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RNA transcription termination factors and persistent R-loops: potential carcinogenic determinants after high or low LET IR**

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ABSTRACT

Neurologic and carcinogenic effects caused by prolonged exposure to high linear energy transfer (LET) ionizing radiation (IR) represent major health-limiting obstacles during an estimated two-three year mission to Mars. Examination of DNA damage caused by high or low LET exposures has shown that high LET IR exposures cause far greater formation of multiply damaged sites, including complex DNA double strand breaks (DSBs) that are more difficult for cells to repair correctly. Presumably, these lesions lead to neurologic (cognitive and visual), and visual (cataracts) defects, as well as increased carcinogenic effects.

This original research article focuses on potential health risks for individuals with defects in factors that function to accurately terminate RNA transcription, a process involving specific key steps in transcription, including: (*i*) accurate mRNA, miRNA, siRNA or lncRNA maturation, (*ii*) RNA polymerase II (RNA Pol II) stalling and dislodging; and (*iii*) subsequent resolution of DNA:RNA:DNA hybrids, known as 'R-loops'. Defects in RNA Pol II processing, in general, lead to persistent R-loop formation and ultimately formation of complex DSBs caused by unstable RNA:DNA structures and collisions between persistent R-loops and DNA replication and/or RNA transcriptional processes. We offer original data as evidence that low LET IR exposures cause delayed formation of persistent R-loops in wild-type cells. Further, we show that these unique and understudied DNA lesions result in indirect complex DSBs, breaks not generated by original deposition of energy. We also show that cells deficient in one RNA termination factor, Kub5-Hera (K-H/RPRD1B) show major defective DSB repair kinetics after high LET IR treatments, consistent with far greater R-loop formation and delayed and slower repair of persistent R-loop-derived DSBs than after low LET IR doses. Thus, haplo-insufficient loss of one essential RNA transcription termination scaffold factor, K-H (aka., RPRD1B), results in simultaneous defective repair of both R-loops and complex DSBs created by low or high LET IR. Whole body low LET IR-exposed haplo-insufficient K-H$^{+/-}$ mice showed hypersensitive carcinogenesis in a dose-dependent manner. These mice also have neurological and visual defects, including loss of hind limb function and

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** *This review summarizes, in part, a mini-symposium, entitled* "Role of R-loop-related factors in DNA damage, genomic instability, and DNA repair" *presented at the Annual Radiation Research Society meeting held in Las Vegas, NV in October, 2014. Please see the summary of that mini-symposium published through the Radiation Research Society (RRS). This review covers our current understanding of the roles of persistent R loops in carcinogenesis after high or low LET IR treatments. As such, published and non-published data are discussed with all unpublished data due to appear in 2015.*

[a] *These authors contributed equally to this peer-reviewed paper.*

eyesight. Importantly, we identified nearly two hundred single nucleotide polymorphisms (SNPs) in K-H, and over 5,000 SNPs in its DSB binding partner, p15RS (RPRD1A) in the human population using two separate databases. While the functional significance of these SNPs is currently being delineated, understanding their roles, as well as defects in other RNA processing proteins (e.g., NONO, SETX) that lead to persistent R-loops, are critical to our understanding of the health effects of high, as well as low, LET IR exposures.

INTRODUCTION

Health risks during space missions. Extended manned space travel is a major mission of National Aeronautics and Space Administrations (NASA). The NASA goal of sending humans to Mars or other destinations outside the earth's magnetosphere (Hellweg and Baumstark-Khan, 2007) comes with a variety of challenges. Along with technology and budget considerations, the potential health risks that astronauts will encounter is of concern. Besides the engineering challenges of launch and return, the biggest threat to human health with extended space travel comes in the form of exposure to solar particle events (SPEs) and galactic cosmic radiation (GSR), for which we currently do not have effective shielding. This risk is an important consideration, particularly for long-duration exploration missions beyond low Earth orbit where the protective magnetic field of the planet no longer provides shielding (Chancellor et al., 2014; Kahn et al., 2014; Moore et al., 2014).

There are two principal components of space radiation outside of low Earth orbit: (a) high-flux, sporadic solar particle events (SPEs); and (b) uniform low-flux/high-energy galactic cosmic radiation (GCR) (Cucinotta and Durante, 2006; Cucinotta and Schimmerling, 2004). SPEs are ejections of low- linear energy transfer (LET) protons, heavier nuclei, and electrons by the Sun during energetic solar events (Cucinotta and Durante, 2006; Cucinotta and Schimmerling, 2004; Dartnell, 2011). The GCR originates from sources outside the Solar System and consists of high-LET protons (85%), alpha-particles (14%), and high atomic number, high energy (HZE) nuclei (1%) (Dartnell, 2011; Ferrari and Szuszkiewicz, 2009; Kronenberg and Cucinotta, 2012). Low-LET SPEs are successfully shielded by current spacecraft materials and exposure is a concern mostly during extra-vehicular activities. Possible health risks following an acute dose of SPE protons involve radiation sickness, vomiting, nausea, fatigue, and blurred vision, which are generally short-term health effects (Kahn et al., 2014; Kronenberg and Cucinotta, 2012). While health risks from low-LET SPEs may be at least partially mitigated by shielding, monitoring, and alert systems, there are currently no dependable ways to protect astronauts from GCR (Moore et al., 2014). Risks from exposure to GCR involve long-term effects like cardiac and neural damage, degenerative tissue effects, cataractogenesis, bone density loss, and carcinogenesis (Blakely et al., 2010; Kronenberg and Cucinotta, 2012; Rola et al., 2008; Soucy et al., 2011; Vlkolinsky et al., 2007; Yu et al., 2011).

