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Transcriptional Upregulation of DNA Damage Response Genes in Bank Voles (*Myodes glareolus*) Inhabiting the Chernobyl Exclusion Zone

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Exposure to ionizing radiation (IR) from radionuclides released into the environment can damage DNA. An expected response to exposure to environmental radionuclides, therefore, is initiation of DNA damage response (DDR) pathways. Increased DNA damage is a characteristic of many organisms exposed to radionuclides but expression of DDR genes of wildlife inhabiting an area contaminated by radionuclides is poorly understood. We quantified expression of five central DDR genes *Atm, Mre11, p53, Brca1,* and *p21* in the livers of the bank vole *Myodes glareolus* that inhabited areas within the Chernobyl Exclusion Zone (CEZ) that differed in levels of ambient radioactivity, and also from control areas outside the CEZ (i.e., sites with no detectable environmental radionuclides) in Ukraine. Expression of these DDR genes did not significantly differ between male and female bank voles, nor among sites within the CEZ. We found a near two-fold upregulation in the DDR initiators *Mre11* and *Atm* in animals collected from the CEZ compared with samples from control sites. As *Atm* is an important regulator of oxidative stress, our data suggest that antioxidant activity may be a key component of the defense against exposure to environmental radioactivity.

Keywords: chernobyl, ionizing radiation, DNA damage, DNA repair, oxidative stress, *Atm*, *Mre11*

INTRODUCTION

Accidental release of radionuclides into the environment presents a potential health risk to humans and wildlife (Møller and Mousseau, 2006; Lourenço et al., 2016). On 26 April 1986, reactor 4 of the Chernobyl Nuclear Power Plant (NPP) exploded, releasing an estimated 9×10^3 to 1×10^4 petabecquerels (Pbq) of radionuclides over much of Eastern Europe, Russia, and Fennoscandia (Dreicer et al., 1996). This accident, together with the accident at the Fukushima Daiichi NPP in 2011, stimulated public and scientific interest in the impacts of environmental radionuclides on natural ecosystems (Wheatley et al., 2016). To limit human exposure to radionuclides, the Chernobyl Exclusion Zone (CEZ) was established at ~30 km radius around the accident site. The CEZ still contains elevated levels of isotopes with long half-lives, notably strontium-90,

caesium-137, and plutonium-239 (about 29, 30, and 24,100 years, respectively). The wildlife inhabiting the CEZ provide the best-studied model of the biological impact of exposure to radionuclides (Møller and Mousseau, 2006).

One harmful effect of exposure to ionizing radiation (IR) from radionuclides is elevated DNA damage, either directly or by generating reactive oxygen species (ROS) through radiolysis of intracellular water (Ward, 1988; Einor et al., 2016). Elevated DNA damage, for example as chromosomal aberrations (Dzyubenko and Gudkov, 2009) or DNA breaks (Bonisoli-Alquati et al., 2010; Fujita et al., 2014), has been observed in wildlife and humans inhabiting areas affected by the accidents at Chernobyl and Fukushima (reviewed by Lourenço et al., 2016). That not all studies find elevated DNA damage in areas with increased radioactivity (e.g., Bonisoli-Alquati et al., 2015) points to interspecific differences in response to environmental radionuclides (Møller and Mousseau, 2015). Somewhat surprisingly, despite the many (>200) studies of DNA damage, few studies have quantified activity of DNA repair pathway genes on organisms exposed to environmental radionuclides (Lourenço et al., 2016).

In eukaryotes, DNA repair is activated and regulated by the DNA damage response (DDR) pathway, which is a multi-branched signaling cascade initiated by the serine/threonine kinases ATR and ATM upon detection of single-strand DNA breaks (SSBs) and double-strand DNA breaks (DSBs), respectively (Giglia-Mari et al., 2011). DSBs typically result from exposure to highly genotoxic agents and IR (Ward, 1990) and pose a serious problem for genomic integrity as they cannot always be repaired without incorporating mutations, thus increasing the risk of cancer (Cannan and Pederson, 2016). DSBs are detected by the MRE11-RAD50-NBS1 complex, which activates ATM. Pathways activated by ATM include p53-mediated cell cycle checkpoint and apoptosis, increased antioxidant production, and DNA repair, where the choice between non-homologous end joining (NHEJ) and homologous recombination (HR) repair is regulated by BRCA1 (Daley and Sung, 2014).

