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14. ABSTRACT

The research covered under this award is designed to further study our original finding that the drug 5-azacytidine increases the responsiveness of prostate cancer cells and xenografts to radiation. The first aim of our research was to identify molecular targets of 5-azacytidine which mediate its radiation sensitizing effect. We originally hypothesized that 5-azacytidine likely suppresses DNA double-strand (DSB) break repair by modulating the non-homologous end-joining pathway, but found no evidence to support this hypothesis. We did identify two targets of 5-azacytidine, Rad50 and MAP2K6, which may suppress DSB repair by modulating homologous recombination and cell cycle arrest. Based on our latest data, however, we conclude that 5-azacytidine predominantly sensitizes prostate cancer cells to radiation by sensitizing the genomic DNA itself to DNA breakage, rather than by suppressing DSB repair. We are currently investigating whether this direct modulation of DNA integrity has an additive or synergistic combined effect with radiation. The second aim of our research was to verify the effects of a combined triple treatment regimen of 5-azacytidine, flutamide, and radiation on local control of xenografted human-derived prostate tumors. Complete local control was observed for a period of 25 days after an initial 5 day treatment block with a combination of 5-azacytidine, flutamide, and radiation. Although tumor growth was eventually restored, the total time needed for triplication of the tumor was 2x longer than observed for the control and 1.5x longer than observed for the group treated with radiation alone. It must be noted that an initial statistical analysis arbitrarily based on linear regression narrowly failed to show a statistical difference between the triple treatment group and the control group treated with radiation alone. However, we believe that the introduction of a second treatment block at day 20 will result in sufficiently prolonged local tumor control to show clear statistical significance. We are currently verifying this hypothesis.

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Introduction

As a component of our ongoing research on DNA double-strand break (DSB) repair mechanisms and their applicability to the improvement of radiation therapy, we have previously discovered a radiation-sensitizing effect of the epigenetic drug Vidaza (5-azacytidine) on the androgen-independent prostate cancer lines PC-3 and DU-145, as well as on PC-3 based xenograft tumors in mice. The project funded by this award is geared towards two main goals: 1) elucidation of the pathways involved in 5-azacytidine mediated radiation-sensitization of prostate cancer cells, and 2) examination of the effectiveness of combing 5-azacytidine with radiation and androgen ablation as a treatment for suppressing xenograft tumor growth.

Short background

It has been shown by other research groups that the drug 5-azacytidine – currently FDA approved for the treatment of myelodysplastic malignancies – restores responsiveness of androgen-insensitive prostate tumors to androgen ablation therapy, even at relatively low doses (1-4). This finding has triggered a strong and contemporary interest in the use of 5-azacytidine as a treatment modality for prostate cancers.

As a result of our own research efforts on DNA repair, we previously observed a statistically significant delay in the repair of radiation-induced DSBs upon treatment of prostate cancer cells with 5-azacytidine, as evidenced by an approximately 4-fold higher level of residual gamma-H2AX foci in 5-azacytidine treated cells versus untreated cells. In addition, we examined the proficiency of the main DSB repair pathway – NHEJ – by measuring the overall efficiency of artificially introduced DSB substrates, and found a 50-55% reduction upon treatment with 5-azacytidine. Finally, we observed a 30-plus day delay in progression of prostate cancer cells (PC-3) based xenograft tumors in nude mice upon dual exposure to radiation and 5-azacytidine. These combined finding, which form the theoretical basis for the project described in this report, strongly argued for a radiation-sensitizing potential of 5-azacytidine, at least in part mediated through the suppression of DSB repair via the NHEJ pathway.

The above discussed ability of 5-azacytidine to potentiate the effects of the two major treatment modalities for prostate cancers – radiation and androgen ablation – could theoretically be utilized to develop a novel treatment strategy for prostate cancer. The facts that 1) 5-azacytidine appears to display its potentiating effects at low concentrations and 2) is an already FDA approved compound, will drastically enhance the practicability of actually implementing a combined 5-azacytidine, radiation, and androgen ablation protocol in a clinical setting.

Summary of research accomplishments over the entire project duration

In order to prevent lengthy interruptions of the text, we have included most data in addenda, presented at the end of this report.

Specific aim 1: To understand the molecular basis for 5-azacytidine induced radio-sensitization.

In our original application for the award which funded this research, we presented a dataset showing that exposure of the prostate cancer cell lines PC-3 and DU-145 to a combined regimen of 5-azacytidine and radiation markedly delayed the clearance rate of gamma-H2AX foci as compared to controls which were exposed to radiation alone. The gamma-H2AX clearance rate is generally accepted as a standard measurement for repair efficiency of DNA double-strand breaks (DSBs). In addition, we demonstrated that repair of an artificial DNA substrate mimicking a DSB, was decreased 2-fold as a result of exposure to 5-azacytidine. Based on this evidence, we hypothesized that 5-azacytidine repressed DSB repair. Because the artificial DNA substrate we utilized can only be repaired through the Non-Homologous End-joining (NHEJ) pathway of DSB repair, we further hypothesized that 5-azacytidne represses DSB repair at least partially through impairment of NHEJ.

The nucleoside analog 5-azacytidne is incorporated by the cell's nucleotide metabolism into DNA as well as RNA. When incorporated into DNA, the drug is known to impair the activity of DNA-methyltransferases, which blocks promoter cytosine methylation and alters gene expression on an epigenetic level. In addition, 5-azacytidine incorporation into RNA species can interfere with transcription/translation. Therefore, 5-azacytidine can exert an effect on different levels and we set out to examine the effects of 5-azacytidine on known DSB repair factors on both an epigenetic and a transcriptional/translational level. In the past three years we have rigorously analyzed cytosine methylation status and steady state mRNA levels of common DNA repair genes. In addition, we have analyzed the effects of 5-azacytidine exposure on regulatory miRNA levels in prostate cancer cells. In the section below we will summarize results obtained and reported in the first two years, as well as hitherto unreported results which were obtained in the third, final year.

Effects of 5-azacytidine on epigenetic modulation of DNA repair genes.

As discussed in our previous reports, we first analyzed the effects of 5-azacytidine on cytosine methylation levels of a panel of 24 key DNA repair genes involved in Base Excision Repair, Nucleotide Excision Repair, Mismatch Repair, and DSB Repair, utilizing the SA Biosciences Human DNA Repair EpiTect Methyl qPCR Array MeAH-421C. This array quantifies the relative percentages of low-,

intermediate-, and hyper-methylated copies of each gene in a total genomic DNA extract loaded onto the array. We utilized total DNA from PC-3 cells treated with radiation alone as a base-line control and compared the control values to those obtained with total DNA from PC-3 cells treated with either 1 or 10 uM 5-azacytdine, plus or minus radiation (Appendix 1). Most genes in the panel displayed only minor responses to 5-azacytidine. However, very significant methylation changes were reproducibly detected in **Ligase 3** and **Rad50**. 5-Azacytidne exposure (10 uM) with concurrent radiation decreased the overall cytosine methylation status of Rad50 by increasing the percentage of un-methylated DNA from 47% to 92%, decreasing the percentage of intermediate-methylated DNA from 40 to 0%, and decreasing the percentage of hyper-methylated DNA from 13 to 8%. The overall cytosine methylation levels of the Ligase 3 gene, on the other hand, increased as a result of 5-azacytidne exposure. The percentage of un-methylated DNA decreased from 97% to 65%, the level of intermediate-methylated DNA remained unaltered at 0%, and the levels of hyper-methylated DNA increased from 3% to 35%.

Ligase 3 is a gene encoding for a key protein in the process of Nucleotide Excision Repair (NER), which mediates the repair of mainly U-induced lesions like pyrimidine dimers. This process is typically not involved in the repair of DSBs introduced by ionizing radiation and therefore the effects of 5-azacytidine on this gene are not likely to mediated increased responsiveness of prostate cells to radiation. It must be noted that the effect of 5-azacytidine on ligase 3 is somewhat remarkable because 5-azacytidine is thought to generally suppress DNA methyltransferases, thereby decreasing, rather than increasing cytosine methylation.

The effects of 5-azacytidine on Rad50, however, are quite relevant to our research. Rad50 is an integral component of DSB repair through Homologous Recombination (HR). The Rad50 protein forms a complex with Mre11 and Nbs1, which is called the MRN complex. The MRN complex binds to DNA termini and is currently thought to be involved in resection of the DNA ends prior to HR-mediated repair. Although controversial, several authors have suggested a role for MRN in NHEJ-mediated repair as well, in which pathway MRN would function to tether DNA termini in lieu of repair. Clearly, the Rad50 gene is of great importance for DSB repair, at least through HR and potentially via NHEJ as well. Therefore, the altered cytosine methylation status of Rad50 might explain (at least in part) the effects of 5-azacytidine on overall DSB repair in PC-3 prostate cancer cells.

At present, however, it is unclear how Rad50 de-methylation would work to suppress DSB repair and additional investigation into this subject is warranted. In addition, it must be noted that the effects of low doses of 5-azacytidine on Rad50 (1 uM instead of 10 uM) appeared to have an opposite effect, increasing the overall methylation status of Rad50, by decreasing the levels of un-methylated DNA (47% to 19%), increasing the levels of intermediate-methylated DNA (from 0% to 29%), and decreasing the levels of hyper-methylated DNA (13% to 9%). Clearly, the effects of 5-azacytidine on Rad50 methylation are dose-dependent and a clear understanding of this effect will require elaborate dose-

titrations. However, **our observation that Rad50 cytosine methylation is significantly altered by 5-azacytidine certainly identifies this gene as a potential mediator for 5-azacytidine-induced DSB repair suppression**.

Next, we focused our attention on studying the effects of 5-azacytidine on promoter cytosine methylation levels of known NHEJ genes. These experiments were completed in the third year and we here discuss these results for the first time. In this analysis we included the general DSB recognizing PI-3 kinases ATM and ATR, as well as all known NHEJ core enzymes (Ku70, KU80, DNA-PK_{CS}, XRCC4, Ligase IV, and XLF) and the HR core genes Rad 51 and Rad 52. For this experiment we treated 4 different prostate cell lines (PC-3, DU-145, LNCaP, and the control line PCS-440-010) with 6 different modalities: 1) control, 2) 4 Gy radiation, 3) 1 uM 5-azacytidine, 4) 10 uM 5-azacytidine, 5) 1 uM 5-azacytidine + 4 Gy radiation, 6) 10 uM 5-azacytidine + 4 Gy radiation. Cell cultures treated with 5-azacytidine received a fresh dose of 5-azacytidine every day, for a period of 3 days. Radiation was delivered by means of a Cobalt-60 teletherapy unit on the third day of the procedure. Triplicate biological repeats of each treatment group were performed. Genomic DNA was extracted from the cell cultures 1-2 hrs post irradiation. Genomic DNA of biological repeats were pooled in equimolar amounts into one sample. Therefore, we obtained 6 samples (6 treatment groups) of each cell line, totaling 24 samples.

Analysis was performed by state of the art massARRAY technology. Although slightly different in design than the originally proposed pyrosequencing technique, this methodology is superior in sensitivity and accuracy and recently became available at the University of Arizona Cancer Center, thereby providing us with a unique and cost-effective opportunity to accommodate our research needs. In short, sodium bisulfite-treated genomic DNA was prepared according to the standard protocol of the Zymo Research company. Sodium bisulfite-treated DNA (5 ng) was seeded into a region-specific PCR incorporating a T7 RNA polymerase sequence as described by the manufacturer (Sequenom). Primer sequences for the above mentioned genes were designed using EpiDesigner 6. The resultant PCR product was then subjected to in vitro transcription and RNase A cleavage using the MassCLEAVE T-only kit, spotted onto a Spectro CHIP array, and analyzed using the MassARRAY Compact System matrix-assisted laser desorption/ionization-time-of-flight mass spectrometer (Sequenom). Each sodium bisulfite-treated DNA sample was processed in two independent experiments. Data were analyzed using Sequonom's EpiTyper software. A summary of the results for each individual gene is presented in Appendix 2.

The data presented in Appendix 2 show that promoter cytosine methylation levels of ATR, XLF, Ku70, Ligase IV, Rad 51, and Rad52 were uniformly low and were not significantly affected by 5-azacytidine or 5-azacytidine with combined radiation treatment. The genes XRCC4, Ku80, DNA-PK_{CS},

and ATM had higher overall promoter cytosine methylation levels. Apparent fluctuation in methylation levels are therefore easier to detect, but do not necessarily reflect a true biological effect. For instance, the XRCC4 gene displayed a decrease in methylation levels in the LNCaP cells treated with 10 uM 5-azacytidine and no radiation, as well as in LNCaP cells treated with 1 uM 5-azacytidine plus radiation. However, methylation levels of XRCC4 in LNCaP cells showed no change in the corresponding treatment groups 1 uM 5-azacytidine and 10 uM 5-azacytidine plus radiation. Moreover, this effect was not observed in the other cell lines DU-145, PC-3, and PCS-440-010. Therefore, it appears that these modulations represent in vitro artefacts rather than true effects. The same can be said for variations in methylation levels of the Ku80, DNA-PK_{CS}, and ATM genes. None of these variations can be attributed to a certain dose or treatment group and they appear to be inconsistent between cell lines as well. Therefore, we were not able to demonstrate a consistent, reproducible, dose-dependent or treatment-dependent effect of 5-azacytidine on the cytosine methylation levels of the examined genes.

Based on the combined results of the Methyl qPCR Array and massARRAY experiments discussed above, we here report that we did not find evidence for a direct effect of 5-azacytidine on promoter cytosine methylation levels of the known NHEJ genes (Ku70, Ku80, DNA-PK_{CS}, XRCC4, Ligase IV, and XLF), the upstream regulators ATM and ATR, as well as the HR genes Rad51 and Rad52. We did, however, find evidence that 5-azacytidine targets cytosine methylation levels of the Rad50 gene, which is a key factor in HR-mediated DSB repair. Based on these results, we **conclude that 5-azacytidine does not have a direct effect on cytosine methylation of NHEJ genes, but does influence the cytosine methylation status of the HR gene Rad50.**

Effects of 5-azacytidine on steady state mRNA levels of DNA repair genes.

In order to test the effects of 5-azacytidine on expression levels of DNA repair genes, we have conducted several investigations into the steady state mRNA levels of common general DNA repair genes and all of the genes involved in NHEJ-mediated DSB repair. Some of these results have been described in experimental detail in our previous reports. First, we analyzed steady state mRNA levels of DNA repair genes, utilizing an SA Biosciences RT^2 profiler Human DNA Damage Signaling Pathway PCR array. In order to accomplish this, PC-3 prostate cancer cells were either mock treated, treated with radiation alone, treated with either 1 uM or 10 uM 5-azacytidine alone , or with 1 uM or 10 uM 5-azacytidine with concurrent radiation. Total RNA extracts of these treated cells were converted to cDNA and loaded on the PCR array. The calculated fold up- or down-regulation of each examined gene is presented in Appendix 3.

