



**HAL**  
open science

## **Homozygous deficiency of ubiquitin ligase RNF168 defines a radiosensitivity syndrome mimicking ataxia-telangiectasia**

Jiri Bartek, Sue Devgan, Carsten Doil, Kotoka Nakamura, Kelly Pettijohn,  
Shareef Nahas, Claudia Lukas, Jiri Lukas, Richard Gatti

► **To cite this version:**

Jiri Bartek, Sue Devgan, Carsten Doil, Kotoka Nakamura, Kelly Pettijohn, et al.. Homozygous deficiency of ubiquitin ligase RNF168 defines a radiosensitivity syndrome mimicking ataxia-telangiectasia. *Cell Death and Differentiation*, 2011, 10.1038/cdd.2011.18 . hal-00621616

**HAL Id: hal-00621616**

**<https://hal.science/hal-00621616>**

Submitted on 11 Sep 2011

**HAL** is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

**Homozygous deficiency of ubiquitin-ligase ring-finger protein RNF168 mimics the radiosensitivity syndrome of ataxia-telangiectasia**

**Running title: Clinical and cellular impact of RNF168 deficiency**

Sue S. Devgan<sup>1</sup>, Ozden Sanal<sup>2</sup>, Carsten Doil<sup>3</sup>, Kotoka Nakamura<sup>1</sup>, Shareef A. Nahas<sup>1</sup>,  
Kelly Pettijohn<sup>1</sup>, Jiri Bartek<sup>3,4\*</sup>, Claudia Lukas<sup>3</sup>, Jiri Lukas<sup>3</sup>, Richard A. Gatti<sup>1,5,\*</sup>

1. University of California Los Angeles, Department of Pathology and Laboratory Medicine, Los Angeles, California, USA

2. Immunology Division, Hacettepe University Children's Hospital, Ankara, Turkey

3. Institute of Cancer Biology and Centre for Genotoxic Stress Research, Danish Cancer Society, Strandboulevarden 49, DK-2100 Copenhagen, Denmark

4. Palacky University, Institute of Molecular and Translational Medicine, Olomouc, Czech Republic

5. University of California Los Angeles, Department of Human Genetics, Los Angeles, California, USA

Corresponding authors: Jiri Bartek: Institute of Cancer Biology and Centre for Genotoxic Stress Research, Danish Cancer Society, Strandboulevarden 49, DK-2100 Copenhagen, Denmark. Tel: +45-35257357; Fax: +45-35257721; e-mail [jb@cancer.dk](mailto:jb@cancer.dk) and Richard A. Gatti: Department of Pathology & Laboratory Medicine, David Geffen School of Medicine, University of California-Los Angeles, Los Angeles, Ca 90095-1732. Tel/Fax 310-825 7618; e-mail [rgatti@mednet.ucla.edu](mailto:rgatti@mednet.ucla.edu)

**Abstract**

Maintaining genomic integrity is critical to avoid life-threatening disorders such as premature aging, neurodegeneration and cancer. A multiprotein cascade operates at sites of DNA double strand breaks (DSBs) to recognize, signal, and repair damage. RNF168 (ring-finger nuclear factor) contributes to this emerging pathway of several E3 ubiquitin ligases that perform sequential ubiquitylations on damaged chromosomes, chromatin modifications essential for aggregation of repair complexes at the DSB sites. Here we report the clinical and cellular phenotypes associated with a newly identified homozygous nonsense mutation in the *RNF168* gene of a patient with a syndrome mimicking ataxia-telangiectasia (A-T). The mutation eliminated both of RNF168's ubiquitin-binding motifs, thus blocking progression of the ubiquitylation cascade and retention of repair proteins including tumor suppressors 53BP1 and BRCA1 at DSB sites, consistent with the observed defective DNA damage checkpoints/repair and pronounced radiosensitivity. Rapid screening for RNF168 pathway deficiency was achieved by scoring patients' lymphoblastoid cells for irradiation-induced nuclear foci containing 53BP1, a robust assay we propose for future diagnostic applications. The formation of radiation-induced DSB repair foci was rescued by ectopic expression of wild-type RNF168 in patient's cells, further causally linking the RNF168 mutation with the pathology. Clinically, this novel syndrome featured ataxia, telangiectasia, elevated alpha-fetoprotein, immunodeficiency, microcephaly and pulmonary failure and has implications for the differential diagnosis of autosomal recessive ataxias.

**Key words:** DNA damage signaling and repair, RNF168 and BRCA1 ubiquitin ligases, ionizing radiation-induced 53BP1 foci, Ataxia-telangiectasia mimicking syndrome, alpha-fetoprotein, microcephaly.

## Introduction

The human genome is continuously exposed to free radicals, ionizing radiation, and other genotoxic agents that cause DNA double strand breaks (DSBs)<sup>1-3</sup>. Over one hundred genes are involved in recognition, signaling and repairing DSBs in order to maintain genomic stability and avoid accumulation of disease-predisposing mutations<sup>2</sup>. At the cellular level, functions of the DNA damage sensors, signal transducers and effectors ultimately modulate the cell-fate decisions under genotoxic stress conditions, including decisions whether to undergo cell death or survive, or possibly take the route towards oncogenic transformation<sup>2-4</sup>. At the level of the whole organism, mutations in a subset of such genes are known to predispose to diverse types of cancer, neurodegenerative disorders, premature aging and/or immunodeficiency syndromes<sup>2-4</sup>. On the other hand, the majority of the DNA damage response (DDR) genes are neither completely characterized nor linked to human disease phenotypes<sup>2-5</sup>.

The *RNF168* gene is located on chromosome 3q29 and encodes a 571 amino acid protein that is a member of the “ring finger nuclear factor” (RNF) family of ubiquitin ligases. These enzymes modify functional properties of other proteins by covalent attachment of ubiquitin polypeptides, and emerging evidence suggests that this type of modification plays a key role in the hierarchically-structured genome surveillance pathways, including responses to DSBs<sup>3,6-8</sup>. Within seconds and minutes after DSB generation, ATM (ataxia-telangiectasia mutated) protein kinase<sup>3,4,9</sup> phosphorylates histone H2AX to become  $\gamma$ -H2AX.  $\gamma$ -H2AX acts as a beacon for identifying sites of DNA damage and initiates a cascade of chromatin modulation and DNA repair events by recruiting MDC1 (Mediator of DNA damage Checkpoint 1)<sup>9</sup>. This is followed by accumulation of two closely-related RNF ubiquitin ligases, RNF8 and RNF168<sup>6-13</sup>, in concert with the functionally related HECT-domain protein HERC2<sup>14</sup>. These proteins, along with SUMO-ligases PIAS1 and PIAS4<sup>14-17</sup> then trigger and/or amplify binding of ubiquitin and SUMO onto histones near the DNA breaks, thereby forming a scaffold for

the local accumulation of essential repair factors, including 53BP1 and yet another ubiquitin ligase, the tumor suppressor BRCA1<sup>3,6-16</sup>.

Although disruption of this DNA damage-activated ubiquitin ligase cascade undermines genome integrity and cell survival in experimental models, the translational significance of the individual components of this pathway can only be appreciated by identifying patients who either lack or harbor deficient versions of, one of them. Arguably the best example of pathophysiological impact of a malfunctional component within this cascade are tumors associated with loss-of-function mutations of BRCA1<sup>3,9,14,16</sup>. Another well-known example is the complex syndrome of ATM deficiency, known as ataxia-telangiectasia (A-T), a life-threatening disease characterized by a combination of developmental defects, including immune deficiency, neurodegeneration, radiosensitivity and enhanced probability to develop cancer<sup>3,17,18</sup>.

