When cells respond to light

All you need is LOV

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CHAPTER 1

General introduction

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Living cells have developed intricate ways to sense their surroundings and process these stimuli into appropriate responses. The most important entity in this process is the plasma membrane (PM), as it acts as a protective barrier, holds all the extracellular receptors, and links them to a range of internal signaling protein complexes. The PM is the initial information processing hotspot, a crucial piece to understanding the workings of a cell as a whole. Yet our knowledge of the mechanism behind the transfer of stimuli from the PM throughout the cell, via signaling cascades and self-regulatory loops, is still far from complete. It is known however, that the general mechanisms involved in all these type of processes, e.g. cytosis, migration, differentiation, are generally the same. These mechanisms involve cooperativity, feedback, active transport, and diverse diffusion. Many processes don't rely on just one of these mechanisms to form local signaling patterns, but rather combine several or all of them to create a robust system[1-2]. If we want to better understand the spatio-temporal patterns of protein activity at the PM, we should aim to understand the underlying mechanisms, which remains difficult within living cells due to their complex interplay of innumerable proteins and structures.

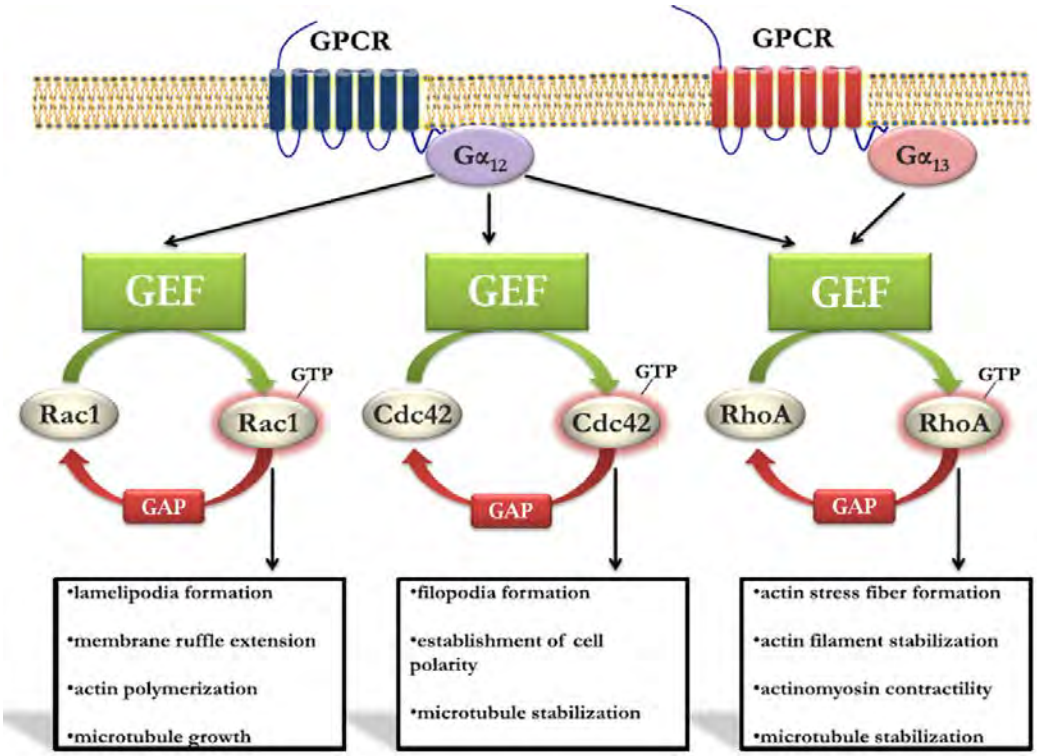

Figure 1. G_α protein mediated regulation of the cytoskeleton. A concise schematic representation indicates how extracellular signals can be translated into cytoskeletal changes through G_α proteins. Adapted from Schappi, *et al.* 2014.[3]

From plasma membrane to cytoskeleton

The process of active transport directly relates to microtubules, which can be viewed as highways of the cell that are used by motor proteins to transport cargo. It is therefore important to understand how signaling related to the cytoskeleton, and microtubules in particular, is normally regulated. The PM receives stimuli that can affect a range of different structures. Many signals that induce cytoskeletal changes start at G-protein coupled receptors (GPCRs). There is a big variety of GPCRs and there are even more different ligands, but the output is the same for all, the activation of membrane associated G-protein alpha subunits[3]. These transmembrane receptors will function as guanine nucleotide exchange factors (GEFs) when ligands are bound, thereby exchanging GDP for GTP of associated G-proteins and allowing the G_{α} subunit to become active by restructuring or dissociating from the other subunits[4]. Many of the different G_{α} subunit types can affect the cytoskeleton in some way, either directly or through downstream effectors.

