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VRK1 phosphorylates and protects NBS1 from ubiquitination and proteasomal degradation in response to DNA damage

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ABSTRACT

NBS1 is an early component in DNA-Damage Response (DDR) that participates in the initiation of the responses aiming to repair double-strand breaks caused by different mechanisms. Early steps in DDR have to react to local alterations in chromatin that are induced by DNA damage. NBS1 participates in the early detection of DNA damage and functions as a platform for the recruitment and assembly of components that are sequentially required for the repair process. In this work we have studied whether the VRK1 chromatin kinase can affect the activation of NBS1 in response to DNA damage induced by ionizing radiation. VRK1 is forming a basal preassembled complex with NBS1 in non-damaged cells. Knockdown of VRK1 resulted in the loss of NBS1 foci induced by ionizing radiation, an effect that was also detected in cell-cycle arrested cells and in ATM (−/−) cells. The phosphorylation of NBS1 in Ser343 by VRK1 is induced by either doxorubicin or IR in ATM (−/−) cells. Phosphorylated NBS1 is also complexed with VRK1. NBS1 phosphorylation by VRK1 cooperates with ATM. This phosphorylation of NBS1 by VRK1 contributes to the stability of NBS1 in ATM (−/−) cells, and the consequence of its loss can be prevented by treatment with the MG132 proteasome inhibitor of RNF8. We conclude that VRK1 regulation of NBS1 contributes to the stability of the repair complex and permits the sequential steps in DDR.

1. Introduction

Genetic damage is occurring continuously in cells as a consequence of multiple types of genetic insults, either of endogenous or exogenous origin [1–3]. Genetic damage is one of the main problems facing cells during their life that has to be overcome to avoid its deleterious consequences. Therefore, during evolution cells have acquired multiple mechanisms to detect these different types of DNA damage, and to trigger the immediate protective and specific DNA repair responses [3]. These mechanisms must also function in non-proliferating cells, since most of the DNA damage occurs independently of cell division. Alterations in these detection and repair mechanisms can have very serious consequences for cells making them more sensitive to genotoxic damage and facilitating the accumulation of mutations, but this characteristic can also be exploited for therapeutic purposes [4,5]. Also the organism can suffer important consequences, since defects in DNA repair can facilitate aging [6], cancer [2] and neurodegeneration [7].

The initial alteration after DNA damage is a local distortion of the chromatin structure, and in which histones and chromatin kinases might be very early and initiating components in triggering the process. Chromatin alteration can activate chromatin kinases, as is the case of VRK1 [8–10], which can also regulate and coordinate the different sequential steps required for successful DNA repair. VRK1 is a Ser–Thr kinase that has been associated to the regulation of cell cycle progression [11], proliferation [8,12–14] and DNA damage responses mediated by p53 [15,16]; and also to proteins directly involved in DDR [9]. VRK1 is a chromatin kinase [8,10,17] that is implicated in processes requiring chromatin remodeling, such as gene transcription and responses to DNA damage. Thus VRK1 depletion results in defective formation of γH2AX foci [10] and 53BP1 foci [5,9] in response to DNA damage induced by ionizing radiation, even in situations of non-dividing cells [9,10]. DNA damage responses have to be performed in each individual cell independently of their particular situation, either cell cycle arrested or proliferating, and of their local heterogeneous microenvironment and cellular interactions.

NBS1/nibrin integrates the input signal from a locally altered chromatin due to DNA damage and participates in early events of the

Abbreviations: VRK1, vaccinia-related kinase 1; NBS1, Nijmegen breakage syndrome 1; DDR, DNA damage response; IR, ionizing radiation.

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DNA damage response. NBS1 is an early component in DDR. Nijmegen breakage syndrome (NBS) is a recessive autosomal disease that includes microcephaly, growth retardation, immune deficiency and increased sensitivity to ionizing radiation among other symptoms [18]. The Nijmegen syndrome is caused by mutations in the *NBS1* gene [19], whose protein forms part of the MRN complex (MRE11–RAD50–NBS1) [20], and also plays a role in prevention of ataxias [21] and cerebellar effects [21]. Following DNA damage, ubiquitinated NBS1 activates ATM [22,23], a protein that is later downregulated by the ubiquitin ligase RNF8 [24]. NBS1 is also regulated by phosphorylation mediated by activated ATM [25].

Homozygous mutation in the *VRK1* gene causes a new neurodegenerative syndrome known as pontocerebellar hypoplasia with spinal muscular atrophy (PCH1-SMA) [26]. This is a complex syndrome that in addition to the pontocerebellar hypoplasia and muscular atrophy there are additional symptoms such as microcephaly, mild growth retardation and ataxia, but their manifestation and severity are variable depending on the specific *VRK1* mutation [26–28]. More recently additional point mutations in the *VRK1* gene have been identified also presenting a complex neurological phenotype, but sharing their main characteristics [27]. Some of these neurological symptoms are also manifested in patients with *NBS1* mutations [18].

The roles of VRK1 and NBS1 in DDR independently identified share effects suggest that there is a functional link between these two proteins in response to DNA damage. In this work we have studied the relation between VRK1 and NBS1/nibrin in the context of DNA damage induced by double strand breaks in DNA.

2. Results

2.1. VRK1 knockdown causes a loss of NBS1 foci induced by ionizing radiation independent of ATM

NBS1 forms nuclear foci after induction of DNA damage [29], and also the VRK1 kinase is activated by DNA damage [9,10,30]. Therefore, we decided to study whether VRK1 knockdown might have an effect on the formation of NBS1 foci in response to ionizing radiation. This effect was determined in several cell lines, including A549 (ATM+/+) (Fig. 1A) and HT144 (ATM−/−) (Fig. 1B) lung carcinoma cells. Depletion of VRK1 resulted in a significant loss of NBS1 nuclear fluorescence induced by IR in both cell lines (Fig. 1). A similar result was also obtained using a different siVRK1 (Fig. S1). Since VRK1 can arrest cells in G0/G1 [12], and in order to rule out a consequence of growth arrest, the formation of NBS1 was also determined in serum deprived cells. Cells were also able to form normal NBS1 foci induced by IR in the absence of serum (Fig. 1A, B, bottom). Therefore, the effect of VRK1 depletion in non-dividing cells is not a consequence of cell cycle arrest, and is also independent of ATM.

