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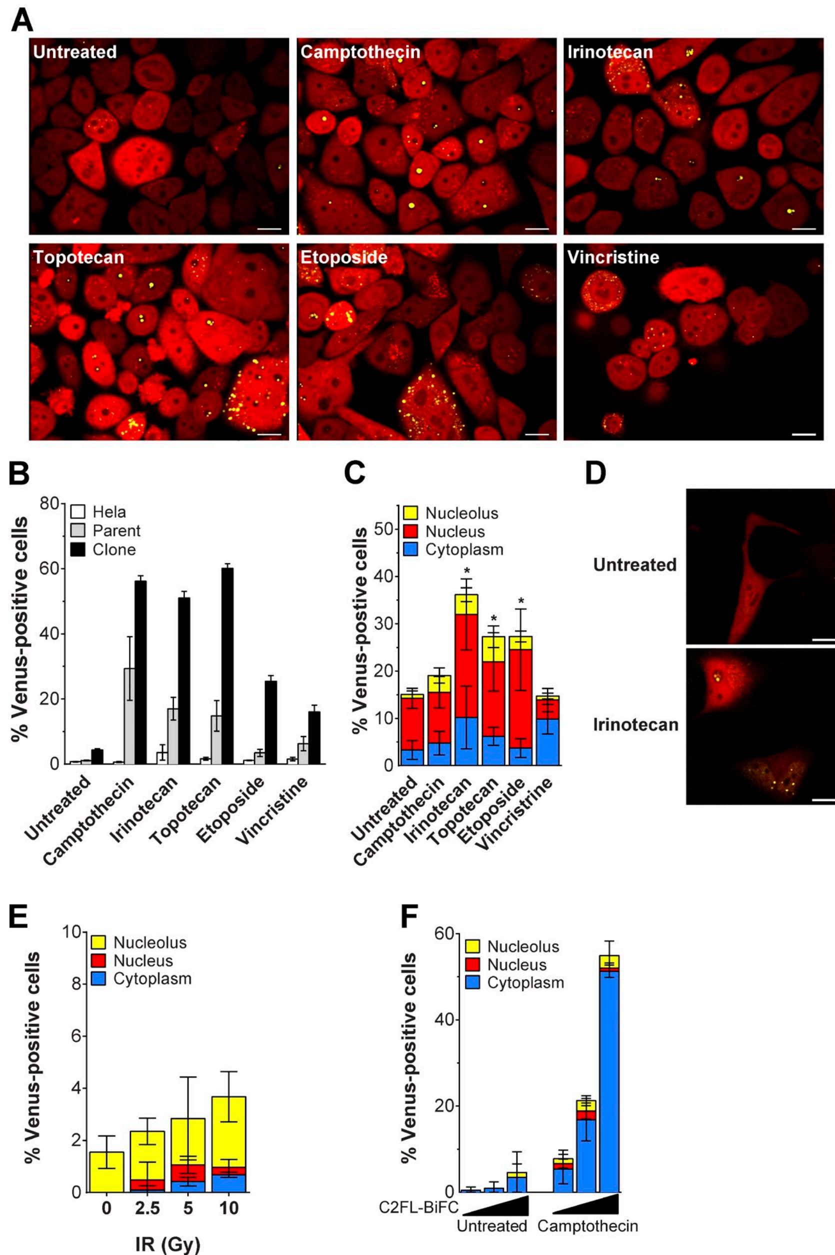


Figure S1. **DNA damaging agents induce caspase-2 BiFC in the nucleolus.** (A) Representative confocal images of HeLa.C2 Pro-BiFC cells that were untreated or treated with camptothecin (100 µM), irinotecan (100 µM), topotecan (100 µM), etoposide (100 µM), or vincristine (100 µM), in the presence of qVD-OPH (20 µM) show mCherry expression throughout the cell (red) and caspase-2 BiFC (yellow). Bars, 10 µm. (B) HeLa or HeLa.C2 Pro-BiFC parent or clone cells treated as in A were analyzed by flow cytometry. (C) U2OS cells stably expressing the C2 Pro-VC-2A-C2 Pro-VN-2A-mCherry were treated with camptothecin (100 µM), irinotecan (100 µM), topotecan (100 µM), etoposide (100 µM), or vincristine (10 µM), in the presence of qVD-OPH (20 µM). The percentage of Venus-positive cells and the proportion of Venus-positive cells that showed caspase-2 BiFC in the cytoplasm, nucleus, or nucleolus were determined at 16 h from microscopy images of a minimum of 50 confocal microscopy images. Results are the mean of four independent experiments ± SD. *, $P < 0.05$. (D) Representative images of cells from C with caspase-2 BiFC shown in yellow. Bars, 10 µm. (E) HeLa.C2 Pro-BiFC cells were treated with or without the indicated doses of IR in the presence of qVD-OPH (20 µM). The percentage of Venus-positive cells and the proportion of the Venus-positive cells that showed caspase-2 BiFC in the cytoplasm, nucleus, or nucleolus were determined at 16 h from microscopy images of a minimum of 50 confocal microscopy images. Results represent triplicate counts ± SD. (F) HeLa cells were transiently transfected with plasmids encoding C2-FL(C320S) BiFC components (0.25, 0.5, or 1 µg) for 24 h followed by treatment with camptothecin (100 µM). The percentage of Venus-positive cells and the proportion of the Venus-positive cells that showed caspase-2 BiFC in the cytoplasm, nucleus, or nucleolus were determined at 16 h from a minimum of 50 microscopy images. Results represent triplicate counts ± SD.