The most common pathways that alter the cytoskeleton involve Gα12/13 which are responsible for coordinating the large structural changes that occur in migration, proliferation, differentiation, etc. (Figure 1)[5-6]. $G_{\alpha 12/13}$ subunits interact with a variety of downstream effectors and can be activated by a range of different receptors, making them a bottleneck which indicates other influences of these receptors on regulation of the cytoskeleton as well[7-8]. Focusing on $G_{\alpha 12}$, we know that it displays slow kinetics due to the long term processes it is involved in[9], and affects downstream small GTPases (20-25kDa), including Rac1, RhoA, and Cdc42. The GTPases have several specific Rho GEF mediators that pass on signals from $G_{\alpha 12/13}$ which they hydrolyze[10]. GTPase activity is further controlled by GTPase activating proteins (GAP) that can inactivate these Rho GTPases by exchanging GTP for GDP, and by guanine nucleotide dissociation inhibitors which can prevent spontaneous activation[11]. The Rho GTPases have more than 50 identified downstream effectors, a handful of which relate to microtubules. RhoA is a critical regulator of cell migration and morphology, with conditional downstream effects[11]. With the help of proteins like mDia1 and the Rho-associated protein kinase (ROCK), microtubules could become stabilized through detyrosination or depolymerized depending on the surrounding signals[12-14]. Cdc42 is known to be important during cell polarization and arranging the microtubule organizing center for example[15]. Rac1 is another crucial regulator for both actin and microtubules that shares some downstream effectors with cdc42, e.g. p21-activated kinase 1 (PAK1) which can alter microtubule growth rates through stathmin, and CLIP-170 which promotes microtubule plus end capture[16-18].

$G_{\alpha 12/13}$ clearly command a large pool of microtubule effectors, however, other G_{α} subunits also play a role in microtubule organization, sometimes through direct interactions. $G_{\alpha s}$ can bind directly to microtubule plus ends and induce hydrolysis of GTP-tubulin, thereby increasing the microtubule's catastrophe rate[19-20]. Certain isoforms of the G protein β/γ subunits on the other hand are able to promote microtubule polymerization[3,21]. $G_{\alpha s}$ can also be transactivated by GTP-bound tubulin, in a process whereby GTP is transferred onto $G_{\alpha s}$[3]. Active Gαs results in increased cAMP production via adenylyl cyclase, which could create a feedback loop as cAMP has been reported to promote microtubule growth[22]. $G_{\alpha q}$ is responsible for activating phospholipase C and the subsequent increase in intracellular Ca^{2+} concentration, resulting in destabilized

microtubules, though binding of $G_{\beta/\gamma}$ can stabilize them[23]. It becomes clear that microtubules can be regulated from many different, cross-regulated, upstream factors. As a result, any alterations to the system involve complex balance adjustments of numerous components, making it hard to study and even harder to gain precise outside control over.

Of microtubules & motor proteins

Microtubules are highly dynamic structures, owing to the myriad of forces acting on it. They self-assemble into a hollow tube of thirteen parallel protofilaments, consisting of αβ-tubulin dimers. Unpolymerized dimers display a nicked configuration, which gets straightened during incorporation into the microtubule lattice[24]. Tubulin has a bound guanine nucleotide, and upon hydrolysis of GTP at the microtubule end, another dimer can elongate the filament[24]. The stability of microtubules is related to the presence of tubulin-GTP at the end which forms a cap. A single cap appears to be sufficient to stabilize the filament, but when the cap is lost, the microtubule can depolymerize[25]. The parallel protofilaments come together in a 'seam' where lateral interactions occur between α- and β-tubulin, rather than the homotypic lateral interactions in the rest of the microtubule, making this the weak point[26]. The protofilaments at the plus end typically have varying lengths, creating a ragged tip with curved outer solo filaments[27]. When a growing microtubule runs into an obstacle, it will exert a pushing force of a couple pN onto it which places the microtubule under compression stress that can alter its dynamic instability[28]. Microtubules run in a strictly organized network, as a radial aster or an elongated polarized network. One way to promote polarization is by anchoring the microtubules to organelles or the cell cortex[29]. This mechanism is actually required to maintain microtubule filaments at the cell edge amid actin retrograde flows[29]. The capture site can also become a recruiting platform microtubule stabilizing factors to further strengthen the emerged structure[30].

