Final Progress Report for Research Projects Funded by Health Research Grants

Instructions: Please complete all of the items as instructed. Do not delete instructions. Do not leave any items blank; responses must be provided for all items. If your response to an item is "None", please specify "None" as your response. "Not applicable" is not an acceptable response for any of the items. There is no limit to the length of your response to any question. Responses should be single-spaced, no smaller than 12-point type. The report **must be completed using MS Word**. Submitted reports must be Word documents; they should not be converted to pdf format.

1. **Grantee Institution:** The Pennsylvania State University

2. **Reporting Period (start and end date of grant award period):** 1/1/2010 - 12/31/2013

3. **Grant Contact Person (First Name, M.I., Last Name, Degrees):** John Anthony, MPA

4. **Grant Contact Person's Telephone Number:** 814 935 1081

5. **Grant SAP Number:** 4100050904

6. **Project Number and Title of Research Project:** 3. Cell Signaling Mechanisms Necessary for High-fidelity Replication of Repetitive Sequences

7. **Start and End Date of Research Project:** 1/1/2010 - 12/31/2010

8. **Name of Principal Investigator for the Research Project:** Kristin Eckert, PhD

9. **Research Project Expenses**.

   9(A) Please provide the total amount of health research grant funds spent on this project for the entire duration of the grant, including indirect costs and any interest earned that was spent:

   $ 42,574

   9(B) Provide the last names (include first initial if multiple individuals with the same last name are listed) of **all** persons who worked on this research project and were supported with health research funds. Include position titles (Principal Investigator, Graduate Assistant, Post-doctoral Fellow, etc.), percent of effort on project and total health research funds expended for the position. For multiple year projects, if percent of effort varied from year to year, report in the % of Effort column the effort by year 1, 2, 3, etc. of the project (x% Yr 1; z% Yr 2-3).

Last Name, First Name	Position Title	% of Effort on Project	Cost
Baptiste, Beverly	Graduate Assistant	100%	$21, 924

9(C) Provide the names of **all** persons who worked on this research project, but who *were not* supported with health research funds. Include position titles (Research Assistant, Administrative Assistant, etc.) and percent of effort on project. For multiple year projects, if percent of effort varied from year to year, report in the % of Effort column the effort by year 1, 2, 3, etc. of the project (x% Yr 1; z% Yr 2-3).

Last Name, First Name	Position Title	% of Effort on Project
Eckert, Kristin	PI	< 2%

9(D) Provide a list of **all** scientific equipment purchased as part of this research grant, a short description of the value (benefit) derived by the institution from this equipment, and the cost of the equipment.

Type of Scientific Equipment	Value Derived	Cost
None		

10. Co-funding of Research Project during Health Research Grant Award Period. Did this research project receive funding from any other source during the project period when it was supported by the health research grant?

Yes_____ No__X_____

If yes, please indicate the source and amount of other funds:

11. Leveraging of Additional Funds

11(A) As a result of the health research funds provided for this research project, were you able to apply for and/or obtain funding from other sources to continue or expand the research?

Yes_____ No____X_____

If yes, please list the applications submitted (column A), the funding agency (National Institutes of Health—NIH, or other source in column B), the month and year when the application was submitted (column C), and the amount of funds requested (column D). If you have received a notice that the grant will be funded, please indicate the amount of funds to be awarded (column E). If the grant was not funded, insert "not funded" in column E.

Do not include funding from your own institution or from CURE (tobacco settlement funds). Do not include grants submitted prior to the start date of the grant as shown in Question 2. If you list grants submitted within 1-6 months of the start date of this grant, add a statement below the table indicating how the data/results from this project were used to secure that grant.

A. Title of research project on grant application	B. Funding agency (check those that apply)	C. Month and Year Submitted	D. Amount of funds requested:	E. Amount of funds to be awarded:
None	☐NIH ☐ Other federal (specify:_____) ☐ Nonfederal source (specify:_)		$	$

11(B) Are you planning to apply for additional funding in the future to continue or expand the research?

Yes____X_____ No_____

If yes, please describe your plans:

Competing renewal of NIH/R01 grant GM087472, "Computational and Biochemical Analysis of Microsatellite Life Cycle", K. A. Eckert (MPI) and K. Makova, (MPI); planned submission July, 2014.

12. Future of Research Project. What are the future plans for this research project?

We plan to pursue our mechanistic studies of mutational biases caused by mismatch repair protein defects, and the impact of DNA polymerase/mismatch repair protein defects on microsatellite instability in cancer progression.

13. New Investigator Training and Development. Did students participate in project supported internships or graduate or post-graduate training for at least one semester or one summer?

Yes___X___ No_____

If yes, how many students? Please specify in the tables below:

	Undergraduate	Masters	Pre-doc	Post-doc
Male				
Female			1	
Unknown				
Total			**1**	

	Undergraduate	Masters	Pre-doc	Post-doc
Hispanic				
Non-Hispanic			1	
Unknown				
Total			**1**	

	Undergraduate	Masters	Pre-doc	Post-doc
White			1	
Black				
Asian				
Other				
Unknown				
Total			**1**	

14. Recruitment of Out-of–State Researchers. Did you bring researchers into Pennsylvania to carry out this research project?

Yes_____ No____X_____

If yes, please list the name and degree of each researcher and his/her previous affiliation:

15. Impact on Research Capacity and Quality. Did the health research project enhance the quality and/or capacity of research at your institution?

Yes____X_____ No_____

If yes, describe how improvements in infrastructure, the addition of new investigators, and other resources have led to more and better research.

This grant was a Graduate Research Supplement Award. The awardee, Dr. Beverly Baptiste, received her Ph.D. degree in Genetics from The Pennsylvania State University in August, 2013. The research supported by the CURE funds directly supported her doctoral dissertation work. Dr. Bapiste is now a postdoctoral fellow at the NIH/National Institute on Aging (Baltimore, MD).

16. Collaboration, business and community involvement.

16(A) Did the health research funds lead to collaboration with research partners outside of your institution (e.g., entire university, entire hospital system)?

Yes____X_____ No_____

If yes, please describe the collaborations:

Part of this research was published in collaboration with the laboratory of Dr. Kateryna Makova, Department of Biology, Penn State College of Science, University Park campus.

16(B) Did the research project result in commercial development of any research products?

Yes_____ No__X_____

If yes, please describe commercial development activities that resulted from the research project:

16(C) Did the research lead to new involvement with the community?

Yes_____ No_____X_____

If yes, please describe involvement with community groups that resulted from the research project:

17. Progress in Achieving Research Goals, Objectives and Aims.

List the project goals, objectives and specific aims (as contained in the grant agreement). Summarize the progress made in achieving these goals, objectives and aims for the period that the project was funded (i.e., from project start date through end date). Indicate whether or not each goal/objective/aim was achieved; if something was not achieved, note the reasons why. Describe the methods used. If changes were made to the research goals/objectives/aims, methods, design or timeline since the original grant application was submitted, please describe the changes. Provide detailed results of the project. Include evidence of the data that was generated and analyzed, and provide tables, graphs, and figures of the data. List published abstracts, poster presentations and scientific meeting presentations at the end of the summary of progress; peer-reviewed publications should be listed under item 20.