2.2. VRK1 forms a basal protein complex with NBS1

NBS1 [29] and VRK1 [10] are participants that play an early role in reactions to DNA damage. Therefore, we hypothesized that based on the functions of these two proteins; both are likely to form a preassembled basal DDR complex in the absence of DNA damage, which is ready to be activated when DNA damage occurs. To test this possibility we first determined whether endogenous VRK1 and NBS1 proteins could be forming a stable protein-complex in non-damaged cells, or in cells in which DNA damage was induced by treatment with doxorubicin. Four cell lines were used for this aim, two ATM (+/+) (HEK293T and A549) and two ATM (−/−) (HT144 and GM9607). The endogenous NBS1 protein was immunoprecipitated, and the bound VRK1 protein in the immunoprecipitate was detected by immunoblot. VRK1 was present in the NBS1 immunoprecipitate independently of treatment with doxorubicin, suggesting that they form a stable preassembled complex in cells in the absence of DNA damage (Fig. 2A). This VRK1–NBS1 complex is also independent of ATM because it was also detected in two ATM −/− cell lines. In addition, a similar protein complex was also detected in HeLa cells in which immunoprecipitation of transfected Flag-NBS1 brought down endogenous VRK1 independently of DNA damage induced by doxorubicin (Fig. 2B). To further confirm this NBS1–VRK1 basal interaction, HEK293T cells were transfected with plasmid expressing myc-VRK1 and its immunoprecipitation brought down endogenous NBS1 independently of treatment with doxorubicin (Fig. S2A, left). The reciprocal immunoprecipitation of transfected-myc-VRK1 also brought down endogenous NBS1 (Fig. S2A, right). A similar result was also obtained in cells transfected with Flag-NBS1 and HA-VRK1 (Fig. S2B). The interaction between VRK1 and NBS1 was independent of doxorubicin treatment (Fig. S2B). Next, we determined whether the kinase activity of VRK1 was necessary to form the complex. Cells were transfected with kinase-active or kinase-dead VRK1 (K179E). The NBS1–VRK1 interaction is independent of the kinase activity of VRK1, as shown by its interaction, with both kinase-active VRK1 (Fig. S2C) and kinase-dead VRK1 (KD: K179E) in pulldown experiments (Fig. S2D). All these results confirmed the formation of a stable and basal NBS1–VRK1 protein complex in the absence of DNA damage.

2.3. VRK1 phosphorylates NBS1 in Ser343

The possible effect of VRK1 on NBS1 is likely to be a consequence of its kinase activity as a result of VRK1 activation by DNA damage [9]. Thus, in order to identify the NBS1 amino acid residue phosphorylated in vitro by VRK1, the phosphorylation of NBS1 was studied with a phosphospecific antibody recognizing that NBS1 is phosphorylated in Ser343, with and without treatment with doxorubicin or ionizing irradiation. NBS1-Ser343 was phosphorylated in response to treatment with doxorubicin and ionizing radiation in both A549 (Fig. 3A) and HT144 (ATM−/−) cells (Fig. 3B). Depletion of VRK1 with siRNA resulted in the loss of NBS1 phosphorylation in Ser343 induced by doxorubicin or IR in both cell lines (Fig. 3A, B). In cells that were neither irradiated nor treated with doxorubicin there was no phosphorylation of NBS1 in Ser343 (Fig 3A, B, left panel).

Next we determined if both NBS1 and its Ser-343 phosphorylated form could be detected forming a stable protein complex with VRK1. For this aim, endogenous NBS1 protein from non-irradiated and irradiated cell extracts were immunoprecipitated and the presence of VRK1 and NBS1 phosphorylated in Ser343 determined in the immunoprecipitate. Phosphorylation of NBS1 in Ser343 was only detected in irradiated cells, but the presence of VRK1 was detected in the NBS1 immunoprecipitate independent of its treatment (Fig. 3C).

To confirm the phosphorylation of NBS1, cells were transfected with empty vector (AU5) or a plasmid expressing AU5-NBS1. The transfected protein was immunoprecipitated and incubated with bacterially expressed GST-VRK1. The GST-VRK1 protein phosphorylated NBS1, which was detected with an anti Ser343-P phosphospecific antibody (Fig. 3D). Next we determined if the loss of VRK1 kinase activity affected this phosphorylation of NBS1. For this aim, kinase-dead GST-VRK1 (K179E) was used. Kinase active, but not kinase-dead VRK1 phosphorylated NBS1 (Fig. 3E).

2.4. VRK1 depletion reduces the level of NBS1 protein in ATM −/− cells that is prevented by proteasome inhibitors

The depletion of VRK1 could affect the formation of NBS1 foci by two alternative mechanisms, either altering gene expression or protein stability. The first mechanism will result in a reduction of the *NBS1* gene expression and consequently of its protein level.

The effect that VRK1 depletion has on the level of NBS1 protein and RNA after DNA damage induced by ionizing radiation or doxorubicin was determined in HT144 (ATM−/−) (Fig. 4A) and A549 (ATM+/+) cells (Fig. 4B). Loss of VRK1 did not affect NBS1 protein levels in A549 cells (Fig. 4B), thus VRK1 effect on NBS1 foci is not due to NBS1 stability.

