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Improving Therapeutics In Head And Neck Cancer

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Improving therapeutics in head and neck cancer

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Abstract

Patients with head and neck cancer suffer not solely from disease but also from sequelae of radiation and chemotherapy treatment, and there is a large, unmet need to both develop novel therapies and improve existing ones to decrease deleterious, life-long side effects and improve patient survival. In the last several years, growing evidence has suggested that head and neck cancer represents two distinct disease entities based on Human Papilloma Virus (HPV) status. Unfortunately, HPV status is currently only used for prognosis and not for guiding management. Subsequently, this work focuses on 1) developing a novel targeted therapy for HPV-positive (HPV(+)) head and neck cancers and 2) improving the efficacy of current treatments for HPV-negative (HPV(-)) head and neck cancers. In the first part of this study, using HPV(+) and HPV(-) head and neck cancer cell lines, we show that roscovitine, a cyclin-dependent kinase (CDK) inhibitor that inhibits CDK-1, CDK-2, CDK-5, CDK-7, and CDK-9 due to competitive binding at the kinase ATP site causes significant DNA damage followed by p53-dependent cell death in HPV(+), but not in HPV(-), head and neck cancer cells. We also show that low dose roscovitine administration significantly inhibits the growth of HPV(+) xenografted tumors in mice without causing any detectable side effects, further reinforcing the potential of roscovitine as a targeted therapy for HPV(+) HNSCC. In the second part of the study, we use a number of different cancer cell lines with variable p53 status (either wild-type, mutant, or null p53), along with embryonic fibroblasts derived from genetically engineered mice, to show that knockdown of leucine zipper-containing ARF-binding protein (LZAP) eliminates the p53 protein independently of its mutation status, subsequently protecting wild-type p53 cells (i.e., healthy tissue) from DNA damage-induced cell death (e.g. such as that caused by radiation), while rendering cells expressing mutant p53 (i.e., HPV(-) HNSCCs) more sensitive to the treatment. Our study highlights the need to develop different therapeutic strategies for HPV(+) and HPV(-) HNSCC patients, specifically taking into account their individual genetic defects and "Achilles' heels."

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Table of Contents

Introduction

Head and neck cancer

Head and neck cancer is the 6th most common cancer in the world, with an estimated incidence of about 600,000 cases annually [1, 2]. In the United States, head and neck cancers represent about 4% of all malignancies, and in 2017, about 65,000 people are expected to be newly diagnosed with the disease [3]. Head and neck cancers are comprised of a diverse set of tumors originating from either the oral cavity, pharynx (including the oropharynx, the nasopharynx, and the hypopharynx), larynx, paranasal sinuses, nasal cavity, or salivary glands. Despite the anatomical diversity of origin, greater than 90% of head and neck cancers are histologically squamous cell cancers arising in the upper aerodigestive tract and are thus termed head and neck squamous cell carcinoma (HNSCC) [4].

Major risk factors for developing head and neck cancer include the consumption of alcohol and the use of tobacco products, as well as exposure to high-risk strains of human papilloma virus (HPV). Symptoms associated with head and neck cancer depend on the location and size of the primary tumor, but frequently include a mass or lump, dysphagia, odynophagia, and/or hoarseness of the voice, along with systemic symptoms such as low-grade fever and weight loss. Tumors originating from the oral cavity may additionally present with leukoplakia or cause bleeding from the mouth, while tumors of pharyngeal origin may cause difficulties with respiration and speaking, headaches, tinnitus, or problems hearing. Unfortunately, head and neck cancers tend to be aggressive and present late in the course of disease, and despite surgical resection and/or chemotherapy and fractionated radiation, relative 5-year relative survival has been poor,

ranging from 54.7 – 65.9% [5-7]. Patients also frequently suffer debilitating side effects as a result of treatment including xerostomia, change in taste, dysphagia, altered speech, difficulties with using dentures, increased tooth decay, pain, and depression [8-11]. These morbidities highlight the functional role of the head and neck anatomy in mastication, swallowing, and speech and underline the need for treatments to conserve native tissue structure and function. Given the relatively poor prognosis and serious sequelae of present treatment, there is a large, unmet need to both develop new, less morbid targeted therapies and to improve existing therapies to maximize treatment efficacy and minimize unwanted side-effects for head and neck cancer patients. This thesis focuses on exploring these latter two points.

HPV-driven head and neck cancers – a different disease

It is becoming increasingly accepted that tumors associated with HPV, or HPV-positive (HPV(+)) tumors, represent a unique disease entity from HPV-negative (HPV(-)) ones. Specifically, patients with HPV(+) cancers have displayed unique epidemiological trends, as well as etiological, clinical, and molecular differences when compared to those with HPV(-) disease. These differences are summarized below.

Though the overall incidence of head and neck cancer has been declining worldwide for the last several decades, likely mirroring a decrease in tobacco and alcohol use, there has been a steady and worrisome rise in the incidence of HPV(+) head and neck cancers in economically developed countries such as the United States, Australia, Canada, Denmark, the Netherlands, Norway, and Sweden [12-15]. The United States Center for Disease Control (CDC) estimates an average annual incidence of 15,738 new

cases from 2008-2012 of oropharyngeal cancers (OPCs), a subset of head and neck cancers, about 70% of which are thought to be directly caused by HPV infection with a high risk strain [16]. This number is particularly striking when compared to an average incidence of 11,771 for cervical cancer (about 90-95% associated with HPV) during the same time period, indicating that new HPV(+) head and neck cancers now occur more frequently than cervical cancers in the United States. These alarming increases in OPC incidence have led numerous declarations of a new epidemic of HPV(+) head and neck cancers [17-19].

Patients with HPV(+) tumors are a clinically distinct population than those with HPV(-) disease. Specifically, HPV(+) patients tend to be younger, less exposed to tobacco and alcohol, of higher relative socioeconomic status, and better educated [20-22]. Furthermore, the main risk factors for cancer development differ between the two populations. Risk of HPV(+) head and neck cancer is predicated on exposure to unsafe sexual practices, and risk factors include an increasing number of sexual partners, engagement in casual sex, and sporadic use of condoms, while the main risk factors for the development of HPV(-) disease are tobacco and alcohol use [23, 24]. Differences in demographics and risk factors support two independent pathways for the development and progression of head and neck cancers: one via HPV infection and transformation, and one through a more classic carcinogen-induced mutagenesis pathway. Interestingly, the combined use of alcohol and tobacco has a synergistic effect on the development of HPV(-) negative head and neck cancers, where years of continued heavy use of both increases risk by 35 to 50 fold [25, 26].

HPV(+) head and neck cancer patients also differ clinically from HPV(-) ones with regards to prognosis and response to treatment. The Eastern Cooperative Oncology Group (ECOG) 2399 prospective clinical trial first demonstrated that patients with HPV(+) head and neck cancers have improved prognosis and increased response to treatment, including higher response rates after chemotherapy and chemoradiation, improved overall survival, lower risk of disease progression, and lower all-cause mortality than patients with HPV(-) head and neck cancers [27]. Several meta-analyses have corroborated these findings, showing that patients with HPV(+) head and neck cancers have lower all-cause mortality, better response to chemo and radiation therapy, and lower risk of recurrence than patients with HPV(-) negative head and neck cancers, and numerous studies since have confirmed the improved prognosis associated with HPV(+) disease [28-33] . Subsequently, HPV positivity is now accepted as a favorable prognostic factor for overall survival in patients with head and neck cancer, most notably by the National Comprehensive Cancer Network (NCCN) [20, 29, 34, 35]. The differences in treatment responsiveness of the two subsets of HNSCC likely reflect the distinct molecular disturbances that ultimately lead to carcinogenesis and give us a unique insight into the pathogenesis of these cancers.

HPV(+) tumors also differ in sites of origin and stage of presentation from HPV(-) ones. The vast majority of HPV(+) cancers arise from the tonsils and base of the tongue (subsites of the oropharynx), while HPV(-) lesions can occur in all locations within the upper aerodigestive tract, including the oral cavity, oropharynx, hypopharynx, and larynx [20, 24]. Furthermore, patients with HPV(+) head and neck cancers tend to present with

early T stages (T1-T2) and higher rates of regional and distant lymph node metastasis, while HPV(-) negative cancers vary in their presentation [36-38].

From a molecular standpoint, The Cancer Genome Atlas (TCGA) for head and neck cancer, a landmark study published in 2015, elucidated key genomic differences between HPV(+) and HPV(-) tumors [39]. HPV(+) head and neck cancers nearly always harbor the wild-type tumor suppressor protein p53, express high levels of the tumor suppressor p16INK4a (an upstream regulator of pRb), more frequently have activating mutations in the PIK3CA oncogene, contain unique alterations involving the loss of TRAF3, and have amplifications of the growth factor E2F1. Conversely, virtually all (determined by TCGA as about 84%) of HPV(-) head and neck cancers have a mutated p53 and also have inactivation of CDK2NA (a cell cycle gene that encodes p16INK4a) [39-41]. In addition to these mutational differences, HPV(+) cancers have a specific epigenetic profile that is distinct from HPV(-) negative tumors and normal tissue. Specifically, HPV(+) head and neck tumors contain hypermethylated CpG islands at tumor suppressor gene promotors, as well as higher global levels of DNA methylation when compared to HPV(-) tumors [42-44]. Interestingly, the genomic DNA of cervical precancerous lesions, which is the major source of our understanding of HPV-driven carcinogenesis, shows increasing levels of DNA methylation as the lesions progress to cancer, lending evidence that DNA methylation may be a crucial part of HPV-induced carcinogenesis [45]. The gene silencing and global changes to the genome due to DNA hypermethylation in HPV(+) head and neck cancer reflects yet another difference between HPV(+) and HPV(-) tumors, and likely represents differences in the process of carcinogenesis. Finally, numerous studies have noted that HPV(-) negative cancers

express high levels of epidermal growth factor receptor (EGFR), and that EGFR expression is inversely correlated to HPV status [46-51].

Taken together, clinical, etiological, demographic and molecular differences reveal that HPV(+) and HPV(-) negative head and neck cancers represent two unique disease entities. Although HPV status is currently used for prognosis, it is not a factor for treatment selection, as most patients receive some combination of primary surgical resection, cervical lymphadenectomy, and fractionated radiation therapy, either alone or with platin-based chemotherapy or the monoclonal antibody cetuximab. Though patients with HPV(+) tumors have better prognosis compared to patients with HPV(-), up to 25% of HPV(+) patients suffer recurrence or metastases following aggressive and morbid therapy. Encouragingly, de-escalation of therapy has been suggested as a tool to reduce treatment-related morbidity, and the ECOG 3311 trial is currently evaluating such de-escalation specifically for patients with HPV(+) disease. Nevertheless, it is apparent that there is not only a large need, but also an opportunity to harness new findings about molecular differences between HPV(+) and HPV(-) disease to design targeted therapies for head and neck cancer patients.

Targeting therapies for HPV(+) cancers

Pathogenesis of HPV(+) head and neck cancers

HPV is an 8kb double-stranded, non-enveloped DNA virus that is the primary etiological agent of cervical cancer and has been shown to cause other cancers including head and neck, and particularly OPSCC [52-54]. Though there are more than 150 different types of HPV currently recognized, the "high-risk" subtypes, or those associated

with persistent infection and malignant transformation, include HPV 16, 18, 31, 33, 34, 35, 39, 45, 51, 52, 56, 58, 59, 66, 68, and 70 [55]. Of these, HPV 16 and 18 are accountable for approximately 70% of cervical cancer cases; in contrast, HPV 16 alone is responsible for close to 90% of HPV(+) head and neck cancer cases, while HPV18 was never reported as being present in this cancer [56, 57]. The HPV genome consists of the structural genes, L1 and L2, and of the non-structural regulatory genes E1, E2, E4, E5, E6, and E7. Of the HPV genes, E6 and E7 are recognized as the most important for transformation and immortalization of the cell [58]. E6 induces ubiquitin-mediated proteolysis of p53, thereby deregulating the cell-cycle, inhibiting p53-mediated cell-cycle arrest and apoptosis, and leading to the increased frequency of somatic mutations. E7 mediates ubiquitination and degradation of the RB family of proteins, including pRB, p107, and p130, which frees the E2F transcription factor, causes further deregulation of the cell cycle and induces hyperproliferation [59, 60]. The negative effects of E6 and E7 on their respective tumor suppressors help promote genomic instability in the infected cell, an important process that is necessary for malignant transformation [55]. Indeed, most infected cells expressing HPV E6 and E7 have numerous chromosomal abnormalities, including aneuploidy and chromosomal rearrangements, along with centrosome aberrations, including centrosome accumulation and disruption of the centrosome replication cycle [60-62].

Much of our current understanding of HPV-induced carcinogenesis comes from research within the field of uterine cervical cancer, where there are known detectable pre-malignant lesions, and thus, the process of carcinogenesis can be rigorously studied. In the canonical cervical model for HPV-induced carcinogenesis, the important step in

progression from a pre-malignant lesion to cancer is the integration of the HPV genome that often disrupts the HPV gene E2, which increases expression of viral major oncogenes E6 and E7 and thereby allows for malignant transformation. However, the role of viral genome integration in HPV(+) head and neck cancer is not as clear. The TCGA head and neck cancer study determined that about one third of HPV(+) head and neck tumors lack an integrated HPV and instead contain the viral genome in an episomal form [57]. Tumors with integrated and episomal HPV have distinct viral and cellular gene expression and DNA methylation patterns; however, the clinical significance of this has yet to be fully appreciated [39, 57]. Given the differences in cell type, HPV subtypes that cause cancer, and the physical status of the HPV genome, it stands to reason that many assumptions regarding HPV-induced carcinogenesis based on cervical cancer studies may not accurately reflect the HPV-dependent carcinogenic process within the oropharynx.

Previous efforts – tumor suppressor restoration

In HPV(+) carcinomas, the viral oncoprotein E6 induces degradation of p53 through ubiquitin-mediated proteolysis, leading to the loss of p53 tumor suppressor activity. Since the HPVE6 oncoprotein inhibits p53, restoring both the protein expression and tumor suppressing function of p53 has been suggested as a promising strategy to combat HPV(+) cancer. Indeed, numerous studies have shown that restoring p53 function in tumors leads to tumor regression [63-66]. In one study, HPV(+) cancer cells treated with leptomycin B and actinomycin D reduced levels of E6 at the transcriptional level, restored endogenous p53 levels, and subsequently induced p53-dependent apoptosis [67].

Another study utilized a chemical library and reported that 2 small molecules upregulated p53 and induced apoptosis in HeLa (HPV18(+) cervical cancer) cells [68]. The small molecule RITA (reactivation of p53 and induction of tumor cell apoptosis) has been shown to prevent p53 degradation and to induce apoptosis in cervical cancer cells [69, 70]. The relative sensitivity to radiation and chemotherapeutic drugs of HPV(+) HNSCC could be, at least partially, attributed to the activation of DNA damage response pathways that stabilizes p53 in HPV(+) cancer cells. Nevertheless, though theoretically and preclinically promising, no drugs specifically aimed at p53 restoration in HPV(+) tumors have been approved as anti-cancer agents to date.

Similar to p53 restoration, promising preclinical studies have looked at restoring the pRB pathway in HPV(+) cancers to induce tumor death [71, 72]. One study identified a peptide that specifically bound to HPV16 E7, restored pRB levels and function, and inhibited the proliferation of SiHa (HPV16(+) cervical cancer cell line) cells in vitro and in a xenografted mouse model [73]. Another study demonstrated that the compound Wogonin decreased the expression of E6 and E7 and induced apoptosis in CaSki (HPV16(+) cervical cancer) and SiHa cells [74]. In addition, shRNA-mediated suppression of E6 and E7 transcription restored p53 and pRB levels and induced apoptosis in HPV16 head and neck cancer cell lines [75]. However, similarly to p53 restoration, reactivation of pRb was more effective in theory and research laboratory practice thus far yielding no approved pRb-restoring anti-cancer agents.

Current efforts – targeted therapies and personalized medicine

More recently, drugs targeting specific genomic characteristics of HPV(+) tumors have been suggested as potentially efficacious in pre-clinical and clinical studies as part of a continuing effort to utilize concepts in precision medicine to improve cancer treatment. To date, Cetuximab, a humanized murine monoclonal anti-EGFR antibody, is the only targeted, selective therapy approved by the Food and Drug Administration (FDA) for treatment of head and neck cancers, though the specific molecular features of the tumor (i.e. HPV(+) status, genomic differences, etc) are not taken into consideration when administering the drug, despite some evidence that it is potentially more efficacious for patients with HPV(+) disease [76-79]. Several other monoclonal anti-EGFR antibodies, including panitumumab and nimotuzumab, along with small molecule EGFR inhibitor erlotinib, have been studied as well [80].

Besides the EGFR pathway, numerous other targets in HPV(+) head and neck cancer have been explored. Several studies have demonstrated that HPV(+) head and neck cancers express elevated levels of human epidermal growth factor 2 (HER2), a member of the human epidermal growth factor receptor (HER/EGFR/ERBB) family, compared to HPV(-) tumors [81, 82]. Subsequently, dual HER2/EGFR inhibitors, such as afatinib and lapatinib, have been proposed as targeted therapies for HPV(+) head and neck cancers, and pre-clinical studies have been promising [82, 83]. HPV(+) tumors also overexpress poly (ADP-ribose) polymerase-1 (PARP-1), a DNA damage repair protein involved in repair of double-stranded DNA (dsDNA) and single-stranded DNA (ssDNA) damage along with nucleotide-excision repair, and the repair of stalled replication forks, and are exquisitely sensitive to DNA damage [84]. Inhibitors of PARP-1 have also shown promise as targeted therapies for HPV(+) head and neck cancers through pre-clinical

studies both by our laboratory (unpublished data) and the work of another group [85].

Finally, given that HPV(+) tumors also harbor a hypermethylated epigenome compared

to HPV(-) tumors, demethylation therapy with agents such as 5-azacitidine (a cytidine

analogue) represents another potential target for precision medicine. Perhaps most

encouragingly, the results of a clinical window trial of 5-azacytidine as an adjunctive

treatment for head and neck squamous cell carcinoma conducted at the Yale Smilow

Cancer Center were recently published by our group, which demonstrated encouraging

antitumor effects on HPV(+) tumors [86]. These large and varied efforts at developing

targeted therapies are certainly encouraging, though more work is needed to bring such

drugs to patients as the accepted standard of care.

