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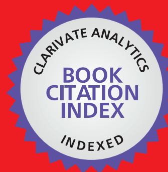

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Molecular Machinery Regulating Exocytosis

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1. Introduction

Exocytosis is the major intracellular route for the delivery of proteins and lipids to the plasma membrane and the means by which vesicular contents are released into the extracellular space. The anterograde trafficking of vesicles to the plasma membrane is vital for membrane expansion during cell division; cell growth and migration; the delivery of specialised molecules to establish cell polarity; cell-to-cell communication; neurotransmission and the secretion of response factors such as hormones, cytokines and antimicrobial peptides. There are two major trafficking routes in eukaryotic cells, which are referred to as constitutive and regulated (Ory & Gasman, 2011). Constitutive exocytosis involves the steady state delivery of secretory carrier vesicles from the endoplasmic reticulum via the Golgi apparatus to the plasma membrane (Lacy & Stow, 2011). Regulated or granule-mediated exocytosis involves a specific trigger, usually a burst of intracellular calcium following an extrinsic stimulus. This system is utilized for secretion in neuronal cells and other specialist secretory cells, such as neuroendocrine, endocrine and exocrine cells (Burgoyne & Morgan, 2003; Jolly & Sattentau, 2007; Lacy & Stow, 2011). Regulated exocytosis enables a rapid response from a subpopulation of vesicles already primed and competent for fusion (Manjithaya & Subramani, 2011; Nickel & Seedorf, 2008; Nickel, 2010). Regulated exocytosis is also used for polarised traffic of vesicular membrane and cargo to specific spatial landmarks and this is particularly important during times of dramatic change in cell morphology, such as cell division, cell motility, phagocytosis and axonal outgrowth.

Regulated exocytosis involves the shuttling of carrier vesicles between vesicular compartments, as they are transported towards the plasma membrane. Each step in this process requires the fission of a vesicle from a donor compartment. This carrier vesicle is then targeted/trafficked to an acceptor compartment where docking and fusion takes place, and the cargo is either unloaded or further processed (Bonifacino & Glick, 2004). These fission and fusion steps are repeated until the cargo reaches the plasma membrane (Bonifacino & Glick, 2004). This sequential trafficking of secretory vesicles is orchestrated by a complex set of molecular machinery including: small GTPases of the Ral, Rab and Rho subfamilies that regulate the processes of vesicle formation, traffic and fusion; the

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exocyst complex for vesicle assembly and membrane tethering; and soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) proteins for vesicular fusion. At the target domain of the plasma membrane, cross-talk between the exocyst complex and SNARE proteins culminates in vesicle-to-plasma membrane fusion, and thereby delivery of membrane proteins and luminal cargo. There are many post-translational modifications of the vesicular machinery that facilitate exocytosis. These include the addition of lipid moieties to increase membrane binding affinity, the switching of GTPase activity by nucleotide exchange factors, phosphorylation, and ubiquitination. Phosphorylation is of particular importance as it incorporates the vesicular trafficking machinery into a circuit of cellular signaling cascades. This chapter focuses on the process of exocytosis and the regulatory role that post-translational modification has on the exocytic machinery. Because the small GTPases and the exocyst complex have multiple inter-connected functions during vesicle formation, trafficking and fusion, we have focused discussion here to the final steps of the exocytic process, which occur in close proximity to the plasma membrane.

2. The exocyst complex and vesicle interaction with the plasma membrane

The exocyst is a scaffolding complex that is required for the final steps of regulated, and constitutive exocytosis (Hsu, *et al*., 2004). The exocyst complex is attached to the cytosolic face of the exocytic vesicular membrane, and tethers the vesicle to specific domains of the plasma membrane (Brymora, *et al*., 2001; X. W. Chen, *et al*., 2011a; Fukai, *et al*., 2003; Inoue, *et al*., 2003; Li, *et al*., 2007; Moskalenko, *et al*., 2002) (Figure 1). The pioneering studies of the early 1990's discovered that there are six yeast secretion (Sec) proteins; Sec3, Sec5, Sec6, Sec8, Sec10 and Sec15; and two exocyst (Exo) subunit proteins; Exo70 and Exo84, which form the exocyst complex (TerBush, *et al*., 1996). The constituents of the exocyst complex are conserved between yeast and mammals (He & Guo, 2009) and there are striking structural and topological similarities in the C-terminal domains of Sec6, Sec15, Exo70 and Exo84, despite there being less than 10% sequence identity between the individual proteins. These C-terminal domains consist of multiple rod-like helical bundles, which appear to be evolutionarily related molecular scaffolds that have diverged to create functionally distinct exocyst proteins (Sivaram, *et al*., 2006). The interaction between these helical structures may create the framework that is necessary for the assembly of the exocyst complex (Munson & Novick, 2006).

There is some evidence that the exocyst complex may be present as distinct sub-complexes on vesicular and plasma membranes. In yeast, two members of the complex are associated with the plasma membrane; Sec3 and Exo70, while in mammals only Exo70 appears to be found on the plasma membrane (He, *et al*., 2007; He & Guo, 2009; Inoue, *et al*., 2003; J. Liu, *et al*., 2007). It is likely that the membrane localisation of Sec3 and Exo70 controls targeting of secretory vesicles to distinct domains of the plasma membrane, thereby defining the sites of active exocytosis and membrane growth during cell migration and cytokinesis (Liu & Guo, 2011). It has been suggested that the Sec3 and Exo70 plasma membrane complex also contains Sec5, Sec8 and Sec6, while Exo84, Sec10 and Sec15 are complexed to the vesicle membrane (Moskalenko, *et al*., 2003). By binding to the vesicular membrane, Sec15 initiates the assembly of the vesicular exocyst sub-complex, while Sec3 and Exo70 mediate assembly of the plasma membrane sub-complex. Sec3 relies on a Rho-mediated targeting

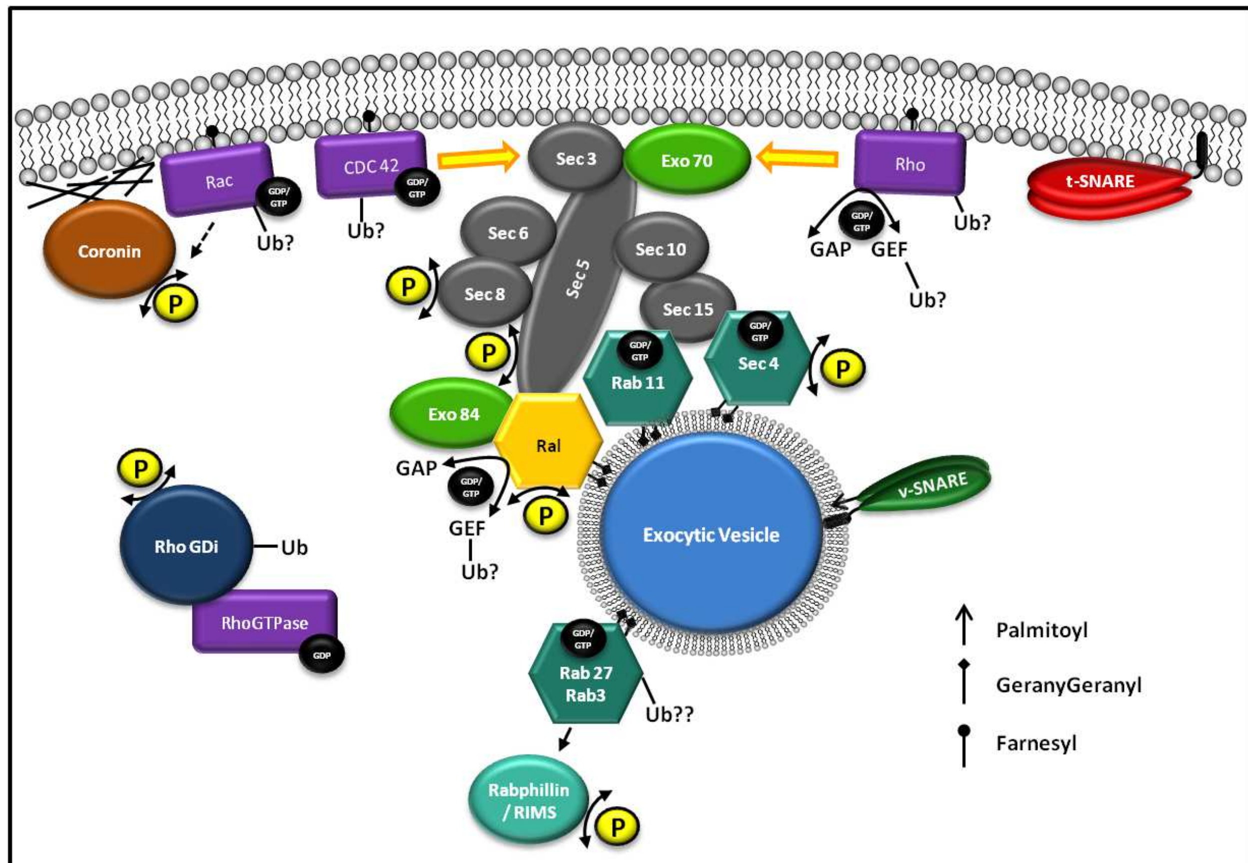

Fig. 1. Post-translational regulation of exocytic vesicle tethering via the exocyst complex

mechanism for its plasma membrane localization (He, *et al*., 2007; Moskalenko, *et al*., 2002; H. Wu, *et al*., 2010), which is distinct from the Rab-dependent targeting of Sec15 to the vesicular membrane (Guo, *et al*., 1999; Langevin, *et al*., 2005; S. Wu, *et al*., 2005; Zhang, *et al*., 2004). Co-assembly of these two exocyst sub-complexes to form the entire complex is governed by Ral-GTPase via its interaction with Sec5 (Hohlfeld, 1990). Prior to membrane fusion, SNAREs (e.g. Sec1, Sro7p and Sro77p) interact with the exocyst complex (via Sec6, Exo84) to facilitate fusion between the vesicle and plasma membranes (Morgera, *et al*., 2012; Zhang, *et al*., 2005).

3. Small GTPases as regulators of exocytosis

While the exocyst complex has a clear role in exocytosis, the factors promoting the final orchestration of exocytosis are yet to be characterized. Emerging data highlights that small GTPases of the Ras super-family, including the Ras homologous (Rho), Ras-associated binding proteins (Rabs), adenosine ribosylation factors (Arfs), and Ras-like proteins (Ral) subfamilies, are involved in regulating distinct steps during exocytosis, some of which are mediated via interaction with the exocyst (reviewed in (Csepanyi-Komi, *et al*., 2011; Hutagalung & Novick, 2011; Segev, 2011)). Thus, there appears to be stage-specific requirements for small GTPase subfamily members during exocytosis (Figure 1).

The unique feature of the small GTPase superfamily (G-protein family) is the presence of a 20 kDa, catalytic domain (Bourne, *et al*., 1991; Pai, *et al*., 1990). Through guanosine

nucleotide-dependent conformational transitions within their G-protein domain (Pereira-Leal & Seabra, 2000), these GTPases act as molecular switches; cycling between the inactive GDP bound form and a GTP-bound active form, the process which regulates the activity of downstream effectors. This activity switch can be triggered by a variety of intracellular stimuli, most notably calcium ions (Khvotchev, *et al*., 2003; Zajac, *et al*., 2005). The current dogma suggests that guanosine-triphosphate-dependent activation is essential for Rho, Ral and Rab relocation to target membranes, which then triggers their function.