This response should be a DETAILED report of the methods and findings. It is not sufficient to state that the work was completed. Insufficient information may result in an unfavorable performance review, which may jeopardize future funding. If research findings are pending publication you must still include enough detail for the expert peer reviewers to evaluate the progress during the course of the project.

Health research grants funded under the Tobacco Settlement Act will be evaluated via a performance review by an expert panel of researchers and clinicians who will assess project work using this Final Progress Report, all project Annual Reports and the project's strategic plan. After the final performance review of each project is complete, approximately 12-16 months after the end of the grant, this Final Progress Report, as well as the Final Performance Review Report containing the comments of the expert review panel, and the grantee's written response to the Final Performance Review Report, will be posted on the CURE Web site.

There is no limit to the length of your response. Responses must be single-spaced below, no smaller than 12-point type. If you cut and paste text from a publication, be sure symbols print properly, e.g., the Greek symbol for alpha (α) and beta (ß) should not print as boxes (□) and include the appropriate citation(s). DO NOT DELETE THESE INSTRUCTIONS.

The long-term goal of this project is to elucidate the polymerases and cell signaling mechanisms that are necessary for high-fidelity replication of microsatellites. Our working model is that the faithful replication of microsatellite sequences necessitates coordination of several polymerases, because replicative polymerases are inhibited by microsatellite sequences, and because other DNA sequences that form non-B structures require Pol κ and/or Pol η for effective replication. The *specific aim* is to test the hypothesis that Y-family polymerases, Pol κ and Pol η, are required for accurate microsatellite replication, using a human cell culture model system.

Subaim 1: To determine the effects of altered Y-family polymerase levels on microsatellite mutagenesis.

Subaim 2: To determine the effect of mutations in UBZ domains of Pol κ on microsatellite mutagenesis.

The proposed research will clarify the roles of DNA polymerases κ and η in human genome replication.

A. Altered Y-family polymerase expression and microsatellite mutagenesis.

A.1. *Summary of cell lines created for use in the model*

Three cell lines were used in this study: two colon carcinoma cell lines, HCT-116 and DLD-1, and one osteosarcoma cell line, U2OS. Gene expression vectors were created for three Y family polymerase genes: POLH (Pol η), POLI (Pol ι) and POLK (Pol κ). All genes were cloned into the pTRE-tight, doxycycline regulated vector (Clontech). HCT-116 and DLD-1 cells were stably transfected with the pTetOn regulation vector; U2OS cells carrying the regulatory vector were purchased from Clontech. All experimental treatments with doxycycline were performed in media containing Tet-free fetal bovine serum.

A.2. *Overexpression of Y family polymerases in human cancer cell lines.* A summary of our experimental results is given in Table 1. We successfully isolated and characterized one stable, U2OS/POLI doxycycline-inducible clone (Figure 1A). Although doxycycline-inducible POLI expression was observed transiently after transfection of HCT-116 cells with pTRE/POLI (Figure 1A), no stable, dox-inducible HCT116 clones have yet to be isolated (Table 1). We were unable to observe doxycycline-induced overexpression of either POLH or POLK, transiently or stably, in either U2OS or HCT116 cell lines. An example of the lack of induction is shown in Figure 1B. We did not isolate any DLD-1 clones that displayed inducible expression of our pTRE-GFP plasmid; therefore, this cell line was not evaluated

Table 1. Summary of Y family polymerase overexpression experimental results

Experiment	U2OS	HCT-116	DLD-1
Transient expression			
pTRE-GFP	**Yes**	**Yes**	No
Transient overexpression			
pTRE/POL I (Pol ι)	**Yes**	**Yes**	n.d.
pTRE/POLK (Pol κ)	No	No	+/-
pTRE/POLH (Pol η)	No	No	n.d.
Stable overexpression			
pTRE/POL I (Pol ι)	**Yes**	No	n.a.
pTRE/POLK (Pol κ)	No	No	n.a.
pTRE/POLH (Pol η)	No	No	n.a.

n.d.,not done; n.a., not applicable

Protein expression levels were assessed by fluorescence microscopy (GFP) or immunoblot analyses (polymerase)

further. Consequently, we were unable to proceed with Subaim 2- to determine the effect of mutations in UBZ domains of Pol κ on microsatellite mutagenesis.

We examined several reasons for the lack of POLK and POLH overexpression results. First, we sequenced the corresponding pTRE vectors, but found the DNA sequence to be correct for each construct. Second, we analyzed POLH mRNA levels with and without doxycycline treatment, in U2OS clones stably transfected with the pTRE/POLH vector by RT-PCR. We observed no differences between untreated and treated cells. Third, we examined the genomic DNA of the U2OS clones and verified integration of the pTRE/POLH vector, using PCR and primers specific to the vector sequence.

A.3. *Knock-down of Y family polymerases in human cancer cell lines.* To test the ability of HCT-116 cells to undergo siRNA-mediated gene silencing, we purchased siGLO RNAs to Lamin A/C from Dharmacon. Transfection of HCT-116 cells with this control siRNAs was successful, as determined by cellular fluorescence, and successful knockdown of Lamins A/C was observed by Immunoblot analyses 3 days after transfection. Time and funding limitations precluded us from attempting siRNA knockdown of the polymerases.

A.4. *Discussion.* Overexpression of particular polymerases in cell lines may provide insight into the roles of those enzymes in genomic stability. Increased abundance of polymerases may increase their activity in replication, though this may be limited by regulatory

mechanisms that control DNA access. One of our goals was to determine whether altered Y family DNA polymerase levels are tolerated in tumor cells. Our results clearly demonstrate that increased levels of Pol ι can be sustained (Figure 1). Increased expression of pol ι in human tumors has been shown to increase spontaneous mutations, which is reversible by knockdown of pol ι, consistent with an increased access to DNA replication due to overexpression (Zhou et al., 2012). In contrast, our studies suggest that overexpression of either Pol η or Pol κ may not be sustainable with tumor cell viability. Future studies are clearly warranted to definitively test the role of these polymerases in tumor cells.

