A Conserved RAS/Mitogen-Activated Protein Kinase Pathway Regulates DNA Damage–Induced Cell Death Postirradiation in Radelegans

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Abstract

Although the epidermal growth factor receptor (EGFR) signaling pathway is overactive in more than half of human cancers and mediates resistance to cytotoxic therapy, the molecular mechanisms of EGFR pathway–mediated resistance have remained elusive in cancer research. This difficulty partly stems from the lack of tissue models enabling clear separation of the many forms of cell death that the downstream signaling pathways of EGFR affect. We have created a model in *Caenorhabditis elegans* of radiation-induced reproductive cell death ("Radelegans") in isolation of all other forms of cell death. We have employed Radelegans to genetically define the role of the EGFR signaling pathway in protection from reproductive cell death, the primary form of tumor stem or clonogen cell death postirradiation. We have found that the RAS/mitogen-activated protein kinase (MAPK) downstream signal transduction pathway of EGFR is critical for protection from reproductive cell death in Radelegans. In addition, we have shown that RAS/MAPK pathway signaling is genetically linear with the DNA damage response pathway and acts downstream of the DNA damage checkpoint in the radioresponse, implicating this pathway in DNA repair post-cytotoxic therapy. These findings support the hypothesis that enhanced repair is a mechanism of RAS/MAPK pathway–mediated resistance to cytotoxic therapy through its interaction with the DNA damage response pathway postirradiation. We postulate that these findings also help explain why current treatment strategies, based on the presumption that tumors have ineffective repair compared with normal tissues, are ineffective in EGFR/RAS/MAPK pathway–mediated tumors. Radelegans is a platform to further define the genetic basis of the radiation response in tissues. (Cancer Res 2006; 66(21): 10434-8)

Introduction

More than 700,000 people will be treated with radiation in 2006 as part of their definitive therapy for newly diagnosed cancer, with more than half of these people failing curative treatment and receiving additional radiation for palliation. Tumor and tissue "clonogens" are the critical targets of radiation (1, 2), with their elimination leading to both normal tissue damage and tumor cure. Clonogens die via reproductive cell death (3, 4), which results primarily from the induction of DNA double-strand breaks (5). The inherent sensitivity to radiation therefore depends on the ability of tissue clonogens to regulate progression through cell cycle checkpoints in coordination with the DNA repair process, referred to collectively as the DNA damage response pathway (6, 7). This theory has been recently validated in a tissue model of reproductive cell death, referred to as Radelegans (3).

Because human cancers often arise secondary to mutations in the DNA damage response pathway (8), they are hypothesized to be more sensitive to cytotoxic therapy–induced reproductive cell death than normal tissues. Whereas this is the paradigm around which cytotoxic therapy is delivered, with incorporated breaks or "fractionation" to allow normal tissue recovery and simultaneous tumor elimination, this treatment regimen fails in certain tumor types. An example are tumors mediated by activating mutations or overexpression of components of the epidermal growth factor receptor (EGFR) signaling pathway, accounting for more than half of newly diagnosed cancers, all of which are notoriously resistant to cytotoxic therapy (9, 10). In fact, the two primary downstream signal transduction pathways of EGFR, RAS/mitogen-activated protein kinase (MAPK) and phosphatidylinositol 3-kinase/AKT, are both implicated in the radiation response. Activation of the EGFR/RAS/MAPK pathway by radiation has been described: radiation causes phosphorylation of EGFR (11); the association of growth factor receptor binding protein 2 with SOS; an increase in Ras-GTP binding; and activation of Raf (12), MAPK/extracellular signal-regulated kinase kinase (MEK), and then MAPK.

The downstream mechanisms of EGFR pathway signaling that lead to enhanced cell survival post-cytotoxic therapy remain poorly defined. A central hypothesis is that EGFR signaling modulates the DNA damage response pathway (13), yet how, when, and which EGFR downstream signaling pathways interact with the DNA damage response is unknown. One theory is that EGFR signaling affects the DNA damage checkpoint, based on a correlation between radiation-induced EGFR signaling and the length of the G_2 checkpoint (14). Others propose that EGFR signaling postirradiation instead directly affects DNA repair, based on the following evidence: DNA damage repair genes are up-regulated after EGFR/RAS/MAPK signaling (15, 16); there is a measurable decrease in DNA repair with EGFR pathway inhibition (17–21); and EGFR signaling leads to activation of the nonhomologous DNA repair gene complex, DNA-dependent protein kinase, after translocation to the nucleus postirradiation (22–24). However, genetic evidence of the molecular order and the components of the EGFR and DNA damage response pathways that interact in the radiation response does not exist, primarily due to the inherent difficulty in doing

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epistasis analysis in mammalian cells or tissues, the paucity of appropriate knockout or transgenic mutants, and the previous lack of a genetic model of radiation-induced reproductive clonogen cell death.