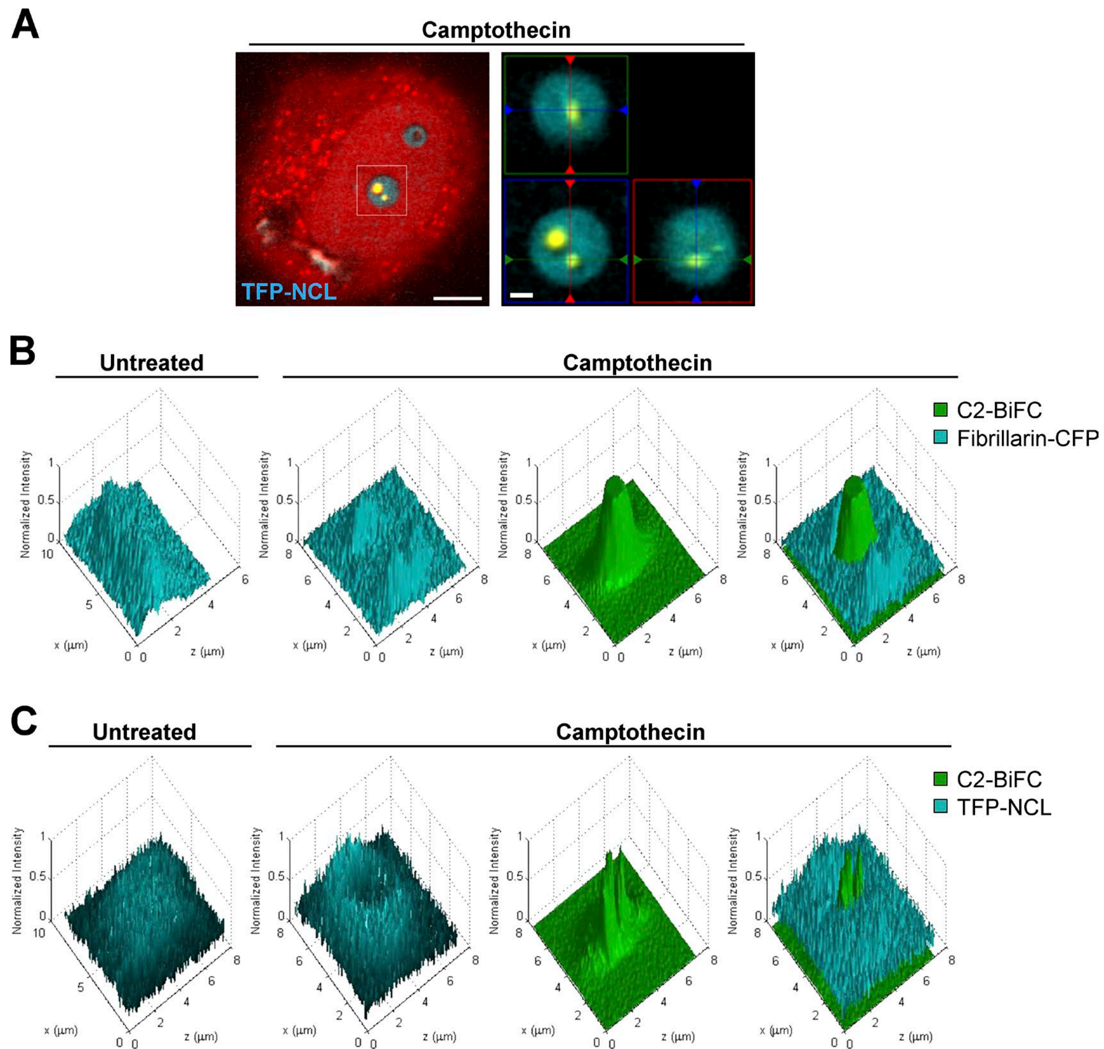


Figure S2. **Localization of caspase-2 activation platforms within the nucleolus.** (A) Representative image of HeLa.C2 Pro-BiFC cells transfected with TFP-NCL (2 μg) followed by treatment with camptothecin (10 μM) in the presence of qVD-OPH (20 μM) showing a cell (red) with two caspase-2 BiFC puncta (yellow) in the nucleolus (marked by NCL fluorescence [blue]). Bar, 10 μm. 3D reconstructions composed from 0.2-μm serial confocal images through the *z*-plane of the cell of the white boxed regions were made. The orthogonal-slice view is shown (right). The middle panel is the *xy* plane, the right panel is the *yz* plane, and top panel is the *xz* plane. The *yz* and *xz* planes intersect according to the crosshairs. Bar, 2 μm. 3D graphs of pixel intensities of Fibrillarin-CFP and C2 BiFC signals (B) and TFP-NCL and C2 BiFC signals (C) in representative untreated and camptothecin-treated HeLa.C2 Pro-BiFC cells.

