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Cell-type specific role of the RNA-binding protein, NONO, in the DNA double-strand break response in the mouse testes

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Abstract

The tandem RNA recognition motif protein, NONO, was previously identified as a candidate DNA double-strand break (DSB) repair factor in a biochemical screen for proteins with end-joining stimulatory activity. Subsequent work showed that NONO and its binding partner, SFPQ, have many of the properties expected for bona fide repair factors in cell-based assays. Their contribution to the DNA damage response in intact tissue in vivo has not, however, been demonstrated. Here we compare DNA damage sensitivity in the testes of wild-type mice versus mice bearing a null allele of the NONO homologue (Nono gt). In wild-type mice, NONO protein was present in Sertoli, peritubular myoid, and interstitial cells, with an increase in expression following induction of DNA damage. As expected for the product of an X-linked gene, NONO was not detected in germ cells. The Nono gt mice had at most a mild testis developmental phenotype in the absence of genotoxic stress. However, following irradiation at sublethal, 2–4 Gy doses, Nono gt mice displayed a number of indicators of radiosensitivity as compared to their wild-type counterparts. These included higher levels of persistent DSB repair foci, increased numbers of apoptotic cells in the seminiferous tubules, and partial degeneration of the blood-testis barrier. There was also an almost complete loss of germ cells at later times following irradiation, evidently arising as an indirect effect reflecting loss of stromal support. Results demonstrate a role for NONO protein in protection against direct and indirect biological effects of ionizing radiation in the whole animal.

Keywords

NONO; double-strand break repair; NHEJ; ionizing radiation; knockout mouse; testes

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Conflict of Interest. The authors declare that there are no conflicts of interest.

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1. Introduction

NONO (p54nrb), SFPQ (PSF), and PSPC1 are multifunctional nuclear proteins that together make up the DBHS protein family. Family members dimerize in all possible combinations. These dimers bind to regulatory noncoding RNAs, inosine-containing RNAs, pre-mRNA 5′ splice sites, and other targets [1, 2]. Among many reported biological functions, they regulate the expression of genes involved in brain synapse function and in progression of tumors of neuroectodermal origin [3–5].

Evidence suggests that NONO SFPQ dimers have additional functions in DNA repair. They interact with DNA via a motif located within an intrinsically disordered region, unique to SFPQ, that is separate from the RNA binding domain [6, 7]. Purified NONO SFPQ dimers stimulate DNA end joining by ten-fold or more in a reconstituted \textit{in vitro} assay system containing DNA ligase IV and other c-NHEJ factors [8–10]. This stimulation evidently arises as the result of direct interaction of the NONO SFPQ complex with the DNA substrate. Purified NONO SFPQ binds to DNA \textit{in vitro}, cooperates with other c-NHEJ proteins to form a committed pre-ligation complex, and stimulates the activity of the DNA-dependent protein kinase, an important regulator of c-NHEJ activity [8–10]. Recent work suggests that NONO SFPQ and XLF, a core c-NHEJ factor, function redundantly \textit{in vitro} to promote DNA substrate pairing [11]. The same study shows, however, that NONO SFPQ has additional non-redundant functions in cell-based assays, which could involve RNA interaction.

Deficiency in NONO and SFPQ in cultured cell models is associated with radiosensitivity and delayed DSB repair [7, 12–14]. Although not as well developed, various lines of evidence also link NONO and SFPQ to homologous recombination repair, nucleotide excision repair, interstrand cross-link repair, and DNA damage-dependent cell cycle checkpoint control [11, 15–21]. Consistent with a physiological role in DNA repair, NONO and SFPQ migrate to sites of DNA damage in cells in a PARP-1-dependent manner [7, 13, 22]. NONO binds PAR noncovalently via its RNA binding domain, and proteome-wide studies suggest that it is also a target of covalent PARylation [13, 23, 24].

