Studies on Intracellular Logistics

Spits, M.

Chapter Three

THE EGFR ODYSSEY – FROM ACTIVATION TO DESTRUCTION IN SPACE AND TIME

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Abstract
When cell surface receptors engage their cognate ligands in the extracellular space, they become competent to transmit potent signals to the inside of the cell, thereby instigating growth, differentiation, motility and many other processes. In order to control these signals, activated receptors are endocytosed and thoroughly curated by the endosomal network of intracellular vesicles and proteolytic organelles. In this Review, we follow the epidermal growth factor (EGF) receptor (EGFR) from ligand engagement to its voyage on endosomes and, ultimately, its destruction in the lysosome. We focus on the spatial and temporal considerations underlying the molecular decisions that govern this complex journey and discuss how additional cellular organelles—particularly the ER—play active roles in the regulation of receptor lifespan. In summarizing the functions of relevant molecules on endosomes and the ER, we cover the order of molecular events in receptor activation, trafficking and downregulation and provide an overview of how signaling is controlled at the interface between these organelles.

Introduction
Multicellular life necessitates communication between distantly located cells in a manner that is straightforward to incite, decode and act upon. To serve these universal needs, cell surface receptors have evolved to recognize and respond to environmental cues with exquisite specificity and precision. In mammalian cells, some of the most vital cellular signaling pathways, including proliferation and differentiation, fall under the purview of growth factor receptors. Imbedded in the plasma membrane, these proteins extend ligand-interacting sensory platforms into extracellular space and receptor tyrosine kinase (RTK) response modules into the cytosol. This arrangement couples environmental inputs received via growth factor binding to signaling cascades transduced inside the cell upon kinase activation. Because stimulatory ligands for these receptors are produced at a distance, their activation is inducible on demand. Crucially, once the receptors become turned 'on', their signals must be terminated in order for cells to regain equilibrium and maintain responsiveness to future inputs. This balance between activation and downregulation is managed largely by the uptake of receptors from the cell surface into the vesicular network of the endocytic pathway, where timing and directionality of transport modulate signal duration and determine receptor fate. Adding further complexity to the matter, receptors such as EGFR signal not only at the cell surface, where ligand engagement oc-

curs, but continue signaling on endosomes for a comparable period (Haugh et al., 1999; Leonard et al., 2008; Foley et al., 2012; Francavilla et al., 2016). EGFR has also been reported to localize to the nucleus, where it is suggested to function as a transcription factor in association with cancer disease progression (Kamio et al., 1990; Brand et al., 2013). From ligand encounters to receptor degradation in the lysosome, in this Review we discuss how EGFR navigates the endosomal system, toggling its signaling switch in cellular space and time.

What happens at the cell surface (doesn't always stay there)

EGFR—the model RTK

EGFR is the first identified member of the receptor tyrosine kinase (RTK) family (Burgess et al., 2003; Bublil et al., 2007) and, in accordance with its plethora of functions, is expressed on the surface of numerous cell types (Chen et al., 2016). When in its active or 'on' state, EGFR transduces signals to the cell interior that instigate key processes of life, such as growth, differentiation, proliferation and motility (Ceresa and Peterson, 2014; Li et al., 2017). Given these profound effects, the association of EGFR with cancer is self-evident and exemplified by the vast number of studies that link deregulated expression and degradation of EGFR, as well as its activating mutations, with transformation (Shan et al., 2012; Tomas et al., 2014). Because many of the basic principles of EGFR biology are shared by its lesser-studied family members and beyond, EGFR represents the model growth factor RTK.

Activate me

EGFR can be activated by a number of ligands, of which EGF is most extensively studied (Cohen, 1962; Cohen and Carpenter, 1975; Harris et al., 2003; Singh et al., 2016). These ligands are produced as transmembrane precursors whose juxtacrine, paracrine and/or endocrine origins vary depending on the biological cues that instigate activity of EGFR. Typically, EGF production is locally controlled, as opposed to being delivered systemically, such as in the case of hormones, which makes it possible for different organs to conduct their own EGF-mediated programs (Singh and Harris, 2005; Conte and Sigismund, 2016). Once released into the extracellular milieu, EGF and related ligands begin the search for their cognate receptors, thereby setting in motion cellular programs of survival and growth (Massague and Pandiella, 1993; Sahin et al., 2004; Li et al., 2015; Chen et al., 2017). Specificity of EGFR activation is mediated through the establishment of defined contacts between the ligand and the binding groove of the receptor located on its extracellular face (Bajaj et al., 1987; Lax et al., 1988; Ferguson et

al., 2003; Jorissen et al., 2003; Zhu et al., 2017). Variations in sidechain features between different ligands, as well as post-translational modifications present on the extracellular EGFR domain, determine the strength of engagement (Azimzadeh Irani et al., 2017). Solid-state NMR experiments have demonstrated that in the absence of ligand, the intracellular region of EGFR exists in a rigid conformation, while the extracellular domain remains highly dynamic. Ligand binding sharply restricts this flexibility, providing a stable platform for ligand-mediated dimerization—a key event in receptor activation and initiation of downstream signaling (Ogiso et al., 2002; Kaplan et al., 2016). Within the receptor dimer, rotation of the transmembrane segment transduces a conformational change to the intracellular kinase domains, resulting in their asymmetric positioning, which in turn promotes cross-phosphorylation of cytoplasmic receptor tails (Honegger et al., 1989; Moriki et al., 2001; Kourouniotis et al., 2016; Purba et al., 2017). Depending on the type and degree of phosphorylation, the latter can now recruit specific signaling complexes and thus harbor the potential to initiate a wide variety of downstream signaling cascades associated with EGF-dependent responses (Foley et al., 2012; Wagner et al., 2013; Ceresa and Peterson, 2014; Li et al., 2017).

