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Evidence of Y Chromosome Long Non-Coding RNAs involved in the Radiation Response of Male Non-Small Cell Lung Cancer Cells

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**Evidence of Y Chromosome Long Non-Coding RNAs involved in the Radiation Response
of Male Non-Small Cell Lung Cancer Cells**

Tayvia Brownmiller

Dissertation submitted
to the West Virginia University School of Medicine
at West Virginia University

in partial fulfillment of the requirements for the degree of

Doctor of Philosophy in
Cancer Cell Biology

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ABSTRACT

Evidence of Y Chromosome Long Non-Coding RNAs involved in the Radiation Response of Male Non-Small Cell Lung Cancer Cells

Tayvia Brownmiller

Non-small cell lung cancer (NSCLC) is the number one cause of cancer related mortality in the United States and worldwide. Advanced and therapeutically resistant lung tumors contribute to the high rate of mortality from NSCLC, therefore there is a need for new methods of diagnosing and treating this disease. Long non-coding RNAs (lncRNAs) have been shown to be a crucial component of human molecular biology, regulating nearly every cellular pathway from chromatin condensation to transcription and translation. Furthermore, many lncRNAs have been classified as oncogenes or tumor suppressors, highlighting the various molecular mechanisms they are involved in regarding the formation and progression of cancer and their use as prognostic and diagnostic biomarkers has been proposed in numerous cancers, including NSCLC.

Our group has discovered, for the first time, a three member family of Y chromosome lncRNAs (linc-SPRY3-2, lin-SPRY3-3, and linc-SPRY3-4) which regulate NSCLC cell response to ionizing radiation (IR). Briefly, the linc-SPRY3 family demonstrated a dose dependent induction of expression following exposure to IR in male radiosensitive NSCLC cell lines, but not in male radioresistant cell lines. This difference was revealed to be due to loss of the Y chromosome in the radioresistant cell lines. Using gain-of-function and loss-of-function experiments, we demonstrated statistically significant changes in cell viability and apoptosis *in vitro*. Furthermore, *in vivo* tumor growth delay assays showed a more radioresistant phenotype in tumors with knockdown of the linc-SPRY3 RNAs versus control tumors. We hypothesize linc-SPRY3-2/3/4 mediate their tumor suppressive effect via sequestration of the RNA binding protein IGF2BP3 demonstrated by CLIP and RNA degradation assays. Moreover, DNA FISH and bioinformatic analysis revealed a trending negative correlation in patient survival between loss of the Y chromosome and linc-SPRY3-2/3/4. These findings suggest that the linc-SPRY3 RNAs function as tumor suppressors by promoting cell death following IR through interactions with IGF2BP3, and could have potential as biomarkers in male NSCLC.

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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

I. Non-coding RNA

The accepted central dogma of biology that has been taught to us has been as follows: DNA is transcribed to RNA, which is then translated to protein, and proteins have long been considered the primary functional units of cellular and molecular biology. However, over the past several decades, we have learned that RNA plays a much larger role in the cell than previously thought (Fig. 1). There is actually an immense universe of non-coding RNA (ncRNA), RNA that do not code for a protein, which carry out regulatory roles once attributed to proteins (1). The two earliest examples of ncRNAs are transfer RNAs (tRNAs) and ribosomal RNAs, both of which are extremely important in translation. Transfer RNAs (tRNAs) are carriers for amino acids that are incorporated into a peptide. Ribosomal RNAs (rRNAs) make up the ribosome, including the ribozyme, an enzymatic RNA which is responsible for processing other RNA molecules such as the tRNA or splicing of nuclear pre-mRNA (2). While these types of RNAs have been extensively studied, they are only a small part of an expansive RNA world. Thanks to the continuing advancement of sequencing technologies and completion of The Human Genome Project followed

Figure 1: Timeline of discoveries of RNAs in biological regulation. (Republished with permission of Annual Reviews, Inc. from Genome Regulation by Long Noncoding RNAs, John L. Rinn and Howard Y. Chang, Volume 81, *Annual Review of Biochemistry*, 2012; permission conveyed through Copyright Clearance Center, Inc.)

by the Encyclopedia of DNA Elements (ENCODE), we have learned that roughly 70-80% of human genome is transcribed, but less than 2% of that RNA is translated to protein. This means that nearly 98% of the transcripts produced in a human cell are ncRNA. Originally, this 98% of RNA was considered “junk” or transcriptional noise, with no function and therefore was considered unimportant, but we now have a much better understanding of this “dark matter” of the genome (3,4).

Interestingly, they offer the connection to organism complexity that was once unknown. What we know now is that the more complex the organism, the larger the noncoding RNA content of that organism, with the human genome harboring the largest percentage of noncoding information compared to other genomes (5). We also know that ncRNAs are involved in nearly every facet of cellular biology, and are categorized in numerous ways, beginning with regulatory or housekeeping ncRNAs. Housekeeping ncRNAs are those important for translation, such as tRNAs and rRNAs. The rest are grouped into regulatory ncRNAs, a much larger group which is categorized first by size. NcRNAs larger than 200 nucleotides are classified as long non-coding RNAs (lncRNAs), which have wide ranging functions. Any ncRNAs smaller than 200 nucleotides are classified as small ncRNAs encompassing microRNAs (miRNAs), small nucleolar RNAs (snoRNAs), small interfering RNAs (siRNAs), small nuclear RNAs (snRNAs), and PIWI-interacting RNAs (piRNAs), each of which have their own specific functions (Fig. 2) (6).

Figure 2: The Major classes of RNAs. (Modified from – Major Tumor Suppressor and Oncogenic Non-Coding RNAs: Clinical Relevance in Lung Cancer, Kentaro Inamura, *Cells*, 2017)

As previously mentioned, ncRNAs function in a myriad of ways which are important for every cellular process. Because they are so intricately woven into our basic biological functions, their dysregulation leads to the development of disease, including cancer (7). While the roles of

the small RNAs, especially miRNAs, have been relatively well studied in regards to cancer cell biology, lncRNAs remain poorly characterized in comparison.

II. Long Non-coding RNA

A. Overview

Long non-coding RNAs (lncRNAs) are those RNAs greater than 200 nucleotides long and lack an open reading frame, and due to their emergence over the past 15 years, there has been a fundamental shift in the landscape of molecular genetics (8). lncRNAs are comprised of intergenic transcripts, enhancer RNAs, and sense or antisense transcripts that overlap coding genes and can function in *cis* or *trans*. They also show incredible cell type and tissue specificity. Those that have been functionally characterized have revealed a myriad of roles, many of which are dictated primarily by sequence and/or cellular localization (9). As it stands, there are currently over 120,000 identified lncRNAs in the human genome (10). Unfortunately, only a handful of these have been functionally characterized leaving hundreds of thousands left to be studied.

B. Characterization

Through advancements in RNA sequencing technologies, we have learned that lncRNAs are abundant and extremely diverse. In general, a non-coding RNA (ncRNA) is classified as a lncRNA if they are larger than 200 nucleotides, but their sizes are very heterogeneous ranging from a few hundred to even larger than a few thousand nucleotides. Furthermore, they originate from nearly every part of the human genome, can be found in virtually every cellular compartment, and carry out numerous functions (8,11). Unfortunately, all of these factors combined with a limited understanding of lncRNAs has resulted in poorly defined system for classification.

LncRNA – Separating non-coding from coding transcripts

The major defining factor for any ncRNA is its lack of coding potential, or its inability to be translated into a protein, but quantitatively determining this characteristic is not an easy task. Moreover, lncRNAs have begun to emerge as a gray area in terms of coding potential, as many lncRNAs have been found to contain small open reading frames (smORF) which could produce

micropeptides. It is also important to note that some mRNAs could have non-coding functions (12).

The primary method for determining coding potential of a RNA is ribosome profiling followed by deep sequencing (RiboSeq). Ribosomes are the cornerstone of protein translation. Briefly, when ribosomes associate with an RNA during translation (protein synthesis), they also protect that piece of RNA from digestion by exonucleases. That protected RNA can be recovered and then be used for deep sequencing and the sequencing data can be aligned to a genome which determines the translational profile of a given RNA (Fig. 3) (13). LncRNAs typically have no to low enrichment of ribosome occupancy and therefore are classified as non-coding (14). However, as sequencing technologies advance and become more sensitive, we are able to better detect and identify small ORFs such as those attributed to micropeptide encoding lncRNAs (15).

There are also computational methods that can be utilized. A few of the more popular pipelines are phylogenetic analysis of codon substitution frequencies based on sequence alignment (PhyloCSF) scoring, sORF finder, coding region identification tool invoking comparative analysis (CRITICA), and micro-peptide detection pipeline (micPDP) (16–19). It is important to note that some controversy still surrounds this grey area as micropeptide potential is a very new field in the realm of lncRNAs. Furthermore, just because a lncRNA contains a small ORF and can produce a micropeptide, does not mean its primary function isn't that of a non-coding RNA.

Biogenesis

Figure 3: Overview of Ribosome Profiling (Adapted by permission from Springer Nature Customer Service Centre GmbH: Springer Nature, *Nature Reviews Molecular Cell Biology*, Ribosome profiling reveals the what, when, where and how of protein synthesis, Gloria A. Brar and Jonathan S. Weissman, 2015.)

In general, lncRNAs are very similar to messenger RNAs (mRNAs). Their promoters display similar epigenetic markers, they are transcribed via Pol II, undergo splicing, and can include both a 5' cap and a Poly A tail. However, that is where the similarities end (20). Every gene is controlled first by epigenetic markers such as histone methylation and acetylation, essentially signaling if a RNA should be transcribed. Both mRNAs and lncRNAs are controlled by promoter enrichment of trimethylation of lysine 4 on histone 3 (H3K4me3), but lncRNAs typically show much higher enrichment of acetylation of lysine 27 of histone 3 (H3K27ac), with more recruitment of transcriptional regulators like Swr1, Isw2, Rsc, and Ino80 resulting in repression (21). This would explain the relatively low expression of most lncRNAs across human tissues.

As previously mentioned, lncRNAs are primarily transcribed by Pol II, and processed similarly as mRNAs by splicing and 3' polyadenylation. However, there are some mechanisms of post-transcriptional regulation that affect lncRNAs and not mRNAs. For example, the lncRNA NEAT1 can be processed like a mRNA to produce what is known as its short form, but alternatively its long form contains a pre-tRNA like structure on its 3' end which is recognized and cleaved by the ribonucleoprotein complex RNaseP (responsible for processing mature tRNAs). The resulting free end then forms a triple helix structure, stabilizing the lncRNA strand (22). Some other forms of alternative RNA processing that affect lncRNAs includes splicing events that result in the formation of circular RNAs (circRNAs). This includes products from canonical splicing called lariat loops, formed by a 2',5'-phosphodiester bond, or the non-sequential splicing of introns, otherwise known as backsplicing. The difference between the two being that circRNAs formed from backsplicing are typically more stable since there are no free ends for exonucleases to recognize and degrade (20,23).

Complexities of Classification

Due to the ever-growing field of lncRNA biology, there is no one unified way to classify a lncRNA other than the basic attributes that they are longer than 200 nucleotides and lack an open reading frame. This cutoff of length is an arbitrary number, set to separate lncRNAs from their short ncRNA counterparts (miRNA, snoRNA, piRNA, etc.). Other than this, there are a number of subclasses of lncRNAs which divides them based on characteristics such as genomic location,

association with DNA elements, tandem repeat content, sequence or structure conservation, cellular localization, and function, just to name a few (24). This has led to some confusion in regards to lncRNA characterization, classification, and even naming. However, as the field of lncRNA biology advances one thing is constant. This species of complex RNAs is extremely important and nearly all aspects of cellular and molecular biology.

Genomic Location

The most commonly used method of classification is based on the genomic location relative to a protein coding gene (8). LncRNAs can be classified as sense or antisense, intronic, divergent (bidirectional), or intergenic (Fig. 4). Sense lncRNAs are transcribed in the same direction of a

protein coding gene and contain one or more exons, and sometimes overlap or cover an entire sequence of a protein coding gene. These overlapping lncRNAs are considered non-

Figure 4: Classes of non-coding transcripts based on genomic location relative to a coding gene. (Reprinted/Adapted by permission from Springer Nature Customer Service Centre GmbH: Springer Nature, Springer eBook: *History, Discovery, and Classification of lncRNAs* by Julien Jarroux, Antonin Morillon, Marina Pinskaya, 2017)

coding transcript variants of the protein coding gene as they originate from the same promoter but lack an open reading frame. Antisense lncRNAs are transcribed in the opposite direction of a protein coding gene, usually initiate inside or downstream of a protein coding gene, and include at least one exon from the protein coding gene. Their expression typically correlates with an mRNA of the same promoter and have been shown to be very important in cancer, usually regulating the expression of tumor suppressor or oncogenic proteins (25). Intronic lncRNAs, as the name suggests, are lncRNAs that initiate from an intron, are composed entirely of introns and can be transcribed in either direction of a protein coding gene. These intronic transcripts have not been well studied, but have been implicated in regulating RNA splicing and stability (26,27). Divergent, or bidirectional, lncRNAs are transcripts that initiate in the opposite direction as its protein coding gene counterpart, typically around a few hundred bases to 1 kilobase from a neighboring protein coding gene promoter (11,28). Finally, intergenic lncRNAs (lincRNAs) are transcripts that

originate from their own transcriptional unit which is found between protein coding units. LincRNAs are by far the most studied among the above listed classes. They have been shown to function in both *cis* and *trans*, affecting transcription, translation and splicing. They have also shown high conservation between species (28,29).

Cellular Localization

LncRNAs can also be classified by their cellular localization. The two main methods primarily used to identify a lncRNA's primary compartment are cellular fractionation and RNA fluorescent *in situ* hybridization (FISH). Cellular fractionation involves lysing cells and separating the nuclear fraction from the cytoplasmic fraction, primarily by multiple centrifugation steps, followed by methods like quantitative reverse transcriptase PCR (qRT-PCR) or even RNA-seq (30). RNA FISH is a method in which a fluorescent probe complementary to an RNA of interest is designed and incubated with cells or tissue of interest. The probe should hybridize to its target RNA and this can be imaged via fluorescent microscopy (31).

Identifying a perspective lncRNA's compartment is a very important part of characterizing a lncRNA, as it narrows the potential functions inside a cell. Unlike mRNAs, the vast majority of lncRNAs are localized to the nucleus due to a number of factors such as inefficient splicing and polyadenylation. Many lncRNAs essentially retained to the nucleus due to their interactions with nuclear structures and proteins. Nuclear lncRNAs typically regulate epigenetic markers, chromatin structure, transcription, and post-transcriptional processing (32,33). To a much lesser extent, there are some lncRNAs which localize to the cytoplasm. Cytoplasmic lncRNAs have been shown to regulate translation, post-translational processing and interact directly with other regulators in the cytosol like microRNAs (miRNAs) and even other mRNAs. Furthermore, a few cytoplasmic lncRNAs have been shown to produce micropeptides, though this function needs more study to fully understand its role in the cell (33,34).

Biological Functions

Unlike proteins, the extent of possible lncRNA functions has yet to be elucidated, nonetheless it has emerged as a way to classify a lncRNA. Those handful of lncRNAs that have been well characterized have revealed a number of important functions, nearly all of which are

regulatory. As mentioned before, a lncRNA's function is highly determined by the cell compartment in which they are found. Furthermore, their functions can be categorized as *cis*, those that directly regulate neighboring genes, or as *trans*, those that translocate to another part of the cell to perform its function. The main classifications have been previously described in four major

Figure 5: Four Major Groups of LncRNA Functions [Reprinted from *Molecular Cell*, Volume 43, Kevin C. Wang and Howard Y. Chang, Molecular Mechanisms of Long Noncoding RNA, 904-914, Copyright (2011), with permission from Elsevier]

groups: signals, decoys, guides, and scaffolds (Fig. 5). However, these four groups are much more complex and a single lncRNA can have multiple functions within the cell (Fig. 6) (35–37).

Figure 6: LncRNA Molecular Mechanisms (Modified from – Long non-coding RNAs: new players in hematopoiesis and leukemia, Morlando et al, *Front. In Med.* 2015)

eRNA: Enhancer lncRNAs (eRNAs) are a type of nuclear lncRNA which can enhance target gene expression, and can be considered both scaffold and signal lncRNAs (Fig. 6A). They can originate from enhancer regions of DNA and stand apart from most “traditional” lncRNAs as they can be transcribed in either direction and most do not undergo maturation such as splicing and polyadenylation. Most importantly, eRNAs function by regulating transcription by recruiting transcription factors and acting as a bridge to bring two genes close together, one of which is usually an enhancer region, primarily by intrachromosomal interactions via chromosome looping or alternatively by interchromosomal interactions by bringing two different chromosomes together (Fig. 6D) (38). An example of an eRNA is the lncRNA HOTTIP (*HOXA* distal transcript antisense RNA). HOTTIP functions in *cis* and facilitates chromosome looping which brings itself into

proximity with the HOXA gene cluster. From there, it recruits WDR5 and the MLL complex which triggers deposition of H3K4me3 to activate transcription of HOXA genes (39).

Chromatin modifiers: One of the better characterized lncRNA functions is their role in chromatin modification and remodeling (Fig. 6B). They can be categorized as scaffolding or guide lncRNAs, are nuclear, can act in either *cis* or *trans*, and can recruit chromatin modifiers that can either promote transcription or repress it (40). One of the most well studied examples is the lncRNA Xist (X-inactive specific transcript). Xist's primary function is the silencing of one of the two X chromosomes in female cells (X chromosome inactivation). Xist coats the inactive X chromosome with multiple copies of itself which recruits transcriptional repressors such as the polycomb repressive complex 2 (PRC2) triggering accumulation of H3K27me3 (41).

Transcriptional regulators: A number of lncRNAs have been shown to directly interact with protein binding partners and thereby affect gene expression by regulating transcription (Fig. C). Their roles in transcription can happen in *cis* or *trans*, and can be activating or repressive. ANRIL is an antisense lncRNA clustered with the genes *CDKN2A* and *CDKN2B* and represses them in *cis* by recruiting both PRC1 and PRC2 to the gene loci (42). In contrast, the lncRNA Evf-2 (DLX6-AS1) acts as a transcriptional cofactor and forms a complex with DLX2 to activate transcription of genes located at the DLX5/6 locus (43). LncRNAs can also affect transcription by acting as decoys to sequester transcription factors which can inhibit or promote transcription of target genes (44).

Regulation of mRNA splicing, stability, and translation: Alongside regulating transcription, lncRNAs can also function post-transcriptionally by affecting the maturation, translation, and degradation of mRNAs (Fig. 6E-H). For example, MIAT (myocardial infarction associated transcript) sequesters splicing factor 1 (SF1) to inhibit formation of the spliceosome complex during splicing (45). Another example is the famous lncRNA MALAT1, which can bind SR splicing factor [serine-arginine (SR)-rich splicing factor] and affect its distribution, downregulating splicing (46). In terms of affecting mRNA stability, some lncRNAs have been shown to directly interact with mRNAs, either to stabilize the mRNA strand itself (e.g. BACE1-AS) or to act as a guide for proteins necessary to stabilize an mRNA (e.g. TINCR) (47,48).

Similarly but in contrast, lncRNAs can destabilize mRNA strands and downregulate protein expression by inducing degradation of a target mRNA (e.g. 1/2sbsRNAs) (49). Additionally, lncRNAs can influence (e.g. lincRNA-p21) translation of target mRNAs (50). LncRNAs can also function as competing endogenous RNAs (ceRNAs) and act as molecular sponges, not just for proteins but also for miRNAs. miRNAs regulate protein expression by binding the 3' UTR of a target mRNA and inhibiting translation or triggering mRNA degradation. LncRNAs can “sponge” miRNAs, preventing them from targeting an mRNA, which allows that mRNA to be translated (51).

Micropeptide encoding lncRNAs: One of the most recent discoveries in lncRNA biology is the capacity for some lncRNAs to contain small open reading frames (smORFs) which can be translated into functional micropeptides (usually <100 amino acids). These lncRNAs could be their own transcript, or a non-coding variant transcript from a protein coding gene and typically localize to the cytoplasm of the cell (Fig. 6I). Computational analysis has predicted numerous smORFs, but studies have suggested that only around 17% of predicted smORF containing lncRNAs are translated. Moreover, these micropeptides are expressed at very low levels and are typically unstable (12,15,52). However, there are some examples of functional micropeptides derived from putative lncRNAs. DWORF is a muscle specific micropeptide (34 amino acids) which originates from the human lncRNA LOC100507537 and functions by displacing SERCA (sarco/endoplasmic reticulum Ca^{2+} -ATPase) membrane pump inhibitory proteins which allows increased calcium uptake and myocyte contractility (53).

Overall, it can be easily determined that lncRNAs are extremely vital to cellular and molecular biology and offer an explanation for organism complexity that scientists have sought for decades. It is important to note that, while we have made great strides in characterizing lncRNAs, we have barely scratched the surface of their importance and potential and given their broad range of functions we should look to them for answers regarding numerous pathologies, including cancer.

C. LncRNAs and Cancer

LncRNAs have emerged as one of the most important and diverse regulators in the human cell. Considering their widespread involvement in nearly every biological process, it is easy to assume that their dysregulation can lead to the development of disease including cancer. Cancer is a collection of diseases, but nearly all have one or more disruptions in the molecular pathways of what cancer researchers call “the hallmarks of cancer”(54). We now know that lncRNAs are just as intricately involved in these processes as proteins and act as tumor suppressors or oncogenes. However, it is important to note that a tumor suppressive or oncogenic role is highly dictated by the cancer type in which a lncRNA is dysregulated (Fig. 7) (55).

Figure 7: LncRNAs associated with the hallmarks of cancer. (Long non-coding RNAs in cancer: Another layer of complexity, de Oliveria et al, *J. Gene Med.* 2019)

Tumor suppressor genes, as their name suggests, are those genes necessary to suppress carcinogenesis (56). There are a number of tumor suppressor proteins (p53, Rb, BRCA, etc.) and the same can be said for lncRNAs, where their dysregulation via loss or downregulation of their expression results in the promotion of a malignant phenotype. One of the most well characterized tumor suppressive lncRNAs is MEG3. Downregulated in multiple cancer types (renal, liver, lung, and brain) its primary function is regulating the tumor suppressor p53 (57). MEG3 downregulates MDM2 expression, an E3 ubiquitin ligase responsible for targeting p53 for degradation. This allows p53, a transcription factor, to function properly and regulate its target genes which are important in a number of cellular processes such as cell cycle and apoptosis (58). Another well-known example is the lncRNA GAS5 (growth arrest specific transcript 5). It has also been associated with numerous cancer types (breast, renal, prostate, and endometrial) and it is an important regulator of cell metabolism (among other mechanisms) which binds glucocorticoid receptor (GR), inhibiting the activation of GR dependent genes. This leads to metabolic stress and eventually cell death (59,60). There are even lncRNAs that are important for regulating telomeres,

the ribonucleoprotein structures at the end of chromosomes. In healthy cells, telomeres shorten every time a cell divides. Once they reach a certain size, the cell is signaled to stop dividing, either undergoing apoptosis or permanent cell growth arrest. One component of telomeres is a group of lncRNAs called telomeric repeat-containing RNAs or TERRA (61). While the complete mechanisms of TERRA have yet to be elucidated, they have been shown to interact with and inhibit telomerase, an enzyme hijacked by cancer cells to maintain telomeres and essentially mediate immortality (62). Additionally, TERRA transcripts are commonly downregulated in cancer, further supporting their role as tumor suppressors in human cells (55,63).

Oncogenic LncRNAs

Oncogenes are those genes that give rise to proteins or lncRNAs that promote tumorigenesis, and upregulation or activation of oncogenic factors results in phenotypes that are beneficial to cancer (56). Some well-known oncogenic proteins include members of the RAS family (KRAS, NRAS, and HRAS), Myc, and numerous receptor tyrosine kinases (RTKs, e.g. EGFR, VEGF, and RET). These proteins stimulate tumor formation via promoting proliferation, resisting apoptosis, and even reprogramming cancer cell metabolism (64). Not surprisingly, there are lncRNAs that have been shown to promote the same oncogenic phenotypes. One example of a fairly well studied oncogenic lncRNA found upregulated in multiple tumor types is HOX transcript antisense RNA (HOTAIR). It's been associated with many different signaling pathways (proliferation, apoptosis, and invasiveness) across numerous cancers including lung, breast, pancreatic and ovarian cancer. It's a wildly promiscuous lncRNA that can regulate the PI3K/Akt pathway by suppressing the expression of the tumor suppressor PTEN, affect cell adhesion and cancer cell epithelial-to-mesenchymal transition via inhibiting expression of proteins such as Wnt inhibitory factor-1 (WIF-1), and also promote metastasis by silencing of specific genes through PRC2 (63,65). Another example is the lncRNA PCAT1 (prostate cancer-associated transcript 1), which is named for the cancer in which it was originally identified. While it is mostly prominent in prostate cancer cell biology, we now know that it is upregulated in other cancer types and functions primarily by upregulating cell proliferation by post-transcriptionally stabilizing the oncogenic protein c-Myc. Furthermore, it essentially forms a positive feedback loop with c-Myc since c-Myc is the transcription factor that controls the expression of PCAT1 (66–68).

Clinical Relevance

Given their dysregulation in cancer and higher tissue specificity relative to their protein counterparts, lncRNAs have become attractive candidates as potential biomarkers (69). One of the best examples is PCA3, a prostate specific lncRNA that is undetectable in healthy tissue, overexpressed in >90% of prostate cancers and can be detected in the urine of patients (70). In 2012, it became the first lncRNA approved by the US Food and Drug Administration for biomarker use and is now considered more reliable than the standard prostate-specific antigen (PSA) level test since its use is more accurate for prostate cancer diagnosis (71). A recent search of ClinicalTrials.gov revealed 5 ongoing biomarker studies looking at lncRNAs including specific trials investigating HOTAIR for use in thyroid cancer (NCT03469544) and CCAT1 for use in colorectal cancer (NCT04269746). Though their clinical use is slowly emerging, the list of potential biomarker lncRNAs is continuously growing, and with the advancements of less invasive techniques such as the liquid biopsy, it is only a matter of time before more lncRNAs are proven as effective diagnostic and prognostic indicators.

lncRNAs have also been investigated as targets for cancer therapeutics. As mentioned before, lncRNAs are highly tissue and/or cancer specific which makes them ideal candidates as druggable targets. Current methods in development include RNAi (siRNA, miRNA), antisense oligonucleotides (ASOs), and small molecules, however none have yet to enter clinical trials. There are multiple examples of these methods effectiveness *in vitro* and *in vivo*, however the lack of an efficient systemic delivery method has proven challenging for use in humans. The alternative option to directly targeting a perspective lncRNA is identifying novel binding partners or downstream effectors and targeting them instead (69). Considering the multitude of functions attributed to lncRNAs, these strategies could one day prove useful in treating cancers.

III. lncRNAs and Non-Small Cell Lung Cancer (NSCLC)

A. Lung Cancer Overview

In the U.S., lung cancer is the number one cause of cancer related mortality, and is the second most commonly diagnosed cancer amongst both men and women. Currently, the average

overall 5 year survival rate is 18.6%, which can be attributed to the fact that more than half of lung cancer cases are diagnosed at late stages (72).

Lung cancer is typically divided into two major types, small cell lung cancer (SCLC), which makes up ~20% of cases, and non-small cell lung cancer (NSCLC), which makes up ~80% of cases. NSCLC is then further divided into three histological subtypes, lung adenocarcinoma (LUAD), lung squamous cell carcinoma (LUSC), and large cell carcinoma (LCC). Of these subtypes, LUAD is the most common, followed by LUSC and then LCC. Interestingly, the subtype most found in non-smokers is LUAD, while LUSC is mostly found in those who currently smoke or have a history of smoking. While most cases can be linked to smoking, other causes also include genetic changes and carcinogenic environmental factors such as asbestos or radon gas (73). Regardless of the cause, genetic alterations such as activation or overexpression of oncogenes and/or down regulation or loss of tumor suppressors and their affected pathways are crucial in the development of lung cancer. With advancements in genomics and proteomics, there have been over two dozen alterations identified that can be classified as “driver mutations.” These are mutations which occur typically in a tumor suppressor gene or oncogene. For NSCLC, the most common are changes in *TP53*, *KRAS*, *EGFR*, and *CDK2NA*. Alterations in these genes results in drastic consequences, leading to changes in multiple cellular processes such as cell cycle regulation, DNA damage response, growth signaling and even metabolic reprogramming, leading to tumorigenesis (74,75). Additionally, like changes in protein coding genes, changes in lncRNAs can also wreak havoc in the cell.

B. Roles of LncRNAs in NSCLC

Numerous lncRNAs have been found to affect all the major hallmarks of cancer, and thanks to the continuing advancements in RNA sequencing technologies, more and more are being discovered. In NSCLC specifically, there have been a number of dysregulated lncRNAs (oncogenic and/or tumor suppressive) characterized to affect nearly all major hallmark pathways involved in carcinogenesis including epithelial-to-mesenchymal transition (EMT), apoptotic resistance, migration and metastasis, and therapy resistance (76,77).

MALAT1

Arguably one of the most studied lncRNAs associated with not only lung cancer but numerous cancers is the oncogenic Metastasis-Associated Lung Adenocarcinoma Transcript 1 (MALAT1). As its name suggests, it was first characterized in lung adenocarcinoma as a mediator of metastasis (78), but further studies revealed it is actually one of the most abundantly expressed lncRNAs in normal human tissues. Only when is it dysregulated, usually overexpressed, does it become oncogenic (79). Since its discovery, MALAT1 has been shown to carry out multiple functions in NSCLC. The most common functions typically involve regulation of RNA splicing and are associated with pathways that promote invasion and metastasis. Moreover, studies using NSCLC cell lines have shown that depletion of MALAT1 leads to a decrease in migration, invasiveness and metastatic potential (80). Interestingly, MALAT1 can also act as a competing endogenous RNA (ceRNA) by sponging miRNA. One study describes an ER β /MALAT1/miR-145-5p/NEDD9 axis that promotes the phenomenon vasculogenic mimicry (the ability for cancer cells to trans-differentiate into endothelial like cells) and NSCLC invasion. Briefly, estrogen receptor β (ER β) signaling upregulates the expression of MALAT1, which in turn sequesters miR-145, allowing NEDD9 to be expressed and promote cell invasiveness (81). Another mechanism involving sponging of miR-145 promotes resistance to the chemotherapeutic agent cisplatin in NSCLC cell lines. Sequestration of miR-145 lead to an increase in Kruppel-like factor 4 (KLF4), a factor known to regulate stemness in cancer cells and promote resistance to chemotherapeutics (82). MALAT1 has also been shown to promote cisplatin resistance through STAT3 activation which upregulates MDR1 and MRP1, known mediators of drug resistance (83). Additionally, numerous studies have proposed MALAT1 as a prognostic marker for NSCLC. Overall, high expression correlates with worse overall survival and high metastatic potential in NSCLC patients (84,85).

HOTAIR

The lncRNA HOX Transcript Antisense RNA (HOTAIR) is another well studied and characterized oncogenic lncRNA found in numerous cancers. In lung cancer, it has been shown to be important in cell proliferation, invasion, and therapeutic response, typically when overexpressed compared to normal tissue (86). One study found that the tumor suppressor p53 negatively regulates HOTAIR expression by directly binding to and occupying its promoter. Furthermore, overexpression of HOTAIR resulted in downregulation of p53 via recruitment of

PRC2 to the p53 promoter, revealing a feedback loop between HOTAIR and p53 that causes changes in cell proliferation and invasiveness (87). Another study revealed that hypoxia is a major regulator of HOTAIR expression in NSCLC. Briefly, hypoxia turns on expression of the transcription factor HIF-1 α , a major regulator of cellular hypoxia response, which binds to hypoxia response elements within the HOTAIR promoter. The upregulation in HOTAIR expression resulted in enhanced cell proliferation, migration, and invasion under hypoxic conditions (88). HOTAIR can also function as a ceRNA to influence tumorigenesis. For example, one study showed that HOTAIR can sponge the miRNA miR-217 which prevents downregulation of dachshund homolog 1 (DACH1), a cell fate determination factor favorable for tumorigenesis (89). Additionally, HOTAIR has been shown to influence cellular response to chemotherapeutics such as Crizotinib, a tyrosine kinase inhibitor, via promoting autophagy in NSCLC cell lines (90). Lastly, increased expression of HOTAIR is negatively correlated with NSCLC patient outcomes (91,92).

MEG3

MEG3 (maternally expressed gene 3) is one of only a handful of characterized tumor suppressor lncRNAs. It is found expressed in many normal tissues, and its loss is associated with numerous cancer types (57). Furthermore, one of the primary roles of MEG3 is activation and accumulation of the tumor suppressor protein p53. It is suggested that it stabilizes the p53 protein which promotes increased activity of the protein and proper regulation of p53 target genes which control cell cycle and apoptosis (58). In NSCLC, the loss of MEG3 is attributed to apoptotic resistance and increased proliferation. One study demonstrated that exogenous overexpression of MEG3 significantly increased the expression and activity of p53. This resulted in reduced cell proliferation and increased apoptosis *in vitro* and slower tumor growth *in vivo* (93). Another tumor suppressive mechanism attributed to MEG3 involves its regulation of Skp2, a component of the ubiquitin ligase complex responsible for degrading p27, a master cell cycle regulator. Briefly, MEG3 coordinates with miR-3163 to inhibit the translation of Skp2 which allows expression of p27 and eventually inhibits cell growth (94). Additionally, through a miRNA mediated mechanism, MEG3 has been shown to enhance the sensitivity of NSCLC cells to cisplatin. Cells with low MEG3 expression demonstrated resistance to cisplatin, which was reversed via exogenous overexpression of MEG3, mediated by a miR-21-5p/SOX7 signaling axis (95). It is

also important to note that in addition to the mechanisms outlined above, MEG3 has also been suggested as a prognostic marker in NSCLC, as low expression of the lncRNA correlates with poor survival (96).