NONO and other DBHS proteins are of special interest because they represent a point of cross-talk between RNA biology and DNA repair. However, studies of their repair function have so far been limited to biochemical assays and cultured cell models. Here, we describe a cell type-specific DNA damage sensitivity phenotype in NONO-deficient mice. Studies focused on the NONO subunit of the NONO SFPQ complex, because SFPQ is an essential gene in vertebrates and SFPQ-deficient mice are expected to be inviable [25]. The derivation of NONO-deficient mice, which bear a functionally null gene trap allele in the X-linked mouse NONO homolog (Nono\textsuperscript{gt}), has been described previously [14, 19]. They are viable, albeit with abnormalities in growth, wound healing, craniofacial development, and gene regulation [3, 14, 19]. We have previously investigated the DNA damage sensitivity phenotype of MEFs derived from these mice and shown that it is blunted by compensatory up-regulation of another DBHS family member, PSPC1. A strongly radiosensitive phenotype emerges, however, when PSPC1 expression is attenuated by miRNA transfection in the NONO-deficient background [14].
Studies here focus specifically on the role of NONO protein in the testes. The effects of DNA damaging agents on the testes have been well characterized, and other DNA repair deficiencies often lead to a testis phenotype [26, 27]. Prior work has also shown that NONO and other DBHS proteins are expressed in a cell-type specific pattern in the testes [28, 29] and, separately, that the c-NHEJ pathway is active in the testes and provides a major mechanism of protection from ionizing radiation 35. In the course of the work it also emerged that compensatory up-regulation of PSPC1 is not observed in the testes of NONO-deficient mice, reducing concerns over this as a potentially confounding factor.

Our results show that NONO deficiency significantly compromises the response to ionizing radiation-induced DNA damage in the testes, consistent with cell type-specific loss of DSB repair capacity. The results provide evidence that this RNA binding protein significantly contributes to the DNA damage response in vivo.

2. Materials and methods

2.1 Animal methods

Experiments were performed under Animal Use Protocols, specific to this project, approved by the Institutional Animal Care and Use Committees at the Medical College of Georgia, currently Augusta University (#BR06-04-777) and at Emory University (#2001909). Generation of Nono-deficient mice from an embryonic stem cell clone (YHA266, BayGenomics) has been described [14]. Nono is X-linked, and the strain was maintained by backcrossing heterozygous females to C57/Bl6 males. For the overall survival experiment, mice were backcrossed for >5 generations into the C57/Bl6 background. Mice were weighed at least weekly for 180 days. All other experiments were performed with mice that were backcrossed for >10 generations. Testosterone measurements were performed using blood collected by retro-orbital bleeding. Serum testosterone was measured by ELISA (Alpco, 55-TESMS-E01) according to the manufacturer’s protocol. Testis histology was evaluated in sections prepared using Bouin’s Fixative solution (RICCA Chemical Co., Arlington, TX, cat# 1120-32) and processed by standard pathology methods. Sperm counts were determined as described [30] with modifications. Mice were sacrificed and both epididymides were dissected and placed in a Petri dish containing 1 ml MEM medium. Sperm were collected by squeezing with forceps, after which the tissue was minced with iris scissors until no obvious pieces were visible. After 5 min incubation at room temperature, the suspension was diluted 1:10 in PBS. Sperm counts were determined in triplicate for each mouse using a hemocytometer. Total body irradiation was performed using a $^{137}$Cs irradiator at a dose rate of 0.8 Gy/min.

2.2 Immunofluorescence and immunoblotting

Testis sections were prepared by standard methods as described above, dewaxed in xylene and rehydrated in graded alcohols, and boiled in sodium citrate buffer (10 mM Tris-sodium citrate, 0.05% Tween 20, pH6) for 30 min to promote antigen recovery. After blocking with 15% goat serum, 1% BSA in PBS, sections were incubated for 1 h at room temperature with primary antibodies diluted in the same buffer. Primary antibodies were: mouse anti-SFPQ (Sigma-Aldrich, WH0006421M2, 1:500), goat anti-NONO (GeneTex, GTX89315, 1:50),
rabbit anti-PSPC1 (Bethyl Laboratories, A303-205A, 1:500), rabbit anti-SOX9 (EMD Millipore, AB5535, 1:1500), rabbit anti-Connexin 43 (EMD Millipore, AB11370, 1:1000) and rabbit anti-53BP1 (Novus Biologicals, NB100-904, 1:1000). After washing with PBS, sections were incubated with species-specific Alexa Fluor 488 or Alexa Fluor 594-conjugated anti-IgG secondary antibodies for 1 h at room temperature (Thermo Fisher Scientific, A11005, A11008, A11055, A11058 or 21206, 1:500). Immunoblotting was performed using rabbit anti-NONO (Abcam, Ab70335, 1:500), rabbit anti-Connexin 43 (EMD Millipore, AB11370, 1:4000) or mouse anti-β-actin (Sigma, A5060, 1:500) with horseradish peroxidase-conjugated goat-anti-mouse or anti-rabbit IgG secondary antibodies (GE Healthcare, NA 931 or NA 934V, 1:10000). Membranes were developed using Enhanced Chemiluminescence Substrate (GE Healthcare) and immune complexes were visualized using X-ray film.

