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REVIEW

SNARE-associated proteins and receptor trafficking

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A wide variety of receptors that function on the cell surface are regulated, at least in part, through intracellular membrane trafficking including endocytosis, recycling and subsequent degradation. Soluble N-ethylmaleimide sensitive factor (NSF) attachment protein (SNAP) receptors (SNAREs) are essential molecules for the final step of intracellular membrane trafficking, i.e. fusion of transport vesicles with the target membrane. SNAREs on two opposing membranes form a *trans*-SNARE complex consisting of a four-helical bundle and drive a membrane fusion. The resultant *cis*-SNARE complex is disassembled through a process mediated by NSF and SNAPs. Cells contain families of SNAREs, and the interaction of cognate SNAREs at least contributes to the specificity of membrane fusion. The SNARE complex formation and dissociation are modulated by many SNARE-associated proteins at multiple steps including tethering, assembly and disassembly. Diverse molecular mechanisms, such as scaffolding, phosphorylation and ubiquitylation of SNARE proteins, and phosphoinositide production, are utilized for the modulation. In this review, we summarize recent progress in understanding the role of SNARE-associated proteins required for the endocytic recycling and degradation of epidermal growth factor receptor, transferrin receptor and integrins. We also discuss the physiological and pathological relevance of SNAREs and SNARE-associated proteins in the receptor trafficking.

Keywords: syntaxin, NSF, α-SNAP, γ-SNAP, NAPG, EGFR, Tfn, TfR, autophagy

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SNARE proteins: key drivers of membrane fusion --- Intracellular membrane trafficking plays critical roles in a wide variety of cellular processes including cell surface presentation, endocytosis, and recycling and degradation of various types of receptors. Membrane trafficking involves the formation of a vesicle from the donor membrane, transport along the microtubule and actin cytoskeletons, tethering and fusion with the target membrane. The final step, membrane fusion, is mediated by soluble N-ethylmaleimide sensitive factor attachment protein receptors (SNAREs). At least 39 genes encoding SNARE proteins exist in the human genome. All SNARE proteins have one or two SNARE motifs, which are evolutionally conserved ~70 amino acid stretches forming α-helix structures [1, 2]. In addition to the SNARE motifs, most, but not all, SNAREs contain an amino (N)-terminal domain and a carboxy (C)-terminal transmembrane domain (TMD).

SNARE proteins are classified into four groups, Qa, Qb, Qc and R, based on the sequence similarity of the SNARE motifs [1, 2]. In most cases, Qa, Qb, and Qc-SNAREs reside in target membranes and thus are also called target-SNAREs

