Poulsen et al., http://www.jcb.org/cgi/content/full/jcb.201109100/DC1

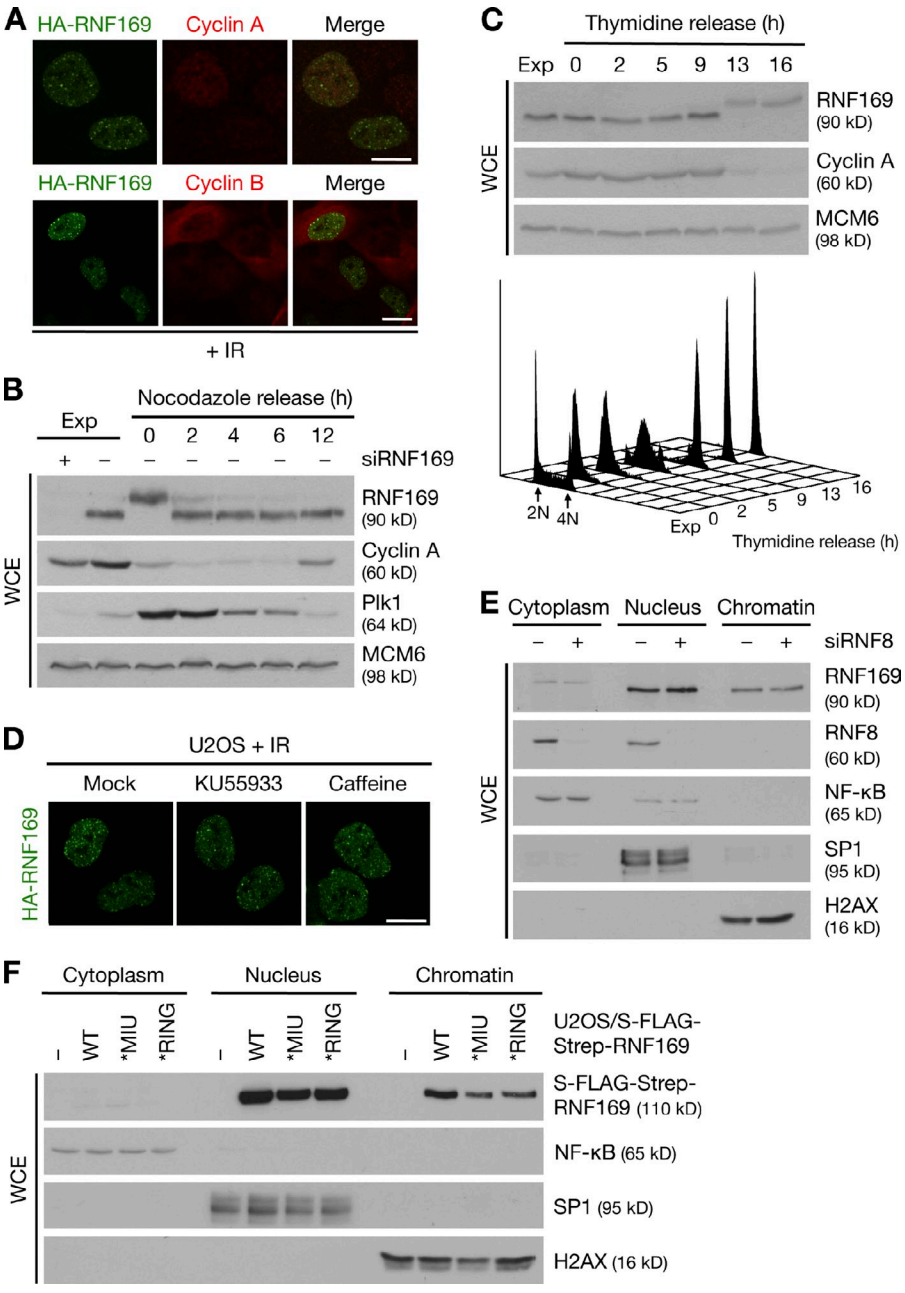

Figure S1. **Expression and subcellular localization of RNF169 during the cell cycle and in response to DNA damage.** (A) RNF169 is recruited to DSB sites throughout interphase. U2OS cells were transfected with the HA-RNF169 construct for 24 h, exposed to IR (4 Gy), and fixed 1 h later. Cells were coimmunostained with HA and Cyclin A (top) or Cyclin B (bottom) antibodies. (B) RNF169 is expressed at constant levels during the cell cycle and hyperphosphorylated in mitosis (see also C). U2OS cells transfected or not transfected with RNF169 siRNA as indicated were grown exponentially (Exp) or arrested in mitosis by treatment with nocodazole for 16 h. Cells were washed extensively, replated in fresh medium, and harvested at the indicated times. Cells were processed for immunoblotting of the indicated proteins in whole-cell extracts (WCE). (C) U2OS cells were synchronized in early S phase by double thymidine block. After extensive washing, cells were incubated in fresh medium containing nocodazole and harvested at the indicated times. Cells were processed for immunoblotting of whole-cell extracts (top) or for flow cytometry analysis of DNA content (bottom). (D) RNF169 is recruited to DSB repair foci in an ATM-independent fashion. U2OS cells treated as in A were mock treated or incubated with the ATM inhibitors KU55933 or caffeine (Sigma-Aldrich) for 30 min before exposure to IR (4 Gy). Cells were then