2.3 TUNEL assay

TUNEL labeling was performed using an ApopTag Fluorescein in situ Apoptosis Detection Kit (EMD Millipore, S7110). Briefly, deparaffinized and hydrated sections were incubated with Proteinase K (20 μg/ml) for 15 min at room temperature. Apoptotic DNA fragments were 3′-end labeled using terminal deoxynucleotidyl transferase and digoxigenin-11-dideoxyuridine triphosphate substrate for 1 h at 37°C and visualized using fluorophore-conjugated anti-digoxigenin antibody with DAPI counterstain. Scoring was determined by counting total TUNEL-positive cells per section and dividing by the number of tubules.

2.4 Electron microscopy for evaluation of tissue ultrastructure

Mouse testis samples were fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4), dissected into small pieces and post-fixed with 1% osmium tetroxide in the same buffer. Samples were dehydrated in graded ethanols to 100%. After infiltration with Epoxy Resin Embed 812 (Electron Microscopy Sciences), samples were embedded in the same resin and incubated at 60°C to allow polymerization. Ultrathin 70–80 nm sections were prepared using a Leica UltraCut S ultramicrotome (Leica Microsystems) and post-stained with uranyl acetate and lead citrate. Ultrathin sections were imaged using a JEOL JEM-1400 Transmission Electron Microscope equipped with a 2k x 2k Gatan US1000 CCD camera.

2.5 Statistical analysis

Statistical analysis was performed using Prism 5 (GraphPad software) using tests specified in the legend to each figure. The threshold for statistical significance was p-value ≤ 0.05.

3. Results

3.1 Overall phenotype of Nonogt mice

The X-linked Nonogt allele was maintained by crossing wild-type males to Nonogt+ heterozygous females. The Nonogt allele confers a slight survival disadvantage: Nonogt- male and Nonogt+ female offspring were under-represented at the time of weaning (Fig. 1A) and continued to exhibit slight excess mortality during the following 18 months of life (Fig. 1B). Nonogt- males were somewhat smaller than their wild-type littermates and exhibited changes in craniofacial morphology as described previously [3] (Fig. 1C). Body weight
showed a significant, 20% reduction at maturity (Fig. 1D). The difference in body weight was not seen between wild type and Nono<sup>gt</sup> females. It was also not seen with a small number of Nono<sup>gt/gt</sup> females that were examined (offspring of a rare, productive cross between a Nono<sup>gt/0</sup> male and Nono<sup>gt/+</sup>female).

3.2 Testis phenotype of Nono<sup>gt</sup> mice in absence of genotoxic stress

We investigated whether Nono gene deficiency was associated with a testis developmental phenotype. Consistent with lower total body weight, the testes of Nono<sup>gt/0</sup> mice were significantly smaller than their wild-type counterparts (Fig. 2A). Sperm counts in Nono<sup>gt/0</sup> mice were significantly lower, even after normalization to body weight (Fig. 2B). Serum testosterone levels were, on average, two-fold lower, although because of the high level of within-group variation, the difference was not significant (Fig. 2C). The histologic appearance of the seminiferous tubules was nearly normal, although some specimens gave the impression of modestly reduced cellularity, consistent with the lower sperm counts (Fig. 2D). Together, results indicate that Nono<sup>gt/0</sup> mice exhibit at most a mild testis developmental phenotype.

