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Bcl-2 Inhibits Nuclear Homologous Recombination by Localizing BRCA1 to the Endomembranes

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Abstract

Genetic stability requires coordination of a network of pathways including DNA repair/recombination and apoptosis. In addition to its canonical anti-apoptotic role, Bcl-2 negatively impacts genome stability. In this study, we identified the breast cancer tumor suppressor BRCA1, which plays an essential role in homologous recombination (HR), as a target for Bcl-2 in the repression of HR. Indeed, ionizing radiation–induced BRCA1 foci assembly was repressed when Bcl-2 was expressed ectopically, in human SV40 fibroblasts, or spontaneously, in lymphoma t(14:18) cells and in HeLa and H460 cancer cell lines. Moreover, we showed that the transmembrane (TM) domain of Bcl-2 was required for both inhibition of BRCA1 foci assembly and the inhibition of HR induced by a double-strand break targeted into an intrachromosomal HR substrate by the meganuclease I-SceI. Fluorescence confocal microscopy, proximity ligation assay, and electron microscopy analyses as well as Western blot analysis of subcellular fractions showed that Bcl-2 and BRCA1 colocalized to mitochondria and endoplasmic reticulum in a process requiring the TM domain of Bcl-2. Targeting BRCA1 to the endomembranes depletes BRCA1 from the nucleus and, thus, accounts for the inhibition of HR. Furthermore, our findings support an apoptosis-stimulatory role for the cytosolic form of BRCA1, suggesting a new tumor suppressor function of BRCA1. Together, our results reveal a new mode of BRCA1 regulation and for HR in the maintenance of genome stability. *Cancer Res; 71(10); 3590–602. ©2011 AACR.*

Introduction

Faithful genome transmission requires coordination of a network of pathways including cell cycle checkpoints, DNA repair/recombination, and programmed cell death. Members of the Bcl-2 family are important agonist/antagonist regulators of apoptosis. In parallel with its canonical anti-apoptotic role, the involvement of Bcl-2 in genome instability has now emerged from a number of studies. Bcl-2 inhibits several DNA repair mechanisms involved in maintaining genome stability, including sensitivity of DNA synthesis to UV-C, base excision repair (BER), mismatch repair (MMR), homologous recombination (HR), and nonhomologous end joining (NHEJ; refs. 1–7). Consistent with defect in error-free repair systems, Bcl-2 expression leads to high levels of mutagenesis induced by a wide variety of genotoxic stresses (2, 8, 9). However, no unifying model can be proposed. Indeed, Bcl-2 affects different DNA repair processes (e.g., MMR, BER, and NHEJ) by different mechanisms. Moreover, none of these mechanisms accounts for HR repression by Bcl-2, which we discuss further in this article.

HR is a fundamental, evolutionarily conserved process that plays a pivotal role in controlling genomic plasticity. It is crucial for the recovery of blocked replication forks as well as for DNA repair [e.g., double-strand break (DSB) repair, interstrand cross-link repair], DNA damage tolerance, meiosis, molecular evolution, and genetic diversity or rearrangement. The repression of HR by Bcl-2 could have important consequences for essential biological processes. Thus, it is essential to determine the mechanisms by which Bcl-2 affects HR.

Bcl-2 localizes to mitochondrial, endoplasmic reticulum (ER), and external nuclear envelope membranes through its C-terminal, α-helical transmembrane (TM) domain (10, 11). In this study, we analyzed the role of the Bcl-2 TM domain in HR. Importantly, we identified the tumor suppressor BRCA1 as an essential molecular target of Bcl-2 for HR repression. Notably, BRCA1 is the most frequently mutated gene in hereditary breast or ovarian cancer and is essential for HR (12).

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Materials and Methods

DNA manipulations

All DNA manipulations were done as described previously (13).

Cell cultures

The cell line RG37, which contains a single, chromosomally integrated copy of the recombination reporter plasmid p*DR-GFP*, has already been described (4). RG37 and HeLa cells were cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal calf serum (FCS) and 2 mmol/L glutamine. Lymphoblastoid and H460 cell lines were cultured in RPMI 1640 supplemented with 20% FCS and 2 mmol/L glutamine. Lymphoblastoid cells GM3657, CRL-2261, and CRL-2632 were obtained from the American Type Cell Culture in 2007; Priess was provided by Dr. D. Papadopoulo (Curie Institute, Paris, France). Translocations t(14:18) in CRL-2261 and CRL-2632 were characterized by cytogenetics and expression of Bcl-2 detected by Western blot assay. H460 cells were provided by Dr. E. Deutsch (Institut Gustave Roussy, Villejuif, France) in 2010, and HeLa cells were historically present in the laboratory. All these cell lines were tested for endogenous Bcl-2 expression by Western blot analysis, and the last test was done during the revision process.

Western blot analysis

Western blot analysis was carried out using anti-Bcl-2 (sc-509; Santa-Cruz), anti-c-Myc (sc-40; Santa Cruz) or anti-actin (Sigma-Aldrich) antibodies. Antibodies were visualized using an ECL Detection Kit (Amersham Biosciences).

Recombination assays

Recombination induced by I-SceI was measured in RG37 cells. These cells were transfected with jetPEI reagent with I-SceI. To maintain similar DNA concentrations among all conditions, control cells were transfected with an empty expression vector 72 hours post transfection, and the cells were fixed in PBS/2% paraformaldehyde (PFA) for 15 minutes at room temperature.

Immunofluorescence analysis

For all immunofluorescence experiments, cells were washed in PBS, fixed with PBS/2% PFA for 15 minutes at room temperature and then permeabilized for 10 minutes in PBS/0.1% saponin. After 30 minutes of saturation in PBS/2%–BSA/0.05%–Tween-20 at room temperature, the cells were incubated with the primary antibodies for 2 hours at 37°C in PBS/0.5% BSA/0.05% Tween-20 and then for 1 hour with the secondary antibodies (Cy2-, TRITC-, or Alexa-coupled secondary antibodies; Molecular Probes). After washing in PBS/0.05% Tween-20, and subsequent 4′,6-diamidino-2-phenylindole (DAPI) staining, the slides were analyzed by fluorescence microscopy. For mitochondrial detection, the cells were either stained with an anti-COX IV antibody (1/100, 20E8; Abcam) or Mito-tracker (Invitrogen), 50 nmol/L for 30 minutes. For ER detection, cells were either stained with ER-tracker 600 nmol/L for 30 minutes or an anti-calnexin antibody. For Bcl-2 detection, the cells were stained with a monoclonal anti-Bcl-2 antibody (1:1,000; sc-509, Santa Cruz) or a polyclonal anti-Bcl-2 antibody (1:1,000, 554160; Becton Dickinson Pharmingen). For BRCA1 foci analysis, the cells were stained with anti-BRCA1 (1:100; C-20, Santa Cruz) or a mouse anti-BRCA1 (1:50; Bioscience) antibody after γ-irradiation and 10 hours of recovery. Cells were imaged with a fluorescent microscope (Olympus BX51 with a camera Spot inside color 320) using a 63× lenses. Picture acquisition was done at room temperature with Spot Advanced 4.3 software and processed with ImageJ software. At least 50 cells were counted in 3 or more independent experiments. The percentage of cells is reported as the mean ± SD. The scale bars represent 10 μm.

Coimmunoprecipitation

Cells were washed in ice-cold PBS and suspended in modified radioimmunoprecipitation assay (RIPA) buffer (20 mmol/L, Tris–HCl, pH 7.5, 150 mmol/L NaCl, 0.5% NP-40, 1% sodium deoxycholate) supplemented with 1 tablet of protease inhibitor cocktail (Complete Mini; Roche) and with phosphatase inhibitor cocktails (Sigma). Homogenates were incubated on ice for 1 hour before centrifugation at 12,000 × *g* for 30 minutes. Freshly prepared cell lysates were diluted in modified RIPA buffer and were precleared with Dynabeads protein G (50 μL per 500 μL lysate) for 1 hour at 4°C. Flag antibodies (ref. F3165; Sigma) were linked to Dynabeads protein G (1 μg antibody per 50 μL beads) for 1 h at 4°C. The cleared lysate was then incubated with the antibody-linked beads and complexes formed between Bcl-2 and BRCA1 were captured for 1 hour at 4°C. Beads were washed 4 times with modified RIPA buffer. Protein complexes were uncoupled from the beads and denatured (70°C for 10 minutes) in 3-(N-morpholino)propanesulfonic acid, sodium dodecyl sulfate sample buffer with reducing agent (Invitrogen).

In situ proximity ligation assay

Anti-BRCA1 and anti-Bcl-2 antibodies were used as described above for immunodetection analysis. The *in situ* proximity ligation assay (PLA; refs. 14, 15) was conducted using the DuoLink Kit (OLINK Bioscience) according to the manufacturer's instructions.

