“Nibrin and promyelocytic leukemia proteins in sensing and signaling of the DNA double strand breaks”

“La nibrina e la proteina della leucemia promielocitica nel riconoscimento e segnalazione delle rotture al doppio filamento del DNA”

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“La mancanza di complessi, una notevole tenacia nel perseguire la strada che ritenevo giusta e la noncuranza per le difficoltà che avrei incontrato nella realizzazione dei miei progetti ...... mi hanno enormemente aiutato a far fronte agli anni difficili della vita”

Rita Levi Montalcini
INTRODUCTION

The DNA is the most vulnerable among the cell macromolecules and is constantly subjected to different both endogenous and exogenous genotoxic agents action (Kotula et al., 2013). Among these, reactive oxygen and nitrogen species, ionizing radiation (IR), ultraviolet radiation, and chemical mutagens cause different types of damage, from single base mutations to double-stranded breaks, considered the most lethal type of DNA damage (Friedberg et al., 2006; Wanotayan et al., 2015).

The integrity of the genome is of prime importance to ensure its stability and changes in the nucleotide sequence are continually monitored by mechanisms that prevent the occurrence of mutations and chromosomal rearrangements, thus preserving the original message in the DNA; in addition to these sophisticated mechanisms of DNA damage "sensing", the cell has developed others in which signal transducing and effector proteins regulate cell cycle progression, damage repair and apoptosis; overall, these mechanisms are known as "DNA damage response" (DDR) (Zhang et al., 2006; Rupnik et al., 2010; Brandsma and Gent, 2012). In particular, DNA repair is coordinated through the cell cycle checkpoint: in the presence of DNA damage, the activation of specific proteins leads to cell cycle arrest, giving the cell the time required to repair the damage before that DNA replication and mitosis have beginning (Sancar et al., 2004; Bernstein and Rothstein, 2009; Humpal et al., 2009). The repair of DNA in eukaryotic cells requires multiprotein repair complexes recruitment and assembly in situ, controlled through post-translational modifications. Among these, the phosphorylation signal is crucial in the regulation of protein-protein interactions (Williams et al., 2005; Glover, 2006).

Mutations in genes involved in the DDR are at the root of the chromosome instability observed in many human diseases characterized by the presence and/or increased susceptibility to cancer (Khanna and Jackson, 2001; Peterson and Côté, 2004).

1.1 The DNA double strand breaks response

The DNA Double Strand Breaks (DSBs) are the most pernicious forms of DNA damage, because an intact complementary strand is not used as a template for the DNA repair. The DSBs can be formed in response to the action of exogenous agents, such as IR, radio-
mimetic drugs, products of oxidative metabolism and mechanical stress (Khanna and Jackson, 2001). However, these breaks also occur as a result of physiological events such as replication, meiotic and V(D)J recombination, immunoglobulins class exchange, apoptosis and retroviral integration (Friedberg et al., 2006; Jackson and Bartek, 2009). If unrepaired or improperly repaired, the damaged sequences are neither transcribed nor replicated (Yu et al., 1999; Polo and Jackson, 2011). The cells have adapted to tolerate low levels of damage, however, if a single DSB occurs within an essential gene, it determines its inactivation and leads, eventually, to cell death (Yoshida and Miki, 2010; Souliotis and Sfikakis, 2014). Eukaryotic cells have evolved multicomponent macromolecular systems specializing in DNA damage sensing, response and repair (Jeggo and Löbrich, 2006; Shrivastav et al., 2008; Bernstein and Rothstein, 2009; Pardo et al., 2009, Gullotta et al., 2010; Lafrance-Vanasse et al., 2015).

The DSBs sensing requests the activation of the signaling pathway to amplify and transduce the damage signal and to generate the appropriate biological responses that include the block of the cell cycle in G1/S phase (G1/S checkpoint), or in the G2/M phase (van den Bosch et al., 2003), the slowdown of DNA synthesis (intra S-phase checkpoint) and the induction of the transcriptional program. For example, the small ribosomal protein RPS3 can regulate ribosome biogenesis in response to DNA damage (Kim et al., 2013) while RPS27a can activate cellular checkpoints via p53 to inhibit cell cycle (Xiong et al., 2011).

An example of a macromolecular complex specialized in the DNA damage sensing and in the early response to the DSBs is the MRE11-RAD50-NBN (MRN) complex, formed by dimers of the RAD50 and MRE11 subunits and by the NBN monomer. The MRN complex acts as a specific DSB sensor and effector involved in the cross-talk between the machinery repair and cell cycle checkpoints (Williams et al., 2010). It binds directly the broken DNA ends (Yuan and Chen, 2010) and once stabilized the injury, recruits the Ataxia-Telangiectasia Mutated (ATM) protein kinase, a master regulator of the DNA damage response. In fact ATM coordinates checkpoint activation, DNA repair, and metabolic changes in eukaryotic cells in response to DSBs and oxidative stress (Hopfner, 2014; Paull, 2015). In addition, MRN complex has a role in the processing and repair of the DSBs through the Homologous Recombination Repair (HRR) and the Non Homologous End Joining (NHEJ), in the telomere maintenance, in the reactivation of stalled replication (Slijpecevic,
2006; Borde, 2007; Williams et al., 2007; Chapman et al., 2012; Srivastava et al., 2015) (Fig. 1.1).

Figure 1.1: Kinetics of DDR protein recruitment and modification at IR-induced DSBs (Vignard et al., 2013).
The MRN complex importance is highlighted by several genetic pathologies existence, caused by mutations in genes codifying for MRE11, RAD50 and NBN protein. In individuals with ataxia telangiectasia-like disorder (ATLD; OMIM #604391), mutations in MRE11 have been identified (Stewart et al., 1999; Taylor et al., 2004); in patients with the Nijmegen breakage syndrome (NBS; OMIM #251260) mutations in NBN have been identified (Carney et al., 1998; Matsuura et al., 1998; Varon et al., 1998); finally, only one case of an individual suffering from the syndrome defined as NBS-like disorder (NLD; OMIM #613078), associated with a heterozygous mutation in the RAD50 gene, has been reported (Waltes et al., 2009). All the above-mentioned genetic syndromes are characterized by hypersensitivity to IR, genomic instability and immunodeficiency (The International Nijmegen Breakage Syndrome Study Group, 2000; Dizikiewicz-Krawczyk, 2008).

In response to the DSBs formation, PARP1 protein rapid accumulates on DSBs, induces its own PARylation together with the PARylation of the surrounding chromatin and many DDR actors; it promotes also the recruitment of MRE11 and NBN (Petritini and Stracher, 2003; Lisby and Rothstein, 2004; Haince et al., 2008; Helmink et al., 2009; Porter-Goff and Rhind, 2009; Shikazono et al., 2009; Vignard et al., 2013). The MRN complex localization on the DNA damage, determines ATM auto-phosphorylation at the Ser1981 residue, with the consequent dissociation of ATM dimers and ATM kinase activation (Bakkenist and Kastan, 2003; Kozlov et al., 2006). ATM in the monomeric active form phosphorylates NBN at Ser278 and Ser343 residues, and H2AX histone at the Ser139 residue (the phosphorylated form of H2AX being named γ-H2AX) (Rogakou et al., 1998; Lavin, 2007; Lavin and Kozlov, 2007; Lee, 2007; Vasireddy et al., 2010). MDC1 protein is required to maintain the link between the MRN complex and chromatin and to allow the accumulation (Hari et al., 2010, Tobias et al., 2013). Indeed, through the interaction between the MDC1 Ser-Asp-Thr (SDT) repeated motif (Chapman and Jackson, 2008) and the NBN FHA and BRCT domains (see section 1.2), the MRN complex is recruited on the chromatin to activate additional molecules of ATM kinase, which phosphorylates other molecules of histone H2AX, along several megabases away on both sides from the lesion site. This signal amplification determines the recruitment, on the damage site, of several proteins involved in DDR including 53BP1, BRCA1, p53, CHK1, CHK2, CDC25A, SMC1, RNF8/RNF168/HERC2 (Yuan and Chen, 2010; Hu et al., 2013; Muñoz et al., 2014).
The γ-H2AX histone generates an initial signal, on the DNA damage site, represented by Ionizing Radiation Induced Foci (IRIF) formation (Bassing et al., 2003; Celeste et al., 2003), large subnuclear macromolecular aggregates that facilitate the DNA damage repair, being the site where proteins involved in DDR cause cell cycle arrest and DSBs repair (Lowndes and Murguia, 2000; Masi et al., 2008b; Lee et al., 2010; Nakamura et al., 2010; Bauerschmidt et al., 2011).

The HRR and the NHEJ repair systems (Fig. 1.2) are respectively error-free and error-prone and operate optimally in different circumstances (Sancar et al., 2004; Benada et al., 2015; Liu et al., 2015; Wanotayan et al., 2015). In bacteria and unicellular eukaryotes the main mechanism of DSBs repair is the HRR, whereas in mammals is the NHEJ (Christmann et al., 2003; Sancar et al., 2004; Jeggo and Lobrich, 2006; Polo and Jackson, 2011; Brandsma and Gent, 2012). The HRR path predominates in S and G2 phases of the cell cycle and allows a faithful repair of the lesion using the DNA of homologous chromosomes or sister chromatid as template for new strand synthesis (Khanna and Jackson, 2001; van Gent et al., 2001; Symington, 2002; Williams et al., 2007; Woodbine et al., 2014). The NHEJ repair system prevails in the cells that are in the G0/G1 phase (Christmann et al., 2003). In the NHEJ system both broken ends are first recognized and then bound by the heterodimer Ku70/Ku80, which recalls the catalytic subunit of the DNA-dependent protein kinase (DNA-PK) (Ciccia and Elledge, 2010). If the protrusions are not compatible, they can be modified by Artemis nuclease or the Polμ and Polλ DNA-polymerases in order to generate blunt end (Lieber, 2010). Finally, the ligation complex made up of DNA ligase IV (LIG4), XRCC4 and XLF cofactors catalyzes ends joining (Yu et al., 1999; Christmann et al., 2003; Ahnesorg et al., 2006; Buck et al., 2006; Jeggo and Lobrich, 2006). The failure of these repair systems can induce the formation of chromosomal aberrations that can cause aneuploidy and consequently lead to developmental defects, neurodegeneration, immunodeficiency, radiosensitivity, predisposition to cancer, and cell death (Dikomey et al., 1998; Pfeiffer et al., 2000; Lips and Kaina, 2001; van den Bosch et al., 2003; Jackson and Bartek, 2009).
Figure 1.2 Schematic representation of the two main repair systems used by eukaryotes to repair DSBs: NHEJ and HR. HR is predominant in S and G2 phases of the cell cycle because of the proximity of the sister chromatid. NHEJ is active throughout the cell cycle, playing a major role during G1 and M phases (Srivastava et al., 2015).

1.2 Nibrin

Nibrin, also named NBS1 or p95, is involved in the initial DSBs sensing, but it is also a mediator of the DNA damage response signal because it interacts with downstream effectors involved in DNA repair and cell cycle checkpoint activation. Furthermore it is involved in chromosomal integrity maintenance (Zhang et al., 2006). NBN is localized in the region 21 of the chromosome 8 long arm (8q21) (Carney et al., 1998 and Varon et al., 1998) and is composed of 16 exons (Matsuura et al., 1997). The NBN protein, ubiquitously expressed in different tissues (Kobayashi et al., 2004; Zhang et al., 2006), is long 754 amino acids (aa), has a molecular weight of about 85 kDa and is composed of three functional regions: an N-terminal region, a central region and a C-terminal region (Fig. 1.3).
Figure 1.3 NBN main structural components. In N-terminal region, the FHA, BRCT1 and BRCT2 mediate the interaction with phosphoproteins through the binding to pThr (FHA) and pSer (BRCT) residues. The C-terminal region presents sites for MRE11 and ATM recognition. The yellow spheres indicate sites phosphorylated by ATM (Williams et al., 2010).

1.2.1 Nibrin N-terminal and central region

Nibrin N-terminal region (Fig. 1.4) contains the Fork Head Associated Domain (FHA) (aa 24-109) and two Breast Cancer 1 (BRCA1) Carboxy-Terminal (BRCT) domains (BRCT1: aa 114-183; BRCT2: aa 221-291) separated from a linker region of 18 amino acids (Bork et al., 1997; Callebaut and Mornon, 1997; Becker et al., 2006). This region of NBN plays a key role in the recognition of DNA damage through the interaction with γ-H2AX histone and is responsible for the MRN complex localization in IRIF (Tauchi et al., 2001; Zhao et al., 2002; Rupnik et al., 2010) (see section 1.1).

In particular some authors argue that the NBN protein possesses the structural determinants (i.e. the BRCT domains) to interact directly with γ-H2AX histone (phosphorylated H2AX) (Kobayashi et al., 2002; di Masi et al., 2008a; di Masi et al., 2012; Mendez et al., 2012;), others suggest that NBN binds directly only MDC1, which in turn, through its tandem BRCT (tBRCT) domains, binds directly to γ-H2AX C-terminal (Lee et al., 2005; Stucki et al., 2005; Coster and Goldberg 2010).

The FHA domain is a protein-protein interaction domain that specifically recognizes the phosphorylated Thr residues of several target proteins involved in numerous biological processes, such as growth and cell division, differentiation, apoptosis, transcription, proteins transport, DNA repair, and protein degradation (Durocher et al., 2000; Li et al., 2000; Liang and Van Doren, 2008). Ohara et al. (2014) suggest that the expression of a FHA-mutated nibrin causes radiosensitization, suggesting a FHA domain role in preventing the generation of DNA mutations caused by ionizing radiations.
Furthermore the FHA interacts with phosphorylated Thr residues located in the Ser-Asp-Thr (SDT) motif of MDC1, and is necessary for the IRIF and multiprotein complexes formation that assemble after DSBs formation (Zhao et al., 2002). The BRCT domains have been identified in many proteins involved in the DDR and may be present in single or multiple copies (Callebaut and Mornon, 1997; Leung and Glover, 2011). Tandem BRCT domains, described for the first time in the BRCA1 protein (Futreal et al., 1994; Miki et al., 1994), function as a single structural unit in the phosphorylated peptides recognition (Mesquita et al., 2011). NBN tBRCT domains are characterized by a globular α/β folding made up of central 4-β filaments flanked by 3 α-helices (α1 and α2 on one side and α3 on the opposite side).

Figure 1.4 Structural details of the NBN N-terminal region (modified from Williams et al., 2009).
The large hydrophobic interface that is formed between the BRCT1 domain C-terminal region (helices α1 and α3) and the BRCT2 domain N-terminal (helix α2) represents the recognition site for phosphoproteins and phosphopeptides (Williams et al., 2001; Glover et al., 2004). The residues placed in the α1 and α3 helices and less in the α2 helix, are preserved, suggesting that the packaging "head-tail" is stored in the members of the BRCT superfamily (Finn et al., 2008). Moreover, it is interesting to note that their tight proximity allows the NBN FHA domain and N-terminal BRCT domain to be considered as a single and compact globular structure required for the MRN complex localization on chromatin after DNA damage (Hari et al., 2010). Two conserved Ser residues (Ser278 localized at the BRCT2 C-terminal and Ser343 in the central region) are phosphorylated by ATM in response to IR and are associated with both p53 dependent- apoptosis regulation (Iijima et al., 2008) and the NBN role in the cell cycle S phase checkpoint (Lim et al., 2000; Wu et al., 2000; Zhao et al., 2000; Zhang et al., 2006; Rupnik et al., 2010).

1.2.2 Nibrin C-terminal region

The nibrin C-terminal region contains a motif for the binding with MRE11 (aa 689-691) (Desai-Mehta et al., 2001) and ATM, (aa 734-754) (Falck et al., 2005).

1.2.3 The Nijmegen breakage syndrome

It is fundamental that nibrin preserves its structural integrity since mutations within nibrin are responsible of cancer proneness. In particular mutations at the homozygous status are responsible for NBS, a rare genetic disorder characterized by an autosomal recessive inheritance whose signs are, among the others, immunodeficiency, microcephaly, hypersensitivity to IR, and predisposition to the development of lymphomas and solid tumors (Weemaes et al., 1981; van der Burgt et al., 1996; Chrzanowska et al., 2012). NBS is a rare autosomal recessive disease characterized by chromosomal instability, described for the first time in 1981 in patients living in Nijmegen (Netherlands) (Demuth and Digweed, 2007). The NBS is manifested at birth with microcephaly, dysmorphic facial features that become more apparent with age, immune deficiency and growth retardation. Late complications involve malignant tumors
development, especially of hematic origin, and infections in various organs (Chrzanowska et al., 2012). From several years NBS diagnostic markers are known: 1) hypomorphic biallelic mutations in \textit{NBN}; 2) spontaneous chromosomal instability in peripheral T lymphocytes, with rearrangements like inversions and translocations, especially involving 7 and 14 chromosomes; 3) cellular sensitivity to IR or radiomimetic agents demonstrated \textit{in vivo}; 4) RDS. The 90\% of NBS patients present the \textit{NBN} 657del5 founder mutation, which occurs mainly in Slav populations (Weemaes et al., 1981; Carney et al., 1998; Varon et al., 1998; Maser et al., 2001; Chrzanowska et al., 2012). NBS was also found in other European countries (including Italy), in North America, Morocco, and New Zealand (Chrzanowska et al., 2012).

\subsection{1.2.3.1 The 657del5 founder mutation}

The 657del5 mutation consists of a 5 bp deletion in the linker region connecting the two NBN BRCT domains. The resultant shift of the reading phase involves the expression of two truncated proteins of 26 kDa (p26) and 70 kDa (p70) and causes the interruption of the two tBRCT. In particular p26 contains the FHA region and the first BRCT domain, and is the product of a protein synthesis premature termination, while the p70 fragment, which includes the second BRCT domain and the entire NBN C-terminal region, originates from an alternative initiation of translation at a cryptic upstream start codon (Maser et al., 2001; Williams et al., 2002; Becker et al., 2006; Alster et al., 2014). With the exception of the first 18 amino acids, the protein p70 has a sequence identical to that of wild-type NBN. It is known that the p26 peptide, preserving the NBN FHA/BRCT1 domains that are essential for both the physical interaction with $\gamma$-H2AX and for the re-location of RAD50 and MRE11 near the site of DNA damage, appears to retain ability to recognize $\gamma$-H2AX (Kobayashi et al., 2002). These events do not seem to take place efficiently in cells from patients expressing both p26 and p70 fragments, as demonstrated by unrepaired DSBs persistence 24 hours after treatment with IR (di Masi et al., 2008a). However truncated p70-nibrin can form the MRN complex (Kruger et al., 2007; Maser et al., 2007). This aspect suggests that the fragments present a residual retention of the pivotal protein interactions, generally established by wild-type nibrin.

To date, in \textit{NBN} ten other mutations have been identified (Fig. 1.5) in the homozygous or compound heterozygous status and considered
responsible for the syndrome development (Tupler et al., 1997; Varon et al., 1998; Maraschio et al., 1986 and 2001; Nakanishi et al., 2002; Resnick et al., 2002; Gennery et al., 2004; New et al., 2005).

Figure 1.5 NBN gene schematic representation and localization of the mutations responsible for NBS development (Modified from Demuth and Digweed, 2007).

In NBS patients with the 657del5 mutation, an NBN loss of function is observed. The loss of an efficient DSBs repair mechanism, observed in cells prepared from NBS patients, causes severe clinical characteristics in NBS patients, displaying the important NBN role in DSBs signaling and repair, fundamental for the cellular homeostasis maintenance. In particular, the cancer beginning are associable with the chromosomal aberrations high frequency (Chrzanoswska et al., 2012); the radiosensibility is imputable to the NBN role in the response to the IR-induced damage and to its non-phosphorylation by ATM (Gatei et al., 2000; Wu et al., 2000); the microcephaly is a characteristic of the syndromes associated with defects in DDR and may be caused by an increased cell death, associated with an inefficient DNA repair (the microcephaly seems to be caused by a decreased neurogenesis derived from an increased cellular apoptotic death or from the reduced neuronal precursors proliferative capacity (O’Driscoll and Jeggo, 2008)).
2.1 PML and PML-Nuclear Bodies

The ProMyelocytic Leukemia (PML) human gene is localized in the q22 region of chromosome 15. It codifies for PML, an oncosuppressor of almost 70 kDa. The PML alternative splicing generates 6 nuclear and one cytoplasmic isoforms (Jensen et al., 2001; Bernardi and Pandolfi, 2007). The PML-NBs are non-membrane bound spherical organelles of 0.1-1 µm of diameter, made up of an intricate protein system (Melnik et al., 1999). PML is the NBs regulator protein and together with SP100 represents the permanent component of these macromolecular aggregates. The PML-NBs are stable structures and highly dynamic and their distribution changes highly in relation to cell type, cell cycle phase or to the possible exposition to stress factors (Jensen et al., 2001; Bernardi and Pandolfi, 2007).

2.2 PML-NBs post-translational modifications

The PML post-translational modifications regulate several PML-NBs functions, as also their assembly or disassembly during the cell cycle phase. The PML phosphorylation events involve mostly the N-terminal region of the protein and are associated with PML-NBs role in DDR.

Figure 2.1 Particular of a PML-NB formation. At first SUMO-1 was distributed more sparsely and also more aggregated than PML. A partial colocalization of PML and SUMO-1 in the same spherical shell was evident. By contrast, SUMO-2/3 was located also in the interior of the PML-NB. Sp100 was distributed similarly to PML in the outer shell of spherical shape (Lang et al., 2010)
The PML phosphorylation sites are localized near the Real Interesting New Gene (RING) domain, in the Nuclear Localization Signal (NLS) sequence, and in the SUMO-Interacting Motif (SIM) (Bernardi and Pandolfi, 2007; Gresko et al., 2009; Schmitz and Grishina, 2012). During the DDR, the kinases ATM, ATR and CHK2 act phosphorylating these PML sites, regulating their stability and of the proteins constituting PML-NBs (Dellaire et al., 2006).

The PML SUMOilation regulates PML stability, dissociation of the transcription factors from PML-NBs, PML regulation of apoptosis, PML-NBs structure maintainance, protein-protein interaction (Ishov et al., 1999; Zhong et al., 2000; Nacerddine et al., 2005; Geiss et al., 2007; Imani-Saber et al., 2014). The PML-NBs formation, also known as nucleation, happens during cell cycle interphase and disappears in mitosis when the activity level of SUMO enzymes lowers and the PML-NBs become more little (Everett et al., 1999) (Figure 2.1).

2.3 PML-NBs role in DNA damage sensing and repair

PML-NBs are constituted by a complex protein network represented by a “basket”, in which are concentrated several proteins, involved also in DNA damage repair (D’Orazi et al., 2002; Hofmann et al., 2002; Dellaire et al., 2006; Yang et al., 2006;). These proteins, being concentrated in the environment delimited by PML-NBs, are readily available for an efficient and rapid DNA repair (Carbone et al., 2002; Dellaire and Bazett-Jones, 2004; Bernardi and Pandolfi, 2007; Kepkay et al., 2011). PML-NBs integrity allows tumor suppressive functions via diverse biologic mechanisms such as growth inhibition, apoptosis induction and suppression of migration and angiogenesis (Chen et al., 2012).

It is important to underline that changes in PML-NBs morphology and number have been observed in response to DNA damage (Mirzoeva and Petrini, 2001; Carbone et al., 2002; Hofmann et al., 2002; Kurki et al., 2003; Seker et al., 2003; Conlan et al., 2004; Eskiew et al., 2004). In fact the stress induced in the chromatin is transmitted to PML-NBs that reply breaking and forming more little nuclear bodies named microbodies (Eskiew et al., 2004; Dellaire et al., 2006). Several DDR proteins are localized in the PML-NBs both constitutively and conditionally, including the DSBs sensing proteins (e.g. MRN complex, ATM, ATR, BRCA1, CHK2, p53, and TOPBP1) and the proteins participating to HRR (Yeager et al., 1999;
Since the repair proteins are localized within PML-NBs, it has been suggested that the PML-NBs may have a role in DSBs sensing and in DNA damage repair events coordination (Dellaire and Bazett-Jones, 2004). It is important to consider that after DSBs induction the Ser824 residue phosphorylation of the KAP1 protein associated to the heterochromatin actives ATM kinase (Kruhlak et al., 2006; Ziv et al., 2006), with the consequent PML-NBs number increment. It has been suggested that this phosphorylation, inducing other nuclear bodies’ formation, facilitates the repair factors access to damage site on chromatin (Kepkay et al., 2011). Also CHK2 kinase protein has a key role in the DNA damage transduction. It is phosphorylated by ATM at Thr68 residue, depending from NBN, and phosphorylates PML protein localized in the nuclear bodies (Stolz et al., 2011).

2.4 PML-RARα and the Acute Promyelocytic Leukemia

The Acute Promyelocytic Leukemia (APL) is a rare malignant tumour characterized by hemorrhagic manifestation caused by altered blood coagulation (Breccia and Lo Coco., 2014). The disease evolves rapidly with unlucky prognosis. The main disease cause is the differentiation block of the myeloid precursor cells, at the promyelocytes stadium, that so proliferate in an uncontrolled way till they invade bone marrow (de Thè et al., 1991; Kakizuka et al., 1991; Nisole et al., 2013).

APL patients are characterized by PML-RARα gene fusion, caused by the t(15;17) (q22;q21) chromosomal translocation, at the heterozygous status, where RARα codifies for the Retinoic Acid Receptor Alpha localized on chromosome 17 (Rowely et al., 1977; de Thè et al., 1990; de Thè et al., 1991, Brown et al., 1997). The translocation involves one of the three breakpoint chromosomal regions (bcr), located within PML gene and the chromosome 17 breakpoint located within the RARα gene second intron (Miller et al., 1992; Pandolfi et al., 1992). The resultant isoforms maintain the PML DNA binding domain and the binding domain to co-activators/co-repressors, ligands and RARα Retinoid-X-receptor (RXR) (Brown et al., 1997) (Figure 2.2). In APL the PML-NBs are substituted by little nuclear bodies named microspeckles (Melnick and Licht, 1999). While in normal condition RXR (the other retinoic acid nuclear
receptor class (Bushue e Wan, 2010)) binds RARα and the RXR-RARα complex is associated to the Retinoic Acid Response Element (RARE) gene sequence, regulating the expression of the genes involved in the myeloid cells last differentiation in granulocytes (de Thé et al., 1991; Brown et al., 2009), on the contrary, the PML-RARα homodimerizes, binds the DNA, blocking the RARα dependent expression of differentiation genes, and forms aggregates with PML wild-type isoforms, forbidding the normal PML interaction (through a competitive way) (Melnik et al., 1999; Brown et al., 2009; Yeung et al., 2012). The loss of these functions contributes to leukemic promyelocytes proliferation and survival (Brown et al., 2009). The PML-RARα dominant negative effect on the PML-NBs formation and damage repair is responsible for a high chromosomal instability in APL patients (Lanotte et al., 1991; Bishof et al., 2001; Yeung et al., 2012). In fact, it has been demonstrated that the HR mechanism to repair DNA depends on the wild type PML protein presence and on its correct organization in PML-NBs (Yeung et al., 2012).

2. 5 APL treatments

The APL patients are cared with All-Trans Retinoic Acid (ATRA) and/or Arsenic Trioxide (ATO) (Khanna-Gupta et al., 1994; Yang et al., 2006; Brown et al., 2009; de Thé et al., 2012; Yeung et al., 2012). The two molecules destroy the PML-RARα oncoprotein, induce the promyelocytes differentiation and the transient reversion to wild type phenotipe, suppressing the dominant negative effect induced by the fusion protein expression (di Thé et al., 2012; Vitaliano-Prunier et al., 2014) (Figure 2.2). In particular, ATRA binds the AF-2 motif in the RARα protein C-terminal region, determining the dissociation of PML-RARα complexes bound to DNA RARE sequences (Tate et al., 1994; de Thé et al., 2012). Furthermore, ATRA restores the RARα target genes activation. ATRA treatment allows new normal NBs formation, from PML proteins produced by non-mutated allele (de Thé et al., 2012).

The importance of the effect of PML-NBs integrity loss on the DNA damage signal transduction has allowed to study this relationship and to discover that PML-RARα suppresses CHK2 kinase protein phosphorylation, inhibiting the CHK2-p53 apoptotic pathway in APL. On the contrary the ATO treatment of cell lines prepared from APL patients determines promyelocytes maturation and/or apoptosis through the direct binding with PML protein and PML-RARα
(Zhang et al., 2010). The ATO in fact stimulates the fusion protein degradation, promoting its iperSUMOilation (Lallemand-Breitenbach et al., 2001; Lallemand-Breitenbach et al., 2008; Thatam et al., 2008; Jeanne et al., 2010). The arsenic induces also new PML-NBs formation.

Recent studies performed in mice have been demonstrated that the ATRA and ATO combination as care for APL increases the complete reversion and the number of survivors. For this reason APL is among the first cancer example for which a care with a specific target exists (de Thè et al., 2012). Finally another valid therapy for the care of acute promyelocytic leukemia derives from a study of Luo et al. (2014) that tested a siRNA targeting PML-RARα mRNA, demonstrating that the cell growth of siRNA treated groups was inhibited, and the apoptosis of APL human cell line NB4 could be induced.

Figure 2.2 In a) PML-RARα fusion protein and the main PML and RARα functional domains are represented. The ATRA target is localized in a RARα motif, while the arsenic target is localized in a PML motif. The black triangles indicate the different fusion points. In b) the leukemic cells differentiation after ATRA treatment are observed. In c) the action model relative to the differentiation or transcriptional control through gene activation (up) or repression mediated by degradation (low) is schematized (modified from de Thè et al., 2012).
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Publications
Research Communication

Cleavage of the BRCT Tandem Domains of Nibrin by the 657del5 Mutation Affects the DNA Damage Response Less Than the Arg215Trp Mutation

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657del5 nibrin mutation; Arg215Trp nibrin mutation; BRCT tandem domains; DNA damage response; DNA double-strand breaks; MRN complex; nibrin; Nijmegen breakage syndrome.