GAS5

Growth arrest specific transcript 5 (GAS5) is another tumor suppressor lncRNA found expressed in multiple tissues, but downregulated in a number of cancer types including NSCLC. Overall, it has been associated with a number of signaling pathways such as cell cycle, cancer cell metabolism, proliferation, and apoptosis (59). One study revealed GAS5 was downregulated in NSCLC tissues when compared to normal adjacent tissues suggesting its function as a tumor suppressor. Further investigation in the same study revealed that exogenous expression of GAS5 in NSCLC cell lines slowed cell proliferation and decreased colony formation ability as well as induced apoptosis (97). Another tumor suppressive mechanism in NSCLC attributed to GAS5 is its ability to regulate glucose signaling. In summary, GAS5 functions in a delicate feedback loop with glucose signaling. It was shown that high glucose fed NSCLC cells downregulated the expression of GAS5 which increased proliferation and migration, while exogenous overexpression of GAS5 attenuated the affect through direct interaction with TRIB3, an important mediator of glucose signaling (98). GAS5 has also been shown to interact with miRNAs to affect NSCLC cell proliferation, metastasis and radiation sensitivity. For example, GAS5 can function as a ceRNA for miR-205. In short, miR-205 suppresses the translation of the tumor suppressor protein PTEN. GAS5 therefore promotes the expression of PTEN by sponging miR-205. Expression of PTEN results in slower proliferation and decreased migration and invasion in NSCLC cells (99). Additionally, GAS5 has been shown to sponge miR-135b, inhibiting tumorigenesis and promoting radiation sensitivity both *in vitro* and *in vivo* (100). Lastly, thanks to technological advances and the development of liquid biopsy methods, low levels of circulating GAS5 has been proposed as a potential biomarker for the prognosis and diagnosis of NSCLC (101,102).

Overall, it can be concluded that lncRNAs are an extremely important factor in the tumorigenesis and treatment of NSCLC. Unfortunately, a lot more work needs to be done in regards to the clinical applicability of lncRNAs.

IV. Radiation Therapy

A. Overview

Radiation therapy (RT) is a major component of cancer therapy. Currently, there are multiple modalities (intensity modulated radiotherapy, image-guided radiotherapy, stereotactic body radiotherapy, etc.) utilized to administer radiation to tumors, typically combined with a chemotherapeutic agent which is dictated by the cancer type and location of the tumor. The clinically approved modalities use one of two kinds of radiation, photon radiation (x-rays and gamma rays) or particle radiation (electron, proton, or neutron beams). The major difference between photon radiation and particle radiation is the rate of energy emission. Overall, particle radiation has a higher linear energy transfer (LET), resulting in more DNA damage compared to photon radiation. However, photon radiation is more commonly used because the equipment needed to administer particle radiation is difficult to manufacture and much more expensive (103). Regardless of their differences, they both have proven effective in the treatment of most cancers, whether through curative or palliative care.

The component shared amongst all types of radiation therapy is the mechanisms by which it kills cancer cells. The primary mechanism is by direct DNA damage, inducing double stranded (DSB) and single stranded breaks (SSB) (Fig. 8A). The secondary mechanism is by the creation of free radicals which signals cell stress and also causes DNA damage (104). DNA damage then triggers a number of molecular signaling cascades which attempt to fix the damage [non-

Figure 8: The effects of radiation-induced DNA damage. (Reprinted/Adapted from *Clinical Cancer Research*, 2015, Volume 21, Issue 13, 2898-2904, Meredith A. Morgan and Theodore S. Lawrence, Molecular Pathways: Overcoming Radiation Resistance by Targeting DNA Damage Response Pathways, with permission from AACR)

homologous end joining (NHEJ), alternative end joining (alt-EJ), and homologous recombination (HR)] and slow/stop cell cycle progression, however the mass amount of stress and genomic instability typically overwhelms these repair mechanisms and results in senescence (permanent cell growth arrest), or cell death via apoptosis or necrosis (Fig. 8B and 8C) (104,105). Unfortunately, like most therapies, there are molecular mechanisms of resistance that can make treating cancers with radiation very difficult (Fig. 9).

Figure 9: Mechanisms of resistance against radiotherapy. (Therapeutic Implications for Overcoming Radiation Resistance in Cancer Therapy, Kim et al, *Int. J. Mol. Sci.*, 2015)

B. *LncRNAs and Mechanisms of Cancer Cell Radiation Resistance*

While RT has proven to be an effective strategy for treating multiple types of cancers, cancer cells are notorious for adapting to circumvent cell death. These mechanisms of resistance span multiple molecular pathways (Fig. 9) and can be intrinsic or acquired, which makes overcoming them very difficult in terms of therapy and leads to poor prognosis. Some well-known examples include apoptosis resistance, hijacking of the DNA damage repair pathway, expression of stem cell like factors, and epithelial-to-mesenchymal transition (106). Additionally, as previously stated, lncRNAs play a role in nearly every molecular pathway so it is no surprise that lncRNAs have been shown to respond to and regulate RT sensitivity in multiple cancer types (Fig. 10) (107). For instance, in glioblastoma, colorectal, and gastric cancers lincRNA-p21 has strong interactions within the Wnt/β-catenin pro-survival pathway which mediates resistance to radiation with respective mechanisms within the pathway specific to each cancer type (108–110). LincRNA-ROR has been associated with radioresistance in hepatocellular carcinoma by sequestering miR-145 which allows upregulation of RAD18, a factor responsible for DNA damage repair (111). It's also been shown to regulate the same miRNA in colorectal cancer, and promotes radioresistance via negative regulation of miR-145/p53 pathway resulting in apoptotic resistance (112). The

lncRNA NEAT1 has been shown to influence EMT in nasopharyngeal carcinoma via negative regulation of miR-204 leading to the upregulation of ZEB1, promoting EMT and enhancing radiation resistance *in vitro* and *in vivo* (113). Furthermore, the lncRNA TUG1 has also been shown to promote EMT via sponging of miR-145 and upregulating ZEB2 in bladder cancer (114). While these examples are only a small sample of the studies investigating the roles of lncRNAs in cancer radiotherapy, it is clear that lncRNAs are a potential area of therapeutic and prognostic value for multiple cancer types.

Figure 10: LncRNAs involved in radiosensitivity of various cancers. (Molecular mechanisms of lncRNAs in regulating cancer cell radiosensitivity, Zhu et al, *Bioscience Reports*, 2019)

C. LncRNAs in NSCLC Radiation Response

While there have been great strides in understanding the mechanisms of lncRNAs in radiation response, only a handful have been characterized in non-small cell lung cancer (NSCLC). The tumor suppressive lncRNA GAS5 (Growth Arrest Specific Transcript 5) has shown the ability to sensitize NSCLC cell lines to radiation. One of these studies proposed a mechanism where

GAS5 is able to work as a competing endogenous RNA (ceRNA) and sequester or “sponge” the miRNA miR-135b. Another study showed that GAS5 functions through a miR-21/PTEN/Akt signaling axis. Interestingly, both studies showed increased ability for cells to undergo apoptosis following exogenous expression of GAS5(100,115). The lncRNA PVT1 (Plasmacytoma Variant Translocation 1) has also shown potential in regulating radioresistance in NSCLC by acting as a ceRNA for the miRNAs miR-195 and miR-424-5p. While there is no further mechanism described in regards to miR-195 other than increased apoptosis following administration of RT when PVT1 was knockdown, PVT1 sponges miR-424-5p and stabilizes the protein CARM1, promoting an anti-apoptotic phenotype. This results in upregulated Bcl-2 (anti-apoptosis protein) and matrix-metalloproteinases MMP-2 and MMP-9 (important for cell adhesion, migration and invasion), which was all attenuated upon knockdown of PVT1 or overexpression of miR-424-5p, increasing radiosensitivity (116,117).

In addition to GAS5 and PVT1, there are other examples of lesser known lncRNAs which have been shown to regulate the radiosensitivity of NSCLC cells. For instance, the lncRNA CYTOR (Cytoskeleton Regulator RNA) can also sponge miR-195 similarly to PVT1. Briefly, high expression of CYTOR correlates with poor patient survival and functions as an oncogene by promoting increased proliferation, migration, invasion, and radioresistance in NSCLC cell lines by acting as a molecular sponge for miR-195. By sequestering miR-195, CYTOR allows for the upregulation of miR-195 targets such as, YAP and WNT3A which are regulators of stem-cell/developmental molecular pathways implicated in radioresistance(118). Another example is the lncRNA FAM201A (LncRNA Family with Sequence Similarity 201-member A). Along with high expression being associated with radiation resistance and poor prognosis in NSCLC patients, FAM201A functions in mediating radioresistance by sequestering miR-370, which promotes expression of EGFR and HIF-1 α , both of which have been associated with pathways involved in mediating resistance to RT (119).

In conclusion, lncRNAs are clearly an important factor in regulating cancer cell radiosensitivity. However, more studies are needed to fully understand the plethora of mechanisms these complex RNAs can elicit in cancer cells. Furthermore, many have been proposed as

prognostic indicators in some cancers (120), but more clinical studies are necessary to fully classify them as relevant molecular biomarkers.

V. The Y Chromosome in Cancer

A. Overview

The human Y chromosome (ChY) has long been considered a genetic wasteland with no value beyond its roles in male development and fertility (121). This is due in large part to the miniscule number of genes found on ChY compared to other chromosomes in the human genome. The total number of annotated genes currently reported is 568, of which only 71 have been described as protein-coding, and of those 71, many of the proteins belong to the same family resulting in a minor 27 distinct protein coding genes. The rest are considered to be potential non-protein-coding genes and pseudogenes (122).

Structurally, ChY is split into three major regions: MSY, PARs and the heterochromatic block (Fig. 11). The male specific region (MSY) is comprised of euchromatin and contains the majority of the annotated coding genes along with some pseudogenes. The pseudoautosomal regions (PAR1 and PAR2), which contain a small number of coding genes, are the only portions of ChY that share homology with the X chromosome, and are the only parts of ChY which undergo meiotic recombination. The remaining region is typically referred to as the heterochromatic block, comprises roughly half of the q arm of ChY, and has been described as transcriptionally inactive (123).

Figure 11: Schematic diagram of the human Y chromosome. (Modified from: Roles of the Y chromosome genes in human cancers, Tatsuo Kido and Yun-Fai Chris Lau, *Asian J. Andr.*, 2015).

Interestingly, ChY is commonly lost in aging somatic cells, otherwise referred to as mosaic loss of chromosome Y (LOY). This phenomenon has been observed for decades and was first considered a simple physiological consequence of aging in male tissues, and considering the limited number of non-essential genes, it has been largely ignored in terms of human biology (124).

However, recent evidence suggests a prognostic or pathological role for LOY. Just within the last two decades there has been an increase in research surrounding ChY. Many have found negative correlations with changes in ChY or LOY and pathologies such as cardiovascular disease, Parkinson's, schizophrenia, age related macular degeneration, cancer and overall mortality in men (125–129). Unfortunately, more information is still needed in regards to the genetic information of the ChY in order to acquire a complete understanding of the full potential of ChY in human disease.

B. ChY in Cancer

Overall, men are more likely to develop and die from cancer (72). Many theories have been offered to explain the disparities in cancer incidence and survival between the sexes, most of which are attributed to differences in hormone regulation, immune surveillance, and external factors (e.g. tobacco use). Numerous studies have also shown associations with genome instability, aneuploidy and cancer risk and mortality primarily for somatic chromosomes, enabling the identification of strengths and weaknesses in different cancers (130,131). However, while all of these have been fairly well elucidated, it is only recently that changes in ChY, especially LOY, has emerged as an additional factor in regards to male cancer susceptibility, tumorigenesis and survival.

Studies done in prostate cancer have eluded that LOY is beneficial for tumorigenesis and could potentially be used as a biomarker. Utilizing the prostate cancer cell line PC-3, one study revealed that restoration of ChY to the cells resulted in less tumorigenic phenotypes *in vitro* and *in vivo* (132). Another study showed that different ChY haplotypes could potentially be used to assess prostate cancer risk (133). More recently, a group identified the ChY specific histone demethylase *KDM5D* as a potential ChY linked tumor suppressor as its loss resulted in more aggressive prostate tumors (134). LOY has also been implicated in increased risk for developing testicular germ cell tumors (135). Additionally, in male breast cancer, it's been shown that the ChY gene *TMSB4Y* is a possible tumor suppressor and that loss of the gene via loss of ChY promotes a more tumorigenic phenotype (136). An additional study determined that clonal ChY in male breast cancer can begin as early as ductal carcinoma *in situ* (DCIS), and is primarily associated with Estrogen Receptor (ER) and Progesterone (PR) negative breast cancer subtypes (137). In addition to prostate, testicular, and male breast cancer, LOY has been observed in other

cancers including head and neck squamous cell carcinoma, urothelial bladder cancer, clear cell renal cell carcinoma, pancreatic adenocarcinoma, and NSCLC, all of which describe LOY as beneficial for the respective tumor types (138–141). Additionally, there have been multiple studies investigating LOY in the blood as prognostic indicator for patient survival or a biomarker for cancer risk. The overall findings within these studies conclude that LOY negatively correlates with patient survival and carcinogenesis (142–144). Interestingly, it was also noted that smoking is an external factor that can accelerate LOY, which adds to the already known fact that smoking has a major role in the development of multiple types of cancers (145).

C. ChY lncRNAs in Cancer

Due to incomplete sequencing data, the non-coding regions of ChY still remain an elusive source of potential in cancer cell biology. However, there are a few studies that have sought to begin identifying and characterizing ncRNAs, specifically lncRNAs from ChY. Currently, however, there are only 2 publications describing a role for the ChY lncRNA TTTY15. Interestingly, in prostate cancer TTTY15 behaves in an oncogenic capacity and is found upregulated in patient tumor samples, but the opposite is seen in NSCLC (146,147). Since these are the only two studies available, it is obvious that more information is needed to fully elucidate the effects of ChY lncRNAs in cancer.

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Chapter 2

Evidence of Y Chromosome lncRNAs involved in Radiation Response of Male Non-Small Cell Lung Cancer Cells

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Running Title: Radiation responsive Y chromosome lncRNAs in male NSCLC

Keywords: Non-small cell lung cancer, radiation therapy, Y chromosome, lncRNA

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DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

Abstract

Numerous studies have implicated changes in the Y chromosome in male cancers, however few have investigated the biological importance of Y chromosome non-coding RNAs. Here we demonstrate a group of Y chromosome-expressed long non-coding RNAs (lncRNAs) involved in male non-small cell lung cancer (NSCLC) radiation sensitivity. Radiosensitive male NSCLC cell lines demonstrated a dose-dependent induction of *linc-SPRY3-2/3/4* following irradiation, not observed in radioresistant male NSCLC cell lines. Cytogenetics revealed the loss of chromosome Y (LOY) in the radioresistant male NSCLC cell lines. Gain- and loss-of-function experiments indicated that *linc-SPRY3-2/3/4* transcripts affect cell viability and apoptosis. Computational prediction of RNA binding proteins (RBPs) motifs and UV Crosslinked Immunoprecipitation (CLIP) assays identified IGF2BP3 (an RBP involved in mRNA stability) as a binding partner for the *linc-SPRY3-2/3/4* RNAs. The presence of *linc-SPRY3-2/3/4* reduced the half-life of known IGF2BP3 binding mRNAs, such as the anti-apoptotic HMGA2 mRNA, as well as the oncogenic c-MYC mRNA. To assess the clinical relevance of these findings, we examined the presence of the Y chromosome in NSCLC tissue microarrays and the expression of *linc-SPRY3-2/3/4* in NSCLC RNAseq and microarray data. We observed a negative correlation between the loss of the Y chromosome or *linc-SPRY3-2/3/4*, and overall survival. Thus, *linc-SPRY3-2/3/4* expression and LOY could represent an important marker of radiation therapy in NSCLC.

Graphical Abstract

Statement of Significance

This study describes previously unknown Y chromosome-expressed lncRNA regulators of radiation response in male NSCLC. Furthermore, our findings show a correlation between loss of chromosome Y and radioresistance.

I. Introduction

Lung cancer is the most commonly diagnosed cancer worldwide and is the number one cause of cancer-related mortality (1). While there have been great strides in advancing treatment modalities for NSCLC, radiation therapy (RT) resistance remains a significant challenge. Previous studies have demonstrated mechanisms of radioresistance that include alterations in proteins involved in cellular response to DNA damage, the repair of DNA, apoptosis, hypoxia, and cell cycle regulators, among others (2,3). Regulatory non-coding RNAs have also been implicated in the development of radioresistance in NSCLC (4).

Regulatory non-coding RNAs include mature microRNAs (miRNAs) of around 22 nucleotides and long non-coding RNAs (lncRNAs) of >200 nucleotides (5). Studies have shown that most lncRNAs do not contain an open reading frame and could regulate a wide variety of processes through diverse mechanisms, including acting as signals (e.g. enhancers of transcriptional regulation), guides (e.g. recruitment of chromatin modifiers enzymes), or competing endogenous RNAs (e.g. sequestering miRNAs or RNA binding proteins) (6). The dysregulation of the expression and function of lncRNAs has been implicated in numerous cancer types, suggesting their potential use as biomarkers, prognostic indicators, or therapeutic targets (7). In NSCLC, lncRNAs have been associated with nearly every facet of cancer cell biology (8). Several lncRNAs have been associated with the development of resistance to radiotherapy (9). Examples of this include *MALAT1* and *HOTAIR*, which are responsible for inhibiting apoptosis and promoting epithelial to mesenchymal transition thereby aiding in radioresistance (10).

Non-coding RNA genes, including those coding for lncRNAs have been mapped to every human chromosome, including the X and Y chromosomes (11). lncRNAs that map to the X chromosome include *XIST*, which is critical for the initiation of X-inactivation. The dysregulation of *XIST* has been shown in multiple cancers (7,12). Other X chromosome lncRNAs include *TSIX* and *XACT*, which also function in regulating X chromosome inactivation (12). In contrast, few studies have considered the possibility that the Y chromosome contains genes encoding regulatory RNAs (13-15). Only 27 distinct protein-coding genes are present on the Y chromosome, most of which function in sex determination and male fertility (16,17). The remaining are considered

potential non-coding genes and pseudogenes (18). This suggests the Y chromosome is an untapped source of non-coding information, and could potentially provide new insights into numerous diseases - including NSCLC.

Here we report, for the first time, the importance of the lncRNAs *linc-SPRY3* (also known as lnc-BPY2C): *linc-SPRY3-2*, *linc-SPRY3-3*, *linc-SPRY3-4* (*linc-SPRY3-2/3/4*) in male NSCLC radiation response. Gain and loss of function experiments demonstrated that *linc-SPRY3-2/3/4* are involved in radiation vulnerability of male NSCLC cells and tumor samples containing chromosome Y or *linc-SPRY3-2/3/4*. Together our findings suggest a role of male-specific lncRNAs in NSCLC radiation sensitivity.

II. Materials and Methods

Cell Culture

Non-small cell lung cancer cell lines H460, H820, H157, H1299, A549, and WVU-Ma-0005 were cultured with DMEM (Sigma-Aldrich, St. Louis, MO, USA, D7777). H1650 and Ma-ALK-0001 cells were grown in RPMI (Corning, Corning, NY, USA, 50-020-PC). DMEM and RPMI media was supplemented with 10% fetal bovine serum (Gemini Bio-products, Sacramento, CA, USA, 100-106), L-glutamine (Gibco-LifeTech, Grand Island, NY, USA, 25030-081), HEPES (GE Healthcare Life Sciences, Logan, UT, USA, SH30237.01), penicillin–streptomycin (Gibco, 15140-122), and amphotericin B (Gibco, 15290-026). Human bronchial epithelial cells (HBEC) were cultured with keratinocyte-SFM supplemented with bovine pituitary extract, human recombinant epidermal growth factor, and L-glutamine (Gibco, 10724-011). All cells were grown in a humidified incubator at 37°C in the presence of 5% CO₂. Cell lines HBEC, H460, H820, H157, A549, H1299, and H1650 were kindly provided by Dr. Erik A. Bey (Indiana University) and patient derived cell lines WVU-Ma-0005 and Ma-ALK-0001 were kindly provided by Dr. Patrick C. Ma (Pennsylvania State University).

Microarray analysis

For global lncRNA expression, we used the GeneChip® Human Transcriptome Array 2.0 (ThermoFisher Scientific, Affymetrix, Santa Clara, CA, USA) that assesses 22,829 non-protein

coding human genes. Microarray processing was carried out in the West Virginia University Genomics Core Facility. Briefly, 1 µg of total RNA was labeled using the Flashtag RNA labeling kit (Genisphere, Hatfield, PA, USA) according to the manufacturer's instructions. Each sample was hybridized to the array at 48°C and 60 rpm for 16 hours then washed and stained on Fluidics Station 450 (Fluidics script FS450_0003) and finally scanned on a GeneChip® Scanner 3000 7G (Affymetrix, Santa Clara, CA, USA). Feature intensities were extracted by using GeneChip® Human Transcriptome Array 2.0 library files. Array data was deposited into the NCBI Gene Expression Omnibus (GEO) database (GEO accession #GSE147708).

Quantitative RT-PCR

Total RNA was extracted using Trizol Reagent (Ambion, Austin, TX, USA, 15596026) per manufacturer's instructions, then treated with Turbo DNase (Invitrogen, Carlsbad, CA, USA, AM1907) for 25 minutes at 37°C. RNA concentrations were determined with a Nanodrop 2000 Spectrophotometer. 0.5 – 1 µg of total RNA was converted to cDNA using the iScript cDNA synthesis kit (Bio-Rad, Hercules, CA, USA, 170-6891), followed by qRT-PCR using the SsoAdvanced Universal SYBR Green Supermix (Bio-Rad, 172-5271) and several pair of primers to specifically amplify the genes of interest in this study (Supplementary Table S4). Relative expression was calculated using the double delta CT method (relative expression = $2^{-\Delta\Delta CT}$; where $\Delta CT = CT \text{ (Target RNA)} - CT \text{ (endogenous control RNA)}$), where the endogenous control for lncRNA and mRNA was GAPDH and/or UBC.

Virtual Northern Blot

Total RNA (60µg) from 8Gy treated H460 cells was resolved on a 1.2% formaldehyde denaturing agarose gel overnight. The gel was imaged (to serve as ladder) and then fragmented into 25 fractions of equal size and RNA was recovered from each fraction by gel extraction. The recovered RNA was then used for qRT-PCR analysis of each individual lncRNA. The values generated by qRT-PCR were plotted as a fraction of the total RNA. Plotted values were a mean of three technical replicates per band.

Clonogenic Cell Survival Assay

Cells were seeded in triplicate 60mm gridded dishes containing 4 mL of media at 250 cells per dish. Cells were treated with sham or a single dose of radiation (2Gy, 4Gy, or 8Gy), and then colonies were allowed to form for 10-15 days. Colonies were fixed and stained with crystal violet in 33% methanol, and manually counted using a microscope. Surviving fraction values relative to the sham controls were plotted as a function of radiation dose.

Viral Transduction of shRNAs

HEK-293T cells were transfected with the lentiviral plasmid pLKO-shScramble (Addgene #1864) or pLKO.1-TRC cloning vector (Addgene #10878) containing shRNA sequences against linc-SPRY3-2, linc-SPRY3-3, or linc-SPRY3-4 and lentiviral packaging plasmids (PsPax2 Addgene #12260, and VSV-G Addgene #8454) using calcium phosphate transfection. Lentivirus was collected after 48 hours, filtered (0.45 μ m) and supplemented with polybrene (1 μ g/mL, MilliporeSigma, Burlington, MA, USA, TR-1003-G). WVU-Ma-0005 or H460 cells were exposed to lentivirus for 16-72 hours and allowed to recover for 48 hours post infection. Infected cells were stably selected via puromycin treatment (1.0-2.5 μ g/mL) for 72 hours. Efficiency of shRNA knockdown was measured using qRT-PCR.

Nucleofection of Y Chromosome BAC

The bacterial artificial chromosome (BAC) RP11-88F4 clone, from the RPCI-11 Human Male BAC Library (containing a 95,171bp insert from ChrY:58,819,440-58,914,611 [GRCh37/hg19]), was purchased from the Children's Hospital Oakland Research Institute and introduced to cells by nucleofection. A549 cells (4×10^6 cells/cuvette) were briefly resuspended in nucleofection buffer, and 16 μ g of RP11-88F4 or pBACe3.6 (control) plasmid DNA was added. The cuvette was placed on the Amaxa Nucleofector II device and the appropriate program was selected (Nucleofector® Program X-001). Immediately after the Nucleofection process ended, cells were resuspended in rescue media and transferred into 6-well plates and incubated at 37°C under 5% CO₂. Expression of *linc-SPRY3-2/3/4* was measured 48 hours post transfection by qRT-PCR.

Flow Cytometry

Cell viability and apoptosis were evaluated via Guava Easycyte HT Flow Cytometer (Millipore). Guava Viacount (Luminex, Austin, TX, 4000-0040) reagent was used for analysis of cell viability

and Guava Nexin (Luminex, 4500-0455) reagent for apoptosis per manufacturer's instructions. Cells were plated at equal densities in a 6-well plate, then sham treated or treated with a single dose of radiation (8Gy). Cells were trypsinized and collected 24, 48 and 72 hours after treatment. Parameters for the flow cytometer were set using sham controls.

Tumor Growth Delay Assay (TGD)

shControl or shlinc-SPRY3-2 (sh1) cells were trypsinized and suspended at 5×10^6 cells/mL in Matrigel. 1×10^6 cells were injected subcutaneously into the right flanks of male nude mice (Nu/Nu-088, Charles River, Wilmington, MA, USA). Once tumors reached $\pm 150 \text{ mm}^3$, RT was initiated. Treatment groups (3 animals each) included sham treated control (0Gy) and radiation (20Gy, 5 fractions). Tumor volume and depth was measured using ultrasound imaging (Vevo 2100, FUJIFILM VisualSonics Inc.). Measurements were quantified using the Vevo LAB 2.2.0 software. Tumors were assessed until they reached $>800 \text{ mm}^3$, day 21 after initiation of treatment, or mice showed evidence of morbidity. Relative tumor volume was determined by normalizing measured volumes to the starting volume (Day 0) of each respective animal.

UV Crosslinking and Immunoprecipitation (CLIP)

CLIP was performed according to the original protocols with some modifications (19,20). The detailed method is described in the Supplementary data.

RNA Stability Assay

Cells were treated with $3 \mu\text{g/mL}$ of Actinomycin D and then collected at 0 hours, 3 hours, 6 hours and 9 hours post treatment. RNA was then extracted and used for subsequent qRT-PCR analysis. *C. elegans* RNA was added as an exogenous spike for qRT-PCR ($70 \text{ ng}/\mu\text{L}$) to amplify the worm specific gene *Ama-1*. Each time point was normalized to the 0 hour control.

siRNA Transfection

Cells were seeded at 3.5×10^5 cells per well in a 6-well dish. siRNAs were transfected utilizing the RNAiMax Lipofectamine reagent according to the manufacturer's standard transfection protocol. 300pmol of scramble control siRNA (Horizon ON-TARGETplus Non-targeting Control siRNA

#2, D-001810-02-05) was used. For the targeted lncRNA siRNAs, 100pmol of each linc-SPRY3 specific siRNA was used to create a pool with a total concentration of 300pmol.

Irradiation

Radiation protocols were carried out on an Xstrahl XenX small animal radiation research platform (Suwanee, GA, USA). The XenX was commissioned following the AAMP Task Group 61 protocol for clinical irradiators of 40-300 kV (11439485). Dosage of experimental design was confirmed using Gafchromic EBT3 film (31198412, 29923495). Mice were anesthetized via isoflurane inhalation for the duration of each treatment. Each tumor was treated to a total dose of 20Gy over 5 fractions given every other day with a 1.2 cm circular fixed collimator at a dose rate of 3.62Gy/min corrected for tumor volume per mouse. For cells, a similar confirmation of dose rate for the experimental setup was determined by Gafchromic EBT3 film. Cells were treated in single fraction doses of 2Gy, 4Gy, or 8Gy with a variable collimator at a dose rate of 1.7Gy/min.

DNA FISH AND Tissue Array Analysis

DNA FISH in cell lines was performed and quantified by the WVU Cytogenetics Laboratory. DNA FISH for the tissue arrays was performed as previously described with the following modifications (21). Slides were first baked at 60°C before proceeding through the protocol. Slides were deparaffinized with Skipdewax (Insitus Biotechnologies, Albuquerque, NM, USA, #T213) for 20 minutes at 80°C followed by three washes with distilled water for 2 minutes each. This process was repeated three times. The SRY probe (Cytocell Ltd., Tarrytown, NY, USA, LPU 026-A) was allowed to hybridize for 22 hours. All tissue arrays were purchased from US Biomax, Inc. (Derwood, MD, USA, Lung: OD-CT-RsLung01-009, Testis: T231a, Cervix: T103). Imaging for representative images for the tissue array FISH was performed via epifluorescent microscopy on a Nikon A1R/N SIM-E microscope. Imaging for quantification of Y chromosome in the lung tissue array was performed on an Olympus VS120 Slide Scanner microscope. Quantification of Y chromosome FISH in the lung TMA was done using IMARIS software. Kaplan-Meier method was used to estimate the survival curves and log-rank test was used to assess the difference of survival curves between groups. TMA data for each patient was evaluated by percentage of Y chromosome positive cells out of the total cells examined, where the cut-off point to define high and low was obtained from the classification and regression tree.

RNA Seq and microarray Analysis from NSCLC patient data

RNA-Seq data for patients diagnosed with NSCLC were obtained from the Gene Expression Omnibus (GEO) database (accession #GSE81089) (22). The expression of linc-SPRY3-2 was measured by TPM (transcripts per millions reads) by salmon (23). A TPM value below 0.1 was deemed as not-expressed. The threshold was determined by comparing to the values obtained from female patients, where linc-SPRY3-2 should not express. The survival period was estimated as days between surgery and vital date (i.e., day of death or latest contact per the definition from GSE81089). For the microarray data, the expression of linc-SPRY3-2 from male Early Stage Lung Squamous Cell Carcinomas patients were obtained from GEO database (accession #GSE74777) based on Affymetrix Human Transcriptome Array 2.0. The survival analysis was done by comparing the top 10% high expressing patients of linc-SPRY3-2. The survival curves were made with the survival R package, and p-value was calculated by log-rank test.

Cell Fractionation

Cells were pelleted and immediately processed for cell fractionation to extract RNA (protocol adapted from previous publication (24)). Pelleted cells were disrupted using an autoclaved glass Dounce homogenizer. Homogenate was centrifuged to pellet the nuclear fraction and the cytoplasmic fraction (supernatant) was collected. Each fraction was then treated with DNase I (New England Biolabs) for 20 minutes at 37°C and then RNA was extracted using Trizol Reagent (Life Technologies) per manufacturer's instructions. Once the RNA was extracted it was treated with Turbo DNase (Ambion) for 20 minutes at 37°C. RNA concentrations were obtained using a Nanodrop 2000 Spectrophotometer (Thermo Scientific). qRT-PCR for cell fractionations was normalized using an exogenous spike of *C. elegans* RNA (70 ng/μL) and the worm specific gene *Ama-1*.

Statistical Analysis

Data are presented as mean $\pm$ SD, *P < 0.05; **P < 0.01; ***P < 0.001. The statistical significance between experimental groups was determined by ANOVA followed by Tukey's multiple comparisons test. Analyses were performed using GraphPad Prism V8 and Microsoft Excel 2013.

III. Results

Identification of radiation-responsive lncRNAs expressed from the Y chromosome

To identify lncRNAs potentially involved in radiation response of NSCLC cells, we profiled the expression of over 20,000 human lncRNAs in the male NSCLC H460 cell line irradiated with 7 Gy of ionizing radiation (IR), compared to unirradiated (0 Gy) H460 cells 24 hours post-treatment. Using a change in gene expression of 1.5-fold or more, we identified 106 lncRNAs as up-regulated following exposure to radiation, while 59 lncRNAs exhibited down-regulation (**Supplementary Fig. S1, Supplementary Table S1**). Among the highest upregulated lncRNAs, we observed changes in transcripts annotated as *linc-SPRY3-2*, *linc-SPRY3-3*, and *linc-SPRY3-4*, also known as *lnc-BPY2C-4*, *lnc-BPY2C-25*, and *lnc-BPY2C-2*, respectively. Surprisingly, the *linc-SPRY3-2/3/4* transcripts map to a region of the Y chromosome heterochromatic block known as the *DYZ1* region that contains a single H3K27Ac mark suggesting transcription in this region (**Fig. 1A**). The *DYZ1* region has been described as genetically inert since it is composed of heterochromatin and, due to its highly repetitive nature, remains poorly sequenced (16). Previously attempted sequence analyses of the human Y chromosome have revealed numerous copies of the pentanucleotide repeat motif 5'-TTCCA-3' in the *DYZ1* region (25,26). Only one previous publication has shown expression of two non-coding RNAs (AK128024.1 and AY598346.2) from the *DYZ1* region in a testis-specific manner (27). The *linc-SPRY3-2/3/4* sequence fragments obtained from the online database LNCipedia (28) contain several 5'-TTCCA-3' repeat motifs (**Fig. 1B**) and partially overlap with AK128024.1 and AY598346.2 (**Supplementary Fig. S2**), further supporting that these lncRNAs are transcribed from the *DYZ1* region of the Y chromosome. Using the *linc-SPRY3-2/3/4* sequence fragments, we designed primers to specifically amplify each *linc-SPRY3* transcript in order to perform virtual northern blot analysis. Using RNA from 8Gy treated H460 cells, we found two peaks of amplification migrating around 5Kb and 1Kb regardless of which *linc-SPRY3* primers were used. This suggests *linc-SPRY3-2/3/4* could be partial sequences of a longer lncRNA transcript(s). (**Supplementary Fig. S3**).

We next developed qRT-PCR assays of the *linc-SPRY3* transcripts to assess their expression in H460 cells following exposure to a single dose of IR (8Gy) at different time points

post-treatment versus non-irradiated controls. We observed a 5-fold induction in the expression of the *linc-SPRY3-2/3/4* transcripts four hours post-exposure to radiation, that increased to almost 15-fold after eight hours and remained elevated for at least twelve hours after treatment (**Fig. 1C**). Furthermore, H460 cells exhibited a dose-dependent increase from 5-fold (2Gy) to 50-fold (8Gy) in the expression of *linc-SPRY3-2/3/4* after 72 hours post-exposure to IR (**Fig. 2B**).

