Supplemental information inventory

Supplemental Figures

S1 – This provides data supporting the idea that the DNA double strand break reporter described recapitulates findings seen in other DNA damage and repair assays. It also provides an important control arguing that DNA end resection is not responsible for the silencing phenotype described.

S2 – These controls demonstrate that global nuclear damage in the form of ionizing radiation does not silence the transcriptional reporter at early time points after the induction of transcription. Additionally, it describes an in situ transcriptional reporter demonstrating ionizing radiation-induced silencing at endogenous loci.

S3 – These data support the data in Figure 3, demonstrating that ATM inhibition prevents transcriptional silencing. A control is included demonstrating that inhibition of the ATM-related kinase, DNA-PK, does not rescue transcription.

S4 – This is a control demonstrating the efficacy of the transcriptional inhibitor DRB.

S5 – These data support the claim that uH2A is responsible for DSB-induced transcriptional silencing, and that uH2A and K63Ub are distinct signals at sites of damage. Also included is a control experiment demonstrating that siRNA-mediated ATM knockdown prior to damage reduces both uH2A and K63Ub at sites of damage.

S6 – This figure contains control experiments for various siRNAs used in the study.

Supplemental materials and methods

These describe the methods used in detail, and provide oligo sequences, antibody information, and associated references.

Figure S1. Recruitment of various members of the DSB damage response to FokI-induced breaks; end resection does not cause transcriptional silencing (related to Figure 1)

(A) Immunofluorescent detection of BRCA1, Rad51, and RAP80 as they accumulate at the site of FokI WT LacI as compared to control transfections.
(B) IF for single-strand binding protein RPA in cells transfected with control or siRNA against end resection factor CtIP.
(C) YFP-MS2 intensity was analyzed in cells expressing FokI WT, transfected with control or anti-CtIP siRNA, and treated with DMSO or ATMi and 1 μg/ml dox for 3 hours.

Figure S2. DSB-induced silencing occurs in cis to damage (related to Figure 2)

(A) Reporter cells were mock irradiated or treated with 3 Gy IR then induced with dox for 30-60 minutes. Reporter transcript was quantified by qRTPCR.
Error bars represent SEM from 2 independent experiments.
(B) Reporter cells were treated as in (A) and reporter protein (CFP-SKL) was assayed by immunoblot at the indicated times.
(C) Top three rows - HeLa cells were irradiated at the indicated doses, nuclear run-on was performed at the indicated times and IF performed for nascent transcript and 53BP1. Cf values represent Pearson's correlation coefficients for colocalization of BrUTP and 53BP1 in the given image. Line scans at right represent colocalization of 53BP1 and BrUTP signals across the yellow lines shown in the images.
Bottom row – HeLa cells were irradiated with 3 Gy, fixed 0.5 hours later and IF was performed for 53BP1 and γH2AX. Cf value and line scan quantify colocalization of 53BP1 and γH2AX across the entire image and at the depicted yellow line, respectively.

Figure S3. ATM depletion reverses break induced silencing (related to Figure 3)

(A) qRT-PCR analysis of reporter mRNA levels in cells transduced with lentivirus to express FokI WT. Lentiviral transduced cells were treated with either ATMi or vehicle control (DMSO). Error bars represent SD from an experiment performed in triplicate.
(B) YFP-MS2 analysis in the two-transgene reporter system. YFP-MS2 accumulation was quantified at both transgenes in the presence of FokI WT or D450A with either DMSO or ATMi as indicated. The two-transgene reporter is described in the text and in Figure 2. Error bars represent SEM from 2 independent experiments.
(C) IF for γH2AX in the presence of 10μM ATMi or siATM. Cells were treated with DMSO or ATMi for 1 hour, then treated with 10 Gy IR and fixed for IF 15 minutes later.
(D) YFP-MS2 accumulation was monitored in FokI WT expressing cells treated with either vehicle (DMSO), ATM inhibitor Ku55933 (ATMi), or DNA-PK inhibitor

Nu7026 (PKi). Data shown are from a representative experiment.

Figure S4. DRB selectively reduces elongating RNAPII (related to Figure 4)
Reporter cells were treated with Dox, and either DMSO or 100 μM DRB for 3 hours and analyzed for YFP-MS2 accumulation (top). Cells were treated with DMSO or 100 μM DRB for 3 hours and IF performed for total RNAPII (8WG16, middle) and actively elongating RNAPII (H5, bottom).