Carcinogenic Consequences. A connection between HZE particle exposure and carcinogenesis is indicated, as noted from various studies *in vitro* and *in vivo*. HZE particles are directly ionizing resulting in clustered, multiple damaged sites (including complex DSBs) in the genetic material of human cells as they transverse the cell (Asaithamby et al., 2011; Desai et al., 2005). The complexity of this damage and the inability of the cell to fully *and correctly* repair its DNA increases directly with atomic number and LET, due to the confined nature of energy deposition and proximity of DNA

lesions (Desai et al., 2005; Little, 2000). Currently, although the same types of DNA lesions are thought to result from low-LET IR, the sparse nature of damage caused by this exposure may allow for more efficient and accurate DNA repair, and lower frequency of long-term radiobiological effects (RBEs). In agreement with the higher complexity and slower and incomplete repair of damage, HZE particles are more mutagenic, causing much more complex DNA damage and ultimately chromosome translocations (Camacho et al., 2010; Cucinotta and Durante, 2006; Durante and Cucinotta, 2008; Loucas and Cornforth, 2013; Mukherjee et al., 2008). Additionally, HZE particle radiation has been shown to be significantly more efficient in cellular transformation. *Here, we will explore a potentially new source of complex DNA damage created by IR (and more efficiently after high LET IR exposure): the formation of R-loops created in response to IR exposure, and consequent complex DSBs and chromosomal rearrangements (Asaithamby et al., 2011; Desai et al., 2005; Durante et al., 2004; Durante et al., 2005; George et al., 2003). Such DNA lesions are not created as a result of the initial energy deposition, but as a downstream consequence of attempts to repair or resolve R-loops. The complex DNA lesions that result represent an under-explored source of carcinogenesis. Such R-loop-induced DSBs are a known source of cytogenetic rearrangements (Morales et al., 2014), resulting in elevated neoplastic transformation in vivo in IR-exposed heterozygote mk-h$^{+/-}$ knockout (KO) mice (below).*

Carcinogenesis of HZE particles in vivo. Animal studies, mostly in rodents, demonstrated that the differential RBEs of low- *vs* high-LET IR described above result in elevated tumorigenic potential, with HZE nuclei having substantially greater carcinogenic effects (Cucinotta and Durante, 2006). Elevated cancer incidence following exposure to HZE compared to reference electromagnetic radiation has been demonstrated for solid tumors in mice or rats including skin tumors, lung cancers, Harderian and mammary gland tumors, hepatocellular carcinoma, and malignant glioma (Camacho et al., 2014; Cucinotta et al., 2004; Kato et al., 2009; Tao et al., 1993; Weil et al., 2009). Equal doses of reference low LET IR failed to induce tumors with comparable frequency. Estimates of carcinogenic risk from HZE particle exposure using animal models are, therefore, crucial for future mission planning.

DNA repair as a preventive factor in HZE particle-induced carcinogenesis: A role for Artemis. The Artemis protein is a single-strand specific 5'-3' exonuclease and appears to acquire endonuclease activity in response to phosphorylation by DNA-PKcs. Artemis is necessary for V(D)J recombination and mutations in the gene encoding this protein can cause severe combined immunodeficiency (SCID) (Ma et al., 2002; Moshous et al., 2001; Moshous et al., 2000). Artemis also plays a role in repairing complex DSBs through a modified non-homologous end joining (NHEJ) pathway. Exposure to IR, particularly to HZE particles, causes complex DSBs, and cells lacking Artemis show hypersensitivity to radiation and persistent cell-cycle arrest (Ma et al., 2002; Wang et al., 2005). In fact, recent data suggest that repair by DNA-dependent protein kinase (DNA-PK), consisting of the Ku heterodimer and DNA-PK catalytic subunit (DNA-PKcs), is particularly reduced by high-LET IR-induced clustered DSBs, making this pathway of NHEJ less relevant for recovery. In contrast, NHEJ-stimulated DNA processing by the Artemis endonuclease is particularly more important to the repair of these high-LET IR-induced DSBs (Ni et al., 2011; Sridharan et al., 2012). Indeed, Artemis overexpression conferred radio-resistance against high, as well as low, LET IR exposures as measured by survival (Nikjoo and Girard, 2012; Sridharan et al., 2012; Taleei et al., 2012). Few

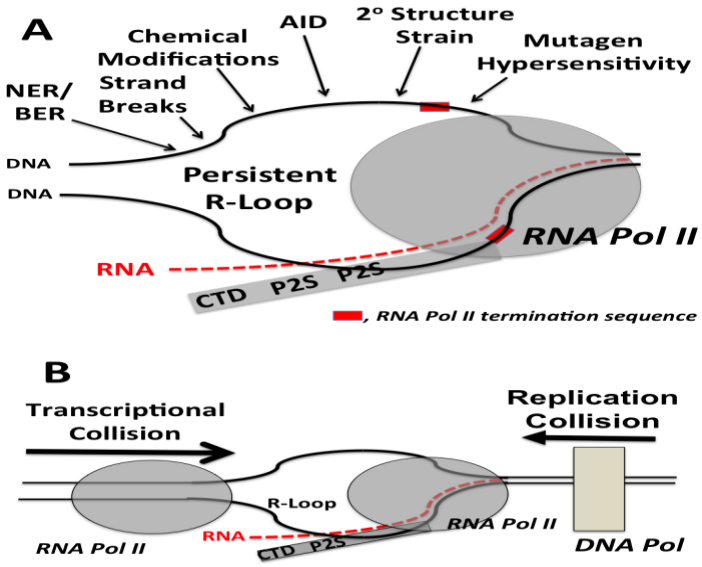
experiments using Artemis KO- or haplo-deficient mice have been performed to date (Sridharan et al., 2012), possibly because Artemis genetic defects are extremely rare. However, since functional K-H is required for maintaining stable steady state Artemis protein levels (Morales et al., 2014), K-H is most likely required for the repair of complex DSBs created directly by high LET IR exposures, as well as for the more delayed R-loop-induced DNA lesions created as a consequence of the initial damage. Thus, while specific genetic loss of Artemis is rare, loss of one functional allele of K-H due to SNPs in the human population appear to be much more common. For example, our analyses indicate that on average each K-H SNP is found in one per thousand persons, raising the possibility that K-H loss in haplo-insufficient individuals could be an important carcinogenic risk factor in individuals due to simultaneous persistent R-loop formation and lost expression of Artemis. We speculate that functional loss of one K-H allele results in (i) increased basal and damage-induced persistent R-loop formation; (ii) subsequent loss of Artemis-dependent DSB repair; and (iii) a dramatic risk of cancer after exposure to high and/or low LET IR treatments.