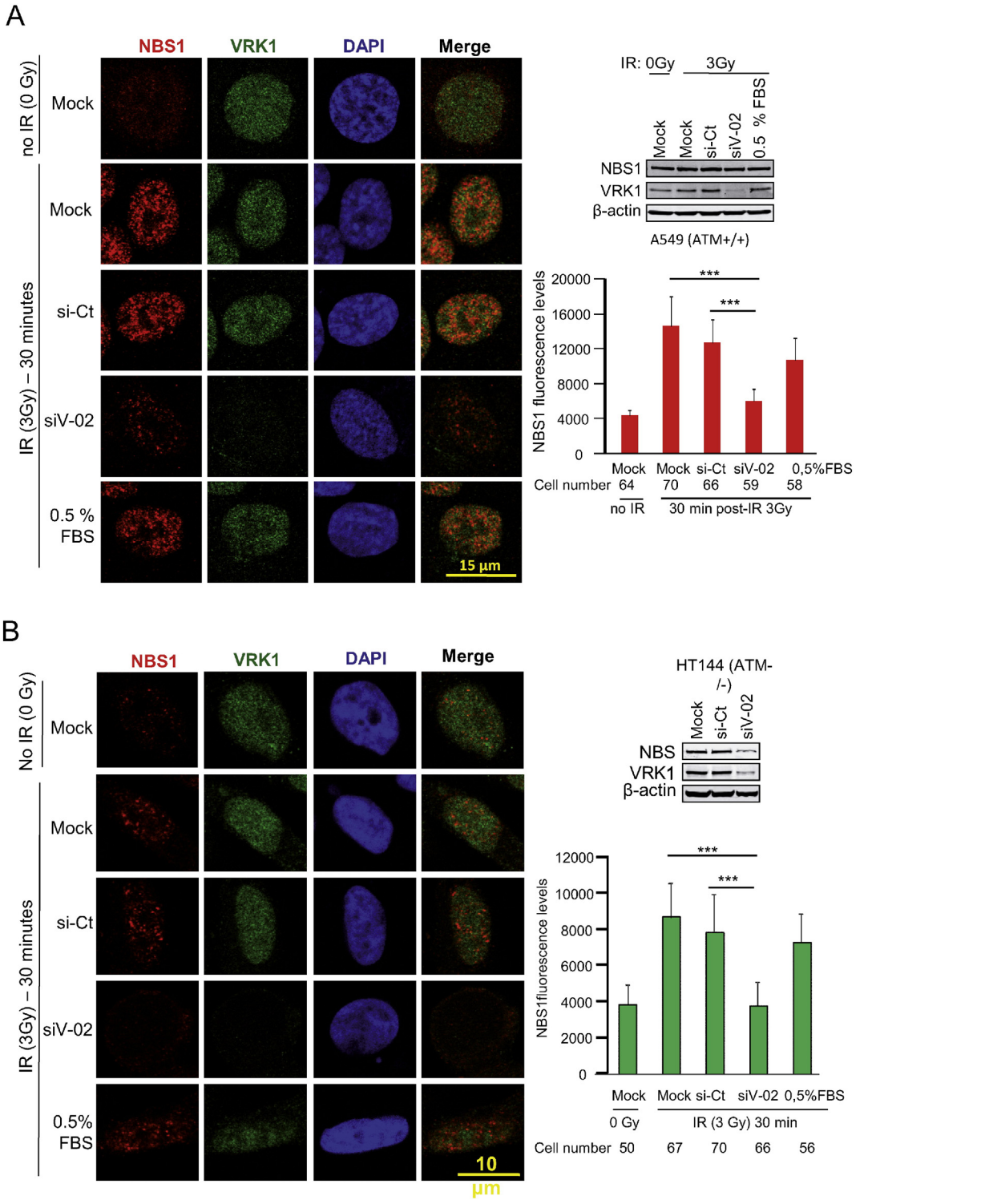

Fig. 1. Effect of VRK1 knockdown on the formation of NBS1 foci induced by ionizing radiation. A. A549 (ATM+/+) cells were either transfected with siControl (si-Ct) or siVRK1 (si-V02) and irradiated. NBS1 and VRK1 were detected with specific antibodies. The immunoblot to the right show the protein levels and the graph the quantification of the fluorescence. The number of cells counted is indicated below. B. HT144 (ATM−/−) cells were either transfected with si-Control (si-Ct) or siVRK1-02 (si-V02) and irradiated. NBS1 and VRK1 were detected with specific antibodies. The immunoblot to the right show the protein levels and the graph the quantification of the fluorescence. The number of cells counted is indicated below. ***P < 0.001.

However, in HT144 (ATM −/−) cells the combination of silencing VRK1 in the absence of ATM resulted in loss of NBS1 protein (Fig. 4A). These data suggested that ATM and VRK1 are necessary for the stability of NBS1, in addition to their role in the formation of NBS1 foci, although this protection can only be achieved by the additional presence of VRK1. We also ruled out that VRK1 depletion did not alter *NBS1* gene expression. The levels of NBS1 gene expression were not affected by VRK1 depletion using different siRNA and were determined by qRT-PCR in RNA from A549 and HT144 cells (Fig. 4C).Therefore, VRK1 knockdown reduced the level of VRK1, but not of NBS1.

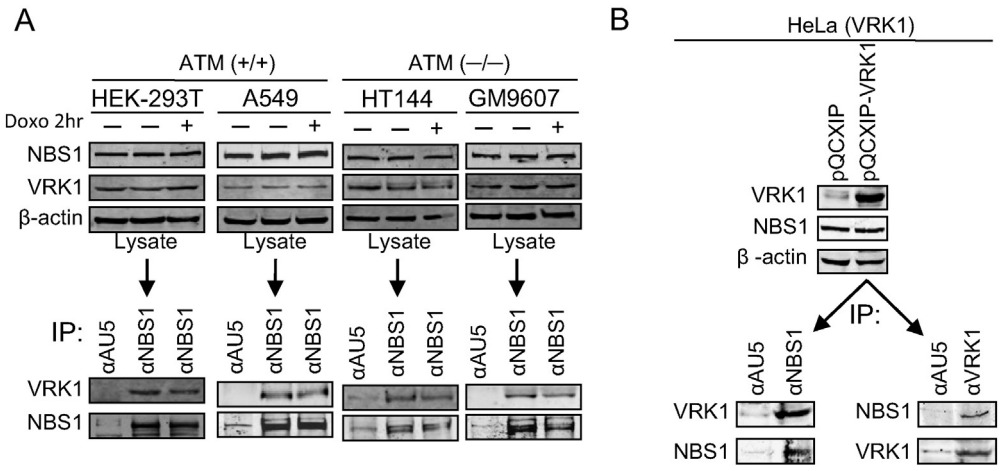

Fig. 2. NBS1 interacts with VRK1 independently of ATM and of DNA damage. A. Detection of the interaction between endogenous proteins in HEK-293T, A549, both ATM (+/+) and HT144 and GM9607 cell lines that are ATM(−/−). Cells were untreated or treated with 1 μM doxorubicina (Doxo) for 2 h. Cell lysates were used for immunoprecipitation with antibody control (anti-AU5) or anti-NBS1. Immunoprecipitates were used for detection of VRK1 with mAb 1B5 and anti-NBS1. B. Reciprocal immunoprecipitation of NBS1 and VRK1. For this experiment a stable HeLa pooled cells overexpressing VRK1 by infection with pQCXIP-VRK1. Reciprocal immunoprecipitation was performed and antiAU5 antibody was used as negative control. The interaction was confirmed in transiently transfected cells with VRK1 and NBS1 plasmids (Fig. S1).

However, an alternative mechanism by which VRK1 can affect formation of NBS1 foci is by altering the stability of the NBS1 protein. The effect of VRK1 knockdown on NBS1 stability could be a consequence of altering its sensitivity to active protein degradation mediated by the proteasome. The use of the proteasome inhibitor MG132 was able to prevent the downregulation of NBS1 as a consequence of VRK1 knockdown only in HT144 cells lacking ATM (Fig. 4D). This means that VRK1 phosphorylation cooperates with the additional phosphorylation of NBS1 mediated by ATM. However, this effect was not affected by lysosomal inhibitors such as chloroquine (not shown). Next, it was determined the protective effect of the proteasomal inhibitor MG132 on NBS1 foci in two ATM −/− cell lines, HT144 (Fig. 5A) and GM9607 (Fig. 5B). The addition of the proteasomal inhibitor MG132 resulted in the recovery of the NBS nuclear fluorescence induced by ionizing radiation in both ATM (−/−) cell lines in which VRK1 was knocked-down (Fig. 5). These observations indicated that phosphorylation of NBS1 in Ser343 protects it from its proteasomal degradation. By this mechanism, the temporal organization of DNA repair foci can be remodeled to facilitate their sequential, but functionally different, steps.