3.3 Expression of DBHS proteins in the testes

To confirm the absence of NONO protein in the Nono<sup>gt/0</sup> mice and to determine the effect of NONO deficiency on expression of other DBHS proteins, we performed immunoblotting using whole-testis protein extracts (Fig. 3A). Nono<sup>gt/0</sup> mice showed no detectable expression of NONO protein in the testes, consistent with prior work showing the absence of Nono mRNA [14]. Nono<sup>gt/0</sup> mice showed little or no increase in levels of PSPC1 in the testes, in contrast to the approximately 5-fold increase over wild type seen in MEFs [14]. There was also little or no increase in SFPQ expression over wild type.

The cell-type specific patterns of DBHS protein expression in Nono<sup>++/0</sup> and Nono<sup>gt/0</sup> mice were characterized by immunofluorescence staining (Fig. 3B, 3C). Patterns of expression in the wild-type background were generally similar to those reported previously [28, 29]. NONO was prominently expressed in a ring of cell nuclei lining the inside of the seminiferous tubule, which were identified as Sertoli cells based on colocalization with a SOX9 marker (Fig. 3B). There was also prominent expression in the nuclei of peritubular myoid and interstitial (Leydig) cells. NONO expression was not detectable in spermatogonia, which lie toward the lumen of the seminiferous tubule, consistent with repression by meiotic sex chromosome inactivation [31]. The Nono<sup>gt/0</sup> mice showed no detectable NONO expression, confirming the specificity of the immunostaining (Fig. 3C).

In wild-type mice, PSPC1 showed a reciprocal pattern of expression to NONO. PSPC1 was strongly expressed in spermatogonia, with weaker staining of the presumptive Sertoli, peritubular myoid, and interstitial cell nuclei (Fig. 3C). The reciprocal pattern of PSPC1 and NONO expression is particularly evident in an enlarged view of a peritubular region of interest (Fig. 3C, inset). SFPQ expression was pan-cell type specific, consistent with the ability of SFPQ to enter into functional complexes with both PSPC1 and NONO (Fig. 3C). The pattern of SFPQ and PSPC1 expression in Nono<sup>gt/0</sup> mice appeared very similar to that in their wild-type counterparts.
3.4 Connexin 43 expression in wild type and NONO-deficient testes

Connexin 43 is a desmosome component that is important for dynamic regulation of the blood-testis barrier, which separates the adluminal and basal compartments of the seminiferous tubules [32, 33]. The effect of NONO deficiency on Connexin 43 expression was of interest because of prior work indicating that NONO acts as a repressor or co-repressor of Connexin 43 expression in cultured melanoma, human embryonic kidney, and myometrial cells [5, 34]. In wild type mice, Connexin 43 was expressed in a regular pattern, luminal to the Sertoli cell nuclei, and consistent with localization to the blood-testis barrier (Fig. 4). In Nono<sup>gt</sup>/0 mice, the pattern and level of expression were not substantially different, as indicated by immunostaining and immunoblotting. The finding that Connexin 43 expression is unaffected in Nono<sup>gt</sup>/0 mice is consistent with observations presented subsequently, that the ultrastructure of the blood-testis barrier is normal in the absence of genotoxic stress.

3.5 Induction of NONO protein by ionizing radiation

As a first step toward characterizing the role of DBHS protein in the DNA damage response, we investigated whether their levels changed following exposure to ionizing radiation. Wild-type and Nono<sup>gt</sup>/0 mice were subjected to a moderate, 4 Gy dose of total body irradiation. Whole-testis protein extracts were prepared after 24 or 48 h of recovery and analyzed by immunoblotting (Fig. 5). In wild-type mice, there was a progressive increase in NONO protein expression of up to 5-fold. In Nono<sup>gt</sup>/0 mice, NONO protein expression remained undetectable following irradiation, as expected. Results were similar in mice subjected to 2 Gy of total body irradiation, although the effect size was less, about 3-fold (data not shown).

In contrast to the results with NONO, there was little change in PSPC1 expression after 24 h, and a decline at 48 h. The reciprocal changes in NONO and PSPC1 expression could reflect greater functional involvement of NONO in the DNA damage response. Alternatively, or in addition, it could arise from selective loss of PSPC1-expressing germ cells, as suggested by subsequent observations. Expression of SFPQ, which forms dimers with both NONO and PSPC1, was relatively stable, differing by only about 2-fold. Changes in PSPC1 and SFPQ expression generally similar in the wild-type and Nono<sup>gt</sup>/0 backgrounds.

