An ER-Associated Pathway Defines Endosomal Architecture for Controlled Cargo Transport

Graphical Abstract

Highlights

- The bulk of the endosomal system and TGN clusters in the perinuclear “cloud”
- The ER-located E3 ubiquitin ligase RNF26 retains vesicles in the perinuclear cloud
- RNF26 employs the ubiquitin scaffold SQSTM1 to capture specific vesicle adaptors
- Opposition between RNF26 and DUB USP15 times release of vesicles for fast transport

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In Brief

The endosomal system exhibits a bilateral architecture, comprised of a relatively immobile perinuclear vesicle “cloud” and a highly dynamic peripheral contingent. How this cloud is organized and what purpose it serves is unknown. Here, we reveal its molecular determinants centered around the ER-located ubiquitin ligase RNF26, capable of retaining the entire endosomal system’s repertoire through a common mechanism operating at the ER membrane. Countered by the deubiquitinating enzyme USP15, RNF26 draws the endosomal system’s architecture, thus orchestrating vesicle maturation and cargo trafficking in space and time.
An ER-Associated Pathway Defines Endosomal Architecture for Controlled Cargo Transport

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SUMMARY

Through a network of progressively maturing vesicles, the endosomal system connects the cell’s interior with extracellular space. Intriguingly, this network exhibits a bilateral architecture, comprised of a relatively immobile perinuclear vesicle “cloud” and a highly dynamic peripheral contingent. How this spatiotemporal organization is achieved and what function(s) it curates is unclear. Here, we reveal the endoplasmic reticulum (ER)-located ubiquitin ligase Ring finger protein 26 (RNF26) as the global architect of the entire endosomal system, including the trans-Golgi network (TGN). To specify perinuclear vesicle coordinates, catalytically competent RNF26 recruits and ubiquitinates the scaffold p62/sequestosome 1 (p62/SQSTM1), in turn attracting ubiquitin-binding domains (UBDs) of various vesicle adaptors. Consequently, RNF26 restrains fast transport of diverse vesicles through a common molecular mechanism operating at the ER membrane, until the deubiquitinating enzyme USP15 opposes RNF26 activity to allow vesicle release into the cell’s periphery. By drawing the endosomal system’s architecture, RNF26 orchestrates endosomal maturation and trafficking of cargoes, including signaling receptors, in space and time.

INTRODUCTION

Visual inspection of a typical cell reveals canonical arrangements of membrane-enclosed organelles. Generally, the endoplasmic reticulum (ER) wraps around the nucleus, extending throughout the cytoplasmic space, while the mammalian Golgi stacks cluster between the nucleus and the microtubule-organizing center (MTOC) (Rios and Bornens, 2003; Thyberg and Moskalewski, 1999; Valderrama et al., 1998). From here, the trans-Golgi network (TGN) vesicles disseminate biosynthetic cargoes to their sites of function throughout the cell (Waguri et al., 2003). On the other side of vesicle traffic, endosomes originating from the plasma membrane carry internalized cargoes to the lysosome for degradation or spare them through diversion to recycling (Raiborg and Stenmark, 2009). The roads traveled by endosomes time intracellular signaling cascades (Scita and Di Fiore, 2010) and tune specialized functions, such as antigen processing and pathogen clearance in immune cells (Blum et al., 2013). To fulfill its myriad responsibilities, the collective endo- and exocytic pathway connects distant organelles (Traub and Bonifacino, 2013) through a progressively maturing network of vesicles (Hucular and Helenius, 2011). How cells sense and manipulate the location of individual vesicles in space and time to suit their global housekeeping and environmental demands is unclear.

Intriguingly, the bulk of lysosomes, early and late endosomes, as well as vesicles of the TGN, locates quizzesently in the perinuclear region of the cell (Anikeeva and Sykulev, 2011; Reed et al., 2013; Rojo Püldö et al., 2011; Sadacca et al., 2013; Wasmieier et al., 2008), poised toward the cell’s periphery. Only select vesicles escape this perinuclear (PN) “cloud” to become subject to fast bidirectional transport (Wubboths et al., 1999) by dynein (Cantalupo et al., 2001; Jordens et al., 2001; Schroeder et al., 2014) and kinesin motors (Raiborg et al., 2015; Rosa-Ferreira and Munro, 2011). What governs acceptance of vesicles into—and their release from—the PN cloud is unknown. Gaining fundamental insights into the way such decisions are made in molecular terms is pivotal to understanding regulation of transport programs in the cell.