Here, we report a novel clinical syndrome, by identification of a patient lacking the RNF168 protein, with a clinical phenotype that mimicks ataxia-telangiectasia<sup>17-19</sup>, including mild gait ataxia, ocular telangiectasia, an elevated alpha-fetoprotein, and terminal respiratory failure. Importantly, this phenotype resulted from homozygous nonsense mutation in the *RNF168* gene that precluded the expression of the two ubiquitin binding domains, MIU-1 and MIU-2 (motifs interacting with ubiquitin)<sup>6,7</sup>. In the proposed operational model, absence of RNF168 should prevent the retention of repair proteins such as 53BP1 and BRCA1 at DSB repair sites, thereby causing a repair defect and increased cell death in response to ionizing radiation. Interestingly, the deficiency detected in our present study was found by screening for irradiation-induced nuclear foci containing not RNF168 but 53BP1, consistent with the model that RNF168 controls the chromatin ubiquitin-binding pathway upstream of 53BP1 and BRCA1<sup>6,7</sup>.

## RESULTS

A 16-year-old Turkish male (RS66) initially presented with concerns about short stature. His birth history was unremarkable; he had no learning difficulties and had completed secondary school. Family history was negative and consanguinity was denied. Past history was noncontributory. He had no allergies, was not on medications, and review of systems was negative. On physical exam, he was diminutive but alert and interactive. His height (153cm/5 ft) and weight (35kg/77 lbs) were below the 5<sup>th</sup> percentile for age. His head circumference was 49.5 cm/19.5 in, more than 2SD below the mean for age and sex. Additional findings were conjunctival telangiectasias and a mild wide-based gait. Cranial nerves, reflexes and sensori-motor exam were normal. He had no neurobehavioral defects and mental status was intact. Congenital malformations were not present; skin exam was normal, and other organ systems were unremarkable. He experienced maxillary sinusitis several times during the following 3-4 years. With the exception of a serum IgA <21 mg/dl (normal range 80-350 mg/dl) and serum AFP 147IU/ml (normal <5.9 IU/ml), laboratory tests were normal, including karyotyping.

Overall, the patient's initial clinical profile, which included mild ataxia, ocular telangiectasia and immunodeficiency, suggested a mild or variant form of A-T, an autosomal recessive neurodegenerative disease caused by mutations in the *ATM* gene<sup>17-19</sup>. In vitro testing of cultured lymphoblastoid cells (LCLs) from RS66 revealed normal amounts of the ATM protein and kinase activity (judged from phosphorylation of several known ATM substrates in response to ionizing radiation), which did not support a conventional diagnosis of A-T (**Figure 1A**, and data not shown). To rule out unconventional forms of A-T, the ATM gene was also sequenced; no mutations were identified.

RS66 cells were then tested by colony survival assay, to assess radiosensitivity and repair of DSBs<sup>20,21</sup>. These cells were poor at forming colonies after irradiation, with a survival fraction (SF) of 16% at 1 Gray (**Figure 1B**); this was within the testing range regarded as “radiosensitive” (0-21 SF%, mean SF $\pm$ 1 S.D. of 13.1 $\pm$ 7.2%), based on responses of cells from 104 A-T patients and 29 normal controls (see ref. 20). The RS66 cells also exhibited an S-phase checkpoint defect when analyzed for the ability to undergo radioresistant DNA synthesis (**Figure 2A**), and showed aberrantly persistent phosphorylation kinetics of SMC1-Ser966 in response to radiation (**Figure 2B**), an ATM-mediated modification of the delayed kinetics that are indicative of impaired DSB repair. In addition, consistent with the aberrantly protracted phosphorylation of SMC1 (Figure 2B), radiation-induced  $\gamma$ H2AX foci remained abnormally elevated for a longer period of time compared to normal control human lymphoblastoid cells. Specifically, by 1 hour after irradiation (1Gy) the percentage of cells with elevated  $\gamma$ H2AX foci increased from the basal level (below 10%) to 65%, 87% and 64% in normal control, A-T and RS66 cells, respectively. This was followed by a drop to 14% in control cells, in contrast to a considerably slower decrease in A-T (to 32%) and RS66 (to 41%) cells, respectively, by 24 h after radiation. Thus, the kinetics of recovery after IR, as monitored by persistence of  $\gamma$ H2AX foci, indicates a cellular DSB repair defect in RS66 cells comparable to ATM deficiency. On the other hand, results of mitochondrial respiration measured in RS66 cells (assessed by a resazurin assay<sup>22</sup>), and the enzymatic machinery used for DNA end joining (assessed by an in vitro plasmid-based assay<sup>23</sup> in RS66 cell extracts) were within the normal range (negative data, not shown).

The patient was subsequently lost to follow-up until age 29 when he was admitted with chronic shortness of breath and cough. He reported having chronic shortness of breath for the previous three years and non-productive cough for the previous five years. He smoked (1/2 pack/day) from 19 to 23 years of age. He had no history of toxic

exposures, chronic pulmonary infections, sarcoidosis, connective tissue diseases, or autoimmune diseases. His skin was very dry (xerosis). He worked in a cafeteria and was married, with one daughter. His height and head circumference were unchanged since age 16. His weight had increased to 39kg (86 lbs). He was intermittently hospitalized with worsening pulmonary function, reduced lung capacity, progressive dyspnea and hypoxemia. By age 28, he had become oxygen dependent. Pulmonary function tests indicated restrictive pathology with FEV1/FVC 100% (FEV1 34- 22% and FVC 31-20%) and a pO<sub>2</sub> of 69-43 mmHg. Thoracic CAT scans revealed peribronchovascular reticular densities, particularly in the upper and middle lobes, intralobular and interlobular septal thickenings, subpleural interstitial and bilateral focal pleural thickenings, irregular thickenings of major fissures, and traction bronchiectasis. Bronchoscopy revealed increased vascularization of the mucosa of both bronchi, with telangiectasias on the right main bronchus. His ataxia was slightly worse than when seen the last time; he never developed apraxia or dysarthria. At age 30, RS66 died from respiratory failure secondary to idiopathic interstitial pneumonia and presumed pulmonary fibrosis.

Given the A-T-like clinical features of the RS66 patient yet wild-type *ATM* gene and normal expression of ATM protein in the RS66 cells (Figure 1A), we argued that a defect of some other component of the DSB-signaling pathway might underlie the observed pathology and cellular radiosensitivity. In addition, we hypothesized that analogous DSB signaling and/or repair defects might also explain also additional cases of radiosensitivity. To search for such potential radiosensitizing molecular defects, we next screened a panel of 80 radiosensitive LCLS of *undetermined* etiology (i.e., excluding *known* DNA DSB repair defects associated with radiosensitivity, such as A-T and Nijmegen Breakage Syndrome) that also included the RS66 cells. For this focused phenotypic screening, we exploited the recent findings of 53BP1 accumulation at sites of double strand DNA breaks<sup>6,7,24-26</sup>. Microscopically, we searched for 53BP1-containing

irradiation (IR)-induced nuclear foci as a reliable surrogate marker for integrity of the ubiquitin ligase-regulated DNA repair pathway<sup>6-16</sup>. Specifically, we exposed the cells to 3Gy IR and 1h later co-immunostained the cells with antibodies to 53BP1 and  $\gamma$ H2AX or MDC1 (the antibodies to  $\gamma$ H2AX and MDC1 served as unbiased indicators of DSB damage). Strikingly, RS66 was the only one of the 80 radiosensitive cell lines that failed to form 53BP1 nuclear foci (**Figure 3A**). Consistent with this finding, IR-induced BRCA1 foci, which also require local chromatin ubiquitinylation<sup>6-9</sup>, were also absent in RS66 cells (**Figure 3B**).