Figure S5. uH2A is important for silencing and is in part a separate signal from K63Ub (related to Figure 5)
(A) Reporter cells were transfected with Flag-H2A WT or Flag-H2A 2KR, then underwent harsh pre-extraction conditions to remove soluble protein (0.05% TritonX-100) before fixing and processing for anti-Flag IF.
(B) Reporter cells were transfected with mCherryLacI and either Flag-H2A WT or Flag-H2A 2KR. 24-48 hours later, they were induced for 3 hours with dox and YFP-MS2 accumulation was quantified. Points represent values for single cells.
(C) Cells were transfected with control siRNA or siRNA against ATM and transduced with lentivirus to express FokI. 36 hours after transduction, uH2A and K63Ub were quantified at FokI induced breaks. Bars represent SEM from 2 independent experiments.
(D) Accumulation of RAP80 at FokI-induced breaks was quantified after 3 hours of treatment with DMSO or ATMi. Bars represent SEM from 2 independent experiments.
(E) HeLa cells were irradiated with 10 Gy, incubated at 37C for 1 hour and then treated with 10 μM ATMi or DMSO for 3 hrs. uH2A and K63Ub IRIF were assessed by immunostaining with specific antibodies. Representative images are shown to display incomplete concordance of each mark in the DMSO control (left column) and that uH2A foci are more sensitive to acute ATMi treatment compared to K63Ub foci (right column).

Figure S6. Opposing activities of the E3 ligases RNF8 and RNF168, and the DUB USP16 (related to Figure 6).
(A) Cells transfected with control or siRNAs against RNF8 and RNF168 were treated with 10Gy IR and fixed for anti-53BP1 IF to assess knockdown efficiency.
(B) Reporter cells were transfected with FokI WT and either WT RNF8, or a RING-finger mutant, RNF8 C403S, which lacks E3 ligase activity. Transcription was induced 12 hours post transfection and YFP-MS2 accumulation was quantified.
(C) Knockdown by DUB siRNAs was assessed by siRNA transfection into HeLa or U2OS cell lines stably expressing each DUB tagged with HA and Flag. Cells were transfected with the indicated siRNA and IF performed with an anti-HA antibody 48 hours later.
(D) 3 different USP16 siRNAs were transfected into a HeLa cell line containing a stably expressed, Flag-HA double tagged USP16. WB was performed 72 hours later.

(E) YFP-MS2 accumulation in FokI expressing reporter cells was quantified in cells transfected with control (Luc) or two different siRNAs against USP16. YFPMS2 RFMI was monitored in each group at 3 hours following addition of either DMSO control or ATMi. Results from experiments with a third siRNA to USP16 are depicted in Figure 6B.
(F) Reporter cells were transfected with siRNA against USP16, and transfected with FokI WT and either WT or a siRNA-resistant allele of USP16. YFP-MS2 accumulation was quantified after treatment with dox and ATMi.

Supplemental Materials and Methods

The following antibodies were used at the indicated dilutions: mouse anti-γH2AX (Upstate) 1:2000 (IF), 10mg (ChIP); rabbit anti-γH2AX (Active Motif) 1:500 (IF); mouse anti-GFP (Roche) 1:333 (WB); mouse anti-total RNAPII (Covance, 8WG16) 1:50 (IF); mouse anti-phospho Ser2 RNAPII (Covance, H5) 1:50 (IF); mouse anti-uH2A (Upstate) 1:500 (IF); mouse FK2 (Biomol) 1:10,000 (IF); human anti-K63-Ub and anti-K48Ub (Genentech) 1:1500 (IF); mouse anti-HA (Covance) 1:1000 (IF); mouse anti-BRCA1 (Santa Cruz, sc6954) 1:25 (IF); mouse anti-Flag (Millipore) 1:1000 (IF); rabbit anti-RAP80 (in-house, Rockland) 1:500 (IF); mouse anti-BrDU (FITC-conjugated) (BD) 1:10 (IF); rabbit anti-RPA (Novus) 1:500 (IF)

The following siRNA sequences were used (sense):

