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ORIGINAL ARTICLE

RNF11 is a GGA protein cargo and acts as a molecular adaptor for GGA3 ubiquitination mediated by Itch

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Ring finger protein 11 (RNF11) is a RING (really interesting new gene)-H2 E3 ligase that is overexpressed in several human tumor tissues. The mature protein, which is anchored to membranes via a double acylation, localizes to early endosome and recycling compartments. Apart from its subcellular localization, additional lines of evidence implicate RNF11 in the mechanisms underlying vesicle traffic. Here we identify two acidic-cluster dileucine (Ac-LL) motifs, which are recognized by the VHS domains of Golgi-localized, gamma adaptin era-containing, ADP-ribosylation factor-binding protein (GGA) adaptors, as the molecular determinants governing RNF11 sorting at the *trans*-Golgi network and its internalization from the plasma membrane. We also show that RNF11 recruits itch to drive the ubiquitination of GGA3. This function is experimentally detectable only in cells overexpressing an RNF11 variant that is inactivated in the RING domain, indicating that RNF11 recruits GGA3 and controls its ubiquitination by regulating itch activity. Accordingly, our data demonstrate the involvement of itch in regulating GGA3 stability. Indeed, we observe that the endogenous levels of GGA3 are increased in cells knocked down for itch and endogenous GGA3 is hyperubiquitinated in an itch-dependent manner in a cell line expressing catalytically inactive RNF11. Our data are consistent with a model whereby the RING E3 ligase RNF11 is a novel GGA cargo actively participating in regulating the ubiquitination of the GGA protein family. The results that we are presenting put RNF11 at the center of a finally regulated system where it acts both as an adaptor and a modulator of itch-mediated control of ubiquitination events underlying membrane traffic.

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INTRODUCTION

Proteins that are associated to vesicular membranes are transported to their destination compartment by finely regulated processes. The key regulators of vesicle traffic are adaptor proteins (APs), which interact with clathrin and recruit cargo proteins to the nascent vesicle.[1] The specificity of this process is mediated by the recognition of sorting signals in the cytosolic tails of transmembrane proteins.[2] The Golgi-localized, gamma adaptin era-containing, ADP-ribosylation factor-binding protein (GGA) proteins are a family of monomeric adaptors involved in trafficking between the *trans*-Golgi network (TGN) and the endosomal compartments.[3,4–6] In mammals, this family comprises three members (GGA1, GGA2 and GGA3) sharing a conserved modular organization consisting of a VHS domain (Vps27, HRS and STAM) at the amino terminus, followed by a GAT domain (GGA and TOM), an unstructured region (hinge) and a carboxy-terminal GAE (γ-adaptin ear) domain (Figure 1a). All these regions have been implicated in functional protein interactions. The VHS domain selects cargo proteins by binding to acidic-cluster dileucine signals (DxxLL).[7–11] The GAT domain mediates the binding with the GTP-bound form of ADP-ribosylation factor and is crucial for the regulated recruitment of GGAs to the TGN and for ubiquitin binding.[12–14] The unstructured hinge binds clathrin,[15] whereas the GAE domain is responsible for the recruitment of accessory proteins that regulate clathrin-mediated endocytosis.[5]

Differently from other APs, GGA-mediated intracellular traffic has been associated with a few receptors, which are typically type-1 transmembrane receptors involved in several endocytic functions. The DxxLL motif was originally identified in the cytosolic domain of the mannose-6-phosphate receptors (CI-MPR and CD-MPR),[16–18] as an essential signal for the anterograde transport from the TGN to the endosome/lysosome system. Later, additional receptors such as sortilin,[19] BACE1[20] and the lipoprotein receptor-related proteins LRP3 and LRP9[18,21] were shown to be dependent on GGAs for proper sorting. In this work, we provide evidence that the RING (really interesting new gene) E3 ligase ring finger protein 11 (RNF11), which we have previously shown to bind to GGA1,[22] is *de facto* a GGA cargo and modulates GGA stability by acting as a scaffold bridging HECT (homologous to E6-associated protein C-terminus)-type E3 ligases.

RNF11 was originally cloned from a library enriched for complementary DNAs (cDNAs) overexpressed in breast cancer cell lines.[23] The gene is highly conserved[24] and codes for a protein of 154 amino acids (aa) containing at least two functional modules: an N-terminal PPPY motif that binds WW domain-containing proteins such as AIP4/itch, Nedd4 and Smurf1/2 (SMAD-specific E3 ubiquitin-protein ligase 1/2) and a C-terminal RING-H2 domain (C3H2C3-type zinc finger)[25,26] that functions as a scaffold for the coordinated transfer of ubiquitin to substrate proteins together with the E2 enzymes UbcH5[27] and Ubc13.[28] Thus, RNF11 has the potential of both acting as a typical RING E3 ligase downstream of an enzymatic cascade for the ubiquitination of specific substrates, and as a molecular adaptor of HECT-type ligases. Much of the evidence reported so far implicates RNF11 in the regulation of

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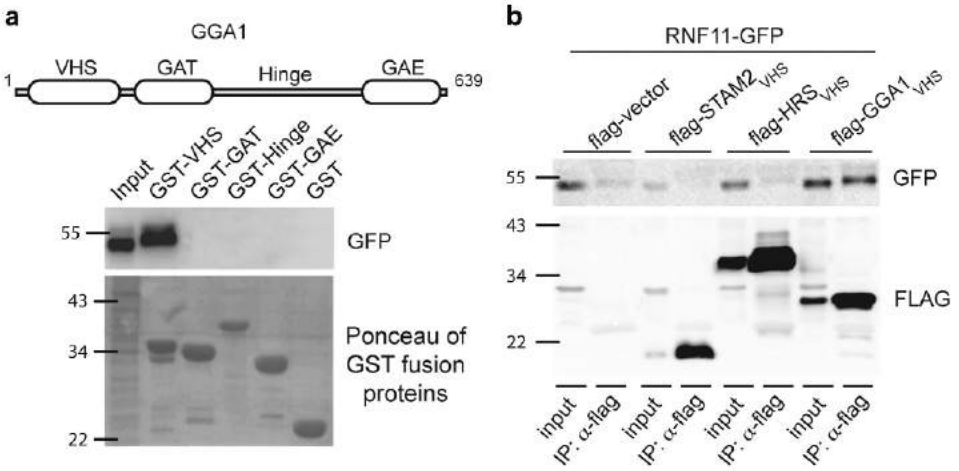

Figure 1. The VHS domain of GGA1 binds RNF11. (**a**) Schematic representation of GGA1 showing its domain composition. The pull-down assay was performed using as bait the GST fusions of the indicated constructs of GGA1, purified as described in Materials and methods. Equal amounts of recombinant proteins were incubated with a cell extract from HEK293 cells transfected with GFP-tagged RNF11. Ponceau staining of the membranes indicates that similar amounts of GST fusions were used. (**b**) The VHS domain of GGA1 immunoprecipitates RNF11. Flag-tagged N-terminal regions of STAM2 (aa 1–152), HRS (aa 1–280) and GGA1 (aa 1–180) including the VHS domain were co-transfected with RNF11-GFP in HEK293 cells. Immunoprecipitates performed with anti-flag M2 affinity gel were resolved by SDS–PAGE and immunoblotted with the anti-GFP antibody. After stripping, the membrane was incubated with anti-flag antibody, to check for successful bait purification.

several signaling pathways. RNF11 enhances the transforming growth factor receptor signaling by both abrogating Smurf2-mediated receptor ubiquitination[29,30] and by promoting the Smurf2-mediated degradation of AMSH (associated molecule with the SH3 domain of STAM), a de-ubiquitinating enzyme that enhances transforming growth factor-β signaling and epidermal growth factor receptor (EGFR) endosomal recycling. Moreover, it has been demonstrated that RNF11 is a critical component of the A20 ubiquitin-editing protein complex that inhibit TNF-mediated NF-κB activation.[31] Finally, RNF11 interacts with SARA (Smad anchor for receptor activation) and the Endosomal sorting complex required for transport (ESCRT)-0 complex, thus participating in the regulation of lysosomal degradation of EGFR.[32]

We have shown that RNF11 localizes to vesicles that, to a large extent, define the endosome compartment, including both the early and recycling compartments. A double acylation of the N-terminus is a generic, albeit necessary, signal for membrane association, whereas the selection of the functional endosomal compartment requires additional signals.[22] In addition, we have provided evidence that a peptide encompassing a dileucine motif at the N-terminal end of RNF11 (aa 7–21) binds the VHS domain of GGA1 in an *in vitro* assay. Here we characterize in detail the interaction between RNF11 and the GGA family of clathrin adaptors and we investigate the functional implication of this association on both RNF11 intracellular sorting and the regulation of GGAs ubiquitination and stability. Our data reveal an intricate and finely regulated mechanism, where the VHS-binding (Ac-LL) and the WW-binding sites (PPPY) of RNF11 govern the correct intracellular sorting of the E3 ligase and its biological function as molecular adaptor for the ubiquitination mediated by itch.

