Rad24 is essential for proliferation of diploid cells in fission yeast

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Abstract The *rad24*$^+$ gene of *Schizosaccharomyces pombe* encodes a ubiquitously expressed 14-3-3 protein. We report here that Δ*rad24* cells displayed a defect in diploid colony formation, although they conjugated efficiently. We found that a cumulative deletion of *mei2*$^+$ gene almost completely suppressed this defect, and demonstrated using two-hybrid analysis that Rad24 protein directly associates with Mei2 protein by recognizing Ser-438 which is a phosphorylation target of Pat1 kinase. We conclude that constitutive progression to meiosis, caused by lack of Mei2 inhibition due to the absence of Rad24 protein, is the primary cause of the proliferative deficiency observed in Δ*rad24* cells.

Key words: Schizosaccharomyces; Rad24; Diploid cells; Mei2; Meiosis

1. Introduction

14-3-3 proteins are a family of highly conserved and ubiquitously expressed proteins which have been implicated in a diverse range of cellular processes [1]. Members of this family function as either homo- or heterodimeric complexes which interact with a conserved phosphorylated motif that has been found in a number of signal transduction proteins [2]. In *Saccharomyces cerevisiae*, two 14-3-3 homologues (*BMH1* and *BMH2*) play roles in Ras and drug-sensitive signalings [3–5]. In mammalian cells, 14-3-3 proteins bind to and activate the enzymes tyrosine hydroxylase and tryptophan hydroxylase, which are involved in neurotransmitter synthesis [1]. Inhibition and activation of protein kinase C and activation of catecholamine exocytosis and Ca^{2+}-induced phospholipase A activity by 14-3-3 proteins have also been described, and biochemical studies have suggested a role for 14-3-3 proteins in Ras/Raf/MAPK cascade signaling in vertebrate cell proliferation and differentiation [1]. Evidence from several organisms indicates that binding of 14-3-3 proteins to the mitotic inducer Cdc25 phosphatase alters its cellular localization and plays a pivotal role in regulating Cdc25 activity at G2/M [6–10]. In *Schizosaccharomyces pombe*, two *14-3-3* genes (*rad24*$^+$ and *rad25*$^+$) have been identified, and a double-null mutant which is inviable [11] as is *S. cerevisiae bmh1bmh2* mutant in most genetic backgrounds [3,4]. *rad24* null mutant, and to a lesser extent *rad25* null mutant, are sensitive to UV and to ionizing radiation and enter mitosis prematurely [11] because of loss of Cdc25 inhibition, which results in a checkpoint defect at G2/M [12].

S. pombe cells are most stable in the haploid state, and are essentially asexual as long as they are not deprived of nutrients. They initiate sexual development only under starvation conditions. When starved of nutrients, especially nitrogen, *S. pombe* cells undergo a process of sexual development which is regulated by two major signal transduction pathways, one of which responds to environmental conditions and the other to mating pheromones [13]. Nutrient depletion and several other stresses (e.g. heat, high osmolarity) lead to the transcriptional induction of *ste11*$^+$, which encodes a key transcription factor that regulates a number of genes required for mating and/or meiosis [14]. Mating and sporulation processes are regulated largely by common signaling pathways and positive feedback with amplifying signals that promote these processes [15]. In this paper, we report a novel function of 14-3-3 proteins, namely, 14-3-3 proteins directly associate with Mei2 protein, recognizing phosphorylated Ser-438 which is known to be phosphorylated by Pat1 kinase [16]. We conclude that failure of Mei2 regulation is the major cause of the proliferative defect in the diploid state of the *14-3-3* mutants.

