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TITLE: **Cutaneous Human Papillomaviruses as Co-Factors in Non-Melanoma Skin Cancer:**

PRINCIPAL INVESTIGATOR: Nicholas A Wallace

CONTRACTING ORGANIZATION: Kansas State University
Manhattan Ks, 66506

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Nicholas A. Wallace

E-Mail: nwallac@ksu.edu

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Kansas State University
1717 Claflin Road
Manhattan Ks, 66506

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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.

15. SUBJECT TERMS
Non-melanoma skin cancer, Cutaneous human papillomavirus infection, Ultraviolet irradiation, Ionizing radiation, DNA damage, Genome fidelity.

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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 Tasks: During the first reporting period, I have made significant advancements toward my goal of establishing myself as a leader in the non-melanoma skin cancer research. This is will allow me to make continued contributions to the PRCRP topic area, "Melanoma and Other Skin Cancers

- **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:** ~85% (detailed in subsequent sections, calculated from percentage of milestones met)

Research Specific Tasks: My research continues to highlight the oncogenic risk of cutaneous human papillomavirus infection co-occurring with exposure to military relevant risk factors UV and ionizing radiation. My findings are consistent with β-HPV infections contributing to non-melanoma skin cancer in military personnel.

- **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:** 100% (detailed in subsequent sections)
    - We have extended our efforts beyond the original task after completing the work proposed in the SOW.
- **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:** ~90% (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:** ~95% (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:** 100% (detailed in subsequent sections)
            - We have extended our efforts beyond the original task after completing the work proposed in the SOW.
    - **Specific Aim 3 Major Task 1:** P300-Independent Disruption of DNA Crosslink Repair
        - **Projected Completion date** (from SOW): August 1, 2018
        - **Completion Percentage:** ~65% (detailed in subsequent sections)
            - We have completed this task as much as possible and are currently examining the other p300-independent phenotypes that we identified during the completion of AIM2. This adheres with the theme of the proposal.
    - **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)
            - We have completed this task as much as possible and are currently examining β-HPV E6 in the context of a common mutation found in NMSC. This adheres with the theme of the proposal.

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

● Last reporting Period Summary: I met with Dr. Giam and Uniformed Services University Health Sciences Microbiology faculty in person. I also presented my research to Dr. Laimonis primarily via email and phone conversations.

● Second Reporting Period: I communicated with Dr. Giam about my progress via email. I met with Dr. Laimonis twice in person to discuss my work.

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

● Last reporting Period Summary: 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.

● Second Reporting Period: I attended the 2018 and 2019 DNA Tumor Virus Meetings and chaired a session in the 2018 meeting. I also presented at the 2019 International Papillomavirus Meeting. I also attended the 18th Annual Symposium in Virology hosted by the University of Nebraska's Center for Virology. In total, my lab and I presented work related to this project 4 times at international conferences and 3 times at a national conference.

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

Dr. Laimonis and I continue discuss my career regularly. This has included regular grant revisions and career guidance. These interactions have been incalculably helpful as I've navigate my pre-tenure career. Thanks to his advice, support from the CDMRP and hard work from my lab

members, my pre-tenure reappointment package received a unanimous vote from my colleagues in the Division of Biology at Kansas State University.

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

With Dr. Clem's support, I was able to move to a newly renovated lab that includes an office next to Dr. Clem and near two other virologists/immunologists. This has dramatically increased the frequency of our informal chats. He also edits my grants and manuscripts before they are submitted.

Milestones Achieved:

(1) Presentation of project data at preeminent meetings annually, (12, 24 36 Months)
   a. In this funding period, project data was presented 7 times at noteworthy national and international meetings. It was also presented several other times at local/regional events.

(2) Submit major grant proposal to extend project by the end of the second budget period (24 Months)
   a. In funding period 1 I submitted the two grants described below:
      i. A proposal to the American Cancer Center was resubmitted 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.
      ii. I am a junior investigator on a COBRE proposal (P20) resubmitted 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.
   b. I also submitted an R15 proposal to the National Institutes of Health entitled, "High Risk Genus Alpha HPV Oncogenes Dysregulate Translesion Synthesis". This grant received a favorable score but was not funded. There is no overlap with this CDMRP funded project.
(3) If necessary, revise and resubmit grant. (24, 36 Months)
   a. All three grants described above were resubmitted during budget period 2 and are awaiting review.
(4) Publish Findings in Peer Reviewed Journal (24, 36 Months)
   a. Three manuscripts were published ahead of schedule during the first funding period.
      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
   b. Two more manuscripts were published during the most recent funding period. References are listed below
      i. Cervical cancer cell lines are sensitive to sub-erthemal UV exposure. Gu W., Sun S., Kahn A., Dacus D., Wendel SO., McMillan N., Wallace NA. 2019 PMID: 30517878 LINK
      ii. mSphere of Influence: the Value of Simplicity in Experiments and Solidarity among Lab Members. Wallace NA. 2019. PMID: 31217299 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 Fly Swat 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.

We anticipate submitting a manuscript describing this Aim's results for review before the end of calendar year (2019).

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

This was completed during the first budget period.

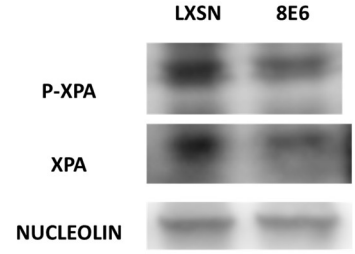

Figure 1: β-HPV 8 E6 blocks XPA phosphorylation.

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 ΔE6 expressing cells.

The majority of this work was completed during the first budget period. In this budget period, we validated our previous reports in primary and hTERT transformed keratinocytes (Figures 1-2). These confirm that β-HPV E6 attenuates XPA stabilization and phosphorylation in biologically relevant cells.

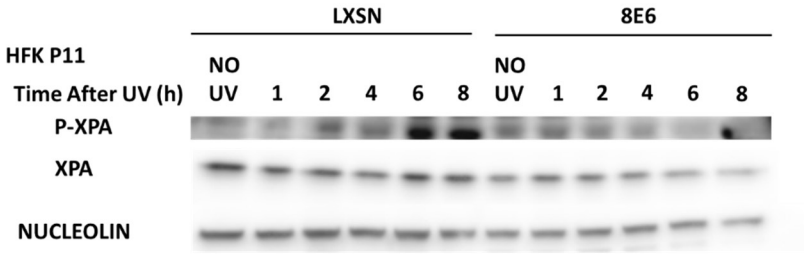

Figure 2: β-HPV 8 E6 hinders XPA phosphorylation and stabilization after UV.

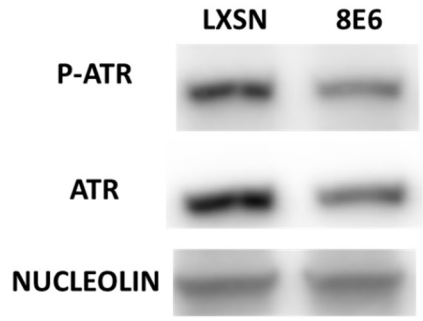

Figure 3: β-HPV 8 E6 decreases ATR phosphorylation/activation.

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

Our first step towards this goal was to define β-HPV E6's ability to hinder ATR phosphorylation/activation a necessary upstream step during XPA stabilization/phosphorylation. We found that β-HPV E6 attenuated ATR activation in untreated cells (Figure 3) and in response to UV (Figure 4).

The results led to our decision not to pursue this subtask further as excess ATR or XPA would not rescue the nucleotide excision repair pathway from β-HPV E6's

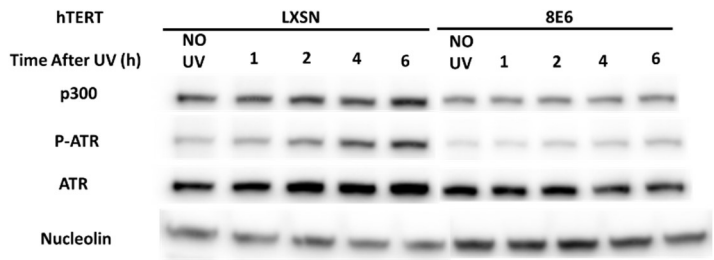

Figure 4: β-HPV 8 E6 prevents ATR activation/phosphorylation in response to UV

inhibition of ATR activation. Instead we focused our efforts on understanding the breadth of β-HPV E6's inhibition of ATR signaling.

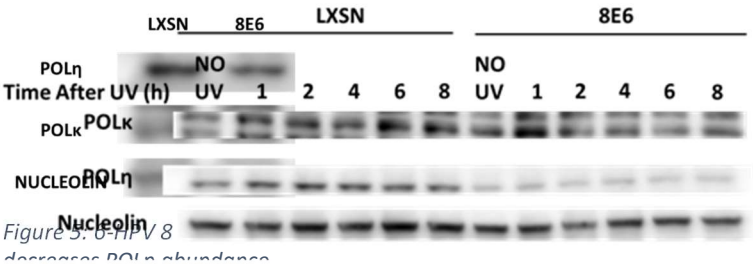

Figure 7: β-HPV E6 abrogates POLη stabilization after UV. POL kapaa, (not a known ATR phosphorylation target was not altered.

These "follow up" experiments extended the list of UV-damage

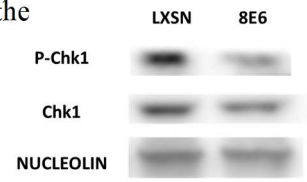

Figure 1: β-HPV E6 decreases CHK1 abundance and activation/phosphorylation.

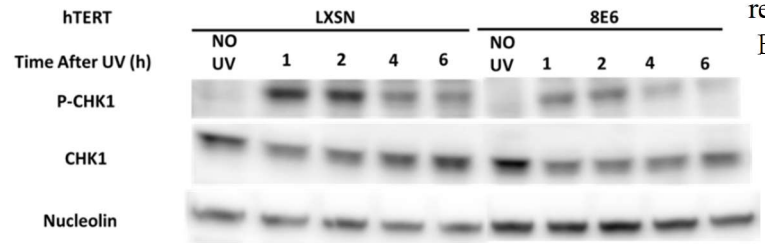

Figure 8: β-HPV E6 decreases CHK1 phosphorylation after UV.

responsive pathways hindered by β-HPV E6 to include translesion synthesis (Figure 5) and cell cycle regulation (Figure 6). β-HPV E6 diminished the abundance of POLη, a translesion synthesis polymerase. POLη stability is regulated by ATR phosphorylation. POLκ is not a known ATR target and was not altered by β-HPV E6. β-HPV E6 also decreases the CHK1 phosphorylation, an established cell cycle mediator. These inhibitory properties extend beyond untreated cells. Following UV, we found POLη levels increased in control cells but not when β-HPV E6 was expressed (Figure 7). As in untreated cells, POLκ does not appear to be altered. CHK1 phosphorylation is also decreased by β-HPV E6 after UV (Figure 8). This did not seem to affect a further downstream target (CDC25A) involved in cell cycle regulation, demonstrating that β-HPV E6 inhibits some but not all ATR signaling events (Figure 9).

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

We demonstrated that β-HPV E6 inhibited nuclear XPA accumulation using immunofluorescence microscopy in our last reporting period. Here, we complete this subtask using subcellular fractionation and immunoblot. This biochemical approach

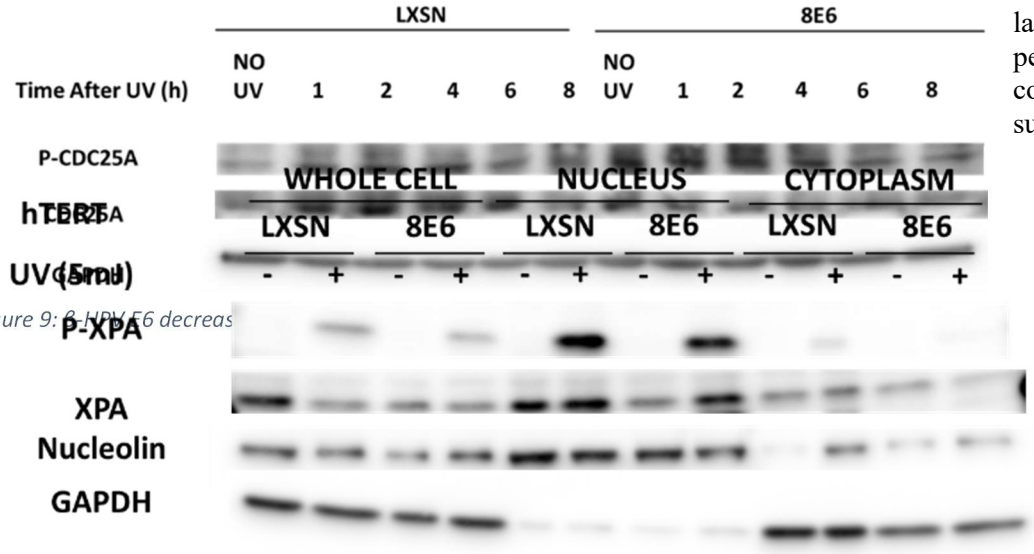

Figure 9: β-HPV E6 decreas

Figure 10: β-HPV 8 E6 does not alter XPA or p-XPA subcellular localization after UV.

demonstrated a mild reduction in nuclear XPA in cells expressing β-HPV E6. This was in line with our previous results. We ascribe the difference in magnitude of the effect to difference between the assays.

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.

To complement the immunoblots described above of UV repair response proteins, we conducted an in silico screen for p300-dependent genes in Nucleotide excision repair and pathways downstream of UV repair (homologous recombination and non-homologous end joining). We did not find evidence that supported further characterization of β-HPV E6 decreasing NER expression in a p300-dependent manner as only XPA was reduced in cells with diminished p300 availability (Figure 11).

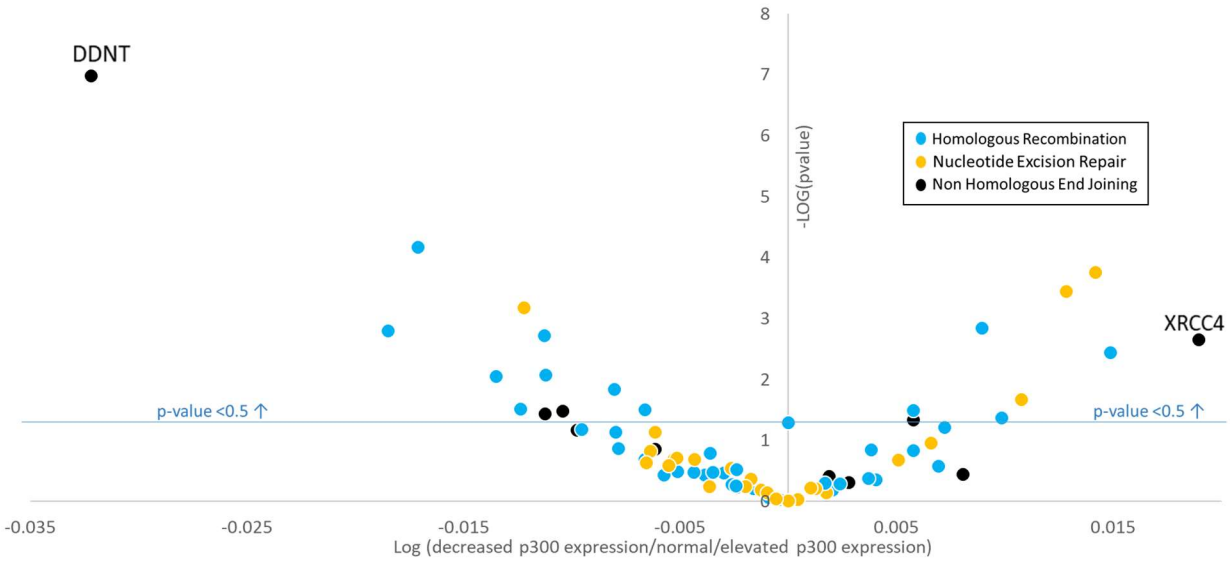

Figure 11: Expression Analysis of Cell Lines with Reduced p300 Expression: X-axis denotes magnitude of expression changes compared to cell lines with unaltered p300 expression. Y-axis depicts significance.