RESULTS

RNF11 contains two dileucine motifs that mediate the binding to the VHS domain of GGA proteins

Li and Seth[29,30] used the yeast two-hybrid method to produce a list of putative RNF11 interactors, several of which are implicated in the modulation of the endocytic traffic. These include Eps15, Epsin, STAM2, GGA1 and GGA3. We have previously shown that RNF11 binds GGA1.[22] We next asked whether the interaction with GGAs might be required for RNF11 correct sorting. By GST pull-down experiments with different GGA1 domains, we identify the GGA1 VHS as the domain that is responsible for binding to RNF11 (Figure 1a). Moreover, when cotransfected with the VHS domains of GGA1, HRS or STAM2 (Figure 1b), RNF11 is co-immunoprecipitated only by the VHS of GGA1, thus confirming the different binding specificities of the members of the VHS-containing protein family.[7,8]

We next precisely mapped the RNF11 peptide that is responsible for GGA1-VHS binding using the SPOT synthesis method (Figures 2a and b; Supplementary Figure 1).[33] Two regions of RNF11 specifically bind to the VHS domain of GGA1. One is close to the amino terminus (10-SD**D**IS**LL**HE-18) and a second is near the C-terminus (142-PV**D**AA**LL**SS-150). Neither the VHS domains of STAM2 and HRS nor the GST bound to these regions. A third cluster of overlapping peptides (from 36 to 44), mapping to the RING domain of RNF11, bind weakly to the VHS domains of HRS and GGA1 and to a lesser extent to STAM2. As these peptides map to regions that are masked in the native protein fold, we consider these signals as not relevant. Both amino- and carboxy-binding motifs conform to the consensus DxxLL.[34,35] Moreover, the amino-terminal motif (11-DDISLL-16) is identical to the sorting motif identified in BACE1, previously described as a recognition site for GGA VHS domains (residues 495–500).[34]

Both RNF11 dileucine motifs mediate the interaction with the VHS domains of GGA1 and GGA2

The DxxLL motif is necessary for VHS binding, however, the sequence context may finally modulate cargo selection. We then performed alanine scanning of the peptides that contain the DxxLL motifs with the aim of identifying residues, flanking the key positions in the binding sites, that modulate binding to the VHS domain of RNF11. Four replicated arrays were probed with the VHS domains of GGA1, GGA2, GGA3 and with GST as a negative control. As shown in Figure 2c and Supplementary Figure 1C, the interaction was sensitive to substitutions of either of the two leucines or the aspartate in the consensus motif. Interestingly, we observed that binding of the amino-terminal motif to the GGA2 VHS domain is markedly reduced by mutating Asp11 and partially affected by alanine substitution of Glu18. Similarly, all the VHS domains bind weakly the C-terminal motif when Glu152, which is four residues downstream the dileucine pair, is mutated to Ala. Glu141, on the other hand, seems to be required for the stable association of the VHS-GGA2 domain with the C-terminal motif.

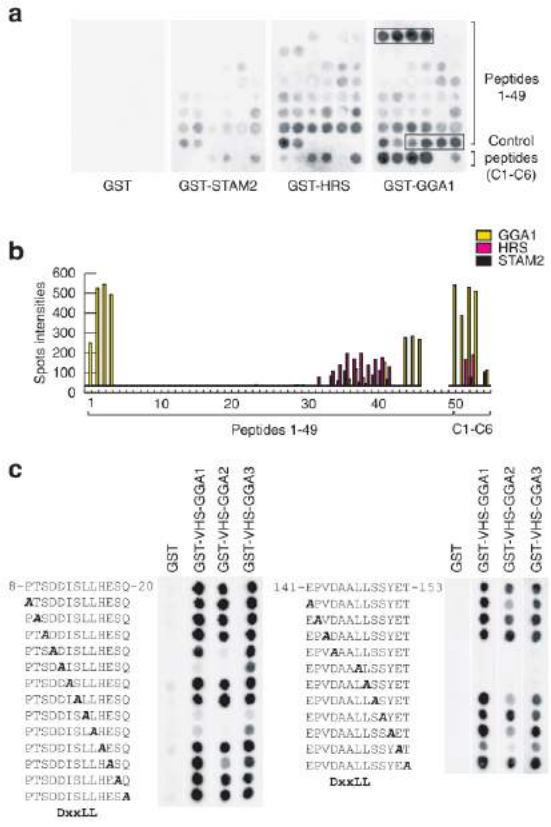

Figure 2. Peptide scan of the RNF11 sequence. (**a**) Full-length human RNF11 was synthesized as 15 amino acid peptides (peptides 1–49), overlapping by 12 amino acids, on a cellulose membrane using the SPOT synthesis method (Intavis, Koeln, Germany).[33] Duplicate arrays were incubated with the VHS domains of STAM2 (aa 1–152), HRS (aa 1–180) and GGA (aa 1–180) fused to the GST or with GST alone. The bound VHS fusion proteins were visualized by anti-GST and anti-goat immunoglobulin G coupled to horseradish peroxidase. Four peptides, synthesized in the bottom row of the spot membrane and containing a dileucine motif, from CI-MPR, CD-MPR, GGA1 and sortilin and two proline-rich sequences were used as positive and negative controls, respectively. Signals considered to be positive were highlighted with squares. (**b**) Spot-signal intensities were quantified by using AIDA Image Analyser (Raytest, Chessington, UK). (**c**) Peptides corresponding to amino acids 8–20 and 141–153 were analyzed by alanine-scanning mutagenesis. Membranes were separately incubated with the VHS domains of GGA1, GGA2 and GGA3 fused to the GST and probed with an anti-GST antibody.

Finally, a significant reduction in binding efficiency of the GGA2-VHS domain is observed when Ser149 is changed to Ala. Differences in residue preference may reflect some *in vivo* specificity in cargo recognition, suggesting that residues other than those within the DxxLL consensus could be important in modulating the selective recruitment of cargos by GGA proteins. The results obtained by the PepSpot (Intavis, Koeln, Germany) analysis were confirmed by pull-down assays. We first engineered three plasmids expressing RNF11 hybrid proteins carrying LL to AA mutations in either the amino- (Ln) or carboxy-terminal (Lc) DxxLL motifs or a third one where both sequence signals were mutated (Lnc) and we tested their ability to bind GGA1 and GGA2 VHS domains (Figure 3). A schematic representation of the LL/AA mutants is shown in Figure 3a. The Ln mutant is affinity purified with an efficiency that is only slightly lower than that of wild type by both VHS domains. Conversely, the Lc mutant exhibits a binding efficiency unexpectedly higher for both VHS domains. The interaction becomes undetectable if both DxxLL motifs are simultaneously mutated (Lnc). Thus, we can assert that the dileucine motifs act as binding sites for GGA VHS domains and binding is abrogated only when both motifs are inactivated. Moreover, the observed increased binding of RNF11 to GGAs when mutated in the C-terminal VHS-binding motif suggests an allosteric mechanism by which the inactivation of the C-terminal binding site remotely affects the affinity of the VHS domain for the N-terminal dileucine motif.

Mutating the dileucine motifs affects the RNF11 distribution to membrane compartments

We next asked whether the disruption of the motifs that are necessary for the interaction with GGAs is important for *in vivo* RNF11 sorting and localization. For this purpose, HeLa cells transfected with the wild-type or the LL mutants were fixed and stained with anti-RNF11 antibody and examined by fluorescence microscopy (Figure 3c). As endogenous RNF11 is expressed at very low levels, we consider the labeling as largely specific for the transfected constructs. The Ln and Lnc mutants were retained at the plasma membrane, a phenotype that was already observed when BACE1 was mutated in the GGA1-binding site.[34,35] Conversely, the Lc mutant has an overall intracellular distribution that is similar to wild-type RNF11, being primary localized in vesicles of the early and recycling compartments, as confirmed by the immunofluorescence analysis carried out with markers of the endosome (EEA1, CD63), recycling (Transferrin-488) and lysosome compartments (LAMP-2; Figure 4a). Nevertheless, when Rab4, Rab5, Rab11 and Rab7 were used as finer compartment markers,[36] we observed that the Lc mutant preferentially localizes in Rab4 and Rab5 over Rab11-positive vesicles, suggesting a prominent peripheral distribution (Figure 4b). These results support a functional and distinct role of both dileucine motifs in the interaction with GGAs and in RNF11 sorting. Particularly, the amino-terminal signal is epistatic over the carboxy-terminal motif, as shown by the observation that the Ln and Lnc mutants share the same intracellular distribution. Therefore, although the amino-terminal sequence DISLL (aa 12–16) acts as a trafficking signal responsible for the correct sorting at the TGN, the C-terminal LL motif modulates the distribution of RNF11 inside the endosome compartment. Interestingly, the dileucine pair 15-LL-16 is also included in the consensus motif for the recognition by the heterotetrameric clathrin adaptor complexes AP [(D/E)XXXL(L/I)][37] (see Figure 2c), thus suggesting that probably functionally redundant proteins could act in the intracellular sorting of the E3 ligase.

RNF11 colocalizes with endogenous GGA3 in HeLa cells

We next examined the colocalization of RNF11 with endogenous GGA3 by confocal microscopy. This was performed using HeLa cells transiently expressing RNF11 wild type or the Ln and Lc mutants (Figure 5). As already described, GGA3 displayed a juxtanuclear and vesicular distribution with vesicles predominantly localized in the TGN but also in more peripheral regions.[3,5,14] We observed that wild-type RNF11 colocalizes with GGA3 primary at perinuclear vesicles; as expected, the degree of colocalization between the RNF11 Ln mutant and GGA3 was markedly decreased; conversely, the Lc mutant, that is distributed also at more peripheral vesicles, shows higher colocalization with GGA3. Finally, the colocalization of RNF11 with GGA1, 2 and 3, performed in cells overexpressing RNF11 together with myc-tagged GGA1/2 or GG3-GFP, showed that RNF11 and GGA proteins colocalize in peripheral vesicles that are close to the plasma membrane (Supplementary Figure 2). The distribution at perinuclear compartments was hard to perform due to the signal intensity of overexpressed GGAs accumulating at the TGN that drowns out the RNF11 signal.

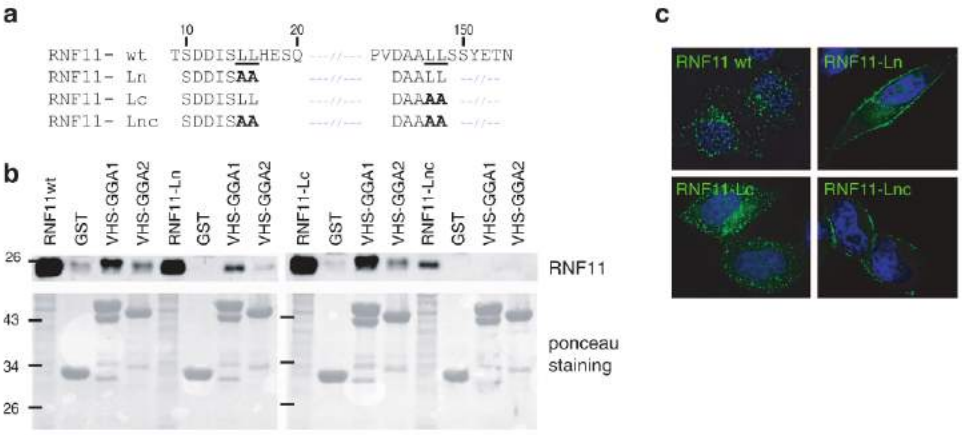

Figure 3. The interaction with the VHS domains of GGA proteins depends on both dileucine motifs in RNF11. (**a**) Schematic representation of the RNF11-LL/AA mutants generated. Residues that have been mutated are underlined in the wild-type RNF11 sequence. (**b**) The dileucine pairs are both involved in the interaction with the VHS domains of GGA1 and GGA2. A pull-down assay was performed with the GST-VHS recombinant proteins from GGA1 and GGA2 and with GST alone. Equal amounts of purified domains were incubated with a cell extract obtained from HEK293 cells transfected with RNF11wt or mutants Ln, Lc and Lnc. Samples were analyzed by SDS–PAGE and immunoblotted with anti-RNF11. Equal amounts of recombinant proteins were used, as shown by ponceau staining in the bottom panel. The experiment is representative of three independent repeats. (**c**) RNF11 mutants LL/AA are defective in sorting. HeLa cells transiently transfected with the indicated constructs were analyzed by immunofluorescence microscopy with the anti-RNF11 antibody.