Ligand or not

In the absence of ligand, most EGFR molecules remain in their monomeric form and are therefore inactive. However, because the arrival of any external signals is difficult to anticipate, EGFR has evolved to be intrinsically poised towards the 'on' state, occasionally giving rise to auto-activation (Ferguson et al., 2003; Burgess et al., 2003; Ceresa and Peterson, 2014). Therefore, while maintaining acute responsiveness to ligands, cells must also guard themselves against aberrant or excessive activation of EGFR. These needs are accommodated through continuous surface sampling and the differential intracellular routing of receptors (**Fig. 1**). Although inactive receptors continuously travel through the endocytic compartment (**Fig. 1, step 1 + route** 1), slow internalization and rapid recycling rates ensure their accumulation on the cell surface. Upon ligand binding (**Fig. 1, step 2**), this equilibrium shifts rapidly (Herbst et al., 1994; Burke and Wiley, 1999; Wiley, 2003; Ceresa and Peterson, 2014; Tomas et al., 2014), causing activated receptors to spend extended periods of time traveling the endocytic route (**Fig. 1, step 3 + route 3**). In this case, signaling continues until receptors are either recycled back to the cell surface or taken up into proteolytic lysosomes, leading to their demise. Understanding how cells control the duration of legitimate ligand-mediated responses, while keeping unwarranted activation at bay in many ways encompasses the crux

of signaling pathways. It appears that cells have taken the 'divide and conquer' approach to solving this problem by segregating the receptor 'on' and 'off' states in cellular space and time. How this is orchestrated to afford proper regulation of EGFR lifespan is discussed in the following sections.

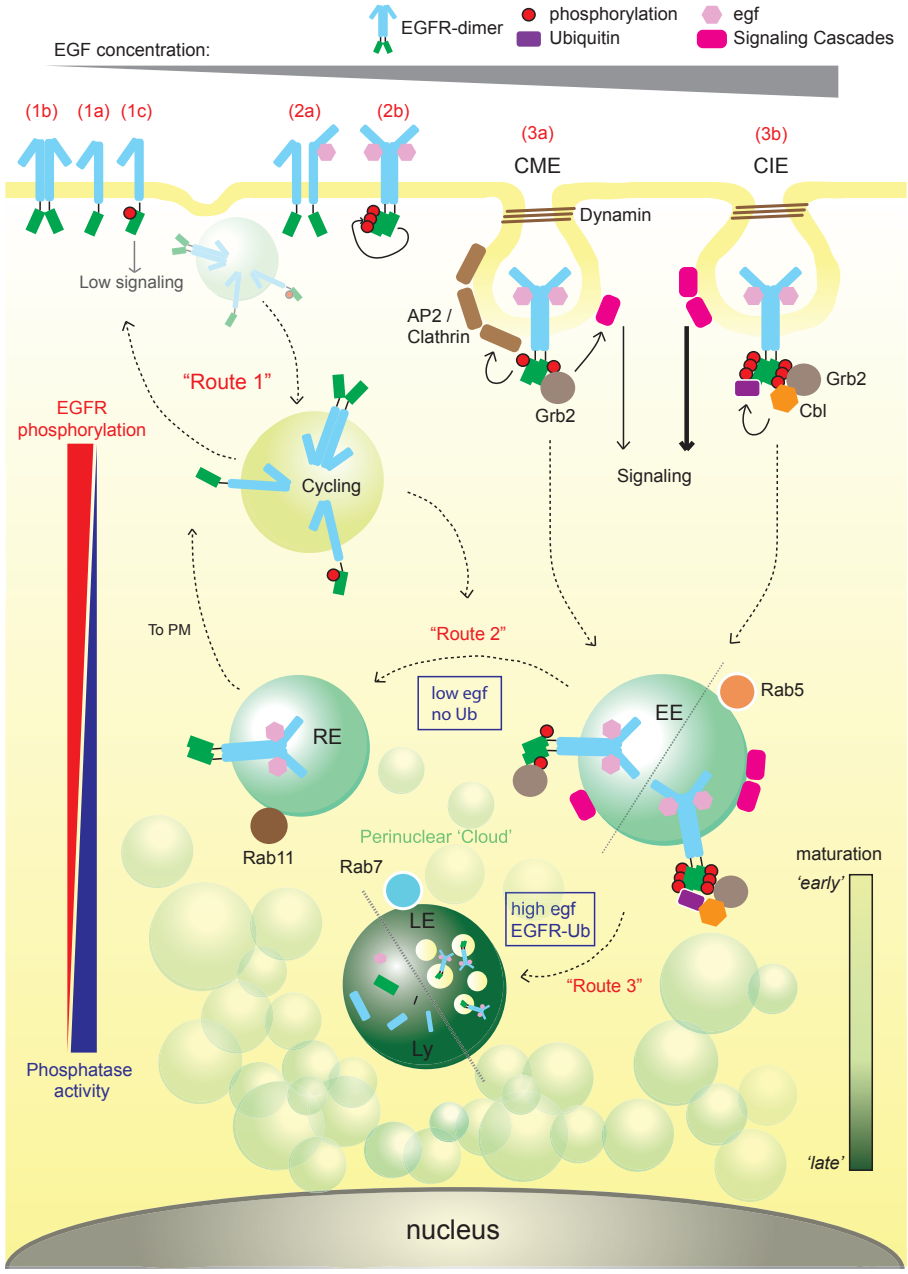

Fig.1: Destinations of activated EGFR: from the cell periphery to the perinuclear 'cloud'
Ligand-free monomers of EGFR, residing primarily on the cell membrane (1a), can be spontaneously internalized and recycled (Route 1). Even in the absence of stimulation, stochastic dimerization (1b) and auto-activation (1c) of EGFR may occur. The latter is kept in check by endocytosis, inactivation and recycling via the Rab11 recycling endosomes (RE) (Route 1). Ligand binding promotes receptor dimerization (2a-b), leading to activation and phosphorylation of the cytoplasmic tails (2b) that mediate recruitment of various adaptor proteins (such as Grb2) for downstream signal transduction cascades (3). The intracellular fate of EGFR depends on the extent of its activation. Under conditions of 'low' stimulation, AP-2 adaptor is recruited for clathrin-mediated endocytosis (CME) (3a), resulting in EGFR-containing early Rab5-positive signaling endosomes (3a-b). As these endosomes mature, they travel to the perinuclear region, where ligand-activated (and auto-activated) EGFR encounters increasing phosphatase activity and is inactivated prior to being recycled (Route 2). By contrast, 'high' levels of EGFR activation result in extensive receptor phosphorylation and ubiquitination by the E3 ligase Cbl, which causes diversion of EGFR, preferentially internalized via clathrin-independent endocytosis (CIE), away from recycling and towards degradation in the lysosome (Ly), located in the perinuclear 'cloud'. This occurs via the Rab7-positive late endosome (LE) (3b), where ubiquitinated EGFR is targeted from the limiting endosomal membrane into intraluminal vesicles (ILV), giving rise to a multivesicular body (MVB) (Route 3). Subsequent LE/Ly fusion delivers EGFR for degradation.

