Regulation of RNF144A E3 Ubiquitin Ligase Activity by Self-association through Its Transmembrane Domain*

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Background: RNF144A is a membrane-associated E3 ubiquitin ligase.

Results: The GXXXG motif within the TM domain of RNF144A mediates its self-association and activation of E3 ligase activity.

Conclusion: The TM domain regulates RNF144A through two independent steps: membrane localization and GXXXG motif-mediated self-association.

Significance: The GXXXG motif is conserved among all RBR-TM proteins, suggesting a similar regulation for other RBR-TM proteins.

RNF144A, an E3 ubiquitin ligase for DNA-dependent protein kinase catalytic subunit (DNA-PKcs), can promote DNA damage-induced cell apoptosis. Here we characterize an important regulation of RNF144A through its transmembrane (TM) domain. The TM domain of RNF144A is highly conserved among species. Deletion of the TM domain abolishes its membrane localization and also significantly reduces its ubiquitin ligase activity. Further evidence shows that the TM domain is required for RNF144A self-association and that the self-association may be partially mediated through a classic GXXXG interaction motif. A mutant RNF144A-G252L/G256L (in the $^{G252}_{XXXG}^{G256}$ motif) preserves membrane localization but is defective in self-association and ubiquitin ligase activity. On the other hand, a membrane localization loss mutant of RNF144A still retains self-association and E3 ligase activity, which can be blocked by additional G252L/G256L mutations. Therefore, our data demonstrate that the TM domain of RNF144A has at least two independent roles, membrane localization and E3 ligase activation, to regulate its physiological function. This regulatory mechanism may be applicable to other RBR (RING1-IBR-RING2) E3 ubiquitin ligases because, first, RNF144B also self-associates. Second, all five TM-containing RBR E3 ligases, including RNF144A and RNF144B, have the RBR-TM (GXXXG) superstructure. Mutations of the GXXXG motifs in RNF144A and RNF217 have also been found in human cancers, including a G252D mutation of RNF144A. Interestingly, RNF144A-G252D still preserves self-association and ubiquitin ligase activity but loses membrane localization and is turned over rapidly. In conclusion, both proper membrane localization and self-association are important for RNF144A function.

Ubiquitination, a posttranslational modification, can regulate cell cycle progression, transcriptional regulation, DNA repair, signal transduction, and protein turnover. This process covalently modifies the 76-residue protein ubiquitin onto lysine residues of target proteins by various E3 ligases (1). Among hundreds of ubiquitin E3 ligases, the RING1-IBR-RING2 (RBR) ubiquitin ligases, which comprise at least 12 complex enzymes, have recently gained attention (2–5). Most, if not all, of them are involved in the pathogenesis of diseases and embryonic development (5–10). For example, dysfunction of the RBR ubiquitin ligase Parkin may cause familial autosomal-recessive juvenile Parkinson disease and sporadic early-onset Parkinson disease (11–13). Therefore, the activities of different RBR ubiquitin ligases are regulated tightly at multiple levels, including transcriptional and posttranslational modification, protein-protein interactions, and autoinhibition (14–16). However, it remains a major challenge to understand how these RBR E3 ligases are activated and regulated at the molecular level (2, 3).

RNF144, an RBR subfamily, comprises RNF144A and RNF144B. In addition to a regular RBR domain, RNF144 subfamily members contain a single transmembrane (TM) domain that is close to their C termini (17–19). Both RNF144A and RNF144B are induced by p53 and promote cell apoptosis under various DNA damages (17, 18, 20, 21). RNF144B can also promote cell death by regulating the stability of p63, p73, and $\Delta$Np73 (19, 22, 23). Furthermore, RNF144B may be involved in the degradation of an aberrantly cytoplasm-dislocalized nucleophosmin (NPMc) protein (24), which has been identified in more than 30% of acute myeloid leukemia patients (25). On the other hand, RNF144A has more than 40 somatic mutations.

