Introduction
1 Prelude

This thesis comprises a set of explorations into various intracellular and intercellular signaling pathways. Throughout these explorations, ‘translocations’ have been a constant theme. Scattered across various cell biological topics, this thesis reflects the discovery of three translocations and the experimental work aimed at unraveling their molecular mechanisms and cell biological relevances. At first glance, the word ‘translocation’ is self-explanatory; moving from one place (‘locus’) to the other (‘trans’ is across). Circulating red blood cells can be considered to undergo continuous translocation, but the work presented here focuses on molecular movements within the micrometer-sized dimensions of the cell.

The interior of a cell is a tangle of continuous activity. On the to-do-list: thousands of simultaneous interactions and enzymatic conversions that cooperate to maintain healthy cell physiology and, in addition, to accomplish cell type-specific functions. The immense ensemble of molecules that constitute a living cell requires strict regulation of all possible interactions and enzymatic activities. We can formulate two basic principles that are prerequisites to control such ‘organized complexity’. First, interactions among constituents, be it proteins, lipids, ions or small molecules, are to be specific. It is not surprising that cell biology has described a vast amount of conserved recognition domains that confer specificity to molecular interactions. The specificity of a recognition domain is accomplished by the unique combination of its three-dimensional structure and charge distribution, fitting to that of its binding partner following the principle of key-and-lock. Second, most enzymatic conversions are regulated. In other words, they can be ‘switched on and off’. For example, many enzymes undergo conformational changes that either silence or release their catalytic activities.

Box 1 Potential pitfalls of fluorescent tagging

A potential pitfall in fluorescence microscopy is the use of fusion proteins. Most fluorophores have molecular weights of ~30 kD. Thus, in the case of CLIC4 (29kD), fusion with a fluorophore doubles the molecular weight. Such bulky tags may sterically hinder protein-protein interactions. In line with this, the choice to tag either the N- or C-terminus could influence subcellular localization of the fusion construct. We have observed the latter for both Epac1 and CLIC4 and used the tagged version whose localization was most comparable to that of the endogenous counterparts. In the case of gap junctional protein connexin43, C-terminal fusion of GFP even disrupted the formation of proper gap junctions. Therefore, a major challenge in fluorescence microscopy is to identify a genetically encoded fluorescent tag of minimal size. The lab of Roger Tsien (Noble Prize, 2008) seemed to catch a glimpse of the holy grail when they introduced the tetracysteine-system [1]. The concept was to insert six residues (Cys-Cys-Pro-Gly-Cys-Cys) into a protein of interest and subsequently label this motif specifically with a fluorescent dye (for comparison: GFP consists of ~240 amino acids). Unfortunately, the labeling conditions await further optimization, since the labeling step seriously compromises cellular viability.

Introduction
Chapter 1

2 Measurement of translocations in living cells using fluorescence microscopy

To monitor a protein of interest by fluorescence microscopy, it is genetically fused to a fluorescent protein moiety. The first, green fluorescent protein (GFP), was derived from the jellyfish *Aequorea victoria* and multiple others have been cloned from other sea organisms. Subsequent mutational modifications have led to optimizations in terms of spectral properties, brightness and stability (reviewed in [2]). The color palette spans the spectral range from blue (BFP) to red (mRFP). As described in Box 1, caution is demanded when tagging a protein of interest with a fluorescent moiety.

Four different techniques have been employed in this thesis:
1) Confocal laser scanning microscopy (CLSM), which yields high spatial resolution by selective rejection of out-of-focus emission from the detectors.
2) Fluorescence Resonance Energy Transfer (FRET), which reports protein-protein interactions.
3) Fluorescence Lifetime Imaging (FLIM), a technique to determine FRET.
4) Fluorescence Recovery After Photobleaching (FRAP), which reveals mobility of fluorescent molecules.

2.1 Fluorescence Resonance Energy Transfer (FRET)

FRET is the radiationless transfer of energy from an excited donor to an acceptor fluorophore. It occurs under stringent conditions. First, the donor emission spectrum must overlap with the absorption spectrum of the acceptor. Second, donor and acceptor fluorophores must be in close proximity, i.e. < 8 nm when using fluorophores in the visible spectrum. A third determinant is formed by the relative orientations of the fluorophore dipoles. FRET is evident from quenched donor fluorescence and gained acceptor fluorescence. Thus, (a change in) FRET can be registered as (a shift in) relative donor and acceptor emission intensities.