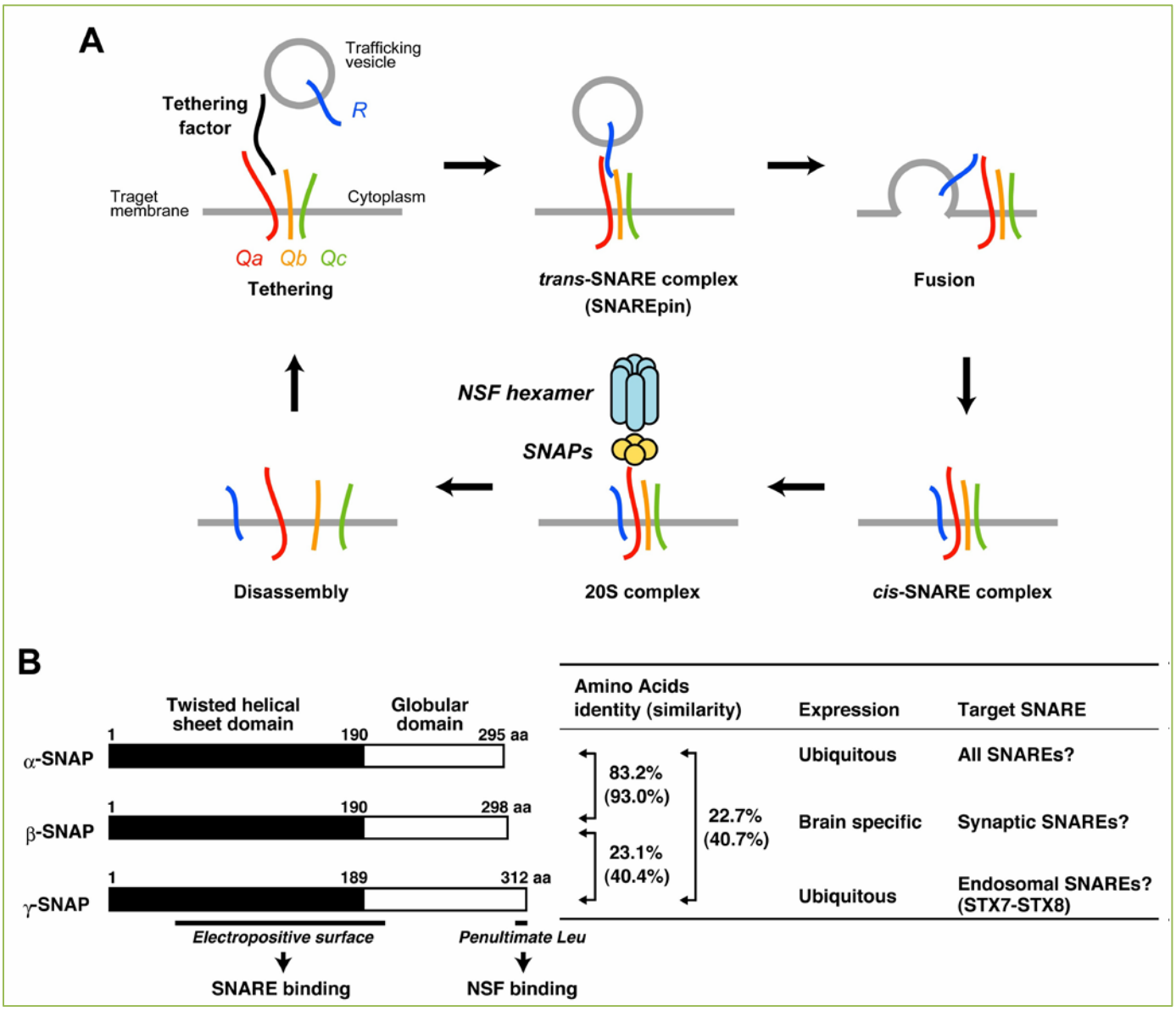

Figure 1. SNARE complex turnover due to SNAP and NSF in membrane fusion. A. SNARE complex assembly and disassembly by SNAPs and NSF. B. Schematic representation and comparison of α-, β- and γ-SNAP

(t-SNAREs). In contrast, most R-SNAREs reside in transport vesicles and thus are also called vesicle-SNAREs (v-SNARE). Three Q-SNAREs (Qa, Qb and Qc) on the target membrane and one R-SNARE on transport vesicles form a specific four-helical bundle complex between opposing membranes *in trans*, which is called the *trans*-SNARE complex or SNAREpin. The energy released during SNARE complex assembly is likely to drive membrane fusion (Fig. 1A).

All Qa-SNAREs are named syntaxin (STX; STX1A, STX1B, STX2, STX3, STX4, STX5, STX7, STX11, STX12/13, STX16, STX17, STX18 and STX19/9) and also called the heavy chain, except for STX6, STX8 and STX10, which are Qc-SNAREs, because Qa-SNAREs have higher molecular weights (approximately 35 to 40 kDa) than Qb, Qc and R-SNAREs (approximately 10 to 30 kDa). By contrast, Qb- and Qc-SNAREs are called the light chain and do not have systematic numerical names (Qb: GOSR2/GS27, GOSR1/GS28, Vti1a, Vti1b and BNIP1/Sec20; Qc: Bet1, Bet1L/GS15 and Use1/p31/Slt1 in addition to STX6, STX8 and STX10). In addition to these SNAREs with a single Qb or Qc SNARE motif, SNAP subfamily SNAREs (SNAP23, SNAP25, SNAP29 and SNAP47) contain both Qb and Qc SNARE motifs in a single molecule. Most R-SNAREs are called VAMPs (VAMP1, VAMP2, VAMP3, VAMP4, VAMP5, VAMP7 and VAMP8), the rest having unique names (Ykt6 and Sec22b). Sec22a and Sec22c are Sec22b