Linc-SPRY3-2/3/4 expression reveals a dose responsive signature to IR

In order to expand our findings, we examined the expression of the *linc-SPRY3* transcripts in several male NSCLC cell lines: the radiosensitive cell lines H820 and H157, and three radioresistant lines: H1299, A549 and H1650 (29-33). We also included two early passage male patient-derived NSCLC cell lines: WVU-Ma-0005 and Ma-ALK-0001. Clonogenic cell survival assays were performed to determine and verify the radiation response of these NSCLC cell lines. Our clonogenic assay data confirmed the radiosensitivity of H460 and H820 as well as the radioresistance of A549 and H1299, which were used as controls for the characterization of the response of the patient-derived cell lines. Our data showed that the WVU-Ma-0005 cell line is radiosensitive and the Ma-ALK-0001 cell line is radioresistant (**Supplementary Fig. S4**). Next, cells were treated with a single dose of IR (2Gy, 4Gy, or 8Gy) and collected 72 hours post-treatment. Controls were established by treating the cells identically in parallel and omitting the irradiation step. We observed that human bronchial epithelial cells (HBEC), which served as a normal control, the radiosensitive cell lines H820, WVU-Ma-0005 and H157 demonstrated a dose-dependent increase in expression of the *linc-SPRY3* RNAs, (**Fig. 2A-D, Supplementary Fig. S5H**) similar to H460 cells. In contrast, the radioresistant cell lines A549, H1299, Ma-ALK-0001 and H1650 showed minimal to no expression of these lncRNAs when compared to no retro-transcription (RT) controls (**Fig. 3E-G and Supplementary Fig. S5I**). To confirm that this expression profile is specific to male radiosensitive NSCLC cells, we investigated the expression of the *linc-SPRY3* transcripts in two established female NSCLC cell lines H1975 (radiosensitive) and H1819 (radioresistant) (29). qRT-PCR revealed no amplification signal above background in the female cell lines, a finding consistent with the Y-specific mapping of the *linc-SPRY3* genes (**Supplementary Fig. S6**). Finally, we assessed the coding potential and cellular location of the *linc-SPRY3* RNAs in H460 and WVU-Ma-0005 cells. In both cell lines, we detected *linc-SPRY3* RNA species exclusively in the nucleus (**Supplementary Fig. S7A and S7B**) and we found no

coding probability in any of the lncRNA transcripts (**Supplementary Fig. S7C**) supporting our hypothesis of their potential non-coding function.

Loss of the linc-SPRY3 transcripts in the radioresistant cell lines is due to loss of the Y chromosome

The differences in the expression of the *linc-SPRY3* transcripts in radiosensitive and radioresistant NSCLC cell lines prompted us to hypothesize that radioresistant lines harbored genetic changes involving chromosome Y. There are reports of changes in the Y chromosome in cancers dating back several decades that include evidence for the mosaic loss of chromosome Y (LOY) (21,34-41). LOY involves the loss of the entire Y chromosome and occurs in human male tissues as a consequence of aging, however, only recent studies have begun validating a correlation between LOY and cancer biology (18,21,36-38,40-45). Using DNA-FISH, we investigated the sex chromosome status of our panel of cell lines. As expected, the immortalized HBECS showed a normal male genotype containing a single copy of the X and Y chromosomes (**Fig. 2H**). The radiosensitive cell lines (H460, H820, and H157) possessed multiple copies of both the X and Y chromosomes, while the patient-derived cell line WVU-Ma-0005 contained a single copy of the X and Y chromosomes (**Fig. 2I-K and Supplementary Fig. S5L**). Interestingly, the radioresistant cell lines A549, H1299, Ma-ALK-0001 and H1650 showed complete loss of the Y chromosome (**Fig. 2L-N and Supplementary Fig. S5M**). Cytogenetic analysis and quantification of LOY in all the NSCLC cell lines verified our DNA-FISH analysis, (**Supplementary Table S2**) suggesting that loss of the *linc-SPRY3* transcripts in radioresistant cell lines is due to LOY, and that these noncoding RNAs and potentially other genes mapping to the Y chromosome could influence the response of cells to radiation.

The DYZ1 region of the long arm of the Y chromosome restores radiosensitivity

As described above, the repetitive nature of the sequences around the gene(s) coding for the *linc-SPRY3* transcript(s) (**Fig. 1B**) means no complete sequence(s) for these RNAs exists hindering gene-specific gain-of-function studies. However, we were interested to determine if a bacterial artificial chromosome (BAC, RP11-88F4) encompassing the *DYZ1* region of the long arm of the human Y chromosome where the *linc-SPRY3* transcript(s) are located (a 95,171bp insert from ChrY:58,819,440-58,914,611 [GRCh37/hg19]) could alter the response of the Y

chromosome negative, radioresistant NSCLC cell line A549 to IR. After 72 hours, we extracted DNA from the nucleofected cells and performed PCR for the BAC plasmid (pBACe3.6) to determine efficiency of the nucleofection (**Supplementary Fig. S8**). Next, we detected the expression of the three *linc-SPRY3* transcripts in the BAC-transfected A549 cells (A549_88F4) by RT-PCR, but not in the A549 BAC control cells (A549_Ctrl) (**Fig. 3A**).

We measured cell viability and apoptosis in the A549_Ctrl and the A549_88F4 cells after a single dose of 8Gy IR. Notably, the A549_88F4 cells showed significantly worse cell viability when compared to control cells in response to treatment (**Fig. 3B**). Additionally, A549_88F4 cells showed increased apoptosis compared to control cells after IR with this difference reaching statistical significance at 48 and 72 hours (**Fig. 3C**). These experiments suggest that the *DYZ1* region of the long arm of the Y chromosome harbors genes that influence the response to IR, which includes the *linc-SPRY3* family. To obtain further evidence to support this hypothesis, we conducted a series of loss-of-function studies using shRNAs to reduce the levels of the *linc-SPRY3* transcripts in NSCLC cells.

First, we designed and validated two shRNAs; one targeting a *linc-SPRY3-2* sequence (sh1) and one a *linc-SPRY3-3* sequence (sh2) (**Supplementary Table S3**). Interestingly, we observed a significant reduction in the levels of all three *linc-SPRY3* RNAs in H460 and WVU-Ma-0005 cells (**Fig. 3D and 3G**), consistent with our hypothesis that *linc-SPRY3* transcripts may be a longer lincRNA transcript. Both H460 and WVU-Ma-0005 cells expressing the *linc-SPRY3* shRNAs exhibited increased survival and reduced apoptosis after 8Gy IR in comparison to control cells (**Fig. 3E-F & 3H-I**); consistent with a loss of *linc-SPRY3* transcripts expression resulting in resistance to radiation.

We next sought to determine if reducing the expression of the *linc-SPRY3* transcripts would result in IR resistance *in vivo*. Briefly, 6-8 week old mice were injected with H460 or WVU-Ma-0005 cells harboring either the shCtrl or sh1 constructs. Once tumors reached a volume of 150mm³, mice were treated with fractionated IR to a total dose of 20 Gy, mimicking the clinical procedures for patients with NSCLC (**Supplementary Fig. S9A**). Ultrasound imaging was used to ensure accurate measurement of both tumor depth and volume, the appropriate settings for IR

administration, as well as the tracking tumor growth (**Supplementary Fig. S9B-C**). Untreated mice showed rapid development of tumors regardless of the type of shRNA used (**Supplementary Fig. S9D-E**). As expected, IR treated mice exhibited slower tumor progression, while sh1 tumors showed larger tumor volumes both during and after IR compared to shCtrl tumors. This suggests that loss of *linc-SPRY3* transcripts results in tumors with higher resistance to RT (**Fig. 3J-K and Supplementary Fig. S9D-E**).

The linc-SPRY3 transcripts interact with the RNA binding protein IGF2BP3 and affect RNA stability in HMGA2 and c-MYC mRNAs

In an attempt to identify a potential mechanism for the *linc-SPRY3-2/3/4* RNAs we utilized a database of RNA binding proteins and Associated motifs (ATtRACT) software (46) to predict RNA binding proteins (RBPs) potentially interacting with the *linc-SPRY3* transcripts. The analysis revealed 13 potential RBPs interacting with the *linc-SPRY3* transcripts but the largest number of predicted binding sites across all three family members was the RBP Insulin Like Growth Factor 2 mRNA Binding Protein 3 (IGF2BP3, also known as IMP3) (**Fig. 4A**). To confirm the predicted association of IGF2BP3 with the *linc-SPRY3* RNAs, we performed UV crosslinking and immunoprecipitation (CLIP) for IGF2BP3 in irradiated H460 cells. RT-PCR of the recovered RNA revealed that IGF2BP3 does indeed directly interact with all three *linc-SPRY3* family members as well as the known binding partner HMGA2 mRNA (47) but not with the control GAPDH (**Fig. 4B**). IGF2BP3 is a known RBP whose functions include stabilizing mRNAs. Two such targets include c-Myc and HMGA2 (48). These two targets were of specific interest to us, since previous studies have shown that downregulation of c-Myc and HMGA2 results in an increase in apoptosis in multiple cancers including lung cancer (49-54).

In order to confirm that the association of IGF2BP3 with the *linc-SPRY3* family members affects its ability to target and stabilize c-Myc and HMGA2, we performed RNA stability assays using Actinomycin D (ActD) to stop global transcription in A549 cells with or without the expression of the *linc-SPRY3* transcripts (A549_Ctrl and A549_88F4 cells) as well as the knockdown of the *linc-SPRY3* transcripts by a pool of siRNAs (siPool). qRT-PCR analysis revealed more rapid degradation of HMGA2 and c-Myc mRNAs in the A549_88F4 siCtrl cells when compared to A549_Ctrl siCtrl cells but GAPDH mRNA was not affected (red bar compared

to black bar) (**Fig.4C**). Furthermore, we observed rescue of c-Myc and HMGA2 mRNAs stabilization in the A549_88F4 siPool cells after the knockdown of the linc-SPRY3 transcripts (**Fig. 4C and Supplementary Fig. S10**). Overall, these data suggest that when the *linc-SPRY3* RNAs are present they could potentially act as competing endogenous RNAs (ceRNAs) for IGF2BP3, thereby preventing it from stabilizing its mRNA targets c-Myc and HMGA2 affecting apoptosis response.

Y chromosome loss and loss of the linc-SPRY3 RNAs are potential poor prognostic indicators for male NSCLC

To assess the potential clinical relevance of the presence or absence of the Y chromosome and radiation sensitivity and resistance, we performed DNA-FISH for the Y chromosome on a lung cancer tissue microarray and quantified LOY for 30 male cores as well as a testicular cancer core (positive control) and a cervical cancer core (negative control). Representative images taken of male lung cancer cores and a testicular cancer core reveal numerous Y chromosome positive cells, while images taken of female lung cancer cores and a cervical cancer core reveal no detectable presence of the Y chromosome DYZ1 (**Supplementary Fig. S11**). Using images from male cores of the NSCLC tissue array, we applied a custom image analysis pipeline to determine the percentage Y chromosome positivity. The representative images demonstrate the DNA-FISH of a primary Y chromosome negative core (**Fig. 5A**, inset is absent of punctate dots) and a heavily Y chromosome positive core (**Fig. 5C**, arrows in inset indicate punctate dots). **Figures 5B and 5D** show the result of the image analysis in which Y chromosome negative cells are artificially colored purple and Y chromosome positive cells are artificially colored red. We next used the supplied clinical data to perform Kaplan-Meier analysis. Our analysis revealed a trend towards a negative relationship between Y chromosome loss and patient survival. Though not statistically significant, the trend revealed worse survival for those patients with < 25% Y chromosome positivity (**Fig. 5E**).

Finally, we accessed the publicly available microarray data (GSE74777) containing 107 Early Stage Lung Squamous Cell Carcinomas (96 male) and the NSCLC short-read RNAseq dataset (GSE81089) containing 198 samples (95 male) to assess the expression of the *linc-SPRY3-2* transcript and clinical outcomes (the expression of the other two lncRNAs was only detected in

limited number of patients in the RNAseq dataset and therefore were not considered). Interestingly, male patients with high *linc-SPRY3* transcripts expression (top 10%) from the microarray data showed a better surviving outcome than the rest of the patients (**Fig. 5F**). Furthermore, in the RNA-seq study, male patients with tumors expressing low levels of *linc-SPRY3-2* exhibited a worse overall survival than those in which their tumor did express *linc-SPRY3-2* (**Supplementary Fig. S11I**). Further studies are needed to validate these initial findings (statistically not significant), but the trends observed in two independent studies suggest that the presence of the Y chromosome and the expression of *linc-SPRY3* RNAs warrants further analysis for their functions in radiation response of male NSCLC tumors.

IV. Discussion

In this study the unbiased expression profiling of lncRNAs, following the irradiation of a NSCLC cell line, revealed the induced expression of three annotated lncRNAs that map to the highly repetitive, heterochromatic DYZ1 region of the Y chromosome: *linc-SPRY3-2*, *linc-SPRY3-3*, and *linc-SPRY3-4*. Further investigation revealed expression of this group of lncRNAs is IR dose dependent and begins approximately 4 hours after administration of IR and maintained through 72 hours (**Fig. 1**). Interestingly, expression of these lncRNAs was only detected in radiosensitive cell lines, but not in radioresistant cell lines, regardless of their oncogenic driver mutations (**Supplementary Table S2**). Cytogenetic analysis of all cell lines showed loss of the Y chromosome in the radioresistant cell lines, while the radiosensitive cells lines retained their Y chromosome, explaining the differences seen in *linc-SPRY3-2/3/4* expression (**Fig. 2**). Gain-of-function and Loss-of-function experiments demonstrated that the *linc-SPRY3* RNAs are important for cellular response to IR as statistically significant changes were seen in cell viability and apoptosis (**Fig. 3**). *In vivo* experiments further confirmed these observations as knockdown of the lncRNAs promoted radioresistance in the NSCLC cellular phenotype.

Additionally, we have provided significant evidence that these lncRNAs could act as competing endogenous RNAs (ceRNAs) for the RNA binding protein IGF2BP3 (also known as IMP3) (**Fig. 4**). In normal tissues, IGF2BP3 is primarily expressed during embryonic development, but its overexpression has been associated with numerous cancer types and as such has been

classified as an oncogenic protein (48,55). More specifically, it has been previously shown that IGF2BP3 is upregulated across all NSCLC subtypes and that its loss leads to increased apoptosis (56). As a RBP, IGF2BP3 regulates several genes primarily by binding and stabilizing target mRNAs in order to increase translation efficiency (48). Two such targets, as mentioned previously, are c-Myc and HMGA2. Interestingly, previous studies have shown that downregulation of c-Myc results in increased radiosensitivity by promoting apoptosis (49,50). Additionally, while there is little to no previous evidence of a role for HMGA2 in radiation sensitivity, there are multiple studies characterizing its relationship to apoptosis (52-54). Moreover, HMGA2 expression is controlled by the transcription factor c-Myc (57) which suggests a potential feedback loop interaction. Based on our data, we propose the hypothesis that the presence of the *linc-SPRY3-2/3/4*, following IR, sequester IGF2BP3 and inhibit the stabilization of c-Myc and HMGA2 which results in downregulation of their proteins making the cells more vulnerable to apoptosis following IR. On the other hand, we cannot discard the possibility that the *linc-SPRY3* transcripts could regulate the expression of c-MYC and HMGA2 at the transcriptional level. Lastly, DNA-FISH analysis, microarray analysis, and RNA-seq revealed a negative correlation between loss of the Y chromosome and loss of *linc-SPRY3-2* expression and overall patient survival, respectively (**Fig. 5**). This establishes, for the first time, a connection between the Y chromosome, the *linc-SPRY3* transcripts, and how male NSCLC cells respond to radiation.

In conclusion, we show for the first time the importance of a Y chromosome derived group of lncRNAs in regulating male NSCLC radiation response. We provide significant evidence that the *linc-SPRY3-2/3/4* transcripts are potentially tumor suppressive and their loss via LOY contributes to radioresistance in NSCLC cells. We also provide evidence that these lncRNAs directly bind IGF2BP3, preventing it from stabilizing its targets HMGA2 and c-Myc which we believe contributes to the radiosensitive phenotype. Moreover, we show a trending negative correlation between Y chromosome and *linc-SPRY3* loss and patient survival. We do, however, acknowledge the limitations of this study. More data is needed to fully validate a clinically relevant relationship of both the Y chromosome and *linc-SPRY3-2/3/4* family in NSCLC patient survival. It would also be interesting to investigate the behavior of these lncRNAs in an acquired resistance model, utilizing isogenic radioresistant cell lines generated from radiosensitive parental cells like the H460 and WVU-Ma-0005 cell lines.

Considering the dose dependent response in expression observed in the radiosensitive cell lines, we speculate that these nuclear lncRNAs could be involved in apoptosis and/or the DNA damage response and repair pathway. We also hypothesize that *linc-SPRY3* transcripts and the Y chromosome could eventually be used as biomarkers for cancer radiotherapy. Overall, we believe that more attention should be given to the Y chromosome, as many of its non-coding regions remain uncharted. This has left a vast untapped resource in the realm of cancer cell biology that has yet to be explored.

V. Acknowledgements

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VII. Figure Legends

Figure 1: Microarray analysis reveals a family of Y chromosome, radiation inducible long non-coding RNAs.

(A) Y chromosome schematic obtained from the UCSC Genome Browser demonstrating the genomic location of the three linc-SPRY3 transcripts in the DYZ1 region (also known as Inc-BPY2C). (B) Linc-SPRY3 transcripts sequences. Highlighted sections indicate pentanucleotide repeats. The black underline regions show the sequences target by the shRNAs and siRNAs used in this study (C) qRT-PCR analysis of the linc-SPRY3 family members validating the radiation induced expression in H460 cells. Time course expression following a single dose of 8Gy IR of linc-SPRY3-2/3/4. GAPDH mRNA was used to normalize qRT-PCR analysis. Error bars represent SD from the mean (n=3).

Figure 2: Radiation sensitive and radiation resistant NSCLC cell lines show significant difference in the expression of the linc-SPRY3 family due to Y chromosome loss.

qRT-PCR analysis of (A) HBEC and radiosensitive cell lines (B) H460, (C) H820, and (D) WVU-Ma-0005, demonstrates a dose dependent response in linc-SPRY3 expression. qRT-PCR analysis of radioresistant cell lines, (E) A549, (F) H1299, (G) Ma-ALK-0001, shows little to no change in linc-SPRY3 expression. Values are relative to untreated control (0Gy) of the same collection day. GAPDH mRNA was used to normalize qRT-PCR analysis. Error bars represent SD from the mean (n=3). (H-N) Cytogenetic analysis via DNA-FISH reveals complete loss of the Y chromosome in the radioresistant cell lines while HBECs and radiosensitive cell lines retain their Y chromosome. Images are a representative from 200 counted cells.

Figure 3: Linc-SPRY3 RNAs are important for cell survival following 8Gy irradiation

(A) RT-PCR analysis shows expression of the linc-SPRY3 family in BAC RP11-88F4 nucleofected A549 cells. (B-C) Overall cell viability and apoptosis were measured by flow cytometry. Y chromosome BAC nucleofection results in lower cell viability and increased apoptosis compared to control in radioresistant A549 cells after 8Gy IR. (D, G) qRT-PCR analysis reveals effective knockdown of the linc-SPRY3 RNAs in radiosensitive cell lines H460 and WVU-Ma-0005. (E-F, H-I) Knockdown of the linc-SPRY3 family in both H460 and WVU-Ma-0005 cells using two different shRNAs against linc-SPRY3 family (sh1 and sh2) resulted in better overall cell viability and increased resistance to apoptosis when compared with shControl (shCtrl) after 8Gy IR. Untreated controls are provided to demonstrate baseline cell viability and apoptosis. (J-K) Tumor growth delay assay (n = 3) shows higher resistance to radiation of the sh1 tumors relative to shCtrl tumors in both H460 and WVU-Ma-0005 animal experiments. Mean relative tumor volumes are plotted. Error bars represent SD from the mean (n=3) (A-I). Error bars represent SD from the mean of 3 mice in each group (J-K). Untreated tumor controls are represented in Supplementary Fig. S9. ANOVA was used to determine statistical significance.

Figure 4: The linc-SPRY3 family act as molecular sponges for IGF2BP3 preventing it from stabilizing HMGA2 and c-Myc.

(A) Predicted binding motifs (using WebLogo3) of IGF2BP3 in each linc-SPRY3 transcript. (B) RT-PCR amplification of the recovered RNA from IGF2BP3 CLIP assays of untreated (No Radiation) or treated (8 Gy) H460 cells. The lanes are as follows (from left to right): Input RNA, IgG Supernatant, IGF2BP3 Supernatant, IgG Immunoprecipitation, IGF2BP3 Immunoprecipitation. HMGA is provided as a positive control and GAPDH as a negative control for IGF2BP3 interaction. Gel images are a representative of duplicate experiments. (C) Actinomycin D (ActD) RNA stability assays show rapid degradation of HMGA2 and c-Myc mRNA when the linc-SPRY3 RNAs are present (A549_88F4 siCtrl). siRNA knockdown rescues mRNA stability of HMGA2 and c-Myc (A549_88F4 siPool). GAPDH is provided as a negative control. Error bars represent SD from the mean (n=3)

Figure 5: The DYZ1 region of the Y chromosome and the linc-SPRY3 family show negative correlations with patient survival.

(A-D) Representative images from the imaging analysis performed on 30 cores from a lung cancer tissue array to determine the percentage of Y chromosome DYZ1 positive cells. Y chromosome positive cells are red and Y chromosome negative cells are purple. (E) Kaplan-Meier analysis of the 30 cores analyzed for Y chromosome DYZ1 positivity. (F) Survival curves for NSCLC male patients (GSE74777).

VIII. Main Figures

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

IX. Supplemental Information

Supplementary Table S1

LncRNA Gene	Transcript Cluster ID	Fold Change	ANOVA p-value	FDR p-value
AF092843	TC12002437.hg.1	4.26	0.000022	0.000414
linc-OCIAD2-1	TC04002544.hg.1	4.17	0.000007	0.000159
linc-LOC100132288-2	TC21000805.hg.1	3.88	0.000028	0.000496
linc-SPRY3-4	TC0Y000283.hg.1	3.17	0.162867	0.440186
linc-SPRY3-3	TC0Y000284.hg.1	3.04	0.09976	0.322973
BC036485	TC03003223.hg.1	2.74	0.00001	0.000226
linc-POTED-9	TC21000560.hg.1	2.73	0.071853	0.258323
linc-ZNF25-4	TC10002568.hg.1	2.55	0.005738	0.036702
linc-CDH6-5	TC05002316.hg.1	2.49	0.000003	0.000094
linc-FCGR1B-9	TC01005759.hg.1	2.43	0.000086	0.001207
BC057782	TC09002209.hg.1	2.3	1.12E-07	0.000007
AK025384	TC16001789.hg.1	2.15	9.63E-07	0.000037
linc-SPRY3-2	TC0Y000285.hg.1	2.13	0.187334	0.479358
AK054573	TC17002305.hg.1	2.11	0.000099	0.001344
S81264	TC17002307.hg.1	2.11	1.37E-08	0.000002
AF085983	TC02004620.hg.1	2.07	6.60E-09	9.36E-07
NR_027299	TC03002817.hg.1	2.07	1.70E-07	0.00001
AX750576	TC17002329.hg.1	2.03	7.14E-07	0.000029
linc-NAA35-2	TC09002052.hg.1	2.01	0.000004	0.000112
NR_026866	TC03002367.hg.1	1.93	7.03E-07	0.000029
BC033874	TC01006039.hg.1	1.89	0.000209	0.002472
BC061593	TC05002175.hg.1	1.89	3.79E-09	6.30E-07
BX537506	TC08002539.hg.1	1.89	5.12E-09	7.85E-07
AY665470	TC12002292.hg.1	1.89	8.29E-10	2.12E-07
NR_044995	TC13001319.hg.1	1.85	2.64E-07	0.000013
BX647541	TC16001788.hg.1	1.85	2.70E-10	1.07E-07
AF172940	TC02003138.hg.1	1.83	1.90E-08	0.000002
M73837	TC10002092.hg.1	1.83	0.000549	0.005416
linc_luo_466	TC06002911.hg.1	1.81	0.313329	0.633221
EF210211	TC07002321.hg.1	1.81	2.36E-08	0.000002
AF086401	TC07002818.hg.1	1.81	0.000009	0.000209
NR_028034	TC10002196.hg.1	1.79	5.54E-07	0.000024
S47380	TC04002187.hg.1	1.78	0.003206	0.022936
NR_033258	TC02004623.hg.1	1.77	1.23E-08	0.000001
linc-C7orf66-2	TC07003112.hg.1	1.77	0.000043	0.000692
AK021785	TC11003067.hg.1	1.77	7.20E-07	0.000029

CR542200	TC04002080.hg.1	1.76	0.000565	0.005548
AF321824	TC08002559.hg.1	1.75	0.000075	0.001082
BC040985	TC03002416.hg.1	1.74	1.03E-08	0.000001
S83545	TC05002797.hg.1	1.74	2.33E-07	0.000012
NR_027140	TC08002257.hg.1	1.74	0.000011	0.000245
AY820830	TC06003084.hg.1	1.73	6.40E-08	0.000005
ENST00000537998	TC12002680.hg.1	1.73	0.000002	0.000054
linc-GRM1	TC06003110.hg.1	1.72	0.000749	0.006988
NR_036684	TC16001604.hg.1	1.71	5.09E-08	0.000004
linc-ZNF626-1	TC19002390.hg.1	1.71	0.017129	0.087898
linc-FCGR1B-6	TC01005756.hg.1	1.7	0.001	0.008855
AK057151	TC07002222.hg.1	1.7	0.000029	0.0005
NR_036431	TC0X002160.hg.1	1.69	0.002693	0.019933
linc-ELTD1-11	TC01005583.hg.1	1.67	0.000003	0.00008
AJ420500	TC06002615.hg.1	1.67	0.000453	0.004635
NR_027783	TC0X001624.hg.1	1.67	9.46E-08	0.000006
AK056630	TC07002487.hg.1	1.66	0.013477	0.073006
AF007193	TC07002493.hg.1	1.65	0.020372	0.100857
X55077	TC12002396.hg.1	1.64	2.21E-08	0.000002
AF086343	TC10002011.hg.1	1.63	0.000542	0.005365
linc-NBPF9-3	TC01006417.hg.1	1.62	0.000033	0.000559
AK026778	TC02004622.hg.1	1.61	0.000051	0.000787
D87470	TC11003246.hg.1	1.61	0.000013	0.000278
uc001gla.1	TC01004846.hg.1	1.6	0.000001	0.000044
AK123435	TC01005963.hg.1	1.6	0.000898	0.008093
linc_luo_506	TC07002386.hg.1	1.6	0.00063	0.006047
BC014926	TC14001766.hg.1	1.6	3.72E-07	0.000018
AF049885	TC04002887.hg.1	1.59	3.44E-11	3.00E-08
L12143	TC06002402.hg.1	1.59	1.06E-07	0.000007
BC006127	TC12002414.hg.1	1.59	0.003111	0.022429
NR_003136	TC15002630.hg.1	1.59	0.028675	0.131215
linc-C1QTNF1-5	TC17002394.hg.1	1.59	1.60E-07	0.000009
M34339	TC17002615.hg.1	1.59	0.000753	0.007018
L26969	TC05003038.hg.1	1.58	0.000017	0.00033
linc-PPP2R3B-2	TC0X001951.hg.1	1.58	0.294838	0.61422
linc-TMEM88B-1	TC01004129.hg.1	1.57	0.133895	0.390383
AK094779	TC01004217.hg.1	1.57	0.000004	0.000117
linc-C5orf43-1	TC05003020.hg.1	1.57	0.000192	0.002313
NR_037623	TC15002291.hg.1	1.57	0.002041	0.015904
linc-FOXF1-4	TC16001680.hg.1	1.57	0.133609	0.389902
NR_036501	TC07002242.hg.1	1.56	0.000008	0.000193

AK125888	TC08002503.hg.1	1.56	0.000873	0.007912
AK026716	TC0X001693.hg.1	1.56	0.003961	0.027195
X69207	TC11002905.hg.1	1.56	0.004246	0.028736
U80232	TC06002661.hg.1	1.55	0.00013	0.00168
AF085942	TC07002488.hg.1	1.55	0.048528	0.194753
linc-MAOA-2	TC0X001666.hg.1	1.55	0.005446	0.035176
linc-ZNF25-1	TC10002567.hg.1	1.54	0.017415	0.088988
AF029311	TC17002638.hg.1	1.54	0.000007	0.00016
linc-NBPF9-7	TC01004681.hg.1	1.53	0.02606	0.121819
AK056155	TC01004870.hg.1	1.53	0.000009	0.0002
AF086224	TC01006049.hg.1	1.53	0.01442	0.076986
AF113008	TC08001925.hg.1	1.53	0.185875	0.477108
DQ088985	TC15002378.hg.1	1.53	0.013512	0.073148
linc_luo_524	TC07003197.hg.1	1.52	0.000422	0.004397
CR626472	TC11003277.hg.1	1.52	0.000022	0.000402
OTTHUMT00000328528	TC02003287.hg.1	1.51	0.122727	0.369369
uc002vef.2	TC02003799.hg.1	1.51	1.17E-09	2.61E-07
AK311114	TC06003872.hg.1	1.51	2.95E-10	1.13E-07
NR_002211	TC17002074.hg.1	1.51	0.000358	0.00385
uc002jdy.1	TC17002746.hg.1	1.51	0.000001	0.000043
NR_027882	TC19002194.hg.1	1.51	0.000001	0.000046
linc_luo_333	TC01004684.hg.1	1.5	0.004694	0.031159
linc_luo_1805	TC01005654.hg.1	1.5	0.190779	0.484473
NR_028308	TC02004133.hg.1	1.5	0.002093	0.016232
uc011hki.1	TC06002389.hg.1	1.5	0.336701	0.656202
AJ459855	TC06003566.hg.1	1.5	0.188301	0.480849
BC040303	TC06003704.hg.1	1.49	0.000003	0.000093
linc-ANKRD20A1-12	TC09001955.hg.1	1.49	0.000091	0.001259
AL049370	TC10002117.hg.1	1.49	0.013632	0.073681
BC033316	TC01004199.hg.1	-1.49	0.001105	0.009591
linc-CCDC80-2	TC03003030.hg.1	-1.49	0.029249	0.133067
DQ459608	TC04002625.hg.1	-1.49	3.08E-07	0.000015
linc-ZFP57-2	TC06003570.hg.1	-1.49	2.17E-07	0.000012
AL833059	TC09002185.hg.1	-1.49	0.003138	0.022584
NR_027280	TC19002180.hg.1	-1.49	0.000034	0.000579
AY211918	TC01004531.hg.1	-1.5	0.005576	0.035907
NR_027157	TC12003031.hg.1	-1.5	0.000005	0.000129
NR_036462	TC01004175.hg.1	-1.51	0.002087	0.016199
linc-PPIAL4F-5	TC01005804.hg.1	-1.51	0.002016	0.015731
linc-AADAT-2	TC04002818.hg.1	-1.51	0.235108	0.545485
D86978	TC07002605.hg.1	-1.51	0.000018	0.000342

CR601575	TC11003430.hg.1	-1.51	0.002021	0.015764
linc_luo_778	TC13001572.hg.1	-1.51	0.000666	0.006327
NR_028323	TC02004129.hg.1	-1.53	0.000013	0.000272
uc002eea.2	TC16001872.hg.1	-1.53	0.013494	0.073083
CR598627	TC07002576.hg.1	-1.54	0.001751	0.013987
linc-NBPF9-14	TC01004671.hg.1	-1.55	0.000047	0.000741
L13804	TC13001407.hg.1	-1.55	0.013766	0.074222
NR_033302	TC01004881.hg.1	-1.56	0.005702	0.036507
NR_037669	TC07002868.hg.1	-1.56	0.009795	0.056413
linc-GRAMD1B-1	TC11002922.hg.1	-1.56	6.54E-07	0.000027
uc010hrq.1	TC03003054.hg.1	-1.57	0.007241	0.044382
Z70768	TC09002468.hg.1	-1.57	0.000078	0.00112
CR614667	TC06003537.hg.1	-1.58	0.001279	0.010817
CR595160	TC14001578.hg.1	-1.58	0.000315	0.003473
NR_026778	TC01006247.hg.1	-1.59	0.011804	0.065552
U87167	TC0X001927.hg.1	-1.59	0.001597	0.012952
BC005221	TC10002200.hg.1	-1.6	0.020556	0.101559
NR_003105	TC15002245.hg.1	-1.6	0.013286	0.07218
AB451448	TC01005350.hg.1	-1.61	0.000522	0.005197
DQ470079	TC04002368.hg.1	-1.61	0.000775	0.007175
linc-DHRS7B-5	TC17002115.hg.1	-1.61	0.010224	0.058437
NR_036608	TC17002792.hg.1	-1.61	0.00002	0.000372
linc-ZNF791-1	TC19001991.hg.1	-1.62	0.098672	0.320528
linc-ZNF131-4	TC05002352.hg.1	-1.63	0.000329	0.003603
ENST00000499627	TC01005364.hg.1	-1.65	0.000279	0.003145
CR595668	TC0X001880.hg.1	-1.65	1.67E-08	0.000002
BC105606	TC10002226.hg.1	-1.65	0.000159	0.00198
AF333388	TC01006221.hg.1	-1.66	7.35E-07	0.00003
NR_034041	TC17002724.hg.1	-1.67	0.00003	0.000515
AL833658	TC06003604.hg.1	-1.68	0.004967	0.032589
S70154	TC06003166.hg.1	-1.69	0.000541	0.005356
linc-PDZD7	TC10002799.hg.1	-1.7	0.0004	0.004211
NR_026978	TC18000697.hg.1	-1.7	0.003329	0.023636
D83986	TC22001090.hg.1	-1.73	4.21E-08	0.000003
NR_024526	TC02003640.hg.1	-1.74	0.005067	0.033116
D82345	TC0X002162.hg.1	-1.74	0.000219	0.00258
AF086045	TC18000953.hg.1	-1.74	0.012803	0.070056
NR_033142	TC01005497.hg.1	-1.77	0.000836	0.007633
CR616786	TC06003578.hg.1	-1.81	0.00002	0.000374
DQ648894	TC02003219.hg.1	-1.85	0.011223	0.063019
uc010fnw.1	TC02003637.hg.1	-1.9	0.023072	0.110835

uc002ufj.3	TC02003679.hg.1	-1.99	0.010288	0.058714
DQ656056	TC09002285.hg.1	-2.04	0.000016	0.000318
AK307939	TC10002686.hg.1	-2.07	0.000458	0.004673
uc002zdz.1	TC21000762.hg.1	-2.2	2.53E-07	0.000013
CR622106	TC12002644.hg.1	-2.34	0.000154	0.001929
AK021443	TC10002227.hg.1	-2.51	0.011029	0.062107