Luciferase – 5'-GCCAUUCUAUCCUCUAGAGGAUG
ATM – 5'-GCGCCUGAUUCGAGAUCCU
Rnf8 – 5'-GGACAAUUAUGGACAACAA
Rnf168 – 5'-GGAGGUGGAUAAAGAGCAA
BRCC36 – 5'-GCCUUCACAUGUUGAUGUU
USP16 – 5'-UUCUCCAUUGCUCCCUUCC
USP16_1 – 5'-CCUCCUGUUCUUACUCUUCAUUUAA
USP16_2 – 5'-CCGGAAAUCUUAGAUUUGGCUCCUU

The following primers for used for the indicated protocols:
ChIP

1F 5' TGTACGGTGGGAGGCCTATATAA
1R 5' GCGTCTCCAGGCGATCTG
2F 5' GGAAGATGTCCCTTGTATCACCAT
2R 5' TGGTTGTCAACAGAGTAGAAAGTGAA
3F 5' GCTGGTGTGGCCAATGC
3R 5' TGGCAGAGGGAAAAAGATCTCA
4F 5' GGCATTTCAGTCAGTTGCTCAA
4R 5' TTGGCCGATTCATTAATGCA

5F 5' CCACCTGACGTCTAAGAAACCAT
5R 5' GATCCCTCGAGGACGAAAGG
ch7F 5' GCTCGTGCCGTTTTGCA
ch7R 5' GGGTTGACCATGGCTAATAGTACA
qRT-PCR

Reporter transcript F
5' TCATTAGATCCTGAGAACTTCA

Reporter transcript R
5' TTTTGGCAGAGGGAAAAAGA

Chromatin Immunoprecipitation (ChIP)

ChIP was carried out as described previously (Vakoc et al., 2006). 10 µg anti γH2AX or control mouse IgG (Santa Cruz) plus 5 µg rabbit anti-mouse crosslinking IgG (Active Motif) were bound overnight to 35 µL 1:1 Protein A slurry (Pierce). The next day, cells were induced with 1 µg/ml dox x 3hrs and approx 3-6 million cells were harvested per IP. Cells were cross-linked with formaldehyde for 10 min at room temp and quenched with glycine. Nuclei were isolated, lysed and sonicated to obtain approx 100-500bp chromatin fragments. Chromatin was precleared with 50 µL Protein A slurry and 50 µg each crosslinking antibody and mouse IgG1κ (Sigma) at 4C for 2-3 hours. Precleared chromatin was IP'd overnight with prebound antibodies at 4C. Unprecipitated aliquots were saved as inputs. IP'd samples were eluted in NaHCO3 and SDS and digested overnight with RNAseA and proteinase K at 65 C. Sample volumes were raised with TE buffer and DNA isolation performed with Qiagen PCR cleanup columns, per manufacterer's instructions.
qPCR was carried out on an ABI 7900HT instrument in triplicate, with SYBR Green chemistry. For each primer set used, serially diluted input samples were used to create standard curves.

Nuclear run-on assay

Run-on was carried out as previously described (Elbi et al., 2002; Solovjeva et al., 2007; Wansink et al., 1993). Cells were grown on round coverslips in 6 cm dishes. At time of experiment, cells were washed twice in PBS at room temp, and incubated in permeabilization buffer containing 0.03% Triton-X 100 for 3 min at room temp. Cells were then washed once with PBS and placed in transcription buffer containing 2 mM ATP, and 0.5 mM each of CTP, GTP, and BrUTP for 3-5 minutes at 37C. Cells were washed, then fixed in formaldehyde for 10 min at room temp, permeabilized, and processed for IF as described.