Milestones Achieved:

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

This was completed during the first budget period.

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

This was completed during the first budget period.

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

In budget period 1, we saw decreased XPA phosphorylation and stabilization after UV in a p300-dependent manner. We confirm this reduction in more biologically relevant cells.

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

We found that β-HPV E6 inhibits ATR activation and expression meaning that it ultimately represses crosslink repair by decreasing ATR expression and activation. An extension of this observation is that β-HPV E6 acts indirectly through XPA.

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

β-HPV E6's ability to change XPA localization appears to be p300-dependent. We confirmed this by subcellular fractionation and immunoblotting.

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

We found that reduced p53, XPA and p-XPA accompanies β-HPV E6 expression, especially after UV exposure. Further, β-HPV E6 decreases the abundance and/or phosphorylation of other proteins essential for UV repair, including CHK1 and POLη.

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.

Most of this subtask was completed during the first budget period. We had continued by showing that while β-HPV E6 does not decrease DNApk expression, it does limit DNApk autophosphorylation in response to DNA damage from Zeocin, a radiation mimetic.

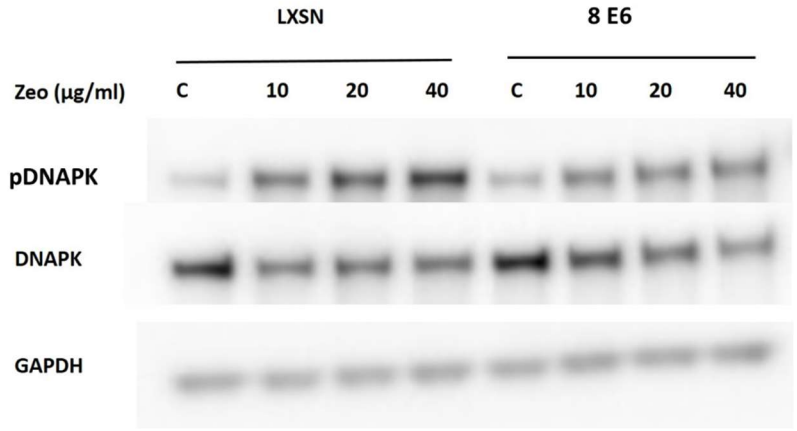

Figure 12: β-HPV E6 decreases pDNApk induction after Zeocin induced DSBs.

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

We have shown that β-HPV E6 does not decrease canonical NHEJ protein abundance by immunoblot and that it does not generally impact NHEJ gene expression using an in silico screen. Indeed, it increases the abundance of phosphorylated DNApk in untreated cells.

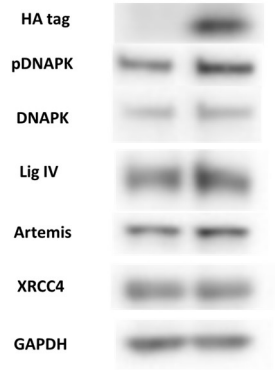

Figure 13: NHEJ protein abundance is not decreased by β-HPV E6.

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

We have generated the cell lines with the reporter construct integrated in cells expressing β-HPV E6. However, a roadblock arose that prevents us from completing this subtask as originally proposed. The necessary cell analyzer housed at the on-campus flow cytometry facility has broken and there is no reliable repair timeline. As a result, we are shift to a plasmid based reporter system that will

utilize an older but equally valid approach. Since this was an unanticipated obstacle, we have not been able to complete this subtask.

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

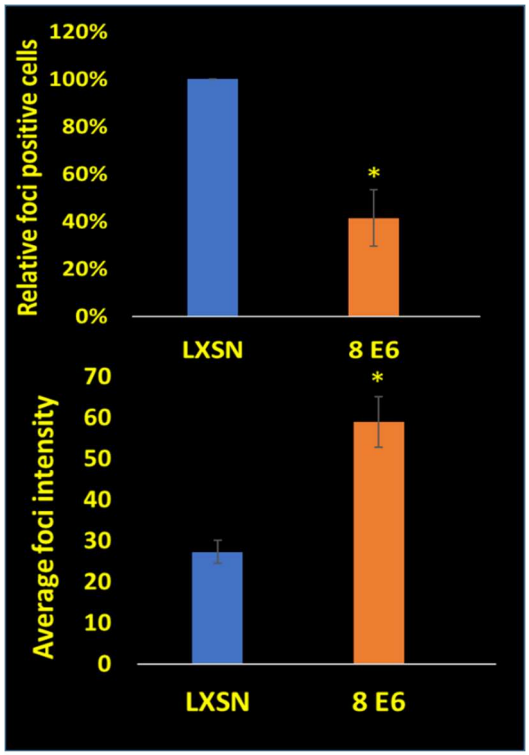

Figure 14: β-HPV E6 causes there to be fewer but brighter pDNAPK foci in cells.

We have completed our analysis of pDNApk foci in two cell lines (HFKs and U2OS cells). In undamaged cells β-HPV E6 lowered the number of pDNApk foci, but those foci had higher intensity (Figure 14 and 15). This results are consistent with hard to repair lesions.

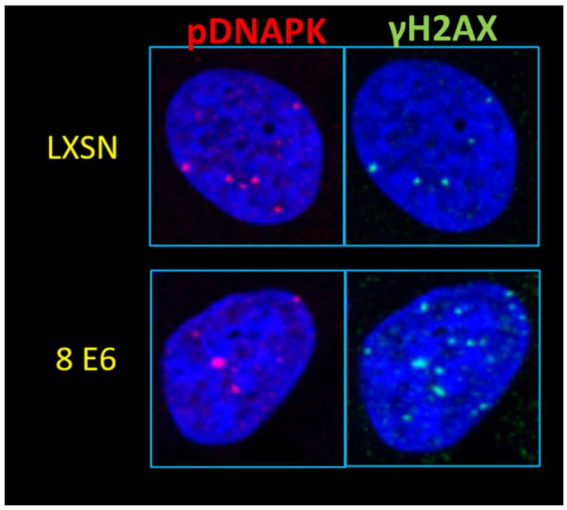

Figure 15: β-HPV E6 decreases the number of pDNApk foci, but the ones that form are larger.

We also measured the impact of β-HPV E6 on repair complex resolution after DSB induction. These data show that β-HPV E6 makes it harder for cells to resolve pDNApk repair complexes. When damage is induced, pDNApk foci remain for at least 24 hours in β-HPV E6 expressing cells (Figure 16). They are resolved by this time in control cells. Consistent with delayed resolution, pDNApk foci are also larger when β-HPV E6 is

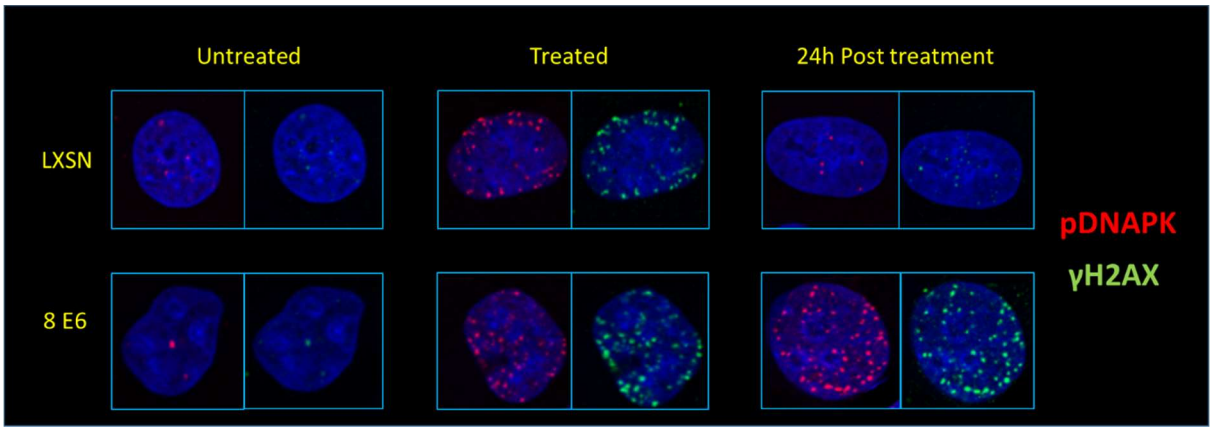

Figure 162: β-HPV E6 increases the persistence of pDNApk foci after Zeocin-induced DSBs.

expressed. We have previously reported that β-HPV E6 attenuates homologous recombination, ultimately leading to persistent RAD51 foci 24 hours after DSB induction. The similarities in this timing led us to hypothesize that β-HPV E6 would cause the same cell to try attempting DSBs with homologous recombination and non-homologous end joining. To test this hypothesis, we costained cells with antibodies against pDNAPK and RAD51. Twenty-four hours after DSB induction, β-HPV E6 signficantly

increased the frequency of cells with both RAD51 and pDNApk foci, suggesting both pathways are both attempting to work at the same time (Figure 17). Moreover, we found a significant induction of colocalization among the repair proteins (Figure 18) when β-HPV E6. This is likely catastrophic for the cell because NHEJ (indicated by pDNApk) removes the type of single strand overhangs that must occur for RAD51 foci to exist. This is predicted to result in large deletions.

To understand why some cells had colocalized foci, but not others we used a C-terminal HA tag β-HPV E6 to define viral gene loads in cells with and without persistent RAD51 foci. This analysis indicates that RAD51 foci are more persistent as β-HPV E6 levels rise.

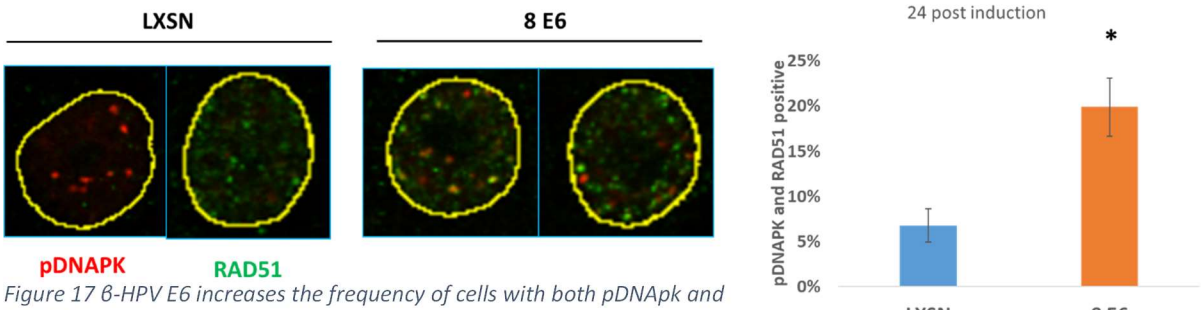

Figure 17 β-HPV E6 increases the frequency of cells with both pDNApk and RAD51 foci.

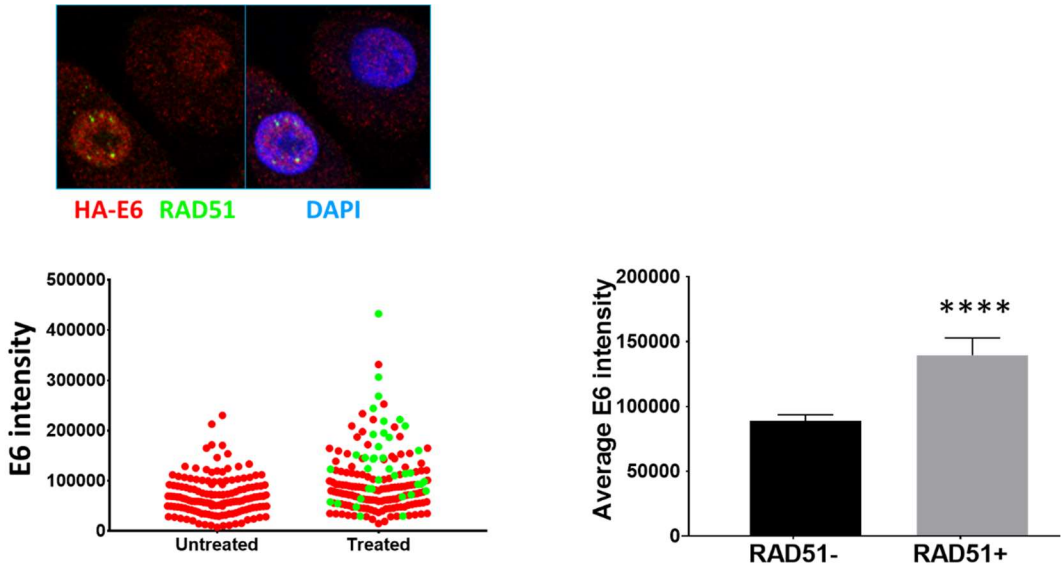

Figure 18: β-HPV abundance (measured by intensity) varies among cells (top and bottom left). Green dots in the bottom left denote cells with persistent RAD51 foci. Increased β-HPV E6 is significantly associated with RAD51 persistence (bottom left and right). Treat cells were analyzed 24 hours after Zeocin-induced DSBs. Bottom right is quantification of β-HPV E6 intensity in cells with and without RAD51 foci 24 hours after treatment.

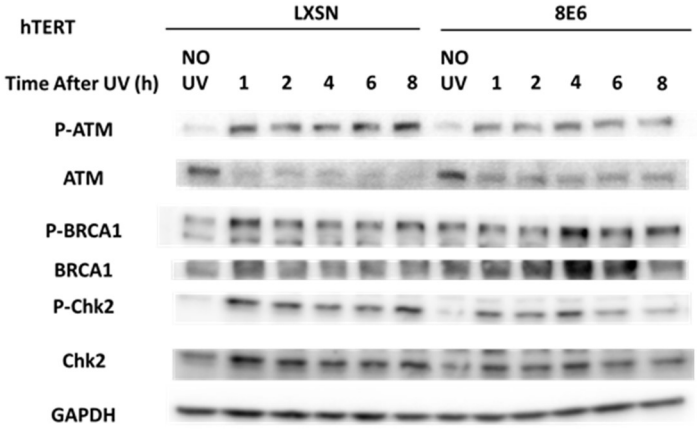

Figure 19: β-HPV 8 E6 prevents ATM activation/phosphorylation and phosphorylation of ATM target proteins after UV exposure.

Finally, we observed ATM signaling in β-HPV E6 expressing cells, since ATM is the primary kinase responsible for inducing cell cycle arrest and coordinating repair during the DSB response. These data demonstrate that β-HPV E6 prevents ATM autophosphorylation and also attenuates the phosphorylation of targets down stream of ATM, including BRCA1 and CHK2 (Figure 19). This is also consistent with impaired DSB signaling and reduced ability to arrest the cell cycle after DNA damage. Together these results support the hypothesis that β-HPV E6 inhibits DSB repair by abrogating signaling events that regulate DNA damage induced cell cycle arrest.

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.*

We found that β-HPV E6 did not inhibit DNApk expression but did impair its activation in response to DNA damage. This seems to create an environment where cells are continually trying but failing to repair DSBs via NHEJ as the basal levels of activated DNApk are elevate with β-HPV E6 expression.

Thus this milestone was met and surpassed.

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

This milestone was achieved during the first budget period

(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.*

Unfortunately, technical difficulties beyond our control or ability to resolve (broken flow cytometer equipment) have prevented this milestone from being met. We are currently working on alternative methods to directly measure NHEJ. We have acquired abundant supporting data that consistent with β-HPV E6 blocking 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.*

This goal was met and surpassed. Specifically, repair foci form at control rates when β-HPV E6 is expressed, but they do not resolve readily. This phenotype is dependent on p300. The persistent pDNApk foci colocalize with a homologous recombination protein (RAD51) potentially leading to large deletions. The persistence of repair lesions correlates with high β-HPV E6 abundance.