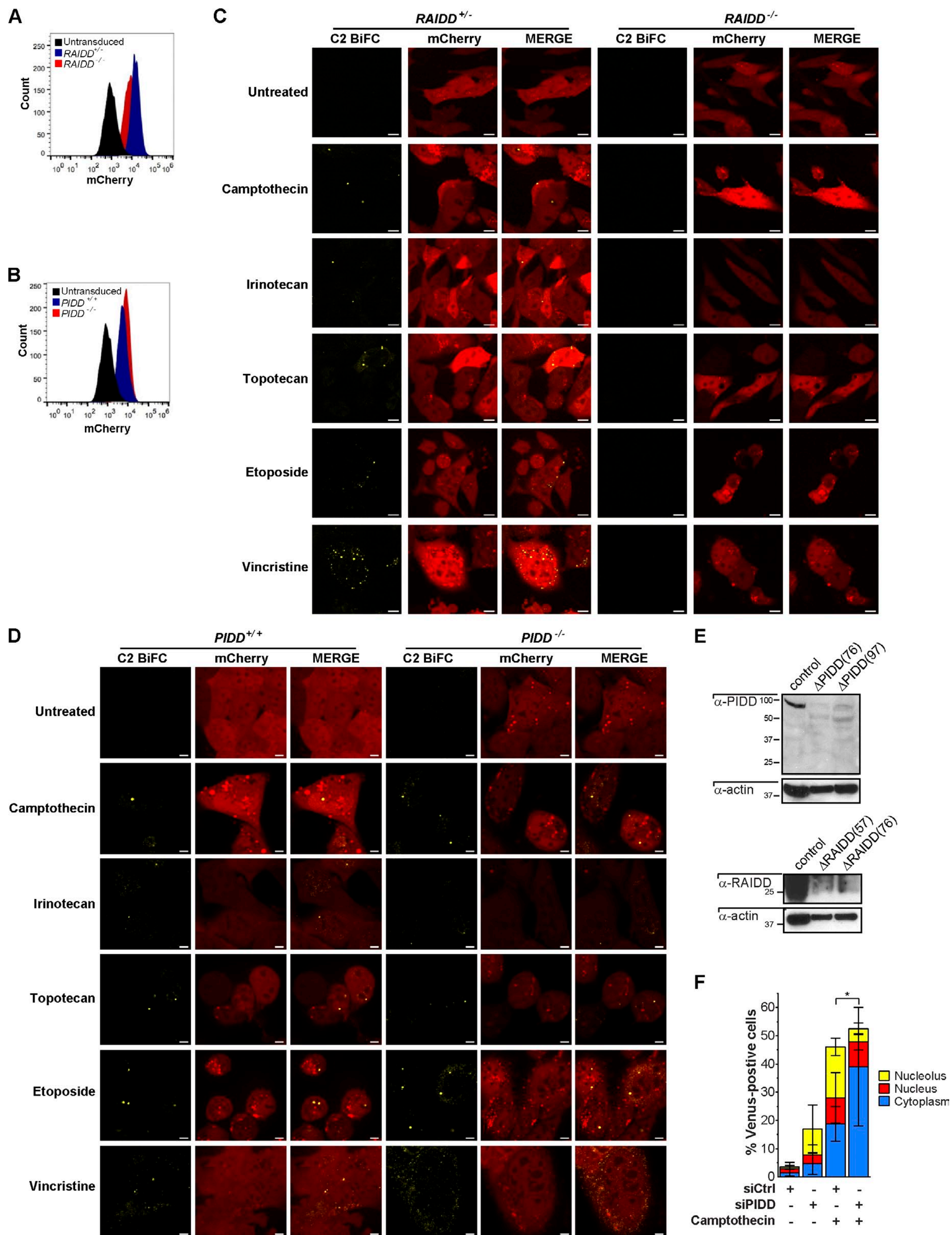


Figure S3. **RAIDD is required for caspase-2 BiFC but PIDD is only required for caspase-2 BiFC in the nucleolus.** *Raidd*$^{+/-}$ and *Raidd*$^{-/-}$ MEFs (A) and *Pidd*$^{+/+}$ and *Pidd*$^{-/-}$ MEFs (B) from littermate embryos were transduced with pRRL.C2 Pro-VC-2A-C2 Pro-VN-2A-mCherry and selected for stable expressors by sorting for equal mCherry fluorescence. Representative confocal images show caspase-2 BiFC (yellow) and mCherry expression (red) in *Raidd*$^{+/-}$ and *Raidd*$^{-/-}$ C2 Pro-BiFC MEFs (C) and *Pidd*$^{+/+}$ and *Pidd*$^{-/-}$ C2 Pro-BiFC MEFs (D) treated as in Fig. 5 (A and C), respectively. Bars, 5 µm. Images representing untreated and camptothecin-treated cells from C are also shown in Fig. 5 B, and images representing untreated cells in D are also shown in Fig. 5 D. (E) Lysates from HeLa.C2 Pro-BiFC cells or HeLa.C2 Pro-BiFC cells with *PIDD* or *RAIDD* deleted by CRISPR/Cas9 were immunoblotted for PIDD (top) or RAIDD expression (bottom). (F) HeLa.C2 Pro-BiFC cells were transfected with the indicated siRNAs in the presence of qVD-OPH (20 µM). 