Receptor endocytosis and the peripheral-perinuclear divide
Endosomes: signaling hubs or traps for destruction?

Although key steps in ligand engagement and nucleation of signaling cascades take place at the cell surface, once activated, EGFR molecules actually spend most of their remaining lifetime in the cell interior, traversing the vesicular network of the endosomal system. Under conditions of low ligand availability, activated EGFR is typically subjected to clathrin-mediated endocytosis (CME) (Sigismund et al., 2005; Robinson, 2015). Although inactive EGFR can move into preformed clathrin-coated pits, phosphorylated receptor accelerates CME by attracting the adaptor AP-2 (Rappoprt and Simon, 2009), which in turn recruits large amounts of clathrin, resulting in receptor clustering and rapid expansion of the budding vesicle (Sorkin et al., 1996; Tomas et al., 2014; Robinson, 2015). Accumulation of receptors in the bud further enhances cross-phosphorylation initiated by ligand binding (Ibach et al., 2015), thereby amplifying low-intensity signals. At the same time, phosphorylation of AP-2 by EGFR helps to initiate internalization, sending EGFR into the endocytic pathway (Fingerhut et al., 2001; Huang et al., 2003; Traub, 2009). The resulting endosomes dwell in the peripheral cytoplasm (**Fig. 1, step 3a**); here, maturation towards the late compartment is 'slow', and numerous recycling pathways are available to spare receptors from degradation (Watanabe and Boucrot, 2017) that takes place in the perinuclear region of the cell, where proteolytic lysosomes abound (Johnson et al., 2016). While, at first, EGFR was considered to predominantly transduce signals at the plasma membrane, recent studies have shown that receptor endocytosis does not interfere with its signaling capabilities (Vieira et al., 1996; Sousa et al., 2012; Conte and Sigismund 2016). Interestingly, it appears

that for certain signaling pathways, such as activation of ERK downstream of EGFR, intracellular localization of signal transduction (i.e. at the plasma membrane versuson endosomes) correlates to the resulting transcriptional response (Sousa et al., 2012; Wu et al., 2012). In this way, spatial compartmentalization of signaling complexes fine-tunes their biological outcomes.

Fast and furious with ubiquitin

When the canonical endocytic route described above is saturated owing to increasing abundance of ligand, 'fast' clathrin-independent endocytosis (CIE) can take over, rapidly routing receptors toward degradation (Sigismund et al. 2005) (**Fig. 1, step 3b**). The decision to rapidly traffic endosomes carrying activated EGFR for degradation appears to be triggered by receptor ubiquitination, as ubiquitination impaired EGFR overwhelmingly travels through the recycling-promoting CME route (Sigismund et al., 2005). Ubiquitination of EGFR is mediated by the E3 ubiquitin ligase Cbl (Huang et al., 2006), brought to the phosphorylated EGFR receptor by the adaptor growth factor receptor-bound protein 2 (Grb2) (Batzer et al., 1994; Levkowitz et al., 1999; Jiang et al., 2003). Once ubiquitinated, EGFR can be recognized by the ubiquitin-dependent adaptors of the endosomal sorting complexes required for transport (ESCRT) and sequestered into the intraluminal vesicles (ILVs) of the multivesicular body (MVB) (Henne et al., 2011). This physically removes the signaling tail of EGFR from the cytosol, effectively terminating the downstream signaling cascade (Eden et al., 2009). Receptor ubiquitination exhibits a sigmoidal response to increasing concentrations of EGF, ensuring that under conditions of low ligand availability activated EGFR will not be marked for destruction (Sigismund et al., 2013). Precisely what sets up this barrier to degradation is not entirely clear. One suggested mechanism postulates that high levels of receptor phosphorylation trigger simultaneous recruitment of Grb2 and Cbl2, resulting in efficient ubiquitination (Sigismund et al., 2013). It is thought that a productive association of Cbl with the receptor is achieved above a certain threshold of phosphorylation, which couples ubiquitination to the intensity of ligand-induced stimulus. In contrast, lower levels of stimulus offer fewer phosphorylated binding sites that are preferentially occupied by signaling molecules, such as Ras and PLCgamma (Chardin et al., 1993; Haugh et al., 1999; Henriksen et al., 2013; Sigismund et al., 2013, Tomas et al., 2014). Thus, by segregating peripheral signaling and recycling pathways from perinuclear degradation in accordance with the degree of stimulation, cells can maximize life-sustaining inputs and effectively cope with overstimulation.

Recycling goes deep

Receptors that are only moderately activated, either owing to low ligand availability or in a ligand-independent manner, are still internalized into endosomes, but their reduced signaling potential does not require degradation. Upon entry into the early endosomal compartment, these receptors are recycled in vesicles characterized by the presence of the GTPase Rab11 (Ullrich et al., 1996; Baumdick et al., 2015). This pathway takes receptors through the perinuclear region, where they become increasingly exposed to the tyrosine-protein phosphatase non-receptor 1 (PTP1B) that resides at the ER. PTP1B dephosphorylates EGFR at ER-endosome contact sites, ensuring that receptors transported back to the plasma membrane are no longer active. This mode of regulation results in an inverse spatial relationship between cellular kinase (peripheral) and phosphatase (perinuclear) activities (**Fig. 1**), which are facilitated by the interactions between endosomes and the ER (as discussed below). In contrast, fully activated EGFR molecules are redirected away from recycling vesicles and traffic toward the late endosomal compartment for degradation (Sabet et al., 2015). Prior to their degradation, these molecules also encounter ER-associated phosphatase PTP1B (Eden et al., 2012), which disables further signaling downstream. Additionally, in response to the intensity of incoming signals, the cell varies the number of signaling vesicles, which helps to maintain a relatively consistent amount of activated EGFR molecules per endosome (Villasenor et al., 2015). This, in turn, keeps the dephosphorylation rate constant and enables the cell to maintain robust responses to the dynamic extracellular environment without becoming vulnerable to overstimulation. The existence of multiple regulated means to abrogate signaling responses (i.e. dephosphorylation and degradation) underscores both the flexibility and rigor of the systems that function to keep cellular signaling cascades in check. Moreover, this complex regulatory framework exemplifies how spatiotemporal regulatory capabilities of the endocytic compartment elegantly serve the greater interests of the cell. How the trafficking and transport of EGFR is orchestrated in molecular terms is discussed in the next section.

