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**Senescence Control in Normoxic and Hypoxic
HPV-Positive Cancer Cells:
The Interplay between B-MYB and A-MYB**

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Summary

Hypoxia is a hallmark of solid tumors, including HPV-positive cervical cancers, and it poses a significant challenge to cancer treatment, as hypoxic tumor regions are often associated with increased therapy resistance and poor patient prognosis. One possible contributing factor to this resistance is the ability of hypoxic cancer cells to evade cellular senescence – a process leading to an irreversible growth arrest. While the repression of the HPV *E6/E7* oncogenes in normoxic HPV-positive cells efficiently induces senescence, hypoxic HPV-positive cells, despite efficiently shutting off *E6/E7* expression, evade senescence and induce a reversible growth arrest that can be overcome by reoxygenation.

The overall aim of this thesis was to uncover mechanisms that enable hypoxic HPV-positive cancer cells to evade senescence. Through comparative gene and protein expression analyses following *E6/E7* repression under normoxic and hypoxic conditions, I identified distinct regulatory patterns of key cell cycle regulatory factors. Notably, B-MYB emerged as a critical factor differentially regulated under these conditions. Its strong downregulation in normoxic cells following *E6/E7* knockdown was associated with the onset of senescence, while its sustained expression under hypoxia correlated with senescence evasion, suggesting a role for B-MYB in controlling this process. To further investigate the functional role of B-MYB, I performed knockdown experiments and assessed the phenotypic consequences using various cellular assays. Interestingly, B-MYB depletion led to a partial senescence induction under normoxia. I further showed that this limited senescence response was due to the compensatory induction of a B-MYB paralog, A-MYB, which could counteract the pro-senescent effects of B-MYB loss. Consequently, the combined repression of both B-MYB and A-MYB was needed for efficient senescence induction under normoxia. Strikingly, I found that this compensatory mechanism is disrupted under hypoxia, rendering hypoxic cancer cells particularly susceptible to the pro-senescent effects of B-MYB loss. This increased vulnerability of hypoxic cells could be reversed by ectopic A-MYB overexpression. Further, my studies revealed that the B-MYB/A-MYB axis also plays a key role for the senescence response of normoxic and hypoxic HPV-negative cancer cells of various histological backgrounds, indicating that this newly discovered mechanism could be broadly conserved across different tumor types.

Collectively, my findings provide novel insights into the regulation of senescence in normoxic and hypoxic cancer cells and identify B-MYB and A-MYB as central factors in these processes. Further, they reveal a particular vulnerability of hypoxic cancer cells to B-MYB inhibition that could be exploited to develop new strategies to target this therapeutically challenging tumor cell population.

Zusammenfassung

Hypoxie ist ein typisches Merkmal solider Tumore, einschließlich HPV-positiver Zervixkarzinome, und stellt eine große Herausforderung für die Krebstherapie dar. Hypoxische Tumorregionen sind häufig mit einer erhöhten Therapieresistenz und ungünstiger Prognose für den Patienten verbunden. Ein möglicher Beitrag zu dieser erhöhten Resistenz hypoxischer Tumorzellen ist ihre Fähigkeit, sich der Induktion zellulärer Seneszenz – einem irreversiblen Wachstumsstopp – zu entziehen. Während die Repression der HPV *E6/E7*-Onkogene in normoxischen HPV-positiven Tumorzellen effizient Seneszenz induziert, entziehen sie sich unter Hypoxie diesem Prozess, obwohl *E6/E7* stark herunterreguliert werden. Stattdessen tritt ein reversibler Wachstumsarrest ein, der durch Reoxygenierung aufgehoben werden kann.

Ziel dieser Arbeit war es, Mechanismen aufzudecken, die es hypoxischen HPV-positiven Krebszellen ermöglichen, der Seneszenzinduktion zu entkommen. Durch vergleichende Analysen der Gen- und Proteinexpression nach *E6/E7*-Hemmung unter Normoxie und Hypoxie wurden unterschiedliche Expressionsmuster zentraler Zellzyklusregulatoren identifiziert. Es zeigte sich, dass u. a. das B-MYB-Protein stark differentiell reguliert wurde: Unter Normoxie war die Seneszenzinduktion mit einer Herunterregulierung von B-MYB assoziiert, während bei der Seneszenzevasion unter Hypoxie die B-MYB-Expression erhalten blieb.

Funktionelle Studien zeigten, dass eine Hemmung der B-MYB-Expression unter Normoxie partiell Seneszenz auslöste. Diese begrenzte Reaktion ließ sich auf eine kompensatorische Hochregulation des A-MYB-Proteins zurückführen, welche die pro-seneszenten Effekte einer B-MYB-Hemmung abschwächt. Entsprechend führte nur eine kombinierte Hemmung von B-MYB und A-MYB zur effizienten Seneszenzantwort unter Normoxie. Bemerkenswerterweise ist diese kompensatorische A-MYB-Induktion unter Hypoxie aufgehoben, wodurch hypoxische Zellen besonders empfindlich gegenüber einer Inhibierung von B-MYB werden. Umgekehrt konnte die erhöhte Sensitivität hypoxischer Zellen gegenüber einer B-MYB-Hemmung durch ektopische Überexpression von A-MYB konterkariert werden. Darüber hinaus zeigte sich, dass die B-MYB/A-MYB-Achse auch in HPV-negativen Krebszellen unterschiedlicher histologischer Herkunft eine zentrale Rolle in der Regulation der Seneszenz unter Normoxie und Hypoxie spielt. Dies deutet darauf hin, dass dieser neu entdeckte Mechanismus möglicherweise in verschiedenen Tumortypen konserviert ist.

Insgesamt liefern die Ergebnisse dieser Arbeit neue Einblicke in die Seneszenzantwort normoxischer und hypoxischer Krebszellen und identifizieren B-MYB und A-MYB als zentrale Regulatoren. Zudem weisen sie darauf hin, dass hypoxische Zellen besonders empfindlich auf eine Hemmung von B-MYB reagieren, was möglicherweise einen neuen Angriffspunkt gegen diese therapeutisch besonders problematische Zellpopulation bieten könnte.

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Publications and presentations

Publications

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Presentations

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CHAPTER 1

INTRODUCTION

1 Introduction

1.1 Human papillomaviruses

Human papillomaviruses (HPVs) are a group of over 400 related DNA viruses from the *Papillomaviridae* family.^{1, 2} These non-enveloped viruses infect epithelial cells of the skin and mucosa.³ Based on their associated clinical outcomes, HPVs are classified into two groups: low-risk types, which cause benign conditions such as cutaneous or anogenital warts, and high-risk types.⁴ The International Agency for Research on Cancer (IARC) categorizes different HPV types into groups based on their carcinogenic potential⁵ (Table 1). Among the high-risk HPV types, twelve are classified as carcinogenic and probably carcinogenic (Group 1, Group 2a), with sufficient evidence for causing cancer, while several additional types are classified as possibly carcinogenic (Group 2b) due to rare associations with cancer.⁵⁻⁷

Table 1. IARC classification of HPV types.

IARC group		HPV types
Group 1	Carcinogenic	HPV16, HPV18, HPV31, HPV33, HPV35, HPV39, HPV45, HPV51, HPV52, HPV56, HPV58, HPV59
Group 2a	Probably carcinogenic	HPV68
Group 2b	Possibly carcinogenic	HPV26, HPV30, HPV34, HPV53, HPV66, HPV67, HPV69, HPV70, HPV73, HPV82, HPV85, HPV97
Group 3	Not classifiable	Low-risk types like HPV6 and HPV11 (commonly associated with benign lesions)

HPV infections are the most common sexually transmitted infections globally, transmitted through direct skin-to-skin contact. Most HPV infections are asymptomatic and are resolved spontaneously within about 2 years by the host immune system, but persistent infections with high-risk types are linked to several cancers.⁸⁻¹⁰ High-risk HPV types are responsible for virtually all cervical cancers and, in addition, a significant portion of other anogenital (including anal, vaginal, vulvar, and penile) and oropharyngeal cancers.^{9, 11-13} Overall, HPV is responsible for 4.5% of all cancers worldwide, accounting for over 600,000 new cases annually, 8.6% in women and 0.8% in men.¹²

1.1.1 HPV biology and life cycle

The HPV genome consists of a double-stranded, circular DNA molecule (approximately 7-8 kilobase (kb) pairs) which is organized into six (in oncogenic HPV-types) early (E) and two late (L) regions, along with a long control region (LCR), also known as the upstream regulatory region (URR) or non-coding region (NCR). The LCR contains crucial transcription factor binding sites and regulatory elements that control viral genome replication and

transcription.^{9, 14} The E region of the HPV genome consists of six open reading frames (ORFs) that encode early proteins: E1, E2, E4, E5, E6, and E7, each of which plays a distinct role in the viral life cycle.¹⁴ *E1* encodes a viral DNA helicase responsible for initiating viral genome replication.^{14, 15} *E2* is a key regulatory protein that not only aids in replication by recruiting E1 to the LCR but also regulates gene transcription.^{14, 16} *E2* can repress *E6/E7* transcription and *E2* gene disruption, caused by viral integration into host DNA, leads to deregulated expression of *E6* and *E7* oncogenes.^{14, 17} The *E4* protein is involved in virion assembly and the release of viral particles from infected cells.^{14, 18} For high-risk HPV types, *E5*, and especially *E6* and *E7*, are the key drivers of HPV-induced carcinogenesis.¹⁹ *E5* contributes to immune evasion and cell proliferation during the early stages of the HPV life cycle.²⁰⁻²² *E6* and *E7* drive cell proliferation, immortalization, and promote senescence and apoptosis evasion by disrupting key cellular regulatory pathways^{19, 23, 24} (please refer to section 1.1.3). The late region of the HPV genome encodes the major and minor capsid proteins, L1 and L2, respectively. These proteins form the viral capsid and are vital for the assembly and release of infectious virions during the late stages of the viral life cycle.²⁵⁻²⁷

During HPV infection, the virus targets the basal layer cells of the skin and mucosal epithelium, as these cells are capable of sustained division.^{3, 9} The expression of *E1* and *E2* early proteins promotes replication of the viral genome at low copy numbers and maintains viral episomes within the basal layer cells.^{3, 28} As these infected cells migrate toward the upper epithelial layers and differentiate into mature keratinocytes, the expression of the viral oncogenes, *E6* and *E7*, is induced, driving viral genome replication and continuous cellular proliferation.^{9, 28, 29} In the most upper layers of the epithelium (terminally differentiated keratinocytes), the *E4* protein, along with the late capsid proteins L1 and L2, promotes virion assembly and release, driving a new productive cycle of infection.^{10, 28}

1.1.2 HPV: from initial infection to cancer

The most common HPV types found in cervical cancers are HPV16 and HPV18, which together account for nearly 80% of HPV-positive cervical cancers (HPV16 being the most prevalent, accounting for approximately 60% of cases worldwide).³⁰

While most HPV infections, including those caused by high-risk types, are cleared by the host immune system, persistent infections with high-risk HPVs can lead to cancer development.^{9, 31} This process typically occurs several decades after the initial infection, indicating that while initial infection is a prerequisite for eventual carcinogenesis, it is not sufficient, and additional factors contribute to the progression to malignancy.^{31, 32} Persistent infection with high-risk HPV types can lead to severe dysplastic changes, characterized by increased proliferation of basal

epithelial cells that eventually replace the squamous epithelium. Initially, this manifests as mild dysplasia, known as cervical intraepithelial neoplasia 1 (CIN1), a precancerous condition.^{3, 33} Most dysplasias resolve spontaneously, but the likelihood of resolution decreases as the severity of the dysplasia increases. If the infection persists and is not cleared by the immune system, it can progress to more advanced stages, such as moderate (CIN2) and severe (CIN3) dysplasia.^{3, 33} These stages are linked to an unproductive HPV life cycle, where progeny virions are no longer produced.³³ Instead, the viral oncoproteins E6 and E7 are continuously expressed, promoting malignant transformation and playing a key role in the progression to cancer.^{3, 19, 33}

The integration of viral DNA into the host genome can enhance viral oncogene expression and drive malignant progression. The integration event typically disrupts the *E2* ORF, leading to the loss of *E2*.^{17, 34} Since *E2* functions as a transcriptional repressor of *E6* and *E7*, its inactivation leads to increased oncogene expression. Moreover, integration can occur in multiple or tandem repeats, forming super-enhancer-like elements, or in close proximity to host enhancer elements, both of which can further amplify HPV oncogene transcription.³⁵ In cancer cells where only episomal copies of the virus are present and the *E2* gene is intact, *E2*-mediated repression of *E6/E7* oncogene transcription can be inhibited by epigenetic modifications, such as DNA methylation of *E2* binding sites in the viral promoter.³⁶

1.1.3 HPV: E6 and E7 oncoproteins

The E6 and E7 oncoproteins from high-risk HPV types play pivotal roles in driving the major characteristics of cancer ("hallmarks of cancer"), including sustained cell proliferation, replicative immortality, evasion of growth suppressors, cell death resistance, and promotion of angiogenesis, tissue invasion, and metastasis.^{23, 37, 38} The expression of *E6* and *E7* genes is regulated by the early promoter located within the LCR. The *E6* and *E7* genes are transcribed as polycistronic mRNAs from the early promoter, with alternative splicing enabling the production of both E6 and E7 proteins.³⁹⁻⁴² Continuous expression of *E6* and *E7* is essential for maintaining the malignant phenotype of cervical cancer cells, which are deemed "oncogene-addicted".²³ The E6 and E7 proteins exert their transforming properties by disrupting key tumor suppressor pathways (Figure 1). As they do not possess intrinsic enzymatic activities, they achieve this by interacting with cellular proteins involved in these pathways.¹⁹

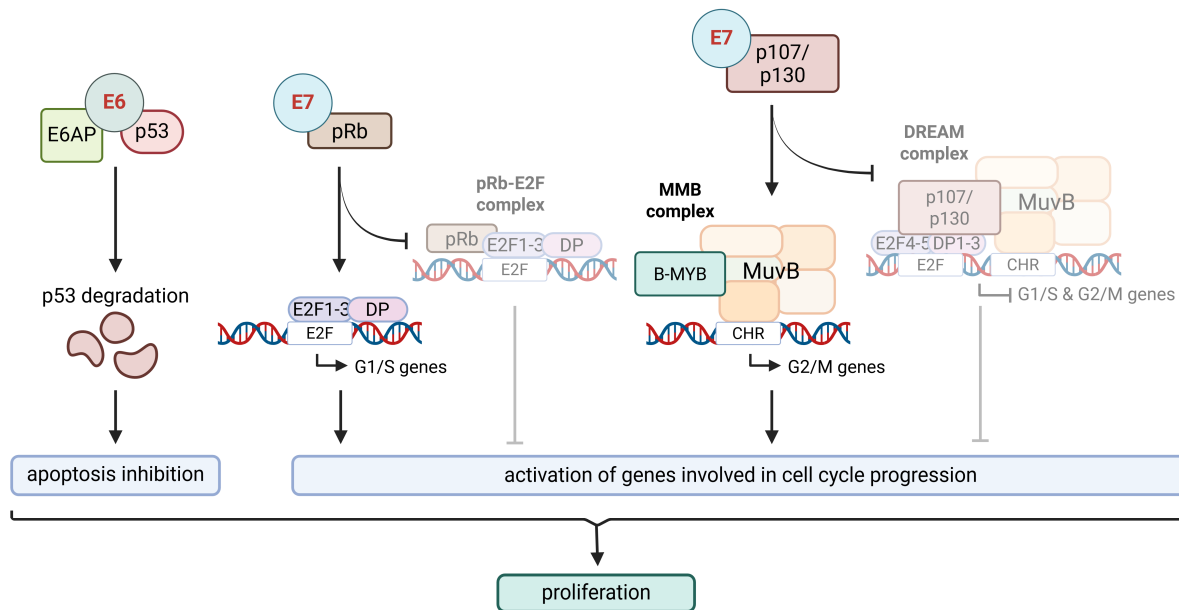


Figure 1. Transforming properties of E6 and E7. The viral oncoproteins E6 and E7 disrupt key tumor suppressor pathways. E6 contributes to oncogenesis by forming a ternary complex with the tumor suppressor p53 and the E3 ubiquitin ligase E6AP, leading to p53's ubiquitin-dependent proteasomal degradation. This prevents p53-mediated cell cycle arrest and apoptosis. E7 targets the retinoblastoma (Rb) protein family and promotes the degradation of Rb protein family members (pRb, p107, p130), thereby disrupting both pRb-E2F and DREAM-mediated gene repression. This leads to inappropriate activation of genes involved in cell cycle progression (G1/S and G2/M genes). Collectively, E6 and E7 drive oncogenesis by simultaneously blocking apoptosis and activating proliferative signaling. Image created with BioRender.

High-risk HPV E6 proteins are approximately 150 amino acids in size.¹⁹ E6 cooperates with E7 to drive the immortalization of human keratinocytes.¹⁹ Unlike their low-risk counterparts, high-risk HPV E6 interacts with the cellular ubiquitin ligase E6-associated protein, E6AP, and the tumor suppressor p53.^{43, 44} They form a ternary complex that leads to the ubiquitin-dependent proteolytic degradation of p53.^{43, 44} Typically, p53 levels rise in response to genotoxic stress or DNA damage, triggering the activation of cell cycle arrest mechanisms which allow cells time to repair DNA or, alternatively, leading to the elimination of damaged cells by inducing apoptosis.⁴⁵ Normally, the transforming activity of E7 would activate this pro-apoptotic response in the absence of E6, e.g. by upregulating the expression of the long noncoding RNA DINO, which stabilizes p53.⁴⁶ E6, however, counteracts this response by facilitating p53 degradation, allowing the cell to evade growth-suppressive and apoptotic signals.

The E7 protein has a size of approximately 100 amino acids and plays a crucial role in driving cell cycle progression.¹⁹ It does so by binding to and promoting the proteasomal degradation of the retinoblastoma protein (pRb) and its related pocket proteins, p107 and p130.^{19, 47-52}

Hypophosphorylated pRb (i.e. the active form) sequesters activator E2F transcription factors (E2F1-3), thereby inhibiting the progression from the G1 phase to the S phase of the cell cycle.⁴⁹ E7 preferentially targets and promotes the degradation of hypophosphorylated pRb leading to the release of activator E2Fs, which subsequently results in unscheduled cell cycle progression.⁴⁷⁻⁴⁹ Moreover, E7, through its interaction with p107 or p130, interferes with the DREAM complex.⁵² The DREAM complex is a multi-subunit transcriptional repressor complex that plays a key role during cellular quiescence (reversible cell cycle arrest in the G0 phase) and early G1 phase by repressing genes involved in cell cycle progression (G1/S and G2/M genes).⁵³ This complex binds to promoters of cell cycle genes, primarily through interactions with E2F sites or cell cycle genes homology region (CHR) sites.^{54, 55} As with its interaction with pRb, E7 binds to the p107 or p130 pocket proteins via its LxCxE motif, leading to the proteosomal degradation of the pocket proteins and therefore disturbing the DREAM complex formation.^{50, 52} This disruption releases the transcriptional repression imposed by the DREAM complex, enabling the activation of cell cycle-promoting genes via activator E2Fs, and the MMB complex (please refer to section 1.4.1 for detailed overview of these complexes), further adding to the uncontrolled proliferation.^{51, 56, 57}

Further, the HPV E6 and E7 oncoproteins likely contribute to malignant transformation through additional mechanisms. For example, E6 targets pro-apoptotic proteins other than p53, such as BAK, and interferes with tumor necrosis factor receptor signaling, further inhibiting cell death.^{58, 59} High-risk HPV E6 contains a unique PDZ (PSD-95/DLG/ZO-1)-binding motif, enabling it to interact with and promote the degradation of various proteins containing the PDZ domain, including several tumor suppressors.⁶⁰ Furthermore, E6 enhances telomerase activity by upregulating the transcription of the catalytic subunit of human telomerase (hTERT), which prevents replicative senescence and supports cellular immortalization.⁶¹ E7 also promotes genomic instability by inducing aberrant centrosome duplication, leading to chromosome missegregation and aneuploidy.⁶² Additionally, E7 inhibits cyclin-dependent kinase inhibitors, such as p21 and p27,⁶³ and can enhance the transcription of cell cycle genes through its interaction with B-MYB⁶⁴ (please refer to section 1.4.2), which further ensures sustained cell proliferation.⁶³ Given the essential roles the E6 and E7 oncoproteins play in attaining cancer hallmarks, they represent promising candidates for therapeutic interventions.⁶⁵

1.1.4 Prevention and treatment of HPV-associated cervical cancer

Prophylactic vaccination and routine screenings, such as the Papanicolaou (Pap) test and HPV testing, are the most effective strategies in preventing HPV-induced cancers. Currently, there are six licensed vaccines that offer protection against the most common high-risk HPV types⁶⁶

(Table 2). These vaccines are based on the recombinantly produced L1 protein of HPV, which forms virus-like particles (VLPs) that trigger the immune system to produce neutralizing antibodies.⁶⁶

Table 2. HPV vaccines.

Vaccine	Valency	HPV type
Cervarix, Cecolin, Walrinvax	Bivalent	HPV16, HPV18
Gardasil, Cervavac	Quadrivalent	HPV6, HPV11, HPV16, HPV18
Gardasil 9	Nonavalent	HPV6, HPV11, HPV16, HPV18, HPV31, HPV33, HPV45, HPV52, HPV58

Although cervical cancer is almost entirely preventable, the disease continues to be a major health concern, primarily due to the limited implementation of widespread vaccination and screening programs, particularly in low- and middle-income countries. Some of the key obstacles are the high cost of HPV vaccines and the need for continuous refrigeration during vaccine distribution, as disruptions can compromise vaccine efficacy.^{12, 67} To this end, researchers are working on developing thermostable, single-shot vaccines.^{68, 69} Moreover, new vaccine candidates aim to provide broader protection across HPV types by targeting conserved regions of the L2 protein.⁷⁰⁻⁷⁴ Of note, currently available prophylactic vaccines do not prevent cancer development in individuals with an established persistent HPV infection.^{71, 75} Consequently, despite the availability of preventive measures, cervical cancer, along with other HPV-related cancers, is expected to represent a major health challenge for decades to come. Therefore, the development of new and improved therapeutic strategies is urgently required.

Treatment options for cervical cancer vary depending on the stage of the disease. In the early stages, surgical excision of the lesion is typically the first-line treatment.⁷⁶ For more advanced cases, treatment involves a combination of surgery and adjuvant therapy: either radiotherapy, chemotherapy (CT), or chemoradiotherapy.⁷⁶⁻⁷⁸ Platinum-based chemotherapies, particularly Cisplatin, are widely used.⁷⁹ For individuals with advanced or recurrent cervical cancer, the concurrent use of Cisplatin with other agents such as Paclitaxel, Topotecan, 5-Fluorouracil (5-FU), or Gemcitabine is a frequently employed chemotherapeutic protocol in order to improve the clinical outcome.^{80, 81} As the disease progresses, additional strategies are being explored. For example, the use of targeted therapies, such as anti-angiogenic therapies using vascular endothelial growth factor (VEGF)-inhibitors. Bevacizumab, VEGF-targeting antibody, that blocks angiogenesis, has been integrated into treatment regimens for advanced stages of cervical cancer.⁸² Moreover, immunotherapeutic approaches are also being increasingly incorporated into treatment protocols. For example, Pembrolizumab is an immune checkpoint inhibitor that works by targeting the programmed death receptor-1 (PD-1) thereby reinstating the immune system's ability to recognize and effectively target cancer cells. It is being used to

treat recurrent or metastatic cervical cancers that express the programmed death ligand 1 (PD-L1).⁸³ Immunotherapeutic approaches also include therapeutic vaccines^{70, 73, 84} and adoptive T-cell therapies^{84, 85} as potential treatments for HPV-positive precancerous lesions and cervical cancer. Therapeutic vaccines aim to stimulate the immune response against HPV-infected cells by delivering HPV-specific antigens to antigen-presenting cells (APCs) which in turn activate cytotoxic and helper T cells.^{73, 86} Currently, there are several types of therapeutic HPV vaccines, including nucleic acid-, peptide and protein-, live vector-based, and whole cell vaccines, which are under development or undergoing preclinical and clinical trials.⁷⁰ Another novel approach is targeting the altered metabolism of HPV-positive cancer cells, which differs significantly from that of normal cells. Drugs like Metformin or Ciclopirox have shown promise in preclinical studies by repressing E6/E7 expression and inhibiting cancer cell proliferation and warrant further exploration under clinical conditions.⁸⁷⁻⁹⁰

Despite the progress in treatment strategies, challenges remain, particularly in addressing drug resistance, improving therapeutic efficacy, and developing alternative treatments for advanced cervical cancer. Therefore, ongoing research on combination therapies, novel targeted treatments, and immunotherapies is important to overcome current limitations and provide more effective treatment options for cancer patients.

1.2 Tumor hypoxia

Oxygen availability plays a critical role in shaping the malignant phenotype of cancer cells.⁹¹ Solid cancers, such as cervical cancer, often contain regions with oxygen concentrations well below physiological levels (<1.5-2% O₂), a condition defined as “hypoxia”.⁹²⁻⁹⁵ Hypoxia is recognized as a hallmark of solid tumors, as it is present in approximately 90% of solid cancers. Yet, it remains poorly represented in most *in vitro* studies, which are typically conducted under the conditions of higher oxygen levels (approximately 21% O₂) referred to as “normoxia”.⁹⁶ This discrepancy between experimental and pathological conditions represents a major limitation in many laboratory-based studies which fail to consider the oxygen levels found in solid tumors.

Two principal forms of tumor hypoxia have been identified: chronic hypoxia and cycling hypoxia.^{93, 97, 98} Chronic hypoxia arises due to oxygen diffusion limitations, typically affecting tumor cells situated more than 100 µm from the nearest functional blood vessel.^{93, 94} Vessels with irregular architecture and frequent vascular collapse following neoangiogenesis, combined with chemotherapy-induced vascular damage, collectively contribute to the development of chronically hypoxic regions within tumors.⁹²⁻⁹⁴ This form of hypoxia may also result from systemic factors such as anemia, and it can last multiple hours to days.^{94, 99} In contrast, cycling hypoxia is driven by transient perfusion deficits caused by abnormal tumor

vasculature, such as vessel occlusions and subsequent reperfusions, leading to oscillating episodes of hypoxia and reoxygenation over variable time intervals ranging from minutes to days.^{97, 98}

Hypoxic stress initiates a broad range of adaptive cellular responses that collectively promote tumor progression and resistance to therapy. A key mediator of these responses are the hypoxia-inducible factors (HIFs), transcription factors composed of a stably expressed β subunit, and an α subunit (HIF-1 α , HIF-2 α , or HIF-3 α) which is stabilized under low oxygen conditions.¹⁰⁰ HIFs promote the transcription of genes involved in cell survival, angiogenesis, immune modulation, and metabolic reprogramming under hypoxia.^{100, 101} One major cellular adaptation is a metabolic shift from oxygen-dependent oxidative phosphorylation under normoxic conditions to enhanced anaerobic glycolysis under hypoxia. This shift is primarily mediated by HIF-1 α -driven upregulation of glycolytic enzymes and glucose transporters, particularly glucose transporter 1 (GLUT1), thereby promoting Warburg-like metabolism.¹⁰¹⁻¹⁰⁶ Glycolysis is characterized by a significantly lower ATP yield, compared to oxidative phosphorylation, therefore, the typical metabolic signature of hypoxic cells entails increased glucose uptake to meet cellular energy demands.¹⁰⁷ This way tumor cells maintain ATP production under oxygen-deprived conditions, albeit at the cost of increased lactate production and acidosis.^{101, 103, 108, 109} Over time, this adaptation contributes to a microenvironment deprived of both oxygen and glucose. Beyond metabolic changes, hypoxic stress also drives cancer stem cell (CSC) maintenance,^{110, 111} angiogenesis,¹¹² and genomic instability,¹¹³ represses apoptosis¹¹⁴ and senescence pathways,^{115, 116} and promotes immune evasion¹¹⁷ by inducing immunosuppressive signaling and inhibiting the activity of effector immune cells.

1.2.1 Hypoxia and therapy resistance

Tumor cells in hypoxic regions undergo a range of adaptive changes that ultimately contribute to their resistance to therapy.⁹⁶ These changes are linked to both metabolic reprogramming and non-metabolic factors. One of the primary consequences of hypoxia-induced metabolic reprogramming is the acidification of the tumor microenvironment due to a shift towards increased lactate production.¹⁰⁹ This results in lower extracellular pH which enables a mechanism known as “ion trapping” where weakly basic chemotherapeutic agents become protonated and are subsequently sequestered in lysosomes upon entering the cell.¹⁰¹ Moreover, many chemotherapeutic agents depend on the presence of oxygen to exert their cytotoxic effects and therefore show reduced efficacy in hypoxic environments.¹¹⁸ Further, due to a hypoxia-induced proliferative halt, hypoxic cells are less susceptible to CT, which preferentially targets proliferating cancer cells.¹¹⁸ Another significant factor contributing to

chemoresistance in solid tumors is poor vascular perfusion, which results in hypoxia and subsequently limited drug delivery to these tumor regions.¹¹⁸ Hypoxia also induces reactive oxygen species (ROS) production, which in turn activates pathways that ultimately contribute to therapy resistance.^{119, 120} In the case of radiotherapy, the lack of molecular oxygen reduces the fixation of difficult-to-repair DNA lesions, contributing to radioresistance.¹²¹ Hypoxic regions also harbor CSCs, which are inherently more resistant to treatment compared to differentiated cells. This is in part due to hypoxia indirectly upregulating the expression of transporters involved in drug efflux via HIF-1 α activity, reducing drug efficacy in CSCs.^{118, 122} Hypoxia also contributes to the development of an immunosuppressive tumor microenvironment, which ultimately reduces the effectiveness of immune-based therapies, such as immune checkpoint inhibitors.^{123, 124} Moreover, emerging evidence indicates that, in addition to contributing to therapy resistance, hypoxic tumor cells are more likely to metastasize and cause recurrence following treatment.¹²⁵⁻¹³⁰ For instance, the capacity of hypoxic cancer cells to re-enter the cell cycle and continue proliferating upon reoxygenation, such as that occurring due to neovascularization¹³¹ or therapy-induced tumor shrinkage,¹³² may contribute to disease recurrence.

The challenges of targeting the therapeutically problematic population of hypoxic tumor cells have driven the development of treatment strategies designed to exploit the unique biology of the hypoxic microenvironment. Among the most promising are hypoxia-activated prodrugs (HAPs), a class of bioreductive agents activated in hypoxic conditions, allowing them to selectively target oxygen-deprived regions of solid tumors.¹³³ Other strategies under investigation include the use of certain bacteria that are capable of delivering drug-loaded nanoliposomes into hypoxic tumor regions or lysing hypoxic tumor cells, and oxygen-self-produced sonodynamic therapies.¹³⁴⁻¹³⁶ Moreover, small-molecule inhibitors that directly disrupt hypoxia-induced factors, such as HIF-1 α and HIF-2 α , or metabolic pathways regulated by these factors, may offer a complementary strategy to counteract therapy resistance associated with hypoxic tumors.¹³⁷ Collectively, these strategies are designed to counteract the inherent treatment resistance posed by hypoxia and to improve outcomes following cancer therapy.

