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RAB7 and TSG101 are Required for the Constitutive Recycling of Unliganded EGFRs via Distinct Mechanisms

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Abstract

Both constitutive and ligand-mediated membrane trafficking regulate Epidermal Growth Factor Receptor (EGFR) signaling. The constitutive endocytosis and recycling of the unliganded EGFR is a critical determinant of cell surface EGFR expression and the cell's sensitivity to ligands. We report that two proteins with established roles in trafficking the EGF:EGFR complex to the lysosome also regulate the recycling of the unliganded EGFR. Knock down of either Tumor suppressor gene 101 (TSG101) or RAB7 causes the endosomal accumulation of the inactive, unliganded receptor in morphologically and biochemically distinct organelles. Knock down of TSG101 causes the EGFR to accumulate in low density endosomes whereas RAB7 knock down results in EGFR accumulation in high density endosomes. Knock down of either protein caused the receptor to co-localize primarily with LAMP-1, but not EEA1. These two proteins regulate EGFR slow, perinuclear recycling, via distinct mechanism and are new molecular targets that regulate cell surface EGFR expression.

Keywords

EGFR; endocytosis; recycling endosome; late endosome; RAB7; TSG101

1. Introduction

The epidermal growth factor receptor (EGFR) is the prototypical receptor tyrosine kinase (RTK) that is nearly ubiquitously expressed throughout the body. Signaling by the EGFR is critical for an organism's development (Zambruno et al., 1990) and tissue homeostasis (Schneider et al., 2008; Yu et al., 2010) as revealed by animal models engineered with attenuated expression of the receptor (Threadgill et al., 1995) and ligands (Luetke et al., 1999). Further, many cancers are characterized by the overexpression and/or hyperactivation of the EGFR (Rowinsky, 2004). Thus, understanding the molecular mechanisms that regulate the activity of the EGFR have important implications in developmental biology, wound healing, and cancer biology.

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One of the principal mechanisms by which EGFR signaling is regulated is via the endocytic pathway (Herbst et al., 1994; Wiley et al., 1991). Constitutive internalization of the unliganded EGFR occurs via clathrin-coated pits at a relatively slow rate and rapidly recycles back to the plasma membrane. At steady-state, the majority of the receptors are on the cell surface and a small percentage of the receptors are in transit in the endocytic pathway. In contrast, when the receptor is bound to epidermal growth factor (EGF), the kinetics of endocytosis change such that the rate of internalization increases approximately 8–10 fold and recycling is dramatically decreased. Under most physiological conditions, the internalized ligand:receptor complex progresses from early endosomes to late endosomes/multivesicular body (LE/MVB) to lysosomes for degradation. Studies of ligand-mediated EGFR endocytic trafficking have provided important insights into how EGFR degradation and signaling are regulated. However, despite being less well studied, the constitutive membrane trafficking of the EGFR is an equally, if not more, important component of receptor signaling. Membrane trafficking of the unliganded receptor determines the cell surface expression of EGFRs and their potential to signal when presented with ligand.

In an effort to identify new regulatory proteins, we examined three proteins with known roles in regulating the trafficking of the liganded EGFR – c-Cbl (Joazeiro et al., 1999), TSG101 (Razi and Futter, 2006), and RAB7 (Vanlandingham and Ceresa, 2009). Of these three proteins, RAB7 and TSG101 are required for the constitutive recycling of the EGFR. In both cervical adenocarcinoma cells (HeLa) and corneal epithelial (hTCEpi) cells, knock down of TSG101 or RAB7 causes a dramatic re-distribution of the EGFR from the cell surface to endosomes due to a defect in constitutive EGFR recycling. Under either knock down condition, extracellular antibodies that bind the amino terminus of the EGFR co-localize with the late endosome, indicating the receptor is inserted in the plasma membrane prior to endosomal accumulation and there is no defect in protein biosynthesis. Further, these receptor-containing compartments are still functional, as the remaining cell surface EGFRs can bind ligand and traffic into these compartments. However, there are several biochemical and morphological differences. Endosomes that arise following TSG101 knock down have a perinuclear distribution and are less dense. In contrast, RAB7 knock down cells yield endosomes that are dispersed through the cytosol, are more dense, and larger in size. Knock down of either protein causes the EGFR to co-localize with proteins associated with the late endosome, but not the early endosome. Together, these data reveal unexpected and distinct roles for TSG101 and RAB7 in the constitutive recycling of EGFRs and regulation of EGFR cell surface expression.

2. Materials and Methods

Cell culture

HeLa cells were obtained from American Type Culture Collection. Cells were maintained in Dulbecco Minimal Essential Media (DMEM) supplemented with 5% fetal bovine serum, 100 units/ml penicillin and 100 units/ml streptomycin and grown at 37°C in 5% CO₂. Human telomerase-immortalized corneal epithelial cells (hTCEpi) were obtained from Geron Corp. (Menlo Park, CA) and described previously (Robertson et al., 2005). Cells were maintained in growth media (Defined Keratinocyte Media with growth supplement; Life Technologies, Carlsbad, CA) containing 100 units/ml penicillin and 100 units/ml streptomycin at 37°C in 5% CO₂.