Markedly altered steady state mRNA levels were noticed for DDIT3 (up), ERCC1 (down), IP6K (up), and MAP2K6 (down). The up-regulation of DDIT3 (DNA Damage Inducible Transcript 3) is not very surprising, since this gene of relative unknown function responds primarily to DNA damage induction. A direct involvement in DSB break repair, however, is unlikely based on current knowledge. ERCC1 (Excision Repair Cross Complementary group 1) is involved in canonical Nucleotide Excision Repair, which mediates repair of UV-induced damages. The involvement of this gene in repair of ionizing-radiation-induced DSBs is unlikely. **MAP2K6** and **IP6K3** are both protein kinases. IP6K3 is a member of the IP-3 like kinases, like ATM and ATR. Both ATM and ATR are canonical upstream regulators of DSB repair, signaling the onset of a DSB event to the repair pathways. IP6K3 has never been identified as an upstream DSB repair regulator, but because it belongs to the same family as ATM and ATR it is theoretically possible that the protein plays a redundant role in DSB repair signal transduction. MAP2K6 facilitates cell cycle arrest by activating p38. Cell cycle arrest is an important component of DSB repair, since the halt of cell cycle progression allows the cell to perform proper repair before initiation of mitosis. Consequently, **the 5-azacytidine-mediated down-regulation of MAP2K6 may increase responsiveness of cells to ionizing-radiation-induced DSBs**. It is therefore possible that MAP2K6 is a target of 5-azacytidine which can, at least in part, explain the increased sensitivity of 5-azacytidine-treated prostate cancer cells to ionizing radiation.

Next, we analyzed the steady state mRNA levels of the known NHEJ genes Ku70, Ku80, DNA-PK_{CS}, XRCC4, Ligase IV, and XLF. This was done in PC-3 cells by means of standard RT-PCR technology. In order to accomplish this, PC-3 prostate cancer cells were either mock treated, treated with radiation alone, treated with either 1 uM or 10 uM 5-azacytidine alone , or with 1 uM or 10 uM 5-azacytidine with concurrent radiation. Total RNA extracts of these treated cells were converted to cDNA and RT-PCR was performed utilizing primers designed to recognize the above mentioned NHEJ genes. Data values (Cycle Threshold [Ct] values) were calculated from each assay with the SDS v2.0 software tool (Applied Biosystems). Expression fold changes from the mock treated control were calculated using the $\Delta\Delta$CT method and presented in Appendix 4. Surprisingly, none of the NHEJ core genes were down-regulated by 5-azacytidine. Rather, a mild up-regulation (although markedly below one order of magnitude) can be observed. Although at this point we do not have an unambiguous explanation for this finding, the data clearly does **not support regulation of NHEJ genes by 5-azacytidine on a transcriptional level.**

In the third year, we conducted a genome-wide analysis of the effects of 5-azacytidine on transcription levels of individual genes. We therefore utilized the GeneChip Human Genome ST 1.0 Array from

Affymetrix, which allows for analysis of over 30,000 coding RNA transcripts. We hypothesized that utilizing this broad approach could potentially identify 5-azacytidine-targeted genes that were not investigated in our previous experiments. We here discuss the results of this approach for the first time.

For this experiment we treated PC-3 cells with 4 different modalities: 1) control, 2) 2 Gy radiation, 3) 10 uM 5-azacytidine, 4) 10 uM 5-azacytidine + 2 Gy radiation. Cell cultures treated with 5-azacytidine received a fresh dose of 5-azacytidine every day, for a period of 3 days. Radiation was delivered by means of a Cobalt-60 teletherapy unit on the third day of the procedure. Triplicate biological repeats of each treatment group were performed. Total RNA was extracted from the cell cultures 1-2 hrs post irradiation. Total RNA of biological repeats were pooled in equimolar amounts into one sample. Total RNA was processed according to the protocol recommended by Affymetrix (Santa Clara, CA). In all, 20 µg of labeled cRNA from each sample was hybridized to the arrays. Microarray hybridizations of untreated control and drug or x-ray treated cells were performed in triplicate. The gene expression data were analyzed using TAC software for data analysis following the manufacturer's recommended filters and settings (Affymetrix freeware).

We subsequently compared expression levels of individual genes between the treatment modality groups and generated lists of differentially expressed genes with an adjusted p value <0.05. Only genes with a difference in expression larger than 2-fold linear were included in these lists. A total of 33,297 transcripts were analyzed and 296 genes in total were found to be differentially expressed between all treatment groups:

Group 1	Group 2	Up-regulated	Down-regulated
0 Gy, 0 uM 5-aza	0 Gy, 10 uM 5-aza	87	40
0 Gy, 0 uM 5-aza	2Gy, 0 uM 5-aza	10	3
0 Gy, 0 uM 5-aza	2 Gy, 10 uM 5-aza	68	47
0 Gy, 10 uM 5-aza	2 Gy, 0 uM 5-aza	62	77
0 Gy, 10 uM 5-aza	2 Gy, 10 uM 5-aza	5	17
2Gy, 0 uM 5-aza	2Gy, 10 uM 5-aza	57	87

These numbers indicate that 2 Gy radiation exposure resulted in altered levels of only a handful transcripts, most of them currently of unknown functionality. Exposure to 10 uM of 5-azacytidine, however, did alter expression of several dozens of genes. Appendix 5 contains a list of genes which were differentially expressed between the 2Gy, 0 uM 5-azacytidine and the 2Gy, 10 uM 5-azacytidine groups (digital files of the other 5 comparative lists, as well as heatmaps are available upon request but not included for space considerations). This list is the most important to consider, because it present the genes which were influenced by 5-azacytidine in the context of radiation exposure and the resultant

DSB damage. Not surprisingly, most genes identified were cell cycle regulators and transcription factors. Much more surprisingly, however, is the absence of any canonical DNA repair factor from the list of transcripts with altered expression levels. The only DNA repair factor identified was DDB2 (DNA Damage specific Binding protein 2), which was up-regulated a modest 2.5 times. Some authors have hypothesized DDB2 to play a role in repair of UV-induced DNA damages. However, this factor is unlikely to play a role in the repair of DSBs. None of the known DSB repair genes - either NHEJ factors or HR factors – or the upstream regulating PI-3 kinases were identified as modulated by 5-azacytidine.

Based on the above discussed experiments on steady state mRNA levels and RNA transcript levels, we here conclude that **we found no evidence for a direct effect of 5-azacytidine on transcription of any of the NHEJ and HR genes**. It must be noted that the absence of a positive identification of Rad50 in these experiments does not completely fit in with our finding that Rad50 cytosine methylation levels are affected by 5-azacytidine, since a modulation of cytosine methylation is expected to also alter expression levels of the RNA transcript. In addition, we expected that we would find MAP2K6 in the list of modulated gene expressions as well, since we had previously identified this target utilizing the SA Biosciences PCR array. However, this did not appear to be the case. The cause of this discrepancy is as of yet unknown.

Effects of 5-azacytidine on expression levels of regulatory microRNA species.

Since 5-azacytidine incorporates into all RNA species, it is possible that the effects of 5-azacytidine on radiation-sensitivity of prostate cancer cells are mediated through regulatory miRNA's, rather than through modulation of mRNA levels. In order to test this hypothesis we isolated total RNA from PC-3 cells exposed to 5-uM 5-azacytidine. miRNA levels were analyzed utilizing the Sanger13 Human MicroRNA Array, which provides a very comprehensive quantification of 369 human miRNA probes. Rather surprisingly, 5-azacytidine appeared to target only a few specific miRNA species. Out of 369 probes, only 3 miRNA species were found to be modulated over a factor of 3-fold: These were miR-155 (20.5 fold up-regulated), miR-200c (4.1 fold up-regulated), and miR-27a (3.2 fold up-regulated). Appendix 6 presents the 14 probes with the lowest FDR (False Discovery Rate).

MiRNA's usually function as post-translational regulators that repress mRNA translation. Although the function of most miRNA species is ill understood, there exists a clearly established link between miR-155 and immune cell development and the humoral immune response. Because NHEJ is not only a critical pathway for the repair of radiation-induced DSBs, but also for V(D)J recombination during immune development, it is tempting to speculate that there may be a link between miR-155 and NHEJ efficiency. Although this hypothesis clearly needs a more direct means of verification (including direct

measurement of NHEJ in miR-155 deficient cells, which is outside the scope of this project), our data suggests that 5-azacytidine may, in part, regulate NHEJ efficiency through modulation of Mir-155.

An alternative hypothesis: direct effects of 5-azacytidine on DNA breakage.

Our original hypothesis assumed a direct effect of 5-azacytidine on DSB repair pathways, most likely the NHEJ pathway, as a mediator of the increased radiation-sensitivity observed in 5-azacytidine-treated prostate cancer cells and xenografts. As described in the sections above, we conducted extensive experiments to methodically verify this hypothesis. However, surprisingly few canonical DSB repair related genes were found to be influenced by 5-azacytidine. Only Rad50 and MAP2K6 were identified as possible targets and no NHEJ genes appeared to be responsive to 5-azacytidine exposure. This lack of actionable targets urged us to revise our original hypothesis. It appeared to us that the incorporation of 5-azacytidine into the genomic DNA of the cells could theoretically increase the sensitivity of the DNA molecule to radiation insult. Such a direct effect on DSB induction could explain the radiation-sensitizing effect of 5-azacytidine just as well as a more indirect effect on DSB repair efficiency, as we previously hypothesized to be responsible.

In order to verify our new hypothesis we exposed mock-treated and 5-azacytidine-treated PC-3 cells to increasing doses of ionizing radiation. Cells were treated with 0 or 10 uM 5-azacytidine for 3 consecutive days, with radiation treatment occurring on the third day, utilizing a Cobalt-60 teletherapy unit. Total DNA was then extracted from the cells and submitted to Pulsed Field Gel electrophoresis (PFGE). Genomic DNA was embedded in agarose plugs, which were subsequently incubated overnight in lysis buffer (0.5M EDTA, 1% N-Lauryl-Sarcosine) supplemented with Proteinase K (20µg/ml) at 50C. Plugs were then transferred to 0.5 M EDTA (pH 8.0) and stored at 48C until electrophoresis. Prior to electrophoresis, the plugs were dialyzed in 0.5X TBE buffer and loaded into the wells of a 1% agarose gel. The gel was then placed into the electrophoresis chamber of a CHEF DR II (Bio-Rad) apparatus and separation of chromosome fragments took place at pulse intervals of 1 s at 6 V for 20 hrs, then 50 s at 6 V for 20 hrs. Utilizing this methodology, small DNA fragments resulting from accumulated DSBs were separated from undamaged genomic DNA. The total intensity of fragmented DNA bands was measured and plotted as a function of radiation dose in Appendix 7.

As can be seen in Appendix 7, cells exposed to 5-azacytidine and concurrent radiation accrued more fragmented DNA than cells exposed to radiation alone. This result clearly indicates that **more DSBs are introduced by radiation in 5-azacytidine exposed cells than in mock-treated cells**. It is therefore reasonable to conclude that 5-azcytidine sensitizes the DNA molecule to radiation, likely by weakening the DNA structure through direct incorporation. This latter statement will obviously need further experimental verification. Because PFGE is a relatively insensitive methodology, there must be

a substantial difference in the absolute number of generated DSBs between 5-azacytidine-treated and mock-treated cells and it is therefore **likely that the direct sensitization of DNA molecules to radiation will have a greater effect on radiation-sensitization by 5-azacytidine than actual suppression of DSB repair**. We are currently verifying whether the effects of 5-azacytidine on DSB induction are additive to or synergistic with radiation. In addition, we are verifying whether the direct incorporation of 5-azacytidne into genomic DNA only increases the actual induction rate of DSBs or also affects the repair rate of these DSB.

Conclusions for aim 1: molecular targets of 5-azacytidine.

- Contrary to our original hypothesis, we have not found any evidence for an effect of 5-azacytidine on any of the NHEJ enzymes involved in DSB repair, neither on an epigenetic, nor on a transcriptional level. Consequently, it does not seem likely that 5-azacytidne exerts a radiation-sensitizing effect on prostate cancer cells by modulating NHEJ-mediated DSB repair.
- Although not altogether unequivocal, we have identified Rad50 and MAP2K6 as potential targets for 5-azacytidine. High doses of 5-azacytidine (10 uM) reduced the cytosine methylation levels of the HR gene Rad50 (but not the steady state mRNA levels). Steady state mRNA levels of the cell-cycle-regulating protein kinase MAP2K6 were down-regulated by 5-azacytidine. We therefore conclude that 5-azacytidine may increase radiation sensitivity in prostate cancer cells by modulating HR-mediated DSB repair through Rad50 and by suppressing cell cycle arrest through MAP2K6.
- Due to the surprising lack of DSB repair related targets for 5-azacytidine, we have investigated the alternative hypothesis that 5-azacytidine incorporation sensitizes the genomic DNA to ionizing radiation. We have recently found evidence to support this hypothesis and we conclude that 5-azacytidine is likely to sensitize prostate cancer cells to radiation by increasing the likelihood of DSB occurrence. This direct effect of 5-azacytidne on DNA breakage may be stronger than any effect of 5-azacytidine on actual DNA repair we have observed during the course of our research. We are currently investigating whether this effect is additive or synergistic and whether it has an effect on DSB repair efficiency as well.

Specific aim 2: To investigate the influence of a combined regimen of 5-azacytidine, radiation, and androgen ablation on tumor control.

We had previously demonstrated that 5-azacytidine sensitizes not only prostate cancer cell lines, but also PC-3 based xenografts to radiation treatment. These xenograft tumors were obtained by injecting PC-3 cells in the flanks of male athymic nude mice, which generated growing tumors based on human-derived prostate cancer cells. Mice with developing tumors were subsequently treated with ip injection of 5-azacytidine (2.5 mg/kg/day for 5 consecutive days) and localized irradiation (2.5 Gy/day X-rays for 5 consecutive days). As can be seen in Appendix 8, treatment with radiation alone delayed the growth of tumors for approximately 40 days, whereas untreated or 5-azacytidine-treated tumors grew in an almost linear fashion in time. Combining 5-azacytidine with radiation resulted in additional retardation of tumor growth past the 40 day delay observed with radiation alone. Although eventual relapse of tumor growth was observed, the growth rate was markedly delayed as compared to tumors treated with radiation alone. Based on these preliminary data, we concluded that 5-azacytidine increases sensitivity of prostate cancer xenografts to radiation treatment. Because 5-azacytidine has been reported to enhance sensitivity of prostate tumors to androgen ablation therapy as well, we speculated that combining 5-azcytidine with both radiation and androgen ablation might impair or delay tumor growth in a synergistic fashion. The purpose of aim 2 of this project is to verify the results of our preliminary data on PC-3 xenografts, which were based on a relatively small sample size of 6 mice per treatment group, and to investigate the influence of androgen ablation by flutamide administration on combined 5-azacytidine/radiation treatment.

As described in our previous report, we tested the influence of a combined 5-azacytidine, flutamide, and radiation regimen on PC-3 xenograft tumor growth by injecting 5 groups of male athymic nude NCr Nu/Nu mice (16 animals per group) with approximately 4.10^6 PC-3 cells. No matrigel was used for this inoculation. The injections resulted in formation of PC-3 based xenografts on the flank of the animals. When most of the xenograft tumors reached an average volume of 500 mm3 at the 25^{th} day post injection, the 5 groups were treated as follows (see figure 1): (1) control group, (2) 5-azacytidine treatment alone, (3) flutamide treatment alone, (4) radiation treatment alone, (5) combination treatment with 5-azacytidine, flutamide, and radiation.

