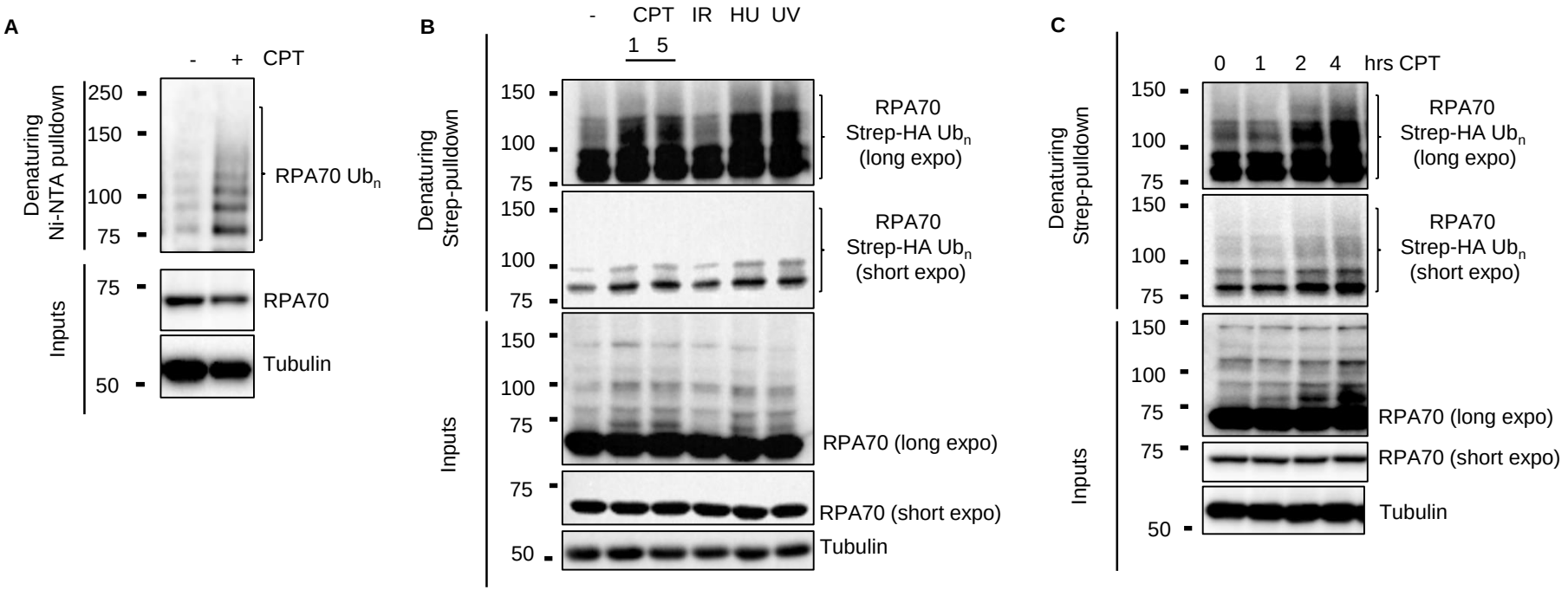
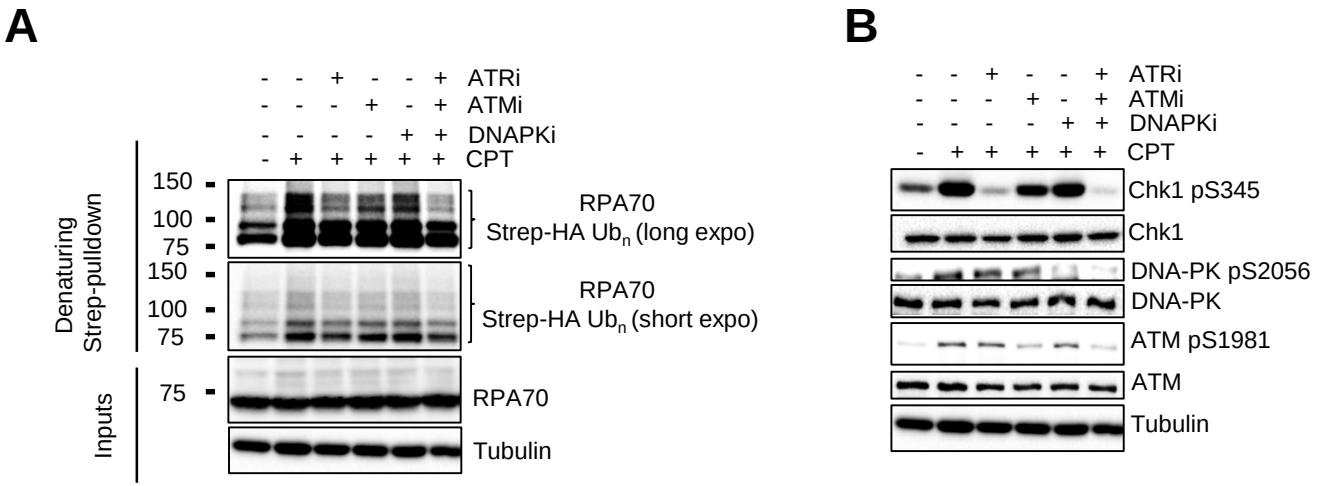
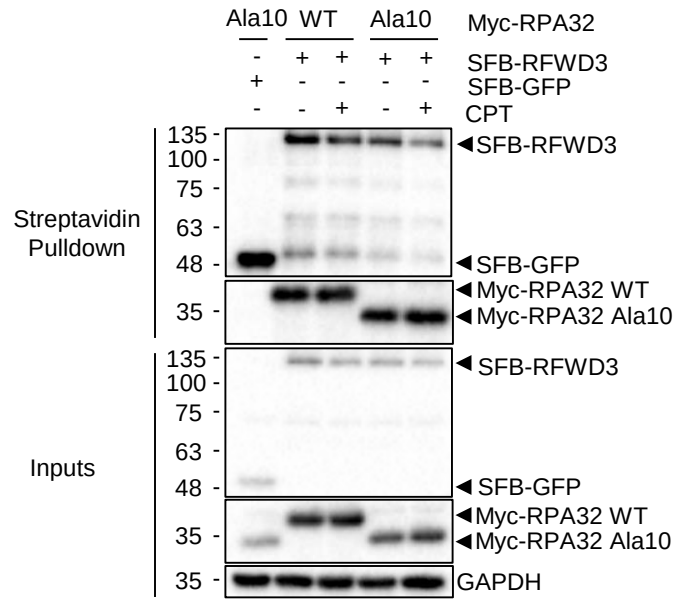


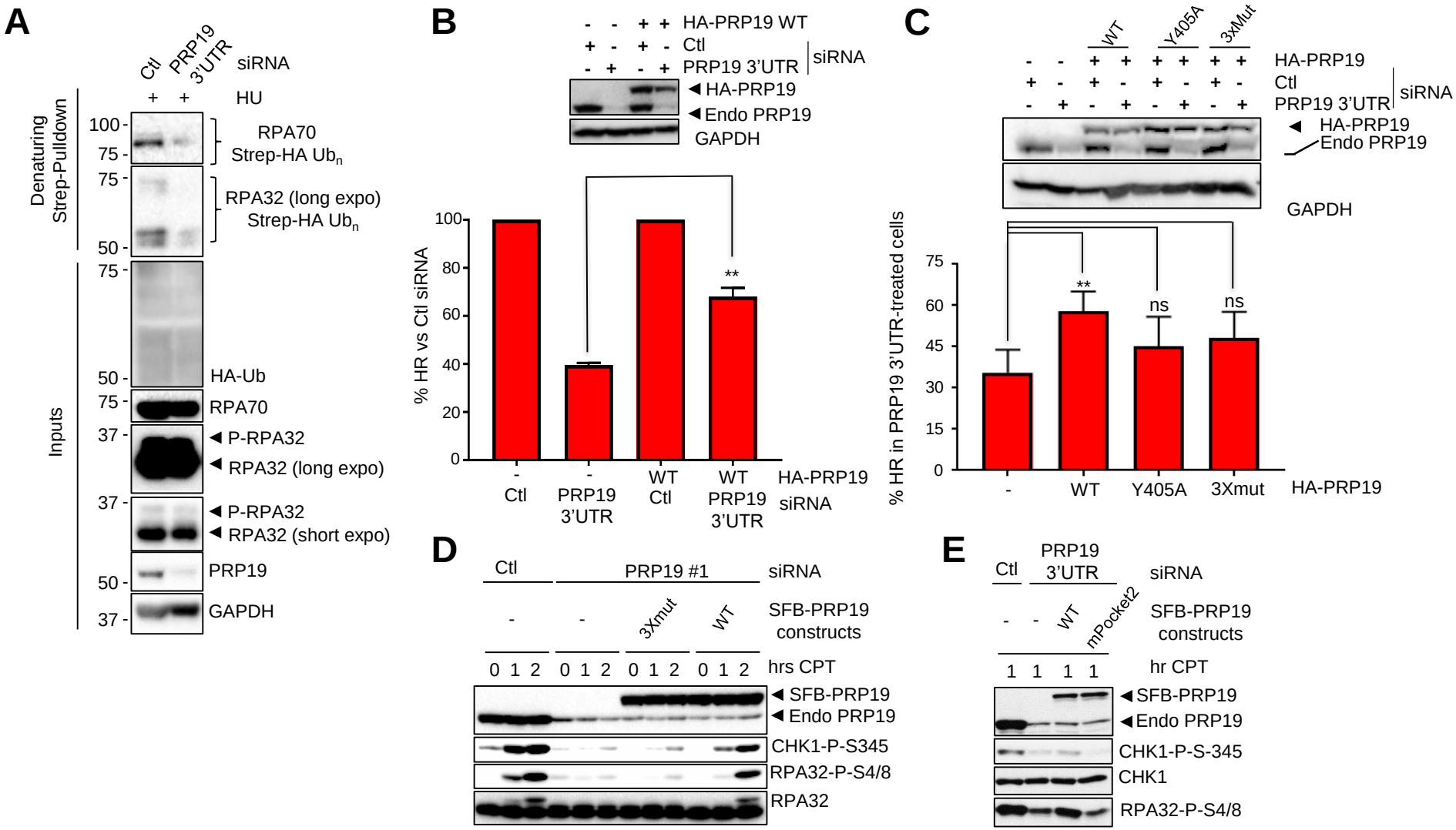
Supplementary Figure S1. RPA70 is ubiquitylated in response to DNA damage. (A) Cells were transfected with a vector expressing His_6-ubiquitin and lysed under denaturing conditions. Ni-NTA pulldown was performed to isolate ubiquitylated proteins. The indicated proteins were detected with specific antibodies. **(B)** Cells were transfected with a vector expressing Strep-HA ubiquitin and treated with 1 or 5 μM CPT, 10 γ IR, 4 mM HU or 50 J/m^2 UV for 4 hrs. Ubiquitylated proteins were isolated by Strep-Tactin pulldown under denaturing conditions. **(C)** Cells transfected as in **(B)** were treated with 1 μM CPT for the indicated times and RPA70 was detected after Strep-Tactin pulldown.