Association of GGA3 and RNF11 at endogenous levels visualized by *in situ* proximity ligation assay

We next analyzed the interaction between endogenous RNF11 and GGA3 by *in* situ proximity ligation assay (PLA) in HeLa cells (Figure 6). The *in situ* PLA was developed to enable detection of individual proteins and protein–protein interactions in cell lines and tissues.[38] The technique is a selective and sensitive method that is based on antibodies and combines the dual recognition of a probe-targeted assay with a split-reporter approach.[39,40] To establish a suitable PLA protocol for the detection of GGA3–RNF11 interaction, we made use of antibodies that specifically stain the two proteins in conventional indirect immunofluorescence. We validated the selectivity of the assay by comparing the *in situ* PLA performed with anti-GGA3 or anti-RNF11 separately (negative controls), with the experiment using both antibodies. Moreover, we analyzed the percentage of blobs localizing in the fast- and slow-recycling compartments, where the majority of the RNF11 signal can be detected. To this end, we performed the PLA assay in cells starved for 1 h and incubated with 488-conjugated tranferrin (Tf) for 10 or 60 min, to visualize the fast- and slow-recycling intracellular routes, respectively. Samples were then analyzed by confocal immunofluorescence microscopy. As shown in Figure 6a (left panel), *in situ* PLA signals were significantly increased in cells analyzed with both antibodies, compared with the negative controls (see Figure 6b for quantification). Unexpectedly, we observed a strong increment in the number of blobs in cells incubated with Tf for 10 min, whereas the number of events tends to a gradual return to the steady-state condition after 60 min of Tf uptake (see Figure 5a right panel and quantification graph in figure 6B). Finally, the colocalization analysis that measures the number of PLA dots localizing in vesicles that are positive for 488-conjugated Tf (Figure 6b, right graph) confirmed that the majority of events of GGA3–RNF11 interaction occurring in the recycling compartment are concentrated in vesicles of the fast-recycling route, as only a small increment of dots (~20%) colocalizing with transferrin can be appreciated after 60 min of incubation. Concluding, this result provides evidence that an interaction between RNF11 and GGA3 occurs in HeLa cells at steady state. Complex formation is somehow promoted by the internalization of the Tf receptor (TfR) from the plasma membrane, but a more generalized mechanism extendible to other membrane receptors cannot be excluded. Although further experiments are needed to fully characterize the molecular mechanism, we could suggest that GGA3–RNF11 interaction is modulated by variations in post-translation modifications occurring during early events of endocytosis and presumably dependent on the recruitment of the internalization machinery.

The RNF11 catalytical activity is involved in the internalization of TfR from the plasma membrane

We then asked whether RNF11 catalytical activity could have a role in regulating its intracellular sorting. To this end, we generated two mutants predicted to affect RNF11 functions that may be more directly involved in the ubiquitination process: the RING mutant (RINGm) where two Cys-to-Ser substitutions inactivate the RING domain (Cys99,102/Ser) and the PA mutant where the mutation Tyr40Ala in the WW domain-interacting motif (PPPY) affects the interaction with HECT ligases. We first transfected HeLa cells with these mutants and analyzed them by western blotting and immunofluorescence. As shown in Figure 7a, the expression levels of RNF11 mutants differ when transiently expressed in cells. Particularly, the PA mutant that is affected in the association with HECT ligases is stabilized when compared with the wild-type protein; conversely, the RINGm is hardly detectable, suggesting that the catalytic activity of RNF11 is involved in the regulation of its protein stability. Moreover, the two mutations have a different impact on RNF11 intracellular localization when analyzed by confocal microscopy (Figure 7b), as the PA mutant primary localizes in the early and recycling endosomes while the intracellular distribution of the RINGm is partly shifted to vesicles of the degradative route (CD63- and LAMP-2-positive compartments). Strikingly, we observed that the uptake of transferrin is markedly reduced in cells overexpressing the RINGm. We conclude that the RNF11 catalytic activity is essential for the internalization of the TfR. We then asked whether the RINGm mutant was also affected in the internalization from the plasma membrane. To this end, we transfected a plasmid vector expressing the dominant-negative dynamin mutant (K44A) in two different inducible cell lines expressing RNF11wt or the RINGm mutant. The dynamin K44A interferes with the function of endogenous dynamin and blocks vesicle internalization before membrane scission.[41] In this condition, we observed the accumulation of wild-type RNF11 at the cell membrane, clearly

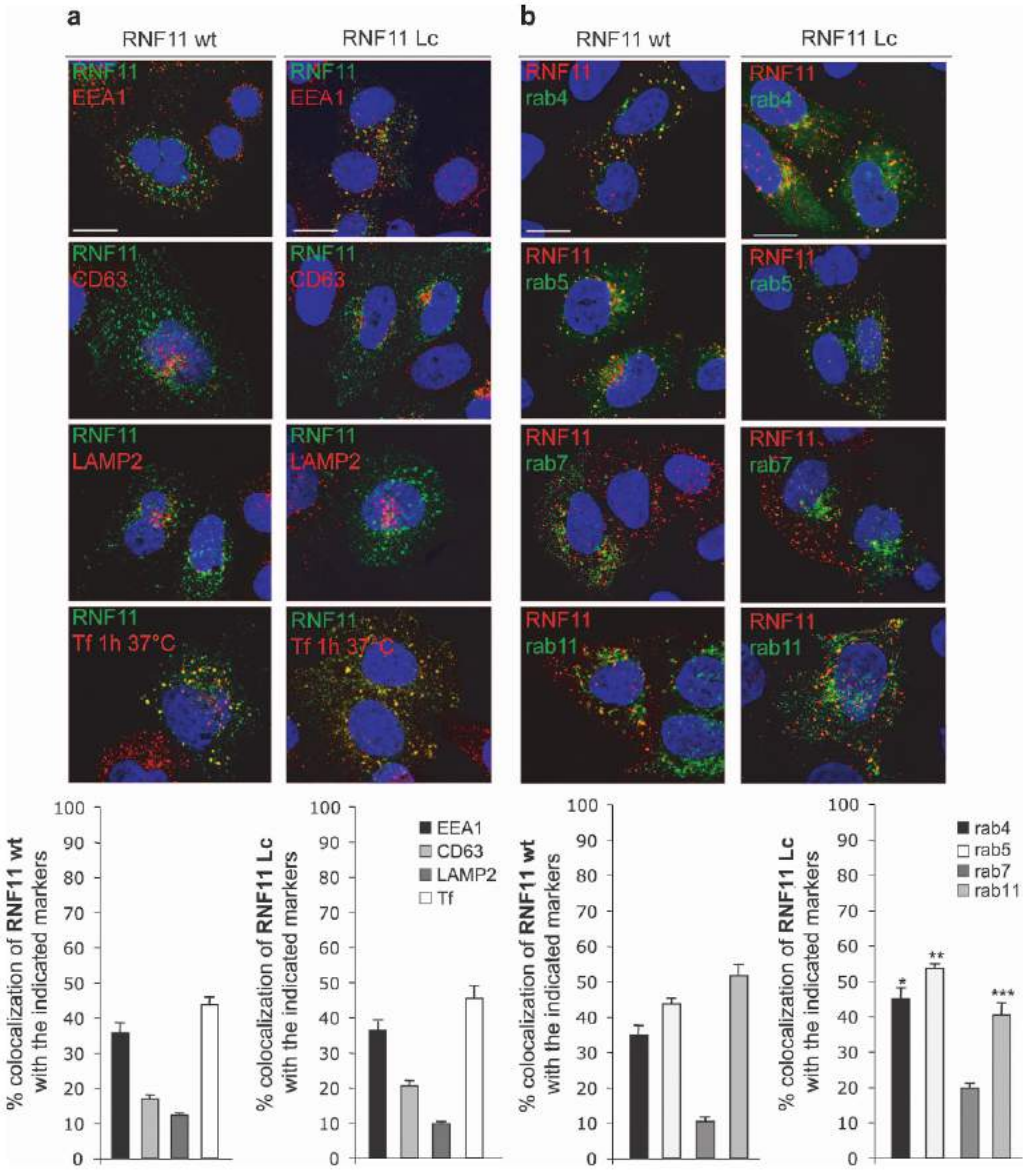

Figure 4. The intracellular localization of the Lc mutant shows significant changes compared with the wild-type protein, revealed by the immunostaining with different endocytic markers. (**a**) HeLa cells were transiently transfected with untagged RNF11 wild-type and the Lc mutant and analyzed by immunofluorescence with anti-RNF11 and anti-EEA1, anti-CD63 and anti-LAMP2 to evaluate the colocalization with early-, late-endosome and lysosome markers. Uptake of transferrin (1 h at 37 °C) was carried out to measure the intracellular localization of RNF11 in the fast- and slow-recycling compartments. (**b**) The intracellular localization of RNF11wt and the LL/AA mutant Lc was analyzed by immunofluorescence microscopy and compared with Rab4, Rab5, Rab7 and Rab11 expressed as GFP fusion proteins (green). Rab4 and Rab5 are localized in the early endocytic compartments, Rab11 participates in controlling traffic through the perinuclear recycling endosome, whereas Rab7 is involved in early-to-late endosome traffic. Twenty-four hours post transfection, HeLa cells were fixed and stained with the anti-RNF11 antibody (red). Images obtained by three-dimensional reconstruction of a selection of three out of the total number of the serial optical sections are shown: the selected sequential sections are central and crossing the nucleus. Quantitative analysis of the percentage of colocalization was performed. Results are expressed as mean values ± s.e.; the percentage of colocalization was calculated analyzing a minimum of 40 cells randomly taken from three independent experiments. Student's *t*-test was performed and significance level has been defined as follows: $^{*}P < 0.05$ vs RNF11 wt, $^{**}P < 0.001$ vs RNF11 wt, $^{***}P < 0.05$ vs RNF11 wt. Bars: 10 μm.

demonstrating that the active endocytosis from the plasma membrane is required for the correct localization of RNF11 to the endosome compartment (Figure 7c). Conversely, we observed that dynamin K44A has a significantly less pronounced effect on the localization of the catalytically inactive RNF11-RINGm mutant, as most of the fluorescent signal is distributed inside the cell. Moreover, although the Lnc mutant is missorted to the plasma membrane (Figure 7d), the RINGm-Lnc mutant still localizes in intracellular and perinuclear vesicles. Taken together, these data indicate that a functional RING domain is essential for RNF11 to transit from the TGN to the endosome compartment via the plasma membrane and suggests a more general function of RNF11 in the internalization of proteins from the plasma membrane.