2. Materials and methods

2.1. Yeast strains and manipulations

All the strains of *S. pombe* used in this study are haploid strains. All mutants were derived from a wild-type diploid strain (h^{90}/h^{90}, *ade6-M210/ade6-M216*, *ura4-D18/ura4-D18*, *leu1/leu1*). *rad24*$^+$ was disrupted by one-step replacement of the *rad24*$^+$ *Pst*I–*Fba*I genomic region with an *ura4*$^+$ fragment. *rad25*$^+$ deletion was accomplished by one-step replacement of the *rad25*$^+$ *Sph*I–*Sph*I genomic region with a *ura4*$^+$ fragment. The *ura4*$^+$ fragment was prepared from a Δ*rad25* strain [11]. *mei2*-deleted strains were constructed by crossing with the JZ127 strain [15]. To form diploid colonies, cells were cultured to mid-exponential phase ($4–7\times10^6$ cells/ml) at 33°C in YE medium, and then aliquots containing equal numbers of cells were mixed and spread onto SPA plates. After incubation overnight, cells were spread onto EMM plates. The conjugation capability under nutrient-rich conditions was measured by counting conjugated and vegetative cells under a light microscope after culture to mid-exponential or early stationary phase ($2–3\times10^7$ cells/ml) at 33°C in YPD medium. The Δ*rad24* Δ*mei2* double mutant strain was constructed by crossing the Δ*rad24* mutant strain with the JZ127 (*mei2::ura4*$^+$) strain, which was a gift from Dr. M. Yamamoto. Ura$^+$ descendants were examined for *rad24* and *mei2* deletion by Southern blot analysis.

2.2. Two-hybrid analysis

To perform two-hybrid analysis, we synthesized the oligonucleotides Mei2N (5′-CGGCGCGCCGCATATGATTATGGAAACCGAATCA-3′) and Mei2C (5′-CCTCCAACTGCAAGCAAATGTCCGCGGCCGCCTCGAGTAC-3′) as primers, which encompass the open reading frame (ORF) of Mei2 and correspond to nucleotides 1797–1811 and 4020–4040 (GenBank X07180), respectively. The *Asc*I and *Not*I sites introduced into the oligonucleotides are underlined. Using these oligonucleotides as primers and *mei2*$^+$ genomic DNA as template in a polymerase chain reaction (PCR), we generated a DNA fragment containing the ORF of Mei2. To select a clone free

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from point mutations caused by PCR amplification, we randomly picked at least four clones and determined the DNA sequences of the amplified regions. This DNA fragment was digested with *Asc*I and *Not*I and inserted into the pBD3 vector to allow expression of Gal4bd-Mei2 fusion protein. The vectors pAD3 or pBD3 were derived from pGAD424 or pGBT9 (Clontech), respectively, by insertion of an extra multiple cloning site between the *Eco*RI and *Sal*I sites. We used the same strategy to prepare the pAD3-Rad24 and Rad25 constructs. Plasmids were transformed into *S. cerevisiae* (Y190) cells and the transformants were selected on plates lacking tryptophan and leucine as described by Bai and Elledge [17]. Colonies thus isolated were patched onto medium lacking the same amino acids. After growth, colonies were spotted onto a filter paper (Whatman 3MM) which was then immersed into liquid nitrogen to disrupt the cells. β-Galactosidase activity was scored according to the development of blue color in Z buffer (10 mM KCl, 1 mM $MgSO_4$, 100 mM $Na\text{-}PO_4$, 38 mM β-mercaptoethanol, pH 7.0) in the presence of 0.33 mg/ml X-gal (5-bromo-4-chloro-3-indoyl-β-D-galactopyranoside).

3. Results and discussion

3.1. Deletion of either *rad24*$^+$ or *rad25*$^+$ inhibited proliferation of diploid cells

Cells in which the *rad24*$^+$ gene of *S. pombe* was deleted did not form diploid colonies even if they were transferred to nitrogen-containing medium soon after conjugation and before meiosis (Fig. 1A). A small number of *ade*-independent colonies formed on the selective EMM plates were found to be haploid cells carrying conversions or reversions in the *ade6* allele (data not shown). Deletion of *rad25*$^+$ also caused a deficiency in diploid cell growth, with a slightly leaky phenotype in comparison with loss of *rad24*$^+$ (Fig. 1A). To quantitate the deficiency in diploid colony formation, conjugation-induced h^{90} cells were returned to YE plates containing Phloxine B. Diploid colonies (red) were easily distinguished from haploid colonies (white) on these plates. The Δ*rad24* mutant formed only haploid colonies. In contrast, up to 0.2% of Δ*rad25* colonies and 99% of wild-type colonies displayed the red color indicative of diploidy (Fig. 1B). Because zygotes were frequently observed after induction of conjugation by nutrient depletion, and spores were formed normally when cells were incubated continuously on the sporulation medium, we speculated that 14-3-3 proteins were essential for maintenance of diploid cell proliferation. Levels of *rad24*$^+$ and *rad25*$^+$ mRNA, as determined by Northern blot analysis, remained constant during meiosis in both wild-type and the meiosis defective diploid (Δ*rad24*/Δ*rad24* or Δ*rad25*/Δ*rad25*; data not shown). Western blotting analysis indicated that Rad24 protein levels also remained constant during meiosis (data not shown). Thus, the amount of *rad24*$^+$ or *rad25*$^+$ expression is irrelevant to this phenotype.

