

ORIGINAL ARTICLE OPEN ACCESS

RNF213-Dependent EGFR and HER2 Activation Regulates Specific Downstream Signaling Pathways in Human Cancer Cells

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Received: 14 April 2025 | **Revised:** 26 May 2025 | **Accepted:** 3 June 2025

Transmitting Editor: Eisuke Nishida

Funding: This work was supported by the Japan Society for the Promotion of Science (Grant Numbers 17H06397, 25K02752, 20H03790), and from the Japan Agency for Medical Research and Development (Grant Numbers JP21bm0804032h0001, JP22bm0804032h0002, JP23bm0804032h0003). I.M.F. would like to thank the Ichiro Kanehara Foundation Scholarship for Foreign Students in Japan.

Keywords: angiogenesis | cancer | EGFR | HER2 | Moyamoya disease | phosphorylation | PLC γ | RNF213 | Src | vasculopathy

ABSTRACT

In this study, we reveal a novel relationship between RNF213, an E3 ubiquitin ligase associated with Moyamoya disease (MMD) and the ubiquitination of both endogenous and pathogenic substrates, and EGFR, the epithelial growth factor receptor involved in cell growth, angiogenesis, and cancer. RNF213 knockdown or knockout in HeLa and A549 cells markedly reduces EGFR phosphorylation at key tyrosine sites following EGF and TGF α stimulation. In RNF213 knockout cells, HER2 phosphorylation, typically activated through heterodimerization with EGFR, and Src recruitment and/or phosphorylation are also diminished. Mutations in the RNF213 RING, RZ finger, or AAA+ domains, including the prevalent R4810K mutation in MMD, consistently reduce EGFR phosphorylation. In vivo, EGF injections increase EGFR and HER2 phosphorylation in WT but not in RNF213 knockout mice. Despite the reduced phosphorylation levels of these tyrosine kinases in knockout cells, the activation of downstream signals such as AKT, ERK1/2, and STAT3 remains unaffected, although phosphorylation of PLC γ , a key mediator of Ca²⁺ release, is selectively reduced by RNF213 knockout. These findings demonstrate that RNF213 modulates EGFR-related pathways and specific downstream signal pathways, possibly affecting physiologic and pathogenic angiogenesis, and may have implications for unraveling the etiology of MMD and for developing cancer therapies that target RNF213.

1 | Introduction

Moyamoya disease (MMD) is a rare, progressive cerebrovascular disorder characterized by the narrowing of the internal carotid arteries and compensatory formation of a fragile network of collateral vessels at the brain base, which predisposes

patients to ischemic and hemorrhagic strokes (Ihara et al. 2022). A landmark discovery in this field was the identification of the ring finger protein 213 gene (*RNF213*) as a major susceptibility factor for MMD (Liu et al. 2011). *RNF213* encodes a large and unique protein with both E3 ubiquitin ligase and AAA+ ATPase activities that cooperate to ubiquitinate various intracellular and

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pathogenic factors and target them for degradation or for modulation of cellular signaling (Ahel et al. 2020). The E3 ligase activity is associated with both its RING domain and RZ finger, and the ATPase functions reside in two of its six AAA+ dynein core domains (Bhabha et al. 2014; Ahel et al. 2020).

Mutations in RNF213, such as the p.R4810K variant that is particularly prevalent in East Asian populations, together with the 50 or so other mutations that are predominantly spread in and around its RING and C-terminal regions, are thought to disrupt endothelial cell functions and alter angiogenesis and vascular homeostasis, contributing to the pathological features of MMD (Ihara et al. 2022; Tan et al. 2025). In addition to the cerebral vasculature, however, RNF213 has also been implicated in extracranial vasculopathies (Hiraide et al. 2022), such as in coronary heart disease, pulmonary hypertension, and renal artery stenosis (Fukushima et al. 2016; Morimoto et al. 2017; Kobayashi et al. 2018; Liu et al. 2022), suggesting its systemic role in vascular integrity and angiogenic processes.

Although RNF213 plays a major role in lipid metabolism, Cav-1 modulation, NF- κ B activation, apoptosis, inflammation, and innate immunity (Piccolis et al. 2019; Takeda et al. 2020; Mineharu and Miyamoto 2021; Otten et al. 2021; Thery et al. 2021; Zhang et al. 2023; Bhardwaj et al. 2025; Choi et al. 2025), its functions in angiogenesis appear more directly linked to the development of the moyamoya vessels in MMD (Roy et al. 2023). Angiogenesis, in which new blood vessels develop from the existing vasculature, is primarily mediated by endothelial cells in response to pro-angiogenic stimuli, such as the vascular endothelial growth factor (VEGF) that activates its receptor VEGFR to initiate a series of downstream signaling events (Liu et al. 2023). Emerging evidence also highlights the role of the epidermal growth factor receptor (EGFR) in angiogenesis (Hirata et al. 2002; Liu et al. 2023). Similar to the process in VEGFR, EGFR activation through ligand binding induces dimerization and subsequent autophosphorylation, thereby triggering downstream signaling cascades that include AKT, PLC- γ , and ERK/MAPK (Yarden and Sliwkowski 2001). Such signaling pathways facilitate endothelial cell survival, migration, and proliferation, all of which are essential for neovascularization (Apte et al. 2019). Together, therefore, these mechanisms precisely control vascular growth, wound healing, and tissue regeneration, and ensure a balanced signaling process that maintains proper blood flow and overall vascular health. Furthermore, EGFR and its close ErbB family members, such as HER2, are recognized for their roles in oncogenesis by driving enhanced and sustained activation of the same downstream mitogenic and survival pathways that lead to cellular proliferation and tumor growth (Baselga and Swain 2009; Uribe et al. 2021). This dual functionality of EGFR, in modulating both physiological angiogenesis and pathological cell proliferation, highlights its pivotal role in vascular remodeling as well as in disease progression.

In this study, we provide evidence of a previously unexplored functional relationship between RNF213 and EGFR/HER2 signaling. We demonstrate that alterations in RNF213 levels, or mutations in critical RNF213 domains, modulate the phosphorylation of EGFR and HER2 and result in the activation of specific downstream signaling events. Such modulation may represent a mechanistic link between RNF213 functions, vascular biology, and oncogenic receptor signaling, suggesting that RNF213 could be a prime target for therapeutic intervention with tyrosine kinase inhibitors

currently employed in cancer therapy—a prospect that may also explain the sensitivity of MMD patients carrying the R4810K variant to such inhibitors (Uemura et al. 2020).

2 | Results

2.1 | RNF213 Overexpression Enhances Tyrosine Phosphorylation of EGFR

During our initial studies on the functions of RNF213 that focused on the mechanism by which RNF213 is phosphorylated, especially in view of the finding that RNF213 serves as a substrate of protein tyrosine phosphatase PTP1B (Banh et al. 2016), we investigated various non-receptor and receptor tyrosine kinases that could phosphorylate RNF213. As an example of a receptor tyrosine kinase, we transiently expressed EGFR in WT HEK293T cells and in a HEK293T cell line stably overexpressing RNF213. However, contrary to our expectations, RNF213 was not phosphorylated (Figure 1A) but rather the overexpression of RNF213 greatly enhanced EGFR phosphorylation at tyrosine 1068 (pY1068) in response to 5 and 15 min EGF compared to WT HEK293T cells not expressing RNF213 (Figure 1A). These increased levels of phosphorylated Y1068 EGFR were not the result of increased amounts of cellular EGFR in response to RNF213 expression.

Moreover, as RNF213 appears to promote a low level of EGFR autophosphorylation, even in the absence of EGF stimulation, it may have a basal stimulatory effect on EGFR phosphorylation that is subsequently enhanced by EGF.

2.2 | Knockdown of Endogenous RNF213 in HeLa and A549 Cells Reduces EGF-Induced Y1068 Phosphorylation of EGFR

To unambiguously determine if RNF213 plays a role in EGFR phosphorylation, we chose to knock down RNF213 expression in HeLa and A549 cells that clearly express detectable levels of both RNF213 and EGFR. We optimized the starvation regimes to 0.1% FBS for 18 h for HeLa cells and 0.2% FBS for 12 h for A549 to maximize their responses to 5 and 15 min EGF (100 ng/mL) treatment. Using 3 different siRNAs, targeting different regions of *RNF213*, we observed that RNF213 protein levels were significantly reduced by all three siRNAs by more than 75% compared to the non-treated parental cell lines or those transfected with scrambled siRNA (Figure 1B,C). Although EGF treatment for 5 and 15 min greatly increased the EGFR pY1068 levels by over 30- and 10-fold in the WT HeLa and A549 cells, respectively, RNF213 knockdown greatly reduced these pY1068 levels at both 5 and 15 min, most notably in the HeLa lines. These results clearly indicate that RNF213 is responsible for the majority of the increased endogenous EGFR pY1068 levels in response to EGF stimulation in HeLa and A549 cells.

2.3 | Knockout of Endogenous RNF213 Limits Endogenous EGFR Y1068 Phosphorylation by EGF and TGF α

To confirm these findings, we generated independent RNF213 knockout HeLa and A549 cell series by CRISPR-Cas9 using the

A or B gRNA sequences (as described in Methods and Materials), and generated several independent lines of each. Both the A and B series of RNF213 HeLa knockouts reduced the RNF213 protein levels to undetectable levels compared to the WT HeLa

cells (Figure 1D). Although the levels of detectable total EGFR changed in response to EGF treatment, there were no significant differences in total EGFR between the WT and the A and B knockout series at each respective timepoint. Nevertheless,

