

RESEARCH ARTICLE

UBC13 is an RNF213-associated E2 ubiquitin-conjugating enzyme, and Lysine 63-linked ubiquitination by the RNF213-UBC13 axis is responsible for angiogenic activity

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Abstract

Moyamoya disease (MMD) is a cryptogenic vascular disorder in the intracranial arteries. RING protein 213 (RNF213) is the susceptibility gene for MMD, and encodes a RING domain and a Walker motif. Herein, we identified UBC13 (UBE2N) as an E2 ubiquitin-conjugating enzyme for RNF213 E3 ubiquitin ligase by yeast two-hybrid screening with a fragment containing RNF213 RING domain as bait, and the immunocomplex of RNF213-UBC13 was detected *in vivo*. Analysis of the ubiquitin chain on RNF213 by monitoring autoubiquitination showed that RNF213 was autoubiquitinated in a K63 chain fashion, but not in a K48 chain fashion. Finally, this RNF213 ubiquitination in a UBC13-dependent manner was required for cell mobility and invasion activity for HUVEC cells in UBC13 knock-down and ubiquitination-dead RNF213 mutant expressing experiments. These findings demonstrated that RNF213 is a K63-linked E3 ubiquitin ligase, and UBC13 is responsible for RNF213 dependent ubiquitination. The RNF213-UBC13 axis may be associated with angiogenic activity and MMD.

KEY WORDS

angiogenic activity, lysine-linked ubiquitination, Moyamoya disease, RNF213

1 | INTRODUCTION

Moyamoya disease (MMD) is a disorder caused by internal dysfunctions in the internal carotid artery and branches in the Willis' circle.¹⁻⁴ A couple of mechanisms, including inflammation, upregulation of various angiogenic factors, and

abnormalities of endothelial progenitor cells, are thought to cause MMD.⁵⁻⁹

In our previous study, we identified RNF213/Mysterin as a causative gene for MMD. *RNF213* encodes a huge protein that possesses two functional domains: two pairs of Walker motifs and a RING domain.^{10,11} The p.R4810K variant

Abbreviations: 3-AT, 3-amino-1, 2, 4-triazole; AAA, ATPases Associated with diverse cellular Activities; AAVS1, Adeno-associated virus integration site 1; CHX, cycloheximide; CRISPR-Cas9, clustered regularly interspaced short palindromic repeat-CRISPR-associated proteins 9; DMEM, Dulbecco's Modified Eagle's Medium; EC, endothelial cells; GFP, green fluorescence protein; HA, hemagglutinin; His, Histidine; HUVEC, Human Umbilical Vein Endothelial Cells; K48, Lysine 48; K63, Lysine 63; Leu, Leucine; Lys, Lysine; MMD, Moyamoya disease; NP-40, Nonidet P-40; PBS, phosphate-buffered saline; PMSF, phenylmethanesulfonyl fluoride; RING, Really Interesting New Gene; RNF213, RING protein 213; RPMI, Roswell Park Memorial Institute medium; SD, Synthetic Dropout; tetR, Tet Repressor protein; Trp, Tryptophan; UBC13, Ubiquitin-conjugating enzyme 13; Wt, wild-type.

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(rs112735431) of the RNF213 gene was a founder polymorphism related to MMD in East Asia.^{1,10,12} A couple of rare RNF213 variants have been identified and reported in MMD patients in ethnically diverse populations.^{6,12,13}

The knockdown of Rnf213 gene expression in zebrafish caused irregular wall formation in the trunk arteries and abnormal sprouting vessels.¹⁰ Although the HUVEC cells expressing the RNF213 R4810K mutant showed defects in tube formation and decreased wound healing activities, which influenced angiogenic activity in endothelial cells, RNF213 knockdown HUVEC cells using siRNAs showed no distinct changes in angiogenic activity.^{10,14–16} These phenomena indicated that the RNF213 R4810K variant might dominant-negatively play a role in MMD endothelial cells.¹²

Posttranslational protein modifications by mono- or polyubiquitin are involved in diverse cellular signaling pathways and are tightly regulated to ensure cellular processes.^{17–20} Ubiquitin molecules are conjugated at the ϵ -amino group of lysyl residues of target proteins through isopeptide bonds. This process occurs by three types of enzymatic activities, namely ubiquitin-activating enzymes (E1), ubiquitin-conjugating enzymes (E2), and ubiquitin-protein ligases (E3). An initial step catalyzed by E1 activates the C-terminus of ubiquitin for subsequent reactions. An intermediate step catalyzed by E2 transfers the activated ubiquitin from E1 to the E2 enzyme. E3 ligases facilitate the final attachment steps of ubiquitin to target proteins. Individual E2 enzymes dictate specific biological functions of ubiquitin because the E2-E3 interaction determines the last substrates of ubiquitin covalently bound by the E2 enzyme.^{17,21,22} The ϵ -amino group of seven lysyl residues of ubiquitin (Lys6, Lys11, Lys27, Lys29, Lys33, Lys48, and Lys63) can also be attached to ubiquitin through an isopeptide bond. The nature of a ubiquitin-ubiquitin isopeptide bond appears to determine the subsequent fate of ubiquitinated proteins.^{18,19,21,23–25} Lys48 (K48)-linked polyubiquitination implies recognizing the conjugated protein by proteasome molecules and subsequent proteolytic degradation of the target proteins. On the contrary, Lys63 (K63)-linked polyubiquitination appears to be involved in critical cellular processes, such as DNA repair, regulation of the I-kappaB kinase/NF-kappaB cascade, or T cell receptor signaling pathway. However, it does not appear to imply proteolytic degradation.^{19,21,26} The UBC13-Mms2 E2 heterodimer can build the K63-linked ubiquitin chains selectively.^{27,28}

RING domain-dependent homo- or heterodimerization has been reported.^{9,19,20} The heterodimer formation between the RING domain of MDM2 and MDMX1 activates the ubiquitination activity,^{20,29–31} as does BRCA1 with BARD1^{22,32,33} and Ring1b with Bmi1.³⁴ Zinc centers of the RING domain of MDM2 are required for E3 ligase activity,

and the five C-terminal residues of the domain are essential for both dimer formation and E3 activity.^{20,29–31} The α -helical flanking region outside the RING domain of BRCA1 is responsible for dimerization with BARD1,^{22,32,33} and heterodimerization activates the ubiquitination activity. Some RING-type E3 ligases form dimers through RING domains or oligomers through a distinct RING domain. Prp19 E3 ligase has been shown to oligomerize with multiple RING dimers as a tetramer.³⁵

To address MMD and the candidate RNF213 functions, we identified UBC13 (UBE2N) as the binding partner of RNF213 protein in yeast two-hybrid screening using E2 enzyme-specific libraries.^{30,36} This interaction was also observed by co-immunoprecipitation using HeLa cells extract. By testing the possibility of ubiquitination on RNF213, K63-linked polyubiquitination was detected, but not K48. The K63-linked polyubiquitination was suppressed in Ubc13 knockdown HeLa cells. The RNF213 mutant, which showed a weak interaction with UBC13 in yeast two-hybrid assay, was not ubiquitinated.

Moreover, the RING domain of RNF213 protein formed a homodimer, and this dimer formation appeared to be required for the ubiquitination. The interaction with UBC13 and the homodimer formation of RNF213 protein were necessary for autoubiquitination and angiogenic, motility, and invasion activities in HUVEC cells. These findings indicate that the homodimer RNF213 protein might have E3 ligase activity in a specific manner of K63-linked ubiquitination with UBC13 as an E2 enzyme in endothelial cells.

2 | MATERIALS AND METHODS

2.1 | Directed yeast two-hybrid screens

All procedures and materials were as in previous papers.^{22,30,36} A DNA fragment corresponding to a 3951–4107 amino acid of RNF213 was amplified with the following primers: 5'-GGGC CATGGCCGGCAGCGGGAGCCTGGCCC-3', 5'-CCCGT CGACCTATTAGCATGCTTTTCAATGGCT-3'. The resulting PCR products were cloned into pGBKT7 (Takara Bio USA, Mountain View, CA) using *NcoI* and *SalI* sites. The I3999A mutation was introduced with a PCR-based site-directed mutagenesis system using *Pfu* Turbo (Agilent Technologies, Santa Clara, CA). E2 enzymes used in Figure S2(A)–(D) were amplified with each specific primer (Table 1) using total RNA from HCT116 cells and cloned into pENTR-D-Topo (Thermo Fisher Scientific, Waltham, MA) according to the manufacturer's instructions. The cloned E2 genes were transferred into pAct2-gtwy (Addgene #11346) using LR Clonase (Thermo Fisher Scientific). The bait plasmid and pGBKT7 were transformed into the yeast strain AH109 (Takara Bio USA) on selection media (SD –Trp).