3.1 Rab GTPases and vesicular tethering

The Rab family of small GTPases is defined by the presence of at least one of five characteristic Rab motifs, with the RabF1 motif frequently positioned within the effector binding domain, and the RabF2 motif usually in the GTPase domain (Pereira-Leal & Seabra, 2000). Recent bioinformatic analysis of the Rab family using the “Rabifier” and “RabDB” tools have uncovered an interesting phenomenon, namely the highly dynamic evolution of this family, with a significant expansion and specialization of the Rabs involved in the secretory pathway (Diekmann, *et al*., 2011). The repertoire of secretion-related Rabs includes 14 subfamilies, which co-evolved with *Metazoan* multicellularity and may reflect either unique roles in tissue-specific membrane trafficking events or restricted trafficking of specialist vesicles (Diekmann, *et al*., 2011). The animal-specific subfamilies have purported roles in the regulation of secretion (e.g. Rab3, Rab26, Rab27, Rab33, Rab37, Rab39), while there are also Golgi-specific Rabs (Rab30, Rab33, Rab34, Rab43) and Rabs relating to the traffic to (Rab43) and from (Rab10) the Golgi. Rab proteins usually play positive roles in anterograde membrane trafficking, but the exact nature of their involvement (in vesicle budding, biogenesis, transport, docking, priming and fusion) depends on the particular pathway, and is yet to be defined. One of the most evolutionary conserved proteins, Rab11, appears to be associated with both constitutive and regulated secretory pathways, as shown in mammalian and insect cells (Chen, *et al*., 1998; Shandala, *et al*., 2011; Urbe, *et al*., 1993; Ward, *et al*., 2005).

There have been mechanistic links established between some Rab proteins and components of the exocyst complex. For example, the interaction of Sec15 with Rab proteins appears to be essential for the tethering of exocyst components to designated membranes. In yeast, the small Rab GTPase Sec4 (orthologous to mammalian Rab10) may bring Sec15 to the vesicular membrane (Guo, *et al*., 1999), which is an essential step in the tethering and assembly of the exocyst complex (Zajac, *et al*., 2005). Metazoan Sec15 is a known effector of Rab11 (S. Wu, *et al*., 2005; X. M. Zhang, *et al*., 2004). Through its C-terminal domain, *Drosophila* Sec15 can interact with Rab11 (and is found co-localized with Rab11 in *Drosophila* photoreceptor and sensory organ precursor cells (Jafar-Nejad, *et al*., 2005; S. Wu, *et al*., 2005)), as well as with Rab3, Rab8, and Rab27 (S. Wu, *et al*., 2005). The functional relationship between Sec15 and Rab11 also exists in mammalian cells (Langevin, *et al*., 2005; Zhang, *et al*., 2004), where the interaction with Rab11 is involved in sequestering the exocyst complex to the endosome recycling compartment. Interestingly, there is a functional co-dependence of Rab11 and Sec15, where the loss of Sec15 function affects the intracellular localisation of Rab11, and mimics a phenotype of abnormal Rab11 function. Evidence of this interaction can be observed during the dramatic changes that occur in photoreceptor cell development (S. Wu, *et al*., 2005). More specifically, the mutant *Sec15* phenotype involves impaired trafficking

from recycling endosomes to the plasma membrane, and restricts cargo trafficking, e.g. DE-Cadherin in *Drosophila* (Langevin, *et al*., 2005).

Rabs have important interactive functions at different stages of exocytosis. Protein sorting in recycling endosomes depends upon the function of the small GTPase Rab4, a close homologue of Rab11 (Li, *et al*., 2008; Ward, *et al*., 2005). Rab3 also has a role in anterograde traffic between the trans-Golgi network and recycling endosomes (Mohrmann, *et al*., 2002; van der Sluijs, *et al*., 1992). The Rab small GTPases, Sec4 in yeast and Rab11 in metazoans, facilitate trafficking of secretory vesicles carrying Sec15-exocyst components from recycling endosomes to the plasma membrane (Langevin, *et al*., 2005). Sec15 does not appear to interact with mammalian Rab4a, and therefore does not function as a Rab4 effector (Zhang, *et al*., 2004). This suggests a unique role for Rab11 in the final delivery of exocyst carrying secretory vesicles, to the plasma membrane (Chen, *et al*., 1998; Shandala, *et al*., 2011; Urbe, *et al*., 1993; Ward, *et al*., 2005). Sec15 does however interact with Rab3, a closely related homologue of Rab11, both of which appear to play a critical role in: secretory vesicle biogenesis; docking and priming of specialised secretory vesicles; delivery of synaptic and dense core vesicles to the active zone of exocytosis; and in maintaining a primed pool of vesicles available for rapid release (Schonn, *et al*., 2010; S. Wu, *et al*., 2005). Thus, the loss of Rab3 led to a reduction in the total number of synaptic vesicles as well as the number recruited to the active zone of the neural synapse (Gracheva, *et al*., 2008). Similarly, there was a reduction in the number of dense core vesicles docked at the plasma membrane in adrenal chromaffin cells, isolated from a mouse quadruple knockout lacking all four Rab3 A to D paralogues (Schonn, *et al*., 2010). An increased number of docked dense core vesicles was observed in PC12 and in adrenal chromaffin cells following Rab3 overexpression and this correlated with a strong inhibition of secretion (Holz, *et al*., 1994; Johannes, *et al*., 1994). Interestingly, there is evidence of some functional redundancy between Rab3 and its closest homologues, Rab27A and Rab27B, which are involved in the delivery of vesicles near the exocytic site (Fukuda, 2008; Gomi, *et al*., 2007; Ostrowski, *et al*., 2010). Studies of melanosome dynamics have indicated that Rab27A has a role in vesicular recruitment and this is mediated by its interaction with a specific effector called Melanophilin, which in turn binds an actin motor protein, MyosinVa (Hume & Seabra, 2011; Seabra & Coudrier, 2004). There appears to be a further functional divergence of Rabs, where Rab27A and Rab27B control different steps of the secretion pathway (Ostrowski, *et al*., 2010). Rab25, a close homologue of Rab11 with a different C-terminus, shows co-localization with Rab11 in exocytic/recycling vesicle membranes of some cells, and may function as a tissue specific tethering factor (Calhoun & Goldenring, 1997; Khandelwal, *et al*., 2008).

Recent studies have implicated Rabs in the movement of transport vesicles from their site of formation to their site of fusion, and several Rabs have been linked to specific microtubule- or actin-based motor proteins (Hammer & Wu, 2002; Lapierre, *et al*., 2001) The role of Rabs in docking of secretory vesicles to the plasma membrane is mediated by their effectors (Fukuda, 2008). Thus, the small GTPases of the Rab family, through interplay with their specialist effector molecules, cooperatively target secretory vesicles from the trans-Golgi network (TGN) to the plasma membrane, and facilitate their docking at the active site of exocytosis (Fukuda, 2008; Orlando & Guo, 2009). This poses the question: what is the molecular mechanism defining the plasma membrane docking sites?

3.2 Rho GTPases and assembly of the plasma membrane exocyst complex

The most highly conserved and best studied members of the Rho family, Rho1/A, Rac1 and Cdc42, play a crucial role in tethering and fusion of vesicles during regulated exocytosis (Ory & Gasman, 2011; Ridley, 2006; Williams, *et al.*, 2009). Most Rho GTPases transiently localize at the plasma membrane, after being targeted to specific phosphoinositide-containing sub-domains. On the one hand, the Rho GTPases, Rho1/A and Rac1, are thought to regulate secretion by remodelling microtubules and the membrane-associated actin cytoskeleton (Ory & Gasman, 2011; Williams, *et al.*, 2009). On the other hand, recent findings have implicated yeast Cdc42, Rho1/A and Rho3/C and mammalian TC10 in actin-independent regulation of exocytosis by anchoring the plasma membrane exocyst components, Sec3 and Exo70, to specific plasma membrane microdomains (Bendezu & Martin, 2011; Guo, *et al.*, 2001; He, *et al.*, 2007; He & Guo, 2009; Inoue, *et al.*, 2003; J. Liu, *et al.*, 2007; Moskalenko, *et al.*, 2002; Novick & Guo, 2002; Wu & Brennwald, 2010; H. Wu, *et al.*, 2010; Xiong, *et al.*, 2012; Zajac, *et al.*, 2005). However, in this case, the normal functioning of the Sec3 and Exo70 plasma membrane exocyst components is a prerequisite for the correct localization of cell polarity regulators such as Cdc42 (Zajac, *et al.*, 2005). This might be due to the fact that Sec3 and Exo70 could independently bind to phosphatidylinositol-4,5-bisphosphate (PI(4,5)P2), via their C-terminal D domain, thereby forming a plasma membrane targeting patch for exocytic proteins (He, *et al.*, 2007; He & Guo, 2009; Inoue, *et al.*, 2003; J. Liu, *et al.*, 2007). Moreover, Exo70 was found to be directly associated with type Iγ phosphatidylinositol phosphate kinase (PIPKIγ), which facilitates the generation of a PI(4,5)P2 phospholipid microdomain and recruitment of Exo70 to the plasma membrane (Xiong, *et al.*, 2012). Thus, the cooperation between the Sec3 and Exo70 exocyst components and Rho small GTPases defines competent sites for exocytosis. The next question is: how are secretory vesicles targeted to these sites?

3.3 Ral small GTPases and the exocyst complex

Ras-like (Ral) small GTPase was first discovered in human platelet cells in 1991 (van der Meulen, *et al.*, 1991), where its association with dense granules suggested a potential regulatory role in the release of storage contents from these granules (Mark, *et al.*, 1996). Subsequently, Ral small GTPases were linked to exocytosis in neural, epithelial, endothelial endocrinal, and pancreatic tissues (Hazelett, *et al.*, 2011; Lopez, *et al.*, 2008; Moskalenko, *et al.*, 2003; Polzin, *et al.*, 2002; Rondaij, *et al.*, 2004; Rondaij, *et al.*, 2008; Takaya, *et al.*, 2007). The two mammalian Ral homologues RalA and RalB share 85% protein sequence identity, and are well conserved in evolution (van Dam & Robinson, 2006). The bulk of the Ral protein comprises a conserved nucleotide phosphate-binding motif (Marchler-Bauer, *et al.*, 2011; van Dam & Robinson, 2006). RalA, but not RalB, contains a short amphipathic helix that binds the Ca^{2+}-sensing protein Calmodulin, conferring Ca^{2+}-dependent activation of RalA during regulated exocytosis (van Dam & Robinson, 2006). Ral functions as an essential component of the cellular machinery, regulating the post-Golgi processing of secretory vesicle membrane, via activation of the exocyst complex (X. W. Chen, *et al.*, 2011a; Feig, 2003; Kawato, *et al.*, 2008; Mark, *et al.*, 1996; Mott, *et al.*, 2003). It has been suggested that GTP-bound Ral, through its effectors Sec5 and Exo84, brings together the plasma and vesicular membrane exocyst subunits, as the loss of RalA, or mutation of its effector binding motif,

leads to the disassembly of the exocyst complex (Moskalenko, *et al*., 2003). Activation of the exocyst complex is initiated by the binding of Ral to Sec5 and Exo84 (Mott, *et al*., 2003). This is followed by the assembly of the full octameric exocyst complex at the interface of the vesicular and plasma membranes (Moskalenko, *et al*., 2003). Thus, the interaction of the exocyst complex with Ral is an essential step in anchoring secretory vesicles to the exocytosis-competent microdomains of the plasma membrane (Angus, *et al*., 2003; Fukai, *et al*., 2003; Jin, *et al*., 2005; Mark, *et al*., 1996; Moskalenko, *et al*., 2002).