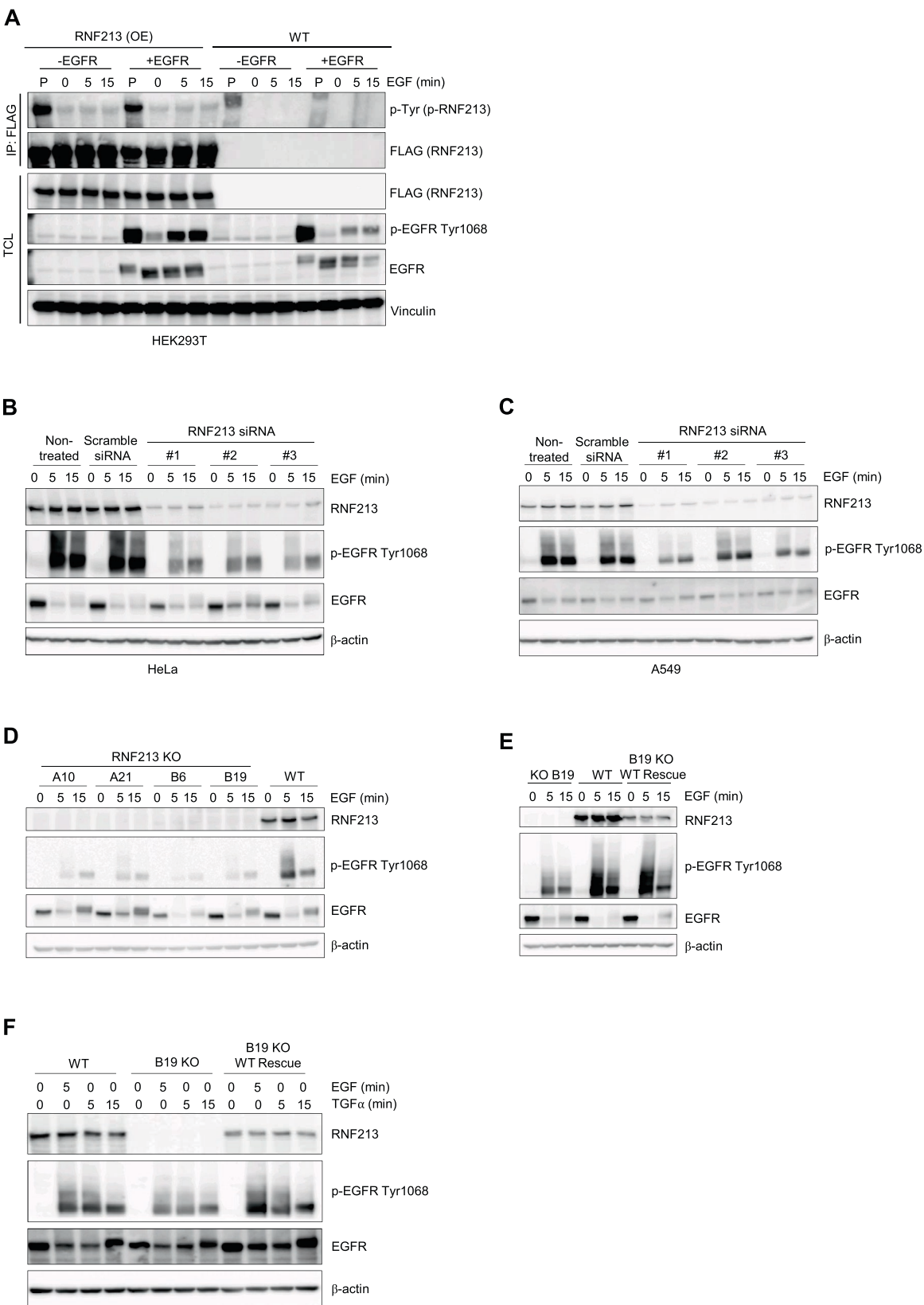


FIGURE 1 | Legend on next page.

FIGURE 1 | RNF213 overexpression, knockdown and knockout modify the EGFR phosphorylation status in HEK293T, HeLa and A549 cells in response to EGF or TGF α . (A) Overexpression of RNF213 in HEK293T cells enhances phosphorylation of EGFR Y1068 following EGF treatment. WT HEK293T cells (WT) or those with stable overexpression of WT FLAG-RNF213 (OE), in which EGFR was (+EGFR) or was not (–EGFR) transiently expressed, were serum starved for 18 h and then treated with 100 ng/mL EGF for 0, 5 or 15 min. Total cell lysates (TCL) of samples were directly used for Western blot analyses with the indicated antibodies against total EGFR, phosphorylated EGFR at Y1068, or FLAG (RNF213). Alternatively, lysates were immunoprecipitated (IP) with anti-FLAG antibody and then used for Western blotting using anti-FLAG or anti-phosphotyrosine (p-Tyr, 4G10) antibodies. RNF213 (FLAG or p-Tyr-reactive) migrates at 591 kDa, while EGFR and its Y1068-phosphorylated form appear at 175 kDa. Pervanadate (P), a protein tyrosine phosphatase inhibitor, was used as a positive control for phosphorylation. (B, C) Knockdown of RNF213 in HeLa (B) and A549 (C) cells reduces EGFR Y1068 phosphorylation in response to EGF. Cells were not transfected (non-treated) or transfected with control (Scramble) siRNA or with 3 different siRNAs (#1, #2 and #3) targeting RNF213. Cells were serum starved, treated with EGF, and samples used for Western blotting as above. (D, E) Knockout of RNF213 in HeLa cells suppresses EGFR Y1068 phosphorylation in response to EGF (D) but can be rescued by stable expression of WT RNF213 (E). In (D) two different series of RNF213 knockout HeLa cells (A, B) each represented by two different clones (i.e., A10, A21 and B6, B19) together with WT HeLa cells were serum starved, treated with EGF, and samples used for Western blotting as above. In (E) the B19 knockout line was rescued by stable transfection with WT RNF213 and then treated with EGF and used for Western blotting as above. (F) RNF213 knockdown reduces Y1068 phosphorylation of EGFR by its alternative ligand, TGF α , but is rescued by stable WT RNF213 expression. WT HeLa, the B19 RNF213 knockout line, and the B19 knockout line rescued by WT RNF213 overexpression were serum starved, untreated or treated with EGF for 5 min or with 100 ng/mL TGF α for 5 and 15 min and then subjected to Western blotting as above.

while the WT HeLa showed a marked increase in pY1068, particularly in response to 5 min but also to 15 min EGF treatment, the A10, A21, B6, and B19 lines showed 75% and 64% lower pY1068 levels compared to WT at 5 and 15 min EGF treatment, respectively (Figure 1D). In order to confirm the direct effects of RNF213 on these pY1068 levels, we rescued the B19 knockout cell lines by stable transfection with WT RNF213 and observed that although restored RNF213 expression had no effect on total EGFR levels, the pY1068 levels were over 3-fold higher than the knockout B19 cells following 5 min EGF treatment (Figure 1E). Interestingly, when we rescued the same B19 knockout cell line with the RNF213 RING-dead C3977A mutant, which has reduced RNF213 E3 ligase activity (Takeda et al. 2020), we were unable to observe the same level of pY1068 as in lines rescued with WT RNF213 (Figure S1). These data clearly indicate that RNF213 is predominantly responsible for the Y1068 phosphorylation of EGFR in response to EGF stimulation in HeLa cells, and that its RING-dependent E3 ligase activity is essential for this response.

As TGF α also functions as a ligand for EGFR activation, we tested whether knockout of RNF213 also reduces the extent of EGFR phosphorylation in response to 5 and 15 min TGF α treatment. Indeed, TGF α induced the same level of pY1068 as EGF in WT HeLa cells, whereas the pY1068 levels were reduced in the B19 knockout cell lines and restored to normal levels in the B19 lines rescued by WT RNF213 expression (Figure 1F).

2.4 | RNF213 Knockout in HeLa and A549 Cells Enhances Phosphorylation of Multiple Key Tyrosine Residues of EGFR

As EGFR can be transphosphorylated and autophosphorylated at various and multiple tyrosine residues in response to EGF, we questioned whether RNF213-induced EGFR phosphorylation was limited to only Y1068 or also occurred at other key tyrosine residues. Using various A and B series RNF213 knockout HeLa cell lines, untreated or treated with 100 ng/mL of EGF for 5 or 15 min, we observed clear reductions in EGF-induced EGFR phosphorylation at Y845 (Figure 2A), Y992, and Y1045

(Figure 2B), and Y1173 (Figure 2C) compared to those in WT. As with Y1068, rescue of the B19 knockout cell line with WT RNF213 greatly increased pY1173 levels, whereas rescue with the RING-dead C3977A mutant RNF213 was unable to restore the phosphorylation levels (Figure 2C). In addition, experiments using WT A549 and three independent A549 RNF213 knockout cell lines showed the same reductions in phosphorylation of tyrosine residues Y845, Y992, Y1045, as well as Y1068, with up to a 15 min exposure to EGF in the three knockout lines (Figure S2). To determine whether there was a difference between the WT and RNF213 knockout cells in their absolute tyrosine phosphorylation levels rather than just in their time of onset, we performed a time-course of EGF stimulation for up to 120 min. The results demonstrated that whereas WT HeLa cells displayed enhanced EGFR phosphorylation for 30 min or longer, depending on the particular phosphorylation site, the phosphorylation of the knockout B19 cells peaked at 5 to 15 min after EGF stimulation but became quite undiscernible soon thereafter (Figure S3). These data confirm that a functional RNF213 is necessary to enhance and to prolong the phosphorylation status of numerous critical tyrosine residues in EGFR in response to EGF stimulation.

2.5 | MMD-Related and Other Mutations in Key RNF213 Domains Abrogate Its Effect on EGFR pY1068

As expression of the C3977A mutant of RNF213 was unable to restore the pY1068 levels in the RNF213 knockout HeLa lines, we questioned whether various point mutations or deletions of key residues or regions of RNF213 were able to enhance the autophosphorylation of EGFR to the same extent as WT RNF213. For this, we co-expressed FLAG-tagged WT RNF213, or RNF213 constructs carrying various mutations or deletions, with or without co-expressed WT EGFR in starved HEK293T cells that were either untreated or treated with 100 ng/mL EGF for 5 min. Although the expression levels of the various RNF213 mutants varied somewhat from that of WT RNF213, presumably due to differences in protein stability (Takeda et al. 2020), all the RNF213 mutations and deletions,