Just three studies, all on plants, have quantified DNA repair activity in wildlife affected by the Chernobyl and Fukushima accidents. After exposure to high doses (750 Gy) of IR, DNA repair activity in pollen and seed from two species of plant depended upon the composition of radionuclides in the soil in which the parental plants had grown (Boubriak et al., 2008). Progeny of *Arabidopsis* inhabiting areas around Chernobyl exhibited low recombination rates, despite X-ray induced upregulation of homologous recombination repair-related *Rad54-like* in samples from a contaminated area (Kovalchuk et al., 2014). Unfortunately these studies within the CEZ did not use replicate samples from contaminated and uncontaminated sites, making it challenging to determine whether radiation or a third variable accounts for these effects. In rice (*Oryza sativa*) seedlings, exposure to low-dose IR affected expression of stress response and DNA repair associated genes (Hayashi et al., 2014), with some single-strand break repair genes initially upregulated but suppressed after 24 h of IR exposure. Ultimately, plants have been found to be sensitive to environmental radiation, and it has been suggested that they could serve as bioindicator species for assessing radiation risk (Nikitaki et al., 2017). The DDR of wild vertebrates exposed to environmental radionuclides has rarely been studied, although, for example, upregulation in the *p53* gene has been observed in wood mice (*Apodemus sylvaticus*) inhabiting a former uranium mine area (Lourenço et al., 2013).

The subject of the physiological and ecological effects of low-dose IR is highly controversial, as evidenced by several conflicting studies concerning species diversity and abundance in the CEZ. Populations of large mammals, such as the wild boar (*Sus scrofa*), appear especially abundant (Deryabina et al., 2015), but it is difficult to quantify the effect of human absence on these populations. Here, we quantify mRNA transcription in five DDR and repair genes in livers of the bank vole *Myodes glareolus,* a small rodent that inhabits areas within and outside the CEZ. Multiple populations from both experimental and control regions were studied to ensure spatial replication of study sites that differ in contamination levels. The bank vole is abundant (typically 10–80 animals per hectare) in forest habitats in much of Europe and Asia (Hutterer et al., 2016) and was one of the first mammals to re-colonize the CEZ after the nuclear accident (Baker et al., 1996). Bank voles inhabiting the CEZ show an increased frequency of chromosomal aberrations (Goncharova and Ryabokon, 1995) and increased oxidative stress in the form of cataracts (Lehmann et al., 2016), although estimates of DNA damage have returned conflicting results (Cristaldi et al., 1991; Rodgers and Baker, 2000). We hypothesize that exposure to low-dose IR stimulates the expression of key genes in the DDR pathway.

MATERIALS AND METHODS

Sample Collection

Animals were caught using Ugglan Special live traps (Grahnab, Sweden), with sunflower seeds and potato as bait, during 6th−11th May 2015, at 14 locations within the CEZ that differed in levels of soil radionuclides (**Figure 1**). Bank voles were also caught during 16th−27th August 2015 at three separate regions outside the CEZ, where elevated levels of soil radionuclides have not been detected: Brody (50°0594 N, 25°10752 E), Lubny (50°05564 N, 32°98566 E), and Korostyshev (50°34422 N, 29°23673 E) in order to control for habitat effects. At each trapping location, 20 traps were placed in a line, with each trap separated by about 10 m and with trapping locations separated by at least 500 m. Procedures were performed in accordance with relevant guidelines and regulations, approved by the Finnish Animal Experiment Board and the Finnish Ministry of the Environment (under the authorization ESAVI/3834/04.10.03/2011 and ESAVI/7256/04.10.07/2014). Ambient radiation levels at the trapping locations were measured at 1 cm above the ground with a hand-held GM dosimeter (Inspector, International Medcom INC, Sebastopol, CA, USA) calibrated to measure Sieverts (Sv); such measurements of radiation are repeatable among days and even years (Møller and Mousseau, 2013). Mean ambient radiation levels varied among trapping locations from 0.1 to 12.3 μSv/h within