3.6 Effect of Nono gene deficiency on DSB repair and radiation-induced apoptosis

We evaluated DSB repair efficiency based on persistence of DSB repair foci in irradiated wild-type and Nono<sup>gt</sup>/0 mice. Prior work in cultured cells has shown that the initial number of repair foci, as measured 30 min post-irradiation, is unaffected by NONO deficiency [12, 14]. Although most foci resolve eventually, resolution is slower in NONO or NONO/PSPC1-deficient cells, with the greatest difference between normal and deficient cells seen at about 4 h post-irradiation [12, 14].

For experiments in the testes, we used 53BP1 as a marker of repair foci. Prior work has shown that 53BP1 is a more specific marker of DNA damage in the testes than γ-H2AX, which accumulates at programmed DSBs associated with meiosis in developing germ cells [35, 36]. The kinetics of 53BP1 foci formation have been characterized in cultured wild type
and c-NHEJ deficient Sertoli cells. As in other cell types, a 4 h post-irradiation timepoint provides good discrimination between genotypes [35].

Mice were subjected to 2 Gy total body irradiation, a dose that is sufficient to induce about 60 DSBs per diploid genome, and were allowed to recover for 4 h before tissue collection. Testes of wild-type mice showed relatively few 53BP1-positive cells, whereas Nono<sup>gt/0</sup> mice showed many more (Fig. 6A). The 53BP1 signal was most prominent in cells lining the perimeter of the seminiferous tubule, which presumably correspond to Sertoli cells. There were very few, if any, 53BP1-positive cells in non-irradiated control mice of either genotype.

To examine the effect of NONO deficiency on radiation-induced cell death, mice were irradiated and allowed to recover for 24 h before tissue collection. Apoptotic cells in the testes were visualized by TUNEL assay (Fig. 6B). Significantly greater numbers of TUNEL-positive cells were present in samples from Nono<sup>gt/0</sup> mice compared to their wild-type counterparts (Fig. 6C). The TUNEL-positive cell nuclei were distributed toward the periphery of the tubules and may correspond to Sertoli cells, although morphology was not sufficiently preserved to be certain. Only sparse TUNEL-positive cells were present in non-irradiated control mice of either genotype. Together, results are suggestive of loss of DSB repair capacity associated with increased radiosensitivity in Nono<sup>gt/0</sup> mice.

**3.7 Breakdown of blood-testis barrier and germ cell depletion in irradiated Nono<sup>gt/0</sup> mice**

We compared testis ultrastructure and histology in irradiated wild type and Nono<sup>gt/0</sup> mice. At 48 h post-irradiation, wild-type mice maintain an intact blood-testis barrier, visible as a densely staining, double-membrane structure in electron micrographs (Fig. 7A, arrowheads). By contrast, Nono<sup>gt/0</sup> mice displayed areas of degeneration (Fig. 7A, asterisks). Non-irradiated control mice showed an intact blood-testis barrier regardless of genotype.

We also examined histology of the seminiferous tubules. At 48 h post-irradiation, wild-type mice showed mild degenerative changes relative to non-irradiated controls: seminiferous tubules appeared narrow with slightly reduced spermatogenesis. In Nono<sup>gt/0</sup> mice, however, there was gross depletion of germ cells with central stromal hyalinization. This evidently arises as an indirect radiation effect, given that germ cells do not themselves express NONO. Plausibly, it results from Sertoli cell dysfunction and loss of stromal support.

**4. Discussion**

In the present study we characterized the DNA damage sensitivity phenotype of hemizygous male mice bearing a null allele of the X-linked Nono gene. We confirmed that, in wild-type mice, NONO protein is expressed in all cells of the testes except for germ cells, where the gene is presumed to be silenced by meiotic sex chromosome inactivation. We also showed that NONO protein expression is undetectable in the testes of Nono<sup>gt/0</sup> mice. Despite the myriad of gene regulatory functions attributed to NONO in various cultured cell models, loss of expression in vivo led to at most a mild testis developmental phenotype. Although sperm count was reduced about two-fold, morphology of the seminiferous tubules was nearly normal, the blood-testis barrier appeared to be intact, and there did not appear to be changes in the pattern or level of expression of Connexin 43, which is a target of NONO.
regulation in other settings [5, 34]. In contrast to NONO-deficient MEFs [14], the mild testis phenotype cannot be attributed to up-regulation of the PSPC1 paralog, expression of which was largely unaffected by Nono genotype. The mild phenotype suggest either that NONO does not play a general role in RNA transcription, processing, and transport in the testes, or that RNA biogenesis is sufficiently buffered by other proteins as to minimize the effects of NONO deficiency.