To address these issues, we have recently developed a model of reproductive clonogen cell death in the nematode *Caenorhabditis elegans*, referred to as Radelegans (3). Reproductive cell death in Radelegans occurs in the vulva, a tissue that arises from 22 cells that are the descendants of three vulval precursor cells, P5.p, P6.p, and P7.p (25). These vulval precursor cells, which mimic mammalian tissue clonogens, respond to an inductive signal, LIN-3/EGF, and their divisions are mediated via an EGFR/RAS/MAPK pathway. The EGFR/RAS/MAPK signaling pathway in *C. elegans* is highly conserved with human EGFR/RAS/MAPK signaling, as shown by the high degree of homology between the key proteins in this pathway, such as *C. elegans* EGFR and human erbB-4 (E value = 7.8×10^{-134}, >89.7% of the sequence); *C. elegans* RAS and human KRAS (E value = 2.2×10^{-75}, >99.5% of the sequence length); *C. elegans* RAF and human B-RAF (E value = 1.6×10^{-123}, >74.5% of the sequence length); *C. elegans* MEK-1 and human MEK-7 (E value = 7.3×10^{-87}, >87.6% of the sequence length); *C. elegans* MEK-2 and human MEK-2 (E value = 3.7×10^{-103}, >97.2% of the sequence length); and *C. elegans* MPK-1 and human MAPK-1 (E value = 1.3×10^{-154}, >76.1% of the sequence).[4] The E value represents the probability due to chance that there is another alignment with a similarity greater than that given for a tested alignment and is approximately equivalent to a P value when $E < 0.01$.[5] Thus, an E value of $>10^{-5}$ is considered statistically significant.[6]

We have previously shown that delivering radiation to vulval precursor cells before their three synchronized cell divisions results in cell death after completion of these divisions, which is morphologically and genetically consistent with reproductive cell death, and results in abnormal vulval structures of which the proportion represents radiosensitivity (3). Reproductive cell death in Radelegans is dependent on the *C. elegans* DNA damage response pathway, as would be predicted from historical modeling of reproductive cell death (3, 5, 7). The *C. elegans* DNA damage response pathway is also highly conserved to the mammalian DNA damage response pathway, as evidenced by functional genomic mapping (26) and the requirement of the *C. elegans* DNA damage response for cell cycle arrest postirradiation (27).

We have now employed Radelegans to define the role of the EGFR downstream signal transduction pathways in reproductive cell death and to genetically order the EGFR and DNA damage response pathways in the radiation response in tissues. Radelegans allows the genetic dissection of the molecular mechanisms of the radiation response, with the potential for identifying and testing means to manipulate this response in tumors as well as in normal tissues.

Materials and Methods

Mutations and strains. Methods for culturing, handling, and genetic manipulation of *C. elegans* were as described by Brenner (28) unless otherwise indicated. The animals referred to here as wild-type *C. elegans* correspond to the Bristol strain N2. Strains used in this study were obtained from the *C. elegans* Genetics Center unless otherwise noted. EGFR pathway mutants were *soc-2(ku167)IV*; *sur-6(ku123)I*; *ksr-1(n2682)X*; *ksr-2(dx27)I/hT2[qIs48](I;III)*; *lin-45(n2520);unc-24(e138)IV* (Kerry Kornfeld); *mek-1(ks54)X*; *mek-2(ku114)I* (Min Han); *mpk-1(ku1);unc-32(e189)III*; *pmk-3(ok169)*; *akt-1(ok525)V*; and *akt-2(ok393)X*. Double mutants were *cdc25.3(ok358); hus-1(op241)* (David Eisenmann) and *cdc25.3(ok358); mek-2(ku114)*. Many alleles used in these studies are hypomorphic, rather than null alleles.

