WASp modulates RPA function on single-stranded DNA in response to replication stress and DNA damage

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Perturbation in the replication-stress response (RSR) and DNA-damage response (DDR) causes genomic instability. Genomic instability occurs in Wiskott-Aldrich syndrome (WAS), a primary immunodeficiency disorder, yet the mechanism remains largely uncharacterized. Replication protein A (RPA), a single-strand DNA (ssDNA) binding protein, has key roles in the RSR and DDR. Here we show that human WAS-protein (WASp) modulates RPA functions at perturbed replication forks (RFs). Following genotoxic insult, WASp accumulates at RFs, associates with RPA, and promotes RPA:ssDNA complexation. WASp deficiency in human lymphocytes destabilizes RPA:ssDNA-complexes, impairs accumulation of RPA, ATR, ETAA1, and TOPBP1 at genotoxin-perturbed RFs, decreases CHK1 activation, and provokes global RF dysfunction. las17 (yeast WAS-homolog)-deficient S. cerevisiae also show decreased ScRPA accumulation at perturbed RFs, impaired DNA recombination, and increased frequency of DNA double-strand break (DSB)-induced single-strand annealing (SSA). Consequently, WASp (or Las17)-deficient cells show increased frequency of DSBs upon genotoxic insult. Our study reveals an evolutionarily conserved, essential role of WASp in the DNA stress-resolution pathway, such that WASp deficiency provokes RPA dysfunction-coupled genomic instability.

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Wiskott-Aldrich syndrome (WAS) is an inborn error of immunity (IEI) caused by deficiency of WASp. WAS lymphocytes show some combination of actin-cytoskeletal defect, gene transcription defect, and genome instability, manifesting clinically in immunodeficiency, atopy/ autoimmunity, and lymphoid malignancy. WASp supports ARP2/3-mediated actin-polymerization in the cytoplasm and RNA Pol II-mediated transcription in the nucleus. Recent evidence links WASp to preserving genome integrity by modulating the cellular load of DSBs in human T and B lymphocytes, cells critical for adaptive immunity. WASp does so by suppressing the ectopic accumulation of pathologic R loops (formed by DNA-RNA hybrid and displaced ssDNA) that cause DSBs, and by enabling the early step of transporting DSB-ends for repair by the homology-directed repair (HDR) pathway. Therefore, WASp-deficient lymphocytes manifest both the afferent arm (increased DSBs) and efferrant arm (decreased DSB-repair efficiency) of the genome instability circuit. Furthermore, WASp deficiency undermines the nucleus-to-Golgi signaling elicited by DNA damage that is essential for cell survival after genotoxin-induced genome instability circuit. Furthermore, WASp deficiency renders WASp-de®cient cells critical for adaptive immunity. WASp does so by suppressing the ectopic accumulation of pathologic R loops (formed by DNA-RNA hybrid and displaced ssDNA) that cause DSBs, and by enabling the early step of transporting DSB-ends for repair by the homology-directed repair (HDR) pathway. Therefore, WASp-deficient lymphocytes manifest both the afferent arm (increased DSBs) and efferrant arm (decreased DSB-repair efficiency) of the genome instability circuit. Furthermore, WASp deficiency undermines the nucleus-to-Golgi signaling elicited by DNA damage that is essential for cell survival after genotoxin-induced genome instability circuit. Furthermore, WASp deficiency renders WASp-de®cient cells critical for adaptive immunity. WASp does so by suppressing the ectopic accumulation of pathologic R loops (formed by DNA-RNA hybrid and displaced ssDNA) that cause DSBs, and by enabling the early step of transporting DSB-ends for repair by the homology-directed repair (HDR) pathway. Therefore, WASp-deficient lymphocytes manifest both the afferent arm (increased DSBs) and efferrant arm (decreased DSB-repair efficiency) of the genome instability circuit. Furthermore, WASp deficiency undermines the nucleus-to-Golgi signaling elicited by DNA damage that is essential for cell survival after genotoxin-induced genome instability circuit. Furthermore, WASp deficiency renders WASp-de®cient cells critical for adaptive immunity. WASp does so by suppressing the ectopic accumulation of pathologic R loops (formed by DNA-RNA hybrid and displaced ssDNA) that cause DSBs, and by enabling the early step of transporting DSB-ends for repair by the homology-directed repair (HDR) pathway. Therefore, WASp-deficient lymphocytes manifest both the afferent arm (increased DSBs) and efferrant arm (decreased DSB-repair efficiency) of the genome instability circuit. Furthermore, WASp deficiency undermines the nucleus-to-Golgi signaling elicited by DNA damage that is essential for cell survival after genotoxin-induced genome instability circuit.

**Results**

Genotoxins promote WASp accumulation at stressed RFs and its co-association with the markers of stressed DNA.

To investigate a possible role of WASp in the RSR and DDR, we employed hydroxyurea (HU: 1 mM, 2 h; causes reduction of RF-velocity and RF-stalling) and camptothecin (CPT: 2 µM or 5 µM, 2 h; causes ssDNA nick, which after replication would become a single-ended DSB) as genotoxins. Using proximity ligation assay (PLA), we show in human T cells and B cells that these genotoxins induce WASp association in vivo with γH2A.X and RPA2 (pSer33) (Fig. 1a), both known to co-accumulate at stressed RFs. In contrast, WASp does not associate spontaneously (i.e., without damage) with these DNA stress markers. We next performed the quantitative in situ analysis of protein interactions at RFs (SIRF)21, a technique that combines principles of iPOND (isolation of proteins at nascent DNA)22 and PLA to visualize WASp enrichment at RFs at the single-cell resolution. We first verified in human T cells that PCNA (proliferating cell nuclear antigen) and GINS-CDC45 (replisome component) are constitutively present at unperturbed RFs, as SIRF positive controls (Fig. 1b). Like PCNA and CDC45, WASp is also constitutively present at unperturbed RFs, albeit at a lower frequency, in T and B cells (Fig. 1b), suggesting a role for WASp in normal DNA replication. However, following HU- or CPT-treatment, WASp enrichment at perturbed RFs is increased significantly in the WT T cells and B cells, reported by SIRF (Fig. 1b).

Because ssDNA stretches generated at stressed RF could be long and thus some of the RPA on ssDNA may be far from EdU-labeled DNA, which could potentially underestimate SIRF foci data, we next verified the SIRF data by iPOND/Western blot. This showed that the enrichment of WASp and RPA2 at perturbed RFs is significantly increased over the steady-state levels following HU-induced RF perturbation, even at an early time-point of 30 min post-HU (Fig. 1c). Similarly, by Western blot we show that the relative abundance of WASp expression in the nucleus of human B cells also increases upon HU-stress relative to unstressed control (Supplementary Fig. S1). The collective findings suggest that WASp is part of the molecular apparatus that regulates both normal DNA replication and the RSR.

**WASp directly binds RPA.** Since RPA is a central component of the RSR and DDR apparatus, and because WASp and RPA co-accumulate at perturbed RFs (Fig. 1a-c), we asked if WASp and RPA interact directly. Using ELISA-based protein-protein binding assays, we show that native purified human WASp directly binds native purified heterotrimeric human RPA protein (RPA1, 2, 3; aka, RPA70, RPA32, RPA14) in vitro, in a dose-dependent manner (Fig. 2a). WASp binds in vitro to both the RPA heterotrimer-ssDNA mixture and RPA heterotrimer alone (Fig. 2a), but does not bind to ssDNA, dsDNA, or ssRNA alone (Supplementary Fig. S2a), denoting that WASp and RPA can interact directly. Moreover, purified human WASp does not bind purified human MAX (Myc-associated factor X), Bovine serum albumin (BSA), or Saccharomyces ScRpa protein (Fig. 2a), confirming specificity of WASp-RPA physical interaction. These in vitro findings align with the in-situ proximity of WASp and RPA2 (likely <10 nm distance from each other) in intact T and B cells by PLA, following HU- or CPT-induced DNA stress (Fig. 1a). Together, these results demonstrate that WASp is an RPA-interacting protein, in vitro and in vivo.