Microtubule dynamics are tightly influenced by microtubule associated proteins (MAPs), as for instance the plus end tracking EB proteins. EB will transiently bind close to the microtubule plus end where tubulin dimers are being integrated into the lattice, and they have a smaller affinity for the lattice itself as well, leaving only the very tip of curved filaments as an EB-free space[31-32]. Its function is to aid GTP hydrolysis in the lattice, which can lead to increased catastrophe rates[27,31]. Other MAPs like XMAP215 act as microtubule polymerases by preferentially binding to nicked tubulin dimers. Thanks to this, they can recruit unpolymerized dimers to the microtubule plus end and release them upon straightening from interactions with the lattice[33]. This XMAP215 characteristic also leads to an opposite depolymerizing effect in the absence of free tubulin dimers, by stabilizing more curved and dissociation-prone configurations of tubulin at the microtubule plus end[34]. Specific microtubule depolymerases, e.g. kinesin13, exist as well which only favor catastrophe induction[35].

Some of the microtubule associated domains are dynamically moving along the lattice, called motor proteins. Two main types of motor proteins are kinesins and dyneins, of which the former migrates towards the plus end while the latter moves to the opposite minus end of the

microtubule. Movement requires hydrolysis of ATP to make one of its two globular heads take a 'step', and the resulting speed is dependent on the size of its cargo load[36]. Motor proteins can transport proteins, vesicles, even mRNA, across the cell bound to the outer end of its elongated structure. These motor proteins and their microtubule tracks are the driving force behind the active transport mechanism, as one of the crucial regulators in the spatio-temporal pattern formation of protein activity at the PM. An example of this is transport of Rac1 and upstream GEFs to the microtubule plus ends at PM protrusions. The growing microtubules trigger GEF and subsequent Rac1 activation via cooperative interaction with MAPs like APC[37], leading to membrane ruffling and further stimulation of microtubule growth by downstream Rac1 effectors. The positive feedback loop that is created here, is influenced by many other Rac1 regulating systems. This concise representation of the microtubule signaling pathway shows the complexity of subcellular signaling patterns and the need for simplified systems if we are to fully understand everything about these intertwined regulating mechanisms.

Stathmin the microtubule specific effector

There are some proteins that affect microtubule dynamics in a relatively isolated manner such as stathmin / oncoprotein 18 (OP18). This microtubule associated protein directly interacts with tubulin as its only downstream target, and is regulated in a non-cooperative manner through phosphorylation alone[38]. Stathmin is the most studied variant of the stathmin protein family which includes proteins like SCLIP and RB3. All members of the stathmin family have a similar microtubule destabilizing function, and are found mostly in the nervous system, though stathmin itself is also expressed in any cell with proliferative potential[39-40]. The 149 amino acid sequence of stathmin is highly conserved among all vertebrates, as this small protein contains many structurally important regions. Even though uncomplexed stathmin displays an equilibrium between a disordered secondary structure and partly formed α-helices, that balance gets shifted towards one fully formed extended α-helix when it is complexed by αβ-tubulin dimers[41]. Only the N terminus of stathmin remains an unstructured domain that stretches about 40 amino acids and acts as a capping mechanism by wrapping around α-tubulin[42]. The helical secondary structure at the C-terminus displays great affinity towards the curved conformation of αβ-tubulin dimers and can bind up to two dimers simultaneously[41]. While the binding sites of stathmin are well understood, there is no consensus on the binding affinity for tubulin, with reports fluctuating between three orders of magnitude[43-45].

The microtubule destabilizing effect stems from two mechanisms that arise from cooperation of stathmin's tubulin binding sites (Figure 2). The microtubule catastrophe promoting mechanism for instance, is initiated by the C-terminal domain binding to exposed protofilaments at the microtubule ends and destabilizing them through the subsequent forced induction of a curved conformation and inhibition of lateral protofilament interactions[46]. The binding to the microtubule lattice can then be strengthened by the N terminus which could cap the outer α-tubulin tips at the minus end and prevent further incorporation of tubulin dimers. At the microtubule plus end, stathmin's N-terminal domain is able to promote severing of the

protofilament by binding to the interdimer surface[47]. Released stathmin molecules are then complexed with two αβ-tubulin dimers, preventing them from reincorporating into the microtubule lattice which leads to the second mechanism of microtubule destabilization, diminishing the pool of free, unpolymerized αβ-tubulin dimers[41,47]. Due to the more curved conformation of free tubulin dimers versus incorporated ones, stathmin's activity will mostly comprise of sequestering these free tubulin dimers, until the interaction is diminished through phosphorylation of stathmin.

