BBA - Molecular Cell Research 1871 (2024) 119743

Contents lists available at ScienceDirect

BBA - Molecular Cell Research

journal homepage: www.elsevier.com/locate/bbamcr

Research paper

E3 ubiquitin ligase RNF2 protects polymerase ι from destabilization

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ARTICLE INFO

Keywords:
DNA polymerase iota
RNF2
Y-family DNA polymerase
Post-translational modification (PTM)
Ubiquitylation (ubiquitination)

ABSTRACT

Human DNA polymerase ι (Polι) belongs to the Y-family of specialized DNA polymerases engaged in the DNA damage tolerance pathway of translesion DNA synthesis that is crucial to the maintenance of genome integrity. The extreme infidelity of Polι and the fact that both its up- and down-regulation correlate with various cancers indicate that Polι expression and access to the replication fork should be strictly controlled. Here, we identify RNF2, an E3 ubiquitin ligase, as a new interacting partner of Polι that is responsible for Polι stabilization *in vivo*. Interestingly, while we report that RNF2 does not directly ubiquitinate Polι, inhibition of the E3 ubiquitin ligase activity of RNF2 affects the cellular level of Polι thereby protecting it from destabilization. Additionally, we indicate that this mechanism is more general, as DNA polymerase η, another Y-family polymerase and the closest paralogue of Polι, share similar features.

1. Introduction

The correct functioning and precise regulation of translesion (TLS) polymerases, a special class of polymerases able to replicate DNA on a damaged template, is crucial in genomic integrity maintenance. Although these enzymes play a pivotal role in permitting cell survival after DNA damage, their activity is often mutagenic, particularly when synthesizing an undamaged template [1]. Due to the potential of diverse performance in promoting and preventing genomic instability TLS polymerase activity must be strictly controlled [2]. It is particularly important in view of the fact that reduced genomic integrity is one of the common features of cancer cells. Human DNA polymerases η, ι, κ, and Rev1 belong to the Y-family of polymerases renowned for their TLS activity [3]. While their expression can be regulated at the transcriptional level [4–7], protein-protein interactions and posttranslational modifications also seem to play a key role in the control of their functioning and access to the replication fork [8,9]. Y-family polymerases are subject to ubiquitination, phosphorylation, acetylation, and sumoylation (reviewed in [8]). However, ubiquitination appears to perform the most versatile function, impacting the cellular levels and interacting partners of these polymerases. Ubiquitination is a multistep process involving sequentially the activity of ubiquitin-activating enzyme (E1), one of 50 ubiquitin-conjugating enzymes (E2), and one of several hundred ubiquitin ligases (E3) to conjugate ubiquitin to a specific lysine residue in a targeted protein. Repeated ubiquitination cycles of the monoubiquitinated protein can result in protein multi-ubiquitination or the forming of polyubiquitin chains of various topologies attached to the protein [10]. Different types of ubiquitination elicit distinct outcomes; *e.g.*, monoubiquitination often alters protein interactions and affects complex formation, localization, or activity of the modified protein. The most abundant polyubiquitin chains, in which ubiquitin molecules are linked *via* K48, mark targeted proteins for proteasomal degradation, while K63-conjugated chains have rather signaling functions regulating activity or guiding complex formation. K11-linked chains can also serve as a proteasome degradation signal, often for the cell cycle regulating proteins [11]. K6-, K27-, K29-, and K33-conjugated chains are less common.

DNA polymerase ι (Polι) is the most error-prone among all known human DNA polymerases, misincorporating bases on an undamaged template with a very high rate [12]. It was also shown to promote migration and invasion of breast cancer cells [13]. On the other hand, the results of *in vitro* experiments show that Polι can bypass several types of DNA lesions, reduces DNA replication stress caused by a defect in Fanconi anemia [14], and prevents chromosome instability by promoting optimal checkpoint activation [15]. Moreover, Polι's deficiency can sensitize cells to oxidative agents [16] and the lack of Polι stimulates mesenchymal tumorigenesis in mice [17]. The duality of the influence of

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https://doi.org/10.1016/j.bbamcr.2024.119743
Received 15 November 2023; Received in revised form 22 April 2024; Accepted 25 April 2024
Available online 3 May 2024

Abbreviations

TLS	translesion synthesis
Pol	DNA polymerase
PCNA	proliferating cell nuclear antigen
PIP	PCNA Interacting Peptide
RIR	Rev1 Interacting Region
UBM	Ubiquitin Binding Motifs

Polι on genomic stability implies that the enzyme should be kept at a low cellular level with access to the replication fork strictly limited to the time of need. Indeed, in most tissues, the enzyme is expressed at low or medium levels [12]. However, little is known about the regulation of Polι's cellular abundance. It has been shown that in cancer cells Polι is induced at the transcription level under hypoxia condition [18]. There is much more information about the regulation of Polι at the post-translational level by protein modifications and protein-protein interactions. Polι, like other Y-family polymerases, is recruited to sites of DNA damage by interacting with the essential replication processivity factor - proliferating cell nuclear antigen (PCNA) and the scaffold protein, Rev1 [19–21]. Polι interacts with its partners involved in TLS, such as PCNA, Rev1, and ubiquitin or ubiquitinated PCNA, through specific domains; PIP (PCNA Interacting Peptide), RIR (Rev1 Interacting Region), and two UBMs (Ubiquitin Binding Motifs) domains, respectively, localized in the C-terminal half of the protein [12]. Additionally, Polι, similar to other Y-family polymerases, is monoubiquitinated which facilitates its interaction with its closest paralog Polη [22]. Other roles of monoubiquitinated Polι are yet unknown but possible [23]. In contrast to Polη, which only has four amino acids that are ubiquitinated in its C-terminus [24], Polι has multiple possible ubiquitination sites located in different functional domains [8]. In response to p300/CBP acetyltransferase inhibition Polι can undergo polyubiquitination and polyubiquitin chains of Polι are formed *via* K11 and K48 links that are known to target proteins for proteasomal degradation [23]. So far, the enzymes performing Polι ubiquitination have not been identified. It has also been shown that Polι can be acetylated by p300/CBP acetyltransferase and the main acetylation site is K550 located in the RIR motif, and this acetylation is induced in response to alkylating and oxidating agents [25].

RNF2, also known as Ring1B or Ring2, is a RING type E3 ubiquitin ligase essential for the function of PRC1, one of the two distinct polycomb repressive complexes involved in epigenetic gene silencing. RNF2 monoubiquitinates histone H2A at K119 what consequently might lead to suppressing gene expression at the transcription level through induction of chromatin compaction [26]. RNF2 forms a heterodimer with Bmi1 that, despite containing a RING domain, does not possess E3 ubiquitin ligase activity by itself. However, it significantly stimulates RNF2 activity [27]. Being a part of a general transcription regulator, RNF2 impacts multiple processes during embryonic development and cell proliferation, as well as in adult life to maintain cell fate and prevent oncogenic cell transformations [28]. In addition to its role as an epigenetic transcriptional regulator, RNF2, through ubiquitination and/or interaction with various proteins such as p53, AMBRA1, SBDS, RRM1 and S6' ATPase, affects many other cellular processes [29–32]. Interestingly, it has been shown that RNF2 is highly expressed in many tumors and lack of RNF2 inhibits proliferation and induces apoptosis in cancer cells [33].

Here, we identify Polι as an interacting partner of RNF2. We show that Polι is not a direct substrate of RNF2 E3 ubiquitin ligase activity. Nonetheless, the activity, but not the ability of RNF2 to interact with Polι, is important for the maintenance of Polι cellular levels and prevents its destabilization. Furthermore, we report that the proteasome indirectly affects Polι abundance and its inhibition reduces the amount of Polι in the cell. Additionally, we present evidence that Polη, the closest paralogue of Polι, also interacts with RNF2, and similarly, its cellular level is affected by RNF2.

2. Materials and methods

2.1. Reagents

PRT4165 was purchased from LifeSensors and was dissolved in DMSO at a concentration of 50 mM, aliquoted, and kept at −80°C. Other reagents were purchased from the following vendors: anti DYKDDDDK Affinity Gel (Bimake), protein A/G PLUS-Agarose (Santa Cruz), DMSO (BioShop), Turbofectin 8.0 (OriGene), normalized universal human Mate & Plate™ Library (Clontech), protease inhibitor cocktail (Sigma-Aldrich), BCA Reagent (Thermo Fisher Scientific).

2.2. Cell cultures, plasmid transfection and protein expression

HEK293T and HCT116 cells were purchased from ATCC and cultured in DMEM containing 10% FBS at 37°C and 5% CO_2. All cell lines used for experiments were tested negative for mycoplasma contamination. When indicated, plasmids were transfected into cells using Turbofectin 8.0 according to the manufacturer's instructions (OriGene). Twenty-four to forty-eight hours post transfection cells were harvested and lysed as described previously [23]. Briefly, cells were scraped from plates, washed twice with cold Dulbecco's PBS without calcium or magnesium and suspended in RIPA buffer (25mM Tris-HCl, pH 7.6, 150mM NaCl, 1% NP-40, 1 mM EDTA, 1 mM AEBSF, 1 mM sodium orthovanadate, and protease inhibitor cocktail, followed by 20 s sonication at 50% amplitude 1 s ON, 1 s OFF. After sonication lysates were centrifuged for 10 min at maximum speed at +4 °C and supernatants were transferred to fresh tubes. The protein concentration of the cell extract was measured using the BCA assay. The presence of expressed proteins of interest was verified by western blot.

2.3. Plasmids

Plasmid pJRM92 was obtained in a BP recombination reaction with pDONOR221 and attB PCR fragment of full length RNF2 using the Gateway cloning system (Invitrogen). pJRM92 was used to generate yeast two-hybrid plasmid pJRM95 expressing full-length RNF2 in an LR Gateway cloning reaction with pGADCg. pGADCg was a gift from Peter Uetz (Addgene plasmid # 20161 ; http://n2t.net/addgene:20161 ; RRID:Addgene_20161) [55]. Plasmids pMF3 and pMF5, expressing N- or C-terminal fragments of RNF2 protein were generated by sub-cloning the respective PCR fragments into pJRM95.

