

Schizosaccharomyces pombe Rad9 contains a BH3-like region and interacts with the anti-apoptotic protein Bcl-2

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Abstract Here we report that the *Schizosaccharomyces pombe* Rad9 (SpRad9) protein contains a group of amino acids with similarity to the Bcl-2 homology 3 death domain, which is required for SpRad9 interaction with human Bcl-2 and apoptosis induction in human cells. Overexpression of Bcl-2 in *S. pombe* inhibits cell growth independently of *rad9*, but enhances resistance of *rad9*-null cells to methyl methanesulfonate, ultraviolet and ionizing radiation. These observations suggest that SpRad9 may represent the first member of the Bcl-2 protein family identified in yeast, though the cell death pathways in *S. pombe* may differ from those found in mammals. © 2000 Federation of European Biochemical Societies. Published by Elsevier Science B.V. All rights reserved.

Key words: Bcl-2; Bcl-2 homology 3 domain; Apoptosis; Rad9; DNA damage; *Schizosaccharomyces pombe*

1. Introduction

The Bcl-2 family of proteins controls a distal step in an evolutionarily conserved pathway for programmed cell death [1–4]. Some proteins within this family, including Bcl-2 and Bcl-x_L, function as suppressors of apoptosis, whereas others, such as Bax and Bad, act as promoters of cell death. Bcl-2 family proteins share conserved regions, termed Bcl-2 homology (BH) domains: BH1, BH2, BH3 and BH4, which can be important for the interactions between Bcl-2 family members and for their function [2]. The BH1 and BH2 domains of the anti-apoptotic proteins such as Bcl-2 and Bcl-x_L and the BH3 domain of the pro-apoptotic proteins such as Bax and Bak appear to be essential for homo- and heterodimerization between the Bcl-2 family members [5–7]. In addition, several proteins, including Bik, Bim, Bid, Hrk, Bad and nematode Egl-1, contain only the BH3 domain and promote apoptosis, thus constituting a new category called the BH3-only subfamily of the Bcl-2 family [2,8]. Most of these BH3-only proteins can form heterodimers with anti-apoptotic Bcl-2 family mem-

bers such as Bcl-2 and Bcl-x_L and inhibit their cell survival activity.

Recently, apoptosis-like features have been observed in some unicellular organisms and non-metazoan multicellular eukaryotes [9–13]. The human Bcl-2 protein can protect some mutant yeast from cell death caused by oxidative injury [14]. Moreover, as in mammalian cells, expression of pro-apoptotic Bcl-2 family proteins Bax and Bak in the yeasts *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* induces cell death that can be suppressed by anti-apoptotic Bcl-2 family proteins, including Bcl-2 and Bcl-x_L [15–19]. These findings imply that the cell suicide pathway controlled by the Bcl-2 family of proteins in mammalian cells may have very primitive evolutionary origins.

We have previously reported that the human homologue of *S. pombe* Rad9 (SpRad9), which is involved in the control of cell cycle checkpoints, contains a BH3 domain, and promotes apoptosis by binding to and inhibiting Bcl-2 and Bcl-x_L in cells [20]. Here, we show that SpRad9 can also interact with Bcl-2, and induce apoptosis in mammalian cells through a region similar to the BH3 domain, thus providing evidence that the Bcl-2 family of proteins may be evolutionarily conserved from fission yeast to human.

2. Materials and methods

2.1. Plasmids

S. pombe rad9 cDNA [21,22] was employed as template for the polymerase chain reactions (PCR) to amplify DNA. Mutations were created using a two-step PCR method [23]. The *rad9* DNA fragments with engineered *EcoRI* and *XhoI* sites were cloned in frame into the *EcoRI/XhoI*-digested yeast two-hybrid vectors pGilda and pJG4-5 [24], or the *EcoRI/SalI*-digested FLAG-tag vector pFLAG-CMV2 [24]. The cDNA fragments encoding green fluorescence protein (GFP) and human Bcl-2 were blunt-ended and subcloned into the *NdeI*-digested/blunted pREP41 (LEU2 marker) vector, which contains a thiamine repressible *mtt* promoter for conditional expression in *S. pombe* [25]. All other plasmids have been described [20].

2.2. Yeast strains and media

S. pombe SP223 (*h⁻ leu1-32 ade6-162 ura4*) and the isogenic *rad9::ura4⁺* derivative were cultured in YEA media [21]. *S. pombe* transformations were performed using the lithium acetate method [26], and cultured in pombe medium (PM) lacking leucine to select cells retaining plasmids [21].