Cell cycle deregulation and Roscovitine – a cyclin-dependent kinase inhibitor for

HPV(+) HNSCC

One common feature of all cancers is uncontrolled cellular proliferation resulting

from cell cycle deregulation. The progression of the cell through cell checkpoints

between G1, S, G2, and M – the 4 major phases of the cell cycle – is regulated through

the activation of different cyclin-dependent kinases (CDKs) and expression their

respective cyclin binding partners. CDK-cyclin binding triggers a cascade of downstream

signaling events that prompt the cell to synthesize DNA, initiate mitosis, and ultimately

complete the cell cycle. CDK4 and 6 are active during the G1 phase of the cell cycle,

CDK2 during the G1/S phase transition and S phase, and CDK1 is active during the

G2/M transition and during mitosis [87]. In cancer, cell cycle checkpoints are frequently

deregulated leading to uninhibited proliferation and cellular division, and in HPV(+)

HNSCC, cell cycle deregulation is driven by E6-mediated p53 degradation and E7-mediated pRB degradation, as discussed previously. Subsequently, CDK inhibition has been an attractive target for cancer therapies for the last several decades, though a CDK inhibitor has never previously been suggested as a drug to treat HPV(+) HNSCC [88, 89]. Over 100 different CDK inhibitors are in clinical trials for cancers including melanoma, mantle cell lymphoma, liposarcoma, neuroblastoma, and non-small cell lung cancer, and encouragingly, Palbociclib (an inhibitor of CDK4/6) was recently FDA approved to be used for estrogen receptor-positive, HER2-negative advanced breast cancer [89-91].

Roscovitine (CY-202, (R)-Roscovitine, Seliciclib) is a small molecule CDK-inhibitor of CDK2, CDK1, CDK5, CDK7, and CDK9 that inhibits CDKs by competitively blocking ATP binding at the CDK ATP binding site [87, 92, 93]. Roscovitine has been shown to have antitumor effects in different cancers, including non-small cell lung cancer and nasopharyngeal carcinoma, and is currently in a number of Phase I and II clinical trials (Table 1) [94]. Furthermore, outside of oncology, roscovitine is a drug of interest in treating a wide variety of diseases including cystic fibrosis, polycystic kidney disease, glomerulonephritis, glaucoma, acute graft-versus-host disease, and herpes simplex infection, among others [94, 95].

Roscovitine was of particular interest to our study because of the aforementioned antitumor properties and specifically as a targeted therapy for HPV(+) HNSCC because 1) Roscovitine has been shown to be highly cytotoxic to HPV-associated cancer cell lines including SiHa (HPV16(+) cervical cancer) and HeLa (HPV18(+) cervical cancer) and 2) Roscovitine interferes with viral replication of other viruses, notably Herpes Simplex Virus (HSV) [96-98]. Thus, we hypothesized that roscovitine would be an efficacious,

targeted therapy for HPV(+) HNSCC, and investigated both its efficacy and mechanism of action as for Specific Aim 1 of this thesis.

Table 1. A summary of current oncological clinical trials for the CDK-inhibitor roscovitine.

Disease	Stage of development
Nasopharyngeal cancer	Phase II clinical trial
NSCLC	Phase II clinical trial
Breast cancer	Phase II clinical trial
Cystic Fibrosis	Phase II clinical trial
Cushing's disease	Phase II clinical trial
RA	Phase II clinical trial
Solid tumors, unspecified	Phase I clinical trial
Pancreatic cancer	Phase I clinical trial
Ovarian cancer	Phase I clinical trial

Improving existing treatments for HPV(-) cancers

Targeting therapies for HPV(-) head and neck cancers – previous efforts

Though our group and others have focused on bringing targeted therapies to patients suffering from HPV(+) HNSCC, patients with HPV(-) disease have a worse response to current treatments and poorer prognosis compared to patients with HPV(+) tumors, suggesting that they have a greater need for novel therapies or improvement of

existing therapies. Given this, several groups have sought to develop novel therapies for HPV(-) HNSCC using the underlying unique genomic features of the tumors as a guide for targeting drugs.

As mentioned previously, The TCGA head and neck cancer study determined that in a majority of cases, HPV(-) HNSCCs were driven by p53 mutations, suggesting that restoration of wild-type p53 function through targeted therapy may represent a rational and viable therapeutic approach in such patients [39]. P53 is unique among tumor suppressors in that most genetic alterations (>80%) are missense point mutations, which yield protein that has lost its wild-type structure and its function [99-102]. Researchers have posited that drugs capable of binding such p53 mutants and altering their three-dimensional structure may be used to restore wild-type p53 structure and subsequent function in such cancer cells; this approach has yielded some promising pre-clinical results in head and neck cancer. One study noted that the small molecules PRIMA-1 and CP-31398 restored wild-type p53 activity and induced cell cycle arrest and apoptosis in HNSCCs driven by mutant p53 [103]. Another study showed that the administration of the small molecule drug piperlongumine sensitized HNSCC cells to APR-246 (a methylated form of PRIMA-1) and caused both apoptosis and autophagic cell death in these cells [104]. However, much like efforts aimed at restoring wild-type p53 function in HPV(+) HNSCCs, no therapies have currently been approved for clinical administration to cancer patients.

Another intriguing target in HPV(-) HNSCC, among other malignancies, is the Programmed Cell Death 1 (PD-1) pathway. PD-1 receptors are expressed on the surface of activated T cells and bind to Programmed Cell Death Ligand 1 (PD-L1), a substrate

constitutively expressed in low levels on the surface of antigen presenting cells (APCs),

and importantly, one that is upregulated on the surface of cancer cells. The binding of

PD-1 to PD-L1 induces apoptosis in the PD-1 expressing cell and represents one

mechanism by which cancer cells are able to evade surveillance by the immune system

[105]. Importantly, it has recently been shown that the presence of CD8(+) tumor

infiltrating lymphocytes confers an improved prognosis in both HPV(+) and HPV(-) head

and neck cancer patients treated with adjuvant chemoradiation, suggesting that the

antitumor activity of these T-cells plays an important role in the survival of head and

neck cancer patients [106]. HNSCCs express higher levels of both PD-1 and PD-L1 than

healthy tissue, though this elevated expression does not appear to be dependent on the

HPV status of the tumor [107]. However, recent evidence demonstrated that increased

PD-L1 expression in HPV(-) HNSCC was associated with resistance to radiation and

with treatment failure, suggesting that such tumors should become more sensitive to

radiation treatment after inhibition of the PD-1 pathway, and that drugs targeting the PD-

1/PD-L1 pathway may be particularly efficacious for HPV(-) head and neck cancers

[108]. Although promising, clinical applications of drugs targeting the PD-1/PD-L1

pathway in HNSCC have had mixed results to date: a multicenter clinical trial that

administered pembrolizumab (a humanized monoclonal anti-PD-1 antibody) for patients

with recurrent or metastatic HNSCC resulted in a 14% response rate for patients with

HPV(-) HNSCC, demonstrating that despite relatively high levels of PD-1 expression,

many patients did not respond to the treatment [109]. Furthermore, the response rate for

HPV(+) HNSCC patients was actually *higher* (25%) than those for HPV(-) patients,

suggesting that this drug may actually be more effective for patients with HPV(+) disease.

Enhancing DNA damaging therapies with LZAP manipulation for HPV(-) HNSCC

Despite huge efforts made in the field of targeted anticancer therapy, DNA damaging therapies, such radiation alone, or in combination with chemotherapeutic drugs (such as platin-based agents), remain one of the most effective anticancer treatment strategies. Given the small number of targets described by TCGA and very modest success of targeted therapies for HPV(-) HNSCC to date (as described in the previous section), we chose to focus Specific Aim 2 of this thesis work on exploring strategies to enhance the efficacy of existing DNA damaging therapies for patients with HPV(-) HNSCC.

Acute side effects targeting the hematopoietic system (as a result of p53-mediated apoptosis in such healthy cells in response to DNA damage) often dramatically limit the application of DNA damaging therapies, and this pitfall has attracted researchers to develop (1) specific radioprotectors of normal tissue; and/or (2) drugs that radiosensitize tumors without affecting normal tissue [110, 111]. The potential combination of both strategies in one drug must generate the optimal therapeutic window of radio- and/or chemotherapy leading to a successful treatment of cancer without causing harmful side effects. As radiation results in massive normal cell death due to p53-dependent apoptosis, one promising radioprotective tactic relies on the temporal inhibition of wild-type p53 (wtp53) activity. Small molecule p53 inhibitors, as well as genetic mouse models, have confirmed the safety and efficacy of this approach [112-118]. On the other hand, p53 is

nearly always inactivated in human cancers through varied mechanisms. Mutations in the TP53 gene are found in ~ 50% of all human tumors and are often associated with poor prognosis [119-121]. As mentioned in the previous section, an exclusive feature of the TP53 gene, distinguishing it from other tumor suppressors, is the type of cancer-related genetic alterations, with the majority (>80%) of them being missense point mutations resulting in the accumulation of stable mutant protein that has lost its original wild-type activity [99-102]. In addition to loss of wild-type function, many p53 mutations convey oncogenic activity that increases resistance to radiation and DNA damaging therapy, suggesting downregulation and/or inhibition of mutant p53 (mtp53) as a therapeutic strategy to enhance response to conventional chemotherapeutic drugs or radiation [99, 102, 122-127]. Much effort has been applied toward restoring wild-type p53 functions in mutant p53-expressing cells; however, the temporal decrease of both mutant (present in cancer cells) and wild-type (expressed in normal surrounding cells) p53 has not been extensively addressed [128-131]. The strategy of simultaneous downregulation of mutant and wild-type p53 should decrease the resistance of tumors with mutant p53 (which includes most HPV(-) HNSCCs) to radiation and chemotherapy, while simultaneously protecting normal tissues from severe side effects. Though theoretically applicable to all p53 mutation driven cancers, we thought such a strategy would be particularly efficacious for HPV(-) HNSCC, given that the vast majority harbor mutant p53.

LZAP (LXXLL/leucine zipper-containing ARF-binding protein), also known as CDK5RAP3, C53, IC53 and HSF-27, was initially identified as a binding partner of the Cdk5 activator p35 [132]. Our laboratory furthered insight into the activity of LZAP by showing that it binds the alternative reading frame protein (p14ARF, ARF) to activate

p53, arrest cellular proliferation and inhibit clonogenic growth [133, 134]. Interestingly, we also demonstrated that LZAP expression activated p53 and induced p53-dependent cell-cycle arrest in the absence of ARF, suggesting that LZAP has both ARF-dependent and ARF-independent effects on p53 [133]. Our group showed that besides its effects on p53, LZAP selectively inhibited the transcription factor NF-kB and that LZAP levels were inversely correlated with the expression of genes regulated by NF-kB, such as IL-8 [134]. Data from our laboratory and others link LZAP to a decrease in phosphorylation of its binding partners, including p38 MAPK, Chk1/2 and RelA, that is, at least partially, explained by the ability of LZAP to enhance WIP1 phosphatase activity [134, 136-139]. However, detailed mechanisms of LZAP functions, particularly ARF-independent LZAP effects on p53, remain unclear. Importantly, as part of our preliminary LZAP studies, our laboratory discovered that depletion of LZAP resulted in depletion p53 regardless of mutational status. Subsequently, we hypothesized that transient depletion of LZAP would be effective in sensitizing cancer cells driven by mtp53 to DNA damaging therapies while at the same time protecting normal cells (which harbor wtp53).

Statement of Purpose:

The goal of this thesis work was to use concepts in precision medicine to tailor treatments to head and neck cancer patients based on their HPV status. Specifically, this involved 1) designing a novel targeted therapy for patients suffering from HPV(+) HNSCC and 2) improving existing DNA damaging therapies for patients suffering from HPV(-) HNSCC. In doing so, we hope that these studies will lead to improvements in current treatments. In particular, we hope that discovery of new therapeutic approaches

specific to HPV(+) or HPV(-) HNSCC will result in guidelines so that the HPV status of the tumor is considered before a specific therapy is chosen. This work is subdivided into two specific aims as listed below.

Specific Aims:

1.   Determine the efficacy of Roscovitine as an antitumor agent for HPV(+) HNSCC and its mechanism of action.

2.   Explore the role of LZAP in protecting healthy cells and sensitizing tumor cells with mutant p53 to DNA damaging therapies and determine the mechanism of action.

Specific Aim 1 – Methods:

Cell lines, constructs and chemicals

We used four HPV(-) (SCC61, SCC35, UNC7 and FaDu) and three HPV(+) (SCC090, SCC104 and UMSCC47) HNSCC cell lines. All HPV(-) cells were cultured in (DMEM)/F12 medium supplemented with 0.4 μg/mL hydrocortizone, and all HPV(+) cell lines were grown in DMEM with nonessential amino acids. All media was supplemented with 10% FBS (Invitrogen), 50 μg/mL penicillin, and 50 μg/mL streptomycin (Invitrogen). All cell lines were tested and found to be negative for mycoplasma and microsatellites were authenticated as well. P-super and p-super p53 shRNA expressing vectors were a gift from Galina Selivanova. Cells were transfected using Lipofectamine 2000 (Invitrogen) according to manufacturer recommendations. Roscovitine and the the specific CDK 4/6 inhibitor (PD 0332991) were obtained from

Sigma. Flavopiridol and the selective CDK1/2 inhibitor III were from Santa Cruz
Biotechnology.

Immunoblotting

Cells were collected by trypsinization and lysed in radioimmunoprecipitation
assay (RIPA) lysis buffer (Sigma) with the addition of protease inhibitors (Roche) and
phosphatase inhibitors (Sigma) for 30 minutes on ice. Insoluble material was removed by
centrifugation at 14,000 rpm for 15 minutes at 4°C. Proteins were separated in 4% to 20%
Tris-glycine polyacrylamide gels (Mini-PROTEAN; Bio-Rad) and electrophoretically
transferred onto polyvinylidene fluoride membranes. The membranes were blocked with
3% BSA in PBS and incubated with antibodies against YH2AX and pATM (Abcam),
p53 and p21 (Santa Cruz), pp53 Ser15 (Cell Signaling) and tubulin (Sigma). After
incubation with primary antibodies, membranes were washed, incubated with secondary
DyLight anti-mouse and anti-rabbit antibodies (Thermo Scientific), and signals was
visualized using a Bio-Rad imager.

Survival assays

All cells lines, except SCC090 and UMSCC104, were seeded in 12-well plates at
a density of 1000 cells/well in duplicates and treated with increasing doses of CDK
inhibitors the following day. SCC090 and UMSCC104 were plated at a density 10,000
cells/well. After 7 days, we used Cell Titer Glo reagent (Promega) to determine the
number of live cells. The data presented in Figure 1A was obtained from 4 independent
experiments.

Immunofluorescence

Cells were grown in chamber slides, treated, fixed, immunostained, and analyzed as previously described [140]. Cells with more than 10 foci were determined as positive. The primary antibodies used were mouse anti-γH2AX (Abcam) at a dilution of 1:2,000, rabbit anti-53BP1 (Cell Signaling) at a dilution of 1:500, and rabbit anti-RPA70 (Cell Signaling) at a dilution of 1:500. Secondary anti-mouse Alexa 555 and anti-rabbit Alexa 488 were from Invitrogen and were used at a dilution 1:1000.

Comet assay

Cells were grown in 6 well plates, treated with roscovitine, and processed for DNA damage detection using a Comet Assay Reagent Kit (Trevigen) according to their protocol. For quantification, nuclear diameter and tail length were measured in at least 50 cells using the ImageJ program.

Fluorescence-activated cell sorting (FACS)

Cells were collected by trypsin and fixed in ice-cold 70% ethanol over night at −20°C. The ethanol was removed by centrifugation and the cells were rehydrated in PBS and pelleted. The pellets were resuspended in 25 µg/ml propidium iodide (PI) (Sigma) in PBS containing 100 µg/ml RNase A (Invitrogen) and stained for 30 minutes at room temperature. The DNA content was analyzed by a FACSCalibur flow cytometer (BD Biosciences). Samples were gated on the single cell population, and 10,000 cells were collected for each sample.

RNA extraction and quantitative RT-PCR

Total RNA was extracted using a Qiagen RNA extraction kit and cDNA was synthesized using an iScript cDNA Synthesis Kit (Bio-Rad) according to the manufacturer's instructions. Quantitative real-time reverse transcription (qRT-PCR) was done using iQ SYBR Green Supermix (Bio-Rad) and the following primer pairs: CDKN1A from Origene; Forward 5′AAGCAACAGTTACTGCGACGTGAG3′ and Reverse 5′ CGGTCCACCGACCCTTATATT3′ for HPV16 E6; Forward 5′ ACCGGACAGAGCCCATTACA3′ and Reverse 5′ GCCCATTAACAGGTCTTCCAA3′; Forward 5′ AGGGCTGCTTTTAACTCTGGT3′ and Reverse 5′ CCCCACTTGATTTTGGAGGGA3′ for human GPDH using the iCycler iQ Real-Time PCR Detection System (Bio-Rad). Each qRT-PCR reaction was done in at least duplicate, and the ΔCt method was used to analyze the data.

In vivo experiments

The *in vivo* study was approved by Yale University animal experimental ethics committee. Exponentially growing UMSCC47 cells were injected subcutaneously into the sacral area of female NUDE mice. Each mouse was inoculated with $2x10^5$ cells in 50% matrigel and 50% PBS at a volume of 100 μL. Body weight, tumor growth, and general behavior were monitored. Tumor volumes were measured every 3 days. Mice were sacrificed when the tumor exceeded a size of $0.5cm^3$.

Statistical analysis

The Kaplan–Meier method was used to generate survival curves, and log-rank test analysis was used to compare roscovitine treated and untreated mouse groups. Other statistical analyses were done using Fisher exact and χ2 for trend tests.