Plasmid pJRM70, expressing GST-RNF2 recombinant protein was generated by sub-cloning the synthesized RNF2 gene (Genscript) into the pGEX-4T-1 vector. pT3-EF1a-Bmi1 was a gift from Xin Chen (Addgene plasmid # 31783; http://n2t.net/addgene:31783; RRID: Addgene_31783) [34]. BMI1 C-terminally tagged with HA epitope was subcloned into pGEX-4T-1 to create pMF4 expressing BMI1 N-terminally tagged with GST and C-terminally tagged HA.

pMF10 expressing N-terminally HA-tagged full-length wild-type RNF2 protein was generated by subcloning respective PCR fragments into the pCMV6AN-HA vector (OriGene). pJRM147 expressing full-length wild-type ubiquitin protein was generated by subcloning the respective PCR fragments into the pCMV6AN-HA vector (OriGene). pMF9 expressing 429–715 aa fragment of Polι tagged at the N-terminus with FLAG epitope was generated by subcloning respective PCR fragment into the pCMV6AN-DDK vector (OriGene). The full list of all the plasmids used in this study is shown in Table 1.

2.4. Antibodies

The following commercial primary antibodies were used to visualize

Table 1
Plasmids used in this study.

Plasmid	Description	Source/Ref.
pCMV6AN-DDK	pCMV6AN-DDK	OriGene
pJRM46	pCMV6AN-DDK-Polι 1-715aa	[22]
pJRM258	pCMV6AN-DDK-Polι 1-419aa	[25]
pJRM259	pCMV6AN-DDK-Polι 1-470aa	[25]
pJRM260	pCMV6AN-DDK-Polι 1-537aa	[25]
pJRM261	pCMV6AN-DDK-Polι 1-676aa	[25]
pJRM262	pCMV6AN-DDK-Polι 409-715aa	[25]
pMF9	pCMV6AN-DDK-Polι 429-715aa	This work
pJRM160	pCMV6AN-DDK-Polη	[25]
pCMV6AN-HA	pCMV6AN-HA	OriGene
pMF10	pCMV6AN-HA-RNF2 wt	This work
pJRM147	pCMV6AN-HA-Ub	This work
pGADCg	Yeast two hybrid vector with Gal4 activating domain	[55]
pJRM95	pGADCg-RNF2	This work
pMF3	pGADCg-RNF2 1-128aa	This work
pMF5	pGADCg-RNF2 109-336aa	This work
pGBKT7	Yeast two hybrid vector with DNA binding domain	Clontech
pAR110	pGBKT7 Polι wt	Woodgate lab
pAR122	pGBKT7 Polι 1-167aa	Woodgate lab
pAR124	pGBKT7 Polι 1-278aa	Woodgate lab
pAR126	pGBKT7 Polι 1-492aa	Woodgate lab
pAR128	pGBKT7 Polι 448-715aa	Woodgate lab
pAR130	pGBKT7 Polι 492-715aa	Woodgate lab
pAVR65	pGBKT7 Polη	[35]
pJM868	His-Ec-CO-polι_wt	[36]
pJRM70	pGEX-4T-1-RNF2	This work
pMF4	pGEX-4T-1-BMI1-HA	This work
pJRM92	pDONOR221-RNF2	This work
pAV27	pET22-Ub	Woodgate lab
pT3-EE1a-Bmi1		[34]

the recombinant proteins: anti-His (Genscript, cat. no. A00186), anti-GST (Santa Cruz, cat. no. sc-459), anti-HA (Abcam, 9110), anti-FLAG (Abnova, cat. no. MAB2094), and native proteins anti-RNF2 (MBL, cat no D139–3 and Thermo Fisher Scientific 703252), anti-tubulin (Abcam, cat. no. ab6160), anti-GAPDH (Thermo Fisher Scientific, cat. no. PA1–987), anti-β-Actin (Cell Signaling, cat. no. 5057), anti LC3B (Cell Signaling, cat. no. 2775), anti-p53 (Santa Cruz, cat. no. sc-98), anti-Polι (Invitrogen, cat. no. PA5102527). Polyclonal rabbit antibodies raised to the N-terminus, or C-terminus, of Polι were made as a custom service (Covance) and have been previously described [37]. For detection we used HRP-conjugated anti-rabbit and anti-mouse secondary antibodies (Thermo Fisher Scientific, cat. no. 31458 and Daco, cat. no. P0447, respectively), as well as fluorescent secondary antibodies anti-rat Alexa 488 (Abcam, cat. no. ab150157), anti-rabbit Alexa488 (Invitrogen, cat. no. A11034) and anti-mouse Alexa 546 (Invitrogen, cat. no. A11030).

2.5. Yeast two-hybrid analysis

The interaction between human Polι and RNF2 proteins was analyzed *in vivo* using the *Saccharomyces cerevisiae* two-hybrid Matchmaker III system (Clontech). The AH109 strain (*MAT a, trp 1-901, leu2-3, 112, ura3-52, his3-200, Δgal4, Δgal80, LYS2: GAL1$_{UAS}$-GAL1$_{TATA}$-HIS3, GAL2$_{UAS}$-GAL2$_{TATA}$-ADE2, URA3: MEL1$_{UAS}$-MEL1$_{TATA}$-lacZ*) was co-transformed with GAL4 DNA binding domain fusion constructs (pGBKT7-derivative plasmids) and GAL4 activation domain fusion constructs (pGADCg derivative plasmids), as indicated in the figures. Co-transformants were selected on selective media lacking tryptophan and leucine. Colonies were subsequently replica-plated on media lacking tryptophan, leucine, adenine and histidine to verify the activation of the reporter genes.

The screen for Polι-interacting proteins was performed using normalized universal human Mate & Plate™ Library (Clontech).

2.6. Human RNF2 knock-out by CRISPR/Cas9

An RNF2 knock-out in the HCT116 cell line was generated using the RNF2CRISPR/Cas9 KO and RNF2HDR plasmids purchased from Santa Cruz (sc-404633-KO-2 and sc-404633-HDR, respectively). The knock-out procedure was performed according to the manufacturer's recommendations. Briefly, HCT116 cells were co-transfected with RNF2CRISPR/Cas9 KO and RNF2HDR. 48h post transfection the medium was removed and replaced with one containing puromycin and cells were selected on the medium with the antibiotic for another 5 days with fresh medium replacement every 2 days. Next, single cell colonies were isolated and complete knock-outs were confirmed by western blot with antibodies against RNF2.

2.7. Protein purification

Full-length recombinant Polι protein N-terminally tagged with a His-epitope was purified as described previously [36]. Briefly, plasmid pJM868 was expressed in *E. coli* strain RW644 [38]. The His-Polι protein was purified on HisPur Ni-NTA Superflow Agarose (Thermo Fisher Scientific) as recommended by the manufacturer. The eluate containing Polι was dialyzed in buffer containing 20 mM sodium phosphate pH 7.3, 10 mM sodium chloride, 10 % glycerol, 10 mM 2-mercaptoethanol, and applied to HP Q Sepharose (GE Healthcare). Polι was eluted in a step gradient of sodium chloride. A similar strategy was used to purify N-terminally His-tagged ubiquitin and p53.

GST tagged full-length RNF2 was purified along with GST tagged Bmi1 protein because Bmi1 has been reported to elevate E3-ligase activity of RNF2 over 100-fold [39]. Moreover, published data has shown that purification of the RNF2/Bmi1 complex is more efficient than purification of the individual proteins [40]. GST-RNF2/GST-Bmi1 complex was overexpressed in the *E.coli* BL21 strain transformed with pJRM70 vector (carrying GST-RNF2), pMF4 vector (carrying GST-BMI1) and pRARE plasmid. The complex was purified by affinity chromatography

on a glutathione agarose resin column (Agarose Bead Technologies) followed by dialysis in buffer containing 20mM Na-phosphate buffer (pH 7.3), 10% glycerol and 10 mM NaCl.

2.8. Immunoprecipitation and western blot

For immunoprecipitation of RNF2 and Polι or Polη, respective cell extracts including FLAG-tagged proteins were incubated for one hour to overnight at 4°C with protein A/G PLUS-Agarose (Santa Cruz) coated with anti RNF2 antibodies or normal IgG (Cell Signaling, cat. no 2729) as a negative control or with anti-FLAG agarose for FLAG-tagged proteins immunoprecipitation. Post incubation resins were washed five times and proteins were separated by SDS-PAGE. Proteins were transferred to a PVDF membrane and analyzed by western blotting using appropriate antibodies and detected by chemiluminescence or fluorescence.

2.9. Proximity ligation assay

HCT116 fibroblasts were fixed with methanol for 10 min at −20°C. In the next step, cells were washed in PBS and incubated with antibodies against Polι and RNF2 (Invitrogen, PA5102527 and MBL, D139–3, respectively), diluted 1:300 with PBS plus 1% donkey serum and 0.2% TritonX-100 (overnight 4°C). Then, after primary antibodies were washed with PBS, the proximity ligation assay (PLA; Sigma DUO92004-100RXN) was performed. The procedure was carried out according to the manufacturer's instructions. Slides were incubated with PLA secondary antibodies diluted 1:5 in the appropriate solution provided in the PLA kit (60 min 37°C). After washing with buffer A, the ligation step was performed (30 min 37°C). It was then followed by washing with buffer A and an amplification solution (100 min 37°C) was applied. Final washing was performed with buffer B also provided by the manufacturer. Sections were then mounted with VECTASHIELD Mounting Medium with DAPI to stain nuclei. Samples were visualized with a Zeiss LSM800 Exciter microscope. The signal was excited with 405, 488, and 568 nm wavelengths. Observations were performed under 40×/1.3 magnification, and 0.4 μm thick scans were taken for analysis.