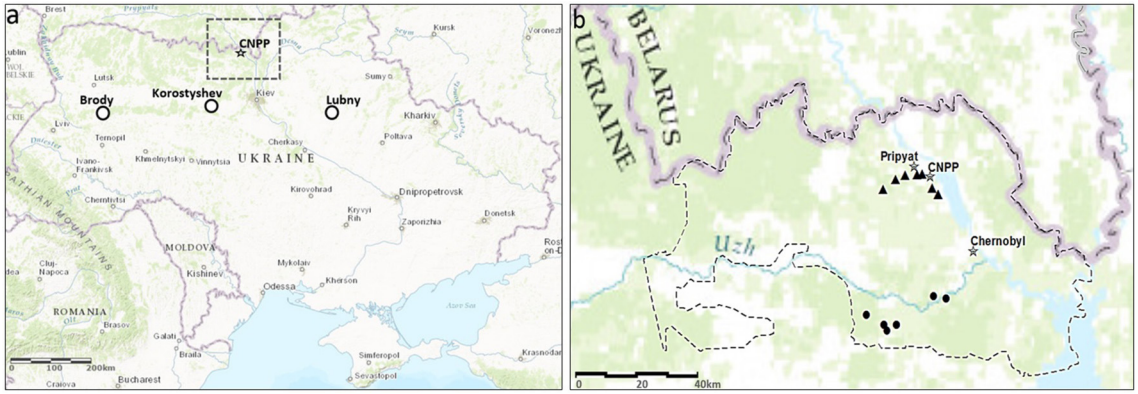

FIGURE 1 | (a) Location of control population trapping sites in Ukraine. **(b)** Bank vole trapping sites in the Chernobyl Exclusion Zone (CEZ). Background radiation intensity is shown as circles (<1.0 μGy/h) and as triangles (up to 12.3 μGy/h). Created using ESRI ArcGIS 10.5. Satellite imagery©.

the CEZ (**Figure 1**) and was 0.135 μSv/h at the three areas outside the CEZ. To estimate lifetime external doses, animals were allocated to two age groups based on head width, which is often used as a proxy of age in small mammals (Kallio et al., 2014): juveniles (<12.0 mm) and adults (≥12.1 mm). Accumulated external doses were calculated for 1 month (juveniles, ranging from 0.07 to 9.01 mGy) and for the range of 2–5 months (adults, ranging from 0.20 to 28.64 mGy; **Datasheet 1**).

After measuring head width (to 0.1 mm) and body mass (to 0.1 g), animals were euthanized by cervical dislocation and liver tissue samples transferred to Allprotect Tissue Reagent (Qiagen). We selected bank voles with 10–14 mm head width to reduce variation associated with maturation. Samples were stored at −80°C until processing.

TABLE 1 | qPCR primer information.

Gene	Primer sequence (5′ –> 3′)	Size (bp)	E
p53	CCA ACA CAA GCT CCT CTC CC	145	1.900
	ATT CGC GTC CTG AGC ATC C		
Brca1	AGT TCC AGC CAC AAC CTT CAG	156	1.900
	CCT CTT GAG ATG GGC AGT TCC		
Atm	GGA TGG CAT TGT GGT GAA GC	121	1.944
	AGG ACC TAT TTC TCC CAA ACA CC		
Mre11a	GGC ACA ACA TCT AGC AAA CGG	101	2.038
	TGG CTG CTC ATG AAA GGG TC		
Actb	TGC GTG ACA TCA AAG AGA AG	197	1.906
	GAT GCC ACA GGA TTC CAT A		
Rer1	GGC CGA TCC TGG TGA TGT AC	132	1.986
	CCA CGT CCT CCT TCC CTT TG		

Quantitative PCR (qPCR)

Intron-exon boundaries in five DSB damage response genes *Atm, Mre11, Brca1, p53,* and *p21* were identified using sequences from a draft bank vole genome (Genbank accession no. GCA_001305785). Bank vole gene sequences were aligned using online BLAST (Altschul et al., 1990) with default parameters against their putative mRNA homologs in the prairie vole (*Microtus ochrogaster*) available in Genbank: *p53* (XM_005349777), *Brca1* (XM_013354463), *Atm* (XM_013346263), *Mre11a* (XM_013355380), and *p21* (XM_005360336). For normalization of gene expression, we amplified two reference genes: beta-actin (*Actb*) and retention in endoplasmic reticulum sorting receptor 1 (*Rer1*). Beta-actin primers are based on a sequence from mouse (*Mus musculus*) and primers for *Rer1* were based on the putative gene sequence derived from bank vole genome. Primers were designed using Primer3 (Rozen and Skaletsky, 2000; **Table 1**). The mouse nucleotide database in Genbank was used to identify common transcripts and avoid potentially rare splice variants.

