Supplementary Information for:

A FORWARD CHEMICAL GENETIC SCREEN REVEALS AN INHIBITOR OF THE MRE11-RAD50-NBS1 COMPLEX

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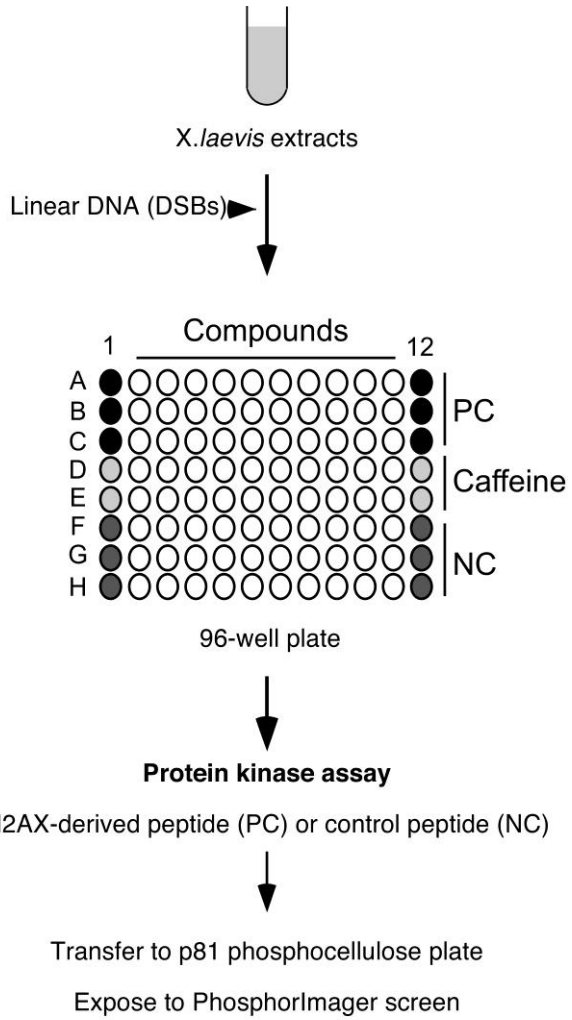
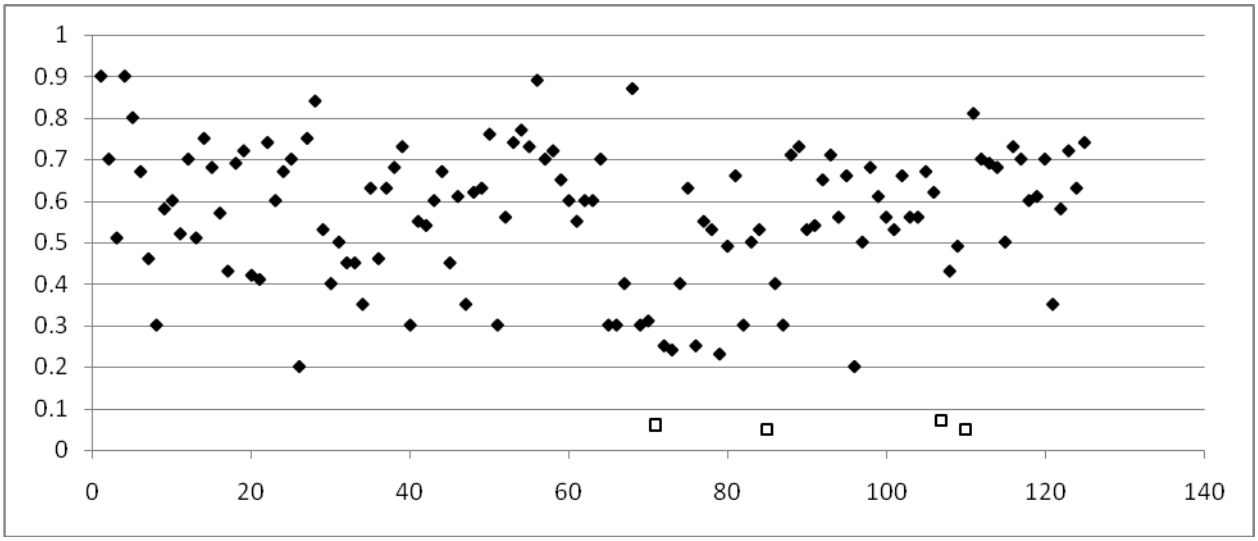
Supplementary Figure 1. Screening protocol. *Xenopus* extracts were treated with DSB-containing DNA (5 ng μl^{-1}). Aliquots of extracts were then transferred to 96-well plates containing 80 compounds (columns 2-11) per plate. Positions A1, A12, B1, B12, C1 and C12 were used for positive controls (PC; no compound). Caffeine, an ATM inhibitor, was used as a control for inhibition in positions D1, D12, E1 and E12. Positions F1, F12, G1, G12, H1 and H12 were used as negative controls (NC) with non-phosphorylatable peptide (AVGKKAAQAAQEY). The activity of ATM was assayed by measuring the incorporation of radio-labeled γ^{32}P-ATP into a peptide derived from Histone H2AX (AVGKKASQASQEY). The reactions were transferred to a 96-well P81 phosphocellulose plate, washed, dried and exposed to a PhosphorImager screen.



