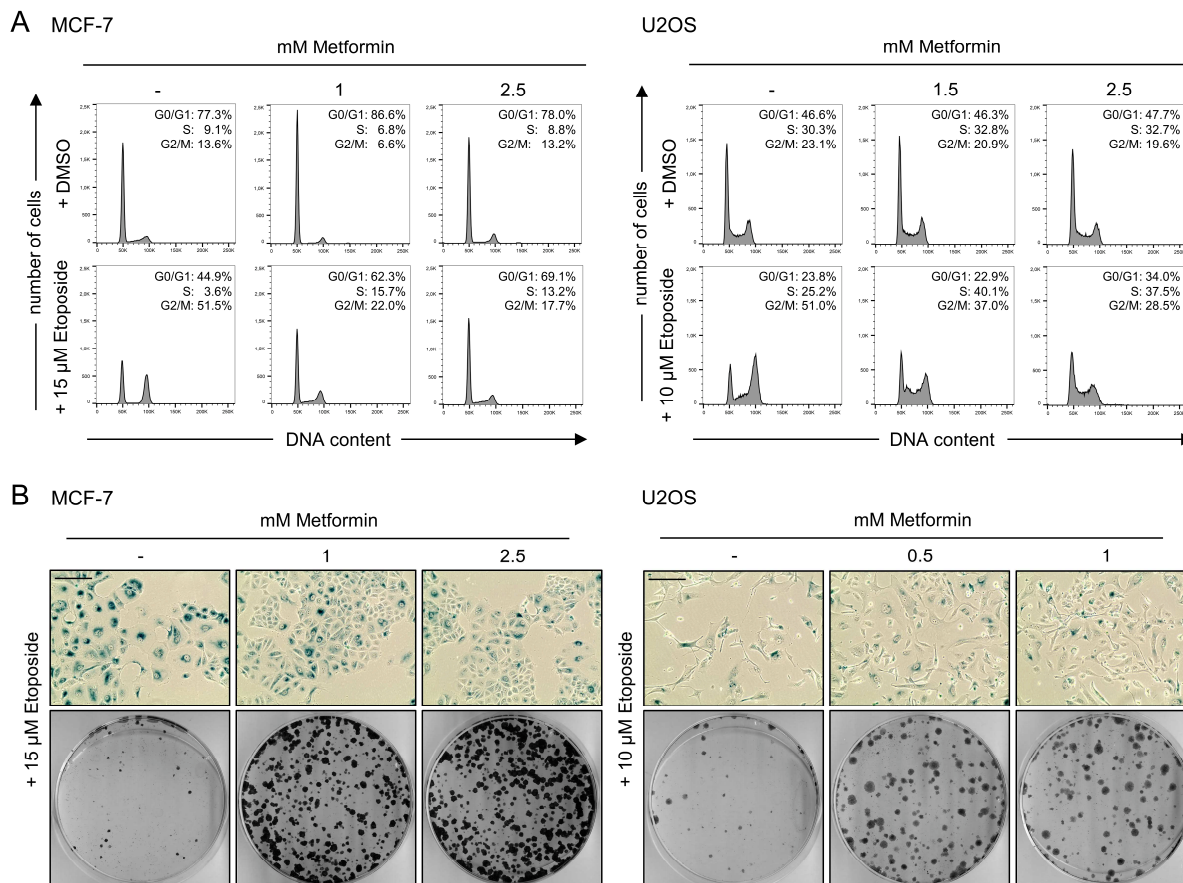


Figure 26. Metformin treatment counteracts Etoposide-induced cell cycle perturbations and senescence induction in HPV-negative cancer cells.

(A) MCF-7 or U2OS cells were treated with the indicated concentrations of Metformin for 48 h. Etoposide at a final concentration of 15 μ M (MCF-7) or 10 μ M (U2OS), or DMSO (solvent control) was added for the last 24 h of treatment. Cell cycle profiles and quantifications of the percentages of cell populations in the individual cell cycle phases are shown. (B) MCF-7 or U2OS cells were treated with the indicated concentrations of Metformin for 72 h. Etoposide at a final concentration of 15 μ M (MCF-7) or 10 μ M (U2OS) was added for the last 48 h of treatment. Subsequently, cells were split and further cultivated in drug-free medium. Upper panels for each cell line: Senescence assays, performed on day 4 after splitting (SA- β -Gal staining, blue; scale bar: 200 μ m). Lower panels for each cell line: Colony formation assays, performed on day 12 after splitting. Subfigure (B) and the corresponding legend have been adapted from Avenhaus et al., *J Med Virol* (2025).³⁶⁶

Taken together, my data show that both Metformin-treated and glucose-deprived HPV-positive cancer cells can evade Etoposide-induced senescence, and that HPV-negative cells exhibit a similar evasion upon Metformin co-treatment. These findings suggest that these anti-senescent effects depend on the growth-inhibitory properties of both conditions.

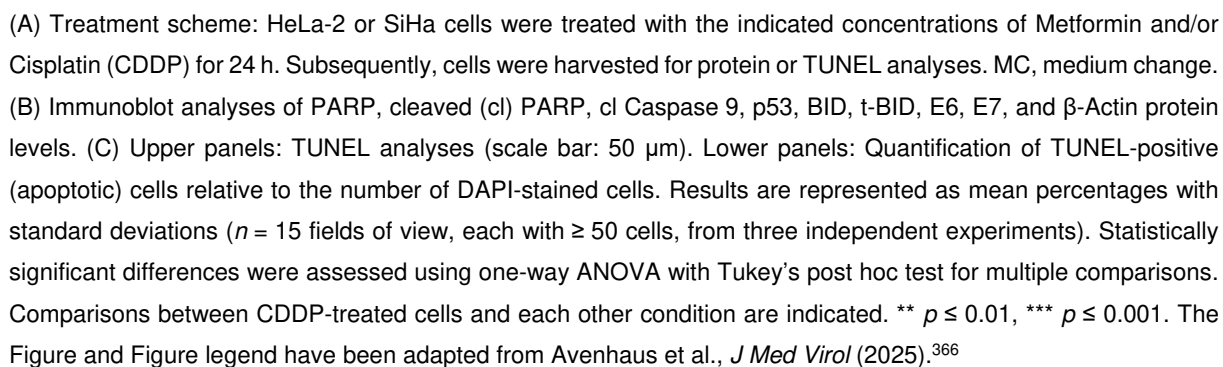
2.2.4 Metformin can either inhibit or enhance Cisplatin-induced apoptosis in HPV-positive cancer cells

As shown above, combining Metformin with the pro-senescent chemotherapeutic Etoposide leads to cellular outgrowth instead of senescence (Figures 25, 26), an effect that is undesirable for cancer treatment. Notably, a broad range of preclinical and clinical studies describes both protective and additive effects of Metformin in combination with other anticancer agents.^{317-323,338} Hence, to further investigate the interactions of Metformin with chemotherapeutics, I next evaluated its effects in combination with Cisplatin, a drug that is recommended as part of the standard care to treat locally advanced and recurrent cervical cancer^{147,148} and can act both pro-senescent and pro-apoptotic.¹⁵⁵⁻¹⁵⁸

2.2.4.1 Simultaneous treatment with Metformin and Cisplatin prevents Cisplatin-induced apoptosis

In a first experimental setup, I treated HeLa or SiHa cells simultaneously with Metformin and/or Cisplatin for 24 hours, according to the treatment scheme in Figure 27A.

Cisplatin monotreatment induced apoptosis, as evidenced by increased levels of the apoptosis markers cleaved PARP and cleaved Caspase 9 (Figure 27B), as well as increased numbers of TUNEL-positive cells (Figure 27C) in both cell lines. This was accompanied by increased levels of p53 as well as cleavage of the BID protein (Figure 27B). The resulting t-BID acts as a pro-apoptotic factor¹⁶⁸ and has been recently shown to play a critical role in Cisplatin-mediated apoptosis in HPV-positive cancer cells.¹⁶⁷ In addition, levels of E6 and E7 were decreased upon Cisplatin monotreatment. Strikingly, co-treatment with Metformin reduced the Cisplatin-mediated induction of apoptosis in both HeLa and SiHa cells in a dose-dependent manner, as reflected by reduced levels of cleaved PARP, cleaved Caspase 9, p53, and t-BID (Figure 27B), as well as significantly decreased numbers of TUNEL-positive cells (Figure 27C), when compared to cells treated with Cisplatin alone. This indicates that Metformin protects cells against Cisplatin-induced apoptosis when both drugs are applied simultaneously.



2.2.4.2 Metformin pre-treatment enhances Cisplatin-mediated apoptosis

In a second experimental setup, HeLa or SiHa cells were treated as depicted in Figure 28A. Instead of simultaneously treating the cells with Metformin and Cisplatin, the cells were pre-treated with Metformin for 24 hours before Cisplatin was added for another 24 hours.

In this experimental condition, Cisplatin monotreatment led to limited apoptosis, as evidenced by low levels of cleaved PARP and cleaved Caspase 9 (Figure 28B), as well as only few TUNEL-positive cells (Figure 28C) in both cell lines. This could be explained by suboptimal growth conditions in this experimental setup caused by the medium change 24 hours prior to Cisplatin treatment. Further, Cisplatin alone induced little to no expression of t-BID and the expression levels of both E6 and E7 remained sustained (Figure 28B). However, despite the low pro-apoptotic response, p53 levels were also strongly increased under this treatment condition.

Interestingly, pre-treatment with Metformin followed by Cisplatin treatment induced apoptosis significantly stronger compared to Cisplatin treatment alone, at least at certain Metformin concentrations. Specifically, immunoblot analyses of HeLa or SiHa cells co-treated with Cisplatin and 1 or 2.5 mM (HeLa), or 5 mM Metformin (SiHa) showed higher levels of cleaved PARP and cleaved Caspase 9 as well as t-BID, than cells treated with Cisplatin alone (Figure 28B). Furthermore, the proportion of TUNEL-positive cells was significantly higher upon co-treatment with Cisplatin and 2.5 mM (HeLa) or 5 mM Metformin (SiHa) than upon Cisplatin monotreatment in both cell lines (Figure 28C). In line, light microscopy images of HeLa cells revealed a pronounced increase in cells exhibiting apoptotic morphology upon co-treatment with Cisplatin and 1 or 2.5 mM Metformin compared to Cisplatin monotreatment (Figure 28D). Notably, this effect was accompanied by a dose-dependent decrease in p53 levels (Figure 28B). It is further noteworthy that the enhancement of Cisplatin-mediated apoptosis by Metformin co-treatment was not observed at higher Metformin doses (3.75 mM [HeLa] or 7.5 and 10 mM [SiHa]). At these concentrations, levels of cleaved PARP, cleaved Caspase 9, and t-BID remained low (Figure 28B) and light microscopy images revealed an only small number of apoptotic HeLa cells (Figure 28D). Overall, these findings show that pre-treatment with certain concentrations of Metformin can enhance the pro-apoptotic effects of Cisplatin.

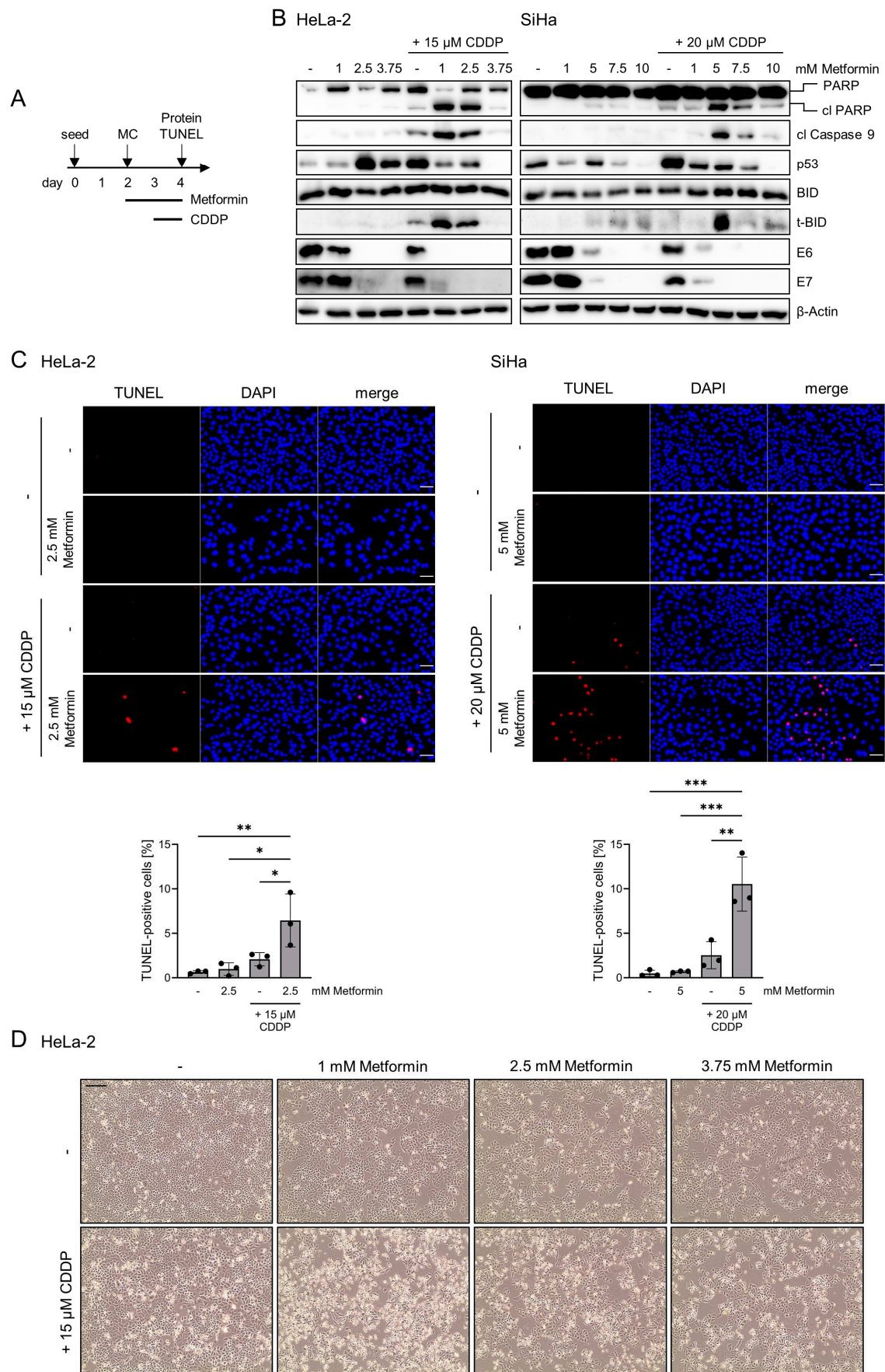


Figure 28. See Figure legend on the next page.

Figure 28. Pre-treatment with Metformin enhances Cisplatin-induced apoptosis in HPV-positive cancer cells.

(A) Treatment scheme: HeLa-2 or SiHa cells were treated with the indicated concentrations of Metformin for 48 h. Cisplatin (CDDP) was added for the last 24 h of treatment, if indicated. Subsequently, cells were harvested for protein and TUNEL analyses. MC, medium change. (B) Immunoblot analyses of PARP, cleaved (cl) PARP, cl Caspase 9, p53, BID, t-BID, E6, E7, and β -Actin protein levels. (C) Upper panels: TUNEL analyses (scale bar: 50 μ m). Lower panels: Quantification of TUNEL-positive (apoptotic) cells relative to the number of DAPI-stained cells. Results are represented as mean percentages with standard deviations ($n = 15$ fields of view, each with ≥ 50 cells, from three independent experiments). Statistically significant differences were assessed using one-way ANOVA with Tukey's post hoc test for multiple comparisons. Comparisons between Metformin + CDDP-treated cells and each other condition are indicated. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$. (D) Light microscopy images of HeLa-2 cells taken at the time of protein harvest with an EVOSxl Core Cell Imaging System at 10 $\times$ magnification (scale bar: 200 μ m). The Figure and Figure legend have been adapted from Avenhaus et al., *J Med Virol* (2025).³⁶⁶

2.2.4.3 The effects of Metformin on Cisplatin-induced apoptosis cannot be mimicked by glucose deprivation

As demonstrated above, the protective effects of Metformin against Etoposide-induced senescence were mimicked by glucose deprivation, likely due to the shared ability to induce growth arrest (Figure 25). This raised the question whether the anti-proliferative response to Metformin may also be involved in its interplay with Cisplatin and, for instance, could be the reason for Metformin's protective effects against Cisplatin-induced apoptosis.

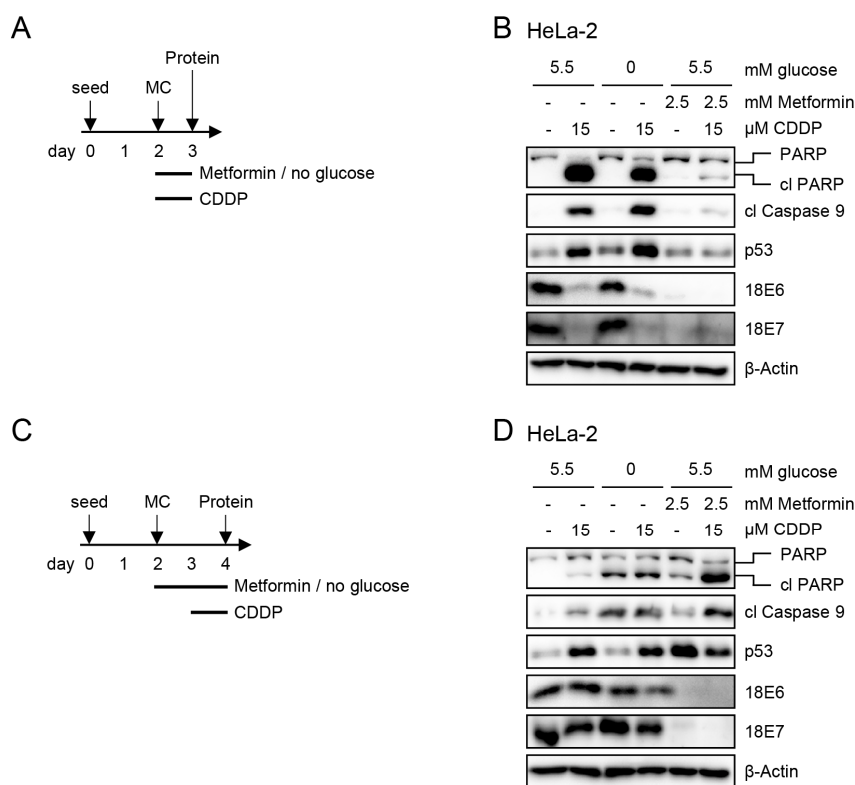


Figure 29. See Figure legend on the next page.

Figure 29. Glucose deprivation does not mimic the effects of Metformin on Cisplatin-induced apoptosis in HPV-positive HeLa cells.

(A) Treatment scheme for Subfigure (B): HeLa-2 cells were treated with 2.5 mM Metformin and/or 15 μ M Cisplatin (CDDP) and/or were cultivated in medium containing no glucose (0 mM) for 24 h. Subsequently, cells were harvested for protein analyses. MC, medium change. (B) Immunoblot analyses of PARP, cleaved (cl) PARP, cl Caspase 9, p53, 18E6, 18E7, and β -Actin protein levels. (C) Treatment scheme for Subfigure (D): HeLa-2 cells were treated with 2.5 mM Metformin or cultivated in medium containing no glucose (0 mM) for 48 h. CDDP at a final concentration of 15 μ M was added for the last 24 h of treatment, if indicated. Subsequently, cells were harvested for protein analyses. (D) Immunoblot analyses of PARP, cl PARP, cl Caspase 9, p53, 18E6, 18E7, and β -Actin protein levels. The Figure and Figure legend have been adapted from Avenhaus et al., *J Med Virol* (2025).³⁶⁶

Therefore, I investigated the impact of glucose withdrawal on apoptosis induction following Cisplatin treatment using the same experimental setups as described in the Chapters 2.2.4.1 and 2.2.4.2. Notably, in contrast to Metformin treatment, glucose deprivation neither prevented the Cisplatin-mediated increase in cleaved PARP, cleaved Caspase 9, and p53 protein levels when applied at the same time (Figure 29A,B), nor enhanced Cisplatin-induced apoptosis in HeLa cells cultivated in glucose-free medium 24 hours prior to Cisplatin treatment (Figure 29C,D).

In summary, these observations imply that Metformin treatment, but not glucose deprivation, can either prevent or enhance Cisplatin-induced apoptosis, depending on specific treatment conditions.

2.2.5 p53 and BID are critically involved in the effects of Metformin on Cisplatin-induced apoptosis in HPV-positive cancer cells

Curiously, while p53 levels were dose-dependently downregulated upon 24 hours of Metformin treatment (Figures 21A, 27B), I observed that after 48 hours of Metformin monotreatment, p53 levels were inconsistently regulated across doses in both HeLa and SiHa cells (Figure 28B). Since p53 is a pro-apoptotic factor, which has been described as an important player in Cisplatin-mediated apoptosis,^{155,166,388} and is discussed to play a role in the anti-cancer properties of Metformin,³⁸⁹⁻³⁹¹ I examined the role of p53 in the effects of Metformin, Cisplatin, and combinatorial treatment regimens in more detail.

