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Supplemental Data

Distinct Roles of Chromatin-Associated Proteins MDC1 and 53BP1 in Mammalian Double-Strand Break Repair

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Supplemental Experimental Procedures:

siRNA oligos and shRNA constructs

Control RNAi duplex (AAUAACAGUGACCUUUAUGGAdTdT), RNAi duplex against mouse *BRCA1* (AACCAGAAGAAAGGGCCUUCAdTdT), human *53BP1* (GCCAGGUUCUAGAGGAUGAdTdT), and RNAi smart-pool against mouse *BRCA2* and *Rad51* were purchased from Dharmacon. pSUPER-MDC1 and LacZ shRNA constructs have been described (Stucki et al, 2005). Two pSUPER-mouse 53BP1 shRNA constructs were created by replacing MDC1-targeting sequence with mouse 53BP1-targeting sequences (#1: GTGGTCATCCAATGGCTAC or #2: GCCAGGTTCTGGAAGAAGA). The shRNA-expression cassettes were cloned into retroviral vector MSIHyg to generate MSIH-mouse 53BP1 shRNA constructs.

Cell lines

Doxycylin (Dox)-inducible I-SceI expression system in U2OS reporter cells was established according to BD^{TM} Tet-ON Gene Expression Systems User Manual (BD Biosciences) and will be described in detail in a later manuscript. Mouse reporter ES

cells stably expressing control and mouse 53BP1 shRNA were generated by transfecting cells with MSIH-shRNA constructs and selecting in hygromycin (400μg/mL).

Antibodies

Commercial antibodies used include mouse monoclonal anti-CHK1 antibody (Santa Cruz Biotechnology) and rabbit polyclonal anti-53BP1 (Novus Biologicals) and anti-myc (Abcam). Mouse monoclonal anti-human 53BP1 antibody (BP13) and sheep polyclonal anti-human MDC1 antibody have been described previously (Rappold et al., 2001; Stucki et al., 2005).

Supplemental References:

Stucki, M., Clapperton, J.A., Mohammad, D., Yaffe, M. B., Smerdon, S. J., and Jackson S. P. (2005). MDC1 directly binds phosphorylated histone H2AX to regulate cellular responses to DNA double-strand breaks. Cell *123*, 1213-1226.

Rappold, I., Iwabuchi, K., Date, T., and Chen, J. (2001). Tumor suppressor p53 binding protein 1 (53BP1) is involved in DNA damage-signaling pathways. J. Cell. Biol. *153*, 613-620.

Supplemental figure legends:

Figure S1. Influence of H2AX residue 142 on MDC1-BRCT suppression of HR. A. H2AX Y142F or Y142W, but not Y142A, stably expressed in *H2AX*$^{-/-}$ ES cells restores efficient HR. Paired *t*-test between "Y142A" and "WT": P < 0.2%; between "Y142A" and "Y142F": P < 0.003%; between "Y142A" and "Y142W": P < 0.08%; between "WT" and "Y142W": P < 4.35%. **B.** Transient over-expression of MDC1 BRCT suppresses I-SceI-induced HR function of H2AX Y142F or Y142W mutant stably expressed in *H2AX*$^{-/-}$ ES cells. Paired *t*-test between "mMDC1 BRCT" and "mMDC1 BRCT K1554M": P < 0.12% in WT, P < 0.88% in Y142F, P < 4.74% in Y142W, and not significant in Y142A.

Figure S2. Depletion of MDC1 by shRNA reduces SCR in U2OS cells. A. I-SceI-induced GFP$^+$ frequencies (left panel), BsdR$^+$ frequencies (middle panel), and ratio of BsdR$^+$ to GFP$^+$ frequency (right panel) in U2OS reporter cells, transiently transfected with lacZ- and MDC1-targeting shRNA constructs. Bars represent mean of a representative experiment with triplicates. Error bars indicate SEM. Paired *t*-test between "MDC1" and "control": P < 0.22% in %GFP$^+$ cells (left panel), P < 1.47% in %BsdR$^+$ cells (middle panel), and not significant in %BsdR$^+$/GFP$^+$ (right panel). **B.** Abundance of MDC1 protein in cells transfected with either lacZ- or MDC1-targeting shRNA plasmids.