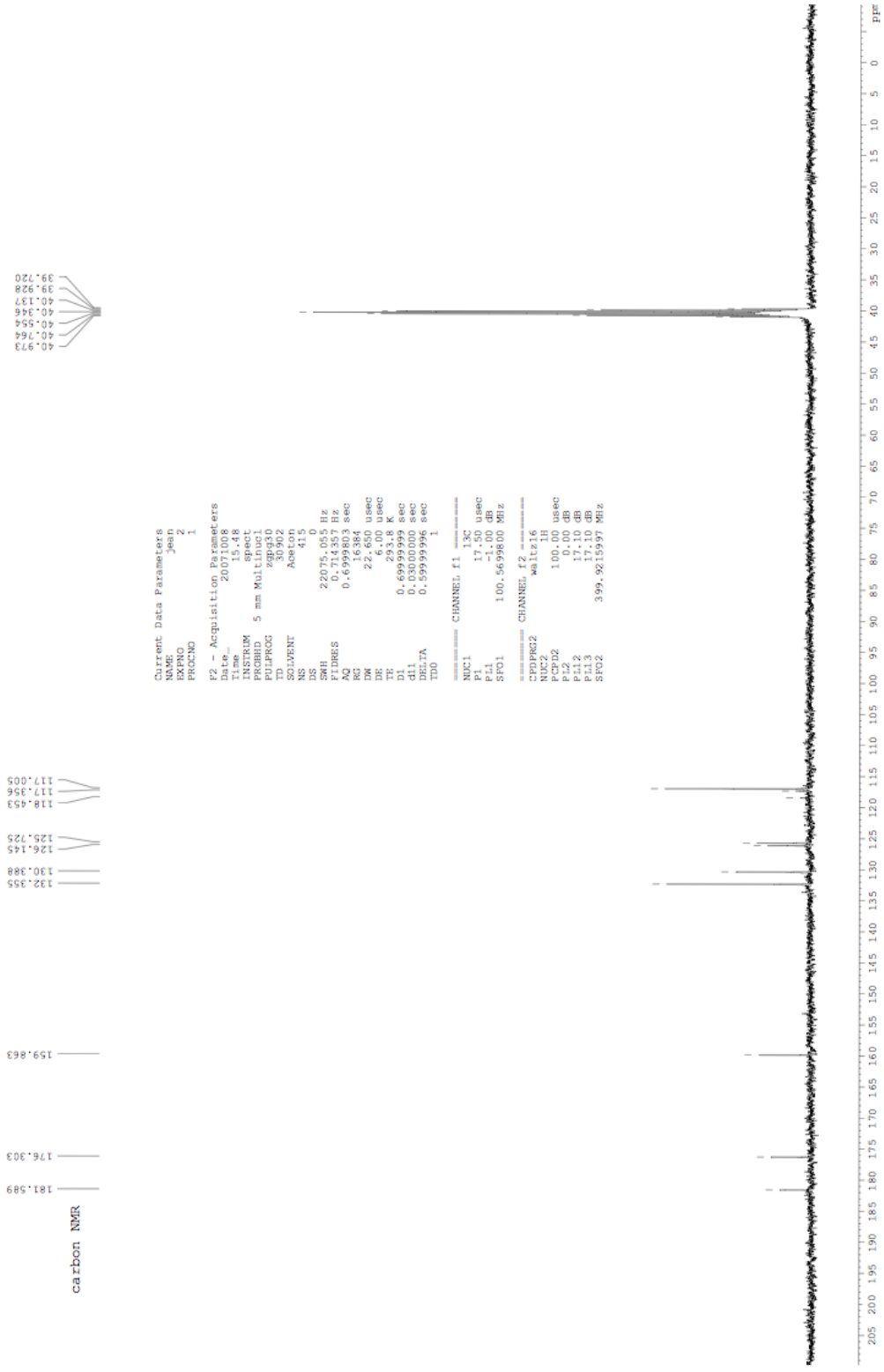
Supplementary Figure 2. Z' factor calculated for the H2AX peptide phosphorylation assay screen (125 plates).

