Bel-2 inhibits nuclear homologous recombination by localizing BRCA1 to the endomembranes

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Abstract

Genetic stability requires coordinating a network of pathways, including DNA repair/recombination and apoptosis. In addition to its canonical anti-apoptotic role, Bcl-2 also negatively impacts genome stability. Here 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 trans-membrane domain (TM) of Bcl-2 was required for both the 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, and western blot analysis of subcellular fractions showed that Bcl-2 and BRCA1 co-localized to mitochondria and endoplasmic reticulum, in a process requiring the TM of Bcl-2. Targeting BRCA1 to the endomembranes depletes BRCA1 from the nucleus accounting for the inhibition of HR. Our findings also 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.

PRECIS:

Findings suggest a new tumor suppressor function and new mode of regulation for BRCA1, with general implications for understanding the role of homologous recombination in the maintenance of genome stability.
INTRODUCTION

Faithful genome transmission requires coordinating a network of pathways, including cell cycle checkpoints, DNA repair/recombination, and programmed cell death. The Bcl-2 family members 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 non-homologous end joining (NHEJ) (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 (MMR, BER, NHEJ) by different mechanisms. Moreover, none of these mechanisms accounts for HR repression by Bcl-2, which we discuss further herein.

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 (double-strand break repair, interstrand crosslink repair), DNA damage tolerance, meiosis, molecular evolution, and genetic diversity or rearrangement. The repression of HR by Bcl-2 should 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). Here we analyzed the role of the Bcl-2 TM in HR. Importantly, we identify the tumor suppressor BRCA1 as an essential molecular target of Bcl-2 for HR repression. Noteworthy, BRCA1 is the most frequently mutated gene in hereditary breast or ovarian cancer and is essential for HR (12).
MATERIALS AND METHODS

**DNA manipulations.** All DNA manipulations were performed as described previously (13).

**Cell cultures.** The cell line RG37, which contains a single, chromosomally integrated copy of the recombination reporter plasmid pDR-GFP, has already been described (4). RG37 and HeLa cells were cultured in DMEM supplemented with 10% fetal calf serum (FCS) and 2 mM glutamine. Lymphoblastoid and H460 cell lines were cultured in RPMI 1640 supplemented with 20% FCS and 2 mM glutamine. Lymphoblastoid cells GM3657, CRL-2261 and CRL-2632 were obtained from ATCC in 2007, Priess was given 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 by western blot. H460 cells were provided by Dr. E. Deutsch (Institut Gustave Roussy, Villejuif, France) in 2010, HeLa cells were historically present in the laboratory. All these cell lines were tested for endogenous Bcl-2 expression by western blot, and the last test was done during the revision process.

**Western blot analysis.** Western blot were performed 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, the cells were fixed in PBS/2% paraformaldehyde for 15 minutes at room temperature.

**Immunofluorescence analysis.** For all immunofluorescence experiments, cells were washed in PBS, fixed with PBS/2% paraformaldehyde (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 and subsequent DAPI staining, the slides were analyzed by fluorescence microscopy. For mitochondrial detection, the cells were either stained with an anti-CoxIV antibody (1/100; 20E8, Abcam) or Mitotracker (Invitrogen), 50nM 50min. For ER detection, cells were either stained with ER-tracker 600nM 50 min or an anti-calnexin antibody. For Bcl-2 detection, the cells were stained with a monoclonal anti-Bcl-2 antibody (1/1000; sc-509, Santa-Cruz) or a polyclonal anti-Bcl-2 antibody (1/1000; 554160, BD 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 63X lenses. Picture acquisition was made at room temperature with a Spot Advanced 4.3 software and processed with ImageJ software. At least fifty 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.
Co-immunoprecipitation. Cells were washed in ice-cold phosphate-buffered saline and suspended in modified RIPA buffer (20 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.5% NP-40, 1% sodium deoxycholate) supplemented with 1 tablet of protease inhibitor cocktail (Complete Mini, Roche, Indianapolis, IN, USA) and with phosphatase inhibitor cocktails (Sigma, St Louis, MO, USA). Homogenates were incubated on ice for 1 h before centrifugation at 12000g for 30 min. Freshly prepared cell lysates were diluted in modified RIPA buffer and were pre-cleared with Dynabeads protein G (50 μL per 500 μL lysate) for 1 h 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 h at 4 °C. Beads were washed 4× with modified RIPA buffer. Protein complexes were uncoupled from the beads and denatured (70 °C for 10 min) in MOPS LDS sample buffer with reducing agent (Invitrogen).