**WASp-RPA interaction is mediated by RPA1-binding motif in WASp.** We next sought to identify the WASp-domain(s) involved in RPA interaction. Because RPA1-interacting proteins express a consensus motif: D-ϕ-x-ϕ-D-ϕ-x-D (D: Aspartic acid; ϕ: hydrophobic or charged side-chain amino acid; x: any amino acid)23,24 (Fig. 2b), we inspected the primary sequence of human WASp and found a putative RPA1-binding motif (RBM1) (939-D-E-D-E-D-E-W-D-502), located in the 3’-end acidic (A)-region of WASp’s VCA-domain, which we show is evolutionarily-conserved down to yeast, and resembles the RBM1-consensus in ATRIP, ETTA1, and RAD9A, other known...
RPA1-binding proteins (Fig. 2b). To test the functionality of RBM1, we generated WASp-mutant lacking aa:493-502 (ΔRBM1*WASp) (Supplementary Fig. S3a). We purified WT*WASp and ΔRBM1*WASp proteins, and by ELISA method show that the in vitro binding of ΔRBM1*WASp to purified heterotrimeric-RPA is dramatically reduced relative to WT*WASp (Fig. 2c). Notably, ΔRBM1*WASp mutant can still bind purified Arp2 protein (Fig. 2c), the latter previously shown to bind VCA-domain with higher affinity than Arp325, thus providing subdomain-delimited separation-of-function for actin-polymerization versus RPA-activity. Next, we expressed GFP-tagged ΔRBM1*WASp-mutant or WT*WASp into WASp-

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**Fig. 1 WASp is a DNA stress-response protein.** **a** Proximity ligation assay (PLA). Confocal immunofluorescence (IF) microscopy showing a representative set of collapsed composite IF images from a z-stack of 30-40 images acquired per optical field. Shown are the PLA signals between the indicated interacting proteins induced by the indicated genotoxins or no damage control in human T and B cells, along with their corresponding PLA statistics in box-and-whisker plots (whiskers @10–90%, horizontal bar denotes median, “−” denotes mean). In both panels, unpaired, two-tailed, Mann-Whitney nonparametric p values ****<0.0001, n = 150 cells/condition. Scale bar: 3 μm. **b** In situ analysis of protein interactions at replication forks (SIRF). **Right**, Representative z-stack collapsed composite confocal IF images showing SIRF signals between 5-ethynyl-2′-deoxyuridine (EdU)-labeled nascent DNA and the indicated protein in steady-state (no damage) or after HU- or CPT-induced DNA perturbation, along with their corresponding statistics (n = 100 cells). **Left**, representative SIRF images of positive and negative controls (ctrl), under replicative stress or no stress/damage. Statistical details as per a. Scale bar: 2 μm. **c** Isolation of proteins on nascent DNA (iPOND). **Left**, schematic of nascent DNA labeling, in which red line denotes DNA labelled with EdU, followed by black line denoting chase into media containing either hydroxyurea (replication stressor) or thymidine (control for true replication proteins that will not enrich in this sample due to EdU displacement). **Middle**, shows Western blots of input and iPOND purified proteins under indicated conditions, including no EdU sample (no-click negative control). **Right**, bar graphs depict gel densitometric quantitation of iPOND/Western blot signals normalized to their respective inputs. n = 3, ±SEM. Mann-Whitney unpaired two-tailed nonparametric p-value *<0.01. Source data are provided as a Source Data file.
deficient (WKO) T cells (Supplementary Fig. S3b) and show by the PLA method in the FACS-enriched GFP-expressing T cells that the in vivo binding of transfected ΔRBM1*WASp with endogenous RPA1 is significantly lower relative to that of transfected WT*WASp after 2 h of CPT treatment (Fig. 2d). In contrast, transfected ΔRBM1*WASp can still bind endogenous Arp2 in the cytoplasm and nucleus (PLA signals captured in both subcompartments; cytoplasmic PLA signals appear to be concentrated in the region typically occupied by Golgi and/or microtubule-organizing center) (Fig. 2d). Together, these results indicate that RBM1 primarily mediates WASp interaction with RPA, in vitro and in vivo.

**WASp enhances the ssDNA-binding activity of RPA.** To elucidate a functional relevance of the WASp-RPA association, we investigated how WASp modulates RPA’s innate function of binding ssDNA. In EMSA-binding assays, we show that adding
Fig. 2 WASp directly binds RPA and enables RPA binding to ssDNA. a. ELISA. In vitro protein-protein interaction monitored by ELISA for the indicated purified proteins at the indicated concentrations. 1st protein is coated onto the plate at a fixed concentration; 2nd protein is added at the indicated increasing concentrations. RPA1-3, human heterotrimeric complex of RPA1, 2, 3; hMAX, human Mucx-associated factor X; BSA, bovine serum albumin; ScRPA, Saccharomyces Rpa; WT−WASp, wild-type WASp. The displayed data are mean±SEM, n = 5 independent assays. The intrinsic property of WASp molecules to spontaneously oligomerize (via VCA/VCA-domain interaction) served as +ve control, and is shown for the highest concentration (1μg) of WT−WASp protein. b. Schematic of the multi-modal domain structure of human WASp showing WH1-domain, Basic-domain containing the NLS (B), GT-Pase-binding domain (GBD), Polyproline-domain (Pro), VCA-domain (WH2, C, A subdomains), in which the location of evolutionarily conserved RPA1-binding motif (RBM1) within the VCA-domain is shown. WH2 (aka, V region) binds monomeric G-actin, C and A regions bind Arp2/3-complex. Amino-acid alignments of human RPA1’s RWP1 with those of other species are shown, in which residues that are conserved (in evolution) and common (with other RPA1-binding proteins) are highlighted in blue. The prototypical RBM1-consensus is shown at the top; D: Aspartic acid; P: hydrophobic charged side-chain amino acid; x: any amino acid. c. ELISA. Shown is the binding efficiency of purified heterotrimeric RPA1-3 protein (on left) or purified Arp proteins (on right) with either purified WT−WASp or RWP1-deleted WASp mutant (ΔRBM1*WASp) at the indicated concentrations. Physical interactions of WT−WASp:WT−WASp and mutant ΔRBM1*WASp:ΔRBM1*WASp (from spontaneous oligomerization of their respective VCA-domains) served as +ve controls. n = 3 independent assays, mean±SEM. p-value: ****<0.0001; ns, nonsignificant. Mann-Whitney unpaired two-tailed nonparametric. d. Proximity ligation assay. Z-stack collapsed composite confocal IF images showing PLA signals (dots) between the indicated protein pairs after transfecting WASp-deficient human T cells with FLAG-tagged WT−WASp or ΔRBM1*WASp mutant after CPT-induced damage. The images/data are for the GFP+ cells enriched by FACS-sorting. Box plots are from n = 150 cells, Mann-Whitney unpaired two-tailed nonparametric p-value: ****<0.0001; *= 0.01; ns, nonsignificant. DAPI (in blue) demarcates the nucleus. Scale bar: 4 μm. EMSA. Assays performed using the indicated purified proteins at the indicated concentrations (nM) against fixed concentration of ssDNA (panel e) or other 3'-ended DNA structures (panel f). Data is representative of at least 3 replicates per condition. Red arrows indicate antibody super-shifted RPA band; other arrows indicate location of non-shifted RPA bands. Other related EMSA results are shown in Supplementary Fig. S2.

increasing concentrations of purified WASp to a fixed mixture of ssDNA oligo-probe (61-nt) plus either purified-RPA1 or heterotrimeric-RPA protein, results in an augmented RPA:ssDNA complexation (Fig. 2e). Maximum RPA1:ssDNA complexation is observed with equal molar concentrations of WASp, i.e., 25 nM (Fig. 2e). In contrast, addition of purified FANCA, XRCC2, DNMT1, MYC, NF-kB1, STAT1, or BSA, does not change the binding affinity of RPA1 to ssDNA (Supplementary Fig. S2b), denoting unique effect of WASp on RPA:ssDNA interaction. These results suggest that a direct interaction between WASp and RPA is modulating RPA interaction with ssDNA, either by increasing affinity or promoting the stability of the RPA:ssDNA-complex.

