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.

Materials and Methods

Strains. We used the following C. elegans strains: wild-type Bristol N2, NL1832(pk732), and TY1774 yh2[zol-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 μg/ml ampicillin were induced with 0.25 mg/ml isopropyl-β-D-thiogalactoside at 37°C for 4 h and then seeded on 4-cm nematode growth-medium plates containing 50 μg/ml ampicillin and 200 μg/ml isopropyl-β-D-thiogalactoside. 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深渊-blocks with 500 μl of Luria broth medium containing 50 μg/ml 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 0.1 μg/ml ampicillin/50 μg/ml isopropyl-β-D-thiogalactoside/1 μg/ml 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-fold-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
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 P0 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 growth-medium plate containing the corresponding RNAi food.

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 a strain (NL1832) that displays a mutator phenotype.

<table>
<thead>
<tr>
<th>Category</th>
<th>Cosmid no.</th>
<th>C. e.</th>
<th>NL1832</th>
<th>Irradiation</th>
<th>Camptothecin</th>
<th>H. s.</th>
<th>S. c.</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>DSB response</td>
<td>Y43C5A.6</td>
<td>rad-51</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>RADS1</td>
<td>RADS1</td>
<td>binds single-stranded DNA during DSB repair by homologous recombination.</td>
</tr>
<tr>
<td>DSB response</td>
<td>M04F3.1</td>
<td>—</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>RPA2</td>
<td>RPA2</td>
<td>plays essential role in DNA replication, nucleotide-excision repair, and homologous recombination.</td>
</tr>
<tr>
<td>DSB response</td>
<td>F37D6.1</td>
<td>—</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>TOPBP1</td>
<td>—</td>
<td>DNA topoisomerase I1β-binding protein, colocalizes with DNA DSBs, substrate of ATM kinase.</td>
</tr>
<tr>
<td>Protein degradation</td>
<td>Y65B4BR.4A</td>
<td>—</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>WWP2</td>
<td>RSP5</td>
<td>E3 ubiquitin ligase, S. pombe homologue is involved in targeted degradation of cdc25.</td>
</tr>
<tr>
<td>Protein degradation</td>
<td>Y41C4a.10</td>
<td>elb-1</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>—</td>
<td>TCEB2</td>
<td>Ubiquitin-like protein, binds von Hippel-Lindau tumor-suppressor complex and thereby inhibits transcription elongation.</td>
</tr>
<tr>
<td>Protein degradation</td>
<td>H19N07.2A</td>
<td>—</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>USP7</td>
<td>UBP15</td>
<td>Ubiquitin specific protease, stabilizes p53 levels.</td>
</tr>
<tr>
<td>Protein degradation</td>
<td>Y67D8C.5</td>
<td>—</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>UREB1</td>
<td>LASU1</td>
<td>E3 ubiquitin ligase, family of proteins, part of E3 ubiquitin ligase complex.</td>
</tr>
<tr>
<td>Protein degradation</td>
<td>C52D10.9</td>
<td>skr-8</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>SKP1A</td>
<td>SKP1*</td>
<td>Member of the SKP1 family of proteins, with a HECT domain.</td>
</tr>
<tr>
<td>Other</td>
<td>F33H1.3</td>
<td>—</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>WBP11</td>
<td>SNP70</td>
<td>Contains a WW binding domain.</td>
</tr>
<tr>
<td>Other</td>
<td>K03H1.2</td>
<td>mog-1</td>
<td>—</td>
<td>+</td>
<td>—</td>
<td>PRP16</td>
<td>DDX38</td>
<td>Protein required for switch from spermatogenesis to oogenesis.</td>
</tr>
<tr>
<td>Other</td>
<td>C27F2.10</td>
<td>—</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>FLJ11305</td>
<td>THP1</td>
<td>Contains a transcription associated recombination domain. S. c. THP1 shows strong hyperrecombination phenotype.</td>
</tr>
</tbody>
</table>

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.
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 II 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). 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. 2a 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. 2b). RNAi against the 32 previously identified genes yielded 6 genes that were sensitive to camptothecin (Fig. 2c and 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.
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

Fig. 3. Knockdown of genes conferring radiation sensitivity results in increased radiation-induced X-chromosomal nondisjunction. Xol-1::lacZ (y633) 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.
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 a 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, 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-damage 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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