Figure S1 The effect of the multiplicity of infection (MOI) of the I-SceI-expressing adenovirus AdNGUS24i on the HR assay, and HTP HR rescreening analyses of candidate siRNAs. (a) DR-GFP cells transfected with the indicated siRNAs in HTP were infected with AdNGUS24i at the estimated MOIs, and % GFP+ cells were determined by high-throughput imaging. Assay results plateaued at an MOI of ~10. Error bars represent ± s.d. across three replicates. (b) The percentage of Dharmacon siRNA pools against candidate HR mediators that validated (3-4 siRNAs rescored with a relative HR ratio >1.5 s.d. from primary screen mean) in 4 subgroups. Subgroups are defined by the strength of the corresponding HR phenotype from the primary screen. Number of pools is indicated. (c) Distributions of relative cell growth ratios (as log2 values) for three sets of screened siRNAs: primary screen pools (black), deconvolved Dharmacon siRNAs against candidate HR mediators (red), individual Ambion siRNAs against candidate HR mediators (orange). (d) Scatter plot comparing the relative HR and relative cell growth ratios (as log2 values) for 2563 Dharmacon siRNAs against 641 candidate HR mediators. No direct correlation between the phenotypes is observed. siRNAs indicated in red target BRCA2, CtIP, DSS1, PALB2, BARD1, RPA2, ATR, BRCA1, Rad51, RUVBL2, TIP60, FANCA, RUVBL1, FANC1, Rad51L1, ATM. (e) Same analysis as in d but for 1401 Ambion siRNAs against 467 candidate HR mediators. siRNAs indicated in red target the same genes as in d. (f) Percentage of candidate HR mediator genes that scored with 0, 1, 2, or 3 Ambion siRNAs in categories of those that scored with 1, 2, 3, or 4 Dharmacon siRNAs. Weak cutoff was used for scoring. Number of candidates indicated. Twenty-two known HR and DDR genes were excluded from analysis.
Figure S2. Gene categories and interaction networks identified among HR candidates by Ingenuity Pathway Analysis (IPA). (a) Functional gene categories enriched among candidate HR suppressors. (b) Phosphatase network from candidates that scored as HR suppressors in the primary screen. Dharmacon rescreen data are indicated by color. (c) Transcription factor network from candidates that scored as HR mediators in the primary screen. Dharmacon and Ambion rescreen data are indicated by color. Color key: red indicates a candidate that rescored with >2 deconvolved siRNAs (out of 4 Dharmacon and 3 Ambion), pink indicates that 1 siRNA rescored, gray indicates that 0 siRNAs rescored, and white indicates that a candidate was not rescreened. Line key: solid lines indicate direct interactions, dashed lines indicate indirect interactions, arrows indicate the direction of interactions and lines without arrowheads indicate binding. Shape key: ovals indicate transcription regulators, diamonds indicate enzymes, triangles indicate phosphatases, inverted triangles indicate kinases, circles indicate other, double circles indicate groups of proteins or complexes.
**Figure S3** HIRA, UBN1, and CAIN localize to DNA lesions caused by laser microirradiation, but siRNAs against HIRA, UBN1, and CAIN do not all cause HR defects. (a) Cells were microirradiated and prepared for immunofluorescence with antibodies against HIRA, UBN1 or CAIN and γH2AX within 30 minutes. Nuclei were stained with DAPI. Scale bars indicate 10 μm. Images were prepared from three separate experiments and are not intended for comparison. (b) RT-qPCR of HIRA mRNA (normalized to beta-actin mRNA) from DR-U2OS cells transfected with the indicated siRNAs. Data represent the mean of two replicates. (c) HR assay results from DR-U2OS cells transfected with HIRA- or CAIN-targeting siRNAs. A range of HR phenotypes is observed. Error bars represent ± s.d. across three replicates. (d) HR assay results from DR-U2OS cells transfected with siRNAs against UBN1. Error bars represent ± s.d. across three replicates. (e) Whole-cell extracts from U2OS cells transfected with the indicated siRNAs were immunoblotted with antibodies against UBN1, Rad51 or Actin / vinculin (loading controls). siCtrl does not target UBN1 and was used as a negative control. Two western blots of the same extracts are presented and panels are grouped accordingly. (f) Whole-cell extracts from U2OS cells transfected with the indicated siRNAs were immunoblotted with antibodies against Rad51 or vinculin (VCL, loading control). Analysis shows siCAIN-2 off-target depletion of Rad51. Full scans of blots in e and f are shown in Supplementary Information, Fig. S13.
**Figure S4** GESS analysis of Dharamcon and Ambion siRNA rescreens, and Rad51 off-target analysis of siRNAs against HIRIP3. (a) Scatter plots representing the percentage of siRNAs in 2 groups that have at least one 7-nucleotide seed sequence match (either guide or passenger strand) to 39,338 human CDSs (coding sequences). Left plot: compares Dharamcon siRNAs that rescored for decreased HR with a strong phenotype (y-axis) to those that did not (x-axis). Right plot: compares the same 2 groups of siRNAs after scrambling the seed sequences and serves as a control. No 3'UTR significantly enriched. (b) Scatter plots representing the percentage of siRNAs in 2 groups that have at least one 7-nucleotide seed sequence match (either strand) to 27,534 human 3'UTRs. Left plot: compares the set of Dharamcon siRNAs that rescored for increased HR with a strong phenotype (y-axis) to those that did not (x-axis). Right plot: compares the same 2 groups of siRNAs after scrambling the seed sequences and serves as a control. Three 3'UTRs (labeled) significantly enriched for seed matches and are indicated in red. (c) Scatter plots representing the percentage of siRNAs in 2 groups that have at least one 7-nucleotide antisense seed sequence match to 27,534 human 3'UTRs. Left plot: compares Ambion siRNAs that rescored for decreased HR with a strong phenotype (y-axis) to those that did not (x-axis). Right plot: compares the same 2 groups of siRNAs after scrambling the seed sequences and serves as a control. No 3'UTR significantly enriched. (d) RT-qPCR of Rad51 mRNA (normalized to beta-actin mRNA) from DU-145 cells transfected with indicated siRNAs. Primer set used against Rad51 mRNA recognizes four transcript variants. Error bars represent ± s.e.m. across three replicates.