The ER is the only intracellular organelle that occupies every corner of cytosolic space. Not surprisingly, it has been shown to participate in various functional contacts with other membranous compartments, mediating exchange of metabolites and controlling transport and fusion processes (Helle et al., 2013). Currently, contact sites between the ER and endosomes are emerging as potent regulatory hubs for vesicle transport (Raiborg et al., 2015; Rocha et al., 2009), fusion (van der Kant et al., 2013), and fission (Rowland et al., 2014) events. Here, we describe how an ER-associated protein network, organized
Figure 1. RNF26 Depletion Disrupts Spatiotemporal Organization of Endosomes

(A) Intracellular distribution of LEs (CD63, green) in various cell lines. Representative maximum z projection (3D) overlays with nuclear DAPI (blue) and their corresponding z cross sections along the demarcated line are shown. Cell boundaries are depicted in dashed lines. For other markers, see Figure S1.
by the E3 ubiquitin ligase Ring finger protein 26 (RNF26), serves as a platform for perinuclear positioning of the entire endosomal system. Localized in the ER membrane, RNF26 extends its catalytic determinants into the cytosol, restricting fast microtubule-based transport of early, recycling, and late endosomes/lysosomes and the TGN. We show that RNF26 utilizes the ubiquitin scaffold p62/sequestosome 1 (SQSTM1) as its substrate to attract ubiquitin-binding domains (UBDs) of various vesicle membrane adaptors. The resulting molecular bridge restrains cognate vesicles in the perinuclear region and organizes the endosomal pathway for efficient cargo transfer and ligand-induced clearance of signaling receptors. Vesicles can then be released for fast transport into the cell’s periphery from their perinuclear positions by way of RNF26-associated deubiquitinating enzyme (DUB) USP15, thus completing the dynamic cycle. Collectively, the RNF26-based protein network elucidates a key paradigm for functional control of intracellular architecture and organelle dynamics, highlighting the importance of inter-compartmental regulation in membrane cell biology.

RESULTS

RNF26 Regulates Endocytic Compartment Architecture and Dynamics

Across cell types, a wide variety of endosomal maturation stages—late (CD63, Figure 1A), early (EEA1) and recycling (TrfR) endosomes, as well as the vesicular arm of the TGN (TGN46) (Figure S1)—tend to cluster into a “cloud” near the nucleus, with only a fraction of each subset extending into the cell’s periphery. How such organization is established and controlled and what purpose it may serve is largely unknown. Given that late endosomes (LEs) constitute central nodes within the endo- and exocytic vesicular network (Huotari and Helenius, 2011), we mined a genome-wide small interfering RNA (siRNA)-based screen for novel factors controlling LE biology (Paul et al., 2011), where silencing the RING finger ubiquitin ligase RNF26 was shown to severely disrupt the intracellular LE organization, leading to marked dispersal of LEs throughout the cytoplasm and even to the tips of cells, without significantly impacting cell shape (Figures 1B–1D; Movies S1A and S1B). These observations cast RNF26 as a potent candidate for control of the LE compartment architecture, prompting us to investigate the role of RNF26 in the organization and function of the perinuclear (PN) cloud.

In live cells, we observed a striking relationship between the LE compartment architecture and its dynamics. The majority of acidified vesicles marked by Lysotracker (LTVs) were positionally restricted to the PN cloud, while the sparsely populated periphery (PP) remained dynamic over time (Figure 1F, top right panels; Movie S2A). On the contrary, cells depleted of RNF26 exhibited an expanded periphery and increased mobility of the LTV contingent relative to control, thus blurring the PN/PP distinction (Figures 1E and 1F, bottom right panels; Movies S2B and S2C, Lysotracker; Movies S2D and S2E, mCherry-CD63).