To further refine this finding, we tested WASp modulation of RPA1-binding to multiple other DNA conformations. Because RPA1 binds ssDNA with a preference for 3'-protrusions (3'-ended tail, 3'-ended flap, 3'-spliced array) over 5'-protrusions26,27, we first verified these RPA1-binding preferences by EMSA. We show that RPA1 binding to the 3'-ended ssDNA protrusions of 30-nt (considered both optimum and stable ssDNA-binding length for hRPA)27 is also increased by WASp in a dose-dependent manner (Fig. 2f). Notably, WASp increases binding of RPA1 to DNA-structures containing ssDNA-protrusion even at a low concentration (25 nM RPA1), which otherwise show a modest binding on its own, i.e., without added WASp (Fig. 2f). These data suggest that WASp improves the efficiency of RPA1 binding to ssDNA. In contrast, WASp does not ectopically enable RPA binding to nucleic-acid structures that normally do not efficiently bind RPA, e.g., ssRNA or DNA Holliday junction26,27 (Supplementary Fig. S2c). Together, the data demonstrate specificity of WASp effect on the intrinsic activity of RPA to bind ssDNA.

Finally, since RPA-binding to ssDNA is dynamic, involving cycles of association-dissociation-reassociation of RPA-subunits to ssDNA28–30, we tested if the effect of WASp on RPA involves modulating the DNA-binding-activity of individual RPA-subunits. To this end, we used purified RPA-subunits containing DNA-binding domain (DBD) DBD-F/A/B (RPA1), DBD-A/B (RPA1), and DBD-D/wh/E (RPA2/RPA3), (Supplementary Fig. S2d), which we had previously generated and characterized29–31. In EMSA-binding assays we show that purified WASp increases the ssDNA-binding activity of RPA1-subunits DBD-F/A/B and DBD-A/B (Supplementary Fig. S2e), considered high-affinity DNA-binding domains (DBDs)29–31. In contrast, WASp does not ectopically induce DNA-binding of RPA2/RPA3-subunits DBD-D/wh/E (Supplementary Fig. S2e), considered the trimerization core that intrinsically has low-affinity for binding ssDNA. This data suggests that WASp likely participates in optimizing the interaction of RPA’s high-affinity DBDs with the available binding sites on ssDNA, i.e., by modulating RPA’s conformational state that favors more OB-fold domains to bind ssDNA32. Together, our results indicate that WASp directly enhances the binding and/or stabilization of RPA to multiple ssDNA conformations.

WASP-deficiency disrupts genotoxin-induced RPA:ssDNA-complex formation. To investigate the role of WASp in RPA-dependent RSR and DDR, we tested how WASP-deficiency resulting from patient-derived mutation in B cells (WAS03) or CRISPR/Cas9-mediated WASp depletion in T cells (ND1-WKO) (Supplementary Fig. S2f, WASp-expression profile) influences RPA activity at perturbed RFs. By SIRF, we show that the enrichment of endogenous RPA2(pSer33) at HU- and CPT-perturbed RFs (4 h post-treatment) is significantly increased relative to unperturbed RFs in WT T cells (Fig. 3a), which is consistent with an essential role of RPA in the RSR and DDR signaling. In contrast, the enrichment of RPA2(pSer33) is significantly decreased in WKO T cells relative to WT control, this despite a significant increase in γH2A.X enrichment at perturbed RFs relative to unperturbed RFs in both WT and WKO T cells (Fig. 3a). Notably, expression of total cellular RPA1 protein as well as the nuclear localization of RPA1 in WKO T cells is comparable with WT T cells in the steady-state in vitro culture condition (Supplementary Figs. S2g, S2h), which rules out the possibility that the decreased occupancy of RPA on stressed RFs is due to an overall reduction in the amount of RPA present in WASP-deficient cells. Similarly, a significant reduction in the SIRF-enrichment of RPA at perturbed RFs is observed also in WAS03 B cells relative to normal B cells (Fig. 3a).

We next verified the SIRF data by iPOND/Western blot. This showed that the enrichment of endogenous RPA2 at HU-perturbed RFs is significantly lower in WKO T cells relative to WT (Fig. 3b). Furthermore, in EMSA-binding assays we show...
that the ssDNA-binding activity of endogenous heterotrimeric-RPA protein in the nuclear lysates of CPT-treated (5 µM; 2 h) WKO T-cells and patient-derived WAS03 B-cells is decreased relative to WT cells (Fig. 3c). Finally, we show by PLA that the co-association of RPA2(pSer33) with γH2A.X in situ is also impaired in HU-treated WASp-deficient T-cells compared to WT T-cells (Fig. 3d). Together, the data suggest that the targeting and/or binding of endogenous RPA to the sites of DNA-damage or replication-stress is compromised in the absence of WASp.

WASp-deficiency impairs activation of ATR/CHK1-signaling by disrupting TOPBP1 and ETAA1 enrichment at perturbed RFs. Since ATR recruitment to RPA-coated ssDNA is required
for the upregulation of ATR-kinase activity and phosphorylation of its substrate CHK1\(^{33,34}\), we tested if the observed RPA:ssDNA association defect is sufficient to subvert ATR signaling at perturbed RFs in WASp-deficient cells. By SIRF, we show that ATR enrichment is increased at genotoxic-perturbed RFs compared to unperturbed RFs, in both WT (normal) T cells and B cells (Fig. 4a). In contrast, ATR enrichment at perturbed RFs is significantly decreased in WASp-deficient T and B cells (Fig. 4a). Moreover, enrichment of both ETA1 and TOPBP1, the 2 canonical ATR-kinase activators, at perturbed RFs is also significantly reduced in WASp-deficient T cells relative to WT (Fig. 4a).

To directly test how WASp influences ATR-ETA1 and ATR-TOPBP1 co-associations under replication-stress or DNA damage, we performed PLA experiments. These show that HUATR:TOPBP1 co-associations under replication-stress or DNA non-canonical ATR-kinase activators, at perturbed RFs is also significantly reduced in WASp-deficient cells. Box-and-whisker plots, \(n=150\) cells, Mann-Whitney unpaired two-tailed nonparametric \(p\)-value: \(* <0.01\). Source data are provided as a Source Data file. c. EMSA/super-shift assays. Experiments performed using purified nuclear extracts from the indicated cell types, treated (\(\gamma\)) or not (\(-\)) with CPT at indicated dose/duration. The location of endogenous heterotrimeric RPA bands, as verified by anti-RPA2 antibody mediated super-shifted band (left panel, red arrow denotes super-shifted band). Red hatched box denotes the general location of the endogenous RPA1-3 bands, verified by supershift. p, probe only lane. Data is representative of \(n=3\) independent assays. d. PLA. Representative z-stack collapsed composite confocal IF images and their quantification showing RPA2 localization at HU-mediated replication-stress sites (monitored by \(\gamma H2A-X\) post-2h in T cells, wild-type (WT) and WASp-deficient (WKO)). Box-and-whisker plots, \(n=150\) cells, Mann-Whitney unpaired two-tailed nonparametric \(p\)-value: \(* * * <0.0001\). Scale bar: 3\(\mu\)m.

