Supporting Online Material

Supplemental Text

Supplemental Results

It is clear that higher organisms contain a number of DDR components not shared in lower eukaryotes, e.g. MDC1, RNF8, BRCA1, ABRA1 and p53, and many substrates of DDR kinases have no lower eukaryotic counterparts (*1, 2*) RNA interference now makes unbiased genetic screening for DDR genes possible in human cells. In order to identify novel participants in the DDR, we developed a high throughput (HTP) microscopy-based assay following siRNA depletion to measure inappropriate cell cycle progression after ionizing radiation (IR) induced DNA damage.

Primary screen. We initially attempted to develop a 384 well HTP assay based on the traditional G2/M cell cycle arrest assay measured one hour after IR (*3*). However, the low percentage of cells able to transit mitosis (phospho-S10 Histone H3 positive) within one hour makes the dynamic range of this traditional assay too low for a genome-wide study. Increasing the time following IR and using a microtubule depolymerizing drug, such as nocodazole, to trap cells that inappropriately enter into mitosis broadens the dynamic range and allows for cells that incurred damage during S phase (as well as G2) to be scored positive in this lengthened assay when cells are cell cycle arrest-deficient. This is demonstrated by the time course experiment shown in Fig. S1A. S phase cells are identified by a short pulse of BrdU, prior to IR, and followed during this lengthened G2/M checkpoint assay. This S phase population normally undergoes cell cycle arrest for as long as 18 h after 10 Gy. However, in the presence of a DNA damage response inhibitor, such as caffeine, cells bypass the cell cycle arrest and a significant increase in double positive BrdU and phospho-H3 cells can be detected (Fig. S1A upper right quadrant, 18 h). Cells experiencing damage in G2 cannot sustain an arrest during this lengthened assay as judged by the transit of G2 cells that lack BrdU incorporation into mitosis even in the absence of caffeine (Fig. S1A, upper left quadrant, 18 h). Therefore, siRNAs targeting genes important for DDR activation after DSBs induced in S-phase score robustly as phospho-H3 positive mitotic cells when assayed 18 hours after IR in the presence of nocodazole. These conditions represent the basis of our lengthened G2/M cell cycle arrest assay.

Consistent with the assay development studies, the ATR pathway that is triggered by DSBs during S phase, scores in the assay to varying degrees depending on the particular component targeted (see Fig. 1D). ATR itself is the strongest hit in the screen often resulting in greater than a 20-fold effect (Fig. S1B). Given the known role of the ATR pathway in controlling Cdc25A stability in response to damage (*4*), inappropriate entry into mitosis after ATR or Chk1 depletion is not unexpected, due to high levels of sustained Cdk1 activity. For these reasons, ATR and Chk1 were chosen as positive controls used throughout the primary screen.

The screen was carried out in the human osteosarcoma cell line, U2OS using Dharmacon's siGenome Smartpool technology. Pools of siRNAs (50 nM) were introduced into cells by reverse transfection on a well-by-well basis in duplicate, using a 384 well format. Three days after transfection, plates were subjected to 10 Gy IR and

nocodazole was added after one hour to trap cells that bypass G2 arrest. Eighteen hours after IR, plates were fixed and stained for phospho-H3 and DNA content and subsequently imaged. The mitotic index (MI) was calculated for each well (more than 1000 cells per well on average) using the MetaExpress software package to identify wells that scored above negative control levels. All wells demonstrating >2 fold increased MI were identified and these images were manually inspected to verify the accuracy of the MI calculations (Fig. 1B and see text for additional information).

A significant number of pools (8%) showed toxicity. i.e. >4 fold decrease in cell number. Analysis of the biological processes among this group revealed an enrichment of proteasomal and ribosomal gene products within the protein metabolism and modification classification (Fig. S2A, B and C). One component of the ATR pathway, Chk1 was in this class. Since both Chk1 and ATR are essential genes and presumably other important DDR factors might be also, cytotoxic siRNA pools were rescreened at a 10 nM siRNA concentration to rescue bypass phenotypes of essential DDR genes (Fig. S1D). This resulted in the identification of 98 additional siRNA pools (Table S1 and 2).

Prioritization of the candidates for secondary screening. All strong and medium candidates were taken for further analysis along with a subset of the weak scoring candidates. Weak candidates were ranked for further study based on a higher level of cell cycle arrest bypass (denoted Weak+, Table S1) or their identification as potential ATM/ATR kinase substrates as described in the text. Using these criteria, 720 genes were chosen for secondary screening efforts. Pools of siRNAs were deconvoluted into 4 individual siRNAs and retested for the cell cycle arrest bypass phenotype using a 20 nM final siRNA concentration (Fig. S3A). Greater than 75% of the genes recapitulated the primary screen phenotype with at least 1 siRNA (Fig. 1C, Table S3). However, genes critical for progression through mitosis could also display an increased MI, regardless of the presence of DNA damage. To exclude these genes from further study, the candidates were screened in the absence of DNA damage or nocodazole. siRNAs resulting in >2 fold increase in MI were considered potential mitotic regulators and discarded from the screen (Fig. 1C and Fig. S3B). Anaphase promoting complex (APC) components (ANAPC5, CDC16 and CDC26) as well as proteins important for microtubule dynamics (Kif11, TPX2 and Aurora A) are examples of genes with multiple siRNAs that result in mitotic arrest and increased MI in the absence of DNA damage (Table S3, MI untreated). Approximately 9% of the positives were eliminated from DNA damage pathway consideration by this process. Analysis of the cytotoxic gene list from the primary screen (Table S1) also reveals an abundance of mitotic cell cycle regulators (Fig. S2A).