2.3. Analysis of *S. pombe* cell growth and sensitivity to DNA-damaging agents

Transformants were grown in PM medium containing thiamine (5 µg/ml) overnight, then washed three times with water. Cultures were diluted to a final concentration of 5 × 10⁵ cells/ml in fresh PM medium with or without thiamine, to either repress or induce, respectively, the *mtt* promoter in the pREP41 plasmid, and cultured overnight. Cells were again diluted to 5 × 10⁵ cells/ml and cultured in fresh

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Abbreviations: SpRad9, *Schizosaccharomyces pombe* Rad9; BH3, Bcl-2 homology 3; PCR, polymerase chain reaction; GFP, green fluorescence protein; ONPG, *o*-nitrophenyl β-galactosidase; PM, pombe medium; UV, ultraviolet; MMS, methyl methanesulfonate

PM medium with or without thiamine, and the number of cells was determined at various times by using a hemocytometer. For DNA damage assays, the twice diluted cells were grown overnight in PM medium with or without thiamine to mid-log phase, then treated with ultraviolet (UV) light, γ -rays or 0.5% methyl methanesulfonate (MMS). The percentage of viability was calculated as the numbers of colonies formed relative to mock-treated controls.

3. Results

3.1. SpRad9 interacts with Bcl-2 in a BH3-like domain-dependent manner

Sequence comparisons revealed that SpRad9 (accession number X64648), like human Rad9, contains a group of 15 amino acids (¹⁶IFTNLSRIDDAVNWE³⁰) with homology to the BH3 domains of pro-apoptotic Bcl-2 family proteins [20]. Initially, yeast two-hybrid assays were employed to test the ability of SpRad9 to interact with Bcl-2 family proteins. This test indicated that SpRad9, whether fused to the transactivation or binding domain, strongly interacted with Bcl-2, to a degree comparable with the interactions between Bcl-2 and Bax, as demonstrated by a quantitative *o*-nitrophenyl

A

LexA (BD)	B42 (AD)	β -Galactosidase Activity
Bcl-2	X SpRad9	27.4
Bcl-2	X SpRad9 Δ BH3	3.6
Bax	X SpRad9	2.2
SpRad9	X Bcl-2	41.3
SpRad9	X Bax	4.7
SpRad9 Δ BH3	X Bcl-2	1.0
Bax	X Bcl-2	32.5
Bcl-2	X v-Ras	1.9

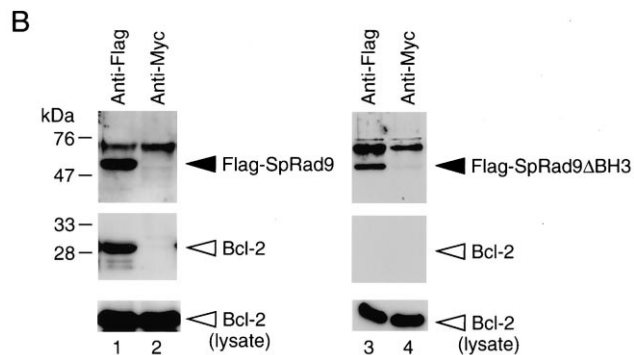


Fig. 1. SpRad9 interaction with Bcl-2 is dependent on the BH3-like domain. (A) Strain EGY48 *S. cerevisiae* cells were co-transformed with various pGilda expression plasmids encoding LexA DNA-binding domain fusion proteins in combination with various pJG4-5 plasmids that produce B42 transactivation domain fusion proteins [38]. The strength of protein–protein interactions was determined by the quantitative ONPG assay [39]. Immunoblotting with polyclonal rabbit antisera specific for SpRad9 confirmed expression of all deletion mutants that failed to interact with Bcl-2 (not shown). (B) 293T cells were transiently co-transfected with pRC/CMV-Bcl-2 and pFLAG-CMV2 plasmids encoding SpRad9 or SpRad9 Δ BH3 mutant proteins. Two days after transfection, immunoprecipitations were performed as described [20] using either anti-Myc antibody as a negative control or anti-FLAG M2 antibody followed by immunoblot analysis of the resulting immune complex using either anti-SpRad9 (top) or anti-Bcl-2 (middle) polyclonal antisera. In addition to immune complexes, 25 μ g of the cell lysates was analyzed directly