B. Development of shuttle vector assay for human epithelial cell lines

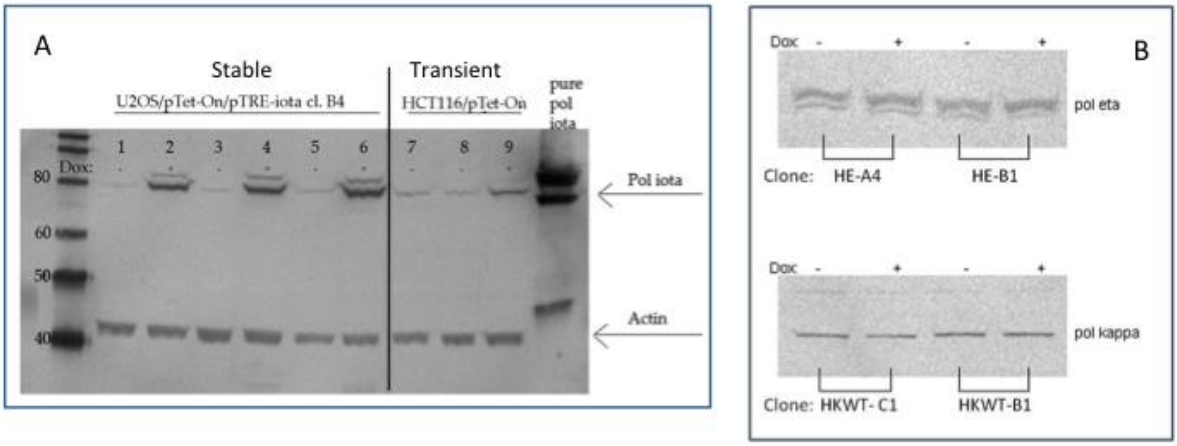


Figure 1. Immunoblot analyses of doxycycline-induced Y family DNA polymerase protein overexpression in tumor cell lines. (A). Overexpression of POLI (Pol ι) in U2OS cells (lanes 1-6, stable expression) and HCT-116 cells (lanes 7-9, transient expression). Lanes 3 and 4 are cytoplasmic fractions; the remaining lanes are nuclear fractions. Lanes 1, 3, 5 7, and 8 show endogenous expression levels; lanes 2, 4, 6 and 9 shown increased levels in the presence of doxycycline. (B). Representative HCT-116 cell line clones that were stably integrated with pTRE-POLH (top) or pTRE-POLK (bottom) expression vectors. No doxycycline-inducible clones for these two polymerases were isolated from either HCT-116 or U2OS cell lines.

Our second goal was to establish a mutational assay to monitor the effects of alterations in Y family polymerase regulation on microsatellite mutagenesis. As described below, we successfully established a new mutagenesis assay and used this approach to examine the effects of specific mismatch repair protein loss on microsatellite mutagenesis.

B.1. *Gap-filling microsatellite mutagenesis assay.* The gap-filling TLS mutagenesis assay described by Avkin et al. was modified to assess microsatellite mutagenesis (Avkin et al., 2002). As a quantitation for TLS, the number of gapped plasmids recovered after cellular TLS are compared with the amount of supercoiled plasmid recovered from the same cells using plasmids with different bacterial selection markers. We utilized gapped duplex molecules containing the HSV-*tk* reporter with a $[GT]_{13}$ microsatellite. Extracted plasmids were analyzed for mutation frequency in *E. coli* and independent mutants were sequenced. Of 24 mutants isolated from HCT116 cells, seven were the same size as untransfected gap, 6 kB. The remaining 17 were

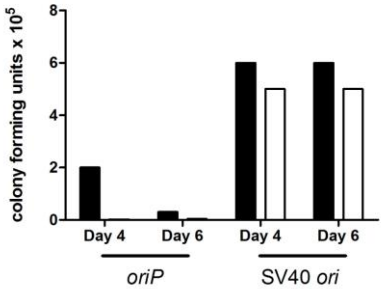
between 1.2 and 4.5 kB. Only eleven of 24 could be sequenced, presumably due to deletion or disruption of the sequencing primer site. Of those eleven, seven contained large deletions and three had no DNA sequence changes in the gapped region. Only one contained a potential polymerase error, a base substitution outside of the microsatellite allele. One mutant plasmid contained a sequence that was not homologous to any portion of the plasmid. Instead, a Basic Local Alignment Search Tool (BLAST) search revealed perfect homology to a region on the human chromosome. These data suggest that gapped DNA duplex molecules are not stable when transfected into human cells.

B.2. Choice of origin of replication. Our lab has previously utilized plasmids containing the Epstein-Barr Virus (EBV)-derived *oriP* origin of replication and the HSV-*tk* forward mutational target in EBV-infected lymphoblastoid cells. The EBV genome is maintained in multiple copies as an episomal plasmid in latently infected cells (Lindahl et al., 1976). Plasmids containing the *oriP* origin of replication are replicated in a cell-cycle dependent manner when cells express the EBV nuclear antigen, EBNA-1 (Yates et al., 1985). Cell-cycle controlled replication of an episomal plasmid is advantageous in mimicking replication on a chromosome, but establishing an *oriP*-based replicon, even in EBNA-1 expressing cells is a rare event (Leight and Sugden, 2001).

The other commonly used origin of replication in mammalian cells is derived from the papovavirus simian virus 40 (SV40). The SV40 genome can also be maintained episomally as a circular molecule. The SV40 A gene, encoding the large tumor antigen (TAg), is required for initiation of replication. TAg binds the SV40 *ori* and acts as a DNA helicase (Fairman and Stillman, 1988). Cellular polymerases and many associated factors are utilized in replicating an SV40 plasmid, enabling inferences to cellular mutation frequencies and specificities. One advantage of an SV40 *ori*-based replication system is that it can be established easily in any cell expressing TAg (Tegtmeyer et al., 1975). A disadvantage of the SV40 system is that multiple rounds of replication can occur within a single cell cycle, and replication can occur outside of S phase, which means that it is not under the same cell cycle controls as chromosomal replication (Yamaguchi and DePamphilis, 1986).

To determine which origin was most effective for this assay, we used two green fluorescent protein (GFP)-expressing plasmids with either *oriP* or SV40 *ori* origin of replication. HEK293+EBNA-1 cells were compared with HEK293+SV40 TAg cells. Both cell lines showed approximately equal levels of transfection based on visual comparison of GFP expression. After 4 or 6 days, cells were harvested and plasmids were digested with *Dpn*I to eliminate unreplicated plasmids. *Dpn*I selectively recognizes plasmids that were replicated and methylated in bacteria. Plasmids were transformed into bacteria and plated to determine the number of *Dpn*I-resistant plasmids for each origin. Use of the SV40 *ori* resulted in over 100-fold more colonies than the *oriP* origin of replication (500 x 10^3 and 4 x 10^3,

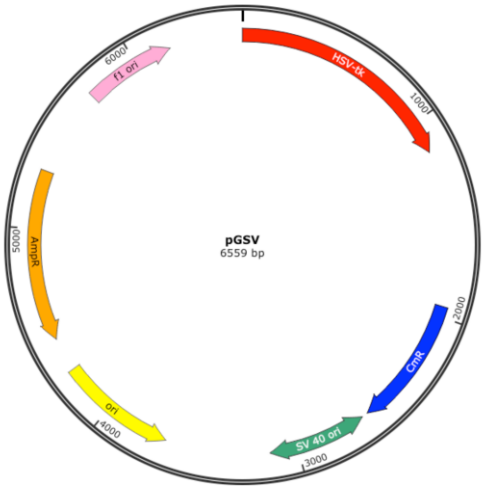
Figure 2. SV-40 origin is more efficient than EBV-oriP for replication in human epithelial cells. HEK293E and HEK293T were transfected with plasmids containing an *oriP* or SV40 *ori* origin of replication, respectively, for 4 or 6 days. Recovered plasmids, uncut (black) or *Dpn*I-digested (white) were electroporated into bacteria and plated to determine the number of intact plasmids. Data shown are the results from one experiment.



respectively) (Figure 2). These data demonstrate that SV40 *ori* is a more efficient origin of replication than *oriP*. The low frequency of mutagenesis requires abundant recovery of replication products and the low yield of *oriP* plasmids is insufficient for robust mutagenesis data.