In contrast to the minimal phenotype observed in the absence of genotoxic stress, the testes of irradiated Nono<sup>gt<sup>0/0</sup> mice were compromised with respect to DSB repair and cell survival. NONO function appeared to be particularly important in Sertoli cells, which have relatively high levels of NONO expression in the wild-type background, as reported here and elsewhere [29]. Evidence of a compromised DNA damage response in Nono<sup>gt<sup>0/0</sup> Sertoli cells includes the presence of persistent radiation-induced repair foci in cells at the periphery of the seminiferous tubule, where Sertoli cell nuclei reside, and radiation-induced changes to the ultrastructure blood-testis barrier, which is dependent on Sertoli cell function for dynamic maintenance of its integrity. In addition to Sertoli cells, germ cells of Nono<sup>gt<sup>0/0</sup> mice are hypersensitive to radiation. This must arise as a non cell-autonomous, indirect effect, given that the Nono gene is not expressed in germ cells. Most likely, germ cell radiosensitivity arises as a secondary consequence of compromised Sertoli cell function. The germ cell toxicity of cisplatin has been similarly attributed to compromised Sertoli cell function [37].

Prior work has shown that Sertoli cells are reliant on the c-NHEJ pathway for DSB repair [35, 38]. Results here suggest that DSB repair is compromised in the Nono<sup>gt<sup>0/0</sup> Sertoli cells in vivo. The results are consistent with the proposed mechanism that has emerged from biochemical and cell-based studies, where NONO-containing protein complexes interact with damaged DNA and directly promote c-NHEJ repair [7–10, 12–14, 22]. Another possibility, not mutually exclusive, is that deficiency in NONO, without compensatory expression of PSPC1, leads to an excess of SFPQ over other family members and, perhaps, to formation of non-physiological SFPQ dimers [1, 39]. In principle, these could have a dominant negative effect on DSB repair. It is also possible that deficiency in NONO compromises PAR binding or affects the PARylation of the other DBHS proteins. Although potentially important, such effects would be challenging to detect directly in the whole mouse model, given that PARylation is an early and transient response to DNA damage and that high levels of steady-state NONO SFPQ PARylation have so far been observed only in settings where the degradatory enzyme, poly ADP-ribose glycohydrolase, is reduced or absent [23, 24].

Studies here also did not address whether gene expression regulatory activities of NONO contribute to the radiosensitive phenotype. NONO and SFPQ are multifunctional proteins with a multitude of effects on gene regulation. Prior work has not identified DNA repair or DNA damage response genes that are specifically dependent on NONO for their expression [3, 14]. These studies were performed using neurons and fibroblasts, however, and a testis-specific role in gene regulation cannot be excluded.
It is noteworthy that the Nono<sup>gt</sup>/0 mice displayed no obvious block to germ cell maturation, indicating that meiosis is not seriously impaired. The result contrasts with findings in DNA polymerase β conditional knockout mice, where a DNA repair deficiency presents a specific block to meiotic synapsis [40]. The finding that Nono<sup>gt</sup>/0 mice are proficient in meiosis is of interest given that the NONO binding partner, SFPQ, has been described as an activator of the meiosis-specific recombinase DMC1 in vitro [18]. If there is a physiological role of SFPQ in meiosis, it evidently does not require interaction with NONO.

Among the many biological functions that have been reported for NONO protein is an ability to function as a co-activator of androgen receptor-mediated transcription [29]. Neither the Nono<sup>gt</sup>/0 mice nor NONO-deficient patients exhibit the features of androgen insensitivity syndrome, which indicates that NONO cannot be essential for androgen receptor function in vivo [3]. It remains possible that there is a partial or tissue-specific androgen insensitivity, which could factor into the reduced overall body size of Nono<sup>gt</sup>/0 male mice. Consistent with this explanation, we did not observe reduced body size in three NONO<sup>gt/gt</sup> female mice that were characterized early in the course of the study, although further work using more individuals will be needed to determine whether the growth deficiency is indeed sex-specific.