A central characteristic of mutations defective in the DDR is sensitivity to DNA damage. Therefore, sensitivity to mitomycin C (MMC), which causes replication bocks and can result in DSBs, was assessed after depletion by siRNAs to provide further support for a gene's role in the DDR. Examples of the performance of control siRNAs in the 384 well survival assay are shown over a range of MMC concentrations (Fig. S3C). MMC doses of 25 and 50 nM were chosen and siRNAs resulting in decreased survival >2 SD from on plate negative controls were deemed MMC sensitive. We found that over half of the genes that scored with at least 2 siRNAs in the checkpoint assay also scored with two or more siRNAs in the MMC-sensitivity assay (97 genes).

Repair choices affect the maintenance of the DDR. Bioinformatic analysis revealed a strong enrichment for components of the ATR pathway and, surprisingly, numerous components of the Fanconi anemia (FA) pathway and homologous recombination (HR) pathway (Fig. 1D and Fig. S4C). At first blush this result seems counterintuitive since DSBs remain unrepaired in the absence of HR, and therefore ATR signaling should persist until the repair process is complete. However, as has been describe in the text, examination of P-Chk1 over time after IR indicates that, in the absence of core HR components such as BRCA2, ATR signaling is not sustained. Similar results were obtained also after depletion of BRCA1. BRCA1 has been previously reported to have a role in promoting Chk1 activation (*5*). The defect in sustaining the cell cycle arrest in the presence of damaged DNA in BRCA1 mutated cells has been attributed to this DDR mediator function (*3*). In contrast to this previous result, we did not observe a defect in Chk1 activation 1h post IR, but we observe a defect in maintenance of the activation. Although HR is the major repair pathway for cells that sustain a DSB in S phase, other repair pathways such as non-homologous end-joining (NHEJ) are also functional at this stage of the cell cycle. Thus, we wondered whether the defect in sustaining ATR activation was potentially due to the removal of signaling ends by NHEJ repair. To test this hypothesis, we inhibited NHEJ and observed a decrease in the cell cycle arrest bypass in BRCA1-depleted cells in both conditions as described in the text, suggesting that in the absence of BRCA1, NHEJ processes the breaks and prevents maintenance of DDR signaling. Interestingly we did not observe a rescue of the BRCA2 cell cycle arrest defect, suggesting that either the processed DSB intermediates that accumulate in these cell lines are not channeled into NHEJ or that BRCA2 has an additional role in the DDR to promote arrest.

The enrichment of the Fanconi Anemia (FA) pathway further suggests that the primary screen effectively identified components necessary for the sensing and repair of DNA damage incurred during S phase. Eight of the 14 known FA genes form the FA core E3 ubiquitin ligase complex that, in response to DNA damage, is responsible for the ATR-dependent monoubiquitination and subsequent chromatin targeting of the ID complex, a heterodimer of FANCD2 and FANCI (*6*). Half of the FA core components (FANCE, G, L and M) as well as all of the FA genes that act downstream of or in parallel to the ID complex, BRCA2 (FANCD1), PALB2 (FANCN) and BRIP1 (FANCJ) scored positive in the screen with 3 or more siRNA (Fig. 1D and Fig. S4C). Inactivation of NHEJ decreased the cell cycle arrest bypass in cells depleted of two major components of the core complex (FANCM and FANCL) and of FANCJ (a BRCA1 interacting protein) These data are consistent with the notion that FA components are involved in processing DNA ends, tilting the repair choice towards HR as opposed to NHEJ. Consistent with this observation, suppression of NHEJ suppresses the repair defect and sensitivity to genotoxic agents in FA deficient cells (*7, 8*).

Candidate Characterization. To further validate candidates from the screen, 100 high priority genes plus 14 recognized DDR controls were chosen for independent siRNA testing using Dharmacon's On target plus (OTP) technology which has been purported to show reduced off target effects. Four siRNA per gene were tested in the G2/M arrest -/+ DNA damage and mitomycin C sensitivity assays, (Table S4). The vast majority of OTP siRNA are unique sequences compared to the siRNAs used in the initial screen. This

allowed us to collect data for at least 80% of the selected genes with 7 or more independent siRNAs (Table S3 and 4). Of this set of 144 genes, 50% score positive in the cell cycle arrest assay with half or more of all unique siRNAs per gene supporting the robustness of the screen. Additionally, to test the role of the candidates in DNA repair, specifically HR, reconstitution of a GFP reporter after an I-SceI induced DSB was carried out after target gene depletion (*9*). A 384 well assay was developed using adenoviral I-SceI delivery and high content microscopy to assess the GFP percentage as a marker of HR capacity. Depletion of core HR proteins, such as Rad51 and BRCA2, results in at least a 5-fold decrease in relative repair capacity (Supp. Fig. S4A, Table S4). Depletion of genes resulting in >2 SD decrease compared to the plate controls were consider as candidates with a potential role in modulation of the DSB repair process (Table S4).

As noted in the text, 24 genes were tested for localization to sites of DNA damage and three (INTS7, Clock and MGC13204/RHINO) showed co-localization with γ-H2AX (Fig. 2A).