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

We are preparing a manuscript for submission to the Journal of Virology based on the data from Specific Aim 2. This is the highest ranked journal in virology. We expect to submit the manuscript in early Fall.

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

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

This was completed during the first budget period.

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

This was completed during the first budget period.

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

This was completed during the first budget period.

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

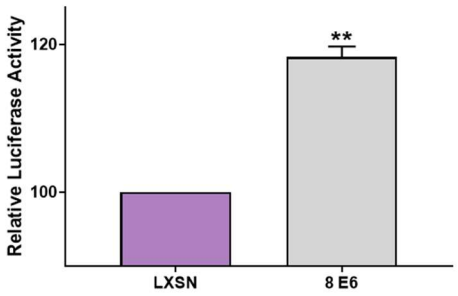

Figure 20: β-HPV 8 E6 increases TEAD promoter activity as seen by luciferase reporter

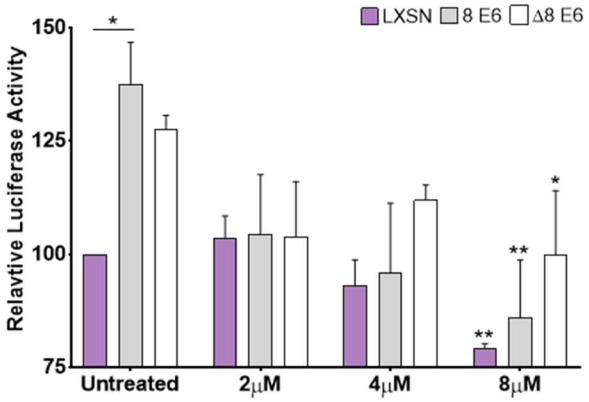

Figure 21: Although β-HPV E6 increases TEAD promoter activity in untreated cells, it does not prevent H2CB-mediated reduction of TEAD promoter activity.

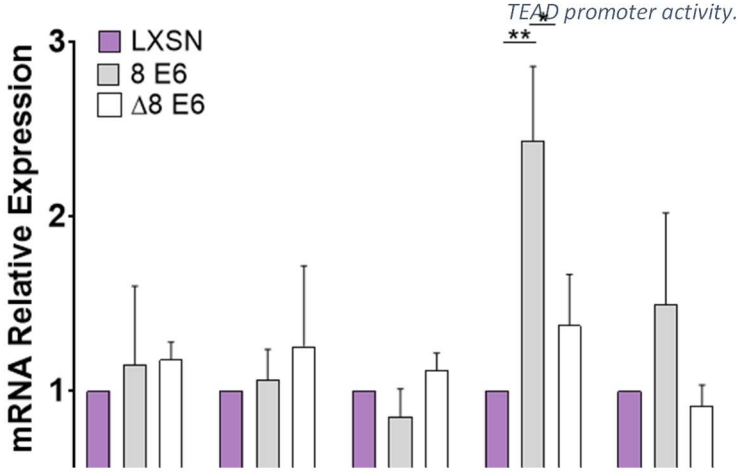

Figure 22: Although β-HPV E6 does not alter HP gene expression, it raises TEAD responsive gene expression.

Most of this subtask was completed in the first budget period, but we have expanded it. We have confirmed that β-HPV E6 increases TEAD promoter activity in untreated cells (Figure 20, but causes significant drops in TEAD promoter activity after failed cytokinesis (Figure 21). These results were independently verified using rtPCR to measure the expression of TEAD responsive genes (Figure 22). We also performed two silico

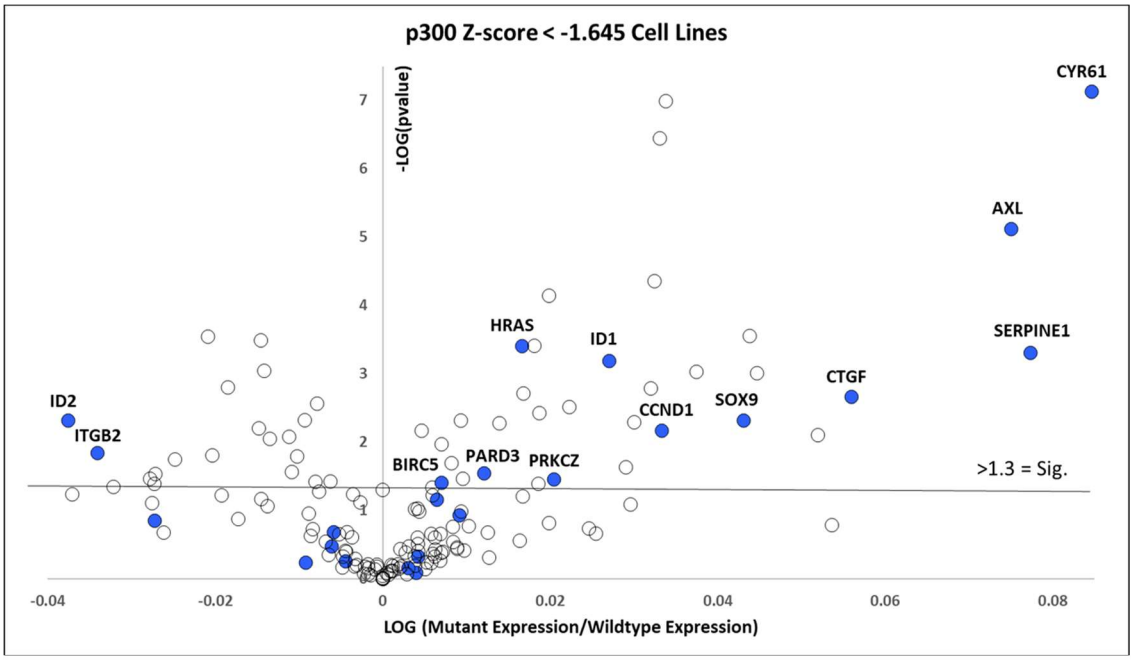

Figure 23: Cells with less p300 have higher expression of TEAD responsive genes (highlighted in blue). The horizontal line denotes statistically significant changes.

screens of cells with reduced p300 expression. Canonical hippo pathway genes were a reasonably close mix of being up and down regulated by p300 (Figure 23). However, TEAD responsive genes show signs of being significantly downregulated by p300 (Figure 24). p300 regulation of a gene is interpreted as being opposite of what happens to the gene when there is less p300 in cells. A subset of these changes

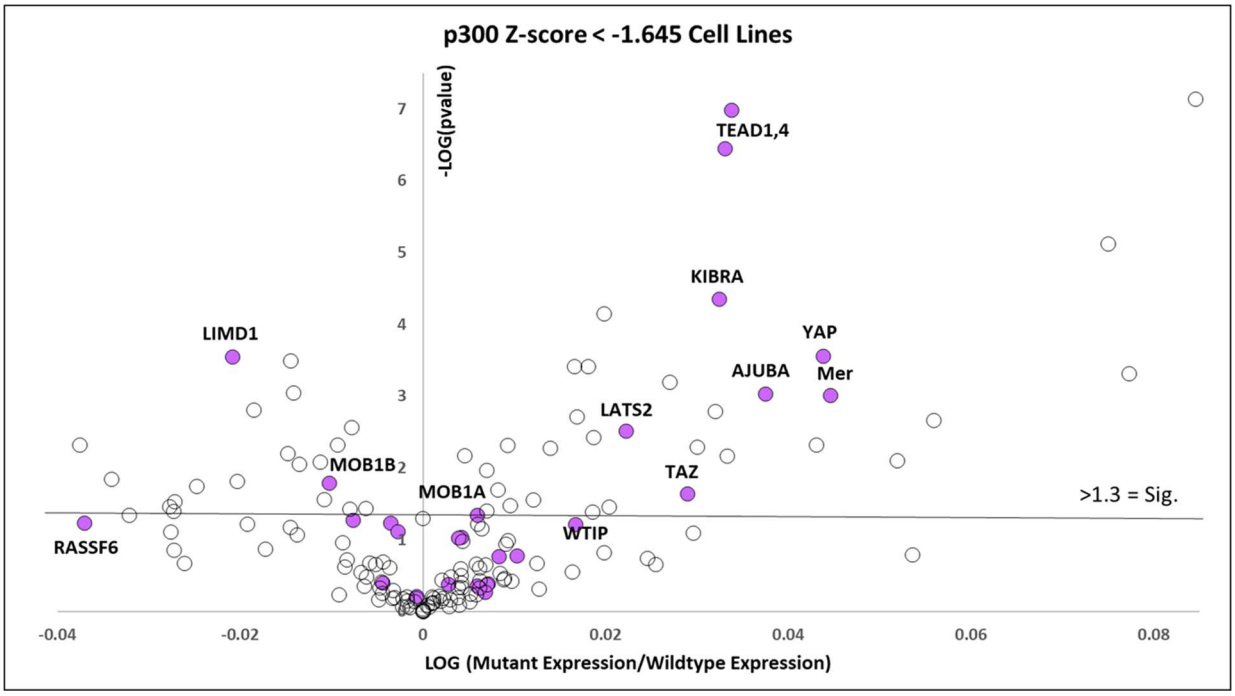

Figure 24: Cells with less p300 have altered expression of Canonical Hippo Pathway genes (highlighted in purple). The horizontal line denotes statistically significant changes.

were confirmed by immunoblot (Figure 25). We performed gene ontology analysis on genes that were upregulated when p300 was downregulated using GOrilla software and found the hippo pathway significantly altered (Figure 26)

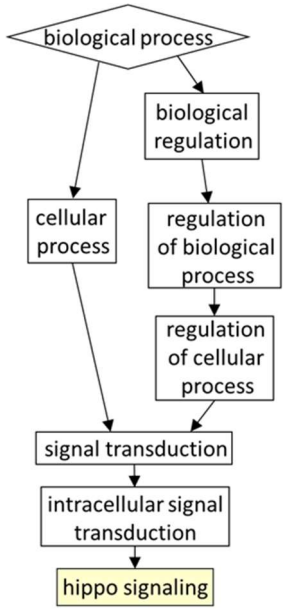

Figure 26: Gene Ontology of pathway changes when p300 expression is reduced.

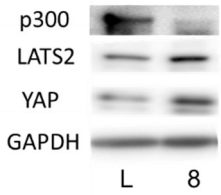

Figure 25: β-HPV E6 does not decrease HP protein abundance.

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

We used immunofluorescence microscopy to demonstrate that β-HPV E6 increases the prevalence of micronuclei (Figure 27). This results in increased ploidy and the resolution of these nuclei correlated with increased ploidy (Figure 28). Mitotic cells were too rare to observe under normal conditions so we are currently planning experiments to arrest cells during mitosis to better understand β-

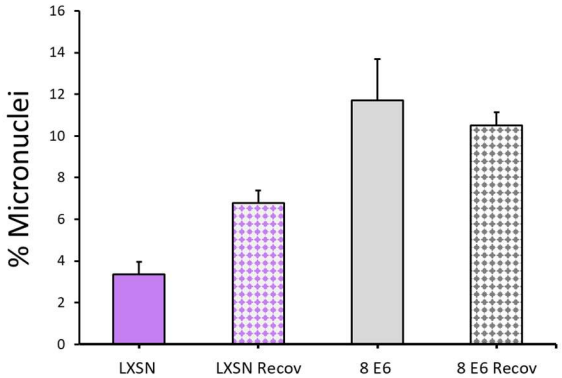

Figure 27: β-HPV E6 increase micronuclei with and without failed cytokinesis induction.

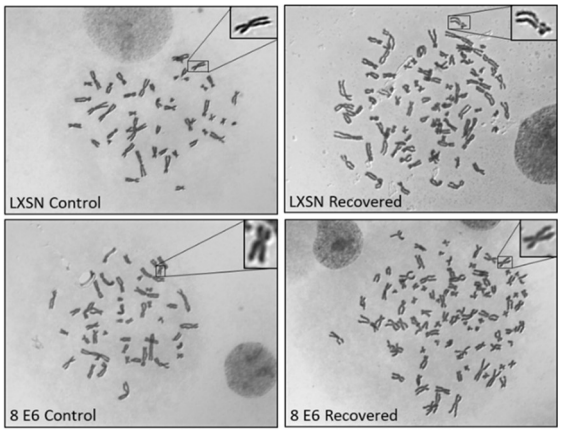

Figure 28: Increased ploidy in cells after H2CB recovery

HPV E6's impact on multipolar mitosis. Since micronuclei are the result of irregular mitosis, our data is consistent with the viral protein also increasing aberrant mitosis. Further supporting the idea that β-HPV E6 induces abnormal mitoses, we found that aneuploidy was significantly increased in cells expressing β-HPV E6. This was true with and without H2CB treatment (Figure 29 and 30).

Milestones Achieved: We will…

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

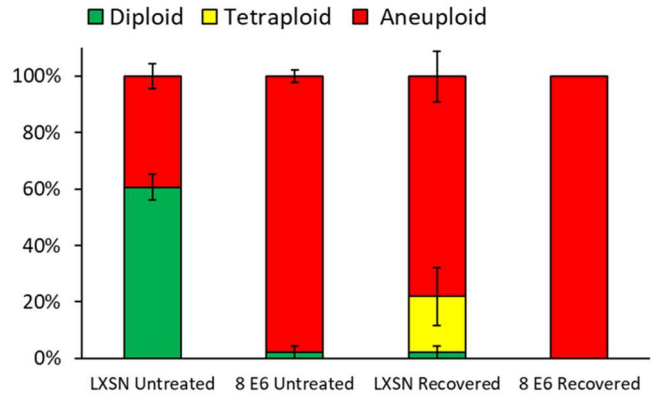

Figure 29: β-HPV E6 increases aneuploidy with and with H2CB treatment.

This was completed during the first budget period.

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

This was completed during the first budget period.

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

This was completed during the first budget period.

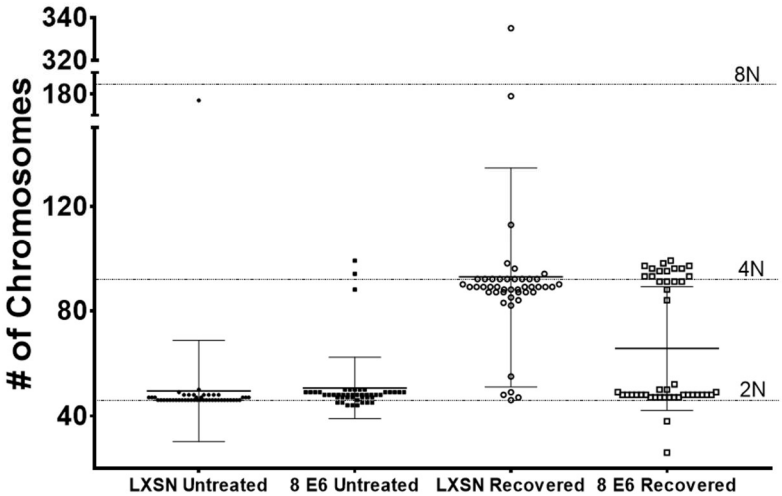

Figure 30: β-HPV E6 increases aneuploidy with and without H2CB-treatment.

(3) 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.

We completed this task, showing that TEAD promoter activity was increased in basal cells in a p300 dependent manner by β-HPV E6 however after failed cytokinesis TEAD promoter activity was decreased when β-HPV E6 was expressed. We have independently confirmed these results using rtPCR and an *in silico* screen.

(4) 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.

This subtask is mostly complete. We have observed an increase in micronuclei and aneuploidy associated with β-HPV E6 expression. We had difficulty detecting mitotic cells of any kind and adjusting our experimental design to observe cells arrested in mitosis to circumnavigate this issue.

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 ΔE6 expressing cells.

This was completed during the first budget period.