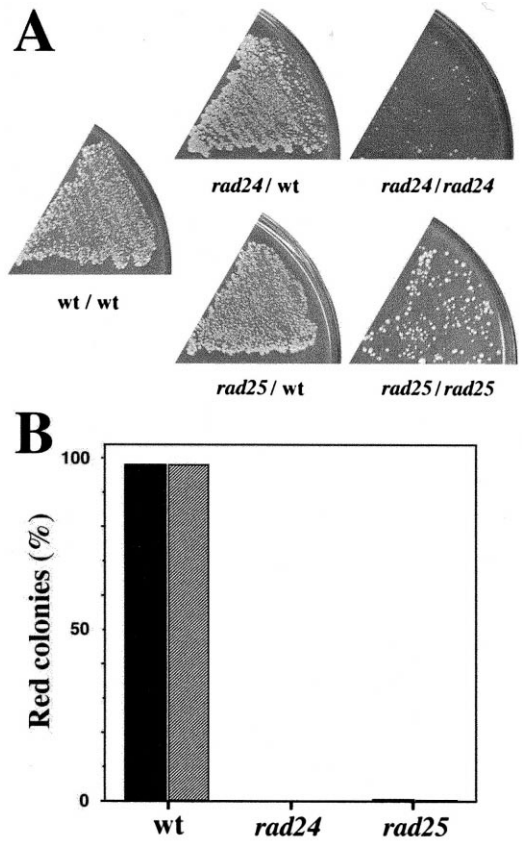

Fig. 1. Deletion of *14-3-3* genes interfered with maintenance of diploidy. (A) *ade6-M210* and *ade6-M216* cells were mixed at a 1:1 ratio and starved on SPA plates, and were returned onto selective plates to form *ade*-independent diploid colonies after 15 h of nutrient deprivation. (B) Mated mixtures were returned onto YE plates containing Phloxine B (20 μg/ml). Diploid colonies were colored red, while haploid cells formed white colonies. Black and gray bars represent the results of independent tests.

3.2. *rad24*$^+$ deletion induced transcription of *ste11*$^+$ and *mei2*$^+$*genes*

Because the absence of one 14-3-3 protein permitted the haploid cells to proliferate, we surmised that a diploid cell carrying a *14-3-3* deletion would be strongly inclined to meiosis even in the absence of environmental signals for meiosis, and that this might be the main reason why the mutant cells were unable to proliferate as diploid. To test whether meiotic induction without nutrient depletion occurred in these mutants, expression of the *ste11*$^+$ gene was examined by Northern blot analysis. Ste11 protein is a key transcriptional factor regulating sexual development, and the expression of many genes depends on the function of *ste11*$^+$ during mating and meiosis [13,14]. As expected, expression of *ste11*$^+$ was found to be induced in Δ*rad24* cells, even in exponentially growing haploid cells without nutrient depletion (Fig. 2A). In contrast to the Δ*rad24* mutant, *ste11*$^+$ expression was only slightly increased in the Δ*rad25* mutant, suggesting that Rad24 and Rad25 proteins are involved in somewhat different regulatory mechanisms. In this report, we concentrate on Rad24 rather than the distinction between Rad24 and Rad25 proteins, and present data concerning the Δ*rad24* mutant in most of our functional analyses. Transcription of the *mei2*$^+$ gene, another key regulatory gene involved in promoting meiosis, was also induced in Δ*rad24* cells (Fig. 2B) and activation of *ste11*$^+$ and *mei2*$^+$ genes was observed in a heterothallic Δ*rad24* mutant (data not shown). Since transcription of *mei2*$^+$ is dependent on Ste11 protein [14], the activity of Ste11 appeared to be up-regulated in the Δ*rad24* mutant by induction of the *ste11*$^+$ gene in the absence of pheromone signaling.