Flutamide treatment, when applicable, consisted of subcutaneous implantation of a commercially available slow-release pellet (Innovative Research of America, Cat # SA-152 25 mg/pellet 60 day release) resulting in a 50 mg/kg accumulative dose over 60 days. This pellet was implanted 2 days before the start of 5-azacytidine and radiation treatments. Groups that did not receive flutamide treatment received an inert placebo pellet (Innovative Research of America Cat# SC-111) instead. 5-

Azacytidine) was administered by intravenous injection following a 2.5 mg/kg/day dosing schedule for 5 consecutive days. Animals not treated with 5-azacytidine received a placebo injection consisting of saline. Radiation treatment, when applicable, was delivered with a Cobalt-60 teletherapy unit and given concurrent with 5-azacytidine (or saline) administration, following a delivery schedule of 2.5 Gy/day for 5 consecutive days. In order to deliver radiation dose localized to the tumor, animals were placed within a custom made shielding tube, which allowed protrusion (and therefore irradiation) of the xenograft tumor. Tumor volumes were recorded during the treatment period and during a 45 day period after treatment, in which no further treatment of any kind was rendered. The results are plotted in Appendix 9.

As expected, administration of 5-azacytidine or flutamide as single modalities did not result in significant deviation of the tumor growth characteristics from the control group, indicating that neither modality by itself was successful in controlling tumor progression. Localized delivery of radiation to the tumors did result in modest reduction of tumor volumes, but tumor progression was never halted and therefore tumor control was never obtained with radiation delivery as a single modality under the chosen conditions. This result is somewhat dissimilar from our original data (Appendix 8), which indicated a sustained reduction tumor growth by radiation alone for up to 40 days. At present, we have no unequivocal explanation for this discrepancy. It is possible that the larger sample size and different source of radiation (gamma-radiation, instead of X-rays), might partially the dissimilar shape of graph obtained with radiation alone. It should also be noted, that animals in the group treated with radiation alone were euthanized at around day 30, because the tumor burdens started to exceed humane limits. Therefore, the graph for the radiation treated group reaches an artificial plateau, just like graphs for the control, 5-azacytidine, and flutamide treated groups. This does not indicate that radiation treatment ceased to reduce tumor growth rates after the 30 day mark. For this reason, our data are best interpreted before the 30 days point.

Importantly, we **observed complete control of tumor volume progression in the first 25 days post treatment in the triple modality group, treated with a combination of flutamide, 5-azacytidine, and radiation**. During this period, the median tumor volume did not appear to markedly increase beyond the initial 500 mm^3 volume that was present pre-treatment, in sharp contrast to the control and single modality groups in which the tumor burden rapidly increased during this period. After the 25 day period of complete tumor control, a relapse in tumor control was observed and tumor volumes started increasing again. However, it did take an additional 20 days past the relapse point for tumors to reach the 1750 mm3 cut-off mark where the animals were euthanized. Based on these observation we conclude that 1) complete control of PC-3 based xenograft tumors can be achieved during a period of approximately a month after an initial administration of a triple modality treatment consisting of 5-azacytidine, flutamide, and radiation treatment, and that 2) the **time needed for a**

triplication of tumor volume is almost doubled as a result of a single administration of this triple modality.

The results presented in Appendix 9 are very interesting and appear to indicate that a combination treatment of 5-azacytidine, flutamide, and radiation is more effective than any of the single modalities. In the third year, we have requested the assistance of our Biometrics core facility to perform a statistical analysis of the data set presented in Appendix 9. We here discuss these results for the first time. In order to compare the graphs of each treatment group, the cube root of the mean tumor burden was plotted and the data points were subsequently fitted with linear regression. The slopes of these linear fitted curves were then compared for significant differences. Because the graphs for all treatment modalities ultimately merge as a result of an artificial plateau (euthanasia of mice due to tumor burden), we requested to focus the analysis on the first weeks post treatment, where the difference between the triple treatment and single treatment modalities appears to be maximal. The choice was made by the biometrics core to focus on the period between days 0 and 17. The analysis report is included as Appendix 10. The slope of the triple modality treatment group was found to be significantly different than the slope of the control, 5-azacytidine, and flutamide treatment groups. However, with the chosen analysis method, the **difference between the slope of the triple treatment group and slope of the radiation treatment group narrowly escaped significance**. We explain this lack of calculated significance by the unusual large error bars at day 14 and day 17 in the radiation treated graph, which markedly skew the p-values for the difference.

Clearly this issue will have to be resolved before we can reach a final conclusion regarding the significance of the difference in tumor control between the triple treatment and the radiation treatment groups. **The experiment will have to be repeated with several modifications.** It is highly likely that the introduction of a second treatment event around day 20 will further increase the difference between the radiation treatment group and the triple modality treatment group. This will make it possible for statistical analysis to be based on more data points. In addition, in order to calculate for synergism in the event of a statistically significant difference between the triple treatment group and the single treatment groups, we will also need to measure tumor progression rates for double modality treatments, rather than just single modality treatments. We therefore set out to repeat the experiment discussed above and presented in Appendix 9. The basic set up was identical, with the following modifications: 1) a second radiation and/or 5-azacytidine treatment event will commence at day 20, consisting of 2.5 mg/kg/day 5-azacytidine injection for 5 consecutive days and/or 2.5 Gy/day radiation for 5 consecutive days, and 2) a total of 8 treatment groups will now be included: a) control, b) radiation

alone, c) flutamide alone, d) 5-azacytidine alone, e) radiation and flutamide, f) radiation and 5-azacytidine, g) flutamide and 5-azacytidine, h) radiation, 5-azacytidine, and flutamide.

In the third year, this experiment was initiated but had to be terminated prematurely due to the unfortunate circumstance that a large number of animals started to experience necrosis of the xenograft tumor tissue. We never experienced this phenomenon before and have attributed it to an unknown infection of the PC-3 culture that was utilized to initiate the tumors. For humane reasons, the animals experiencing necrosis were euthanized and the number of remaining animals was insufficient to allow for an acceptable statistical power. We therefore terminated the experiment altogether and started again. This repeat experiment is progressing without problems, but is unfortunately not finished yet. We could therefore not include it in this report. Upon request, we will be happy to submit an addendum with the results of the experiment when completed.

We initially planned on investigating whether 5-azacytidine has an effect on the metastatic potential of prostate cancer tissue. In order to pursue this aim, we have generated several cell lines based on the mouse prostate tumor line TRAMP-C2, which stably express GFP, thereby allowing for detection by fluorescence microscopy. The premise of the experiment is to generate subcutaneous tumors based on one of these TRAMP-GFP cell lines, by injecting mice with these cells as described above. Metastases derived from these tumors can then be followed throughout the body based on its fluorescent signature. In order to test the capability of the TRAMP-GFP cells to form tumors and to compare the results to the original TRAMP-C2 line, we conducted a pilot experiment in which we injected either the original TRAMP-C2 cells or the fastest proliferating clone of the TRAMP-GFP cells in the flanks of 5 C57BL/6J mice. Tumor progression was monitored for 80 days and presented in Appendix 11.

The overall rate of tumor formation by TRAMP-C2 cells was significantly slower than observed for PC-3 cells. However, all mice injected with the original TRAMP-C2 cells eventually developed tumors. Only 3 out 5 mice injected with the TRAMP-GFP cells developed tumors, which equals a tumor formation percentage of 60%. In order to increase this rather low percentage, we have obtained cells from the fastest developing TRAMP-GFP tumor (see Appendix 11), which we will use as the basis for subsequent inoculations.

Because at present it is still somewhat unclear whether the local tumor control obtained by a combination therapy of 5-azacytidine, flutamide, and radiation is significantly improved over the local tumor control obtained by radiation alone, we have postponed the execution of this experiment on metastasis until we can draw clear conclusions on the effects of 5-azacytidine on a local level. If the repeat experiment on local control of PC-3 based tumors reveals a significant difference, we will analyze the effects of 5-azacytidine on metastatic spread as well.

Conclusions for aim 2: effects of a combined treatment of 5-azacytidne, flutamide, and radiation.

- Complete local control of PC-3 based xenograft tumors in mice was observed for a period of 25 days after an initial 5 day treatment block with a combination of 5-azacytidne, flutamide, and radiation. Although tumor growth was eventually restored, the total time needed for triplication of the tumor was 2x longer than observed for the control and 1.5x longer than observed for the group treated with radiation alone.
- An initial statistical analysis based on linear regression of the cube root of the tumor burden, followed by comparison of the slopes of the regression lines narrowly failed to show a statistical difference between the groups treated with radiation alone and treated with a combination of 5-azacytidine, flutamide, and radiation. However, we believe that the introduction of a second treatment block at day 20 will result in sufficiently prolonged local tumor control to show clear statistical significance. We are currently verifying this hypothesis.

Key Research Accomplishments

- Contrary to our original hypothesis, we have not found any evidence for an effect of 5-azacytidine on any of the NHEJ enzymes involved in DSB repair, neither on an epigenetic, nor on a transcriptional level. Consequently, it does not seem likely that 5-azacytidne exerts a radiation-sensitizing effect on prostate cancer cells by modulating NHEJ-mediated DSB repair.
- Although not altogether unequivocal, we have identified Rad50 and MAP2K6 as potential targets for 5-azacytidine. High doses of 5-azacytidine (10 uM) reduced the cytosine methylation levels of the HR gene Rad50 (but not the steady state mRNA levels). Steady state mRNA levels of the cell-cycle-regulating protein kinase MAP2K6 were down-regulated by 5-azacytidine. We therefore conclude that 5-azacytidine may increase radiation sensitivity in prostate cancer cells by modulating HR-mediated DSB repair through Rad50 and by suppressing cell cycle arrest through MAP2K6.
- Due to the surprising lack of DSB repair related targets for 5-azacytidine, we have investigated the alternative hypothesis that 5-azacytidine incorporation sensitizes the genomic DNA to ionizing radiation. We have recently found evidence to support this hypothesis and we conclude that 5-azacytidine is likely to sensitize prostate cancer cells to radiation by increasing the likelihood of DSB occurrence. This direct effect of 5-azacytidne on DNA breakage may be stronger than any effect of 5-azacytidine on actual DNA repair we have observed during the

course of our research. We are currently investigating whether this effect is additive or synergistic and whether it has an effect on DSB repair efficiency as well.

- Complete local control of PC-3 based xenograft tumors in mice was observed for a period of 25 days after an initial 5 day treatment block with a combination of 5-azacytidine, flutamide, and radiation. Although tumor growth was eventually restored, the total time needed for triplication of the tumor was 2x longer than observed for the control and 1.5x longer than observed for the group treated with radiation alone.
- An initial statistical analysis based on linear regression of the cube root of the tumor burden, followed by comparison of the slopes of the regression lines narrowly failed to show a statistical difference between the groups treated with radiation alone and treated with a combination of 5-azacytidine, flutamide, and radiation. However, we believe that the introduction of a second treatment block at day 20 will result in sufficiently prolonged local tumor control to show clear statistical significance. We are currently verifying this hypothesis.

Reportable Outcomes

Manuscript '**The nucleoside analog 5-azacytidine sensitizes prostate cancer cells to radiation-induced DNA breaks and delays the growth of xenografted prostate cancer tumors**' by Alfred Gallegos, Pamela Dino, Suzanne Regan, Bernard Futscher, Giuseppe Pizzorno and Eric Weterings. This manuscript is currently being compiled and will be published contingent on the outcomes of the repeat experiment on local control of PC-3 based tumors with two treatment blocks, as discussed in the sections above.

Conclusion

The research covered under this award is designed to further study our original finding that the drug 5-azacytidine increases the responsiveness of prostate cancer cells and xenografts to radiation. The first aim of our research was to identify molecular targets of 5-azacytidine which mediate its radiation sensitizing effect. We originally hypothesized that 5-azacytidine likely suppresses DNA double-strand (DSB) break repair by modulating the non-homologous end-joining pathway, but found no evidence to support this hypothesis. We did identify two targets of 5-azacytidine, Rad50 and MAP2K6, which may suppress DSB repair by modulating homologous recombination and cell cycle arrest. Based on our latest data, however, we conclude that 5-azacytidine predominantly sensitizes prostate cancer cells to radiation by sensitizing the genomic DNA itself to DNA breakage, rather than by suppressing DSB

repair. We are currently investigating whether this direct modulation of DNA integrity has an additive or synergistic combined effect with radiation. The second aim of our research was to verify the effects of a combined triple treatment regimen of 5-azacytidine, flutamide, and radiation on local control of xenografted human-derived prostate tumors. Complete local control was observed for a period of 25 days after an initial 5 day treatment block with a combination of 5-azacytidine, flutamide, and radiation. Although tumor growth was eventually restored, the total time needed for triplication of the tumor was 2x longer than observed for the control and 1.5x longer than observed for the group treated with radiation alone. It must be noted that an initial statistical analysis arbitrarily based on linear regression narrowly failed to show a statistical difference between the triple treatment group and the control group treated with radiation alone. However, we believe that the introduction of a second treatment block at day 20 will result in sufficiently prolonged local tumor control to show clear statistical significance. We are currently verifying this hypothesis.

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List of salaried personnel on the award

- Eric Weterings, Principal Investigator, Feb 2011 - Feb 2014.
- Andrea McKinney, Research Specialist, Nevada Cancer Institute, Feb 2011 - Jan 2012.
- Steven Brotman, Senior Research Specialist, University of Arizona, Feb 2012 – Feb 2013.
- Alfred Gallegos, Senior Research Specialist, University of Arizona, Dec 2012 – Feb 2014.
- Suzanne Regan, Research Specialist, University of Arizona, March 2013 – Feb 2014.

Appendix 1

Regulation of cytosine methylation levels of general DNA Repair genes in PC-3 prostate cancer cells in response to 5-azacytidine and radiation exposure as single or combined treatment modalities. Numbers reflect the relative percentage of high, low, and intermediate level methylated DNA of each gene. Experiments were done in triplicate.