Cruising in the endosome: how mature!

Ready, set, phosphoinositides

Reversible association of proteins and complexes with specific vesicular membranes underlies the membrane dynamics throughout the endosomal system. To ensure recruitment and exclusion at the right place and time, vesicles undergo continuous maturation, with their dif

ferent stages characterized by the presence of distinct phosphoinositides (PIs). These derivatives of phosphatidylinositol are anchored to the membrane and acquire different phosphorylation states, which then direct the differential recruitment of proteins associated with early or late stages of endosomal maturation (as expertly reviewed by Schink et al., 2016). Not surprisingly then, progress of EGFR along the endocytic route closely depends on the PI contents of its carrier vesicles (Tan et al., 2015; Schink et al., 2016; Henmi et al., 2016). In fact, activated EGFR can itself influence membrane composition through the recruitment of PI3-kinase II alpha, which increases the concentration of phosphatidylinositol 3-phosphate (PI(3)P). The presence of this lipid, in turn, stimulates the recruitment of the small GTPase Rab5—the central organizer of early endosomes (Zerial and McBride, 2001; Jordens et al., 2005; Zeigerer et al., 2012)—and thus marks the start of endosomal maturation (Leevers et al., 1999; Ceresa and Peterson, 2014). Therefore, by manipulating membrane features, EGFR effectively accelerates its own trafficking and downregulation.

Rab5 is on

Once EGFR, residing on the surface of the cell, turns 'on' and moves into newly budding vesicles, it sets in motion an orderly chain of arrivals and departures of membrane-associated proteins that facilitate and control its progress along the endocytic track. This begins with recruitment of factors responsible for the establishment of early endosomal character, marked by the presence of the Rab5 GTPase. Firstly, the guanine nucleotide exchange factor (GEF) RME-6, which activates the Rab5 GTPase, associates to the budding membrane to promote Rab5 recruitment towards the nascent endosome (Sato et al., 2005) (**Fig. 2, step 1a**). After the EGFR-containing endosome buds off to begin its intracellular journey, it acquires another Rab5 GEF, Rin-1 (Balaji et al., 2012) (**Fig. 2, step 1b**). This likely leads to increased levels of Cbl associated with EGFR and consequently stimulates receptor ubiquitination (Barbieri et al., 2004). Ubiquitinated EGFR, in turn, recruits yet another Rab5 GEF, Rabex-5 (**Fig. 2, step 1c**). Collectively, these steps create a positive feedback loop of GTP-loaded (and thus active) Rab5 membrane occupancy (Penengo et al., 2006; Mattera et al., 2006; Zhang et al., 2014), thereby stabilizing Rab5-associated machineries responsible for early endosome fusion and transport. Specifically, tethering factor EEA1 is recruited to Rab5 (Simonsen et al., 1998; Dumas et al., 2001; Navarolli et al., 2012), which, together with the class C Core Vacuole/Endosome Tethering (CORVET) complex, promotes fusion between early endosomes (Balderhaar et al., 2013; Van der Kant et al., 2015) (**Fig. 2, step 2**). At the same time, Rab5-positive endosomes move away from

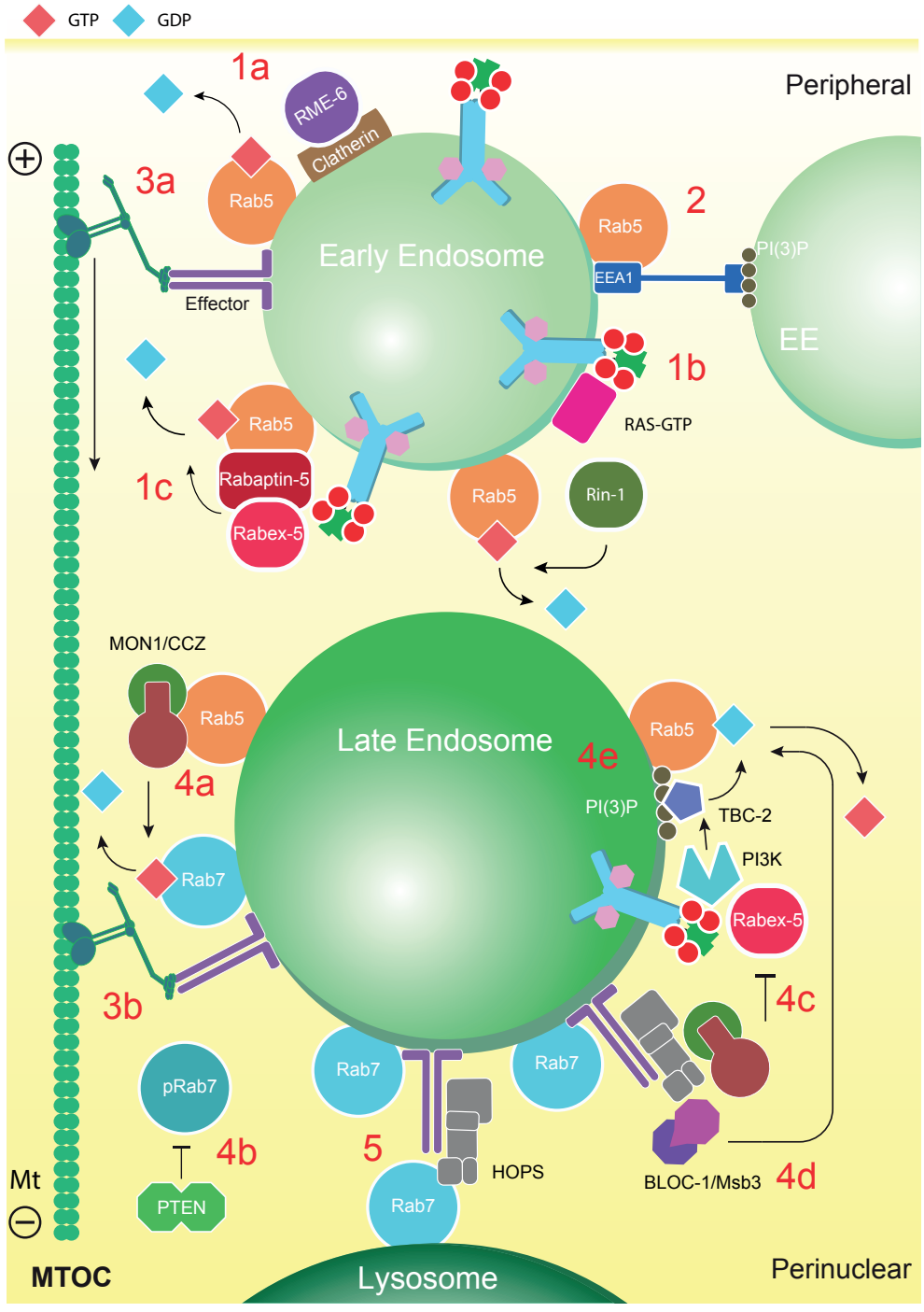
GTP
GDP
Peripheral
1a
RME-6
Clatherin
3a
Rab5
Early Endosome
Rab5
2
EEA1
PI(3)P
EE
Effector
1b
RAS-GTP
Rab5
Rabaptin-5
1c
Rabex-5
Rab5
Rin-1
MON1/CCZ
Rab5
Late Endosome
4e
Rab5
4a
PI(3)P
TBC-2
PI3K
Rab7
Rabex-5
3b
4c
Rab7
Rab7
pRab7
4b
5
HOPS
PTEN
Rab7
BLOC-1/Msb3
4d
Mt
MTOC
Lysosome
Perinuclear

