Supporting Information

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SI Text

Materials and Methods. ***Mice, surface-spread chromosomes, and immunostaining.*** Surface-spread chromosomes from UBR2$^{-/-}$ (1) *Brca*1$^{\Delta 11/\Delta 11}$*p*53$^{+/-}$ (2) mice were used for immunohistochemistry with antibodies listed in Table S1.

Quantitative RT-PCR. Quantitative RT-PCR. Total RNA was isolated from +/+ and UBR2$^{-/-}$ testes at 4 weeks of age using RNeasy Protect Midi Kit (Qiagen) after DNase treatment to prevent genomic DNA contamination. Approximately 2 μg of total RNA was subjected to reverse-transcription reactions using SuperScript III reverse transcriptase (Invitrogen) and oligo-dT primers. Quantitative PCR was performed in duplicates using Platinum SYBR Green (Invitrogen) with ABI 7300/7500 real time PCR system and primer sequences listed in Table S2. β-actin was used as a control to normalize the data.

Fluorescence in situ hybridization. FITC-labeled Y chromosome probe and Cy3-labeled X chromosome probes from Cambio (Cambridge, UK) were used for FISH according to the manufacturer's instruction. After FISH, X–Y painted slides were fixed in 4% paraformaldehyde for 5 min at 4 °C and rinsed in PBS, followed by immunostaining for SCP3.

Purification of endogenous UBR2. Endogenous UBR2 was partially purified from extracts prepared from rat or mouse testes using synthetic degrons essentially as described (3), with major modifications. Briefly, a pair of C-terminally biotin-conjugated peptides (X-Ile-Phe-Ser-Thr-Ile-Glu-Gly-Arg-Thr-Tyr-Lys), bearing either N-terminal Phe (type-2 destabilizing) or Val (stabilizing), were synthesized and immobilized on streptavidin-sepharose beads (GE Healthcare) to a ratio of 1∶1.5 μmol peptides per 1 mL beads. The residues 2–9 of X-peptides were derived from residues 2–9 of Sindbis virus polymerase nsP4, which is degraded through the N-end rule pathway (3). X-peptide-beads were used as an affinity ligand to purify N-recognins at low salt concentrations (75–150 mM NaCl) coupled with other procedures not described in detail here (Y.H.J. and Y.T.K., unpublished data). Immobilized E3s were eluted with 10 mM Trp-Ala, followed by dialysis at 0.1 M NaCl and 10 mM HEPES (pH 7.9) and concentration using Amicon Ultra (Millipore).

In vitro ubiquitination assay. Partially purified endogenous UBR2 (100 ng per 20 μL reaction) was incubated with 1 μg purified histone H2A (New England Biolabs) or 5 μg chicken oligonucleosome in the presence of 30 ng purified human UbcH2/HR6B (Biomol), 100 ng human recombinant E1 (Biomol), 1 μg Ub tagged with ha or FLAG (Boston Biochem), and 5 mM Mg-ATP for 60–90 min at 37 °C. Proteins were separated by SDS-PAGE and subjected to immunoblotting for uH2A, H2A, UBR2, or HA.

Ubiquitination-coupled E3 binding assay. Endogenous N-recognins immobilized on Phe-peptide-beads were mixed with HR6B (Boston Biochem) or H2A (New England Biolabs) in the presence or absence of E1 and Ub activating reagents, followed by incubation at 37 °C for 1 hr. Bound proteins were washed three times with washing buffer (20 mM HEPES, pH 7.9, 150 mM NaCl, and 0.05% Tween20), separated by 4–20% gradient SDS-PAGE, and subjected to immunoblotting.

GST-pulldown assay. In a reaction of 300 μl, E3-F (equivalent to 250 ng UBR2) was mixed with 200 ng of GST-HR6B (Boston Biochem) immobilized on GST-beads and/or 1 μg H2A (BioLabs) in the presence or absence of E1 and Ub activating reagents, followed by incubation at 37 °C for 1 hr. Proteins bound to GST-HR6B were washed three times with phosphate buffered saline containing 0.5% Triton X-100 and separated by 4–20% gradient SDS-PAGE, followed by immunoblotting.