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The abbreviations used are: RBR, RING1-IBR-RING2; DNA-PKcs, DNA-dependent protein kinase catalytic subunit; IBR, in-between-ring; TM, transmembrane; IP, immunoprecipitation; a.a., amino acids; Ub, ubiquitin; TCGA, The Cancer Genome Atlas.
that were discovered in many clinical human tumor samples (26–28). Most of those important functions are dependent on the ubiquitin ligase activity of the RNF144A subfamily. However, the mechanisms that regulate RNF144A activity remain unknown.

RNF144A, RNF19, and RNF217 are three RBR ubiquitin ligase subfamilies that contain a TM domain. However, the function of these TM domains is not well characterized. Most TM domains do not just restrict the localization of these TM-containing proteins on the membrane but also have other important functions (such as self-association) (29). Our previous study has demonstrated that RNF144A is an E3 ubiquitin ligase and that the TM domain is required for its membrane localization. Deletion of the C-terminal TM domain of RNF144A significantly reduces its auto-ubiquitination. However, the mechanism was not known (18). In this study, we show that the TM domain is required for RNF144A self-association-dependent activation of ubiquitin ligase activity at least in part through a classic GXXXG motif. Deletion of the TM domain of RNF144B also abolishes its self-association, suggesting that the TM domain-dependent activation of E3 ligase activity might be a common mechanism in the RNF144 subfamily.

**Experimental Procedures**

**Cell Culture and Transfection**—HEK293T cells or human osteosarcoma U2OS cells were maintained in DMEM or McCoy’s 5A medium supplemented with 10% FBS, penicillin (50 IU/ml), and streptomycin (50 μg/ml). All cells were grown in a humidified incubator at 37 °C with 5% CO2 and 95% air. A (50 IU/ml), and streptomycin (50 μg/ml). All cells were grown in a humidified incubator at 37 °C with 5% CO2 and 95% air. A standard Lipofectamine® 2000 (Life Technologies) method was used for transfection of HEK293T or U2OS cells.

**Reagents, Chemicals, and Antibodies**—All reagents, chemicals, and antibodies were purchased from Sigma-Aldrich unless indicated otherwise. The antibodies specific to RNF144A were purchased from Abcam (catalog nos. ab75054 and ab89260) and Novus Biological (catalog no. NBP1-01049). DNA-PKcs Ab-4 antibody was purchased from NeoMarkers, GAPDH (catalog no. sc-47724), GST (catalog no. sc-138, Ub (catalog no. sc-9133), GFP (catalog no. sc-8334), EGF receptor (catalog no. sc-03), and HA (catalog no. sc-805) antibodies were purchased from Santa Cruz Biotechnology. The p84 (SE10) antibody was purchased from GeneTex.

**Plasmid Construction and Mutagenesis**—GST-tagged and FLAG-tagged RNF144A and RNF144B have been described previously (18). In brief, the mammalian expression pcMV-Tag2B vector was used for cloning of the FLAG-RNF144A and FLAG-RNF144A truncated mutants. Mutations of FLAG-RNF144A and RNF144B were generated using QuikChange II site-directed mutagenesis kits (Agilent Technologies). The primers for site-directed mutagenesis were as follows: RNF144A-DTM-F, 5′-ctg gca tcg gac aca gca cac ctc ctt tgt tct tgt tcg ac-3′; RNF144A-DTM-R, 5′-gta caa agg gag tgg cct gtg gat gcc aga g3′; RNF144A-3L259F-R, 5′-tgc agg att tgt gcg gcc gcg ctt ggt ggc ctc ac-3′; RNF144A-3L259-R, 5′-tgt gcg agg cca cca ggc gcc gcg cca aac atc ctc ca-3′; RNF144A-G252L/G256L-F, 5′-ctg cat cgg aca cag gtt gtc tta att tgt gca tta ttt ggg ctt c-3′; RNF144A-G252L/G256L-R, 5′-gca gcc cca ata agt caa aaa tta