incubated in the presence of the drugs for 1 h, fixed, and processed for immunostaining with the HA antibody. (E) Overall, RNF169 chromatin association is independent of the RNF8/RNF168 pathway. HeLa cells were transfected with control (−) or RNF8 siRNAs for 72 h, separated into cytoplasmic, nuclear, and chromatin-enriched fractions, and immunoblotted with the indicated antibodies. (F) Association of RNF169 with nondamaged chromatin does not require its ubiquitin-binding MIU domain. U2OS cells (−) or U2OS/S-FLAG-Strep–RNF169 cell lines induced to express the transgenes by treatment with doxycycline for 24 h were fractionated and processed as in E. Bars, 10 µm.

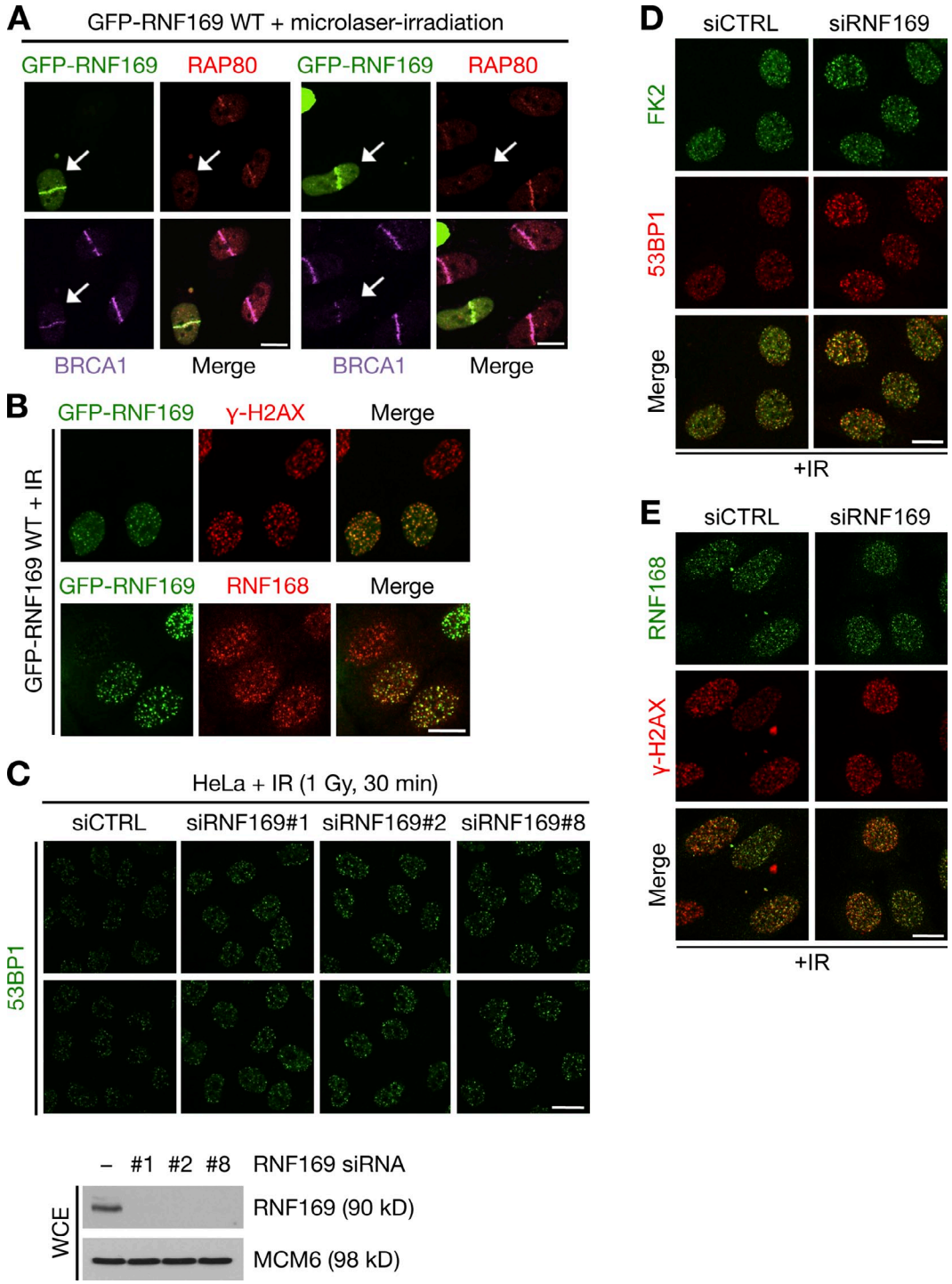

Figure S2. **RNF169 negatively regulates accumulation of factors downstream of RNF168 at DSB-flanking chromatin.** (A) RNF169 overexpression reduces recruitment of RAP80 and BRCA1 to DSB-modified chromatin (indicated by arrows). U2OS cells transfected with GFP-RNF169 WT construct for 24 h were subjected to microlaser irradiation, fixed 1 h later, and coimmunostained with BRCA1 and RAP80 antibodies. Representative images are shown. (B) Overexpression of RNF169 does not affect accumulation of γ-H2AX and RNF168 at DSB sites. U2OS cells were transfected with GFP-RNF169 WT expression plasmid for 24 h, exposed to IR (4 Gy), and incubated for an additional 1 h and then fixed and immunostained with the γ-H2AX (top) or RNF168 (bottom) antibody. (C) Enhanced 53BP1 foci after RNF169 knockdown by three independent