To elucidate the cause of the observed defects in recruitment of the DNA repair proteins at sites of DNA damage, we analyzed protein extracts from control and RS66 cells by western blotting with a panel of antibodies to the key chromatin-associated DSB regulators<sup>3,6-9,16</sup> (**Figure 1A**). RNF168 protein was completely absent in RS66 cell lysates. Since only one antibody to RNF168 was commercially available at the time of these studies, we also raised a new, mouse monoclonal antibody to RNF168 (DCS-402) to validate the lack of the protein in RS66 cells. As shown in Figure 1A, the absence of RNF168 was reproducible and confirmed by the two independent antibodies to RNF168, suggesting that the lack of this factor might be the molecular explanation for the inability of RS66 cells to retain the 53BP1 and BRCA1 repair proteins around DNA repair break sites.. Interestingly, immunoblotting also confirmed that other components of this DSB signaling and repair pathway, such as MDC1, 53BP1, BRCA1, and RNF8, were present in normal amounts in RS66 cells (**Figure 1A**).

DNA sequencing of the entire *RNF168* gene identified two homozygous nonsense mutations, 391C>T, in exon 3 (**Figure 4A**). These mutations are predicted to create a premature stop codon during translation of the RNF168 protein that would eliminate the key structural domains of the mature protein required for its function as a ubiquitin

ligase, MIU-1 and MIU-2 (motif interacting with ubiquitin)<sup>6-8</sup>. The parents of the RS66 patient were no longer alive for follow-up sequencing.

Although the above results strongly implicated RNF168 in RS66-associated DNA repair, it was possible that mutations in other genes might cause the disease phenotype. Such possibility was excluded by genetic complementation of RS66 cells, whereby reintroduction of a full length RNF168 cDNA construct that was tagged with green fluorescent protein (GFP)<sup>6</sup> robustly restored both 53BP1 and BRCA1 retention in IR-induced nuclear foci (**Figure 4B**).

## DISCUSSION

Collectively, our analyses provide evidence that the absence of the RNF168 protein and its associated catalytic activity prevent retention of 53BP1 and BRCA1 proteins at sites of DSB repair, in accordance with the recently proposed model<sup>5,6,7</sup>, and thereby is the major cause of the DSB repair malfunction in RS66 cells. Our present data further indicate that insufficient repair of DNA breaks translates into the pathophysiological phenotype observed in this patient, thereby identifying a novel syndrome that resembles the clinical features and cellular phenotypes associated with A-T.

RS66 succumbed to respiratory failure at an early age. His clinical course was accompanied by ataxia, growth retardation, microcephaly, immunodeficiency and an elevated serum alphafetoprotein (AFP). An elevated serum AFP has only been described in two other inherited ataxias: A-T and AOA2 (Ataxia with Qculomotor Apraxia Type 2)(a.k.a. senataxin deficiency)<sup>18,27</sup>. Elevated AFP is also found in gastric cancers and in the amniotic fluid of fetuses carrying open neural tube defects. However, the pattern of differential glycosylation for the elevated AFP of A-T patients resembles that of hepatic not fetal, gastric, or neural origin<sup>19</sup>. The mechanism for an elevated serum AFP is unclear for any of these conditions. The absence of apraxia and dysarthria would not have

supported a diagnosis of A-T or AOA2, although this patient was followed for many years as an “A-T variant”, a term that is often used by clinicians as an operational working diagnosis when no other is more specific. Similarly, a diagnosis of Nijmegen Breakage syndrome (NBS)<sup>5</sup> would not have been supported by the normal intelligence and the presence of an abundant level of nibrin (NBS1/NBN) protein observed on immunoblots.

Genetic causes of idiopathic pulmonary fibrosis (IPF) remain unclear although surfactant and interleukin genes have been linked to a few familial cases<sup>28-30</sup>. Accumulation of DNA fragments can trigger an inflammatory cascade<sup>1,28</sup>. Thus, the respiratory failure and suggested pulmonary fibrosis in this patient may have been caused by chronic inflammation from unrepaired DNA damage. Further clarification of RNF168 function(s) may expand our understanding of IPF. Alternatively, it is possible that the progressive and fatal respiratory failure observed in this single patient may have had another etiology. Severe lymphocytic infiltration with pulmonary failure is often reported in the later stages of A-T<sup>31</sup> (**Table 1**).

We show that Patient RS66 carried homozygous nonsense mutations in the RNF168 gene, which abolished production of RNF168 protein. Interestingly, the only other patient with RNF168 deficiency (15-9BI) reported to date was heterozygous for two nonsense mutations that both truncated the protein but left one ubiquitin-interacting motif (MIU1) to possibly continue the formation of chromatin ubiquitylation<sup>7,26</sup>. In contrast to RS66, this patient did not manifest ataxia, telangiectasia, microcephaly, nor respiratory failure. In common were growth retardation, serum IgA deficiency, and radiosensitivity (**Table 1**). No mention was made of AFP<sup>1,26</sup>. Of great translational interest, both patients were lacking IR-induced 53BP1 and BRCA1 nuclear foci (i.e., the ability to localize and retain these repair factors at the DSB sites was lost). This strongly

supports the critical role of the RNF8-**RNF168**-HERC2-BRCA1 chromatin ubiquitin ligase cascade<sup>8,16</sup> in the maintenance of genome integrity.

Considering the two patients together, RNF168 deficiency may provide insights into the pathogenesis of IgA Deficiency and Common Variable Immune Deficiency, which together encompass a large number of immunodeficiency patients with a wide range of clinical manifestations but few defined genetic causes<sup>32</sup>. As a direct regulator of BRCA1 retention at DSB damage sites, RNF168 may also play a role in breast cancer<sup>33</sup>. A role for RNF168 in class switch recombination is also suggested by the presence of IgA deficiency in both RNF168 deficiency patients and the recent studies of Ramachandran et al<sup>12</sup>. Clinically, the RNF168-deficiency syndrome we describe here is closer to A-T than to the overall milder phenotype of the 15-9BI patient<sup>26</sup> (**Table 1**). Whether these clinical differences are attributable solely to the differential severity of RNF168 mutations themselves, or modulated by genetic background of the individual patients and/or interplay with environmental factors, would require identification of additional patients and future studies.

A further implication of our results reflects the specific localization of the RNF168 gene to chromosome 3q29. Deletion of this genomic region is associated with a syndrome of microcephaly, growth retardation short stature, ataxia, chest wall deformities, skin abnormalities and recurrent infections (“3q29 Microdeletion Syndrome”)<sup>34</sup>. These features overlap substantially with those of our new RNF168 deficiency patient, RS66, suggesting the possibility that RNF168 deficiency may contribute to the pathogenesis of 3q29 Microdeletion Syndrome. Furthermore, 3q29 is a region where large (>100kb) copy number variants (large CNVs) commonly exist, even in normal individuals. These are thought to change gene dosages and be highly penetrant and are reported to be hotspots of human genetic disease, autism, and schizophrenia.<sup>35-37</sup>

An important next step for understanding the role of RNF168 in disease will be to identify and evaluate additional patients with radiosensitivity, neurodegeneration, cancer,

immunodeficiency, microcephaly, autism, growth defects and/or pulmonary fibrosis. As we have demonstrated, the immunocytochemical detection of 53BP1 nuclear foci is a robust functional surrogate screening assay for identifying patients with deficiencies of the chromatin ubiquitin-dependent cascade of DNA repair. The rapid identification of two patients with RNF168 deficiency further suggests that the recently defined pathway may herald a new category of disorders associated with malfunctioning of DNA repair and processing. Last but not least, the fact that RNF168 operates downstream of tumor suppressors such as ATM and the Mre11/Rad50/Nbs1 complex, and upstream of additional tumor suppressors such as BRCA1 and 53BP1<sup>16</sup>, raises the issue of whether the RNF168-deficiency syndrome may include a predisposition to cancer. Given that cancer incidence among patients suffering from the A-T syndrome, is approximately 30% (see also Table 1), it is important that this question be addressed as well for the RNF168 deficiency syndrome, which will require clinical follow up and family history studies of a larger number of patients. While there was only limited information about the family history of the RS66 patient, the father of the other patient with RNF168 deficiency<sup>7</sup> developed chronic B cell lymphocytic leukemia, a malignancy that also occurs in A-T patients. Overall, considering the role of the DSB signaling, repair and checkpoint machinery as an intrinsic barrier against activated oncogenes and tumor progression<sup>38-40</sup>, it is reasonable to speculate that RNF168 itself represents a plausible candidate for a potential tumor suppressor.