1.3 Senescence

Cellular senescence classically refers to a stable, irreversible cell cycle arrest, typically occurring in the G1 phase (G1 exit) or potentially in the G2 phase (G2 exit).^{138, 139} Senescence can be triggered in response to a variety of stimuli, including telomere shortening or dysfunction (replicative senescence), exposure to chemical or physical stress such as genotoxic drugs and

radiation (stress-induced senescence), oncogene activation (oncogene-induced senescence), loss of a tumor suppressor gene, epigenetic events, and mitochondrial dysfunction.^{139, 140} Senescence can arise under both physiological and pathological conditions.¹³⁹ Unlike quiescence, which is a reversible cell cycle arrest in the G0 phase, e.g. triggered by limited nutrient or growth factor availability, senescence is associated with irreversible morphological and physiological changes, such as enlarged cell size, flattened cell morphology, and elevated lysosomal activity.^{141, 142} A commonly employed biomarker for detecting senescent cells is the senescence-associated β -Galactosidase (SA- β -Gal).¹⁴³ Although this enzyme is normally active in lysosomes at acidic pH, senescent cells exhibit SA- β -Gal activity at near-neutral pH due to its overexpression.¹⁴³ Since its introduction, SA- β -Gal has become a standard tool for identifying senescent cells.

Senescence serves as one of the crucial tumor-suppressive mechanism by halting the proliferation of cells that have undergone potentially oncogenic changes, such as irreparable DNA damage or oncogene activation.¹⁴⁴⁻¹⁴⁶ There are several pathways involved in senescence response. In the p53-p21 pathway, the stabilization of p53 induces the expression of the cyclin-dependent kinase (CDK) inhibitor p21.¹⁴⁷ p21 inhibits CDK2/4/6, thereby blocking phosphorylation of the Rb family members, pRb, p107 and p130. Hypophosphorylated p107 and p130 are part of the DREAM complex which inhibits transcription of cell cycle-promoting genes, halting cell cycle progression, particularly at the G1/S checkpoint⁵³ (please refer to section 1.4.1). While p21 is a key mediator of p53-dependent growth arrest and senescence, p53 also independently regulates a large set of other target genes linked to senescence. For instance, during oncogene-induced senescence, p53-driven upregulation of E2F7 contributes to the stable repression of E2F-dependent genes and reinforces the growth arrest.^{148, 149} The p16-pRb-E2F pathway represents another crucial senescence mechanism. p16 is a CDK inhibitor that targets CDK4/6, thereby maintaining pRb in its hypophosphorylated, active state, similar to the action of p21.¹⁵⁰ Active pRb sequesters E2Fs and inhibits the transcription of E2F-dependent genes required for G1/S transition.¹⁵⁰ In HPV-associated malignancies, however, the viral oncoproteins E6 and E7 actively disable the senescence-promoting functions of p53, pRb, and the DREAM complex, respectively (please refer to section 1.1.3).

1.3.1 Senescence and hypoxia in HPV-positive cervical cancer cells

Hypoxic tumor cells are more resistant to the induction of cellular senescence, an important consideration given that cervical cancers have a median oxygen concentration of approximately 1.2%.^{93, 151, 152} Previous studies of our group demonstrated that chronic hypoxia disrupts the virus/host cell crosstalk in HPV-positive cancer cells by repressing the viral E6 and

E6/E7 oncogene expression.¹¹⁶ HPV oncogene expression is, however, promptly restored upon reoxygenation.¹¹⁶ Mechanistically, this reversible suppression is linked to the activation of the PI3K/mTORC2/AKT signaling axis under hypoxia.¹⁵³ Both AKT isoforms (AKT1 and AKT2), which are phosphorylated by its upstream activators PIK3 and mTORC2 under hypoxia, mediate the repression of *E6/E7*, at least in part at the transcriptional level.¹⁵³ Importantly, hypoxic repression of *E6/E7* oncogenes is also dependent on glucose availability and is efficiently counteracted by culturing cells in media containing high glucose concentrations (25 mM).¹⁵³ Interestingly, hypoxia-mediated *E6/E7* repression does not induce cellular senescence, in stark contrast to experimental *E6/E7* silencing under normoxic conditions, e.g. by RNA interference (RNAi) or by ectopic expression of the HPV E2 protein (which suppresses *E6/E7* transcription).^{116, 154-156} One reason is the concurrent suppression of mTORC1 signaling (via the REDD1/TSC2 axis) under hypoxia.¹¹⁶ This enables hypoxic HPV-positive cancer cells to bypass senescence and enter a reversible dormant state, which is marked by a proliferative halt that can be overcome by reoxygenation.¹¹⁶ In contrast, senescence induction on *E6/E7* inhibition in normoxic HPV-positive cancer cells relies, at least partially, on mTORC1 activity.¹¹⁶ Lastly, hypoxic HPV-positive cells can also efficiently evade CT-induced senescence.^{97, 116}

Given that senescence is an important mechanism through which chemotherapy and radiotherapy can exert their anti-proliferative effects, disruption of this pathway under hypoxia presents a barrier to effective treatment.¹⁵⁷⁻¹⁶⁰ Moreover, the *E6/E7* repression in hypoxic HPV-positive cancer cells could play a role in promoting resistance to antiviral therapeutic strategies, such as small-molecule inhibitors of E6 and E7.^{161 162} Additionally, immunotherapies directed against *E6/E7*-derived peptides may be less effective in hypoxic niches as hypoxia could impair the viral antigen presentation.^{162, 163}

1.3.2 Senescence: dual roles in cancer

A defining feature of many senescent cells is the senescence-associated secretory phenotype (SASP), which plays a crucial role in facilitating the effects of senescence on the surrounding tissue microenvironment.¹⁶⁴ The SASP comprises a diverse array of bioactive molecules, such as growth factors, cytokines, chemokines, and proteases, which are released by senescent cells into the extracellular space.¹⁶⁵ In the context of cancer biology, SASP can exert both beneficial and detrimental effects.¹⁶⁶ Initially, SASP factors contribute to tumor suppression by facilitating immune-mediated clearance of senescent cancer cells.^{158, 164, 166} This anti-tumor effect is particularly evident during oncogene-induced senescence or after therapy-induced senescence.¹⁵⁸

However, the effects of the SASP are highly context-dependent. While it exerts tumor-suppressive functions during early tumorigenesis, accumulating evidence suggests that chronic or unresolved SASP activity may hold tumor-promoting effects.¹⁶⁶ SASP-driven remodeling of the tumor microenvironment can induce stem-like phenotype in neighboring cells, enhancing their tumorigenicity, invasiveness, and resistance to therapy.¹⁶⁷ Moreover, SASP contributes to an immunosuppressive microenvironment that facilitates immune evasion and promotes tumor growth.^{164, 166} These dual roles underscore the complexity of senescence and the need for careful consideration when designing senescence-inducing cancer therapies. One promising strategy could be the use of senolytic agents to selectively eliminate therapy-induced senescent cells and mitigate their pro-tumorigenic effects.¹⁵⁸

1.4 MYB transcription factors

The MYB family of transcription factors is evolutionarily conserved across eukaryotes, underscoring their importance in essential biological functions. In humans and other vertebrates, the MYB family consists of three members: A-MYB (*MYBL1*), B-MYB (*MYBL2*), and C-MYB (*MYB*).^{168, 169} *MYB* was the first identified member of the MYB family in humans, recognized as a cellular homolog of the *v-Myb* oncogene, the transforming gene of avian myeloblastosis virus (AMV) causing acute myeloblastic leukemia in chickens.¹⁷⁰ Structurally, all MYB proteins share several features, including a highly conserved helix-turn-helix (HTH) N-terminal DNA-binding domain (DBD) that contains three tryptophan-rich tandem motifs (R1, R2, and R3), a central transactivation domain (TAD), and a C-terminal negative regulatory domain (NRD).¹⁶⁹⁻¹⁷¹ The DNA recognition is primarily driven by the R2 and R3 motifs, with the R1 motif being dispensable.^{171, 172} MYB proteins recognize and bind to the common DNA consensus sequence (the MYB recognition or MYB-binding site (MBS): 5'-(YAACG/TG)-3'), with "Y" indicating a pyrimidine base (C or T), in order to transactivate gene expression.^{168, 170} The C-terminal regulatory domain of these proteins is conserved across species, with particular homology in its central region. This domain has a leucine zipper motif that can form a homodimer, thereby exerting a negative regulatory effect by interfering with DNA binding. Moreover, post-translational modifications in the C-terminal regulatory domain can disrupt its interaction with the DBD, inhibiting transcriptional activity.¹⁷⁰ Mutations or truncations of the C-terminal domain can result in the constitutive activation of MYB proteins, contributing to their oncogenic potential.¹⁷³

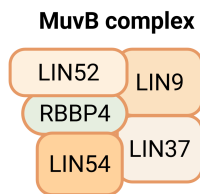
Despite their structural similarities, MYB family proteins associate with distinct co-factors, and their expression typically does not overlap, implying that they have different biological functions. *MYB* expression is predominantly restricted to colonic crypts, hematopoietic tissues,

and neural stem cells, while *MYBL1* is mainly expressed in the developing mammary glands, gonadal tissue, B lymphocytes, and the central nervous system. On the other hand, *MYBL2* has a more ubiquitous expression, being present in virtually all proliferating cells.^{170, 174, 175} Aberrant expression of *MYB* genes, e.g. due to gene amplification or transcriptional upregulation, is frequently observed in various cancers. Additionally, alternative splice variants and gene fusions of *MYB*, *MYBL1*, and *MYBL2* have been documented in certain cancers.^{168, 170}

1.4.1 B-MYB: a master cell cycle regulator

MYBL2 (MYB proto-oncogene like 2), which encodes B-MYB protein, is classified as an early G1/S cell cycle gene and is, accordingly, repressed by the multi-subunit DREAM complex during G0 and early G1 phases to prevent premature cell cycle entry.^{176, 177} The DREAM complex consists of dimerization partners (DP1, DP2, and DP3), pRb-like proteins (p107 or p130), E2Fs (E2F4-5), and the MuvB core complex (LIN9, LIN37, LIN52, LIN54, and RBBP4).¹⁷⁷ The DREAM complex binds to promoters of cell cycle genes through four distinct modes, most notably through E2F-binding sites and CHR sites.^{54, 55} In addition, two other cis-regulatory elements, the cell cycle-dependent element (CDE) and the CHR-like element (CLE) further support the DREAM complex binding and enhance its regulatory function.⁵³ Upon cell cycle initiation, DYRK1A-mediated phosphorylation of LIN52 (the MuvB core component) leads to the dissociation of p107/p130 and repressor E2Fs (E2F4-5) from the MuvB core, thereby disrupting the DREAM complex.¹⁷⁷ This allows activator E2Fs (E2F1-3) to bind to promoters of early G1/S cell cycle genes and initiate their transcription, including that of *MYBL2* (coding for B-MYB).^{177, 178} B-MYB can also act as a transcription factor independently by directly binding to MBS elements in target gene promoters, or it can act as a co-activator of CHR-responsive genes within the MMB complex.^{176, 179-181} B-MYB binds to the MuvB core (specifically LIN9 and LIN52) through its C-terminal domain and forms the MMB (B-MYB-MuvB) complex during the late G1/early S phase.^{182, 183} The MMB complex binds exclusively to genes containing CHR promoter elements via LIN54.^{54, 184, 185} As cells progress into the G2 and M phases, FOXM1 is recruited to the MMB complex, forming FOXM1-MMB and FOXM1-MuvB complexes.^{186, 187} The interaction between B-MYB and MuvB is essential for the recruitment of FOXM1 to the MuvB core. All three of these complexes activate the expression of G2/M cell cycle genes (Figure 2). Formation of FOXM1-MuvB complexes is followed by a phosphorylation-dependent proteasomal degradation of B-MYB.^{186, 187} In addition to cell cycle-specific regulation, the MMB complex can bind nucleosomes and initiate chromatin remodeling, facilitating DNA exposure and promoter accessibility.^{188, 189}

A



B

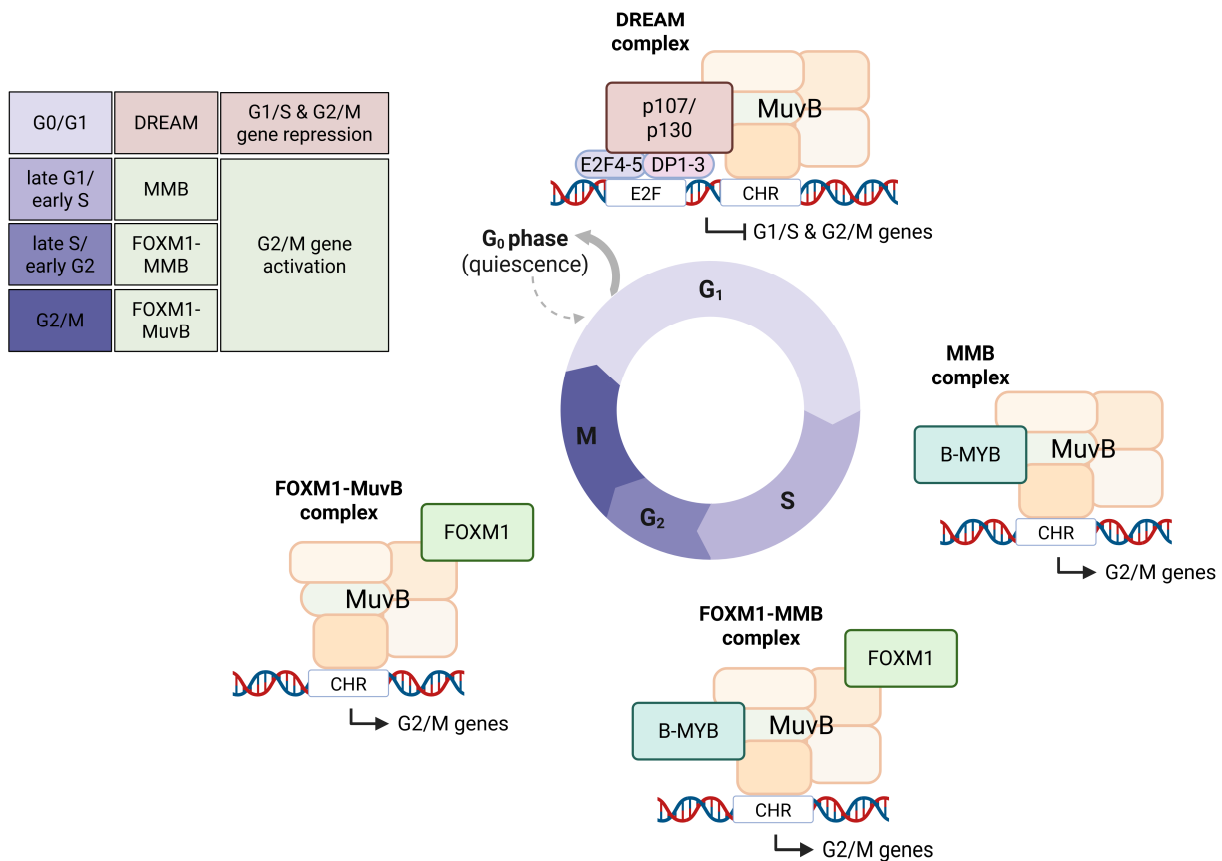


Figure 2. The MuvB cell cycle regulatory complexes. (A) MuvB core complex consists of LIN9, LIN37, LIN52, LIN54 and RBBP4 proteins. The MuvB complex associates with different co-factors and forms distinct transcriptional complexes which regulate cell cycle progression. (B) During G₀ (quiescence) and early G₁ phase, the MuvB core complex recruits the dimerization partners (DP1, DP2, and DP3), pRb-like proteins (p107 or p130), and repressor E2Fs (E2F4-5), forming the DREAM complex which binds to E2F and CHR promoter elements and represses genes involved in cell cycle progression (G₁/S and G₂/M genes). Upon cell cycle entry, the MuvB core complex disassociates from the DREAM components and recruits B-MYB, facilitating the switch from the repressor DREAM to the activator MMB (B-MYB-MuvB) complex. The MMB complex binds to CHR promoter elements and transactivates G₂/M genes. The binding of B-MYB to MuvB is a prerequisite for the recruitment of FOXM1 during the late S/early G₂ phases, forming the FOXM1-MMB complex. Subsequently, B-MYB undergoes phosphorylation-dependent proteasomal degradation, while FOXM1 remains bound to the MuvB core complex, forming the FOXM1-MuvB complex during the G₂ and M phases. Like the MMB complex, FOXM1 complexes activate and maintain the expression of G₂/M genes. Activator MuvB complexes (MMB, FOXM1-MMB, and FOXM1-MuvB) ensure proper cell cycle progression. Image created with BioRender.

Apart from the DREAM-mediated regulation, *MYBL2* expression can be regulated post-transcriptionally by microRNAs during cellular senescence.¹⁹⁰ Beyond transcriptional regulation, the activity of B-MYB is also controlled by post-translational modifications and protein-protein interactions.¹⁹¹ During the late G1 and S phases, B-MYB undergoes phosphorylation by Cyclin A/E-CDK2 complexes, which promotes its transactivation activity.¹⁹² Interestingly, while Cyclin A-dependent phosphorylation activates B-MYB, Cyclin A also promotes its degradation via ubiquitin-mediated proteolysis, potentially to ensure proper progression through the cell cycle.¹⁹³ The transcriptional co-activator p300 can enhance B-MYB activity through acetylation, particularly when B-MYB is phosphorylated by Cyclin A.¹⁹⁴ In contrast, Cyclin D1 negatively regulates B-MYB activity by directly interacting with it and inhibiting its transactivation potential.¹⁹⁵

In addition to its role in cell cycle regulation, B-MYB can also contribute to the maintenance of genomic stability. Specifically, B-MYB can form a complex with Clathrin and Filamin (Myb-Clafl complex), which is needed for Clathrin localization at the mitotic spindle and the stabilization of kinetochore fibers.^{176, 196} Loss of B-MYB in embryonic stem cells disrupts DNA replication by causing replication fork stalling and DNA double-strand breaks, suggesting that B-MYB plays a protective role against genomic damage by supporting proper cell cycle progression.^{176, 197} Moreover, B-MYB knockdown has been linked to structural chromosomal instability, as evidenced by features such as chromosomal fragmentation, end-to-end fusions, altered chromatid morphology, and chromatid loss.¹⁷⁶

1.4.2 B-MYB and cancer

B-MYB has emerged as a well-established oncogene. Deregulation of B-MYB has been strongly linked to tumorigenesis, with its overexpression observed across various cancer types including colorectal cancer,¹⁹⁸ hepatocellular carcinoma,¹⁹⁹ cervical cancer,²⁰⁰ breast cancer,²⁰¹ neuroblastoma,²⁰² and lung cancer.²⁰³ Elevated B-MYB expression correlates with poor clinical outcomes and is associated with aggressive tumor behavior. In addition, amplification of its chromosomal locus 20q13 is found in several cancers.²⁰⁴⁻²⁰⁶ B-MYB contributes to malignancy by bypassing cell cycle checkpoints and driving deregulated cell cycle progression and proliferation.²⁰⁷ Moreover, B-MYB is associated with therapy resistance due to its ability to transactivate the expression of anti-apoptotic genes and indirectly inhibit DNA damage response, thereby enhancing cell survival.^{208, 209}

The HPV E7 oncoprotein may contribute to the deregulation of host cell cycle control by modulating B-MYB activity through both indirect and direct mechanisms. Indirectly, E7 activates *MYBL2* transcription by disrupting the pRb-E2F and DREAM repressor complexes.⁴⁷

By binding to pRb, E7 releases activator E2Fs, thereby enhancing E2F-dependent transcription, including that of *MYBL2*.⁴⁷ Additionally, E7 prevents the assembly and function of the repressive DREAM complex, further promoting *MYBL2* expression.^{52, 207} Beyond these indirect effects, E7 has also been reported to directly interact with B-MYB and enhance mitotic gene expression through the MMB complex.⁶⁴ Due to its central role in driving cancer cell proliferation, survival, and therapy resistance, B-MYB and its downstream effectors are being explored as potential therapeutic targets in B-MYB-driven malignancies.²⁰⁸

1.4.3 B-MYB and senescence

Accumulating evidence has revealed that B-MYB can also play a significant role in cellular senescence control since it prevents cellular senescence by maintaining cell cycle progression and proliferation, and suppressing senescence-associated pathways.^{210, 211} For instance, B-MYB can bind to the promoter of the *CDKN2A* gene (encoding p16), thereby downregulating its expression.²¹² As a consequence, B-MYB could, indirectly, interfere with p16-pRb-mediated senescence. Moreover, other studies have demonstrated that high B-MYB expression can also bypass senescence through the reconstitution of the MMB complex and promote the DREAM complex disassembly, possibly by interfering with the phosphorylation of LIN52, although the precise molecular mechanism remains to be elucidated.^{213, 214} B-MYB has also been reported to rescue *ras*-induced senescence in primary mouse embryonic fibroblasts.²¹⁵ Importantly, several studies have demonstrated that B-MYB expression is strongly repressed during the onset of senescence. Senescence-associated microRNAs, miR-29 and miR-30 target and repress B-MYB expression during pRb-E2F-induced senescence.¹⁹⁰ Targeted knockdown of B-MYB using RNA interference has been sufficient to trigger senescence in both primary and cancer cells.^{216, 217}

Together, these findings underscore the potential of B-MYB to act as a key negative regulator of cellular senescence and imply that B-MYB may serve as a promising therapeutic target for inducing senescence in cancer cells, particularly in tumors that rely on its sustained expression.

1.4.4 A-MYB

A-MYB, encoded by the *MYBL1* gene, is a member of the MYB transcription factor family that exhibits the most restricted, tissue-specific expression pattern among its paralogs.¹⁶⁸ It is highly expressed in spermatocytes where it functions as a master regulator of meiosis, and it is also involved in mammary gland development. *MYBL1*-null mice are viable but males display

infertility due to the arrest of spermatogenesis. Female *MYBL1*-null mice display defects in breast tissue development following pregnancy.²¹⁸ In human hematopoietic cells, high A-MYB expression is largely confined to a subset of mature tonsillar B lymphocytes, characterized by the CD38⁺, CD39⁻, and predominantly sIgM⁻ phenotype, suggesting A-MYB's localization within the germinal centers.²¹⁹ Studies examining B cell development and malignancy have further shown that A-MYB is strongly expressed in Burkitt's lymphoma cell lines, as well as sIg⁺-B acute lymphoblastic leukemia, and in a subset of B-chronic lymphocytic leukemias.²²⁰ A-MYB also serves as a marker of centroblasts (proliferating B-cells that undergo clonal expansion).²²¹ Furthermore, A-MYB is highly expressed in the central nervous system.²²² Truncations and rearrangements of the *MYBL1* gene have been implicated in various pediatric and low-grade gliomas, such as angiocentric glioma, isomorphic diffuse glioma, and diffuse astrocytoma. These *MYBL1* alterations are now considered molecular hallmarks of specific glioma subtypes and are of increasing diagnostic relevance.²²³⁻²²⁶ Moreover, *MYBL1* rearrangements have been identified in rare type of triple-negative breast cancer (TNBC),^{227, 228} as well as within rare vulvar malignancies.²²⁹ *MYBL1* has been shown to promote angiogenesis and therapy resistance in hepatocellular carcinoma.²³⁰ Additionally, gene fusions involving *MYBL1* have been reported in a rare form of lung cancer,²³¹ and elevated *MYBL1* expression has been observed in clear cell renal carcinoma.²³² Moreover, a recent study revealed an emerging pro-proliferative and anti-apoptotic role for A-MYB in the absence of B-MYB, further supporting its broader oncogenic potential in diverse tissues.²³³ This effect may stem from A-MYB's ability to bind and activate target genes also regulated by B-MYB.

1.5 Research objectives

Tumor hypoxia is a hallmark of solid tumors and is associated with increased resistance to therapy and poor patient outcomes. Hypoxia enables HPV-positive cancer cells to escape from senescence, despite effective growth inhibition and *E6/E7* oncogene repression, a condition that typically induces senescence under normoxia. Additionally, hypoxic tumor cells can evade pro-senescent CT.

The main aim of my studies is to gain insights into the mechanisms underlying these differential phenotypic responses of normoxic and hypoxic HPV-positive cancer cells. A particular emphasis will be placed on the role of the senescence regulator B-MYB and its paralog A-MYB. The following key questions will be addressed:

- (1) What are the differences in the growth response and cell cycle regulation between normoxic and hypoxic cancer cells? This objective will include comparative analyses of HPV-positive cervical cancer cells cultivated under normoxia, hypoxia, or upon *E6/E7* silencing under normoxia. The expression of key cell cycle regulators will be investigated, complemented by flow cytometry-based cell cycle analyses. It further will be studied whether hypoxia-mediated effects are limited to HPV-positive cells, whether external factors (e.g., glucose availability) can modulate these effects, and whether these effects may influence the response to the pro-senescent chemotherapeutic agent Etoposide.
- (2) What is the expression pattern of B-MYB in normoxic and hypoxic cells and what is the functional relevance of B-MYB for the senescence response of HPV-positive (and HPV-negative) cancer cells? This objective will focus on the phenotypic consequences of B-MYB silencing, such as its effects on cell proliferation, expression of B-MYB target genes, cell cycle regulation and senescence, both under normoxia and hypoxia.
- (3) Does A-MYB contribute to senescence regulation, and can it compensate for the loss of B-MYB? A-MYB will be silenced individually or in combination with B-MYB, and downstream effects on cell proliferation, target gene expression, cell cycle distribution, and senescence will be analyzed under normoxia. These analyses will be extended to hypoxic conditions and will include investigating the expression pattern of A-MYB in hypoxic cells and determining whether it affects the phenotypic response to B-MYB repression.

Overall, these studies aim to gain novel insights into the senescence regulation in normoxic and hypoxic HPV-positive cancer cells, with a focus on elucidating the roles of B-MYB and A-MYB in these processes. It is further hoped that a better understanding of these regulatory phenomena may ultimately allow the identification of novel options for more effectively targeting the therapeutically highly problematic population of hypoxic cancer cells.

CHAPTER 2

RESULTS

2 Results

2.1 Cell cycle regulation in HPV-positive and HPV-negative cancer cells under hypoxia

2.1.1 E6/E7 repression by RNAi or by hypoxia differentially affects cell cycle regulators in HPV-positive cancer cells

Previous studies have shown that cell cycle arrest and growth inhibition triggered by E6/E7 knockdown become irreversible through rapid senescence induction under normoxia.^{154, 156, 234, 235} In contrast, whereas hypoxia also leads to effective E6/E7 repression and growth inhibition, these effects are reversible by reoxygenation.¹¹⁶ In order to explore the mechanisms behind these distinct phenotypic responses, I compared the expression of key factors involved in cell cycle regulation and growth control in HPV-positive cancer cells, following E6/E7 repression by RNA interference (RNAi) or by hypoxia (Figure 3).

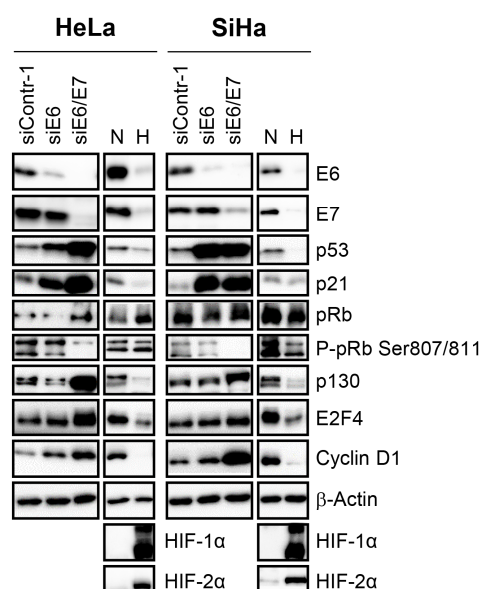


Figure 3. Repression of E6/E7 by RNAi or by hypoxia: differential expression of cell cycle regulators in HPV-positive cancer cells. HeLa or SiHa cells were transfected with siE6, siE6/E7, or control siRNA (siContr-1) and grown for 72 h (left panels for each cell line) or were cultivated under normoxia (N; 21% O₂) or hypoxia (H; 1% O₂) for 24 h (right panels for each cell line). Immunoblot analyses of E6, E7, p53, p21, pRb, P-pRb Ser807/811, p130, E2F4, Cyclin D1, hypoxia markers HIF-1α and HIF-2α, and β-Actin protein levels. Figure content and corresponding legend adapted from Velimirović et al., 2025.²³⁶

HPV18-positive HeLa and HPV16-positive SiHa cells were transfected with siRNA pools targeting either *E6* oncogene expression alone or the *E6/E7* oncogene expression in combination. In line with previous findings,²³⁴ E6/E7 repression, similar to E6 repression alone, resulted in a pronounced increase in protein levels of p53 and its downstream effector, p21, compared to control siRNA-transfected cells. A key distinction between silencing the expression of E6/E7 versus E6 alone is the persistence of the E7 protein in the latter case,

which can interfere with the function of the pocket proteins pRb^{48, 49} and p130.⁵⁰⁻⁵² Through this mechanism, E7 disrupts two major tumor suppressive pathways, the pRb-E2F pathway, and the DREAM complex-mediated repression pathway. Accordingly, E6/E7 silencing was accompanied by a marked decrease in phosphorylated pRb (P-pRb, i.e. the inactive form) levels and increased levels of the key DREAM complex components, p130 and E2F4. Moreover, Cyclin D1 protein levels, commonly upregulated in senescent cells,²³⁷ were elevated (Figure 3, left panels for each cell line).

Next, the expression of the same regulatory factors was analyzed in HPV-positive HeLa and SiHa cells exposed to hypoxia (H, 1% O₂) for 24 h. Remarkably, and in strong contrast to RNAi-mediated E6/E7 repression under normoxia, E6/E7 repression by hypoxia did not result in increased levels of p53, p21, p130, E2F4, and Cyclin D1, with most rather showing reduced expression, compared to normoxia (N, 21% O₂). Levels of P-pRb (inactive form) were only slightly, if at all, decreased compared to normoxia (Figure 3, right panels for each cell line).