Results

Sensitivity of head and neck cancer cells to roscovitine depends on HPV status

Since previous studies suggested that uterine cervical cancer cells were sensitive to roscovitine and upon treatment experienced both significant inhibition of proliferation and increased caspase-mediated apoptosis, we first tested whether HPV status had an effect on the sensitivity of head and neck cancer cells to roscovitine [96, 97]. A survival assay was performed to gauge the response of four HPV(-) (SCC61, SCC35, FaDU, and UNC7) and three HPV(+) (UMSCC47, SCC090 and SCC104) HNSCC cell lines to increasing roscovitine concentrations. HPV(+) cancer cell lines, but not HPV(-) cells responded to roscovitine with decreased clonogenic survival in a dose-dependent manner. Among HPV(-) cells, the sensitivity to roscovitine was not dependent on p53 mutational status, as there was no significant differences between wild type p53 expressing UNC7 cells and mutant p53 carrying SCC61, SCC35, and FaDU cell lines. Furthermore, the greatest differences in sensitivity to roscovitine between HPV(+) and HPV(-) cancer cell lines were found at lower concentrations of roscovitine.
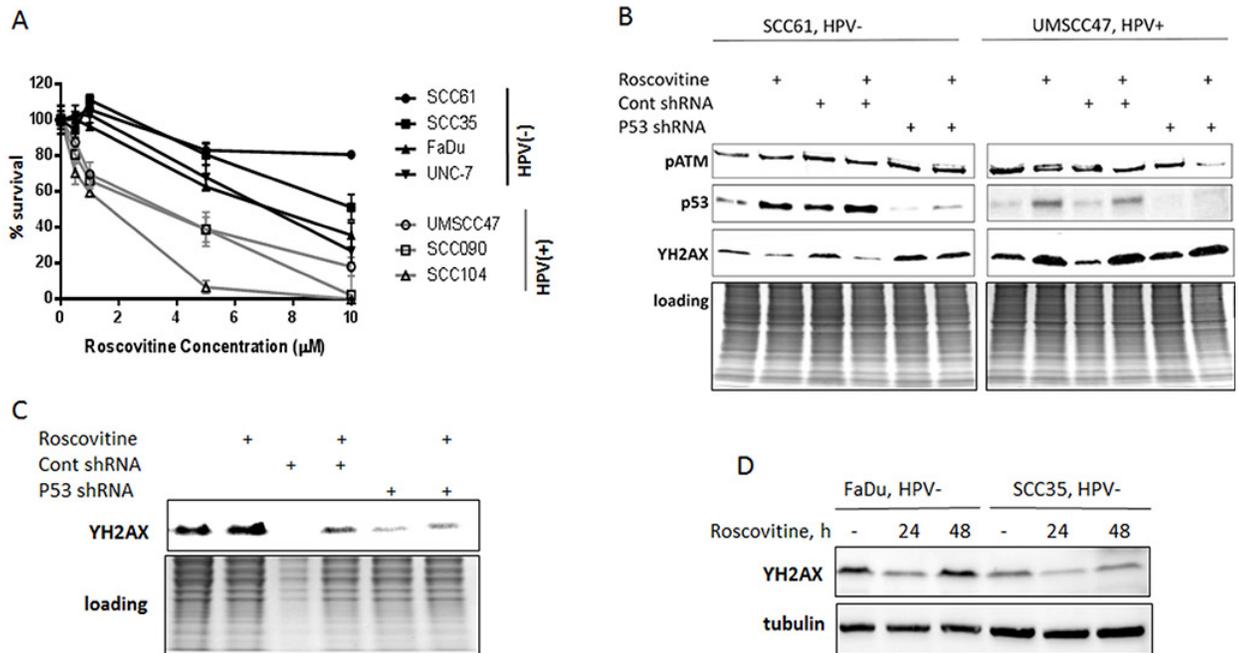
Figure 1: Roscovitine induces p53- and ATM-independent phosphorylation of H2AX and selectively inhibits growth in HPV(+) head and neck cancer cells. A. Survival after increasing doses of roscovitine was determined in HPV(-) SCC35, SCC61, FaDu and UNC7 (labeled in black) and HPV(+) UMSCC47, SCC104 and SCC090 (labeled in grey) head and neck cancer cell lines; standard deviations are calculated from four independent experiments. B. HPV(-) SCC61 and HPV(+) UMSCC47 cells expressing either control, or p53 shRNAs, were treated with 20μM of roscovitine; immunoblotting with indicated antibodies was performed 24 hours after the treatment. C. HPV(+) SCC090 cells transfected with control or p53 shRNA were treated with roscovitine for 24h and immunoblotted with YH2AX antibody. D. Two HPV(-) cells lines, FaDu and SCC35, were treated with 20μM of roscovitine for 24 and 48 hours and immunoblotted with indicated antibodies.

Roscovitine promotes p53- and ATM- independent stimulation of DNA damage response

selectively in HPV(+) head and neck cancer cells

Given that HPV status correlated with sensitivity to roscovitine in head and neck cancer cells, we next investigated the potential mechanism of this sensitivity. Roscovitine stabilizes and activates wild type p53 and induces apoptosis in multiple human cancer cell lines, including HPV18(+) cervical cancer HeLa cells [97, 141-143]. Furthermore,

roscovitine activates DNA damage response pathways and inhibits DNA damage repair

machinery, although whether roscovitine treatment damages cellular DNA remains

unclear [144, 145]. We found that roscovitine upregulates p53 in head and neck cancer

cells regardless of p53 mutation and HPV status (Figure 1B; HPV(-) negative SCC61

cells harbor mutant p53, while HPV(+) UMSCC47 cells have wild type p53).

Intriguingly, roscovitine activated the DNA damage response, as detected by

phosphorylation of H2AX (γH2AX), in HPV(+) UMSCC47 cells only (Figure 1B). In

contrast, we found a significant decrease in H2AX phosphorylation in HPV(-) SCC61

head and neck cancer cells after roscovitine treatment (Figure 1B). Depletion of p53 with

p53 shRNA neither abrogated γH2AX induction in HPV(+) nor redacted H2AX

phosphorylation in HPV(-) cells (Figure 1B). Interestingly, the DNA damage-responsive

kinase, ATM, was not activated by roscovitine treatment in any cells tested (Figures 1B,

2C), suggesting that stimulation of DNA damage response by roscovitine treatment

proceeded via an ATM-independent pathway in HPV(+) cells.

Similar results were obtained in another HPV(+) cell line, SCC090, in which

roscovitine treatment resulted in activation of DNA damage response, as indicated by

elevated phosphorylation of H2AX, independently of the presence or absence of p53

(Figure 1C). In addition, analogous to the results obtained with HPV(-) SCC61 cells, 24

hours of treatment with roscovitine downregulated phosphorylation of H2AX in two

other HPV(-) head and neck cancer cells (SCC35 and Fadu) with γH2AX levels restored

back to levels found in control untreated cells 48 hours after the treatment (Figure 1D).

Thus, roscovitine activated the DNA damage response selectively in HPV(+), but not in

HPV(-), head and neck cancer cells.

Roscovitine treatment activates p53 and induces p53-dependent HPV(+) cell death

The tumor suppressor wild type p53 is a powerful inducer of cell death in response to diverse stress signals, including DNA damage. In HPV(+) cancer cells, the HPV oncoprotein E6 induces degradation of p53 through ubiquitin-mediated proteolysis, leading to the loss of p53 activity. As described earlier, we found that HPV-positivity conferred sensitivity to roscovitine and that roscovitine treatment increased p53 levels in HPV(+) head and neck cancer cells (Figure 1B). In order to determine whether the increased sensitivity of HPV(+) cells to roscovitine was due to upregulation and activation of wild type p53, we transiently transfected UMSCC47 cells with psuper control or psuper p53 shRNAs. We found that depletion of p53 increased survival of UMSCC47 cells (Figure 2A) after roscovitine treatment. The classical p53 target gene, CDKN1A, was upregulated by roscovitine in HPV(+) SCC090 cells at the mRNA (Figure 2B) and protein levels (Figure 2C), suggesting that roscovitine-elevated p53 is transcriptionally active. Depletion of p53 with shRNA partially abolished p21 induction after roscovitine treatment (Figure 2C), further confirming roscovitine-induced p53 transcriptional activation in HPV(+) HNSCC cells.

Next, we attempted to find a mechanism of p53 induction in HPV(+) head and neck cancer cells after roscovitine treatment. First, we determined the expression of the p53 negative regulator HPV E6 gene. Interestingly, roscovitine treatment differentially affected HPV E6 levels in two HPV(+) cell lines; while roscovitine decreased HPV E6 expression in UMSCC47 cells, it upregulated HPV E6 mRNA in SCC090 cells (Figure 2D, top).

Despite the opposite effect on HPV16 E6 mRNA levels, roscovitine treatment

induced p53 in both cell lines (Figure 2D, bottom). Thus, p53 was upregulated by roscovitine independently of HPV E6 expression. To begin determining if DNA damage would stabilize p53 in HPV(+) head and neck cancer cells, we treated SCC090 cells the with radiomimetic drug zeocin. As expected, zeocin induced DNA damage, as indicated by increased phosphorylation of H2AX, and despite the HPV-positive status of these cells, DNA damage was associated with stabilization of p53 protein (Figure 2E). In contrast to roscovitine, zeocin activated ATM, resulting in phosphorylation of p53 at Ser15. Similar to UMSCC47 cells (Figure 1B), roscovitine triggered the DNA damage response (γH2AX) and upregulated the total level of p53; however, roscovitine therapy did not activate ATM and did not induce p53 phosphorylation at Ser15 in SCC090 cells (Figure 2E).

Together, our data suggest that roscovitine activates ATM-independent DNA damage response and that this response may stabilize p53 to promote p53-dependent cell death in HPV(+) head and neck cancer cells.
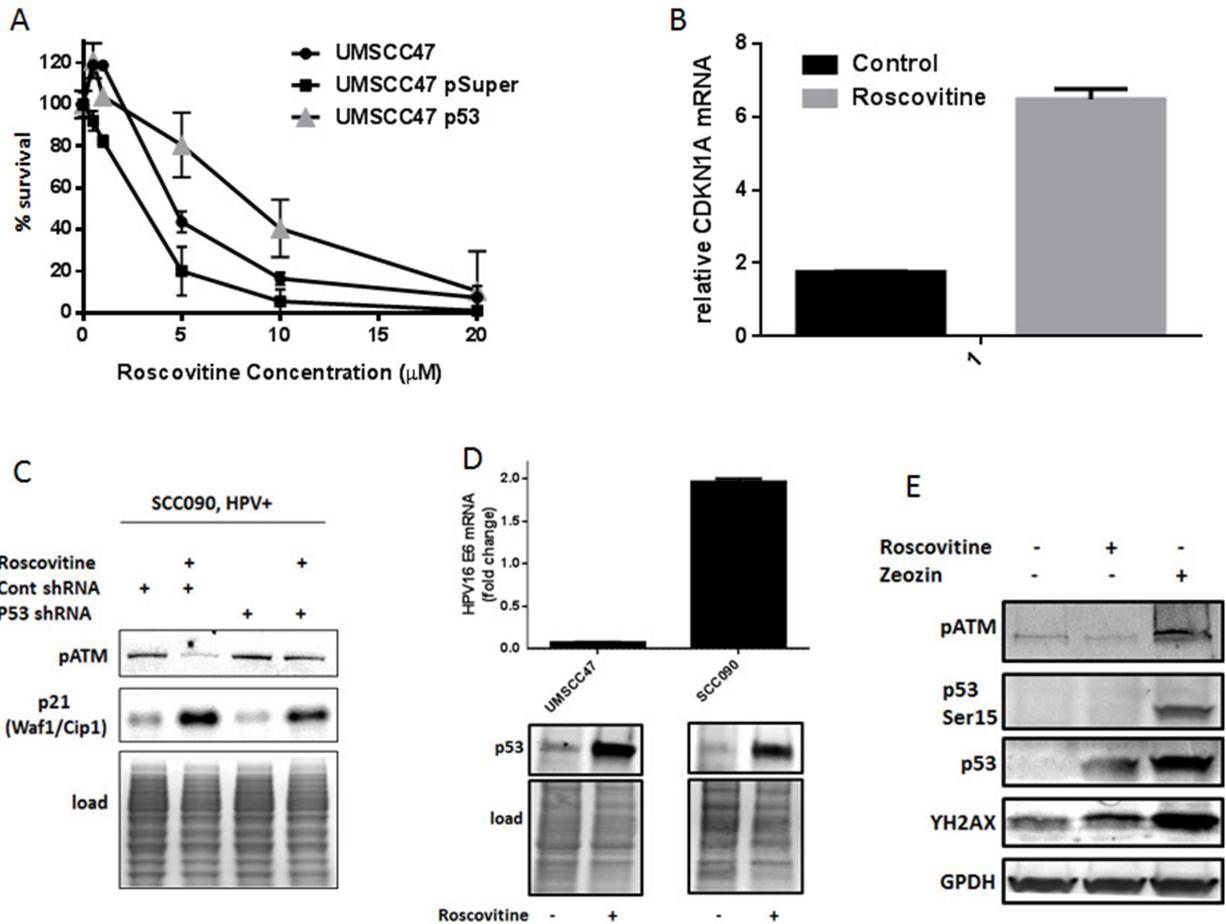
Figure 2: Roscovitine treatment activates p53 and induces p53-dependent suppression of HPV(+) cells' growth. A. HPV(+) UMSCC47 cells were transiently transfected with control or p53 shRNA and plated for survival after treatment with increasing doses of roscovitine; standard deviations were calculated from two independent experiments. B. Relative mRNA levels of the p53 target gene CDKN1A (p21) in HPV(+) SCC090 cells treated or not with 20 μM roscovitine for 24 hours; standard deviations were calculated from two independent experiments. C. SCC090 cells were treated or not with roscovitine, lysed, and immunoblotted with indicated antibodies. D. HPV(+) UMSCC47 and SCC090 cells were treated or not with roscovitine; the cells were collected and HPV16 mRNA levels were determined in qRT-PCR (top), or p53 protein levels were determined in Western Blot (bottom). E. HPV(+) SCC090 cells were treated for 24 hours with roscovitine, zeocin, or left untreated as a control, then lysed and immunoblotted with indicated antibodies.

Roscovitine does not induce DNA double strand breaks

Phosphorylation of H2AX at Ser139 is commonly used as a marker for general DNA damage; however, it is also elevated in the process of apoptosis, during progression of replication forks, and in G2/M arrest [146-148]. To determine which particular events caused the phosphorylation of H2AX in HPV(+) head and neck cancer cells after roscovitine treatment, we tracked the formation of the 53BP1 foci as a marker for DNA double strand breaks (DSBs), a particular type of DNA damage [149, 150]. Confirming our immunoblotting data which showed changes in H2AX phosphorylation (Figure 1B, 1C and 1D), treatment with roscovitine induced the formation of γH2AX foci in HPV(+) cells (UMSCC47 and SCC090) and reduced the number of γH2AX-positive cells in the HPV(-) cell line SCC61 (Figure 3A and 3B). However, no there was not a significant difference in the number of 53BP1-positive cells in control versus roscovitine treated samples in any of cell lines tested (Figure 3A and 3B). These data suggest that roscovitine treatment does not induce formation of DNA DSBs.
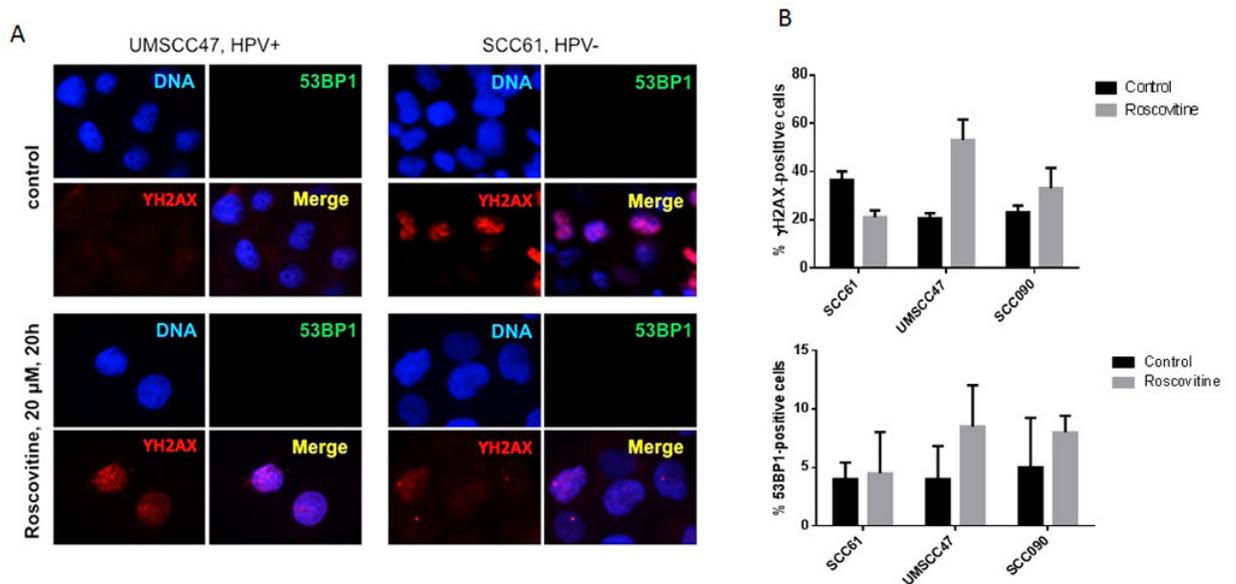
Figure 3: Roscovitine does not induce DNA double strand breaks as indicated by the absence of 53BP1 foci. A. HPV(-) SCC61 and HPV(+) UMSCC47 cells were treated with 20μM of roscovitine for 24 hours. Cells were fixed and immunostained with γH2AX and 53BP1 antibodies; representative images are shown. B. Quantification of γH2AX and 53BP1 positive cells from two independent experiments.

Roscovitine induces RPA foci formation in HPV(+), but not in HPV(-), head and neck cancer cells

Due to its strong affinity to single stranded DNA (SSD) and its ability to attract other proteins to these sites, the Replication Protein A (RPA) complex is an essential player in transcription, replication, and repair [151-154]. Because of the rapid accumulation of RPA at DNA single strand breaks (SSBs) and resected DSBs, an increased number of cellular RPA foci indicates an accumulation of SSD [155]. Interestingly, a substantial rise in RPA-positive cells, as well as partial co-localization of RPA and γH2AX foci, were found 24 hours after roscovitine treatment of HPV(+) UMSCC47 cells (Figure 4A and 4B). Conversely, roscovitine neither induced the formation of RPA foci nor the colocalization of RPA and γH2AX in HPV(-) SCC61 cells, again suggesting that roscovitine did not induce DNA damage in HPV(-) cancer cells.
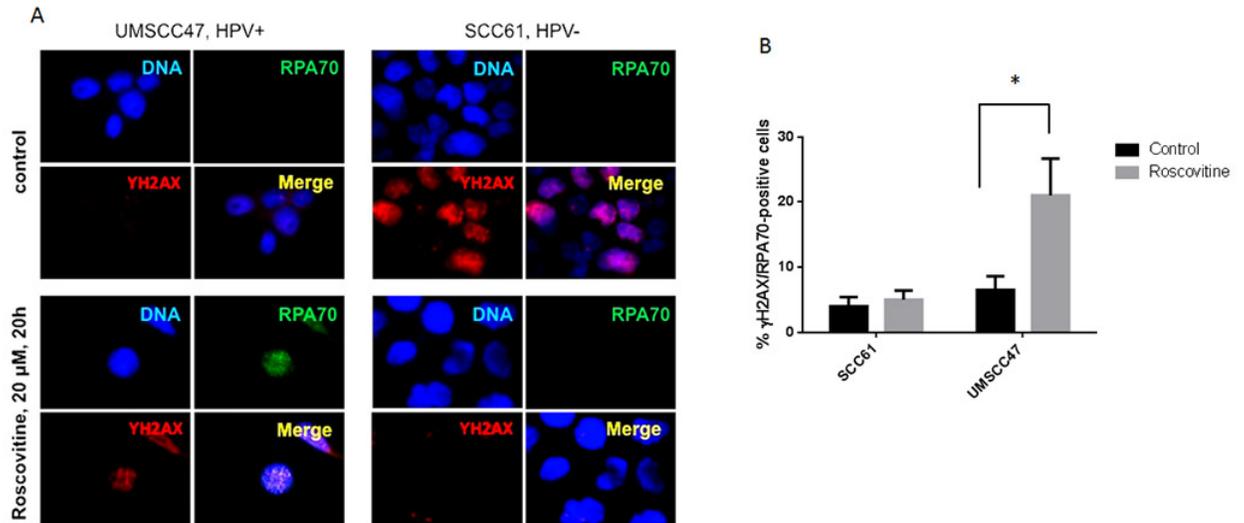
Figure 4: Roscovitine induces RPA foci formation in HPV(+), but not in HPV(-), head and neck cancer cells. A. HPV(-) SCC61 and HPV(+) UMSCC47 cells were treated with 20μM of roscovitine for 24 hours. Cells were fixed and immunostained with γH2AX and RPA70 antibodies; representative images are shown. B. Quantification of RPA-positive cells from two independent experiments.

