Merlin/ERM proteins regulate growth factor-induced macropinocytosis and receptor recycling by organizing the plasma membrane:cytoskeleton interface

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The architectural and biochemical features of the plasma membrane are governed by its intimate association with the underlying cortical cytoskeleton. The neurofibromatosis type 2 (NF2) tumor suppressor merlin and closely related membrane:cytoskeleton-linking protein ezrin organize the membrane:cytoskeleton interface, a critical cellular compartment that both regulates and is regulated by growth factor receptors. An example of this poorly understood interrelationship is macropinocytosis, an ancient process of nutrient uptake and membrane remodeling that can both be triggered by growth factors and manage receptor availability. We show that merlin deficiency primes the membrane:cytoskeleton interface for epidermal growth factor (EGF)-induced macropinocytosis via a mechanism involving increased cortical ezrin, altered actomyosin, and stabilized cholesterol-rich membranes. These changes profoundly alter EGF receptor (EGFR) trafficking in merlin-deficient cells, favoring increased membrane levels of its heterodimerization partner, ErbB2; clathrin-independent internalization; and recycling. Our work suggests that, unlike Ras transformed cells, merlin-deficient cells do not depend on macropinocytic protein scavenging and instead exploit macropinocytosis for receptor recycling. Finally, we provide evidence that the macropinocytic proficiency of NF2-deficient cells can be used for therapeutic uptake. This work provides new insight into fundamental mechanisms of macropinocytic uptake and processing and suggests new ways to interfere with or exploit macropinocytosis in NF2 mutant and other tumors.

[Keywords: Merlin/ERMs; membrane:cytoskeleton interface; macropinocytosis; EGFR/ErbB2; cholesterol-rich domains]

Supplemental material is available for this article.

The interface between the plasma membrane and the cortical cytoskeleton is dynamically maintained by proteins that link membrane lipids and/or proteins to the underlying actomyosin meshwork (Gauthier et al. 2012; Kusumi et al. 2012). By spatially organizing both, these linkages establish the architectural and biochemical features of the cell surface. One key function of this cellular compartment is to both modulate the activity of membrane receptors and respond to their activation. For example, membrane:cytoskeleton linkages can direct local interactions between receptors and their effectors and/or regulators, impact receptor dimerization, aggregation, and signal propagation; and control multiple aspects of endocytic trafficking (Jaqaman and Grinstein 2012; Kusumi et al. 2012). Conversely, receptor activation can trigger rapid local changes in membrane lipids and the associated cortical cytoskeleton (Chiasson-MacKenzie and McClatchey 2018). Details of this dynamic functional interrelationship and how it influences cell behavior are poorly understood.

The epidermal growth factor receptor (EGFR) is a member of the ErbB family of receptor tyrosine kinases (RTKs) whose activities are tightly controlled in normal tissues and deregulated in many cancers (Lemmon et al. 2014). Ligand-activated EGFR homodimerizes or heterodimerizes with other ErbBs to activate key mitogenic and survival signals, including the phosphatidylinositol 3′ kinase (PI3K)–Akt and MEK–Erk pathways. EGFR/ErbB activation can also elicit membrane lipid and cortical cytoskeletal remodeling that drive essential changes in cell shape

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and adhesion during morphogenetic processes (Chiasson-MacKenzie and McClatchey 2018). Activated receptors are internalized via several mechanisms and either down-regulated via lysosomal trafficking or recycled back to the plasma membrane (Tomas et al. 2014). The endocytic trafficking of ErbBs is profoundly influenced by membrane lipids—particularly specific phospholipids, sphingolipids, and cholesterol—and the dynamic organization of the associated cortical cytoskeleton (Bertelsen and Stang 2014). Despite the immense effort dedicated to therapeutically blocking the mitogenic activity of ErbB receptors, particularly EGFR and ErbB2, remarkably little is known of how they elicit remodeling of the membrane:cytoskeleton interface, how that interface controls ErbB dimerization and endocytic fate, or how either of these biological activities impacts the behavior of normal and tumor cells.

Dramatic remodeling of the membrane:cytoskeleton interface is associated with membrane ruffling that enables macropinocytosis, an evolutionarily ancient mechanism by which cells obtain nutrients, sample their external environment, and control plasma membrane turnover (Buckley and King 2017). Successful macropinocytosis is associated with the exaggerated vertical extension of the actin-rich rims of membrane ruffles that flop over and fuse, entrapping fluid and extracellular nutrients that are then actively internalized in macropinocytic vesicles. In mammalian cells, macropinocytosis can be constitutive or triggered by growth factors such as EGF; however, growth factors do not always elicit ruffling, and ruffles do not always lead to successful macropinocytosis. Molecular studies and modeling indicate that the property of the membrane:cytoskeleton interface as an excitable medium is central to both, but it is not clear how this is tuned (Devreotes et al. 2017). It is also not clear how the processing of internalized macropinosomes is regulated. Much recent attention has focused on the importance of macropinocytosis as a mechanism by which Ras transformed tumor cells acquire and lysosomally process exogenous nutrients to meet their increased metabolic needs; however, macropinosomes are not always delivered to the lysosome (Buckley and King 2017). The recent appreciation that macropinocytosis is also an important conduit for the delivery of macromolecular cancer therapeutics to tumor cells further highlights the need to better understand this important cellular process (Ha et al. 2016).

We previously uncovered a critical role for the neurofibromatosis type 2 (NF2) tumor suppressor merlin in controlling the interrelationship between cortical actomyosin and EGFR (Curto et al. 2007; Chiasson-MacKenzie et al. 2015). Merlin is closely related to the membrane:cytoskeleton-linking ERM proteins (ezrin, radixin, and moesin) and has an important role in limiting their cortical distribution (Fehon et al. 2010; Hebert et al. 2012). Our studies showed that, in confluent cells, EGFR is immobilized on the cell surface via a mechanism that depends on an intact cortical actomyosin cytoskeleton and occurs rapidly in response to activation of EGFR itself; in the absence of merlin, increased cortical ezrin alters the configuration of cortical actomyosin and disables this mechanism (Chiasson-MacKenzie et al. 2015). Here we show that, instead, merlin deficiency renders the membrane:cytoskeleton interface markedly excitable in response to growth factors, “priming” cells for dramatic ruffling and macropinocytosis—features that are rescued by the restoration of Nf2WT expression or elimination of ezrin. Our data also suggest that Nf2−/− liver and Schwann cells (SCs) do not depend on macropinocytic protein scavenging, unlike Ras-activated cells (Commissio et al. 2013). In contrast, we provide evidence that the actin-dependent stabilization of cholesterol/sphingolipid-rich membranes in the absence of merlin stabilizes the surface levels of the EGFR heterodimerization partner ErbB2 and favors macropinocytic recycling of EGFR. Importantly, we found that macropinocytic priming, accumulation of membrane cholesterol/sphingolipids and ErbB2, and altered EGFR trafficking are signatures of Nf2 deficiency in multiple tumor-relevant cell types, suggesting novel biomarkers and therapeutic insight into these intractable tumors.

