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Role of Rabex-5 in the sorting of ubiquitinated cargo at an early stage in the endocytic pathway

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The covalent modification of transmembrane receptors by ubiquitin (Ub) is a key biological mechanism controlling their internalization and endocytic sorting to recycling and degradative pathways to attenuate their signaling potential. In this Ub-dependent endocytic trafficking pathway, Ub-binding proteins (UBPs) play a critical role in the sorting of these ubiquitinated transmembrane proteins at the plasma membrane, early endosomes, and multivesicular bodies. We recently reported that Rabex-5, a UBP and guanine nucleotide exchange factor for Rab5, is translocated to the plasma membrane in an extracellular ligand-dependent manner to regulate the internalization of ligand-induced ubiquitinated transmembrane proteins upon stimulation with extracellular ligands. Here, we show that Rabex-5 predominantly localizes on Rab5- and syntaxin 13-positive endosomes, but not on Rab11-positive recycling endosomes before stimulation with extracellular ligands. We further discuss the significance of Rabex-5-mediated sorting of ubiquitinated transmembrane proteins as cargo at an early stage of the endocytic pathway.

Following binding to their cognate ligands, a variety of cell-surface receptors undergo ubiquitination on the plasma membrane to promote their endocytosis for lysosomal degradation, thereby ensuring the termination of receptor signaling.[1] The ubiquitin (Ub) moieties of the ubiquitinated membrane proteins are recognized by compartment-specific Ub-binding protein (UBP)-containing Ub-binding domains (UBDs) inherent to key adaptor proteins at the plasma membrane, early endosomes, and *trans*-Golgi network (TGN) (**Fig. 1A**). At the plasma membrane, epidermal growth factor (EGF) receptor pathway substrate clone 15 (EPS15), EPS15-interacting protein 1 (epsin-1), and their yeast homologs mediate the internalization of many ubiquitinated transmembrane proteins via Ub-interacting motifs.[2] At the endosomes, the endosomal sorting complex required for transport machinery plays an important role in the sorting of ubiquitinated transmembrane proteins into the interior of multivesicular bodies (MVBs) and in MVB biogenesis.[3] At the TGN in yeast, Golgi-localized gamma-ear-containing ARF-binding (GGA) proteins regulate the Ub-dependent sorting from the TGN to endosomes via GGA and Tom1 (target of Myb 1) (GAT) domain-mediated direct interactions with Ub.[4]

To target these UBPs to subcellular compartments properly, the different domain structures outside of their UBDs could be involved. For example, Rabex-5 requires the early endosomal targeting (EET) domain to localize to early endosomes.[5] In addition to this EET domain, the tandem UBDs such as zinc finger (ZnF) and motif interacting with Ub (MIU)[6,7] at the N-terminus of Rabex-5 are required for recruiting Rabex-5 to the membrane fractions, including the endosomal membrane and/or plasma membrane.[8,9] Intriguingly, Rabex-5 mutants with impaired Ub-binding activity in these UBDs also reduced Rab5-binding activity.[10] Although a comprehensive proteomic screen for proteins interacting with Rabex-5 via its UBDs has not been reported yet, the ubiquitinated EGF receptor[7] and the neural cell adhesion molecule L1[9] were shown to interact with Rabex-5 after their ligand-induced ubiquitination. Importantly, Rabex-5 is translocated to the plasma membrane upon stimulation with these extracellular ligands.[7,9] This ligand-dependent translocation of Rabex-5 might be required for internalization of the ubiquitinated cargo from the plasma membrane.