Un-Methylated

	mock	radiation	low aza	low aza/rad	high aza	high aza/rad
OSGEP	82.61%	98.76%	98.51%	55.98%	96.88%	99.02%
ATM	98.69%	96.64%	96.00%	58.43%	94.91%	96.13%
BRCA1	98.29%	97.27%	96.93%	76.43%	95.87%	95.17%
BRCA2	82.29%	96.70%	96.37%	72.35%	89.93%	94.46%
CCNH	96.32%	95.59%	96.05%	72.94%	91.38%	96.52%
C11orf10	99.89%	99.26%	99.23%	73.83%	99.69%	99.05%
LIG1	80.99%	96.95%	97.49%	58.36%	97.30%	96.43%
LIG3	98.67%	96.86%	91.88%	56.99%	89.99%	64.69%
MLH1	99.15%	99.25%	98.39%	71.83%	99.09%	99.34%
MLH3	74.88%	65.13%	81.58%	32.49%	78.06%	72.97%
ANKRD49	94.19%	96.77%	95.94%	44.87%	86.12%	98.15%
MSH2	91.10%	72.38%	87.88%	29.83%	87.53%	75.89%
PARP1	82.88%	99.21%	98.83%	47.03%	99.03%	99.08%
PARP3	80.45%	95.70%	96.28%	52.10%	94.20%	93.79%
AIMP2	99.76%	82.19%	98.88%	55.39%	99.52%	99.28%
POLB	99.38%	99.32%	98.03%	77.35%	99.17%	98.98%
POLD3	98.91%	98.09%	97.29%	71.16%	97.44%	98.17%
RAD23A	96.77%	97.54%	95.99%	67.01%	94.58%	93.09%
RAD23B	99.10%	99.22%	97.09%	55.83%	99.11%	99.58%
RAD50	46.94%	47.35%	39.73%	19.08%	86.28%	91.76%
RAD51	80.70%	93.17%	94.14%	62.30%	90.81%	93.00%
UNG	93.88%	90.42%	92.74%	42.23%	87.96%	84.50%
XPC	98.86%	97.81%	97.28%	71.52%	96.88%	96.20%
XRCC1	72.91%	52.00%	69.37%	26.24%	72.87%	64.63%

Intermediate-Methylated

	mock	radiation	low aza	low aza/rad	high aza	high aza/rad
OSGEP	0.00%	0.00%	0.00%	41.49%	0.00%	0.00%
ATM	0.00%	0.00%	0.00%	36.64%	0.00%	0.00%
BRCA1	0.00%	0.00%	0.00%	18.76%	0.00%	0.00%
BRCA2	16.98%	0.00%	0.00%	21.72%	0.00%	0.00%
CCNH	0.00%	0.00%	0.00%	22.99%	0.00%	0.00%
C11orf10	0.00%	0.00%	0.00%	23.64%	0.00%	0.00%
LIG1	18.07%	0.00%	0.00%	35.93%	0.00%	0.00%
LIG3	0.00%	0.00%	0.00%	37.89%	0.00%	0.00%
MLH1	0.00%	0.00%	0.00%	25.47%	0.00%	0.00%
MLH3	14.68%	10.06%	0.00%	46.61%	0.00%	0.00%
ANKRD49	0.00%	0.00%	0.00%	44.75%	0.00%	0.00%
MSH2	0.00%	14.91%	0.00%	55.86%	0.00%	12.39%
PARP1	16.61%	0.00%	0.00%	48.79%	0.00%	0.00%
PARP3	18.13%	0.00%	0.00%	42.88%	0.00%	0.00%

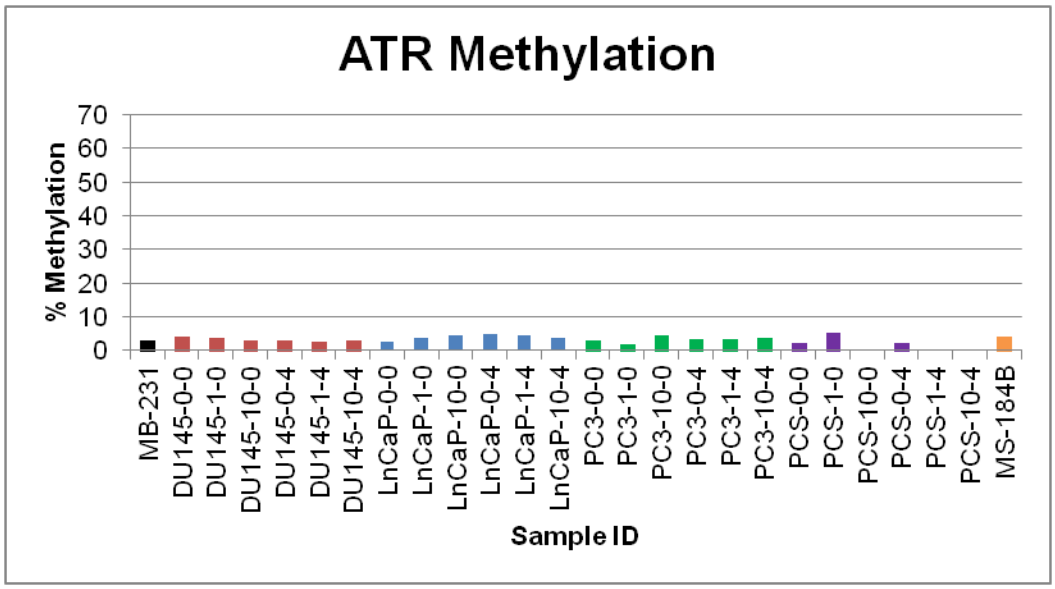
AIMP2	0.00%	17.44%	0.00%	42.12%	0.00%	0.00%
POLB	0.00%	0.00%	0.00%	21.09%	0.00%	0.00%
POLD3	0.00%	0.00%	0.00%	23.01%	0.00%	0.00%
RAD23A	0.00%	0.00%	0.00%	23.76%	0.00%	0.00%
RAD23B	0.00%	0.00%	0.00%	42.36%	0.00%	0.00%
RAD50	41.81%	39.80%	48.46%	70.07%	0.00%	0.00%
RAD51	16.15%	0.00%	0.00%	28.55%	0.00%	0.00%
UNG	0.00%	0.00%	0.00%	43.64%	0.00%	0.00%
XPC	0.00%	0.00%	0.00%	24.82%	0.00%	0.00%
XRCC1	17.24%	33.88%	16.73%	59.80%	11.43%	20.04%

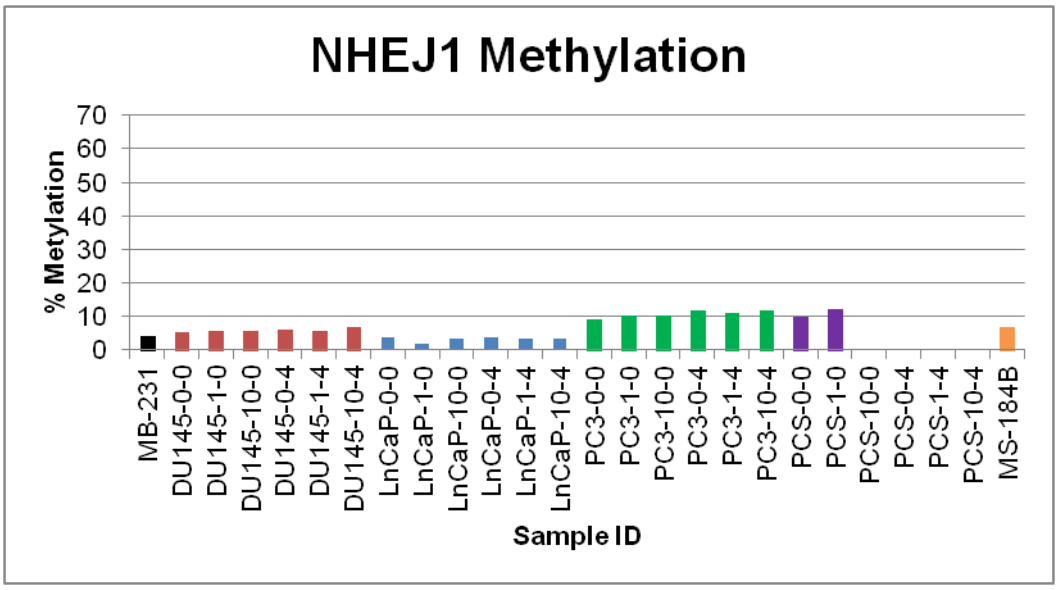
Hyper-methylated

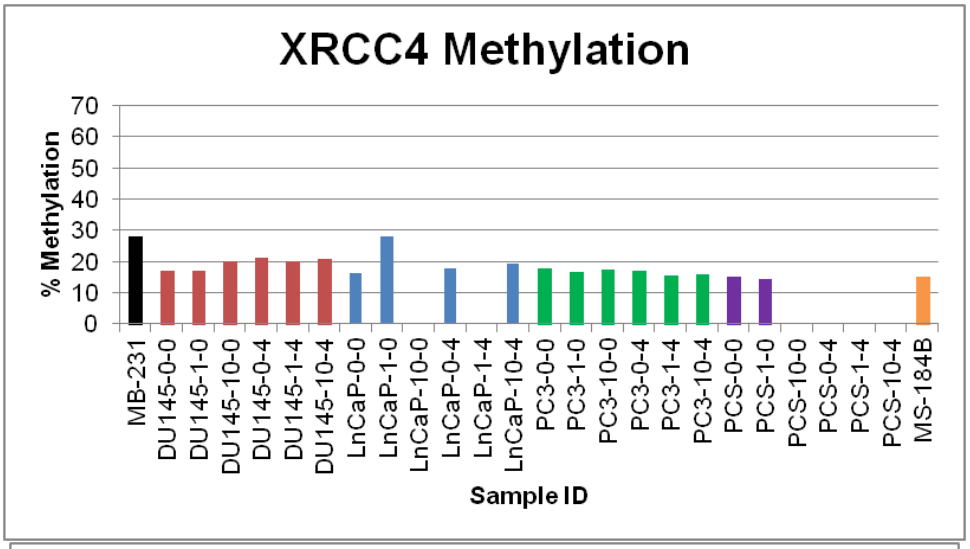
	mock	radiation	low aza	low aza/rad	high aza	high aza/rad
OSGEP	17.39%	1.24%	1.49%	1.88%	3.12%	0.98%
ATM	1.31%	3.36%	4.00%	4.21%	5.09%	3.87%
BRCA1	1.71%	2.73%	3.07%	3.35%	4.13%	4.83%
BRCA2	0.73%	3.30%	3.63%	3.83%	10.07%	5.54%
CCNH	3.68%	4.41%	3.95%	3.68%	8.62%	3.48%
C11orf10	0.11%	0.74%	0.77%	1.44%	0.31%	0.95%
LIG1	0.94%	3.05%	2.51%	3.43%	2.70%	3.57%
LIG3	1.33%	3.14%	8.12%	6.06%	10.01%	35.31%
MLH1	0.85%	0.75%	1.61%	2.10%	0.91%	0.66%
MLH3	10.44%	24.81%	18.42%	14.48%	21.94%	27.03%
ANKRD49	5.81%	3.23%	4.06%	6.08%	13.88%	1.85%
MSH2	8.90%	12.71%	12.12%	9.24%	12.47%	11.72%
PARP1	0.51%	0.79%	1.17%	2.28%	0.97%	0.92%
PARP3	1.41%	4.30%	3.72%	3.64%	5.80%	6.21%
AIMP2	0.24%	0.36%	1.12%	1.70%	0.48%	0.72%
POLB	0.62%	0.68%	1.97%	1.98%	0.83%	1.02%
POLD3	1.09%	1.91%	2.71%	3.56%	2.56%	1.83%
RAD23A	3.23%	2.46%	4.01%	5.17%	5.42%	6.91%
RAD23B	0.90%	0.78%	2.91%	3.09%	0.89%	0.42%
RAD50	11.25%	12.85%	11.81%	8.73%	13.72%	8.24%
RAD51	3.15%	6.83%	5.86%	6.06%	9.19%	7.00%
UNG	6.12%	9.58%	7.26%	8.88%	12.04%	15.50%
XPC	1.14%	2.19%	2.72%	2.67%	3.12%	3.80%
XRCC1	9.85%	14.12%	13.90%	18.58%	15.70%	15.33%

Appendix 2

Regulation of promoter cytosine methylation levels of NHEJ genes in PC-3, DU-145, LNCaP, and PCS-440-010 cells in response to 5-azacytidine and radiation exposure as single or combined treatment modalities. Data were obtained by massARRAY. Utilized gene name analogs: PRKDC = DNA-PK$_{CS}$, XRCC6 = Ku70, XRCC5 = Ku80, NHEJ1gene = XLF.






XRCC4 Methylation
% Methylation
70
60
50
40
30
20
10
0
MB-231
DU145-0-0
DU145-1-0
DU145-10-0
DU145-0-4
DU145-1-4
DU145-10-4
LnCaP-0-0
LnCaP-1-0
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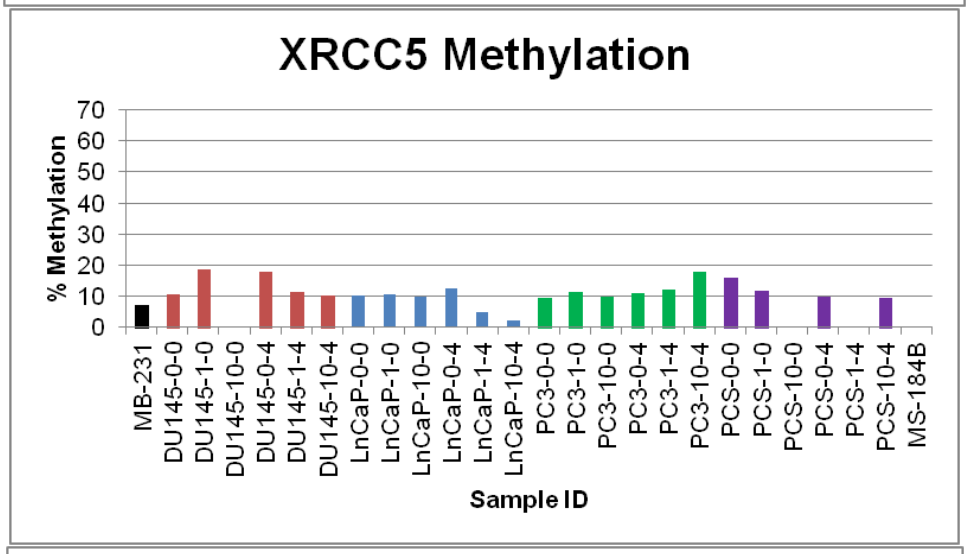
MS-184B
Sample ID