2.5. RNF8 is part of the NBS1–VRK1 complex

NBS1 is a known target of the RNF8 ubiquitin ligase [24]. Therefore, we tested whether NBS1–RNF8 complexes were also containing VRK1. For this aim we tested the formation of a protein complex by transfection of different combinations of tagged-plasmids expressing NBS1, RNF8 and VRK1 proteins. The immunoprecipitation of RNF8 by itself was able to bring down NBS1, and also VRK1 when it was included (Fig. 6A). Next, we determined whether the VRK1–NBS1–RNF8 complex was altered in cells treated with doxorubicin. The RNF8 immunoprecipitate always contained VRK1, independent of DNA damage treatment (Fig 6B). This observation is consistent with the existence of a preassembled NBS1–VRK1 complex.

Because RNF8 is an ubiquitin ligase we determined if it affected VRK1 and NBS1 levels. In HT144 (ATM −/−) cells both proteins were sensitive to degradation induced by RNF8 in a dose dependent manner (Fig. 6C, left). However, in A549 (ATM +/+) cells, both proteins were resistant to RNF8 and their levels remained stable (Fig. 6C, right), indicating that an additional contribution by ATM was necessary to maintain the stability of NBS1 protein.

3. Discussion

The immediate response to DNA damage requires that its initial participants form a preassembled complex on chromatin, which can be immediately reorganized and relocated to the site of DNA damage. This relocation can initiate and trigger the recruitment of the proteins participating in the sequential steps of the specific DNA repair process. The formation of a preassembled protein complex between p53 and VRK1 has already been detected and is critical for the cellular responses to DNA damage response mediated by p53 activation [31,32]. The preassembled complex leads to the immediate activation of p53 by phosphorylation in Thr18 [30] resulting in a switch to binding transcriptional cofactors [33]. Therefore, among early participants in DDR, chromatin kinases and scaffold proteins are likely candidates for this function since they can integrate the assembly of consecutive signaling complexes. Among these, the formation of a basal protein complex between NBS1 and VRK1 is a very likely component for this early role in DDR. VRK1 as chromatin kinase can participate in the processes requiring dynamic modifications of chromatin that are associated to specific chromatin functions, such as DDR, chromatin condensation and telomere assembly and protection. The direct interaction of VRK1 with histones [8,10] and its regulation [17] can function as the initial step that permits reacting to local alterations of chromatin in response to DNA damage, and as part of the signal that activates early components in DDR, among which NBS1 is a key player [34]. Therefore, VRK1 can be integrated in the cellular interactome reacting to DNA damage at different functional levels (Fig. 7), such as chromatin components at the local site of DNA damage, as well as the response to cellular damage mediated by p53, which is a target of VRK1 [16,35–37] and that also forms a preassembled complex with VRK1 [30].

The role of VRK1 on NBS1 foci induced by ionizing radiation can have different roles. One is that NBS1 foci formation is prevented because VRK1 is acting as an upstream component by participating in the formation of γH2AX foci induced by IR [10], which is a trigger to initiate specific DDR responses. NBS1 interacts by its FHA/BRCT domain with γH2AX [38]. The permanence of VRK1 in the complex with the NBS1 phosphorylated molecule suggested that VRK1 is also likely to participate at later, or downstream, steps in DDR. This is consistent with the observation that VRK1 is also able to regulate the formation of 53BP1 foci, an intermediate participant in DNA repair by NHEJ [9]. The other is acting downstream and thus permitting the sequential

