Gene interactions in the DNA damage-response pathway identified by genome-wide RNA-interference analysis of synthetic lethality

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Here, we describe a systematic search for synthetic gene interactions in a multicellular organism, the nematode *Caenorhabditis elegans*. We established a high-throughput method to determine synthetic gene interactions by genome-wide RNA interference and identified genes that are required to protect the germ line against DNA double-strand breaks. Besides known DNA-repair proteins such as the *C. elegans* orthologs of TopBP1, RPA2, and RAD51, eight genes previously unassociated with a double-strand-break response were identified. Knockdown of these genes increased sensitivity to ionizing radiation and camptothecin and resulted in increased chromosomal nondisjunction. All genes have human orthologs that may play a role in human carcinogenesis.

DNA double-strand break | synthetic gene interactions | *Caenorhabditis elegans*

The availability of genome-wide gene inactivation approaches in model organisms such as *Saccharomyces cerevisae* (1, 2) and *Caenorhabditis elegans* (3, 4) has contributed immensely to the understanding of gene function, but a large fraction of genes remain unclassified. One explanation for the absence of apparent abnormal phenotypes upon gene inactivation is genetic redundancy, resulting from functional complementation by a similar gene or a parallel pathway (5), which can be uncovered by inactivation of two genes simultaneously, resulting in a so-called synthetic phenotype.

Synthetic genetic analysis has proved to be a powerful method to build gene-interaction networks in yeast (6–8). The nematode *C. elegans* is used widely as a multicellular model organism and is evolutionarily closer to humans than yeast, which, for example, lacks the main regulators of apoptosis, such as p53. Synthetic interactions have been identified successfully in *C. elegans* by using mutagenesis (9–11). However, this approach requires construction of a rescuing transgene and subsequent identification of the second-site mutation by means of time-consuming positional cloning, which hampers a broad applicability.

Currently, *C. elegans* genes can be inactivated systematically by feeding animals genetically engineered *Escherichia coli* clones that express double-stranded RNA for 86% of the genes encoded by the *C. elegans* genome (3, 4, 12). In principle, this powerful genetic tool allows for (semi-)automated high-throughput analysis of biological function.

Here, we report the use of high-throughput RNA interference (RNAi) for systematic identification of synthetic gene interactions. We identified genes that are involved in the cellular response to DNA double-strand breaks (DSBs); cells respond to DSBs through the actions of systems that detect the DNA damage, subsequently triggering various downstream events, including repair. Such genes are of great clinical importance: inaccurate repair of DSBs can lead to mutations or to larger-scale genomic instability (i.e., translocations or aneuploidy) with accompanying tumorigenic potential. Indeed, many genes involved in repair and/or signaling of DSBs are causally implicated in cancer (13).

Materials and Methods

Strains. We used the following *C. elegans* strains: wild-type Bristol N2, NL1832(*pk732*), and TY1774 *yIs2*[*xol-1*::*lacZ rol-6*(pRF4)] IV.

Synthetic Lethality Assay. In the pilot experiment, we tested 74 DNA damage-response genes that were used as "bait" by Boulton *et al.* (14), which were present in the Ahringer RNAi library (4). RNAi bacteria from an overnight culture in Luria broth medium containing 50 $\mu g \cdot ml^{-1}$ ampicillin were induced with 0.25 $mg \cdot ml^{-1}$ isopropylthiogalactoside at 37°C for 4 h and then seeded on 4-cm nematode growth-medium plates containing 50 $\mu g \cdot ml^{-1}$ ampicillin and 200 $\mu g \cdot ml^{-1}$ isopropylthiogalactoside. We placed ≈30 synchronized L1 larvae on fresh RNAi plates and transferred 3 × 3–5 animals to a single RNAi plate after 3 days of growth at 20°C. These animals were allowed to lay eggs for 1 day. We removed the parents and counted dead eggs after 24 h and offspring after 48 h.

