The general aim of this thesis was to uncover molecular mechanisms that are critical in regulated secretion of large dense-core vesicles in adrenal CCs: from the biogenesis of vesicles at the ER and the Golgi apparatus to their final destiny at release sites at the PM. We have focused our efforts on the role of Vti1a in the biogenesis of secretory vesicles and the Munc18-1 dependent regulation of the cortical F-actin network.

Biogenesis and maturation of secretory vesicles are key aspects of the first experimental chapter. We identified Vti1a, thus far known as endosomal SNARE protein (Kreykenbohm et al., 2002; Mallard et al., 2002) and suggested to play a functional role in spontaneous release in neurons (Ramirez et al., 2012), as essential regulator of secretory vesicles biogenesis in adrenal CCs. We showed that the impaired secretion, which was observed in the absence of \textit{vti1a}, was not attributable to a function of Vti1a in steps prior to fusion, such as docking and priming, or fusion itself. Instead, the deletion of \textit{vti1a} resulted in a decrease in the number of secretory vesicles, which led to the conclusion that Vti1a plays an essential role in the biogenesis of secretory vesicles (Chapter 2).

In the studies described in the following chapters, we focused on regulated exocytosis and PM localized processes. We first developed an automatic and therefore user-bias free algorithm that allowed us to quantify PM localized fluorescent signals in a highly objective and less time-consuming manner than previously described methods. In Chapter 3 we present this algorithm called PlasMACC and a selection of potential applications. The comparison between PlasMACC and earlier methods illustrated not only its functionality, but also emphasized improvements.

In Chapter 4, we used PlasMACC to quantify Munc18-1 dependent cortical F-actin alterations. We confirmed the increase in the cortical F-actin network in the absence of \textit{munc18-1}, which was described earlier (Toonen et al., 2006; Kurps and de Wit, 2012; Kurps et al., 2014). Due to the improved resolution of this analysis method, we were able to identify Munc18-1s domain 3 in general and amino acid V263 specifically as essential for Munc18-1s role in F-actin regulation. Alignment studies led us to conclude that especially the hydrophobicity at this position is a necessary requirement to facilitate Munc18-1 dependent cortical F-actin regulation.
Finally, we suggested a potential role of the PM localized phosphoinositide PI(4,5)P$_2$ in functional linking the SM protein Munc18-1 to the subplasmalemmal F-actin network. We found an increase in PI(4,5)P$_2$ levels on the PM of munc18-1 null CCs compared to wild type CCs. We suggest that the observed F-actin phenotype in munc18-1 null CCs is a result of the dysregulation of the lipid metabolism in the absence of munc18-1 (Chapter 5).

Munc18-1 dependent cortical F-actin regulation and the role of PI(4,5)P$_2$

*Munc18-1 Interactome:*
*An overview of candidates connecting Munc18-1 to PI(4,5)P$_2$ and/or F-actin*