## **Materials and Methods**

All studies were conducted in accordance with Institutional Review Board requirements and appropriate approvals were obtained.

### **Colony Survival Assay**

We assessed the radiosensitivity of LCLs using CSA, as previously described<sup>20</sup>. Briefly, after the LCLs were exposed to varying doses of IR from 0 to 2 Gy, they were

incubated at 37° C. for 10-14 days, depending on cell growth. After MTT staining, each well was scored for the presence/absence of colonies of >32 cells (5 divisions) and colony survival fractions (%CSF) were calculated. Based on an earlier study comparing 104 bona fide A-T cell lines to 29 wild type cells, the %CSF was interpreted as “radiosensitive” if below 21%SF (13±7%) for 1 Gy exposure. Radiosensitive and radionormal controls were included in each experiment.

### **Immunocytochemistry and $\gamma$ -H2AX foci kinetics**

Lymphoblastoid cell lines from RS66 and controls were exposed to 3 Gy radiation to induce double strand DNA breaks. After one hour incubation, cells were fixed in 4% phosphate buffered formalin, permeabilized with 0.5% Triton X-100 and immunostained with antibodies to  $\gamma$ -H2AX (Upstate #05-636), MDC1 (Abcam #ab11171), 53BP1 (Santa Cruz #sc-22760), or BRCA1 (Santa Cruz #sc-6954), as specified in Figure legends, and followed with Alexa Fluor 488 and Alexa Fluor 568 secondary antibodies (Invitrogen). Slides were mounted in DAPI Vectashield (Vector laboratories, USA) and analyzed by a confocal fluorescence microscopy as described<sup>6,13</sup>. To assess the kinetics of  $\gamma$ -H2AX foci, exponentially growing cells were irradiated with a dose of 1Gy, and samples for immunocytochemical estimation were taken at times 0 (pre-irradiation state), 1 and 24 h post radiation. At least 300 cells were counted per sample, and cells with more than 4  $\gamma$ -H2AX foci were regarded as positive.

### **Western blotting**

Protein extracts were resolved by 7.5% SDS-PAGE, transferred to the nitrocellulose or PVDF membranes, blocked with 5% evaporated milk (BioRad, Hercules, CA), and probed with the following antibodies: mouse monoclonal antibody to RNF168 (DCS-402; generated for the purpose of this study by standard hybridoma technology); rabbit polyclonal antibodies to RNF168 (gift from Drs. Daniel Durocher and

Stephanie Panier), ATM (abcam #ab31842), sheep polyclonal antibody to MDC1 (gift from Dr. Stephen Jackson), RNF8 (ref. 7), and mouse monoclonal antibody to MCM7 (DCS-141; ref. 6).

### **Radioresistant DNA synthesis**

LCLs were incubated with  $^{14}\text{C}$  thymidine for 24 h to establish background synthesis of DNA. The medium was replaced with fresh media, and the cells were exposed to varying doses of IR (0, 2, 5, 10, and 25Gy). The cells were returned to the incubator for 60 min and pulse labeled in medium containing tritiated thymidine  $\text{H}^3\text{T}$  for an additional 60 min. The samples were harvested and counted in a Packard 2900TR scintillation counter. The ratio of incorporated  $\text{H}^3\text{T}$  to  $^{14}\text{C}$  was used as a measure of the amount of DNA synthesis after IR exposure. Experiments were performed in triplicate.

### **SMC1 phosphorylation kinetics**

Nuclear extracts from 5-10 million RS66, WT or A-T LCLs were prepared following the manufacturer's protocol (NE-PER Nuclear and Cytoplasmic Extraction Reagents, Pierce, Rockford, IL). LCLs were harvested 1, 4 and 24 hours after 10Gy or mock treatment. Twenty-five ug of nuclear lysate were electrophoresed on a 7.5% SDS-polyacrylamide gel (PAGE), transferred onto PVDF membrane (Bio-Rad, Hercules, CA), and blocked with 5% dry milk (Bio-Rad, Hercules, CA). Blots were incubated overnight at 4°C with the following antibodies: anti-SMC1s966, (Novus, Littleton, CO), and SMC1, (Novus, Littleton, CO). An HRP-conjugated anti-rabbit or anti-mouse secondary antibody was incubated at room temperature for 40 min for detection by enhanced chemiluminescence (ECL) (Amersham Pharmacia, Piscataway, NJ).

### **DNA sequencing**

Genomic DNA was isolated from RS66 and PCR amplified using 6 primer sets (Forward and Reverse) for each exon. After the PCR amplification, the products were

visualized using agarose gel electrophoresis and purified (Qiagen PCR purification kit). The purified PCR products were used to sequence each exon, in addition to 200bp upstream and downstream of the flanking regions. DNA sequences were compared to wildtype sequences using blast 2 program from the NCBI website (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>).

### **Acknowledgements**

Dr. Nermin Capan provided pulmonary care for the patient at the Atatürk Chest Disease and Chest Surgery, Education and Research Hospital, Ankara, Turkey. We thank Drs. Daniel Durocher, Stephanie Panier and Stephen Jackson for reagents. C.D., J.B., C.L. and J.L. were supported by the Danish Cancer Society, the Danish National Research Foundation, the Lundbeck Foundation (R13-A1287 and R44-A4400), the Czech Ministry of Education (MSM6198959216) and the European Community (projects: GENICA, Infla-Care and CZ.1.05/2.1.00/01.0030). S.S.D., K.N., K.P., S.A.N. and R.A.G. were supported by U.S.P.H.S grants (AI 067769, NS052528), the A-T Ease Foundation, the A-T Medical Research Foundation, the Joseph Drown Foundation, and by a fellowship (S.A.N.) from the University of California Toxic Substances Research and Teaching Program.