Confocal microscopy

Image acquisition was done with a Leica DMRxA2 confocal microscope SPE (Leica Microsystems) using ACS APO 63× 1.3 OIL lenses. Images were processed with Leica and ImageJ software programs.

Subcellular fractionation

Subcellular protein extraction was done using the ProteoExtract Subcellular Proteome Extraction Kit (Calbiochem) according to the manufacturer's instructions provided by the manufacturer.

Electron microscopy

Immunogold labeling of BRCA1 and Bcl-2 was done on ultrathin cryosections (Tokoyasu's method), as previously reported (16). The primary antibody (rabbit polyclonal;

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1:200 dilution for anti-Bcl-2 and 1:50 dilution for anti-BRCA1) was applied for 1 hour in BSA/cacodylate medium, followed by 3 washes of 5 minutes each. Protein-A gold (10 nm) was used for labeling. The sections were washed in water before staining in 2% uranyl acetate for 5 minutes, followed by a 10-minute incubation period in methylcellulose–uranyl acetate. After drying, the samples were analyzed using a Philips CM12 electron microscope (16).

Results

The TM domain of Bcl-2 is required for inhibition of HR

The carboxyl-terminus of Bcl-2 contains the TM domain, which is necessary for the anchoring of Bcl-2 to intracellular membranes, such as the ER and outer mitochondrial membranes. Such membrane anchoring is essential for the role of Bcl-2 in apoptosis regulation (17). To test whether the TM domain of Bcl-2 plays a role in the inhibition of gene conversion, we deleted this domain (Fig. 1A). Two different forms of Bcl-2 were expressed in the RG37 cell line. This cell line contains a single, chromosomally integrated copy of a substrate that specifically monitors gene conversion induced by double-stranded cut targeted within the recombination substrate by the meganuclease I-SceI (18; Fig. 1B). The impact of Bcl-2 family members on HR has already been characterized in these cells (4). We used ^{G145A}Bcl-2 as the full-length Bcl-2 control because, although it cannot inhibit apoptosis, it retains its anti-HR function at wild-type (WT) levels (2, 4). This form allowed us to focus on the anti-recombination role of Bcl-2 while avoiding potential interference from apoptosis regulation. As expected, ^{G145A}Bcl-2 was colocalized with the mitochondrial or ER network, unlike Bcl-2ΔTM (Fig. 1C). Notably, there is intense labeling of Bcl-2ΔTM in the nucleus. After cotransfection of I-SceI and the Bcl-2 forms, the cells were stained with an anti-Bcl-2 antibody, and the number of green fluorescent protein (GFP)-positive cells was measured (to estimate levels of gene conversion, i.e., HR) among those cells expressing Bcl-2 (Fig. 1D). This analysis confirms the repression of gene conversion by ^{G145A}Bcl-2 expression. Notably, overexpression of Bcl-2 did not alter cell cycle distribution (Supplementary Fig. S1), confirming that the repression of HR by Bcl-2 is independent of its impact on the cell cycle, as already shown (2). Interestingly, Bcl-2ΔTM failed to inhibit gene conversion (Fig. 1D), showing that the TM domain is required for HR repression.

To test the specificity of Bcl-2 for HR inhibition, we fused the TM domain of Bcl-2 to an enhanced cyan fluorescent protein (eCFP; Supplementary Fig. S2). Whereas WT eCFP exhibits a diffuse localization throughout all cell compartments, eCFP-TM exhibits a cytoplasmic localization with a pattern characteristic of the membrane network. Neither eCFP protein was found to affect gene conversion (Supplementary Fig. S2B).

These data show that the TM domain of Bcl-2 is required, but not sufficient by itself, for HR repression, indicating the specificity of Bcl-2 for HR suppression.

Bcl-2 inhibits the formation of BRCA1 foci

The tumor suppressor BRCA1, which plays key roles in the response to DNA damage, is essential for HR regulation (19, 20). In response to DNA damage, BRCA1 assembles into nuclear foci, and these structures appear to be a prerequisite for efficient HR. Monitoring the formation of BRCA1 foci is frequently used to estimate DNA repair/recombination in different situations, including in tumor cells (21–26). Because Bcl-2 impacts HR, we investigated the effects of Bcl-2 on the formation of endogenous BRCA1 foci induced by ionizing radiation (IR). In control cells, BRCA1 efficiently assembled into nuclear foci following irradiation (Fig. 2A and B). In contrast, in Bcl-2-transfected cultures, cells expressing Bcl-2 failed to assemble BRCA1 foci (Fig. 2A and B and Supplementary Fig. S3A). Importantly, expression of Bcl-2ΔTM did not impair BRCA1 foci formation; this shows that the TM domain is required for suppression of BRCA1 foci formation. These data establish BRCA1 as a molecular target of Bcl-2.

BRCA1 overexpression compensates for the negative effects of Bcl-2 on HR

The aforementioned data suggested that the BRCA1 pathway is affected by Bcl-2, resulting in a decrease in HR. In the RG37 cell line used in this study, we have previously shown that overexpression of BRCA1 stimulates HR efficiency (23). In this study, we confirmed that cotransfection of BRCA1 with I-SceI led to a significant stimulation of HR (Fig. 2C). This strategy allowed us to focus specifically on BRCA1-controled HR and the impact of Bcl-2 on this pathway. When cotransfected with BRCA1, Bcl-2 significantly reduced the frequency of HR compared with cells overexpressing BRCA1 alone, whereas Bcl-2ΔTM had no effect (Fig. 2C). These data confirm that Bcl-2 affects BRCA1-induced HR.

Nonetheless, BRCA1 overexpression increased HR frequency even when Bcl-2 was expressed (Fig. 2C). This reveals that increasing the amount of BRCA1 can saturate Bcl-2 and limit HR repression. However, this bypass remains partial, as the levels of HR did not reach those obtained in the absence of Bcl-2. These data suggest that a stoichiometric-like equilibrium between the amounts of BRCA1 and Bcl-2 proteins regulates the efficiency of HR.

Impact of endogenous Bcl-2 expression on BRCA1 foci in cancer cells

To assess the aforementioned data in a physiopathologic context, we first analyzed lymphoma cells that spontaneously exhibit very high levels of endogenous Bcl-2 due to a t(14:18) translocation (Fig. 3A). Endogenous BRCA1 efficiently assembles into nuclear foci after IR in WT cells, but not in the 2 independent t(14:18) lymphoma cell lines analyzed (Fig. 3B and Supplementary Fig. S3B). HR and NHEJ are the 2 main DNA DSB repair processes. Interestingly, the 2 t(14:18) lymphoma cell lines, which constitutively overexpress Bcl-2, exhibited strong spontaneous γ-H2AX labeling compared with WT cells, which is consistent with a general deficiency in DSB repair (Supplementary Fig. S4A).

Many cancer cells express Bcl-2, but, in general, to a lower level than t(14:18) lymphoma cells. However, because the final impact on HR results from the relative amounts of BRCA1 and

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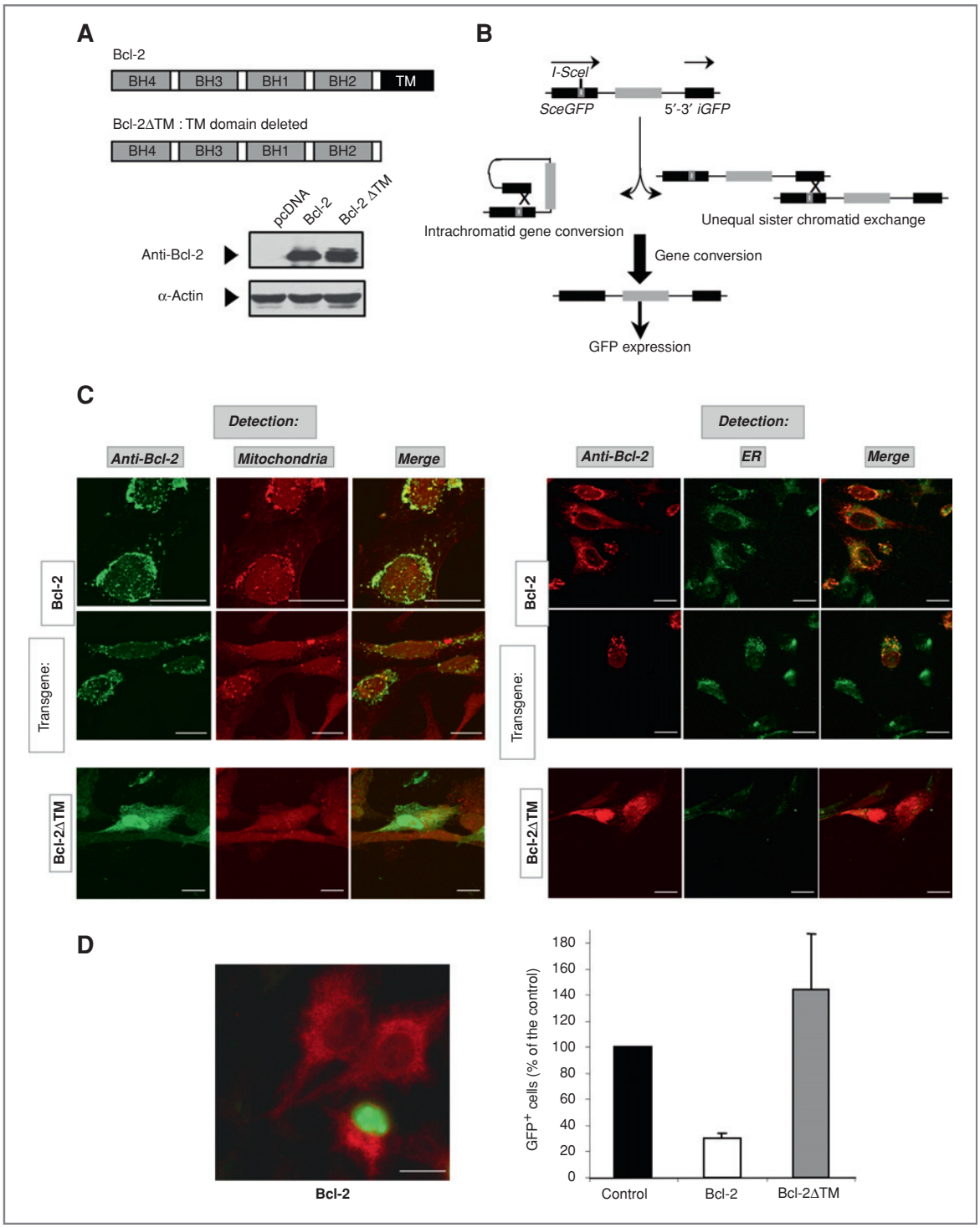