The distance constraint is most important for the application of FRET in cell biology. When using common fluorophore combinations (e.g. CFP/YFP or GFP/mRFP), FRET is strictly limited to distances of less than 8 nm. Thus, FRET registers intermolecular distances comparable to the sizes of the studied proteins themselves (5-15 nm) and has therefore been termed a ‘molecular ruler’ (to put this in perspective: confocal microscopy is limited to a resolution of ~200 nm). FRET can be measured by several experimental approaches, each with its own advantages and disadvantages. As outlined in Box 2, the technique of choice depends on the question to be addressed.

Besides its use in revealing interactions between proteins, FRET is also employed in a growing num-

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Box 2 Three approaches to measure FRET

FRET between CFP and YFP can be measured in several ways. The three major ones are:
1) Monitoring of YFP/CFP ratio. Excite CFP and detect total CFP and total YFP emission from a single cell. The pooling increases signal-to-noise ratio (S/N), allowing sub-second sampling.
2) Sensitized YFP emission. Excite CFP and record CFP- and YFP-images. Post-acquisition analysis calculates YFP emission resulting from FRET by subtraction of false signals.
3) Measure donor lifetime (FLIM). Excite donor and follow kinetics of fluorescence decay (fluorescence lifetime, $\tau$). FRET accelerates fluorescence decay and can thus be registered as a decrease in $\tau$.

<table>
<thead>
<tr>
<th>Technique</th>
<th>Advantages</th>
<th>Disadvantages</th>
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<tbody>
<tr>
<td>Monitor YFP/CFP</td>
<td>* good signal-to-noise</td>
<td>* no spatial information</td>
</tr>
<tr>
<td></td>
<td>* rapid sampling</td>
<td>* not quantitative</td>
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<td></td>
<td>* experiments are easy and fast</td>
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</tr>
<tr>
<td></td>
<td>* very low exposure (bleaching, toxicity)</td>
<td></td>
</tr>
<tr>
<td>Imaging sensitized YFP</td>
<td>* spatial information with confocal resolution</td>
<td>* best for high fluorophore expression</td>
</tr>
<tr>
<td>emission</td>
<td>* quantitative</td>
<td>* high exposure</td>
</tr>
<tr>
<td>FLIM</td>
<td>* spatial information</td>
<td>* labor intensive</td>
</tr>
<tr>
<td></td>
<td>* quantitative</td>
<td>* slow image acquisition</td>
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number of gene-encoded sensors that report intracellular signaling events. These are typically small molecules that can not be made fluorescent directly, such as the second messengers calcium, PI(4,5)P₂, Reactive Oxygen Species (ROS), cAMP or cGMP [3,4]. Other FRET sensors have been designed to report kinase/phosphatase activities towards specific consensus domains (for e.g. PKA [5] or PKC [6]) or to monitor activation of GTPases (e.g. RhoA [7] or Rap [8]). Because FRET sensors have been extensively applied in this thesis, the most important ones are here briefly introduced.

2.2 FRET-based sensors for calcium, PI(4,5)P₂ and cAMP

One of the first genetically encoded FRET-based sensors was the Yellow Cameleon calcium sensor [11]. Its core is composed of the C-terminus of Calmodulin (CaM) and the CaM-binding domain of MLC-kinase M13. This core is flanked by CFP and YFP. The increased interaction between CaM and M13 upon calcium binding shortens the distance between the fluorophores and results in enhanced FRET. In this manner, the Yellow Cameleon sensor can register calcium changes with high spatial and temporal resolution.

Other FRET assays for signaling molecules require the co-expression of two separate constructs. For instance, a CFP- and a YFP-tagged version of the Pleckstrin Homology (PH) domain of PLCδ1 (CFP- and YFP-PH) can be combined to measure PI(4,5)P₂ [12]. In resting cells, the PH-domains rapidly shuttle between cytosol and PM because PI(4,5)P₂ binding is characterized by fast on/off rates. Their partial presence at the PM suffices to induce FRET between the fluorescent moieties. PI(4,5)P₂ hydrolysis results in translocation of the PH domains into the cytosol and dilution of the fluorophores is detected as a loss of FRET. This assay has been important to define the role of PI(4,5)P₂ in the ligand-induced gating of connexin43-based gap junctions (chapter 7).