Supplementary Table S1: Differentially expressed lncRNAs after IR in H460 cells

Supplementary Figure S1

Supplementary Figure S1: Microarray analysis of differentially expressed lncRNAs after IR in NSCLC cell line H460. Heat map of lncRNAs microarray data comparing H460 no radiated (Control) against H460 irradiated (7Gy) after 24 hrs. Only lncRNAs differentially expressed are shown

Supplementary Figure S2

>AK128024.1 Homo sapiens cDNA FLJ46143 fis, clone TESTI2053561 (1986bp)
(with AY598347.3 fragment is called AK47)

CCATTCCTTTTCGAGCCCTTTCAATTTGAGTCCATTCTTTCCAGTCCATTTCACTCCAGTCCATTACTAT
CCATTCCATACCATTCCATCCCATTCCATTCCATTCCATTCCATTCCATTCCATTCCATTCCATTCCATT
CCATTCCATTGCATTCAATTCCATTCCATTCCATTGCACCTGCACTCCATTCCATTACATTCTACTCTATC
TGAGTCGGTTTTATTGCATTAGATTCTATTCCATTGGATTACTTTCCATTTCGATTACCTTCCATTCATGT
ACATTCCATTCCAGTCAATTACATTTCGAGTTCATTACATTACATTCCAGTATATTCCATTGTATTTCGATC
CCATTCCTTTCAATTCATTTCATTTCGACTCCATTATATTCCATTCCATTCCACTCGAATCCATTCCATT
AGAGGACATTCCATTCCAATGCATTCCCTTCCATTCCATAGCATTCCATTGCATTTCGATTCCATTCCATT
TGATGCCATTCCATTTGATGCCATTCCATGACATTCCATTCCATTTCGAGTCCATTCCGTTCCAATTCATT
CCATTCCGTTTCATGAAATTCGAGTCCCTTTCCAGTACATTTTCATTCCAATCCCATCCAATCCAATCTACT
CCATTCAATTCCTTTCCATTCCATTTCGATTGATTCCATTGACTTGATTCCATTTCAGTTTGATTCCATT
CGTGAAATTTTCGTTCCATTCTATTCCATTGCATTACTTTCCATTCAATTCCATTCCATTTCATTTCAGTC
CATTCGCTTCCTTTTCCTTTTCGATTCAATTCCATTTCGATTCCACTCCATTCTATGCGATTTTCATTCCAATC
GATTCAATTCCATTTCGATGACATTCTTTTCGTTTCATTCCATTTCGAGTCCATTCAATTTGAGCATTCGT
GTCCATTCTATTTCGAGTCCATTCCATTACCGTCTATTCTATTCCCTTCCATTCCCTGTTGATTCAATTTCA
TTCCCTTCCATTTCGATTCTTTCCATTTCGATTCCATTCTTTCCATTCCATTCCATTTCGTTCCCACTCCA
TGTGATTTTCATTCCATTCCAGTCCATTATATTGCACTCCACTCCACTCCATTCTATTACATTCAATTCCT
TTTGAGTCCGTTCCATAACACTCCATTTCATTTCGATTCCATTCTTGCCAGTTTTCTTCCATTTTATTCC
ATTCCGTTTCGATTCCATTCCATTTCGATTGCATTCCATTTCGAATCCTTTCCATTCCATTTCATTCCATTCC
TTTCTATTCCATTCCATTTCATTTCGATTTCGATTCCATTCTGCTCTATTCCATTCAATTCCTTTTCATTCC
ATTTCGAATCCTTTCTATTGAGTCCATTCTATTTCGAGTCCATTCCAATCCCTTCCATTCCATTCAATTAC
AGTCCATTCCAATAGATTCCATTCTTTGCTTCCATTTCGAATCCATTCCATTCTAGTCCATTCCATTTCG
AGTCCATTCCATTCCATTCCATTCTATTCTTTCCAATCCATTTCGATTCCATTTCGATTCAATTCCATTTCG
ATTCTCTTTTCATTCTATTTTATTCCATGCCATTTGATTGCATTGCATTCCATTCCGTTTGATTGCAGTCC
ATTCAAGAAAGTTCATTCCAGTCCATTGCTTTCCAGTCCATTCCATTCCACTCTAGTCTATTCCACTCC
ATTCTTTTCATTCCATTCCATACTATTCCATTCCATTCTTTGCTTCCGTTTCCAATCTATTTCGAGTC
CATTGCATTCCAGTCCAATCCATTTCGATTACATTCTTTTGATTCCCTGCCAGTTCGATTGCATTGCATAC
TAGACCATTCCAAAGGAGTCCATTCCATTCTATCTCAACACTTTCCATTCCACTCTGTTTCGAGTCCATT
CATTCCAGTCCATTTAATTCAAGGGCATTCCATTCCATTCCATTCTATTCCATTCCATTCCATTTCATAT
TATTCCATTCCATTCAATTCCATCTCTCCAGATGATTCCATTCCATTCTATACCATTTGCTCTCTGTTCCA
TTCCATTCCATCTGTCTCCATTCTTTTCGTTTCGATTCTTTCCATTCCATTCCATTACATTTGATCCTA
TTTTATTAAATTGCATTCTATTTCGAGTGATTTCATTTCGAGTCTTTCCATTTCGATTCCATTCCCACTG
ACGCCGGCGCTACTTACAGTTGGCGGGGCACAAAAAAAAAAAAA

Lnc-SPRY3-2

Lnc-SPRY3-4

AY598347.3 sequence (591-816)

Splicing region

>AY598346.2 Homo sapiens heterochromatic block map Yq12 transcribed
DYZ1 sequence mRNA, partial sequence (1441)

GGTTCCGTACGATTCCATTCCCTTTGGAATCCATTCCATTGGAGTCCATTCACTTCCAGAACATTCCATTCC
CAGTCGAATCCATTTCGAGTACATTCCATTCCGTGGCTGTCCATTCCATTCCGTTTGATGCCATTCCATAC
GATTCCATTCAATTTCGAGACCATTCTATTCCCTGTCCATTCCCTTGTGGTTCGATTCCATTTCACTCTAGTC
CATTCCATTCCATTCAATTTCAATTTCGACTCTATTCCGTTCCATTCAATTCCATTCCATTTCGATTCCATTT
TTTTTCGAGAACCTTCCATTACACTCCCTTCCATTCCAGTGCATTCCATTCCAGTCTCTTCAGTTCGATTCC
CATTCCATTTCGTTTCGATTCCCTTTCCATTCCAGCCCATTCCATTCCATTCCATTCCCTTTCCCTTTCCGTTT
CATTAGATTCCATTGCATTCCATTTCAATTCAATTCCGTGCTATTCAATTTGATTCCATTTCCATTT
AATTCCATTCCATTAGATTCCATTCCGTACGATTCCATTCCCTTTGGAATCCATTCCATTGGAGTCCATTCC
ACTTCCAGAACATTCCATTCCAGTGAATCCATTTCGAGTACATTCCATTAAAGTTCATAACATTCTAATA
CATTCCATTCCATTGCATTCCATTCCATTCCATTTCGATGCCATTTCGATTCCATTCCATGCCAAATCATTG
CATTCCCTTTCCATTCCGTTCCCTATCAATTCCATTCCATTTCGATTTAGTTTCGATTCTATTCACTTCCATTCC
CATTTCGATTCCATTCCATTGGAGTCAATTCCCTTTCGACACCCAGCCTTTCCAGTCAATGATTTTGGATTCC
CATTTTTTTGCATTCCATTACATTCTATGACATTTCGATTTCGTTTCATTGCATTCCATTCCATACATTTT
TATTCCATTTCGAGACCGTAGCATTCCACTTTATTCCAGGCTGTCCATTACACTACATTCCCTTCCATTCC
CAATGAATTCCATTCCATTCCATTCCATTCCATTCCATTCCATTCCATTCCATTCCATTCCATTCCATTCC
CATAAATTTCACTCCATTTCGTTCCCATTTCCATTCAATTTCGATTCCACTCAATTCCATTCCATTTCGTTTC
CATTCTCTTCGATTCCATTTCTTTATATTCCATGCCATTTCGATTCCATTCTATTGGATTGCATTACATTCC
GTGTTCCATTCCATTCCAGACCATTCCATTTGACTCCATTCCCTTTTCGAGCCCTTTCAATTTGAGTCCATTCC
CTTTCCAGTCCATTTCCTCCAGTCCATTACTATCCATTCCACACCATTCCATTCCATTCCATTCCATTCCATTCC
CATTCCATTCCATTCCATTCCATTCCATTCCATTCCATTCCATTCCATTCCATTCCATTCCACTGCACTGCACTGCAGTC
GGACTACGCGAGTGAACCTCCCTAAAAAAAAAAAAAAAAAAAAA

Lnc-SPRY3-3

Splicing region

Supplementary Figure S2: Overlapping sequences between linc-SPRY3-2/3/4 and clones AY598347.3 and AY598346.2

Supplementary Figure S3

Supplementary Figure S3: Virtual northern blot analysis of linc-SPRY3-2/3/4 in H460 cells demonstrates potential transcript size. (A) Schematic outlining the virtual northern blot method. **(B)** Virtual northern blot reveals two large peaks, one at ~5kb and one at ~1kb. **(C)** These two peaks align when the individual graphs are merged. Similar peaks are seen when a second set of primers for linc-SPRY3-2 and linc-SPRY3-3 are used. GAPDH was used as a control.

Supplementary Figure S4

Supplementary Figure S4: Clonogenic cell survival assay shows the radiation response of a panel of male NSCLC cell lines. Clonogenic cell survival assay shows the relative radiation response of a panel of male NSCLC cell lines. Surviving fractions are plotted as a function of dose. Error bars represent SD from the mean of triplicate measurements from a single experiment. Shown here is a representative of two independent experiments with similar result.

Supplementary Table S2

Cell Line	Driver Mutation	Observed Radiation Response	% Y Chromosome (+) Cells
H460	KRAS	Sensitive	98%
H820	EGFR	Sensitive	100%
H1299	NRAS	Resistant	0%
A549	KRAS	Resistant	0%
WVU-Ma-0005	Unknown	Sensitive	100%
Ma-ALK-0001	ALK Fusion	Resistant	0%

Supplementary Table S2: Cell lines used in this study.

Outline of the observed radiation response of each cell line used in this study color coded to correspond with Supplementary Fig. 2. Cytogenetic analysis and quantification were performed by the WVU Cytogenetics Laboratory in the Department of Pathology, Anatomy and Laboratory Medicine. The percentages shown are a result of the quantification of a population of 200 cells.

Supplementary Figure S5

Supplementary Figure S5: Radiation response of cell line panel normalized to the housekeeping gene UBC and two additional NSCLC cell lines. (A-G) qRT-PCR analysis of the linc-SPRY3 family normalized to UBC. (H-I) qRT-PCR analysis of the linc-SPRY3 family in the additional radiosensitive cell line H157 and radioresistant cell line H1650 normalized to GAPDH. (J-K) qRT-PCR analysis of the linc-SPRY3 family in the additional radiosensitive cell line H157 and radioresistant cell line H1650 normalized to UBC. (L-M) Y chromosome DNA FISH of H157 and H1650 cells.

Supplementary Figure S6

Supplementary Figure S6: Female NSCLC cell lines show no expression of the linc-SPRY3 family. qRT-PCR analysis of the linc-SPRY3 family in female NSCLC cell lines (A) H1975 and (B) H1819. Cells were plated at equal density and treated with one dose of radiation (2Gy, 4Gy, or 8Gy) and collected 72 hours after treatment. Values are relative to untreated control of the same collection day (0Gy). GAPDH mRNA was used to normalize qRT-PCR analysis. Error bars represent SD from the mean of triplicate experiments.

Supplementary Figure S7

Supplementary Figure S7: Cell fractionation of radiosensitive cell lines reveals nuclear localization of the linc-SPRY3 family. qRT-PCR analysis of the linc-SPRY3 family in fractionated (A) H460 and (B) WVU-Ma-0005 cell lines. The dotted line represents the total relative RNA of each experiment. Mature β -Actin mRNA was used as a cytoplasmic control and U6 small nucleolar RNA was used as a nuclear control. Error bars represent SD from the mean of triplicate measurements from a single experiment. Shown here is a representative of three independent experiments with similar result. Normalization was done using *C. elegans* total RNA as an exogenous spike for the amplification of worm specific *ama-1* gene. (C) Coding probability (CP) scores of the *linc-SPRY3-2/3/4* showing no coding probabilities for these lncRNAs (a CP of 0.364 and above is considered a coding RNA). Coding-Potential Assessment Tool (CPAT) was used for these calculations.

Supplementary Figure S8

Supplementary Figure S8: PCR of the pBACe3.6 plasmid backbone in A549 cells

Lane 1: A549 Parental cells, Lane2: A549 cells nucleofected with the empty vector pBACe3.6, Lane 3: A549 cells nucleofected with the BAC clone RP11-88F4. GAPDH is provided as a loading control. DNA was extracted using the Thermo Scientific GeneJET Genomic DNA Purification Kit (#K0722) following the manufacturers protocol for adherent cells. PCR was run using the SsoAdvanced Universal SYBR Green Supermix (Bio-Rad, 172-5271). PCR products were resolved on a 1% agarose gel with ethidium bromide.

Supplementary Figure S9

Supplementary Figure S9: *In vivo* study scheme and untreated controls for the tumor growth delay assays. (A) Schematic detailing the tumor growth delay assay. (B and C) Representative images of ultrasound measurements utilized in determining tumor volume and depth respectively. (D) Tumor growth delay assay with untreated controls for H460 shCtrl and sh1 cells. (E) Tumor growth delay assay with untreated controls for WVU-Ma-005 shCtrl and sh1 cells.

Supplementary Figure S10

Supplementary Figure S10: siRNA knockdown of linc-SPRY3 RNAs in A549 cells nucleofected with the BAC clone RP11-88F4. Representative replicate from triplicate experiments. Error bars represent SD from the mean of 3 technical replicates.

Supplementary Figure S11

Supplementary Figure S11: Representative images of DYZ1 fluorescent *in situ* hybridization in tissue microarrays. (A-C) Images taken of male cores from the lung cancer tissue microarray. (D-F) Images taken of female cores from the lung cancer tissue microarray. (G) Image taken of a core from the testis tissue microarray as a positive control. (H) Image taken of a core from a cervical cancer tissue microarray as a negative control. (I) Survival curves for NSCLC male patients (GEO GSE81089) which express and do not express linc-SPRY3-2. P-value by log rank test.

Supplementary Table S3

shRNA/siRNA Sequences

ID	Target Sequence	Hairpin oligo sequences	
sh1 linc-SPRY3-2	GAAUCCAUUCCA UUAGAGGAC	FWD	CCGGGAATCCATTCCATTAGAGGACCTCG AGGTCCTCTAATGGAATGGATTCTTTTTG
		REV	AATTCAAAAAGAATCCATTCCATTAGAGGA CCTCGAGGTCCTCTAATGGAATGGATTC
sh2 linc-SPRY3-3	GCAUUCCAUUA CAUUCUAUGA	FWD	CCGGGCATTCCATTACATTCTATGACTCG AGTCATAGAATGTAATGGAATGCTTTTTG
		REV	AATTCAAAAAGCATTCCATTACATTCTATG ACTCGAGTCATAGAATGTAATGGAATGC
For cloning: Agel/EcoRI			
ID	Target Sequence	siRNA sequences	
si-linc-SPRY3-2	GAAUCCAUUCCA UUAGAGGAC	5'-GTCCTCTAATGGAATGGATTC-3'	
si-linc-SPRY3-3	GCAUUCCAUUA CAUUCUAUGA	5'-TCATAGAATGTAATGGAATGC-3'	
si-linc-SPRY3-4	GAGUCCGUUCC AUAACACUCC	5'-GGAGTGTTATGGAACGGACTC-3'	

Supplementary Table S4

qRT-PCR/PCR Primer Sequences

Primers	Sequence (5'-3')
linc-SPRY3-2 FWD	CATCCAATCCCATCTACTCCAT
linc-SPRY3-2 REV	TAGAATGGAACGAAATTTACAG
linc-SPRY3-3 FWD	TCCAGTCAATGATTTTGGATTC
linc-SPRY3-3 REV	GGAATAAAGTGGAATGCTACGG
linc-SPRY3-4 FWD	GTCCACTCCAC CCATTCTA
linc-SPRY3-4 REV	CGAACGGAATGGAATAAAAT
linc-SPRY3-2 FWD (2 nd set)	TCCAGTCCATTTCACTCCAG
linc-SPRY3-2 REV (2 nd set)	TGCAATAAAATCGACTCAGATAGA
linc-SPRY3-3 FWD (2 nd set)	CCGTTTCATTGCATTCCA
linc-SPRY3-3 REV (2 nd set)	GCAAGCGAAAGGAAAGGA
GAPDH FWD	CCACTCCTCCACCTTTGAC
GAPDH REV	ACCCTGTTGCTGTAGCCA
UBC FWD	GATTTGGGTCGCAGTTCTTG
UBC REV	CCTTATCTTGGATCTTTGCCTTG
Beta-Actin FWD	AGCACAGAGCCTCGCCTTT
Beta-Actin REV	CCACGATGGAGGGGAAGAC
U6 FWD	GTGCTCGCTTCGGCAGCACATAT
U6 REV	AAAAATATGGAACGCTTCACGAA
<i>C. elegans</i> ama-1 FWD	GGAGCTCGAGTGGATCTTCG
<i>C. elegans</i> ama-1 REV	GCGCAGAGAGTATCCTGGAC
pBACe3.6 FWD	TTGAGTCTGCAAAAGGACTTGA
pBACe3.6 REV	GATTGATGTGTCATCAGCGTCT
HMGA2 FWD	GCCCCAGGAAGCAGCAA
HMGA2 REV	TCGAACGTTGGCGCCCCCTA
C-MYC FWD	
C-MYC REV	

Ultra-Violet Radiation Crosslinking and Immunoprecipitation (CLIP)

Reagents

NET-2 Buffer (Nuclease-free) stored at 4C

(150 mM NaCl; 0.05% NP-40; 50 mM Tris-HCl, pH7.4)	500
mL	
2 M Sodium Chloride (NaCl)	37.5 mL
Surfact-Amps NP-40 (10% Nonidet P-40)	2.5 mL
1 M Tris, pH 7.4 (tris(hydroxymethyl)aminomethane)	25
mL	
Bring volume up to 500 mL with Nuclease-free water, autoclave	
Add Protease inhibitor and RNasin just before using	

Proteinase K Buffer (Nuclease-free)

(50 mM Tris-Cl, pH 7.5, 5 mM EDTA, 1.5% SDS, 300 mM NaCl)	100mL
1 M Tris-Cl (Tris(hydroxymethyl)aminomethane hydrochloride), pH 7.5	5 mL
0.5 M EDTA (Ethylenediaminetetraacetic acid)	1 mL
10% SDS (sodium dodecyl sulfate)	15 mL
2 M NaCl (Sodium Chloride)	15 mL
Mix and bring volume up to 100 mL with Nuclease-free water; autoclave.	

BSA Solution (Nuclease-free) stored at 4

(2 mg/mL)	100 mL
BSA (Bovine serum albumin)	200 mg
Mix and bring volume up to 100 mL with Nuclease-free water; autoclave.	

1X PXL (wash buffer made fresh)

1x PBS (tissue culture grade; no Mg++, no Ca++)	9.89 mL
0.1% SDS (0.01 ml of 10% SDS in 0.99 ml water)	0.01 mL
0.5% sodium deoxycholate (5 mg in 1 ml water)	0.05
mL	
0.5% NP-40 (4.7 ul in 1 ml water)	0.05 mL

Protocol

- 1. 72 hours before Day 1 (if using treated cells)**
 - Treat cell lines with 8Gy radiation (6cm dishes)
 - Let incubate for 72 hours before beginning.
- 2. Day 1 – UV X-linking, BCA assay and Bead Prep (complete this for all samples)**
 - Aspirate media from cells. Rinse with tissue culture grade PBS, remove, and replace PBS.
 - UV irradiate using Stratalinker (On → energy → value → start) with the dish lid off. Can irradiate 2 6cm dishes at a time. MUST be on ice. Irradiate one time for 400 mJ/cm², rotate the plate 90° and irradiate an additional 200 mJ/cm².

- Collect suspension (scrape) for all dishes, pellet cells at 2500 rpm for 5 min at 4°.
- Resuspend pellet in (~3x dry volume) of tissue culture grade PBS; quick spin at 4°, remove supernatant and freeze pellets at -80° until use.
- Resuspend each tube of cross-linked lysate using 700 µl of 1X PXL + 300 µl of HALT protease.
- Add 15 µL RNasin and let sit on ice for 10 min.
- Add 30 µl of RQ1 DNase or DNase 1 (Promega, M6101 or NEB) to each tube; incubate at 37° for 5 min, 1000 rpm.
- Supernatant was collected after 10 min centrifugation at 16,000Xg at 4 C
- Quantitate protein with BCA assay...100 ug of each lysate was used for IP

***Clip end of pipette tip before pipette beads**

- 600 µL Protein G-Sepharose beads in 1.5mL tube; wash beads (spin at 4° C for 3 min at 1000 rpm, then remove supernatant) 2 times with about 500 µL PXL buffer each wash (protein G beads are stored at 4°C).
- Block Protein G Sepharose beads with 900 uL of 2 mg/mL BSA for 1hr or overnight at 4°C (BSA stored at 4°C).

3. Day 2 - Immunoprecipitation

- Wash beads 2 times with 500uL PXL Buffer each time (Did not add RNasin/PI during the washes to save reagents). After last wash, add 600 µL of PXL, 6.0 µl HALT, and 15 µl RNasin.
- Measure out the volume of lysate (100 ug) determined from BCA assay. Divide supernatants into 2 1.5mL tubes (2 antibodies – IGF2BP3, Abcam #177942 and IgG, Abcam #172730).
- Preclear lysates by adding 90uL of blocked/washed beads to each lysate; incubate for 1hr at 4°C on rotator.
- Spin down precleared beads/lysate at 4°C 1,000 rpm for 3 min (low speed when working with beads).
- Transfer supernatant into new tubes and discard beads used for preclearing; bring up to equal volume (500 µl).
- Add desired antibody to remaining lysates (8 ug antibody/lysate). Incubate for 3 hrs at 4°C on rotator.
- Add 50uL of blocked/washed beads to each lysate/antibody tube; Incubate for 1hr at 4°C on rotator.
- Spin down lysate/antibody/beads at 4°C 1,000 rpm for 3 min (low speed when working with beads).
- Divide supernatant into 2 tubes (IP supernatant for RNA and protein).
- Wash pelleted beads 5 times with about 200 uL each wash with NET2-Buffer (Spin down beads at 4°C 1,000 rpm for 3 min. add the following volumes of RNasin to each wash:
 - Wash 1: 14 uL RNasin
 - Wash 2: 14 uL RNasin
 - Wash 3: 6 uL RNasin

- Wash 4: 3 uL RNasin
- Wash 5: 3 uL RNasin
- Take 25% (125uL) of beads for Western Blot analysis -> Spin down beads at 4°C 1,000 rpm for 3 min.
 - Spin → remove supernatant → resuspend in 50 µl PXL buffer
 - Add 10 µL 5X LSB and boil at 100°C for 5 min to remove protein from beads
- Spin down remaining 75% (375uL) of beads at 4°C 1,000 rpm for 3 min. Incubate beads with Proteinase K Buffer (150ul) (w/ 300 mM NaCl) and 1.5mg/mL proteinase K (11.25uL of 20mg/mL) for 30 min at 50°C.
- Then incubate beads for 60 min at 70°C (Add 0.5 uL RNasin/sample).
- RNAs (from beads and supernatant) were recovered with phenol/chloroform extraction
 - Add same volume of Phenol-chloroform-isoamyl alcohol/tube.
 - Vortex 20 seconds (10 up and 10 angled).
 - Centrifuge 10 minutes at 11,400 rpm at RT.
 - Transfer aqueous phase to another centrifuge tube w/ 50 µL of 3M Sodium Acetate (NaOAc), at pH 5.2.
 - Add 1 mL of 100% Ethanol (precooled to -20° C), fill to top of tube, add 1 µL of GlycoBlue (15 µg/µL) Store at -20° C overnight or for a few days to increase yield.

4. **Day 3 – Finish RNA extraction**

- Spin down RNA for 15 minutes at 11,400 rpm in micro-centrifuge at 4°C.
- Aspirate Ethanol (leave a little).
- Wash with 500 uL of 75% Ethanol (precooled to -20°C); don't shake just add.
- Spin down for 1 minute at 11,400 rpm.
- Aspirate Ethanol, remove any remaining with pipette.
- Re-suspend RNA pellet in 20 µL of Nuclease-Free water (Ambion).
- DNase treat samples.
- Collect supernatant.
- Quantify samples.
- Store RNA in -80 until use.

CHAPTER 3

DISCUSSION AND FUTURE DIRECTIONS

For decades, biomedical research has focused on the central dogma of molecular biology; DNA is the blueprint, RNA is the messenger, and proteins are the functional mediators. However, thanks to the Encyclopedia of DNA Elements (ENCODE), which determined that <3% of the human genome gives rise to functional proteins, this dogma has started to be challenged (1,2). The emergence of functional non-coding RNAs (ncRNAs) has revealed that the realm of human biology is much more complex than previously thought, and researchers have only begun to scratch the surface of their impact in molecular biology and disease. We now know that a single ncRNA can function in numerous ways, in numerous pathways, which makes their study imperative to understanding human disease (3,4).

Long non-coding RNAs (lncRNAs) are ncRNAs >200 nucleotides long, lack a functional open reading frame, and have emerged as vital regulators of many important genes and signaling pathways in human cells. Furthermore, their dysregulation has been implicated in multiple cancer types, and current studies are focused around identifying mechanisms of carcinogenesis and their potential as biomarkers for cancer diagnosis and/or prognosis (5). Lung cancer is the number one cause of cancer related mortality and in late stages is notoriously resistant to therapy (6,7). Our group's overall goal is to elucidate the roles of ncRNAs in regulating radiation therapy responsive genes and signaling pathways in an attempt to identify potential therapeutic targets or biomarker candidates. Therefore, we set out to investigate potential ncRNAs, specifically lncRNAs, involved in lung cancer radiation therapy response.

Not only have previous studies showed radioresponsive lncRNAs, but many lncRNAs have been implicated in the radioresistance of cancers (8,9). A few of the mechanisms identified include regulation of anti-apoptotic pathways, DNA damage repair pathways, and promotion of epithelial-to-mesenchymal transition (EMT). Therefore, in the initial stages of the project presented in Chapter 2, we began with a microarray analysis utilizing RNA from a well-known non-small cell lung cancer (NSCLC) cell line (NCI-H460) treated with ionizing radiation to identify candidates

for study. Not surprisingly, we found hundreds of differentially expressed lncRNAs and among the highest upregulated genes we discovered a three-member family; linc-SPRY3-2, linc-SPRY3-3, and linc-SPRY3-4 (also known as lnc-BPY2C-4, lnc-BPY2C-25, and lnc-BPY2C-2, respectively) (Chapter 2, Fig. S1 and Table S1). Unexpectedly, further investigation into the linc-SPRY3 transcripts on LNCipedia revealed that they were transcribed from the Y chromosome (ChY), and to our knowledge, were the first examples of Y chromosome linked lncRNAs (Chapter 2, Fig. 1A).

It was determined that the linc-SPRY3 family maps specifically to the DYZ1 region of the heterochromatic block of the q arm of ChY. Interestingly, this region has been traditionally described as genetically inert, and is characterized by a 3.4 kilobase pentanucleotide tandem repeat sequence which can vary in the number of copies from 2000-4000 within the heterochromatic block (10). Beyond structural characterizations, investigations into its use in forensics, gender verification in sports, or as a tool for monitoring genomic instability in males, there is little known about the DYZ1 region (11–14). This is not surprising considering its suggested lack of transcriptional potential. Our study challenges this notion and proposes that more attention should be paid not only to this region but to ChY in general.

We demonstrate that the linc-SPRY3 transcripts are upregulated after exposure to radiation in cells that have retained ChY (Chapter 2, Fig. 1C, Fig. 2A-D, and Fig. S5A-D, H and I). It is well-known that radiation induces a signaling cascade to trigger cell death. Amongst these signals is changes in chromatin structure to turn specific genes on and off in order to promote the necessary gene expression for cellular responses (15). In the context of the linc-SPRY3 RNAs, it would be interesting to tease out changes in the chromatin marks of the DYZ1 region in response to radiation. As there is currently little to no sequencing data available for the q arm of ChY, using tools such as the UCSC Genome Browser has proven difficult and unsuccessful. Therefore, identifying chromatin changes in the DYZ1 region would allow for the identification of potential promoter sites within the region. Furthermore, specifically identifying the promoter(s) of the linc-SPRY3-2/3/4 transcripts would allow for investigation into upstream regulators of their expression.

One of the major limitations to the study presented in Chapter 2 is the lack of full sequences for linc-SPRY3 transcripts. The sequence fragments we were able to mine from LNCipedia.org gave us enough information to design primers for quantitative reverse transcriptase PCR (qRT-PCR), but without the full sequences we were unable to perform methods such as, northern blotting, RNA fluorescent in situ hybridization (FISH), and cloning to exogenously express the lncRNAs. This also prevents us from determining if the linc-SPRY3 RNAs are a family of lncRNAs or one large transcript. An attractive option for attempting to sequence the linc-SPRY3 family is the Oxford Nanopore minION platform. This technology is one of few that can perform direct RNA long read sequencing. Based on the data we acquired from virtual northern blot analysis, we predict that the SPRY3 lncRNAs are roughly 5 kilobases long, and the near perfect alignment of peaks between all three family members further supports the hypothesis that these three family members could be one large transcript, originally classified as 3 transcripts due to realignment errors because of the highly repetitive nature of their sequences (Chapter 2, Fig. S3).

Long read sequencing has shown much higher accuracy at reading highly repetitive sequences, and the ability to perform direct RNA sequencing is much more ideal as it removes the need to generate a cDNA library, a step which usually involves fragmenting the DNA which is then read and aligned to a reference genome (16–18). If successful, minION sequencing would provide the full sequence(s) of the linc-SPRY3 RNAs. This would then allow us to perform more rigorous studies on their structure and function and ultimately better understand their roles and potential clinical relevance in NSCLC which are further explained in the below paragraphs.

An extremely useful tool to studying RNA is RNA fluorescent in situ hybridization (FISH), and it has become the gold standard for identifying transcript localization (19). Unfortunately, successful RNA FISH relies on sequence specificity in order for the fluorescent probes to hybridize to a transcript of interest. This has made it a challenge to visualize the compartmentalization of linc-SPRY3-2/3/4 using fluorescent microscopy. We did attempt to utilize the sequence fragments that we currently have to design Stellaris RNA FISH probes, but were not successful. Therefore, it is important that we define the complete sequence(s) in order to be able to move forward with this method. However, we have been able to identify that these lncRNAs are nuclear via cellular fractionation (Chapter 2, Fig. S7). An even more important use for this method is its applications

in determining clinical relevance. Successful RNA FISH would enable us to use patient tissue samples such as a tumor microarrays to better elucidate if the linc-SPRY3-2/3/4 correlate with patient therapeutic response and/or survival. Considering the tumor suppressive characteristics we have observed regarding the linc-SPRY3-2/3/4 *in vitro* and *in vivo*, and the survival trends seen using microarray and RNA-seq data (Chapter 2), we predict that a more in depth analysis with a large patient cohort would demonstrate a negative correlation between loss of the linc-SPRY3 RNAs and patient outcomes.

Another important method used for characterizing novel RNAs is northern blotting. Briefly, northern blotting is a method used to visualize RNA expression and determine transcript size, somewhat similar to the way western blots are used to look at protein expression. Northern blots traditionally use radiolabeled (e.g. ^{32}P) oligonucleotide probes (cDNA or RNA) which are complementary to a transcript of interest. In order to design a probe, the sequence of a target RNA is necessary, therefore identifying the full sequence of the linc-SPRY3 RNAs is needed in order to perform this method. Like RNA FISH, we attempted to synthesize northern blot probes based on the sequence fragments mined from LNCipedia, but were unsuccessful at detecting the linc-SPRY3 transcripts. qRT-PCR has been essential in assaying the expression of these lncRNAs, however, how large these transcripts are or if they are a family or a single large transcript is still uncertain. The ability to perform traditional northern blots would complement the data from the previously mentioned virtual northern blots and allow us to better ascertain the potential size(s) of the linc-SPRY3 transcript(s).

Identifying the full sequence(s) of linc-SPRY3-2/3/4 would also be imperative for designing expression vectors (retroviral or lentiviral vectors) to better characterize the effect and molecular functions of these RNAs. A standard in most molecular biology based projects is the ability to knockdown and overexpress genes of interest. While we were very successful at suppressing the expression of the SPRY3 lncRNAs via short-hairpin RNA (shRNA) lentiviral constructs, the opposite proved to be more difficult. However, we were able to circumvent the traditional methods of exogenous expression such as cloning the lncRNAs into a viral vector by utilizing an old tool in a new way: transient expression of the linc-SPRY3 RNAs by transfecting a

bacterial artificial chromosome containing the human Y-chromosome fragment where these lncRNAs are expressed.

Bacterial artificial chromosomes (BACs), bacterial plasmids which contain large fragments of DNA, are tools that were vital for the completion of the Human Genome Project and the completion of sequencing numerous other genomes (20,21), but we were able to take advantage of this technology in a different way. Since we could not clone linc-SPRY3-2/3/4 into an expression vector, we acquired two ChY BAC clones from bacpacresources.org, RP11-80F8 and RP11-88F4. These BACs were chosen because they both contain large fragments of the q arm from the human ChY which includes the DYZ1 region. Fortunately, we were able to introduce these BACs to the radioresistant NSCLC cell line A549 via nucleofection and subsequently detect robust expression of the SPRY3 lncRNAs by qRT-PCR. Additionally, we determined that restoration of linc-SPRY3-2/3/4 expression via BAC nucleofection resulted in a more radiosensitive phenotype *in vitro* (Chapter 2, Fig. 3A-C). While these data were very exciting, there are some drawbacks to the method. This system is highly transient, meaning lengthy studies such as *in vivo* tumor growth delay assays are not possible. Also, there is the potential of introducing unknown ChY factors that might be present in the ChY fragment within the BACs which could influence cellular response to radiation. Therefore, fully sequencing linc-SPRY3-2/3/4 would enable us to generate a more stable and pure expression system and allow us to investigate their functions more specifically and more in depth.