Munc18-1 interacts with several proteins that can be linked directly or indirectly to PI(4,5)P$_2$ and/or the actin cytoskeleton (fig6.1). The interaction between Munc18-1 and the SNARE protein Syntaxin1a is the most studied and best understood. The only hint that suggest a link between Syntaxin1a and the phospholipid PI(4,5)P$_2$ is their co-existence in clusters in lipid membranes. This electrostatic interaction was shown in synthetic lipid rafts, giant unilamellar vesicles (GUVs) and membrane sheets of PC12 cells, but not in intact cells (Murray and Tamm, 2009; van den Bogaart et al., 2011). Furthermore, PI(3,4,5)P$_3$ has an even higher ability to induce Syntaxin1a clusters than PI(4,5)P$_2$; in GUVs as well as in *Drosophila melanogaster* neuromuscular junction (NMJ) boutons (Khuong et al., 2013). The binding between Syntaxin1a and Myosin Va is a direct link between the fusion machinery and the cytoskeleton (Watanabe et al., 2005). This interaction was shown to be essential for the transport of secretory vesicles to their release sites, but not for the regulation of the subplasmalemmal F-actin network. However, the involvement of the interaction between Munc18-1 and Syntaxin1a in the Munc18-1 dependent regulation of the levels of PI(4,5)P$_2$ or the cortical F-actin network seems rather unlikely, since there are no strong functional links between Syntaxin1a and the lipid metabolism or the cytoskeleton. Also the existence of a Munc18-1 mutant (Munc18-V263T) that, when expressed in munc18-1 null MCCs, rescues the Syntaxin1a phenotype (50% reduction of Syntaxin1a and equal distribution between cytoplasm and PM in munc18-1 null MCCs (Voets et al., 2001; Kurps et al., 2014), but not the cortical F-actin phenotype, strengthens the assumption that Syntaxin1a is not involved in the Munc18-1 dependent PI(4,5)P$_2$ or F-actin regulation (Chapter 4). The expanded F-actin network that is observed in the munc18-1 null CCs cannot be explained via the interaction of Syntaxin1a with Munc18-1.

The Ca$^{2+}$ binding protein 5 (CaBP5) is a neuronal calmodulin-like Ca$^{2+}$ binding protein and another candidate to functionally link Munc18-1 to cytoskeletal components. CaBP5 interacts with Munc18-1 as well as Myosin VI. The expression of CaBP5 in PC12 cells results in increased neurite outgrowth and dopamine secretion (Sokal and Haeseleer, 2011). In contrast to Myosin V, Myosin VI plays a functional role in Actin dynamics by recruiting actin-regulating factors such as Arp2/3 and Cortactin (in *Drosophila melanogaster*) (Rogat and Miller, 2002). Studies in fibroblasts showed that the absence of Myosin VI resulted in a decrease in the size of the Golgi apparatus (Warner et al., 2003), a phenotype that was also observed in hippocampal neurons from munc18-1 null mice (unpublished data, T. Cerveira).
Even though the involvement of CABP5 in the Munc18-1 dependent cortical F-actin regulation is a plausible scenario, further research is necessary to fully understand its role.

Besides the two interactions described above, another potential Munc18-1 effector has been identified in our lab. Genetic interaction studies in our lab suggest that Dynamin is an additional Munc18-1 interactor, since Dynamin levels are decreased in brains of \textit{munc18-1} null mice. Dynamin is primarily known for its role in endocytosis (Chen et al., 1991). This function is highly dependent on the recruitment of Dynamin to PI(4,5)P\textsubscript{2} clusters at the PM. Lower levels of Dynamin might result in an increase of available PI(4,5)P\textsubscript{2}, which in turn recruits more Actin-stabilizing proteins such as MARCKS. Furthermore, Dynamin directly regulates Actin dynamics and leads to Actin filament elongation (Gu et al., 2010). Decreased Dynamin levels would therefore result in impaired elongation and a reduction in Actin filament length which is not in line with the increased F-actin network in \textit{munc18-1} null MCCs. Based on those opposing findings, a role of Dynamin in the F-actin regulating role of Munc18-1 cannot be excluded. Therefore the exact nature of the potential link with Dynamin needs to be further investigated.

Apart from proteins that directly bind Munc18-1, kinases and phosphatases that regulate the phosphorylation state of Munc18-1 are directly or indirectly involved in F-actin regulation. Examples are the tyrosine kinase Src (Olivares et al., 2014), PKC (Larsson, 2006) or calcineurin (PP2B) (Craig et al., 2003; Singh et al., 2014). The interactions of kinases or phosphatases with Munc18-1 are probably transient. Since Munc18-1 is one of several substrates, its absence is unlikely to severely affect the kinase or phosphatase function. Therefore these interactions are not likely to underlie the severe cortical F-actin phenotype, which is observed in \textit{munc18-1} null MCCs.