TABLE 1 Primer list

Fw		Rv	
RNF213_B	GGG CCA TGG CCA TTC AGC CGT GCT CC	RNF213_A	CCC GTC GAC AGT TAA ACA GTA GGG GC
RNF213_C	GGG CCA TGG CCA GCC GCG TCC CCG AG	RNF213_D	GGG GTC GAC CTG AAT CCC AAA CCT GC
RNF213_Y2H_ F3924A	GTGGAGTCGGATTGCTCCACCGCACTC	RNF213_Y2H_ F3924A	CTCCACGAAGAGTGCGGTGGAGGC AATCCG
RNF213_Y2H_ H3932A	CTCTTCGTGGAGGCCGTGCTCCTAGGAA	RNF213_Y2H_ H3932A	CTCTCGGTTCTAGGAGCACGG CCTCCAC
RNF213_Y2H_ H3951A	CTGGTGACCGAGGCCGTCTTCTACTAGAC	RNF213_Y2H_ H3951A	GACACTTGTCTAGTAAGAAGACGG CCTCGGT
RNF213_Y2H_ F3971A	ACGCACGGGCCTGCTGAGGCCGTGATGCG	RNF213_Y2H_ F3971A	GAGAGTGCGCATCACGGCCTCAG CAGGCC
RNF213_Y2H_ F3992A	GACCCTCAGCAGGGCTGGGATTCAGCCGTG	RNF213_Y2H_ F3992A	GGAGCACGGCTGAATCCCAGCCC TGCTGAG
RNF213_Y2H_ P4040A	TTAACTGCCTTGGCAGACGAATTCTCTCCA	RNF213_Y2H_ P4040A	AAACAGCTGGAGAGAATTCGT CTGCCAAGG
RNF213_Y2H_ F4043A	CTTGCCAGACGAAGCCTCTCCAGCTG	RNF213_Y2H_ F4043A	GGAAACAGCTGGAGAGGCTT CGTCTG
RNF213_Y2H_ F3953A	GACCGAGCACGTCGCCTTACTAGACAA	RNF213_Y2H_ F3953A	GAAGACACTGTCTAGTAAGGCG ACGTGCT
RNF213_Y2H_ F3929A	CCACCGCACTCGCCGTGGAGCACGTG	RNF213_Y2H_ F3929A	CTAGGAGCACGTGCTCCACGGC GAGTGCG
RNF213_Y2H_ N3962D	GTCTTCGAGAGGACTCTGACGTGAAG	RNF213_Y2H_ N3962D	CGTGCGTCTTCACGTCAGAGT CCTCTCGA
RNF213_Y2H_ D4013 N	CTGTCTGCCCTGCAACCACGTGCACTGC	RNF213_Y2H_ D4013 N	GCAGGCAGTGCACGTGGTT GCAGGGCAG
UBE2B_F	caccATGTCGACCCCGGCCCGG	UBE2B_R	TTATGAATCATTCCAGCTTTG
Ube2C_F	caccATGGCTTCCAAAACCGC	Ube2C_R	TCAGGGCTCCTGGCTGGTGAC
Ube2D1_F	caccATGGCGCTGAAGAGGATT	Ube2D1_R	TTACATTGCATATTTCTGAGT
Ube2D2_F	caccATGGCTCTGAAGAGAATC	Ube2D2_R	TTACATCGCATACTTCTGAGT
UBE2D3_F	caccATGGCGCTGAAACGGATT	UBE2D3_R	TCACATGGCATACTTCTGAGT
UBE2E1_F	C ACCATGTTCGGATGACGATTTCG	UBE2E1_R	TTATGTAGCGTATCTCTTGGT
Ube2E2_F	C ACCATGTCCACTGAGGCACAA	Ube2E2_R	CTATGTGGCGTACCGCTTGGT
Ube2E3_F	C ACCATGTCCAGTGATAGGCAA	Ube2E3_R	TTATGTTGCGTATCTCTTGGT
UBE2F_F	caccATGCTAACGCTAGCAAGT	UBE2F_R	TCATCTGGCATAACGTTTGTAT
UBE2G1_F	caccATGACGGAGCTGCAGTCG	UBE2G1_R	TCACTCAAAGCAGTCTCTTG
UBE2H_F	caccATGTCATCTCCAGTCCG	UBE2H_R	CTACAACCTCCATATCTGGGC
Ube2I_F	caccATGTTCGGGATCGCCCTC	Ube2I_R	TTATGAGGGCGCAAACCTTCTT
Ube2K_F	caccATGGCCAACATCGCGGTG	Ube2K_R	TCAGTTACTCAGAAGCAATTC
UBE2L3_F	caccATGGCGGCCAGCAGGAGG	UBE2L3_R	TTAGTCCACAGTTCGCTTTTC
Ube2L6_F	caccATGATGGCGAGCATGCGA	Ube2L6_R	TTAGGAGGGCCGGTCCACTCC
UBE2M_F	caccATGATCAAGCTGTTCTCG	UBE2M_R	CTATTCAGGCAGCGCTCAAA
Ube2N_F	caccATGGCCGGGCTGCCCCGC	Ube2N_R	TTAAATATTATTCATGGCATA
Ube2q2_F	caccATGTCCGTGTCAGGGCTC	Ube2q2_R	TTAGCCATCTTCTTTGGAGG
UBE2R1_F	CACCATGGCTCGGCCGCTAGTGCCC	UBE2R1_R	TCAGGACTCTCCGTGCCAGAGTC
UBE2S_F	C ACCATGAACTCCAACGTGGAGAAC	UBE2S_R	CTACAGCCGCCGACGCGCCC
UBE2T_F	caccATGCAGAGAGCTTCACGT	UBE2T_R	CTAAACATCAGGATGAAATTT

(Continues)

TABLE 1 (Continued)

Fw		Rv	
UBE2U_F	caccATGCACGGCAGAGCTTAC	UBE2U_R	TCATTTTAAATTCCATTCTTT
Ube2v1_F	caccATGCCAGGAGAGGTTCAA	Ube2v1_R	TTAATTGCTGTAACACTGTCC
UBE2V2_F	caccATGGCGGTCTCCACAGGA	UBE2V2_R	TTAATTGTTGTATGTTTGCC
Ube2v3_F	caccATGAAGGAAGACTTGAAC	Ube2v3_R	TTAATTGCTGTAACACTGTCC
UBE2W_F	caccATGGCGTCAATGCAGACC	UBE2W_R	TCAACAAGTATCATCATGATA
Ube2z_F	caccATGGCGGAGAGTCCGACT	Ube2z_R	CTAAACCCTCAGGCTCCCATG
Ft1_F	caccATGAACCCTTTCTGGAGC	Ft1_R	TTAAGTCGCCACTGTTTCTC
TSG101_F	caccATGGCGGTGTCGGAGAGC	TSG101_R	TCAGTAGAGGTCCTGAGACC

Good-growing colonies were selected and transformed with the prey plasmids and pAct2-gtwy and selected on selection media (SD –Trp/–Leu). Three independent colonies were mixed and suspended in water, and the mixture was spotted onto selection media (SD –Trp/–Leu) and incubated at 30°C for 24 h and replicated on selection media (SD –Trp/–Leu) and (SD –Trp/–Leu/–His plus 1 and 5 mM 3-amino-1, 2, 4-triazole (3-AT; Merk, Darmstadt, Germany)), and incubated for 3 days.

2.2 | Cell culture and plasmid transfection, and siRNA transfection

HeLa cells were grown under standard conditions in DMEM supplemented with 10% fetal bovine serum (FBS), penicillin, and streptomycin. U2OS cells were grown under standard conditions in RPMI media supplemented with 10% FBS and penicillin and streptomycin. U2OS AAVS1-tetR cells were generated in the AAVS1 locus using the CRISPR-Cas9 system using pZDonor-AAVS1 Puromycin plasmid (Merk) containing tet repressor (tetR) and puromycin resistant gene cassette between AAVS1 exons. Stable cells expressing RNF213 protein were the selected antibiotic, and protein expression was confirmed by western blotting.

HUVEC cells were purchased from Thermo Fisher Scientific (lot # 883826) medium 200 supplemented with low serum growth supplement (Thermo Fisher Scientific). Lipofectamine LTX (Thermo Fisher Scientific) was used for plasmid transfection. Lipofectamine RNAi Max (Thermo Fisher Scientific) was used for siRNA treatment. siRNA duplexes to repress RNF213 (sc-94184, Santa Cruz Biotechnology, Dallas, TX), control (sc-37007, Santa Cruz Biotechnology), and Ubc13 (sc-43551, Santa Cruz Biotechnology) were transfected using Lipofectamine RNAiMax (Thermo Fisher Scientific) according to the manufacturer's instructions. The cells were seeded into 6-well plates, and after 6 or 12 h, the 1.5×10^5 of cells were replated into 6-well plates, and then, analyzed 30–60 h after transfection.