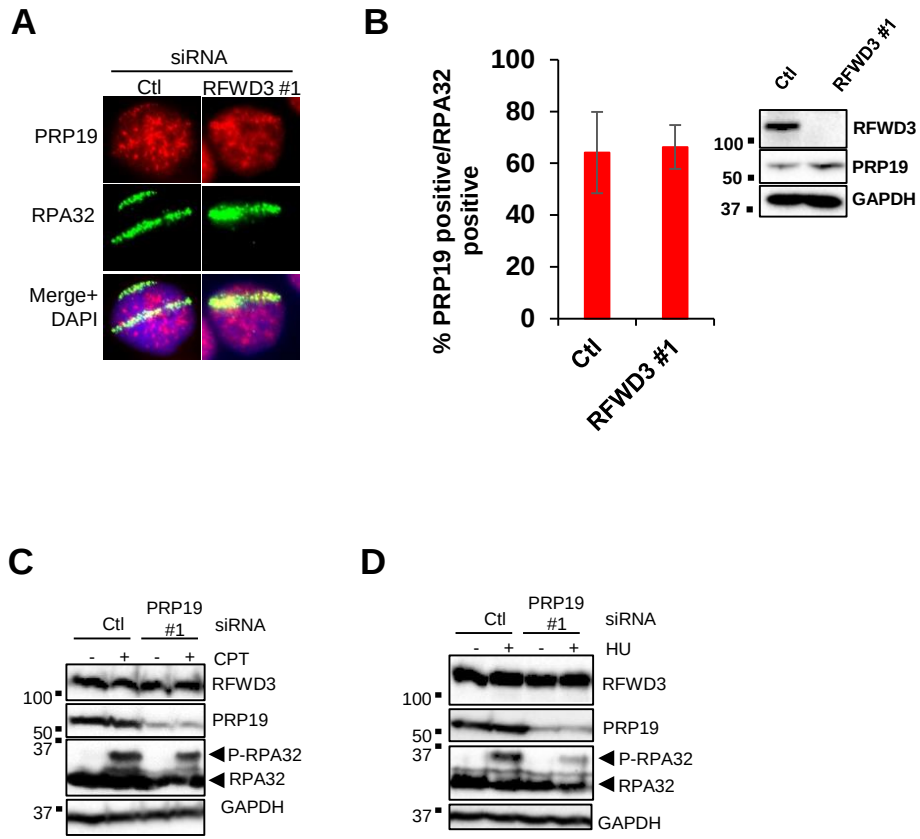
Supplementary Figure S2. RPA70 ubiquitylation is regulated by PI3K-like kinases. (A) Cells were transfected with a vector expressing Strep-HA ubiquitin, pre-treated for 1 hr with VE-821 (ATRi, 10 μM), KU55933 (ATMi, 10 μM), NU7441 (DNAPKi, 2 μM) or a combination of all 3 inhibitors, treated with 1 μM CPT for 3 hrs and lysed under denaturing conditions. Strep-Tactin pulldown was performed to isolate ubiquitylated proteins. **(B)** U2OS cells were treated as in **Figure 2 C,D**, lysed and the efficiency of the various inhibitors was assessed by monitoring DNA-PK, CHK1 and CHK2 phosphorylation levels.



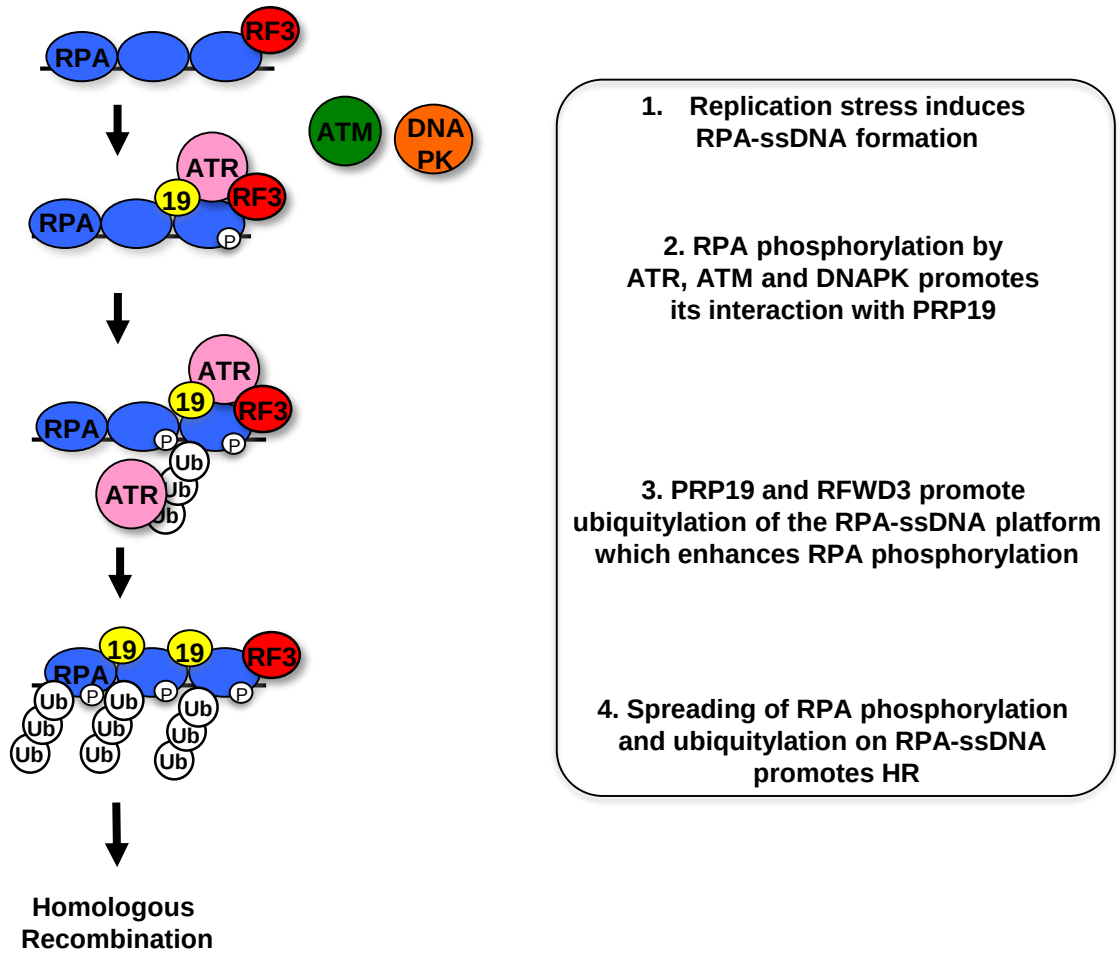
Supplementary Figure S3. Disruption of RPA32 phosphorylation does not affect its constitutive interaction with RFWD3. Cells were transfected with a vector expressing SFB-RFWD3 or SFB-GFP along with a vector expressing myc-RPA32 WT or myc-RPA32 Ala10. 24 hrs later, cells were treated or not with 1 uM CPT for 3 hrs and streptavidin pulldown was performed to isolate SFB-proteins and their interactors. The indicated proteins were detected by immunoblotting.



Supplementary Figure S4. The PRP19-RPA binding pocket and ubiquitin ligase activity are required for homologous recombination and checkpoint activation. (A) HEK293T cells were transfected with the indicated siRNA and a Strep-HA ubiquitin vector. Cells were subsequently treated with 2 mM HU for 4 hours lysed under denaturing conditions and strep-HA ubiquitin conjugated proteins were purified. **(B,C)** U2OS DR-GFP cells stably expressing