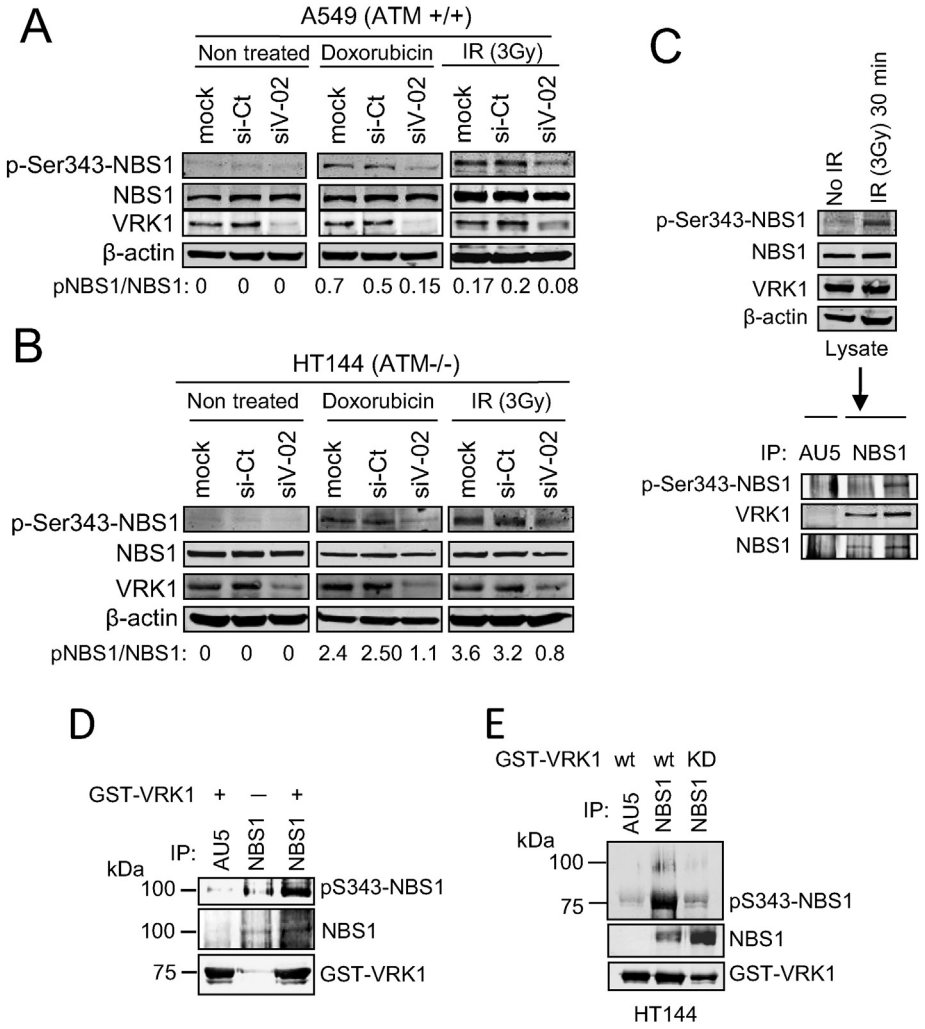

Fig. 3. Effect of VRK1 depletion on the induction of NBS1 phosphorylation in Ser343 induced by DNA damage. A. A549 (ATM+/+) cells were used for depletion of VRK1 and treated with doxorubicin or ionizing radiation. The levels of VRK1, NBS1 and NBS1-Ser343-P were determined with specific antibodies in cell lysates. B. HT144 (ATM−/−) cells were used for depletion of VRK1 and treated with doxorubicin or ionizing radiation. The levels of VRK1, NBS1 and NBS1-Ser343-P were determined with specific antibodies in cell lysates. C. Both NBS1 non-phosphorylated and NBS1 phosphorylated in Ser343 form a complex with VRK1. Cells were used as controls or treated with ionizing radiation. Cell lysates were used for immunoprecipitation of NBS1. In input lysate and in the immunoprecipitate the presence of VRK1, NBS1 and NBS1-Ser343P was determined. D. Phosphorylation of NBS1 by VRK1 in in vitro kinase assay. Bacterially expressed and purified GST-VRK1 was used in kinase assays with immunoprecipitated endogenous NBS1 protein. In the assay the specific VRK1, NBS1 and NBS1-Ser343P were detected with specific antibodies. E. In vitro kinase assay with VRK1 active and kinase-dead (KD: kinase dead, substitution K179E) proteins. Endogenous NBS1 was immunoprecipitated and used as substrate. VRK1, NBS1 and NBS1-Ser343P were detected with specific antibodies.si-Ct: control; siV-02: si-VRK1-02. pNBS: NBS1 phosphorylated in Ser343.

functional steps in DDR that have different components. In this context the removal of NBS1 is necessary to allow other proteins to function in a correct and organized sequential order. In this context, the role of VRK1 on NBS1 Ser343 phosphorylation is a contributor to a downstream regulation.

NBS1 phosphorylation in Ser343 has been associated to S phase progression [39,40], although it has been proposed to be mediated by ATM this kinase downstream of NBS1 [22] and 53BP1 [41]. Nevertheless, the phosphorylation of this Ser343 by VRK1 in cells lacking ATM suggests that VRK1 is the initial upstream kinase implicated since it phosphorylates NBS1 in the absence of ATM, and is activated in cell cycle progression, a role also associated to VRK1 [9,10]. This implies that the two kinases play a cooperative role in DNA damage response. In addition to ATM, NBS1 is also phosphorylated in Ser432 by CDK in cell cycle progression [42]. This phosphorylation participates in the recession of the DNA lesion, a later stage in the process of DNA repair [42]. VRK1 depletion also prevents the activating phosphorylation of ATM in Ser1981 [9,10]. This ATM phosphorylation is mediated by autophosphorylation of ATM and is thus a likely consequence of the response to a chromatin that is locally altered by DNA damage, which is an activator of VRK1 [10,30].

NBS1 is ubiquitinated by several ubiquitin ligases in different residues that can play different functional roles. NBS1 ubiquitination in Lys735 and Lys751 by Skp2 facilitates ATM activation in homologous recombination [43]. NBS1 ubiquitination in Lys435 is a key residue targeted by RNF8, which forms a complex with NBS1 [24]. The ubiquitination of NBS1 by RNF8 has been associated to DNA repair by homologous recombination [24], however the role of NBS1 ubiquitination in non-dividing cells that repair DNA double-strand breaks by NHEJ is not known. In this work we show that the phosphorylation of NBS1 by VRK1 is necessary to protect NBS1 from degradation that will facilitate progression to the assembly of complexes with specific repair functions, such as 53BP1 that is a selector of the NHEJ pathway [9,41]. Thus, in the absence of DNA damage and cell division there is a preassembled repair complex that includes VRK1–NBS1 and RNF8 that might be a key component for the activation and early steps in DDR. Formation of a basal complex that contains NBS1, RNF8 and VRK1, suggested that it is likely to be a complex playing an immediate response system in case DNA