RNAi in Liquid 96-Well Culture. We inoculated 96-well-deep blocks with 500 μl of Luria broth medium containing 50 $\mu g \cdot ml^{-1}$ ampicillin per well, grew the cultures overnight at 37°C, and induced the bacteria as described above. Worm cultures were synchronized by bleaching and hatching in M9 at 20°C overnight. For RNAi cultures in liquid, we put 10–20 L1 larvae in 50 μl of $M9^+$ per well of a flat-bottom 96-well tissue-culture plate. $M9^+$ buffer consists of M9 buffer with 10 $\mu g \cdot ml^{-1}$ cholesterol/50 $\mu g \cdot ml^{-1}$ ampicillin/12 $\mu g \cdot ml^{-1}$ tetracycline/200 $\mu g \cdot ml^{-1}$ isopropylthiogalactoside/0.1 $\mu g \cdot ml^{-1}$ fungizone. We added 100 μl of induced bacterial suspension per well and grew the RNAi cultures at room temperature while shaking at 150–200 rpm. We scored worm cultures after 5 days for growth. Genome-wide, we found 929 foods that give a reduction of growth. This set showed an overlap with 68% (588 of 865) of the foods that have been reported to give sterility and/or >50% lethality (4). Screening 16,757 clones resulted in a total of 32 genes that scored positive in the synthetic lethality assay (Table 1 and Table 2, which is published as supporting information on the PNAS web site). The identity of all positive clones was verified by sequencing. To exclude an *rrf-3*-like hypersensitivity to RNAi, we compared the published RNAi phenotypes of the 32 positive genes in N2 and *rrf-3*(*pk1426*) background and found only 5 of 32 foods to give a >50% lethality in *rrf-3*. Given the fact that the set of 32 was preselected against lethality in N2 and the variance in RNAi screens, these data indicate that NL1832 does not show an *rrf-3*-like hypersensitivity to RNAi.

Radiation-Sensitivity Assay. Synchronized wild-type L1 worms were grown on RNAi foods in liquid for 2 days at 20°C, as

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Abbreviations: DSB, double-strand break; RNAi, RNA interference.

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Table 1. Genes that show synthetic lethality with the mutator phenotype with ionizing radiation and camptothecin

Category	Cosmid no.	*C. e.*	NL1832	Irradiation	Camptothecin	*H. s.*	*S. c.*	Description
DSB response	Y43C5A.6	*rad-51*	+	+	+	RAD51	RAD51	Binds single-stranded DNA during DSB repair by homologous recombination.
DSB response	M04F3.1	—	+	+	+	RPA2	RFA2	Plays essential role in DNA replication, nucleotide-excision repair, and homologous recombination.
DSB response	F37D6.1	—	+	+	+	TOPBP1	—	DNA topoisomerase IIβ-binding protein, colocalizes with DNA DSBs, substrate of ATM kinase.
Protein degradation	Y65B4BR.4A	—	+	+	+	WWP2	RSP5	E3 ubiquitin ligase, *S. pombe* homologue is involved in targeted degradation of cdc25.
Protein degradation	Y41C4a.10	*elb-1*	+	+	—	TCEB2	—	Ubiquitin-like protein, binds von Hippel-Lindau tumor-suppressor complex and thereby inhibits transcription elongation.
Protein degradation	H19N07.2A	—	+	+	—	USP7, HAUSP	UBP15	Ubiquitin specific protease, stabilizes p53 levels.
Protein degradation	Y67D8C.5	—	+	+	—	UREB1, LASU1	TOM1	E3 ubiquitin ligase with a HECT domain.
Protein degradation	C52D10.9	*skr-8*	+	+	—	SKP1A*	SKP1*	Member of the SKP1 family of proteins, part of E3 ubiquitin ligase complex.
Other	F33H1.3	—	+	+	—	WBP11, SNP70	—	Contains a WW binding domain.
Other	K03H1.2	*mog-1*	+	—	+	PRP16, DDX38	PRP16	Protein required for switch from spermatogenisis to oogenesis.
Other	C27F2.10	—	+	+	+	FLJ11305	THP1	Contains a transcription associated recombination domain. *S. c.* THP1 shows strong hyperrecombination phenotype.