Fig. 2: The order of molecular events in the maturation and transport of EGFR-containing endosomes.
Following endocytosis of activated EGFR, early endosomes (EE) acquire the GTPase Rab5, which is activated by its GEFs RME-6 (1a) and Rin-1 (1b). Meanwhile, ubiquitinated EGFR recruits the Rab5 effector Rabaptin-5 and another Rab5 GEF, Rabex-5 (1c). Once stably associated with the endosomal membrane, Rab5 can recruit effector proteins EEA1 and FHF, which respectively mediate early fusion events (2) and transport (3) along microtubule tracks, carried out by the dynein motor complex toward the nucleus (the minus-end of microtubules). As EEs mature, they acquire the GTPase Rab7 and 'kick' off Rab5 (4). First, Rab5 recruits the Rab7 GEF complex, Mon1/Ccz1 (4a), which activates Rab7, resulting in a hybrid Rab5/Rab7 endosome. Mon1/Ccz1 also displaces Rabex-5 (4b). Recruitment of Rab7 is further modulated by PTEN dephosphorylation activity (4c). To complete the Rab5-to-Rab7 hand-over, Rab5 GAPs Msb3 (via the BLOC-1 complex) (4d) and TBC-2 (4e) associate with Rab7 to promote inactivation and release of Rab5. Through its effector RILP, Rab7 can recruit the dynein motor for minus-end-directed transport (5) and the HOPS tethering complex for fusion (6), thereby coupling LE transport towards and fusion with the lysosome in order to efficiently deliver activated EGFR for degradation.

the plasma membrane towards the perinuclear region, where their fusion with later-stage endosomes is more likely. This transport is accomplished by the minus-end-directed dynein motor complex, adapted to Rab5 through its effector Fused TOES (FTS)-Hook-FTS and HOOK-interacting protein (FHIP) (FHF) (Driskell et al., 2007; Guo et al., 2016) (**Fig. 2, step 3**). Taken together, the processes orchestrated by the Rab5 GTPase enable early endosomes to grow in size, in preparation for their transition into the late compartment, where cargo proteolysis occurs.

Hand it over to Rab7

Late endosomal vesicles are typically marked by the GTPase Rab7 and devoid of Rab5. Occurring through an elegant hand-over mechanism, the conversion from Rab5 to Rab7 constitutes the hallmark of endosomal maturation (Pols et al., 2013; Balderhaar et al., 2013; Lin et al., 2014; van der Kant et al., 2015; McEwan et al., 2015) (**Fig. 2, step 4**). This begins with the arrival of the Mon1-ccz1 complex (**Fig. 2, step 4a**), which interacts with both Rab5 and Rabex-5 (Poteryaev et al., 2010; Nordman et al., 2010; Huotari and Helenius, 2011). Subsequent dephosphorylation of PI3P on the endosomal membrane (Shinde and Maddika, 2016) enables Mon1-ccz1 to attract and activate Rab7 (by loading it with GTP; Fig. 2, step 4C) (Nordman et al., 2010; Yasuda et al., 2016), as well as to displace Rabex-5 (**Fig 2, step 4b**) (Rink et al., 2005), resulting in a hybrid vesicle harboring both Rab5 and Rab7. At this point, the GTPase-activation protein (GAP) Msb3 can be recruitedto expel Rab5 from the endosomal membrane (**Fig. 2, step 4d**) (John Peter et al., 2013). Finally, interaction of the GAP TBC-2 with PI(3)P stimulates the removal of Rab5 from the maturing endosomal membrane (**Fig. 2, step 4e**) (Law et al., 2017). Taken together, this interconnected cascade of molecular events organizes the conversion of a Rab5-positive early endosome into a later one marked by Rab7 (John Peter et al., 2013; Rana et al., 2015). Owing

to the presence of Rab7, the late endosome can now acquire the dynein motor machinery via the Rab7 effector protein Rab Interacting Lysosomal Protein (RILP) and move along microtubules towards the perinuclear region (Cantalupo et al., 2001; Jordens et al., 2001;) (**Fig. 2, step 5**). This Rab7-assocaited transport complex also recruits the homotypic fusion and vacuolar protein-sorting (HOPS) complex, effectively coupling minus-end-directed transport to fusion of late endosomes with one another or with lysosomes carrying Rab7/HOPS (Ungermann et al., 2000; Van der Kant et al., 2015) (**Fig. 2, step 6**). Along their journey, late endosomes receive key inputs and direction from the ER, our current understanding of which is discussed below.