Results

Merlin limits EGF-induced remodeling of cortical actomyosin

Our previous studies revealed that merlin and ezrin influence the poorly understood interdependency between EGFR and the cortical cytoskeleton (Curto et al. 2007; Chiasson-MacKenzie et al. 2015). To better understand this relationship, we studied the cortical changes that occur in response to EGF stimulation in Nf2−/− and Nf2WT-expressing liver-derived epithelial cells (LDCs) (Chiasson-MacKenzie et al. 2015). We found that EGF elicited dramatic F-actin-rich circular dorsal ruffles (CDRs) within 10 min of stimulating Nf2−/− cells but only modestly affected cortical actin in the presence of merlin despite activating equivalent levels of EGFR (Fig. 1A; Supplemental Fig. 1A). Both Nf2−/− and Nf2WT-expressing LDCs are nonmotile and grow in small colonies when subconfluent. In Nf2−/− colonies, EGFR-induced CDRs were prominent on interior cells and cells at the colony perimeter, which also exhibited marked peripheral ruffling. Nf2−/− cells exhibited a constitutive accumulation of F-actin at the colony edge (Fig. 1A), and F-actin and myosin IIa accumulated in both dorsal and peripheral ruffles upon EGFR stimulation (Fig. 1A; Supplemental Fig. 1B). Treatment with latrunculin A or jasplakinolide, which disrupt F-actin in different ways (Rotsch and Radmacher 2000), abolished dorsal and peripheral ruffling as well as the edge accumulation of cortical actomyosin (Supplemental Fig. 1C).

To understand why EGF elicited such different cortical cytoskeletal responses in the presence and absence of merlin, we focused on the earliest response to EGF stimulation. We found that within 2 min, EGF triggered the formation of cortical actin patches or tiny rings that also contained ezrin and the Arp2/3 activator N-WASP and likely represent nascent CDRs in Nf2−/− cells but not Nf2WT-expressing cells (Fig. 1B; Supplemental Fig. 1D). Notably, Texas red-labeled EGF ligand (TR-EGF), which marks activated EGF:EGFR complexes, often formed a
“bull’s-eye” at the center of these patches [Fig. 1B]. While ~75% of the Nf2−/− cells had one to two initiating patches or mature structures per cell, few Nf2WT-expressing cells had any dorsal structure, and those that did form were much smaller [Fig. 1A–C]. Most striking was the very different vertical architectures achieved by the structures that did form. The cortex of Nf2WT-expressing cells remained largely flat, with only minor actin-containing surface “ripples” (<1–4 µm) and little colocalization of F-actin and N-WASP [Fig. 1C]. In contrast, Nf2−/− cells formed large actin-enriched vertical membrane extensions (3–8 µm) with a strong accumulation of N-WASP at the tips [Fig. 1C]. Consistent with the ability of ectopic cortical ezrin to drive other phenotypes in Nf2−/− cells, shRNA depletion of ezrin eliminated dorsal and peripheral ruffles as well as the edge accumulation of actomyosin before (starved) and after EGF stimulation for 10 min in Nf2−/− and Nf2WT-expressing colonies. [Middle] The percentage of cells that formed at least one CDR after EGF stimulation. Note that many CDRs in Nf2WT-expressing cells were small cortical “ripples.” [Right] Quantitation of edge-accumulated F-actin in Nf2−/− and Nf2WT-expressing cells, measured as peak F-actin intensity using a line scan analysis [Smutny et al. 2010]. [B] Cortical ezrin localization to tiny circular rings that encircle Texas red-labeled EGF ligand (TR-EGF) at 5 min after stimulation of Nf2−/− cells. (C) Distribution of F-actin and N-WASP across the cortex (X–Y sections; left) and within the vertical extensions (Y–Z sections; middle) of Nf2−/− and Nf2WT-expressing cells. [Right] The height of vertical extensions on the surface of Nf2−/− and Nf2WT-expressing cells. (D, left) Confocal images showing F-actin-rich CDRs in Nf2−/− cells infected with shScr-expressing [control] or shEzrin-expressing lentiviruses [Hebert et al. 2012]. [Middle] The percentage of shScr- or shEzrin-expressing Nf2−/− cells with CDRs. [Right] Quantitation of F-actin at the free edge of Nf2−/− and Nf2−/−;shEzrin-expressing cells. (***) P < 0.001, Mann-Whitney U-test. Values shown are mean ± SEM. Bars, A, 20 µm; B–D, 10 µm.

Merlin deficiency primes cells for macropinocytosis

Both CDRs and peripheral ruffles can initiate the process of macropinocytosis when their vertical membrane extensions fold over and entrap extracellular fluid [Buckley and King 2017]. Macropinocytosis has been particularly well studied in the amoeba Dictyostelium discoideum, where it has been shown to initiate from surface patches of Ras and PI3K-generated P(3,4,5)P3 that recruit a surrounding rim of Arp2/3-driven actin remodeling and vertical membrane extension to form a “macropinocytic cup” [Veltman et al. 2014, 2016]. The structures that form on the surface of EGF-stimulated Nf2−/− cells mirror that of classic macropinocytic cups [Fig. 1C].

To determine whether Nf2−/− cells execute successful macropinocytic internalization, we monitored the uptake of the large dye dextran, an established marker of macropinocytosis. We found that fluorescently labeled dextran (dextran-488, 70,000 MW [molecular weight]) was internalized into large vesicular structures by Nf2−/− cells within 30 min of EGF stimulation; in contrast, few vesicles were marked by dextran-488 in EGF-stimulated Nf2WT-expressing cells [Fig. 2A]. Similarly, fluorescently labeled bovine serum albumin (BSA-488) was readily internalized by Nf2−/− but not Nf2WT-expressing cells [Fig. 2B]. Importantly, dextran-488 uptake by Nf2−/− cells was completely blocked by the macropinocytosis inhibitor 5-(N-ethyl-N-isopropyl) amiloride (EIPA), which blocks actin remodeling by inhibiting the Na+/H+ exchanger and lowering submembranous pH [Fig. 2C; Koivusalo et al. 2010].