C. e., C. elegans; H. s., Homo sapiens; S. c., S. cerevisiae.
*Homolog instead of ortholog. In this case, the reciprocal BLAST did not return the original *C. elegans* gene as a first hit.

described above. L4 animals were subsequently irradiated at 60 Gy, and five or six animals were transferred to a nematode growth-medium plate containing the corresponding RNAi food. These animals were allowed to lay eggs for 2 days. We removed P_0 animals, and we counted eggs after 24 h and offspring after 48 h to calculate the percentage of lethality. As a control experiment, we tested 40 genes from plate 72 of the Ahringer library (including C27F2.10), and we found one food other than the positive control giving increased radiation sensitivity.

Camptothecin-Sensitivity Assay. We performed RNAi and determined the percentage of lethality exactly as was done in the radiation-sensitivity assay (described above). L4 animals were exposed to 0.14 mM camptothecin (Sigma) in M9 containing 0.5% DMSO for 2 h. Control animals were treated with 0.5% DMSO in M9. Pictures were taken 24 h after exposure to camptothecin. In a control experiment (see above), we found two additional foods yielding camptothecin sensitivity.

Chromosomal-Instability Assay. We grew ≈50 *yIs2* [*xol-1*::*lacZ rol-6*(pRF4)] L1 larvae in liquid RNAi until they reached late L4 stage. These animals were irradiated at 60 Gy and transferred to fresh RNAi agar plates. These plates were checked for the absence of males. We stained for β-galactosidase activity with X-gal 24 h after irradiation, counted the number of blue eggs per worm, and photographed the animals. Only animals containing >10 eggs were included. We choose the *xol-1* assay as an assay for chromosomal loss after irradiation instead of counting percentage of males or directly observing chromosomes in oocytes because it is quantitative, scalable, and usable in combination with dead or dying embryos.

Results

In our experimental setup, we screened for synthetic lethality by using a *C. elegans* strain (NL1832) that displays a mutator phenotype (an increased level of spontaneous mutations) as a result of DNA transposons jumping freely in its germ-line lineage (15). The *C. elegans* genome contains many active DNA transposons that are normally silenced in the germ line; loss of this silencing causes increased levels of DSBs as transposons excise from the genome (15, 16). Most transposon mutators are defective for RNAi; however, NL1832 is the strongest mutator strain that is completely RNAi proficient. This strain contains a mutation in the gene T24C4.1, which has been identified as a mutator gene by means of RNAi (17). We found a mutation in a highly conserved amino acid in the peptidase family M16 domain (G51R): a drastic amino acid change that is likely to affect protein function. How T24C4.1 plays a role in transposon silencing is unclear. As a consequence of enhanced DSBs in the mutator background, we hypothesized that inactivation of a gene essential for DSB repair by RNAi could lead to lethality or a severely reduced brood size in NL1832 animals but not in a wild-type background.

To test this hypothesis, we performed RNAi against a broad spectrum of DNA damage-response genes (74 in total), such as nucleotide-excision repair, mismatch repair, base-excision repair, nonhomologous end joining, homologous recombination, and checkpoint pathways (14). Three genes known to be required specifically for DSB repair were found to show synthetic lethality