Z' factor is used for determining validity of an assay, and Z' factor scores between 0.5 and 1 indicate an excellent screening assay. The Z' factor for each plate was calculated based on the following formula: 1– ((standard deviation positive sample values + standard deviation negative sample values) / (average of positive sample values – average negative sample values)). Open squares indicate Z' values below 0.2. Data from these plates were eliminated and these plates were not considered in the calculation of the average Z' factor for the screening assay. The average for Z' factor for 121 plates in our screen was 0.57, indicating that the assay is highly robust. Mirin was identified from plate number 68, with a Z' factor of 0.87.

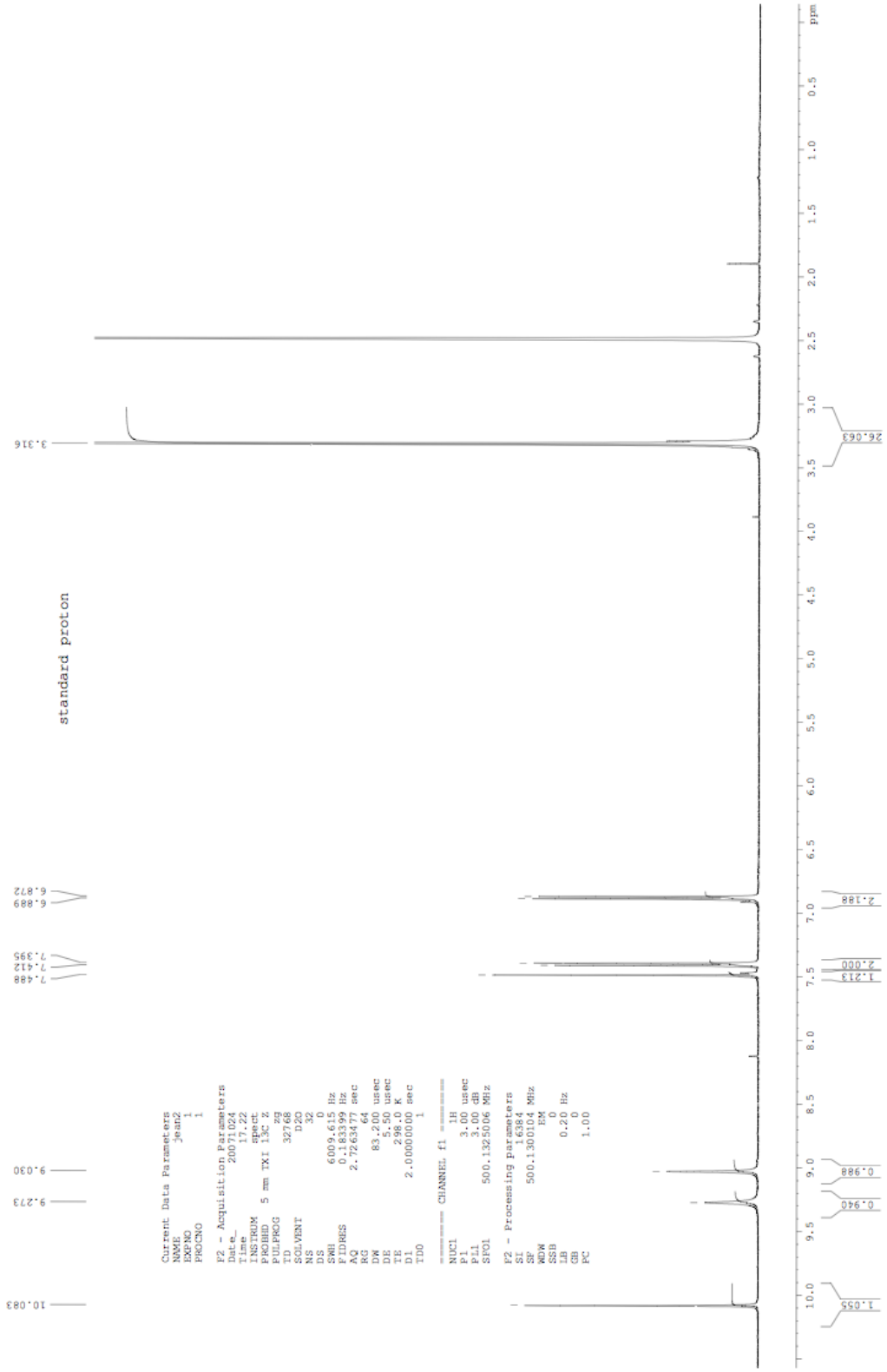


Supplementary Figure 3. Purity of Mirin by NMR

(a) ^{13}C NMR

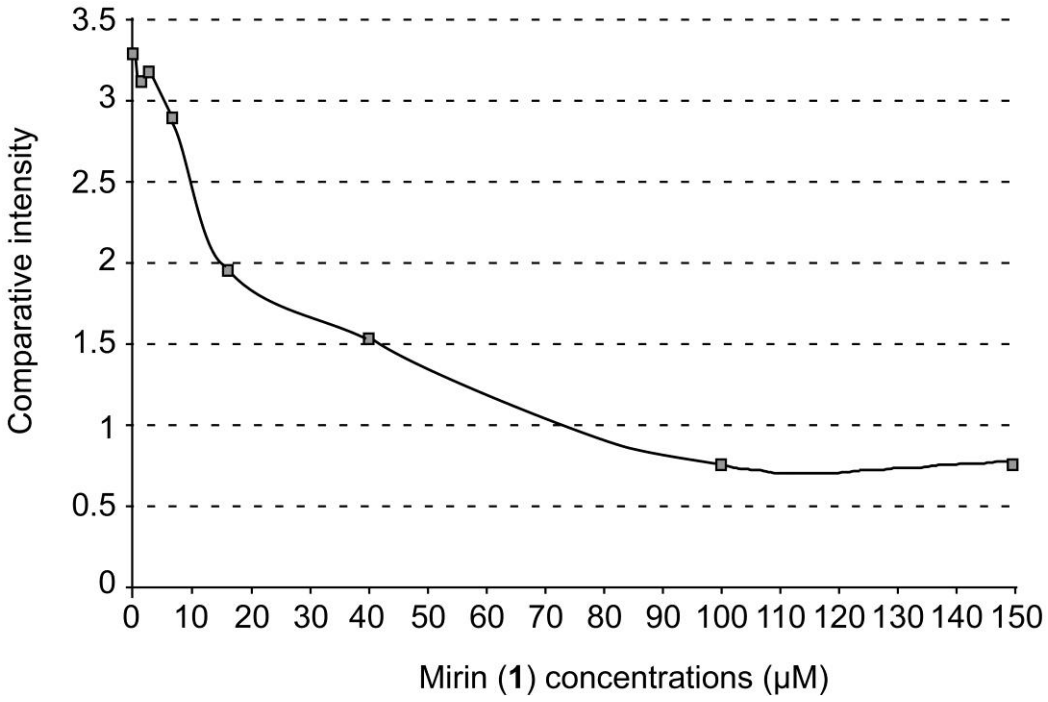


(b) ^{1}H NMR

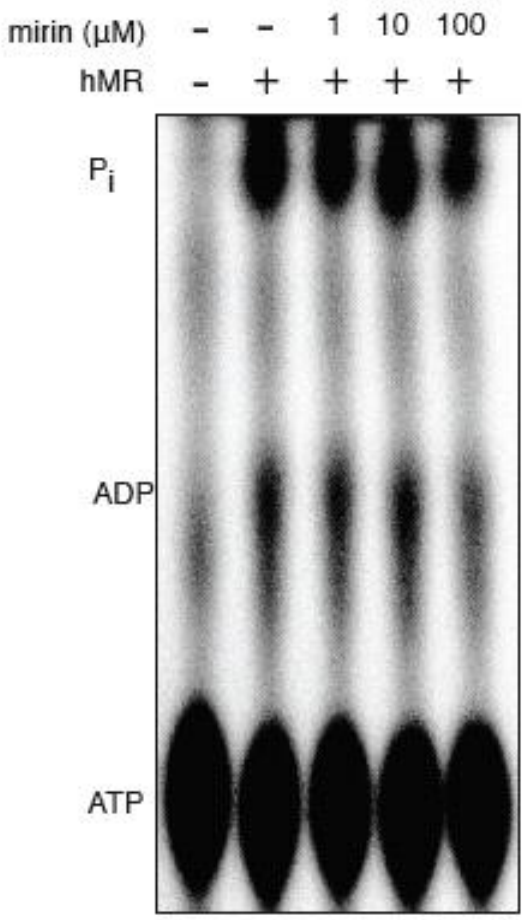

standard proton
Current Data Parameters
NAME jean2
EXPNO 1
PROCNO 1
F2 - Acquisition Parameters
Date_ 20071024
Time 17.22
INSTRUM spect
PROBHD 5 mm TXI 13C %
PULPROG zg
TD 32768
SOLVENT CDCl3
NS 32
DS 0
SWH 6009.615 Hz
FIDRES 0.183399 Hz
AQ 2.7263477 sec
RG 64
DW 83.200 usec
DE 5.50 usec
TE 298.0 K
D1 2.00000000 sec
TD0 1
======== CHANNEL f1 ========
