POLDIP3 facilitates the activation and maintenance of DNA damage checkpoint in response to replication stress

Sufang Zhang
New York Medical College
Ernest Y. C. Lee
New York Medical College
Marietta Y. W. T. Lee
New York Medical College
Dong Zhang (dzhang12@nyit.edu)
New York Institute of Technology

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Abstract

The replication stress response is crucial for the maintenance of a stable genome. POLDIP3 was initially identified as one of the DNA polymerase δ (Pol δ) interacting proteins almost twenty years ago. Using a variety of in vitro biochemical assays, we previously established that POLDIP3 is a key regulator of the enzymatic activity of Pol δ. However, the in vivo function of POLDIP3 has been elusive for a long time. Here, we first generated POLDIP3 knockout (KO) cells using the CRISPR technology and investigated its biological functions in vivo. We showed that though the POLDIP3-KO cells manifest no pronounced defect in the global DNA synthesis under non-stress conditions, they are sensitive to a variety of replication fork blockers. Intriguingly, we found that POLDIP3 plays a crucial role in the activation and maintenance of the DNA damage checkpoint in response to exogenous as well as endogenous replication stress. Our results thus indicate that when the DNA replication fork is blocked, POLDIP3 can be recruited to the stalled replication fork and functions to bridge the early DNA damage checkpoint response and the later replication fork repair/restart.

Introduction

DNA replication is one of the most important cellular processes, the fidelity of which is critical for the fitness of the organism. For example in humans, errors arising from DNA replication of stem cells may account for two-thirds of the cancer incidence [1, 2]. Therefore, organisms, from yeast to human, have developed elaborate pathways to cope with the replication stress and to ensure the accurate and complete replication of DNA during each cell cycle [3–6].

In eukaryotes, three DNA polymerases are involved in replicating DNA during the S phase: DNA polymerase alpha (α), DNA polymerase delta (δ), and DNA polymerase epsilon (ε) [6]. DNA polymerase α, or Pol α, initiates DNA synthesis by synthesizing a short RNA primer. DNA polymerase ε, or Pol ε, is mainly responsible for the synthesis of the leading strand while DNA polymerase δ, or Pol δ, is mainly responsible for the synthesis of the lagging strand.

In order to identify novel Pol δ interacting proteins, we performed a two-hybrid screen using the second subunit of human Pol δ, p50, as the bait [7]. Two of the p50 interacting proteins identified were novel and with no known functions. We thus named them Pol δ interacting protein 38 (PDIP38) and polymerase δ interacting protein 46 (PDIP46) based on their sizes. PDIP46 is later renamed as DNA polymerase delta interacting protein 3, or POLDIP3. Subsequently, Richardson and colleagues reported the identification of SKAR, which is the mouse homologue of POLDIP3, as a binding partner and substrate of the S6 kinase 1[8]. It was also reported later that the human enhancer of rudimentary, or ERH, is also a binding partner of POLDIP3 [9]. Using a variety of in vitro biochemical assays, we established that POLDIP3 is a robust activator of Pol δ [10, 11]. Using purified recombinant proteins, we showed that POLDIP3 directly binds PCNA and p50 in vitro. Using a variety of in vitro functional assays, we further demonstrated that POLDIP3 stimulates the enzymatic activity of Pol δ in the primer extension and strand displacement assays as well as its ability to overcome a hairpin secondary structure during DNA synthesis. However,

the in vivo function(s) of POLDIP3 in DNA replication and DNA damage response (DDR) remains a mystery.

To elucidate the function of POLDIP3 in DNA replication and DDR in vivo, we first deleted *POLDIP3* gene in a variety of human cancer cell lines using the CRISPR technology. We found that the POLDIP3 knockout (KO) cells are sensitive to a variety of DNA replication blockers, including Cisplatin [cis-Diamminedichloroplatinum (II), or CDDP] and Mitomycin C (MMC). Surprisingly, we also found that POLDIP3 plays a crucial role in activating and maintaining the DNA damage checkpoint in response to the exogenous as well as the endogenous replication stress. Our results thus indicate that when DNA replication is blocked, POLDIP3 is recruited to the stalled replication fork and functions to bridge the early DNA damage checkpoint response and the later replication fork repair/restart.

Results

POLDIP3 deficient cells are sensitive to replication stress inducers

POLDIP3 was first identified as a Pol δ interacting proteins in a two-hybrid screen. [7]. In our previous studies using purified recombinant POLDIP3, we have demonstrated that POLDIP3 can robustly stimulate the various enzymatic activities of Pol δ in vitro [10]. However, the in vivo function of POLDIP3 in DNA replication and DDR is still unclear.