Here comes the ER for a meet-and-greet

Endosomes in the cloud

Once early endosomes begin to mature, they are increasingly guided by interactions with the ER (Friedman et al., 2013). Transient physical contacts between these two organelles coordinate long-range vesicle transport, regulate membrane dynamics within the maturing endosome and influence the receptor signaling status (Eden et al., 2012). In the fast-paced world of endosomal flux, knowing where and when to go is crucial (Neefjes et al., 2017). To achieve this task, cells partition their endosomal compartment into two fractions—a motile peripheral pool of vesicles and a comparatively stationary perinuclear 'cloud' of endosomes that is located around the Golgi complex (Jongsma et al., 2016). This organization is critical for endosomes to efficiently meet each other and mature. The perinuclear endosomal pool is kept in place by the ER-located ubiquitin ligase RNF26 (**Fig. 3, step 1**) (Jongsma et al., 2016), which recruits and ubiquitinates SQSTM1 (also known as p62), a protein best known for its function as an autophagy adaptor. The resulting complex is able to position specific endosomes at the ER by attracting EPS15, which is present on the earliest vesicles, and TOLLIP, located on later endosomes, through their ubiquitin-binding domains. When endosomes need to leave the cloud, the deubiquitinating enzyme USP15 releases them from the 'grip' of the ER, allowing their long-range transport (Jongsma et al., 2016). Inhibition of this positioning mechanism dislocates the entire endosomal system, which results in the failure of endosomes to progressively mature, attenuates cargo degradation and leads to continued EGFR signaling (Jongsma et al., 2016). Consequently, through the activity of RNF26, the ER promotes trafficking of activated EGFR and enables timely termination of its signaling.

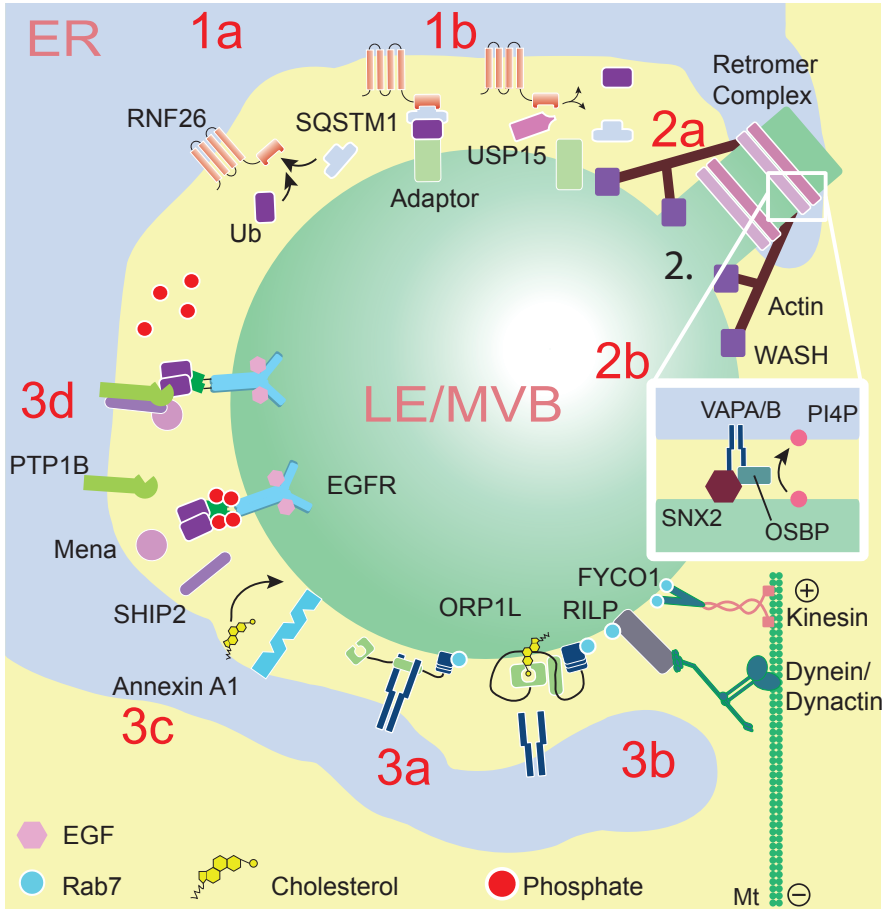

Fig. 3. ER-mediated regulation of the EGFR-containing endosome.
Upon ligand challenge, EGFR-containing endosomes travel from the cell periphery to the perinuclear vesicle 'cloud', where their maturation and degradation of activated receptors occur. (1) The perinuclear cloud is regulated by the ER-located E3 ligase RNF26, which recruits and ubiquitinates SQSTM1 (1a). The resulting ER-associated complex then positions endosomes by attracting various ubiquitin-binding endosomal adaptors. Deubiquitination of SQSTM1 by the DUB USP15 can release positioned endosomes for continued transport (1b). (2) Maturation of endosomes requires them to expel cargoes not intended for degradation. This recycling process is supported by the ER, where ER-bound proteins VAP-A and -B interact with the retromer complex subunit SNX2 (2a). At this ER-endosome contact site, the WASH complex induces local actin polymerization (2b) to promote fission of recycling tubules away from the maturing endosome. (3) The maturing endosome travels toward the lysosome. This transport is mediated by the Rab7/RILP/dynein motor complex and controlled by the cholesterol sensor ORP1L. When cholesterol is abundant in the endosomal membrane, minus-end transport is uninhibited. Conversely, if cholesterol is depleted, ORP1L can interact with VAP-A, resulting in release of the dynein motor (3a). At this juncture, facilitated by the ER-associated protrudin, Rab7 may be able to switch direction of transport by acquiring the effector FYCO1 and the kinesin-1 motor (3b). At the ORP1L/VAP ER-endosome contact site, the Annexin 1A tether mediates cholesterol transfer from the ER to the endosome, which promotes incorporation of EGFR into the ILVs for degradation (3c). Prior to targeting of EGFR to ILVs, activated receptor is dephosphorylated by the phosphatase PTP1B, with the help of the phosphatase SHIP2 and actin nucleating protein Mena (3d).