Another future route in regards to studying the linc-SPRY3 family, is fully vetting the molecular mechanism(s) by which they regulate NSCLC cell response to radiation. Our group has previously shown that cell lines that express the SPRY3 lncRNAs are more sensitive to ionizing radiation, thus we sought to determine a potential mechanism by which they mediate this effect (Chapter 2, Fig. 2, Fig. S4, Fig. S5, Table S2). Through *in silico* analysis followed by UV-crosslinked immunoprecipitation (CLIP) we identified the RNA binding protein (RBP) insulin growth factor 2 binding protein 3 (IGF2BP3; also known as IMP3) as a direct binding partner of linc-SPRY3-2/3/4 (Chapter 2, Fig. 4A and 4B). IGF2BP3 presented as an interesting target because previous studies have demonstrated that it is consistently upregulated in numerous tumor types when compared to normal adjacent tissue controls, including lung adenocarcinoma (ADC,

LUAD) and lung squamous cell carcinoma (SCC, LUSC) (Fig.1 and Fig. 2 black box), as well as mediates multiple oncogenic mechanisms in cancer including radioresistance (22–24).

Figure 1: IGF2BP3 expression is significantly upregulated in NSCLC. (Modified from: Expression profile, clinical significance and biological function of insulin-like growth factor messenger RNA-binding proteins in non-small cell lung cancer. Shi et al, *Tumor Biology*, 2017)

While CLIP is a good start at demonstrating the interaction between linc-SPRY3-2/3/4 and IGF2BP3, additional experiments to

confirm the interaction are still needed. In addition to confirming the CLIP experiments in other NSCLC cell lines, it would be complementary to perform multiplexed RNA FISH for linc-SPRY3-2/3/4 and IF for IGF2BP3 to visualize co-localization of the RNA with this RBP. Furthermore, as linc-SPRY3-2/3/4 expression is induced upon treatment with radiation, RNA FISH/IF could reveal clues towards confirming our ceRNA hypothesis as we predict there would be higher enrichment

Figure 2: IGF2BP3 gene expression across human tissue and cancer types. (Modified from: IGF2BP3 From Physiology to Cancer: Novel Discoveries, Unsolved Issues, and Future Perspectives, *Front. Cell Dev. Biol.*, Caterina Mancarella and Katia Scotlandi, 2020)

of IGF2BP3 in the nucleus versus cytoplasm in treated cells as linc-SPRY3-2/3/4 would sequester the RBP to the nucleus.

According to previous studies, IGF2BP3 has multiple targets, and among them is HMGA2 and the oncogene c-Myc, both of which have ties to regulating cell viability and apoptosis (25,26). IGF2BP3 regulates these targets by stabilizing their mRNA which allows for their translation (27–29). We hypothesized that when the linc-SPRY3 RNAs are expressed, they act as ceRNAs to sponge IGF2BP3. This in turn prevents stabilization of its targets, c-Myc and HMGA2, resulting in a shorter half-life of their mRNA and subsequent downregulation in expression. To confirm this, we performed actinomycin-D RNA degradation assays which revealed that expression of the SPRY3 lncRNAs resulted in faster degradation of the mRNAs of HMGA2 and c-Myc (Chapter 2, Fig. 4C). The RNA degradation assay data was obtained from manipulated A549 cells without radiation, and while these data provided valuable results, they only revealed the tip of the iceberg of a potential mechanism for the SPRY3 lncRNAs. Future experiments should include the same RNA degradation assay in other cell lines and confirm downregulation in expression of HMGA2 and c-Myc at the protein level (Western blots). Furthermore, since we provide evidence that increased mRNA degradation of HMGA2 and c-Myc is as a result of IGF2BP3 being sequestered by the SPRY3 lncRNAs, which are only upregulated in radiosensitive cell lines following radiation, it is imperative that we perform knockdown experiments of IGF2BP3 and look at mRNA degradation of HMGA2 and c-Myc as well as cellular response (e.g. viability and apoptosis) to radiation. If our hypothesis is correct, and the linc-SPRY3 transcripts do sequester IGF2BP3 as its primary function, then exogenous overexpression of IGF2BP3 should promote a more radioresistant phenotype in radiosensitive cell lines similar to what was seen upon knockdown of linc-SPRY3-2/3/4, and knockdown of IGF2BP3 in radioresistant cell lines should lead to increased sensitivity to radiation.

It is also important to consider the other functions of IGF2BP3 which could be regulated by the linc-SPRY3 RNAs. We provide significant evidence of a downstream effect on the ability of IGF2BP3 to stabilize mRNA targets such as c-Myc and HMGA2, two targets that were chosen because of their proven roles in regulating cell viability and apoptosis in cancer cells. However, IGF2BP3 is a prolific RBP with thousands of predicted targets and multiple functions aside from

mRNA stability which include miRNA biogenesis, RNA degradation, and RNA localization (Fig. 3) (30). These pathways would provide alternative avenues of study regarding the potential molecular functions mediated downstream of the linc-SPRY3/IGF2BP3 interactions.

Figure 3: Mechanisms of IGF2BP3 (IGF2BP3 From Physiology to Cancer: Novel Discoveries, Unsolved Issues, and Future Perspectives, *Front. Cell Dev. Biol.*, Caterina Mancarella and Katia Scotlandi, 2020)

Numerous studies investigating lncRNAs have revealed that many of them have multiple functions, and moreover interact with more than one binding partner which can be DNA, RNA, or proteins (31). We have provided evidence of a direct interaction between linc-SPRY3-2/3/4 and the RBP IGF2BP3, but we cannot discount other potential interactions of linc-SPRY3 RNAs. In order to investigate other potential interactions, we could employ the method of chromatin isolation by RNA purification (ChIRP). Briefly, biotinylated probes are designed complementary to a transcript of interest (in our case the linc-SPRY3 RNAs) and incubated with lysates from crosslinked cells. Next, magnetic streptavidin beads are used to recover hybridized RNA and anything potentially bound to that RNA due to crosslinking. The recovered material can then be

subjected to sequencing for DNA elements or RNA binding partners, or mass spectrometry for protein binding partners. As mentioned previously regarding other suggested methods, the linc-SPRY3-2/3/4 sequence(s) will need to be determined first so effective probes can be designed to pull down the lncRNAs. In our hands it would be interesting to perform this using lysates from cells either treated or untreated with radiation and then compare the recovered DNA/RNA/protein to identify primary interactions in response to radiation.

An additional aspect of the project presented in Chapter 2 that should be investigated in more depth is the use of ChY as a predictive marker for patient response to radiation therapy as well as overall survival. Previous studies have implicated loss of ChY (LOY) as a marker for poor prognosis/survival in multiple cancer types (32–37). Furthermore, data we collected utilizing ChY DNA FISH from a NSCLC tumor microarray (TMA) revealed a trending negative correlation between LOY and overall survival in males, though the sample size was too small to provide statistical significance (Chapter 2, Fig. 5). We intend on increasing the sample size by adding more TMAs in an attempt to reach statistical significance. Additionally, in an attempt to build on this, we initiated a retrospective study in collaboration with Dr. Malcolm Mattes of Rutgers Cancer Institute of New Jersey (formerly WVU Cancer Institute) to examine LOY using ChY DNA FISH in treatment naïve NSCLC tumor samples. The goal of this study is to determine if LOY could be used as a predictive biomarker for patient response to radiation therapy. Our current hypothesis is that patients whose tumors exhibit high LOY will respond worse to radiation than those whose tumors have managed to retain ChY. If correct, this could eventually lead to a clinical trial to validate LOY as a predictive biomarker for radiation therapy use in male NSCLC patients.

While our group has primarily focused on ChY and linc-SPRY3-2/3/4 in NSCLC, we also have an interest in pursuing similar studies outlined above and in Chapter 2 in other cancer types. It is well documented that disparities between the sexes, independent of race, exist in both incidence and survival for the vast majority of cancer types (Fig. 4). Many mechanisms have been proposed to explain these disparities, though most are primarily attributed to differences in sex hormone function and regulation (38,39). It is only recently that the sex chromosomes and their resident genes have gained significant attention in regards to their roles in regulating carcinogenesis, though the X chromosome has received far more attention than ChY. A recent

PubMed search using the keywords X chromosome and cancer resulted in nearly 4,000 publications, versus Y chromosome and cancer which yielded less than half that at ~1,700 publications.

Figure 4: Major cancer types, in organs unrelated to reproductive function, with gender difference in incidence and mortality. (Reprinted by permission from Springer Nature Customer Service Centre GmbH: Springer Nature, *Nature Reviews Cancer*, Sexual dimorphism in cancer. Clocchiatti et al, 2016)

Overall, we believe that ChY as a whole and its genes, discovered and undiscovered, deserve a lot more attention. Additional studies into their relationship to cancer could provide a better understanding of the rampant sex disparities seen in incidence and mortality across multiple cancers leading to the development of precision medicine not just informed by diagnostic standards such as cancer type and driver mutations but by inherent sexual differences.

Project Summary

1. Identification of Candidate LncRNAs

- Treat cells with radiation and extract RNA
- Send for microarray analysis
- Identify significantly up and downregulated genes

2. Characterization of Linc-SPRY3-2/3/4

- UCSC Genome Browser reveals the linc-SPRY3 RNAs originate from the Y chromosome
- qRT-PCR Analysis reveals a dose dependent increase in expression of linc-SPRY3-2/3/4 in radiosensitive male NSCLC cell lines but not in radioresistant cell lines
- DNA FISH Reveals Y chromosome loss in radioresistant male NSCLC cell lines

3. Manipulation of linc-SPRY3-2/3/4 expression changes NSCLC cell line radiosensitivity

- Exogenous expression of linc-SPRY3-2/3/4 via Y chromosome BAC results in increased radiosensitivity in A549 cells

3. Manipulation of linc-SPRY3-2/3/4 expression changes NSCLC cell line radiosensitivity (cont.)

- Knockdown of linc-SPRY3-2/3/4 via shRNAs results increased radioresistance *in vitro* and *in vivo* in H460 and WVU-Ma-0005 cells

4. Evidence suggests the linc-SPRY3 RNAs function through sequestration of the RBP IGF2BP3

5. The Y chromosome and linc-SPRY3 RNAs have potential clinical relevance by showing trending negative correlations with male NSCLC patient survival.

Future Directions

- Sequence the linc-SPRY3 RNAs via minION nanopore sequencing
- Fully vet the linc-SPRY3/IGF2BP3 interaction and proposed mechanism
- Perform ChIRP to identify potential binding partners, which will allow us to further investigate the Linc-SPRY3 molecular functions
- Use DNA/RNA FISH to correlate Y chromosome presence and Linc-SPRY3 expression with patient survival and validate their potential use as a biomarker for male NSCLC
- Determine if these lncRNAs have a role in other cancers

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Long non-coding RNA FAM83H-AS1 is regulated by human papillomavirus 16 E6 independently of p53 in cervical cancer cells

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I. Abstract

High-risk human papillomavirus (HPV) infection is one of the first events in the process of carcinogenesis in cervical and head and neck cancers. The expression of the viral oncoproteins E6 and E7 are essential in this process by inactivating the tumor suppressor proteins p53 and Rb, respectively, in addition to their interactions with other host proteins. Non-coding RNAs, such as long non-coding RNAs (lncRNAs) have been found to be dysregulated in several cancers, suggesting an important role in tumorigenesis. In order to identify host lncRNAs affected by HPV infection, we expressed the high-risk HPV-16 E6 oncoprotein in primary human keratinocytes and measured the global lncRNA expression profile by high-throughput sequencing (RNA-seq). We found several host lncRNAs differentially expressed by E6 including GAS5, H19, and FAM83H-AS1. Interestingly, FAM83H-AS1 was found overexpressed in HPV-16 positive cervical cancer cell lines in an HPV-16 E6-dependent manner but independently of p53 regulation. Furthermore, FAM83H-AS1 was found to be regulated through E6-p300 pathway. Knockdown of FAM83H-AS1 by siRNAs decreased cellular proliferation, migration and increased apoptosis. FAM83H-AS1 was also found to be altered in human cervical cancer tissues and high expression of this lncRNA was associated with worse overall survival, suggesting an important role in cervical carcinogenesis.

II. Introduction

High-risk HPV infection (e.g. HPV-16) is one of the most common causes of cervical cancer (1-3), as well as a subset of head and neck squamous cell carcinoma (HNSCC) (1). The HPV oncoproteins E6 and E7 have been shown to contribute to carcinogenesis by modulating the degradation of human proteins, such as the tumor suppressors p53 (4) and Rb (5) as well as a plethora of other cellular proteins (2,3,6-8). The HPV-16 E6 protein can abrogate p53 function by proteasomal degradation as it forms a complex with E6-associated protein (E6AP) (9), or by targeting the p53 coactivator CBP-p300 (8,10). Upon transmission, HPV infects the undifferentiated keratinocytes at the basal layer of the stratified epithelia and its genome remains episomal maintaining low copy numbers. During the course of cancer development, the viral genome frequently becomes integrated into the host cell DNA (11).

The recent discovery of different classes of non-coding RNAs (ncRNAs) expressed in human cells has opened a new chapter in the understanding of cellular processes, such as chromatin remodeling, transcriptional control, and post-transcriptional regulation. One of these classes of ncRNAs called long non-coding RNAs (lncRNAs) are defined as RNAs larger than 200 nucleotides that are not translated into proteins. Recent findings indicate that lncRNAs are involved in gene regulation at the transcriptional level by functioning as signal, guide, decoy, or scaffold RNAs (12-14). Dysregulation of lncRNAs occurs in a variety of cancers, suggesting a potential use of these ncRNAs as biomarkers for diagnosis, prognosis, stage of cancer, and response to therapy (15-18). lncRNAs have been shown to be altered in cervical cancer (19-21), however, only a few publications have studied lncRNAs that are specifically regulated by the HPV E6 oncoprotein, such as MALAT1 and CCEPR (22,23). These lncRNAs were found altered in cervical cancer but it is unknown if these alterations are part of the early events in cervical carcinogenesis. A few studies have looked at aberrant expression of lncRNAs in progression from pre-malignant cervical intraepithelial neoplasia (CIN) to cervical cancer (24,25). For these reasons, it is important to understand if certain lncRNAs are important in the first stages of immortalization and transformation caused by HPV infections.

In this study, we demonstrated that the lncRNA FAM83H-AS1 (also known as onco-lncRNA-3) is up-regulated in primary keratinocytes expressing HPV-16 oncogene E6 as well as HPV-16 positive human cervical cancer cell lines and cervical tumor samples. We show that FAM83H-AS1 is regulated by HPV-16 E6 through the presence of p300 instead of the tumor suppressor p53. Finally, we show that FAM83H-AS1 is involved with cellular proliferation, migration, and apoptosis and is associated with worse overall survival in cervical cancer patients.

III. Results

A. High-risk HPV-16 E6 oncoprotein alters host long non-coding RNAs in primary keratinocytes.

As an initial screen to identify host lncRNAs that are regulated specifically by HPV-16 E6, we developed a system to look specifically at the effect of E6 expression alone in primary human

foreskin keratinocytes (HEKa). HEKa were infected with a retroviral vector expressing HPV-16 E6 oncogene or GFP as a control. After puromycin selection and stable expression of HPV-16 E6 was confirmed by RT-PCR (Fig. S1), RNA was extracted, and samples were analyzed by RNA high-throughput sequencing (RNA-seq) to determine gene expression alterations in long non-coding RNAs (lncRNAs). Following bioinformatics analysis, we found 151 up- and 100 down-regulated host lncRNAs altered greater than 1.5-fold change when HPV-16 E6 was expressed in HEKa cells compared to GFP control (Fig. 1A, Table S1). From these host lncRNAs, we randomly chose 8 up- and 8 down-regulated host lncRNAs to validate our RNA-seq data by qRT-PCR. The expression of all the lncRNAs selected for validation followed the same trend of up- or down-regulation found by RNA-seq (Fig. 1B). In order to determine the importance of these 251 lncRNAs in cervical carcinogenesis, a variety of filtering methods were utilized to reduce our scope (see Methods section). After filtering, we performed preliminary experiments with many lncRNAs, however, some of our top altered lncRNAs from our RNAseq analysis of foreskin keratinocytes (HEKa) were not altered in cervical cells (HCK) with HPV-16 E6 expression (e.g. SNHG15). This is not surprising, as lncRNAs are known to typically be tissue specific (26). We also found differences in the expression of some lncRNAs between pre-malignant and cancerous cervical cells CaSki and W12/201402 to HCK (e.g. miR205HG). One of the up-regulated lncRNAs in our RNA-seq dataset, FAM83H-AS1 was intriguing. Its expression recently was found to correlate with poor overall survival in a variety of human cancers (27-32) but had not previously been shown in the context of cervical cancer. In addition, it was recently shown to be involved in regulating cellular processes associated with the hallmarks of cancer (e.g. proliferation and migration) (28,31,32). To ensure that expression of FAM83H-AS1 is comparable to lncRNAs with well-known functions, we utilized our RNAseq dataset to compare their RPKM values (Table S2).

B. FAM83H-AS1 expression is higher in HPV-16+ pre-malignant and cancerous samples.

Because many lncRNAs are known to be tissue specific (26) and our RNA-seq screening was performed with foreskin keratinocytes expressing HPV-16 E6 oncogene, it was critical to confirm the expression changes of FAM83H-AS1 in epithelial keratinocytes of the cervix where HPV naturally infects (11). Additionally, we considered that the E6 and E7 oncogenes can be

synergistic with each other (33), so we needed to develop a model that closely mimics HPV infection by using the entire HPV-16 genome. Therefore, primary human cervical keratinocytes (HCK) were transfected with the entire HPV-16 genome (by releasing the viral genome from a plasmid construct and circularizing it by ligation before transfection), then passaged several times (around 10-15 divisions) for growth selection of HPV positive immortalized cells. HPV-16 E6 and E7 expression was confirmed by RT-PCR (Fig. S2A), as well as p53 degradation through the HPV-16 E6 pathway by Western Blot (Fig. S2B). After confirmation of HPV-16 oncogene expression in these cells, we named them JAMM-16. It was confirmed by qRT-PCR that FAM83H-AS1 is also up-regulated in JAMM-16 cervical keratinocytes expressing the entire HPV-16 genome in comparison to the parental cervical keratinocytes (Fig. 2A). We then used HPV-16 positive low-grade cervical (W12/20863 [episomal HPV-16], W12/201402 [integrated HPV-16]) and carcinoma (CaSki [integrated HPV-16]) cell lines to investigate the expression of FAM83H-AS1. As shown in Figure 2B, we found higher expression of FAM83H-AS1 in all the HPV-16 positive cell lines in comparison to HCK cells. Interestingly, FAM83H-AS1 was expressed at lower levels in HPV-negative cervical cancer C33A cells compared to HCK as well as HPV-16 positive cervical cells (Fig. 2B). Furthermore, HPV-16 positive and HPV-negative head and neck squamous cell carcinoma (HNSCC) cell lines were compared, and higher expression of FAM83H-AS1 was observed in the HPV-16 positive versus the HPV-negative HNSCC (Fig. 2C). Overall, we conclude that presence of HPV-16 correlates with elevated levels of FAM83H-AS1 expression in early stages of cervical carcinogenesis (newly immortalized JAMM-16 cells and cervical low-grade pre-malignant cell lines) as well as cervical cancer and HNSCC cell lines.

C. FAM83H-AS1 expression is regulated by HPV-16 E6 in a p53-independent, p300-dependent manner.

As shown in Figure 1, foreskin keratinocytes (HEKa) expressing HPV-16 E6 up-regulated FAM83H-AS1 expression. To further confirm HPV-16 E6 regulation of FAM83H-AS1 in cervical cells, cervical keratinocytes (HCK) stably expressing HPV-16 E6 were developed (Fig. S3). To measure if FAM83H-AS1 regulation could be affected by HPV-16 E7 oncogene in synergistic or antagonistic manner, we generated HCK stable cell lines expressing HPV-16 E7 or co-expressing HPV-16 E6 and E7 (Fig. S3). FAM83H-AS1 was up-regulated when cells expressed HPV-16 E6

or HPV-16 E6 and E7, but not when they expressed HPV-16E7 alone (Fig. 3A) suggesting a specific regulation by E6. To further confirm these findings, HPV-16 E6 was knocked down in CaSki (Fig. 3B, Fig. S4A) and W12/201402 (Fig. 3C, Fig. S4B) cell lines by two different siRNAs against HPV-16 E6-E7. FAM83H-AS1 expression was down-regulated after the reduction of HPV-16 E6 expression confirming the regulation of this lncRNA by HPV-16 E6. It is well known that one of the major HPV E6 targets is the tumor suppressor p53 (34), which is involved in the regulation of cell proliferation, DNA repair, and apoptosis (35). Interestingly, when we knocked down p53 in HCK by using two different siRNAs, we observed that FAM83H-AS1 expression did not change (Fig. 3D, Fig. S4C), suggesting a regulation by HPV-16 E6 in a p53-independent manner. It is also known that HPV-16 E6 is able to regulate the expression of other important genes in carcinogenesis such as hTERT through the regulation of transcriptional coactivators such as p300 (36). By using the UCSC Genome Browser (<https://genome.ucsc.edu/>) to investigate potential p300 binding site in the promoter region of FAM83H-AS1, we found three predicted p300 binding sites (Fig. 3E). In order to measure the potential regulation of FAM83H-AS1 by p300, we used two siRNAs against p300 and quantified FAM83H-AS1 expression. When p300 was knocked down in primary cervical keratinocytes, FAM83H-AS1 expression was also reduced (Fig. 3F, Fig. S4D) suggesting direct and/or indirect p300 regulation of FAM83H-AS1. Previous publications have shown a greater affinity of HPV-16 E6 to interact with p300 in comparison with other high-risk HPV E6 such as HPV-18 E6(37). Interestingly, FAM83H-AS1 expression was found to be up-regulated in HPV-16 positive cell lines (Fig. 2), but down-regulated in HPV-18 positive HeLa cells and HPV-31b positive CIN-612 cells (Fig. S4E). Altogether, we found that FAM83H-AS1 is regulated by HPV-16 E6 independently of p53 but influenced by the presence of p300.

D. FAM83H-AS1 is localized to the nucleus and does not regulate transcription of nearby FAM83H.

The cellular localization of lncRNAs can provide information on their potential function. Nuclear lncRNAs can regulate at the transcriptional level by interacting with critical epigenetic regulators and enhancing chromatin looping, as well as interact with splicing factors to regulate splicing (38). Meanwhile cytoplasmic lncRNAs have been found to function at the post-

transcriptional level as competing endogenous RNAs by acting as microRNA sponges and binding to mRNAs leading to the recruitment of RNA binding proteins that promote decay, suppress translation, or factors that initiate translation (39). For this reason, we investigated the cellular localization of FAM83H-AS1 in two HPV-16 positive cervical cell lines by cellular fractionation. We used U6 as a nuclear RNA control and β -actin mature mRNA as a cytoplasmic RNA control. We found significantly higher amplification of FAM83H-AS1 in the nuclear fractions in comparison to the cytoplasmic fractions by qRT-PCR in CaSki (Fig. 4A) and W12/201402 (Fig. 4B) cells, suggesting that FAM83H-AS1 is a nuclear lncRNA. Because many nuclear lncRNAs can act in cis (38), we hypothesized that FAM83H-AS1 could regulate its nearby protein coding gene FAM83H. The lncRNA FAM83H-AS1 and protein coding gene FAM83H share a promoter region but are transcribed in opposite directions (27) (Fig. 3E, Fig. S5). FAM83H is required for the organization of the keratin cytoskeleton in epithelial cells (40) and has been shown over-expressed in different tumor samples compared to their matching normal tissues (41). Interestingly, we found increased expression of FAM83H in HCK expressing HPV-16 E6 in comparison to parental HCK cells (Fig. S5B), but when we transfected an siRNA against FAM83H-AS1 in HPV-16 positive CaSki cells, we were unable to detect changes in FAM83H expression (Fig. S5C), suggesting that FAM83H-AS1 is not involved in regulation of FAM83H expression.

E. FAM83H-AS1 knockdown in cervical cancer cells causes reduced cellular proliferation and migration, as well as induction of apoptosis.

To understand the significance of FAM83H-AS1 in cervical cancer cells, we analyzed the effects on cellular proliferation when FAM83H-AS1 was knocked down by siRNA in CaSki and W12/201402 cervical cells. First, knockdown efficiency of a pool of 4 individual siRNAs (SMARTpool), as well as each of the individual siRNAs, was evaluated by qRT-PCR. Knockdown of FAM83H-AS1 in CaSki with the siRNA SMARTpool was maintained over a time-course from 24 to 120 hours, which was sufficient for all functional assays conducted (Fig. S6). All of the individual siRNAs, as well as the SMARTpool showed greater than 51% knockdown of FAM83H-AS1 in CaSki (Fig. 5A) and greater than 49% in W12/201402 cells (Fig. S7A). Therefore, we decided to randomly choose two of the individual siRNAs and SMARTpool to knockdown

FAM83H-AS1 and monitor cellular functional changes. Two siRNAs against FAM83H-AS1 were transfected independently into CaSki and W12/201402 cells, cultured for 48 hours, and replated to measure cell proliferation by cell counting. In both CaSki and W12/201402 cells, we observed a decrease ($\geq 48\%$) in cell number with knockdown of FAM83H-AS1 compared to control (Fig. 5B, Fig. S7B). Cellular proliferation assay (CCK-8) showed that there was a decrease in cellular proliferation when FAM83H-AS1 is knocked down in CaSki cells and W12/201402 as monitored from 48 hours to 96 hours after replating. We found significantly less proliferation in CaSki (64% decrease) and W12/201402 (73% decrease), in the siRNA FAM83H-AS1 knockdown compared to siRNA control cells at the 96-hour time point (Fig. 5C, Fig. S7C). We observed similar functional changes between the two individual siRNAs and the SMARTpool so for future functional assays we only used the siRNA SMARTpool. In order to identify changes in cell cycle that could explain the differences found in cellular proliferation after FAM83H-AS1 knockdown, we performed cell cycle flow cytometry analysis. CaSki and W12/201402 cells had a significant reduction (43% and 56%, respectively) of cells in S-phase when FAM83H-AS1 was knocked down in comparison to control suggesting that FAM83H-AS1 is important in the G2/S-phase transition (Fig. 5D, Fig. S7D). Other important hallmarks of cancer such as migration and resistance to apoptosis were measured after FAM83H-AS1 knockdown. We found that cellular migration was significantly decreased in CaSki and W12/201402 after siRNA knockdown of FAM83H-AS1 compared to siRNA control (Fig. 5E, Fig. S7E). Also, we measured a significant increase in early and late apoptosis in CaSki and W12/201402 cells with knockdown of FAM83H-AS1 compared to control cells by using Annexin V/PI staining and flow cytometry (Fig. 5F, Fig. S7F and S7G-H). W12/201402 cells with knockdown of FAM83H-AS1 showed a significant increase in necrosis (Fig. S7F), while only a slight increase in necrosis was observed in CaSki cells (Fig. 5F, Fig. S7H). Altogether, we observed significant alterations in cellular proliferation, cell cycle, migration, and apoptosis by the absence of FAM83H-AS1, suggesting an important role in cervical carcinogenesis.

F. FAM83H-AS1 expression is increased in cervical cancer human tissues & is associated with worse overall survival.

In order to extrapolate our findings into a more clinically relevant setting, we extracted RNA from pre-malignant and cervical cancer patient samples and analyzed the expression of FAM83H-AS1 by qRT-PCR. We found high expression of FAM83H-AS1 in the pre-malignant sample (CIN3) as well as the cervical cancer (CaCx) samples in comparison to normal cervix tissue (Fig. 6A). These findings corroborate our *in vitro* data suggesting an importance of FAM83H-AS1 in clinical tumor samples at different stages of carcinogenesis. We also took advantage of the cervical cancer samples deposit in the TCGA database to compare the expression of FAM83H-AS1 between normal cervix and cervical cancer samples obtained from different cancer stages (120 Stage I, 35 Stage II, 30 Stage III, 7 Stage IV, 4 Stage unavailable). The TCGA data showed elevated expression of FAM83H-AS1 (RPKM values) in cervical cancer patients compared to normal cervix control (Fig. 6B). This coincides with our previous observations of FAM83H-AS1 expression being higher in cervical cancer cells lines (Fig. 2B). Finally, we used the TCGA data set from the TANRIC database to divide cervical cancer patients into high versus low expression groups and measured overall survival based on FAM83H-AS1 expression. Interestingly, we found that patients with higher FAM83H-AS1 expression yielded a worse overall survival than patients with lower FAM83H-AS1 expression suggesting a biological importance of this lncRNA in cervical cancer reflected in patients' clinical outcomes (Fig. 6C). Overall, we conclude that FAM83H-AS1 expression is elevated in cervical cancer patients and high expression correlates with overall poor survival.

IV. Discussion

4.5% of all cancers worldwide are attributable to HPV infection. Almost all cervical cancers and a substantial amount of other anogenital and oropharyngeal cancers have been found to be infected by high-risk HPVs. HPV-16 and -18 contribute to 73% of HPV-associated cancers (42), implying a higher ability to induce tumorigenesis in comparison to other types of HPVs. Although the prevalence of HPV-associated cancers has decreased due to development of the preventative vaccine and early detection screening methods (2), there is still a great need for prognostic and therapeutic options specially to people already infected with HPV as well as those affected in less developed countries where the access to the vaccine is limited.

Long noncoding RNAs (lncRNAs) have been shown to regulate a variety of critical cellular processes, including transcription and chromosome remodeling (12-14). Dysregulation of lncRNAs has been shown to be associated with the development and progression of many cancers (15-17), and interestingly they are commonly tissue specific (26) and only altered in one cancer type (32). Therefore, lncRNAs are currently being studied in the context of biomarkers for diagnosis and prognosis of cancer, as well as therapeutic targets (15-18).

Previous studies have shown that the high-risk HPV E6 protein expressed is clearly involved in the progression of carcinogenesis. HPV E6-regulation of non-coding RNAs such as microRNAs has been well studied (43), however long non-coding RNA regulation by high-risk HPV E6 needs to be studied further; there are only a couple lncRNAs shown to be specifically HPV-16 E6 regulated, including MALAT1 and CCEPR (22,23). To add to this field of study, we sought out to identify an HPV-16 E6 regulated gene that was altered from the early stages of HPV infection until carcinogenesis and therefore we considered to be important in both the development and progression of carcinogenesis. Thus, we developed a new HPV-16 positive cell line referred to as JAMM-16 to represent early infection, but also analyzed expression in established HPV-16 positive cervical cell lines such as CaSki and W12 cells as well as pre-malignant and malignant cervical tumor samples. We found FAM83H-AS1 overexpression in W12/20863 and W12/201402 (which came from a CIN2 tumor) similar to CaSki cells (cervical carcinoma). Additionally, in Figure 6A, the CIN3 (considered Stage 0 cervical carcinoma) patient sample shows similar expression to later stage cervical carcinoma (CaCx) samples. For this reason, we believe that FAM83H-AS1 up-regulation in pre-malignant cervical samples could be linked to the expression of viral oncogenes in early HPV infection.

From the host lncRNAs altered by HPV-16 E6 in our RNA-seq analysis (Fig. 1), we found several of these lncRNAs previously described to be altered in cervical cancers, confirming that our data aligned with former studies. For example, it was previously found that decreased expression of GAS5 is associated with poor prognosis of cervical cancer patients (44,45) as well as is tumor suppressive in other types of cancer such as breast cancer (46,47) and prostate cancer (47). Furthermore, GAS5 expression was also found altered *in vitro* in HPV-16 positive CaSki cells (44) and HPV-18 positive HeLa cells (45). Another lncRNAs affected in our study was H19.

It was shown previously that DNA methylation alterations at the IGF2/H19 imprinted domain may mediate the association between HPV and invasive cervical cancer (48) and high H19 expression has also been shown to be predictive of poor prognosis in cervical cancer (49) as well as in a variety of other human cancers, including HNSCC (50) and breast cancer (51).

A class of lncRNAs known as onco-lncRNAs were also interesting to us because, as the majority of lncRNAs are tissue specific (26), this group of lncRNAs exhibit differential expression across multiple cancers and are hypothesized to have conserved oncogenic or tumor suppressive functions. One such onco-lncRNA which was found in our RNA sequencing analysis of an E6-regulated lncRNA is onco-lncRNA-3, referred to as FAM83H-AS1. This lncRNA is transcribed from chromosome 8 and its function in normal cells is unknown. It was first characterized in 2015 (32), and as of now multiple publications have shown increased expression of FAM83H-AS1 in breast (29,32), lung (31,32), colorectal (28,30,32), kidney (32), bladder (32), and pancreatic cancers (27) and increased expression correlates with worse overall survival in most of these cancers (27,29-31). Our findings show for the first time that FAM83H-AS1 is overexpressed in human cervical cancer (CESC) tissues and high expression in patients correlates with poor overall survival (Fig. 6).

According to previous studies, FAM83H-AS1 is an epithelial lncRNA (27) supporting our data obtained from foreskin and cervical keratinocytes. Determining the localization of a lncRNA can predict functionality of the lncRNA; our findings that FAM83H-AS1 is localized in the nucleus of cervical cancer cells (Fig. 4) is consistent with previous findings of its nuclear localization in lung cancer cells (14). Functionally, it has been found to be co-expressed with protein coding genes that were enriched for cell cycle-related genes (32), and knockdown of FAM83H-AS1 altered cell cycle (31,32), proliferation (28,31), migration (28,31), invasion (31), and apoptosis (28) in certain cancers. Our group shows here that in the context of cervical cancer, FAM83H-AS1 is involved in cell cycle, proliferation, migration, and apoptosis (Fig. 5). It is unknown if FAM83H-AS1 elicits its functions in cis or trans, but our findings suggest that FAM83H-AS1 does not elicit cis regulation on the nearby protein coding gene FAM83H (Fig. S5), which is up-regulated in a variety of human cancers. For this reason, it will be interesting in future

studies to identify the protein, RNA, and/or DNA interactions of FAM83H-AS1 in cervical cancers.