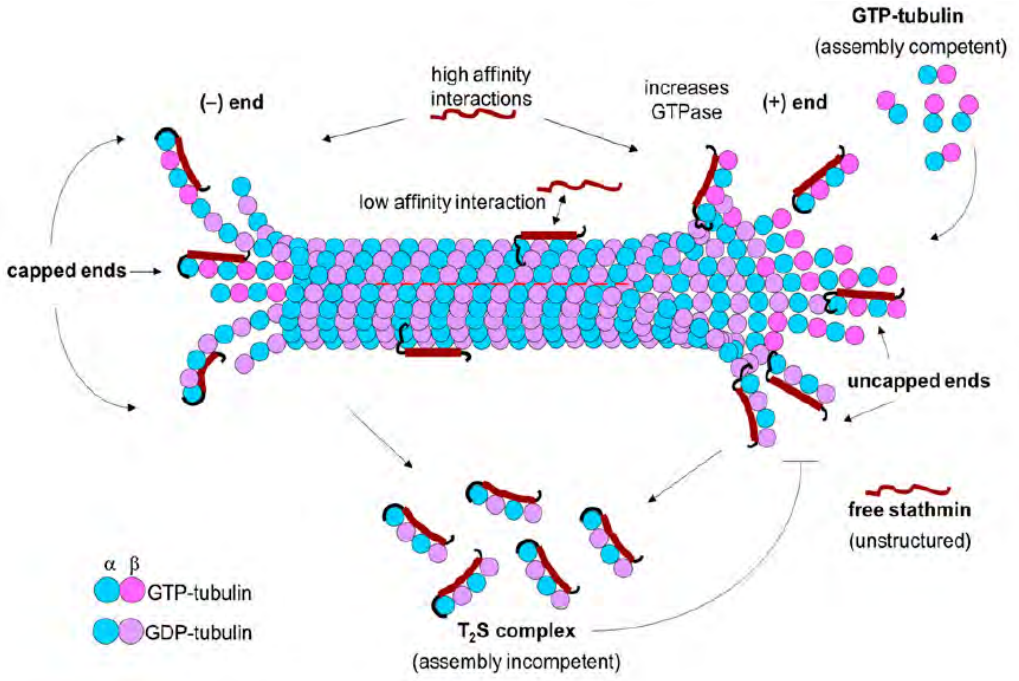

Figure 2. Complex forming configurations of stathmin and tubulin. Cartoon of the various possible interactions between stathmin and tubulin dimers. The microtubule catastrophe promoting ability of stathmin is achieved via a mix of direct binding of the microtubule ends, and sequestering of free αβ-tubulin dimers.[47]

Stathmin has four serine residues that can be phosphorylated, Ser16, Ser25, Ser38, and Ser63. The outer two residues, Ser16 and Ser63, appear to have the biggest impact on stathmin's affinity for tubulin, as phosphorylation at either one of these sites is sufficient to strongly impair stathmin's tubulin sequestering ability[48]. On a structural basis, the phosphoryl group at Ser16 or Ser 63 results in steric hindrance for α-tubulin or disruption of the α-helix backbone respectively[45,49]. Phosphorylation of residue 25 or 38 alone is not sufficient to abolish the stathmin-tubulin complex, yet these residues are accessible in the tubulin bound state and the resulting local perturbation of added phosphoryl groups is thought to promote phosphorylation of the neighboring Ser16 and Ser63 which are more occluded in complex with tubulin[50-51]. Due

to their dissimilar effect, it is not surprising that different kinases phosphorylate Ser16/Ser63 and Ser25/Ser38. CDK1 and MAPK are two kinases that are responsible for phosphorylating the middle serine residues[52-53]. Some of the most common regulators are specific to Ser16 alone, e.g. PAK1 and Ca^{2+}/calmodulin kinases, which underscores the regulatory importance of this residue[54-55]. Kinases that phosphorylate Ser63 usually target Ser16 as well, like PKA for instance[56]. The dephosphorylation of stathmin is less residue specific, with the PP2A phosphatase acting on all four serines[57]. The straightforward effect and phosphorylation based regulation of stathmin could make this protein an ideal target for gaining empirical control over microtubule dynamics with minimal unwanted side activity, with the help of some new emerging molecular tools like optogenetics.