Subtask 2: Determine if p300 is involved in β-HPV E6-induced changes to the abundance of HP proteins using β-HPV ΔE6 mutant and immunoblot.

This was completed during the first budget period.

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

We completed the planned qPCR experiments but did not see any change in HP gene expression (Fiugre 22). As a result, we did not continue with the planned chromatin immunoprecipitation experiment.

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.

This was completed during the first budget period.

Milestones Achieved: We will learn…

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

This was completed during the first budget period.

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

Our rtPCR data demonstrate that HP gene expression is not decreased by β-HPV E6. As a result, there was no value in determining the mechanism of β-HPV E6's non-existent modification of HP expression. We do see an increase in LATS2 protein abundance suggesting an increase in protein stability.

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

This was completed during the first budget period.

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.

This was completed during the first budget period.

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

We have regrettably had difficulty reproducing the previously observed p300-independent repair inhibition. When we were able to reproduce the results, they were too small of p300-indpendent repair defects to characterize robustly. Instead, we are exploring the p300-independent induction of aneuploidy and polyploidy by β-HPV E6. Not only do these increases in ploidy and aneuploidy occur in β-HPV ΔE6 (p300 binding site deleted), but the phenotype is conserved across species of β-HPV (Figures 31 and 32).

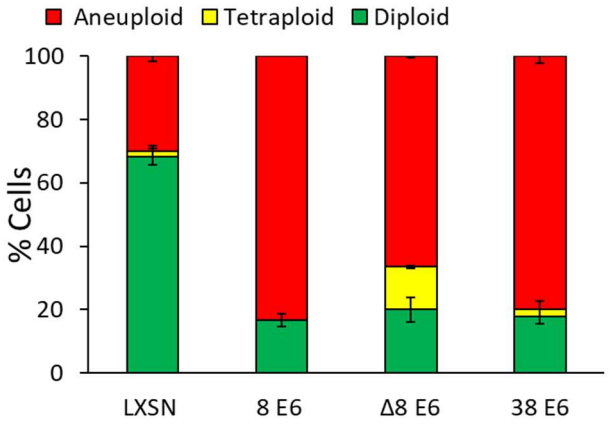

Figure 31: β-HPV E6 increases aneuploidy in an p300-independent manner (neither Δ8 E6 nor 38 E6 destabilize p300.)

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 discovered p300 independent induction of micronuclei (Figure 33) and aneuploidy and have finished the majority of work in Aims 1 and 2. As a result, we will continue to devoted our efforts to understanding how β-HPV E6 causes micronuclei. Specifically, our next step is going to be characterize the micronuclei induced by β-HPV E6. When micronuclei form it is small, γH2AX positive, 53bp1 negative and has high intensity DAPI staining. Over time DAPI and γH2AX staining fades and is replaced by 53bp1. Also the size of the micronucleus increases over time. This information will help us determine if β-HPV E6 is inducing micronuclei formation or increasing the cell's tolerance of these aberrant subcellular structures.

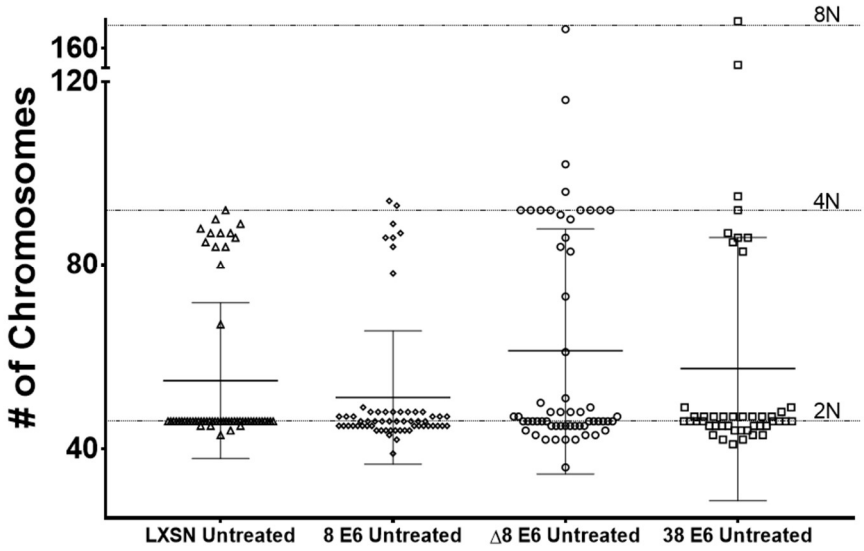

Figure 32: β-HPV E6 increases aneuploidy in an p300-independent manner (neither Δ8 E6 nor 38 E6 destabilize p300.)

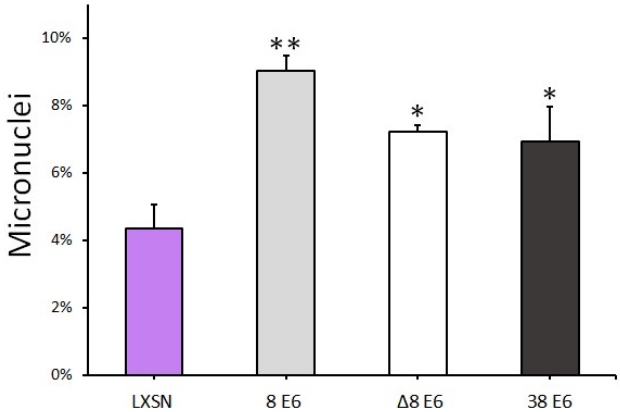

Figure 33: β-HPV E6 increases micronuclei in a p300-independent cross-genus manner.

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).

We will also characterize how these micronuclei are forming by testing the hypothesis that chromosome segregation errors are the root cause of micronuclei formation. This will be accomplished by observing cells stained with DAPI and arrested in mitosis by nocodazole treatment. We will also test our hypothesis that both telomerase activation and p300 inhibition act synergistically to increase aneuploidy and micronuclei. To this end, we are in the process of obtaining cell lines expressing other β-HPV genus oncogenes that represent diverse abilities to impair p300 and activate telomerase.

Milestones Achieved: We will…

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

This was completed during the first budget period.

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

This was completed during the first budget period.

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

We were unable to perform any meaningful manipulation of the p300-independent mechanisms we identified, because the phenotypes were too mild. Having followed the goals of this proposal to their natural end, we have begun dissecting other p300-independent phenotypes. Specifically, describing the pan-species ability of β-HPV E6 to introduce aneuploidy and micronuclei.

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

Kansas State University and International Agency for Research on Cancer have negotiated the terms on an MTA that will allow us to acquire the means of identifying other members of the β-HPV genus that induce p300-independent induction of aneuploidy and micronuclei. In the next budget period, we will complete these efforts expanding the evidence that cutaneous HPV infections destabilize the genome\.

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

As described above, we have refocused our attention. Remaining within the framework of our proposal, we are now extending the efforts of AIM 2 by defining how β-HPV E6 expressing cells recover from failed cytokinesis.

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.

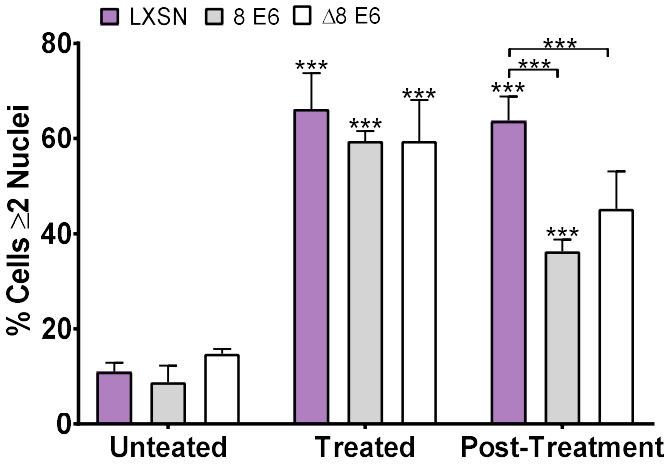

Figure 34: β-HPV E6 reduces the prevalence of cells with supernumerary nuclei after H2CB treatment.

Our first goal was to understand how β-HPV E6 expressing cells recovered from H2CB-induced failed cytokinesis. To this end, we washed the drug off and tracked cells via bright field microscopy. While control cells did not significantly reduce the prevalence of cells with too many nuclei, β-HPV E6 expression did (Figure 34). This was independent of

p300 as deletion of the p300 binding domain did not alter this phenotype. This suggests that β-HPV E6 is allowing cells to replicate or otherwise resolve their increased nuclei.

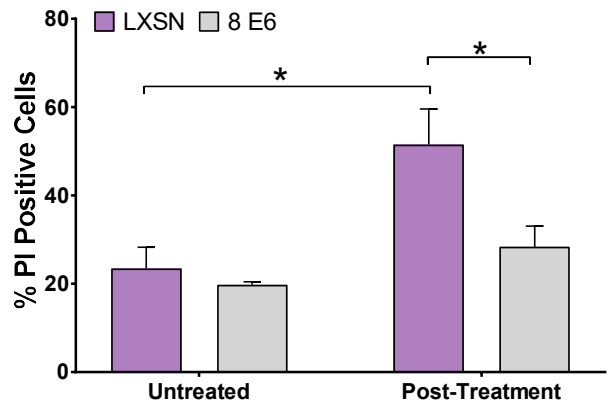

Figure 35: β-HPV E6 prevents H2CB-induced apoptosis.

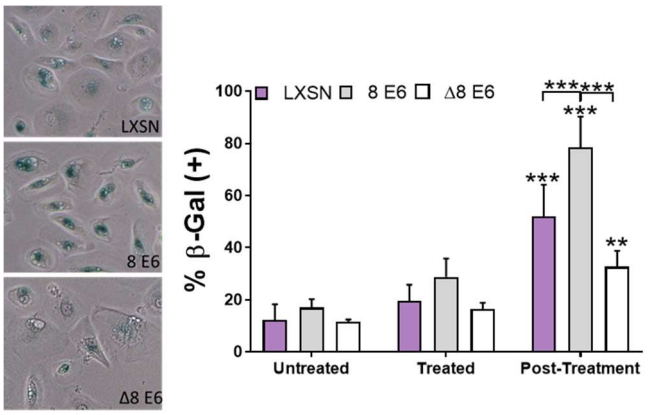

Figure 36: β-HPV E6 increases senescence after H2CB-induced failed cytokinesis as shown by increased senescence associated β-Gal activity.

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

Our next action was to determine how β-HPV E6 was allowing cells to resolve their supernumerary nuclei. We found that β-HPV E6 hindered H2CB induced apoptosis (Figure 35), but actually increased H2CB induced senescence (Figure 36). This is notable as it provides evidence that β-HPV E6 can selectively alter one branch point in the HP, while increasing the pathway's activity in another branch.

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

To identify common mutations in NMSCs that could allow β-HPV E6 expressing cells to bypass senescence, we analyzed sequencing data from 39 tumors available through web-based software at Cbioportal.org. We performed gene ontology analysis on the top 10% most common mutations and found replicative senescence was among the significantly altered pathways. Specifically, this was the result of telomerase activating mutations.

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

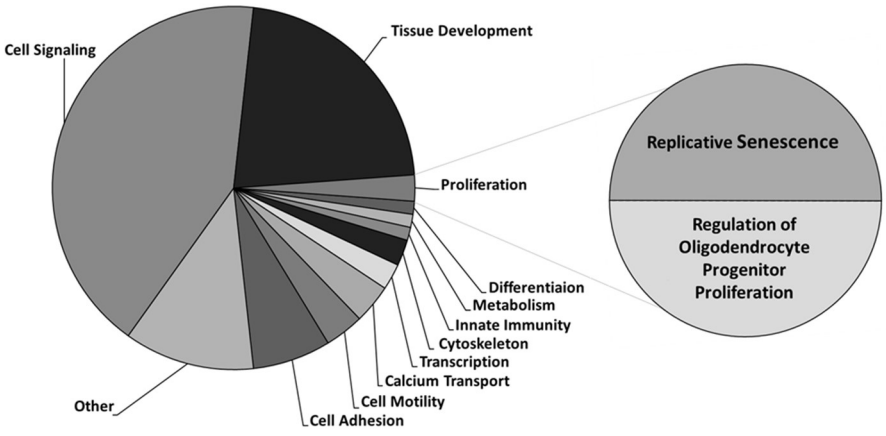

Figure 37: Replicative senescence was identified among commonly altered pathways in non-melanoma skin cancer (particularly telomerase activating mutations).

Telomerase activity in NMSCs has been previously separately reported giving us confidence to begin modeling this phenomenon with *in vitro* systems.

Specifically, we obtained vector control or β-HPV E6 expressing HFKs that have been immortalized by hTERT activation. When treated with H2CB, similar percentages of these cells

became bionucleated. Our next step was to compare the recovery of hTERT-immortalized HFKS after H2CB treatment. On its own β-HPV E6 was not capable of allowing cells to proliferate after 3 days of H2CB treatment. hTERT activation alone however was capable of rescuing these cells from H2CB-induced growth arrest and apoptosis (Figure 38). When cells were treated with H2CB for 6 days simulating a harsher cytokinesis failure, hTERT activation alone rarely allowed cells to survive (1 out of every 6 times). However, hTERT combined with β-HPV E6 allowed cells to recover everytime (6 of 6). Moreover, the combination of hTERT immortalization and β-HPV E6 expression resulted in a more rapid recovery the one time that hTERT alone allowed cells to survive (Figure 39).

Cell Type	% Recovered
LXSN	0% (3/3)
8 E6	0% (3/3)
LXSN + *hTERT*	100% (3/3)
8 E6 + *hTERT*	100% (3/3)

Figure 38: β-HPV E6 and telomerase activation act synergistically to promote recovery from failed cytokinesis.

<u>Subtask 5: Identify novel β-HPV E6 interacting proteins by mass spectrometry with validation by co-immunoprecipitation.</u>

We have obtained a series of dominant negative mutants and shRNA expression constructs targeting the HP upstream of LATS2. We intend to use these in our continuing efforts to understand how β-HPV E6 dysregulates the pathway.

Milestone(s) Achieved: We will learn…

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

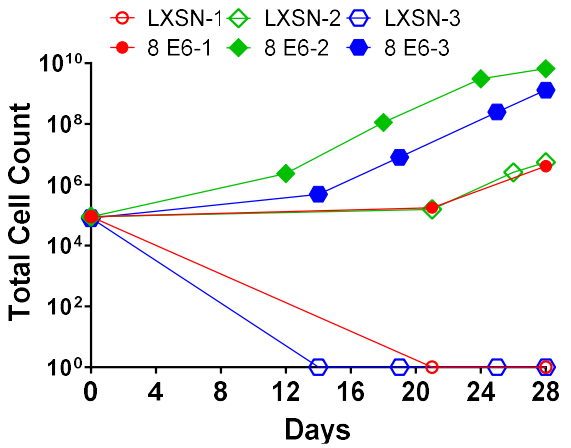

Figure 39: β-HPV E6 accelerates and stimulates growth after H2CB-induced failed cytokinesis.

We have not been able to complete the originally proposed milestones, because our BCL6 result produced a phenotype that was too small/variable for further characterization. To honor our commitment to the award, we have instead extended our work in two ways. First, we have determined how the oncogenic effects of a common mutation in NMSCs are augmented by β-HPV E6. Second, we are characterizing β-HPV E6's p300-independent induction of genome instability by promoting micronuclei formation and inducing aneuploidy.

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

See above.

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

See above.

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

See above.

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

See above.

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

See above.