Supplemental References

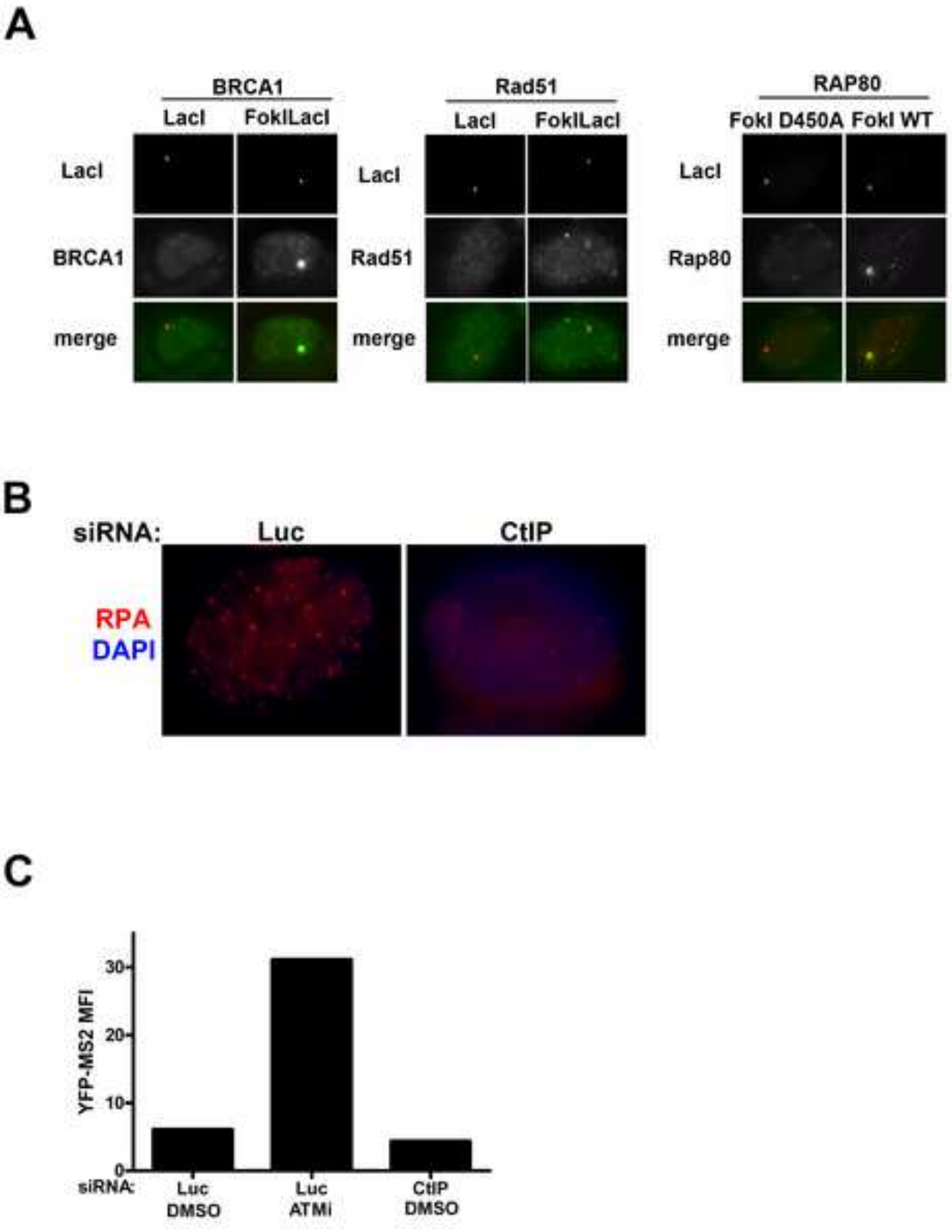
Elbi, C., Misteli, T., and Hager, G.L. (2002). Recruitment of dioxin receptor to active transcription sites. Mol Biol Cell *13*, 2001-2015.

Solovjeva, L.V., Svetlova, M.P., Chagin, V.O., and Tomilin, N.V. (2007). Inhibition of transcription at radiation-induced nuclear foci of phosphorylated histone H2AX in mammalian cells. Chromosome Res *15*, 787-797.