**References:**

1. Westbrook AM, Schiestl RH. Atm-deficient mice exhibit increased sensitivity to dextran sulfate sodium-induced colitis characterized by elevated DNA damage and persistent immune activation. *Cancer Res.* 2010; **70**:1875-1884.
2. Wood RD, Mitchell M, Sgouros J, Lindahl T. Human DNA Repair Genes. *Science* 2001; **291**:1284-1289.
3. Jackson SP, Bartek J. The DNA damage response in human biology and disease. *Nature.* 2009; **461**:1071-1078.
4. Kastan BM, Bartek J. Cell-cycle checkpoints and cancer. *Nature* 2004; **432**:316-323.
5. Nahas SA, Gatti RA. DNA double strand break repair defects, primary immunodeficiency disorders, and 'radiosensitivity'. *Curr Opin Allergy Clin Immunol.* 2009; **9**: 510-516.
6. Doil C, Mailand N, Bekker-Jensen S, et al. RNF168 Binds and Amplifies Ubiquitin Conjugates on Damaged Chromosomes to Allow Accumulation of Repair Proteins. *Cell* 2009; **136**:435-446.
7. Stewart G, Panier S, Townsend K, et al. The RIDDLE Syndrome Protein Mediates a Ubiquitin-Dependent Signaling Cascade at Sites of DNA Damage. *Cell* 2009; **136**:420-434.
8. Al-Hakim A, Escibano-Diaz C, Landry MC, O'Donnell L, Panier S, Szilard RK, Durocher D. The ubiquitous role of ubiquitin in the DNA damage response. *DNA Repair* 2010; **9**:1229-1240.
9. Bartek J, Lukas J. DNA damage checkpoints: from initiation to recovery or adaptation. *Curr Opin Cell Biol.* 2007; **19**:238-245.
10. Mailand N, Bekker-Jensen S, Faustrup H, et al. RNF8 ubiquitylates histones at DNA double-strand breaks and promotes assembly of repair proteins. *Cell* 2007; **131**:887-900.
11. Kolas NK, Chapman JR, Nakada S, et al. Orchestration of the DNA-damage response by the RNF8 ubiquitin ligase. *Science* 2007; **318**:1637-1640.
12. Ramachandran S, Chahwan R, Nepal RM, et al. The RNF8/RNF168 ubiquitin ligase cascade facilitates class switch recombination. *Proc Natl Acad Sci U S A.* 2010; **107**:809-814.
13. Bekker-Jensen S, Rendtlew Danielsen J, Fugger K, Gromova I, Nerstedt A, Lukas C, Bartek J, Lukas J, Mailand N. HERC2 coordinates ubiquitin-dependent assembly of DNA repair factors on damaged chromosomes. *Nat Cell Biol.* 2010; **12**:80-86.

14. Galanty Y, Belotserkovskaya R, Coates J, et al. Mammalian SUMO E3-ligases PIAS1 and PIAS4 promote responses to DNA double-strand breaks. *Nature*. 2009; **462**:935-939.
15. Morris JR, Boutell C, Keppler M, et al. The SUMO modification pathway is involved in the BRCA1 response to genotoxic stress. *Nature*. 2009; **462**:886-890.
16. Bartek J, Hodny Z. SUMO boosts the DNA damage response barrier against cancer. *Cancer Cell* 2010; **17**:9-11.
17. Shiloh Y, Kastan MB. ATM: genome stability, neuronal development and cancer cross paths. *Adv Cancer Res*. 2001; **83**:209-254.
18. Chun HH, Gatti RA. Ataxia-telangiectasia, an evolving phenotype. *DNA Repair* 2004; **3**:1187-1196.
19. Ishiguro T, Taketa K, Gatti RA. Tissue of origin of elevated alpha-fetoprotein in ataxia-telangiectasia. *Dis Markers*. 1986; **4**:293-297.
20. Sun X, Becker-Catania SG, Chun HH, et al. Early diagnosis of ataxia-telangiectasia using radiosensitivity testing. *J Pediatr*. 2002;**140**:724-731.
21. Pollard J, Gatti RA. Clinical radiation sensitivity with DNA repair disorders: an overview. *Int J Radiat Oncol Biol Phys*. 2009; **74**: 1323-1331.
22. Ambrose M, Goldstine JV, Gatti RA. Intrinsic mitochondrial dysfunction in ATM-deficient lymphoblastoid cells. *Hum Mol Genet*. 2007; **16**:2154-2164.
23. Perrault RB, Wang H, Wang M, Rosidi B, Iliakis G. Backup pathways of NHEJ are suppressed by DNA-PK. *J Cell Biol*. 2004; **92**:781-794.
24. Schultz LB, Chehab NH, Malikzay A, Halazonetis TD. P53 binding protein 1 (53BP1) is an early participant in the cellular response to DNA double strand breaks. *J Cell Biol*. 2000; **151**:1381-1390.
25. DiTullio RA Jr, Mochan TA, Venere M. et al. 53BP1 functions in an ATM-dependent checkpoint pathway that is constitutively activated in human cancer. *Nat Cell Biol*. 2002; **4**:998-1002.
26. Stewart GS, Stankovic T, Byrd PJ, et al. RIDDLE immunodeficiency syndrome is linked to defects in 53BP1-mediated DNA damage signaling. *Proc Natl Acad Sci USA*. 2007; **104**:16910-16915.
27. Asaka T, Yokoji H, Ito J, Yamaguchi K, Matsushima A. Autosomal recessive ataxia with peripheral neuropathy and elevated AFP: novel mutations in SETX. *Neurology* 2006; **66**:1580-1581.

28. Rigby RE, Leitch A, Jackson A. Nucleic acid mediated inflammatory diseases. *Bioessays* 2008; **30**:833-842
29. Lawson WE, Loyd JE. The genetic approach in pulmonary fibrosis: can it provide clues to this complex disease? *Proc Am Thorac Soc.* 2006; **3**:345-349.
30. Scotton C, Chambers R. Molecular Targets in Pulmonary Fibrosis The Myofibroblast in Focus. *Chest* 2007; **132**:1311-1321.
31. Tangsinmankong N, Wayne AS, Howenstine MS, et al. Lymphocytic interstitial pneumonitis, elevated IgM, and hepatosplenomegaly in ataxia-telangiectasia. *J Pediatr.* 2001; **138**: 939-941. 32. Bonilla FA, Geha RS. Common variable immunodeficiency. *Pediatr Res.* 2009; **65**: 13R-19R.
33. Tan D, Marchiò C, J S Reis-Filho. Hereditary breast cancer: from molecular pathology to tailored therapies. *J. Clin Pathol.* 2008; **61**:1073-1082
34. Willatt L, Cox J, Barber J, Cabanas ED, Collins A, Donnai D. Microdeletion Syndrome: Clinical and Molecular Characterization of a New Syndrome. *Am J Hum Genet.* 2005; **77**:154–160.
35. Itsara A, Cooper GM, Baker C, Girirajan S, Li J, Absheer D, Krauss RM, Myers RM, Ridker PM, Chasman DI et al. Population analysis of large copy number variants and hotspots of human genetic disease. *Am J Hum Genet.* 2009; **84**:148-161.
36. Mulle JG, Dodd AF, McGrath JA, Wolyniec PS, Mitchell AA, Shetty AC, Sobreira NL, Valle D, Rudd MK, Satten G, Cutler DJ, Pulver AE, Warren ST. Microdeletions of 3q29 Confer High Risk for Schizophrenia. *Am J Hum Genet.* 2010; **87**:229-236.
37. Quintero-Rivera F, Sharifi-Hannauer P, Martinez-Agosto JA. Autistic and psychiatric findings associated with the 3q29 microdeletion syndrome: case report and review. *Am J Med Genet A.* 2010;152:2459-67.
38. Gorgoulis VG, Vassiliou LV, Karakaidos P, Zacharatos P, Kotsinas A, Liloglou T, Venere M, Ditullio RA, Kastrinakis NG, Levy B, Kletsas D, Yoneta A, Herlyn M, Kittas C, Halazonetis TD. Activation of the DNA damage checkpoint and genomic instability in human precancerous lesions. *Nature* 2005; **434**:907-913.
39. Bartkova J, Horejsi Z, Koed K, Kramer A, Tort F, Zieger K, Guldborg P, Sehested M, Nesland JM, Lukas C, Ørntoft T, Lukas J, Bartek J. DNA damage response as a candidate anti-cancer barrier in early human tumorigenesis. *Nature* 2005; **434**:864-870.
40. Halazonetis TD, Gorgoulis VG, Bartek J. An oncogene-induced DNA damage model for cancer development. *Science* 2008; **319**:1352-1355.

## Figure legends

### **Figure 1. Analysis of protein levels of selected DDR factors and radiosensitivity of RS66 cells.**

(A). Cell lysates from RS66 and control (Choc26) cells were analyzed by immunoblotting with the indicated antibodies (see Methods). The red box highlights the lack of RNF168 protein in RS66 cells.