2.3 | Immunoprecipitation and western blotting

Harvested cells were washed once with PBS (pH 7.2) without calcium and magnesium and lysed in NP-40 lysis buffer (50 mM HEPES, pH 7.4, 250 mM NaCl, 1% NP-40, 5 mM β -glycerophosphate, 1 mM PMSF, 2 mM Na_3VO_4 , 0.2 mM EDTA, 10 mM NaF, 1 mM dithiothreitol and protease inhibitor cocktail (Nacalai Tesque, Kyoto, Japan). Cell lysates were incubated at 4°C for 20 min and centrifuged at 13,200 rpm for 15 min. For immunoprecipitation of EGFP-tagged proteins, the supernatants were incubated with anti-GFP antibody conjugated to agarose beads (MBL, Nagoya, Japan) for 4 h at 4°C. The immunoprecipitates were washed once with NP-40 lysis buffer, twice with NP-40 lysis buffer without NaCl, and subjected to western blotting. Antibodies against RNF213¹⁴ or GFP (Roche, Basel, Switzerland) were used at a concentration of 0.5 $\mu\text{g}/\text{ml}$. The antibody against β -Actin (FUJIFILM Wako Pure Chemistry, Osaka, Japan) was used at the recommended dilution. For immunoprecipitation of the RNF213 protein, the supernatants were incubated with anti-RNF213 antibody at 4°C. After 4 h, the precleaned beads (Santa Cruz Biotechnology) were added and incubated for 4 h at 4°C. The immunoprecipitates were washed once with NP-40 lysis buffer, twice with NP-40 lysis buffer without NaCl, and subjected to western blotting. For immunoprecipitation of RNF213 protein to observed ubiquitinated RNF213 protein, solubilized cell lysates by SDS-sample buffer were incubated with anti-TurboGFP (Evrogen, Moscow, Russia) antibody at room temperature. After 4 h, the precleaned beads (Santa Cruz Biotechnology) were added and incubated for 4 h at room temperature. The immunoprecipitates were washed three times with SDS-sample buffer without dye and subjected to western blot. Other antibodies, anti-TurboGFP, anti-UBC13 (Cell Signaling Technology, Danvers, MA), anti-Flag (Nacalai Tesque), anti-HA (Covance, Denver, CO), anti-K63-linkage-specific polyubiquitin (Cell Signaling Technology), and anti-K48-linkage-specific polyubiquitin antibody (Cell Signaling Technology) were used at a concentration of 1 $\mu\text{g}/\text{ml}$.

2.4 | Assessment of angiogenic activity

Endothelial tube formation was assessed as described previously^{37,38} HUVEC cells (10,000 cells/well) were seeded onto Geltrex Reduced Growth Factor Basement Membrane Matrix-coated (Thermo Fisher Scientific) μ -Slide Angiogenesis (ibid, Martinsried, Germany). Cells were incubated for 18 h at 37°C, and digital images of the tubes were captured at the indicated time points. For quantification, the tube area, total tube length, and the number of tube branches were calculated using ImageJ software (National Institute of Health, Bethesda, MD). Three independent tube formation assays were conducted.

Migration assays were performed as previously described.³⁹ HUVECs or U2OS cells were grown to over-confluence in Culture-Inserts 2 Well (ibid), and then, incubated overnight. After removing the insert to create a wound, the medium was added, and the wound was allowed to narrow for healing for 18 h. Digital images were obtained every 2 h, and the area of re-endothelialization was calculated using Image J software.

2.5 | Invasion assay

The cell culture inserts in the invasion chamber plate (Thermo Fisher Scientific) were coated with Geltrex hESC-qualified Ready-To-Use Reduced Growth Factor Basement Membrane (Thermo Fisher Scientific) following the manufacturer's manual. HUVEC cells (10,000 cells/insert) were seeded onto each insert and incubated for 48 h at 37°C. After two days, the media was aspirated from the inside of the insert. The interior of the inserts was gently wiped with cotton-tipped swabs to remove noninvasive cells. The inserts were transferred to a fresh well plate and fixed with 5% glutaraldehyde solution and stained with crystal violet (1% crystal violet in 2% ethanol). The stained inserts were washed with tap water several times. The inserts were air-dried, and digital images of invaded cells were captured.⁴⁰ For U2OS cells, the cell culture inserts in the invasion chamber plate (Thermo Fisher Scientific) were coated with collagen type I (Nippi, Tokyo, Japan) following the manufacturer's manual. The cells (10,000 cells/insert) were seeded onto each insert and incubated for 48 h at 37°C.

3 | RESULTS

3.1 | Structure of the RNF213 RING domain

RNF213 gene was initially characterized as a susceptibility gene and encoded a 591 kDa protein that possesses two Walker motifs and a RING domain.^{10,11} Sequence

alignment of the RING domain of RNF213 orthologs indicated that the RING domains of RNF213 belonged to RING-HC subclass C3HC4-type (Figure S1(A)).^{36,41} Amino acids with bulky side chains in the RING domain were relatively conserved in RNF213 orthologs and E3 ligase with C3HC4-type RING like BRCA1. In our previous work, ectopic-expressed RNF213 in HEK293 cells was efficiently ubiquitinated, but not the RING mutant.¹⁰ These data indicate that RNF213 is an E3 ubiquitin ligase and may interact with specific E2 enzymes to ubiquitinate the target protein and/or itself.

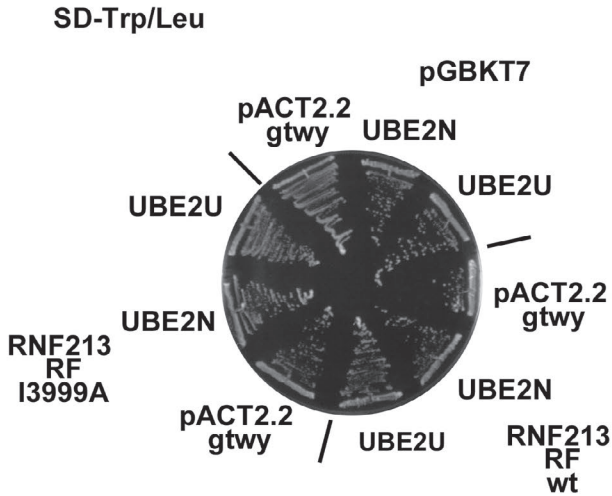
3.2 | Identification of E2 enzymes bound to the RNF213 RING domain

Functional and selective E2-E3 interactions are required for the efficient ubiquitination of the target protein. A growing number of reports show that the E2-E3 selective interaction can achieve specific mono- and/ or polyubiquitination.^{17,21,25,30,33,41} To understand RNF213 cellular functions and MMD, we used the fragment containing the RING domain (3951-4107 amino acid region of RNF213) of RNF213 as bait in a directed yeast two-hybrid screen with 23 human E2 enzymes as prey [Figure S2(A)-(D)]. The 23 plasmids cloned E2 enzymes were transformed with RNF213 RING in pGBKT7 or empty vector (pGBKT7), respectively, and these yeast clones grew on selective media [SD –Tryptophan (Trp)/ –Leucine (Leu)]. Good-growing independent colonies were replicated onto another selective media [SD –Trp/–Leu and –Histidine (His) plus 1 or 5 mM 3-AT (3-amino-1,2,4-triazole)]. Two E2 enzymes, UBE2N (UBC13) and UBE2U, introduced into yeast with RNF213 RING could grow on the selective media (SD –Trp/–Leu/–His +1 mM 3-AT) well, although the other E2 enzymes and the empty vector introduced into yeast with RNF213 RING or empty vector could not grow anymore [Figure S2(A)-(D)]. These data indicated that UBE2N (UBC13) and UBE2U are good candidates for specific E2 enzymes interacting with RNF213 E3 ligase.