Cell Lysate Preparation and Immunoblotting

Cell lysates were generated as described previously (Rush et al., 2012). Protein concentrations were determined by BCA assay (Pierce), and samples were diluted in SDS-sample buffer. Equivalent amounts of protein (indicated in the figure legends) were

separated by the indicated percentage SDS-PAGE and transferred to nitrocellulose. Proteins were detected using antibodies against EGFR (SC-03, Santa Cruz), Transferrin Receptor (BD Biosciences), LAMP-2 (University of Iowa Hybridoma Bank), c-Cbl (Cell Signaling), Ub (SC-8017, Santa Cruz), RAB7 (Sigma), TSG101 (Genetex, Irvine, CA), α -tubulin (Sigma), RAB11 (Cell Signaling). HRP-conjugated goat anti-mouse or goat anti-rabbit secondary antibodies were obtained from Pierce. Detected proteins were visualized by enhanced chemiluminescence using a UV Products imaging system or Fotodyne imaging system.

siRNA oligos and transfection

RAB7 and c-Cbl siRNAs were siGenome SMARTpool preparations (catalog # M-010388-00-0005 and L-003003-00-0005, respectively) obtained from Dharmacon (Lafayette, CO). siRNA for TSG101 was obtained from Integrated DNA Technologies (Coralville, IA) using the published sequence (Razi and Futter, 2006). Transfection of HeLa cells was performed using Interferin® following the manufacturer's direction.

Nucleofection was used to introduce siRNA into hTCEpi cell and was performed using the AMAXA Basic Nucleofector Kit for Primary Mammalian Epithelial Cells. hTCEpi cells were harvested in serum free media and resuspended at a concentration of 2×10^6 cells/100 μ l of Nucleofector Solution. The indicated siRNA (0.1 nMoles) were mixed with 100 μ l of cells, placed in the transfection cuvette, and electroporated using program U-20. Immediately after nucleofection, 600 μ l of serum free media was added to the cells and transferred to a 1.5 ml Eppendorf tube that was placed at 37°C for 20 minutes. Cells were plated at the desired density in growth media and returned to 37°C for 72 hours.

Shown in Figure 1 are representative immunoblots to indicate the level of knock down using each technique. In subsequent experiments, the knock down of the target proteins was confirmed by immunoblot, but is not shown.

Percoll Gradient Fractionation

Analysis of steady-state receptor distribution was performed as described by Kornilova *et al.* (Kornilova et al., 1996; Vanlandingham and Ceresa, 2009). Following centrifugation, the 17% isotonic Percoll gradient was fractionated into 10-drop fractions (~30 fractions/gradient) from the bottom. Samples were resuspended in SDS sample buffer and every other fraction was immunoblotted for EGFR, an early endosomal marker [transferrin receptor (TfnR)], and a late endosome/lysosome marker lysosome-associated membrane protein 2 (LAMP-2)) as indicated in the figure.

Indirect Immunofluorescence

Localization of the EGFR was examined as previously described (Dinneen and Ceresa, 2004a). Briefly, cells were fixed in 4% paraformaldehyde and permeabilized with 0.1% saponin/0.5% fetal bovine serum/0.25M CaCl₂/0.25M MgCl₂/PBS pH 7.4. Cells were incubated first with the primary antibody indicated in the figure [EGFR (Ab-1, Sigma), TfnR (Invitrogen), LAMP1 (Cell Signaling), EEA1 (Cell Signaling)] followed by incubation with any appropriate secondary antibody - either Alexa488- or Alexa568-labeled (Life Technologies). Following extensive washing, cells were mounted on microscope slides using Prolong with DAPI (Life Technologies). Images were captured using a Nikon Eclipse Ti-E microscope using Nikon NIS Elements software or Olympus Confocal Microscope using Fluoview Software. The images were then exported to Adobe Photoshop for preparation of figures.

Treatment of cells with anti-EGFR monoclonal antibody Ab-1 (clone 528)

Anti-EGFR mouse monoclonal antibody (mAb) Ab-1 (Sigma, clone 528) has been well characterized (Sobol et al., 1987). In experiments monitoring the internalization of the Ab-1 mAb bound to EGFR, HeLa cells were transfected with siRNA as described above. Twenty-four hours after recovery, cells were replated on coverslips and incubated with 1 µg/ml of Ab-1 for an additional 24 or 48 hours. To monitor localization of the Ab-1 antibody, cells were fixed and permeabilized as described for “indirect immunofluorescence”. Cells were incubated for an hour with an Alexa488 conjugated goat anti-mouse secondary antibody (Life Technologies (Carlsbad, CA)). After six 10 minutes washes with PBS⁺⁺, coverslips were rinsed in Millipore water and mounted on a slide with Prolong Antifade (Life Technologies). Images were collected on a Nikon Eclipse TE2000-U microscope.