In view of this discrepant cellular response observed under normoxic E6/E7 repression by RNAi or hypoxia-linked E6/E7 repression, I investigated whether these differences were reflected in transcriptional repression programs associated with cell cycle regulation. Specifically, I focused on the transcriptional outputs of the p53-p21, the pRb-E2F, and the DREAM pathways. These pathways are disrupted during HPV infection, but their reconstitution upon E6/E7 repression can induce senescence by downregulating cell cycle-promoting genes and enforcing cell cycle arrest.²³⁴ Of note, these pathways are a part of a tightly interconnected cell cycle gene regulatory network and many of their downstream targets overlap.¹⁸⁴ To this end, I analyzed two independently generated transcriptome data sets from HeLa cells, in which E6/E7 expression was either blocked by E6/E7-targeting siRNAs under normoxia²³⁸ or by hypoxia (unpublished data), using gene set enrichment analyses (GSEA) and the molecular signature database (MSigDB) (Figure 4). In line with functional reconstitution of the DREAM complex following RNAi-mediated E6/E7 knockdown, GSEA revealed a downregulation of the target genes of the DREAM complex (gene set: FISHER_DREAM_TARGETS),¹⁸⁴ indicated by a strong negative enrichment, with a normalized enrichment score (NES) of -4.42 (Figure 4A, upper panel). Further, knockdown of E6/E7 led to a negative enrichment of the FISHER_G1_S_CELL_CYCLE¹⁸⁴ (NES: -1.94) (Figure 4B, upper panel) and FISHER_G2_M_CELL_CYCLE¹⁸⁴ gene sets (NES: -3.53) (Figure 4C, upper panel), which are associated with G1/S and G2/M checkpoint regulation, respectively. These results are consistent with the efficient and rapid G1 cell cycle arrest observed upon E6/E7 knockdown²³⁴ and in accordance with the concept that p53-p21, pRb-E2F, and the DREAM signaling axes mediate cell cycle arrest through transcriptional repression of genes promoting cell cycle progression. Remarkably, while hypoxia also leads to a strong downregulation of E6/E7

expression, the response of genes involved in cell cycle progression strongly differed as hypoxic HeLa cells exhibited positive enrichment of all three gene sets (NES: +1.33, +1.17, and +1.58, respectively) (Figure 4A, B, C, lower panels).

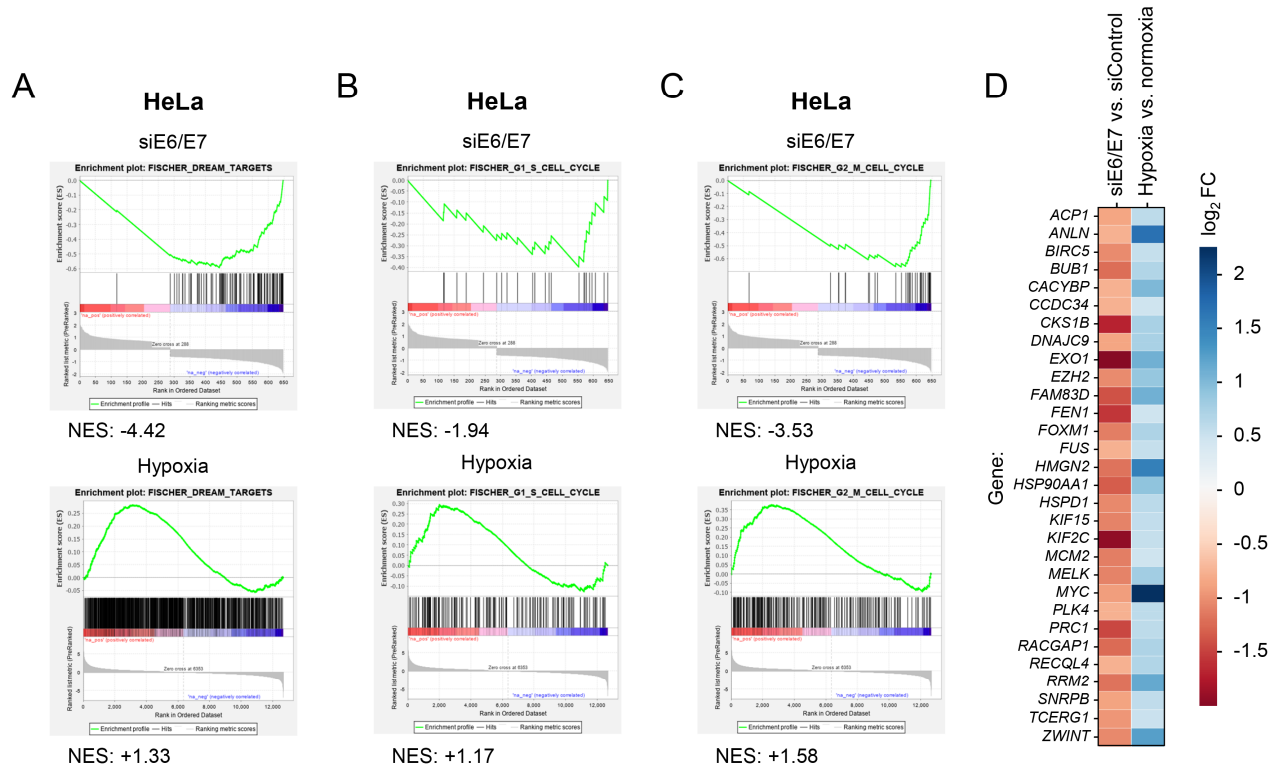


Figure 4. GSEA reveals distinct transcriptomic signatures upon E6/E7 repression by RNAi or by hypoxia in HPV-positive cancer cells. Gene set enrichment analysis (GSEA) was applied to available transcriptomic data from HeLa cells transfected with siE6/E7 and grown for 72 h, as well as to unpublished transcriptomic data from HeLa cells cultivated under hypoxia (1% O₂) for 16 h. Log₂-transformed fold change (log₂FC) values were used as input for pre-ranked GSEA. Enrichment plots for the gene sets (A) FISCHER_DREAM_TARGETS (NES: -4.42, FDR: <1x10⁻³; NES: +1.33, FDR: <1x10⁻³), (B) FISCHER_G1_S_CELL_CYCLE TARGETS (NES: -1.94, FDR: 6x10⁻³; NES: +1.17, FDR: 1.6x10⁻¹), and (C) FISCHER_G2_M_CELL_CYCLE (NES: -3.53, FDR: <1x10⁻³; NES: +1.58, FDR: <1x10⁻³) for each condition are shown. NES, normalized enrichment score; FDR, false discovery rate. (D) A heat map depicting log₂FC values of leading-edge genes from the FISCHER_DREAM_TARGETS set that are common between both conditions (siE6/E7 vs. siControl and hypoxia vs. normoxia) is shown. The color scale reflects gene downregulation (red) and upregulation (blue).

To enable the comparison between the two independent transcriptome data sets, one comparing siRNA-mediated E6/E7 knockdown to control (siE6/E7 vs. siControl)²³⁸ and the other comparing hypoxia to normoxia, leading-edge genes (contributing to the enrichment of the FISCHER_DREAM_TARGETS gene set) which were shared between both data sets, were extracted from the GSEA outputs and visualized in a heat map (Figure 4D). Notably, while these genes were downregulated following E6/E7 knockdown under normoxic conditions, their expression was maintained or even elevated under hypoxia. This suggests that, despite both

experimental conditions resulting in effective E6/E7 repression, the downstream transcriptional programs modulated by these viral oncogenes are differentially affected.

To validate the GSEA results, I further assessed the expression of several key DREAM and/or pRb target genes involved in cell cycle progression by mRNA and protein analyses (Figure 5). Accordingly, genes such as *MYBL2* (coding for B-MYB), *FOXM1*, *CCNA2* (coding for Cyclin A2), *CCNB1* (coding for Cyclin B1), *CCNB2* (coding for Cyclin B2), *CDC25C*, and *RRM2* were significantly downregulated following RNAi-mediated E6/E7 repression, but not E6 repression alone, under normoxia in both HeLa and SiHa cells. Remarkably, under hypoxia-induced E6/E7 repression, these genes were either not, or much less downregulated compared to normoxia (Figure 5A). These regulatory differences were similarly mirrored at the protein level (Figure 5B).

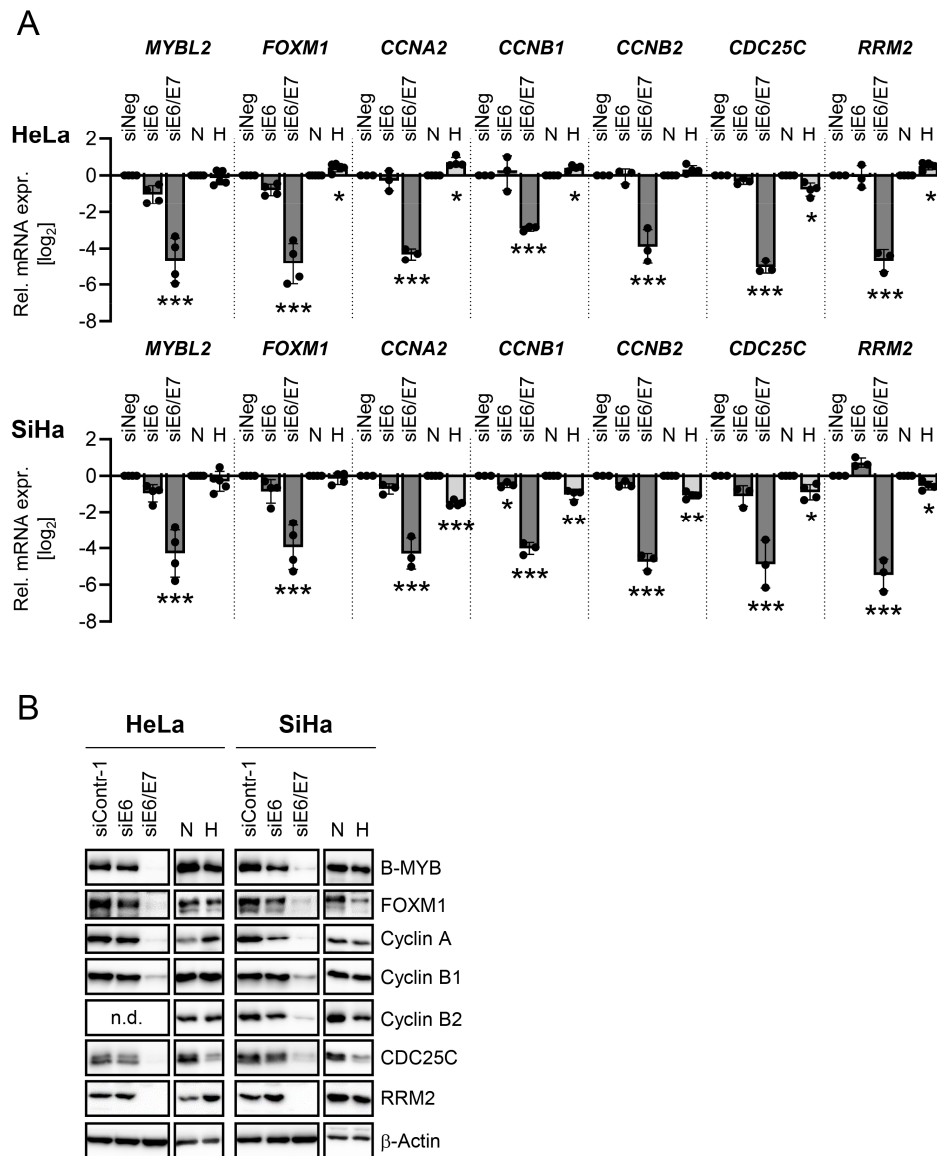


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Figure 5. Repression of E6/E7 by RNAi or by hypoxia: differential expression of factors involved in cell cycle progression in HPV-positive cancer cells. HeLa or SiHa cells were transfected with siE6, siE6/E7, or control siRNA (siContr-1 or siNeg) and grown for 72 h or were cultivated under normoxia (N; 21% O₂) or hypoxia (H; 1% O₂) for 24 h. (A) qRT-PCR analyses of *MYBL2* (coding for B-MYB), *FOXM1*, *CCNA2* (coding for Cyclin A2), *CCNB1* (coding for Cyclin B1), *CCNB2* (coding for Cyclin B2), *CDC25C*, and *RRM2* mRNA levels. Shown are individual data points and mean expression levels with standard deviation (SD) ($n \geq 3$) relative to the expression of siNeg or under normoxia, respectively ($\log_2FC$). Statistical significance was assessed using one-way ANOVA (siNeg, siE6, siE6/E7) or a two-sided *t*-test (N, H). * $p \leq 0.05$, ** $p \leq 0.01$, and *** $p \leq 0.001$. (B) Immunoblot analyses of B-MYB, FOXM1, Cyclin A, Cyclin B1, Cyclin B2, CDC25C, RRM2, and β -Actin protein levels. n.d., not determined. Figure content and corresponding legend adapted from Velimirović et al., 2025.²³⁶

Together, these findings indicate that although E6/E7 expression is strongly repressed under normoxia, via RNAi, or by hypoxia, downstream factors that can mediate G1 cell cycle arrest and senescence through repression of cell cycle-promoting genes are efficiently induced only under normoxia, but remain largely unaffected under hypoxia.

2.1.2 Hypoxia inhibits the growth of HPV-positive cancer cells without inducing pronounced changes in their cell cycle profiles

Based on the discrepant regulation of cell cycle-promoting factors observed following E6/E7 repression by RNAi or hypoxia, I next conducted comparative cell cycle analyses in HeLa and SiHa cells using flow cytometry to determine whether these findings translate into differences in cell cycle regulation (Figure 6). Consistent with mRNA and protein analyses, which demonstrated the downregulation of genes involved in cell cycle progression (Figure 5), RNAi-mediated E6/E7 silencing led to a profound G1 arrest in both cell lines (Figure 6A, upper panels for each cell line). In stark contrast, hypoxia caused only a modest increase in G1 and/or G2 populations, despite E6/E7 repression (Figure 6A, upper panels for each cell line). To further examine the effects on cell cycle progression, cells were treated with Nocodazole,²³⁹ which induces a G2/M cell cycle arrest in proliferating cells by inhibiting microtubule polymerization. As expected, Nocodazole treatment of (proliferating) control siRNA-transfected or untransfected normoxic cells resulted in a marked accumulation of cells in the G2/M phase. Conversely, following RNAi-mediated repression of E6/E7, no substantial change in the cell cycle profile was observed upon Nocodazole treatment, further confirming that these cells were efficiently growth arrested (Figure 6A, lower panels for each cell line). Remarkably, the response of hypoxic HeLa and SiHa cells to Nocodazole treatment was also limited, showing only slight alterations in cell cycle distribution (Figure 6A, lower panels for each cell line).

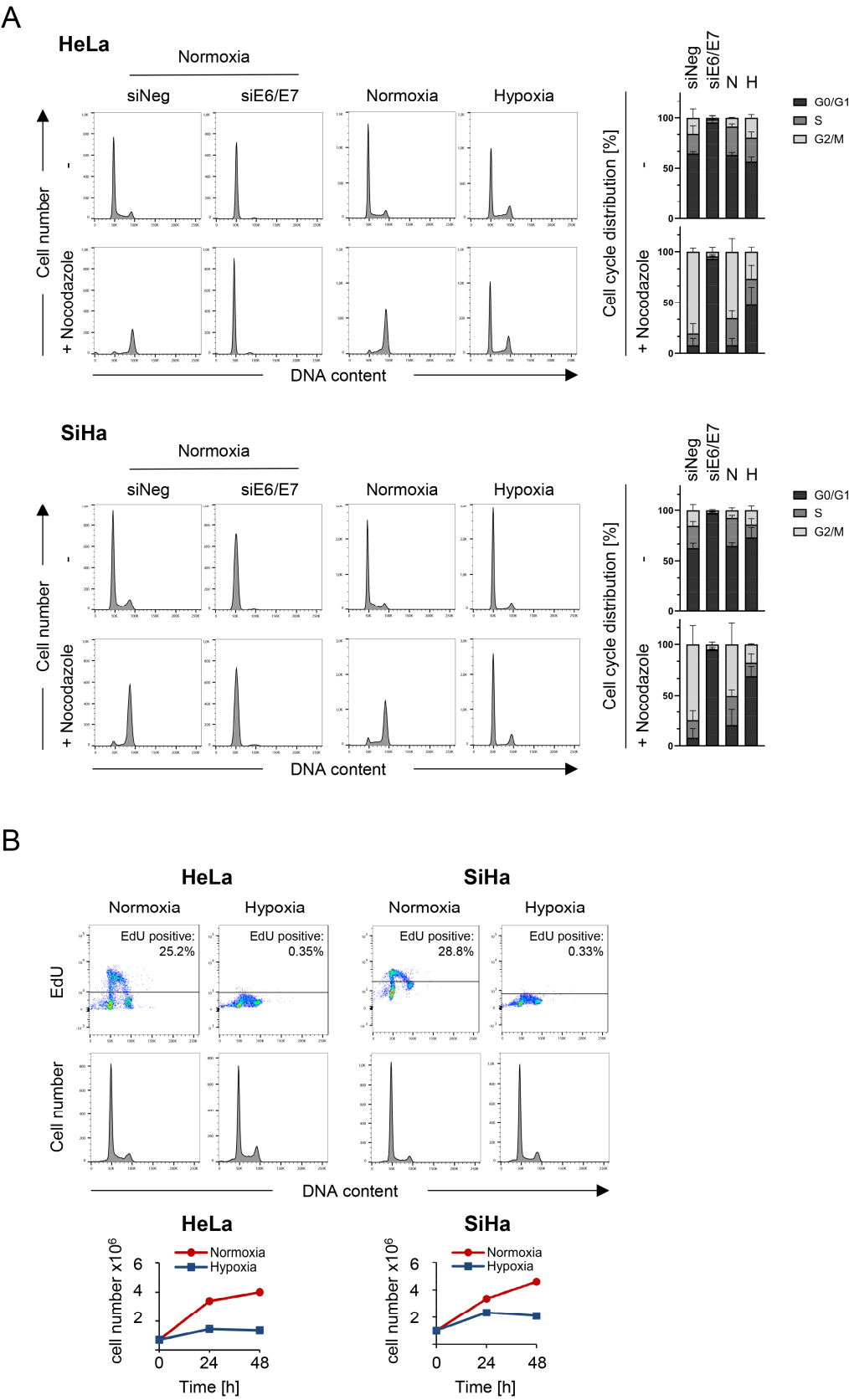


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Figure 6. Hypoxia inhibits the growth of HPV-positive cancer cells without inducing pronounced changes in their cell cycle profiles. (A) HeLa or SiHa cells were transfected with siE6/E7 or control siRNA (siNeg) and grown for 72 h or were cultivated under normoxia (N; 21% O₂) or hypoxia (H; 1% O₂) for 48 h. Nocodazole (0.1 µg/mL) or solvent control were added during the last 20 h of cultivation. Cell cycle distribution was assessed using flow cytometry. Shown are cell cycle profiles from representative experiments (left panels) and averaged percentages of cell populations in the individual cell cycle phases from 3 independent experiments with SD (right panels). (B) EdU incorporation assays and corresponding cell cycle profiles of HeLa and SiHa cells cultivated under normoxia or hypoxia for 24 h (upper panels). Growth curves of HeLa and SiHa cells obtained in parallel to the respective EdU assays. Viable cell numbers were determined at the indicated time points using the trypan blue exclusion test (lower panels). Figure content and corresponding legend adapted from Velimirović et al., 2025.²³⁶

This suggests that despite exhibiting cell cycle profiles similar to those of proliferating, normoxic cells, hypoxic cells are growth-arrested, albeit without pronounced changes in their cell cycle profile. In order to directly assess their proliferative capacity, EdU cell proliferation assays were performed (Figure 6B). This method identifies actively dividing cells by detecting the incorporation of 5-Ethynyl-2'-Desoxyuridin (EdU), a thymidine analog, into newly synthesized DNA during replication²⁴⁰ (please refer to section 4.3.8). EdU assays revealed that DNA replication was halted in the S phase under hypoxia in both HeLa and SiHa cells, as evidenced by a reduction in EdU-positive cells relative to normoxia, indicating that these cells were no longer actively synthesizing DNA despite exhibiting comparable cell cycle profiles (Figure 6B, upper panels for each cell line). Further, the anti-proliferative effect of hypoxia was also supported by the results of proliferation assays conducted in parallel, revealing that hypoxic HeLa and SiHa cells exhibited marked growth inhibition over time (Figure 6B, lower panels for each cell line).

Taken together, these findings show that in contrast to the pronounced G1 arrest observed upon RNAi-mediated E6/E7 repression in normoxic cells, hypoxia – which also blocks E6/E7 expression – inhibits the proliferation of HPV-positive cancer cells without causing a substantial cellular accumulation in a specific cell cycle phase. This suggests that the mechanisms driving growth arrest under hypoxic conditions differ from those triggered by RNAi-mediated silencing of E6/E7 under normoxia.

2.1.3 Hypoxia-mediated cell cycle regulation is independent of HPV status

Next, I was interested in whether the above-described effects of hypoxia on cell cycle regulation are restricted to HPV-positive cells only. Interestingly, mirroring my observations in HPV-positive cells, hypoxia limited the G2/M cell cycle arrest typically induced by Nocodazole treatment in normoxic (proliferating) HPV-negative osteosarcoma U2OS cells (Figure 7A). This suggests that U2OS cells also undergo growth inhibition under hypoxic conditions.

Furthermore, immunoblot analyses revealed that hypoxia induced a similar response of the investigated cell cycle regulators in U2OS cells, as observed in HPV-positive cancer cells. These included B-MYB, Cyclin A, Cyclin B1, Cyclin B2, and CDC25C (Figure 7B). A similar response was also observed in additionally tested HPV-negative cell lines, including the colon carcinoma HCT116, breast cancer MCF-7, non-small cell lung carcinoma H1299, human embryonic kidney HEK293T, and the spontaneously immortalized keratinocyte HaCaT cell lines (Figure 7C).

Collectively, these results indicate that the hypoxia-mediated alterations of cell cycle-promoting factors and the associated proliferation arrest are not restricted to HPV-positive cells. Rather, these findings likely reflect a more general response to low oxygen levels that is conserved across cells of different histopathological origin.

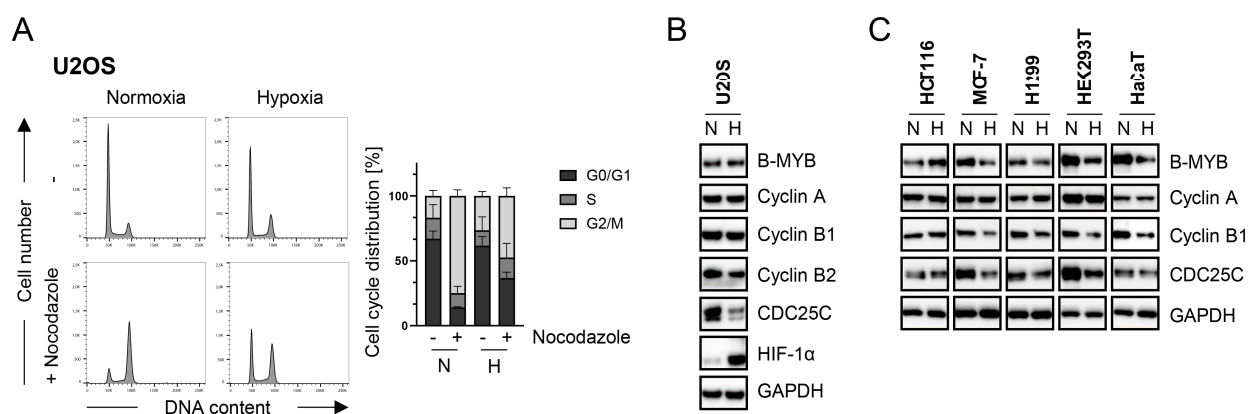


Figure 7. Hypoxia-mediated cell cycle regulation is independent of HPV status. (A) U2OS cells were cultivated under normoxia (N; 21% O₂) or hypoxia (H; 1% O₂) for 48 h and treated with Nocodazole (0.1 µg/mL) or solvent control for the last 20 h of cultivation. Cell cycle distribution was assessed using flow cytometry. Shown are cell cycle profiles from representative experiments (left panels) and averaged percentages of cell populations in the individual cell cycle phases from 2 independent experiments with SD (right panels). (B) U2OS cells were cultivated under normoxia or hypoxia for 24 h. Immunoblot analyses of B-MYB, Cyclin A, Cyclin B1, Cyclin B2, CDC25C, HIF-1α, and GAPDH protein levels. (C) HCT116, MCF-7, H1299, HEK293T, or HaCaT cells were treated as described in (B). Immunoblot analyses of B-MYB, Cyclin A, Cyclin B1, CDC25C, and GAPDH protein levels. Figure content and corresponding legend adapted from Velimirović et al., 2025.²³⁶

2.1.4 Glucose availability modulates hypoxia-induced growth arrest

Hypoxia induces metabolic reprogramming by shifting cells from oxidative phosphorylation, an oxygen-dependent and energy-efficient process, to anaerobic glycolysis (Warburg-like metabolism) via HIF-1α activation.²⁴¹ Since the ATP yield of anaerobic glycolysis is only 2 ATP per glucose molecule, 18 times less than oxidative phosphorylation, hypoxic cells compensate by increasing glucose uptake to meet their energy demands.¹⁰⁷ To investigate the influence of

glucose availability on the hypoxia-induced growth arrest, HPV-positive HeLa and SiHa cells were cultivated under normoxic and hypoxic conditions in media containing either physiological serum glucose concentrations (5.5 mM) or medium containing unphysiologically high glucose concentrations (25 mM) (Figure 8). In line with a previous study,¹⁵³ immunoblot analyses revealed that while hypoxia consistently stabilized hypoxia markers, HIF-1 α and HIF-2 α , a marked downregulation of the E6/E7 oncoproteins occurred only under conditions when both oxygen (H) and glucose (5.5 mM) were limited, whereas glucose limitation alone under normoxic conditions did not impact E6/E7 expression (Figure 8A). In contrast, E6/E7 levels remained stable under hypoxia when cells were cultivated under high glucose conditions (25 mM), indicating that hypoxic repression of viral oncoprotein expression depends on glucose supply, with lower glucose levels facilitating hypoxic E6/E7 repression.

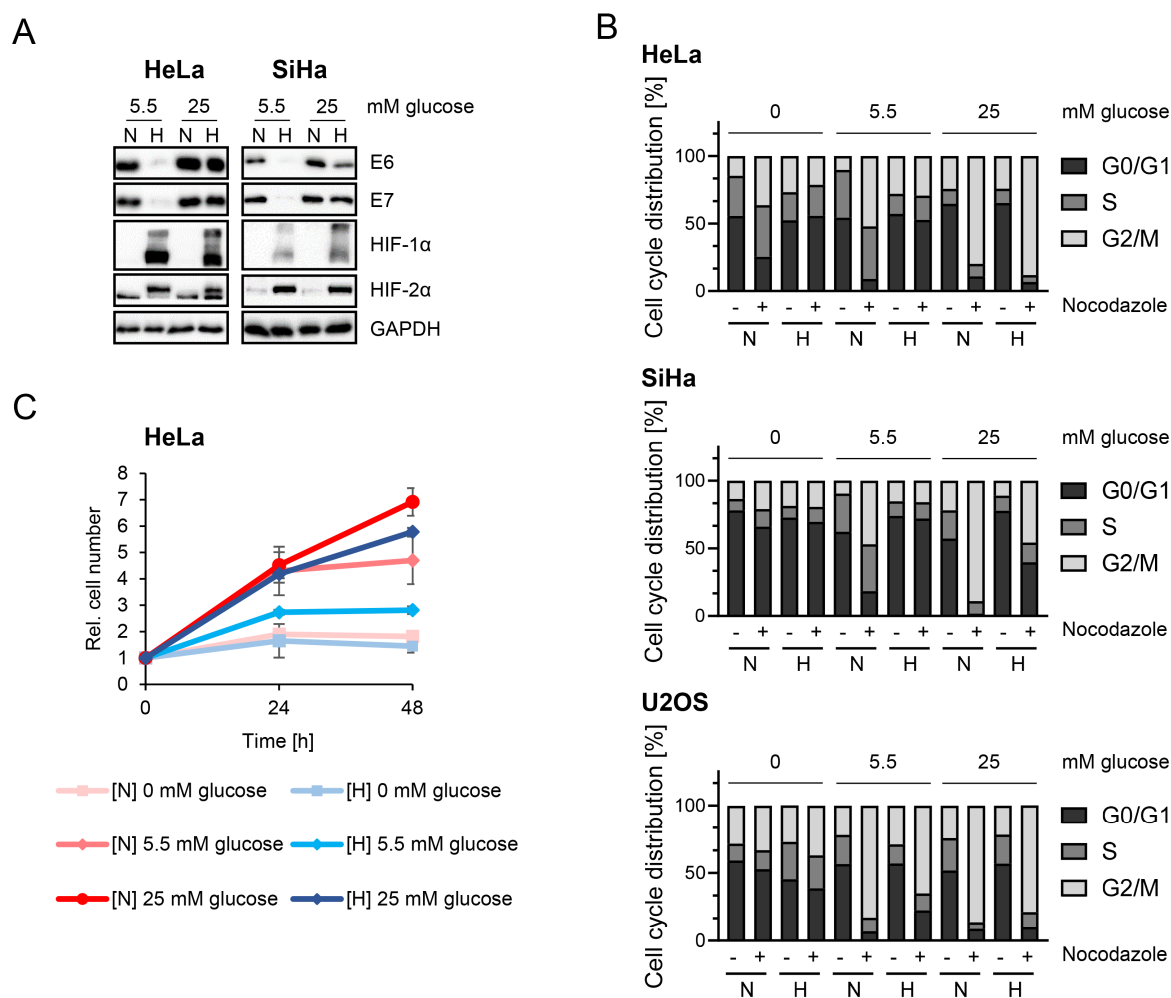


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Figure 8. Glucose availability modulates hypoxia-induced growth arrest. (A) HeLa or SiHa cells were cultivated in media containing either 5.5 mM or 25 mM glucose, under normoxia (N; 21% O₂) or hypoxia (H; 1% O₂) for 24 h. Immunoblot analyses of E6, E7, HIF-1 α , HIF-2 α , and GAPDH protein levels. (B) HeLa, SiHa, or U2OS cells were cultivated in media containing either 0 mM, 5.5 mM, or 25 mM glucose under normoxia or hypoxia for 48 h and treated with Nocodazole (0.1 μ g/mL) or solvent control for the last 20 h of cultivation. Percentages of cell populations in the individual cell cycle phases from representative experiments are shown. (C) Growth curves of HeLa cells cultivated in media containing either 0 mM, 5.5 mM, or 25 mM glucose under normoxia or hypoxia. Viable cell numbers were determined using the trypan blue exclusion test. Cell numbers at the initial time point (0 h) were normalized to 1 and relative cell numbers at the indicated time points are shown with SD ($n=2$).