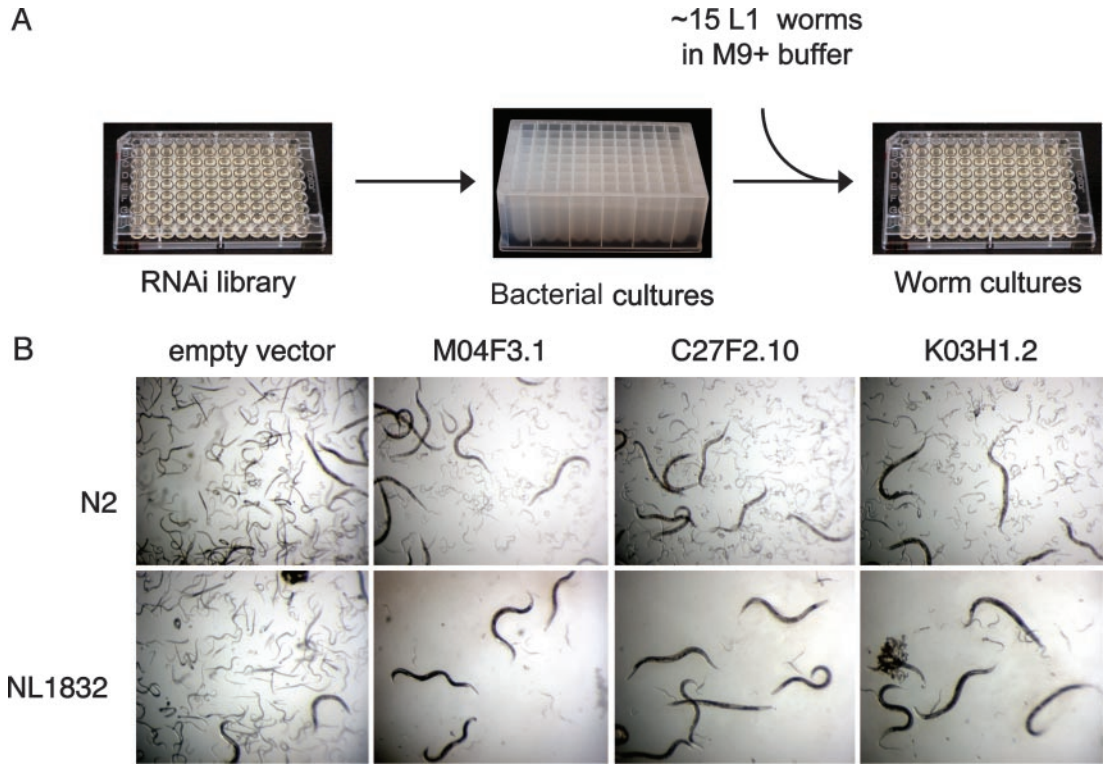

Fig. 1. RNAi by feeding in 96-well-format liquid cultures. (*A*) Schematic representation of the liquid RNAi-feeding protocol. The bacterial RNAi library is in a 96-well format, which is used to inoculate bacterial cultures overnight. This bacterial suspension is added to ≈15 L1 larvae in $M9^{+}$ buffer. The worm cultures are shaken at 20°C for 4–5 days and scored by visual inspection for wells with reduced growth. To facilitate scoring, N2 control and NL1832 are loaded in wells next to each other. (*B*) Examples of worm cultures showing synthetic lethality. (*Upper*) Wild-type worms fed on empty vector (M04F3.1, C27F2.10, and K03H1.2 double-stranded RNA producing clones). (*Lower*) NL1832 worms.

with DNA transposition, namely, *rad-51*, M04F3.1, and F37D6.1 (see Fig. 4, which is published as supporting information on the PNAS web site). *rad-51* is the *C. elegans* homolog of mammalian RAD51, which binds single-stranded DNA during repair of DSBs by means of homologous recombination (13). M04F3.1 is the homolog of human RPA2, a subunit of the heterotrimeric replication protein A. Replication protein A is known to enhance the single-stranded DNA-binding activity of RAD51 (18). M04F3.1 is the only subunit of the *C. elegans* replication protein A for which RNAi knockdown results in a viable phenotype. F37D6.1 is the *C. elegans* homolog of TopBP1, a protein that interacts with DNA topoisomerase IIβ and colocalizes with DNA DSBs (19).

We next used RNAi on a high-throughput, genome-wide scale by culturing animals in liquid 96-well format, with each well containing an *E. coli* strain expressing double-stranded RNA directed against a different *C. elegans* gene (Fig. 1*A*). Because glycerol stocks, bacterial cultures, and worm cultures are in a 96-well format, the number of practical steps was reduced to a minimum. All liquid handling was done with regular 12-channel and repeating pipettes. This setup enables one to screen the RNAi library (4), consisting of *E. coli* strains producing double-stranded RNA against 16,757 of the 19,427 predicted individual *C. elegans* genes within 5 weeks. To compare the effectiveness of RNAi via liquid culture with culturing on conventional solid agar plates, we scored clones that reduced the brood size of wild-type N2 animals in liquid culture and compared these with published data for solid culturing (4). This comparison led to an overall confirmation rate of 68% for clones inducing a strong nonviable phenotype. Although this result may suggest that the liquid approach is less efficient, similar levels (75%) of interexperimental fluctuations have been reported in comparing solid-plate-based genome-wide RNAi screens (12).