siRNAs. (top) HeLa cells transfected with control (CTRL) or RNF169 siRNAs for 72 h were exposed to IR (1 Gy), fixed, or harvested 30 min later and processed for 53BP1 immunostaining. Representative images acquired with identical microscope settings are shown. (bottom) Immunoblot analysis of RNF169 knockdown efficiency is shown. The sequences of the RNF169 siRNAs were RNF169 #1, 5′-GGUCCUCUCUGAGUAUACU-3′; RNF169 #2, 5′-GGAUACAGAAACAGGGAAA-3′; and RNF169 #8, 5′-GAGAAAGCUGAGAGAAGAA-3′. (D) Depletion of RNF169 does not affect formation of conjugated ubiquitin (FK2) species at DSB sites. U2OS cells were transfected with control (CTRL) or RNF169 siRNAs and exposed to IR (1 Gy) 72 h later. After an additional 30 min, cells were fixed and coimmunostained with FK2 and 53BP1 antibodies. (E) Depletion of RNF169 does not affect accumulation of γ-H2AX and RNF168 at DSB sites. U2OS cells treated as in D were coimmunostained with RNF168 and γ-H2AX antibodies. WCE, whole-cell extract. Bars, 10 µm.

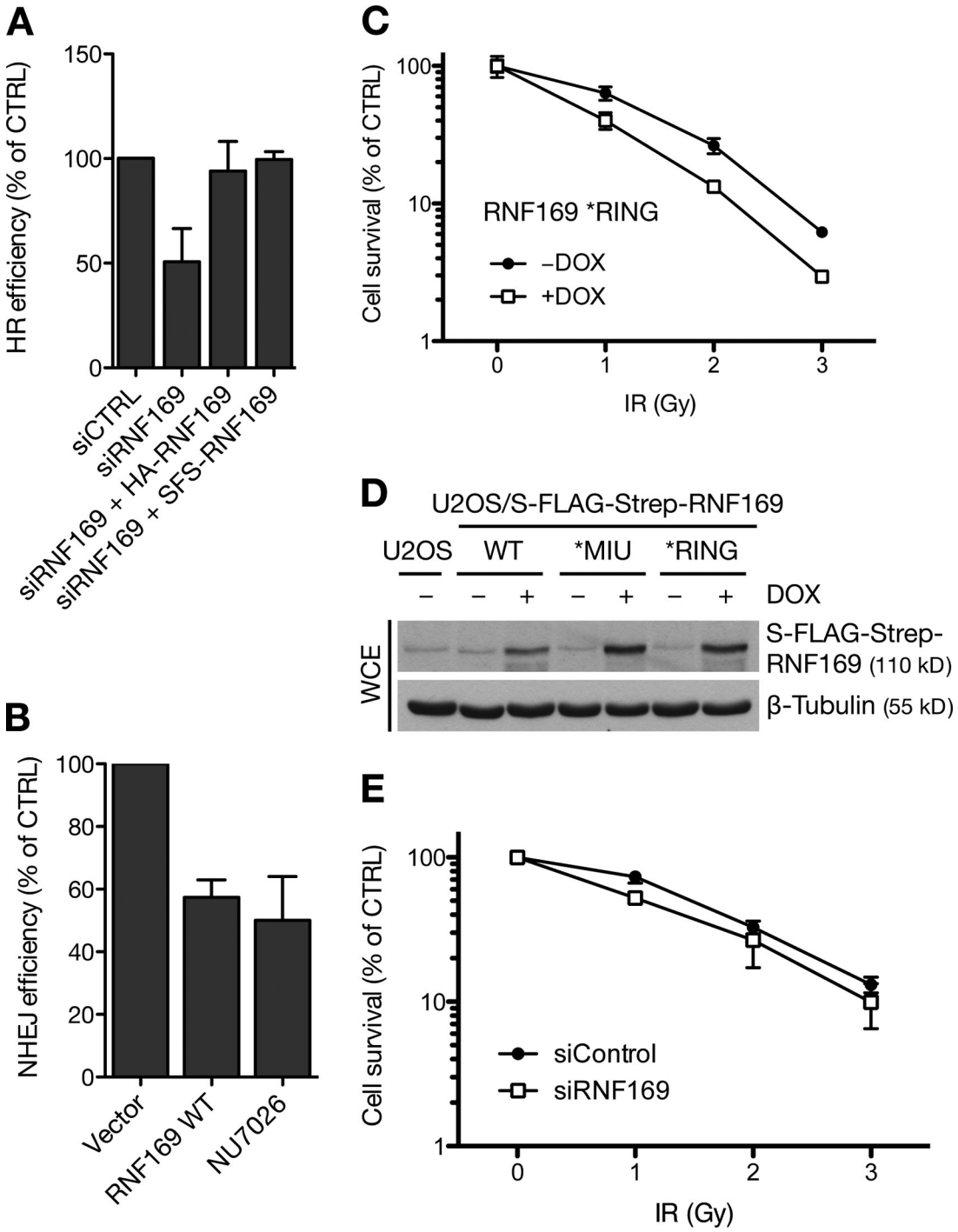

Figure S3. **Impact on DSB repair pathway choice and cell survival by modulation of RNF169 functional status.** (A) Expression of siRNA-insensitive ectopic RNF169 rescues the HR defect in cells depleted of endogenous RNF169. U2OS/DR-GFP cells were transfected with the indicated siRNAs for 24 h and then cotransfected with plasmids encoding I-SceI, RFP, and HA- or S-FLAG-Strep (SFS)–tagged RNF169 WT for 48 h. Cells were processed by flow cytometry, and the GFP/RFP ratio was used as a measure of HR