1. Kwon YT, et al. (2003) Female lethality and apoptosis of spermatocytes in mice lacking the UBR2 ubiquitin ligase of the N-end rule pathway *Mol Cell Biol* 23:8255–8271.
2. Xu X, Aprelikova O, Moens P, Deng CX, Furth PA (2003) Impaired meiotic DNA-damage repair and lack of crossing-over during spermatogenesis in BCRA1 full-length isoform deficient mice. *Development* 130:2001–2012.
3. Tasaki T, et al. (2005) A family of mammalian E3 ubiquitin ligases that contain the UBR box motif and recognize N-degrons.*Mol Cell Biol* 25:7120–7136.

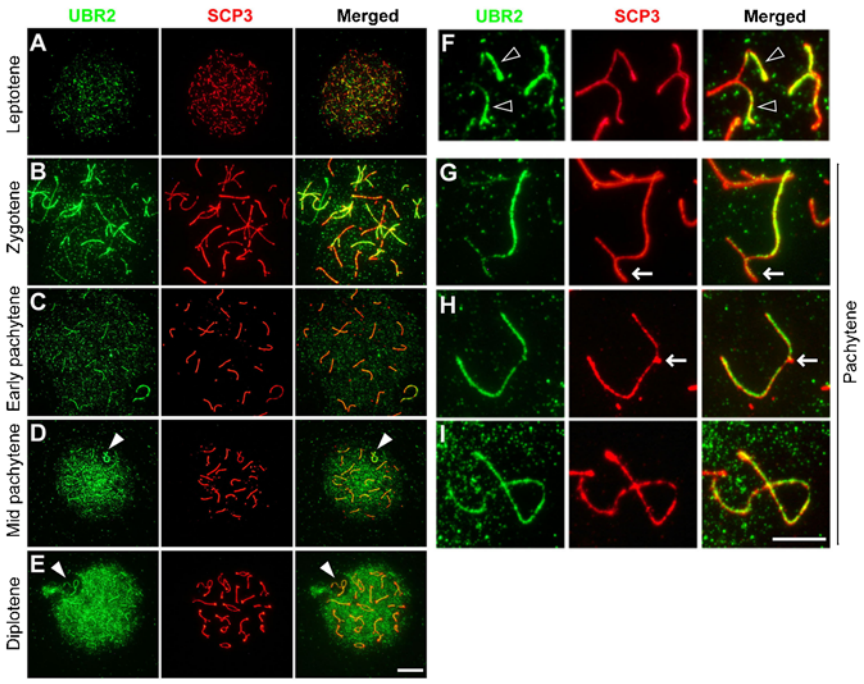

Fig. S1. Localization of UBR2 on meiotic chromosomes. (*A*) At leptotene, UBR2 staining (green) appears as foci in the chromatin and as segment-like staining along the newly emerging axial elements. SCP3 (red) was costained to monitor meiotic substages. (*B* and *F*) At zygotene, UBR2 staining is enriched on unsynapsed axial regions (arrowhead). (*C*) At early pachytene, UBR2 staining gradually disappears from the axes that have achieved synapsis and is enriched on the unpaired axes of the X and Y chromosomes. (*D* and *E*) At mid-pachytene, the number of UBR2 foci surge throughout the entire chromatin, except for the XY chromatin domain (arrowhead) (*D*), a pattern that is maintained through diplotene (*E*). (*G–I*) UBR2 localization on unsynapsed axial regions of the sex chromosomes during early pachytene (*G*), mid-pachytene (*H*), and late pachytene (*I*). Arrow, PAR. The specificity for the UBR2 staining was confirmed by competing with peptides used to raise anti-UBR2 antibody, which did not affect the SCP3 signal (data not shown). [Scale bar (*A–E*), 10 μm; (*F–I*), 5 μm.]