XRCC5 Methylation
% Methylation
70
60
50
40
30
20
10
0
MB-231
DU145-0-0
DU145-1-0
DU145-10-0
DU145-0-4
DU145-1-4
DU145-10-4
LnCaP-0-0
LnCaP-1-0
LnCaP-10-0
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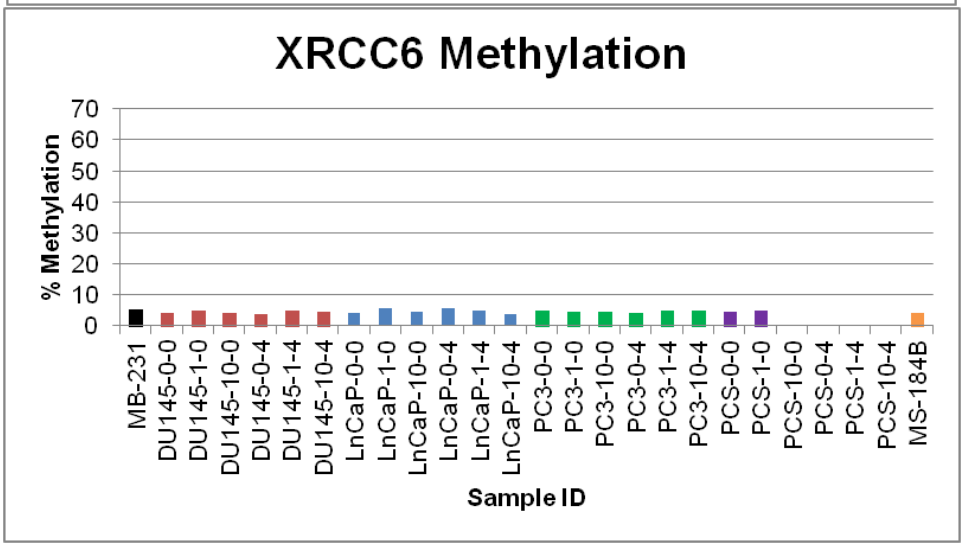
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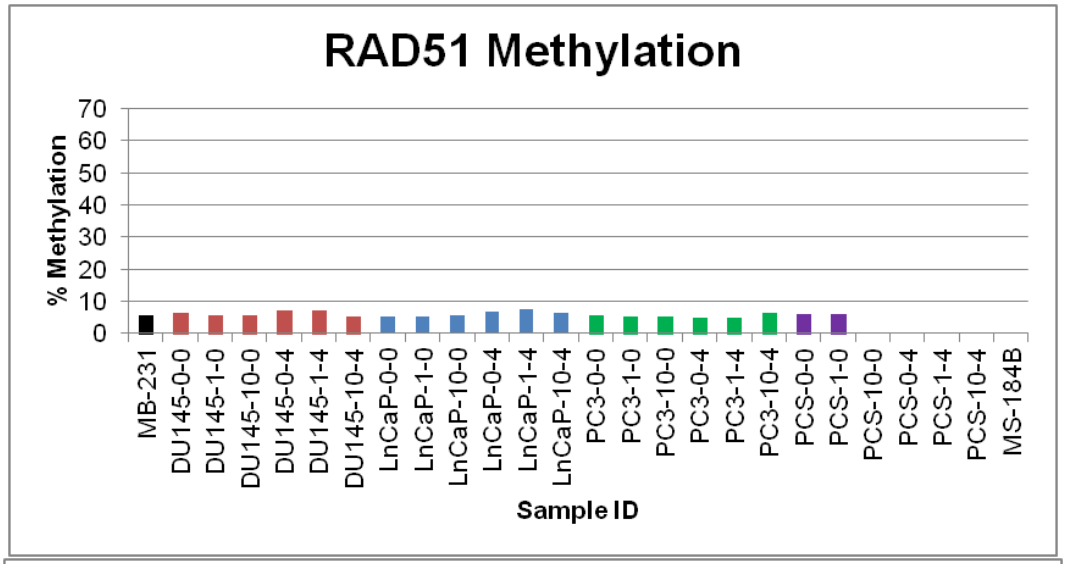
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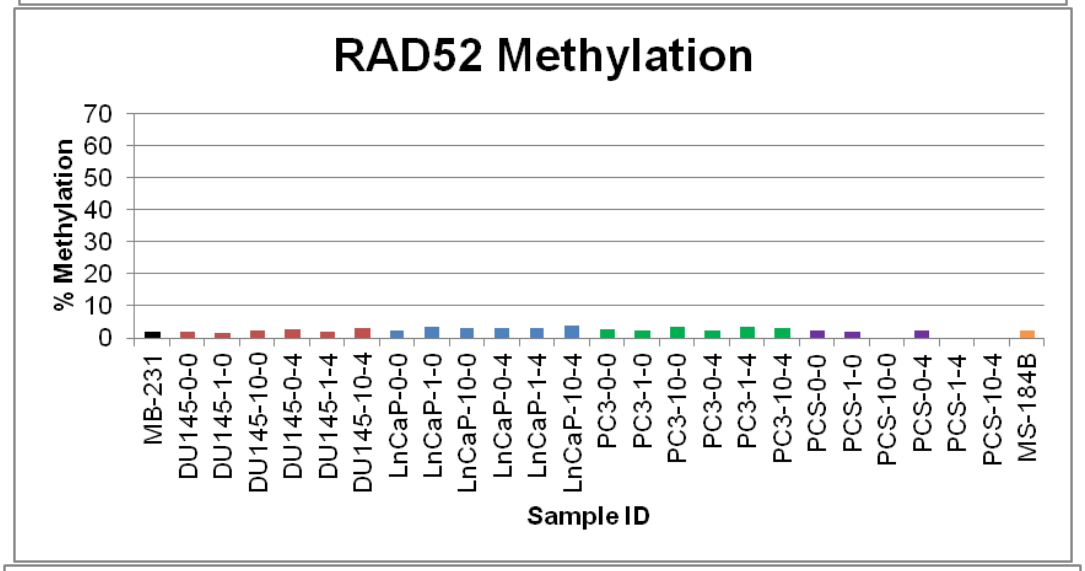
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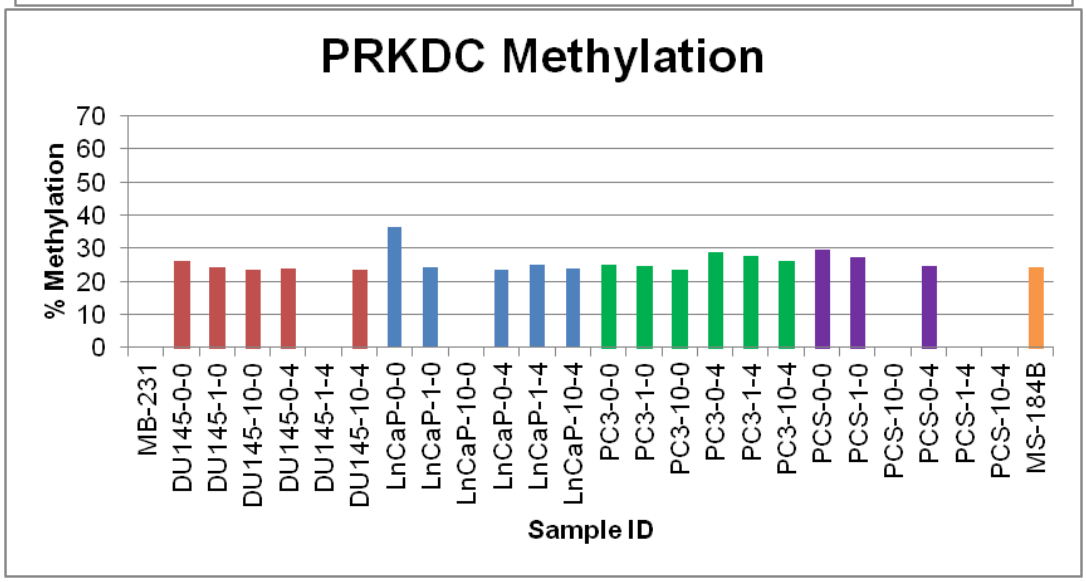
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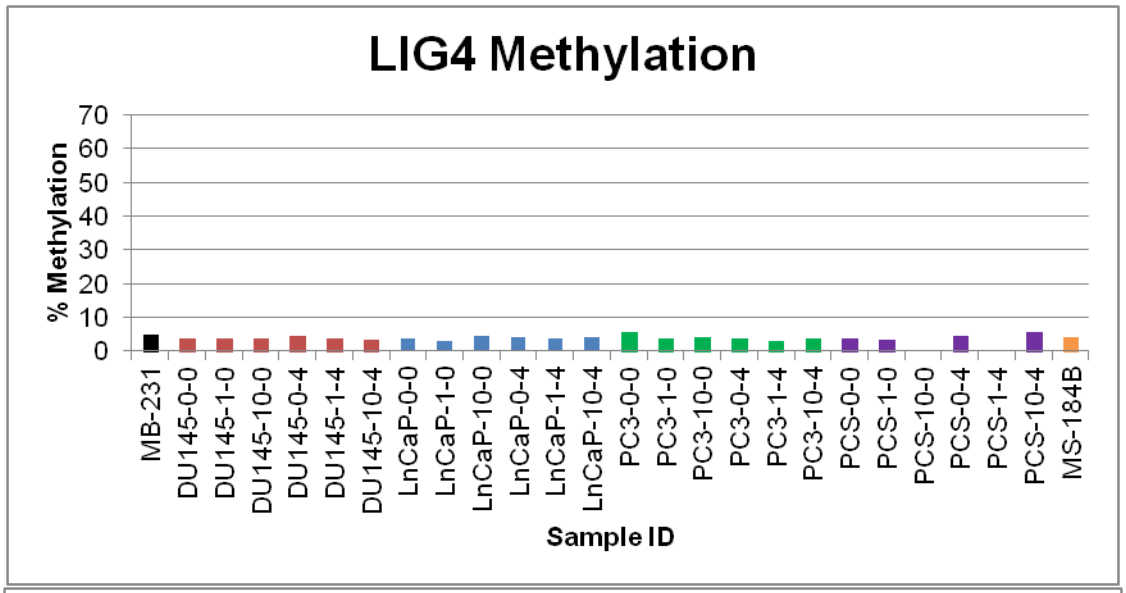
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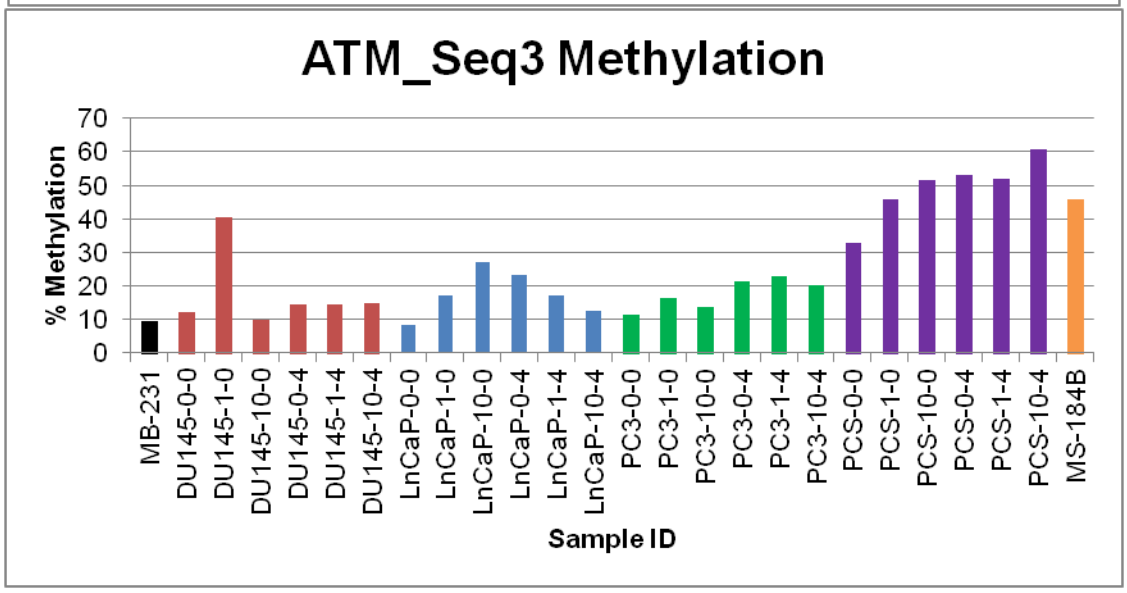
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MS-184B
Sample ID

Appendix 3

Regulation of steady state mRNA levels of general DNA Repair genes in PC-3 prostate cancer cells in response to 5-azacytidine and radiation exposure as single or combined treatment modalities. Numbers reflect the relative fold up-or down regulation of each gene. Experiments were done in triplicate.

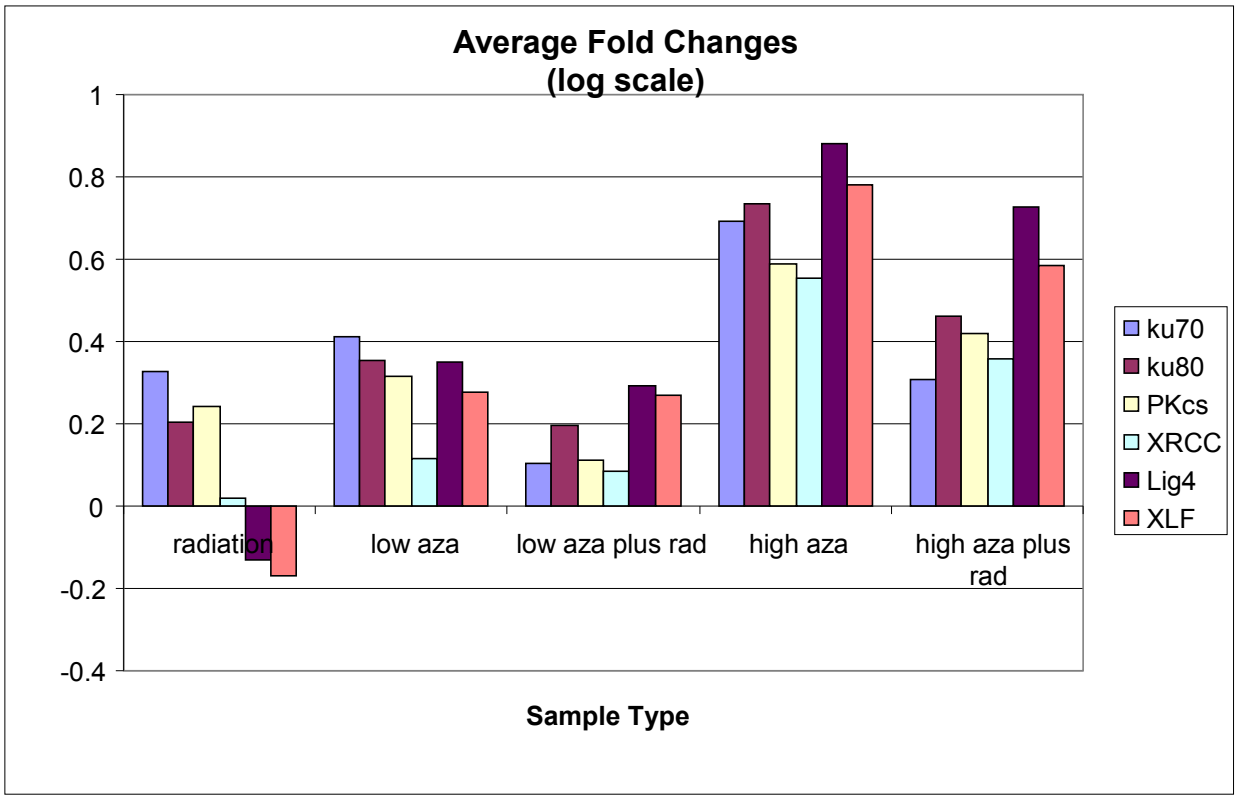
	Fold Up- or Down-Regulation				
Symbol	radiation	low aza	low aza + rad	high aza	high aza + rad
ABL1	-1.86	-2.92	-1.46	-1.77	-1.04
ANKRD17	1.11	1.03	2.10	2.09	1.18
APEX1	1.18	-1.73	-1.23	-1.36	-1.20
ATM	1.07	-1.21	1.87	1.19	1.44
ATR	1.21	1.90	1.77	3.01	-1.58
ATRX	1.49	2.05	1.76	1.87	1.29
BRCA1	1.72	1.11	1.83	1.60	1.96
BTG2	-1.28	1.18	3.31	3.07	3.80
CCNH	1.13	1.65	1.67	2.71	1.27
CDK7	1.30	1.55	1.78	2.76	1.39
CHEK1	1.26	1.75	1.49	1.56	1.60
CHEK2	1.20	-1.10	1.56	1.60	-1.41
CIB1	1.24	1.28	1.29	1.49	1.60
CIDEA	1.33	1.14	3.09	1.78	2.63
CRY1	1.06	1.24	1.71	1.68	1.20
DDB1	-1.60	-2.96	-1.50	-3.37	1.02
DDIT3	1.55	6.24	9.28	16.54	5.49
DMC1	1.07	1.09	-1.77	-1.19	-2.95
ERCC1	-2.89	-9.00	-3.68	-10.98	-3.68
ERCC2	-1.77	-3.85	-1.21	-2.68	-1.16
EXO1	-1.21	1.16	1.13	1.37	-1.19
FANCG	-1.07	-1.71	-1.11	-1.66	-1.16
FEN1	1.57	-3.23	1.06	-4.79	1.10
XRCC6	-1.02	-1.71	1.13	-1.32	-1.37
GADD45A	1.32	1.95	3.18	4.63	3.60
GADD45G	-1.03	-1.26	2.34	2.44	2.18
GML	1.10	-1.24	1.65	1.92	2.13
GTF2H1	1.30	2.63	2.34	4.19	1.67
GTF2H2	1.13	1.13	1.34	1.73	-1.41
GTSE1	1.05	-1.07	-1.94	-1.32	-1.49
HUS1	-1.44	-1.33	3.67	1.65	1.40
IGHMBP2	-1.29	-3.02	1.18	-1.49	1.59
IP6K3	1.77	1.40	2.98	8.22	9.21
XRCC6BP1	-1.34	-2.31	-1.99	-1.62	-3.25
LIG1	-1.73	-5.75	-2.41	-6.71	-1.66
MAP2K6	-1.27	-3.94	-6.66	-12.37	-12.39