Lastly, Munc18-1 inhibits phospholipase D (PLD) (Lee et al., 2004), which is strongly involved in both lipid metabolism (Liscovitch et al., 1994) and regulation of the cytoskeleton (Lee et al., 2001). Based on those functional links, this interaction forms the foundation of our current working model and will be explained in detail in the following paragraph.

\textbf{The pathway hypothesis}

Munc18-1 was shown to inhibit PLD by direct interaction (Lee et al., 2004). Yeast-3-hybrid studies confirmed the direct interaction and showed its PKC-independency (Angeli Möller, unpublished data). PLD directly interacts with actin, whereby actin inhibits PLD activity (Lee et al., 2001). Furthermore, PLD catalyzes the hydrolysis of phosphatidylcholine (PC) resulting in the production of the second messenger phosphatidic acid (PA) and choline (Exton, 1997). PA generated by PLD has been shown to be a key activator for PI(4)P\textsubscript{5} kinase \textgamma (PI4P5K\textgamma), which is the primary kinase to increase PI(4,5)P\textsubscript{2} levels at the PM (Jenkins et al., 1994). The phospholipid PI(4,5)P\textsubscript{2} has a multitude of intracellular functions, including membrane anchoring of the Actin cytoskeleton and Actin regulating proteins (for review see (McLaughlin et al., 2002; Logan and Mandato, 2006)). PI(4,5)P\textsubscript{2} stabilizes the cortical F-actin network in several manners: the actin-filament-crosslinking protein MARCKS is activated when it is bound to PI(4,5)P\textsubscript{2}. Under stimulated conditions, MARCKS is phosphorylated by PKC, thereby dissociates from PI(4,5)P\textsubscript{2} and stops crosslinking Actin fibers (Glaser et al., 1996). Another example is the Actin severing protein Scinderin, which is inhibited when bound to PI(4,5)P\textsubscript{2} (Zhang et al., 1996).
Figure 6.1: Munc18 interactome showing interactors of Munc18 which directly or indirectly can be linked to PI(4,5)P$_2$ and/or the cortical F-actin network.
Based on those findings, we formulate the current hypothesis for the increased cortical F-actin in munc18-1 null CCs: the absence of Munc18-1 results in a reduced inhibition and therefore over-activation of PLD, which leads to increased levels of PA in the cell. PA in turn activates PI(4)P5Kγ, which phosphorylates its substrate PI(4)P which is localized at the PM. The increase in available PI(4,5)P₂ at the PM allows more actin-stabilizing proteins to localize at or near the PM, which in turn results in an increase in the subplasmalemmal F-actin network (fig6.2). This model can plausibly explain the observed Munc18-1 dependent alterations in PI(4,5)P₂ levels at the PM and the cortical F-actin network.
**Future experiments to test our hypothesis**