[¹²⁵I]-Transferrin internalization

Following transfection with either siCON or RAB7 or TSG101 specific siRNA, cells were allowed to recover in growth media for 72 hours at 37°C. On the day of the experiment, media was removed and replaced with 37°C binding media containing 180 nMol [¹²⁵I]-transferrin (0.7 Ci/mg, Perkin Elmer). Cells were placed at 37°C for the indicated times. Following incubation, the media was collected (free [¹²⁵I]-Tfn) and cell-surface associated radioligand was removed by twice incubating the cells in 0.5M NaCl/0.2M acetic acid (pH 2.8) buffer for eight minutes each. Cells were solubilized in 1 M NaOH and the associated radioactivity was measured using a Perkin-Elmer Wizard² Gamma counter (Ceresa et al., 1998).

Texas Red EGF labeling

HeLa cells transfected with the indicated siRNA were replated onto coverslips 24 hours later and 1 µg/ml of Ab-1 antibody was added to the media. Cells were allowed to recover for an additional 48 hours. Coverslips were incubated with 4 ng/ml Texas Red EGF (Tx-Red EGF) in DMEM for 15 minutes at 37°C. Free TX-Red EGF was removed with two washes of ice cold PBS⁺⁺⁺⁺ (0.5 mM MgCl₂/0.5 mM CaCl₂/0.2% BSA/5mM glucose/PBS pH7.4). Membrane bound Tx-Red EGF was removed with three washes in ice cold citrate buffer (25.5 mM Citric Acid/24.5 mM Sodium Citrate/ 280 Sucrose/pH 4.6) and cells were re-equilibrated with two additional washes in ice cold PBS⁺⁺⁺⁺. Cells were returned to 37°C DMEM. At the indicated times, cells were washed and fixed in 4% p-formaldehyde and processed for indirect immunofluorescence as described above.

Inhibition of EGFR phosphorylation with AG1478

HeLa cells transfected with the indicated siRNA were replated onto coverslips and 60 mm dishes 24 hours after transfection and immediately treated with 1µM AG1478 inhibitor (Cayman) in growth media. Media was changed every 12 hours with 1µM AG1478 in 2.5% serum media. Two hours prior to EGF treatment media was replaced with fresh media containing 1µM AG1478. Cells were treated with or without 10ng/mL EGF as indicated. Coverslips were then fixed and processed for “Indirect-Immunofluorescence” using the primary antibody EGFR (Ab-1) as described above. EGF (10 ng/ml) treated and untreated 60 mm dishes were harvested as described above, and immunoblotted for either pY1068 or EGFR and -Tubulin.

3. Results

3.1 RAB7 and TSG101 are required for surface EGFR distribution

To identify molecular mechanisms that regulate the cell surface expression of EGFRs, proteins known to regulate the endocytic trafficking of the liganded EGFR were knocked

down and the steady-state distribution of EGFRs was examined. HeLa (human cervical adenocarcinoma) cells were transfected with control siRNA (siCON) or siRNA specific for RAB7, TSG101, or C-Cbl. After recovery for 72 hours in growth media, the cells were fixed and processed for indirect immunofluorescence using an anti-EGFR antibody (Figure 1A).

Control cells exhibited a distribution of EGFRs that was primarily along the plasma membrane, as did the c-Cbl knock down cells. In contrast, a significant population of EGFRs from cells with attenuated levels of RAB7 or TSG101 was redistributed into endosomal compartments, although with subtle differences in their patterns of distribution (discussed below) and vesicle size. The lack of an effect with c-Cbl knock down is consistent with previous reports that indicate c-Cbl is only required for trafficking of liganded receptors (Baldys et al., 2009). Knock down of the three proteins was confirmed by immunoblot (Figure 1B).

Importantly, this change in receptor distribution was not specific to HeLa cells. The same experiment in immortalized human corneal epithelial cells (hTCEpi cells) yielded qualitatively similar results (Figure 1C). In addition to the differences between the tissue of origin for these cell lines, hTCEpi cells express $\sim 1.4 \times 10^6$ EGFRs/cell¹. In comparison, HeLa cells express $\sim 50,000$ EGFRs/cells (Berkers et al., 1991). Similar to HeLa cells, knock down of RAB7 or TSG101 in hTCEpi cells resulted in the intracellular, endosomal accumulation of EGFRs with a similar patterns of distribution (Figure 1C and D). EGFR distribution in hTCEpi cells transfected with siCON or c-Cbl siRNA was primarily along the plasma membrane. Although the extent of EGFR redistribution does not appear to be as great in hTCEpi cells as HeLa cells, this may be due to the differences in receptor density. Nevertheless, these data indicate that the role of RAB7 and TSG101 in maintaining surface EGFR distribution is independent of cell type and receptor density.

