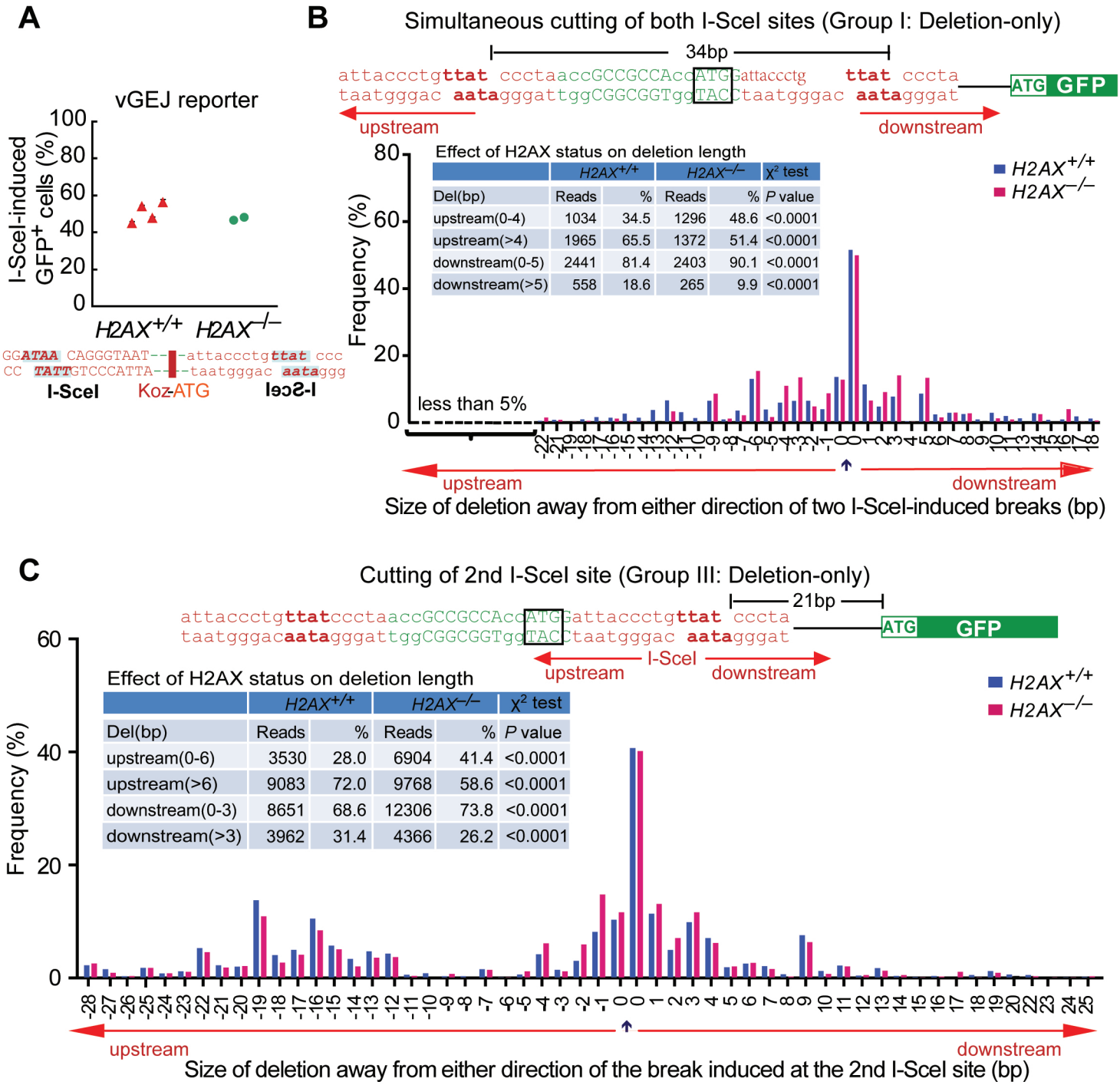
Supplementary Figure 1

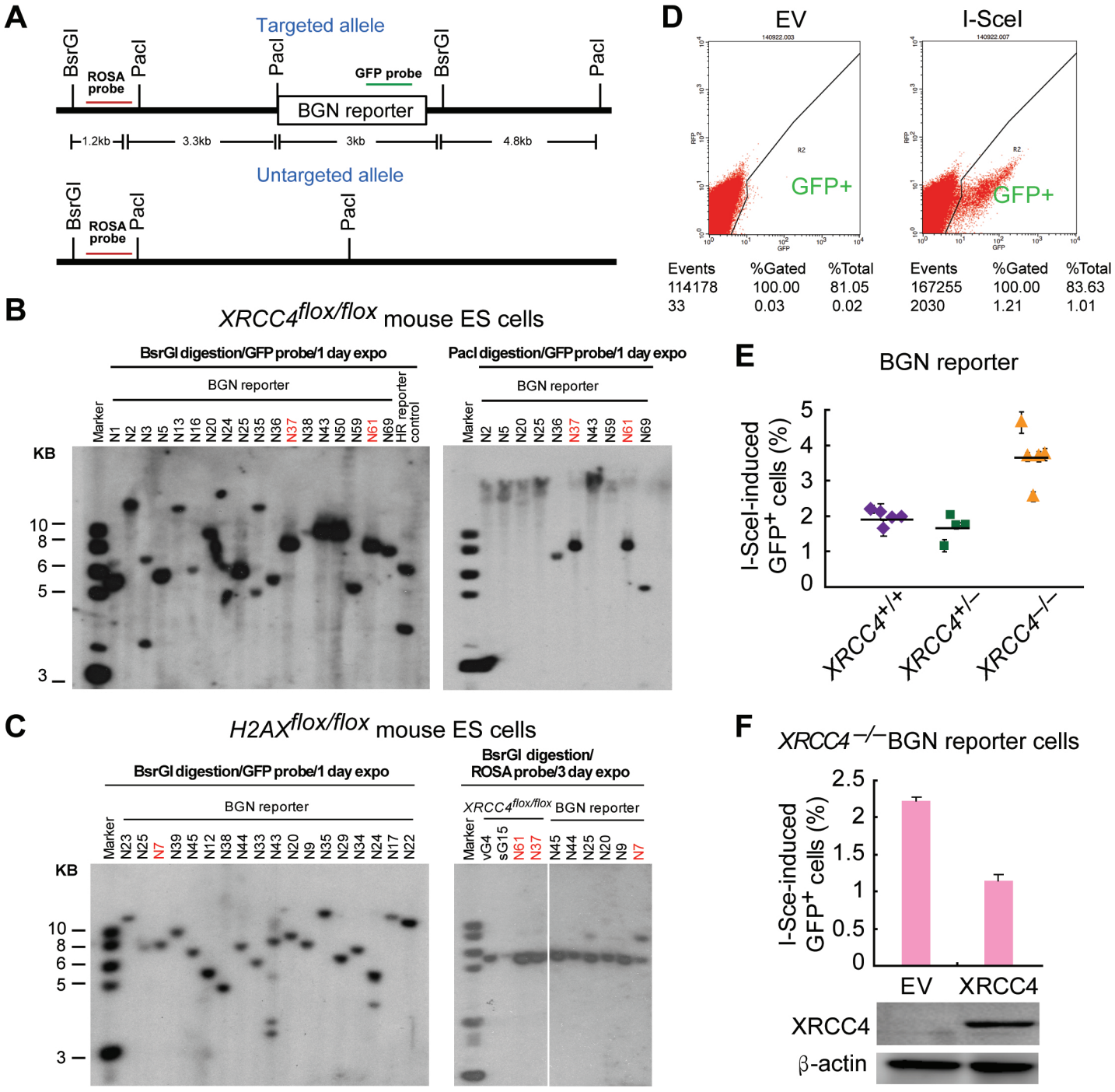


	H2AX+/+		H2AX−/−		χ² test
Del(bp)	Reads	%	Reads	%	*P* value
upstream(0-4)	1034	34.5	1296	48.6	<0.0001
upstream(>4)	1965	65.5	1372	51.4	<0.0001
downstream(0-5)	2441	81.4	2403	90.1	<0.0001
downstream(>5)	558	18.6	265	9.9	<0.0001

	H2AX+/+		H2AX−/−		χ² test
Del(bp)	Reads	%	Reads	%	*P* value
upstream(0-6)	3530	28.0	6904	41.4	<0.0001
upstream(>6)	9083	72.0	9768	58.6	<0.0001
downstream(0-3)	8651	68.6	12306	73.8	<0.0001
downstream(>3)	3962	31.4	4366	26.2	<0.0001

Supplementary Figure 1. The bias towards shorter deletion caused by *H2AX* deficiency is bi-directional. **(A)** Percentage of I-SceI-induced GFP$^+$ cells from *H2AX*$^{+/+}$ and *H2AX*$^{-/-}$ vGEJ reporter mouse ES cells. Tandem I-SceI sites in vGEJ are invertedly positioned as indicated. (**B** and **C**) Frequency of deletions with different deletion sizes away from either direction of the breaks induced simultaneously at both I-SceI site (Group I; **B**) and at the second I-SceI site (Group III; **C**) from mouse *H2AX*$^{+/+}$ and *H2AX*$^{-/-}$ ES cells. Only deletion-only events are shown here. "↑" indicates the site of the break. Deletion directions "upstream" and "downstream" are also indicated. The deletion-only NHEJ events were grouped into upstream (0-4bp), upstream (>4bp), downstream (0-5bp), and downstream (>5bp) in (**B**) and upstream (0-6bp), upstream (>6bp), downstream (0-3bp), and downstream (>3bp) in (**C**). The combined reads and frequencies of these groups were summarized in inset. *P* values from a χ^2 test are indicated. The sequence around two I-SceI sites is shown on the top of each chart with two I-SceI sites in red and the "Koz-ATG" start codon highlighted with black box.