Inactivation of the RING domain reveals a role for RNF11 as a bridge for GGA3 ubiquitination mediated by the HECT-type E3 ligase itch

To investigate a possible involvement of RNF11 in the regulation of GGA ubiquitination and stability, we analyzed the *in vivo*

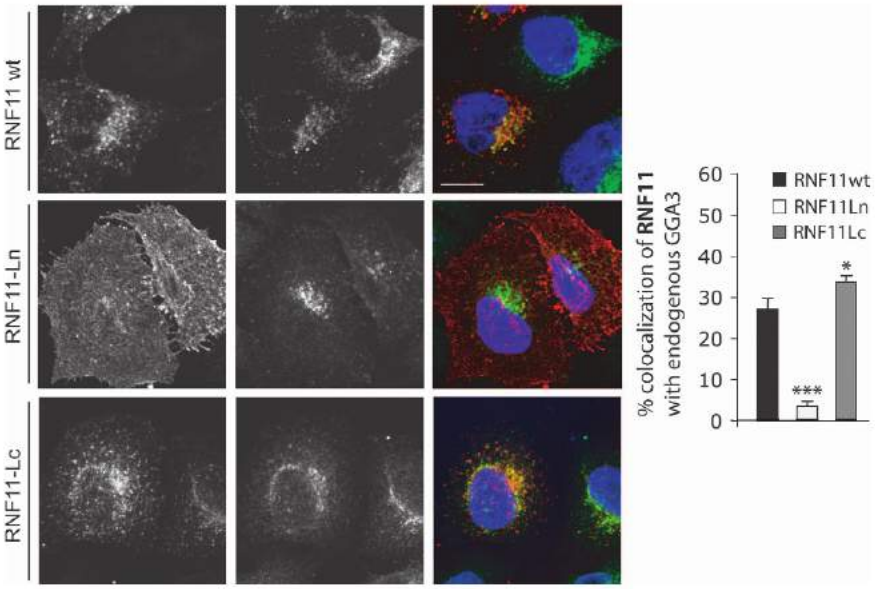

Figure 5. RNF11 and GGAs colocalize at intracellular vesicles in HeLa cells. Confocal microscopy of HeLa cells transiently transfected with wild-type RNF11. Quantitative analysis of the percentage of colocalization was performed as previously shown. Results (mean ± SEM) are shown in the bar graphs. The statistical analysis was performed with a Student's t test. $^{*}P < 0.05$, $^{***}P < 0.0001$. Bars: 10 μm.

ubiquitination of GGA1 and GGA3 in HeLa cells (Figure 8a). As shown, with the exception of the RINGm mutant, the overexpression of RNF11 caused little change in the ubiquitination levels of GGA1 and GGA3, with a range of variability going from no to little increment (see also Figure 8c, lane 3). Conversely, a hyperubiquitination of GGA1 and GGA3 was detected in cells expressing the RNF11 catalytically inactive mutant. We then asked whether the inactivation of the GGA-binding sites in the RINGm mutant could revert the hyperubiquitination of GGA3. To this end, we generated the RNF11 mutants RINGm-Ln, RINGm-Lc and RINGm-Lnc and we overexpressed them in HeLa cells together with GGA3-GFP and HA-ubiquitin (Figure 8b). As shown, the stimulation of GGA ubiquitination could only be observed if the GGA-binding motifs were in their wild-type form. Thus, RNF11 does not act as the ubiquitin ligase promoting the ubiquitination of GGAs, but it is required for their modification. We then tested whether RNF11 could bridge HECT E3 ligases to GGAs. We opted for itch that we have previously identified as an RNF11-associated E3 enzyme responsible for its ubiquitination. Consistently, the observed RNF11-scaffolding activity must be dependent on the motifs, PY and dileucine, which bind the itch E3 ligase and its target GGAs, respectively (Figure 8c). To this end, we co-transfected GGA3-GFP together with RNF11 and myc-itch and we monitored the ubiquitination of GGA3. Wild-type RNF11 causes a small but consistent increase in GGA3 ubiquitination; this effect is abrogated in cells transfected with the PA mutant while it is increased with the RINGm mutant (Figure 8c, compare lanes 1, 3, 5 and 7). Moreover, neither ectopically expressed itch nor the co-expression of itch with wild-type RNF11 or with the PA mutant that is unable to bind itch affected GGA3 ubiquitination (lanes 2, 4 and 6). Conversely, the hyperubiquitination caused by RINGm overexpression was further increased in cells co-expressing itch (lane 8), but not the catalytically inactive mutant (itch-KD, lane 10), whereas it is abrogated in cells where itch wild type was co-transfected with the RINGm lacking GGA-binding sites (RINGm-Lnc, lane 9). Finally, we observed that itch co-immunoprecipitated with GGA3 and that this interaction was substantially decreased in cells expressing RNF11 PA or RINGm-Lnc mutants (compare lanes 4, 6, 8 and 10). Attempts to detect an RNF11 GGA complex either by immunoprecipitation with GGAs or with RNF11 antibodies were unsuccessful. Nevertheless, collectively, these experiments demonstrate that the integrity of the signals that promote binding of RNF11 to GGA and itch is essential for the ubiquitination of GGA proteins mediated by itch. Moreover, our data indicate that the catalytic activity of RNF11 somehow antagonizes the enzymatic activity of itch, as the ubiquitination mediated by the HECT ligase is only detected when the RNF11 RING domain is inactivated. Accordingly, the small increment in GGA3 ubiquitination observed following the overexpression of RNF11wt alone is abrogated in cells overexpressing RNF11 together with itch (compare lanes 3 and 4), suggesting that the functionality of the RNF11/itch complex is strictly inhibited by the reciprocal activity of both E3 ligases.

Itch affects the endogenous levels of GGA3

The results so far are consistent with RNF11 forming a bridge that promotes the ubiquitination of GGA proteins by HECT-type E3 ligases. As most of the features of the model are based on experiments where the main players were transiently overexpressed, we developed a cell system that could support the model in more physiological conditions. The poor expression of RNF11 in all the cell systems that we have tested did not allow monitoring RNF11 protein levels with the available tools. Thus, we engineered T-Rex-derived 293 HEK cell lines containing a single copy of gene constructs expressing wild-type, or the two mutants PA and RINGm, RNF11 under the control of a tetracycline-inducible promoter (Supplementary Figure 3).

To establish whether RNF11 could affect GGA stability, we incubated the cells for 18 h with doxycycline to induce the expression of RNF11 and we monitored the impact on GGA3 protein concentration after 8 h treatment with cycloheximide (Figure 9a). Wild-type RNF11 or the PA mutants do not affect GGA3 stability. Conversely, lower levels of endogenous GGA3 can be observed in the T-Rex RINGm cell line particularly after cycloheximide treatment, indicating that a functional RNF11 catalytic activity promotes GGA3 stability. We also observed that the band corresponding to the monoubiquitinated form of GGA3 rapidly disappears after cycloheximide treatment (see the longer exposure in Figure 9a), suggesting that monoubiquitinated GGA3 is preferentially targeted for degradation. The instability of GGA3 observed in the T-Rex RINGm cell line is in agreement with the