An alternative or additional explanation for the smaller body size is that it reflects syndromic DSB repair deficiency. Growth retardation, frequently accompanied by microcephaly and craniofacial abnormalities, is seen in patients who are deficient in LIG4, XRCC4, or NHEJ1, which encode subunits of the complex responsible for the ligation step in c-NHEJ [41–43], and in patients who are deficient in NBS1, which encodes a different DSB repair factor [44]. Reduced body size is also seen in mice lacking XRCC5 and XRCC6, which encode the two subunits of Ku protein [45, 46]. Although DBHS proteins are clearly involved in many aspects of gene regulation, syndromic DSB repair deficiency should not be overlooked as an explanation for phenotypes associated with loss of NONO function in vivo.

The present work serves to validate NONO as an important determinant of the radiation response in vivo. Studies were, however, limited to the testis as a model organ and to short term endpoints. It will be of interest to extend the work to other radiosensitive cells and tissues, such as hematopoietic stem cells or intestinal crypt cells, which figure prominently in determining overall radiation survival.

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Abbreviations

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<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>NONO</td>
<td>Non-POU domain-containing octamer-binding protein</td>
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<tr>
<td>SFPQ</td>
<td>Splicing Factor Proline/Glutamine-rich</td>
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<tr>
<td>PSPC1</td>
<td>Paraspeckle Component 1</td>
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DBHS  Drosophila behavior, human splicing

