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14. ABSTRACT We have made substantial strides in our efforts to better define β -HPV E6's ability to augment the mutagenic potential of genome destabilizing events. We show that β -HPV E6 changes signaling events in 3 pathways (Nucleotide Excision Repair or NER, Hippo Pathway or HP and Non-Homologous End Joining or NHEJ) that protect genome fidelity. It attenuates NER signaling by decreasing XPA phosphorylation, accumulation and nuclear translocation. It decreases LATS2 phosphorylation in the HP, resulting in aberrant responses to failed cytokinesis. β -HPV E6 also makes cells more reliant on DNAPk and the NHEJ pathway. These findings are largely dependent on the viral oncogene's ability to bind and destabilize p300. We also found p300 independent inhibition of the cellular DNA repair response. Specifically, we show p300-independent sensitivity to Zeocin, a radiation mimetic. We also have preliminary data suggesting that this could be due to β -HPV E6's ability to increase BCL6 abundance.					
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1. **INTRODUCTION:** Genus β human papillomaviruses (β -HPVs) are believed to contribute to non-melanoma skin cancer by acting as a cofactor in UV-induced destabilization of the host genome. The purpose of this project is to test this hypothesis by measuring the ability of the E6 protein from β -HPV to disrupt cellular signaling in response to challenges to genome fidelity. We are examining non-homologous end joining, nucleotide excision repair, and the Hippo Pathway. Mechanistically, we are testing disruptions of this pathway that are either dependent or independent of β -HPV E6's degradation of p300, a cellular histone acetyltransferase.
2. **KEYWORDS:** Non-melanoma skin cancer, Cutaneous human papillomavirus infection, Ultraviolet irradiation, Ionizing radiation, DNA damage, Genome fidelity.
3. **ACCOMPLISHMENTS:** *The PI is reminded that the recipient organization is required to obtain prior written approval from the awarding agency Grants Officer whenever there are significant changes in the project or its direction.*
 - **Career Development Specific Major Task 1:** Receive Career and Research Advice from Experts in Cancer Causing Viruses
 - **Projected Completion date** (from SOW): August 1, 2020
 - **Completion Percentage:** ~45% (detailed in subsequent sections, calculated from percentage of milestones met)
 - **Specific Aim 1 Major Task 1:** Characterize β -HPV E6's attenuation of DNA crosslink repair.
 - **Projected Completion date** (from SOW): May 1, 2019
 - **Completion Percentage:** ~60% (detailed in subsequent sections)
 - **Specific Aim 1 Major Task 2:** Determine the Extent to which β -HPV E6 Attenuates Non-Homologous End Joining Repair (NHEJ) of DNA Lesions
 - **Projected Completion date** (from SOW): August 1, 2020
 - **Completion Percentage:** ~35% (detailed in subsequent sections)
 - **Specific Aim 2 Major Task 1:** Defining β -HPV E6's inhibition of the Hippo Pathway (HP)
 - **Projected Completion date** (from SOW): February 1, 2019
 - **Completion Percentage:** ~60% (detailed in subsequent sections)
 - **Specific Aim 2 Major Task 2:** Determine the mechanism of β -HPV E6's inhibition of the HP.
 - **Projected Completion date** (from SOW): August 1, 2020
 - **Completion Percentage:** ~66% (detailed in subsequent sections)
 - **Specific Aim 3 Major Task 1:** P300-Independent Disruption of DNA Crosslink Repair
 - **Projected Completion date** (from SOW): August 1, 2018
 - **Completion Percentage:** ~50% (detailed in subsequent sections)
 - **Specific Aim 3 Major Task 2:** Determine the mechanism of β -HPV E6's p300-independent inhibition of DNA crosslink repair.
 - **Projected Completion date** (from SOW): August 1, 2020
 - **Completion Percentage:** ~15% (detailed in subsequent sections)

What was accomplished under these goals?

CAREER DEVELOPMENT-SPECIFIC TASKS

Major Task 1: Receive Career and Research Advice from Experts in Cancer Causing Viruses

Subtask 1: Present Research Annually to Drs. Laimonis and Giam's Research Teams

I met with Dr. Giam's research teams on April 2nd, 2018 at the Uniformed Services University Health Sciences. I presented my work at USUHS as part of their weekly seminar series. In addition to my time public discussing work relevant to this project and meeting with Dr. Giam and his research staff, I had one on one meetings with 6 other professors and 5 graduate students.

I have presented my research to Dr. Laimonis primarily via email and phone conversations because I met with his group just before the reporting period began (July 17-22nd 2017). As a result, it was not prudent to meet with them in person in the last 11 months.

Subtask 2: Attend and Present Research at Marquee HPV Research Conferences

The Marquee 2017 and 2018 HPV Research Conferences fell just outside of the reporting period. The DNA Tumor Virus Meeting concluded in late July 2017, while the International Papillomavirus Meeting was held from Feb 28th to March 4th. I presented the lab's research at both conferences. I was a chair and panelist for the Basic Science workshop at the International Papillomavirus Meeting. I will attend the 2018 additions of each of these conferences, notably the DNA Tumor Virus Meeting that begins on July 31st 2018. Finally, I attended several regional conferences in between these meetings highlighted by 17th Annual Symposium in Virology hosted by the University of Nebraska's Center for Virology.

Subtask 3: Monthly Discussion of Career Progress and Grant Submission Strategy with Dr. Laimonis.

Dr. Laimonis and I discuss my career regularly. This has included regular grant revisions and career guidance. These interactions have been incalculably helpful as I've navigated my pre-tenure career.

Subtask 4: Discuss Scientific and Professional Progress with Dr. Clem (KSU Faculty Advisor).

Dr. Clem and I are colleagues in the Division of Biology at Kansas State University. As a result, we see each other very frequently. Rollie's check-ins during these encounters often lead to unscheduled advising sessions. He edits my grants and manuscripts before they are submitted. I have improved as an instructor by discussing teaching philosophies with Dr. Clem.

Milestones Achieved:

- (1) Presentation of project data at preeminent meetings annually, (12, 24, 36 Months)
 - a. Project data was presented at these meetings, but they occurred outside funding period. In combination with other members of the lab, we gave research talks at regional meetings. Two oral presentations of our work will be made at the DNA tumor virus meeting beginning on July 31, 2018. Additional presentations will be made at the international papillomavirus meeting in October 2018.
- (2) Submit major grant proposal to extend project by the end of the second budget period (24 Months)
 - a. Both of the grant proposals described below are 1 year ahead of schedule.
 - b. A proposal to the American Cancer Center was submitted on 4/26/2018 titled, "Genus Beta Human Papillomavirus E6 Impairs Genome Fidelity". This grant would provide support to expand the efforts funded by the CDMRP from 2019-2023. Notably, there is no overlap to the work supported by the CDMRP and this milestone was completed over 1 year earlier than projected.
 - c. I am a junior investigator on a COBRE proposal (P20) submitted to the National Institute of General Medical Sciences. This work would also extend the efforts funded by the CDMRP with complimentary analysis of the oncogenic potential of cutaneous HPV infections. There is no overlap with the CDMRP funded project.

- (3) If necessary, revise and resubmit grant. (24, 36 Months)
 - a. N/A
- (4) Publish Findings in Peer Reviewed Journal (24, 36 Months)
 - a. Three manuscripts were published on work produced with support from the CDMRP. This is significantly ahead of schedule as the first publication were not anticipated until the second budget period. References are listed below
 - i. Loss of Genome Fidelity: Beta HPVs and the DNA Damage Response. Wendel SO, Wallace NA Front. Microbiol. 2017 PMID:29187845 [LINK](#)
 - ii. Characterizing DNA Repair Processes at Transient and Long-lasting Double-strand DNA Breaks by Immunofluorescence Microscopy. Murthy V, Dacus D, Gamez M, Hu C, Wendel SO, Snow J, Kahn A, Walterhouse SH, Wallace NA. 2018 PMID: 29939192 [LINK](#)
 - iii. The Curious Case of APOBEC3 Activation by Cancer Associated Human Papillomaviruses. Wallace NA, Munger K. 2018 PMID: 29324878 [LINK](#)
- (5) Gain career advice from experts in viral oncology
 - a. I have had extensive interactions with my formal mentors and given talks at 3 regional conferences (**Kansas IDEA Network of Biomedical Research Excellence's Annual Symposium; Kansas University Medical Center's Viral Pathogenesis Symposium; University of Nebraska's Center for Virology Flyswat Meeting**).

RESEARCH-SPECIFIC TASKS

Specific Aim 1: To define the p300 inhibition of DNA repair by β -HPV E6

Major Task 1: Characterize β -HPV E6's attenuation of DNA crosslink repair.

Subtask 1: Obtain HRPO approval to isolate keratinocytes from neonatal foreskins and complete onboarding of Changkun Hu.

HRPO approval was obtained on 6/16/17. HRPO Log Number A-20356. Changkun Hu was enrolled at Kansas State University at the beginning of Fall Semester 2017. He completed safety training via the Collaborative Institutional Training Initiative Program (CITI).

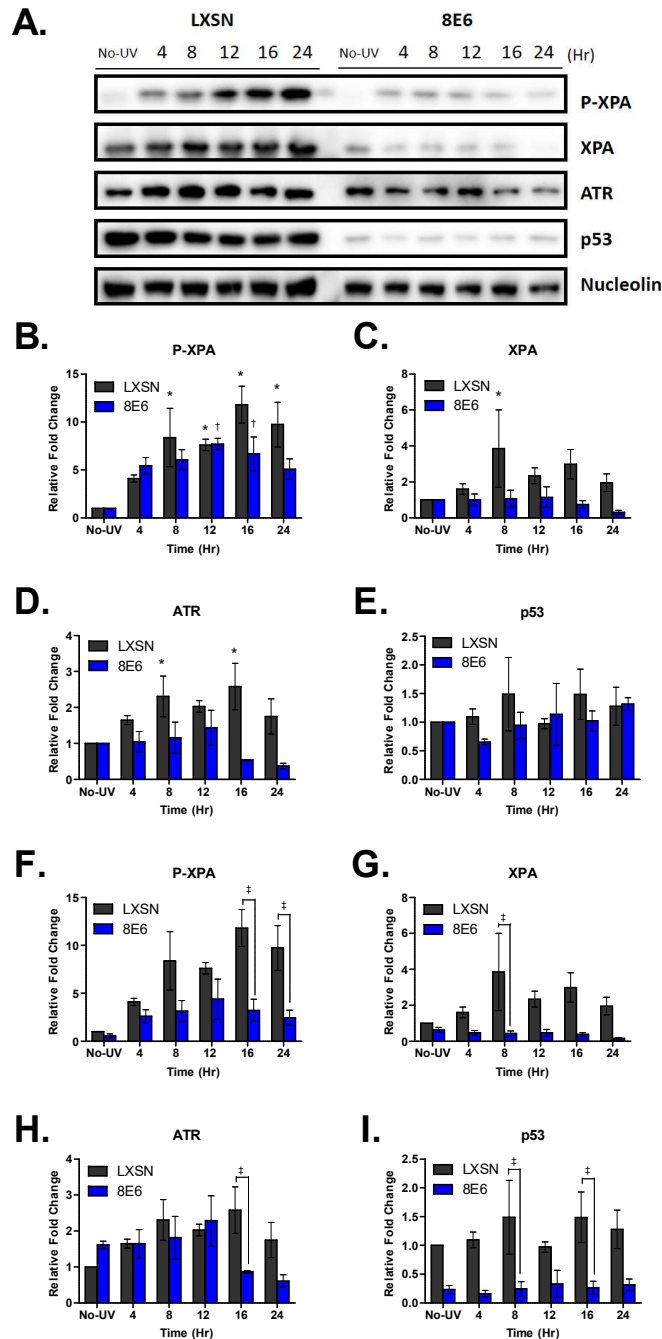


Figure 1: β -HPV E6 (8 E6) attenuates the NER response to UV. A. Representative Immunoblots at indicated times after UV. Nucleolin serves as a loading control. B-E. Densitometry of indicated protein levels normalized to untreated cells. F-I. Densitometry of indicated protein levels normalized to control untreated cells. $n \geq 3$. Error bars represent SEMs. Significant differences as determined by Student t-test are indicated

repeating them in TERT-immortalized keratinocyte cell lines. We will also determine if they are p300-dependent by repeating essential aspects of the experiment in cells expressing HPV $\Delta 8$ E6.

Subtask 2: Examine XPA phosphorylation and Stabilization using Immunoblot at representative time points following UV exposure. This will be done in vector control, β -HPV E6 and β -HPV Δ E6 expressing cells.

We determined changes in the abundance and phosphorylation of XPA after UV in cells with and without β -HPV E6 (**Figure 1**). Consistent with our working hypothesis, the accumulation of XPA seen in control cells was attenuated by HPV 8 E6. There was a slight decrease of XPA in 8 E6 expressing untreated cells compared to control, but these differences become significant after UV exposure (**Figure 1 A, C and G**). While XPA peaks 8 hours after UV in control cells, total XPA decreases in cells expressing HPV 8 E6. To begin determining if these changes were driven by ATR's phosphorylation of XPA, we probed for phospho-XPA (p-XPA). The steady p-XPA increase in control cells was muted by HPV 8 E6. We measured ATR levels in these cells having previously seen that HPV 8 E6 impairs ATR's response to UV. Consistent with our published work, HPV8 E6 decreased ATR abundance before and after UV exposure (**Figure 1A and H**). We examined another ATR phosphorylation/stabilization target (p53). To our surprise, p53 levels were decreased with and without UV in cells expressing HPV 8 E6. We have repeated these experiments at 1 and 4 hours after UV with a gradient of UV doses and found consistent results (**Figure 2** and not shown). We are currently working to determine if these observations are cell lines specific by

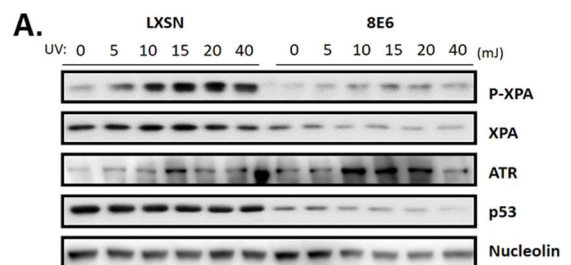


Figure 2: β -8 E6 inhibits the dose dependent increase in XPA-phosphorylation. Representative immune blot 4 hours after UV.

Subtask 3: Determine if DNA crosslink repair is rescued by exogenous expression of XPA and ATR.

We obtained the ATR expression vector. We transformed bacteria with this vector, before amplifying and purifying the plasmid. The resulting working stock (1µg/ml concentration) and frozen stock are now available for exogenous expression or “rescue” experiments. We have signed to a material transfer agreement with www.addgene.org and obtained an XPA expression plasmid. We are currently preparing a

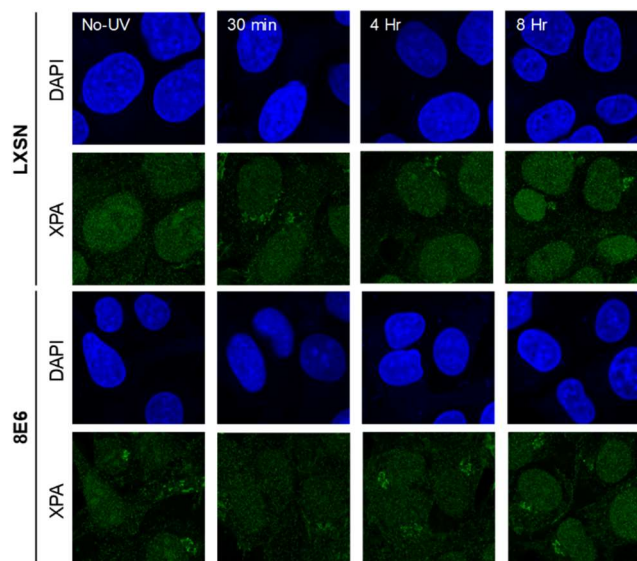


Figure 3: HPV 8 E6 hinders accumulation of XPA in the nucleus after UV exposure. DAPI stain delineates nuclei. XPA is seen localizing to the nucleus and shown in green. Notably, little green aka XPA is seen in cells expressing HPV 8 E6 other than non-specific aggregates that we believe are non-functional.

idea that HPV 8 E6 blocks the nucleotide excision repair pathway by preventing the typical increases in XPA localization to the nucleus. Unfortunately, we cannot make this conclusion more strongly because we believe our no UV treated samples were not protected from irradiation.

Subtask 5: Determine if β -HPV E6 changes the abundance of other crosslink repair proteins using immunoblotting to detect the abundance of NER proteins before and after UV.

We are repeating these experiments in keratinocytes, dissecting the molecular mechanism using the HPV Δ 8 E6 mutant. Exogenous XPA and ATR expression will provide further insight.

Milestones Achieved:

- (1) obtain oversight from HRPO necessary to avoid unintentional or unethical mistreatment of the human subjects.

HRPO approval was obtained before the start of the reporting period (6/16/17). HRPO Log Number A-20356.

- (2) get the staff necessary to complete this aim.

working stock.

Subtask 4: Define the subcellular localization of XPA using immunofluorescence microscopy and immunoblots, before and after UV.

We detect XPA via immunofluorescence microscopy. IN control cells, UV exposure caused XPA to accumulate in the nucleus (**Figure 3**). This is consistent with the increased p-XPA seen by immunoblot (**Figure 1A and 2**). HPV 8 E6 reduces the increase of nuclear XPA (**Figure 3**). We have adapted protocol to quantify these immunofluorescence microscopy data using ImageJ. This analysis found an increase in nuclear XPA in control cells after UV that was minimally seen in cells expressing HPV 8 E6 (**Figure 4**). This experiment bears repeating so that statistical analysis can be done on the nuclear intensity. Our data are consistent with the

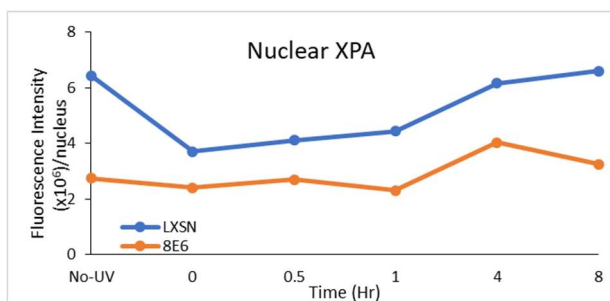


Figure 4: Reduced fluorescent staining for XPA in cells expressing HPV 8 E6 after UV.

A graduate student, Changkun Hu was recruited to work on this project. He has been focused on the efforts listed in Major Task 2 of this Aim. We also recruited a Research Assistant, Vaibhav Murthy. Mr. Murthy produced the data discussed here.

- (3) *learn whether β -HPV E6 alters XPA phosphorylation and stabilization after UV and whether any changes are p300-dependent.*

We have seen decreased XPA phosphorylation and accumulation after UV in cells expressing β -HPV E6. We are currently working to determine whether these phenotypes are p300 dependent and if the increased abundance is due to increased stability.

- (4) *learn whether β -HPV E6 acts through XPA- and ATR-dependent mechanisms to prevent crosslink repair.*

Our results are consistent with an XPA and ATR dependent disruption of crosslink repair. Not only does β -HPV E6 result in significant decreases in both of these repair proteins, the viral oncogene as decreases the amount of p53 in cells. Because p53 is stabilized by ATR-dependent phosphorylation after UV, our data is consistent with the broad impact to cellular repair expected with ATR's role as a keystone damage kinase. We are in the process of confirming the ATR- and XPA-dependence by exogenous expression of these genes in HPV 8 E6 expressing cells.

- (5) *learn whether β -HPV E6 changes the subcellular localization of XPA following UV and whether any changes are p300-dependent.*

We have seen striking changes in subcellular localization of XPA after UV in control cells that are attenuated by HPV 8 E6. Notably, our immunofluorescence microscopy does not show a build-up of XPA in the nuclei of cells expressing HPV 8 E6. This a remarkable finding as XPA is the rate limiting protein for nucleotide excision repair and required to be at the site of damaged DNA for repair. Because of their significance, we are actively working to confirm these results. We are also testing our hypothesis that these phenotypes are p300-dependent.

- (6) *learn if β -HPV E6 changes the abundance of NER proteins in cells with and without UV exposure.*

We reduced p53, XPA and p-XPA accompanies cells expressing β -HPV E6, whether or not they are exposed to UV. ATR is also reduced when they are treated with UV. The differences only become significant statistically after UV. This likely reflects the reduced need for nucleotide excision repair proteins without stimulating the pathway with UV. This makes HPV 8 E6's impact less apparent in untreated cells.

Major Task 2: Determine the Extent to which β -HPV E6 Attenuates Non-Homologous End Joining Repair (NHEJ) of DNA Lesions

Subtask 1: Define β -HPV E6's ability to disrupt DNAPk expression and autophosphorylation by immunoblot.

Our initial hypothesis was that HPV 8 E6 would decrease DNAPk expression limiting its autophosphorylation and thus blocking NHEJ. We have found that the ability of HPV 8 E6 to influence DNAPk signaling is more complex than we initially believed.