Previously, it was shown that FAM83H-AS1 regulates MET/EGFR signaling in lung cancer cells (31) and that when FAM83H-AS1 was downregulated it exhibited an anti-proliferative role by suppressing the Notch signaling pathway in colorectal cancer (28). To elucidate additional downstream targets of FAM83H-AS1, a group recently conducted RNA-seq on a pancreatic cancer cell line with siRNA knockdown of FAM83H-AS1 compared to control and identified gene alterations (78 activated and 68 inhibited targets) (27). Our group plans to determine if these downstream regulators are also involved in FAM83H-AS1 mediated functional changes observed in cervical cancer cells.

HPV-16 E6 and -18 E6 are well known to contribute to the degradation of p53, however, it is important to note that HPV-16 and HPV-18 vary in their interactions with other proteins to regulate carcinogenesis. For example, previous studies have shown that HPV-16 E6 directly interacts with CBP/p300 (8,10,37), but HPV-18 E6 appears to be unable to interact with p300 (37). This could be a possible explanation for variation in FAM83H-AS1 expression between HPV-16 and -18 positive cancers observed in our study (Fig. 2, Fig. S4E). Supporting our data, another publication previously showed low expression of FAM83H-AS1 in HeLa (HPV-18 positive) cells (27). This information led us to elucidate the mechanism of FAM83H-AS1 up-regulation by E6 in a p53-independent and p300-dependent manner (Fig. 3, Fig. S4). This regulation is interesting because the majority of E6 regulation of several coding and non-coding genes is primarily through the p53 pathway. Interestingly, a previous study showed that overexpression of cyclooxygenase (COX-2) gene was the result of the recruitment of p300 to its promoter region via the overexpression of HPV-16 E6 protein in CaSki cells as well as the exogenous expression of HPV-16 E6 in HPV-negative C-33A cells (52). Recently, p300 inhibitors such as C646 have been shown to be good candidates as anti-cervical cancer drugs, demonstrating the importance of p300 not only in the regulation of host genes but also of HPV viral genes (53).

In summary, the identification of FAM83H-AS1 up-regulation in the early stages of cervical carcinogenesis, correlation with overall survival in cervical cancer, and involvement in

different hallmarks of cancer contributes further evidence of the importance of this lncRNA in cancer. Further studies on this lncRNA could enhance the use of FAM83H-AS1 as a potential biomarker or therapeutic target in multiple cancers.

V. Methods

Detailed experimental protocols are described in the Supplementary Methods section. All experiments were performed in compliance with the Institutional Biosafety Committee at West Virginia University, number 15-03-03.

A. Cells

The following cell lines were used: human primary foreskin keratinocytes (HEKa) (Invitrogen, C-005-5C); human primary cervical keratinocytes (HCK) and J2-3T3 murine fibroblast feeder cells (obtained from Dr. Alison McBride's laboratory, NIH, Bethesda, MD); 3T3M murine fibroblast feeder cells, as well as CaSki (HPV-16 positive), HeLa (HPV-18 positive), and C-33A (HPV negative) cervical carcinoma cells (obtained from Dr. Daniel DiMaio's laboratory, Yale University, New Haven, CT); W12/201402 (HPV-16 positive) and W12/20863 (HPV-16 positive) pre-malignant cervical cells (obtained from Paul F. Lambert, University of Wisconsin-Madison, Madison, WI); CIN-612 (HPV-31b positive) (obtained from Dr. Laimonis A. Laimins' laboratory, Northwestern University, Chicago, IL); UMSCC-1 (HPV negative), UMSCC-47 (HPV-16 positive), and UMSCC-104 (HPV-16 positive) head and neck squamous cell carcinoma (HNSCC) cell lines (obtained from Dr. Scott A. Weed's laboratory, West Virginia University, Morgantown, WV). Further details are in the Supplementary Methods section.

B. High-throughput RNA sequencing

Three replicates each of human foreskin keratinocytes (HEKa) stably expressing HPV-16 E6 or GFP were sent for RNA high-throughput sequencing (Illumina). FASTQ files were subsequently imported into Strand NGS suites for analysis. Reads were aligned to the human hg19 reference genomes using the Bowtie algorithm. These were then quantified against Ensemble

transcript and including small and lncRNA annotations. Any lncRNA that were detected in human subjects were used for further analysis. Raw lncRNA counts were then normalized to the total number of lncRNA reads per sample and expression values calculated against the control samples. Further Mapping rate visualization done using Strand NGS software. In order to determine the importance of the lncRNAs obtained from the RNA-seq analysis, we used the following filtering strategy: First, only lncRNAs with reasonable expression (RPKM greater than 1) were analyzed further. Then, we used The Atlas of Noncoding RNAs in Cancer (TANRIC, MD Anderson Cancer Center) (54), which contains 297 sequenced human cervical squamous cell carcinoma and endocervical adenocarcinoma (CESC) patient data from The Cancer Genome Atlas (TCGA), to analyze the expression and clinical outcomes of these lncRNAs in patient samples. Finally, we searched previous publications to identify lncRNAs altered in other types of cancer and/or involved in hallmarks of cancer. To increase our novelty, we eliminated lncRNAs that were previously shown to be involved specifically in cervical cancer (e.g. H19).

C. Functional Analysis

For cell counting experiments, CaSki cells were transiently transfected with Lincode Human FAM83H-AS1 siRNA SMARTpool (Dharmacon, R-188909-00-000), Individual: Lincode FAM83H-AS1 siRNA (N-188909-02-0002 and N-188909-04-0002), or Lincode Non-targeting siRNA #1 (Dharmacon, D-001320-01-05) using Lipofectamine® RNAiMAX according to manufacturer's instructions (Invitrogen).

CaSki cells were cultured with siRNA-containing media for 48 hours, re-plated in equal cell numbers (200,000 cells/well of 6-well), cultured for another 48 hours, and attached cells were re-counted with a hemocytometer.

For all other functional assays (CCK-8 cell proliferation, FACS cell cycle, transwell migration, annexin V-FITC/PI apoptosis), CaSki cells were transiently transfected with Lincode Human FAM83H-AS1 siRNA SMARTpool or Lincode Non-targeting siRNA #1 (Dharmacon, D-001320-01-05) using Lipofectamine® RNAiMAX according to manufacturer's instructions (Invitrogen). Cells were incubated with siRNA-containing media for 24 hours then re-plated in

equal cell numbers for to initiate experiments described below. To monitor cell proliferation, transfected cells were plated in 96-well plates and after 24, 48, 72, and 96 hours analyzed with CCK-8 kit (Sigma-Aldrich) according to manufacturer's protocol. Alterations in cell cycle were determined by flow cytometry propidium iodide DNA staining. Transfected cells were plated in media containing 10% fetal bovine serum (FBS). Cells were allowed to attach and then were serum-starved for 24 hours. Samples were then fixed with ethanol, stained with propidium iodide, and analyzed by flow cytometry (Fortessa S10). For transwell migration assay, transfected cells were seeded onto upper chambers of transwell inserts (8µm pore size) with 20% FBS chemoattractant in the lower chamber of 24-well plate. After 48 hours, migrated cells located on the underside of the transwell insert were stained with 0.5% crystal violet in methanol. Migrated cells were quantified using ImageJ software. To monitor apoptosis, transfected cells were plated and incubated 24, 48, and 72 hours. At desired time point, attached and floating cells were pelleted and co-stained with annexin V-FITC and propidium iodine and immediately analyzed by flow cytometry (Fortessa S10).

VI. Data Availability

The RNA-seq raw data generated during and/or analyzed during the current study are available in the Gene Expression Omnibus (GEO-NCBI) repository, accession number: GSE115334.

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IX. Author Contributions:

J.A.B., K.E.H. and I.M. designed research; S.K. provided initial analysis and collected cervical tissue samples; J.A.B, K.E.H., T.B., A.D.H, and I.M. performed research; J.A.B, K.E.H., T.B., A.D.H, R.J. P.R.L., and I.M. analyzed data; J.A.B. and I.M. wrote the paper.

X. Additional Information:

The authors declare no competing interests.

XI. Figure Legends:

Figure 1: Differential expression of host lncRNAs after expression of HPV-16 E6 in primary foreskin keratinocytes. (A) Waterfall plot of host lncRNAs altered 1.5-fold or greater in primary foreskin keratinocytes (HEKa) expressing HPV-16 E6 compared to uninfected HEKa by high-throughput RNA sequencing (RNA-seq) analysis. Triplicates for each sample were sent for RNA-seq. (B) qRT-PCR validation of representative differentially expressed host lncRNAs found by RNA-seq analysis. Red bars represent the lncRNAs up-regulated with HPV-16 E6 expression, and green bars represent the lncRNAs down-regulated with HPV-16 E6 expression. GAPDH mRNA was used to normalize the qRT-PCR analyses, which are shown relative to uninfected HEKa (grey bars).

Figure 2: Increased FAM83H-AS1 expression in primary cervical keratinocytes containing the HPV-16 genome as well as in HPV-16 positive cervical cancer and head and neck squamous cell carcinoma cell lines. (A) qRT-PCR analysis showing the increase of FAM83H-AS1 expression in newly immortalized cervical keratinocytes expressing entire HPV-16 genome (JAMM-16) compared to uninfected primary cervical keratinocytes (HCK). (B) qRT-PCR analysis showing the increase of FAM83H-AS1 expression in HPV-16 positive cervical cell lines (CaSki, W12/20863, W12/201402) and decrease of FAM83H-AS1 in HPV negative cervical cancer cell line (C-33A) compared to uninfected cervical keratinocytes (HCK). (C) qRT-PCR analysis showing the increase of FAM83H-AS1 expression in HPV-16 positive head and neck squamous cell carcinoma (HNSCC) cell lines (UMSCC-47 and UMSCC-104) compared to HPV negative HNSCC cell line (UMSCC-1). All graphs in the figure show the average of two individual experiments. Similar results were obtained in at least three independent experiments. GAPDH mRNA was used to normalize the qRT-PCR analyses. Two-tailed t test results are indicated as ** $p \leq 0.01$.

Figure 3: Regulation of FAM83H-AS1 expression by HPV-16 E6 in a p53-independent, p300-dependent manner. (A) FAM83H-AS1 expression by qRT-PCR analysis in cervical keratinocytes (HCK) stably individually expressing HPV-16 E6 or E7, or co-expressing E6/E7 compared to GFP control. (B-C) qRT-PCR analysis of HPV-16 E6 and FAM83H-AS1 expression in HPV-16

positive CaSki (B) and W12/201402 (C) cervical cell lines transfected with an siRNA against HPV-16E6 compared to siRNA control. (D) qRT-PCR analysis of p53 and FAM83H-AS1 expression in HCK transfected with an siRNA against p53 compared to siRNA control. (E) Genome representative image showing location of FAM83H, FAM83H-AS1, and three predictive p300 binding sites in FAM83H-AS1 promoter region. (F) p300 and FAM83H-AS1 expression in HCK transfected with an siRNA against p300 compared to siRNA control. All graphs in the figure show the average of two individual experiments. Similar results were obtained in at least three independent experiments. GAPDH mRNA was used to normalize the qRT-PCR analyses. Two-tailed t test results are indicated as * $p \leq 0.05$ and ** $p \leq 0.01$. CTRL, control.

Figure 4: FAM83H-AS1 is localized in the nucleus in cervical pre-malignant and cancerous cell lines. (A) qRT-PCR of FAM83H-AS1 expression in fractionated HPV-16 positive cervical cancer CaSki (A) and pre-malignant W12/201402 (B) cell lines. U6 small nuclear RNA (snRNA) was used as a nuclear control RNA and mature Beta Actin was used as a cytoplasmic control RNA. Representative images; similar results were obtained in at least three independent experiments. Normalization was done using *C. Elegans* total RNA as an exogenous spike for the amplification of worm-specific *ama-1* gene. Two-tailed t test results are indicated as ** $p \leq 0.01$.

Figure 5: FAM83H-AS1 knockdown altered cell proliferation, migration, and apoptosis in CaSki cells. (A) Knockdown efficiency of individual and SMARTpool siRNA against FAM83H-AS1 in HPV-16 positive CaSki cell line, measured by qRT-PCR analysis. Because of variations in the expression of GAPDH after the knockdown of FAM83H-AS1, we used UBC mRNA to normalize the qRT-PCR analyses. The graph shows average of two individual experiments. (B) CaSki cells were transfected with individual siRNAs against FAM83H-AS1, siRNA SMARTpool against FAM83H-AS1, or siRNA control for 48 hours. Cells were then re-plated in equal numbers (200,000 cells/well, represented by dashed line in graph) and cultured another 48 hours prior to re-counting attached cells. Data were obtained in triplicate, and the graph shows the average of two individual experiments. (C) CaSki cells were transfected with individual siRNAs against FAM83H-AS1, siRNA SMARTpool against FAM83H-AS1, or siRNA control for 24 hours then plated in equal numbers. Transfected cells were analyzed for cellular proliferation assessment by CCK-8 assay at 48, 72, and 96 hours post-plating. The graph shows the average of two individual

experiments; similar results were obtained in three independent experiments. (D-F) CaSki cells were transfected with siRNA SMARTpool against FAM83H-AS1 or siRNA control for 24 hours then plated in equal numbers for experiments. (D) Transfected cells were analyzed for cell cycle alterations by FACS analysis. CaSki cells with knockdown of FAM83H-AS1 exhibit less cells in S-phase of cell cycle compared to control cells. The graph shows the average of two individual experiments; similar results were obtained in three independent experiments. (E) Transwell migration of transfected cells was analyzed 48 hours post-plating in upper chamber with chemoattractant in lower chamber. The graph shows the average of three individual experiments. (F) Transfected CaSki cells were collected at 1, 2, and 3 days post-plating, stained with Annexin V/PI, and analyzed by flow cytometry to show alterations in apoptosis compared to siRNA control. The graph shows the average of three individual experiments. Two-tailed t test results are indicated as * $p \leq 0.05$ and ** $p \leq 0.01$.

Figure 6: FAM83H-AS1 expression is increased in human cervical cancer tissues and correlates with poor overall survival. (A) Increased FAM83H-AS1 expression in human cervical cancer and cervical intraepithelial neoplasia (CIN) stage 3 patient samples compared to non-cancerous cervical tissue as measured by qRT-PCR analysis. (B) The Cancer Genome Atlas (TCGA) analysis of FAM83H-AS1 RPKM values in cervical cancer tissues (n=196) compared to non-cancerous tissues (n=3). (C) Survival plot of cervical squamous cell carcinoma and endocervical adenocarcinoma (CESC) patient data mined from TCGA with low (n=22) vs. high (n=174) expression of FAM83H-AS1. High expression of FAM83H-AS1 expression correlates with worse overall survival in CESC patients.

Figure 1:

Figure 2:

Figure 3:

Figure 4:

Figure 5:

Figure 6:

XI. Supplementary Figures

Supplementary Figure S1

Figure S1: Validation of stable HPV-16 E6 expression in primary foreskin keratinocytes. RT-PCR was used to confirm stable HPV-16 E6 expression in primary foreskin keratinocytes (HEKa) compared to HEKa expressing GFP control and HPV-16 positive CaSki cell line.

Supplementary Figure S2

Figure S2: Validation of HPV-16 expression in primary cervical keratinocytes. RT-PCR was used to confirm HPV-16 E6 and E7 expression in cervical keratinocytes transfected with entire HPV-16 genome (JAMM-16) compared to uninfected primary cervical keratinocytes (HCK) and HPV-16 positive CaSki and W12/201402 cell lines. GAPDH was used as loading control. (B) Western blot analysis was used to confirm p53 degradation in HPV-16 positive JAMM-16, CaSki, W12/20863, W12/201402 cell lines compared to HCK and HPV negative, p53-mutated C-33A cell line. Note: N/A represents a cervical cell line that was not used in this study.

Supplementary Figure S3

Figure S3: Validation of stable HPV-16 E6 and E7 expression in primary cervical keratinocytes. RT-PCR was used to confirm stable HPV-16 E6 and E7 expression, as desired, in primary cervical keratinocytes (HCK) compared to HCK expressing GFP control.

Supplementary Figure S4

Figure S4: FAM83H-AS1 expression is regulated by HPV-16 E6 in a p53-independent, p300-dependent manner. (A-B) qRT-PCR analysis of HPV-16 E6 and FAM83H-AS1 expression in HPV-16 positive Caski (A) and W12/201402 (B) cell lines transfected with a second siRNA (siRNA #2) against HPV-16 E6 compared to siRNA control. (C) qRT-PCR analysis of p53 and FAM83H-AS1 expression in primary cervical keratinocytes (HCK) transfected with a different siRNA (siRNA #2) against p53 compared to siRNA control. (D) p300 and FAM83H-AS1 expression in HCK transfected with a different siRNA (siRNA #2) against p300 compared to siRNA control. (E) qRT-PCR analysis of FAM83H-AS1 expression in HPV-18 positive HeLa cells and HPV-31b positive CIN-612 cervical cell lines compared to primary cervical keratinocytes (HCK). (A-D) The graphs show the average of two independent experiments. Similar results were obtained in at least three independent experiments. GAPDH mRNA was used to normalize the qRT-PCR analyses. Two-tailed t test results are indicated as ** $p \leq 0.01$. CTRL, control.

Supplementary Figure S5

Figure S5: FAM83H expression is not regulated by FAM83H-AS1. (A) Schematic diagram depicting possible cis-regulation of protein coding gene FAM83H by lncRNA FAM83H-AS1. (B) qRT-PCR analysis of FAM83H protein coding gene expression in primary cervical keratinocytes (HCK) stably expressing HPV-16 E6 compared to GFP control. (C) qRT-PCR analysis of FAM83H and FAM83H-AS1 expression in CaSki transfected with siRNA against FAM83H-AS1. The graphs shows the average of two independent experiments. Similar results were obtained in at least three independent experiments. GAPDH mRNA was used to normalize the qRT-PCR analyses. Two-tailed t test results are indicated as ** $p \leq 0.01$. CTRL, control.

Supplementary Figure S6

Figure S6: FAM83H-AS1 expression at 24, 48, 72, 96, and 120 hours post-transfection with FAM83H-AS1 siRNA SMARTpool by qRT-PCR analysis.

Supplementary Figure S7

F**W12/201402**

G

201402

H

CaSki

Figure S7: FAM83H-AS1 knockdown altered cell proliferation, migration, and apoptosis in W12/201402 cells. (A) Knockdown efficiency of individual and SMARTpool siRNA against FAM83H-AS1 in HPV-16 positive W12/201402, measured by qRT-PCR analysis. Because of variations in the expression of GAPDH after the knockdown of FAM83H-AS1, we used GUSB mRNA to normalize the qRT-PCR analyses. The graph shows average of two individual experiments. (B) W12/201402 cells were transfected with individual siRNAs against FAM83H-AS1, siRNA SMARTpool against FAM83H-AS1, or siRNA control for 48 hours. Cells were then re-plated in equal numbers (200,000 cells/well, represented by dashed line in graph) and cultured another 48 hours prior to re-counting attached cells. The graph shows the average of two individual experiments. (C) W12/201402 cells were transfected with individual siRNAs against FAM83H-AS1, siRNA SMARTpool against FAM83H-AS1, or siRNA control for 48 hours then plated in equal numbers. Transfected cells were analyzed for cellular proliferation assessment by CCK-8 assay at 48, 72, and 96 hours post-plating. The graph shows the average of two individual experiments. (D-G) W12/201402 cells were transfected with siRNA SMARTpool against FAM83H-AS1 or siRNA control for 24 hours then plated in equal numbers for experiments. (D) Transfected cells were analyzed for cell cycle alterations by FACS analysis. W12/201402 cells with knockdown of FAM83H-AS1 exhibit less cells in S-phase of cell cycle compared to control cells. The graph shows the average of two individual experiments. (E) Transwell migration of transfected cells was analyzed 48 hours post-plating in upper chamber with chemoattractant in lower chamber. The graph shows the average of two individual experiments. (F-H) Transfected W12/201402 or CaSki cells were collected at 1, 2, and 3 days post-plating, stained with Annexin V/PI, and analyzed by flow cytometry to show alterations in apoptosis compared to siRNA control. (F) Quantitative analysis of Annexin V/PI flow cytometry data in W12/201402 cells. The graph shows the average of two individual experiments. (G) Dot plot analysis of Annexin V/PI flow cytometry data in W12/201402 cells. (H) Dot plot analysis of Annexin V/PI flow cytometry data in CaSki cells. Representative images shown for siRNA FAM83H-AS1 and siRNA control at each time point. Two-tailed t test results are indicated as * $p \leq 0.05$ and ** $p \leq 0.01$.

XII. Supplementary Tables

Table S1: Host lncRNAs altered after expression of HPV-16 E6 in primary foreskin keratinocytes.

Table shows details for each host lncRNA that was altered 1.5-fold or greater in primary foreskin keratinocytes (HEKa) expressing HPV-16 E6 compared to uninfected HEKa. Triplicates for each sample were sent for high-throughput RNA sequencing analysis.

Gene ID	Fold Change ([HPV 16E6] vs [Control])	Gene Symbol	Chromosome	Start	End	Strand	RPKM GFP Control -1	RPKM GFP Control -2	RPKM GFP Control -3	RPKM HPV-16 E6-1	RPKM HPV-16 E6-2	RPKM HPV-16 E6-3
ENSG00000130600	4.99	H19	chr11	2016406	2022700	-	2.1290493	2.1426256	5.6363993	19.229809	10.783182	15.414708
ENSG00000254236	3.54	KB-1639H6.2	chr8	104032415	104033656	-	0	0	0.2873624	3.250996	3.96886	3.4498966
ENSG00000244528	3.31	AC134873.1	chr2	243064438	243064620	+	1.2767934	2.8750565	2.7582934	6.186408	6.602485	9.019768
ENSG00000273413	3.07	RP11-96C23.15	chr10	88729994	88730583	-	8.393546	0	0	4.7901816	7.0136943	7.2466874
ENSG00000228626	3.06	RP11-495P10.9	chr1	147760107	147761057	-	2.0288084	0.88392633	2.2444494	6.9817734	5.0385094	3.6965914
ENSG00000230937	2.94	MIR205HG	chr1	209602165	209606183	+	61.548264	49.323284	62.656292	218.4987	181.15729	121.917755
ENSG00000254860	2.73	TMEM9B-AS1	chr11	8986222	8999074	+	0.8062374	1.7021289	0.6363604	4.052271	3.1377754	2.730444
ENSG00000249641	2.69	HOXC-AS5	chr12	54329112	54333427	-	0.9593882	1.8041867	0.9305784	3.716362	3.7588704	2.5167224
ENSG00000270168	2.56	LA16c-380H5.4	chr16	3051301	3052017	+	6.4768987	0.8551222	4.0893016	9.19451	7.0534296	6.8577585
ENSG00000235314	2.53	LINC00957	chr7	44078770	44081905	+	0.35596678	0.73328674	0.70044947	1.9380348	2.741936	3.0452104
ENSG00000230409	2.50	TCEA1P2	chr3	37317087	37318089	+	7.469508	9.592129	10.218497	22.008438	24.913902	20.846043
ENSG00000233223	2.45	AC113189.5	chr17	7485282	7487390	-	1.4596063	2.9885232	2.0333843	3.720071	5.5129123	6.3233705
ENSG00000254192	2.36	CTC-558O2.2	chr5	168081518	168094766	+	1.2354965	0.90355885	1.6458715	3.684764	2.9198492	2.4772859
ENSG00000251580	2.35	RP11-539L10.3	chr4	6672452	6675557	-	0.83309	1.3336157	1.0557044	2.159164	2.0041137	4.219327

ENSG00000220804	2.29	AC093642.5	chr2	243030784	243102304	+	1.02285 7	1.6583 912	2.8178 344	4.230 8583	2.589 8807	5.207 1075
ENSG00000201428	2.28	RN7SKP71	chr12	112704881	112705198	+	0.05682 099	0.2640 6604	0.3352 5583	0.033 64112 6	0.464 84584	11.79 032
ENSG00000238107	2.27	RP11- 495P10.5	chr1	147767287	147769663	+	1.19644 03	0.5992 599	0.7781 85	3.452 5478	1.896 4818	2.127 1327
ENSG00000228463	2.25	AP006222.2	chr1	227615	267253	-	0.92179 984	1.8147 042	2.4698 875	3.840 908	3.520 3686	3.766 3238
ENSG00000263934	2.22	SNORD3A	chr17	19091329	19092027	+	56.5595 66	96.541 93	87.400 215	115.8 12065	101.6 3637	442.5 1657
ENSG00000267984	2.21	CTD- 2616J11.9	chr19	51848423	51856547	+	0.03900 8502	0	0	0.093 65486	0	10.77 701
ENSG00000200488	2.18	RN7SKP203	chr2	76672205	76672536	-	0.39458 07	2.4766 135	0.8919 96	1.181 4926	0.604 2596	21.76 185
ENSG00000255717	2.13	SNHG1	chr11	62619460	62623386	-	7.09001 87	3.8827 178	5.6702 17	13.07 7036	11.90 0302	9.669 952
ENSG00000259001	2.12	RPPH1	chr14	20811207	20811844	-	15.0019 23	52.592 74	31.222 376	27.47 0306	36.77 239	231.4 2776
ENSG00000234741	2.10	GAS5	chr1	173833038	173838020	-	75.5498 6	34.682 1	63.601 818	141.4 7461	116.2 6347	93.72 997
ENSG00000215039	2.08	CD27-AS1	chr12	6548167	6560733	-	0.66829 19	0.8538 55	0.8496 1987	3.074 375	1.768 162	1.665 7759
ENSG00000259970	2.06	AC099668.5	chr3	49721913	49722416	-	0.57804 507	1.3075 932	0.8779 387	2.843 681	2.354 5742	1.696 4713
ENSG00000254578	2.04	CTD- 2517M22.16	chr8	145689200	145690484	+	2.56431 48	1.6997 473	2.1004 875	4.821 358	4.543 132	3.554 0457
ENSG00000202198	2.04	RN7SK	chr6	52860418	52860748	+	77.7898 2	202.92 421	175.02 835	174.8 5051	183.0 6963	733.0 992
ENSG00000255831	2.04	AL139385.1	chr13	111291555	111292340	+	0	2.5623 991	0	0	5.166 7576	4.200 331
ENSG00000271992	2.02	RP11-42O15.3	chr1	70910754	70911219	+	0.34897 357	0.0900 998	0.8007 2904	2.295 6822	1.722 0101	2.083 4737
ENSG00000227195	2.01	MIR663A	chr20	26167556	26232162	-	0.89503 71	1.0255 735	1.4251 992	1.545 9784	1.247 556	6.125 4115
ENSG00000239002	1.98	SCARNA10	chr12	6619388	6619717	+	0.54754 776	1.9084 775	1.3999 47	1.167 0413	2.879 6296	6.134 6283
ENSG00000263917	1.97	RP11-53I6.2	chr18	29598792	29691742	+	1.69259 02	0.7714 8676	1.5012 345	1.865 241	3.775 4347	2.767 804
ENSG00000266929	1.96	RP11- 400F19.8	chr17	40688528	40714080	+	2.64025 28	2.2248 166	2.0022 075	4.118 1316	4.623 1112	4.685 91
ENSG00000223891	1.96	OSER1-AS1	chr20	42839600	42854667	+	0.42706 716	1.3540 938	1.7394 654	2.074 9772	3.062 2957	2.806 612

ENSG00000244879	1.96	GABPB1-AS1	chr15	50647525	50659636	+	1.49664 08	1.0193 752	1.1708 221	2.835 0017	2.036 269	2.337 8665
ENSG00000225976	1.96	RP11- 192N10.2	chr10	29986832	29987157	+	4.10156 9	1.4167 225	3.0522 678	4.856 7057	5.441 2627	5.069 306
ENSG00000259827	1.95	RP11- 343H19.2	chr16	56716382	56721719	+	3.48532 18	2.4112 535	2.5835 383	3.920 711	3.862 1109	10.60 9867
ENSG00000235499	1.95	AC073046.25	chr2	74212259	74213470	+	4.49410 3	3.1759 024	2.5058 224	6.528 4495	5.577 129	7.256 5813
ENSG00000258377	1.94	RP11-649E7.5	chr14	50087533	50090198	-	0	3.7360 387	4.1599 88	5.070 155	4.762 3515	4.735 923
ENSG00000233825	1.94	RP11- 135A24.4	chr10	32635427	32636107	+	2.00523 7	0.9287 9003	1.8029 468	2.122 1492	3.477 26	3.551 1832
ENSG00000269926	1.93	RP11- 442H21.2	chr10	74034673	74035738	-	37.1678 16	17.135 89	27.302 435	56.37 6045	53.58 8455	41.68 311
ENSG00000261824	1.91	LINC00662	chr19	28175488	28284848	-	1.77218 08	0.9460 5726	1.4761 61	3.744 9877	2.442 1446	1.996 9538
ENSG00000267317	1.90	CTB-25B13.12	chr19	1457664	1462764	-	0.77052 76	2.7577 708	2.0533 23	3.240 5212	3.709 7907	3.223 024
ENSG00000179859	1.89	AC025335.1	chr17	7816642	7819271	-	2.48756 43	3.6358 418	4.2272 625	7.583 128	6.336 298	5.399 1914
ENSG00000257653	1.87	RP11-579D7.2	chr12	49159977	49161106	+	0.70771 77	1.0421 093	1.3791 414	2.705 9877	1.943 5432	1.797 2413
ENSG00000238271	1.87	IFNWP19	chr9	21455483	21456048	+	6.09118 27	4.1468 25	6.1276 43	10.78 6538	8.897 233	10.57 8037
ENSG00000224032	1.87	EPB41L4A-AS1	chr5	111496223	111499973	+	7.39583 83	3.9629 142	7.1221 61	9.977 508	12.22 6742	11.20 5975
ENSG00000257270	1.87	RP11- 521B24.5	chr14	105934130	105936954	-	6.40190 6	3.2108 22	3.5815 47	8.843 35	7.365 5524	7.390 7046
ENSG00000257084	1.86	U47924.27	chr12	7072409	7073610	+	31.198	36.405 44	20.322 99	53.07 6824	61.99 1566	44.91 036
ENSG00000225377	1.85	RP5-1103G7.4	chr20	300957	328868	-	2.56064 6	3.4069 042	5.1782 656	6.993 363	5.695 6306	7.200 583
ENSG00000239470	1.84	RP11-16F15.2	chr11	9681946	9682503	+	6.97289 23	3.0191 91	4.1343 21	10.61 4801	8.628 567	5.930 6784
ENSG00000198496	1.84	NBR2	chr17	41277627	41305688	+	0.83190 143	1.0633 509	1.4072 425	2.247 7791	2.288 2934	1.808 5096
ENSG00000203499	1.84	FAM83H-AS1	chr8	144816310	144828507	+	9.59697 8	3.3299 105	4.7020 435	12.95 5384	9.471 8895	7.570 58
ENSG00000177410	1.83	ZFAS1	chr20	47894715	47905797	+	211.102 16	139.73 38	252.70 905	418.7 3697	367.0 7626	297.5 4745
ENSG00000224891	1.83	AC007899.3	chr2	37375673	37376447	+	4.01025 72	2.9299 183	3.7727 127	6.872 386	6.110 306	6.431 074
ENSG00000214293	1.82	RSBN1L-AS1	chr7	77286977	77325582	-	1.88850	1.6980	1.8871	3.833	3.262	2.897

							9	832	909	9822	9604	0351
ENSG00000250902	1.81	RP11-301H24.4	chr4	146435767	146438270	-	6.9897666	3.0939543	5.392143	10.337118	8.141412	8.172936
ENSG00000259375	1.81	RP11-815J21.2	chr15	86122277	86123409	-	1.8337024	1.1494807	1.1902504	1.5972154	2.8849528	3.210236
ENSG00000012171	1.80	SEMA3B	chr3	50304990	50314977	+	0.9493543	0.34291044	1.0639977	2.2476633	1.7905937	1.5402615
ENSG00000273344	1.79	PAXIP1-AS1	chr7	154795158	154797413	+	1.0732518	2.633462	2.4573538	3.0064073	3.0608828	4.358569
ENSG00000260552	1.79	RP11-49I11.1	chr18	33759959	33767411	-	1.3054833	2.1372445	4.726611	3.9699843	6.0681834	3.1545815
ENSG00000243004	1.79	AC005062.2	chr7	19958604	20180076	-	4.8755813	7.298544	3.9218314	6.9311867	14.130893	8.209532
ENSG00000196756	1.78	SNHG17	chr20	37049235	37063996	-	2.5424457	1.8823376	4.6329513	4.3709865	5.7459593	5.0056214
ENSG00000272906	1.78	RP11-533E19.7	chr1	179850742	179851730	-	0.2989431	1.278876	1.0368878	1.5778978	1.703776	2.7940536
ENSG00000261771	1.78	DYX1C1-CCPG1	chr15	55647446	55790558	-	2.0968938	2.6955016	2.898272	5.290535	4.608052	3.8043263
ENSG00000204054	1.78	LINC00963	chr9	132245730	132275947	+	1.0122313	0.81810194	0.75098056	2.6907895	1.8905445	1.1253008
ENSG00000269893	1.78	SNHG8	chr4	119199864	119200978	+	95.683075	85.64051	110.784874	193.31099	208.99217	126.607704
ENSG00000269900	1.78	RMRP	chr9	35657748	35658015	-	61.719086	117.54486	105.27629	99.84465	121.56224	352.01825
ENSG00000265735	1.76	RN7SLSP	chr9	9442060	9442347	+	89.34267	171.01642	175.36769	146.4969	160.55365	623.5067
ENSG00000229413	1.75	AC018638.1	chr7	128293740	128293989	-	2.0811276	0.85338426	0.6139542	2.203358	1.4593241	3.445703
ENSG00000250299	1.74	MRPS31P4	chr13	53191693	53211581	+	0.9976758	0.6966341	0.8349367	2.1997466	1.8856709	1.2795835
ENSG00000226419	1.74	RP11-31F15.1	chr1	113499037	113542118	+	3.0520382	2.3166275	2.4479396	5.745393	4.4174857	3.588944
ENSG00000245060	1.74	LINC00847	chr5	180257957	180262726	+	2.517983	1.9512683	1.9726743	3.6677196	3.6637437	3.780457
ENSG00000110811	1.73	LEPREL2	chr12	6937572	6949018	+	1.4318174	1.6433642	1.4975213	3.065626	2.1591876	2.762544
ENSG00000262160	1.73	RP11-96D1.11	chr16	68259872	68263048	-	1.0164231	1.7172104	1.3368715	2.8964238	2.1146343	1.9768672
ENSG00000250251	1.73	PKD1P6	chr16	15219099	15248421	-	4.1067576	2.8266087	4.433204	4.759571	5.149509	10.823436
ENSG00000203512	1.72	AL353662.2	chr9	34195642	34195883	+	1.7316197	0.80911475	1.1475528	3.1647897	1.5251368	2.1087782