- **What opportunities for training and professional development has the project provided?**
  - Both graduate students funded in this project presented their work at an international conference (DNA tumor virus meeting). The PI received significant mentoring from Drs. Laimins, Gao and Clem. Further, he was invited to give 8 seminars on his work at Universities across the country and internationally.
- **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 (@wallacehpvlab) that we use to communicate our work to a network of followers.
  - We again 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 Enginnering).
  - The PI also gave a public lecture as part of the local "Science on Tap" series.
- **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 two international meetings in the next reporting period (the DNA tumor virus meeting in Montreal and as an invited guest to a meeting on emerging oncogenic viruses in Italy). 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, and encouraged that we were able to identify p300-independent chromosomal abrogations induced by β-HPV E6. This greatly extends the known ability of the viral protein to destabilize the host genome as predicted by the "hit and run" mechanism of oncogenesis that we are testing.
  - We expect three research publications stemming from this support in the next budget period
  - Though not funded, we received a favorable review of our resubmission to the American Cancer Society, which we will resubmit in April. Notably, our score improved from 1.85 to 1.63 on a 5 point scale, with a funding line set at or near 1.5. An R01 grant application to the National Institutes of Health is in progress and we expect to hear receive reviews of our P20

and R15 proposals in the coming months. This represents ~$780,000 in potential research funds.

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. Moreover, we discovered the virus's ability to cause aneuploidy and polyploidy as well as micronuclei. Finally, we delineated the impact of β-HPV E6 on the multiple signaling pathways (Hippo, ATR, ATM, CHK1 and CHK2). 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, chromosome segregation and maintenance, double strand break repair, cell cycle regulation and crosslink repair.
    - **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 were unable to extend our studies into p300-independent inhibition of DNA repair due to technical issues (phenotypes too small for further characterization. Instead, we have made notable progress characterizing p300-independent abrogation of chromosome maintenance and segregation.*

        - We also chose to more completely characterize β-HPV E6's abrogation of the hippo pathway.
        - As a result, we have functionally completed the third aim, although without the desired results and have expanded other projects described in our original proposal beyond their initial scope but within the frame work of our proposal.
    - **Actual or anticipated problems or delays and actions or plans to resolve them**
        - Damage to a piece of shared equipment (flow cytometry sorter) has caused us to adjust our plans to measure NHEJ. We are now using a more classical approach.
    - **Changes that had a significant impact on expenditures**
        - none
    - **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.**
            - Cervical cancer cell lines are sensitive to sub-erthemal UV exposure. Gu W., Sun S., Kahn A., Dacus D., Wendel SO., McMillan N., Wallace NA. 2019 PMID: 30517878 LINK
                - Acknowledgement of Federal Support: Yes
            - mSphere of Influence: the Value of Simplicity in Experiments and Solidarity among Lab Members. Wallace NA. 2019. PMID: 31217299 LINK
        - **Books or other non-periodical, one-time publications.**
            - Nothing to report
        - **Other publications, conference papers, and presentations.**
            - Griffith University, Gold Coast, Australia, 09/2018 "Cutaneous HPV infections and Skin Cancer"
            - University of Kansas, Lawrence, KS, 09/2018 "HPV Oncogenes Induce and Disrupt Translesion Synthesis"
            - Wake Forest University School of Medicine Microbiology and Immunology 11/2018 "HPV Oncogenes Induce and Disrupt Translesion Synthesis"
            - Virginia Commonwealth University Philips Institute for Oral Health Research, Richmond VI, 12/2018 "HPV Oncogenes Induce and Disrupt Translesion Synthesis"

        - Center for Molecular Medicine's Symposium on the missing links in HPV-Biology: Focus on Head & Neck cancer and Skin Cancer, Cologne, Germany 05/2019 "Interference of HPV with DNA damage and repair pathway"
    - **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 over 600 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:	*Dalton Dacus*
Project Role:	*Graduate Student*
Researcher Identifier (e.g. ORCID ID):	*Not Applicable*
Nearest person month worked:	*12*
Contribution to Project:	Mr. Dacus performed most of analysis of the hippo pathway (Aim 2) and some of the work for AIM3
Funding Support:	*CDMRP and Wallace Startup funds*

    -

Name:	*Changkun Hu*
Project Role:	*Graduate Student*
Researcher Identifier (e.g. ORCID ID):	*Not Applicable*
Nearest person month worked:	*12*
Contribution to Project:	*Mr. Hu has performed the analysis of the NHEJ pathway.*
Funding Support:	*CDMRP and Wallace Startup Funds*

-

Name:	*Jazmine Snow*
Project Role:	*Research Assistant*
Researcher Identifier (e.g. ORCID ID):	*Not Applicable*
Nearest person month worked:	*4*
Contribution to Project:	*Ms. Snow has performed the characterization of ATM and ATR signaling described above in Specific AIM 1.*
Funding Support:	*Wallace Startup funds*

-

Name:	*Nicholas Wallace*
Project Role:	*Primary Investigator*
Researcher Identifier (e.g. ORCID ID):	*0000-0002-3971-716X*
Nearest person month worked:	*3*
Contribution to Project:	*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.*
Funding Support:	*CDMRP Support and Kansas State University Salary Support*

-

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

- **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
   - **APPENDICES:** PDF versions of the three manuscripts published during this reporting period are appended below.

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Research paper

Cervical cancer cell lines are sensitive to sub-erythemal UV exposure

Wenyi Gu[a,1], Surong Sun[a,c], Andrew Kahn[b], Dalton Dacus[b], Sebastian O. Wendel[b], Nigel McMillan[a,*], Nicholas A. Wallace[b,*]

[a] School of Medical Sciences, Griffith University, Gold Coast Campus, Australia
[b] Division of Biology, Kansas State University, Manhattan, KS, United States of America
[c] Xinjiang Key Laboratory of Biological Resources and Genetic Engineering, College of Life Science and Technology, Xinjiang University, Urumqi 830046, China

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ABSTRACT

High risk human papillomavirus (HPV) infections are the causative agent in virtually every cervical cancer as well as a host of other anogenital and oropharyngeal malignancies. These viruses must activate DNA repair pathways to facilitate their replication, while avoiding the cell cycle arrest and apoptosis that can accompany DNA damage. HPV oncoproteins facilitate each of these goals, but also reduce genome stability. Our data dissect the cytotoxic and cytoprotective characteristics of HPV oncogenes in cervical cancer cells. These data show that while the transformation of keratinocytes by HPV oncogene leaves these cells more sensitive to UV, the oncogenes also protect against UV-induced apoptosis. Cisplatin and UV resistant cervical cancer cell lines were generated and probed for their sensitivity to genotoxic agents. Cervical cancer cells can acquire resistance to one DNA crosslinking agent (UV or cisplatin) without gaining broad tolerance of crosslinked DNA. Further, cisplatin resistance may or may not result in sensitivity to PARP1 inhibition.

1. Introduction

Cervical cancers are the third most common and second deadliest cancer in women worldwide. Over half a million new cases of cervical cancer are diagnosed annually killing nearly 300,000 people (Parkin and Bray, 2006). Access to healthcare is a major factor in the development of cervical cancer with as many as 80% of cases occurring in developing countries (Sherris et al., 2001; Siegel et al., 2012; Tota et al., 2011). In addition to differences in screening and care, environmental factors (smoking and UV exposure for instance) increase the risk of cervical cancer (Fonseca-Moutinho, 2011; Godar et al., 2014). Nearly every cervical cancer is the result of a human papillomavirus infection (zur Hausen, 2002). This very large family of double stranded circular DNA viruses is divided into 5 genera based on sequence differences in their L1 major capsid gene (Bernard et al., 2010). While members of both the genus alpha and beta of human papillomaviruses are associated with cancer (Godar et al., 2014; Howley and Pfister, 2015; Wendel and Wallace, 2017), members of the genus alpha human papillomaviruses are further divided into high risk and low risk papillomaviruses based on the relative ability to cause cancer. Among the high risk alpha papillomaviruses, human papillomaviruses 16 and 18 cause 70% of cervical cancers (Winer et al., 2006). In this manuscript, we will refer to these two viruses as simply as HPV.

Much is known about the molecular basis of how HPV manipulates the host cell both to promote its life cycle and to cause malignant transformation. Although the E5 protein from these viruses has oncogenic potential, the two canonical HPV oncogenes are HPV E6 and HPV E7 (Roman and Munger, 2013; Wallace and Galloway, 2015). HPV E6 binds a cellular ubiquitin ligase (E6AP) and uses it to promote p53 degradation (Huibregtse et al., 1991; Scheffner et al., 1993, p. 53). HPV E6 also activates the catalytic subunit of telomerase (Klingelhutz et al., 1996). HPV E7 disengages cell cycle checkpoints by degrading RB and RB family proteins (Dyson et al., 1989; Roman and Munger, 2013; Zhang et al., 2006). The lack of evolutionary pressure to mutate or otherwise inactivate these tumor suppressors results in tumors that are dependent on continued HPV E6 and E7 expression (Chang et al., 2010).

In addition to their well-characterized ability to inactivate p53 and RB, HPV oncogenes have a complicated relationship with cellular response to UV (Wallace and Galloway, 2014). HPV E7 prevents HPV-

Abbreviations list: HPV, human papillomavirus; UV, ultraviolet radiation; E6AP, E6 associated protein; RB, retinoblastoma protein; TLS, translesion synthesis; NER, nucleotide excision repair; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

* Corresponding authors.
E-mail addresses: n.mcmillan@griffith.edu.au (N. McMillan), nwallac@ksu.edu (N.A. Wallace).
[1] Current address: Australian Institute for Bioengineering and Nanotechnology (Building 75), The University of Queensland, Cooper Rd., St Lucia, Brisbane QLD 4072, Australia.

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transformed cells from undergoing the G1 arrest after UV necessary to avoid replication fork collapse (Flynn and Zou, 2011; Gujuluva et al., 1994; Morandell et al., 2012; Wieringa et al., 2016). HPV E6's degradation of p53 prevents the induction of p53 target genes (Gu et al., 1994). HPV E6 blocks the induction of NER proteins (McKay et al., 2001) correlating with limited NER and more mutations after UV exposure (El-Mahdy et al., 2000; Havre et al., 1995; Rey et al., 1999). Both HPV oncogenes hinder the homologous recombination pathways that respond to double strand DNA breaks that can result from UV-induced replication fork collapse (Wallace et al., 2017). The repair mechanisms that respond to UV are also critical for repairing lesions caused a number by genotoxic drugs that cause DNA crosslinks (Alt et al., 2007; Furuta et al., 2002; Ho et al., 2006). It is not surprising then that HPV E6 and E7 sensitize cells to this class of chemotherapeutics (Koivusalo et al., 2002; Liu et al., 2000; Wallace et al., 2012). The dependence of cervical cancers on these viral oncogenes suggests that these drugs would be particularly lethal to cervical malignancies.

However, HPV oncogenes also activate a plethora of DNA repair proteins including those in the pathways that respond to UV damage; nucleotide excision repair (NER), Fanconi anemia repair (FA) and translesion synthesis (TLS) (Alan and D'Andrea, 2010; Knobel and Marti, 2011; Schärer, 2013). HPV depends on DNA repair pathways to replicate its genome (Anacker et al., 2014; Gillespie et al., 2012; Hong et al., 2015; Moody and Laimins, 2009; Spriggs and Laimins, 2017a). In addition to creating an environment that is seemingly primed to respond to crosslinked DNA, HPV oncogenes inhibit the apoptosis induced by these lesions (Garnett et al., 2006; Garnett and Duerksen-Hughes, 2006; Jackson et al., 2000; Leverrier et al., 2007). Thus in at least certain scenarios, HPV E6 can protect against DNA crosslinking agents (Koivusalo et al., 2002).

The balance between chemo-sensitization and chemo-resistance is critical in the management of any cancer. DNA repair and damage tolerance pathways are particularly important with regard to cervical cancer as a DNA crosslinking agent, cisplatin, is currently the standard of care (Lorusso et al., 2014). Resistance to cisplatin in advanced and recurrent cervical cancers results in only a 10–20% chance of living through the year (Diaz-Padilla et al., 2013). As a result, substantial effort has been made to identify potential mechanisms of cisplatin resistance as well as ways to re-sensitize cervical cancer cells (Kilic et al., 2015; Roy and Mukherjee, 2014; Zhu et al., 2016). The efficacy of cisplatin is at least partially determined by gene expression patterns in cervical cancer cells, with multiple DNA repair genes being identified as important predictors of cisplatin sensitivity (Garzetti et al., 1996; Hasegawa et al., 2011; Henríquez-Hernández et al., 2011; Kitahara et al., 2002; Saito et al., 2004; Zhu et al., 2016). Because cisplatin induces DNA damage, increased repair capability likely accounts for the resistance (Woods and Turchi, 2013).

Conversely, if HPV oncogenes block repair in a predictable manner, these defects could be leveraged therapeutically. The sensitivity of BRCA1 or BRCA2 deficient breast and ovarian cancers is the most publicized link between known repair deficiencies and improved cancer treatments (D'Amours et al., 1999; Jasin, 2002). A similar relationship exists for cisplatin sensitivity. Specifically, the response of TLS and NER to UV predicts the lethality of cisplatin exposure (Gueranger et al., 2008; Marteijn et al., 2014; Rosell et al., 2003; Srivastava et al., 2015). In this manuscript, we characterize the interplay between HPV oncogene-induced sensitivity to crosslinking agents and their protection from these genotoxins in the context of cervical cancer cell lines. We show that, compared to control cells, cervical cancer cells are more sensitive to sub-erythemal UV exposure and low doses of cisplatin. We demonstrate that these cells have a greater tendency to undergo apoptosis after UV. Interestingly, HPV E6 and E7 also block apoptosis after UV. We generate UV- and cisplatin-resistance cervical cancer cell lines and define their cross sensitivity. Finally, we determine the toxicity of a chemotherapeutic agent (olaparib) with a different mechanism of action (PARP1 inhibition) in cervical cancer cells that are resistant to cisplatin.

2. Methods and materials

2.1. MTT assays

LD50s were determined by MTT assay. HeLa cells were seeded in a 96 well plates and treated with UV, cisplatin, or olaparib at specified doses. 48 h after treatment, MTT solution was added to the cells and solubilized 24 h later. Plates were then read for optical density by spectrophotometer with a dual reading at frequencies 550 and 655 nm.

2.2. Cell culture

HeLa cells are HPV 18 positive *Homo sapiens* adenocarcinomas grown in Dulbecco's Modified Eagle Medium (ThermoFisher Scientific, catalog number: 12100046), supplemented with 10% fetal bovine serum (VWR, catalog number: 89510-194) and Pen-strep (Calsson Labs, catalog number: PSL02-6X100ML). Human foreskin keratinocytes or HFKs were derived from neonatal human foreskins and were grown in EpiLife medium supplemented with calcium chloride (60 μM), human keratinocyte growth supplement (Cascade Biologics, Portland, OR), and penicillin-streptomycin. Multiple cell lines were derived from several donors for this work. The cells were incubated at 37 °C in a 5% CO_2 environment. Cells were washed with 0.1% EDTA (Invitrogen catalog #15-576-028) and raised from plate with 0.05% Trypsin (Sigma-Aldrich catalog # T4049-500ML).

2.3. Immunoblotting

HeLa Cells transfected by different plasmids (PLL3.7, 18E6-1, 16E7-1) were cultured for different times (0 h, 3 h, 6 h, 24 h) in 6 cell-well plate after using UV light dose (8 mJ/cm^2) treatment, then both adherent and floating cells were collected and lysed in ice-cold RIPA buffer (50 mM Tris–HCl, 150 mM NaCl, 1 mM ethylene diamine tetraacetic acid (EDTA), 1 mM ethylene glycol tetraacetic acid (EGTA), 20 mM NaF, 1% Nonidet P-40 (NP-40), 1 mM phenylmethylsulfonyl fluoride (PMSF), 100 mM Na3VO4, 1% Triton X-100, 10 mg/mL Aprotinin and 10 mg/mL Leupeptin) for 30 min. The lysates were centrifuged at 12,000 rpm for 10 min and the supernatant was collected. Equal amount of protein lysates were electrophoresed on a 12% SDS polyacrylamide gel and transferred onto nitrocellulose filter (NC) membrane. The membrane was washed 3 times for 10 min in TBST (Tris-Buffered Saline and Tween-20), then after blocking with 2.5% skim milk suspended in TBST for 1 h, membranes were individually incubated respectively with the appropriate primary antibodies Tubulin, P53, Bax, HPV E7 (1:1000) overnight followed by secondary antibodies (1:1000) for 1 h at room temperature. Protein bands were detected using ECL assay kit (Invitrogen, Australia) and exposed using a Kodak medical X-ray processor (Kodak, USA). Anti-human p53 and β-tubulin monoclonal antibodies were purchased from Sigma (Sydney, Australia). Anti-HPV 18E7 polyclonal antibody was purchased from Santa Cruz Biotechnology. Anti-human Bax polyclonal antibody was from Cell Signaling Technology.