Figure 1. A, schematic representation and detection of Bcl-2 and Bcl-2ΔTM in RG37 cell extracts. pcDNA corresponds to transfection with an empty expression vector. B, recombination substrates. RG37 immortalized fibroblasts contain 1 copy of the p*DR-GFP* substrate and a tandem repeat of 2 inactive eGFP genes—*SceGFP* and *iGFP*. When the *I-SceI* endonuclease is expressed, a DSB is introduced at the *I-SceI* site in the *SceGFP* gene. Recombination restores a functional *eGFP* gene, and cells can then be monitored using fluorescence detection methods (18). C, localization of Bcl-2 and Bcl-2ΔTM in RG37 cells. Cells were stained with an anti-Bcl-2 antibody. Mitochondrial staining was done using an anti-COX IV antibody (left), and ER staining was carried out with an anti-Calnexin antibody (right). D, effects of the TM domain of Bcl-2 on I-SceI–induced HR. eGFP-positive recombinant cells were detected by fluorescent microscopy. Left, an example of Bcl-2-expressing cells (red) also expressing eGFP (green) after a recombination event. Right, recombination frequency relative to control cells (transfected with HA-I-SceI and an empty expression vector). Transgenes cotransfected with HA-I-SceI are indicated, and the values correspond to the means of at least 3 independent experiments.

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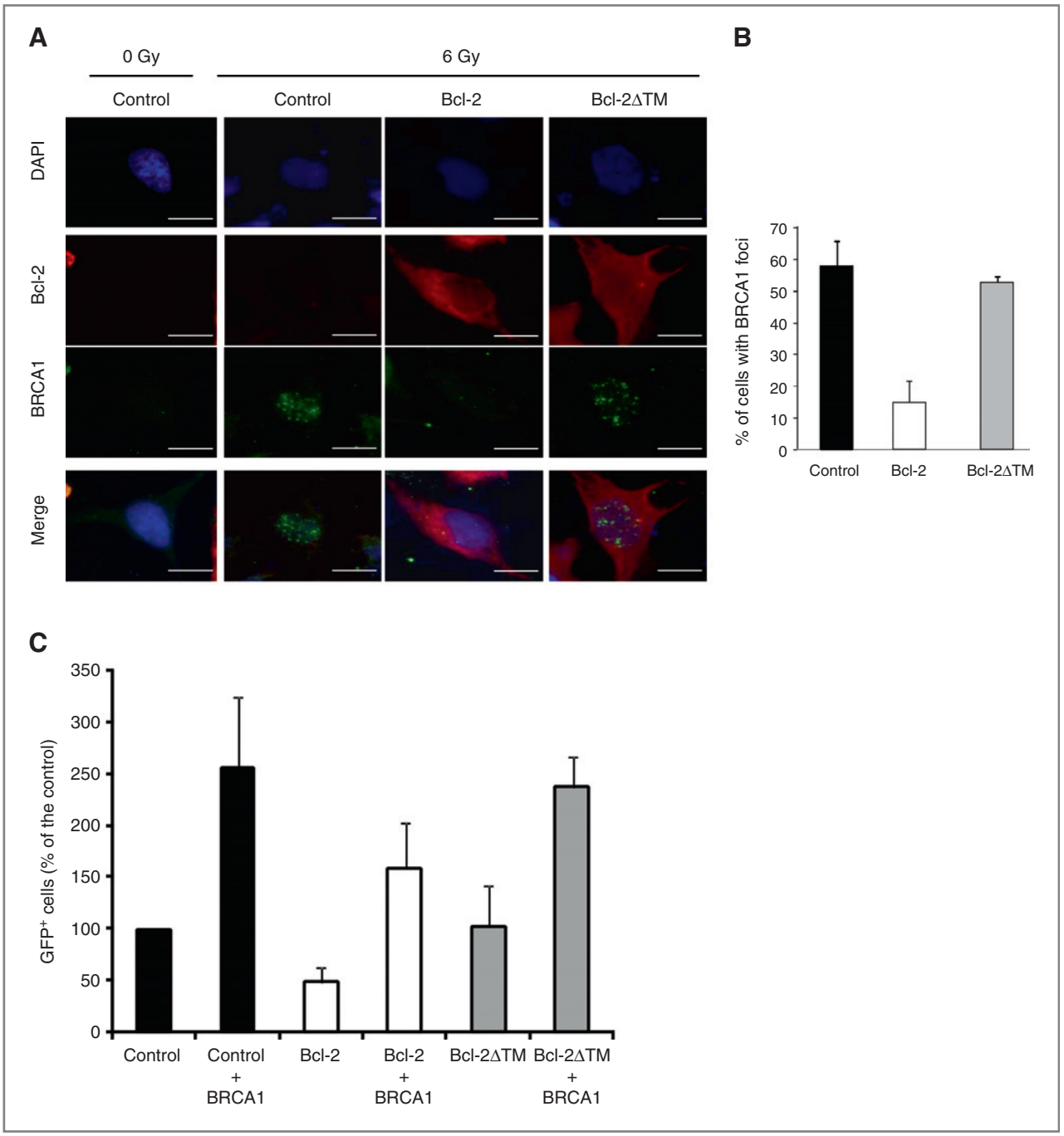

Figure 2. Bcl-2 affects BRCA1 foci formation induced by IR. A, human RG37 cells were transfected either with Bcl-2 or Bcl-2ΔTM expression plasmids, or with empty pcDNA3puro plasmid (control), and then irradiated. The images shown represent endogenous BRCA1 foci visualized by immunofluorescence after immunostaining with an anti-Bcl-2 antibody (red) and an anti-BRCA1 antibody (green). B, percentage of cells with BRCA1 foci. The values correspond to the means of at least 3 independent experiments. C, impact of BRCA1 overexpression on HR, with or without Bcl-2 or Bcl-2ΔTM.

Bcl-2 proteins (see Fig. 2C), an intermediate response is expected. We used HeLa and H460 cells in which Bcl-2 is expressed to detectable levels (Fig. 3C). Interestingly, silencing Bcl-2 (Fig. 3C) stimulated the formation of IR-induced BRCA1 foci 3- and 6-fold in HeLa and H460 cells, respectively (Fig. 3D). These data confirm that endogenous Bcl-2 expression represses the assembly of BRCA1 foci after irradiation. Notably, the presence of Bcl-2 did not impair the efficient activation of ATM and p53 phosphorylation in $p53^{+}$ H460 cells (Supplementary Fig. S5).

