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Supplementary Information

Chemical proteomics-based identification of artesunate as a POLH-PHB1 interaction disruptor

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Table of Contents

Experimental procedures

Materials and cell culture

All the oligodeoxyribonucleotides (ODNs), enzymes and chemical reagents used in this study, unless otherwise specified, were purchased from Sangon Biotech (Shanghai, China), New England Biolabs and Sigma-Aldrich, respectively (Table S2). The HEK293T human embryonic kidney epithelial cells (ATCC) and CRISPR/Cas9-mediated POLH knockout ($POLH^{-/-}$) cells were cultured at 37℃ with 5% CO2 in Dulbecco's modified Eagle's medium (Thermo Fisher Scientific) supplemented with 10% fetal bovine serum (Zeta Life) and 1% penicillin/streptomycin antibiotics (Invitrogen).[1,2]

Plasmid construction and transfection experiments

For construction of the expression vector of POLH-APEX2 fusion protein, the coding sequence of POLH was amplified by re-verse-transcription PCR and subcloned into the BamHI and NotI sites of the Mito-V5-APEX2 vector (Addgene plasmid # 72480).[3] For construction of PHB1-FLAG fusion expression vector, the coding sequence of PHB1 was PCR amplified and inserted into the BamHI and XbaI sites of the pRK7-3×FLAG vector. The plasmids were transfected into HEK293T cells by lip2000 transfection rea-gent (Thermo Fisher Scientific). For siRNA-mediated knockdown of PHB1, HEK293T cells were transfected with siRNA duplexes against PHB1 (siPHB1) or negative control siRNA (siControl) using lip2000 transfection reagent according to the manufacturer's procedures.

Western blotting

Cellular proteins were extracted using CellLytic M lysis reagent containing 1% protease inhibitor cocktail (Sigma-Aldrich), and quantified by the Bradford assay. Western blotting was performed as previously described [4] using the following antibodies: an-ti-POLH antibody (Sangon, D225422), anti-PHB1 antibody (Sangon, D194798), anti-V5 antibody (Beyotime, AF2894), anti-FLAG anti-body (Beyotime, AF519), anti-WNT antibody (Sangon, D263202), anti-β-CATENIN antibody (Sangon, AF0066), anti-α-TUBULIN antibody (Beyotime, AF5012). The secondary antibodies include HRP-conjugated goat anti-mouse IgG (Proteintech, SA00001-1) and goat anti-rabbit IgG antibodies (Proteintech, SA00001-2). Protein expressions were detected by ECL system (Beyotime) ac-cording to the manufacturer's protocols.

Proximity labeling, dimethyl labeling and LC–MS/MS analysis

The POLH-APEX2 plasmids were transfected into HEK293T cells. After 24 h of transfection, the cells were equally divided into two flasks and incubated for 48 h with either 0.5 mM ART (Aladdin, dissolved in DMSO) or an equivalent volume of DMSO (vehicle control). The cells were then subjected to proximity labeling as previously described with some modifications.[5,6] Briefly, the cells were incubated with medium containing 0.5 mM biotin phenol for 30 min. Subsequently, the cells were treated with 1.0 mM hydrogen peroxide for 1 min. The reaction was halted by rinsing the cells with a buffer composed of 5 mM 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), 10 mM sodium ascorbate and dissolved in DPBS solution.

The cellular proteins were extracted by CelLytic M lysis buffer (Sigma-Aldrich), and equal amounts of ART-treated and DMSO-treated proteins were incubated with Pierce™ High Capacity Streptavidin Agarose (Thermo Scientific) at 4°C for 2 h. The beads were washed 3 times with binding buffer (50 mM HEPES, 150 mM NaCl, 1% NP40, 1 mM DTT) and washed another 3 times with washing buffer (100 mM TEAB, 150 mM NaCl). The samples were then reduced with 5 mM tris (2-carboxyethyl phosphine (TCEP) in 100 mM TEAB solution at 37°C for 1 h, followed by alkylation by 10 mM methyl methanethiosulfonate (MMTS) at room temperature for 10 min. Proteolysis was then carried out using trypsin at 37°C for 18 h. For dimethyl labeling, the tryptic digested peptides were dissolved in 100 µl 100 mM TEAB and incubated with 4 µl 4% heavy or light formaldehyde (CD2O or CH2O) and 4 µl 600 mM cyanoborohydride (NaBH3CN) at room temperature for 1 h, followed by addition of 16 µl 1 % ammonia to stop the reaction. The ART-treated heavy peptides and DMSO-treated light peptides were then mixed as forward sample, whereas the ART-treated light peptides and DMSO-treated heavy peptides were mixed as reverse sample. The samples were then desalted using C18 ZIP-TIP desalting columns (Agilent, 5188-5239), lyophilized in a SpeedVac, and analyzed by liquid chromatography-tandem mass spectrometry (LC-MS/MS) as previously described.[7]

Co-immunoprecipitation (Co-IP) assay

For Co-IP analysis of POLH-PHB1 interaction, the POLH-APEX2 and PHB1-FLAG plasmids were co-transfected into HEK293T cells. Cells were harvested at 48 h after

transfection and lysed by CellLytic M lysate reagent supplemented with 1% protease inhibitor cocktail (Sigma-Aldrich). For Co-IP assay, equal amounts of proteins were separately incubated with pre-washed anti-FLAG affinity gel (Beyotime) at 4°C for 2 h. The bound proteins were then eluted by 3×FLAG peptide solution (Beyotime) and analyzed by western blotting. For Co-IP analysis of the effect of ART treatment on the POLH-PHB1 interaction, cells were co-transfected with POLH-APEX2 and PHB1-FLAG plasmids for 48 h, followed by exposure to either 0.5 mM ART (dissolved in DMSO) or an equal volume of DMSO (vehicle control) for an additional 48 h. Then, cells were lysed and affinity purified with anti-FLAG agarose for western blotting analysis as described above.