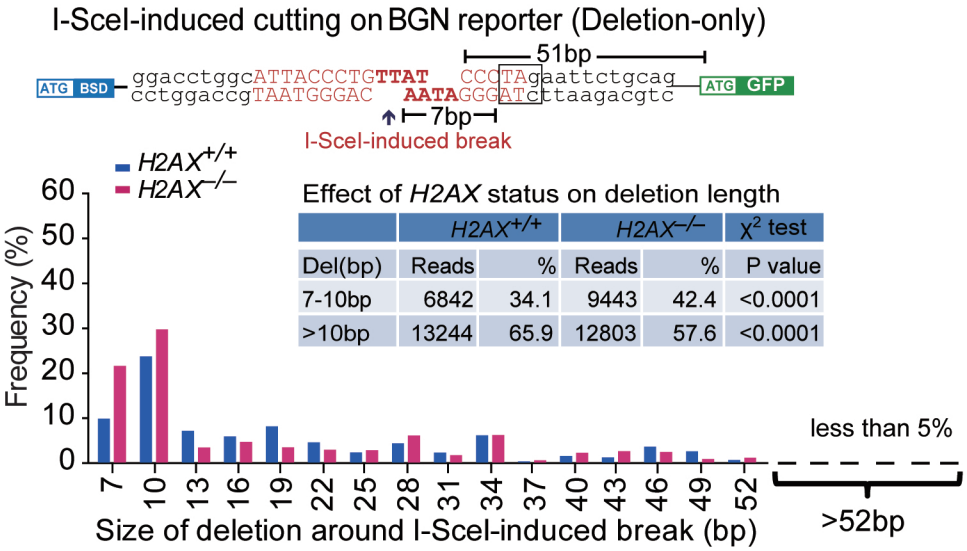
Supplementary Figure 2



Supplementary Figure 2. Generation of *XRCC4*$^{flox/flox}$ and *H2AX*$^{flox/flox}$ BGN reporter mouse ES cells. **(A)** Schematic diagram of mouse *ROSA26* alleles targeted and untargeted with the BGN reporter. *ROSA* probe (red) and *GFP* probe (green) for Southern blot were indicated. **(B)** Southern blot demonstrating the integration of single-copy, intact BGN reporter into the genome of *XRCC4*$^{flox/flox}$ mouse ES cells. gDNA were digested with BsrGI or PacI and hybridized with *GFP* probe. Clone N37 and N61 generated a single band indicating integration of single-copy BGN reporter. **(C)** Southern blot illustrating the targeting of the BGN reporter into the *ROSA26* locus of *H2AX*$^{flox/flox}$ mouse ES cells. gDNA were digested with BsrGI and hybridized by the *GFP* and *ROSA* probe. Clone N7 generated a ~8kb single band with the *GFP* probe and two bands (~5.5kb and ~8kb) with the *ROSA* probe indicating targeting of single-copy BGN reporter into the *ROSA26* locus. *XRCC4*$^{flox/flox}$ mouse ES clones N61 and N37 generated only a single band (~5.5kb) with the *ROSA* probe indicating no *ROSA26* targeting of the BGN reporter. **(D)** Examples of flow cytometry analysis of I-SceI-induced mNHEJ in mouse ES cells carrying the BGN reporter.

Reporter cells were transfected with I-SceI expression plasmids and empty vector (EV) as control and analyzed by flow cytometry 72h post-transfection. The percentages along with analysis parameters are indicated under the graph. (**E**) Percentage of I-SceI-induced GFP^+ cells from *XRCC4*$^{+/+}$, *XRCC4*$^{+/-}$ and *XRCC4*$^{-/-}$ BGN reporter cell clones. Bars represent the mean±S.D. of three independent experiments, each performed in triplicates. One-way Anova: NS between "*XRCC4*$^{+/+}$" and "*XRCC4*$^{+/-}$"; *P*=0.001 between "*XRCC4*$^{+/+}$" and "*XRCC4*$^{+/-}$". (F) Percentage of I-SceI-induced GFP^+ cells from *XRCC4*$^{-/-}$ BGN reporter cells transiently transfected with mouse *XRCC4* expression plasmids. Bars represent the mean±S.D. of three independent experiments, each in triplicates. Student's paired t-test: *P*=0.004 between "EV" and "XRCC4". Expression of exogenous *XRCC4* was detected by Western blot as indicated.

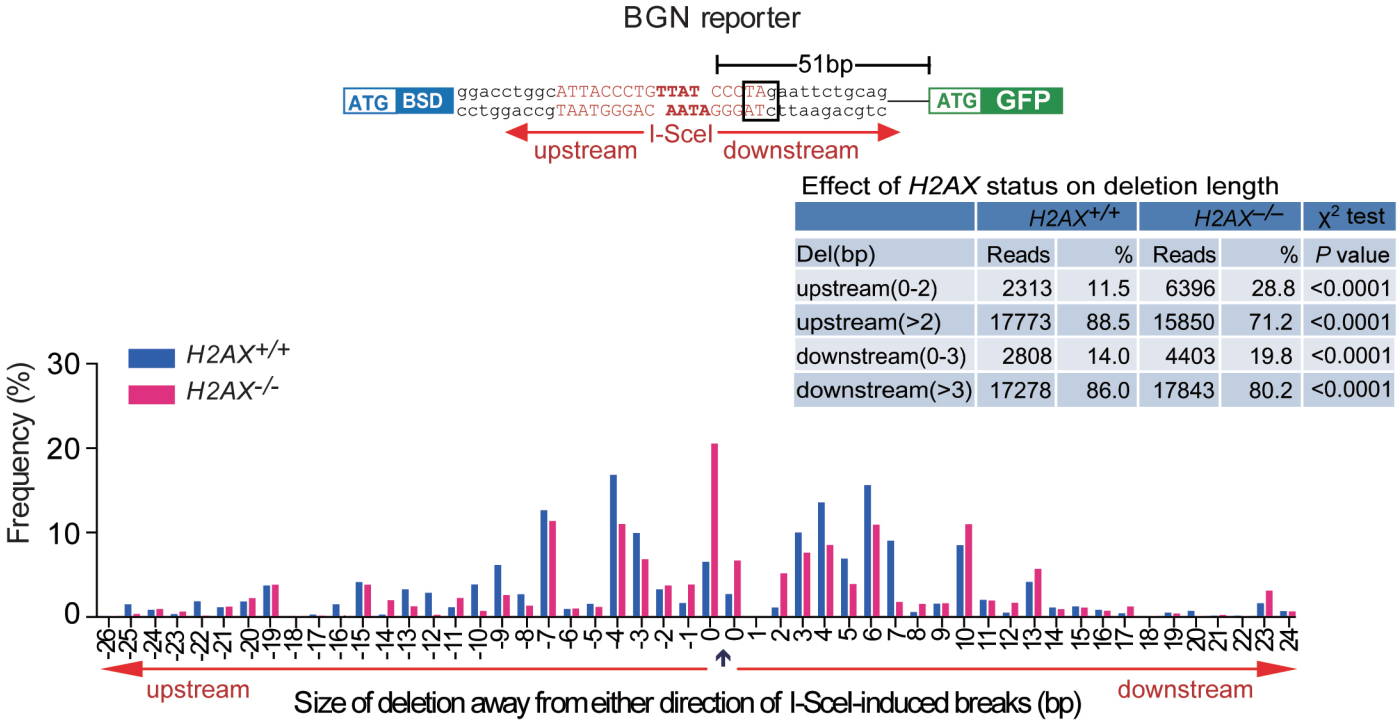
Supplementary Figure 3



	$H2AX^{+/+}$		$H2AX^{-/-}$		χ^2 test
Del(bp)	Reads	%	Reads	%	P value
7-10bp	6842	34.1	9443	42.4	<0.0001
>10bp	13244	65.9	12803	57.6	<0.0001

Supplementary Figure 3. Frequency of deletions with different deletion length in "Del" events of I-SceI-induced mNHEJ at the BGN reporter from *H2AX*$^{+/+}$ and *H2AX*$^{-/-}$ mouse ES cells. The mNHEJ events were grouped into 7-10bp and >10bp, and their combined reads and frequencies summarized in inset with *P* values from a χ^2 test indicated. The sequence surrounding the I-SceI site (red) in the BGN reporter is shown with the "TAG" stop codon highlighted in black box. The distance between I-SceI-induced breakage site and the GFP start codon "ATG" is 51bp as indicated.