Synchronization and radiation of *C. elegans*. For synchronization, gravid hermaphrodites are digested with a NaOH and bleach solution; embryos are plated and grown at 20°C for 14 hours; larvae are transferred to *E. coli* OP50-seeded plates (1 hour old on placement on food). For radiation, *C. elegans* are placed in a 15-mL conical tube (Falcon) with OP50-seeded agarose and treated in the high-dose rate position in a ^{137}Cs irradiator (Mark I Model 68). After irradiation, *C. elegans* are immediately transferred to a fresh OP50-seeded plate and grown at 20°C to adulthood without disturbance and with adequate food.

Phenotypic characterization. Animals are anesthetized with 5 mmol/L levamisole HCl, placed onto 2% agarose pads, and examined using 40× Nomarski optics. All strains are normalized to their 0-Gy data point to rule out any vulval defects independent of irradiation.

Dose-response curves. Dose-response curves are generated at the first S-phase radioresistance peak (determined as previously described ref. 3) by dividing synchronized *C. elegans* populations into individual feeding plates and treating each dose point sequentially, with a start and an end same-dose control sample. For each dose, a minimum of 100 animals are treated and scored per experiment and experiments are repeated two to four times.

Strain construction and genetic analyses. Double mutants were generated using standard genetic methods (28). When necessary, the presence of both mutant alleles was confirmed by single-worm PCR and/or restriction enzyme digestion. Primers for *cdc-25.3*(*ok358*) and *hus-1(op241)* were designed based on *C. elegans* Genetics Center recommendations. A restriction digest with Hpy188 I was used to identify the *mek-2(ku114*) mutation.

RNA interference of genes of interest. After synchronization, animals are placed on plates with the appropriate bacterial strain containing the plasmid that overexpresses the gene of interest and grown until appropriate time for radiation. After irradiation, animals are placed on plates with the same bacterial strain and grown until phenotypic analysis.

Table 1. Significance of gene mutations on radiosensitivity

Protein (genotype)	Orthologue	*P*	*N*
Sensitive strains			
ksr-1(ku68)	KSR1	0.015	2,118
ksr-2(dx27)	KSR2	0.015	2,254
soc-2(ku167)	SHOC-2	0.042	1,517
lin-45(n2520)	RAF	0.012	2,672
mek-1(ks54)	MEK-7	0.010	2,133
mek-2(ku114)	MEK-2	0.001	2,668
mpk-1(ku1)	MAPK-1	0.004	2,194
Nonsensitive strains			
sur-6(ku123)	PP2A-B	0.288	1,681
pmk-3(ok169)	p38	0.100	1,824
akt-1(ok525)	AKT1	0.462	2,075
akt-2(ok393)	AKT2	0.443	1,927

NOTE: EGFR signaling pathway mutants. Significance was derived from the comparison of N2 animals (N = 2,658) and the listed strains exhibiting the WT phenotype across dose-response curves postirradiation. *N,* number of animals analyzed.

[4] http://www.wormbase.org/.
[5] http://www.ncbi.nlm.nih.gov/BLAST/tutorial/Altschul-1.html.
[6] http://www.osc.edu/research/bioinformatics/FAQ/evalue.shtml.