2.2.5.1 Metformin induces apoptosis and dynamically regulates p53 over time

In order to analyze the regulation of p53 upon treatment with Metformin alone, I conducted dose-time kinetics in HeLa or SiHa cells (Figure 30A). p53 levels were not affected by treatment with 1 mM Metformin in both cell lines over time. Strikingly, upon higher doses of Metformin, p53 was downregulated at 24 hours of treatment but strongly upregulated after

48 hours (HeLa) or 72 hours (SiHa), respectively. This effect was most pronounced at 2.5 mM (HeLa) or 5 mM Metformin (SiHa). At higher doses, p53 was only slightly upregulated in HeLa cells, and even downregulated in SiHa cells. Further, these effects were similarly observed for post-translationally modified p53 forms (P-p53 Ser15 and Ac-p53 Lys382) and indicate that p53 levels can fluctuate over time in a dose-dependent manner. Moreover, as expected, E6 and E7 were efficiently downregulated at doses exceeding 1 mM Metformin. In line with previous work from our group,⁹² increasing doses of Metformin further induced apoptosis after 72 hours of treatment in both HeLa and SiHa cells, as evidenced by elevated levels of cleaved PARP at this time point. In SiHa cells, the induction of apoptosis upon Metformin treatment was consistently accompanied by increased levels of t-BID. This was also observed in HeLa cells treated with 2.5 mM Metformin, but not at higher concentrations (Figure 30A).

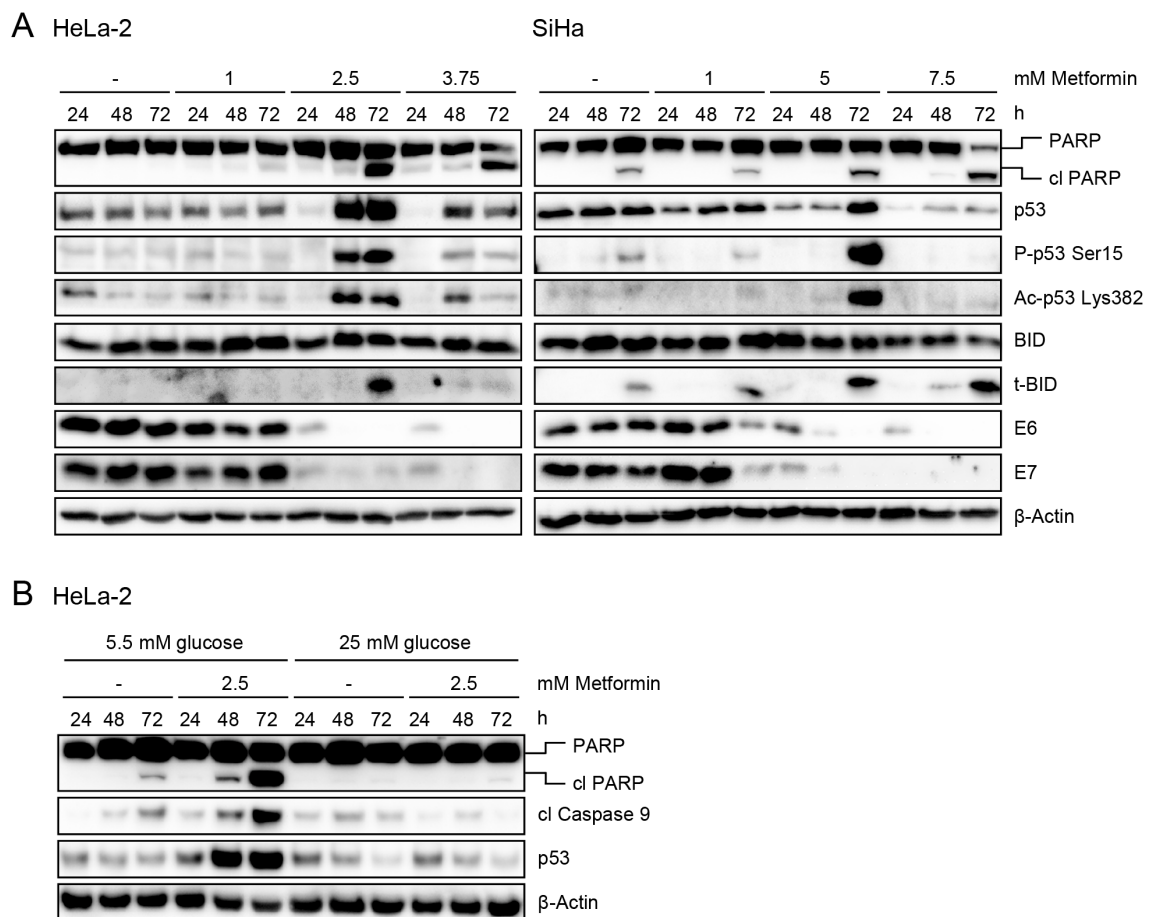


Figure 30. Metformin can decrease or increase p53 levels in HPV-positive cancer cells in a time-, dose-, and glucose-dependent manner.

(A) HeLa-2 or SiHa cells were treated with the indicated concentrations of Metformin for up to 72 h and examined by immunoblot for PARP, cleaved (cl) PARP, p53, P-p53 Ser15, Ac-p53 Lys382, BID, t-BID, E6, E7, and β-Actin protein levels. (B) HeLa-2 cells were cultivated under either 5.5 or 25 mM glucose in the cell culture medium, treated with 2.5 mM Metformin for up to 72 h, if indicated, and examined by immunoblot for PARP, cl PARP, cl Caspase 9, p53, and β-Actin protein levels. Subfigure (A) and the corresponding legend have been adapted from Avenhaus et al., *J Med Virol* (2025).³⁶⁶

Since the effects of Metformin are frequently described as glucose-dependent,^{92,392-394} I additionally investigated whether the impact of Metformin on p53 levels not only depends on the treatment duration but could also be influenced by the amount of glucose available. To address this, I compared the effects of Metformin on p53 levels in HeLa cells cultivated in medium containing 5.5 or 25 mM glucose, respectively. Both Metformin-induced apoptosis and the fluctuating p53 regulation were found to be glucose-dependent, as cultivation in 25 mM glucose abolished the Metformin-mediated increase in cleaved PARP and cleaved Caspase 9, as well as the initial downregulation and subsequent upregulation of p53 (Figure 30B).

Collectively, p53 levels can underlie strong fluctuations upon Metformin treatment, which depend on the Metformin concentrations used, the length of treatment, as well as glucose availability.

2.2.5.2 Apoptosis induction upon Metformin treatment depends on p53 and BID

As the upregulation of p53 and/or t-BID in response to Metformin treatment was accompanied by the induction of apoptosis in both HeLa and SiHa cells, I next assessed whether there is a causal link between the increase of those factors and apoptosis induction. To this end, the apoptosis induction upon Metformin treatment in control siRNA-transfected HeLa or SiHa cells was compared to that in cells where p53 was silenced using RNAi. Strikingly, the induction of apoptosis by Metformin, as indicated by upregulated levels of cleaved PARP and cleaved Caspase 9 (Figure 31A) as well as high numbers of TUNEL-positive cells (Figure 31B), was significantly reduced by repression of p53 in both cell lines. Furthermore, p53 silencing abolished the cleavage of BID upon Metformin treatment (Figure 31A). Likewise, siRNA-mediated silencing of BID efficiently reduced Metformin-induced apoptosis in SiHa cells, as indicated by a pronounced decrease in levels of cleaved PARP and cleaved Caspase 9 (Figure 31C). This effect, however, was only marginally observable in HeLa cells, indicating that BID may play a more critical role in Metformin-induced apoptosis in SiHa than in HeLa cells.

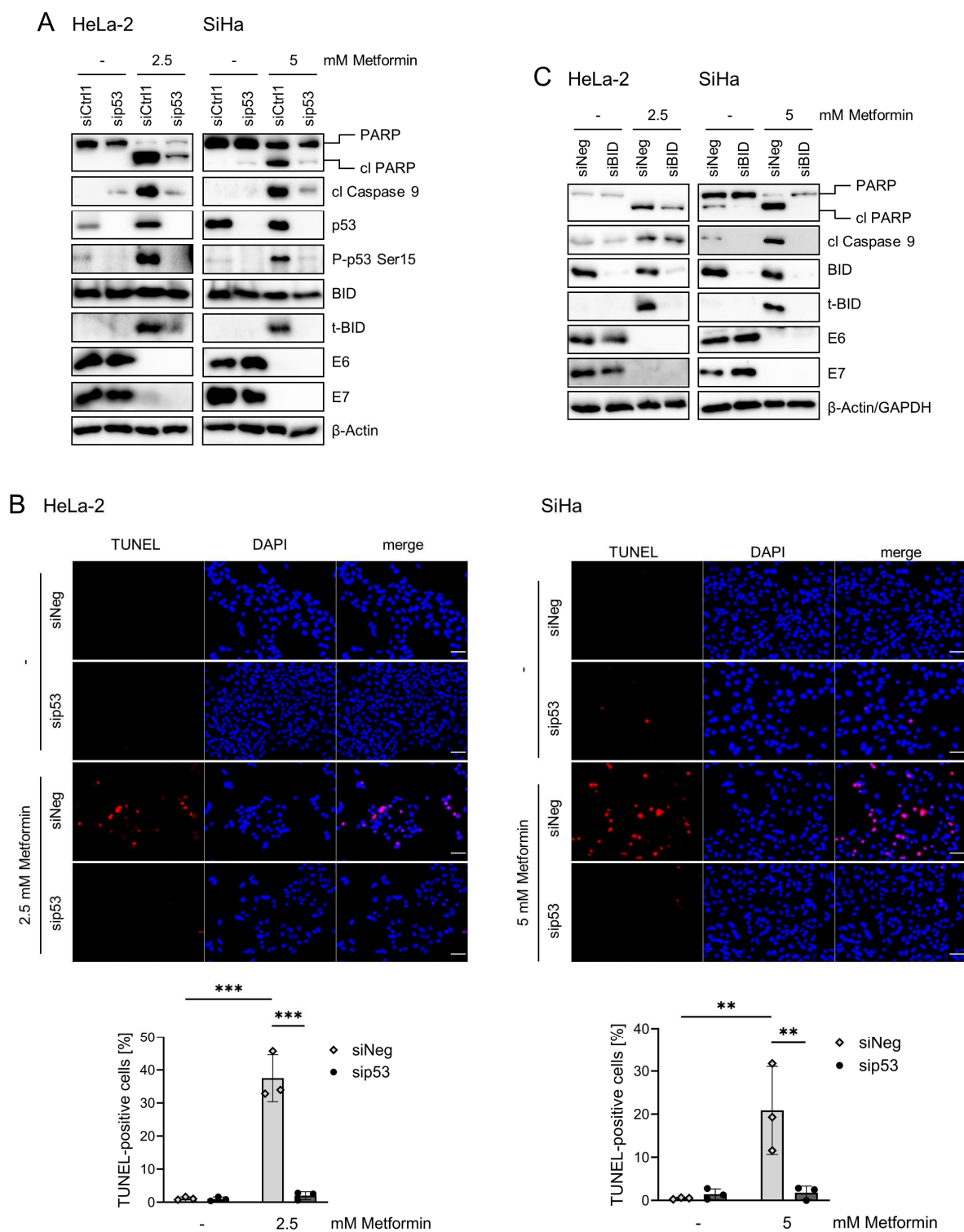


Figure 31. See Figure legend on the next page.

2.2.5.3 Metformin counteracts p53 induction, which is critical for Cisplatin-induced apoptosis

Next, I examined whether p53 is also essential for Cisplatin-induced apoptosis in HPV-positive cancer cells. Notably, Cisplatin-induced apoptosis, which was accompanied by an increase in the levels of cleaved PARP, cleaved Caspase 9, p53, P-p53 Ser15, Ac-p53 Lys382, and t-BID, was abrogated upon siRNA-mediated silencing of p53 in both HeLa and SiHa cells (Figure 32A). Similarly, as shown earlier in Figure 27B, simultaneous treatment of these cells with Cisplatin and Metformin for 24 hours according to the treatment scheme in Figure 27A inhibited the induction of apoptosis and efficiently counteracted the increase in p53 and t-BID levels induced by Cisplatin (Figure 32B). Moreover, the co-treatment also counteracted the increase in post-translationally modified p53 forms (P-p53 Ser15, Ac-p53 Lys382). This could indicate that Metformin exerts its protective effect against Cisplatin-induced apoptosis by inhibiting the increase in p53, which is essential for apoptosis induction upon Cisplatin treatment in HPV-positive cancer cells.

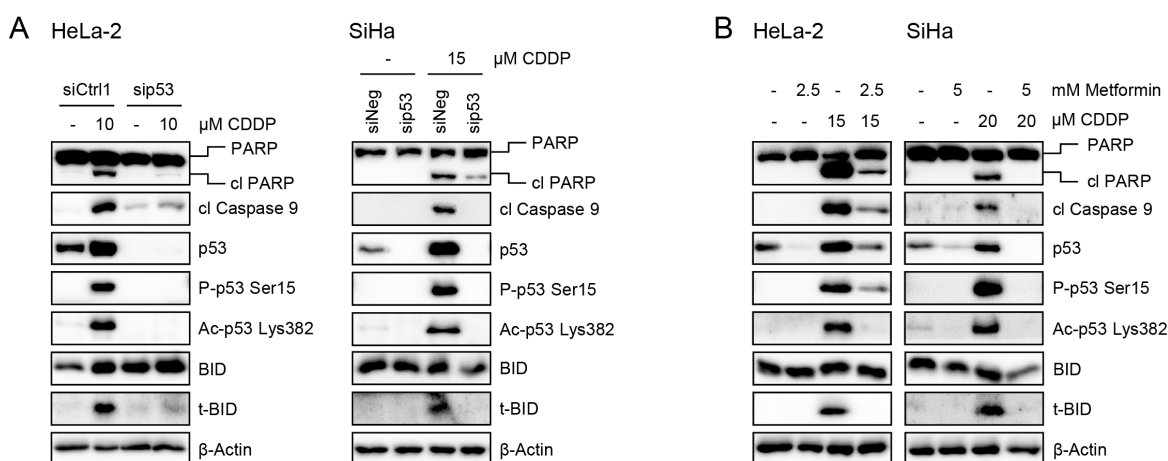
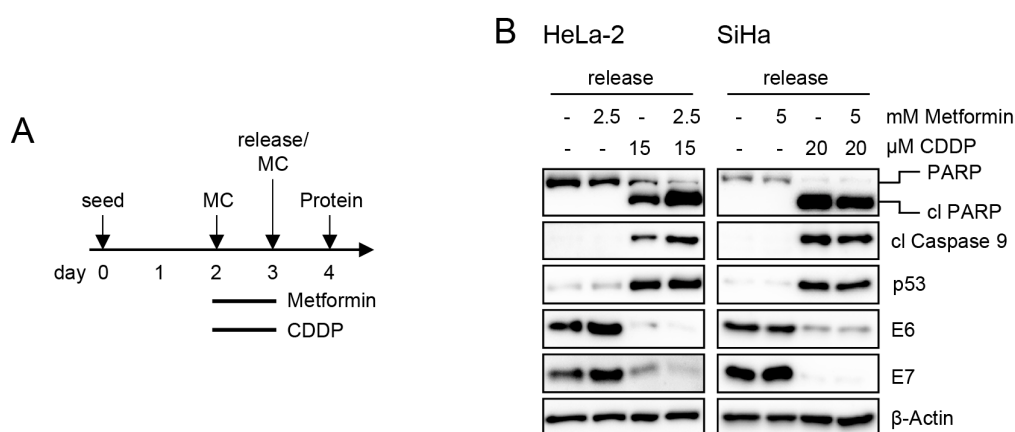


Figure 32. See Figure legend on the next page.

Figure 32. Metformin treatment counteracts p53 induction by Cisplatin in HPV-positive cancer cells.

(A) HeLa-2 or SiHa cells were transfected with sip53 or control siRNA (siCtrl1 [HeLa-2] or siNeg [SiHa]). Forty-eight hours after transfection, the medium was changed and cells were treated with the indicated concentrations of Cisplatin (CDDP) for 24 h. Cells were then examined by immunoblot for PARP, cleaved (cl) PARP, cl Caspase 9, p53, P-p53 Ser15, Ac-p53 Lys382, BID, t-BID, and β -Actin protein levels. (B) HeLa-2 or SiHa cells were simultaneously treated with the indicated Metformin and/or CDDP concentrations for 24 h following the treatment scheme depicted in Figure 27A, and examined by immunoblot for PARP, cl PARP, cl Caspase 9, p53, P-p53 Ser15, Ac-p53 Lys382, BID, t-BID, and β -Actin protein levels. The Figure and Figure legend have been adapted from Avenhaus et al., *J Med Virol* (2025).³⁶⁶

This hypothesis was further supported by the finding that the protective effect of Metformin against Cisplatin-induced apoptosis was lost when its inhibitory effect on p53 levels diminished. In specific, HeLa or SiHa cells were either treated with Metformin or Cisplatin alone, or co-treated with both drugs simultaneously for 24 hours. After treatment, the drugs were removed from the cell culture medium and the cells were cultured in drug-free medium for an additional 24 hours before harvesting (Figure 33A). Interestingly, immunoblot analyses revealed a strong increase in the levels of the apoptosis markers cleaved PARP and cleaved Caspase 9 not only following Cisplatin monotreatment, but also after Metformin and Cisplatin co-treatment (Figure 33B). The induction of apoptosis was accompanied by an upregulation of p53 levels in both cell lines. These results suggest that the protection conferred by Metformin against Cisplatin-mediated apoptosis, which was observed under similar experimental conditions when the cells were harvested directly at the end of treatment (Figures 27, 32), is transient and persists only as long as p53 remains suppressed by Metformin.

**Figure 33. The expression of apoptosis markers and p53 in HPV-positive cancer cells increases 24 hours after release from co-treatment with Metformin and Cisplatin.**

(A) Treatment scheme: HeLa-2 or SiHa cells were treated with the indicated concentrations of Metformin and/or Cisplatin (CDDP) for 24 h. Subsequently, the medium was replaced with drug-free medium (release), and the cells were cultivated for another 24 h before harvesting for protein analyses. MC, medium change. (B) Immunoblot analyses of PARP, cleaved (cl) PARP, cl Caspase 9, p53, E6, E7, and β -Actin protein levels. The Figure and Figure legend have been adapted from Avenhaus et al., *J Med Virol* (2025).³⁶⁶

2.2.5.4 Metformin enhances Cisplatin-induced apoptosis in a p53- and BID-dependent manner

Given the dependency of both Metformin- and Cisplatin-induced apoptosis on p53 and BID (Figures 31, 32A),¹⁶⁷ I further investigated whether the enhanced apoptosis rate upon Metformin and Cisplatin co-treatment is also dependent on these factors. HeLa or SiHa cells were transfected with sip53, siBID, or control siRNA and treated according to the scheme in Figure 34A. Strikingly, in both cell lines, the enhancement of Cisplatin-induced apoptosis by Metformin was both p53- and BID-dependent, as the inhibition of either factor led to a pronounced reduction in cleaved PARP and cleaved Caspase 9 levels (Figure 34B), as well as strongly decreased numbers of TUNEL-positive cells (Figure 35). Moreover, the increased expression of t-BID, which was observed upon Metformin and Cisplatin co-treatment, was diminished by concurrent silencing of p53 (Figure 34B).

Altogether, these data suggest that apoptosis induced by either Metformin or Cisplatin alone, as well as the enhanced apoptosis observed upon their combined application, depends on both p53 expression and cleavage of BID.

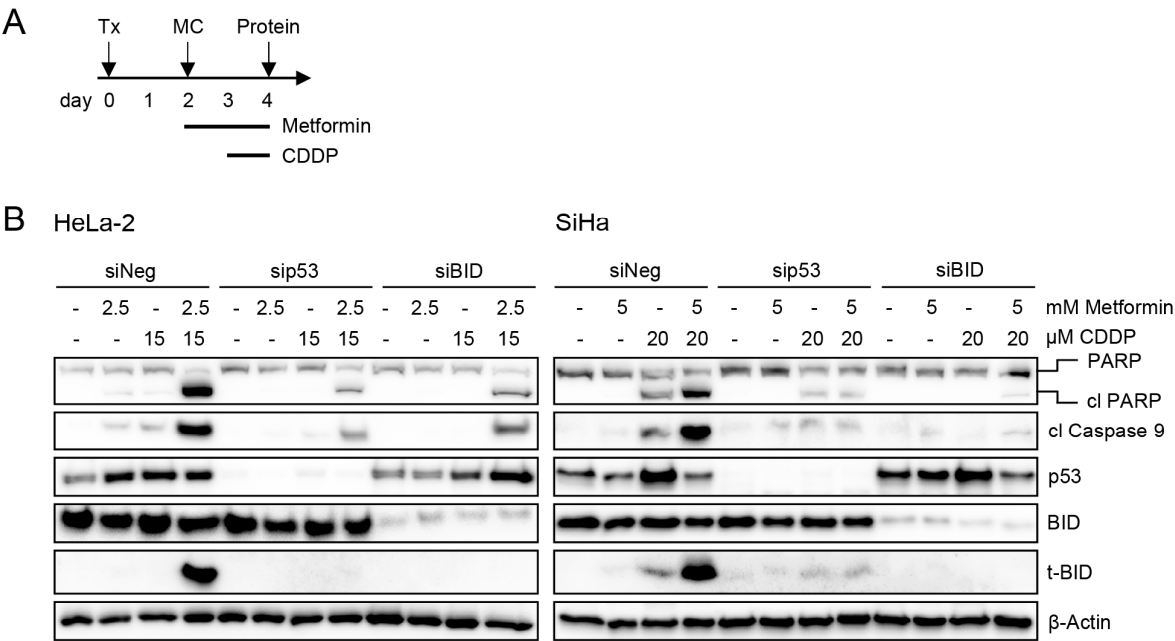


Figure 34. p53 and BID/t BID are critical for the enhancement of Cisplatin-induced apoptosis by Metformin in HPV-positive cancer cells: analyses of apoptosis markers by immunoblot.