The functional interaction of Ral and the exocyst complex is highlighted by their co-involvement in multiple exocytic processes. The exocyst complex has well-established roles in anterograde trafficking of membrane receptors from recycling endosomes (Langevin, *et al*., 2005; Xiong, *et al*., 2012; Yeaman, *et al*., 2004); membrane delivery in cell growth (Chernyshova, *et al*., 2011; Genre, *et al*., 2011); and the translocation of glucose transporters in response to insulin (Ljubicic, *et al*., 2009; Lopez, *et al*., 2008). Each of these processes has been linked to a functional requirement for a member of the Ral protein family. RalA is required for establishing neuronal cell polarity (Lalli, 2009), and the regulation of readily releasable pools of synaptic vesicles (Lee, *et al*., 2002; Li, *et al*., 2007). In the epithelium, RalB is required for delivery of membrane to the dynamic leading edge of migrating cells (Rosse, *et al*., 2006); while RalA is involved in polarised delivery of the membrane protein, E-Cadherin, to the basolateral surface of epithelial cells (Shipitsin & Feig, 2004). Exocytosis of vesicular content, such as hormones, chemokines, enzymes, and adhesion molecules from Weibel-Palade bodies (endothelial cell-specific storage organelles), occurs in response to a specific agonist that requires Ral regulation (Kim, *et al*., 2010; Rondaij, *et al*., 2008). RalA is required in glucose regulation where it mediates insulin secretion from pancreatic cells (Ljubicic, *et al*., 2009; Lopez, *et al*., 2008), and translocation of the glucose transporter GLUT4 in adipocytes (Chen, *et al*., 2007). Ral is also required for dense granule secretion from platelets (Kawato, *et al*., 2008) and cell growth and migration, all of which have been shown to be reliant on Ral for lipid raft trafficking to the plasma membrane (Balasubramanian, *et al*., 2010; Spiczka & Yeaman, 2008).

Given that multiple GTPases regulate the assembly of the full octameric exocyst complex, which is necessary for vesicular tethering to the site of fusion, the assembly of the exocyst complex might represent the integration of various cellular signaling pathways that ensure tight control of exocytosis (Sugihara, *et al*., 2002).

4. Exocyst, SNARE complexes and membrane fusion machinery

The exocyst mediated tethering of secretory vesicles to specific sites of the plasma membrane precedes the assembly of SNARE complexes and membrane fusion (He & Guo, 2009; Novick & Guo, 2002) (Figures 1 & 2). In the early 1980s, Rothman and colleagues used an *in vitro* trafficking assay to identify the soluble factors; *N*-ethylmaleimide-sensitive factor (NSF) and Soluble NSF Attachment Protein (SNAP) (Balch, *et al*., 1984). This was followed by isolation of their membrane receptors termed SNAREs (for SNAP receptors) (Sollner, *et al*., 1993). SNAREs were initially isolated from mammalian brain cells, as factors crucial for vesicle fusion–mediated release of neurotransmitters at synapses. It soon became evident that SNAREs are involved in most, if not all vesicular fusion events (Malsam, *et al*., 2008).

SNAREs comprise evolutionarily conserved families of membrane-associated proteins (including the Synaptobrevin/vesicle associated membrane protein (VAMP), Syntaxin and SNAP, families), which are characterized by the presence of a 60–70 amino acid long SNARE motifs located, in most cases, immediately adjacent to a C-terminal trans-membrane anchor (Weimbs, *et al*., 1997). In some cases (e.g. Sec9), this trans-membrane motif is absent, and the membrane binding is mediated by lipid modifications or by the presence of domains capable of binding lipid head groups (Grote, *et al*., 2000). A coiled-coil structure formed by SNARE motifs is known to mediate protein-protein interactions between different SNAREs, and this is believed to create the membrane curvature required for membrane fusion (Groffen, *et al*., 2010). It has been suggested that the SNARE-induced membrane curvature becomes fusion competent when decorated by two Ca^{2+} sensors, Synaptotagmin1 and double C2 domain (DOC2) protein (Groffen, *et al*., 2010; Hui, *et al*., 2009).

SNAREs act at all levels of the secretory pathway, although individual family members tend to be compartment-specific and thus, are thought to contribute to the specificity of docking and fusion events (Gerst, 1999). The exocyst complex may facilitate vesicular targeting to the plasma membrane by direct interaction with SNARE proteins, as is the case with the exocyst component Sec6 and the target t-SNAREs Sec1 and Sec9 (Morgera, *et al*., 2012). In some cases this interaction may be indirect and mediated by adaptor proteins. For example, the yeast exocyst component Exo84 is capable of interacting with t-SNAREs though the WD40 domain adaptor proteins Sro7p and Sro77p (Sivaram, *et al*., 2005; Zhang, *et al*., 2005). In addition, during the process of regulated exocytosis involved in neurotransmitter release, a unique set of components (Synaptotagmin, Complexin, Munc13, RIM, - also called the trans-SNARE complex), assemble between v-SNAREs on the transport vesicle and t-SNAREs on the fusion target, to ensure efficient membrane fusion (Li & Chin, 2003). Some elements of this SNARE complex can also act as negative regulators of membrane fusion: as is the case during sperm exocytosis, where α-SNAP prevents docking of the acrosome by sequestering monomeric Syntaxin (Rodriguez, *et al*., 2011).

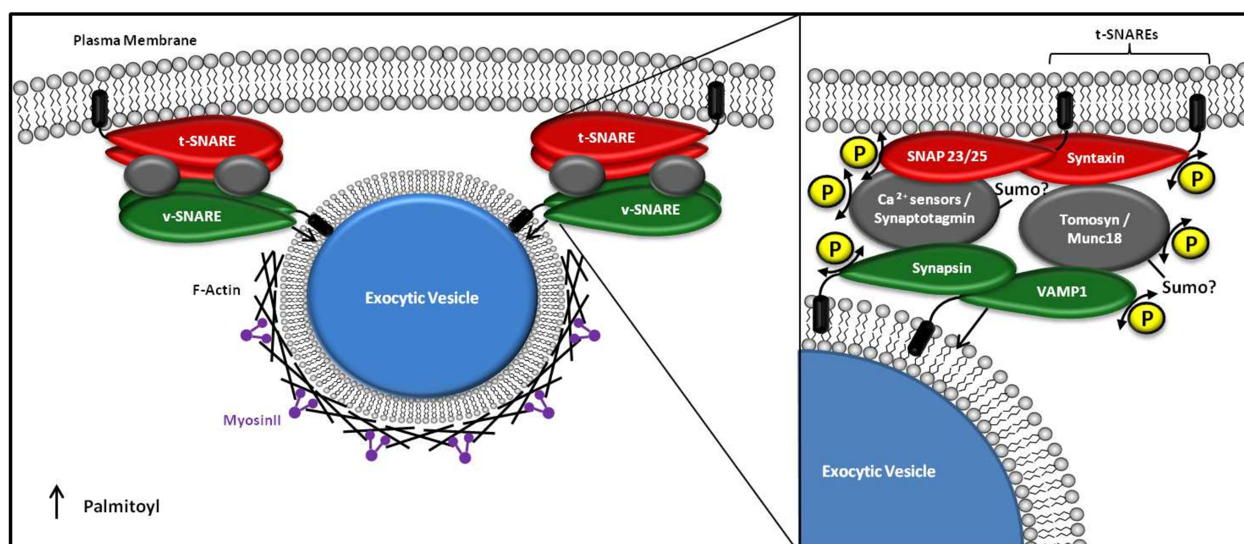

Fig. 2. Post-translational regulation of docking and fusion at the plasma membrane

5. Post-translational modifications that regulate components of the exocytic machinery

5.1 Regulation of GTPases: Activity by nucleotide exchange

As mentioned above, the small GTPases involved in exocytosis are regulated by switching between an active (GTP-bound) and inactive (GDP-bound) state (Ali & Seabra, 2005; Barr & Lambright, 2010; Csepanyi-Komi, *et al*., 2011; Stenmark, 2009; Uno, *et al*., 2010) (Figure 1). This exchange between two states is regulated by guanine nucleotide exchange factors (GEFs) and GTPase activating proteins (GAPs) (Csepanyi-Komi, *et al*., 2011). GEF proteins activate GTPases by a mechanism involving the destabilization of the interaction between the GEF and guanosine di-phosphate (GDP), resulting in the fast release of the GDP and generation of a nucleotide-free GTPase-GEF intermediary complex (Liao, *et al*., 2008). As guanosine tri-phosphate (GTP) is much more abundant in the cell than GDP (Gamberucci, *et al*., 1994), GTP will preferentially bind to the nucleotide binding pocket of the GTPase inducing dissociation of the GEF, leaving the GTP-loaded GTPase ready to perform its function. The subsequent hydrolysis of the GTP by the GTPase provides the energy required for the GAP enzyme to accomplish its specific task. In some cases, the membrane affinity of GEFs and GAPs has been linked to the binding modules of the Phox-homology domain (PX domain in TCGAP, a protein with intrinsic GAP activity towards Rho GTPases as shown *in vitro*), and the Pleckstrin-homology domain (PH domain in Trio, a GEF for Rho GTPases). These domains target the proteins to specific phosphoinositide-containing sub-domains of intracellular membranes (Chiang, *et al*., 2003; Hyvonen, *et al*., 1995; Kutateladze, 2007; van Rijssel, *et al*., 2012; Zhou, *et al*., 2003).

Once activated by GEFs, the small GTPases (Rho, Rab, Arf, and Ral) translocate to their destination membrane in order to carry out their specific function (Csepanyi-Komi, *et al*., 2011). In exocytosis, the GEFs identified so far control the function of members of three of the GTPase families, the Rabs, Rals, and the Rhos. In the budding yeast *S. cerevisiae*, the Rab GTPase Sec4 and its GEF Sec2 were found to be associated with the polarized transport of vesicles from the TGN to the site of bud formation at the plasma membrane. Sec2 catalyzes the guanine nucleotide exchange and subsequent activation of Sec4, a key mediator of the docking and fusion steps (Dong, *et al*., 2007; Medkova, *et al*., 2006; Ortiz, *et al*., 2002). The Rab GTPase Rab3a performs a similar role in mammalian neuroendocrine cells, with its function being required for docking and fusion of hepatocyte growth factor granules in chromaffin cells, as demonstrated by the use of mutated forms of the protein (Holz, *et al*., 1994; Luo, *et al*., 2001; Macara, 1994). The GEF for Rab3a, called GRAB, has been shown to be essential for the targeting of secretory vesicles to the plasma membrane (Luo, *et al*., 2001; Sato, *et al*., 2007). The small Rho GTPase Cdc42 is involved in remodeling of the actin cytoskeleton of the cell including some of the events that occur in Ca^{2+} mediated regulated exocytosis. Recent studies in pancreatic β-cell islets, using a combination of *in vitro* binding assays and RNAi silencing screens in cell culture, have identified Cool-1/β-pix as a GEF for Cdc42 (Kepner, *et al*., 2011). A number of GEF proteins have been proposed to mediate Ral function in exocytosis, which include Ral guanine nucleotide dissociation stimulator (RalGDS) (Albright, *et al*., 1993) and RalGDS-like 2 (Rgl2)/RalGDS-like factor (Rlf) (Wolthuis, *et al*., 1996). The activity of RalGEFs, are themselves regulated by the Ras GTPase protein. Active GTP-bound Ras facilitates activation by binding to RalGEFs (Spaargaren & Bischoff, 1994), and delivering them to Ral in the plasma membrane. RalGDS has been shown to be required for the activation of RalA

dependent exocytosis from Weibel-Palade bodies in endothelial cells and the secretion of insulin in β-pancreatic cells (Kim, *et al.*, 2010; Ljubicic, *et al.*, 2009; Rondaij, *et al.*, 2008), while calcium induced exocytosis from endosomes is predicted to be regulated by Rgl2/Rlf – mediated activation of Ral (Takaya, *et al.*, 2007). Thus, the regulation of Ral dependent exocytosis may be at the nexus of a number of upstream signaling pathways. Often GEFs will display a certain level of promiscuity, being capable of activating more than one GTPase family member. For example, the Rab3a GEF GRAB is also capable of activating Rab27a (Baisamy, *et al.*, 2005; Baisamy, *et al.*, 2009). This might reflect a need for simultaneous activation of more than one GTPase in order to coordinate the progression of exocytosis. Moreover, individual GTPases may be regulated by multiple GEFs (for example Cdc42 is regulated by Cool-1/β-pix (Kepner, *et al.*, 2011), FGD1 (Olson, *et al.*, 1996), FGD3 (Pasteris, *et al.*, 2000), and PEM-2 (Reid, *et al.*, 1999)), although this may reflect a mechanism for the tissue-specific activation of these uniformly expressed proteins.