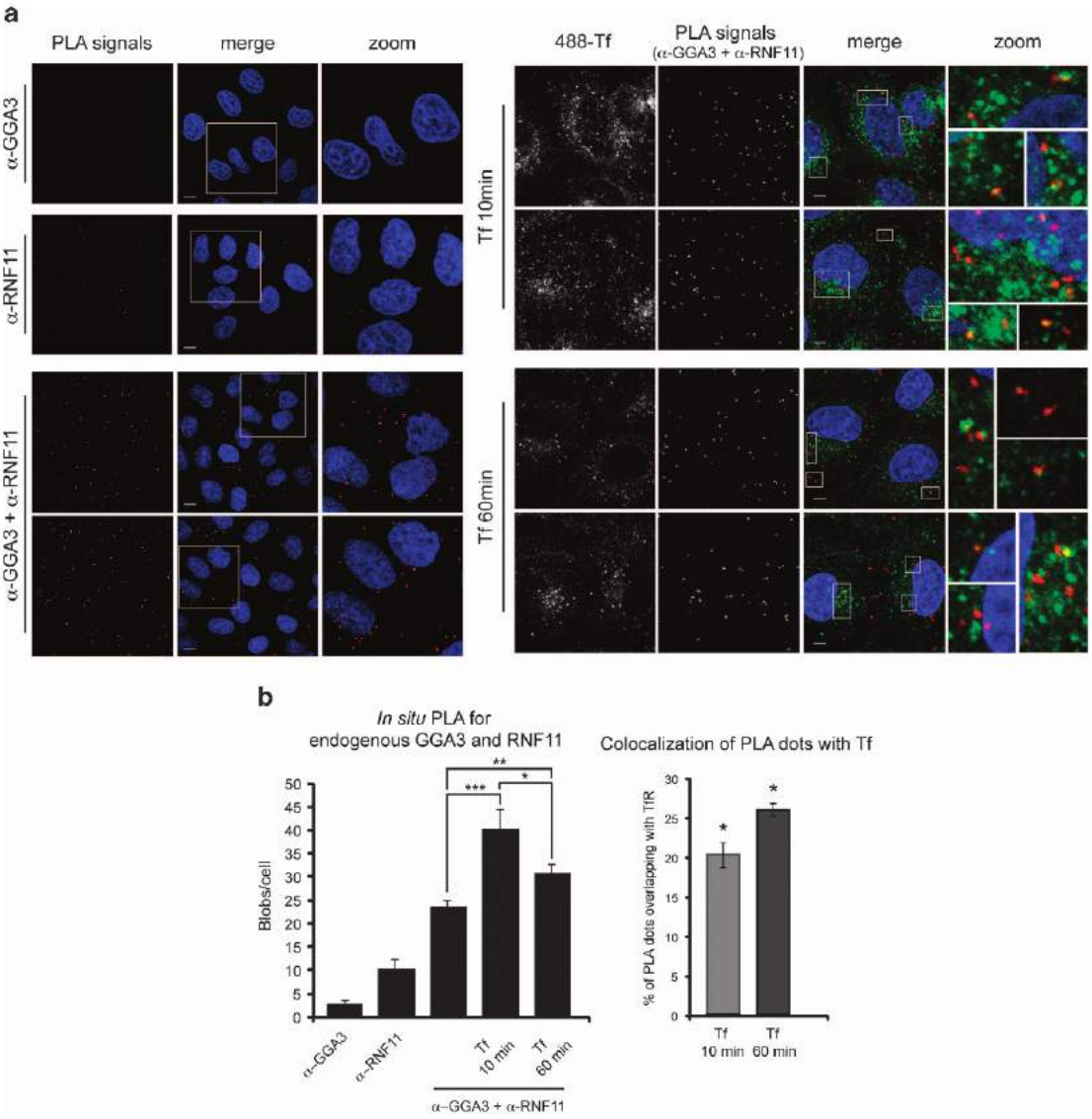

Figure 6. Intracellular localization of endogenous RNF11 and GGA3 complexes with *in situ* PLA. (**a**) HeLa cells were fixed, permeabilized and incubated with anti-GGA3 and anti-RNF11, or with only one of these antibodies as negative control. Then, PLA was performed to visualize GGA3–RNF11-interacting complexes (each red spot represents a single protein–protein interaction), as described in Materials and methods. Nuclei were stained with DAPI. Cells were analyzed by confocal immunofluorescence microscopy. Images for each condition are shown and representative regions were selected and magnified. (**b**) Quantitative analysis. The number of fluorescent red dots was quantified using the Blobfinder V3.2 software. Quantitative analysis of colocalization between PLA dots and Tf was performed by measuring Mander's Coefficient. Stack colocalization analysis tool provided by Genome Damage and Stability Centre (GDSC) colocalization ImageJ plugin (NIH, Bethesda, MD, USA) was used. Results (mean ± s.e.m.) are shown in the bar graphs. Statistical analysis was performed with Student's *t*-test. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.0001$. Bars: 10 μm.

results of the experiments in HeLa cells (see Figure 8) and supports a model whereby RNF11 acts as a bridge to direct itch activity to the physiological targets. To gain more evidence, we knocked down itch in the three T-Rex RNF11 cell lines and monitored the endogenous levels of GGA1 and GGA3 (Figure 9b). As shown, GGA3 levels, but not GGA1, increase after knocking down itch, thus confirming the involvement of itch in modulating GGA3 stability. Both the ability of RNF11 to bind HECT ligases and the catalytic activity of the RING domain are important for GGA3 stabilization, as both the PA and the RINGm mutants are less effective in GGA3 stabilization (compare lanes 1, 3 and 4 in Figure 9c).

Finally, we analyzed the ubiquitination of GGA3 following itch knockdown (Figure 10). As observed in the previous experiment, the knockdown of itch affects GGA3 stability. As far as concern the ubiquitination of GGA3, when both RNF11 and itch are expressed (lanes 2, 4 and 6), the western blot analysis of immunoprecipitated GGA3 revealed a clear increase in GGA3 ubiquitination only in the T-Rex RINGm cell line (lane 6). The ubiquitin ladder is abrogated if itch is knocked down (compare lanes 5 and 6), clearly demonstrating that itch is responsible for the increase in endogenous GGA3 ubiquitination in presence of RNF11 RINGm. In this experiment, we also assayed GGA3 immunoprecipitates with anti-itch and we detected endogenous itch in complex with GGA3 only in the T-Rex RNF11 RINGm cell line. This is in accord with the observation that GGA3 is strongly ubiquitinated by itch in this context. Interestingly, the ubiquitinated form of itch is also efficiently recruited in the immunoprecipitate. Therefore, our results indicate that both RNF11 and itch are involved in the ubiquitination and stabilization of GGA3. More specifically, our

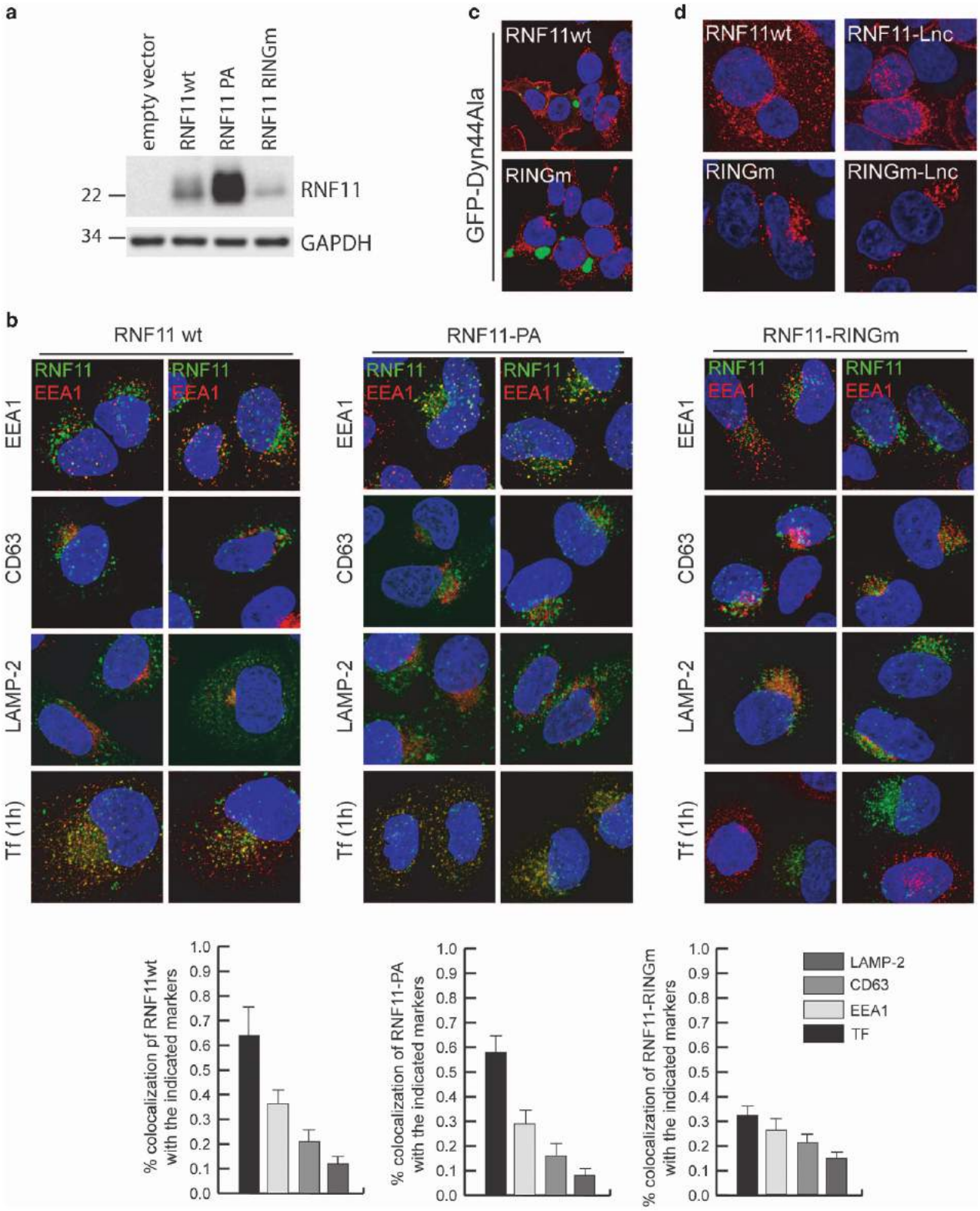

Figure 7. Functional motifs in RNF11 analyzed by western blotting and immunofluorescence in HeLa cells. (**a**) RNF11 wild type, the mutant in the WW-binding site (PA), and the catalytically inactive RNF11 (RINGm) were transiently transfected in HeLa cells. Cells were incubated with doxycycline for the indicated times and equal amounts of cellular extracts were analyzed by western blotting with anti-RNF11 antibody. Signal intensities were quantitated by densitometry using ImageQuant software (GE Healthcare Europe GmbH, Freiburg, Germany) and normalized to GAPDH. Data are representative of three independent experiments. (**b**) HeLa cells transiently transfected as in **a** were analyzed by confocal microscopy with markers of the endocytic route (EEA1, CD63, Tf and LAMP-2). Quantitative analysis of the percentage of colocalization is shown in the graph. (**c**) HeLa cells were transfected with GFP-DynK44A and, after 24 h, with RNF11wt or RINGm. Fixed cells were analyzed by conventional microscopy. (**d**) Plasmids coding for RNF11wt, RINGm, Lnc and RINGm-Lnc were transfected in HeLa and analyzed by conventional microscopy. The catalytically inactive RNF11, in the absence of the dileucine signals (RINGm-Lnc)—a condition that irreversibly target RNF11 to the plasma membrane—still localizes in a perinuclear compartment, differently from the Lnc mutant that is missorted to the plasma membrane.