Fig. 3 WASp-deficiency impairs RPA occupancy at perturbed RFs. a. SIRF. Shown are the representative z-stack collapsed composite confocal IF images and their box-and-whisker plots quantifying the enrichment of the indicated proteins at RFs, unperturbed or perturbed by the indicated genotoxins (post-4h treatment) in human T and B cells. \(n=150\) cells, Mann-Whitney unpaired two-tailed nonparametric \(p\)-value: \(* * * <0.0001\). WKO T cells and WAS03 B cells are WASp-deficient (see Supplementary Fig. S2f for WASp expression profiles). a.u. denotes arbitrary units. Scale bar: 2\(\mu\)m. b. iPOND. Left panel, schematic of nascent DNA labeling, description as per the legend for Fig. 1c. Middle panel, shows Western blots of input and iPOND purified proteins under indicated conditions for wild-type (WT) and WAS knock-out (WKO) isogenic pair of human T cells. iPOND signal for WASp in WKO T cells served as a negative control. Right panel, bar graphs depict gel densitometric quantitation of iPOND/Western blot band signals normalized to their respective inputs. \(n=3\), ±SEM. Mann-Whitney unpaired two-tailed nonparametric \(p\)-value: \(* <0.01\). Source data are provided as a Source Data file. c. EMSA/super-shift assays. Experiments performed using purified nuclear extracts from the indicated cell types, treated (+) or not (-) with CPT at indicated dose/duration. The location of endogenous heterotrimeric RPA bands, as verified by anti-RPA2 antibody mediated super-shifted band (left panel, red arrow denotes super-shifted band). Red hatched box denotes the general location of the endogenous RPA1-3 bands, verified by supershift. p, probe only lane. Data is representative of \(n=3\) independent assays. d. PLA. Representative z-stack collapsed composite confocal IF images and their quantification showing RPA2 localization at HU-mediated replication-stress sites (monitored by \(\gamma H2A-X\) post-2h in T cells, wild-type (WT) and WASp-deficient (WKO)). Box-and-whisker plots, \(n=150\) cells, Mann-Whitney unpaired two-tailed nonparametric \(p\)-value: \(* * * <0.0001\). Scale bar: 3\(\mu\)m.
**WASp-deficient cells in the in vitro culture conditions**. Furthermore, employing confocal immunofluorescence imaging of DAPI-labelled cells, we show increased micronuclei formation, likely due to sequestered damaged DNA caused by unresolved stressed RFs, in WASp-deficient T and B cells compared to WT controls treated with CPT (Fig. 5f). Together, the data imply increased DNA damage in WASp-deficient cells.

Because cell-cycle phase influences DSB repair pathway choice (SSA vs. HDR vs. NHEJ), dependent partly on whether end-resection is activated (S/G2 phase) or not (G1 phase), we next analyzed the effect of fork dysfunction on cell-cycle distribution profiles. Flow-cytometry analyses revealed that, like ETA1A1-deficiency in human HCT116 and HeLa cells, WASp-deficiency also does not markedly alter the overall cell-cycle distribution in human T and B cells (Supplementary Fig. S5). Under low-dose HU, both WT and WASp-deficient T and B cells show intra-S checkpoint activation. Under high-dose HU and CPT, WT and WASp-deficient T and B cells show a combination of G1 arrest, intra-S, and/or G2/M checkpoint activation with some notable differences in WT and WKO T-cells. Specifically, under CPT-Fig. 4 WASp deficiency disrupts ATR signaling at perturbed RFs and impairs global CHK1 activation. a, SIRF. Shown are the representative z-stack collapsed composite confocal IF images and their box-and-whisker plots quantifying the enrichment of the indicated proteins at RFs, unperturbed or perturbed by the indicated genotoxins and doses (post-4h treatment) in human T and B cells, WT or WASp-deficient (WKO T cells; WAS03 B cells). In box-and-whisker plots, whiskers @10-90%, horizontal bar denotes median, "+" denotes mean. The box-and-whisker plots are from n = 150 cells analyzed, Mann-Whitney unpaired two-tailed nonparametric p-value: **** <0.0001; *** <0.001; ns, nonsignificant. a.u. denotes arbitrary units. Arrows show WASp-deficient T and B cells with micronuclei formation (See Fig. 5f for additional data on micronuclei). Scale bar: 2μm. b, PLA. Representative z-stack collapsed composite confocal IF images and their quantification plots for the indicated protein:protein interactions in T cells, wild-type (WT) and WASp-deficient (WKO), treated with the indicated genotoxin (post-4h treatment), or no treatment. In box-and-whisker plots, whiskers @10-90%, horizontal bar denotes median, "+" denotes mean. The box-and-whisker plots are from n = 150 cells analyzed, Mann-Whitney unpaired two-tailed nonparametric p-value: **** <0.0001; ** <0.001; ns, nonsignificant. Scale bar: 4μm. c, Western blot. Representative images of the indicated proteins expressed in total cell extracts of human T cells, WT and WKO (panel c), B cell lines (Normal donor and WAS03 patient) (panel d), and WASp-deficient (WKO) human T cells stably-transfected to re-express GFP-tagged WT*WASp or RPA1-binding domain-deleted mutant of WASp (ΔRBP1*WASp) (panel e) treated with the indicated genotoxin for 4 h or untreated (control, ctrl), along with their gel densitometric analyses. In box-and-whisker plots, whiskers @10-90%, horizontal bar denotes median, "+" denotes mean. n = 4 independent assays. In box plots shown in panels d and e, data is for mean±SEM. n = 3 independent assays. Mann-Whitney unpaired two-tailed p-value: <0.01. **<0.001. In panel c, the p-values for HU (0.2 low-dose) and untreated (no damage) comparing WT and WKO T cells were not significant. Source data are provided as a Source Data file.
induced damage, WKO T cells showed a higher percentage of G1-arrested cells (~70% at 24 h; ~44% at 48 h) compared to WT T cells (~37% at 24 h; ~20% at 48 h). These cell-cycle profiles suggest that WASp likely influences DSB repair pathway choices in a context and cell-type dependent manner, as further evidence from our yeast studies described later. Together, our results indicate that WASp is important for maintaining genome stability in human lymphocytes.

Yeast Las17-inactivation manifests RPA and recombinational DNA repair defects. Since WASp has an ortholog in *Saccharomyces cerevisiae*, Las17, we did a genetic analysis to test whether results in human cells can be extrapolated to yeast to establish conservation of function in eukaryotes. Because in *S. cerevisiae*, homologous recombination is the primary mechanism for repairing DSBs, we used Las17-deficient *Saccharomyces* mutants carrying the *las17-14* allele (see Methods section) to directly test WASp (Las17) role in HDR. In addition, we generated a *las17-14* auxin-inducible degron strain (*las17-aid*) (see Methods section) to be able to conditionally deplete cells of Las17 expression after auxin (IAA) addition. By Western blot, we show that the degron allele effectively depleted the Las17 protein expression after IAA addition (Supplementary Fig. S6), thus validating the auxin-based degron system. Notably, hypersensitivity to DNA-damaging agents HU and Methyl methanesulfonate (MMS) is seen in both the *las17-14* and *las17-aid* mutants, the latter in the presence of IAA that activates the degron (Fig. 6a). This is accompanied by an increase in Rad52 foci (Fig. 6b), denoting an accumulation of recombinogenic DSBs, and thus confirming a defect in HDR under replication-stress and DNA damage-inducing conditions. Since *las17-14* and *las17-aid* (+IAA) mutants exhibited similar phenotypes for the DNA-damage readouts of our interest, we employed *las17-14* mutant for all subsequent studies.