When driven by the intrinsic enzymatic activity of small GTPases, the hydrolysis of GTP to GDP is a slow process (Crechet & Parmeggiani, 1986; Liao, *et al.*, 2008; Trahey & McCormick, 1987), but this can be stimulated by GTPase activating proteins (GAPs) (Csepanyi-Komi, *et al.*, 2011; Trahey & McCormick, 1987). In yeast for example, once GTP-bound Sec4 reaches the plasma membrane, its GAPs, Msb3 and Msb4, promote hydrolysis of the GTP to switch it to its GDP-bound inactive form and translocate it away from the plasma membrane (Gao, *et al.*, 2003). Yeast cells with deletions of Msb3 and Msb4 display defective exocytosis as indicated by the accumulation of abnormally large numbers of vesicles within the cells and a reduction in secretion (Gao, *et al.*, 2003). As is the case with GEFs, there is also a certain promiscuity of RhoGAP activity. For example, the neuron specific GAP Nadrin has been demonstrated to have activity towards Rho1/A, Cdc42, and Rac1 (Harada, *et al.*, 2000). In doing so, Nadrin appears to modulate the actin related function of Rho GTPases, as its overexpression in NIH3T3 fibroblast cells resulted in abnormal reorganization of the actin stress fibers (Harada, *et al.*, 2000). Biochemical studies have identified Rab3GAP1/Rab3GAP2 as a GAP for Rab3, and EV15 as a GAP for Rab11, but the physiological relevance of their function is yet to be fully defined (Csepanyi-Komi, *et al.*, 2011; Dabbeekeh, *et al.*, 2007; Fukui, *et al.*, 1997). The RalGAP complex (RGC), which is made up of RalGAP complex 1 (RGC1) and a catalytic subunit RalGAP complex 2 (RGC2) has been identified for its role in Ral dependent exocytosis of GLUT4 (Chen & Saltiel, 2011). RCG interacts with RalA under steady state conditions, acting as its inhibitor. Insulin induced activation of the PI3-kinase/Akt pathways leads to Akt-mediated phosphorylation of the RGC2 catalytic subunit, disengaging it from the GTPase and reversing the inhibitory effect of RGC on RalA activity. This allows RalA to associate with the exocyst and facilitate exocyst complex assembly (Chen & Saltiel, 2011).

After GTPases complete their function on the membrane, they are returned to the cytosol in an inactive GDP-bound form, terminating the exocytic process and preventing inappropriate activity of the enzyme and its effectors. This sequestration and inactivation is mediated by members of the guanine dissociation inhibitor (GDI) family. Notably, GDIs have dual modes of GTPase binding. One domain of the protein binds to and occludes the nucleotide binding site of the GTPase (Gosser, *et al.*, 1997; Grizot, *et al.*, 2001; Hoffman, *et al.*, 2000; Keep, *et al.*, 1997; Longenecker, *et al.*, 1999; Scheffzek, *et al.*, 2000), preventing the release of the nucleotide and interaction with GTPase effector proteins. The second domain of the protein forms an immunoglobin-like hydrophobic pocket into which the prenyl group of the GTPase can be

inserted (see section 5.2), preventing it from interacting with membranes (Gosser, *et al.*, 1997; Grizot, *et al.*, 2001; Hoffman, *et al.*, 2000; Keep, *et al.*, 1997; Longenecker, *et al.*, 1999; Scheffzek, *et al.*, 2000). This dual mode of interaction suggests that GDIs could play a negative regulatory role in the GTPase cycle; holding a GTPase in an inactive GDP-bound state, while still bound to the membrane via a lipid moiety, then transporting it away from the membrane after the lipid moiety is engaged (DerMardirossian & Bokoch, 2005; Johnson, *et al.*, 2009). This has been demonstrated most clearly in studies examining the mechanism of action of RhoGDI on Cdc42. In solution, RhoGDI exhibits an equal affinity to GDP or GTP bound Cdc42 (Nomanbhoy & Cerione, 1996). However, when Cdc42 is membrane associated, RhoGDI shows a much higher affinity to GDP bound Cdc42 (Johnson, *et al.*, 2009). During its normal activation/hydrolysis/inactivation cycle, Cdc42 translocates from membrane bound to freely solvent GDI-bound states. In the latter state, the immunoglobin-like hydrophobic pocket of RhoGDI prevents Cdc42 from re-associating with the plasma membrane while the rest of the protein prevents the release of GDP from Cdc42 (Johnson, *et al.*, 2009).

GDIs have been shown to have important regulatory roles during exocytosis. In yeast, once Sec4 is converted from a GTP to a GDP-bound form, it is removed from the plasma membrane by the GDI, Sec19 (Garrett, *et al.*, 1994). The loss of Sec19 in mutant yeast strains leads to the accumulation of Sec4 at the site of budding, indicating a block of Sec4-GDP recycling (Zajac, *et al.*, 2005). Also, members of the rat Rab GDI family, GDI α and β, were demonstrated to block nucleotide exchange of both Rab3 and Rab11 in *in vitro* assays (Nishimura, *et al.*, 1994), and RNAi mediated knockdown of RhoGDI in pancreatic β-cells was found to result in a defect in Rac1/Cdc42 mediated insulin secretion (Kowluru & Veluthakal, 2005; Wang & Thurmond, 2010).

In general, GEFs function in large membrane associated complexes, and serve to translate signals from membrane bound receptors to specific members of the small GTPase superfamily (reviewed in (Csepanyi-Komi, *et al.*, 2011)). This mechanistic link ensures that the activation of small GTPases is dependent on major cellular signaling events. GEFs also provide a link between the exocyst complex and the vesicle. This is demonstrated by the interaction between the GTPase Sec4 and the exocyst component Sec15, an interaction that relies upon the activation of Sec4 by its GEF Sec2 (Guo, *et al.*, 1999). Phosphorylation also plays a key role in regulating the activity of the GEFs, GAPs and GDIs and examples of this regulatory role in exocytosis will be discussed in section 5.3.

5.2 Lipid modifications as a mean to control intracellular localization of proteins

The covalent attachment of lipids to exocytic proteins provides an important mechanism for regulating their location and in doing so, their activity. In exocytosis, two major lipid modifications, prenylation and palmitoylation (Yalovsky, *et al.*, 1999), facilitate the targeting of proteins to the plasma membrane and intracellular membrane compartments. Although somewhat controversial, modification by specific lipid moieties appears to play a role in controlling the strict compartmentalisation of the modified protein to specialist intracellular organelles (Figure 1&2).

5.2.1 Prenylation

Protein prenylation involves the attachment of either of two isoprenoids (C15 farnesyl or the C20 geranylgeranyl) to cysteine residues at the C terminus of the protein (Seabra, 1998;

Sebti, 2005; Zhang & Casey, 1996). The choice of isoprenoid in Rho proteins is dictated by the amino acid composition of a conserved C*AAX* motif, where C is cysteine, *A* is an aliphatic amino acid residue and *X* represents any amino acid (Seabra, 1998; Sebti, 2005; . Zhang & Casey, 1996). However, in Rho1/A, Rho2/B, Rac1, Rac2, Cdc42 and Ral, where *X* is a leucine residue, the protein becomes a substrate for geranylgeranyl transferase I (GGTase I), which attaches a 20-carbon (geranylgeranyl) isoprenoid moiety (Berzat, *et al*., 2006; Matsubara, *et al*., 1997). In Rho proteins where the *X* is a methionine, serine, alanine or glutamine (e.g. Rho2/B), a 15-carbon (farnesyl) isoprenoid will be attached by the enzyme farnesyl transferase (FTase) (Adamson, *et al*., 1992; Zhang & Casey, 1996). There is some evidence to suggest that prenylation triggers the cleavage of the *AAX* peptide by Rce1 (Ras-converting enzyme 1) endoprotease and the addition of a methyl group to the prenylated cysteine residue (catalyzed by isoprenylcysteine-*O*-carboxyl methyl transferase (Icmt)), which enhances membrane association of the prenylated protein (Roberts, *et al*., 2008; Sebti & Der, 2003).

In contrast to the Rho small GTPases, the C terminal peptide motif for the majority of Rab proteins contains two cysteine residues (e.g. *XXX*CC, *X*CC*XX*, *XX*C*X*C, CC*XXX*, *or XX*CC*X*), both of which can be prenylated resulting in mono- or di-geranylgeranylated proteins (Pereira-Leal & Seabra, 2000, 2001). This modification is catalyzed by geranylgeranyl transferase II (GGTase II, also called Rab geranylgeranyl transferase, RabGGTase), acting in conjunction with a chaperone Rab escort protein (human REP/yeast Yip-1) (Anant, *et al*., 1998; Andres, *et al*., 1993; Calero, *et al*., 2003; Seabra, *et al*., 1992).

5.2.1.1 Prenylation of Rho GTPases

Our understanding of the role of lipid modification in protein targeting to a particular membrane comes from studies using either specific enzyme inhibitors or genomic mutants (Mitin, *et al*., 2012). Rho2/B GTPase is known to undergo modification with both farnesyl (Rho2/B-F) and geranylgeranyl groups (Rho2/B-GG). In one study, treatment of HeLa cells with FTase inhibitors resulted in a loss of Rho2/B association with the plasma membrane, although association with the endosomal membranes remained unaffected (Lebowitz, *et al*., 1997). This suggested that the farnesyl moiety was specific for Rho2/B targeting to the plasma membrane and that the geranylgeranyl moiety controlled targeting to the endosomal membranes. However, for experiments performed in mouse embryonic fibroblasts or yeast cells, Rho1/A and Rho2/B (normally found on the cell surface and in cytoplasmic vesicles) accumulated in the nucleus when treated with a GGTase-I inhibitor (Rho1/A) or a combination of a GGTase-1 and FTase inhibitor (Rho2/B), presumably because they were unable to traffic to their target membranes (Roberts, *et al*., 2008). In this study, treatment with FTase inhibitor alone had a minimal impact on the membrane localisation of Rho2/B (Roberts, *et al*., 2008). Although conflicting, the results of these inhibitor studies can be explained by the inhibitors exhibiting a competitive effect on the substrate proteins and thereby partially affecting both enzymes (Lobell, *et al*., 2002). These results also suggest a potential functional redundancy between farnesyl and geranylgeranyl isoprenoids (Sjogren, *et al*., 2007), which is further complicated by the suggested equilibrium between the two modifications of Rho2/B (Lebowitz, *et al*., 1997). Evidence for functional redundancy between the isoprenoids comes from studies using mouse embryonic fibroblasts (MEFs) genetically depleted for GGTase-I function (Sjogren, *et al*., 2007). Loss of GGTase-1 in MEFs isolated from mutant mice resulted in reduced actin polymerization

leading to impaired cell migration. Transfection of these MEFs with plasmid vectors, driving the expression of farnesylated Rho1/A and Cdc42, restored the actin cytoskeleton, and the cells' ability to migrate and proliferate. This phenotypic rescue could be due to similar functionality of these two variants, as well as compensatory up-regulation of farnesylated variants, similar to that observed in COS cells, where treatment with an FTase inhibitor led to the accumulation of the geranylgeranylated form Rho2/B-GG (Du, *et al*., 1999; Lebowitz, *et al*., 1997).