Experimental testing of this hypothesis is the next crucial step in unraveling the molecular mechanism underlying the Munc18-1 dependent cortical F-actin regulation. In future experiments, the inhibitory function of Munc18-1 on PLD should be further investigated. It will be particularly interesting to study the function of amino acid V263 (and strong hydrophobicity at this position respectively) and examine whether this point mutation disturbs the interaction with PLD. Furthermore, PLD inhibitors (such as 1-butanol (Hu and Exton, 2005) or FIPI (Su et al., 2009) (overview and comparison of effects by different PLD inhibitors: (Scott et al., 2009; McDavid et al., 2014)) should be used on munc18-1 null CCs to determine whether the cortical F-actin network will be decreased. Furthermore, intermediate steps between the Munc18-1 PLD interaction and the cortical F-actin network need to be investigated. Quantification and comparison of levels of free PA (e.g., by HPLC (Holland et al., 2003) or enzymatic measurements (Morita et al., 2009)) in wild type as well as munc18-1 null MCCs could be an additional indicator for the validity of our current working model. A comparison of amount, activity and localization of PI4P5Kγ in both genotypes will also be informative. Based on our working model, higher levels and/or an increased activity of the kinase are expected. The levels of PI4P5Kγ in wild type and munc18-1 null MCCs can be determined with Western blots. A shift in the localization of PI4P5Kγ towards the PM in munc18-1 null MCCs could explain the observed increase in levels of PM localized PI(4,5)P₂. The localization can be studied using immunocytochemistry approaches in fixed cells or fluorescently labeled PI4P5Kγ in live cells. To test whether the increased amount of PI(4,5)P₂ in munc18-1 null MCCs directly causes the increase in the cortical F-actin network, several strategies can be used. Overexpression of PI4P5Kγ in wild type MCCs is likely to increase PI(4,5)P₂ levels and result in an increased F-actin network when both phenotypes are directly linked. The overexpression of the phosphatase-domain of Synaptojanin reduces PM localized PI(4,5)P₂ (Milosevic et al., 2005). Therefore the overexpression of this domain in munc18-1 null MCCs might rescue the cortical F-actin phenotype. Besides overexpression of PI kinases or phosphatases, pharmacological methods can be used to alter PI(4,5)P₂ levels acutely and investigate the resulting effects on the cortical F-actin network. Furthermore, it needs to be investigated which of the known PI(4,5)P₂-binding/actin-regulating proteins are involved in this mechanism in CCs. The identification of a specific actin-regulating protein (or a set of proteins) that is involved in the cortical F-actin regulation by Munc18-1 via PI(4,5)P₂ will therefore be a key aspect. The proposed experiments will help validating our current pathway hypothesis.

**Translation from adrenal chromaffin cells to neurons**

If we are able to confirm the existence of this particular pathway in CCs, and show that it represents the molecular mechanism underlying the described F-actin phenotype in munc18-1 null CCs, it will be interesting to examine whether the same mechanism occurs in neurons. This might be a challenging aim for at least two reasons: (I) the function of Actin filaments in the synapse, especially at the active zone, is still not fully understood, (II) visualization of PI(4,5)P₂ via PH-PLCδ1-eGFP in neurons is much more problematic than in CCs as the LV-mediated expression of PH-PLCδ1-eGFP induces apoptosis in neurons. Circumventing this methodological difficulty will be another challenge in the future.
Addressing these issues will result in an improved basic understanding of fundamental processes in neurons and potentially uncover implications of this pathway in several pathologies, e.g., Alzheimer’s disease (AD). The destabilization of Actin filaments is one of the key symptoms of AD (Penzes and van Leeuwen, 2011). The Actin-severing and PI(4,5)P$_2$-binding protein Cofilin was shown to mediate Amyloid-beta induced neurodegeneration through the destabilization of neuronal Actin (Minamide et al., 2000; Heredia et al., 2006; Maloney and Bamburg, 2007). An increased PI(4,5)P$_2$ metabolism was suggested to be linked to AD (Landmann et al., 2006) and PLD was clearly shown to play a role during AD (Oliveira and Di Paolo, 2010). Overexpression of PLD promotes the cell surface delivery of the Amyloid precursor protein (APP, (Cai et al., 2006)). The cleavage of surface-localized APP by $\alpha$-secretase generates soluble sAPP$\alpha$, which is believed to be neuroprotective (Sisodia, 1992; Furukawa et al., 1996). Therefore, it might be possible that decreased levels of Munc18-1 or a reduced activity, might play a role in AD.

Integrated approaches

A key aspect of all the studies presented here is the necessity of integrated approaches. It is absolutely vital to combine knowledge from different fields of research in order to understand complex biological systems. We applied this in two ways: (I) we integrated knowledge from three biological systems with distinct cellular functions (exocytosis machinery, lipid metabolism and cytoskeleton) and (II) we connected data from several steps of the secretory vesicle pathway, from biogenesis and maturation to the regulated secretion at the PM.