(A) Treatment scheme: HeLa-2 or SiHa cells were transfected with sip53, siBID, or control siRNA (siNeg). Forty-eight hours after transfection (Tx), cells were treated with the indicated concentrations of Metformin for 48 h. Cisplatin (CDDP) was added for the last 24 h of treatment, if indicated. Subsequently, cells were harvested for protein analyses. MC, medium change. (B) Immunoblot analyses of PARP, cleaved (cl) PARP, cl Caspase 9, p53, BID, t-BID, and β-Actin protein levels. The Figure and Figure legend have been adapted from Avenhaus et al., *J Med Virol* (2025).³⁶⁶

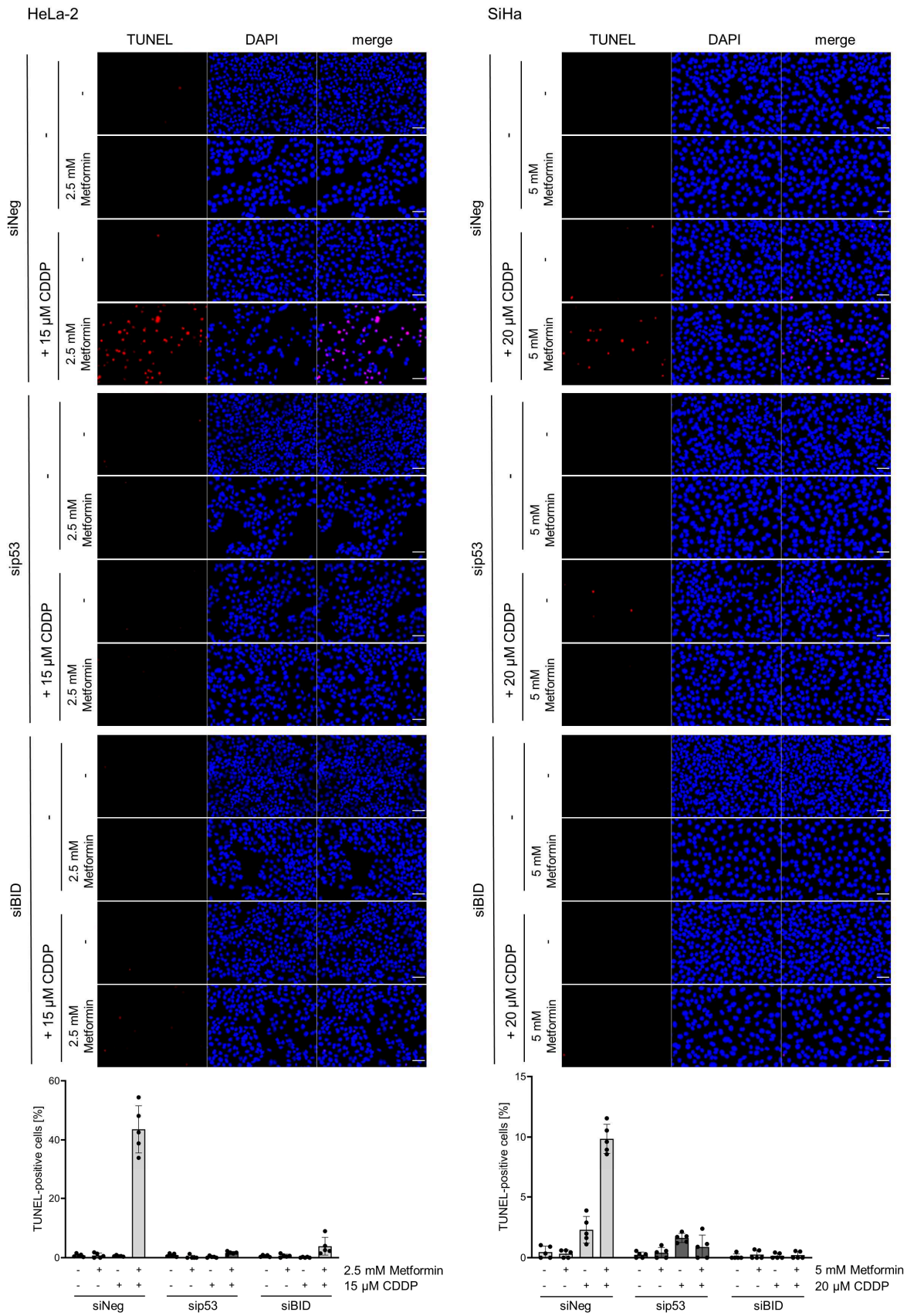


Figure 35. See Figure legend on the next page.

Figure 35. p53 and BID/t BID are critical for the enhancement of Cisplatin-induced apoptosis by Metformin in HPV-positive cancer cells: TUNEL analyses.

HeLa-2 or SiHa cells were transfected with sip53, siBID, or control siRNA (siNeg) and pre-treated with the indicated Metformin concentrations and subsequently, if indicated, treated with Cisplatin (CDDP), following the treatment scheme depicted in Figure 34A. Cells were harvested for TUNEL assays at the time point of protein harvest. Upper panels: TUNEL analyses (scale bar: 50 μ m). Lower panels: Quantification of TUNEL-positive (apoptotic) cells relative to the number of DAPI-stained cells. Results are represented as mean percentages with standard deviations ($n = 5$ fields of view, each with ≥ 50 cells, from one experiment).

2.2.6 The long-term growth inhibitory effects of Cisplatin can be enhanced by Metformin in both HPV-positive and HPV-negative cancer cells

As observed in short-term analyses, Metformin surprisingly appears to be able to either prevent or enhance Cisplatin-mediated apoptosis when cells are analyzed directly at the end of treatment. Still, it remains unclear whether these differential effects persist in the long term.

2.2.6.1 Metformin treatment does not confer long-term protection against Cisplatin-induced growth inhibition

To analyze the long-term effects of Metformin and Cisplatin co-treatment in the two experimental setups described in Chapter 2.2.4, I first treated HeLa or SiHa cells simultaneously with increasing doses of Metformin and/or Cisplatin for 24 hours and examined their phenotypic responses over time, following the treatment scheme in Figure 36A. Senescence and colony formation assays demonstrated a dose-dependent induction of senescence upon Cisplatin monotreatment in both cell lines. This was indicated by progressively increasing numbers of senescent and decreasing amounts of proliferating, non-senescent cells (Figure 36B), as well as reduced colony formation capacity (Figure 36C) with increasing doses of Cisplatin. These findings are in accordance with Cisplatin's pro-senescent potential.¹⁵⁶⁻¹⁵⁸ At the highest Cisplatin doses used (15 μ M [HeLa] or 20 μ M [SiHa]), a marked loss of viable cells was evident (Figure 36B), which is consistent with Cisplatin's pro-apoptotic effects under these treatment conditions (Figure 27). Interestingly, the protective effects that I observed when cells were harvested immediately after 24 hours of simultaneous treatment with Metformin and Cisplatin (Figure 27) did not persist over time. Instead, the combination of sublethal Cisplatin doses (1 μ M) with 2.5 mM (HeLa) or 5 mM Metformin (SiHa) even led to a further reduction in colony outgrowth compared to either drug alone (Figure 36C). At higher Cisplatin doses, which strongly impaired colony formation on their own, co-treatment with Metformin did not induce any detectable cooperative or protective effects (Figure 36B,C), being in line with the observation that the initial protective effect of Metformin is lost 24 hours after drug removal (Figure 33). Therefore, although Metformin co-treatment transiently protects

HPV-positive cancer cells against Cisplatin-induced apoptosis, it ultimately fails to confer long-term protection and may even enhance the cytotoxic effects of Cisplatin over time when both drugs are administered simultaneously.

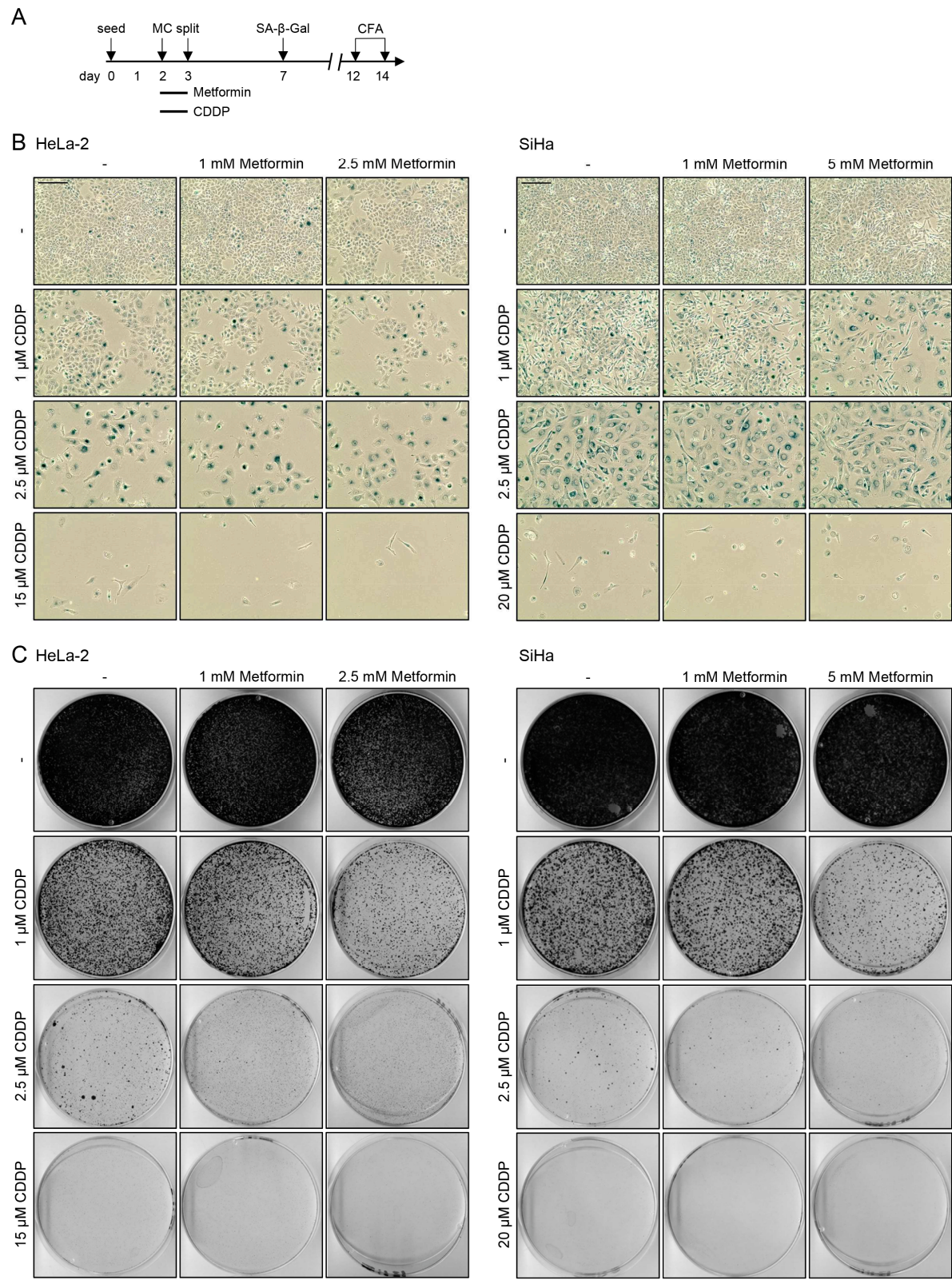


Figure 36. See Figure legend on the next page.

Figure 36. The anti-apoptotic effect of Metformin is only temporary and does not prevent Cisplatin-induced long-term growth inhibition in HPV-positive cancer cells.

(A) Treatment scheme: HeLa-2 or SiHa cells were treated with the indicated concentrations of Metformin and/or Cisplatin (CDDP) for 24 h. Subsequently, cells were split and further cultivated in drug-free medium for senescence assays (SA- β -Gal staining) or colony formation assays (CFAs) after the indicated time periods. MC, medium change. Corresponding (B) senescence assays (SA- β -Gal staining, blue; scale bar: 200 μ m) and (C) CFAs. The Figure and Figure legend have been adapted from Avenhaus et al., *J Med Virol* (2025).³⁶⁶

2.2.6.2 Metformin enhances the growth-inhibitory effects of Cisplatin and induces a shift from senescence to cell death

Next, I aimed to determine the long-term consequences of Metformin and Cisplatin combination following Metformin pre-treatment, a condition that led to enhanced induction of apoptosis in the short term (Figure 28). To this end, HeLa or SiHa cells were treated following the scheme in Figure 37A. Cisplatin monotreatment primarily acted pro-senescent, as reflected by increasing numbers of senescent cells (Figure 37B) as well as reduced colony outgrowth in CFAs (Figure 37C) with increasing doses of Cisplatin. Remarkably, co-treatment with Metformin led to pronounced cooperative effects, as it induced a shift from proliferation to senescence and from senescence towards apoptosis. Specifically, combining 1 mM Metformin with sublethal doses of Cisplatin (2.5 μ M) resulted in a shift from proliferation to senescence (Figure 37B) and a corresponding substantial reduction in colony formation capacity (Figure 37C). When higher concentrations of Metformin (2.5 mM [HeLa] or 5 mM [SiHa]) were combined with sublethal Cisplatin doses, a pronounced reduction in overall cell numbers (Figure 37B) and outgrowing colonies (Figure 37C), as well as a marked increase in levels of cleaved PARP (Figure 37D) were observed, indicating the induction of apoptosis. Furthermore, the combination of high-dose Cisplatin with only 1 mM Metformin efficiently shifted the cellular response from Cisplatin-induced senescence to apoptosis. This was evidenced by a strong reduction in overall cell numbers in both HeLa and SiHa cells (Figure 37B) and elevated levels of cleaved PARP in HeLa cells at these concentrations (Figure 37D). Interestingly, I found that treatment with 15 μ M Cisplatin led to an upregulation of BCL-xL, an anti-apoptotic member of the BCL-2 protein family,²³⁰ in this treatment condition, whereas concurrent Metformin treatment counteracted the induction of BCL-xL in HeLa cells (Figure 37E). Moreover, protein levels of p21 were reduced upon Metformin and Cisplatin co-treatment. This could suggest an involvement of both BCL-xL and p21 in the Metformin-mediated shift from Cisplatin-induced senescence to apoptosis, which warrants further exploration.

Taken together, my data indicate that, irrespective of the specific experimental setup, co-treatment with Metformin enhances Cisplatin-mediated long-term growth inhibition in HPV-positive cancer cells. Yet, Metformin pre-treatment appears to be particularly effective, as it

not only enhances the induction of either senescence or apoptosis, but also promotes a phenotypic shift from proliferation to senescence and from senescence to cell death.

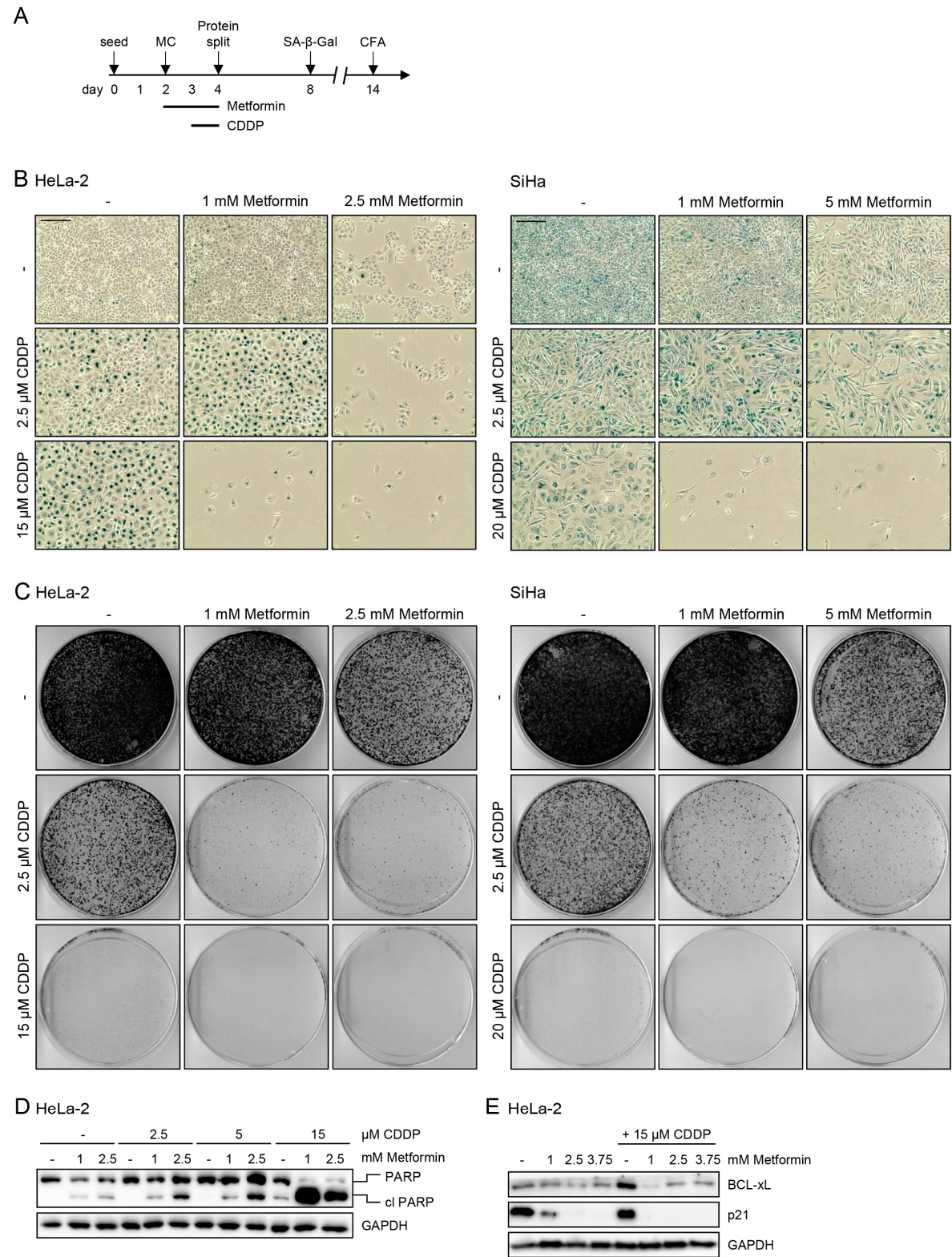


Figure 37. See Figure legend on the next page.

Figure 37. Metformin pre-treatment enhances the Cisplatin-induced long-term growth inhibition in HPV-positive cancer cells.

(A) Treatment scheme for Subfigures (B-D): HeLa-2 or SiHa cells were treated with the indicated concentrations of Metformin for 48 h. Cisplatin (CDDP) was added for the last 24 h of treatment, if indicated. Subsequently, cells were either harvested for protein analyses (HeLa-2) or split (HeLa-2 and SiHa) and further cultivated in drug-free medium for senescence assays (SA- β -Gal staining) or colony formation assays (CFAs) after the indicated time periods. MC, medium change. Corresponding (B) senescence assays (SA- β -Gal staining, blue; scale bar: 200 μ m), (C) CFAs, and (D) immunoblot analyses of PARP, cleaved (cl) PARP, and GAPDH protein levels. (E) HeLa-2 cells were pre-treated with the indicated Metformin concentrations and subsequently, if indicated, treated with CDDP, following the treatment scheme depicted in Figure 28A. Cells were then examined by immunoblot for BCL-xL, p21, and GAPDH protein levels. Subfigures (A-C) and their corresponding legends have been adapted from Avenhaus et al., *J Med Virol* (2025).³⁶⁶

2.2.6.3 Cisplatin and Metformin cooperate to impair the long-term growth of HPV-negative cancer cells

Several *in vitro* studies report discrepant effects of co-treatment with Metformin and Cisplatin across different cell lines.^{318,319,321-327} In view of the distinct short- and long-term outcomes that I observed in HPV-positive cancer cells following the combination treatment, I investigated whether these effects also occur in HPV-negative cancer cells.

To this end, I treated four HPV-negative cell lines, derived from various histopathological origin, following the different experimental setups described in Chapter 2.2.4. U2OS osteosarcoma, HCT116 and RKO colon cancer, or A549 non-small-cell lung cancer cells were first simultaneously treated with Metformin and/or Cisplatin according to the treatment scheme in Figure 27A. Similar to the response in HPV-positive cancer cells (Figure 27), co-treatment with Metformin and Cisplatin resulted in reduced levels of cleaved PARP and cleaved Caspase 9 compared to Cisplatin monotreatment (Figure 38A), indicating that Metformin can also protect HPV-negative cancer cells from Cisplatin-induced apoptosis. This response was accompanied by a moderate reduction in p53 protein levels in all four cell lines. Next, U2OS, HCT116, RKO, or A549 cells were pre-treated with Metformin before adding Cisplatin (following the scheme in Figure 28A), a condition in which Metformin enhanced Cisplatin-induced apoptosis in HPV-positive cancer cells at certain concentrations (Figure 28). Notably, in contrast to the response observed in HPV-positive cancer cells, all four HPV-negative cell lines remained consistently protected against Cisplatin-induced apoptosis by Metformin at the tested concentrations in this experimental setup. This was reflected by lower levels of cleaved PARP and cleaved Caspase 9, as well as strongly reduced levels of p53 upon the co-treatment, compared to Cisplatin monotreatment (Figure 38B).