In an effort to better describe the compartments in which the EGFRs were re-distributing, we used indirect immunofluorescence to probe the knock down cells with markers of the endocytic pathway. Early Endosome Autoantigen-1 (EEA1) and Lysosome Associated Marker Protein (LAMP-1) were used to identify the early endosome and late endosomes, respectively. Seventy-two hours after introduction of the siRNA, cells were fixed and co-stained for either EGFR (green) and EEA1(red) (Figure 2A) or EGFR (green) and LAMP-1(red) (Figure 2B).

In the four conditions tested, EEA1 antibody staining was punctate and distributed uniformly throughout the cytoplasm (Figure 2A). In siCON and c-Cbl knock down cells, the EGFR is primarily localized to the cell surface and exhibited minimal co-localization with EEA1. In the RAB7 and TSG101 knock down cells, EGFR and EEA1 antibodies stained distinct compartments. Thus, the intracellular EGFRs (for RAB7 and TSG101 knock down cells) were not accumulating in early endosomes.

In siCON and c-Cbl transfected cells, the distribution of LAMP-1 positive endosomes was primarily perinuclear and uniform in size. As with EEA1, there was minimal co-localization of EGFR staining with LAMP-1 staining in siCON and c-Cbl knock down cells. However, RAB7 knock down cells produce LAMP1 positive endosomes that were larger in size and extensively co-localized with the EGFR. In addition, LAMP-1 and EGFR staining was localized through out the cytosol. In TSG101 knock down cells, LAMP-1 staining had a perinuclear distribution and the endosomes were smaller in size, although larger than control and c-Cbl knock down cells. The changes in endosomal size associated with RAB7 and

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TSG101 knock down are consistent with previous reports (Doyotte et al., 2005; Tu et al., 2010; Vanlandingham and Ceresa, 2009).

To be certain that the immunofluorescent experiments reflected the entire population of cells and not just a subset, we used a biochemical approach to complement the microscopy experiments (Figure 3). HeLa cells were transfected with the same experimental and control siRNAs as described in the previous experiments. After recovery, a post-nuclear supernatant was prepared and resolved on a 17% isotonic Percoll gradient to separate endosomes based on density, and fractionated. Every other fraction was resolved by SDS-PAGE and immunoblotted for the presence of EGFR, Transferrin receptor (TfnR), Late endosome Associated Membrane Protein (LAMP2), and RAB11. The differences in endosomal density and presence of marker proteins are two independent ways to characterize the endosomal populations.

The distribution of TfnR (early and recycling endosomes), LAMP2 (late endosome), and RAB11 (recycling endosome) in the siCON and c-Cbl transfected cells is consistent with previous reports (Dinneen and Ceresa, 2004b; Sheff et al., 1999; Vanlandingham and Ceresa, 2009). Under these conditions, there are low levels of EGFRs that are coincident with early endosomal markers and likely represent the constitutively recycling EGFR.

In the absence of RAB7, there is an apparent redistribution of EGFRs into very high density fractions ($R_f \sim 0.9$; = 1.080–1.109 g/ml) and co-localize with LAMP2, characteristic of late endosomes (Kornilova et al., 1992). The presence of TfnR in these high-density fractions provides additional evidence of a perturbation in the composition of the endosome. TSG101 knock down cells exhibit a relative distribution of EGFRs that is similar to control cells, but there is a significant increase in the amount of EGFR in low-density endosomes ($R_f \sim 0.2$; = 1.080–1.109 g/ml) relative to the levels of TfnR. In addition, TfnR is broadly distributed into more dense fractions. This is consistent with a previous observation that the loss of TSG101 results in the formation of fully mature endosomes (Rush et al., 2012). The differences in spatial distribution in EGFR immunostaining (Figure 2) and in density of EGFR-positive endosomes with RAB7 and TSG101 knock down, supports the notion that each condition causes the EGFR to accumulate in a different compartments and each protein fulfills a unique role in regulating constitutive EGFR trafficking.

None of the conditions tested dramatically altered the distribution of RAB11, a small molecular weight G-proteins with a well-described role in fast recycling (Sheff et al., 1999). These data provide evidence that the RAB11-positive, rapidly recycling endosomes are largely unperturbed. This is supported by the data in Figure 6A.

3.2 The redistribution of EGFRs is due to a failure of unliganded EGFRs to recycle

One explanation for the endosomal accumulation of EGFRs is knock down of RAB7 or TSG101 causes a defect in the biosynthetic pathway and newly made receptors never reach the cell surface. To determine if this is the case, 24 hours after transfection with control (siCON), RAB7, TSG101, or c-Cbl specific siRNA, cells were incubated with an anti-EGFR mouse monoclonal antibody, Ab-1, that recognizes the extracellular, amino terminus of the receptor (Sobol et al., 1987). After 24 or 48 hours with the antibody, cells were fixed, permeabilized, and probed and an Alexa488 conjugated goat anti-mouse secondary antibody, and visualized by fluorescent microscopy (Figure 4). Since the antibody will remain extracellular until it binds an EGFR that has properly trafficked to the plasma membrane and then internalizes, this experiment allows us to distinguish between endosomal receptors that internalized from the plasma membrane (positive for intracellular staining) from those that accumulate as the result of a biosynthetic defect (no intracellular

staining). Staining of cell surface receptors reflects EGFRs that have made it to and were retained on the cell surface.