ENSG00000227615	1.72	RP11-864N7.2	chr11	74456761	74457159	-	6.18152 57	1.2890 593	4.9579 773	6.729 743	6.448 0925	4.633 6823
ENSG00000257086	1.72	RP11- 783K16.13	chr11	64014411	64016966	-	1.58257 52	1.1539 651	1.8851 668	3.347 7898	2.183 432	2.384 6471
ENSG00000230795	1.72	HLA-K	chr6	29894236	29897009	+	0.61804 247	1.1913 046	1.5108 755	1.330 7873	2.285 3572	2.992 5754
ENSG00000180672	1.71	AC007362.1	chr2	206642540	206644433	+	1.37003 06	0.6789 9704	1.1776 617	3.312 5985	1.914 6636	1.281 505
ENSG00000254682	1.71	RP11- 660L16.2	chr11	71159720	71163203	+	1.14260 32	0.7100 659	0.4964 7444	1.667 6105	1.925 3993	1.792 3354
ENSG00000267023	1.71	LRRC37A16P	chr17	66121918	66148609	-	1.45405 14	1.6921 17	1.4601 423	2.523 746	2.944 7954	2.423 118
ENSG00000227081	1.71	RP11- 543P15.1	chr12	3320775	3321096	-	7.24820 5	1.5755 806	5.4170 194	7.375 556	8.514 681	4.918 4346
ENSG00000223745	1.71	RP4-717I23.3	chr1	93727743	93811582	-	0.95840 377	1.4749 708	1.4199 601	2.833 7555	1.944 4739	1.893 2604
ENSG00000226752	1.70	PSMD5-AS1	chr9	123587106	123616651	+	0.90399 02	0.6311 066	1.0164 516	2.319 7796	1.464 0397	1.481 2256
ENSG00000227632	1.70	AC018804.6	chr2	130959885	130970201	+	1.50575 63	2.2844 598	2.4223 318	2.553 2963	4.621 544	3.451 2596
ENSG00000228327	1.69	RP11- 206L10.2	chr1	700237	714006	-	3.12584 38	1.0653 372	3.5199 823	3.532 1116	3.393 6765	4.746 0814
ENSG00000228638	1.69	FCF1P2	chr3	48332283	48332865	-	0.71284 515	0.7201 8015	0.2133 4462	2.789 1552	1.738 6442	0.814 9583
ENSG00000258186	1.69	SLC7A5P2	chr16	21531151	21531686	-	8.26003	6.4758 79	14.699 861	13.65 3822	13.00 4153	21.40 1606
ENSG00000225648	1.68	SBDSP1	chr7	72300004	72307909	+	10.7959 62	11.587 327	13.133 697	19.68 8213	19.81 655	19.96 0356
ENSG00000272711	1.68	RP11- 259N19.1	chr2	75059782	75061114	-	1.45040 58	1.1969 146	1.6662 19	3.884 3012	2.328 7628	1.503 2032
ENSG00000256462	1.67	RP11-116G8.5	chr10	5566939	5567705	-	3.94108 49	0	0	3.097 3866	5.978 923	0
ENSG00000236439	1.67	RP11-175B9.3	chr1	202440937	202441258	-	6.70856 76	3.0561 595	4.4550 18	7.306 8976	8.068 728	7.226 4347
ENSG00000233016	1.67	SNHG7	chr9	139615818	139622636	-	3.80191 66	2.5988 54	3.9963 634	4.894 757	6.888 8636	5.446 209
ENSG00000234975	1.67	FTH1P2	chr1	228823162	228823574	+	2.36462 16	8.7332 3	5.5768 6	7.877 958	8.395 835	8.092 968
ENSG00000228998	1.66	RP11-697E2.7	chr15	90818266	90820841	+	1.66241 1	1.0105 448	1.4692 17	2.458 5187	2.098 612	2.181 2184
ENSG00000251022	1.66	THAP9-AS1	chr4	83814162	83822113	-	2.55584 17	2.0884 433	4.3881 38	5.688 368	4.508 133	4.144 217
ENSG00000256633	1.64	RP11-169D4.2	chr11	72295616	72299023	+	0.36736	0.2219	0.2809	0.585	1.582	2.773

							822	0487	7868	01613	57	093
ENSG00000266754	1.64	RN7SL524P	chr10	105734277	105734572	-	0.67657 28	0.7092 3144	1.0905 253	2.662 4227	0.618 2988	1.791 2405
ENSG00000260269	1.63	CTD- 2323K18.1	chr15	75819491	75893546	-	1.65046 27	0.9741 242	0.6995 654	3.534 106	1.676 849	1.202 5416
ENSG00000260088	1.62	RP11-92G12.3	chr1	200638635	200663378	+	1.84138 01	1.6593 088	3.4227 7	4.249 6977	2.985 226	3.536 2139
ENSG00000267519	1.62	MIR24-2	chr19	13945330	13947173	-	4.72304 44	4.5993 896	8.6144 75	9.827 661	7.844 5435	10.23 9091
ENSG00000254681	1.61	PKD1P5	chr16	18458147	18495797	-	1.31439 34	1.0079 812	1.4375 087	1.722 2397	2.123 2183	2.183 325
ENSG00000197989	1.61	SNHG12	chr1	28905050	28909495	-	3.87161 02	2.7489 998	4.3954 32	5.069 179	5.840 756	6.601 1386
ENSG00000247315	1.61	AL034548.1	chr20	278214	280961	+	3.02354 24	2.0685 76	2.8194 673	4.320 936	4.353 7407	3.915 0994
ENSG00000251260	1.61	WDFY3-AS1	chr4	85724411	85731544	+	1.00389 2	1.4533 712	2.2153 144	3.214 5927	1.323 8585	3.147 409
ENSG00000250182	1.60	EEF1A1P13	chr5	14652047	14653438	-	9.46529	6.2075 24	6.9742 58	12.66 7569	11.56 31275	11.53 3177
ENSG00000251143	1.60	RP11-849H4.4	chr11	71725337	71731956	+	3.97319 3	6.8831 76	5.2716 713	9.131 103	7.891 925	8.129 692
ENSG00000272888	1.59	AC013394.2	chr15	93425937	93441975	+	0.84985 04	1.4388 658	1.8384 619	2.750 0532	2.498 7977	1.538 8129
ENSG00000173295	1.58	FAM86B3P	chr8	8086117	8102387	+	0.99347 425	0.8375 4015	1.0657 19	1.652 3628	1.481 0578	1.727 7136
ENSG00000271581	1.58	XXbac- BPG248L24.12	chr6	31324424	31325414	+	10.9642 39	42.648 693	30.244 503	26.87 288	45.12 537	46.04 9137
ENSG00000234694	1.58	RP1-92O14.3	chr1	43820355	43824329	-	1.44880 64	2.5090 265	1.8279 302	3.333 9834	4.057 3297	1.937 6101
ENSG00000231970	1.58	RP11- 452K12.7	chr10	99160872	99179281	+	4.40716 4	1.8063 688	1.6439 916	2.576 6385	4.841 898	4.122 6187
ENSG00000258824	1.58	CTD- 2555O16.2	chr14	64889653	64915275	-	0.54871 875	0.3577 88	0.8857 678	2.461 6747	1.143 732	1.395 1521
ENSG00000224886	1.58	BEND3P3	chr10	81444250	81444739	-	0.14750 265	1.2424 577	0.5076 731	1.309 9444	0.603 35094	3.709 9042
ENSG00000259623	1.57	RP11-156E6.1	chr17	40004770	40007699	-	1.19021 55	0.7809 776	1.3159 648	2.417 0635	1.441 4529	1.755 5282
ENSG00000214184	1.57	AC012487.2	chr2	109123971	109150652	-	1.13318 16	1.5004 897	1.6879 808	2.188 2014	1.909 4998	2.645 182
ENSG00000231864	1.57	RP11- 229P13.23	chr9	139957987	139959033	+	2.14776 64	0.5192 181	1.6303 065	3.468 3156	2.224 2556	1.746 7672
ENSG00000259948	1.56	RP11- 326A19.5	chr15	89744322	89744999	-	0	0	0	0	2.250 7296	1.690 6314

ENSG00000203875	1.56	SNHG5	chr6	86370710	86388451	-	102.081 635	49.028 21	72.932 594	136.5 7454	115.0 8786	87.77 891
ENSG00000262944	1.56	RP11-473I1.9	chr16	9198705	9200618	+	4.37061 83	3.5058 978	5.2434 583	9.294 138	6.550 2	4.981 1077
ENSG00000223361	1.56	FTH1P10	chr5	17353804	17354733	-	1.12084 73	1.9096 965	3.1168 904	3.494 8602	3.568 4345	2.014 9157
ENSG00000234797	1.55	RPS3AP6	chr15	60060543	60061347	+	8.03755 8	4.6485 057	6.0571 284	10.87 507	9.480 915	8.230 892
ENSG00000186831	1.55	KRT17P2	chr17	18330175	18335162	+	2.67693 57	5.9414 525	8.2313 9	8.073 757	7.960 8884	7.615 3316
ENSG00000235298	1.55	RP11-575L7.8	chr9	86587148	86590692	+	0.99603 415	0.1577 4745	1.2684 056	1.084 6498	1.106 6335	3.923 4712
ENSG00000244398	1.55	RP11-466H18.1	chr11	16996240	16996560	-	210.415 99	118.90 0734	159.80 867	323.5 744	266.5 1407	171.5 4987
ENSG00000232956	1.54	SNHG15	chr7	45022622	45026560	-	4.96357 63	4.2213 054	6.0738 254	7.899 4136	7.568 8367	7.776 898
ENSG00000265688	1.54	MAFG-AS1	chr17	79885705	79888629	+	2.63169 65	1.0524 323	1.6549 61	2.523 4575	3.253 9558	2.027 5886
ENSG00000205746	1.54	RP11-1212A22.1	chr16	18428257	18488396	-	2.70093 97	1.9503 261	2.9362 893	3.497 657	3.365 603	4.758 7376
ENSG00000205763	1.53	RP9P	chr7	32956427	32982788	-	1.58876 7	2.6736	4.2468 457	3.486 325	3.794 6262	4.901 2694
ENSG00000260107	1.53	AC005606.15	chr16	2047655	2048375	+	9.51927 2	5.6106 744	7.2765 503	8.348 566	12.49 4355	13.30 5827
ENSG00000232445	1.53	RP11-132A1.4	chr7	100951627	100954266	+	6.63814 2	1.0719 961	3.6041 183	4.977 168	4.163 5494	4.395 196
ENSG00000204194	1.52	RPL12P1	chr6	33367836	33368333	-	8.81644 3	4.4001 51	10.382 515	8.702 698	12.67 3401	12.93 3394
ENSG00000254473	1.52	RP11-522I20.3	chr9	86322509	86328293	+	0.62335 21	0.6170 2996	0.9024 317	2.064 1985	1.439 8504	1.178 33
ENSG00000261971	1.52	RP11-473M20.7	chr16	3101992	3109371	-	0.38817 36	0.9558 703	1.3753 506	1.559 6504	1.193 0581	2.581 4805
ENSG00000235725	1.51	AC007389.3	chr2	65816700	65867311	-	0.91346 866	0.7893 489	1.0001 628	1.792 3195	1.434 173	1.351 0778
ENSG00000229212	1.51	RP11-561C5.4	chr15	85747141	85778026	-	1.54617 49	2.1187 642	2.2953 222	2.896 4856	2.832 387	3.181 3629
ENSG00000259328	1.51	RP11-152F13.7	chr15	82944773	82974312	+	1.00161 61	1.3405 782	1.1754 336	1.238 5976	1.825 4333	2.403 051
ENSG00000223396	1.51	RPS10P7	chr1	201487831	201499602	+	3.12924 72	1.9355 738	2.9419 267	4.337 2602	4.088 839	3.454 8473
ENSG00000269032	1.51	AC016629.7	chr19	59097525	59097723	+	0.32712 79	0.8532 135	0.6416 285	0	2.120 2471	1.611 3639
ENSG00000203394	1.51	RP5-930J4.4	chr1	21069480	21070455	+	4.33596	2.2852	2.7163	5.946	4.018	3.844

							37	726	084	6834	1866	3263
ENSG00000225151	1.50	AC103965.1	chr15	84867600	84898888	-	7.19354 3	9.0674 305	8.6491 41	13.35 8168	12.31 3677	11.67 2823
ENSG00000244257	1.50	PKD1P1	chr16	16404198	16428047	+	2.76064 6	1.8415 892	2.8608 568	3.244 7884	3.438 1177	4.434 0997
ENSG00000267580	1.50	CTD- 2540B15.11	chr19	33790840	33792074	+	1.09239 59	2.0792 82	1.7315 762	2.528 3904	2.831 5682	1.866 6612
ENSG00000267151	1.50	MIR2117	chr17	41522075	41528568	+	1.46409 5	1.9914 546	1.7382 286	2.071 921	3.630 837	2.286 1967
ENSG00000270055	1.50	CTD- 3092A11.2	chr15	30780166	30782516	+	1.17078 79	0.9375 974	1.4183 607	2.055 2423	1.595 0989	1.714 8618
ENSG00000228989	1.50	AC133528.2	chr2	242629829	242633704	+	1.82867 69	1.5681 707	0.6850 529	3.815 1476	2.544 251	0.348 43844
ENSG00000262791	1.50	RP11- 961A15.1	chr17	1629042	1641879	-	0.61560 89	0.1723 9359	0.3134 515	1.525 6633	0.680 255	2.214 0858
ENSG00000248092	1.50	NNT-AS1	chr5	43571696	43603332	-	2.29409 2	2.9645 55	2.8210 022	4.673 5754	3.726 381	3.718 8199
ENSG00000270232	-1.51	RP11-475J5.7	chr5	99381632	99382078	-	14.4310 055	8.3597 29	7.1948 752	7.179 785	5.739 9325	6.123 285
ENSG00000255125	-1.51	RP11- 685M7.5	chr11	10804860	10823172	+	2.00767 5	1.9517 535	4.4939 42	1.263 7898	0.861 9299	4.028 4314
ENSG00000265415	-1.52	CTD-2510F5.4	chr17	57280038	57281190	-	2.19017 36	1.3541 911	1.1740 087	0.709 3079	0.546 59224	0.912 0858
ENSG00000232358	-1.52	RP5- 955M13.4	chr20	49615907	49626556	+	10.9595 83	13.578 551	15.932 661	5.993 6986	10.71 9287	10.58 0886
ENSG00000231298	-1.52	LINC00704	chr10	4692377	4720346	-	3.50088 14	0.8419 1686	0.5089 952	0.122 57986 5	0	0.204 42243
ENSG00000269834	-1.52	CTD- 3018O17.3	chr19	52892095	52901019	-	0.42566 574	3.0581 512	1.1532 478	0.938 63595	0.609 15244	0.861 5535
ENSG00000228649	-1.53	AC005682.5	chr7	22893797	22901021	+	2.84680 3	1.1739 081	1.4650 004	1.375 8489	0.801 59485	0.919 04426
ENSG00000250234	-1.53	CTD- 2024P10.1	chr5	34656517	34657355	-	2.84991 8	3.6838 043	2.7612 894	1.368 9165	1.759 3302	3.366 3306
ENSG00000260549	-1.53	MT1L	chr16	56651388	56652730	+	32.0605 55	7.0488 276	13.661 485	8.433 363	8.580 506	11.88 6822
ENSG00000269983	-1.53	RP11- 497H16.9	chr5	69745463	69746180	+	2.89407 18	2.6753 237	1.4972 115	1.057 8682	2.014 671	1.510 466
ENSG00000232656	-1.54	IDI2-AS1	chr10	1068606	1090138	+	8.97962	2.5590 303	4.5844 545	4.037 7965	2.612 7467	2.731 6613
ENSG00000273132	-1.54	RP11- 350J20.12	chr6	150173598	150174328	+	4.93389 4	3.8961 434	1.3101 063	0.214 81651	2.748 0006	2.503 8617
ENSG00000262413	-1.54	RP11-498C9.3	chr17	79825597	79826428	+	131.380	83.971	65.720	57.11	68.61	50.39

							23	855	39	3407	178	401
ENSG00000233144	-1.55	RP11-537A6.9	chr10	75141191	75143254	+	1.73480 49	1.7642 145	2.0247 85	0.579 00864	1.376 7893	1.206 9995
ENSG00000226963	-1.55	AC078883.4	chr2	173292502	173293331	-	3.65149	3.3694 608	3.6925 917	2.460 7828	1.833 3695	2.698 5145
ENSG00000234327	-1.55	AC012146.7	chr17	5014763	5017674	+	1.95390 83	2.4199 314	1.5286 9	0.984 7564	1.408 148	1.369 486
ENSG00000259153	-1.56	RP6-65G23.3	chr14	71276922	71282120	+	1.99365 48	2.7267 768	1.2656 376	1.045 882	0.530 8814	1.731 0688
ENSG00000258791	-1.58	LINC00520	chr14	56247864	56263406	-	0.64874 68	2.9121 616	2.3695 24	0.322 98782	1.232 0567	1.418 7404
ENSG00000267776	-1.58	AC006116.24	chr19	56888073	56888653	-	2.15047 86	3.7224 712	2.8212 99	1.832 4789	1.608 6241	1.933 2796
ENSG00000257181	-1.58	RP11-611O2.5	chr12	69235068	69237017	-	5.82705 8	7.4925 43	6.0171 41	4.169 096	3.725 6134	4.259 7303
ENSG00000267774	-1.58	RP11-2N1.2	chr18	57363691	57364644	+	4.9585	2.7897 93	5.2442 584	1.467 508	3.439 1992	3.611 8386
ENSG00000241749	-1.59	RPSAP52	chr12	66151800	66220754	-	3.20631 31	1.0826 937	1.4755 359	0.093 51292	0.572 23415	1.282 0605
ENSG00000268854	-1.59	CTD- 2545M3.2	chr19	50983376	50986608	-	4.72710 04	2.3693 736	1.3470 087	3.467 783	1.066 7965	1.013 8669
ENSG00000224818	-1.59	RP11-134G8.8	chr1	201433511	201434274	-	3.14244 77	7.4775 295	3.8924 434	2.826 5297	2.930 994	2.728 9903
ENSG00000232815	-1.59	LINC00537	chr9	68413482	68414196	-	3.58854 34	3.3276 02	2.7501 92	2.034 8413	2.185 565	1.820 1643
ENSG00000241494	-1.61	RP11-796G6.1	chr14	102144280	102144717	+	3.58287 45	1.8213 323	2.3123 465	1.848 1115	1.181 2179	1.666 4281
ENSG00000258017	-1.62	RP11- 386G11.10	chr12	49521565	49541652	+	27.8705 02	18.402 964	17.180 283	12.05 1238	12.65 8115	13.56 6694
ENSG0000034063	-1.62	UHRF1	chr19	4903092	4962165	+	6.74429 46	1.2855 517	2.7702 923	2.075 5224	1.767 3001	1.536 0543
ENSG00000254258	-1.62	RP11-398H6.1	chr8	140472305	140475259	-	4.54776 14	7.7839 007	6.3626 404	3.925 3066	3.584 0328	3.740 049
ENSG00000213412	-1.64	HNRNPA1P33	chr10	47133338	47133898	-	14.4520 4	8.3262 005	6.1332 52	8.466 77	4.197 107	4.750 102
ENSG00000126005	-1.64	MMP24-AS1	chr20	33804265	33865934	-	9.15239 3	7.7007 318	6.1065 493	5.459 143	5.335 1436	3.377 0957
ENSG00000172965	-1.65	MIR4435-1HG	chr2	111953927	112252677	-	3.55057 67	3.3823 805	3.6901 011	1.780 8807	2.279 2993	2.452 1575
ENSG00000253161	-1.65	RP11- 150O12.1	chr8	37278859	37411701	-	11.0290 21	12.441 466	9.9816 79	6.311 8887	8.075 069	6.012 6433
ENSG00000261068	-1.65	RP11-7K24.3	chr6	42059976	42061997	-	1.63533 17	8.6070 25	3.6644 359	1.894 0854	2.506 5024	2.411 0541

ENSG00000187653	-1.66	TMSB4XP8	chr4	91759652	91760263	-	8.07511 6	19.661 818	14.503 13	6.456 596	7.568 5825	10.31 9572
ENSG00000226005	-1.66	RP11- 464C19.3	chr10	3976711	3978005	+	1.07008 05	1.8116 003	2.9764 59	0.289 62108	0.678 89744	1.258 3201
ENSG00000250318	-1.67	CTA-963H5.5	chr22	31049864	31050801	-	1.83993 59	2.2982 81	1.8083 909	0.734 8674	0.672 6246	1.651 6936
ENSG00000240225	-1.67	ZNF542	chr19	56879468	56891197	+	2.40770 34	2.6245 768	2.5352 726	1.434 9543	1.558 2225	1.541 5956
ENSG00000240875	-1.68	LINC00886	chr3	156465135	156534851	-	8.04174 8	2.8910 108	2.4221 4	2.591 8982	2.259 8019	2.029 531
ENSG00000256663	-1.72	RP11- 424C20.2	chr12	20704524	20705946	+	4.26013 66	0.7228 8775	1.1924 789	0.947 2472	0.934 9184	0.820 1998
ENSG00000241990	-1.72	RP6-109B7.3	chr22	46449585	46453090	+	7.39844 27	3.9867 473	4.8413 033	3.091 525	3.469 6312	2.616 5955
ENSG00000245112	-1.74	SMARCA5-AS1	chr4	144434625	144435788	-	0.00956 0357	5.2469 225	0	0	0.159 80777	0
ENSG00000259330	-1.74	LINC00984	chr15	40617417	40618916	+	4.89751	3.6402 433	3.2306 47	2.593 4253	2.204 161	1.912 7241
ENSG00000235385	-1.75	GS1-600G8.5	chrX	13284167	13321571	-	4.73486 38	22.813 477	19.080 942	3.339 2224	14.33 58755	8.066 61
ENSG00000261065	-1.75	RP11-74C13.4	chr1	204100190	204101094	+	1.99908 89	2.6730 783	0.4170 3206	0.414 32646	0.868 4934	0
ENSG00000253618	-1.76	GRPEL2-AS1	chr5	148727679	148737205	-	1.68886 54	3.2317 665	0	0.215 5406	0.803 7886	0
ENSG00000258232	-1.77	RP11- 161H23.5	chr12	49658939	49667089	-	33.3097 57	28.819 185	28.912 59	14.44 1423	19.74 7879	17.62 5807
ENSG00000272734	-1.79	ADIRF-AS1	chr10	88725102	88731068	-	5.43516 25	12.950 257	11.457 844	4.700 653	5.038 381	5.980 5155
ENSG00000234546	-1.81	RP3-510D11.2	chr1	9242263	9252148	+	11.2780 56	4.5766 697	5.7239 7	2.883 4605	5.230 489	3.284 4615
ENSG00000258908	-1.82	RP11- 203M5.8	chr14	20942948	20945248	-	7.68502 6	3.8135 834	4.4676 06	2.660 3262	2.985 3904	2.748 8363
ENSG00000272159	-1.83	RP11- 350N15.6	chr8	38265566	38266260	-	7.30503 03	2.4662 013	2.9248 87	1.585 6465	2.061 9986	2.634 9046
ENSG00000226721	-1.84	EEF1DP2	chr9	95599108	95599950	-	0	6.2755 63	0	0	0	0
ENSG00000204556	-1.86	CTD-2514C3.1	chr20	25999468	26001320	+	3.08402 75	2.3788 395	1.0570 529	1.181 9186	0.448 66753	1.024 0932
ENSG00000197358	-1.86	BNIP3P1	chr14	28733596	28735180	+	4.02136 66	6.1853 94	4.5065 994	2.335 3097	2.544 7326	2.919 4083
ENSG00000235237	-1.88	RP1-151B14.6	chr22	37562797	37578890	+	5.69870 1	5.5882 27	3.9706 326	2.796 2503	2.296 2286	2.966 6011
ENSG00000237499	-1.89	RP11-356I2.4	chr6	138144810	138189370	-	2.07288	2.8674	2.2776	0.910	1.268	1.577

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ENSG00000235847	-1.89	LDHAP7	chr2	85004383	85005347	-	2.74156 7	2.0703 194	1.2152 584	0.508 1032	1.017 5685	0.249 74425
ENSG00000273038	-1.90	RP11- 479G22.8	chr10	33176189	33178239	-	2.26414 06	2.7482 634	1.5507 42	0.526 8092	1.410 5644	0.987 04404
ENSG00000231991	-1.91	ANXA2P2	chr9	33624223	33625532	+	134.588 53	110.10 068	128.57 472	52.56 764	66.60 7285	77.69 0636
ENSG00000256929	-1.91	AC067852.1	chr17	40673736	40674065	+	3.25574	8.0733 17	4.7597 175	3.042 4023	3.687 5687	1.590 6979
ENSG00000255959	-1.92	RP11- 804A23.2	chr11	60603469	60610438	-	5.55156 56	2.3799 477	2.5062 263	1.125 2497	1.753 554	2.367 571
ENSG00000251292	-1.93	RP11- 380P13.2	chr4	23724885	23735202	-	38.6029 8	68.663 376	43.137 657	25.19 0517	34.78 7884	18.08 7368
ENSG00000262410	-1.96	RP11- 388C12.8	chr17	80702944	80703585	+	2.04051 07	3.6950 738	4.6220 4	2.332 8707	0.493 39446	1.994 9535
ENSG00000266088	-1.97	RP5-1028K7.2	chr17	38673278	38683254	+	4.31365 35	1.3317 922	1.3348 67	0.232 17432	0.229 15247	0.586 2707
ENSG00000255234	-1.98	RP11- 727A23.10	chr11	82997171	83134559	+	74.2644 35	131.67 998	93.626 11	40.38 3236	64.51 9875	45.11 061
ENSG00000231826	-1.98	AC016735.2	chr2	43254992	43266686	-	16.923	4.0733 547	5.2401 91	4.893 341	3.788 711	2.493 266
ENSG00000235823	-2.00	LINC00263	chr10	102133372	102143125	+	2.90408 5	3.2670 798	2.4657 273	1.211 951	1.841 8641	1.311 8396
ENSG00000265992	-2.00	ESRG	chr3	54666149	54673884	-	233.581 45	439.66 51	314.18 79	138.6 1157	202.8 8977	142.8 2372
ENSG00000260834	-2.02	RP11-256I9.2	chr16	65224876	65268817	-	56.8772 7	96.663 605	70.333 88	29.44 8397	46.84 315	33.75 8797
ENSG00000256940	-2.06	RP11- 783K16.5	chr11	64013436	64015689	+	13.2824 1	9.2834 835	8.4336 85	6.031 071	4.409 4415	4.459 4874
ENSG00000273387	-2.07	RP3-412A9.16	chr22	31478142	31479551	-	3.50702 29	1.8962 02	1.4603 726	1.100 5665	0.395 10265	0.518 1117
ENSG00000255021	-2.08	RP11-536I6.2	chr3	14313873	14345345	-	14.2297 64	26.088 594	15.773 34	7.262 8064	12.21 7939	7.326 7016
ENSG00000100181	-2.08	TPTEP1	chr22	17082777	17179632	+	20.2850 84	40.960 05	27.098 47	11.95 8863	17.23 5292	12.10 646
ENSG00000236673	-2.09	RP11-69I8.2	chr6	132223103	132241705	+	8.28539 9	16.666 319	10.295 293	4.810 559	6.592 9637	4.929 272
ENSG00000226792	-2.09	LINC00371	chr13	51656984	51746524	-	9.05261 9	16.953 245	10.620 668	5.284 7075	6.886 0707	4.875 6647
ENSG00000236824	-2.12	BCYRN1	chr2	47558199	47571656	+	12.5237 26	24.176 477	18.561 161	5.118 2065	7.327 3034	15.67 111
ENSG00000224081	-2.12	LINC01057	chr1	95104017	95285837	-	59.7651 8	112.70 979	71.806 335	32.73 587	50.64 0713	30.50 0008

ENSG00000225383	-2.15	SFTA1P	chr10	10826400	10836943	-	33.8832 36	11.625 587	16.437 605	5.999 579	6.570 4207	16.63 6505
ENSG00000219445	-2.15	RP11-3B12.3	chr7	124869633	124904345	-	5.30390 45	10.288 663	7.6753 13	3.506 465	4.159 0476	2.902 5939
ENSG00000233218	-2.25	SNX18P16	chr1	142688379	142688925	-	1.71771 82	2.6481 433	3.3620 58	0.430 26203	1.351 1974	0.339 88443
ENSG00000233461	-2.25	RP11- 295G20.2	chr1	231658134	231664302	-	4.34471 65	5.2603 03	4.8380 613	0.901 0945	2.709 4634	3.601 3868
ENSG00000269495	-2.34	CTB-147C22.8	chr19	51453441	51466905	+	4.91188 86	14.511 923	12.695 248	1.724 4989	4.951 8948	8.315 971
ENSG00000257219	-2.41	RP11-54A9.1	chr12	76653619	76698911	+	3.86657 3	2.7792 904	2.3597 717	0.302 85415	0.949 48655	1.806 0194
ENSG00000230439	-2.41	RP11-488P3.1	chr1	94218480	94241000	-	16.7174 1	2.3367 712	4.8807 71	2.343 1618	2.641 2401	2.188 129
ENSG00000214110	-2.44	LDHAP4	chr9	14921335	14922332	-	6.77137 7	6.3298 79	5.6765 62	2.142 0772	2.575 1235	3.043 416
ENSG00000267279	-2.45	RP11- 879F14.2	chr18	59252979	59274149	-	6.17703 3	1.2901 783	1.8376 158	0.892 23325	0.914 65175	0.947 58815
ENSG00000254740	-2.63	RP11-334E6.3	chr11	119243416	119252323	+	1.65935 97	5.2611 43	5.3788 548	1.041 0688	1.639 0346	1.514 5406
ENSG00000268621	-2.68	AC006262.5	chr19	46692423	46706340	-	2.93583 7	5.8567 724	3.7140 47	1.001 9716	2.637 1474	1.250 5352
ENSG00000271573	-2.69	RP11- 96C23.12	chr10	88786941	88787234	+	1.96670 2	9.9967 87	7.0107 24	0.436 6481	2.154 825	3.302 377
ENSG00000257042	-2.69	RP11- 993B23.3	chr12	28111450	28122746	+	25.1891 9	26.207 75	21.461 538	4.820 6534	9.942 112	15.11 63
ENSG00000255198	-2.71	SNHG9	chr16	2014960	2015510	+	5.08295 3	5.4102 4	2.8378 108	1.320 2466	1.149 7614	2.586 8635
ENSG00000261780	-2.74	CTD- 2354A18.1	chr18	70992176	71017113	+	7.45266 5	13.354 17	8.3805 11	2.986 0015	4.862 3896	2.805 7775
ENSG00000258177	-2.75	RP11-394J1.2	chr12	96616575	96617751	-	11.3985 49	7.2211 123	5.6029 59	1.443 8808	3.685 8037	4.163 6434
ENSG00000255729	-2.78	AC005618.1	chr5	140699661	140700339	-	3.83701 63	7.4735 03	7.0763 206	0.756 8286	4.717 6375	1.997 0802
ENSG00000238266	-2.79	LINC00707	chr10	6821560	6884868	+	3.58438 56	4.5337 21	3.8257 384	1.909 5888	1.506 412	0.787 2698
ENSG00000239492	-2.98	FAM25HP	chr10	47740330	47747200	+	15.1145 64	31.412 348	26.541 6	5.972 542	9.727 91	8.178 021
ENSG00000253746	-3.17	RP11- 527N22.2	chr8	37262957	37264242	-	3.75136 5	4.0315 757	2.6437 647	0.703 32074	0.949 91237	1.250 8819
ENSG00000256288	-3.22	RP11- 277P12.10	chr12	10485460	10490891	-	6.18079 3	7.4155 67	4.6269 43	0.939 5672	3.611 7387	1.750 629
ENSG00000242147	-4.40	RP13-	chr10	5636954	5638081	-	18.6134	1.4156	3.2376	0.521	0.030	0.474

		463N16.6					9	137	003	8478	29739 3	20192
ENSG00000229647	-4.79	AC007879.7	chr2	208104374	208110611	+	31.3377 27	7.7880 12	10.499 597	3.835 9287	3.087 454	1.968 523

Table S2: Comparison of FAM83H-AS1 RPKM (Reads Per Kilobase Million) with other previously published functional LncRNAs

LncRNA	ENSEMBL ID	Avg. RPKM GFP	Avg. RPKM 16E6	Known function
FAM83H-AS1	ENSG00000203499	5.876310667	9.9992845	
XIST	ENSG00000229807	8.137879	12.21193233	miRNA sponge in cervical cancer (Zhu et al 2018)
HOTAIR	ENSG00000228630	1.817852513	1.5785181	miRNA sponge in cervical cancer (Liu et al 2018)
NEAT1	ENSG00000245532	34.23374033	27.70111733	miRNA sponge in cervical cancer (Han et al 2018)
TINCR	ENSG00000223573	16.26229083	16.07113433	miRNA sponge in gastric cancer (Chen et al 2017) and breast cancer (Liu et al 2018)
H19	ENSG00000130600	3.3026914	15.14256633	miRNA sponge in cervical cancer (Ou et al 2018)

Table S3: Primers used in this study for qRT-PCR.