efficiency. Data represent the means (± SD) from three independent experiments. (B) RNF169 inhibits NHEJ. H1299dA3-1 cells were transfected with plasmids expressing I-SceI and S-FLAG-Strep–RNF169 WT or empty vector for 48 h and harvested. Where indicated, 20 μM DNA–protein kinase catalytic subunit inhibitor NU7026 (Sigma-Aldrich) was added to cells at the time of I-SceI transfection. The proportion of GFP-positive cells (as a measure of NHEJ activity) was determined by flow cytometry. Data represent the means (± SD) from three independent experiments. (C) Clonogenic survival of U2OS/S-FLAG-Strep–RNF169 *RING cells induced or not induced with doxycycline (DOX) for 24 h and exposed to the indicated doses of IR. Results depict the means (± SD) of three replicates from one representative experiment. (D) Expression of ectopic RNF169 alleles in inducible cell lines. U2OS/S-FLAG-Strep–RNF169 cell lines were left untreated or induced to express the transgenes by treatment with doxycycline for 48 h. Cell extracts were subjected to immunoblotting with FLAG and β-tubulin antibodies. The extract of parental U2OS cells was included as a control. Note that a cross-reacting band co-migrates with the transgenes in immunoblots. (E) Clonogenic survival of RNF169-depleted cells in response to IR. U2OS cells were transfected with control or RNF169 siRNAs for 48 h and exposed to the indicated doses of IR. Results depict the means (± SD) of three replicates from one representative experiment. CTRL, control. WCE, whole-cell extract.