The first FRET-based sensors designed to measure the second messenger cAMP were based on the tetrameric Protein Kinase A (PKA) [9,13,14]. The concept was to register the cAMP-dependent dissociation of the two catalytic subunits (YFP-PKA-Cat) from the two regulatory subunits (CFP-PKA-Reg). This sensor has been useful in the demonstration of cAMP oscillations [15,16]. However, this first-generation sensor displays some serious disadvantages.

The recent discovery of Epac [17] provided an interesting alternative for the generation of a FRET-sensor for cAMP, since binding of cAMP has a dramatic effect on Epac’s conformation. The concomitantly increased distance between the two termini can be measured as a loss of FRET between terminally fused fluorophores. In this manner, Epac-based FRET sensors have been developed in our lab (chapter 5) and, independently, by the groups of Lohse [18] and Zhang [10]. Epac-based cAMP sensors have several advantages over the earlier PKA-based sensor (chapter 5).

CFP-Epac-YFP was further optimized by removal of its PM-targeting DEP domain (ΔDEP) and rupture of the catalytic activity by double point mutagenesis in the GEF domain (T781A/F782A). The resultant CFP-Epac(ΔDEP-C.D.)-YFP is cytosolic, catalytically dead (C.D.) and therefore our cAMP sensor of choice.

To further improve the Epac-based cAMP sensor, its fluorescent moieties have been optimized. For this, a series of sensor variants containing variable donor and acceptor fluorophores (in various combinations) has been generated and compared in their ‘cAMP-sensorship’ (chapter 8). The lessons learned from these comparative analyses may be useful for the generation of other gene-encoded FRET sensors in the future.
3 Diffusion-based translocations

Translocation events are common to signaling cascades. Several examples will be discussed in section 4 and this thesis describes the translocation of signaling molecules CLIC4 (chapter 2) and Epac1 (chapters 3 and 4). We will here describe the functional significance of translocations in signaling cascades and explain how they can result from diffusion.

3.1 Plasma membrane translocation leads to strong enrichment of signaling proteins

Translocations of signaling molecules add a dimension of regulation to signaling cascades: translocation to the PM may bring signaling protein \( P \) in the vicinity of PM-localized effectors, whereas it is physically separated from other binding partners. Importantly, the density of \( P \)-molecules increases dramatically when moving from the relatively voluminous cytosol (\( m^3 \)) to the limited surface (\( m^2 \)) of the PM. As briefly explained in Box 3, the increase in molecular density may amount to \( >2 \) orders of magnitude. Obviously, such enrichment has a huge impact on the reaction rates of signaling processes at the PM. Therefore, translocations are a major determinant of the switch-like activation patterns of signaling proteins.

The impact of enrichment can be illustrated by the targeting technology that makes use of the ‘switchable’ interaction between domains of the FK506-binding protein (FKBP) and the FKBP-rapamycin binding (FRB) protein (see Fig. 1). The interaction is triggered by the membrane-permeable drug rapamycin. Rapamycin binds FKBP with high affinity and the subsequent FKBP-rapamycin tandem has dramatically increased affinity for FRB. Thus, the drug links the two domains together. For the inducible hydrolysis of the phospho-inositide PI(4,5)P\(_2\), a 5-phosphatase has been fused to FKBP (FKBP-5-phosphatase) and coexpressed with a PM-anchored FRB domain (FRB-CAAX) [19]. Thus, addition of rapamycin triggers translocation of the cytosolic FKBP-5-phosphatase to the PM, resulting in complete depletion of membrane PI(4,5)P\(_2\). Key to the success is the enrichment of the phosphatase enzymes. In the absence of rapamycin, PI(4,5)P\(_2\) levels remain unaffected, because the phosphatases remain diluted in the cytosol. Rapamycin-induced translocation ‘turns the switch’. For this thesis, this assay has been used to establish the central role of PI(4,5)P\(_2\) in gating of connexin43-based gap junctions (chapter 7).

Box 3 “Concentration by translocation”

A simple calculation suffices to estimate the increase in density of a particle that translocates from the cytosol to the PM. Two conditions are compared:

1) All molecules locate to the cytosol, or
2) All molecules locate to the PM.