B.3. SV40-origin based assay. A shuttle vector for epithelial cells (pGSV) containing an SV40 *ori* and the HSV-*tk* reporter gene (Figure 3) was constructed, containing GT/CA microsatellite repeats. In a proof of principle experiment, MMR-deficient HCT116 cells and MMR-proficient U2OS cells were transfected with $[GT/AC]_{10}$ or $[GT/AC]_{19}$ pGSV plasmids (pGSV-GT10 and pGSV-GT19, respectively), along with pPVU-o containing the SV-40 T-Antigen (TAg). Three days post transfection, harvested plasmids were digested with *Dpn*I, electroporated into *E. coli* to determine mutation frequency. The expectation was that the frequency of mutation of pGSV-GT10 would be greater than pGSV-GT19 because dinucleotide microsatellites mutate at higher frequency as the repeat tract length increased. In addition, we expected that plasmids replicated in HCT116 cells would have a higher mutation frequency than plasmids replicated in U2OS cells, because MMR is an important factor in microsatellite stability. The results were surprising in two ways. First, in U2OS cells, the frequency of mutation for pGSV-GT19 (29×10^{-4}) was lower than pGSV-GT10 (64×10^{-4}). Second, the mutation frequency for the pGSV-GT10 plasmid after replication in HCT116 was within 2-fold of that measured in U2OS cells, a difference that is not biologically significant (Table 2).

Figure 3: Plasmid map of pGSV. Numbers correspond to HSV-*tk* gene. Microsatellite is inserted at position 112. Sequencing primer is a reverse primer that begins at position 309.



To determine mutational events, independent mutants were isolated and characterized by several methods. First, we used gel electrophoresis to determine the size of the isolated plasmids (Figure 4). The pGSV plasmids are 6.7 kB, but most of the plasmids recovered from mutants were smaller than expected. To determine if the size discrepancy resulted from plasmid rearrangement that eliminated the target microsatellite sequence, colony hybridization was performed using an oligonucleotide containing $[GT/AC]_9$ as a probe. Only 7/24 (29%) of the pGSV-GT10 mutants and 12/24 (50%) of the pGSV-GT19 mutants hybridized to the probe, indicating that less than half of the mutants isolated contained the microsatellite target (Figure 5).

Figure 4: Gel electrophoresis of a selection of supercoiled wild type and mutant pGSV-GT19 plasmids. Lane SC is a supercoiled DNA ladder, sizes indicated to the left of the gel. Lane 1 is the wild-type plasmid, 6.7 kB. Lanes 2-16 are independent mutants of pGSV-GT19 isolated after replication in HCT116 cells. Asterisks mark plasmids that migrate at the same molecular weight as wild-type plasmid. Arrow points to the expected migration of pGSV-GT19.

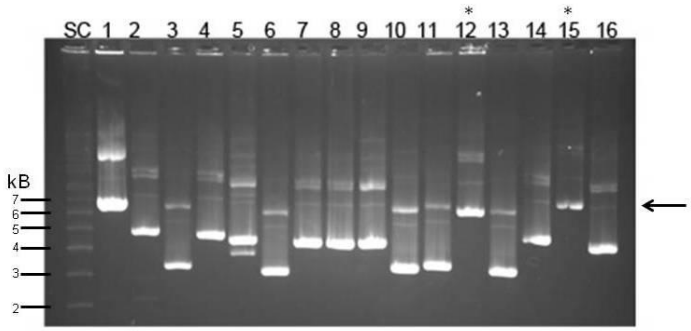
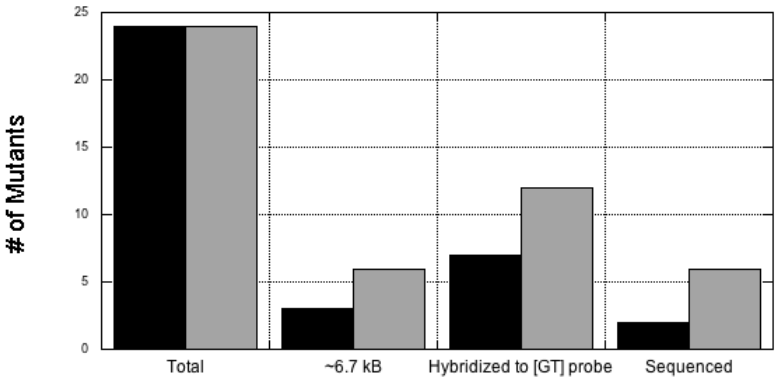


Table 2: HSV-*tk* mutation frequencies in bacteria, HCT116 (MMR-) cells and U2OS (MMR+) cells.

Vector	Mutation Frequency x 10^{-4}	
	pGSV-GT10	pGSV-GT19
Bacteria[a]	0.49	2.2
HCT116	110	243
U2OS	62	29

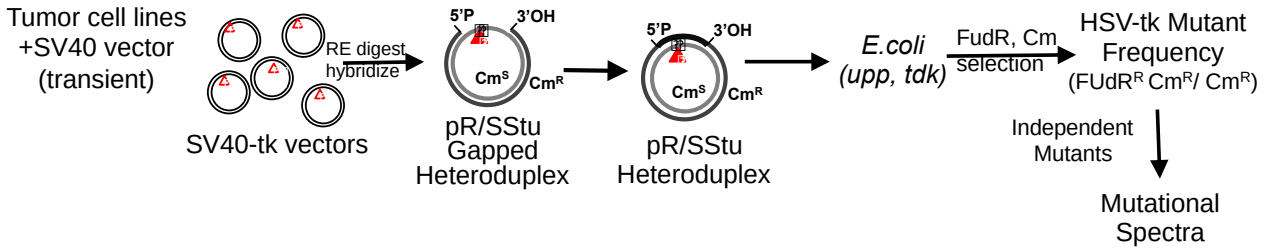
[b] Transfection of E. coli directly, without replication in human cells.

Figure 5: The pGSV plasmids harvested from HCT116 cells vary in size, ability to hybridize to a microsatellite-specific probe, and ability to be sequenced with sequencing primer *TK*-309. Graph shows the number of mutants that were shown to be ~6.7 kB after electrophoresis, that hybridized to a probe of the microsatellite, and that sequenced with a primer within the HSV-*tk* gene. Black bars = pGSV-GT10, gray bars = pGSV-GT19.



To decrease the number of rearranged, non-informative mutants in the assay, we used a small fragment approach. Here, replicated plasmids are digested to produce a fragment containing the microsatellite sequence and then hybridized to a gapped duplex DNA (Figure 6). This eliminates

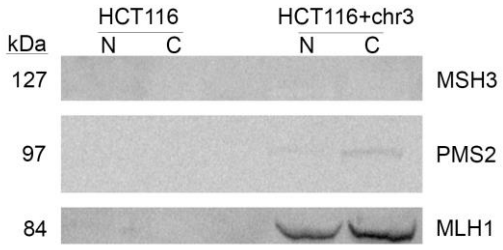
Figure 6. Schematic of transient *ex vivo* HSV-*tk*, SV40-based mutagenesis assay for use in epithelial cells (Baptiste et al. 2013).



non-informative mutant plasmids in which the microsatellite region has been deleted. In addition, we removed the chloramphenicol resistance markers (making psGSV plasmids) to eliminate uncut plasmids. Utilizing this approach, 64% of the plasmids isolated from mutants sequenced properly.