Abstract

The Nijmegen breakage syndrome (NBS) is a genetic disorder caused by mutations in NBN gene and characterized by chromosomal instability and hypersensitivity to ionizing radiations (IR). The N-terminus of nibrin (NBN) contains a tandem breast cancer 1 (BRCA1) carboxy-terminal (BRCT) domain that represents one of the major mediators of phosphorylation-dependent protein-protein interactions in processes related to cell cycle checkpoint and DNA repair functions. Patients with NBS compound heterozygous for the 657del5 hypomorphic mutation and for the Arg215Trp missense mutation (corresponding to the 643C>T gene mutation) display a clinical phenotype more severe than that of patients homozygous for the 657del5 mutation. Here, we show that both the 657del5 and Arg215Trp mutations, occurring within the tandem BRCT domains of NBN, although not altering the assembly of the MRE11/RAD50/NBN (MRN) complex, affect the MRE11 IR-induced nuclear foci (IRIF) formation and the DNA double-strand
break (DSB) signaling via the phosphorylation of both ataxia-telangiectasia-mutated (ATM) kinase and ATM downstream targets (e.g., SMC1 and p53). Remarkably, data obtained indicate that the cleavage of the BRCT tandem domains of NBN by the 657del5 mutation affects the DNA damage response less than the Arg215Trp mutation. Indeed, the 70-kDa NBN fragment, arising from the 657del5 mutation, maintains the capability to interact with MRE11 and γ-H2AX and to form IRIF. Altogether, the role of the tandem BRCT domains of NBN in the localization of the MRN complex at the DNA DSB and in the activation of the damage response is highlighted. © 2012 IUBMB, IUBMB Life, 64(10):853–861, 2012

Abbreviations

DAPI, 40,6-diamidine-2-phenylindole; 53BP1, p53 binding protein 1; ATM kinase, ataxia-telangiectasia mutated kinase; BRCA1, breast cancer 1; BRCT, BRCA1 carboxy terminal domain; DDR, DNA damage response; DSB, DNA double-strand break; FHA, forkhead associated domain; IR, ionizing radiation; IRIF, IR-induced nuclear foci; MDC1, mediator of DNA-damage checkpoint 1; MRN, MRE11/RAD50/NBN complex; NBN, nibrin; NBS, Nijmegen breakage syndrome; NLS, nuclear localization signal; PVDF, polyvinylidene fluoride; SMC1, structural maintenance of chromosomes protein 1A; γ-H2AX, phosphorylated H2AX.

INTRODUCTION

Nibrin (NBN), a member of the trimeric complex formed by MRE11, RAD50, and NBN (MRN), is involved in several phases of the DNA double-strand break (DSB) damage response, including sensing, signaling, and repair of DNA lesions (1, 2). After DNA damage, several proteins involved in the damage response (including MRN) accumulate in large subnuclear structures, called ionizing radiation-induced nuclear foci (IRIF) (3–5). In mammalian cells, the histone H2AX represents a crucial component of IRIF. In fact, in response to DSB induction, H2AX is phosphorylated at the Ser139 residue (the phosphorylated H2AX protein being named γ-H2AX) by members of the phosphoinositide-3-kinase-related protein kinase family [e.g., ataxia-telangiectasia-mutated (ATM) kinase and the DNA-dependent kinase (DNA-PK)] (6). The p53 binding protein 1 (53BP1) functions downstream of a γ-H2AX-dependent hierarchy of proteins (e.g., the MRN complex, ATM, and the mediator of DNA-
damage checkpoint 1 protein (MDC1)) that collectively establish IRIF at DSB sites (7–11). NBN is composed of three regions. The N-terminal region contains the fork-head-associated (FHA) domain (amino acid residues 24–109) and two breast cancer 1 (BRCA1) carboxy-terminal (BRCT) tandem domains (i.e., BRCT1, amino acid residues 114–183; and BRCT2, amino acid residues 221–291) (12, 13). Notably, the BRCT domains are the major mediators of phosphorylation-dependent protein-protein interactions in cell cycle checkpoint and DNA repair mechanisms (14–20). The central region of NBN contains a consensus sequence encompassing the Ser343 residue that, together with the Ser278 residue located within the BRCT2 domain, is phosphorylated by ATM kinase in response to IR (21–24). Lastly, the C-terminal region of NBN contains the MRE11-binding domain and the ATM-binding motif (12, 25). Mutations at the homozygous or compound heterozygous status within the NBN gene are responsible for the Nijmegen breakage syndrome (NBS; OMIM #251260), a rare genetic disorder characterized by an autosomal recessive inheritance, whose signs are microcephaly, immunodeficiency, spontaneous chromosomal instability, and sensitivity to IR (25). The majority of patients with NBS are homozygous for the 657del5 hypomorphic mutation in exon 6 of NBN (1, 25). This mutation determines the synthesis of two fragments of NBN with molecular weights of 26 and 70 kDa. The 26-kDa fragment includes the FHA and the BRCT1 domains, whereas the 70-kDa fragment contains the BRCT2 domain and the C-terminal region (13, 26). A further relevant mutation located within the NBN tandem BRCT domains is the 643C>T mutation (corresponding to the Arg215Trp substitution), which affects the relative orientation of the tandem BRCT domains, impairing γ-H2AX binding (27). Remarkably, both the 657del5 and Arg215Trp NBN mutations are responsible for cancer susceptibility both at the homozygous and heterozygous status (17, 20, 25, 28–30).

Here, we show that the 657del5 and Arg215Trp mutations, affecting the NBN tandem BRCT domains, do not alter the NBN ability to form the MRN complex and to be efficiently phosphorylated. However, these mutations affect the NBN-dependent relocalization of MRE11 from the cytoplasm to the nucleus and the phosphorylation of both ATM and ATM downstream targets (i.e., the structural maintenance of chromosomes protein 1A (SMC1) and p53). Remarkably, the cleavage of the BRCT tandem domains of NBN by the 657del5 mutation affects the DNA damage response less
than the Arg215Trp mutation. In fact, the 70-kDa fragment, arising from the 657del5 mutation, maintains the capability to interact with MRE11 and γ-H2AX to form IRIF, even if this complex does not allow the full activation of the DSB repair and related signaling pathways.

**EXPERIMENTAL PROCEDURES**

**Cells, Culture Conditions, and Irradiation**

The SV40-transformed fibroblasts established from a normal donor (here named MRC-5), from the GM7166VA7 NBS patient (here named NBS) homozygous for the 657del5 founder mutation, and from the NBS cell line stably transfected with the vector pLXIN-NBN carrying the 643C>T mutation (corresponding to the amino acidic substitution Arg215Trp; here named R215W) (27), were grown in DMEM (VWR International, Milan, Italy) supplemented with 10% fetal bovine serum (VWR International), 100 µg/mL penicillin and 100 µg/mL streptomycin (VWR International). Transfected cells stably expressing the Arg215Trp NBN variant were maintained in the above media with 800 µg/mL G418 (Calbiochem Merck, Darmstadt, Germany). As a control, NBS cells were also separately transfected with the vector alone (here named NBS-pLXIN), and the clones obtained were grown in the presence of 800 µg/mL G418.

DSBs were induced by exposing cells to X-rays, using a MGL 300/6-D apparatus (250 kV, 6 mA, Cu filter, dose rate 0.53 Gy/min; Gilardoni, Mandello Lario, Italy).

**Immunoblotting**

Total cell lysates were prepared by lysing cells in 20 mM Tris-HCl pH 8, 137 mM NaCl, 10% glycerol (v/v), 1% NP-40 (v/v), 10 mM EDTA, 1 µg/mL aprotinin, 1 µg/mL leupeptin, 1 µg/mL pepstatin, 1 mM orthovanadate, and 2 mM PMSF. The protein concentration was determined by the Bradford protein assay.

Thirty micrograms of protein extracts were loaded onto a SDS-PAGE and then transferred to polyvinylidene fluoride (PVDF) membrane (Bio-Rad, Hercules, CA). Membranes were blocked in a solution composed of PBS, 3% BSA (w/v), and 0.5% Tween-20 (v/v) for 40 min at room temperature. Primary antibodies were incubated overnight at 4°C, and final concentrations used were as follows: 1 µg/mL anti-NBN rabbit polyclonal antibody (Abcam,
Cambridge, UK), 1 µg/mL anti-ATM-phosphoSer1981 (Abcam), 0.5 µg/mL anti-ATM mouse monoclonal antibody (Novus Biologicals, Littleton, CO), 2 µg/mL anti-SMC1-phosphoSer966 rabbit polyclonal antibody (Abcam), 1 µg/mL anti-SMC1 rabbit polyclonal antibody (Chemicon, Temecula, CA), 1 µg/mL anti-p53-phosphoSer15 mouse monoclonal antibody (Cell Signaling Technology, Danvers, MA), 1 µg/mL anti-p53 mouse monoclonal antibody (Santa Cruz, Santa Cruz, CA), 1 µg/mL anti-RAD50 mouse monoclonal antibody (Abcam), and 1 µg/mL anti-MRE11 monoclonal antibody (Abcam). Primary antibodies were detected by incubating the membranes for 1 h at room temperature with secondary anti-mouse or anti-rabbit antibodies HRP-conjugated (GE Healthcare, Fairfield, CT) and visualized using the enhanced chemiluminescence detection system (Amersham Biosciences).

Coimmunoprecipitation

Five hundred micrograms of the whole protein extracts were purified with protein A-agarose (GE Healthcare) for 1 h at 4°C on a rotating wheel. Precleared protein lysates were immunoprecipitated overnight at 4°C on a rotating wheel, using 3 µg of anti-NBN antibody directed against the C-terminus of NBN (amino acid residues 700–754; Abcam) and protein A-agarose. The immunocomplexes were washed four times in lysis buffer, loaded onto a 10% SDS-PAGE, and finally transferred to polyvinylidene fluoride membrane. Filters were probed with either 1 µg/mL anti-RAD50 mouse monoclonal antibody (Abcam) or 1 µg/mL anti-MRE11 mouse monoclonal antibody (Abcam).

The interaction between γ-H2AX and the NBN fragments arising from the 657del5 NBN mutation was analysed using 1.5 mg of whole protein extracts that were purified with protein G-Sepharose (GE Healthcare) and immunoprecipitated using 5 µg of anti-γ-H2AX antibody (Upstate, Millipore, Billeica, MA). The immunocomplexes were loaded onto a 10% SDS-PAGE, and the filters were probed with either 1 µg/mL anti-NBN rabbit polyclonal antibody (Abcam) or 1 µg/mL anti-NBN rabbit polyclonal antibody (amino acid residues 700–754; Abcam).

Immunofluorescence

The NBN, MRE11, γ-H2AX, and 53BP1 localization analyses were performed as previously described (31). Briefly, cells were grown on glass cover slips, irradiated, and fixed with 4% paraformaldehyde by
maintaining cells for 10 min on ice. After permeabilization with PBS/0.2% Triton X-100 (v/v), cells were blocked in PBS/1% BSA. NBN and MRE11 localizations were detected by costaining overnight at 4°C with 10 µg/mL anti-NBN (amino acid residues 700-754; Abcam) and 5 µg/mL anti-MRE11 (Abcam) primary antibodies. The localizations of γ-H2AX and the 70-kDa NBN fragment were detected by incubating overnight at 4°C with 10 µg/mL anti-γ-H2AX (Upstate) and 10 µg/mL anti-NBN (amino acid residues 700-754; Abcam) primary antibodies. After the incubation of primary antibodies, cells were washed in PBS/1% BSA and incubated for 1 h at 37°C with either an anti-mouse FITC-conjugated (Vector, Burlingame, CA) or an anti-rabbit Alexa 610 Fluor-conjugated secondary antibodies (Invitrogen Molecular Probes, Madison, MA). For 53BP1 foci analysis, the 53BP1 protein was detected using 10 µg/mL anti-53BP1 primary antibody (Abcam) and an anti-rabbit Alexa 610 Fluor-conjugated secondary antibody. DNA was counterstained with 0.2 µg/mL 4′,6-diamidine-2-phenylindole (DAPI) (Sigma-Aldrich), and the slides were mounted with an anti-fade solution (Vectashield, Vector). Slides were analyzed by means of an Axio Imager M1 fluorescent microscope (Zeiss, Germany) equipped with a charged coupled device camera. Images were re-elaborated with Adobe Photoshop 7.0 software (Adobe Systems, Mountain View, CA). All the immunofluorescence experiments have been repeated three times. Quantitative analysis was carried out by counting foci in at least 50 cells per experiment.

RESULTS

The 657del5 and Arg215Trp NBN Mutations Do Not Alter the Formation of the MRN Complex but Affect the Proper Recruitment of MRE11 to the DSB Sites

To determine whether mutations within the tandem BRCT domains of NBN may affect the MRN complex formation, NBN binding to MRE11 and RAD50 has been assessed. As shown in Fig. 1, in NBS cells the 70-kDa fragment of NBN (arising from the 657del5 mutation) is able to interact with both MRE11 and RAD50. Similarly, also the Arg215Trp NBN mutation does not affect the proper interaction between NBN and the two other members of the MRN complex.
Figure 1. The tandem BRCT domains of NBN are not involved in the formation of the MRN complex. MRC-5, NBS, and R215W cells were treated with 10 Gy of X-rays and lysed after 1 h. Five hundred micrograms of whole protein extracts have been immunoprecipitated with 3 µg of anti-NBN antibody directed against the C-terminal region of NBN. Filters were probed with anti-MRE11 and anti-RAD50 antibodies. Figures show representative blots of two independent experiments. For details, see the text.

To study whether the tandem BRCT domains of NBN may have a role in the recruitment of MRE11 from the cytoplasm to the nucleus, as well as in MRE11 IRIF formation, double-immunofluorescence analysis has been performed. Data shown in Fig. 2A indicate that in MRC-5 untreated fibroblasts, NBN and MRE11 are mainly localized into the nucleus and to a lesser extent into the cytoplasm, both with a diffuse pattern. After IR treatment, the two proteins colocalize forming discrete foci within 1 h (mean value: 30 foci per cell); 6 h after IR, almost all the foci disappear (mean value: 10 foci per cell); 24 h after the IR treatment, the number of MRE11 and NBN foci per cell returned to the baseline level (Fig. 2B).

On the contrary, in both NBS and R215W-untreated fibroblasts, MRE11 shows a completely different localization with respect to the MRC-5 cells (Fig. 2A). Indeed, in NBS fibroblasts MRE11 localizes mainly in the cytoplasm and to a lesser extent into the nucleus (26, 32), whereas in R215W cells MRE11 localizes completely into the cytoplasm. Remarkably, in NBS fibroblasts, and more severely in the R215W cells, an altered pattern and time course of IRIF formation occurs after IR treatment (Fig. 2B). Indeed, in NBS cells, 1 h after IR, MRE11 completely localizes into the nucleus without forming IRIF. NBN and MRE11 colocalization occurs after 6 h from the IR treatment (mean value: 27 foci per cell); after 24 h, the number of residual IRIF is significantly higher with respect to the MRC-5 cell line (mean value: 8 foci per cell). In contrast, in the R215W fibroblasts, MRE11 is still cytoplasmatic at 6 h from the IR treatment (signals are extremely diffused and difficult to be quantified).
Figure 2. The tandem BRCT domains of NBN play a role in the proper recruitment of MRE11 to the DSB sites. A: MRC-5, NBS, and R215W untreated cells. B: MRC-5, NBS, and R215W cells exposed to 1 Gy of X-rays and fixed after 1, 6, and 24 h from treatment. Cells have been analyzed by double-immunofluorescence staining, using an anti-NBN antibody directed against the C-terminal region of NBN and an anti-MRE11 antibody. Fluorescence microscopy images, magnification ×100. For details, see the text. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

After 24 h from the IR treatment, a high number of IRIF is still present (mean value: 18 foci per cell) indicating the presence of residual damage (Fig. 2B). In both cell lines, IRIF completely disappears after 48 h from the IR treatment (data not shown).

Both the 26- and the 70-kDa Fragments Arising From the 657del5 NBN Mutation Are Able to Colocalize With γ-H2AX

Next, we asked whether the 26- and the 70-kDa NBN fragments, arising from the 657del5 NBN mutation, are able to localize at the DSB sites, thus allowing the observed nuclear localization of MRE11. Data reported in Fig. 3A indicate that both the 26- and the 70-kDa NBN fragments coimmunoprecipitate with γ-H2AX. Although it is well known that the FHA/BRCT1 domains, present in the 26-kDa NBN fragment, are essential for IRIF formation (32, 33), these data represent the first evidence for the interaction of the 70-kDa NBN fragment (containing only the second BRCT domain) to γ-H2AX. Remarkably, the Arg215Trp NBN mutant does not coimmunoprecipitate with γ-H2AX (27).
Coimmunoprecipitation results (Fig. 3A) match to coimmunofluorescence analysis (Fig. 3B). In MRC-5 fibroblasts, γ-H2AX and NBN colocalize within 6 h, and a complete foci disappearance is observed at 24 h from the IR treatment, thus indicating a complete repair of DSBs. Remarkably, in NBS cells, γ-H2AX and the 70-kDa NBN fragment are able to colocalize at 6 h from IR treatment (mean value: 23 foci per cell), IRIF persisting also after at 24 h (mean value: 8 foci per cell). Interestingly, γ-H2AX and NBN colocalization occurs only at 24 h in R215W fibroblasts (mean value: 13 foci per cell) (27). Overall, data presented here indicate that the 70-kDa NBN fragment, arising from 657del5 NBN mutation, is able to localize at the DSB sites and to interact with MRE11.

Data shown in Figs. 2B and 3A, concerning NBN ability to localize on the damaged site 1 h after IR, seem to be apparently contradictory. However, it should be noted that the mechanism of the IRIF formation is based on an amplification process. IRIF may be visible by fluorescence microscopy only when a sufficient number of proteins localize at the DSB. Therefore, when even one of the key proteins (i.e., NBN) is defective, this mechanism results delayed, or possibly impaired, and therefore detectable by fluorescence microscopy only after several hours from IR. On the contrary, the coimmunoprecipitation technique allows to concentrate the protein of interest (i.e., γ-H2AX) and to detect the interaction with the partner (i.e., NBN) also at short time from IR, but using higher doses of IR (i.e., 10 Gy).

The 657del5 and Arg215Trp NBN Mutations Do Not Affect the Recruitment of 53BP1 at the DSB Sites

As shown in Fig. SI-1, the 53BP1 focus formation and dispersion after exposure to 1 Gy of X-rays occur in all the cell lines considered. In fact, in MRC-5, NBS, and R215W cell lines, the average number of 53BP1 foci per cell peaked at 30 min (35 foci per cell), after 6 h of the IR treatment more than half of the foci are dispersed, and 24 h after the IR treatment, the number of foci per cell returns to the baseline level. According to the literature (34), no differences were observed in 53BP1 IRIF disappearance after IR between MRC-5 and NBS cell lines.
The 657del5 and Arg215Trp Mutations Impair NBN Ability to Properly Activate ATM, SMC1, and p53

To evaluate the proper activation of the DDR signal transduction pathway, the NBN phosphorylation status after IR has been examined by a gel mobility assay. The MRC-5 cell line has showed
slower electrophoretic mobility after IR because of the phosphorylation of both Ser278 and Ser343 residues (Fig. 4A). Similarly, the slower electrophoretic mobility of the NBN protein has been observed in the R215W fibroblasts, thus indicating that the Arg215Trp mutation does not affect the phosphorylation of neither Ser278 nor Ser343. Because of the hypomorphic nature of the 657del5 mutation of NBN, in NBS cells, no signal at 95 kDa has been observed (Fig. 4A).

We next investigate the effects of the Arg215Trp mutation on the NBN-dependent ATM activation and on the ATM-dependent SMC1 and p53 phosphorylation, both promoted by NBN. As shown in Fig. 4B, phosphorylation of ATM occurs in MRC-5 cells after irradiation with 0.5, 1, and 2 Gy. According to the literature, ATM phosphorylation in NBS fibroblasts has not been detected at doses below 1 Gy (35), whereas in the R215W fibroblasts ATM phosphorylation is absent at 1 Gy and barely detectable at 2 Gy. These results indicate that the Arg215Trp NBN-mutated protein is less able to sustain IR-induced ATM activation than the NBN fragments arising from the 657del5 NBN mutation. Similar data were observed for both SMC1 and p53 phosphorylation (Fig. 4B). In particular, SMC1 and p53 are phosphorylated in a dose-dependent manner in MRC-5 cell line, are slightly modulated in NBS fibroblasts, and are not phosphorylated at all in R215W fibroblasts.

To analyze whether in the R215W fibroblasts, the phosphorylation of ATM, SMC1, and p53 may take place at longer time from IR, further experiments have been performed. As shown in Fig. 4C, ATM and SMC1 phosphorylation in MRC-5 cells reaches a maximum after 1 h from 1 Gy of IR, whereas in the R215W fibroblasts, neither ATM, nor SMC1, nor p53 are phosphorylated at longer time from IR.
Figure 4. The Arg215Trp mutation strongly impairs NBN ability to properly activate ATM, SMC1, and p53. A: MRC-5, NBS, and R215W fibroblasts have been exposed to 10 Gy of X-rays and harvested after 1 h. Filters have been probed with anti-NBN and anti-α-tubulin antibodies. B: MRC-5, NBS, and R215W cells have been treated with 0.5, 1, and 2 Gy of X-rays and fixed after 0.5 h. As a control, NBS cells were also separately transfected with the vector alone (here named NBS-pLXIN). C: MRC-5 and R215W fibroblasts have been exposed to 1 Gy of X-rays and cells lysed after 0.5, 1, and 3 h. The pictures show representative blots of two independent experiments. For details, see the text.

DISCUSSION

The patients with NBS characterized by the compound heterozygosity for the 657del5 and 643C>T (Arg215Trp) mutations within the NBN gene display a more severe phenotype than patients with NBS homozygous for the 657del5 mutation (35). Despite the moderate frequency of the Arg215Trp heterozygotes among the Eastern Europe population, to date no NBS cases caused by the
homozygosity for the Arg215Trp mutation have been described (28). This leads to the hypothesis that the homozygosity for the Arg215Trp mutation may lead to a very severe phenotype, probably lethal in the early development (35, 36).

Here, the role of the 657del5 and Arg215Trp mutations, both affecting the tandem BRCT domains of NBN, in the DNA damage response has been investigated. Data obtained indicate that (i) the 70-kDa NBN fragment, arising from the 657del5 NBN mutation, forms IRIF together with MRE11; (ii) both the 657del5 and Arg215Trp mutations do not affect the interaction of NBN with MRE11 and RAD50 and thus the MRN complex assembly; however, both these mutations affect NBN and MRE11 IRIF formation; and (iii) both the 26- and the 70-kDa NBN fragments are able to form immunocomplexes with γ-H2AX.

The ability of the 70-kDa NBN fragment to form IRIF together with MRE11 could be at the root of the less severe phenotype of patients homozygous for the 657del5 mutation when compared with the compound heterozygous ones (36). Indeed, although it has been reported that the 70-kDa NBN fragment predominantly localize into the nucleus and that MRE11 is equally distributed between the nucleus and the cytoplasm (26), IRIF formation in NBS cells homozygous for the 657del5 mutation has never been reported before. This divergence may lie in the fact that kinetics of NBN and MRE11 localization has been investigated here over a long time after IR treatment, thus allowing highlighting the delayed, and partially impaired, IRIF formation. Indeed, in NBS cells homozygous for the 657del5 mutation, MRE11 and NBN colocalize in the nucleus at 6 h from the IR treatment, IRIF being almost completely resolved at 24 h from IR. On the contrary, in cells compound heterozygous for the 657del5 and Arg215Trp mutations, MRE11 results still cytoplasmatic at 6 h from the IR treatment, and after 24 h a high number of IRIF is still observed, thus indicating the presence of residual DNA damage. Remarkably, the number of residual foci observed at 24 h from the IR treatment agrees with our previously published results (27). The difference between the NBS cells homozygous for the 657del5 mutation and the R215W cells compound heterozygous for the 657del5 and Arg215Trp seems to lie in the different localization of MRE11 in the untreated cells. Indeed, in NBS fibroblasts, MRE11 has been found to localize mainly in the cytoplasm and only partially into the nucleus, whereas in the R215W cells MRE11 is completely localized into the cytoplasm.
Both the 26- and the 70-kDa NBN fragments are able to form immunocomplex with γ-H2AX, as also confirmed by double-immunofluorescence experiments. The ability of the N-terminal region of NBN, containing the FHA and BRCT1 domains (corresponding to the p26 fragment), to interact with γ-H2AX has been already demonstrated (33). The absence of a nuclear localization signal (NLS) within the p26 fragment seems not to be so relevant for its localization within the nucleus (26, 33), as demonstrated by the evidence that C-terminal truncation mutants of mouse NBN fused to GFP and transfected into NIH-3T3 cells where able to localize within the nucleus (37). Although the FHA and BRCT1 domains are pivotal for both IRIF formation (32, 36, 38) and for the interaction with γ-H2AX (33), data reported here indicate that the 70-kDa NBN fragment, containing only the BRCT2 domain, binds to γ-H2AX. Thus, the 70-kDa NBN fragment is somehow able to localize at the DSB, bringing on the damaged site also to the MRN complex, even if with a delayed time course. This result agrees with data indicating that the NBN fragment comprising amino acids 401-754 is able to interact with MRE11 (32, 39). Indeed, the 70-kDa NBN fragment may fold independently, thus binding MRE11 and maintaining many important cellular functions, as demonstrated by the inverse correlation between the 70-kDa NBN fragment levels and the lymphoma risk (40). Remarkably, the Arg215Trp mutation determines a defective interaction between the Arg215Trp NBN mutant and γ-H2AX, thus supporting the notion that the integrity of the tandem BRCT domains of NBN is fundamental for the proper localization of NBN at the DSB (see 27 and present data).

As the N-terminus region of NBN is necessary for the localization at the DSB sites (33), we suggest that the 26- and the 70-kDa NBN fragments arising from the 657del5 mutation may somehow interact, thus enabling the MRN nuclear localization on the damaged site. Despite the severe amino acid sequence variation induced by the 657del5 mutation in the linker region between tandem BRCT domains, elements crucial for the structural integrity of the BRCT2 domain are preserved (13). Therefore, the BRCT domains present in both the 26- and the 70-kDa NBN fragments are involved in the dimerization of the two NBN fragments. Interestingly, several proteins containing BRCT domains interact with specific protein partners by BRCT–BRCT homointeractions and heterointeractions (27, 41–43). Remarkably, the 657del5 and Arg215Trp NBN mutations do not affect the recruitment of 53BP1 into IRIF, thus supporting the notion
that NBN is not involved in the 53BP1 incorporation into IRIF (44). It can be hypothesized that the persistence of γ-H2AX, NBN, and MRE11 IRIF may be associated to the complexity of the lesion, these proteins marking either damaged sites not yet properly repaired or still unrepaired. Indeed, although 53BP1 is recruited to the open chromatin and is excluded when a DSB is rejoined, γ-H2AX may persist on the repaired damage, as dephosphorylation after DSB rejoining requires a phosphatase; this enzymatic event probably taking place later (45). Therefore, present data may suggest that the presence of unrepaired or misrepaired DNA damage in human cells carrying mutations at the BRCT tandem domains of NBN does not impact on the H2AX-independent recruitment by the 53BP1 DNA damage sensor protein, but rather determines the persistence of γ-H2AX IRIF (27).

MRE11, RAD50, and NBN are phosphorylated after DNA damage (46–48). In particular, after IR treatment, ATM phosphorylates NBN at Ser278 and Ser343 (21–24). These two Ser residues are crucial for the proper activation of the intra-S phase checkpoint in response to the DNA damage (46). Accordingly to the localization of the Arg215 residue of NBN at the C-terminus of the BRCT1 domain, right before the linker region that connects the two BRCT domains, the Arg215Trp mutation does not affect the ATM-dependent phosphorylation of Ser278 and Ser343, located in the BRCT2 domain and in the central region, respectively. Once phosphorylated, NBN promotes the ATM-mediated phosphorylation of SMC1 (at residues Ser957 and Ser966) and p53 (at the Ser15 residue). Notably, SMC1 is involved in the activation of the intra-S cell cycle checkpoint and in the entry into mitosis, while p53 regulates the G1/S checkpoint (49–51). Data obtained indicate that the proper orientation and integrity of the tandem BRCT domains of NBN seems to be crucial for the activation of both ATM and its downstream effectors. In fact, in the R215W fibroblasts, the phosphorylation of ATM, SMC1, and p53 results strongly compromised at several X-ray doses and times from IR. Similarly, also in NBS cells homozygous for the 657del5 mutation, the impairment of ATM, SMC1, and p53 phosphorylation has been observed, even if at a lower degree when compared to R215W cells. These data can be explained considering that NBN acts in both the ATM- and the ataxia-telangiectasia and Rad3-related protein (ATR)-dependent signaling (52). Although ATM responds to the presence of DSBs, ATR appears to be activated by single-stranded DNA, arising at stalled replication forks or generated during processing of
bulky lesions (53, 54). ATR phosphorylates many of the same damage response proteins as ATM, including NBN (21–24), which localizes to stalled replication forks (55).

Overall, these results support a dominant-negative function of the Arg215Trp mutation with respect to the 657del5 “founder mutation” (27, 35), which might explain also the phenotypic differences between the twin NBS brothers compound heterozygous Arg215Trp/657del5 and the patients with NBS homozygous for the 657del5 mutation (35). However, the reason because both the heterozygous carriers of the Arg215Trp NBN mutation and the twin brothers compound heterozygous Arg215Trp/657del5 do not show chromosomal instability remains still unclear (35, 56).