Gaining control over signaling with optogenetics

In the early 2000s, some people had the brilliant idea of taking light-responsive protein domains found in plants or bacteria, and utilizing their activity as a means of acquiring easy experimental control over cellular signaling. This new technology was called optogenetics and originated in neurobiology with light-responsive ion channels[58]. These microbial opsins revolutionized neuroscience, as fast activation or deactivation of neurons was now possible with only a simple pulse of light in a single component system, which quickly led to optogenetic control of live mammals[59]. These light-responsive ion channels work by the same basic principal as all other optogenetic domains. They absorb light from within a certain range of wavelengths and use that energy to induce a conformational change that affects the protein's activity, which in the case of opsins translates to opening or closing the ion channel[60-61]. The opsins paved the way for optogenetics to become a widely recognized, revolutionary tool thanks to their easy applicable inherent function. Other light-responsive domains often have functions that are not generally applicable as empirical tools, e.g. cryptochrome-2's (CRY2) photoperiodic control of floral initiation[62], yet people quickly realized that the domains itself could be modified and applied to different functions. In the case of CRY2, by extracting its PHR domain and pairing it with the calcium- and integrin-binding protein 1 (CIB1) binding partner, a light-induced dimerizing system was created which could be fused to any desired target protein[63]. For instance, by tethering CRY2 to the PM and combining it with an inositol phosphatase fused to cytosolic CIB1, it becomes possible to alter the phosphoinositide distribution thanks to a blue light induced conformational change in CRY2 that creates a binding site for CIB1 and thereby recruits the inositol phosphatase[64].

There are several known light-induced effects, including the aforementioned ion pump regulation, and dimerization which can trigger translocation or protein fragment complementation. Others include dissociation, aggregation, and G protein release[65-67]. Each of these categories contains several different optogenetic domains and the list continues to grow, ever-expanding the possibilities of this new technique. The varying sensitivities of these domains stretch from the UV until the infrared wavelengths, although most are responsive to just the blue end of the spectrum[68]. The light-induced conformational changes all spontaneously revert back

to the ground state over time, though with highly domain-dependent half-lives, and some can even be actively reverted back via the absorption of a different wavelength of light[69]. While nearly all optogenetic domains require a cofactor, some are luckily ubiquitous in all cells like FAD, making these domains more popular than others which require for example the less common phycocyanobilin[69]. The advantages of optogenetics are clear, being able to obtain the gain or loss of a function in a simple, reversible, and target-specific manner with high spatiotemporal precision is allowing unparalleled control over cellular systems. No other technique is currently capable of precisely altering signaling activities on a subcellular scale, and with a plethora of domains to choose from, optogenetics will certainly continue to make a big impact in the biological sciences.

The LOV domain

One optogenetic domain stands out as one of the most commonly used, the Avena sativa Light Oxygen Voltage 2 (asLOV2) domain, also referred to as simply LOV when discussing optogenetic systems. This is partly due to the fact that its cofactor FMN is present in all cells, and due to its peak activation at 450nm being outside of the harmful UV range, but mostly due to the versatility of this domain. AsLOV2's function *in vivo* is related to phototropism, yet optogenetics has modified and applied this domain for recruitment, complementation, dissociation, and even allosteric enzyme activation purposes[70-72]. These effects are all made possible by its special conformational change (Figure 3). The core asLOV2 structure is a part of the Per-Arnt-Sim (PAS) domain family, containing a central five-stranded antiparallel β-sheet with GXNCRFLQ consensus motif and α-helices to capture the FAD cofactor[73]. In the ground state, the oxidized cofactor can absorb blue light and trigger the formation of a covalent bond between FMN and the cysteine within the GXNCRFLQ sequence of asLOV2[74]. This structural change at the protein's core will propagate all the way to the α-helices at both the N- and C-terminus, resulting in completely unfolded helices that have turned into long flexible linkers[75]. The conformational change causes terminal fusion proteins to become less sterically hindered by the LOV domain and become further removed from domains at LOV's other end, both of which are being used for optogenetic strategies[70,76].