For both conditions we determine the particle concentration that allows an average intermolecular distance of 8 nm, which approximates the distance at which protein-protein interactions and FRET occur. For the dimensions of a cell we estimate that:

* its volume is 1 picoliter \((10^{-12} \text{ liter} = 10^{-15} \text{ m}^3)\)
* its surface is 600 \( \text{um}^2 \) \((\approx 6 \times 10^{-10} \text{ m}^2; \text{precisely determined parameter in electrophysiology})\)

1) **All particles locate to the cytosol**
   - We subdivide the cytosolic volume in hypothetical volumes that are occupied by a single molecule: 
     - each molecule occupies \((8 \text{nm} \times 8 \text{nm} \times 8 \text{nm}) = (8 \times 10^{-9})^3 \text{ m}^3\)
     - the cell \((10^{-15} \text{ m}^3)\) contains \(1.9 \times 10^3\) of such cubic sub-volumes, thus \(1.9 \times 10^6\) molecules
     - these are \((1.9 \times 10^6) / (6 \times 10^{23}) = 3.3 \times 10^{-17} \text{ moles}\)
     - intracellular concentration: \((3.3 \times 10^{-17}) / (10^{-12}) = 3.3 \times 10^5 = 3.3 \text{ mM}\)

2) **All particles locate to the PM**
   - We subdivide the membrane surface in hypothetical surfaces that are occupied by a single molecule: 
     - each molecule occupies \((8 \text{nm} \times 8 \text{nm}) = (8 \times 10^{-9})^2 \text{ m}^2\)
     - the surface \((6 \times 10^{-12} \text{ m}^2)\) contains \(9.4 \times 10^6\) square sub-surfaces, thus \(9.4 \times 10^6\) molecules
     - these are \((9.4 \times 10^6) / (6 \times 10^{23}) = 1.6 \times 10^{-17} \text{ moles}\)
   - If these \(1.6 \times 10^{-17}\) moles of particles translocate back to the cytosol...
     - intracellular concentration: \((1.6 \times 10^{-17}) / (10^{-12}) = 1.6 \times 10^5 = 16 \text{ mM}\)

These two concentrations are in a mutual ratio of \(~200 x\).

In other words, translocation to the PM induce a \(~200\)-fold particle enrichment.
3.2 Diffusion-based translocation: a few scenarios

Because free diffusion (also known as Brownian motion) directs signaling molecules to all corners of the cell, signaling molecules encounter their downstream effectors by coincidence. Whether they truly transfer signals depends on binding specificity and on activation status, which is under the regulation of alternative signaling events. To explain translocations of signaling modules, one element must be added: one of the interactors is tethered to a static subcellular compartment. We assume the PM-anchored target $T$ and the cytosolic protein $P$. In resting conditions, there is no binding affinity between $P$ and $T$. Brownian motion dictates the stochastic movement of $P$ in an equilibrium shuttling between cytosol and PM. However, when mutual affinity is ‘switched on’, the PM-localized target $T$ becomes a sink for $P$. Diffusion drives $P$ to accumulate, or translocate, to target $T$.

The ‘switch’ can be turned on at the level of the diffusible protein or that of the anchor at the PM. At the molecular level, switching can be done in several ways. One of the binding partners can be post-translationally modified. For instance, phosphorylation of protein $P$ may define a new recognition domain that triggers translocation to $T$. Conformational changes may also expose affinity domains that are otherwise inaccessible. Such conformational rearrangements may, on their turn, be triggered by phosphorylation but also by binding of (upstream) signaling components. An example of the latter is the cAMP-induced conformational change of Epac1 that triggers PM translocation (chapter 3). An affinity domain may further be released by dissociation from a complex in which it was previously shielded. Conversely, mechanisms where binding capacity is acquired by association into a protein complex can be considered ‘coincidence detectors’.

These scenarios are summarized in Table 1 and are referred to in the next section. It must be stressed that this limited list represents only a fraction of all possible mechanisms in cell signaling.

<table>
<thead>
<tr>
<th>Modify diffusible protein $P$</th>
<th>What turns the switch?</th>
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<tbody>
<tr>
<td></td>
<td>1a) post-translational modification</td>
</tr>
<tr>
<td></td>
<td>1b) conformational change</td>
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<td></td>
<td>1c) association to protein $X$</td>
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<td>1d) dissociation from protein $X$</td>
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<td>... or combinations thereof</td>
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</table>

| Modify anchor target $T$ | 2a) post-translational modification |
|--------------------------| 2b) conformational change |
|                          | 2c) association to protein $X$ |
|                          | 2d) dissociation from protein $X$ |
|                          | ... or combinations thereof |

**Table 1** Scenarios of switchable interactions that underly translocation
Chapter 1

4 GPCR signaling is full of translocations

This section surveys a subset of signaling pathways that are under the control of G Protein-Coupled Receptors (GPCRs). Selected are those that have been subject of study in the course of this thesis: via the G protein subunits Gα13, Gαs and Gαq. Meanwhile, these pathways illustrate the occurrence of translocations in cell signaling. Thus, the focus lies on localizations and translocation mechanisms rather than on the extensive treatment of each and every signaling protein.