To test whether other components of the endo- and exocytic vesicular repertoire also fall under the RNF26 purview, we investigated the effect of RNF26 silencing on distribution of various vesicle markers. Without exception, localization of all post-Golgi vesicles examined was susceptible to RNF26 depletion in two different cell lines tested, while distribution of the Golgi remained unaffected (Figures 2A, S2A, and S2B; Movies S3A-S3D). Given that intracellular organization and its associated compartmentalization of transport apply across diverse vesicle types, we hypothesized that the PN cloud may serve as a meeting hub for maturation and cargo exchange. In support of this, we found that vesicles endocytosed by fluid-phase, as monitored using uptake of the extracellular dye sulforhodamine (SR101) (Wubboldt et al., 1996), readily encountered LT-positive structures residing primarily in the PN cloud. By contrast, acquisition of SR101 by the disorganized acidified compartments in RNF26-depleted cells was markedly restrained (Figures 2B and 2C; Movies S4A and S4B), while SR101 internalization rate remained unaffected (Figure 2C), suggesting that endosomes mature in the PN cloud. We further explored whether trafficking of specific cargoes to the proteolytic compartment is affected by the endosomal system’s architecture. Following acute stimulation with EGF, ligand-containing vesicles distributed to the PN cloud over time in control cells, but not in those compromised for RNF26 (Figure 2A). In the latter case, trafficking of EGF-positive vesicles to the LE compartment was severely impaired (Figures 2D and 2E), while availability of EGF receptor (EGFR) on the cell surface, as well as total receptor levels, remained unaffected (Figures S2C and S2E). Consistent with the above, ligand-induced degradation of EGFR was attenuated with RNF26 depletion, leaving activated receptors (detected as pY) to linger at late time points following stimulation (Figures 2F and 2G). Taken together with the SR101 experiments, these findings imply that the PN cloud and its architect RNF26 facilitate efficient vesicle maturation and transit of cargo through the endosomal system, with implications for ligand-induced receptor signaling.

(B) Effect of RNF26 depletion on distribution of LEs, represented as fractional distances of CD63 vesicles from center of nucleus (distance of pixels from nucleus = fraction of distance from nucleus to the plasma membrane [1.0]; mean shown in red). For 3D view, see Movies S1A and S1B.

(C) Cell shape analysis for samples in (B), showing total cell area and eccentricity calculated in an automated fashion as described in the Supplemental Experimental Procedures.

(D) mRNA levels of RNF26 targeted by two different siRNAs (siRNF26_1 and siRNF26_2) as assessed by qPCR are expressed relative to sC; n = 3.

(E) Quantification of the mobile fraction of acidified Lysotracker (LT)-positive vesicles (LTVs) as a function of RNF26; n = 3. For details, refer to the Supplemental Experimental Procedures.

(F) Organization and dynamics of LTVs (white) in control (sC) versus RNF26-depleted (siRNF26_1) MeJuSo cells. Left panels: representative single confocal plane fluorescence images taken at the start of time lapse. Right panels: vesicle displacement rates (blue, immobile; red, max mobility) observed over the 297-s time interval. Nuclei and cell boundaries are depicted in dashed lines, and zoom-ins highlight peripheral (PP) and perinuclear (PN) boxed regions. Quantification appears in (E). For LT time lapses, see Movies S2A–S2C. For CD63 time lapses see Movies S2D and S2E.

Scale bars, 10 μm. For all figures: n, # of cells analyzed per condition; n, # independent experiments; error bars, SD.
ER Localization and Ubiquitin Ligase Activity of RNF26
Mediate Endosomal Positioning

Having determined that loss of RNF26 incurs detrimental effects on endosomal organization and function, we turned to ask whether this ER-located ubiquitin ligase (Qin et al., 2014) actively positions endosomes in the PN cloud. Ectopic expression of full-length RNF26, but not its catalytic RING domain (ΔRING) truncation, substantially restricted mobility of LT-positive vesicles (Figures 3A and 3B). Mirroring the PN position of the vesicle cloud, RNF26 localized predominantly to the region of the ER proximal to the nucleus, while its ΔRING mutant distributed throughout the ER (Figures 3C and S3A–S3C; ER marked by VAP-A), indicating that the RING domain drives retention of the ligase in the perinuclear ER subdomain.