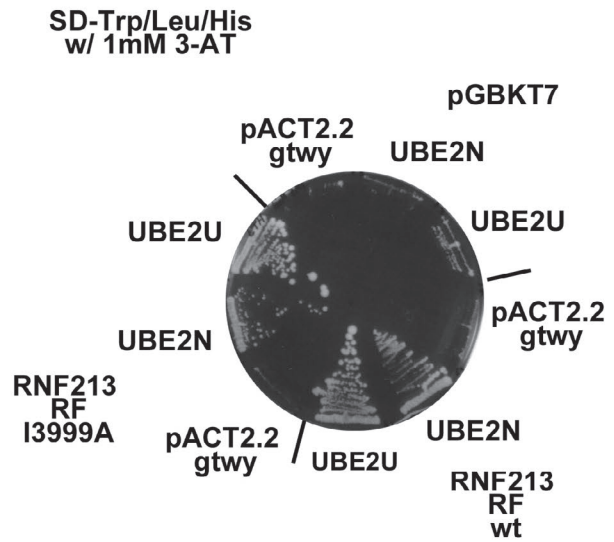
3.3 | The specificity of interaction between two E2 enzymes and RNF213

Structural and functional analysis showed that several mutations in the RING domain disrupt the E2-E3 interaction and result in the loss of E3 ligase activity^{22,30,32,34}). To confirm the specific E2-E3 interaction, Isoleucine at the 3999 amino acid position in the RNF213 RING domain [Figure S1(A),(B)], corresponding to the BRCA1 RING mutant,^{29,30,34,41–43} was changed into Alanine. This mutant in the two-hybrid assay with UBE2 N (UBC13) reduced

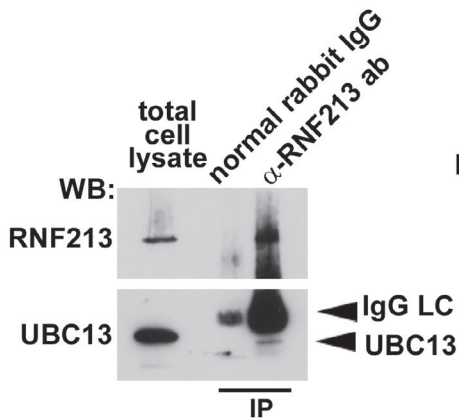
(A)



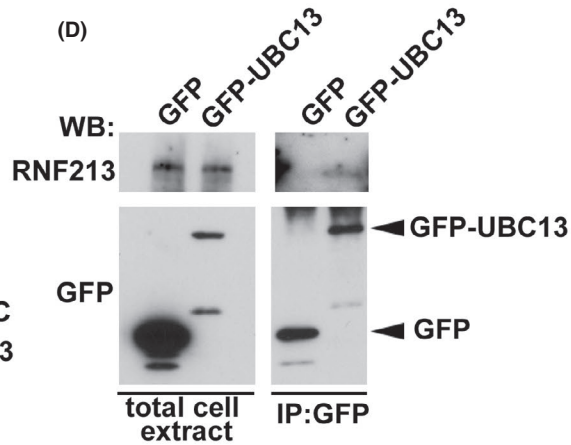
(B)



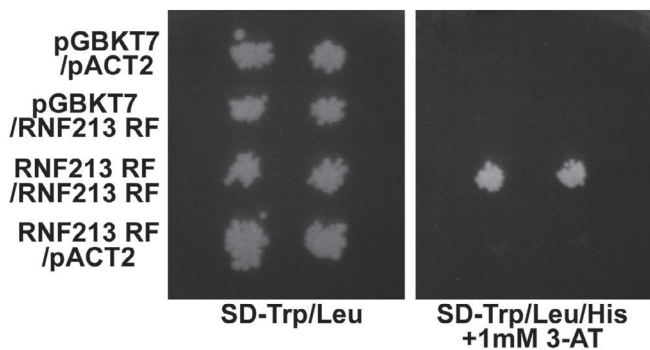
(C)



(D)



(E)



(F)

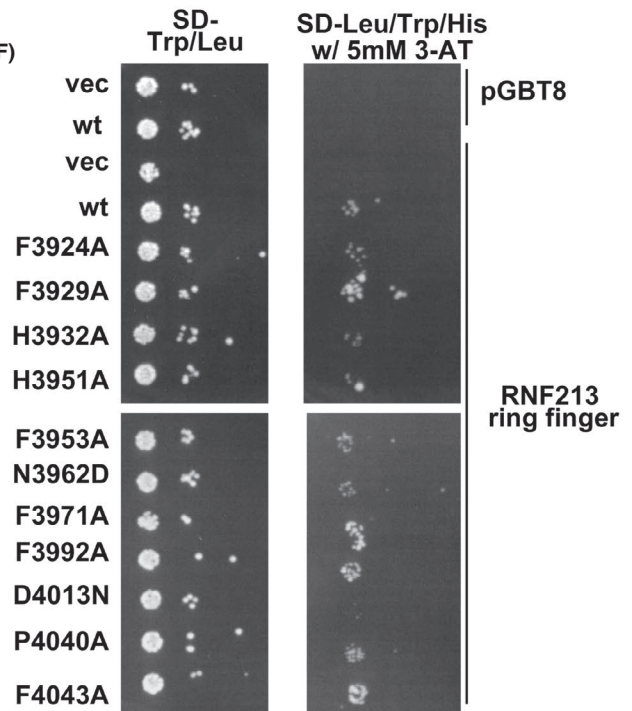


FIGURE 1 RNF213 can bind to UBC13 E2 enzymes. (A) Confirmation of principle E2 enzyme screening. (B) Each positive (UBE2N and UBE2U) transformants with wt of RNF213 RING fragment or I3999A mutant or empty vector as bait were streaked on SD media (–Trp/–Leu) (left), and replica plated on selective media (–Trp/–Leu/–His +1 mM 3-AT)(right). (B) UBC13 binding to RNF213 protein. The cell extracts were prepared for immunoprecipitation with anti-RNF213 antibody or normal rabbit IgG, followed by western blotting with anti-UBC13 antibody. The expression level of each protein was monitored by western blotting with anti-RNF213 and UBC13 antibodies. (C) RNF213 binding to UBC13 protein. GFP, GFP-tagged UBC13 was overexpressed in HeLa cells, and the expression level of each protein was monitored by western blotting with anti-GFP antibody for GFP-tagged proteins, with anti-RNF213 antibody for loading controls. The cell extracts overexpressed in each protein were prepared for immunoprecipitation with anti-GFP antibody-conjugated beads, followed by western blotting with anti-RNF213 and GFP antibodies. (D) RNF213-RNF213 homo-dimer formation. Yeast two-hybrid assay was performed with the RNF213 RING fragment as bait and prey plasmids. Each positive (wt of RNF213 RING fragment and empty vector) transformant with wt of RNF213 RING fragment or empty vector as bait were spotted onto SD media (–Trp/–Leu) (left), and replica plated on selective media (–Trp/–Leu/–His +1 mM 3-AT)(right). (E) Mutation scanning assay for RNF213-RNF213 homo-dimer formation. Yeast two-hybrid assay was performed with the RNF213 RING fragment as bait and the mutated fragment prey plasmids. Each positive (wt of RNF213 RING fragment and empty vector) transformant with wt of RNF213 RING fragment or each mutated fragment or empty vector as prey were spotted onto SD media (–Trp/–Leu) in the three stepwise diluted conditions (left), and replica plated on selective media (–Trp/–Leu/–His +1 mM 3-AT) (right)

the growth on selective media, but not with UBE2U. [Figure 1(A),(B)] This result indicated that UBE2 N (UBC13) could specifically interact with the RNF213 RING domain, and UBE2U may interact with RNF213 in a RING-independent manner.

3.4 | RNF213-UBC13 interaction in HeLa cells

To further confirm RNF213-UBC13 interaction *in vitro*, undenatured cell extracts from HeLa cells were subjected to co-immunoprecipitation assay with anti-RNF213 antibody. UBC13 was detected in the immune complex of RNF213 but not in the control rabbit IgG [Figure 1(C)]. Moreover, undenatured cell extracts from Rnf213 or Ubc13-knockdown cells and EGFP-tagged UBC13 expressed cells were subjected to immunoprecipitation. The level of UBC13 protein decreased in the RNF213 immune complex using Rnf213- or Ubc13-knockdown cell extract compared with control siRNA-transfected cells (Figure S3(G)). Furthermore, using undenatured cell extracts from EGFP-UBC13 expressing HeLa cells, RNF213 was detected in the EGFP-UBC13 complex, but not in the EGFP complex [Figure 1(D)]. Using undenatured cell extract from 3xFlag-tagged RNF213 expressing HeLa cells, UBC13 was detected reciprocally in the anti-Flag IgG immune complex but not in the control rabbit IgG [Figure S3(H)]. These results indicated that RNF213 could form the complex with UBC13 *in vivo* and *in vitro*.

3.5 | RNF213 can form a homo-dimer in vitro

Many E3 ubiquitin ligases can form homo- and heterodimers for ubiquitination activities.^{9,19,20,22,28,32,34} To address the homo-dimer formation of RNF213 protein, yeast two-hybrid assay using RING fragment of RNF213 as bait and

prey plasmids was performed under the same conditions as the E2 enzyme screen condition [Figure 1(E)]. The yeast clone with the RING fragment of RNF213 could grow with the RING fragment as prey on SD media (–Trp/–Leu/–His +1 mM 3-AT), but not with the empty vector as prey. To determine the region responsible for the homo-dimer formation of RNF213 protein, yeast two-hybrid assay was performed using a series of deletion mutants of RNF213 [Figure S2(E),(F)]. The yeast cells with RNF213 RING fragment containing 97 amino acids (3939–4036 amino acid of RNF213) could only grow on selective media, but not with RNF213 RING fragment containing 95 amino acids (3902–3996 amino acid of RNF213), 63 amino acids (3996–4058 amino acid of RNF213), or 41 amino acids (3996–4036 amino acid of RNF213). The mutation scanning experiment using two-hybrid assay with the RING fragment of RNF213 showed that D4013N amino acid substitution remarkably reduced the cell growth on selective media [Figure 1(F)]. These results indicated that RNF213 protein could form a homo-dimer through the RING domain, and amino acids between the third and fourth Zn²⁺ coordinating residues of the RING domain were responsible for dimer formation.