To investigate the in vivo function of POLDIP3, we first genetically deleted *POLDIP3* using the CRISPR technology in multiple cancer and transformed cell lines (A549, DU145, and HEK293T) (**Figs 1A** and **S1**). We are able to successfully recover multiple POLDIP3 knockout (KO) clones from each cell line. The growth rates of the POLDIP3-KO cells are indistinguishable from their parental cells, indicating that POLDIP3 is dispensable for the viability of these cells. We then performed cell cycle analysis of two independent POLDIP3-KO clones of the A549 cells and found no pronounced difference in G1, S, and G2 distribution when compared to the wild-type (WT) A549 cells (**Fig. 1B**), indicating that POLDIP3 is also dispensable for the cell cycle regulation under the non-stressed conditions.

Next, we tested whether the POLDIP3-KO cells are sensitive to DNA damaging agents. Interestingly, as shown in **Figs 1C** and **1D**, POLDIP3-KO cells are sensitive to the treatment of CDDP and MMC, both of which are known to form intra- and inter-strand crosslinks with DNA and block the progression of DNA replication forks. These data suggest that POLDIP3 is likely involved in the regulation of the DNA replication stress response and/or repair/restart of the stalled replication forks.

POLDIP3 deficient cells are defective in the activation and maintenance of DNA damage checkpoint

To further investigate the role of POLDIP3 in the DNA replication stress response, we first performed a focused screening of known DDR proteins that may interact with POLDIP3 by immunoprecipitation using cell lysates. Interestingly, we detected strong interactions between POLDIP3 and RPA34 and between POLDIP3 and Tipin (**Fig 2A**). Furthermore, we also observed strong interactions between POLDIP3 and

RPA34 and between POLDIP3 and Tipin using purified recombinant proteins (**Fig 2B**), suggesting that the in vivo interactions seen in **Fig 2A** are most likely through direct protein-protein interaction. RPA34 is part of the heterotrimeric complex, called replication protein A (RPA), which selectively binds and protects the single-stranded DNA (ssDNA). Tipin is part of the so-called Fork Protection Complex (FPC), which is a key regulator of the replisome. In addition to their functions in DNA replication, RPA34 and Tipin are also required for the DNA damage checkpoint activation in response to replication stress [12, 13].

Since POLDIP3-KO cells grew normally and manifested no pronounced cell cycle defects (**Fig 1B**), we speculated that POLDIP3 is not required for global DNA replication. Indeed, when we performed the DNA fiber assay, we did not observe any pronounced difference between the POLDIP3-WT and POLDIP3-KO cells (**Fig S2**), indicating that under non-stressed conditions, POLDIP3 is dispensable for the genome-wide DNA synthesis.

To test whether POLDIP3 is involved in DNA damage checkpoint activation, we treated both the POLDIP3-WT and POLDIP3-KO cells with different doses of UVC, another replication stress inducer, and examined the checkpoint activation by immunoblotting the whole cell lysate with antibodies recognizing γH2AX and the phosphorylated serine-345 of Chk1 (pChk1) (**Fig 2C**). Interestingly, we found a profound reduction of both γH2AX and pChk1 in the POLDIP3-KO cells, suggesting that POLDIP3 plays a critical role in the activation of the replication stress checkpoint. Consistently, when POLDIP3 was depleted using shRNA, the UVC induced DNA damage checkpoint response was also severely compromised compared to the Control-shRNA transfected cells (**Fig S3**). Similar to the treatment with UVC, when the POLDIP3-KO cells were treated with different doses of camptothecin (CPT), a topoisomerase I inhibitor that also blocks the progression of the replisome, we found that the induction of pChk1 is also attenuated (**Fig 2D**). To further test if POLDIP3 also plays a role in the maintenance of DNA damage checkpoint, we first treated both the POLDIP3-WT and POLDIP3-KO cells with 1 μM CPT for one hour. After the removal of CPT, cells were then allowed to recover in normal growth medium for 24 or 48 hours. As shown in **Fig 2E**, POLDIP3-KO cells failed to sustain the DNA damage checkpoint in both timepoints. This strongly indicates that POLDIP3 is also required for the maintenance of the replication stress checkpoint.

Collectively, our data strongly indicate that though POLDIP3 is dispensable for routine DNA replication under non-stressed conditions, it does play a critical role in the initial activation as well as the maintenance of replication stress checkpoint in vivo.

In response to the endogenous replication stress at ALT telomeres, POLDIP3 is recruited to the stalled replication forks and facilitates the activation of DNA damage checkpoint

We recently reported that depletion of FANCM induces a drastic increase of replication stress at the telomeres in cells that have adopted the Alternative Lengthening of Telomere (ALT) pathway due to the accumulation of the TERRA R-loops [14, 15]. We thus referred to this endogenous replication stress model as MR-SAT (FANCM deficiency induced Replication Stress at ALT Telomeres). We proposed that the MR-SAT model can be used to elucidate the molecular mechanism of replication stress responses at a specific endogenous locus. Intriguingly, POLDIP3 was previously identified as one of the ALT telomere

associated proteins [16, 17]. However, the exact function of POLDIP3 at the ALT telomeres was not further investigated.