Cell viability assay

Cell viability was assessed using the Cell Counting Kit-8 (CCK-8) assay following the manufacturer's instructions. In brief, cells were seeded into 96-well plates for 24 h and ART was then added at a concentration gradient of 0, 0.5, 1.0, 1.5 and 2.0 mM for 48 h. Cell viability was then determined using CCK-8 solution (Solarbio) on a Synergy 2 microplate reader (Biotek).

Cellular thermal shift assay (CETSA)

CETSA experiments were performed as previously described with some modifications.[8,9] Briefly, the cell lysates were incubated with or without 1 mM ART at room temperature for 2 h. The mixture was subsequently divided into equal aliquots and incubated at 37°C, 40°C, 45°C, or 50°C for 3 min, followed by centrifuging at 17,000

g for 20 min. The supernatants were then analyzed by western blot using POLH antibody.

Molecular docking analysis

The initial structure of POLH was extracted from the X-ray structure (PDB ID: 4Q8E). The missing hydrogen atom was added at pH 7.0. ART was reconstructed using Open Babel GUI and optimized using AutoDock Vina software. The docking poses of ART were determined by AutoDock Vina, where an iterative local search blob optimizer was applied to locate the most favorable binding sites. Semi-flexible molecular docking was performed by considering POLH as a rigid body. The search for the best binding site in a 52 × 58 × 52 Å box covered the entire protein. The top 50 poses for ART were picked up using the scoring function of Auto-Dock Vina.

Table S1: List of differential interactors of POLH upon ART treatment. The data were based on LC-MS/MS analysis of forward and reverse dimethyl-labeled samples.

Protein Names	Ratio (ART/Control) Forward	Ratio (ART/Control) Reverse	ART/Control Enrichment Ratio (Mean ± S.D.)
HIST1	0.00	0.02	0.01±0.01
HSP90	0.00	0.02	0.01±0.01
TUBAIB	0.01	0.05	0.03±0.03
EIF4A1	0.01	0.06	0.03±0.04
RPL18	0.01	0.06	0.03±0.04
ACTB	0.02	0.04	0.03±0.02
PPIA	0.03	0.03	0.03±0
EEF1A1	0.01	0.06	0.04±0.03
TUBB	0.02	0.06	0.04±0.03
TUBB4B	0.02	0.07	0.05±0.04
NPM1	0.02	0.07	0.05±0.03
TRAP1	0.04	0.09	0.06±0.04
IMPDH2	0.11	0.04	0.07±0.05
GANAB	0.03	0.13	0.08±0.07
ILF3	0.07	0.09	0.08±0.01
EEF2	0.09	0.07	0.08±0.01
PHB2	0.07	0.11	0.09±0.03
HSP90B1	0.09	0.10	0.09±0.01
SLC25A6	0.15	0.07	0.11±0.06
PHB1	0.02	0.25	0.13±0.17
CHCHD3	0.16	0.11	0.13±0.04
ATP5B	0.09	0.19	0.14±0.07
PKM2	0.18	0.10	0.14±0.06
RPS3	0.20	0.08	0.14±0.09
HSPD1	0.06	0.29	0.17±0.17
DDX21	0.27	0.10	0.18±0.12
HNRNPR	0.12	0.29	0.2±0.12
LARS	0.30	0.10	0.2±0.14
HSPA9	0.17	0.25	0.21±0.06
UBA1	0.34	0.08	0.21±0.19

DHX9	0.18	0.26	0.22±0.05
RPL13	0.39	0.05	0.22±0.24
CAD	0.32	0.14	0.23±0.13
DYNCH1	0.15	0.35	0.25±0.15
FLNA	0.39	0.14	0.27±0.18
TARS1	0.31	0.28	0.3±0.02
PRKDC	0.19	0.42	0.31±0.16
MTHFD1	0.27	0.36	0.32±0.07
NUP155	0.36	0.28	0.32±0.05
SNRNP200	0.40	0.30	0.35±0.07
RARS	0.54	0.23	0.38±0.22
HSPA5	0.13	0.68	0.4±0.38
AARS	0.28	0.63	0.46±0.25
DARS	0.59	0.35	0.47±0.17
PSMD3	0.39	0.62	0.5±0.16
RPN1	0.70	0.33	0.52±0.26

Table S2: Primers sequences used in this study.