This hypothesis was also supported by measuring the internalization of L1 in N2a cells expressing small interfering RNA (siRNA) for Rabex-5. In control cells, a fluorescence-conjugated L1 antibody was rapidly internalized and accumulated around perinuclear endosomal structures, with increasing incubation time (**Fig. 1B**, left panels). In contrast, a fluorescence-conjugated L1 antibody was observed only on the plasma membrane in cells expressing Rabex-5 siRNA, even after 30 min incubation (**Fig. 1B**, right panels), indicating that the internalization of L1 was dramatically inhibited by largely eliminating Rabex-5 expression, which is consistent with our previous report.[9]

Rabex-5-mediated sorting of ubiquitinated cargo on the plasma membrane might be surprising since Rabex-5 localizes predominantly on syntaxin 13, which is involved in homotypic early endosomal fusion as Q-soluble N-ethylmalemide-sensitive fusion

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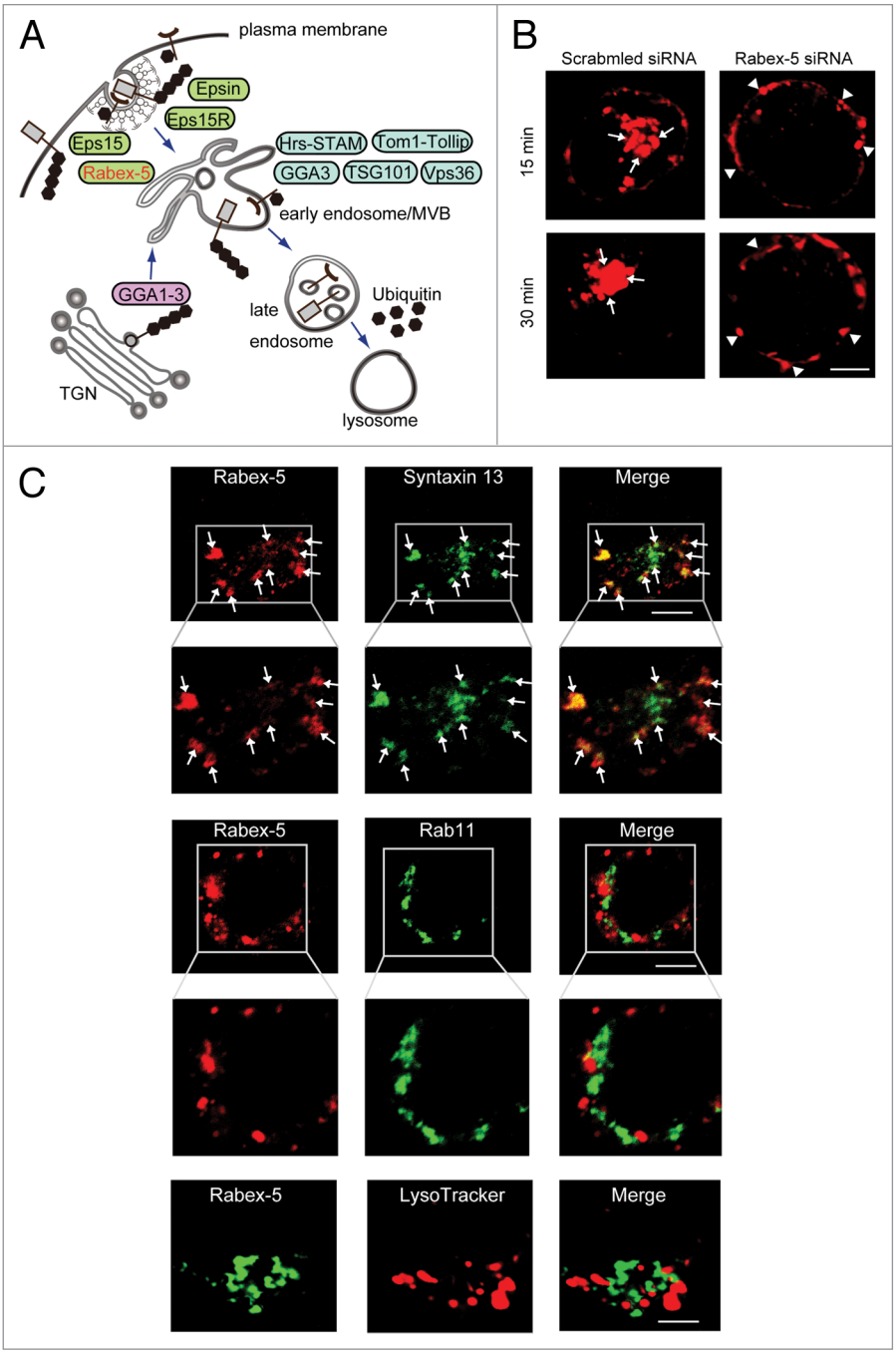