Figure S5 RBMX localization to DNA damage and anti-stripe formation of RNA binding proteins. (a) U2OS cells were microirradiated and processed for immunofluorescence with antibodies against RBMX and γH2AX within 35 minutes. Cells were fixed directly or pre-extracted with 0.5% TritonX-100. Nuclei were stained with DAPI. Scale bars indicate 10 μm. Images were prepared from two separate experiments (panels are grouped accordingly) and none are intended for direct comparison. Exposure and processing were adjusted to best demonstrate stripes and anti-stripes. (b) U2OS cells expressing Flag/HA (FHA)-tagged RBMX were microirradiated and processed for immunofluorescence with antibodies against HA and γH2AX within 25 minutes. Cells were fixed directly or pre-extracted with 0.5% TritonX-100. Nuclei were stained with DAPI. Scale bars indicate 10 μm. Images were prepared from two separate experiments and are not intended for direct comparison. (c) Live cell imaging of GFP-RBMX recruitment to DNA damage at room temperature. U2OS cells expressing GFP-RBMX were laser microirradiated and imaged at indicated times after damage. Scale bar indicates 25 μm. (d) The Y-chromosome homolog of RBMX (RBMY) and two hnRNP proteins (hnRNP C and hnRNP K) form anti-stripes after microirradiation. U2OS cells expressing the indicated HA fusion proteins were microirradiated and processed for immunofluorescence with antibodies against γH2AX and HA within 25 minutes. HA-tagged histone H3 does not form anti-stripes and serves as a negative control. Images were prepared from two separate experiments (panels are grouped accordingly) and each image was adjusted during exposure and processing to best demonstrate localization. Scale bars indicate 10 μm.
**Figure S6.** RBMX recruitment to DNA damage requires PARP1 activity, but not H2AX or ATM signaling. (a) U2OS cells expressing GFP-RBMX were treated with an inhibitor against ATM (ATMi) or PARP (PARPi), or DMSO (untreated) and after 1 hour were microirradiated (one at a time for 5 minutes). At the indicated times cells were processed for immunofluorescence with an antibody against γH2AX. Damaged cells (as indicated by γH2AX staining) were counted for GFP-RBMX stripes (left) or anti-stripes (right) (approx. 80-160 cells / condition, n=1). (b) RBMX stripe formation was inhibited by PARP1-targeting (but not PARP2-targeting) siRNAs and stabilized by siRNAs against PARG. U2OS cells expressing GFP-RBMX and transfected with the indicated siRNA pools were microirradiated (one at a time for 5 minutes) and processed immediately for imaging with an antibody against γH2AX. GFP was observed directly. Nuclei were stained with DAPI. The percentages of cells with GFP-RBMX accumulation at γH2AX stained laser tracks are indicated (approx. 90-300 cells / condition). Data represent the mean of two replicates except for siFF, which was performed in triplicate. Scale bars indicate 20 μm. (c) H2AX−/− mouse embryonic fibroblasts (MEFs) and an isogenic cell line reconstituted with H2AX (H2AX+/+) expressing GFP-RBMX were microirradiated (one at a time for 5 minutes) and immediately processed for imaging with an antibody against PARP. GFP was observed directly. Nuclei were stained with DAPI. The percentages of cells with GFP-RBMX accumulation at γH2AX stained laser tracks are indicated (approx. 80-140 cells / condition, n=1). Scale bars indicate 10 μm. (d) GFP-RBMX stripe formation was coincident with accumulation of PARP and PAR at laser tracks but prior to RPA accumulation. U2OS cells were microirradiated (one at a time for 5 minutes) and processed for immunofluorescence with antibodies against γH2AX and PARP, PAR or RPA at the indicated times. U2OS cells expressing GFP-RBMX were similarly microirradiated and processed for immunofluorescence with an antibody against γH2AX. GFP was observed directly. Damaged cells were evaluated for the indicated stripes at γH2AX stained laser tracks (approx. 90-210 cells / condition, n=1).
**Figure S7** RBMX depletion by RNAi does not effect I-SceI expression from the AdNGUS24i virus, decrease Rad51 mRNA, nor substantially alter the cell cycle. (a-b) Whole-cell extracts from cells transfected with the indicated siRNAs were immunoblotted with antibodies against RBMX, Rad51 or vinculin (VCL, loading control). Data in a and b represent independent experiments. Additional data from the blot in b is presented in Supplementary Information, S12d (RBMX and VCL panels are reproduced as controls in that figure). (c) Whole-cell extracts from DR-U2OS cells transfected with the indicated siRNAs and infected with AdNGUS24i were immunoblotted with antibodies against HA and vinculin (VCL, loading control) to determine levels of HA-tagged I-SceI expression. The same cells were also assayed for HR efficiency (n=1). (d) RT-qPCR of RBMX mRNA (normalized to beta-actin mRNA) from undamaged DR-U2OS cells transfected with indicated siRNAs or transduced with indicated shRNAs. Error bars represent ± s.e.m. across three replicates. (e) RT-qPCR of Rad51 mRNA (normalized to beta-actin mRNA) from undamaged DR-U2OS cells transfected with indicated siRNAs or transduced with indicated shRNAs. The three primer sets against Rad51 mRNA have different specificities for four transcript variants of Rad51 (v. 1-4). Error bars represent ± s.e.m. across three replicates. (f) Cell cycle analysis of undamaged DR-U2OS cells transfected with the indicated siRNAs or transduced with indicated shRNAs (n=1). (g) Cell cycle profiles from data in f. The same siRNA transfected or shRNA transduced cells were used for experiments in d-g, as well as Fig. 6a and Supplementary Information, Fig. S8c. Full scans of blots in a-c are shown in Supplementary Information, Fig. S13.
Supplementary Figure 8, S. J. Elledge