2.4. Resistant colony generation and isolation

Colony isolation was performed using sterile cloning disks (SP Scienceware catalog # F37847-0001). Cells were grown at low confluence in a 10 cm plate and treated 4 times every 2 weeks for a 2-month period. Cells were observed from single cells, with their growth monitored microscopically. They were washed with 0.1% EDTA, before colonies were isolated by placing a sterile cloning disks soaked in trypsin on them. Individual disks were then put in a well of a 24-well plate containing growth media, allowing cells to detach from the disk. Cells were then expanded, and acquisition of resistance was assessed.

2.5. Colony formation assays

Colony formation assays were performed by seeding low concentrations of cells on six well plates. They were then treated with UV 48 h after seeding and allowed to grow until colonies appeared. Twenty-four hours after the first colonies with 15 or more cells were seen, colonies were stained with a crystal violet solution (0.2% crystal violet, 5% ascetic acid, and 2.5% 2-proponol) and hand counted.

2.6. shRNA transfections

Cell transduction was basically according to the method described previously (Gu et al., 2006). Briefly, HeLa cells were maintained in complete DMEM medium (Gibco-Invitrogen) and were plated in T75 flasks (4×10^5/flask) overnight culture. LV-shRNAs was diluted in 2.0 mL cultural medium containing polybrene (8 μg/mL) and added to the cells for culture for 1 h at 37 °C. After this, 8 mL of fresh polybrene medium was added to the cells and incubation continued for 24 h. Polybrene medium was then replaced with fresh DMEM culture medium, and the cells were further cultured for assays.

2.7. UV irradiation

To prepare cells for UV irradiation the media was aspirated, and the cells were washed with 1× PBS (Bio Basic catalog # PD8117). A thin layer of PBS was added to the wells or plate and then cells were irradiated in a UV Stratalinker 2400 (Stratagene Catalog # 400075-03) at specified doses. Media was then added, and the cells were incubated at 37 °C.

2.8. Flow cytometry

Flow cytometry analysis was carried out with the FACSCalibur™ (Becton Dickinson). The acquisition and analysis of data was done with the program Cell Quest Pro.

2.9. Apoptosis assay

The Dead Cell Apoptosis Kit (ThermoFisher Scientific catalog #V13242) and the Countess™ II FL Automated Cell Counter (ThermoFisher Scientific catalog #AMQAF1000) were used to measure apoptosis. Cells were seeded at 50,000 cells/well in a 6-well plate and incubated at 37 °C overnight. Cells were then UV irradiated at specified intervals and incubated at 37 °C for 48 h. Cells were then washed with 0.1% EDTA and incubated with Trypsin for 3 min at 37 °C. DMEM was then added to cells and they were incubated at 37 °C for 30 min. Cells were then pelleted by centrifuging at 188 ×*g* for 5 min. After aspiration of DMEM, they were resuspended in ~1×10^6 cells/mL in 1× annexin binding buffer and incubated with propidium iodide for 15 min at RT. Cells then were loaded onto slides and imaged using appropriate fluorescent filters on Countess™ II FL Automated Cell Counter.

2.10. Reagents

Cisplatin: cisplatin (Sigma-Aldrich catalog # 479306-1G) is a platinum based antineoplastic agent that forms cytotoxic adducts with the DNA dinucleotide d(pGpG) that induces intrastrand cross-links.

Olaparib: olaparib (LC laboratories catalog # O-9201) is a selective inhibitor of PARP1/2 with IC50 of 5 nM/1 nM in cell-free assays.

3. Results

3.1. HPV+ cervical cancer cell lines are sensitive to sub-erythemal doses of UV

Cells mitigate the toxicity of low dose UV by pausing the cell cycle and inducing a highly coordinated signaling response. HPV oncogenes transform keratinocyte cells in part by disrupting both processes. HPV E7 degrades the master cell cycle regulator, RB. While both HPV E6 and E7 induce aberrant activation of DNA damage proteins. We hypothesize that the continued expression of HPV E6 and E7 in cervical cancer cell lines will make them less able to respond to UV and as a result more sensitive to UV exposure. This hypothesis was tested by defining the

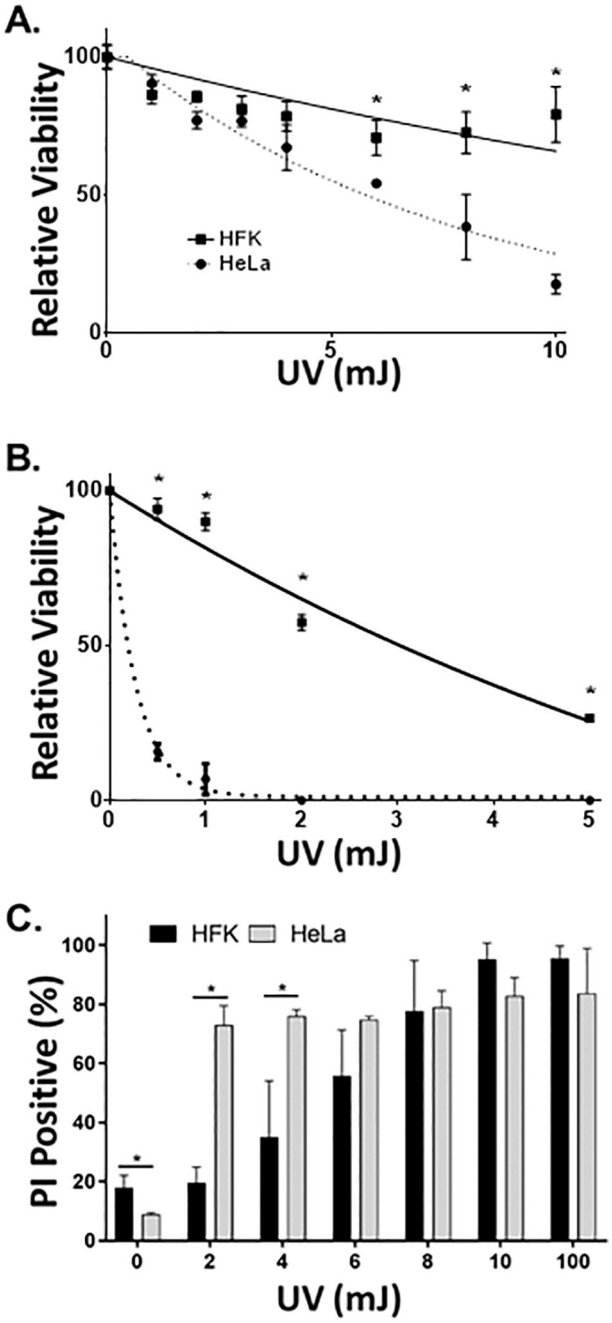

Fig. 1. Sensitivity of cervical cancer to UV. A. This graph depicts the sensitivity of cervical cancer cell lines to ultraviolet radiation when compared to primary human foreskin keratinocytes (HFK) as measured by MTT. The black square points and solid line represent HFKs. The black circle points and dotted line represent HeLa cells, a cervical cancer cell line. B. This graph depicts the combined results of the colony formation assays. The solid black line and solid black squares represent HeLa and the dotted line and dot points are non-cancer HFK. C. This graph depicts the percentage of apoptotic (propidium iodide positive) cells 48 h after increasing doses of UV. For all, $n = 3$, $^*p < 0.05$ by unpaired *t*-test and error bars represent mean ± SD.

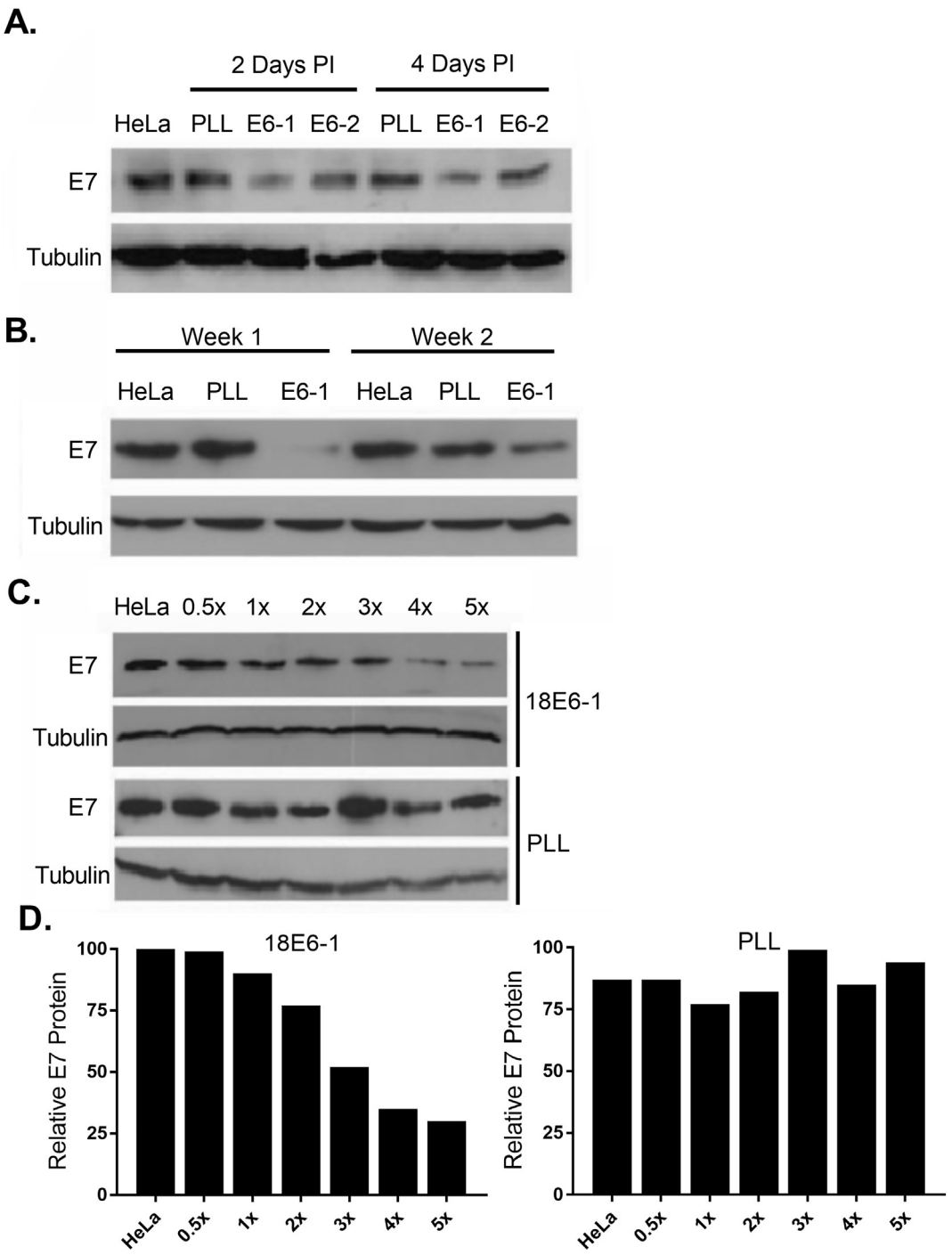

Fig. 2. Confirmation of lentiviral transduction. A. Representative immunoblot showing E7 levels in HeLa cells transduced with the empty vector PLL, shRNA E6-1, or shRNA E6-2. Lysates were collected 2 days post-lentiviral infection (PI) or 4 days PI. Tubulin is the loading control. B. Representative immunoblot showing E7 levels in HPV18 HeLa cells transduced with empty vector PLL or a gradient of shRNA 18E6-1. C. Representative immunoblot showing E7 levels in HeLa cells transduced with the empty vector PLL or shRNA E6-1. Lysates were collected 1 and 2 weeks PI. Tubulin is the loading control. D. Graphs showing relative E7 protein determined by densitometry.

sensitivity of a HPV+ cervical cancer cell line (HeLa) to UV. As a control, untransformed primary keratinocytes derived from neonatal foreskins (HFKs) were also observed because HPV infects and transforms keratinocytes. HeLa cells were significantly more sensitive to a gradient of low dose UV (0–10 mJ/cm^2) than HFKs when viability was measured by MTT assay (Fig. 1A).

As a complimentary measure of UV sensitivity, a colony formation assay was used to measure the toxicity of the same range of UV doses. These results confirm the data obtained by the MTT assay. Indeed, HeLa cells were significantly more sensitive to all exposures of UV in the colony formation assay, with virtually no colonies visible after 1 mJ/cm^2 of exposure (Fig. 1B and Supplemental Fig. 1). Conversely, HFKs formed readily detectable colonies at all observed doses of UV.

The sensitivity resulting from an inability to properly respond to UV

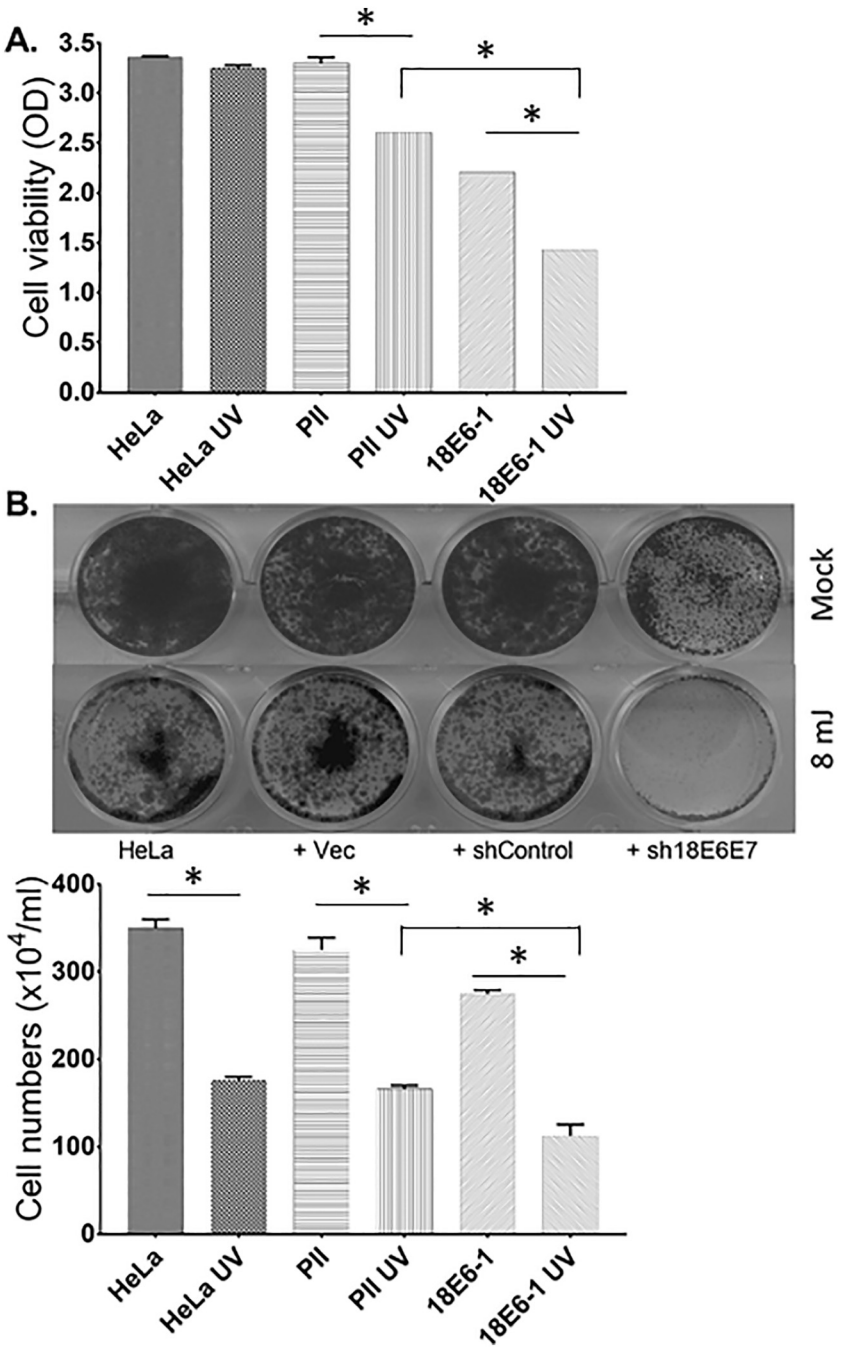

Fig. 3. Increased UV sensitivity in E6/E7 knockdown HeLa cells. A. This graph depicts UV sensitivity in HeLa cells transduced with the empty vector PLL or shRNA E6-1 as measured by MTT assay. B. This image depicts UV sensitivity in HeLa cells transduced with empty vector, a shRNA Control, or a shRNA 18E6E7 by colony formation assay (CFA). C. This graph depicts UV sensitivity in HeLa cells transduced with the empty vector PLL or shRNA 18e6-1 by cell counting. For all, $n = 3$, $^*p < 0.05$ by unpaired *t*-test and error bars represent mean ± SD.