We first examined whether POLDIP3 can be recruited to the ALT telomeres that experience replication stress using the FANCM depleted U2OS cells as previously described [14, 15]. U2OS cells lack telomerase activity and depend on the ALT pathway to maintain their telomeres. We induced robust telomeric replication stress using two different siRNAs targeting FANCM. Cells were then co-stained with antibodies recognizing POLDIP3 and TRF2, which is a component of the Shelterin complex and is often used as the marker for telomeres [18]. As previously reported [14], depletion of FANCM in the wild-type (WT) U2OS cells induces a drastic increase of DNA damage and replication stress at the telomeres, as reflected by the dramatically increased appearance of TRF2 foci colocalized with the γH2AX foci (**Fig 3C**) and the pChk1 foci (**Fig 3E**), respectively. Using this MR-SAT model, we observed a robust recruitment of POLDIP3 to the ALT telomeres experiencing severe replication stress (**Fig 3A**). Thus, POLDIP3 joins a cohort of DDR, DNA repair, and DNA replication proteins that have been shown to be recruited to these foci upon FANCM depletion [14, 15].

To further investigate the potential role of POLDIP3 in the replication stress response at ALT telomeres, we first deleted *POLDIP3* gene in U2OS cells using the CRISPR technology (**Fig 3B**) and then examined the replication stress response by further depleting FANCM using siRNAs. Interestingly, the increase in both γH2AX and pChk1 telomeric foci that was usually seen after FANCM depletion was severely suppressed (**Figs 3C** to **3F**), indicating that POLDIP3 also plays an important role in the activation of the replication stress response at ALT telomeres.

Taken together, these experiments reveal a novel and important additional facet of POLDIP3 function in regulating the replication stress response at ALT telomeres.

Discussion

Replication stress response is vital for the accurate and complete replication of eukaryotic genome in every cell cycle. Dysfunction or mis-regulation of replication stress response will lead to a variety of human diseases including cancers [3]. Here we report a novel function of POLDIP3 in vivo. Our data demonstrates that POLDIP3 plays an important role in the initial activation as well as the subsequent maintenance of the DNA damage checkpoint induced by either the exogenous or the endogenous replication blockage. These findings are somewhat unexpected since POLDIP3 was initially identified as a Pol δ interacting protein [7]. In light our new findings, we propose that in cells experiencing replication stress, POLDIP3 is uniquely positioned to bridge the early DNA damage response events, such as the DNA damage checkpoint activation, with the later events, such as the repair/re-start of replication forks as discussed below (**Fig 4**).

How does POLDIP3 facilitate the activation of the DNA damage checkpoint?

One of the important questions related to our new findings is: how does POLDIP3 facilitate the activation and maintenance of DNA damage checkpoint? We showed that POLDIP3 directly interacts with RPA34, which is part of the trimeric RPA complex, as well as Tipin, which is part of the FPC (**Figs 2A** and **2B**). RPA facilitates the recruitment of ATR-ATRIP to the vicinity of stalled replication forks and activate the DNA damage checkpoint [12]. Recent cryo-EM and reconstitution studies using the yeast homolog of FPC suggest that the FPC situates ahead of the CMG helicase complex to modulate the rate of the replisome [19]. It is thus conceivable that the binding of POLDIP3 to RPA and Tipin facilitates its recruitment to the stalled replication forks. Our findings that POLDIP3 depletion leads to the attenuation of H2AX and pChk1 renders these protein-protein interactions functionally significant since it implicates POLDIP3 in the DDR and the replication stress checkpoint activation.

The process of assembly and activation of the replication stress response at the stalled replication forks is complex, but mainly involves the recruitment and activation of ATR/ATRIP and subsequently the recruitment and activation of Chk1 [20, 21]. Together they prevent the stalled forks from collapsing and are also involved in the subsequent events of repair and restart of the stalled forks. Our studies reveal here that *POLDIP3* deletion greatly affects the activation of Chk1 at the stalled replication forks, indicating that it plays a crucial role in the replication stress checkpoint activation, and thus opens up a new perspective of its functions that goes beyond its functions in relation to Pol . Clearly much further work still needs to be done. A minimal model of what our findings have demonstrated in terms of its recruitment to stalled replication forks and the implications of its interactions for the DNA damage checkpoint response is shown in **Fig. 4**. Further investigation to understand the multi-faceted functions of POLDIP3 in the DNA replication stress response is certainly warranted.

Besides this present work, which implicates POLDIP3 in the replication stress induced DNA damage checkpoint activation in the context of the ALT telomere model system, i.e., the MR-SAT, other recent studies have also pointed to an involvement of POLDIP3 in the replication stress. Most recently, Bjorkman and colleagues reported that POLDIP3 associates with RTEL1, an important DNA helicase, and together they facilitate the disruption of R-loops ahead of a moving replication fork [22]. Moreover, RTEL1 is also implicated in the abundance and localization of TERRA RNA [23]. We and others have shown that TERRA R-loops are the major replication barriers at ALT telomeres [15, 24, 25]. Here it is also noted that POLDIP3 possesses a RNA recognition motif (RRM), which can directly bind different RNA [8]. We thus speculate that POLDIP3 may also be involved in regulating the biogenesis of TERRA, or the localization of TERRA to telomeres, or the formation of TERRA R-loops by directly binding to TERRA RNA.