**Immunoprecipitation (IP), Western Blot Analysis, and Immunofluorescence**—For co-IP, the transfected cells were harvested 24–48 h after transfection with TNN buffer (50 mM Tris, 0.25 mM NaCl, 5 mM EDTA, and 0.5% Nonidet P-40) supplemented with 1 mM dithiothreitol, 1 mM NaF, 1 mM sodium orthovanadate, 20 mM microcystin, and a mixture of protease inhibitors. A 10% aliquot of the cell lysates was saved for protein input control, and immunoprecipitation was carried out using FLAG M2 beads (Sigma-Aldrich) overnight and resolved by 10% or 4–15% gradient SDS-PAGE (Bio-Rad). Specific signals were detected with the appropriate antibodies. Cells prepared for direct Western blot analysis were lysed in radioimmunoprecipitation assay buffer (50 mM Tris (pH 8.0), 150 mM NaCl, 0.5% sodium deoxycholate, 1% Nonidet P-40, and 0.1% SDS). Equal amounts of proteins were resolved by SDS-PAGE and then probed with the indicated antibodies. For immunofluorescence, the cells were stained with a rabbit FLAG antibody (catalog no. F7245, Sigma-Aldrich) and a fluorescein isothiocyanate-conjugated secondary antibody. The nuclei were stained with Hoechst 33258. Images were captured on a Zeiss fluorescence microscope equipped with ApoTome 2 (Axio Observer inverted microscope).

**Protein Purification and in Vitro Ubiquitination Assay**—GST-tagged RNF144A constructs were subcloned from FLAG-tagged RNF144A using BamHI/XhoI sites. Protein purification was performed as described previously (18). Briefly, Escherichia coli strain DH5α transformed with either the pGEX-6P1 vector, pGEX-6P1-RNF144A-WT (aa. 1–292), the pGEX-6P1-RNF144A-RING1 mutant (C20A/C23A), pGEX-6P1-RNF144A-DTM (Δaa. 250–270), and pGEX-6P1-RNF144A-RBB (aa. 1–229) were cultured in Luria-Bertani broth medium containing ampicillin at 37 °C to an A600 value of 0.6. GST fusion proteins were induced by 0.5 mM isopropyl-β-d-galactopyranoside at 37 °C for 3 h. GST-tagged proteins were purified using glutathione-Sepharose 4B (GE Healthcare). The *in vitro* auto-ubiquitination assay was performed in 50 μl of reaction buffer (50 mM Tris (pH 8), 1 mM dithiothreitol, 5 mM...
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MgCl₂, 100 mM NaCl, and 5 mM ATP or 5 μl 10× energy regeneration solution (Boston Biochem). In each reaction, 15 mM human recombinant E1 (Boston Biochem), 0.5 mM UbCH7 (Boston Biochem), and 5 mM HA-ubiquitin (Boston Biochem) were mixed with 0.77–1 mM GST-RNF144A or GST-RNF144A mutants in the reaction buffer and incubated at 37 °C for 90 min. Samples were then washed with 1× PBS five times and subjected to SDS-PAGE for Western blot. The membranes were blotted using the indicated antibody.

In Vivo Ubiquitination Assay—The in vivo ubiquitination assay was performed as described previously (18). Briefly, HEK293T cells were cotransfected with HA-tagged ubiquitin and FLAG-tagged RNF144A (WT or mutants) for 24 h as indicated. Cells were treated with 20 μM MG132 for 6 or 8 h before collection. Cells were then lysed in radioimmunoprecipitation assay buffer or SDS lysis buffer (1% SDS and 60 mM Tris (pH 6.8)), followed by boiling for 5 min at 95 °C. The denatured cell lysates were reconstituted to 0.1% SDS by 1:10 dilution in TNN buffer supplemented with a mixture of protease inhibitors. The lysates were then sonicated and clarified by centrifugation at 12,000 rpm (16,000 × g) for 10 min in a microfuge. Equivalent amounts of lysates were incubated overnight with DNA-PKcs antibody/protein G-agarose at 4 °C. Beads were washed five times with radioimmunoprecipitation assay buffer and subjected to SDS-PAGE, followed by Western blot analysis using anti-HA and anti-DNA-PKcs antibodies.