Figure S3. Transient expression of MDC1 rescues HR in *MDC1*$^{-/-}$ cells. A. I-SceI induced GFP$^+$ frequencies in *MDC1*$^{-/-}$ MEF cells containing the SCR reporter, transiently transfected with myc-tagged mouse *MDC1* expression plasmid. Bars represent mean of a representative experiment with triplicates. Error bars indicate SEM. Paired *t*-test between "MDC1" and "Vector": P < 0.36%. Steady state level of mouse MDC1 protein is shown

under the graph. **B.** I-SceI induced GFP^+ frequencies in *MDC1*$^{-/-}$ MEF reporter cells transiently transfected with myc-tagged mouse wt*MDC1* or mutant *MDC1* expression plasmids. Bars represent mean of a representative experiment with triplicates. Error bars indicate SEM. Paired *t*-test between "WT" and "Vector": P < 1.14%; between "WT" and "K1554M": P < 1.14%; between "WT" and "ΔFHA": P < 1.18%; between "WT" and "ΔPST": P < 1.77%; between "K1554M" and "Vector": P < 4.21%; between "WT" and "ΔSQ": not significant.

Figure S4. BRCA1, Rad51, and BRCA2 HR functions do not require *H2AX*. The percentage of I-SceI-induced GFP^+ cells from ES cell lines transfected with RNAi duplex. *H2AX*$^{+/+}$ and *H2AX*$^{-/-}$ ES cell lines as indicated were transiently transfected with control RNAi or RNAi against BRCA1, Rad51, or BRCA2, together with I-SceI expression plasmid. GFP^+ cells were quantified by flow cytometry analysis 3 days post-transfection. Bars represent mean of a representative experiment with triplicates. Error bars indicate SEM. Paired *t*-test between "siCtrl" and "siBRCA1": P < 0.05% in *H2AX*$^{+/+}$ cells and P < 0.45% in *H2AX*$^{-/-}$ cells; between "siCtrl" and "siRad51": P < 0.004% in *H2AX*$^{+/+}$ cells and P < 0.30% in *H2AX*$^{-/-}$ cells; between "siCtrl" and "siBRCA2": P < 0.028% in *H2AX*$^{+/+}$ cells and P < 0.67% in *H2AX*$^{-/-}$ cells.

Figure S5. Depletion of 53BP1 stimulates HR. A. The percentage of I-SceI-induced GFP^+ cells from U2OS reporter cells containing Dox-inducible I-SceI expression system transfected with control RNAi duplex or RNAi against 53BP1. Dox (1μg/mL) was administrated to induce I-SceI expression 24 hours post-transfection. Mock treatment was

also indicated. GFP^+ cells were quantified by flow cytometry 72 hours post-transfection. Bars represent mean of a representative experiment with triplicates. Error bars indicate SEM. Paired *t*-test between "control siRNA" and "53BP1 siRNA" for Dox treatment: P < 0.14%. Protein levels with siRNA treatment were shown under the chart. **B.** Dox-inducible I-SceI expression in U2OS reporter cells used in (**A**). Cells were administrated with Dox (1μg/mL), lysed at indicated time points, and analyzed for myc-I-SceI by Western blotting with anti-myc antibody. Non-specific protein bands detected by anti-myc were used as a loading control. **C.** The percentage of I-SceI-induced GFP^+ cells from mouse ES reporter cells stably expressing control or mouse 53BP1 shRNAs transfected with I-SceI expression plasmid. Bars represent mean of a representative experiment with triplicates. Error bars indicate SEM. Paired *t*-test between "ctrl shRNA" and "53BP1 shRNA #1": P < 0.5%; between "ctrl shRNA" and "53BP1 shRNA #2": P < 3.1%. Protein levels of 53BP1 with shRNA treatment were shown under the chart with protein levels of CHK1 as a loading control.