To further assess the functional impact of metabolic and hypoxic stress, HeLa and SiHa cells were cultivated under normoxic and hypoxic conditions in media with glucose concentrations of 0 mM (i.e. devoid of glucose), 5.5 mM, or 25 mM, followed by treatment with Nocodazole. Cell cycle analyses revealed that combined oxygen deprivation (H) and glucose deprivation/limitation (0 mM, 5.5 mM) induced anti-proliferative effects, as evidenced by an impaired G2/M arrest in Nocodazole-treated HeLa and SiHa cells (Figure 8B) and a corresponding reduction of proliferation in HeLa cells (Figure 8C). Specifically, glucose deprivation (0 mM) strongly inhibited proliferation irrespective of oxygen availability, while physiological serum glucose concentrations (5.5 mM) induced growth arrest, especially under hypoxic conditions. In contrast, high glucose concentrations (25 mM) mitigated hypoxia-induced growth arrest and supported continued cell proliferation, as evidenced by the increased G2/M phase accumulation in Nocodazole-treated hypoxic cells (Figure 8B) and the continued growth of hypoxic HeLa cells (Figure 8C). Notably, this glucose-mediated rescue was not limited to HPV-positive cancer cells, as similar effects were observed in HPV-negative U2OS cells (Figure 8B). Together, these findings highlight the critical role of glucose availability in modulating the cellular response to hypoxia.

2.1.5 Hypoxia-induced growth arrest protects HPV-positive cancer cells from Etoposide-induced senescence

The chemotherapeutic agent Etoposide exerts its anti-cancer effects by inhibiting topoisomerase II, leading to replication-associated DNA damage in proliferating cells, and, consequently, senescence induction.^{242, 243} Etoposide has been explored in clinical settings as an adjuvant therapy for cervical cancer.^{244, 245} Our group has previously demonstrated that hypoxic HPV-positive cervical cancer cells can efficiently escape from Etoposide-induced senescence.¹¹⁶ Therefore, I hypothesized that hypoxia-mediated growth arrest may underlie this protective effect by limiting S phase progression and, as a result, DNA damage accumulation during Etoposide treatment. To test this, HPV-positive HeLa and SiHa cells were treated with Etoposide under normoxia or hypoxia (Figure 9). Consistent with previous

reports,^{87, 242, 246-248} Etoposide treatment led to a marked accumulation of both HeLa and SiHa cells in the S phase as well as a pronounced G2/M arrest under normoxia. However, these cell cycle alterations were substantially attenuated in hypoxic cells, where the distribution of cells across cell cycle phases remained largely unaltered.

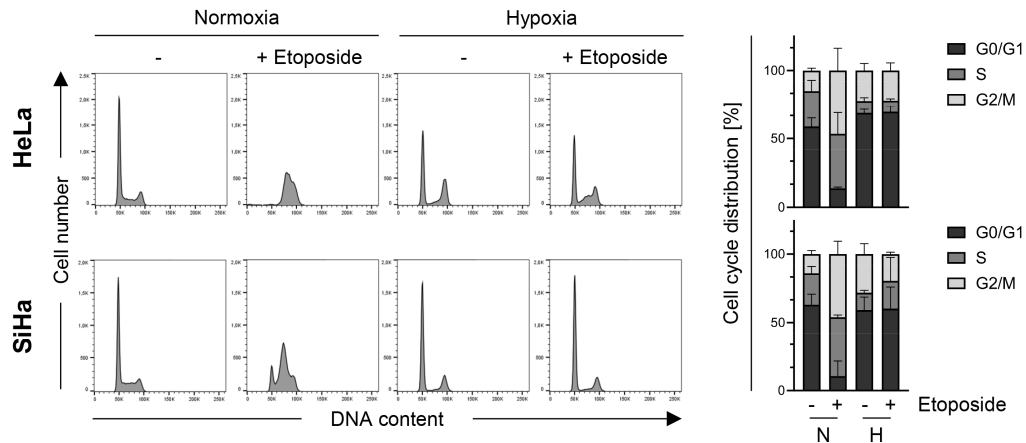


Figure 9. Hypoxia-induced growth arrest protects HPV-positive cancer cells from Etoposide-induced senescence. HeLa or SiHa cells were cultivated under normoxia (N; 21% O₂) or hypoxia (H; 1% O₂) for 72 h and treated with Etoposide (15 μ M) or solvent control during the last 48 h of cultivation. Cell cycle distribution was assessed using flow cytometry. Shown are cell cycle profiles from representative experiments (left panels) and averaged percentages of cell populations in the individual cell cycle phases from 3 independent experiments with SD (right panels).

Collectively, these data indicate that the impairment of Etoposide-induced senescence under hypoxic conditions is a consequence of hypoxia-mediated inhibition of cellular growth and cell cycle progression, which in turn protects cells from the Etoposide-triggered DNA damage during the S phase passage.

2.2 The interplay between B-MYB and A-MYB in normoxic cancer cells

I observed that RNAi-mediated E6/E7 silencing, which induces senescence in HPV-positive cancer cells,^{156, 234} was accompanied by a significant reduction of B-MYB mRNA and protein levels (Figure 5), in agreement with previous studies suggesting that B-MYB loss can promote senescence in normoxic cells.^{190, 216} In strong contrast, B-MYB expression levels were sustained under hypoxia, which promotes senescence evasion¹¹⁶ (Figure 5). This maintenance of B-MYB expression raises the possibility that B-MYB might play a critical functional role in the mechanism by which hypoxic cancer cells could bypass senescence. In this context, it is further noteworthy that a B-MYB paralog, A-MYB, has been recently reported to possess the potential to functionally compensate for the loss of B-MYB, in certain contexts.²³³

2.2.1 Functional redundancy between B-MYB and A-MYB determines the proliferation rate of HPV-positive cancer cells

To analyze the possible interplay between B-MYB and A-MYB in the proliferation control of HPV-positive cancer cells, three different B-MYB-specific siRNAs (siB-MYB-1, -2, -3) and two different A-MYB-specific siRNAs (siA-MYB-1, -2) were employed (Figure 10). Each of these siRNAs individually led to an effective reduction of transcript levels and a downregulation of protein levels of their respective targets (Figure 10A). For all subsequent experiments, equimolar pools of three B-MYB or two A-MYB siRNAs were utilized (referred to in the text as siB-MYB or siA-MYB).

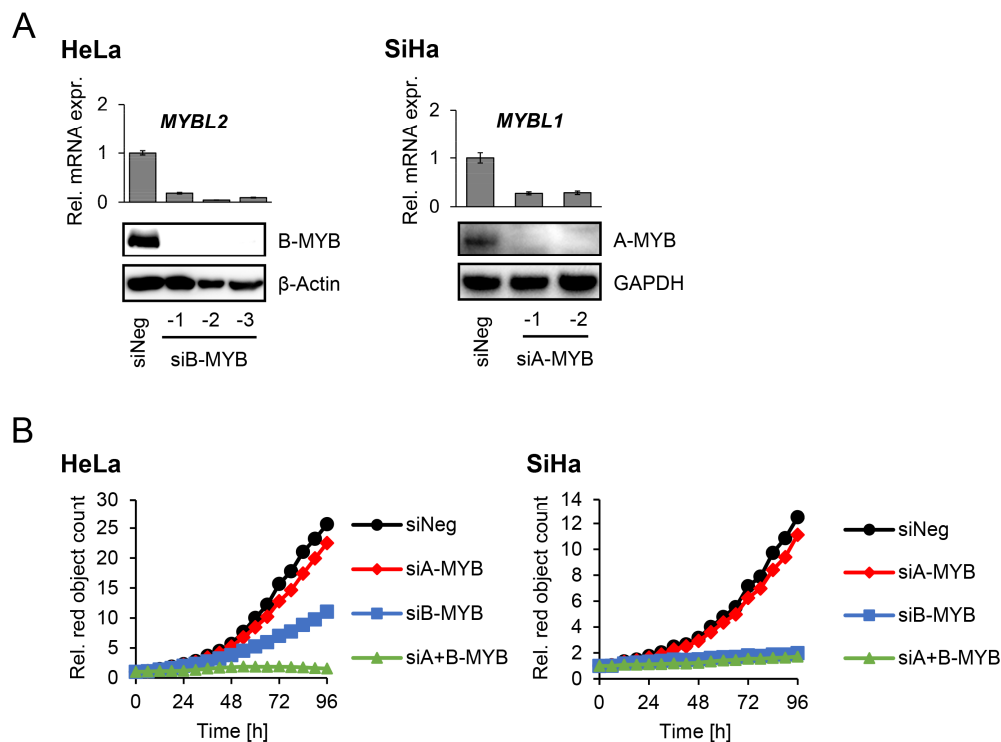


Figure 10. Functional interplay between B-MYB and A-MYB regulates growth of HPV-positive cancer cells.

(A) HeLa or SiHa cells were transfected with three different B-MYB-specific siRNAs (siB-MYB-1, -2, -3) and two different A-MYB-specific siRNAs (siA-MYB-1, -2) individually and grown for 72 h. qRT-PCR analyses of *MYBL2* (coding for B-MYB) and *MYBL1* (coding for A-MYB) mRNA levels (upper panels) and corresponding immunoblot analyses of B-MYB, A-MYB, β -Actin, and GAPDH protein levels (lower panels). (B) HeLa-mKate2 or SiHa-mKate2 cells transfected with siA-MYB, siB-MYB, either individually or in combination, or control siRNA (siNeg). Cells were imaged using the Incucyte S3 live-cell imaging system and cell proliferation was determined by quantifying viable cells (red objects) for up to 96 h using the Incucyte software package. Figure content and corresponding legend adapted from Velimirović et al., 2025.²³⁶

HeLa and SiHa cells were transfected with siB-MYB, siA-MYB, either individually or in combination, or with control siRNA (siNeg), and cultivated under normoxia for up to 96 h. Proliferation curves were obtained using live-cell imaging. Consistent with the well-established

growth-promoting functions of B-MYB,^{176, 211} B-MYB repression resulted in a marked inhibition of proliferation in both HeLa and SiHa cells (Figure 10B). Remarkably, the anti-proliferative effect of B-MYB silencing in HeLa cells was further enhanced when A-MYB was concomitantly silenced (Figure 10B, left panel), suggesting that A-MYB can partially compensate for the loss of B-MYB in terms of proliferation. In contrast, SiHa cells exhibited a higher reliance on B-MYB, as evidenced by their substantial sensitivity to B-MYB depletion alone and the absence of a notable additive effect upon simultaneous A-MYB silencing.

Collectively, these findings indicate that B-MYB and A-MYB can exhibit functional redundancy in regulating the proliferation of HPV-positive cancer cells, however, the extent of this compensatory relationship appears to be cell line-dependent.

2.2.2 B-MYB loss induces compensatory induction of A-MYB

In order to further elucidate the potential interplay between B-MYB and A-MYB, siRNA-mediated knockdowns of each paralog individually, or in combination, were performed and their protein expression was assessed relative to control siRNA-transfected cells (Figure 11). Interestingly, immunoblot analyses revealed a clear upregulation of A-MYB protein levels upon B-MYB silencing in both HeLa and SiHa cells (Figure 11A). Of further note, the increase in A-MYB protein expression was similarly observed in HPV-negative U2OS osteosarcoma and HCT116 colon cancer cells (Figure 11B), indicating a conserved regulatory response to B-MYB loss. These findings show that normoxic HPV-positive, as well as HPV-negative cells, react to B-MYB repression with a strong increase in A-MYB levels, which is corroborated by findings from a recently published study.²³³

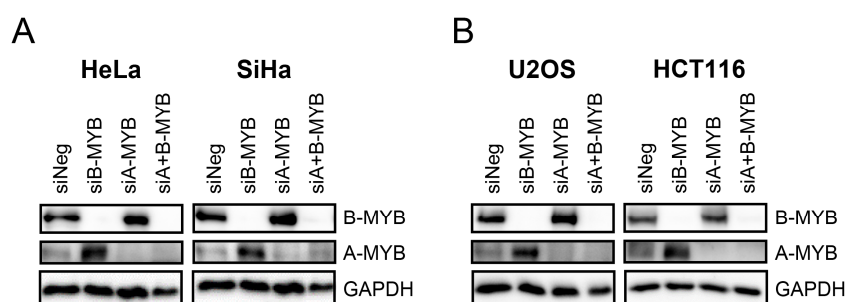


Figure 11. B-MYB loss induces a compensatory induction of A-MYB. (A) HeLa or SiHa cells were transfected with siA-MYB, siB-MYB, either individually or in combination, or control siRNA (siNeg) and grown for 48 h (HeLa) or 72 h (SiHa). Immunoblot analyses of B-MYB, A-MYB, and GAPDH protein levels. (B) U2OS or HCT116 cells were transfected with siA-MYB, siB-MYB, either individually or in combination, or control siRNA (siNeg) and grown for 72 h. Immunoblot analyses of B-MYB, A-MYB, and GAPDH protein levels. Figure content and corresponding legend adapted from Velimirović et al., 2025.²³⁶

2.2.3 A-MYB compensates for B-MYB loss by sustaining G2/M gene expression

B-MYB typically interacts with the MuvB core complex to form the MMB complex, which binds to CHR promoter elements to activate the transcription of late cell cycle genes (G2/M genes) essential for mitotic entry and cell cycle progression¹⁸² (Figure 2). Given this role, the compensatory upregulation of A-MYB observed upon B-MYB depletion raised the question of whether A-MYB might substitute for B-MYB in supporting the transcriptional activation of G2/M genes in the absence of B-MYB.

To investigate this hypothesis, siRNA-mediated knockdowns were performed to silence B-MYB, A-MYB, or both, and the expression of canonical B-MYB target genes involved in the G2/M progression,^{51, 184} such as *CCNA2*, *CCNB1*, *CCNB2*, *CDC25C*, and *PLK1*, was assessed in HPV-positive (HeLa, SiHa) and HPV-negative (U2OS, HCT116) cancer cells (Figure 12). The cellular response to B-MYB silencing alone varied to some degree across the different cell lines, with some showing downregulation of G2/M genes, while others exhibited little to no change in their expression. Further, the silencing of A-MYB alone did not result in significant changes in the mRNA expression levels of G2/M genes. Importantly, however, the combined silencing of both B-MYB and A-MYB led to a substantial reduction in the expression of G2/M genes compared to control siRNA-transfected cells, or siB-MYB-transfected cells in almost all tested cell lines. An exception was observed in SiHa cells, where A-MYB co-silencing did not significantly contribute to a further decrease in the expression of G2/M genes compared to B-MYB silencing alone. (Figure 12A). Immunoblot analyses corroborated the results of the mRNA analyses, revealing a marked reduction in the protein levels of B-MYB targets following dual B-MYB/A-MYB knockdown, compared to either individual knockdowns or control siRNA-transfected cells (Figure 12B). Notably, *CDC25C* and *PLK1*, two factors that are critical for G2/M phase transition,²⁴⁹⁻²⁵² were consistently downregulated across all examined cell lines, indicating that they are particularly susceptible to the combined repression of B-MYB and A-MYB. Taken together, these data strongly suggest that A-MYB can, at least partially, compensate for the loss of B-MYB and preserve transcriptional activation of G2/M genes required for cell cycle progression.

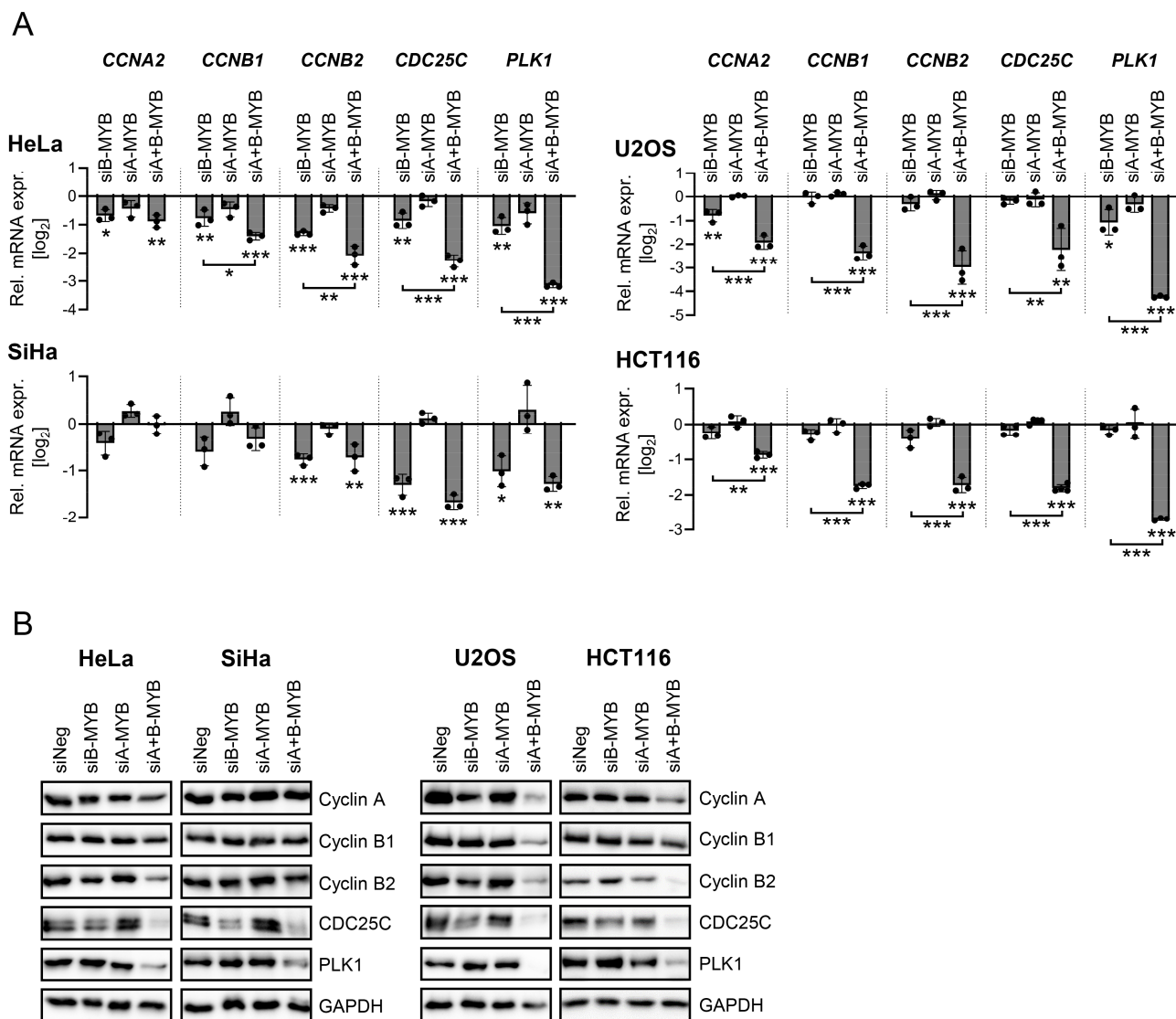


Figure 12. A-MYB compensates for B-MYB loss by sustaining G2/M gene expression. (A) HeLa, SiHa, U2OS, or HCT116 cells were transfected with siA-MYB, siB-MYB, either individually or in combination, or control siRNA (siNeg) and grown for 48 h (HeLa) or 72 h (SiHa, U2OS, HCT116). qRT-PCR analyses of *CCNA2* (coding for Cyclin A2), *CCNB1* (coding for Cyclin B1), *CCNB2* (coding for Cyclin B2), *CDC25C*, and *PLK1* mRNA levels. Shown are individual data points and mean expression levels with SD ($n=3$) relative to the expression of siNeg ($\log_2$ FC). Statistical significance was assessed using one-way ANOVA. $*p \leq 0.05$, $**p \leq 0.01$, and $***p \leq 0.001$. (B) Corresponding immunoblot analyses of Cyclin A, Cyclin B1, Cyclin B2, *CDC25C*, *PLK1*, and GAPDH protein levels. Figure content and corresponding legend adapted from Velimirović et al., 2025.²³⁶

2.2.4 Combined silencing of B-MYB and A-MYB induces a robust G2/M cell cycle arrest

Since the compensatory induction of A-MYB appeared to mitigate the loss of B-MYB by sustaining the expression of factors critical for G2/M progression, I next examined the impact of B-MYB and A-MYB silencing on the cell cycle distribution using flow cytometry (Figure 13).

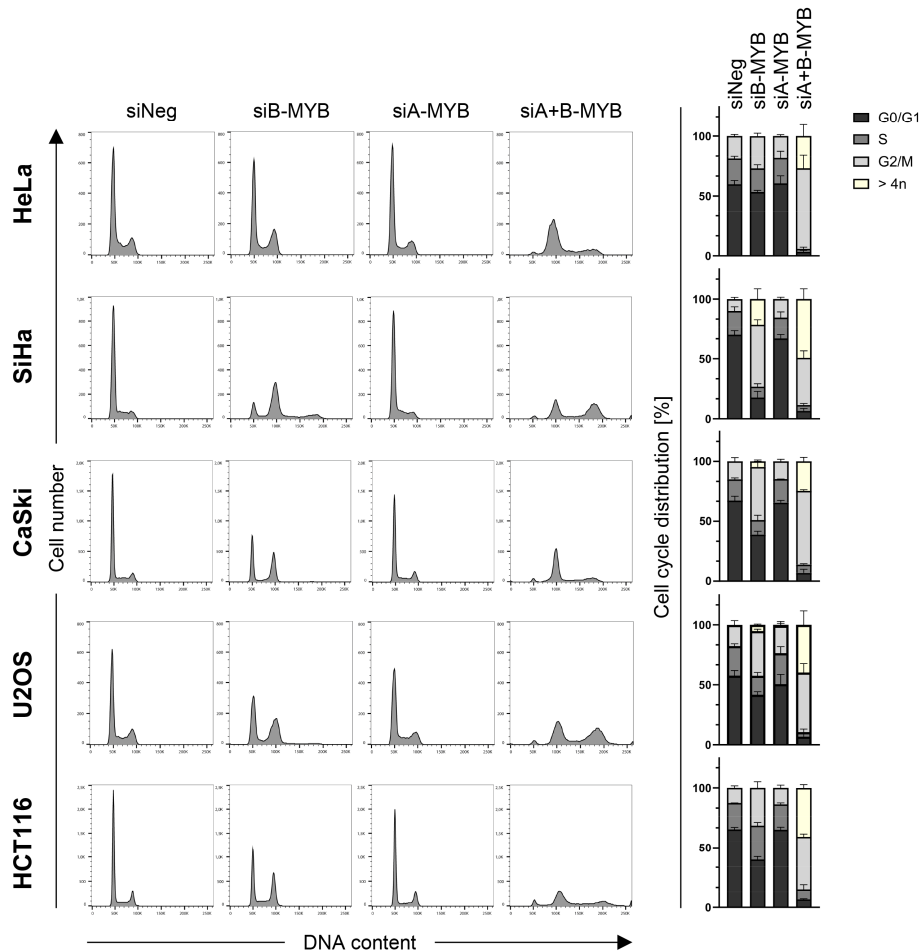


Figure 13. Combined silencing of B-MYB and A-MYB induces a robust G2/M cell cycle arrest. HeLa, SiHa, CaSki, U2OS, or HCT116 cells were transfected with siA-MYB, siB-MYB, either individually or in combination, or control siRNA (siNeg) and grown for 48 h (HeLa) or 72 h (SiHa, CaSki, U2OS, HCT116). Cell cycle distribution was assessed using flow cytometry. Shown are cell cycle profiles from representative experiments (left panels) and averaged percentages of cell populations in the individual cell cycle phases from 3 independent experiments with SD (right panels). Figure content and corresponding legend adapted from Velimirović et al., 2025.²³⁶

As supported by earlier studies,^{198, 253, 254} B-MYB knockdown led to an accumulation of cells in the G2/M phase, which was particularly evident in HPV-positive SiHa and CaSki cells, as well as in HPV-negative U2OS and HCT116 cells. This effect was less pronounced in HeLa cells, consistent with previous studies reporting that the cell cycle response to B-MYB loss can vary in a cell line-dependent manner.^{198, 233, 255} In contrast, silencing of A-MYB alone had little to no effect on cell cycle distribution across all examined cell lines. Strikingly, combined depletion of

both B-MYB and A-MYB induced a much more pronounced G2/M arrest compared to either individual knockdowns alone or to control siRNA-transfected cells. Moreover, the dual knockdown of B-MYB and A-MYB led to a marked increase in endoreduplication events and accumulation of polyploid cells (> 4n DNA content), as visualized by the emergence of an additional peak in the cell cycle profiles (Figure 13). In SiHa cells, B-MYB depletion alone was sufficient to induce a strong G2/M arrest, and concomitant silencing of A-MYB had only marginal effects, further supporting the notion that the compensatory role of A-MYB can be cell type-dependent and that SiHa cells are more B-MYB “addicted”, as discussed above.

2.2.5 Silencing of A-MYB enhances the pro-senescent effect of B-MYB loss

As outlined earlier, senescence induction following RNAi-mediated silencing of E6/E7 was associated with a marked downregulation of B-MYB (Figure 5), consistent with prior reports linking B-MYB repression to the onset of senescence.^{190, 216, 217} While the potential of B-MYB to bypass senescence is well-documented,^{214, 215} the involvement of A-MYB in this process is unexplored. Therefore, I first examined A-MYB expression during siE6/E7-induced senescence in HPV-positive HeLa and SiHa cells (Figure 14). Notably, and in contrast to E6 repression alone (phenotype: no senescence), E6/E7 repression (phenotype: senescence) led to a marked reduction in A-MYB expression at both the transcript (Figure 14A) and protein (Figure 14B) levels in both cell lines.

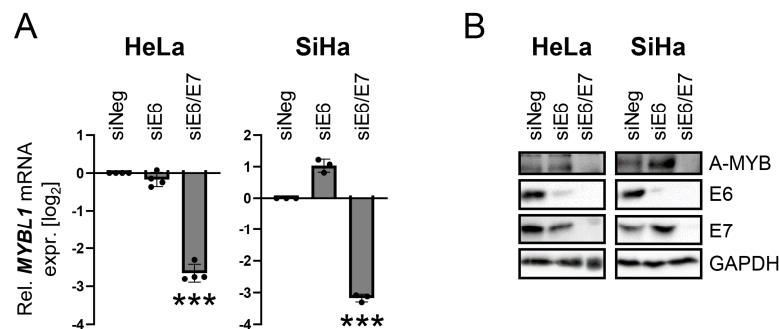


Figure 14. siE6-E7-induced senescence is linked to a strong downregulation of A-MYB. HeLa or SiHa cells were transfected with siE6, siE6/E7, or control siRNA (siNeg) and grown for 72 h. (A) qRT-PCR analyses of *MYBL1* (coding for A-MYB) mRNA levels. Shown are individual data points and mean expression levels with SD ($n=3$) relative to the expression of siNeg (log₂FC). Statistical significance was assessed using one-way ANOVA. * $p \leq 0.05$, ** $p \leq 0.01$, and *** $p \leq 0.001$. (B) Corresponding immunoblot analyses of A-MYB, E6, E7, and GAPDH protein levels.

This suggests that the downregulation of A-MYB, alongside B-MYB repression, may contribute to the establishment and/or maintenance of the senescent phenotype upon E6/E7 silencing. Given my earlier findings that A-MYB can functionally compensate for B-MYB loss by

sustaining G2/M gene expression and cell cycle progression, I next sought to explore whether A-MYB may similarly play a role in modulating the senescence response in the absence of B-MYB.

HPV-positive HeLa, SiHa, and CaSki cells, as well as HPV-negative U2OS and HCT116 cells, were subjected to individual or combined siRNA-mediated knockdowns of B-MYB and A-MYB, and treated as outlined in Figure 15A. Consistent with prior findings, B-MYB silencing alone exhibited pro-senescent effects across all tested cell lines, as evidenced by positive staining for the well-known senescence marker senescence-associated β -Galactosidase (SA- β -Gal) and characteristic senescent cell morphology, such as cell flattening and enlargement^{142, 143} (Figure 15B, left panels for each cell line). Accordingly, loss of B-MYB was associated with a reduction in colony formation capacity in all examined cell lines (Figure 15B, right panels for each cell line). Importantly, while B-MYB silencing was efficient (Figure 11), senescence induction was only partial, with non-senescent cells (i.e. SA- β -Gal-negative and without the characteristic morphological signs of senescence) persisting alongside senescent cells. This outcome could be explained by the compensatory induction of A-MYB, which I found to affect cell proliferation (Figure 10B), B-MYB target gene expression (Figure 12A, B), and cell cycle progression (Figure 13). Loss of A-MYB alone did not induce senescence, as supported by both senescence and colony formation assays. Strikingly, however, the pro-senescent response to B-MYB silencing was substantially enhanced when A-MYB was silenced in combination. This was demonstrated by a marked reduction in the number of cells evading senescence (Figure 15B, left panels for each cell line), as well as a further decline in colony formation capacity in comparison to siB-MYB-transfected cells (Figure 15B, right panels for each cell line).

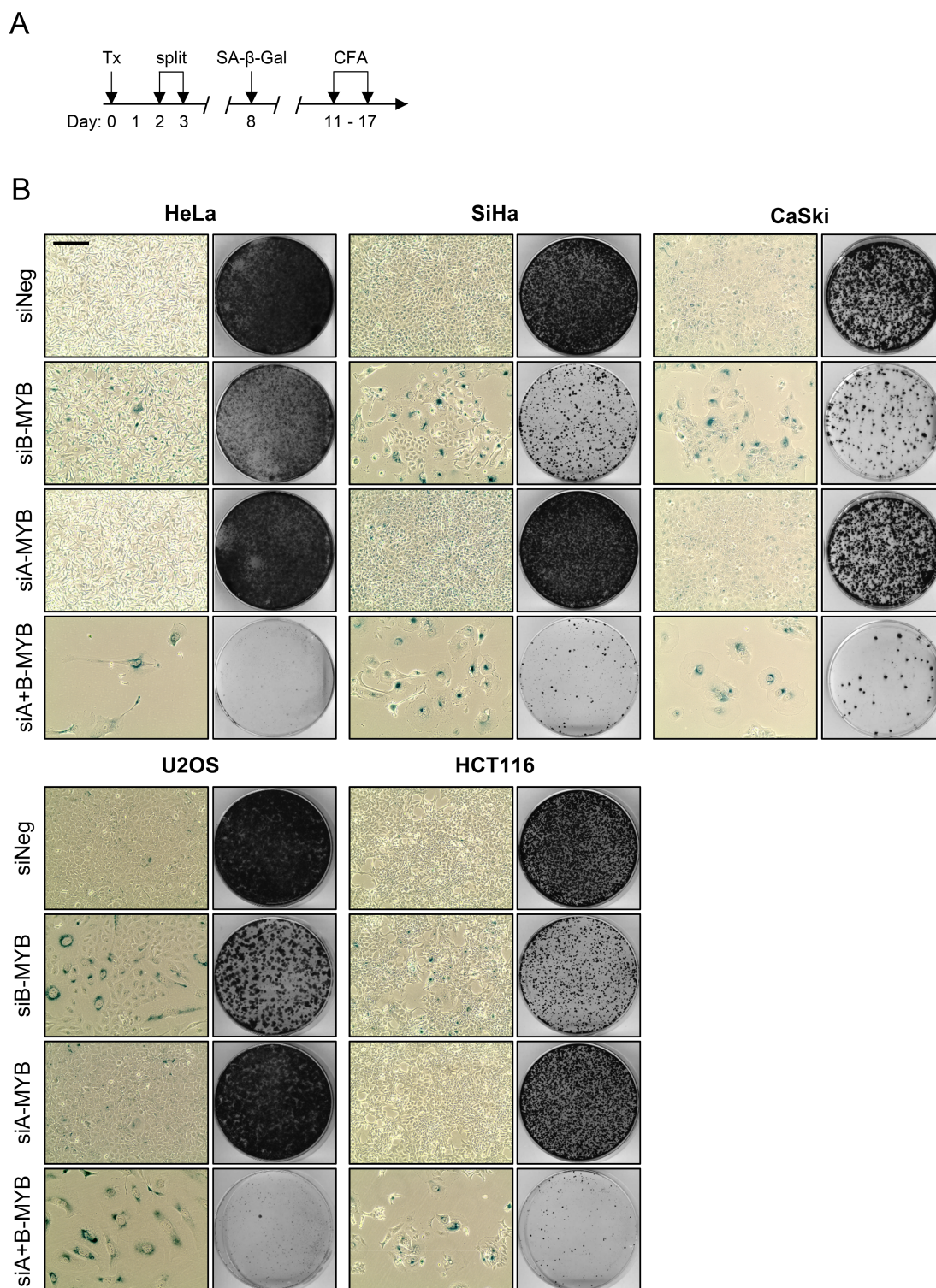


Figure 15. Silencing of A-MYB enhances the pro-senescent effects of B-MYB loss. (A) Treatment scheme: HeLa, SiHa, CaSki, U2OS, or HCT116 cells were transfected (Tx) with siA-MYB, siB-MYB, either individually or in combination, or control siRNA (siNeg). Cells were split after 48 h (HeLa) or 72 h (SiHa, CaSki, U2OS, HCT116), and fixed after the indicated times for senescence assays (SA-β-Gal staining) or colony formation assays (CFAs). (B) Senescence assays (left panels for each cell line; SA-β-Gal positive cells staining blue) (scale bar: 200 μm) and corresponding CFAs (right panels for each cell line). Figure content and corresponding legend adapted from Velimirović et al., 2025.²³⁶

These results support the notion that the compensatory A-MYB induction, at least partially, counteracts the pro-senescent effects of B-MYB silencing under normoxia. Further, they show that co-silencing of A-MYB can strongly enhance the pro-senescent efficacy of B-MYB silencing and that efficient induction of senescence requires concomitant repression of both B-MYB and A-MYB.