NUC1 1H
P1 3.00 usec
PL1 3.00 dB
SFO1 500.1325006 MHz
F2 - Processing parameters
SI 16384
SF 500.1300104 MHz
WDW EM
SSB 0
LB 0.20 Hz
GB 0
PC 1.00
10.083
9.273
9.030
7.488
7.412
7.395
6.889
6.872
3.316
1.055
0.988
0.940
1.213
2.000
2.188
26.063
ppm

Supplementary Figure 4. Titration curve of Mirin inhibition of dimeric ATM activation.

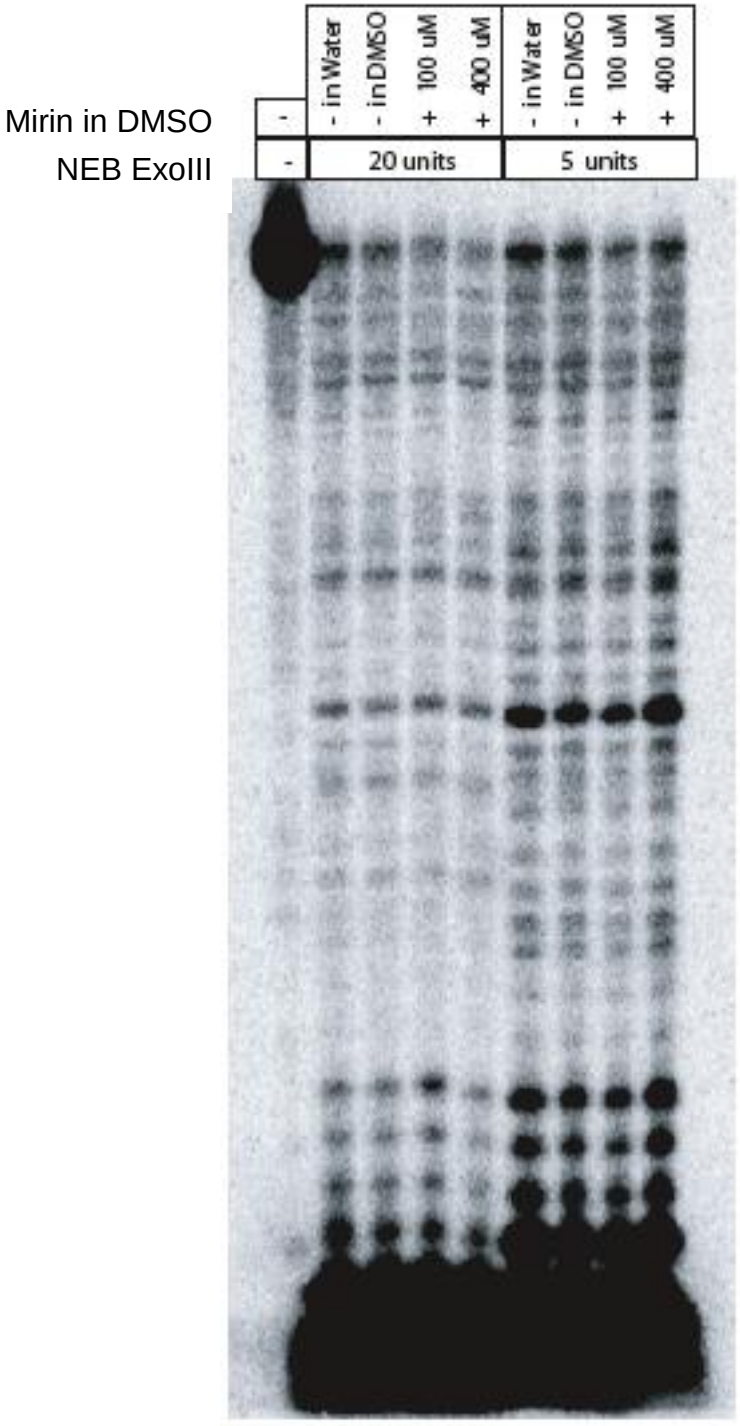
Dimeric recombinant ATM was incubated at the indicated concentrations of Mirin in presence of DNA and MRN. Activation of ATM was then monitored using phosphorylation of p53 on serine 15 as a read-out. ATM activation was inhibited by Mirin with an IC_{50} of 12 µM.