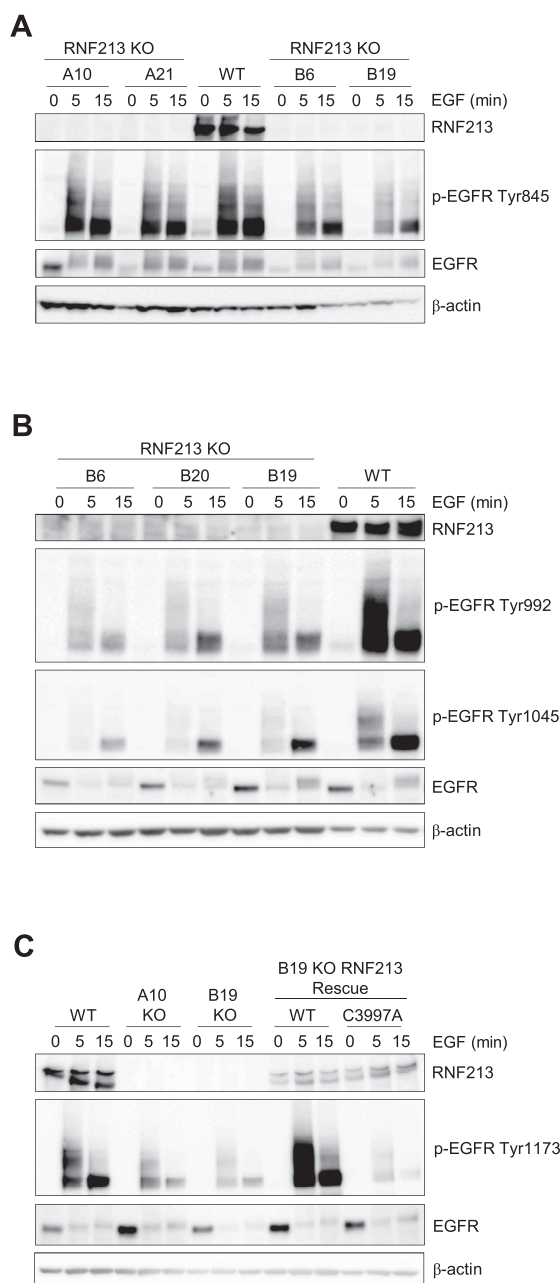


FIGURE 2 | Knockout of RNF213 in HeLa cells reduces phosphorylation at multiple tyrosine residues of EGFR and can be rescued by stable expression of RNF213 WT but not the RING-dead mutant. WT HeLa cells together with (A) the RNF213 A10, A21, B6 and B19 knockout cell lines, (B) the B6, B19 and B20 knockout lines, and (C) the A10 and B19 knockout lines as well as the B19 knockout line rescued by stable expression of the RNF213 WT or the RING-dead C3997A mutant were serum starved, and treated with 100 ng/mL EGF for 0, 5 or 15 min. Samples were used for Western blot analyses with the indicated antibodies targeting EGFR tyrosine phosphorylation sites at Y845, Y992, Y1045 and Y1173.

whether with a premature stop codon in the N-terminal region (δ ORF), deletions of the 3rd AAA+ domain (Δ AAA, necessary for RNF213 functionality) or the entire RZ finger (Δ RZ, necessary for E3 ligase activity), or point mutations in the 3rd and 4th AAA+ domains (C19), MMD mutations in the RING domain (C3997Y, D4013N, C4017S, R4019C, necessary also

for E3 ligase activity), or mutations in the RZ finger (H4509A, C4525A) or the C-terminal (S4118F, V4146A, and notably R4810K the primarily allele for MMD in Asians) domains reduced the level of pY1068 EGFR in comparison to WT RNF213 both in the absence of added EGF (–EGF) and following EGF treatment for 5 min (Figure 3A,B). It is important to note that mean total EGFR levels did not significantly differ between WT cells and those expressing various deletions or mutations in the absence of EGF treatment. However, cells expressing the Δ AAA deletion or the C19 mutant exhibited significantly higher EGFR levels ($p < 0.05$), whereas cells with the C3997Y RING-dead mutation showed significantly lower EGFR levels ($p < 0.05$) compared to WT after 5 min of EGF stimulation. Despite these alterations in total EGFR, the results clearly confirm that mutations in key residues or deletions of essential RNF213 domains can significantly impair EGFR Y1068 autophosphorylation, underscoring the requirement of a functional and catalytically active RNF213 in EGFR activation.

2.6 | RNF213-Dependent EGFR Phosphorylation Promotes Activation of the Tyrosine Kinase Family Member HER2

HER2, also known as ErbB-2, a member of the ERBB receptor tyrosine kinase family, is not activated by ligands or growth factors but rather by heterodimerization with other activated family members such as EGFR, and its hyperactivation is a major cause of the development and progression of various cancers, including ovarian and breast tumors. As expression of a functional RNF213 is clearly necessary to enhance EGFR activation, we sought to clarify whether RNF213 is also necessary to promote transactivation of HER2 in response to EGF. Indeed, treatment of WT HeLa cells with 100 ng/mL EGF for 5 or 15 min clearly promoted HER2 autophosphorylation at Y1221/1222 and Y1248, sites that are necessary for downstream signaling. However, the RNF213 knockout HeLa cell lines A10 and B19 showed up to 75% reduced HER2 phosphorylation levels without a reduction in the total cellular HER2 levels (Figure 4A). Consistent with EGFR phosphorylation, HER2 activation in the B19 knockout line could be rescued by stable overexpression of WT RNF213 but not by the RNF213 RING-dead C3997A mutation. These results also confirm that a functional RNF213 is necessary for transactivation of HER2 by EGFR phosphorylation in response to EGF.

2.7 | EGF-Upregulated EGFR Phosphorylation Enhances Src Y416 Phosphorylation in RNF213 WT but Not Knockout HeLa Cells

As EGF-induced EGFR dimerization and autophosphorylation is known to promote the recruitment, Y416 phosphorylation, and activation of the non-receptor tyrosine kinase Src, which in turn phosphorylates EGFR Y845 and also amplifies downstream signaling pathways, we examined whether RNF213 knockout would also downregulate Src phosphorylation in response to EGF. In the absence of EGF and the Src inhibitor PP2, the baseline levels of Src Y416 phosphorylation in the RNF213 B19 knockout cells were generally equal to or lower than those of the WT cells. However, when induced by EGF, there was a

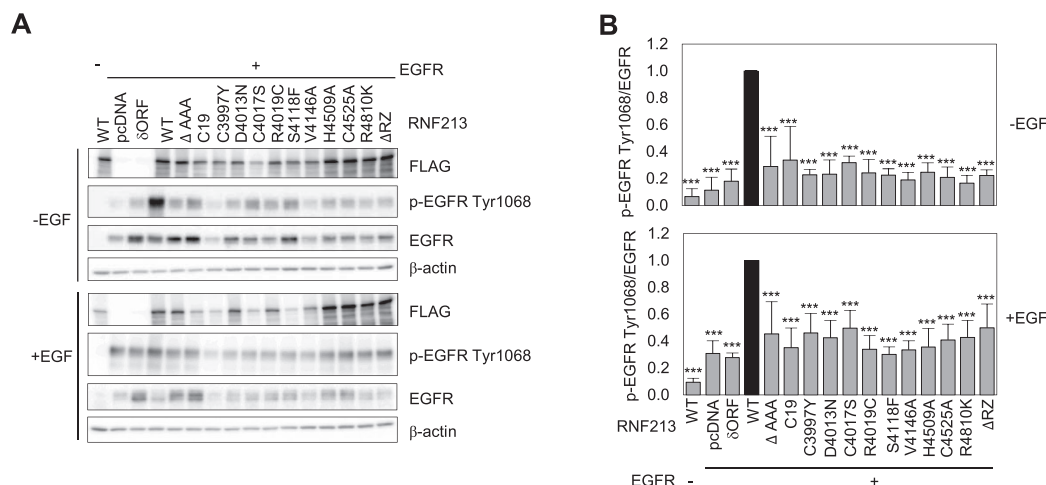


FIGURE 3 | Deletions and mutations of key domains and residues of RNF213 reduce EGFR Y1068 phosphorylation in HEK293T cells. (A) HEK293T cells were co-transformed with (+) or without (–) EGFR together with WT RNF213 or RNF213 harboring individual deletions and mutations in key domains and residues essential for RNF213 functionality or related to MMD (see Materials and Methods for full descriptions). Cells were serum starved for 18h, untreated (–EGF) or treated (+EGF) with 100 ng/mL EGF for 5 min, and samples used for Western blot analyses with the indicated antibodies. (B) Quantitative analysis of (A) showing the ratios of phosphorylated EGFR Y1068 over total EGFR levels for each of the various RNF213 WT and deletion/mutation clones. WT RNF213 co-expressing EGFR is given the arbitrary value of 1.0. Data from three biological replicates were analyzed by one-way ANOVA followed by Tukey's test with *** indicating $p < 0.0001$.

marked increase in Src Y416 phosphorylation in the WT but not in the RNF213 B19 knockout cells (Figure 4B). Furthermore, the Src Y416 phosphorylation levels in the WT HeLa lines could be reduced by increasing levels of PP2, whereas PP2 had no effect on Src phosphorylation in the RNF213 knockout lines. These results indicate that Src phosphorylation, like EGFR phosphorylation, is suppressed in the absence of a functional RNF213.

2.8 | RNF213-Dependent Activation of EGFR and HER2 in Response to EGF Does Not Enhance pAKT, pERK1/2, or pSTAT3 Levels nor Alter HeLa Cell Growth or Wound Healing

The phosphorylated tyrosine residues of EGFR and HER2 serve as docking sites for a number of scaffolding and adapter proteins that subsequently trigger numerous downstream signaling pathways, including PI3K/AKT, RAS/ERK, JAK/STAT, and PLCγ/PKC, which then regulate various cellular processes such as cell growth, differentiation, migration, apoptosis, and angiogenesis. We therefore assumed that one or more of these signal pathways would be activated by RNF213 in response to EGF stimulation. Unexpectedly, however, whereas the pAKT (pS473), pERK1/2 (pT202/pY204) and pSTAT3 (Y705) levels were clearly enhanced in response to 5 and 15 min of EGF treatment, there was no clear reduction in the phosphorylation levels of these proteins in the RNF213 knockout B19 cell line (Figure 5A). We therefore addressed the possibility that differences between these cell lines could arise not at the onset but rather in the duration of the phosphorylation states of these proteins, and we therefore set out to monitor the changes in response to EGF over a 120 min period. Although once again, there were clear increases in the phosphorylation levels of pAKT, pERK1/2, and pSTAT3 in response to EGF, we observed no consistent differences between

the RNF213 WT and knockout cell lines in their phosphorylation states (Figure 5B).

To confirm whether these findings had any impact on the cell characteristics, we used the WT and RNF213 B19 knockout HeLa cell lines to evaluate their relative growth rates and wound healing potentials as these are often affected by the aforementioned downstream signaling pathways. Although EGF appeared to reduce cell growth in both the WT and knockout cells, consistent with the response of A431 cancer cell lines treated with nanomolar concentrations of EGF (Graness et al. 2000), the only significant effect was the difference between the knockout cells without treatment and that after 5 days of EGF treatment (Figure S4A). In the migration assays, EGF generally increased the wound healing rates, although the only significant effect was the faster migration of the knockout cells than the WT cells in the absence of EGF treatment (Figure S4B). Interestingly, RNF213 knockdown in human brain microvascular endothelial cells was recently shown to promote cell proliferation and migration (Ye et al. 2023).