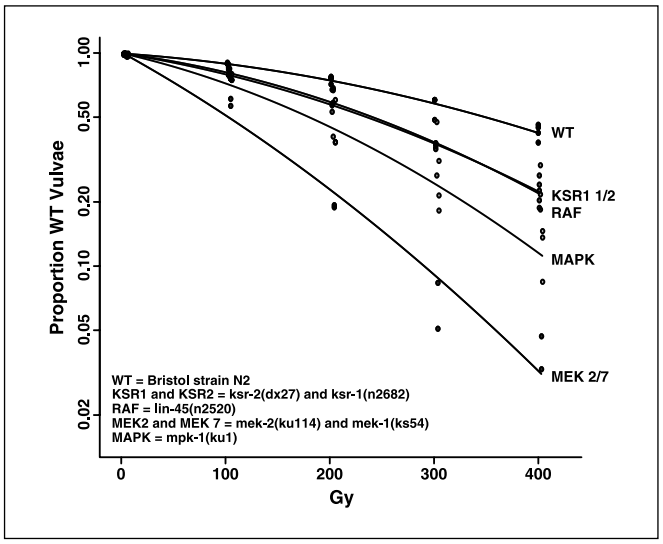

Figure 1. RAS/MAPK pathway mutants are sensitive to reproductive cell death. Mutations in proteins of the RAS/MAPK pathway lead to radiosensitivity, as depicted in a dose-response curve with percent WT vulvae postirradiation plotted against increasing dose. Results of the two KSR mutant strains and of the two MEK mutant strains were combined. *Points,* averaged values of individual sample results. Mammalian protein orthologues are listed at the end of the curves and *C. elegans* strain names are listed in the bottom left corner. $P < 0.01$, for all strains compared with wild-type animals.

Statistical analysis. Dose-response curves were obtained using a linear fit to the data (proportion of WT) after Box-Cox transformation. For each of the strains, the logarithm of the proportion of WT worms was modeled as a quadratic function dose of the form log (prop WT) = $A \times \text{dose} + B \times \text{dose}^2$. The least-squares fit of the coefficients A and B was used to draw the dose-response curves in the figures. Each of the mutant strains was compared against the WT using a stratified two-sample Wilcoxon rank sum test, which uses the test statistic obtained by taking the sum of the two sample rank sums for comparing the two strains within each dose level and standardizing them appropriately. Stratified t tests were done to analyze significance for all other cases.

Results and Discussion

Radelegans allows study of EGFR signaling in reproductive cell death. The EGFR/RAS/MAPK pathway is critical for normal vulval development in *C. elegans*, with its disruption leading to lack of vulval cell divisions resulting in vulval abnormalities. Because these types of vulval abnormalities resemble the radiation-induced cell death phenotypes found in Radelegans, to determine if we could test all EGFR pathway mutants in Radelegans, we began by studying a mild loss-of-function (*lof*) EGFR/RAS/MAPK pathway mutant strain, *mek-2(ku114)* (MEK-2). To determine any baseline abnormalities in vulval development in this EGFR/RAS/MAPK signaling mutant, we carefully evaluated *mek-2(ku114)* in the absence of radiation. We first did lineage analysis of the vulval cells in *mek-2(ku114)* ($N = 30$) to document that vulval cells are normally produced in this strain. We then studied *mek-2(ku114)* animals isolated at the L4 stage (post-vulval cell divisions and morphogenic movements) to determine if these cells underwent normal movements after divisions ($N = 50$). We found that normal vulval cell divisions and morphogenic movements occurred in *mek-2(ku114)*, even in the presence of its *lof* mutation in the EGFR/RAS/MAPK signaling pathway, suggesting that vulval cells develop normally and that these strains can be tested in Radelegans.

We then irradiated *mek-2(ku114)* animals and found this strain was significantly radiosensitive as compared with wild-type (N2) animals ($P < 0.001$). To confirm that *mek-2(ku114)* vulval cells die via reproductive cell death, we first examined *mek-2(ku114)* at the L4 stage postirradiation and found that the majority of L4 structures were normal (89%, $n = 100$), like wild-type animals postirradiation (3). This indicates that the great majority of vulval cells die after all cell divisions and morphogenic movements postirradiation in *mek-2(ku114)* and is consistent with reproductive cell death (3). Vulval cells in *mek-2(ku114)* animals were then followed postirradiation and found to die 3 to 5 days postirradiation, also consistent with reproductive cell death, as has previously been shown in Radelegans (3). This gradual postmitotic vulval cell death following irradiation is unlike the decreased vulval cell production one would see with insufficient EGFR/RAS/MAPK signaling. Therefore, these findings confirm that vulval cells in *lof* EGFR signaling pathway mutants die via reproductive cell death in Radelegans, thus indicating that these mutants can be appropriately tested in this system.