c-NHEJ  canonical nonhomologous end joining

PAR  poly ADP-ribose

PARP-1  PAR polymerase-1

gt  gene trap

MEFs  murine embryonic fibroblasts

SOX9  SRY (sex determining region Y)-box 9

DSB  DNA double strand break

53BP1  Tumor Protein p53 Binding Protein 1

γ-H2AX  phosphorylated H2A Histone Family, Member X

XRCC5  X-Ray Repair Complementing Defective Repair In Chinese Hamster Cells 5

XRCC6  X-Ray Repair Complementing Defective Repair In Chinese Hamster Cells 6

LIG4  DNA Ligase 4

NBS1  Nijmegen breakage syndrome 1

BLM  Bloom Syndrome RecQ Like Helicase

TUNEL  Terminal deoxynucleotidyl transferase dUTP nick end labeling

MEM  Minimum Essential Medium

ELISA  Enzyme-linked immunosorbent assay

PBS  Phosphate-buffered saline

BSA  Bovine serum albumin

DAPI  4′,6-diamidino-2-phenylindole

Cx43  Connexin 43

TEM  Transmission electron microscopy

H & E  Hematoxylin and eosin

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Highlights

- NONO is an RNA binding protein previously implicated in NHEJ repair.
- DNA damage sensitivity was investigated in testes of NONO knockout (KO) mice.
- In the absence of genotoxic stress, KO mice had at most a mild testes phenotype.
- Sertoli cells of irradiated KO mice showed a number of markers of radiosensitivity.
- Germ cells, which do not normally express NONO, died from loss of stromal support.
Fig. 1.
Overall phenotype of Nono<sup>+/0</sup> mice. (A) Fraction of mice of each genotype at weaning, based on 301 consecutive genotypings. Deviation from Mendelian ratios was significant by \( \chi^2 \) analysis (p < 0.01). (B) Overall survival of mice of indicated genotypes. (n ≥ 20 per group). Survival differences were evaluated by Log-rank (Mantel-Cox) test (*, p < 0.05). (C) Example of wild-type (left) and Nono<sup>+/0</sup> (right) littermates. (D) Weights at postnatal day 120 for mice of indicated genotypes. Bars represent mean values. The difference between wild-type and NONO-deficient male mice was evaluated by an unpaired, two-tailed t-test (*, p < 0.05).
Fig 2.
Testes phenotype of Nono\textsuperscript{gt0} mice. (A) Left and right testes were weighed individually and each symbol represents the average for one individual. Bars represent mean for each group. Differences between genotypes were evaluated by an unpaired, two-tailed t-test (*, \( p < 0.05 \); **, \( p < 0.01 \)). (B) Sperm counts in mice of indicated genotypes, normalized to body weight. Each symbol denotes mean of triplicate measurements for one individual. Bars denote mean values. Differences were evaluated by a two-tailed Mann-Whitney test (*, \( p < 0.05 \)). (C) Serum testosterone levels. Each symbol denotes one individual. Bars denote mean for each group. Difference was evaluated by unpaired, two-tailed t-test and was not significant. (D) H & E sections were prepared from three mice of each genotype. Representative images are shown.
Fig. 3.
DBHS protein expression patterns in wild-type and Nono<sup>et/0</sup> mice. (A) Immunoblot of protein extract from testes of wild-type and NONO-deficient mice and probed for NONO, PSPC1, and SFPQ expression. Blots were probed for β-actin as a loading control. Plot at right shows quantification of protein levels, normalized to β-actin and expressed relative to expression of each protein in the wild type (marked by dotted line). Samples were analyzed for three mice of each genotype; a representative pair is shown. (B) Testes section from wild-type mouse stained for NONO protein expression and for SOX9 protein as a Sertoli cell marker. Scale bar: 40 μm. Images were collected for samples from three mice and one representative image is shown. (C) Testes sections from wild-type and NONO-deficient mice, stained for NONO, PSPC1, and SFPQ protein expression. Inset for wild-type mice show an enlarged region of interest. Scale bar: 40 μm. Images were collected for samples from three mice of each genotype; representative images are shown.
Fig. 4.
Expression of Connexin 43 protein in testes of adult wild-type (+/0) and NONO-deficient (gt/0) mice. (A) Sections stained for Connexin 43 expression (red) and counterstained with DAPI as a nuclear marker (blue). Scale bar: 30 μm. Images were collected two mice of each genotype; one representative image is shown. (B) Immunoblot of protein extract from testes of wild-type and NONO-deficient mice probed for NONO and Connexin 43 (Cx43) expression. Blots were probed for β-actin as a loading control. Samples were analyzed for three mice of each genotype; a representative pair is shown. (C) Plot showing quantification of Cx43 levels, normalized to β-actin and expressed relative to non-irradiated Nono+/0 control (marked by dotted line).
Fig. 5.
Induction of NONO protein following total body irradiation. (A) Wild type (Nono<sup>+/0</sup>) or NONO-deficient (Nono<sup>gt/0</sup>) mice were irradiated and allowed to recover as indicated. Immunoblots of total testes protein were probed for NONO protein and for β-actin as a loading control. (B) Plots show quantification of protein levels, normalized to β-actin and expressed relative to the non-irradiated wild-type individual (marked by dotted line). A total of four experiments were performed; representative results are shown.
Fig. 6.
Persistent DSB repair foci and apoptosis in testes of irradiated wild-type and NONO-deficient mice. Mice were irradiated and allowed to recover before sacrifice as indicated. Experiments were performed with 3–4 mice for each experimental group. Representative images are shown. (A) Sections of mouse testes stained for 53BP1 (red) and counterstained with DAPI as a nuclear marker (blue). Scale bar: 30 μm. Inset shows an enlarged view of a region of interest. (B) Sections of mouse testes stained using the TUNEL method (green) and counterstained with DAPI. Scale bar: 50 μm. Inset shows an enlarged view of a region of interest. (C) Quantification of TUNEL staining. Quantification was performed for entire testis cross sections from each mouse. Mean and standard deviations are shown. Statistical analysis was performed using an unpaired t-test with Welch’s correction (*, p < 0.05).
Fig. 7.
Breakdown of blood-testes barrier and germ cell depletion in irradiated wild-type and Nono<sup>A0/A0</sup> mice. (A) Transmission electron micrographs. Mice were irradiated and allowed to recover before sacrifice as indicated. Arrowheads indicate densely staining double-membrane structure of blood-testis barrier. Asterisks indicate region of breakdown. Inset shows enlarged view of indicated region. Scale bar: 100 nm. The experiment was performed using two mice for each genotype/dose combination. Representative images are shown. (B) Testis histology. Mice were irradiated and allowed to recover as indicated. Arrows indicate depletion of spermatocytes and central stromal hyalinization. Scale bar: 20 μm. The experiment was performed using six mice for each genotype/dose combination and representative images are shown.