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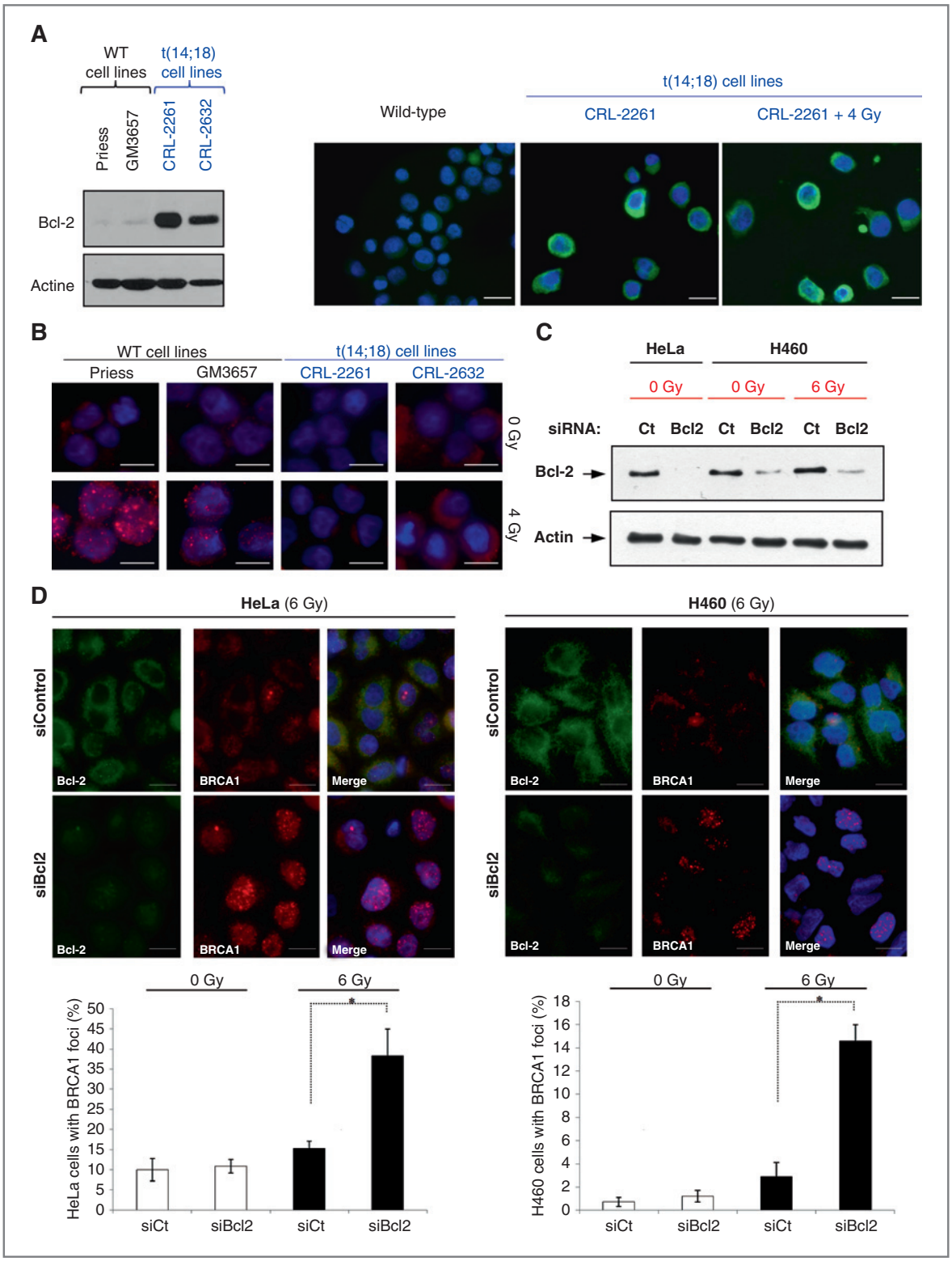

Figure 3. IR-induced BRCA1 foci in cancer cells. A, expression of endogenous Bcl-2 in 2 lymphoblastoid cell lines from patients with t(14:18) translocation versus 2 control lymphoblastoid cell lines. Left, Western blot using an anti-Bcl-2 antibody. Right, immunofluorescence detection of Bcl-2 in WT cells (left), without IR (middle), or after irradiation (right). Nuclei were labeled using DAPI. B, IR-induced BRCA1 foci in the 2 control and 2 t(14:18) cell lines. Top, nonirradiated cells. Bottom, cells after irradiation (4 Gy). C, expression of Bcl-2 in HeLa and H460 cells and efficiency of siRNA extinction. D, impact of Bcl-2 silencing on IR-induced BRCA1 foci in HeLa (left) and H460 (right) cells.

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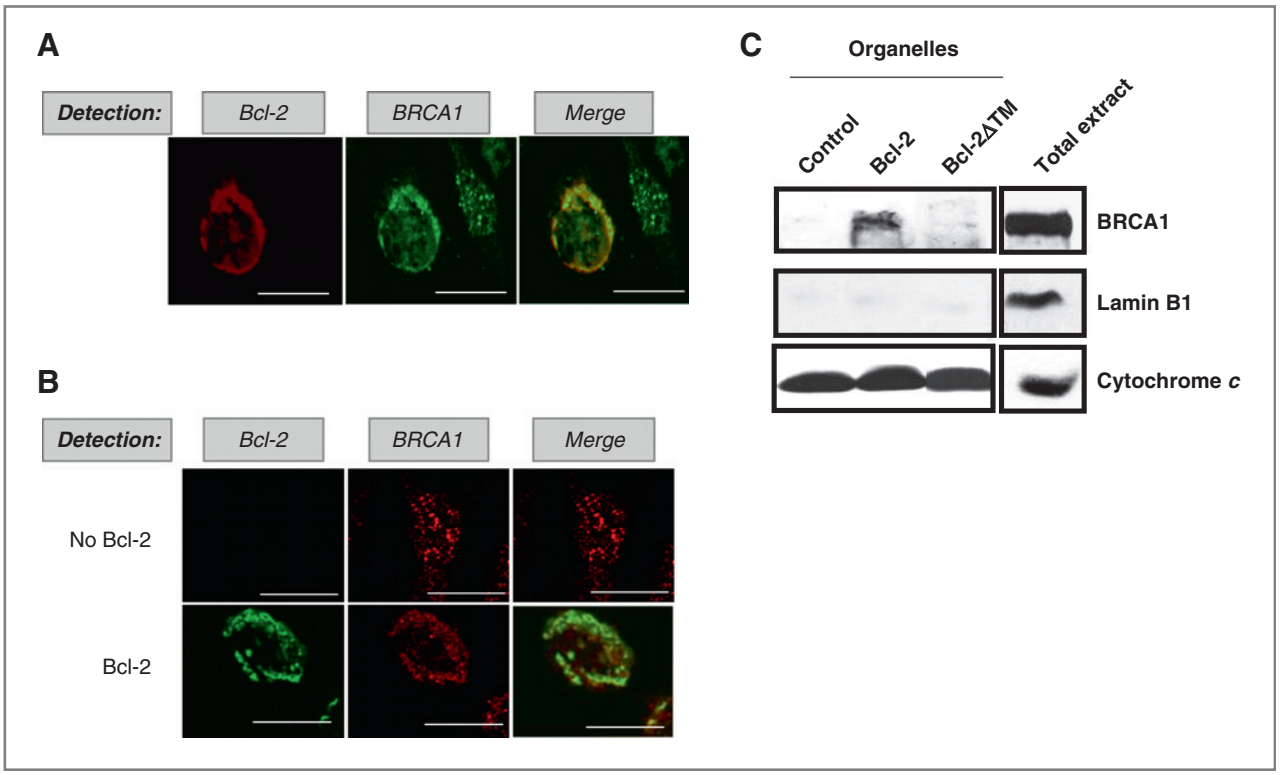

Figure 4. Colocalization of endogenous BRCA1 and expressed Bcl-2 in cytoplasmic organelles (RG37 cells). A, example of colocalization of Bcl-2 (red) and endogenous BRCA1 (green) after IR. (Note the BRCA1 foci in the cell that did not express Bcl-2.) B, confocal microscopic analysis of endogenous BRCA1 and expressed Bcl-2. Note the formation of IR-induced BRCA1 foci in the absence of Bcl-2 (top). C, detection of BRCA1 by Western blotting in the organelle fraction. The purity of the fraction was confirmed by detection of lamin B1 (nuclear marker) or cytochrome *c* (cytoplasmic marker).

BRCA1 colocalizes with Bcl-2 in cytoplasmic membranes

In the course of the above experiments, we obtained immunofluorescence data suggesting that endogenous BRCA1 colocalized with expressed Bcl-2 (Fig. 4A). This prompted us to establish the colocalization of Bcl-2 and endogenous BRCA1 by confocal microscopic analysis in RG37 cells (Fig. 4B). After IR, we confirmed the formation of endogenous IR-induced BRCA1 nuclear foci in the absence of Bcl-2. Interestingly, endogenous BRCA1 colocalized with Bcl-2 in the cytoplasmic subcellular structures (Fig. 4B).

Given that Bcl-2 localizes to the mitochondrial and ER membranes (see Fig. 1), the data above imply that BRCA1 should also localize to endomembranes. To test this hypothesis, we analyzed subcellular fractions by Western blotting (Fig. 4C). In nontransfected control cells, BRCA1 was undetected in the organelle fraction. In contrast, a significant amount of BRCA1 was present in the organelle fraction when Bcl-2 was expressed (Fig. 4C). Importantly, expression of Bcl-2ΔTM did not result in the detection of BRCA1 in the organelle fraction. These data show that expression of Bcl-2 directs BRCA1 to organelle membranes in a process requiring the TM domain of Bcl-2, consistent with the inhibition of BRCA1 foci assembly and HR.