Supplementary Figure 4

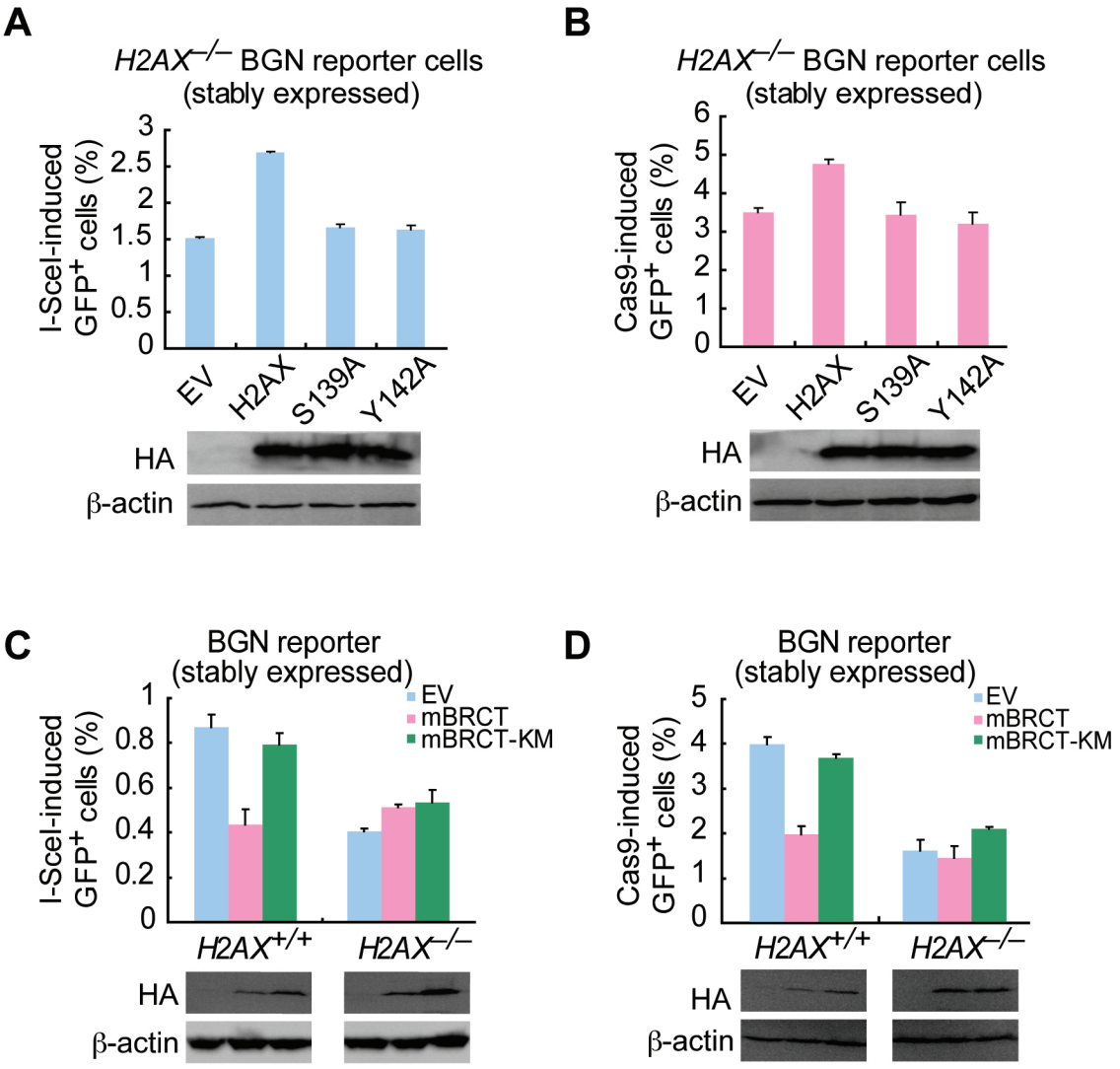


Effect of *H2AX* status on deletion length

	$H2AX^{+/+}$		$H2AX^{-/-}$		χ^2 test
Del(bp)	Reads	%	Reads	%	*P* value
upstream(0-2)	2313	11.5	6396	28.8	<0.0001
upstream(>2)	17773	88.5	15850	71.2	<0.0001
downstream(0-3)	2808	14.0	4403	19.8	<0.0001
downstream(>3)	17278	86.0	17843	80.2	<0.0001

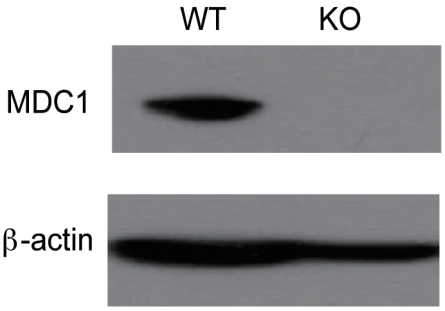
Supplementary Figure 4. Frequency of deletions with different deletion sizes away from either direction of the I-SceI-induced break at the BGN reporter from $H2AX^{+/+}$ and $H2AX^{-/-}$ mouse ES cells. The sequence around the I-SceI site of the BGN reporter is shown on the top of the chart with the I-SceI site in red and the "TAg" stop codon in black box. The 51bp distance between I-SceI-induced breakage site and the GFP start codon "ATG" is indicated, and the deletion distributions demonstrate the sizes of most deletions are within 51bp from either direction. Only deletion-only events are shown here. "↑" indicates the site of the break. Deletion directions "upstream" and "downstream" are also indicated. The deletion-only NHEJ events were grouped into upstream (0-2bp), upstream (>2bp), downstream (0-3bp), and downstream (>3bp). The combined reads and frequencies of these groups were summarized in inset. *P* values from a χ^2 test are indicated.