To explore the contribution of catalytic activity to RNF26 localization and function, we mutated a conserved isoleucine 382 to Arginine (I382R, Figure S3A), thereby inhibiting expected interactions with E2 enzyme(s) without incurring deleterious effects on RING domain architecture, such as by mutating key Zn²⁺ coordinating modules (Deshales and Joazeiro, 2009). RNF26-I382R markedly reduced the enzyme’s ubiquitin ligation capacity (Figure S3B), similar to the previously reported C401S mutant (Qin et al., 2014). Ubiquitin ligase activity was further illustrated by strong colocalization of wild-type RNF26 with ubiquitin-labeled species, relative to its catalytically dead mutants showing only marginal overlap with ubiquitin (Figures 3C and S3C). We next tested whether ubiquitin ligase activity afforded by RNF26 is critical to perinuclear endosome positioning. The RNF26 depletion phenotype, scored on the basis of LE scattering away from the nucleus, was robustly rescued by re-expression of RNF26, but not its mutants deficient in either ubiquitination or ER transmembrane segments (Figures 3D and 3E), implying that RNF26-mediated ubiquitination must take place at the ER membrane to effectively position vesicles in the PN cloud.

RNF26 Interacts with a Network of Vesicle-Associated Adaptor Proteins

To understand how an ER-located protein exerts control over the endosomal system and the TGN, we sought out interacting partners of the cytosolic tail of RNF26. Mass spectrometric analysis of proteins co-precipitating with either GST-ΔTM or GST-RING (Figures 4A and S4A) identified three membrane-associated adaptor proteins functioning in sorting and trafficking of endocytic vesicles—EPS15 (Benmerah et al., 1999), T6BP/TAX1BP1 (Morrison et al., 2007), and TOLLIP (Ankem et al., 2011), a ubiquitin scaffold p62/SQSTM1 (Ciani et al., 2003) known for its role in autophagy (Lippai and Low, 2014) and a DUB USP15, which localizes to the nucleus and cytosol, targeting the transforming growth factor β (TGF-β) and nuclear factor kappa-light-chain enhancer of activated B cells (NF-κB) pathways (Eichhorn et al., 2012; Schweitzer et al., 2007). Collectively, cargo specificities of the three former proteins afford broad coverage of both endocytic and biosynthetic vesicle trajectories (Figures 4A and S4A), implying that by associating with different vesicle-targeting adaptors, RNF26 may influence positioning of a wide range of endosomes and the TGN. Silencing the above proteins (excluding USP15) produced marked LE dispersion (Figures 4B and 4C). By contrast, TGN vesicle dispersion resulted only from depletion of the TGN-associated adaptor TAX1BP1 and SQSTM1, but not of endocytic adaptors EPS15 and TOLLIP (Figures 4B and 4C), and overall cell shape parameters were profoundly altered only by depletion of TAX1BP1 (Figure S4B). Further, co-silencing multiple adaptors resulted in additive effects on CD63 distribution (Figure S4C), underscoring the contribution of multi-directional traffic to the global architecture of the LE compartment.

To assess whether specific adaptors can influence localization and dynamics of their cognate vesicles, we followed the mobility of LTVs in cells ectopically expressing GFP-TOLLIP. Double-positive vesicles were found to localize primarily in the PN cloud (Figure 4D), and overall LT movement was dramatically restricted relative to control (Figure 4D; Movies S5A and S5B). Additionally, exogenous TOLLIP restored PN localization of vesicles marked by CD63 in an exceptional cell line (RKO, Figure S4D), which exhibits natural dispersion of the LE compartment (Figure S1). Mirroring the dysfunction in endosomal maturation incurred by depletion of RNF26 (Figures 2B and 2C), silencing TOLLIP inhibited access of SR101-containing endosomes to the acidified compartments, without affecting the internalization rate (Figure 4E). Taken together, the above
observations illustrate the capacity of a specific adaptor to position its chosen vesicles in the PN cloud.

Catalytically Active RNF26 Attracts Ubiquitin-Binding Domains of Endocytic Adaptors