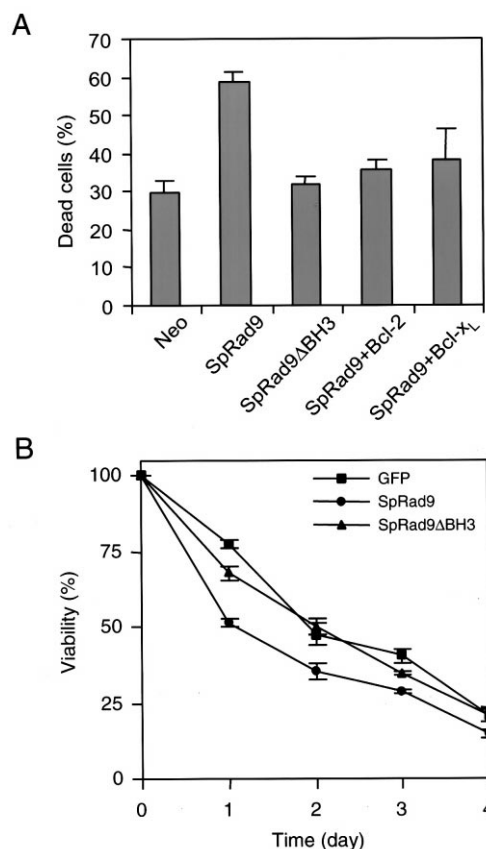


Fig. 2. Overexpression of SpRad9 kills human cells. (A) 293T cells in 6-well plates were transiently co-transfected by a calcium-phosphate precipitation method with 1 μ g of pFLAG-CMV2 encoding SpRad9 or SpRad9 Δ BH3 and 1 μ g of pcDNA3-Bcl-2, pcDNA3-Bcl-x_L or parental plasmid DNA, together with 0.2 μ g of pCMV- β -gal plasmid as a marker for transfected cells. The total amount of plasmid DNA was normalized to 2.2 μ g for each transfection by adding control vector pcDNA3. 24 h after transfection, floating and adherent cells were fixed with 2% formaldehyde and stained with X-gal as described [20]. The percentage of transfected cells (blue) with apoptotic morphology (round dark blue) was determined (data shown are means \pm S.D.; *n* = 3). (B) MCF7 cells were transfected with the control vector pEGFP-C2 (GFP), or pEGFP-C2 encoding SpRad9 or the SpRad9 Δ BH3 mutant proteins by lipofectin (Gibco BRL) and selected in 400 μ g/ml G418. Neomycin-resistant cells were cultured without fetal calf serum for various times, and the percentage of viable cells was determined by exclusion of trypan blue dye (data shown are means \pm S.D.; *n* = 3).

β -galactoside (ONPG) assay (Fig. 1A). In contrast, SpRad9 Δ BH3, a deletion mutant of Rad9 protein lacking critical residues in the BH3-like region (amino acids 20–27), failed to form two-hybrid interactions with Bcl-2. The specificity of the association between Bcl-2 and SpRad9 was confirmed by the failure of these proteins to form two-hybrid interactions with Bax or v-Ras.

To determine whether similar interactions could occur between SpRad9 and Bcl-2 in mammalian cells, human 293T epithelial cells were transiently co-transfected with plasmids encoding Bcl-2 and FLAG-epitope tagged full-length SpRad9 or SpRad9 Δ BH3. Immunoprecipitations were performed with anti-FLAG or anti-Myc antibody as a control, followed by an SDS-PAGE immunoblot assay using antisera specific for SpRad9 or Bcl-2. Under these conditions, Bcl-2 could be coimmunoprecipitated with the wild-type SpRad9 pro-