Subcellular Fractionation Assay—HEK293T cells were transiently transfected with FLAG-tagged RNF144A-WT or mutants for 24 h, and then harvested for cytosol soluble enriched fractionation in the Tris:EDTA 20:2 solution on ice for 15 min. The insoluble fraction (nuclei, subcellular organelles, and plasma membrane) was spun down in a microfuge at 16,000 × g for 60 min. The enriched nuclei were dissolved in TNN lysis buffer. The total cell lysates, nuclear extract, and supernatant were subjected to immunoblotting. The blot was probed with anti-GAPDH, anti-p84, and anti-RNF197 (or anti-EGF receptor) antibodies as cytosolic, nuclear, and membrane markers, respectively.

Cross-linking Assay—HEK293T cells were transiently transfected with FLAG-RNF144A-WT or empty vector for 24 h and then harvested for anti-FLAG IP. The immunoprecipitated proteins were aliquoted equally into two tubes. The tubes were washed and incubated with either 20 mM freshly prepared dimethyl pimelimidate-2HCl (Thermo Scientific) in one tube or vehicle control in the other tube for 60 min. An equal volume of 50 mM NH₄Cl in 1× PBS was added for 10 min to quench the reaction (30). The samples were subjected to SDS-PAGE and Western blot analysis.

Results

The Transmembrane Domain Is Required for RNF144A Autoubiquitination—To determine whether the single-spanning TM of RNF144A is required for its autoubiquitination, we constructed GST-RNF144A-WT (a.a. 1–292), the GST-RNF144A-RING1 mutant (C20A/C23A), GST-RNF144A-ΔTM (Δa.a. 250–270), and the GST-RNF144A-RBR mutant (a.a. 1–229) and performed an in vitro autoubiquitination assay (Fig. 1). Consistent with our previous study, GST-RNF144A-WT, but not the GST-RNF144A-RBR mutant, was able to exhibit clear monoubiquitination in this assay (Fig. 1B) (18). Furthermore, this monoubiquitination also disappeared in both the GST-RNF144A-RING1 mutant and GST-RNF144A-ΔTM, indicating that both RING1 and the TM domain are required for RNF144A ubiquitin ligase activity (Fig. 1C). RNF144A is heavily ubiquitinated in vivo (18), which is characteristic of most ubiquitin ligases. However, RNF144A-ΔTM dramatically lost this ubiquitination (Fig. 1D). Because there is no lysine residue for ubiquitination on the TM domain (Fig. 2A), these data suggest that the TM domain of RNF144A is important for its E3 ligase activity.

RNF144A Possesses Ubiquitin Ligase Activity Independent of Its Membrane Localization in Vivo—To address whether membrane localization or some other undefined properties in the TM domain are important for its ubiquitin ligase activity, we generated a membrane localization loss mutant, RNF144A-3L259R, by mutating three leucine residues to arginine on the TM domain (Fig. 2A). Membrane localization loss of RNF144A-3L259R was confirmed by immunofluorescence and Western blot analysis (Fig. 2, B–D). Indeed, both RNF144A-3L259R and RNF144A-ΔTM lost the membrane localization restriction that is characteristic of wild-type RNF144A (Fig. 2, B and C). In the Western blot experiment, cell lysates were fractionated to a soluble cytosolic fraction and an insoluble fraction containing membranes. Unlike wild-type RNF144A, which mainly localized at the insoluble fraction, RNF144A-3L259R mainly localized at the soluble fraction (Fig. 2D).

To further determine whether membrane localization is important for full RNF144A ubiquitin ligase activity, we checked the ubiquitination of DNA-PKcs, which is a target of RNF144A through its minimal interaction domain (RNF144A, a.a. 185–249) in vivo (18). Consistent with the previous study, overexpression of RNF144A-WT was able to promote the ubiquitination of DNA-PKcs (Fig. 2E). However, RNF144A-ΔTM failed to promote the ubiquitination of DNA-PKcs, confirming that the ΔTM mutant loses its E3 ligase activity. To our surprise, the RNF144A-3L259R mutant was also able to promote DNA-PKcs ubiquitination, suggesting that membrane localization is not necessary for the full activation of RNF144A ubiquitin ligase activity. In fact, there was more DNA-PKcs ubiquitination by RNF144A-3L259R compared with the wild type, suggesting that membrane localization of RNF144A restricts access to its potential substrate(s). Although the TM domain regulates both the membrane localization and ubiquitin ligase activity of RNF144A, RNF144A ubiquitin ligase activity is independent of its membrane localization. These results suggest that the TM domain may regulate RNF144A ubiquitin ligase activity through other membrane localization-independent mechanism(s).