2.9 | RNF213 Is Necessary for pEGFR-Mediated Phosphorylation of PLCγ

Despite the lack of any visible effect of RNF213 knockdown on the phosphorylation levels of AKT, ERK1/2, or STAT3, we observed an almost 5-fold reduction in the level of PLCγ Tyr 783 phosphorylation in the RNF213 B19 knockout HeLa cells in response to EGF (100 ng/mL) over a 120-min period (Figure 5B). This reduced PLCγ phosphorylation pattern was also observed in the other RNF213 knockout lines, A10, A21, and B6 (Figure 5C). Furthermore, as with phosphorylation of EGFR, the level of PLCγ Tyr 783 phosphorylation could be restored to the WT HeLa level following rescue of the B19 knockout line by stable expression of WT RNF213 (Figure 5D).

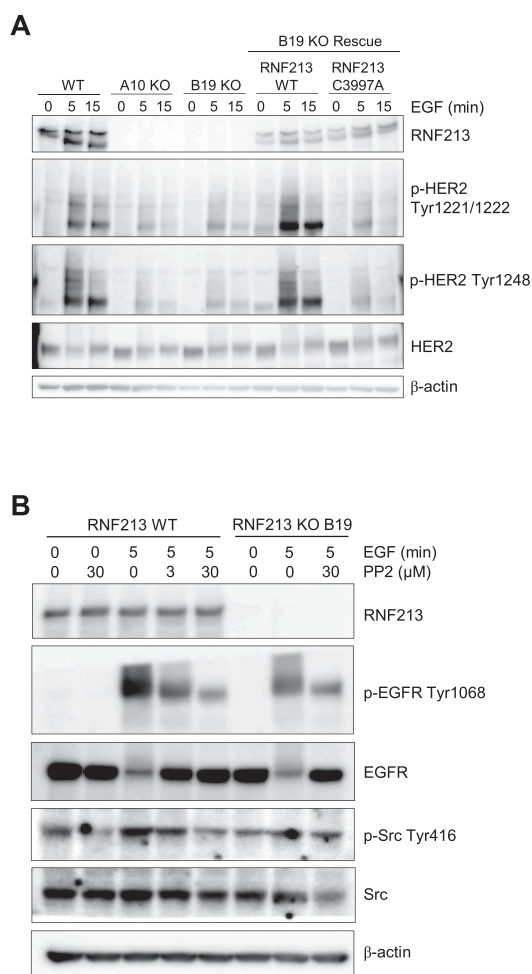


FIGURE 4 | Knockout of RNF213 in HeLa cells reduces tyrosine phosphorylation of HER2 and Src and can be rescued by stable expression of WT RNF213. (A) WT HeLa cells, and the A10 and B19 knockout lines, as well as the B19 knockout line rescued by stable expression with RNF213 WT or with the RING-dead C3997A mutant were serum starved, and treated with 100ng/mL EGF for 0, 5 or 15 min. (B) WT HeLa cells and the RNF213 B19 knockout lines as well as the knockout line rescued by stable expression of WT RNF213 were serum starved, treated with 0, 3 or 30 μM of the Src inhibitor PP2 for 1 h, followed by treatment with 100ng/mL EGF for 0 or 5 min. Samples were used for Western blot analyses with the indicated antibodies targeting phosphorylated HER2 at Y1248 and Y1221/1222, or phosphorylated Src at Y416.

2.10 | RNF213 Knockout Mice Show Reduced EGFR and HER2 Phosphorylation in Liver But no Differences in the Phosphorylation States of Downstream Signaling Factors

We next addressed the possibility that differences in the phosphorylation states of AKT, ERK1/2, and STAT3 may not be apparent in the cell lines we used but could be discernible in *in vivo* tissues. We therefore used WT and RNF213 knockout male mice and administered a single (*i.p.*) dose of EGF for 5 min and immediately isolated liver tissue for Western blot analysis (Figure 6A). EGF induced up to a 30-fold increase in EGFR pY1068 and up to a 5-fold increase in HER2 pY1248 without significantly affecting total cellular EGFR or HER2 contents in any of the mice tested. However, the liver tissue of RNF213

knockout mice exhibited significantly attenuated EGFR pY1068 (Figure 6B) and HER2 pY1248 (Figure 6C) levels in response to EGF treatment as compared to that from WT mice, indicating that RNF213 is required for full receptor activation. However, at this time point, we observed no differences in the levels of phosphorylated AKT, ERK1/2, STAT3, or even PLCγ between the WT and RNF213 knockout mice (Figure 6A).

3 | Discussion

RNF213, originally identified as the main susceptibility gene for Moyamoya disease (MMD), is a unique E3 ubiquitin ligase that has also been implicated in various cellular processes, including lipid metabolism, Cav-1 modulation, NF-κB activation, and innate immune responses (Piccolis et al. 2019; Takeda et al. 2020; Zhang et al. 2023; Bhardwaj et al. 2025; Choi et al. 2025). Our study now uncovers a novel and critical role for RNF213 in modulating the phosphorylation status of the epidermal growth factor receptor (EGFR) and its downstream signaling partners.

EGFR (HER1), a member of the HER/ErbB family of transmembrane receptor tyrosine kinases that also includes HER2/ErbB2, HER3/ErbB3, and HER4/ErbB4, is composed of an extracellular ligand-binding domain, a transmembrane region, a tyrosine kinase domain, and a cytoplasmic COOH-terminal tail with multiple phosphorylation sites (Arteaga and Engelman 2014; Kovacs et al. 2015). Binding of ligand to the binding domain stimulates EGFR homo- or heterodimerization and subsequent activation of the receptor kinase, followed by autophosphorylation of specific tyrosine residues in the receptor cytoplasmic tail, including Y992, Y1045, Y1068, and Y1173. This activation recruits the non-receptor tyrosine kinase Src, which first undergoes autophosphorylation at Y416 and then catalyzes transphosphorylation of EGFR at Y845. Activation of EGFR thus creates docking sites for downstream adapters, substrates, and signaling molecules, including GRB2, c-CBL, and PLCγ that can then trigger intracellular signaling pathways that promote cell proliferation and survival (Klapper et al. 2000; Di Marco et al. 1989).

In addition to the RNF213 overexpression experiments in HEK293T cells, our RNF213 knockdown and knockout studies in HeLa and A549 cells, which endogenously express both RNF213 and EGFR, unequivocally demonstrate that endogenous RNF213 is required for the full phosphorylation response of EGFR to its ligands EGF and TGFα. Indeed, knockout of RNF213 reduces Y1068 phosphorylation by up to 75% in response to these ligands, confirming that the major control of EGFR activation in these cells is mediated through RNF213. The significance of RNF213 in this activation process was further validated by the RNF213 knockout mice that show a significantly attenuated phosphorylation response to EGF administration. These reduced EGFR phosphorylation levels, in both knockout lines and mice, could not be attributed to either a delay in the time of onset of phosphorylation or to a reduced level of total EGFR proteins, arguing for a mechanism by which RNF213 modulates the absolute levels of EGFR phosphorylation. Indeed, even in the absence of EGF treatment, we observed a background increase in EGFR phosphorylation by overexpression of RNF213. Furthermore, the finding that RNF213 influences phosphorylation at multiple EGFR

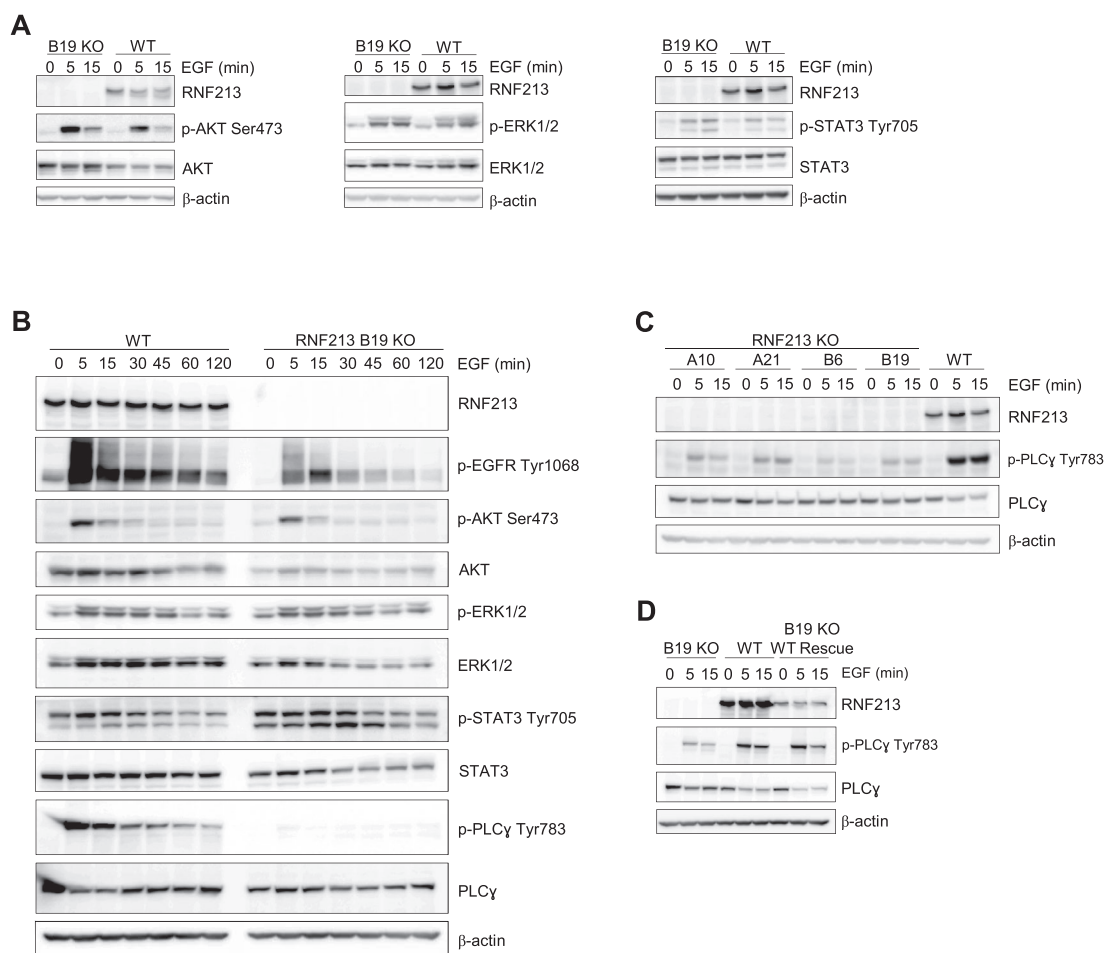


FIGURE 5 | RNF213 knockout in HeLa cells has no effect on several key EGFR downstream signaling factors but reduces phosphorylated PLC γ levels. (A) RNF213 B19 knockout has no effect on downstream regulatory kinases and signal transducers. RNF213 WT and B19 knockout cells were serum starved, treated with 100 ng/mL EGF for 0, 5 or 15 min, and samples used for Western blot analyses with the indicated antibodies targeting phosphorylated AKT, ERK1/2, and STAT3. (B) RNF213 B19 knockout has no effect on phosphorylated AKT, ERK1/2, or STAT3 levels over an extended 120 min period, but greatly reduces phosphorylated PLC γ levels. RNF213 WT and B19 knockout cells were serum starved, treated with 100 ng/mL EGF for 0 to 120 min, and samples used for Western blot analyses with the indicated antibodies. (C, D) Different RNF213 knockout lines show reduced phosphorylated PLC γ (C), but this can be rescued by stable expression of WT RNF213 in the B19 knockout line (D). WT, knockout and rescued cell lines were serum starved, treated with 100 ng/mL EGF for 0, 5 or 15 min, and samples used for Western blot analyses with antibodies targeting phosphorylated PLC γ .