Figure S6. Effect of 53BP1 on NHEJ-dependent recircularization of a transfected linearized plasmid.

(A). Plasmid pEGFP-N3 as an NHEJ substrate in a plasmid-based assay. pEGFP-N3 (Clontech) contains unique HindIII and KpnI sites between the CMV promoter and the EGFP open reading frame (ORF) and is linearized as the NHEJ substrate by HindIII or HindIII/KpnI. Expression of GFP is abolished in these linear plasmids and restored by NHEJ-dependent re-circularization of linear DNA. pEGFP-N3 linearized by StuI serves as a control for transfection efficiency. HindIII, KpnI, and StuI sites are indicated. (B).

Percentage of GFP+ cells from mouse *53BP1*$^{+/+}$ and *53BP1*$^{-/-}$ embryonic cells, transiently transfected with either one of NHEJ substrates (as indicated), corrected for transfection efficiency. Bars represent mean of triplicate samples; error bars indicate SEM. Paired *t*-test between *53BP1*$^{+/+}$ and *53BP1*$^{-/-}$ embryonic cells: not significant for both NHEJ substrates ($P > 0.1$ and $P > 0.07$ respectively). (C). Percentage of GFP+ cells from mouse *XRCC4*$^{+/+}$ and *XRCC4*$^{-/-}$ embryonic stem cells, transiently transfected with linear pEGFP-N3-HindIII plasmid and expression plasmids for empty vector, HA-tagged F-53BP1 or F-D1521R as indicated. All data was corrected for transfection efficiency. Bars represent mean of triplicate samples; error bars indicate SEM. Paired *t*-test between "empty vector" and "F-53BP1" and between "F-53BP1" and "F-D1521R": not significant (all $P > 6\%$). (D). Similar experiment to (C), but with transiently transfected linear pEGFP-N3-HindIII/KpnI plasmid as substrate. Paired *t*-test between "empty vector" and "F-53BP1" and between "F-53BP1" and "F-D1521R": not significant (all $P > 0.2$).

Figure S1

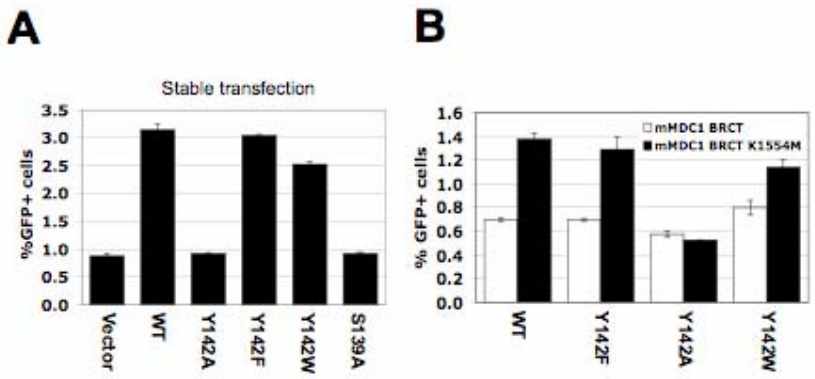

A
Stable transfection
%GFP+ cells
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
Vector
WT
Y142A
Y142F
Y142W
S139A
B
% GFP+ cells
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
mMDC1 BRCT
mMDC1 BRCT K1554M
WT
Y142F
Y142A
Y142W