Roscovitine induces DNA damage selectively in HPV(+) head and neck cancer cells

Given the upregulation of γH2AX (Figures 1B, 1C, 3 and 4) and formation of RPA foci (Figure 4) after roscovitine treatment in HPV(+), but not in HPV(-), head and neck cancer cells, we sought to examine whether roscovitine did truly selectively induce DNA damage in HPV(+) cells.

The presence of DNA damage was determined in SCC61 and UMSCC47 cell lines using a Comet assay (Figure 5). After roscovitine treatment, HPV(-) SCC61 cells had a significant reduction in the average tail length/nuclear diameter ratio, corroborating with previously observed decreased H2AX phosphorylation (Figures 1B and 1D, 3 and 4), and signifying that roscovitine did indeed reduce the amount of damaged DNA present in HPV-negative SCC61 cells (Figure 5). In contrast, HPV(+) UMSCC47 cells

showed an increase in the average tail length/nuclear diameter ratio and a substantial

right shift towards a higher ratio in the tail length/ nuclear diameter distribution

histogram after roscovitine treatment, confirming induction of H2AX phosphorylation

(Figures 1B and 1C, and 3), and demonstrating that roscovitine treatment induced DNA

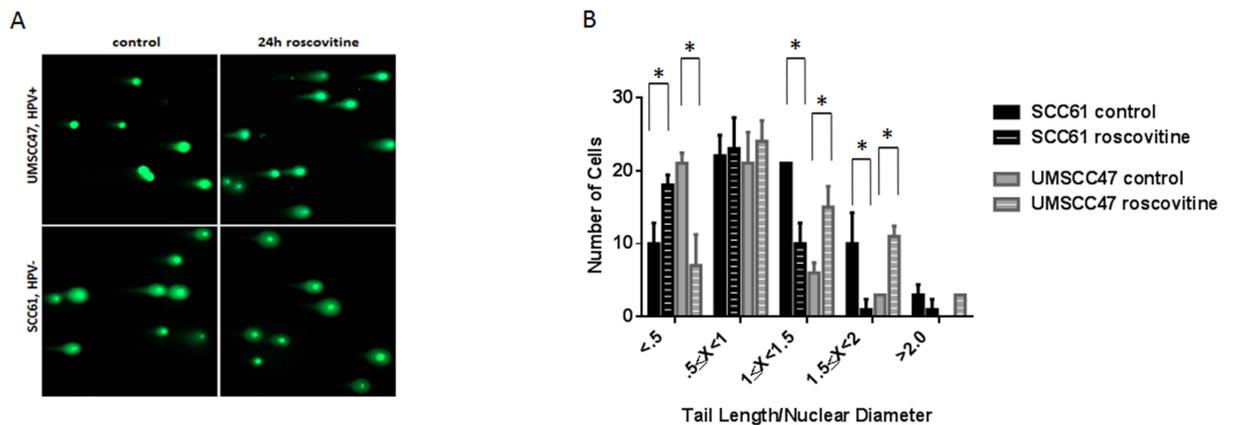damage selectively in HPV(+) UMSCC47 cells.

Figure 5: Roscovitine induces DNA damage exclusively in HPV(+) head and neck cancer cells. A. Representative images of a neutral Comet assay from HPV(-) SCC61 and HPV(+) UMSCC47 cells untreated or treated with roscovitine for 24 hours. B. Quantification of the Comet assay from two independent experiments.

Roscovitine treatment results in HPV(+) cell death

Since roscovitine is a CDK inhibitor and has been shown to arrest cells in the G1

and G2/M phases of the cell cycle, we investigated if HPV status would confer a different

cell cycle distribution in cancer cells after roscovitine treatment. Fluorescence activated

cell sorting (FACS) was performed on HPV(-) SCC61 and HPV(+) UMSCC47 cells

treated with 20μM roscovitine for 24 and 48 hours (Figure 6). HPV(-) SCC61 cells

experienced a time-dependent increase in the number of G2/M cells, decrease in the S

population and a moderate increase in the sub-G1 population upon roscovitine treatment, indicating that about 16% of SCC61 cells had died 48 hours after roscovitine. In contrast, HPV(+) UMSCC47 cells showed a significant decrease in the G1 population 24 and 48 hours after roscovitine application and a lesser decrease in the G2 population 48 hours after the treatment. Importantly, HPV(+) cells experienced a major escalation of the sub-G1 population with about 36% and 45% of dead cells 24 and 48 hours after roscovitine treatment, respectively. Thus, roscovitine induced pronounced cell death in HPV(+) cells, while transiently arresting and moderately killing HPV(-) head and neck cancer cells.
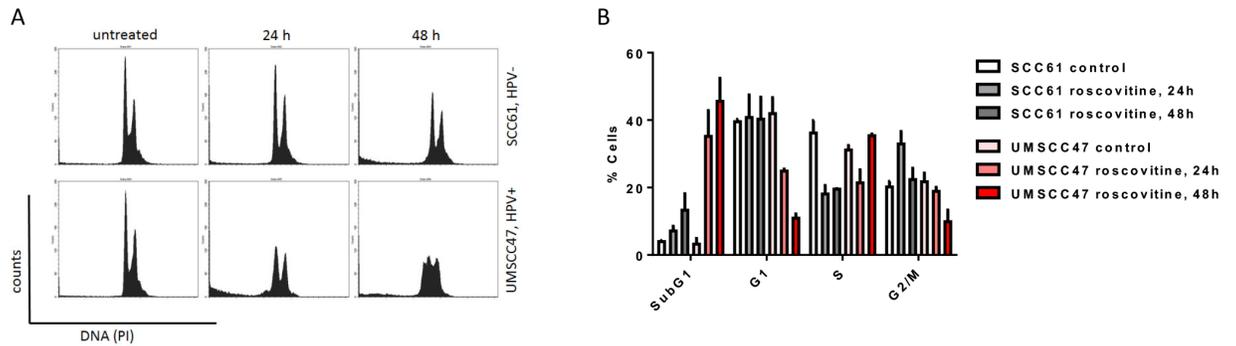

Figure 6: Roscovitine induces massive HPV(+) cell death. A. Cells were untreated or treated with roscovitine, collected and fixed at indicated time points, stained with propidium iodide (PI), and analyzed by flow cytometry. B. Percentage of cells in each phase of the cell cycle was quantified in two independent experiments.

Roscovitine inhibits the growth of HPV(+) head and neck cancer cells in vivo

To test the potential of roscovitine as a selective agent against HPV(+) head and neck cancers in an *in vivo* model, a NUDE mouse-based xenograft assay was utilized. Mice were injected with HPV(+) UMSCC47 cells, and after tumors reached a measurable size, the mice were treated with 16.5 mg/kg doses of intraperitoneal roscovitine or

vehicle injections. Tumor size was measured two times per week and mice were

sacrificed when tumor volumes reached or exceeded 0.5 cm^3. Roscovitine significantly

reduced the rate of tumor growth (Figure 7A) and increased survival (Figure 7B) of

treated mice. Strikingly, roscovitine treatment led to complete tumor regression in one

mouse (25%); moreover, no tumor regrowth in this mouse was found 5 months after

completion of the treatment (Figure 7B). Mouse weights did not differ significantly

between mice treated with roscovitine and control mice, and behavioral differences

between the two groups were also negligible. These results suggest that roscovitine

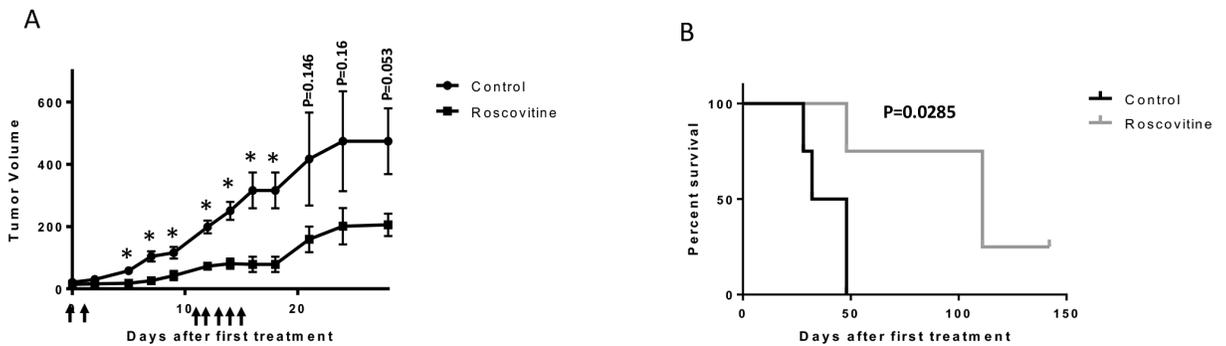effectively inhibits tumor growth in HPV(+) head and neck cancer.

Figure 7: Roscovitine suppresses HPV(+) tumor growth in vivo. A. HPV(+) UMSCC47 head and neck cancer cells were inoculated into NUDE mice. When tumors became palpable, mice were treated with 16.5 mg/kg of roscovitine or vehicle (4 mice in each group) at days indicated with arrows; tumor volume is presented. B. Mice were sacrificed when tumors reached a volume of 500 mm^3; the survival of mice in the control and roscovitine-treated groups is presented.

HPV status does not determine the sensitivity of head and neck cancer cells to avopiridol

or CDK1/2 inhibitor

Roscovitine is a broad CDK inhibitor; however, it also has activity toward the

extracellular regulated kinases, erk1 and erk2, as well as the pyridoxal kinase (PDXK)

38

that is responsible for the phosphorylation and activation of vitamin B6 [156]. To begin

determining whether HPV(+) head and neck cancer cells are sensitive to roscovitine due

to specific CDK inhibition, we assessed the response of HPV(+) and HPV(-) cells to

another broad CDK inhibitor, avopiridol, as well as to specific CDK1/2 and CDK4/6

inhibitors [157-160]. HPV(+) cancer cells are known to overexpress endogenous CDK

4/6 inhibitor p16^{ink4A}; moreover, a high p16 protein level is used as a surrogate marker

for HPV status in clinic [55]. Therefore, it was not surprising that HPV(+) head and neck

cancer cells were completely resistant to chemical inhibition by the CDK4/6 inhibitor

(Figure 8). Interestingly, although cell lines used in our study showed different responses

to both avopiridol and the CDK1/2 inhibitor, their sensitivity was not dependent on HPV

status. Thus, HPV(+) UMSCC47 cells displayed the highest sensitivity to avopiridol,

while another HPV(+) cell line, SCC090, was the most resistant to the same treatment

(Figure 8). In contrast, UMSCC47 cells were relatively resistant to CDK1/2 inhibition,

whereas SCC090 cells exhibited significantly increased sensitivity (Figure 8). These data

strongly suggest that at least CDK1/2 inhibition is not responsible for the HPV-dependent

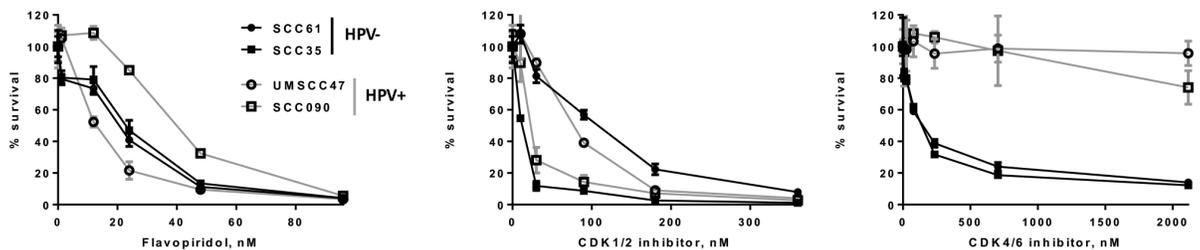sensitivity of cells to roscovitine.

Figure 8: Survival after increasing doses of flavopiridol, selective CDK1/2 inhibitor III, and specific CDK 4/6 inhibitor. Survival was determined in HPV(-) SCC35 and SCC61 (labeled in black) and HPV(+) UMSCC47 and SCC090 (labeled in grey) head and neck cancer cell lines; standard deviations are

calculated from three independent experiments.

Discussion:

Cytotoxic drugs, such as platin-based agents, and radiation that are widely used in cancer therapy cause various types of DNA damage through different mechanisms of action [161]. However, systemic drug administration damages DNA not only in cancer, but also in normal healthy cells, leading to the development of severe side effects and limiting efficacy of the treatment. Therefore, drugs that cause DNA damage selectively in cancer cells should improve outcomes and decrease treatment-associated morbidity as well as reduce the instances of premature termination of therapy due to intolerance of side effects. Discovery of such drugs is particularly important for patients with HPV-associated OPSCC due to two reasons. First, it is well established that these patients respond better to currently used radio- and chemotherapy, as compared to similarly staged HPV(-) head and neck cancer patients, indicating that HPV(+) OPSCCs are in general more sensitive to DNA damage. Second, as no HPV status therapy de-escalation is currently used outside of clinical trials, patients treated with DNA damaging therapy are loaded with lifelong-associated morbidity that includes pronounced swallowing and speech dysfunction, mandibular osteoradionecrosis, accelerated dental decay, and lymphedema. In addition, about 20% of patients with HPV(+) HNSCC suffer from recurrent cancer and distant metastases, for which effective therapies are absent.

In this study, we investigated the potential of roscovitine as a novel therapeutic agent against HPV(+) HNSCC. Roscovitine is a CDK inhibitor and antineoplastic agent that has been shown to exhibit cytotoxic effects towards multiple human cancer cells lines including colon, uterine, breast, Ewing's Sarcoma, as well as HPV18(+) cervical

cancer HeLa and HPV16(+) cervical cancer SiHa cells, among others [96, 143, 162, 163]. Interestingly, though roscovitine induces cell cycle arrest at the G1 and G2/M phases, previous studies reported that roscovitine appears to exert its antitumor effects by inducing apoptosis in cancer cells [92, 143, 164-167]. Roscovitine has also been associated with uncoupling replication proteins and inhibiting non-homologous end-joining DNA damage repair machinery, suggesting that the cytotoxic properties of roscovitine may be associated with the induction and/or accumulation of DNA damage [144, 145]. Though roscovitine is currently in clinical trials for a wide variety of cancers, it has never previously been suggested as an agent that selectively targets HPV(+) HNSCC.

Here, we first determined whether the HPV status of HNSCC would confer a heightened sensitivity to roscovitine, and subsequently investigated the preliminary mechanism behind HPV status-dependent sensitivity. A NUDE mouse-based xenograft assay was also employed to test if roscovitine had effects on tumor growth rate *in vivo*. A clonogenic survival assay (Figure 1A) demonstrated that three HPV(+) HNSCC cell lines (UMSCC47, SCC090 and SCC104) displayed a significantly increased sensitivity to roscovitine, as compared to four HPV(-) head and neck cell lines (SCC61, SCC35, FaDu, UNC7). We used flow cytometry to investigate whether the elevated sensitivity of HPV(+) cells was due to roscovitine-induced cytotoxicity, and demonstrated that roscovitine triggered a much greater degree of cell death in HPV(+) HNSCC cells, when compared to HPV(-) HNSCC cells (Figure 6). These results suggested that roscovitine toxicity was dependent on HPV status, and strengthened the potential of roscovitine as a selective agent against HPV(+) HNSCC. Importantly, roscovitine was able to exert its

selective cytotoxic effects on HPV(+) HNSCC cell lines and in a xenografted mouse model (Figure 7) at relatively low concentrations, supporting its therapeutic potential in this subset of cancers, as doses could be kept low enough to minimize off-target side effects in the patient.

Roscovitine was found to upregulate the phosphorylation of H2AX in HPV(+) but not in HPV(-) cells (Figures 1B, 1C, 1D, 3 and 4). This result corroborated previous studies that suggested that roscovitine treatment upregulated γH2AX in HPV(+) cancer cells [168]. Since phosphorylated H2AX is a marker of DNA damage, our findings suggested that roscovitine induces DNA damage in HPV(+), but not HPV(-) cancer cells, which was verified with the Comet assay (Figure 5), providing one possible mechanistic explanation for HPV(+) HNSCC sensitivity.

Interestingly, depletion of p53 with p53 shRNA resulted in significant improvement of HPV(+) cell survival after treatment with roscovitine (Figure 2A). In addition, roscovitine upregulated p53 in both HPV(+) and HPV(-) cells (Figure 1B). Moreover, the elevated level of p53 after roscovitine treatment was transcriptionally active in HPV(+) cells (Figure 2B and 2C). Cancer cells are usually very sensitive to reactivation of wild-type p53 and respond to ectopic p53 by apoptosis or growth arrest. Importantly, restoration of p53 function in established tumors results in tumor regression [63-66]. Restoring p53 expression has been suggested as an effective strategy to combat HPV(+) cancer. Indeed, several studies have shown that p53 stabilization in HPV(+) cervical carcinoma by silencing E6 or E6AP activates the tumor suppressor function of p53 and kills cancer cells. The combination of leptomycin B and actinomycin D reduced expression of *E6* mRNA and induced apoptosis via p53 upregulation [67]. A chemical

library screen identified two small molecules that suppress the growth of cervical carcinoma cells by inhibiting E6 [68]. In addition, a synthetic peptide that binds E6 and inhibits its activity has been identified [169]. The small molecule RITA protected p53 from degradation and killed cervical cancer cells [69, 70].