Target gene	Sequence (5'-3')
POLH-APEX2-F	CTATAGGGAGACCCAAGCTTATGAAGCTCGTGCATTTGGA
POLH-APEX2-R	GGGTTGGGGATGGGCTTGCCATGTGTTAATGGCTTAAAAA
PHB1-FLAG-F	ATGACAAGCTGCAGGTCGACATGGCTGCCAAAGTGTTTGA
PHB1-FLAG-R	CCATCGATTGAATTCCCCGGTCACTGGGGCAGCTGGAGGA
siPHB1-F	GCGACGACCUUACAGAGCGUUTT
siPHB1-R	AACGCUCUGUAAGGUCGUCGCTT
siControl-F	UUCUCCGAACGUGUCACGUTT
siControl-R	ACGUGACACGUUCGGAGAATT

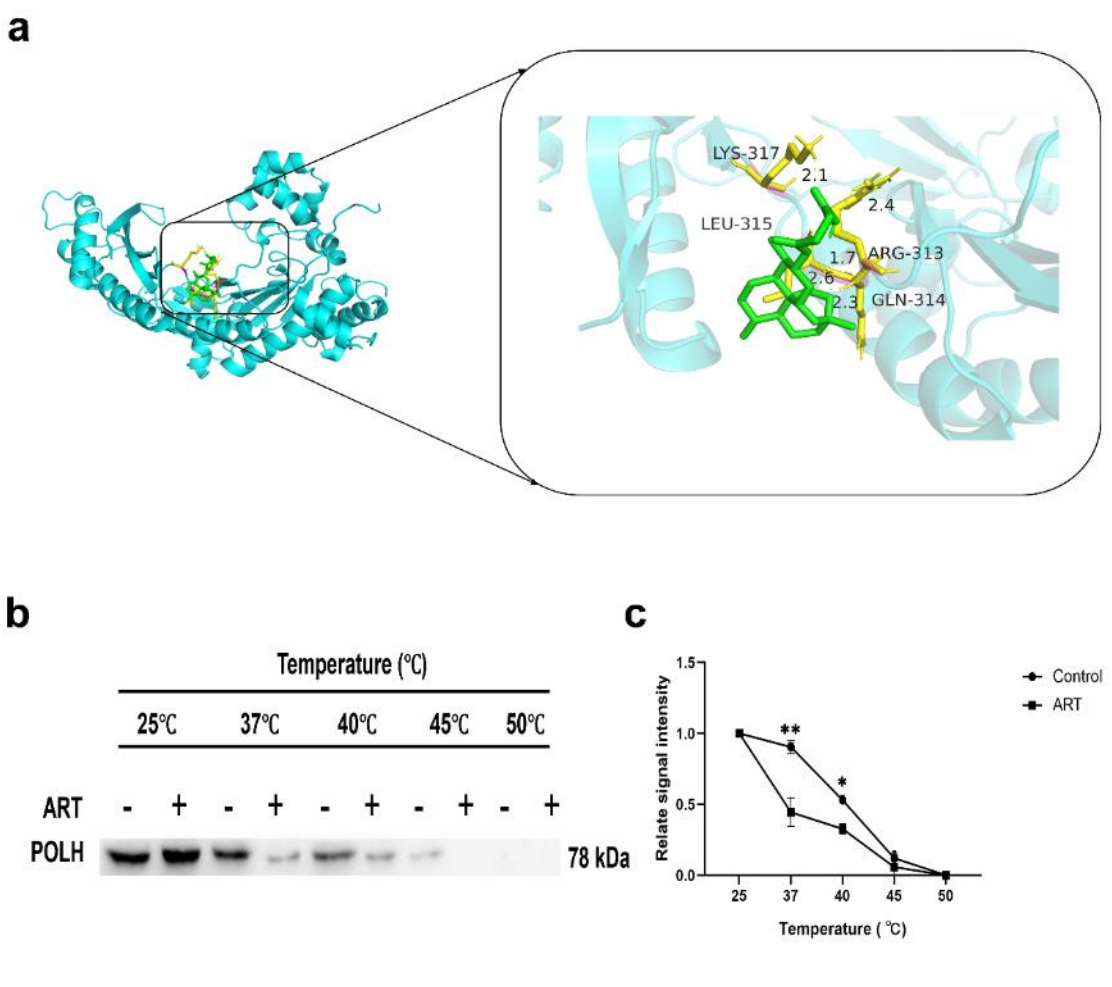

Fig. S1 Identification of an interaction between ART and POLH protein. (**a**) Molecular docking revealing the binding model of ART with POLH (left: full view, right: partial view). (**b**) Western blot-based CETSA assay showing POLH protein levels at different temperatures with or without ART. (**c**) Quantitative data of CETSA assay. The data represent mean ± SD (n=3). *p<0.05, **p<0.01. The p value was calculated using the paired two-tailed Student's t-test.