RHINO a novel player in the DDR. MGC13204, referred to hereafter as RHINO, is currently annotated as a noncoding RNA, although we previously found a RHINO peptide inducibly phosphorylated on T33 in response to IR (*1*). Depletion of RHINO, led to cell cycle arrest bypass (6/7 siRNAs) and MMC sensitivity (3/7 siRNAs) as well as sensitivity to IR and camptothecin in subsequent testing (Fig. 2D). The level of RHINO depletion strongly correlated with the cell cycle arrest defect phenotype in response to IR (Fig. 2B and Fig. S6B) and could be rescued by reintroduction of a siRNA (#9)-resistant RHINO cDNA (Fig. S7A) supporting the on-target nature of the phenotype. Two of 4 siRNAs resulted in decreased HR and like the checkpoint defect and damage sensitivity and the HR defect also correlated with the level of RHINO depletion. Cell cycle distribution can strongly impact the readout of the HR assay since G1 cells do not perform HR. However, we found the cell cycle distribution after RHINO depletion was unperturbed (Fig. S8A). To exclude the possibility that depletion of RHINO was affecting I-SceI restriction endonuclease activity or site accessibility, we amplified the chromosomal I-SceI site in GFP after I-SceI expression and repair, cloned it into a plasmid vector and evaluated the proportion of the breaks that were imprecisely repaired versus those retaining the parental sequence indicating uncut sites (see Materials and Methods for more details). No significant difference in the proportion of mutant versus WT I-SceI sites was found between the negative control and RHINO depleted cells, suggesting that RHINO is not affecting HR by altering the efficiency of the cut (Fig. S8B and C). Furthermore we were able to restore HR repair by expressing siRNA (#9)-resistant RHINO (Fig. S7B, C). We were also able to observe a partial rescue by expression of WT RHINO which reached ~30% of endogenous levels in the presence of the siRNA (Fig. S7B, C) indicating that a small level of RHINO is sufficient for HR. As noted in the text, mass spec analysis if immunoprecipitated RHINO identified Rad9, Rad1, Hus1, TopBP1, Rad18 and Ubc13. Independent mass spec analysis of Hus1 containing complexes after IR revealed an interaction with endogenous RHINO (data no shown).

Species-specific BLAST searches identified clear orthologs of RHINO in mouse, rat, chicken and frog, with the highest conservation in the N and C terminal regions (Supp. Fig 8A). Examination of human RHINO revealed 3 potential ATM/ATR

phosphorylation sites (S11, T33, S232), one of which (T33) we previously identified as a DNA damage inducible site (*1*). In addition to the mutations we described in the text, we also mutated all the potential ATM/ATR phospho-sites. We performed IP-westerns after expression of FLAG-HA-tagged full length and mutant RHINO and mutation of all potential ATM/ATR sites did not affect the interaction with either 9-1-1 or TopBP1 (Fig. 4B).

Supplemental Discussion.

In this study we have explored the persistence of the DDR signaling response to DNA damage occurring during S phase using a prolonged cell cycle arrest assay. We discovered that cells that incur DNA damage via IR in G2 can arrest for a short time but by 18 hours have entered mitosis, while cells damaged in S phase show a prolonged arrest. The ability of G2 relative to S phase cells to enter mitosis may reflect the differing signaling capacities of the cell cycle stages or possibly the different repair choices available. For example, DSBs in G2 can be efficiently repaired by HR, while a portion of the DSBs incurred in S phase cells in DNA that has not yet replicated are likely repaired much less efficiently by HR and may persist longer in a signaling state.

Our high-through-put genetic screen identified multiple genes and pathways required to maintain prolonged cell cycle arrest in response to DNA damage in S phase. In principle, our genes could act to promote DDR signaling or to inhibit an adaptation pathway, although by the classical definition these are checkpoint defective because when inhibited they lose a dependency and no adaptation pathway has been described in WT human cells. Bioinformatic analysis revealed an unexpected role for the homologous recombination and Fanconi Anemia pathways in promoting cell cycle arrest. This is likely to reflect, in part, differential funneling of repair intermediates into different pathways. In the absence of FA or HR components, it is possible that unrepaired breaks are acted upon by the NHEJ pathway, resulting in the removal of these lesions from further signaling (Supp. Fig 10). This hypothesis is supported by the finding that signaling defects in response to depletion of FA components and BRCA1 can be suppressed by blocking NHEJ. This suppression could be explained if more DSBs remain to signal when NHEJ is blocked. However, this does not explain the fact that blocking NHEJ fails to suppress the defect in BRCA2 depleted cells. Instead this suggests that BRCA2 is promoting DDR signaling in a manner distinct from preventing the removal of signaling lesions through NHEJ. In the case of signaling in the absence of BRCA1, the model would predict that the reduced flux of DBSs through the BRCA2 branch allows the accumulation of intermediates that can be healed by NHEJ. If NHEJ is inhibited, these DSBs will remain and can eventually enter the BRCA2 pathway where they will generate a persistent signal. This interpretation depends upon the notion that cells retain the capacity to signal if they process lesions through the HR pathway. Currently there are two ssDNA binding proteins required for DDR signaling, the RPA complex and the SSB1 and SSB2 proteins. The HR pathway, which replaces RPA with another ssDNA binding protein, Rad51, may represent a third (Supp. Fig 10). We were unable to further test this possibility due to extreme toxicity of Rad51 depletion but analysis of Rad51 and its paralogs could shed light on this possibility. It makes biological sense that cells would evolve the capacity to sense ongoing repair, as it is the completion of repair once initiated that is the relevant biological event with respect to turning off the

DDR. Additionally, it is possible that, in the absence of BRCA2, ssDNA-RPA complexes might accumulate and could be removed by the single strand annealing (SSA) repair pathway to terminate signaling (Supp. Fig 10). BRCA2 mutant cells are known to show increased SSA (*10*).

This study has identified a large number of new players in DDR signaling that should provide many new leads for proteins involved in human diseases such as Fanconi anemia, familial breast cancer, Seckel syndrome and other microcephaly disorders. Three of these localize to sites of DNA damage. Identification of CLOCK, a transcription factor to which has recently been found to possess a histone acetyltransferase activity (*11*), as a protein that localizes to sites of DNA damage suggests a more direct connection to DNA repair and warrants further studies aimed at uncovering acetylation targets impinging on the DDR. In addition, the presence of INTS7 along with INTS3 and SSB proteins raises the possibility that the Integrator complex as a whole participates in the DDR, which is intriguing given its known endonuclease activity.