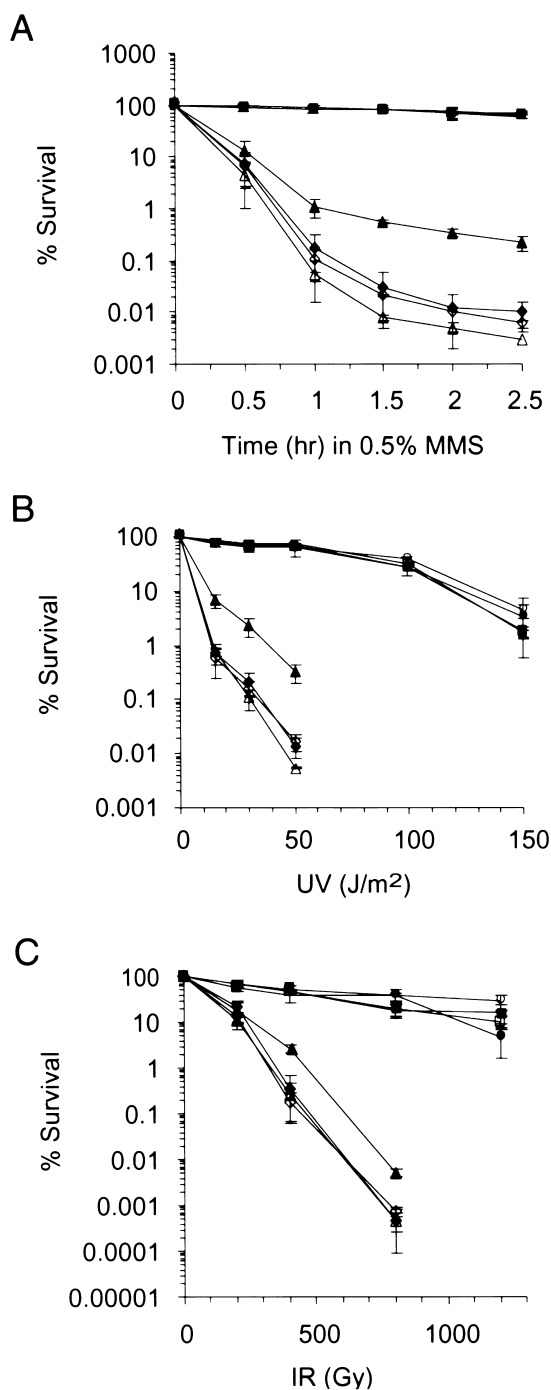


Fig. 3. Bcl-2 rescues *rad9::ura4⁺* *S. pombe* cells from DNA damage. *S. pombe* strains *rad9⁺* and *rad9::ura4⁺* were transformed with pREP41 plasmids encoding the human Bcl-2 protein or GFP as a control. Transformants were grown in selective medium with (+) or without (–) thiamine to either repress or induce, respectively, the *mt* promoter. Exponentially growing cells were exposed to 0.5% MMS for various times (A), or UV light (B) or γ -rays (C) at the indicated doses. An equal number of cells were plated on PM minimal media lacking leucine and containing thiamine, and viability was assayed by the ability to form colonies relative to mock-treated cells (data shown are means \pm S.D.; $n=3$). Symbols represent: (○), *rad9⁺/bcl-2⁺*+thiamine; (●), *rad9⁺/bcl-2⁻*-thiamine; (□), *rad9⁺/gfp⁺*+thiamine; (■), *rad9⁺/gfp⁻*-thiamine; (△), *rad9::ura4⁺/bcl-2⁺*+thiamine; (▲), *rad9::ura4⁺/bcl-2⁻*-thiamine; (◇), *rad9::ura4⁺/gfp⁺*+thiamine; and (◆), *rad9::ura4⁺/gfp⁻*-thiamine.

tein (Fig. 1B). In contrast, the BH3 deletion mutant (SpRad9 Δ BH3) failed to coimmunoprecipitate with Bcl-2; immunoblot analysis confirmed the presence of the Bcl-2 protein in the cell lysates. Taken together, these findings indicate that Bcl-2 associates with SpRad9 and that the BH3-like region of SpRad9 is required for this interaction.

3.2. SpRad9 induces human cell death only when the BH3-like domain is intact

To elucidate the effects of SpRad9 on cell death, we transiently transfected FLAG-tagged SpRad9 into 293T cells together with a *lacZ* reporter plasmid. 24 h after transfection, nearly 60% of the cells transfected with SpRad9-expressing plasmid died, compared to \sim 30% of the cells receiving the Neo control plasmid (Fig. 2A). Co-expression of Bcl-2 or Bcl-x_L essentially abrogated the effects of SpRad9 on cell death. In contrast to the wild-type SpRad9 protein, removal of the BH3-like region dramatically reduced the ability of SpRad9 to induce cell death.