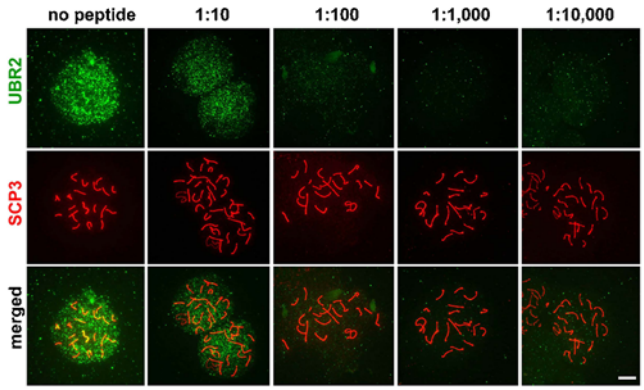

Fig. S2. UBR2 signals, but not SCP3 signals, on meiotic chromatin are inhibited by the peptide that has been used to generate anti-UBR2 antibody. Immunohistochemistry was performed as described in Fig. S1 in the presence of the synthetic peptide RGPNPFPPLKEDT that has been used to generate UBR2(3-1) antibody (1). Indicated are the molar ratios between the peptide and anti-UBR2 antibody.

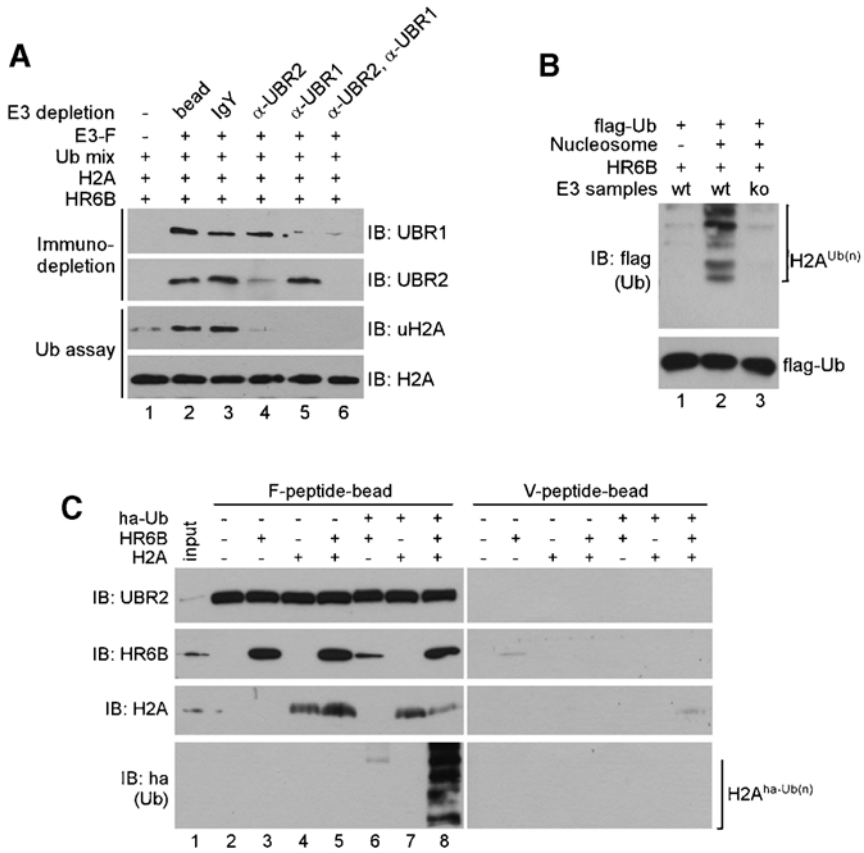

Fig. S3. UBR2, in combination with HR6B, mediates ubiquitination of H2A. (*A*) In vitro ubiquitination assay with 100 ng E3-F prepared from rat testes, immunodepleted with antibodies to UBR1 and/or UBR2. (*B*) In vitro ubiquitination assay with 5 μg nucleosome and 100 ng E3-F prepared from +/+ (wt) and UBR2-deficient mouse tissues (ko). (*C*) Cooperative interactions between UBR2, HR6B, and H2A. UBR2 (as a mixture with UBR1) immobilized on F-peptide-beads were mixed with HR6B or H2A in the presence or absence of E1 and Ub activating reagents, followed by immunoblotting of proteins (HR6B, H2A, and Ub conjugated H2A) retained by X-peptide.