HA-PRP19 constructs were transfected with Ctl or PRP19 3'UTR siRNAs and subsequently co-transfected with mCherry and I-Sce-I expressing plasmids. % HR is plotted relative to Ctl siRNA transfected cells. Graph represents the mean obtained from 4 independent experiments (** = p<0.01, Student's t-test). **(D,E)** HeLa cells were co-electroporated with PRP19-targeting siRNA and plasmids expressing siRNA-resistant WT, 3Xmut (C5A, V17E, Y33A (U-box defective) or mPocket2 mutants SFB-PRP19. 40 hrs later, cells were treated with 1 μM CPT for the indicated times and CHK1 and RPA phosphorylation were assayed.



Supplementary Figure S5. RFWD3 depletion does not impede PRP19 relocalization to laser stripes and PRP19 Knockdown does not significantly affect RFWD3 protein levels (A) Cells were transfected with the indicated siRNAs and microirradiated. 3 hours post-microirradiation, cells were processed for immunofluorescence and PRP19-positive RPA32-positive stripes were counted. Representative cells are shown. **(B)** 3 independent replicates were scored for a total of 176 Ctl and 218 RFWD3 siRNA transfected and microirradiated cells. No significant difference in PRP19 accumulation at sites of damage could be observed upon RFWD3 knockdown. **(C, D)** HEK293T cells were transfected with the indicated siRNAs and 48 hrs later were treated or not with 1 mM CPT or 2 mM HU for 3 hrs. Cells were collected, lysed and immunoblots against the indicated proteins were performed.



Supplementary Figure S6. A working model for the regulation of RPA ubiquitylation during replication stress.