2.2.6 Combined silencing of B-MYB and A-MYB can exert pro-apoptotic effects

Importantly, while co-silencing A-MYB alongside B-MYB resulted in a pronounced reduction in cell proliferation consistent with an enhanced senescence phenotype, the contribution of additional mechanisms, such as cell death, cannot be excluded. As most evident in HeLa cells, combined silencing of B-MYB and A-MYB resulted in a substantial reduction in cell numbers, leaving only a few residual senescent cells (Figure 15B). This effect was further confirmed in colony formation assays, where the dual B-MYB/A-MYB knockdown markedly impaired colony formation capacity compared to both control siRNA-transfected and siB-MYB-transfected cells (Figure 15B). These findings pointed to a possible pro-apoptotic component of combined B-MYB and A-MYB silencing. Therefore, I investigated the expression of established apoptosis markers, cleaved PARP (Poly(ADP-ribose) polymerase) and cleaved Caspase-9,^{256, 257} following the respective individual and combined knockdowns, starting with HPV-positive HeLa and SiHa cells. In HeLa cells, dual silencing of B-MYB and A-MYB induced a strong upregulation of both cleaved PARP and cleaved Caspase-9 (Figure 16A), whereas B-MYB depletion alone only moderately increased apoptosis markers and A-MYB knockdown alone had no detectable effect. Consistent with prior observations, this apoptotic response was somewhat cell line-dependent: in SiHa cells, apoptosis markers also increased upon co-silencing of B-MYB and A-MYB, but to a much lesser extent than in HeLa cells. In order to quantify late-stage apoptotic cells following the respective knockdowns, TUNEL assays were performed. TUNEL assays corroborated the increased sensitivity of HeLa and SiHa cells to the combined silencing of B-MYB and A-MYB, as the percentage of TUNEL-positive cells was markedly increased, compared to individual knockdowns and control siRNA-transfected cells (Figure 16B). Next, I extended my analyses to HPV-negative HCT116 and U2OS cells. HCT116 cells exhibited increased sensitivity to the combined B-MYB and A-MYB knockdown, whereas U2OS cells displayed no induction of cleaved PARP or cleaved Caspase-9 (Figure 16C).

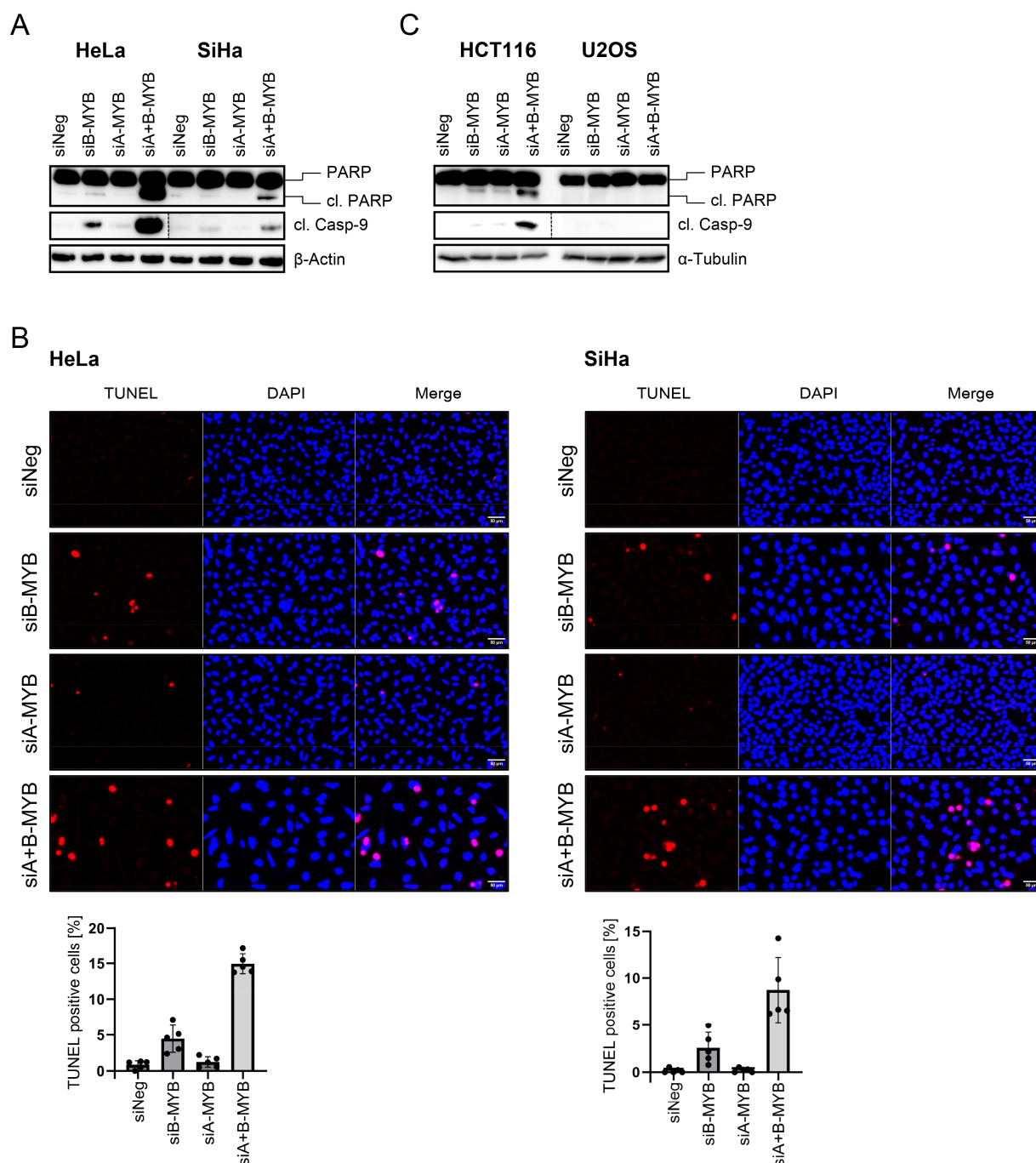


Figure 16. Combined silencing of B-MYB and A-MYB can exert pro-apoptotic effects. (A) HeLa or SiHa cells were transfected with siA-MYB, siB-MYB, either individually or in combination, or control siRNA (siNeg) and harvested after 48 h (HeLa) or 72 h (SiHa). Immunoblot analyses of cleaved PARP, cleaved Caspase-9 and β -Actin protein levels (B) HeLa or SiHa cells were treated as described in (A) and harvested for TUNEL analyses. Shown are representative images from 2 independent experiments which produced consistent results (upper panels for each cell line) (scale bar: 50 μ m). TUNEL-positive (apoptotic) cells were quantified relative to DAPI-stained cells (reflecting total cell numbers) and are presented as averaged percentages from 5 different images per condition (lower panels for each cell line). (C) HCT116 or U2OS cells were transfected with siA-MYB, siB-MYB, either individually or in combination, or control siRNA (siNeg) and harvested after 72 h. Immunoblot analyses of cleaved PARP, cleaved Caspase-9 and α -Tubulin protein levels. Figure content and corresponding legend adapted from Velimirović et al., 2025.²³⁶

In summary, these findings demonstrate that the effects of B-MYB loss can be substantially enhanced by concomitant A-MYB silencing, resulting in an amplified senescence and/or apoptotic response in a cell type-dependent manner under normoxia. This further substantiates the functional redundancy between B-MYB and A-MYB and suggests that their combined repression is leading to a more efficient induction of growth arrest in normoxic cancer cells.

2.3 B-MYB and FOXM1 have distinct roles in regulating cell fate

The binding of B-MYB to the MuvB core complex is crucial for the recruitment of the transcription factor FOXM1 during the late G2 phase of the cell cycle, facilitating the formation of FOXM1-MMB and FOXM1-MuvB complexes^{186, 187} (Figure 2). Together, B-MYB and FOXM1 drive the expression of late cell cycle genes (G2/M genes) and play a pivotal role in G2/M phase transition.^{184, 186, 187} FOXM1 was, alongside B-MYB, significantly downregulated upon siRNA-mediated E6/E7 repression under normoxia – consistent with the onset of senescence. In contrast, FOXM1 expression remained largely preserved when E6/E7 were repressed under hypoxic conditions (Figure 5A). Given the shared role of both B-MYB and FOXM1 in MuvB-mediated transcriptional activation, this raised the question of whether FOXM1, like B-MYB, can exert anti-senescent effects.

To evaluate whether FOXM1 downregulation is sufficient to induce senescence, I employed an equimolar pool (referred to in the text as siFOXM1) of two different siRNAs targeting FOXM1 (siFOXM-1, -2), each of which efficiently reduced FOXM1 expression (Figure 17A). Senescence induction was subsequently examined in HPV-positive HeLa and SiHa cells. Despite the effective FOXM1 knockdown, pro-senescent effects were not detectable in either HeLa or SiHa cells (Figure 17B), suggesting that FOXM1 depletion is not sufficient to trigger a senescence response in these cells.

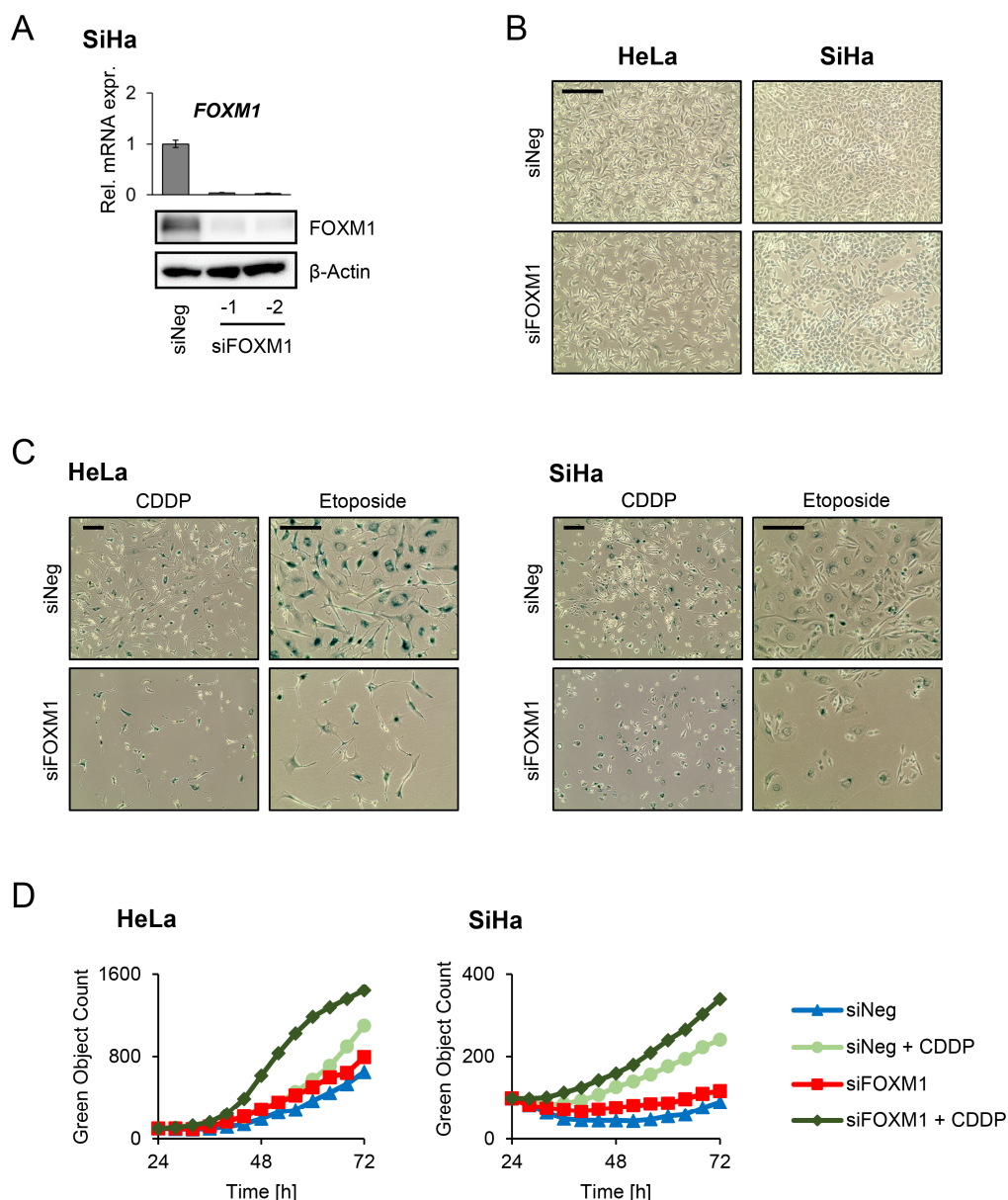


Figure 17. Silencing of FOXM1 does not induce senescence but enhances CT-induced cell death in HPV-positive cancer cells. (A) SiHa cells were transfected with two different FOXM1-specific siRNAs (siFOXMX1-1, -2) individually and grown for 72 h. qRT-PCR analyses of *FOXM1* mRNA levels (upper panel) and corresponding immunoblot analyses of FOXM1 and β -Actin protein levels (lower panel). (B) HeLa or SiHa cells were transfected with siFOXMX1 or control siRNA (siNeg), grown for 72 h, split and cultivated for senescence assays (SA- β -Gal positive cells staining blue) (scale bar: 200 μ m). (C) HeLa or SiHa cells were transfected with siFOXMX1 or control siRNA (siNeg). 24 h post-transfection, cells were either treated with CDDP (2.5 μ M) for 24 h or Etoposide (10 μ M) for 48 h, split and cultivated in drug-free medium for senescence assays (SA- β -Gal positive cells staining blue) (scale bar: 200 μ m). (D) HeLa-mKate2 or SiHa-mKate2 cells were transfected with siFOXMX1 or control siRNA (siNeg). 24 h post-transfection, cells were treated with CDDP (2.5 μ M) in the presence of Cytotox Green Dye (100 nM), which was added concurrently. Cells were imaged using the Incucyte S3 live-cell imaging system and cell death was determined by quantifying dead cells (green objects) starting at 24 h post-CDDP treatment using the Incucyte software package.

Moreover, FOXM1 is implicated to play a role in cancer therapy resistance. Its overexpression is commonly observed in chemoresistant tumors, where it contributes to treatment failure.²⁵⁸⁻²⁶³ Inhibition of FOXM1 has been shown to restore sensitivity to chemotherapeutic agents.^{264, 265} Based on these findings, I next investigated whether FOXM1 loss modulates the cellular response to chemotherapy-induced senescence. To this end, HeLa and SiHa cells were treated with two agents, Cisplatin (CDDP) and Etoposide, at concentrations that induce senescence (please refer to the figure legend). Interestingly, loss of FOXM1 enhanced the cytotoxic effects of both drugs, shifting the cellular response from senescence towards cell death, as indicated by a marked reduction in cell numbers and the persistence of only a small population of residual senescent cells (Figure 17C). These observations were further validated using live-cell imaging and Incucyte Cytotox Green reagent (please refer to section 4.3.6). Quantification of cytotox-positive green cells revealed that FOXM1 knockdown enhanced Cisplatin-induced cell death in both cell lines (Figure 17D).

Given that hypoxia represents a major barrier to chemotherapy,²⁶⁶ next I investigated whether FOXM1 depletion could sensitize HPV-positive cervical cancer cells to Cisplatin treatment under hypoxic conditions. HeLa and SiHa cells were cultured under normoxia or hypoxia and subsequently treated with pro-apoptotic concentrations of Cisplatin (please refer to the figure legend). As expected, Cisplatin treatment of normoxic cells led to a strong increase of apoptosis markers, cleaved PARP, and cleaved Caspase-9, as well as increased γ -H2AX levels, indicative of DNA double-strand breaks (Figure 18A). Moreover, FOXM1 expression was upregulated in response to Cisplatin treatment, consistent with its reported role in promoting cell survival.^{258, 259} Notably, FOXM1 knockdown under normoxic conditions increased the sensitivity of HPV-positive cancer cells to Cisplatin-induced apoptosis. Similarly, FOXM1 knockdown partially restored sensitivity to Cisplatin in hypoxic HeLa and SiHa cells, although the effect was less pronounced than under normoxia. Further, TUNEL analyses confirmed the heightened sensitivity of normoxic and hypoxic SiHa cells to Cisplatin-induced apoptosis upon FOXM1 knockdown compared to the respective control siRNA-transfected cells, as evidenced by an increase in the percentage of TUNEL-positive cells (Figure 18B).

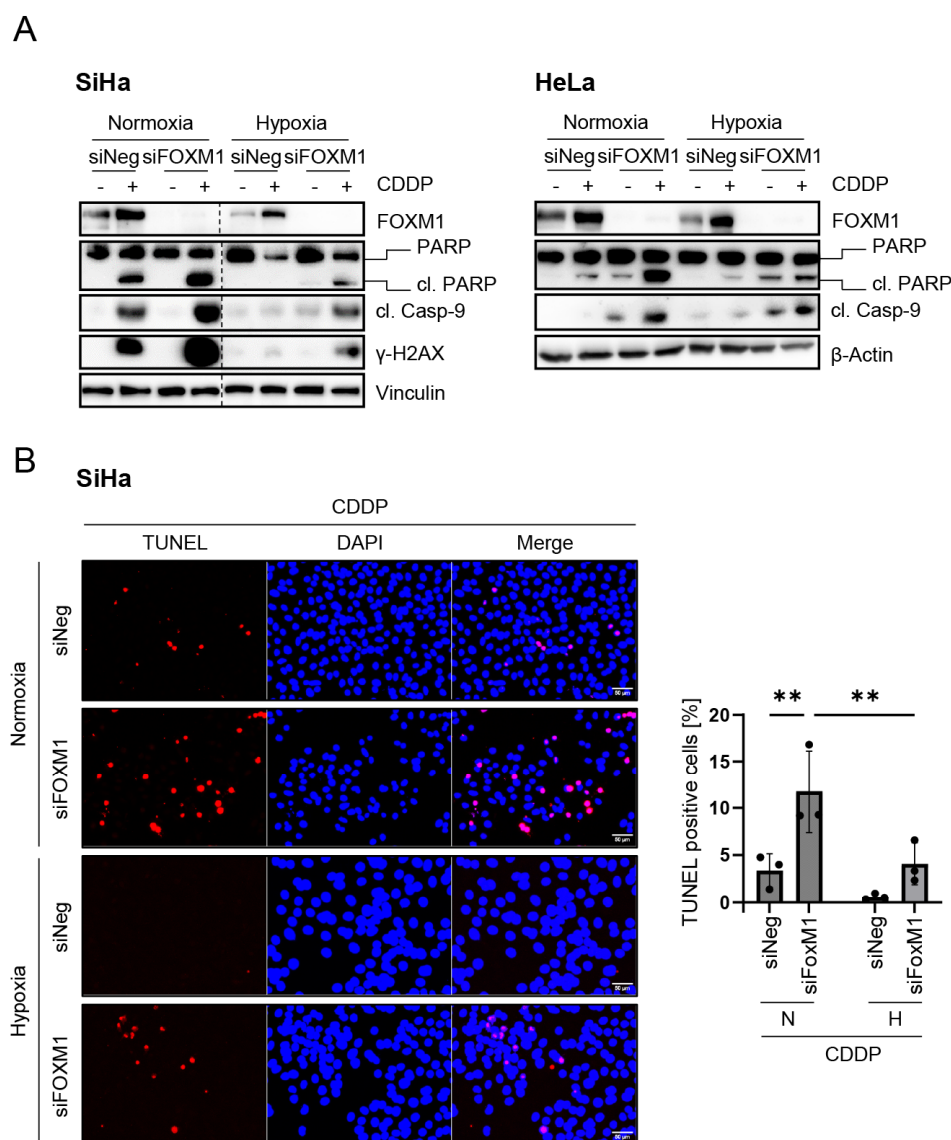


Figure 18. Silencing of FOXM1 sensitizes HPV-positive cancer cells to Cisplatin-induced apoptosis. SiHa or HeLa cells were transfected with siFOXM1 or control siRNA (siNeg). 24 h post-transfection, cells were cultivated under normoxia (N; 21% O₂) or hypoxia (H; 1% O₂) for 48 h and treated with 30 μ M or 15 μ M CDDP, respectively, for the last 24 h of cultivation. (A) Immunoblot analyses of cleaved PARP, cleaved Caspase-9, γ -H2AX, Vinculin, and β -Actin protein levels. (B) TUNEL analyses of SiHa cells (left panel) (scale bar: 50 μ m). TUNEL-positive (apoptotic) cells were quantified relative to DAPI-stained cells (reflecting total cell numbers). Shown are the averaged percentages with SD ($n = 15$ fields of view; each with ≥ 50 cells from 3 independent experiments) relative to the siNeg under normoxia (right panel). Statistical significance was assessed using two-way ANOVA. * $p \leq 0.05$, ** $p \leq 0.01$, and *** $p \leq 0.001$.

In summary, the presented data provide no evidence that FOXM1 knockdown induces senescence in the examined cell lines, highlighting a functional distinction between the two MuvB-associated factors, B-MYB and FOXM1. Of note, FOXM1 repression, particularly under normoxic conditions, markedly enhanced chemotherapy-induced cell death, suggesting that its inhibition could serve as a potential strategy to improve therapeutic efficacy in cancer cells.

2.4 The interplay between B-MYB and A-MYB in hypoxic cancer cells

2.4.1 The compensatory induction of A-MYB in response to B-MYB loss is impaired under hypoxia

Given the critical role of B-MYB and A-MYB in senescence regulation under normoxic conditions, in both HPV-positive and HPV-negative cancer cells (Figure 15), I next aimed to investigate the interplay between these two factors under hypoxia. To this end, HPV-positive HeLa and SiHa, and HPV-negative U2OS cells were transfected with either siB-MYB or control siRNA (siNeg), cultured under normoxic or hypoxic conditions, and subsequently harvested for mRNA and protein analyses (Figure 19).

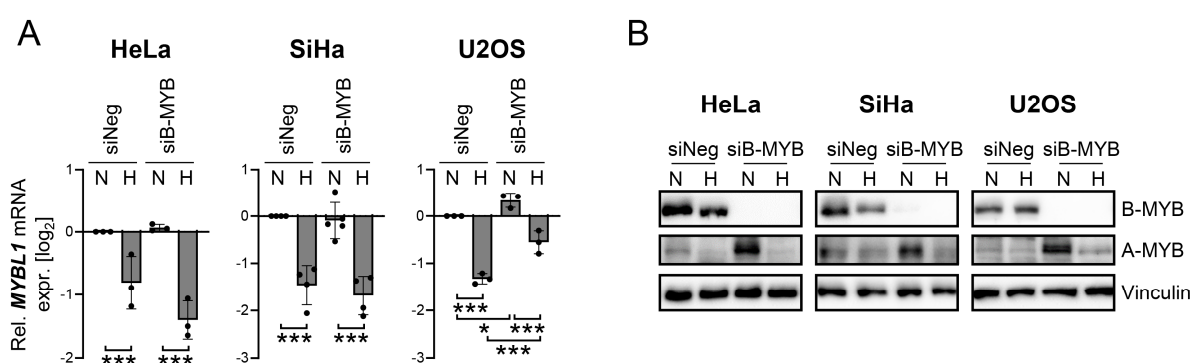


Figure 19. The compensatory induction of A-MYB in response to B-MYB loss is impaired under hypoxia. HeLa, SiHa, or U2OS cells were transfected with siB-MYB or control siRNA (siNeg). 24 h post-transfection, cells were cultivated under normoxia (N; 21% O₂) or hypoxia (H; 1% O₂) and subsequently harvested after 48 h for RNA and protein analyses, with the exception of SiHa cells (harvested after 24 h for RNA analyses). (A) qRT-PCR analyses of *MYBL1* (coding for A-MYB) mRNA levels. Shown are individual data points and mean expression levels with SD ($n \geq 3$) relative to the expression of siNeg under normoxia (log₂FC). Statistical significance was assessed using two-way ANOVA. * $p \leq 0.05$, ** $p \leq 0.01$, and *** $p \leq 0.001$. (B) Immunoblot analyses of B-MYB, A-MYB, and Vinculin protein levels. Figure content and corresponding legend adapted from Velimirović et al., 2025.²³⁶

Remarkably, and in striking contrast to the substantial increase of A-MYB levels upon B-MYB repression under normoxia, A-MYB was not induced in hypoxic cells upon B-MYB silencing, neither at the transcript (Figure 19A) nor at the protein level (Figure 19B). Of further note, while A-MYB protein levels were markedly elevated following B-MYB knockdown under normoxia across all tested cell lines (Figure 19B), transcript levels of A-MYB remained largely unchanged, with the exception of U2OS cells (Figure 19A), indicating that the regulation of A-MYB in response to B-MYB loss occurs predominantly at the protein level. These results uncover that hypoxic cells are strongly impaired in their capacity to compensate for the loss of B-MYB through the upregulation of A-MYB expression.

2.4.2 The lack of compensatory A-MYB induction enhances G2/M gene repression under hypoxia

The observed lack of compensatory upregulation of A-MYB in response to B-MYB depletion implies that A-MYB cannot functionally substitute for B-MYB in hypoxic cells. To further investigate this hypothesis, B-MYB expression was silenced in HPV-positive HeLa and SiHa cells, as well as HPV-negative U2OS cells. Following siRNA transfections, cells were cultured under normoxia or hypoxia. In order to evaluate the functional consequences of B-MYB loss under hypoxic conditions, I analyzed the expression of selected B-MYB target genes (G2/M genes), *CCNA2*, *CCNB1*, *CCNB2*, *CDC25C*, and *PLK1*, and their corresponding protein products (Figure 20). Notably, B-MYB knockdown under hypoxia led to a more pronounced downregulation of G2/M genes compared to hypoxic control siRNA-transfected cells and normoxic B-MYB-depleted cells, as evidenced by qRT-PCR (Figure 20A) and immunoblot analyses (Figure 20B). These findings support the notion that – unlike in normoxic cells – A-MYB can no longer functionally compensate for the loss of B-MYB in hypoxic cells to maintain the expression of B-MYB target genes.

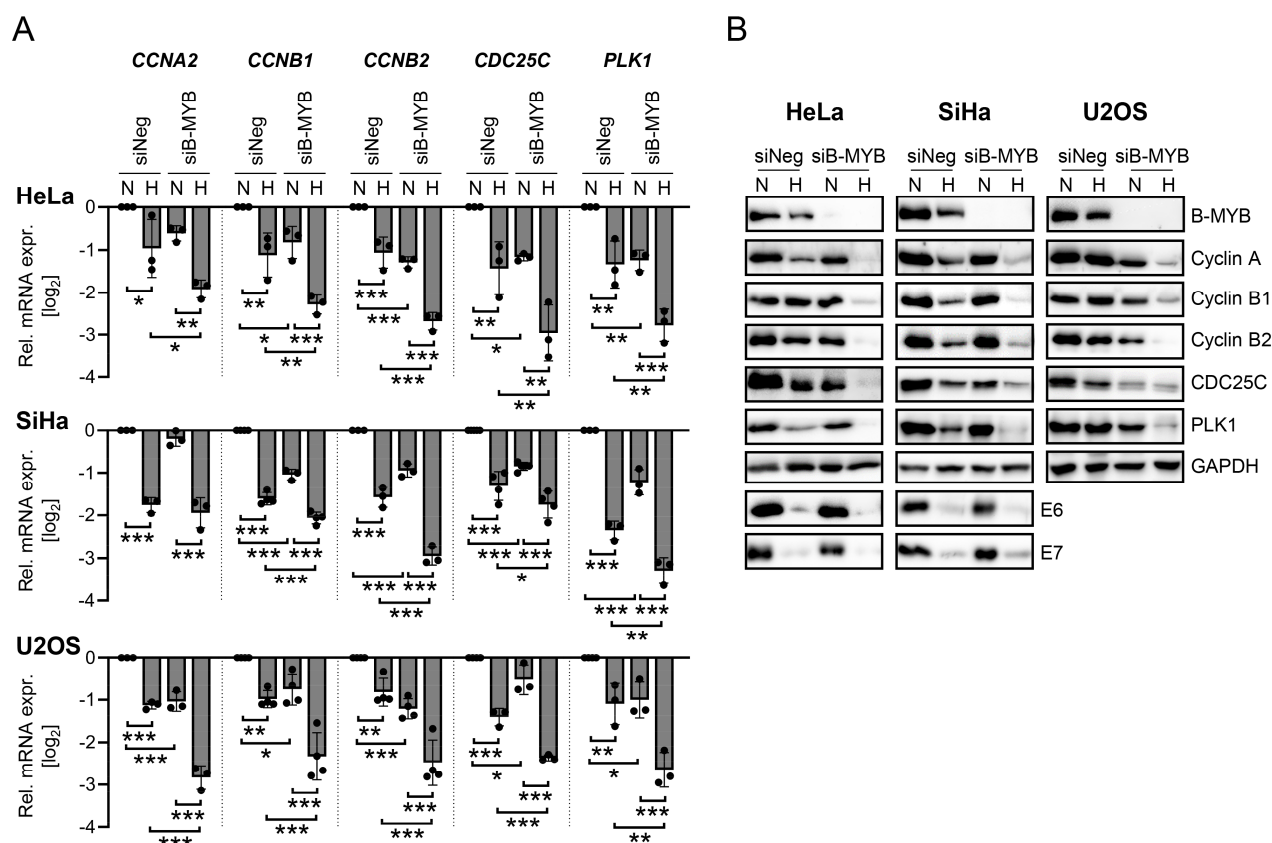


Figure 20. See the figure legend on the next page.