Next, since accumulating evidence indicate that a natural source of replication stress and DNA damage is the formation of ectopic DNA-RNA hybrids (R loops) and we have previously shown that WASp deficiency leads to accumulation of such structures and that RPA facilitates ribonuclease H1 (RNH1) action in suppressing ectopic R loops, we tested if Las17 has a role in preventing R loop accumulation in yeast. Indeed, the *las17-14* mutant accumulates significantly more R loops at two previously validated genes, as determined by DNA-RNA immunoprecipitation (DRIP) using the antibody S9.6. The specificity of DRIP-signal was further verified by in vitro treatment with RNH1, which eliminated the DRIP-signals (Fig. 6c). Importantly, accumulation of DNA breaks as determined by Rad52 foci were also suppressed by RNH1 overexpression in vivo (Fig. 6d), confirming that R loops in these mutants are a natural source of replication stress and DNA damage. This implies that Las17, like human WASp, is required to maintain a healthy R loop balance and prevent R-loop-mediated DSBs in yeast.
Next, to directly test how the Las17 protein may influence HDR, we analyzed recombination by single-strand annealing deletion events in the plasmid-born recombination system L-lacZ based on two 0.6-kb leu2 repeats with a 3-kb lacZ sequence in between (Fig. 6e). Notably, we found that the frequency of recombination in las17-14 mutant was not higher than that in wild-type yeast (Fig. 6e), which we would have expected if the high levels of Rad52 foci observed in these las17 mutants were a consequence of an increase in DSBs (Fig. 6b). Instead, these results imply that DSBs are not efficiently processed by HDR in the las17-14 mutant, and consequently Rad52 foci accumulate at high levels. To formally test this possibility, we determined the capacity of the las17 mutants to repair DSBs by single-strand annealing (SSA), a repair pathway that does not depend on the
Rad51 strand exchange protein for homologous recombination. For this, we generated a DSB between the two 0.6-k leu2 direct-repeats harbored by a CEN-plasmid at a unique PstI site (Fig. 6f) and determined the level of repair by SSA. We show that las17-14 mutant displays a significantly higher level of DSB-induced SSA (cut) as determined by the ratio of Leu+ recombinants versus Leu- non-recombinants (Fig. 6f) compared to the spontaneous (cut) as determined by the ratio of Leu+ overexpression in vivo. Mean + SEM, n = 5 independent experiments. ***p < 0.001, ****p < 0.0001 by Paired Student’s t-test. A.U. denotes arbitrary units.

Together, human and yeast data suggest a defect in RPA loading/retention at DNA damaged sites as causative of altered DSB-repair (Fig. 7), consistent with an evolutionarily conserved role of WASp in the DDR and RSR.

**Discussion**

Our study demonstrates that WASp is a required component of the DNA stress-resolution apparatus, with an obligatory role in the formation/stabilization of the RPA:ssDNA-complex, a crucial step in the DNA repair and replication cascades. WASp helps coordinate a core RPA function of binding ssDNA. Because RPA innately binds ssDNA tightly, “chaperons” like WASp are needed to modulate the RPA:ssDNA-complex and thus improve the efficiency of protein-DNA “handoffs” needed to complete DNA repair (Fig. 7). This genome-stabilizing role of human WASp is conserved down to yeast, in which Las17 deficiency also causes RPA dysfunction and DNA damage. Collectively, our findings in human lymphocytes and yeast models propose that WASp:RPA signal integration likely co-evolved to support physiologic DNA replication, RSR, and DSB repair, whose integrity is fundamental to organismal fitness and in the prevention of disease. Since homologous recombination (HR) is an essential mechanism for efficient DNA replication and DSB repair (homology-directed repair, HDR), and since we show that Las17-deficient cells favor DSB repair via the SSA pathway, which is relatively mutagenic, our findings propose mis-regulated HR/HDR as an important driver of genome instability in WASp-deficient cells in both higher and lower eukaryotes, and a mechanism for oncogenesis in human WAS. Since WASp is expressed in all hematopoietic-derived cell lineages, the deleterious effects on the genome we have uncovered in WASp-deficient lymphocytes could also potentially manifest in other immune cells. We therefore propose that the degree of combinatorial defects in both the adaptive and innate immune cells contribute to the development of different clinical severity phenotypes in different WAS patients.

From a cell-biological standpoint, since nuclear Arp2/3 and F-actin are essential in DNA repair and ATR-linked RSR, our results propose a region-specific, dual-actions of WASp in genome-stability, one that is RPA-mediated (via a small segment of A-region of the VCA-domain) and another F-actin-mediated (via rest of the VCA-domain). Hence, the VCA-domain function of WASp, historically known only for supporting F-actin cortical-cytoskeletal remodeling, extends to DNA replication and repair in the nucleus. Moreover, due to the intrinsic nature of WASp to oligomerize through VCA:VCA-domain co-association, our results suggest a unique molecular mechanism for focally concentrating the enrichment of both Arp2/3:F-actin (via multiple
VC-regions) and RPA (via multiple A-regions) at DNA-damage/RS sites where WASp accumulates. As such, WASp oligomerization provides a means to enrich multiple RPA-DBDs to ssDNA, the latter resulting in a more stable RPA:ssDNA complex needed for efficient DNA repair35,36. Conversely, in the steady-state, allosteric autoinhibitory conformation of WASp, which buries the VCA-domain in the body of WASp, provides a mechanism to prevent constitutive/persistent activation of both F-actin- and RPA-linked signaling in the nucleus. These findings suggest a model in which localized actin (oligomeric or polymeric) assembly at RFs provides a pool of RPA at the right time (Rsr-induced) and at the right place (stressed RFs, e.g., slow-moving or arrested). Because WASp has a role in modifying chromatin34, our findings do not rule out the possibility that nuclear WASp may influence RSR also by regulating the activity of chromatin-remodelers at stressed RFs. To wit, disruption of actin dynamics at the nuclear envelope, by causing global chromatin reorganization, could also indirectly contribute to RF dysfunction in WAS cells. Furthermore, since our yeast studies revealed that las17Δ deficiency results in a more pronounced damage phenotype compared to arp2Δ3 deficiency, propose that WASp (Las17Δ) role in genome stability is both Arp2/3-dependent and -independent. It should be noted that since scLas17 has not yet been shown in the nucleus of budding yeast, Las17 may influence these events directly or indirectly, the latter for example by altering the mono/oligomeric vs. polymeric actin balance in the nucleus. Accordingly, WASp is part of the rheostat dial that regulates the measured activity of the RSR and DDR by dynamically modulating RPA-activity at the RF, likely by multiple mechanisms.