R-Loops, complex DSBs and genetic instability. Normal cells contain repair systems to nullify mutations and avoid genomic instability that may lead to chromosomal rearrangements (Ciccia and Elledge, 2010). Some acquired chromosomal alteration may ultimately contribute to tumor formation (Zhang et al., 2010). The most dangerous DNA lesion that a cell can encounter is a DSB, where one unrepaired DSB can cause lethality (Zhou and Elledge, 2000). On the other hand, mis-repaired DSBs are a prominent source of chromosomal rearrangements, such as translocations within the genome (Chiarle et al., 2011). Thus, DSBs are a constant threat to genomic stability, as they can naturally arise during normal metabolic, replication, and developmental processes (Iyama and Wilson III, 2013). However, as discussed above, low LET IR-induced DSBs are qualitatively different from those created after HZE particle radiation exposures, where complex DSBs with multiple damaged sites are lethal or highly mutagenic when repair is attempted. In cells that do repair, the risk for carcinogenesis is high in cells that ultimately survive.

R-Loops. The formation of persistent DNA:RNA:DNA hybrids, (R-loops) (Aguilera and García-Muse, 2012), are a potent source of genomic instability, yet mechanisms for their resolution and/or repair are not fully understood. Transient R-loop formation is required during all transcription-related processes, such as class switch recombination (Zarrin et al., 2004) and transcription termination by RNA Polymerase II (RNAPII) (Ginno et al., 2013; Skourti-Stathaki et al., 2011; Wahba et al., 2011). In contrast, unresolved persistent R-loops (that greatly expand over time due to the inability to dislodge RNAPII) can lead to complex DSBs and genetic instability (Aguilera and García-Muse, 2012; Li and Manley, 2005). These lesions ultimately, by poorly understood mechanisms, lead to the creation of complex DSBs. Several mechanisms for R-loop-mediated DSB formation have been proposed (Gomez-Gonzalez et al., 2011; Kim and Jinks-Robertson, 2012), mostly centered on R-loops acting as a physical barrier for replication/transcription machinery. Although R-loops can form throughout the genome wherever RNAPII operates, we will focus specifically on R-loops created due to defects at RNA transcription termination sites. We will show evidence that R-loops can increase in response to IR exposures. *Note that these assessments of R-loop formation represent a likely underestimation of their frequency and repair throughout the genome, as we are only assessing lesions at RNA termination sequences.* An example of R-loops formed at termination sequences near poly(A) mRNAs is illustrated in Figure

1A. When cells are deficient in specific termination factors (see below) required to associate with the C-terminal domain (CTD) of RNAPII at repetitive phospho-serine 2 (pS2) residues to terminate and dislodge RNAPII, a persistent R-loop forms. The absence of RNAPII termination allows its progression, generating a long stretch of DNA:RNA:DNA sequence that represents a blockage to subsequent RNA transcription and/or DNA replication processes (Figure 1B). While it is clear that complex DSBs are formed in a delayed manner from persistent R-loop formation, which can be resolved by Artemis (Morales et al., 2014), the exact mechanisms by which these DSBs form from these structures is not clear. Chemical modifications, mutagen hypersensitivity, secondary structural strain, modification by enzymes (e.g., Adenosine Deaminase or nucleotide excision repair (NER)) and DNA strand breaks due to various endonuclease activities of the free DNA strand have been proposed (Figure 1A).

Figure 1. **Persistent R-loop formation and consequential collision with DNA and RNA synthetic processes result in complex DSBs**. (**A**) Specific DNA lesions and/or loss of specific RNA processing factors, such as RNA transcription termination factors, can result in the formation of persistent R-loops. RNA polymerase II (RNA Pol II or RNAPII, text) proceeds along its DNA coding template, releasing the mRNA for poly(A) processing. At the RNA transcriptional termination sequence, RNAPII stalls awaiting a specific sequence of RNA transcription termination factors. K-H and p15RS are essential. If K-H, p15RS or a host of other factors (see **Figure 2**) are not present, RNA Pol II (RNAPII) fails to stall and a persistent DNA:RNA:DNA structure (an R-loop) is formed. This structure is hypersensitive to secondary damage or mis-repair from nucleotide excision repair (NER) or base excision repair (BER). (**B**) Persistent R-loops represent a barrier to RNA or DNA synthetic forks, creating collisions that create even more complex DSBs, which can be repaired by Artemis (Morales et al., 2014).



Factors required for RNAPII displacement and termination. Transcription termination by RNAPII is a complex process requiring multiple protein factors (Richard and Manley, 2009). As RNAPII reaches the 3'-end of a gene, it releases a poly(A)-containing pre-RNA, but continues to transcribe the coding strand until it reaches a natural pause site. It is at these pause sites that RNAPII stalls, allowing time for active recruitment of a series of factors (Figure 2A) that: (*i*) bind the enzyme's CTD domain and stall RNAPII; (*ii*) allows XRN2-mediated digestion of the remaining RNA, destroying the residual RNA:DNA (R-loop) hybrid; and (*iii*) displacing RNAPII and ultimately resolving a transient R-loop and eliminating any chance for DSBs due to persistent R-loop formation. A main protein factor that mediates transcription termination is K-H, which acts as a scaffold to recruit PSF, p54(nrb) and XRN2 within the RNA termination complex (Morales et al., 2014).

