DNA Repair 8 (2009) 803–812

Contents lists available at ScienceDirect

DNA Repair

journal homepage: www.elsevier.com/locate/dnarepair

DNA damage responses in Drosophila *nbs* mutants with reduced or altered NBS function

Sushmita Mukherjee[a], Matthew C. LaFave[b], Jeff Sekelsky[a,c,*]

[a] Department of Biology, University of North Carolina, Chapel Hill, NC 27599, United States
[b] Curriculum in Genetics and Molecular Biology, University of North Carolina, Chapel Hill, NC 27599, United States
[c] Program in Molecular Biology and Biotechnology, University of North Carolina, Chapel Hill, NC 27599, United States

ARTICLE INFO

Article history:
Received 26 May 2008
Received in revised form 16 March 2009
Accepted 17 March 2009
Available online 22 April 2009

Keywords:
Double-strand break repair
NBS
Checkpoints
Haploinsufficiency

ABSTRACT

The MRN complex, composed of MRE11, RAD50 and NBS, plays important roles in responding to DNA double-strand breaks (DSBs). In metazoans, functional studies of genes encoding these proteins have been challenging because complete loss-of-function mutations are lethal at the organismal level and because NBS has multiple functions in DNA damage responses. To study functions of Drosophila NBS in DNA damage responses, we used a separation-of-function mutation that causes loss of the forkhead-associated (FHA) domain. Loss of the FHA domain resulted in hypersensitivity to ionizing radiation and defects in gap repair by homologous recombination, but had only a small effect on the DNA damage checkpoint response and did not impair DSB repair by end joining. We also found that heterozygosity for an *nbs* null mutation caused reduced gap repair and loss of the checkpoint response to low-dose irradiation. These findings shed light on possible sources of the cancer predisposition found in human carriers of *NBN* mutations.

1. Introduction

Cells have evolved multiple ways to preserve genome integrity. Successful genome maintenance ensures high-fidelity transmission of genetic material to daughter cells. This maintenance can be interrupted by DNA damage, which can be incurred either exogenously by genotoxic agents or endogenously due to metabolic errors. DNA double-strand breaks (DSBs), because they involve both strands, are highly toxic if unrepaired or repaired inaccurately. Mechanisms for repairing DSBs fall into two categories. In homologous recombination (HR), information from a homologous template is used to repair the DSB accurately. In contrast, in non-homologous end joining (NHEJ) the broken ends are joined with little or no use of homology. Cells lacking proteins required for either of these repair types manifest genome instability; in mammals, this can lead to cancer [1–3].

Prior to activation of repair, the DNA damage response is initiated through a cascade of events. Sensor proteins recognize the damage and trigger transducers, including checkpoint proteins that arrest the cell cycle. This is thought to allow time for repair processes to act. A given DNA damage response protein may be involved in one or more aspects of the response. A good example is the MRN/MRX complex, composed of MRE11, RAD50, and NBS (Xrs2 in *S. cerevisiae*), which participates in damage sensing, checkpoint activation, and DNA repair [4,5].

Null mutations in genes encoding MRN components are lethal in metazoans [6–8]. While lethality illustrates the importance of these genes, it also poses challenges for *in vivo* studies of their molecular functions. Nonetheless, substantial progress has been made in characterization of the component proteins of the MRN complex and of functions of the complex as a whole. The MRE11 subunit is an exonuclease [9,10]. Studies in *S. cerevisiae* have demonstrated that MRE11 is essential during repair of meiotic DSBs, but MRE11 is partially redundant with other exonucleases during DSB repair in vegetative cells [11–14]. In humans, mutations in *MRE11* cause ataxia telangiectasia-like disease (ATLD) [15], which is characterized at the cellular level by chromosome instability.

The RAD50 subunit has important enzymatic and structural functions, including ATPase activity and DNA bridging activity, both of which are required for DSB repair [16–19]. No genetic disorders have been associated with mutation in *RAD50*, but mouse *Rad50* mutants exhibit cancer predisposition and hematopoetic failure [20].