As expected, there is no appreciable intracellular staining of the EGFR in the control (siCON) or c-Cbl knock down cells. However, both RAB7 and TSG101 knock down cells have very pronounced intracellular staining. Importantly, this pattern of staining is similar to what was seen in Figures 1 and 2 and is consistent with this staining reflecting the steady-state EGFR population.

We eliminated the possibility that knock down of RAB7 and TSG101 enhanced the production, secretion, or processing of naturally occurring EGFR ligands to promote the internalization of ligand:receptor complexes using two separate assays. First, the Ab-1 anti-EGFR antibody binds the amino terminus of the receptor and previously has been reported to antagonize the binding of other EGFR ligands (Dinneen and Ceresa, 2004a; Kawamoto et al., 1983). Therefore, there is little likelihood that the receptor redistribution is due to ligand mediated endocytosis via an endogenous ligand. This notion is supported by the absence of any detectable phosphorylated EGFR in RAB7 or TSG101 knock down cells as measured by western blot (Figure 4B). Therefore, the accumulation of intracellular EGFRs can not be due to increased ligand-mediated endocytosis.

Second, following transfection of the cells with the appropriate siRNA, the cells were treated with the commercially available AG1478, an EGFR-specific small molecule inhibitor in growth media. After 48 hours, the cells were fixed and processed for immunofluorescence using an anti-EGFR antibody (Figure 5A). Despite the presence of AG1478, EGFRs redistributed into endosomes in RAB7 and TSG101 knock down cells. The inhibition of kinase activity was confirmed by immunoblot analysis (Figure 5B).

Thus, based on the absence of receptor phosphorylation, and the inability of the antagonizing antibody and kinase inhibitor to block the accumulation of EGFRs in endosomes, we conclude the receptor internalization is ligand-independent.

3.3 Knockdown of RAB7 blocks the slow perinuclear recycling of the transferrin receptor

The TfnR is a well-characterized, constitutively internalized cell surface receptor that traffics through two distinct endosome populations with different kinetics (Sheff et al., 1999). The first is a more rapid, peripheral recycling route. The second route is slower and takes the receptor through a perinuclear recycling endosome. We performed experiments to determine whether each of these routes of trafficking were affected by RAB7 or TSG101 knockdown.

To determine whether RAB7 and TSG101 were necessary for receptor recycling through the rapid, peripheral, compartment, we examined the uptake and recycling of radiolabeled transferrin [^{125}I -transferrin (^{125}I -Tfn)] in RAB7 and TSG101 knock down cells (Figure 6A). Three days after the introduction of the indicated siRNAs, we performed a kinetic analysis of ^{125}I -Tfn over the course of 60 minutes. The kinetics of ^{125}I -Tfn uptake and recycling in TSG101 and RAB7 knock down were indistinguishable from the siCON control.

The steady-state distribution of the TfnR was analyzed to determine if the slower, perinuclear endosomal population was affected. Knock down cells were incubated in growth media for 72 hours, fixed and processed for immunofluorescence using TfnR and LAMP1 antibodies (Figure 6B). Both siCon and c-Cbl knock down cells had a uniform distribution of both the TfnR and LAMP1 with minimal co-localization. RAB7 KD cells had the previously observed enlarged, LAMP1-positive endosomes that co-localized with the TfnR. TSG101 knock down cells had LAMP-1 endosomes that were slightly enlarged. These

endosomes had minimal co-localization with the TfnR. These data are consistent with the Percoll gradient data in Figure 3 and support the notion that RAB7 and TSG101 have distinct roles in the constitutive recycling of receptors.

3.4 Liganded cell surface EGFRs traffic through the same endosomes as the redistributed EGFRs

The endocytic pathway is a well-regulated, homeostatic process whose equilibrium can easily be altered with the loss of any single protein. Having observed an unexpected distribution of EGFRs with the knock down of RAB7 and TSG101, we wanted to determine whether this reflects the formation of a new, but not functional, compartment or represents the inability of the receptor to traffic through existing organelles.

To determine whether the redistributed EGFRs were localized into compartments that were still functional, we examined whether liganded EGFRs would traffic into the organelles that contained the redistributed EGFRs (Figure 7). Following transfection with RAB7 or TSG101 siRNA, cells were allowed to recover for 24 hours. The transfected cells were then replated onto coverslips in the presence of the Ab-1 EGFR antibody as previously described to mark the redistributed receptors. After 48 hours, the cells were treated with Texas Red-EGF to monitor the internalization of the liganded EGFR for either 15 minutes or 15 minutes followed by a 45 minute chase with ligand free media. Cells were fixed, permeabilized, and stained with an Alexa488 conjugated goat anti-mouse antibody to detect the Ab-1 antibody. This experiment allows for a comparison of the localization of new internalized EGFRs (shown in red) as compared to the redistributed, endosomal EGFRs (shown in green).