Transcription termination defects, R-loops, and cancer. Importantly, transcription termination factors, such as Senataxin (SETX), p54(nrb), PSF, and XRN2 have been linked with several different disease states, where missense, single nucleotide

polymorphisms (SNPs) and copy number alterations (CNAs) were noted. Missense mutations in a putative DNA:RNA helicase, SETX, is highly associated with neurological pathologies, such as amyotrophic lateral sclerosis 4 and Ataxia-Oculomotor Apraxia 2 (Chen et al., 2004; Chen et al., 2006). Polymorphisms and gene expression alterations in *XRN2*, a 5'-3' exoribonuclease, PSF, and p54(nrb) are associated with cases of solid tumors such as spontaneous lung cancer in non-smokers (Lu et al., 2009) (XRN2), colon and prostate cancer (PSF, together with p54(nrb), functions in recruitment of XRN2 (Kaneko et al., 2007)) (Takayama et al., 2013; Tsukahara et al., 2013)., and the development and progression of malignant melanoma (Schiffner et al., 2011) (p54(nrb)). Our research has focused on K-H (Rtt103 in yeast, RPRD1B in humans) that is required for accurate recruitment of the PSF-p54(nrb)-XRN2 complex for transcription termination.

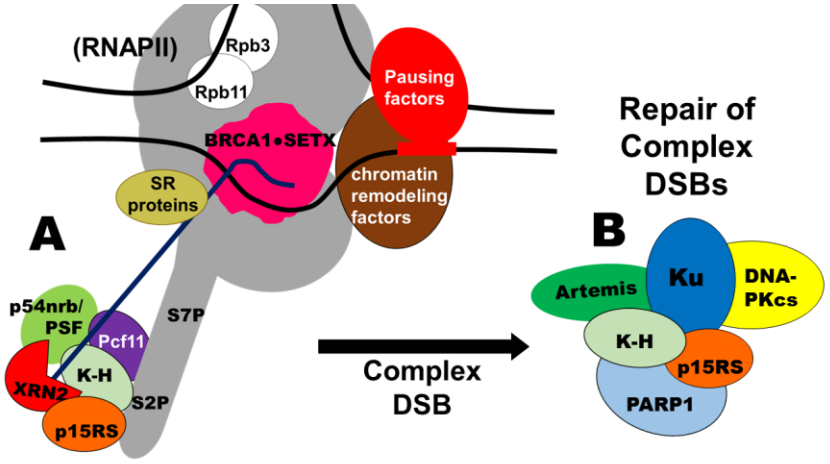


Figure 2. **Kub5-Hera (K-H) is an essential scaffolding protein required for RNA transcription termination, RNA transcription and DSB repair complex formation.** (**A**) K-H is an essential protein for the functional assembly of RNA termination factors to dislodge RNA Pol II (RNAPII) and mediate XRN2 degradation of the RNA component of an R-loop, thereby resolving persistent R-loops (Adapted from Richard, Genes and Dev., 2009). (**B**) K-H forms a complex with specific non-homologous end joining recombination factors and is required for stabilization of Artemis to mediate repair of complex DSBs (Morales, NAR, 2014).

Interestingly, loss of K-H, PSF, p54(nrb), and SETX in cells were shown to impair DSB repair, linking transcription termination to the DNA Damage response (DDR). These proteins form complexes involved in transcription termination and/or DNA repair. PSF and p54(nrb) play functional roles in both NHEJ and homologous recombination (HR) pathways of DSB repair (Bladen et al., 2005; Morozumi et al., 2009). Loss of either PSF or p54(nrb), and K-H abrogates DNA repair and leads to increased chromosomal aberrations (Salton et al., 2010). The role of SETX in the DDR, in particular, centers on the resolution of transcriptional R-loops after DNA damage (Becherel et al., 2013; Wang et al., 2013). It must be noted that losses of SETX or K-H, result in persistent R-loop formation, as well as defective DSB repair complexes. When exposed to IR, a very complicated DDR results in normal or tumor epithelial or fibroblast cells deficient in K-H *(Morales et al., 2014).* Thus, we hypothesize that loss of K-H is highly mutagenic, causing chromosomal aberrations and carcinogenesis in mouse or human cells. Since numerous SNPs in K-H/RPRD1B and p15RS/RPRD1A, a close binding partner of K-H, have been identified (summarized below) in the human population, we posit that changes in K-H may be important carcinogenic predictive risk factors in individuals exposed to high or low LET IR.

Kub5-Hera (K-H) discovery and functions

K-H interacts with NHEJ factors. K-H was isolated in a screen to identify Ku70 binding proteins, presumably involved in NHEJ (Yang et al., 1999). The interaction of K-H protein with Ku70 was comparable to the association of SV40 large T to p53 *(Morales et*

al., 2014). Deletion analyses suggested that a coiled-coil domain in K-H is crucial for interaction with, perhaps, the coiled-coil domain within Ku70, also known to associate with nuclear clusterin (Leskov et al., 2003). The K-H protein has two highly conserved functional domains: an amino-terminal CTD interacting domain (CID), that mediates interactions with the C-terminal domain of RNAPII (Ni et al., 2004)(Kim et al., 2004) and a carboxy-terminal coiled-coil domain that is required for Ku70 interaction. A specific point mutation, L276A, within the coiled-coil domain in K-H abolished its binding to Ku70. Human (hK-H) and mouse (mK-H) K-H share significant regions of homology with yeast Rtt103 as noted by Clustal Omega analyses. In fact, a single amino acid change separates human and mouse K-H *(Morales et al., 2014).*