In untreated U2OS cells and in contrast to our preliminary data, DNAPk abundance was increased in cells

expressing HPV 8 E6 (**Figure 5A**). This was also true for the autophosphorylation of DNAPk (p-DNAPk). We are currently exploring an alternative hypothesis based on our previous observation that HPV 8 E6 attenuates homologous recombination (HR), the other major repair mechanism for double strand breaks in DNA (DSBs). We hypothesize that inhibiting HR cells more dependent on NHEJ. The mechanism of HPV 8 E6's inhibition support this idea. HPV 8 E6 prevents the expression of BRCA1 by destabilizing p300. Davis et al 2014 reports that BRCA1 hinders DNAPk autophosphorylation and that decreased BRCA1

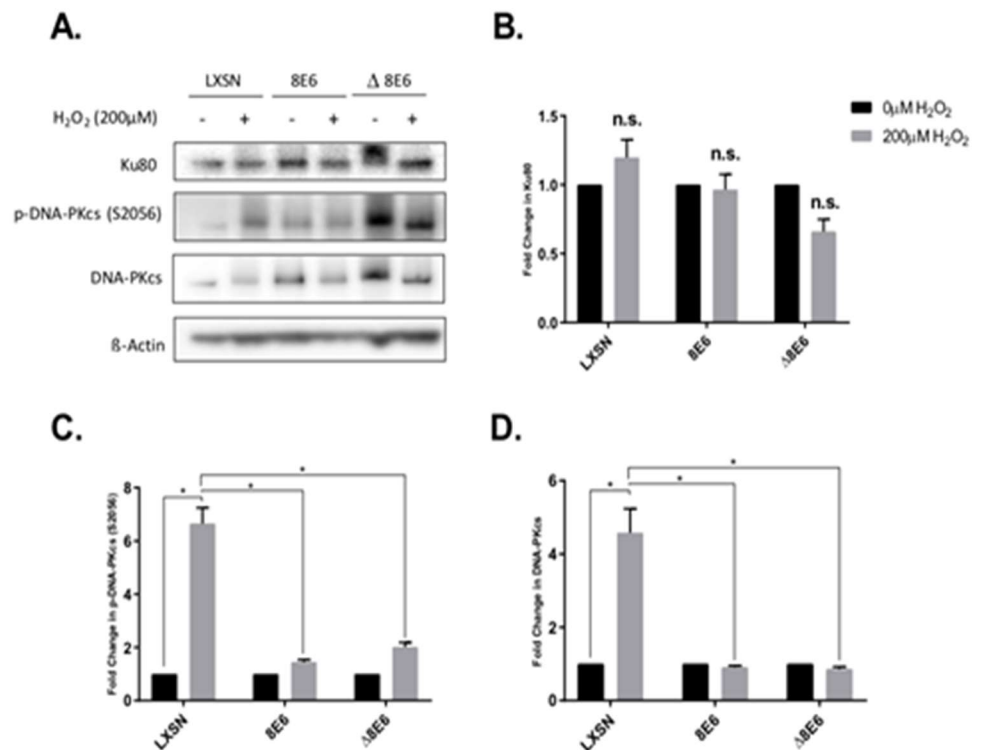


Figure 5: HPV 8 E6 increases basal p-DNAPk but impairs further activation of the repair kinase upon hydrogen peroxide (H₂O₂) exposure. A. Representative Immunoblot. B. Densitometry of Ku80 protein abundance normalized to untreated control. C. Densitometry of p-DNAPk protein abundance normalized to untreated control. D. Densitometry of total DNAPk protein abundance normalized to untreated control. Statistical significance is noted with lines and asterisks ($p \leq 0.05$ by Student T-test). n.s. denotes no significant difference from control was observed. $n \geq 3$.

expression results leads to elevated p-DNAPk. We exposed cells expressing vector control or HPV 8 E6 to hydrogen peroxide (H₂O₂) to activate the NHEJ pathway. As expected, both total DNAPk and p-DNAPk levels were increased by H₂O₂ in control cells (Figure 5A, C and D). However, HPV 8 E6 blocked both DNAPk and p-DNAPk accumulation induced by H₂O₂. Since H₂O₂ can produce inconsistent results due to its short half-life, we confirmed these results by exposing cells to a radiation mimetic, Zeocin. HPV 8 E6 expressing cells were also unable to induce p-DNAPk to control levels after Zeocin exposure (data not shown and **Figure 7**). We interpreted these results as consistent with HPV 8 E6 activating DNAPk signaling in untreated cells. Also, HPV 8 E6 prevents further activation of DNAPk in response to DNA damage. If this is the case, we predicted HPV 8 E6 would increase zeocin toxicity. We tested this hypothesis using standard colony formation assays to define the extent that HPV 8 E6 caused sensitivity to Zeocin. Supporting our hypothesis, we found a sizable and significant increase in Zeocin sensitivity accompanied HPV 8 E6 expression (**Figure 6**).

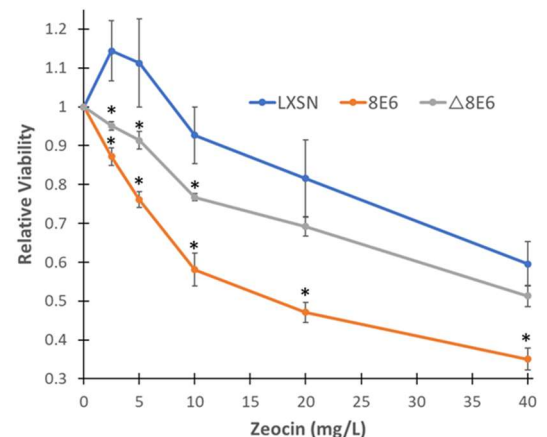


Figure 6: HPV 8 E6 sensitizes cells to zeocin in p300-dependent and -independent manners. Asterisks denote significant differences from vector control (LXSN) cells as determined by Student T-test (p value < 0.05). $n = 9$

To test the hypothesized increased dependence on DNAPk more directly, we used a specific small molecular DNAPk inhibitor (NU7441). Two sets of cells were treated in parallel, protein lysates were harvested from one and the other was used to measure viability. As seen with H₂O₂ treatment, untreated HPV 8 E6 expressing cells had higher p-DNAPk levels but these levels did not significantly increase after Zeocin treatment in HPV 8 E6. Vector control cells had significantly higher p-DNAPk after Zeocin exposure. p-DNAPk levels in Zeocin treated cells also did not significantly differ among the cell lines. Inhibiting DNAPk's autophosphorylation capability, made p-DNAPk levels indistinguishable among all cell lines, both with and

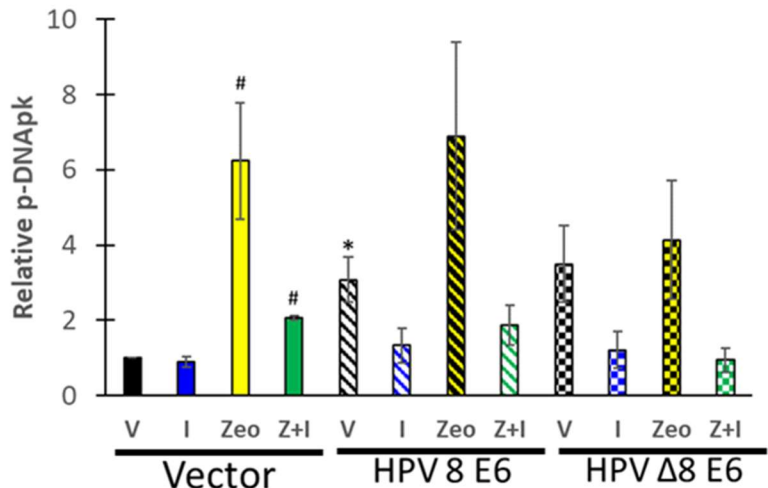


Figure 7: Increased p-DNAPk in cells expressing HPV 8 E6 can be mitigated by DNAPk inhibition: Indicated cells were treated with either vehicle (V), Nu7441 (I), Zeocin (Zeo) or Zeocin and Nu7441 (Z+I). Asterisks denote significant differences from similarly treated vector controls (p-value < 0.05). # denote significant differences from untreated cells within the same cell line (p-value < 0.05). Significance calculated by Student T-Test. n=3.

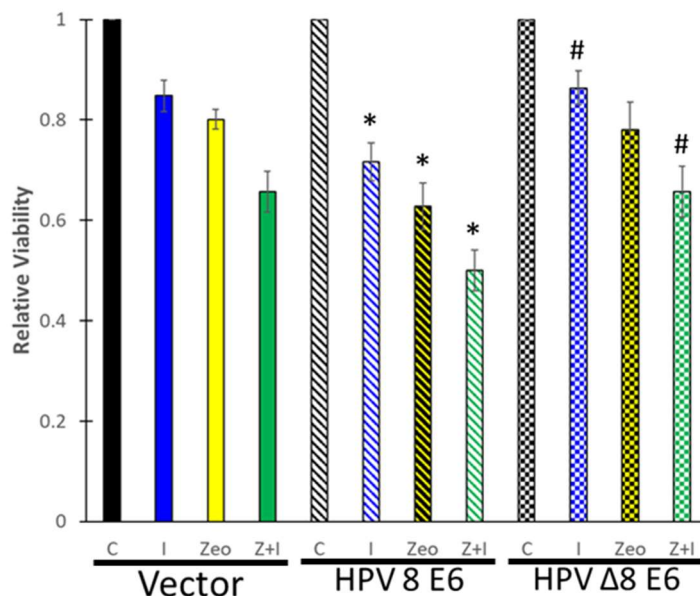


Figure 8: HPV 8 E6 makes cells more sensitive to DNAPk inhibition. Indicated cells were treated with either vehicle (V), Nu7441 (I), Zeocin (Zeo) or Zeocin and Nu7441 (Z+I). Asterisks denote significant differences from similarly treated vector controls (p-value < 0.05). # denote significant differences between HPV 8 E6 and HPV Δ8 E6 expressing cells (p-value < 0.05). Significance calculated by Student T-Test. n=3.

without Zeocin treatment. HPV 8 E6 expression made cells significantly more sensitive to Nu7441 with and without Zeocin (**Figure 8**).

We determined the ability of HPV Δ8 E6 to alter DNAPk signaling. To our surprise, HPV Δ8 E6 maintained the ability to increase the basal levels of DNAPk and p-DNAPk, while suppressing their induction after H₂O₂ (**Figure 5 A, C and D**). Similar results were observed in cells treated with Zeocin (**Figure 7**). The mutated viral protein also increased sensitivity to Zeocin (**Figure 6**). Ablating HPV 8 E6's ability to destabilize p300 did significantly decrease Zeocin sensitivity compared to wt HPV 8 E6. Compared to control cells, HPV Δ8 E6 expressing cells were not significantly more sensitive to DNAPk inhibition with or without Zeocin. We take this as evidence supporting p300-dependent and p300-independent abrogation of the DSB repair response by HPV 8 E6. Further, the differential

sensitivity to DNAPk inhibition (HPV 8 E6 vs HPV Δ8 E6) lead us to hypothesize that HPV 8 E6 attenuates DSB repair through DNAPk-dependent and DNAPk-independent mechanism. Our efforts to examine p300-independent inhibition of DSB repair are discussed in more detail in Specific Aim 3. Because the deletion mutation in HPV Δ8 E6 prevents it from blocking homologous recombination, these

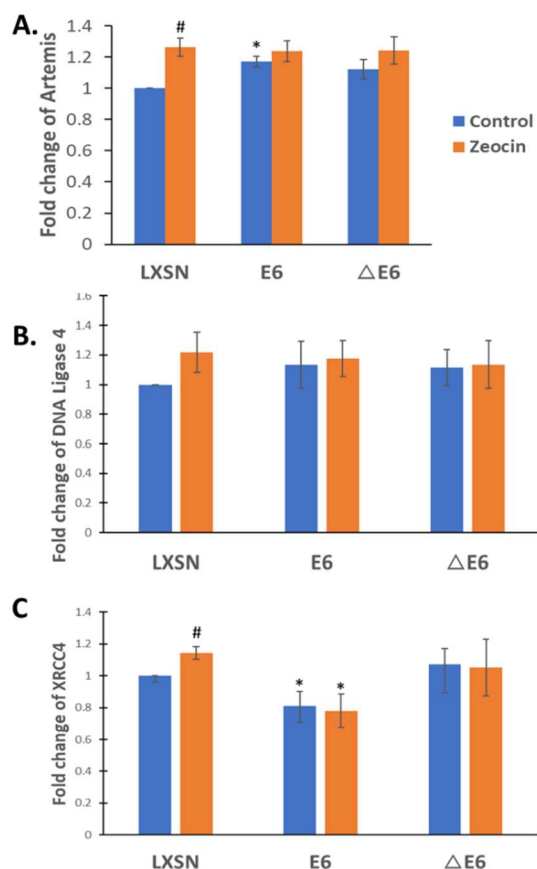


Figure 9: Zeocin and HPV 8 E6 induced minimal changes to other NHEJ proteins. Densitometry of NHEJ proteins. Asterisks denote significant differences from similarly treated vector controls (p -value < 0.05). # denote significant differences from untreated cells within the same cell line (p -value < 0.05). Significance calculated by Student T-Test, $n=3$.

data are also consistent with our hypothesis that homologous recombination inhibition causes increased DNAPk dependence.

Subtask 2: Determine the effect of β -HPV E6 on the expression of NHEJ proteins by immunoblot.

Subtask 2 was not expected to be accomplished until the 6th month of year 2. However, we have made notable progress on it. We used immunoblots to define the abundance of NHEJ proteins with and without the induction of DSBs. Other than DNAPk, HPV 8 E6 causes minimal although occasionally significant changes to other NHEJ proteins (**Figure 9**). Artemis abundance is significantly elevated, but by only 15-20%. XRCC4 is significantly decreased, but again only by about 20%. After exposure to Zeocin, vector control cells had minimal increases in DNA ligase 4, XRCC4 and Artemis, but only the latter two reached statistical significance. These protein levels do not change after Zeocin treatment in HPV 8 E6 expressing cells. We interpret these results as evidence against the regulation of these proteins by increased or decreased abundance.

We next took an unbiased and broader approach by performing a computational analysis of publically available expression 1040 cell lines (**Figure 10**). To identify relevant changes caused by reduced p300 abundance, we compared NHEJ gene expression between cell lines that had significant p300 reduction (Z-score ≤ 1.645). This analysis was consistent with our previous reports that p300 was needed for the expression of ATM, ATR, BRCA1 and BRCA2 as each of these genes was downregulated in cells with less p300.

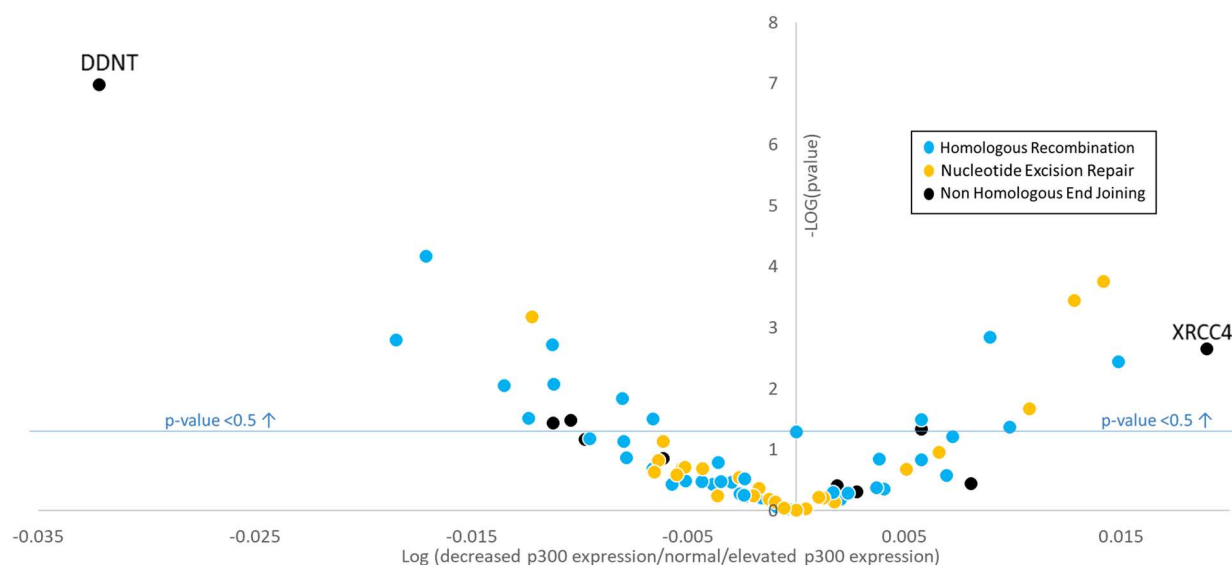


Figure 10: Expression Analysis of Cell Lines with Reduced p300 Expression: X-axis denotes magnitude of expression changes compared to cell lines with unaltered p300 expression. Y-axis depicts significance.

When we examined all genes defined by KEGG to be involved in the NHEJ pathway, we only found 4 differentially expressed genes, DCLER1C, LIG4, XRCC4 and DNTT. DCLER1C and LIG4 are marginally significant. DNTT is highly significant and robustly decreased, but falls outside of the canonical NHEJ pathway. In contrast, canonical genes in the homologous recombination pathway are down regulated, supporting our hypothesis that loss of p300 makes cells less capable of repairing DSBs using this pathway. These results are consistent with our immunoblot analysis and lead us to conclude that HPV 8 E6 does not notably decrease the expression of NHEJ genes by destabilizing p300. Rather, HPV 8 E6 likely increases DNAPk phosphorylation as an indirect result of decreasing BRCA1 expression. We have similarly analyzed nucleotide excision repair pathway genes expression.

Subtask 3: Define the ability of β -HPV E6 to impair NHEJ using a fluorescence based reporter system (traffic light reporter assay).

Although we are proud to be significantly ahead of schedule on Major Task 2, Subtask 3 has not been started. Please note the SOW has this work beginning in the second year of support.

Subtask 4: Determine the ability of β -HPV E6 to prevent NHEJ repair foci formation by immunofluorescence microscopy.

We anticipate that subtask 4 will be completed by the end of the third reporting period as noted in the SOW. However, we have begun pilot experiments detecting DNAPk by immunofluorescence microscopy and find that it is induced by Zeocin and specifically localized to DSB (Figure 11).

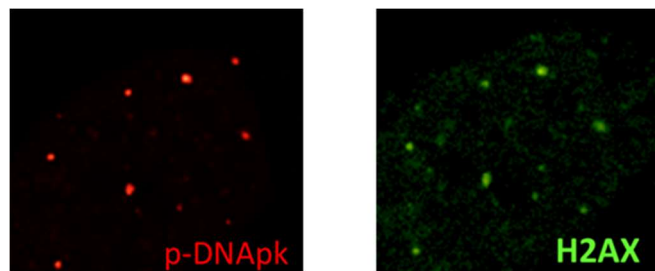


Figure 11: p-DNAPk co-localizes with H2AX foci, a marker of DSBs.

Milestones Achieved: We will...

- (1) *learn the extent to which β -HPV E6 prevents DNAPk expression and activation as well as whether this phenotype is p300-dependent.*

Contrary to our initial working hypothesis, we have found that at least in some cell types and protein extraction conditions, β -HPV E6 elevates basal DNAPk activation. This correlates to an increase dependence on DNAPk manifest by enhanced sensitivity to DNAPk inhibition. While this is not the outcome we anticipated, it is perhaps more consistent with our overall hypothesis that β -HPV infections contribute to skin cancer by destabilizing the host genome, because NHEJ is a mutagenic repair pathway. This could destabilize the host genome with and without UV. This phenotype is at least partially independent of p300 degradation. HPV 8 E6 and HPV Δ 8 E6 both increase p-DNAPk and total DNAPk, but only the wild type protein increases sensitivity to DNAPk inhibition. We are repeating these results in keratinocytes.

- (2) *learn whether β -HPV E6 decreases the abundance of NHEJ proteins and whether these changes are dependent on p300 degradation.*

We found that β -HPV E6 did not decrease the abundance of other NHEJ proteins. Further, an unbiased computation analysis found that loss of p300 expression was primarily manifest in decreases in mRNAs related to homologous recombination rather than NHEJ.

- (3) *learn whether β -HPV E6 inhibits the repair of double strand breaks by the non-homologous end joining pathway as well as the role of p300 in any inhibition.*

We have not met this milestone, but were not expected to begin working on this task until the 15th month of the project. Our current data suggest that there will not be a decrease in NHEJ.

- (4) learn the extent to which β -HPV E6 prevents non-homologous end joining proteins from forming repair foci and the p300-dependence of any such phenotype.

We have not met this milestone, but were not expected to begin working on this task until the 21st month of the project. We have successfully detected DNAPk repair foci and are actively comparing them among control cells and those expressing HPV 8 E6 and HPV Δ 8 E6.

Specific Aim 2: Determine the breadth and mechanism of β -HPV E6's Hippo Pathway (HP) Inhibition

Major Task 1: Defining β -HPV E6's inhibition of the HP

Subtask 1: Obtain HRPO approval to isolate keratinocytes from neonatal foreskins.

HRPO approval was obtained before the start of the reporting period (6/16/17). HRPO Log Number A-20356.

Subtask 2: Determine the impact of β -HPV E6 on the phosphorylation of HP proteins by immunoblot.

Our efforts to learn how β -HPV E6 prevents the stabilization of p53 when cells do not divide after mitosis center on the Hippo pathway (HP). Since the HP kinase, LATS2 phosphorylates and stabilizes p53 in response to failed cytokinesis, we hypothesized that β -HPV E6 could hinder LATS2 activation. Since LATS2 phosphorylation is needed for its activation and phosphorylation, we tested this hypothesis by immunoblotting against total and phosphorylated LATS2 (p-LATS2). In cells where dihydrocytochalasin (H2CB) was used to block cell division after replication, vector control cells had an increased ratio of p-LATS2 to total LATS2 indicating elevated activation of the pathway (**Figure 12**). Although total LATS2 levels increased in cells expressing HPV 8 E6, less LATS2 was phosphorylated. LATS2 is one of the kinases that phosphorylates YAP causing it to be sequestered in the cytoplasm. Unlike LATS2, YAP phosphorylation was not decreased by HPV 8 E6. Instead, it was increased by H2CB treatment in both

cell lines. This leads us to hypothesize that YAP is phosphorylated by kinases other than LATS2 in cells expressing HPV 8 E6. This is consistent with YAP as a broad mediator of proliferation. Consistent with our previous reports and with reduced LATS2 activity, p53 was less abundant in HPV 8 E6

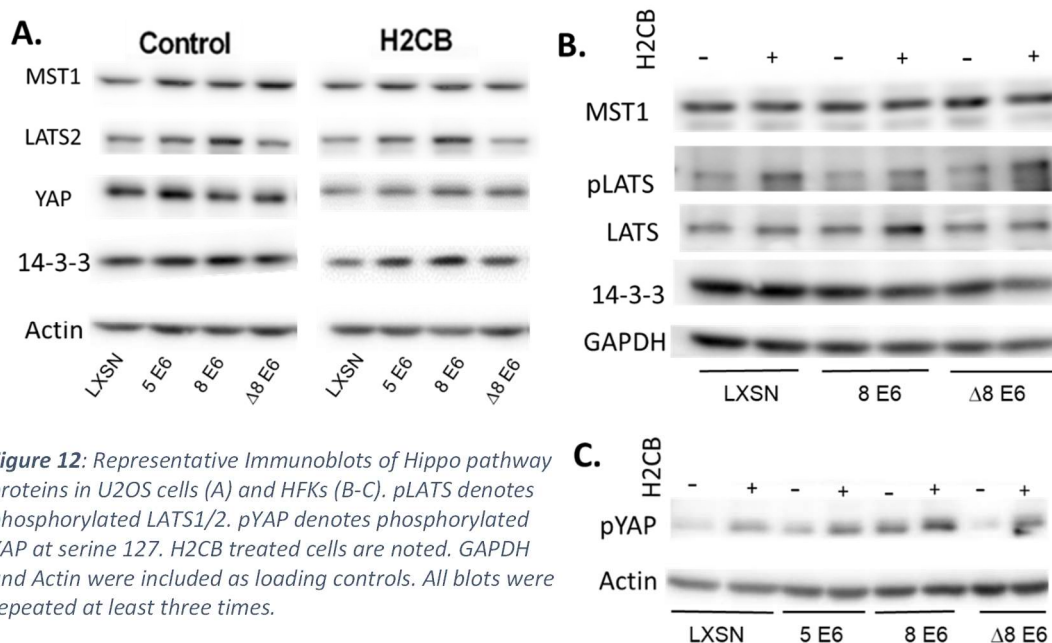


Figure 12: Representative Immunoblots of Hippo pathway proteins in U2OS cells (A) and HFKs (B-C). pLATS denotes phosphorylated LATS1/2. pYAP denotes phosphorylated YAP at serine 127. H2CB treated cells are noted. GAPDH and Actin were included as loading controls. All blots were repeated at least three times.

expressing cells after H2CB treatment (**Figure 13**). We extended this analysis to include 2 other canonical HP proteins (14-3-3 and MST1), but did not see a difference in their abundance. These results were true in both transformed (U2OS) and primary (human foreskin keratinocytes) cells.