3.6 | Stability of RNF213 protein in vivo

K11, K27, K29, K33, and K48-linked polyubiquitinations are recognized by the proteasome and subsequently lead to degradation, but not K63-linked autoubiquitination.^{18,21,23} To test the stability of RNF213 protein, RNF213 protein level was monitored using soluble extracts from cycloheximide (CHX)-treated HeLa cells. The protein levels of RNF213 was not affected by CHX treatment for 4 h [Figure S3(A)]. Furthermore, the protein level of RNF213 was also monitored using the soluble extract from MG132 proteasome inhibitor-treated HeLa cells. The RNF213 protein level also did not change after the treatment [Figure S3(B)]. These data indicated that RNF213 is a stable protein and may not be

ubiquitinated through K11, K27, K29, K33, and K48-linked ubiquitination.

3.7 | K63-linked ubiquitination of RNF213 dependent on UBC13

Yeast-two-hybrid analysis indicated that RNF213 might ubiquitinate the substrates through the K63-linked polyubiquitin chain. The RNF213 was a stable protein and was not affected by proteasome pathway. Moreover, using denatured total cell lysate, slow-migrating smear bands reacted with anti-RNF213 antibody, and the reactivity was dependent on the amount of cell lysate [Figure S3(D)]. These observations suggested that these slow-migrating smear bands corresponded to RNF213 protein, and RNF213 might be ubiquitinated through K63 ubiquitin. To test this possibility, immunoprecipitation assay using denatured total cell lysate was performed with K48- and K63-linkage-specific polyubiquitin antibodies and subjected to western blotting with anti-RNF213 antibody. Reciprocally, immunoprecipitation assay with anti-RNF213 antibody was performed and subjected to western blotting with K48- and K63-specific polyubiquitin antibodies under similar conditions [Figure S3(I)]. The immunoprecipitates by K63-linkage-specific polyubiquitin antibody could react with anti-RNF213 antibody, compared with K48-linkage-specific polyubiquitin antibody and normal IgG [Figure 2(A)]. It might not be easy to separate the modified from unmodified bands of RNF213 protein because the protein is a high molecular weight protein (over 500 kDa). The highest reactivity band of RNF213 might be ubiquitinated in addition to the slow migrating bands because the immunoprecipitates were pull down by the polyubiquitin antibody. However, p53 protein, which is well known as ubiquitinated through the K48-linked ubiquitin chain, was detected efficiently in the K48-linkage-specific polyubiquitin antibody. Using USP2 deubiquitinase to remove the linked-ubiquitin chain of RNF213 protein, the slow migrated smearing K63-specific bands in immunoprecipitates with anti-RNF213 antibody

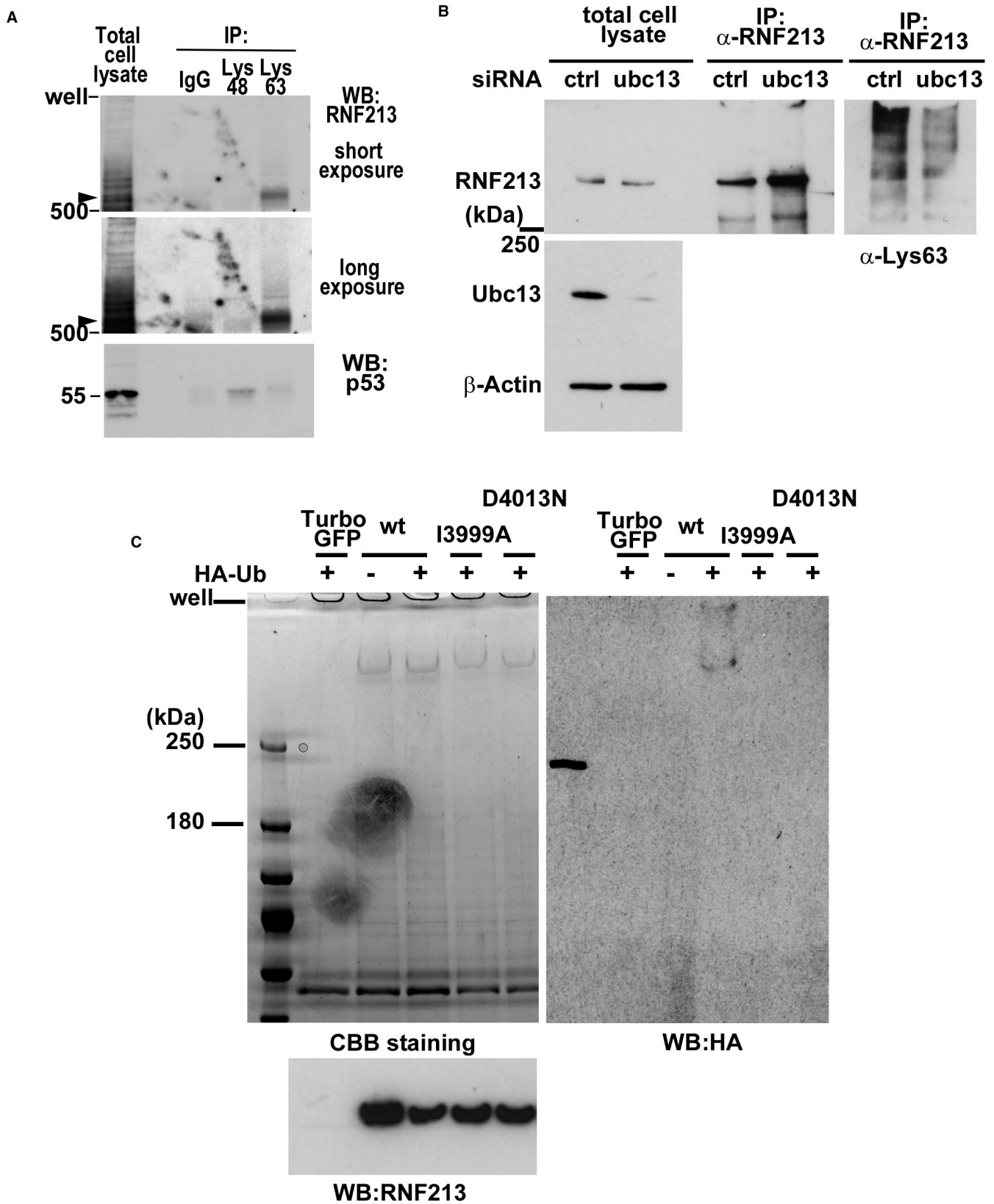
was dramatically reduced [Figure S3(E)]. The incomplete deconjugation of the modification bands might be due to using denatured protein or the accessibility to the substrate of USP2 enzyme. The optimization will be required to understand the ubiquitination pattern. To confirm the K63-linked ubiquitination of RNF213, denatured extracts from Ubc13-siRNA-treated HeLa cells were subjected to immunoprecipitation with anti-RNF213 antibody. Treatment of HeLa cells with Ubc13 siRNA reduced the level of UBC13 by up to 10% of the control level [Figure 2(B)]. The K63-linked ubiquitin chain band intensity from Ubc13 siRNA treated cells was lower than that from the control siRNA treatment (slow migration bands/RNF213 band ratio; control siRNA: Ubc13 siRNA = 1: 0.16) [Figure 2(B)].

Furthermore, *in vitro* auto-ubiquitination assay using purified Myc-tagged RNF213 protein was performed to test RNF213 autoubiquitination. The ubiquitination signal of Myc-tagged RNF213 protein was detected in the presence of His-tagged UBC13 and GST-fusion UBE2V1 proteins as E2 conjugating enzymes, but not in the absence of the E2 enzymes or ATP/magnesium [Figure S3(F)]. These results indicated that this ubiquitination was dependent on UBC13-UBE2V1, and RNF213 might be autoubiquitinated through K63 of ubiquitin.