We found that roscovitine-induced p53 upregulation was not due to inhibition of HPV E6 (Figure 2D). We therefore suggested a model in which roscovitine selectively induces DNA damage in HPV(+) head and neck cancer cells only, which in turn stabilizes and activates p53, and finally induces substantial HPV(+) cell death (Figure 9). Our model may not completely cover all the effects of roscovitine on p53, however, since we observed induction of p53 after the treatment in the absence of DNA damage in HPV(-) SCC61 cells carrying mutant p53 (Figure 1B). However, HPV(-) UNC7 cells that harbor wild type p53 were resistant to roscovitine treatment, as compared to HPV(+) cells (Figure 1A), suggesting that in the absence of DNA damage, elevated p53 is either transient, or not transcriptionally active, and therefore does not stimulate HPV(-) cell death machinery. In addition, our model most likely illustrates one of several pathways that leads to selective toxicity of roscovitine in HPV(+) head and neck cancer cells. We recently found that knockdown of an important player in DNA damage response, SMG-1, in cancer cells leads to increased sensitivity to roscovitine [170]. Furthermore, expression of SMG-1 was diminished in HPV(+) HNSCCs due to SMG-1 promoter hypermethylation that may contribute to the sensitivity of HPV(+) head and neck cancer cells to roscovitine [171].
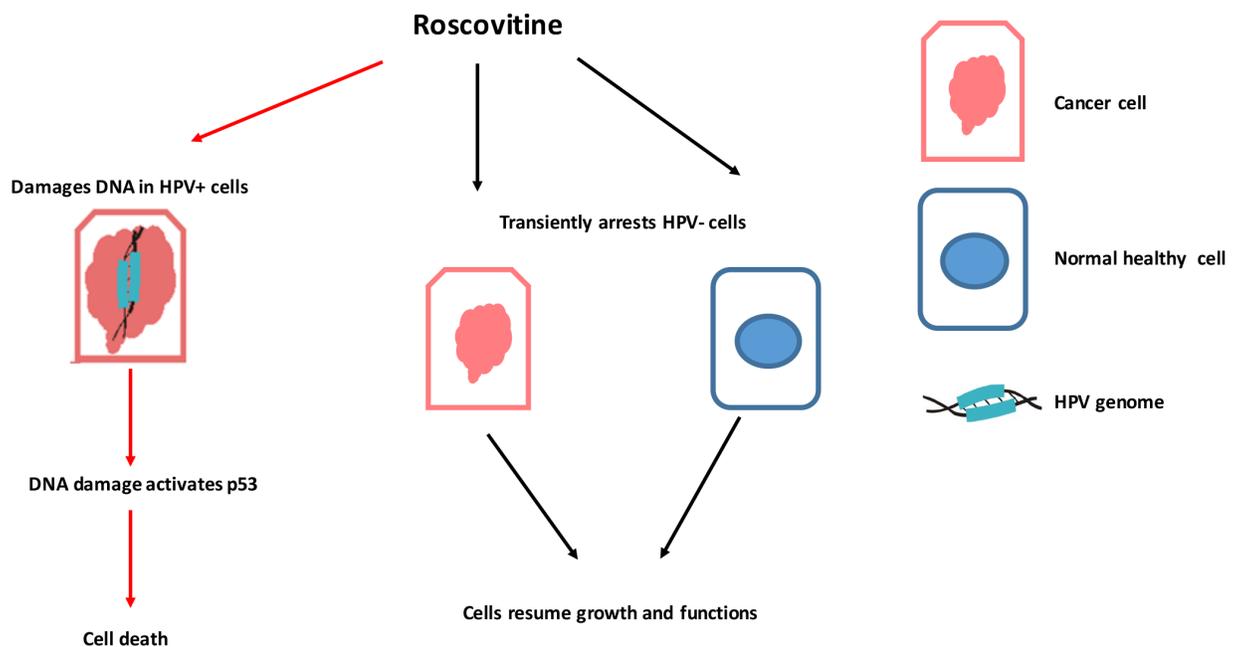
Figure 9: Proposed model of roscovitine dependent toxicity in HPV-associated HNSCC.

The exact mechanism and type of DNA damage induction by roscovitine in HPV(+) cells remains unclear. It is apparent that the phosphorylation of H2AX proceeds via an ATM-independent pathway (Figure 1B), corroborating our finding that roscovitine did not induce DNA DSBs in HPV(+) cells, as indicated by the lack of p53BP1 foci formation (Figure 3). Instead, we found a significant increase in the number of RPA-positive HPV(+) cells after roscovitine treatment (Figure 4), suggesting an elevated amount of single stranded DNA. Moreover, the partial co-localization of RPA and γH2AX foci suggest the persistence of single stranded cellular DNA after roscovitine treatment. The moderate decrease in the number of cells in S phase of the cell cycle, accompanied by the reduction of G1 and massive induction of cell death 24 hours after

roscovitine treatment (Figure 6) suggested that roscovitine causes stalling of replication

forks associated with the formation of unresolved SSD regions marked with

phosphorylated H2AX. However, the exact mechanism deserves further detailed

investigation. The strong HPV dependent activity of roscovitine cannot be attributed to

the inhibition of CDK1/2, since the sensitivity of head and neck cancer cells to the

selective CDK1/2 inhibitor was not dependent on HPV status (Figure 8). Three HPV(+)

head and neck cancer cell lines showed similar sensitivity to roscovitine with IC50

concentrations between 2 and 3.5 μM (Figure 1A). The remarkable response pattern to

the broad CDK inhibitor avopiridol, with one HPV(+) cell line being the most resistant

(IC50~45 nM) and another one demonstrating significantly increased sensitivity

(IC50~10 nM), together with a comparable response to avopiridol in two HPV(-) cell

lines (IC50~22 nM) (Figure 8), suggests that selective roscovitine toxicity toward

HPV(+) head and neck cancer cells may not be due to inhibition of CDKs, but most

likely represent roscovitine-specific effect.

In conclusion, our study revealed selective HPV-dependent toxicity of roscovitine

to head and neck cancer cells and proposed its underlined molecular mechanism. The

profound HPV(+) head and neck tumor growth delaying effects of roscovitine *in vivo*

further emphasize the potential of roscovitine as an anti-HPV(+) HNSCC agent.

Specific Aim 2 – Methods:

Cell lines, transfection and retroviral infection

The human cell lines U2OS, Saos-2 and Phoenix were obtained from Yue Xiong

in 1998 (University of North Carolina). The UNC10 cell line was created by David

Witsell in 1997 (University of North Carolina). Cells were cultured in complete growth media recommended by the American Type Culture Collection (ATCC) at 37 °C in 5% CO_2.

Non-targeting siRNA (Origene, Rockville, MD, USA), siHDM2-1 and siHDM2-2 (Origene), siLZAP-1 and siLZAP-2 (Dharmacon, Lafayette, CO, USA), and siWip1 (Dharmacon) were transfected using Lipofectamine RNAiMAX (ThermoFisher, Waltham, MA, USA) per manufacturer's instructions. For shRNA-mediated knockdown of LZAP in HCT116 cells, the LZAP-1 siRNA sequence was inserted into the pRetro-Super retroviral vector. Control and shLZAP constructs were transfected into Phoenix cells, and supernatant containing viral particles was harvested. Stable cell lines were generated by infecting with retrovirus and selecting with puromycin (InvivoGen, San Diego, CA, USA) followed by clonal expansion. Stable LZAP CRISPR clonal cell lines were created by transfection with CRISPR constructs (Santa Cruz Biotechnology, Dallas, TX, USA) targeting LZAP and selection with puromycin.

Transfections of plasmid DNA were performed using Fugene 6 (U2OS) (Promega, Madison, WI, USA) or Lipofectamine 2000 (Phoenix; Thermo- Fisher) per manufacturer's protocol. The total amount of DNA (and siRNA) transfected was kept equal by adding appropriate amounts of empty vector (pcDNA3.1) or non-targeting siRNA. pcDNA3-Flag-LZAP and pcDNA3-Myc3-LZAP were cloned as previously described. GFP-p53- expressing vectors, as well as psuper and psuper p53 plasmids, were a gift from G Selivanova (Sweden).

Antibodies and reagents

Primary antibodies for immunoblotting include Flag (Sigma, St Louis, MO, USA; M2), phospho-p53 (Ser15) (Cell Signaling, #9284; Danvers, MA, USA), GAPDH (FL-335), β-actin (N-21), GFP (B-2), Myc (9E10), Wip1 (F-10), MDM2 (SMP14 and 2A10) and p53 (DO1 and FL393) (Santa Cruz Biotechnology). LZAP custom antiserum was previously described. Other reagents include normal IgG (Promega), HRP-conjugated secondary antibodies (Promega), goat anti-mouse IgG (H+L) secondary antibody (Dylight 550 conjugate) and goat anti-rabbit IgG (H+L) secondary antibody (DyLight 650 conjugate) (ThermoFisher), zeocin (Invitrogen, Carlsbad, CA, USA), MG132 (Sigma), carboplatin (Sigma), doxorubicin (Selleckchem, Houston, TX, USA), paclitaxel (Sigma), and nutlin (Santa Cruz Biotechnology).

Recombinant proteins

Recombinant human GST-HDM2 and recombinant human p53 proteins were obtained from R&D systems (Minneapolis, MN, USA). GST was from Abcam (Cambridge, MA, USA). Recombinant LZAP was purified from Escherichia coli BL21(DE3) as described in Wamsley et al [139].

Immunoprecipitation and immunoblotting

For immunoprecipitation, cells were lysed in RIPA buffer (Sigma) supplemented with Complete Mini EDTA-free Protease Inhibitor cocktail (Roche, Basel, Switzerland) and PhosStop (Roche). About 200 μg of lysates were pre-cleared for 30 min using normal mouse or rabbit IgG (Promega) and 20 μl protein A/G beads (Santa Cruz Biotechnology)

prior to incubation with agarose beads conjugated to antibodies recognizing Flag- or Myc-conjugated beads (Sigma, 15 µl). Immunoblotting was performed as described previously.

About 100 ng of indicated recombinant proteins (Figures 6e and f) were incubated in phosphate-buffered saline supplemented with Complete Mini EDTA-free Protease Inhibitor cocktail (Roche), 0.05% Triton X100, 100 mM of NaCl and 0.05% bovine serum albumin for 1 h at 4°C. About 20 µl of DO1 (p53 antibody) conjugated to agarose (Santa Cruz Biotechnology, sc-126 AC) or 20 µl of LZAP custom antiserum conjugated to Protein A/G agarose (Santa Cruz Biotechnology) were added and incubated overnight at 40°C. The beads were spun down at 3000 r.p.m. for 1 min, washed four times with phosphate-buffered saline supplemented with 0.05% Triton X100 and 100 mM of NaCl, resuspended in 2 × Laemmli sample buffer (Bio-Rad, Hercules, CA, USA) and boiled for 3 min. Immunoblotting was performed as described [172].

Caspase activity

This assay was performed using a Caspase 3 Activity Assay Kit (Cell Signaling, #5723) according to manufacturer instructions.

Cell viability assays

Cell viability assays were performed using Cell Titer Glo (Promega) as previously described [135]. Alternatively, 10,000 cells per well were plated in 24 well plates, treated with indicated drugs the next day, and stained with 0.5% methylene blue in methanol after 6–8 days. Pictures were taken and/or the dye was extracted from stained cells with

3% HCl solution for absorbance quantification.

Creation of LZAP heterozygous mice

LZAP was targeted in murine embryonic stem cells by homologous recombination using a LZAP floxed construct targeting the first two exons of murine LZAP. After selection, clones were screened by PCR and Southern blotting with two independent recombinant clones (2A2 and 2G5) identified. Mice were crossed with B6.FVB-Tg (EIIa-cre) C5379Lmgd/J mice (Jackson Laboratories, Bar Harbor, ME, USA) and then crossed for six generations with C57Bl/6 mice (Jackson Laboratories). The genotype of mice was confirmed by PCR; see Table 2 for oligonucleotide sequences. The studies were approved by the Yale Institutional Animal Care and Use Committee prior to initiation.

Table 2. Oligonucleotide sequences: oligonucleotides for confirming LZAP mice genotype (5′-TGTGCCACCACGCAACTTTT-3′; 5′-CATGAAG ACAGAACCAAAC-3′).

qRT-PCR oligonucleotides	
Gene	Sequence (5′-3′)
H. sapiens CDK5RAP3 F	CAATGCTGCCATCCAGGACATG
H. sapiens CDK5RAP3 R	ATCCGCTGTGAAGAGTATCGGC
H. sapiens TP53 F	CCTCAGCATCTTATCCGAGTGG
H. sapiens TP53 R	TGGATGGTGGTACAGTCAGAGC

H. sapiens CDKN1A F	AAGACCATGTGGACCTGT
H. sapiens CDKN1A R	GGTAGAAATCTGTCATGCTG
H. sapiens PPM1D F	GAAGAAACTGGCGGAATGG
H. sapiens PPM1D R	TTGTGAGTGAGTCGAGGTCGT
H. sapiens BAX F	TGGAGCTGCAGAGGATGATTG
H. sapiens BAX R	GAAGTTGCCGTCAGAAAACATG
H. sapiens APAF1 F	GCTCTCCAAATTGAAAGGTGAAC
H. sapiens APAF1 R	ACTGAAACCCAATGCACTCC
H. sapiens BBC3 F	GCAGGCACCTAATTGGGCT
H. sapiens BBC3 R	ATCATGGGACTCCTGCCCTTA
H. sapiens PIDD F	TCTGACACGGTGGAGATGTTCG
H. sapiens PIDD R	AGGTGCGAGTAGAAGACAAAGCAG

Primary cultures of mouse embryonic fibroblasts

MEFs were isolated from 12 day postcoitus embryos by breeding LZAP +/−
females and males. The embryos were individually trypsinated in 0.05% trypsin
(Invitrogen), plated and cultured in DMEM supplemented with 10% fetal bovine serum
penicillin/streptomycin. Passage 2 or 3 MEFs were used in the assays.
DNA was isolated from each culture using the Qiagen DNA purification kit. The
genotype of the embryos was determined by PCR using primers from Table 2.

Bone marrow mononuclear cell colony-forming assay

C57Bl/6 wild-type and LZAP heterozygous mice were treated with total body irradiation (6 Gy) or left untreated (four mice in each group). Four hours after total body irradiation, bone marrow mononuclear cells were isolated from femurs and tibias of each mouse and plated (4×10^4 cells/ml in 35mm diameter plates) in MethoCult M3231 medium (StemCell Technologies, Vancouver, BC, Canada) supplemented with 10ng/ml recombinant mouse GM-CSF (StemCell Technologies) and IMDM growth medium (Invitrogen). Colony formation (megakaryocyte erythrocyte progenitor (MEP) and granulocyte-monocyte progenitor (GMP)) was scored after 7 days of culture at 37 °C in the presence of 5% CO_2. Data are analyzed using a one-tailed Student's t-test.

Quantitative reverse transcriptase real-time PCR

Total RNA was extracted from the cells using a RNeasy Mini Kit (Qiagen, Hilden, Germany) and reverse transcribed into cDNA using the iScript cDNA Synthesis kit (Bio-Rad). qRT-PCR was performed using iQ SYBR Green Supermix (Bio-Rad) and CFX96 Real-Time System (Bio-Rad); see Table 2 for oligonucleotide sequences used. The expression of the mRNA of interest was normalized to the expression of GPDH.

Immunohistochemistry

Specimens were fixed with 10% formalin and embedded in paraffin per routine of the surgical pathology division. Sectioning and immunostaining were performed by the Yale Tissue Microarray Core using antibodies recognizing LZAP (HPA022141, Sigma) and p53 (DO7, Santa Cruz Biotechnology). Informed consent was obtained from each

subject, and human investigations were performed after approval by the Yale institutional review board.

Results:

LZAP loss decreases p53 expression regardless of p53 mutational status

We previously showed that LZAP activates p53 through both ARF-dependent and ARF-independent mechanisms [133, 134]. To test whether loss of LZAP could inactivate wild-type p53 in human cells, LZAP was depleted in U2OS osteosarcoma cells (Figure 10a, left) by small interfering RNA (siRNA). Downregulation of LZAP remarkably decreased p53 protein in U2OS cells. p53 is an important player for cellular processes such as progression through the cell cycle, differentiation, the immune response, metabolism, DNA repair, and senescence, and is a potent inducer of apoptosis; therefore, p53 protein levels are tightly regulated by multiple mechanisms [173, 174]. Observed downregulation of p53 at the protein level following LZAP depletion could result from decreased TP53 transcription, mRNA stability, mRNA translation and/or protein stability. To begin exploring these possibilities, TP53 mRNA levels were measured by qRT-PCR with and without LZAP depletion in U2OS. LZAP downregulation decreased p53 mRNA levels in U2OS cells (Figure 10a, right).

As loss of LZAP was associated with downregulation of wtp53, we next determined if depletion of LZAP similarly affected mutant p53 protein. Endogenous mutant p53 (R248Q) was downregulated in UNC10 cells at protein (Figure 10b, left) and mRNA (Figure 10b, right) levels after transfection with LZAP, but not control, siRNAs. Thus, depletion of LZAP diminished p53 levels independently of p53 mutation

status. Similar to U2OS cells, depletion of LZAP decreased p53 expression in colon

cancer HCT116 cells (Figure 10c). To confirm the observed association between LZAP

and p53 expression levels regardless of p53 mutation status, p53 null osteosarcoma Saos-

2 cells were co-transfected with control or LZAP siRNAs and wild-type or mutant

(R175H) p53. As found with endogenous wild-type (Figures 10a and c) and mutant

(Figure 10b) p53, depletion of LZAP downregulated exogenous expression of p53

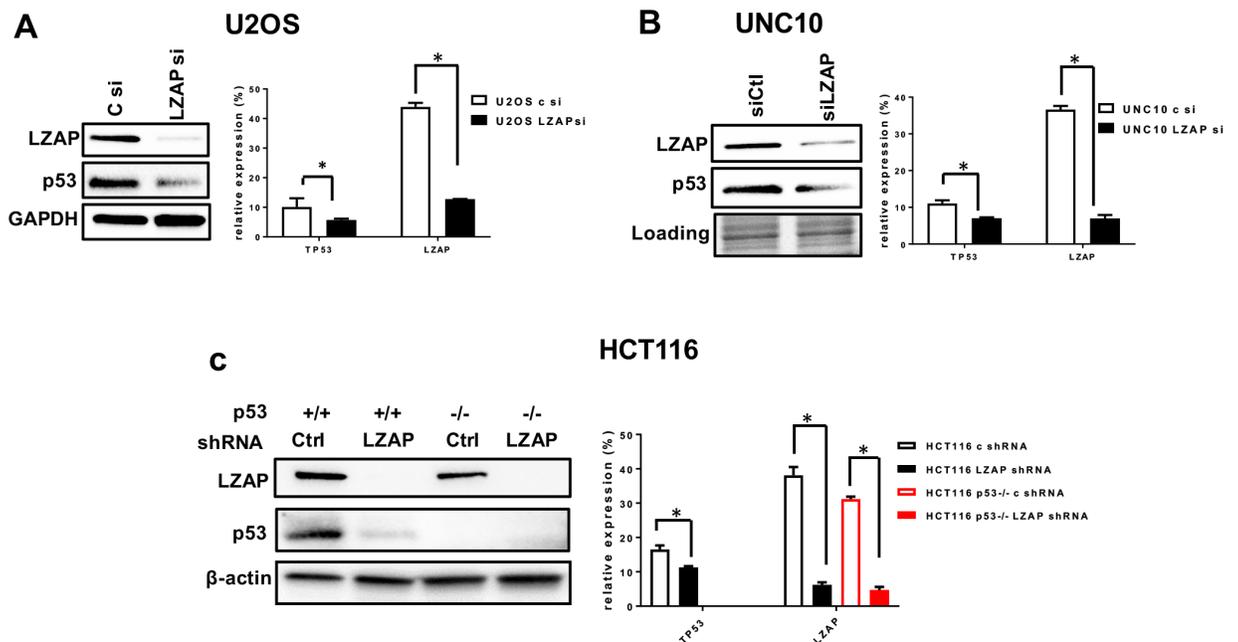independently of mutation status (Figure 11a).