The role of post-translational prenylation for the appropriate targeting of the lipid modified Rho protein to intracellular membranes has been further clarified by the mutational analysis of the enzyme recognition C*AAX*-motif as well as of the respective enzymes. Changing the C*AAX*-motif cysteine to a serine, or deleting the AAX component, rendered members of the Rho GTPase family unable to be prenylated and disrupted their ability to associate with the appropriate membranes (Winter-Vann & Casey, 2005). In addition, the loss of the prenylation enzyme GGTase-I in murine bone marrow macrophages (in *Pggt1b$^{fl/fl}$* mutants) resulted in high levels of the activated Rho small GTPases, Cdc42, and Rho1/A, which predominantly accumulated in the cytoplasm, and Rac1 which was found preferentially associated with the plasma membrane. Unexpectedly, this was correlated with increased secretion of the pro-inflammatory cytokines IL-1β and TNF-α and hyper-activation of macrophages, resulting in erosive arthritis in GGTase-I deficient mice (Khan, *et al*., 2011) . The authors proposed that this may be due to the lack of isoprenyl moieties in these small GTPases interfering with their recognition by endogenous regulatory proteins, such as RhoGDIs, which are responsible for GTPase inactivation and sequestration from the plasma membrane to the cytoplasm (Berzat, *et al*., 2006). While similar studies in yeast have suggested that prenylation may have a regulatory role in the targeting of vesicles to specific sites on the plasma membrane (such as the site of yeast budding), this is yet to be fully explored (H. Wu, *et al*., 2010).

5.2.1.2 Prenylation of Rab GTPases

To date, there have been no effective inhibitors identified for RabGGTase (El Oualid, *et al*., 2005), so the functional studies of Rab GTPase prenylation have been carried out using genetic manipulation, either of the enzyme recognition sites for the Rab proteins, or involving genomic RabGGTase knock-out mutants. Experiments in yeast have demonstrated that the mode of geranylgeranylation can alter which organelle membrane the Rab proteins associate with (Calero, *et al*., 2003). For instance, the wild-type di-geranylgeranylated yeast Rab proteins, YPT1 and Sec4, localize to the Golgi stack and the yeast bud tip respectively (Calero, *et al*., 2003). In yeast cells expressing Rab proteins, carrying a C-terminal peptide with one cysteine mutated, mono-geranylated YPT1 and Sec4 were often mis-localized to a reticular structure (possibly the endoplasmic reticulum) or to the cytoplasm (Calero, *et al*., 2003). Similarly, Rab27a, which is normally associated with melanosomes in mammals, became mis-localized when one of the cysteine residues in the C-terminal peptide motif was mutated (Gomes, *et al*., 2003). Genomic mutations in the catalytic subunit of RabGGTase (in *gunmetal* (*gm/gm*) mutant mice) resulted in reduced prenylation and vesicular membrane association of Rab27a and reduced Rab11 prenylation in mouse platelets and melanocytes (Detter, *et al*., 2000; Zhang, *et al*., 2002). The *gunmetal* mutant showed a phenotype indicative of defective exocytosis in affected cells; defective killing capability by cytotoxic T-cells (Stinchcombe, *et al*., 2001), hypopigmentation,

macrothrombocytopenia and reduced platelet synthesis leading to extended bleeding time/excessive bleeding (Detter, *et al*., 2000; Novak, *et al*., 1995; Swank, *et al*., 1993). Secretion defects in resorptive osteoclasts and bone forming osteoblasts were also observed in this mutant, resulting in imbalanced bone homeostasis (Taylor, *et al*., 2011). Both of these cell populations exhibited problems with anterograde vesicular trafficking. In osteoclasts, plasma membrane delivery of proteases and ion pumps was defective, and in osteoblasts there was a reduced mineralisation potential, possibly due to a failure to deliver vesicles containing bone-matrix (Taylor, *et al*., 2011). The underlying molecular cause is likely to be under-prenylation of many RabGTPases, including Rab3d, and possibly Rab27a.

Together, these *in vitro* and *in vivo* data indicate a significant role for prenylation in the function and activity of small GTPases of the Rho and Rab families, most likely through stable localization in the target membrane. Interestingly, there is a lipid-sensitive step at the secretory vesicle and plasma membrane fusion stage, which is proposed to be mediated by geranylgeranyl modification of SNAREs (Grote, *et al*., 2000; Tong, *et al*., 2009). In yeast, the exocytic SNARE Sec9 contains a geranylgeranyl lipid anchor (GG). When SNARE-GG is overexpressed, this inhibits exocytosis at a stage after SNARE complex assembly, possibly due to the generated membrane curvature blocking the merger of the secretory vesicle and plasma membrane. This inhibitory effect could be partially rescued by changing the curvature, by for example inserting an inverted cone–shaped lipid (e.g. lysophosphatidylcholine).

5.2.2 Palmitoylation

In contrast to prenylation, palmitoylation is reversible, allowing for a cycle of palmitoylation/depalmitoylation to occur in either a constitutive or regulated manner, such as in response to a specific extracellular signal (Fukata & Fukata, 2010). The palmitoylated proteins do not share an easily recognisable palmitate-binding motif, and palmitoylation can occur at the N-terminal, C-terminal or mid regions of proteins (Aicart-Ramos, *et al*., 2011; Baekkeskov & Kanaani, 2009; Resh, 2004). Palmitoylation often occurs at cysteine residues (S-palmitoylation), where a 16-carbon saturated fatty acid (palmitate) is added via a thioester linkage with the aid of the Golgi localized palmitoyl acyltransferase (PAT), an enzyme belonging to a DHHC (Asp-His-His-Cys) protein family. Although there is no known consensus sequence for palmitoylation, a pre-existing prenylation may promote the attachment of a palmitoyl motif. For instance, impaired Rac1 prenylation caused by mutation of cysteine to serine in the C*AAX* motif (S*AAX*), also prevents the incorporation of [H^3]-palmitic acid at other sites on the Rac1 protein. This leads to the mis-localization of Rac1 to the cytosol and nucleus, instead of the plasma membrane (Navarro-Lerida, *et al*., 2011). Depalmitoylation is carried out by an acyl protein thioesterase (APT), which releases protein from membranes in the cell periphery to traffic back to the Golgi membranes (Baekkeskov & Kanaani, 2009).

Disruption of palmitoylation prevents anterograde traffic to the target membrane. For example, the treatment of COS-7 cells and mouse embryonic fibroblasts (MEFs) with the palmitoylation inhibitor 2-bromo-palmitate (2BP) consistently led to an accumulation of GFP-tagged Rac in the perinuclear region, resulting in a partial loss from the plasma membrane and an exclusion from the nucleus (areas where the wild-type protein normally resides) (Navarro-Lerida, *et al*., 2011). In addition, this treatment abolished the attachment of a palmitate moiety to the protein in living cells (blocking incorporation of [H^3]-palmitate).

Furthermore, mutation of the conserved amino acid residue at position 178 in Rac1 from cysteine to serine ($Rac1^{C178S}$) prevented the incorporation of radiolabeled [H^3]-palmitate and plasma membrane localisation of GFP-tagged Rac1. The loss of Rac1 palmitoylation in $Rac1^{C178S}$ mutants affected cell motility, with mutants showing reduced cell spreading and delays in wound closure in scratch wound-healing assays (Navarro-Lerida, *et al*., 2011). In *Drosophila* palmitoyl transferase (Huntington-interacting protein 14, *hip14*) null mutants, the resulting loss of palmitoylation significantly impaired neurotransmitter release from neuromuscular junctions isolated from 3rd instar larvae as indicated by fluorescent dye uptake and electrophysiology studies (Ohyama, *et al*., 2007). The *hip14* mutant phenotype was linked to the mis-localization of two exocytic components, cysteine string protein (CSP) and SNAP25 at neural synapses, due to the loss of palmitoylation.

The equilibrium between palmitoylation and depalmitoylation is essential for the dynamic association of proteins with the plasma membrane, and for recycling back to the Golgi. A deficiency in the depalmitoylation enzyme palmitoyl thioesterase1, in *PPT1*-knockout neurons, evoked the retention of the palmitoylated SNARE proteins VAMP2 and SNAP25 on synaptic vesicles, making neural synapses non-responsive to depolarisation signals and stopping synaptic vesicles from releasing their cargo (Kim, *et al*., 2008). In humans, the essential role for depalmitoylation in the recycling of vesicular proteins was confirmed by the early onset neurodegenerative pathology detected in brain tissue from patients with infantile neuronal ceroid lipofuscinosis, a disease linked to a mutation in *PPT1* gene (Mitchison, *et al*., 1998; Vesa, *et al*., 1995). This indicated that palmitoylation, is as essential as prenylation for targeting of these proteins to the plasma membrane. Notably, palmitoylation favors association of proteins with lipid rafts (Delint-Ramirez, *et al*., 2011), which are specialized microdomains of plasma membrane containing distinctive arrangement of lipids and signaling molecules (Simons & Ikonen, 1997). It is this preferential association with lipid rafts which may bring palmitoylated proteins into close proximity with specific signaling cascades.

5.2.3 Multiple lipid modifications are associated with spatial and functional diversity in the Rho GTPase family

It has been previously proposed that prenylated *CAAX* proteins associate initially with the endomembrane prior to trafficking to the plasma membrane, and this process requires a secondary targeting motif (Choy, *et al*., 1999). Now there is emerging evidence that modification with different lipid moieties could localize a protein to different microdomains within the plasma membrane, thereby diversifying the functions within a protein family. In *Saccharomyces cerevisiae*, Cdc42 and Rho3 are found at distinct sites on the plasma membrane, with Cdc42 restricted to the tip of the bud in a budding yeast cell and Rho3 found across the plasma membrane with only a slight enrichment at the bud (Wu & Brennwald, 2010). These differences in localisation correlate with the unique functions of these two proteins in regulating polarized exocytosis and overall cell polarity (Wu & Brennwald, 2010). Curiously, both Cdc42 and Rho3 contain the C-terminal C*AAX* prenylation motif, with Cdc42 known to be geranylgeranylated and Rho3 predicted to be farnesylated (Moores, *et al*., 1991). However, Rho3 contains a long N-terminal extension with a cysteine residue that can be palmitoylated by the Ankyrin repeat-containing protein (Akr1) palmitoyl transferase, whereas Cdc42 does not (Wu & Brennwald, 2010). In yeast mutation studies, the diverse localization and functional differences for Rho3 and Cdc42 are

imparted by the N-terminal region, which contains palmitoylated residues (Wu & Brennwald, 2010).

Another member of the Rho family of GTPases is the Wnt-regulated Cdc42 homolog-1 (Wrch-1). Although sharing functional properties with Cdc42, it is also present on some intracellular membranes, which is distinct from Cdc42. Wrch-1 contains an unusual CCFV C-terminal motif, (Berzat, *et al.*, 2005). This non canonical CCFV motif of Wrch-1 more readily incorporated [^{3}H]-palmitate rather than isoprenyl moieties ([^{3}H]-FPP or [^{3}H]-GGPP). In addition, the plasma membrane localization of Wrch-1 was found to be more sensitive to a palmitoylation inhibitor (2-Bromo-palmitate) as opposed to prenyl trasferase inhibitors (GGTase I and/or FTase). Mutation of the second cysteine residue (CCFV to CSFV) demonstrated that the second cysteine of this non-conventional motif was palmitoylated, as CSFV-mutant Wrch-1 resulted in a failure to incorporate [^{3}H]palmitate, and HA-tagged mutant Wrch-1protein was re-distributed from the plasma membrane to the cytosol and nucleus (Berzat, *et al.*, 2005). Palmitoylation of the *CCFV*-motif on Wrch-1, in addition to other possible sequences within its C terminus, may serve as a secondary membrane targeting signal, which may define a distinct Wrch-1 plasma membrane localization, and thus account for its divergent roles in cell physiology.