Despite the redistribution of a substantial population of EGFRs to endosomes, there were sufficient levels of cell surface EGFRs in both RAB7 and TSG101 knock down cells to take up Texas Red-EGF (Figure 7). After a 15-minute incubation with the fluorescent ligand, both RAB7 and TSG101 knock down cells exhibit co-localization of the Texas Red-EGF with the redistributed EGFRs, although there appears to be more co-localization in the RAB7 knock down cells. However, after 60 minutes (15 minutes labeling with ligand and 45 minute chase) almost all Texas Red-EGF is localized to the EGFR containing endosomes in both conditions. These data indicate that the EGFR containing endosomes have retained the ability to traffic into them. However, the absence of RAB7 or TSG101 precludes trafficking out of those endosomes.

4. Discussion

Despite being an important regulatory mechanism in EGFR signaling, the mechanisms that govern endocytic trafficking of the unliganded receptor have been largely overlooked. This process determines the receptor's steady-state distribution, cell surface expression, and the potential for signaling. In this study, we find that RAB7 or TSG101 knock down causes the receptor to accumulate in endosomal compartments that arise due to a failure of constitutively internalizing receptors to recycle back to the plasma membrane. In both instances, the intracellular receptors fail to accumulate with markers of the early endosomes (EEA1) but localize with late endosomal markers. Taken together, this suggests a new role for RAB7 and TSG101 in EGFR recycling and causes reconsideration of the molecular mechanisms that control recycling endosomes.

The different functions of these proteins in EGFR recycling are revealed in the morphological and biochemical properties of the receptor-containing endosomes. The differences in the endosome size and distribution when RAB7 versus TSG101 is knocked down are evident in the high magnification micrographs in Figures 2A and 2B. The Percoll

gradients in Figure 3 also demonstrate the EGFR distribute into endosomes with different densities following RAB7 and TSG101 knock down. These data have led us to the conclusion that TSG101 has a different role than RAB7 in regulating EGFR recycling. It has been previously reported that TSG101 regulates recycling of the liganded EGFR (Baldys and Raymond, 2009). However, recycling of the unliganded receptor was not examined in these studies. Since the loss of TSG101 increases LE/MVB size even without stimulation of the EGFR (Razi and Futter, 2006), the role of TSG101 in EGFR recycling may be independent of ligand, as well.

Perhaps the easiest explanation for these findings is that knock down of either RAB7 or TSG101 alters the equilibrium of the endocytic pathway and causes the formation of aberrant organelles. The increased size of the LAMP-1 positive compartment supports that argument. However, the defects in trafficking can not be explained that simplistically. The endocytic trafficking of those EGFRs remaining on the cell surface into endosomes containing redistributed EGFRs offers some additional insight. The analysis of Texas Red-EGF:EGFR localization relative to the distribution of unliganded EGFRs, reveals that in both RAB7 and TSG101 knock down cells, cell surface EGFRs associate with endosomal EGFRs. These newly internalized EGF:EGFR complexes can traffic to and co-localize with the same endosomes housing the re-distributed EGFRs indicating that these organelles permit trafficking of cargo into them. Therefore, the changes in size and/or composition of the endosome that accompany RAB7 and TSG101 knock down are likely due to the inability of cargo to traffic out of the organelle. While the changes in endosome morphology are likely secondary to a defect in membrane trafficking, the inability of receptors to recycle can not be strictly attributed to a perturbation in homeostasis of the endocytic pathway.

The two experiments examining TfnR trafficking and distribution provide important insights into RAB7 and TSG101 function. Sheff et al. (Sheff et al., 1999) has reported there are two distinct endosome populations that are associated with recycling. The first is the peripheral early endosome and the second is perinuclear endosome population. The ¹²⁵I-Tfn trafficking data verifies that this endosome population does not require RAB7 or TSG101. Conversely, the RAB7- and TSG101-dependent changes in TfnR steady-state distribution, as assessed by indirect immunofluorescence, mimic that of the EGFR, providing strong evidence that the receptor is recycling through the perinuclear population.

RAB proteins have multiple, well established roles in regulating EGF:EGFR membrane trafficking (Ceresa, 2006). As stated previously, the regulation of constitutive trafficking of unliganded EGFRs has been less well studied. However, it has been demonstrated that constitutive RAB5 activity can affect the distribution of unliganded EGFRs (Dinneen and Ceresa, 2004a). Interestingly, expression of dominant negative canine RAB7 does not alter the distribution of the unliganded EGFR (Ceresa and Bahr, 2006), suggesting the endogenous levels of wild type RAB7 may be sufficient for receptor recycling.

The finding that RAB7 is needed for constitutive EGFR recycling is unexpected because of 1) where RAB7 localizes in the endocytic pathway and 2) where the EGFRs accumulate. Our previous work used a series of morphological and biochemical assays to demonstrate that knock down of RAB7 in HeLa cells, causes an accumulation of enlarged LE/MVBs with an increase in the number of intraluminal vesicles (Vanlandingham and Ceresa, 2009). Together, these data are consistent with constitutively recycling EGFRs passing through the LE/MVB before returning to the plasma membrane.