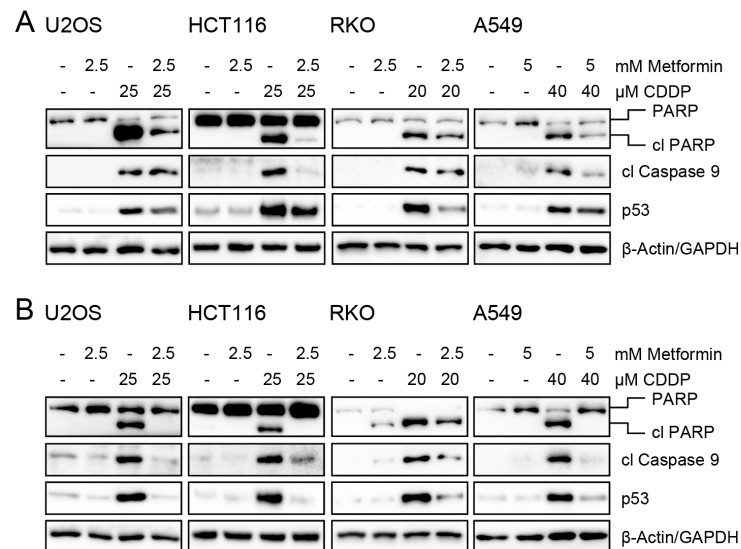


Figure 38. Metformin acts anti-apoptotic when combined with Cisplatin in HPV-negative cancer cells.

(A) U2OS, HCT116, RKO, or A549 cells were simultaneously treated with the indicated Metformin and/or Cisplatin (CDDP) concentrations for 24 h following the treatment scheme depicted in Figure 27A, and examined by immunoblot for PARP, cleaved (cl) PARP, cl Caspase 9, p53, and β -Actin (U2OS, HCT116, RKO) or GAPDH (A549) protein levels. (B) U2OS, HCT116, RKO, or A549 cells were pre-treated with the indicated Metformin concentrations and subsequently, if indicated, treated with CDDP, following the treatment scheme depicted in Figure 28A. Cells were then examined by immunoblot for PARP, cl PARP, cl Caspase 9, p53, and β -Actin (U2OS, HCT116, RKO) or GAPDH (A549) protein levels.

Subsequently, I analyzed the long-term effects of co-treatment with increasing doses of Metformin and Cisplatin in U2OS cells. First, U2OS cells were simultaneously treated with Metformin and/or Cisplatin following the treatment scheme in Figure 36A. Consistent with the findings in HPV-positive cells (Figure 36), the protective effect observed when analyzing the cells directly after the end of treatment (Figure 38A) did not translate into long-term protection against Cisplatin-induced growth-inhibition. In specific, both senescence assays (Figure 39A) and CFAs (Figure 39B) revealed a moderate enhancement of the pro-senescent response to 2.5 μ M Cisplatin upon Metformin co-treatment compared to Cisplatin monotreatment. Moreover, Metformin did not protect the cells from the strong cytotoxic effects observed upon treatment with 25 μ M Cisplatin in this experimental setup. Notably, although pre-treatment with Metformin also protected U2OS cells from Cisplatin-induced apoptosis when the cells were analyzed immediately after the end of treatment (Figure 38B), this protective effect was again lost over time. U2OS cells that were pre-treated with Metformin (following the scheme in Figure 37A) showed a similar enhancement of Cisplatin-induced senescence and apoptosis, as well as a shift from senescence to cell death (Figure 39C) as seen in HPV-positive cancer cells (Figure 37B). This was further supported by CFAs, showing a strong dose-dependent

enhancement of Cisplatin-induced growth inhibition when sublethal Cisplatin doses were combined with Metformin (Figure 39D).

Collectively, these findings demonstrate that, despite consistently providing a protection against Cisplatin-induced apoptosis directly at the end of treatment, Metformin enhances the Cisplatin-induced growth inhibitory effects over time also in HPV-negative cancer cells. This indicates that the cooperative anti-proliferative effects of Metformin and Cisplatin co-treatment are not limited to HPV-positive cancer cells, but reflect a phenotypic response, which is also observed in HPV-negative cancer cells.

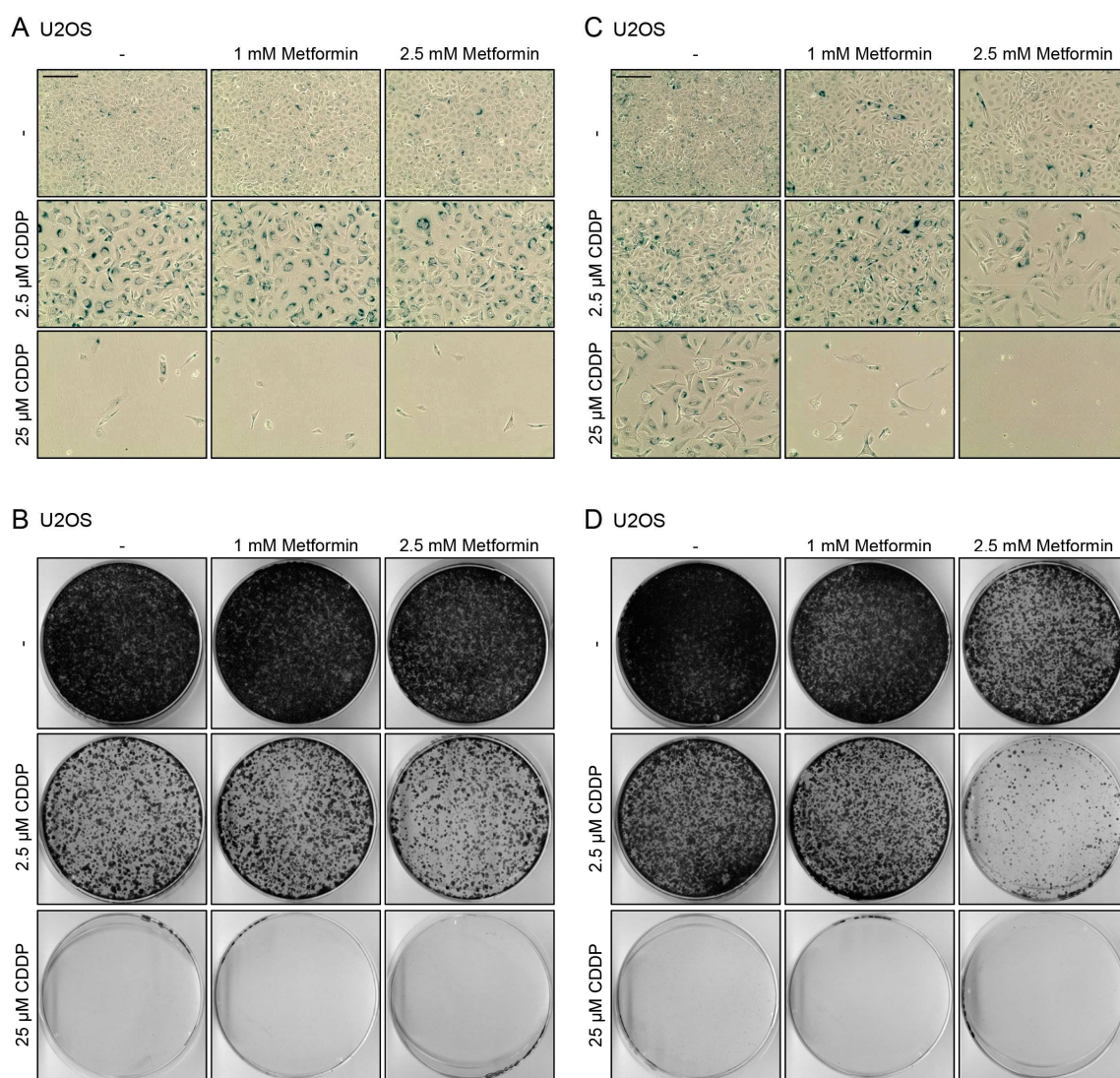


Figure 39. Metformin and Cisplatin cooperate to inhibit the long-term growth of HPV-negative U2OS cells.

(A,B) U2OS cells were simultaneously treated with the indicated Metformin and/or Cisplatin (CDDP) concentrations for 24 h and examined following the treatment scheme depicted in Figure 36A. Corresponding (A) senescence assays (SA-β-Gal staining, blue; scale bar: 200 μm) and (B) colony formation assays are shown. (C,D) U2OS cells were pre-treated with the indicated Metformin concentrations, subsequently, if indicated, treated with CDDP, and examined following the treatment scheme depicted in Figure 37A. Corresponding (C) senescence assays (SA-β-Gal staining, blue; scale bar: 200 μm) and (D) colony formation assays are shown.

CHAPTER 3

DISCUSSION

3 Discussion

Effective prophylactic vaccines against HPV, including those targeting high-risk types, have been available for more than 15 years. Nevertheless, HPV infections continue to cause about 690 000 cancer cases annually, and their treatment remains challenging.^{117,149} Although the viral *E6/E7* oncogenes have long been considered promising HPV-specific therapeutic targets, strategies to interfere with *E6/E7* expression or function have not been successfully implemented in the clinic thus far.^{69,75} This underscores the need to identify alternative therapeutic targets and novel treatment strategies capable of interfering with the growth of HPV-positive cancer cells. The experiments conducted in this thesis identified E6AP as a potential novel therapeutic target in HPV-positive cancer cells that plays an essential role in their proliferative capacity. Furthermore, they provide evidence that the anti-diabetic drug Metformin – a known inhibitor of *E6/E7* expression with debated potential for cancer therapy^{92,305} – may have therapeutic value in HPV-positive malignancies, particularly when combined with Cisplatin, a standard chemotherapeutic agent in the treatment of cervical cancer.^{147,148}

3.1 E6AP as a potential therapeutic target in HPV-positive cancer cells

To exert their transforming activities, the HPV *E6* and *E7* oncoproteins interact with multiple host proteins, some of which may also serve as therapeutic targets.³⁹⁵ One of the well-established interaction partners of the *E6* oncoprotein is the cellular E3 ubiquitin ligase E6AP. Through its interaction with *E6*, E6AP mediates the proteasomal degradation of the anti-proliferative and pro-apoptotic tumor suppressor p53, thereby promoting continuous cellular growth.^{43,69} Several studies have reported that E6AP inhibition can trigger a pro-apoptotic response in HPV-positive cancer cells, but the amount of apoptosis induction differs between studies.^{94,98-100} Notably, however, the fates of cells surviving this pro-apoptotic stimulus have remained unexplored. Strikingly, my experiments showed that cells surviving the initial pro-apoptotic stimulus of E6AP silencing cease to proliferate and efficiently undergo cellular senescence. This senescence response was not observed in HPV-negative cancer cells, suggesting that E6AP may function as a therapeutic target that is specific for HPV-positive cancer cells.

3.1.1 Comparison of the cellular consequences resulting from E6AP, E6/E7, or E6 silencing

To evaluate the therapeutic potential of E6AP inhibition as an alternative to targeting the viral *E6/E7* oncogenes, I conducted detailed comparative analyses of the phenotypic consequences and underlying mechanisms following E6AP, E6/E7, or E6 silencing. Similar to E6AP depletion, silencing of E6 acts pro-apoptotic in HPV-positive cancer cells, but the reported extent of apoptosis induction is also inconsistent between studies.^{80-83,93-97} One possible explanation for these discrepancies could be my finding that the partial pro-apoptotic response to both E6AP and E6 silencing is glucose-dependent. Specifically, the induction of apoptosis following E6AP or E6 inhibition was strongly impaired in cells growing in medium containing 25 mM glucose (concentrations that are often employed in cell culture) compared to 5.5 mM glucose (corresponding to physiological human blood glucose levels), an effect that may contribute to the conflicting reports in the literature. Further, the observed increase in apoptosis under lower glucose supply might be clinically relevant, since glucose levels in the tumor interstitium have been reported to be significantly lower (up to 10-fold) than in blood or healthy tissue.³⁹⁶

Remarkably, my data showed that the fates of cells surviving the pro-apoptotic stimulus of either E6AP or E6 repression can differ profoundly. While E6AP-depleted cells efficiently underwent senescence, cells escaping the pro-apoptotic effects of E6 silencing continued to proliferate after a transient growth delay. This is especially interesting given that siRNA-mediated silencing of E6AP led to a concomitant downregulation of E6, in line with previous studies showing that E6 stability critically depends on the presence of E6AP.^{100,361,362} This raised the theoretical possibility that senescence observed upon E6AP silencing could, at least partially, be supported by E6 downregulation. Yet, even upon repeated transfections or the use of higher concentrations of E6-targeting siRNAs, only limited senescence was observed upon E6 depletion, while E6AP levels remained unaffected. These findings suggest that E6 repression alone is insufficient to induce a strong senescence response in HPV-positive cancer cells.

Moreover, although the efficiency of senescence induction observed upon E6AP silencing was comparable to that of the well-established pro-senescent response following E6/E7 inhibition,⁷¹⁻⁷³ my data indicate that the associated cell cycle regulation differs fundamentally. As expected, silencing of E6/E7 induced a highly efficient G1 arrest in HPV-positive cancer cells.^{207,365} This was accompanied by a pronounced downregulation of the pRb and/or DREAM targets B-MYB, FOXM1, E2F1, Cyclin A, Cyclin B1, CDC2, CDK2, and CKS1. These findings can be explained by the ability of E7 to interfere with both pRb and the DREAM complex components p130 and p107.^{52,53,58} Consequently, upon E7 inhibition, the repressor activities

of pRb-E2F and DREAM complexes are restored, thereby leading to the transcriptional repression of various cell cycle-promoting genes.^{56,58,201} In strong contrast, my results suggest that the anti-proliferative effect of E6AP silencing is linked to a deceleration of cell cycle progression across multiple cell cycle phases. In particular, senescence induction upon E6AP depletion was not associated with an accumulation of cells in a distinct cell cycle phase, and the expression levels of pRb and/or DREAM targets were only marginally affected, if at all, although efficient growth arrest was induced. Since many genes regulated by pRb and the DREAM complex are expressed in a phase-specific manner during the cell cycle,^{56,58,201} the slowdown of cell cycle progression in various cell cycle phases could explain why their downregulation is not readily detectable.

3.1.2 Mechanisms underlying the senescence induction upon E6AP silencing

Besides the observed differences in cell cycle regulation, my studies imply distinct mechanisms underlying the pro-senescent responses following E6AP or E6/E7 silencing, as they differ substantially in their dependence on the p53-p21 axis. While E6/E7 silencing induced senescence largely independently of the p53-p21 axis, E6AP silencing triggered a pro-senescent response that was strongly p53- and p21-dependent. Interestingly, there is evidence that p21 can mediate cell cycle arrest in G1, S, and G2 phases,^{397,398} and that cells can enter a senescent state not only from G1 but also from G2 in a p21-dependent manner.²⁰⁹ This may provide a mechanistic explanation for my observation that growth arrest and senescence induction upon E6AP silencing, in contrast to E6/E7 silencing, were not linked to a pronounced G1 arrest but rather to an arrest in different cell cycle phases. Importantly, my data further revealed that, despite the observed upstream differences, the pro-senescent responses upon both E6AP and E6/E7 silencing ultimately require the activity of the pocket proteins pRb and p130. This is highly remarkable since E7, which is known to interfere with pocket protein function,^{52,53} remains expressed upon E6AP depletion. Thus, although E7 would be expected to inactivate pRb and p130, their activity appears to be necessary for senescence induction following E6AP inhibition.

Notably, the strong activation of the p53-p21 axis appears to represent a key driver of senescence upon E6AP silencing and could potentially explain the phenotypic and mechanistic differences compared to E6/E7 or E6 silencing. In specific, the pronounced induction of the potent CDK inhibitor p21^{399,400} following E6AP inhibition likely contributes to reduced phosphorylation of the pocket proteins pRb and p130,^{56,401,402} thereby promoting senescence via activation of the pRb-E2F and DREAM complexes (Figure 40).^{56,201,403} Supporting this hypothesis, my data showed that E6AP depletion leads to decreased levels of hyperphosphorylated (inactive) pRb in a p53- and p21-dependent manner. This mechanistic

model is further reinforced by my observation that E6AP silencing results in substantially higher protein levels of p53 (both total and post-translationally modified forms) and p21, as well as higher transcript levels of the p21-encoding *CDKN1A* gene, a transcriptional target of p53,²⁵⁰ compared to E6/E7 or E6 silencing. This could be explained by the concomitant suppression of both E6AP and E6 following E6AP silencing. In contrast, E6AP remains expressed when only E6 or E6/E7 are silenced, which may allow residual E6 protein to associate with E6AP, thereby possibly promoting continued p53 degradation – a pathway that might be more strongly inhibited when E6AP and E6 are downregulated simultaneously upon E6AP silencing. Consequently, the increased levels of hypophosphorylated (active) pocket proteins following the particularly strong activation of p53 and p21 upon E6AP silencing may be sufficient to override the potential of E7 to interfere with pocket protein activity. This could explain how E6AP silencing is able to induce senescence despite the continued expression of the potentially anti-senescent E7 protein.⁵¹

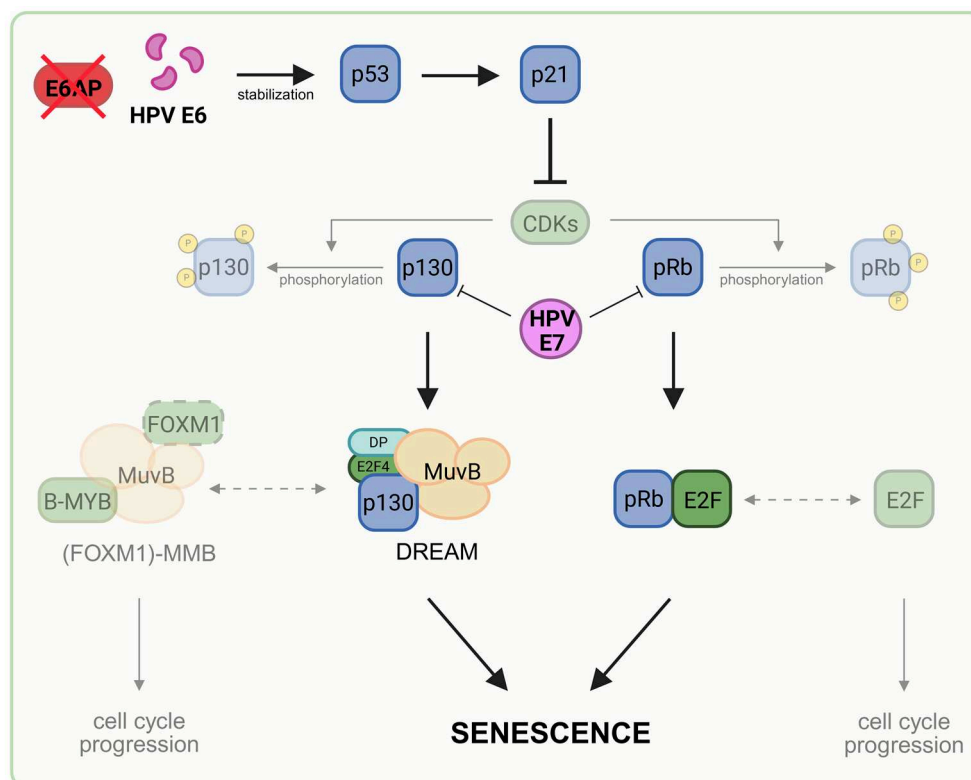


Figure 40. Proposed model of the mechanisms underlying the pro-senescent effects of E6AP silencing in HPV-positive cancer cells.

Silencing of E6AP in HPV-positive cancer cells abolishes the formation of the trimeric E6AP/E6/p53 complex and destabilizes E6. This strongly increases the levels of p53 and p21, and subsequently inhibits the phosphorylation of the pocket proteins pRb and p130 through p21-mediated suppression of CDKs. Both pRb and p130 play a critical role for senescence induction upon E6AP silencing, as they are key components of the repressive pRb-E2F and DREAM complexes, thereby preventing cell cycle progression mediated by free E2Fs or MMB/FOX M1-MMB complexes. Created with BioRender.com. The Figure and Figure legend have been adapted from Avenhaus et al., *PLOS Pathog* (2025).⁷⁰

In contrast to the scenario discussed above, E7 expression is inhibited upon E6/E7 silencing, thereby releasing pRb and p130 from their inhibitory interactions with E7 and allowing them to induce the efficient repression of cell cycle genes.⁵⁸ In support of this notion, my results demonstrated that combined repression of pRb and p130 reversed the downregulation of multiple pRb/DREAM targets following E6/E7 repression. Thus, it is conceivable that the direct reactivation of the pocket proteins through loss of E7 drives senescence upon E6/E7 silencing, while activation of the upstream p53-p21 axis is, in contrast to senescence upon E6AP silencing, largely dispensable in this scenario. Additionally, the proposed mechanism underlying senescence following E6AP silencing might provide a rationale for why E6 silencing alone fails to trigger senescence. Although E6 knockdown leads to the stabilization of p53, it may not result in a sufficiently strong activation of the p53-p21 axis to override the continued inhibitory effects of E7 on pRb and p130 – possibly because of the sustained expression of E6AP.