(B). Colony survival assay demonstrates AT-like radiosensitivity of RS66 cells. Asterisk refers to SF% ranges defined in Ref. 20.

### **Figure 2. Functional analysis of DNA damage response in RS66 cells.**

(A). RS66 cells exhibit an intra-S phase cell cycle checkpoint defect similar to A-T cells. WT, A-T and RS66 LCLs were labeled with  $^{14}\text{C}$  thymidine for 24 h, irradiated with the doses indicated, and then chased with  $^3\text{H}$  thymidine ( $\text{H}^3\text{T}$ ) for 1 h. Cells were fixed and the  $\text{H}^3\text{T}/^{14}\text{C}$  ratio, indicative of S-phase inhibition, was calculated by using a scintillation counter. Error bars represent the mean  $\pm$  s.d. N=3.

(B). RS66 cells maintain increased levels of phosphorylated SMC1s966 24 hours post-IR. RS66 LCLs were mock-irradiated or exposed to 10 Gy of IR and fixed at the times indicated after irradiation (bottom). WT (top) and A-T (middle) LCLs controls were included in each experiment. Nuclear extracts were prepared and subjected to SDS/PAGE/Western blot analysis using the antibodies indicated.

### **Figure 3. Immunocytochemical analysis of 53BP1 and BRCA1 foci formation in response to ionizing radiation.**

(A). 53BP1 IRIF formation. RS66 and control (Choc26) cells were exposed to 3 Gy of IR and 1 h later immunostained with the antibodies to 53BP1 and gamma-H2AX. The red-framed field shows cells with impaired focal accumulation of 53BP1.

(B). BRCA1 IRIF formation. RS66 and control (Choc26) cells were treated as in (A) and immunostained with antibodies to BRCA1 and MDC1. The green-framed field shows cells with impaired focal accumulation of BRCA1.

**Figure 4. Identification of the *RNF168* gene mutation in RS66, and phenotypic rescue of DNA damage signaling by wild-type RNF168 in the RS66 cells.**

- (A) Sequence of genomic DNA from RS66 cells, showing a homozygous nonsense mutation at c.391C>T (R131X) (shaded column).
- (B) 53BP1 and BRCA1 foci formation in RS66 cells reconstituted by ectopic wild-type RNF168. RS66 cells were transfected with the control plasmid coding for free nuclear GFP (nucGFP, upper row) or the expression plasmid for wild-type GFP-RNF168 (bottom row). The productively transfected cells were identified according to the nuclear GFP signal (green channel) and scored for 53BP1 (left panel) or BRCA1 (right panel) focus formation. Red arrows (upper row) point to control cells lacking nuclear foci for either 53BP1 or BRCA1; green arrows (lower row) mark RNF168-complemented cells that regained the ability to accumulate 53BP1 and BRCA1 in nuclear foci. The merged images contain cells with yellow foci (red on a green background).