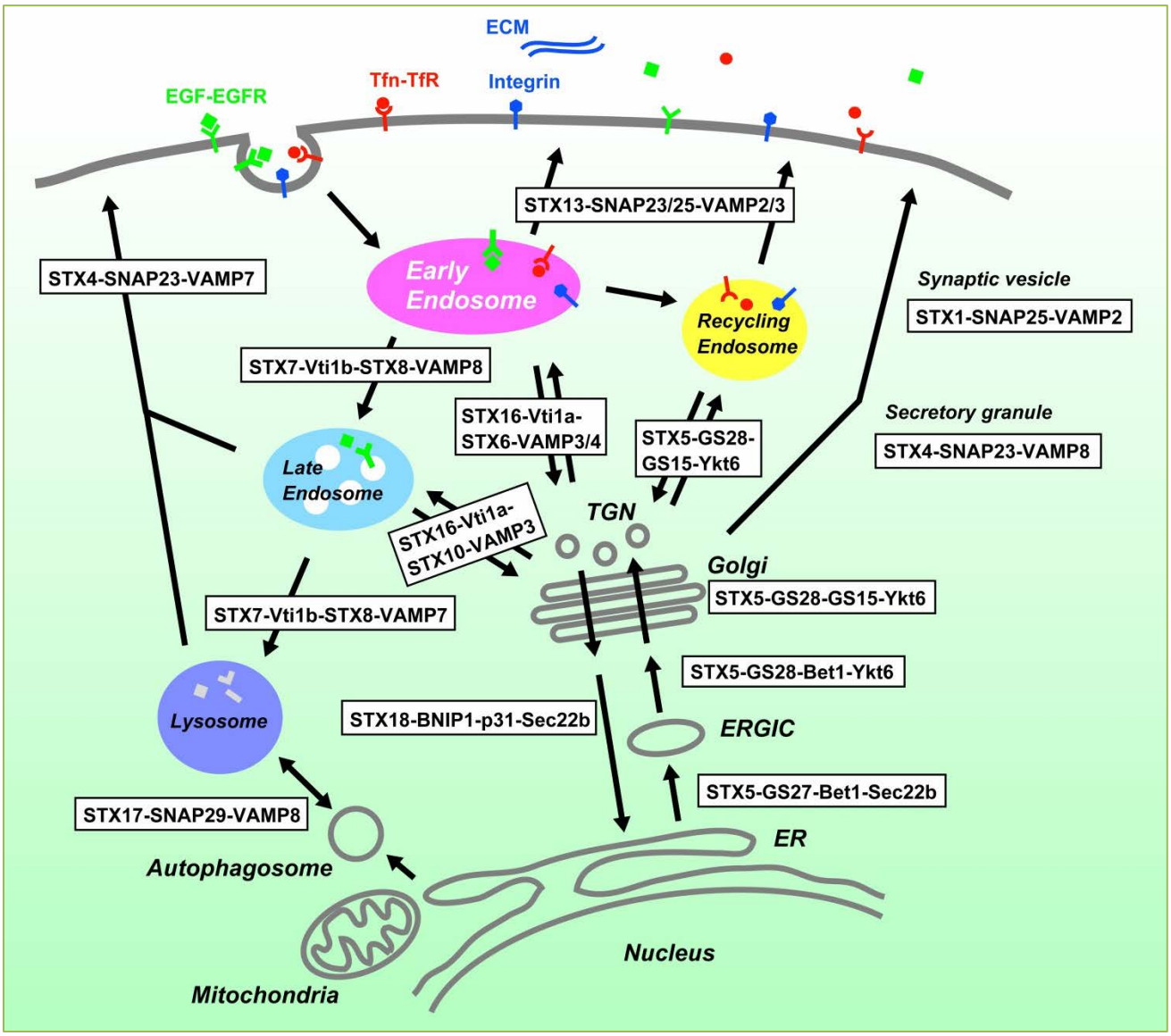

Figure 2. SNAREs in membrane trafficking. Major SNARE complexes are mapped on biosynthetic and endocytic recycling pathways in which the SNARE complexes function. In each complex, SNAREs are in the order of Qa-Qb-Qc (or Qbc)-R from left to right.

homologues but seem to lack a SNARE motif.

The C-terminal TMD anchors SNAREs to the membrane. Some SNAREs (STX11, STX19, SNAP23, SNAP25, SNAP29, SNAP47 and Ykt6) lack the C-terminal TMD, and instead are palmitoylated (and Ykt6 is also prenylated) and anchored to proper organelle membranes through the lipid-modifications [3]. Two types of N-terminal domains, Habc and longin domains, have been relatively well characterized. The Habc domain is found in all Qa- and Qb-SNAREs except BNIP1. The domain forms a three-helical bundle and interacts intramolecularly with its own SNARE motif and/or intermolecularly with SNARE-associated proteins such as Sec1/Munc18 (SM) proteins [4-7]. The interactions both positively and negatively regulate the assembly of the SNARE complex and the consequent membrane fusion. A longin domain exists in some R-SNAREs (VAMP7, Ykt6, Sec22a, Sec22b and Sec22c), and has a profilin-like fold consisting of three α-helixes and a five-stranded β-sheet. Similar to Habc domains, some longin domains interact with their own SNARE motifs and associated proteins [8, 9].