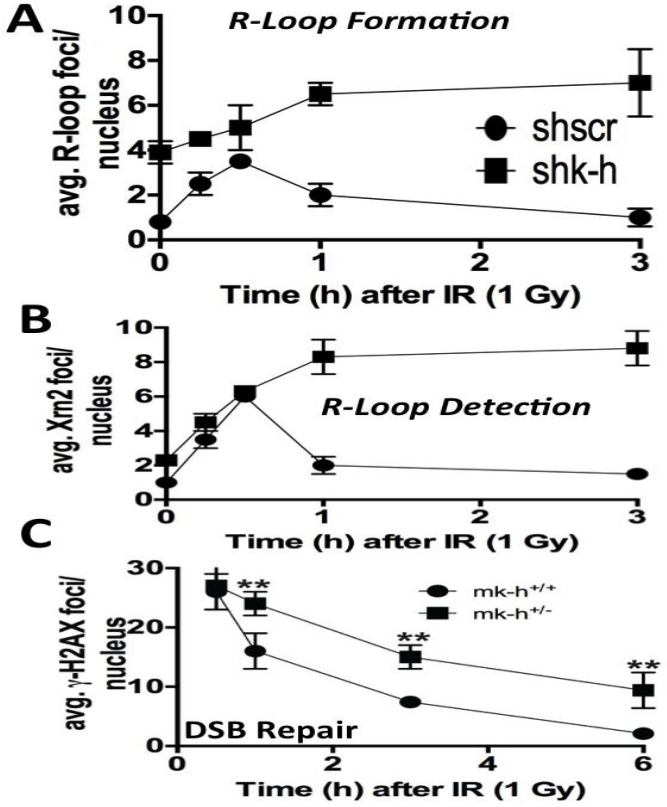
Investigation of complexes associated with K-H using gel filtration chromatography revealed two higher-order protein associations containing K-H: one complex associated with RNAPII and another separate complex closely associated with p15RS (Morales et al., 2014) – an association between K-H and p15RS was previously reported (Ni et al., 2011). Additionally, K-H co-eluted with numerous NHEJ factors, including Ku70, Ku86 and Artemis, consistent with the yeast two-hybrid data suggesting an association of K-H with Ku70 (Figure 2B). Importantly, co-Immuno-Precipitation (Co-IP) analyses using a K-H-specific antibody generated in our lab that recognizes K-H, but not the closely related p15RS protein, revealed that K-H associated with Ku70 and Artemis. This association was not dependent on DNA interaction, since it was observed in the presence or absence of ethidium bromide (Morales et al., 2014). Moreover, we showed that K-H, Ku70, Ku86 and Artemis are present in higher molecular weight protein complexes separate from RNAPII (Morales et al., 2014).

K-H loss elevates basal DSBs and increases genomic instability. Evidence supporting the role of K-H in DNA repair was demonstrated using a variety of cellular models, including stable sh*k-h*-mediated knockdown in human foreskin fibroblasts (sh*k-h* fibroblasts), triple-negative MDA-MB-231 (sh*k-h* 231) breast cancer cells, and mouse embryonic fibroblasts (MEFs) derived from heterozygote (het) mice (*mk-h*$^{+/-}$) versus wild-type cells. The *mk-h*$^{+/-}$ MEFs were used in these studies since complete loss of K-H led to early embryonic lethality in mice, and *mk-h*$^{+/-}$ cells were haplo-insufficient for a range of phenotypes examined. In all three cellular systems, loss of K-H led to increased basal levels of γ-H2AX and 53BP1 foci formed without genomic insult (Morales et al., 2014). Furthermore, K-H-deficient cells displayed slow clearance of DDR indicators following exposure to IR (Morales et al., 2014). The defect in DDR foci regression was confirmed by a delay in neutral comet tail regression after IR treatment in sh*k-h* 231 cells (stable short hairpin RNA-mediated knockdown of k-h in MDA-MB-231 cells) compared to sh*Scr* 231 cells (short hairpin RNA control containing scrambled/non-targeting sequence) or sh*k-h* 231 cells reconstituted with RNAi-resistance K-H cDNA (Morales et al., 2014). As neutral comet analyses examine DSBs specifically, we concluded that loss of K-H impairs the cell's ability to repair these types of lesions. We also noted a significant increase in chromosomal aberrations, by metaphase spread, before and after exposure to IR in sh*k-h* knockdown fibroblasts *vs* sh*Scr* control cells. Elevated levels of chromatid aberrations were also observed in irradiated cells depleted for K-H. In contrast, the extent of other genomic aberrations, such as di-centric, tri- and tetra-radial chromosomes, were not statistically different between K-H depleted and control cells. Importantly, re-expression of K-H in all three cell systems corrected all of the defects noted. Collectively, these data indicated that K-H plays a role in facilitating specific DSB repair processes. The absence of radial

chromosomes in K-H-depleted cells, commonly seen in cells lacking HR-mediated DSB repair (Cheung et al., 2002; Tomimatsu et al., 2012), suggested that the primary DSB repair defect in K-H-deficient cells was NHEJ. However, potential defects in HR are still being delineated as these assays are not entirely definitive to rule out HR.

K-H loss sensitizes cells to DSB-inducing agents. Cells deficient in selective DSB repair pathways typically show unique hypersensitivities to specific cytotoxic agents in long-term colony forming (survival) assays (Chalasani and Livingston, 2013; Mladenov et al., 2013); e.g., NHEJ-deficient Ku70 KO cells are hypersensitive to agents that create DSBs (Mahaney et al., 2009). Cells depleted for K-H were hypersensitive to IR treatments *vs* wild-type (*mk-h*$^{+/+}$) MEF cells (Morales et al., 2014). sh*k-h* MDA-MB-231 (231) breast cancer cells were also hypersensitive to other DSB-inducing agents tested, including cisplatin, H_2O_2, etoposide, doxorubicin, and topotecan *vs* genetically matched sh*Scr* 231 cells (Morales et al., 2014). In contrast, K-H knockdown cells were not sensitive to ultraviolet (UV) light. To control for shRNA off-target effects, sh*k-h* 231 cells were reconstituted with human *k-h* cDNA, which restored resistance to IR, cisplatin, and H_2O_2 at levels comparable to sh*Scr* 231 cells.

