Figure S1. Ku foci formation requires Ku’s ability to bind DNA. (A) Structures of wild-type (WT) and Mut6E Ku heterodimers with a colored mesh representation of their electrostatic surfaces as computed by Swiss-PdbViewer using Ku structure (Protein Data Bank accession no. 1JEQ; Walker et al., 2001). (B) U2OS Tet-On cells stably expressing doxycycline-inducible GFP-FLAG–tagged wild-type (clone 8) or Mut6E (clone 7) Ku70 resistant to siRNA were subjected to two consecutive rounds of siRNA against endogenous Ku70 or control (Ctrl), 3 d before being induced with doxycycline for an additional 2 d. A double-strand DNA pull-down was performed from corresponding extracts, and the retained proteins were analyzed by immunoblotting. Inputs correspond to 50% of extracts used for pull-down. (C) U2OS stably expressing GFP-FLAG only (clone 2; GFP) and U2OS Tet-On cells stably expressing doxycycline-inducible GFP-FLAG–tagged wild-type or Mut6E Ku70 resistant to siRNA were induced with doxycycline for 2 d. Extracts were collected from corresponding cells, and IPs were performed against GFP. Inputs correspond to 10% of extracts used for IPs. Immunoprecipitated proteins were loaded on two gels, one for GFP staining and one for Ku80 staining. Ponceau staining was used as a loading control. (D) U2OS Tet-On cells stably expressing doxycycline-inducible GFP-FLAG–tagged wild-type or Mut6E Ku70 resistant to siRNA were subjected to two consecutive rounds of siRNA against endogenous Ku70 or control, 3 d before being induced with doxycycline for an additional 2 d. Whole-cell extracts were analyzed by immunoblotting. (E) Cells were prepared as in D and processed for immunofluorescence using an anti-GFP antibody to boost the signal. (F) Cells were prepared as in D, submitted to laser microirradiation, postincubated for 5 min, and preextracted with CSK+R before being processed for immunofluorescence using an anti-GFP antibody to boost the signal. Mut6E corresponds to Ku70 K282E K287E T300E K331E K338E R403E. The position of each nucleus, as defined by DAPI staining, is highlighted by a dotted line. Bars, 10 µm.
Figure S2. Ku IRIF can be detected in various human cell types and on the compacted chromatin of mitotic chromosomes. (A) RPE-1 (left) and HT1080 cells (right) were untreated (top) or treated (bottom) with 10 Gy of IR, postincubated for 5 min, and preextracted with CSK+R before being processed for immunofluorescence. (B) U2OS cells were treated with 10 Gy of IR, postincubated for 5 min, and preextracted with CSK+R before being processed for immunofluorescence. NT, not treated. The position of each nucleus, as defined by DAPI staining, is highlighted by a dotted line. Bars, 10 µm.
Figure S3. **CSK+R extraction does not affect nuclear architecture, chromatin organization, or 53BP1 IRIF and is compatible with analyzing HR events.** (A) Localizations of lamin A/C and nucleolin were analyzed in undamaged U2OS cells by immunofluorescence without CSK preextraction (no CSK), with pre-extraction using CSK only (CSK), or CSK combined with RNase A (CSK+R). Lamin A/C is part of the nuclear lamina. (B) Localizations of a marker of heterochromatin, H3K9me3 (histone H3 K9 trimethyl), and Ku80 were analyzed in undamaged U2OS cells by immunofluorescence without CSK (no CSK), with preextraction using CSK only (CSK), or CSK combined with RNase A (CSK+R). (C) U2OS cells were treated with 3 Gy of IR, postincubated for 15 min, and processed for immunofluorescence without CSK preextraction (no CSK), with preextraction using CSK only (CSK), or CSK combined with RNase A (CSK+R). Fibrillarin is a nucleolar protein used as a control of the RNase preextraction. (D) U2OS cells were microirradiated, postincubated for 1 h, and fixed without CSK (no CSK) or with preextraction using CSK+R. Antibodies against RPA2 and RAD51 were used as markers of sites of early and late HR events, respectively. Note that only a subset of γ-H2AX laser tracks is showing RPA2/RAD51 staining, in accordance with HR being restricted to the S and G2 phases of the cell cycle. The position of each nucleus, as defined by DAPI staining, is highlighted by a dotted line. Bars, 10 µm.
Figure S4.  