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CHAPTER 4

Cancer Proneness in Nijmegen Breakage Syndrome Carriers

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Abstract: Biallelic mutations in the NBN gene are responsible for the Nijmegen breakage syndrome (NBS), a rare autosomal recessive disorder characterized by chromosome instability and hypersensitivity to ionising radiation (IR). Epidemiological data evidence that the NBN gene can be considered a susceptibility factor for cancer development, as demonstrated by the fact that almost 40% of NBS patients have developed a malignancy before the age of 21. Interestingly, also NBN heterozygotes, which are clinically asymptomatic, display an elevated risk to develop some types of malignant tumours, especially breast, prostate and colorectal cancers, lymphoblastic leukaemia, and non-Hodgkin’s lymphoma (NHL). So far, nine mutations in the NBN gene have been found, at the heterozygous state, in cancer patients. Among them, the 657del5, the I171V and the R215W mutations are the most frequently described. The pathogenicity of these mutations is presumably connected with their occurrence in the highly conserved BRCT tandem domains of the NBN protein, which are present in a large superfamily of proteins and are recognized as major mediators of processes related to cell-cycle checkpoints and DNA repair. This review will focus on the current state of-knowledge regarding the correlation between carriers of NBN gene mutations and the proneness to the development of malignant tumours.

Keywords: NBN, nijmegen breakage syndrome, NBN carriers, 657del5 founder mutation, R215W, I171V, BRCT domain, DNA repair.
INTRODUCTION

Mutations at the homozygous status in the NBN gene (formerly known as NBS1) are responsible for a rare disease known as Nijmegen breakage syndrome (NBS; OMIM 251260), an autosomic recessive disorder whose signs are a distinct facial appearance, microcephaly, immunodeficiency, chromosome rearrangements and sensitivity to ionising radiation (IR) [1]. In NBS, a defective response to DNA damage is associated with chromosomal instability, and in turn with a strong predisposition to develop malignancy, in particular lymphomas [2, 3]. The majority of them are non-Hodgkin lymphoma (NHL) (with a high incidence of diffuse large B-cell lymphomas), lymphoblastic anaemia, and Hodgkin lymphoma (HL) [3, 4]. Among solid tumours, medulloblastoma has been observed in four patients [5], and rhabdomyosarcoma of the perianal region in three others [6]. Since the latter is extremely uncommon among children, a strong association with NBS has been suggested [6]. All the 11 disease-causing mutations so far identified in NBN gene have been found within exons 6-10 (Fig. 1), and eight of them result in premature truncation of the NBN protein, with the possible synthesis of NBN variants of lower molecular weight [7, 8].

![Coding sequence variants of NBN gene identified to date (modified from http://www.nijmegenbreakagesyndrome.net). Grey: high frequency polymorphisms; grey*: low frequency polymorphisms; black: mutations found in NBS patients; black*: mutations also found in cancer patients at the heterozygous state; black: mutations found in cancer patients only; grey: mutation found both in cancer patients and in healthy controls.](image)

As a measure of cancer incidence in NBS, the Polish registry report that 40% of patients suffering for NBS developed lymphoma within the first two decades of life [3]. Though NBS is a recessive disease and one would not expect any cellular feature or clinical symptom, a growing number of papers report higher spontaneous and induced
chromosome instability and an increased incidence of tumours among NBS carriers. The FISH chromosome painting analysis revealed that NBS carriers display a 3-fold higher rate of chromosome translocations compared with non-carriers [9]. Furthermore, the same authors reported that after irradiation, the response of cells from heterozygous carriers was intermediate between that of NBS homozygous and normal individuals, and could be clearly differentiated from those of the other groups in double-coded studies. Moreover, NBS heterozygosity can be distinguished from other genotypes by the number of the long-lived stable aberrations in NBS cells [10]. Radiation hypersensitivity in NBS carriers seems anyhow restricted to cells irradiated in the G1-phase, whereas the number of chromatid-aberrations scored in G2-phase-treated NBS heterozygous cells is in the range of normal cells or slightly higher [8].

The NBN protein consists of 754 amino acids, has a molecular weight of ~95 kDa, and is composed of three regions (Fig. 2). The N-terminus contains the fork-head associated (FHA) domain (amino acids 24-109), and two breast cancer 1 (BRCA1) carboxy-terminal (BRCT) tandem domains (i.e., BRCT1, amino acids 114-183; and BRCT2, amino acids 221-291) [11]. The central region of NBN contains a consensus sequence encompassing the Ser343 residue that, together with the Ser278 residue located within the BRCT2 domain, is phosphorylated by the ataxia-telangiectasia mutated (ATM) kinase in response to ionizing radiation (IR) [12-15]. Finally, the C-terminus of NBN contains the MRE11-binding domain and the ATM-binding motif [16-18]. Notably, the BRCT domains are present as a tandem repeat in a large super-family of proteins that are the major mediators of phosphorylation-dependent protein-protein interactions in processes related to cell cycle checkpoints and DNA repair functions [4, 19-24]. Mutations within tandem BRCT domains of NBN are responsible for cancer susceptibility, both at the homozygous and heterozygous status [4, 22, 25, 26].

Nine mutations localized in the coding sequence of the NBN gene have been found, at the heterozygous state, in cancer patients (Fig. 1). The 657del5 (or founder mutation), the 511A>G (I171V), the 643C>T (R215W), and the 742insGG mutations were found both in NBS patients and in cancer patients. Four mutations have been found in cancer patients only (278C>T, 381C>T, 448G>T, and 628G>T). The 283G>A (D95N) mutation, has been identified both in cancer
patients and in healthy controls [27], but is not listed as known NBN polymorphism by the NCBI website [28].

![Image](99x275 to 377x506)

**Fig. (2).** Wild type and mutated NBN proteins. (A) Structure of the NBN wild-type protein. (B) The 657del5 mutation, which splits up the tandem BRCT domains, determines the expression of two truncated proteins of 26 and 70kDa. (C) The R215W mutation occurs in the linker region that connects the two BRCT domains, and determines the substitution at position 215 of arginine (R) with a tryptophan (W). (E) The I171V missense mutation occurs in the first BRCT domain, and determines the substitution at position 171 of an isoleucine (I) with a valine (V). (MBD: MRE11 binding domain; ABD: ATM binding domain).

**NBN MUTATIONS AND CANCER RISK**

The first evidence of a possible correlation between NBN carriers and cancer risk came from family data studies, indicating that blood relatives of NBS patients with the 657del5 founder mutation had a high probability to develop malignancy [29]. From 1998, several
studies evaluated the frequency of the NBN mutations in cancer patients. In Table 1, we have collected all the existing data relative to the frequency of cancer onset in carriers of three of the most frequent NBN mutations, i.e. 657del5, R215W, and I171V. Remarkably, all these mutations have been defined susceptibility factor in cancer development. In Table 2 we have performed a statistical elaboration of the whole epidemiological data summarized in Table 1: all the carriers for each NBN mutation among tumor patients were compared with a control population derived from the sum of all the healthy carriers of each mutation. Adults and children were considered separately.

The 657del5 mutation

The 657del5 hypomorphic germ line mutation in exon 6 of NBN gene, accounts for more than 90% of all mutant alleles in NBS. The highest frequency of heterozygous carriers of the 657del5 mutation has been found in the Slavic population of Central Europe, with an average frequency of 1/177 [30]. A total number of 130 carriers of 11725 cancer patients (1.1%), have been so far identified (Table 1). Among heterozygous carriers of the 657del5 mutation the following type of cancers were observed: gastrointestinal lymphoma (10.8%), familial prostate cancer (8.9%), medulloblastoma (2.9%), NHL (2.4%), non familial prostate cancer (2.3%), ovarian (1.6%), ALL (1.6%), melanoma (1%), astrocytic cancer (0.8%), breast cancer (0.9%), colorectal cancer (0.6%) (Table 2) [31-47].

The evidence of a strong correlation between carriers of the 657del5 mutation and cancer risk, has been strengthened by a large study on 344 blood relatives (first- through fourth-degree) of NBS patients, in 24 different NBS families of Czech Republic and Slovakia, from 1998 to 2003 [48]. Thirteen blood relatives developed malignancies of any type, among them 11 were carriers of the 657del5 NBN mutation, compared with six expected. In this study, the most frequently observed type of cancer was stomach cancer and colorectal cancer. Breast cancers were also reported, though at a lower frequency [48].
Table 1. Frequency of the 657del5, R215W, and 1171V NBN mutations among NBN carriers, affected or not by cancer (NHL: non-Hodgkin lymphoma; ALL: acute lymphoblastic leukaemia; HL: Hodgkin lymphoma; OR: odd ratio) (nad: not available data). Data were considered significant when \( p<0.05 \).

<table>
<thead>
<tr>
<th>NBN Mutation</th>
<th>Patient Age</th>
<th>Cancer Type</th>
<th># of NBN Mutation Carriers Among Tumour Patients</th>
<th># of NBN Mutation Carriers Among Control Subjects</th>
<th>Statistical Analysis</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>657del5</td>
<td>adult</td>
<td>colorectal</td>
<td>3/234 (1.3%)</td>
<td>10/1620 (0.6%)</td>
<td>OR: 2.091, ( p=0.2197 )</td>
<td>[35]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>breast</td>
<td>3/750 (0.4%)</td>
<td>5/1411 (0.35%)</td>
<td>OR: 0.95, ( p=0.946 )</td>
<td>[43]</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>4/224 (1.8%)</td>
<td>10/1620 (0.6%)</td>
<td>OR: 2.297, ( p=0.0795 )</td>
<td>[35]</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>11/562 (2%)</td>
<td>1/162 (0.6%)</td>
<td>OR: 3.21, ( p=0.0107 )</td>
<td>[66]</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>1/477 (0.2%)</td>
<td>1/856 (0.1%)</td>
<td>OR: 1.8, ( p=0.76 )</td>
<td>[31]</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>7/873 (0.8%)</td>
<td>2/692 (0.3%)</td>
<td>OR: 2.8, ( p=0.32 )</td>
<td>[36]</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>17/2012 (0.8%)</td>
<td>18/4000 (0.5%)</td>
<td>OR: 1.9, ( p=0.09 )</td>
<td>[37]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>melanoma</td>
<td>2/150 (1.3%)</td>
<td>3/530 (0.6%)</td>
<td>( \chi^2 ) test, ( p&lt;0.0001 )</td>
<td>[37]</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>3/80 (3.8%)</td>
<td>3/530 (0.6%)</td>
<td>( \chi^2 ) test, ( p&lt;0.0001 )</td>
<td>[37]</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>2/270 (0.7%)</td>
<td>2/295 (0.7%)</td>
<td>OR: 1.11, ( p=1.00 )</td>
<td>[42]</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>16/2664 (0.6%)</td>
<td>1/2031 (0.05%)</td>
<td>OR: 12.26, ( p=0.98 )</td>
<td>[41]</td>
</tr>
<tr>
<td>1376 (0.3%)</td>
<td></td>
<td></td>
<td>nad</td>
<td>nad</td>
<td></td>
<td>[40]</td>
</tr>
<tr>
<td>NHL</td>
<td></td>
<td></td>
<td>2/42 (4.8%)</td>
<td>10/1620 (0.6%)</td>
<td>OR: 2.05, ( p=0.0351 )</td>
<td>[35]</td>
</tr>
<tr>
<td>8228 (3.5%)</td>
<td></td>
<td></td>
<td>10/1620 (0.6%)</td>
<td>5/85 (0.0001)</td>
<td>OR: 5.85, ( p=0.0001 )</td>
<td>[66]</td>
</tr>
<tr>
<td>prostate</td>
<td></td>
<td></td>
<td>7/305 (2.2%) (non familial)</td>
<td>9/1500 (0.6%)</td>
<td>OR: 3.9, ( p=0.01 )</td>
<td>[34]</td>
</tr>
<tr>
<td>5/56 (9%)</td>
<td></td>
<td></td>
<td>9/1500 (0.6%)</td>
<td>OR: 16, ( p=0.0001 )</td>
<td></td>
<td>[34]</td>
</tr>
<tr>
<td>gastrointestinal lymphoma</td>
<td></td>
<td></td>
<td>4/37 (10.8%)</td>
<td>10/1620 (0.6%)</td>
<td>OR: 19.52, ( p=0.0002 )</td>
<td>[66]</td>
</tr>
<tr>
<td>ovarian</td>
<td></td>
<td></td>
<td>1/64 (1.6%)</td>
<td>nad</td>
<td></td>
<td>[44]</td>
</tr>
<tr>
<td>children</td>
<td></td>
<td>NHL</td>
<td>1/68 (1.5%)</td>
<td>11/2261 (0.5%)</td>
<td>( \chi^2 ) test, ( p&lt;0.0001 )</td>
<td>[33]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>ALL</td>
<td>2/212 (0.9%)</td>
<td>42/6984 (0.6%)</td>
<td>OR: 1.57, ( p=0.041 )</td>
<td>[38]</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>3/270 (1.1%)</td>
<td>42/6984 (0.6%)</td>
<td>OR: 1.85, ( p=0.035 )</td>
<td>[38]</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>1/68 (1.5%)</td>
<td>11/2261 (0.5%)</td>
<td>( \chi^2 ) test, ( p&lt;0.0001 )</td>
<td>[33]</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>8/400 (0.2%)</td>
<td>53/7705 (0.6%)</td>
<td>OR: 3.01, ( p=0.004 )</td>
<td>[47]</td>
</tr>
</tbody>
</table>
The cancer risk of the 657del5 mutation carriers has been also assessed in cancer patients with no NBS cases in the family. It was found that carriers of the 657del5 mutation were about twice more frequent among cancer patients than among matched controls [35]. In this study, Steffen and co-workers observed that most of the 657del5 carriers were found among patients with melanoma (3.8%; OR: 6.376, p=0.0081), NHL (4.8%; OR: 8.05, p=0.0351), breast cancer (1.8%; OR: 2.927, p=0.0795), and colorectal cancer (1.3%; OR: 2.091, p=0.2197) [35]. Moreover, malignant tumors among parents and siblings of 657del5 carriers were twice more frequent (14/77) than in control population [35]. Interestingly, in a study on 2400 healthy NBN heterozygous Polish women, emerged a frequency of 96/10603 (8.8%) malignant tumors among parents and siblings. This suggested that first-degree relatives of the 657del5 mutation carriers may have an elevated risk of cancer [35]. Heterozygotes for the 657del5 mutation are about three times more

<table>
<thead>
<tr>
<th>NBN Mutation</th>
<th>Patient Age</th>
<th>Cancer Type</th>
<th>n° of NBN Mutation Carriers Among Tumour Patients</th>
<th>n° of NBN Mutation Carriers Among Control Subjects</th>
<th>Statistical Analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>R215W adult</td>
<td></td>
<td>ALL+HL</td>
<td>5/545 (0.9%)</td>
<td>42/6984 (0.6%)</td>
<td>OR: 1.48, p=0.037</td>
</tr>
<tr>
<td></td>
<td></td>
<td>ALL+NHL</td>
<td>5/482 (1.03%)</td>
<td>42/6984 (0.6%)</td>
<td>OR: 1.73, p=0.029</td>
</tr>
<tr>
<td></td>
<td></td>
<td>medulloblastoma</td>
<td>3/104 (2.5%)</td>
<td>74/12484 (0.6%)</td>
<td>OR: 4.86, p=0.0028</td>
</tr>
<tr>
<td></td>
<td></td>
<td>astrocytic</td>
<td>1/127 (0.8%)</td>
<td>74/12484 (0.6%)</td>
<td>OR: 1.33, p=0.77</td>
</tr>
<tr>
<td></td>
<td></td>
<td>colorectal</td>
<td>3/234 (1.3%)</td>
<td>4/1620 (0.2%)</td>
<td>OR: 5.247, p=0.0472</td>
</tr>
<tr>
<td></td>
<td></td>
<td>breast</td>
<td>18/2664 (0.7%)</td>
<td>7/2031 (0.3%)</td>
<td>OR: 1.9, p=0.18</td>
</tr>
<tr>
<td></td>
<td></td>
<td>melanoma</td>
<td>1/376 (0.3%)</td>
<td>nad</td>
<td>nad</td>
</tr>
<tr>
<td></td>
<td></td>
<td>NHL</td>
<td>2/186 (1.1%)</td>
<td>10/1620 (0.6%)</td>
<td>nad</td>
</tr>
<tr>
<td></td>
<td></td>
<td>prostate</td>
<td>6/338 (1.8%)</td>
<td>3/208 (1.4%)</td>
<td>OR: 1.24, p=0.77</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(non familial)</td>
<td>2/139 (1.4%)</td>
<td>3/208 (1.4%)</td>
<td>OR: 1, p=1</td>
</tr>
<tr>
<td>children</td>
<td></td>
<td>HL</td>
<td>1/39 (2.6%)</td>
<td>nad</td>
<td>nad</td>
</tr>
<tr>
<td></td>
<td></td>
<td>ALL</td>
<td>1/47 (2.1%)</td>
<td>nad</td>
<td>nad</td>
</tr>
<tr>
<td></td>
<td></td>
<td>astrocytic</td>
<td>1/127 (0.8%)</td>
<td>7/2875 (0.3%)</td>
<td>OR: 3.2, p=0.254</td>
</tr>
<tr>
<td>II71V adult</td>
<td></td>
<td>larynx cancer</td>
<td>4/176 (2.3%)</td>
<td>1/500 (0.2%)</td>
<td>OR: 11.7, p=0.0175</td>
</tr>
<tr>
<td></td>
<td></td>
<td>breast</td>
<td>20/1636 (1.2%)</td>
<td>18/1014 (1.8%)</td>
<td>OR: 0.68, p=0.3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>10/1048 (0.9%)</td>
<td>7/1017 (0.7%)</td>
<td>OR: 1.39, p=0.7</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>5/270 (1.8%)</td>
<td>1/500 (0.2%)</td>
<td>OR: 9.42, p=0.02</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>head and neck</td>
<td>5/81 (6.2%)</td>
<td>1/600 (0.2%)</td>
<td>OR: 39.41, p=0.0001</td>
</tr>
<tr>
<td></td>
<td></td>
<td>colorectal carcinoma</td>
<td>3/131(2.3%)</td>
<td>1/600 (0.2%)</td>
<td>OR: 14.39, p=0.0196</td>
</tr>
<tr>
<td>children</td>
<td></td>
<td>medulloblastoma</td>
<td>4/104 (3.8%)</td>
<td>54/4227 (1.3%)</td>
<td>OR: 3.0, p=0.0241</td>
</tr>
</tbody>
</table>

[35]
frequent among non-selected breast cancer patients than expected. Since an elevated risk of breast cancer has been also observed among carriers of mutations in the BRCA1, p53 and ATM genes [49-51], and because these gene products interact with each other and with NBN [52], these findings suggest that NBN is another gene that might be associated with increased risk of breast cancer in heterozygotes [35].

The R215W mutation

The R215W mutation has been considered for a long time a polymorphism of NBN, and only recently its severe pathogenicity is emerged with the identification of compound heterozygous 657del5/R215W NBS patients [53].

The R215W missense mutation was first described in a case of acute lymphoblastic leukemia (ALL) and in 9 probands of Slavic origin from a population-based study [27]. Subsequently, a high frequency of heterozygous carriers of the R215W mutation was found not only among children affected by ALL (2.1%), but also by HL (2.6%) (Table 1) [54].

To date, the R215W mutation has been detected in 35 of 4150 individuals with tumours (0.8%) (Table 1). Several studies conducted among Poland, Germany, Czech Republic and United Kingdom reported that among carriers of the R215W missense mutation the following type of cancers were observed: ALL (2.1%), HL (2.6%), prostate cancer (1.7%, both familial and non familial forms), colorectal cancer (1.3%), NHL (1.1%), atrocytic cancer (0.8%), and breast cancer (0.7%), melanoma (0.3%) (Table 2) [35, 39-41, 46, 55].

The I171V mutation

The 511A>G (I171V) germ-line mutation was identified for the first time in five of 47 children with ALL [27]. These children were all characterized by late prognoses due to a late relapses [27]. The same mutation, at the homozygous status, was detected in a Japanese patient with aplastic anaemia, but with no other clinical signs of NBS [56]. In a large study aimed to assess the frequency of NBN mutations in patients with larynx cancer and multiple primary
tumours, is emerged that the frequency of the I171V mutation carriers is significantly higher than in control populations (2.3% in larynx cancer patients; 5.4% in multiple primary tumours) (Table 1 and 2). These results imply that the I171V mutation contributes significantly to the overall incidence of larynx carcinoma [57].

To date, the I171V mutation has been detected in 56 of 3539 individuals with tumours (1.6%) (Table 1). Among heterozygous carriers of the I171V missense mutation, the following type of tumors were observed: head and neck cancers (6.2%), multiple primary tumors (5.4%), medulloblastoma (3.8%), colorectal carcinoma (2.3%), larynx cancer (2.3%), breast cancer (1.2%) (Table 2) [42, 45, 57-60].

An investigation among the Polish population has provided evidence that the I171V mutation could be associated with an increased breast cancer risk (1.8% of I171V carriers). In particular, this association concerns patients with breast cancer, whose first-degree relatives also had diagnosis of those malignancies [58]. Furthermore, it has been observed that the I171V mutation occurred more frequently in the breast cancer group than in the control one, thus indicating that this mutation represent a significant risk factor for breast cancer development [42]. However, in an association study in two large hospital-based case-control settings from Germany and Belarus, is emerged that the I171V missense mutation does not significantly increase the breast cancer risk (0.9%) [59].

Remarkably, the percentage of the I171V mutation carriers is particularly high among patients with neck and head tumours (6.2%) [60], and with medulloblastoma (3.9%) [45]), thus suggesting that the I171V mutation in NBN gene may be susceptibility factor in solid tumours.

**BIOCHEMICAL EFFECTS OF THE NBN MUTATION ON THE STRUCTURE AND FUNCTION OF THE NBN PROTEIN**

Low expression of abbreviated polypeptides of both N-terminal and C-terminal NBN has been demonstrated in NBS lymphoblastoid cell lines with different mutations. Particularly, C-terminal peptides of lower molecular weight than 95kDa, which maintain the ability to interact with MRE11, has been detected by means of a co-immunoprecipitation assay in lymphoblastoid NBS cell lines [7, 8].
NBS cells characterized by the presence of the classical mutation 657del5 in the *NBN* gene show two alternative forms of nibrin with a lower molecular weight, of approximately 26 and 70 kDa. In particular, the 5bp deletion in position 657 splits the BRCT tandem domain exactly in the linker region that connects the two BRCT domains. The 26kDa protein includes the region 1-218 of the NBN protein, and comprises the FHA and the first BRCT domains. The 70kDa protein is produced by an alternative initiation of translation upstream the 5bp deletion: after a 18 residue extension at the N-terminus, the sequence is identical to that of the wild type NBN, from the amino acid 221 to the end, and contains the second BRCT domain and the C-terminal half of NBN [11, 61] (Fig. 2B).

The R215W missense mutation determines the substitution at position 215 of an arginine (R) with a tryptophan (W). The 215 residue of NBN protein is located at the C-terminus of the BRCT1 domain, right before the linker region which connects the two BRCT domains, and seems to be pivotal for the relative orientation of the NBN BRCT domains [62] (Fig. 2C). Since tryptophan is a hydrophobic and bulky residue, it could lead to a perturbation of the relative geometry of the tandem BRCT domains. It has been demonstrated, in fact, that the R215W mutation in NBN impairs histone γ-H2AX binding after induction of DNA damage, leading to a delay in DNA-DSB rejoining [62].

The pathogenic character of the I171V mutation is presumably connected with its occurrence in the BRCT1 domain of NBN (Fig. 2D). So, similarly to the R215W mutation, the I171V may perturb the proper geometry of the tandem BRCT domain, thus impairing the binding to γ-H2AX and the delocalization of the MRN complex to the vicinity of the DNA damage site [57, 63].

**CONCLUSIONS**

Epidemiological data so far collected point to an increased risk of cancer incidence in heterozygous carriers of the 657del5, R215W, and I171V mutations of the NBN gene. The pathogenicity of these NBN cancer-related mutations is presumably connected with their occurrence in the BRCT tandem domains of NBN. Interestingly, cytogenetic analysis revealed that NBN heterozygotes exhibit increased spontaneous chromosomal aberrations respect to normal
individuals [14]. These data indicate that carriers of NBN mutations are less effective in DSBs repair. Notably also a single DSB not repaired or inaccurately repaired can have profound effects, producing aberrant chromosomal rearrangements and chromosomal instability.

The only 657del5 founder mutation frequency among newborns is 1:154 in Czech Republic, 1:182 in Ukraine (Lvov region), 1:190 in Poland, with a mean prevalence of 1:177 for the three populations tested [30], whereas the R215W mutation frequency among Czech newborns is 1:234 [64]. A moderately elevated risk in heterozygous carriers would result in hundreds of new cancer cases in these populations every year. Because it cannot be excluded that cancer patients who carry germ line NBN mutations may show a specific sensitivity to treatment with IR or cytostatic drugs, as recently shown [10], systematic studies are now under way to protocol their responses to radio- and chemotherapy.

Experimental support that NBN heterozygosity predispose cells to malignancy, come from a study in which the mouse homologue of the human NBN gene, Nbn, was disrupted in mice [65]. Nbn+/- mice showed a significantly increased occurrence of spontaneous solid tumours (epithelial tumours affecting the liver, prostate and mammary glands, and gonad malignancy) in addition to lymphoma. Moreover, IR dramatically increased cancer formation in Nbn+/- mice, especially thyroid tumours. These data provide a clear relationship between NBN heterozygosity, radiation sensitivity and increased cancer risk. Interestingly, examination of the tumours gave no evidence for loss or mutation of the wild-type allele, suggesting that haploinsufficiency is the presumed pathogenic mechanism. In contrast, for human heterozygotes, the possible existence of a truncated protein produced by alternative translation [7], and capable of interaction with MRE11 would be compatible with a dominant negative mechanism.

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ABBREVIATIONS

ALL = Acute lymphoblastic leukaemia

ATM = Ataxia telangiectasia mutated gene

BRCT = BRCA1 C-terminal domain

FHA = Forkhead associated domain

HL = Hodgkin lymphoma

IR = Ionising radiation

NBS = Nijmegen breakage syndrome

NHL = Non Hodgkin lymphoma

OR = Odds ratio

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Do tandem BRCT domains of NBN mediate the interaction with γ-H2AX?

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ABSTRACT
The BRCA1 Carboxy-Terminal (BRCT) tandem domains are present in several proteins (e.g., NBN, BRCA1, and MDC1) involved in the response to the DNA double-strand breaks (DSBs) sensing and signaling. These domains are pivotal in phosphorylation-dependent protein-protein recognition, and epidemiological data indicate a relationship between mutations within BRCT domains and cancer susceptibility. Human nibrin (NBN) forms a trimer with MRE11 and RAD50 (MRN complex) involved in nearly every aspect of DNA damage response (DDR), including sensing, signaling, and repair of DNA lesions. In mammalian cells, the histone H2AX represents a crucial component of the DDR. In fact, in response to DSB induction, H2AX is phosphorylated at the Ser139 residue (the phosphorylated H2AX protein being named γ-H2AX), serving as a platform for the assembly of proteins involved in DNA repair and checkpoint response, like NBN, BRCA1, and MDC1. To date, the exact role of BRCT domains of NBN in all the phases of the DDR is still a matter of debate, and controversial data are available regarding protein-protein recognition and timing of BRCT-containing protein localization on the DSB. Indeed, while some authors demonstrated that NBN can directly interact by its tandem BRCT domains with γ-H2AX, others suggest that tandem BRCT domains of NBN mediate the interaction with phosphorylated MDC1, rather than with γ-H2AX. Here, structural and functional aspects of NBN recognition by either γ-H2AX or MDC1 are discussed to shed light on timing of BRCT-containing proteins localization on the DSB.

KEYWORDS: DNA damage response, double strand breaks, MDC1, NBN, tandem BRCT domains, γ-H2AX

ABBREVIATIONS
BRCA1, breast cancer 1; BRCT, BRCA1 carboxy terminal domain; DDR, DNA damage response; DSB, DNA double-strand break; FHA, forkhead associated domain; HR, homologous recombination; IR, ionizing radiation; IRIF, IR-induced nuclear foci; MRN, MRE11/RAD50/NBN complex; NBN, nibrin; NBS, Nijmegen breakage syndrome; NHEJ, non-homologous recombination and joining; (PST)-rich repeats, (Pro-Ser-Thr)-rich repeats; phosphor-Ser, pSer; SDT repeats, Ser-Asp-Thr repeats; SIRT1, deacetylase sirtuin-1; SMCA, the structural maintenance of chromosomes 1 protein; TQX² repeats, Thr-Gln-Xxx-Phe repeats; γ-H2AX, phosphorylated H2AX

INTRODUCTION
Eukaryotic cells have evolved specialized multi-component macromolecular systems to sense, respond to, and repair DNA damage [1–4]. When DNA double strand breaks (DSBs) are induced by the exposure to ionizing radiations (IR), many DNA damage-signaling proteins are recruited to
the damaged sites forming discrete nuclear IR-induced foci (IRIF) [5-7]. The hierarchy and timing of protein (e.g., ATM, MDC1, MRE11, NBN, and RAD50) recruitment to form IRIFs are thought to be critical for the checkpoint response and the DNA damage response (DDR) [7-9]. Indeed, the hierarchy and timing of IRIF formation provides the order of the molecular events resulting from DNA damage detection and signal transduction [7, 10, 11] (Figure 1).