How do we reconcile the previous morphological and biochemical findings with this current report? One explanation is that RAB7 has multiple roles in the LE/MVB. If this model is correct, then loss of RAB7 would prevent both necessary LE/MVB interactions that lead to

recycling and degradation. Alternatively, these data may suggest that in the absence of RAB7, the LE/MVB never fully matures to progress to subsequent steps – either recycling or lysosomal degradation. It has been previously reported that a RAB5/RAB7 switch occurs in the early endosome, but to date, there has been no reported function of RAB7 in the early endosome. This model would be consistent with complete maturation being a RAB7-dependent process that is required for either receptor recycling or delivery to the lysosome.

4. Conclusion

These data reveal that both the liganded and unliganded EGFR traffic through similar endocytic compartments and only very late in the endocytic pathway does the EGFR get sorted for degradation versus recycling. There are multiple lines of evidence that indicate that ubiquitylation is needed for sorting to the lysosome. Constitutively internalized, unliganded EGFRs were thought to recycle because they were not ubiquitylated. As such, they were not targeted to the ESCRT machinery which would direct them toward lysosomal degradation. This idea is supported by a recent finding that a lysine null mutant of the EGFR that can not be ubiquitylated (Eden et al., 2012) preferentially recycles. However, since recycling of the unliganded and non-ubiquitylated receptor is blocked in the absence of RAB7 or TSG101, then EGFR recycling must be regarded as a membrane sorting event, rather than a default pathway.

The data presented here demonstrate that a significant portion of EGFRs pass through the LE/MVB before recycling to the plasma membrane. Further, these findings provide the groundwork for selectively targeting receptors such that their route of endocytic trafficking can be directed in order to favorably modulate receptor signaling.

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Abbreviations

siCON	Control siRNA
EEA1	Early Endosome Autoantigen 1
ESCRT	Endosomal sorting complex required for transport
EGF	Epidermal Growth Factor
EGFR	Epidermal Growth Factor Receptor
G-protein	Guanosine nucleotide binding protein
LE/MVB	Late endosome/multivesicular body
LAMP-1/2	Lysosome Associated Membrane Protein-1/2
TSG101	Tumor Suppressor Gene 101
TfnR	transferrin receptor

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Highlights

- Constitutive trafficking determines the cell surface expression of the EGFR.
- Knock down of either RAB7 or TSG101 decreases cell surface EGFR expression.
- Attenuation of TSG101 causes EGFRs to localize to smaller, low density, LAMP-1 positive endosomes.
- RAB7 knock down causes EGFRs to redistribute into larger, high density, LAMP-1 positive endosomes
- Both RAB7 and TSGS101 are required for recycling of the EGFR from perinuclear endosomes.

A. IF: EGFR-HeLa Cells

C. IF: EGFR-hTCEpi

B. Protein Knock Down-HeLa

D. Protein Knock Down-hTCEpi

Figure 1. Knockdown of RAB7 or TSG101 causes the EGFR to re-distribute into endosomes
 A) HeLa cells or C) hTCEpi cells were transfected with either control siRNA (siCON) or siRNA specific for RAB7, TSG101, or c-Cbl as described in Materials and Methods. After recovery (72 hours), cells were fixed and processed for indirect immunofluorescence using an EGFR specific mouse monoclonal antibody (green). The nucleus was stained with DAPI (blue). Images were collected with an Olympus Confocal Microscope. B) and D) Immunoblots from cell lysates prepared from either the HeLa or hTCEpi samples, respectively. Equivalent amounts of cell lysate (10 μ g) were resolved by SDS-PAGE and immunoblotted with the indicated antibody. Size bar = 10 μ m. Shown are representative images and immunoblots from an experiment performed at least three times.

Figure 2. Knockdown of RAB7 or TSG101 causes EGFRs to accumulate in LAMP-1 positive endosomes

HeLa cells were transfected with either control siRNA (siCON) or siRNA specific for RAB7, TSG101, or c-Cbl as described in Materials and Methods. After recovery (72 hours), cells were fixed and co-stained for indirect immunofluorescence using an EGFR mouse monoclonal antibody and either A) an EEA1 rabbit polyclonal antibody or B) a LAMP-1 rabbit polyclonal antibody. In the larger image, EGFR staining is shown in green, endosomal marker proteins (EEA1 or LAMP-1) are shown in red, and DAPI-stained nuclei are blue. The boxed region is cropped and magnified to show the individual staining of the EGFR and marker proteins. Images were collected with a Nikon Ti-E Microscope. Size bar = 10 μ m. Shown are representative images from an experiment performed at least three times.