Figure S2

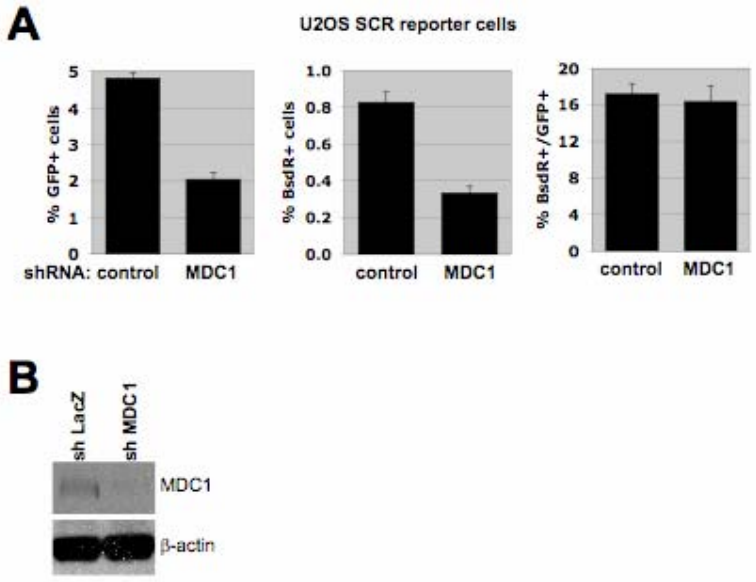

A
U2OS SCR reporter cells
% GFP+ cells
5
4
3
2
1
0
shRNA: control
MDC1
% BsdR+ cells
1.0
0.8
0.6
0.4
0.2
0.0
control
MDC1
% BsdR+/GFP+
20
16
12
8
4
0
control
MDC1
B
sh LacZ
sh MDC1
MDC1
β-actin

Figure S3

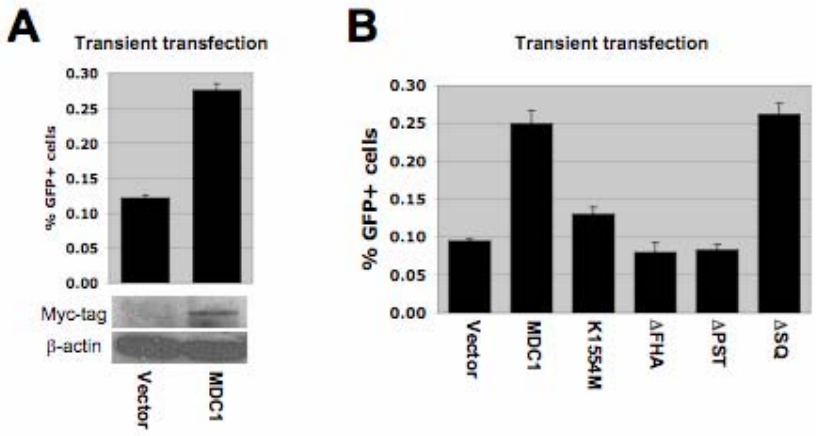

A
Transient transfection
% GFP+ cells
0.30
0.25
0.20
0.15
0.10
0.05
0.00
Myc-tag
β-actin
Vector
MDC1
B
Transient transfection
% GFP+ cells
0.30
0.25
0.20
0.15
0.10
0.05
0.00
Vector
MDC1
K1554M
ΔFHA
ΔPST
ΔSQ

Figure S4

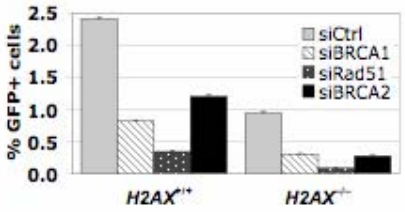

% GFP+ cells
2.5
2.0
1.5
1.0
0.5
0.0
siCtrl
siBRCA1
siRad51
siBRCA2
H2AX+/+
H2AX-/-