Each SNARE forms a complex with specific, "cognate" SNAREs, and mediates vesicle fusion through specific routes in biosynthetic and endocytic recycling pathways (Fig. 2). Most SNAREs operate not only in one SNARE complex, but also in multiple complexes comprising different SNAREs, e.g. STX4, STX5, STX7, STX8, STX16, SNAP23, SNAP25, Vti1a, Vti1b, GS15, Bet1, VAMP2, VAMP3, VAMP7,

VAMP8, Ykt6 and Sec22b (Fig. 2). As mentioned above, although most v-SNAREs are classified as R-SNAREs, *in vitro* assays involving purified yeast SNAREs have revealed that Qc-SNARE Bet1 functions as a v-SNARE in the complexes of STX5-GS28-Bet1-Sec22b and STX5-GS27-Bet1-Ykt6 in the anterograde trafficking from the ER to the ER-Golgi intermediate compartment (ERGIC), and from the ERGIC to the Golgi, respectively [10]. Similarly, GS15 (also known as Bet1L) appears to be a v-SNARE in the STX5-GS28-GS15-Ykt6 complex in the endosome-Golgi retrograde trafficking pathway [11]. The two Bet1-type Qc-SNAREs might share a common feature in their primary sequences as R-SNAREs.

The endocytic recycling pathways are mainly governed by STX13- and STX16-containing SNARE complexes (Fig. 2). The SNARE complex comprising STX13, SNAP23/25 and VAMP2/3 may participate in the trafficking of cargos from recycling endosomes to the plasma membrane [12,13]. The STX13 complex also contributes to homotypic fusion of early endosomes [14]. The STX16-Vti1a-STX6-VAMP3/4 SNARE complex functions between early endosomes and the *trans*-Golgi network (TGN), whereas the STX16-Vti1a-STX10-VAMP3 complex operates between late endosomes and the TGN [15], suggesting that Qc-SNAREs STX6 and STX10 distinguish the transport routes and determine the specificity of their complexes.

Two distinct STX7-containing complexes are involved in the lysosomal degradation pathway. Both complexes include STX7, Vti1b and STX8, but differ in R-SNARE, VAMP7 and VAMP8; the former acts in late endosomes to lysosomes, whereas the latter acts in early to late endosomes, and homotypic fusion of late endosomes [16]. The complex comprising STX4-SNAP23-VAMP7 functions in the fusion of late endosomes and lysosomes to the plasma membrane, and the secretion of exosomes, which play important roles particularly in the immune system and tumor cells [17, 18]. In autophagy, the STX17-SNAP29-VAMP8 complex catalyzes the fusion of lysosomes and autophagosomes [19]. STX17 also mediates autophagosome formation and mitochondrial fission through interactions with ATG14L and mitochondrial fission factor Drp1 at ER-mitochondria contact sites [20, 21].

NSF and SNAPs as SNARE complex disassembly factors ---- After membrane fusion, the four-bundled SNARE complex on a membrane, called the *cis*-SNARE complex, needs to be disassembled and recycled for the next fusion event (Fig. 1A). The disassembly of the *cis*-SNARE complex is mediated by *N*-ethylmaleimide sensitive factor (NSF) and α-soluble NSF attachment protein (α-SNAP). NSF, a member of the AAA-ATPase superfamily, is a chaperone-like ATPase with a homohexameric structure. Three or four α-SNAPs bind to the *cis*-SNARE complex, recruiting NSF and stimulating its ATPase activity, which leads to disassembly of the *cis*-SNARE complex [22-26]. The supercomplex of the SNARE bundle, α-SNAPs and the NSF hexamer is called the "20S complex". α-SNAPs have been considered to mediate the NSF binding to all SNAREs, although there is a slight preference for Qa-SNAREs. α-SNAPs consist of two domains, an N-terminal twisted helical sheet domain and a C-terminal globular domain, and the former domain interacts with the electronegative convex surface of the four-helical bundle of the SNARE complex through its electropositive concave surface [27, 28]. On the other hand, the extreme C-terminal region of α-SNAP plays an important role in the functional interaction with the NSF hexamer because an α-SNAP mutant with the substitution of its penultimate leucine to alanine fails to stimulate the ATPase activity of NSF [29]. Recent cryo-electron microscopy (cryo-EM) studies of the 20S complex revealed that the cluster of acidic residues adjacent to the penultimate leucine interacts with the basic residue cluster in the N-terminal domain of NSF [25, 26].