3.8 | RING mutations of RNF213 abolished the ubiquitination activity of RNF213 protein

The substitutions of an amino acid (I3999A and D4013N) within the RING domain of RNF213 protein reduced the UBC13 interaction and homo-dimer formation, respectively. Isoleucine's position at the 3999 amino acid is responsible for ubiquitination and autoubiquitination corresponding to the BRCA1 RING mutant [Figure S1(A),(B)].^{19,30,41,42,44} These mutants tagged with TurboGFP protein were ectopically expressed with HA-tagged ubiquitin in U2OS cells. The total cell extracts were used for immunoprecipitation with anti-TurboGFP antibody, and the immunoprecipitates were subjected to western blotting with anti-HA antibody

FIGURE 2 RNF213 protein has K63-linked ubiquitination activity. (A) RNF213 was ubiquitinated through K63-linked polyubiquitin. The cell lysates were prepared for immunoprecipitation with anti-K63- or K48-linkage-specific polyubiquitin antibodies or normal rabbit IgG, followed by western blotting with anti-RNF213 antibody. The time for Long exposure was ten times longer than that for short exposure. The bottom panel showed the blotting with anti-p53 antibody (DO-1) using the same blotting membrane. (B) K63-linked polyubiquitination of RNF213 was dependent on UBC13. The cell lysates from siRNA-treated cells were prepared for immunoprecipitation with anti-RNF213 antibody, followed by western blotting with anti-K63-linkage-specific polyubiquitin antibodies. The expression and immunoprecipitant levels of each protein were monitored by western blotting with the anti-RNF213 and anti-UBC13 antibodies for UBC13 knockdown efficiency, with anti- β -Actin antibody for loading controls. (C) The ubiquitination of RNF213. The cell lysates from TurboGFP, TurboGFP-tagged wild-type, or I3999A or D4013N overexpressing cells with HA-tagged ubiquitin were prepared for immunoprecipitation with anti-TurboGFP antibody, followed by western blotting with anti-HA antibody (right). CBB staining showed the immunoprecipitants with anti-TurboGFP antibody (left), and the immunoprecipitants were normalized by western blotting with anti-RNF213 antibody



to detect RNF213 ubiquitination [Figure 2(C)]. The immunoprecipitant from the TurboGFP-tagged mutants (I3999A and D4013N) did not react with anti-HA antibody similar to that from negative control TurboGFP. However, the TurboGFP-tagged wild-type (wt) immunoprecipitant reacted

with anti-HA antibody [Figure 2(C) right]. These amino acids might be essential for catalyzing the ubiquitination of substrates of RNF213. These results indicated that the disruption of the binding with UBC13 and the homo-dimer formation, abolished the ubiquitination.

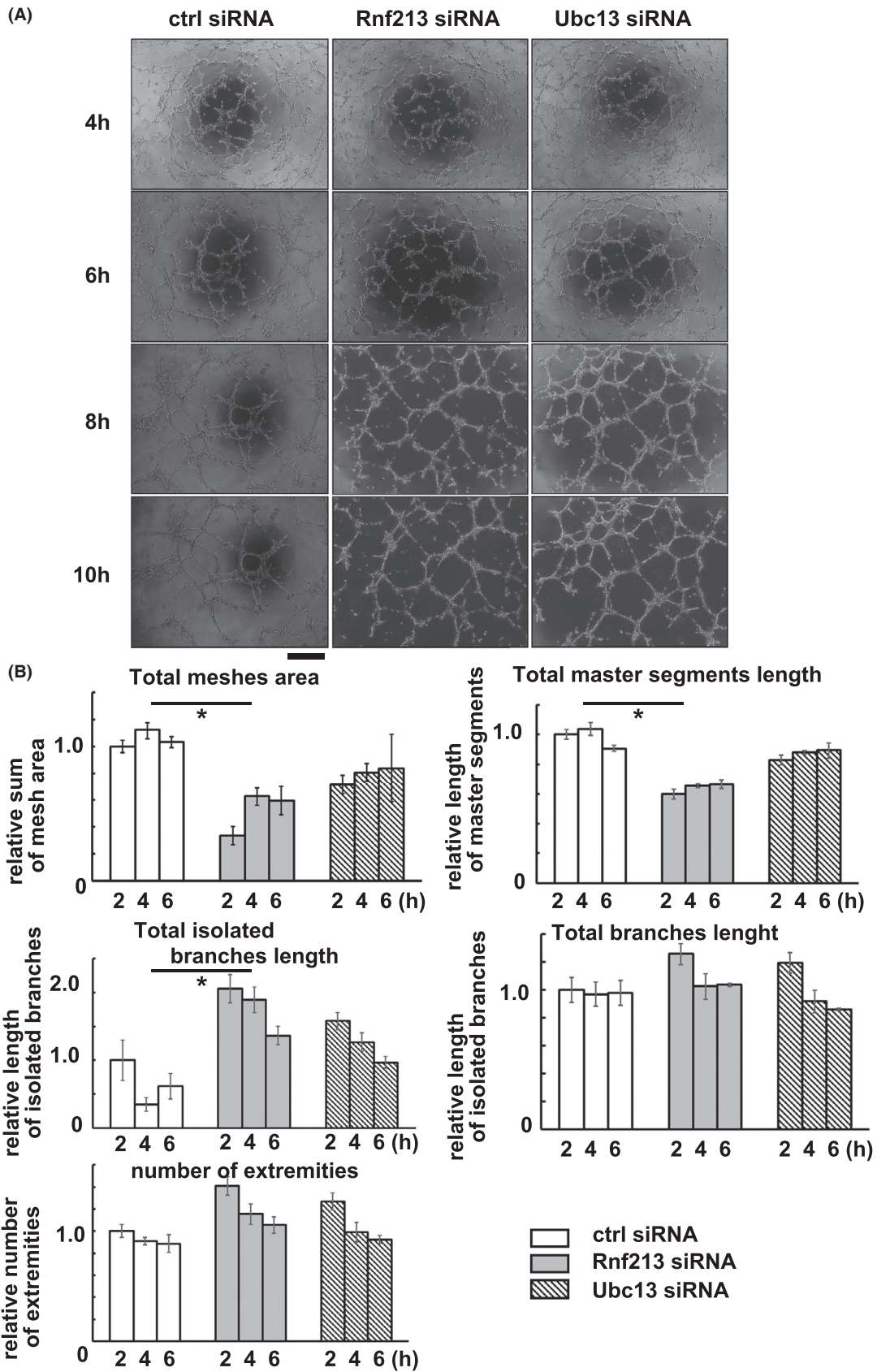


FIGURE 3 The time course of tube formation activity by UBC13-RNF213 axis in HUVECs. (A) Tube formation assays for siRNA-treated HUVECs. The siRNA-treated HUVEC cells were seeded onto the matrix, and digital images were captured at the indicated times. The scale bars indicate 100 μm . (B) Analysis of tube formation assays. The tube areas per field of the digital image were analyzed using ImageJ software ($n = 3$; $*p < 0.05$, by Student's t -test). Similar results were obtained for the total tube length and number of branches

3.9 | Ubiquitination activity was responsible for the angiogenic activity of endothelial cells

To address the responsibility of ubiquitination activity of the RNF213 protein, Ubc13 or Rnf213 siRNA-treated HUVEC cells were subjected to a tube formation assay on a matrix, and the time-dependent tube formation was monitored at 4, 6, 8, and 10 h from the start of the test for 18 h [Figure 3(A),(B)]. Ubc13 and Rnf213 siRNA-treated HUVEC cells showed reduced tube network formation compared with the control siRNA-treated HUVEC cells (ctrl) between 2 and 8 h from the assay start point [Figure 3(A)]. Image analysis using ImageJ software showed that network mesh and total master segments length decreased compared with the control experiment between 2 and 8 h. On the contrary, total isolated branches length and the number of extremities increased in Ubc13 or Rnf213 siRNA-treated HUVEC cells between 2 and 6 h [Figure 3(B)]. Flag-tagged RNF213 wt, ubiquitination-defective I3999A, and D4013N mutants were ectopically expressed in HUVEC cells [Figure S4(A),(B)] and subjected to a tube formation assay on a matrix. Ubiquitination defective I3999A and D4013N mutants expressing HUVEC cells showed reduced mesh network formation and increased branch length and number between 2 and 4 h from the assay start point, compared with the wild-type Flag-tagged RNF213 protein-expressing HUVEC cells.

Furthermore, a wound healing assay was performed using siRNA-treated HUVEC cells, Flag-tagged RNF213 wt, ubiquitination-defective I3999A, and D4013N mutants expressing HUVEC cells. The Ubc13 or Rnf213 siRNA-treated HUVEC cells showed lower healing activity than control siRNA-treated HUVEC cells [Figure 4(A),(B)]. The rate of tube formation in Ubc13 and RNF213 siRNA-treated cells was similar to each other. The ubiquitination-defect mutants also showed lower wound healing activity than the wild-type RNF213 expressing HUVEC cells [Figure 4(C),(D)]. These results indicated that RNF213 dependent K63-linked ubiquitination might influence angiogenic activity in HUVEC cells. The RNF213-UBC13K63-linked ubiquitination axis may influence angiogenic activity. Although MMD shows endothelial cell-specific defects, these RNF213 dependent ubiquitination effects were studied using U2OS cells (Human bone osteosarcoma epithelial cells) by wound healing assay [Figure 4(E),(F)]. Surprisingly, ubiquitination defect mutants expressing U2OS cells showed increased migration activity compared with a wild-type of RNF213 or control U2OS cells [Figure 4(E),(F)]. These findings indicated that RNF213

dependent K63-linked ubiquitination might play different roles between endothelial cells and epithelial cells.