Of note, while E6AP silencing efficiently triggered senescence in HPV-positive cancer cells, I did not observe senescence induction upon E6AP repression in HPV-negative, p53 wild-type cancer cells. This specificity likely reflects the distinct mechanisms of p53 degradation in these cells: in HPV-positive cancer cells, p53 is targeted by the E6AP/E6 complex, whereas in HPV-negative cancer cells, p53 degradation is mediated by other E3 ligases, most notably Mdm2.^{111,112} As a result, only HPV-positive cancer cells respond to E6AP silencing with a pronounced p53 stabilization and activation of the senescence-associated p53-p21 axis, which could explain the lack of senescence induction in HPV-negative cancer cells.

It is further important to highlight that the E6AP/E6 complex targets substrates other than p53,^{44,114,116} and E6AP alone also has numerous cellular ubiquitination substrates.^{107,404} Thus, despite the observed strong dependency on the p53-p21 axis, my data cannot rule out that additional E6AP-mediated mechanisms, independent of its role in p53 degradation, might contribute to the induction of senescence upon E6AP silencing. For instance, the E6AP/E6 complex promotes the degradation of NHERF1, a negative regulator of the Wnt/ β -catenin signaling pathway, which can interfere with the induction of senescence.^{44,405,406} Moreover, E6AP mediates proteasomal degradation of the tumor suppressor PML, which has also been implicated in the regulation of senescence.^{104,407} In line with this, downregulation of E6AP has been reported to elevate PML expression and induce senescence in human Burkitt lymphoma derived cell lines and prostate cancer xenograft models.^{408,409} This might indicate that E6AP is involved in senescence control in certain HPV-negative cancer cells, but stands in contrast to my analyses, which consistently showed no signs of senescence induction upon E6AP silencing in an arbitrary selection of HPV-negative cancer cells of different histological backgrounds. Further studies with larger collections of cell lines are required to clarify this

issue. Finally, although HPV E7 protein levels were not affected by E6AP silencing, it cannot be formally excluded that E6AP silencing interferes with the anti-senescent activity of E7 without reducing its expression – for example through modulation of PTMs, such as polyamination or phosphorylation, which are known to regulate E7 function.⁴¹⁰⁻⁴¹²

3.1.3 Clinical potential of targeting E6AP

My experiments revealed that the growth of HPV-positive cancer cells depends not only on the continuous expression of the viral *E6/E7* oncogenes, but also on the presence of E6AP. These findings carry important clinical implications, as they identify E6AP as a potential host-derived therapeutic target with specificity for HPV-positive cancer cells and provide a rationale for the future development of strategies to inhibit E6AP.

Targeting E6AP could offer several advantages over direct E6/E7-targeting strategies. First, inhibition of E6AP simultaneously destabilizes E6, which appears to be more effective than silencing E6 alone since p53 is upregulated more efficiently. Furthermore, it not only promotes apoptosis, but ultimately blocks the growth of surviving cancer cells by efficiently inducing senescence. Second, therapeutic approaches directly targeting E6/E7 may be HPV type-restricted due to the heterogeneity of oncogenic HPV-types. In contrast, cellular E6AP interacts with E6 proteins of different high-risk HPV types,⁴² making E6AP inhibition an HPV type-independent therapeutic approach that might even be beneficial in multiple-type infections.⁴¹³ Third, because E7 remains expressed upon E6AP silencing, it could be targeted in parallel, for example by HPV-specific, E7-directed immunotherapeutic strategies.¹³⁵

An alternative strategy to targeting E6AP directly could involve drug-mediated disruption of the interactions within the E6AP/E6/p53 complex, a feasible approach since the crystal structure of the complex was solved.^{109,414} Importantly, my data suggest that such strategies may only be effective if they induce sufficiently strong p53 upregulation, as senescence induction upon E6AP repression critically depends on robust activation of the p53-p21 axis.

Nevertheless, it is important to note that the therapeutic use of senescence induction should be generally approached with caution. While therapy-induced senescence has emerged as a promising strategy for cancer treatment,^{415,416} experimental evidence also shows that senescent tumor cells, while being growth arrested, may still possess potential to exert cancer-promoting activities, as detailed in Chapter 1.5.2. Moreover, it also should be considered that targeting cellular proteins, such as E6AP, inherently carries the risk to exert unwanted effects in non-target cells. Although my data did not reveal pronounced effects of E6AP silencing in HPV-negative cells, this potential limitation has to be carefully addressed in future studies, which should also include analyses in untransformed and primary cells.

In this context, it is further interesting that my results revealed that Metformin can counteract p53 upregulation and senescence induction in response to E6AP silencing. Thus, the administration of Metformin or other drugs with anti-senescent properties could possibly interfere with therapies targeting E6AP, as well as with other pro-senescent treatment strategies. This is especially noteworthy since Metformin is widely used and is currently being investigated for its potential in cancer therapy.³⁰⁵

3.2 Metformin exerts pleiotropic effects on the phenotype and chemotherapy response in HPV-positive cancer cells

The repurposing potential of Metformin for the treatment of various cancer types, including cervical cancer,^{350,352,355} has been discussed for many years, but preclinical and clinical studies have yielded controversial results.^{278,305,317} Previous work from our group demonstrated that Metformin efficiently inhibits viral E6/E7 expression and induces reversible growth suppression in HPV-positive cancer cells.⁹² Thus, Metformin may possess potential for targeting HPV-related malignancies. However, despite the fact that E6/E7 inhibition typically acts pro-senescent, Metformin treatment does not trigger senescence in HPV-positive cancer cells.⁹² Instead, my results showed that Metformin can even counteract the pro-senescent effects of either E6/E7 or E6AP repression. These findings add to the controversy around Metformin's anti-cancer potential. Therefore, I aimed to better understand the phenotypic effects of Metformin in HPV-positive cancer cells, both applied as single agent or in combination with other therapeutic approaches (Figure 41).

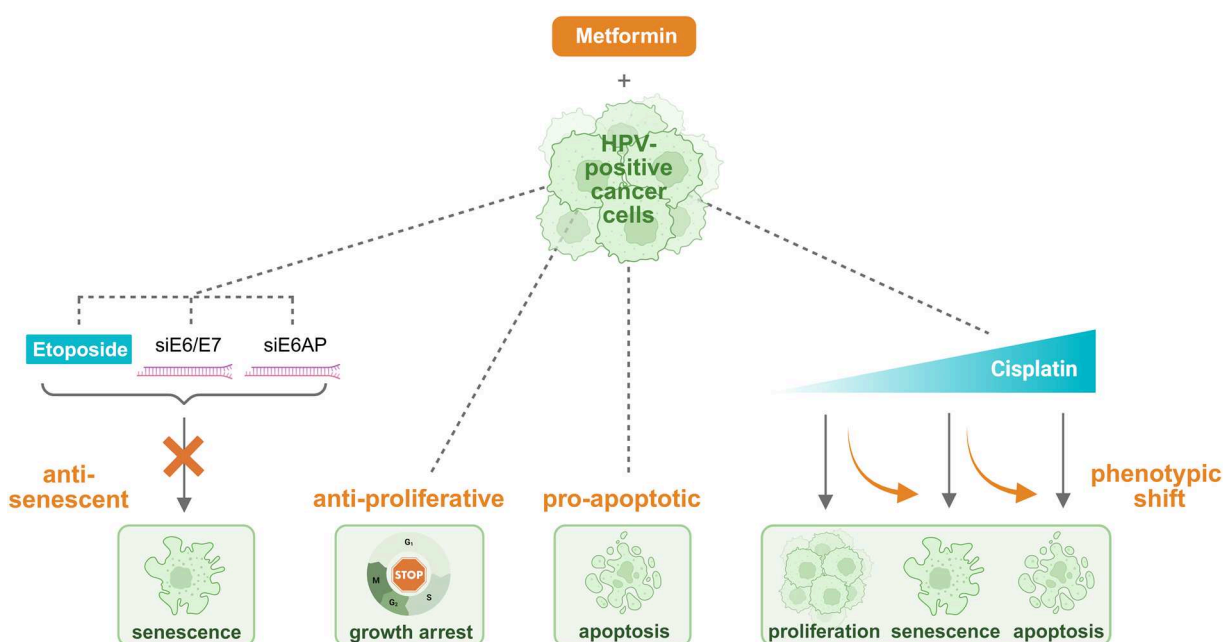


Figure 41. See Figure legend on the next page.

Figure 41. Metformin exerts pleiotropic phenotypic effects in HPV-positive cancer cells.

From left to right: Metformin can prevent the induction of senescence triggered either by pro-senescent chemotherapeutics like Etoposide or by RNAi-mediated silencing of E6/E7 or E6AP. As a monotreatment, Metformin reversibly suppresses proliferation and inhibits cell cycle progression across all phases of the cell cycle. Moreover, Metformin treatment can promote apoptosis. In combination with Cisplatin – which has minimal effects on proliferation at low doses but triggers either senescence or apoptosis with increasing doses – Metformin can enhance the growth-inhibitory effects of Cisplatin and support phenotypic shifts, either from proliferation to senescence or from senescence to apoptosis. Created with BioRender.com.

3.2.1 Comparison of the phenotypes resulting from Metformin- or siRNA-induced E6/E7 inhibition

The observation that Metformin downregulates viral oncogene expression suggests that its anti-proliferative effects in HPV-positive cancer cells result from the inhibition of E6/E7 and their growth-promoting activities. Interestingly, however, my studies challenge this notion.

In particular, in comparative analyses, I observed major differences in the mechanisms underlying growth arrest induced by Metformin or by RNAi-mediated silencing of E6/E7. While E6/E7 silencing using siRNAs induced a strong G1 arrest and efficient senescence, it also led to increased levels of the pocket proteins pRb and p130 and downregulated the expression of cell cycle genes involved in the G1/S transition as well as genes under negative control of pRb-E2F and/or DREAM complexes, as expected.^{56,58,201} In contrast, Metformin treatment did not induce a phase-specific cell cycle arrest. Moreover, even at doses that efficiently downregulated E6/E7, Metformin did not upregulate the pocket proteins or repress pRb/DREAM targets. Notably, these findings could explain the anti-senescent properties of Metformin. Metformin treatment *per se* did not promote reactivation of the pocket proteins or repression of their target genes, and my data further showed that it might even counteract pRb and p130 induction upon E6/E7 silencing. Thus, by interfering with the activation of the pRb-E2F and DREAM pathways,^{56,58,403} Metformin could suppress senescence despite E6/E7 downregulation. In addition, consistent with previous reports,^{92,340-342} my data demonstrated that Metformin efficiently inhibits mTOR signaling, a pathway known to play an important role in senescence induction.^{367,368} In fact, pharmacological inhibition of mTOR using Rapamycin or KU-0063794 has been previously shown to interfere with senescence induction upon RNAi-mediated E6/E7 silencing.³⁶⁹ This raises the possibility that mTOR inhibition upon Metformin treatment could play a role in its anti-senescent activity. However, under my experimental conditions, the anti-senescent effects of classical mTOR inhibitors towards senescence induced by siRNA-mediated E6/E7 or E6AP silencing were weak to negligible compared to those of Metformin. This suggests that Metformin-mediated mTOR inhibition may only marginally contribute to its ability to suppress senescence.

Furthermore, and in agreement with reports showing that Metformin can exert growth-inhibitory effects also in HPV-negative cancer cell lines,^{371,372,417,418} my data strongly indicate that Metformin-induced growth arrest does not depend on its ability to downregulate E6/E7. Supporting this notion, I found that low doses of Metformin (1 mM), which do not repress E6/E7 expression, still acted anti-proliferative and interfered with Etoposide-induced senescence in HPV-positive cancer cells. A similar anti-proliferative effect was observed upon glucose deprivation, which likewise did not repress E6/E7 levels. Since relatively high (millimolar) doses of Metformin, such as those used in my experiments and commonly employed in *in vitro* studies, are widely accepted to inhibit complex I of the mitochondrial respiratory chain, the resulting energy stress and its downstream effects may be one explanation for the proliferation arrest observed across different cancer cell lines.^{305,339,419,420} However, the fact that the depletion of a main energy source, such as glucose, does not lead to E6/E7 downregulation could suggest that energy depletion *per se* is not sufficient to repress HPV oncogene expression. Therefore, the E6/E7 downregulation observed with increasing Metformin doses may involve additional mechanisms beyond energy stress, which remain to be identified. It further should be noted that some studies have questioned the direct inhibitory effects of Metformin on complex I,^{421,422} and alternative mechanisms mediating the anti-proliferative effects of Metformin in cancer cells, such as increased ROS production, direct inhibition of mTOR signaling, or reduction of Cyclin D1 expression, have been proposed.^{371,423-425} Overall, while the exact mechanisms underlying the growth-inhibitory effects of Metformin treatment warrant further exploration, my data indicate that it acts independently of HPV oncogene repression and suggest an alternative mode of action.

3.2.2 Interactions of Metformin with chemotherapeutic drugs

Many preclinical and clinical studies have evaluated the anti-cancer effects of Metformin not only as a single agent but also in combination with other anti-cancer treatments, such as CT and RT. Notably, discrepant results have been obtained in these studies, ranging from improved to unchanged or even reduced therapeutic efficacy.^{305,317-322,338,426,427} Previous studies from our group indicated that Metformin might interfere with CT in HPV-positive cancer cells as it enables the cells to escape senescence induction upon treatment with the pro-senescent chemotherapeutic drug Etoposide.⁹² My experiments revealed that pre-treatment with Metformin attenuates the Etoposide-induced enrichment of cells in S and G2/M phases. Since Metformin induces growth arrest and impairs cell cycle progression, this reduces the number of cells passing through the S phase. As Etoposide induces DNA damage primarily in the S phase by inhibiting topoisomerase II in proliferating cells,³⁸⁵⁻³⁸⁷ these findings indicate that Metformin limits Etoposide-induced DNA damage and thereby prevents senescence

induction (Figure 41). Further, due to the reversible nature of Etoposide-induced topoisomerase II inhibition,³⁸⁵ cells protected from DNA damage by Metformin can restore their proliferation upon drug withdrawal. In support of this mechanistic concept, the protective effects were not restricted to Metformin, since growth-inhibition through glucose deprivation also enabled HPV-positive cancer cells to evade Etoposide-induced senescence. Furthermore, hypoxia – which, similar to Metformin, blocks proliferation and induces a cell cycle arrest across different cell cycle phases²⁰⁷ – has been recently shown by our group to confer resistance to Etoposide-induced senescence in HPV-positive cancer cells.^{167,369} Importantly, I further observed that these protective activities of Metformin against the pro-senescent effects of Etoposide were not limited to HPV-positive cancer cells, but also detectable in HPV-negative cancer cells of diverse histological origin. These findings could be informative for the ongoing discussion on the use of Metformin in cancer therapy, as they indicate that Metformin has the potential to protect cancer cells against CT.

Consequently, this raised the question of whether Metformin-induced cell cycle arrest also protects cancer cells from chemotherapeutic agents other than Etoposide. Cisplatin, a drug that forms inter- and intrastrand crosslinks in the DNA,¹⁵³ is particularly relevant in this context, as it represents a key component of standard therapy regimens for cervical cancer.^{147,148} Moreover, its combination with Metformin is especially interesting in view of a large number of studies reporting conflicting results on the anti-tumorigenic effects of Cisplatin when combined with Metformin in different cancer types. On the one hand, many preclinical and clinical studies describe improved treatment outcomes upon this combination therapy.^{318,319} On the other hand, however, several studies report no benefit, or even antagonistic effects and reduced Cisplatin-induced apoptosis upon co-treatment with Metformin.^{321-327,338,428} Surprisingly, my data revealed strong differences in the effects of Metformin on Cisplatin-induced apoptosis in HPV-positive cancer cells, depending on specific treatment conditions. While simultaneous treatment with Metformin and Cisplatin for 24 hours reduced Cisplatin-induced apoptosis when assessed directly after the end of treatment, pre-treatment with certain Metformin concentrations prior to Cisplatin exposure enhanced apoptosis induction. Therefore, my observation that even relatively subtle differences between experimental conditions can strongly affect the outcome of Metformin and Cisplatin co-treatment could provide a possible explanation for the discrepant results reported in the literature.

Another interesting finding was that the protective effects observed immediately after simultaneous treatment with Metformin and Cisplatin for 24 hours were no longer detectable at later time points and did not translate into long-term survival advantages, likely due to persistent Cisplatin-induced DNA lesions. Moreover, cells pre-treated with Metformin prior to Cisplatin exposure exhibited an enhanced long-term growth inhibition. Depending on the

employed Cisplatin dose, these cooperative effects were linked to a Metformin-induced phenotypic shift from proliferation towards senescence and from senescence towards apoptosis (Figure 41). Importantly, similar effects were also observed in HPV-negative U2OS osteosarcoma cells. Overall, these findings indicate that combined treatment with Metformin and Cisplatin effectively inhibits the growth of both HPV-positive and HPV-negative cancer cells over time, with particularly pronounced effects upon Metformin pre-treatment.

3.2.3 The role of p53 in the interplay of Metformin and Cisplatin

Although it seemed possible that Metformin-induced cell cycle arrest may underlie its transient protective effects against Cisplatin-induced apoptosis, glucose-deprived cells, which also undergo growth arrest similar to Metformin-treated cells, did not show diminished nor enhanced Cisplatin-induced apoptosis. This indicates that the growth arrest alone does not, or not fully, explain the observed effects upon Metformin co-treatment.

Instead, my studies point towards a central role for p53 in mediating the effects of Metformin and Cisplatin co-treatment in HPV-positive cancer cells across all tested treatment conditions. On the one hand, under conditions where Metformin conferred protection against apoptosis induced by Cisplatin, it efficiently counteracted the Cisplatin-mediated p53 upregulation. On the other hand, under conditions where Metformin pre-treatment resulted in enhanced Cisplatin-induced apoptosis, concomitant silencing of p53 could abrogate the effect. This indicates that both the anti- and pro-apoptotic effects of Metformin in combination with Cisplatin are p53-dependent and aligns with previous studies describing p53 as a central factor for the effects of either Metformin³⁸⁹⁻³⁹¹ or Cisplatin^{155,166,388} treatment alone.

Additionally, my analyses uncovered that BID, a pro-apoptotic member of the BCL-2 family, is likewise essential for the observed effects. Notably, the gene encoding BID is transcriptionally activated by p53, and BID has been shown to act pro-apoptotic downstream of p53.^{429,430} My results revealed that, similar to p53 silencing, BID knockdown also counteracted Metformin and/or Cisplatin-induced apoptosis. In line with this latter observation, it has been previously reported that Cisplatin-induced apoptosis can critically depend on BID, which is cleaved into the pro-apoptotic t-BID protein upon activation.^{167,168} Of note, my experiments showed that t-BID expression in response to apoptosis induction by Metformin or Cisplatin treatment, either alone or in combination, was abolished when p53 was silenced, whereas total BID levels remained unchanged. This could indicate that the p53-linked regulation of t-BID levels is not primarily mediated through changes in total BID expression, but rather suggests that p53 directly or indirectly facilitates BID cleavage into t-BID, thereby promoting apoptosis. Since BID cleavage typically occurs via Caspase 8 activation,¹⁶⁸ and p53 has been implicated in the

regulation of Caspase 8,^{430,431} p53 may, for example, contribute to BID cleavage by activating Caspase 8. The observation that Metformin and Cisplatin, independently from each other, appear to be able to activate the same p53- and BID/t-BID-dependent pro-apoptotic pathway may be one possible explanation for the cooperative pro-apoptotic effects of Metformin and Cisplatin.

Furthermore, my findings revealed dose-, time-, and glucose-dependent fluctuations in p53 levels following Metformin monotreatment. p53 levels decreased dose-dependently after 24 hours of treatment. This is surprising given that Metformin concurrently downregulated E6/E7 expression in HPV-positive cancer cells, which would be expected to result in increased p53 levels. However, I observed a similar response in HPV-negative cells, which indicates that Metformin-mediated p53 downregulation occurs in an HPV-independent manner. Moreover, since *TP53* mRNA levels were unaltered following Metformin treatment, a post-transcriptional regulation of p53 seems likely. For example, it could be linked to inhibition of mRNA translation or altered protein stability, mechanisms that have been previously reported to be potentially affected by Metformin.^{340,432,433} Notably, upon longer Metformin treatment (48 or 72 hours), p53 levels in HPV-positive cancer cells were upregulated, indicating an only transient block of p53 expression. The increase in p53 levels would be consistent with the expected outcome of an E6 inhibition and could explain the enhancement of Cisplatin-induced apoptosis upon Metformin pre-treatment.