4.1 G Protein-Coupled Receptors

GPCRs constitute one of the largest mammalian protein families, with nearly 1000 members in the human genome [20]. They are characterized by seven transmembrane domains and coupling through heterotrimeric G proteins. GPCRs occur in all cells of the body, but expression depends on cell type, function and developmental stage. They can be activated by a wide variety of stimuli, including neurotransmitters, peptide hormones, lipids, ions, and light. GPCR functions vary from transmission of sensory information (e.g. >200 family members expressed in the olfactory organ [21]) to stimulation of proliferation and migration [22].

The heterotrimeric G proteins consist of an alpha subunit (Gα), and a dimer beta/gamma subunit (Gβγ) [23]. For the many different signaling cascades, the human GPCR family utilizes a repertoire of at least 20 Gα, 5 Gβ and 12 Gγ isotypes [24]. In the absence of receptor stimulation, Gα and Gβγ are tightly associated in the tripartite complex [25] and achieve PM localization by prenylation of Gγ and palmitoylation of Gα [20,25]. The GTPase activity is contained by the larger Gα subunit. Like all GTPases, Gα cycles between a GDP-bound, inactive state and a GTP-bound, active state (Fig. 2A). Guanine Exchange Factors (GEFs) enhance transition to the GTP-bound state, whereas GTPase Activating Proteins (GAPs) enhance GTP hydrolysis. The association with Gβγ stabilizes the inactive state of Gα [25].

Ligand binding, however, induces the activated conformation of the GPCR that binds Gα/Gβγ via the third intracellular loop [26] and serves as a GEF for the recruited Gα subunit. Using a rapid fluorescent readout, Vilardaga et al. showed that α2a-adrenergic receptors undergo this shape change within 50 milliseconds [27]. Activation of Gα is, on its turn, accompanied by a conformational change that induces dissociation from Gβγ [28]. Next, Gα redistributes laterally along the PM (it remains palmitoylated) to find its downstream effector(s). Although not fully understood, some reports have claimed that dissociation from Gβγ relieves the protection from depalmitoylation. As a consequence, the Gα subunit may rapidly lose its PM localization and the subsequent translocation towards the cytosol may constitute a shut-off mechanism for signaling towards PM-localized effectors [20].

Figure 2 The GTPase cycle

A) Cycling of GTPase proteins between the inactive, GDP-bound and the active, GTP-bound conformation is regulated by guanine exchange factors (GEFs) and GTPase activating proteins (GAP). See text for details

B) In its inactive conformation, the small GTPase RhoA is kept in the cytosol by RhoGDI, which shields the fatty acid moiety.
4.2 Gα13 signaling

Activated Gα13 subunits mainly signal through Guanine Exchange Factors (GEFs) for the small GTPase Rho. These include PDZ-RhoGEF, AKAP-Lbc [35], p115RhoGEF and LARG [25,36]. Together with Ras, Rac, Cdc42 and Rap, RhoA belongs to the family of small Rho-GTPases (21kD) [37], which, similar to the larger Gα subunit (43kD), cycle between the GDP- and GTP-bound state (Fig. 2A).

RhoA activation has dramatic effects on cellular morphology. It does so mainly via its effector Rho-kinase (or ROCK), which induces actomyosin contraction by phosphorylation of myosin light chain (MLC). Pronounced Rho-dependent morphological changes can best be studied by stimulating the Gα13-coupled receptors for lysophosphatidic acid (LPA) in N1E-115 cells (e.g. see chapter 2).

The cycling of RhoA is accompanied by dynamic shuttling between cytosol and PM, contributing to switchable signaling behavior. PM localization is accomplished by prenylation of its very C-terminus. The attached fatty acid tail tends to insert into the inner leaflet of the PM (scenario 1a), unless this is prevented by Guanine nucleotide Dissociation Inhibitor (GDI) proteins, which locate to the cytosol (Fig. 2B). As long as RhoA is complexed to GDI, which shields the C-terminus and the prenyl modification, it remains in the cytosol. LPA receptor stimulation dissociates this complex and thus triggers translocation of RhoA to the PM (scenario 1d). Interestingly, this translocation is independent of RhoA activation itself, since also the inactive mutant Rho(N19) translocates upon LPA stimulation [38]. Thus, LPA induces translocation of prenylated RhoA towards the PM, where its transition to the active state is catalyzed by Gα13-activated Rho-GEFs.