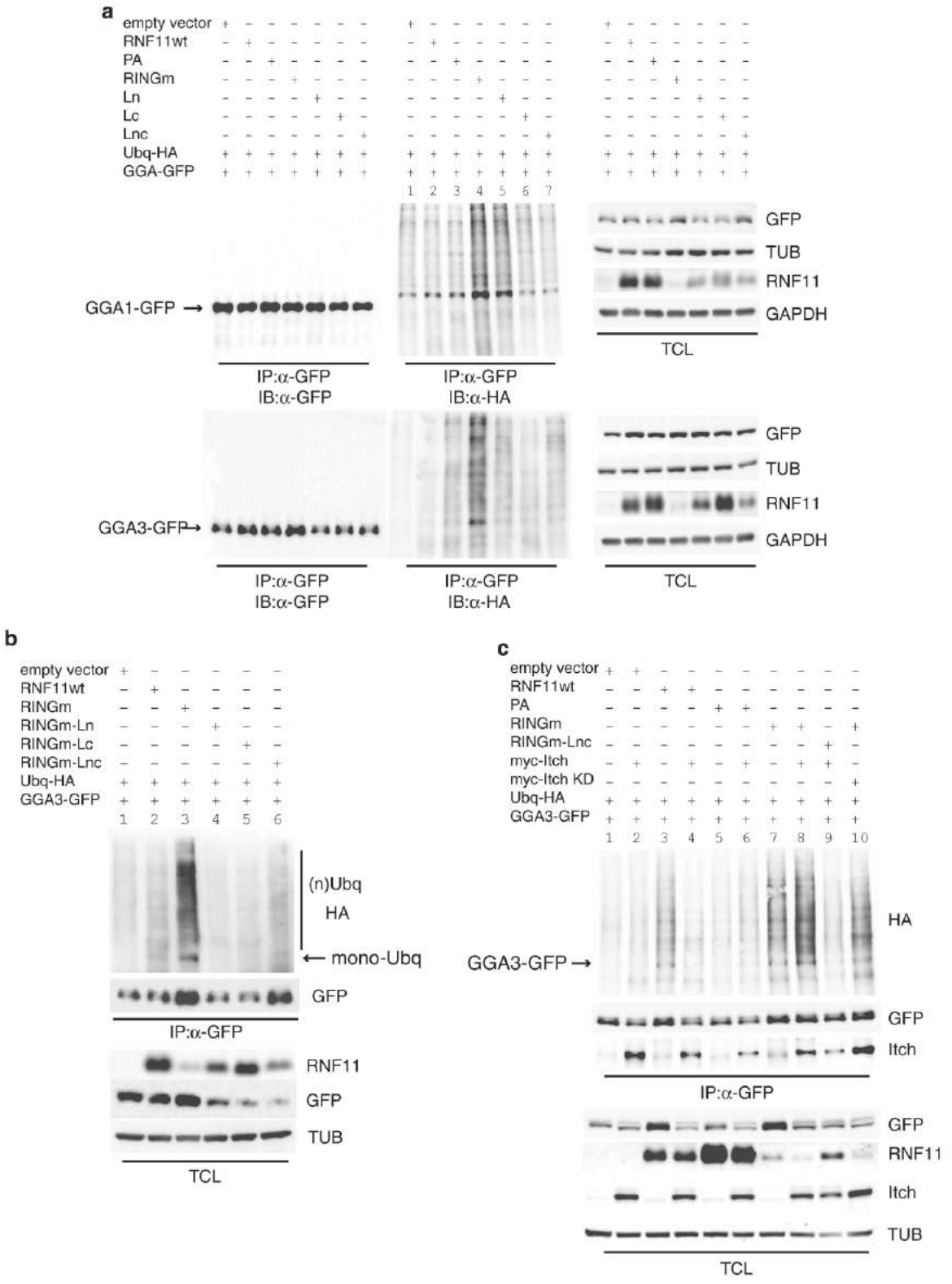

Figure 8. The RINGm mutant affects the ubiquitination of GGA3. (**a**) HeLa cells where co-transfected with GGA3-GFP and HA-ubiquitin together with different constructs of RNF11 (Ln, Lc, Lnc) affected in the interaction with GGA VHS domain. After immunoprecipitation with anti-GFP, samples were analyzed by SDS–PAGE and immunoblotted with anti-HA to reveal the presence of the ubiquitinated isoforms. The arrow indicates the GGA3-GFP mono-ubiquitinated isoform. The levels of each transfected construct in the cell lysates, and a fraction of immunoprecipitated GGAs, are shown. The overexpression of RINGm mutant but not wild-type RNF11 increases mono- and polyubiquitination of GGA3. The experiment is representative of three independent repeats. (**b**) Hyperubiquitination of GGA3 by RNF11 RINGm requires the dileucine motifs. HeLa cells were transfected with the RNF11 mutants indicated in figure together with GGA3-GFP and Ubq-HA. Samples were analyzed as in **a**: the arrow indicates the position of unmodified GGA3-GFP. (**c**) Itch increases the ubiquitination of GGA3 in the presence of RNF11 RINGm mutant. The experiment described in **a** was performed by overexpressing the RNF11 mutants RINGm and RINGm-Lnc together with itch.

data suggest that the adaptor function of RNF11 is essential for HECT-mediated ubiquitination of substrates; on the other hand, the catalytic activity of RNF11 negatively regulates the HECT ligase function, as only after RNF11 RING inactivation, the enzymatic activity of itch can be clearly highlighted, thus suggesting a cross-talk between the activities of the two E3 ligases. Finally, RNF11 was recently shown to be associated with the Endosomal sorting complex required for transport (ESCRT-0) complex and with SARA in the endosome compartment.[32] We then asked whether RNF11 had a regulatory function on this complex by inhibiting itch activity (Figure 11). We observed that itch ubiquitinates RNF11 as previously demonstrated.[22] Moreover, overexpressed itch also induces the hyperubiquitination of STAM2 and HRS, an effect that is completely abrogated when also RNF11 is co-expressed. These data further confirm the inhibitory activity exerted on itch by RNF11.

DISCUSSION

The ubiquitination process involves the action of an enzymatic cascade (E1, E2, E3) and terminates with the transfer of ubiquitin to lysine residues in a target molecule. The E3 ubiquitin ligase contains either a HECT or a RING domain and performs a critical role because it selectively binds the target protein and directly or indirectly catalyzes its ligation to ubiquitin. Ubiquitin itself can act as a substrate for the addition of more ubiquitin moieties, so that the ubiquitination signal is typically amplified and differentiated based on linkage type and chain length.[42] It has been recently suggested that monoubiquitination acts as a reversible modification for the activation of substrates among a pool of otherwise identical proteins and that specialized factors, referred as E4 (ubiquitin chain assembly factor) enzymes, are needed for ubiquitin chain elongation and subsequent degradation of the activated substrates.[43]

RNF11 is a RING E3 ligase that is anchored, via a double acylation, to intracellular vesicles primary corresponding to the endosome compartment. Membrane anchoring is essential for RNF11 to be ubiquitinated by itch.[22] In this work, we investigated the interaction between RNF11 and the GGA protein family. We identified two DxxLL sorting signals in RNF11, the recognition of which represents a critical step in the mechanism underlying selection of the intracellular routes taken by RNF11 following its insertion into the endoplasmic reticulum and the vesicular distribution in specific 'districts' of the endosome compartment. Intriguingly, the amino-terminal sequence of RNF11 spanning residues 11–16 (DDISLL) presents both an intracellular sorting motif recognized by VHS domains, as clearly established by our binding assays, and a putative binding site for the AP2 complex. The observation suggests that, as frequently happens in cellular biology where the same function can be fulfilled by redundant mechanisms, other APs could also govern the intracellular distribution of RNF11. Unfortunately, we attempted with no success the simultaneous depletion of GGA1, 2 and 3 so that the lack of any significant difference in the RNF11 sub-cellular localization pattern cannot be univocally interpreted (data not shown).

The catalytic activity of the RING domain is also involved in RNF11 intracellular sorting. Indeed, the mutation in the RING domain is epistatic over the absence of functional sorting motifs, as RNF11 RINGm-Lnc, as the single mutant RINGm, is sequestered in enlarged perinuclear vesicles and does not reach the plasma membrane (Figures 7c and d). Moreover, cells overexpressing the RINGm are markedly affected in the uptake of transferrin, and in the same experimental condition GGA1 and GGA3 are hyper-ubiquitinated (Figures 8 and 10), suggesting that trafficking of specific GGA cargo proteins could also be affected. This evidence prompted us to highlight a novel function of RNF11 in the regulation of the GGA-dependent trafficking machinery. We showed that GGA3 is ubiquitinated by itch and that this modification requires the functional motifs that mediate the binding of GGA3 and itch to RNF11. In other words, RNF11 acts as an adaptor, connecting itch to the trafficking machinery responsible for protein sorting and transport. Several lines of evidence support this statement: (i) the absence of functional sorting motifs in RNF11 disrupts itch-mediated ubiquitination of GGA3 (Figure 8), (ii) GGA3 accumulates in cells depleted of itch (Figures 9b and 10), (iii) the hyperubiquitination of endogenous GGA3 is abrogated if itch is knocked down (Figure 10) and (iv) this mechanism seems to be highly specific for GGA3 as GGA1 level is unaffected in the same experimental condition. Our experiments also showed that RNF11 inhibits itch, as (i) only when RNF11 is mutated in the RING domain the catalytical activity of itch can be revealed and (ii) itch-mediated ubiquitination of ESCRT-0 proteins is abrogated when RNF11 is expressed together with itch. Others have also shown the involvement of adaptors in the recognition of HECT ligase substrates. The transmembrane lysosomal protein LAPTM5 recruits, via its PY motif, the HECT ligase Nedd4 and promotes Nedd4-mediated ubiquitination of GGA3,[44] whereas N4BP1 has been shown to associate with and negatively regulate itch by interfering with the E3 binding to its substrates.[45] Even though the mechanistic details are not fully clarified, we can speculate that RNF11, by interacting with Ubc13 and UbcH5, antagonizes itch activity by regulating its access to substrates. A possible mechanism could be dependent on RNF11 autoubiquitination and/or phosphorylations, so that post-translational modifications in the RING E3 ligase could be responsible for the temporally and spatially regulated recruitment of catalytically active complexes on the trafficking machinery.

The observation that GGAs could be involved in the endocytosis of RNF11 is also intriguing. It has been demonstrated that the endocytosis and the transport to endosome of Memapsin2 is dependent on GGA-binding motifs.[20] Moreover, GGA3 has been shown to act in cargo selection of receptors destined to recycling[46] and RNA interference of GGA3 expression impairs the degradation of internalized EGF.[14] Puertollano and Bonifacino demonstrated that the population of GGAs localized to early endosomes is associated with components of the ubiquitin-dependent sorting machinery, such as TSG101, and with ubiquitinated cargo. This pool of GGA proteins seems to spatially control receptors' internalization.[47] The evidence that the GGA3-RNF11 complex, detected by *in situ* PLA, is promoted by the internalization of TfR suggests the presence of a dynamic complex where post-translation modifications activated by the endocytic mechanism regulate the localization and strength of the interaction. The involvement of RNF11 catalytic activity in the uptake of transferrin and the hyperubiquitination of endogenous GGA3, dependent on both itch activity and RNF11 recruitment as demonstrated by our experiments, is in good agreement with our model indicating RNF11 as a key molecule that links the ubiquitination machinery to membrane trafficking. Accordingly, recent reports show that RNF11 promotes EGFR and ErbB2 degradation and that its activity is negatively modulated by the interaction with the HECT ligase WWP1 (WW domain-containing E3 ubiquitin protein ligase).[48] It has also been proposed that RNF11 downregulates EGFR by targeting the deubiquitinating enzyme AMSH for degradation by forming a complex with Smurf2.[49] Finally, RNF11 is heavily ubiquitinated and it is also a ubiquitin receptor as suggested by the presence of a ubiquitin-interacting motif in its amino acid sequence.[30] Taken together, all these observations suggest that the correct intracellular trafficking and the activity of RNF11 could have a role in the downregulation of activated membrane receptors by interacting with proteins of the endosome sorting machinery and by recruiting HECT ligases that are essential for the spatial and temporal regulation of both signaling and downregulation pathways.