In the genome-wide screen for synthetic lethality, assaying the wild-type N2 control and NL1832 animals side-by-side facilitated scoring by visual inspection. We scored RNAi foods as positive if the brood size of the NL1832 culture was reduced significantly compared with the wild-type control (Fig. 1*B*). Positives were repeated in triplicate, omitting genes whose knockdown by RNAi is already known to result in sterility or high embryonic lethality in wild-type animals (3, 4) to avoid effects of cumulative lethality. Positive clones were subsequently quantified for synthetic lethality on agar plates (Fig. 4). Screening 16,757 clones resulted in a total of 32 genes showing synthetic lethality with the mutator phenotype (Tables 1 and 2), including two of the three genes identified in the pilot screen.

To test which genes are genetically downstream of transposon-induced DSBs, we generated DSBs in two other ways: by ionizing radiation and by camptothecin. Although ionizing radiation induces a broad spectrum of DNA lesions, DSBs are considered to be the main cytotoxic lesions (20). We found that inactivation of 10 of the 32 genes synthetic to the mutator phenotype caused a clear increase in embryonic lethality after irradiation (Fig. 2*a* and Table 1). Camptothecin inhibits the release of DNA topoisomerase I from DNA, leaving a single-strand break. When a DNA replication fork collides with this complex, the single-strand break is converted to a DSB. Because active replication is required to generate camptothecin-induced DSBs, its main cytotoxic effects take place during S phase (21). In yeast, camptothecin induces a strong cell-cycle arrest (22). We found that camptothecin also induces a cell-cycle arrest in *C. elegans* (Fig. 2*b*). RNAi against the 32 previously identified genes yielded 6 genes that were sensitive to camptothecin (Fig. 2*c* and

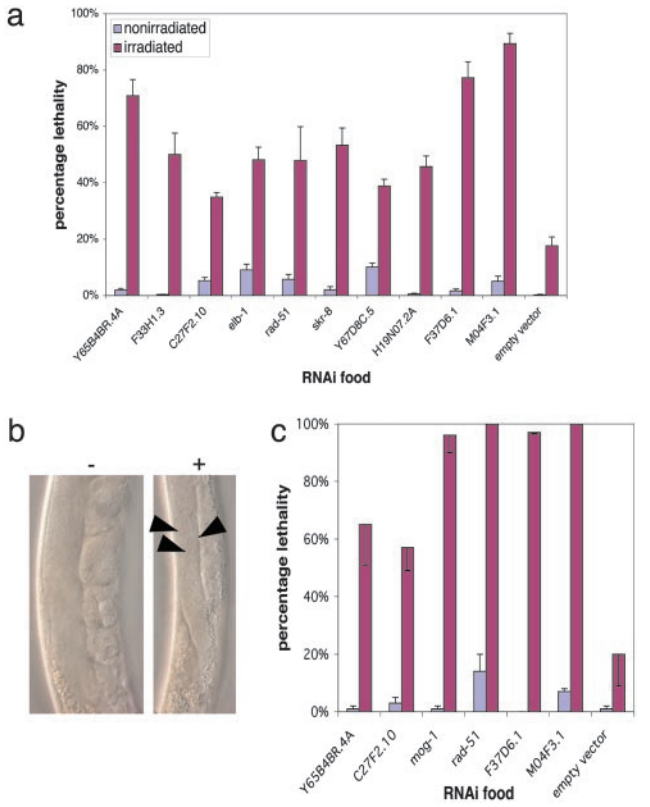

Fig. 2. Genes showing synthetic lethality with NL1832 are also sensitive to DNA breaks induced by radiation and camptothecin. (*a*) Radiation sensitivity. L4 worms fed on RNAi foods were irradiated with 60 Gy. The percentage of lethality was determined by counting the number of dead eggs in the total brood. All 32 genes that show synthetic lethality with NL1832 were tested. Only genes with an increased sensitivity to ionizing radiation are shown. Data represent mean ± SD of five to six experiments. (*b*) Camptothecin induces cell-cycle arrest in *C. elegans*. Staged animals treated with 0.14 mM camptothecin for 2 h were photographed after a 24-h recovery. Camptothecin-treated worms show less germ-cell nuclei and the size of remaining nuclei and their cytoplasm becomes greatly enlarged (arrows). Furthermore, the absence of mature oocytes is evident in camptothecin-treated worms. (*c*) Camptothecin sensitivity. Worms were treated, and the percentage of lethality was determined as described above. Blue bars indicate lethality in broods from mock-treated animals, and red bars represent lethality in broods from camptothecin-treated worms.