Finally, it is remarkable that p53 protein levels did not consistently correlate with the extent of apoptosis induction following Metformin and Cisplatin co-treatment, albeit apoptosis was shown to be strictly p53-dependent. This suggests that p53 abundance alone may not determine the apoptotic outcome. This could, for example, be explained by the fact that the response of cells to p53 activation can be affected by the interplay of additional, both pro- and anti-apoptotic factors that collectively define the cellular apoptosis threshold.²⁴⁷ For instance, Metformin has been described to downregulate pro-survival proteins of the BCL-2 family,⁴³⁴⁻⁴³⁶ which could sensitize cells to p53-mediated apoptosis.^{247,437} Consistent with this possibility, my experiments revealed that Metformin co-treatment counteracts the Cisplatin-induced upregulation of the anti-apoptotic protein BCL-xL. Moreover, my studies showed that Metformin reduces p21 levels when combined with Cisplatin. Interestingly, p21 expression has been reported to be essential for the survival of senescent cells.⁴³⁸ Thus, Metformin-induced downregulation of p21 could not only impair senescence but also promote a shift towards apoptosis. Together, these results support the idea that the cell fate following p53 activation is not defined by p53 levels alone, but likely by a complex regulatory network²⁴⁷ that might be affected by Metformin treatment.

3.2.4 Implications of Metformin treatment

The results of my studies suggest that Metformin interacts differently with chemotherapeutic agents, depending on the experimental conditions and the specific drug used. These findings may have important clinical implications, especially in light of numerous clinical studies investigating Metformin as an adjuvant to CT.³⁰⁵ For instance, my data indicate that Metformin-induced growth inhibition and cell cycle arrest protects cancer cells from Etoposide-induced senescence. This raises the possibility that certain pro-senescent chemotherapeutic agents might be less effective in diabetic patients receiving Metformin therapy. In contrast, Metformin demonstrated promising long-term cooperative effects with Cisplatin, particularly upon pre-treatment. Interestingly, my data further showed that Cisplatin can induce either senescence or apoptosis, depending on differences in cell culture conditions. Since tumor tissues are typically heterogeneous in nutrient and oxygen availability,^{439,440} it therefore is possible that both responses might occur simultaneously in different tumor regions. In this context, Metformin may enhance the therapeutic efficacy of Cisplatin by shifting senescent cell populations towards apoptosis, thereby avoiding unwanted effects of the SASP (see Chapter 1.5.2 for more details). Furthermore, my results suggest that lower Cisplatin doses may be sufficient when used in combination with Metformin, which could potentially reduce Cisplatin-related toxicity.

However, it is important to emphasize that translating these findings into the clinic might be challenging, since my experiments investigated the direct effects of Metformin on the cellular phenotype under well-defined cell culture conditions. In the clinical context, by contrast, indirect mechanisms could also influence Metformin's anti-tumorigenic activity. For example, Metformin has been shown to lower systemic glucose and insulin levels.³⁴⁶ This could also contribute to reduced tumor growth, as these factors possess potential to support tumor progression.^{347,441} Additionally, Metformin can modify the tumor immune microenvironment and reduce tumor hypoxia,^{348,442} mechanisms which could further influence its therapeutic effectiveness.

Moreover, as in many other preclinical studies that investigate the anti-cancer properties of Metformin, the concentrations employed in my experiments were supra-pharmacological and may therefore not be clinically achievable without causing severe side effects.²⁸² Yet, some studies have reported preferential accumulation of Metformin in tumor tissue,^{443,444} suggesting that pharmacologically active concentrations of Metformin may potentially be achieved within tumors at lower doses, which can be administered to patients. Further, it has been shown that the high concentrations of glucose, serine, and glutamine commonly used in *in vitro* studies can decrease the sensitivity of cells to Metformin.⁴⁴⁵ This could also indicate that lower

Metformin concentrations might be therapeutically relevant *in vivo*, but further clinical exploration is required.

Nevertheless, despite the above discussed limitations regarding direct clinical translation, my findings provide important insights that may help explain the often controversial and inconsistent results reported in preclinical studies investigating the anti-cancer efficacy of Metformin in combination with CT.^{318,319,321-327} This is particularly relevant for the ongoing discussion about repurposing Metformin for cancer therapy, for which the outcome of preclinical studies is of utmost significance. My results further emphasize that the experimental results obtained in *in vitro* analyses can be strongly influenced by several critical factors, including the duration of treatment, the employed Metformin concentration, glucose availability, the type of chemotherapeutic agent used, as well as subtle aspects of the experimental setup such as timing of medium changes. Further, the time point of analysis, whether directly at the end of treatment or at later time points after drug removal, is particularly relevant for interpreting the cellular responses to Metformin.

3.3 Conclusions

Overall, my findings may serve as a foundation for the development of novel therapeutic strategies to inhibit the growth of HPV-positive cancer cells. The identification of E6AP as an anti-senescent factor, which is essential for HPV-positive cancer cell proliferation, highlights its potential as a new therapeutic target in HPV-induced cancers. Additionally, my results suggest that targeting E6AP could be a strategy to specifically target HPV-positive cancer cells, independently of the HPV type. Moreover, my findings demonstrated that Metformin can efficiently inhibit the proliferation of HPV-positive cancer cells and enhance the pro-apoptotic efficacy of Cisplatin in combinatorial treatment regimens. These beneficial effects, which were not limited to HPV-positive cancer cells, indicate that Metformin could have the potential to be valuable in cancer therapy, provided that pharmacologically effective Metformin concentrations can be achieved in the patients' tumor tissue.

CHAPTER 4

MATERIALS AND METHODS

4 Materials and Methods

The experiments described in this thesis were performed using standard laboratory materials and molecular biology grade reagents, where applicable. Reagents and consumables were obtained from the following suppliers: AppliChem (Darmstadt, Germany), BD Biosciences (San Jose, CA, USA), Bio-Rad (Hercules, CA, USA), Biozym Scientific (Hessisch Oldendorf, Germany), Carl Roth GmbH (Karlsruhe, Germany), Corning (Corning, NY, USA), Enzo Life Sciences (Farmingdale, NY, USA), Eppendorf (Hamburg, Germany), Gabler-Saliter Milchwerk (Obergünzburg, Germany), GE Healthcare (Chicago, IL, USA), Gerbu Biotechnik (Heidelberg, Germany), Greiner Bio-One (Kremsmünster, Austria), Merck (Darmstadt, Germany), Nerbe Plus (Winsen, Germany), New England Biolabs (Ipswich, MA, USA), Promega (Madison, WI, USA), Qiagen (Hilden, Germany), Roche Diagnostics (Basel, Switzerland), Santa Cruz Biotechnology (Dallas, TX, USA), Sartorius (Göttingen, Germany), Sigma-Aldrich (St. Louis, MO, USA; now part of Merck), Thermo Fisher Scientific (Waltham, MA, USA), and Vector Laboratories (Newark, CA, USA). The manufacturers of non-standard reagents are specified in the corresponding sections. Solutions and buffers were prepared with ultrapure water obtained from a TKA GenPure water purification system (Thermo Fisher Scientific), unless stated otherwise.

4.1 Cell-based methods

4.1.1 Cell culture, cryopreservation, and thawing

HPV18-positive HeLa (RRID: CVCL_0030), HPV16-positive SiHa (RRID: CVCL_0032) and CaSki (RRID: CVCL_1100) cervical cancer cells, as well as HPV-negative A549 (RRID: CVCL_0023) non-small-cell lung cancer, HCT116 (RRID: CVCL_0291) and RKO (RRID: CVCL_0504) colon cancer, MCF-7 (RRID: CVCL_0031) breast cancer, and U2OS (RRID: CVCL_0042) osteosarcoma cells, all of human origin, were obtained from the tumor bank of the German Cancer Research Center (DKFZ, Heidelberg, Germany). Two distinct laboratory strains of HeLa cells (referred to as HeLa-1 and HeLa-2, both authenticated as HeLa) were used in different experiments. HeLa-1 cells are better transfectable but more sensitive to Metformin treatment than HeLa-2 cells. Therefore, HeLa-2 cells were used in all experiments involving Metformin treatment, unless stated otherwise. In the main text, both strains are referred to as “HeLa” and the specific strain used is indicated in the corresponding Figures and Figure legends. HeLa-mKate2, SiHa-mKate2, and CaSki-mKate2 cells were generated as described previously⁴⁴⁶ and stably express the nuclear-restricted fluorescent protein mKate2. HeLa “p53 null” cells (HeLa A1, HeLa C2) lack p53 expression due to stable expression of an

shRNA targeting *TP53* mRNA.⁹⁸ All cell lines were routinely authenticated by single-nucleotide polymorphism profiling (Multiplexion GmbH, Heidelberg, Germany) within the last three years and regularly tested negative for mycoplasma contamination.

Cells were cultivated in Dulbecco's Modified Eagle Medium (DMEM) (used for HeLa, SiHa, CaSki, MCF-7, U2OS, HeLa-mKate2, SiHa-mKate2, CaSki-mKate2, HeLa A1, and HeLa C2), RPMI 1640 medium (A549, RKO), or McCoy's 5A medium (HCT116) (all Gibco, Thermo Fisher Scientific). For experiments involving Metformin, media containing 1 g/L (5.5 mM) glucose (which corresponds to physiological human blood glucose levels) were used, unless specified otherwise. In all other experiments, media containing 4.5 g/L (25 mM) glucose were used, unless indicated differently. All media were supplemented with 100 U/mL penicillin, 100 µg/mL streptomycin, 2 mM L-glutamine (PSG; all Sigma-Aldrich), and 10% fetal calf serum (FCS; PAN-Biotech, Aidenbach, Germany). Stock cultures of HeLa-mKate2, SiHa-mKate2, and CaSki-mKate2 cells were additionally supplemented with 1 µg/mL puromycin (Enzo Life Sciences), and those of HeLa A1 and HeLa C2 cells with 0.7 mg/mL G418 (Carl Roth GmbH). For experiments, the selection antibiotics were omitted from the culture media. All cells were maintained at 37 °C, 5% CO₂, and 21% O₂ in a humidified incubator.

Cell stock cultures were routinely split every 3 to 4 days upon reaching confluency. For passaging, cells were washed with phosphate-buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 4.3 mM Na₂HPO₄, 1.4 mM KH₂PO₄; pH 7.4) and detached using 0.25% Trypsin-EDTA (Gibco, Thermo Fisher Scientific). Before seeding for experiments, viable cells were stained with Trypan Blue Stain 0.4% (Gibco, Thermo Fisher Scientific) and counted using the Countess™ Automated Cell Counter (Invitrogen, Thermo Fisher Scientific). About 6 x 10⁵ to 1 x 10⁶ cells were seeded in 6 cm dishes containing 3 mL of the respective medium.

To enable long-term preservation, cells were detached using Trypsin-EDTA, collected by centrifugation at 800 g for 3 min, and resuspended in freezing medium composed of 30% FCS and 10% dimethyl sulfoxide (DMSO). The cell suspensions were aliquoted into cryogenic vials and placed in an isopropanol-filled freezing container (Nalgene, Thermo Fisher Scientific) to ensure gradual cooling at -80 °C over several days before being transferred to liquid nitrogen storage. For re-culturing, frozen cells were quickly thawed in a 37 °C water bath, resuspended, and transferred into a new cell culture flask. The medium was replaced the next day to remove residual DMSO.

4.1.2 Synthetic siRNA transfections

RNAi-mediated inhibition of gene expression was carried out using synthetic Silencer® Select siRNAs (Thermo Fisher Scientific), listed in Table 1, at a final concentration of 6-10 nM, if not indicated otherwise. For co-transfection experiments, siRNAs were combined in equimolar ratios, and the final siRNA concentration was equalized across all conditions by adding control siRNA siNeg where necessary.

Table 1. Synthetic siRNAs and target sequences.

Target transcript	siRNA	pool	siRNA target sequence (5' – 3')
-	siNeg	-	UACGACCGGUCUAUCGUAG
-	siCtrl1	-	CAGUCGCGUUUGCGACUGG
<i>UBE3A/E6AP</i>	siE6AP-1	siE6AP	GAAUUUGUUCAUUAUCGUA
	siE6AP-2		CCAAUGAUGUAUGAUCUAA
	siE6AP-3		CGGCUAGAGAUGAUCGCUA
<i>HPV18 E6</i>	si18E6-1	si18E6	GACAUUAUUCAGACUCTGU
	si18E6-2		CAGACUCUGUGUAUGGAGA
	si18E6-3		CUCUGUGUAUGGAGACACA
<i>HPV16 E6</i>	si16E6-1	si16E6	ACCGUUGUGUGAUUUUGUUA
	si16E6-2		GGGAUUUAUGCAUAGUAUA
	si16E6-3		UUAGUGAGUAUAGACAUUA
<i>HPV18 E6/E7</i>	si18E6/E7-1	si18E6/E7	CCACAACGUCACACAAUGU
	si18E6/E7-2		CAGAGAAACACAAGUAUAA
	si18E6/E7-3		UCCAGCAGCUGUUUCUGAA
<i>HPV16 E6/E7</i>	si16E6/E7-1	si16E6/E7	CCGGACAGAGCCCAUUACA
	si16E6/E7-2		CACCUACAUUGCAUGAAUA
	si16E6/E7-3		CAACUGAUCUCUACUGUUA
<i>TP53</i>	siP53	-	GACUCCAGUGGUAAUCUAC
<i>CDKN1A</i>	siP21	-	CAAGGAGUCAGACAUUUUA
<i>RBL2</i>	siP130	-	GAGCAGAGCUUAAUCGAAU
<i>RB1</i>	siPRb	-	GCGUGUAAAUUCUACUGCA
<i>BID</i>	siBID	-	CUUGCUCGUGAUGUCUUU

siRNA stock solutions (10 μ M) were diluted in improved Minimal Essential Medium (Opti-MEM™, Gibco, Thermo Fisher Scientific) to a final volume of 250 μ L. At the same time, 5 μ L of the transfection reagent Lipofectamine RNAiMAX (Invitrogen, Thermo Fisher Scientific) were mixed with 245 μ L Opti-MEM™. Both solutions were incubated separately at room temperature (RT) for 5 min and then mixed and incubated for another 20 min at RT.

For forward transfections, which were used in experiments involving simultaneous transfection and treatment with compounds such as Metformin, Rapamycin, or KU-0063794 (Figures 19 and 20), cells were seeded in 6 cm dishes the day before transfection. On the day of transfection, the medium was replaced with 2 mL of fresh medium containing 10% FCS but no PSG, and supplemented with the respective compounds, as indicated, and 500 μ L transfection mix was gently added to the cells dropwise. For reverse transfection experiments, the transfection mix was added to an empty 6 cm dish (500 μ L per dish), after which cells resuspended in 2 mL of FCS-containing, PSG-free medium were seeded directly onto the mix. In both forward and reverse approaches, the medium was changed to 3 mL of standard medium 24 h after transfection, followed by further treatment as described in the Figures and their corresponding legends. For reverse transfection experiments in 96-well plates, 0.2 μ L RNAiMAX was used per well in 100 μ L FCS-containing, PSG-free medium. After 24 h, 200 μ L of standard medium were added directly without prior removal of the existing medium.

The employed siRNAs were used either individually or in equimolar pools (Table 1), as specified in the Figures and their corresponding legends. The pool of E6AP-targeting siRNAs was designed to target all known transcript variants of E6AP according to the National Center for Biotechnology Information (NCBI) RefSeq database⁴⁴⁷ and the target sites of the individual siRNAs are visualized in Figure 42A. The siRNAs targeting HPV E6 or HPV E6/E7 have been well characterized in previous studies^{80,369,373,448-450} and are shown with their target sites in Figure 42B,C. siNeg and siCtrl1 were used as non-targeting controls, each containing four or more mismatches to the transcripts of all known human genes.

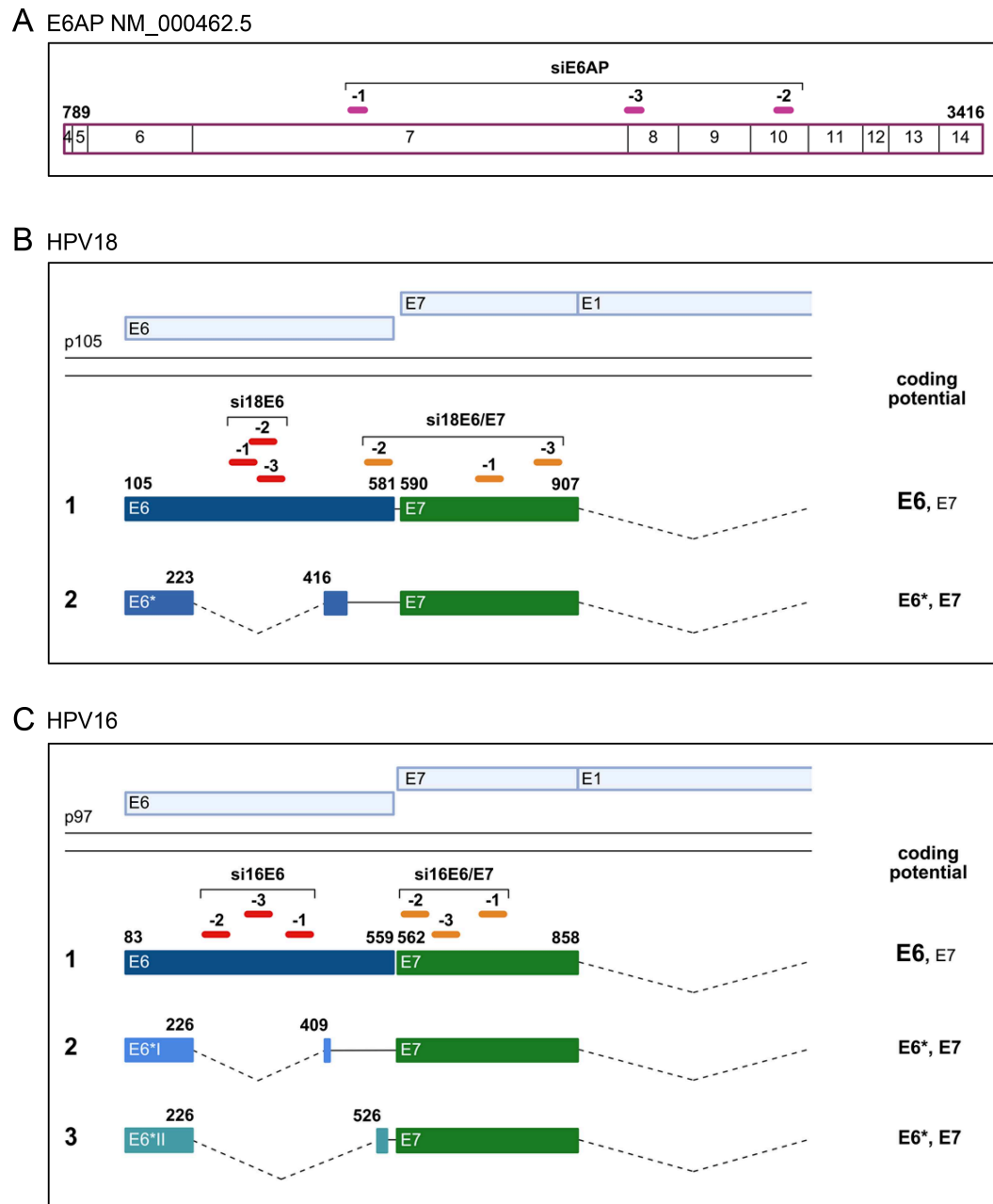


Figure 42. The target sites of the E6AP-, HPV E6-, and HPV E6/E7-targeting siRNAs employed in this thesis.

(A) Schematic representation of the exons of the *UBE3A/E6AP* gene targeted by the three E6AP-targeting siRNAs (siE6AP-1, -2, -3). NM_000462.5, the transcript variant that produces the longest protein isoform, is used as reference transcript. (B,C) Schematic representation of (B) HPV18 and (C) HPV16 transcripts, which code for E6 and E7.^{451,452} Boxes represent ORFs, with their respective nucleotide positions indicated. Spliced introns are illustrated by dashed lines. The coding potential of each transcript is specified on the right. Type 1 transcripts have the coding potential for both E6 and E7. However, they produce E6, but little or no E7, due to inefficient translational reinitiation. Type 2 transcripts (and type 3 transcripts for HPV16) produce E7 and E6*.^{451,452} Therefore, siRNAs targeting the intronic E6 sequences selectively suppress E6, whereas siRNAs targeting sequences present in all transcript classes suppress both E6 and E7. The specific target sites for 18E6 siRNAs (si18E6-1, -2, -3), 18E6/E7 siRNAs (si18E6/E7-1, -2, -3), 16E6 siRNAs (si16E6-1, -2, -3), and 16E6/E7 siRNAs (si16E6/E7-1, -2, -3) are visualized. Created with BioRender.com. The Figure and Figure legend have been adapted from Avenhaus et al., *PLOS Pathog* (2025).⁷⁰

4.1.3 Plasmid transfections for CRISPR/Cas9-mediated E6AP silencing

CRISPR plasmids (see Chapter 4.2.3) were transfected using the calcium phosphate co-precipitation method, following the protocol by Chen and Okayama, *Mol Cell Biol* (1987).⁴⁵³ In short, cells were seeded into 6 cm dishes in 3 mL standard medium one day prior to transfection. To prepare the transfection mix, 6 µg of plasmid DNA were mixed with 150 µL of sterile-filtered 0.25 M CaCl₂. Next, 150 µL sterile-filtered 2x BES buffer (50 mM BES, 280 mM NaCl, 1.5 mM Na₂HPO₄; pH 6.95) were added and the mix was incubated at RT for 20-30 min. The transfection mix was gently added dropwise to the cells, which were then incubated at 35 °C and 3% CO₂ in a humidified incubator. After 16-18 h, cells were washed with PBS, 3 mL of fresh standard medium were added, and cells were returned to standard incubation conditions (37 °C, 5% CO₂). Six hours after the medium change, puromycin was added at a final concentration of 1 µg/mL for 48 h to select for transfected cells. Subsequent treatment steps were carried out as described in Figure 5 and the corresponding legend.