ER goes in for a hug

EGFR-containing endosomes that travel toward the lysosome must ex pel any cargoes, which are not destined for degradation. This type of recycling intimately involves the ER (**Fig. 3, step 2**). To this end, the retromer complex subunit SNX2 that is bound to PI3P on endosomal membrane, interacts with the vesicle-associated protein A/B (VAPA/B) on the ER membrane; the resulting complex couples recycling tubule formation with the transient WASH-mediated assembly of a localized actin cytoskeleton, which is required for fission (Dong et al., 2016). In effect, this 'embrace' of the recycling tubule by the ER dictates both the exact location and timing of fission (Rowland et al., 2014). As soon as maturing endosomes acquire Rab7, they begin to contact the ER for guidance on directionality of their transport throughout the cell. As mentioned above, Rab7 mediates dynein-dependent transport of late endosomes toward the nucleus through its effector RILP (Jordens et al., 2001), which is needed to bring late endosome cargo, such as the EGFR, to the lysosome (Driskell et al., 2007). However, Rab7 can also 'choose' to recruit the effector FYCO1, and subsequently the kinesin-1 motor, which enables microtubule-based transport of late endosomes in the opposite (plus-end) direction, i.e. toward the periphery of the cell (Pankiv et al., 2010). In order for Rab7 to change course from one direction to the other, it needs to disengage from one motor complex, while recruiting another. Interestingly, both release of the dynein motor and acquisition of kinesin-1 involve help from the ER. To achieve the former, Rab7 interacts with the cholesterol sensor Rab7-associated oxysterol-binding protein, ORP1L (**Fig. 3, step 3**) (Rocha et al., 2009). As long as endosomal cholesterol is available, ORP1L remains in a closed conformation, which is compatible with maintenance of the dynein transport complex on Rab7/RILP. Conversely, under conditions of cholesterol depletion from the endosomal membrane, ORP1L opens up to interact with the ER-bound VAPA/B (Rocha et al., 2009; Van der Kant et al., 2013; Wijdeven et al., 2016). This results in release of dynein from the Rab7-RILP complex and temporarily halts transport of the endosome toward the microtubule minus-end. Incidentally, VAPA/B also interacts with an ER-associated protein protrudin, which is capable of loading kinesin-1 motor onto Rab7/FYCO1 (Raiborg et al., 2015). It has been speculated that this scenario presents an opportunity for Rab7 to switch direction of endosomal transport away from the nucleus (Wijdeven et al., 2015; Raiborg et al., 2016). Although EGFR-containing late endosomes have not been shown to travel via this plus-end-directed route, whether and how Rab7, or its associated proteins, may 'guard' against the misdirection of EGFR is an important issue that re

mains largely unexplored.In addition to modulating endosomal transport, ER-endosome contact sites established by the ORP1L-VAPA/B interaction allow endosomal cholesterol to be replenished directly from the ER by way of the Annexin 1A tether (Eden et al., 2016). Meanwhile, the first steps of signal inactivation also take place at ER-endosome contact sites. It is here that EGFR encounters phosphatase PTP1B, which resides on the ER membrane, and the subsequent dephosphorylation of its cytoplasmic tail renders the receptor inactive (Eden et al., 2012). Interaction between phosphorylated EGFR and PTP1B is likely regulated by two adaptor proteins, Mena and Ship (Hughes et al., 2015). Both receptor dephosphorylation and replenishment of late endosomal cholesterol promote the incorporation of EGFR into the ILVs of a maturing MVB (Raiborg and Stenmark, 2009; Eden et al., 2010; Eden et al., 2012), which physically removes the tail of EGFR from the cytosol, effectively terminating its signaling. This spatially and temporally links receptor inactivation to its degradation. The details of how EGFR finds its way inside the MVB are discussed below.

The final act: inactivation and destruction

In the final throes of EGFR's life, late endosomes, arriving in the perinuclear region of the cell, fuse with the proteolytic lysosome stationed here (Luzio et al., 2007; Johnson et al., 2016). To get into the lysosome, ubiquitinated EGFR molecules are escorted to the site of ILV formation by four sequentially operating ESCRT complexes, ESCRT-0, -I, -II, and -III (Chirst et al., 2017) (**Fig. 4**). In a first selection step, taking place on early endosomes, The ESCRT-0 complex, comprised by the hepatocyte growth factor-regulated tyrosine kinase substrate (Hrs) and signal transduced adaptor molecule (STAM), recognizes and sequesters ubiquitinated EGFR away from recycling domains. Interestingly, STAM and Hrs are both phosphorylated by EGFR following its kinase domain activation and dephosphorylated by PTP1B (Eden et al., 2010; Stuible et al., 2010). Co-regulation of ESCRT-0 with the EGFR activity cycle temporally synchronizes peak sorting activity with sharply increasing demand following ligand-mediated receptor activation. Once ubiquitinated EGFR traffics to the late endosome, ESCRT I, II and III are sequentially recruited to sort and package the chosen cargoes into ILVs. In conjunction with Flotillin-1, ESCRT-I transfers ubiquitinated receptors to ESCRT-II, which results in accumulation of degradation substrates, invagination of the limiting endosomal membrane and ESCRT-III-dependent formation of ILVs (Meister et al., 2017; Christ et al., 2017). EGFR can escape ubiquitin-dependent sorting into ILVs, either early on in the endosomal pathway through deubiquitination by the STAM-associated DUB, USP8 (also referred to as UBPY) (Niendorf et al., 2007; Berlin

et al., 2010) or at the limiting membrane of the MVB (Eden et al, Traffic 2012). In addition to binding ESCRT-0, USP8 also interacts with ESCRT-III components further down the sorting pathway (Row et al., 2007), and a recent report suggests that USP8 can promote the switch between ESCRT complexes on the EGFR substrate through an ESCRT-0 accessory protein HD-PTP (Ali et al., 2013). Ubiquitination does not only control the fate of cargoes, such as EGFR, but also regulates the function of ESCRT proteins themselves. For instance, the oncogene LAPTM4B promotes the ubiquitination of Hrs by the E3 ligase NEDD4, which renders this adaptor unable to recognize ubiquitinated receptors (Hoeller et al., 2006; Persaud et al., 2009; Tan et al., 2015). By contrast, the accumulation of phosphatidylinositol 4,5-bisphosphate (PI(4,5)P2) and the resulting recruitment of SNX5 to the endosomal membrane inhibits Hrs ubiquitination and instead promotes recognition of ubiquitinated cargoes by the ESCRT-0 complex (Tan et al., 2015). Because PI exchange on the endosomal membrane coincides with maturation, this the above regulatory module couples sorting of EGFRs that are marked for destruction to the physical progression of receptor-containing vesicles along the endocytic route. Finally, to complete its life cycle, EGFR must be delivered to the lysosome. To accomplish this, the MVB must fuse with the lysosome, depositing its ILVs into the proteolytic lumen of the latter organelle (Luzio et al, 2007). Here, the luminal part of EGFR (i.e., its ligand-binding domain) is degraded after an unfolding step, which likely requires first the reduction of the cysteine bridges by the protein GILT (Arunachalam et al., 2000), followed by the action of multiple glycosidases and proteases of the cathepsin family. Further, the transmembrane domain of EGFR is cleaved by the transmembrane aspartate proteases of the Rhomboid family (Lemberg and Freeman, 2007). However, the fate of the remaining cytoplasmic tail remains unclear. While it has been postulated that the tail may be expelled in the cytosol experimental demonstration thereof is yet to be reported. Although the pathway towards degradation of EGFR is at this time fairly clear, the mechanisms of its actual destruction are much less understood.