**Ku IRIF analysis by STED microscopy.** (A) U2OS cells were treated with 10 Gy of IR, postincubated for 5 min, preextracted with CSK+R, and processed for immunofluorescence. The same cells were imaged using conventional confocal microscopy (top left) and STED microscopy (top right). The bottom images show higher magnification of the region of the cell highlighted by a dashed square to highlight the resolution gain between high (left)- and super (right)-resolution microscopy. (B) Graph showing a representative fluorescence profile, in arbitrary units (AU), of an individual Ku focus, corresponding to the dashed line represented in A, as analyzed by STED microscopy. The data shown are representative of 10 measured foci in two independent experiments. The position of each nucleus, as defined by DAPI staining, is highlighted by a dotted line. Bars: (white) 10 µm; (red) 0.2 µm. STED allows a greater x-y resolution but requires higher illumination power than 3D-SIM, which impedes 3D imaging in the case of a weak signal.
Validation of the activity and specificity of ATMi and DNA-PKi. (A) γ-H2AX total nuclear intensity is a measure reflecting the number of DSBs as revealed by a graph representing a dose–response analysis of the intensity of γ-H2AX in cells analyzed in Fig. 5 A. The sum of γ-H2AX intensity in each cell analyzed in Fig. 5 A was computed using Volocity, and for normalization, the average γ-H2AX intensity in the untreated condition was subtracted from the γ-H2AX intensity in each condition. Values were divided by a correcting factor to get intensities in the range of 0–300 AU, arbitrary units. (B) DNA-PKi induces a persistence of γ-H2AX as compared with DMSO, in contrast to ATMi, which blocks most of the γ-H2AX formation, as revealed on a graph representing a kinetic analysis of γ-H2AX intensity in cells analyzed in Fig. 5 C. γ-H2AX intensities were calculated and normalized as in A. (C) ATMi strongly reduces phosphorylation of ATM substrates H2AX (S139), KAP1 (S824), and CHK2 (T68), whereas DNA-PKi induces persistence of these phosphorylations, reflecting a repair defect, as revealed by immunoblotting. U2OS cells were preincubated with DMSO, DNA-PKi, and/or ATMi, untreated (NT) or treated with 10 Gy of IR and postincubated for 30 min or 2 h before whole-cell extracts were collected. Extracts were analyzed by immunoblotting for DNA damage–induced phosphorylations. Asterisks indicate a nonspecific band recognized by the anti-CHK2 antibody. Ub indicates a band at the size of the monoubiquitinated form of H2AX and that we found to disappear upon MG-132 treatment (not depicted). Ph, phosphorylated form.
Video 1. **γ-H2AX foci colocalize with Ku foci when observed by high-resolution imaging.** Video showing 3D rendering of the nucleus of a U2OS cell treated with 2 Gy of IR and postincubated 5 min before being preextracted with CSK+R and processed for immunofluorescence and staining for Ku80 (green) and γ-H2AX (red). Z stacks were collected every 0.125 µm on a DeltaVision OMX V3 microscope in conventional mode using a 100× U Plan S Apochromat/1.40 NA oil objective. A 3D rendering was created for a single U2OS nucleus using Imaris 7.6 (Bitplane).

Video 2. **γ-H2AX foci colocalize with Ku foci when observed by high-resolution imaging.** Video showing 3D rendering of an individual γ-H2AX focus from a U2OS cell treated with 2 Gy of IR and postincubated for 5 min before being processed for immunofluorescence and staining for Ku80 (green) and γ-H2AX (red). Z stacks were collected every 0.125 µm on a DeltaVision OMX V3 microscope in conventional mode using a 100× U Plan S Apochromat/1.40 NA oil objective. A 3D rendering was created for an individual γ-H2AX focus using Imaris 7.6 (Bitplane).

Reference