(I) A triangular system of exocytosis regulation in chromaffin cells

The triangular system contains three overlapping nodes: the "exocytosis machinery", the "lipid metabolism" and the "cytoskeleton" (fig6.3). The "exocytosis machinery" hereby includes the SNARE proteins (Syntaxin1a, SNAP-25 and Synaptobrevin2) and proteins that are involved in the regulation of docking, priming and/or fusion. Membrane lipids as well as regulatory enzymes (e.g., kinases, phosphatases and lipases) are grouped as "lipid metabolism". We defined actin, myosins and a variety of regulating factors as the "cytoskeleton". The triangular representation of the groups is based on interactions shown in fig6.1. The lipid metabolism determines the composition of the PM and is therefore essential for the localization of release sites. Membrane lipids (e.g., PI(4,5)P$_2$) function as membrane anchors for cytoskeletal components and recruit actin regulating proteins to the PM. Cytoskeleton components enable transport of secretory vesicles to the PM and play a regulatory role during exocytosis. Exocytosis of secretory vesicles alters the lipid composition of the PM and the organization of the cytoskeleton. Examples for the interactions between different cellular components are listed in fig6.3. The functional synergy of these three groups is an indispensable presupposition for understanding regulated exocytosis in CCs.
(II) Importance of vesicle biogenesis on regulated secretion in chromaffin cells

Impaired secretory vesicle biogenesis can cause reduced exocytosis in CCs. For example, the absence of the SNARE protein Vti1a results in a decrease in the number and size of secretory vesicles, which causes a reduction in the number of docked vesicles (Chapter 2). This docking phenotype causes a decrease in the amount of catecholamines that is released. The same phenotype is observed in MCCs lacking the BAR protein PICK1 (Pinheiro et al., 2014). However, a reduced number of secretory vesicles does not always lead to a decrease in the number of morphologically docked vesicles. MCCs lacking all four Rab3 isoforms show a 50% reduction in the number of secretory vesicles compared to rab3a null MCCs, but docking is normal (Schonn et al., 2010). Due to an additional priming defect, secretion is reduced in MCCs from the quadruple KO. The knock down of adaptor protein AP-3 by siRNA also results in a decreased number of secretory vesicles. However, in this case regulated secretion is reduced while constitutive secretion is increased (Asensio et al., 2010; Sirkis et al., 2013). MCCs from synaptobrevin-2 null mice have an increased number of secretory vesicles, but no change in docking is observed (Gerber et al., 2008). Those studies show that impaired biogenesis can modify secretion in different directions and that these processes are not simply correlated linearly. When focusing on the correlation between the total number of secretory vesicles and the number of docked vesicles, two scenarios can be observed. In vti1a null and pick null MCCs, the reduction of the total number of secretory vesicles is reflected by a decreased number of docked vesicles, hinting at the number of vesicles as the determining factor of the docking (Walter et al., 2014; Pinheiro et al., 2014). In the second scenario, altered total numbers of secretory vesicles do not result in docking phenotypes. Even though the total number of vesicles is strongly reduced (50%) in the Rab3 quadruple knockout, the number of docked vesicles is slightly, but not significantly, decreased, showing that Rab3 isoforms are not per se essential for the docking mechanism (Schonn et al., 2010). This observation might be explained by a smaller reserve pool, which, however, is still big enough to saturate the readily-releasable pool (RRP). In CCs from synaptobrevin-2 null mice, the total number of vesicles is almost doubled compared to wild type MCCs, whereas the number of docked vesicles is only slightly, but not significantly increased. This can be explained by either a bigger reserve pool or a faster release and docking rate, which results in a faster refill of the RRP. However, for the later explanation, no indication was found when exocytosis in synaptobrevin-2 null mice was studied (Borisovska et al., 2005). The number of docked vesicles is not determined by the total number of vesicles in MCCs deficient of Rab3 isoforms or Synaptobrevin-2. Therefore an alternative explanation might be that instead of the total number of secretory vesicles, the number of available docking sites is determining the amount of docked vesicles in those cells. One could imagine a minimal amount of secretory vesicles that needs to be present to occupy the existing docking sites and only if that minimal amount is not met, the total number of vesicles becomes the determining factor for the amount of docked vesicles. However, the phenotype of the Rab3 quadruple knockout argues against this hypothesis, since those MCCs contain a smaller total number of secretory vesicles than MCC deficient of Vti1a or PICK (Schonn et al., 2010; Walter et al., 2014; Pinheiro et al., 2014).
The difference between both scenarios might also be dependent on the sample preparation. In the first scenario, in which the number of docked vesicles is directly dependent on the total number of vesicles (e.g., \textit{vti1a} null and \textit{pick} null MCCs), the secretory vesicles were analyzed in single cells that were not enclosed by other cells. In the second scenario, in which the number of docked vesicles was probably dependent on the number of docking sites, sections from whole adrenal glands were analyzed (e.g., \textit{rab3} null and \textit{synaptobrevin-2} null MCCs). The number of docking sites might be more restricted in CCs that are enclosed by other CCs (in the whole adrenal gland preparation) than in individual cells without other cells attached. To test this hypothesis, the PM composition of single cells and cells in adrenal glands need to be analyzed and compared.