Figure 10. LZAP depletion results in downregulation of endogenous wild-type and mutant p53. LZAP and p53 protein (left) or relative to GPDH mRNA levels (right) in U2OS (a), UNC10 (b) orHCT p53 wild-type and isogenic p53 null cells (c) were transfected with control or siRNAs specific to LZAP. mRNA levels were determined using qRT-PCR; the mean from two experiments is shown, error bars represent the standard deviation.; P-values were calculated using unpaired t-test.

LZAP loss inhibits p53 induction and transactivation in response to DNA damage

Following DNA damage, p53 accumulates and transactivates pro- apoptotic and proliferation inhibitory genes. In addition, p53 induces apoptosis through transcriptional repression of another set of genes, and through direct activation of the mitochondrial apoptotic pathway [173, 175-178]. As the transcriptional transactivation function of p53 is very important for the induction of apoptosis, we investigated whether downregulation of p53 following LZAP depletion inhibited p53 transactivation. LZAP was depleted by shRNA in HCT116 cells and the cells were treated with zeocin to induce DNA damage. shRNA- induced downregulation of LZAP significantly inhibited upregulation of p53 at several time points after zeocin treatment (Figure 11b, left). Importantly, induction of the p53 pro-apoptotic target genes BAX, PUMA, PIDD and APAF1 was attenuated in cells expressing LZAP shRNA, as compared to control shRNA cells (Figure 11b, right).

The stress responsive kinases ATM and ATR are rapidly activated after DNA damage and phosphorylate the p53 protein at different sites, including Ser15, leading to the disruption of the interaction between p53 and HDM2 with resultant p53 stabilization and activation [179]. To determine whether depletion of LZAP reduced p-p53 (Ser15) levels, CRISPR constructs targeting LZAP were stably transfected into U2OS cells prior to zeocin treatment. Following DNA damage, LZAP loss decreased both p-p53 (Ser15) and total p53 levels (Supplementary Figure S2). Taken together, these data suggest that depletion of LZAP results in decreased levels of total p53 protein, as well as p53 transcriptional transactivation following DNA damage.
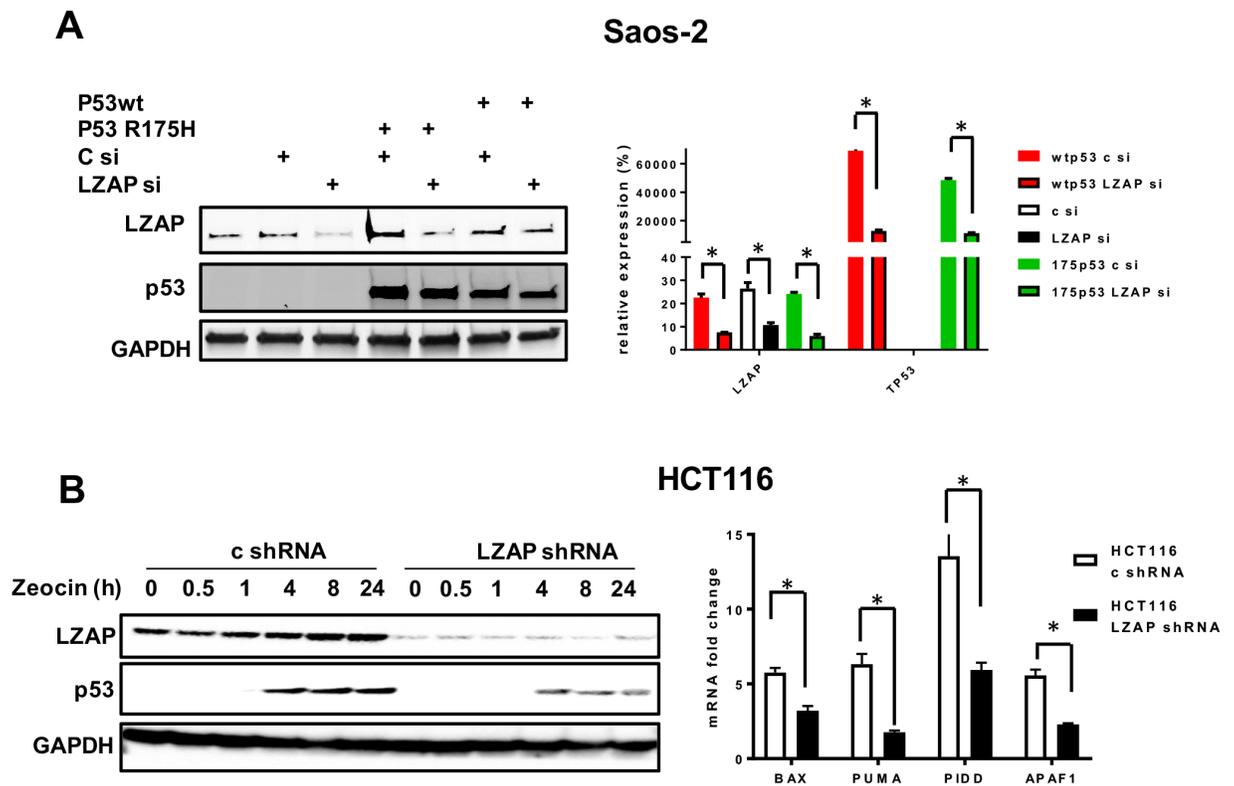
Figure 11. LZAP depletion downregulates exogenously expressed p53 and attenuates p53 induction and transactivation in response to DNA damage. (a) LZAP and ectopically expressed wild-type or mutant p53 R175H protein levels (left) or mRNA relative to GPDH (right) in p53 null Saos-2 cells expressing control or LZAP siRNA. mRNA levels were determined using qRT-PCR; the mean from two experiments is shown, error bars represent the standard deviation.; P-values were calculated using an unpaired t-test. (b) Immunoblot detecting LZAP and p53 in HCT116 cells infected with retrovirus containing control or shRNA specific to LZAP prior to treatment with zeocin (200 μg/ml) for the indicated times (left); right: expression of p53 pro-apoptotic transcriptional targets in HCT116 cells stably transfected with control LZAP shRNA before zeocin treatment (200 μg/ml) for 24 h, as measured by qRT-PCR and relative to GPDH. The mean from three experiments is shown and error bars represent the standard deviation.

Downregulation of LZAP confers resistance to DNA damage in wild-type p53-expressing

cells, but renders mutant p53 cells more sensitive to a treatment

P53 is a primary regulator of the cellular response to standard anticancer therapies [173, 174, 180]. Therefore, transient suppression of wtp53 has been proposed as a protective strategy to spare normal cells consequences of treatment [112, 113, 117, 181, 182]. On the other hand, mutations in the p53 gene frequently have gain-of-function activity associated with increased resistance to DNA damage. Inhibition of gain-of-function p53 mutants is an attractive target for anticancer therapy, particularly in combination with radiation and chemotherapy [123, 125, 127]. As LZAP depletion downregulated both wild-type and mutant p53 (Figures 10 and 11), we suspected that LZAP depletion may protect cells with wtp53 and sensitize cells with mtp53 to DNA damage.

To explore if LZAP depletion and the resultant decrease in wtp53 levels and activity protects cells from DNA damage, U2OS LZAP CRISPR and parental cells were treated with increasing doses of carboplatin (DNA/DNA and DNA/protein crosslinker), doxorubicin (DNA-intercalating agent), paclitaxel (microtubule stabilizer and anti-mitotic agent) and radiomimetic zeocin. Indeed, loss of LZAP protected U2OS cells from these DNA- damaging agents (Figure 12a). Similar results were observed following zeocin treatment in HCT116 LZAP shRNA cells (Figure 12b). Remarkably, in contrast to wild-type p53-harboring cells, LZAP loss in mutant p53-expressing UNC10 cells increased sensitivity to zeocin (Figure 12c).
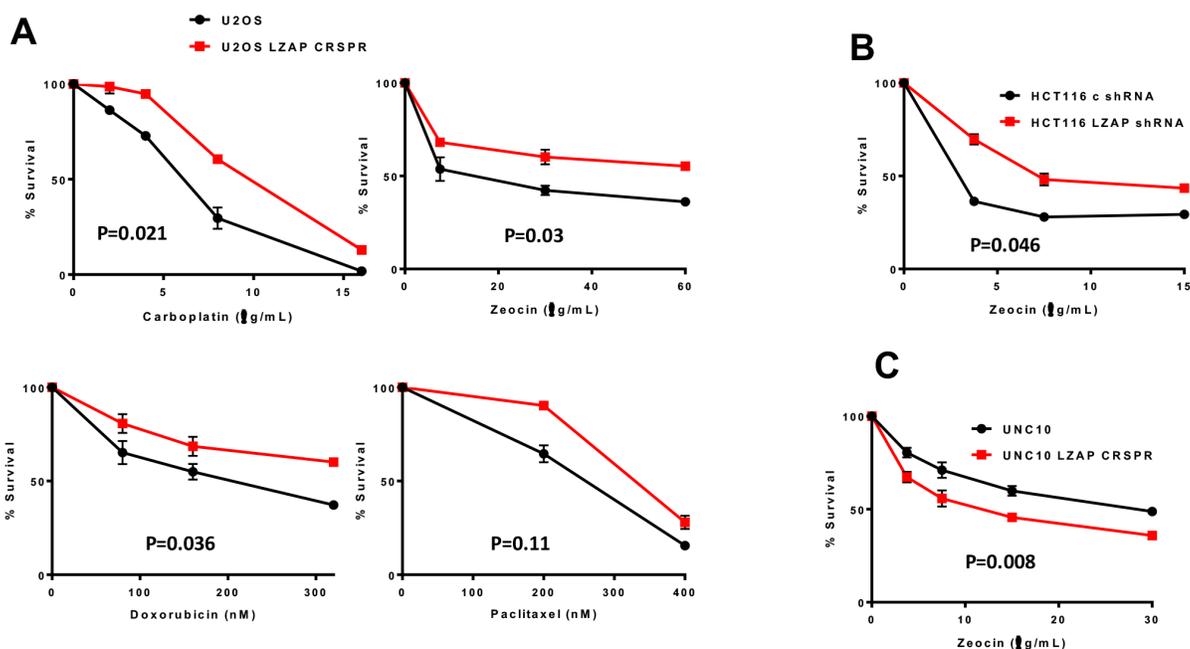
Figure 12. LZAP depletion protects wild-type p53-expressing cells from DNA damage, while sensitizing mutant p53 cells to the treatment. (a) U2OS parental and LZAP CRISPR cells were plated (1000 cells per well of 96 well plates) prior to treatment with the indicated DNA-damaging agents. Six days later, viability was measured using Cell Titer Glo (Promega). (b) HCT116 stable LZAP shRNA control or knockdown cells , and UNC10 parental, or LZAP CRISPR cells (c) were treated with zeocin for 6 days prior to viability analysis. Mean is shown and the error bars represent the standard deviation., N = 3; P-values were calculated using a paired t-test.

Cancer cells expressing wtp53 were protected from DNA damage-induced cell death; however, the potential clinical relevance relies on determining the effect of LZAP loss on normal, non-cancer cells. Because of early embryonic lethality observed in zebrafish (before epiboly) and in mice (3.5 days, data not shown), mice with homozygous loss of LZAP were not available for the study [135]. However, mouse embryonic fibroblasts (MEFs) derived from LZAP+/ – mice (Supplementary Figure S3) expressed

lower LZAP protein levels as compared to LZAP+/+ MEFs (Figure 13a). Zeocin

treatment activated caspases in wild-type, but not in LZAP+/− MEFs (Figure 13b).

Importantly, LZAP+/+ MEFs were significantly more sensitive than LZAP+/ − MEFs to

DNA damage induced by zeocin or carboplatin treatment (Figures 13c and d).

Bone marrow mononuclear cells are exquisitely sensitive to radiation through

mechanisms largely attributed to p53-associated apoptosis. Bone marrow sensitivity is

the major cause of organismal demise following whole-body irradiation and is the major

dose limiting factor for many chemotherapy regimens; however, p53 inhibition abrogates

this acute radiation syndrome [118, 183]. To begin exploring the effect of LZAP loss on

bone marrow cell survival after radiation, wild-type (LZAP+/+) and LZAP heterozygous

(LZAP+/−) mice were irradiated with sublethal doses of total body irradiation, and

clonogenic growth of isolated bone marrow mononuclear cells was determined. Total

body irradiation decreased colony-forming capacity in cells derived from both wild-type

and LZAP+/− mice; however, bone marrow progenitor cells derived from LZAP+/− mice

were significantly protected compared to cells derived from wild-type mice (Figure 13e).

These data suggest that lower LZAP expression driven by a loss of a single

Cdk5rap3/Lzap allele in LZAP heterozygous mice is sufficient to render embryonic

fibroblasts or bone marrow mononuclear cells resistant to DNA damage. Taken together,

our results demonstrate that LZAP down-regulation protects cells carrying wtp53 from

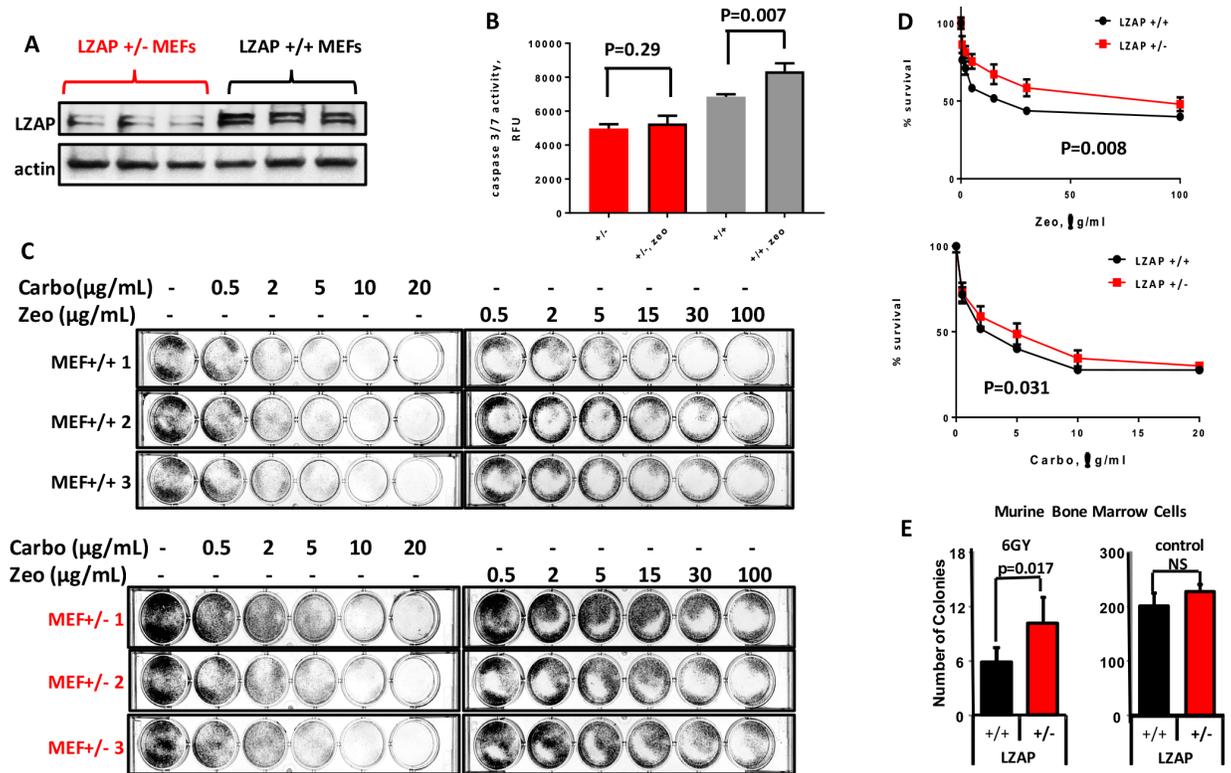DNA-damaging agents, while sensitizing those with mtp53.

Figure 13. Loss of a single Cdk5rap3/Lzap allele in LZAP heterozygous mice results in increased resistance of cells to DNA damage. (a) LZAP protein levels in LZAP+/− and LZAP+/+ MEFs (genotyping of MEFs is shown in Supplementary Figure S2). (b) Caspase 3/7 activity (cleavage of the fluorescent substrate) in LZAP+/− and LZAP+/+ MEFs treated with zeocin for 6 h; the experiment was performed twice using three LZAP+/− and three independent LZAP+/+ MEF cultures; P-values are calculated with an unpaired t-test. (c) LZAP+/− and LZAP+/+ MEFs were treated with increasing concentrations of carboplatin or zeocin, and alive cells were visualized by methylene blue staining 7 days after treatment. (d) Viability of MEFs from (c) was determined by methylene blue extraction, followed by quantification of absorbance. Percent survival is shown relative to control cells; error bars show the standard error of the mean; assays were performed in duplicate, P-values were calculated with a paired t-test. (e) Survival of bone marrow progenitor cells derived from untreated wild-type or LZAP+/− mice or littermates treated with 6 Gy total body irradiation was determined after 7 days of *in vitro* growth. Data are presented as mean ± the standard deviation (n = 2 mice per group).

Depletion of LZAP alters cellular response to DNA damage in a p53-dependent manner

The role of LZAP in DNA damage response is well documented. It was reported

that LZAP modulates the G2/M checkpoint and enhances DNA damage-induced cell death [136]. In addition, activation of checkpoint kinases Chk1 and Chk2 was partially inhibited by LZAP overexpression [137]. To confirm that LZAP depletion regulated cell survival after DNA damage in a p53-dependent manner, p53 was depleted with shRNA in U2OS LZAP CRISPRand parental cells (Figure 14a). U2OS LZAP CRISPR cells transiently transfected with control shRNA survived significantly better than U2OS cells expressing control shRNA after zeocin treatment (Figure 14b). Depletion of p53 increased resistance of U2OS, but not U2OS LZAP CRISPR cells to zeocin (Figure 14b). In fact, survival curves after zeocin treatment of cells with shRNA-mediated depletion of p53 was similar to survival of LZAP CRISPR cells suggesting that survival of cells with LZAP depletion was primarily due to LZAP-mediated p53 downregulation (Figure 14b).