2.10. In vitro ubiquitination reaction

GST-RNF2, GST-Bmi1, His-ubiquitin and His-Polι were purified as described above. E1 and E2s were purchased from LifeSensors and Boston Biochem, respectively. *In vitro* ubiquitination reactions were performed at 37°C, as described previously [41]. Briefly, a typical reaction containing buffer (40 mM Tris-HCl pH 7.6, 5mM $MgCl_2$, 2mM ATP and 0.1 mM DTT), 5nM E1, E2s (mix of UbcH5a, UbcH5b, UbcH5c and UbcH1, 100nM of each), GST-RNF2/Bmi1, His-ubiquitin and His-Polι or His-p53 purified from bacteria, was terminated after one hour with 5mM EDTA and separated by SDS-PAGE, followed by western-blot with anti-Polι or anti-p53 and anti-RNF2.

2.11. Total RNA extraction and real-time quantitative PCR (RT-qPCR) analysis

Total RNA was extracted from cell cultures by Blood/Cell RNA Mini Kit (Syngen) according to the manufacturer's instructions. RNA samples were stored at −80°C until further analysis. Removal of contaminating genomic DNA and reverse transcription (RT) using 1 μg of total RNA as a template were performed using a Maxima First-Strand cDNA Synthesis RT-qPCR kit with DNase (Thermo Fisher Scientific) according to the manufacturer's recommendations. Control PCR was performed without prior RT to ensure that the RNA samples were not contaminated with genomic DNA. The reactions were performed with RT HS-PCR mix SYBR® A (A&A Biotechnology) and a LightCycler® 96 system (Roche) according to the manufacturer's protocol. The following primers were used for RT-qPCR (for ACTB [to normalize the data] and POLI:ACTB forward 5'-GTGGACATCCGCAAAGAC3'- and ACTB reverse 5'-AAAGGGTGTAACGCAACTAA3'-; POLI forward 5'-ATGGTCGTGAGAGTCGTCAG3'- and POLI reverse 5'-CTTGCCAGAGCGTGAAGTAG3-. The amplification curves were analyzed using Roche LightCycler ® 96 software (version 1.1). The crossing point (Cp) values were determined by the second derivative method and melting curve analysis were performed. Relative Expression Software Tool (REST 2009) was used for analysis of the fold change of gene expression levels [42]. RT-qPCR was performed with at least three biological and three technical replicates.

2.12. Statistical analysis

Statistical analysis was performed using GraphPad Prism 10 software. Differences between two groups were identified using *t*-test analysis.

3. Results

3.1. Identification of RNF2 as Polι-binding protein

To identify proteins involved in the direct regulation of Polι, we utilized a yeast-two hybrid screen and searched a normalized universal human Mate & Plate™ Library (Clontech) for potential interacting partners of Polι. Interestingly, among the positive clones we identified, three were independent clones encoding N-terminal regions of the ubiquitin ligase, RNF2. Initially, we were unable to confirm this interaction in the yeast two-hybrid system, using Polι and full length RNF2, due to the possible disturbance of the interaction by the Gal4 activation, or DNA binding domain, attached to the N-terminus of the recombinant RNF2 protein. However, by attaching the Gal4 activation domain to the C-terminus of RNF2, we have demonstrated the interaction between Polι and full length RNF2 in the yeast two-hybrid system (Fig. 1A).

To further confirm the interaction between Polι and RNF2, we performed a GST pull-down assay using recombinant proteins, GST-RNF2 and His-Polι expressed in *E. coli*. GST-RNF2, but not GST itself was able to pull-down His-Polι, demonstrating that the two proteins bind to each other (Fig. 1B). Additionally, we confirmed this interaction by immunoprecipitation of native RNF2 and N-terminally FLAG-tagged Polι expressed in HEK293T cells (Fig. 1C). We have observed that not only does RNF2 interact with Polι itself, but also with its mono-ubiquitinated form. Moreover, we have demonstrated, by performing a proximity ligation assay (PLA), that the endogenous Polι and RNF2 proteins are in close proximity in the cell. This result strengthens the evidence presented in previous experiments showing the interaction between Polι and RNF2 (Fig. 1D). Negative controls for the PLA assay are presented in Fig. S1.

RNF2, besides the RING ubiquitin ligase domain which is located in the N-terminus portion of the protein, and is responsible for substrate ubiquitination, also possesses the ubiquitin-like domain (RAWUL) in the C-terminus half of the protein (Fig. 2A). Since Polι has two ubiquitin binding domains (UBM1 and UBM2) that could possibly bind ubiquitin-like domains, we used the yeast two-hybrid system to examine which part of RNF2 protein binds Polι. As shown in Fig. 2A, only cells expressing constructs with the N-terminal half of the protein, containing the RING domain, grow on selective medium, suggesting that the ubiquitin-like RAWUL domain is not required for the RNF2 and Polι interaction.

Having identified a region in RNF2 that appears necessary for the interaction with Polι, we were interested in pinpointing the reciprocal region in Polι that interacts with RNF2. Again, we used the yeast two-hybrid system and a series of N- and C-terminus deletion constructs of Polι. As shown in Fig. 2B, RNF2 interact with both N- and C-terminally truncated Polι proteins suggesting interactions *via* more than one region. The shortest identified fragment sufficient to interact with RNF2 contained the first 167 residues expressing part of the catalytic domain. Curiously, cells expressing a longer Polι construct (1–492 amino acids),

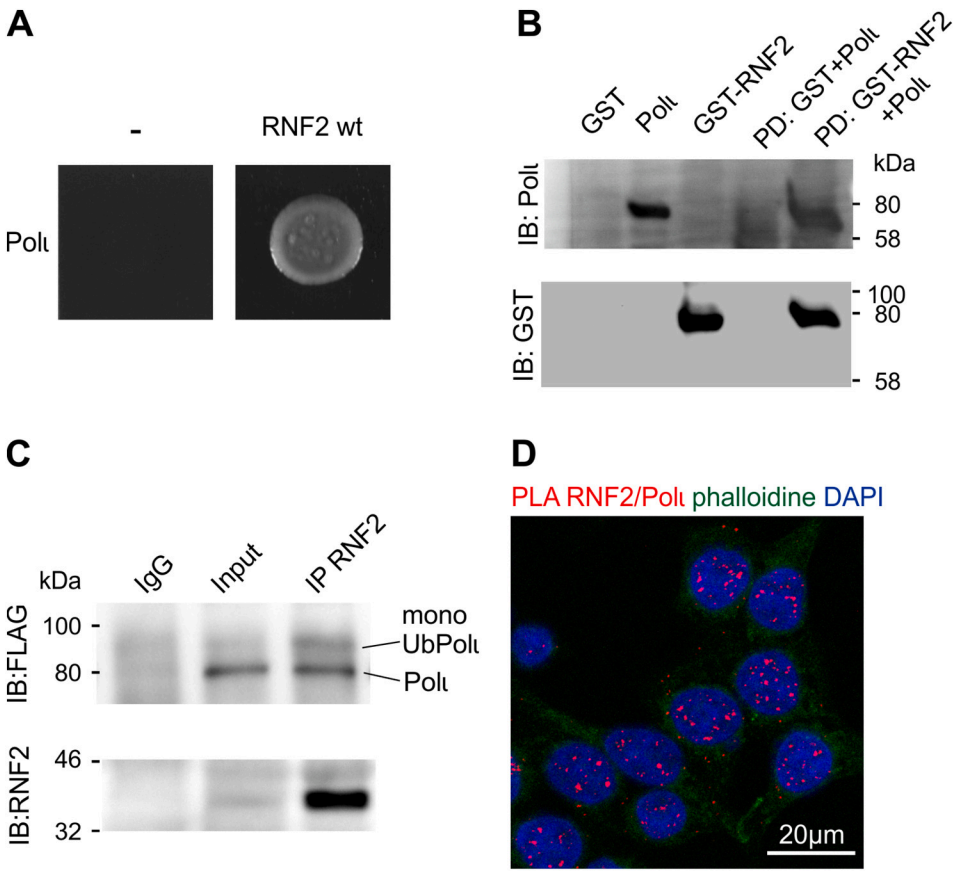

Fig. 1. Polι interacts with E3 ubiquitin ligase, RNF2. (A) Yeast two hybrid system assay showing the interaction between full-length Polι and full-length RNF2. *Saccharomyces cerevisiae* strain AH109 was co-transformed with pGADCg-RNF2 (pJRM95) and pGBKT7-Polι (pAR110). Images were taken after 6 days of incubation at 30°C. (B) Pull-down assay showing the interaction between GST-tagged RNF2 and His-tagged Polι. His-Polι was incubated with GST- or GST-RNF2. GST was captured on glutathione sepharose beads, washed and separated by SDS-PAGE parallel to 5% of protein input, followed by immunoblotting with polyclonal antibodies raised to the C-terminus of Polι [37] and anti-GST antibodies (Covance and Santa Cruz, respectively). (C) Immunoprecipitation (IP) with anti-RNF2 antibodies (MBL) of RNF2 from HEK293T cells expressing FLAG-tagged Polι. Input was 10% of cell extract used for immunoprecipitation. Control reaction was performed with normal IgG (Cell Signaling) antibodies. (D) Proximity ligation assay (PLA), signal (red) provides evidence that endogenous Polι and RNF2 are close together in the cell, actin cytoskeleton is shown in green (Alexa488 Phalloidin staining), nucleus in blue (Hoechst staining). Each experiment was repeated at least three times.

comprising the full-length catalytic domain followed by the PIP box, failed to grow on selective media in the two-hybrid assay. Still, the remaining C-terminal part of Polι including the UBM1, RIR and UBM2 motifs exhibited a strong interaction with RNF2.

To confirm the observations obtained in the yeast two-hybrid system, we performed an RNF2 immunoprecipitation assay in HEK293T cells transiently expressing a variety of truncated versions of N-terminally FLAG-tagged Polι (Fig. 2C). The immunoprecipitation results showed that RNF2 interacted with the Polι constructs containing N- and C-terminally located domains. It suggests that, on one hand, the catalytic domain is sufficient for interaction with RNF2. On the other hand, the C-terminal half, including the UBM1, RIR and UBM2 motifs, but not the PIP box, also seems to interact with RNF2, while the fragment of a protein with the N-terminally located PIP box seemed to lose the interacting ability. Additionally, it is worth noting that the construct expressing the first 470 amino acids encompassing the catalytic domain with the PIP box exhibited a relatively weaker interaction than the catalytic domain itself, or the catalytic domain with the PIP box and UBM1. Together, those results confirm that RNF2 can interact with more than one region of Polι, one located in the catalytic domain, and one in its C-terminal half.