Next, we confirmed that the pro-apoptotic activity of SpRad9 was not unique to 293T cells by introducing a GFP-tagged SpRad9 into MCF7 human mammary carcinoma cells. Cells that received the GFP-SpRad9 plasmids died faster compared to the control GFP-transfected cells when deprived of serum from the cultures (Fig. 2B). In the experiment shown, for example, half of the cells expressing GFP-SpRad9 died from apoptosis after serum withdrawal for 1 day, compared to \sim 20% of the MCF7-GFP control cells. In contrast, expression of the deletion mutant GFP-SpRad9 Δ BH3 protein has no effect on the viability of MCF7 cells and the kinetics of cell death were essentially the same as in the control cells expressing GFP alone.

To date, we have been unable to generate any stable cell lines derived from FL5.12, Jurkat, HeLa and NIH3T3 that express the SpRad9 transgene at high levels in the absence of exogenous Bcl-2 or Bcl-x_L, providing evidence that overexpression of SpRad9 is also lethal to these cells (not shown).

3.3. Bcl-2 enhances resistance of *rad9*-null *S. pombe* cells to DNA-damaging agents

It has been reported that the human Bcl-2 protein can pro-

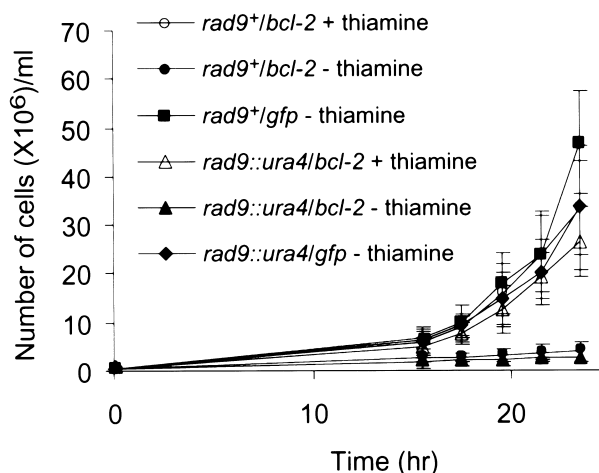


Fig. 4. Bcl-2 inhibits cell growth of *S. pombe*. Cells were transformed and grown as described in Fig. 3. The rates of cell growth were determined by counting the number of cells using a hemocytometer during a 24 h period.

fect some mutant strains of budding yeast that lacked superoxide dismutase from cell death induced by oxidative stress [14]. SpRad9 is essential for the resistance of *S. pombe* to γ -rays and UV light [21,27]. To determine whether Bcl-2 also protects *rad9* deficient fission yeast cells from the genotoxic effects of DNA-damaging agents, we introduced the human *bcl-2* gene or *gfp* as a control into *rad9*⁺ and *rad9::ura4*⁺ *S. pombe* strains under the regulation of a thiamine repressible *nmt* promoter for conditional expression. Transformants were exponentially cultured in PM medium with or without thiamine, to either repress or induce, respectively, expression of the transgenes, and treated with MMS, UV or radiation. As shown in Fig. 3, expression of Bcl-2 significantly increased the resistance of *rad9::ura4*⁺ cells to cell death induced by MMS, UV and γ -rays, compared to the cells expressing the GFP control protein. In contrast, there are no differences in the sensitivity profiles of transformants containing the Bcl-2 or control plasmids when grown in thiamine-supplemented medium. However, the human Bcl-2 protein has no effect on the survival of *rad9*⁺ *S. pombe* cells exposed to these DNA-damaging agents. When treated with MMS, UV or γ -rays, the *rad9*⁺ *S. pombe* transformants showed similar viability in medium containing or devoid of thiamine.

3.4. Bcl-2 inhibits cell growth in both *rad9*⁺ and *rad9::ura4*⁺ strains

It has been reported that overexpression of the anti-apoptotic Bcl-2 protein causes human cells to delay entry into the S phase of the cell cycle [28–31], but the biochemical mechanism is unclear. We therefore employed fission yeast as a model to examine whether Bcl-2 can also inhibit *S. pombe* cell growth and, if so, whether the interaction of Bcl-2 with Rad9 is necessary. Both *rad9*⁺ and *rad9::ura4*⁺ *S. pombe* cells were transformed with conditional expression plasmids encoding the human Bcl-2 protein or GFP as a control, and cultured in growth media in the presence or absence of thiamine. Induction of Bcl-2 expression markedly inhibited cell growth in both *rad9*⁺ and *rad9::ura4*⁺ cells, suggesting that the *rad9* gene is not essential for Bcl-2 to inhibit cell growth in this yeast (Fig. 4). In contrast, expression of the control GFP protein did not influence the rates of cell growth in both *rad9*⁺ and *rad9::ura4*⁺ fission yeast cells. Growth of the GFP-carrying but non-expressing cells was the same as for the other cells not expressing their respective transgenes (not shown).