17% Percoll Gradient-HeLa Cells

A. siCON

B. RAB7 siRNA

C. TSG101 siRNA

D. c-Cbl siRNA

Figure 3. Knockdown of RAB7 or TSG101 alters the endosomal distribution of EGFRs
 HeLa cells were transfected with either A) control siRNA (siCON) or siRNA specific for B) RAB7, C) TSG101, or D) c-Cbl. After recovery (72 hours), post-nuclear supernatants were resolved on a 17% isotonic Percoll gradient and fractionated, as described in Materials and Methods. For each condition, every other fraction was resolved by SDS-PAGE and immunoblotted with antibodies against the EGFR, TfnR, LAMP2, and RAB11. R_f values indicate the relative density of a fraction in the Percoll gradients with $R_f = 0.0$ indicating the least dense fraction and $R_f = 1.0$ representing the most dense fraction. Shown are representative immunoblots from an experiment performed at least three times.

Figure 4. The endosomal accumulation of EGFRs in RAB7 and TSG101 knockdown cells is due to a defect in unliganded EGFR recycling

HeLa cells were transfected with the indicated siRNAs. A) Following recovery (24 hours), cells were replated onto coverslips in a 24 well dish and incubated with 1 μ g/ml of a mouse monoclonal EGFR antibody (Ab-1, Sigma). After an additional 24 or 48 hours in culture, extracellular antibody was washed away, cells were fixed, permeabilized and incubated with a secondary antibody (goat anti-mouse) conjugated to Alexa488 (Invitrogen). Images were collected with a Nikon Ti-E Microscope. Size bar = 10 μ m. Shown are representative images from an experiment performed at least three times. B) Untreated cell lysates were prepared from cells transfected in (A). Equivalent amount of cell protein (50 μ g) was resolved by

7.5% SDS-PAGE, transferred to nitrocellulose and immunoblotted for either EGFR tyrosine phosphorylated at position 1068 (EGFR pY1068), total EGFR, or α -tubulin. Equivalent amount of cell lysate from parental HeLa cells treated for 15 minutes with or without 10 ng/ml of EGF were used as controls. Shown is a representative blot from an experimental performed at least three times.

Figure 5. The endosomal accumulation of EGFRs in RAB7 and TSG101 knock down cells is independent of receptor kinase activity

Cells were transfected with siRNA as previously described. Twenty-four hours after transfection, cells were replated and incubated without with 1 μ M AG1478 for an additional 48 hours. A) Cells were fixed and processed for indirect immunofluorescence using an anti-EGFR antibody (Ab-1). Shown are representative images of cells from each siRNA treatment in the presence of AG1478. Size bar = 10 μ m B) Parallel dishes of transfected cells were treated with or without EGF as indicated. Cell lysates were prepared and 40 μ g was resolved by 7.5% SDS-PAGE, and immunoblotted for phosphoEGFR (pY1068), total EGFR, and α -tubulin.

Figure 6. RAB7 and TSG101 are required for slow, perinuclear, transferrin receptor recycling HeLa cells were transfected with control (siCON), RAB7, or TSG101 specific siRNA. A) After recovery (72 hours), cells were assayed for ^{125}I -Tfn uptake and recycling. Briefly, cells were incubated with ^{125}I -Tfn on ice for 60 minutes. Unbound radioligand was removed with extensive PBS washes. Cells were incubated with 37°C media and incubated at 37°C for the indicated times. Secreted, cell surface associated, and intracellular ^{125}I -Tfn was collected and measured as described in Materials and Methods. Data are plotted as either the average percentage ($\pm$ S.E.M.; $N=4$) of internalized radioligand (left graph) or the average percentage ($\pm$ S.E.M.; $N=4$) of secreted radioligand (right graph). Inset shows a representative immunoblot for the indicated proteins. B) HeLa cells were transfected with

the indicated siRNAs as described above. Seventy-two hours after recovery, cells were fixed and processed for indirect immunofluorescence using antibodies against the transferrin receptor (TfnR - green) or lysosomal associated membrane protein 1 (LAMP1 - red). Nuclei were stained with DAPI. The boxed region is cropped and magnified to show the individual staining of the EGFR and marker proteins. Images were collected with a Nikon Ti-E Microscope. Size bar = 10 μ m. Shown are representative images from an experiment performed at least three times.

Figure 7. Organelles with redistributed EGFRs are functional

HeLa cells were transfected with RAB7 or TSG101 specific siRNA as indicated. Following recovery (24 hours), cells were incubated with 1 $\mu\text{g/ml}$ of a mouse monoclonal EGFR antibody that recognizes the amino terminus (Ab-1, Sigma). After an additional 48 hours in culture, extracellular antibody was washed away and cells were pulse-labeled with 4 ng/ml Texas Red-EGF for 15 minutes, extracellular ligand was removed, and the cells were either fixed immediately or chased for an additional 45 minutes prior to fixation. Cells were stained with a goat anti-mouse antibody conjugated to Alexa488 and DAPI, and visualized with a Nikon Ti-series confocal microscope. Size bar = 5 μm . Shown are representative images from an experiment repeated three times showing the distribution of liganded EGFRs (red), redistributed EGFRs (green), and nuclei (blue).