damage is likely manifest in elevated apoptosis. Unfortunately, neither a colony formation assay nor a MTT detect apoptosis directly and thus they cannot distinguish apoptosis from other types of cell death. To determine if HPV+ cervical cancers have an increased propensity to undergo apoptosis after UV exposure, we measured propidium iodide (PI) staining as a standard indicator of apoptosis (Fig. 1C). PI is a membrane impermeable dye that intercalates between DNA base pairs. We used a fluorescence-based assay to detect PI. Cell membranes become permeable during apoptosis allowing PI uptake measurable at 617 nm. Taking the ratio of cells that are stained with PI to the total cell count allows us to determine the percentage of apoptotic cells after UV exposure. We found HeLa cells had significantly higher levels of PI staining compared to HFKs when both were exposed to either 2 or 4 mJ/cm^2 of UV (Fig. 1C).

3.2. HPV oncogenes decrease sub-erythemal UV-induced apoptosis

This increased sensitivity is likely due to HPV E6 and E7 disrupting cell cycle regulation and causing abnormal activation of DNA damage response genes. However, HPV oncogenes also mitigate the deleterious consequences of DNA damage by inhibiting apoptosis. To define the ability of HPV oncogenes to block the apoptosis in the context of a cervical cancer cell line exposed to UV, a shRNA system (PLL E61) was used to partially deplete HPV oncogenes in HeLa cells. HeLa cells with

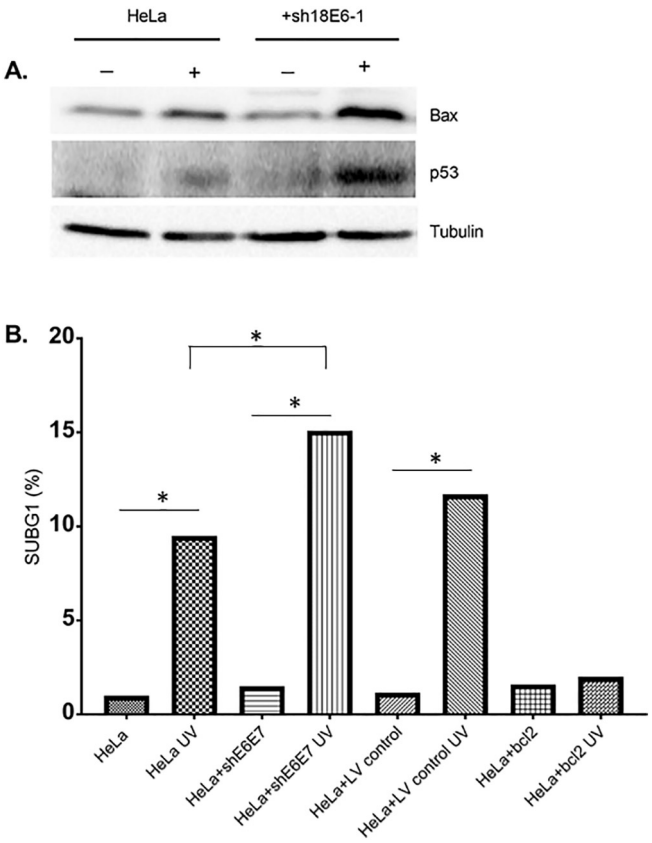

Fig. 4. Increased cell cycle arrest and apoptosis due to UV sensitivity in E6/E7 knockdown HeLa cells. A. Representative immunoblot showing BCL-2 related X protein (Bax) and p53 levels in shRNA 18E6-1 transduced HeLa cells with and without UV. Tubulin is the loading control. B. This graph depicts the percentage of HeLa cells in sub G1 after or without UV treatment. Cells were transduced with shRNA E6E7, lentiviral control, and BCL-2 exogenously expressed. For all, $n = 3$, $^*p < 0.05$ by unpaired *t*-test and error bars represent mean ± SD.

PLL E61 cells had decreased HPV E7 levels 2 and 4 days after treatment with shRNA (Fig. 2A). Because these genes share a promoter, their expression is known to be linked. Indeed, a recent study was unable to achieve knockdown of HPV E6 or HPV E7 individually (Wechsler et al., 2018). Thus, decreased HPV E7 abundance is a reliable marker of HPV E6 for which a dependable antibody does exist. To determine if this knockdown was persistent, oncogene levels were measured at 1 and 2 weeks after transfection with shRNA. Transfection with PLL E61 was able to detectably decrease the abundance of HPV E7 at these late time points (Fig. 2B). As a final measure of the PLL E61's ability to diminish HPV oncogene levels, the abundance of HPV oncogenes was determined after transfection with increasing amounts of PLL E61. Immunoblot analysis shows a dose dependent decrease in HPV E7 levels (Fig. 2C). Since there is no viable commercially available antibody to HPV E6, the degradation of its target protein, p53, is used as a surrogate for HPV E6 expression. P53 levels were determined by immunoblot after treatment with PLL E6. Notably, the reduction in oncogene abundance remains below the levels that result in apoptosis (data not shown).

Having characterized PLL E61's ability to decrease the amount of HPV oncogenes in HeLa cells, the approach was used to define the role of HPV oncogenes in the apoptosis induced by low doses of UV. Supporting a role for HPV oncogenes in repressing UV-induced apoptosis, both MTT and colony formation assays demonstrated that UV was more toxic to HeLa cells when HPV E6 and E7 were targeted by shRNA (Fig. 3A–B). Specifically, the knockdown of HPV oncogenes combined with exposure to a low dose of UV resulted in lower cellular viability and fewer colonies than either knockdown or UV exposure alone.

As noted, these assays do not directly measure apoptosis, so the abundance of BAX, a pro-apoptotic protein, was defined by immunoblot. After exposure to a sub-erythemal amount of UV, HeLa cells demonstrated a mild induction of both BAX and p53 (Fig. 4A). In contrast, UV-induce increases in BAX and p53 abundance were enhanced by HPV E6 and E7 knockdown. Notably, PLL E6.1 also increased the abundance of p53 in untreated HeLa cells further confirming the knockdown of HPV E6. To more directly detect the role of HPV oncogenes in protecting cervical cancer cells against apoptosis, flow cytometry was used to define the population of sub-G0/G1 cells after UV. The results from this assay are consistent with HPV oncogene-mediated inhibition of apoptosis as the percentage of sub-G0/G1 cells after UV was significantly increased by oncogene knockdown (Fig. 4B). As a control and to further confirm that the increase in the sub-G0/G1 population was due to BAX-induced apoptosis, BCL2, an anti-apoptotic BAX antagonist was transfected into HeLa cells. BCL2 expression prevented the accumulation of sub-G0/G1 cells after UV (Fig. 4B).

3.3. Generation of UV- and cisplatin-resistant HPV+ cervical cancer cells

The ability of HPV oncogenes to both sensitize cells to UV and protect them from UV-induced apoptosis portrays the complexity of their manipulation of the cellular damage response. To further probe this relationship, UV-resistant HeLa cell lines were generated by repeated exposure to UV followed by a recovery period (Fig. 5A). This process was repeated at least 4 times and until individual resistant colonies could be isolated using trypsin soaked cloning rings. These colonies were then expanded and their sensitivity to UV was compared to parental HeLa cells via MTT assay. The LD50 was calculated for each resistant cell line and used to select highly resistant cells for further analysis (Fig. 5B and Table 1).

Cisplatin is a genotoxic agent that is quite effective at killing HPV+ cervical cancers. Cisplatin works by inducing DNA crosslinking that activate many of the same cellular responses induced by UV exposure (McKay et al., 2001). For comparison with the UV-resistant HeLa cells, cisplatin-resistant HeLa cells were generated using the previously described treatment-recovery cycle (Fig. 5A). Resistance was again established using MTT assays to determine LD50 values (Fig. 5C and Table 1). This approach allowed resistant cells to be identified and selected for further analysis. UV resistant HeLa A and B as well as cisplatin resistant HeLa A were chosen because they had the most robust resistance.

3.4. Sensitivity of UV- and cisplatin-resistant HPV+ cervical cancer cells to other genotoxic agents

We postulated that resistance to one type of crosslinking agent may not necessary result in resistance to agents that similar damage DNA. To begin testing this hypothesis and to characterize the mechanisms of resistance in these cell lines, MTT assays were conducted to define the LD50 for UV for the most cisplatin-resistant HeLa lines (Fig. 6A and Table 1). Although individual matched data points were not significantly between resistant and parental cell lines, a statistical analysis comparing the full data sets to each other revealed that a significant increase in UV resistance accompanied acquired cisplatin resistance in HeLa cells (Wilcoxon Matched Pairs Test, $p < 0.05$). The average LD50 increased from 65.47 in parental HeLa to 108.6 in the cisplatin resistant colony demonstrating an almost 2-fold difference in sensitivity. Next, MTT assays defined the LD50 for cisplatin in the most UV-resistant HeLa lines (Fig. 6B and Table 1). This analysis demonstrated that cells that acquired UV resistance could become more sensitive to other crosslinking agents. Finally, a recent report indicated that acquired cisplatin resistance could sensitize cells to PARP inhibition (Michels et al., 2013). To determine if this was true for the cisplatin-resistant HeLa cells described in this report, MTT assays were used to measure sensitivity to olaparib, a commercially available inhibitor of PARP1

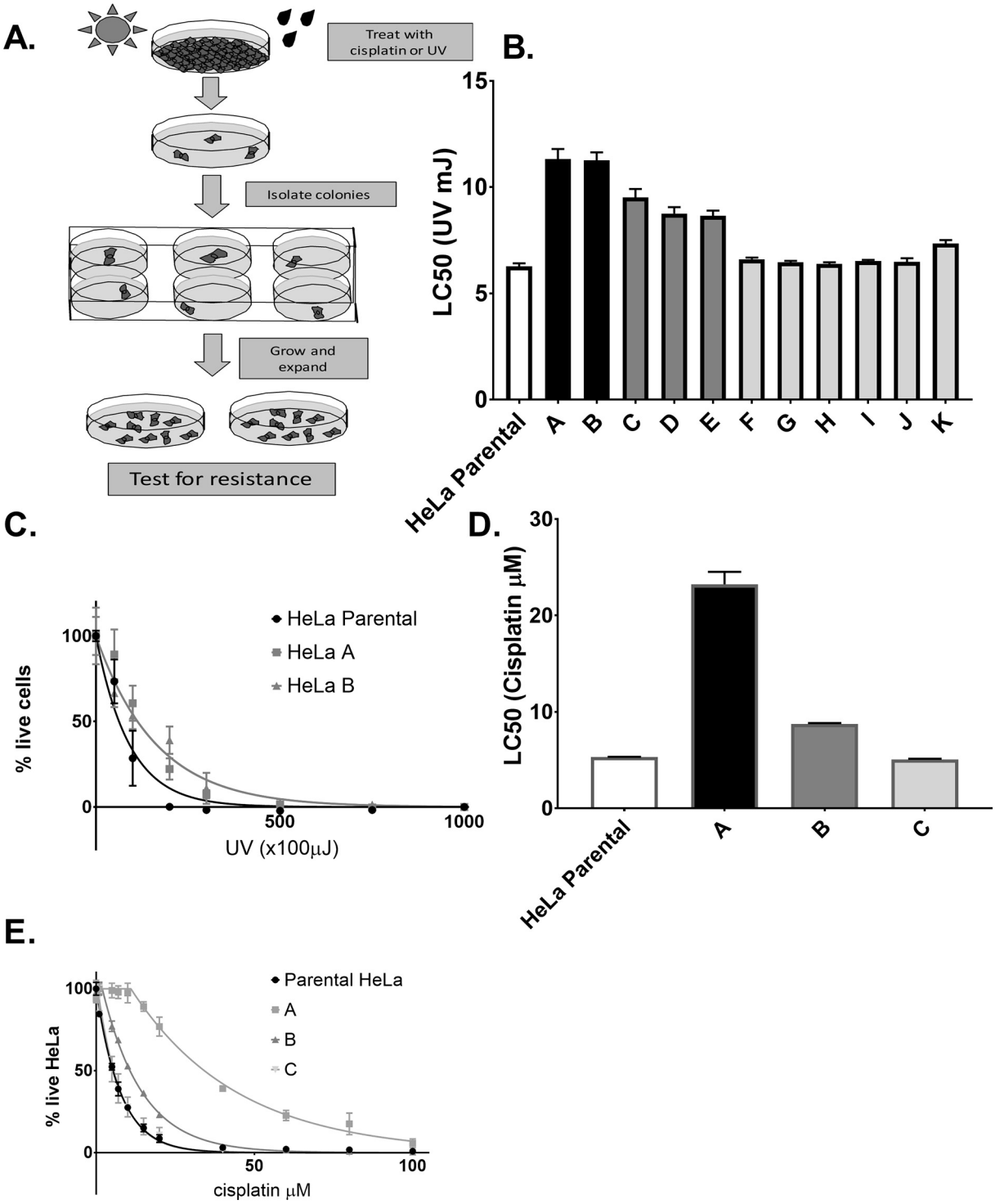

Fig. 5. Creating cross-linker resistant HeLa and SiHa cell lines. A. This image depicts the steps used to generate the cross linker resistant cell lines. Briefly, cells were treated 4 times with either 10 μM cisplatin or 5 mJ UV and given recovery periods between treatments. The resulting colonies were then isolated and transferred to individual wells of a 6-well plate for expansion. The resulting clonal populations were then tested for acquired resistance by MTT. B. This chart depicts the LC50s (amount required to kill 50% of cells) for each isolated cell line. The color gradient represents increasing resistance with black being the most resistant. C. This graph depicts UV sensitivity of the 2 most UV resistant HeLa cell lines as measured by MTT. The solid black line and circles represent the parental HeLa. The grey line, square, and triangle represent the 2 most resistance colonies. D. This chart depicts the LC50s for each isolated cell line with error bars representing the 95% confidence intervals. The color gradient represents increasing resistance with black being the most resistant. E. This graph depicts the cisplatin sensitivity of the isolated cell lines as measured by MTT. The solid black line and circle points represent Parental HeLa. The light grey line and square points represent the most resistant colony. For all, $n = 3$, $^{*}p < 0.05$ by unpaired *t*-test and error bars represent mean ± SD.