To dissect the molecular basis of communication between RNF26 and its partners, we interrogated their respective interaction determinants. Without exception, EPS15, TAX1BP1, TOLLIP, USP15 (Figure 5A), and SQSTM1 (Figure 5B, right panels) exhibited a strong binding preference for catalytically competent RNF26, relative to the inactive I382R mutant. Given the critical role of RNF26 ubiquitination activity in the establishment of endosomal system’s architecture described in Figure 3, we proceeded to investigate ubiquitin-mediated recognition in this context. Most interacting partners of RNF26 described here harbor ubiquitin-binding domains (UBDs) (Figure 5C), and point mutations targeting the CUE domain of TOLLIP (Mitra et al., 2013), UIM domain of EPS15 (Klapiz et al., 2002), and UBZ2 domain of TAX1BP1 (but not UBZ1, which is incapable of ubiquitin interactions) (Iha et al., 2008) strongly affected association of these proteins with wild-type RNF26 (Figures 5B, left panels, and S5A). Moreover, the UBD-dependent loss of binding was comparable to that observed between wild-type adaptors and mutant RNF26 (Figure 5D). Strikingly, SQSTM1 did not follow suit, displaying no significant reliance on its UBS domain for productive interaction with RNF26 (Figures 5B, right panels, and 5D). Mechanistically, these observations set SQSTM1 apart from the other adaptors within the RNF26 network.

Because the known specificity of SQSTM1 for autophagic membranes is unlikely to account for its broad effects on positioning of both endocytic and biosynthetic systems of vesicles (Figures 4A–4C and S4A), we hypothesized that it may instead function by attracting ubiquitin-dependent partners to RNF26. To test this, we investigated spatial localization of SQSTM1 versus the RNF26-interacting vesicle adaptors relative to the ligase and its associated ubiquitin signals. As expected based on the coimmunoprecipitation (coIP) data, the LE adaptor GFP-TOLLIP readily colocalized with RNF26 at the corresponding sites of endogenous ubiquitin accumulation, while its ubiquitin-binding-deficient point mutant (CUE*) did not (Figures 5E).

Importantly, lack of its recruitment to the ligase had no discernable effect on ubiquitin enrichment at RNF26 (Figures 5E and 5F). By contrast, UBA domain truncation of SQSTM1 (∆UBA) was still able to occupy RNF26-positive structures, but significantly suppressed accumulation of associated ubiquitinated species (Figures 5G and 5H), thus implicating SQSTM1, along with its ubiquitin interactions, in the assembly of ubiquitinated species at RNF26.

RNF26 and the DUB USP15 Share a Substrate in SQSTM1

To elucidate the unique mechanism of SQSTM1 function within the RNF26 protein network, we delved into the contribution of its UBA domain. Given previously reported connections between ubiquitin binding and ubiquitination of UBD-containing proteins (Sorkin, 2007), we considered whether SQSTM1 constitutes a substrate for RNF26. Indeed, major enhancement in short ubiquitin conjugates on SQSTM1 was observed in response to ectopic expression of wild-type but not catalytically inactive RNF26 relative to vector control (Figures 6A and 6B). Importantly, RNF26 was unable to ubiquitinate truncated SQSTM1 lacking its UBA domain (Figures 6A and 6B), indicating that only SQSTM1 in possession of its ubiquitin-binding faculties can serve as a substrate for the ER-located ubiquitin ligase. Taken together with the findings presented in Figure 5, the above evidence suggests that ubiquitinated SQSTM1 comprises the Ub-rich signals observed at sites of RNF26 activity.

If ubiquitination afforded by RNF26 restricts vesicles in the PN cluster, a deubiquitinating activity may then complete the biochemical cycle to allow release of vesicles for rapid transit in the cell’s periphery. This function could be served by USP15, which preferentially associates with catalytically competent RNF26 (Figures 5A and 5D). We therefore tested whether USP15 deubiquitinates the RNF26 substrate, SQSTM1. Indeed, overexpression of wild-type USP15 dramatically decreased short-chain modification of SQSTM1 with ubiquitin in a manner dependent on its catalytic Cys 269 residue (Figures 6A and 6B). Furthermore, expression of wild-type (but not inactive) USP15 reduced the degree of colocalization of RNF26 with SQSTM1 (Figures 6C, 6D, and S5B), indicating that USP15 activity modulates occupancy of ligase-positive sites. Consistent with the notion that USP15 functionally rivals RNF26, silencing USP15 essentially ablated the highly mobile peripheral contingent marked by Lysotracker (Figure 6E; Movies S5A and S5C), resulting in an overall decrease in mobility of acidified organelles (Figure 6F)—a phenotype opposite to that observed with...
A

GST-RING
MJS Lysate Pull-down → LCMS/MS → ID Interacting Proteins (S4A) → Localization (S4A) → Phenotypic Validation (4B-E; S4B-D)