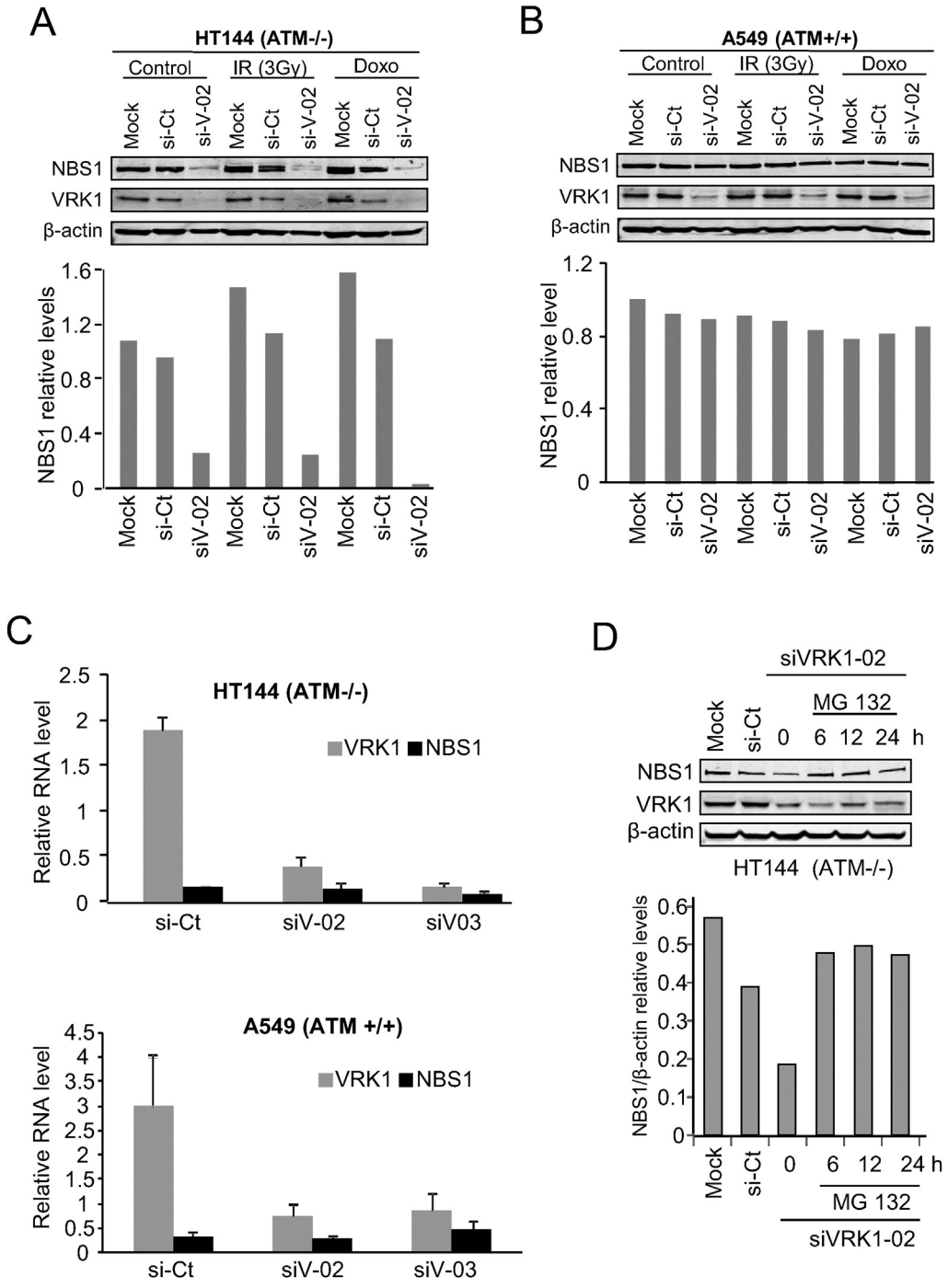

Fig. 4. VRK1 depletion alters the levels of NBS1 protein only in ATM (−/−) cells. A. Effect of VRK1 knockdown in A549 (ATM+/+) cells on the level of NBS1 protein after induction of DNA damage by doxorubicin or ionizing radiation. NBS1 and VRK1 were detected by immunoblots of cell lysates. B. Effect of VRK1 knockdown in HT144 (ATM−/−) cells on the level of NBS1 protein after induction of DNA damage by doxorubicin or ionizing radiation. NBS1 and VRK1 were detected by immunoblots of cell lysates. C. Effect of VRK1 knockdown in HT144 (ATM−/−) cells on the level of NBS1 RNA determined by qRT-PCR. D. Effect of the MG123 proteasome inhibitor on the reduction of NBS1 levels induced by VRK1 knockdown in HT144 (ATM−/−) cells.

damage occurs. Therefore, high levels of either VRK1 [10] or NBS1 [24,43] contribute to increased resistance to DNA damage induced by drugs or IR.

The role of VRK1 in the regulation of NBS1 in the context of DNA damage response indicates that the targeting of VRK1 can be of potential use for therapeutic purposes. The loss of VRK1, in addition to its interference with cell proliferation [11,12], also results in defective formation of γH2AX [10] and 53BP1 [5,9] foci in response to DNA damage and in that way sensitizes cells to gamma irradiation or chemotherapy. The atypical structure of VRK1 makes feasible the design of highly specific inhibitors with little or no cross reactivity with other kinases [44–46]. Future development of specific VRK1 inhibitors would greatly facilitate a reduction in tumor proliferation and facilitate cell death by sensitizing cells to DNA damage, which implies a likely reduction of the dose for radiotherapy or chemotherapy [5]. This is important because kinase inhibitors are better tolerated than chemotherapeutic drug based DNA damage. Thus the combination of these drugs with specific kinase inhibitors could reduce the toxicity of the treatment. However, the structural characteristics of the VRK1 protein [44] make most currently available inhibitors unsuitable [46], since they only have a

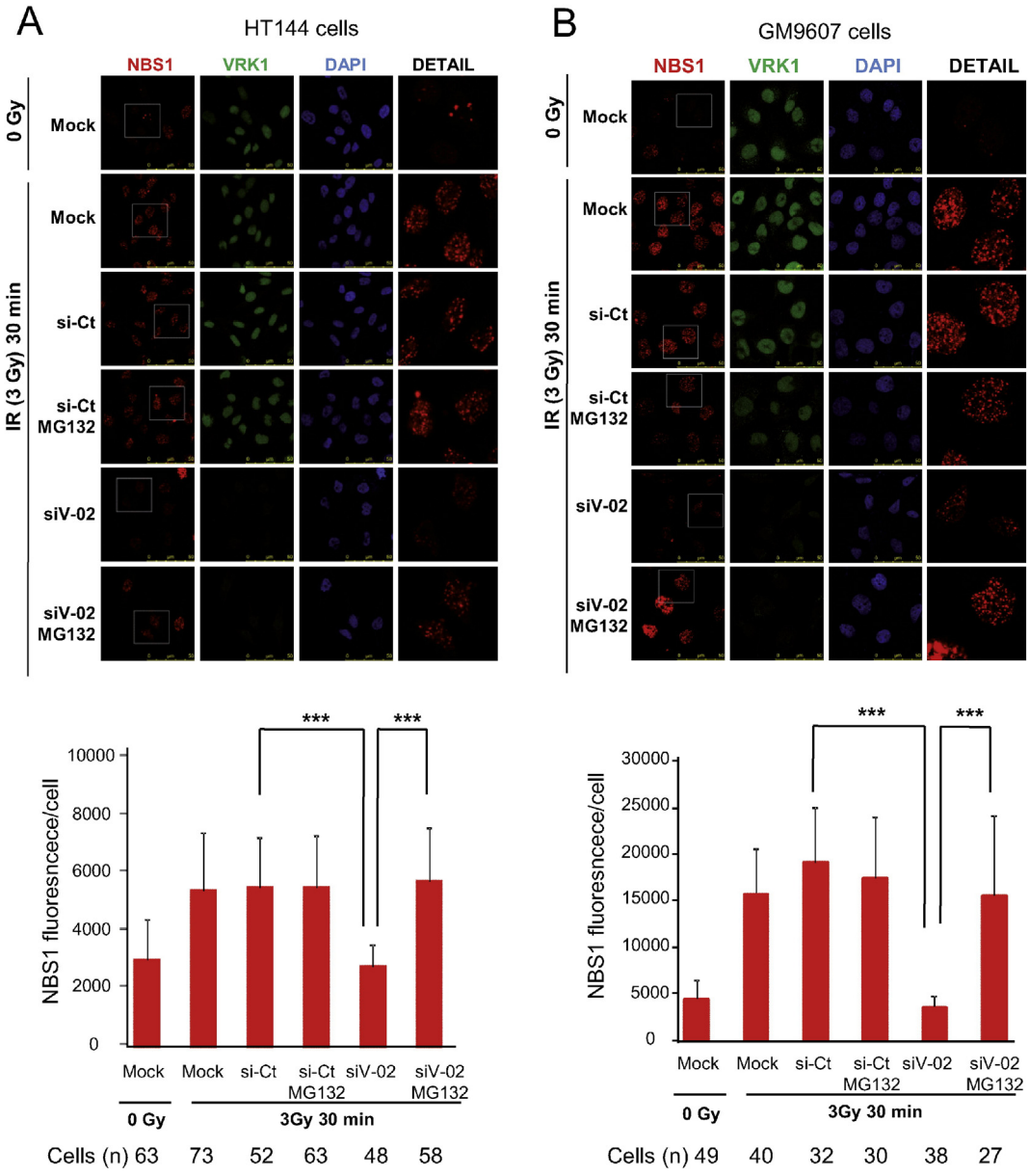

Fig. 5. Loss of NBS1 foci induced by IR after VRK1 knockdown is prevented by proteasomal inhibition in ATM −/− cells. A. HT144 (ATM −/−) cells were transfected with si-Control (siCt) or siVRK1-02 (siV-02) and 96 h later cells were irradiated with 3 Gy. The proteasomal inhibitor MG132 (50 μM) was added to cultures 6 h before irradiation. At the bottom is shown the quantification of NBS1 fluorescence. ***P < 0.001. B. GM9607 (ATM −/−) cells were transfected with si-Control (siCt) or siVRK1-02 (siV-02) and 96 h later cells were irradiated with 3 Gy. The proteasomal inhibitor MG132 (50 μM) was added to cultures 6 h before irradiation. At the bottom is shown the quantification of NBS1 fluorescence. ***P < 0.001. NBS1 was detected with a monoclonal antibody and VRK1 with a policlonal antibody. Nuclei were identified by staining with DAPI.