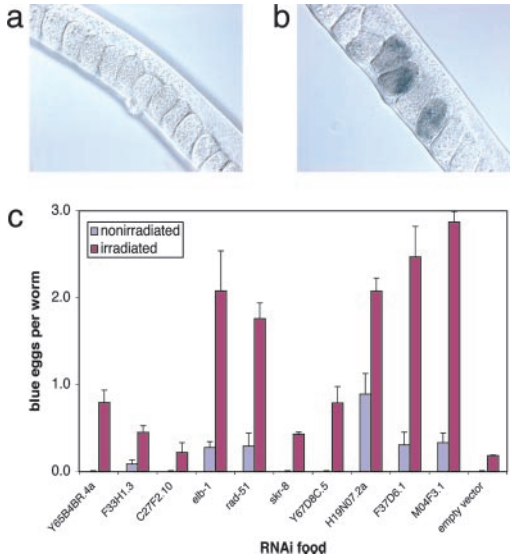

Fig. 3. Knockdown of genes conferring radiation sensitivity results in increased radiation-induced X-chromosomal nondisjunction. *Xol-1*::*lacZ* (*yIs33*) worms were fed RNAi foods that have been found to cause radiation sensitivity. (*a* and *b*) Worms were irradiated at L4 stage and stained for galactosidase activity after 24 h. As an example, worms fed on empty-vector control (*a*) and M04F3.1 food (*b*) are shown. (*c*) The number of blue eggs per worm was counted for all 10 RNAi foods that cause radiation sensitivity. Data represent mean ± SD of three experiments.

Table 1). Knockdown of five of these six genes causes sensitivity to both ionizing radiation and camptothecin. As a negative control, 40 randomly picked genes were tested for ionizing radiation and camptothecin sensitivity, yielding one and two positives, respectively, demonstrating the specific enrichment of DNA damage-response genes in our primary screen.

We next investigated chromosomal aberrations resulting from irradiation in the RNAi knockdowns. Expression of the *C. elegans* gene *xol-1* reflects the X-chromosome-to-autosome ratio during early embryogenesis and triggers male (XO) or hermaphrodite (XX) development (23). In a male, embryo *xol-1* is expressed, but in hermaphrodite embryos, which constitute 99.8% of the wild-type brood, *xol-1* is silent. We used transgenic animals that carry a *LacZ* reporter gene driven by the *xol-1* promoter (23), as a marker for X-chromosomal nondisjunction. We expected that loss of an X-chromosome due to an improper DSB response would activate the *xol-1* gene and, thus, show up as a blue egg upon staining animals for β-galactosidase activity. Indeed, RNAi against 9 of the 10 radiation-sensitive genes resulted in an increased number of embryos with chromosomal aberrations upon irradiation (Fig. 3). This experiment demonstrates that the genes that we have identified in a screen for synthetic lethality with transposon-induced DNA damage are required to prevent chromosomal aberrations after exogenously induced DSBs.