3.2. *RNF2 does not ubiquitinate Polι*

Our result showing that Polι interacts with the N-terminal part of RNF2 protein including the RING ubiquitin ligase domain prompted us to test whether Polι is a substrate of the RNF2 E3 ubiquitin ligase. In the *in vitro* ubiquitination reaction we have demonstrated that, under the tested conditions, RNF2 had no ability to ubiquitinate Polι (Fig. 3A, top panel), even in the presence of Bmi1, which significantly stimulates RNF2 catalytic activity [27]. Nonetheless, in parallel experiments, RNF2 was able to catalyze auto-ubiquitination and ubiquitination of p53 proving that the purified enzyme was active *in vitro* (Fig. 3A, bottom panel). To determine whether RNF2 ubiquitinates Polι *in vivo* we investigated the effect of RNF2 on the status of Polι ubiquitination. First, we co-transfected HEK293T cells with plasmids expressing FLAG-Polι and HA-ubiquitin in the absence or presence of ectopically expressed RNF2 (Fig. S2). The results showed a similar Polι ubiquitination pattern that was independent of RNF2 protein levels. Second, we tested the effect of RNF2 deficiency. Due to the fact that HEK293T cell lines are not particularly good for genome editing because of its heterogeneous and unstable karyotype [43], we generated an RNF2 knock-out in the HCT116 cell line (Fig. S3). Then, we co-transfected both the wild-type

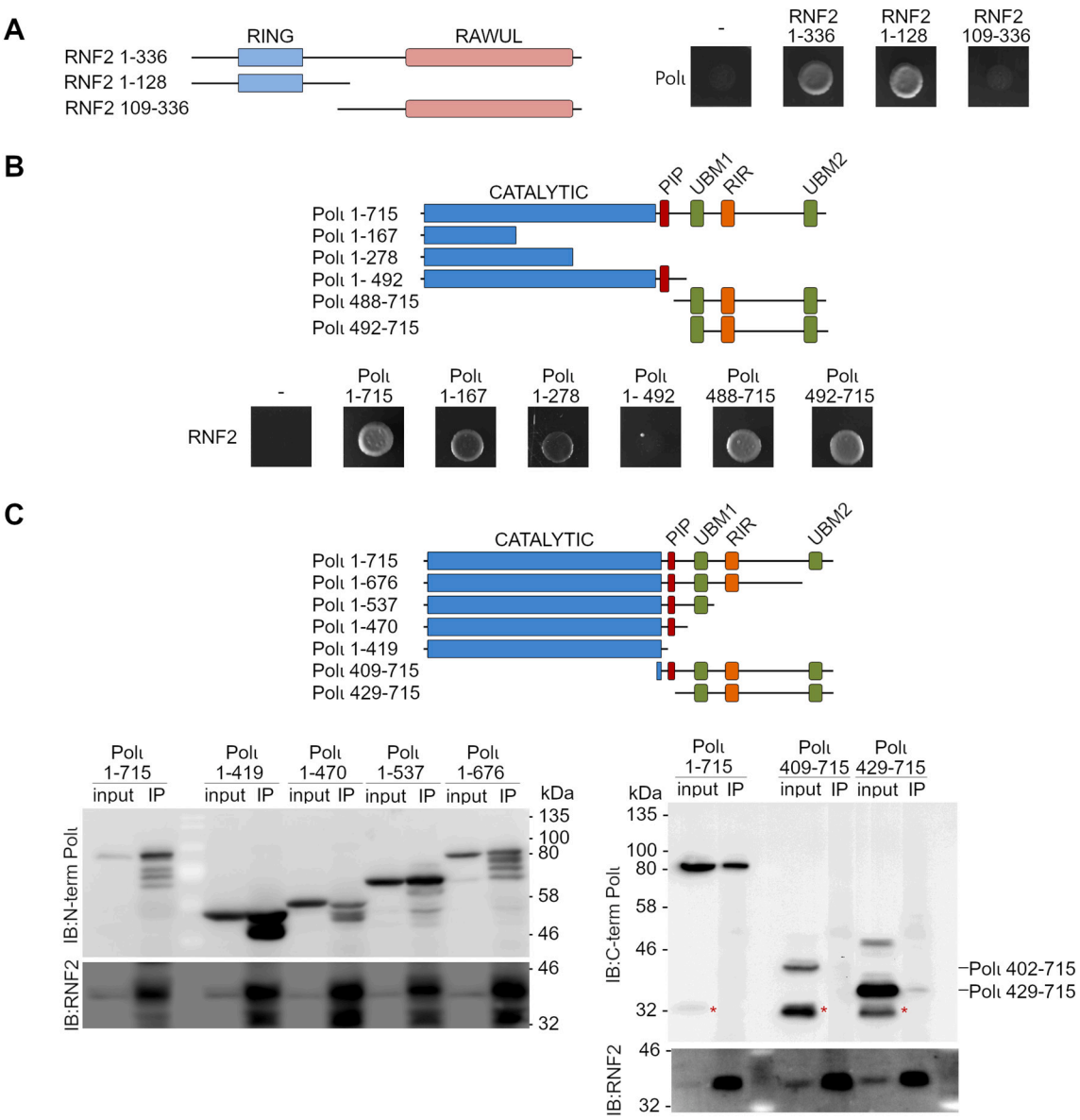

Fig. 2. Mapping the regions of interaction between Polι and RNF2. (A) Schematic representation of RNF2 with indicated domains and deletion constructs (top panel). Yeast two-hybrid assay showing the interaction between full-length Polι and N-, but not the C-terminal half of RNF2 (bottom panel). (B) Schematic illustration of Polι truncation mutants employed in this figure (top panel). Yeast two-hybrid assay showing the interaction between full-length of RNF2 and truncated fragments of Polι (bottom panel). (C) Schematic representation of Polι with indicated domains and deletion constructs (top panel). Immunoprecipitation (IP) from HEK293T cells expressing FLAG-tagged fragments of Polι protein (bottom panel). Input was 10% of cell extract used for immunoprecipitation. Samples were analyzed by SDS PAGE and western blotting using polyclonal rabbit antibodies against N- or C-terminus of Polι [37] (also described in Materials and Methods) as indicated and against RNF2 (MBL). An asterix indicates a non-specific band in cell extracts. Each experiment was repeated at least three times.

and RNF2 knock-out cells with plasmids expressing FLAG-Polι and HA-ubiquitin (Fig. 3B). The results showed not only a dramatic decrease of the level of ubiquitinated forms of Polι in RNF2 deficient cells, but above all, a significant reduction of the cellular amount of Polι. In extracts from RNF2 knock-out cells mono- and polyubiquitinated forms of Polι were still visible, however, only in overexposed blots. Nonetheless, the ratio of unmodified, monoubiquitinated and polyubiquitinated forms of Polι in the wild-type (60.2%, 23.6%, 16.2%) and RNF2 knock-out (65.1%, 20.3%, 14.6%) seems similar. This suggests that the lack of RNF2 does not affect the ubiquitination of Polι *per se*, but rather the cellular levels of Polι. Together, the results of *in vitro* and two cell culture experiments imply that Polι is not a substrate of direct ubiquitination by RNF2, but RNF2 nevertheless seems to have an impact on Polι's cellular abundance.

3.3. *RNF2 influences cellular level of Polι*

Given the substantial effect an RNF2 deficiency had on Polι's cellular levels (Fig. 3B), we were interested in analyzing its cause. As RNF2 is known to be important for transcription regulation of various genes, we first investigated whether RNF2 affects *POLI* gene expression. Using HCT116 cell lines, we compared the level of *POLI* mRNA in wild-type and RNF2 deficient cells. Using real-time RT-PCR, we observed no difference in the *POLI* mRNA level between the wild-type and RNF2 knock-out strains (Fig. 4A) indicating that RNF2 does not influence *POLI* transcription. Next, using the same HCT116 cell lines we performed a western blot with antibodies against Polι using the whole cell extracts of the wild-type and RNF2 deficient cells. The results indicate that an RNF2 deficiency reduces the cellular level of endogenous Polι protein to about 60% of that observed in wild-type RNF2 (Fig. 4B).