Figure 20. The lack of compensatory A-MYB induction enhances G2/M gene repression under hypoxia. HeLa, SiHa, or U2OS cells were transfected with siB-MYB or control siRNA (siNeg). 24 h post-transfection, cells were cultivated under normoxia (N; 21% O₂) or hypoxia (H; 1% O₂) and subsequently harvested after 48 h for RNA and protein analyses, with the exception of SiHa cells (harvested after 24 h for RNA analyses). (A) qRT-PCR analyses of *CCNA2* (coding for Cyclin A2), *CCNB1* (coding for Cyclin B1), *CCNB2* (coding for Cyclin B2), *CDC25C*, and *PLK1* mRNA levels. Shown are individual data points and mean expression levels with SD ($n=3$) relative to the expression of siNeg under normoxia ($\log_2FC$). Statistical significance was assessed using two-way ANOVA. $^*p \leq 0.05$, $^{**}p \leq 0.01$, and $^{***}p \leq 0.001$. (B) Immunoblot analyses of B-MYB, Cyclin A, Cyclin B1, Cyclin B2, CDC25C, PLK1, E6, E7, and GAPDH protein levels. Figure content and corresponding legend adapted from Velimirović et al., 2025.²³⁶

2.4.3 Hypoxic cells are more vulnerable to the pro-senescent effects of B-MYB loss

The more pronounced downregulation of B-MYB target genes under hypoxia (Figure 20), coupled with the earlier observation that A-MYB silencing enhanced the pro-senescent effects of B-MYB loss in normoxic cells (Figure 15), raised the question of whether hypoxic cells might be particularly sensitive to B-MYB depletion owing to their inability to induce A-MYB as a compensatory mechanism. To investigate this possibility, senescence induction was analyzed in hypoxic HPV-positive HeLa, SiHa, and HPV-negative U2OS cells following the treatment protocol outlined in Figure 21A.

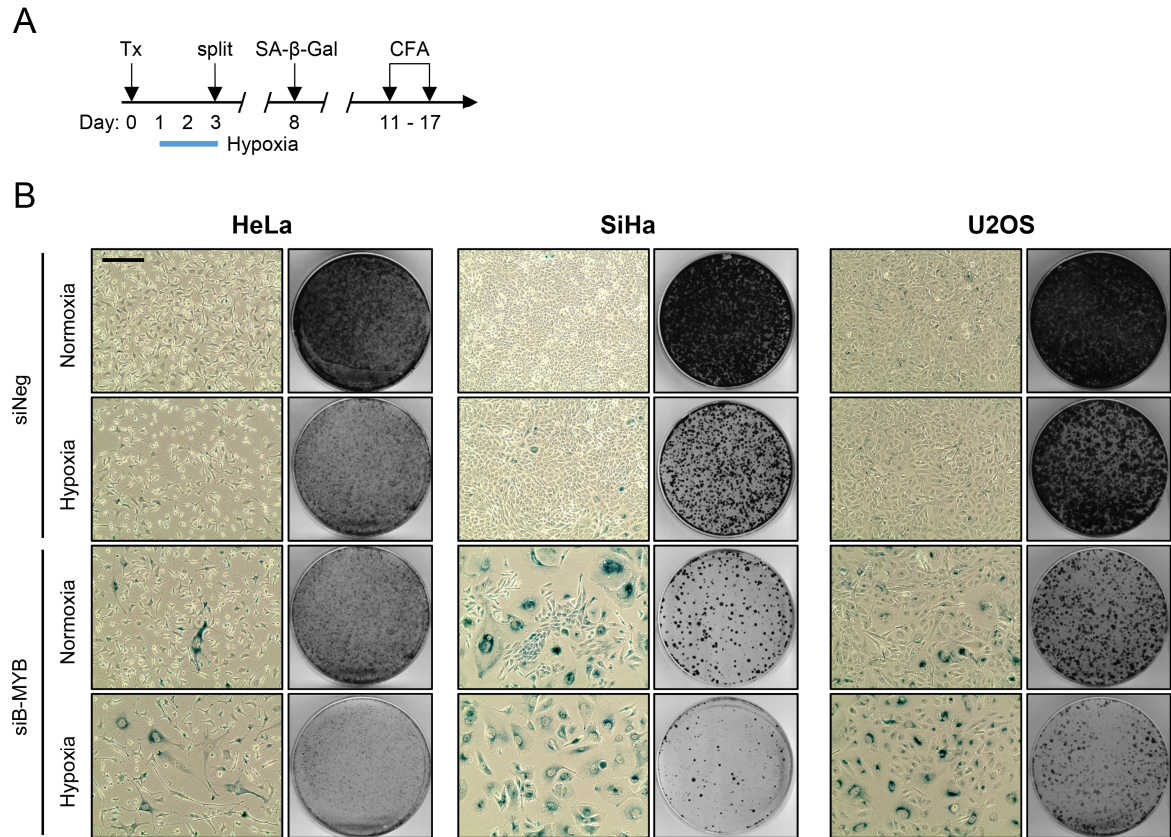


Figure 21. See the figure legend on the next page.

Figure 21. Hypoxic cells are more vulnerable to the pro-senescent effects of B-MYB loss. (A) Treatment scheme: HeLa, SiHa, or U2OS cells were transfected (Tx) with siB-MYB or control siRNA (siNeg). 24 h post-transfection, cells were cultivated under normoxia (21% O₂) or hypoxia (1% O₂) for 48 h. Cells were split and fixed after the indicated times for senescence assays (SA- β -Gal staining) or colony formation assays (CFAs). (B) Senescence assays (left panels for each cell line; SA- β -Gal positive cells staining blue) (scale bar: 200 μ m) and corresponding CFAs (right panels for each cell line). Figure content and corresponding legend adapted from Velimirović et al., 2025.²³⁶

Remarkably, compared to normoxic cells, B-MYB repression under hypoxia resulted in a clear increase in the number of SA- β -Gal-positive cells with the typical morphological changes associated with senescence, as well as a reduction of cells evading senescence (Figure 21B, left panels for each cell line). In accordance with this enhanced pro-senescent response, B-MYB silencing under hypoxia led to a substantial reduction of colony formation capacity, compared to B-MYB silencing under normoxia (Figure 21B, right panels for each cell line). This phenotypic outcome was consistently observed in all three tested cell lines. Taken together, these findings reveal that hypoxic cells are more susceptible to the pro-senescent effects of B-MYB repression, likely due to the lack of compensatory A-MYB induction.

2.4.4 Hypoxia confers resistance to the pro-apoptotic effect of B-MYB silencing

Next, I investigated the role of A-MYB in the apoptosis response. A-MYB was upregulated in response to B-MYB silencing under normoxia, which could counteract the pro-apoptotic effects of B-MYB loss in HPV-positive HeLa and SiHa cells. Conversely, concomitant depletion of both paralogs led to increased apoptosis compared to B-MYB silencing alone (Figure 16). Since my data showed that hypoxia suppresses this compensatory A-MYB induction (Figure 19), it would be expected that B-MYB silencing under hypoxia could similarly increase apoptosis. To further investigate this, I analyzed the expression of the apoptosis markers cleaved PARP and cleaved Caspase-9 in hypoxic HeLa and SiHa cells following B-MYB knockdown (Figure 22). Interestingly, whereas B-MYB silencing under normoxia led to increased expression of both apoptosis markers, hypoxia counteracted this response (Figure 22A). Further analyses using TUNEL assays corroborated the enhanced apoptosis resistance under hypoxia, as the percentage of TUNEL-positive SiHa cells was reduced upon B-MYB knockdown under hypoxia, relative to normoxia (Figure 22B). Collectively, these findings suggest that hypoxic cells, despite lacking the compensatory A-MYB induction, employ other mechanisms that can effectively protect them from the pro-apoptotic effects of B-MYB repression and rather induce a phenotypic switch towards senescence.

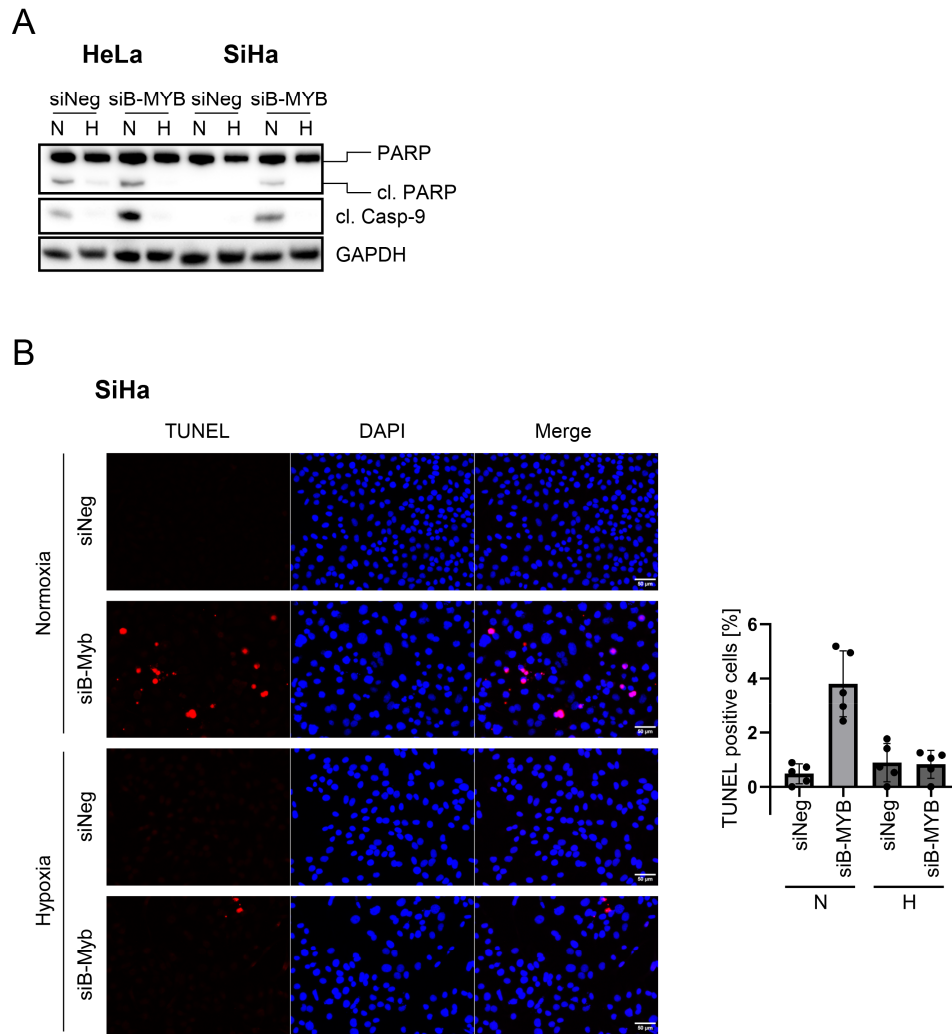


Figure 22. Hypoxia confers resistance to the pro-apoptotic effect of B-MYB silencing. HeLa or SiHa cells were transfected with siB-MYB, or control siRNA (siNeg). 24 h post-transfection, cells were cultivated under normoxia (N; 21% O₂) or hypoxia (H; 1% O₂) and subsequently harvested after 48 h for protein or TUNEL analyses. (A) Immunoblot analyses of cleaved PARP, cleaved Caspase-9, and GAPDH protein levels. (B) TUNEL analyses of SiHa cells. Shown are representative images from 2 independent experiments which produced consistent results (left panel) (scale bar: 50 μ m). TUNEL-positive (apoptotic) cells were quantified relative to DAPI-stained cells (reflecting total cell numbers) and are presented as averaged percentages from 5 different images per condition (right panel).

2.4.5 A-MYB overexpression counteracts the effects of B-MYB loss under hypoxia

My findings indicate that hypoxia efficiently impairs the compensatory mechanism between A-MYB and B-MYB, which results in stronger repression of B-MYB target genes and an enhanced pro-senescent response to B-MYB loss. To test whether the lack of compensatory A-MYB induction under hypoxia is directly responsible for the observed phenotype, rescue experiments were conducted using HeLa cells, which were selected due to their suitability for

highly efficient transfection via calcium phosphate co-precipitation (please refer to section 4.3.4). Cells were transfected with a vector overexpressing A-MYB according to the treatment scheme outlined in Figure 23A.

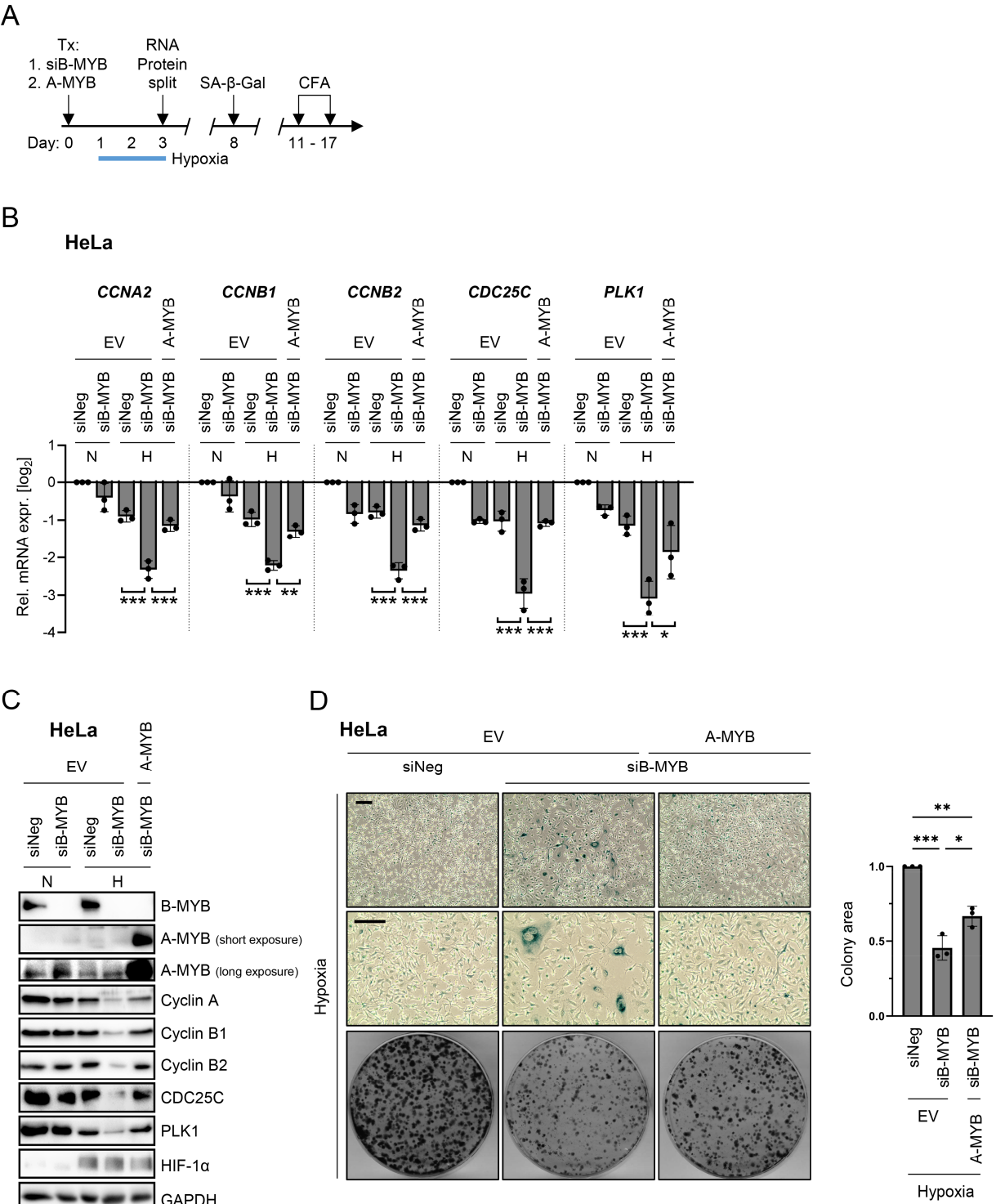


Figure 23. See figure legend on the next page.

Figure 23. A-MYB overexpression counteracts the effects of B-MYB loss under hypoxia. (A) Treatment scheme: HeLa cells were transfected with siB-MYB, or control siRNA (siNeg), followed by a transfection with a vector overexpressing *MYBL2* (A-MYB) or an empty control vector (EV) 8 h later. The next day, cells were placed under normoxia (N; 21% O₂) or hypoxia (H; 1% O₂) and cultivated for 48 h. (B) qRT-PCR analyses of *CCNA2* (coding for Cyclin A2), *CCNB1* (coding for Cyclin B1), *CCNB2* (coding for Cyclin B2), *CDC25C*, and *PLK1* mRNA levels. Shown are individual data points and mean expression levels with SD ($n=3$) relative to the expression of siNeg under normoxia ($\log_2FC$). Statistical significance was assessed using one-way ANOVA and only relevant comparisons are shown. $*p \leq 0.05$, $**p \leq 0.01$, and $***p \leq 0.001$. (C) Immunoblot analyses of B-MYB, A-MYB, Cyclin A, Cyclin B1, Cyclin B2, *CDC25C*, *PLK1*, HIF1- α , and GAPDH protein levels. (D) Senescence assays (upper panels; SA- β -Gal positive cells staining blue) (scale bar: 200 μ m) and corresponding CFAs (lower panels). CFAs were quantified from 3 independent experiments by measuring the percentage of area covered with colonies. Obtained colony area values were normalized to the corresponding control, which was set to 1. Shown are individual data points and mean expression levels with SD ($n=3$). Statistical significance was assessed using one-way ANOVA. $*p \leq 0.05$, $**p \leq 0.01$, and $***p \leq 0.001$.

Ectopic expression of A-MYB in B-MYB-depleted hypoxic HeLa cells restored the expression of B-MYB target genes, such as *CCNA2*, *CCNB1*, *CCNB2*, *CDC25C*, and *PLK1*, at the transcript (Figure 23B) and protein (Figure 23C) level, reaching levels comparable to those observed in hypoxic control-transfected cells. In addition, A-MYB overexpression rescued hypoxic HeLa cells from the pro-senescent effect of B-MYB loss, as evidenced by a reduction of SA- β -Gal-positive cells in senescence assays (Figure 23D, upper panels). Further, this response was accompanied by a partial restoration of their colony formation capacity (Figure 23D, lower panels).

Taken together, these results provide direct experimental evidence that the inability to induce A-MYB under hypoxia contributes to the heightened sensitivity of hypoxic cells to B-MYB depletion. Moreover, they strengthen the notion that A-MYB functionally substitutes for B-MYB in sustaining the expression of cell cycle-promoting genes and, thereby, mitigating the senescence response.

CHAPTER 3

DISCUSSION

3 Discussion

Tumor hypoxia poses a considerable obstacle in cancer therapy and is frequently associated with poor clinical outcomes.⁹⁶ One contributing factor to this is the capacity of hypoxic tumor cells to evade cellular senescence.¹¹⁶ In this study, I explored mechanisms by which hypoxic HPV-positive cancer cells can evade senescence and addressed the question of whether this regulation could form a basis for further therapeutic exploration. By comparing normoxic and hypoxic conditions, I identified a key role for the MYB family members B-MYB and A-MYB in regulating the senescence response. Specifically, hypoxic HPV-positive cancer cells are especially susceptible to the pro-senescent effects of B-MYB silencing, since – other than normoxic cells – they are not able to functionally compensate for B-MYB loss by the induction of A-MYB expression. Importantly, this regulatory mechanism was not restricted to HPV-positive cancer cells, indicating that it is conserved across different cell types. These findings reveal, for the first time, a role for A-MYB in senescence regulation and unravel a distinct vulnerability of hypoxic cancer cells, which indicates that B-MYB could serve as a therapeutic target to attack this critical tumor cell population.

3.1 Hypoxia-mediated phenotypic changes in cancer cells

Repression of E6/E7 expression in normoxic HPV-positive cancer cells results in rapid induction of senescence.^{154, 235, 267} In contrast, our group previously showed that hypoxic HPV-positive cancer cells, which effectively shut off E6/E7 expression, do not undergo senescence, but rather enter a dormant state that can be reversed upon reoxygenation.¹¹⁶ My investigations further explore these observations and uncover pronounced differences in how HPV-positive cancer cells respond to E6/E7 repression under normoxic versus hypoxic conditions, including distinct transcriptomic signatures, differential expression of factors involved in cell cycle regulation, and divergent mechanisms of cell cycle arrest and growth inhibition.

E6/E7 knockdown under normoxia restores the function of pRb and the DREAM complex, two central pathways in enforcing G1 arrest and senescence.²³⁴ pRb controls the G1/S transition by sequestering E2F transcription factors classified as activators (E2F1-3), thereby inhibiting the expression of E2F-responsive genes.²⁶⁸ In parallel, the pRb-related pocket proteins, p107, and p130, cooperate with repressor E2Fs (E2F4-5) and the MuvB core complex to form the DREAM complex, which mediates the repression of genes involved in cell cycle progression (G1/S and G2/M genes)⁵³ (Figure 2). Consistent with reactivation of these pathways following E6/E7 knockdown under normoxia, my findings firstly revealed a strong negative enrichment

of DREAM complex targets, as well as genes involved in G1/S and G2/M checkpoint transitions. Furthermore, several cell cycle-promoting genes, *B-MYB*, *FOXM1*, *CCNA2* (coding for Cyclin A2), *CCNB1* (coding for Cyclin B1), *CCNB2* (coding for Cyclin B2), *CDC25C*, and *RRM2*, which are known targets of pRb and/or the DREAM complex⁵¹ and are typically repressed during senescence,^{269, 270} were downregulated at both the transcript and protein levels. In strong contrast, this regulation was not observed upon E6/E7 repression by hypoxia. Secondly, while RNAi-mediated E6/E7 knockdown led to a highly efficient G1 arrest, consistent with suppression of cell cycle-promoting factors, this response was not detected in hypoxic cells. Yet, hypoxia also effectively suppressed cell proliferation, as evidenced by Nocodazole and EdU incorporation assays, suggesting that growth inhibition under hypoxia occurs via a distinct mechanism. My results indicate that hypoxia-mediated growth inhibition does not result from a marked cell cycle arrest in a specific cell cycle phase but rather reflects a general slowdown of cell cycle progression across multiple cell cycle phases. This likely accounts for the limited response of pRb and the DREAM complex target genes under hypoxia, whose expression is tightly linked to specific cell cycle phases and is, therefore, more profoundly downregulated in response to a synchronized G1 arrest, as seen following E6/E7 repression under normoxia.

Together, these findings demonstrate that, unlike RNAi-mediated E6/E7 silencing, hypoxia does not restore pRb and the DREAM complex function in a comparable manner, and instead enforces growth inhibition without a pronounced G1 arrest. Of note, these findings were mirrored in HPV-negative cancer cells, where hypoxia also suppressed proliferation, but did not induce a marked arrest in a specific cell cycle phase. This further indicates that hypoxia-mediated growth inhibition reflects a more general cellular response that is independent of the HPV status of the cell. One possible explanation for the lack of cell cycle phase-specific accumulation under hypoxia may be limited glucose availability, since hypoxic cells undergo metabolic reprogramming characterized by a shift from oxidative phosphorylation toward anaerobic glycolysis. Due to the substantially lower ATP yield of glycolysis (2 ATP per glucose molecule compared to the much higher output of oxidative phosphorylation, which is 32 ATP per glucose molecule), hypoxic cells adapt by enhancing glucose uptake to sustain adequate energy production, which over time creates a glucose-deprived environment.¹⁰⁷ Indeed, inhibition of proliferation without pronounced cellular accumulation in a distinct cell cycle phase has been similarly observed in cells cultured in a medium devoid of glucose (0 mM).⁸⁷ This was also detected in my studies, where glucose deprivation (0 mM) impaired Nocodazole-induced G2/M arrest, indicating a proliferative halt. In further support of the notion that the anti-proliferative effects of hypoxia in cancer cells are

tightly linked to glucose availability, I found that cultivating hypoxic cells in a high glucose medium (25 mM) restored their proliferative capacity.

Moreover, the observed hypoxia-mediated proliferative halt could explain the lack of senescence¹¹⁶ observed in hypoxic HPV-positive cells following Etoposide treatment. In this context, it should be noted that most chemotherapeutic agents, including Etoposide, a reversible topoisomerase II inhibitor widely used in cancer therapy,²⁴² preferentially target rapidly dividing cells.¹¹⁸ In normoxic, proliferating cells, treatment with Etoposide induces DNA damage during the S phase, and results in S phase accumulation and a strong G2/M arrest, ultimately triggering senescence response.^{242, 243} Notably, I found that hypoxia abrogated the S phase accumulation and G2/M arrest typically observed following Etoposide treatment of normoxic, proliferating cells. These findings suggest that hypoxia suppresses Etoposide-induced senescence by limiting cell cycle progression, which in turn shields the cells from the DNA-damaging effects of Etoposide during the S phase passage. Supporting this concept, similar protective effects against Etoposide were observed under glucose deprivation or Metformin treatment,⁸⁷ implying that other growth-limiting conditions can likewise suppress Etoposide-induced senescence by restricting cell cycle progression and cell growth.

3.2 MYB transcription factors in senescence, cell cycle regulation, and apoptosis

Notably, immunoblot analyses revealed that B-MYB expression is strongly inhibited following siE6/E7-induced senescence under normoxia, whereas it is sustained in hypoxic HPV-positive cells, which also downregulate E6/E7 expression but do not undergo senescence.¹¹⁶ This differential regulation was particularly interesting, as B-MYB is a proto-oncogene known to drive cell cycle progression and promote cellular proliferation.^{176, 271} B-MYB binds to the CHR promoter elements and activates the expression of late cell cycle genes (G2/M genes) through the MMB (B-MYB-MuvB) complex^{182, 184, 185} (Figure 2). Beyond its well-established role in cell proliferation, B-MYB has been implicated in the regulation of cellular senescence, as its downregulation both drives and marks senescence.²¹¹ Furthermore, B-MYB expression has been shown to counteract *ras*-induced senescence²¹⁵ (a form of oncogene-induced senescence), and the concurrent expression of the FOXM1-MMB complex has been reported to efficiently bypass senescence.²¹⁴ Therefore, the sustained expression of B-MYB under hypoxia pointed toward a possible role for B-MYB in facilitating senescence evasion and consequently became a central focus of my studies.

3.2.1 Partial induction of senescence upon B-MYB repression in normoxic cancer cells

The marked downregulation of B-MYB expression following E6/E7 knockdown under normoxia is consistent with the reactivation of pRb and the DREAM complex activity and the establishment of a stable G1 arrest.^{51, 87, 234} B-MYB expression is tightly regulated in a cell cycle-dependent manner, since the *MYBL2* gene (coding for B-MYB) is a transcriptional target of both pRb and the DREAM complex.⁵¹ Therefore, B-MYB is actively repressed during the early G1 phase and becomes expressed during the late G1 and S phases.

In view of the documented anti-senescent properties of B-MYB (please refer to section 1.4.3), including in HPV-positive HeLa cells,¹⁹⁰ it was likely that the loss of B-MYB during siE6/E7-induced senescence was not merely a consequence, but a contributing factor to the establishment of the senescent phenotype. Indeed, I found that B-MYB silencing alone was sufficient to trigger senescence in normoxic HPV-positive cancer cells. The observed senescence induction upon B-MYB silencing could stem from the fact that the loss of B-MYB disturbs MuvB-mediated activation of G2/M genes as it prevents the formation of the MMB, and latter MuvB-related (FOXM1-MMB, FOXM1-MuvB) complexes (Figure 2). Interestingly, however, FOXM1 knockdown did not induce senescence in HPV-positive cells, despite its role in these complexes and its marked downregulation during siE6/E7-induced senescence. This finding was unexpected and contrasts with previous reports linking FOXM1 depletion to the induction of senescence across different cellular models.²⁷²⁻²⁷⁴ One possible reason for this discrepancy may reside in the experimental approach used in my studies, since it cannot be formally excluded that the siRNA-mediated knockdown may have left residual FOXM1 protein levels sufficient to form the FOXM1-MMB and FOXM1-MuvB complexes and bypass senescence.²¹⁴ Alternatively, considering the transforming capacities of the HPV E6 and E7 oncoproteins,¹⁹ it could also be possible that they engage signaling pathways that can sustain proliferation and prevent senescence even in the absence of FOXM1.

Another important consideration is that studies linking FOXM1 repression to the induction of senescence typically involve additional cellular stress. For example, FOXM1-depleted MCF-7 breast cancer cells showed reduced viability and induction of senescence following treatment with the chemotherapeutic drug Epirubicin or γ -irradiation.²⁷² In Panc1 pancreatic cancer cells, combined treatment with the anti-cancer drugs Romidepsin and Tamoxifen led to a marked reduction in FOXM1 levels, with this downregulation identified as a key event for the onset of cellular senescence.²⁷³ In proliferating epithelial cells, silencing of FOXM1 was sufficient to induce a senescent phenotype, although this effect was partially attributed to elevated levels of intracellular ROS,²⁷⁴ suggesting that, in certain cellular contexts, FOXM1 loss may sensitize

cells to stress-induced senescence. This aligns with FOXM1's established role in therapy resistance,^{258-261, 275-277} while its inhibition enhances cellular sensitivity to genotoxic agents and promotes treatment-induced senescence or apoptosis.^{264, 272, 273, 278} Consistent with this, I found that the treatment of HPV-positive cancer cells with Cisplatin, a chemotherapeutic agent commonly used for the treatment of cervical cancer,²⁷⁹ induces FOXM1 expression, corroborating previous observations in other tumor types.^{263, 277} Conversely, FOXM1 knockdown enhances Cisplatin-induced cell death in normoxic HPV-positive cancer cells, further supporting a role for FOXM1 in promoting therapy resistance, including in cervical cancer.^{262, 280-282} Extending these findings, I demonstrate that FOXM1 depletion also sensitizes hypoxic HPV-positive cells to Cisplatin, albeit less effectively, likely due to hypoxia-mediated protective mechanisms, such as growth arrest, which limit the susceptibility of non-cycling cancer cells to DNA-damaging agents such as Cisplatin.