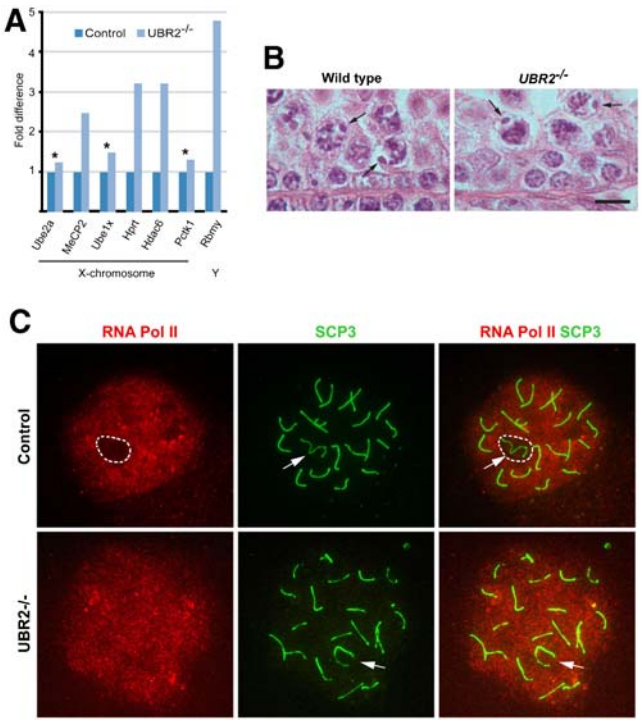

Fig. S4. UBR2 is required for transcriptional silencing of the X and Y chromosomes in male meiosis. (*A*) Real-time RT-PCR analysis was performed for X- or Y-linked genes using control and UBR2$^{-/-}$ testes at 4 weeks of age. Genes that are not subjected to MSCI are indicated by asterisks. The data presented are averages of duplicates or triplicates. (*B*) XY body formation appears to be normal in UBR2-deficient spermatocytes. H/E staining was performed for cross sections from $+/+$ and UBR2$^{-/-}$ testes at 4 weeks. Arrows, the XY body. (Scale bar, 10 μm.) (*C*) UBR2-deficient spermatocytes are impaired in the exclusion of RNA polymerase II from the X and Y chromosomes. Immunostaining was performed for RNA polymerase II on chromosome spreads prepared from $+/+$ and UBR2$^{-/-}$ spermatocytes. Arrows indicate partially paired X and Y chromosomes. (Scale bar, 10 μm.)