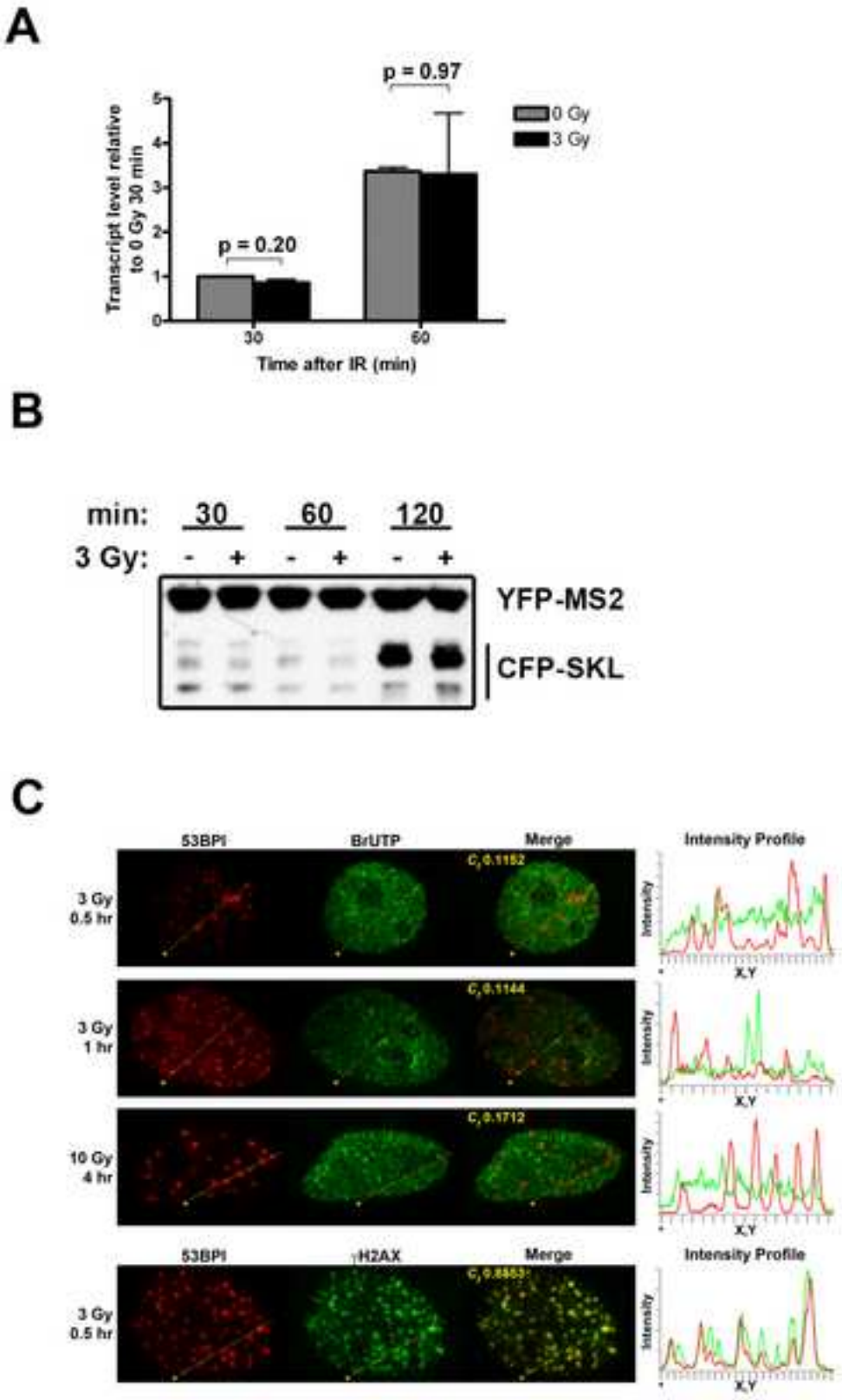
Vakoc, C.R., Sachdeva, M.M., Wang, H., and Blobel, G.A. (2006). Profile of histone lysine methylation across transcribed mammalian chromatin. Mol Cell Biol *26*, 9185-9195.

Wansink, D.G., Schul, W., van der Kraan, I., van Steensel, B., van Driel, R., and de Jong, L. (1993). Fluorescent labeling of nascent RNA reveals transcription by RNA polymerase II in domains scattered throughout the nucleus. J Cell Biol *122*, 283-293.