Importantly, this work also defined a new component of the Rad17/9-1-1/TopBp1 pathway, RHINO, required for full ATR activation (see Text).

Materials and Methods

Cell culture

U2OS and DR-GFP U2OS were cultured in McCoy's 5A media with 10% FBS. MEFs and 293T were passaged in Dulbecco's Modified Eagle's Medium (DMEM) with 10% FBS. N-terminal GFP-fusion stable U2OS cell lines were selected in 1 μg/ml puromycin for 4 days following infection and then maintained without selection. Dox inducible Flag-HA tagged candidates were introduced into TREX-293 cells (Invitrogen) via retroviral transduction, selection with puromycin and maintenance with Tet-approved FBS (Clontech). Induction of these cell lines with 2 μg/ml doxycycline was carried out for 24 hours. All cell lines were grown at 37° C in a humidified incubator with 5% CO_2.

Plasmids, RNAi and RT-qPCR

cDNAs for genes of interest were either obtained from the Orfeome collection (*12*) or TOPO cloned into a directional Gateway Entry vector (pENTR-TOPO) after PCR amplification from oligo-dT or random primed cDNA generated from U2OS RNA or from cDNAs purchased from Open Biosystems. Point mutations were introduced into entry clones using Strategene's multi-site mutagenesis kit as recommended. LR reactions of entry clones were used to generate MSCV based puromycin resistant vectors with N-terminal tags of GFP or Flag-HA. The individual sequences and catalog numbers for all 4 Dharmacon siGenome siRNAs for the 720 cherry picked screen hits can be found in Supp. Table 3 and the sequences of On target plus siRNAs used to further characterized the 114 high confidence hits are listed in Supp. Table 4. Additional siRNAs for Rad18 and Rad17 are listed: D-004591-01 CAUAUUAGAUGAACUGGUA, D-004591-02 GCAGGUUAAUGGAUAAUUU, D-004591-03 GAUAAUAUGACCUCAG UAA, D-004591-04 GCAGUUUGCUUUAGAGUCA, D-003294-05 GAAUC AAGCUUCCAUAUGUUU, D-003294-06 CAACAAAGCCCGAGGAUAUUU, D-003294-07 ACACAUGCCUGGAGACUUAUU, D-003294-08 CUACAUAGA UUUCUUCAUGUU. siRNA transfections for individual experiments (not for the screen)

were performed using Lipofectamine RNAiMAX as suggested by the manufacturer with the final siRNA concentration ranging from 20 to 30 nM for the individual siRNA and of 50 nM for pools. The shRNA targeting 53BP1 is in the MSCV-PM vector (V2HS_56192) from the Hannon-Elledge shRNA collection (Open Biosystem). For RT-qPCR Superscript III reverse transcriptase followed by Platinum cybergreen super mix with Rox dye (11733-046, Invitrogen) was used according to the instructions. β-actin was used as control. Two sets of RHINO primers were used. RHINO RTqPCR set #1 was used in Fig 3C and Supp. Fig. 5C: primer L GCACCATCACTTCCTGGGTA and primer R TTTCTGGTAGGCTGGGAACA. RHINO RTqPCR set #2 was used in Supp. Fig. 5D and Supp. Fig. 8B: primer L CCTACCACCTCCAAGTTTCC primer R CACTG ACATGTTCCCACAGC.

Antibodies and reagents

Phospho-S10 Histone H3 (Upstate 06-570), Phospho-S317 Chk1 (Cell Signaling #2344), Chk1 (Santa Cruz sc-8408), SMC1 (Bethyl A300-055A), γ-H2AX (Upstate 05-636), Chicken GFP (AbCam ab13970), Rad18 (Novus and Bethyl A301-340A), Ubc13 (Invitrogen 37-1100), HA (Covance MMS-101R), Flag-HRP (Sigma A8592), ATR (Santa Cruz sc-1887), PCNA (Santa Cruz sc-56), INTS3 (Bethyl A302-050A), INTS7 (Bethyl A300-271A), SSB1 (Bethyl A301-938A), Rad9 (Bethyl A300-890A), Rad1 (Novus Biologicals NB100-346), Hus1 (Santa Cruz sc-8323) and TopBp1 (Bethyl A300-111A). DNApk inhibitor from Tocris bioscience (NU7441) was used at a final concentration of 1 μM.

Primary screen conditions

All high throughput siRNA screening efforts were carried out at the Institute of Chemistry and Cell Biology-Longwood (ICCB-L). Dharmacon's siGENOME human library containing 21,121 pools of 4 siRNA per gene in individual wells was screened at a 50 nM final concentration in duplicate using a reverse transfection protocol. Nine micro-liters of an Optimem/oligofectamine cocktail was arrayed in microscopy grade 384 well, clear bottom, black plates using an in hood Matrix wellmate. siRNAs were added with the Velocity 11 robot in a Baker Bioprotect II safety cabinet. Positive and negative controls (firefly luciferase, ATR and Chk1) were manually added to each plate using a multichannel pipetman. After a 30 min. room temperature incubation to allow lipid/siRNA complex formation, 900 U2OS cells in 20 microliters of McCoy's media with 15% FBS were added per well using the Wellmate. Plates were briefly spun down for 1 min. prior to incubation for gene knockdown. On plate Plk1 siRNAs served as a positive transfection control, judged by loss in cell viability. After 72 h, plates were irradiated with 10 Gy using a Cesium-137 source. One hour after IR, 10 µl of a 400 ng/ml nocodazole-McCoy's media mixture was added to achieve a final concentration of 100 ng/ml. 18 h after IR, plates were fixed with 3.7% formaldehyde for 10 min. and permeabilized with 0.2% Triton X-100 in PBS. Mitotic cell staining was carried out with anti-Phospho-S10 Histone H3 (Upstate 06-570) primary antibody and AlexaFlour 488 secondary antibody (Invitrogen). Hoescht 33342 DNA staining was carried out simultaneously with secondary antibody incubation.