B

\begin{align*}
\text{siC} & \quad \text{CD63} \\
\text{siC} & \quad \text{TGN46}
\end{align*}

\begin{tabular}{|c|c|c|c|c|}
\hline
Fractional Distance & n=12 & 12 & 12 & 13 & 11 & 9 & 14 & 13 & 14 & 13 & 13 & ns & ns & ns & ns \\
\hline
\end{tabular}

C

\begin{tabular}{|c|c|c|c|c|c|}
\hline
EPS15 & TAX1BP1 & TOLLIP & SQSTM1 & USP15 & Actin \\
\hline
siEPS15 (1) & siTAX1BP1 (2) & siTOLLIP (3) & siSQSTM1 (4) & siUSP15 (5) & \\
\hline
\end{tabular}

D

\begin{align*}
V \text{ (relative to control)}
\end{align*}

\begin{tabular}{|c|c|c|c|c|}
\hline
& Control & & & & \\
\hline
GFP-TOLLIP & & & & & \\
\hline
\end{tabular}

E

\begin{align*}
\text{Overlap LT\textsuperscript{101}; SR101}
\end{align*}

\begin{tabular}{|c|c|c|}
\hline
\text{Time (min)} & 0 & 30 & 60 & 90 & 120 & 150 \\
\hline
\text{siC} & 0.1 & 0.4 & 0.6 & 0.8 & 0.2 & 0 \\
\text{siTOLLIP} & 0.2 & 0.5 & 0.7 & 0.9 & 0.3 & 0 \\
\hline
\end{tabular}

(legend on next page)
depletion of RNF26 (Figures 1E and 1F). As expected, based on their catalytic opposition, co-depletion of USP15 and RNF26 partially restored the PN/PP balance (Figure 6D), implying that USP15 promotes release of vesicles captured and restrained by the active RNF26 complex.

**The ER-Located RNF26/SQSTM1 Complex Controls Vesicle Positioning and Dynamics**

Consistent with the proposed role of SQSTM1 in bridging adaptor-selected vesicles to catalytically competent RNF26, we observed colocalization of all three specific membrane adaptors—EPS15, TAX1BP1, and TOLLIP—with endogenous SQSTM1 at sites of wild-type, but not inactive RNF26 (Figures S6A and S6B). Additionally, the interaction between TOLLIP and SQSTM1 was exquisitely sensitive to mutation in the UBD domain of the former (Figure S6C), recapitulating ubiquitin-mediated recognition of RNF26 complexes by adaptor proteins (Figure 5). Importantly, structures positive for RNF26 and SQSTM1 did not overlap with the autophagy marker LC3 (Figure S6A, right panels), indicating that the function of SQSTM1 in this context is unrelated to autophagy.

To test whether SQSTM1 dictates endosome positioning at RNF26, we monitored vesicle dynamics via specific membrane adaptors in living cells co-expressing fluorescent SQSTM1. We observed stable PN contacts between GFP-adaptors (“green”) and RFP-RNF26 (“red”) that were overwhelmingly positive for TRQ-SQSTM1 (“blue”; Figure S7A, Movie S6A), with tripartite complex formation (appearing “white” in the overlay) strongly correlated to fixed positional residence of vesicles marked by EPS15, TAX1BP1, and TOLLIP (Figures 7A–7C). By contrast, the vast majority of only GFP-positive “green” vesicles remained subject to fast transport (Figure 7C; Movie S6A). As expected, RNF26 lacking its RING domain could not mediate stable contacts with TRQ-SQSTM1 and failed to stabilize vesicles in position over time (Figures 7C and S7A; Movie S6B). Similar to TRQ-SQSTM1, TRQ-ubiquitin was found at contacts between GFP-TOLLIP-positive vesicles retained by RFP-RNF26 (but not RFP-ΔRING), while highly mobile vesicles were free of ligase and ubiquitin contacts (Figures S7B and S7C). Interestingly, while most “white” vesicles stayed docked at the RNF26/SQSTM1 complex over time, occasional release was observed following disappearance of TRQ-SQSTM1 (Figures 7A and 7B, vesicles 1 and 2, respectively; Movie S7), and depletion of SQSTM1 markedly reduced distribution of TOLLIP-positive structures to RNF26 (Figure 7D). Considering diminished RNF26 occupancy by SQSTM1 in the presence of USP15 (Figures 6C and 6D), these findings suggest that assembly of SQSTM1 contacts at sites of RNF26 positions vesicles in the PN cloud, while disintegration of such complexes mediates vesicle release.