3.3. *mei2* inactivation suppressed the proliferative deficiency and enhanced the conjugation rate of Δ*rad24* cells

Having noticed that *mei2*$^+$ gene expression was up-regulated in the Δ*rad24* cells (Fig. 2B), we reasoned that lack of Rad24 protein may cause abnormal modulation of Mei2, resulting in constitutive progression to meiosis. To examine this

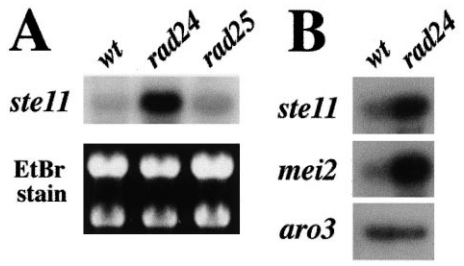

Fig. 2. Northern analysis on the effects of null mutation of *14-3-3* genes. (A) Expression of the *ste11*$^+$ gene was induced in Δ*rad24* cells. Total RNA prepared from mutant cells cultured to exponential growth phase ($4–7\times10^6$ cells/ml) was probed with ^{32}P-labeled *ste11*$^+$ DNA fragment. The ethidium bromide stain is also presented as a loading control. (B) *rad24*$^+$ deletion caused up-regulation of *mei2*$^+$ as well as *ste11*$^+$ induction. Northern blots were probed with ^{32}P-labeled *ste11*$^+$, *mei2*$^+$ and *aro3*$^+$ DNA fragments.

possibility, we constructed a Δ*mei2* Δ*rad24* double disruptant and analyzed the phenotype. By complementation testing using *ade6-M210* and *ade6-M216* mutations, we found that the defect in the maintenance of diploidy during vegetative growth of the Δ*rad24* cells was completely blocked by *mei2*$^+$ deletion (Fig. 3A, vi). This result strongly suggests that constitutive activation of Mei2 protein in the Δ*rad24* mutant cells may be the major cause for compulsion to meiosis without nutrient depletion.

We next quantitatively analyzed the induction profile of sexual development in h^{90}Δ*rad24* cells, by counting zygotes and spores during exponential growth and at early stationary phase. One zygote and one ascus counted as two conjugation-induced cells, and one spore, which was seldom observed in any strain, counted as 0.5. As shown in Fig. 3B, when wild-type cells showed almost no conjugation, the proportion of conjugated cells in the Δ*rad24* mutant in YPD-rich medium was found to be elevated not only at the mid-point of exponential growth (4×10^6cells/ml) but also at early stationary phase (1×10^7 cells/ml). This result suggests that anomalous induction of conjugation in haploid cells of the Δ*rad24* cells occurred under nutrient-rich conditions, indicating that sexual development in this mutant was constitutively active. In contrast, the proportion of conjugated cells in Δ*rad24* Δ*mei2* double mutant was very small, both at mid-exponential growth and at early stationary phase (Fig. 4). As expected from the results of flow cytometry (FACS) analysis (data not shown), conjugation of the Δ*rad24* Δ*mei2* double mutant was not induced in YPD. To exclude the possibility that diploid cells were miscounted as haploid cells in Δ*mei2* and wild-type strains, aliquots were taken from cultures and examined on YPD containing Phloxine B. No diploid colonies were observed among more than 10^3 colonies of either strain after 3 days incubation (data not shown), indicating that the level of miscounting was negligible. Taken together, these results indicate that inactivation of Mei2 almost completely suppressed the enhanced conjugation rate of Δ*rad24* cells.

3.4. Mei2 is a direct association target of Rad24

The results shown above suggest two possibilities: Mei2 may be a regulator of the putative downstream target of Rad24 and Rad25 proteins, or it may be the target itself. The presence in Mei2 protein of a consensus binding motif for 14-3-3 proteins [18] seems to favor the latter possibility. We found two candidate 14-3-3 binding motifs, RSSS(179)LN and RTES(438)SP, in Mei2 protein; the putative phosphorylated target serine residues are underlined and their positions in the primary sequence indicated in parentheses. It is of note