Supplementary Figure 5



Supplementary Figure 5. Function of H2AX in mNHEJ requires γH2AX/MDC1 signaling. (**A** and **B**) Percentage of I-SceI (**A**) or Cas9 (**B**)-induced GFP$^+$ cells from *H2AX*$^{-/-}$ BGN reporter cells stably transfected with mouse H2AX expression plasmids. Bars represent the mean±S.D. of three independent experiments, each performed in triplicates. In I-SceI-induced NHEJ assays (**A**), Student's paired t-test: *P*=0.014 between "EV" and "WT"; *P*=0.039 between "WT" and "S139A"; and *P*=0.004 between "WT" and "Y142A". In Cas9-induced NHEJ assays (**B**), Student's paired t-test: *P*=0.047 between "EV" and "WT"; *P*=0.039 between "WT" and "S139A"; and *P*=0.048 between "WT" and "Y142A". Exogenous HA-tagged H2AX and its variants were detected by Western blot as indicated. (**C** and **D**) Percentage of I-SceI (**C**) or Cas9 (**D**)-induced GFP$^+$ cells from *H2AX*$^{+/+}$ and *H2AX*$^{-/-}$ BGN reporter cells stably transfected with EV, HA-tagged mBRCT or mBRCT-KM expression plasmids. Bars represent the mean±S.D. of three independent experiments, each performed in triplicates. Student's paired t-test between "EV" and "mBRCT" in *H2AX*$^{+/+}$ BGN reporter cells: *P*=0.005 in I-SceI-induced NHEJ assays (**C**) and *P*=0.0003 in Cas9-induced NHEJ assays (**D**); between "EV" and "mBRCT" in *H2AX*$^{-/-}$ BGN reporter cells and between "EV" and "mBRCT KM" in both *H2AX*$^{+/+}$ and *H2AX*$^{-/-}$ BGN reporter cells: NS. Exogenous HA-tagged mBRCT proteins detected by Western blot as indicated.

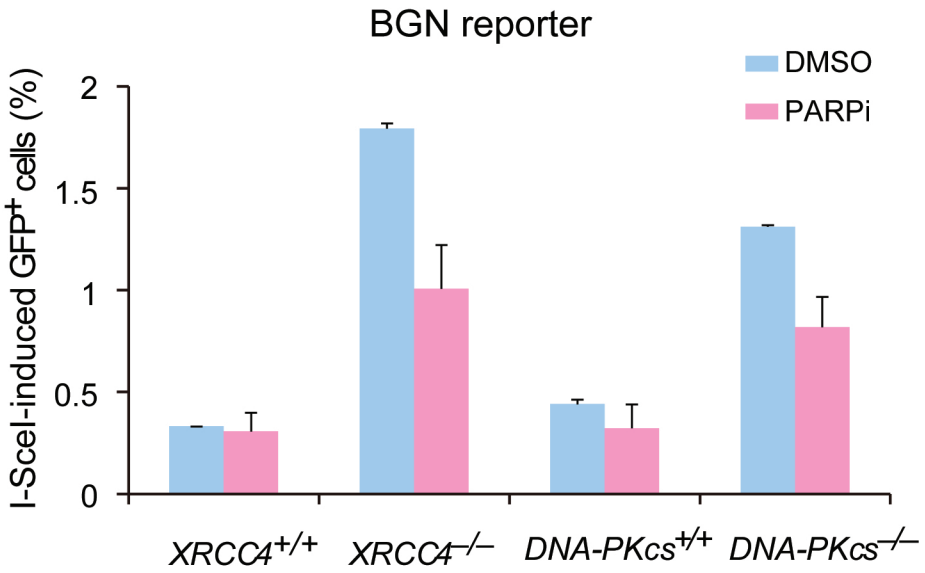
Supplementary Figure 6



WT. CAGGTGATTGAC.....ACTATCCAGTGGCTCCTT
KO Δ52bp. CAGG--------.....----------CTT

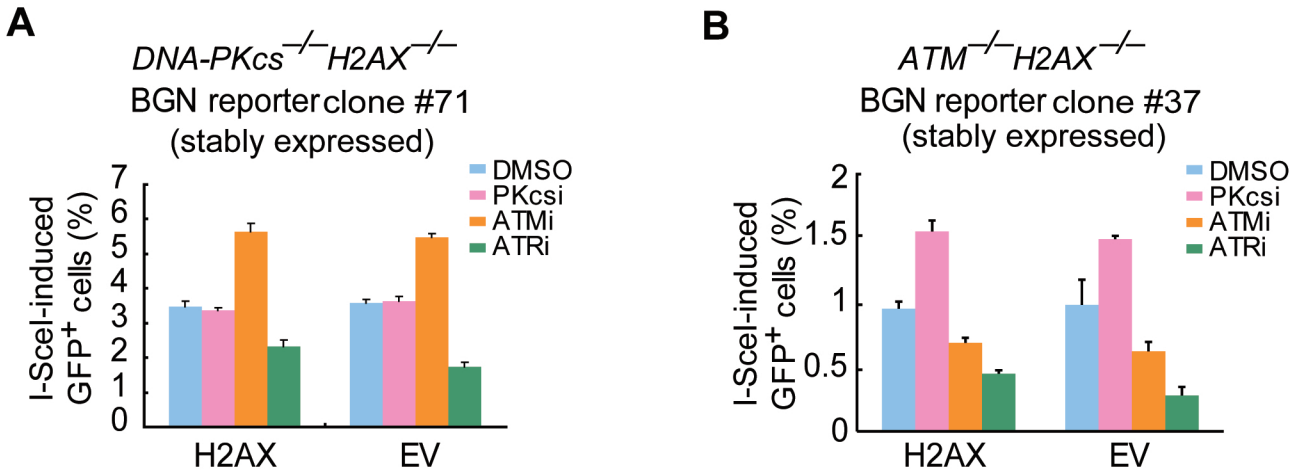
Supplementary Figure 6. Generation of *MDC1* knockout mouse ES cells by CRISPR/Cas9. Mouse ES cells were transfected twice with Cas9 expression plasmids and *MDC1* gRNA plasmids, and then plated on MEF. In about 2 weeks, individual clones were picked, and deletion of *MDC1* in one *MDC1* knockout clone was verified by Western blot (upper) and by Sanger sequencing of the edited site (lower).