MAPK12	-2.57	-4.81	-1.76	-3.84	-1.04
MBD4	1.36	1.46	1.82	2.65	1.25
MLH1	1.38	1.88	1.57	2.42	1.06
MLH3	1.51	1.91	2.59	3.28	2.18
MNAT1	1.09	1.20	1.23	1.87	-1.04
MPG	-1.50	-2.61	-1.85	-1.90	-1.90
MRE11A	1.20	-1.05	1.76	1.11	1.63
MSH2	1.13	1.21	1.05	1.49	-1.66
MSH3	1.23	1.02	1.71	1.71	1.54
MUTYH	-1.00	-1.45	1.51	-1.00	1.78
N4BP2	1.02	-1.21	1.68	1.62	1.65
NBN	1.85	1.67	3.25	2.26	1.17
NTHL1	-1.54	-2.40	-1.92	-2.04	-1.72
OGG1	-1.37	-2.26	-1.38	-2.26	-1.29
PCBP4	-2.38	-5.79	-2.01	-2.88	-1.38
PCNA	-1.09	-1.50	-1.24	-1.19	-1.56
AIFM1	1.82	1.76	1.87	2.47	1.08
PMS1	-1.01	1.52	1.79	2.28	-1.24
PMS2	1.18	1.26	2.52	1.41	2.91
PMS2P3	-1.08	1.03	1.45	1.58	2.18
PNKP	-1.58	-4.10	-2.33	-6.62	-1.37
PPP1R15A	-1.38	-1.08	3.88	2.08	3.66
PRKDC	1.12	-1.77	-1.01	-1.44	1.05
RAD1	1.36	2.29	1.77	2.55	1.77
RAD17	1.24	1.53	1.33	2.27	1.31
RAD18	-1.29	-1.63	1.09	-1.05	1.07
RAD21	-1.07	-1.58	-1.12	-1.14	-2.34
RAD50	-1.19	-2.97	2.28	-1.32	1.83
RAD51	1.69	1.54	2.23	2.59	2.11
RAD51B	1.25	1.29	1.43	1.23	2.24
RAD9A	1.10	-1.56	1.45	1.05	1.45
RBBP8	1.23	2.20	1.94	2.92	1.23
REV1	1.36	1.36	1.52	2.24	-1.15
RPA1	1.13	-1.56	1.18	-1.13	1.08
SEMA4A	-1.31	-3.22	-1.15	-2.18	2.04
SESN1	1.59	1.73	3.24	3.03	1.16
SMC1A	-1.48	-1.98	-1.02	-2.26	1.57
SUMO1	1.11	-1.17	-1.10	1.13	-1.24
TP53	-1.16	-1.73	1.38	-1.07	-1.03
TP73	1.11	-2.27	2.15	1.66	1.66
TREX1	1.16	-1.82	1.01	-1.73	1.10
UNG	1.05	1.29	1.08	1.79	1.16
XPA	1.12	1.81	1.64	2.26	1.22
XPC	1.21	1.43	2.08	3.21	1.28
XRCC1	-1.24	-2.39	-1.37	-2.27	-1.44
XRCC2	-1.04	-1.05	1.59	-1.03	1.41
XRCC3	-1.89	-2.97	1.06	-1.41	2.99
ZAK	1.36	-2.06	-1.19	-1.25	-1.19
B2M	-1.10	1.07	1.03	1.06	-1.74

HPRT1	1.33	1.26	-1.01	1.33	1.05
RPL13A	-1.21	1.31	1.17	1.40	-1.09
GAPDH	1.06	-1.35	-1.25	-1.43	1.42
ACTB	-1.05	-1.31	1.04	-1.38	1.27

Appendix 4

Regulation of steady state mRNA levels of the core NHEJ genes Ku70, Ku80, DNA-PKCS, XRCC4, Ligase IV, and XLF in PC-3 prostate cancer cells in response to 5-azacytidine and radiation exposure as single or combined treatment modalities. Numbers reflect the relative fold up-or down regulation of each gene. Numbers are based on quadruplicate measurements of biological duplicates: n=8 .Low aza = 1 uM 5-azacytidine, high aza = 10 uM 5-azacytidine.



Appendix 5

Modulation of RNA expression levels in PC-3 prostate cancer cells in response to 5-azacytidine (10 uM) and radiation exposure (2 Gy) as compared to radiation exposure alone. Table list genes that were modulated more than 2 fold as compared to the control (radiation alone). Expression levels of over 30,000 RNA transcripts were analyzed utilizing the GeneChip Human Genome ST 1.0 Array from Affymetrix. Meaning of the columns, from left to right: Transcript Cluster ID, control Bi-weight Avg Signal (log2), sample Bi-weight Avg Signal (log2), Fold Change (linear), control vs. sample ANOVA p-value, control vs. sample FDR p-value, Gene Symbol, Description.

7927710	9.83	7.33	5.68	0.003342	0.999947	CDK1	cyclin-dependent kinase 1
8168794	9.10	6.85	4.76	0.018982	0.999947	CENPI	centromere protein I
8018849	10.26	8.04	4.65	0.031495	0.999947	TK1	thymidine kinase 1, soluble
7960702	7.58	5.38	4.58	0.028127	0.999947	CDCA3	cell division cycle associated 3
7979307	10.73	8.53	4.57	0.000704	0.999947	DLGAP5	discs, large (Drosophila) homolog-associated protein 5
8172220	6.59	4.54	4.14	0.011259	0.999947	NDP	Norrie disease (pseudoglioma)
8120932	7.41	5.37	4.13	0.024496	0.999947	PRSS35	protease, serine, 35
8112260	8.49	6.49	4.01	0.043076	0.999947	DEPDC1B	DEP domain containing 1B
7947110	7.33	5.35	3.95	0.024549	0.999947	E2F8	E2F transcription factor 8
7974404	10.55	8.58	3.92	0.012123	0.999947	CDKN3	cyclin-dependent kinase inhibitor 3
7915592	9.43	7.46	3.91	0.048330	0.999947	RNU5D-1	RNA, U5D small nuclear 1
8062571	9.22	7.31	3.75	0.037180	0.999947	FAM83D	family with sequence similarity 83, member D
8040223	10.00	8.10	3.72	0.024417	0.999947	RRM2	ribonucleotide reductase M2
8040712	9.27	7.41	3.63	0.035396	0.999947	CENPA	centromere protein A, chromosome 2 open reading frame 18
8168161	4.89	3.06	3.56	0.024750	0.999947	RNY4P23	RNA, Ro-associated Y4 pseudogene 23
7982358	10.25	8.44	3.51	0.040166	0.999947	ARHGAP11A	Rho GTPase activating protein 11A
7955736	7.91	6.17	3.34	0.032728	0.999947	ESPL1	extra spindle pole bodies homolog 1 (S. cerevisiae)
8061471	9.12	7.39	3.33	0.046613	0.999947	GINS1	GINS complex subunit 1 (Psf1 homolog)
7941587	7.49	5.77	3.30	0.043794	0.999947	CNIH2	cornichon homolog 2 (Drosophila)
8063043	8.58	6.91	3.18	0.002607	0.999947	UBE2C	ubiquitin-conjugating enzyme E2C
8008784	10.90	9.24	3.17	0.008801	0.999947	PRR11	proline rich 11
8041422	6.15	4.54	3.05	0.031820	0.999947	RASGRP3	RAS guanyl releasing protein 3 (calcium and DAG-regulated)
7933855	7.98	6.43	2.92	0.037151	0.999947	RTKN2	rhotekin 2
8178399	9.02	7.48	2.90	0.004203	0.999947	NRM	nurim (nuclear envelope membrane protein)
8179683	9.02	7.48	2.90	0.004203	0.999947	NRM	nurim (nuclear envelope membrane protein)
7966878	9.37	7.84	2.88	0.008014	0.999947	CIT	citron (rho-interacting, serine/threonine kinase 21), microRNA 1178
8018860	5.67	4.20	2.77	0.015197	0.999947	BIRC5	baculoviral IAP repeat containing 5
8124806	8.91	7.46	2.73	0.006091	0.999947	NRM	nurim (nuclear envelope membrane protein)
8089372	9.76	8.33	2.69	0.020140	0.999947	KIAA1524	KIAA1524
8017133	10.22	8.82	2.64	0.029097	0.999947	SKA2	spindle and kinetochore associated complex subunit 2
8061579	10.11	8.70	2.64	0.049103	0.999947	TPX2	TPX2, microtubule-associated, homolog (Xenopus laevis)
7916432	11.69	10.30	2.61	0.022350	0.999947	DHCR24	24-dehydrocholesterol reductase
8123920	7.26	5.88	2.60	0.041878	0.999947	ELOVL2	ELOVL fatty acid elongase 2
8138504	6.87	5.50	2.59	0.002646	0.999947	RAPGEF5	Rap guanine nucleotide exchange factor (GEF) 5
7955195	7.93	6.57	2.58	0.044434	0.999947	TROAP	trophinin associated protein (tastin)
7939738	8.53	7.20	2.51	0.003922	0.999947	DDB2	damage-specific DNA binding protein 2, 48kDa
7940600	8.98	7.68	2.46	0.039055	0.999947	INCENP	inner centromere protein antigens 135/155kDa
8113773	9.83	8.54	2.44	0.012249	0.999947	ALDH7A1	aldehyde dehydrogenase 7 family, member A1
8044804	9.41	8.13	2.42	0.001695	0.999947	DBI	diazepam binding inhibitor (GABA receptor modulator, acyl-CoA binding protein)
8144153	9.86	8.60	2.40	0.037665	0.999947	NCAPG2	non-SMC condensin II complex, subunit G2
7919560	9.55	8.32	2.34	0.013646	0.999947		
7982287	8.99	7.78	2.31	0.010201	0.999947	ARHGAP11B	Rho GTPase activating protein 11B, OTU domain containing 7A pseudogene
7919556	9.73	8.52	2.31	0.014123	0.999947		
8064844	10.22	9.04	2.27	0.034130	0.999947	PCNA	proliferating cell nuclear antigen
7904953	5.08	3.91	2.25	0.027967	0.999947		
8104234	9.42	8.26	2.24	0.041554	0.999947	TRIP13	thyroid hormone receptor interactor 13

8072876	11.44	10.27	2.24	0.010907	0.999947	LGALS1	lectin, galactoside-binding, soluble, 1
8063536	8.65	7.53	2.18	0.021948	0.999947	TFAP2C	transcription factor AP-2 gamma (activating enhancer binding protein 2 gamma)
7926821	8.44	7.34	2.14	0.009707	0.999947	MASTL	microtubule associated serine/threonine kinase-like
8035236	7.12	6.03	2.14	0.030561	0.999947	HAUS8	HAUS augmin-like complex, subunit 8
8100578	6.70	5.61	2.13	0.033079	0.999947	EPHA5	EPH receptor A5
8149281	3.89	2.79	2.13	0.044866	0.999947		
8010673	8.73	7.66	2.10	0.005688	0.999947	SLC25A10	solute carrier family 25 (mitochondrial carrier; dicarboxylate transporter), member 10
7991374	8.83	7.76	2.09	0.008842	0.999947	IDH2	isocitrate dehydrogenase 2 (NADP+), mitochondrial
8081620	5.46	4.41	2.07	0.036601	0.999947	TAGLN3	transgelin 3
7975066	5.07	4.03	2.06	0.012839	0.999947	AKAP5	A kinase (PRKA) anchor protein 5
7919390	6.62	5.60	2.03	0.016291	0.999947		
8055096	7.66	8.67	-2.01	0.043056	0.999947	AMMECR1L	AMME chromosomal region gene 1-like
7893506	7.89	8.91	-2.02	0.047477	0.999947		
8135480	5.43	6.45	-2.02	0.040414	0.999947	DNAJB9	DnaJ (Hsp40) homolog, subfamily B, member 9
8094476	6.44	7.46	-2.03	0.032964	0.999947	TBC1D19	TBC1 domain family, member 19
7895098	9.20	10.23	-2.05	0.029157	0.999947		
8043583	5.26	6.29	-2.05	0.013790	0.999947	LOC285033	uncharacterized LOC285033
8082965	5.76	6.80	-2.06	0.021968	0.999947	MRAS	muscle RAS oncogene homolog
7993580	8.03	9.09	-2.07	0.035405	0.999947	PKD1P1	polycystic kidney disease 1 (autosomal dominant) pseudogene 1, nuclear pore complex-interacting protein-like 1-like
7980547	8.73	9.78	-2.07	0.027554	0.999947	SEL1L	sel-1 suppressor of lin-12-like (C. elegans)
7959023	8.91	9.97	-2.08	0.037364	0.999947	MAP1LC3B	microtubule-associated protein 1 light chain 3 beta, microtubule-associated protein 1 light chain 3 beta 2
7957433	3.90	4.96	-2.08	0.015153	0.999947	LRRIQ1	leucine-rich repeats and IQ motif containing 1
7893057	2.40	3.47	-2.10	0.020754	0.999947		
8048772	8.16	9.24	-2.11	0.041578	0.999947	RHBDD1	rhomboid domain containing 1
8110569	10.80	11.88	-2.11	0.009540	0.999947	SQSTM1	sequestosome 1
7894831	3.33	4.43	-2.14	0.028258	0.999947		
7895530	3.38	4.48	-2.14	0.035731	0.999947		
7996569	3.37	4.47	-2.15	0.046907	0.999947		
8063337	8.30	9.41	-2.16	0.004222	0.999947	ZNFX1-AS1	ZNFX1 antisense RNA 1 (non-protein coding)
8050367	7.84	8.95	-2.16	0.049306	0.999947	NBAS	neuroblastoma amplified sequence
7913001	9.24	10.34	-2.16	0.000275	0.915251	UBR4	ubiquitin protein ligase E3 component n-recognin 4
8089247	3.28	4.40	-2.17	0.029885	0.999947		
7894663	2.80	3.93	-2.18	0.036305	0.999947		
8139656	8.70	9.83	-2.18	0.023361	0.999947	GRB10	growth factor receptor-bound protein 10
8162019	5.51	6.64	-2.18	0.006972	0.999947	KIF27	kinesin family member 27
7999841	8.72	9.87	-2.21	0.013904	0.999947	SMG1	smg-1 homolog, phosphatidylinositol 3-kinase-related kinase (C. elegans), uncharacterized LOC100506830, SMG1 homolog, phosphatidylinositol 3-kinase-related kinase (C. elegans) pseudogene
8042310	7.72	8.87	-2.22	0.025563	0.999947	SLC1A4	solute carrier family 1 (glutamate/neutral amino acid transporter), member 4
7993546	8.57	9.74	-2.25	0.021618	0.999947	NPIP	nuclear pore complex interacting protein, nuclear pore complex-interacting protein-like 1-like, polycystic kidney disease 1 (autosomal dominant) pseudogene 1
8039692	6.81	7.98	-2.26	0.038876	0.999947	ZNF552	zinc finger protein 552, zinc finger protein 587B, zinc finger protein 814, zinc finger protein 587
8000537	7.81	8.99	-2.28	0.047373	0.999947	LOC100507607	nuclear pore complex-interacting protein-like 2-like
7896521	7.13	8.32	-2.29	0.029171	0.999947		
7968577	6.23	7.42	-2.29	0.019028	0.999947	NBEA	neurobeachin
7999769	8.47	9.67	-2.29	0.017830	0.999947	LOC399491	GPS, PLAT and transmembrane domain-containing protein, nuclear pore complex interacting protein, nuclear pore complex-interacting protein-like 1-like, polycystic kidney disease 1 (autosomal dominant) pseudogene 1
7993404	8.86	10.05	-2.30	0.030078	0.999947	NPIP	nuclear pore complex interacting protein, nuclear pore complex-interacting protein-like 1-like, polycystic kidney disease 1 (autosomal dominant) pseudogene 1
7997239	7.76	8.96	-2.30	0.045557	0.999947	LOC100507607	nuclear pore complex-interacting protein-like 2-like
7994371	7.35	8.56	-2.30	0.044311	0.999947	LOC728741	uncharacterized LOC728741, nuclear pore complex-interacting protein-like 2-like
8139873	4.95	6.16	-2.31	0.012936	0.999947		
7895300	4.03	5.26	-2.35	0.035431	0.999947		
7895450	3.70	4.93	-2.35	0.022566	0.999947		
7940582	4.67	5.91	-2.37	0.031539	0.999947	BEST1	bestrophin 1
7895836	3.51	4.76	-2.38	0.011358	0.999947		
8156164	7.07	8.32	-2.38	0.015804	0.999947	KIF27	kinesin family member 27, kinesin family member 27 pseudogene
7999614	8.52	9.79	-2.40	0.038218	0.999947	LOC399491	GPS, PLAT and transmembrane domain-containing protein, nuclear pore complex interacting protein pseudogene 1
7956785	9.52	10.79	-2.42	0.048359	0.999947	XPOT	exportin, tRNA (nuclear export receptor for tRNAs)
8032392	8.09	9.37	-2.42	0.049501	0.999947	MKNK2	MAP kinase interacting serine/threonine kinase 2