POLDIP3 and the Difficult-to-Replicated regions

It is somewhat surprising to find that POLDIP3 is not require for the viability of the four human cell lines tested here (A549, DU145, HEK293T, and U2OS) given that it plays such an important role in the regulation of Pol δ [10, 11]. On the other hand, though the three larger subunits of Pol δ, p125, p68, and p50, are required for the viability of mammalian cells [26-30], the smallest subunit of Pol δ, p12, is not [31], suggesting that the Pol δ3, consisting of p125, p68, and p50, may be sufficient to support the DNA

replication under non-stressed conditions and the survival of most, if not all, mammalian cells. The logical question then becomes what is the in vivo function of Pol δ4. Our recent studies has shed some new lights on that. We found that loss of p12 led to a defect in the homologous recombination (HR) repair pathway as well as sensitization to PARP inhibitors, suggesting that the major in vivo function of Pol δ4 is to facilitate the homology-dependent repair (HDR) [31]. It is well established that certain genomic regions are more difficult to replicate (DTR) than others, including centromeres, common fragile sites, rDNA loci and telomeres. A unique HDR pathway, called break-induced replication (BIR), has been implicated in replicating through many of the DTRs, including the ALT telomeres [32]. We thus speculate that both the checkpoint function of POLDIP3 and its function as the key activator of Pol δ4 will likely be important for the replisome to overcome various replication barriers in the DTRs.

Material And Methods

Expression and purification of protein reagents

For the pull-down assays, wild type GST-POLDIP3 was expressed in pGEX-5X-3 (Amersham Pharmacia Biotech) as previously described [10]. His-tagged RPA34 was subcloned in the PET21b vector and expressed in E. coli BL21DE3 (pLysS). His-tagged Tipin was purchased from Addgene and expressed in Sf9 cells. GST fusion or his-tagged proteins were, and purified by using either glutathione beads (GE Healthcare Life Sciences) or Ni-NTA agarose (Qiagen).

Cell culture

Non-small cell lung cancer cell line A549 and Human Bone Osteosarcoma Epithelial Cell line U2OS were from ATCC. Both A549 and U2OS cells were passed in Dulbecco's modified Eagle's medium (Fisher Scientific) supplied with 10% of fetal bovine serum (Atlanta biologicals). All cells were grown in a humidified 5% CO_2 incubator in 37°C as previous reported [31].

Western blotting, Co-immunoprecipitation and GST pull-down assays

Western blotting for POLDIP3 and other proteins were performed as described previously [10]. A549 cells were treated with 10 J/m^2 UVC or without UVC for 4 hours, then used to prepare protein lysates separately. Coimmunoprecipitation was performed using POLDIP3 antibody or control IgG. The lysates were centrifuged at 14,000rpm for 10 min. Primary antibodies were added overnight followed by addition of A/G agarose beads for 1 h (Santa Cruz Biotechnologies). The beads were spun down and washed 8 times with RIPA buffer followed by suspension in 2x SDS loading buffer The bound proteins were analyzed using SDS-PAGE and Western blotted with antibody against RPA34 (cell signaling) or Tipin (Santa Cruz Biotechnology) or POLDIP3[10]. 1 mg GST-POLDIP3 or GST were incubated with 1 mg of His-RPA34 or His-Tipin in 800 ml binding buffer (50 mM Tris-HCl, pH 7.8, 1 mM EDTA, 150 mM NaCl, 0.1% NP-40 and 0.2 mM PMSF) for 4 hours with rotation at 4˚C. 15 ml of packed glutathione beads was added and further rotated for another hour at the same temperature. The beads were spun down at 2,500 rpm for 5 minutes and washed 8 times with the binding buffer followed by suspension in 1XSDS loading

buffer. The bound proteins were analyzed by SDS-PAGE and Western blotted with either antibody against RPA34 or against Tipin.

CRISPR/Cas9-mediated or shRNA-mediated POLDIP3 gene knockout or knockdown

CRISPR/Cas9 mediated human POLDIP3 knockout in A549 and U2OS cells were generated according to the manufacturer's instructions (Santa Cruz Biotechnology, Inc). Briefly, 1.5×10^5 cells/well were seeded onto 6-well culture plates in 2.5 ml of antibiotic-free medium per well and allowed to grow to 70% confluency. Cells were transfected with 2 μg of human POLDIP3 CRISPR/Cas9 KO Plasmid (sc-413603) and POLDIP3 HDR Plasmid (sc-413603-HDR-2) (Santa Cruz Biotechnology, Inc.) using 8 ml UltraCruz® Transfection Reagent (Santa Cruz Biotechnology, Inc.) and incubated at 37°C, 5% CO_2. Two days after transfection, successful co-transfection of the CRISPR/Cas9 KO Plasmid and HDR Plasmid was visually confirmed by detection of the red fluorescent protein via a fluorescence microscope. Stably transfected clones were selected by adding puromycin (1.0 μg/ml). Selected clones were transfected with Cre vector (sc-418923) for the removal of genetic material flanked by LoxP sites. Control CRISPR/Cas9 Plasmid (sc-418922) containing a non-targeting 20 nt scramble guide RNA (gRNA) was used as a negative control.

