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 cerevisiae (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 large-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).
Table 1. Genes that show synthetic lethality with the mutator phenotype with ionizing radiation and camptothecin

<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 II-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>TCEB2</td>
<td></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>HAUSP</td>
<td>Ubiquitin specific protease, stabilizes p53 levels.</td>
</tr>
<tr>
<td>Protein degradation</td>
<td>Y67D08C.5</td>
<td>—</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>URE81</td>
<td>LASU1</td>
<td>E3 ubiquitin ligase with a HECT domain.</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, part of E3 ubiquitin ligase complex.</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>mag-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.

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 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 specifically for DSB repair were found to show synthetic lethality.
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. 1A). 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...

![Image](image_url)
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.

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 hermaphroditic 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 Schizosaccharomyces pombe 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/Cull1-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 inverse 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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