NBS/Xrs2 is the major regulator of the complex [21,22]. This protein has a nuclear-localization signal (NLS), and in its absence the MRN complex remains cytoplasmic even in the presence of DNA damage [6,23,24]. For this reason, functional studies carried out in *nbs* mutants can be used to understand the nuclear

* Corresponding author at: Department of Biology, CB #3280, University of North Carolina at Chapel Hill, Chapel Hill, NC 27599, United States. Tel.: +1 919 843 9400; fax: +1 919 962 8472.
E-mail address: sekelsky@unc.edu (J. Sekelsky).

1568-7864/$ – see front matter © 2009 Elsevier B.V. All rights reserved.
doi:10.1016/j.dnarep.2009.03.004

function of the complex as a whole. Specific mutations in *NBN* (the gene that encodes human NBS) that allow expression of a partially functional protein cause Nijmegen Breakage Syndrome [25–27]. Clinical features manifested by these patients include microcephaly, immunodeficiency, and lymphorecticular malignancies, and cells from these patients are hypersensitive to IR and have defects in DNA damage responses. In addition, linkage analyses have shown that heterozygous carriers of *NBN* mutations, which may be as frequent as 1 in 150 to 1 in 190 in some populations, are predisposed to several types of cancer [28–32]. Cells from *NBN* carriers also show gross genome instability. This may be explained by a recent study that found that the checkpoint protein ATM is not activated normally in response to low-dose ionizing radiation in cells heterozygous for an *NBN* mutation [33].

The MRN/MRX complex has been proposed to function during early steps of DSB repair. Models for meiotic recombination and repair of DSBs by homologous recombination (HR) in mitotic cells require that the 5′ ends of DSBs are resected to produce intermediates with 3′-ended single-stranded overhangs, which are then bound by strand invasion proteins. Meiotic cells from *S. cerevisiae mre11* mutants show an accumulation of unprocessed DSBs [13], leading to the proposal that the MRX complex participates in resection. *In vitro* studies demonstrated both exonuclease and endonuclease activities in the Mre11 protein [34]. It was suggested that coupling of these two activities might be required for resection, since the exonuclease activity had the opposite polarity of that required for resection. More recently, CtIP/Sae2, Exo1, and BLM/Sgs1 have been found to have important roles in resection, sometimes acting redundantly [35–37].

The role of the MRN/MRX complex in NHEJ remains unclear. Several studies have found a role for MRX in NHEJ in *S. cerevisiae* [38–41]. Experiments using HeLa cell extracts have implicated MRN in NHEJ [42], and studies of skin fibroblasts from NBS patients found a defect in microhomology-directed NHEJ [43]. In contrast, genetic experiments in the fission yeast *Schizosaccharomyces pombe*, studies with cell-free extracts from *Xenopus laevis*, and genetic analysis of chicken DT40 cells all failed to find a requirement for the MRN complex in NHEJ [44–46].

We sought to use Drosophila as another model in which to study roles of the MRN complex in DNA damage responses. We assayed end-joining and homologous repair simultaneously in an *in vivo* assay in mutants with reduced or altered NBS function. We found that either reducing levels of NBS or removing the N-terminal forkhead-associated (FHA) domain caused a defect in gap repair by HR. Among products repaired by NHEJ, loss of the FHA domain was associated with decreased usage of short (1–5 bp) microhomologies and increased usage of a 10-bp microhomology. Reducing the level of NBS also resulted in a profound defect in the DNA damage-dependent cell cycle checkpoint. These finding provides further insights into *in vivo* NBS functions in metazoans.