From a clinical standpoint, our study proposes WAS as a genotoxin-sensitive genome-stabilizing immunodeficiency disorder that predisposes to cancer development, in part by misregulated RSR and DDR from the RPA defect. Although, loss-of-function of the RPA1-subunit is not causally-linked to cancer development in humans, RPA1 haplo-insufficient mice develop lymphoid cancers47, and somatic RPA1 mutations are found in a subset of cancers48. Because genome-instability is a hallmark of cancer, the findings described here support a previously proposed model that WASp functions within the "tumor-suppressor apparatus" in normal lymphocytes49, and that its loss triggers replication dysfunction and genome-instability contributing to oncogenesis in WAS. Notably, immunodeficiencies frequently occur in certain congenital diseases arising from mutations in essential replication genes50. In this connection, multiple WAS mutations/variants (p.D485G; p.E486K; p.D489N; p.D493N; p.D497E; p.D497Y) are reported in the public WAS-databases that occur in/or around RBM1, which could potentially disrupt RPA1-activity directly. Moreover, disease-causing mutations are found in the nuclear-localizing and nuclear-export sequences (NLS and NES) located in the N-terminal region of WASp5, which can potentially affect RPA1-activity by perturbing entry and/or retention of WASp in the nucleus. Thus, establishing the cause-and-effect relationships between patient-derived WAS alleles and RPA-linked defects in DNA replication/repair will provide insightful models that can be predictive of patient disease severity and clinical outcomes.

Methods

Cells. Human CD4 T and CD19 B lymphocytes were employed. T cell line (ND1-WT, HTLV1-immortalized) was established from the PBMCS of a normal donor by MACS cell separation (Miltenyi Biotec, Germany). To generate an isogenic WASp-deficient T cell-line (ND1-WKO), we performed CRISPR-mediated KO of WAS, as described previously31. Briefly, 2 mg of WASP-CRISPR/CAS9-GFP plasmid (sc-400712-KO-2) (Santa Cruz Biotechnology, Santa Cruz, CA) was transfected into ND1-WT T cells using Amaxa® Cell Line Nucleofector® Kit V (Lonza Biotec, USA). FACS-sorted GFP+ cells were seeded into 96-well plates by serial dilution and screened by PCR to identify clones exhibiting complete WASp KO. Successful WASp depletion was verified by RT-PCR for mRNA expression and Western blot for protein expression, and only such WASp KO T cell clones were expanded and used for downstream assays. Immortalized Epstein-Barr virus (EBV)-transformed B-lymphoblastoid cell lines (BLCLs), control BLCL harboring WT WAS (Com.S719; ND03719) and WAS patient-derived BLCL harboring E133K (WAS03; ID00063) mutation, were purchased from Coriell Institute (Camden, NJ). These cell lines were periodically tested for mycoplasma contamination.

Genotoxins. For human T and B cells, we employed hydroxyurea (HU), low dose (0.2 mM, known to induce replication stress without DSBS) and high dose (4 mM, known to induce replication stress with DSBS), and camptothecin (CPT) (1nM-5 mM concentration range, known to induce single-ended DSBS that are classically linked to fork lesions). For S. cerevisiae, both HU and methyl methanesulfonate (MMS) (0.01% or 0.02% concentration), the latter known to induce replication block linked DSBS.

Recombinant WASp, RPA, and RPA mutants. Recombinant human WT*WASp and ARBM1*WASp mutant proteins were produced commercially employing proprietary methods (GenScript USA Inc., Piscataway, NJ). Briefly, for recombinant human WT*WASp protein, codon-optimized WASp gene was cloned into NdeI-HindIII site of pET30a vector (pET30a-WT*WASp). For generating recombinant human ΔRBM1*WASp protein, WASp gene from pET30a-WT*WASp plasmid was cut out by NdeI-HindIII digestion and then cloned into NdeI-HindIII site of pET30a vector after RPA-binding motif (RBM1, amino acids 493-502 of RPA1) was deleted from 5’-directed mutant (ΔRBM1*WASp). Plasmids (pET30a-WT*WASp or pET30a-ΔRBM1*WASp) were transfected into E. coli DE3, and transformed colonies were screened to identify correct colonies by restriction enzyme digestion and sequencing. A single colony was used for large scale culture for protein generation after expression optimization and verification. Target protein was purified using Flag-tag by BacPower® Customized Protein Service in E. coli expression system (SC1318, GenScript). The protein purity and their molecular weights were determined by standard SDS-PAGE and Western blot. Integrity of target protein was confirmed by LC-MS/MS and peptide mapping. Human recombinant proteins RPA1 (TP302066), RPA2 (TP305715), MAX (TP320343), ARPS (TP308460), FANCA (TP716475), TRC2 (TP308330), DNMT1 (TP326414), MYC (TP301611), NF-kB (TP308384) and STAT1 (TP313858) were purchased from OriGene (Rockville, MD). ARP2 (ab217837) was purchased from Abcam (Cambridge, MA). HeterotrimERIC RPA and RPA1 mutant forms of RPA were expressed as recombinant protein E. coli and then purified over Affi-gel blue, hydroxyapatite, and Mono-Q columns as described previously31,51. Purifications of RPA1-FAB and RPA1-AB were adjusted as described in Ref. 52 (Walther et al. 1999) because of altered elution profiles. The purification of RPA2.3-Dwhē substituted DEAE-fastflow for Affi-gel blue as the first column as described in ref. 51. All forms of RPA were purified to greater than 95% purity as determined by SDS-PAGE.

ELISA protein-protein binding assay. 1 µg of 1st protein diluted in H2O (volume: 50 µl/well) was coated onto 96 well plate and incubated for 1 h at 25 °C, and washed 3 times with washing buffer (1X PBS, 0.2% Tween-20), thereafter blocked with 300 µl of blocking buffer (1X PBS with 5% milk) for 30 min at 25 °C and washed 3 times with washing buffer. 50 µl of 2nd protein diluted in blocking buffer was added, incubated for 1 h at 25 °C and then washed 3 times with wash buffer. 50 µl of 1:1000 dilution of 1st antibody (anti-WASp antibody; protitechTECH:109877-1-AP), Rosemont, IL) in blocking buffer was added, incubated for 30 min at 25 °C and then washed 3 times with wash buffer. 50 µl of freshly prepared 0.8 mg/ml of O-phenylenediamine (Sigma: P5451-50TAB, St. Louis, MO) in 0.05 M phosphate-citrate buffer containing 0.03% sodium perborate (Sigma: P4922-100CAP, St. Louis, MO) was added, incubated at 25 °C for 10 min in dark and then the color change (absorbance) was measured at OD450 nm on microplate reader (FLUOstar omega, BMG LABTECH, Cary, NC). To coat ssDNA-bound 1st protein, 1 µg of 1st protein was incubated with 1 nM ssDNA for 20 min at room temperature (final volume 25 µl), and then transferred to 96 well plate for coating.

Electrophoretic Mobility Shift Assay (EMSA). For preparation of nuclear extract (NE), 10 × 10⁶ cells were harvested, lysed with 400 µl of buffer A (10 mM KCl, 0.2 mM EDTA, 1.5 mM MgCl₂, 0.5 mM DTT, and 0.2 mM PMSF) and incubated at chilled temperature for 15 min. Nuclei were sedimented by centrifugation for 5 min at 14,000 × g. Pellet was resuspended in 100 µl of buffer C (20 mM HEPES [pH 7.9], 420 mM NaCl, 1.5 mM MgCl₂, 20% glycerol, 0.2 mM EDTA, 0.5 mM DTT, and 0.2 mM PMSF), incubated at 4 °C for 20 min, centrifuged for 6 min at 14,000 × g, and then the supernatants were collected as nuclear extract (NE). Concentration of NE was determined using a concentrated advanced reagent protein assay kit (cytoleukin ADV02-A, Denver, CO). For preparation of RNA or DNA probes, 20 µl of reaction mixture (2.0 µl RNA, ssDNA, or DNA substrates [2 µl], 2 µl 10X T4-polyadenylate kinase buffer,
1.5 µl T4-polyribonucleotide kinase [Promega M4108, Madison, WI], 2.5 µl γ-[32P]-ATP [BLU502A250UC; 3000 Ci/mmol, 250 µCi/25 µl, Perkin-Elmer, Waltham, MA], 12.5 µl dH2O was incubated at 4°C for 10 min. Probe (10 µl CFIP) was added to reaction mixture, incubated at room temperature for 20 min and then resolved on 6% non-denaturing polyacrylamide gels. Gels were dried and subjected to autoradiography. For super-shift assay, 2 µg antibody was added to reaction mixture after the reaction was completed at room temperature for 30 min. RPA1 (TA309716) and RPA2 (TA500786) antibodies were purchased from Origene (Rockville, MD), WASP antibody (10987-1-AP) was purchased from Proteintech (Rosemont, IL) and control IgG antibody (sc-66931) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). All recombiant proteins, antibodies and reagents used in this study are listed in Supplementary Table S1.