Three sites per well were captured at a 10X magnification using the Image Xpress high content automated microscope (Molecular Devices). Approximately 1000 cells per

well were analyzed and the mitotic index was calculated using the MetaExpress software package by comparing the total number of nuclei per well to the total number of phospho-H3 positive cells. All wells yielding a 2-fold increase in mitotic index or greater compared to the negative control (FF) were manually inspected to verify the mitotic index calculation. Hits were grouped into strong, medium and weak categories based on a greater than 8-, 4- or 2-fold increase in mitotic index, respectively. Wells demonstrating a >4-fold decrease in cell number as compared to FF were deemed cytotoxic. 1657 pools of cytotoxic siRNAs were cherry picked and rescreened at a 50 and 10 nM concentration in an attempt to rescue lethality and uncover checkpoint defects. This cytotoxic rescreen was carried out as described for the primary screen.

Secondary screen conditions

SiRNAs for 720 genes were rearrayed in 384 well plates for testing of the 4 individual siRNAs for each candidate. Sequences and Dharmacon catalog numbers for each siRNA of the 720 candidate genes can be found in Supp. Table 3. Individual siRNAs were rescreened at a final concentration of 20 nM. The checkpoint assay was carried out as described for the primary screen. To assess mitotic accumulation independent of DNA damage, the mitotic index after 3 days of depletion was calculated in the absence of IR and nocodazole. siRNAs resulting in a 2-fold increase in mitotic index without damage were excluded from further consideration as novel DNA damage checkpoint components.

MMC sensitivity assays were conducted in 384 well plates using Cell titer glow (Promega) as a read-out for cell survival after siRNA depletion and drug treatment. 600 U2OS cells were reversed transfected with 20 nM individual siRNAs in duplicate for untreated, 25 nM and 50 nM MMC conditions. MMC was added one day after siRNA transfection and viability was assessed 5 days later. Cell titer glow luminescent measurements of cell viability were read on an EnVision plate reader (Perkin Elmer). MMC treatments for each siRNA were compared to untreated siRNA samples to adjust for siRNA mediated toxicity. siRNAs that were >2SD away from the on plate negative controls in 3 out of 4 replicates (2 at 25 nm and 2 at 50 nM) were scored as MMC sensitive.

High throughput candidate testing using On target Plus siRNAs

The 114 genes (including positive controls) chosen for further analysis are listed in Supp. Table 4 along with the sequences and catalog numbers for the On-target plus (OTP) siRNAs. These siRNAs were used in the aforementioned 3 assays as described above. The only modification was to the MMC assay, in which one drug concentration (50 nM) was used compared untreated, each of which was done in triplicate. In this case, siRNAs that were >2SD away from the on plate negative controls in 2 out of 3 replicates at 50 nM were scored as MMC sensitive.

HR assay

The U2OS DR-GFP cell line was used to monitor the efficiency of homologous recombination after a site-specific double strand break (*13*). Adenoviral delivery of I-SceI resulted in a single DSB per cell and accurate repair by HR could be scored by the production of GFP. A high throughput microscopy based assay was developed to

interrogate the 100 gene candidate set with OTP siRNAs. Six hundred DR-GFP U2OS cells were reverse transfected with individual OTP siRNAs in 384 well plates. Following 3 days of gene knockdown, cells were infected at an MOI of 10 for 48 h based on the estimated cell number following 3 days of cell division. Plates were fixed and stained for DNA content. GFP and DNA images were captured on the Image Express micro and the percent GFP positive cells per well was calculated using the MetaExpress software platform. Results from 36 on plate negative controls were used to calculate the average HR and standard deviation. siRNAs >2SD above or below this negative control average are considered to have an HR defect and are denoted on Supp. Table 4 in the HR assay column (column H) as either increased or decreased HR.

For genes of particular interest, FACS analysis was carried out to rule out any cell cycle perturbations that could lead to the altered HR efficiency. Follow-up studies on specific genes, such as those shown in Fig. 3D, were transfected in 6 well dishes and cell numbers were counted prior to I-SceI infection to ensure equal MOI delivery to cells. These experiments were done in triplicate and analyzed traditionally by FACS analysis for GFP positive cells. The output is illustrated as a relative HR score that was determined for each siRNA by comparing to the FF control that was set to 100%.

Cut efficiency was evaluated by PCR amplification the I-SceI site in GFP after I-SceI expression and repair. The set of primers used were: L-CCGGCTGCAGCT GCTAACCATGTTCATGCC and R-GGCTCGAGAAGTCGTGCTGCTTCATGTG. The PCR was performed at a TM of 48º C for the first 5 cycles and then at 60º C for 30 cycles with Platinum Taq DNA Polymerase High Fidelity (Invitrogen). The PCR product was cloned into the Pst1/XhoI sites in the Bluescript II ks (+) vector, E. coli transformed. 100 colonies from each condition (negative control and after depletion of RHINO) were mini-prepped and the plasmid digested with I-SceI and consecutively with SfaNI (New England BioLabs). If the I-SceI site was cleaved and the DSB repaired the I-SceI site would be lost. DNA repair through HR or SSA will additionally result in the replacement of the I-SceI site with a SfaNI restriction site. In this way we were able to evaluate the percent of cells that were uncut (colonies that have the I-SceI site intact), the percent of cells that were repaired through NHEJ (colonies that lack the I-SceI or SfaNI site) and the percent of cells that were repaired through HR or SSA (colonies that lack the I-SceI but have the SfaNI site).