In conclusion, prenylation, palmitoylation and depalmitoylation might be interdependent processes, and the precise control over these lipid modifications is critical for traffic to and from the plasma membrane and or the shuttling membrane and content between intracellular compartments (Fukata, *et al.*, 2006). The importance of this precise control is borne out by the many examples of defects in post-translational lipid modifications that are linked to neurodegenerative diseases, such as Schizophrenia, X-linked mental retardation, Huntington disease, as well as colorectal and bladder cancers (Buff, *et al.*, 2007; Fukata & Fukata, 2010; Williams, 1991). Despite significant progress in identifying the enzymes involved in post-translational lipid modification, their specific protein targets and the relationship between lipid modifications and membrane targeting remain unclear. Recent advances in proteomics together with improved bioinformatics prediction algorithms (CSS-Palm, PrePS) have shed some light on these lipid related post-translational modifications in some proteins (Adamson, *et al.*, 1992; Fukata, *et al.*, 2006; Kang, *et al.*, 2008; Shmueli, *et al.*, 2010), although they don't provide.

5.3 Phosphorylation as a means of controlling protein activity

In the past decade, a significant regulatory role has been revealed for post-translational phosphorylation as a means of controlling exocytosis. Phosphorylation involves the covalent addition of a phosphate group to a specific protein residue (namely serine, threonine, tyrosine or histidine), and is catalysed by one of the many protein kinases found within the cell (Alberts, *et al.*, 2002). Phosphorylation can affect a protein in two ways; conformational change that can alter the proteins activity through an allosteric affect; and the attached phosphate can form part of a recognition domain that facilitates protein-protein interactions. These types of modifications are transient, and are often followed by de-phosphorylation, where the phosphate can be removed by the catalytic activity of a specific phosphatase. The analysis of protein activities and interactions that are controlled by phosphorylation and de-phosphorylation has been empowered by technologies such as: genetic manipulation in animal models; the generation and expression of phosphomimetic mutant proteins; and the

generation of phosphospecific antibodies. This has facilitated the functional study of individual proteins as targets of particular protein kinases, whilst uncovering the significance of phosphorylation/dephosphorylation events in the regulation of exocytosis, such as in: synaptic transmission and cell plasticity (Amin, *et al*., 2008; Barclay, *et al*., 2003; Boczan, *et al*., 2004), neuronal morphogenesis (Chernyshova, *et al*., 2011), insulin secretion (Butelman, 1990; Sugawara, *et al*., 2009; Wang & Thurmond, 2010), insulin stimulated GLUT4 transport (Aran, *et al*., 2011; X. W. Chen, *et al*., 2011a; Sano, *et al*., 2011), mast cell and platelet degranulation (Fitzgerald & Reed, 1999; Foger, *et al*., 2011), exocytosis of factors required for neutrophil adhesion (Fu, *et al*., 2005), acrosomal exocytosis in sperm (Castillo Bennett, *et al*., 2010; Zarelli, *et al*., 2009) and lung surfactant exocytosis (Gerelsaikhan, *et al*., 2011). It has now become obvious that phospho-regulation of exocytosis is a complex and dynamic process implicated at almost all points along the exocytic route, from recruitment and transport of vesicles to their ultimate fusion at the plasma membrane (Figure 1&2).

5.3.1 Phospho-regulation of small GTPases, their effectors and regulators

Recruitment and tethering of secretory vesicles that are destined to fuse with the plasma membrane involves GEFs, GAPs and small GTPases, as well as their effector proteins, all of which have been reported to undergo phospho-regulation by a number of different kinases. Phosphorylation appears to add an additional level of regulation to small GTPases beyond the GTP/GDP cycle (see 5.1 section above) and lipid modification/s (see 5.2 section above) modulating the intracellular localization of the protein and its function.

Aikawa and Martin identified a role for the plasma membrane bound GTPase Arf6 in specifying exocytosis competent plasma membrane microdomains (Aikawa & Martin, 2003). This process depends on the site-specific localization of the phospholipid kinase phosphatidylinositol 4-phosphate 5-kinase (PIP_5K), which itself is regulated by phosphorylation, and is stimulated by agonists and cell stresses. Arf6 regulates various cellular functions by activation of PIP_5K including the exocytosis of insulin and dense core vesicles, and neurotransmitter release (Funakoshi, *et al*., 2011). Arf6 interacts with the dephosphorylated form of PIP_5Kc in PC12 cells when stimulated by depolarization of the cell (Aikawa & Martin, 2003). This interaction activates PIP_5Kc resulting in increased levels of the lipid messenger phosphatidylinositol 4,5-biphosphate (PI(4,5)P) at the plasma membrane, triggering exocytosis of dense core vesicles (Aikawa & Martin, 2003; Funakoshi, *et al*., 2011).

Altered localization due to phosphorylation has been shown for the Rab3A and Rab27A effector, Rabphilin. Rabphilin, together with Rab3A and Rab27A, has been implicated in the modulation of the docking step for dense core and synaptic vesicles, destined for exocytosis (Deak, *et al*., 2006; Lin, *et al*., 2007; Tsuboi & Fukuda, 2006). The modulation of this docking step has recently been attributed to the interaction of Rabphilin with the plasma membrane SNARE protein, SNAP25 (Tsuboi, *et al*., 2007; Tsuboi, 2009). Rabphilin can be phosphorylated by the kinases PKA, PKC and CaMKII. Further investigation revealed that calcium-mediated phosphorylation of non-vesicle bound Rabphilin at Ser234 and Ser 274 reduced its affinity for the vesicular membrane. Therefore, phosphorylation of Rabphilin modulates its membrane localization during synaptic transmission (Foletti, *et al*., 2001; Lonart & Sudhof, 2001).

Recently, studies of pancreatic, colorectal, and other cancers identified RalGEFs as key effectors of Ras signaling cascades (Feldmann, *et al.*, 2010; Neel, *et al.*, 2011). Phosphorylation of constitutively active RalA ($RalA^{G23V}$) at a conserved C-terminal S194 residue, by Aurora A kinase, promotes collagenI-induced cell motility and anchorage-independent growth in MDCK epithelial cells (J. C. Wu, *et al.*, 2005). This phosphorylation caused RalA relocation from the plasma membrane to endomembranes, where RalA associated preferentially with its effector Ral binding protein 1 RalBP1/RLIP76, thereby promoting cell motility during human cancer cell metastasis (Lim, *et al.*, 2010; Z. Wu, *et al.*, 2010). Similarly, phosphorylation of RalB by PKC at the C-terminal S198 residue led to its translocation from the plasma membrane to the perinuclear region of the cell. S198 phosphorylation of a constitutively activated G23V RalB mutant enhanced tumor growth in experimental lung metastasis of T24 or UMUC3 human bladder cancer cells (Wang, *et al.*, 2010).

RalA and RalB are present on synaptic vesicles, platelet granules and glucose transporting GLUT4 vesicles, and are involved in the assembly of the octameric exocyst complex. During insulin stimulated GLUT4 vesicle transport, phosphorylation of the RalGAP complex by protein kinase Akt2 (downstream of PI3-Kinase) relieves its inhibitory effect on RalA activity, and thereby increases GLUT4 exocytosis at the plasma membrane (Chen, *et al.*, 2011b; Chen & Saltiel, 2011). Intriguingly, phosphorylated RalGAP complex was shown to be capable of binding RalA *in vitro*, and the authors suggested that in cells this phosphorylation may act through sequestering RalGAP in the cytoplasm, away from site of RalA action (X. W. Chen, *et al.*, 2011b). Once activated, GTP-bound RalA protein interacts with Sec5 protein to assemble vesicular and plasma membrane subunits of the exocyst complex. Protein kinase C (PKC) then promotes exocytosis though the phosphorylation of the G-protein binding domain of Sec5 to disengage RalA from the exocyst, allowing GLUT4 exocytosis in adipocytes (X. W. Chen, *et al.*, 2011a). Phosphorylation has been implicated in the modulated function of another exocyst subunit, Sec8 (B. H. Kim, *et al.*, 2011). The FGF receptor mediated tyrosine phosphorylation of Sec8 was required for efficient recruitment of the exocyst complex to growth cones of growing neurites. However, the exact mechanism by which phosphorylation modulates Sec 8 function still remains to be elucidated.

In a similar scenario to the RalGAP complex, phosphorylation of the RabGAP, AS160, relieves its inhibitory activity on Rab10. Insulin activates the PI3 kinase/Akt signaling pathway where Akt phosphorylates AS160 and inhibits its GAP activity, leading to increased levels of active Rab10-GTP and triggering movement of GLUT4 containing vesicles to the plasma membrane ready for fusion (Sano, *et al.*, 2011). Vasopressin–stimulated activation of Akt leads to phosphorylation of AS160 at Ser473 in kidney collecting duct cells, and this phosphorylation enhances the translocation of the Aquaporin water channel (AQP2) to the apical membrane (H. Y. Kim, *et al.*, 2011). Although it is clear that phosphorylation plays a role in modulating GAP function, its role in modulating GEF function remains unclear and there are relatively few examples in the literature (Schmidt & Hall, 2002). However, phosphorylation of the GEF Vav by Src and Syk tyrosine kinases, has been shown to result in stimulation of its catalytic activity (Aghazadeh, *et al.*, 2000). Phosphorylation of Tyr174 induced a conformational change in Vav's N-terminal region, subsequently allowing access to Rac GTPase.

Rho GTPases, particularly Rac2, also play a crucial role in actin remodeling in response to pathogen invasion, regulating neutrophil degranulation, and the release of antimicrobial

mediators. A recent proteomic analysis of Rac2-mediated degranulation in neutrophils identified several actin-binding proteins as novel downstream effectors of this pathway, including Coronin1A. This Rac2 function is mediated by phosphorylation of Coronin1A at Thr418, after secretagogue-stimulation (Eitzen, *et al*., 2011). Phosphorylation of Coronin1A at Ser2 has also been implicated in modulating degranulation in mast cells (Foger, *et al*., 2011).

Thus, specific phosphorylation events modulate the engagement and disengagement of small GTPases with their regulators and effector proteins, adding an additional tier of temporal and spatial control over this vesicular machinery.

5.3.2 Phospho-regulation of SNARE complex assembly

There is emerging evidence to suggest that phosphorylation plays a significant role in the regulation of the final steps of exocytosis through the membrane bound tethering of SNARE family proteins and their accessory proteins (Figure 1&2). For example, phosphorylation modulates the association of SNARE accessory proteins with v- and t-SNAREs adding another level of regulation to SNARE complex formation.

A number of phosphorylation events have been found to regulate the interaction of the SNARE accessory proteins Sec1/Munc18c with SNARE proteins. In most, but not all systems, Munc18 has an inhibitory role in SNARE complex formation, and phosphorylation of the Munc18 isoforms by different kinases alleviates this inhibitory role. Exocytosis of Weibel-Palade bodies (WPBs) by endothelial cells in response to inflammatory mediators, such as Thrombin, is important for initiating the adhesion of neutrophils. Thrombin, acting in conjunction with an influx of intracellular calcium, activates PKCα, resulting in the phosphorylation of Munc18c and Syntaxin4. This leads to the dissociation of the inhibitory complex, allowing SNARE complex formation and fusion of WPBs at the plasma membrane (Fu, *et al*., 2005). In another cell system, phosphorylation of Munc18c at Tyr521 by the insulin receptor tyrosine kinase in pancreatic β-cells was shown to inhibit its interaction with Syntaxin4 and VAMP2 (Aran, *et al*., 2011). Similarly, in neurotransmitter release, phosphorylation of Munc18a at Ser 306 and Ser313 by PKC reduces its affinity for Syntaxin and the phosphorylation state of Ser 313 alone was sufficient to alter the dynamics of vesicle release events (Barclay, *et al*., 2003). In support of the importance of Ser313 phosphorylation, the phosphomimetic mutant S313D increased vesicle docking and enhanced the ready releasable vesicle pool in adrenal chromaffin cells (Nili, *et al*., 2006). In contrast to these examples of Munc18 as a negative regulator of SNARE complex formation, there have been reports of Munc18 promoting fusion by stabilising SNARE complex formation. In parietal epithelial cells, gastric acid exocytosis involved Cdk5 phosphorylation of Munc18b at Thr572, which enhanced its association with the Syntaxin3 - SNAP25 complex, and facilitated vesicle docking and fusion (Y. Liu, *et al*., 2007). This functional difference might be attributed to the distinct modes by which Munc18 binds with SNARE proteins. In its inhibitory role, Munc18 binding to the SNARE motif of monomeric Syntaxin disables SNARE complex formation. In its fusion promoting role, the N-terminal domain of Munc18 binds to the N-terminal Habc-domain of Syntaxin, which allows it to fold back onto the SNARE complex. When v-SNARE proteins are present, Munc18 exhibits a binding capacity that enables it to clasp the SNARE complex and promote fusion (reviewed in (Burgoyne, *et al*., 2009; Sudhof & Rothman, 2009)). It is obvious that phosphorylation is an important modulator of Munc18 and that Munc18 activity can differ depending on the isoform and cell system involved.