We next asked whether exaggerated cortical actomyosin dynamics and macropinocytosis are features of merlin deficiency in other tumor-relevant cell types. Nf2 mutations underlie the development of schwannomas, meningiomas, and mesotheliomas in humans [Petrelli and Fernandez-Valle 2016]. We found that EGF-induced ruffling and macropinocytosis were prominent in Nf2-deficient human mesothelioma and mouse meningioma cells and significantly reduced upon re-expression of Nf2WT [Fig. 2D; Supplemental Fig. 2A]. We then examined primary normal (Nf2lox/lox) and derivative Nf2−/− SCs from Nf2lox/lox mice [Giovannini et al. 2000]. Primary SCs express negligible levels of EGFR and instead depend on Neuregulin [Nrg]-activated ErbB2/3 heterodimers
tive macropinosomes in growth factor stimulation, the enlarged dextran-488-positive macropinosomes in EGF-stimulated Nf2−/− and Nf2WT-expressing cells (Supplemental Fig. 3A). In contrast, upon Nf2 deficiency broadly sensitizes cells to growth factor-in-
suced cortical excitability and macropinocytosis. In addition to an increase in number, we noted that the

Figure 2. Nf2−/− cells are primed for macropinocytosis. [A] Confocal images [left] and quantitation [right] of dextran-488+ macropinosomes in EGF-stimulated Nf2−/− and Nf2WT-expressing cells. [B] Quantitation of BSA-488+ macropinosomes after EGF stimulation. [C] Quantitation of dextran-488+ macropinosomes in EGF-stimulated Nf2−/− cells treated with 50 μM EIPA or vehicle [DMSO]. [D, left] Confocal images of F-ac-
tin-labeled cortical ruffles [arrowheads] in EGF-stimulated Nf2−/− and Nf2+ mesothelioma cells. [D, right] Quantitation of dextran-488+ macropinosomes in Nf2−/− and Nf2WT-expressing mesothelioma cells. [E, left] Confocal images of F-actin-labeled cortical ruf-
fles [arrowheads] in starved and Nrg-stimulated primary Nf2−/− and wild-type [Nf2lox/lox] SCs. [Right] Quantita-
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A role for ERM proteins in stabilizing cholesterol-rich domains in the plasma membrane has been proposed [Prag et al. 2007; Martinelli et al. 2013; Santos et al. 2013]. We found that CTxB-488 was particularly enriched at the base of macropinocytic cups prior to their internal-
ization; in fact, surface CTxB-488 levels were markedly el-
levated in Nf2−/− cells of all three types relative to controls [Fig. 3D,E, Supplemental Fig. 3C]. These data suggest that internalized macropinosomes in Nf2−/− cells are formed from surfac-derived cholesterol-enriched membrane. Similarly, the levels of membrane cholesterol itself [as measured by filipin labeling] and of flotillin [an independ-
ent marker of cholesterol-rich membranes] were elevated in Nf2−/− cells [Supplemental Fig. 3D,E]. Actomyosin
cytoskeletal networks can control the spatial distribution and stability of cholesterol-rich membrane domains [Gowrishankar et al. 2012; Kusumi et al. 2012]. Indeed, dextran-488+ and Rab7+ macropinosomes in Nf2−/− cells were specifically encircled by actin, and shEzrin expres-
sion blocked macropinocytosis and reduced the size of Rab7+ vesicles in Nf2−/− cells [Fig. 3F–H, Supplemental Fig. 3F]. While it is clear that most macropinosomes un-
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sence of merlin, ectopic ezrin and altered cortical actin stabilize cholesterol-rich membrane domains, influencing the uptake and processing of macropinosomes.

We compared macropinosomes formed by Nf2−/− SCs with those of SCs lacking the NF1 tumor suppressor, which encodes an established Ras inhibitory GTPase-acti-
vating protein [RasGAP] [Cichowski and Jacks 2001]. Like Ras transformed mammalian cells, strains of

Altered macropinosome processing in Nf2-deficient cells

In addition to an increase in number, we noted that the
dextran-488-positive macropinosomes in Nf2−/− cells were much larger than the few that formed in control cells [Fig. 3A]. The small GTPase Rab5, the earliest marker of macropinosomes, marked vesicles of a similar size and distribution in EGF-stimulated Nf2−/− and Nf2WT-expressing cells [Supplemental Fig. 3A]. In contrast, upon growth factor stimulation, the enlarged dextran-488-positive macropinosomes in Nf2−/− cells of all three cell types accumulated Rab7, which replaces Rab5 and is involved in trafficking macropinosomes to the lysosome [Fig. 3B; Supplemental Fig. 3B; Kerr and Teasdale 2009].

An enlarged Rab7 vesicle compartment is frequently as-

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vating protein [RasGAP] [Cichowski and Jacks 2001]. Like Ras transformed mammalian cells, strains of
**Dictyostelium** carrying a mutation in the **NF1** ortholog exhibit excessive macropinocytosis [Bloomfield et al. 2015]. Familial NF1, like NF2, features the development of SC-derived tumors known as neurofibromas but has distinct features or anatomical distribution relative to **NF2** mutant schwannomas [Lin and Gutmann 2013]. We found that, in contrast to control or **Nf2**−/− SCs, **Nf1**−/− SCs exhibit constitutive rather than growth factor-induced macropinocytosis [Fig. 4A; Supplementary Fig. 4]. Furthermore, macropinosomes in **Nf1**−/− SCs, like control SCs [Fig. 3B], were markedly smaller than those of Nrg-induced **Nf2**−/− SCs and lacked Rab7 accumulation [Fig. 4B,C]. Finally, in striking contrast to **Nf2**−/− SCs, **Nf1**−/− SCs exhibited little to no plasma membrane or vesicular CTxB-488 [Fig. 4D].