AsLOV2 is a fast cycling LOV protein, meaning it requires higher intensities of light in order to saturate its transition than other proteins from the same family with slower kinetics[77]. The domain therefore mostly responds to daylight level intensities, and not dusk or dawn intensities, which is actually good for experimental setups. Due to the big interest in the LOV domain, many mutations have been discovered than can fine-tune the lifetime of its photochemistry, resulting in lifetime variations between 2-4300s[78]. These modifications are especially useful in cases where very transient activation is required, or where the diffusion of activity from a small number of activated proteins is of interest for example. Since the LOV domain's structure-to-function relation is so well characterized, it becomes easier to design stabilizing mutations for terminal fusion domains as well, e.g. L531E in iLID[70]. On top of that, single point mutations that trap the

LOV domain in the dark or active state are available as well. Taken together, it becomes clear that the asLOV2 domain is a major asset in the optogenetic toolbox.

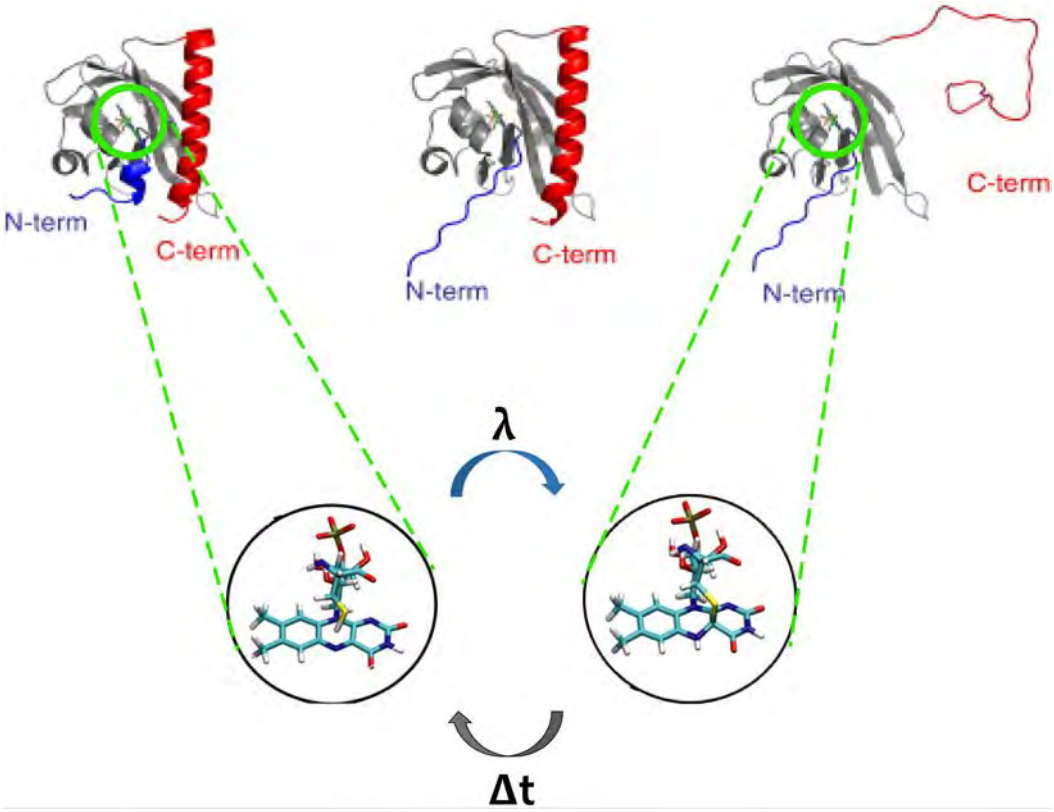

Figure 3. Light-induced conformational change of asLOV2. Cartoons of the LOV domain visualize the unfolding of both the N- and C-terminal helices in response to blue light exposure. Enlarged sections show the formation of a covalent bond between the FMN cofactor and a backbone cysteine which triggers the entire conformational change.

Bottom-up reconstitution of microtubule signaling

The powerful effect of optogenetic domains allows for acquiring control over a signaling pathway through a much further downstream target than traditional approaches[72]. This already eliminates many unwanted side effects in an *in vivo* setting, yet far from all due to the complexity of cellular signaling networks. Even a handful of components *in vivo* will have a multitude of interacting partners, displaying non-linear dynamics and containing feedback loops across multiple spatial and temporal hierarchies[79-80]. The fact that many of these interactions are likely unknown at this point or irrelevant to the research question at hand, make traditional top-down experiments problematic. Thus, if we truly want to understand the interacting mechanisms behind the formation of membrane signaling patterns and their relation to membrane deformations, a less complex system is required by utilizing a bottom-up approach. Such an *in vitro* system additionally provides the opportunity to quantify the relation between conditions like protein concentration or membrane properties and the resulting signaling gradients and membrane deformations.