minor effect at very high doses [46]. Nevertheless, some VRK1 inhibitors targeting VRK1 have been identified, such a luteolin [47] or ursolic acid [48], but in both cases the specificity is unknown, and doses in millimolar range are required to have an effect.

In this report we have identified a preassembled NBS1–VRK1 complex that is activated by DNA damage and that contributes to the stability of NBS1 thus facilitating the sequential steps in DDR.

4. Materials and methods

4.1. Plasmids

NBS1 constructs pCDNA3-myc-NBS1 [24]; pIRES2-NBS1-GST and pIRES2-NBS1-FLAG were obtained from J. Chen [49].VRK1 constructs pGEX4-GST-VRK1 [35], pCEFL-GST-VRK1 [50,51], and pCEFL-HA-VRK1 [37,50] have already been reported. Empty plasmid vectors with specific epitope tags, such as AU5, Flag, myc or GST, were used in the appropriate transfection experiment as controls of plasmids expressing tagged proteins.

4.2. Cell lines, culture and transfections

A549, H1299 (p53 −/−), HT144 (ATM −/−), GM9607 (ATM −/−), HeLa and HEK293T cell lines were from the ATCC. Cells were grown as recommended by the supplier in DMEM supplemented with antibiotics, 10% FBS and 5 mM glutamine. Plasmid transfections were performed using the Jet-Pei reagent (Polytransfection Plus, Illkirch, France) according to the manufacturer's instructions [52].

4.3. RNA interference

Specific silencing of VRK1 was performed using two different siRNA: siVRK1-01 (siV1-01) and siVRK1-02 (siV1-02) from Dharmacon (Dharmacon RNA Technologies). The sequence target of the two VRK1

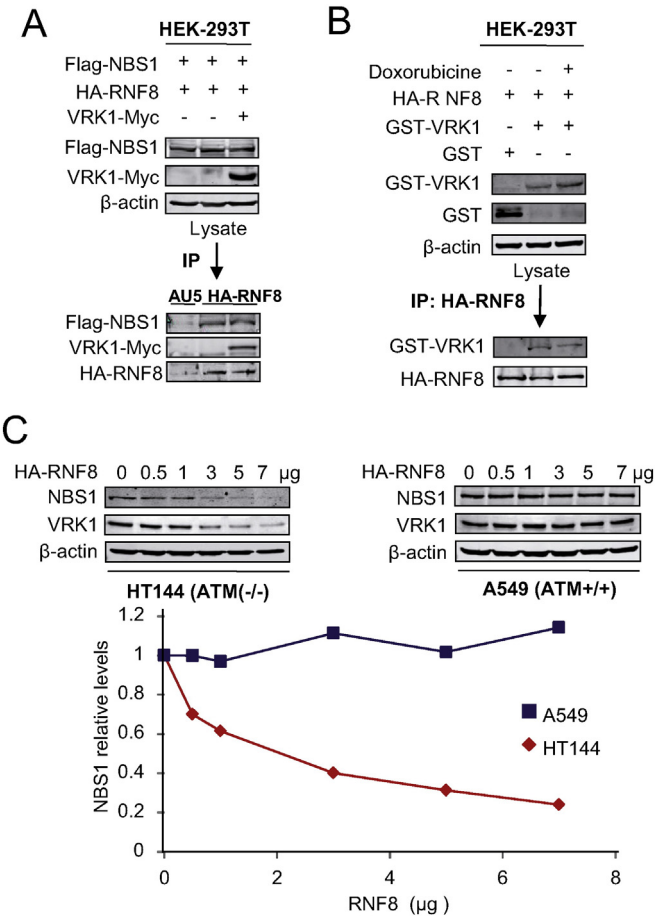

Fig. 6. NBS1, RNF8 and VRK1 interactions. A. HEK-293T cells were transfected with plasmids pCMV-FLAG-NBS1, pcDNA3.1-HA-RNF8 and pCDNA3.1-VRK1-Myc as indicated. HA-RNF8 was immunoprecipitated with an anti-HA policlonal antibody and NBS1 and VRK1 were detected in an immunoblot blot with antibodies specific for their tags. B. HEK-293T cells were transfected with plasmids pcDNA3.1-HA-RNF8 and pCEFL-GST-VRK1. HA-RNF8 was immunoprecipitated with an anti-HA policlonal antibody; the presence of VRK1 in the immunoprecipitate was determined with an anti-GST antibody. C. Dose dependent effect of RNF8 on endogenous NBS1 and VRK1. Plasmid pcDNA3.1-HA-RNF8 was transfected in HT144 (left) and A549 (right) cells. Protein levels were determined in immunoblots with polyclonal antibodies specific for NBS1 or VRK1. The total amount of plasmid in each transfection was always constant by completing it with the corresponding empty vector.

siRNA oligos was siVRK1-01: GAAAGAGAGTCCAGAAGTA and siVRK1-02: CAAGGAACCTGGTGTTGAA. As negative control, indicated as siCt in experiments, the "ON-TARGETplus siCONTROL Non-targeting siRNA" from DHARMACON was used. The efficiency of RNAi transfection was determined with "siGLO RISC-free siRNA" (Dharmacon).

Briefly, cells were transfected with the indicated siRNA at a concentration of 20 nM using Lipofectamine 2000 Reagent (Invitrogen) according to the manufacturer's instructions. After transfection, cells were processed at the times indicated in specific experiment that were performed as previously reported [9,15]. Targeted protein and plasmid expression was analyzed 36 h after transfection.