In this thesis, we demonstrate that LPA-induced Gα13/RhoA signaling triggers an additional PM translocation: that of chloride intracellular channel 4 (CLIC4).

Below: catch a glimpse at chapter 2

Chapter 2: CLIC4 translocates to activated, Gα13-coupled receptors at the plasma membrane

Chloride intracellular channels (CLICs) are a family of six proteins that are thought to function as chloride channels in intracellular organelles and, also, the PM [29]. Chloride conductances have been reported, but only in in vitro experiments using artificial membranes [30]. Altogether, the cellular roles of CLIC proteins are poorly understood.

Using time-lapse microscopy, we showed that LPA stimulation triggers the rapid translocation of CLIC4 from the cytosol to the activated receptor at the PM. CLIC4 recruitment is transient (lasting 5-10 min) and strictly dependent on Gα13 and RhoA activation. In apparent contradiction to its nomenclature, we find no evidence that CLIC4 can insert into the PM or modulate chloride currents.

Although the biological function of CLIC4 translocation has not been resolved to date, we speculate that CLIC4 translocates to the receptor complex to regulate signal transfer from Gα13 towards RhoA (activation) at the PM. Interestingly, CLIC4 has been reported to interact with the scaffold protein AKAP350 [31], while a family member of the latter, AKAP-Lbc is a RhoGEF, that directly links Gα12 to RhoA [32]. Furthermore, CLIC4 binds the adaptor protein 14-3-3 [33], which is an activator of AKAP-Lbc [34]. Therefore, it is tempting to hypothesize that CLIC4 translocates to a protein complex containing the activated receptor, Gα13 and an AKAP scaffold with GEF activity towards RhoA. In this model, the spatial regulation of CLIC4 will undoubtedly add to the switch-like regulation of RhoA activity.
4.3 $G_{i\alpha}$ signaling

Activated $G_{i\alpha}$ subunits stimulate adenylyl cyclases (ACs) to synthesize the second messenger cyclic adenosine monophosphate (cAMP) from ATP. The second messenger cAMP is involved in numerous cellular functions, depending on cell type and activated receptor. For example, cAMP stimulates cell growth in many cell types while inhibiting it in others [41-43]. The diverse effects of cAMP are mediated by protein kinase A (PKA), exchange protein directly activated by cAMP (Epac) and cyclic nucleotide-gated channels (CNGCs). The classical cAMP effector PKA is a tetramer consisting of two regulatory and two catalytic subunits [44]. The catalytic subunits are released when cAMP binding to the regulatory subunits dissociates the complex (scenario 1d). The released catalytic domains phosphorylate various substrates on typical PKA-consensus motifs. cAMP-mediated cell differentiation is characterized by the induction of specific genes through the transcription factor CREB (cAMP responsive element binding protein), which translocates to the nucleus upon phosphorylation by PKA [41] (scenario 1a).

Given that PKA is involved in numerous parallel signaling cascades, it is a challenge to understand how the kinase is activated in the right place and at the right time. It is generally believed that specificity is achieved, in part, through the compartmentalization of PKA at different subcellular locations through interaction with so-called A Kinase Anchoring Proteins (AKAPs) [35,45-47]. At least 50 different AKAP isoforms target the regulatory subunits of PKA to various intracellular compartments (reviewed in [35]).

To restrict cAMP signaling to these compartments, AKAP anchors also cluster the cAMP-degrading phosphodiesterases (PDEs) [48,49], implying that cAMP levels are reduced in the vicinity of PKA. Interestingly, PKA phosphorylation is known to upregulate PDE activity [50], establishing a feedback-loop that rapidly terminates the cAMP signal. Thus, the AKAP scaffold restricts cAMP turnover to the micrometer scale [51,52]. Indeed, using a FRET reporter for PKA activity, Saucerman et al (2006) demonstrated that the occurrence of local cAMP gradients is enhanced by PDE activity [53]. Another study used an Epac-based cAMP sensor to show that following receptor stimulation, cAMP elevations in the nucleus follow with a lag time and with decreased amplitude as compared to elevations near the PM [10].

CAMP is a small messenger molecule that can pass through gap junctions [54-57]. We have studied this gap junctional communication (GJC) of cAMP and observed that PDE activity determines the degree of cAMP exchange between adjacent cells (chapter 6).