Figure 1. Model of the hierarchy and timing of IRIF formation. After a DSB induction (A) the MRN complex, made up of MRE11, RAD50 and NBN, localizes at damage site (B). This event determines ATM auto-phosphorylation at the Ser1981 residue, with the consequent dissociation of ATM dimers and ATM activation (C). ATM phosphorylates H2AX histone that, as γ-H2AX, serves as a platform for the assembly of proteins involved in DNA repair, cell checkpoint response, and transcription (D). Note that all these proteins are phosphorylated by ATM. Depending on the severity of the DNA damage and on the cell type involved, cells may undergo apoptosis.
NBN interaction with γ-H2AX

In mammalian cells, one of the main players in IRIF formation is the H2A histone family member X (H2AX). Phosphorylation of H2AX is a variant of histone H2A, constituting approximately 10-15% of the histone H2A pool [12, 13]. In particular, histone H2AX is characterized by the long C-terminal tail that contains the conserved Ser139-Gln140-Glu141-Tyr142-COOH motif [14]. The phosphorylation of Ser139 by members of the PI3K family (e.g., ATM and DNA-PK) represents an early and important event in DDR [14, 15]. The epigenetic signal generated by γ-H2AX is then recognized by sensor proteins [15-17]. Indeed, γ-H2AX marks the chromatin region at or near the DSB site and serves as a platform for the recruitment of DNA checkpoint signaling and BRCT-containing repair proteins, including 53BP1, BRCA1, MDC1, and NBN [5, 8, 18-26]. The association of the MRE11/RAD50/NBN (MRN) complex to DSBs represents the first event in the DSB response [10, 27-30].

Suddenly after the localization of the MRN complex at the DSB, ATM undergoes to auto-phosphorylation at the Ser1981 residue, with the consequent dissociation of ATM dimers and ATM kinase activation [31]. Once activated, ATM phosphorylates several substrates, many of them involved in cell cycle checkpoints (e.g., p53, CHK1, CHK2, CDC25A, SMC1, and BRCA1) [31, 32]. This determines the execution of DSB responses, including cell cycle arrest, DNA repair, and induction of a transcriptional program [26, 33]. Depending on the severity of the DNA damage and on the cell type involved, cells may undergo apoptosis instead of attempting to repair the DNA damage [34] (Figure 1).

Here, structural and functional aspects concerning the BRCT-dependent recognition of NBN with either γ-H2AX or MDC1 are discussed to shed light on the timing of BRCT-containing proteins localization on the DSB.

The BRCT domains as mediators of the interaction with phosphoproteins

Many DNA sensing and repair proteins contain a conserved globular domain termed breast cancer 1 (BRCA1) Carboxy-Terminal (BRCT) domain (e.g., 53BP1, DNA Ligase IV, MDC1, MCFH1, NBN, and XRCC4) have been characterized [35-39]. BRCT domains are pivotal in phosphorylation-dependent protein-protein recognition [39-41] and their mutations are responsible for cancer susceptibility, both at the homozygous and heterozygous status [40, 42, 43]. The BRCT domains are composed of 85-95 amino acids, comprising several clusters of conserved residues forming the core of the domain. They can be found either as single or multiple tandem domain(s) within individual proteins in a variety of different arrangements; in the tandem BRCT domains, the repeats are separated by variable linker regions [40, 44, 45].

The BRCT motifs are arranged in a parallel four-stranded β-sheet flanked on one side by a pair of α-helices (named α1 and α3), and on the other side by a single α-helix (named α2). In Figure 2A, the structure of the tandem BRCT domains BRCA1 are represented (PDB code: 1JNX) [46]. The structure of the BRCT motif is well conserved, with the exception of the α2-helix, whose structure is poorly conserved or absent (Figure 2B, C) [40, 46]. Two highly conserved motifs are present in BRCT domains. Motif-1 consists of the Gly-Gly-Gly-Ala pair prior to the β2-strand, corresponding to a sharp turn between the α1-helix and the β2-strand. Motif-2 occurs within α3-helix near to the C-terminus of the domain, and contains the Tyr-Xxx-Xxx-Cys/Ser sequence, where the two variable residues after the Tyr residue are small and hydrophobic [40, 44, 45].

Most of the BRCT domains occur in multiples, the pair of domains packs in a head-to-tail manner involving a large hydrophobic interface and represents the recognition site of phosphoproteins and phosphopeptides [40]. The interface comprises the α2-helix of the N-terminal domain, which is packed against the α1- and α3-helices of the C-terminal domain, and portions of the linker region that connect the two domains [40, 46] (Figure 2A). The interface residues located in the α1- and α3-helices, and to lesser extent the α2-helix, are conserved in the BRCT domains, suggesting that the head-to-tail packing mode is conserved in the members of the BRCT superfamily [47].

BRCT domains bind phosphoproteins and phosphopeptides containing the pSer-Xxx-Xxx-Phe recognition motif [40]. The overall location of phosphopeptides is similar in BRCA1 and...
MDC1 proteins [45, 48, 49]. Both proteins bind the phosphate moiety by direct interactions with side- and main-chain atoms of three structurally conserved residues (i.e., Ser1655, Gly1656, and Lys1702 in BRCA1; Thr1898, Gly1899, and Lys1936 in MDC1) (Figure 3) [45, 48]. The same behavior has been postulated for NBN (the corresponding amino acid residues being Ser118, Cys119, and Lys160) (Figure 3) [49]. The Phe residue present phosphoproteins and phosphopeptidases is recognized by a hydrophobic groove located at the interface between the two BRCT domains [45, 50, 51].

Molecular bases for MDC1: γ-H2AX recognition

MDC1 represents an important protein that takes part to the early phases of the DDR. Remarkably, MDC1 is recruited to the damaged sites rapidly after DSBs induction, playing a pivotal role in the recruitment of other DDR proteins (e.g., 53BP1, the MRN complex, CtIP, and BRCA1) (Figure 1) [8, 23, 15, 52, 53].

MDC1 is a large protein composed of 2089 amino acids, comprising the N-terminal forhead-associated (FHA) domain (amino acids 33-131), the C-terminal tandem BRCT domain (i.e., BRCT1, amino acid residues 1891-1972; and BRCT2, amino acid residues 1998-2082) and the long linker region without a known structural domain [54]. While FHA domain mediates the phosphorylation-dependent dimerization of MDC1 in response to DNA damage [55], tandem BRCT domain specifically binds the C-terminal phosphorylated region of γ-H2AX [56, 57]. The linker region harbors several repetitive sequence motifs, i.e. the Ser-Asp-Thr (SDT), the Thr-Glu-Xxx-Phe (TQXF), and the Pro-Ser-Thr (PST)-rich repeats. The dual-phospho SDT motif recruits the MRN complex via both the FHA and BRCT domains of NBN [33-63]. This interaction is important for MRN focus formation, DSB repair, and the checkpoint activation. The TQXF repeats are phosphorylated by ATM in response to DNA damage, inducing an ubiquitination cascade that involves the E3 ubiquitin ligases RNF8, RNF168, and BRCA1 [64-68].

The tandem BRCT domains of MDC1 show a strong selectivity for a Tyr or Phe residues at the +3 position, yielding the pSer-Xxx-Xxx-Tyr/Phe consensus binding motif [69]. Remarkably, histone H2AX contains the C-terminal pSer-Glu-Glu-Tyr
NBN interaction with γ-H2AX

| NBN | 108 KKEEYEPVVRSCDLVSSGKLMAQAILGQVF | ZRLLWNE 146 |
| BCC1 | 1406 STETUNQHYYVNYLILEPHNRYVHPLNHGRTYH 1493 |
| MDC1 | 1591 TASKVTIGVGDFRSVAVLAIGS | I-AGEASE 1922 |

| NEN | 147 CTYLVMS-----VYQVTIVKTLICLGGPRIVFEPYVEQPFKATESSQPPQIESFY-PFFL 203 |
| BCC1 | 1634 ERKVRMARTDEYRYECCNINGTVGGTVWSQKEQKM---NEGSENCFRLEYDV 1797 |
| MDC1 | 1792 ASKYNR----NTYKVTIGVGDFRSVAVLAIGS | FSPDIEVV-TIEE 1874 |

| NEN | 201 DPEISGSHNVLSDGRFQKEQHCTGLTIILNHQYHLRSAYNTGGEARELITEEERE 246 |
| BCC1 | 1746 HERN----HQQPRXAEKKDEEITLIEYIVIY 1976 |
| MDC1 | 1798 QINFGESIQALPARRALEGTE | ------ 2010 |

| NEN | 261 NPELAPGTCVQQHITGNSQTLFEDQGGSHQKSMQGCGGQGQGQIQ----RPPFEAEI--- 813 |
| BCC1 | 1776 ------SS-----PDQLERQGVLSSQFVRKLLRSTTVYFIEEPFHSSYQGAEHTM--- 1916 |
| MDC1 | 2001 YYTLPQVQPP----FQGKTQSCGCGTYLPEQRRGQVQQR-VYRCPQHCTEFLP 2038 |

| NEN | 311 ----GLAIV-----FTKTYQPGQPSHI---- 810 |
| BCC1 | 1220 ----G--------FAIQQGCEAFYTR---- 1185 |
| MDC1 | 2084 RGGLFSSEELT-GLYQKPEAFVLS 2382 |

Figure 3. Amino acids sequence alignment of the BRCT domains of NBN, BCC1, and MDC1 proteins. The protein sequences were obtained from the NCBI database (http://www.ncbi.nlm.nih.gov). The amino acid sequence alignment was performed by Clustal W (from http://www.ebi.ac.uk/Tools/msa/clustalw2). The "*" symbol means that the residues are identical in all the aligned sequences; the "-" symbol means that conserved substitutions have been observed; the "-" symbol means that semi-conserved substitutions have been observed. The residues in bold are those constituting the BRCT domains.

The tandem BRCT domains of MDC1 show high specificity for the C-terminus of histone H2AX only in its phosphorylated form, thus allowing MDC1 to function as a specific sensor for γ-H2AX [56, 57]. Interestingly, it has been shown that the tandem BRCT domains of MDC1 bind to the motif that requires an adjacent free C-terminus, leading to the definition of a more specific binding consensus sequence of pSer-Xxx-Xxx-Tyr/Pho-COOH [56, 57]. In particular, the recognition of γ-H2AX by MDC1 is mediated by key interactions involving the last amino acid of histone H2AX, i.e., Tyr142. This residue binds to the interface formed by the two BRCT domains of MDC1 and is in close contact with the Pro209 residue of MDC1 located within the second BRCT domain, while H2AX carboxyl group forms an essential salt bridge with the guanidinium group of the Arg1932 residue of MDC1 placed within the first BRCT domain [56, 57].

The Ser/Thr-protein phosphatase PP1A and PP4 have been implicated in the dephosphorylation of γ-H2AX [70, 71]. It was proposed that MDC1 protects γ-H2AX from dephosphorylation by its strong binding, thereby mediating downstream γ-H2AX signals and preserving γ-H2AX itself [57].

Does NBN bind directly to γ-H2AX?

NBN is involved in several phases of the DSBs damage response, including sensing, signaling, and repair of DNA lesions [10, 19, 72].

NBN is composed of three regions. The N-terminal region contains the FHA domain (amino acids 24-109), and two BRCT tandem domains (i.e., BRCT1, amino acid residues 114-183; and BRCT2, amino acid residues 221-291) [73, 74]. The central region of NBN contains a consensus sequence encompassing the Sar343 residue that, together with the Ser278 residue located within the BRCT1 domain, is phosphorylated by the ATM kinase in response to IR [73-78]. Lastly, the C-terminal region of NBN contains the MRE11-binding domain and the ATM-binding motif [73, 79].

The FHA and the tandem BRCT domains can each mediate protein-protein interactions on their own, showing specificity for Thr or Ser, respectively [80].
However, NBN seems to have combined both domains to yield a third domain. Two very recent structural and functional studies shed some light on this matter [62, 63]. The solved structures of the FHA-tandem BRCT domain of fission yeast and NBN show a conserved structure in which the FHA and tandem BRCT domains retain their fold. However, the FHA domain becomes fused to the first BRCT domain. The distance between the two binding sites of the FHA and tandem BRCT domains of NBN is such that a single molecule would only be able to bind both sites at once if at least 22 amino acids separate the recognition sites [91].

Several experimental evidence indicate that NBN is able to bind γ-H2AX [49, 82, 83], the FHA and tandem BRCT domains of NBN having a crucial role in mediating this interaction. The NBN interaction with γ-H2AX was investigated by in vitro experiments with recombinant proteins. After H2AX phosphorylation by ATM kinase, the immunoprecipitate obtained using anti-H2A antibody contained recombinant NBN and γ-H2AX, thus suggesting that NBN binds directly to H2AX and that this recognition process is specific for the phosphorylated form of the histone [82]. Furthermore, it has been observed that the Arg215Trp mutation, located within the tandem BRCT domains of NBN, impairs NBN ability to bind to γ-H2AX [49]. Indeed, immunoprecipitation and co-immunofluorescence experiments revealed that the Arg215Trp mutated NBN is unable to bind γ-H2AX shortly after IR, co-localization of NBN with γ-H2AX being observed only 24 hours after IR [49]. Molecular modeling of the NBN tandem BRCT domains revealed that the Arg215Trp mutation could affect the relative orientation of the two BRCT domains that appears to be critical for γ-H2AX binding. Indeed, the presence of the hydrophobic and bulky Trp215 residue could lead to a perturbation of the relative geometry of the two BRCT domains, impairing γ-H2AX binding. These findings indicate that despite the presence of an intact FHA domain, the perturbation of the BRCT tandem repeats affects the ability of NBN to bind the γ-H2AX tail, demonstrating that the correct geometry of the two BRCT domains is essential for recognition of the γ-H2AX tail. Interestingly, the GG136-137EE point mutations in the conserved motif of the BRCT domain impair the IR-induced nuclear foci formation even in the presence of the intact FHA domain [84]. On the basis of the similarity between NBN and MDC1 BRCT tandem domains and in the light of the functional results here reported, it can be speculated that also in NBN these domains play a major role in γ-H2AX recognition [49].

Further experimental data supporting the direct interaction between NBN and γ-H2AX have been recently obtained [83]. The Δ57/Δ65 mutation within NBN gene is located in the linker region that connects the tandem BRCT domains of NBN and causes the synthesis of two fragments of 26 and 70 kDa, each one containing one integral BRCT domain [74, 85]. Remarkably, both NBN fragments are able to co-localize with γ-H2AX after the DSB induction, thus suggesting that also the single BRCT domain present in the 70 kDa NBN fragment is somehow able to localize on the DSB, probably through a dimerization mechanism involving the two NBN fragments and mediated by the BRCT domains [49, 50, 83, 86, 87]. This result further supports a key role of the tandem BRCT domain of NBN in the proper DDR, arises a question: if the interaction between NBN and MDC1 is mediated by both the FHA and tandem BRCT domains, how can be explained the ability of the 70 kDa NBN fragment do co-localize with γ-H2AX?

From structure and amino acid sequence comparison between Xenopus laevis NBN homologue (XNbs1) and MDC1, Xu and coworkers suggested that it is unlikely that NBN tandem BRCT domains would recognize γ-H2AX in the same manner as MDC1 [88]. Indeed, the Arg1933 residue of MDC1 is replaced by the Val1153 residue in XNbs1 and Val1157 in NBN. From the three-dimensional structure-based alignment of MDC1 and XNbs1 BRCT2, Pro2009 of MDC1, which contacts Tyr142 of γ-H2AX, occupies a position close to that of the Lys233 residue of XNbs1 and Lys233 in NBN. Therefore, the typical phosphopeptide motif (i.e., pSer139 of γ-H2AX) recognized by MDC1 [57] and BRCA1 [89] may not apply to NBN tandem BRCT domains. In the case of NBN, it has been speculated that an Asp or a Glu (instead of Phe or Tyr)
at +3 position to pSer would be a more favored residue as it could form a charge or salt bridge interaction with Lys233 of NBN or Lys231 of XRNs1. Remarkably, the Lys233 residue of NBN is acetylated by the deacetylase SIRT1 as part of the DDR [90]. Acetylation and deacetylation of the Lys233 residue may therefore regulate NBN binding to the phosphorylated target [88].

However, some important points need to be highlighted. To date, all published results support a direct interaction between MDC1 and γ-H2AX, but no experimental data exclude the direct binding of NBN to γ-H2AX. The only data supporting the hypothesis of an indirect binding derived from bioinformatics studies [88]. Conversely, the three-dimensional structure of the tandem BRCT domains of NBN modeled on the basis of the multiple amino acid sequence alignment of 25 homologs of NBS1, and using as the template the structure of MDC1 tandem BRCT domains evidences the presence of a groove in which, by homology with the MDC1 protein, the phosphorylated tail of γ-H2AX may bind directly through molecular contacts provided by both BRCT domains [49]. The molecular determinants of γ-H2AX recognition by MDC1 appear to be conserved in NBN. In fact, the electrostatic interaction between the Lys1936 residue of the first BRCT domain and the phosphate group of the γ-H2AX tail, observed in the MDC1 three-dimensional structure [57], is conserved in NBN (corresponding to the Lys160 residue) (Figure 4). Further, it is also conserved the interaction between the Tyr142 residue of the histone tail, located three residues away from the phosphorylated Ser139 residue, and a hydrophobic patch made up by residues contributed by both BRCT domains (Val1935, Met2014, Leu2066 in MDC1; and Ile159, Leu238, and Leu312 in NBN) (Figure 4). The similar binding mode of the histone tail to MDC1 and NBN extends up to the conservation of a hydrogen bond between the histone tail residue Tyr142 and the Glu234 residue of the second BRCT domain [49]. Furthermore, the interaction between the Glu141 of the γ-H2AX and the Arg1932 residue of MDC1 may be replaced by the Lys156 residue in NBN (Figure 4).

In recent studies, it was shown that a direct interaction between NBN N-terminal FHA domain and phosphorylated MDC1 is necessary for the retention of the MRN complex at DNA damage sites [99–101]. MDC1 is constitutively phosphorylated by casein kinase 2 (CK2) at Ser and Thr residues in SDT repeats. In human MDC1 there are five such SDT repeats (from residues 299 to 456), all having an Asp at position pSer +3

![Figure 4. Residues involved in γ-H2AX recognition by MDC1 (panel A; PDB: 2AZM) [57] and NBN (panel B) [49]. The pSer139, Glu141 and Tyr142 residues of γ-H2AX are highlighted. Tyr142 and pSer139 residues of γ-H2AX contact Val1935 and Lys1936 of MDC1 and Ile159 and Lys160 of NBN, respectively. Remarkably, the interaction between Arg1932 of MDC1 and Glu141 of γ-H2AX is conserved in NBN (Lys156). Further, it is also conserved the interaction between the Tyr142 residue of γ-H2AX and the hydrophobic patch made up by Val1935, Met2014, Leu2066 residues in MDC1, and Ile159, Leu238, and Leu312 in NBN. Molecular graphics images were produced using the UCSF Chimera package [92].](image-url)
(i.e., pSer-Asp-pThr-Asp), what was predicted to be favorable for binding NBN tandem BRCT domains, as the side-chain ammonium group of NBN Lys333 or Xb51 lys233 could participate in a charge interaction with the Asp. Noticeably, in the pSer-Asp-pThr-Asp sequence the +3 position to pSer is an Asp, which could form a charge interaction with Lys233 of NBN. Furthermore, the large number of residues separating the MDC1 repeats suggests a possible dual-binding mode with pThr repeats interacting with the FHA domain of NBN and pSer repeats interacting with the tandem BRCT domains of NBN [88]. Therefore, the re-localization of NBN to DNA damage sites may be in part mediated by the direct interaction of NBN tandem BRCT domains with phosphorylated MDC1 [88].

CONCLUSIONS AND PERSPECTIVES
Data here presented brought to speculate that experimental data supporting a direct interaction between NBN and γ-H2AX represents “false positives”, MDC1 being the real mediator that links NBN to γ-H2AX. Furthermore, since it has been demonstrated that NBN interaction with MDC1 is mediated by both FHA and tandem BRCT domains, mutations within the FHA and BRCT domains of NBN, affecting γ-H2AX binding, might be attributable to a defect in the proper binding of NBN to MDC1 [82].

Remarkably, even though the tandem BRCT domains of MDC1 and NBN exhibit only 16% sequence identity, the direct protein-phosphate interactions are essentially conserved. This extraordinary stereochemical similarity in phosphate binding by two such highly diverged BRCT tandem domains shows that phospho-dependent binding is an important and ancient activity of BRCT tandem proteins. Overall, it remains of primary importance to determine whether or not NBN is able to interact directly with γ-H2AX, in order to shed light on the timing of BRCT-containing proteins localization on the DSB.

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NBN interaction with y-H2AX


Abstract

Nibrin (also named NBN or NBS1) is a component of the MRE11/RAD50/NBN complex, which is involved in early steps of DNA double strand breaks sensing and repair. Mutations within the NBN gene are responsible for the Nijmegen breakage syndrome (NBS). The 90% of NBS patients are homozygous for the 657del5 mutation, which determines the synthesis of two truncated proteins of 26 kDa (p26) and 70 kDa (p70). Here, HEK293 cells have been exploited to transiently express either the full-length NBN protein or the p26 or p70 fragments, followed by affinity chromatography enrichment of the eluates. The application of an unsupervised proteomics approach, based upon SDS-PAGE separation and shotgun digestion of protein bands followed by MS/MS protein identification, indicates the occurrence of previously unreported protein interacting partners of the full-length NBN protein and the p26 fragment containing the FHA/BRCT1 domains, especially after cell irradiation. In particular, results obtained shed light on new possible roles of NBN and of the p26 fragment in ROS scavenging, in the DNA damage response, and in protein folding and degradation. In particular, here we show that p26 interacts with PARP1 after irradiation, and this interaction exerts an inhibitory effect on PARP1 activity as measured by NAD$^+$ levels. Furthermore,
the p26-PARP1 interaction seems to be responsible for the persistence of ROS, and in turn of DSBs, at 24 h from IR. Since some of the newly identified interactors of the p26 and p70 fragments have not been found to interact with the full-length NBN, these interactions may somehow contribute to the key biological phenomena underpinning NBS.

**Introduction**

Human nibrin (also named NBN or NBS1), a component of the MRE11/RAD50/NBN (MRN) complex, has been reported to participate to cell cycle checkpoint activation, to the early steps of DNA damage sensing, and to double strand breaks (DSBs) repair [1–8]. The MRN complex accumulates at sites of DSBs in large microscopically discernible sub-nuclear structures, usually referred to as ionizing radiation (IR)-induced foci (IRIF) [9]. The foci formation around the DNA damaged sites, marked by the presence of the H2AX histone phosphorylated at the Ser139 residue (i.e., γ-H2AX), is mediated by a direct interaction between NBN and the phosphorylated mediator of the DNA damage checkpoint 1 (MDC1) [10–15]. Among others, NBN is known to interact also with ATM [10, 16-20], CtIP (also named RBBP8) [13, 21–27], Tip60 [28, 29], BRCA1 [23, 30, 31], and SMC1 [31].

NBN consists of 754 amino acids and is composed of three regions (Fig. 1). The N-terminus contains the fork-head associated (FHA) domain (amino acids 24-109) and two BRCA1 C-terminal (BRCT) tandem domains (i.e., BRCT1: amino acids 114-183; BRCT2: amino acids 221-291) [32]. The central region of NBN contains two consensus sequences encompassing the Ser278 and Ser343 residues, which undergo phosphorylation by ATM in response to IR [33, 34]. The C-terminus of NBN contains two MRE11-binding motifs and the ATM-binding motif [13, 18, 19, 35].

Mutations at the homozygous or compound heterozygous status within the NBN gene are responsible for the Nijmegen breakage syndrome (NBS; OMIM #251260) [36, 37], a rare genetic disorder characterized by an autosomic recessive inheritance, whose signs are, among others, microcephaly, humoral and cellular immunodeficiency, and radiosensitivity [3, 6, 38–40]. Remarkably, one of the main clinical features of NBS patients is the cancer predisposition. By the age of 20 years, over 40% of NBS patients develop a malignant disease, predominantly of lymphoid origin.
(Non-Hodgkin lymphomas of B and T cells are the most common) [40].

Figure 1. Schematic representation of the wild type and mutated NBN proteins. (A) Structure of the NBN wild type protein, having a molecular weight of approximately 85 kDa. (B) The 657del5 mutation, which splits up the tandem BRCT domains, determines the expression of two truncated proteins of 26 and 70 kDa. The Ser278 (S278) and Ser343 (S343) residues are phosphorylated by ATM in response to the DNA damage induction. (FHA: fork-head associated domain; BRCT: breast cancer 1 (BRCA1) carboxy-terminal domain; MBD: MRE11 binding domain; ABM: ATM binding motif). For details, see the text.

The 90% of NBS patients are homozygous for the hypomorphic 657del5 mutation in NBN [3, 40]. This mutation determines the synthesis of two truncated proteins of 26 kDa (p26) and 70 kDa (p70) (Fig. 1) [41]. The p26 protein includes the region encompassing amino acids 1-218 of the NBN protein, thus comprising the FHA and the BRCT1 domains. The p70 protein is produced by an alternative initiation of translation upstream the 5 base pair deletion; after a 18 residue extension at the N-terminus, the sequence is identical to that of the wild type NBN from the amino acid 221 to the end, and contains the BRCT2 domain and the C-terminal region of NBN [32]. Remarkably, a significant correlation between the p70 expression levels and lymphoma incidence has been observed; in fact, patients displaying high intracellular levels of the p70 truncated protein are at lower risk for lymphoma than those with low levels of p70 [42].
The aim of the present work was to identify, by SDS-PAGE and mass spectrometry approaches, the interactors of both the full-length NBN protein and of the p26 and p70 NBN fragments, under basal condition and after X-rays induced DNA damage. Data obtained allowed the identification of several previously unreported interacting partners of NBN. A high number of interactors of the p26 (containing the FHA/BRCT1 domains) and p70 (containing the BRCT2 domain and the MRE11/ATM binding motifs) NBN fragments were isolated after DNA damage induction, most of them not interacting with the full-length NBN. Results obtained shed light on new possible roles of NBN in ROS scavenging, in the DNA damage response, as well as in protein folding and degradation.

**Materials and Methods**

*Amplification and cloning*

The p26 and p70 coding sequences were obtained from the total RNA of a lymphoblastoid cell line established from a NBS patient homozygous for the 657del5 mutation. Two micrograms of the RNA sample were reverse-transcribed using the SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen, Carlsbad, USA). The oligo-dT primer was used to prime the reverse transcription, which was performed according to the manufacturer’s instructions. PCR was performed using the Exact Polymerase (5 Prime GmbH, Hamburg, Germany), using as templates the cDNA obtained from the reverse transcription. For the amplification of the NBN full-length sequence, the pLXIN-NBN retroviral vector was used as template [43]. The primer sequences, all carrying at the 59 and 39 ends the BsaI restriction enzyme recognition sites, were designed using the “Primer D’Signer Software” (IBA, Goettingen, Germany). Oligonucleotide sequences used were:

p26_FW: 5’ - ATGGTAGGTCTCAGCGCCATGTGGAAACTGCTGCCCGCC3’;

p26_RV: 5’ - ATGGTAGGTCTCATATCATGCTGTTTGGCATTCAAAAATAT AAAT- 3’;
p70_FW: 5’ -
ATGGTAGGTCTCAGCGCTTCTTCTCCTTTTTAAATAAGGAT
TGTA – 3’;

p70_RV: 5’ -
ATGGTAGGTCTCAGCGCTTCTTCTCCTTTTTAAATAAGGAT
TGTA- 3’;

NBN_FW: 5’ -
ATGGTAGGTCTCAGCGCCATGTGGAAACTGCTGCCCGCC-
3’;

NBN_RV: 5’ -
ATGGTAGGTCTCAGCGCTTCTTCTCCTTTTTAAATAAGGAT
TGTA- 3’.

The resulting amplicons were digested with the BsaI restriction
enzyme (New England Biolabs, Ipswich, USA) and ligated into the
BsaI pre-digested pEXPRIBA105 vector (IBA) using the Quick
Ligation kit (New England Biolabs). The resulting plasmids were
sequenced to check the proper cDNA sequence insertion, amplified
in the JM109 E. coli strain, and purified using the PerfectPrep
Endofree Maxi kit (5 Prime).

Cell culture and transient transfection

HEK293 cells were grown in Dulbecco Modified Eagle’s medium
supplemented with 10% fetal bovine serum (VWR, Milan, Italy), 2
mM L-glutamine (VWR), 100 μg/ml penicillin and 100 μg/ml
streptomycin (VWR). Transient transfection was performed using
the calcium chloride method [44]. Briefly, HEK293 cells were
seeded at a density of 2 X 10^5 cells/ml. One hour prior transfection
the medium was refreshed and then 10 mg of plasmid DNA (either
p26_pEXPRIBA105 or p70_pEXPR-IBA105 or NBN_pEXPR-
IBA105 or empty pEXPRIBA105) was mixed together with 0.25 M
CaCl2 in Hepes buffer (HBS: 25 mM Hepes, 10 mM KCl, 12 mM
Dextrose, 280 mM NaCl, and 1.5 mM Na2HPO4 X 7H2O). To check
the expression of the recombinant proteins fused to the Strep-tag
sequence, HEK293 cells were lysed after 24 and 48 h from
transfection (lysis buffer: 20 mM Tris-HCl pH 8.0, 137 mM NaCl,
10% glycerol (v/v), 1% NP-40 (v/v), 10 mM EDTA, 1 μg/mL
aprotinin, 1 μg/mL leupeptin, 1 μg/mL pepstatin, 1.0 mM orthovanadate, and 2.0 mM PMSF). Thirty micrograms of protein lysates were analyzed by immunoblotting using a StrepMAB-Classic horse radish peroxidase conjugated antibody (IBA).

**X-ray treatment**

In order to induce DSBs, cells were exposed to 2 Gy of X-rays 48 h after transfection (MGL 300/6-D apparatus, Gilardoni, Italy; 250 kV, 6 mA, Cu filter; dose rate 0.53 Gy/min).