48 h later, cells were left untreated or treated with camptothecin (100 µM). The percentage of Venus-positive cells and the proportion of the Venus-positive cells that showed caspase-2 BiFC in the cytoplasm, nucleus, or nucleolus was determined at 24 h from a minimum of 50 microscopy images per well. Results represent the mean of three independent experiments ± SD. *, $P < 0.05$.

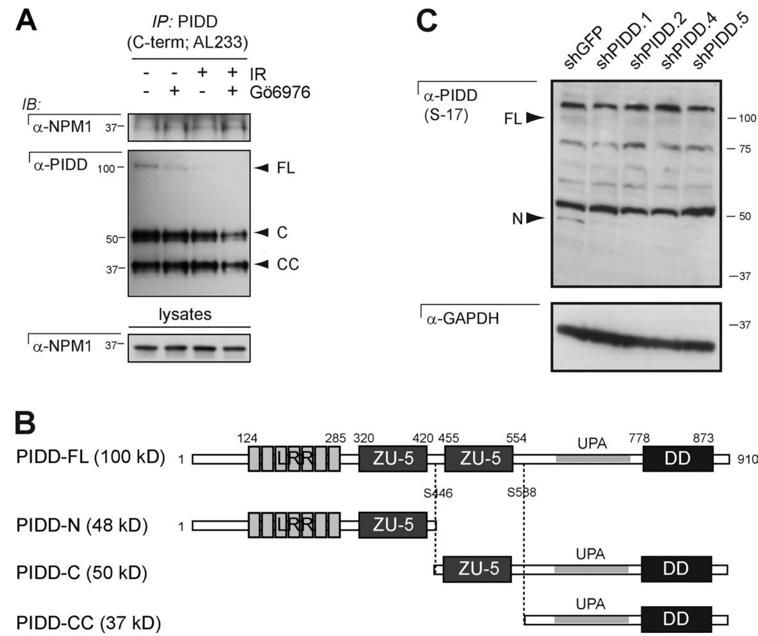


Figure S4. **NPM1 interacts with PIDD after DNA damage.** (A) HeLa cells treated with or without Gö6976 (1 µM) ± IR (10 Gy) were harvested 24 h after IR, lysed, and immunoprecipitated with C-terminal PIDD antibody (clone AL233). Immunoprecipitates were analyzed by Western blot. (B) Schematic representation of PIDD-FL and PIDD autocleavage products. Indicated are molecular weights of cleavage sites (Tinel et al., 2007). LRR, LRR domain; ZU-5, ZO-1 and UNC5-like; UPA, uncharacterized protein domain in UNC5, PIDD, and Ankyrin family of proteins (designates the putative PIDD oligomerization domain; Janssens and Tinel, 2012). (C) HeLa cells stably expressing the indicated shRNAs were analyzed by Western blot with indicated antibodies.