Human POLDIP3 shRNA, and control shRNA were purchased from Thermo Scientific. The shRNAs were packaged into lentivirus by co-transfection with the packaging plasmids Δ8.9 and VSVG into HEK293T cells. Forty-eight hours after transfection, the supernatant was filtered through a sterile 0.45-μm filter and used for infection of target cell A549. Following infection, knockdown cells were selected by addition of 2–3 μg/ml puromycin, and the cultures were grown for 1 week and further maintained in 0.5 μg/ml puromycin for experimental use.

Clonogenic cell survival assays

Clonogenic cell survival assays were performed as previously described [31]. Briefly, A549 cells were seeded into 100mm cell culture dishes (1200 cells/dish) to allowed cells to attach the plastic overnight. After attached cells were treated with CDDP (Sigma-aldrich) or MMC(Sigma-aldrich) in different concentrations for three hours, each concentration has triplets; DMSO treatment as control. After the treatment, the medium was removed, cells washed once with PBS and replaced by fresh complete medium. The dishes were incubated until cells in control dishes formed sufficiently large colonies (11 to 14 days). Subsequently, the media from each plate, was removed, then washed with PBS. The colonies were fixed with 5ml acetic acid/methanol 1:7(vol/vol) for 20 min, and stained with 5 ml 0.5% (w/v) crystal violet (Sigma-Aldrich) in dH_2O for 4 hours. Finally, excess crystal violet was removed with dH_2O and allow to dry. Colonies containing more than 50 individual cells were counted using a stereomicroscope. Digital images of the colonies are obtained using a scanning device.

Immunofluorescence Microscopy

U2OS and U2OS-POLDIP3KO cells (2.0×10^5 cells/well) were seeded onto 6 well plates and grown to 60% confluency. After 24 hours, cells were transiently transfected twice with different siRNAs and then re-

plated on coverslips. Cells were then used for immunostaining 72 hours later. Briefly, cells were first fixed in 4% paraformaldehyde for 15 min, then permeabilized at room temperature for 15 min with 0.1% Triton-X-100 in PBS followed by blocking with 2% BSA in PBS for 1 h. Cells were labeled with different primary antibodies (as indicated below) and the appropriate Alexa-488 (Invitrogen) and Alexa-546 (Invitrogen) conjugated secondary antibodies. Finally, cells were washed 3× times PBS for 10 min each, then dried and mounted with ProLong Antifade reagent with DAPI (Invitrogen). Slides were visualized and imaged using a Zeiss Axiovert 200M. All the images were taken at a magnification of 40X. Antibodies used for immunofluorescent staining include: Chk1-pS345 (Cell Signaling, 2348); TRF1 (abcam, ab10579); TRF2 (Millipore, 05-521 and Novus, NB110-57130); γ-H2AX (abcam, ab81299) and POLDIP3 [10].

Graphing and statistics

Results were presented as means ± standard errors of the mean (SEM). All statistical tests and graphing were performed with the aid of GraphPad Prism version 7.01. Unpaired t test was applied to statistical analysis. P values £0.05 were considered statistically significant.

DNA fiber assay

Cells (1.0 × 105 cells/well) were seeded onto 6 well plates separately to grow to 70% confluency. DNA fiber assay was performed as described [33]. Briefly, IdU (Sigma-aldrich) was added to the cell culture to a final concentration of 25μM. Cells were incubated for 30 min for A549 and A549-POLDIP3-KO cells, the media was then removed, the cells were washed three times with serum-free media and media containing 100 μM CldU (Sigma-aldrich) was added to the plates, and cells were incubated for the same time as Idu. After double labelling, cells were washed with ice cold PBS, harvested and resuspended in ice cold PBS to the 200 to 400 cells /μl. 2μl of the cell suspension were spotted at the end of the microscope slides (Fisherbrand 15-188-48) and air dried for 5 min. Subsequently, 7 μl of the lysis buffer (200mM Tris-HCl, pH 7.5, 50mM EDTA and 0.5%SDS) were applied on the top of the cell suspension, then gently mixed with a pipette tip and incubated for 2 min. Following cell lysis, slides were tilted to 15 ^{0}C to allow the DNA fibers spread along the slides. Once the spread fibers had dried, slides were fixed in methanol/acetic acid (3:1) solution in a staining jar for 10 min. Subsequently, slides were washed in distilled water and immersed with 2.5 M HCl for 80 min. After DNA denature, slides were washed with 3 times PBS for 5 min each, then blocking solution (5% BSA in PBS) was applied for 20 min. After removal of the blocking buffer, the slides were incubated with primary antibodies monoclonal anti-IdU (Sigma-aldrich, SAB3701448) and BrdU antibody (BU1/75) (Thermo Scientific, MA1-82088) for 2 hours at room temperature. Subsequently, the slides were washed three times with PBS for 5 min. The second antibodies (1:500 goat anti-mouse Alexa Fluor 594, 1:200 goat anti-rat Alexa Fluor 488) were applied on each slide for 1 hour. After removing the second antibodies, slides were washed three times with PBS as before and subsequently a drop of mounting medium was spotted onto each slide, coverslips were applied by gently pressing down. Slides were sealed with transparent nail polish, let dry and stored at -20 °C until being analyzed. Slides were visualized and imaged using a Zeiss Axiovert 200M with a black and white CCD AxioCam and pseudo-colored with Axiovision 4.8 software. All the images were taken at a