EGFR/RAS/MAPK pathway mutants are radiosensitive in Radelegans. We next surveyed all available strains with *lof* genetic mutations in the EGFR pathway without disruption of normal vulval development, including SHOC-2/*soc-2(ku167)*; KSR/*ksr-2(dx27)*; *ksr-1(n2682)*; RAF/*lin-45(n2520)*; MEK-2/*mek-2(ku114)*; MEK-7/*mek-1(ks54)*; MAPK-1/*mpk-1(ku1)*; PP2A (subunit B)/*sur-6(ku123)*; p38/*pmk-3(ok169)*; and AKT/*akt-1(ok525)*; *akt-2(ok393)*. For each strain, we did a spot check at the L4 stage to confirm normal vulval formation in the unirradiated control and normalized the radiated samples to the control untreated sample. We found that the RAS/MAPK pathway strains tested were significantly radiosensitive in our system (Table 1; Fig. 1). In contrast, strains with mutations in other EGFR downstream signaling transduction pathways (p38 or AKT) were not radiosensitive in Radelegans (Table 1).

A *C. elegans* strain containing a *lof* mutation in the RAS protein (with normal vulval development) does not exist. We therefore tested the ability of using RNA interference (RNAi) for

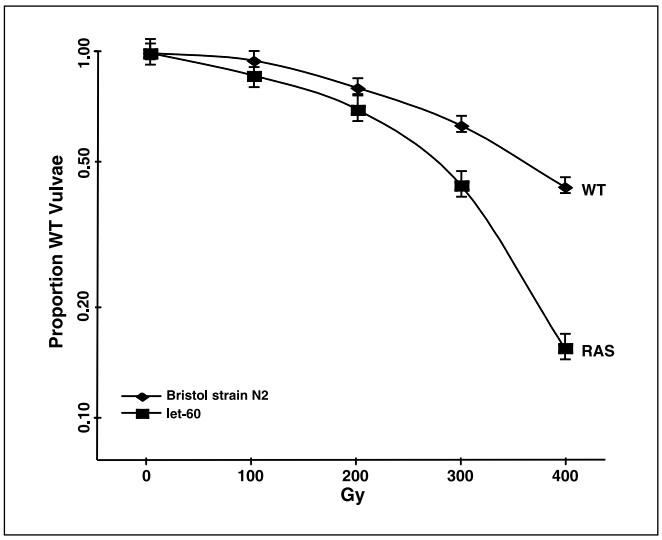

Figure 2. RNAi shows that RAS is required for radioprotection in Radelegans. RNAi was done against the RAS protein and animals underwent dose-response experiments. *Points,* mean of individual sample results; *bars,* SD. $P < 0.01$, for RAS RNAi compared with wild-type N2 animals fed a control vector.