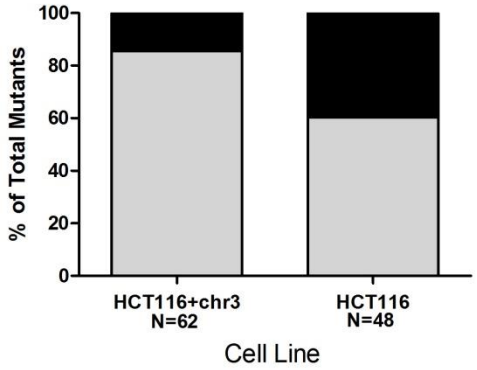
B4. Role of MMR in generating a mutational bias towards expansion of $[GT/CA]_n$ alleles.
Human cells have specification in MMR correction of DNA replication errors, which is achieved through the combination of different heterodimers of the MutS and MutL components of the MMR machinery. We examined mutational specificity in the HCT116 human colon cancer cell line, which carries loss-of-function mutations in both the MLH1 and MSH3 genes. This cell line has been shown previously to be deficient in repair of DNA substrates containing two unpaired bases, which are similar to the two-nucleotide loops caused by slippage intermediates of dinucleotide repeats. Because these cells do not express the MLH1 protein, they are deficient in both MutLα and MutLγ repair complexes. Therefore, although HCT116 cells express MSH2 and MSH6 proteins (MutSα), they are functionally MMR-deficient because they lack a functional MutL heterodimeric complex. We expected that the mutational events observed in HCT116 cells would reflect errors generated by DNA polymerases, which we have shown here and previously to be balanced between expansions and deletions (Abdulovic et al., 2011). We also analyzed mutational biases in HCT116 cells complemented with chromosome 3 (HCT116+chr3). This complementation restores MLH1 protein expression, but does not affect expression of the MSH3 protein (Figure 7). HCT116+chr3 cells are MutSα proficient, but MutSβ deficient. Thus, comparison of HCT116+chr3 cells to the HCT116 parental cell line allows us to determine the role that MutSα plays in generating mutational bias.

Figure 7. HCT116 cells lack MLH1, PMS2 and MSH3. HCT116+chr3 cells express MLH1 and PMS2, but lack MSH3. Western analysis of 40 µg of nuclear and cytoplasmic extracts of HCT116 and HCT116+chr3 cells.



Utilizing the modified shuttle vector assay shown in Figure 6, three days following transient transfection of the $[GT/CA]_{19}$ construct, plasmids were recovered from HCT116 cells and analyzed for mutation frequency and mutational specificity. As predicted for cells containing no functional MMR complexes, we measured high mutation frequencies. The mutation frequency of plasmids after replication in HCT116+chr3 cells (5.7×10^{-3}) was ~two-fold lower than the frequency after replication in HCT116 cells (1.2×10^{-2}), suggesting that MutSα contributes to MMR of replication errors of mature $[GT/CA]_n$ dinucleotide repeats. The percentage of total mutants with microsatellite expansions was greater for HCT116 (40%) than for HCT116+chr3 cells (15%)(Figure 8). Specifically, we observed that the frequency of expansion mutations was six-fold higher in HCT116 cells (4.8×10^{-3}) than in HCT116+chr3 cells (8.0×10^{-4}). Because loss of MutSα results in a higher expansion mutation frequency, our data suggest that the MutSα heterodimer preferentially repairs pre-mutational expansion intermediates.

Figure 8: GT/CA microsatellite mutation rates and directional biases observed in mismatch repair-deficient human cells. Mutational biases of GT/CA_{19} microsatellite replication in HCT116+chr3 and HCT116 cells. Black bars, expansions of 1 or more units; gray bars, deletion of one or more units.



To understand the role of MMR in mutagenesis of mature microsatellites, we compared the data from HCT116 cells and HCT116+chr3 cells to published results from two other assays (Baptiste et al., 2013). First, *in vitro* data obtained using DNA pol β was examined to determine the error specificity of DNA polymerases. Second, cellular data obtained using the Eckert lab *oriP*-based mutational assay from MMR-proficient LCL721 cells and MMR-deficient LCL1261 cells was compared to assess the importance of specific MMR proteins in the mutational specificity of mature microsatellites. This analysis revealed that MMR is a source of the directional bias in microsatellite mutagenesis that results in more expansions than deletions in human cells (Baptiste et al., 2013). Total absence of cellular MMR mimics the expansion *versus* deletion balance seen in our *in vitro* results (Table 3). Comparisons of MutSα-deficient/proficient cell line pairs identified this repair complex as having greater activity towards removing pre-mutational expansion intermediates (Table 3). A constant balance of expansions and contractions of microsatellites may play a role in maintaining their genetic stability over time. Interestingly, our results show that for dinucleotide alleles in the 13 to 20 repeat number range, expansions occurred more frequently than deletions, not only in MMR-proficient cells, but also in cells deficient for MutSα. In contrast to the previous conclusions of Yamada *et al.* (2002) using a reversion assay, we find no directional bias for dinucleotide microsatellite mutations in functionally MMR-deficient HCT116 cells, using a forward assay (Table 3). In fact, the mutational specificity that we observed after replication of the $[GT/CA]_{19}$ vector in HCT116 cells is highly similar to *in vitro* observations of errors produced by Pol β, pol κ and Pol δ at the same microsatellite allele (Baptiste and Eckert, 2012). One explanation for the different conclusions of the two studies may be the fact that we compared our HCT116 results to chromosome 3-complemented HCT116 cells as a control for MMR, while Yamada *et al.* (2002)

used MMR-proficient mouse cells as the control comparison. Our complemented cells still lack MutSβ, while the mouse cells are presumably proficient for both MutS complexes. Also, mouse cells may replicate and repair looped intermediates differently than human cells.

Table 3. Comparison of $[GT/CA]_{19}$ mutagenesis using *ex vivo* and *in vitro* assays.

MMR heterodimer		*Ex vivo* assay		*In vitro* assay (Pol β)[b]
		HCT116+chr3	HCT116	
MutS	α	+	+[a]	-
	β	-	-	-
MutL	α	+	-	-
	γ	+	-	-
Expansion MF		8.0×10^{-4}	4.8×10^{-3}	5.6×10^{-3}
Deletion MF		4.9×10^{-3}	7.3×10^{-3}	1.2×10^{-2}
Expansion:Deletion		1:6	1:1.5	1:2

[a]Components of the MutSα mismatch recognition complex are present in HCT116 cells, but cannot function in MMR because the absence of MLH1 renders the cells devoid of any MutL complex.
[b]Data are derived from 4 independent pol β reactions and sequence analysis of 103 independent mutants. Data taken from Baptiste *et al.*, 2013.