tyrosine residues, notably Y845, Y992, Y1045, and Y1173, in addition to Y1068, defines a broad requirement for RNF213 in both autophosphorylation and transphosphorylation events following receptor dimerization.

An extension of our study is the finding that RNF213-mediated modulation of EGFR phosphorylation also impacts the asymmetric transactivation of HER2 (ErbB2), the key ERBB family member that preferentially forms heterodimers with EGFR (Cheng 2024). Both RNF213 knockout HeLa cells and mice exhibit a substantial reduction in HER2 phosphorylation without altering the total amount of HER2 protein. As HER2 lacks a ligand binding domain and is activated only through heterodimerization (Cheng 2024), the reduced HER2 transphosphorylation likely reflects the levels of RNF213-mediated EGFR phosphorylation. Moreover, the lower levels of EGFR Y845 transphosphorylation observed in the RNF213 knockout cells in response to EGF suggest a substantial reduction in Src recruitment to the activated homo- or heterodimer EGFR complexes (Belli et al. 2020). In RNF213 WT cells,

EGF markedly increases Src Y416 phosphorylation whereas this phosphorylation is attenuated in the knockout cells, providing evidence that RNF213 modulation of EGFR activation is critical for EGFR-directed activation of Src.

Several hypotheses may explain how RNF213 is involved in regulating EGFR and subsequent HER2 and Src phosphorylations. The simplest prospect is that RNF213 ubiquitinates negative regulators of EGFR signaling, such as protein tyrosine phosphatases and, by targeting them toward proteosomal degradation, affects their cellular amounts or even their spatial distribution. Our analyses of EGFR-associated tyrosine phosphatases PTP1B, SHP1, and SHP2 (Tenev et al. 1997; Agazie and Hayman 2003; Haj et al. 2003), which bind to docking sites on EGFR, show that their total protein levels remained unchanged in RNF213 knockout cells, although PTP1B was unexpectedly elevated in knockouts rescued with the RING-dead mutant (Figure S5). Nonetheless, it is still possible that RNF213 may regulate EGFR phosphorylation by modulating the activities of these phosphatases. RNF213 may

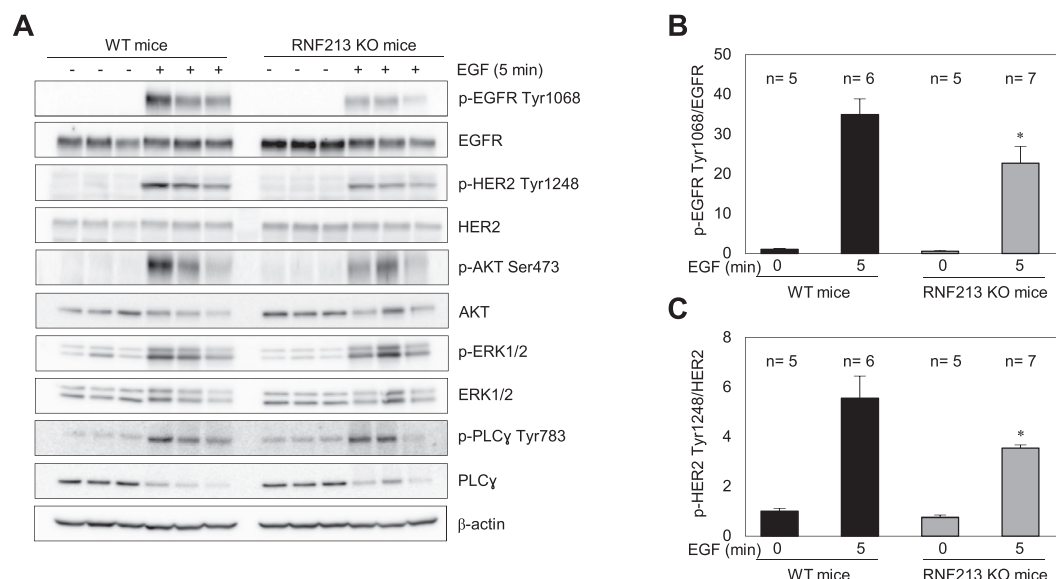


FIGURE 6 | RNF213 knockout mice show reduced EGFR and HER2 phosphorylation in liver in response to EGF but no effects on downstream signaling factors. (A) WT and RNF213 knockout (KO) mice at 6–9 weeks of age were starved for 24 h before injections (i.p.) with either saline (–EGF) or 1 μ g/g body weight EGF (+EGF). Five minutes post-injection, mice were anesthetized, and their liver tissues were harvested. Tissue samples were then lysed and centrifuged, and the resulting supernatants analyzed by Western blotting using the indicated antibodies. (B, C) Quantitative analyses of (A) showing the ratios of phosphorylated EGFR Y1068 to total EGFR (B) and phosphorylated HER2 Y1248 to total HER2 (C) for the WT and RNF213 KO mice at 0 and 5 min EGF treatment. WT RNF213 mice without EGF are given the arbitrary value of 1.0. Data were analyzed by two-way ANOVA followed by Tukey's test with * indicating $p < 0.05$ for the difference between WT and KO mice at 5 min EGF treatment.

also limit the activity of other negative regulators of EGFR, for example MIG6 (ERRFI1) that functions by binding to the EGFR kinase domain, preventing its dimerization and inhibiting autophosphorylation (Zhang et al. 2007). Alternatively, RNF213 could influence receptor trafficking and recycling (Schultz et al. 2023), for example, by modifying the activity of the E3 ligase c-CBL, thereby maintaining a pool of activated receptors at the cell surface or promoting recycling pathways that favor prolonged signaling rather than rapid degradation. Additionally, RNF213 may promote non-degradative K63-linked ubiquitination of EGFR or its associated adaptor proteins, as it does with FOXO1 (Yang et al. 2024), thereby promoting receptor clustering or dimerization and stabilizing receptor interactions at the plasma membrane so as to facilitate autophosphorylation. Another potential mechanism could involve alterations in the lipid microenvironment through which RNF213 might affect the composition or organization of membrane microdomains, including lipid rafts or caveolae, where EGFR dimerization and activation occur, thereby enhancing the likelihood of receptor interactions (Abe et al. 2024).

Regardless of how RNF213 modulates EGFR activation, our results also show that a functional RNF213 is indispensable in this process. In our RNF213 knockout experiments, reintroduction of WT RNF213 but not the C3997A RING-dead mutation fully restored EGFR and HER2 phosphorylation. This clearly demonstrates that the RING E3 ligase activity is critical for EGFR phosphorylation. Moreover, mutations or deletions of other essential domains of RNF213, such as the RZ finger, which also possesses E3 ligase activity and ubiquitinates substrates including CYLD/SPATA2 or components of bacterial and viral pathogens (Zhang et al. 2023; Bhardwaj et al. 2025), as well as mutations in the active AAA+ domains, which are

important for RNF213 oligomerization and regulation of its E3 ligase activities (Takeda et al. 2020; Ahel et al. 2020), also significantly reduce EGFR phosphorylation. It is not surprising, therefore, that mutations in or near these key domains and especially near the C-terminus of the RING domain not only impair RNF213 functionality and EGFR phosphorylation but are also associated with the onset of MMD and other vasculopathies (Ihara et al. 2022).

Although phosphorylation of EGFR, HER2, and Src is evidently reduced in the RNF213 knockdown cells, activation of downstream canonical signaling pathways, including PI3K/AKT, ERK1/2, and STAT3, remains unexpectedly unaltered in both cell lines and mice in response to EGF. Similar observations have been reported, for example, following knockdown of AIB1 (amplified in breast cancer 1, a p160 steroid receptor coactivator member), where reductions in EGFR tyrosine phosphorylation did not translate into changes in phosphorylated AKT and ERK1/2 levels (Lahusen et al. 2007). This apparent disconnect suggests that while RNF213 plays a critical role in modulating proximal EGFR events, such as receptor phosphorylation and immediate effector activation, the downstream signaling events involving PI3K/AKT, ERK, and STAT may be buffered by redundant signaling or compensatory mechanisms. Alternatively, RNF213 may selectively regulate a specific subset of EGFR-mediated outputs, as evidenced by the 5-fold higher phosphorylation of PLC γ in WT cells compared to the RNF213 knockouts following EGF stimulation. PLC γ , which is directly phosphorylated by EGFR, hydrolyzes the membrane lipid phosphatidylinositol 4,5-bisphosphate (PIP2) into inositol-1,4,5-trisphosphate (IP3) and diacylglycerol (DAG), triggering an increase in intracellular calcium and activating protein kinase C (PKC) (Mandal et al. 2021). Given

its central role in intracellular calcium signaling, PLC γ may have significant implications for cellular processes dependent on this pathway. This raises the possibility that RNF213 may selectively influence downstream pathways depending on cellular context or the availability of specific adaptor proteins. Notably, in the mouse liver, phosphorylation levels of AKT, ERK1/2, STAT3, and even PLC γ do not substantially differ between WT and RNF213 knockout tissues, suggesting some tissue-specific or compensatory process that maintains the PLC γ phosphorylation levels.