In mammals, there are three SNAP isoforms: α-SNAP, β-SNAP and γ-SNAP (Fig. 1B). In contrast to ubiquitous expression of α-SNAP, β-SNAP is specifically expressed in the brain and endocrine cells [30]. They exhibit more than 80% amino acid sequence identity, and thus β-SNAP appears to bind to and catalyze disassembly of the SNARE complex, and they may act together in regulated exocytosis in neuronal cells [31, 32]. γ-SNAP is ubiquitously expressed as α-SNAP, and is only ~25% identical to α-SNAP and β-SNAP. In contrast to the involvement of α-SNAP and β-SNAP in membrane trafficking events, the biochemical aspect and cellular function of γ-SNAP have not been fully characterized. Recently, we reported that γ-SNAP binds to endosomal SNAREs such as STX6, STX7, STX8, Vti1a and Vti1b, and, like α-SNAP, facilitates the disassembly of SNARE complexes comprising STX7 and STX8 in an NSF-dependent manner [33]. In γ-SNAP, some of the positively charged residues in the N-terminal concave sheet domain of α-SNAP are replaced by neutral or negatively charged residues, possibly explaining why the two SNAPs exhibit different preferences for SNAREs. γ-SNAP directly and indirectly interacts with NSF and α-SNAP, respectively, and the interaction between γ-SNAP and NSF exhibits higher affinity than that of α-SNAP and NSF [31, 33]. Although the C-terminal penultimate leucine and the adjacent acidic residue cluster are conserved in γ-SNAP, the acidic cluster of γ-SNAP includes more residues than that of α-SNAP (six aspartates/glutamates in γ-SNAP vs. four in α-SNAP). Furthermore, the C-terminal tail of γ-SNAP is extended by an

Table 1. SNARE-associated proteins that are involved in endocytosis and recycling of EGFR, TfR and integrins

SNARE-associated Protein	Interacting protein	Cargo	Method	Cell	Reference
NSF	PTP1B	EGFR, Met (HGF receptor)	RNAi, Mutant overexpression	HeLa	Sangwan *et al*., 2011
NSF	VAMP3	α5β1 Integrin	Mutant overexpression	CHO-K1	Skalski and Coppolino, 2005
α-SNAP		Integrin	RNAi	SK-CO15	Naydenov *et al*., 2014
γ-SNAP	STX6, STX7, STX8	EGFR, TfR	RNAi	HepG2	Inoue *et al*., 2015
Bves	VAMP3	TfR, β1 Integrin	Morpholino	MDCK, Xenopus embryo	Hager *et al*., 2010
CytLEK1	SNAP25, STX4, VAMP2	TfR	Morpholino	NIH3T3	Pooley *et al*., 2006
EHD1	SNAP29, syndapin II	TfR	Overexpression	HeLa	Xu *et al*., 2004
MARCH-II	STX6	TfR	Overexpression	COS-7	Nakamura *et al*., 2005
Mig-6	STX8	EGFR	RNAi, Overexpression	U87, LN319	Ying *et al*., 2010
NEEP21	STX13	TfR, GluR1	RNAi, Overexpression	PC12, Primary neuron	Steiner *et al*., 2002
PI4K2A	VAMP3	TfR	RNAi	COS-7	Jovic *et al*., 2014
RNF167	VAMP3	TfR	Overexpression	HEK293	Yamazaki *et al*., 2013
SHIP164	STX6, GARP complex	TfR, M6PR	Overexpression with STX6	HeLa	Otto *et al*., 2010
Snapin	ATG14L	EGFR	Knockout, RNAi	MEF, HeLa	Kim *et al*., 2012
Syndetin	STX6, EARP complex	TfR	RNAi	HeLa	Schindler *et al*., 2015
Vps45	STX16, Rabenosyn-5	β1 Integrin	Neutropenia patients	Neutrophil	Vilboux *et al*., 2013
Vps45	STX16, Rabenosyn-5	β1 Integrin	RNAi	HeLa	Rahajeng *et al*., 2010

insertion of ~20 residues that precedes the acidic cluster, and the insertion has a disordered structure that makes the C-terminal tail flexible [34]. These structural features of γ-SNAP might confer the ability to bind to NSF with a higher affinity.

RNA interference (RNAi)-mediated depletion of γ-SNAP affects the size and intracellular localization of endosomes in human hepatoma cell line HepG2 [33]. Early and late endosomes, and lysosomes are partially swollen, and recycling endosomes are redistributed to the cell periphery upon γ-SNAP depletion. Consistent with these morphological changes, degradation of epidermal growth factor (EGF) receptor (EGFR), and the endocytosis and recycling of transferrin (Tfn) receptor (TfR) are partially delayed in γ-SNAP-depleted cells, and the endocytosed receptors are retained in the swollen early endosomes. Although the molecular mechanisms underlying these phenotypes have yet to be elucidated in detail, γ-SNAP may contribute to the receptor trafficking through the disassembly of SNARE complexes in endosomal membranes. Additionally, because γ-SNAP interacts with Rab11 effector FIP5/Rip11/Gaf-1 [35-37], the depletion of γ-SNAP might affect the endocytic recycling compartments through a Rab11-dependent pathway. Interestingly, γ-SNAP-depleted HepG2 cells exhibit enhanced cell spreading and reduced intercellular adhesion [33], suggesting that γ-SNAP is also involved in intracellular trafficking, e.g. cell surface targeting and proper recycling, of extracellular matrix receptors such as integrins and intercellular adhesion molecules.