Oligonucleotides, RNA and DNA substrates. Oligonucleotides used to create ssDNA (61-mer) and dsDNA (61 bp) were adopted from a design reported previously25, DNA was chemically synthesized by Integrated DNA Technologies, Inc. (Iowa City, IA) using the same sequence as the 61-mer ssDNA. Sequences of the oligonucleotides used in this study are listed in Supplementary Table S3. Combbinations of oligonucleotides annealed to create the DNA substrates used in this study are also listed in Supplementary Table S3. Annealing was performed in a water bath in 0.1X SSPE for 2 min, then heated at 95°C for 2 min, cooled to room temperature and 100 µl of Amaxa cell line Nucleofactor Kit V (VCA-1003) or L (VCA-1005) (Lonza, Basel, Switzerland) were added to cell suspension and incubated at room temperature for 10 min, transferred to cuvette and transfection performed using nuclease 2b device (Lonza, Basel, Switzerland). Transfected cells were cultured for 1 day and then subjected to FACs for sorting GFP-enriched cell population.

DNA fiber assay. The assay was performed as previously described25,26. Briefly, polyclonal and asynchronous population of human T cells (Wild-type and WASp-deficient (WKO) isogenic pair) or human B cells (normal donor and WASO3 patients) were propagated in vitro culture and labelled with 25h4 iododeoxy- yridine (IDU) (1st label) followed by 250m chlorideoxyuridine (CdU) (2nd label) for different durations under genotoxic (HU or CPT)-perturbed conditions or unperturbed control. Finally, 2 µl of labelled cells were placed onto Superfrost glass slides and lysed with 12 µl lysis buffer 0.2 (0.5 Tris–HCl, pH 7.5, 50 mM EDTA, 0.5% SDS) for 12 min at room temperature, and DNA fibers slowly spread onto 15-20 µm thick slides, fixed with methanol:acetic acid solution at 3:1 ratio, denatured with freshly prepared 2.5 M HCl for 1 hr at RT, washed with PBS, and then blocked with 37°C pre- warmed 5% BSA in PBS for 30 min. The IDU- and CdU-labelled DNA was detected by 2-color immunofluorescence after staining with primary (mouse and rat anti-BrdU) and secondary antibodies (Alexa Fluor 546 and 488) to detect IDU (red) and CdU (green), images captured by Zeiss 710 confocal imaging system with 63X magnification, and –35–40 µm size tracks captured at 0.3 µm level using Image J software. The YMK-L17-D strains containing the ΔRBM1 for transfection) was added to cell suspension and incubated at room temperature for 10 min, then blocked with 37°C pre-warmed 5% BSA in PBS, and then re-incubated with HPJ-conjugated secondary antibody for 1 h at RT. The slides were then incubated with the primary antibody overnight at 4°C, washed with PBS, and then re-incubated with the secondary antibody for 1 h at RT. The images of protein bands, developed by incubation with Clarity Western ECL substrate (Bio-Rad), were acquired by UVP image system, and gel densitometric analyses performed using Image J software. Cell cycle analyses was performed after staining 2 × 10⁶ cells with propidium iodide (PI) at 37°C for 30 min, and cell cycle distribution analyzed by flow cytometry (Becton Dickinson LSR II).

SIRF and PLA. SIRF assay was performed as described10. Briefly, T and B cells, WT and WASp-deficient, grown in vitro culture were labelled with 125I at 37°C for 30 min, washed with PBS and then treated with different genotoxins for 4 h. Treated cells were fixed with 4% PFA on a poly-L-lysine pretreated slide, permeabilized with 0.3% Triton X-100, washed with PBS, and then followed by Click-iT reaction. For performing Click-iT reaction, the click cocktail containing 100 mM sodium ascorbate (Sigma Aldrich), 2 mM copper sulfate, and 25 µCi iodide-125IDNB in PBS was freshly made for each reaction. The slide was placed in a humid chamber and the click cocktail was added to the slide area containing fixed cells (~30 µl/sample) and incubated at room temperature for 40 min. After the click reaction, the slide was washed for 5 min with PBS containing 3% BSA. Primary antibodies were immobilized in the clickable alkaline phosphatase (Sigma Aldrich) and incubated with mouse or rabbit primary antibodies, and incubated at 4°C overnight in the humid chamber. Subsequent steps were according to the proximity ligation assay (PLA) per the Duolink in situ labeling kit PLA protocol from the manufacturer (Sigma Aldrich). Briefly, the slides were washed twice with 80 µl Buffer A (10 mM Tris–HCL pH 7.5, 150 mM NaCl, and 0.05% Tween-20) in a Coplin jar. The slides were then incubated with secondary PLA probes (~25 µl per sample of anti-mouse minus and anti-rabbit plus) in the humid chamber at 37°C for 1 h. The excess secondary PLA antibodies were removed by tapping the slide, and slides were washed with Buffer A. Ligation mix was freshly prepared by diluting 5X Ligation Stock and 40X ligase in high water in a Coplin jar, and applied onto the slides, which were incubated in a humid chamber at 37°C for 30 min. After washing, amplification mix containing Amplification buffer (dilute 1:5) and Polymerase (dilute 1:80) in high water quality was then applied onto the slides and incubated in a humid chamber at 37°C for another 100 min. Finally, Slides were washed with 80 µl Buffer B (0.2 M Tris and 0.1 M NaCl) in a Coplin jar twice, 10 min each, and then with 100X diluted Buffer B for another 10 min. Slides were air dried and applied with DAPI and cover slips. Slides were imaged using Zeiss 710 confocal imaging system with 63X magnification, and –35–40 µm size tracks acquired at 0.3 µm step-size were analyzed using combination of Duolink, Fiji Image J, and Prism 8 software.