magnification of 100X. Pictures were then analyzed using an appropriate image analysis software ImageJ (http:rsbweb.nih.gov/ij).

Declarations

Data availability Statement: All data generated or analysed during this study are included in this published article and its supplementary information files.

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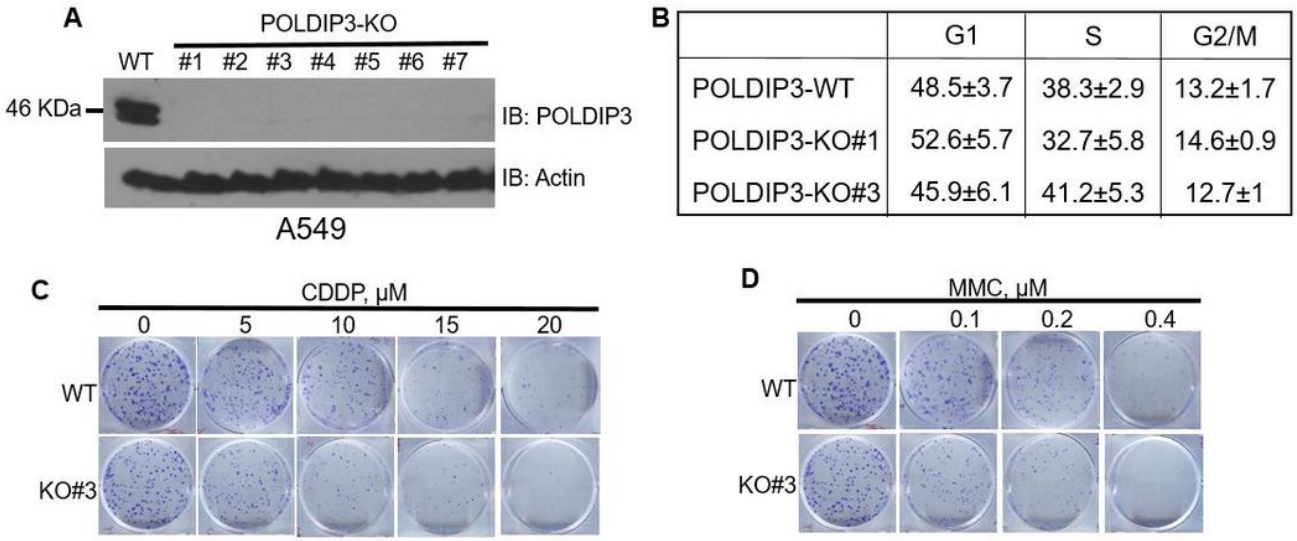
Figures



B	G1	S	G2/M
POLDIP3-WT	48.5±3.7	38.3±2.9	13.2±1.7
POLDIP3-KO#1	52.6±5.7	32.7±5.8	14.6±0.9
POLDIP3-KO#3	45.9±6.1	41.2±5.3	12.7±1

Figure 1

POLDIP3 deficient cells are sensitive to replication stress inducers, CDDP and MMC. (**A**) Cell lysates from the wild-type (WT) A549 cells and POLDIP3 knockout (KO) A549 clones #1 to #7 were immunblotted (IB) with different antibodies as indicated on the right. (**B**) Cell cycle analysis of the WT A549 cells (POLDIP3-WT) and two POLDIP3-KO clones (#1 and #3). (**C** and **D**) Equal number of POLDIP3-WT and POLDIP3-KO#3 cells were treated with different concentration of CDDP (**C**) and MMC (**D**) for three hours, and allowed to grow in fresh growth medium for eleven more days. Cells were finally stained with crystal violet.