In addition to promoting Munc18 and Syntaxin interaction, Cdk5 can also phosphorylate the filamentous protein Septin5 at Ser327 and Ser161, with the former site resulting in dissociation of Septin5 from Syntaxin at the synapse (Amin, *et al*., 2008). It has been suggested that Septin5 filaments may act as tethers that prevent access of exocytic vesicles to the membrane, until there is appropriate signaling to fuse and release their contents (Beites, *et al*., 2005). Phosphorylation of the Ser327 and Ser161 sites can affect Septin–Septin filament assembly (Amin, *et al*., 2008), and Cdk5 phosphorylation of Septin5 results in its dissociation from the vesicle associated protein Synapsin. This may play an important role in modulating vesicle interaction with the SNARE complex (Amin, *et al*., 2008).

Syntaphilin, a Syntaxin1 clamp that controls SNARE complex assembly, competes with SNAP25 for Syntaxin1 binding, and inhibits SNARE complex formation by regulating the availability of free Syntaxin during synaptic vesicle exocytosis. PKA phosphorylation of Syntaphilin at Ser43 in PC12 cells modulates its interaction with Syntaxin1 and annuls its inhibitory affect on SNARE complex formation and exocytosis (Boczan, *et al*., 2004). Another SNARE regulatory protein that interacts with Syntaxin1 and inhibits SNARE complex formation is Tomosyn (Fujita, *et al*., 1998). PKA phosphorylation of Tomosyn at Ser724 reduces its association with Syntaxin1 and promotes SNARE complex formation increasing the size of the readily releasable pool of synaptic vesicles in neurons (Baba, *et al*., 2005). In contrast, phosphorylation of Syntaxin1 by the Rho-associated coiled-coiled forming kinase (ROCK) at Ser14, positively regulates the Syntaxin1-Tomosyn association by increasing Syntaxin1 affinity for Tomosyn. This inhibits SNARE complex formation during neurite extension in hippoccampal cultured neurons (Sakisaka, *et al*., 2004).

Protein phosphorylation of the membrane fusion machinery plays a central role sperm activation in keeping acrosomal exocytosis on hold until the sperm contacts the egg. N-ethylmaleimide-sensitive factor 5 (NSF5), which is necessary for the disassembly of fusion incompetent cis-SNARE complexes, and the calcium sensor SynaptotagminVI, are phosphorylated in resting sperm and inactive. Dephosphorylation of NSF5 by protein-tyrosine phosphatase PTP1B results in its activation and allows disassembly of cis-SNARE complexes, a requirement for subsequent fusion competent trans-SNARE complex assembly and membrane fusion (Zarelli, *et al*., 2009). Similarly dephosphorylation of SynaptotagminVI by the calcium-dependent phosphatase Calcineurin, was shown to be a requirement for acrosomal exocytosis (Castillo Bennett, *et al*., 2010). Snapin is a SNARE-binding protein that enhances the association of Synaptotagmin with the SNARE complex. Phosphorylation of Snapin at Ser50 by PKA enhances its interaction with SNAP25 and resulted in increased binding of Synaptotagmin to the SNARE complex in chromaffin cells (Chheda, *et al*., 2001). In contrast to Snapin, phosphorylation of the synaptic vesicle membrane protein CSP (a cysteine string protein) by PKA (on Ser10) inhibited its interaction with Syntaxin and Synaptotagmin (Evans, *et al*., 2001; Evans & Morgan, 2002). More recently synapse specific localisation of phosphorylated CSP was shown in rat brain suggesting a role in synapse specific regulation of neurotransmitter release (Evans & Morgan, 2005).

There are clearly a multitude of SNARE accessory proteins that regulate SNARE complex assembly, particularly in the central nervous system. Phosphorylation/dephosphorylation modulates their association with SNARE complex proteins and this involves a number of signaling pathways.

5.3.3 Phospho-regulation beyond fusion

A recent and elegant *intra-vital* study demonstrated a role for filamentous actin and non-muscle Myosin2 in the secretion of cargo from vesicles. In the salivary glands of transgenic mice, real-time live imaging of secretory vesicles showed that shortly after a vesicle docks at the plasma membrane it becomes coated with actin filaments (Masedunskas, *et al.*, 2011). Furthermore, it was found that to release the content of these vesicles, Myosin2a and 2b recruitment and activity were required to provide the contractile force necessary to complete fusion of the vesicle with the plasma membrane (Masedunskas, *et al.*, 2011). Similar observation has been reported for the secretion of von Willebrand factor from human endothelial cells (Nightingale, *et al.*, 2011).

Phospho-regulation of the non-muscle associated actin molecular motor, Myosin2, plays a role in modulating its function at the post fusion level during exocytosis. Inhibition of phosphorylation by the myosin light chain kinase (MLCK) at sites Tyr18 and Ser19 of Myosin2, slowed down the opening of the fusion pore, during the release of catecholamines and peptide transmitters in chromaffin cells (Doreian, *et al.*, 2008; Doreian, *et al.*, 2009; Neco, *et al.*, 2008). Similarly, phosphorylation at Ser 19 of Myosin2 by MLCK was shown to be necessary for maintaining the opening of the fusion pore in pancreatic cells (Bhat & Thorn, 2009). In addition to Myosin2, phosphorylation by PKC of Myristoylated alanine-rich C-kinase substrate (MARCKS), another actin-associated protein, has been implicated in regulating the activity dependent rearrangement of the actin cytoskeleton. Phosphorylation of both MARCKS and Myosin2 have been implicated in modulating the transition from an omega kiss-and-run mode of exocytosis involving a narrow fusion pore, to the full granule collapse mode involving fusion pore expansion (Doreian, *et al.*, 2009).

5.3.4 Multiple phosphorylation sites modulated by single or multiple kinases

There is increasing evidence of multiple phosphorylation sites within individual proteins, which are phospho-regulated by single or multiple kinases/phosphatases. This brings to light the intriguing possibility of a convergence of different signaling events on key components of the vesicular machinery. The dynamic interplay of phosphorylation events can alter a proteins' physiological function. An example of this is phosphorylation of the Rab11 effector, Rab11 interacting protein (Rip11), where phosphorylation at Ser 357 by PKA modulates the recruitment of insulin granules to the plasma membrane (Sugawara, *et al.*, 2009). In addition, a non-PKA dependent phosphorylation at a serine/threonine site by an as yet unidentified kinase was shown to be important for its role in apical membrane recycling in MDCK cells (Prekeris, *et al.*, 2000; Sugawara, *et al.*, 2009). Phosphorylation of SNAP25 by the kinases PKA (at Thr138) and PKC (at Ser187) respectively modulated the size of the releasable vesicle pool and rate of refilling after the pools were emptied in chromaffin cells (Nagy, *et al.*, 2004). Similarly, differential phosphorylation of the synaptic vesicle protein Synapsin has been implicated in modulating its various roles including neurotransmitter release, vesicle clustering, maintaining the reserve pool, and vesicle delivery to the active zones. These processes are regulated via a dynamic phospho-regulation cycle which involves multiple phosphorylation sites and several kinases including cAMP-dependent protein kinase A, PKA (at site 1 (Ser9)) (Angers, *et al.*, 2002; Menegon, *et al.*, 2006), Ca2+/calmodulin-dependent kinase CaMKII and VI (at sites 1, 2 and 3 (Ser9, Ser566 and ser603)) (Chi, *et al.*, 2003), mitogen-activated kinase MAPK (sites 4, 5, 6

and 7 (ser62, Ser67 and Ser549 and Ser551)) (Chi, *et al*., 2003; Giachello, *et al*., 2010), and tyrosine kinase Src (site 8 (Tyr301)) (Messa, *et al*., 2010). Phosphorylation on serine residues upon activation of PKA, CaMK and MAPK signaling pathways, promotes the dissociation of Synapsin from synaptic vesicles and/or the actin network which results in trafficking of synaptic vesicles from the reserve pool to the ready releasable pool for exocytosis (Chi, *et al*., 2003; Giachello, *et al*., 2010; Menegon, *et al*., 2006). In contrast, Src kinase-mediated phosphorylation of Synapsin enhances its oligomerization and increases its association with synaptic vesicles and the cytoskeleton, stimulating the re-clustering of recycled vesicles and subsequent recruitment to the reserve pool (Messa, *et al*., 2010). In addition, phosphorylation at different Synapsin sites can occur concurrently through the selectivity of kinase/phosphatase activation, which is dependant on the stimulus and the signaling pathways implicated. For example, in synaptosomal preparations, calcium entry stimulated bidirectional phospho-regulation of Synapsin involving phosphorylation at CaMKII dependent and PKA dependent sites and dephosphorylation at MAPK/Calcineurin sites (Cesca, *et al*., 2010; Jovanovic, *et al*., 2001; Yamagata, *et al*., 2002). Synapsin phospho-regulation involving multiple signaling pathways allows Synapsin to control synaptic vesicle mobilisation and trafficking.

Phosphorylation at different sites within an individual protein can also modulate a proteins' function through the differential kinetics of phosphorylation/dephosphorylation events, as in the case of Rabphilin. Rab3A recruits Rabphilin to synaptic vesicles where it can undergo phosphorylation during membrane depolarisation stimulated calcium influx. Rabphilin Ser234 and Ser274 are phosphorylated by different kinases (PKA for the former site and PKA, PKC, CaMKII for the latter) and can show distinct regulatory effects on Rabphilin during synaptic transmission, depending upon the extent of phosphorylation at these two sites and the kinetics of dephosphorylation after stimulus removal (Foletti, *et al*., 2001).

Regulation of a proteins' function by phosphorylation may occur in a hierarchical sequence. For instance, RhoGDI undergoes sequential phosphorylation in response to glucose stimulation in β-cells (Wang & Thurmond, 2010). The first phosphorylation event involves Tyr156 and coincides with RhoGDI-Cdc42 dissociation. This is then followed by Ser101/ Ser174 phosphorylation coinciding with RhoGDI-Rac1 dissociation. The sequential phosphorylation of RhoGDI allows differential temporal activation of the Rho GTPases, Cdc42 and Rac1, during insulin secretion from pancreatic β-cells. (Wang & Thurmond, 2010). The yeast tethering Rab GTPase, Sec4, has multiple phosphorylation sites (Ser8, Ser11, Ser201 and Ser204), and phosphorylation of the N-terminal serines (Ser8, Ser11) prevents binding to its effector, the Sec15 exocyst subunit, and hinders polarised exocytosis (Heger, *et al*., 2011). The authors also identified protein phosphatase 2A (when containing the regulatory subunit Cdc55) as the phosphatase responsible for alleviating the inhibitory affect of Sec4 phosphorylation (Heger, *et al*., 2011). Structural analysis of Sec4 suggests a clustering and physical proximity of the N- and C-terminal phosphorylation sites. This has led the authors to postulate that the impact of the N-terminal serines can be modulated by phosphorylation at the C-terminal serines and may involve phosphorylation at these sites in a hierarchical manner (Heger, *et al*., 2011), although further studies are required to verify this assertion.