Primers	Sequence (5'-3')
FAM83H-AS1 FWD	TCCAATAAACAGGGCAGAC
FAM83H-AS1 REV	CAAGATCACCACACCCCTCT
Gapdh FWD	CCACTCCTCCACCTTTGAC
Gapdh REV	ACCCTGTTGCTGTAGCCA
UBC FWD	GATTGGGTGCGAGTCTTG
UBC REV	CCTTATCTTGGATCTTGCCTTG
Beta-Actin FWD	AGCACAGAGCCTCGCCTTT
Beta-Actin REV	CCACGATGGAGGGGAAGAC
U6 FWD	GTGCTCGCTTCGGCAGCACATAT
U6 REV	AAAAATATGGAACGCTTCACGAA
<i>C. Elegans</i> ama-1 FWD	GGAGCTCGAGTGGATCTTCG
<i>C. Elegans</i> ama-1 REV	GCGCAGAGAGTATCCTGGAC
HPV-16E6 FWD	AATGTTTCAGGACCCACAGG
HPV-16E6 REV	ACTGTTGCTTGCAGTACACACA
HPV-16E7 FWD	ACAAGCAGAACCGGACAGAG
HPV-16E7 REV	GCCCATTAACAGGTCTTCCA
p53 FWD	TTTGGGTCTTTGAACCCTTG
p53 REV	CCACAACAAAACACCAAGTGC
p300 FWD	AGCGGCCTAAACTCTCATCTC
p300 REV	CACCATTGGTTAGTCCCAATTC
FAM83H FWD	CGACAAGTGCCGTGTCAACC
FAM83H REV	ACTTCCCAGTGCGGCAGTAG
H19 FWD	AGTGGACTTGGTGACGCTGTAT
H19 REV	CTCCTGAGAGCTCATTCACTCC
MIR205HG FWD	TCATTAAAGAGAGAAATCAACTATTCA
MIR205HG REV	TAAAGCACCCAGTTCAGCAG

HOXC-AS5 FWD	CTCCTTGGAGCAGTACACCTG
HOXC-AS5 REV	GTCCATTTGCTCTCCACTTGA
GAS5 FWD	CTTCTGGGCTCAAGTGATCCT
GAS5 REV	TTGTGCCATGAGACTCCATCAG
LINC00963 FWD	AACTGCCTTTGGAAGCAAGTAG
LINC00963 REV	ACTGCTCTAGTCCAGGAGTTCG
SNHG15 FWD	GCTGAGGTGACGGTCTCAA
SNHG15 REV	GCCTCCCAGTTTCATGGACA
MAFG-AS1 FWD	AGGACTCGGGAGGAAGATAAAC
MAFG-AS1 REV	AACACCTTCAGCTCTCTGCTG
RP6-65G23.3 FWD	GAGATAGGAGGCCATAATGTTTC
RP6-65G23.3 REV	CTGCAACCAGGTGGAAGTCA
GS1-600G8.5 FWD	ACTGTCCGGTTCCTGTGGG
GS1-600G8.5 REV	CGGTGCCACTGGGTCATTTT
RP3-510D11.2 FWD	CCAGACCGACGGGACAGCG
RP3-510D11.2 REV	GCTTCCCTGTCCTCCTCCTA
RP11-479G22.8 FWD	TGGGAAACTAAAACCATTTAAGC
RP11-479G22.8 REV	GCTCAGAACATGGTTTTCTCTG
LINC01057 FWD	GTGAATTCCTTGAAGATGAGG
LINC01057 REV	TCATTCACTCAACCACTGAACC
SFTA1P FWD	CAGCATTCCAGGTGGGCTTT
SFTA1P REV	CCTTGTTTGGCTTACTCGTGC
RP13-463N16.6 FWD	TTGGAAATCACTCCTTCCACTT
RP13-463N16.6 REV	CAGAGACAATTCAACTCCCACA
AC007879.7 FWD	ACAGGGAGCCAGGACACC
AC007879.7 REV	GGAACCAGCACCAGGAACC

XIII. Supplementary Methods

A. Cell Culture

Primary human foreskin keratinocytes (HEKa) were cultured as described by supplier (EpiLife® Medium supplemented with Human Keratinocyte Growth Supplement (HKGS)). Primary human cervical keratinocytes (HCK) and J2-3T3 mouse fibroblast feeders were cultured as described by Alison McBride's laboratory (NIH, Bethesda, MD)(55). HCK were maintained in F-media [3:1 F12:DMEM with 5% Fetal Bovine Serum (FBS), 0.4ug/mL Hydrocortisone, 5ug/mL Insulin, 8.4ng/mL Cholera Toxin, 10ng/mL Epidermal Growth Factor, 24ug/mL Adenine, 10U/mL Penicillin, 10ug/mL Streptomycin, 2mM L-Glutamine, Amphotericin B] containing 10uM Y-27632 ROCK Inhibitor (Tocris). HCK cells were co-cultured with J2-3T3 feeder cells rendered mitotically inactive in 8ug/mL Mitomycin C for 3 hours; these growth-arrested feeder cells were replenished every 3-4 days. J2-3T3 stock cells were cultured in Feeder media [Dulbecco's Modified Eagles Medium (DMEM), high glucose, 10% Newborn Calf Serum, 2mM L-Glutamine, 10U/mL Penicillin, 10ug/mL Streptomycin, Amphotericin B]. J2-3T3 feeder cells are sensitive to trypsin; HCK, J2-3T3 stock, and growth-arrested J2-3T3 cells were centrifuged to remove the trypsin when passaged. HPV-16 positive CaSki cells were cultured in Roswell Park Memorial Institute (RPMI)-1640 medium containing 10% FBS, Streptomycin-Penicillin, HEPES Buffer, Amphotericin B. HPV-16 positive W12/20863 and W12/201402 and HPV-31b positive cervical (CIN-612) were co-cultured in E-media (1:1 DMEM:Ham's Nutrient Mix F12 medium supplemented with 5% FBS, 10mM HEPES Buffer, Penicillin-Streptomycin, Amphotericin B, 0.02uM Triiodothyronine, 0.4ug/mL Hydrocortisone, 0.1ug/mL Cholera Toxin, 5ug/mL Transferrin, 180uM Adenine, 5ug/mL Insulin) with murine 3T3 fibroblast cells (3T3M) that were rendered mitotically inactive with 4ug/mL Mitomycin C for at least 2 hours. HPV-negative cervical C33A cells, HPV-18 positive cervical (HeLa), HPV-16 positive HNSCC cell lines UMSCC-47 and -104, HPV negative HNSCC cell line UMSCC-1, HEK293T, and 3T3M cells were all cultured in DMEM supplemented with 10% FBS, Penicillin-Streptomycin, HEPES Buffer, and Amphotericin B. To passage stock cells, 0.25% trypsin was utilized. Growth-arrested J2-3T3 and 3T3M fibroblasts were removed with Versene solution and gently pipetting, prior to

pelleting and when otherwise desired. Cells were cultured at 37°C in a humidified 5% CO₂ cell culture incubator.

B. Generation of stable cell lines

Plasmids MSCV-N-GFP (Plasmid #37855), MSCV-N-16E6 (Plasmid #37875), and MSCV-N-16E7 (Plasmid #37881) were purchased from Addgene. For retroviral production, these plasmids were co-transfected with packaging (pCL-ECO) and envelope (pVSV-GF) plasmids into HEK293T cells using calcium phosphate co-precipitation. 12-16 hours later, HEK293T media was replaced with fresh target cell media. After 48-hour incubation, retrovirus-containing target cell media was harvested, filtered (0.45µm), and mixed with polybrene (4µg/mL final concentration) to increase infection efficiency. 24 hours before infection, co-cultured J2-3T3 fibroblasts were removed with Versene solution and gentle pipetting from HCK cells. HEKα and HCK cells were infected with recombinant retroviruses for 8 hours, virus was removed and replaced with fresh target cell media. Growth arrested J2-3T3 were added to infected HCK and replenished every 3 days. Cells were allowed to recover for 24 hours prior to 72-hour puromycin selection (HEKα-2.5µg/mL; HCK-3µg/mL). Cells were allowed to recover and expression of exogenous HPV-16 E6 and E7, as desired, was confirmed by RT-PCR (Fig. S1, Fig. S3).

C. Generation of HPV-16 positive cervical JAMM-16 cell line

According to Buck et al. protocol described previously (56), the HPV-16 insert was cut out of pBR322 HPV-16 plasmid by performing a restriction digestion of two separate 25ug plasmid reactions in 225uL each with the restriction enzyme BamHI (NEB) according to manufacturer's recommended conditions (37°C for 2 hours). Digested samples were then PCR purified by QIAquick® PCR Purification Kit (Qiagen) resuspended in 200uL Buffer TE. Purified samples were then ligated under dilute conditions (9mL total volume) by adding 1X Ligase Buffer and 6uL of high concentration (2m U/mL) T4 DNA Ligase (NEB) and incubated at 16°C overnight. <25ug in 9mL ligation reactions were conducted to avoid concatemer formation. The ligated samples were then treated with 4.5mL of 7.5M Ammonium acetate and mixed. 35mL of 95% ethanol was added to each, tubes were mixed, and incubated at 4°C overnight. The next day, the samples were

brought back to room temperature and centrifuged at $\sim 5,000 \times g$ at room temperature for 1 hour. Pellets were washed with 70% ethanol, spun briefly, and washed again with 70% ethanol. The pellets were spun one last time to remove any residual ethanol, then air dried for several minutes. Pellets were resuspended in 100uL Buffer TE each, pellets were combined, and Nanodrop was used to calculate concentration retrieved. The entire product was run in 1% agarose gel for 3 hours. Desired band [expect to see supercoiled and relaxed circular bacterial backbone (~ 1.8 and ~ 3.2 kb) and supercoiled and relaxed circular (nicked) HPV genome (~ 6.2 and >16 kb)] was cut out and gel extracted with QIAquick® Gel Extraction Kit (Qiagen). Before transfection of the HPV-16 genomes, growth-arrested J2-3T3 fibroblasts were removed by Versene solution and pipetting from co-cultured primary normal cervical keratinocytes (HCK). HCK were plated in a 6-well plate on day prior to transfecting according to manufacturer's protocol for Lipofectamine LTX (Invitrogen). On day of transfection, HCK were at about 70% confluency. Media was replenished on each well. For each well, 2.8ug of re-ligated HPV-16 insert was diluted in 150uL Opti-MEM I Reduced Serum Media with 2.5uL PLUS Reagent, 15uL Lipofectamine LTX was diluted in Opti-MEM I Reduced Serum Media. Diluted DNA and diluted Lipofectamine LTX/Plus complex were combined and incubated for 5 minutes at room temperature prior to adding to well. The transfected cells were passaged at least 10-15 times so HPV-16 immortalized cells could overgrowth the untransfected HCK cells prior to experimentation.

D. RNA Extraction

Total RNA of cultured cell lines and human tissues was purified with TRIzol reagent (Invitrogen) and treated with Turbo DNA-free DNase (20 minutes at 37°C , Ambion) according to manufacturers' protocols. RNA concentration was determined with a Nanodrop 2000 Spectrophotometer (Thermo Scientific).

E. cDNA synthesis, qualitative or quantitative RT-PCR

RNA was reverse transcribed to complementary DNA (cDNA) under standard iScript cDNA synthesis kit (Bio-Rad) instructions in T100TM Thermal Cycler (Bio-Rad). Exogenous spike of *C. Elegans* (70ng) was added for normalization of results if GAPDH mRNA levels were altered

during processing (cellular fractionation). RT-PCR or qRT-PCR using SsoAdvanced™ Universal SYBR® Green enzyme was performed according to manufacturer's protocol (Bio-Rad) in CFX Connect™ Real-Time System (Bio-Rad). The oligonucleotide primer sequences can be found in Supplementary Table S3. GAPDH was primarily used as housekeeping gene, however Ubiquitin C (UBC) or Glucuronidase Beta (GUSB) were used if GAPDH mRNA levels were altered with significant apoptosis (siRNA FAM83H-AS1 vs. siRNA-CTRL) and ama-1 was used for cellular fractionation studies. To validate cell fractionation efficiency, U6 snRNA was used as nuclear control and mature mRNA beta-actin as cytoplasmic control. Qualitative RT-PCR product was resolved by agarose gel electrophoresis to visualize or quantitative RT-PCR (qRT-PCR) relative expression was calculated using the double delta CT method (relative expression = $2^{-\Delta\Delta CT}$; where $\Delta CT = CT(\text{Target RNA}) - CT(\text{mRNA endogenous GAPDH/UBC control or } C. \text{ Elegans mRNA Ama-1 was exogenous spike control})$). Fold changes were calculated relative to control siRNA or the mean value of normal samples.

F. Western Blot analysis

Cell lysates were boiled in sample buffer, separated by SDS-PAGE, and transferred onto Immobilon-Fl polyvinylidene difluoride membranes. After blocking for 1 hour with 5% milk-TBST buffer (5% non-fat dry milk, 25mM Tris-HCl pH 8.0, 125mM NaCl, 0.5% Tween-20), the membranes were incubated with primary antibodies against human p53 (Cell Signaling, #9282) or Actin (C-11) (Santa Cruz, sc-1615) overnight at 4°C. The membranes were then washed with TBST, incubated with appropriate horseradish peroxidase (HRP)-conjugated secondary antibodies [anti-goat IgG (Santa Cruz, sc-2020); anti-mouse IgG (Thermo Scientific)] for 1 hour at room temperature, washed with TBST, and the proteins were detected on the membrane using Pierce SuperSignal West Pico or Femto Maximum Sensitivity Substrate (Thermo Scientific) chemiluminescence.

G. Transient transfection

The following siRNAs were used to knock down FAM83H-AS1: Lincode Human FAM83H-AS1 siRNA - Set of 4 (Dharmacon, RU-188909-00-0002), Lincode Human FAM83H-

AS1 siRNA SMARTpool (Dharmacon, R-188909-00-000), Lincode Non-targeting siRNA #1 (Dharmacon, D-001320-01-05). Two different siRNA against HPV-16E6: 5'-GAGGUAUAUGACUUUGCUUTT-3' (Dharmacon) and 5'-UCCAUAUGCUGUAUGUGAUTT-3' (siRNA 209, Dharmacon), two different siRNA against p53 (NEB, #2011S; Thermo Scientific Dharmacon®, J-003329-16), two different ONTARGETplus Human siRNA against p300 (Dharmacon, J-003486-11-0002; Dharmacon, J-003486-12-0002), and CTLsiRNA (Ambion, AM16104) were used.

HCK, CaSki, and W12/201402 cells were transiently transfected using standard Lipofectamine® RNAiMAX protocol (siRNA FAM83H-AS1 and siRNA HPV-16E6 #1). W12/201402 and CaSki cells were transfected with reverse transfection protocol (siRNA HPV-16 E6 #2 to improve transfection efficiency. For standard transfection, cells were plated at appropriate confluency to be about 70% confluent for transfection 24 hours post-plating. Growth-arrested J2-3T3 fibroblasts were co-cultured with HCK, and on day of transfection removed with Versene solution and gently pipetting. On day of CaSki, W12/201402, and HCK standard transfection, media was replenished on each well. For each well, Opti-MEM I Reduced Serum Media was used to dilute Lipofectamine RNAiMAX (9uL per well of 6-well), as well as siRNA (40pmol per well of 6-well). Diluted RNAiMAX was combined with diluted siRNA, incubated 5 minutes at room temperature and added to each well. Cells were incubated 48 hours in media containing siRNA prior to harvesting, unless otherwise stated.

H. Cellular fractionation

Growth arrested 3T3M cells were removed by Versene solution and pipetting the day prior to fractionation for the W12/201402. Cellular fractionation protocol was modified from previous publication (57). On ice, CaSki (~70% confluent) were washed two times with cold PBS, scraped, pelleted, and resuspended in RSB Buffer (10-1.25mM Tris, pH 7.4; 10-1.25mM NaCl; 3-0.38mM MgCl₂), incubated on ice for 5 minutes, centrifuged (1500rpm for 4 minutes at 4°C). RSB Buffer was removed and swollen pellets were resuspended in RSB-G40 Buffer [2.5-0.63mM Tris, pH 7.4; 2.5-0.63mM NaCl; 0.75-0.19mM MgCl₂; 2.5-0.63% glycerol; 0.125-0.03% NP-40; 0.125-0.03mM DTT; 100U/mL RNasin Plus RNase Inhibitor (Promega)] in ≥ 4 times volume of pellet.

Cells were disrupted with Dounce homogenizer; homogenate was centrifuged (4000 rpm, 4 minutes) to pellet nuclear fraction. The cytoplasmic fraction (supernatant) was collected. The pelleted nuclear fraction was resuspended in RSB-G40 Buffer, 3.3% of sodium deoxycholate and 6.6% of Tween20 were added to the volume of RSB-G40 (10% of final volume was sodium deoxycholate and 10% was Tween 20), samples were tapped to mix, incubated on ice for 5 minutes, and pelleted by centrifugation (7000rpm, 3 minutes). The supernatant was collected and combined with first cytoplasmic fraction, and nuclear pellet was washed two times with RSB-G40 Buffer, and then resuspended in RSB-G40 Buffer. Fractionated cells were Turbo DNase I-treated, according to manufacturer's instructions (NEB), prior to RNA extraction to remove bound DNA and improve recovery of RNA. Total cell (second pellet), cytoplasmic, and nuclear RNA was extracted using TRIzol Reagent (Life Technologies) per manufacturer's instructions (Glycoblue was added during isopropanol precipitation step due to small amounts of RNA recovered from nuclear fraction) and treated with Turbo DNase (Ambion) for 20 minutes at 37°C. qRT-PCR analysis was performed using cDNA generated using equal concentration of RNA with exogenous *C. elegans* RNA (70ng) added for normalization.

I. Cell proliferation assay

Cell proliferation was monitored using Cell Counting Kit - 8 (CCK-8) (Sigma-Aldrich). 24 hours (CaSki) or 48 hours (201402) after transfection with FAM83H-AS1 siRNA, CaSki cells were detached with 0.05% trypsin and W12/201402 cells were detached with 0.25% trypsin. After cell detachment, trypsin was inactivated with trypsin neutralizer (CaSki) or E-media containing serum (201402), pelleted, resuspended in appropriate media containing serum, and plated at desired concentration (3E3 cells per well and of four separate 96-well plates; technical triplicates). In equal density per well, 3T3M fibroblasts treated with mitomycin C were added to the W12/201402 cells and fibroblast alone control wells were plated. At desired time (48, 72, or 96h post-plating), CCK-8 dye (100uL/well) was added to each well, incubated for 2 hours (CaSki) or 3 hours (201402). Then, absorbance was read with a spectrophotometer (BioTek Synergy H1 Hybrid Reader) (OD₄₅₀) according to manufacturers' protocols. Absorbances of feeders alone were subtracted from W12/201402 co-cultured with feeders. Biological replicate was conducted.

J. Flow-cytometric analysis of cell cycle.

3T3M fibroblasts were stained with CellTrace™ Far Red Cell Proliferation Kit (Invitrogen) the day before plating transfected 201402 cells. 24 hours (CaSki) or 48 hours (201402) after transfection with FAM83H-AS1 siRNA, cells were detached with 0.05% trypsin (CaSki) or 0.25% trypsin (201402). After cell detachment, trypsin was inactivated with trypsin neutralizer (CaSki) or media containing serum (201402), and cells were plated at desired concentration (4E6 for CaSki and 2.3E6 for 201402) in 100mm dishes in media containing fetal bovine serum (FBS). Mitomycin C treated, far red stained 3T3M cells were added to the 201402 cells. Cells were allowed to attach (about 5 hours) then were washed 3 times with 1X D-PBS to remove residual FBS. Cells were then serum-starved for 24 hours. After incubation, cells were trypsinized with 0.25% trypsin, suspended in serum-free media (to avoid stimulation by serum), washed with 1X PBS, pelleted, and resuspended in PBS (200μL). Harvested cells were then fixed by adding 70% cold ethanol (1.8mL) while vortexing the cells and stored at 4°C until processing. Cells in ethanol were pelleted, washed with PBS, centrifuged, and resuspended in room temperature 0.2% Tween 20 in PBS (300-1,000μL, depending on pellet size). 100uL of each sample was placed in a U-bottom 96-well plate (3 wells/technical triplicates) and incubated 15 minutes at 37°C. PBS (100uL) was added to each well, cells were pelleted, resuspended in 10uL of RNase A-PBS (180ug/mL stock), tapped gently to mix, and incubated at room temperature for 15 minutes. After incubation, 20uL of PI-PBS (final concentration of 50μg/mL) was added to each well, pipetted to mix, and incubated for 15 minutes at room temperature. After incubation, the volume was brought up to 300uL and analyzed in the dark by flow cytometry (Fortessa S10). Gates were placed around 201402 population. Biological replicate was conducted.

K. Transwell migration assay

24 hours (CaSki) or 48 hours (201402) after transfection with FAM83H-AS1 siRNA, cells were detached with 0.05% trypsin. After cell detachment, trypsin was inactivated with trypsin neutralizer, pelleted, resuspended in serum-free media, and cells were plated [1E5 (CaSki) or 0.75E5 (201402), 200uL well, technical duplicates] in upper chambers of 24-well transwell (8mm pore size) with 800uL serum-free media in lower chamber. In equal density per well, 3T3M

fibroblasts treated with mitomycin C were added to the W12/201402 cells and fibroblast alone control wells were plated. Cells were then allowed to attach for ~3 hours prior to adding fetal bovine serum (final concentration of 20% for CaSki and 5% for W12/201402) to lower chamber. After 24 hours, media in upper chamber was replenished with appropriate fresh serum free media and lower chamber with appropriate fresh media containing with FBS chemoattractant. 48 hours after cells were plated, lower chamber/underside of transwell and upper chamber of transwell were washed with 25% D-PBS. D-PBS was removed and cells from the top of the upper chamber transwell membrane (non-migrated cells) were wiped away using a cotton swab. The lower chamber/underside of the transwell and upper chamber of transwell were washed again with 25% D-PBS and then the underside of the transwell (migrated cells) was fixed with 4% formaldehyde in 25% D-PBS in the lower chamber for 5 minutes at room temperature. The formaldehyde solution was removed and the lower chamber/underside of transwell and upper chamber of transwell were washed with 25% D-PBS. The cells on the underside of the transwell were then stained using 0.5% crystal violet in 20% ethanol in water. The stain was added to the lower chamber, submerging the transwell for 20 minutes at room temperature. The stain was removed and the lower chamber/underside of the transwell and upper chamber of transwell washed 3 times with 25% D-PBS. The top of the upper chamber transwell membrane was then cleaned with a cotton swab. The transwells were allowed to dry overnight and then the underside of the transwell imaged with an Olympus MVX10 microscope. Migration was quantitated with ImageJ. Feeders alone were subtracted from W12/201402 co-cultured with feeders. Biological replicate was conducted.

L. Flow-cytometric analysis of apoptosis

3T3M fibroblasts were stained with CellTrace™ Far Red Cell Proliferation Kit (Invitrogen) the day before plating transfected 201402 cells. 24 hours (CaSki) or 48 hours (201402) after transfection with FAM83H-AS1 siRNA, cells were detached with 0.05% trypsin (CaSki) or 0.25% trypsin (201402). After cell detachment, trypsin was inactivated with trypsin neutralizer (CaSki) or media containing serum (201402), pelleted, resuspended in appropriate media containing serum, and cells were plated in 100mm dishes at desired concentration (1.75E6 for CaSki and 1.1E6 for 201402). At 24, 48, and 72h later post-plating, attached and floating cells were collected. On day of harvest, media containing dead cells was collected, PBS was added to

the plates, collected, and added to the media. Attached cells were trypsinized with 0.25% trypsin, cells were pelleted ($2 \text{ to } 5 \times 10^6$) at 4°C , resuspended in PBS, and 100uL of each sample was aliquoted into 3 wells of a 96 well plate (technical triplicates). Cells were pelleted, PBS was removed, Annexin V Binding Buffer (100uL; diluted with autoclaved milliQ water) was added to each tube, Annexin V-FITC (5uL) was added to each tube, and incubated in the dark at room temperature for 10 minutes. After incubation, cold Binding buffer (200uL) was added to each tube, cells were pelleted, washed with cold Binding buffer two times, incubated on ice for 5 minutes in propidium iodine in Binding buffer (2ug/mL final concentration, 300uL), and analyzed by flow cytometry (Fortessa S10) immediately. Gates were placed around 201402 population. Biological replicate was conducted.

M. Human cervical tissue specimen

10 human specimens were obtained from patients under the tissue collection protocol [Prognostic Marker (IRB0406147)] at the University of Pittsburgh. Patient samples were categorized as cervical intraepithelial neoplasia stage 3 (CIN3), invasive cervical cancer (CaCx), or non-cancerous.

N. TCGA Analysis

The Cancer Genome Atlas (TCGA) (<https://cancergenome.nih.gov/>) contains patient survival and RNA sequencing data from 196 cervical squamous cell carcinoma and endocervical adenocarcinoma (CESC) human patient samples. TCGA-CESC reads per kilobase million (RPKM) data was extracted, and average RPKM for all patients combined was calculated. TCGA also contains RPKM data from 3 individual non-cancerous cervical samples that were averaged together to compare to CESC RPKM values. The Atlas of non-coding RNA in Cancer (TANRIC) (MD Anderson Cancer Center) (54) that utilizes TCGA-CESC dataset to characterize the expression profiles of long non-coding RNAs (lncRNAs) was used to extract FAM83H-AS1 expression in each of the TCGA-CESC patient samples. Classification and Regression Trees (CART) analysis was used to statistically define high/low lncRNA expression groups. The cut-off z score value was 3 to discern between high and low expression groups. Survival curves were

estimated by the Kaplan-Meier method. The log-rank test was used to estimate statistical differences between survival curves. GraphPad software was used to make the survival plots.

O. Statistics

Student's t-test was utilized to determine mean values differences between groups examined and significance was determined at $p \leq 0.05$ (*) and $p \leq 0.01$ (**). Scale bars represent Standard Deviations (SD).

XIV. Supplemental References

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Education

West Virginia University School of Medicine, Morgantown, WV 08/2013 – 05/2020
PhD in Biomedical Sciences – Cancer Cell Biology
Current GPA: 3.73
PhD conferral: 05/2020

Henderson State University, Arkadelphia, AR 08/2009 – 05/2013
Bachelor of Science in Biology
Final GPA: 3.44

Research Experience

West Virginia University School of Medicine 08/2013 – 05/2020
Principal Investigator: Ivan Martinez, PhD
Project: The Importance of the Lnc-SPRY3 Family in Non-Small
Cell Lung Cancer Therapeutic Response

Henderson State University Spring 2012 – Spring 2013
Mentor: Martin Campbell, PhD
Project: Nicotinic Acid Ester-Based Ionic Liquids

Baylor College of Medicine Summer 2012
Summer Medical and Research Training (SMART) Program
Mentor: Hoang Nguyen, PhD
Project: Sox9 and Sox11 Inhibit Differentiation of Skin Epithelial Cells

Relevant Skills

Laboratory-based:	
RNA isolation and purification	Bacterial Cell Culture/plasmid purification
Mammalian cell culture	Xstrahl Xenex cell irradiation
RT-PCR/qRT-PCR analysis	RNAi techniques
Northern Blotting	CRISPR
Western Blot analysis	Retro/Lentiviral transduction
Immunoprecipitation	Guava easyCyte flow cytometry
Cloning	Cellular Fractionation

Mouse Based:
Cell line derived xenografts
Xstrahl Xenex small animal irradiation
Tumor ultrasound imaging via Vevo 2100

Other:
Microsoft Office
ImageJ and GraphPad Prism
Scientific writing/presentation
Mentoring undergraduate/fellow
graduate students

Publications

Barr, J.A., Hayes, K.E., Harold, A., Lovern, M., **Brownmiller, T.**, Jagannathan, R., Lockman, P., Martinez, I. Long non-coding RNA FAM83H-AS1 is regulated by human papillomavirus 16 E6 independently of p53 in cervical cancer cells. *Scientific Reports*, **9**:3662 (2019) <https://doi.org/10.1038/s41598-019-40094-8>.

Brownmiller, T., Juric, J.A., Ivey, A.D., Stevens, A.M., Hayes, K.E., Sprowls, S.A., Ammer, A.G., Walker, M., Wu, X., Lim, Z., Zhu, L., Bey, E.A., Hu, G., Wen, S., Ma, P.C., Martinez, I. Evidence of Y Chromosome LncRNAs involved in Radiation Response of Male Non-Small Cell Lung Cancer Cells. (undergoing revision for *Cancer Research*)

Hayes, K.E., Barr, J.A., Harold, A., **Brownmiller, T.**, Wilusz, J.E., Martinez, I. Discovery of Novel Non-Coding Circular RNAs Generated by High-Risk Human Papillomaviruses. (Manuscript in preparation)

Honors/Awards

Awards:

- Graduate:
 - First Place – Poster: Basic Science, 2019 WVU Cancer Institute Annual Meeting
 - WVU Biomedical Sciences PhD Programs – Cancer Cell Biology Graduate Student of the Year Award 2019
 - 2019 WVU Three Minute Thesis Competition Finalist
 - WVU Office of Research and Graduate Education, Robert C. Byrd Health Sciences Center Doctoral Student Travel Award – Spring 2019
 - Second Place – Poster: Clinical Science, 2018 WVU Cancer Institute Annual Meeting
 - WVU Office of Research and Graduate Education, Robert C. Byrd Health Sciences Center Doctoral Student Travel Award – Spring 2018
 - Outstanding Graduate Student Award – 2016 Team Award, Mad Scientists ACS Relay for Life Team
 - First Place – Poster: Basic Science 2013-Group 2, 2015 Van Lier Memorial Convocation & HSC Research Day
- Undergraduate:
 - Arkansas Academy of Science Undergraduate Research Award
 - Arkansas Space Grant Consortium Undergraduate Research Grant
 - Poster – American Chemical Society Excellence in Undergraduate Polymer Research

Scholarships:

- Henderson State University Presidential Scholarship: Fall 2009 – Spring 2013
- Arkansas Academic Challenge Scholarship: Fall 2010 – Spring 2013
- Parkers Chapel Scholarship: Fall 2009
- Henderson State University Match Fall: 2009
- Hartsell Pest Control Scholarship: Fall 2009
- Academic Honor Roll: Fall 2009 – Spring 2013

Memberships**National:**

RNA Society	2019 - Present
American Association of University Women (AAUW)	2018 - Present
American Association for the Advancement of Science (AAAS)	2015 – Present
American Association for Cancer Research (AACR)	2015 – Present
AACR Women in Cancer Research	2015 – Present

West Virginia University:

Graduate Student Organization	Fall 2013 – Present
• President: Summer 2015 – Summer 2016	
• Vice President: Fall 2014 – Summer 2015	
Cell Biology Training Program	Fall 2014 – Present
First Year Student Mentorship Program	Fall 2014 – Present

Henderson State University:

Biology Club	Spring 2010 – Spring 2013
• President: Fall 2012- Spring 2013	
Chemistry Club	Spring 2011 – Spring 2013
Tri Beta Biological Honor Society	Spring 2011 – Spring 2013
• President: Fall 2012 – Spring 2013	
Gamma Beta Phi Academic Honor Society	Spring 2011 – Spring 2013
American Chemical Society	Fall 2012 – Spring 2013

Professional Development**Meetings/Conferences and Abstracts:**

October 2019 - WVU Cancer Institute Annual Scientific Meeting, Morgantown, WV. Poster presented: “A Family of Y Chromosome LncRNAs is Involved in the Radiation Response of Male Non-Small Cell Lung Cancer Cells”

April 2019 – NIH Symposium “RNA Biology 2019” at NCI, Bethesda, MD. Poster presented: “The Importance of the Lnc-SPRY3 Family in Non-small Cell Lung Cancer Therapeutic Response.”

March 2019 – Van Liere Research Day: West Virginia University’s yearly celebration of the biomedical research being conducted at its Health Sciences Center campus. Flash Talk and

Poster presented: “The Importance of the Lnc-SPRY3 Family in Non-small Cell Lung Cancer Therapeutic Response.”

February 2019 – Keystone Symposia, Long Noncoding RNAs: From Molecular Mechanism to Functional Genetics, Whistler, British Columbia, Canada. Poster Presented: “The Importance of the Lnc-SPRY3 Family in Non-small Cell Lung Cancer Therapeutic Response.”

October 2018 – WVU Cancer Institute Annual Scientific Meeting, Morgantown, WV. Poster presented: “The Importance of the Lnc-SPRY3 Family in Non-small Cell Lung Cancer Therapeutic Response.”

April 2018 – American Association for Cancer Research Annual Meeting, Chicago, IL. Poster presented: “The Importance of a Family of Long Non-coding RNAs from the Y Chromosome in Radiotherapy and Chemotherapy Response of Male Non-small Cell Lung Cancer.”

April 2017 – NIH Symposium “RNA Biology 2017” at NCI, Bethesda, MD. Poster presented: “The Importance of the Lnc-SPRY3 Family in Non-small Cell Lung Cancer Therapeutic Response.”

March 2017 – Van Liere Research Day: West Virginia University’s yearly celebration of the biomedical research being conducted at its Health Sciences Center campus. Poster presented: “The Importance of the Lnc-SPRY3 Family in Non-small Cell Lung Cancer Therapeutic Response.”

June 2016 – 6th Biennial National IDeA Symposium of Biomedical Research Excellence (NISBRE). Poster presented: “The Importance of the PRYP4 Long Non-coding RNA family in Non-small Cell Lung Cancer Radiation Response.”

March 2016 – Van Liere Research Day: West Virginia University’s yearly celebration of the biomedical research being conducted at its Health Sciences Center campus. Poster presented: “The Importance of the PRYP4 Long Non-coding RNA family in Non-small Cell Lung Cancer Radiation Response.”

December 2015 – AACR Special Conference on “Non-coding RNAs and Cancer: Mechanisms to Medicine,” Boston, MA.

October 2015 – “RNA and the New Genetics” Symposium at University of Toronto, Toronto, ON.

March 2015 - NIH Symposium “RNA Biology 2015” at NCI, Bethesda, MD.

February 2015 - Van Liere Research Day: West Virginia University’s yearly celebration of the biomedical research being conducted at its Health Sciences Center campus. Poster presented entitled “The Importance of miRNAs in the Cancer Resistant Animal Model: The Naked Mole-rat”

August 2014 – “Regulatory and Non-coding RNAs” Meeting at Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

Other:

September 2019 – Workshop on Long-read Sequencing, The Jackson Laboratory for Genomic Medicine – JAX Genomic Medicine, Farmington, CT.

Spring 2019 – WVU Three Minute Thesis Competition, West Virginia University, Morgantown, WV.

Fall 2018 – Guest lecturer for first year graduate students Cellular Methods course: “*In Vitro* Methods of Genetic Manipulation”

Fall 2017 – Spring 2018: West Virginia University, Student representative to the Cancer Cell Biology Graduate Program faculty meetings

Fall 2015 – Spring 2016: West Virginia University PhD in Biomedical Sciences admissions committee member

June 2014 – “Setting the PACE” Writing Workshop by Laura Brady, Nathalie Singh-Corcoran, Andrea Bebell, and James Holsinger at West Virginia University Health Sciences Center, Morgantown, WV.

References

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