Our findings have implications for both vascular biology and oncology. Previous studies indicate that EGFR contributes to physiologic and pathologic angiogenesis (Hirata et al. 2002; Makki et al. 2013). The rise in intracellular calcium following PLC γ and PKC activation and the downstream cascades leading from the EGFR pathway may limit the synthesis of anti-angiogenic factors and promote the synthesis and secretion of pro-angiogenic factors, including the vascular endothelial growth factor (VEGF), the master regulator of angiogenesis (Moccia et al. 2019; Liu et al. 2023). Earlier studies have shown that EGF stimulation of glioma cells enhances VEGF production (Goldman et al. 1993) whereas inhibition of EGFR signaling downregulates VEGF (Petit et al. 1997; Riedel et al. 2002). In fact, many of the key downstream regulators of angiogenesis are shared by EGF and VEGF, and they can therefore induce the same angiogenic responses that promote the proliferation, migration, and organization of endothelial cells into new vessels, a key component of angiogenesis (Ciardiello et al. 2006; Moccia et al. 2019). It is therefore tempting to speculate that RNF213 mutations, notably variants such as R4810K linked to MMD, may have impaired EGFR signaling in endothelial cells that, under appropriate cellular conditions such as hypoxia, lead to aberrant autocrine VEGF production and angiogenic responses that form the fragile, compensatory vasculature that is characteristic of MMD. Indeed, VEGF has been shown to be downregulated in RNF213-deficient proliferative but not in confluent endothelial cells (Roy et al. 2023). In addition, it will be important to confirm whether a reduced or modified RNF213 functionality can, as with selective EGFR and/or VEGF inhibitors such as cetuximab and bevacizumab, inhibit tumor-induced angiogenesis that often deregulates VEGF signaling and promotes the development of blood vessels with anarchic organization and leads to the enhanced growth and metastasis of various malignant tumors (Hirata et al. 2002; Liu et al. 2023). In addition to the potential effects of RNF213-dependent EGFR phosphorylation on angiogenesis, the modified activation states of EGFR, HER2, Src, and PLC γ could amplify various downstream signal cascades and lead to unregulated cell proliferation and enhanced cell cycle progression and survival, which are found in many tumor types, such as glioblastomas or cervical, lung, breast, and colorectal cancers (Uribe et al. 2021; Yan et al. 2015; Raji et al. 2023). Furthermore, the determination that RNF213 mutations can function as oncogenic drivers in various cancers, including EGFR/HER2-associated gastric cancer (Ge et al. 2017) and lung cancer (Jiang et al. 2021), and that these mutations are associated with resistance to tyrosine kinase inhibitors such as nilotinib (Uemura et al. 2020), raises the possibility that targeting RNF213 or its downstream effectors may offer a new therapeutic approach in oncology.

4 | Experimental Procedures

4.1 | Cell Culture, Transfections and siRNA Knockdowns

Human Embryonic Kidney 293T (HEK293T, Dharmacon), HeLa, and A549 cells were cultured in Dulbecco's Modified Eagle's Medium (Nacalai Tesque) with 10% Fetal Bovine Serum (Thermo Fisher) and 1% penicillin/streptomycin (Nacalai Tesque). The cells were maintained in a humidified chamber at 37°C and 5% CO₂. Media were changed every 2 days, and cells were passaged at 80%–90% confluence. Cells were transfected with plasmids using Viofectin (Viogene) and gene knockdowns with siRNA were performed with Lipofectamine RNAiMAX (Thermo Fisher) as suggested by the manufacturers.

4.2 | Plasmids, DNA Clones, siRNAs and Modified Cell Lines

The 3xFLAG-RNF213 in pcDNA3, point mutations in the RNF213 sequence resulting in amino acid changes C3997Y, D4013N, C4017S, R4019C, V4146A, and R4810K, and the Δ AAA clone with deletion of the Walker A domain of the 3rd AAA+, as well as mutations within the Walker A and Walker B motifs of the 3rd and 4th AAA+ domains (i.e., mutations K2426A, E2488A, K2775A, and E2845A, respectively and denoted as C19) were derived from our previous studies (Kobayashi et al. 2015, 2016; Takeda et al. 2020). New point mutations in the 3xFLAG-RNF213 sequence resulting in mutations S4118F, H4509A, and C4525A, and deletion of the entire RZ finger from C4505 to H4537 (denoted Δ RZ) of RNF213, and a full-length transcript of RNF213 with a frameshift stop codon after G222 (denoted δ ORF) were developed in the current study by PCR-mediated mutagenesis and cloning using the In-Fusion HD cloning system (Clontech, Takara). For stable expression experiments, 3xFLAG-RNF213 WT or the C3997A RING-dead mutation coding sequences were cloned into the pHULK PiggyBac mammalian transposon system (DNA2.0 Inc., CosmoBio) for constitutive RNF213 expression in HEK293T cells or in RNF213 knockout HeLa cells for rescue experiments. All new constructs were subjected to complete DNA sequence analysis to confirm their modifications. Knockdowns were performed in HeLa and A549 cells with three different RNF213 siRNAs that targeted sequences (1) CGtAtGAGCtCACAAACCGA and (2) CAGCtGcTtGAAAAACGA (Sugihara et al. 2019, Sigma) and (3) GGACtGGtGCCtttGCAtt (Sigma) or with scramble Universal Control siRNA (Sigma). Knockout of RNF213 in HeLa and A549 cells was performed by CRISPR-Cas9-mediated gene targeting using the pX459v2 vector (Addgene #62988, gift of F. Zhang) into which the RNF213-specific gRNA sequences A (sense, caccTTGGACGGTCCAGGAGGCTT and anti-sense, aaacAAGCCTCCTGGACCGTCCAA oligos) or B (sense, caccACAATGGCGTTCGGCTCGGA and anti-sense, aaacTCCGAGGCCGACGCCATTGT oligos) (Sugihara et al. 2019; Banh et al. 2016) were cloned. Human EGFR in plasmid pME was as previously described (Kim et al. 1999).

4.3 | Cell Stimulation, Harvesting and Preparation

HEK293T, HeLa, and A549 cells were starved for 12–18 h in Dulbecco's Modified Eagle's Medium with either 0.1% or

0.2% FBS before stimulation with recombinant human EGF (Fujifilm Wako) at final concentrations of 10 to 100 ng/mL for the appropriate times of 5–120 min. Alternatively, cells were treated with 100 ng/mL TGF α (Peprotech, Thermo Fisher) for 5 or 15 min. For Src inhibition experiments, cells were treated with the Src inhibitor PP2 (Cayman Chemical) at 0, 3, or 30 μ M for 1 h prior to EGF treatment. After stimulation, cells were immediately placed on ice, gently scraped off the plates, and washed three times with ice-cold PBS. Cells were lysed with TNE Buffer (150 mM NaCl, 2 mM EDTA, 50 mM Tris-HCl pH 8.0, 1% NP-40) containing Protease Inhibitor Cocktail (Nacalai Tesque) and PhosSTOP phosphatase inhibitor (Roche).

4.4 | Immunoprecipitation

HEK293T cells stably expressing FLAG-tagged RNF213 were transfected with EGFR and stimulated with EGF as described above. The cells were washed with ice-cold PBS, lysed in ice-cold TNE Buffer as described for 30 min at 4°C on a rotator, and centrifuged at 10,000g for 15 min at 4°C. Protein concentrations in the supernatants were measured using the BCA Protein Assay Reagent (Pierce). Cellular extracts were then incubated overnight at 4°C with anti-FLAG antibody, followed by a 1 h incubation with pre-washed Protein A/G beads (Millipore, USA) at 4°C. Complexes were pelleted by centrifugation at 5000 $\times$ rpm for 1 min at 4°C, washed five times with lysis buffer, resuspended in 6 $\times$ SDS loading buffer, and boiled at 99°C for 10 min.

4.5 | Mice Experiments

RNF213 WT and knockout C57BL/6 mice, developed as previously described (Kobayashi et al. 2013), were maintained in the Kyoto University Animal Facility Center with ad libitum food and water under controlled temperature (24°C) and humidity (50%) and a 12-h light/12-h dark cycle. Homozygous male mice at 6–9 weeks of age were starved for 24 h prior to i.p. injections of either saline or 1 μ g/g body weight (Yang et al. 2017) of recombinant human EGF (Fujifilm, Wako), and anesthetized 3 min later with Sevofrane (Maruishi Pharmaceutical Co). Liver tissues were harvested after 5 min and instantly frozen in liquid nitrogen and stored at –80°C until use. Tissue samples were lysed by sonication in RIPA buffer (Thermo Fisher), supplemented with protease and phosphatase inhibitors as above, centrifuged at 10,000g for 15 min at 4°C, and the supernatants aliquoted and stored at –80°C until use. The breeding and care of the mice used in these studies followed the Animal Welfare Guidelines of Kyoto University, and the experimental protocols used were approved by the Animal Care, Use and Ethics Committee at Kyoto University (Approval number: Med Kyo 22520).

4.6 | Western Blotting

Protein concentrations of lysates were determined using the BCA Protein Assay Reagent (Pierce) as directed, and 60 μ g aliquots were separated on 5%–20% gradient SDS-PAGE

gels (Nacalai Tesque). Proteins were transferred onto PVDF membranes (Merck) by standard protocols, which were then blocked in Blocking One solution (Nacalai Tesque) for 2–4 h at room temperature and incubated with appropriate dilutions of primary antibodies overnight at 4°C. Membranes were washed in TBS-T, and then incubated with the appropriately diluted secondary antibodies for 1 h at room temperature. Chemiluminescent signals were developed with ECL Prime (GE Healthcare) or Chemi-Lumi One (Nacalai Tesque) for HRP, analyzed using a LAS3000 imager (Fujifilm) and quantified with Multi Gauge software (GE Healthcare). At least 3 biological replicates of each Western were performed to confirm results.

4.7 | Cell Growth and Migration

WT HeLa and RNF213 B19 knockout cells were seeded at 5×10^3 cells on 48-well plates for 24 h, serum starved on 0.1% FBS for 18 h, and either not treated with EGF (–EGF) or treated with 10 or 100 ng/mL EGF (+EGF). For relative growth assays, the treated cells were grown on 1% FBS-containing plates, harvested at the appropriate time intervals, and the number of viable cells directly determined using the CCK-8 method according to the manufacturer's instructions (Dojindo) using a Wallac multi-label microplate reader (Arvo SX 1420, PerkinElmer). For the migration assays, the EGF-treated cells were grown on 5% FBS-containing plates, wound streaks were made on the cell layer using a 200 μ L-pipette tip, and cell debris was washed off with culture medium. Photographs of the wounded regions were taken under phase-contrast microscopy immediately (0 h) and after 24 h of wound closure. The distance of the gap covered by the migrating cells after 24 h was quantified using Image-J software (NIH, USA).