To further investigate the p53-dependent effect of LZAP loss after DNA damage, p53 null Saos-2 cells were transiently transfected with control or LZAP siRNAs together with wild-type or mutant p53 R175H (Figure 11a). Elevated expression of the p53 target gene CDKN1A (p21) in cells expressing wild-type p53 and control siRNA was diminished in cells expressing LZAP siRNA (Figure 14c). In contrast, depletion of LZAP did not change p21 expression in cells expressing mutant or no p53 (Figure 14c). Notably, Saos-2 cells expressing mutant p53 and LZAP siRNA were more sensitive to zeocin treatment than Saos-2 cells transfected with mutant p53 and control siRNA (Figure 14d). Opposite to mutant p53-expressing cells, Saos-2-wild-type p53-LZAP siRNA cells were more resistant to zeocin, as compared to Saos-2-wtp53-control siRNA cells (Figure 14d). Interestingly, we found a moderate, but significant, sensitization to zeocin in p53 null Saos-2 cells expressing LZAP siRNA compared to Saos-2 cells

expressing control siRNA (Figure 14e).

These data show that LZAP depletion sensitized cells to DNA damage in the
absence of p53 (Figure 14e) or in the presence of mutant p53 (Figure 14d, green),
whereas LZAP depletion in cells expressing wtp53 was protective (Figure 14d, red).
Together, our experiments confirmed that the effect of LZAP depletion on cell survival
after DNA damage depends on p53 status, with cells expressing wild-type p53 being
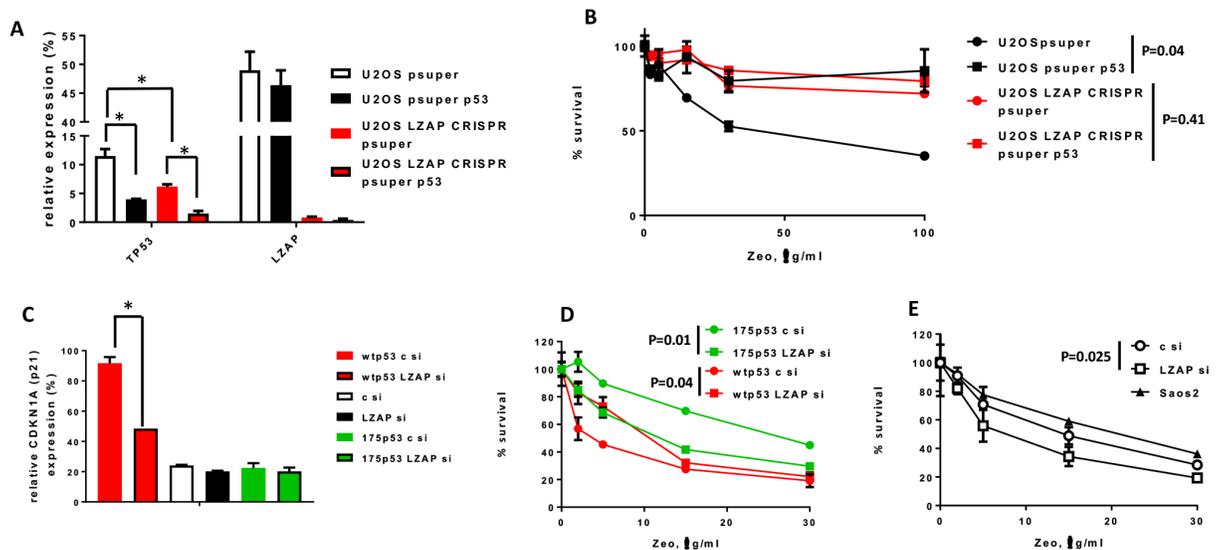protected and cells with mutant p53 or without p53 being sensitized.

**Figure 14. LZAP depletion affects survival after DNA damage in a p53-dependent
manner.** (a) mRNA levels, relative to GPDH, of LZAP or TP53 in U2OS or U2OS
LZAP CRISPR cells transiently transfected with psuper vectors expressing control or p53
shRNAs as determined by qRT-PCR. (b) Survival after increasing concentrations of
zeocin of cells from (a) as determined by methylene blue staining, extraction and
absorbance measurement, 7 days after the treatment. (c) CDKN1A expression, relative to
GPDH, in p53 null Saos-2 cells transfected with control or LZAP siRNAs and wild-type
or mutant p53, as determined by qRT-PCR; mean from two experiments is shown, error
bar represents the standard deviation; P-values were calculated using an unpaired t-test.
Survival after increasing doses of zeocin of Saos-2 cells-co-transfected with control or
LZAP siRNA and wild-type or mutant p53 (d), or Saos-2 cells transfected or not with
control or LZAP siRNAs (e), P-values were calculated using a paired t-test.

LZAP binds p53 and HDM2

The p53 protein stability is regulated by phosphorylation and degradation by the 26S proteasome followed by its polyubiquitination; therefore, we examined the ability of proteasomal inhibition to restore p53 levels that accompany LZAP depletion. U2OS cells were transfected with either control or LZAP-specific siRNAs prior to 4h treatment with MG132 or vehicle. Lysates harvested from cells treated with DMSO showed a significant decrease in p53 protein levels following LZAP depletion (Figure 15a); however, this reduction was completely reversed by inhibition of the 26S proteasome (Figure 15a). Moreover, blocking protein synthesis with cycloheximide treatment following knockdown of LZAP in U2OS cells revealed a moderate decrease in the half-life of p53 (Supplementary Figures S4A and B). These findings suggest that in addition to regulation of p53 mRNA levels (Figures 10 and 11), LZAP regulates p53 at the level of protein stability.

p53 is almost always inactivated in human cancers, either by mutation, indirectly through binding to viral proteins, or as a result of alterations in genes, whose products either activate, stabilize, or carry signals from p53 including ARF, Wip1 and HDM2 [173, 174, 180, 184]. As neither U2OS nor HCT116 cells express ARF due to promoter methylation (Supplementary Figure S5), LZAP regulation of p53 does not require ARF, as we previously reported [185, 186]. Recently, our laboratory found that LZAP binds the phosphatase Wip1, a negative regulator of p53. Wip1 dephosphorylates p53 at Ser15, resulting in its destabilization and inactivation [139, 187]. To determine if Wip1 was required for the regulation of p53 levels observed upon loss of LZAP, U2OS CRISPR cells were transfected with control siRNA or siRNA targeting Wip1. LZAP loss resulted

in downregulation of p53 levels in the presence or absence of Wip1 (Figure 15b), suggesting that the effect of LZAP loss on p53 levels is independent of Wip1.

HDM2 is the most prominent negative regulator of p53, as indicated by its amplification and overexpression in human cancers and by p53-mediated embryonic lethality observed upon HDM2 deletion (refs, donehower??). HDM2 binds p53, inhibits its transactivation activity and directly ubiquitinates p53, ultimately leading to its proteasomal degradation [188, 189]. Importantly, we recently reported that LZAP directly binds HDM2 [139]. To determine if HDM2 was essential for downregulation of the p53 protein observed following LZAP loss, LZAP was depleted by siRNA transfection in U2OS cells in the presence or absence of two different siRNAs targeting HDM2. As expected, HDM2 depletion increased p53 levels when compared to transfection with non-targeting siRNA (Figure 15c). Surprisingly, we also noted that HDM2 siRNA-mediated loss upregulated LZAP levels, suggesting that HDM2 may work as E3 ubiquitin ligase to destabilize LZAP. Expectedly, depletion of LZAP decreased p53 levels in control siRNA-expressing cells, but this effect was abrogated by HDM2 knockdown (Figure 15c).

Because LZAP regulated p53 protein stability (Figure 15a; Supplementary Figure S3), we hypothesized that LZAP may directly bind p53 to promote its stabilization. To explore possible interactions, we ectopically expressed Flag-LZAP in the presence or absence of GFP-wtp53 and immunoprecipitated LZAP with anti-Flag affinity agarose gel. Overexpression of LZAP increased GFP-p53 levels (Figure 15d, inputs), as we previously noted, and GFP-p53 proteins were readily detectable in LZAP immunoprecipitates (Figure 15d). These data show that exogenously expressed LZAP

and p53 interact in mammalian cells, providing a potential mechanism for LZAP's regulation of p53 protein stability.

To confirm and further examine LZAP binding to HDM2 and p53, complex formation was investigated using recombinant proteins in a cell-free system. LZAP was found to bind p53 alone, GST-HDM2 alone, as well both p53 and GST-HDM2, but not GST protein (Figure 15e, immunoprecipitation with LZAP antibody). Likewise, p53 was found in the complex with LZAP, GST-HDM2 or both LZAP and GST-HDM2, but not GST (Figure 15e, immunopre- cipitation with p53 antibody). These results suggested that LZAP and p53 independently bind different parts of HDM2. Confirming this hypothesis, addition of the specific HDM2 inhibitor nutlin disrupted the interaction between p53 and GST-HDM2 but did not influence the binding of LZAP to p53 in the same reaction (Figure 15f) [190]. Moreover, nutlin did not alter the interaction between LZAP and HDM2 (Figure 15f). These data allowed us to suggest that LZAP binds to different parts of HDM2 than p53 and that all three proteins may exist in one complex. Taken together, our results propose that LZAP binds both HDM2 and p53, and regulates p53 levels in a HDM2-dependent manner.
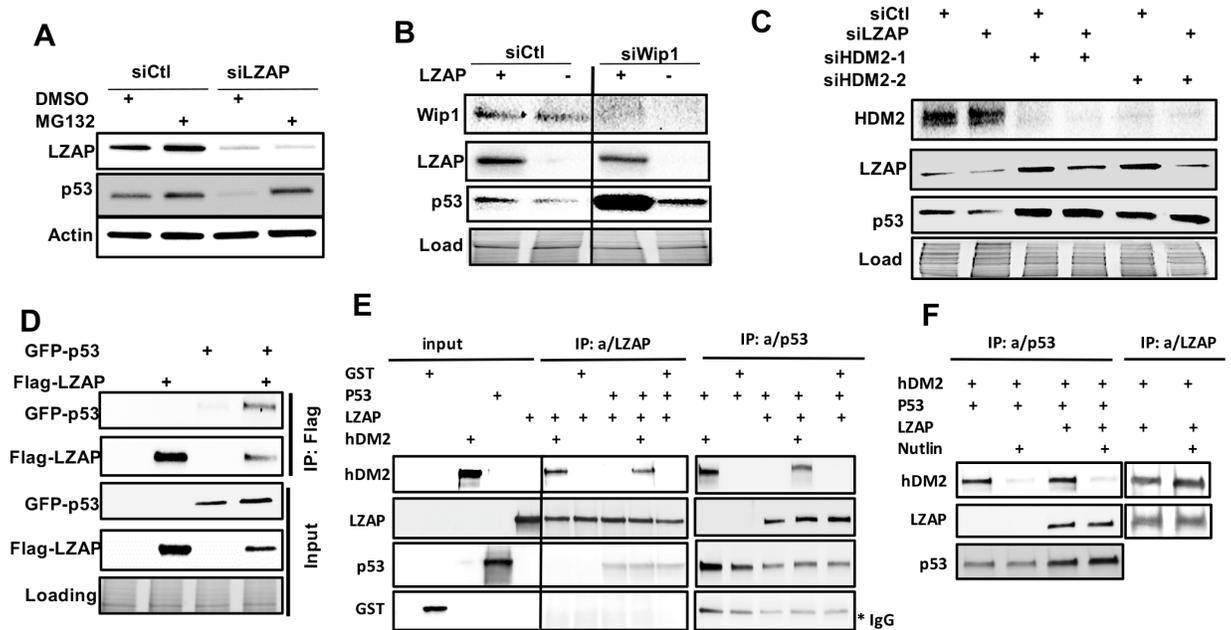
Figure 15. LZAP binds both p53 and HDM2. (a) Immunoblots of LZAP and p53 of U2OS lysates following transfection with control or LZAP siRNA, and treatment with vehicle or MG132 for 4 h. (b) Immunoblots of LZAP, p53 and Wip1 of U2OS (parental and LZAP CRISPR) lysates following transfection with control or Wip1 siRNA. All lanes were run on the same gel; solid line indicates where the images were cropped. (c) Immunoblots of LZAP, p53 and MDM2 of U2OS lysates following transfection with combinations of non-targeting siRNA, LZAP siRNA or one of two siRNAs targeting MDM2. (d) U2OS cells were transfected with indicated plasmids encoding tagged Flag-LZAP or GFP-p53. Immunoprecipitates were prepared using Flag affinity matrix to pulldown LZAP, resolved by SDS–polyacrylamide gel electrophoresis, and immunoblotted with antibodies recognizing Flag(-LZAP) or GFP(-p53). Expression of LZAP and p53 was confirmed by immunoblotting whole- cell lysates with Flag or GFP antibodies, respectively. (e) Purified LZAP was incubated with p53 alone or together with GST-HDM2 or GST proteins followed by pulldown with agarose beads conjugated with LZAP or p53 antibodies and detected with HDM2, LZAP, p53 or GST antibodies (see materials and methods section). (f) Purified p53 was incubated with GST-HDM2 alone or together with LZAP in the presence or absence of nutlin. Similarly, LZAP was incubated with GST-HDM2 in the presence or absence of nutlin, followed by pulldown with agarose beads conjugated with LZAP or p53 antibodies and detection with HDM2, LZAP or p53 antibodies.

Loss of LZAP represents a new mechanism of p53 inactivation in cancer

The p53 protein does not function properly in human cancers, being inactivated directly by mutations in the TP53 gene or indirectly by viral proteins. Alternatively, p53 function can be inhibited by alterations in genes, whose products regulate p53 itself or signaling to or from p53 [173, 174, 180]. Altered genes in human cancer that impact p53 function include, but are not limited to: amplification and overexpression of the major negative p53 regulator, HDM2; loss of expression of p14ARF, a negative regulator of HDM2; overexpression of ΔNp73 (NH2-terminally truncated, transactivation-deficient, dominant-negative isoform of p53 homolog p73), which blocks p53 activities, mutations in tumor suppressor PTEN, and disruption of Chk1/2 signaling [185, 188, 189, 191-196]. Our data suggest that depletion of LZAP downregulated steady-state p53 levels and inhibited radiation-induced stabilization and activation of wild-type p53 (Figures 10 and 11). We previously reported that LZAP protein expression is decreased in ~30% of head and neck squamous cell carcinomas. These findings led us to hypothesize that loss of LZAP may represent a novel mechanism of p53 inactivation in human cancer.

To provide support for this hypothesis, human NSCLC specimens (n = 178; Table 3) were examined to determine if decreased expression of LZAP correlated with decreased levels of p53 protein. A tissue microarray consisting of NSCLC tumors was stained with antibodies recognizing LZAP and p53. Slides were scored as 'low' for LZAP and p53 if fewer than 20% of tumor cells stained positively; others were designated as 'high' (Figure 16a). Remarkably, LZAP and p53 levels positively correlated with one another (Figure 16b). Only 18% of tumors within 'high LZAP' group expressed low p53 levels, whereas 44% of 'low LZAP' tumors had low p53 staining

intensity (Figures 15b, P=0.0002 as analyzed by two-tailed Fisher's test). Together, these data show that LZAP levels correlate with p53 levels in NSCLC, suggesting that LZAP may regulate p53 not only in experimental cell culture conditions, but also *in vivo* in human cancers.
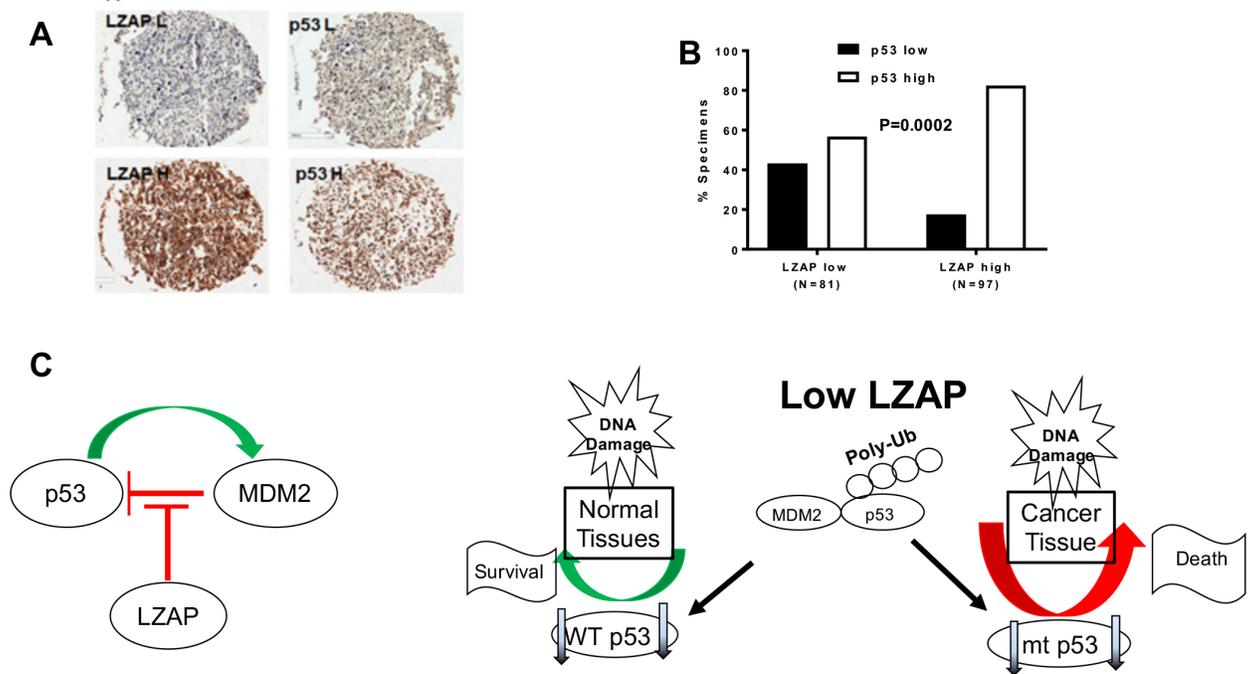

Figure 16. LZAP and p53 protein levels correlate in non-small-cell lung cancer. (a) Left: representative photomicrographs of LZAP and p53 IHC NSCLCs. (b) Quantification of IHC (primary NSCLC, n = 178); high LZAP/p53 group, strong staining in 420% of tumor cells, others designated as low. The proportions of low and high p53 staining were divided based on low and high LZAP staining. P=0.0002 analyzed by 2×2 contingency table (Fisher's two-tailed test). (c) Left: schematic for LZAP regulation of p53 through HDM2. When LZAP is present, it stabilizes p53. Right: LZAP loss downregulates p53 in a MDM2-dependent manner. Following DNA damage, LZAP depletion decreases wild-type p53 in normal tissues resulting in protection, while sensitizing tumor cells harboring mutant p53 to the treatment. IHC, immunohistochemistry.