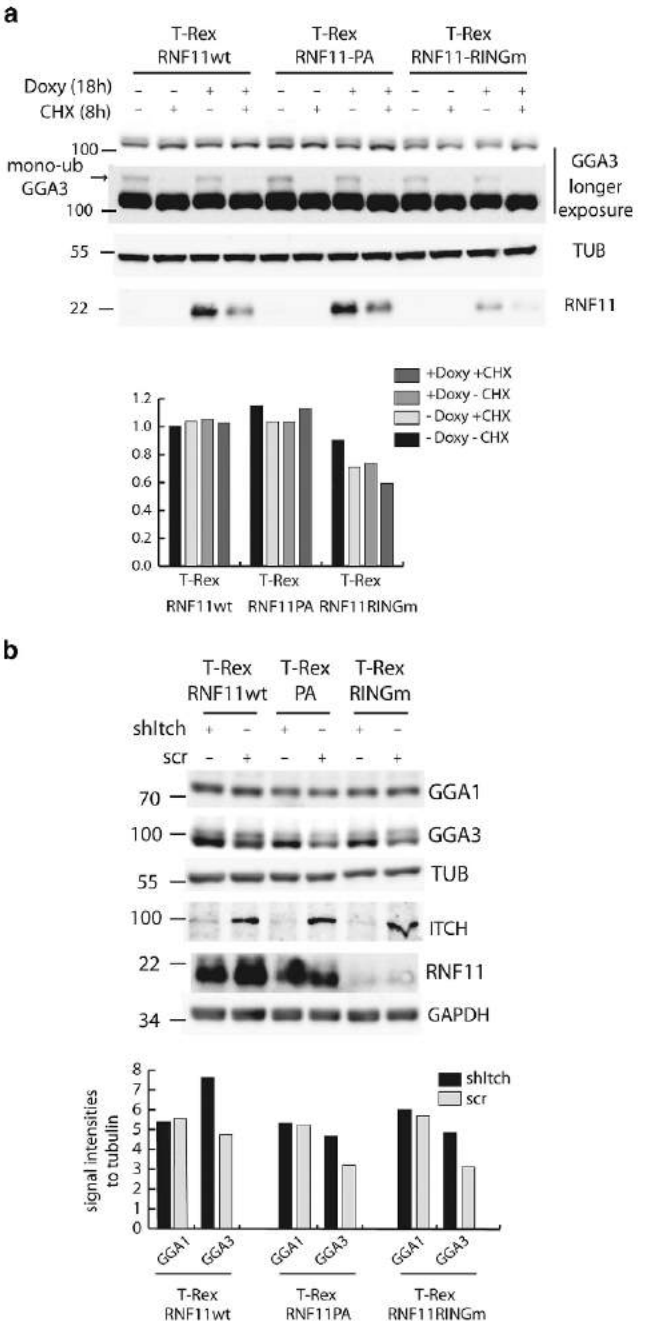

Figure 9. (**a**) The endogenous levels of GGA3 are reduced in the T-Rex RNF11 RINGm cell line. T-Rex RNF11 cells induced with doxycycline, as shown, were incubated with cycloheximide and cell extracts were nalyzed by western blotting for endogenous GGA3 levels. Two different exposures are shown for GGA3 and the signal intensities normalized to tubulin are shown in the graph. (**b**) The interference of RNF11 in the T-Rex RNF11wt cell line does not affect GGA3 stability. T-Rex RNF11wt cells were transfected with a mix of shRNA plasmid vectors to knockdown RNF11 or with a scramble control vector. After 72 h, cell extracts were analyzed with the indicated antibodies and signal intensities reported in the graph were normalized to tubulin or GAPDH. (**c**) The endogenous levels of GGA3 but not GGA1 are reduced in cells where itch has been knocked down. T-Rex RNF11 cells were transfected with a mix of shRNA plasmid vectors to knockdown itch or with a scramble control vector. After 72 h, cell extracts were analyzed with the indicated antibodies and GGA1 and GGA3 signal intensities were normalized to tubulin and reported in the graph.

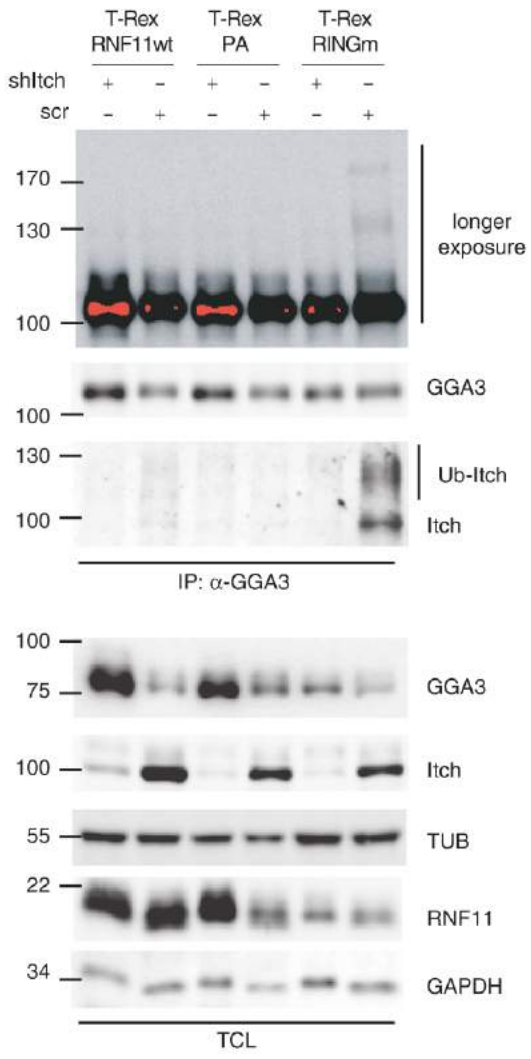

Figure 10. GGA3 is hyperubiquitinated in T-Rex RNF11 RINGm cells at endogenous levels and this effect is itch dependent. The ubiquitination of GGA3 was analyzed in T-Rex RNF11 RINGm cells' knocked down for itch. Cellular extracts were immunoprecipitated with anti-GGA3 and immunoprecipitates were analyzed with anti-GGA3 and itch.

The association of RNF11 with proteins of the trafficking machinery (that is, GGAs, STAM2 and SARA) is mediated, at least in the case of GGAs, by the interaction of their VHS domains with sorting motifs in RNF11, but we can speculate that the presence of a UIM motif in RNF11 could be involved in the recognition of distinct ubiquitinated proteins at the endosome membranes. In our model, RNF11 interacts with HECT ligases and recruits them to the endosome compartment (Figure 12). At these sites, RNF11 associates to proteins that traffic from/to the plasma membrane and/or the TGN. When a specific signal is given, that is, the stimulation with growth factors, RNF11 acts an E4 enzyme that promotes the polyubiquitination and degradation of substrates (that is, cargo proteins) and the inhibition of enzymes involved in ubiquitin metabolism (that is, other E3 ligases and de-ubiquitinating enzymes). Thus, the role of RNF11 could be essential for regulating the 'switch' from recycling to degradation, controlling route, time and duration of the internalization process.

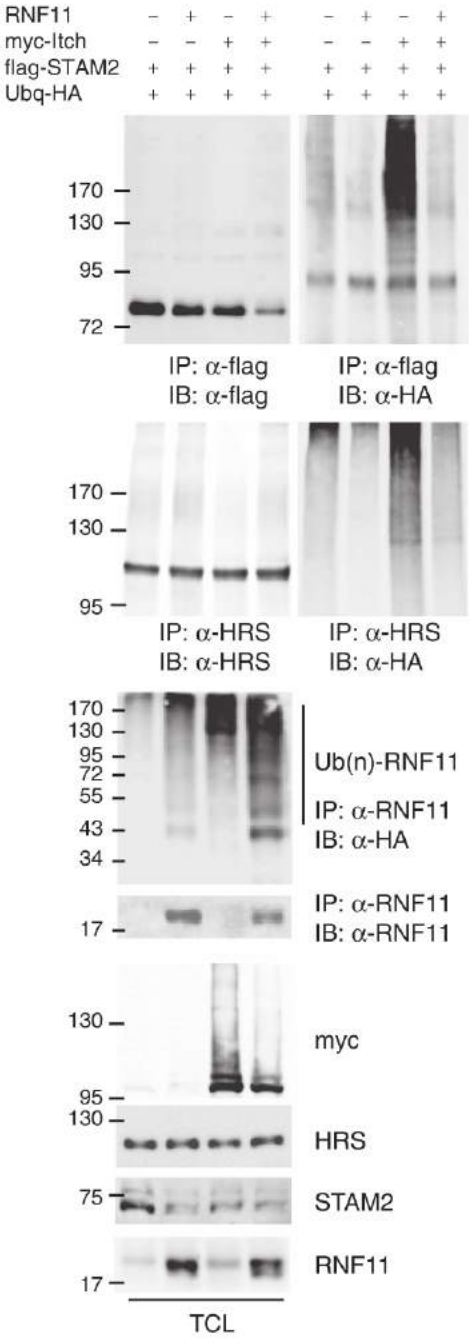

Figure 11. RNF11 inhibits the mediated ubiquitination of ESCRT-0 proteins. HEK293 cells were transiently transfected with RNF11, myc-itch, flag-STAM2 and HA-Ubq. Twenty hours post transfection, cells were lysed and equal amounts of cell extract were immunoprecipitated with anti-flag, anti-HRS and anti-RNF11. Immunoprecipitates were analyzed by western blotting with the indicated antibodies.