4.1.4 Drug treatments

The compounds listed in Table 2 were used to treat cells according to the treatment schemes described in the Figures, their respective legends, and the main text. Final concentrations are provided within the corresponding Figures and their legends. All drugs were directly pipetted into the cell culture medium, and equal volumes of the appropriate solvent were included as controls.

Table 2. Chemical compounds and their corresponding solvents.

Compound	Solvent	Manufacturer
Etoposide	DMSO	Enzo Life Sciences
Metformin	DMEM, RPMI 1640, or McCoy's 5A	
Nocodazole	DMSO	Cayman Chemical Company, Ann Arbor, MI, USA
Nutlin-3	DMSO	
Cisplatin	0.9% NaCl/H ₂ O	Sigma-Aldrich
KU-0063794	DMSO	
Rapamycin	DMSO	AdipoGen, San Diego, CA, USA

4.1.5 Live-cell imaging

The Incucyte® S3 Live-Cell Analysis Instrument (Sartorius) was used to count viable cells over time. To this end, 2000 to 4000 mKate2-labelled cells per well were seeded into 96-well plates and either reverse transfected or treated as indicated. Four images per condition were acquired at 10× magnification every 6 h starting 24 h post-transfection, or every 24 h following compound treatment. Each condition was assessed in triplicates. The Incucyte® v2021C Software was used to determine the average red object counts (= number of mKate2-positive nuclei, corresponding to viable cell counts) across 12 images per time point per condition. Red object counts were normalized to the initial time point (i.e., 24 h post-transfection or the time of drug addition), which was set to 1.

To monitor cell death, the Incucyte® Cytotox Green Dye (Sartorius, Cat. No. 4633), which binds to DNA upon loss of plasma membrane integrity, was added at a final concentration of 50 nM 24 h after transfection. Green object counts (= number of dead cells) were acquired analogously, with images taken every 4 h following dye addition. Counts were normalized to the time point 24 h after dye addition (i.e., 48 h post-transfection), which was set to 1.

4.1.6 Cell cycle analyses

In order to analyze cell cycle profiles via flow cytometry, cells were washed with PBS, trypsinized, and collected by centrifugation at 1000 *g* and 4 °C for 5 min. Pellets were washed with cold PBS, centrifuged again, and then fixed by adding 300 µL cold PBS and 900 µL ice-cold 100% ethanol under constant vortexing. The fixed cells were stored at -20 °C until further use. To proceed with staining, the samples were centrifuged again at 1000 *g* and 4 °C for 5 min. The supernatant was discarded and the cells were resuspended in 900 µL cold PBS and stained with propidium iodide (PI; Sigma-Aldrich) at a final concentration of 25 µg/mL in the presence of RNase A (Roche Diagnostics) at a final concentration of 500 µg/mL. Following incubation at RT for 30 min in the dark, the cells were filtered through a cell strainer into round-bottom tubes (Corning). Cell cycle profiles were assessed by flow cytometry recording 20 000 events per condition using a BD LSRFortessa™ flow cytometer (BD Biosciences) and the BD FACSDiva™ v8.0.1 Software (BD Biosciences). Data analysis and image generation was performed with the FlowJo™ v10.8.1 Software (BD Life Sciences, Ashland, OR, USA) and the Dean-Jett-Fox model was applied for quantification of cell cycle phase distribution.⁴⁵⁴

4.1.7 TUNEL assays

Apoptotic cells were identified by detecting DNA fragmentation through terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) analyses, in which fluorescently labeled nucleotides bind to 3'-hydroxyl termini of damaged DNA.^{242,455} To this end, cells were grown on glass coverslips placed in 6 cm dishes. Following the respective treatments, coverslips were transferred into 4% paraformaldehyde in PBS and fixed at RT for 30 min. After a PBS wash, the coverslips were stored in 80% ethanol at -20 °C until further use. For processing, coverslips were washed again in PBS and cells were permeabilized in a solution of 0.1% Triton X-100 and 0.1% sodium citrate in PBS at 4 °C for 2 min. After washing with PBS again, the coverslips were placed in a humidified chamber and stained using the *In Situ* Cell Death Detection Kit, TMR red (Roche Diagnostics), according to the manufacturer's protocol. In brief, the enzyme and label solutions were mixed in a 1:10 ratio to generate the TUNEL reaction solution, of which 25 µL were applied per coverslip. Incubation was carried out at 37 °C for 60-90 min in the dark. Afterwards, coverslips were washed twice for 10 min in PBS and then the nuclei were stained with 30 µL of 1 µg/mL 4',6-diamidino-2-phenylindole (DAPI, Roche Diagnostics) in PBS for 5 min in the dark. Following two additional PBS washes for 10 min, coverslips were briefly dipped in H₂O and in 100% ethanol. Dried coverslips were mounted on microscope slides using VECTASHIELD® Antifade Mounting Medium (Vector Laboratories). Imaging of the cells was performed on a Zeiss Cell Observer microscope (Zeiss, Oberkochen, Germany) using a 20× objective. Quantification of TUNEL-positive cells was performed with the Fiji/ImageJ 1.52e Software⁴⁵⁶ (NIH, Bethesda, MD, USA) and a Fiji/ImageJ macro (kindly provided by Dr. Damir Krunić from the Light Microscopy Core Facility, DKFZ, Heidelberg, Germany). For each condition, at least five images were analyzed, and the proportion of TUNEL-positive cells was calculated relative to the total number of DAPI-stained nuclei.

4.1.8 Senescence assays

The presence of senescent cells can be determined by measuring SA-β-Gal activity. Specifically, due to high amounts of lysosomal β-galactosidase in senescent cells, activity of this enzyme can be measured at pH 6.0 through *in situ* staining with its chromogenic substrate 5-bromo-4-chloro-3-indolyl-β-D-Galactopyranoside (X-Gal), which forms a blue precipitate when cleaved.^{223,224} To perform senescence assays following the respective treatments, cells were trypsinized, seeded into new 6 cm dishes at defined cell densities, and grown under standard cell culture conditions in drug-free medium. In general, the medium was replaced with fresh medium three days after splitting, and SA-β-Gal staining was carried out on day four, unless stated otherwise. An exception was made for cells that were forward transfected and

simultaneously treated with Metformin, Rapamycin, or KU-0063794 (Figures 19 and 20), which were not split prior to staining. For staining, cells were washed with PBS and fixed in 1 mL fixation buffer (2% formaldehyde and 0.2% glutaraldehyde in PBS) per dish at RT for 3 min. After removal of the fixation buffer, cells were washed with PBS. Subsequently, senescence assay buffer (40 mM citric acid, 150 mM NaCl, 2 mM MgCl₂, adjusted to pH 6.0 with Na₂HPO₄) was freshly supplemented with 1 mg/mL X-Gal (Carl Roth GmbH) in dimethylformamide (DMF), 5 mM K₃[Fe(CN)₆], and 5 mM K₄[Fe(CN)₆], and 1.5 mL of the buffer was added to each dish. After an incubation in a humidified chamber at 37 °C for 16-24 h, cells were washed with PBS and bright-field images were taken using the EVOSxl Core Cell Imaging System (Invitrogen, Thermo Fisher Scientific) at 20× magnification.

4.1.9 Colony formation assays

To assess colony outgrowth as a proof of cell survival and proliferation following different treatments, cells were trypsinized and split as described in Chapter 4.1.8. The medium was replaced with fresh medium every 3-4 days. 7-14 days after splitting, cells were washed with PBS, and colonies were fixed and simultaneously stained with 350 µL of formaldehyde-crystal violet solution (12 mM crystal violet, 29 mM NaCl, 3.7% formaldehyde, 22% ethanol) per dish at RT for 5 min while shaking. The colonies were washed with H₂O, dried at 37 °C, and imaged with an Epson Perfection 4990 Photo Scanner (Epson, Suwa, Japan).

4.2 DNA-based methods

4.2.1 Agarose gel electrophoresis and DNA fragment purification

Agarose gel electrophoresis was used to separate and purify DNA fragments for cloning purposes. A 1% agarose gel was prepared using electrophoresis buffer (40 mM Tris, 5 mM sodium acetate, 1 mM EDTA; pH 7.8) and supplemented with PeqGREEN non-toxic DNA/RNA dye (Avantor, Radnor, PA, USA) at a 1:20000 dilution. The gel was placed into a PerfectBlue™ gel system (VWR International GmbH, Darmstadt, Germany) filled with electrophoresis buffer. DNA samples were mixed with 6x DNA loading buffer (0.25% bromophenol blue, 0.25% xylene cyanol, 30% glycerol) and loaded alongside the DNA SmartLadder (Eurogentec, Seraing, Belgium). Electrophoresis was performed at 80-100 V for 45-90 min. DNA bands were visualized under UV light using a gel documentation system (Intas Science Imaging Instruments, Göttingen, Germany) and DNA was extracted from the gel with the QIAquick Gel Extraction Kit (Qiagen) according to the manufacturer's protocol.

4.2.2 Bacteria transformation and plasmid DNA isolation

Bacteria transformation for plasmid amplification was carried out according to the protocol of D. Hanahan, *J Mol Biol* (1983).⁴⁵⁷ Briefly, to generate competent bacteria of the *Escherichia coli* (*E. coli*) strain TG2, bacteria were grown overnight at 37 °C in Lysogeny Broth (LB) medium (170 mM NaCl, 1% Bacto tryptone, 0.5% yeast extract; pH 7.0). The bacterial culture was then centrifuged for 5 min at 800 *g* and the pellet was resuspended in transformation buffer (10% polyethylene glycol 8000, 15% glycerol, 5% DMSO, and 50 nM MgCl₂ in LB medium; pH 6.5). Flash-frozen aliquots of TG2 bacteria suspension were stored at -80 °C until use. For transformation, an aliquot of competent bacteria was thawed on ice, 100 µL of the suspension were mixed with plasmid DNA and incubated on ice for 30 min, followed by heat-shock at 42 °C for 1 min in a water bath. This was immediately followed by another 3 min incubation on ice before adding 900 µL of LB medium. The suspension was incubated at 37 °C on a shaker. After 1 h, 100 µL of the bacterial suspension were plated on LB agar supplemented with 100 µg/mL ampicillin (Sigma-Aldrich). The agar plates were incubated overnight at 37 °C to allow for colony growth. The following day, individual colonies were picked and grown in LB medium containing ampicillin overnight at 37 °C while shaking. Plasmid DNA was isolated using the QIAprep Spin Miniprep Kit (Qiagen) or the PureLink™ HiPure Plasmid Filter Midiprep Kit (Invitrogen, Thermo Fisher Scientific) following the manufacturer's instructions. DNA concentrations were measured with the NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific).

4.2.3 Cloning of E6AP-targeting gRNAs

In order to target the *UBE3A/E6AP* gene using the CRISPR/Cas9 system,⁴⁵⁸ E6AP-specific gRNA sequences were designed using the online platform E-CRISP (<http://www.e-crisp.org>, version 5.4) from the Boutros lab (DKFZ, Heidelberg)⁴⁵⁹ or were adapted from Cosper et al., *Proc Natl Acad Sci U S A* (2023).⁴⁶⁰ For cloning into the LentiCRISPRv2 vector (Addgene plasmid #52961; RRID: Addgene_52961, kind gift from Feng Zhang),⁴⁶¹ a CACC overhang was added to the 5'-terminus of the sense strand, and an AAAC overhang to the 5'-terminus of the antisense strand of the 20 nt gRNA sequence. A list of the plasmids used is provided in Table 3.

Table 3. CRISPR plasmids and target sequences.

Plasmid	Target transcript	gRNA target sequence (5' – 3')	Source
pLentiCRISPRv2	-	no gRNA inserted	
pLentiCRISPRv2 gE6AP-1	<i>UBE3A/E6AP</i>	GGTTTACTATGCAAATGTAG	E-CRISP
pLentiCRISPRv2 gE6AP-2	<i>UBE3A/E6AP</i>	GAAGGATAGGTGATAGCTCAC	⁴⁶⁰

Cloning of the LentiCRISPR constructs was performed according to the protocol by Sanjana et al., *Nat Methods* (2014).⁴⁶¹ The empty LentiCRISPRv2 vector was linearized by digestion at 55 °C for 1 h using the restriction enzyme BsmBI (New England Biolabs). This was followed by 5'-dephosphorylation with calf intestinal alkaline phosphatase (CIP; New England Biolabs) at 37 °C for 1 h. The digested vector was purified by agarose gel electrophoresis (see Chapter 4.2.1). In parallel, the oligonucleotides for the respective gRNAs were phosphorylated with the T4 polynucleotide kinase (Thermo Fisher Scientific) in the presence of 1 mM ATP at 37 °C for 30 min in an MJ Research PTC-200 Thermal Cycler (Marshall Scientific, Hampton, NH, USA). After denaturation at 95 °C for 5 min, the oligonucleotides were annealed by step-wise cooling to 25 °C (5 °C/min). The purified vector was ligated with a 3- to 7-fold molar excess of annealed oligonucleotides using T4 DNA ligase (Thermo Fisher Scientific). The reaction was incubated at 21 °C for 1 h and heat inactivated at 65 °C for 5 min. Subsequently, the ligation product was transformed into competent *E. coli* TG2 cells for amplification (see Chapter 4.2.2). To confirm successful cloning, plasmids were sequenced by Eurofins Genomics, Ebersberg, Germany using the primer hu6_promoter_for (5'-ACGATACAAGGCTGTTAGAGAGA-3'). Sequence data were analyzed with the Basic Local Alignment Search Tool provided by NCBI.

4.3 RNA-based methods

4.3.1 RNA extraction

In order to measure mRNA expression levels, total RNA was first isolated from cells with a column-based PureLink™ RNA Mini Kit (Invitrogen, Thermo Fisher Scientific) following the manufacturer's instructions. In brief, cells were washed with PBS, resuspended in 600 µL RNA lysis buffer (Invitrogen, Thermo Fisher Scientific), which was freshly supplemented with 100 µL β-mercaptoethanol (Sigma-Aldrich) per 10 mL of buffer, and stored at -20 °C until RNA isolation. For purification, 600 µL 70% ethanol was added to each cell lysate before transferring to a silica-membrane spin column. Subsequently, the PureLink DNase Set (Invitrogen, Thermo Fisher Scientific) was used to remove genomic DNA contamination, followed by a series of

wash steps and final elution of total RNA from the column in 30-50 μL RNase-free H_2O . RNA concentrations of the samples were determined using the NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific), and purified samples were stored at $-80\text{ }^\circ\text{C}$ until further processing.

4.3.2 Reverse transcription

The purified RNA was reversed transcribed into complementary DNA (cDNA) using the ProtoScript® II First Strand cDNA Synthesis Kit (New England Biolabs) following the manufacturer's instructions. Briefly, RNase-free H_2O was used to adjust an amount of 500 ng RNA in 3 μL , which was then mixed with 0.5 μL oligo-dT primers and 0.5 μL random primers. The mixture was denatured at $70\text{ }^\circ\text{C}$ for 5 min and briefly cooled on ice. Subsequently, 5 μL of 2x M-MuLV Reaction Mix (containing dNTPs and optimized buffer) and 1 μL 10x M-MuLV Reverse Transcriptase Enzyme Mix were added. Reverse transcription was carried out in an MJ Research PTC-200 Thermal Cycler (Marshall Scientific, Hampton, NH, USA) following the program described in Table 4. The resulting cDNA was diluted with 40 μL RNase-free H_2O and stored at $-20\text{ }^\circ\text{C}$ until further use.

Table 4. Reverse transcription program.

Temperature	Time
70 $^\circ\text{C}$	5 min
- Addition of enzyme and reaction mix -	
25 $^\circ\text{C}$	5 min
42 $^\circ\text{C}$	60 min
80 $^\circ\text{C}$	5 min

4.3.3 Quantitative real-time polymerase chain reaction (qRT-PCR)

For each qRT-PCR reaction, 10 μL SYBR™ Green PCR Master Mix (Applied Biosystems, Thermo Fisher Scientific), 7.2 μL nuclease-free H_2O , as well as 0.4 μL each of forward and reverse primers (final concentration: 100 nM per primer) were mixed with 2 μL of cDNA in a MicroAmp™ Optical 96-Well Reaction Plate (Applied Biosystems, Thermo Fisher Scientific). Each sample was analyzed in technical duplicates, and a non-template control (H_2O) was included for each primer pair to monitor for contamination. qRT-PCR was carried out on a 7300 Real Time PCR System (Applied Biosystems, Thermo Fisher Scientific) following the program detailed in Table 5.

Table 5. qRT-PCR program.

Step	Temperature	Time
Initiation	50 °C	2 min
Polymerase activation	95 °C	10 min
Denaturation	95 °C	15 s
Annealing and elongation	60 °C	1 min
Dissociation curve	95 °C	15 s
	60 °C	1 min
	95 °C	15 s
	60 °C	15 s

To quantify relative target gene expression, the comparative C_t ($2^{-\Delta\Delta C_t}$) method⁴⁶² was employed and cycle threshold (C_t) values were normalized to the internal reference *TMBIM6*. The fold change values were $\log_2$ -transformed if indicated in the Figures and their corresponding legends. Forward and reverse primers used for qRT-PCR analyses are listed in Table 6.

Table 6. qRT-PCR primers and sequences.

Target	Forward primer (5' – 3')	Reverse primer (5' – 3')
<i>CCNA2</i>	CCCCCAGAAGTAGCAGAGTTT	ACTTGAGGTATGGGTCAGCATC
<i>CDKN1A</i>	GACCATGTGGACCTGTCACT	GCGGATTAGGGCTTCCTCTT
<i>E2F1</i>	GACGGCTTGAGGGGTTGAC	ATGCTACGAAGGTCCTGACAC
<i>FOXM1</i>	GGCAGCAGGCTGCACTATC	TCGAAGGCTCCTCAACCTTAAC
<i>MYBL2</i>	AGGCTGGCATCGAACTCATC	CTTGGGCAGTGTGGACATCA
<i>UBE3A/E6AP</i>	CGGTGGCTATACCAGGGACT	CTCTGTCTGTGCCCGTTGT
<i>TMBIM6</i>	GTGGTCATGTGTGGCTTCGT	GGAAAGGCTGGATGGTCACT
<i>TP53</i>	CTGAGGTTGGCTCTGACTGT	CAAAGCTGTTCCGTCCCAGT

4.4 Protein-based methods

4.4.1 Protein extraction for immunoblot analyses

To extract total protein for immunoblot analyses, cells cultured in 6 cm dishes were washed and scraped in ice-cold PBS. Following centrifugation at 16000 *g* and 4 °C for 15 s, cell pellets were either stored at -80 °C for later use or directly resuspended in CSK-1 lysis buffer (300 mM NaCl, 300 mM sucrose, 10 mM PIPES pH 6.8, 1 mM EDTA, 1 mM MgCl₂, 0.5% Triton X-100), freshly supplemented with 100 µL PhosSTOP phosphatase inhibitor cocktail (Roche Diagnostics), 25 µL Pefabloc serine protease inhibitor (Merck), and 10 µL P8340 protease inhibitor cocktail (Sigma-Aldrich) per 900 µL CSK-1 buffer. Depending on pellet size, 25-110 µL of this complete lysis buffer were added to each sample. Cells were lysed for 30 min on ice, followed by centrifugation at 16000 *g* and 4 °C for 5 min. The protein-containing supernatant was transferred to a fresh microreaction tube. Protein concentration was determined by mixing 1 µL of the lysate with 1 mL of 1:5 diluted Bio-Rad Protein Assay Dye Reagent (Bio-Rad) in H₂O in a BRAND® semi-micro disposable cuvette (Sigma-Aldrich). After an incubation at RT for 5 min, absorbance was measured at 595 nm using a BioPhotometer D30 (Eppendorf). Protein concentrations were calculated using a bovine serum albumin (BSA) standard curve. The samples were then adjusted to the desired protein concentration by adding the appropriate amount of 4x protein loading buffer (250 mM Tris-HCl, 8% SDS, 20% β-mercaptoethanol, 40% glycerol, 0.008% bromphenol blue). The protein extracts were denatured by boiling at 95 °C for 5 min and stored at -80 °C.

4.4.2 Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

To separate the extracted proteins by molecular weight, protein samples were loaded onto polyacrylamide gels. These gels were prepared between two glass plates sealed with 1% agarose following the recipe described in Table 7, placed into an XCell SureLock Mini-Cell Electrophoresis System (Invitrogen, Thermo Fisher Scientific), and covered with 500 mL Tris-glycine SDS running buffer (2.5 mM Tris, 19.2 mM glycine, 0.1% SDS). Alternatively, NuPAGE™ 4-12% Bis-Tris Mini Gels (Invitrogen, Thermo Fisher Scientific) were used in the same electrophoresis system and covered with 500 mL 1x NuPAGE™ MES SDS Running Buffer (Invitrogen, Thermo Fisher Scientific). Equal amounts of total protein (10-30 µg per lane) were loaded onto the gels, alongside 2 µL of PageRuler™ Prestained Protein Ladder (Thermo Fisher Scientific) as a size reference. Electrophoresis was carried out for approximately 2 h at 100-120 V.

Table 7. Recipe for 2 SDS-polyacrylamide gels.