B5. *Discussion.* The mutagenesis assay developed in this grant is an efficient forward mutational assay for use in epithelial cells. The assay has been optimized to minimize the number of rearranged plasmids that result in a mutant phenotype, yet are not informative regarding replication errors. The transient nature of the assay is advantageous for studying the effects of knockdown of specific replication or repair proteins on mutagenesis. Here, we utilized the assay to examine the role of MMR in the mutagenesis of mature microsatellite alleles

An expansion bias has long been observed in mammalian cells. This directional bias is not seen in *in vitro* polymerase reactions, indicating that another aspect of DNA metabolism is responsible. Our data demonstrate that in the absence of MMR, the expansion bias does not exist. This is significant because it indicates that MMR as a whole is more effective on premutational deletion intermediates than expansion intermediates. Interestingly, the two components of MMR examined in this study, PMS2 and MLH3, both contribute more towards correction of premutational expansion intermediates. Further studies are needed to determine which MMR components or combination of components function to selectively remove deletion intermediates and the mechanism by which this occurs.

C. Scientific Presentations resulting from this research

Doctoral dissertation

B.A. Baptiste. "Frameshift Mutagenesis: From Test Tubes to Tumor Cells", August 2013

Poster Presentations

B. A. Baptiste and K. A. Eckert, "Y family polymerases: heroes or villains?" EMS Meeting, Fort Worth, TX, 11/2011.

B. A. Baptiste and K. A. Eckert, "The Battle of the Bulge: How DNA Polymerase Kappa Stabilizes Microsatellites," Gordon Research Conference on Mutagenesis, Newport, R.I., 8/19-24/2012

D. Literature Cited

Abdulovic, A.L., Hile, S.E., Kunkel, T.A., and Eckert, K.A. (2011). The in vitro fidelity of yeast DNA polymerase delta and polymerase epsilon holoenzymes during dinucleotide microsatellite DNA synthesis. DNA Repair 10, 497-505.

Avkin, S., Adar, S., Blander, G., and Livneh, Z. (2002). Quantitative measurement of translesion replication in human cells: evidence for bypass of basic sites by a replicative DNA polymerase. Proc Natl Acad Sci U S A 99, 3764-3769.

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Baptiste, B.A., Ananda, G., Strubczewski, N., Lutzkanin, A., Khoo, S.J., Srikanth, A., Kim, N., Makova, K.D., Krasilnikova, M.M., and Eckert, K.A. (2013). Mature Microsatellites: Mechanisms Underlying Dinucleotide Microsatellite Mutational Biases in Human Cells. G3: Genes Genomes Genetics **3**, 451-463.

Fairman, M.P., and Stillman, B. (1988). Cellular factors required for multiple stages of SV40 DNA replication in vitro. The EMBO Journal **7**, 1211.

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Tegtmeyer, P., Schwartz, M., Collins, J., and Rundell, K. (1975). Regulation of tumor antigen synthesis by simain virus 40 gene A. Journal of Virology 16, 168-178.

Yamada, N.A., Smith, G.A., Castro, A., Roques, C.N., Boyer, J.C., and Farber, R.A. (2002). Relative rates of insertion and deletion mutations in dinucleotide repeats of various lengths in mismatch repair proficient mouse and mismatch repair deficient human cells. Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis 499, 213-225.

Yamaguchi, M., and DePamphilis, M.L. (1986). DNA binding site for a factor(s) required to initiate simian virus 40 DNA replication. Proceedings of the National Academy of Sciences 83, 1646-1650.

Yates, J.L., Warren, N., and Sugden, B. (1985). Stable replication of plasmids derived from Epstein-Barr virus in various mammalian cells. Nature 313, 812-815.

Zhou, J., Zhang, S., Xie, L., Liu, P., Xie, F., Wu, J., Cao, J., and Ding, W.Q. (2012). Overexpression of DNA polymerase iota in esophageal squamous cell carcinoma. Cancer Sci

103, 1574-1579.

18. Extent of Clinical Activities Initiated and Completed. Items 18(A) and 18(B) should be completed for all research projects. If the project was restricted to secondary analysis of clinical data or data analysis of clinical research, then responses to 18(A) and 18(B) should be "No."

18(A) Did you initiate a study that involved the testing of treatment, prevention or diagnostic procedures on human subjects?

_____Yes
___X__No

18(B) Did you complete a study that involved the testing of treatment, prevention or diagnostic procedures on human subjects?

_____Yes
__X___No

If "Yes" to either 18(A) or 18(B), items 18(C) – (F) must also be completed. (Do NOT complete 18(C-F) if 18(A) and 18(B) are both "No.")

18(C) How many hospital and health care professionals were involved in the research project?

_____Number of hospital and health care professionals involved in the research project

18(D) How many subjects were included in the study compared to targeted goals?

_____Number of subjects originally targeted to be included in the study
_____Number of subjects enrolled in the study

Note: Studies that fall dramatically short on recruitment are encouraged to provide the details of their recruitment efforts in Item 17, Progress in Achieving Research Goals, Objectives and Aims. For example, the number of eligible subjects approached, the number that refused to participate and the reasons for refusal. Without this information it is difficult to discern whether eligibility criteria were too restrictive or the study simply did not appeal to subjects.

18(E) How many subjects were enrolled in the study by gender, ethnicity and race?

Gender:
_____Males
_____Females
_____Unknown

Ethnicity:
_____Latinos or Hispanics
_____Not Latinos or Hispanics
_____Unknown

Race:
_____American Indian or Alaska Native
_____Asian
_____Blacks or African American
_____Native Hawaiian or Other Pacific Islander
_____White
_____Other, specify:_____
_____Unknown

18(F) Where was the research study conducted? (List the county where the research study was conducted. If the treatment, prevention and diagnostic tests were offered in more than one county, list all of the counties where the research study was conducted.)

19. Human Embryonic Stem Cell Research. Item 19(A) should be completed for all research projects. If the research project involved human embryonic stem cells, items 19(B) and 19(C) must also be completed.

19(A) Did this project involve, in any capacity, human embryonic stem cells?
_____Yes
__X__ No

19(B) Were these stem cell lines NIH-approved lines that were derived outside of Pennsylvania?
_____Yes
_____No

19(C) Please describe how this project involved human embryonic stem cells:

20. Articles Submitted to Peer-Reviewed Publications.

20(A) Identify all publications that resulted from the research performed during the funding period and that have been submitted to peer-reviewed publications. Do not list journal abstracts or presentations at professional meetings; abstract and meeting presentations should be listed at the end of item 17. **Include only those publications that acknowledge the Pennsylvania Department of Health as a funding source** (as required in the grant agreement). List the title of the journal article, the authors, the name of the peer-reviewed publication, the month and year when it was submitted, and the status of publication (submitted for publication, accepted for publication or published.). Submit an electronic

copy of each publication or paper submitted for publication, listed in the table, in a PDF version 5.0.5 (or greater) format, 1,200 dpi. Filenames for each publication should include the number of the research project, the last name of the PI, and an abbreviated title of the publication. For example, if you submit two publications for Smith (PI for Project 01), one publication for Zhang (PI for Project 03), and one publication for Bates (PI for Project 04), the filenames would be:

Project 01 – Smith – Three cases of isolated
Project 01 – Smith – Investigation of NEB1 deletions
Project 03 – Zhang – Molecular profiling of aromatase
Project 04 – Bates – Neonatal intensive care

If the publication is not available electronically, provide 5 paper copies of the publication.