(a) Whole-cell extracts from DR-U2OS cells transduced with the indicated cDNAs (FHA-tagged) and then transfected with the indicated siRNAs were immunoblotted with antibodies against RBMX, Rad51, the FLAG epitope, and vinculin (VCL, loading control).

(b) Cell cycle analysis of cells evaluated in a. Data represent the mean of two replicates.

(c) DR-U2OS cells transfected with the indicated siRNAs were treated with 10 Gy IR and processed for immunofluorescence with antibodies against Rad51 and γH2AX at the indicated times. The 0 hour time point was taken immediately after damage induction. Cells with Rad51 foci were counted by eye; because transfection with siRNAs against RBMX causes some changes to nuclear morphology, only normal shaped nuclei were counted (approx. 70-130 cells / condition). Data represent the mean of two replicates. Representative images of this experiment are presented in Fig. 6a.

(d) Representative images of cells evaluated for IR-induced Rad51 foci in Fig. 6c. Scale bars indicate 20 μm. Full scans of blot in a are shown in Supplementary Information, Fig. S13.
Figure S9 RBMX is not required for damage-induced RPA2 or Chk1 phosphorylation. (a-b) DR-U2OS cells transfected with the indicated siRNAs were treated with 10 Gy IR or 1μM camptothecin (CPT) and after ~6.5 hours whole-cell extracts were collected and immunoblotted with the indicated antibodies. UNT indicates untreated. Data from a and b represent two independent experiments, with two western blots of corresponding extracts presented in both (panels are grouped accordingly). (c) RT-qPCR of RBMX mRNA (normalized to beta-actin mRNA) from undamaged DR-U2OS cells evaluated in b. Error bars represent ± s.e.m. across three replicates. The same siRNA transfected cells used in b-c were analyzed in Fig. 6b. Full scans of blots in a-b are shown in Supplementary Information, Fig. S13.
**Figure S10** Structure-function analysis of RBMX. RBMX is composed of four identifiable regions: an N-terminal RNA recognition motif (RRM), a centrally located RBM1CTR region identified as common among RBMY-like hnRNPs, a C-terminus rich in serine, arginine, glycine, and tyrosine residues, and a putative second RNA binding domain at the C-terminal end (C-RBD)\(^1\). The canonical RRM of RBMX preferentially binds CC(A/C)-rich single-stranded RNA; and although RBMX can influence alternative splicing in an RRM-independent manner, evidence suggests that some splicing is directly facilitated through the RRM\(^2\). (a) A graphical representation of RBMX mutants and their corresponding phenotypes. Flag/HA-tagged mutants were evaluated for capacity to attenuate the defect in HR caused by depletion of endogenous RBMX (data in b-d). NT indicates not tested. GFP-tagged mutants were evaluated for localization to DNA damage after microirradiation (data is from 12 separate experiments performed with subgroups of the mutants). The ptRNP mutant carries point mutations of four aromatic residues important for the RNA binding of conserved RNA recognition motifs (RRMs): F11A, R49A, F51A and F53A. The ΔRNP mutant has deletions of two ribonucleoprotein (RNP) domains (amino acids 10-15 and 49-56) containing these conserved residues. Nuclear localization of all GFP-RBMX mutants except for the 1-93 amino acid truncation was observed. An NLS was added to the 1-93 and 218-341 amino acid fragments to aid nuclear localization; 218-341 GFP tracks were rarely observed without the NLS. +/- indicates that GFP damage tracks were rarely observed without depletion of endogenous RBMX. (b) DR-U2OS cells transduced with the indicated cDNAs and then transfected with either siRBMX-3 or siFF were evaluated for HR efficiency. % GFP+ data from cells with the same cDNA