Figure 3. **Low LET IR treatment results in persistent R-Loops, R-loop/DSB hybrids and DSBs that are repaired in a defective manner in cells depleted of K-H expression**. (**A-C**) MDA-MB-231 breast cancer cells were stably knocked down for K-H expression (sh*k-h*) or transfected with nonsense, scrambled sequence (shScr) and exposed to various doses of low LET IR. Cells were then analyzed for R-loops (**A**), R-loop/DSB hybrids (**B**), or DSBs (**C**) using the S9.6 R-loop-specific antibody, XRN2 antibody that detects R-loops being processed, or anti-γH2AX DSB foci.



Artemis over-expression rescued K-H-deficient cells. Artemis loss impairs DNA repair (specifically in the very late phase of DSB repair illustrated by foci regression analyses), resulting in genomic instability and sensitivity to IR (Evans et al., 2006). Since K-H loss led to a concomitant loss of Artemis expression and function, our lab examined the effects of Artemis re-expression on DNA repair capacity in sh*k-h* knockdown normal and cancer cells. Artemis restored DSB repair and hypersensitivities to DNA damaging agents, while K-H protein levels remained repressed. Importantly, while cells lacking K-H expression showed only a slight decrease in the ability to repair compatible ends, there was pronounced deficiency in the ability of these cells to repair incompatible DNA ends, which is similar to results found in *Rtt103Δ* yeast (Morales et al., 2014). Moreover, cells defective in K-H expression were far more hypersensitive to DNA damaging agents and demonstrated more dramatic DSB repair defects than cells deficient in Artemis alone.

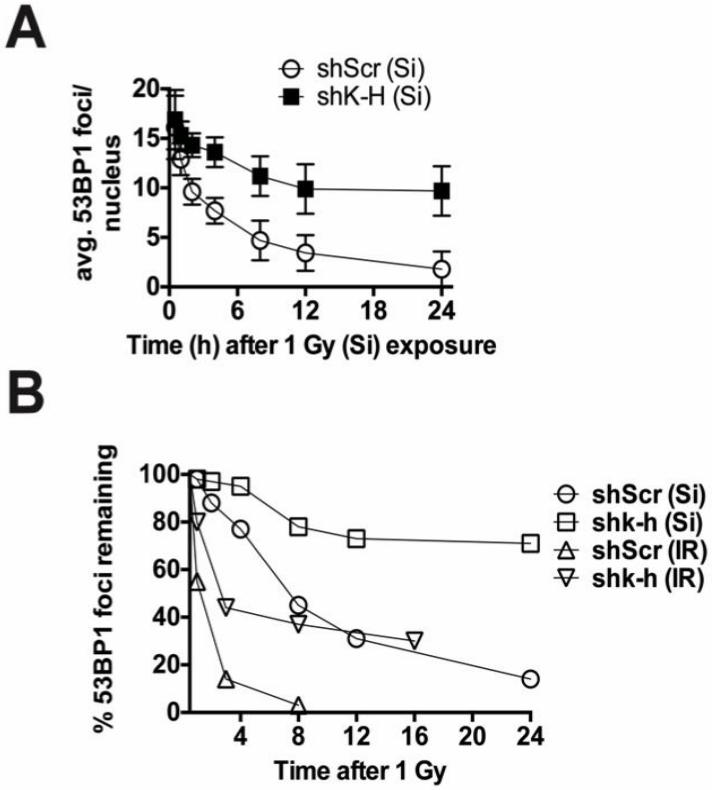
K-H loss leads to persistent R-loop formation. Given prior observations linking K-H to RNAPII transcriptional regulation (Lu et al., 2012; Ni et al., 2011), our lab examined whether persistent R-loops, in combination with loss of Artemis expression, may explain the qualitative and quantitative differences between the DNA repair capacities of haplo-insufficient m*k-h*$^{+/-}$ or K-H knockdown cells directly compared to Artemis (*Art*$^{-/-}$) KO cells. Indeed, a considerable increase (~4-fold) in the number of basal R-loop foci/nucleus was detected in sh*k-h vs* sh*Scr* cells, whereas only minor increases in m*Art*$^{-/-}$ cells were noticed (Morales et al., 2014). Artemis overexpression in mK-H+/- or human K-H-depleted cells had no effect on basal or IR-induced R-loop levels. In contrast, R-loops were completely resolved after forced GFP-RNase H expression (Morales et al., 2014). Overexpression of GFP-RNase H, which alleviates R-loops (Sordet et al., 2009; Wang et al., 2013), significantly decreased basal 53BP1 foci/nucleus in sh*k-h* knockdown fibroblasts to levels detected in sh*Scr* cells, ± RNase H over-expression (Morales et al., 2014); similar effects of RNase H over-expression on R-loop formation and foci representing DSBs were reported (Shanbhag et al., 2010; Wang et al., 2013). GFP overexpression alone did not spare defects noted in K-H depleted defects. We then over-expressed GFP or GFP-RNase H in Scr or K-H knockdown cells and treated these cells with IR (1 Gy) and monitored γ-H2AX foci regression over time. Collectively, our data strongly suggested that R-loops directly affected the rate of 53BP1 and γ-H2AX foci regression in early phases of DSB repair in K-H-deficient cells. Thus, R-loop formation played a significant (but confounding) contribution to DNA repair kinetics after IR, which is likely to be further amplified in response to high LET IR exposures. Artemis was able to significantly reduce both basal and IR-induced DSBs in K-H-depleted or haplo-insufficient cells, whereas GFP-RNase reduced both. These data strongly suggest that R-loop formation, which increased after IR treatment and whose levels were further enhanced in cells deficient in K-H expression, are a major source of complex DSBs after IR treatment and that Artemis is specifically required for their repair. *It is most likely not a coincidence that Artemis-mediated NHEJ is a major repair systems of both high and low LET IR, as well as R-loop-generated complex DSBs, and that K-H is a major non-genetic source of Artemis deficiency in the normal human population due to numerous infrequent SNPs in either K-H or p15RS. Further research on these SNPs and the roles of K-H and p15RS in complex DSB repair are warranted.*