To identify the subcellular structure containing BRCA1 following Bcl-2 expression, we carried out immunogold labeling for electron microscopy. In the absence of Bcl-2, BRCA1 was primarily localized to the nucleus and could not be detected in cellular membranes (Fig. 5A). In contrast, full-length Bcl-2 was localized to the ER and mitochondrial membranes and was excluded from the nucleus. Bcl-2 expression resulted in intracellular membrane aggregation, forming characteristic structures called organized smooth endoplasmic reticulum (OSER) that contained large amounts of Bcl-2 protein (Fig. 5B). However, Bcl-2 was also present in membranes not involved in OSER. In contrast, Bcl-2ΔTM did not localize to membranes and did not stimulate OSER formation (Fig. 5C). Furthermore, we found Bcl-2ΔTM in inclusion bodies and in the nucleus, as shown by immunofluorescent labeling (see Fig. 1C). Finally, Bcl-2ΔTM molecules were frequently clustered, showing that this protein retains its capacity to interact with partners, as expected, due to the presence of the BH domains.

Importantly, when full-length Bcl-2 was expressed, BRCA1 was clearly localized to membranes, including those of the ER, mitochondria, and OSERs (Fig. 6A). In contrast, when Bcl-2ΔTM was expressed, BRCA1 did not localize to endomembranes (Fig. 6B).

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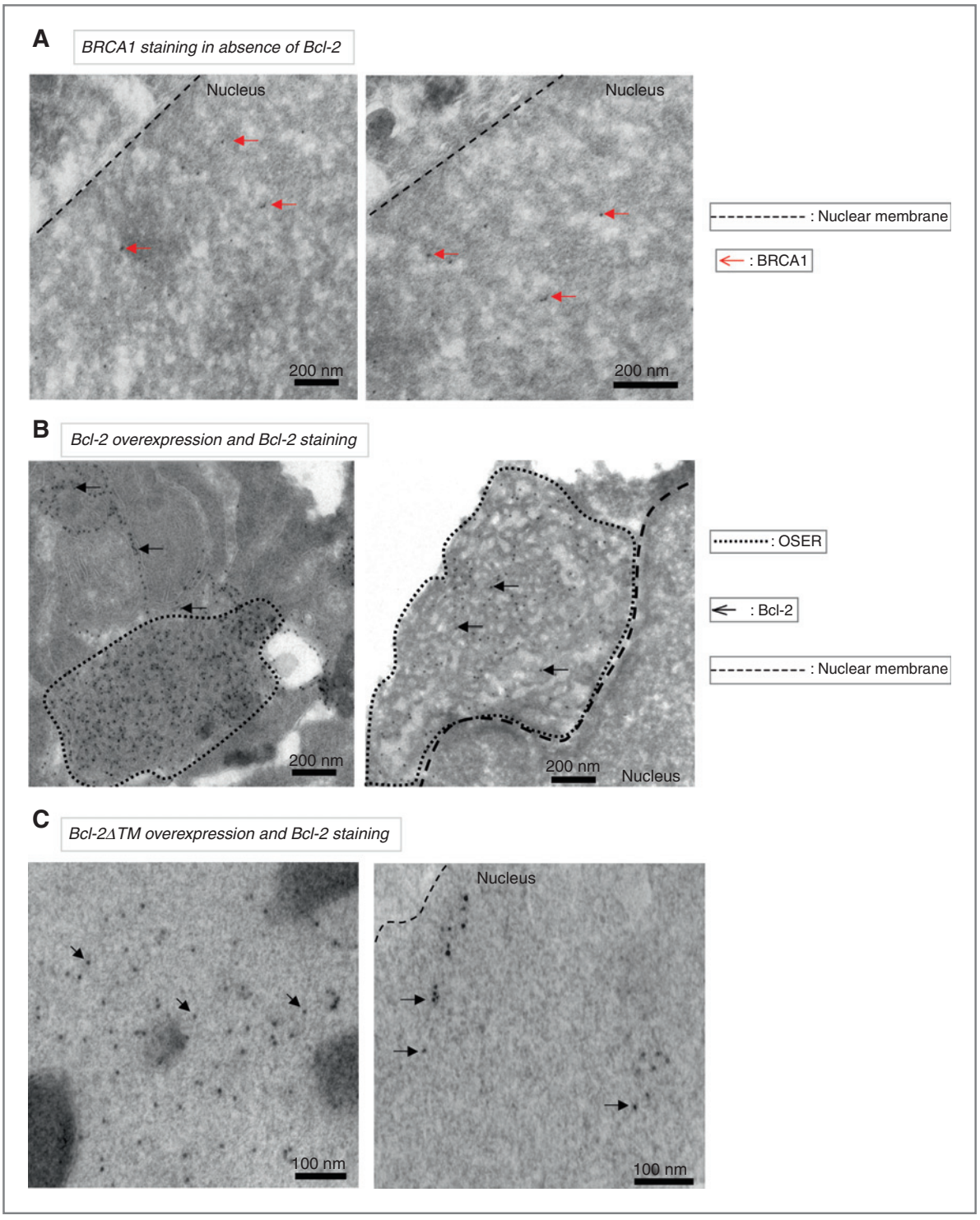

Figure 5. BRCA1 and Bcl-2 immunogold labeling for electron microscopy. A, detection of BRCA1 in the absence of Bcl-2. B, detection of Bcl-2. OSERs (stacked ER membranes) are within dotted lines. C, detection of Bcl-2ΔTM.

These data show that the TM domain of Bcl-2 is required both to assemble OSERs and to localize BRCA1 to membranes.

In situ interaction of Bcl-2 and BRCA1

To address the question of a Bcl-2–BRCA1 *in situ* interaction, we used the PLA, which allows the *in situ* detection of

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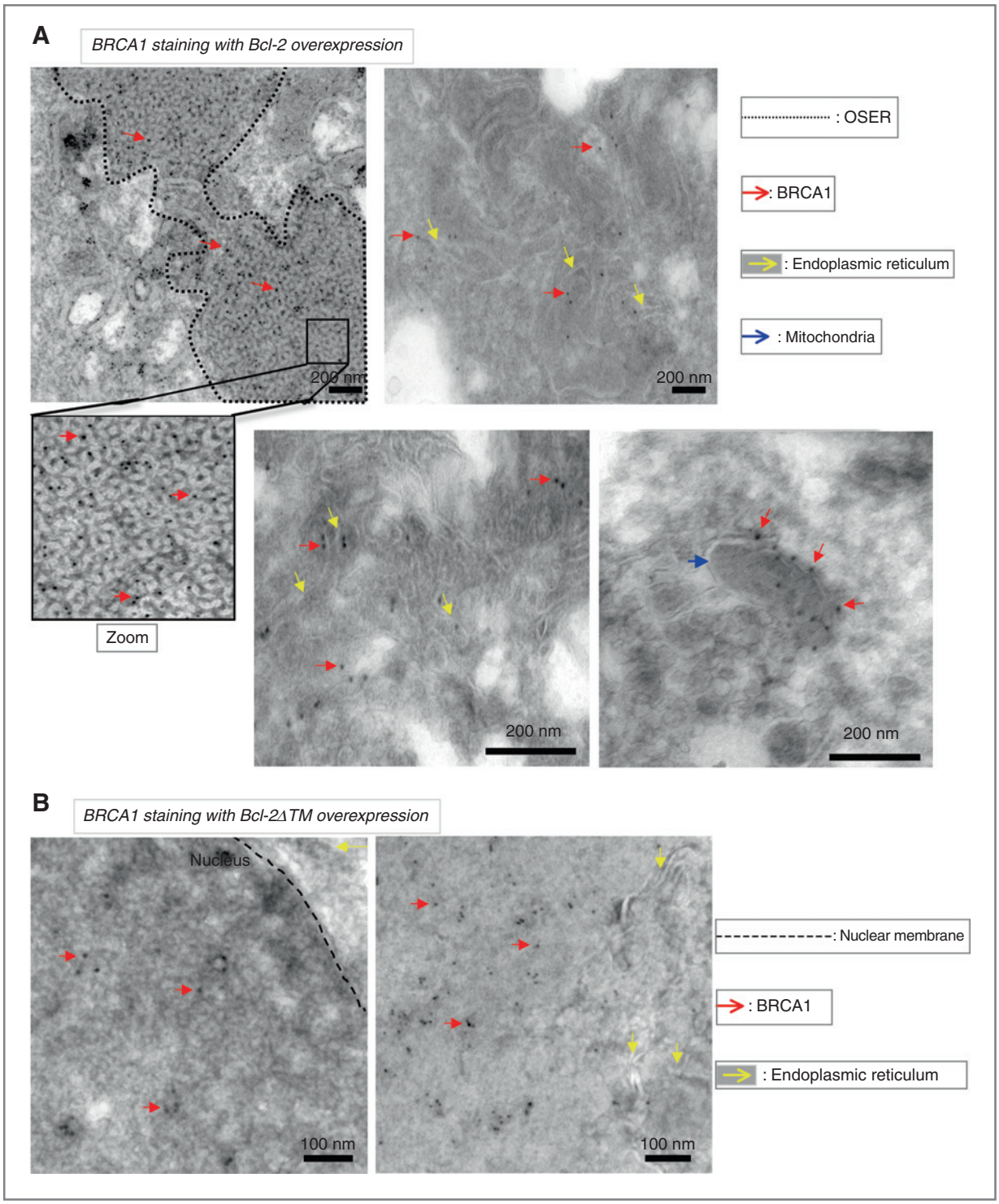