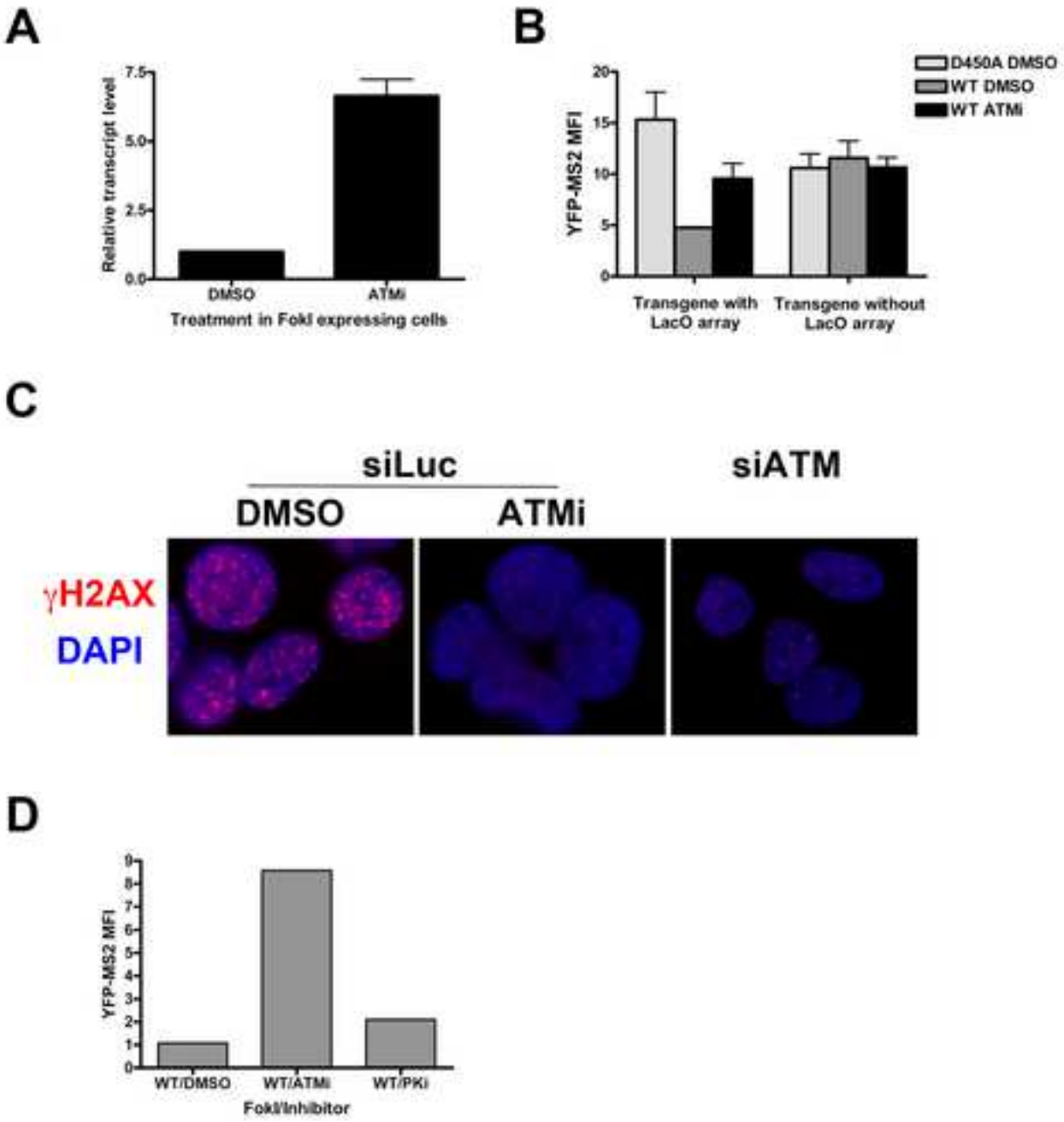
Supplemental Figure 1



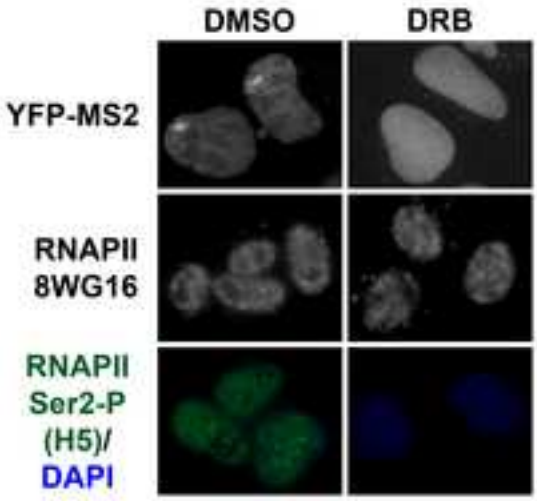
Supplemental Figure 2