Up to 30 mg liver tissue per sample was homogenized using TissueLyser II (Qiagen) bead mill (2 × 2 min at 25 Hz), and total RNA was extracted with RNeasy Mini Kit (Qiagen) that incorporated a DNase digestion step according to the manufacturer's protocol. Four hundred nanograms of total RNA per sample was used for reverse transcription in 20 μl reaction volumes using iScript cDNA synthesis kit (Bio-Rad) according to the manufacturer's protocol.

Quantitative PCRs were completed for each individual sample in 16 μl final reaction volumes that contained 4 ng cDNA template, 400 nM both forward and reverse primers and 8 μl LightCycler 480 SYBR Green I Master (Roche). Thermal cycling profiles were: 95°C for 5 min followed by, 95°C for 10 s, 60°C for 15 s (for all primers), and 72°C for 10 s (with recording), using a LightCycler 480 Real-Time PCR System (Roche). Primer specificity was determined by melt curve analysis and PCR efficiencies were calculated from standard curves using five-fold serial dilutions of mixed-sample liver cDNA (**Table 1**). All qPCRs were run as three technical replicates and a sample was re-analyzed if the standard deviation among replicates was >0.4. *Actb* showed slightly greater variation in expression ($SD = 0.97$ cycles, $n = 25$) than *Rer1* ($SD = 0.65$ cycles, $n = 25$) across samples.

Data Analysis

Raw data were imported into GenEx v.6.1 to allow PCR efficiency correction ($Cq = CqE\frac{\log 1+E}{\log 2}$, where CqE is the uncorrected C_q value and E is the PCR efficiency). Each sample was normalized against the geometric mean expression of two internal reference genes *Actb* and *Rer1* ($Cq_{norm} = CqGOI - \frac{1}{n}\sum_{i=1}^{n} CqRGi$) to control for outlying values (Vandesompele et al., 2002). C_q values were converted to relative linear scale with average expression of the control groups set as the reference level, after which all data were converted to $\log_2$ scale (**Datasheet 2**). Subsequent statistical analyses were performed in SPSS v.24.0 (IBM Corp. 2016).

First, we examined differences in gene expression between three groups of samples: (1) animals within the CEZ caught from areas with elevated ambient radiation dose rates (>1.0 μGy/h, mean 4.8 μGy/h, conferring a yearly external radiation dose of between ∼10 and 110 mGy at the most irradiated location), (2) animals from within the CEZ where ambient radiation was not dramatically elevated (<1.0 μGy/h, mean 0.20 μGy/h) and (3) animals from outside the CEZ (mean 0.13 μGy/h). While the levels of soil radiation do not differ significantly between the latter two groups, bank voles inhabiting the CEZ may have moved among areas prior to capture. Variation in gene expression among the three groups and possible sex interaction was analyzed using two-way analysis of variance (ANOVA), followed by a Tukey's *post-hoc* test. Second, as no significant differences in gene expression could be attributed to an animal's sex or among samples within the CEZ (see *Results*), we made a comparison in gene expression between animals collected (1) from within the CEZ and (2) the three control areas, using a Student's *t*-test.

RESULTS

We quantified gene expression in five DDR genes *Atm, Mre11, p53, Brca1,* and *p21* in bank voles populating the CEZ. We chose these genes for their positions and signaling roles at the top of the DDR cascade to investigate possible activation of DDR response in a natural low-dose radiation environment. Our analyses were based on data from 57 animals: 30 (14 males, 16 females) from the CEZ, and 27 (14 m, 13 f) from Brody, Lubny, and, Korostyshev. Samples from the CEZ were further divided into two groups of "elevated" (7 m, 5 f) and "near-background" (7 m, 11 f) levels of environmental radioactivity as described above. Lifetime external doses within the "elevated" group were estimated to range between 1.14 and 9.01 mGy for juveniles and 2.82 to 28.64 mGy for adults, while in "near-background group" ranges were 0.07–0.34 mGy for juveniles and 0.13–1.68 mGy for adults. Gene expression levels were found to be similar between the three external control locations (**Datasheet 2**), and thus were considered as a single control group. Liver was selected as the tissue of interest as it is radiosensitive in a clinical context (Christiansen et al., 2007; Stryker, 2007); moreover, liver tissue appears sensitive to DNA damage, with liver tissue from bank voles exposed to environmental radionuclides having shorter telomeres compared with samples from control areas (Kesäniemi et al. unpublished).