Note: The grant agreement requires that recipients acknowledge the Pennsylvania Department of Health funding in all publications. Please ensure that all publications listed acknowledge the Department of Health funding. If a publication does not acknowledge the funding from the Commonwealth, do not list the publication.

Title of Journal Article:	Authors:	Name of Peer-reviewed Publication:	Month and Year Submitted:	Publication Status (check appropriate box below):
1. Mature microsatellites: Mechanisms underlying dinucleotide mutational biases in human cells	Baptiste, B., G. Ananda, N. Strubczewski, A. Lutzkanin, S.J. Khoo, A. Srikanth, N. Kim, K.D. Makova, M.M. Krasilnikova, and K.A. Eckert	G3: Genes, Genomes, Genetics G3 vol. 3: pages 451-463.	October 2012	☐Submitted ☐Accepted X Published

20(B) Based on this project, are you planning to submit articles to peer-reviewed publications in the future?

Yes_____ No__X_____

If yes, please describe your plans:

21. Changes in Outcome, Impact and Effectiveness Attributable to the Research Project. Describe the outcome, impact, and effectiveness of the research project by summarizing its impact on the incidence of disease, death from disease, stage of disease at time of diagnosis, or other relevant measures of outcome, impact or effectiveness of the research project. If there were no changes, insert "None"; do not use "Not applicable." Responses must be single-spaced below, and no smaller than 12-point type. DO NOT DELETE THESE INSTRUCTIONS. There is no limit to the length of your response.

None.

22. Major Discoveries, New Drugs, and New Approaches for Prevention Diagnosis and Treatment. Describe major discoveries, new drugs, and new approaches for prevention, diagnosis and treatment that are attributable to the completed research project. If there were no major discoveries, drugs or approaches, insert "None"; do not use "Not applicable." Responses must be single-spaced below, and no smaller than 12-point type. DO NOT DELETE THESE INSTRUCTIONS. There is no limit to the length of your response.

None.

23. Inventions, Patents and Commercial Development Opportunities.

23(A) Were any inventions, which may be patentable or otherwise protectable under Title 35 of the United States Code, conceived or first actually reduced to practice in the performance of work under this health research grant? Yes_____ No___X_____

If "Yes" to 23(A), complete items a – g below for each invention. (Do NOT complete items a - g if 23(A) is "No.")

a. Title of Invention:

b. Name of Inventor(s):

c. Technical Description of Invention (describe nature, purpose, operation and physical, chemical, biological or electrical characteristics of the invention):

d. Was a patent filed for the invention conceived or first actually reduced to practice in the performance of work under this health research grant?
Yes_____ No____

If yes, indicate date patent was filed:

e. Was a patent issued for the invention conceived or first actually reduced to practice in the performance of work under this health research grant?
Yes_____ No____
If yes, indicate number of patent, title and date issued:
Patent number:
Title of patent:
Date issued:

f. Were any licenses granted for the patent obtained as a result of work performed under this health research grant? Yes_____ No____

If yes, how many licenses were granted?_____

g. Were any commercial development activities taken to develop the invention into a commercial product or service for manufacture or sale? Yes___ No____

If yes, describe the commercial development activities:

23(B) Based on the results of this project, are you planning to file for any licenses or patents, or undertake any commercial development opportunities in the future?

Yes_____ No___X_____

If yes, please describe your plans:

24. Key Investigator Qualifications. Briefly describe the education, research interests and experience and professional commitments of the Principal Investigator and all other key investigators. In place of narrative you may insert the NIH biosketch form here; however, please limit each biosketch to 1-2 pages.

BIOGRAPHICAL SKETCH

Provide the following information for the Senior/key personnel and other significant contributors.
Follow this format for each person. **DO NOT EXCEED FOUR PAGES.**

NAME Eckert, Kristin A.	POSITION TITLE Professor of Pathology
eRA COMMONS USER NAME (credential, e.g., agency login) KAEckert	

EDUCATION/TRAINING *(Begin with baccalaureate or other initial professional education, such as nursing, include postdoctoral training and residency training if applicable.)*

INSTITUTION AND LOCATION	DEGREE *(if applicable)*	MM/YY	FIELD OF STUDY
Pennsylvania State University, University Park, PA	B.S.	05/82	Microbiology
University of Wisconsin-Madison, Madison, WI	Ph.D.	12/88	Oncology
National Institute of Environmental Health Sci, RTP, NC	Postdoctoral	06/93	Molecular Genetics

A. Personal Statement

The experimental focus of my research career has been mechanisms of mutagenesis in somatic human cells, particularly in relation to neoplastic cell evolution. I have 24 years of basic research experience studying damage-induced mutagenesis, DNA repair, DNA polymerase fidelity, and repetitive DNA sequence biochemistry. My Ph.D. training focused on the relationship of chemical carcinogenesis, mutagenesis and tumor initiation. As a postdoctoral fellow, I studied DNA polymerase structure/function in relation to DNA polymerase error discrimination mechanisms. At the Penn State College of Medicine, my lab developed complementary *in vitro* and *ex vivo* approaches to study mutagenesis in human cells caused by endogenous DNA lesions (alkylation and oxidative lesions), using an HSV-tk gene-based assays. Beginning in 1995, the primary focus of my independent laboratory became mechanisms of microsatellite DNA mutagenesis. Microsatellite DNA instability is a well-known genetic signature found in colorectal cancer. We have used both nontumorigenic and tumorigenic human cell lines to elucidate the contributions of DNA polymerase fidelity and mismatch repair to microsatellite instability, within the colorectal cancer model. My research program includes an interdisciplinary, collaborative research project with Dr. Makova's computational group (Penn State University Park) to study microsatellite DNA evolution in the human genome. Recently, my laboratory embarked on the in-depth examination of replicative DNA polymerases and microsatellites present within common fragile sites (CFS), regions of the genome prone to chromosomal instability.