iPOND assay. iPOND assay was performed as described previously27. Briefly, T cells (~80 million cells per sample) were incubated with 20 µM EdU in 30 µl culture medium at 37°C for 20 min and then washed with fresh medium at 37°C. For HU or thymidine plus, cells were resuspended either with 30 µl fresh medium at 37°C as the control or 4 ml HU or 20 µl thymidine in fresh medium. These cells were further cultured at 37°C for another 2 hrs. After labeling/treatments, each cell samples were washed with PBS, and crossed-linked in 1% Formaldehyde in PBS for 20 min at RT, quenched with glycine at the final concentration of 0.125 M for another 5 min, and washed 2 times in PBS. Cell pellets were permeabilized with 10 µl permeabilizing buffer (0.3% Triton X-0.5% BSA in PBS) at 30 min at RT and washed with 0.5% BSA/PBS. Each cell pellet was resuspended in 10 ml PBS as the control, or 10 ml fresh prepared Click buffer (10 mM Sodium ascorbate, 2 mM CuSO4, and 20 µM Biotin-PEPG7-azide) and incubated for 1-2 hr at room temperature (RT). Cells were washed with 0.5% BSA/PBS and then pellets either frozen at –80°C or immediately used for lysis. Each cell pellet was resuspended in 0.8 lysis buffer (25 mM NaCl, 2 mM EDTA, 50 mM Tris pH 8, 1% IGEVAL CA630, 0.2 % SDS, 0.5% sodium deoxycholate, and 1X Halt protease and phosphatase inhibitor cocktail (Thermo Fisher)) and incubated for 10 min on ice. Samples were sonicated with a Branson 250 using the settings, 20-25 W, 20 sec constant pulse, and 40 sec pauses for a total of 4 min on ice. Cell lysates were centrifuged at 18,000 X g for 10 min at RT. The supernatants were collected and diluted with the lysis buffer (the lysis buffer without SDS or sodium deoxycholate). Streptavidin-agarose beads (Millipore Sigma) (80µl/sample) were washed with the dilution buffer 3 times, and then incubated with the diluted samples overnight at 4°C. The beads were again washed 3 times washed with RIP buffer. Captured proteins were separated from beads by incubating beads in 50 µl 2+ Laemmli Sample Buffer (Bio-Rad) at 95°C for 25 min. The supernatant was collected, and proteins resolved on 4-15% SDS-PAGE and detected by immunoblotting.

Neutral COMET assays. The neutral COMET assays were conducted according to the manufacturer’s ( Trevigen) specifications, and as we described previously47. Briefly, CometSlide (Trevigen, Gaithersburg, MD) plated with cells (2.5 × 10⁶/ml) were plated in the Sub-Cell Gel Electrophoresis System (Bio-Rad Laboratories, Hercules, Cali) for 45 min at 30 V in neutral electrophoresis buffer. Slides were stained with SYBR-Gold dye, and ~35–50 z-stack images acquired by the Zeiss LSM-710 confocal microscopy. Comet images were analyzed for tail moment statistics calculated using OpenComet software.

Yeast strains, plasmids, and spotting studies. Yeast strains used in this study are indicated in Supplementary Table S4. The las17-ts allele used is a lack of function mutation in the las17C gene, and all other yeast strains used are indicated in Supplementary Table S4. Yeast strains, plasmids, and spotting studies are indicated in Supplementary Table S4. The las17-ts allele used is a lack of function mutation in the las17C gene, and all other yeast strains used are indicated in Supplementary Table S4. Yeast strains, plasmids, and spotting studies are indicated in Supplementary Table S4. The las17-ts allele used is a lack of function mutation in the las17C gene, and all other yeast strains used are indicated in Supplementary Table S4.
constructed using a yeast strain in which the only copy of the TIR1 gene was under the constitutive ADH1 promoter\(^6\) and the plasmid pHis AID-9myc\(^7\). Mid-log cultured yeast cells were grown in YPAD medium. 10-fold serial dilutions of the culture were prepared with sterile water and 3 μl of each dilution was spotted in solid plates. Plates were incubated 2-3 days at 30 °C.

Rad52 and RPA foci detection. Yeast strains were transformed with pWJ1344 and pRS315-GAL:RNH1 or pRS315 plasmids\(^{58-60}\). Resulting transformants were grown in glucose- or galactose-containing selective media until exponential growth and then cells were fixed with 2.5% formaldehyde in 0.1 M KHPO pH 6.4 during 10 min and washed twice in 0.1 M KHPO pH 6.6. Afterwards, cells were washed in 0.1 M KHPO pH 7.4 and permeabilized with 80% ethanol during 10 min, followed by resuspension in 1 μg/mL DAPI for staining nuclei. More than 200 nuclei for each experiment were visualized and counted in a fluorescence microscopy Leica DC 350 F microscope. For RPA foci detection, mid-log cultures were grown in YPAD medium with or without 0.005% MMS during 1 hour. More than 200 nuclei were counted and visualized in a fluorescence microscopy Leica DC 350 F microscope.

Recombination and SSA assays. Single strand annealing (SSA) was determined by the frequency of deletions obtained with the leu2 direct-repeat recombination systems L or L-lacZ based on the constitutive expression of the reporter leu2 gene in a CEN-URA3 plasmid as described previously\(^6\). The pRS316-L-lacZ plasmid was introduced into the cells by transformation after which cells were plated on SC-ura to isolate independent transformants. Recombination frequencies were calculated as the median of six independent colonies for each transformation. The average of the median values of four independent transformants was plotted. Recombinants were obtained by plating appropriate dilutions in applicable selective medium. To calculate the total number of cells, these were plated in the same media used for transformation. All plates were grown for 3-7 days at 30 °C.

For DSB-induced SSA, the L system was used. Leu- SSA deletions or Leu+ papillating colonies (Leu\(^\oplus\)) carrying the parental direct-replicator were determined among the Ura- transformants by subsequent replica plating onto SC-leu. Experiments were performed under two conditions, either using the intact uncult plasmid or cut with a double-strand break (DSB) in between the repeats. For each transformation, the average of the median values of four independent transformants was plotted. Recombinants were obtained by plating appropriate dilutions in applicable selective medium. To calculate the total number of cells, these were plated in the same media used for transformation. All plates were grown for 3-7 days at 30 °C.

DNA-RNA immunoprecipitation (DRIP) assay. DRIP experiments were performed as described\(^6\). Briefly, cells coming from exponential growth were pelleted by centrifugation and resuspended in 2.4 mL of spheroplasting buffer (1 M sorbitol, 2 mM Tris–HCl pH 8.0, 100 mM EDTA pH 8.0, 0.1% v/v β-mercaptoethanol, 2 mg/mL Zymoliase 20 T). Samples were incubated at 30 °C during 30 min. After centrifugation, the pellet was resuspended in 1.125 mL of buffer G (0.8 mM Guanidine HCl, 30 mM Tris–HCl pH 8.0, 30 mM EDTA pH 8.0, 5% Tween 20, 0.5% Triton X-100) to which 10 mg/mL RNase A and incubated at 37 °C during 30 min. Then, 75 μL of 20 mg/mL proteinase K were added and samples stood at 50 °C for 1 hour. DNA was purified by chloroform:isoamyl alcohol (24:1) and precipitated with 1 volume of isopropanol. With the help of a glass Pasteur pipette, DNA was transferred to a new eppendorf where resuspended in 150 μL of 1X TE (1 mM Tris–HCl pH 7.5, 0.5 mM EDTA pH 8.0) and digested overnight with 50 U of HindIII, EcoRI, BglII, XbaI and SalI. Half of the DNA was treated with 8 μl of each dilution was spotted in solid plates. Plates were incubated 2-3 days at 30 °C.

Data availability

The data supporting the findings of this study are available in the Nature Research Reporting Summary linked to this article.

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Author contributions

Y.M.V. and W.N. conceived the project. S.S.H. generated all CRISPR/Cas9-mediated WASP gene KO cellular models and WASP domain-deleted mutant, performed ELISA assays, EMSA assays, and cell-cycle flow studies. K.K.W. performed SIRF, PLA, IPOND, Comet assay, Western blots, and DNA fiber assays. M. L. G. –R. performed Saccharomyces studies. M. V. provided recombinant RPA proteins and RPA domain-deleted mutants. Y.M.V. and A.A. designed experiments and interpreted the data. Y.M.V. wrote the manuscript with inputs from A.A., and editing contributions from W.N. and M.W. All authors reviewed the manuscript.

Competing interests

The authors declare no competing interests.

Additional information

Supplementary information

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