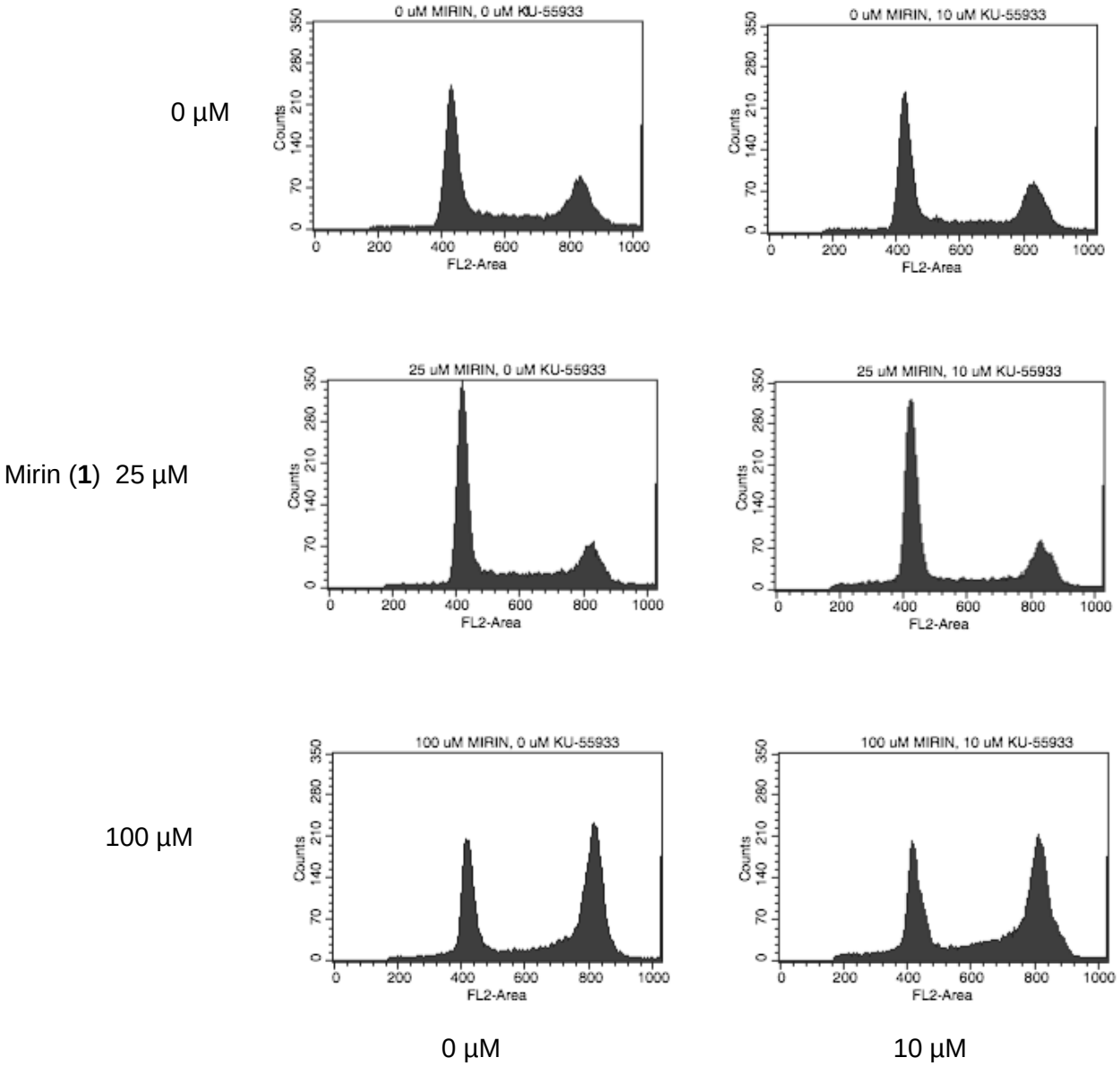
Supplementary Figure 5. Rad50-associated adenylate kinase (AK) is not inhibited by Mirin. Adenylate kinase assays were performed with human MR (~300 nM), γ^{32}P-ATP (50 µM) and AMP (50 µM). The reaction products were separated by TLC. Positions of the P_i product of ATPase activity and ADP product of AK activity are indicated.