Besides their total number, other characteristics (e.g., diameter and core density) of secretory vesicles might influence exocytosis. Especially the size of secretory vesicles and their core composition were shown to be altered as result of genetic modifications (Walter et al., 2014; Pinheiro et al., 2014; Hao et al., 2015). To fully understand the effect of those alterations on regulated exocytosis of secretory vesicles in neuroendocrine cells, more research is essential. Furthermore, it will be necessary to integrate knowledge from different intracellular pathways to fully understand cellular functions of secretory vesicle biogenesis and maturation.

Given the functional synergy of cellular components (fig6.3), the biggest challenge in future experiments will be the dissection of specific functions of molecules that are involved in a variety of processes and/or connect a multitude of interactors. One example is phosphatidic acid (PA), which is part of our hypothesis to explain the Munc18-1 dependent regulation of PI(4,5)P$_2$ and cortical F-actin. In this pathway, PA is involved in the regulation of secretion. However, PA is also implicated in vesicle biogenesis. Inhibition of PLD-dependent PA synthesis results in the fragmentation of the Golgi apparatus (Siddhanta et al., 2000). Depletion of the SM protein Vps45 in HeLa cells and fibroblasts leads to a condensation of the Golgi apparatus (Rahajeng et al., 2010), similar to the munc18-1 null phenotype in neurons. This might be explained by the ability of Vps45 to regulate F-actin (Chapter 4). Furthermore, Vps45 interacts with Syntaxin6, which forms a SNARE complex with Vti1a and is involved in the secretory vesicle maturation (Bock et al., 1997). These examples emphasize the necessity to combine data and knowledge from different biological systems as well as distinct cellular pathways.
Figure 6.3: Functional synergy: top: Venn diagram illustrating the close functional relation between exocytosis machinery (blue), lipid metabolism (green) and the cytoskeleton (orange) in the secretory pathway; bottom: overview of functions and examples of interactions between the groups in the Venn diagram.
6. General Discussion

Concluding remarks and perspective

The studies presented in this thesis might be a first step towards a holistic approach that includes several interwoven intracellular pathways (such as biogenesis and regulated secretion) as well as multiple molecular systems that are highly connected and crucial for regulated exocytosis in CCs: the fusion machinery (especially Munc18-1), lipid metabolism (focus on the role of PI(4,5)P₂) and the cytoskeleton (especially the cortical F-actin network). This process can only be fully understood when all parts and their impact on each other will be integrated. This is not only crucial for the understanding of this specific pathway, but a more general conclusion: integrated approaches that analyze the interplay of proteomics and lipidomics will be essential to unravel molecular pathways in the future.
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