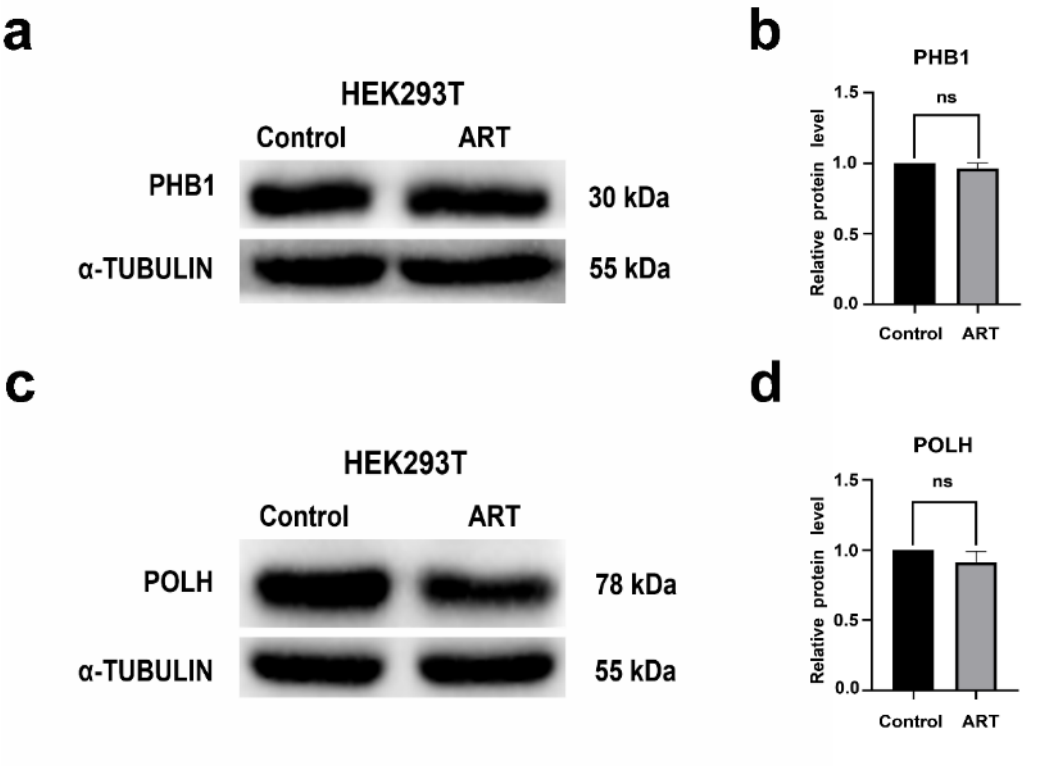

Fig. S2 ART treatment does not affect POLH and PHB1 expression in HEK293T cells. **(a, b)** Western blot **(a)** and quantification **(b)** of PHB1 expression. **(c, d)** Western blot **(c)** and quantification **(d)** of POLH expression. α-TUBULIN served as loading control. The data represent mean ± SD (n=3). ns, $p>0.05$. The p value was calculated using the unpaired two-tailed Student's t-test.

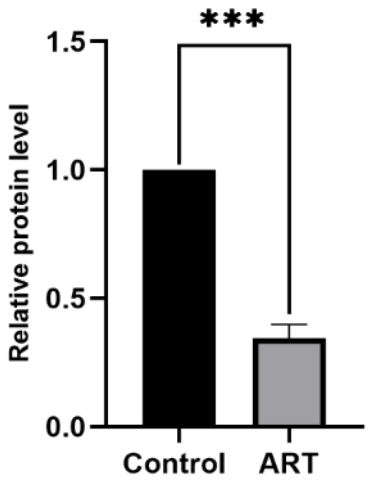

Fig. S3 Quantification of the POLH-PHB1 co-immunoprecipitation level after ART treatment (corresponding to Fig. 3c). α-TUBULIN served as loading control. The data represent mean ± SD (n=3). ***p<0.001. The p value was calculated using the unpaired two-tailed Student's t-test.

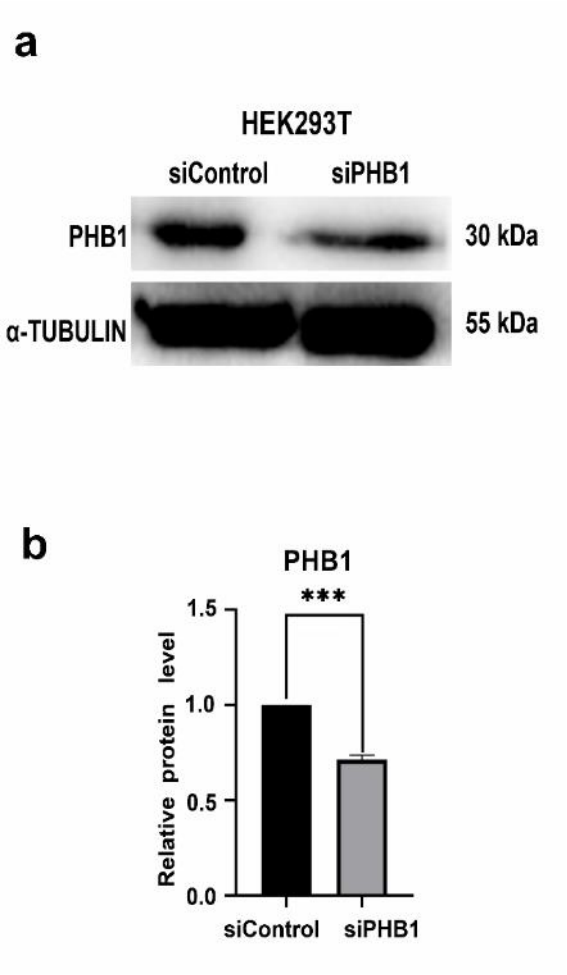

Fig. S4 SiRNA-mediated knockdown of PHB1 in HEK293T cells. **(a, b**) Western blot **(a)** and quantification **(b)** of PHB1 expression. α-TUBULIN served as loading control. The data represent mean ± SD (n=3). ***p<0.001. The p value was calculated using the unpaired two-tailed Student's t-test.