Ras transformed pancreatic tumor cells undergo constitutive macropinocytosis and exploit it to acquire amino acids via the lysosomal degradation of internalized extracellular protein. As such, cultured Ras transformed cells can survive and proliferate in the absence of free extracellular glutamine by breaking down exogenously provided BSA (Commissio et al. 2013). However, we found that **Nf2**−/− LDCs and SCs were not able to exploit exogenous BSA to survive under conditions of glutamine deprivation [Fig. 4E]. Moreover, **Nf2**−/− cells were relatively insensitive to the lysosomal acidification inhibitor hydroxychloroquine (HCQ) or inhibition of PIKfyve localization [FTY720], which is important for the lysosomal processing of both autophagosomes and macropinosomes [Fig. 4F; Kim et al. 2016]. Together, these data suggest that, unlike Ras transformed pancreatic cells, **Nf2**−/− LDCs and SCs do not depend on or receive benefit from the lysosomal processing of exogenous protein. These results are consistent with a model in which stabilization of actin-associated cholesterol-rich membranes in the absence of merlin alters the processing of growth factor-induced macropinosomes.

**Increased EGFR recycling in the absence of merlin**

Cells undergoing significant macropinocytosis internalize a considerable portion of their membrane and must have an efficient mechanism for recycling membrane and receptors back to the surface [Buckley and King 2017]. In fact, neurons depend on macropinocytic recycling to maintain sufficient surface receptors during high synaptic power images of dextran-488+ macropinosomes in EGF-stimulated **Nf2**−/− and **Nf2**WT-expressing cells. (Right) The diameter of dextran-488+ vesicles in EGF-stimulated **Nf2**−/− and **Nf2**WT-expressing cells. (B, left) Confocal images showing enlarged Rab7+ and dextran-488+ macropinosomes in EGF-stimulated **Nf2**−/− and **Nf2**WT-expressing cells. (C, left) Confocal images showing the distribution of cholera toxin B (CTXB-488) and Rab7 to macropinocytic vesicles in **Nf2**−/− cells after incubation with CTXB-488 and EGF for 30 min at 37°C. (Right) Quantitation of Rab7+ vesicle size in **Nf2**−/− cells treated with vehicle (water) or 10 mM MjCD. (D) Confocal images (left) and quantitation (right) of surface-labeled CTXB-488 after 30 min at 4°C in unstimulated **Nf2**−/− and **Nf2**WT-expressing cells. (E) X–Z confocal images showing CTXB-488 and N-WASP localization on the dorsal surface of EGF-stimulated **Nf2**−/− cells. (F) Confocal images showing the distribution of F-actin relative to Rab7 in EGF-stimulated **Nf2**−/− and **Nf2**WT-expressing cells. (G) Quantitation of dextran-488 uptake in **Nf2**−/− cells infected with shScr- or shEzrin-expressing lentiviruses. (H) Quantitation of Rab7+ vesicle size in **Nf2**−/− cells infected with shScr- or shEzrin-expressing lentiviruses. Error bars indicate mean ± SD. (A,B,C,H) or SEM. (***) P < 0.001, Mann-Whitney U-test. Bars, 10 μm.
mechanisms controlling whether macropinosomes and their associated receptors are recycled or lysosomally processed are not understood, but exploitation of this balance to favor recycling of EGFR or other mitogenic receptors could provide an advantage to Nf2−/− cells [Tomas et al. 2014; Francavilla et al. 2016]. We observed that EGFR trafficking proceeded in strikingly different ways in Nf2−/− and Nf2WT-expressing cells. In addition to concentrating in nascent dorsal ruffles/macropinocytic cups [Fig. 1B], TR-EGF and EGFR accumulated at the free edge of Nf2−/− cells [a site of vigorous peripheral ruffling and macropinocytosis] within 2 min of stimulation but localized to individual dispersed puncta by 30 min [Fig. 5A; Supplemental Fig. 5A]. In contrast, in Nf2WT-expressing cells, TR-EGF labeled tiny dispersed puncta that increased in size by 30 min [Fig. 5A]. Acid wash removed little of the accumulated TR-EGF at 2 min in Nf2−/− cells, indicating that TR-EGF:EGFR complexes rapidly accumulate in a submembranous endocytic compartment [Supplemental Fig. 5B]. This pattern of rapid TR-EGF accumulation in Nf2−/− cells was independent of the concentration of EGFR used and provided a readily quantitative assay for investigating mechanisms of EGFR trafficking in Nf2−/− cells [Supplemental Fig. 5C].

Disruption of actin or elimination of ezrin abolished the early submembranous accumulation of TR-EGF:EGFR in Nf2−/− cells without blocking internalization [Fig. 5B; Supplemental Fig. 5D]. We observed previously that activated EGFR biochemically fractionates with cholesterol-rich membranes and exhibits a cholesterol-sensitive change in mobility in the absence of merlin [Curto et al. 2007; Chiasson-MacKenzie et al. 2015]. Indeed, treatment of Nf2−/− cells with MβCD or simvastatin, which inhibits cholesterol production, also eliminated the unique pattern of TR-EGF internalization in Nf2−/− cells [Fig. 5C; Supplemental Fig. 5E]. In contrast, the internalized transferrin receptor, which undergoes clathrin-mediated endocytosis (CME), did not accumulate submembranously in Nf2−/− cells and was unaffected by MβCD [Supplemental Fig. 5F]. This is consistent with the exclusion of clathrin-labeled vesicles from this region of Nf2−/− cells [Supplemental Fig. 5G]. Conversely, in Nf2WT-expressing cells, TR-EGF internalization was unaffected by MβCD but was blocked by the CME inhibitor chlorpromazine (CPZ), which did not affect TR-EGF in Nf2−/− cells [Supplemental Fig. 5H, I]. As expected, CPZ blocked the internalization of the CME cargo transferrin in Nf2WT-expressing cells [Supplemental Fig. 5I]. Together, these data suggest that in the absence of merlin, changes in the membrane:cytoskeleton interface direct clathrin-independent trafficking of EGFR.

Macropinocytosis is clathrin-independent and has been linked to high membrane cholesterol [Grimmer et al. 2002; Mercer and Helenius 2009]. Indeed, EIPA markedly reduced internalized TR-EGF in Nf2−/− cells, supporting the idea that a substantial fraction of EGFR internalizes via macropinocytosis [Fig. 5D]. To determine whether the fate of internalized EGFR is altered in the absence of merlin, we monitored EGFR turnover after EGF stimulation in the presence of cyclohexamide. While EGF...
stimulation led to a rapid loss of surface EGFR in both
N{\textsuperscript{2}−/−} and N{\textsuperscript{2}WT}-expressing cells, EGFR quickly reappeared
at the plasma membrane in N{\textsuperscript{2}−/−} cells but not
N{\textsuperscript{2}WT}-expressing cells (Fig. 5E). Similarly, immunoblotting
revealed a marked down-regulation of total EGFR in
N{\textsuperscript{2}WT}-expressing cells but not N{\textsuperscript{2}−/−} cells, also consist-
tent with the idea that EGFR is preferentially recycled
in the absence of merlin (Supplemental Fig. 5J).