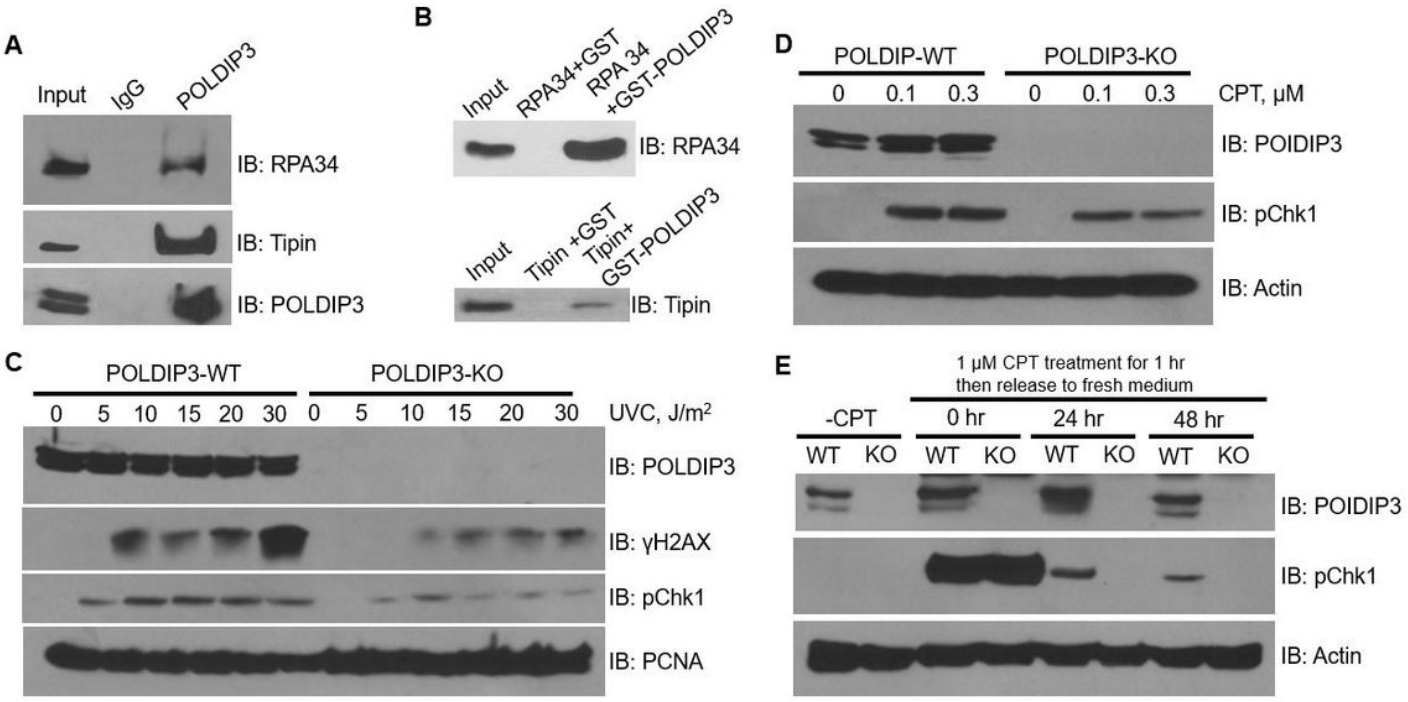


Figure 2

POLDIP3 promotes and sustains the DNA damage checkpoint in response to replication stress. (**A**) A549 whole cell lysates were used for immunoprecipitation using either normal rabbit IgG or POLDIP3 antibody and then immunoblotted (IB) with different antibodies as indicated on the right. (**B**) GST pull-down assays using purified recombinant proteins as indicated on the top. (**C**) Wild-type (POLDIP3-WT) and POLDIP3 knockout (POLDIP3-KO) A549 cells were treated with different doses of UVC for four hours. (**D**) POLDIP3-WT and POLDIP3-KO A549 cells were treated with different doses of CPT for one hour. (**E**) POLDIP3-WT and POLDIP3-KO A549 cells were first treated with 1 μM CPT for one hour, and then grew in the fresh medium without any CPT for the indicated time. Cell lysates were prepared and immunoblotted (IB) with different antibodies as indicated on the right.