These experiments were also performed in cells expressing HPV Δ8 E6 (**Figure 12**). The ratio

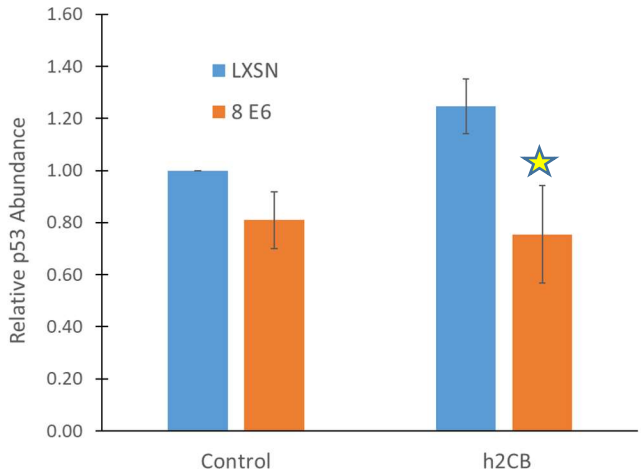


Figure 13: H2CB-induced accumulation of p53 is attenuated by HPV 8 E6. Average densitometry of 3 immunoblots detecting p53 levels in HFKs. Error bars denote standard error of the mean. Stars denote statistical differences from similar treated LXSN cells.

of p-LATS2 to total LATS2 was higher in HPV Δ8 E6 than HPV 8 E6, but less than the amount observed in vector control cells. HPV Δ8 E6 also did not significantly change the amount of phosphorylated YAP compared to either HPV 8 E6 or vector control cells. Loss of the p300 binding site from HPV 8 E6 prevented

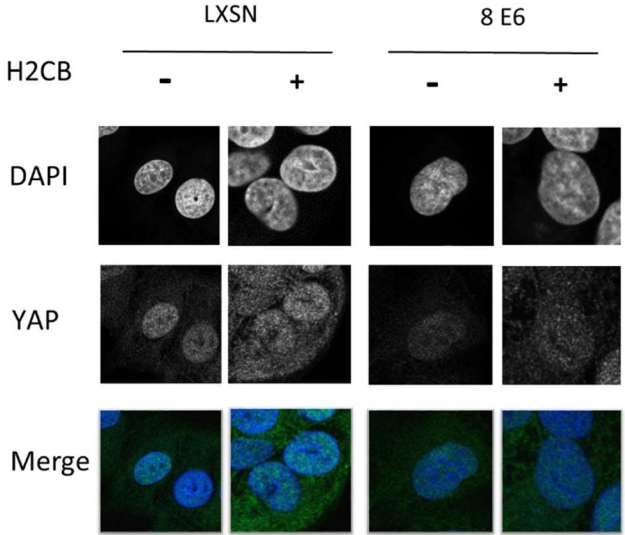


Figure 14: Representative images of YAP in U2OS cells after H2CB treatment

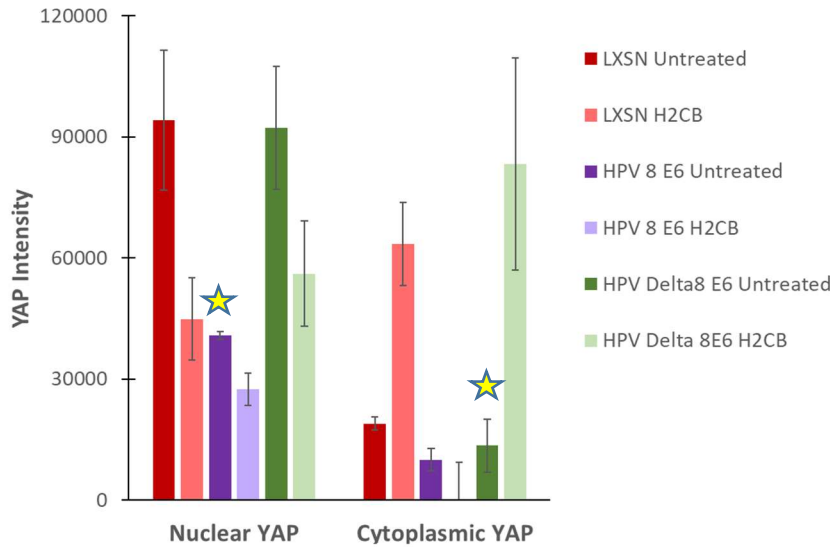


Figure 15: Subcellular YAP Localization is regulated by HPV 8 E6. YAP was monitored by IF in U2OS treated with H2CB. Compared to controls, cells expressing HPV 8 E6 have less YAP in the cytoplasm after H2CB, but also have less YAP overall. n=3. Error bars denote standard errors of the mean. Stars indicate significant differences from similar treated controls by Student T-test, p-value < 0.05. Similar results were obtained for HFKs.

the reduction in p53 accumulation after H2CB treatment. We interpret this as evidence for p300-dependent and -independent abrogation of HP signaling by HPV 8 E6.

Subtask 3: Define the subcellular localization of HP proteins in cells by immunofluorescence microscopy.

The HP regulates cell growth by controlling the subcellular localization of YAP. Phosphorylation of YAP causes the transcription factor to be sequestered in the cytoplasm or degraded. This prevents it from interacting with promoters and driving expression of genes involved in proliferation. The decreased phosphorylation of LATS2 that we observed would be consistent with less YAP translocating to the cytoplasm, but the equivalent amount

of YAP phosphorylation suggests that YAP will still get sequestered. To determine which of these occurred, we determined the subcellular localization of YAP using immunofluorescence microscopy to detect YAP before and after H2CB exposure (**Figure 13-14**). We found HPV 8 E6 reduced the abundance

of YAP in the cytoplasm in cells that failed to divide. Although less YAP is located in the cytoplasm, total YAP is reduced in HPV 8 E6 expressing cells. We interpret this to be the result of LATS2-independent phosphorylation. These results were true in both transformed (U2OS) and primary (human foreskin keratinocytes) cells.

We also measured the accumulation of p53 by immunofluorescence microscopy in cells expressing 4 different HPV E6s under these same conditions (Figure 15). Cells expressing β -HPV E6's that bound and destabilized p300 (HPV 5 and 8 E6) prevented the accumulation of p53, whereas cells expressing a β -

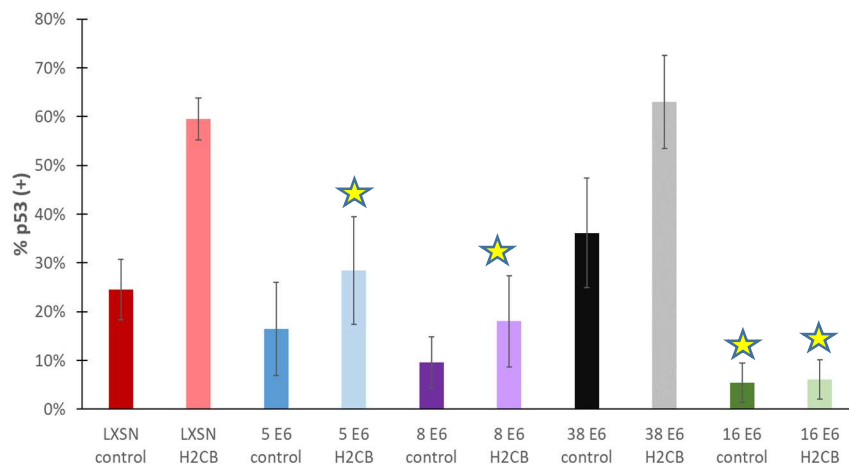


Figure 16: Beta HPV E6 prevents the accumulation of p53 in cells treated with H2CB. Star denotes statistically significant differences from similarly treated control cells. Student T-test, p -value < 0.05. Immunofluorescence microscopy was repeated at least 3 times. 38 E6 was included as a negative control as it does not destabilize p300 and HPV 16 E6 was included as a positive control as it degrades p53.

HPV E6 that does not bind p300 did not have significantly different p53 levels from vector controls. As a positive control, HPV 16 E6 expressing cells were examined. Consistent with its well-documented ability to destabilize p53, HPV 16 expression prevented p53 accumulation. This is consistent with our hypothesis that p53 accumulation is p300-dependent and that H2CB induces the HP in a manner consistent with naturally occurring failed cytokinesis. We will use HPV Δ 8 E6 expressing cells to

confirm p300-dependence of these observations. Elevated p53 is expected to cause apoptosis. Interestingly, neither cell line underwent significantly increased H2CB-induced apoptosis during exposure (Figure 17). Instead, vector control cells had significantly increased apoptosis after H2CB. This was muted by HPV 8 E6.

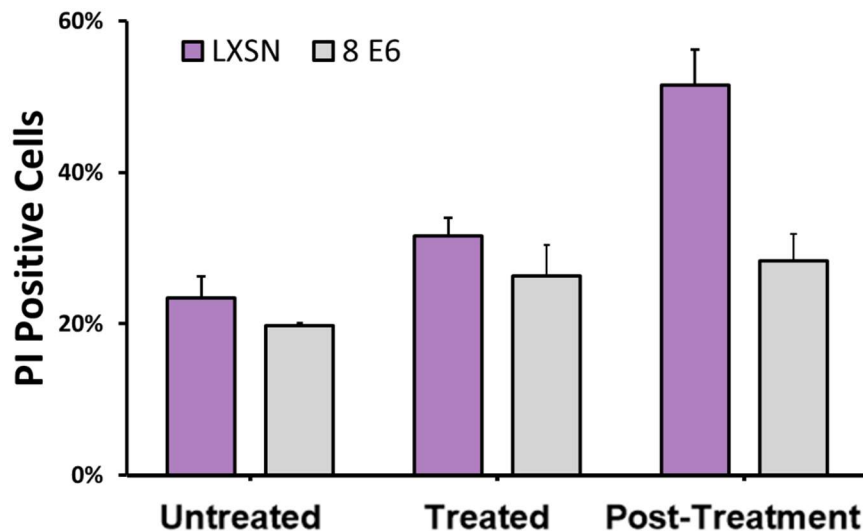


Figure 17: HPV 8 E6 prevents apoptosis after H2CB treatment. We used the Countess FL instrument purchased in this budget period to measure propidium iodide stained cells after H2CB treatment as a measure of apoptosis. Neither cell line underwent apoptosis during treatment. HPV 8 E6 blocked the apoptosis seen in control cells after the drug was removed.

Subtask 4: Determine the extent to which β -HPV E6 promotes TEAD promoter activity by luciferase reporter assay.

We hypothesize that reduced YAP abundance will result in diminished TEAD promoter activity. To test this, we transfected a Luciferase reporter construct into U2OS cells. The construct consists of a TEAD responsive promoter driving the expression of the firefly luciferase gene. As a transfection control, on the same plasmid, a strong promoter drives renilla luciferase gene expression. Vector control cells responded to increasing amount of H2CB with decreased TEAD activity. HPV 8 E6 augmented this reduction, leading to significantly less TEAD reporter expression (**Figure 18**). The mutant HPV 8 E6 that cannot destabilize p300 also loses its ability to decrease TEAD promoter activity after H2CB. These results were true in transformed (U2OS) cells and will be repeated in primary (human foreskin keratinocytes) cells. Deletion of HPV 8 E6's p300 binding site prevents the decreased TEAD promoter activity. We expect that the loss of TEAD promoter activity will result in decreased proliferative and be further manifest in increased senescence. We tested this using senescence associated β -galactosidase as a marker of senescent cells. Like with apoptosis the most profound effects were seen after treatment when vector control had an elevated number of β -galactosidase positive cells (**Figure 19**).

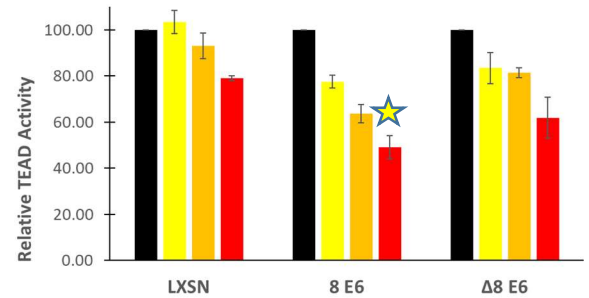


Figure 18: HPV 8 E6 augments H2CB-induced depression of TEAD Reporter Activity. Star denotes statistically significant difference from similar treated control cells, by student T-test. $p, 0.05$. All experiments were conducted in triplicate.

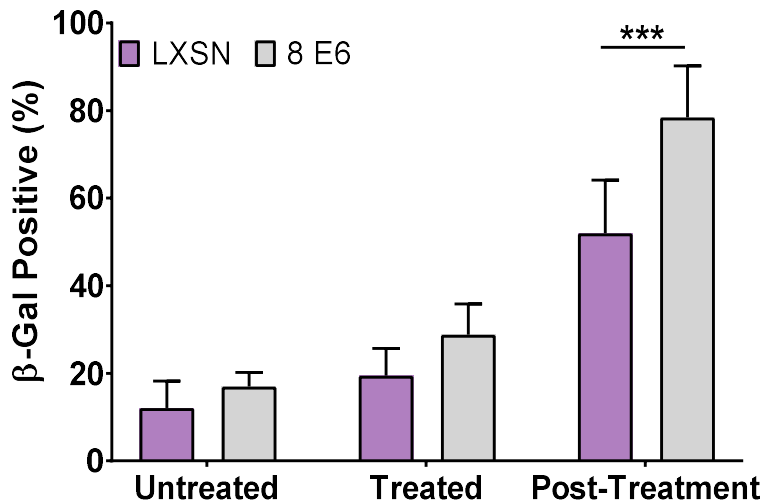


Figure 19: HPV 8 E6 increases senescence associated beta galactosidase positive cells during recovery from H2CB.

HPV 8 E6 significantly increased the amount of senescence during H2CB recovery.

Subtask 5: Define the prevalence of multipolar mitosis and micronuclei formation in β -HPV E6 expressing cells by immunofluorescence microscopy.

We have not begun working on this subtask, but it is not expected to be completed until the 18th month of support.

Milestones Achieved: We will...

- (1) obtain oversight from HRPO necessary to avoid unintentional or unethical mistreatment of the human subjects.

HRPO approval was obtained before the start of the reporting period (6/16/17). HRPO Log Number A-20356.

- (2) learn whether β -HPV E6 alters the phosphorylation of Hippo pathway proteins and if any such changes are p300-dependent.

We have shown that β -HPV E6 attenuates LATS2 activation as evidenced by a diminished proportion of total LATS2 being phosphorylated after H2CB-induced failed cytokinesis. This phenotype appears to be p300-dependent as the ratios remain the same as vector control cells when HPV Δ 8 E6 is expressed. While attenuated LATS2 phosphorylation correlates to a decreased accumulation of p53, it is not associated with any change in the YAP phosphorylation. The different responses of downstream LATS2 targets can be explained in at least 2 ways. Either LATS2 phosphorylation is less necessary for

it to phosphorylate YAP or other kinases are capable of phosphorylating YAP when LATS2 is unable. We favor the later explanation as other reports have already described other kinases such as AKT that phosphorylate YAP in certain conditions.

P53 stabilization after failed cytokinesis is in part so that the abnormal cell undergoes apoptosis. We hypothesized that H2CB will induce apoptosis in control cells and HPV Δ 8 E6 expressing cells to a greater extent than in cells expressing HPV 8 E6. Since propidium iodide staining is a canonical marker of dead/apoptotic cells, we used the Countess II FL (a purchase funded by this PRCRP support) to detect propidium iodide staining in H2CB treated cells. Supporting our hypothesis, HPV 8 E6 expression significantly dampened apoptosis after H2CB compared to vector control.

(3) learn whether β -HPV E6 prevents the subcellular localization of Hippo proteins induced by failed cytokinesis and if any such inhibition is p300-dependent

We have partially completed this Milestone, with only the portion expected to be completed in the second funding period left to pursue. Specifically, we have found that β -HPV E6 decreases the accumulation of p53 and decreases the amount of YAP in the nucleus and cytoplasm after H2CB treatment. Our data also suggest that this is p300 dependent as we do not see these changes in cells expressing HPV Δ 8 E6.

(4) learn whether β -HPV E6 increase TEAD promoter activity after failed cytokinesis and if they can whether or not it is a p300-dependent phenotype.

In contrast to our initial hypothesis, β -HPV E6 seems to block phosphorylation of one LATS2 target (p53) but not another (YAP). As a result, we were not surprised to find that TEAD promoter activity was reduced after H2CB treatment in cells expressing β -HPV E6. As mentioned above and as expected by the decreased TEAD promoter activity, HPV 8 E6 increased H2CB-induced senescence as measured by the presence of senescence-associated beta galactosidase in cells. Our current efforts include repeating the TEAD promoter experiments in HFKs and determining the extent to which HPV 8 E6 abrogates Hippo pathway signaling as cells recover from H2CB treatment.

(5) learn whether β -HPV E6 increases the likelihood of multipolar mitosis and micronuclei formation after failed cytokinesis as well as the role of p300-degradation in any such increases.

We will perform this analysis in the second budget period of this proposal.

Major Task 2: Determine the mechanism of β -HPV E6's inhibition of the HP.

Subtask 1: Define the abundance of HP proteins by immunoblot. This will be done in vector control, β -HPV E6 and β -HPV Δ E6 expressing cells.

We used immunoblots to determine the abundance of HP proteins in both U2OS and HFK cells expressing HPV 8 E6 (**Figure 12**). Although HPV 8 E6 attenuates other signaling pathways by decreasing the abundance of keystone proteins in those pathways, we did not find decrease HP protein abundance associated with HPV 8 E6 expression. Instead, there was more LATS2 in cells expressing HPV 8 E6. Given the observation that HPV 8 E6 decreases LATS2 phosphorylation (described in Major Task 1 of Specific Aim 2), we interpret these results as indicative of a feedback loop induced by failure to complete the Hippo pathway.

Subtask 2: Determine if p300 is involved in β -HPV E6-induced changes to the abundance of HP proteins using β -HPV Δ E6 mutant and immunoblot.

We immunoblotted cells expressing HPV Δ 8 E6 (**Figure 12**). Consistent with a p300-dependent changes in LATS2, deletion of the p300 binding domain prevented HPV 8 E6 from increasing LATS2 abundance.

Subtask 3: Determine if p300 is present at HP gene promoters by chromatin immunoprecipitation and qPCR.

This subtask is not expected to be started until the second funding period, but we have already gained insight into this task. Namely, the observed increased expression of LATS2 suggests that p300 acts as a transcriptional repressor and may be found at the LATS2 promoter.

Subtask 4: Perform an unbiased analysis of β -HPV E6's effect on the HP using HP PCR Array purchased

from Qiagen and validated by qPCR as well as immunoblot.

We found a more cost-effective way of performing an unbiased analysis of the impact that β -HPV E6's degradation of p300 has on Hippo Pathway proteins. The cancer cell line encyclopedia provides publicly available RNAseq data from over 1000 cell lines. We identified the 8% of these cell lines that have significantly reduced p300 expression as

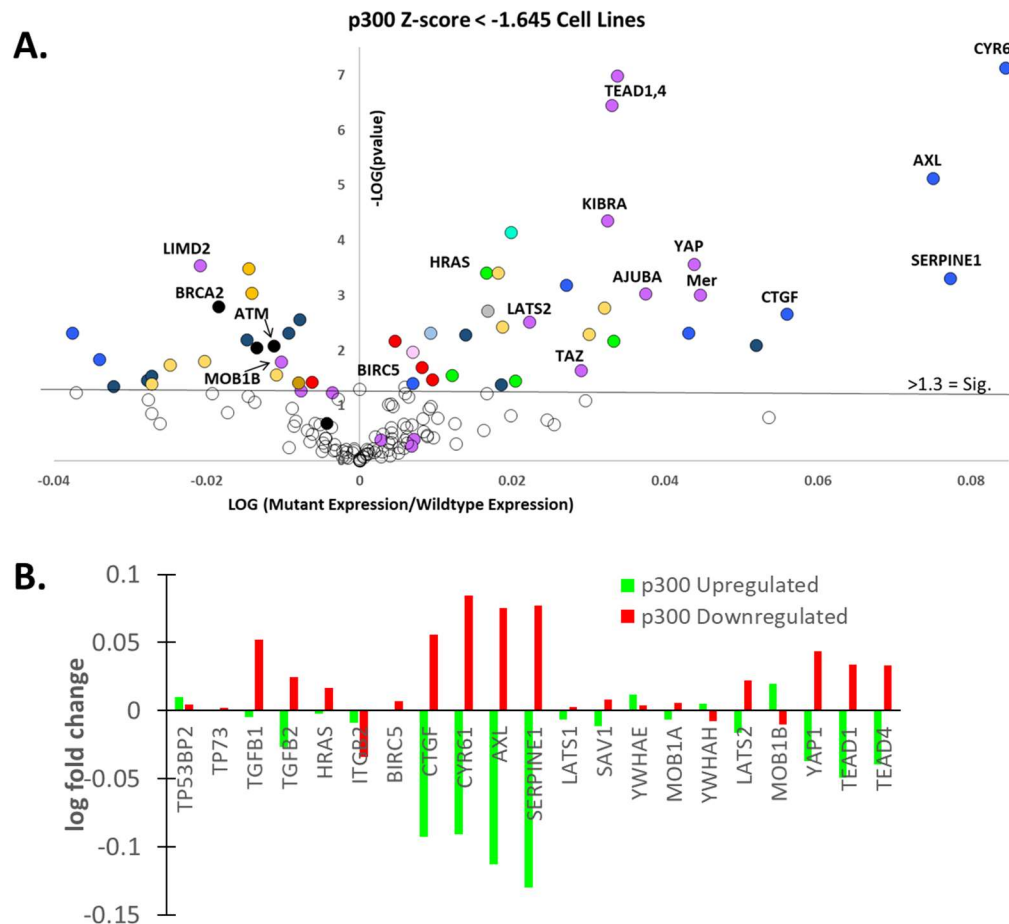


Figure 20: RNAseq analysis of p300-responsive genes: A. Volcano blot depicting changes to Hippo pathway genes when p300 expression is reduced. B. Comparison of the change in expression of notable Hippo pathway genes when p300 is upregulated or downregulated. Purple circles denote canonical Hippo pathway genes, while blue circles note TEAD responsive genes.

well as the 2.7% of these cell lines with significantly increased p300 expression. We next compared HP gene expression among cell lines with reduced p300 expression and those with normal or elevated p300 expression. This analysis shows decreased p300 expression is accompanied by significant increases in canonical TEAD-responsive genes (CYR61, AXL, SERPINE1 and CTGF), LATS2 and YAP/TAZ (Figure 18A). Further, the relationship is nearly universally inverted when we examined HP gene expression in cells where p300 was increased. These data complement our *in vitro* observations, further confirming that p300 degradation does not inhibit downstream TEAD responsive genes. Our analysis of H2CB suggests that the deregulation of the HP pathway created by decreased p300 is manifest most significantly during recovery from H2CB treatment.