RNA termination factors and DSB repair. Recent data highlight the fact that several proteins involved in the process of RNA transcription termination are also involved in DNA repair. Senataxin (SETX), is an RNA:DNA helicase that promotes transcription termination by unwinding the RNA:DNA hybrid, allowing for proper degradation of the RNA moiety by the 5'-3-exonuclease, XRN2 (Skourti-Stathaki et al., 2011). Interestingly, SETX also interacts with several DNA repair factors, such as the tumor suppressor *Brca1* and catalytic subunit of the DNA-dependent protein kinase (DNA-PKcs), and plays a critical role in preventing R-loop mediating DNA damage (Hill et al., 2014; Richard et al., 2013; Yüce and West, 2013). PSF, along with its binding partners, p54nrb and K-H, promotes transcription termination by mediating XRN2 localization (Kaneko et al., 2007). PSF and p54nrb have also been implicated in the DNA damage response. Loss of PSF, K-H or p54nrb led to increased amounts of genomic instability and hypersensitivity to low LET (γ-ray) exposure (Ha et al., 2011; Li et al., 2009). Kub5-Hera (K-H) promotes transcription termination by mediating the distribution of XNR2 along the genome (Morales et al., 2014).

High LET IR and persistent R-loops. In 2010, Chen and colleagues demonstrated accumulation of OGG1 along the high LET particle tracks, suggesting the formation of DNA base damage as part of the multiple damaged sites after high LET IR exposure (Asaithamby and Chen, 2011). In 2010, Greenberg and colleagues found that ATM mediates transcriptional pausing after DSB formation (Shanbhag et al., 2010). It was later found that DNA-PKcs also contributed to RNAPII pausing after DSB formation (Pankotai et al., 2012). The formation of DNA base damage, along with transcription pausing after DSB formation led us to question whether high LET IR exposure caused the formation of R-Loops, since these structures are quite detrimental to genomic stability (Aguilera and García-Muse, 2012; Helmrich et al., 2013).

Low LET IR appears to have little affect on global transcriptional rates (Shanbhag et al., 2010). Cells exposed to this damage halted transcription in areas of the genome adjacent to DSBs (Pankotai et al., 2012). To examine the effects of low compared to high LET IR on R-loops in wild-type or K-H-deficient cells, we compared cells exposed to γ-rays *vs* silicon ions (^{28}Si, 1 Gy Si; 237.8 MeV/n energy; 4.825 cm HDPE; 79.3 keV/μm). Wild-type K-H expressing human fibroblasts exposed to γ-rays demonstrated significant levels of persistent R-loops (detected by S9.6 antibody foci formation, (Figure 3A), XRN2 foci formation (Figure 3B) and DSBs that were repaired quickly and in comparable time-frames. In contrast, genetically matched K-H-deficient fibroblasts exposed to high LET IR induced higher levels of R-loops and DSBs that were not repaired over time (Figures 3A-C). Indeed, R-loop formation increased over time in K-H-depleted cells, plateauing at 6-8 lesions per nuclei (Figures 3A, 3B). When low and high LET IR exposures were compared, we noted that DSB repair rates in high LET-treated K-H-depleted cells were significantly delayed compared to those found in cells exposed to low LET IR (Figures 4A, 4B). Thus, DSB repair capacity was significantly compromised in cells depleted in K-H expression, and high LET exposures caused significantly more DSBs in these cells. More importantly, the repair capacity of DSBs created in shK-H-

Figure 4. **Persistent R-loops are created by low and high LET IR exposures and K-H loss leads to the accumulation and lack of repair of R-loops and corresponding complex DSBs**. (**A**) Stable sh*Scr* or sh*k-h* knockdown cells were exposed to 1 Gy Silicon (Si) ionizing radiation and monitored for DSB formation and repair using 53BP1 foci/nuclei. (**B**) Stable sh*k-h* or sh*Scr* cells were irradiated with 1 Gy low LET gamma rays or 1 Gy Si ions high LET IR and assayed for DSB formation and repair as described in (**A**). Note the inability of sh*k-h* cells to repair DSBs created by Si high LET or γ-ray low LET IR exposures compared to sh*Scr* control cells. DSBs are created by energy deposition as well as by formation of persistent R-loops as outlined in **Figure 3**.



depleted cells after high LET exposures was significantly compromised compared to repair rates in wild-type fibroblasts. Intriguingly, treatment of shK-H-depleted or mouse K-H$^{+/-}$ cells with GFP-RNase H to decrease R-loops increased the DNA repair rates of these cells, aside from the Artemis-mediated slow phase of DSB repair (Morales et al., 2014). Thus, R-loops play a significant role in DSB formation in a delayed manner and have been overlooked in prior studies examining delayed DNA lesions created after high or low LET IR exposures. Indeed, the overall concept delayed (due to mis-repair, repair, transcriptional or replication-dependent processes) represents a major under-explored area of research in radiobiology. Persistent R-loop formation is particularly important because of the complex and potentially carcinogenic DSBs generated. We are currently investigating the formation of R-loops in cells exposed to high vs low LET exposures, as well as the roles of K-H, p15RS and XRN2 in the resolution of R-loops, downstream DSB repair, survival and carcinogenesis in these contexts.