Taken together with the interaction and functional studies, the observations described above are consistent with the following order of molecular events: catalytically competent RNF26 recruits SQSTM1, which becomes subject to UBA-dependent ubiquitination by the ligase. This ubiquitin-rich RNF26/SQSTM1 complex is then poised to attract UBDs of endocytic adaptors (and the DUB USP15) to the PN cloud (proposed model depicted in Figure 7E). Subsequently, deubiquitination by USP15 at these sites determines release of the SQSTM1/Ub/Adaptor complex from the ER membrane, allowing transport of vesicles into the cell periphery. Collectively, our findings illustrate that by attracting diverse membrane-associated vesicle adaptors through a common mechanism, the RNF26/SQSTM1 complex controls the positioning and dynamics of endosomal vesicle transport and so designs the architecture of the endo- and exosomal system.

**DISCUSSION**

Proper control of the biosynthetic and endocytic endomembrane networks is crucial to normal functioning of cells and organisms, and failures therein are known to result in neuronal diseases (van der Kant and Neefjes, 2014) and obstructed immune responses (Watts, 2012), as well as contribute to a variety of cancers (Meilm and Yarden, 2013). The biology of endosomes (and the TGN) relies on cargo acquisition and vesicle transport working together to ensure accurate and timely delivery of select materials to their destinations. While we understand various aspects of cargo selection and vesicle transport, we know very little of the molecular decisions required to negotiate their arrivals and departures in the busy 3D environment of the cell. In the present study, we explored the functional relationship between the endosomal system’s architecture and dynamics, exposing its molecular underpinnings.
Figure 5. RNF26 Couples to Ubiquitin-Binding Domains of Specific Membrane Adaptors

(A) Interactions (assayed by coIP) between RNF26 versus its inactive mutant I382R (IR) and endogenous EPS15, TAX1BP1, TOLLIP, and USP15 in HEK293T cells. WCL, whole-cell lysate.

(B) Effects of mutations in ubiquitin-binding domains (UBDs) of TOLLIP and SQSTM1 on interaction with RNF26 in HEK293T cells (extraneous lanes between 4 and 5, as well as 6 and 7 were excised). For TAX1BP1 and EPS15, see Figure S5A.

(C) Schematic: domain organization of RNF26-interacting proteins, highlighting membrane-targeting domains (gray), UBDs (blue), and USP (red).

(D) Quantification of interactions (normalized as % of WT/WT coIP, black bars) as a function of RNF26 inactivation (I382R white bars) or loss of UBD capabilities (UBA*/ΔUBA, blue bars) for each pair of proteins; n = 3.

(E–H) Colocalization of GFP-tagged (green) (E) TOLLIP (quantified in F; n = 2) and (G) SQSTM1 (quantified in H; n = 2) or their UBD mutants (CUE* and ΔUBA, respectively) with HA-RNF26 (red) and endogenous ubiquitin (blue) in MelJuSo cells. Representative single confocal plane fluorescence overlays and single-channel zooms are shown. Summary is illustrated schematically at the right.

Scale bars, 10 μm.
Figure 6. SQSTM1 Is a Substrate for RNF26 and the DUB USP15

(A) Ubiquitination status of SQSTM1 as a function of catalytic activities of RNF26 and USP15. GFP-SQSTM1 (or GFP-ΔUBA) was isolated from HEK293T cells overexpressing HA-Ub in the presence of vector, RFP-RNF26 versus its catalytic mutant I382R, or USP15 versus its catalytic mutant C269S. Ubiquitination status of GFP-substrate (green) was assessed by immunoblots against HA (red).

(B) Quantification of the ubiquitination assay in (A); n = 4. Schematic on the left depicts proposed catalytic opposition between RNF26 and USP15.

(C) Overexpression of GFP-substrate (green) was assessed by immunoblots against HA (red).

(D) Quantification of RNF26 or I382R occupancy by SQSTM1 (Mander’s overlap) as a function of USP15 catalytic activity; n = 2.