Milestones Achieved: We will learn...

(1) whether β -HPV E6 changes the abundance of select HP proteins by destabilizing p300.

β -HPV E6 increases LATS2 abundance in a p300 dependent manner. We did not find the amount of any other HP protein changed with β -HPV E6 express.

(2) whether p300 is at the promoters of HP genes and whether β -HPV E6 changes the abundance of p300 at these promoters.

We have not begun this milestone, but our immunoblot and computational analysis suggests that p300 acts as a repressor of LATS2 expression and will be found at its promoter.

(3) the comprehensive impact of β -HPV E6 on HP gene expression.

Our bioinformatics analysis of HP gene expression suggests that p300 is a repressor of LATS2 expression and that despite increased LATS2 abundance TEAD responsive genes are expressed at high levels.

Specific Aim 3: Determine how β -HPV E6 induces p300-independent inhibition of DNA repair.

Major Task 1: P300-Independent Disruption of DNA Crosslink Repair

Subtask 1: Finish Onboarding for Dalton Dacus and obtain HRPO approval to isolate keratinocytes from neonatal foreskins.

HRPO approval was obtained before the start of the reporting period (6/16/17). HRPO Log Number A-20356. Dalton Dacus has been successfully onboarded and completed his regulatory and safety training.

Subtask 2: Define ICL-repair in HT1080 cells where β -HPV cannot degrade p300 by immunofluorescence microscopy using antibodies against UV-induced ICLs.

The results obtained in Specific Aim 1 and depicted in Figure 6 have caused us to refocus some of the efforts in Specific Aim 3. This new direction follows directly from new and exciting data, but still squarely falls under the goals of Specific Aim 3. While examining the sensitivity of HPV 8 E6 expressing cells to zeocin-induced DSBs, we noted a statistically significant increase in sensitivity between HPV Δ E6 and vector control. Zeocin is significantly less toxic to cells expressing HPV Δ 8 E6 than it is to cells expressing wildtype HPV 8 E6. These data support the aim's hypothesis that HPV 8 E6 disrupts DNA repair through p300-independent and -dependent mechanisms. We will repeat these experiments in HT1080 to confirm p300-independent sensitization to Zeocin.

Subtask 3: Define ICL-repair when exogenous expression of degradation resistant p300 prevents β -HPV E6 from degrading p300 using immunofluorescence microscopy with antibodies against UV-induced ICLs.

We have delayed these experiments because of the observation that HPV 8 E6 disrupts crosslink and DSB repair through mechanisms not dependent on p300 degradation. Double strand breaks are the most deleterious form of DNA lesion, so we've begun to "follow the data" on this new and exciting path. Our ongoing experiments parallel the ones originally proposed. We are currently measuring the kinetics of DSB repair in cells expressing HPV Δ 8E6 by immunofluorescence microscopy using H2AX as a marker. Once these experiments are completed, we will determine the kinetics of ICL and DSB repair in cells expressing the degradation resistant p300 mutant discussed in this subtask.

Subtask 4: Determine if β -HPV E6 can further impede crosslink repair in cells where ATR and p300 are targeted for RNAi mediated degradation (Assayed by immunofluorescence microscopy).

These mechanistic studies may be expanded to also include targeting BRCA1 and BRCA2 by RNAi because β -HPV E6 blocks DSB repair by decreasing their abundance.

Milestones Achieved: We will...

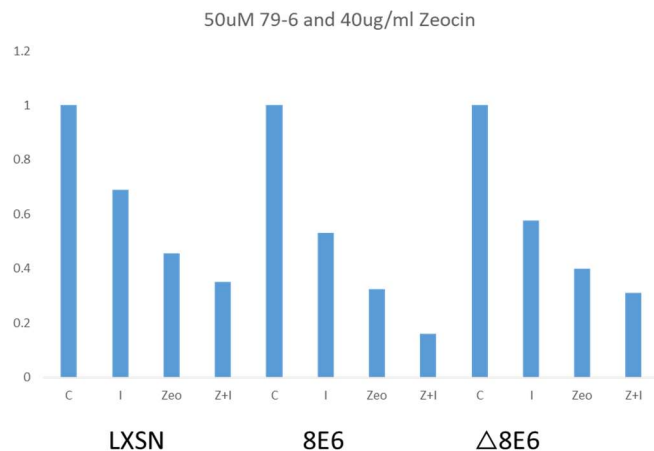


Figure 21: HPV8 E6 sensitizes cells to BCL6 inhibition in a p300 independent manner. Graph depicts relative viability in a p300 independent assays. C denotes mock treated control cells., I denotes cells treated with 79-6, a BCL6 inhibitor. Zeo denotes cells treated with Zeocin. Z+I denotes cells treated with both Zeocin and 79-6. N=1.

independent mechanisms.

We observed an unexpected likely p300-independent inhibition of DSB repair and are working to define the mechanism(s) by which HPV Δ8 E6 sensitizes cells to zeocin-induced DSBs.

(4) *learn the extent to which β-HPV E6 prevents ICL repair through p300- and ATR-independent mechanisms.*

We are excitedly characterizing HPV Δ8 E6's ability to disrupt DSB repair. These result will synergize with the planned efforts to dissect β-HPV E6's disruption of ICL repair, as unrepaired ICLs become DSBs.

Major Task 2: Determine the mechanism of β-HPV E6's p300-independent inhibition of DNA crosslink repair.

Subtask 1: Define the extent to which BCL6 inhibition prevents β-HPV E6 from preventing DNA crosslink repair by immunofluorescence microscopy and chemical inhibition and RNAi-mediated knockdown of BCL6.

We have previously shown that β-HPV E6 increases BCL6 abundance independent of its ability to destabilize p300. BCL6 expression allows B-cells to undergo DSB repair while continuing to replication. While uncoupling DSB repair from cell cycle progression is necessary for B-cell somatic hypermutation, it would be deleterious to a keratinocyte. Therefore, we hypothesize that β-HPV E6 sensitizes cells to Zeocin by increasing BCL6 abundance. We have begun testing this by determining if the BCL6 inhibitor, 79-6, decreases the Zeocin sensitivity caused by HPV Δ8 E6. Since BCL6 also promotes proliferation, we also expect that 79-6 will decrease growth in HFKs expressing HPV 8 E6 or HPV Δ8 E6 more than vector control cells. Our initial results support this. Although the results need to be repeated, 8 E6 expression increased sensitivity to 79-6 independent of its ability to destabilize p300 (**Figure 21**).

Subtask 2: Determine if β-HPV E6 interacts with ATR/ATRIP using Co-immunoprecipitation reactions.

This subtask was not expected to be completed before the beginning of the 3rd funding period. We have not begun the task, but have an agreement in place to acquire HFK cell lines immortalized with hTERT

(1) *obtain oversight from HRPO necessary to avoid unintentional or unethical mistreatment of the human subjects.*

HRPO approval was obtained before the start of the reporting period (6/16/17). HRPO Log Number A-20356.

(2) *get the staff necessary to complete this aim.*

A graduate student, Dalton Dacus, was successfully recruited to our team. He works on this aim as well as Aim2 and has presented his work at local and regional conferences highlighted by an oral presentation at the **University of Nebraska's Center for Virology Flyswat Meeting**.

(3) *learn the extent to which β-HPV E6 prevents ICL repair through p300-*

that stably express HA-tagged HPV 8 E6. These cells will be used for the co-immunoprecipitation proposed here.

Subtask 3: Determine if BCL6 is acting as a transcriptional repressor of ATR expression using chromatin immunoprecipitation.

This subtask was not expected to be completed before the beginning of the 3rd funding period. We have not begun the task.

Subtask 4: Define the impact of β -HPV E6 on BCL6 protein stability and transcription using immunoblots and qPCR.

This subtask was not expected to be completed before the beginning of the 2nd funding period. We have not begun the task.

Subtask 5: Identify novel β -HPV E6 interacting proteins by mass spectrometry with validation by coimmunoprecipitation.

As noted above, we will shortly be receiving the HPV 8 E6-HA tagged cell lines needed to do this experiment. The material transfer agreement between Kansas State University and the University of Texas Medical Branch has been signed by all parties. We expect to have the cells before the end of August 2018.

Milestone(s) Achieved: We will learn...

(1) whether β -HPV E6 inhibits ICL repair through BCL6 inhibition.

We have found that β -HPV E6 sensitizes cells to Zeocin through a p300-independent mechanism. This suggests a defect in DSB repair. Our working hypothesis is that both this inhibition and the inhibition of ICL repair both occur through the induction of BCL6, which prevents cell cycle regulation in response to DNA damage. Our pilot experiment supports this idea as inhibition of BCL6 was more toxic in cells expressing either HPV 8 E6 or HPV Δ 8 E6, suggesting acquired reliance on the increased BCL6 expression.

(2) whether β -HPV E6 interacts with ATR/ATRIP.

We have not begun working on this aim. However, we have obtained the cell lines necessary to begin characterizing the β -HPV E6 interactome.

(3) whether β -HPV E6 induced increases in BCL6 result in transcriptional repression of ATR.

Consistent with our initial assessment, we anticipate working on this milestone in the second or third budget period.

(4) the extent to which β -HPV E6 changes BCL6 protein stability.

Consistent with our initial assessment, we anticipate working on this milestone in the second budget period.

(5) the extent to which β -HPV E6 changes BCL6 transcription.

We expect to begin working on this milestone in later budget periods.

(6) the identity of novel β -HPV E6 interacting proteins.

We have signed an agreement to obtain the cell lines necessary to achieve this milestone.

- **What opportunities for training and professional development has the project provided?**
 - Both graduate students funded in this project presented their work at regional conferences. Dalton Dacus won an award for his oral presentation at the celebration of Kansas State University Division of Biology's 50th anniversary. He was also selected to give an oral presentation at the 2018 DNA tumor virus meeting in August. The PI received significant mentoring from Drs. Laimins, Gao and Clem. Further, he was selected to chair a session at the 2018 DNA tumor virus meeting.
 - **How were the results disseminated to communities of interest?**
 - Our group is highly engaged in the dissemination of our findings to the local regional and international communities of interest.
 - We have a very active twitter account (@wallacehplab) that we use to communicate our work to a network of followers.
 - We also engaged over 100 community members in hands-on tours of our lab. Given our proximity to a US Army installation, this very likely included persons with connections to Fort Riley. We cannot be certain as no identifying information is collected from our guests.
 - We participated in two events organized by the K-State Office for the Advancement of Women in Science and Engineering to promote the participation of women in science (Girls Reaching Our World and EXploring sCIence, Technology and Enginerring)m
 - **What do you plan to do during the next reporting period to accomplish the goals?**
 - Dr. Wallace will continue his career development by presenting at the International Papillomavirus meeting in October. He is chairing a session of the DNA tumor virus meeting and giving an invited seminar at Gold Coast University in Australia. He will also continue to discuss his career and interact with each of his three mentors, with specific plans to visit Dr. Laimins in the spring of 2019.
 - We are happy with our progress towards our goals, but expect even greater strides during the second budget period. Specifically, we will shortly obtain immortalized keratinocyte cell lines expressing tagged β -HPV E6 so that we can define the β -HPV E6 interactome.
 - Both graduate students will have greater experience increasing their productivity
 - Though not funded, we received a favorable review of our submission to the American Cancer Society, which we will resubmit. We will also submit at least 1 R01 grant application to the National Institutes of Health.
4. **IMPACT:** *Describe distinctive contributions, major accomplishments, innovations, successes, or any change in practice or behavior that has come about as a result of the project relative to:*
- **What was the impact on the development of the principal discipline(s) of the project?**
 - β -HPV infections are believed to cause cancer by increasing the ability of sunlight and radiation to cause skin cancer. We have hypothesized that

they do this by preventing the cells they infect from properly responding to damaged DNA. In this period, we have found support for this idea by showing the proteins from this virus hinder cells from fixing UV and radiation damage. They also make it more likely that a cell will experience errors when dividing. These results are important for the military community in particular for two reasons. 1. Military service is a risk factor for skin cancers. 2. Military service is associated with increased sun and radiation exposure.

- **What was the impact on other disciplines?**
 - Our work has broad impacts as it helps clarify the role of p300 in signaling pathways known to suppress tumors. This includes investigators studying the Hippo Pathway, double strand break repair and crosslink repair. Studying the effects of β -HPV E6's ability to increase BCL6 abundance teaches us about BCL6 biology, providing impact to people studying B-cell biology.
- **What was the impact on technology transfer?**
 - *The overall goal of this project is to determine the oncogenic potential of β -HPV infections so that anti-viral drugs or vaccines can be developed. Our results remain supportive of this goal.*
- **What was the impact on society beyond science and technology?**
 - Efforts to prevent cancer will always have the potential to impact society at large. Our work remains impactful in this manner.
 - Our outreach and engagement efforts also help grow lay knowledge of science and encourage participation in science by underrepresented members of society
- 5. **CHANGES/PROBLEMS:** *The Project Director/Principal Investigator (PD/PI) is reminded that the recipient organization is required to obtain prior written approval from the awarding agency Grants Officer whenever there are significant changes in the project or its direction. If not previously reported in writing, provide the following additional information or state, "Nothing to Report," if applicable:*
 - **Changes in approach and reasons for change**
 - *We found that β -HPV E6 has p300-independent functions that inhibit double strand DNA break repair in addition to intrastrand crosslink repair. We have begun characterizing this attenuation of DSB repair.*
 - We found that β -HPV E6 manifest a greater ability to recover from H2CB treatment than it does the initial response to that the treatment. We have completed most of the planned experiments relevant to the Hippo Pathway and are expanding to characterize the ability of β -HPV E6 and related oncogene β -HPV E7 to destabilize cellular genomes after H2CB treatment.
 - We found a publically available online data set that allowed us to perform an unbiased screen of Hippo Pathway gene expression in cells with diminished p300 expression. This cost effective approach was adopted.
 - **Actual or anticipated problems or delays and actions or plans to resolve them**
 - We have made more progress than anticipated on some aspects of this study. Although we endeavored to stay as close to the original SOW, we

have in some cases reprioritized our efforts directing them towards new and exciting findings (Hippo pathway vs p300-independent inhibition of UV repair). Notably, this represents the logical progression from the work funded by this proposal and not a departure from the SOW.

- **Changes that had a significant impact on expenditures**
 - We were fortunate to replace a costly screen of Hippo Pathway genes with a computation approach. The savings from this have been redirected, enabling us to expand the originally conceived work on this signaling cascade.
 - **Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents**
 - Nothing to report
 - **Significant changes in use or care of human subjects**
 - Nothing to report
 - **Significant changes in use or care of vertebrate animals.**
 - Nothing to report
 - **Significant changes in use of biohazards and/or select agents**
 - Nothing to report
6. **PRODUCTS:** *List any products resulting from the project during the reporting period. If there is nothing to report under a particular item, state "Nothing to Report."*
- **Publications, conference papers, and presentations.**
 - **Journal publications.**
 - Wendel SO, **Wallace NA**; Loss of Genome Fidelity: Beta HPVs and the DNA Damage Response; Front Microbiol; 8:2250; 2017 Published ([link](#))
 - Acknowledgement of Federal Support: Yes
 - Murthy V, Dacus D, Gamez M, Hu C, Wendel SO, Snow J, Kahn A, Walterhouse SH, **Wallace NA**. Characterizing DNA Repair Processes at Transient and Long-lasting Double-strand DNA Breaks by Immunofluorescence Microscopy; J Vis Exp; 136; 2018; Published ([link](#))
 - Acknowledgement of Federal Support: Yes
 - **Wallace NA** and Munger K; The Curious Case of APOBEC3 activation by Cancer-Associated Human Papillomaviruses. PLoS Pathogens; 14(1); 2018. Published ([link](#))
 - Acknowledgement of Federal Support: Yes
 - **Books or other non-periodical, one-time publications.**
 - Nothing to report
 - **Other publications, conference papers, and presentations.**
 - "Human Papillomavirus and the DNA Damage Response" **Wallace NA**. 06/15/2018. Poster Presentation at K-State Research Showcase (Local Society)
 - "Human Papillomavirus and the DNA Damage Response" **Wallace NA**. 01/13/2018. Oral Presentation at K-INBRE Symposium (Regional Society)

- Wendel SO. **Wallace NA** “HPV oncogenes disrupt an essential DNA Damage Tolerance Pathway” 03/19/2018 Oral Presentation at University of Nebraska 17th Annual Viral Symposium (Regional Society)
- Dacus DC. **Wallace NA** “ β -HPV E6 disrupts the Hippo pathway after failed cytokinesis” 03/19/2018 Oral Presentation at University of Nebraska 17th Annual Viral Symposium (Regional Society)
- Murthy V. **Wallace NA** “ β -HPV E6 attenuates Nucleotide Excision Repair” 03/19/2018 Poster Presentation at University of Nebraska 17th Annual Viral Symposium (Regional Society)
- **Website(s) or other Internet site(s)**
 - www.WallaceLabKSU.weebly.com
 - **This is our personal lab website. It broadcasts our twitter handle and announces major accomplishments.**
 - @wallaceHPVlab is our twitter handle.
 - This twitter account disseminates the daily activities and science news from our group. We use it to connect with our nearly 500 followers. It is an effective outreach tool.
- **Technologies or techniques**
 - Nothing to report
- **Inventions, patent applications, and/or licenses**
 - Nothing to report
- **Other Products**
 - Nothing to report

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

- **What individuals have worked on the project?**

Name:	<i>Dalton Dacus</i>
Project Role:	<i>Graduate Student</i>
Researcher Identifier (e.g. ORCID ID):	<i>Not Applicable</i>
Nearest person month worked:	<i>12</i>
Contribution to Project:	<i>Mr. Dacus performed most of analysis of the hippo pathway (Aim 2) and some of the work for AIM3</i>
Funding Support:	<i>CDMRP and Wallace Startup funds</i>

▪

Name:	<i>Changkun Hu</i>
Project Role:	<i>Graduate Student</i>

Researcher Identifier (e.g. ORCID ID):	<i>Not Applicable</i>
Nearest person month worked:	<i>12</i>
Contribution to Project:	<i>Mr. Hu has performed the analysis of the NHEJ pathway.</i>
Funding Support:	<i>CDMRP and Wallace Startup Funds</i>

■

Name:	<i>Vaibhav Murthy</i>
Project Role:	<i>Research Assistant</i>
Researcher Identifier (e.g. ORCID ID):	<i>Not Applicable</i>
Nearest person month worked:	<i>4</i>
Contribution to Project:	<i>Mr. Murthy has performed the analysis of the NER pathway. He also was the first author on the methods paper supported by the CDMRP.</i>
Funding Support:	<i>Wallace Startup funds</i>

○

Name:	<i>Nicholas Wallace</i>
Project Role:	<i>Primary Investigator</i>
Researcher Identifier (e.g. ORCID ID):	<i>0000-0002-3971-716X</i>
Nearest person month worked:	<i>3</i>
Contribution to Project:	<i>Dr. Wallace oversaw the work on each projects. He wrote and edited all manuscripts. He also submitted grants to fund future relevant studies. He mentored the graduate students, post doc and research assistant in his lab. He also presented the lab's findings to external and internal audiences.</i>
Funding Support:	<i>CDMRP Support and Kansas State University Salary Support</i>

■

Name:	<i>Sebastian Oliver Wendel</i>
Project Role:	<i>Post-Doctoral Fellow</i>

Researcher Identifier (e.g. ORCID ID):	<i>Not Applicable</i>
Nearest person month worked:	<i>2</i>
Contribution to Project:	<i>Dr. Wendel was the primary author reviewing the mutagenic potential of β-HPVs published this budget period.</i>
Funding Support:	<i>K-INBRE Post-Doctoral Fellowship and Wallace Startup Funds</i>

Name:	<i>Laimonis Laimins</i>
Project Role:	<i>Designated Mentor</i>
Researcher Identifier (e.g. ORCID ID):	<i>0000-0002-6314-623X</i>
Nearest person month worked:	<i>1</i>
Contribution to Project:	<i>Dr. Laimins advised and mentored Dr. Wallace as necessary throughout the budget period.</i>
Funding Support:	<i>Dr. Laimin is supported by 2 RO1's from the NCI and 1 R21 from NIAID.</i>

- **Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?**
 - Nothing to Report.
 - **What other organizations were involved as partners?**
 - Nothing to Report.
8. **SPECIAL REPORTING REQUIREMENTS**
- Nothing to Report
9. **APPENDICES:**
- *PDF versions of the three manuscripts published during this reporting period are appended below.*

Video Article

Characterizing DNA Repair Processes at Transient and Long-lasting Double-strand DNA Breaks by Immunofluorescence Microscopy

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URL: <https://www.jove.com/video/57653>

DOI: [doi:10.3791/57653](https://doi.org/10.3791/57653)

Keywords: Cancer Research, Issue 136, Immunofluorescence microscopy, DNA repair, double-strand breaks, repair kinetics, homologous recombination, non-homologous end joining, H2AX

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Abstract

The repair of double-stranded breaks (DSBs) in DNA is a highly coordinated process, necessitating the formation and resolution of multi-protein repair complexes. This process is regulated by a myriad of proteins that promote the association and disassociation of proteins to these lesions. Thanks in large part to the ability to perform functional screens of a vast library of proteins, there is a greater appreciation of the genes necessary for the double-strand DNA break repair. Often knockout or chemical inhibitor screens identify proteins involved in repair processes by using increased toxicity as a marker for a protein that is required for DSB repair. Although useful for identifying novel cellular proteins involved in maintaining genome fidelity, functional analysis requires the determination of whether the protein of interest promotes localization, formation, or resolution of repair complexes.