Receptor endocytosis and recycling mediated by SNAREs and SNARE-associated proteins ---- In addition to γ-SNAP, recent studies have revealed that many SNARE-associated proteins regulate receptor trafficking through several different mechanisms. In this review, we particularly focus on the recent progress in understanding the roles of SNARE-associated proteins in the endocytic recycling and degradation of EGFR, TfR and integrins (Table 1).

EGFR: EGFR is one of the most characterized receptor tyrosine kinases (RTKs), and is involved in a wide variety of cellular events including cell growth and survival, migration and epithelial morphogenesis. EGFR and its signaling pathways have been attracting keen attention in the field of cancer research. Amplification and overexpression of EGFR

are clinically observed in multiple types of cancers and known to correlate with a poor prognosis [38]. Because overstimulation or abnormal elongation of EGFR signaling leads to tumorigenesis, downregulation of the signaling is critical for cellular homeostasis. One of the major mechanisms underlying the downregulation is endocytosis and subsequent degradation of the receptor-ligand complex; after EGF stimulation the majority of the receptor and the ligand itself are directed to lysosomes via early and late endosomes, and degraded rapidly (within 30 min to several hours typically).

In addition to γ-SNAP, NSF and endosomal SNAREs, e.g. STX7, STX8, Vti1b, VAMP7, VAMP8, and also STX6 and STX19, have been reported to be involved in EGFR trafficking and degradation [39-43]. EGF stimulation triggers the intrinsic tyrosine kinase activity of EGFR, which is followed by the activation of many downstream serine/threonine kinases. In addition to many targets of the kinases, NSF is also phosphorylated after EGF or hepatocyte growth factor (HGF) stimulation in HeLa cells and then dephosphorylated by protein tyrosine phosphatase PTP1B [44]. Dephosphorylation of NSF by PTP1B is required to promote SNARE complex disassembly on RTK-containing trafficking vesicles and endosomes as well as on the acrosome-plasma membrane in sperm [44-46]. Moreover, PTP1B interacts with and dephosphorylates EGFR at the contact site between late endosomes/multivesicular bodies (MVBs) and the ER [47]. PTP1B activity promotes the incorporation of EGFR into intraluminal vesicles in MVBs, although the incorporation has not been so far directly linked to the dephosphorylation of NSF by PTP1B.

Mitogen-inducible gene-6 (Mig-6)/receptor-associated late transducer (RALT) negatively regulates EGFR signaling at multiple steps. Mig-6 was initially identified as a factor that interacts with EGF-stimulated dimerized EGFR and inhibits its tyrosine kinase activity [48, 49]. A recent study revealed that Mig-6 forms a ternary complex with STX8 and EGFR upon EGF stimulation to recruit EGFR to STX8-positive late endosomes, and then facilitates subsequent degradation of the receptor in lysosomes [50]. In addition to STX8, Mig-6 also interacts with AP-2, a clathrin adaptor protein complex in the plasma membrane, and intersectins (ITSNs), adaptor proteins involved in clathrin-coated pit formation and maturation. Thus, Mig-6 may confer on EGFR the ability to be trafficked efficiently from the plasma membrane to lysosomes as a platform for multiple endocytic trafficking proteins [51].

Snapin in a complex with autophagy protein ATG14L mediates EGFR trafficking and degradation in an autophagy-independent manner [52]. Snapin was originally identified as a SNAP25-binding protein that modulates neurotransmission by increasing the interaction between a SNARE complex and Ca^{2+}-sensor protein synaptotagmin [53]. Later studies suggested that Snapin functions in broader membrane fusion events in non-neuronal cells through interaction with SNAP23, a non-neuronal homologue of SNAP25 [54-56]. Moreover, Snapin recruits the dynein minus-end motor to late endosomes and leads to lysosomal maturation [57]. In this context, Kim and colleagues revealed that Snapin with ATG14L promotes endocytic trafficking to lysosomes and degradation of EGFR [52]. Snapin also plays roles in autophagosome maturation and retrograde trafficking of hybrid organelles of autophagosomes and late endosomes, called amphisomes, from axon terminals to the soma in serum-starved neurons [58]. Interestingly, the Snapin-ATG14L interaction is required for late endocytic trafficking, but not for autophagosome maturation [52]. These observations suggest that ATG14L may switch its binding partner to change functions under serum-grown or -starved conditions, as shown for other autophagic proteins including STX17 [21].