It is well established that ErbB2 localizes to membrane
protrusions and ruffles and preferentially distributes
to cholesterol/GM1-rich plasma membrane domains [Nagy
et al. 2002; Hommelgaard et al. 2004; Bertelsen and Stang
2014]. Moreover, ErbB2 is resistant to down-regulation
and can confer that resistance—along with clathrin-inde-
dependent internalization—to EGFR, instead, ErbB2:EGFR
heterodimers preferentially recycle relative to EGFR
homodimers [Nagy et al. 2002; Hendriks et al. 2003; Has-
lekas et al. 2005]. We found that merlin-deficient cells ex-
hibited elevated surface levels of ErbB2 that are sensitive
to MβCD (Fig. 6A,B). In fact, ErbB2 colocalizes with
both CTxB-488 and TR-EGF at 2 min after stimulation,
and ErbB2 and TR-EGF colocalized in discrete vesicles
in N{\textsuperscript{2}−/−} cells at 30 min after stimulation (Fig. 6B; Supple-
mental Fig. 6A). ErbB2 can also impose alterations upon
EGFR signaling [Li et al. 2012]; indeed, we found that
EGF stimulation yielded a strikingly different pattern of
pAkt but not pErk in N{\textsuperscript{2}−/−} and N{\textsuperscript{2}WT}-expressing cells
despite similar stimulated levels (Fig. 6C; Supplemental
Figs. 1A, 6C). In N{\textsuperscript{2}−/−} cells, EGF stimulation yielded a
strong accumulation of pAkt in ruffling areas of the
cell—including specifically in CDRs—that was ezrin-, ac-
tomyosin-, and cholesterol-dependent [Fig. 6C,D; Supple-
mental Fig. 6B]. In contrast, EGF stimulation yielded
diffusely localized pErk in N{\textsuperscript{2}−/−} cells [Supplemental
Fig. 6C]. In N{\textsuperscript{2}WT}-expressing cells, EGF triggered diffuse
localization of both pAkt and pErk [Fig. 6C; Supplemental
Fig. 6C]. The pan-PI3K inhibitor GDC0941 blocked
ruffling and macropinocytosis in N{\textsuperscript{2}−/−} cells but not the
formation of actin-rich initiating patches [Supplemental
Fig. 6D], consistent with the established requirement of
PI3K-induced PI(3,4,5)P3 in mature macropinocytic cup
formation [Maekawa et al. 2014; Veltman et al. 2016]. To-
gether, these data suggest a model in which the altered
configuration of actomyosin- and cholesterol-rich mem-
branes in the absence of merlin promotes EGFR:ErbB2
heterodimerization, locally amplified PI3K activity, cor-
tical cytoskeletal excitation, macropinocytosis, and re-
cycling of a substantial fraction of internalized EGFR.
These signatures of N{\textsuperscript{2}F} deficiency were also evident in
mesothelioma cells [Supplemental Fig. 6E].

Translational implications of merlin-deficient
macropinocytic priming

Our studies have novel translational implications for NF2
mutant tumors. First, a prediction of our studies is that
NF2-deficient cells are “addicted” to the advantage conferred by the recycling of ErbB and perhaps other mitogenic receptors. Our results suggest that altered trafficking of EGFR in NF2−/− cells depends on increased membrane cholesterol (Fig. 6B). Altered cholesterol homeostasis—particularly the availability of hydroxymethylsterols—has been shown to impact EGFR/ErbB2 recycling (Sukhanova et al. 2013). We found that in both LDCs and SCs, NF2 deficiency conferred increased sensitivity to ketoconazole, an inhibitor of sterol biosynthesis upstream of hydroxymethylsterol production (Fig. 6B). Importantly, this sensitivity was associated with a reduction in both Rab7 vesicle size and EGFR recycling (Fig. 6C). Thus, NF2-deficient cells may be particularly vulnerable to modulators of membrane cholesterol and receptor recycling.

Second, it is increasingly appreciated that macropinocytosis can be exploited for the delivery of therapeutics into tumor cells, in particular larger nanoparticles or biologics that are not membrane-permeable (Ha et al. 2016). Another key example is extracellular vesicles (EVs), which can be drug-loaded and delivered into cells via macropinocytosis (Nakase et al. 2015; Costa Verdera et al. 2017). We asked whether NF2 deficiency renders cells preferentially able to internalize fluorescently labeled EVs (PKH26-EVs). As shown in Figure 7D, NF2−/− LDCs internalized PKH26-EVs with significantly...

**Figure 6.** Merlin controls the surface levels of ErbB2 and spatial distribution of pAkt. (A) Confocal images (left) and quantitation (right) of total ErbB2 levels in NF2−/− and NF2WT-expressing cells. (B, left) Distribution of ErbB2 with CTxB-488 following 2 min of EGF stimulation in NF2−/− and NF2WT-expressing cells. Distribution (middle) and quantitation (right) of ErbB2 and TR-EGF 2 min after stimulation in NF2−/− cells treated with vehicle or 10 mM MβCD. (C, left) Distribution of pAkt in starved or TR-EGF-stimulated NF2−/− and NF2WT-expressing cells. (Right) X–Z confocal images showing pAkt and F-actin in CDRs in EGF-stimulated NF2−/− cells. [**P** < 0.001, Mann-Whitney U-test. Bars, 10 μm.

**Figure 7.** Therapeutic implications of macropinocytic priming in NF2−/− cells. (A) Sensitivity of NF2−/− versus NF2WT-expressing LDCs and NF2−/− versus control (NF2lox/lox) SCs to 72 h of treatment with ketoconazole relative to vehicle (DMSO). (B) Quantitation of Rab7+ vesicle size in EGF-stimulated NF2−/− and NF2WT-expressing cells treated with vehicle or ketoconazole. (C) Confocal images (left) and quantitation (right) of EGFR levels in NF2−/− cells pretreated with cyclohexamide and stimulated with EGF for 30 min at 4°C and then transferred for 30 min or 2 h to 37°C following treatment with vehicle or ketoconazole. (D) Confocal images (left) and quantitation (right) of PKH26-labeled extracellular vesicles (EVs) internalized by EGF-stimulated NF2−/− or NF2WT-expressing cells. Error bars represent mean ± SEM. [**P** < 0.01, [***] P < 0.001, Student’s t-test for A and Mann-Whitney U-test for B–D. Bars 10 μm.
improved efficiency relative to $N^2_{(2WT)}$-expressing LDCs (Fig. 7D). Thus, $N^2_{(2WT)}$-deficient cells may be particularly susceptible to targeting via therapeutics that use macropinocytic uptake.