**Strep-tag chromatography**

Thirty minutes after X-ray treatment, untreated and irradiated cells were harvested and lysed using 400 ml (column bed volume, CV) of the lysis buffer. Columns packed with 5.0 ml of Strep-Tactin Sepharose resin (IBA) were equilibrated with 1 CV of buffer W. After centrifugation of soluble extracts (14,000 rpm, 5 minutes, 4°C), each supernatant was added to an equilibrated column. After the cell extracts have completely entered the columns, they were washed 5 times with 0.5 CV, and the eluates were collected in fractions having a size of 1 CV. Finally, 0.5 CVs of buffer E (100 mM TrisHCl, 150 mM NaCl, 1.0 mM EDTA pH 8.0, and 2.5 mM desthiobiotin pH 8.0) were added 6 times to each column and the eluates were collected in 0.5 CV fractions. Twenty mL samples of each fraction were analyzed by SDS-PAGE, using precast gradient polyacrylamide gels (BioRad, Hercules, USA). Proteins were detected by silver staining. Gel lanes were divided into 30 uniform slices and subjected to trypsin digestion for shotgun MS-based identification, as previously reported [45].

**MALDI TOF/TOF and nanoHPLC/Mass spectrometry**

Protein slices were excised from the first dimension SDS-PAGE gels and subjected to trypsin digestion according to literature [46] with minor modifications. The gel pieces were swollen in a digestion buffer containing 50 mM NH₄HCO₃ and 12.5 ng/mL trypsin (modified porcine trypsin, sequencing grade, Promega, Madison, WI, USA) in an ice bath. After 30 min, the supernatant was removed and discarded; then 20 μL of 50 mM NH₄HCO₃ were added to the gel pieces and digestion was allowed to proceed overnight at 37°C. The
supernatant containing tryptic peptides was dried by vacuum centrifugation prior to MALDI-TOF/TOF [47] and nano-liquid chromatography-electrospray ionization-ion trap mass spectrometry/mass spectrometry (nano-LC-ESI-IT MS/MS) identification [48].

MALDI-based identification was performed through an Autoflex II MALDITOF/TOF mass spectrometer. The LIFT module (Bruker Daltonics, Bremen, Germany) was used for mass analysis of peptide mixtures. Twenty microliters of the tryptic protein digests were loaded onto activated (0.1% TFA in acetonitrile) ZipTip columns and washed three times with 10 μL of 0.1% TFA in DD-H2O. The peptides were eluted with 1.0 μL of matrix solution (0.7 mg/mL α-cyano-4-hydroxy-trans-cinnamic acid (Fluka, Seelze, Germany) in 85% acetonitrile, 0.1% TFA and 1.0 mM NH4H2PO4) and spotted directly on the MALDI-TOF target plate for automatic identification (PAC384 pre-spotted anchor chip). Proteins were identified by peptide mass fingerprint (PMF) using the database search program MASCOT (http://www.matrixscience.com/) upon removal of background ion peaks. Accuracy was set within 50 ppm, while the enzyme chosen was trypsin and only 1 missed cleavage was allowed. Fixed carbamidomethyl Cys and variable Met-oxidation was used as optional search criterion. For those proteins for which PMF-based identification was not successful, most abundant peptides were analyzed with MALDI-TOF/TOF-based LIFT mode MS/MS analyses of precursor ions and repeated MASCOT-based database searches. Runs were performed automatically through FlexControl setting and Biotools processing of MS data. When PMF protein identification was unsuccessful, automatic determination of the three most abundant peaks and identification through MS/MS (LIFT analysis) on the three most intense ion peaks was performed. A peptide mixture (Peptide calibration standard I; Bruker Daltonics) was used for external calibration.

Nano-LC-ESI-IT MS/MS identification was performed on those protein bands or spots that could not be successfully identified either through PMF or LIFT (MS/MS) MALDI TOF/TOF analyses. Nano-LC-ESI-IT MS/MS analysis was performed through a split-free nano-flow chromatography separation system (EASY-nLC II, Proxeon, Odense, Denmark) coupled to a 3D-ion trap (model AmaZon ETD, Bruker Daltonik, Germany) equipped with an online ESI nanosprayer (the spray capillary was a fused silica capillary, 0.020 mm i.d., 0.090 mm o.d.). For all experiments, a sample volume of 15 μL was loaded by the autosampler onto a homemade 2 cm
fused silica pre-column (100 μm i.d., 375 μm o.d., Reprosil C18-AQ, 5 μm; Dr. Maisch GmbH, Ammerbuch-Entringen, Germany). Sequential elution of peptides was accomplished using a flow rate of 300 nL/min and a linear gradient from Solution A (2% acetonitrile; 0.1% formic acid) to 50% of Solution B (98% acetonitrile; 0.1% formic acid) in 40 min over the pre-column in-line with a homemade 15 cm resolving column (75 mm i.d., 375 mm o.d., Reprosil C18-AQ, 3 mm; Dr. Maisch GmbH, Ammerbuch-Entringen, Germany). The acquisition parameters were as follows: dry gas temperature, 220°C; dry gas, 4.0 L/min; nebulizer gas, 10 psi; electrospray voltage, 4000 V; high-voltage end-plate offset, 2200 V; capillary exit, 140 V; trap drive: 63.2; funnel 1 in 100 V out 35 V, and funnel 2 in 12 V out 10 V; ICC target, 200,000 and maximum accumulation time, 50 ms. The sample was subjected to the “Enhanced Resolution Mode” at 8100 m/z per second (which allows mono isotopic resolution up to four charge stages) polarity positive, scan range from m/z 300 to 1500, 5 spectra averaged, and rolling average of 1. The “Smart Decomposition” was set to “auto”.

Acquired spectra were processed in DataAnalysis 4.0, and deconvoluted spectra were further analyzed with BioTools 3.2 software and submitted to the Mascot search program (in-house version 2.2, Matrix Science, London, UK). The following parameters were adopted for database searches: NCBI nr database (release date 21/01/2012; 243,775 sequences); taxonomy: Homo sapiens; peptide and fragment mass tolerance: ±0.3 Da; enzyme specificity (trypsin) with 2 missed cleavages was considered; fixed modifications: carbamidomethyl and variable modifications: oxidation. For positive identification, the score of the result of (-10 X Log P) had to be over the significance threshold level (p<0.05) and only peptides with Mascot scores ≥ 30 were considered. Peptide fragmentation spectra were also manually verified, accounting for the mass error, the presence of fragment ion series, and the expected prevalence of C-terminus containing (y-type ions) in the high mass range. All spots required a minimum of two verified peptides to be identified. Moreover, replicate measurements (n=3) have confirmed the identity of these protein hits.

Immunoblotting

To analyze the NBN full-length, the p26 and the p70 interactors identified by mass spectrometry, 30 μg of protein eluates obtained
from the Strep-tag chromatography were loaded onto a polyacrylamide gel and transferred to a PVDF membrane (Immobilon, Millipore, Billerica, USA). The blots were probed with the following antibodies: anti-53BP1 rabbit polyclonal antibody (Novus Biologicals, Atlanta, USA), anti-ATM mouse monoclonal antibody (Abcam, Cambridge, UK), anti-ATM pSer1981 mouse monoclonal antibody (Santa Cruz Biotechnology), anti-CHK2 pThr68 rabbit polyclonal antibody (Santa Cruz Biotechnology), anti-CHK2 mouse monoclonal antibody (Santa Cruz Biotechnology), anti-BRCA1 mouse monoclonal antibody (Santa Cruz Biotechnology), anti-γ-H2AX rabbit polyclonal antibody (Santa Cruz Biotechnology), anti-γ-H2AX rabbit polyclonal antibody (Santa Cruz Biotechnology), anti-γ-H2AX rabbit polyclonal antibody (Santa Cruz Biotechnology), anti-γ-H2AX rabbit polyclonal antibody (Santa Cruz Biotechnology), anti-γ-H2AX rabbit polyclonal antibody (Santa Cruz Biotechnology), anti-γ-H2AX rabbit polyclonal antibody (Santa Cruz Biotechnology), anti-γ-H2AX rabbit polyclonal antibody (Santa Cruz Biotechnology), anti-γ-H2AX rabbit polyclonal antibody (Santa Cruz Biotechnology), anti-γ-H2AX rabbit polyclonal antibody (Santa Cruz Biotechnology), anti-γ-H2AX rabbit polyclonal antibody (Santa Cruz Biotechnology), anti-γ-H2AX rabbit polyclonal antibody (Santa Cruz Biotechnology), anti-γ-H2AX rabbit polyclonal antibody (Santa Cruz Biotechnology), anti-γ-H2AX rabbit polyclonal antibody (Santa Cruz Biotechnology), anti-γ-H2AX rabbit polyclonal antibody (Santa Cruz Biotechnology), anti-NBN mouse monoclonal antibody (Abcam), anti-PP2A rabbit polyclonal antibody (Santa Cruz Biotechnology), anti-PAR1 mouse monoclonal antibody (Santa Cruz Biotechnology), anti-PML rabbit polyclonal antibody (Santa Cruz Biotechnology), anti-SMC1 rabbit polyclonal antibody (Chemicon, Billerica, USA), anti-Strep-tag (IBA). ECL detection solutions (Amersham Pharmacia, Milan, Italy) were used to visualize the antibody reactions.

**Protein-protein interaction analysis**

The STRING 9.1 software [49] has been used to perform functional annotations, through mapping protein interactors, to the experimentally identified protein species. Proteins were uploaded in the software along with indications of the species under investigation (Homo sapiens) in order to exclude false-positive protein-protein interactions and functional annotations derived from investigations on other species. Protein-protein interaction analysis/prediction software, such as String, determines and makes graphs of unbiased networks, in which gene products are represented as nodes, and the biological relationship between two nodes is represented as an edge (line). All edges are supported by at least one reference from the literature, from a textbook, or from canonical information stored in the software internal database (literature and evidence-based). White nodes represent predicted interactors upon matching against the internal database which were not present in the uploaded list (absent in the experimental dataset). The confidence interval was set to 0.400 (high confidence), additional white nodes to 10, and network depth
was kept to the minimum value 1 to exclude as many false positive interactions as possible.

**Quantification of NAD\(^+\) levels**

Cells transfected with either the NBN full-length- or the p26-coding vector were treated with 2 Gy of X-rays and harvested after 0.5, 2, and 24 h. Briefly, 2 X 10\(^5\) cells were washed in PBS and lysed. To remove NADH-consuming enzymes, the extracted samples were filtered through 10 kDa molecular weight cut off filters (Abcam) before performing the assay. The NAD\(^+\)/NADH quantification was performed using the NAD\(^+\)/NADH colorimetric kit, according to manufacturer’s instruction (BioVision, Milpitas, USA). Samples were read at OD 450 nm, using the microplate reader Victor X (PerkinElmer, Waltham, USA).

**Immunofluorescence analysis of γ-H2AX foci**

HEK293 transfected cells were grown on glass coverslips, irradiated with 2 Gy of X-rays, and harvested after 0.5, 2, and 24 h. Cells were fixed in 2% paraformaldehyde, permeabilized on ice for 5 min with 0.2% Triton X-100, and blocked in PBS/1% BSA (v/w) for 0.5 h at room temperature. Slides were incubated over night with 1 μg/mL γ-H2AX mouse monoclonal antibody (Millipore, Billerica, MA), and detected with an anti-mouse FITC-conjugated secondary antibody (Immunological Sciences, Rome, Italy). Confocal analysis was performed using LCS microscope (Leica Microsystems, Heidelberg, Germany). Quantitative analysis of γ-H2AX foci was carried out by counting foci in at least 50 cells/experiment, in two repeated experiments.

**Statistical analysis**

The statistical analysis was performed using the ANOVA test with the InStat version 3 software system (GraphPad Software Inc., San Diego, CA). Data are means of at least two independent experiments ±SD.
Results and Discussion

Recombinant proteins expression

To identify the NBN full-length and the p26 and the p70 interactors, the cDNAs were cloned into the pEXPR-IBA105 plasmid, in frame with the Strep-tag coding sequence. The obtained plasmids, named pEXPR-IBA105_NBN, pEXPRIBA105_p26, and pEXPR-IBA105_p70, respectively, were transiently transfected into HEK293 cells. The expression of the recombinant proteins fused to the Strep-tag was checked after 24 and 48 h from transfection, using the StrepMAB-Classic horse radish peroxidase conjugated antibody (IBA). After 48 h from transfection, the recombinant proteins were expressed at higher levels with respect to HEK293 cell transfected with the empty vector (named pEXPR-IBA105) (Fig. 2A).

Identification of the interactors of NBN and p26 and p70 fragments arising from the NBN 657del5 founder mutation

HEK293 transfected cells were then exposed to 2 Gy of X-rays, and soluble cell extracts were prepared after 30 minutes. The NBN full-length, the p26, and the p70 recombinant proteins, together with the bound proteins present in the cell extracts, were purified using the Strep-tactin gravity flow columns. The obtained eluates were analyzed by mass spectrometry and SDS-PAGE.

Protein bands were excised from the gels and identified either through MALDI TOF/TOF or nanoHPLC-ESI MS/MS. In Tables 1-4 are reported the interactors identified for NBN, p26, p70, and the empty vector, respectively, in both untreated (K) and irradiated (RX) samples, along with the theoretical molecular weight and pI, the overall number of peptides identified, the Mascot score, and the explanation of the biological functions, as gleaned via the UniProt database.

Table 5 summarizes the identified interactors of NBN full-length, and of p26 and p70 fragments, upon exclusion of false positive (hits highlighted in yellow in Table 1-4). This workflow allowed us to determine novel likely interactors of the full-length NBN protein, and of the p26 and p70 fragments, while expanding or complementing existing literature [15, 23, 25, 31, 50-53].

The protein eluates obtained from the Strep-tag chromatography performed on lysates of HEK293 transfected with either NBN full-
length, p26 or p70 were checked for the presence of the recombinant proteins, using an antibody directed against the full-length protein (Fig. 2B).

Figure 2. Evaluation of the Strep-tag recombinant proteins expression and Western blot analysis of NBN, p26 and p70 interactors. (A) Protein conjugated antibody. The highest level of expression was observed after 48 h from transfection, for all the recombinant proteins. Protein extracts obtained from HEK293 cells transfected with the empty vector were used as negative control. (B) Check of the expression of the Strep-tag recombinant proteins. Immunoblots were performed using the protein eluates obtained from HEK293 transfected cells either untreated or exposed to 2 Gy of X-rays, lysed after 30 minutes and purified by Strep-tag chromatography. Filters were probed with the anti-NBN antibody directed against the full-length protein. (C) Protein eluates obtained from HEK293 transfected cells exposed to 2 Gy of X-rays were lysed after 30 minutes, purified by Strep-tag chromatography, and analysed by Western blot using the following antibodies: anti-53BP1 rabbit polyclonal, anti-ATM mouse monoclonal, anti-ATM pSer1981 mouse monoclonal, anti-BRCA1 mouse monoclonal, anti-CHK2 pThr68 rabbit polyclonal, anti-CHK2 mouse monoclonal, anti-CtIP rabbit polyclonal, γ-H2AX rabbit polyclonal, anti-Hsp90 rabbit polyclonal, anti-MRE11 mouse monoclonal, anti-NBN mouse monoclonal, anti-PARP1 mouse monoclonal, anti-PP2A rabbit polyclonal, anti-PML rabbit polyclonal, anti-SMC1 rabbit polyclonal.
Interactors of the full-length NBN

Among the NBN full-length protein interactors, the following categories were identified in untreated and treated samples: protein biosynthesis and degradation, nuclear protein import, control of G2/M cell cycle checkpoint, cell growth regulation, transcription regulation, meiosis, DSBs response, activation of the apoptotic process, and oxidative stress response. The identification of several novel NBN interactors contribute to the improvement of the NBN interactome complexity, as gleaned through graphic representation via the software String [49] (Table 1; Fig. 3). It is worthwhile to note that some of the observed interactors were likely to result from a technical bias of the enrichment analytical workflow, as it emerged by assaying the eluates obtained from the enriched protein extracts obtained from empty vector controls (Table 4).

If we exclude false positive results from Table 1 (hits highlighted in yellow), some interactors of the full-length NBN protein are apparently IR-independent (i.e., ENO1, ACTA1, CKB, EEF1A1, LDHC, PKM2/PKLR, and TRAP1) (Table 5).

Several interactors of the full-length NBN protein have been detected only after IR treatment, such as antioxidant enzymes (i.e., PRDX1 and SOD1), chaperones (i.e., HSPA8 and HSP90AA1), calreticulin, metabolic enzymes (i.e., ALDOA and ATP5B), and proteins involved in protein biosynthesis (i.e., EIF4A2 and RPSA) (Table 5).

Since PRDX1, an antioxidant that scavenges hydroperoxides, exerts a radioprotective role [54], irradiation can induce PRDX1 expression, thus protecting human cells from IR-induced damage [54]. PRDX1 induction is ATM-dependent, indeed ATM is an important sensor of ROS in human cells [55] and ATM-/− osteoblast are characterized by a reduced induction of PRDX1 after oxidative stress [56]. Therefore, we may speculate that the ATM/NBN axis controls PRDX1 expression, with NBN directly interacting with PRDX1 through the FHA and BRCT1 domains, as suggested by the fact that this protein has been found to be an interactor of p26 after IR (see Tables 2 and 5; Fig. 4).
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The identification of SOD1 among the NBN interactors after irradiation supports a role of NBN in the oxidative stress response [57]. Noteworthy, oxidative stress induces cell cycle-dependent MRE11 recruitment, ATM and CHEK2 activation, and histone H2AX phosphorylation, all these events being strictly associated to NBN [58]. Data obtained in conditional null mutant Nbn mice have indicated disturbances in redox homeostasis due to impaired DSBs processing [59]. The persistent up-regulation of ROS-related proteins in the liver of irradiated Nbn mutant mice may explain the increased DNA damage levels, the chromosomal instability and cancer occurrence in NBS, thus supporting a role of NBN in the repair of ROS-induced damage [59].
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26031  8.72  7  68  g|167102783 | Carbonic anhydrase-related protein 13 | CA13 | No catalytic activity |

90405  6.83  11  78  g|19829853 | Rhogtp1 guanine nucleotide exchange factor (GEF) 7, isoform CRA_b | ARHGGEF7 | Cell migration, attachment and cell spreading; possible function as a positive regulator of apoptosis; pulses and synapses formation; promotion in hippocampal neurons |

4250  8.94  4  70  g|57266470 | Fc gamma receptor III-A | FCGRA3 | Binding of complexed or aggregated IgG and also monomeric IgG; mediation of antibody-dependent cellular cytotoxicity (ADCC) and other antibody-dependent responses, such as phagocytosis |

24735  7.86  7  77  g|1958284 | Phosphoglucomutase 5 | PGMS | Adherens-type cell-cell and cell-extracellular matrix junctions formation |
Table 2. Cont.

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<p>|        | 143709| 5.82                     | 5            | 226                    | gi|407399                    | IsocitrylRNA synthetase | IARS       | RNA aminoacylation for protein translation; ATP binding; gene expression |
|        | 115243| 6.87                     | 1            | 175                    | gi|19611543                  | DEAH (Asp-Glu-Ala-His) box polypeptide 9, isoform CRA_c | DHX9       | DNA and protein binding; double-stranded RNA binding; RNA helicase activity; RNA splicing; cellular response to heat |
|        | 144064| 6.59                     | 2            | 62                     | gi|303735                    | Spliceosomal protein SAP 155 | SF3B1      | Chromatin binding; RNA splicing; gene expression                             |
|        | 89631 | 5.60                     | 26           | 468                    | gi|52358                     | HnRNP U protein | HNRNPU     | DNA binding; RNA splicing                                                   |
|        | 113810| 9.02                     | 9            | 186                    | gi|190167                    | Poly(ADP-ribose) polymerase | PARP1      | DNA damage response, detection of DNA damage; base-excision repair; regulation of growth rate; telomere maintenance |
|        | 11366 | 11.36                    | 3            | 225                    | gi|6004301                   | Histone H4 | HIST1H4A | DNA binding; telomere maintenance; phosphatidylinositol-mediated signaling |
|        | 123882| 5.83                     | 3            | 209                    | gi|134133236                 | POTE ankyrin domain family member E | POTE       | ATP binding                                                                    |
|        | 42318 | 5.39                     | 3            | 205                    | gi|3055557                  | Beta-adrenergic protein 2 | ACTBL2     | ATP binding                                                                    |
|        | 47421 | 7.01                     | 4            | 108                    | gi|659533                    | 2-phosphorylurate-hydrolase alpha isoenzyme | ENO1       | Phosphoabsorbate hydrolase activity; regulation of cell growth and transcription; tumor suppression |</p>
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Table 3. p70 fragment interactors.
The alterations in the chaperone and heat shock protein groups also indicate elevated cellular ROS levels [60]. Chaperones and calreticulin, which is involved in the Ca\textsuperscript{2+}-dependent protein folding, have been identified among the NBN interactors after IR treatment. Disturbances in Ca\textsuperscript{2+} homeostasis and protein folding, as well as in the generation of ROS and in the oxidative damage, are essential features of neurodegeneration [61, 62]. Remarkably, among the main clinical manifestations of NBS there are both microcephaly and intellectual disability [3, 40]. Furthermore, both HSPA8 and HSP90AA1 proteins have been demonstrated to be upregulated following genotoxic stress [63, 64] (see below, paragraph "Interactors of the p26 fragment").

Since the role of NBN in the DNA damage response has been well demonstrated [5, 7, 38], we performed Western blot analysis on the chromatographic eluates to verify the interaction of NBN with proteins playing a key role in the DSBs response pathway. Indeed, since the confidence interval of the mass spectrometry data was set to 0.400 (high confidence), in order to exclude as many false positive interactions as possible, well-known NBN interactors, as well as possible p26 and p70 interactors, might have been excluded. Furthermore, this combined approach allowed us to further analyze the role of the N- and C-terminal region of NBN in the recruitment of repair proteins on the DSBs.
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40194  8.56  9  124  g1537756D  Beta actin  ACTB  Cytoskeleton structural constitution; protein folding; cellular component movement

118638  6.67  7  77  g16566655  Topoisomerase binding RS protein  TOPORS  DNA binding; intrinsic apoptotic signaling pathway in response to DNA damage; SUMO ligase activity; regulation of cell proliferation; transcription, DNA-dependent

26528  4.63  14  336  g15003325  14-3-3 protein epsilon  YWHAE  Phosphoprotein binding; protein complex binding; G2/M transition of mitotic cell cycle; apoptotic process; hippocampal development

4995  8.94  4  70  g37726670  Fc gamma receptor II-A  FCGR3A  Binding of complement or aggregated IgG and also monomeric IgG; mediation of antibody-dependent cellular cytotoxicity (ADCC) and other antibody-dependent responses, such as phagocytosis

24135  7.86  7  77  g119522804  Phosphoglucomutase 5  PGM5  Adhesion-type cell-cell and cell-matrix junctions formation
Table 5. Novel interactions of NRR, p28 and p70 fragments.

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Table 5. Cont.

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Immunoblots were performed using the protein eluates obtained from the Strep-tag chromatography (Fig. 2C). Data obtained by Western blot experiments confirmed the NBN full-length protein ability to interact with MRE11, RAD50, ATM, H2AX, CHEK2, BRCA1, SMC1, CtIP, SP100, and 53BP1. These data permitted to verify that the experimental approach allowed the isolation of the DNA repair complex(es) that is(are) known to be assembled in response to the DSBs induction [9].

In particular, NBN full-length interacts with MRE11 and RAD50 (forming the MRN complex) independently from the presence of DNA damage [36, 37, 53, 65]. Similarly, it has been demonstrated that NBN interacts with SMC1 in a BRCA1-dependent fashion, these interactions being fundamental for the proper phosphorylation of both SMC1 and BRCA1 after IR treatment [31]. Remarkably, SMC1 and BRCA1 are involved in a common DNA damage pathway and play a crucial role in the maintenance of chromosomal integrity [31]. Data obtained also confirmed the ability of NBN to interact with the nuclear dots-associated protein SP100 [66], both in basal conditions and after IR treatment. Indeed, the BRCT tandem domains of NBN are responsible for the interaction with SP100 [67], thus allowing their co-localization within the promyelocytic leukemia protein (PML)-nuclear bodies (NB) and in the alternative lengthening of telomere (ALT)-associated PML bodies (APBs) [67–69]. Thus, the interaction of NBN with SP100 may be crucial for the genomic stability and for telomere length maintenance [67]. This interaction requires the integrity of the tandem BRCT domains, since the presence of only one BRCT repeat in both the p26 and p70 fragments does not allow the recognition of SP100.
Figure 3. Interactome of the NBN full-length protein. The interactome was gleaned from SDS-PAGE-MS/MS analysis of the streptavidin chromatography eluates obtained from cells expressing the full-length NBN protein (A) in control conditions or (B) following 2 Gy of X-rays. Colored nodes indicate those proteins that have been identified experimentally in the present study, while grey nodes indicate likely additional interactors that are predicted on the basis of evidences available from the literature. For details, see the text.
Figure 4. Interactome of the p26 fragment. The interactome was gleaned by merging the results obtained through SDS-PAGE MS/MS analysis of the streptavidin chromatography eluates obtained from cells expressing the p26 protein fragment (A) in control conditions or (B) following 2 Gy of X-rays. For details, see the text.

As expected, after DNA damage induction by IR we observed that the NBN fulllength protein formed complexes with pSer1981-ATM,
\( \gamma \)-H2AX, pThr68-CHEK2, BRCA1, CtIP, SMC1, and 53BP1. Remarkably, these interactions involve the recognition of the phosphorylated forms of these proteins, and allow the proper DNA damage signaling and cell cycle arrest [23, 25, 31, 50–52].

**Figure A**

**Figure B**

cont.
Figure 5. (A) Measurement of NAD⁺ levels in HEK293 transfected cells. Cells transfected with either the full length or the p26 NBN fragment were exposed to 2 Gy of X-rays and harvested after 0.5, 2, and 24 h. The concentration of NAD⁺ was determined using 2 X 10⁵ transfected cells. Error bars represent the standard deviation from two independent experiments. (**p<0.01; *p<0.05). (B) DSBs repair analysis evaluated by γ-H2AX foci. HEK293 cells transfected with either the full length or the p26 NBN fragment were exposed to 2 Gy of X-rays and harvested after 0.5, 2, and 24 h. Fixed cells were stained with anti-γ-H2AX, and the number of γ-H2AX foci was counted in 50 cells/experiment, in two repeated experiments. (**p<0.01; *p<0.05). (C) Co-immunoprecipitation experiments aimed at evaluating the interaction of MRE11 with the Strep-tagged proteins and PARP1. HEK293 cells transfected with either the full length or the p26 NBN fragment were exposed to 2 Gy of X-rays and harvested after 0.5 h. Protein eluates were immunoprecipitated with MRE11 and blotted with anti-Strep-tag and anti-PARP1 mouse monoclonal antibodies.

Interactors of the p26 fragment

The analysis of p26 interactors highlighted yet undisclosed role of the N-terminal region of the NBN protein in cell homeostasis. Indeed, p26 partners can be clustered in three main categories: DSBs repair, anti-oxidant responses, and ribosome joining. Fig. 4 and Table 2 show the interactors of the p26 fragment in untreated (K) and irradiated (RX) transfected cells.

Some metabolic enzymes (i.e., ENO1, LDHC, PRDX1, and PKM2/PKLR) were found among the interactors of both NBN and p26, thus bringing to speculate that the FHA and BRCT1 domains, present in the p26 fragment, mediate these interactions (Table 5).
The expression of the p26 fragment in HEK293 resulted in the recruitment of p26-unique IR-dependent partners, as summarized (Tables 2 and 5).