Supplementary Figure 7



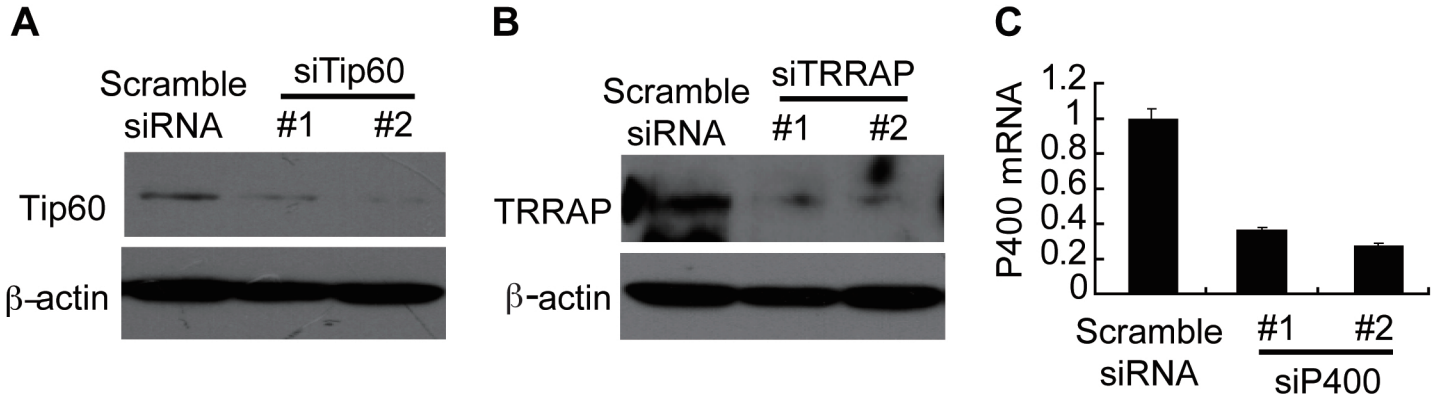
Supplementary Figure 7. Effect of the PARP inhibitor (PARPi) Olaparib (2uM) on I-SceI-induced mNHEJ in *XRCC4*$^{+/+}$, *XRCC4*$^{-/-}$, *DNA-PKcs*$^{+/+}$ and *DNA-PKcs*$^{-/-}$ BGN reporter cells. Bars represent the mean±S.D. of three independent experiments, each in triplicates. Student's paired t-test: $P<0.05$ between "DMSO" and "PARPi" in both *XRCC4*$^{-/-}$ and *DNA-PKcs*$^{-/-}$ BGN reporter cells; and NS between "DMSO" and "PARPi" in either *XRCC4*$^{+/+}$ or *DNA-PKcs*$^{+/+}$ clones.

Supplementary Figure 8



Supplementary Figure 8. Effect of DNA-PKcs inhibitor (PKcsi; NU7441), ATM inhibitor (ATMi; KU60019) and ATR inhibitor (ATRi; VE821) on I-SceI-induced mNHEJ in *DNA-PKcs*$^{-/-}$*H2AX*$^{-/-}$ H2AX and *DNA-PKcs*$^{-/-}$*H2AX*$^{-/-}$EV BGN reporter cells (**A**), and in *ATM*$^{-/-}$*H2AX*$^{-/-}$H2AX and *ATM*$^{-/-}$*H2AX*$^{-/-}$EV BGN reporter cells (**B**). Bars represent the mean±S.D. of three independent experiments, each in triplicates. Student's paired t-test: $P<0.05$ between "DMSO" and "ATMi" and NS between "DMSO" and "PKcsi" in both *DNA-PKcs*$^{-/-}$*H2AX*$^{-/-}$H2AX and *DNA-PKcs*$^{-/-}$*H2AX*$^{-/-}$ EV clones (**A**); $P<0.001$ between "DMSO" and "PKcsi" and $P<0.05$ between "DMSO" and "ATMi" in both *ATM*$^{-/-}$*H2AX*$^{-/-}$H2AX and *ATM*$^{-/-}$*H2AX*$^{-/-}$EV clones (**B**).

Supplementary Figure 9



Supplementary Figure 9. SiRNA-mediated depletion of the chromatin remodeler Tip60-TRRAP-P400 in BGN reporter mouse ES cells. Mouse ES cells were transfected with scramble siRNA and a pair of siRNAs respectively targeting Tip60 (**A**), TRRAP (**B**) and P400 (**C**), along with I-SceI expression plasmid. Cells were collected and lysed 72h post-transfection for Western blot (**A** and **B**) and for qRT-PCR (**C**) as indicated.