In Situ proximity ligation assay (PLA). Anti-BRCA1 and anti-Bcl-2 antibodies were used as described above for immunodetection analysis. The in situ PLA (14, 15) was performed using the DuoLink kit (OLINK Bioscience, Uppsala, Sweden) according to the instructions provided by the manufacturer.

Confocal microscopy. Image acquisition was performed with a Leica DMRxA2 confocal microscope SPE (Leica Microsystems, Wetzlar, Germany) using ACS APO 63X 1.3 OIL lenses. Images were processed with Leica and ImageJ softwares.
**Sub-cellular fractionation.** Sub-cellular protein extraction was performed using the ProteoExtract Subcellular Proteome Extraction kit (Calbiochem) according to the instructions provided by the manufacturer.

**Electron Microscopy.** Immunogold labeling of BRCA1 and Bcl-2 was performed on ultrathin cryosections (Tokoyasu method), as previously reported (16). The primary antibody (rabbit polyclonal; 1:200 dilution for anti-BCL2 and 1:50 dilution for anti-BRCA1) was applied for 1 h in BSA/cacodylate, followed by 3 washes of 5 minutes each. Protein-A gold (10 nm) was used for labeling. The sections were washed in water prior to staining in 2% uranyl-acetate for 5 min, followed by a 10 min incubation 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 carboxy-terminus of Bcl-2 contains the TM domain, which is necessary for the anchoring of Bcl-2 to intracellular membranes, such as the endoplasmic reticulum (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). The 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 G145ABcl-2 as the full length Bcl-2 control because it cannot inhibit apoptosis but retains its anti-HR function at wild-type 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, G145ABcl-2 was co-localized with the mitochondrial or ER network, unlike Bcl-2ΔTM (Fig. 1C). Note the intense labeling of Bcl-2ΔTM in the nucleus. After co-transfection of I-SceI and the Bcl-2 forms, the cells were stained with an anti-Bcl-2 antibody, and the number of 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 G145ABcl-2 expression. Of note, overexpression of Bcl-2 did not alter cell cycle distribution (supplementary data 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), demonstrating that the TM domain is required for HR repression.
To test the specificity of Bcl-2 for HR inhibition, we fused the TM of Bcl-2 to an eCFP protein (Supplementary data S2). While wild-type 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 data S2B).

These data show that TM of Bcl-2 is required, but not sufficient by itself, for HR repression, showing 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 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 2B). In contrast, in Bcl-2-transfected cultures, cells expressing Bcl-2 failed to assemble BRCA1 foci (Fig. 2A, 2B and supplementary data S3A). Importantly, expression of Bcl-2ΔTM did not impair BRCA1 foci formation; this demonstrates 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 above data suggested that the BRCA1 pathway is affected by Bcl-2, resulting in a decrease in HR. In the RG37 cell line used here, we have previously shown that overexpression of BRCA1 stimulates
HR efficiency (23). Here we confirmed that co-transfection of BRCA1 with I-SceI led to a significant stimulation of HR (Fig. 2C). This strategy allowed us to focus specifically on BRCA1-controlled HR and the impact of Bcl-2 on this pathway. When co-transfected with BRCA1, Bcl-2 significantly reduced the frequency of HR compared with cells overexpressing BRCA1 alone, whilst Bcl-2ΔTM had no effect (Fig. 2C). These data confirm that Bcl-2 affects BRCA1-induced HR.

Nevertheless, 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 above data in a physio-pathological 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 wild-type cells, but not in the two independent t(14:18) lymphoma cell lines analyzed (Fig. 3B and supplementary data S3B). HR and NHEJ are the two main DNA double-strand break (DSB) repair processes. Interestingly, the two t(14:18) lymphoma cell lines, which constitutively overexpress Bcl-2, exhibited strong spontaneous γ-H2AX labeling compared to wild-type cells, consistent with a general deficiency in DSB repair (Supplementary data S4A).

Many cancer cells express Bcl-2, but generally 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 Bcl-2 proteins (see Figure 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. Of note, the presence of Bcl-2 did not impair the efficient activation of ATM and p53 phosphorylation in p53+ H460 cells (Supplementary data S5).

**BRCA1 co-localizes with Bcl-2 in cytoplasmic membranes.** In the course of the above experiments, we obtained immunofluorescence data suggesting that endogenous BRCA1 co-localized with expressed Bcl-2 (Fig. 4A). This prompted us to establish the co-localization 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 co-localized with Bcl-2 in the cytoplasmic sub-cellular structures (Fig. 4B).