Below: catch a glimpse at chapter 6

More recently, the discovery of Epac as a direct effector of cAMP [17] has triggered the elucidation of many cAMP-regulated processes that could not be explained by the classical effector PKA. Epac1 and Epac2 act as GEFs for the small G Proteins Rap1 and Rap2. Unlike RhoA, Rap GTPases are permanently anchored to membrane compartments via its prenylated C-terminus [62], including the Golgi network, vesicular membranes and the plasma membrane [63-66]. It plays roles in diverse processes typical for the PM, including cell adhesion [58,64,67], adherens junction formation.
Introduction

Epac GEFs contain a regulatory region with one (Epac1) or two (Epac2) cAMP-binding domains, a D-shauleved, Egl-10, Pleckstrin (DEP) domain, and a cattylic region for GEF activity [60]. Recently, the structures of both the inactive and the active conformation of Epac2 were solved [70,71]. This revealed that in the inactive conformation the regulatory region occludes the Rap binding site and that this is relieved by a conformational change induced by cAMP binding.

Similar to PKA, also Epac signaling is governed by compartmentalization. Interestingly, in cardiomyocytes Epac1 has been found in the same complex as PKA, anchored by the muscle-specific mAKAP [72]. The complex, which also contains the PDE4D3, is a crossroad of cAMP signaling involved in the regulation of ERK5. Specifically Epac2 is part of the Rim2-piccolo complex involved in cAMP-dependent exocytosis [73]. Both Epac1 and Epac2 are targeted to microtubules via an interaction with the Light Chain of Microtubule Associated Protein (MAP-LC) [74]. In addition, Epac translocations can be triggered by hormone stimulation. Li et al. (2006) found that Epac2 translocates to the plasma membrane by binding to the small GTPase Ras, when Ras acquires the active conformation [75] (scenario 2b).

In this thesis, we uncovered two additional mechanisms of Epac1 targeting in response to extracellular signals. Epac1 translocates to the PM upon binding of cAMP (chapter 3) and upon activation of members of the Ezrin/Radixin/Moesin (ERM) family (chapter 4). Both types of PM recruitment appeared important for Epac1-mediated cell adhesion. This is not surprising, since activation of Rap at the PM is crucial for cell adhesion [64]. Thus, not only is Epac1 compartmentalized, several extracellular cues can regulate Epac1 function via dynamic translocations from one compartment to the other.

Below: catch a glimpse at chapters 3 and 4

Glimpse at:

**Chapter 3**: Epac1 translocates to the plasma membrane upon cAMP binding

Epac1 is a GEF for the small G protein Rap and is thereby involved in processes such as integrin-mediated cell adhesion [58] and cell-cell junction formation [59]. In the absence of cAMP, Epac1 localizes to the nuclear envelope, the cytosol and, to a minor extent, the PM. Here, we report that cAMP induces the translocation of Epac1 towards the PM. Combining high-resolution confocal fluorescence microscopy with FRET assays, we observed that Epac1 translocation is a rapid and reversible process. This dynamic redistribution of Epac1 requires both the cAMP-induced conformational change and the DEP domain.

In line with its translocation, Epac1 activation induces Rap activation predominantly at the PM. Furthermore, translocation-deficient Epac1 mutants were impaired in their ability to induce Rap-mediated adhesion of Jurkat T cells. This indicates that the cAMP-induced translocation enhances Rap-mediated cell adhesion.

Interestingly, our data imply that cAMP exerts dual regulation on Epac1. The cAMP-induced conformational change not only elicits GEF activity by Epac1 [60], it also induces its translocation to the compartment where its GEF activity should activate Rap. This dual regulation guarantees that Epac1 acts as a binary switch for Rap activation.

**Chapter 4**: Epac1 is recruited to the PM by activated ERM proteins

Besides the cAMP-induced translocation, we revealed an additional translocation mechanism that targets Epac1 to the PM. This redistribution is mediated by direct interaction with members of the Ezrin/Radixin/Moesin (ERM) family. Recruitment to ERM proteins is distinct from the cAMP-induced translocation in several aspects. First, it is not mediated by the DEP domain but by the N-terminal 49 residues that precede the DEP domain. Second, it is independent of Epac1 conformational state. And third, it targets Epac1 to specific, asymmetrical regions of the PM, whereas cAMP-binding induces uniform PM targeting.