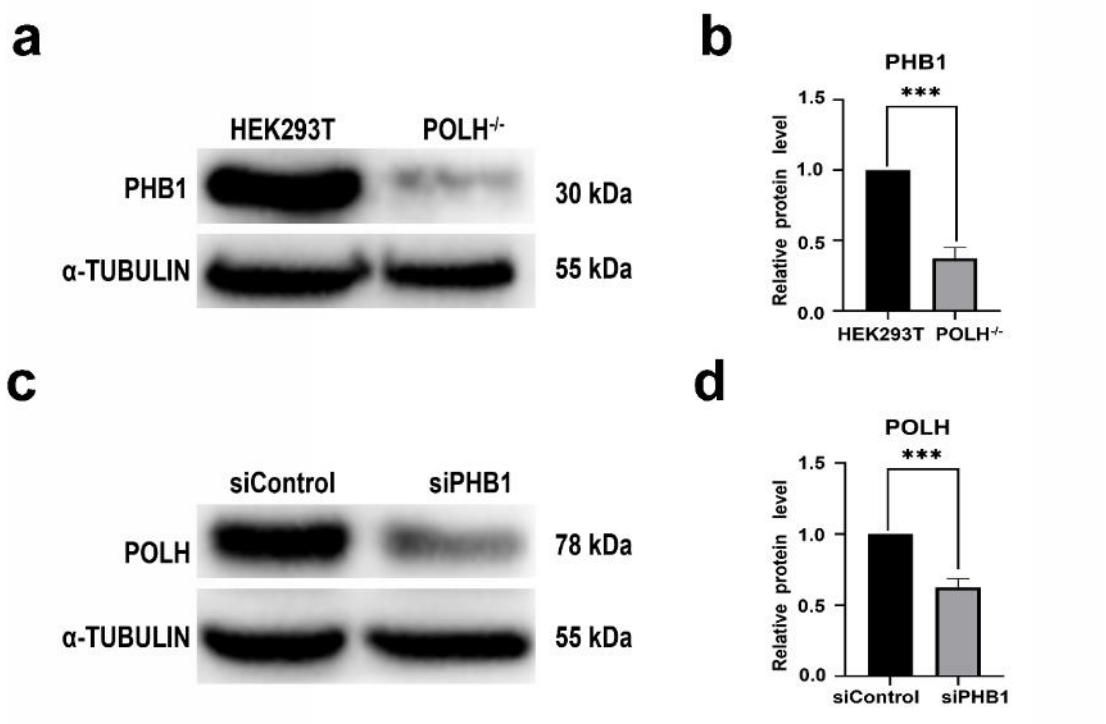

Fig. S5 Depletion of POLH or PHB1 reciprocally reduces the other's protein level. **(a, b**) Western blot **(a)** and quantification **(b)** of PHB1 expression in POLH$^{-/-}$ and control cells. **(c, d**) Western blot **(c)** and quantification **(d)** of POLH expression in siPHB1 and control cells. α-TUBULIN served as loading control. The data represent mean ± SD (n=3). ***p<0.001. The p value was calculated using the unpaired two-tailed Student's t-test.

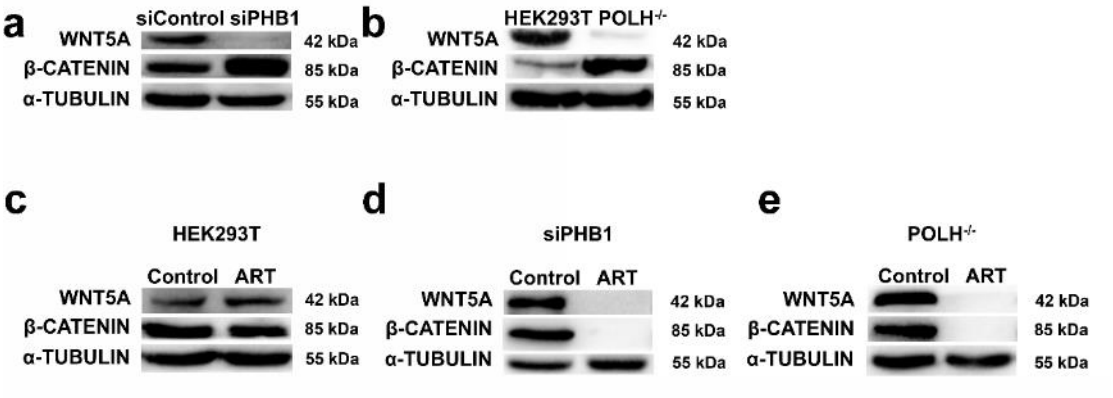

Fig. S6 Western blot analysis of WNT5A and β-CATENIN in POLH or PHB1 deficient cells ± ART. (**a**) WNT5A and β-CATENIN levels in siPHB1 and control cells. (**b**) WNT5A and β-CATENIN levels in POLH$^{-/-}$ and control cells. (**c-e**) WNT5A and β-CATENIN levels in HEK293T (**c**), siPHB1 (**d**), and POLH$^{-/-}$ (**e**) cells with or without ART treatment. α-TUBULIN served as loading control.