In summary, these findings highlight the distinct cellular consequences that arise from targeting different components of MuvB-associated complexes. While FOXM1 depletion does not seem to directly induce senescence in HPV-positive cancer cells, B-MYB plays an active role in triggering senescence in these cells. Notably, despite highly efficient siRNA-mediated B-MYB repression, the resulting senescence response was only partial, as evidenced by the emergence of cells that lack typical morphological signs of senescence and do not stain positive for the senescence marker SA- β -Gal. These findings suggest that while the loss of B-MYB is sufficient to trigger senescence, additional events are required for a complete senescence response. In HPV-positive cancer cells, siE6/E7-induced senescence mobilizes multiple overlapping senescence-inducing signals.²¹⁶ For instance, senescence induction upon E6/E7 repression is supported by the reconstitution of pRb- and DREAM-mediated transcriptional repression of cell cycle-promoting genes,²³⁴ including B-MYB, and intact mTORC1 activity.¹¹⁶ Finally, since I observed that B-MYB knockdown also induces only a partial senescence response in HPV-negative U2OS cells, my results suggest that the limited pro-senescent effect of B-MYB silencing could be mitigated by a compensatory mechanism that is independent of the HPV status of the cell.

3.2.2 Concomitant A-MYB silencing is required for efficient senescence induction upon B-MYB repression in normoxic cancer cells

Interestingly, a recent study by Kohler and Engeland demonstrated that, under normoxic conditions, cells can functionally compensate for anti-proliferative effects of B-MYB loss by inducing the expression of its paralog, A-MYB.²³³ I hypothesized that this compensatory mechanism may be involved in the limited senescence response observed following B-MYB

knockdown. Indeed, A-MYB levels were upregulated following B-MYB silencing in normoxic cells, and their senescence response was substantially enhanced when A-MYB was co-silenced alongside B-MYB. Moreover, co-silencing of B-MYB and A-MYB led to a markedly stronger repression of B-MYB target genes involved in cell cycle progression (G2/M genes) compared to B-MYB silencing alone. Consistent with the repression of these genes, co-silencing of B-MYB and A-MYB led to a pronounced G2/M arrest. This suggests that A-MYB may functionally substitute for the transcriptional activation of G2/M gene expression in the absence of B-MYB, thereby supporting continued cell cycle progression. In line with this hypothesis, a previous study demonstrated that B-MYB knockdown reduces, but does not completely abolish, transcriptional activation via CHR promoter elements in its target gene *CCNB2* (coding for Cyclin B2).¹⁸⁵ The compensatory induction of A-MYB may thus account for this residual transactivation of target genes via CHR sites, potentially substituting for B-MYB within the MMB (B-MYB-MuvB) complex. Supporting this latter notion, A-MYB has been shown to associate with the MuvB core components,²⁸³ and indirectly bind to the CHR sites in *CCNB2*, through the MuvB core complex.²³³ Of note, A-MYB binds to the MuvB complex with a lower affinity compared to B-MYB.²⁸³

Remarkably, the two B-MYB target genes, *CDC25C* (cell division cycle 25c) and *PLK1* (polo-like kinase 1), were consistently strongly downregulated upon co-silencing of B-MYB and A-MYB across all examined cell lines. This could indicate a key mechanism underlying the pronounced G2/M cell cycle arrest observed following combined B-MYB and A-MYB silencing, as both *CDC25C* and *PLK1* are critical factors for G2/M progression.^{250, 252} In specific, the serine/threonine kinase family member *PLK1* phosphorylates a serine residue within the nuclear export signal (NES) sequence of *CDC25C* during prophase, facilitating its nuclear translocation.²⁴⁹ Nuclear localization of *CDC25C*, a critical G2/M checkpoint phosphatase, is essential for its ability to dephosphorylate CDK1 and activate the Cyclin B1/CDK1 complex, triggering mitosis.²⁵² Of note, loss of either *PLK1* or *CDC25C* has previously been associated with G2/M phase arrest.²⁸⁴⁻²⁸⁸ Further, *PLK1* downregulation via siRNAs or using pharmacological inhibitors has been repeatedly linked to senescence induction.²⁸⁹⁻²⁹¹ Although the direct causal role of *CDC25C* in cellular senescence has not been demonstrated yet, several studies have reported its downregulation in senescence-associated contexts, including E6/E7 repression, replicative senescence, and TNF- α -induced senescence, where *CDC25C* gene is transcriptionally repressed as part of the DREAM complex-mediated repression.²⁶⁹ Moreover, *CDC25C* has been identified as a downstream target of at least one senescence-associated microRNA, and it is downregulated during Temozolomide-induced senescence.^{292, 293} Of further note, the pronounced G2/M cell cycle arrest upon combined silencing of B-MYB and A-MYB was accompanied by an increase in endoreduplication

events,²⁹⁴ likely arising from failed mitosis due to the insufficient activation of B-MYB target genes. This results in the accumulation of polyploid cells. Finally, my findings indicate that the senescence induction following combined B-MYB and A-MYB knockdown arises from a G2/M arrest. At first glance, this appears to contrast with the classical view of senescence as an irreversible arrest occurring in the G1 phase (G1 exit). However, emerging evidence indicates that senescence can also be initiated following cell cycle arrest at the G2/M checkpoint.^{138, 295}

In addition to blocking senescence induction, a number of studies suggest that B-MYB can also promote cell survival by upregulating the expression of anti-apoptotic genes and repressing pro-apoptotic pathways.²⁹⁶ For instance, B-MYB overexpression has been shown to confer resistance to apoptosis in CTLL-2 cytotoxic T cells as well as in neuroblastoma cells exposed to DNA-damaging agents like Doxorubicin and UV irradiation.^{297, 298} Conversely, B-MYB depletion has been associated with enhanced apoptotic response. For instance, in colorectal and gastric cancer cells, B-MYB knockdown increased basal and drug-induced apoptosis.^{299, 300} Interestingly, in my studies I found that both HPV-positive and HPV-negative cancer cells exhibit increased apoptotic response upon combined silencing of B-MYB and A-MYB, compared to B-MYB silencing alone, suggesting that compensatory induction of A-MYB following B-MYB loss can also mitigate the pro-apoptotic effects of B-MYB silencing. This observation is corroborated by a recent study showing that simultaneous loss of both B-MYB and A-MYB induces cellular stress and upregulates genes resembling a p53-like transcriptional signature, which the authors linked to apoptosis induction.²³³ Additionally, ectopic expression of A-MYB has been shown to protect murine B-cell lymphomas from anti-IgM-induced apoptosis,³⁰¹ further supporting its potential anti-apoptotic function. However, beyond these observations, the specific role of A-MYB in regulating apoptotic pathways remains poorly understood, warranting further research.

It is further important to note that the phenotypic effect of B-MYB silencing varied to some degree between different cell lines analyzed in my studies. Specifically, SiHa cells appear to be particularly “B-MYB addicted”, since they exhibit a strong reduction in proliferation as well as a pronounced G2/M arrest and substantial senescence induction in response to B-MYB silencing alone, whereas these effects were much less pronounced in HeLa cells. CaSki, U2OS, and HCT116 cells exhibited an intermediate phenotype. These observations align with previous findings indicating a cell type-dependent functional roles of B-MYB, which even include no effects upon B-MYB loss in several cell lines.²³³ To this end, the study by Kohler and Engeland suggested that the variability in cellular responses to B-MYB loss could be attributed to differences in (compensatory) A-MYB expression levels across different cell types.²³³ Specifically, their analysis of the DepMap database revealed a strong link between increased mRNA *MYBL1* (coding for A-MYB) expression and reduced effect of B-MYB

knockout or knockdown on cell proliferation and survival.²³³ Interestingly, in my own experiments, A-MYB upregulation following B-MYB silencing occurred mainly on the protein level, rather than on RNA level, and the increase of A-MYB protein levels was comparable across different tested cell lines, despite their varying sensitivities to B-MYB loss. This raises the possibility that additional factors may modulate A-MYB's compensatory activity in response to B-MYB loss, including, for instance, specific post-translational modifications of A-MYB or the availability of co-factors important for A-MYB function. Moreover, alternative A-MYB-independent compensatory pathways may also contribute to mitigating the impact of B-MYB loss, in a cell type-dependent manner.

3.2.3 Hypoxic cancer cells are more susceptible to B-MYB repression due to impaired compensatory A-MYB induction

Strikingly, while normoxic cancer cells responded to B-MYB depletion with a functionally compensatory upregulation of A-MYB, I found that this adaptive response was absent under hypoxic conditions. Moreover, hypoxia itself markedly reduced basal *MYBL1* mRNA levels. These findings point to an oxygen-dependent regulation of *MYBL1* at the transcript level. However, the mechanisms governing the expression and stability of MYB family members under hypoxia are thus far poorly understood. So far, HIF-2 α has been indicated to play a role in stabilizing B-MYB via physical interaction with its C-terminal domain³⁰² although the exact molecular mechanisms underlying this regulation remain to be fully elucidated. Moreover, in pancreatic cancer cells, hypoxia has been shown to upregulate *MYB* (encoding the B-MYB paralog, C-MYB) expression through mRNA stabilization mediated by the RNA-binding protein HuR.³⁰³ Nonetheless, further studies are required to gain a complete understanding of the mechanisms that regulate MYB family members in the hypoxic environment.

The lack of A-MYB induction following B-MYB knockdown under hypoxia suggested that hypoxic cancer cells could be more sensitive to the loss of B-MYB owing to their inability to compensate for it by inducing A-MYB. Indeed, I found that B-MYB depletion under hypoxia led to a stronger senescence response and a more pronounced downregulation of B-MYB target genes compared to normoxia. Supporting the notion that the lack of A-MYB induction under hypoxia is one of the key factors contributing to the heightened sensitivity of hypoxic cancer cells to B-MYB depletion, ectopic expression of A-MYB in hypoxic HeLa cells, in which B-MYB was silenced, restored the expression of B-MYB target genes, attenuated the senescence response, and improved colony formation capacity. Notably, A-MYB overexpression did not fully restore the proliferative capacity of HeLa cells, which could possibly be attributed to the transient nature of the transfection method. As CFAs assess long-term proliferative capacity,

it is conceivable that, for instance, A-MYB expression declined over time while B-MYB inhibition persisted, ultimately limiting the compensatory effects of A-MYB.

The compensatory A-MYB induction was recently reported to mitigate the pro-apoptotic effects of B-MYB loss,²³³ consistent with the observations from my study analyzing the apoptotic response to B-MYB silencing in normoxic cells. Based on these observations, I expected that hypoxic cells, lacking the compensatory A-MYB induction, would also exhibit heightened sensitivity to the pro-apoptotic effects of B-MYB silencing. Surprisingly, however, hypoxic HPV-positive cells were rather protected from the pro-apoptotic effects of B-MYB silencing. This finding aligns with previous reports indicating that hypoxia can exert anti-apoptotic effects.³⁰⁴⁻³⁰⁷ Specifically, in this context, hypoxia may impair apoptotic signaling and shift the cellular response toward senescence.

In conclusion, my findings demonstrate that the absence of compensatory A-MYB induction underlies the heightened sensitivity of hypoxic cancer cells to pro-senescent effects of B-MYB loss. A proposed model summarizing the findings of this study is presented in Figure 24.

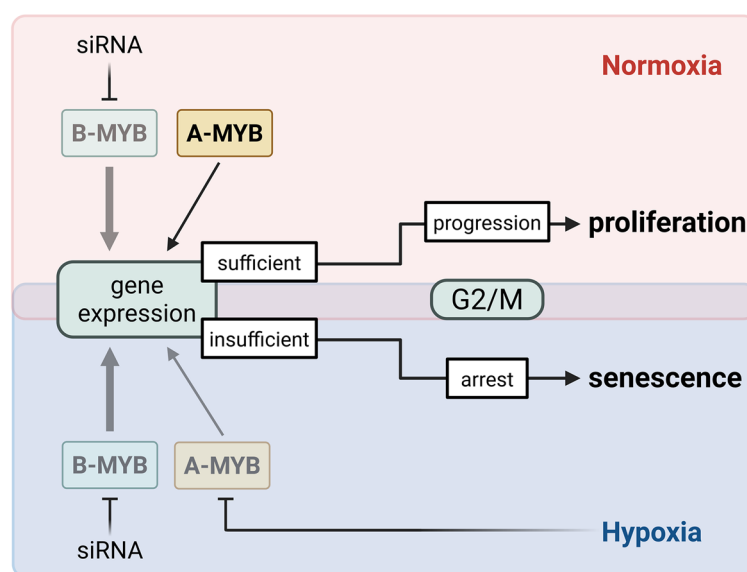


Figure 24. Proposed model illustrating the enhanced vulnerability of hypoxic cancer cells to B-MYB depletion. Under normoxia (upper part), silencing of B-MYB triggers a compensatory induction of its paralog A-MYB, which can partially substitute for the loss of B-MYB by maintaining the expression of key cell cycle-promoting genes essential for G2/M progression, thereby supporting continued cell proliferation. Under hypoxia (lower part), however, cells are impaired in their ability to induce A-MYB in response to B-MYB loss. This results in insufficient expression of cell cycle-promoting genes, impaired cell cycle progression and an increased senescence response. Image created with BioRender. Figure content and corresponding legend adapted from Velimirović et al., 2025.²³⁶

3.3 MYB inhibitors: opportunities and challenges

Tumor hypoxia often stems from disorganized, poorly structured tumor vasculature, which fails to adequately supply the growing tumor tissue with oxygen.⁹² Hypoxia can enhance the malignant phenotype of cancer cells, supporting their survival and promoting cell migration, invasion, and metastasis.³⁰⁸ Moreover, hypoxia is linked to therapeutic resistance of cancers towards standard anti-cancer treatments in the clinic. For example, limited oxygen availability reduces the efficacy of radiotherapy by interfering with the fixation of radiation-induced DNA damage.⁹⁶ Further, hypoxic cancer cells often remain shielded from treatment due to the compromised tumor vascularization that can hinder drug delivery, diminishing the impact of chemotherapeutics.¹¹⁸ Additionally, the effectiveness of many chemotherapeutic agents depends on the presence of molecular oxygen.¹¹⁸ Hypoxia can also induce the expression of drug efflux transporters, lowering the intracellular concentration of drugs, which further reduces their potency.^{96, 118} Lastly, due to the hypoxia-mediated growth arrest, hypoxic cells tend to be less sensitive to chemotherapies that preferentially target rapidly dividing cells.¹¹⁸ Therefore, there is an urgent need for therapeutic strategies that more effectively target the hypoxic cancer cell populations. Currently, intensively studied approaches include the development of HAPs (hypoxia-activated prodrugs) and HIF-1 inhibitors as well as the intratumoral injection of hypoxia-targeting bacteria.^{137, 309, 310} However, many of these approaches are either still in the preclinical phases or have resulted in only limited success in clinical studies.³¹¹ Thus, it is of utmost importance to identify vulnerabilities of hypoxic cancer cells, which could be explored for therapeutic purposes.

My findings reveal that hypoxic cancer cells exhibit a heightened sensitivity to the pro-senescent effects of B-MYB depletion, since they lack the ability to compensate for B-MYB repression by A-MYB induction. B-MYB has been increasingly recognized as a potential target for cancer therapy due to its role in tumorigenesis and association with poor clinical outcomes across a wide spectrum of malignancies, including cervical,²⁰⁰ ovarian,²⁰⁵ breast,²⁰¹ colon,¹⁹⁸ bladder,³¹² liver,¹⁹⁹ and non-small cell lung cancer.²⁰³ Moreover, MYB transcription factors, including B-MYB, have emerged as mediators of resistance to a wide range of anti-cancer therapies, such as chemotherapy, targeted therapy, epigenetic drugs, and immunotherapy.²⁰⁸ Therefore, several strategies aimed at targeting MYB family members are currently under evaluation in preclinical trials.²⁰⁸ These include small-molecule inhibitors, MYB-targeting polypeptides, antisense oligonucleotides, drug repurposing, and even MYB-based vaccines.²⁰⁸ Importantly, to date, most efforts in the development of MYB-targeted therapies have focused predominantly on C-MYB, and the majority of MYB inhibitors described in the literature primarily refer to agents targeting C-MYB activity.³¹³

Moreover, it must be considered that targeting transcription factors is inherently challenging due to the lack of well-characterized drug-binding sites and the structural complexity that makes it difficult to disrupt their interactions with the DNA or other proteins.³¹⁴ Additionally, MYB proteins, particularly B-MYB, which is expressed in virtually all proliferating cells, play essential roles in cell cycle regulation,¹⁷⁵ raising concerns about potential side effects when targeting them therapeutically (“therapeutic window”). Further, B-MYB has also been shown to contribute to genome stability by facilitating the repair of DNA damage in hematopoietic cells, therefore, systemic inhibition of B-MYB could lead to hematopoietic toxicity.³¹⁵ Lastly, whereas combining agents that target MYB transcription factors with standard anti-cancer therapies has been shown to enhance the sensitivity of cancer cells to treatment,^{316, 317} potential toxicities from such combinations must be carefully considered.

My findings have two major implications for the ongoing development of B-MYB targeting therapeutic strategies. Firstly, since hypoxic cancer cells lack the compensatory A-MYB induction, they are particularly vulnerable to B-MYB inhibitors. This strikingly differs from the effects of most classical anti-cancer agents (chemotherapy, radiotherapy) to which hypoxic cells are usually more resistant. Therefore, B-MYB inhibitors could not only have anti-cancer potential per se, but also may possess the capacity to serve as adjuvants during chemo- or radiotherapy. Secondly, my results indicate that, under normoxia, the pro-senescent and pro-apoptotic efficacy of B-MYB inhibitors can be substantially impaired by a reactive A-MYB induction. Thus, the design of B-MYB targeting strategies could benefit from co-targeting A-MYB in normoxic cancer cells.

It is also important to note that my studies focused on the direct effects of the interplay between B-MYB and A-MYB under tightly controlled *in vitro* conditions. It is likely that the situation in the tumor microenvironment *in vivo* is far more complex, and additional parameters could affect phenotypic outcomes. These include the heterogeneous distribution of glucose and oxygen within tumors.¹⁰³ For instance, my data indicate that the senescence response of hypoxic cancer cells is favored under glucose levels lower than those in serum. Yet, in this context, it is also noteworthy that the tumor interstitium was found to contain glucose concentrations up to tenfold lower than those in human serum.³¹⁸ Similarly, the varying oxygen levels that are typically found in different tumor subregions⁹³ are likely to impact the efficacy and overall outcome of therapeutic strategies that depend heavily on tumor oxygenation status.

Collectively, my findings provide novel insights into the senescence response of normoxic and hypoxic HPV-positive cancer cells. Moreover, the critical interplay between B-MYB and A-MYB in regulating cellular senescence is not limited to HPV-positive cancer cells, indicating mechanistic conservation across different cell types. Importantly, the lack of compensatory

A-MYB induction makes hypoxic cancer cells particularly sensitive to B-MYB depletion, revealing a potential “Achilles heel” for targeting this therapeutically highly problematic cancer cell population.

CHAPTER 4

MATERIALS AND METHODS

4 Materials and methods

4.1 Reagents and materials

Whenever applicable, molecular biology grade reagents and standard laboratory materials were obtained from established suppliers, including: AppliChem (Darmstadt, Germany), Bio-Rad (Hercules, CA, USA), Biozym Scientific (Hessisch Oldendorf, Germany), BD Biosciences (Franklin Lakes, NJ, USA), Carl Roth (Karlsruhe, Germany), Corning (Corning, NY, USA), Enzo Life Sciences (Farmingdale, NY, USA), Eppendorf (Hamburg, Germany), GE Healthcare (Chicago, IL, USA), Gerbu Biotechnik (Heidelberg, Germany), Greiner Bio-One (Kremsmünster, Austria), Gabler-Saliter Milchwerk (Obergünzburg, Germany), Merck (Darmstadt, Germany), Nerbe Plus (Winsen, Germany), New England Biolabs (NEB) (Ipswich, MA, USA), Promega (Madison, WI, USA), Roche Diagnostics (Basel, Switzerland), Santa Cruz Biotechnology (Dallas, TX, USA), Sigma-Aldrich (St. Louis, MO, USA), Sartorius (Göttingen, Germany), Thermo Fisher Scientific (Waltham, MA, USA), Vector Laboratories (Newark, CA, USA), Qiagen (Hilden, Germany). Unless specified otherwise, all buffers and stock solutions were prepared using double-distilled water (ddH₂O). When non-standard reagents were used, manufacturers are indicated directly in the respective sections.

4.2 DNA-based methods

4.2.1 Plasmid preparation and validation

For ectopic A-MYB overexpression, plasmid TFORF3370 was used. TFORF3370 was a gift from Feng Zhang (Addgene plasmid #144846; http://n2t.net/addgene:144846;RRID:Addgene_144846).³¹⁹ To generate a corresponding empty control vector (EV), the *MYBL1* insert was excised from the TFORF3370 plasmid by double digestion with NheI and MluI restriction enzymes. The resulting sticky ends were subsequently blunted using T4 DNA polymerase, enabling efficient ligation. Validation of the control vector was performed by restriction analysis using the enzymes: SacII and HindIII (all from NEB), followed by agarose gel electrophoresis. Agarose gels (1%) were prepared by dissolving agarose in an electrophoresis buffer (pH 7.8; 40 mM Tris, 1 mM EDTA, 5mM sodium acetate) and heating the mixture in a microwave until fully melted. To enable visualization of DNA, PeqGREEN, a nucleic acid stain (VWR, Avantor, PA, US), was added to the solution prior to casting. A 6x loading buffer (0.25% bromophenol blue, 0.25% xylene cyanol, 30% glycerol) was added to the DNA samples and samples were loaded on gel alongside 5 µL of DNA SmartLadder (Eurogentec, Seraing, Belgium) for size reference. The gels were run in a PerfectBlue

electrophoresis system (VWR, Avantor, Radnor, PA, US) and electrophoresis was carried out at 80 V for 90 min. For visualization of the DNA fragments, a gel documentation system with UV illumination (Intas Science Imaging Instruments, Göttingen, Germany) was used.

4.2.2 Plasmid transformation

Competent *E. coli* TG2 cells were prepared according to the protocol of D.Hanahan.³²⁰ In short, bacteria were grown overnight in Lysogeny Broth (LB) medium (pH 7.0; 1% NaCl, 1% bacto trypton, 0.5% yeast extract) at 37 °C. The following day, cells were pelleted by centrifugation at 800 *g* for 5 min and resuspended in the transformation buffer (pH 6.5; 5% dimethyl sulfoxide (DMSO), 10% polyethylene glycol 8000, 15% glycerol, 50 nM MgCl₂, in LB medium). For transformation, plasmid DNA was mixed with 100 µL of competent TG2 cells and incubated on ice for 30 min. This was followed by a 1 min heat shock at 42 °C in a water bath. The suspension was immediately returned to the ice, then supplemented with 900 µL of LB medium and incubated at 37 °C for 1 h while shaking. In the next step, 100-200 µL of the transformation mixture were plated on LB agar containing 100 µg/mL ampicillin and incubated overnight at 37 °C. Bacterial colonies from the agar plate were used to inoculate an overnight culture of LB medium containing 100 µg/mL ampicillin. For the isolation of plasmid DNA from overnight LB cultures, the QIAprep Spin Miniprep Kit (Qiagen) was used following the manufacturer's protocol. DNA concentration was assessed using the NanoDrop ND-1000 (Peglab, Erlangen, Germany), measuring absorbance at 260 nm.

4.3 Cell-based methods

4.3.1 Cell culture

The following cell lines of human origin were acquired from the tumor bank of the German Cancer Research Center (DKFZ, Heidelberg, Germany): 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: U2OS (RRID: CVCL_0042) osteosarcoma, HCT116 (RRID: CVCL_0291) colon cancer, MCF-7 (RRID: CVCL_0031) breast cancer, NCI-H1299 (RRID: CVCL_0060) non-small cell lung carcinoma, HEK293T (RRID: CVCL_0063) human embryonic kidney, and HaCaT (RRID: CVCL_0038) spontaneously immortalized keratinocyte cells. HeLa-mKate2 and SiHa-mKate2 cells, stably expressing mKate2, a nuclear-restricted fluorescent protein, were generated as previously described.⁹⁰ Cells were maintained in Dulbecco's minimal essential medium (DMEM) containing 1 g/L glucose (5.5 mM) (HeLa, SiHa, CaSki, U2OS, MCF-7, HEK293T, and HaCaT cells),

McCoy's 5A medium (HCT116 cells) or RPMI 1640 medium (NCI-H1299 cells) (all Gibco, Thermo Fisher Scientific), supplemented with 10% fetal calf serum (FCS) (PAN-Biotech, Aidenbach, Germany), 2 mM L-glutamine, 100 U/mL penicillin, and 100 µg/mL streptomycin (all Sigma-Aldrich). For experiments involving no or high glucose conditions, DMEM with no (0 mM) or containing 4 g/L glucose (25 mM) was used. Cell authentication was performed by single-nucleotide polymorphism profiling (Multiplexion, Heidelberg, Germany), and cells were certified negative for mycoplasma contamination via PCR.

Cells were incubated at 37 °C and 5% CO₂ under normoxia (21% O₂) or hypoxia (1% O₂). Normoxic conditions were achieved in a humidified incubator. Hypoxic conditions were achieved using an InvivoO₂ 400 physiological oxygen workstation (Ruskin Technology, Bridgend, UK). For routine passage, cell stock cultures were split using 0.25% Trypsin-EDTA (Thermo Fisher Scientific) every 3-4 days, when they reached approximately 80% confluency. Cell viability was determined using the trypan blue exclusion method, and cells were counted using the Countess Automated Cell Counter (Thermo Fisher Scientific) for experiments involving seeding of a certain number of cells or cell quantification.

4.3.2 Long-term storage and thawing of cells

Cells were first trypsinized, centrifuged at 800 g for 3-5 minutes, and then resuspended in a cryopreservation medium containing 30% FCS and 10% DMSO. The cell suspension was aliquoted into cryovials, which were placed in isopropanol-filled freezing containers (e.g., Nalgene or Mr. Frosty, Thermo Fisher Scientific) for gradual freezing at -80 °C over the course of several days. Once frozen, vials were subsequently transferred to liquid nitrogen. For thawing, the cryovials were rapidly warmed in a 37 °C water bath. Once thawed, the cells were gently resuspended in fresh culture medium and transferred into a new cell culture flask. To eliminate any remaining DMSO, the medium was replaced the following day.

4.3.3 siRNA transfection

Chemically synthesized Silencer Select small interfering RNAs (siRNAs) (Thermo Fisher Scientific) were used to silence gene expression via RNA interference (RNAi). For transfection, siRNAs were diluted at a final concentration of 10-20 nM by pre-incubating 2.5-5 µL of the 10 µM siRNA stock with Opti-MEM medium (Thermo Fisher Scientific) in a total volume of 250 µL. The siRNA solution was incubated for 5 min at room temperature (RT). Simultaneously, 5 µL of Lipofectamine RNAiMAX transfection reagent (Thermo Fisher

Scientific) was mixed with Opti-MEM medium in a total volume of 250 μ L, incubated for 5 min at RT, combined with the siRNA solution and incubated for an additional 20 min at RT.

For forward transfections, cells were seeded in 6 cm dishes, and 24 h after seeding, the medium was exchanged with 2 mL of DMEM supplemented with 10% FCS (without antibiotics or L-glutamine). The transfection mix (500 μ L) was added to the 6 cm dishes. For reverse transfections, 500 μ L of the transfection mix was first added to a 6 cm dish. Cells from stock cultures were trypsinized, resuspended in DMEM supplemented with 10% FCS (without antibiotics or L-glutamine), and plated onto the transfection mixture in a final volume of 2.5 mL. In both cases, 24 h post-transfection the medium was exchanged with fresh standard cell culture medium. For reverse transfections in 96-well plates, 0.2 μ L of RNAiMAX was used in a total volume of 100 μ L per well. Following the initial 24 h incubation, 200 μ L fresh medium was added to each well, and cells were treated as indicated in the respective figure legend or text.

siRNA target sequences are listed in Table 3. The non-targeting control siRNAs (siContr-1 and siNeg) both contain at least four mismatches to all known human genes. In co-transfection experiments, control siRNA (siNeg) was added across all samples to maintain the constant total siRNA concentration. When indicated, equimolar pools of several different siRNAs were used in order to diminish off-target effects.

Table 3. Synthetic siRNAs.

pool	siRNA	siRNA target sequence (5' – 3')
	siContr-1	CAGUCGCGUUUGCGACUGG
	siNeg	UACGACCGGUCUAUCGUAG
si16E6	si16E6-1	ACCGUUGUGUGAUUUGUUA
	si16E6-2	GGGAUUUAUGCAUAGUAUA
	si16E6-3	UUAGUGAGUAUAGACAUUA
si18E6	si18E6-1	GACAUUAUUCAGACUCTGU
	si18E6-2	CAGACUCUGUGUAUGGAGA
	si18E6-3	CUCUGUGUAUGGAGACACA
si16E6/E7	si16E6/E7-1	CCGGACAGAGCCCAUUACA
	si16E6/E7-2	CACCUACAUUGCAUGAAUA
	si16E6/E7-3	CAACUGAUCUCUACUGUUA
si18E6/E7	si18E6/E7-1	CCACAACGUCACACAAUGU
	si18E6/E7-2	CAGAGAAACACAAGUAUAA
	si18E6/E7-3	UCCAGCAGCUGUUUCUGAA
siA-MYB	siA-MYB-1	GGAAGAAGAUAUUCGGGAA
	siA-MYB-2	CGAGCACACUAGUGAGUUU

siB-MYB	siB-MYB-1	CUGGAACUCUACCAUCAA
	siB-MYB-2	GAAACAUGCUGCGUUUGUA
	siB-MYB-3	GAUCUGGAUGAGCUGCACU
siFOX1	siFOX1-1	AACAUCAGAGGAGGAACCU
	siFOX1-2	UGGGAUCAAGAUUAUUAAC

4.3.4 Plasmid transfection

Plasmids (described in section 4.2.1) were transfected into cells using the calcium phosphate co-precipitation method, as described previously.³²¹ In detail, cells were seeded in 3 mL of standard cell culture medium in 6 cm dishes at approximately 30% confluency and transfected the following day. For optimal transfection, 6 µg of total DNA was used: 0.5 µg of TFORF3370 or EV was adjusted to 6 µg by adding pBluescript II vector (Agilent, Santa Clara, CA, US). To prepare the transfection mixture, 150 µL of 0.25 M CaCl₂ was mixed with the DNA, followed by the addition of 150 µL of 2x BES buffer (pH 6.95; 50 mM BES, 280 mM NaCl, 1.5 mM Na₂HPO₄, filter-sterilized). The transfection mixture was incubated at RT for 20-30 min before being gently added onto the cells. The cells were incubated for 18 h in a humidified incubator set to 35 °C and 3% CO₂. Cells were subsequently washed once with phosphate-buffered saline (PBS), followed by the addition of 3 mL of fresh standard cell culture medium. Subsequent treatments were carried out as described in the respective figure legend or text.