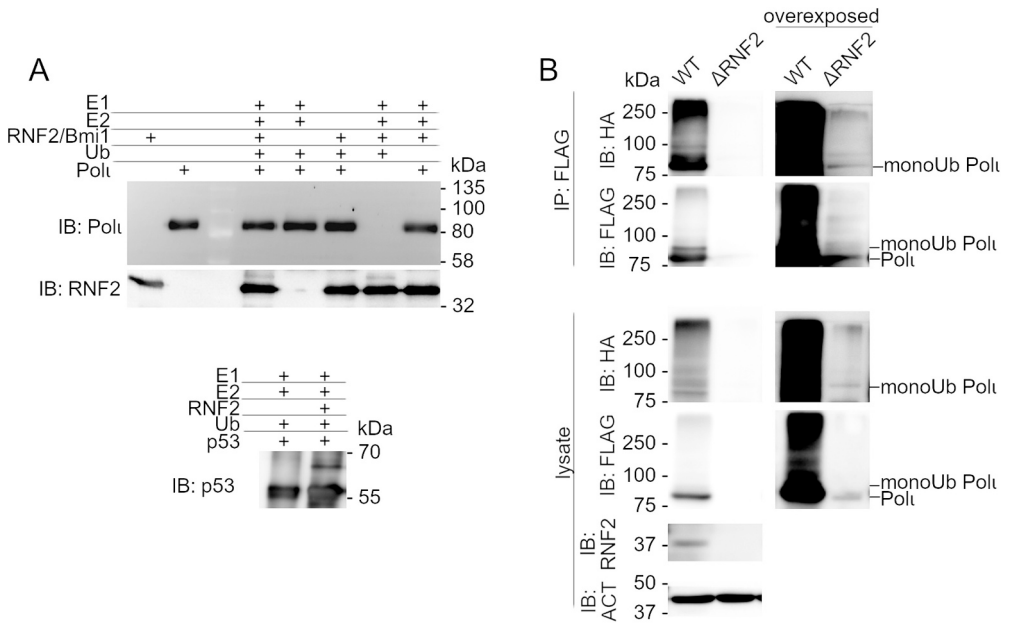

Fig. 3. RNF2 does not ubiquitinate Polι *in vitro* or *in vivo*. (A) *In vitro* ubiquitination of recombinant His-tagged Polι by GST-tagged RNF2 with supplement of GST-Bmi1 (top panel) and control *in vitro* ubiquitination of p53 (bottom panel) performed in parallel under similar conditions as described in the Materials and Methods section. The reaction products were resolved by SDS-PAGE and analyzed by western blot using antibodies against C-terminal Polι (Covance) and RNF2 (MBL) or p53 (Santa Cruz). (B) Immunoprecipitation assay performed with 10 μl of anti-FLAG resins that were incubated with equal amounts of extracts from HCT116 wild-type (WT) and RNF2 knock out cells (ΔRNF2) co-expressing FLAG-Polι and HA-ubiquitin. Samples were resolved by SDS-PAGE and analyzed by western blotting using antibodies against FLAG (Abnova), HA (Abcam), RNF2 (MBL) and β-actin (Cell Signaling) as a loading control IP- immunoprecipitation. The input controls contain 10 μg of protein extracts. Panels on the right present results of longer exposition of the blots on the left. The *in vitro* ubiquitination assay was repeated 7 times. Immunoprecipitation was repeated three times.

Subsequently, we further investigated whether an RNF2 deficiency, or its overproduction affects the abundance of Polι at the protein level. To do so, we co-expressed FLAG-Polι and an empty vector or HA-tagged RNF2 in HCT116 wild-type and RNF2 knock-out cells and monitored the level of Polι *via* western blotting. The results confirmed that cells lacking RNF2 exhibited much lower levels of FLAG-Polι than wild-type cells. Additionally, in the wild-type cells overexpressing RNF2, the steady state level of Polι was two-fold higher than in cell lines without RNF2 overexpression (Fig. 4C) and the ectopic overexpression of RNF2 complemented the phenotype exhibited in the RNF2 knock-out. We have also confirmed that the transcription levels of overexpressed HA-RNF2 is comparable in the wild-type and RNF2 deficient cells (Fig. S4).

Having discovered that RNF2 affects the cellular level of Polι we evaluated the impact of an RNF2 deficiency on the stability of Polι protein by estimating its half-life (Fig. 5). HCT116 wild-type and RNF2 knock-out cells transfected with plasmid pJRM46 expressing FLAG-tagged Polι were treated with cycloheximide blocking protein synthesis. In the wild-type cells we have not observed any significant difference in the protein level within 6 h of the experiment indicating that Polι is a rather stable protein. However, in cells lacking RNF2 the half-life of FLAG-tagged Polι protein decreased to about 4 h. These data show that RNF2 normally limits the degradation of Polι.

Together, our results reveal that RNF2 influences the intracellular concentration of Polι by securing its stability.

3.4. Ubiquitin ligase activity of RNF2 is important in the control of Polι cellular levels

To further corroborate the regulation of Polι's cellular abundance by RNF2, we checked whether RNF2 protein itself, or specifically its E3 ubiquitin ligase activity, is crucial for the maintenance of Polι cellular levels. To answer the question, we used the PRT4165 that inhibits the ubiquitin ligase activity of RNF2 [44] and performed both time course and concentration course experiments in HEK293T cell lines transfected with FLAG-Polι (Fig. 6). The Polι level was monitored by immunoblotting with anti-FLAG antibodies.

We noted that a 1h treatment with increasing concentration of PRT4165 resulted in a reduction of FLAG-Polι cellular levels (Fig. 6A). Similarly, prolonged treatment of HEK293T cells with 100μM PRT4165, caused a substantial reduction in FLAG-Polι (Fig. 6B). Interestingly, we observed that, while decreasing Polι cellular levels, PRT4165 did not cause a significant change in Polι's ubiquitination pattern and mono-ubiquitinated forms were observed at all time points and PRT4165 concentrations. Together, the results support the hypothesis that inhibition of ubiquitin ligase activity of RNF2 affects the cellular abundance of both unmodified and ubiquitinated Polι.

3.5. Proteasome activity affects the cellular abundance of Polι

To explore the mechanism involved in RNF2-dependent regulation of Polι, we investigated whether the reduction of Polι abundance in cells lacking RNF2 is caused by proteasomal degradation. We used the MG132 proteasome inhibitor to evaluate if proteasome activity is responsible for Polι destabilization in RNF2 deficient cells (Fig. 7). As previously observed, in non-treated cells overexpression of HA-tagged RNF2 resulted in about a two-fold increase in the level of FLAG-tagged Polι, while in RNF2 deficient cells dropped to about 60%. However, to our surprise, in both the wild-type, as well as in RNF2 knock-out cells expressing FLAG-tagged Polι, MG132-induced inhibition of the proteasome caused an ~50% reduction in the amount of Polι. The results indicate that proteasomal degradation is not directly responsible for the decreased cellular level of Polι in cells lacking RNF2. However, this effect strongly suggests that the proteasome is involved in an indirect stimulation of Polι's cellular abundance.

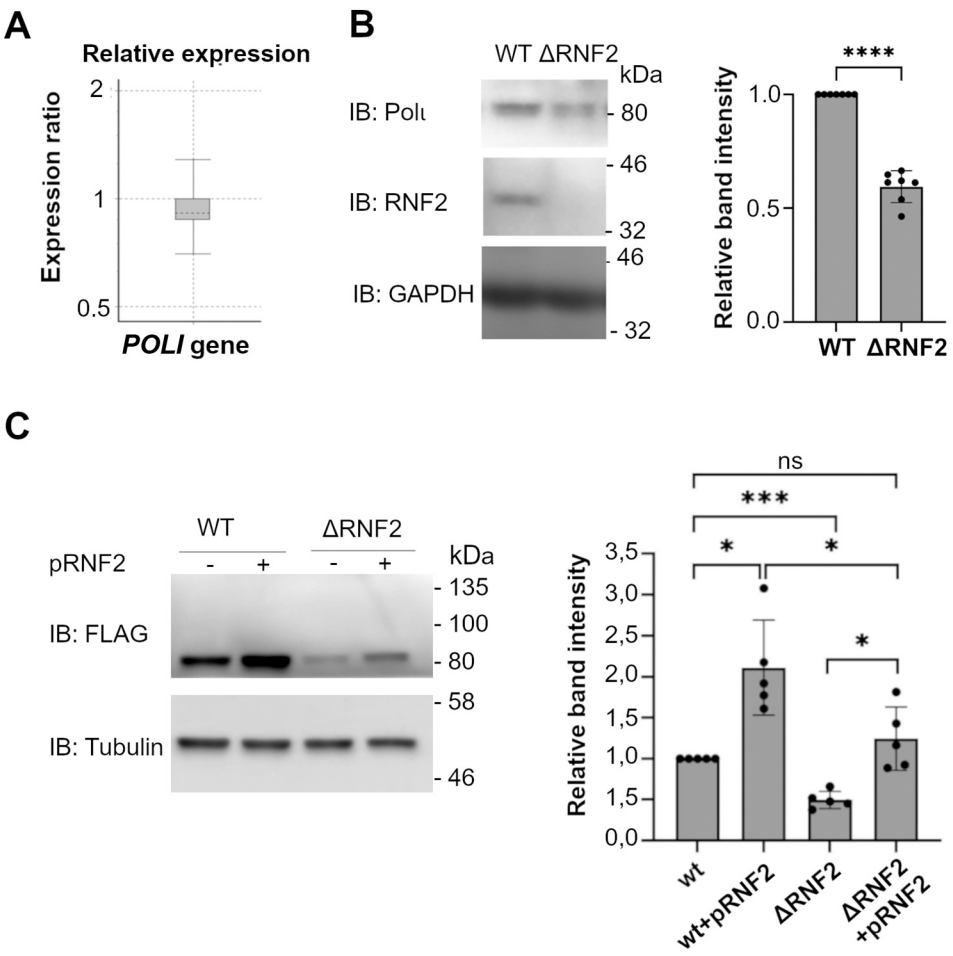

Fig. 4. RNF2 impacts Polι cellular level by regulation of protein, but not mRNA levels. (A) RT-qPCR analysis of *POLI* expression in RNF2 knock-out in HCT116 cell line. Expression values for the *POLI* gene were first normalized to expression values of reference gene *ACTB*, and then compared with similarly normalized values obtained for wild-type HCT116 cell line. Expression ratios were calculated using the REST 2009 software tool. Results are presented as whisker-box plot with the box area encompassing 50% of all observations, the dashed line representing the sample median and the outer 50% of observations represented by the whiskers. The black solid line defines the value of no change in relative expression. As the $p = 0.147$ the level of *POLI* in RNF2 knock-out sample group is not different to the control wild-type group. Three independent experiments were performed each with three replicates. (B) Analysis of endogenous Polι protein level in HCT116 wild-type or RNF2 knock-out cells. 50 μg of cell extracts were resolved by SDS-PAGE and analyzed by western blot using antibodies against Polι (OriGene), RNF2 (MBL) and GAPDH (Thermo Fisher Scientific) as a loading control. The Polι band was normalized to the GAPDH band. The number of independent experiments $n = 7$. Error bars represent standard deviations. (C) HCT116 wild-type or RNF2 knock-out cells transfected with pJRM46 (FLAG-Polι) and pMF10 (HA-RNF2, marked as pRNF2) or an empty pCMV6AN-HA vector, as indicated by pRNF2 addition. 10 μg of cell extracts were resolved by SDS-PAGE and analyzed by western blot using antibodies against FLAG (Abnova) and tubulin (Abcam) as a loading control (left panel). Relative band intensity was calculated based on the ratio of FLAG to tubulin signals and normalized to the wild-type control. The graph represents mean value of quantifications from 5 independent experiments. Error bars represent standard deviations. Student *t*-tests were used to assess differences in relative band intensities. * = $p < 0.05$, *** = $p < 0.0005$, **** = $p < 0.00005$.