Figure 1. (**A**) Ub functions as a sorting signal in intracellular trafficking pathways. The diagram shows 3 sites at which Ub is known to act as a sorting signal for membrane proteins, i.e., the TGN, plasma membrane, and endosomes. Some known components of the Ub-interacting sorting machinery are indicated. (**B**) Impact of Rabex-5 siRNA-mediated knockdown on L1 endocytosis in N2a cells. Cells expressing Rabex-5 siRNA (right panels) or control scrambled siRNA (left panels) were incubated with an Alexa 568-conjugated L1 antibody (Ab), followed by visualization using confocal microscopy. The arrows and arrowheads indicate the internalized Alexa 568-conjugated L1-Ab on the endosomes and the accumulated Alexa 568-conjugated L1-Ab on the plasma membrane, respectively. The scale bar represents 10 μm. (**C**) (upper and middle panels) Colocalization of Rabex-5 (red) with GFP-syntaxin 13- and GFP-Rab11-positive endosomal compartments (green) in the presence of L1-Ab in N2a cells. (Bottom panels) Colocalization of Rabex-5-GFP (green) with LysoTracker red DND-99 (red). The arrows indicate colocalization (yellow). The scale bar represents 5 μm.

attachment protein receptor (SNARE)[11]-positive early endosomes but not on Rab11-positive recycling endosomes or LysoTracker-positive lysosomes before incubating the cells with extracellular ligands (**Fig. 1C**). Rabex-5 is required for the functions of early endosomes through the activation of Rab5.[12] The formation of a complex with Rabaptin-5 promotes the guanine nucleotide exchange factor (GEF) activity of Rabex-5 via a coiled-coil domain at the C-terminal region of Rabex-5, resulting in the establishment of a feedback loop by which Rab5-GTP promotes further Rab5 binding[13] and recruitment of Rab5 effectors such as phosphatidylinositol (PtdIns)-3-OH kinase.[14] As a result, Rab5 and PtdIns-(3)-P serve the platform for effectors involved in early endosomal membrane docking and fusion through interactions with SNAREs.[15]

In contrast to the functional role of Rab5 on early endosomes, the localization of Rab5 on the plasma membrane has been detected,[16] and the expression of Rab5A resulted in the enhancement of EGF–EGF receptor internalization, whereas a dominant-negative mutant of Rab5, Rab5^{S34N}, inhibited this internalization.[17] Although Rab5 isoforms are functionally redundant at the internalization step of EGF and/or transferrin (Tf) receptors trafficking,[18] these results suggest that the extracellular ligand-dependent activation of Rab5 by Rab5GEFs on the plasma membrane is required for operating the cargo-specific sorting at the early step in these endocytic trafficking pathways. Intriguingly, total internal reflection fluorescence microscopy (TIRF-M) revealed pre-endocytic sorting of EGF and Tf on the plasma membrane.[19] Although it remains unclear whether Rab5GEFs are involved in such pre-endocytic sorting, Rabex-5 might simultaneously make it possible to regulate pre-endocytic sorting of the ubiquitinated cargo and drive the internalization from the plasma membrane.

In light of these considerations, the molecular machinery underlying the translocation of Rabex-5 to the plasma membrane needs to be determined. It will also be crucial to identify key proteins that recruit Rabex-5 to the plasma membrane via its ZnF and MIU domain.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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