Integrin: Integrins are adhesion receptors for components of the extracellular matrix (ECM) such as collagens, laminins and fibronectin [59]. Integrins are implicated in various physiological and pathological processes including cell spreading, migration, invasion, and cell growth. Integrins function as heterodimers of α and β subunits; eighteen α and eight β subunits exist in mammals and are assembled into 24 different complexes. Both α5β1 and αVβ3 integrins have received considerable attention in the cancer research field because they are highly related to the motility and invasiveness of cancer cells. Integrins form adhesion machineries such as focal adhesions and invadopodia, and then link ECM structures to intracellular actin microfilaments [60]. Integrins also function as signaling stations together with integrin-associated kinases and scaffold proteins, and evoke signaling cascades to induce several cellular responses including cytoskeletal rearrangement [61]. The integrin signaling is triggered and downregulated in several ways including through conformational changes, clustering and endocytosis and recycling of integrins themselves [62, 63]. Integrins, as well as TfR, have been regarded as typical recycling cargos. Many SNAREs that function in the endocytic recycling pathway, e.g. STX3, STX4, STX6, STX13, SNAP23, SNAP29, VAMP2 and VAMP3, have been shown to be involved in the trafficking of integrins [64-75].

Blood vessel/epicardial substance (Bves) positively regulates the recycling of integrin β1 and Tfn through direct interaction with VAMP3 [76]. Bves is expressed in myocytes and epithelial cells, in which it is localized to the lateral plasma membrane and endocytic recycling vesicles. Bves is a

three-transmembrane protein with short extracellular N-terminal and long intracellular C-terminal regions. The C-terminal region contains an evolutionally conserved Popeye domain, which binds to cAMP [77]. Depletion of Bves or overexpression of its dominant negative form attenuates the spreading and adhesion of cultured cells on fibronectin, and impairs integrin-mediated cell movement during gastrulation in *Xenopus*. In addition to VAMP3, Bves interacts with a wide variety of proteins including cardiac potassium channel TREK-1, tight junction anchor protein ZO-1, Rac1/Cdc42 guanine nucleotide exchange factor GEFT and N-myc downstream regulated gene NDRG4 [77-80]. Although most of the interacting partners are known to be involved in integrin-dependent cellular events through regulation of the actin and microtubule cytoskeletons and/or membrane trafficking, the significance of the interaction with Bves in these events has not been fully elucidated. Likewise, whether Bves regulates the trafficking of VAMP3, or vice versa, is unknown.

The second example of a SNARE-associated protein involved in integrin trafficking is Vps45; it is an SM protein for a SNARE complex comprising STX16, Vti1a, STX6 and VAMP4, which functions in retrograde trafficking from endosomes to the TGN [81]. Vps45 also interacts with Rab4/Rab5-effector Rabenosyn-5, and knockdown of Vps45 decreases Rabenosyn-5 and STX16 at the protein level. Furthermore, the knockdown compromises integrin β1 recycling and cell migration on fibronectin as the knockdown of Rabenosyin-5 does [82]. More recently, it was revealed that Vps45 is a causative factor for a neutrophil defect syndrome, which confers a predisposition to severe infections [83]. Homozygous mutations, Thr-224 to Asn or Glu-238 to Lys, in Vps45 identified in seven patients caused reductions of not only the Vps45 protein itself but also the Rabenosyn-5 and STX16 proteins. The patients' neutrophils and fibroblasts showed decreased integrin β1 expression at the cell surface and reduced cell motility, respectively. These observations clearly indicated a strong relationship between the genetic disorder and integrin trafficking by a SNARE-associated protein.

TfR: TfR plays pivotal roles in iron absorption into cells under physiological and pathological conditions [84, 85]. TfR, which binds an iron-binding protein transferrin (Tf), is internalized into the cell through clathrin-dependent endocytosis and then is initially delivered to early endosomes, also known as sorting endosomes. From this compartment, some of the receptor-ligand complex is directly recycled back to the plasma membrane (which usually takes from several minutes to less than 30 minutes), but the others are sorted into recycling endosomes and then back to the plasma membrane (usually from 30 minutes to a few hours); thus, it is regarded as a typical recycling cargo like integrins. It has been shown that SNAREs, STX4, STX6, STX10, STX13, SNAP23, SNAP25, SNAP29, VAMP2 and VAMP3, are involved in the trafficking pathways [12, 13, 64, 86-91].

The Golgi-associated retrograde protein (GARP) complex is a tethering complex for SNARE-mediated membrane fusion in the trafficking pathway from endosomes to the TGN, and is composed of Vps51, Vps52, Vps53 and Vps54. Syndetin is a structural homologue of Vps54, and comprises a complex together with Vps51, Vps52 and Vps53, instead of Vps54, called the endosome-associated recycling protein (EARP) complex [86]. The EARP complex is localized in recycling endosomes, whereas the GARP complex is localized in the TGN. Knockdown of syndetin decreases the protein levels of other EARP subunits and delays the recycling of internalized Tfn, although knockdown of Vps54 has only a partial effect on the recycling. Interestingly, both the EARP and GARP complexes interact with STX6 and its cognate SNAREs, STX16, Vti1a and VAMP4. In addition, EARP also interacts with STX13 and VAMP3, both of which are known to function in recycling endosomes. The STX6 Habc domain-interacting protein of 164 kDa (SHIP164) is another STX6- and GARP subunit-binding protein. Overexpression of SHIP164 enhances the inhibitory effect of STX6 overexpression on Tfn uptake. However, the precise mechanisms by which the novel endosomal tethering complex and the GARP-associated protein regulate the recycling of the cargos have not been fully elucidated.