Given that Bcl-2 localizes to the mitochondrial and ER membranes (see Fig. 1), the data above implies that BRCA1 should also localize to endomembranes. To test this hypothesis, we analyzed sub-cellular fractions by western blotting (Fig. 4C). In non-transfected 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 performed immunogold labeling for electron microscopy. In the absence of Bcl-2, BRCA1 was

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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 OSER (organized smooth endoplasmic reticulum) 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). We also 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, demonstrating 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).

These data demonstrate that the TM 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 proximity ligation assay (PLA), which allows the in situ detection of 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.
In non-transfected cells, although BRCA1 and Bcl-2 were both poorly expressed, PLA labeling was detected, showing an in situ cytoplasmic interaction between endogenous wild-type 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 should 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 Fig. 1, 5 and 7A), co-immunoprecipitation analysis becomes feasible using this particular Bcl-2 construct. Indeed, in RG37 cells, immunoprecipitation of BRCA1 resulted in co-precipitation 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 non-specific 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 pre-clear 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 wild-type Bcl-2 and BRCA1, in the physio-pathological model of the t(14:18) lymphomas (Fig. 7C). A small but significant signal was seen in the wild-type cells, consistent with the faint band of Bcl-2 detected by western blot (see Fig. 3A); importantly, the signal increased in the t(14:18) lymphoma cells.
Finally, we analyzed the \textit{in situ} interaction of endogenous wild-type 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 Mitotracker, and the ER was labeled by ER-tracker. Bcl-2/BRCA1 PLA was performed in cells labeled with both Mitotracker 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 data 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 data 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 ionizing radiation (IR) (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 patho-physiological t(14:18) lymphoma model (see Fig. 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 transmembrane domain (TM) 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 \textit{in situ} interaction of Bcl-2 and BRCA1 in cell endomembranes, resulting in sequestration of BRCA1 outside the nucleus.

\textbf{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; they also 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 endoplasmic reticulum-Golgi complex, in invaginations of the nucleus (32), and in the mitochondrial matrix (33). Here we show 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-2\textsubscript{ΔTM}, expression of full-length Bcl-2 results in the formation of aggregated membrane structures termed OSERs. Such structures were first
described following expression of an artificially engineered GFP protein fused to a TM domain (36). GFP exhibits self-aggregation properties; after anchoring to the membrane via the TM region, the interaction between two aggregating molecules leads to folding of the attached membrane and consequent formation of OSERs. Therefore, OSER formation reflects two protein properties: membrane anchoring and the capacity to aggregate with other membrane proteins. Bcl-2 family members possess both properties. Moreover, OSER assembly has been reported upon overexpression of the BH3-only protein BNIP (37). Here we provide the first description of OSER formation resulting from Bcl-2 overexpression.

Nevertheless, 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 two 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); it also controls BER through the transcription control of OGG1, NTH1 and the REF1/APE1 genes (41, 42). This suggests that trapping of BRCA1 in the membrane should be sufficient to affect a large variety of DNA repair and DNA damage response 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 (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 pre-cancerous 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.
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References


Figure legends

**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 one copy of the pDR-GFP substrate and a tandem repeat of two 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 performed using an anti-CoxIV antibody (left panels), and ER staining was performed with an anti-Calnexin antibody (right panels) D/ Effects of the TM domain of Bcl-2 on I-SceI-induced HR. EGFP-positive recombinant cells were detected by fluorescent microscopy. Left panel shows an example of Bcl-2-expressing cells (red) also expressing EGFP (green) after a recombination event. The right panel depicts recombination frequency relative to control cells (transfected with HA-I-SceI and an empty expression vector). Transgenes co-transfected with HA-I-SceI are indicated, and the values correspond to the means of at least three independent experiments.

**Figure 2.** Bcl-2 affects BRCA1 foci formation induced by ionizing radiation. 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 three independent experiments. C/ Impact of BRCA1 overexpression on HR, with or without Bcl-2 or Bcl-2ΔTM.