(E) Organization and dynamics of acidified LT-positive vesicles (LTVs, white) in control (siC) versus USP15-depleted (siUSP15) HeLa cells. Left panels: representative single confocal plane fluorescence images at the start of time lapse are shown. Right panels: corresponding vesicle displacement rates (blue, immobile; red, max mobility) observed during the 343-s time interval; zoom-ins highlight boxed PP and PN regions. For time lapses, see Movies S6A and S6C. Scale bar, 10 μm.

(F) Effect of USP15 depletion on LTV dynamics (displacement/s relative to cells transfected with control siRNA); n = 2.

(G) Functional interplay between siUSP15 and siRNF26. Quantification of mobile LTV fraction as a function of indicated siRNA perturbations (+) in MelJuSo cells; n = 2.
Figure 7. RNF26/SQSTM1 Complex Positions and Retains Adaptor-Selected Vesicles

(A) Overlay zooms of frames selected from a time lapse (Movie S7) of vesicles marked by GFP-TOLLIP (green) in the presence of TRQ-SQSTM1 (blue) and RFP-RNF26 (red) in HeLa cells. Arrowheads point to two vesicles profiled in (B). Scale bar, 2.5 μm.

(B) Vesicle #1

(Vesicle #2)

0 sec 60 120 150 330

Overlay

TRQ-

SQSTM1

GFP-

TOLLIP

RFP-

RNF26

164

Cell 166, 152–166, June 30, 2016

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from our observations on delays in endosomal progression and cargo trafficking under conditions of PN cloud breakdown afforded by silencing RNF26. Furthermore, as a consequence of temporarily restricting vesicle mobility, the ER-located RNF26 could assist in the logistics of complex molecular processes, such as fission and/or fusion, both reportedly dependent on ER-endosome contact sites (Rowland et al., 2014; van der Kant and Neefjes, 2014). Our findings take a key step toward understanding how cells determine and manipulate the location of their highly mobile endosomal constituents and unveil a new facet of influence the ER exerts over the endosomal system.

**EXPERIMENTAL PROCEDURES**

Descriptions of cell lines, culture conditions, reagents, antibodies, and DNA constructs can be found in the Supplemental Experimental Procedures.

**siRNA Delivery**

Silencing was performed as previously described (Paul et al., 2011) using siRNA oligos purchased from Dharmacon. For protocol and sequence details refer to the Supplemental Experimental Procedures.

**Light Microscopy**

Samples were prepared as described in the Supplemental Experimental Procedures. Fixed and live samples were imaged using 63× lenses on Leica SP5 confocal microscopes adapted with a climate control chamber. To calculate fractional distances, fluorescent intensities along multiple line ROIs (assessed on maximum z projections using the line profile tool in LAS-AF software) were background corrected based on signal thresholds and normalized to median. Fractional distances were reported relative to the maximum distance from the center of the nucleus to the cell perimeter along a given trajectory. Vesicle tracking during time lapses was performed using TrackMate for Fiji. Fluid phase endocytosis was performed using SR101 as previously described (Wubbolts et al., 1996). Colocalization was reported as Mander’s coefficients calculated using JACOp for ImageJ. All error bars correspond to SD of the mean. Statistical evaluations report on Student’s t test (analysis of two groups) or one-way ANOVA analyses (analysis of three or more groups), with *p < 0.05, **p < 0.01, and ***p < 0.001 (ns, not significant).

For additional details and descriptions of endocytosis and EGFR degradation assays, as well proteomic and biochemical methods, refer to the Supplemental Experimental Procedures.

**SUPPLEMENTAL INFORMATION**

Supplemental Information includes Supplemental Experimental Procedures, seven figures, and seven movies and can be found with this article online at http://dx.doi.org/10.1016/j.cell.2016.05.078.

**AUTHOR CONTRIBUTIONS**

M.J. and I.B. designed, conducted, and interpreted the majority of the experiments and prepared the manuscript. R.H.W. performed the experiments in Figures 3C and S3C and advised on data presentation throughout the manuscript. P.V. and G.J. performed mass spectrometry on prepared samples. L.J. and M.M. provided technical support. H.J. advised on endosome morphology. M.G. contributed to the study of TOLLIP function on late endosomes. R.S. discussed the results throughout the project. J.N. supervised the project. All authors edited the manuscript.

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