were normalized to the siFF condition (% Relative HR). Error bars represent ± s.d. across three replicates. (c) Whole-cell extracts from cells in b were immunoblotted with antibodies against FLAG and vinculin (VCL, loading control) to confirm expression of RBMX (wild-type and mutant) transgenes. (d) Cells evaluated in b were processed for immunofluorescence with an antibody against HA to confirm nuclear localization of RBMX (wild-type and mutant) transgenes. Scale bar indicates 20 μm. Full scans of blot in c are shown in Supplementary Information, Fig. S13.
Figure S11 Neither inhibition nor depletion of PARP1 affects HR. (a) HR assay results and corresponding PARP1, RBMX western blot analysis from DR-U2OS cells transfected with the indicated siRNAs at 20 nM. siRad51, siPARP1, siPARP2, and siPARG indicate pools of 4 siRNAs. siPARP1/2 indicates two pools of 4 siRNAs each targeting PARP1 and PARP2. siFF, siRBMX-1, and siRBMX-3 indicate individual siRNAs. Error bars represent ± s.d. across three replicates. (b) HR efficiency in DR-U2OS cells treated with a PARP inhibitor (PARPi), ATM inhibitor (ATMi), or DMSO (untreated, negative control). Inhibitors were added to cells at the same time as the I-SceI carrying adenovirus and again 12 hours later; cells were submitted to FACS analysis 36 hours post infection. Error bars represent ± s.d. across three replicates. (c) HR assay results and corresponding PARP1, RBMX western blot analysis from DR-U2OS cells transfected with the indicated siRNAs at the indicated concentrations (20 or 40 nM). siPARP1 indicates a pool of siRNAs. siFF, siRBMX-1, and siRBMX-3 indicate individual siRNAs. Error bars represent ± s.d. across three replicates. Full scans of blots in a and c are shown in Supplementary Information, Fig. S13.
Figure S12  Expression of DDR proteins after depletion of RBMX and other pre-mRNA processing factors. (a-b) Whole-cell extracts from DR-U2OS cells transfected with RBMX siRNAs were immunoblotted with the indicated antibodies. Four western blots of the same extracts are presented in a-b and panels are grouped accordingly. Additional data from the blot in b are presented in Fig. 6d (the Lamin B panel is reproduced as a control in that figure). (c) DR-U2OS cells from a-b were irradiated with 10 Gy IR and processed for immunofluorescence with antibodies against BRCA1 and 53BP1 after 4 hours. Nuclei were stained with DAPI. Scale bars indicate 20 μm. (d) Whole-cell extracts from DR-U2OS cells transfected with the indicated siRNAs were immunoblotted with the indicated antibodies. Additional data from this blot are presented in Supplementary Information, S7b (RBMX and VCL panels are reproduced as controls in that figure). (e) Whole-cell extracts from DR-U2OS cells transduced with the indicated cDNAs (FHA-tagged) and then transfected with the indicated siRNAs were immunoblotted with antibodies against ATR and vinculin (VCL, loading control). The same cells were evaluated in Figure 6c, e. (f) HR assay results from DR-U2OS cells transfected with the indicated siRNAs that (except for those against hnRNPUL1) target pre-mRNA processing genes that scored as candidate HR mediators. (g-j) Whole-cell extracts from cells in f were immunoblotted with antibodies against BRCA2 and vinculin (VCL, loading control). Full scans of blots in a-b, d-e, g-j are shown in Supplementary Information, Fig. S13.
**Figure S13** Full scans of western blots from Figure 3b-c, Figure 3e, Figure 5c, Figure 5e and Figure 6d-e; Supplementary Information, Figure S3e, Figure S3f, Figure S7a-c, Figure S8a, Figure S9a-b, Figure S10c, Figure S11a, Figure S11c, Figure S12a-b, Figure S12d-e, Figure S12g-j. Molecular weight markers (kDa) are included.
Supplementary table legends