Irradiation of HEK293 cells expressing the p26 fragment promoted the formation of a complex involved in protein biosynthesis (i.e., RPS14, RPS8, RPS9, RPL19, RPL22, and RPL24) and RNA splicing/DNA binding (i.e., HNRNPU, HIST1H2AK, HIST1H4A, SF3B1, DHX9, PARP1, and PRKDC), as gleaned from the protein-protein interaction map in Fig. 4. The formation of these p26-unique IR-dependent complexes might hold potential biological pitfalls in patients suffering of NBS. The binding of PARP1 to damaged DNA, including singlestrand breaks (SSBs) and DSBs, through its double zinc finger DNA-binding domain potently activates PARP1 enzymatic activity. Of note, the enzymatic activity of PARPs requires a ready supply of NAD\(^+\), which is hydrolyzed to produce ADP-ribose units for the PARylation of protein targets [70]. The regulated availability of NAD\(^+\) may represent a key point of control for PARP1, and the concentration of NAD\(^+\) has been shown to affect the length of PAR synthesized by PARP1 in vitro [71]. NAD\(^+\) depletion represents PARP1 activity, which is induced by DNA damage and allows its proper signaling and repair [72–74]. Here we show that in HEK293 cells over-expressing the p26 fragment a significant increase in NAD\(^+\) levels was observed at 0.5 h from IR compared to HEK293 cells over-expressing the NBN protein (Fig. 5A). Similarly, it has been reported that NBN-silenced cells, as well as cells established from NBS patients, showed a significant decrease of NAD\(^+\) depletion following the treatment with either H2O2 or methyl methanesulfonate, thus demonstrating that PARP1 activity is dependent upon NBN expression [57]. Remarkably, the targets of PARP1 enzymatic activity include PARP1 itself, which is the primary target in vivo, core histones, the linker histone H1, and a variety of transcription-related factors that interact with PARP1 [75, 76]. The automodification domain of PARP1 contains several Glu residues that are likely targets for automodification and a BRCT motif that functions in protein-protein interactions [75, 77]. Therefore, we hypothesize that the interaction of p26 with PARP1 after irradiation exerts an inhibitory effect on PARP1 activity, as measured by NAD\(^+\) levels. Possibly, the interaction of the p26 fragment with PARP1 involves the BRCT domain present in both of them. The BRCT motif is important in protein-protein interaction associated to DNA repair and cell signaling pathways, and several proteins containing BRCT domains
interact with specific protein partners by BRCT-BRCT homo- and hetero- interactions. Of note, the BRCT domain of PARP1 is known to mediate the interaction with the BRCT domain of XRCC1, thus allowing an efficient DNA repair [78–81]. However, since following irradiation the p70 fragment of NBN undergoes phosphorylation at the Ser278 residue placed within the BRCT2 [41, 82] (see Fig. 1), it might be speculated that this phosphorylation inhibits the interaction of the p70 fragment with PARP1 (see Tables 3 and 5). According to data reported by Digweed’s group [83], we speculate that the p26-PARP1 interaction may be responsible for the persistence of ROS, as demonstrated by the NAD⁺ depletion at 24 h from IR in cells over-expressing p26 compared to those over-expressing the full length NBN (Fig. 5A). Noteworthy, this NAD⁺ depletion correlates with the persistence of unrepaired DSBs after 24 h from IR (Fig. 5B). Accordingly with the observation that in NBS patients the truncated p70 NBN fragment is unable to prevent the hyperactivation of PARP1 (with the consequent loss of cellular anti-oxidant capacity) [83], our results suggest that also the p26 NBN fragment may contribute to the NBS phenotype, being responsible for the persistence of high levels of ROS at long time from irradiation. Remarkably, PARP1 has been suggested also as a contributing factor in the pathogenesis of Ataxia telangiectasia, an autosomal recessive disease caused by mutation in ATM [84, 85]. Considering the key role played by NBN in the DSBs sensing and repair, it is interesting to note that among p26-unique IR-dependent interactors a whole subset of proteins involved in DNA/RNA binding and DNA repair (i.e., DHX9, HNRNPU, PARP1, PRKDC, HIST1H2AK, HIST1H4A, and SF3B1) was detected. Notably, DHX9 is an ATP-dependent RNA helicase A that is capable of unwinding double strand DNA and RNA in a 39 to 59 direction, and thus functions as a transcriptional activator [86]. Besides, RNA helicase A mediates the association of CBP with RNA polymerase II [87], and the association of BRCA1 to the RNA polymerase II holoenzyme [88], thus influencing DNA transcription. Of note, BRCA1 is a known interacting partner to NBN [31] (see Fig. 2C). On the other hand, the DNA-PK catalytic subunit isoform 1 (PRKDC) is a serine/threonine-protein kinase that acts as a molecular sensor for DNA damage [27, 89]. PRKDC is involved in the non-homologous end joining (NHEJ) repair mechanism required for both DSB repair and the V(D)J recombination, and may also act as a scaffold protein to aid the localization of DNA repair proteins to the site of damage [90].
Finally, among the p26 interactors, the histones HIST1H2AK and HIST1H4A have been identified, as further validated by immunoblot (Fig. 2C). Many histone covalent modifications have been shown to play key regulatory roles in eukaryotic transcription, DNA damage repair, and replication. The chromatin structure has been proposed to make HIST1H4A inaccessible in the absence of DNA damage, with passive relaxation of chromatin at DSBs and the targeted recruitment of histone acetylation, ubiquitination, and chromatin remodeling activities acting to facilitate focal accumulation of 53BP1 [91–94]. While the key role of the HIST1H2AK histone in the DSBs response has been well clarified [95, 96], recently new data are highlighting an important role played also by the HIST1H4A [97, 98]. Competitive binding by the hybrid tandem Tudor domains of the JMJD2A and JMJD2B lysine demethylases has been suggested to prevent binding of 53BP1 in the absence of DNA damage, with RNF8-mediated ubiquitination, proteasomal degradation, and rapid depletion of JMJD2A/B from chromatin at damaged sites facilitating focal accumulation of 53BP1 [97]. Recently, it has been demonstrated that transient HIST1H4A deacetylation is an early response to DSBs, which facilitates 53BP1 foci formation, DSB repair by NHEJ, and repression of transcription [98]. HIST1H4A deacetylation may also coordinate with other ATM-and NBN-dependent events to facilitate focal accumulation of 53BP1 at DNA damage sites [99–101].

The analysis of p26-unique IR-dependent interacting partners also suggested the yet undisclosed role of this portion of the NBN protein in mediating ribosomal subunit joining (i.e., 40S ribosomal protein S8, S9, and S14, as well as 60S ribosomal protein L19, L22, and L24), in like-fashion to the Shwachman-Diamond Syndrome protein (SBDS) [102, 103]. However, it is also important to stress that several ribosomal proteins have extra-ribosomal functions, including replication and DNA repair. Therefore, either mutations in ribosomal proteins or alterations to their interacting partners may have effects that are independent of the protein translation machinery [104].

Interestingly, when looking at the interactors of the p26 fragment, we observed the presence of proteins able to bind, either directly or indirectly, the FHA and BRCT domains (Fig. 2C). While some authors suggested that tandem BRCT domains of NBN mediate the interaction with phosphorylated MDC1 [95, 105], several experimental evidences indicate that NBN is able to bind directly \( \gamma \)-H2AX, the FHA and tandem BRCT domains of NBN having a crucial role in mediating this interaction [15, 53, 106, 107].
Therefore, it was not surprising that our data indicate that only the p26 fragment of NBN was able to interact with γ-H2AX. Remarkably, it has been previously demonstrated that the p26 and p70 NBN fragments were able to co-immunoprecipitate with c-H2AX [53]. These evidences brought to the hypothesis that the BRCT domains present in p26 and p70 are involved in the dimerization of the two NBN fragments, thus recreating the pocket that allows the interaction with γ-H2AX [15, 53, 108]. Remarkably, our results indicate that while the FHA and BRCT1 domains present in the p26 fragment seem sufficient to bind (either directly or indirectly) γ-H2AX, the BRCT2 domain present in the p70 fragment does not. Indeed, differently from NBS cells, HEK293 cells were transfected either with the p26 or the p70 fragment, and H2AX has been identified among the interactors of the p26 fragment upon irradiation, but not among the interactors of the p70 fragment.

Specifically, γ-H2AX [53, 106, 107], BRCA1 [23, 30, 31], and CtIP [13, 21–27] were found among the interactors of the p26 fragment, thus confirming that the FHA and the BRCT1 domains maintain the capability to interact with proteins involved in the DNA damage response (Fig. 2C). Remarkably, our data agree with previous reports indicating that the interaction of the MRN complex with BRCA1 is ATM- and NBN-dependent [31]. In particular, IR enhances ATM binding to BRCA1 [33]. However, both the N- and C-terminal domains of NBN seem to be required for the proper interaction with BRCA1 [31], as further supported by our data indicating that both the p26 and p70 fragments bind BRCA1. Noteworthy, looking at the p26 interactors, also MRE11 and RAD50 were detected by Western blot analysis. This result appears surprising since p26 lacks both the MRE11- and ATM-binding motifs. Indeed, the absence of both the MRE11-interaction motifs identified in NBN (i.e., the interaction motif 2 comprised between amino acids 638–662, and the interaction motif 1 comprised between amino acids 682–693) [35], prompted us to further investigate why MRE11 was identified among the p26 NBN interactors. Of note, MRE11 has been described to interact with PARP1 at sites of DSBs, and this interaction is required for rapid accumulation of MRE11 protein, and in turn of the MRN complex, at DSBs [72]. Furthermore, in mammalian cells the presence of PARP1 and MRE11 proteins in a functional complex has been described as an important mechanism in resolving DNA lesions [108]. Remarkably, after irradiation, we observed that p26 interacts with PARP1 (see Table 2 and Fig. 2C), and, in turn, MRE11 interacts with both the
Streptagged p26 NBN fragment and PARP1 (Fig. 5C). Of note, while MRE11 coimmunoprecipitated with the Strep-tagged full length NBN both in basal conditions and after irradiation, the interaction with the p26-tagged fragment, as well as with PARP1, took place only after DSBs induction (Fig. 5C). At the light of these results, our hypothesis is that the observed interaction between the p26 NBN fragment and MRE11 is mediated by the PARP1 common interactor.

Hsp90 chaperone machinery ensures the function of proteins important for DNA repair, recombination, and chromosome segregation [109]. It has been recently demonstrated that BRCA1 and Hsp90 cooperate in homologous and non-homologous DSBs repair, as well as in the G2/M checkpoint activation [110]. Hsp90 is known to associate with the MRN complex, although the molecular mechanism is yet unknown [111]. In particular, Hsp90 seems to associate with NBN to facilitate the NBN/ATM interaction, and the use of the Hsp90 inhibitor 17-(dimethylaminoethylamino)-17-demethoxygeldanamycin (17DMAG) reduces the interaction between NBN and ATM, although no degradation of the MRN complex has been detected. The diminished radiation-induced activation of ATM in 17DMAG-treated cells seems to be the result of a compromised function of the MRN complex [111]. Results obtained by mass-spectrometry indicate Hsp90 as an interactor of both the NBN full length and the p26 fragment after IR. This result has further validated by immunoblot experiments (Fig. 2C). Of note, Hsp90 does not interact with the 70 kDa NBN fragment (Fig. 2C). These results strongly suggest that at least the N-terminal region of NBN is involved in the recruitment of Hsp90 in the MRN complex in response to the IR-induced damage. Overall, data from literature support our mass spectrometry data, indicating that Hsp90 may somehow contribute to the maintenance of the genome stability, although the molecular mechanism needs to be further clarified.

As already known, the phosphorylated form of CtIP is recruited to the DNA damaged site by direct interaction with the FHA domain of NBN [13, 23], as confirmed by the identification of this protein among the p26 interactors (Fig. 2C). However, the absence of ATM, CHEK2, and SMC1 among the interactors of p26 and p70 fragments after IR impairs the proper DNA damage signaling, thus allowing the proliferation of unrepaired or misrepaired cells.
Interactors of the p70 fragment

The p70 protein fragment showed a limited number of shared interactors with respect to the full-length NBN protein (Fig. 6). The p70 interactors can be clustered in two main categories: helicase activity and regulation of protein import into nucleus. Among these interactors ALDOA, CKB, and EIF4A2 were identified (Tables 3 and 5). The reduced complexity of the p70 interactome after irradiation allow hypothesizing that the single BRCT2 domain and the MRE11- and ATM- binding motifs are not involved in the recognition of the full-length NBN interactors.

Very low amounts of p70 in lymphoblastoid B-cell lines correlate with B-cell lymphoma development in NBS patients [42]. Similarly, the disease-causing Arg215Trp NBN mutation is associated with low levels of the NBN mutated protein in cell lines, and with a significant predisposition to cancer development in the heterozygous carriers [112, 113]. Therefore, it has been postulated that even loss of one NBN allele increases cancer risk [114], this hypothesis being in line with the concept of DNA repair is a primary cancer avoidance pathway, in which repair enzyme levels are critical for lifetime cancer risk [115]. Remarkably, p70 variation among NBS patients is not determined at the transcription level, but rather by protein stability. Indeed, in vitro inhibition of the proteasome increases cellular levels of p70 [116]. Lastly, the irradiation of cells transfected with p26 caused its interaction with two proteasomal components (i.e., proteasome HSPC and proteasome subunit Y). This might suggests that p70 clearance might be driven by p26, though further studies are mandatory.

As expected from the presence of the MRE11-binding motif [35–37, 65, 117–119], MRE11 and RAD50 were identified among the p70 interactors (Fig. 2C). Notably, this represents an important clinical feature, since patients whose cells display high levels of the truncated p70 protein are at a low risk for lymphoma than those patients with low levels of p70 in their cells [42]. Furthermore, ATM [10, 16–20, 120], CHK2 [50, 51, 52, 65] and BRCA1 [23, 30, 31] bind directly or indirectly to the ATM-binding motif present at the C-terminus of the 70 kDa fragment (Fig. 2C).
Figure 6. Interactome of the p70 fragment. The interactome was gleaned by merging the results obtained through SDS-PAGE-MS/MS analysis of the streptavidin chromatography eluates obtained from cells expressing the p70 protein fragment (A) in control conditions or (B) following 2 Gy of X-rays. For details, see the text.

Conclusions

Several relevant clinical and experimental evidences prompted us to evaluate the residual activity of p26 and p70 fragments through a proteomic characterization of the interactors, both in basal condition and after irradiation.
1. The 657del5 mutation is a hypomorphic mutation causing only a partial loss of the NBN gene function. Remarkably, in mice null mutation of NBN leads to embryonic lethality [121, 122] and only animals with hypomorphic mutations survive past early embryogenesis [123, 124]. Remarkably, transduction of inducible knock-out mice cells with a cDNA containing the 657del5 mutation, rescued cells from death in culture [2]. This clearly indicate that the 26 and 70 kDa fragments arising from the 657del5 mutation retain a residual function, supporting the notion that NBS patients survive owing to the expression of these fragments [2].

2. NBS patients displaying high intracellular levels of the p70 truncated protein are at lower risk for lymphoma than NBS patients expressing low levels of p70 [42].

3. Cells established from NBS patients survive following irradiation, but retain γ-H2AX foci due to a subtle DSBs repair defect, albeit at a lower level compared to Ataxia telangiectasia (AT) lymphocytes (characterized by the absence of the ATM protein) [95, 125, 126]. The slightness of the defect observed in NBS cells compared to AT ones might reflect the expression of the p26 and p70 NBN proteins that, maintaining residual protein activity may contribute, although with a reduced efficiency, to the genome integrity. Indeed, even if in NBS cells the defect of the overall rejoining of DSBs is subtle, a more substantial defect in the correct rejoining of DSBs has been demonstrated [126].

4. Though NBS is a recessive disease and one would not expect any cellular feature or clinical symptom, a growing number of papers report higher spontaneous and induced chromosome instability and an increased incidence of tumors among NBS carriers [127–129]. Remarkably, Nbn+/- mice showed a significantly increased occurrence of spontaneous solid tumors in addition to lymphoma. Moreover, IR dramatically increased cancer formation in Nbn+/- mice, especially thyroid tumors. These data provide a clear relationship between NBN heterozygosity, radiation sensitivity and increased cancer risk. Interestingly, examination of the tumors gave no evidence for loss or mutation of the wild-type allele, suggesting that haploinsufficiency is the presumed pathogenic mechanism [121]. In human heterozygotes, the existence of two truncated proteins produced by alternative translation induced by the 657del5
mutation is compatible with a dominant negative mechanism [41, 129].

The present study reports for the first time a proteomic analysis of the interactors of both the full-length NBN protein and of its fragments arising from the 657del5 founder mutation, responsible for the development of NBS. The application of an unsupervised proteomics approach suggested previously unreported protein interacting partners to the NBN protein and to the p26 and p70 fragments (possessing the FHA/BRCT1 domains, and the BRCT2 domain/MRE11- and ATM- recognition domain, respectively). The phenotype caused by the expression of two proteins normally absent within a cell may arise from a gain-of-function that causes the pathological phenotype deriving from their expression at the homozygous status in NBS patients. Furthermore, since the tandem BRCT domains are the major mediators of phosphorylation-dependent protein-protein interactions, the loss of their integrity is expected to affect the interaction with a number of proteins. Our results revealed that approximately the 30% of the interactors are shared by the full length NBN and the p26 fragment, while approximately the 41% of the interactors are shared by the full length NBN and the p70 fragment. These results highlight several relevant aspects: (i) the two fragments possess a residual protein activity; (ii) the disruption of NBN allows a partial maintenance of the wild-type functions; and (iii) each fragment acquire a gain-of-function determined by the domains and motifs present.

In particular, results obtained shed light on new possible roles of NBN and of its p26 fragment in ROS scavenging, in the DNA damage response, and in protein folding and degradation. Furthermore, the N-terminus of NBN might enroll protein partners involved in the DSBs repair and in the anti-oxidant response. Since some of the newly identified interactors of the p26 and p70 fragments have not been found to interact with the full-length NBN, this suggests that these interactions may somehow contribute to the key biological phenomena underpinning the Nijmegen breakage syndrome. Overall, further studies will be necessary to clarify the biological significance of the newly identified interactors of NBN in cell homeostasis and in the DNA damage response.
Author Contributions
Conceived and designed the experiments: ADA AdM LZ PA. Performed the experiments: AdM CM DC RP VP. Analyzed the data: AdM CM DC. Contributed reagents/materials/analysis tools: AA AdM LZ PA. Wrote the paper: AdM.

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Oncogenic disruption of promyelocytic leukemia nuclear bodies in acute promyelocytic leukemia cells results in defects in DNA double strand breaks sensing and repair
(non published)

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Running title: PML nuclear bodies and DSBs repair

ABSTRACT

Promyelocytic leukemia nuclear bodies (PML-NBs) are non membrane-bound organelles present within the nuclei of mammalian cells. They are implicated in many cellular processes relevant to tumor suppression, including DNA damage response (DDR) and repair. Many proteins involved in the DNA double strand breaks (DSBs) damage response localize to PML-NBs either constitutively or conditionally. The disruption of PML-NBs consequent to the expression of the oncogenic PML-RARα fusion protein is at the root of the acute promyelocytic leukemia (APL) pathogenesis. Despite the growing body of correlative observations, it is yet unclear to what extent the ionising radiation (IR)-induced DSBs response requires PML-NBs integrity. Results here reported suggest that the expression of the PML-RARα fusion protein in APL and APL-like
human cell lines (i.e., NB4 and U937/PR9 cells, respectively), in human primary cells freshly established from RA-untreated APL patients, and in a pre-blastic PML-RARα knock-in mouse model, causes a defective sensing and signaling of the IR-induced DSBs. Specifically, the disruption of PML-NBs due to the expression of PML-RARα caused a delayed DSBs repair, as revealed by the kinetic of disappearance of both the phosphorylated form of H2AX and of 53BP1 foci, but did not cause chromosomal damage. Furthermore, PML-NBs disruption affects the proper activation of the ATM kinase and, consequently, of its substrates H2AX, NBN and CHK2. In turn, these events cause a delayed signaling of the DSBs, and consequently, a delayed repair. Overall, data obtained highlighted the PML-NBs function in coordinating and regulating of the DDR, and further shed light on the pathogenesis and progression of APL.

INTRODUCTION

To ensure the high-fidelity transmission of genetic information, cells have evolved mechanisms to monitor genome integrity. Cells respond to DNA damage by activating a complex DNA damage response (DDR) pathway that includes cell cycle arrest, the transcriptional and post-translational activation of a subset of genes including those associated with DNA repair, and, under some circumstances, the triggering of programmed cell death. An inability to respond properly to, or to repair, DNA damage leads to genetic instability, which in turn may enhance the rate of cancer development. Indeed, it is becoming increasingly clear that deficiencies in DNA damage signaling and repair pathways are fundamental to the etiology of most, if not all, human cancers (Hoeijmakers, 2001; Madhusudan and Middleton, 2005; Bridge et al., 2014).

Among several types of lesion, the DNA double-strand break (DSB) is one of the most deleterious and harmful (O’Driscoll and Jeggo, 2006; Chistiakov et al., 2008; Pardo et al., 2009; Gullotta et al., 2010). The elimination of DSBs involves the spatio-temporal orchestration of a high number of proteins. Briefly, the sensing phase of the DSBs repair includes their recognition by the MRE11/RAD50/NBN complex, the Ataxia-telangiectasia mutated (ATM) protein activation, histone H2AX phosphorylation at Ser139 (the phosphorylated form is named γ-H2AX), MDC1 recruitment, and MRE11/RAD50/NBN retention (leading to further ATM
activation and γ-H2AX spreading). Γ-H2AX is believed to be the initial DSB sensor for subsequent accumulation of various signaling and repair proteins to DNA breaks to form the so-called ionizing radiation (IR)-induced foci (IRIF) (Rogakou et al., 1998; Rogakou et al., 1999; Sedelnikova et al., 2002; Bassing et al., 2003; Rothkamm and Lobrich, 2003; Scully and Xie, 2013). All the above mentioned proteins are physically and functionally required to recruit the p53 binding protein 1 (53BP1) to the DSB sites (Panier et al., 2014). After sensing the damage, a myriad of post-translational modifications that alter the catalytic activities and the specificity of protein interactions took place. Finally, two different, but partially redundant, DSBs repair are involved in the physical removal of the breakage, i.e. the non-homologous end-joining (NHEJ) and homologous recombination repair (HRR) (Hoeijmakers, 2001; Chapman et al., 2012; Gupta et al., 2014).

Promyelocytic leukemia (PML)-nuclear bodies (PML-NBs) are nonmembrane-bound organelles within the nuclei of mammalian cells (de Thé et al., 2012). PML-NBs are implicated in many critical cellular processes relevant to tumor suppression (e.g., induction of apoptosis, cell cycle checkpoints control, transcriptional regulation, post-translational modification, chromatin remodeling, DDR, repair, and senescence (Bernardi and Pandolfi, 2007; Lallemand-Breitenbach and de Thé, 2010; Yeung et al., 2012). PML-NBs are believed to be one of the key regulators in IR-induced DDR (Dellaire et al., 2006a; Dellaire and Bazett-Jones, 2007). Indeed, PML-NBs increase in number and change their sub-nuclear distribution in response to DNA damage, and this response is dependent upon early DNA break-sensing proteins (Dellaire et al., 2006a; Dellaire et al., 2006b). Many proteins involved in DDR localize to PML-NBs either constitutively or conditionally, including the DNA damage-sensing proteins ATM, ATR, BRCA1, CHK2, p53, the MRE11/RAD50/NBN complex, and TopBP1, as well as multiple proteins that participate in DSBs by the homologous recombination repair mechanism (Jensen et al., 2001; Carbone et al., 2002; Bernardi et al., 2004; Dellaire et al., 2006a; Yang et al., 2006; Dellaire and Bazett-Jones, 2007; Viale et al., 2009; Boichuk et al., 2011; Yeung et al., 2012). This supports the hypothesis that PML-NBs may represent DNA damage sensors, their physical disruption, following DNA damage, representing a mechanism of DNA damage signaling (Dellaire et al., 2006 JCB; Dellaire and Bazett-Jones, 2007; Boichuk et al., 2011; Yeung et al., 2012).
At least seven PML isoforms exist due to alternative splicing of the C-terminal exons, thus generating different protein interaction domains (Jensen et al., 2001; Nisole et al., 2013). The PML isoform V, together with DAXX and Sp100, are constitutive components of PML-NBs (Bernardi and Pandolfi, 2007; Lallemand-Breitenbach and de Thé, 2010). Complete or partial loss of PML protein expression or PML-NBs integrity has been reported in several tumor types in addition to acute promyelocytic leukemia (APL) (Gurrieri et al., 2004). The PML gene was originally cloned from breakpoints of the characteristic t(15;17) chromosomal translocation that occur in over 95% of APL patients. The translocated PML gene becomes fused to the retinoic acid (RA) receptor α (RARA) gene, leading to the expression of PML-RARα fusion protein (de Thé et al. 1991; Kakizuka et al., 1991; Fagioli et al., 1992; Pandolfi et al., 1992). Typically only one chromosome 15 and one chromosome 17 are translocated, and wild-type PML and RARα proteins continue to be expressed from the unaffected homologs. The comparison of the cDNA structures of the different APL cases shows variations in the amount of the PML sequence included in the fusion protein (Pandolfi et al., 1992), caused by heterogeneous breakpoint cluster regions as well as by alternative splicing (Chang et al., 1992; Pandolfi et al., 1992; Dong et al., 1993; Geng et al., 1993). About 70% of APL patients exhibit the longest variant named PML(L)-RARα (Chomienne et al., 1996; Melnick and Licht, 1999). The leukemogenic effects of PML-RARα fusion proteins are thus dominant-negative. In APL cells, PML-RARα fusion proteins form homodimers that bind RA response elements on chromosomal DNA (Minucci et al., 2000), leading to the blockage of RA target gene transcription (Grignani et al., 1998; Vitoux et al., 2007). PML-RARα fusion proteins also competitively inhibit binding between wild-type PML proteins, leading to disruption of discrete PML-NBs into a characteristic pattern of dispersed nuclear “microspeckles” (Dick et al., 1994).

Despite this growing body of data, it is yet unclear to what extent the DSBs repair is dependent upon PML and PML-NBs function. Here, the relationship existing between PML-NBs integrity and IR-induced DSBs sensing, signaling and repair has been investigated in APL and APL-like cell lines, and validated in primary cells freshly established from RA-untreated APL patients, as well as in a PML-RARα pre-blastic knock-in mouse model. Data obtained highlighted the PML-NBs function in coordinating and regulating of the DDR.
MATERIALS AND METHODS

Reagents and antibodies

All the products were from Sigma-Aldrich (St. Louis, MO). Analytical- or reagent-grade products, without further purification, were used. Bradford protein assay was obtained from Bio-Rad (Hercules, CA). Antibodies against phospho-Ser1981-ATM (10H11.E12, mouse), phospho-Thr68-CHK2 (Thr68-R, rabbit), CHK2 (A-12, mouse), H2AX (H-124, rabbit), phospho-Ser139-H2AX (Ser139, rabbit), phospho-Ser343-NBN (Ser343-R, rabbit), PML (H-238, rabbit), RARα (C-20, rabbit), and tubulin (TU-02, mouse) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies against 53BP1 (NB100-305, rabbit), ATM (2C1 (1A1), mouse), and NBN (NBS1-501, mouse) were purchased from Novus Biologicals (Cambridge, UK). The antibody against phospho-Ser139-H2AX (JBW301, mouse) was obtained from Millipore (Billerica, MA). Secondary antibodies were purchased from Immunological Sciences (Rome, Italy). The chemiluminescence reagent for Western blot was obtained from Thermo Fisher Scientific (Waltham, MA).

Cell lines, culture conditions, and treatments

The human myeloid leukemia cell lines used in this study were the following: APL-derived NB4 cell line (Lanotte et al., 1991) that contains the t(15;17) and expresses both RARα and PML-RARα proteins; the human promonocytic U937 cell line (U937/WT); the U937 cell line stably transfected with the PML-RARα coding sequence under the control of the zinc-inducible mouse MT-I promoter (U937/PR9) (Grignani et al., 1993); the U937 cell line transfected with the empty vector (U937/MT) (Grignani et al., 1993); the human myeloblastic leukemia cell line HL-60 (Gallagher et al., 1979). All the cell lines were maintained in RPMI 1640 medium (GIBCO Life Technologies, Monza, Italy) supplemented with 10% fetal calf serum (VWR International, Milan, Italy), 100 µg/ml penicillin, and 100 µg/ml streptomycin (VWR International, Milan, Italy). For each experiment, 2X10^5 cells were seeded in a 6-well culture plate. The expression of PML-RARα was induced in U937/PR9 cells with 100 µM of ZnSO₄·7H₂O for 8 h. To suppress the expression of PML-RARα, cells were treated for 96 h with
1×10^{-6} \text{M} \text{ of all-trans retinoic acid (RA). Where indicated, U937/PR9 cells expressing PML-RAR}_\alpha \text{ were exposed for 0.5 h to 10 } \mu\text{g/ml of cycloheximide (CHX) and then irradiated. DSBs were induced by exposing cells to X-rays, using a MGL 300/6-D apparatus (250 kV, 6 mA, Cu filter, dose rate 0.53 Gy/min) (Gilardoni, Mandello Lario, Italy). Human primary cells were isolated from the peripheral blood of RA-untreated APL patients by conventional methods (Diverio et al., 1992; Grimwade et al., 2000).}

**Flow Cytometry**

For cell cycle analysis, after each treatment, 1 X 10^6 cells was washed twice with PBS, fixed dropwise with ice cold ethanol (70%) and rehydrated with PBS. DNA staining was performed by incubating cells for 30 min at 37°C in PBS containing 0.18 mg/ml propidium iodide (PI) and 0.4 mg/ml DNase-free RNase (type 1-A). Samples were analysed with a Dako Galaxy Flow Cytometer equipped with a 488nm laser source. Cell cycle analysis was performed using a FloMax v2.4e software. Double discrimination was performed by an electronic gate on FL3-Area vs. FL3-Height. For hypodiploid peak analysis, Nicoletti’s protocol was followed (Riccardi and Nicoletti, 2006). Briefly, cell pellet was resuspended in 500 µl of PBS, fixed by adding 4.5 ml of 70% cold ethanol, washed twice and resuspended in 500 µl of PBS and 500 µl of DNA extraction buffer (0.19M Na2PO4, 0.004% Triton X-100, pH 7.8). Cells were incubated 5 min RT. Pellet was resuspended in 1 ml of DNA staining solution (20 µg of propidium iodide, 0.2 mg of RNaseA, in PBS) and incubated once again for 30 min RT. Finally, was acquired 20.000 total events in logarithmic scale and percentage of hypodiploid peak was calculated by a proper electronic marker.

**Immunofluorescence analysis**

Cells were washed twice in PBS and seeded on a glass coverslip using a cytocentrifuge Shandon Cytospin III (Thermo Fisher Scientific Inc., Walthman, MA). Slides were then fixed in cold methanol for 15 min and in cold acetone for 2 min, and finally air-dried. The detection of nuclear foci was performed after blocking in 10% BSA dissolved in PBS (w/v), and hybridization over night at 4°C with primary antibodies. The primary antibodies were detected
hybridizing slides for 1 h at 37°C with either 10 µg/ml of Alexa Fluor 488 goat anti-mouse or Alexa Fluor 610 goat anti-rabbit secondary antibodies (Immunological Science, Rome, Italy). Confocal analysis was performed using the LCS Leica confocal microscope (Leica Microsystems, Heidelberg, Germany). Quantitative analysis of γ-H2AX and 53BP1 foci was carried out by counting foci in at least 50 cells/experiment, in three repeated experiments.