Figure S5

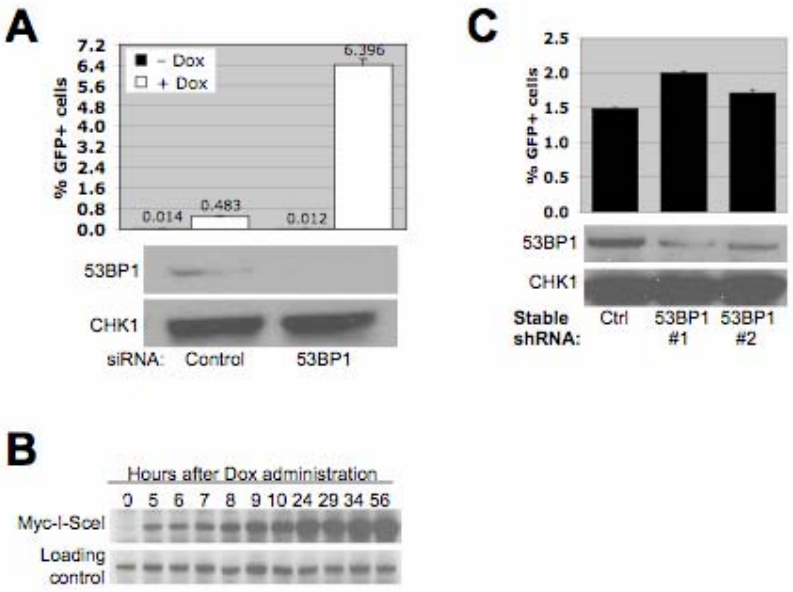
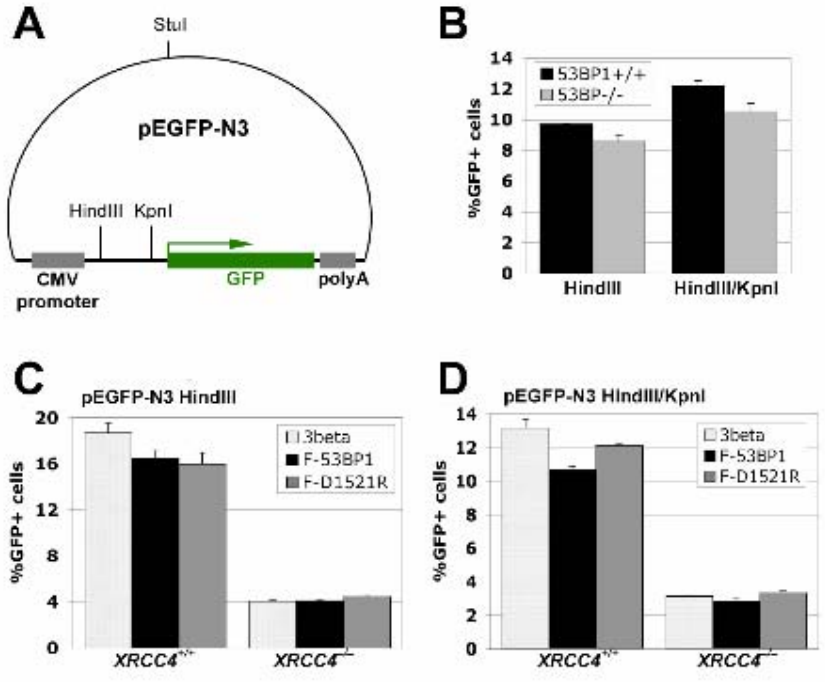

A
7.2
6.4
5.6
4.8
4.0
3.2
2.4
1.6
0.8
0.0
% GFP+ cells
- Dox
+ Dox
6.396
0.014
0.483
0.012
53BP1
CHK1
siRNA: Control 53BP1
C
2.5
2.0
1.5
1.0
0.5
0.0
% GFP+ cells
53BP1
CHK1
Stable shRNA: Ctrl 53BP1 #1 53BP1 #2
B
Hours after Dox administration
0 5 6 7 8 9 10 24 29 34 56
Myc-I-SceI
Loading control

Figure S6


A
StuI
pEGFP-N3
HindIII
KpnI
CMV promoter
GFP
polyA
B
53BP1+/+
53BP-/-
%GFP+ cells
HindIII
HindIII/KpnI
C
pEGFP-N3 HindIII
3beta
F-53BP1
F-D1521R
%GFP+ cells
XRCC4+/+
XRCC4-/-
D
pEGFP-N3 HindIII/KpnI
3beta
F-53BP1
F-D1521R
%GFP+ cells
XRCC4+/+
XRCC4-/-