The Transmembrane Domain Is Required for RNF144A Self-association—To investigate other mechanisms responsible for the TM domain-mediated autoubiquitination of RNF144A, we examined the self-association of RNF144A, which is important for the activation of many transmembrane proteins. By co-IP assay, FLAG-RNF144A was able to pull down a significant fraction of GFP-RNF144A but not GFP protein (Fig. 3A), whereas FLAG-RNF144A-ΔTM lost its interaction with GFP-
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RNF144A (Fig. 3B) and Myc-RNF144A (Fig. 3C), indicating that the TM domain is required for RNF144A self-association. We also took an independent approach to detect RNF144A self-association. Proteins were cross-linked by treating cell lysates with dimethyl pimelimidate-2HCl in vitro, and protein self-association was determined by Western blot assay (30). High molecular weight RNF144A signals were only observed after dimethyl pimelimidate-2HCl treatment (Fig. 3D), supporting the self-association of RNF144A. An identical result was observed in RNF144B. FLAG-RNF144B only interacted with GFP-RNF144B-WT but not GFP-RNF144B-/H9004 TM (a.a. 265–287 of human RNF144B) (Fig. 3E). We used the membrane localization loss mutant RNF144A-3L259R to determine whether membrane localization is required for self-association. FLAG-RNF144A-3L259R was able to pull down GFP-RNF144A-3L259R, suggesting that RNF144A self-association does not require membrane localization (Fig. 3F). Therefore, the TM domain is responsible for two independent properties of RNF144 proteins, membrane localization and self-association. Because membrane localization is not required for the E3 ligase activity of RNF144A, these data imply that the self-association property of the TM domain is responsible for its role in ubiquitin ligase activity.

RNF144A Self-associates Partially through the GXXXG Interaction Motif—Among species, RNF144A has a highly conserved TM domain containing a classic GXXXG interaction motif (Fig. 4A). The (G/A)XXX( G/A) motif on many TM domains can support the homo- and heterotypic association of TM helices, and replacement of both glycine residues with leucine residues can significantly reduce these associations (31–33). To determine the effect of G252XXXG256 on RNF144A function, we mutated the GXXXG motif of RNF144A (G252L/G256L). Like RNF144A-WT, RNF144A-G252L/G256L also mainly localized on plasma membrane (Fig. 4, B and C). However, RNF144A-G252L/G256L significantly reduced its interaction with wild-type RNF144A (Fig. 4D). This result suggests that RNF144A self-association occurs at least in part through its GXXXG motif. Furthermore, RNF144A-G252L/G256L dramatically reduced its high molecular weight forms, which were also absent in RNF144A-/H9004 TM but were reproducibly seen in RNF144A-WT (Fig. 4E), suggesting that the mutation in the GXXXG motif may affect its autoubiquitination. This was confirmed by probing with ubiquitin antibody (Fig. 4F). To further investigate the role of self-association in the E3 ligase activity of RNF144A, we examined its activity in promoting DNA-PKcs ubiquitination. Unlike RNF144A-WT, overexpression of RNF144A-G252L/G256L failed to promote ubiquitination of DNA-PKcs (Fig. 4G). To further verify whether the effect of the GXXXG motif on RNF144A function is independent of its membrane localization, we generated a G252L/G256L mutation on membrane loss mutant 3L259R, i.e. the RNF144A-