The accumulation of repair proteins can be readily detected as distinct nuclear foci by immunofluorescence microscopy. Thus, association and disassociation of these proteins at sites of DNA damage can be accessed by observing these nuclear foci at representative intervals after the induction of double-strand DNA breaks. This approach can also identify mis-localized repair factor proteins, if repair defects do not simultaneously occur with incomplete delays in repair. In this scenario, long-lasting double-strand DNA breaks can be engineered by expressing a rare cutting endonuclease (e.g., I-SceI) in cells where the recognition site for the said enzyme has been integrated into the cellular genome. The resulting lesion is particularly hard to resolve as faithful repair will reintroduce the enzyme's recognition site, prompting another round of cleavage. As a result, differences in the kinetics of repair are eliminated. If repair complexes are not formed, localization has been impeded. This protocol describes the methodology necessary to identify changes in repair kinetics as well as repair protein localization.

Video Link

The video component of this article can be found at <https://www.jove.com/video/57653/>

Introduction

Each day, every cell in the human body is bombarded with an estimated 10,000 DNA lesions¹. This existential threat puts us at risk for mutations, oncogenesis as well as cell death. To protect genome fidelity, mammalian cells have evolved to respond to DNA damage with a complex series of protein associations and modifications. This response is organized into multiple pathways, collectively known as the DNA damage response (DDR)^{2,3}. The DDR consists of the accumulation of DNA repair proteins at DNA lesions, coordinated both temporally and spatially. DDR frequently induces cell cycle arrest to avoid the propagation or intensification of damage that can occur during the replication of damaged DNA^{2,4,5}. In turn, it is also necessary for the cellular viability to turn off cell cycle arrest by disassociating repair complexes after repair has been completed.

Among the various types of DNA damage, DSBs are the most deleterious. Failure to repair DSBs can result in chromosome rearrangements or large-scale deletions such as the loss of entire chromosome arms. The repair of DSBs is divided into two pathways^{6,7,8}. Homologous recombination (HR) requires a sister chromosome to use as a DNA template and thus is limited to late S and G2/M phases of the cell cycle^{9,10}. Non-homologous end joining (NHEJ) does not have these restrictions but can cause small deletions when repairing DSBs^{11,12}.

DSB repair specifically and the DDR in general are active areas of investigation. Despite being organized into conveniently separated pathways, there is a great deal of redundancy. Indeed, many proteins (BRCA1, BRCA2, and the RPA complex for example) are involved in multiple pathways^{13,14,15,16}. The repair of a lesion by one pathway, can lead to a damage intermediate that must be repaired by another pathway¹⁴. The

intertwining of these pathways, combined with their complex task of recruiting the right proteins to the correct place for the precise amount of time necessary, requires a multi-tiered regulatory process.

A recent report highlights the intricacies of DDR by demonstrating that repair complex formation, resolution, and localization can each separately be impaired¹⁷. The overall goal of the following protocol is to definitively dissect the ability of cells to repair DSB. Using immunofluorescence microscopy, accumulation of repair proteins at the sites of damage can be visualized at the representative time points following the induction of DSBs.

This technique has several advantages to commonly used approaches. Frequently, repair is investigated at single time points and incapable of representing the dynamic process of assembly and dissociation of repair complexes. Observing the full range of repair from the initial activation to the full resolution ensures that a delay in repair is not misidentified as complete inhibition. Conversely, it assures that the induction of a repair response that is unable to inactivate said response is not misidentified as the normal or the excessive activation.

Delayed protein complex formation and the mis-localization of repair proteins, however, may not be unambiguously distinguished with this approach. To determine if repair proteins are mis-localized versus delayed in their localization, a "long-lasting" DSB can be introduced through enzymatic cleavage of cellular DNA. The resulting lesion is recut each time it is repaired, resulting in a distinct large nuclear repair focus and removing the temporal restriction from recruitment. This can be achieved by modifying the existing approach with the use of a rare cutting endonuclease (e.g., I-SceI) to induce a long-lasting DSB. The longevity of DSBs enables the visualization of elusive repair proteins by immunofluorescence microscopy. The enhanced abundance could also improve the visualization when detection is hindered by limitation in the antibody quality, a feature that could be useful when lesser studied proteins are identified as having an impact on DNA repair.

Notably, we provide explicit instructions for a free image processing and analysis software (e.g., ImageJ). This removes a major financial barrier in image analysis, opening the interrogation of DNA damage repair to a wider audience.

Protocol

Please note, this protocol is written for U2OS cells containing an I-SceI recognition site¹⁸. The cells need not be U2OS but must contain the I-SceI site. The protocol may need to be adjusted (e.g., number of cells seeded and incubation times) depending on the type of cells used.

1. Defining the Kinetics of DSB Repair Complex Formation

1. Grow U2OS-DRGFP cells on a 10 cm tissue culture plate until 85–90% confluent.
2. Remove the media and incubate at room temperature (RT) in 3 mL of EDTA for 2 min.
3. Replace EDTA with 1 mL of trypsin and incubate at 37 °C for 5 min. Ensure that the cells are detached from the surface of the plate by viewing under a microscope. Neutralize trypsin with 5 mL of Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin.
4. Determine the concentration of cells in this suspension using a hemocytometer and the trypan blue exclusion method.
5. Seed 6×10^3 cells/well in 200 μ L of a glass-bottom 96-well plate. Alternatively, seed 4×10^6 cells in 8 mL on 12 mm coverslips placed in a 10 cm plate.
NOTE: Each well or coverslip represents a single time point, including the control.
6. Grow the cells overnight at 37 °C and 5% CO₂.
7. **Inducing DSBs**
 1. Replace the media from the 96-well plate/10 cm dish with H₂O₂ diluted to a concentration of 25 μ M with DMEM + 10% FBS and incubate at 37 °C for 1 h.
 2. Wash the cells 3x with 1x PBS and replace with fresh DMEM supplemented with 10% FBS and 1% penicillin/streptomycin. Incubate the cells at 37 °C for 0, 1, 4, 8, 24, and 48 h. Ensure that fresh dilutions of H₂O₂ are used for each experiment.
NOTE: To avoid killing cells at the H₂O₂ concentration used here, ensure that the dose of damage is low enough such that the bulk of cells will avoid apoptosis and senescence. It is critical to observe the recovery process. This is best achieved by measuring sensitivity to a range of doses. Perform an MTT assay or other cell viability assay to determine the minimal concentration of hydrogen peroxide that can be used.
8. **Fixing and permeabilizing cells**
 1. Wash the cells 3 times with 1x PBS. Remove a coverslip from the 10 cm plate for each time point and place it in a single well of a 24-well plate for fixing. Use the needle and forceps to carefully remove the coverslip and avoiding scratching cells off the coverslip surface.
 2. Fix the cells by incubating for 15 min with 4% paraformaldehyde (PFA) at designated times after 1 h of H₂O₂ exposure and fresh DMEM replacement (0, 1, 4, 8, 24, and 48 h).
 3. Wash the fixed coverslips 3 times with PBS. Permeabilize the cells with 0.5% non-ionic detergent diluted in 1x PBS. Incubate for 15 min at RT, before washing 3x with PBS.
NOTE: After permeabilization, storage of coverslips is possible in PBS at 4 °C for at least a week.
9. **Repair complex visualization**
 1. Block the 96-well plate/coverslips in a solution of 3% bovine serum albumin (BSA) and 0.1% non-ionic detergent diluted in 1x PBS (3% BSA solution) for 1 h at RT.
 2. Incubate with primary antibodies that target the repair protein of interest, diluted according to the manufacturer's specifications in 3% BSA solution for 1 h at RT, before washing 3 times with 1x PBS.
 3. Incubate the 96-well plate/coverslips with secondary antibody diluted according to the manufacturer's protocol in 3% BSA solution. Confirm that the secondary fluorophores do not have overlapping excitation or emission spectra.
 4. After washing the cells with PBS 3 times add DAPI, diluted according to the manufacturer's specifications in 1x PBS and incubate at RT for 5 min.

5. Mount the coverslips onto slides with a drop of mounting agent, making sure the cell-side faces down. Press the coverslips carefully and soak up any excess mounting agent with a wipe.
6. Seal the coverslips with fast-drying transparent nail polish and ensure a complete seal of the coverslip with the slide.
7. Take the confocal microscope images by focusing on the DAPI channel, before other channels (with signals from DNA repair gene of interest) to avoid introducing bias into the experiment.
8. Alternatively, images can be acquired by taking Z-sections of the desired region and condensing the image into a single plane to visualize all detectable foci.
NOTE: If DSB resolution or protein of interest is not observed at time points chosen, determine when DSBs resolve by selecting a wide range of time points initially (0–48 h post DSB induction). The time points of interest will vary by the method of DSB induction and the repair protein of interest.

10. Use free ImageJ software to define the nuclei in the immunofluorescence microscopy images.

NOTE: To open microscope images, the Bio-Formats plugin must be installed in ImageJ.

1. Open the confocal images (.czi or .tif) by dragging the image file into the ImageJ window, or by selecting "Bio-Formats Importer" from the "Plugin" toolbar in ImageJ.
NOTE: The "Bio-Formats Import Option" window will appear.
2. Select "Split Channels" to split the image into constitutive DAPI and fluorescent images within the "Bio-Formats Import Option" window.
3. Select "Colorized" in "Color options" from the drop-down menu. Select "OK" to open the DAPI and Histone H2AX (H2AX) images as separate windows and choose the DAPI image.
4. Select the "Image" toolbar and "Adjust Threshold" option to select nuclei.
NOTE: A "Threshold" window will appear.
5. Adjust the image threshold by sliding the bottom bar of the "Threshold" window completely to the right. Adjust the top bar until the nuclei appear completely red with distinct outlines, and the background black. DO NOT select apply. Close the "Adjust Threshold" window.
6. Select the "Analyze" toolbar and "Analyze Particles" option. See an "Analyze Particles" window appearing on the screen.
7. Input the minimum size of a nucleus.
NOTE: Particles below the size inputted will not be counted as nuclei.
8. From the "Show" dropdown menu, select "Outlines". Select the "Add to Manager" option. Click "OK" to open an "ROI Manager" window.
NOTE: Outlines of nuclei will appear in a separate window.
9. Assign numbers to the nuclei that can be selected from the "ROI Manager" window.

11. Use ImageJ to quantify H2AX foci in immunofluorescence microscopy images.

1. Select the "H2AX" foci window (stained with fluorescently conjugated secondary antibody). Then, select the "Process" toolbar and choose "Find Maxima" to find the foci within the nuclei.
NOTE: A "Find Maxima" window will open.
2. Choose the "Single Points" option from the "Output Type" dropdown menu and select "Preview point selection" to visualize the maxima/foci. Input a "Noise Tolerance" value depending on the fluorescence intensity of the image (**Figure 1**). Check for the appearance of a white window with small black dots.
NOTE: These dots represent the H2AX foci/maxima that have been detected. The noise tolerance value must be maintained constant for all images being analyzed. A low/high noise tolerance will depict too many/few maxima (foci) within the nucleus and will not be an accurate representation of the foci within the nucleus.
3. Select the nuclei for which H2AX foci must be quantified for from the "ROI Manager" window. Select all the nuclei by checking the "show all" option.
4. Select "Measure" to measure the number of maxima/foci within the selected nuclei.
NOTE: The number of maxima/foci appear as multiples of 255, *i.e.*, one maximum has a "RawIntDen" (Integrated density) reading of 255.
5. Copy the "RawIntDen" values and paste them into software for data analysis.

2. Analyzing Localization to a Long-lasting Double-strand DNA Break

1. Seed cells on 96-well plates/coverslips as described in sections 1.1 and 1.2.
2. **Induction of double-strand DNA breaks**
 1. Transfect cells with the I-SceI expression vector using a lipid-based transfection reagent according to the manufacturer's specifications. Wait 24–72 h after transfection to maximize the endonuclease expression. Determine if the transfection was successful by locating cells with a singular large nuclear γ -H2AX focus.
3. **Acquisition of images**
 1. Fix, permeabilize, block, and wash the 96-well plate/coverslips as described in section 1.8.
 2. Co-incubate cells with an antibody against γ -H2AX and the repair protein of interest (here, a primary antibody against RAD51 was used) diluted according to the manufacturer's specifications in 3% BSA solution.
 3. Wash the 96-well plate/coverslips 3 times with 1x PBS. Incubate the 96-well plate/coverslips with secondary antibody diluted according to the manufacturer's protocol in 3% BSA solution. Confirm that the secondary fluorophores do not have overlapping excitation or emission spectra.
 4. Identify cells that have a large nuclear γ -H2AX focus. Only take pictures of these cells to avoid bias.
4. **Analysis of long-lasting I-SceI induced foci**

1. Analyze the long-lasting foci manually, by counting both the number of cells that have the large γ -H2AX foci and co-localized foci of the repair protein of interest.

Representative Results

Figure 1 depicts the selection of the correct noise discrimination for maxima/foci quantification using ImageJ. The merged images of DAPI and the repair protein of interest are on the left panel. **Figure 1A** shows a noise discrimination of 90 and marks the correct number of foci. Nuclei on the edge (depicted with a pink arrow) and foci outside the nuclei (depicted with a yellow arrow) are not counted during the quantification. **Figure 1B** depicts a low noise tolerance of 50. Numerous maxima are identified outside the nucleus and within the nucleus. **Figure 1C** shows a high noise tolerance of 220. Only a single focus (a white arrow) was detected. To offer the reader a sense of "positive" and "negative" results, we have also compiled representative results in **Figure 2**. Specifically, **Figure 2A** depicts co-localization between a γ -H2AX focus (green) and a repair protein of interest (red). Similarly, an untransfected cell is shown in the upper left of this panel and a cell with a non-nuclear (false) γ -H2AX focus is shown in the upper right. Please note the micronuclei associated with DNA damage machinery are a likely source of these non-nuclear foci¹⁹. **Figure 2B** provides a higher magnification of the two right-most cells from **Figure 2A** to provide clarity in how they were determined to be interior and exterior to the nucleus, respectively. An example of a nuclear γ -H2AX focus without co-localization of a repair protein is shown in **Figure 2C**. A schematic of this protocol is provided in **Figure 3** that summarizes this approach to characterizing DNA repair.

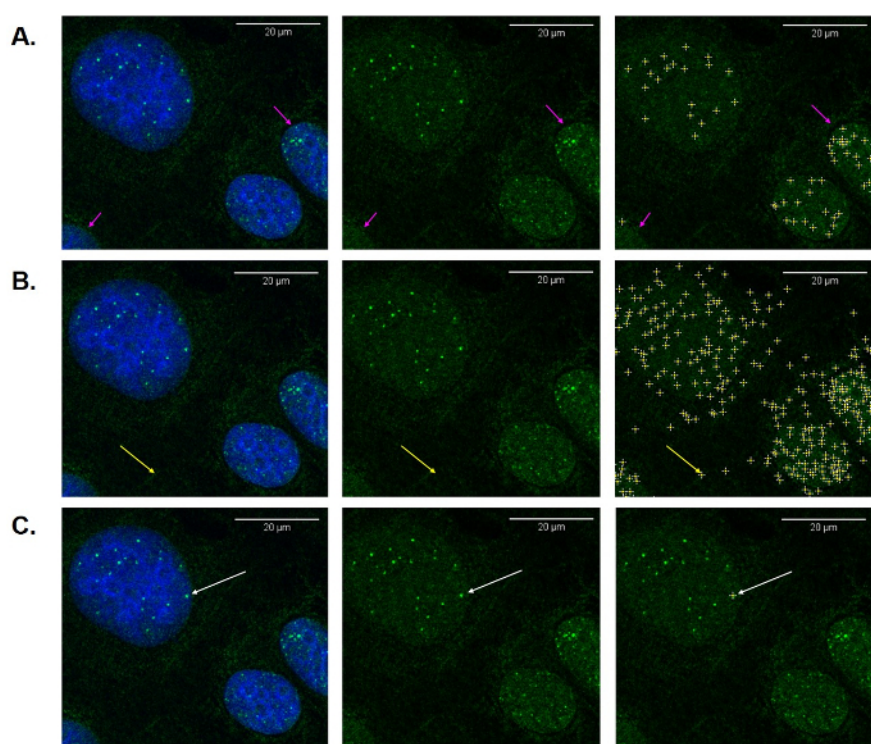


Figure 1: Representative analysis of double-strand DNA break repair kinetics. Representative images of γ -H2AX foci quantification using ImageJ (with Bio-Formats plugin) software. The left panels are composite images of nuclei (40X magnification), stained blue for DAPI and green for γ -H2AX. Center panels show reference un-analyzed nuclei with γ -H2AX foci/maxima. (A) The right panel shows maxima/foci detection with a noise tolerance of 90 and is representative of the actual estimation of the number of foci within the nucleus. (B) The right panel shows maxima quantification with a low noise tolerance (50). Maxima/foci can be detected outside the nucleus (yellow arrow), while majority of the maxima located within the nucleus are non-specific background signals. (C) The right panel shows maxima quantification with high noise tolerance (220). Only a single maximum/focus (white arrow) is detected within the nucleus and is not representative of the foci within the nucleus. [Please click here to view a larger version of this figure.](#)

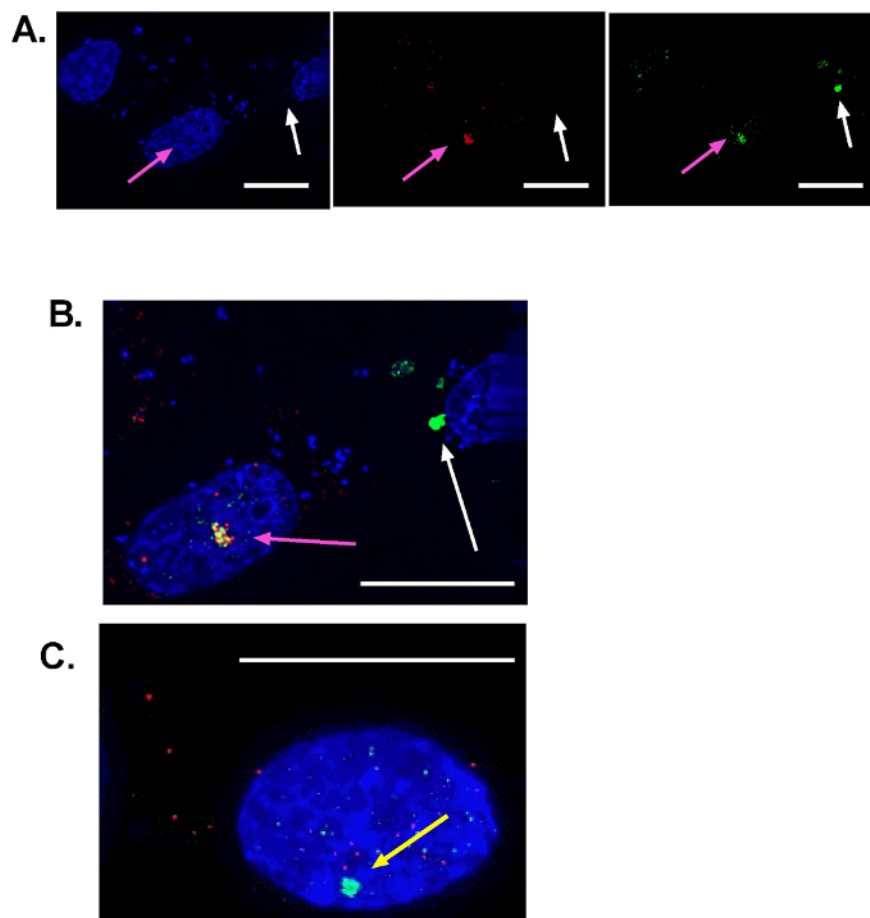


Figure 2: Representative I-SceI induced double-strand DNA breaks. Representative images of nuclei from cells grown on coverslips (40X magnification). Nuclei are stained blue. A repair protein (RAD51) is stained red and γ-H2AX is stained green. **(A)** The three panels show the same three cells. In the left most panel, nuclei are visible. In the middle panel, staining for RAD51 is shown. On the right panel, γ-H2AX staining is shown. The nucleus in the upper right corner does not show any sign of I-SceI induced DSBs. The nucleus in the center (pink arrow) represents a true positive event as it has both a large γ-H2AX nuclear focus and a co-localized RAD51 nuclear focus. The nucleus on the right side has a false large γ-H2AX focus as it is extranuclear. Foci outside of the nucleus should not be used for co-localization analysis. **(B)** This is a higher magnification of the merged image in part A (center and right most cells). The large nuclear yellow focus indicating overlap of RAD51 and H2AX signal is denoted by the pink arrow. **(C)** A representative image of a nuclear γ-H2AX focus without co-staining of the DNA repair protein of interest. Unlike the focus denoted with a white arrow in (A) and (B), the yellow arrow illustrates typical results when repair proteins are prevented from localizing to sites of damage. Scale bar = 15 μm. [Please click here to view a larger version of this figure.](#)

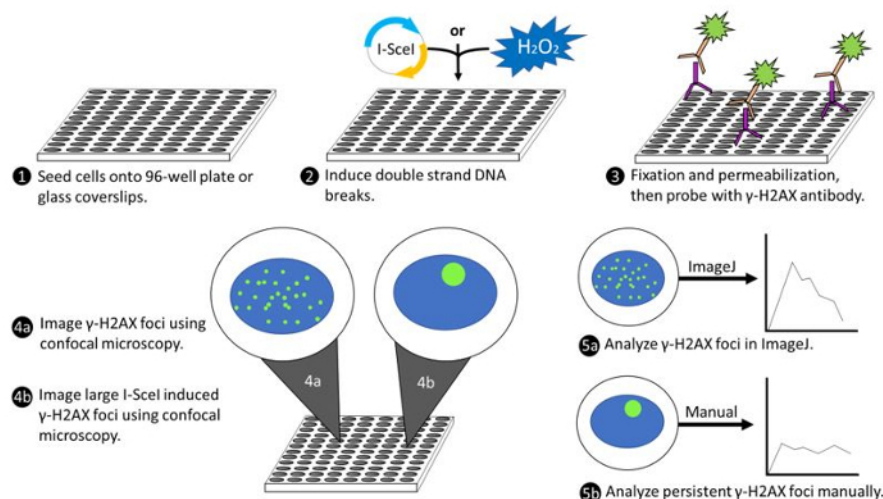


Figure 3: Visual depiction of protocol for analyzing double-strand DNA break repair. (1) Representation of protocol steps 1.1 for seeding cells onto coverslips/glass bottom plates. (2) Illustration of the induction of double-strand breaks by treating with hydrogen peroxide (right) or inducing long-lasting double-strand breaks by transfecting 3 μg of I-SceI. (3) Representation of protocol steps 1.4 to 1.5.3 for visualization of double-strand breaks. (4a) Image representing confocal microscopy of cell stained with DAPI (blue) and γ-H2AX foci (green). (4b) Image of singular large γ-H2AX foci (green) again nuclei stain DAPI (blue). (5a) Depiction of the analysis of γ-H2AX foci using ImageJ (protocol steps 1.10.1–1.11.5). (5b) Depiction of manual count of large long-lasting γ-H2AX foci (protocol step 2.4). [Please click here to view a larger version of this figure.](#)

Discussion

The analysis of DNA damage repair in general and the repair of double-stranded DNA breaks specifically is an active area of research because its consequences span tumorigenesis to basic biology^{6,20}. This manuscript details an approach that accurately dissects the contribution of RAD51 and γ-H2AX proteins to the resolution of DSBs through HR. Looking forward, this method can be used to elucidate additional functions of repair proteins at DSBs¹⁴. The comparison of repair kinetics and the recruitment of repair machinery to I-SceI induced DSBs between control cells and cells with the protein of interest knocked out, would define the contribution of that protein to the recruitment, localization, and resolution of repair factors to DSBs.