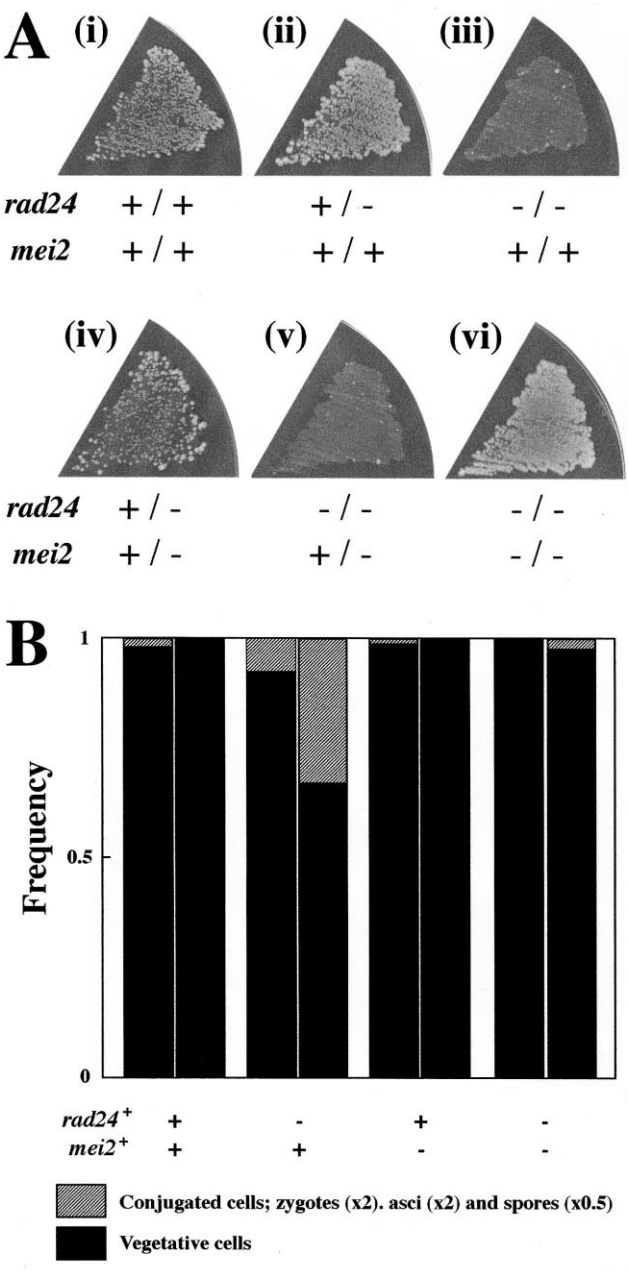

Fig. 3. Effect of *mei2*$^+$ deletion in Δ*rad24* strain on the proliferative deficiency and enhanced conjugation rate. (A) Deletion of *mei2*$^+$ completely suppressed the proliferative deficiency of Δ*rad24* diploid cells. Plus or minus denotes that the relevant gene is intact or deleted, respectively. Formation of zygotes and return to growth phase were examined as described in Fig. 1. (B) Deletion of the *rad24*$^+$ gene caused vegetative cells to habituate to conjugation, and this effect was almost completely suppressed by cumulative *mei2*$^+$ deletion. The proportions of conjugated cells in mid-exponential (left columns) and early stationary phase (right columns) cultures in YPD medium are shown. A zygote, an ascus and four spores were counted as two conjugation-induced cells. Conjugated and vegetative cells are indicated by diagonal and solid bars, respectively. Plus or minus shown below signify that the indicated gene is intact or deleted, respectively.

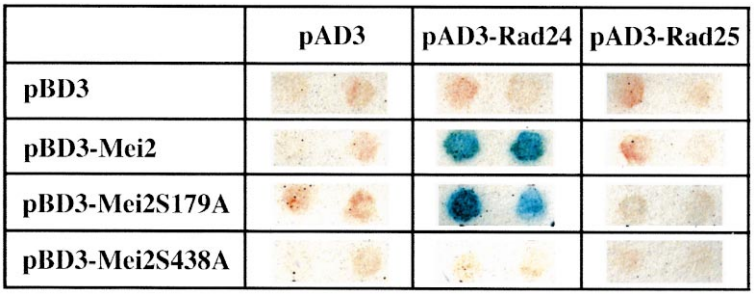

Fig. 4. Rad24 but not Rad25 directly associates with Mei2 at Ser-438. Plasmid DNA carrying *rad24*$^+$ (pAD3-Rad24), *rad25*$^+$ (pAD3-Rad25) or vector alone (pAD3) was co-transfected into *S. cerevisiae* (Y190) cells with plasmids carrying *mei2*$^+$ (pBD3-Mei2, pBD3-Mei2S179A or pBD3-Mei2S438A) or vector alone (pBD3). Cells transformed with each pair of plasmids were assayed for β-galactosidase activity.

that Ser-438 has been identified as one of the phosphorylation targets of Pat1 [16].