Figure 6. A, detection of BRCA1 in the presence of Bcl-2. Left, BRCA1 located in OSER (within dotted lines). Right, BRCA1 outside OSERs but localized within endomembranes. Bottom left, BRCA1 outside OSERs and localized within mitochondria. B, detection of BRCA1 with Bcl-2ΔTM overexpression.

physical protein interactions by immunofluorescence microscopy (14, 15). We first analyzed the *in situ* Bcl-2–BRCA1 proximity/interaction in the RG37 cell line, in which HR experiments were carried out. Because endogenous levels of both BRCA1 and Bcl-2 are very low in these cells (23), we overexpressed both proteins; additionally, this strategy gave us the opportunity to analyzed engineered Bcl-2 proteins, such as Bcl-2ΔTM.

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In nontransfected cells, although BRCA1 and Bcl-2 were both poorly expressed, PLA labeling was detected, showing an *in situ* cytoplasmic interaction between endogenous WT Bcl-2 and BRCA1 (Fig. 7A). Expression of exogenous transgenes strongly increased the PLA signal, mostly in the cytoplasm, supporting the fact that Bcl-2 and BRCA1 are members of a common complex. Interestingly, Bcl-2ΔTM also maintained *in situ* interaction/proximity with BRCA1, consistent with the fact that Bcl-2ΔTM retains protein association capacity (see Fig. 5). Because BRCA1 and Bcl-2 interact at the endomembrane, physical interaction would be difficult to analyze by classical immunoprecipitation methods because extraction of soluble proteins from the membranes requires techniques that generally disrupt protein complexes. However, because BRCA1 appears to also interact with Bcl-2ΔTM outside endogenous membranes (see Figs. 1, 5, and 7A), coimmunoprecipitation analysis becomes feasible using this particular Bcl-2 construct. Indeed, in RG37 cells, immunoprecipitation of BRCA1 resulted in coprecipitation of Bcl-2ΔTM (Fig. 7B). No Bcl-2 signal was seen when cells were transfected with an empty expression vector (the faint band seen in the immunoprecipitated fraction corresponds to a nonspecific signal). In contrast, when Bcl-2ΔTM was expressed, a strong Bcl-2 expression was observed in the extract. Importantly, the Bcl-2–specific band was present in the fraction precipitated with BRCA1 and absent in the preclear fraction (Fig. 7B). These data confirm the physical interactions between BRCA1 and Bcl-2 observed *in situ* by PLA (see Fig. 7).

We then analyzed endogenous WT Bcl-2 and BRCA1 in the physiopathologic model of the t(14:18) lymphomas (Fig. 7C). A small but significant signal was seen in the WT cells, consistent with the faint band of Bcl-2 detected by Western blotting (see Fig. 3A); importantly, the signal increased in the t(14:18) lymphoma cells.

Finally, we analyzed the *in situ* interaction of endogenous WT BRCA1 and Bcl-2 proteins in HeLa and H460 cells and their localization to the ER and the mitochondria (Fig. 7D). Mitochondria were labeled by Mito-tracker, and the ER was labeled by ER-tracker. Bcl-2–BRCA1 PLA was carried out in cells labeled with both Mito-tracker and ER-tracker and analyzed by confocal analysis (Fig. 7D). Most positive PLA signals colocalized with either mitochondria or ER staining (Fig. 7D and Supplementary Fig. S6).

Discussion

Bcl-2 regulates HR via a novel mechanism

Bcl-2 inhibits different DNA repair systems by different mechanisms; however, none of these mechanisms can account for HR repression. Bcl-2 inhibits MMR by a pathway involving the E2F transcription factor (3) and by directly affecting MSH2–MSH6 heterodimer formation (27). Consequently, the mutant ^{Y28A}Bcl-2 fails to repress MMR. In contrast, ^{Y28A}Bcl-2 does repress HR (2). In addition, the effect of Bcl-2 on HR cannot result from MMR inhibition because this would, conversely, stimulate HR (28).

Bcl-2 inhibits BER via c-Myc regulation (5) and by altering the interaction between APE1 and XRCC1 (6). We analyzed the expression of Myc following the transfection of different forms of Bcl-2 (Supplementary Fig. S7). In these experiments, we confirmed the stimulation of Myc expression following Bcl-2 transfection. Although Bcl-2ΔTM seemed to stimulate Myc expression less efficiently, it still substantially increased Myc expression without affecting HR efficiency. This suggests that HR repression and Myc stimulation are not correlated.

It has been reported that Bcl-2 inhibits NHEJ via the interaction and titration of KU70 following a massive nuclear translocation of Bcl-2 after IR exposure (7). This cannot account for HR repression by Bcl-2. First, the inhibition of NHEJ should stimulate HR (29–31). Second, in our systems, we did not observe massive translocation of Bcl-2 into the nucleus following IR in transfection experiments or in the physiopathologic t(14:18) lymphoma model (see Figs. 2C and 3A). Finally, Bcl-2 localization to cytoplasmic membranes is essential for HR repression.

In the present work, we identified important aspects of the regulation of HR by Bcl-2, including the requirement for the TM domain of Bcl-2 for HR repression and BRCA1 as a molecular target. The impact of Bcl-2 on HR appeared to be dependent on the relative amounts of Bcl-2 and BRCA1 proteins. These data argue in favor of a process involving physical interaction rather than regulation by signaling, which should amplify the signal and, thus, should not be regulated in a stoichiometric way. Consistent with this hypothesis, we describe a novel mechanism of BRCA1 regulation by Bcl-2 that involves the *in situ* interaction of Bcl-2 and BRCA1 in cell endomembranes, resulting in sequestration of BRCA1 outside the nucleus.

Bcl-2 represses HR via retention of BRCA1 in organelles

Our data show that membrane localization of Bcl-2 results in BRCA1 mislocalization and inhibition of nuclear HR; furthermore, they strongly support the idea that Bcl-2 and BRCA1 interact at the membranes. Remarkably, membrane localization is essential for the canonical anti-apoptotic role of Bcl-2. Localization of BRCA1 to cytoplasmic organelles has been reported, specifically in the perinuclear compartment of the ER–Golgi complex, in invaginations of the nucleus (32), and in the mitochondrial matrix (33). In this study, we showed that BRCA1 localizes to both ER and external mitochondrial membranes when full-length Bcl-2 is expressed. Interestingly, it has recently been shown that DNA-damage induced cytotoxicity is dependent on cytosolic BRCA1 localization (34). Consistent with these data, BRCA1 has been shown to facilitate apoptosis induced by DNA-damaging agents, through its cytosolic p90, caspase3-induced fragment (35). These data are, thus, highly consistent with those presented here. Reciprocally, our data provide molecular support to these observations. Indeed, the Bcl-2 family members play a pivotal role in the control of apoptosis. Therefore, it is tempting to speculate that the cytosolic form of BRCA1 can facilitate apoptosis through its interactions with the Bcl-2 family members, constituting an additional tumor suppressor role for BRCA1.