VAMP3 is one of the key SNARE proteins that acts in early and recycling endosomes. In addition to Bves described above, two other VAMP3-interacting proteins, ring finger protein 167 (RNF167) and phosphatidylinositol 4-kinase II alpha (PI4K2A), have been determined to be involved in Tfn trafficking. RNF167 and its *Drosophila* orthologue Godzilla are E3 ubiquitin ligases, and VAMP3 is a target of these enzymes [92, 93]. Overexpression of RNF167, but not its ligase activity-dead mutant, leads to the accumulation of Rab5-positive giant endosomes and this effect is abolished by VAMP3 depletion. Endocytosed Tfn is trapped in the giant endosomes and its recycling to the cell surface is blocked in RNF167-overexpressing cells. The possibility suggested by the authors is particularly intriguing, i.e. that the ubiquitylation of the SNARE may act as a molecular switch for the recycling endosome pathway [93]. Another E3 ubiquitin ligase, MARCH-II/RNF172, which interacts with STX6 and modulates the trafficking of TfR from early to recycling endosomes, may have a similar role [94]. Another VAMP3-interacting protein, PI4K2A, is an enzyme that produces phosphatidylinositol 4-phosphate (PI4P) in the Golgi apparatus and endocytic recycling compartments

including early, late and recycling endosomes. Knockdown of PI4K2A impairs the trafficking of VAMP3 to a perinuclear compartment, decreases the complex of VAMP3 with its cognate SNARE Vti1a, and inhibits recycling of Tfn to the cell surface [92]. Moreover, acute depletion of its enzymatic product, PI4P, on the TGN and endosomes using an elegant gene-engineered tool with Sac1 PI4P phosphatase revealed the requirement of endosomal PI4P for trafficking of VAMP3, suggesting that the lipid is a critical component for the trafficking of VAMP3 and Tfn.

Concluding remarks ---- SNARE complexes are central machineries for intracellular membrane fusion. Given their fundamental importance, it is reasonable that they serve as a molecular hub that modulates the intracellular trafficking of various receptors. A wide variety of mechanisms for the regulation of receptor trafficking have been adopted by SNARE-interacting proteins, as described above, e.g. modulation of the tethering, formation and disassembly of the SNARE complex, ubiquitylation of SNAREs and other proteins, and modification of membrane lipids. In addition, many of the SNARE-interacting proteins discussed above have other binding partners. They may act as scaffolds for the SNARE complexes and other machineries.

Although over the past few decades our knowledge of receptor trafficking and membrane fusion has been greatly expanded, detailed understanding of their molecular mechanisms is still limited. Open questions and future directions in this field include the following. First, it is potentially important to identify more SNARE-interacting proteins that are involved in the regulation of receptor trafficking. Such efforts may reveal the global regulatory systems in intact cells. Second, the coordination of the fusion machinery comprising the SNARE complex with other cellular machineries such as cytoskeletons remains poorly understood. As receptor trafficking is a highly regulated and vectorial process, cytoskeletons and many other cellular machineries should be correlated to receptor trafficking and membrane fusion. Third, defining the spatiotemporal regulation of the interaction between SNAREs and their associated proteins is an exciting prospect. Photic stimulation technologies such as fluorescence resonance energy transfer should become key techniques, and the development of new bioprobes is also critical. Fourth, the regulation of the interactions with SNAREs and their biochemical activities of the SNARE-interacting proteins through post-translational modifications such as phosphorylation are potentially important. Several examples of regulation of the complex formation between SNAREs and their interacting proteins, e.g. STX1, SNAP25 and Munc-18, through phosphorylation in regulated exocytosis of neurotransmitters are well known [95]. In contrast, the regulation of SNARE-interacting proteins that act in the degradation and endocytic recycling pathways, which are described in this review, is poorly understood. Given the importance of the spatiotemporal regulation of degradation and endocytic recycling of receptors, these SNARE-interacting proteins may be regulated through phosphorylation and other modifications. Further investigations including on these issues may reveal the correlation between receptor trafficking, and physiological and pathological events.

Conflicting interests

The authors have declared that no conflict of interests exist.

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Dedication

This review is dedicated to the memory of Dr. Katsuko Tani who prematurely died on March 27, 2016.

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