Discussion

The membrane:cytoskeleton interface has a central but poorly understood role in regulating and responding to growth factor receptors. Our studies identified merlin/ERMs as fundamental architects of this crucial cellular compartment and uncovered a novel role for them in regulating growth factor-induced macropinocytosis. Macropinocytosis is evolutionarily rooted in primitive means of nutrient acquisition that center on the interrelationship between nutrient sensing and morphological changes in the cell surface; its rediscovery as both a cellular behavior exploited by tumor cells and a conduit for drug delivery begs a deeper mechanistic understanding of the cortical actomyosin and associated membrane lipids, but it is not well established but poorly understood (Gowrishankar et al. 2012; Kusumi et al. 2012). Ezrin has been reported to influence this interface and could stabilize it by binding to and aggregating membrane PI(4,5)P$_2$, which clusters within cholesterol-rich membranes (Barret et al. 2000; Kwik et al. 2003; Prag et al. 2007; Martinelli et al. 2013; Santos et al. 2013; Senju et al. 2017). EGFr-stimulated PI(4,5)P$_2$ is essential for membrane ruffle formation and localizes to the vertically protruding rims of remodeling actin that surround PI3K-catalyzed PI(3,4,5)P$_3$ and form the walls of the macropinocytic cup (Araki et al. 2007). Cholesterol-rich domains may also amplify the signal from the activated EGFR itself, as has been shown for Fc receptors that initiate the related process of phagocytosis (Swanson 2008). A diffusion barrier must exist to prevent signal expansion and enable such a discrete structure to form, but the nature of such a barrier is unknown (Swanson 2008; Veltman et al. 2016). Local organization of the membrane:cytoskeleton interface is a clear candidate.

It is well known that internalized macropinosomes can undergo lysosomal processing, which is associated with the degradation of extracellular proteins as a source of amino acid nutrients, a feature exploited by Ras transformed pancreatic tumor cells (Commesso et al. 2013; Kamphorst et al. 2015; Davidson et al. 2017). We provide evidence that $N^2_{(2WT)}$-cells do not exploit macropinocytosis for the acquisition and lysosomal processing of exogenous protein and instead preferentially recycle macropinosomes back to the cell surface, as can occur in certain settings (Buckley and King 2017). Internalized macropinosomes in $N^2_{(2WT)}$-cells are enlarged and accumulate Rab7 and the cholesterol-rich membrane marker CtxB-488. Enlarged Rab7-positive endocytic structures are associated with impaired lysosomal trafficking and decreased EGFR degradation (Jaber et al. 2016; Laviolette et al. 2017). Rab7 recruits clusters of dynein motors to cholesterol-rich membrane patches on internalized phagosomes to power their transport to the lysosome; however, cholesterol overloading, as in Neimanick Pick disease, impedes this directed lysosomal transport and may favor the default kinesin-powered delivery back to the cell surface (Rocha et al. 2009; Rai et al. 2016). Our data are consistent with the notion that increased plasma membrane cholesterol in $N^2_{(2WT)}$-cells favors macropinosome recycling rather than lysosomal transport.

We propose that the stabilization of cholesterol-rich membranes in $N^2_{(2WT)}$-cells further impacts the signaling linking could dramatically impact the formation and amplification of actin waves that underlie the dorsal and peripheral ruffles that initiate macropinocytosis via the differential recruitment of actin modifiers or modulation of membrane tension; those differences also impact receptor mobility and the diffusion of signals emanating from an activated receptor (Clayton and Cousin 2009; Gauthier et al. 2012; Kusumi et al. 2012; Bermitt et al. 2017).

The concomitant stabilization of cholesterol-rich domains within the plasma membrane of $N^2_{(2WT)}$-cells likely also influences the excitability of both cytoskeletal and signaling responses. The coordination between cortical actomyosin and cholesterol-rich domains is well established but poorly understood (Gowrishankar et al. 2012; Kusumi et al. 2012). Ezrin was originally identified as a target of EGF-induced tyrosine phosphorylation that localizes to dorsal ruffles, supporting an active role in the membrane:cytoskeleton remodeling that enables macropinocytosis (Gould et al. 1986; Bretscher 1989; D’Angelo et al. 2007). How do alterations in the membrane:cytoskeleton interface translate into significant changes in the “excitability” of cortical topography and signaling? The formation of a macropinocytic cup involves the establishment of a discrete domain of membrane:cytoskeleton dynamics and signaling that is sculpted by feedback (Swanson 2008). Like band 4.1, the prototype of the FERM protein family, merlin/ERMs likely simultaneously tether membrane proteins or lipids to the cortical cytoskeleton, impact cortical actin organization, and assemble protein complexes that locally regulate actin, membrane lipids, and/or receptors (McClatchey 2014). They are therefore poised to simultaneously impact both cytoskeletal and signaling responses to growth factor stimulation. A molecular understanding of how merlin and ezrin differentially configure the membrane:cytoskeleton interface awaits a dynamic high-resolution analysis of the cortical meshwork and associated membrane lipids, but it is notable that they interact quite differently with actin. The ERMs bind directly to actin filaments and are thought to align them parallel to the membrane (Thery and Bornens 2008; Fehon et al. 2010). Merlin lacks the ERM actin-binding domain but can interact with a-catenin, an actin-binding protein that influences Arp2/3-driven actin branching (Drees et al. 2005; Gladden et al. 2010). Differences in cortical actin configuration and membrane:actin cross-
response to EGF by increasing the surface availability of ErbB2 and favoring the formation of EGFR:ErbB2 heterodimers that internalize via clathrin-independent endocytosis, transiently accumulate in a submembranous compartment, and recycle. Consistent with this model, increased ErbB2, which is known to prefer cholesterol-rich membrane domains and can be stabilized at the surface via the ERM-binding adapter NHERF1, can promote recycling rather than degradation of EGFR and drive membrane-proximal PI3K activity (Hommelgaard et al. 2004; Offerdinger and Bastiaens 2008; Jeong et al. 2017). This feed-forward signaling, together with the appropriate actin configuration, could further optimize conditions for macropinocytosis and provide a distinct advantage to NF2−/− cells.