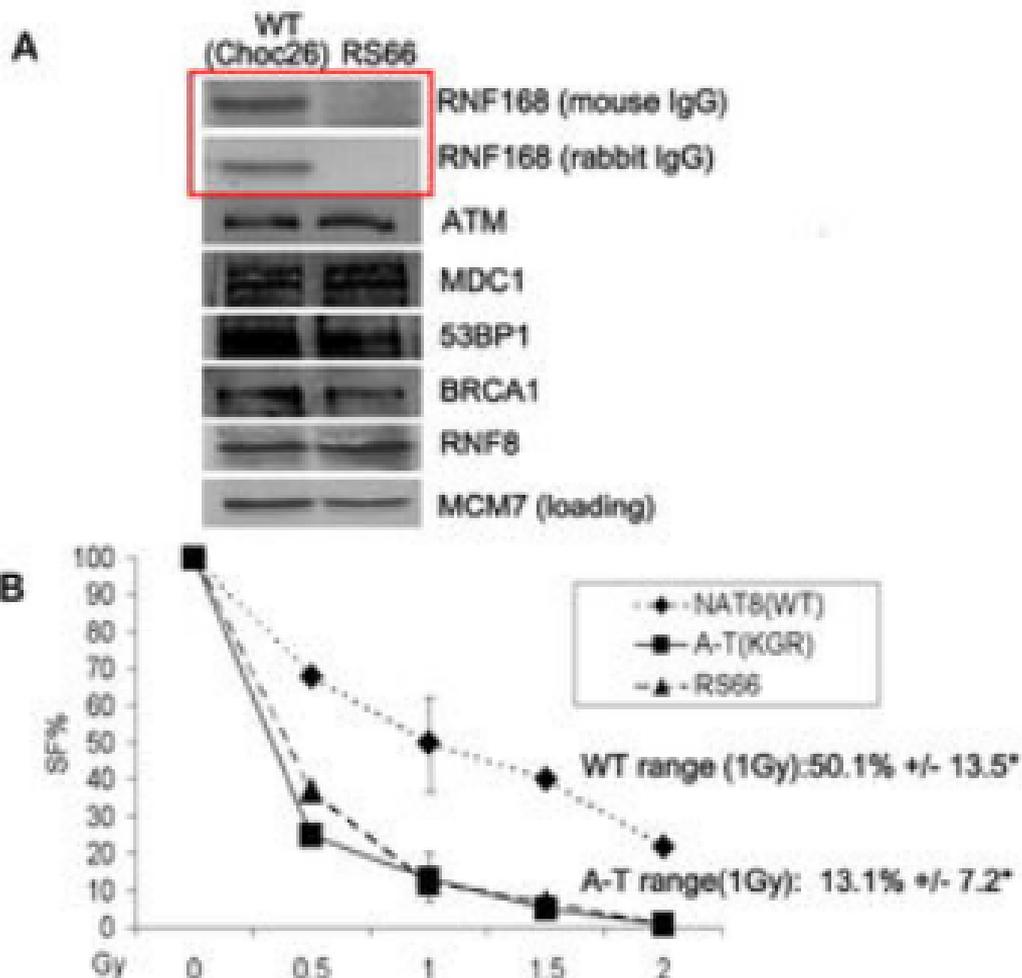


Figure 1

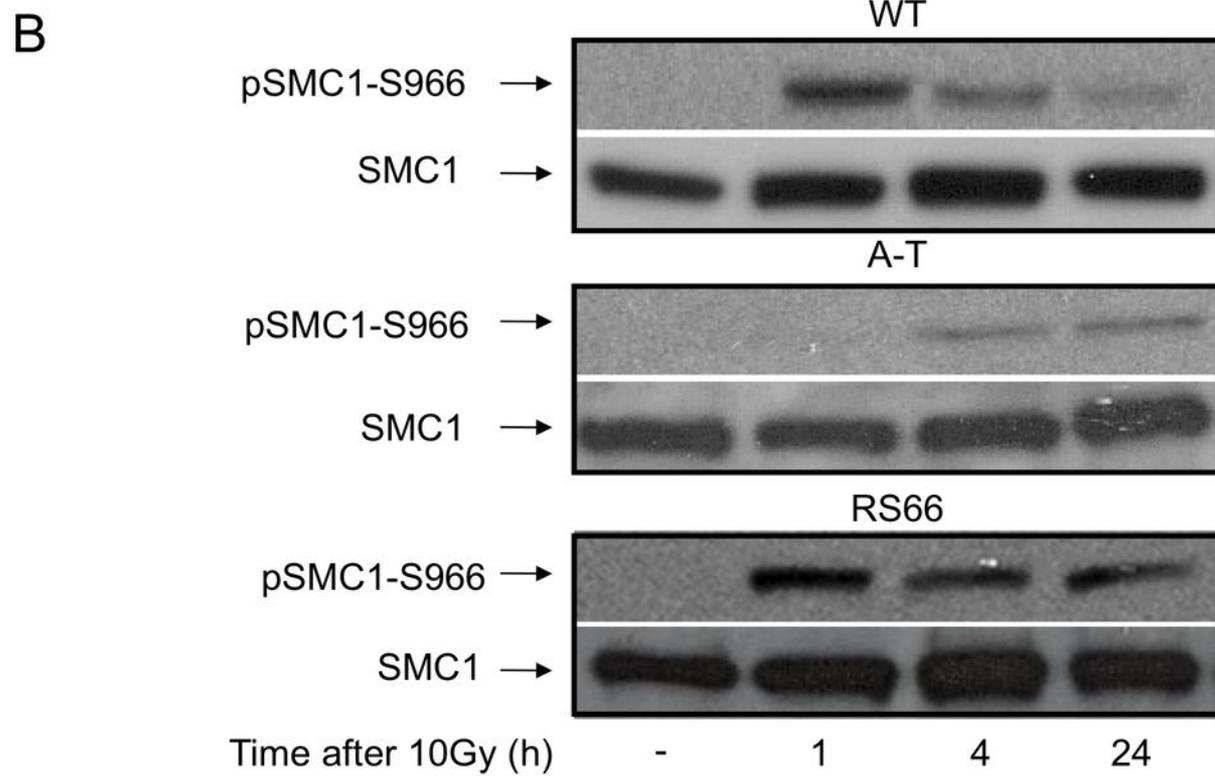
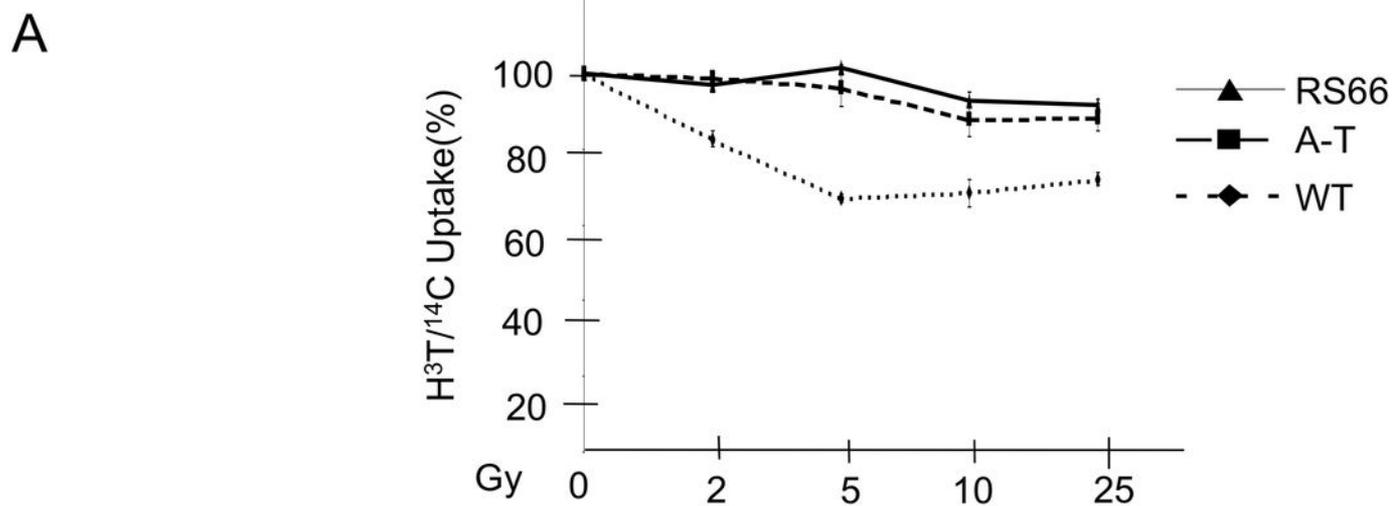


Figure 2

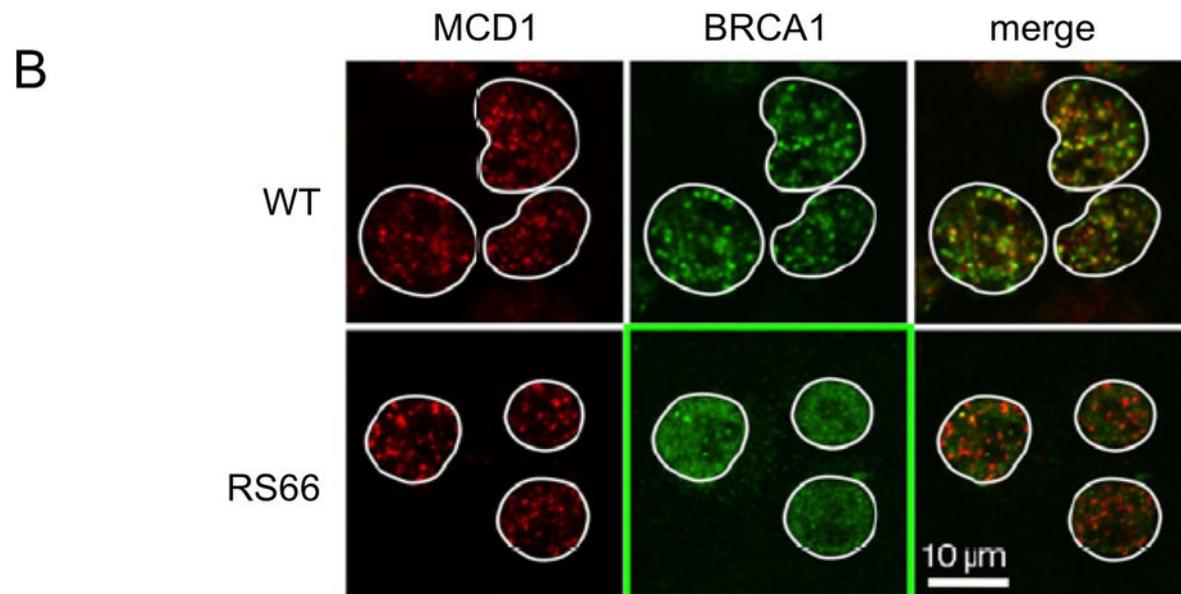
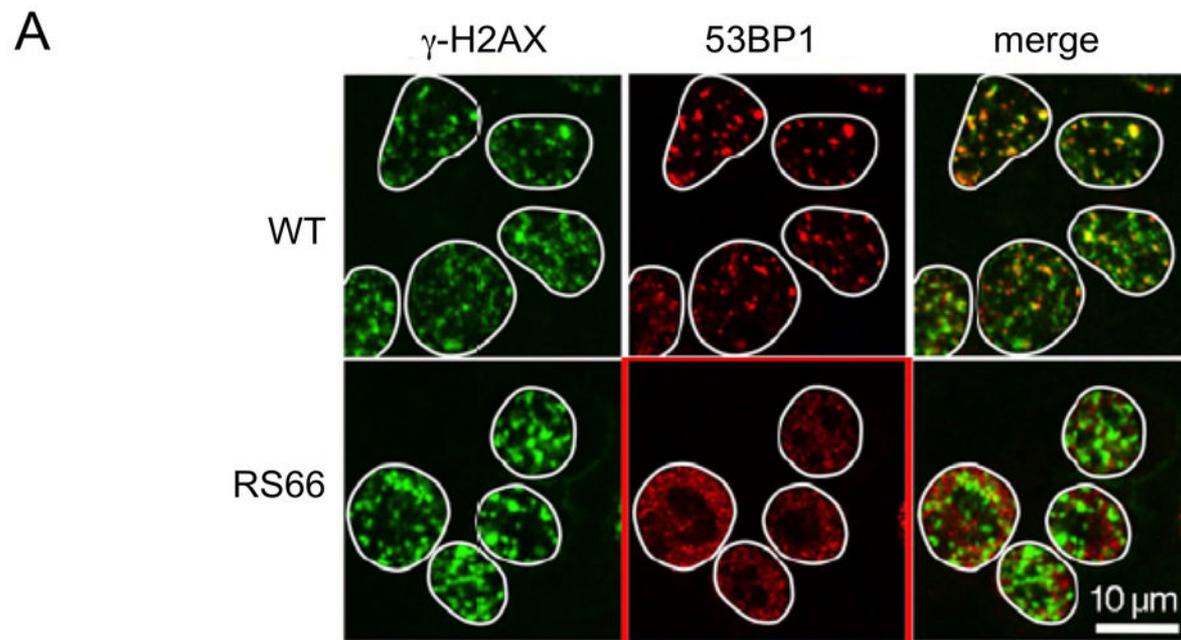