MATERIALS AND METHODS

Antibodies and reagents

Antibodies for immunoprecipitation and western blot were: monoclonals (mAb) anti-HA and anti-flag (M2-Affinity Gel), and polyclonal (pAb) anti-flag (Sigma, St Louis, MO, USA), mAb anti-GFP clone 86/38 (UC Davis/NIH NeuroMab Facility, Department of Neurobiology, Physiology and Behavior, UC Davis, Davis, CA, USA), pAb anti-GFP and anti-tubulin (Santa Cruz Biotechnology, Santa Cruz, CA, USA), pAb anti-GST (Amersham Pharmacia Biotech, Uppsala, Sweden), pAb anti-RNF11 (described in Santonico *et al.*[22]),

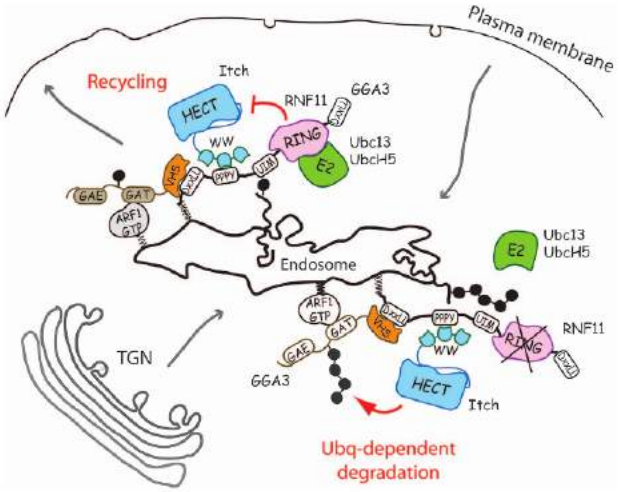

Figure 12. Cartoon showing the domain organization and protein–protein interactions involving RNF11, ITCH, GGA3 and the E2 enzymes Ubc13 or UbcH5. The linear motifs in the amino acid chain of RNF11 are also shown. At steady state, RNF11 associates with itch and GGA3, respectively, via the PPPY and DxxLL motifs, and inhibits the polyubiquitination and degradation of GGA3, through a mechanism that requires the RING catalytic activity of RNF11. When a specific signal is given, RNF11 catalytic activity is inhibited and it promotes the polyubiquitination mediated by itch and the ubiquitin-dependent degradation of GGA3.

mAb anti-Itch (BD Transduction Laboratories Pharmingen, San Diego, CA, USA), mAb anti-myc (Invitrogen, Paisley, UK), mAb anti-GAPDH (Millipore MAB374, Billerica, MA, USA), anti-rabbit and anti-mouse peroxidase conjugate (Jackson ImmunoResearch, West Grove, PA, USA). Antibodies for immunofluorescence were: mAb anti-EEA1, and anti-CD63 (BD Transduction Laboratories), mAb anti-LAMP2 (BD Biosciences, Franklin Lakes, NJ, USA), anti-mouse Alexafluor-555-conjugated secondary antibody, anti-rabbit rhodamine Alexafluor-555-conjugated secondary antibody and anti-rabbit Alexafluor 488-conjugated secondary antibody (Invitrogen).

Construction of mammalian cell expression vectors

The plasmid vectors encoding for ubiquitin $(HA)_6$, myc-itch and myc-itchC830S were a gift from M Rossi. pEGFP-N3-RNF11 plasmid vectors have been described in Santonico *et al.*[22] The cDNA encoding for RNF11 (Swiss-Prot: Q9Y3C5) was amplified by PCR from a human brain cDNA library (Novagen, Merck KGaA, Darmstadt, Germany) and cloned in pcDNA3 vector (for transient transfections) or in pcDNA5/FRT/TO vector (to create stable cell lines; Invitrogen). Mutants were generated with QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA, USA). The sequences of the DNA constructs generated by PCR were systematically verified. The cDNAs encoding for GGA1, GGA2 and GGA3 were amplified by PCR from expression vectors kindly provided by Dr Juan Bonifacino and cloned in pEGFP-N3. The plasmid vectors encoding for GFP-Rab4, GFP-Rab5, GFP-Rab7 and GFP-RAB11 were a gift from M Zerial and the GFP-dynamin-K44/A vector was kindly provided by G Cestra. Constructs encoding STAM2-VHS (residues 1–152), HRS-VHS (residues 1–280) and GGA1-VHS (1–224) were generated by standard PCR methods and subcloned into pcDNA-CMV14-3 × Flag expression vector (Sigma).

Mammalian cell culture and transfection

HeLa and HEK293 cell lines were grown at 37 °C and 5% CO_2, in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, and were transfected with Lipofectamine 2000 (Life Technologies, Carlsbad, CA, USA).

The doxycycline-inducible RNF11wt and RNF11-RINGm stable T-Rex 293 cell lines were created using the Flp-In T-Rex system (Invitrogen). Protein expression was induced by doxycyclin addition (0.1 μg/ml) to the growth medium.

Preparation of the GST fusion protein and pull-down procedures

GST constructs encoding GGA1-VHS (residues 1–170), GGA1-GAT (residues 171–299), GGA1-Hinge (residues 300–509), GGA1-GAE (residues 510–631), GGA2-VHS (residues 1–165), GGA3-VHS (residues 1–150), STAM2-VHS (residues 1–152) and HRS-VHS (residues 1–160) were generated by standard PCR methods and subcloned into pGEX expression vector. GST fusion proteins were prepared as previously described.[50] Immobilized recombinant proteins (50 μg) were incubated with 1 mg of cell extracts in pull-down experiments. The beads were washed with cold buffer (25 mm HEPES, pH 7.5, 125 mm NaCl, 1% glycerol, 1 mm $MgCl_2$, 1 mM phenylmethylsulfonyl fluoride (PMSF), 5 mM NaF, 5 mM N-ethylmaleimide (NEM), 0.5% NP-40 and protease inhibitors), boiled in 1 × Laemmli buffer and separated by SDS–PAGE (SDS–polyacrylamide gel electrophoresis). Gel was stained with 0.1% (wt/vol) Coomassie blue R350 solution (Sigma) or transferred onto nitrocellulose membrane for immunoblotting.

Cell lysates, immunoprecipitation and western blot analysis

Subconfluent cultures of HeLa or HEK293 cells were lysed in lysis buffer (25 mm HEPES, pH 7.5, 125 mm NaCl, 1% glycerol, 1 mm $MgCl_2$, 1 mM PMSF, 5 mM NaF, 5 mM NEM, 0.5% Triton X-100, 0.5% NP-40, 0.1% sodium deoxycolate and protease inhibitors cocktail). Cleared supernatants were mixed with 4 × Laemmli sample buffer, boiled, resolved by SDS–PAGE and transferred to nitrocellulose membrane (Schleicher and Schull, Dassel, Germany). Membranes were blocked with 5% non-fat dry milk in phosphate-buffered saline (PBS) and 0.1% Tween-20, incubated with the indicated primary and secondary antibodies and then detected with SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific, Rockford, IL, USA).

For immunoprecipitation experiments, 1 mg of total protein was immunoprecipitated, at 4 °C for 2 h, with agarose-conjugated anti-Flag M2-Agarose, or using a mouse anti-GFP monoclonal antibody and Protein G-Sepharose beads (Invitrogen). After washing, pellets were resolved by SDS–PAGE and transferred to nitrocellulose. Densitometric analysis was performed using ImageQuant.

Immunofluorescence microscopy, internalization assays and confocal microscopy

Transfected HeLa cells, grown on coverslips, were fixed with 4% paraformaldehyde for 15 min at 25 °C, followed by treatment with 0.1 M glycine for 10 min at 25 °C and with 0.1% Triton X-100 for additional 5 min at 25 °C to allow permeabilization.

To study transferrin internalization and recycling, transfected HeLa cells were grown on glass coverslips, serum starved for 2 h in serum-free medium, followed by incubation with Tf-TxRed (50 μg/ml; Molecular Probes, Eugene, OR, USA) for 1 h at 37 °C before fixation. Incubations were stopped by placing the dishes on ice and cells were processed for immunofluorescence as described above. Nuclei were stained with 4,6-diamidino-2-phenylindole (DAPI; 1:1000 in PBS; Sigma). Coverslips were mounted with 90% glycerol in phosphate-buffered saline for observation by epifluorescence microscope. Cells were scanned in a series of 0.5 μm sequential sections with an ApoTome System (Zeiss, Hertfordshire, UK) connected with an Axiovert 200 inverted microscope (Zeiss); image analysis was then performed by the Axiovision software (Zeiss) and three-dimensional reconstruction obtained. Quantitative analysis of the extent of colocalization was performed using Zeiss KS300 3.0 Image Processing system (Zeiss). The mean ± s.d. percent of colocalization was calculated by analyzing a minimum of 30 cells for each treatment randomly taken from three independent experiments. Single-plane images were also acquired with a confocal laser scanner microscope (Olympus Fluoview 1000, Tokyo, Japan), whereas for conventional microscopy analysis, single-plane images were captured and deconvoluted with a DeltaVision microscope (Applied Precision, San Francisco, WA, USA) using the SoftWoRx-2.50 software (Applied Precision).

In situ PLA

HeLa cells were grown on glass coverslips, left untreated or starved for 2 h in serum-free medium, followed by incubation with Oregon green 488-conjugated-Tf (50 mg/ml; Molecular Probes) for 10 min or 60 min at 37 °C. Cells were fixed in 4% paraformaldehyde, permeabilized with 0.1% Triton X-100-PBS1x, incubated with a blocking solution (BSA1x, PBS1x, 0.1% Triton X-100) for 1 h and then with primary antibodies targeting GGA3 (1:100) and RNF11 (1:100). Negative controls consisted of the same process but without one of the two antibodies. Duolink *in situ* PLA was performed according to manufacturer's protocol (Olink Bioscience, Uppsala, Sweden). Briefly, following primary antibody treatment, cells were incubated with oligonucleotide-linked PLA secondary antibodies for 1 h at 37 °C followed by hybridization, ligation and amplification. Cell nuclei were stained and slides mounted on coverslips using the manufacturer's mounting medium containing DAPI. Slides were analyzed by confocal microscopy. PLA dots were quantified with BlobFinder V3.2 image analysis software (Uppsala University, Stockholm, Sweden).

Peptide array synthesis

Peptides were synthesized according to standard SPOT synthesis protocols using an automatic spot synthesizer as described elsewhere.[50]

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGEMENTS

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