In view of the fact that Polι does not seem to be short-lived protein and the proteasome is not directly involved in Polι degradation we investigated whether autophagy, known to be responsible for degradation of long-lived proteins [45], is involved in Polι protein elimination. We employed chloroquine, an autophagy inhibitor, that impairs autophagosome fusion with lysosomes [46], and compared its impact with the effect of the proteasome blocking by MG132 and to untreated controls. As previously reported inhibition of proteasome by MG132 diminished the cellular level of Polι by 50% (Fig. 8). Chloroquine treatment increased the lipidated form of LC3B indicating autophagy perturbations. Interestingly, after treating cells with chloroquine about 2.5-fold higher level of Polι compared to untreated control cells was observed. Together the results indicate that both proteasome and autophagy affect the cellular level of Polι, however in different manners. While the proteasome is involved in rather indirect stabilization of Polι, autophagy seems to be responsible for the protein's degradation. Further work needs to be performed to establish this effect more clearly.

3.6. A direct protein interaction is not required for RNF2 to regulate the levels of Polι

Given the fact that Polι interacts with RNF2 but does not seem to be directly ubiquitinated by the enzyme, we were interested in verifying whether the interaction of RNF2 and Polι is necessary for its RNF2-dependent stabilization. To do so, we tested if the inhibition of RNF2 ubiquitin ligase activity by PRT4165 alleviates the level of the Polι fragment that has reduced ability to interact with RNF2 compared to the full-length protein. We hypothesized that if the interaction between the proteins is required for RNF2-dependent Polι protein level maintenance, the level of Polι fragments that are unable to interact with RNF2 will not be affected by PRT4165 inhibition. We transfected HEK293T cells with full-length FLAG-Polι or fragments encompassing amino acid residues 1–470 or 402–715 that have a reduced ability to bind RNF2 (as shown in Fig. 2C), and treated, or not, with PRT4165. As seen in Fig. 9 the protein levels of full-length and N-terminal 470 (Fig. 9A) or C-terminal 313 amino acids of Polι (Fig. 9B), are significantly decreased upon treatment

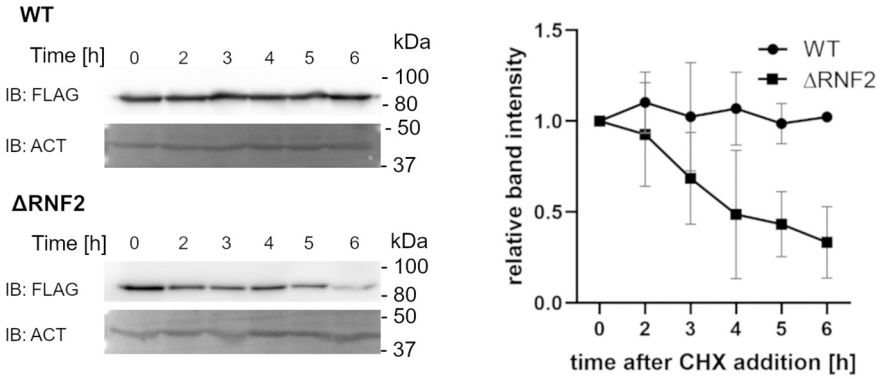

Fig. 5. RNF2 stabilizes Polι protein. Analysis of the stability of FLAG-Polι in HCT116 wild-type and RNF2 knock-out cells. Cells transfected with pJRM46 (FLAG-Polι) were incubated with the protein synthesis inhibitor - cycloheximide (CHX) 10μg/ml for the indicated time. Whole cell extracts were subjected to western blotting with anti-FLAG (Abnova) and anti β actin (Cell Signaling) antibodies as a loading control. Comparison of Polι stability in wild-type and RNF2 knock-out cells (left top and bottom panel). The FLAG intensity normalized by the β actin signal is plotted in the graph (right panel). The graph represents the mean of three experiments. Error bars represent standard deviations.

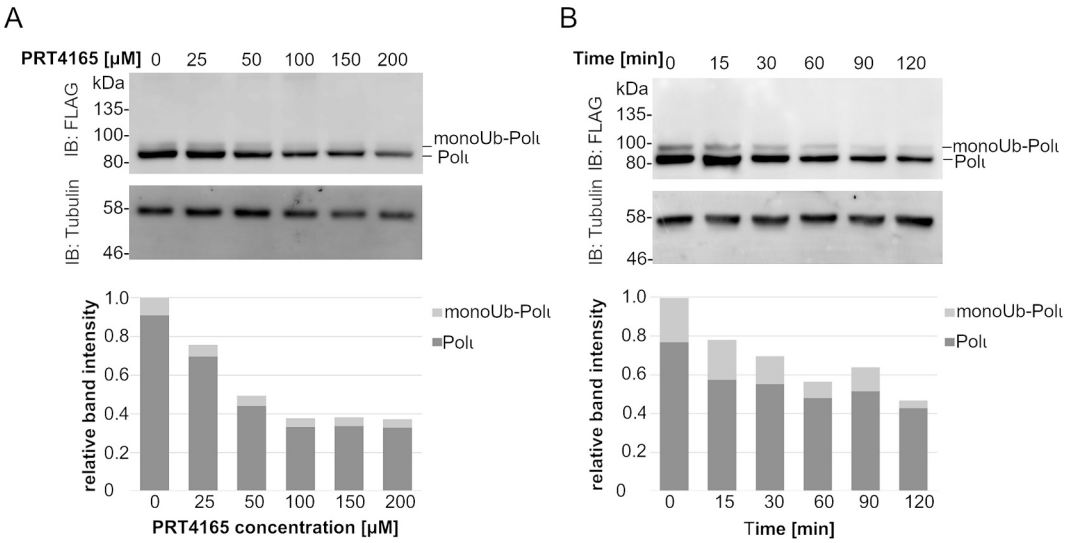

Fig. 6. RNF2 inhibition by PRT4165 affects the cellular level of Polι. (A) Concentration course. HEK293T cells transfected with pJRM46 (FLAG-Polι) were treated for 1 h with increasing concentrations of PRT4165. (B) Time course. HEK293T cells transfected with pJRM46 (FLAG-Polι) were treated with 100 μM PRT4165 for up to 2 h. In (A) and (B) 10 μg of cell extracts were resolved by SDS-PAGE and analyzed by western blot using antibodies against FLAG (Abnova) and tubulin (Abcam) as a loading control (top panels). Densitometric analysis of FLAG-Polι western blots. The FLAG-Polι bands were normalized to the respective tubulin bands and to "0" concentration or time points, respectively. Unmodified Polι (dark grey), monoubiquitinated Polι (light grey). The graphs represent the mean values of three independent experiments.

with RNF2 inhibitor, compared to non-treated cells. This suggests that a direct RNF2-Polι interaction is unnecessary for RNF2-dependent regulation of Polι's cellular levels.

3.7. *RNF2 regulates Polη similar to Polι*

The presence of a close paralogue of Polι – Polη in mammalian cells tempted us to verify whether RNF2 specifically regulates the level of Polι, or it rather functions more generally in regulation of Y-family polymerases. We investigated both the interaction of RNF2 and Polη as well as the impact of RNF2 on the cellular level of Polη. First, by using yeast two hybrid system we have shown that Polη, similar to Polι, interacts with full length and the N-terminal part of RNF2, but not the C-terminal portion containing the ubiquitin-like RAWUL domain (Fig. 10A). We confirmed the results of the RNF2 and Polη interaction by *co*-immunoprecipitation of native RNF2 and N-terminally FLAG-tagged polymerase expressed in HEK293T cells (Fig. 10B).

To test the effect of RNF2 on the cellular level of Polη and compare it with its effect on Polι, we checked the steady state level of FLAG-tagged polymerases in HCT116 wild-type cells in comparison to the RNF2 knock-out cells (Fig. 10C). We can clearly see that lack of RNF2 causes reduction of the cellular level of both tested polymerases. Altogether, these studies suggest that RNF2-dependent regulatory mechanism is not limited to Polι and can affect other Y-family polymerases.

4. Discussion

The extreme infidelity of Polι together with the fact that both its up- and downregulation correlates with various cancers indicates that the expression and access of Polι to the replication fork should be strictly controlled. As with the regulation of other TLS proteins, protein interactions and posttranslational modifications, particularly ubiquitination, play an important role in the control of Polι. In the current study, we were originally interested in identifying the E3 ubiquitin ligase(s) involved in Polι ubiquitination. A candidate, RNF2, was selected as a potential interacting partner of Polι. Here, we present multiple evidence