Changes in the membrane:cytoskeleton interface, as occur in NF2−/− cells, could trigger more stable cellular adaptation in differentiated tissues or disease states. Plasma membrane cholesterol and GM1 levels decrease in response to cellular crowding (Snijder et al. 2009). We showed previously that merlin/ERMAs play an essential role in controlling EGFR trafficking and signaling in response to the mechanical forces imposed by high cell density (Chiasson-MacKenzie et al. 2015). It is tempting to speculate that, in addition to preferentially recycling, EGFR:ErbB2 heterodimers differentially signal to maintain an adapted state, perhaps by impacting cholesterol biosynthesis or transport (Nagy et al. 2002; Chung et al. 2010). Such broad changes in the plasma membrane:cytoskeleton interface conferred by merlin/ERMAs likely impact other receptors; indeed, defects in other mitogenic and adhesion receptors have been reported in NF2−/− cells (Petrilli and Fernandez-Valle 2016). Our findings may help to explain the pleiotropic consequences of NF2 deficiency on multiple signaling pathways.

NF2 gene mutations occur in a range of human tumors (Petrilli and Fernandez-Valle 2016). Our work suggests new and broad ways to target NF2 mutant tumors. First, it may be possible to exploit their macropinocytic proficiency as a vulnerability. Unlike Ras transformed pancreatic tumor cells, NF2-deficient SCs and liver cells do not seem to depend on macropinocytic uptake and lysosomal degradation of extracellular protein as a source of nutrients. A better understanding of how the membrane:cytoskeleton interface is used for macropinocytic internalization and processing will have broad consequences for NF2 mutant and other tumor types.

Materials and methods

Cell culture

LDCs are HB-like cells derived from hyperplastic liver lesions in a 12-wk-old Alb-Cre;Nf2lox/lox mouse as described (Chiasson-MacKenzie et al. 2015). Cells were cultured in DMEM with 10% fetal bovine serum [FBS]. Adenovirus infection was used for Nf2 re-expression [Ad5-CMV-Nf2WT]. Primary murine SCs [Nf2lox/lox or Nf1lox/lox] were cultured on poly-L-lysine/laminin-coated plates in N2 medium [DMEM/F12-HAM, 1× N2 supplement [Thermo Fisher], 50 µg/mL gentamicin [Thermo Fisher], 2 µM forskolin [Calbiochem], and 10 ng/mL Nrg [HRG-β1 EGF domain] [R&D Systems]; provided by Marco Giovannini] [Giovannini et al. 2000; Manent et al. 2003]. Nf2 or Nf1 was deleted from fox/lox SCs via adenovirus infection with Cre-recombinase [Ad5-CMV-Cre]. Human mesothelioma cell line HP-1 (provided by David Kwiatkowski, Brigham and Women’s Hospital, Boston) was grown in 10% FBS-DMEM. H2731 and NCI-H28 (provided by Cyril Benes, Massachusetts General Hospital Center for Molecular Therapeutics, Boston) were cultured in 10% FBS-DMEM and 10% FBS-RPMI, respectively. Nf2WT-expressing mesothelioma cells were prepared via adenoviral expression of Nf2WT. 293A cells for adenovirus production (Agilent) and 293T cells for lentivirus production (American Type Culture Collection) were cultured in 10% FBS-DMEM. All cell culture media were supplemented with 1% penicillin/streptomycin [Thermo Fisher].

Growth factors and pharmacological inhibitors

EGF (Peprotech) was used at 100 ng/mL, and Nrg was used at 10 ng/mL. Drug pretreatment was as follows: 50 µM EIPA [Sigma-Aldrich] for 60 min, 25 µM HCQ [Sigma-Aldrich] for 48 h, 2.5 µM FTY720 [Cayman] for 48 h, 1 µM GDC0941 [Selleck] for 1 h, 40 µM ketoconazole [Sigma-Aldrich] for 72 h [sensitivity] or 24 h [re-cycling], 1 µM BBW2992 [Selleck] for 1 h, 10 mM MJ6CD [Sigma-Aldrich] for 30 min, 10 µM latrunculin A [Cayman] for 15 min, 5 µM jasplakinolide for 15 min, and 50 µg/mL cyclohexamide [Sigma-Aldrich] for 1 h. These doses were maintained throughout the experiment.

Plasmids and shRNA constructs

The Nf2WT expression construct was generated by PCR amplification of the mouse Nf2-coding region and cloned into a pAdCMV vector as described (Chiasson-MacKenzie et al. 2015). The shRNA constructs targeting mouse eznin [5′-ATTTCCT TGTATTATAATCTCCG-3′] in a pLKO.puro.1 vector were from GE Healthcare and were described in Hebert et al. (2012). The control [shScr, 5′-CAGTCCGCTTTTGCAGCTG-3′] in a pLKO.puro.1 vector was provided by Marianne James (Massachusetts General Hospital, Boston) [James et al. 2008].

Virus production and infection

Nf2WT-expressing and Cre-recombinase adenoviruses were generated using the AdEasy system (Agilent) as described in Chiasson-MacKenzie et al. (2015). Cells were infected 24 h before the start of the experiment to induce Nf2 gene expression. An empty adenoviral vector was used as a control (EV). shRNA-expressing lentiviruses were generated by cotransfecting 293T cells with pLKO.puro.1 vectors and the packaging vectors ΔVPR and VSVG (Fugene). Viruses were harvested 24–48 h after transfection. shScr- or shEzrin-expressing lentiviruses were stably expressed in LDCs and selected in 4 µg/mL puromycin.
Antibodies
The following primary antibodies were used: anti-ezrin mouse monoclonal antibody (mAb; 1:500 for immunofluorescence; Neo-markers), anti-N-WASP rabbit polyclonal antibody (pAb; 1:100; Cell Signaling Technology), anti-Rab7 rabbit mAb (1:100; Cell Signaling Technology), anti-pAkt (S473) rabbit mAb (D9E, 1:100; Cell Signaling Technology), anti-pERK1/2 (T202/Y204) rabbit mAb (D13.14.4E, 1:100; Cell Signaling Technology), anti-ErbB2 rabbit pAb (1:100; Dako), and anti-Merlin rabbit pAb (1:500; Cell Signaling Technology). Alexa fluor 488-phalloidin or rhodamine-phalloidin (1:500; Thermo-Fisher) was used to label F-actin. Alexa fluor 488-CTxB (Thermo Fisher) was used to label membrane GM1. Species-specific secondary antibodies conjugated to Alexa fluor 488, 555, or 647 (Thermo Fisher) were used for immunofluorescence. DAPI was used to label nuclei.