4.8 | Data Analysis

Wherever appropriate, results are presented as mean $\pm$ standard error of the mean (SEM). Statistical significance was evaluated by one-way or two-way ANOVA and, when significant ($p < 0.05$), then Tukey's honestly significant difference test was performed. Statistical analyses used PRISM software (Graphpad). A value of $p < 0.05$ indicates statistical significance. All experiments were repeated at least three times to confirm the validity of the results.

Author Contributions

Intisar M. Fouad, Akio Koizumi, Tohru Tezuka, and Shohab Youssefian: conceptualization. **Intisar M. Fouad, Seiji Masuda, Minsoo Kim, Tohru Tezuka, and Shohab Youssefian:** methodology. **Intisar M. Fouad, Qianying Huang, Jungmi Choi, Minsoo Kim, Tohru Tezuka, and Shohab Youssefian:** investigation. **Intisar M. Fouad, Jungmi Choi, and Shohab Youssefian:** writing – original draft preparation. **Intisar M. Fouad, Jungmi Choi, James A. Hejna, Minsoo Kim, Akio Koizumi, Yohei Mineharu, Tohru Tezuka, and Shohab Youssefian:** writing – review and editing. **James A. Hejna, Tohru Tezuka, Shohab Youssefian:** supervision. **Tohru Tezuka, Akio Koizumi, Yohei Mineharu, Shohab Youssefian:** funding acquisition. All authors have read and agreed to the published version of the manuscript. The other authors declare no conflicts of interest.

Acknowledgments

We thank Amanda P. Elvandari, Lirong Yang, and Dr. Luc Grinand for their technical support.

Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

References

- Abe, M., M. Yanagawa, M. Hiroshima, T. Kobayashi, and Y. Sako. 2024. "Bilateral Regulation of EGFR Activity and Local PI(4,5)P₂ Dynamics in Mammalian Cells Observed With Superresolution Microscopy." *eLife* 13: e101652. <https://doi.org/10.7554/eLife.101652>.
- Agazie, Y. M., and M. J. Hayman. 2003. "Molecular Mechanism for a Role of SHP2 in Epidermal Growth Factor Receptor Signaling." *Molecular and Cellular Biology* 23: 7875–7886. <https://doi.org/10.1128/MCB.23.21.7875-7886.2003>.
- Ahel, J., A. Lehner, A. Vogel, et al. 2020. "Moyamoya Disease Factor RNF213 Is a Giant E3 Ligase With a Dynein-Like Core and a Distinct Ubiquitin-Transfer Mechanism." *eLife* 9: e56185. <https://doi.org/10.7554/eLife.56185>.
- Apte, R. S., D. S. Chen, and N. Ferrara. 2019. "VEGF in Signaling and Disease: Beyond Discovery and Development." *Cell* 176, no. 6: 1248–1264. <https://doi.org/10.1016/j.cell.2019.01.021>.
- Arteaga, C. L., and J. A. Engelman. 2014. "ERBB Receptors: From Oncogene Discovery to Basic Science to Mechanism-Based Cancer Therapeutics." *Cancer Cell* 25: 282–303. <https://doi.org/10.1016/j.ccr.2014.02.025>.
- Banh, R. S., C. Iorio, R. Marcotte, et al. 2016. "PTP1B Controls Non-Mitochondrial Oxygen Consumption by Regulating RNF213 to Promote Tumour Survival During Hypoxia." *Nature Cell Biology* 18, no. 7: 803–813. <https://doi.org/10.1038/ncb3376>.
- Baselga, J., and S. Swain. 2009. "Novel Anticancer Targets: Revisiting ERBB2 and Discovering ERBB3." *Nature Reviews Cancer* 9: 463–475. <https://doi.org/10.1038/nrc2656>.
- Belli, S., D. Esposito, A. Servetto, A. Pesapane, L. Formisano, and R. Bianco. 2020. "C-Src and EGFR Inhibition in Molecular Cancer Therapy: What Else Can we Improve?" *Cancers* 12, no. 6: 1489. <https://doi.org/10.3390/cancers12061489>.
- Bhabha, G., H.-C. Cheng, N. Zhang, et al. 2014. "Allosteric Communication in the Dynein Motor Domain." *Cell* 159: 857–868. <https://doi.org/10.1016/j.cell.2014.10.018>.
- Bhardwaj, A., M. C. Panepinto, B. Ueberheide, and B. G. Neel. 2025. "A Mechanism for Hypoxia-Induced Inflammatory Cell Death in Cancer." *Nature* 637: 470–477. <https://doi.org/10.1038/s41586-024-08136-y>.
- Cheng, X. 2024. "A Comprehensive Review of HER2 in Cancer Biology and Therapeutics." *Genes* 15: 903. <https://doi.org/10.3390/genes15070903>.
- Choi, J., R. Inoue, Y. Masuo, et al. 2025. "RNF213 Acts as a Molecular Switch for Cav-1 Ubiquitination and Phosphorylation in Human Cells." *Cells* 14, no. 11: 775. <https://doi.org/10.3390/cells14110775>.
- Ciardiello, F., T. Troiani, R. Bianco, et al. 2006. "Interaction Between the Epidermal Growth Factor Receptor (EGFR) and the Vascular Endothelial Growth Factor (VEGF) Pathways: A Rational Approach for Multi-Target Anticancer Therapy." *Annals of Oncology* 17, no. Suppl 7: vii109–vii114. <https://doi.org/10.1093/annonc/mdl962>.
- Di Marco, E., J. H. Pierce, T. P. Fleming, et al. 1989. "Autocrine Interaction Between TGF Alpha and the EGF-Receptor: Quantitative Requirements for Induction of the Malignant Phenotype." *Oncogene* 4, no. 7: 831–838.
- Fukushima, H., T. Takenouchi, and K. Kosaki. 2016. "Homozygosity for Moyamoya Disease Risk Allele Leads to Moyamoya Disease With Extracranial Systemic and Pulmonary Vasculopathy." *American Journal of Medical Genetics, Part A* 170: 2453–2456. <https://doi.org/10.1002/ajmg.a.37829>.
- Ge, S., B. Li, Y. Li, et al. 2017. "Genomic Alterations in Advanced Gastric Cancer Endoscopic Biopsy Samples Using Targeted Next-Generation Sequencing." *American Journal of Cancer Research* 7, no. 7: 1540–1553.
- Goldman, C. K., J. Kim, W. L. Wong, V. King, T. Brock, and G. Y. Gillespie. 1993. "Epidermal Growth Factor Stimulates Vascular Endothelial Growth Factor Production by Human Malignant Glioma Cells: A Model of Glioblastoma Multiforme Pathophysiology." *Molecular Biology of the Cell* 4, no. 1: 121–133. <https://doi.org/10.1091/mbc.4.1.121>.
- Graness, A., S. Hanke, F. D. Boehmer, P. Presek, and C. Liebmann. 2000. "Protein-Tyrosine-Phosphatase-Mediated Epidermal Growth Factor (EGF) Receptor Transactivation and EGF Receptor-Independent Stimulation of Mitogen-Activated Protein Kinase by Bradykinin in A431 Cells." *Biochemical Journal* 347, no. 2: 441–447. <https://doi.org/10.1042/0264-6021>.
- Haj, F. G., B. Markova, L. D. Klamann, F. D. Bohmer, and B. G. Neel. 2003. "Regulation of Receptor Tyrosine Kinase Signaling by Protein Tyrosine Phosphatase-1B." *Journal of Biological Chemistry* 278: 739–744. <https://doi.org/10.1074/jbc.M210194200>.
- Hiraide, T., H. Suzuki, M. Momoi, et al. 2022. "RNF213-Associated Vascular Disease: A Concept Unifying Various Vasculopathies." *Lifestyles* 12, no. 4: 555. <https://doi.org/10.3390/life12040555>.
- Hirata, A., S. Ogawa, T. Kometani, et al. 2002. "ZD1839 (Iressa) Induces Antiangiogenic Effects Through Inhibition of Epidermal Growth Factor Receptor Tyrosine Kinase." *Cancer Research* 62: 2554–2560.
- Ihara, M., Y. Yamamoto, Y. Hattori, et al. 2022. "Moyamoya Disease: Diagnosis and Interventions." *Lancet Neurology* 21: 747–758. [https://doi.org/10.1016/s1474-4422\(22\)00165-x](https://doi.org/10.1016/s1474-4422(22)00165-x).
- Jiang, N., J. Zhou, W. Zhang, et al. 2021. "RNF213 Gene Mutation in Circulating Tumor DNA Detected by Targeted Next-Generation Sequencing in the Assisted Discrimination of Early-Stage Lung Cancer From Pulmonary Nodules." *Thoracic Cancer* 12, no. 2: 181–193. <https://doi.org/10.1111/1759-7714.13741>.
- Kim, M., T. Tezuka, Y. Suzuki, S. Sugano, M. Hirai, and T. Yamamoto. 1999. "Molecular Cloning and Characterization of a Novel Cbl-Family Gene, Cbl-c." *Gene* 239, no. 1: 145–154. [https://doi.org/10.1016/s0378-1119\(99\)00356-x](https://doi.org/10.1016/s0378-1119(99)00356-x).
- Klapper, L. N., M. H. Kirschbaum, M. Sela, and Y. Yarden. 2000. "Biochemical and Clinical Implications of the ErbB/HER Signaling Network of Growth Factor Receptors." *Advances in Cancer Research* 77: 25–79.
- Kobayashi, H., M. Brozman, K. Kyselová, et al. 2016. "RNF213 Rare Variants in Slovakian and Czech Moyamoya Disease Patients." *PLoS One* 11, no. 10: e0164759. <https://doi.org/10.1371/journal.pone.0164759>.
- Kobayashi, H., R. Kabata, H. Kinoshita, et al. 2018. "Rare Variants in RNF213, a Susceptibility Gene for Moyamoya Disease, Are Found in Patients With Pulmonary Hypertension and Aggravate Hypoxia-Induced Pulmonary Hypertension in Mice." *Pulmonary circulation* 8: 2045894018778155. <https://doi.org/10.1177/2045894018778155>.
- Kobayashi, H., Y. Matsuda, T. Hitomi, et al. 2015. "Biochemical and Functional Characterization of RNF213 (Myosin) R4810K, a Susceptibility Mutation of Moyamoya Disease, in Angiogenesis in Vitro and in Vivo." *Journal of the American Heart Association* 4. <https://doi.org/10.1161/jaha.115.002146>.