We therefore looked for direct physical interaction between Mei2 and Rad24 proteins using two-hybrid analysis [17]. The ORF of Rad24 was fused to the Gal4 activation domain of pAD3 (a modified form of the pGAD424 vector). The ORF of Mei2 was fused to the DNA binding domain of Gal4 in pBD3 (a modified pGBT9 vector). We also prepared two Mei2 mutants in which either Ser-179 (pBD3-Mei2S179A) or Ser-438 (pBD3-Mei2S438A) was replaced with alanine. These plasmids were introduced into *S. cerevisiae* Y190 cells and the expression of the *LacZ* reporter gene was tested. As shown in Fig. 4, when pAD3-Rad24 and pBD3-Mei2 were co-transformed, the resulting colonies turned blue in the presence of X-gal, suggesting a direct physical interaction between Rad24 and Mei2 proteins. When Ser-438 was replaced with alanine, the colony color did not change (viz. no *LacZ* expression was observed), whereas transformation of the S179A construct resulted in the same color change as wild-type Mei2, suggesting that Rad24 associated with Mei2 at Ser-438 but not at Ser-179. From these results, we conclude that Mei2 is the downstream target of Rad24, and that the compulsion to meiosis observed in Δ*rad24* cells is a result of the failure of inhibition of Mei2 activity and is the primary cause of the proliferation-deficient phenotype of these mutant cells. It appears that this putative inhibitory regulation of Mei2 by Rad24 plays an important role in progression to meiosis, since Ser-438 was previously identified as one of the phosphorylation targets of Pat1 kinase [16]. In normal *S. pombe* cells, Rad24 protein associates with the phosphorylated form of Mei2 and inhibits its ability to promote meiosis. Since Mei2 is a nuclear protein, Rad24 protein may recruit associated Mei2 away from the nucleus, in a manner similar to the regulation of another 14-3-3 association target, Cdc25 [6,8].

3.5. Rad25 is not involved in Mei2 regulation

Rad24 and Rad25 are very similar at the amino acid sequence level, and there is believed to be considerable functional overlap between these two proteins. Both *rad24*$^+$ and, to a lesser degree, *rad25*$^+$ deletion mutants are sensitive to UV and γ-irradiation, and Δ*rad24* Δ*rad25* double mutants are inviable [11]. It has been reported that Rad24 associates with a phosphorylated form of Cdc25, recruiting it from nucleus to cytoplasm by enhancing its nuclear export, and the two amino acid residues of Rad24 which are essential for nuclear export have been identified [10]. Although it remains to be determined whether Rad25 has a similar function with respect to Cdc25, Rad25 appears likely to act as a nuclear export signal since 39 sequential amino acid residues, including the two residues shown to be essential for nuclear export of Cdc25, are absolutely conserved between Rad24 and Rad25. Thus, it appears that Rad24 and Rad25 proteins operate in identical pathway(s) and have complementary functions in the regulation of common targets.

However, the function of Rad25 with respect to inhibition of Mei2 seems to be distinct from that of Rad24, since no association between Rad25 and Mei2 was observed in two-hybrid analysis (Fig. 4). Why then did the *rad25* deletion mutant also show a very low level of diploid colony formation (Fig. 1)? One possible explanation is that Rad24 may regulate Mei2 in vivo primarily as a heterodimeric complex with Rad25, in which Rad24 is essential to association with Mei2 while Rad25 plays a subsidiary role which is partly replacable by Rad24. In this model, no association of Rad25 homodimeric complex with Mei2 would occur in Δ*rad24* cells, and while Rad24 homodimers could associate with Mei2 in Δ*rad25* cells, this complex would not fully inhibit Mei2 function and would result in a leaky proliferative defect phenotype in Δ*rad25* cells. Rigorous testing of this model will be a future target of our research.

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