In contrast to the effects observed for Bcl-2ΔTM, expression of full-length Bcl-2 results in the formation of aggregated membrane structures termed OSERs. Such structures were

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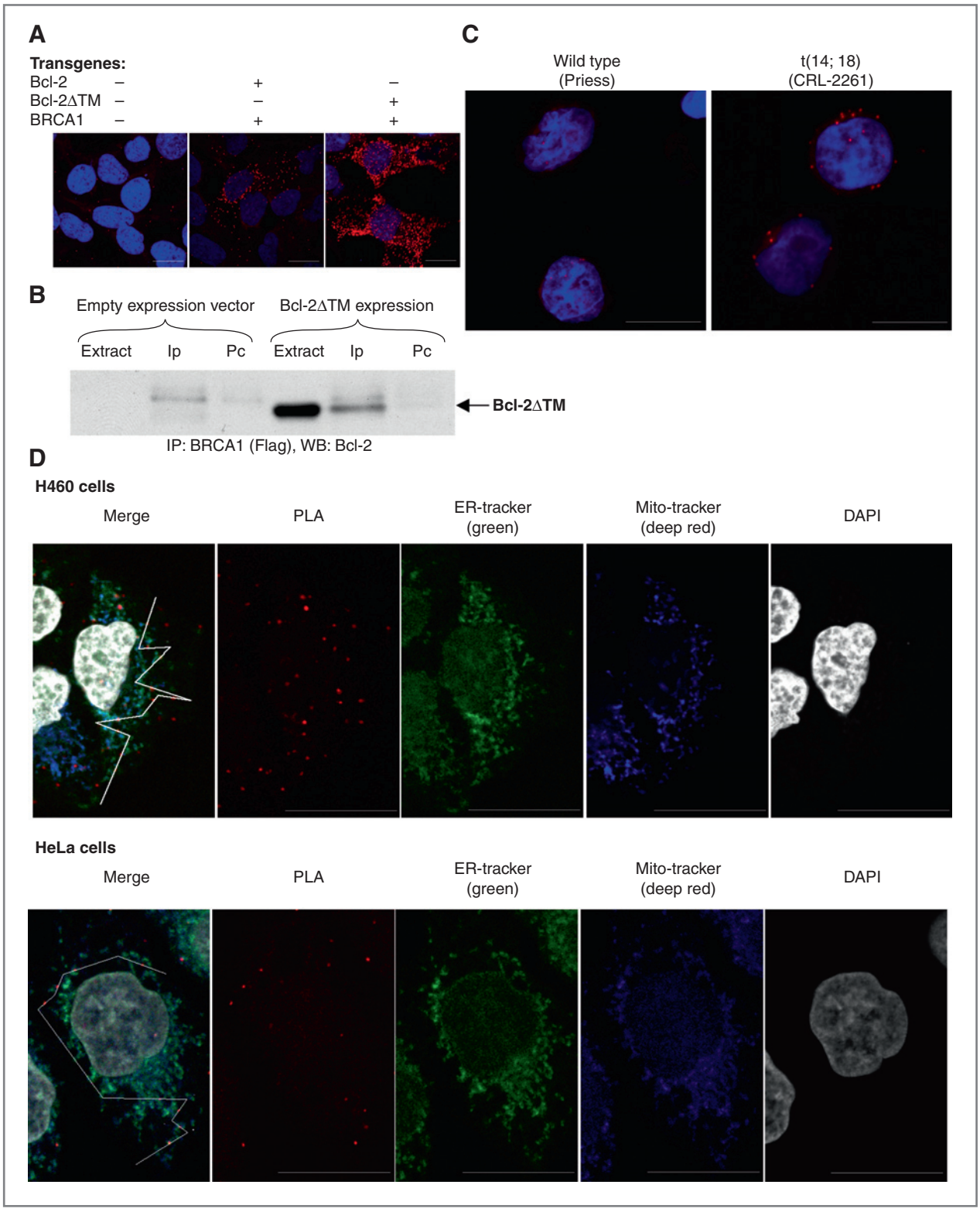

Figure 7. A, *in situ* PLA in RG37 cells, after expression of the different transgenes. B, coimmunoprecipitation. BRCA1-Flag and Bcl-2ΔTM were coexpressed in RG37 cells and immunoprecipitation was conducted using an anti-Flag antibody. As a control, in the left part, the Bcl-2ΔTM vector was replaced by an empty expression vector. Ip, immunoprecipitated fraction; Pc, preclear. C, PLA on endogenous Bcl-2 and BRCA1 in lymphoblastoid cell lines, WT versus t(14:18). D, PLA on endogenous Bcl-2 and BRCA1 and colocalization with Mito-tracker or ER-tracker in H460 and HeLa cells. White lines on the merge panels correspond to the path for the fluorescence quantification and colocalization (Supplementary Fig. S6).

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first described following expression of an artificially engineered GFP fused to a TM domain (36). GFP exhibits self-aggregation properties; after anchoring to the membrane via the TM region, the interaction between 2 aggregating molecules leads to folding of the attached membrane and consequent formation of OSERs. Therefore, OSER formation reflects 2 protein properties: membrane anchoring and the capacity to aggregate with other membrane proteins. Members of the Bcl-2 family possess both properties. Moreover, OSER assembly has been reported upon overexpression of the BH3-only protein BNIP (37). In this article, we provide the first description of OSER formation resulting from Bcl-2 overexpression.

Nonetheless, while BRCA1 is present at high levels in OSERs, such structural formations are not required for localization of BRCA1 to endomembranes (Fig. 6). Our data support a model in which anchoring Bcl-2 to the membrane results in 2 independent consequences: aggregation with other Bcl-2 family members resulting in OSER formation and trapping of BRCA1 (which, incidentally, can be embedded in OSERs), resulting in inhibition of its nuclear function.

Impact on maintenance of genome stability and tumorigenesis

Affecting BRCA1 nuclear localization should alter several DNA repair pathways. Indeed, BRCA1 plays a pivotal role in the DNA damage response (DDR) and, thus, in the maintenance of DNA stability. It is involved both in HR and NHEJ (19, 38–40); in addition, it controls BER through the transcription control of *OGG*1, *NTH*1, and the *REF*1/*APE*1 genes (41, 42). This suggests that trapping of BRCA1 in the membrane should be sufficient to affect a large variety of DNA repair and DDR processes.

In mammalian cells, replication arrest produces DSBs, which can be processed by both HR and NHEJ (43). Thus, BRCA1 inhibition should lead to an accumulation of spontaneous DSBs resulting from endogenous and/or replication stress. Bcl-2 expression, via repression of both HR and NHEJ, should inhibit DSB processing and lead to DSB accumulation. Consistent with this conclusion, unchallenged t(14:18) cells, which express high levels of Bcl-2, show spontaneous, high levels of γ-H2AX foci (present data and ref. 44). The significance of these points is underscored by the fact that the presence of DSB and activation of DNA damage signaling are detected in precancerous tissues, interpreted as a consequence of endogenous replication stress, and proposed as initial events of tumorigenesis (45, 46).

BRCA1 plays a central role in the maintenance of genomic stability and is involved in fundamental biological processes, such as cell cycle control, X chromosome inactivation, and transcriptional regulation (47–49). By affecting BRCA1 functions, Bcl-2 targets a pivotal regulatory protein and may alter multiple pathways involved in DNA metabolism and genomic stability. Additionally, because BRCA1 is a tumor suppressor, these data support the idea that Bcl-2 exerts oncogenic functions in addition to its anti-apoptotic role.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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References

1. Liu Y, Naumovski L, Hanawalt P. Nucleotide excision repair capacity is attenuated in human promyelocytic HL60 cells that overexpress BCL2. Cancer Res 1997;57:1650–3.
2. Saintigny Y, Dumay A, Lambert S, Lopez BS. A novel role for the Bcl-2 protein family: specific suppression of the RAD51 recombination pathway. EMBO J 2001;20:2596–607.
3. Youn CK, Cho HJ, Kim SH, Kim HB, Kim MH, Chang IY, et al. Bcl-2 expression suppresses mismatch repair activity through inhibition of E2F transcriptional activity. Nat Cell Biol 2005;7:137–47.
4. Dumay A, Laulier C, Bertrand P, Saintigny Y, Lebrun F, Vayssière JL, et al. Bax and Bid, two proapoptotic Bcl-2 family members, inhibit homologous recombination, independently of apoptosis regulation. Oncogene 2006;3196–205.
5. Jin Z, May WS, Gao F, Flagg T, Deng X. Bcl2 suppresses DNA repair by enhancing c-Myc transcriptional activity. J Biol Chem 2006; 281:14446–56.
6. Zhao J, Gao F, Zhang Y, Wei K, Liu Y, Deng X. Bcl2 inhibits abasic site repair by down-regulating APE1 endonuclease activity. J Biol Chem 2008;283:9925–32.
7. Wang Q, Gao F, May WS, Zhang Y, Flagg T, Deng X. Bcl2 negatively regulates DNA double-strand-break repair through a nonhomologous end-joining pathway. Mol Cell 2008;29:488–98.
8. Cherbonnel-Lasserre C, Gauny S, Kronenberg A. Suppression of apoptosis by Bcl-2 or Bcl-xL promotes susceptibility to mutagenesis. Oncogene 1996;13:1489–97.
9. Kuo ML, Shiah SG, Wang CJ, Chuang SE. Suppression of apoptosis by Bcl-2 to enhance benzene metabolites-induced oxidative DNA damage and mutagenesis: A possible mechanism of carcinogenesis. Mol Pharmacol 1999;55:894–901.
10. Krajewski S, Tanaka S, Takayama S, Schibler MJ, Fenton W, Reed JC. Investigation of the subcellular distribution of the bcl-2 oncoprotein: residence in the nuclear envelope, endoplasmic reticulum, and outer mitochondrial membranes. Cancer Res 1993;53:4701–14.
11. Nguyen M, Millar DG, Yong VW, Korsmeyer SJ, Shore GC. Targeting of Bcl-2 to the mitochondrial outer membrane by a COOH-terminal signal anchor sequence. J Biol Chem 1993;268:25265–8.
12. Walsh T, King MC. Ten genes for inherited breast cancer. Cancer Cell 2007;11:103–5.
13. Ausubel F, Brent R, Kingston RE, Moore DD, Seidman JG, Smith JA, et al. Current Protocols in Molecular Biology. Boston: John Wiley & Sons Inc.;1999.
14. Fredriksson S, Gullberg M, Jarvius J, Olsson C, Pietras K, Gústafsdóttir SM, et al. Protein detection using proximity-dependent DNA ligation assays. Nat Biotechnol 2002;20:473–7.