8008564	5.56	6.84	-2.43	0.006890	0.999947	TOM1L1	target of myb1 (chicken)-like 1
7994559	7.85	9.14	-2.44	0.044964	0.999947	LOC100507607	nuclear pore complex-interacting protein-like 2-like
7894184	4.69	5.98	-2.45	0.041816	0.999947		
7993359	8.51	9.81	-2.46	0.032453	0.999947	NPIP	nuclear pore complex interacting protein, GPS, PLAT and transmembrane domain-containing protein, polycystic kidney disease 1 (autosomal dominant) pseudogene 1, nuclear pore complex-interacting protein-like 1-like, nuclear pore complex interacting protein pseudogene 1
8126629	8.14	9.44	-2.47	0.014398	0.999947	GTPBP2	GTP binding protein 2
8002347	9.93	11.23	-2.47	0.008066	0.999947	AARS	alanyl-tRNA synthetase
7993349	8.48	9.79	-2.47	0.032599	0.999947	NPIP	nuclear pore complex interacting protein, GPS, PLAT and transmembrane domain-containing protein, polycystic kidney disease 1 (autosomal dominant) pseudogene 1, nuclear pore complex-interacting protein-like 1-like, nuclear pore complex interacting protein pseudogene 1
8128977	5.80	7.12	-2.50	0.041882	0.999947	TUBE1	tubulin, epsilon 1
7940717	9.69	11.02	-2.51	0.026109	0.999947	SLC3A2	solute carrier family 3 (activators of dibasic and neutral amino acid transport), member 2
7896669	5.60	6.94	-2.52	0.019002	0.999947		
7893686	4.11	5.46	-2.55	0.031960	0.999947		
8110032	6.32	7.68	-2.57	0.022078	0.999947	CREBRF	CREB3 regulatory factor
8075182	8.93	10.31	-2.60	0.043989	0.999947	XBP1	X-box binding protein 1
7999634	8.38	9.77	-2.61	0.035068	0.999947	LOC399491	GPS, PLAT and transmembrane domain-containing protein, nuclear pore complex interacting protein, polycystic kidney disease 1 (autosomal dominant) pseudogene 1, nuclear pore complex-interacting protein-like 1-like
7894465	3.19	4.57	-2.62	0.005006	0.999947		
7904226	5.17	6.57	-2.63	0.009560	0.999947	SLC22A15	solute carrier family 22, member 15
7995895	8.81	10.23	-2.67	0.011205	0.999947	HERPUD1	homocysteine-inducible, endoplasmic reticulum stress-inducible, ubiquitin-like domain member 1
7957338	6.76	8.21	-2.73	0.004854	0.999947	SYT1	synaptotagmin I
7895134	2.96	4.42	-2.75	0.026179	0.999947		
7974090	6.38	7.85	-2.77	0.034037	0.999947	CTAGE5	CTAGE family, member 5
7893741	2.65	4.13	-2.78	0.046441	0.999947		
7909610	5.23	6.71	-2.80	0.038462	0.999947	ATF3	activating transcription factor 3
7945803	8.05	9.54	-2.81	0.006976	0.999947	CARS	cysteinyl-tRNA synthetase
7895555	4.37	5.89	-2.88	0.025918	0.999947		
8134051	3.97	5.52	-2.94	0.038569	0.999947	C7orf63	chromosome 7 open reading frame 63
7893482	3.44	5.02	-2.98	0.014240	0.999947		
7898750	7.12	8.70	-3.00	0.015418	0.999947	ZBTB40	zinc finger and BTB domain containing 40
7973530	6.34	7.98	-3.11	0.036518	0.999947	PCK2	phosphoenolpyruvate carboxykinase 2 (mitochondrial)
7894360	3.24	4.91	-3.17	0.040941	0.999947		
7902290	6.54	8.34	-3.49	0.046824	0.999947	CTH	cystathionase (cystathionine gamma-lyase)
8042503	5.70	7.66	-3.89	0.003508	0.999947	MXD1	MAX dimerization protein 1
8154381	7.22	9.23	-4.05	0.000074	0.857937	LURAP1L	leucine rich adaptor protein 1-like
7893241	2.71	4.78	-4.21	0.013122	0.999947		
8141150	8.16	10.29	-4.36	0.032486	0.999947	ASNS	asparagine synthetase (glutamine-hydrolyzing)
7893496	2.24	4.42	-4.53	0.022623	0.999947		
8143341	4.67	6.91	-4.73	0.021529	0.999947	JHDM1D	jumonji C domain containing histone demethylase 1 homolog D (S. cerevisiae)
8000574	8.77	11.14	-5.17	0.011947	0.999947	NUPR1	nuclear protein, transcriptional regulator, 1
7899436	6.12	8.69	-5.93	0.022861	0.999947	SESN2	sestrin 2
7982868	7.52	10.17	-6.26	0.038173	0.999947	CHAC1	ChaC, cation transport regulator homolog 1 (E. coli)
8115851	7.15	9.90	-6.75	0.037484	0.999947	STC2	stanniocalcin 2
7964460	6.34	9.10	-6.79	0.009288	0.999947	DDIT3	DNA-damage-inducible transcript 3
8027002	8.05	10.92	-7.28	0.015526	0.999947	GDF15	growth differentiation factor 15
8060344	6.68	9.68	-8.02	0.004567	0.999947	TRIB3	tribbles homolog 3 (Drosophila)

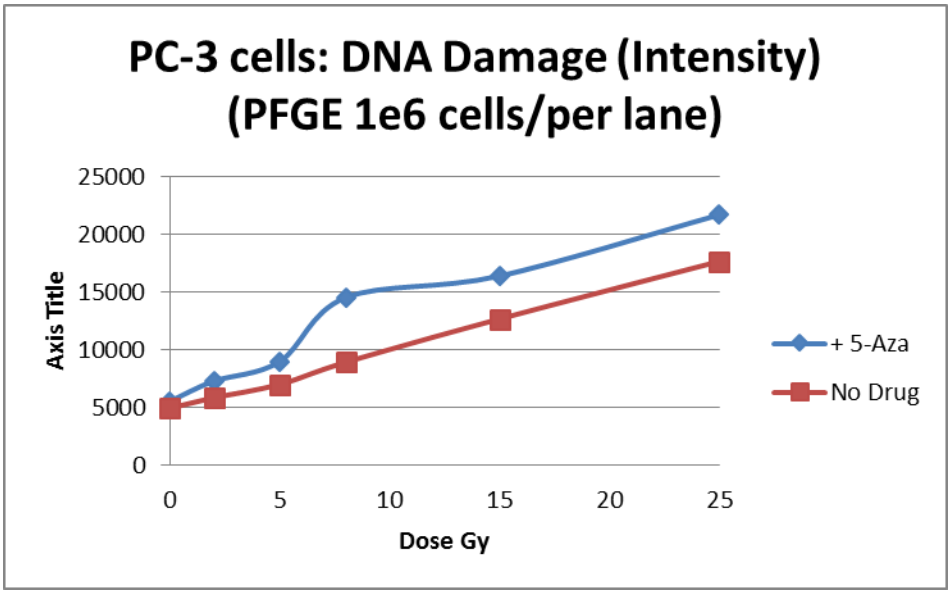
Appendix 6

Regulation of human micro RNA species in PC-3 prostate cancer cells as an effect of 5-azacytidine exposure (selection of miRNA species out of a 369 species data set, based on most significant low FDRs).

Probe ID	S13_hsa_miRNA name	S13_hsa_miRNA_Sequence	Fold Change vs Control	FDR	rank
3274	hsa-miR-155	UUAAUGCUAAUCGUGAUAGGGGU	20.53	0.000	1
3128	hsa-miR-200c	UAAUACUGCCGGGUAAUGAUGGA	4.05	0.003	2
3232	hsa-miR-661	UGCCUGGGUCUCUGGCCUGCGCGU	1.83	0.017	3
ORB__0908003	hsa-miR-1469	CUCGGCGCGGGGCGCGGGCUCC	1.74	0.042	4
2088	hsa-miR-601	UGGUCUAGGAUUGUUGGAGGAG	1.45	0.042	5
3240	hsa-miR-206	UGGAAUGUAAGGAAGUGUGUGG	0.54	0.042	6
2199	hsa-miR-662	UCCCACGUUGUGGCCCAGCAG	1.64	0.042	7
2123	hsa-miR-506	UAAGGCACCCUUCUGAGUAGA		0.042	8
ORB_0409010	hsa-miR-1978	GGUUUGGUCCUAGCCUUUCUA	0.54	0.047	9
2996	hsa-miR-27a*	AGGGCUUAGCUGCUUGUGAGCA	3.18	0.047	10
3268	hsa-miR-933	UGUGCGCAGGGAGACCUCUCCC	1.85	0.047	11
ORB__0908015	hsa-miR-1911*	CACCAGGCAUUGUGGUCUCC	0.79	0.047	12
2342	hsa-miR-375	UUUGUUCGUUCGGCUCGCGUGA	0.90	0.047	13
1014	hsa-miR-132	UAACAGUCUACAGCCAUGGUCG	1.75	0.047	14

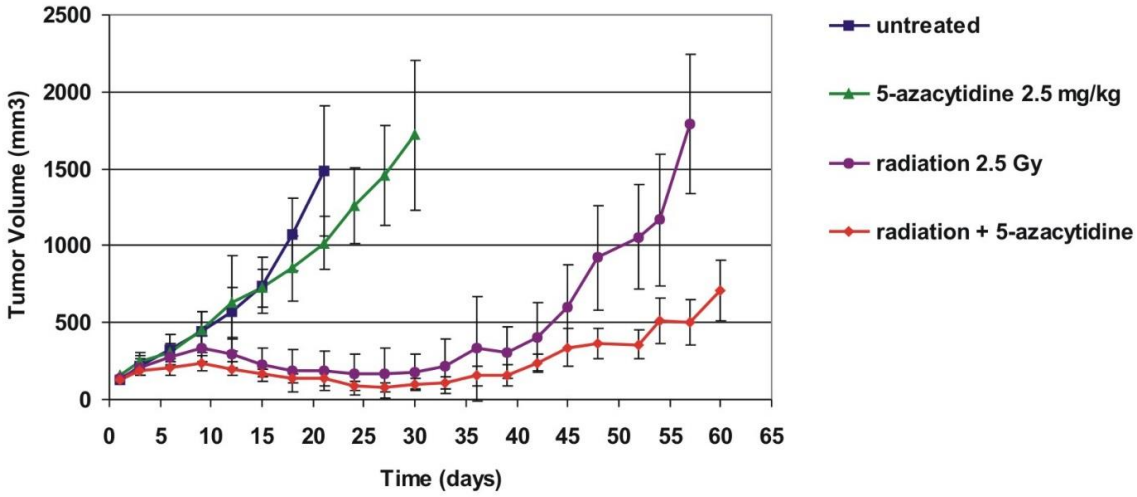
Appendix 7

5-azacytidine sensitized genomic DNA of prostate cancer PC-3 cells to radiation-induced DNA breaks. DNA fragmentation, as measured by Pulsed Field Gel Electrophoresis (PFGE) is plotted as a function of radiation dose. Treatment with a 10uM dose of 5-azacytidine resulted in increased levels of radiation-induced DNA fragmentation.