UV striping and Immunofluorescent foci detection

Laser striping of GFP-fusion expressing U2OS cells was carried out as previously described (*14*). Cells were fixed with 3.7% formaldehyde (10 min.) and permeabilized with 0.2% TritonX100 (10 min.) 45 min. after damage and co-stained with GFP and γ-H2AX to assess recruitment to sites of damage. Foci formation was assessed 4 h after 10 Gy using the same fix/permeabilization conditions. Alexa-Fluor conjugated secondary (Invitrogen) were used for detection.

Cell lysis, immunoprecipitation and western blot analysis

Cells were harvested by trypsinization, and cell pellets were washed with PBS and lysed in TNXG buffer (50 mM Tris pH 7.5, 150 nM NaCl, 0.5% Triton X100, 10% glycerol) supplemented with Roche Complete protease inhibitors, Calbiochem Phosphatase inhibitor cocktails I and II, 1mM PMSF and 10 mM NEM. Equivalent

protein amounts were separated using 4-12% gradient Bis-Tris gels (Invitrogen), run in MOPS or MES buffer and transferred to PVDF membrane (Millipore) overnight using a BioRad wet transfer apparatus.

Immunoprecipitations for Mass spec analysis were carried out in after overnight Dox induction of Flag-HA tagged proteins in TREX-293 cells and 4 hours after 10 Gy as previously described (*15*). Resulting protein pellets were processed as previously described and mass spec data was analyzed using ComPASS (*16*). Verification of protein interactions was independently repeated using a smaller scale IP protocol in regular 293T. HA bead complexes were directly analyzed by western blot analysis for the presence of proteins of interest.

Multicolor competition assay (MCA)

MCA assays were carried out as previously described (*17*) using IR (5 Gy), MMC (100 nM) and camptothecin (5 nM) treatments.

Cell cycle distribution

$BrdU^+$ cells and DNA content were detected using an APC BrdU Flow Kit (BD Pharmingen), according to the manufacturer's protocol.

Supplemental Table legends

Supplemental Table 1. Primary screen results.

Genes are listed in the categories of Strong, Medium or Weak. Higher scoring weak hits are denoted as Weak+. Cytotoxic hits resulted in >4-fold decrease in cell number compared to on plate negative controls. Cytotoxic siRNAs still resulting in a checkpoint bypass phenotype are denoted cytotoxic bypass. Known DDR proteins and putative ATM/ATR substrates are marked.

Supplemental Table 2. Additional genes identified in the cytotoxic rescreen at 10 nM.

98 genes that resulted in checkpoint bypass (strong, medium and weak) after decreasing the concentration of pooled siRNA to 10 nM.

Supplemental Table 3. Secondary screen results for individual siRNAs.

Results are shown for the 720 cherry picked genes (4 siRNAs per gene) for the checkpoint assay with and without DNA damage as well as MMC sensitivity. Genes are ranked by the number of siRNAs out of 4 that scored in the DNA damage checkpoint assay. siRNA sequences and catalog numbers are provided.

Supplemental Table 4. OTP siRNA testing of 114 high confidence screen hits.

Results are shown for the 114 high confidence screen hits (4 OTP siRNAs per gene) for the checkpoint assay with and without DNA damage as well as MMC sensitivity. Additionally, results of the high throughput HR assay are provided for each siRNA. siRNA sequences and catalog numbers are provided. The number of siRNAs scoring in the secondary screen as well as performance in primary screen is listed. Chromosomal locations are also given for each gene.

Supplemental Figure legends

Supplemental Figure 1. Development of the long DNA damage checkpoint assay.
(**A**) DNA damage-induced cell cycle arrest assessment using BrdU/pH3 flow cytometry. U2OS cells were treated with a 30 min. pulse of BrdU for identification of the S-phase population and then irradiated with 10 Gy, and after 1 h treated with nocodazole to prevent cells from exiting mitosis. In a parallel experiment, caffeine pretreatment was used to inactivate the ATM/ATR pathways. Cells were collected 1, 8 and 18 h after IR and processed for BrdU incorporation (X-axis) and mitotic entry (pH3+, Y-axis). The classical short G2/M checkpoint assay readout is demonstrated in upper left quadrant (pH3+, BrdU-) while this lengthened assay identifies cells that experienced DNA damage in S-phase and enter mitosis in the upper right quadrant (pH3+, BrdU+)
(**B**) ATR depletion is shown as a control in cells in cells fixed 18 h post 10 Gy.

Supplemental Figure 2. Analysis of cytotoxic siRNAs from the primary screen.
(**A**) Panther gene analysis of 1657 siRNA pools resulting in cellular cytotoxicity. Protein metabolism and modification represented the largest subclass (besides unclassified) due to the large number of ribosomal and proteasomal components whose depletion lead to cell death. Of note, the cell cycle category was also enriched.
(**B**) siRNA library efficacy as judged by expected toxicity resulting from depletion of essential genes such as ribosomal, proteasomal and RNA polymerase core components. The number of genes in each category contained in the library is listed as well as toxicity results for the primary screen and when rescreened again at 50 nM. The high reproduction rate when rescreened diminishes the likelihood of plate position dependent cell death effects.
(C) Statistics for cytotoxic siRNA pools are shown.
(**D**) The effects of Chk1 siRNA titration on viability and the checkpoint assay. Depletion of Chk1 with four independent siRNAs (6, 7, 8 and 9) was carried out at 50, 20 and 5 nM. Cell number (left Y-axis) and the IR induced cell cycle arrest assay mitotic index score (right Y-axis) demonstrates rescue of viability and checkpoint bypass phenotype at lower siRNA concentrations. In this experiment the negative control (FF) result in an average of 2500 cells and an average 1.5 % of mitotic cells.