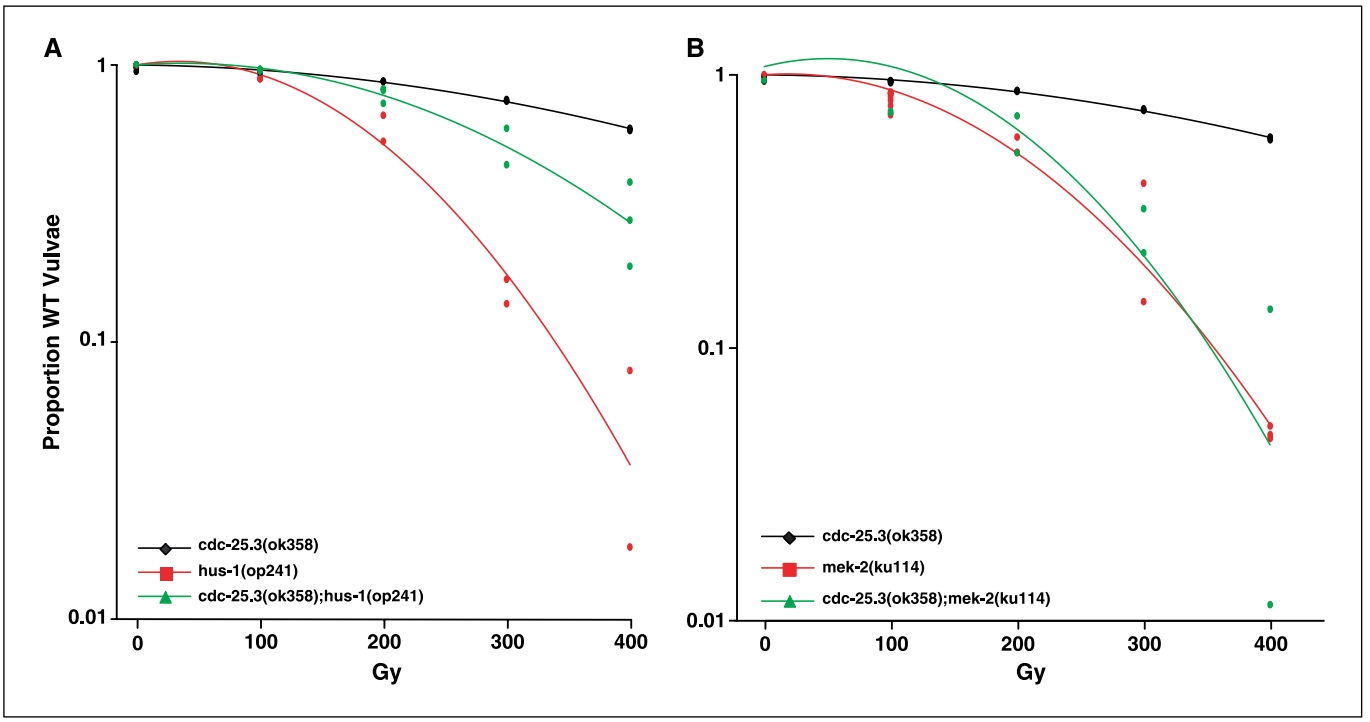

Figure 3. Epistasis analysis genetically orders the radioresponse. *A,* epistasis between the radiosensitive HUS-1 mutation and the radioresistant CDC25 mutation shows that CDC25 is epistatic to HUS-1. *B,* epistasis between the radiosensitive MEK-2 mutation and the radioresistant CDC25 mutation shows that MEK-2 is epistatic to CDC25.

this protein in Radelegans. RNAi was originally discovered in *C. elegans* and is a technology where the introduction of double-stranded RNA into the developing animal causes specific inactivation of the corresponding gene (for review, see ref. 29). Advances in RNAi delivery methods allow feeding *C. elegans* bacteria containing the RNA of interest. We used RNAi specifically for the RAS protein and found that the knockdown of RAS led to significant radiosensitivity in Radelegans (Fig. 2; $P < 0.01$). These findings indicate that RNAi can be used to determine the role of individual proteins in the radiation response in Radelegans.

Because many of the above strains do not contain null mutations of the genes of interest, their role in protection from reproductive cell death cannot be ruled out using Radelegans. However, the significant radiosensitive phenotype in strains exhibiting mild *lof* mutations or after RNAi of proteins of the RAS/MAPK pathway supports the hypothesis that RAS/MAPK is a critical downstream EGFR signaling pathway responsible for radioprotection from reproductive cell death in Radelegans.

Ordering of the RAS/MAPK and DNA damage response pathways. Because Radelegans is a model of reproductive cell death (which depends on the DNA damage response pathway; ref. 3), the finding that proteins of the RAS/MAPK signaling pathway are necessary for radioprotection in Radelegans supports the hypothesis that this pathway affects the DNA damage response pathway postirradiation. We therefore applied epistasis analysis to order these pathways in the radiation response.

We have previously ordered proteins of the DNA damage response pathway by creating and doing epistasis analysis in the double-mutant strain *cdc25.3(ok358); hus-1(op241)* (ref. 3) with the radioresistant *lof* mutant *cdc25.3(ok358)* and the radiosensitive *lof* cell cycle checkpoint mutant *hus-1(op241)*. Based on mammalian studies, we would predict that a *lof* CDC25 mutation (with a prolonged checkpoint) would be epistatic to a *lof* HUS-1 mutation. We found that *cdc25.3(ok358); hus-1(op241)* is in fact radioresistant, not significantly different from the *cdc25.3(ok358)* parental strain but significantly different from *hus-1(op241)* ($P < 0.02$; Fig. 3*A*),