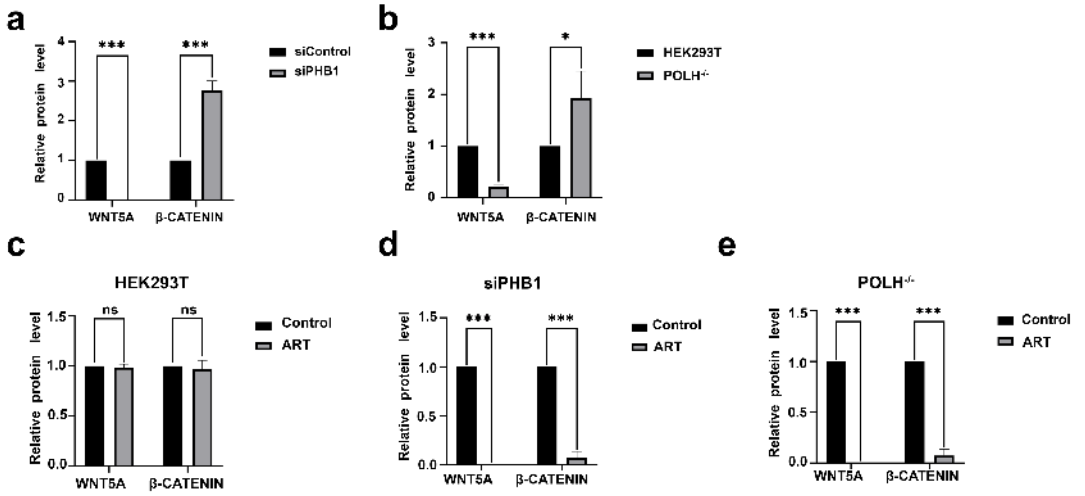

Fig. S7 Quantification of WNT5A and β-CATENIN expression in POLH or PHB1 deficient cells ± ART (corresponding to Fig. S6). (**a**) WNT5A and β-CATENIN levels in siPHB1 and control cells. (**b**) WNT5A and β-CATENIN levels in POLH$^{-/-}$ and control cells. (**c-e**) WNT5A and β-CATENIN levels in HEK293T (**c**), siPHB1 (**d**), and POLH$^{-/-}$ (**e**) cells with or without ART treatment. α-TUBULIN served as loading control. The data represent mean ± SD (n=3). ns, $p>0.05$, *$p<0.05$, ***$p<0.001$. The p value was calculated using the unpaired two-tailed Student's t-test.

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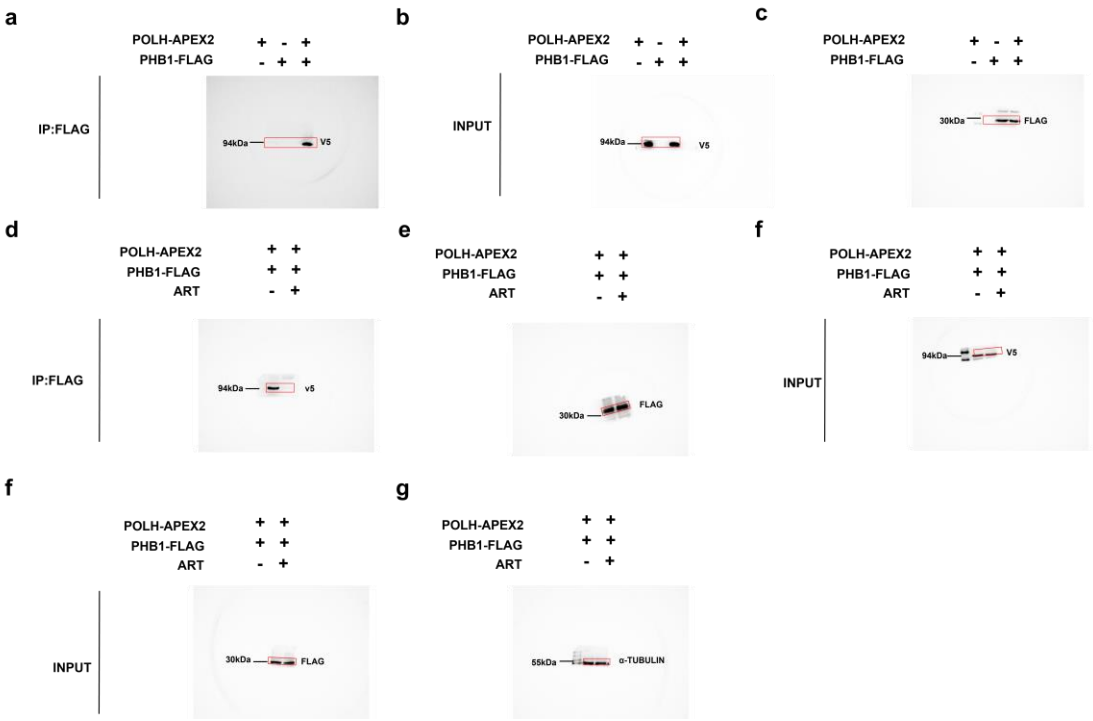

Fig. S8 Co-IP confirmation of the POLH–PHB1 interaction and its inhibition by ART treatment in human cells. **(a-c**) Uncropped WB films of Fig. 3b. (**d-h**) Uncropped WB films of Fig. 3c.