Supplemental Figure 3. Assay conditions for the secondary screens.
(A) Individual ATR siRNAs were tested to ensure phenotypic penetrance at lower siRNA concentrations. Based on these experiments as well as with other genes (data not shown), secondary screens were carried out at 20 nM final siRNA concentration, which allowed us to develop additional assays (untreated mitotic index assay and mitomycin C sensitivity) to test our siRNAs for these additional DDR phenotypes.
(B) Example of secondary screen controls in the absence of DNA damage. The APC component Cdc16 is used as a positive control for mitotic accumulation. siRNA oligo number is shown in parentheses.
(C) MMC sensitivity curve after siRNA depletion. High throughput secondary screening was carried out with individual siRNAs, performed at 25 and 50 nM MMC concentrations compared to untreated controls and assayed for cell viability.

Supplemental Figure 4. The behavior of different DDR genes in the HR and checkpoint assays, and biological categorization of the 97 high confidence hits.
(A) Example of the 384 well microscopy based HR assay. HR efficiency is represented as a percentage relative to the negative control (FF), which is set at 100%. Data for siRNA pools of ATR, BRCA1, BRCA2 and Rad51 as well as individual siRNA results for CtIP, TopBP1, FANCM and SHFM1 are shown.
(**B**) Significant enrichment for biological categories as determined by Ingenuity pathway analysis for the 97 high confidence hits scoring with 2 or more siRNAs from the cell cycle arrest and MMC assays.
(C) Percentage of mitotic cells 18 hours post 10 Gy after depletion with 4 individual siRNAs of ATR, BRCA1, BRCA2 and 4 genes of the FA pathway. All of the genes scored with 3 or more siRNA, the threshold is set at three standard deviation above the negative control (FF).

Supplemental Figure 5. H2AX-independent recruitment of Clock, INTS7 and RHINO to sites of DNA damage and INTS7 interaction with SSB1.
(**A**) WT and H2AX null MEFs were infected with GFP fusions to either Clock, INTS7 and RHINO, striped with a UV laser and stained with P-RPA2 to identify sites of DNA damage. All three proteins localize to DNA damage in the absence of H2AX.
(**B**) Endogenous SSB1 immunoprecipitation demonstrating interaction with endogenous INTS3 and INTS7.

Supplemental Figure 6. Localization of RHINO at DDR foci after IR and measurement of depletion of RHINO mRNA levels.
(A) U2OS GFP-RHINO cells were left untreated (UNT) or irradiated (10 Gy) to examine RHINO localization. Depletion of RHINO foci formation by RHINO siRNA demonstrates siRNA specificity as compared to FF depletion.
(B) Real time PCR of RHINO levels following depletion using individual siRNAs (9, 10, 11 and 12) at 30 nM in U2OS cells. Actin mRNA was used as an internal normalization control. siRNA number 9 and 11 result in greater than 80% depletion of RHINO.

Supplemental Figure 7. Re-expression of RHINO rescues both the checkpoint and homologous recombination defects in RHINO depleted cells.
(A) Cell cycle arrest can be restored after re-expression of RHINO. After three days of siRNA transfection, cells were infected with a MOI of 2 with a retrovirus expressing the negative control or a full length siRNA-resistant cDNA version of RHINO, 18 h after infection cells were irradiated with 5 Gy, MI was calculated 8 h post IR.
(B) HR after re-expression of RHINO. As in Supp. Fig. 5A, cells were infected with a retrovirus expressing the negative control a wild type version and siRNA-resistant cDNA version of RHINO, three day post siRNA transfection and 18 h prior I-SceI cleavage.
(C) Levels of RHINO expression for the experiment shown in (B). Depletion of RHINO was achieved with siRNA #9 indicated as (9). The vector inserts are indicated below "N.C." stands for the negative control fragment, "R." stands for RHINO**.**

Supplemental Figure 8. Cell cycle distribution and efficiency of I-SceI cleavage after RHINO depletion.
(A) Depletion of RHINO does not effect cell cycle distribution. Three days after transfection of the indicated siRNAs, cells were pulse labeled with BrdU for 30' prior to fixation.
(B) Depletion of RHINO does not affect the efficiency of I-SceI cleavage. The table indicates the percent of I-SceI sites that were found uncut, repaired by imprecise non-homologous end joining (NHEJ), or repaired by homologous recombination (HR) or single strand annealing (SSA) in cells transfected with the indicated siRNAs.
(C) HR frequency for the experiment shown in (B).

Supplemental Figure 9. Localization of RHINO to sites of DNA damage depends upon the 9-1-1 complex.
(**A)** RHINO localization to DNA damage depends on Rad17. U2OS cells expressing GFP-RHINO were transfected with the indicated siRNAs and localization to UV laser stripes was quantified.
(B) Hus1$^{-/-}$ MEFs, and Hus1$^{-/-}$- MEFs reconstituted with Hus1 were infected with the GFP-RHINO expressing virus used in Fig. 4D. Lysates were probed to assess the level of expression of RHINO GFP and vinculin as indicated.

Supplemental Figure 10. RHINO depletion has no effect on PCNA monoubiquitination. siRNA transfected U2OS cells were untreated or subjected to 25 J/m2 (UV) and harvested 2 h later, as indicated. Lysates were immunoblotted to assess PCNA ubiquitination (PCNA-Ub).