4.4. Antibodies

VRK1 was detected with monoclonal antibody 1F6 [53], polyclonal antibody VC1 [53] or polyclonal antibody (HPA017929, Sigma). NBS1 (residues 692–706) with murine monoclonal (611871, BD Bioscience, San José, CA), or polyclonal antibody (N3162, Sigma, St. Louis, MO). NBS1-Ser-343-P with rabbit polyclonal antibody (3001, Cell Signaling). NBS1-Ser432-P with rabbit monoclonal antibody (LS-c105367, LifeSpan Biosciences, Seattle, WA). Myc epitope with murine monoclonal or rabbit polyclonal antibodies (EMD-Millipore, Darmstadt, Germany). The flag epitope was detected with a polyclonal antibody (F7425, Sigma) or monoclonal antibodies (clones M2 or M5, Sigma). The HA epitope was detected with a murine monoclonal (sc-7392, Santa Cruz, Santa Cruz, CA) or rabbit polyclonal antibody (H6908, Sigma). GST with detected with a rabbit polyclonal antibody (sc-138, Santa Cruz).

4.5. Immunoprecipitations

Immunoprecipitations were prepared using 1.5 mg of whole cell extracts. Cells were lysed and to avoid nonspecific interactions, cell extracts were preincubated with 30 μL of "GammaBind Plus Sepharose" beads (GE Healthcare) equilibrated in the same buffer as the extracts, and incubated for 1 h at 4 °C with orbital rotation. The beads were removed by centrifugation and extracts were incubated with the specific antibody indicated in the experiment for 3 h. Afterwards, 40 μL of "GammaBind Plus Sepharose" beads, which have been previously blocked with seroalbumin (BSA), was added and incubated for 2–3 h or overnight at 4 °C with rotation. Next, the resin was washed several times in lysis buffer, before processing for gel loading. The immunoprecipitate was fractionated by SDS-PAGE, and the gel transferred to a PDVF membrane, Immobilon-P (Millipore), for immunoblot analysis. All methods have been previously reported [15,52–55]. The VRK1–NBS1 protein interaction has been assigned the identifier IM-24744 by the IMEX consortium [56].

4.6. DNA damage

DNA damage was induced by cell irradiation with 3 Gy using a Gammacell 1000 Elite irradiator (Theratronics, Ottawa, Canada) with a ^{137}Cs source. Cells were treated with doxorubicin (Sigma, St. Louis, MO) for the time and dose indicated in individual experiments [5,9,10].

4.7. Immunofluorescence and confocal microscopy

Cells were grown on cover slides treated with 0.01% poly-lysine, to improve their adherence, washed in cold PBS and fixed with 3% paraformaldehyde in PBS for 30 min at room temperature. After fixation, they were washed with 0.2 M glycine followed by permeabilization with 0.2% Triton X-100 in PBS for 30 min and blocking with 1% BSA in PBS for 30 min at room temperature. Following antibody incubations cells were washed three times with PBS for 5 min. Secondary antibodies labeled with Cy2 or Cy3 were diluted in blocking buffer and incubation was performed for 1 h at room temperature. Nuclei were stained with DAPI (4′,6′-diamidine-2-fenilindol), diluted in PBS and incubated for 10 min at room temperature. Cells were washed three times for 5 min in PBS and a final wash in distilled water. Coverslips were mounted on a slide with MOWIOL 4-88 (Calbiochem). Subcellular localization was determined by confocal microscopy. The acquisition of images by confocal microscopy has already been reported [5,9,10] using a Leica TCS SP5 DMI-6000B confocal microscope (Leica), using the following lasers: Argon (488 nm), DPSS (561 nm) and UV diode (405 nm). Fluorescent images were captured with a 63.0× lens zoomed in 1.5–3× with a 1024 × 1024 frame and 600 Hz scanning speed. Images were analyzed with Leica LAS AF (Leica) and ImageJ (NIH, http://rsb.info.nih.gov/ij) software.

4.8. Kinase assays

The radioactive kinase assay was performed using bacterially expressed and purified GST-VRK1 and using as substrate immunoprecipitated NBS1. Specific phosphorylation was detected with a monoclonal antibody. The kinase assay conditions have been previously reported [46,57].

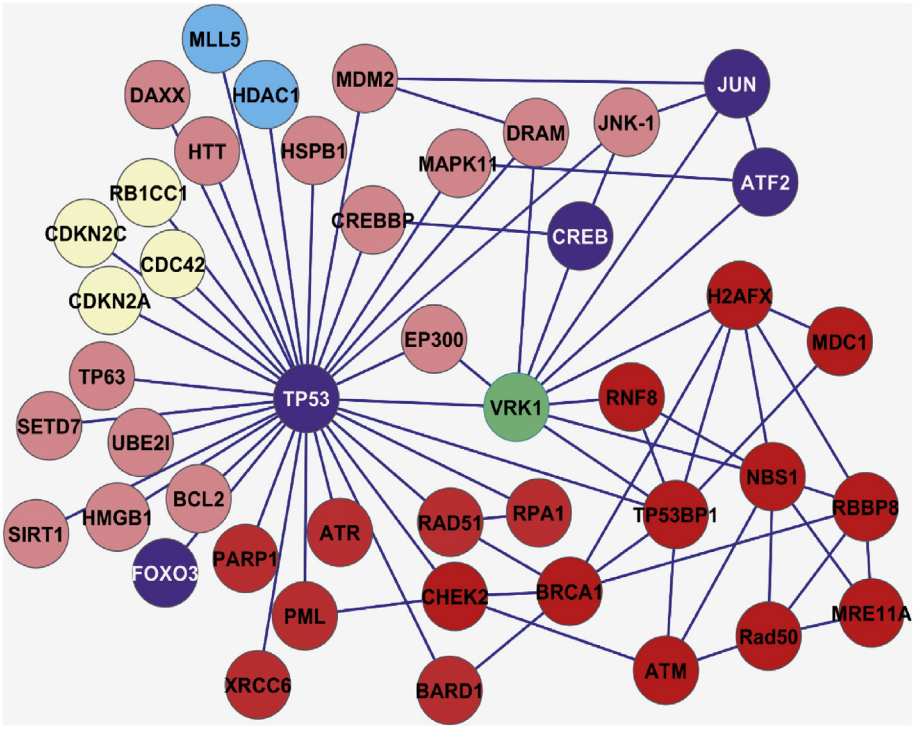

Fig. 7. The interactome of VRK1. The protein interaction network of VRK1 has been generated with the Cytoscape program (www.nih.gov) using validated protein–protein interactions from the IntAct Molecular Interaction Database (www.ebi.ac.uk/intact).

4.9. Statistical analysis

Quantitative experiments were performed at least three times, the number of cells is indicated and statistical analysis was performed by the ANOVA two-way test using the IBM SPSS 21 statistics package.

4.10. Database of protein interactions

The protein interactions from this publication have been submitted to the IMEx.

(http://www.imexconsortium.org) consortium through IntAct database and assigned the identifier IM-24744 [56].

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbamcr.2016.02.005.

Competing interests

The authors have no competing interests to declare.

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