We find that the Epac1-ERM interaction is regulated by conformational opening of ERM proteins. In agreement with this, thrombin receptor signaling, which induces the conformational opening of ERM proteins [61], triggers the recruitment of Epac1 to asymmetrical zones of the PM.

Also ERM-mediated recruitment is important for activation of Rap signaling. Epac1(Δ49), which is deficient in ERM binding, was impaired in its ability to induce adhesion upon Epac1-activating stimuli. Considering that both the cAMP-dependent Epac1 translocation and the interaction with ERM proteins contribute to Rap activation (and hence cell adhesion), we suggest that these two targeting mechanisms synergistically cooperate to effectively couple GEF activity of Epac1 to its effector Rap. Within the context of translocations as enrichment mechanisms for signaling molecules (section 2.4), it is tempting to speculate that the marked asymmetrical PM distribution imposed by ERM proteins may further enrich Epac1 to the appropriate compartment for Rap activation, thereby increasing its efficiency of action.
4.4 $G_{\alpha_q}$ signaling

Activated $G_{\alpha_q}$ subunits trigger the activity of phospholipase C (PLC$\beta$), which hydrolyzes the PM-specific phospho-inositol PI(4,5)P$_2$. The cleavage generates the second messengers inositol-triphosphate (IP$_3$) and diacylglycerol (DAG). PI(4,5)P$_2$ itself is also a second messenger involved in a vast amount of PM-localized processes, many of which are related to actin polymerization. In this way, PI(4,5)P$_2$ plays roles in processes ranging from actin cytoskeletal remodeling to regulation of ion transporters [76].

In unstimulated cells, PLC$\beta$ is anchored to the PM via electrostatic interactions between its C-terminal domain (CT-domain) and negative charges at the inner leaflet (Singer et al). When activated $G_{\alpha_q}$ interacts with PLC$\beta$ [77], it elicits the PI(4,5)P$_2$ hydrolyzing activity. The loss of negatively charged PI(4,5)P$_2$ induces translocation of PLC$\beta$ to the cytosol [78]. Probably, this cytosolic translocation is further enhanced by GAP activity of the PLC$\beta$ CT-domain [79], which inactivates $G_{\alpha_q}$ and thereby disrupts the interaction. Thus, hydrolysis of its substrate PI(4,5)P$_2$ and inactivation of its upstream signal form the switch to induce PLC$\beta$ translocation to the cytosol (scenario 1d; here translocation is away from the PM). Obviously, this negative feedback mechanism may contribute to the transient kinetics of $G_{\alpha_q}$-mediated PI(4,5)P$_2$ hydrolysis.

PI(4,5)P$_2$ hydrolysis is involved in the gating of several PM-localized channels, such as the KCNQ potassium channel [80] and the transient receptor potential cation channels TRPM7 [81] and TRPM8 [82]. In this thesis, we established the crucial role of PI(4,5)P$_2$ in ligand-induced closure of connexin43-based gap junctions (this thesis, chapter 7).

Below: catch a glimpse at chapters 7

Glimpse at chapter 7

Chapter 7: PI(4,5)P$_2$ regulates communication via connexin43-based gap junctions

Cell-cell communication through connexin43 (Cx43)-based gap junction channels is rapidly inhibited upon activation of GPCRs, that couple through $G_{\alpha_q}$. However, the mechanism has long remained unknown. Here, we show that Cx43-based cell-cell communication is inhibited by depletion of PI(4,5)P$_2$ from the plasma membrane. Knockdown of phospholipase C$\beta$3 (PLC$\beta$3) inhibits PtdIns(4,5)P$_2$ hydrolysis and keeps Cx43 channels open after receptor activation. Furthermore, when PI(4,5)P$_2$ is overproduced by overexpressed PI(4)P-5-kinase, Cx43 channel closure is impaired.

Next, we showed that PI(4,5)P$_2$ depletion is not only required but also sufficient for gap junction closure. For this, we used the translocatable FKBP-5-phosphatase described in section 3.1. Rapamycin-induced recruitment of FKBP-5-phosphatase towards PM-anchored FRB-CAAX induced immediate PI(4,5)P$_2$ depletion without generation of second messengers IP$_3$ and DAG [80]. Thus, the observed reduction in gap junctional communication implies that hydrolysis of PI(4,5)P$_2$ itself is a direct signal for channel closure.
References


31. Shanks R.A., Larocca M.C., Berryman M., Edwards J.C., Urushidani T., Navarre J., and Goldenring...
Introduction