FIGURE 1. The transmembrane domain is required for RNF144A autoubiquitination. A, schematic of GST-tagged human RNF144A WT and mutants used in this study. IBR, in-between-ring. B and C, Western blot analysis showing autoubiquitination of GST-RNF144A-WT but not GST-RNF144A-RBR, GST-RNF144A-RING1 mutant, and GST-RNF144A-ΔTM in vitro. The in vitro ubiquitination assay was performed as described under “Experimental Procedures.” IB, immunoblot. D, Western blot analysis showing a significantly reduction of FLAG-tagged RNF144A-ΔTM ubiquitination in vivo. Different FLAG-tagged constructs were transfected into HEK293T cells. After 24 h, cell lysates were harvested for FLAG IP, followed by Ub antibody immunoblotting to detect FLAG-RNF144A ubiquitination in vivo.
G252L/G256L-3L259R double mutant (referred to as GL/3L259R hereafter). Indeed, the GL/3L259R mutant also reduced its self-association (Fig. 4H). Moreover, as shown in Fig. 4I, RNF144A-3L259R induced more DNA-PKcs ubiquitination than the wild type (consistent with Fig. 2E), but RNF144A-GL/3L259R failed to promote ubiquitination of DNA-PKcs. Taken together, we conclude that the E3 ligase activity of RNF144A requires GXXXG motif-dependent self-association.

The GXXXG motif mutant RNF144A-G252L/G256L Is Stable in Vivo—Like most ubiquitin ligases, RNF144A is unstable and is degraded by the ubiquitin-proteasome system (18). This could be mediated by autoubiquitation. We determined whether the

FIGURE 2. RNF144A possesses ubiquitin ligase activity independent of its membrane localization in vivo. A, schematic of the membrane localization loss human RNF144A mutant RNF144A-3L259R (259LLL261 to 259RRR261) used in this study. B and C, immunofluorescence study showing that FLAG-RNF144A-3L259R and FLAG-RNF144A-ΔTM redistributed to the cytoplasm (green positive dots). Different FLAG-tagged RNF144A plasmids were transfected into U2OS cells. The next day, cells were fixed and stained with anti-FLAG antibody (green) and Hoechst 33258 (blue; DNA). Immunofluorescence images (×100) were acquired by a Zeiss inverted microscope. D, Western blot analysis showing that FLAG-RNF144A-3L259R mainly localized at the cytoplasmic soluble fraction. FLAG-RNF144A WT and RNF144A-3L259R were transfected into HEK293T cells. After 24 h, cell fractionation was performed. Soluble or insoluble fraction extracts were then immunoblotted (IB) with the indicated antibodies. E, both RNF144A-WT and RNF144A-3L259R promoted ubiquitination of DNA-PKcs in vivo. The wild-type, ΔTM mutant (Δaa. 250–270), and membrane localization loss mutant of RNF144A (3L259R) were cotransfected with HA-ubiquitin into HEK293T cells. After 24 h, cells were treated with MG132 for 6 h, and cell lysates were harvested and immunoprecipitated by anti-DNA-PKcs antibody, followed by immunoblotting.
GXXXG motif mutant RNF144A-G252L/G256L also affected its protein stability. We measured protein half-lives by cycloheximide treatment. RNF144A-WT was unstable, with a short half-life of about 2 h (Fig. 5, A and B). However, RNF144A-G252L/G256L was more stable and had a long half-life of more than 5 h. These results indicate that the mutation in the GXXXG motif reduces RNF144A self-association and its E3 ligase activity and also reduces its self-degradation. A similar result was observed in the whole TM domain deletion mutant, which lost its self-association and E3 ligase activity. RNF144A-ΔTM had a long half-life of more than 5 h (Fig. 5, C and D). In contrast, RNF144A-3L259R was extremely unstable, with a half-life of about 10 min (Fig. 5, E and F). The double mutant RNF144A-GL/3L259R only partially rescued its protein stability and had a half-life of about 25 min, suggesting that a proper transmembrane helix structure may also contribute to RNF144A protein stability. Taken together, the TM domain is important for RNF144A function, and the GXXXG motif in the TM mediates RNF144A self-association to activate its ubiquitin ligase activity.