**Multicolor FISH (mFISH)**

Fixed cells were dropped onto glass slides and hybridized with the 21X Human Multicolor FISH (mFISH) Probe Kit (MetaSystems, Germany) as described elsewhere (Nieri et al., 2013). Briefly, slides were denatured in 0.07 N NaOH and then rinsed in 70-100% ethanol series. Meanwhile, the probe mix was denatured in a MJ mini personal thermal cycler (Bio-Rad laboratories, USA) as follows: 5 min 75°C, 30 sec 10°C, and 30 min 37°C. Samples were then hybridized in a humidified chamber at 37°C for 48 h followed by one wash in 1X saline-sodium citrate (SSC) buffer for 5 min at 75°C, and finally counterstained with DAPI-containing Vectashield®. Metaphases were visualized and captured using the Axio Imager M1 microscope (Carl Zeiss, Germany) equipped with six filter sets specific for the applied fluorochromes. Karyotyping and cytogenetic analysis of each single chromosome was performed by means of the ISIS software. In total, at least 50 cells for each sample were analyzed by the mFISH-technique in two independent experiments.

**Immunoblotting**

Total cell lysates were prepared by lysing human cell lines in 20 mM Tris-HCl pH 8, 137 mM NaCl, 10% glycerol (v/v), 1% NP-40 (v/v), 10 mM EDTA, 1 µg/ml aprotinin, 1 µg/ml leopeptin, 1 µg/ml pepstatin, 1 mM orthovanadate, and 2 mM PMSF. Lin- cells were lysed in 8 M urea, 25 mM Tris-HCl pH 6.8, 10% glicerol. The protein concentration was determined by the Bradford protein assay. Thirty micrograms of protein extracts were loaded onto a SDS-PAGE and then transferred to polyvinylidene fluoride membrane (Bio-Rad, Hercules, CA). Membranes were blocked in PBS, 3% BSA (w/v), and 0.5% Tween-20 (v/v), for 40 min at room temperature. Primary antibodies were incubated over night at 4 °C,
detected incubating the membranes for 1 h at room temperature with secondary antibodies HRP-conjugated (Immunological Science), and visualized using the enhanced chemiluminescence detection system.

**Purification of lin− cells from APL transgenic mouse**

APL mice were generated by retroviral transduction of PML-RARα into Lin− cells, as previously described (Minucci et al., 2002). C57BL/6 wild-type and transgenic 14-week-old mice were irradiated with 5.5 Gy of X-rays, and sacrificed after 0.5, 3, 6, and 24 h. Lin− cells were purified from the bone marrow. Briefly, after centrifugation through a Ficoll gradient, mononucleate cells were enriched for progenitors by depletion of cells presenting myeloid, erythroid, and lymphoid differentiation markers using commercially available reagents (Stem Cell Technologies, Vancouver, BC, Canada).

**Statistical analysis**

The statistical analysis was performed using the ANOVA test with the InStat version 3 software system (GraphPad Software Inc., San Diego, CA). Data are means of at least three independent experiments ± standard deviations.

**RESULTS**

The integrity of PML-NBs is required for the proper DSBs rejoining

To evaluate whether the IR treatment may modulate RARα and PML-RARα levels, we irradiated NB4, U937/PR9, and U937/WT cells, either treated or not with ZnSO₄ and RA. Results obtained showed that the irradiation with 1 Gy of X-rays did not cause any variations in the expression levels of both RARα and PML-RARα (Figure 1).

Next, to address the role of PML-NBs integrity on the DSB sensing and rejoining efficiency, double immunofluorescence staining with anti-γ-H2AX and anti-PML antibodies was performed in untreated and irradiated cells (Figure 2, panels A-C). Figure 2A compares the γ-H2AX de-phosphorylation profile following irradiation in NB4 cells either expressing the PML-RARα fusion protein (i.e., NB4) or in which the expression of PML-RARα was repressed by the RA treatment (i.e., NB4 +RA cells).
Figure 1. Western blot analysis of RARα and PML-RARα expression in NB4, U937/PR9 and U937/MT cells. Cells were exposed to 1 Gy of X-rays and lysed after 0.5 h. Filters were probed with anti-RARα antibody. Tubulin was used as loading control.

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Figure 2. DSBs repair analysis evaluated by γ-H2AX de-phosphorylation. (A) Exemplificative double immunofluorescence imaging of NB4 –RA and NB4 +RA cells non-irradiated or exposed to 1 Gy of X-rays, and fixed after 0.5, 3, 24 e 48 h from IR. Fixed cells were stained with anti-γ-H2AX and anti-PML antibodies. Counterstain: DAPI. Confocal microscopy images, magnification × 63.
Figure 2 cont. (B) Quantification of γ-H2AX foci/cell in NB4 and U937/PR9 reported as the mean value of γ-H2AX foci at each time point. Error bars represent the standard deviation from the analysis of 50 cells from three independent experiments. Student’s t-test: *p<0.05. (C) Measure of the DSBs rejoining capability in NB4 and U937/PR9 cells; the mean number of γ-H2AX foci obtained 0.5 h after IR was taken as 100%, and the percentages remaining at various times after this were graphed.

First, we observed that, according to data in literature (Yeung et al., 2011), the expression of PML-RARα in NB4 cells did not affect H2AX phosphorylation in response to DSBs induction. Second, we detected an IR-induced increase in PML-NBs both in NB4 and NB4 +RA cells. In particular, in NB4 +RA cells the increase of PML-NBs number and size correlated with the peak of IR-induced DSBs and consequently of the γ-H2AX foci number (Dellaire et al., 2006). However, the possibility that the co-localization between PML and γ-H2AX occurs randomly could not be fully excluded. Lastly, as
discussed below, NB4 cells showed a higher number of $\gamma$-H2AX foci persisting at 24 and 48 h compared to NB4 +RA cells. The DSBs rejoining profile has been reported both as the mean value of $\gamma$-H2AX foci number/cell (Figure 2B) and as the percentage of residual DSBs at each time point (Figure 2C). In the latter, to allow comparison between replicate experiments, the mean $\gamma$-H2AX foci number scored at 0.5 h after IR was taken as 100%, and the percentages remaining at various times after this were graphed. In NB4 cells, a higher number of $\gamma$-H2AX foci (mean value: 70 foci/cell) compared to NB4 +RA cells (mean value: 60 foci/cell) was scored after 0.5 h from DSBs induction; after 3, 24, and 48 h from IR the number of persisting DSBs was significantly higher in NB4 cells (mean values: 56 foci/cell at 3 h; 16.4 foci/cell at 24 h; 10 foci/cell at 48 h) compared to NB4 +RA cells (mean values: 34 foci/cell at 3 h; 5.4 foci/cell at 24 h; 3.2 foci/cell at 48 h) (Figure 2, panels B and C). U937/PR9 cells expressing PML-RAR$\alpha$ (i.e., U937/PR9 +ZnSO$_4$) showed a DSBs repair kinetics very similar to that of NB4 cells (Figure 2, panels B and C). After 0.5, 3, 24, and 48 h from IR, U937/PR9 +ZnSO$_4$ cells displayed a mean value of 55, 45, 7.6 and 3.7 foci/cell, respectively. In comparison, U937/PR9 +ZnSO$_4$ +RA cells displayed a mean value of 52, 28 1.3, and 1 foci/cell after 0.5, 3, 24, and 48 h from IR, respectively. Overall, zinc-induced U937/PR9 cells showed a repair profile comparable to that of NB4 cells, i.e. slower, than cells expressing the PML wild-type protein (Figure 2, panels B and C). Data obtained were compared with the DSBs rejoining profiles of U937/MT cells (Figure 2, panels B and C), and U937/WT and HL-60 cells (Figure 1 Supplementary Material). Overall, results obtained highlight a relationship existing between the expression of the PML-RAR$\alpha$ fusion protein and a delayed DSBs repair, thus suggesting that the DSBs rejoining proficiency is associated to the integrity of PML-NBs.

Since the 53BP1 protein accumulates within PML-NBs (Foltánková et al., 2013) and is actively involved in the DSB sensing (Panier et al., 2014), the relationship between PML-NBs integrity and 53BP1 localization at DSBs was analyzed. Data obtained indicate that PML-NBs integrity is required for 53BP1 localization into the nuclei, as well as for 53BP1 foci formation after DSBs induction. In fact, while 53BP1 was almost not visible in non-irradiated NB4 cells, probably due to a dispersion of the protein into the nucleus, it co-localized with PML within the nuclei of non-irradiated NB4 +RA cells (Figure 3, panels A and B). After IR-induced damage, in cells expressing the PML-RAR$\alpha$ protein, the 53BP1 foci number, as well as 53BP1 co-
Localization with PML, was significantly lower compared to NB4 +RA cells. On the contrary, in NB4 +RA cells, the number of 53BP1 foci increased in irradiated cells, fully co-localizing with PML (Figure 3, panels A and B). This supports the hypothesis that 53BP1 nonrandomly associate with PML-NBs at the DSB foci. The analysis of the DSBs rejoining was performed by counting the number 53BP1 foci/cells (Figure 3B). Overall, after 0.5 h from irradiation, cells expressing the PML-RARα showed a lower number of 53BP1 foci compared to cells expressing the PML wild-type protein (i.e., 41.4 foci/cell in U937/PR9 +ZnSO₄ versus 67.1 foci/cell in U937/PR9 +ZnSO₄ +RA, 59.9 foci/cell in U937/PR9, and 62.7 foci/cell in U937/PR9 +RA; 48.4 foci/cell in NB4 versus 63.9 foci/cell in NB4 +RA) (Figure 3B). After 24 and 48 h from IR, residual 53BP1 foci were scored (i.e., mean value at 24 h: 9.5 foci/cell in U937/PR9 +ZnSO₄, 10.4 foci/cell in NB4; mean value at 48 h: 3.1 foci/cell in U937/PR9 +ZnSO₄, 4.3 foci/cell in NB4). After treatment with RA, both in NB4 and U937 +ZnSO₄ cells the number of 53BP1 foci was comparable to that observed in U937/PR9 cells expressing the PML wild-type protein. The number of 53BP1 foci/cell was scored also in U937/MT, U937/WT, and HL-60 cells (Figure 2 Supplementary Material). Overall, results obtained strongly indicate a role of the PML-NBs integrity in 53BP1 localization at the DSBs.

![Figure 3](image)

**Figure 3.** DSBs rejoining analysis by 53BP1 foci/cell disappearance. (A) Exemplificative double immunofluorescence imaging of NB4 and NB4 +RA cells untreated or exposed to 1 Gy of X-rays and fixed after 0.5 h. Fixed cells were stained with anti-53BP1 and anti-PML antibodies. Confocal microscopy images, magnification × 63.
Figure 3 cont. (B) Quantification of 53BP1 foci association events with PML in control and irradiated NB4 cells, untreated or exposed to 1 µM RA. (C) Quantification of 53BP1 foci/cell in NB4 and U937/PR9 cells, reported as the mean value of 53BP1 foci at each time point. Cells were exposed to 1 Gy of X-rays and fixed after 0.5, 3, 24 e 48 h from IR. Error bars represent the standard deviation from the analysis of 50 cells from three independent experiments. Student’s t-test: **p<0.01; *p<0.05.

The disruption of PML-NBs does not affect genomic stability

Since some chromosomal rearrangements are characterized by DSBs as intermediate and unrepaired DSBs lead to chromosome damage (Pfeiffer et al., 2000; Pfeiffer et al., 2004; Durante et al., 2013), we evaluated whether the residual γ-H2AX and 53BP1 foci observed after 24 and 48 h from IR in NB4 cells may correlate with chromosome instability. The mFISH analysis was performed in NB4 cells, either untreated to exposed to RA, in combination with
irradiation with 1 Gy of X-rays. The karyotype of NB4 was established considering conserved translocations that appear in more than 90% of the cells analyzed in controls. Karyotype found was hypotetraploid and can be summarized as follows: 80 XX, -X, -X, -1, -3, -5, +7, -8, -14, -15, -18, -19, -19, -19, -21, -22, t(8’-9) (9’-8), t(10-19), t(10-19), t(14-19), t(15’-17), t(16-5) t(17’-15), t(17’-15) (Figure 4A).

Analysis of chromosomal exchanges in each sample was performed ignoring basal translocations observed in control cells. Results obtained indicated that RA alone did not exert any effect on the frequency of chromosome aberrations over control values and did not modify X-ray-induced breaks frequency (Figure 4B). In particular, although breaks frequency remain unchanged, RA pre-treatment reduced the frequency of radiation-induced chromosome exchanges (from 2 to 0.9 exchanges/cell in -RA and +RA irradiated samples, respectively) whereas increased the frequency of chromosomal excess fragments (from 1.8 to 3.9 fragments/cells in -RA and +RA irradiated samples, respectively) (Figure 4B). Overall, the only difference observed in NB4 +RA versus NB4 -RA samples was in terms of quality of the radiation-induced chromosome damage rather than in terms of quantity (total breaks).

Figure 4. Evaluation of chromosomal damage in NB4 cells (A) Representative image of a mFISH stained NB4 karyotype. Karyotype was established considering conserved translocations that appear in more than 90% of the cells analyzed in controls.
Figure 4 cont. (B) Frequency per cell of chromosomal aberrations (i.e., fragments, exchanges and breaks) after exposure to 1 Gy of X-rays in NB4 cells untreated or exposed to 1 X 10⁻⁶ M RA.

The integrity of PML-NBs is required for the DNA damage signal transduction

ATM kinase acts as a critical regulator of the cellular DDR. Indeed, ATM is rapidly phosphorylated in response to IR, and this active form in turn phosphorylates H2AX (Rogakou et al., 1998; Rogakou et al., 1999), as well as several component of the DDR (e.g., NBN and CHK2) (Gatei et al., 2003).

The role of PML-NBs integrity on ATM activation in response to DSBs induction was evaluated in cells exposed to 1 Gy of X-rays, fixed after 0.5 and 3 h, and analysed by double immunofluorescence using anti-pSer1981-ATM and anti-PML antibodies (Figure 5A). In cells expressing the PML wild-type protein, PML-NBs number increased in response to DSBs, and pSer1981-ATM foci, co-localizing with PML, were observed both in NB4 +RA and in U937/PR9 cells. In cells expressing the PML-RARα (i.e., NB4 and U937/PR9 +ZnSO₄ cells), a reduced phosphorylation of ATM was observed, and pSer1981-ATM foci poorly co-localized with PML. These data indicate that the proper phosphorylation and activation of ATM is dependent upon PML-NBs integrity, the phosphorylation
possibly taking place at the PML-NBs itself. As control, the U937/MT cells were analyzed (Figure 3 Supplementary Material). To study the role of PML-NBs in the ATM-dependent DNA damage signaling, co-immunofluorescence experiments were performed using anti-pSer1981-ATM and anti-pSer343-NBN antibodies (Figure 5B). Results obtained indicated that in NB4 cells ATM and NBN phosphorylation were significantly reduced after 0.5 and 3 h from IR when compared NB4 +RA cells. Similarly, U937/PR9 cells expressing the wild-type PML showed a high number of pSer1981-ATM foci at 0.5 h from IR, almost all co-localizing with pSer343-NBN foci, both in the absence and presence of RA treatment. After 3 h from IR exposure, pSer1981-ATM foci disappeared, while NBN foci persisted. On the contrary, U937/PR9 cells expressing PML-RARα, ATM was fairly phosphorylated both at 0.5 h and 3 h from IR. As a control, the U937/MT cells were tested (Figure 3 Supplementary Material.).

**Figure 5.** (A) Double immunofluorescence analysis of pSer1981-ATM (green) and PML (red) localization, performed in NB4 and U937/PR9.
The role of PML-NBs integrity in the DDR signaling was further investigated by western blot (Figure 6). Cells were irradiated with 1 Gy of X-rays and lysed after 0.5, 3, and 24 h. Data obtained indicated that in cells expressing the PML-RARα protein (i.e., NB4 and U937/PR9 +ZnSO4 cells), ATM, NBN, and CHK2 proteins were phosphorylated, but with a delayed kinetic.
Figure 6. Phosphorylation of ATM kinase and its substrates. (A) Western blot analysis of ATM, NBN, and CHK2 phosphorylation in NB4 and U937/PR9 cells. Cells were exposed to 1 Gy of X-rays and lysed after 0.5 and 3 h. (B) Phosphorylation of ATM at Ser1981 residue after 24 h from exposure to 1 Gy of X-rays, in NB4 and U937/PR9 cells. Filters were probed with anti-pSer1981-ATM, anti-ATM, anti-pSer343-NBN, anti-NBN, anti-pThr68-CHK2, and anti-CHK2 antibodies. Blots presented are exemplificative of the results obtained from two independent experiments. (C) To evaluate the dependence of the DDR signaling on de novo protein synthesis, U937/PR9 cells were previously treated with 100 µM of ZnSO₄ for 8 h, then exposed for 0.5 h to 10 µg/ml CHX, and finally irradiated with 1 Gy. Filters were probed with anti-γ-H2AX, anti-pSer343-NBN, anti-NBN, anti-pThr68-CHK2, and anti-CHK2 antibodies. Blots presented are exemplificative of the results obtained from two independent experiments.

Indeed, the phosphorylation signal reached a peak after 3 h from irradiation, ATM phosphorylation being visible up to 24 h from irradiation (Figure 6A). On the contrary, in cells expressing the PML wild-type protein, ATM, NBN, and CHK2 were rapidly...
phosphorylated after IR (i.e., 0.5 h), and the phosphorylation signal almost completely disappeared after 3 h (Figure 6B). These data further support the notion that PML-NBs integrity is fundamental for the activation of the ATM-CHK2 axis, the DDR signaling defect observed being independent on de novo protein synthesis, as observed by western blot analysis of γ-H2AX, pSer343-NBN, and Thr68-CHK2 expression in U937/PR9 cells treated with 100 µM of ZnSO4 for 8 h, then exposed for 0.5 h to 10 µg/ml CHX, and finally irradiated (Figure 6C).

Figure 7. In vivo validation of the DDR after DSBs induction in human primary cells established from an APL patient. (A and B) DSBs repair analysis evaluated by both γ-H2AX de-phosphorylation and 53BP1 foci disappearance. Cells were irradiated with 1 Gy of X-rays and fixed after 0.5, 3, 24, and 48 h. Quantification of γ-H2AX foci/cell was reported as the mean value of either γ-H2AX or 53BP1 foci at each time point. Error bars represent the standard deviation from the analysis of 50 cells from two independent experiments.
Figure 7 cont. (C and D) Double immunofluorescence analysis of pSer1981-ATM (green) and PML (red) foci (left panels) and pSer1981-ATM (green) and pSer343-NBN (red) foci (right panels). Cells were treated with 1 Gy of X-rays and fixed after 0.5 and 3 h. (E) Western blot analysis of NBN and CHK2 phosphorylation in cells untreated or irradiated with 1 Gy and lysed after 0.5, 3, and 6 h. Cell image: bright field. Confocal microscopy images, magnification × 63.

Figure 8. In vivo validation of the DDR after DSBs induction in APL transgenic mice. Wild-type and APL transgenic mice were irradiated with 5.5 Gy of X-rays and sacrificed after 0.5, 3, 6, and 24 h. Lin− cells were isolated from the bone marrow of three pooled mice. (A) DSBs repair analysis evaluated by both γ-H2AX de-phosphorylation and 53BP1 foci disappearance. Quantification of γ-H2AX and 53BP1 foci/cell were reported as the mean value of foci at each time point. Error bars represent the standard deviation from the analysis of 100 cells.
Figure 8 cont. (B) Immunoblot analysis of PML and PML-RARα expression, ATM phosphorylation at the Ser1981 residue, and of ATM substrate phosphorylation (i.e., H2AX, NBN and CHK2). (C) Double immunofluorescence of pSer1981-ATM (green) and pSer345-NBN (red) foci in cells fixed after 0.5, 3, and 6 h from irradiation. Cell image: bright field. Confocal microscopy images, magnification × 63.

In vivo validation

Data obtained in NB4 and U937/PR9 cells were validated in human primary cells established from RA-untreated APL patients (Figure 7). Results obtained confirmed the delay in DSBs repair, and the persistence of unrepaired DSBs after 24 and 48 h from IR, as evaluated by counting γ-H2AX and 53BP1 foci/cell (Figure 7, panels A and B, respectively). The analysis of the DNA damage signal transduction indicated that ATM is poorly activated at 0.5 h from IR,
its phosphorylated form being clearly visible only at 3 h from IR (Figure 7C). Similarly, NBN was phosphorylated only after 3 h from irradiation (Figure 7D), whereas CHK2 activation was evident after 6 h from IR (Figure 7E). Overall, these results confirmed a defect in the IR-induced DSBs sensing and signaling in APL patients.

To further evaluate the role of PML-RARα expression in the DSBs damage sensing and signaling, experiments were performed using APL mice generated by retroviral transduction of PML-RARα into Lin− cells (i.e., PR mice). This mouse model allowed us to study the effect of PML-RARα in a pre-blastic model in the absence of any epigenetic and/or genetic alteration responsible for the clonal expansion typical of leukemic cells. First, we confirmed that also in this mouse model the expression of RARα and PML-RARα was not modulated by irradiation (Figure 8, panel B). When looking at the DSBs response, we observed that in PR mice the kinetic of both γ-H2AX de-phosphorylation and 53BP1 recruitment at the DSBs was delayed compared to wild-type animals (i.e., WT), with the persistence of a significantly higher number of unrepaired damage after 24 h from IR (p<0.05) (Figure 8, panels A and B). In addition, the ATM activation, as well as the phosphorylation of its substrates NBN and CHK2, was impaired in PR mice compared to WT ones (Figure 8, panels B and C).

DISCUSSION

PML protein and PML-NBs are known to be implicated in the DDR, although it remains still unclear whether and to what extent the DSBs repair is dependent upon them. In particular, it has been suggested that PML-NBs serve as DNA damage sensors, DNA repair compartments, and physical sites where the DDR events are coordinated and monitored (Carbone et al., 2002; Varadaraj et al., 2007). Recently, using HT1885 and HEK293 cell models in which PML was either depleted or over-expressed or in which the PML-RARα fusion protein was transfected, it has been demonstrated that the DSBs repair is dependent upon PML protein (Yeung et al., 2012). However, the complexity of the hematopoietic compartments cannot be fully mimicked by gene silencing or overexpression strategies. Therefore, to shed light on the relationship between PML NBs integrity and the response to the DSBs, here we investigated the role of PML and PML-NBs in DSBs sensing, signaling, and repair using the APL-derived NB4 cell line (Lanotte et al., 1991) and the
human promonocytic U937 cell line stably transfected with the PML-RARα coding sequence (i.e., U937/PR9; Grignani et al., 1993). Notably, results obtained were validated in vivo, using human primary cells established from RA-untreated APL patients and a PML-RARα knock-in pre-blastic mouse model.

The harmful effects of just one DSB underline the importance of a sensitive damage detection system and a rapid response. DSBs are the major lethal lesion from ionizing radiation, with serious consequences for genomic stability and cancer. The importance of H2AX phosphorylation for the DDR has been highlighted in H2AX−/− mice that although being viable, display several abnormalities including accumulation of chromosomal aberrations and enhanced radio-sensitivity (Bassing et al., 2002; Celeste et al., 2002; Fernandez-Capetillo et al., 2002). Here, the analysis of H2AX phosphorylation both in vitro and in vivo revealed a high basal level of γ-H2AX foci in NB4 cells, as well as in APL patients and in mice expressing the PML-RARα protein. However, the same result was not observed in U937/PR9 +ZnSO₄ cells. Although γ-H2AX has been reported to increase in the S and G2 phases of the cell cycle independently from irradiation (Huang et al., 2005; Pluth et al., 2007), the NB4 and U937/PR9 cell lines were similar regarding their proliferation properties, showing a very similar percentage of cells distributed between the S and G2 phases of the cell cycle (Figure 4 Supplementary Material; Benedetti et al., 1998). Noteworthy, high levels of γ-H2AX foci have been reported in human tumors and cultured cells (e.g., astrocytoma, fibrosarcoma, melanoma, cervical and colon carcinoma, glioblastoma) (Rogakou et al., 1999; Sedelnikova et al., 2002; Warters et al., 2005; Nakamura et al., 2009), such endogenous DNA damage resulting from telomere dysfunction, replication and transcription errors, reactive oxygen species, and genome instability. However, the mFISH analysis here performed in NB4 cells did not reveal a higher induction of chromosomal damage after irradiation, comparing RA-untreated and -treated cells. It has been suggested that substantial fraction of the endogenous γ-H2AX and 53BP1 foci might be marking uncapped telomeres rather than DSBs (Takai et al., 2003; Nakamura et al., 2009).

The second relevant aspect emerged is that the expression of the PML-RARα fusion protein, both in NB4 and U937/PR9 +ZnSO₄ cells, slow down the DSBs rejoining kinetics, causing the persistence of a higher number of unrepaird or misrepaird DSBs at long times from IR, i.e. 24 and 48 h, compared to wild-type cells. These data are
consistent with previous DSBs rejoining studies performed in NBN-deficient cells that revealed no defect at early times from DNA damage induction, but persistence of DSBs at 24 h from IR (Kraakman-van der Zwet et al., 1999; Girard et al., 2000; Porcedda et al., 2006; Pluth et al., 2007). Such persistence agreed qualitatively with an observed increase in both chromosome fragments and translocations in these cells, thus bringing to the speculation that NBN-deficient cells have a DSBs rejoining defect in a process specific for a subclass of DSBs that preferentially give rise to chromosome breaks (Pluth et al., 2007). A similar finding was also been reported for ATM- and Artemis-defective cells, which proved incapable of rejoining a small fraction of IR-induced DSBs, the so-called ‘dirty’ end breaks (Riballo et al., 2004; Porcedda et al., 2006). The persistence of γ-H2AX foci in cells expressing the fusion protein may be ascribed to a reduced ability to resolve a subclass of DSBs. Since PML-NBs are defined as baskets that contains proteins involved in the DSBs sensing and repair (Yeager et al., 1999; Henson et al., 2002; Dellaire et al., 2003; Xu et al., 2003; Spardy et al., 2008), their disruption may cause a dispersion of these proteins and consequently a defect in the DSBs tethering (Friedberg et al., 2006; Jackson and Bartek, 2009).

Although the phosphorylated form of H2AX is required for the retention of DNA damage and repair proteins in the vicinity of DNA lesions, H2AX phosphorylation is dispensable for the initial recognition of DNA breaks because the repair and signaling factors, such as the MRN complex, 53BP1, and BRCA1, can be initially and transiently recruited to DSBs in the absence of H2AX (Celeste et al., 2003). However, although γ-H2AX represents a biomarker of the DSB but not a functional component of the DSB signaling and repair (Cleaver et al., 2011), we further analysed the DSB sensing using a protein directly involved in DSBs repair, such as 53BP1. The 53BP1 protein is an established player in the cellular response to DNA damage and is a canonical component of IRIF. It has been recently demonstrated that 53BP1 localize at the PML-NBs (Foltánková et al., 2013), and significantly affects the outcome of DSBs repair by modulating chromatin structure surrounding the break site (Noon and Goodarzi, 2011). DSBs rejoining analyzed by 53BP1 foci disappearance highlighted that 53BP1 nonrandomly associate with PML-NBs at the DSB foci, and confirmed a defective recruitment of 53BP1 protein to the damaged sites in cells expressing the PML-RARα fusion protein that was re-established by treating cells with RA. These results further support a direct role of PML-NBs in the
DSB sensing, regulating and controlling the availability of protein directly involved in the damage sensing and repair (Viale et al., 2009). Remarkably, it has been recently demonstrated that the expression of PML-RARα increases the histone deacetylase inhibitors (HDI)-induced DNA damage and apoptosis, by downregulating the expression of genes involved in the base excision repair mechanism (Petruccelli et al., 2013).

It has been reported that HT1885 cells, derived from the fibrosarcoma cells HT1080 stably transfected with the homologous recombination repair (HRR) reporter construct (Lio et al., 2004) and expressing the PML-RARα protein, were characterized by a 10-fold reduction of DSBs repair by the HRR pathway (Yeung et al., 2012). Interestingly, the same authors reported that the PML protein is not required for the initial sensing of DNA lesions at the level of H2AX phosphorylation (Yeung et al., 2012). On the contrary, our results strongly suggest a relationship between PML protein and DSBs sensing, as measure by both γ-H2AX and 53BP1 residual foci counted at long time from irradiation. As previously suggested, such discrepancy may be ascribed to the cellular model used. In conclusion, the reduced ability of cell expressing the PML-RARα fusion protein to repair DSBs may reflect a defect arising from the disruption of the PML-NBs, as well as the dominant negative effect of the fusion protein with respect to both the PML protein and the proteins involved in the DNA repair.