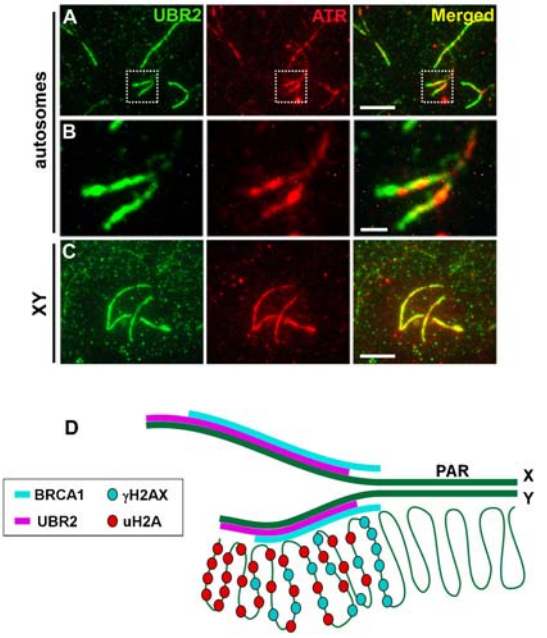

Fig. S5. The localization pattern of UBR2 to unsynapsed axial regions of autosomes is distinct from that of ATR. (*A* and *C*) Localization of UBR2 (green) and ATR (red) on unsynapsed axes of the autosomes at zygotene (*A*) and the sex chromosomes at pachytene (*C*). (*B*) Enlarged images for chromosomal regions indicated by the insets in (*A*). [Scale bar (*A* and *C*), 5 μm; (*B*), 1 μm.] (*D*) A model for the localizations of UBR2 and BRCA1 on meiotic chromosomes. UBR2 and BRCA1 show distinct spatial localization patterns along the unsynapsed axes, in particular in autosomes, in that the UBR2 staining on unsynapsed axes is virtually excluded from the synaptic folk, whereas the axial staining of BRCA1 and ATR is relatively weaker in the region proximal to the telomere.

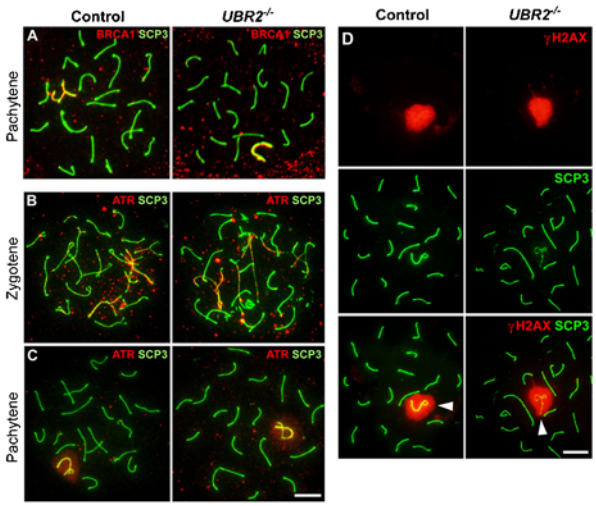

Fig. S6. The localizations of BRCA1 and ATR on meiotic chromosomes are not significantly altered in UBR2-deficient spermatocytes. (*A–C*) Chromosome spreads from control and UBR2-deficient spermatocytes were subjected to immunostaining for BRCA1 (*A*) and ATR (*B* and *C*). (Scale bar, 10 μm.) (*D*) Phosphorylated H2AX (γH2AX) normally localizes to the XY body of pachytene-like UBR2-deficient spermatocytes. Chromosome spreads from control and UBR2-deficient spermatocytes were subjected to immunostaining for γH2AX (red) and SCP3 (green). (Scale bar, 10 μm.)

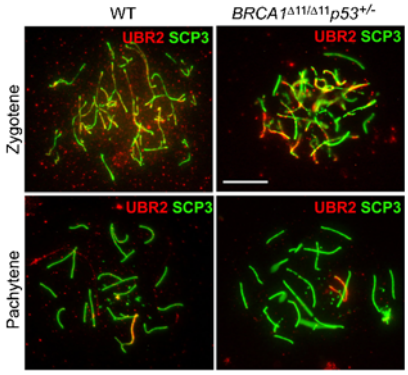

Fig. S7. The localization of UBR2 on meiotic chromosomes is not significantly altered in *Brca1*$^{\Delta 11/\Delta 11}$*p53*$^{+/-}$ spermatocytes at the zygotene stage. Chromosome spreads from control and *Brca1*$^{\Delta 11/\Delta 11}$*p53*$^{+/-}$ spermatocytes were subjected to immunostaining for UBR2 (red) and SCP3 (green). (Scale bar, 10 μm.)

Other Supporting Information

Table S1 (DOC)

Table S2 (DOC)