Conclusions and perspectives

At the time of writing, a Pubmed search for the term 'EGFR' returned over 44,000 publications, of which the vast majority considers primarily the immediate steps in the life of an activated receptor—those occurring at the cell surface. Yet, an EGFR molecule that has been turned 'on' likely spends more time traveling the endosomal system than residing at the cell surface. Meanwhile, its cytoplasmic tail remains exposed and available for signaling. Interestingly, the quality of signaling may be dif

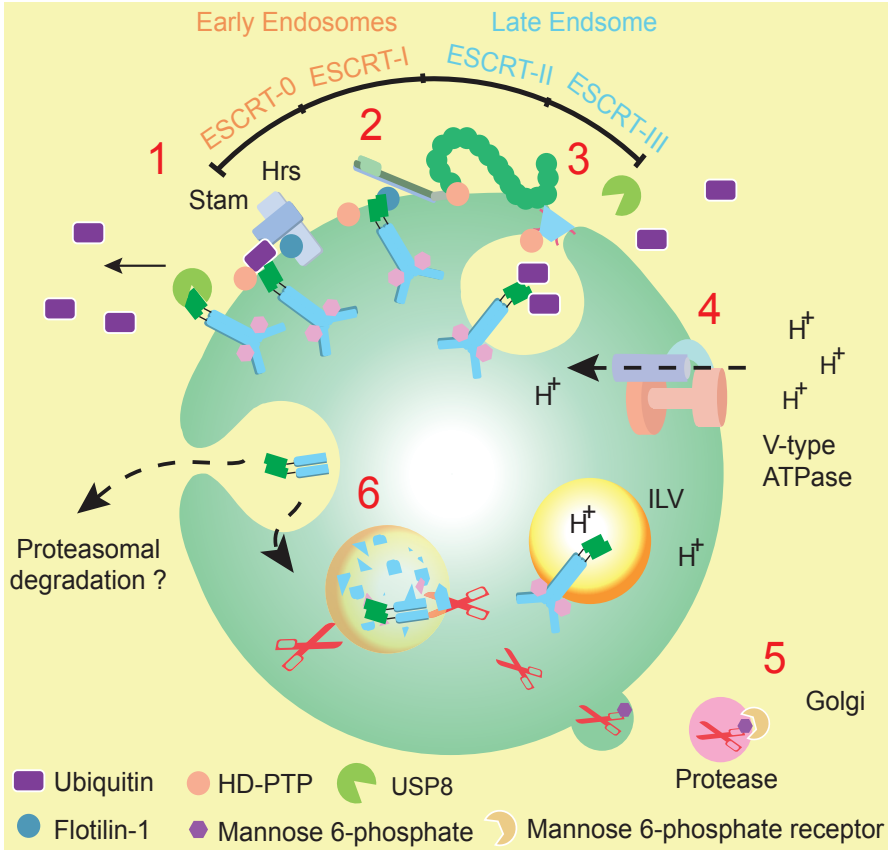

Fig. 4. In or out: sorting and degradation of EGFR.
Degradation of activated EGFR necessitates its sorting and incorporation into the ILVs of a maturing MVB, which are orchestrated by the ESCRT system. This begins on early endosomes, where the ESCRT-0 complex, consisting of the ubiquitin-binding adaptor proteins Hrs and STAM, sorts ubiquitinated EGFR to the MVB (1). Assisted by Flotilin-1, EGFR is subsequently transferred to the ESCRTs -I, -II, and -III (2). ESCRT-III deforms the limiting membrane of the MVB, resulting in ILV formation and sequestration of EGFR therein. Deubiquitination of EGFR by the ESCRT-0-associated DUB USP8 can spare the receptor from degradation. USP8 can also interact with ESCRT-III and, in the presence of the phosphatase HD-PTP, may promote transfer of EGFR down the ESCRT pathway. (3) Proteolytic capabilities of late endosomes and lysosomes require an acidic environment, provided by V-type ATPases (4), which is optimal for denaturation and degradation by the lysosomal proteases, which are transported by the Mannose 6-Phosphate Receptor from the golgi (5). While the transmembrane section of EGFR is thought to be degraded by the rhomboid proteases, how—and whether—the cytoplasmic tail of EGFR is degraded remains unclear (6).

ferent in the cell interior than at the plasma membrane. However, due to the transient nature of endosomes and their ability to move swiftly through the cell, it has been challenging to understand what happens to EGFR on this complex journey, and when. Recent advances in imaging tools and techniques have enabled us to make substantial progress in addressing these questions and have revealed the intricate molecular mechanisms at play, as well as the subtle ways in

which EGFR influences them to promote its own demise. As it moves in endosomes towards the perinuclear cloud, en route to its final destination in the lysosome, active EGFR is subjected to regulation by the ER at the ER-en-dosome contact sites. As EGFR, marked for destruction with ubiquitin, reaches the multivesicular bodies, termination of its signaling is ensured by dephosphorylation and subsequent inclusion into the ILVs. But what if the receptor thus committed could escape the ILVs back to the limiting membrane of the MVB? Could its signaling from endosomes resume? Or would the receptor still be recycled and reused at the cell surface? Perhaps these provocative questions will find answers in the next phase of the discovery regarding endocytosis and the management of key cellular cargoes, with EGFR at their forefront.

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