Resolving gel (12.5%)		Stacking gel (5%)	
H ₂ O	4.5 mL	H ₂ O	3.5 mL
3 M Tris-HCl (pH 8.9)	1.2 mL	0.47 M Tris-HCl (pH 6.7)	620 µL
10% SDS	100 µL	10% SDS	50 µL
10% APS	10 µL	10% APS	100 µL
30% Acrylamide/bisacrylamide	4.2 mL	30% Acrylamide/bisacrylamide	830 µL
TEMED	50 µL	TEMED	5 µL

4.4.3 Western blot and detection of proteins

To assess protein levels after SDS-PAGE, semi-dry blotting was used to transfer the proteins from the gel to an Immobilon-P PVDF membrane (Merck). Prior to transfer, the PVDF membrane was activated in 100% methanol. Both the membrane and Whatman filter papers were soaked in Towbin transfer buffer (19.2 mM glycine, 2.5 mM Tris, 20% methanol; pH 8.3). The transfer stack was assembled in the following order (from anode to cathode): four layers of Whatman paper, PVDF membrane, protein gel, and another four layers of Whatman paper. Protein transfer was performed using a Trans-Blot® SD Semi-Dry Electrophoretic Transfer Cell (Bio-Rad) for 1 h at 20 V. At the end of protein transfer, the membrane was incubated on a shaker for 1 h in blocking buffer (0.2% Tween-20, 5% skim milk powder, 1% BSA in PBS) to block unspecific binding sites. Subsequently, the membrane was incubated with primary antibody (Table 8), diluted in blocking buffer, at 4 °C overnight.

The following day, the membrane was washed three times with PBS-T (0.2% Tween-20 in PBS) for 10 min per wash and then incubated with the appropriate horseradish peroxidase (HRP)-conjugated secondary antibody (Table 9), diluted in blocking buffer, for 1 h at RT. This incubation was followed by an additional three wash steps in PBS-T and subsequent protein detection. To this end, the membrane was covered with the enhanced chemiluminescence (ECL) reagents Amersham ECL Prime (Cytiva, Marlborough, MA, USA) or WesternBright Sirius (Advansta, San Jose, CA, USA) according to the manufacturer's instructions. Chemiluminescent signals were visualized using a Fusion SL Gel Detection System (Vilber Lourmat, Eberhardzell, Germany).

Table 8. List of primary antibodies.

Target	Source	Dilution	MW [kDa]	Clone, product	Supplier
HPV18E6	mouse	1:2000	18	AVC 399	kind gifts from Dr. J. Schweizer (Arbor Vita Corporation, Fremont, CA, USA)
HPV16E6	mouse	1:3000	14	AVC 843	
HPV18E7	chicken	1:1000	12	E7C	kind gift from Prof. Dr. H. Zentgraf (DKFZ)
HPV16E7	mouse	1:1000	16	NM2, sc-65711	kind gift from Prof. Dr. M. Müller (DKFZ)
B-MYB	mouse	1:10	110	LX015.1	from Dr. R. Watson, kindly provided by Prof. Dr. S. Gaubatz, University of Würzburg, Würzburg, Germany
β-Actin	mouse	1:50000	40	AC-74, A2228	Sigma-Aldrich
Cyclin B1	mouse	1:1000	58	GNS3 (8A5D12), 05-373	
E6AP	mouse	1:500	100	E6AP-300, E8655	
CDC2 p34	mouse	1:500	34	B-6, sc-8395	Santa Cruz Biotechnology
CDK2	mouse	1:500	34	D-12, sc-6248	
Cyclin A	rabbit	1:2000	54	H-432, sc-751	
Cyclin B2	mouse	1:1000	51	A-2, sc-28303	
Cyclin D1	mouse	1:500	37	DCS-6, sc-20044	
E2F1	mouse	1:500	60	KH95, sc-251	
GAPDH	rabbit	1:4000	37	FL-355, sc-25778	
p21	mouse	1:500	21	SX118, sc-53870	
p53	mouse	1:2000	53	DO-1, sc-126	
PCNA	mouse	1:1000	36	PC10, sc-56	
PLK1	mouse	1:500	66	F-8, sc-17783	
R1 [RRM1]	mouse	1:500	94	A-10, sc-377415	
R2 [RRM2]	mouse	1:500	45	A-5, sc-398294	

Table 8. (continued)

Target	Source	Dilution	MW [kDa]	Clone, product	Supplier
Vinculin	mouse	1:4000	117	7F9, sc-73614	Santa Cruz Biotechnology
Phospho-4E-BP1 (S65)	rabbit	1:1000	15-20	#9451	Cell Signaling Technology, Boston, MA, USA
BID	rabbit	1:1000	15, 22	#2002	
cleaved Caspase-9 (D330)	rabbit	1:1000	37	D2D4, #7237	
FOXm1	rabbit	1:500	110	D3F2B, #20549	
cleaved PARP (D214)	mouse	1:1000	89	#9546	
Acetyl-p53 (K382)	rabbit	1:1000	53	#2525	
Phospho-p53 (S15)	rabbit	1:1000	53	#9284	
Phospho-p53 (S20)	rabbit	1:1000	53	#9287	
Phospho-p70S6K (T389)	rabbit	1:1000	70, 85	#9205	
pRb	mouse	1:1000	110	4H1, #9309	
Phospho-pRb (S807/811)	rabbit	1:1000	110	#9308	
RBL2 [p130]	rabbit	1:1000	130	D9T7M, #13610	
Phospho-S6 (S235/236)	rabbit	1:1000	32	#2211	
BCL-xL	rabbit	1:500	28	Ab-1, PC67	Merck
CKS1	rabbit	1:250	10	36-6800	Invitrogen, Thermo Fisher Scientific

Table 9. List of HRP-conjugated secondary antibodies.

Target species, specificity	Source	Dilution	Product	Supplier
chicken IgY	goat	1:5000	sc-2428	Santa Cruz Biotechnology
mouse IgG	goat	1:10000	W4021	Promega, Madison, WI, USA
rabbit IgG	goat	1:10000	W4011	

4.4.4 Tandem mass tag (TMT) mass spectrometry (MS)-based proteomics

MS-based proteome analyses of SiHa cells depleted of HPV16 E6/E7 by RNAi were performed in collaboration with Dr. Bianca J. Kuhn in the lab of Prof. Dr. Jeroen Krijgsveld (DKFZ, Heidelberg, Germany) in order to compare global proteomic alterations upon E6/E7 silencing with previously by our group published proteome data from SiHa cells treated for 24 h with 7.5 mM Metformin or DMSO (ProteomeXchange data set identifier PXD011095).^{92,376}

Sample preparation for proteome analyses of three independent biological replicates of E6/E7-depleted cells was kindly performed by Claudia Lohrey. SiHa cells were seeded in 6 cm dishes and forward transfected with either control siRNA (siCtrl1) or si16E6/E7 (pool) at a final concentration of 10 nM using 6 µL of the transfection reagent DharmaFECT (Dharmacon, Lafayette, CO, USA) per dish. To this end, 194 µL Opti-MEM™ were mixed with 6 µL DharmaFECT. In parallel, 2 µL siRNA (10 µM stock) were mixed with 198 µL Opti-MEM™. After 5 min incubation at RT, both mixes were combined and forward transfection was performed as described in Chapter 4.1.2. Cells were harvested 72 h after transfection by washing with ice-cold PBS, scraping in 500 µL ice-cold PBS, centrifugation, and flash freezing of the resulting cell pellets in liquid nitrogen, followed by storage at -80 °C.

Subsequent sample processing as well as MS analysis were kindly performed by Dr. Bianca J. Kuhn, following a protocol previously described.^{167,376} Briefly, proteins were extracted using *RapiGest SF* (Waters, Milford, MA, USA) in triethylammonium bicarbonate buffer (TEAB; pH 8.0) and digested with sequencing-grade modified trypsin (Promega). Isobaric labeling of the peptides was performed using TMTpro™ 16plex label reagents (Thermo Fisher Scientific) according to the manufacturer's protocol. After confirmation of labeling efficiency and pooling of the samples, high-pH reverse-phase liquid chromatography (LC) using a 1260 Infinity HPLC system (Agilent, Santa Clara, CA, USA) with a Gemini C18 column (Phenomenex, Torrance, CA, USA) was performed for sample fractionation. Peptides were then separated by nanoLC using an EasynLC 1200 System (Thermo Fisher Scientific) and analyzed on an Orbitrap Fusion™ Tribrid™ (Thermo Fisher Scientific) mass spectrometer using the MS3 method.

Raw data were processed with MaxQuant version 1.6.17.0⁴⁶³ and the Andromeda search engine.⁴⁶⁴ Searches were performed against the UniProtKB/Swiss-Prot *Homo sapiens* (obtained 6 November 2020) and UniProtKB/Swiss-Prot *Human papillomavirus type 16* (obtained 15 May 2021) databases, along with the default contaminants database. The MaxQuant output was processed in R version 4.0.4 (R Core Team, 2021) operated in RStudio version 1.1.453 (RStudio Team, 2020). Peptide intensities were log₂-transformed and median normalized. Proteins identified only by site, reverse hits, and potential contaminants were excluded. Differential expression analysis was performed using “linear Models for Microarray

Data" (limma) moderated test statistics⁴⁶⁵ in R, with Benjamini-Hochberg correction applied to control for multiple testing. Proteins with an adjusted p value < 0.05 were considered significantly differentially expressed. The MS proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE⁴⁶⁶ partner repository under the data set identifier PXD061861.

The final processed data set containing $\log_2$ fold changes and adjusted p values was provided by Dr. Bianca J. Kuhn for comparative analysis against the Metformin vs. DMSO data set. Gene set enrichment analysis (GSEA) was conducted using the GSEA v4.3.3 Software,^{377,378} employing gene sets from the C2 collection (curated gene sets) of the Molecular Signature Database (MSigDB) v2024.1 (file: c2.all.v2024.1.Hs.symbols.gmt).^{379,380} Pre-ranked enrichment analysis was performed based on signed $\log_{10} p$ values derived from proteomic analyses of si16E6/E7 vs. siCtrl1 (4649 detected proteins) or Metformin vs. DMSO treatment (5929 detected proteins),⁹² each based on $n = 3$ biological replicates per condition. The following parameters were applied: number of permutations, 1000; enrichment statistic, weighted; gene set size filters, min = 15, max = 600; normalized mode, meandiv; seed for permutation, timestamp. Signed $\log_{10} p$ values were calculated using the following formula: signed $\log_{10} p$ value = $\text{abs}(\log_{10}(p \text{ value}) \times 10) \times \text{sign}(\log_2 \text{FC})$.

Heatmaps illustrating the differential expression of genes (proteins) from the FISCHER_G1_S_CELL_CYCLE²⁰² gene set or of leading-edge genes from the FISCHER_DREAM_TARGETS²⁰² gene set detected in both proteome data sets (si16E6/E7 vs. siCtrl1 and Metformin vs. DMSO) were generated using R version 4.4.2 (R Core Team, 2024) within RStudio version 2024.09.1 (RStudio Team, 2024). The leading-edge genes from the FISCHER_DREAM_TARGETS gene set were identified using the GSEA "Core Enrichment: Yes" classification. Venn diagrams visualizing the overlap between proteins from these two gene sets identified in both or uniquely in one of the analyzed data sets were also generated using R within RStudio. R scripts were developed with the help of OpenAI's ChatGPT.

4.5 Statistical analyses

Unless stated otherwise, all experiments were performed with at least three independent biological replicates. Statistical analyses were conducted using GraphPad Prism v10.2.3 (Dotmatics, Boston, MA, USA). Data are presented as mean $\pm$ standard deviations. Where appropriate, $\log_2$ transformation was applied prior to analysis. Statistical significance was determined using two-sided unpaired t -tests or one-way/two-way analysis of variance (ANOVA), as appropriate. ANOVA was followed by Sidak's or Tukey's post hoc test for multiple

comparisons, as indicated. MS data analysis is described in Chapter 4.4.4. Statistical significance levels are indicated in the Figures as * $p \leq 0.05$, ** $p \leq 0.01$, and *** $p \leq 0.001$.

4.6 Software

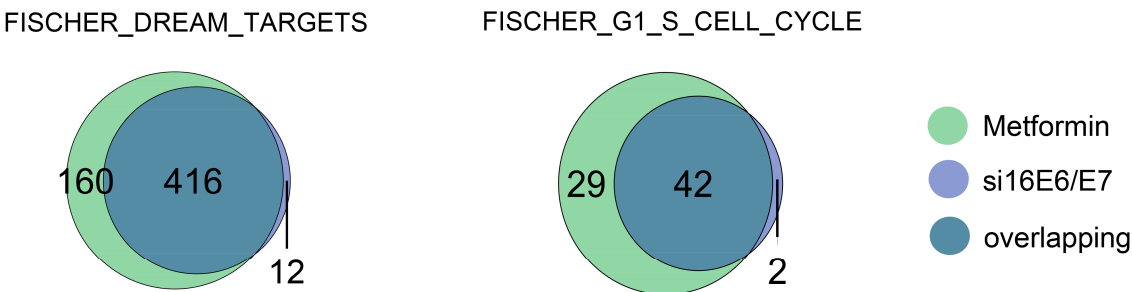
Table 10. Software used for data visualization.

Software	Version	Supplier
BioRender	web platform	BioRender, Toronto, Canada
ChatGPT	model GPT-4o	OpenAI, San Francisco, CA, USA
Fiji is just ImageJ	1.52e	NIH, Bethesda, MD, USA
FlowJo™	10.8.1	BD Life Sciences, Ashland, OR, USA
GraphPad Prism	10.2.3	Dotmatics, Boston, MA, USA
GSEA	4.3.3	Broad Institute, Cambridge, MA, USA
Microsof Office 365 ProPlus	Professional Plus 2016	Microsoft Corporation, Redmond, WA, USA
R	4.4.2	R Core Team
RStudio	2024.09.1	Posit PBC, Boston, MA, USA

APPENDIX

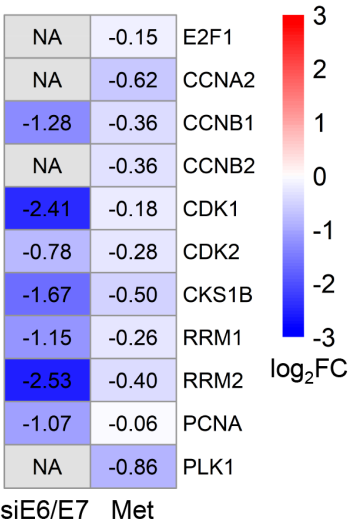
Appendix

Supplementary Figures



Supplementary Figure 1. Substantial overlap of proteins detected in the two proteomic data sets from analyzed cell cycle gene sets in Figure 22A,B.

Venn diagrams illustrating the overlap of proteins detected in two proteomic data sets from SiHa cells (siE6/E7 vs. siCtrl1 and Metformin vs. DMSO) that are annotated to the FISCHER_DREAM_TARGETS²⁰² gene set (left panel) or the FISCHER_G1_S_CELL_CYCLE²⁰² gene set (right panel). Numbers indicate proteins uniquely or commonly detected in each condition within the respective gene set. Light green: Metformin vs. DMSO, light blue: si16E6/E7 vs. siCtrl1, dark blue: overlapping proteins.



Supplementary Figure 2. Proteomic changes of proteins validated by immunoblotting.

Heatmaps showing log₂ fold change (log₂FC) values of selected proteins (E2F1, CCNA2 [Cyclin A2], CCNB1 [Cyclin B1], CCNB2 [Cyclin B2], CDK1 [CDC2], CDK2, CKS1B [CKS1], RRM1, RRM2, PCNA, and PLK1) detected in the proteomic data sets from SiHa cells (siE6/E7 vs. siCtrl1 and Metformin vs. DMSO) that were additionally analyzed by immunoblotting (Figure 22D). Expression changes are coded by color: red, upregulation; blue, downregulation. NA, not detected. Met, Metformin.

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Abbreviations

4E-BP1	Eukaryotic initiation factor 4E-binding protein 1
ADP	Adenosine diphosphate
AMP	Adenosine monophosphate
AMPK	AMP-activated protein kinase
ANOVA	Analysis of variance
APAF-1	Apoptotic protease-activating factor 1
APS	Ammonium persulfate
ATM	Ataxia telangiectasia mutated
ATP	Adenosine triphosphate
ATR	ATM- and Rad3-related
BCL-2	B-cell lymphoma 2
BCL-xL	B-cell lymphoma-extra-large
BID	BH3-interacting domain death agonist
BSA	Bovine serum albumin
CDDP	Cisplatin
CDK	Cyclin-dependent kinase
cDNA	complementary DNA
CFA	Colony formation assay
CHK	Checkpoint kinase
CHR	Cell cycle genes homology region
CIN	Cervical intraepithelial neoplasia
CIP	Calf intestinal alkaline phosphatase
cl	cleaved
CO ₂	Carbon dioxide
CT	Chemotherapy
DAPI	4',6-diamidino-2-phenylindole
DDR	DNA damage response
DMEM	Dulbecco's Modified Eagle Medium
DMF	Dimethylformamide
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleoside triphosphate
DP	Dimerization partner
DREAM	Dimerization partner, Rb-like, E2F, and multi-vulval class B
dsDNA	double-stranded DNA
E6AP	E6-associated protein
<i>E. coli</i>	<i>Escherichia coli</i>
ECL	Enhanced chemiluminescence
EDTA	Ethylenediaminetetraacetic acid
ER	Endoplasmic reticulum
expr.	Expression
FACS	Flow cytometry analyses
FADD	Fas-associated death domain

FC	Fold change
FCS	Fetal calf serum
FDA	US Food and Drug Administration
FDR	False discovery rate
FOXM1	Forkhead box protein M1
GLP1	Glucagon-like peptide 1
gRNA	guide RNA
GSEA	Gene Set Enrichment Analysis
H ₂ O	Water
H3K9	Histone 3 lysine 9
HECT	Homologous to the E6AP carboxyl terminus
HHR23A	Human homologue of yeast Rad23
HIV	Human immunodeficiency virus
HPV	Human papillomavirus
HRP	Horseradish peroxidase
HSPG	Heparan sulfate proteoglycan
hScrib	Human Scribble
hTERT	Human telomerase reverse transcriptase
KU	KU-0063794
LB	Lysogeny Broth
LC	Liquid chromatography
LCR	Long control region
limma	Linear Models for Microarray Data
LKB1	Liver kinase B1
MAPK	Mitogen-activated protein kinase
MATE	Multidrug and toxin extrusion
MC	Medium change
Mdm2	Murine double minute 2
MEM	Minimal Essential Medium
Met	Metformin
miRNA	micro RNA
MMB	Myb-MuvB
MOMP	Mitochondrial outer membrane permeabilization
mRNA	messenger RNA
MS	Mass spectrometry
MSigDB	Molecular Signature Database
mTOR	Mechanistic/mammalian target of rapamycin
mTORC1/2	mTOR complex 1/2
MuvB	Multi-vulval class B
MW	Molecular weight
NB	Nuclear body
NCBI	National Center for Biotechnology Information
NES	Normalized enrichment score
NHERF1	Na ⁺ /H ⁺ exchanger regulatory factor 1
nt	Nucleotide

O ₂	Oxygen
OCT	Organic cation transporter
ORF	Open reading frame
OXPHOS	Oxidative phosphorylation
P-	phospho
p70S6K	p70-S6 kinase
Pap	Papanicolaou
PARP	Poly(ADP-ribose) polymerase
PBM	PDZ binding motif
PBS	Phosphate-buffered saline
PBS-T	PBS with Tween20
PDZ	PSD-95/DLG/ZO-1
PD-1	Programmed death 1
PD-L1	PD ligand-1
PMAT	Plasma membrane monoamine transporter
PML	Promyelocytic leukemia
PSG	Penicillin, streptomycin, glutamine
PTM	Post-translational modifications
PVDF	Polyvinylidene difluoride
PI	Propidium iodide
pRb	Retinoblastoma protein
Prot	Protein
qRT-PCR	Quantitative real-time polymerase chain reaction
Rapa	Rapamycin
rel.	relative
RNA	Ribonucleic acid
RNAi	RNA interference
ROS	Reactive oxygen species
RT	Radiotherapy
RT	Room temperature
S6	S6 ribosomal protein
SA- β -Gal	Senescence-associated β -galactosidase
SAHF	Senescence-associated heterochromatin foci
SASP	Senescence-associated secretory phenotype
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
siRNA	small-interfering RNA
T2DM	Type 2 diabetes mellitus
t-BID	Truncated BID
TEAB	Triethylammonium bicarbonate buffer
TEMED	Tetramethylethylenediamine
TF	Tissue factor
TMT	Tandem mass tag
TRAIL	Tumor necrosis factor-related apoptosis inducing ligand
TUNEL	Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling
Tx	Transfection

VEGF	Vascular endothelial growth factor
VLP	Virus-like particle
WHO	World Health Organization
X-Gal	5-bromo-4-chloro-3-indolyl- β -D-Galactopyranoside

Units and prefixes

Units

Symbol	Unit
%	percent
°C	degree Celsius
C _t	cycle threshold
Da	Dalton
<i>g</i>	gravitational acceleration
g	gram
h	hour
L	liter
m	meter
M	Molar
min	minute
s	second
U	Unit (international unit)
V	volt

Prefixes

Symbol	Prefix	Factor
n	nano	10 ⁻⁹
μ	micro	10 ⁻⁶
m	mili	10 ⁻³
c	centi	10 ⁻²
k	kilo	10 ³

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