Supplementary Table 1. Genome-wide screen data. Screened siRNA pools with corresponding gene symbol, gene ID, gene accession number, Dharmacon vendor ID, and relative HR and cell growth ratios [relative HR and relative cell growth]. The relative HR and cell growth ratios are accompanied by standard deviations calculated for technical replicates [s.d.(relative HR) and s.d.(relative cell growth)]. Pools without standard deviations had two replicates deleted from analysis after visual inspection of screening images. Pools that scored –based on a 2 s.d. cutoff from the screen-wide relative HR ratio mean– and / or were submitted to rescreening analysis are indicated.

Supplementary Table 2. Rescreen data for deconvolved Dharmacon siRNAs against candidate HR mediators. Rescreened siRNAs with corresponding gene symbol, gene ID, gene accession number, Dharmacon vendor ID, sequence, and relative HR and cell growth ratios [relative HR and relative cell growth]. The relative HR and cell growth ratios are accompanied by standard deviations calculated for technical replicates [s.d.(relative HR) and s.d.(relative cell growth)]. siRNAs that scored by strong and / or weak cutoffs, are predicted to off-target Rad51, and / or have an identical sequence to a screened Ambion siRNA are indicated.

Supplementary Table 3. Rescreen data for deconvolved Dharmacon siRNAs against candidate HR suppressors. Rescreened siRNAs with corresponding gene symbol, gene ID, gene accession number, Dharmacon vendor ID, sequence, and relative HR and cell growth ratios [relative HR and relative cell growth]. The relative HR and cell growth ratios are accompanied by standard deviations calculated for technical replicates [s.d.(relative HR) and s.d.(relative cell growth)]. siRNAs that scored by a strong and / or weak cutoff are indicated.

Supplementary Table 4. Data for Ambion siRNAs screened against candidate HR mediators. Screened siRNAs with corresponding gene symbol, gene ID, gene accession number, Ambion vendor ID, sequence, and relative HR and cell growth ratios [relative HR and relative cell growth]. The relative HR and cell growth ratios are accompanied by standard deviations calculated for technical replicates [s.d.(relative HR) and s.d.(relative cell growth)]. siRNAs that scored by a strong and / or weak cutoff, and / or have an identical sequence to a screened Ambion siRNA are indicated.

Supplementary Table 5. GFP fusion proteins evaluated for localization after microirradiation by UV laser.

Supplementary Table 6. 121 candidate HR mediators that scored with at least 3 of 7 Ambion and Dharmacon siRNAs after eliminating Dharmacon siRNAs predicted to off-target Rad51 by at least one 7mer antisense seed match to the 3’UTR. Scoring siRNAs met a weak cutoff (59% relative HR). Candidates are listed with corresponding gene symbol, gene ID, and gene accession number.

Supplementary Table 7. Antibodies. Antibodies are listed with corresponding host animal, source, catalog number, and dilution. WB indicates dilutions used for western blots and IF indicates dilutions used for immunofluorescence.

Supplementary Table 8. siRNAs. Sequences of siRNAs used that are not listed in Supplementary Tables 2-4. Positive and negative (siFF) controls used in each experiment were (for the most part) from the same vendor as the experimental siRNAs.

Supplementary Table 9. shRNAs.

Supplementary Table 10. RT-qPCR primers.

Supplementary references