We found significant differential expression in *Mre11* and *Atm*, which could be explained by radioactivity [2-way ANOVA, Atm $F_{(2, 51)} = 9.52$, $P < 0.001$; Mre11 $F_{(2, 51)} = 10{,}18$, $P < 0.001$; p53 $F_{(2, 51)} = 0.34$, $P > 0.05$; Brca1 $F_{(2, 51)} = 0.04$, $P > 0.05$; p21 $F_{(2, 51)} = 0.62$, $P > 0.05$]. No significant differences in the expression of any of the five genes could be explained by sex [*Atm* $F_{(2, 51)} = 0.19$, $P > 0.05$; *Mre11* $F_{(2, 51)} = 0.60$, $P > 0.05$; *p53* $F_{(2, 51)} = 0.07$, $P > 0.05$; *Brca1* $F_{(2, 51)} = 1.82$, $P > 0.05$; *p21* $F_{(2, 51)} = 0.03$, $P > 0.05$]. Significant upregulation (compared with samples from control areas) of *Atm* and *Mre11* was observed in both "elevated" ($P_{Atm} < 0.01$, $P_{Mre11} < 0.05$) and "near-background" ($P_{Atm} < 0.001$, $P_{Mre11} < 0.001$) groups of samples from the CEZ, despite the significantly lower amount of environmental radiation in the latter group of samples (**Figure 2**); upregulation of *Mre11* was in fact slightly stronger (but non-significantly so) in the "near-background" treatment.

Hence, relative quantification of five DDR genes among samples (males and females) from (1) the CEZ and (2) the control areas outside the CEZ revealed significant upregulation in *Atm* and *Mre11* in bank voles inhabiting the CEZ compared with bank voles taken from control areas: transcription of mRNA increased almost two-fold in *Atm* (1.93-fold increase, $t_{(55)} = 4.51$, $P < 0.001$) and *Mre11* (1.87-fold increase, $t_{(55)} = 4.56$, $P < 0.001$), whereas no significant differences in mRNA levels of *p53, Brca1,* or *p21* were detected (**Figure 2**).

DISCUSSION

Exposure to IR causes DNA damage, and elevated DNA damage is characteristic of wildlife inhabiting areas affected by nuclear accident sites. An appropriate response from DNA repair pathways is an expected response to exposure to environmental radioactivity. We identified a significant, almost two-fold, upregulation in expression of *Atm* and *Mre11* in the livers of bank voles living within the CEZ, which is the first evidence that expression of DNA repair-related genes is stimulated in vertebrates exposed to low-dose environmental radioactivity.

A notable feature of these data is the similar gene expression in animals caught from within the CEZ, irrespective of the level of soil radionuclides at the trapping location. The CEZ comprises a mosaic of radionuclide contamination (Chesser et al., 2004) where the variation in radionuclide levels in soil and vegetation introduce small scale (few hundred meters) heterogeneity in the overall received doses for bank voles (Chesser et al., 2000). One explanation for the common upregulation effect is that bank voles can readily disperse over 1 km during the breeding season (Kozakiewicz et al., 2007) and thus individuals trapped from the areas with little or no soil radionuclides within the CEZ could have been exposed to substantial levels of radionuclides prior to capture. Bank voles inhabit burrows and have an opportunistic diet (Butet and Delettre, 2011), and are thus exposed to a wide variety of ingested radionuclides including the common fission product caesium-137. Caesium-137 is particularly problematic for ecosystems due to its capacity to form various water-soluble salts. Bank voles can carry potentially high amounts of