B. Positions

Positions and Employment

1993-1999 Assistant Professor, Departments of Pathology and Biochemistry and Molecular Biology, Pennsylvania State University College of Medicine, Hershey, PA
1999-2007 Associate Professor (with tenure), Penn State College of Medicine
2007-present Professor, Penn State College of Medicine
2006-present Member, Penn State Hershey Cancer Institute

Other Experience and Professional Memberships

2000-2008 Member, Genetics SSSY Study Section, NIH/CSR
2000-2006 Member, American Cancer Society CNE Study Section (Chair, 2006)
2006-2010 Member, Radiation Therapeutics and Biology Study Section, NIH/CSR

C. Selected Peer-reviewed Publications (Selected from over 50 peer-reviewed publications)

1. Sweasy, J.B., J.M. Lauper and **Eckert KA**. DNA Polymerases and human disease, *Rad Res* 2006; 166: 693-714.
2. Hile SE and **Eckert KA.** DNA Polymerase κ produces interrupted mutations and displays polar pausing within mononucleotide microsatellite sequences. *Nucleic Acids Res*. 2008; 36: 688-696
3. Shah SN, **Eckert KA**. Human postmeiotic segregation 2 exhibits biased repair at tetranucleotide microsatellite sequences. *Cancer Res*. 2009; 69(3):1143-9.
4. **Eckert KA**, Hile SE. Every microsatellite is different: Intrinsic DNA features dictate mutagenesis of common microsatellites present in the human genome. *Mol Carcinog*. 2009; 48(4):379-88.
5. Shah SN, Hile SE, **Eckert KA**. Defective mismatch repair, microsatellite mutation bias, and variability in clinical cancer phenotypes. *Cancer Res*. 2010; 70(2):431-5.
6. Shah SN, Opresko PL, Meng X, Lee MY, **Eckert KA**. DNA structure and the Werner protein modulate human DNA polymerase delta-dependent replication dynamics within the common fragile site FRA16D. *Nucleic Acids Res*. 2010; 38:1149-1162.
7. Damerla RR, Knickelbein K, Kepchia D, Jackson A, Armitage B, **Eckert KA**, and Opresko PL. Telomeric repeat mutagenicity in human somatic cells is modulated by repeat orientation and G-quadruplex stability, *DNA Repair* 2010; 9: 1119-1129.
8. Kelkar, YD, Strubczewski, N. Hile, SE, Chiaromonte, F**, Eckert KA***, and **Makova KD***. What is a microsatellite: A computational and experimental definition based upon repeat mutational behavior at A/T and GT/CA repeats, *Genome Biology and Evolution*. 2010; 2: 620-635. ***Co-corresponding authors**
9. Abdulovic, AL, Hile SE, Kunkel TA, and **Eckert KA**. The in vitro fidelity of yeast DNA polymerase δ and polymerase ε holoenzymes during dinucleotide microsatellite DNA synthesis, *DNA Repair* 2011; 10: 497-505.
10. Hile, SE, Wang XX, Lee MYWT, and **Eckert KA.** Beyond Translesion Synthesis: Polymerase κ Fidelity as a Potential Determinant of Microsatellite Stability, *Nucleic Acids Res.* 2012; 40: 1636-47.
11. Kelkar, YD., **Eckert KA**, Chiaromonte F, and Makova KD. A matter of life and death: How microsatellites emerge in and vanish from the human genome. *Genome Res.* 2011; 21: 2038-48.
12. Fungtammasan, A., Walsh E, Chiaromonte F*, **Eckert KA***, and **Makova** KD* A Genome-wide Analysis of Common Fragile Sites: What Features Determine Chromosomal Instability in the Human Genome? *Genome Research* 2012; **22**: 993-1005. ***Co-corresponding**
13. Baptiste, B. and **Eckert KA**. DNA Polymerase kappa microsatellite synthesis: two distinct mechanisms of slippage-mediated errors. *Environ. Mol. Mutagenesis* 2012; **53:** 787-796
14. Walsh, E., X. Wang, M. Lee and **K.A. Eckert**. Mechanism of replicative DNA polymerase δ pausing and a potential role for DNA polymerase κ in common fragile site replication. *J Mol Biol.* 2013. 425: 232-43.
15. Hile, S.E., S. Shabashev, and **K.A. Eckert**. Tumor-specific microsatellite instability: Do distinct mechanisms underlie the MSI-L and EMAST phenotypes? *Mutation Research - Fundamental and Molecular Mechanisms of Mutagenesis*, 2013; 743-744: 67-77.

NAME Beverly A. Baptiste	POSITION TITLE Graduate Student
eRA COMMONS USER NAME (credential, e.g., agency login)	

EDUCATION/TRAINING *(Begin with baccalaureate or other initial professional education, such as nursing, and include postdoctoral training.)*

INSTITUTION AND LOCATION	DEGREE *(if applicable)*	YEAR(s)	FIELD OF STUDY
San Francisco State University, CA	B.A.	1992	Political Science
Eastern New Mexico University, Portales, NM		1996-1998	Biology
Penn State College of Medicine, Hershey, PA	M.S.	2003	Cell and Molecular Biol.
Penn State College of Medicine, Hershey, PA	Ph.D.	2008-13	Genetics

A. Positions and Honors

Professional Experience

2003-2008 Adjunct Faculty, Department of Chemistry, Harrisburg Area Community College
2008-2013 Graduate Assistant, Pennsylvania State University College of Medicine

Honors and Awards

1998-1999 The Pennsylvania State University Graduate Fellowship
2010 Environmental Mutagen Society Travel Award
2010 Karl H. Beyer, Jr., MD/PhD Award, Penn State University College of Medicine

B. Publications and Presentations

Publications

1. L.K. Palmer, J.L. Shoemaker, **B.A. Baptiste**, D. Wolfe, and R.L. Keil, *Inhibition of translation initiation by volatile anesthetics involves nutrient-sensitive GCN-independent and -dependent processes in yeast.* Mol Biol Cell, 2005. **16**(8): p. 3727-39.
2. L.K. Palmer, **B.A. Baptiste**, J. Fester, J. Perkins, R.L. Keil, *RRD1, a component of the TORC1 signaling pathway affects anesthetic response in Saccharomyces cerevisiae.* Yeast, 2009, p. 655-661.
3. **B. A. Baptiste** and K. A. Eckert, *DNA polymerase kappa microsatellite synthesis: Two distinct mechanisms of slippage-mediated errors.* Environmental and Molecular Mutagenesis, 2012. **53** (9): 787-796.
4. **B. A. Baptiste**, Guruprasad Ananda, Noelle Strubczewski, Su Jen Khoo, Abhinaya Srikanth, Nari Kim, Kateryna D. Makova, Maria M. Krasilnikova, Kristin A. Eckert, *Mature Microsatellites: Mechanisms Underlying Long Dinucleotide Microsatellite Mutational Biases In Human Cells*. G3: Genes Genomes Genetics **3**, 451-463.

Conference Presentations

1. **B. A. Baptiste** and R. L. Keil, "Lights Out and Still aWAKe: Anesthetic Resistant Yeast," International Yeast Meeting, Seattle, WA, 7/25-7/30/2000.
2. L. K. Palmer, **B. A. Baptiste**, J. C. Fester, J. C. Perkins, and R. L. Keil, *"RRD1*, a Component of the TOR Signaling Pathway, Affects Anesthetic Response in *Saccharomyces cerevisiae,"* International Yeast Meeting, London, England, 7/21/09.
3. **B. A. Baptiste** and K. A. Eckert, "Y family polymerases: heroes or villains?" EMS Meeting, Fort Worth, TX, 11/2011.
4. **B. A. Baptiste** and K. A. Eckert, "The Battle of the Bulge: How DNA Polymerase Kappa Stabilizes Microsatellites," Gordon Research Conference on Mutagenesis, Newport, R.I., 8/19-24/2012