Discussion

The first class of genes, which is introduced in Table 1, consists of genes known to play a role in the DSB response, namely *rad-51*, M04F3.1, and F37D6.1 (described above). Interestingly, most of the newly identified genes are expected to play a role in targeted protein degradation. Y65B4BR.4A is a ubiquitin E3 ligase. The *Schizosaccharomyces pombe* homolog of Y65B4BR.4A is involved in the targeted degradation of CDC25 (24), which is an important effector in the DNA-damage-checkpoint response. H19NO7.2a is the *C. elegans* homolog of mammalian USP7/HAUSP, a ubiquitin-specific protease that stabilizes p53 levels (25). Aberrations in both p53 and CDC25A are found in many types of human cancer (26). The three other members of the protein degradation class are *elb-1*, Y67D8.5, and *skr-8*. *elb-1* encodes a ubiquitin-like protein, the *S. cerevisiae* homolog of Y67D8.5 is an ubiquitin E3 ligase (27), and *skr-8* is a member of the SKP1-related family. Mammalian Skp1 functions as a core component of the Skp1-Cdc53/Cul1-F-box (SCF) protein E3 ubiquitin ligase complexes, which mediate the degradation of a range of proteins such as cell-cycle regulators and transcription factors (28). Several DSB response factors are regulated by ubiquitination, such as RAD51, histones, CDC25A, and p53 (29). Our data support the hypothesis that DSB-response pathways are regulated by means of such degradation (29), perhaps with proteasomal targeting serving as an important on/off switch; lack of regulation of the DSB response is only harmful at a threshold amount of DNA damage, which explains the wild-type phenotypes being observed without DNA damage.

In the genome-wide screen, we identified 32 genes that show synthetic lethality with the mutator phenotype. Of these genes, 11 genes were confirmed in secondary assays, namely, radiation

sensitivity (10 genes), camptothecin sensitivity (6 genes), and increased chromosomal nondisjunction after irradiation (9 genes). There are several explanations for the fact that not all RNAi knockdowns of the genes that we identified in the primary screen are sensitive to exogenously induced DSBs. First, these genes do not necessarily function genetically downstream of transposon-induced DSBs; for example, knockdown of a gene involved in chromosome organization might result in a higher accessibility of the DNA to the transposase, resulting in increased lethality in an transposon-activated background. In addition, DSBs induced by transposition, radiation, and camptothecin have different characteristics, such as cell-cycle phase and the time window in which they are induced. It is unknown at which stage during the cell-cycle transposons excise from the genome; camptothecin induces DSBs during S phase, whereas radiation is expected to induce breaks at all cell phases, explaining both the smaller subset of camptothecin resistance genes (6 vs. 10 genes) and the large overlap with the radiation resistance genes (5 of 6 genes). Also, we cannot exclude the possibility that some of the observed synthetic lethal interactions are due to synthetic effects with mutations in the mutator background and, thus, are not related directly to activated transposition in the germ line.

It is difficult to speculate on the ''success rate'' of this screen and how many genes were not identified. Obviously, because RNAi is a knockdown and not a knockout approach, genes have been missed. However, using RNAi could also be considered as an advantage because some of the genes that we identified are expected to be essential and would have been overlooked (for example, in reverse genetic approaches). We also compared our results with a study that used phylogenetic comparison and two-hybrid interaction data to identify *C. elegans* genes that act in response to DNA damage (14), and we found four genes to be present in both data sets. Apart from biological differences, the limited overlap also could result from the relatively mild stress induced by transposon hopping. Perhaps a broader range of DNA damage-response genes would result from screening with more severe DNA-damaging conditions, such as ionizing radiation.

To our knowledge, many of the genes that we identified have not been found previously in screens for sensitivity to DNA damage in yeast (or bacteria). In some cases, this absence of overlap is explained by the lack of a clear *S. cerevisiae* ortholog. However, another reason could be that complete loss of the gene product is incompatible with growth. Indeed, four genes proved to be essential in yeast, and the absence of such essential genes in yeast knockout arrays is a recognized drawback (6). Because RNAi is temporal and, perhaps more important, not completely penetrant, a higher fraction of genes can be tested in *C. elegans*. Reverse genetic approaches in yeast and worms are complementary, and a future cross-species comparison of synthetic gene relations will help to identify highly conserved interactions, as seen for two-hybrid data (30). Furthermore, genome-wide high-throughput RNAi permits efficient detection of chemical–genetic interactions, as shown for camptothecin in this study.

We have set up a protocol for screening for synthetic gene interactions in *C. elegans* and provided proof of concept by the identification of 11 genes that protect cells against genomic instability. The molecular nature of these genes implies that specific targeting of protein degradation is an important regulator of the DSB response. Further understanding of these genes may help us to understand mechanisms underlying genomic instability in cancer and yield putative anticancer drug targets. In principle, this protocol is applicable in combination with any viable knockout and allows the simultaneous screening of multiple strains, thus providing a platform for the construction of gene-interaction networks.

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