Finally, the unique combination of tandem phosphorylated sites within a protein can act as recognition sites for phosphoprotein-binding proteins, such as 14-3-3. An increasing number of proteins are being identified with tandem sites that can act as 14-3-3 dimer binding sites

(Chen, *et al*., 2011). These sites can be phosphorylated by distinct protein kinases and the combination of phosphorylated sites can alter the effect of 14-3-3 on the protein target. 14-3-3 has been shown to bind to the Rab GAPs, AS160 (Ser341 and Thr642) and TBC1D1 (Ser237 and Thr596), which are involved in the regulation of GLUT4 trafficking to the plasma membrane, and 14-3-3s' interaction with these two proteins occurs in response to insulin and energy stress respectively (reviewed (Chen, *et al*., 2011)). 14-3-3 binding sites have been identified on Rab3A effectors - the Rab3A interacting molecules Rim1 and Rim2 and Rabphilin3 (Sun, *et al*., 2003). The physiological relevance of the 14-3-3 interaction with these proteins in neuroendocrine exocytosis and synaptic transmission is still being investigated.

Exocytosis requires the orchestrated actions of distinct exocytic machinery that is governed by multiple signaling pathways, and this occurs in a temporally and spatially regulated manner that is dependent on the types of cells and their stimuli. Phospho-regulation of the exocytic machinery is one of the mechanisms by which the cell coordinates the function of these proteins during exocytosis and it is implicated at each stage of the process. It is clear from the literature that the proteins implicated in exocytosis, which contain multiple phosphorylation sites, can be differentially modulated by single or multiple kinases. With the identification of an increasing number of phosphorylation targets and the elucidation of the precise functional roles of the exocytic machinery, the physiological significance of these phospho-regulation events may be determined. A huge task lays ahead to delineate the functional role of each phosphorylation site for all the exocytosis players and then integrate this information into a comprehensive model that can define the signaling pathways that are responsible for modulating these events.

6. Ubiquitin and small ubiquitin-like modifier in exocytosis

6.1 Ubiquitination

Post-translational modification with ubiquitin has also been recognised as an important sorting signal on cargo transported by the endosomal network (specifically as a signal for internalisation), particularly at the late endosome (LE)/multivesicular body (MVB) and at the trans-Golgi apparatus. For example, at the LE/MVB, the proteins that make up the endosomal sorting complex required for transport (ESCRT) machinery (ESCRT I and ESCRT II) are known to contain ubiquitin-binding domains that enable them to recognise ubiquitinated cargo proteins and sort them into internalised vesicles destined for lysosomal degradation or for secretion events (reviewed in (Hurley, 2010)).

Ubiquitin has an established role in regulating protein relocation and targeted destruction at the proteasome (see (Hershko & Ciechanover, 1998; Hershko, 2005) for some excellent reviews). The three Rho GTPases, Rho1/A, Rac1, and Cdc42 have now been demonstrated to be ubiquitinated and degraded under certain stimuli (de la Vega, *et al*., 2011) (Figure 1&2). Furthermore, both Rac1 and Cdc42 ubiquitination and protein levels are increased when cells are treated with protease inhibitors (Doye, *et al*., 2006). Inactive Rho1/A was shown to be ubiquitinated by the E3 ubiquitin ligase Smurf1 and degraded in migrating Mv1Lu epithelial cells (H. R. Wang, *et al*., 2003). Ubiquitination of Rho1/A may be required to prevent the Rho1/A mediated formation of actin stress fibres at the leading edge of migrating cells, and to allow the Cdc42 and Rac1 mediated dynamic actin rearrangement necessary for anterograde delivery of membranes to the leading edge of migrating cells (Y. Wang, *et al*., 2003). This site

specific ubiquitination/degradation of Rho1/A appears to be restricted to the lamellipodia and filopodia of the leading edge, where Smurf1 is recruited through atypical protein kinase C zeta (aPKC)-mediated phosphorylation (H. R. Wang, *et al.*, 2003). The latter is activated by the Cdc42/Rac1 polarity complex (H. R. Wang, *et al.*, 2003). Hence the polarised exocytosis during cell migratory activity is regulated by a hierarchy of post-translational modifications, where ubiquitination of Rho1/A appears to be important for switching between the competing actin modifying activities of Rho1/A and Cdc42/Rac1, which in turn controls phosphorylation dependent ubiquitination activity of Smurf, and thereby Rho degradation. A further level of complexity is added by the down-regulation of Rho1/A in migrating cells by another E3 ligase, the Cul3/BACURD complex (Chen, *et al.*, 2009). The Cul3/BACURD complex is a ring finger E3 ubiquitin ligase complex that has been shown to ubiquitinate Rho1/A in a diverse range of organisms from human cell lines (293T and HeLa fibroblasts), insect cells (*Drosophila melanogaster* S2 cells) and amphibians (*Xenopus laevis* embryos). Depletion of the Cul3 and BACURD ligase complex by siRNA results in defective migration of HeLa cells and mouse embryonic fibroblasts, and in embryonic abnormalities resulting from defective cell migration in *Xenopus* embryos (Chen, *et al.*, 2009). There is also evidence for the ubiquitination of Rac1 by the ubiquitin E3 ligase POSH2 but the purpose of this ubiquitination is not yet clear (Karkkainen, *et al.*, 2010). A proteasomal degradation resistant and thus constitutively active mutant of Rac1 (Rac1b), is found in colorectal and breast cancer tumour cells (Jordan, *et al.*, 1999; Schnelzer, *et al.*, 2000). Interestingly, RNAi mediated silencing of this mutant results in a failure of cancer cells to undergo an epithelial to mesenchymal transition (Radisky, *et al.*, 2005) suggesting a role for ubiquitination of Rac1 in controlling cell motility (Visvikis, *et al.*, 2008).

Rho GTPases can also be regulated by the ubiquitination of their GEF activators. Activation of Rho1/A via ubiquitination of PDZ-RhoGEF, was found to be initiated by Cul3/KLHL20 (Lin, *et al.*, 2011). Likewise, Cdc42 can be activated via ubiquitination of its GEF, hPEM-2 (Yamaguchi, *et al.*, 2008). It is yet to be established whether Smurf–mediated regulation these two Rho GTPases occurs in a coordinated manner. In addition to Smurf, Cdc42 activity could be regulated by ubiquitination and or proteasomal degradation of its GEFs, FGD1 and FGD3 by E3 ligase $SCF^{FWD1/\beta\text{-}TrCP}$ (Hayakawa, *et al.*, 2005; Hayakawa, *et al.*, 2008). There is an interesting interplay between the regulatory effects of ubiquitination and phosphorylation. At the leading edge of migrating cells, $SCF^{FWD1/\beta\text{-}TrCP}$ ligase recognises only forms of GEFs inactivated by GSK-3β kinase phosphorylation; the latter kinase could in turn be inactivated by aPKC-mediated phosphorylation (Etienne-Manneville & Hall, 2003; H. R. Wang, *et al.*, 2003). Therefore, phosphorylation by aPKC appears to be at the nexus of regulation of ubiquitination of small Rho GTPases, promoting degradation in Rho1/A and preventing it in CDC42. Finally, two of the small Rab GTPase GEFs, Rabex5 and Rabring7 (Xu, *et al.*, 2010; Yan, *et al.*, 2010) are known to have ubiquitin E3 ligase activity (Sakane, *et al.*, 2007), and Rabex5 cellular localisation is regulated by its ability to bind a ubiquitin signal (Mattera, *et al.*, 2006). As yet there are no known Rab proteins that are themselves ubiquitinated.

Ubiquitination of the negative regulators of small Rho GTPases, RhoGDIs, has also been shown. RhoGDI is ubiquitinated by the E3 ubiquitin ligase GRAIL which, while not resulting in its proteasomal degradation, did appear to increase the stability the RhoGDI protein (Su, *et al.*, 2006). This results in sequestration of Rho molecules in the cytosol, blocking their activation and initiation of the Rho signaling pathway, and thereby impairing cytoskeletal polarization or actin polymerization. It is yet to be defined why, in the context

of GRAIL-mediated ubiquitination, RhoGDI inhibition is restricted to Rho1/A, but not Rac1 or Cdc42 (Su, *et al*., 2006).

6.2 Sumoylation

Sumo is a small ubiquitin-like modifier that, like ubiquitin, can be covalently attached to a protein via an internal lysine residue on the target and can serve to modify its function (for recent reviews see (Wang & Dasso, 2009; Wilkinson & Henley, 2010)). Sumoylation is emerging an additional level of control over the proteins that regulate exocytosis (Figure 2). At least two SNARE proteins are believed to be sumoylated. Sumoylation of the SNARE accessory protein, Tomosyn, relieves its inhibitory effect on SNARE complex assembly, and thereby on exocytosis (Williams, *et al*., 2011). In response to Ca^{2+} signaling, sumoylation is known to inhibit exocytosis of insulin granules following their docking at the plasma membrane, and this is most likely to occur through SynaptotagminVII (Dai, *et al*., 2011). In addition, the Rho GTPase Rac1 was found to be sumoylated in response to hepatocyte growth factor stimulation of a number of cell lines (HEK293T, MDCKII, HeLa, and Cos7 cells (Castillo-Lluva, *et al*., 2010)). Sumoylation of Rac1 resulted in sustained activation of Rac1 which promoted the formation of lamellipodia and cell motility (Castillo-Lluva, *et al*., 2010). Sumoylation as a post-translational modification of the proteins in the exocytic pathway is a new field of research and will undoubtedly be found to regulate many more of these proteins.

7. Concluding remarks

Here we have illustrated that there is a complex array of specialist molecular machinery that is used to control each step in the process of exocytosis. Emerging evidence suggests that there is a highly organised regulatory network required to achieve control of exocytosis. This involves the post-translational modification of the vesicular machinery and membrane associated proteins that orchestrate exocytosis; including the addition of lipid moieties, phosphorylation, and ubiquitination and sumoylation. These post-translational modifications are responsible for mediating protein intracellular localization, protein-protein interactions, complex assembly, and ultimately protein function. The dynamics and precision of exocytosis often require multiple modifications of a single protein in order to tightly control temporal/spatial function. Moreover, to ensure the harmonious reaction of the cell to a specific stimulation, these post-translational modifications respond to a variety of cell-type specific signaling events. The challenge facing researchers in this field is to investigate the cross-talk between different modifications in the context of a specific signal, and to determine how these are coordinated with other cellular functions. Thus, it is tempting to speculate about an even higher point of control in the regulation of exocytosis, involving proteins that recognise post-translational modifications and facilitate appropriate functional interaction.

8. References

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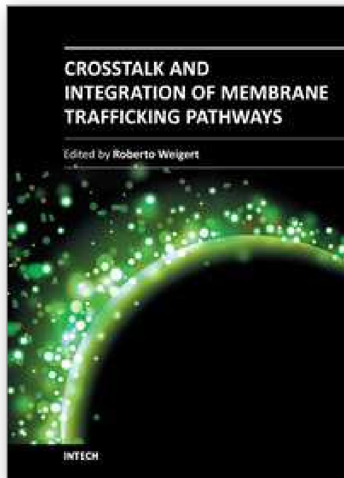

Crosstalk and Integration of Membrane Trafficking Pathways

Edited by Dr. Roberto Weigert

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Membrane traffic is a broad field that studies the complex exchange of membranes that occurs inside the cell. Protein, lipids and other molecules traffic among intracellular organelles, and are delivered to, or transported from the cell surface by virtue of membranous carriers generally referred as "transport intermediates". These carriers have different shapes and sizes, and their biogenesis, modality of transport, and delivery to the final destination are regulated by a multitude of very complex molecular machineries. A concept that has clearly emerged in the last decade is that each membrane pathway does not represent a close system, but is fully integrated with all the other trafficking pathways. The aim of this book is to provide a general overview of the extent of this crosstalk.

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