- Kobayashi, H., S. Yamazaki, S. Takashima, et al. 2013. "Ablation of Rnf213 Retards Progression of Diabetes in the Akita Mouse." *Biochemical and Biophysical Research Communications* 432, no. 3: 519–525. <https://doi.org/10.1016/j.bbrc.2013.02.015>.
- Kovacs, E., J. A. Zorn, Y. Huang, T. Barros, and J. Kuriyan. 2015. "A Structural Perspective on the Regulation of the Epidermal Growth Factor Receptor." *Annual Review of Biochemistry* 84: 739–764. <https://doi.org/10.1146/annurev-biochem-060614-034402>.
- Lahusen, T., M. Fereshteh, A. Oh, A. Wellstein, and A. T. Riegel. 2007. "Epidermal Growth Factor Receptor Tyrosine Phosphorylation and Signaling Controlled by a Nuclear Receptor Coactivator, Amplified in Breast Cancer 1." *Cancer Research* 67, no. 15: 7256–7265. <https://doi.org/10.1158/0008-5472.CAN-07-1013>.
- Liu, E., H. Zhao, C. Liu, X. Tan, C. Luo, and S. Yang. 2022. "Research Progress of Moyamoya Disease Combined With Renovascular Hypertension." *Frontiers in Surgery* 9: 969090. <https://doi.org/10.3389/fsurg.2022.969090>.
- Liu, W., D. Morito, S. Takashima, et al. 2011. "Identification of RNF213 as a Susceptibility Gene for Moyamoya Disease and Its Possible Role in Vascular Development." *PLoS One* 6: e22542. <https://doi.org/10.1371/journal.pone.0022542>.
- Liu, Z. L., H. H. Chen, L. L. Zheng, L. P. Sun, and L. Shi. 2023. "Angiogenic Signaling Pathways and Anti-Angiogenic Therapy for Cancer." *Signal Transduction and Targeted Therapy* 8, no. 1: 198. <https://doi.org/10.1007/s10456-014-9420-y>.
- Makki, N., K. W. Thiel, and F. J. Miller Jr. 2013. "The Epidermal Growth Factor Receptor and Its Ligands in Cardiovascular Disease." *International Journal of Molecular Sciences* 14: 20597–20613. <https://doi.org/10.3390/ijms141020597>.
- Mandal, S., S. Bandyopadhyay, K. Tyagi, and A. Roy. 2021. "Recent Advances in Understanding the Molecular Role of Phosphoinositide-Specific Phospholipase C Gamma 1 as an Emerging Onco-Driver and Novel Therapeutic Target in Human Carcinogenesis." *Biochimica et Biophysica Acta: Reviews on Cancer* 1876, no. 2: 188619. <https://doi.org/10.1016/j.bbcan.2021.188619>.
- Mineharu, Y., and S. Miyamoto. 2021. "RNF213 and GUCY1A3 in Moyamoya Disease: Key Regulators of Metabolism, Inflammation, and Vascular Stability." *Frontiers in Neurology* 12: 687088. <https://doi.org/10.3389/fneur.2021.687088>.
- Moccia, F., S. Negri, M. Shekha, P. Faris, and G. Guerra. 2019. "Endothelial Ca²⁺ Signaling, Angiogenesis and Vasculogenesis: Just What It Takes to Make a Blood Vessel." *International Journal of Molecular Sciences* 20: 3962. <https://doi.org/10.3390/ijms20163962>.
- Morimoto, T., Y. Mineharu, K. Ono, et al. 2017. "Significant Association of RNF213 p.R4810K, a Moyamoya Susceptibility Variant, With Coronary Artery Disease." *PLoS One* 12: e0175649. <https://doi.org/10.1371/journal.pone.0175649>.
- Otten, E. G., E. Werner, A. Crespillo-Casado, et al. 2021. "Ubiquitylation of Lipopolysaccharide by RNF213 During Bacterial Infection." *Nature* 594: 111–116. <https://doi.org/10.1038/s41586-021-03566-4>.
- Petit, A. M., J. Rak, M. C. Hung, et al. 1997. "Neutralizing Antibodies Against Epidermal Growth Factor and ErbB-2/Neu Receptor Tyrosine Kinases Down-Regulate Vascular Endothelial Growth Factor Production by Tumor Cells In Vitro and In Vivo: Angiogenic Implications for Signal Transduction Therapy of Solid Tumors." *American Journal of Pathology* 151: 1523–1530.
- Piccolis, M., L. M. Bond, M. Kampmann, et al. 2019. "Probing the Global Cellular Responses to Lipotoxicity Caused by Saturated Fatty Acids." *Molecular Cell* 74: 32–44.e8. <https://doi.org/10.1016/j.molcel.2019.01.036>.
- Raji, L., A. Tetteh, and A. R. M. R. Amin. 2023. "Role of c-Src in Carcinogenesis and Drug Resistance." *Cancers* 16, no. 1: 32. <https://doi.org/10.3390/cancers16010032>.
- Riedel, F., K. Götte, M. Li, K. Hörmann, and J. R. Grandis. 2002. "EGFR Antisense Treatment of Human HNSCC Cell Lines Down-Regulates VEGF Expression and Endothelial Cell Migration." *International Journal of Oncology* 21: 11–16.
- Roy, V., A. Brodeur, L. T. Deschênes, N. Dupré, and F. Gros-Louis. 2023. "RNF213 Loss-Of-Function Promotes Angiogenesis of Cerebral Microvascular Endothelial Cells in a Cellular State Dependent Manner." *Cells* 12: 78. <https://doi.org/10.3390/cells12010078>.
- Schultz, D. F., D. D. Billadeau, and S. D. Jois. 2023. "EGFR Trafficking: Effect of Dimerization, Dynamics, and Mutation." *Frontiers in Oncology* 13: 1258371. <https://doi.org/10.3389/fonc.2023.1258371>.
- Sugihara, M., D. Morito, S. Ainuki, et al. 2019. "The AAA+ ATPase/Ubiquitin Ligase Mysterin Stabilizes Cytoplasmic Lipid Droplets." *Journal of Cell Biology* 218, no. 3: 949–960. <https://doi.org/10.1083/jcb.201712120>.
- Takeda, M., T. Tezuka, M. Kim, et al. 2020. "Moyamoya Disease Patient Mutations in the RING Domain of RNF213 Reduce Its Ubiquitin Ligase Activity and Enhance NF-κB Activation and Apoptosis in an AAA+ Domain-Dependent Manner." *Biochemical and Biophysical Research Communications* 525: 668–674. <https://doi.org/10.1016/j.bbrc.2020.02.024>.
- Tan, B. Y. Q., C. H. P. Kok, M. B. J. Ng, et al. 2025. "Exploring RNF213 in Ischemic Stroke and Moyamoya Disease: From Cellular Models to Clinical Insights." *Biomedicine* 13: 17. <https://doi.org/10.3390/biomedicines13010017>.
- Tenev, T., H. Keilhack, S. Tomic, et al. 1997. "Both SH2 Domains Are Involved in Interaction of SHP-1 With the Epidermal Growth Factor Receptor but Cannot Confer Receptor-Directed Activity to SHP-1/SHP-2 Chimera." *Journal of Biological Chemistry* 272, no. 9: 5966–5973. <https://doi.org/10.1074/jbc.272.9.5966>.
- Thery, F., L. Martina, C. Asselman, et al. 2021. "Ring Finger Protein 213 Assembles Into a Sensor for Isgylated Proteins With Antimicrobial Activity." *Nature Communications* 12: 5772. <https://doi.org/10.1038/s41467-021-26061-w>.
- Uemura, M., M. Kanazawa, T. Yamagishi, et al. 2020. "Role of RNF213 p.4810K Variant in the Development of Intracranial Arterial Disease in Patients Treated With Nilotinib." *Journal of the Neurological Sciences* 408: 116577. <https://doi.org/10.1016/j.jns.2019.116577>.
- Uribe, M. L., I. Marrocco, and Y. Yarden. 2021. "EGFR in Cancer: Signaling Mechanisms, Drugs, and Acquired Resistance." *Cancers* 13, no. 11: 2748. <https://doi.org/10.3390/cancers13112748>.
- Yan, M., M. Schwaederle, D. Arguello, S. Z. Millis, Z. Gatalica, and R. Kurzrock. 2015. "HER2 Expression Status in Diverse Cancers: Review of Results From 37,992 Patients." *Cancer Metastasis Reviews* 34, no. 1: 157–164. <https://doi.org/10.1007/s10555-015-9552-6>.
- Yang, X., X. Zhu, J. Sheng, et al. 2024. "RNF213 Promotes Treg Cell Differentiation by Facilitating K63-Linked Ubiquitination and Nuclear Translocation of FOXO1." *Nature Communications* 15, no. 1: 5961. <https://doi.org/10.1038/s41467-024-50392-z>.
- Yang, Y. P., H. Ma, A. Starchenko, et al. 2017. "A Chimeric Egfr Protein Reporter Mouse Reveals Egfr Localization and Trafficking in Vivo." *Cell Reports* 19, no. 6: 1257–1267. <https://doi.org/10.1016/j.celrep.2017.04.048>.
- Yarden, Y., and M. J. Sliwkowski. 2001. "Untangling the ErbB Signalling Network." *Nature Reviews Molecular Cell Biology* 2: 127–137. <https://doi.org/10.1038/35052073>.
- Ye, F., X. Niu, F. Liang, et al. 2023. "RNF213 Loss-Of-Function Promotes Pathological Angiogenesis in Moyamoya Disease via the Hippo Pathway." *Brain* 146, no. 11: 4674–4689. <https://doi.org/10.1093/brain/awad225>.
- Zhang, X., K. A. Pickin, R. Bose, N. Jura, P. A. Cole, and J. Kuriyan. 2007. "Inhibition of the EGF Receptor by Binding of MIG6 to an Activating

Kinase Domain Interface.” *Nature* 450, no. 7170: 741–744. <https://doi.org/10.1038/nature05998>.

Zhang, Y., Y. Yuan, L. Jiang, Y. Liu, and L. Zhang. 2023. “The Emerging Role of E3 Ubiquitin Ligase RNF213 as an Antimicrobial Host Determinant.” *Frontiers in Cellular and Infection Microbiology* 13: 1205355. <https://doi.org/10.3389/fcimb.2023.1205355>.

Supporting Information

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