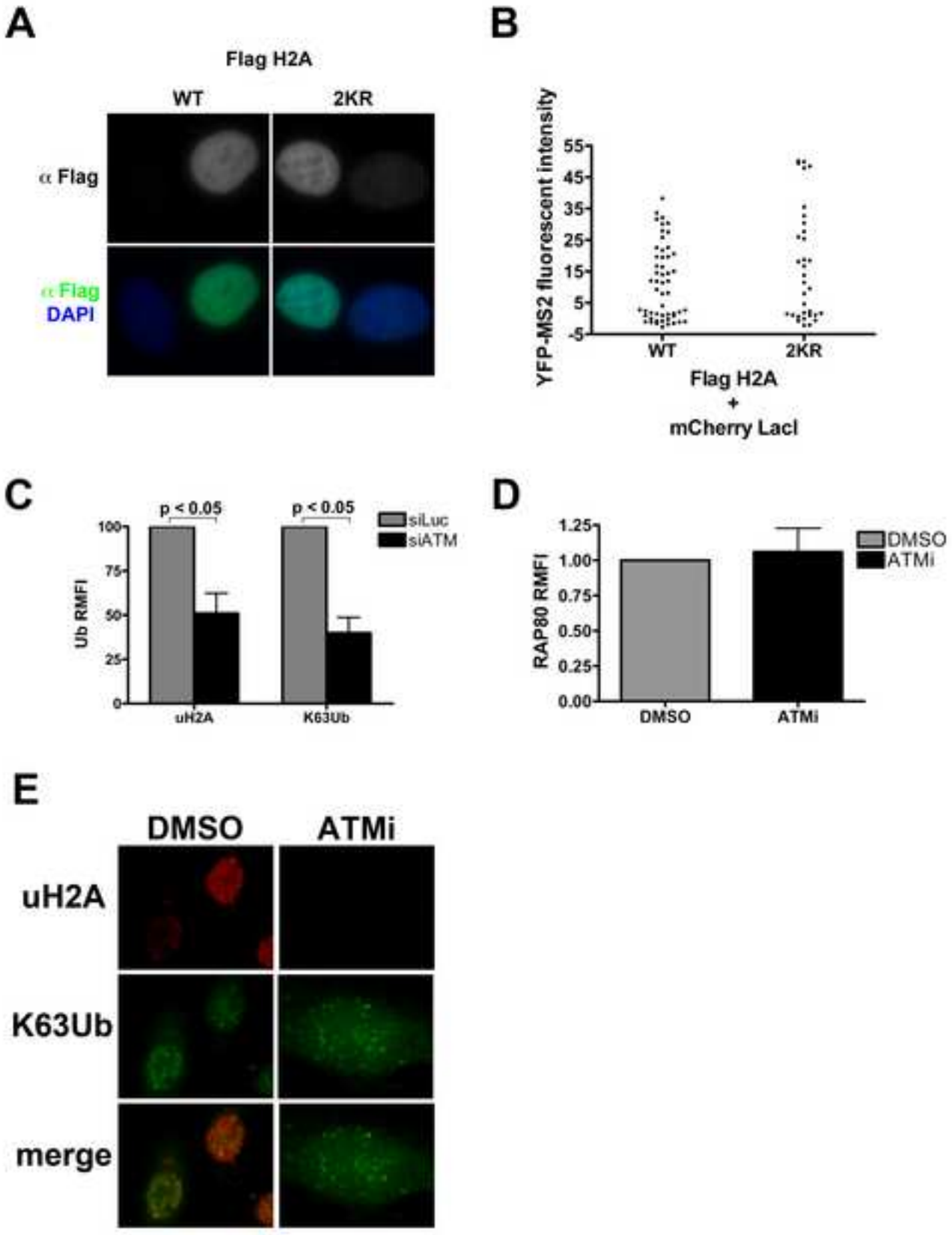
Supplemental Figure 3



Supplemental Figure 4



Supplemental Figure 5



Supplemental Figure 6