Figure 3

**A****RNF168 c.391C>T (R131X)<sup>hom</sup> mutation**

Query	376	AAGGTGGCGGCAGAGCGACGGGCCAGCGAGGAAGAAGAAAACAAAGCCAGTGAAGAATAC	435
Sbjct	132	..... <b>T</b> .....	191

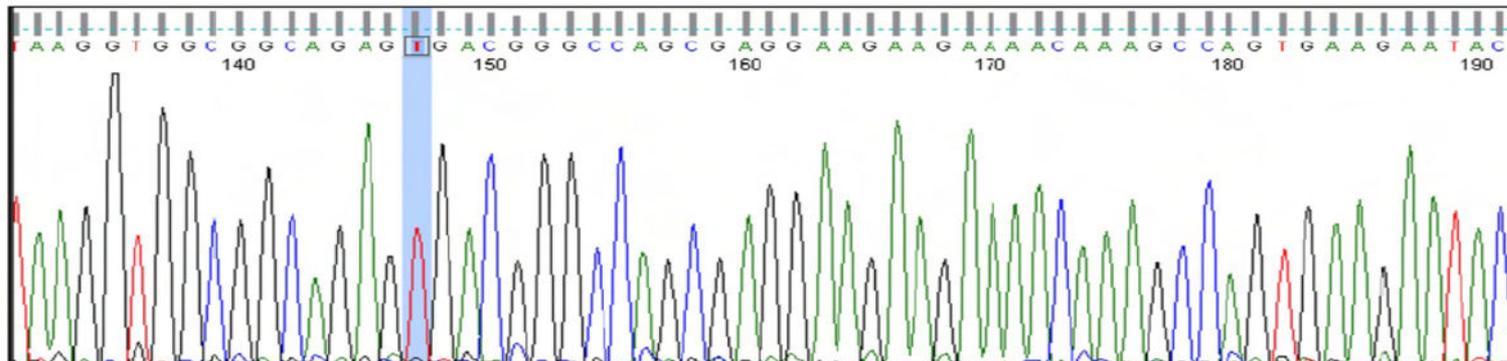
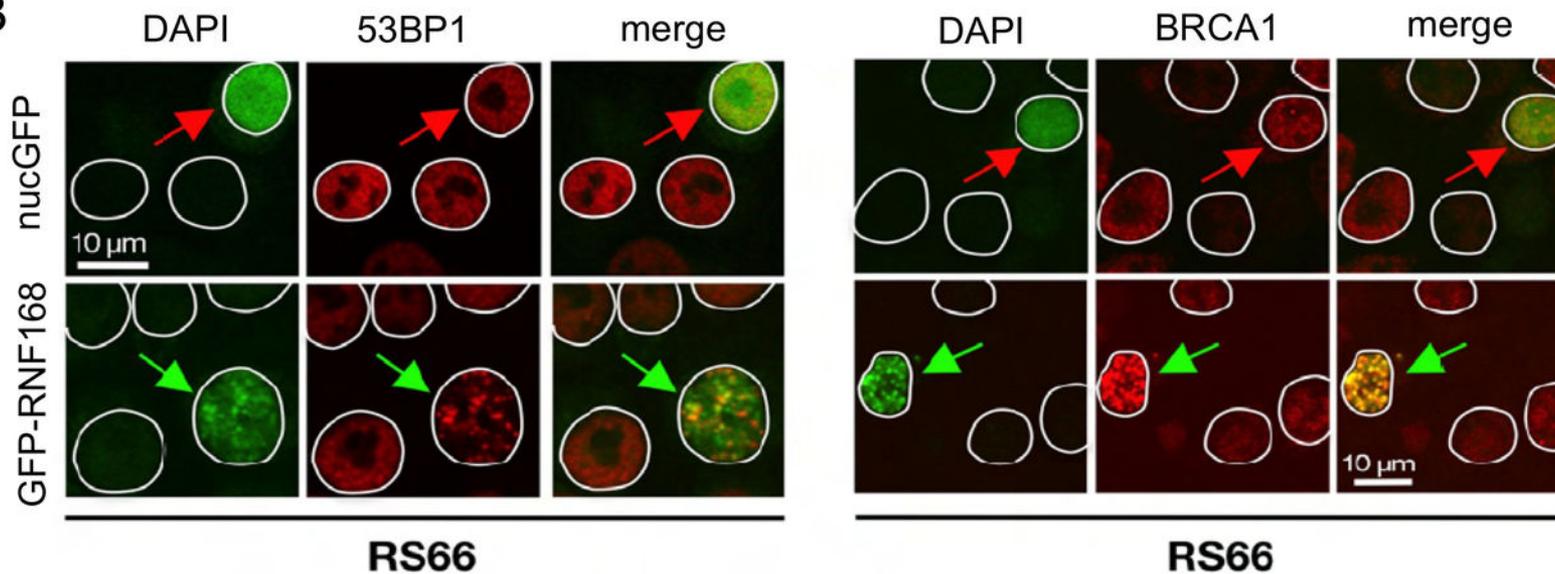
**B**

Figure 4

**Table 1. RNF168 deficiency phenotypes\*, compared to A-T**

<b>Patient 1 (15-9BI)<sup>26</sup></b>	<b>Patient 2 (RS66)<sup>(this study)</sup></b>	<b>Ataxia-telangiectasia</b>
<b>Short stature</b>	<b>Short stature</b>	<b>Occasional short stature</b>
<b>No telangiectasia</b>	<b>Ocular and bronchial telangiectasia</b>	<b>Telangiectasia</b>
<b>Mild motor impairment</b>	<b>Ataxia, without apraxia or dysarthria</b>	<b>Ataxia, with apraxia and dysarthria</b>
<b>Learning difficulties</b>	<b>Normal intelligence</b>	<b>Normal intelligence</b>
<b>Facial dysmorphism</b>	<b>Microcephaly</b>	<b>Microcephaly (A-T<sub>Fresno</sub> variant)</b>
<b>Hypimmunoglobulinemia</b>	<b>Low serum IgA</b>	<b>Low serum IgA, IgE, IgG2</b>
<b>Cellular radiosensitivity*</b>	<b>Cellular radiosensitivity*</b>	<b>Cellular and clinical radiosensitivity</b>
	<b>Terminal respiratory failure</b>	<b>Terminal respiratory failure</b>
	<b>Elevated serum AFP</b>	<b>Elevated serum AFP</b>
	<b>*</b>	<b>Cancer susceptibility</b>
	<b>Dry skin with fine scaling (xerosis)</b>	

\*Potential cancer susceptibility and clinical radiosensitivity of RNF168 deficiency remain to be assessed (see Discussion)