4.3.5 Treatment of cells with chemical compounds

Cells were treated with the chemical compounds listed in Table 4 (specific concentrations and incubation times are detailed in the respective figure legend or text). Treatments were carried out by directly adding the compounds to the culture medium, with the corresponding volume of solvent used in control treatments.

Table 4. Chemical compounds.

Chemical compound	Solvent	Manufacturer
Cisplatin	0.9% NaCl/H ₂ O	Sigma-Aldrich
Etoposide	DMSO	Enzo Life Sciences
Nocodazole	DMSO	Cayman Chemical Company (Ann Arbor, MI, USA)

4.3.6 Live-cell imaging

The Incucyte S3 device (Sartorius) was used to monitor the proliferation of mKate2-labeled cells. Cells were seeded into 96-well plates at a density of 2000 (HeLa-mKate2) or 3000

(SiHa-mKate2) cells per well and reverse-transfected. Cells were treated as detailed in the respective figure legend or text. Images were acquired every 4 to 6 h, starting from 24 h post-transfection (time point 0), using a 10x magnification (4 images were taken of each well). Each condition was analyzed in triplicates, yielding 12 images per condition per time point. The average cell counts from these 12 images at each time point are shown. Cell proliferation was determined by quantifying viable mKate2-labeled cells (represented as red objects) over a 96 h period using Incucyte 2021C software. Red object counts are shown relative to the counts at time point 0 (set as 1).

For assessing cell death, the Incucyte Cytotox Green Dye (Sartorius, Cat. No. 4633) was used at a final concentration of 100 nM concurrently with the respective treatment. The Cytotox Green Dye selectively enters cells with compromised membrane integrity, binds to the DNA, and becomes fluorescent when excited at the appropriate wavelength, which enables the identification and counting of dead cells (represented as green objects). Green object counts were normalized to the selected reference time point (24 h post-drug treatment).

4.3.7 Cell cycle analyses

For flow cytometry-based cell cycle analyses, cells were washed with PBS to remove any residual culture medium, trypsinized, and pelleted. Centrifugation was consistently conducted at 1000 *g*, 5 min, and 4 °C. Cell pellets were washed with cold PBS, centrifuged again, and the supernatant was discarded. To fix the cells, 300 µL of cold PBS was added, followed by the slow addition of 900 µL ice-cold 75% ethanol while vortexing continuously to avoid cell aggregation. The cells were stored at -20 °C overnight or until further use. In the next step, cells were pelleted by centrifugation and resuspended in 900 µL cold PBS. To stain the DNA, propidium iodide (PI) (Sigma-Aldrich) was added at a final concentration of 25 µg/mL. To ensure that RNA does not interfere with the PI staining and subsequent analysis, RNase A (Roche Diagnostics) was added to degrade RNA, at a final concentration of 500 µg/mL. After incubation for 30 min at RT, data acquisition and initial data analyses were performed using a BD LSRFortessa™ flow cytometer (BD Biosciences), along with BD FACS Diva Software version v8.0.1. A total of 10000-20000 cells were counted per experimental condition. FlowJo™ v10.8.1 Software (BD Biosciences) was used for detailed analyses, including quantification of cell cycle phase populations and generation of cell cycle histograms. Quantification of the cell cycle phases was based on the Dean-Jett-Fox model.³²² Unless specified otherwise, cell cycle analyses were conducted three times, with consistent results.

4.3.8 EdU assays

To confirm growth arrest in cells cultured under hypoxia, a flow cytometry-based EdU assays were performed. These assays utilize EdU (5-Ethynyl-2'-Desoxyuridin), a thymidine analog, that is incorporated into the DNA of actively proliferating cells during DNA synthesis (S phase).²⁴⁰ The incorporation of EdU can be detected using the EdU-Click 647 Kit (baselclick, Neuried, Germany) which employs click chemistry to label EdU with a fluorescent dye. Cells were exposed to normoxia or hypoxia for 24 h, and 10 μ M EdU was added to the culture medium 2 h prior to harvesting. Cells were then harvested and fixed following the protocol used for cell cycle analyses (please refer to section 4.3.7). After fixation with ethanol, cells were washed twice with 2 mL PBS, centrifuged and resuspended in 100 μ L of saponin-based permeabilization and wash buffer. To detect the incorporated EdU, 500 μ L of freshly prepared click assay cocktail was added, and the mixture was incubated for 30 min at RT in the dark. Following incubation, cells were washed again with 2 mL permeabilization buffer and resuspended in 1 mL PBS containing 25 μ g/mL PI and 500 μ g/mL RNase. The suspension was incubated for an additional 30 min at RT in the dark. Analysis and image generation were carried out as detailed for cell cycle analyses (please refer to section 4.3.7).

4.3.9 TUNEL assays

To detect late-stage apoptotic cells, a terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay was employed, which identifies chromatin fragmentation events associated with apoptosis. During apoptosis, cellular endonucleases cleave DNA between nucleosomes, exposing 3'-hydroxyl termini. Fluorescently labeled nucleotides are added to these exposed ends by the enzyme terminal deoxynucleotidyl transferase, allowing for the detection of apoptotic cells.³²³

Cells were cultured on glass coverslips placed in 6 cm plates and treated as indicated in the respective figure legend or text. Subsequently, cells grown on glass coverslips were fixed in 4% formaldehyde for 30 min at RT. After fixation, cells were stored in 70% ethanol at -20 °C until further use. Following this, cells were permeabilized by incubation in a solution of 0.1% Triton X-100 and 0.1% sodium citrate in PBS for 2 min. In Situ Cell Death Detection Kit (Roche) was used to perform TUNEL assays. Coverslips were incubated with 25 μ L of the TUNEL staining solution (enzyme solution:label solution in a 1:10 ratio) for 60-90 min at 37 °C in the dark. After the incubation, coverslips were washed with PBS and stained with 1 μ g/mL 4',6-diamidino-2-phenylindole (DAPI) (Roche) for 5 min at RT in the dark, in order to visualize total cell nuclei. Prior to being air-dried, coverslips were washed with PBS again, dipped in H₂O and 100% ethanol. Coverslips were mounted on microscope slides with

Vectashield (Vector Laboratories, Newark, CA, USA). Images were acquired using a Zeiss Cell Observer microscope (Zeiss, Oberkochen, Germany), at a 20x magnification. Fluorescent images were captured for TUNEL-positive cells (apoptotic cells) and DAPI-stained nuclei (total cells). Image analysis was performed using Fiji/ImageJ³²⁴ software (NIH, Bethesda, MD, USA). TUNEL-positive cells were quantified using Fiji/ImageJ macro (kindly provided by Dr. Damir Kronic from the Light Microscopy Core Facility, DKFZ, Heidelberg, Germany).

4.3.10 Senescence assays

Senescence is classically defined as an irreversible growth arrest.¹³⁹ To assess senescence induction in response to different treatment protocols, senescence assays were performed. Senescent cells are typically recognized by the presence of senescence-associated β -Galactosidase (SA- β -Gal) activity.¹⁴³ β -Gal enzymatic activity can be detected using the chromogenic substrate X-Gal (5-bromo-4-chloro-3-indolyl β D-Galactopyranoside) at pH 6.0 that forms a blue precipitate upon hydroxylation. Following treatments outlined in the respective figure legend or text, cells were trypsinized and reseeded at a defined density in fresh cell culture medium. Cells were cultivated under normoxic conditions, in drug-free medium for 4-7 days with medium changes every 3-4 days. For senescence analysis, cells were washed with PBS and fixed using 1 mL of senescence assay fixation buffer (2% formaldehyde, 0.2% glutaraldehyde in PBS) for 3 min at RT. After fixation, cells were washed with PBS and incubated in 1.5 mL senescence assay buffer (40 mM citric acid, 150 mM NaCl, 2 mM MgCl_2 , adjusted to pH 6.0 with Na_2HPO_4 , supplemented with 1 mg/mL X-Gal, 5 mM $\text{K}_3[\text{Fe}(\text{CN})_6]$, and 5 mM $\text{K}_4[\text{Fe}(\text{CN})_6]$ for 16-24 h at 37 °C in a humidified chamber. Subsequently, the staining solution was replaced with PBS, and cells were imaged using the EVOSxl Core Cell Imaging System brightfield microscope (Thermo Fisher Scientific) at 20x or 40x magnification.

4.3.11 Colony formation assays

Colony formation assays (CFAs) were used to evaluate cell survival and proliferation in response to different treatment protocols. Cells were split as described in section 4.3.10 and allowed to grow for 11-17 days, with medium exchanges every 3-4 days. Prior to staining, the cells were washed with PBS, and colonies were fixed/stained simultaneously with 350 μL of a formaldehyde-crystal violet solution (composed of 12 mM crystal violet, 29 mM NaCl, 3.7% formaldehyde, and 22% ethanol) for 5 min at RT. Colonies were then washed with water, dried at 37 °C, and imaged using an Epson Perfection 4990 Photo Scanner (Epson, Suwa,

Japan). Quantifications were conducted using an ImageJ Plugin, ColonyArea.³²⁵ Obtained colony area values were normalized to the corresponding control, which was set to 1. This was performed across three independent biological replicates, and statistical significance was assessed.

4.4 Protein-based methods

4.4.1 Protein extraction

To prepare total protein extracts for immunoblotting, cells were first washed with ice-cold PBS and then lysed directly using 150 μ L of ice-cold CSK-1 lysis buffer (pH 6.8; 10 mM PIPES, 300 mM NaCl, 1 mM EDTA, 300 mM sucrose, 1 mM $MgCl_2$, 0.5% Triton X-100). PhosSTOP phosphatase inhibitor cocktail (Roche), Pefabloc serine protease inhibitor (Merck), and P8340 protease inhibitor cocktail (Sigma-Aldrich) were freshly added to the CSK-1 lysis buffer. Cell lysates were kept on ice for 30 min, followed by centrifugation at 16000 g for 5 min at 4 °C. The supernatant was transferred into a new 1.5 mL tube on ice. Protein concentrations were quantified by mixing the 2 μ L of the sample with 1 mL of the Bio-Rad Protein Assay Dye Reagent (Bio-Rad) (1:5 dilution) in a cuvette and measuring the absorbance at 595 nm using a BioPhotometer D30 (Eppendorf). Samples were adjusted to the required concentration using 4x SDS-PAGE loading buffer (8% SDS, 250 mM Tris-HCl, 20% β -mercaptoethanol, 40% glycerol, 0.008% bromphenol blue), then heated at 95 °C for 5 min, and stored at -80 °C until further use.

4.4.2 SDS-PAGE

To assess protein expression levels following different experimental treatments, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed. Polyacrylamide gels (Table 5) were cast between glass plates sealed with 1% agarose, mounted into an XCell SureLock Mini-Cell Electrophoresis System (Thermo Fisher Scientific), and covered in Tris-glycine SDS running buffer (2.5 mM Tris, 19.2 mM glycine, 0.1% SDS). Protein samples (12-30 μ g per lane) were loaded on gels and separated based on their molecular weight. A PageRuler Prestained Protein Ladder (Thermo Scientific) was used as a molecular weight reference. Electrophoresis was performed at a constant voltage of 120 V for approximately 2 h.

Table 5. Recipes for SDS-PAGE polyacrylamide gels (2 gels each).

Resolving gel 12%		Stacking gel 5%	
H ₂ O	5.7 mL	H ₂ O	2 mL
Tris 3M pH 8.9	1.8 mL	Tris 0.47M pH 6.7	1.2 mL
10% SDS	137.5 µL	10% SDS	45.8 µL
10% APS	176 µL	10% APS	183.3 µL
30% Acrylamid	5.5 mL	30% Acrylamid	620 µL
TEMED	3.4 µL	TEMED	1.8 µL

4.4.3 Western blotting and protein detection

After SDS-PAGE, proteins were transferred onto a PVDF membrane using a semi-dry blotting method. The membrane was activated in methanol prior to transfer. The membrane and the Whatman filter papers were then pre-soaked in Towbin transfer buffer (pH 8.3; 2.5 mM Tris, 19.2 mM glycine, 20% methanol). The transfer stack was assembled in a Trans-Blot system (Bio-Rad) with the membrane placed beneath the gel and sandwiched between three layers of filter paper on each side. Protein transfer was carried out at 20 V for 60-90 min.

To prevent nonspecific binding, membranes were blocked for 1 h at RT in a blocking solution (5% skim milk, 1% bovine serum albumin (BSA) in 0.2% PBS-Tween 20 (PBS-T)). Depending on the molecular weight of the target proteins, membranes were cut according to the position of the protein ladder and incubated overnight at 4 °C with the appropriate primary antibodies (Table 6), diluted in blocking solution. The membranes were washed three times for 10 min each in PBS-T the next day. They were then incubated for 1 h at RT with horseradish peroxidase (HRP)-conjugated secondary antibodies (Table 6), followed by another series of three 10 min washes in PBS-T. Protein detection was performed using enhanced chemiluminescence (ECL) reagents (WesternBright Sirius, Advansta, San Jose, CA, USA or ECL Prime, Cytiva, Marlborough, MA, USA). HRP catalyzed a luminol-based reaction, generating light, which was visualized using the Fusion SL Gel Detection System (Vilber Lourmat, Eberhardzell, Germany).

Table 6. List of antibodies.

Primary antibodies				
Specificity	Source	Dilution	Clone, product	Supplier
HPV18 E6	mouse	1:2000	AVC 399	kind gifts from Dr. Johannes Schweizer, Arbor Vita Corporation, Fremont, CA, USA
HPV16 E6	mouse	1:3000	AVC 849	
HPV18 E7	chicken	1:1000	E7C	kind gift from Prof. Dr. Hanswalter Zentgraf (DKFZ)

HPV16 E7	mouse	1:1000	NM2, sc-65711	kind gift from Prof. Dr. Martin Müller (DKFZ)
B-MYB	mouse	1:10	LX015.1	from Dr. Roger Watson; kindly provided by Dr. Stefan Gaubatz, University of Würzburg, Würzburg, Germany
β-Actin	mouse	1:50000	C4, sc-47778	Santa Cruz Biotechnology
CDC25C	mouse	1:500	H-6, sc-13138	
Cyclin A	rabbit	1:2000	H-432, sc-751	
Cyclin B2	mouse	1:1000	A-2, sc-28303	
Cyclin D1	mouse	1:500	DCS-6, sc-20044	
E2F4	mouse	1:500	D-7, sc-398543	
GAPDH	rabbit	1:4000	FL-335, sc-25778	
HIF-2α	mouse	1:500	sc-46691	
p21	mouse	1:1000	SX118, sc-53870	
p53	mouse	1:1000	DO-1, sc-126	
PLK1	mouse	1:500	F-8, sc-17783	
RRM2	mouse	1:500	R2 [A-5], sc-398294	
Vinculin	mouse	1:4000	7F9, sc-73614	
cleaved Caspase 9	rabbit	1:1000	#7237	Cell Signaling Technology
FOXM1	rabbit	1:500	D3F2B, #20549	
cleaved PARP	mouse	1:1000	#9546	
RBL2 (p130)	rabbit	1:500	#13610	
pRb	mouse	1:1000	#9309	
phospho-pRb	mouse	1:1000	S807/811, #9308	
Histone H2AX phospho (Ser139)	mouse	1:500	05-636	Merck
Cyclin B1	mouse	1:100	GNS3 [8A5D12], 05-373	
A-MYB	rabbit	1:1000	HPA008791	Sigma-Aldrich
HIF-1α	mouse	1:500	610959	BD Biosciences
α-Tubulin	mouse	1:5000	CP06	Calbiochem, San Diego, CA, USA
Secondary antibodies (HRP-coupled)				
Specificity	Source	Dilution	Product	Supplier
anti-chicken IgY	goat	1:5000	sc-2428	Santa Cruz Biotechnology
anti-goat IgG	donkey	1:5000	sc-2020	
anti-mouse IgG	goat	1:10000	W4021	Promega, Madison, WIS, USA
anti-rabbit IgG	goat	1:10000	W4011	

4.5 RNA-based methods

4.5.1 RNA extraction

Total RNA was extracted using the column-based PureLink RNA Mini Kit (Thermo Fisher Scientific). Cells were cultured in 6 cm dishes and lysed directly using 600 μ L of lysis buffer. The lysis buffer was freshly prepared by supplementing 10 mL of lysis buffer with 100 μ L β -mercaptoethanol (Sigma-Aldrich). Lysates were either stored at -20 °C or immediately processed following the manufacturer's protocol. DNA digestion was performed using the PureLink DNase Set (Thermo Fisher Scientific) to eliminate DNA contamination. After sequential wash steps, total RNA was eluted in 30-50 μ L of nuclease-free H₂O. RNA concentration was assessed by measuring absorbance at 260 nm using the NanoDrop ND-1000 (PepLab, Erlangen, Germany). RNA samples were stored at -80 °C.

4.5.2 Reverse transcription and quantitative real-time PCR (qRT-PCR)

Purified RNA was used to generate cDNA via reverse transcription using ProtoScript II First Strand cDNA Synthesis Kit (NEB) following the manufacturer's protocol. Purified RNA (500 ng, adjusted to a final volume of 3 μ L with nuclease-free H₂O) was mixed with random and oligo-dT primers (1:1 ratio), denatured at 70 °C, for 5 min. Master mix was added (containing the 2x reaction mix (5 μ L) and 10x enzyme mix (1 μ L)) and samples were incubated at 25 °C for 5 min, followed by incubation at 42 °C for 1 h, and at 80 °C for 5 min (MJ Research PTC-200 thermal cycler, Global Medical Instrumentation, Ramsey, MN, USA). cDNA samples were filled up with 40 μ L of nuclease-free H₂O and stored at -20 °C until quantitative real-time PCR (qRT-PCR) analyses.

For qRT-PCR analyses, reactions were performed using a 96-well plate (MicroAmp Optical 96-Well Reaction Plate, Thermo Fisher Scientific) and all samples were analyzed in duplicate. Each reaction contained 7.2 μ L nuclease-free H₂O, 0.4 μ L of forward and reverse primer, each (final primer concentrations were adjusted to 100 nM per primer) (Table 7), 10 μ L SYBR Green PCR Master Mix (Thermo Fisher Scientific), and 2 μ L cDNA. Nuclease-free H₂O was used as a negative control.

Table 7. List of primers.

Target	Forward primer (5' – 3')	Reverse primer (5' – 3')
<i>CCNA2</i>	CCCCCAGAAGTAGCAGAGTTT	ACTTGAGGTATGGGTCAGCATC
<i>CCNB1</i>	GCCTCTACCTTTGCACTTCCT	TGTTGTAGAGTTGGTGTCCATT
<i>CCNB2</i>	AAGTTCCAGTTCAACCCACCA	CCTCAGGTGTGGGAGAAGGA
<i>CDC25C</i>	TGGAAACTTGGTGGACAGTGA	GGGAGCGATATAGGCCACTT
<i>FOXM1</i>	GGCAGCAGGCTGCACTATC	TCGAAGGCTCCTCAACCTTAAC

<i>MYBL1</i>	GCGAACTTAGGGATGGCTCA	TGCCCACAAATAGGGGTTGA
<i>MYBL2</i>	AGGCTGGCATCGAACTCATC	CTTGGGCAGTGTGGACATCA
<i>PLK1</i>	TGTTAGTGGGCAAACCACCTT	GGCAGTGGGATCTGTCTGAA
<i>RRM2</i>	TGCATTGTGAGGTACAGGCG	GGTCTGAGCTGGCAGAAAGTTA
<i>TMBIM6</i>	GTGGTCATGTGTGGCTTCGT	GGAAAGGCTGGATGGTCACT

qRT-PCR was carried out using the 7300 Real-Time PCR System (Thermo Fisher Scientific). The qRT-PCR program is outlined in Table 8. The comparative Ct ($2^{-\Delta\Delta C_t}$) method³²⁶ was used for quantification of mRNA levels, with Cycle threshold (Ct) values normalized to *TMBIM6*, which served as a reference gene due to its suitability for assessing hypoxia-related gene expression.³²⁷ The resulting fold change values were $\log_2$ transformed and statistical analyses were performed (please refer to section 4.6).

Table 8. qRT-PCR program.

	Temperature	Time	
Initiation	50 °C	2 min	
Polymerase activation	95 °C	10 min	
Denaturation	95 °C	15 s	40 cycles
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	

4.5.3 GSEA

Gene Set Enrichment Analysis (GSEA) was performed on two transcriptome data sets derived from HeLa cells under different experimental conditions. The first dataset consisted of previously published²³⁸ genome-wide microarray data from HeLa cells transfected with siRNAs targeting HPV E6/E7 or a non-targeting control (siControl). Cellular gene expression profiles were assessed 72 h post-transfection, a time point at which E6/E7 expression was efficiently repressed and cell cycle arrest was evident, to determine the gene regulatory impact of E6/E7 knockdown.²³⁸ In addition, GSEA was performed on unpublished transcriptome data obtained from HeLa cells cultured under normoxic (21% O₂) or hypoxic (1% O₂) conditions for 16 h. RNA sample preparation was kindly performed by Claudia Lohrey and samples were submitted to the Next Generation Sequencing (NGS) Core Facility (DKFZ, Heidelberg, Germany) for high-throughput sequencing. Library preparation was performed using the TruSeq Stranded RNA protocol and sequencing was conducted on the Illumina HiSeq 4000 platform using single-end 50 bp reads. All samples passed initial, library, sequencing, and data quality control metrics. Following data acquisition, transcripts were filtered based on a $\log_2$ -transformed fold

change ($\log_2\text{FC}$) threshold of ± 0.58 (equivalent to a 1.5x-FC). Only transcripts that consistently met these criteria were included in downstream analyses. I gratefully acknowledge the support of the NGS Core Facility of the DKFZ for sequencing services and data handling.

GSEA was performed using GSEA software version 4.3.3 with gene sets retrieved from the C2 collection (curated gene sets) of the Molecular Signatures Database (MSigDB) v2024.1.³²⁸
³²⁹ Specifically, the following cell cycle-related gene sets were used: *FISCHER_DREAM_TARGETS*, *FISCHER_G1_S_CELL_CYCLE*, and *FISCHER_G2_M_CELL_CYCLE*.¹⁸⁴ For each condition, a pre-ranked enrichment analysis was conducted by loading gene symbols along with their corresponding $\log_2\text{FC}$ values into the GSEA software.³³⁰ The analysis was performed using the following parameters: number of permutations = 1000; enrichment statistic = weighted; gene set size filters (minimum = 15, maximum = 500); normalization mode = meandiv; permutation seed = timestamp. For each analysis, enrichment plots were generated using the default GSEA visualization tools to illustrate the distribution and contribution of gene set members across the ranked gene list, and the leading-edge subset. Following analysis, core enrichment genes (i.e., leading-edge genes contributing to the enrichment signal) were identified for the *FISCHER_DREAM_TARGETS* data set using the GSEA output (Core Enrichment: Yes). Genes that were shared between both data sets (i.e., common to both core enrichment gene lists) were visualized using a heat map. A custom script, originally developed with the assistance of OpenAI's ChatGPT (version 4, accessed via chat.openai.com) was used to generate a heat map of the intersected core enrichment genes. The model was consulted to write an initial script which was subsequently edited by myself. The script was run on the Google Colab platform.

4.6 Statistical analyses

GraphPad Prism 10.1.2 (GraphPad Software, San Diego, CA, USA) was used for statistical tests. Depending on the experimental setup, statistical significance was assessed using a two-sided unpaired *t*-test, one-way analysis of variance (ANOVA), followed by Sidak's or Dunnett's multiple comparisons test, or two-way ANOVA, followed by Fisher's LSD test. Significance levels were reported as follows: * $p \leq 0.05$, ** $p \leq 0.01$, and *** $p \leq 0.001$. All experiments were carefully designed and, unless specified otherwise, conducted with a minimum of three biological replicates.

4.7 Tools

Table 9. Tools used in this thesis.

Tools	Retrieved from	Supplier
Adobe Illustrator	https://adobe.com/products/illustrator	Adobe Inc. (2019)
BioRender.com	https://www.biorender.com/	BioRender (2025)
ChatGPT (GPT-4)	https://chat.openai.com/chat	OpenAI (2025)
Fiji is just ImageJ	-	Multiple contributors
Graph Pad Prism	https://www.graphpad.com/	GraphPad Software
Google Colab	https://colab.research.google.com/	Google (2025)
GSEA	https://www.gsea-msigdb.org/gsea/index.jsp	The Broad Institute, Inc.
Microsoft Office 365 ProPlus		Microsoft

APPENDIX

Appendix

List of figures

Figures 1, 2 and 24 were created with BioRender.com. Experimental results and/or illustrations, as well as the corresponding figure legends, or parts thereof, presented in Figures 3, 5, 6, 7, 10, 11, 12, 13, 15, 19, 20, 21, 24 were taken, extended, or adapted from Velimirović et al., 2025.,²³⁶ as indicated in the respective figure legends.

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Heat map script

```

import pandas as pd
import seaborn as sns
import matplotlib.pyplot as plt

# Set font to Arial
plt.rcParams['font.family'] = 'Arial'

# Gene expression data
data = {
    "Gene": [
        "ACP1", "ANLN", "BIRC5", "BUB1", "CACYPB", "CCDC34", "CKS1B", "DNAJC9",
        "EXO1", "EZH2", "FAM83D", "FEN1", "FOXM1", "FUS", "HMGN2", "HSP90AA1",
        "HSPD1", "KIF15", "KIF2C", "MCM2", "MELK", "MYC", "PLK4", "PRC1", "RACGAP1",
        "RECQL4", "RRM2", "SNRPB", "TCERG1", "ZWINT"
    ],
    "E6/E7 KD": [
        -0.84, -0.76, -1.00, -1.18, -0.76, -0.76, -1.64, -0.84, -1.94, -1.00,
        -1.36, -1.51, -1.09, -0.76, -1.15, -1.29, -1.03, -1.06, -1.89, -1.09,
        -1.06, -0.89, -0.76, -1.43, -1.22, -0.76, -1.15, -0.86, -0.94, -1.03
    ],
    "Hypoxia": [
        0.56, 1.59, 0.50, 0.65, 0.95, 0.44, 0.70, 0.70, 1.01, 0.82,
        1.04, 0.45, 0.66, 0.51, 1.47, 0.86, 0.57, 0.53, 0.51, 0.44,
        0.75, 2.14, 0.56, 0.55, 0.66, 0.65, 1.09, 0.54, 0.48, 1.18
    ]
}

# Create DataFrame
df = pd.DataFrame(data)
df.set_index("Gene", inplace=True)

# Plot heatmap
plt.figure(figsize=(4, 12)) # Adjust width for narrower columns
sns.heatmap(
    df,
    cmap="RdBu",
    linewidths=0.5,
    center=0,
    cbar_kws={"label": "Log2 Fold Change"},
    square=True, # make the cells square
    annot=False # no numbers in boxes
)
plt.title("Gene Expression: E6/E7 KD vs Hypoxia", fontsize=14)
plt.xticks(rotation=45, ha='right')
plt.tight_layout()

# Save as SVG
plt.savefig("heatmap_gene_expression_clean.svg", format='svg')

plt.show()

```

List of abbreviations

AMV	Avian myeloblastosis virus
ANOVA	Analysis of variance
APC	Antigen-presenting cell
APS	Ammonium persulfate
ATP	Adenosine triphosphate
BAK	BCL2 antagonist/killer 1
BCL-2	B-cell lymphoma 2
BSA	Bovine serum albumin
CDC25C	Cell Division Cycle 25C
CDDP	Cisplatin
CDK	Cyclin-dependent kinase
cDNA	Complementary DNA
CFA	Colony formation assay
CHR	Cell cycle genes homology region
CIN	Cervical intraepithelial neoplasia
cl.	cleaved
CO ₂	Carbon dioxide
CSC	Cancer stem cells
CT	Chemotherapy
DBD	DNA-binding domain
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DREAM	Dimerization partner, Rb-like, E2F and multi-vulval class B
E6AP	E6-associated protein
ECL	Enhanced chemiluminescence
EDTA	Ethylenediaminetetraacetic acid
EdU	5-Ethynyl-2'-Desoxyuridin
FC	Fold change
FCS	Fetal calf serum
FDR	False discovery rate
FOXM1	Forkhead Box M1
5-FU	5-Fluorouracil
GLUT1	Glucose transporter
GSEA	Gene Set Enrichment Analysis

H	Hypoxia
HAP	Hypoxia-activated prodrug
HIF	Hypoxia-inducible factor
HPV	Human papillomavirus
HTH	helix-turn-helix
HRP	Horseradish peroxidase
H ₂ O	water
ddH ₂ O	Double-distilled water
IARC	International Agency for Research on Cancer
kb	kilobase
LB	Lysogeny Broth
LCR	Long coding region
log ₂	Binary logarithm
MBS	MYB-binding site
MMB	Myb-MuvB complex
mRNA	Messenger RNA
miRNA	Micro RNA
mTOR	Mechanistic target of rapamycin
mTORC1/2	mTOR complex 1/2
MuvB	Multi-vulval class B
MYB	Myeloblastosis
<i>MYB</i>	MYB proto-oncogene
<i>MYBL1</i>	MYB proto-oncogene like 1
<i>MYBL2</i>	MYB proto-oncogene like 2
NCR	Non-coding region
NES	Normalized enrichment score
NES	Nuclear export signal
NRD	Negative regulatory domain
O ₂	Oxygen
ORF	Open reading frame
PARP	Poly (adenosine diphosphate-ribose) polymerase
PBS	Phosphate-buffered saline
PBS-T	PBS with Tween 20
PDZ	PSD-95/DLG/ZO-1 domain
PD-L1	Programmed death ligand 1
PDVF	Polyvinylidene difluoride

PFA	Paraformaldehyde
PI	Protease inhibitor
PI3K	Phosphoinositide 3-kinase
PLK1	Polo-like kinase 1
pRb	Retinoblastoma protein
qRT-PCR	Quantitative real-time polymerase chain reaction
RNA	Ribonucleic acid
RNAi	RNA interference
ROS	Reactive oxygen species
RT	Room temperature
SA	Senescence assay
SA- β -Gal	Senescence-associated β -Galactosidase
SASP	Senescence-associated secretory phenotype
SD	Standard deviation
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
siRNA	Small interfering RNA
TAD	Transactivation domain
TEMED	Tetramethylethylenediamine
TERT	Telomerase reverse transcriptase
TUNEL	Terminal deoxynucleotidyltransferase-mediated dUTP-biotin nick end labeling
Tx	Transfection
URR	Upstream regulatory region
VEGF	Vascular endothelial growth factor
VHP	Von Hippel-Lindau protein
VLP	Virus-like particles
WHO	World Health Organization
X-Gal	5-bromo-4-chloro-3-indolyl β D-Galactopyranoside

Units and prefixes

Units	
%	percent
°C	degree Celsius
ct	cycle threshold
Da	Dalton
<i>g</i>	gravitational acceleration
g	gram
h	hour
L	liter
M	Molar
min	Minute
V	volt

Prefixes	
Sybmol, prefix	Factor
n, nano	10^{-9}
μ , mciro	10^{-6}
m, milli	10^{-3}
K, kilo	10^3

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