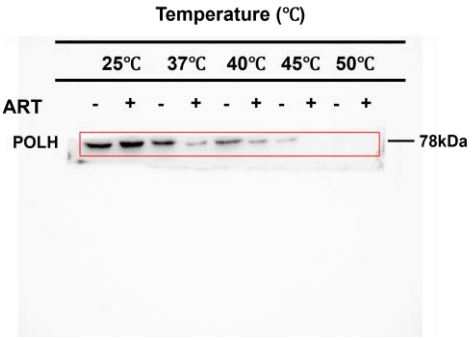

Fig. S9 Identification of an interaction between ART and POLH protein. Uncropped WB films of Fig. S1b.

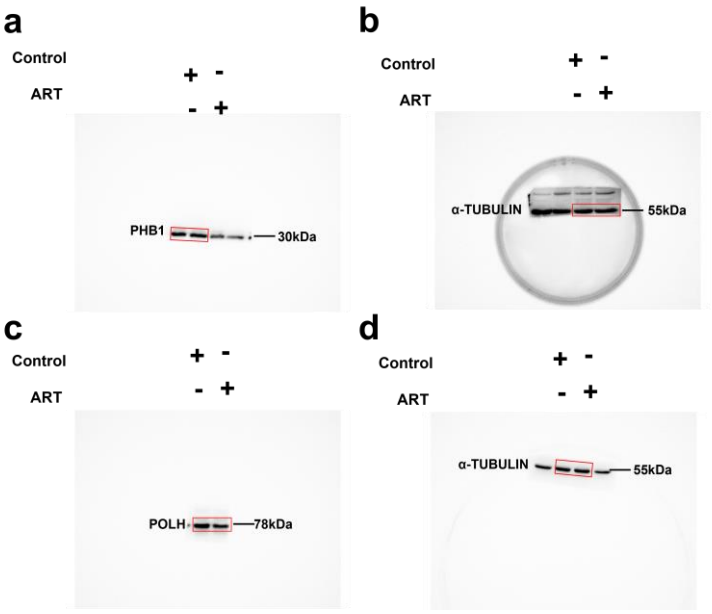

Fig. S10 ART treatment does not affect POLH and PHB1 expression in HEK293T cells.

(**a, b**) Uncropped WB films of Fig. S2a. (**c, d**) Uncropped WB films of Fig. S2c.

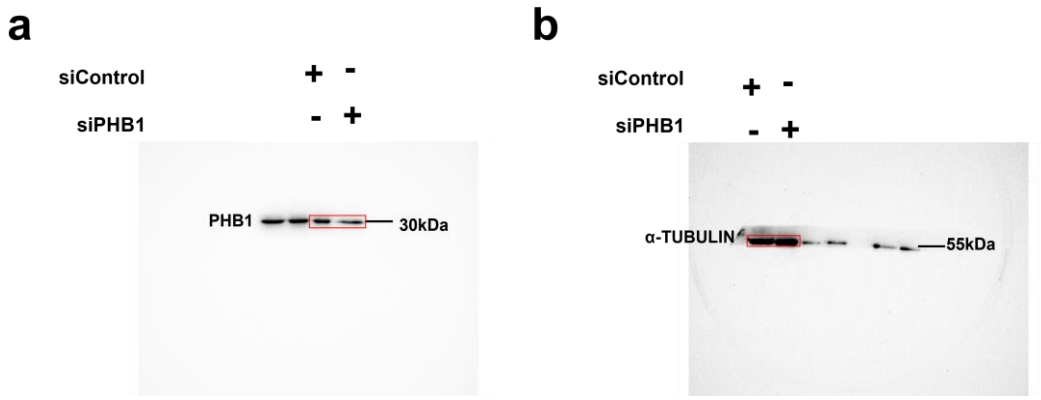

Fig. S11 SiRNA-mediated knockdown of PHB1 in HEK293T cells. (**a, b**) Uncropped WB films of Fig. S3a.