Visualizing repair kinetics has several advantages over commonly used approaches. Frequently, repair is investigated at single time points, which is incapable of representing the dynamic process of assembly and dissociation of repair complexes. Observing the full range of repair from initial activation to full resolution ensures that a delay in repair is not misidentified as complete inhibition. Conversely, it assures that induction of a repair response without the ability to inactivate that response is not misidentified as normal or excessive activation. These are largely changes in the application of an established approach, but the use of a rare cutting endonuclease to induce a long-lasting DSB is a novel tool. It allows the investigator to unambiguously determine if the localization of a repair protein is inhibited instead of its recruitment being delayed a short period of time beyond the conclusion of the experiment. I-SceI induced DSB can last for days and theoretically as long as the enzyme is expressed (should the cell remain viable). The kinetics of DSB repair can also be observed via live cell imaging. However, although live cell imaging allows detection of repair events in real time, its application is hindered by the requirement that proteins be tagged with a fluorophore such as GFP. Such genetic manipulation has the potential to alter protein function and represents an additional technical barrier.

Image analysis via ImageJ software can be cumbersome to master. The step-by-step instruction for repair focus recognition and quantification using this software is provided in hopes of removing this obstacle for other groups. This could lead to increased utilization of the powerful software and eliminate the cost of more expensive programs at a time of shrinking pools of federal grant support.

In principle, the duration of these DSBs should enable the visualization of elusive repair proteins by immunofluorescence microscopy. This could be the case for proteins that are difficult to view because they do not accumulate in a high enough concentration to be detected, *e.g.*, the persistence of the I-SceI induced DSB results in a greater than typical accumulation of repair proteins at the lesion. The enhanced abundance could also improve the visualization when detection is hindered by a limitation in antibody quality; a feature that could be particularly useful when less studied proteins are identified as having an impact on DNA repair. To date, this approach has been verified for 4 DNA repair proteins, namely RAD51, RPA70, BRCA1, and BRCA2 (data not shown, **Figure 2**, and reference¹⁷).

Finally, there are modifications that could be made to this protocol to expand the types of DNA damage that can be interrogated as well as the cellular environments. The most obvious modification would be changing the source or type of induced DNA damage. The proof of principle for using UVB, ionizing radiation, or radiomimetics (phleomycin, bleomycin, and etoposide) to study the kinetics of DNA repair has already been published^{18,19,23,24,25}. With slight modification, this approach can also elucidate repair protein response by NHEJ to DSBs. Alternatively, Britton *et al.* describe a pre-extraction technique that could be used²⁶. Further, there are homing nicking endonucleases (I-Hmul or I-BasI) that have extensive recognition sites, similar to I-SceI²⁷. Distinguishing them from I-SceI, these enzymes cause single-strand DNA breaks (SSBs). Introducing the recognition site for these enzymes into a cell line would allow the analysis of SSB repair that parallels the protocol described in this manuscript for interrogating long-lasting DSBs. Notably, the process of integrating the I-SceI recognition site and verifying the introduction of the site can be laborious. Fortunately advances in genome editing technology (such as CRISPER/Cas9 systems) have eased this burden²⁸.

There is also a field of active research that has succeeded in engineering homing endonucleases to cut at a desired genomic location, which suggests that in the future, these engineering advancements and the use of CRISPR/Cas9 may lead this cloning step to be dispensable^{28,29}.

Disclosures

The authors have nothing to disclose.

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Loss of Genome Fidelity: Beta HPVs and the DNA Damage Response

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While the role of genus alpha human papillomaviruses in the tumorigenesis and tumor maintenance of anogenital and oropharyngeal cancers is well-established, the role of genus beta human papilloviruses (β -HPVs) in non-melanoma skin cancers (NMSCs) is less certain. Persistent β -HPV infections cause NMSCs in sun-exposed skin of people with a rare genetic disorder, epidermodysplasia verruciformis. However, β -HPV infections in people without epidermodysplasia verruciformis are typically transient. Further, β -HPV gene expression is not necessary for tumor maintenance in the general population as on average there is fewer than one copy of the β -HPV genome per cell in NMSC tumor biopsies. Cell culture, epidemiological, and mouse model experiments support a role for β -HPV infections in the initiation of NMSCs through a “hit and run” mechanism. The virus is hypothesized to act as a cofactor, augmenting the genome destabilizing effects of UV. Supporting this idea, two β -HPV proteins (β -HPV E6 and E7) disrupt the cellular response to UV exposure and other genome destabilizing events by abrogating DNA repair and deregulating cell cycle progression. The aberrant damage response increases the likelihood of oncogenic mutations capable of driving tumorigenesis independent of a sustained β -HPV infection or continued viral protein expression. This review summarizes what is currently known about the deleterious effects of β -HPV on genome maintenance in the context of the virus’s putative role in NMSC initiation.

Keywords: beta HPV, DNA-damage response, skin cancer, genomic fidelity, tumorigenesis, epidermodysplasia verruciformis, viral oncogenesis, p300

INTRODUCTION

Human papillomavirus (HPV) is a family of small, non-enveloped double-stranded DNA viruses that infect mucosal and cutaneous epithelia. This family is comprised of five genera (alpha, beta, gamma, nu, and mu) spanning across over 396 different HPV types potentially inhabiting human skin (Bzhalava et al., 2014). Classification of the HPV family is based on the sequence of the L1 capsid protein (Bernard et al., 2010; de Villiers, 2013). The alpha and beta genera are most widely studied because of the pathogenesis associated with some members of these genera. α -HPV causes genital warts (low risk types, HPV 6, and 11 for example) and genital cancer (high risk types, HPV 16, 18, 31, and 45 for example) (Tommasino, 2014; Doorbar et al., 2015). While β -HPV is known to cause flat warts and non-melanoma skin cancer in individuals with compromised immune systems (Cubie, 2013), there is a growing interest in determining if these viruses can cause tumors in the general population. Further, while so called “high risk” α -HPVs have been identified, the relative oncogenic potential among the beta genus of HPV is still being discussed. Moreover, despite already consisting of ~ 50 types of HPV that are sub-classified in five species (Van Doorslaer, 2013), it is likely that a large portion of β -HPV types have yet to be discovered (Ekström et al., 2013;

Bzhalava et al., 2014, 2015). Because the first β -HPV types isolated were HPV 5 and HPV 8 from cutaneous squamous cell carcinomas (cSCC) of patients with the rare disorder Epidermodysplasia Verruciformis (EV) (Pfister et al., 1981; Kremsdorf et al., 1982, 1983), these two β -HPVs have been the most extensively examined.

Because β -HPVs do not generally persist in tumors, they are hypothesized to act through a “hit and run” mechanism (Pfister, 2003; Hufbauer and Akgül, 2017). Specifically, β -HPVs are believed to hinder the repair of DNA damage caused by UV radiation making mutations more likely. For a virus that infects an area frequently exposed to UV radiation and yet requires proliferating cells to complete its lifecycle, it is reasonable that β -HPVs have developed mechanisms to prevent the cell cycle arrest that accompanies repair of UV damaged DNA. However, this could have severe consequences for the host cell as the UV-induced mutations will remain after the viral infection is cleared and could drive the development of non-melanoma skin cancers (NMSCs) without continued expression of β -HPV genes. In this review, we will begin by providing a brief synopsis of the evidence that β -HPV infections cause NMSCs as well as the cellular signaling pathways that respond to UV insults. We will provide a concise discussion of α -HPV associated tumorigenesis, as these more clearly defined mechanisms of transformation provide a useful and relevant comparison for β -HPV associated oncogenesis. Then, we will move on to the *in vitro* and *in vivo* data that demonstrate the ability of β -HPV genes to disrupt the repair of DNA, destabilizing the host genome in a manner consistent with a “hit and run” mechanism of tumorigenesis.

Transformation by Alpha Genus HPV Oncogenes

Extensive evidence has established α -HPV as the causative agent in cervical cancers and in many other malignancies in the oropharynx and throughout the anogenital tract (Hobbs et al., 2006; Moody and Laimins, 2010; Carter et al., 2011; D'Souza and Dempsey, 2011; Tommasino, 2014). Infections with high risk α -HPVs can begin a multi-decade process where cells are immortalized, accumulate destabilized genomes, and are ultimately transformed into deadly malignant tumors. Two oncogenes, α -HPV E6 and E7 proteins drive this progression by activating telomerase and degrading two tumor suppressor, p53 and pRB (Dyson et al., 1989; Münger et al., 1989; Scheffner et al., 1990; Werness et al., 1990; Huibregtse et al., 1991; Boyer et al., 1996; Klingelhutz et al., 1996; Kiyono et al., 1998; Oh et al., 2001). These oncogenes also induce aberrant activation of the DNA damage response (DDR) and in some case, impair the cells ability to repair DNA damage (Patel et al., 1999; Zimmermann et al., 1999; Moody and Laimins, 2009; Sakakibara et al., 2011; Gillespie et al., 2012; Reinson et al., 2013; Hong et al., 2015; Wallace et al., 2017). In notable contrast to β -HPV associated malignancies, tumors caused by α -HPV are dependent on the continued expression of α -HPV E6 and E7 (Hwang et al., 1993; Goodwin and DiMaio, 2000; Goodwin et al., 2000). Because the β -HPV homologs of these α -HPV oncogenes are believed to be the primary contributors to NMSC, β -HPV E6, and E7 are

frequently compared to α -HPV E6 and E7. When informative, we will make similar comparisons.

Beta-HPV in Epidermodysplasia Verruciformis Patients and Organ Transplant Recipients

The oncogenic potential of β -HPV is most firmly established in people with compromised immune systems. β -HPV associated cSCCs occur in 30–60% of people with the rare genetic disorder epidermodysplasia verruciformis (EV) and presented the first link between β -HPV infections and skin carcinogenesis (Orth, 1986). β -HPV+ tumors found in people with EV generally contain a high copy number ($\sim$ 300 copies/cell) of viral DNA (Dell'Oste et al., 2009). In contrast, β -HPV+ tumors of non-EV patients show a very low viral DNA copy number <1 per cell (Weissenborn et al., 2005; Arron et al., 2011). The most frequently β -HPV types associated with EV are β -HPV 5 and 8 (also less often β -HPV 14 and 20) (de Oliveira et al., 2004; Dell'Oste et al., 2009). Tumors in individuals with EV occur predominantly in parts of the body frequently exposed to the sun, suggesting a role for UV in β -HPV associated tumorigenesis (Pfister, 2003). Another group at risk for β -HPV associated cSCCs are people receiving immunosuppressive therapy following organ transplant. Organ transplant recipients (OTRs) (Bouwes Bavinck et al., 2001) show an increased susceptibility to β -HPV infections, a higher prevalence of viral DNA and a greater risk of developing non-melanoma skin cancer (Boyle et al., 1984; Kiviat, 1999). This increased risk is particularly notable if they are seropositive for β -HPV, with a hazard ratio of 2.8 (Genders et al., 2015). Both, EV-patients and OTRs display a significantly elevated viral load than the immunocompetent population (Dell'Oste et al., 2009; Weissenborn et al., 2012). Further, OTRs that have similar β -HPV viral loads to patients with EV show a 100-fold increase of cSCC incidence (Weissenborn et al., 2012). Investigations in to NMSCs in EV and OTR patient groups provide strong evidence that β -HPV infections have oncogenic potential. Additionally, the OTR patient group shows that β -HPV associated cSCCs are not limited to patients with the rare EV disorder (Howley and Pfister, 2015; Tommasino, 2017). These “special” scenarios provide the proof of context specific β -HPV induced oncogenesis, but defining the breath of β -HPV's contribution to NMSC development is a critical area of research as millions of people are diagnosed with these malignancies each year.

Beta-HPV in the General Population

β -HPVs inhabit the cutaneous epithelium and are found in abundance throughout the population (Casabonne et al., 2009; de Koning et al., 2009; Weissenborn et al., 2012; Farzan et al., 2013). A particularly frequent site of infection is the hair follicles of eyebrows (Weissenborn et al., 2012; Neale et al., 2013; Iannacone et al., 2014). Unlike α -HPV infections, which are usually sexually transmitted and occur later in life, β -HPV infections often occur during early childhood through skin to skin contact (Antonsson et al., 2003; Weissenborn et al., 2009). The persistence of α - and

β -HPVs is a further differentiating factor, although the exact mechanisms driving this difference are not fully appreciated. Median β -HPV infection duration is 8.6 months in eyebrow hairs, while infections of the skin are less common but have a median persistence of 11.3 months (Hampras et al., 2014). In contrast α -HPV infections persist longer (18.3 months on average), and upon accidental genome-integration are present for decades (Richardson et al., 2003). Suggesting reinfections, β -HPV infections can persist within a family for several years without manifestation of clinical symptoms (Hsu et al., 2009). While infections first occur in infants, advanced age is a risk-factor for a β -HPV infection (Hazard et al., 2007; Weissenborn et al., 2009). Sunburn is another risk factor, potentially due to local immune suppression (Hampras et al., 2014). The evidence of β -HPV's involvement in cSCCs of EV patients led to the classification of HPV 5 and HPV 8 as possibly carcinogenic by a WHO-IARC 2009 work group (Bouvard et al., 2009).

Epidemiology

Epidemiologically, β -HPV antibody positivity is associated with an increased risk for cSCCs, especially for infections by β -HPV 38 (Bzhalava et al., 2013; Chahoud et al., 2016). There is also a difference of β -HPV prevalence by anatomical site (Hampras et al., 2017). The most common site of infection was genital skin (81.6%), followed by forearm skin (64.4%), eyebrow hairs (60.9%), oral mucosa (35.6%), and anal mucosa (33.3%). The most common type on the sunlight exposed and therefore risk associated sites eyebrows and forearm are β -HPV 38 and β -HPV 12 respectively. High loads of β -HPV DNA are statistically associated with increased cutaneous SCC incidences at an odds ratio of ~ 3 in immunocompetent Australians and immunosuppressed OTRs (Bouwes et al., 2010; Neale et al., 2013). People with cSCCs more frequently test positive for viral DNA in skin as well as anti β -HPV L1 antibodies than the general population (Forslund et al., 2007; Waterboer et al., 2008; Karagas et al., 2010; Iannacone et al., 2012; Farzan et al., 2013). The involvement of β -HPV in cSCC carcinogenesis is somewhat challenged by the low relative incidence rate of β -HPV+ cSCCs considering the high prevalence of 80% for β -HPV infections. Furthermore, unlike α -HPV+ cancers, the expression of β -HPV viral proteins is not required for tumor maintenance and the viral DNA copy-number is <1 per cell in β -HPV associated cSCCs (Meyer et al., 2001; Nindl et al., 2006). Since cSCC tumors typically occur in parts of the body exposed mutagenic UV radiation from the sun, the role of β -HPV is thought to be in the initiation and acceleration of genomic destabilization.

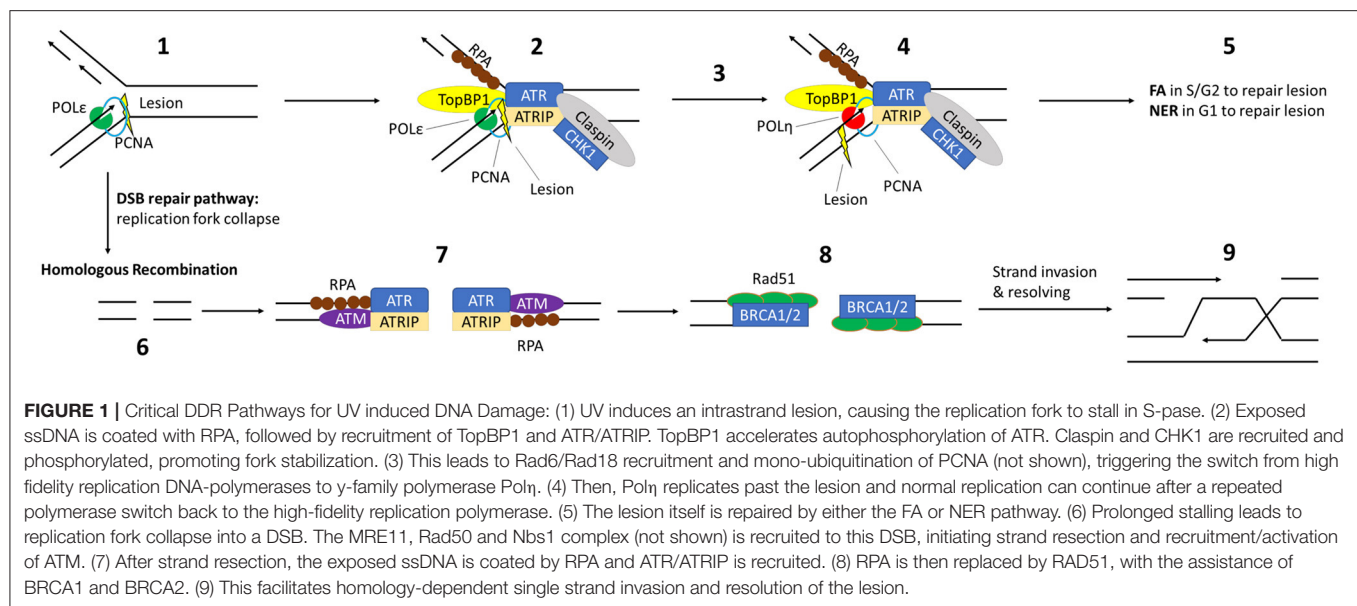
The phrase “hit and run” was coined to describe the hypothesize contribution of β -HPV infections to NMSCs, where the virus acts as a cofactor along with UV during tumor initiation (Bavinck et al., 2008). The destabilization of the host genome caused by sun exposure is augmented by β -HPV's ability to attenuate the cellular response to UV damage and thus increase the risk of oncogenic mutations capable of driving tumor development independent of continued β -HPV gene expression. Epidemiological studies support this hypothesis (Forslund et al., 2007; Iannacone et al., 2012) as there is increased prevalence and frequency of β -HPV in precancerous, actinic keratinosis

lesions (AK). AK, otherwise known as solar keratinosis, is an abnormal growth of the skin, induced by UV exposure (Moy, 2000). Consistent with a role in tumor initiation, despite being found at very low copy numbers in cSCCs, the β -HPV viral load in AK is >50 copies/cell (Weissenborn et al., 2005). Together these data form the foundation that supports the “hit and run” model of β -HPV associated skin cancer, but this hypothesis is dependent on the ability of β -HPV infections to make UV more mutagenic.

DNA Damage Response-Pathways

The most likely way that β -HPV could amplify the destabilization of the host genome induced by UV is through the aberration of the cellular DDR. The primary type of DNA damage caused by UV are DNA intrastrand crosslinks, most often cyclobutane purimidine dimers or CPDs (Yang, 2011). Failure to properly repair these lesions can result in point mutations (Brash et al., 1991; Keohavong et al., 1991). Crosslinked DNA can also cause a replication fork collapse and subsequent double strand break in DNA (DSB) (Jeggo and Löbrich, 2007). If DSBs are not repaired, the damage becomes much more deleterious, including chromosome rearrangement or loss of entire chromosome arms (Pankotai and Soutoglou, 2013). In this section, we will introduce the biochemical pathways that coordinate the cellular response to UV-damage to preserve the integrity of the human genome. A graphical representation of the cellular response to UV induced DNA damage in S-phase can be found in **Figure 1**. During S-phase, translesion synthesis (TLS) prevents UV-induced DNA crosslinks from causing replication fork collapse by facilitating the bypass of the damaged bases (Lerner et al., 2017). Depending on the position in the cell cycle, nucleotide excision repair (NER), and the Fanconi Anemia (FA) pathways repair these crosslinks (Moldovan and D'Andrea, 2009; Marteiijn et al., 2014). While FA requires sister chromosomes to complete repair and is thus limited to cells that have undergone replication, NER is not bound by cell cycle position. UV-induced DSBs are the result of replication fork collapse and therefore must occur during S-phase. Although DSB repair occurs through two major pathways, non-homologous end joining (NHEJ) and homologous recombination (HR), DSBs occurring during S-phase are repaired predominantly by HR (Mao et al., 2008). Repairing DNA lesions usually requires pausing the cell cycle to avoid escalating the damage during replication (Willis and Rhind, 2009). Finally, should the cell receive more damage than can be repaired, the intrinsic apoptosis pathway will initiate program cell death (Offer et al., 2002).

Although recognized as distinct pathways, the individual proteins that make up these repair mechanisms are often shared. This is particularly true for repair kinases (Yang et al., 2003; Yan et al., 2014). For instance, ATR controls the rate limiting step of NER (through phosphorylation mediated stabilization of XPA) and facilitates translesion synthesis by phosphorylating the TLS-specific polymerase, Pol η (Chen et al., 2008; Göhler et al., 2011; Lee et al., 2014). ATR's kinase activity is similarly necessary for the repair of crosslinked DNA by the FA pathway and plays a role in DSB repair via the homologous recombination pathway (Jazayeri et al., 2006; Shiotani and Zou, 2009; Shigechi et al., 2012;



Maréchal and Zou, 2013). A similar situation exists for the related kinase, ATM. ATM phosphorylates proteins critical for the FA, NER, and HR repair pathways (Ray et al., 2013, 2016; Shiloh and Ziv, 2013). It may also play a role in translesion synthesis. Further, both ATM and ATR can stabilize p53 in response to DNA damage, causing p53-dependent DNA repair or apoptosis (Kruse and Gu, 2009; Cheng and Chen, 2010). ATM and ATR are both classically involved in DNA damage induced cell cycle arrest (Reinhardt and Yaffe, 2009). Both of these kinases also help pause the cell cycle progression by phosphorylating/activating cell cycle regulatory proteins (CHK1 and CHK2) (Branzei and Foiani, 2008). Finally, ATM and ATR also facilitate programmed cell death or apoptosis should the cell's DNA be too extensively damaged. Specifically, their phosphorylation of p53 stabilizes the tumor suppressor and can result in p53-dependent apoptosis (Banin et al., 1998; Tibbetts et al., 1999; Shiloh and Ziv, 2013).