(Fig. 6C and Table 1). This analysis demonstrated that the sensitivity to PARP1 inhibition in HeLa cells that acquired cisplatin resistance depended on the concentration of inhibitor. At lower concentrations, cisplatin resistance was associated with increased resistance to PARP1 inhibition, while at very high concentrations of inhibitor the cells were notably more sensitive. Since this result differed from published

Table 1
Lethal Concentrations in UV and cisplatin-resistant HeLa and SiHa. This table depicts the toxicities in cell lines before and after acquisition of resistance to cisplatin and UV. LC50 denotes the concentration or dose required to kill 50% of the cells calculated from MTT data (Fig. 6). 95% CI denotes the 95% confidence intervals. * denotes significance difference compared to parental cell line determined by Student *t*-test (p value ≤ 0.05).

Cell type	LC_{50}	LC_{50} 95% CI
UV toxicity (mJ/cm^2)		
HeLa parental	6.547	5.46 to 7.8
HeLa cisplatin resistant A	10.8	8.49 to 13.73
Cisplatin toxicity (μM)		
HeLa parental	19.08	17.3 to 21.1
HeLa UV resistant A	19.89	16.7 to 23.6
HeLa UV resistant B	6.113*	5.9 to 6.3
Olaparib toxicity (μM)		
HeLa parental	845.3	674.8 to 1084
HeLa cisplatin resistant A	982	650 to 1656
SiHa parental	636	556 to 731.1
SiHa cisplatin resistant pooled	523.6	499.1 to 549.6

reports, we generated a pool cisplatin-resistant cervical cancer cell line using SiHa cells (Supplemental Fig. 2). Unlike clonal populations of resistant cells, this cell line is likely to have gained resistance through multiple mechanisms providing a broader representation of resistance mechanisms. SiHa cisplatin resistant pooled cells were more sensitive to PARP1 inhibition via olaparib (Supplemental Fig. 3 and Table 1), suggesting that the increased dependence on PARP1 activity varies depending on the individual tumor or more likely the mode of resistance.

4. Discussion

We investigated the response of cervical cancer cells to low dose UV exposure. Particularly, our efforts illuminate the increased likelihood of UV-induced apoptosis in these cells. HPV oncogenes seem to counter the inclination towards programed cell death as their shRNA mediated knockdown caused increased p53- and BAX-associated apoptosis, suggesting dying cervical cancer cells sustain a greater abundance of DNA damage. This is somewhat suspected given the ability of HPV oncogenes to impair cellular DNA repair. We also generated clonal populations of cervical cancer cells that were resistant to chemical- or radiation-induced DNA crosslinks and measured their sensitivity to other crosslinking agents and a PARP1-inhibitor.

4.1. HPV oncogenes have both protective and detrimental effects on a cell's response to UV

One realization from this study is that HPV oncogenes sensitize cells to UV while also protecting them from UV-induced apoptosis. HPV oncogenes cause constitutive activation of both ATM and ATR, two keystone repair kinases (Gillespie et al., 2012; Johnson et al., 2017; Moody and Laimins, 2009). While this indicates repair initiation, successful repair also requires resolution of these signaling events as well. HPV E6 also mislocalizes repair proteins from sites of damage allowing the existence of active repair complexes without resolution of the damage they were activated in response to (Mehta and Laimins, 2018; Wallace et al., 2017). Such a disruption of repair signaling is consistent with the increased sensitivity to crosslinked DNA. HPV oncogenes protect the cell from BAK-mediated apoptosis, consistent with the virus's extensive efforts to avoid immune detection by minimizing its cytotoxic effects (Jackson et al., 2000).

While the benefits of evading the immune system are obvious, the evolutionary pressures that drove HPV to sensitize cells to UV are more cryptic. Unlike cutaneous members of the papillomavirus family, infections in the genital tract would not encounter sunlight often, making it unlikely they evolved a mechanism to change the cellular response to UV. The increased toxicity is more likely an unintended consequence of the role repair factors play in replicating the HPV genome. Perhaps being recruited to sites of viral replication prevents ATM and ATR from coordinating the robust response required to avoid the deleterious effect of UV-damage.

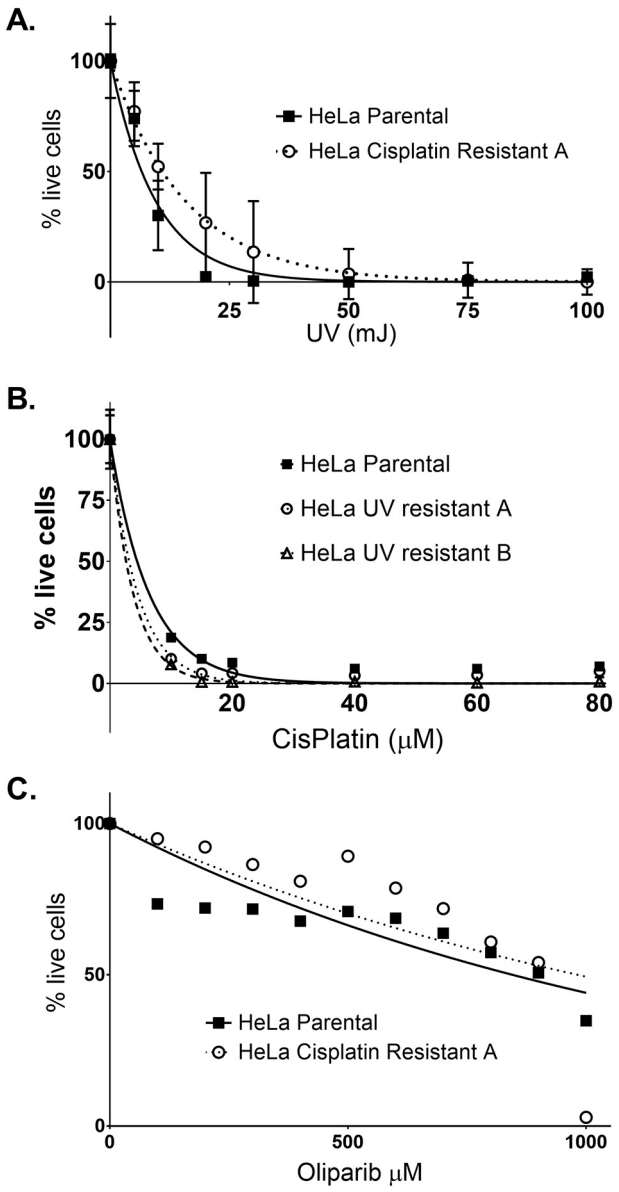

Fig. 6. Sensitivity to cross-linking agents in UV and cisplatin resistant cells. A. This graph depicts the sensitivity of HeLa cells as measured by MTT to ultraviolet radiation before and after acquiring resistance to cisplatin. The square points and solid line represent parental HeLa. The circle points and dotted line represent the Clone A of HeLa cells that acquired resistance to cisplatin. B. This graph depicts the sensitivity of HeLa cells to cisplatin measured by MTT before and after acquiring UV resistance. The square points and solid line represent the parental HeLa. The circle points and dotted line depict data from Clone A of UV resistant HeLa cells. The triangle points and the dashed line represent data from Clone B of UV resistant HeLa cells. C. This graph depicts the PARP1 inhibitor (Olaparib) sensitivity of HeLa cells measured by MTT before and after cisplatin resistance was acquired. Black squares represent the parental HeLa line while open circles depict data from Clone A of cisplatin resistant HeLa cells. The untreated controls are set at 100. For all, $n = 3$, $^*p < 0.05$ by unpaired *t*-test and error bars represent mean ± SD.

4.2. Resistance to crosslinking agents does not guarantee resistance to other genotoxic agents

We generated cervical cancer cell lines that were resistant to two different sources of DNA crosslinks. The clonal populations of resistant cells insured that the acquisition of resistance occurred separately in each cell line. Although we did not determine the mechanism of resistance in these cells, the clonality suggests that cells gained the ability to survived either UV or cisplatin through diverse means. A significant amount is known about the ways that cells become tolerant of genotoxic drugs. Potential resistance strategies include reducing the functional concentration of the drug in the cell by pumping it out of the cell or obtaining a mutation that restores expression of a repair factor (Michels et al., 2013; Rosell et al., 2003; Srivastava et al., 2015; Zhu et al., 2016). Less is known about the ways cells come to be more tolerant of UV, but our observations are in line with previous reports indicating UV-resistance does not confer resistance to other DNA crosslinking agents (Petersen et al., 1995). Instead, UV-resistance appears to have fitness costs as cells grow slower and can be more sensitive to cisplatin (Data not shown, Fig. 6 and (Petersen et al., 1995)). Similarly, sensitivity to small molecule PARP1 inhibitor may or may not accompany resistance to cisplatin (Fig. 6 and Table 1). Moreover, cisplatin resistance was not accompanied with proportionate resistance to UV (Fig. 6 and Table 1). Together, this adds to the evidence that understanding underlying mechanisms of resistance is critical for predicting the response of tumors to other genotoxic drugs.

4.3. Implications for therapeutic intervention

Virtually every cervical cancer is the result of a human papillomavirus infection. HPV transforms cells in part by blocking tumor suppressors (p53 and RB) and by activating the catalytic subunit of telomerase. HPV E6 and E7 also have a well-documented ability to disrupt DNA damage repair (Bristol et al., 2017; Moody, 2018; Spriggs and Laimins, 2017b). HPV transformed cells require continued expression of HPV oncogenes. Since HPV E6 and E7 hinder a cell's ability to protect itself from DNA crosslinks, every cell in HPV-associated tumors are predicted to share this defect. This could explain the efficacy of crosslinking agents like cisplatin in the treatment of malignancies caused by HPV. Therapeutics could be better designed to target HPV-associated cancers if a greater understanding of the mechanisms by which HPV oncogenes induce sensitivity to DNA crosslinks is gained. We show that it is possible to acquire resistance to one source of DNA crosslinks while remaining sensitive to crosslinks from other origins, suggesting that tumors that have acquired resistance to cisplatin could remain sensitive to other crosslinking drugs such as chlorambucil or mitomycin c.

Finally, the importance of measuring the effects of sub-erythemal doses of UV should be noted for studies addressing most clinically relevant questions, particularly those relevant to sensitivity or tumorigenicity. While exposure to high doses of UV or other crosslinking agents may provide interesting molecular insights, they likely induce different repair responses than the levels seen in common exposure to UV or after chemotherapy (Quinet et al., 2016). Further, excessive damage is more likely to induce an apoptotic response rather than a full-fledged effort to repair lesions.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.gene.2018.11.079.

Declaration of interest statement

The authors declare they have no conflicts of interest relevant to this manuscript.

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COMMENTARY
Host-Microbe Biology

mSphere of Influence: the Value of Simplicity in Experiments and Solidarity among Lab Members

Nicholas A. Wallace[a]

[a]Division of Biology, Kansas State University, Manhattan, Kansas, USA

ABSTRACT Nicholas Wallace studies how human papillomaviruses cause cancer throughout the genital and oropharyngeal tracts as well as in the skin. These viruses inhibit host DNA repair to promote their life style and in doing increase the risk of oncogenic mutations. In this mSphere of Influence article, he reflects on how two papers influenced him. "Human Papillomaviruses Activate the ATM DNA Damage Pathway for Viral Genome Amplification upon Differentiation" by C. A. Moody and L. Laimins (PLoS Pathog 5:e10000605, 2009, https://doi.org/10.1371/journal.ppat.1000605) reminded him of the power of straightforward approaches, while "Forty-Five Years of Cell-Cycle Genetics" by B. Reid et al. (B. J. Reid, J. G. Culotti, R. S. Nash, and J. R. Pringle, Mol Biol Cell 26:4307–4312, 2015, https://doi.org/10.1091/mbc.E14-10-1484) gave him the inspiration for his lab management style.

KEYWORDS DNA repair, cervical cancer, human papillomavirus

Every scientist has their own unique approach, generated from a myriad of influences and personal experiences. Mentoring styles are even more individualized. Obviously, research mentors play an outsized role in shaping our approach to science. We are influenced by daily interactions with peers and lab mates, and every so often, there is a paper that changes the way we look at the world around us. The first time I read "Human Papillomaviruses Activate the ATM DNA Damage Pathway for Viral Genome Amplification upon Differentiation" by Cary Moody and Laimonis "Lou" Laimins is a vivid and influential memory (1). I had studied genomic instability induced by retrotransposons as a graduate student and had recently begun work as a postdoc in Denise Galloway's group at the Fred Hutchinson Cancer Research Center (affectionately known as "The Hutch"). The question of how much skin cancer resulted from cutaneous human papillomavirus (HPV) infections fascinated me, but I was struggling to find my footing in this new field. My first ambitious project had plenty of technological bells and whistles. It had also just completely bombed. I felt left behind in the race to develop the fanciest approach to match the experiments that everyone else seemed to be running.

In the midst of these doldrums, Denise casually dropped Cary and Lou's paper on my desk with a passing comment about it seeming like "something up your alley." It was. Not only did the paper help me connect my graduate work to my new interests in viral oncology, it relied on elegantly designed experiments, rather than fancy, but hard to replicate approaches. The results were clear and definitive, relying on Southern and Western blotting combined with immunofluorescence microscopy to dissect HPV's manipulation of DNA repair processes. Using familiar techniques, Cary and Lou had shown undeniable evidence that hyperactivation of a DNA repair signaling pathway facilitated HPV genome amplification. I felt hopeful again. Sure cutting edge techniques can drive a field forward, but there were clearly other paths to success. I poured my energy back into asking important questions and trying to answer them directly and precisely. The impact of Cary and Lou's dissection of altered ATM signaling is easy to

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Address correspondence to nwallac@ksu.edu.

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Getting to talk about two very influential papers was a pleasure. I hope this commentary draws interest to the authors' wonderful work. @wallaceHPVlab

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find in every paper from my lab. Perhaps more generally important, it is a steady reminder that flashiness is not a requirement for good science.

Several years later and at another transition point in my life, I encountered a different kind of article that was similarly impactful. It was not a primary research article or even a review. "Forty-Five Years of Cell-Cycle Genetics" by Brian Reid et al. is a retrospective commentary about the early years in Leland Hartwell's lab that was published almost exactly 1 week after I started my first faculty job (2). I read it about 3 months later, when I was back visiting the Galloway lab over the winter holidays. Brian's lab and office are just down the hall from Denise's at the Hutch, so we had spoken each day for years. It must have surprised him to see me back to my morning routine when I greeted him. I remember being filled with excitement from my new position, but beginning to realize how much I was unprepared for. I peppered him with questions, "Who should I hire?" "What should I do first?" "How do I attract students?" "How can I help undergraduate students do 'good' science?" and on and on. When I paused to breathe, Brian matched my enthusiasm with stories of his time as a student in the Hartwell lab. It was wonderful to see that the memory of 40 years earlier still energized him.

Before I left, he sent me a copy of the article that he and his contemporaries in the Hartwell lab penned about their early days together. It should be required reading for any new principal investigator, especially if they plan to interact with undergraduates. I would love for my students to have half as positive of an experience as Brian and his colleagues had. The article describes the perfect atmosphere for science and is filled with motivational quotes like "although the group was not large, it never seemed small, in part because interaction among the lab members was so constant and intense". Who would not want to work in that environment?!? Life as a scientist has peaks of exhilaration, but it is also brutal. Surviving negative reviews, rejections, misbehaving experiments, and unexplained contaminations are a daunting task that we all face. Your lab environment can make all the difference. I model my lab atmosphere after the one described in "Forty-Five Years of Cell-Cycle Genetics". It is the first paper people read when they join our team. "An atmosphere of total openness and high collegiality, in which no one worried about who would get credit for a particular idea" sounds like science utopia. The camaraderie that Brian Reid, Joseph Culotti, Robert Nash, and John Pringle describe should be the goal of every leader.

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