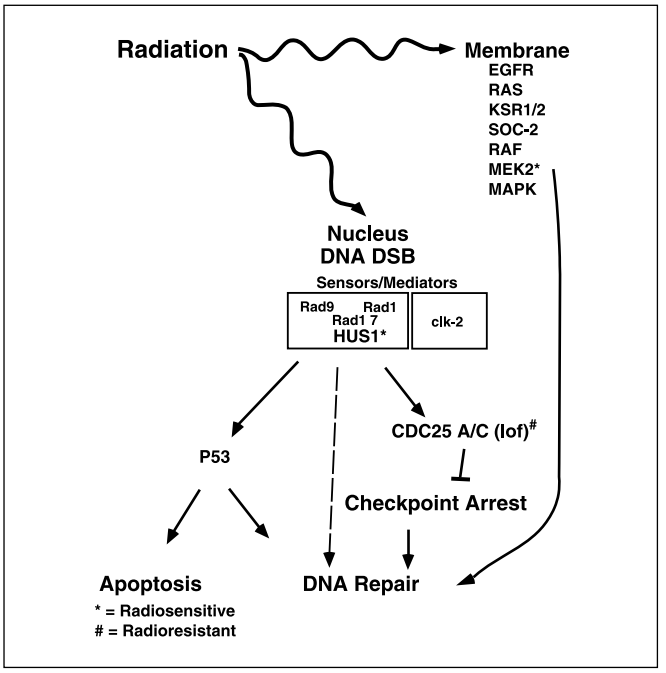

Figure 4. Model of the RAS/MAPK and DNA damage response pathways in reproductive cell death. Findings using Radelegans indicate that the RAS/MAPK and the DNA damage response pathways are genetically linear in the radioresponse and that RAS/MAPK signaling acts downstream of the cell cycle checkpoint in the radioresponse. Of note, HUS-1 is implicated in both the checkpoint and DNA damage repair (*dashed line*).

confirming the usefulness of epistasis analysis in ordering the radiation response in Radelegans.

To determine the existence of genetic ordering and epistasis between the RAS/MAPK and the DNA damage response pathways, we engineered a double-mutant strain harboring the radioresistant *lof cdc25.3(ok358)* mutation and the radiosensitive *mek-2(ku114)* mutation. We did epistasis analysis on this strain and found that *cdc25.3(ok358); mek-2(ku114)* was radiosensitive, not significantly different from the *mek-2(ku114)* parental strain but significantly different from *cdc25.3(ok358)* ($P < 0.02$; Fig. 3*B*; $N = 3{,}724$; Supplementary Table S1).

Our findings using Radelegans in the strain *cdc25.3(ok358); mek-2(ku114)* indicate that in the radiation response (*a*) the RAS/MAPK pathway acts linearly with the DNA damage response pathway; (*b*) prolonging the cell cycle checkpoint does not overcome the requirement of RAS/MAPK pathway signaling; and thus (*c*) RAS/MAPK pathway signaling acts downstream of the cell cycle checkpoint, implicating it in DNA repair (Fig. 4).

In summary, we have employed a tissue model of radiation-induced reproductive cell death in *C. elegans* (Radelegans) to study the role of the EGFR signaling pathway in the radiation response. We have found that the EGFR downstream signal transduction pathway RAS/MAPK is required for protection from radiation-induced reproductive cell death. Further, we have used epistasis analysis and showed that a radiosensitizing mutation in the RAS/MAPK signaling pathway protein MEK is epistatic to a radioresistant mutation in the cell cycle checkpoint protein CDC25, indicating that these pathways are linear. Our findings further indicate that RAS/MAPK signaling acts downstream of the DNA damage checkpoint in the radioresponse, implicating this transduction pathway directly in DNA repair postirradiation. Whereas there is evidence to support the role of the EGFR protein in nonhomologous DNA repair (22–24), there is no such genetic evidence for involvement of the RAS/MAPK signaling pathway in this process.

These findings support the hypothesis that excess signaling through the EGFR/RAS/MAPK pathway in tumors might act as a resistance mechanism via bypassing any inherent tumor sensitivity to cytotoxic therapy caused by ineffective checkpoint signaling. In addition, excess EGFR/RAS/MAPK signaling might enhance DNA damage repair, a hypothesis that should be further tested. Unfortunately, we are unable to directly test this hypothesis in Radelegans, secondary to abnormal primary vulval structures in animals harboring activating EGFR/RAS/MAPK pathway mutations. Regardless, our findings offer the first genetic proof for a proposed mechanism of EGFR/RAS/MAPK pathway–mediated resistance in an *in vivo* tissue model. We will continue to use Radelegans to determine the genetic means of radiation resistance to better define targets and to assist in designing and testing new strategies to overcome these forms of resistance.

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