The RPA complex (RPA14, RPA32, and RPA70), BRCA1 and BRCA2 are similarly important for a myriad of DDRs (Yoshida and Miki, 2004; Maréchal and Zou, 2015). The RPA heterotrimer binds single stranded DNA (ssDNA) protecting it from degradation. Because ssDNA intermediates occur during both homologous recombination and translesion synthesis, RPA proteins are essential for these repair mechanisms. Both the FA and HR pathways require BRCA1 and BRCA2. FA pathway proteins contain the prefix "FAN" in their names indicating that many of these proteins were named after loss of the gene was shown to result in the clinical manifestation (Fanconi Anemia) from which the repair pathway derives its name, for example, FANCA, FANCB, FANCD1. Indicative of its requirement for FA repair an alternative name for BRCA2 is FANCD1. Moreover, BRCA1 was recently shown to be a critical component of the FA pathway (Domchek et al., 2013; Sawyer et al., 2015) and even described as the pathway's "missing link" (D'Andrea, 2013). The requirement of BRCA1 and BRCA2 in repair of DSBs by homologous recombination has been established for over a

decade. Further indicative of how intertwined the cellular DNA response is, ATR and ATM both play regulatory roles in repair by phosphorylating the RPA complex, BRCA1 as well as BRCA2.

EFFECTS OF β -HPV E6 ON DNA DAMAGE RESPONSE

Inhibition of Apoptosis

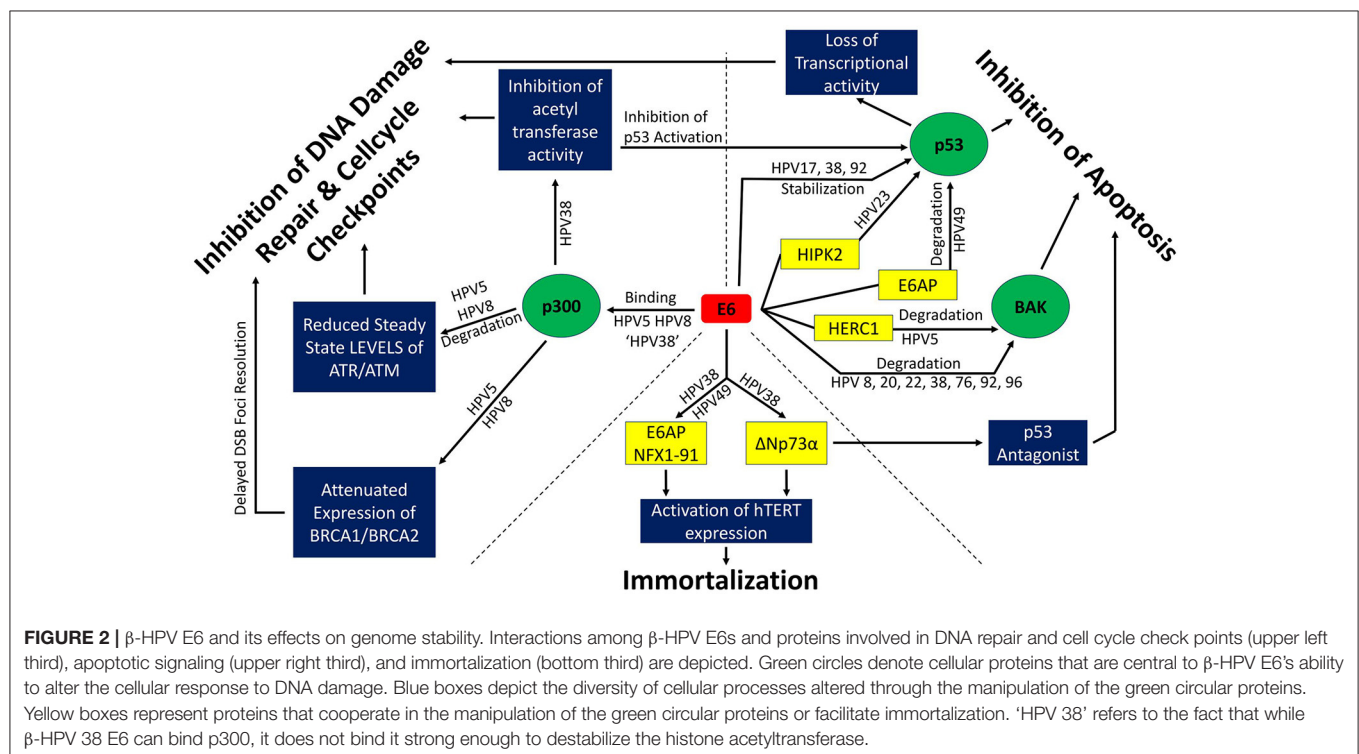
The E6 protein of β -HPV can hinder both DNA repair machinery and apoptotic pathways in response to DNA damage. Unlike high risk α -HPVs, it does not commonly degrade the "guardian of the genome," p53, one of the most prominent high-risk E6 targets, directly (Scheffner et al., 1990; White et al., 2014). The tumor-suppressor, p53, is an important signaling protein that regulates two large subsets of target proteins: negative regulators of cell cycle progression (p21, 14-3-3, GADD45 α) and pro-apoptotic proteins (PUMA, BAX, BAK). In normal cells, p53 has to balance on a fine line between sufficient activity to ensure genome fidelity and tumor suppression as well as avoiding hyperactivity that would induce abnormal aging by depleting stem-cell populations (Vogelstein et al., 2000; Reinhardt and Schumacher, 2012). Compared to high risk α -HPV E6s, β -HPV E6, apart from β -HPV 49, have a lower affinity for a cellular E3 ubiquitin ligase, known as E6AP or UBE3A, and are therefore not able to form the E6-E6AP complex required for proteasomal degradation of p53 (Huibregtse et al., 1991; Cornet et al., 2012). Instead, β -HPV types 17, 38, and 92 have been shown to bind to p53 directly and stabilize it (White et al., 2012), while β -HPV 23 has been shown to interfere with the phosphorylation-dependent activation of p53 by inhibiting HIPK2 (Muschik et al., 2011). β -HPV 5 and β -HPV 8 delay the stabilization and phosphorylation after UV irradiation (Wallace et al., 2014). HIPK2 is a protein kinase that can phosphorylate p53 at Ser 46, which subsequently leads to the acetylation of p53 at Lys 382 and a promotion of p53 target gene expression. More specifically, HIPK2's activity is UV-induced

and UV exposure leads to HIPK2 mediated growth arrest or apoptosis via p53. β -HPV 38 E6 induces the accumulation of Δ Np73, altering p53 functions (Accardi et al., 2006). These effects cause the alteration or loss of p53's activity as a transcriptional co-factor and impact both apoptosis- (Fas, BAX) and cell cycle checkpoint pathway (p21) gene expression (White et al., 2014). p53 is also a known transcription factor for the TLS polymerase Pol η , implying the TLS DDR pathway as a suitable target for investigation (Lerner et al., 2017).

The selection between a p53-mediated checkpoint activation and p53-mediated apoptosis depends on a cell type specific threshold of p53 expression and activation (Kracikova et al., 2013). Mild DNA damage triggers p53-dependent checkpoint activation and subsequent DNA repair while moderate DNA damage causes p53-mediated senescence. Excessive DNA damage or failed cytokinesis induce apoptosis (Chen et al., 1996). β -HPV E6 can also inhibit apoptosis downstream of p53. The interaction of E6 with a pro-apoptotic protein, Bcl2 homologous antagonist killer (BAK), is a highly conserved function across HPV types and genera (Thomas and Banks, 1999; Simmonds and Storey, 2008; Underbrink et al., 2008; Jackson and Bartek, 2009; Holloway et al., 2015; Tomaić, 2016). Increases in BAK abundance are an essential step in the intrinsic apoptotic pathway (Chittenden et al., 1995). β -HPV E6 prevents the accumulation of Bak following UV-irradiation inducing DNA damage. An overview of the different pathways for β -HPV E6 mediated inhibition of apoptosis and the β -HPV types involved can be found in the upper right panel of **Figure 2**.

Interference with Checkpoint Signaling & DNA Damage Repair

Upstream of the inhibition of apoptosis, β -HPV E6 can attenuate G1 to S-phase cell cycle checkpoint induction in response to DNA damage (Wallace et al., 2012; Hufbauer et al., 2015) and continue cellular proliferation while DNA damage repair is attenuated (Giampieri and Storey, 2004). These effects arise from the interaction of β -HPV E6 with the acetyltransferase p300 (Muench et al., 2010; Howie et al., 2011). As an important coactivator of DDR gene transcription, the loss of p300 activity has far reaching consequences. β -HPV 5 and 8 have been shown to facilitate the destabilization of p300 (Howie et al., 2011; Wallace et al., 2012, 2013), while β -HPV 38 has been shown to inhibit p300 acetyltransferase activity (Muench et al., 2010). The steady state levels of the DDR kinases ATM and ATR as well as BRCA1 and BRCA2 are reduced because of the lack of p300 in β -HPV 5 and β -HPV 8 E6 expressing cells (Wallace et al., 2012, 2013, 2015). This attenuates the repair of UV induced cyclobutane pyrimidine dimer (CPD), increases the UV-induced frequency of DSBs and attenuates LINE-1 retrotransposition. Double strand breaks can be a secondary effect of UV-induced DNA damage, occurring when a replication fork collapses at an unrepaired CPD. Both the repair of CPDs (ATR dependent) as well as the repair of DSBs (ATM, BRCA1 and BRCA2 dependent) are significantly delayed by β -HPV 5 and β -HPV 8 E6 (Giampieri and Storey, 2004; Wallace et al., 2012, 2015). γ H2AX is a known marker for DSBs and its foci kinetics can be utilized as indicators for DSB-repair. The (β -HPV 5 E6 and 8 E6) p300 degradation-dependent attenuation of BRCA1/BRCA2 expression and foci formation led



to delayed γ H2AX foci resolution after DSBs were induced by ionizing radiation (Wallace et al., 2015).

ATR and ATM activate cell cycle checkpoints by phosphorylating checkpoint kinases Chk1 and Chk2 respectively. Activation of Chk1/Chk2 induces cell cycle arrest and DDR (Reinhardt and Yaffe, 2009). β -HPV 5 and 8 E6 reduce steady state levels of ATM and ATR *in vitro* as well as reduced pATM and pATR levels *in vitro* and *in vivo* (Hufbauer et al., 2015). This leads to an attenuated Chk1 phosphorylation and impaired G1 to S-phase checkpoint activation, presenting a bypass to an important tumor-suppressing barrier. Furthermore, p300 is an important transcriptional cofactor for BRCA1, BRCA2 (Both essential proteins for HR) (Goodman and Smolik, 2000; Pao et al., 2000; Yoshida and Miki, 2004) and has been shown to be essential for proper cytokinesis and the faithful resolution of mitotic figures (Turnell et al., 2005; Xia et al., 2012). Attenuation of proper checkpoint signaling and the consequent possible unscheduled cell cycle progression leaves pre-existing DNA damage unaddressed. This allows less severe types of damage (for example, UV-induced intrastrand crosslinks) to evolve into more deleterious types of DNA damage like DSBs. β -HPV E6 expressing cells have been shown to contain a significantly larger portion of multinucleated cells *in vitro* (Wallace et al., 2014), pointing to a possible attenuation of the Hippo pathway. The Hippo pathway stabilizes p53, inactivates the oncogene YAP and induces apoptosis in the event of failed cytokinesis (Harvey et al., 2013; Ganem et al., 2014). The mechanisms through which β -HPV E6 disrupts the DDR are depicted in the upper left panel of Figure 2.

In addition to p300-dependent checkpoint inhibition, β -HPV 5E6 and 8E6 can bind the transcription factor SMAD3, thereby inhibiting the transforming growth factor-beta (TGF- β) pathway (Mendoza et al., 2006). TGF- β has a well-described dual role in carcinogenesis. In normal cells and early carcinomas, it acts as tumor suppressor through cytostatic effects. As the cancer progresses, the TGF- β aids proliferation, survival, invasion, and angiogenesis of tumor cells (Lebrun, 2012). Destabilization of the SMAD3 may attenuate these tumor suppressive effects by inhibiting the expression of CDK inhibitors p16, p17, p21, and p27 and possibly promoting unscheduled cell cycle progression from G1- into S-phase (Itoh et al., 2000; ten Dijke and Hill, 2004). Disruption of the TGF- β pathway has also been linked to an impaired function of DSB-repair following ionizing radiation (Kirshner et al., 2006; Bouquet et al., 2011; Kim et al., 2015).

Additional Functions of β -HPV E6

Additional notable functions of β -HPV E6 include hTERT stabilization (β -HPV 5, 20, 22, 38; Bedard et al., 2008; Gabet et al., 2008; Cornet et al., 2012), which may stabilize the genome by preventing unstably short telomers. Conversely, it could decrease genome fidelity by aborting the role of telomers as a “cellular clock” that prevent proliferation of older cells that are more likely to contain mutations (Sharma et al., 2003). Another target of β -HPV 5 and 8 E6 is the Notch signaling pathway. The Notch signaling pathway has been shown to have tumor suppressive functions in epithelial cells (Reichrath and Reichrath, 2012) and can decide cell fate (Lai, 2004). It can also act as

a negative regulator of the ATM-dependent DDR (Vermezovic et al., 2015). β -HPV 5 and 8 interfere with the Notch signaling pathway through interaction with MAML1. The β -HPV E6-mediated repression of Notch signaling delays S-phase cell cycle exit and differentiation (Brimer et al., 2012; Meyers et al., 2013). Prolonged or perpetual existence in S-phase in combination with impaired DNA-damage signaling and attenuated DDR could lead to cross-amplification of these individual effects.

Summary

β -HPV E6 attenuates UV-induced apoptosis and DDR through interaction with multiple targets. BAK degradation and prevention of apoptosis is highly conserved throughout the β -HPV genus. Less common is the inhibition of apoptosis through direct interaction with and degradation of p53 (β -HPV 49, E6AP mediated) or the inhibition of p53 activation (β -HPV 23 through HIPK2 interaction). β -HPV 17, 38, and 92 E6 interact with p53 directly, leading to its stabilization but altering or restricting p53's transcriptional activity. β -HPV 5, 8, and 38 E6 interfere with DDR and checkpoint signaling through interaction with p300. A combination of inhibited apoptosis, impaired DNA damage signaling and attenuated DSB response bears a potential to destabilize the genome over time or cause carcinogenesis in combination with DNA-damaging agents that is consistent with β -HPV's proposed contribution to skin cancer. Notably, although through markedly different mechanisms, high risk α -HPV oncogenes similarly contribute to tumorigenesis by impairing the cellular response to genome destabilizing events.

E7 AND ITS INFLUENCE ON CELL CYCLE PROGRESSION AND DDR

Promotion of Cell Cycle Progression

A canonical function of the high risk α -HPV E7 oncogene is the degradation of the tumor suppressor pRb (Moody and Laimins, 2010). In normal cells, the function of pRb is the suppression of E2F transcription factor dependent genes through binding E2F and inhibiting its association with promoters (Dyson, 1998). E2F-family proteins facilitate cell cycle progression and proliferation by promoting the expression of a variety of genes encoding for proteins involved in G1/S-phase transition (Bertoli et al., 2013). High risk α -HPV E7 oncogenes bind directly to pRb via the highly conserved LXCXE binding motif (Münger et al., 1989). This leads to the disruption of the E2F—pRb complex, allows E2F to bind to its transcriptional targets (Chellappan et al., 1992). E2F target expression (for example cyclin A or cyclin E) then facilitate premature cell cycle progression and S-phase entry (Zerfass et al., 1995). Cell cycle checkpoints are meant to pause the cell cycle in case of DNA damage and allow time to repair the damage, or, if the damage surpasses a threshold, induce senescence or apoptosis. Cell cycle checkpoints are an integral part of a cells quest to maintain genome integrity. Consequently, attenuated checkpoint activation leads to a destabilization of the genome through accumulating DNA damage (Malumbres and Barbacid, 2009). α -HPV E7 allows keratinocytes with damaged DNA to progress through cell cycle check points, even when p53 is induced (Demers et al., 1994). Much like these α -HPV

E7s, several of the β -HPV E7s (β -HPV 5, 8, 38, 49) can interact with Rb (Yamashita et al., 1993; Caldeira et al., 2003; Cornet et al., 2012). However, the β -HPV E7 interactions tend to cause a hyperphosphorylation of Rb in Keratinocytes, rather than its destabilization (Caldeira et al., 2003; Cornet et al., 2012). Hyperphosphorylation of Rb inhibits its ability to bind and inactivate E2F, allowing E2F to promote transcription of its target genes (Bertoli et al., 2013). Additionally, β -HPV 24, 38, and 49 E7 severely attenuate pRb half-life in rodent fibroblasts, while β -HPV 14, 22, 23, and 36 do not affect pRb's half-life (Cornet et al., 2012). Cornet et al. also found that in the context of E6 and E7 expressing cells, β -HPV 14 and 22 can degrade pRb, but do not induce the expression of cyclin A or Cdk1. Cdk1 is essential for cell cycle progression (Santamaria et al., 2007).

Alteration of the p53-Transcriptional Network

The tumor suppressor p53 is an essential factor in the avoidance of inappropriate cell proliferation in the presence of genotoxic stress. It has transcriptional targets in several pathways, including DNA damage tolerance and apoptosis (Espinosa et al., 2003; Yu and Zhang, 2005; Beckerman and Prives, 2010). While the mechanisms of how high Risk α -HPV E7 disrupts p53 activity remain a poorly defined, significantly more is known about how some β -HPV E7 proteins disrupt this essential tumor suppressor. Δ Np73 is an important antagonist of the p53 gene family and participating in a negative feedback loop with p53 (Bailey et al., 2011). β -HPV-38 E7 promotes the accumulation and stability of Δ Np73 α through both transcriptional and post-translational mechanisms (Accardi et al., 2006, p.73; Saidj et al., 2013). β -HPV 38 E7 promotes the accumulation of double monophosphorylated p53 (serine 15 and 392) in the nucleus. These dual p53 phosphorylation events increase Δ Np73 α expression. β -HPV 38 E7 also mediates the nuclear translocation of the I κ B kinase (IKK β) that increases the stability of Δ Np73 α by phosphorylating it at serine 422 (Accardi et al., 2011). Furthermore, IKK β , Δ Np73 α , DNMT1, and EZH2 form a transcription regulatory complex. This complex is able to bind to a subset of p53 regulated promoters and interferes with the expression of DDR pathway- and apoptosis-related genes, but not with pro-survival genes like Survivin. This implies a role of β -HPV-38 E7 in the inhibition of p53 dependent apoptosis and allows for speculation on the influence of β -HPV E7 over DDR-proteins that depend on p53 activity as a transcriptional factor (for example Polh of the TLS pathway and XPC of the NER pathway) (Fischer, 2017).

Other Functions Related to DDR

A systematic screening of E7 interacting protein has shown that E7 from β -HPVs 8, 25, and 92 can interact with the tyrosine phosphatase and tumor suppressor PTPN14. While several high- and low-risk α -HPV types have been shown to degrade PTPN14 (White et al., 2016; Szalmás et al., 2017), the role of the β -HPV E7–PTPN14 interaction is yet to be investigated. PTPN14 is a required regulator of the Hippo signaling pathway, as it is necessary for the translocation of the Hippo transcription factor, YAP1, from the nucleus to the cytoplasm (Wang et al., 2012). This

indicates that β -HPV E7 may attenuate the Hippo pathway, if β -HPV E7 proves to also be capable of PTPN14 destabilization. The Hippo pathway plays a crucial role in the control of proliferation and induces apoptosis in the event of cytokinesis (Ganem et al., 2014). Phosphorylation of Yap1, a part of the hippo pathway, is a critical step in the induction of apoptosis in response to DNA damage (Levy et al., 2008). β -HPV infections occur in the skin and thus in an environment where UV-induced cell cycle arrest and apoptosis occur with some frequency. As a result, it would not be surprising if there yet undiscovered abilities of β -HPV E7 to disconnect the cellular response to UV in manners that promote the β -HPV lifecycle.

MOUSE MODELS: EVIDENCE FOR A ROLE OF β -HPV IN CARCINOGENESIS VIA A HIT AND RUN MECHANISM

Mouse models can be an effective tool for elucidating the oncogenic potential of β -HPVs. The most common of these is the transgenic (tg) mouse model that expresses an individual and groups of β -HPV genes (E2, E6, E7, E6/E7) or the entire early region of the virus under a keratin promoter. Placing the expression under a keratin promoter restricts expression to keratinocytes, the cell type infected by β -HPV. The result is that genes are only expressed at anatomical sites relevant in the context of β -HPV infections. Our review of the literature shows that β -HPV 8 is the most frequently investigated β -HPV type, followed by β -HPV 38, β -HPV 49, and β -HPV 20. Additionally, one study has investigated β -HPV 5 E7 in a nude mouse/artificial skin graft model (Buitrago-Pérez et al., 2012).

β -HPV 8 Transgenic-Mouse Models

Schaper et al. established the first β -HPV tg-model using the Keratin 14 (K14) promoter to express the entire early gene region of β -HPV 8. Transgene expression was the highest for E2, followed by E6 and E7 (Schaper et al., 2005). While the tg-negative littermates did not develop lesions on the skin or other organs, 91% of the β -HPV 8 tg-positive mice developed single- or multifocal benign tumors. Of this population, 25% showed varying degrees of dysplasia and finally 6% developed SCCs spontaneously. The SCCs developed without additional DNA-damaging events. This established an *in vivo* link between β -HPV 8 and carcinogenesis. A caveat, however, is the stable oncogene expression in tg-mice. β -HPV infections, and therefore expression of β -HPV genes in the immunocompetent population, are transient.

In a follow up study, a possible role of β -HPV 8 E2 in skin tumor induction was revealed when 60% of the E2 positive population, but none of the E2 negative population developed spontaneous ulcerous lesions of the skin (Pfefferle et al., 2008). Moreover, 3 weeks after UV irradiation, 87% of a tg-mouse line with high E2 expression levels and 36% of a tg-mouse line with lower E2 expression levels developed skin tumors. On the other hand, irradiation of tg-negative mice did not lead to tumor formation. This could point toward a potential synergy between β -HPV 8 E2, E6, and E7 for their role in carcinogenesis.