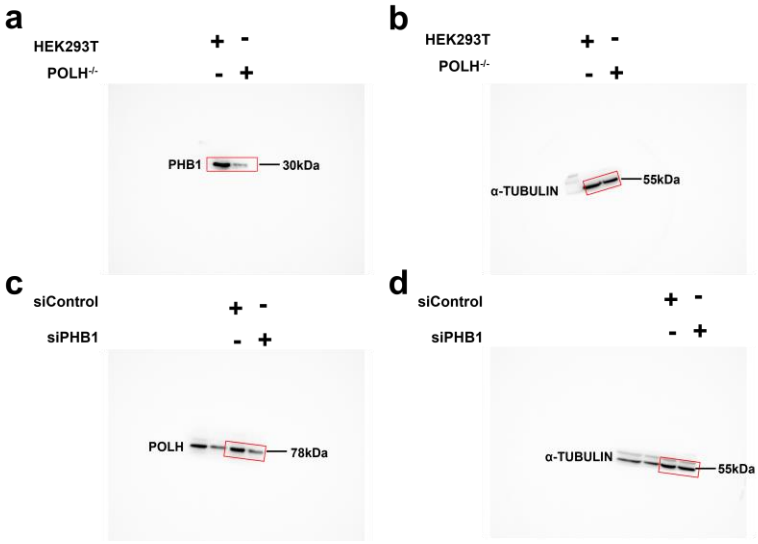

Fig. S12 Depletion of POLH or PHB1 reciprocally reduces the other's protein level. **(a, b**) Uncropped WB films of Fig. S4a. (**c, d**) Uncropped WB films of Fig. S4c.

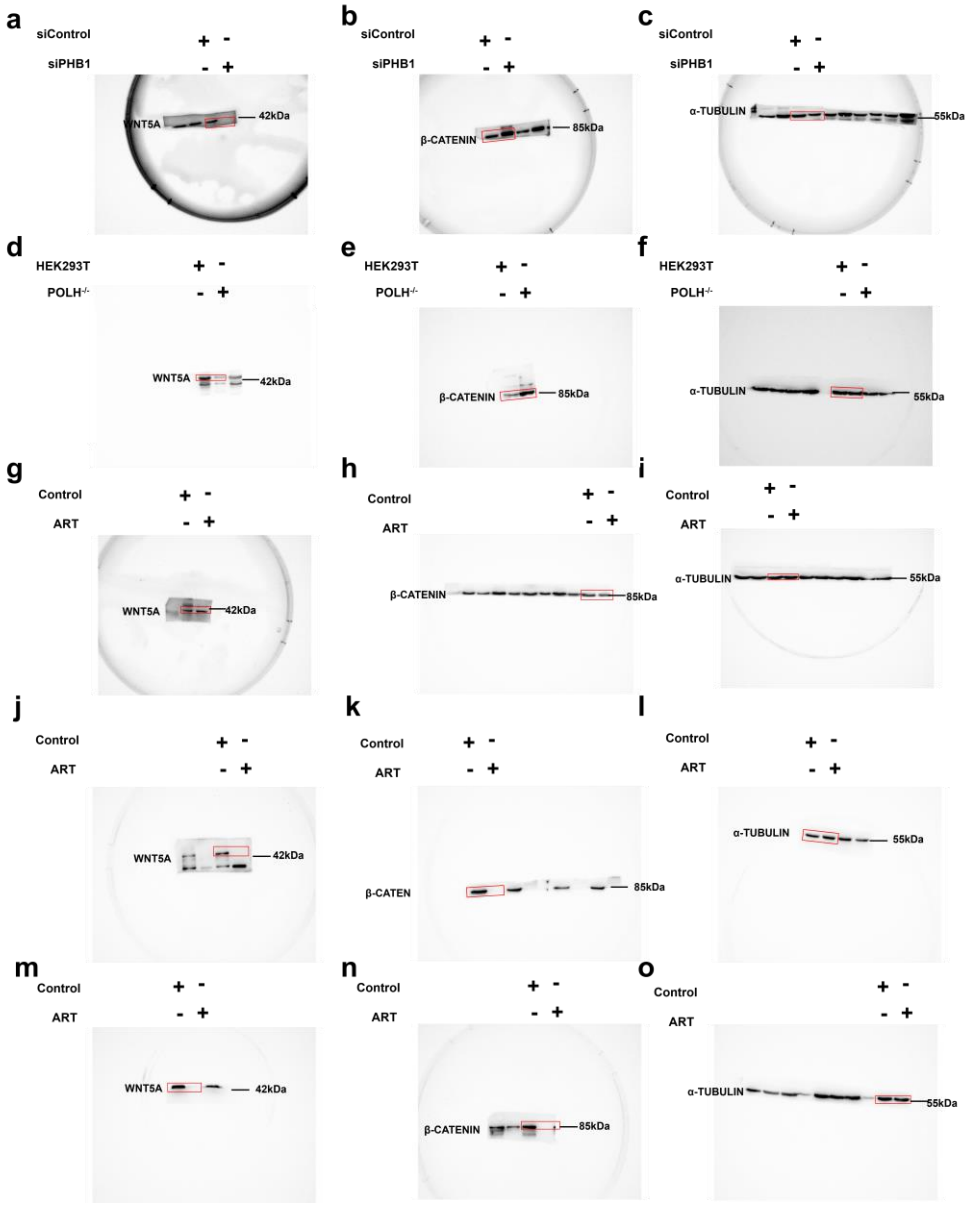

Fig. S13 Western blot analysis of WNT5A and β-CATENIN in POLH or PHB1 deficient cells ± ART. (**a-c**) Uncropped WB films of Fig. S5a (**d-f**) Uncropped WB films of Fig. S5b. (**g-i**) Uncropped WB films of Fig. S5c. (**j-l**) Uncropped WB films of Fig. S5d. (**m-o**) Uncropped WB films of Fig. S5e.

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