3.10 | The ubiquitination activity of RNF213 protein inhibited the invasion activity of endothelial cells

An invasion assay using a matrix-coated two-phase culture dish was performed with Flag-tagged RNF213 wt and mutants expressing HUVEC cells. Flag-tagged mutated RNF213 expressing HUVEC cells could invade efficiently through the matrix-coated membrane, but not the Flag tag only, and Flag-tagged wild-type RNF213 [Figure 5(A),(B)]. These results indicated that the ubiquitination activity of RNF213 protein was required for the movement of endothelial cells but inhibited the invasion activity of HUVEC cells.

Furthermore, invasion assay using a matrix-coated chamber showed that the number of invaded cells through the matrix was reduced by expression of the ubiquitination-defect I3999A mutant compared with control cells, despite the increasing number of invaded cells in wild-type RNF213 expressing U2OS cells. These results showed that the I3999A mutant had lower invasion activity than control or wild-type RNF213 expressing U2OS cells [Figure 5(C),(D)]. These findings indicated that RNF213 dependent K63-linked ubiquitination might play different roles between endothelial cells and epithelial cells, and ubiquitination activity of RNF213 protein might regulate both migration and invasion activity.

4 | DISCUSSION

RNF213 is a large protein that encodes AAA+ATPase (Walker domain) and the RING domain. Our previous work demonstrated that the AAA+domain possessed ATP hydrolysis activity *in vitro*, and ectopically expressed RNF213 protein efficiently ubiquitinated itself, but not RING deleted RNF213.¹⁰ Two functional domains of RNF213 may contribute to its physiological functions. In this study, RNF213 interacted with UBC13 (UBE2N) and UBE2U in yeast two-hybrid assay, and UBC13 could bind to RNF213 in the manner of RING structure, but not UBE2U. The UBC13-RNF213 interaction may contribute to the selective ubiquitination of the target protein. UBC13 forms heterodimers with other E2 enzymes, UBE2V1 (Uev1A) and UBE2V2 (Mms2), and catalyzes the synthesis of polyubiquitin chains that are linked through K63 of ubiquitin.^{26–28,44} The UBC13-UBE2V1

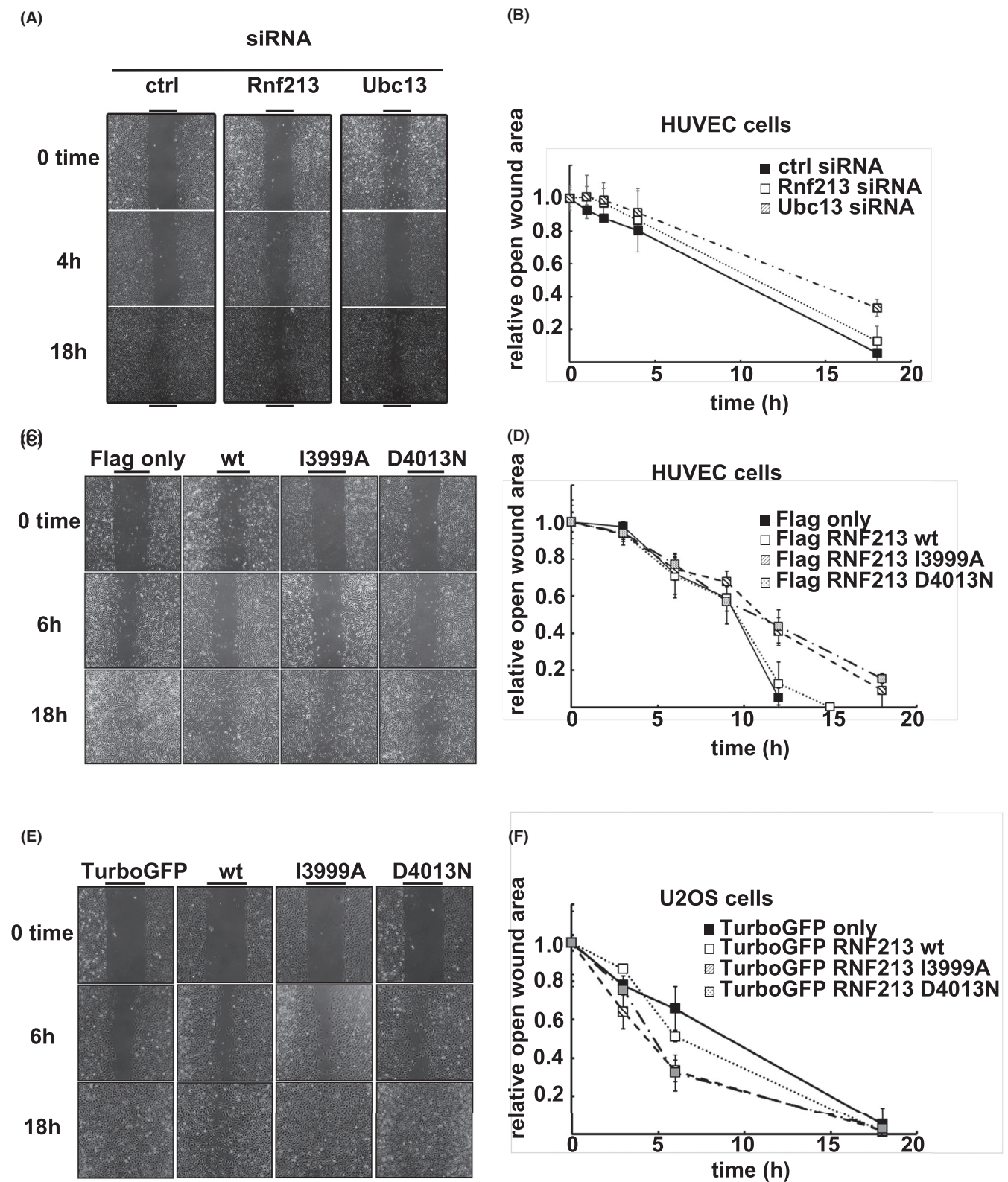


FIGURE 4 The cell migration activities of UBC13-RNF213 axis. Migration assays for HUVEC cells after treatment with siRNAs. The scale bars indicate 100 μ m. (A) Analysis of migration assays for HUVEC cells. The open wound areas were quantified using ImageJ software. Data represent the mean \pm SD ($n = 3$; $*p < 0.05$, by Student's t -test compared with control siRNA-treated cells). (B) Migration assays for HUVEC cells overexpressing Flag, wt, I3999A, or D4013N mutants of RNF213 protein. The scale bars indicate 100 μ m. (C) Analysis of migration assays for HUVEC cells. The open wound areas were quantified using ImageJ software. Data represent the mean \pm SD ($n = 3$). (D) Migration assays for U2OS cells overexpressing TurboGFP, wt, I3999A, or D4013 N mutants of TurboGFP-tagged RNF213 proteins. The scale bars indicate 100 μ m. (E) Analysis of migration assays for U2OS cells. The open wound areas were quantified using ImageJ software. Data represent the mean \pm SD ($n = 3$)