2. Materials and methods

2.1. Drosophila stocks and transgenic constructs

Drosophila stocks were maintained on standard medium at 25°. The null mutation used was nbs^{1} [6,47]. The hypomorphic allele used here was a *P* element insertion generated during the Berkeley Drosophila Gene disruption project and is available at the Bloomington stock center (# 21141) $y^{1}\ w^{67c23}$; $P\{EPgy2\}nbs^{EY15506}$. The *P* element is inserted in the 2nd exon of *nbs*, within coding sequences. A derivative of the $P\{EPgy2\}nbs^{EY15506}$ hypomorphic allele was generated for use in assays that involve *P* transposase. Males carrying $P\{EPgy2\}nbs^{EY15506}$ and *P* transposase were generated and crossed to w^{-} females, and progeny that had lost the w^{+} marker from $P\{EPgy2\}$ were screened for the absence of one end of the *P* element. The derivative we recovered, named nbs^{SM9}, lacks the 3′ *P* element end and has approximately 3 kb of the original 10 kb $P\{EPgy2\}$ remaining.

2.2. RNA analysis

For RNA blots, total RNA was isolated from wild-type w^{1118}, nbs^{P}, nbs^{SM9} and nbs^{1} homozygous larvae by homogenizing 10 larvae in 1 ml Trizol reagent. This was followed by organic phase-separation and RNA precipitation using standard methods. Polyadenylated RNA was selected using the Poly(A)Purist kit (Ambion). A probe from the 5′ end of *nbs* (Fig. 1b) was used to detect *nbs* transcripts. The structure of the transcripts of *nbs* mutants was determined with the 5′ RACE System for Rapid Amplification of cDNA Ends, version 2.0 (Invitrogen).

2.3. IR sensitivity measurements

Males and females balanced with *TM3*, *Sb* were crossed and allowed to lay eggs overnight at 25 °C on grape agar plates. Plates were then changed for a second collection. Second collection plates were incubated for two days, then exposed to gamma irradiation from a ^{137}Cs source; the first collection was used as an unirradiated control. After irradiation the grape agar with larvae was divided into four sectors and transferred to standard medium in bottles, then incubated at 25 °C until adults eclosed. Adults were counted to determine the ratio of mutant to non-mutant. For homozygous and heteroallelic combinations, non-mutants were *nbs*/*TM3*, *Sb* flies, which we had determined were not hypersensitive to IR at the doses used (Fig. 1d). Relative survival of mutants was determined by normalizing the mutant to non-mutant ratio in irradiated bottles to the ratio in unirradiated bottles.

2.4. Double-strand break repair assays

The $P\{w^{a}\}$ assay has been described in detail previously [48–51]. We counted progeny from multiple vials ($n = 53$ for +/+, $n = 67$ for nbs^{1}/+, and $n = 94$ for nbs^{1}/nbs^{SM9}), each with a single male parent. Each vial was treated as a separate experiment. Statistical comparisons were done for each pair of genotypes, using a Mann–Whitney test done with InStat 3.05 (Graphpad Software, Inc.). Tract lengths were compared through Fisher's exact test. Junction sequences from repair events were sequenced to understand the mechanism of joining. PCR was carried out with the forward primer 5′-CCCTGTCTGAAGTTCCGTAG-3′ and reverse primer 5′-CCCTCGCAGCGTACTATTGAT-3′, and products were sequenced with the forward primer.

We measured SSA with the assay of Rong and Golic [51]. In this assay, repair by SSA, which is the most common mechanism in wild-type flies, gives white-eyed progeny. We used a PCR assay to distinguish between SSA and deletion formation, which can also give rise to white-eyed progeny. To detect imprecise NHEJ among red-eyed progeny, PCR was conducted using the forward primer 5′-TGTGTGTTTGGCCGAAGTAT-3′ and the reverse primer 5′-CGCGATGTGTTCACTTTGCT-3′. Products were digested *in vitro* with *I-Sce*I enzyme (New England Biolabs). Those that did not cut were sequenced using the forward primer. Statistical comparisons were done by Kruskal–Wallis non-parametric ANOVA.

2.5. Cell cycle checkpoint assay

We measured the G2/M DNA damage checkpoint using an assay described previously [52]. Wing imaginal discs were dissected out of L3 larvae 1 h after exposure to 1000 or 4000 rads of gamma radiation from a ^{137}Cs source. A rabbit polyclonal antibody to