Carcinogenesis in K-H$^{+/-}$ mice after IR exposures. At this point, little is known about the carcinogenic consequences of persistent R-loops. Recently, we developed a K-H knockout mouse and the phenotypes of this mouse will soon be reported. Briefly, we found that complete inactivation of the K-H gene in K-H$^{-/-}$ mice lead to early embryonic lethality (Morales et al., unpublished data). However, loss of a single K-H allele predisposed K-H$^{+/-}$ mice of both sexes to tumor formation after IR exposure in a dose-dependent (1-7 Gy) manner. The amount of IR exposure dictated the types of tumors formed in K-H$^{+/-}$ mice within ~300 days post-treatment, while wild-type K-H$^{+/+}$ animals failed to develop tumors within the same time-frame. At 1 Gy, 100% of mice formed cancers, but with a changed distribution, including squamous cell sarcomas of head and neck and thymomas in males, while females formed mammary, cervical and ovarian cancers after 1 Gy. In contrast, <1% of wild-type animals formed tumors after 7 Gy, and no cancers were noted in C57/B6;129 mixed background mice exposed to 3 or 1 Gy at ~400 days. Thus far, we have only tested the effects of low LET IR on haplo-insufficient K-H$^{+/-}$ animals. However, considering that loss of K-H was accompanied with dramatic increase in R-loop formation (Figures 3 and 4), DSB repair deficiencies, and concomitant loss of the DNA repair protein, Artemis, we would expect that exposure of these animals to high LET IR would constitute an even greater risk for carcinogenesis. Importantly, we also noted several non-cancer effects in the haplo-insufficient K-H$^{+/-}$ mice after γ-ray treatments, such as fur loss, cataract formation, blindness, and radiation-induced paralysis of the hind legs.

K-H mutational analyses in the human population and in human cancers. Since we demonstrated a link between persistent R-loop formation and dose-dependent low LET IR induced-tumor formation, we are beginning to examine whether loss of a single K-H allele is associated with incidences of human cancers. Alterations in K-H copy number, single nucleotide polymorphisms (SNPs) and methylation-related changes in K-H protein levels could matter, since one copy number loss of K-H in the K-H$^{+/-}$ mice correlated with 50% less protein levels in the at-risk tissues of mice, and this constituted carcinogenic risk. For therapy, loss of K-H correlates with hypersensitivity to agents that induce DSBs and are used for cancer therapies, including IR, cisplatin, and topoisomerase I/II poisons (Morales et al., 2014).

K-H array-CGH analyses. Examination of the cBioPortal website for cancer genomics revealed numerous somatic alterations in the K-H gene in several types of human tumors, where some (but not all) have recently been described (Cerami et al., 2012;

Gao et al., 2013). Interrogation of this database revealed loss of K-H copy number in many types of human tumors, particularly lung cancer. Other cancers with significant loss in K-H levels included breast cancers and lymphomas, cancers found in the K-H$^{+/-}$ mice. While it is not clear why whole-body irradiated K-H$^{+/-}$ mice failed to get lung cancer, the possibility is that they succumb to lymphomas and other cancers before lung cancer could develop. Preliminary analyses of K-H protein levels in carcinogenic-resistant tissues such as colon and brain of these mice showed levels that were not appreciably different from wild-type animals. In contrast, tissues sensitivity to IR-induced cancer demonstrated 50% or less wild-type levels. Determinants of K-H protein levels are under investigation in our lab. Interestingly, little evidence for the regulation of K-H gene expression was found at the methylation or general protein levels, suggesting that in cancers, K-H levels are closely regulated by copy number alterations. Finally, examination of K-H mutations in human tumors indicates numerous alterations in three essential domains within the protein. Since K-H plays essential scaffolding functions in DNA repair, RNA termination and RNA transcriptional promotion (Figure 2), alterations in these and any regions within the protein could alter secondary and tertiary structure, and therefore, essential functions affecting R-loop formation, complex DSB creation, DNA repair and carcinogenesis. The rather high penetration of K-H single allelic loss and high at-risk cancer rates in mice warrant consideration of the genetics of this gene, both for therapy, risk for resistance/hypersensitivity to therapy, as well as for carcinogenesis, especially with respect to IR treatment/exposure, with particular emphasis on heavy charged particle radiotherapy.

Human population K-H SNP analyses and risk assessment in the human population. Closer examination of the K-H gene within the normal human population using 1000G and GO-ESP MAF databases (http://www.ncbi.nlm.nih.gov/variation/view/) revealed a number of naturally-occurring single nucleotide polymorphisms (SNPs). Roughly 1,731 SNPs in the K-H gene (59 coding and 1672 noncoding), and over 2,857 SNPs (17 coding and 2840 noncoding) in the p15RS gene, a close K-H homolog, have been discovered; p15RS is a closely related binding partner of K-H that also plays functional roles in similar scaffolding structures with K-H in RNA transcriptional termination, DSB detection and repair, and R-loop resolution. Each K-H SNP ranged in frequency between 0.001% - 1.5% in the human population. Understanding the function(s) of these SNPs in K-H and/or p15RS genes, and their structure/function alterations, would seem to be warranted given the apparent functional significance of K-H loss in cancer risk in the above preclinical model. Added together, approximately over 6.3% of individuals within the human population may carry one or more SNPs in the coding region of the K-H gene alone, and a slightly lower percentage of individuals have mutations in K-H's binding partner and close homolog, the p15RS gene. Considering noncoding SNPs, which could affect mRNA stability, the combined minor allele frequency of SNPs for each gene is roughly about 30% in the human population. Since both K-H and p15RS play essential scaffolding functions in various cellular processes described above, the consequences of these SNPs to confer loss of function, or potential dominant-negative effects should be examined.

Significance to NASA's mission. We show evidence suggesting that low or high LET IR exposures can significantly increase persistent R-loops, and heretofore, ultimately create unrecognized, and unaccounted for, complex DSBs in a delayed manner following radiation exposure. The complex DSBs formed are delayed compared to initial energy-directed DNA lesions, and their formation and persistence (lack of repair) are

likely more significant after high LET radiation exposures than after low LET IR (Figures 3 & 4). More importantly, loss of expression of key RNA transcription termination factors (e.g., haplo-insufficient loss of K-H) appear to be essential for understanding the carcinogenic consequences of these lesions. Thus, studies to better understand the functional consequences of identified functional (or lack thereof) SNPs encoding specific RNA termination factors, as well as examining changes in gene expression of these factors (e.g., K-H), is important for delineating potential cancer vulnerabilities. More studies focused on the roles of these linked RNA termination-DNA repair complexes are warranted.

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