Table 3 – Patient characteristics

Characteristics	Total	LZAP low	LZAP high	P-value
Number of cases	178	81 (45.5%)	97 (55.5%)	
Gender				.88
Male	86 (48.3%)	38 (46.9%)	48 (49.5%)	
Female	91 (51.1%)	42 (51.9%)	49 (50.5%)	
NA	1 (.6%)	0	1 (1.2%)	
Age at Dx				.34
Median	63	66	64	
Range	35-89	42-89	35-83	
Mean	62.4	65.8	64.3	
Staging				1.00
T				
T1-T2	53 (29.8%)	26 (32.1%)	27 (27.8%)	
T3-T4	19 (10.7%)	10 (12.3%)	9 (9.3%)	
NA	106 (59.6%)	45 (55.6%)	61 (62.9%)	
N				.25
N0	34 (19.1%)	18 (22.2%)	16 (16.5%)	
N1-N3	42 (23.6%)	16 (19.8%)	26 (26.8%)	
NA	102 (57.3%)	47 (58.0%)	55 (56.7%)	
Stage				.10
I-II	124 (69.7%)	62 (76.5%)	62 (63.9%)	
III-IV	53 (29.8%)	19 (23.5%)	34 (35.1%)	

NA	1 (.56%)	0	1 (1.2%)	

Discussion:

Previously, we reported that overexpression of LZAP stabilizes p53 and increases wild-type p53 transcriptional activity [133, 134]. In this study, we discovered that downregulation of LZAP decreased basal p53 protein levels and abrogated p53 phosphorylation, accumulation and transactivation activity, classically observed following DNA damage (Figures 10 and 11; Supplementary Figure S2). Supporting this result, p53 and LZAP protein levels correlated in primary NSCLC (Figure 16). As is typical for many new proteins that are implicated in tumorigenesis, the role of LZAP in cancer development and progression is likely to be dependent on accompanying molecular defects in the tumor, and the complicated nature of these interactions may be beginning to emerge with contradictory reports of LZAP as both an inhibitor of cancer cell growth and invasion, and a promoter of cell proliferation and metastasis. Given the importance of known LZAP-binding partners in human cancer (for example, ARF, p38, Wip1, RelA, Chk1 and Chk2) and the dearth of knowledge concerning functional regulation of LZAP through protein–protein interactions or posttranslational modifications, it is also possible that LZAP may play opposing roles in tumor promotion depending on the surrounding cellular environment and/or genetic defects co-existing in the tumor. Data reported herein further support a context-dependent role for LZAP in cancer, potentially providing tumor suppressor effects by activating wild-type p53, but also oncogenic activities by stabilizing mutant p53 (Figures 10 and 11).

A translational and interesting finding of our studies is that LZAP depletion

regulated DNA damage-induced cell death in a p53- dependent manner (Figures 12, 13, 14). Although a treatment strategy of simultaneous temporal downregulation of mutant and wild- type p53 has not been extensively explored, in theory, this approach should sensitize tumors with mutant p53 (such as most HPV(-) HNSCCs) to radiation and chemotherapy and, at the same time, protect normal, wtp53 expressing, tissues. Support for this potential therapeutic strategy was provided by survival assays, revealing that depletion of LZAP in cells with wild-type p53 expression increased their resistance to DNA damage (Figures 12, 13, 14). Remarkably, the loss of one LZAP allele in a genetically engineered mouse model increased radiation resistance of MEFs and bone marrow progenitors (Figure 13). Interestingly, control untreated LZAP+/− MEFs proliferated faster (Figure 13c, untreated wells; Supplementary Figure S6A). In contrast, LZAP CRISPR osteosarcoma U2OS cells were not as efficient in clonogenic survival as parental U2OS cells (Supplementary Figures S6B and C); a similar effect was observed in other cancer cells expressing LZAP siRNA or shRNA (data not shown). The tendency of partial LZAP depletion to support proliferation of normal cells, while inhibiting survival of cancer cells, is intriguing and will warrant further investigation that will be best addressed with a conditional LZAP knockout mouse that we are creating in our laboratory.

In contrast to wild-type cells, downregulation of LZAP in cells expressing mutant p53 sensitized them to radiomimetic zeocin (Figures 12c and 13d). Although we focused our study on LZAP activities toward p53, LZAP depletion increased the sensitivity of p53 null Saos-2 cells to zeocin (Figure 13e). The mechanism of how LZAP depletion potentiates p53 null cells to DNA damage-induced cell death remains to be elucidated;

however, it is possible that inability of LZAP-depleted cells to arrest cell cycle progression may increase apoptosis in response to stress signals [135]. Recently, it has been discovered that the immediate activation of p53 upon DNA damage mediates many toxic side effects, but is not required for the suppression of carcinogenesis [197]. Therefore, efficient p53 activity is needed for tumor growth suppression during the period following recovery from DNA damage [179]. We suggest that transient LZAP depletion or inhibition of LZAP activities toward p53 before DNA-damaging anticancer therapy could minimize p53-dependent toxicity of the treatment in normal tissues without decreasing the tumor-suppressive p53 function.

Mechanistically, we found that depletion of LZAP down-regulated p53 at multiple levels. LZAP downregulation modestly but significantly decreased wild-type or mutant TP53 mRNA (Figure 10). Moreover, exogenous expression of cytomegalovirus promoter-driven wild-type or mutant TP53 was inhibited in p53 null Saos-2 cells co-transfected with LZAP siRNA, as compared to cells co-transfected with control siRNA (Figure 11a). This result most likely indicated that LZAP regulates TP53 mRNA stability rather than p53 transcription. Regulation of TP53 mRNA expression and stability is an important step in controlling p53. TP53 transcription is regulated by PKCσ, HOXA5, BCL6 and by itself; in addition, several proteins, including RPL26, nucleolin, WRAP53, Wig-1 and HuR, have been implicated in the regulation of TP53 mRNA stability or translation [198-205]. Recently, we found that LZAP binds HuR, therefore it was reasonable to hypothesize that LZAP may regulate TP53 mRNA stability and translation through HuR [139]. Initial studies showed that some HuR targets (for example, Cyclin A) were down-regulated in U2OS cells lacking LZAP expression, while others (Rb1, Myc)

were not (Supplementary Figure S7). Therefore, whether LZAP regulates TP53 mRNA

levels through HuR needs further experimental support.

To date, enzymatic activity of LZAP has not been demonstrated, and diminished

p53 levels associated with LZAP depletion were dependent on the presence of HDM2—a

major p53-negative regulator (Figures 15c and 16c, left). Given that LZAP directly binds

both, p53 and HDM2 (Figures 15d–f), we propose that high levels of LZAP stabilizes

p53 through inhibition of HDM2 activity. This could be through binding of HDM2 and

prevention of its binding to p53, through conformational changes in HDM2 upon LZAP

binding that inactivates its activities toward p53. Our data does not distinguish between

these possibilities and is also consistent with the existence of a trimeric complex of p53,

LZAP and HDM2, where HDM2 activity toward p53 is inhibited within the complex. .

Exact mechanistic clarity will require further analyses, but our data consistently show

that depletion of LZAP results in lower p53 protein levels. Correlation of LZAP and p53

levels in lung cancer specimens was consistent with our experimental data. Observed

upregulation of LZAP upon siRNA-mediated depletion of HDM2 (Figure 15c) suggests

that LZAP may be a target of HDM2 E3 ubiquitin ligase activity and brings another level

of complexity to the LZAP-mediated regulation of p53.

In summary, these studies have identified a new mechanism of p53 inactivation in

human cancer, connecting LZAP loss with downregulation of p53. LZAP depletion was

found to protect normal and tumor cells expressing wild-type p53 from radiation and

chemotherapeutic drugs, while sensitizing cells expressing mutant p53 to the treatment

(Figure 7c, right). These findings raise important therapeutic considerations and suggest

that strategies or drugs that temporarily inhibit LZAP activity toward p53 may be useful

for treating p53-mutant cancers, such as HPV(-) HNSCC, while simultaneously

protecting normal tissues from DNA-damaging therapeutic agents.

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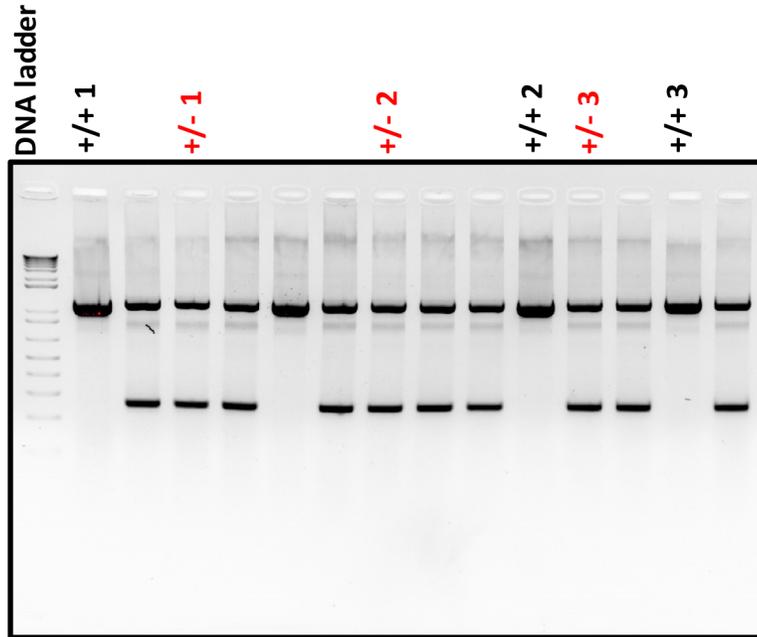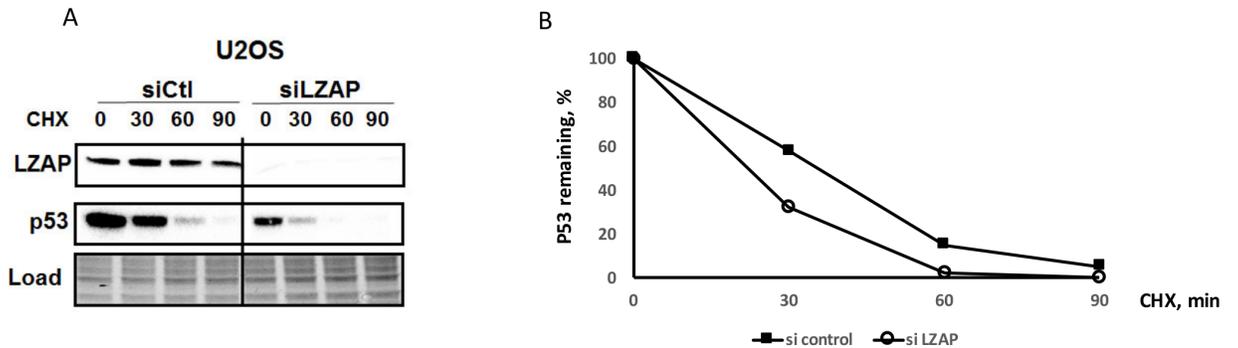
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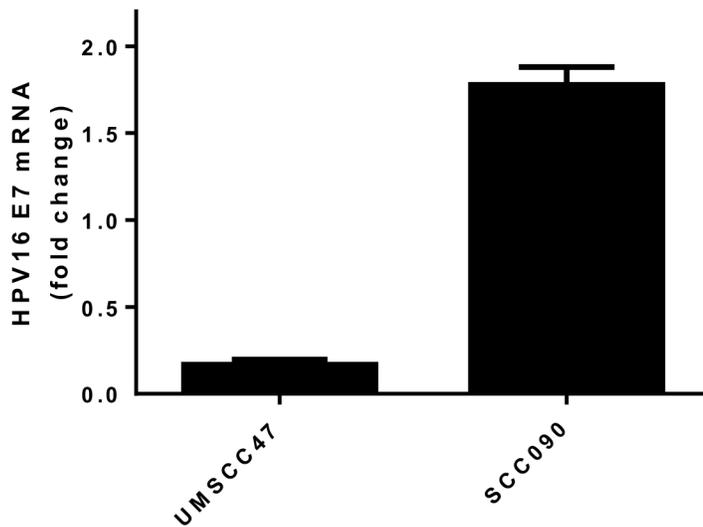
Supplementary Figure 1. Relative to GPDH expression of the HPV16 E7 gene in two HPV(+) cell lines following roscovitine treatment as determined by qRT-PCR.
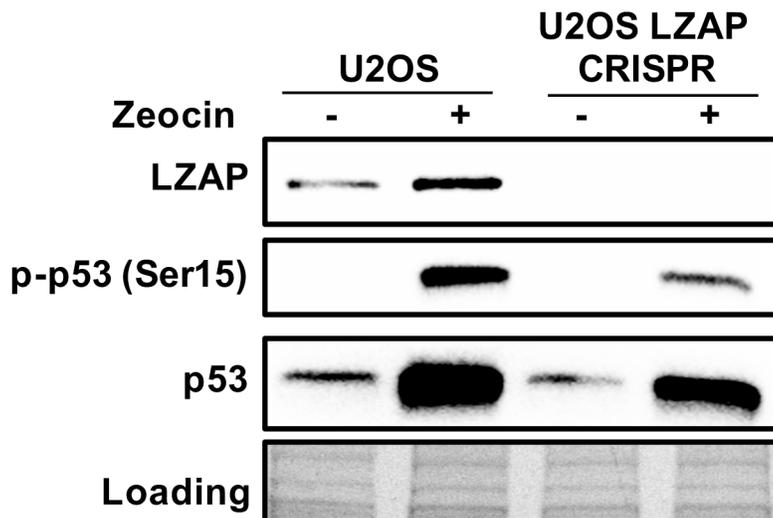
Supplementary Figure 2. Immunoblot detecting LZAP, p53 (pSer15), and total p53 in U2OS cells stably transfected with CRISPR/Cas9 constructs targeting LZAP, before and after zeocin stimulation (200 ug/mL) for eight hours.


Supplementary Figure 3. Genotyping of MEFs. MEFs used in the experiments are labelled.


Supplementary Figure 4. (A) Immunoblot detecting LZAP and p53 in U2OS cells transfected with control or LZAP siRNAs and treated with cycloheximide (CHX) for indicated time points (minutes). (B) Quantification of p53 protein levels (% from 0 min) from immunoblot in (A).
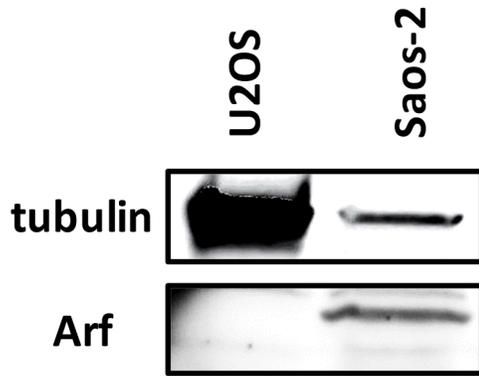
Supplementary Figure 5. Arf protein levels in U2OS or Saos-2 cells.
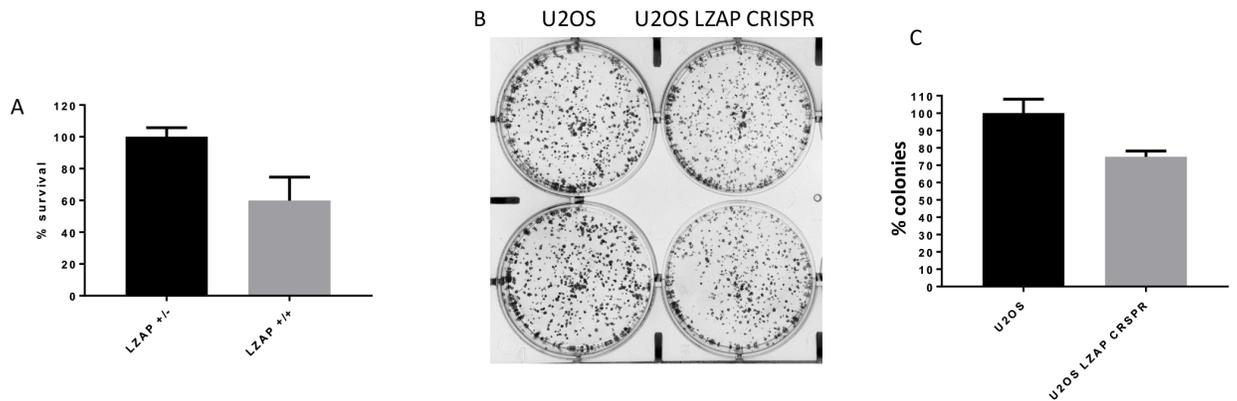
Supplementary Figure 6. (A) Quantification of survival of LZAP +/- or LZAP +/+ MEFs that was determined by methylene blue extraction, followed by quantification of absorbance. (B) Clonogenic survival of U2OS or U2OS LZAP CRISPR cells. (C) Colonies from (B) were counted, and the number of colonies from U2OS LZAP CRISP cells was normalized to U2OS cells.
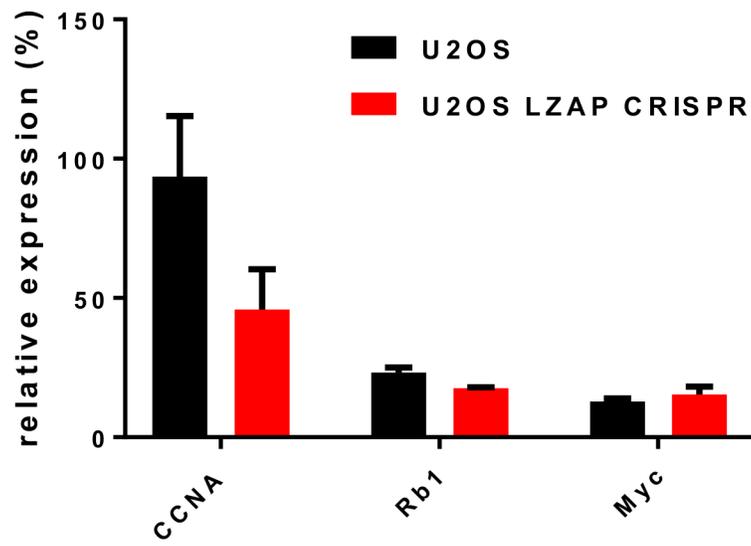
Supplementary Figure 7. Relative to GPDH expression of several HuR targets in U2OS or U2OS LZAP CRISPR cells as determined by qRT-PCR.