Immunofluorescence microscopy
Cells were fixed in 4% paraformaldehyde in PBS for 15 min at room temperature and then permeabilized in 0.2% Triton X-100 for 10 min. Primary and secondary antibodies were diluted in PBS with 1% BSA and incubated for 1 h at room temperature. Alexa fluor-phalloidin was added with secondary antibodies. Coverslips were mounted with Prolong Gold anti-fade mountant (Thermo Fisher). Cells were imaged with an inverted laser-scanning confocal microscope (LSM710, Carl Zeiss) equipped with a 63× oil immersion objective (plan apochromat NA 1.4; Carl Zeiss). Alexa fluor 488, Alexa fluor 555, and rhodamine probes were excited with the 488-nm or 514-nm laser line of an argon laser. Alexa fluor 647 probes were excited with the 633-nm laser line of a helium-neon laser. Images were acquired as single images or z-stacks in sequential mode using Zen Black software (2012 version; Carl Zeiss).

Dextran/BSA uptake
Cells grown on glass coverslips to 30%–40% confluency were serum-starved overnight and then stimulated with EGF or Nrg for 30 min. Dextran-488 (Oregon Green 488; 70,000, anionic, lysine-fixable) or BSA-488 (Alexa fluor 488 conjugate) (Thermo Fisher) was added at a concentration of 0.5 mg/mL with growth factors. Cells were rinsed three times with cold PBS and fixed with 4% paraformaldehyde. To measure constitutive macropinocytosis, dextran-488 was added to complete growth medium for 30 min. For containing with Rab7, cells were permeabilized with 0.2% TX-100 prior to staining.

TR-EGF internalization and recycling
LDCs were plated on 24-mm glass coverslips and grown to a confluency of 30%–40%. TR-EGF internalization experiments were performed as described previously (Chiaisson-MacKenzie et al. 2015). Prior to TR-EGF stimulation, cells were serum-starved overnight and incubated in DMEM/1% BSA for 1 h. Cells were stimulated with 2 μg/mL TR-EGF (Thermo Fisher) for 2, 10, or 30 min at 37°C. Cells were rinsed three times with cold PBS. When noted, 10 μg/mL CtxB-488 was added with TR-EGF.

To measure EGF recycling, cells were serum-starved overnight and treated with 50 μg/mL cyclohexamide for 1 h. Cells were stimulated with 2 μg/mL TR-EGF for 30 min at 4°C. Cells were washed twice with cold PBS and then shifted for 30 min or 2 h to 37°C. Cyclohexamide was maintained in the medium throughout the experiment. Following fixation, cells were permeabilized and stained.

Cell viability/drug treatment
LDCs [5000 cells per well] or SCs [10,000 cells per well] were seeded in triplicate in 24-well plates in appropriate medium and allowed to adhere overnight. The drug was added 24 h after plating and incubated for 48 or 72 h. Drugs were replenished every 24 h. Cells were counted with a hemocytometer, and results were quantified as relative cell number compared with vehicle.

Glutamine deprivation
LDCs [5000 cells per well] or SCs [10,000 cells per well] were seeded in 24-well plates in triplicate. Twenty-four hours after plating, the medium was replaced with glutamine-free growth medium. Where noted, BSA was added to a final concentration of 2%. The medium was replaced every 24 h. Cells were grown for 6 days, and final cell counts were determined using a hemocytometer and quantified as relative cell number compared with Nf2 WT cells in complete growth medium.

EV production and uptake
EVs were harvested as described previously (Reategui et al. 2018) from the conditioned medium of ~1.0 × 10⁸ Nf2−/− LDCs. Forty-eight hours prior to harvesting, 10% FBS/DMEM was replaced with DMEM with 100 ng/mL EGF for 48 h. The medium was collected and centrifuged for 10 min and at 2000 g for 10 min. Supernatants were filtered (0.8 μm; EMD Millipore) and ultracentrifuged at 100,000 g for 90 min at 4°C (Optima L-90K Ultracentrifuge, Beckman Coulter). Pelleted EVs were resuspended in 200 μL of PBS. Purified EVs were labeled with PKH26 dye (Sigma-Aldrich) on a column and rinsed three times by centrifugation at 400 g for 10 min. Labeled EVs were added to Nf2−/− or Nf2 WT-expressing LDCs at ~10⁶ per 0.7 cm² and incubated for 1 h at 37°C. Cells were then fixed and stained.

Image analysis and statistics
ImageJ software (version 2.0, National Institutes of Health) was used for all image processing and analysis. The displayed images were produced from single confocal slices or maximum projections of z-stack images. Background was removed with rolling ball background subtraction. Lookup tables were applied to produce final images. CDRs were analyzed by counting the percentage of cells with CDRs from at least five random fields (20× objective; n > 300 cells). CDR height was measured by drawing a line from the surface of the cell to the tip of a CDR (n = 20). F-actin intensity was measured using the line scan function (Smutny et al. 2010). A line was drawn perpendicular to the free edge of a cell, and pixel intensity along the line was graphed using the plot profile function. The data were fit to a Gaussian curve using nonlinear regression analysis, and the mean amplitude of the curve was graphed (n = 20). Dextran, BSA, or EV uptake was measured using the “analyze particles” function to determine the ratio of total particle area to total cell area from at least five random fields. Vesicle size was analyzed by using a line to measure the diameter of vesicles labeled with dextran-488 or Rab7 from five random fields (n > 300). CtxB and ErbB levels were analyzed by measuring the integrated density of a cell using a thresholding mask within a region of interest (n = 20). For analysis of TR-EGF, CtxB, or cholesterol enrichment at the free edge of cells, the sum of the pixel values [RawIntDen] was measured within a region of
interest. TR-EGF internalization was measured by dividing the sum of the intensity of objects identified with a thresholding mask by the area of the cell \( n = 20 \). Data from all analyses were imported into Prism 7 for plotting graphs and statistical analysis. The unpaired t-test or Mann-Whitney test was used to compare groups. All data are representative of three independent experiments.

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