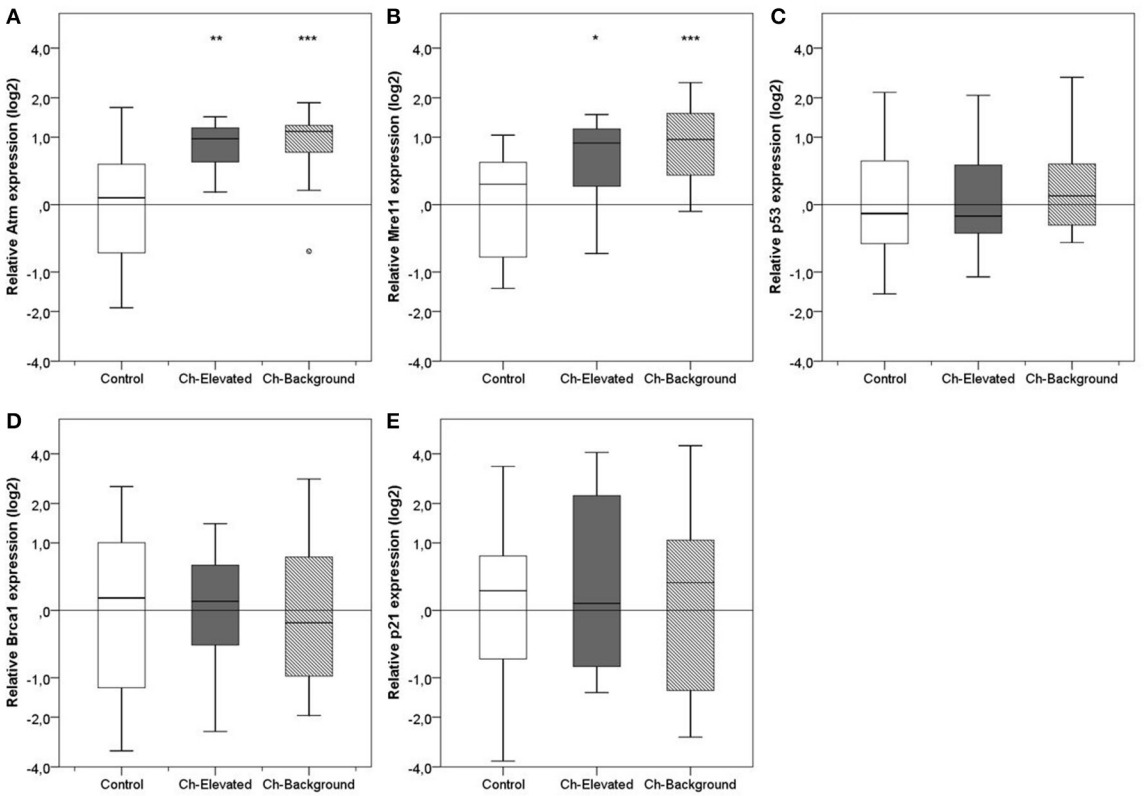

FIGURE 2 | Relative expression of five central DNA damage response (DDR) genes in livers of bank voles collected from the Chernobyl Exclusion Zone (CEZ) (Ch-Background = <1.0 μGy/h, Ch-Elevated = up to 12.3 μGy/h) and control locations outside the CEZ. Gene expression was normalized against two internal reference genes (*Actb* and *Rer1*) with the average expression in the control group set as the reference level. **(A)** *Atm,* **(B)** *Mre11*, **(C)** *p53,* **(D)** *Brca1,* and **(E)** *p21*. $^{*}p < 0.05$; $^{**}p < 0.005$; $^{***}p < 0.001$.

internal radiation sources (Baker et al., 2017). In this scenario, the common upregulation effect between contaminated and non-contaminated areas represents an average expression for inhabiting the CEZ especially if there are carry forward effects from exposure to very high amounts of radionuclides in prior generations. Such effects of past exposures have been reported with respect to mitochondrial mutation rates in these same voles (Baker et al., 2017) and would have the effect of averaging effects over broader geographic scales. Another possibility is that observed patterns reflect local adaptations that have spread from populations at contaminated regions to adjacent areas via gene flow (Møller et al., 2006; Fedorka et al., 2012) which could also account for a "higher than expected" level of expression in less contaminated areas within the CEZ.