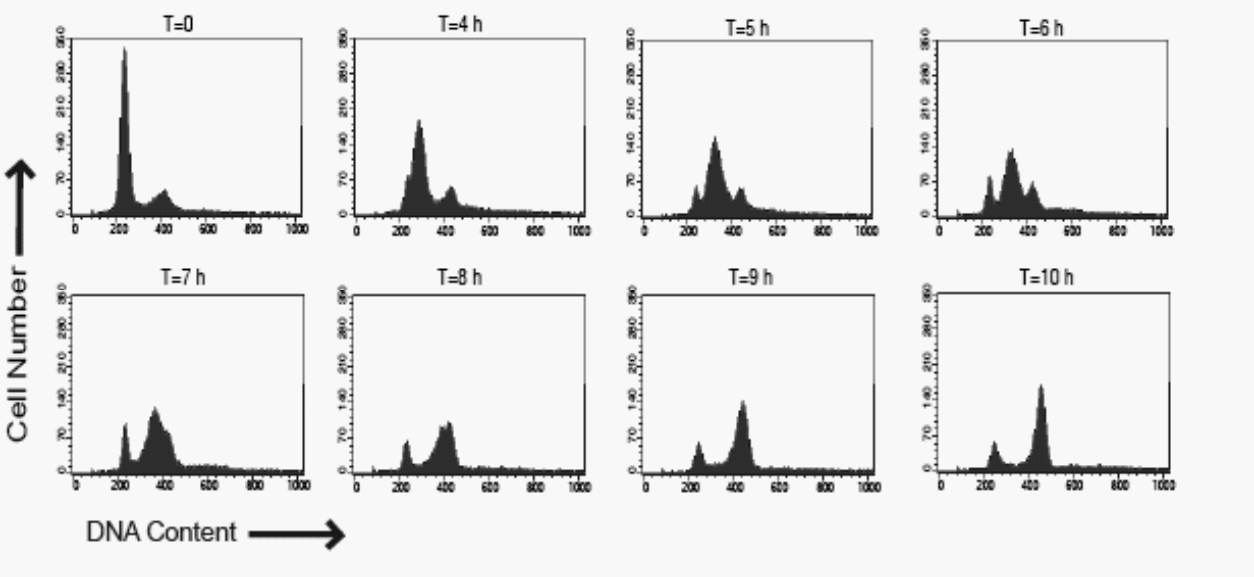
Supplementary Figure 6. Mirin does not inhibit Exonuclease III nuclease activity



Supplementary Figure 7. Effects of Mirin and KU-55933 on G2/M transition. U2OS cells were plated and allowed to attach overnight. Cells were then treated with the indicated concentrations of Mirin and/or KU-55933. After 24 h incubation, cells were harvested, fixed with 70% ethanol, and stained with propidium iodide for cell cycle analysis by FACS. .

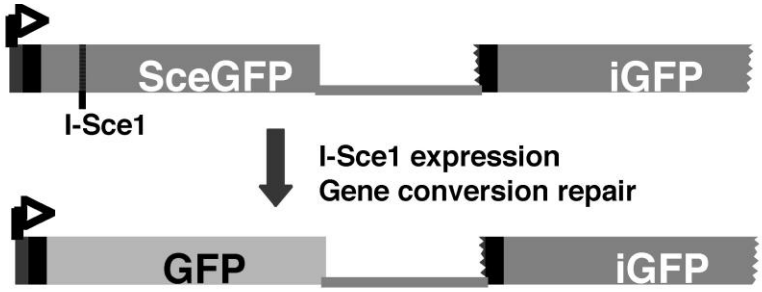


Supplementary Figure 8. Time course of U2OS cells after release from double thymidine block.



Supplementary Figure 9. Scheme of Gene Conversion Repair assay

In this assay, HEK293-derived, TOSA4 cells harbor a single chromosomally-integrated copy of the DR-GFP reporter substrate. The DR-GFP reporter harbors two non-functional copies of the GFP gene, one (*Sce*GFP) that is disrupted by the recognition site for the rare-cutting endonuclease *I-Sce*I and another (*i*GFP) that only encodes an internal region of the GFP polypeptide. Expression of I-*Sce*I in TOSA4 cells results in a chromosomal DSB at the I-*Sce*I site of *SceGFP*, and repair of the induced DSB by gene conversion with *iGFP* yields cells expressing a functional *GFP*$^{+}$ gene that can be scored by flow cytometry.



Supplementary Methods

Preparation of U2OS cell lysates

U2OS cells were synchronized in G1/S using double thymidine block (see below). Thirty min after addition of Mirin, cells were mock-treated or irradiated (10 Gy) from a Cesium-137 source in presence of 2 mM thymidine to maintain cells at G1/S border. Thirty min later, cells were harvested and washed twice with cold 1× PBS. Cell pellets were resuspended in lysis buffer (50 mM Tris, pH 7.6, 100 mM NaCl, 1% SDS, 5 mM DTT, 1X protease inhibitor cocktail (Sigma)). Lysates were heated 5 min at 95°C, protein quantified by Bradford Assay and the equivalent of 30 µg protein was loaded onto 3-8% NuPage Tris-Acetate gel (Invitrogen), followed by Western Blot analysis with the indicated antibodies. The chemiluminescent signal was quantified using densitometer and ImageQuant software.