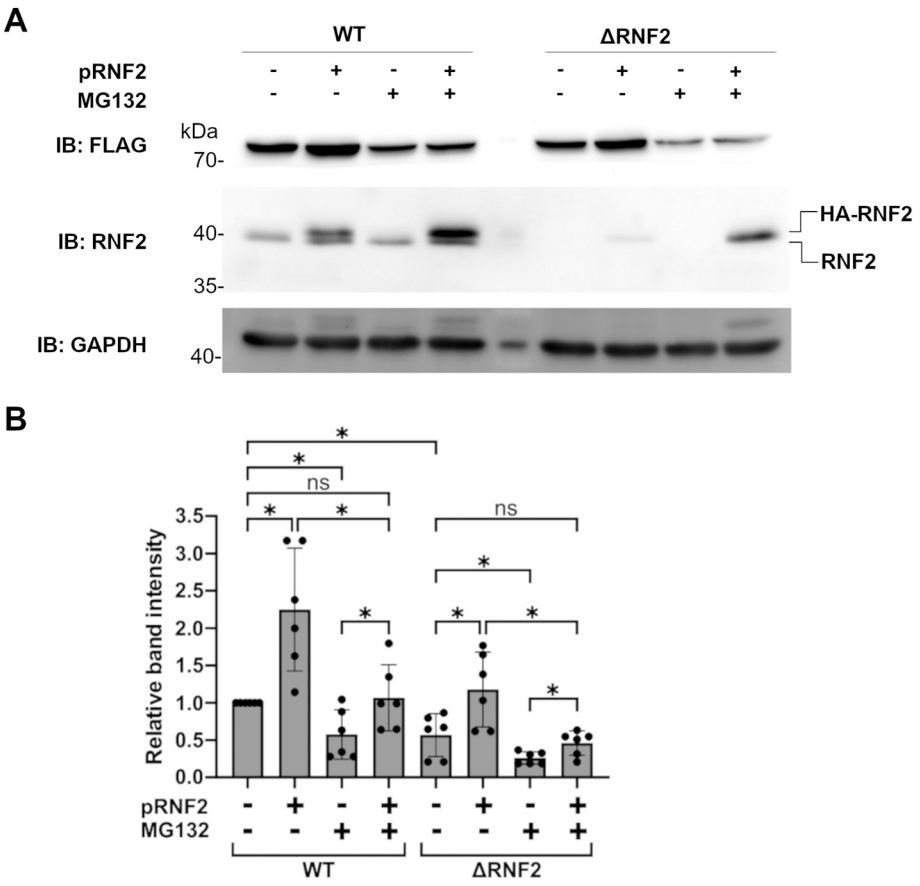

Fig. 7. Proteasome inhibition impacts the cellular level of Polι. (A) Western blot analysis of FLAG-tagged Polι expressed in HCT116 wild-type or RNF2 knock-out cells transfected with pJRM46 (FLAG-Polι) and pMF10 (HA-RNF2, labeled as pRNF2) or an empty pCMV6ANHA vector, and treated for 24 h with 20 μM MG132, as indicated. 10 μg of cell extracts were resolved by SDS-PAGE and analyzed by western blot using antibodies against FLAG (Abnova), RNF2 (MBL) and GAPDH (Thermo Fisher Scientific) as a loading control. (B) Densitometric analysis of FLAG-Polι western blots. Relative band intensity was calculated based on the ratio of FLAG to GAPDH signals. The graph represents mean value of quantifications from 6 independent repeats. Error bars represent standard deviations. Student t-tests were used to assess differences in relative band intensities. * = $p < 0.05$.

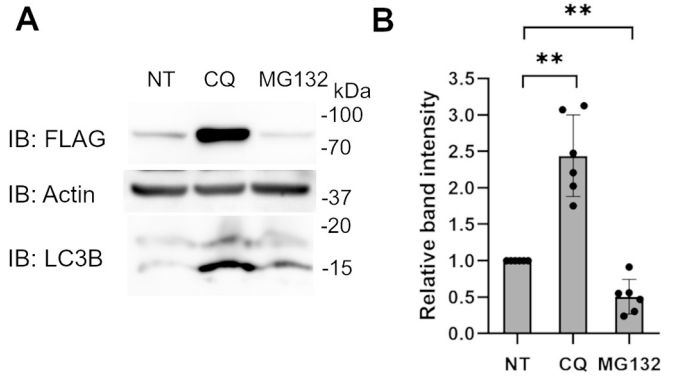

Fig. 8. Autophagy inhibition causes an increase in Polι protein levels. (A) Western blot analysis of FLAG-tagged Polι expressed in HCT116 wild-type cells transfected with pJRM46 (FLAG-Polι) and treated for 9 h with 100 μM chloroquine (CQ) or 20 μM MG132, as indicated. 30 μg of cell extracts were resolved by SDS-PAGE and analyzed by western blot using antibodies against FLAG (Abnova), β-actin (Cell Signaling) as loading control and LC3B (Cell Signaling) as an autophagy perturbations indicator. (B) Densitometric analysis of FLAG-Polι western blots. Relative band intensity was calculated based on the ratio of FLAG to β actin signals. The graph represents mean value of quantifications from 6 independent repeats. Error bars represent standard deviations. Student t-tests were used to assess differences in relative band intensities. ** = $p < 0.005$.

for the interaction between RNF2 and Polι through co-immunoprecipitation, pull-down of purified proteins and yeast two-hybrid assays. Additionally, PLA studies showed co-localization of native RNF2 and Polι proteins, providing evidence of interactions between the endogenous proteins. The results also indicate that more than one site located either at the N- or C-terminus half of Polι is involved in the interaction with RNF2. Interestingly, the N-terminal half of RNF2, including the RING catalytic domain is sufficient to interact with Polι, excluding the possibility that the interaction occurs *via* the ubiquitin-like domain of RNF2 and one of the UBMs of Polι. The interaction of Polι with the N-terminal half of RNF2 might also explain the fact that we were not able to confirm the interactions between the two proteins in the yeast two-hybrid system when RNF2 was N-terminally fused with GAL4 DNA binding domain or GAL4 activating domain. Such big peptides (about 140 amino acids) probably disturb proper folding of the N-terminus of the protein or block the interacting surface. In contrast, a GAL4 peptide located at the C-terminus of RNF2 did not affect the interaction with Polι. The fact that RNF2 connects with Polι by the N-terminal fragment containing RING E3 ubiquitin ligase catalytic domain suggests that the enzymatic activity is important in RNF2-Polι relations. Surprisingly, both *in vitro* ubiquitination assay and the ubiquitination pattern in cells overexpressing or deficient in RNF2, vote against Polι as a direct substrate of the E3 ubiquitin ligase activity of RNF2. Our results indicate, however, that RNF2 nevertheless has an impact on the cellular level of Polι. We show, for the first time, that under normal conditions Polι is a rather stable protein with its half-life being well over 6 h. Intriguingly, our results indicate that this stability is guaranteed by

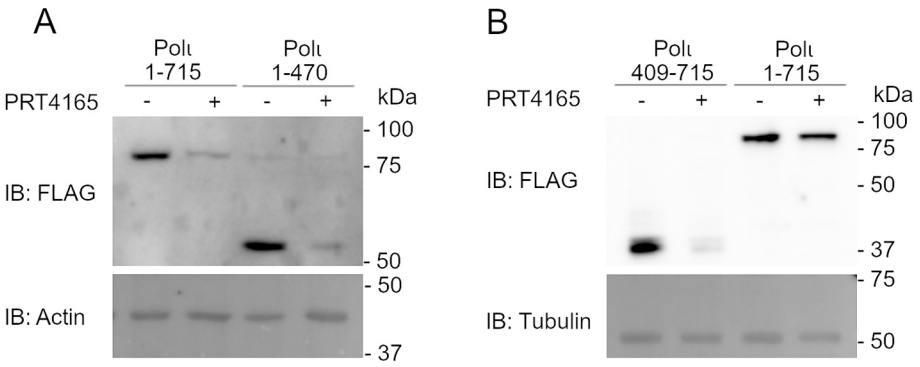

Fig. 9. RNF2 inhibition by PRT4165 affects the cellular level of Polι protein independent of its interaction with RNF2. HEK293T cells transfected with FLAG-Polι full-length (1-715aa), or fragments with a reduced ability to interact with RNF2, 1-470aa (A) or 409-715aa (B) were treated, or not, with 100 μM PRT4165 for 1 h. 10 μg of cell extracts were resolved by SDS-PAGE and analyzed by western blot using antibodies against FLAG (Abnova) and tubulin (Abcam) or β actin (Cell Signaling) as a loading control. The experiment has been repeated three times.

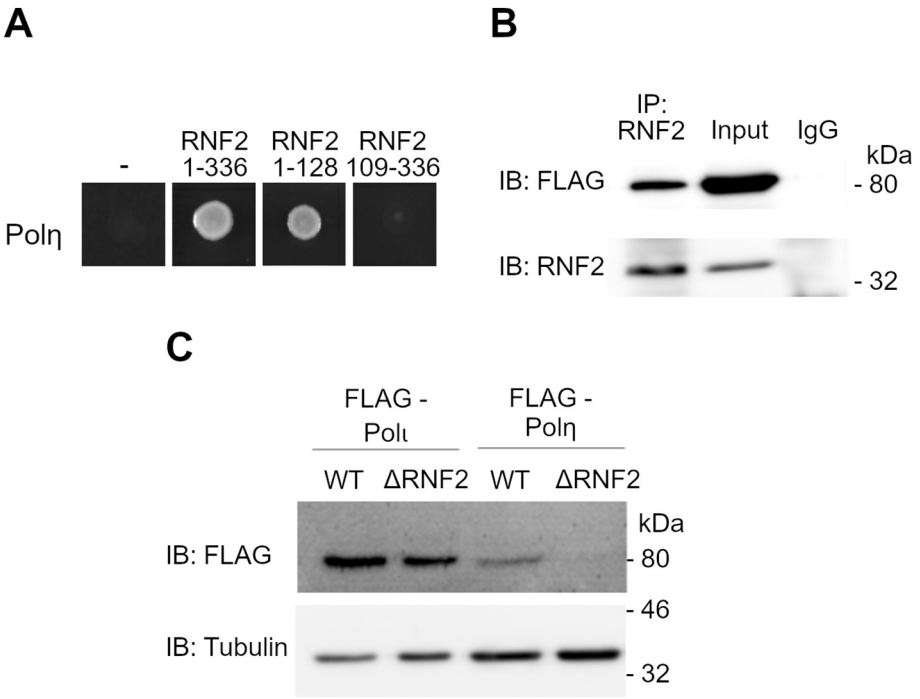

Fig. 10. RNF2 affects Polη similarly to Polι. (A) Yeast two-hybrid assay showing the interaction between full-length of Polη and N-, but not the C-terminal half of RNF2. (B) Cell extract from HEK293T cells expressing FLAG-tagged Polη protein was subject to immunoprecipitation (IP) using control IgG (Cell Signaling) or anti-RNF2 (MBL) antibodies, followed by immunoblotting with anti-FLAG (Abnova) and anti-RNF2 (Thermo Fisher Scientific) antibodies. Input was 10 % of cell extract used for immunoprecipitation. (C) Comparing the impact of RNF2 deletion on the cellular level of Polι and Polη. HCT116 cells, wild-type or with knocked-out RNF2, were transfected with pJRM46 (FLAG-Polι) or pJRM160 (FLAG-Polη) as indicated. 10 μg of cell extracts were resolved by SDS-PAGE and analyzed by western blot using antibodies against FLAG (Abnova) and tubulin (Abcam) as a loading control. Each experiment was repeated at least three times.