Published OnlineFirst March 28, 2011; DOI:10.1158/0008-5472.CAN-10-3119

15. Soderberg O, Gullberg M, Jarvius M, Ridderstråle K, Leuchowius KJ, Jarvius J, et al. Direct observation of individual endogenous protein complexes in situ by proximity ligation. Nat Methods 2006;3:995–1000.
16. Ma T, Frigeri A, Tsai ST, Verbavatz JM, Verkman AS. Localization and functional analysis of CHIP28k water channels in stably transfected Chinese hamster ovary cells. J Biol Chem 1993;268:22756–64.
17. Nguyen M, Branton PE, Walton PA, Oltvai ZN, Korsmeyer SJ, Shore GC. Role of membrane anchor domain of Bcl-2 in suppression of apoptosis caused by E1B-defective adenovirus. J Biol Chem 1994;269:16521–4.
18. Pierce AJ, Johnson RD, Thompson LH, Jasin M. XRCC3 promotes homology-directed repair of DNA damage in mammalian cells. Genes Dev 1999;13:2633–8.
19. Moynahan ME, Chiu JW, Koller BH, Jasin M. BRCA1 controls homology-directed DNA repair. Mol Cell 1999;4:511–8.
20. Scully R, Plug A, Chen J, Xiao Y, Weaver D, Feunteun J, et al. Association of BRCA1 with Rad51 in mitotic and meiotic cells. Cell 1997;88:265–75.
21. Asakawa H, Koizumi H, Koike A, Takahashi M, Wu W, Iwase H, et al. Prediction of breast cancer sensitivity to neoadjuvant chemotherapy based on status of DNA damage repair proteins. Breast Cancer Res 2010;12:R17.
22. Chen H, Ma Z, Vanderwaal RP, Feng Z, Gonzalez-Suarez I, Wang S, et al. The mTOR inhibitor rapamycin suppresses DNA double-strand break repair. Radiat Res 2011;175:214–24.
23. Plo lLaulier C, Gauthier L, Lebrun F, Calvo F, Lopez BS. AKT1 inhibits homologous recombination by inducing cytoplasmic retention of BRCA1 and RAD51. Cancer Res 2008;68:9404–12.
24. Powell SN, Kachnic LA. Therapeutic exploitation of tumor cell defects in homologous recombination. Anticancer Agents Med Chem 2008;8:448–60.
25. Valeri A, Alonso-Ferrero ME, Rio P, Pujol MR, Casado JA, Pérez L, et al. Bcr/Abl interferes with the Fanconi anemia/BRCA pathway: implications in the chromosomal instability of chronic myeloid leukemia cells. PLoS One 2010;5:e15525.
26. Willers H, Taghian AG, Luo CM, Treszezamsky A, Sgroi DC, Powell SN. Utility of DNA repair protein foci for the detection of putative BRCA1 pathway defects in breast cancer biopsies. Mol Cancer Res 2009;7:1304–9.
27. Hou Y, Gao F, Wang Q, Zhao J, Flagg T, Zhang Y, et al. Bcl2 impedes DNA mismatch repair by directly regulating the hMSH2-hMSH6 heterodimeric complex. J Biol Chem 2007;282:9279–87.
28. de Wind N, Dekker M, Berns A, Radman M, te Riele H. Inactivation of the mouse Msh2 gene results in mismatch repair deficiency, methylation tolerance, hyperrecombination, and predisposition to cancer. Cell 1995;82:321–30.
29. Allen C, Kurimasa A, Brenneman MA, Chen DJ, Nickoloff JA. DNA-dependent protein kinase suppresses double-strand break-induced and spontaneous homologous recombination. Proc Natl Acad Sci U S A 2002;99:3558–63.
30. Delacote F, Han M, Stamato TD, Jasin M, Lopez BS. An xrcc4 defect or Wortmannin stimulates homologous recombination specifically induced by double-strand breaks in mammalian cells. Nucleic Acids Res 2002;30:3454–63.
31. Pierce AJ, Hu P, Han M, Ellis N, Jasin M. Ku DNA end-binding protein modulates homologous repair of double-strand breaks in mammalian cells. Genes Dev .2001;15:3237–42.
32. Coene E, Van Oostveldt P, Willems K, van Emmelo J, De Potter CR. BRCA1 is localized in cytoplasmic tube-like invaginations in the nucleus [letter]. Nat Genet 1997;16:122–4.
33. Coene ED, Hollinshead MS, Waeytens AA, Schelfhout VR, Eechaute WP, Shaw MK, et al. Phosphorylated BRCA1 is predominantly located in the nucleus and mitochondria. Mol Biol Cell 2005;16:997–1010.
34. Wang H, Yang ES, Jiang J, Nowsheen S, Xia F. DNA damage-induced cytotoxicity is dissociated from BRCA1's DNA repair function but is dependent on its cytosolic accumulation. Cancer Res 2010;70:6258–67.
35. Dizin E, Ray H, Suau F, Voeltzel T, Dalla Venezia N. Caspase-dependent BRCA1 cleavage facilitates chemotherapy-induced apoptosis. Apoptosis 2008;13:237–46.
36. Snapp EL, Hegde RS, Francolini M, Lombardo F, Colombo S, Pedrazzini E, et al. Formation of stacked ER cisternae by low affinity protein interactions. J Cell Biol 2003;163:257–69.
37. Nakajima K, Hirose H, Taniguchi M, Kurashina H, Arasaki K, Nagahama M, et al. Involvement of BNIP1 in apoptosis and endoplasmic reticulum membrane fusion. EMBO J 2004;23:3216–26.
38. Baldeyron C, Jacquemin E, Smith J, Jacquemont C, De Oliveira I, Gad S, et al. A single mutated BRCA1 allele leads to impaired fidelity of double strand break end-joining. Oncogene 2002;21:1401–10.
39. Zhong Q, Boyer TG, Chen PL, Lee WH. Deficient nonhomologous end-joining activity in cell-free extracts from BRCA1-null fibroblasts. Cancer Res 2002;62:3966–70.
40. Zhong Q, Chen CF, Chen PL, Lee WH. BRCA1 facilitates microhomology-mediated end joining of DNA double strand breaks. J Biol Chem 2002;277:28641–7.
41. Le Page F, Randrianarison V, Marot D, Cabannes J, Perricaudet M, Feunteun J, et al. BRCA1 and BRCA2 are necessary for the transcription-coupled repair of the oxidative 8-oxoguanine lesion in human cells. Cancer Res 2000;60:5548–52.
42. Saha T, Rih JK, Roy R, Ballal R, Rosen EM. Transcriptional regulation of the base excision repair pathway by BRCA1. J Biol Chem 2010;285:19092–105.
43. Saintigny Y, Delacote F, Vares G, Cabannes J, Perricaudet M, Feunteun J, et al. Characterization of homologous recombination induced by replication inhibition in mammalian cells. EMBO J 2001;20:3861–70.
44. Zhang J, Lahti JM, Bruce A, He L, Parihar K, Fan C, et al. Identification of an ataxia telangiectasia-mutated protein mediated surveillance system to regulate Bcl-2 overexpression. Oncogene 2006;25:5601–11.
45. Bartkova J, Horejsi Z, Koed K, Krämer A, Tort F, Zieger K, et al. DNA damage response as a candidate anti-cancer barrier in early human tumorigenesis. Nature 2005;434:864–70.
46. Gorgoulis VG, Vassiliou LV, Karakaidos P, Zacharatos P, Kotsinas A, Liloglou T, et al. Activation of the DNA damage checkpoint and genomic instability in human precancerous lesions. Nature 2005;434:907–13.
47. Ganesan S, Silver DP, Greenberg RA, Avni D, Drapkin R, Miron A, et al. BRCA1 supports XIST RNA concentration on the inactive X chromosome. Cell 2002;111:393–405.
48. Narod SA, Foulkes WD. BRCA1 and BRCA2: 1994 and beyond. Nat Rev Cancer 2004;4:665–76.
49. Starita LM, Parvin JD. The multiple nuclear functions of BRCA1: transcription, ubiquitination and DNA repair. Curr Opin Cell Biol 2003;15:345–50.