PML-NBs increase in number and change their sub-nuclear distribution in response to DNA damage (Chan et al., 1997; Lydall e Whitehall, 2005; Dellaire et al., 2006a; Dellaire et al., 2006b), and this response is dependent upon early DSBs sensing proteins, leading to the suggestion that PML-NB may act to transduce DNA damage signaling. Post-translational modifications regulate multiple biological functions of the PML protein and also the fission, disassembly, and rebuilding of PML-NBs during the DDR (Schmitz and Grishina, 2012). Overall, PML-NBs appear to represent multifunctional structures within the nucleus where proteins and protein complexes, involved in a variety of molecular pathways, are assembled, anchored and/or post-translationally modified. To date, it has been hypothesized that these effects may depend either on a IR-activated kinase, or on the inhibition of a kinase negative regulator, or specifically on the activation of the ATM-CHK2 axis (Barr et al., 2003; Varadaraj et al., 2007). Indeed, PML role in the damage sensing and repair is dependent upon the ATM and ATR kinases (Shiloh, 2001; Pommier et al., 2005; Iijima et al., 2008), although
chromatin structural changes seem to be primarily ATM-dependent (Shiloh, 2001). Exposure of cells to radiation triggers the kinase activity of ATM and enables it to phosphorylate several substrates involved in multiple damage response pathways (Shiloh, 2003). To determine whether cells expressing the PML-RARα protein and characterized by a defect in the DSBs rejoining were proficient in the activation of the ATM-dependent pathway, we analyzed the autophosphorylation of ATM at the Ser1981 residue, an event reflecting the activation of ATM by DNA damage (Bakkenist and Kastan, 2003), and we assessed the phosphorylation of ATM target residues Chk2-Thr68 and NBN-Ser343 (Shiloh, 2003). Results obtained highlighted that PML-NBs play a critical role in the ATM pathway, since their disruption strongly affected both ATM activation and CHK2 and NBN phosphorylation. These defects were clearly associated with the expression of the PML-RARα protein. This support the notion that the PML-NBs represent not only sites for the sequestration and release of proteins involved in signaling and DNA damage repair, but also regions that regulate the post-translational modifications of nuclear proteins (D’Orazi et al., 2002; Hofmann et al., 2002; Yang et al., 2006; Dellaire et al., 2006a; Dellaire and Bazett-Jones, 2007). Furthermore, our results highlighted that the PML protein has itself a regulatory role on the activation of the ATM-CHK2 axis. Indeed, the endogenous or induced expression of the PML-RARα protein affects ATM activation and consequently the phosphorylation of NBN and CHK2. In turn, these PML-dependent effects determine a defect in the DSBs signaling that may be responsible for the progression of APL. Our results agree with data obtained in PML-depleted human fibroblasts, suggesting a functional role for PML-NBs in IRIF formation, maintenance, and clearance during the DSB repair process after low-dose irradiation (Münch et al., 2014). Furthermore, these results are consistent with studies showing that accumulation of many DDR factors at DNA damage foci is abolished in PML-depleted cancer cells (Xu et al., 2003; Boichuk et al., 2011; Liu et al., 2011; Yeung et al., 2012).

Overall, the results here reported suggested that the expression of the PML-RARα fusion protein in APL and APL-like human cell lines, in a pre-blastic PML-RARα knock-in mouse model, and in human primary cells freshly established from RA-untreated APL patients causes: (i) a delayed sensing of the DSBs, thus supporting the notion that the fusion protein causes a DNA damage repair impairment; and (ii) a lower activation of ATM and ATM substrates, especially at...
short time from IR, these events being responsible for a defective DDR. Furthermore, it seems that, at least in NB4 cells (-RA versus +RA), the observed defects in the DSBs sensing and signaling cannot be translated in a higher induction of chromosomal damage, an aspect that needs further investigations. These data highlight the PML-NBs function in coordinating and regulating of the DDR, and further shed light on the pathogenesis and progression of APL.

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Figure 1. DSBs repair analysis evaluated by γ-H2AX de-phosphorylation. (A) Quantification of γ-H2AX foci/cell in U937/MT, U937/WT, and HL60 cells, reported as the mean value of γ-H2AX foci at each time point. Error bars represent the standard deviation from the analysis of 50 cells from three independent experiments. (B) Measure of the DSBs rejoining capability in U937/MT, U937/WT, and HL60 cells; the mean number of γ-H2AX foci obtained 0.5 h after IR was taken as 100%, and the percentages remaining at various times after this were graphed.
Figure 2. DSBs rejoining analysis by 53BP1 foci/cell disappearance in U937/MT, U937/PR9, and HL60 cells reported as the mean value of 53BP1 foci at each time point. Cells were exposed to 1 Gy of X-rays and fixed after 0.5, 3, 24 and 48 h from IR. Error bars represent the standard deviation from the analysis of 50 cells from three independent experiments.

Figure 3. Double immunofluorescence analysis of (A) pSer1981-ATM (green) and PML (red), and (B) pSer1981-ATM (green) and pSer343-NBN (red) localizations in U937/MT cells either untreated or exposed to 100 µM ZnSO₄. Cells were treated with 1 Gy of X-rays and fixed after 0.5 h and 3 h. Cell image: bright field. Confocal microscopy images, magnification × 63.
Figure 4. NB4 (A) and U937/PR9 (B) cells distribution among the G0/G1, S and G2/M phases of the cell cycle.
CONCLUSIONS

Data obtained from this PhD project shed light on the determinant role of nibrin and PML proteins in sensing and signaling of the DSBs. Only a rapid and neat recruitment and assembly of the proteins involved in DDR allows an efficient repair of the DSBs. Of note, DSBs are the most toxic DNA lesions affecting the human genome that, if unrepaired, determine either the accumulation of mutations or the onset of chromosomal aberrations (Stephen et al., 2009; Korwek et al., 2014). In turn, these events promote the onset of human genetic diseases associated with genomic instability, such as cancer (Tian et al., 2015).

It is known that the crucial events in the very early phase of the DSBs sensing are represented by: (i) the phosphorylation of the H2AX histone (i.e., γ-H2AX), (ii) the recruitment of the MRN complex to the damages site (Carney et al., 1998; Spycher et al., 2008), and (iii) by the activation of the DSBs signaling events (Wen et al., 2013). The loss of the structural integrity of the main proteins involved in the DNA damage sensing and repair may affect the DDR. It is known that mutations at the homozygous status within nibrin causes the rare genetic disorder named Nijmegen breakage syndrome (NBS) described for the first time in 1981 in patients living in Nijmegen (Netherlands) (Demuth and Digweed, 2007). More than 50 proteins, containing one or more tandem BRCT (tBRCT) domains, and involved in the DNA repair and in cell cycle progression (e.g., NBN, 53BP1 and MDC1) have been characterized. (di Masi et al., 2008a).

During the first year of the PhD project, we first decided to will focus our attention on the current state-of-knowledge regarding the correlation between heterozygous carriers of nibrin mutations and the proneness to the development of malignant tumors. The analysis performed highlighted that healthy heterozygous carriers of the 657del5 and 643C>T (corresponding to the Arg215Trp substitution) nibrin mutations, which are clinically asymptomatic, display an elevated risk to develop some types of malignant tumors, especially breast, prostate and colorectal cancers, lymphoblastic leukemia, and non-Hodgkin’s lymphoma (NHL). So, it clearly emerged that the expression of a single copy of the wild-type nibrin is not sufficient to allow a proper DDR and to maintain genomic stability, suggesting that both mutations may have a “dominant negative effect” with respect to the wild-type nibrin (di Masi et al., 2012).
Compound heterozygous 657del5/Arg215Trp NBS patients display a clinical phenotype more severe than the majority of NBS patients homozygous for the 657del5 mutation (Seemanova et al., 2007). It has been demonstrated that this phenotype is due to the fact that the Arg215Trp mutation impairs γ-H2AX binding after induction of DNA damage, leading to a delay in DSBs rejoining. Molecular modelling reveals that the 215 residue of nibrin is located between the tBRCT domains, affecting their relative orientation that appears critical for γ-H2AX binding. So, the expression of the Arg215Trp substitution, both at the heterozygous and compound heterozygous status, affects the DSBs repair more seriously than the 657del5 mutation, probably acting with a “dominant-negative effect” with respect to the 657del5 mutation itself (di Masi et al., 2008). Of note, also the 657del5 mutation localizes within nibrin tBRCT domains. These domains are present in more than 50 proteins involved in DNA damage sensing/repair and in cell cycle checkpoint control, suggesting their role in these pathways (di Masi at al., 2008a). At the light of these considerations, we next decided to study the effect of the 657del5 and Arg215Trp mutations in the DDR, being this the main pathway in which nibrin is involved. In particular, through immunoprecipitation and immunofluorescence experiments, we showed that nibrin tBRCT domains are not involved in the MRN complex formation, but rather play a role in the recruitment of MRE11 to the DSBs sites. Furthermore, nibrin tBRCT domains seem necessary to properly activate the DNA damage signaling, through the phosphorylation of effector proteins like ATM, SMC1 and p53 (Mendez et al., 2012). These results agree with the well-known role of the tBRCT domains in phosphorylation-dependent protein-protein interactions (Williams et al., 2005; Glover et al., 2006; di Masi et al., 2011).

However, it is still highly debated in literature whether nibrin directly interacts with γ-H2AX, or rather the binding is mediated by MDC1, an important DSBs sensor protein (Stucky et al. 2005). Indeed, some authors suggest that MDC1 possesses the structural determinants for directly interacting with γ-H2AX on one side, and with nibrin of the other. This allows the proper localization of the MRN complex to the DSBs (Lee et al., 2005; Stucki et al., 2005; Coster and Goldberg 2010). Remarkably, data obtained in our laboratory support the direct interaction of nibrin with γ-H2AX. In fact, through a bioinformatics approach we observed that the molecular determinants that allow the interaction of MDC1 with the phosphorylated histone C-terminal region are conserved also in
nibrin (di Masi et al., 2012; unpublished results, see Supplementary materials). These results were confirmed by spectrofluorimetric analysis indicating that the emission spectrum observed mixing γ-H2AX to the nibrin tBRCT domains is significantly different (i.e., reduced) if compared to that obtained in the presence of the unphosphorylated form of H2AX. Such effect may be due to the γ-H2AX binding-dependent quenching of the Trp and Tyr residues located within the tBRCT domains (unpublished results, see Supplementary materials).

Since the 90% of NBS patients present the 657del5 mutation, during the second year of this PhD project, we decided to investigate the residual functions of the two fragments arising from this nibrin founder mutation. In fact, this mutation determines the synthesis of two truncated proteins of 26 kDa (p26) and 70 kDa (p70) (Maser et al., 2001). The p26 protein includes the region encompassing amino acids 1-218 of the NBN protein, thus comprising the FHA and the BRCT1 domains. The p70 protein is produced by an alternative initiation of translation upstream the 5 base pair deletion; after a 18 residue extension at the N-terminus, the sequence is identical to that of the wild type NBN from the amino acid 221 to the end, and contains the BRCT2 domain and the C-terminal region of NBN (Becker et al., 2006). Remarkably, a significant correlation between the p70 expression levels and lymphoma incidence has been observed; in fact, patients displaying high intracellular levels of the p70 truncated protein are at lower risk for lymphoma than those with low levels of p70 (Kruger et al., 2007). By applying an unsupervised proteomic approach, we identified full length nibrin interactors, as well as the p26 and p70 fragments one (Cilli et al., 2014). In particular, we showed that the full length nibrin plays a role not only in the DDR, but also in the regulation of the G2/M cell cycle transition, in cell growth control, in meiosis, in transcription regulation, in apoptosis activation, in protein biosynthesis and degradation, in nuclear protein import, and finally in the oxidative stress response (Cilli et al., 2014). Indeed, nibrin has been found to interact with anti-oxidant enzymes like PRDX1 and SOD1, these events being associated to the DDR (Zhao et al., 2008). It is known that PRDX1 induction is ATM-dependent, which is considered an important sensor of reactive oxygen species (ROS) in human cells (Guo et al., 2010), protecting them from radiation damage (Chen et al., 2002). It has been speculated that the ATM/NBN axis controls PRDX1 expression, nibrin directly interacting with PRDX1 through
the FHA-BRCT1 domain. In fact, PRDX1 has been identified also among the p26 nibrin fragment (Cilli et al., 2014). The identification of SOD1 among the full-length nibrin interactors, after IR-dependent DSBs induction, supports nibrin role in the oxidative stress response (Sagan et al., 2009; Krenzlin et al., 2012). Remarkably, it is known that oxidative stress induces events strictly connected to the DDR, such as histone H2AX phosphorylation, the cell cycle-dependent MRE11 recruitment, and ATM and CHEK2 activation (Zhao et al., 2008).

A further nibrin interactor after IR treatment is calreticulin, which is involved in the Ca\(^{2+}\)-dependent protein folding; disturbances in Ca\(^{2+}\) homeostasis and protein folding are essential features of neurodegeneration (Federico et al., 2012; Prell et al., 2013). Since one of NBS main clinical manifestations are both microcephaly and intellectual disability (Digweed and Sperling, 2004; Chrzanowska et al., 2012) we suggested a relationship between these different pathways.

Also p26 fragment, containing the nibrin FHA and BRCT1 domains alone, maintains the capability to interact with proteins involved in the DDR like \(\gamma\)-H2AX, CtIP, and PRKDC. Among the other p26 interactors, we identified proteins involved in DNA/RNA binding, in anti-oxidant responses, in ribosome joining, and DNA repair. Of note, PARP1 protein was observed among the p26 fragment interactors after IR. We demonstrated that the BRCT-mediated interaction of p26 with PARP1 exerts an inhibitory effect on PARP1 activity, as measured by NAD\(^+\) levels. Furthermore, the p26-PARP1 interaction seems to be responsible for the persistence of ROS, and in turn of DSBs, at long time from IR (i.e., 24 h).

Since some of the newly identified interactors of the p26 and p70 fragments have not been found to interact with the full-length NBN, these interactions may somehow contribute to the key biological phenomena underpinning NBS, contributing to the characteristic phenotype. Finally, only a short subset of proteins was identified among the p70 interactors, involved in helicase activity and regulation of protein import into nucleus. This result suggested that the single BRCT2 domain and the MRE11- and ATM-binding motifs were not involved in the recognition of all the full-length NBN interactors.

Several proteins involved in DDR, like the MRN complex, BRCA1, 53BP1, TopBP1, are localized within PML-NBs, non-membrane bound spherical organelles within the nuclei of mammalian cells.
(Melnick et al., 1999). This supports the hypothesis that PML-NBs may represent DNA damage sensors, their physical disruption, following DNA damage, representing a mechanism of DNA damage signaling (Dellaire et al., 2006 JCB; Dellaire and Bazett-Jones, 2007; Boichuk et al., 2011; Yeung et al., 2012). Two structural components of PML-NBs are PML and SP100 proteins (Geng et al., 2012). We focalized the attention on the possible role of these nuclear structures in DDR since SP100 was found among the NBN full-length interactors, mostly after DNA damage induction (Cilli et al., 2014). A loss of PML-NBs integrity is reported in several tumour types, among these the APL, caused by the t(15;17) involving the PML protein and the retinoic-acid receptor RARα, with the consequent generation of PML-RARα fusion protein (Gurrieri et al., 2004; (de Thé et al. 1991; Kakizuka et al., 1991; Fagioli et al., 1992; Pandolfi et al., 1992). Even if only one chromosome 15 and one chromosome 17 are translocated and wild-type PML and RARα proteins continue to be expressed from the unaffected homologs, however the leukemogenic effects of PML-RARα fusion proteins are dominant-negative, this mutation determining a PML-NBs disgregation that produces microspeakles, typical of the desease (Dick et al., 1994). During the third year of this PhD project, the join of results obtained from immunofluorescence and western blotting experiments, has allow to understand that: i) the PML-NBs loss of integrity caused by the expression of the PML-RARα fusion protein causes a significant DNA repair kinetic delay, after IR induction, and alters 53BP1 localization at the DSBs sites, ii) PML-NBs disruption affects ATM phosphorylation and activation, the phosphorylation possibly taking place at the PML-NBs itself, determining consequently the failure of the nibrin and CHK2 substrates phosphorylation. The same results were observed in human primary APL cells and validated in vivo through a pre-leukemic mouse model, knock in for PML-RARα. The use of these mice allowed attributing the deficit in the DNA damage repair kinetic only to the PML-RARα expression, excluding the pathological context characterizing APL cells. We confirmed that the repair kinetic is delayed in PR mice, signal of a residual DNA damage unrepaired. Therefore the two possible aspects that may be studied are: i) PML and PML-RARα interactors’ identification, in control situations or after DNA damage induction; and ii) the role of PML and PML-NBs integrity in genome stability.

A study in depth of data obtained from PML proteomics analysis may open new scenario for the development of molecules
interfering, in a specific manner, with the delineated molecular mechanisms. For these reasons we think that the understanding of the close relationship between NBN and PML structure and function is fundamental since the loss of the two proteins function is associated to cancer development.

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SUPPLEMENTARY MATERIALS

INTRODUCTION

Nibrin (also known as NBN) is a member of the trimeric complex formed by MRE11, RAD50 and NBN (MRN), a dynamic macromolecular machine that acts in the first steps of DNA double strand break repair, and of which the components have intrinsic dynamics and flexibility properties, directly linked with their functions (Stracker et al., 2011; Lafrance-Vanasse et al., 2015). After DNA damage, several proteins involved in the DDR (including MRN) accumulate in large sub-nuclear structures, called ionizing radiation-induced nuclear foci (IRIF) (Paull et al., 2000; Rouse et al., 2002; Kobayashi et al., 2008; Tobias et al., 2013). In mammalian cells a crucial component of IRIF is the histone H2AX, which, in response to DSB induction, is phosphorylated at the Ser139 residue (named γ-H2AX) by members of the phosphoinositide-3-kinase-related protein kinase (PI3K) family (Rogakou et al., 1999). Nibrin is composed of an N-terminal region containing the fork-head associated (FHA) domain (amino acids 24-109), and two breast cancer 1 (BRCA1) carboxy-terminal (BRCT) tandem domains (i.e., BRCT1, amino acid residues 114-183; and BRCT2, amino acid residues 221-291) (Kobayashi et al., 2004; Becker et al., 2006). The central region of nibrin contains a consensus sequence encompassing the Ser343 residue that, together with the Ser278 residue located within the BRCT2 domain, is phosphorylated by the Ser/Thr protein kinase ATM in response to ionizing radiation (IR) (Gatei et al., 2000; Lim et al., 2000; Wu et al., 2000; Zhao et al., 2000; Shiloh et al., 2013). Lastly, the C-terminal region of nibrin contains the MRE11-binding domain and the ATM-binding motif (Digweed et al., 2004; Kobayashi et al., 2004) (Fig. 1). Notably, the BRCT domain represents the major mediator of phosphorylation-dependent protein-protein interactions in cell cycle checkpoint and DNA repair mechanisms (Koonin et al., 1996; Manke et al., 2003; Yu et al., 2003; Glover et al., 2004; Williams et al., 2005; Glover et al., 2006; di Masi et al., 2011). This domain was described for the first time in the BRCA1 protein, codified by the breast and ovarian cancer suppressor gene BRCA1 (Koonin et al., 1996). Since then, more than 50 proteins, containing one or more tandem BRCT (tBRCT) domains, and involved in the DNA repair and in cell cycle progression (e.g., nibrin, 53BP1 and MDC1) have been characterized (Glover et al., 2006). The BRCT repeat structure provides a flexible
framework for diverse interactions that are crucial in DNA damage signaling, and may represent scaffolding elements at the heart of large, multi-protein complexes (di Masi et al., 2011; Gerloff et al., 2012).

**Figure 1**: Schematic representation of nibrin structure. FHA: fork head associated domain; BRCT1-2: breast cancer 1 (BRCA1) carboxy-terminal (BRCT) tandem domains.

**MATERIALS AND METHODS**

**Plasmid constructs, site-directed mutagenesis and transformation**

Using specific primers (FW_FHA: 5’ - CGCGGATCCGCGATGGAAACTGCTG - 3’; FW_BRCT: 5’ – CGCGGATCCGCGATAGAGTATGAGCCTTTG - 3’; RV_BRCT2: 5’ – CCGCTCGAGCGGGGCCCTGAGGATCACAGTA – 3’), the cDNA sequences encoding the FHA-BRCT1-2 and the only BRCT1-2 regions of nibrin were mutagenized by adding the BamHI and XhoI restriction sites at the 5’ and 3’ ends, respectively, to enable cloning into the pET-28a(+) expression vector (Novagen, CN Biosciences, San Diego, CA, USA). The amino acidic substitution Lys160Met was introduced into the wild-type cloned sequences by the QuickChange Lightning Site-Direct Mutagenesis system (Stratagene, La Jolla, CA, USA). All codon changes and DNA fragment exchanges were confirmed by sequencing. The expression vectors obtained were transformed and amplified in *E. coli* Rosetta(DE3)pLysS (Novagen). The cells used to express the pET-28a(+)_(FHA)-BRCT1-2 vector were grown in Terrific Broth (2.4% yeast extract, 1.2% tryptone, 0.45% glycerol, 1.3% K$_2$HPO$_4$, 0.38% KH$_2$PO$_4$; Sigma-Aldrich, Saint Louis, MO, USA) supplemented with ampicillin (50 µg/mL) (Sigma-Aldrich) and chloramphenicol (34
µg/mL) (Sigma-Aldrich) at 37°C until the O.D.₆₀₀ reached a value of 0.6.

**Proteins expression**

The wild-type or Lys160Met FHA-BRCT1-2 and BRCT1-2 recombinant proteins were expressed using IPTG 0.1 mM, at 20°C for 20 h. The bacterial cell pellets were harvested by centrifugation at 8,000 rpm and 4°C for 15 min and stored at -80°C. The bacterial cell pellets were lysed at a ratio of 2 ml for 1 gr, by repeated pipetting, using a buffer composed of: 50 mM NaH₂PO₄ pH 8.0, 300 mM NaCl, 10 mM imidazole, and 20 mM β-mercaptoethanol. Then lysozyme was added at a ratio of 1 mg for 1 ml of the resuspended pellet volume. Cell lysates were incubated on ice for 30 min and then sonicated (six cycles, 10 seconds each, with intervals of 10 seconds on ice). The suspension was centrifuged at 11,000 rpm and 4°C for 25 min and the supernatant was quantified with Bradford (AMRESCO LLC, Solon, OH, USA) method.

**Affinity chromatography**

Each clarified lysate was mixed for 1 h at 4°C with a PerfectPro Ni-NTA Agarose resin (5 PRIME Inc., Gaithersburg, MD, USA) at a ratio of 6.4 mg of lysate for 1 ml of resin, previously equilibrated with 3 volumes of 50 mM NaH₂PO₄ pH 8.0, 300 mM NaCl, and 20 mM imidazole (here named wash buffer). The column, to which the lysates were applied, was washed with 20 volumes of wash buffer. Then 5 fractions (1 ml each), were eluted with 50 mM NaH₂PO₄ pH 8.0, 300 mM NaCl, and 100 mM imidazole and 8 fractions were eluted with 50 mM NaH₂PO₄ pH 8.0, 300 mM NaCl, and 300 mM imidazole.

**SDS-PAGE**

Protein extracts were heated at 100°C for 5 min in sample buffer 1X and resolved by SDS polyacrylamide gel electrophoresis (SDS-PAGE). Samples were applied to 12% polyacrylamide gels with a 5% stacking gel and electrophoresed at 100 V for 1 h using the Tris/glycine/SDS (TGS) 1X (Biorad, Hercules, CA, USA) course buffer at pH 8.3. The proteins were stained with Silver nitrate (Fig. 2). The fractions containing the purified nibrin protein were pooled.
and dialyzed for 3 days against 10 mM HEPES/150 mM NaCl (pH 7.4), to remove imidazole. The protein pool was lyophilized to concentrate it.

Figure 2: Analysis through SDS-PAGE of elution fractions obtained by affinity chromatography. Elution fractions derived from affinity chromatography of A) pET-28a(+) BRCT1-2; 1-5: fractions eluted with 100 mM imidazole; 5*: fraction eluted with 300 mM imidazole; and B) pET-28a(+) FHA-BRCT1-2 recombinant bacteria lysates; 2-5: fractions eluted with 100 mM imidazole; 2*: fraction eluted with 300 mM imidazole.
Fluorescence Studies

The peptides γ-H2AX and H2AX were purchased from Abcam (Cambridge, UK). Fluorescence spectra were acquired using a Jasco FP-6500 spectrofluorimeter (Jasco Corporation, Tokyo, Japan) with a 0.5 cm path length cell. The bandwidth for excitation and emission was 5 nm. For equilibrium fluorescence spectra, tryptophan residues were selectively excited at 290 nm and the emission was recorded from 300 to 400 nm. Values of the dissociation equilibrium constants for γH2AX and H2AX (final concentration, 5.0 \times 10^{-8} \text{ M} to 1.0 \times 10^{-5} \text{ M}) binding to wild type (FHA)-BRCT1-2 or K160M (FHA)-BRCT1-2 (final concentration, 1.0 \times 10^{-6} \text{ M}) were obtained at pH 7.0 (5.0 \times 10^{-2} \text{ M phosphate buffer}) and 25.0°C. Under all the experimental conditions, the assay time was 120 min.

RESULTS AND DISCUSSION

Some authors suggest that nibrin tBRCT domains mediates the interaction with the phosphorylated MDC1 protein, rather than with γ-H2AX. MDC1, a tBRCT domains-containing protein, binds the C-terminal region of γ-H2AX and plays a pivotal role in the recruitment of other DDR proteins (e.g. the MRN complex) (Goldberg et al., 2003; Lou et al., 2003; Mochan et al., 2003; Stewart et al., 2003; Xu et al., 2003; Lee et al., 2005; Stucki et al., 2005; Tobias et al., 2013). From structure and amino acid sequence comparison between Xenopus laevis nibrin homologue (XNbs1) and MDC1, it has been suggested that it is unlikely that nibrin tBRCT domains would recognize γ-H2AX in the same manner as MDC1 (Xu et al., 2008). On the contrary, the molecular modeling performed in collaboration with Prof. F. Polticelli (Department of Sciences, University Roma Tre) of the nibrin tBRCT domains revealed that the molecular determinants of γ-H2AX recognition by MDC1 appear to be conserved in nibrin. In fact, the electrostatic interaction between the K1936 residue of the first BRCT domain of MDC1 and the phosphate group of the γ-H2AX tail, is conserved in nibrin (corresponding to the Lys160 residue) (Stucki et al., 2005; di Masi et al., 2008). Remarkably, the role of the nibrin tBRCT domains in the DSBs recognition was supported by our experimental evidences indicating that the R215W mutation, localized within the nibrin tBRCT domains, affects its ability to bind γ-H2AX (di Masi et al., 2008). In order to confirm these data, the molecular bases of
nibrin/γ-H2AX interaction have been further investigated. To do that, the only tBRCT domains were cloned into a His-tag vector, and the Lys160Met mutation was introduced into the so obtained construct, in order to evaluate the role of this residue in the hypothetical recognition of the γ-H2AX phosphate group. Since (i) the FHA and the tBRCT domains can each mediate protein-protein interactions on their own, showing specificity for pThr or pSer respectively (Huang et al., 2011) and (ii) nibrin seems to have combined both domains to yield a third domain (Lloyd et al., 2009; Williams et al., 2009; Coster et al., 2010), we addressed the role of the FHA in the recognition of γ-H2AX. For this reason we cloned the N-terminal region of nibrin (i.e., the FHA and tBRCT domains) into a His-tag vector, producing also in this case the relative Lys160Met mutant. After cloning, the proper expression conditions were set up (e.g., bacterial growth medium, IPTG concentration, expression time-course, and growth temperature). The four nibrin recombinant proteins obtained were purified through Ni-NTA affinity chromatography.

Data obtained from spectrofluorimetric analysis indicate that the emission spectrum observed by adding γ-H2AX to the tBRCT domains is reduced if compared to that obtained in the absence of γ-H2AX. Such effect can be due to the quenching of the Trp and Tyr residues present within the tBRCT domains, and are not observed when the interaction between the tBRCT domains and H2AX is analyzed (Fig. 3). These data allows us to hypothesize that the minimal model of the reaction is characterized by two phases: the first one is extremely rapid, not directly observable by spectrofluorimetric methods, γ-H2AX-independent and BRCT-dependent; the second step is slower, observable by spectrofluorimetric analysis, γ-H2AX-dependent and BRCT-independent. Furthermore, this step may be due to the conformational change of the tBRCT domains pocket. Remarkably, data obtained allow us to determine the dissociation constant of the complex, its value being approximately 7.5 µM.

The study of the interaction between the Lys160Met tBRCT domains and either γ-H2AX or H2AX does not evidence any significant change of the emission spectrum (Fig. 4). These results seem to suggest that the Lys160Met mutation alters the putative BRCT binding pocket, supporting the notion that the integrity of the tBRCT domains is pivotal for the proper interaction with γ-H2AX. Remarkably, preliminary results obtained from the study of the interaction between the recombinant nibrin protein carrying also the
FHA domain and $\gamma$-H2AX seem to indicate that the interaction is too fast to be measured by spectrofluorimetric methods since the phospho-binding domain FHA enhances the velocity of the association of the nibrin N-terminal region with $\gamma$-H2AX.

**CONCLUSIONS AND PERSPECTIVES**

Coimmunoprecipitation experiments conducted in our laboratory (Mendez et al., 2012) and fluorescence studies seem to demonstrate the presence of an interaction between nibrin tBRCT domains and $\gamma$-H2AX. The further validation of results obtained remains of primary importance. Therefore, we decided to perform experiments with the Biacore X-100 system in collaboration with Prof. A. Arcovito (Università Cattolica del Sacro Cuore, Rome). We just cloned the wild-type or K160M nibrin protein forms and protein expression and purification experiments are in course. The experiments with Biacore will consist in a binding assay between biotinylated peptides corresponding to the C-terminal of H2AX or $\gamma$-H2AX, and wild-type or mutated nibrin. The peptides will be immobilized on a typical chip and a flux of nibrin protein will be generated to test a possible *in vitro* interaction with the histones.
Figure 3: Representation of nibrin tBRCT domains emission spectrum in the presence of either the phosphorylated or non phosphorylated histone. These data were obtained by advanced methods of data analysis. The blu line of the A panel represents the emission spectrum of the nibrin tandem BRCT domains in the absence of the histone. By adding the phosphorylated histone (see green line), a reduction in the emission spectrum is observed. Interestingly (B) the emission spectrum representing the interaction between the tBRCT domains and the H2AX increases (see green line).
Figure 4: Representation of nibrin tBRCT (K160M) emission spectrum in the presence of either the phosphorylated or non phosphorylated histone. The blu line of the A panel represents the emission spectrum of the nibrin tBRCT (K160M) domains in the absence of the histone. (A) The addition of the phosphorylated histone (see green line), does not evidence any significant change of the emission spectrum. The same thing is observed with H2AX (B).
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