4. Discussion

The findings reported here suggest that SpRad9 may be a member of the BH3-only family of apoptosis promoting proteins. Like human Rad9, SpRad9 also contains a region near its N-terminus (amino acids 16–30) with similarity to the BH3 domains of pro-apoptotic Bcl-2 family proteins, but lacks critical residues found in BH1, BH2 and BH4. Overproduction of this fission yeast protein in human cells induced cell death, which can be blocked by co-expression of Bcl-2 or Bcl-x_L. Deletion of the BH3-like region from SpRad9 abolished its ability to interact with Bcl-2 and to induce apoptosis in human cells, consistent with properties of other members of the BH3-only pro-apoptotic protein family [2,8,20]. These proteins are unable to form homodimers but can heterodimerize with anti-apoptotic members of the Bcl-2 family and an-

tagonize their function. One mechanism by which the BH3-only proteins may induce apoptosis could involve competitive displacement of a pro-apoptotic partner such as Bax or Apaf-1 from a complex with death antagonists of the Bcl-2 family, freeing it to commit the cell to apoptotic suicide [2,8].

Cell cycle checkpoints control cell cycle progression, allowing more time, for example, to repair damaged DNA prior to progression into the S or G2 phases of the cell cycle. In multicellular organisms, checkpoints play an essential role in maintenance of genomic integrity. To prevent genetic diseases and cancer, the mutated or damaged cells must be eliminated through activation of a physiological process (apoptosis). In relatively simple organisms, checkpoint controls are critical for survival when exposed to DNA-damaging agents or DNA replication inhibitors. *S. pombe rad9* is one of six checkpoint genes in fission yeast, and plays an essential role in mediating resistance to radiation [21,32]. The *rad9* mutant strains of *S. pombe*, which are unable to stop cell cycle progression in response to DNA damage, are more sensitive to γ -rays, UV light or MMS compared to *rad9*⁺ cells. These results are not consistent with those found in human cells, in which expression of an antisense Rad9 RNA blocks apoptosis induced by MMS, suggesting that this organism may use different machinery to control cell cycle progression and cell death processes in response to DNA damage. In mammals, Rad9 may contribute more to activation of apoptosis than to arrest of the cell cycle after DNA damage. In addition, other pathways controlling cell cycle checkpoints, which are Rad9-independent, may exist in humans. In fission yeast, however, the inability to delay cell cycle progression after radiation-induced DNA damage accounts for an essential part of the radiosensitivity of *rad9* mutant cells, though the lack of other cell cycle-independent functions of Rad9 may also contribute [27].

It has been suggested that cell suicide mechanisms may exist even in unicellular organisms [11,33], potentially limiting replication of viruses, reducing competition for nutrients during periods of starvation, or eliminating cells with damaged or mutated DNA to keep up the genomic integrity of future generations. Though apoptosis-like cell death has been observed in several unicellular eukaryotes [10,11,34–36], the specific cell death machinery may differ from that found in multicellular organisms. Our results that expression of Bcl-2 can partially enhance resistance of *rad9* mutant *S. pombe* cells to DNA-damaging agents, however, argue that the cell death pathway controlled by the Bcl-2 family is, at least in part, conserved in yeasts.

In addition to the control of apoptosis, it has been demonstrated that Bcl-2 family proteins can also modulate cell cycle progression. Overexpression of Bcl-2 or Bcl-x_L causes cells to delay entry into S phase [28–31], while production of the pro-apoptotic protein Bax increased the proportion of cells in this phase of the cell cycle [37]. Interestingly, expression of the human Bcl-2 protein in fission yeast inhibits cell growth, although that is Rad9-independent. The potential for Bcl-2 to modulate the cell cycle, causing a slowing of either entry into (G0 to G1) or progression through the cell cycle, may explain, at least in part, why expression of Bcl-2 can protect *S. pombe* cells from genotoxic effects of DNA-damaging agents. Clearly, further studies are required to explore the biochemical mechanism by which Rad9 commits the cell to undergo suicide or DNA repair.

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