The Somatic Mutant RNF144A-G252D Is Mislocalized and Unstable in Vivo—An increasing amount of somatic mutations in human RNF144A have been identified in cancer (68 somatic

FIGURE 3. The transmembrane domain is important for RNF144A self-association. A, Western blot analysis showing that FLAG-RNF144A was able to interact with GFP-RNF144A. IB, immunoblot; WCL, whole cell lysate. B and C, unlike RNF144A, RNF144A-ΔTM was not able to interact with GFP-RNF144A nor with Myc-RNF144A. Different FLAG-tagged constructs and Myc-tagged or GFP-tagged RNF144A-WT were transfected into HEK293T cells. After 24 h, cell lysates were harvested for anti-FLAG IP, followed immunoblotting with the indicated antibody. D, Western blot analysis showing the self-association of FLAG-tagged RNF144A-WT. DMP, dimethyl pimelimidate-2HCl. E, FLAG-RNF144B was able to interact with GFP-RNF144B, but not with GFP-RNF144B-ΔTM (Δa.a. 263–287). F, the membrane localization loss mutant of RNF144A (3L259R) was able to form self-association.
mutations in 292 residues), including V251M, G252D, L261F, and V263M mutations on the TM domain (Fig. 6A). Because a proper TM structure is important for RNF144A localization, function, and stability, we investigated whether any of these mutations might affect RNF144A localization, activity, or stability. Although the V251M, L261F, and V263M mutations did not affect plasma membrane localization, RNF144A-G252D lost its membrane localization and was distributed in the cyto-

![Figure 4. RNF144A self-associates partially through the GXXXG interaction motif.](image)

FIGURE 4. RNF144A self-associates partially through the GXXXG interaction motif. A, alignment of the RNF144A C terminus among species. The TM domain is indicated with a bold underline. A conserved GXXXG motif is indicated by two red columns. B, Western blot analysis showing that FLAG-RNF144A-G252L/G256L mainly localized at the membrane-containing insoluble fraction. C, the immunofluorescence images (×100) of FLAG-RNF144A-G252L/G256L show that they mainly localize on the membrane (green positive dots). D, Western blot analysis showing a reduction of self-association in the FLAG-tagged RNF144A-G252L/G256L mutant. E and F, Western blot analysis showing that a reduction of the high molecular weight species (E) and the ubiquitinated form (F) in the FLAG-RNF144A-G252L/G256L mutant. G, the FLAG-RNF144A-G252L/G256L mutant dramatically lost its ability to promote ubiquitination of DNA-PKcs in vivo. The wild type and the G252L/G256L mutant were cotransfected with HA-ubiquitin into HEK293T cells. After 24 h, cells were treated with MG132 for 6 h, and cell lysates were harvested and immunoprecipitated by anti-DNA-PKcs antibody, followed by immunoblotting. WCL, whole cell lysate.
sol (Fig. 6 B and C). In addition, the G252D mutation strongly reduced its protein stability, with a half-life of less than 1 h (Fig. 6, D and E). These results suggest that G252D may alter normal RNF144A physiological functions at least through its mislocalization and rapid protein turnover. Interestingly, contrary to the aspartic acid mutation, mutation from glycine to alanine or leucine on residue 252 had a similar localization and half-life as wild type RNF144A (Fig. 6, F–H). These data suggest that the
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carboxylic acid side chain of aspartic acid on residue 252 affects its membrane localization. As discussed earlier, a proper transmembrane helix structure may contribute to RNF144A protein stability, and therefore G252D was more unstable.

Because G252D occurs on the GXXXG motif, we also examined whether this mutation had an effect on self-association. As shown in Fig. 6f, RNF144A-G252D still preserved the self-association ability. Mutation of G252 to alanine or leucine alone or mutations of both G252 and G256 to alanine also did not inhibit self-association as G252L/G256L did. This is consistent with the prior observation that mutations of both glycine residues to leucine are needed to significantly block the activity of the GXXXG motif (34). Consistent with its ability in self-association, the G252D mutant was still capable of promoting the ubiquitination of DNA-PKcs in vivo (Fig. 6f). This result supports the hypothesis that membrane localization and E3 ligase activity regulation are two independent functions of the RNF144A TM domain. Interestingly, this mislocalized G252D mutant promoted more DNA-PKcs ubiquitination (Fig. 6k), similar to RNF144A-3L259R (Fig. 2e).

Discussion

Emerging evidence shows that the RBR E3 ligases have different additional domains on their long N termini and may be responsible for other functions or autoinhibition (2, 3). There is no conserved motif in the N termini of RNF144A (a.a. 1–19) and RNF144B (a.a. 1–29). Therefore, there may not be common functions for the N termini of the RNF144 subfamily, such as autoinhibition, which has been seen in other RBR E3 ligases. In this regard, the C terminus and TM domain may be the candidate regions for regulation of the RNF144 subfamily.