Production and purification of recombinant FLAG MRN complex

Human FLAG-MRN complex was expressed from pTP11 (Mre11), pTP17 (Rad50) and pTP38 (FLAG-Nbs1) baculovirus constructs in SF9 insect cells as previously described in[31]. Proteins were eluted from M2-FLAG agarose resin (Sigma) with 20 mM Tris-HCl (pH 8), 100 mM NaCl, 1 mM EDTA, 0.1 % Triton X-100, 0.1% IGEPAL supplemented with 0.1mg ml^{-1} FLAG peptide (Sigma) and dialyzed against ELB buffer.

Immunodepletions and antibodies

For *Xenopus* experiments, Mre11, Nbs1 and ATM antibodies have been described elsewhere[9]. Chk2 antibody was a kind gift of Dr. H. Lindsay (Lancaster University). For immunodepletions, extracts were incubated three times with protein A–Sepharose beads (GE Healthcare) coupled to anti-XMre11 serum (ratio of extract/beads/serum = 1:1:1) or preimmune-serum previously diluted in PBS (Mock depletion).

For mammalian cells, antibodies directed against the phosphorylated form of ATM and total ATM were purchased from Rockland Immunochemicals, Inc. HRP-conjugated secondary antibodies were from Jackson Immunoresearch Laboratories.

Biotinylated DNA pull-down experiments

Non-biotinylated and biotinylated 150bp DNA fragments were generated by PCR using M13 single stranded DNA template and *Pfu* polymerase as previously described[10]. Non-biotinylated radioactive DNA was also produced by PCR in the presence of α-^{32}P-dCTP. Unincorporated nucleotides were removed using Qiagen purification kit. Biotinylated DNA was coupled to streptavidin-coated magnetic beads (M-280, Dynal Biotech) as described[10]. Beads were washed six times with ELB buffer (10mM HEPES, pH 7.7; 2.5mM $MgCl_2$; 0.05mM KCl and 250mM Sucrose) and then incubated with extracts for 10 min at a final concentration of 1.2×10^{11} or 3.6×10^{11} ends μl^{-1}. DNA and extracts were then separated according to the manufacturer's instructions. DNA was washed six times with ELB supplemented with 0.1% (v/v) Triton X-100. Proteins were then analyzed by Western blot in both soluble fractions and DNA fractions using NuPage 3-8% Tris-acetate SDS polycrylamide gels (Invitrogen).

For extract fractionation, 50 µl of extracts were loaded on a Superose 6 gel-filtration column and 1 ml fractions were collected. 200 µl of each fraction was then precipitated with tricholoroacetic acid and analyzed on a 6% SDS-PAGE gel by Western blot using an antibody directed against Mre11.

For FLAG pull down experiment, 5 µl of WT FLAG-MRN was added for 15 min to extracts, in the presence DMSO or Mirin. Recombinant complexes were then isolated with 5 µl of M2-FLAG resin. Resulting supernatants (1 µl) and beads (5 µl) were loaded on an 8% SDS page and immunoblotted using an antibody against FLAG, Mre11 or Rad50.

For DNA tethering assays, streptavidin-bound biotinylated DNA and non-biotinylated radioactive DNA were incubated in extracts for 30 min. Biotinylated DNA was then pulled down, washed six times with ELB buffer supplemented with 0.1% (v/v) Triton X-100, and the radioactivity associated with biotinylated DNA counted by scintillation.

Cytotoxicity assay

HEK293 cells were grown in DMEM with 10% fetal bovine serum and 1% penicillin-streptomycin at 37°C in 5% CO_2. HEK293 cells were plated at approximately 500 cells per 100 mm plate and allowed to attach overnight (Day 0). On the following day, media was removed and 8 ml of fresh media supplemented with the appropriate concentration (0 µM, 10 µM, 25 µM, 50 µM, 100 µM) of Mirin was added to each plate (Day 1). Duplicate plates were set up for each dose. Cells were incubated with the compound for 24 h at 37°C and 5% CO_2, after which media was aspirated and fresh, compound-free media was applied. Fresh media was applied every 3 days until Day 10. On Day 10 cells were fixed with 100% methanol and stained with 10% Giemsa for colony counting. Percentage survival is expressed as the average number of colonies on treated plate divided by average number of colonies on control plate (0 µM of Mirin).