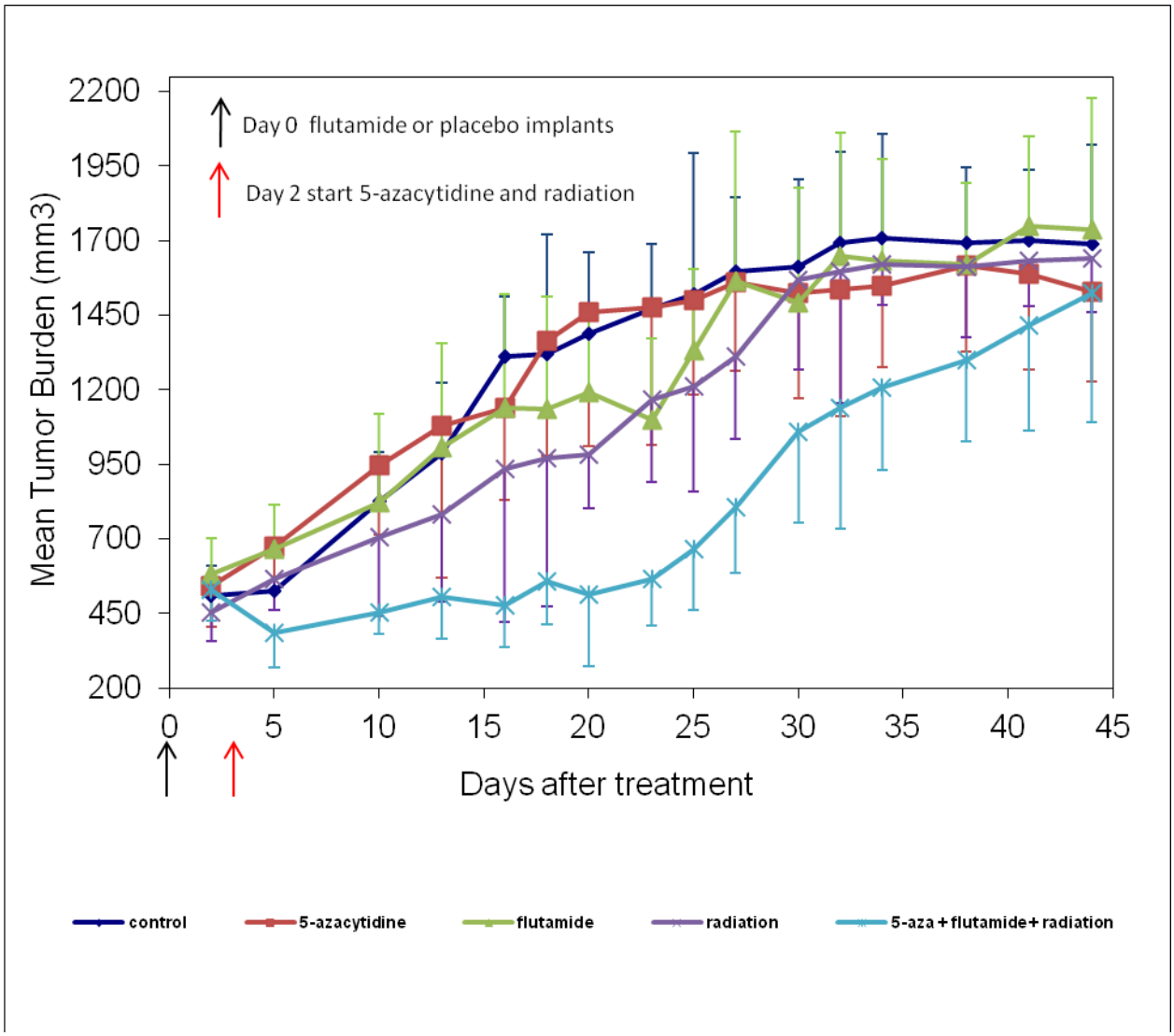
Appendix 8

5-azacytidine delays regrowth of PC-3 tumors in mice after radiation. A minimum of 6 mice per data point was used. Error bars represent standard deviations. Radiation source = X-rays



Appendix 9

Progression of PC-3 based xenograft tumor volumes in athymic nude mice after treatment with single modalities (5-azacytidine, flutamide, and radiation) or with a combined regimen of 5-azacytidine, flutamide, and radiation. Each treatment group consists of 16 animals. Radiation source = Co60 (gamma radiation).



Appendix 10

Combination Effects of Flutamide, 5-Azacytidine and XRT On Tumor Burden in Mice

Technical Report Revised

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Project: Biometry Core
Investigator: Eric Weterings
Consultee: Eric Weterings
Date: July 2013
Distribution: Biometry

0. Summary

A study was performed to investigate the combination effects of flutamide, 5-azacytidine and XRT on tumor burden in mice. When the entire range of time points (24 to 101 days) was considered, there were no statistically significant differences between the combination and any of the individual agents (all $p > 0.05$). When only the data from days 24 to 41 were included in the analysis, the tumor growth rate for Flutamide 50 mg/kg +5-azacytidine 2.5mg/kg+ XRT 2.5Gy was significantly different than that of the positive control ($p = 0.0007$), 5-azacytidine 2.5 mg/kg alone ($p = 0.0014$), and Flutamide 50 mg/kg alone ($p = 0.0034$). However, there was no statistically significant difference in the tumor growth rate for Flutamide 50 mg/kg +5-azacytidine 2.5mg/kg+ XRT 2.5Gy versus XRT 2.5Gy alone ($p = 0.1299$). Thus, although the combination was significantly different from the positive control and each of the individual drugs alone, there was no difference between the combination and radiation alone.

1. Introduction

The effect of the combination of flutamide, 5-azacytidine and XRT on tumor growth was studied in the experiment. 5-azacytidine 2.5mg/kg, Flutamide 50 mg/kg, XRT 2.5Gy and the combination of 5-azacytidine 2.5mg/kg, Flutamide 50 mg/kg, XRT 2.5Gy were studied, with Control & sham XRT as the positive control. For each mouse, the tumor burden was measured at day 24, 27, 30, 35, 38, 41, 43, 45, 48, 50, 52, 55, 57, 59, 63, 66, 69, 71, 73, 76, 79, 84, 87, 91, 94, 98 and 101. The primary goal of the study was to investigate the difference in tumor growth between treatment groups.

2. Statistical Method

The statistical analysis was performed by estimating the tumor growth for each mouse by fitting the least squares regression line of cube root of tumor burden by day. The cube root of the observed tumor burden was used to induce linearity in the values. The slope of the regression line measures the tumor growth rate. One-way analysis of variance model was employed to test for the treatment effects on tumor growth inhibition. The Dunnett adjustment was applied for multiple comparisons.

3. Results

Analysis using all days: The means and standard deviation of the slope for each group are summarized in Table 1. The analysis results (Table 2) showed that the tumor growth rate for Flutamide 50 mg/kg +5-azacytidine 2.5mg/kg+ XRT 2.5Gy was not significantly different from either 5-azacytidine 2.5mg/kg alone, Flutamide 50 mg/kg alone or XRT 2.5Gy alone. The difference between Flutamide 50 mg/kg +5-azacytidine 2.5mg/kg+ XRT 2.5Gy and positive control was not significant.

Analysis using days 24 to 41 only: The means and standard deviation of the slope for each group are summarized in Table 3. The analysis results (Table 4) showed that the tumor growth rate for Flutamide 50 mg/kg +5-azacytidine 2.5mg/kg+ XRT 2.5Gy was significantly different than that of the positive control ($p = 0.0007$), 5-azacytidine 2.5 mg/kg alone ($p = 0.0014$), and Flutamide 50 mg/kg alone ($p = 0.0034$). There was no statistically significant difference in the tumor growth rate for Flutamide 50 mg/kg +5-azacytidine 2.5mg/kg+ XRT 2.5Gy versus XRT 2.5Gy alone ($p = 0.1299$). Thus, although the combination was significantly different from the positive control and each of the individual drugs alone, there was no difference between the combination and radiation alone.

4. Data Files and Programs

aim.sd2	SAS data file
anal .sas	SAS program for all time points
dr analy days 24 to 41 only	SAS program for days 24 to 41 only
tr.doc revised	Technical report

Table 1. The Means and Standard Deviation of Slope by treatment (all days)

treatment	**N**	**mean**	**S.D.**
Positive control	15	0.164	0.034
5-azacytidine 2.5mg/kg	16	0.159	0.033
Flutamide 50 mg/kg	16	0.148	0.033
XRT 2.5Gy	16	0.106	0.033
Flutamide 50 mg/kg +5-azacytidine 2.5mg/kg+ XRT 2.5Gy	16	0.074	0.033

Table 2. Statistical Significance of Difference in Slope between Treatments (all days)

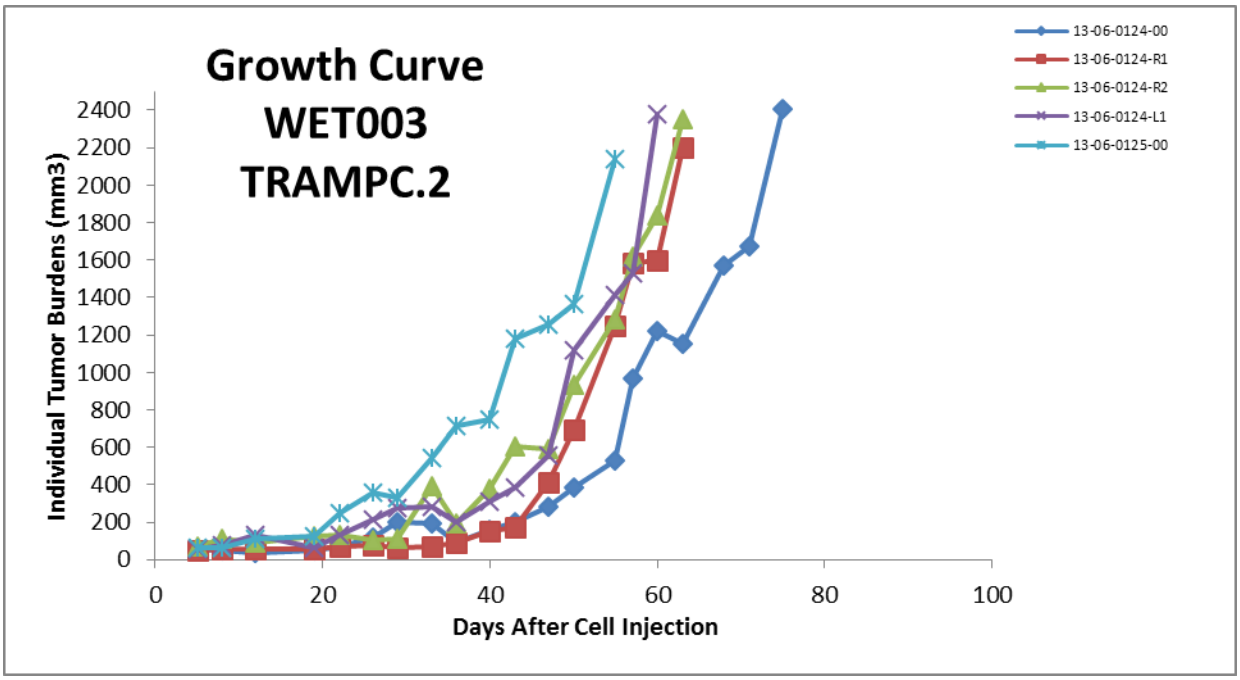
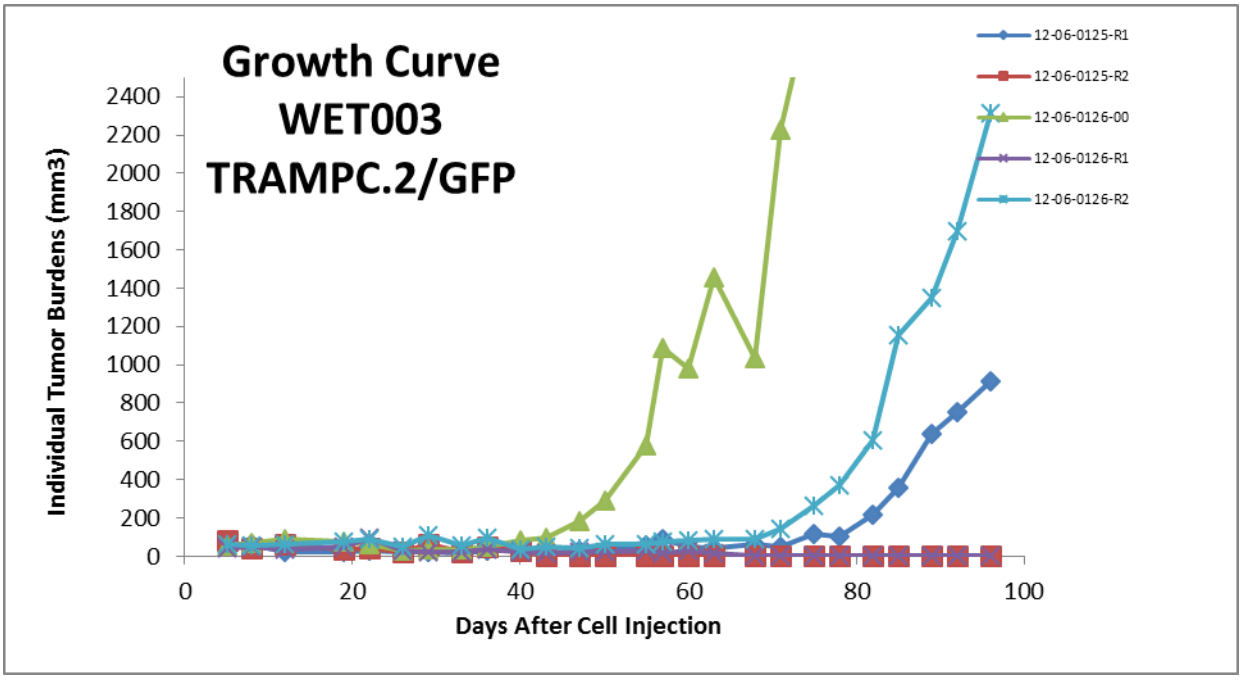
	p-value for the difference
Flutamide 50 mg/kg +5-azacytidine 2.5mg/kg+ XRT 2.5Gy vs. Positive control	0.1931
Flutamide 50 mg/kg +5-azacytidine 2.5mg/kg+ XRT 2.5Gy vs. 5-azacytidine 2.5mg/kg	0.2193
Flutamide 50 mg/kg +5-azacytidine 2.5mg/kg+ XRT 2.5Gy vs. Flutamide 50 mg/kg	0.3312
Flutamide 50 mg/kg +5-azacytidine 2.5mg/kg+ XRT 2.5Gy vs. XRT 2.5Gy	0.9010

Table 3. The Means and Standard Deviation of Slope by treatment (days 24 – 41 only)

treatment	**N**	**mean**	**S.D.**
Positive control	15	0.170	0.114
5-azacytidine 2.5mg/kg	16	0.158	0.137
Flutamide 50 mg/kg	16	0.146	0.151
XRT 2.5Gy	16	0.084	0.138
Flutamide 50 mg/kg +5-azacytidine 2.5mg/kg+ XRT 2.5Gy	16	-0.008	0.077

Table 4. Statistical Significance of Difference in Slope between Treatments (days 24 – 41 only)

	p-value for the difference
Flutamide 50 mg/kg +5-azacytidine 2.5mg/kg+ XRT 2.5Gy vs. Positive control	0.0007
Flutamide 50 mg/kg +5-azacytidine 2.5mg/kg+ XRT 2.5Gy vs. 5-azacytidine 2.5mg/kg	0.0014
Flutamide 50 mg/kg +5-azacytidine 2.5mg/kg+ XRT 2.5Gy vs. Flutamide 50 mg/kg	0.0034
Flutamide 50 mg/kg +5-azacytidine 2.5mg/kg+ XRT 2.5Gy vs. XRT 2.5Gy	0.1299

Appendix 11

Progression of TRAMP-C2 based (top panel) and TRAMP-GFP based (bottom panel) tumors in C57BL/6J mice. Each graph represents an individual tumor.