Supplementary Table 1. Effect of *H2AX* status on I-SceI-induced NHEJ events in sGEJ reporter mouse ES cells (**Del**: mNHEJ events carrying deletions only; **Ins**: mNHEJ events carrying insertions only; **InDel**: mNHEJ events carrying both deletions and insertions.)

	Reads		**%Total of each group**		**%Total reads**		
***H2AX* status**	+/+	–/–	+/+	–/–	+/+	–/–	χ^2 **test**
Total NHEJ	**52231**	**71075**	**–**	**–**	**100**	**100**	
Accurate+naNHEJ	31918	45934	–	–	61.1	64.6	<0.0001
mNHEJ	20313	25141	–	–	38.9	35.4	<0.0001
Group I (Simultaneous)	**35880**	**49822**	**100**	**100**	**68.7**	**70.1**	
Accurate NHEJ	**30255**	**43640**	**84.3**	**87.6**	**57.9**	**61.4**	<0.0001
naNHEJ	**967**	**1008**	**2.7**	**2.0**	**1.9**	**1.4**	<0.0001
mNHEJ	**4658**	**5174**	**13.0**	**10.4**	**8.9**	**7.3**	<0.0001
Del	2999	2779	8.4	5.6	5.7	3.9	
Ins	657	736	1.8	1.5	1.3	1.0	
InDel	1002	1659	2.8	3.3	1.9	2.3	
Group II	**2768**	**2950**	**100.0**	**100.0**	**5.3**	**4.2**	
mNHEJ	**2768**	**2950**	**100.0**	**100.0**	**5.3**	**4.2**	
Del	2767	2950	100.0	100.0	5.3	4.2	
Ins	0	0			0.0	0.0	
InDel	1	0			0.0	0.0	
Group III	**13583**	**18303**	**100.0**	**100.0**	**26.0**	**25.8**	
naNHEJ	**696**	**1286**	**5.1**	**7.0**	**1.3**	**1.8**	<0.0001
mNHEJ	**12887**	**17017**	**94.9**	**93.0**	**24.7**	**23.9**	<0.0001
Del	12613	16672	92.9	91.1	24.1	23.5	
Ins	197	265	1.4	1.4	0.4	0.4	
InDel	77	80	0.6	0.4	0.1	0.1	

Supplementary Table 2. Effect of *H2AX* status on Cas9/g2-2-induced NHEJ events in sGEJ reporter mouse ES cells

	***H2AX*$^{+/+}$**		***H2AX*$^{-/-}$**		**χ^2 test**
	Reads	%	Reads	%	*P* value
Del	390266	83.7	297876	79.6	<0.0001
Ins	51140	11.0	57193	15.2	<0.0001
InDel	24997	5.3	19325	5.2	<0.0001

Supplementary Table 3. Effect of *H2AX* status on I-SceI-induced NHEJ events in BGN reporter mouse ES cells

	***H2AX*$^{+/+}$**		***H2AX*$^{-/-}$**		**χ^2 test**
	Reads	%	Reads	%	*P* value
Del	20086	72.0	22246	65.9	<0.0001
Ins	994	3.6	509	1.5	<0.0001
InDel	6803	24.4	10993	32.6	<0.0001

Supplementary Table 4. Effect of *H2AX* status on Cas9/g4-2-induced NHEJ events in BGN reporter mouse ES cells

	***H2AX*$^{+/+}$**		***H2AX*$^{-/-}$**		**χ^2 test**
	Reads	%	Reads	%	*P* value
Del	931509	69.2	711297	51.9	<0.0001
Ins	292984	21.8	550792	40.1	<0.0001
InDel	120837	9.0	109350	8.0	<0.0001

Supplementary Table 5. Effect of *MDC1* status on paired gRNA-guided Cas9-induced NHEJ events at the *LDHA* locus of mouse ES cells

	Reads		%NHEJ		%Total reads		%Total (normalized with TE)[2]		
***MDC1* status**	+/+	–/–	+/+	–/–	+/+	–/–	+/+	–/–	**χ2 test**
Total	**951189**	**871778**	**–**	**–**	**100**	**100**			
Total NHEJ[1]	**803420**	**656228**	**100.0**	**100.0**	**84.5**	**75.3**	**57.9**	**43.8**	**<0.0001**
Accurate NHEJ	426764	427027	53.1	65.1	44.9	49.0	30.7	28.5	
mNHEJ	376656	229201	46.9	34.9	39.6	26.3	27.1	15.3	<0.0001
Individual cutting	**114655**	**79071**	**–**	**–**	**12.1**	**9.1**	**8.3**	**5.3**	
Del	82482	48727	–	–	8.7	5.6	5.9	3.2	
Ins	3257	7134	–	–	0.3	0.8	0.2	0.5	
InDel	28916	23210	–	–	3.0	2.7	2.1	1.5	
Simultaneous cutting (Group I)	**688765**	**577157**	**100.0**	**100.0**	**72.4**	**66.2**	**49.6**	**38.5**	**<0.0001**
Accurate NHEJ	426764	427027	62.0	74.0	44.9	49.0	30.7	28.5	
mNHEJ	262001	150130	38.0	26.0	27.5	17.2	18.9	10.0	<0.0001
Del	213546	100875	31.0	17.5	22.5	11.6	15.4	6.7	
Ins	17357	24871	2.5	4.3	1.8	2.9	1.2	1.7	
InDel	31098	24384	4.5	4.2	3.3	2.8	2.2	1.6	

[1]. Total NHEJ = Joining of one end of DSB and one end of the other DSB + Joining of two ends of individual DSB.

[2]. TE: transfection efficiency, which reflects the level of DSB induction in each cell line. Because transfection was done twice for site-specific induction of DSBs, %Total is normalized with TE twice and equals (%Total reads / TE) / 2.