Upregulation of DDR pathways of animals residing within the CEZ may seem intuitive, but these pathways have not been studied in wildlife inhabiting areas contaminated by radioactivity. That *Atm* is upregulated is relevant as this gene is positioned at the top of the DBS repair pathway. ATM is primarily regulated at the protein level when subjected to IR (Bakkenist and Kastan, 2003), but *Atm* promoter activity, and protein abundance, is stimulated by exposure to IR in mice tissues, including liver (Gueven et al., 2006); moreover, silencing of ATM decreases radioresistance of glioma *in vitro* and *in vivo* (Li et al., 2016). Perhaps crucially, ATM is an important regulator of cellular oxidative stress (Barzilai et al., 2002), promoting the production of NADPH (Cosentino et al., 2011). Moreover, ATM can be activated by oxidative stress independently from DBS-related activation by the MRN complex (Guo et al., 2010). Antioxidant production likely constitutes a major part of the adaptive response against environmental radioactivity in birds within and around the CEZ (Galván et al., 2014) and in cell lines of fibroblasts from bank voles (Mustonen et al., submitted). Moreover, a change in fur color of bank voles within the CEZ was attributed to downregulation of pheomelanin to reallocate antioxidants to ROS defense (Boratynski et al., 2014). Indeed, upregulation of oxidative stress response genes *Cat* and *Fsd3* in *Arabidopsis* (Kovalchuk et al., 2014) indicate that increased ROS defense could be a key coping strategy against exposure to environmental radionuclides.

MRE11 is a constituent protein of the MRE11-RAD50-NBS1 complex, a multi-purpose maintainer of genomic stability whose tasks include DSB detection and subsequent activation of ATM protein (Lee and Paull, 2005) as well as DNA repair functions and telomeric maintenance (Lamarche et al., 2010). Reduced expression of the *Mre11* gene often leads to genomic instability,

and increased protein levels of ATM and MRE11 in some tumors are associated with resistance against radiotherapy (Tribius et al., 2001; Deng et al., 2011). An apparently low frequency of micronuclei in bank voles from the CEZ might indicate that this species has some degree of radioresistance (Rodgers and Baker, 2000). Given our results, *Atm* and *Mre11* represent important candidate genes that regulate genomic stability in bank voles exposed to environmental radionuclides.

Our observation of no significant variation in expression of *p53* may be explained by this gene being primarily regulated through post-translational modification of cellular protein (Kruse and Gu, 2009), although transcriptional upregulation has previously been detected in the livers of European wood mice inhabiting an abandoned uranium mine area (Lourenço et al., 2013). Significant enrichment of heavy metals (uranium and cadmium) were also detected in these mice, contributing to toxicity alongside radiation. Thus, radionuclide levels comparable to studied areas in the CEZ are not severe enough to induce transcription in *p53*. *P21* is under transcriptional regulation by p53 and is required for DNA damage related cell cycle inhibition (Bunz, 1998). That *p21* expression is not altered in animals inhabiting the CEZ implies cell cycle control pathways are not activated in response to increased *Atm* expression. *Brca1* is upregulated in response to genotoxic stress (De Siervi et al., 2010). A lack of change in expression of *Brca1* in bank voles from the CEZ indicates that the homologous recombination repair pathway is not a key component of the response to environmental radionuclides, consistent with a recent study of expression of DBS repair pathways during exposure to IR (Liu et al., 2016). *Arabidopsis* from the CEZ show reduced recombination, which might prevent gross chromosomal rearrangements (Kovalchuk et al., 2014).

As the expression of *p21* and *Brca1* is not stimulated it is plausible that the low-dose IR environment of Chernobyl does not cause sufficient DNA damage to warrant cell cycle arrest and activation of DSB repair. Nonetheless, some genomic impact is derived from exposure to radionuclides as bank voles from the CEZ have shorter telomeres than bank voles from control areas (Kesäniemi et al., unpublished), likely reflecting an increase in oxidative stress that is widely associated with telomere shortening (von Zglinicki, 2002). Hence is it interesting that ATM can be activated by oxidation independently from MRN interaction. Altered telomere homeostasis may affect the expression of *Mre11* as a telomere maintainer and the role of this gene warrants further investigation.

AUTHOR CONTRIBUTIONS

TJ and PW: designed the outline of the study; AL, ET, AM, TM, TAM, and GM: were involved in setup of infrastructure in the field and animal sample collection, and TJ and JK: conducted molecular analyses and performed calculations; TJ: wrote the manuscript with input from all other authors.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fenvs.2017.00095/full#supplementary-material

Datasheet 1 | Accumulated external dose estimations by age groups.

Datasheet 2 | qPCR raw and normalized quantification cycle data.

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