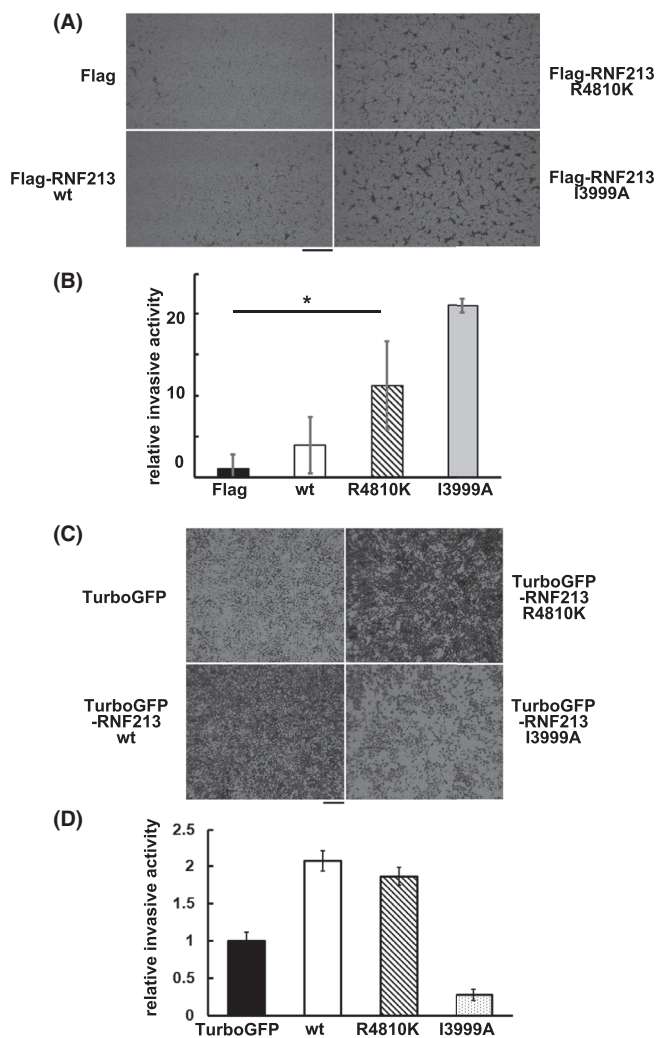


FIGURE 5 The cell invasion activities of UBC13-RNF213 axis. (A) Invasion assays for HUVEC cells overexpressing Flag, wt, I3999A, or R4810K mutants of RNF213 protein. The invaded cells were stained with dye and digital images were captured. The scale bars indicate 100 μ m. (B) Analysis of invasion assays for HUVEC cells. The invaded cells were quantified using ImageJ software. Data represent the mean \pm SD ($n = 3$; $*p < 0.05$, by Student's t -test compared with Flag overexpressing cells). (C) Invasion assays for U2OS cells overexpressing TurboGFP, wt, I3999A, or R4810K mutants of TurboGFP-tagged RNF213 proteins. The invaded cells were stained with dye and digital images were captured. The scale bars indicate 100 μ m. (D) Analysis of invasion assays for U2OS cells. The invaded cells were quantified using ImageJ software. Data represent the mean \pm SD ($n = 3$)

heterodimer forms a complex with TRAF2 and TRAF6 and mediates NF- κ B activation through K63-linked polyubiquitination.^{19,26} The UBC13-UBE2V2 heterodimer forms a complex with E3 protein ligase involving DNA repair and DNA break processing.^{27,28,43,44} The two E2 enzyme variants seem to regulate UBC13-E3 ligase and mediate polyubiquitination through K63 of ubiquitin.^{18,21,43,44} In this two-hybrid screen, interactions between RNF213 and these UBE2V variants

were not observed. Moreover, these variants were not detected in the RNF213 immunocomplex with anti-RNF213 antibody (data not shown). However, *in vitro* ubiquitination assay using recombinant proteins, RNF213 protein was ubiquitinated in the presence of UBC13-UBE2V1 E2 complex. RNF213 protein may bind weakly with these variants or not have an interacting surface with the variants. These indicated that UBE2V1 might be a candidate for E2 enzyme collaborating with UBC13 and RNF213 proteins. Further investigations are required. Studies of the homo-dimer formation through the RING domain of RNF213 and binding with E2 suggested that the asymmetrical RNF213 structure might be necessary for the interaction between RNF213 and the variants. The heterodimer formation with other E3 ligases might facilitate the interaction between RNF213 and UBC13-UBE2V E2 enzymes. The conserved bulky hydrophobic residues were involved in the interaction between the RING domain and E2 as c-Cbl, MDM2, and BRCA1.^{20,30,32,45} Isoleucine at the 3999 amino acid position was also conserved among RNF213 ortholog and RING protein. Mutation of these residues could reduce RING-E2 interaction and lead to decreased ubiquitination activity. This mutation was located between the first and second Zn²⁺-coordinating residues and appeared to lose Zn²⁺ chelating and RING structure. Further studies are required for RNF213-mediated selective E2 binding and ubiquitination activity.

RNF213 protein has two AAA + walker motifs and forms an oligomer, and this hydrolysis activity changes its oligomeric state.⁴⁶ These motifs are located at the N-terminus of RNF213. Like Prp protein, N-terminal walker motifs may involve a higher-order oligomer that has multiple dimerized RING domains.^{9,35} The relationship between oligomerization and RING-mediated dimerization needs to be clarified.

UBC13 can facilitate ubiquitination through K63 ubiquitination and is involved in several signal transduction pathways, unlike K48 linked ubiquitination. RNF213 can interact with UBC13 *in vivo*, *in vitro*, and is autoubiquitinated through K63 well but not K48 in a UBC13-dependent manner. RNF213-UBC13 ubiquitin ligase activity may involve known or unknown signal transduction pathways through K63-linked ubiquitination. In *in vivo* studies, Ubc13 knock-down cells and ubiquitination-deficient mutant expressing cells showed slow tube formation or slow healing in the endothelial cells. Deubiquitinase of the K63-linked ubiquitin chain has been reported to play a critical role in angiogenesis.⁴⁷ The balance between deubiquitinase and RNF213 may be essential for angiogenesis. Ubiquitination linked via the N-terminal methionine and K63 facilitates innate immune signaling initiated by pattern recognition receptors such as toll-like receptors and nucleotide-oligomerization domain-like receptors, and cytokine receptors such as tumor necrosis factor receptor 1 (TNFR-1).^{26,48} The ligands for receptors might be substrates for K63-linked polyubiquitination by RNF213. Indeed,

TNFR-1 controls endothelial inflammatory responses and angiogenic activities.^{49,50}

Matsuoka et al.⁵¹ reported that ATR phosphorylates RNF213 under UV treatment. Indeed, the UBC13-RNF8 or RNF168 ubiquitin ligase complex can facilitate the ubiquitination chain on PCNA and histone H2AX, which are critical molecules in DNA metabolism.^{28,43,44,52} RNF213 collaborating with UBC13 may be involved in DNA replication, DNA repair pathway, and DNA metabolism under control by ATR kinase.^{14,15}

Many motor proteins that encode AAA + domain and Walker motifs, utilize ATP to move along microtubules.^{53–55} For example, Dynein motor protein complex, which is a large multi-subunit protein complex and poses 6 AAA + domains in the large subunit, moves on microtubules with ATP hydrolysis.⁵³ In our previous report, the Walker motif of RNF213 could hydrolyze ATP efficiently *in vitro*.^{10,16} RNF213 protein is a novel AAA + ATPase, forms oligomers, and this hydrolysis activity changes its oligomeric state.⁴⁶ RNF213 encoded two AAA + ATPase modules. The one module regulates the assembly of RNF213 oligomers, while the second one regulates the disassembly of RNF213 oligomers. These modules consist of Walker A and Walker B motifs to hydrolyze ATP. The pR4810K variant of RNF213 forms oligomers like the wild-type, although the variant exhibits lower ATPase activity *in vitro*.⁴⁶ These indicate that this variant does not appear to affect ATP binding and ATP hydrolysis. Previous reports indicated that the R4810K variant affected tube formation and migration activities.^{14,16} In this report, the R4810K variant exhibited higher invasion activity than the wild-type RNF213 in HUVEC cells. Conversely, the ubiquitination defect mutant exhibited more aggressive invasion activity of HUVEC cells, despite the lower migration activity. The relationship between ATPase activity and migration and invasion activity should be investigated further. The AAA + domain of RNF213 may contribute to cell motility by coordinating with K63-linked ubiquitination activity. K63-linked ubiquitination is reported to be required for cell migration.^{26,49,56–58} RNF213 has been reported to surround lipid droplets in fibroblast cells.⁵⁹ RNF213 may play a role in the transportation of cellular lipid droplets. The transport of K63-linked ubiquitinated proteins may contribute to cell motility and invasion.

New blood vessels' growth consists of five steps: tip cell selection, sprout formation, tip cell migration, stalk cell proliferation, and vascular stabilization.^{60–63} A specialized EC, termed the tip cell, exists at the distal end of each sprout. Tip cells have motility, invasion, and high polarization with elongated filopodial protrusions and navigate endothelial sprouts.^{61,62} During angiogenesis, vascular endothelial growth factors (VEGFs)/Notch signaling pathway regulate the tip-stalk cell selection and shuffling.⁶³ Tumor necrosis factor α (TNF- α) induces the tip cell phenotype.^{60,64} The

expression of the tip cell markers platelet-derived growth factor BB (PDGF-BB) and VEGFR2 guides angiogenic sprouting.⁶⁵ A couple of studies have reported that inflammatory cytokines, IFN- γ , TNF- α , and INF- β activate transcription of RNF213.^{16,66} The upregulation of RNF213 transcription by these cytokines in tip cells might activate cell motility and invasion, and in this process, K63-linked ubiquitination might be upregulated dependent on RNF213.

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CONFLICT OF INTEREST

The authors have no conflict of interest directly relevant to the content of this article.

AUTHOR CONTRIBUTIONS

T. Habu and K.H. Harada designed the research and analyzed data; T. Habu performed the research and wrote the paper.

ETHICAL APPROVAL

This article does not contain any studies with human participants or animals performed by any of the authors.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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