Supplementary Table 6. Effect of *H2AX* status on paired gRNA-guided Cas9-induced NHEJ events at the *LDHA* locus of mouse ES cells

	Reads		**%NHEJ**		**%Total reads**		**%Total** (normalized with TE)[2]		
***H2AX* status**	+/+	–/–	+/+	–/–	+/+	–/–	+/+	–/–	**χ2 test**
Total	**1676348**	**1510050**	**–**	**–**	**100.0**	**100.0**	**100.0**	**100.0**	
Total NHEJ[1]	**398845**	**167938**	**100.0**	**100.0**	**23.8**	**11.1**	**40.1**	**18.2**	**<0.0001**
Accurate NHEJ	118986	95194	29.8	56.7	7.1	6.3	12.0	10.3	<0.0001
mNHEJ	279859	72744	70.2	43.3	16.7	4.8	28.2	7.9	<0.0001
Individual cutting	**184854**	**38562**	**–**	**–**	**11.0**	**2.6**	**18.6**	**4.2**	
Del	126547	20870	**–**	**–**	7.5	1.4	12.7	2.3	
Ins	12790	7019	**–**	**–**	0.8	0.5	1.3	0.8	
InDel	45517	10673	**–**	**–**	2.7	0.7	4.6	1.2	
Simultaneous cutting (Group I)	**213991**	**129376**	**100**	**100**	**12.8**	**8.6**	**21.5**	**14.1**	**<0.0001**
Accurate NHEJ	118986	95194	55.6	73.6	7.1	6.3	12.0	10.3	<0.0001
mNHEJ	95005	34182	44.4	26.4	5.7	2.3	9.6	3.7	<0.0001
Del	74428	19939	34.8	15.4	4.4	1.3	7.5	2.2	
Ins	7916	9157	3.7	7.1	0.5	0.6	0.8	1.0	
InDel	12661	5086	5.9	3.9	0.8	0.3	1.3	0.6	

[1]. Total NHEJ = Joining of one end of DSB and one end of the other DSB + Joining of two ends of individual DSB.

[2]. TE: transfection efficiency, which reflects the level of DSB induction in each cell line. Because transfection was done twice for site-specific induction of DSBs, %Total is normalized with TE twice and equals (%Total reads / TE) / 2.

Supplementary Table 7. Sequence list of gRNAs, PCR primers and siRNAs

sgRNA	Sequence (5'-3')	PAM	Length (bp)
mRosa26-1	CATCCACGCACCCCTGACCC	AGG	20
mRosa26-2	GGCTGCAGCATCAGTACAC	AGG	19
mRosa26-3	CCTAAGAATGAGAAAGGCAA	AGG	20
mH2AX	TCTACCTCGTACACTATGTC	CGG	20
mDNA-PKcs-1	TGTTGGCTACTGCAGCTGC	AGG	19
mDNA-PKcs-2	CAGACCGCTGCAGTGCTGC	CGG	19
mATM-1	ACAGAATTTCGCTTAGCTGG	AGG	20
mATM-2	TTGTGTGGGTTCTGATGGCA	AGG	20
mLDHA-1	AGCATCACCAAGTGCAGGCA	AGG	20
mLDHA-2	TAATATGCTAACACCTTGGT	GGG	20
mMDC1-1	AACTATCCAGTGGCTCCTT	GGG	19
mMDC1-2	AAGAGACGTAGCTGCCCTAT	AGG	20
sGEJ2-1	AGGGATAACAGGGTAATCCA	TGG	20
sGEJ2-2	ATAACAGGGTAATCCATGG	TGG	19
sGEJ2-3	TGGATTACCCTGTTATCCCT	AGG	20
BGN4-1	TTCTGCAGTCGACGGTACCG	CGG	20
BGN4-2	CCGTCGACTGCAGAATTCTA	GGG	20

PCR primers	Sequence (5'-3')
BSD-F	CTCAAGCTTAACTAAACCATGGCCAAGCCTTTGTCTCAAGAAG
BSD-R	AGAATTCCAGTAGGGATAACAGGGTAATGCCAGGTCCGCCCTCCC ACACATAACCAGAG
sGEJ-F	CCGCGCTGTTCTCCTCTTC
sGEJ-R	GCCGTACGTAAGGGTGGTCACGAGGGTGG
BGN-F	GCTGGCAACCTGACTTGTATC
BGN-R	GCTTGCCGGTGGTGCAGATG
mRosa26-F	GAGCACAGGAACAATTGGC
mRosa26-R	GAATCCGTGCAAGCCAAG
mLDHA-F	CCACTGAGCCATCTATCTCTCC
mLDHA-R	GGTGCAGACATAAGCGGGAGAA
mH2AX-F	GCGTGTCCAGGGAGTTTATAAGG
mH2AX-R	GTAGTGGCCTTTCCGCAGCAG
mATM-F	TAGGTTGATCCCACAGGAGAG
mATM-R	ATCCAGACAACCAAATGAGG
mDNA-PKcs-F	GAGTGCAAACACCAATCTGTGATATCTCCG
mDNA-PKcs-R	TTTTGTCAAGGTCCTATTGAGTTCAAGCGA
mMDC1-F	GGAAATCATCACCTCATGGT
mMDC1-R	ATAAATGGTTCTACCAACCC
qPCR-mP400-F	CCAGATACAGCAGCTGATGAGCAG
qPCR-mP400-R	TGTATGATGGAGAGCCATGCTC
qPCR-mActin-F	TGAGCGCAAGTACTCTGTGTGGAT
qPCR-mActin-R	ACTCATCGTACTCCTGCTTGCTGA

siRNA targets	Sequence (5'-3')
mTRRAP-1	GCACATGAAGGACCTCTTT
mTRRAP-2	GGAGATGAACTGAAAGCAA
mTip60-1	ACGGAAGGTGGAGGTGGTT
mTip60-2	GGGCCAGTATATCCTAACT
mP400-1	GCGGTTGCCAAAGCTTCAA
mP400-2	GGAACCATCTGTCAGTGAA