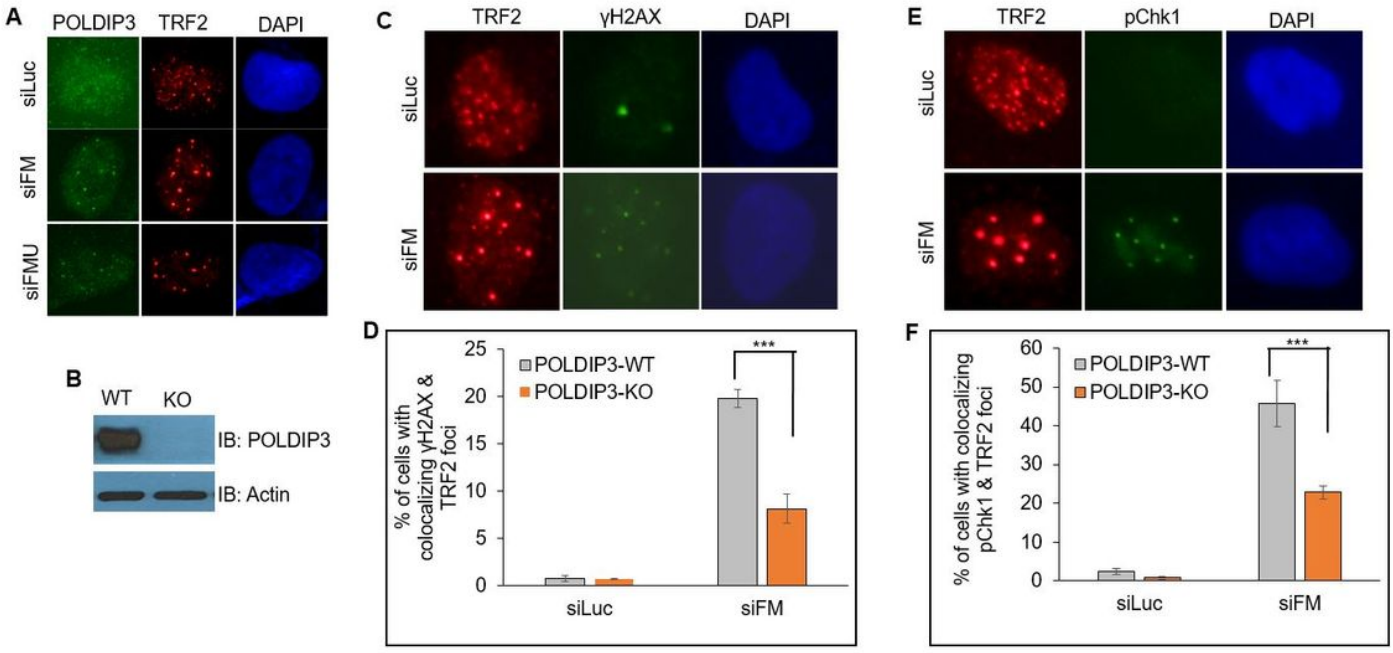


Figure 3

POLDIP3 is recruited to telomeres in FANCM deficient ALT cells and facilities the activation of replication stress induced DNA damage checkpoint. (**A**) U2OS cells were transfected with siRNA targeting either luciferase (siLuc) or FANCM (siFM and siFMU), and then stained with antibodies as indicated on the top. All the nuclei were stained with DAPI. (**B**) *POLDIP3* was deleted in U2OS cells using the CRISPR technology. Cell lysate from wild-type (WT) and POLDIP3 knockout (KO) U2OS cells were prepared and immunoblotted (IB) with different antibodies as indicated on the right. (**C** to **F**) POLDIP3-WT and POLDIP3-KO U2OS cells were transfected with either siLuc or siFM and then stained with antibodies as indicated on the top. All the nuclei were stained with DAPI. More than 200 cells were counted. All error bars are standard deviation of the mean obtained from three different experiments. Standard two-tailed Student's t-test: ***p<0.001.

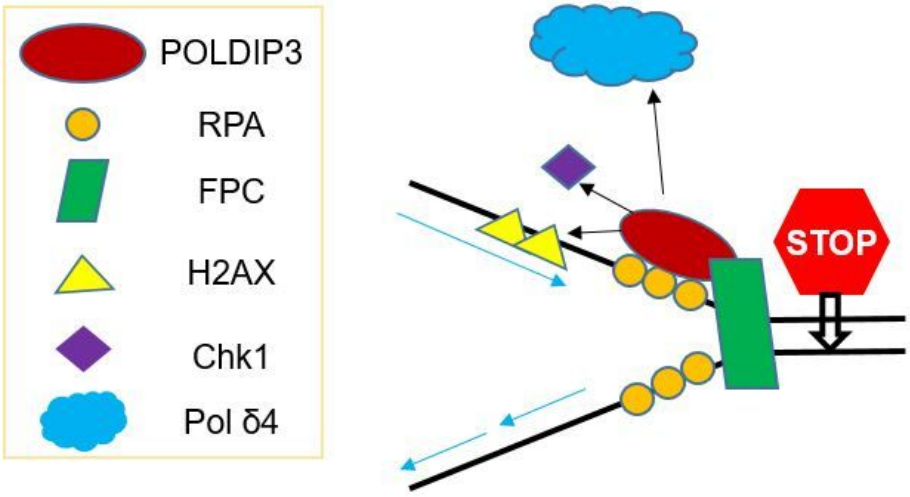


Figure 4

A model of POLDIP3 in replication stress response. In response to replication stress, POLDIP3 functions to bridge the early events (checkpoint activation and maintenance) with the late events (fork repair and restart)

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- FigS1.pptx
- FigS2.pptx
- FigS3.pptx