Most, if not all, TM-containing proteins form self-association for regulation, including activation and target recognition. This self-association of α helix TM-containing proteins is mainly mediated through their TM domains using polar, GXXXG, and proline motifs or others (31–33). Interestingly, all five RBR-TM-containing proteins have a GXXXG motif on their TM domains (Fig. 7b). Furthermore, the space between the second RING domain and the first TM domain of these five proteins is about 34–45 amino acids. Therefore, the mechanism elucidated in this study may be applicable to other RBR-TM proteins. The relatively conserved proximity between RBR and TM domains among all five RBR-TM proteins is intriguing, suggesting RBR-TM as a “superdomain” with potential cooperativity between the RING domain and the TM domain.

Mutation of the 252GiaF256 motif of RNF144A to the 252Liafl256 motif only partially reduced self-association and the ubiquitin ligase activity of RNF144A, suggesting that either Gly → Leu mutations do not completely destroy the interactions or that other residues or mechanism also contribute to self-association. Moreover, the membrane localization loss mutants RNF144A-3L259R and RNF144A-G252D can still self-associate and maintain E3 ligase activity. These data support the hypothesis that membrane localization and self-association are two independent properties of the RNF144A TM domain.

More and more somatic mutations are identified on the TM domain of human RNF144A (26–28). Of the four point mutations examined in our study, only G252D causes mislocalization and rapid protein turnover. Whether the other three mutations can affect other aspects of RNF144A function remains to be determined. Although the G252D mutation is on the GXXXG motif of RNF144A, this single negative charge amino acid substitution is not sufficient to affect its self-association and activity but blocks its translocation to the plasma membrane, again supporting the hypothesis that they are two independent processes. It is possible that additional mechanisms might contribute to the regulation of RNF144A. During the preparation of this manuscript, two more somatic mutations, A255fs*30 and L260M, were identified on the TM domain (37, 38). It would be very interesting to investigate the potential effects of these somatic mutations.

**FIGURE 6.** The somatic mutation RNF144A-G252D is mislocalized and unstable in vivo. A, schematic of four somatic mutations on the TM domain of human RNF144A. B, immunofluorescence study showing that FLAG-RNF144A-G252D redistributed to the cytoplasm (green positive dots). The images shown are ×100. C, Western blot analysis showing that FLAG-RNF144A-G252D mainly localized at the cytoplasmic soluble fraction. D and E, the half-lives of four somatic mutations of RNF144A were determined as in Fig. 5. FLAG-RNF144A-G252D had a short half-life compared with RNF144A-WT and other somatic mutations. IB, Immunoblot; CHX, cycloheximide; F, immunofluorescence images (×100) of FLAG-RNF144A-G252A and FLAG-RNF144A-G252L I and J, Western blot analysis showing that the FLAG-tagged RNF144A-G252D somatic mutation still had the ability to self-associate (I) and to promote ubiquitination of DNA-PKcs in vivo (J). K, the ubiquitinated DNA-PKcs level/DNA-PKcs ratio was quantified from J using ImageJ. The value from the FLAG group was set as 1.
It is worth noting that mutations in the $508^{\text{GXXXG}}512^{\text{G}}$ motif of RNF217 in tumors also occur, including G508E (gastric adenocarcinoma, The Cancer Genome Atlas (TCGA)), A510P (rectal adenocarcinoma, TCGA) and G512V (hepatocellular carcinoma, TCGA) (data obtained via the International Cancer Genome Consortium Data Portal). Although the biological significance of these mutations needs to be determined in future studies, our study predicts a loss of function for these mutations and full activation of its ubiquitin ligase activity. This single transmembrane domain cannot only restrict the localization of RNF144A at plasma membrane and some subcellular vesicles but is also required for its self-association and subsequent activation of E3 ligase activity. Similar results were observed in RNF144B, which has a highly conserved C terminus with RNF144A. Therefore, this TM domain-mediated regulation may be a common feature in the RNF144 subfamily.

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