RNF2. Both effects, of the specific inhibitor of RNF2 (PRT4165), as well as a deficiency of RNF2 cause a decrease in Polι cellular protein levels and suggests that the E3 ubiquitin ligase activity of RNF2 protects Polι from destabilization. The influence of RNF2 on protein levels often has an opposite effect, as RNF2-dependent polyubiquitination of various proteins *e.g.* RRM1 [47], SBDS [31] or AMBRA1 [48] directs the proteins for proteasomal degradation and consequently alleviates their cellular concentration. However, a recent publication by Yuan et al., shows that in breast cancer cells the association with RNF2 increases ERα protein stability by inhibiting its polyubiquitination targeting of the protein to the proteasome [49]. Nevertheless, the mechanism of regulating Polι levels might be different from that of ERα since overexpression or deficiency of RNF2 does not seem to change the ubiquitination pattern of Polι, and proteasome inhibition does not prevent degradation, but, on the contrary, it rather encourages it. Interestingly, it has been shown that RNF2 interacts with S6'/TBP1 ATPase, a subunit of 19S regulatory complex of the proteasome, and the presence of RNF2 increases the ATPase activity of S6' [32]. Yet, this does not explain the destabilizing effect of proteasome inhibition, as such additional role of RNF2 in recruiting substrate to the proteasome would cause RNF2-dependent destabilization, rather than stabilization of the protein, which is why we suggest that the proteasome indirectly affects Polι's cellular abundance. We hypothesize that RNF2 protects Polι protein by limiting a destabilizing effect of Polι by a yet unknown protein factor. We propose a model [Fig. 11], in which Polι's destabilizer is polyubiquitinated by RNF2 and directed to the proteasomal degradation. This would explain why overproduction of RNF2 enhances the quantity of Polι when proteasome activity is undisturbed.

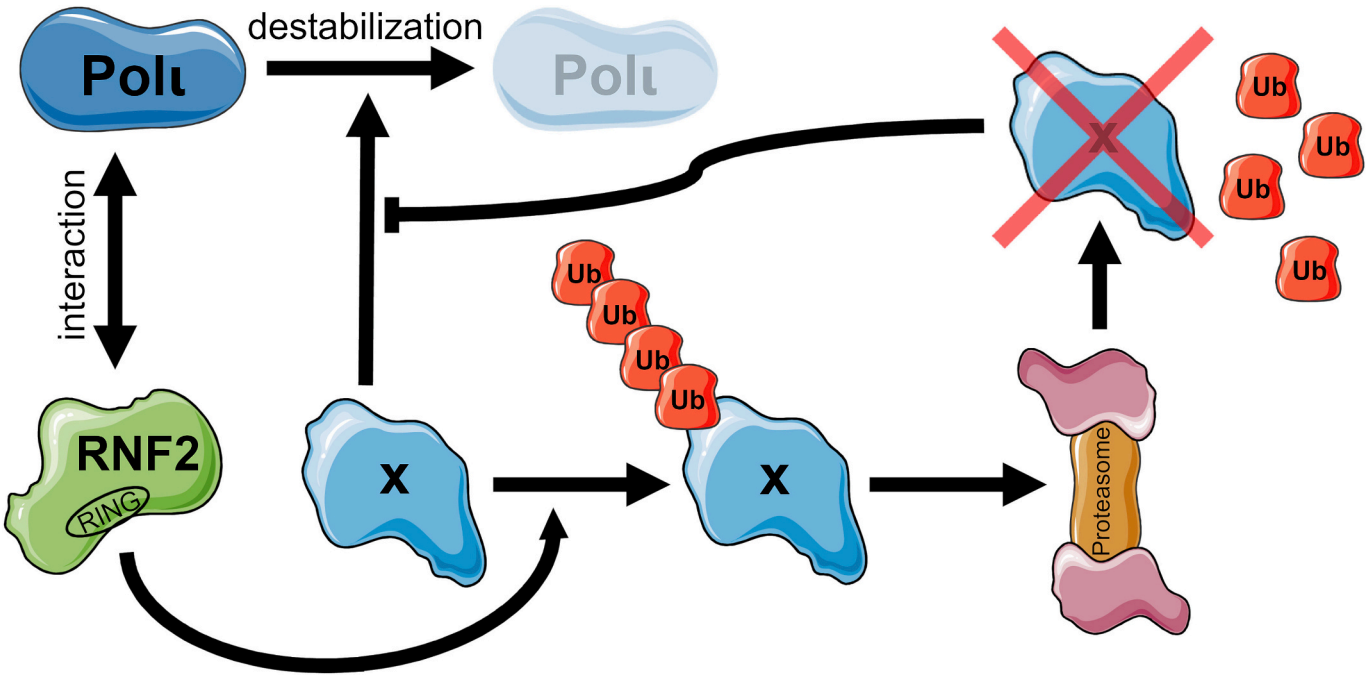

Fig. 11. Cartoon proposing how RNF2 can indirectly protect Polι in a proteasome-dependent manner. RNF2 (green shape) polyubiquitinates yet an unknown protein (light blue shape with X symbol) destabilizing Polι (blue shape) what directs it to degradation by 26S proteasome, and consequently, blocks the destabilization of Polι. Polι interacts with RNF2, but this interaction is not required for RNF2-dependent regulation of the cellular level of Polι. The figure was generated using pictures from Servier Medical Art. Servier Medical Art by Servier is licensed under a Creative Commons Attribution 3.0 Unported License (https://creativecommons.org/licenses/by/3.0/).

An indirect impact of RNF2 on Polι's protein level seems to be independent of the interaction with RNF2 and indicate that the relationship between RNF2 and Polι in on more than one level. The process requiring Polι-RNF2 association, as well as identification of the destabilizing factor will be of our future investigation.

The fact that Polη, another Y-family polymerase, similar to Polι interacts with RNF2 and likewise its level is diminished by an RNF2 deficiency, suggests that RNF2-dependent mechanism involved in protein maintenance is more general. However, despite similar final effect of RNF2 on interaction and cellular level of both Polι and Polη, a direct translation of the RNF2 impact on the two proteins might not be possible at the moment and requires further investigation. The existence of differences in regulation is clear in a view of the fact that Polη is a short-lived protein with its half-life estimated to be 28 min and is degraded by the proteasome [50] while, according to our results, Polι seems to be a stable protein with an opposite proteasomal effect. Additionally, the results show that the cellular level of Polι increases upon autophagy inhibition. At the moment we cannot conclude whether autophagy-related regulation of Polι is direct and whether it is RNF2-dependent. However it is the first evidence of a Y-family TLS polymerase to be regulated in a process dependent on autophagy, thereby adding new evidence for the expanding role of autophagy in genome maintenance [51].

As mentioned in the introduction, many cellular proteins are substrates of RNF2 ubiquitination. Among them are proteins such as RRM1 [47], histone H2A [52] and p53 [29], which are associated with replication stress. Polι, being one of the TLS polymerases, functions as an effector of such stress. Therefore, functional or regulatory links between replication stress proteins and RNF2-dependent Polι control should also be considered. Particularly, the relationship between p53 and Polι in the regulatory loop is worth analyzing due to the fact that the two proteins have been reported to interact [53]. However, the connection might not have simple and direct explanation, as the ubiquitination activity of RNF2 leads to p53 degradation while stabilizing Polι. This implies an inverse correlation between the cellular abundance of Polι and p53, which seems inconsistent with the joint action of those proteins in the HLTF/ZRANB-dependent DTT pathway [53]. In addition, our results suggest that Polη, which does not interact with p53, is stabilized by RNF2 in similar manner to Polι, indicating that other factors can be involved in an indirect regulation of the levels of these TLS polymerases by RNF2.

A stabilizing effect of RNF2 might have various consequences. It has been shown that RNF2 is highly expressed in many tumors, including pancreatic cancer, ovarian carcinoma, urothelial carcinoma, hepatocellular carcinoma, prostate cancer, breast cancer and esophageal carcinoma, suggesting that it might have an oncogenic function [54]. The stabilization of Polι and Polη by RNF2 might be a tissue-specific event, however we cannot ignore the situation that a higher level of RNF2 translates to greater stability of error-prone polymerases and increased genomic instability. The indirect effect of Polι destabilization by the proteasome is intriguing, especially in a view of the anticancer therapies using proteasome inhibitors. TLS polymerases, such as Polι, may hamper anticancer treatment based on introducing DNA lesions into actively dividing tumor cells. Such lesions can be effectively bypassed by TLS polymerases, so, a mechanism limiting the level of TLS polymerases can support effective therapies.

In summary, our results indicate that the proteasome indirectly affects the cellular abundance of Polι, which is counteracted by the E3 ubiquitin ligase activity of RNF2. This points to the complexity of the regulation of the cellular abundance of Polι at the level of protein stability. Additionally, RNF2's direct interaction with Polι suggests yet another level of relation that will also be the subject of future investigation.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbamcr.2024.119743.

CRediT authorship contribution statement

Mikolaj Fedorowicz: Visualization, Investigation, Conceptualization. **Agnieszka Halas:** Writing – review & editing, Methodology,

Investigation. **Matylda Macias:** Methodology, Investigation. **Ewa Sledziewska-Gojska:** Writing – review & editing, Conceptualization. **Roger Woodgate:** Writing – review & editing, Funding acquisition, Conceptualization. **Justyna McIntyre:** Writing – review & editing, Writing – original draft, Supervision, Project administration, Methodology, Investigation, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Acknowledgments

This work was supported by grant number 2015/17/B/ NZ1/00862 to J.M. from the Polish National Science Center and the NICHD/NIH Intramural Research Program to R.W.

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