Later, by expressing E6 individually under the K14 promoter, it was shown that β -HPV 8 E6 is the major driving force behind both spontaneous tumor development in tg-mice and that tumor formation could be prevented via DNA vaccination (Marcuzzi et al., 2009, 2014). Rapid tumor development by induction, either through UV irradiation or wounding, was demonstrated. This observation that persistent β -HPV 8 infections combined with UV exposure and wound healing processes pose a significant risk factor for skin cancer is consistent with observations in individuals with persistent β -HPV 8 infections due to EV. A potential role of the signal transducer and activator of transcription 3 (Stat3) was demonstrated in tg mice expressing the early region of β -HPV 8 that additionally had an epidermis restricted ablation of Stat3 (Andrea et al., 2010, p.3). The Stat3 heterozygous line was significantly less prone to spontaneous tumor development and these tumors did not progress to malignancy. Hufbauer et al. showed that β -HPV 8 E6 expressing tg-mice dysregulate mi-RNA expression (Hufbauer et al., 2011). Following UV induced inflammation and wound healing, the levels of mi-RNA for regulatory targets including cell-cycle (Rb) and apoptosis (PTEN, PDCD4) remained dysregulated, while dysregulation in wt-mice was transient. Hufbauer et al. also demonstrated that β -HPV 8 E6 expression leads to an impaired DNA-damage response in tg-mice following UV irradiation (Hufbauer et al., 2015). β -HPV 8 E6 expressing mice were shown to be thymine-dimer positive for an extended period while wt-mice efficiently repaired the UV induced DNA damage within 48 h. More importantly, probing for the DSB marker, γ H2AX, revealed that the persistence of UV induced lesions in β -HPV 8 E6 expressing cells ultimately led to the formation of highly mutagenic and more dangerous DSBs. Immunohistochemical analysis of tg-mouse skin biopsies showed an increased signal for γ H2AX at both, early (3d, 5d) and later (13d, 24d) timepoints. This not only demonstrates β -HPV 8 E6's attenuation of DDR, but also its ability to enhance the cells tolerance of persistent and increasingly severe DNA-damage. UV radiation typically causes intrastrand lesions that are tolerated by TLS- and repaired by NER-pathway. If this type of damage persists it will lead to increased rates of DSBs. The emergence and persistence of DSBs could point toward a broad influence of β -HPV 8 E6 on the DNA-damage response machinery, stretching from pathways meant to handle minor DNA damage (TLS, NER) to pathways that control and repair highly mutagenic DSBs (NHEJ, HR).

The role of β -HPV 8 E7 has also been investigated in tg-mice (Sperling et al., 2012; Heuser et al., 2016). It was shown that β -HPV 8 E7, but not β -HPV 8 E6 enables an escape from the immune response of β -HPV 8⁺ lesions. β -HPV 8 E7 achieved this by inhibiting chemotactic signaling. β -HPV 8 E7 binds to C/EBP β and inhibits its interaction with the CCL20 promoter, leading to decreased levels of CCL20, a chemoattractant for Langerhans cells. β -HPV 8 E7 is also critical for the invasiveness of hyperproliferating keratinocytes by dysregulating cell-cell interactions.

β -HPV 38 Transgenic-Mouse Models

While β -HPV 8 tg-mice demonstrated spontaneous tumor formation, tg-models of other β -HPV types do not exhibit the

same behavior. Dong et al. showed that a mouse model for β -HPV 38 E6/E7 expressing mice under the Keratin 10 promoter exhibit hyperproliferation of the skin, but require external stimuli for carcinogenesis (Dong et al., 2005). Furthermore, Δ Np73 α has been identified as integral to the attenuation of cell cycle arrest after UV exposure (Dong et al., 2007). Loss of p53 attenuated Δ Np73 α expression and partially restored cell cycle arrest while loss of p73 lead to loss of Δ Np73 α and consequently to high levels of p21 expression and cell cycle arrest after UV exposure. Differential susceptibility to chemically induced (DMBA/TPA) carcinogenesis correlates with differential oncogene expression levels and indicates a dose-dependency for risk of carcinogenesis. Irradiation of wt-mice with a single UV-dose led to accumulation of p21 and cell cycle arrest in the epidermis, while β -HPV 38 E6/E7 expressing mice inhibited cell cycle arrest through attenuation of p21 accumulation. Chronic irradiation of tg-mice with UV led to the formation of AK-like lesions that are considered precursors to SCCs in humans, while wt-mice did not develop lesions. Some of the AK-like lesions progressed to SCCs after 22 weeks (Viarisio et al., 2011). This provides evidence for the carcinogenesis-risk amplifying potential of the β -HPV 38 oncogenes E6 and E7.

Other β -HPV Transgenic-Mouse Models

The K14 tg-model for β -HPV 49 E6/E7 does neither show spontaneous tumor development nor tumor development after UV-irradiation (Viarisio et al., 2016). However, the β -HPV 49 tg-mice lines were susceptible to chemically induced carcinogenesis of the upper digestive tract. When exposed to 4 nitroquinoline 1-oxide (4NQO), 87% of the β -HPV 49 tg-mice developed tumors in the upper digestive tract while 4NQO treatment had little effect on β -HPV 38 tg-mice, which are susceptible to UV-triggered skin carcinogenesis.

A model for β -HPV 20 E6/E7 showed enhanced proliferation and papilloma formation in the evaluated transgenic lines, when compared to non-transgenic controls (Michel et al., 2006). Chronic exposure to UV radiation led to the development of SCCs and proliferation was enhanced for several weeks after UV treatment. The tg-lines also showed a reduced expression of differentiation markers (involucrin & loricrin and irregular patterns of p53 expression post UV, while controls showed a continued expression of proliferation markers and even expression of p53.

Summary

Mouse models provide a valuable *in vivo* tool to dissect the relative ability of β -HPV genes to induce tumorigenesis. They also allow a distinction between different carcinogenic potentials exhibited by E2, E6, or E7 to be accessed. The mouse models make it evident that β -HPV E6 carries a particularly high potential due to its effects on both apoptotic pathways and the DDR. The tg-mouse models also support the "hit and run" hypothesis regarding β -HPV induced malignancies, since most models require external stimuli to induce malignant tumors. β -HPV 8 has the potential for spontaneous transformation in tg-models, but it remains unclear whether the potential persists when β -HPV 8 early gene expression is transient, as it is the

case in most humans. It also should be pointed out that the average daily UV dose of an American is $\sim 94 \text{ J/m}^2$ (Godar et al., 2001), while the dosages used in the β -HPV tg-model studies discussed here reach 45-fold great levels. Although constraints in experimental design may require doses of this magnitude, it is a necessary caveat to be considered when evaluating tg models of β -HPV induced skin cancer.

CONCLUSIONS

The impact of genus β -HPV on the DNA damage as well as the ability of these viruses to induce carcinogenesis is diverse and manifest in both *in vitro* and *in vivo* investigations. Generally, β -HPV viruses effect on DDR may be explained by their interest of uninterrupted proliferation, despite the presence of DNA damage. While most β -HPV E6 inhibit apoptosis by degrading the pro-apoptotic protein BAK, only a subset (β -HPV 5 and 8 E6) bind p300 strongly enough to destabilize the histone acetyltransferase, causing delayed repair of thymine dimers and double strand breaks (Giampieri and Storey, 2004; Muench et al., 2010; Howie et al., 2011; Hufbauer et al., 2011; Wallace et al., 2012, 2013, 2015). A larger subset of β -HPV E6 proteins (β -HPV 5, 8, 17, 23, 38, 49, and 92) interfere with p53 activity, but they do so through different mechanisms. Continuing the theme of diversity among β -HPVs, β -HPV 38 E6 immortalizes cells through a $\Delta\text{Np73}\alpha$ -dependent mechanism, while β -HPV 49 E6 acts more similarly to high risk α -HPV E6, immortalizing cells through interactions with E6AP and NFX1-91 (Caldeira et al., 2003; Gabet et al., 2008; Muench et al., 2010). **Figure 2** depicts these diverse effects of β -HPV E6.

There are notable differences between β -HPV E7 proteins as well. A large cohort of these viral proteins (β -HPV 5, 8, 24, 38, and 49 E7) impair pRB function. The sole mechanism

of pRB interference by β -HPV 5 and 8 E7 is through binding and induction of hyperphosphorylation of pRB. In addition to promoting the phosphorylation of pRB, β -HPV 38 and 49 E7 also decrease the half-life of pRB. β -HPV 24 E7 is only known to reduce pRB's half-life. Further, β -HPV 38 E7 has the ability to interfere with p53 activity by stabilizing the p53 antagonist, $\Delta\text{Np73}\alpha$, and increase inhibitory post-translational modifications of p53.

Not surprisingly, *in vivo* transgenic mouse models of β -HPV associated tumorigenesis reflect the varied ability of β -HPV to degrade the fidelity of host cells (Dong et al., 2005; Michel et al., 2006; Viariso et al., 2011). The tg-mouse models of β -HPV 8 show spontaneous tumor formation (Schaper et al., 2005; Pfefferle et al., 2008; Marcuzzi et al., 2009). While carcinogenesis is augmented by UV exposure in β -HPV 8 mouse models, similar models of other β -HPV induced cancers (β -HPV 20, 38, and 49) require external stimulants to develop tumors. The required stimulants vary as well. Expression of β -HPV 20 and 38 proteins cause cancer in mice exposed to UV, but only β -HPV 49 proteins cause upper digestive tract tumors after exposure to 4NQO. Notably, the transient nature of β -HPV infections in immunocompetent individuals is not reflected in the mouse models discussed above and the ability of persistent β -HPV infection to cause cancer is generally accepted. Further, the amount of UV exposure necessary to cause tumors in β -HPV tg mice is often well above what humans typically receive. Thus, new *in vivo* models will be quintessential to drive the field forward and may include mice infected with the murine papillomavirus (MmuPV1) that recapitulates some but not all of the characteristics of β -HPV (Meyers et al., 2017).

The bulk of evidence for β -HPV infections playing a role in NMSCs suggests that β -HPV 5, 8, and 38 are the most tumorigenic, with a particular and unsurprising emphasis on

TABLE 1 | Characteristics and differences of several β -HPV strands and high risk α -HPVs.

	HPV5	HPV8	HPV38	HPV49	high risk α HPVs
Impact on DDR	•Delay UV repair •Destabilize p300 •ATM, ATR, BRCA1/2 ↓	•Delay UV repair •Destabilize p300 •ATM, ATR, BRCA1/2 ↓	•Delayed UV repair •$\Delta\text{Np73}\alpha$ ↑ •Inhibit p300	•Unknown	•Attenuate and repurpose several DDR proteins
Prevention of Apoptosis	•Degrade BAK	•Degrade BAK	•Stabilize p53 •Alter p53 activity •Degrade BAK	•Degrade p53	•Degrade p53
Proliferation	•Hyperphosphorylate pRb	•Hyperphosphorylate pRb	•Attenuate p21 accumulation •Phosphorylate pRB	•Hyperphosphorylate pRb	•Degrade pRb
Immortalization	•No	•No	•Yes	•Yes	•Yes
Tumor maintenance	•Not required	•Not required	•Not required	•Not required	•Required
E6 motif	•No PDZ domain	•No PDZ domain	•No PDZ domain	•No PDZ domain	•PDZ domain present
Animal Models	•Artificial skin graft model •UV-induced lesion formation	•Spontaneous tumor formation	•UV DNA damage induced tumor formation	•No cSCC formation •Chemically-induced carcinogenesis in digestive tract	•Spontaneous tumor formation

This table compares the impact of several β -HPVs and high risk α -HPVs on DDR and other pathways relevant to carcinogenesis. ↑ and ↓ indicate increased or decreased abundance, respectively.

the homologous of high risk α -HPV oncogenes (E6 and E7). These viruses are expected to promote carcinogenesis via the “hit and run” model that is dependent on their amplification of UV’s genome destabilizing effects and otherwise decreasing genome fidelity. Since the mechanisms of abrogating genome stability vary among β -HPVs, it is reasonable to expect that the carcinogenic potential of individual β -HPVs depends on their cellular environment during infection. As a result, not only is the clear delineation of pathways and proteins impacted by β -HPV necessary to understand their oncogenic potential, but also the relative ability among β -HPVs to disrupt these pathways. A major knowledge gap regarding β -HPV induced oncogenesis is the extent that the reduced availability of ATM, ATR, BRCA1, and BRCA2 affects the activity of these proteins in each of the repair pathways that they participate in. This could expand the known ability of β -HPV proteins to disrupt DNA repair to include nearly every cellular response to damaged DNA. Alternatively, if the impairment of these repair proteins was context specific, it could highlight particularly deleterious situations for a β -HPV infection to occur. Further, there is a diversity of genome destabilizing events beyond DNA damage (failed cytokinesis, centrosome duplication errors, etc.) that if disrupted would fit the ascribed “hit and run” model of tumorigenesis. Defining the mutagenic potential of β -HPV infections is essential to take next steps in preventing disease associated with these viruses. **Table 1** compares β -HPV 5, 8, 38, and 49 as well as high-risk α -HPV oncogenes regarding their oncogenic potential. A possible intervention is the development of β -HPV specific vaccines. While β -HPV infection occurs in early childhood, a vaccine against specific “high-risk” types may prevent re-infection. Additionally, immunity acquired through vaccination may be more effective than immunity through infection. The FDA-approved technology to make the current

vaccine could be readily adapted to prevent β -HPV infections or specific inhibitors of β -HPVs could be developed and added to sunscreens to precisely target the intersection of UV and viral infection. A commonly stated challenge to the development of a vaccine against β -HPV is the fact that these infections are initially acquired soon after birth and that β -HPV infections do not illicit a protective immune response. Although true, vaccination could mitigate disease by preventing reinfection and potential initial infection with particularly oncogenic β -HPVs not necessarily acquired at birth. Further, vaccination is effective against members of the alpha HPV genus despite poor natural adaptive immune responses to those infections. Ultimately, these ambitious goals are dependent on advancements in the dissection of β -HPV biology and its ability to hinder the cellular DDR.

AUTHOR CONTRIBUTIONS

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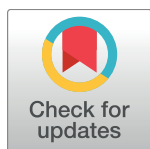
OPINION

The curious case of APOBEC3 activation by cancer-associated human papillomaviruses

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High-risk human papillomavirus (HR-HPV) infections cause approximately 5% of all human cancers worldwide. These include almost all cervical carcinomas, a leading global cause of cancer deaths, as well as a significant percentage of other anogenital tract cancers and a growing fraction of oropharyngeal carcinomas. To accommodate their life cycles, HR-HPVs need to extensively rewire infected cells. The HR-HPV E6 and E7 proteins are the main drivers of this process, and their expression elicits a barrage of cellular defense responses that restrict this unfriendly takeover of the host cell.

Not surprisingly, HR-HPVs have in turn evolved mechanisms to escape or curb antiviral and anti-oncogenic cellular responses. These mechanisms include degradation of the retinoblastoma (RB1) and the p53 (TP53) tumor suppressors by HR-HPV E7 and E6, respectively. If unopposed, RB1 and TP53 would cause cell cycle arrest, senescence, or cell death in response to HR-HPV infection (Fig 1). Other antiviral pathways, including DNA sensing and interferon signaling, are also blunted by HR-HPV E6 and E7 proteins [1].

Surprisingly, HR-HPVs have not evolved strategies to counteract restriction by apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like 3 (APOBEC3, or simply A3). A3s are interferon-regulated DNA cytosine-to-uracil deaminases encoded as a cluster of seven genes (A3A–A3H; there is no A3E) on human chromosome 22, which are all expressed, albeit at vastly different levels, in epithelial cells [2], which are the natural hosts of HPV infection. While their cytidine deaminase activity causes deoxycytidine (C) to deoxythymidine (T) mutations during viral genome synthesis, A3s also restrict viral replication through cytidine deaminase-independent mechanisms [3].

In response, many viruses have evolved mechanisms to evade A3 restriction. The human immunodeficiency virus 1 (HIV1) Viral infectivity factor (Vif) protein targets A3 family members for degradation, and the HIV2 Viral protein X (Vpx) protein targets A3A for degradation [4, 5]. The Hepatitis B Virus X protein impairs this pathway by packaging A3G into exosomes [6]. Human polyomaviruses—including the Merkel Cell Polyomavirus (McPyV)—trigger A3 activity, yet McPyV-positive Merkel cell carcinomas do not show an A3 mutational signature [7, 8]. This strongly suggests that McPyV overrides A3 restriction [8].

While ectopically expressed HR-HPV E7 and E6 have each been reported to increase expression of A3 family members, and A3A can restrict infection with in vitro-generated HPV16 pseudovirions, A3 activity is not blocked by HR-HPVs [3, 9–11]. Despite the fact that HPV genomes contain fewer than predicted A3 recognition sites [12], the mutational drift caused by A3 mutagenesis is extensive; many of the thousands of HPV16 variants that were detected in a recent study exhibit nucleotide changes that are consistent with A3 action [13].

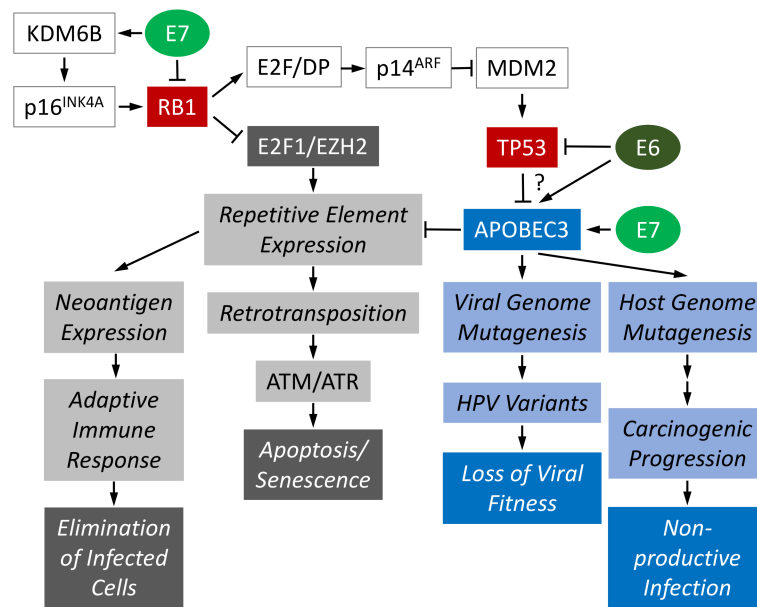


Fig 1. Molecular consequences of APOBEC3 induction in HR-HPV-infected cells. See text for detail. APOBEC3, apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like 3; HR-HPV, high-risk human papillomavirus.

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Furthermore, A3 expression causes nucleotide changes in the host cellular genome. Integration of HPV sequences during malignant progression is accompanied by increased A3A levels [14], and cervical carcinomas and other HPV-associated cancers exhibit A3 mutational signatures [15, 16]. Indeed, A3-mediated host genome destabilization may be one of the mechanisms that drive carcinogenic progression of HR-HPV-associated lesions as evidenced by Phosphatidylinositol-4,5-Bisphosphate 3-Kinase Catalytic Subunit Alpha (PIK3CA) mutations that are consistent with A3 mutagenesis that have been detected in HPV-associated cancers [17]. HPV-associated cancers are generally nonproductive lesions [1], therefore increased carcinogenicity is unlikely to provide an evolutionary benefit to the life cycles of HR-HPVs and does not explain why HR-HPVs have not evolved to block A3 mutagenesis.

Why, then, have HR-HPVs not evolved to subvert this highly active cellular defense response? It has been argued that A3 activation serves to generate viral diversity, and there is no doubt that HPVs are incredibly diverse; the current count stands at more than 300 HPV genotypes, as well as thousands of variants, many of which may have been generated as a result of A3 mutagenesis [13].

The recent discovery by Fred Dick's research group that RB1 plays a key role in the epigenetic silencing of repetitive elements may provide an alternative explanation as to why it may be beneficial for HR-HPVs not to counteract A3 restriction [18]. RB1 silences repetitive elements by associating with a unique E2F1 transcription factor complex that contains the Enhancer Of Zeste 2 Polycomb Repressive Complex 2 Subunit (EZH2) methyl transferase. The degradation of RB1 by HR-HPV E7 proteins is therefore predicted to cause transcription of repetitive elements [18]. Because translation of some of these—most notably the Long Interspersed Element-1 (LINE1 or L1)—results in neoantigen expression, transcription of repetitive elements would put HR-HPV-infected cells at risk of extinction through adaptive immune responses. Moreover, the LINE1 open reading frame 2 (ORF2) encodes an endonuclease that facilitates the mobilization of LINE1 elements through a “copy and paste” mechanism referred to as retrotransposition. Excessive double-strand DNA breaks caused by the ORF2

endonuclease can result in senescence or apoptosis, which would also lead to the elimination of HR-HPV-infected cells (Fig 1). While the majority of the LINE1 copies in the human genome are 5' truncated and do not express functional endonucleases, it has been estimated that the remaining functional LINE1 elements may cause 0.3% of all human mutations [19, 20]. This staggering number likely underestimates the mutational impact of LINE1 element mobilization. LINE1 can also mobilize nonautonomous transposable elements, which only requires its second open reading (ORF2) that frequently remains intact even with 5'-truncated LINE1 copies. A3s are well known to restrict expression of repetitive elements, including LINEs, via deaminase-independent activity [21]. Therefore, HR-HPV-infected cells would gain significant advantages from this A3-dependent restriction of LINE elements. Because even unrestrained A3 activity will not completely prevent retrotransposition, the residual activation of double-strand DNA break sensing and repair machinery may be beneficial for efficient HR-HPV genome replication given its well-documented dependence on these factors, including the Ataxia Telangiectasia Mutated (ATM) and Ataxia Telangiectasia And Rad3-Related Protein (ATR) kinases [22].

In addition, HR-HPV-mediated RB1 degradation causes high-level expression of satellite RNAs that can lead to formation of R-loops, a DNA RNA three-stranded structure, which causes replication forks to stall [18]. A3 target the single-stranded DNA in R-loops and can thereby also activate the DNA damage response [23], which benefits HPV replication. Lastly, HR-HPVs may benefit from a deaminase-independent activity of A3, making the A3 mutational signature in HPV-associated tumors simply a by-product of the viruses' requirement for another function of A3.

Because nearly every unvaccinated, sexually active individual has been infected with an HR-HPV, such an incredibly successful virus should have evolved a defensive strategy against the potent restriction of viral replication and viral persistence by A3 unless it provides them with a selective advantage. HR-HPVs uniquely cause RB1 degradation and thus are predicted to de-repress retrotransposon expression. TP53 is known to restrict retrotransposition [24–26], and HR-HPV E6-mediated TP53 degradation may further increase LINE1 activity. While activation of double-strand DNA sensing and repair pathways induced by retrotransposition triggered by RB1 degradation and R-loop resolution by A3s may stimulate HR-HPV genome replication and progeny synthesis, A3 restriction of repetitive elements may protect HR-HPV-infected cells from undergoing excessive, lethal DNA damage and genomic instability. Moreover, A3 restriction will prevent elimination of HR-HPV-infected cells by adaptive immune responses to neoantigen expression due to expression of repetitive elements.

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