Supplemental Figure 11. Different alignments of RHINO among vertebrates and the N-terminus of RHINO aligned with different virus (Amsacta moorei entomopoxvirus-AMV and Fowlpox virus-FPV), proteobacteria (Xylella fastidiosa-XF) and phage (KilA) all containing the APSES domain.
(A) Homologs were identified in mouse (GeneID: 72440), rat (GeneID: 297627), chicken (GeneID: 426083), and frog (GeneID: 100127313). No homologs were detected in yeast, worm or fly. Yellow dots indicates the SQ and TQ sites, the yellow bar indicates the most conserved region in the hypothetical APSES domain, see (B).
(B) The N-terminus of RHINO (1-70) aligned with three different proteins of the Amsacta moorei entomopoxvirus 'L' (AMV024 (Gene ID: 1494614), AMV110 (Gene ID: 1494700) and AMV112 (Gene ID: 1494702)); two proteins of the Fowlpox virus (FPV124 (Gene ID: 1486672) and FPV236 (Gene ID: 1486808)); and with the protobacterial Xylella fastidiosa XF2294 (Gene ID: 1127847) and the phage KilA (Gene ID: 2777383). The yellow bar indicates the most conserved region in the hypothetical APSES domain**.**

Supplemental Figure 12. Expression of different forms of RHINO in HeLa cells and complementation assays with the different mutants of RHINO.

(A) Levels of expression of WT and mutant GFP-RHINO proteins in the HeLa cells used in the experiment shown in Fig 4C.
(**B**) The RHINO mutants that are unable to bind the 9-1-1 complex are checkpoint defective. Three days after siRNA transfection, cells were infected at a MOI of 2 with vector alone, or a retrovirus expressing the indicated RHINO expressing constructs including an siRNA-resistant derivative of RHINO. Cells were irradiated with 5 Gy, 18 h after infection. MI was calculated 8 h post IR.
(**C**) RHINO binding to the 9-1-1 complex is required for HR. Three days after siRNA transfection, cells were untreated or infected at a MOI of 2 with retroviruses expressing the negative control, WT, SWV mutant or the N-terminal fragment of RHINO. After 18 h, cells were then infected with adenovirus expressing I-SceI to initiate HR, which was assessed by GFP expression.
(D) RHINO levels of expression relative for the experiment in C. The vector inserts are indicated below "N.C." stands for the negative control fragment, "R." stands for RHINO**.**

Supplemental Figure 13. Double depletion of RHINO and Rad17 suggest that they operate in the same HR pathway. HR assays were performed as in Fig. S4A with cells transfected with the indicated siRNAs.

Supplemental Figure 14.
During S phase, DSBs are predominantly repaired through HR. HR is an error-free repair pathway but is likely to be slower than other error-prone mechanisms like NHEJ and SSA. We suggest that the cell can detect ongoing repair by sensing multiple intermediate structures in the repair process that are able to activate the DDR (indicated with a star in the cartoon). In the absence of BRCA1, fewer structures flow through the HR pathway due to reduced CtIP function and, therefore, more intermediates flow into the NHEJ branch. 53BP1 competes with HR under these circumstances by promoting flow through the NHEJ pathway and possibly by directly antagonizing CtIP. Nevertheless, in this scenario DSBs will be predominantly repaired through NHEJ and this would result in a termination of the signal and cell cycle arrest bypass. Loss of 53BP1 in the presence of BRCA1 loss would now allow more flow of intermediates down the partially impaired HR pathway to restore signaling ends. In the absence of BRCA2, intermediates are further along in the HR pathway and therefore are not suitable for processing by NHEJ. Because of this, the effect of BRCA2 on DDR signaling is not suppressed by loss of 53BP1 or DNA-PKcs inhibition. Depletion of BRCA2 would result in reduced signaling presumably for two main reasons: it will reduce the accumulation of signaling structures (possibly Rad51 filaments) and the accumulation of RPA-ssDNA that can signal might be consumed by SSA which is increased as a fraction of repair events in cells lacking BRCA2.

Supplemental References

1. S. Matsuoka *et al*., *Science* **316**, 1160 (May 25, 2007).
2. M. P. Stokes *et al*., *Proc Natl Acad Sci U S A* **104**, 19855 (Dec 11, 2007).
3. B. Xu, S. Kim, M. B. Kastan, *Mol Cell Biol* **21**, 3445 (May, 2001).
4. H. C. Reinhardt, M. B. Yaffe, *Curr Opin Cell Biol* **21**, 245 (Apr, 2009).
5. R. I. Yarden, S. Pardo-Reoyo, M. Sgagias, K. H. Cowan, L. C. Brody, *Nat Genet* **30**, 285 (Mar, 2002).
6. W. Wang, *Nat Rev Genet* **8**, 735 (Oct, 2007).
7. A. Adamo *et al*., *Mol Cell* **39**, 25 (Jul 9, 2010).
8. P. Pace *et al*., *Science* **329**, 219 (Jul 9, 2010).
9. A. J. Pierce, R. D. Johnson, L. H. Thompson, M. Jasin, *Genes Dev* **13**, 2633 (Oct 15, 1999).
10. J. M. Stark, A. J. Pierce, J. Oh, A. Pastink, M. Jasin, *Mol Cell Biol* **24**, 9305 (Nov, 2004).
11. M. Doi, J. Hirayama, P. Sassone-Corsi, *Cell* **125**, 497 (May 5, 2006).
12. P. Lamesch *et al*., *Genomics* **89**, 307 (Mar, 2007).
13. D. M. Weinstock, K. Nakanishi, H. R. Helgadottir, M. Jasin, *Methods Enzymol* **409**, 524 (2006).
14. S. Bekker-Jensen *et al*., *J Cell Biol* **173**, 195 (Apr 24, 2006).
15. A. Ciccia *et al*., *Genes Dev* **23**, 2415 (Oct 15, 2009).
16. M. E. Sowa, E. J. Bennett, S. P. Gygi, J. W. Harper, *Cell* **138**, 389 (Jul 23, 2009).
17. A. Smogorzewska *et al*., *Cell* **129**, 289 (Apr 20, 2007).