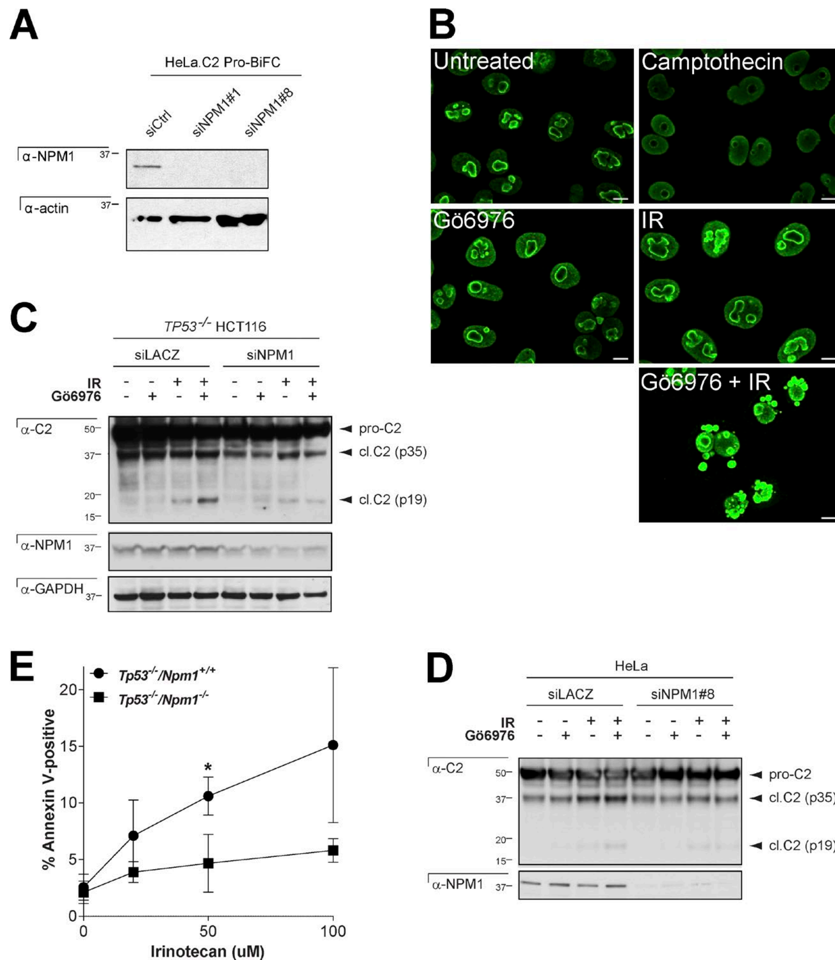
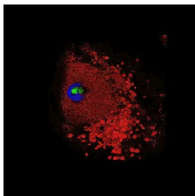


Figure S5. **NPM1 is required for PIDDosome signaling.** (A) Camptothecin-treated HeLa.C2 Pro-BiFC cell lysates transfected with control or NPM1 siRNAs. (B) HeLa cells were untreated, treated with camptothecin (100 µM), or 50 Gy IR ± Gö6976 (1 µM) in the presence of qVD-OPH (20 µM). After 16 h, cells were fixed and stained with anti-NPM1 antibody and Alexa Fluor 488–conjugated secondary antibody. Labeled NPM1 is shown in green. Bars, 10 µm. *Tp53*$^{-/-}$ HCT116 cells (C) or HeLa cells (D) transfected with the indicated siRNAs were treated with or without 10 Gy IR ± Gö6976 (1 µM) and harvested 24 h after IR. Lysates were analyzed by Western blot. (E) *Tp53*$^{-/-}$ MEFs of indicated *Npm1* genotypes were treated with or without the indicated concentrations of irinotecan. Apoptosis was assessed by flow cytometry for Annexin V binding 16 h after treatment. Results represent the mean of three independent experiments ± SD. *, $P < 0.05$.



Video 1. **Camptothecin-induced caspase-2 BiFC localizes to the nucleolus.** 3D isosurface rendering reconstruction, rotated around the *yz*-axis of confocal images through the *x*-plane of a HeLa.C2Pro BiFC cell expressing fibrillarin-CFP treated with camptothecin (10 µM). Video shows caspase-2 BiFC (green), followed by a merge with the nucleolar protein fibrillarin (blue) followed by a merge with mCherry, which is expressed throughout the cell (red).

Provided online as an Excel file is Table S1 showing NPM1 peptides from Flag-PIDD IP (HeLa+IR+Gö6976), Sample 4B (~35–37-kD bands on Coomassie).

References

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