Figure 3. IR-induced BRCA1 foci in cancer cells. A/ Expression of endogenous Bcl-2 in two lymphoblastoid cell lines from patients with t(14:18) translocation versus two control lymphoblastoid cell lines. Left panel: western blot using an anti-Bcl-2 antibody. Right panels: immunofluorescence detection of Bcl-2 in wild-type cells (left), without IR (middle panel), or after irradiation (right panel). Nuclei were labeled using DAPI. B/ IR-induced BRCA1 foci in the two control and two t(14:18) cell lines. Upper panels: non-irradiated cells. Lower panels: 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 panels) and H460 (right panel) cells.

Figure 4. Co-localization of endogenous BRCA1 and expressed Bcl-2 in cytoplasmic organelles (RG37 cells). A/ Example of co-localization 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 microscopy analysis of endogenous BRCA1 and expressed Bcl-2. Note the formation of IR-induced BRCA1 foci in the absence of Bcl-2 (upper panel). C/ Detection of BRCA1 by western blot in the organelle fraction. The purity of the fraction was confirmed by detection of lamin B1 (nuclear marker) or cytochrome C (cytoplasmic marker).

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.
Figure 6. A/ Detection of BRCA1 in the presence of Bcl-2; Left panels: BRCA1 located in OSER (within dotted lines). Right panels depict BRCA1 outside OSERs but localized within endomembranes. Lower left panel depicts BRCA1 outside OSERs and localized within mitochondria. B/ Detection of BRCA1 with Bcl-2ΔTM overexpression.

Figure 7. A/ In situ proximity ligation assay (PLA) in RG37 cells, after expression of the different transgenes. B/ Co-immunoprecipitation. BRCA1-Flag and Bcl-2ΔTM were co-expressed in RG37 cells and immunoprecipitation was performed 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: pre-clear. C/ PLA on endogenous Bcl-2 and BRCA1 in lymphoblastoid cell lines, wild type 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 data S6).
A. Bcl-2

BH4 | BH3 | BH1 | BH2 | TM

Bcl-2ΔTM: TM domain deleted
BH4 | BH3 | BH1 | BH2

Anti-Bcl-2
α-actin

B. 1-Sce
SoeGFP
5'3'IGFP

Intra-chromatid gene conversion
Unequal sister chromatid exchange
Gene conversion
GFP expression

C. Detection:
Anti-Bcl-2
Mitochondria
merge

Detection:
Anti-Bcl-2
ER
merge

Transgene:
Bcl-2
Bcl-2ΔTM

Transgene:
Bcl-2
Bcl-2ΔTM

Bcl-2

D. Bcl-2

GFP+ cells (% of the control)

Control | Bcl-2 | Bcl-2ΔTM

Laulier et al. Figure 1.
A. 

<table>
<thead>
<tr>
<th>0 Gy</th>
<th>6 Gy</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>Control</td>
</tr>
<tr>
<td>Bcl-2</td>
<td>Bcl-2ΔTM</td>
</tr>
</tbody>
</table>

- DAPI
- Bcl-2
- BRCA1
- merge

B. 

% of cells with BRCA1 foci

- Control
- Bcl-2
- Bcl-2ΔTM

C. 

GFP+ cells (% of the control)

- Control + BRCA1
- Bcl-2 + BRCA1
- Bcl-2ΔTM + BRCA1
- Bcl-2ΔTM + BRCA1

Laulier et al. Figure 2.
Laulier et al. Figure 4
A. **BRCA1 staining in absence of Bcl-2**

B. **Bcl-2 overexpression and Bcl-2 staining**

C. **Bcl-2ΔTM overexpression and Bcl-2 staining**

Laulier et al. Figure 5
A. BRCA1 staining with Bcl-2 overexpression

[Images showing BRCA1 staining with Bcl-2 overexpression]

B. BRCA1 staining with Bcl-2ΔTM overexpression

[Images showing BRCA1 staining with Bcl-2ΔTM overexpression]
A. Transgenes:

<table>
<thead>
<tr>
<th>Transgene</th>
<th>Bcl-2</th>
<th>Bcl-2ΔTM</th>
<th>BRCA1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild Type</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>t(14;18)</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
</tbody>
</table>

B. Empty expression vector vs. Bcl-2ΔTM expression

C. Wild Type (P1025) vs. t(14;18) (CRL-2261)

D. H460 Cells

- Merge
- PLA
- ER-tracker (green)
- Mito-tracker (Deep red)
- DAPI

HeLa Cells

- Merge
- PLA
- ER-tracker (green)
- Mito-tracker (Deep red)
- DAPI

Laulier et al. Figure 7.
Bcl-2 inhibits nuclear homologous recombination by localizing BRCA1 to the endomembranes

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