The basic components for such a bottom-up approach are either water-in-oil emulsion droplets or liposomes, filled with a microtubule organizing center and tubulin dimers. These setups are capable of inducing membrane deformations[81], and are ideal for finding and studying a minimal set of regulatory proteins that can control the microtubule dynamics locally. The simplest artificial signaling pathway would require only one microtubule effector like stathmin and a way to turn its activity on or off. Such a system can be used to first characterize the exact influence of diffusion on the signaling gradient and the resulting microtubule alterations, through tweaking and comparing diffusion rates of free and membrane bound molecules. Next, the system could be expanded to include active transport of signaling molecules along the microtubules and even introduce feedback loops (Figure 4). Understanding how all the physical mechanisms influence the biological interactions in this *in vitro* system would be a major step towards explaining the complexities of cellular signaling pathways. In order to achieve this, a minimal set of regulating proteins need to be identified. This will involve the use of optogenetics to decrease the amount of intermediary proteins from endogenous pathways as much as possible. The first step will therefore be the creation of new optogenetic fusions and optogenetic-compatible tools, tested *in vivo* before assembling everything *in vitro*, which will be the focus of this thesis.

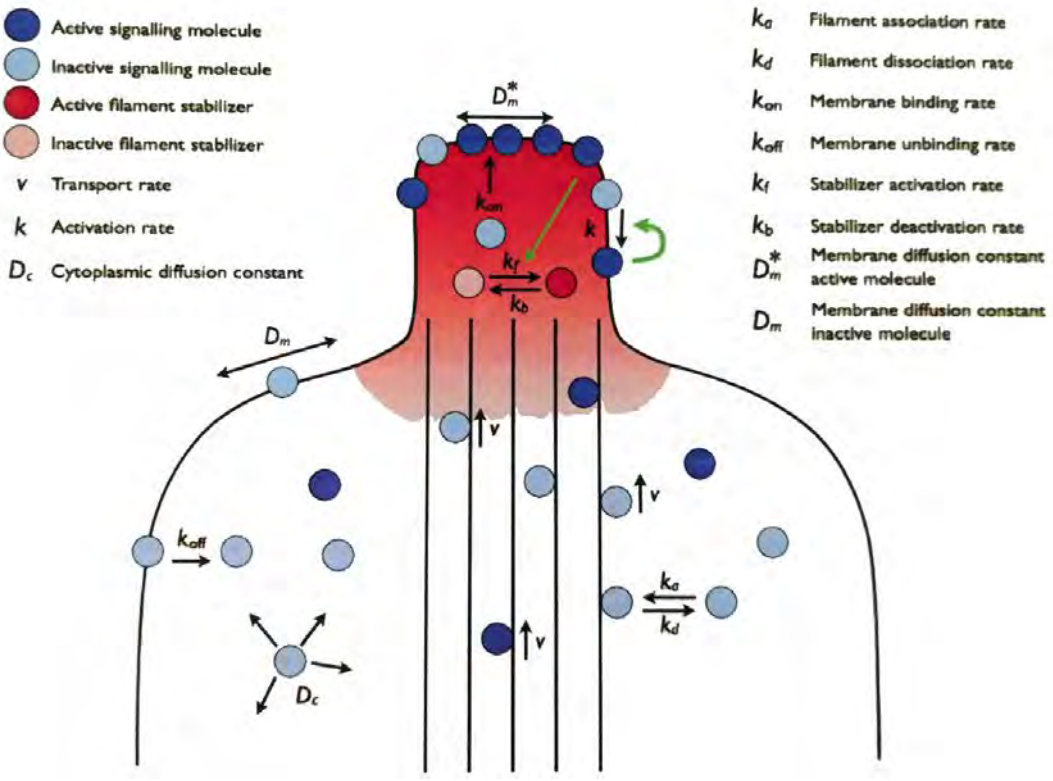

Figure 4. Interplay of forces affecting localized signaling. Cartoon of a possible design for creating an artificial membrane system capable of forming localized signaling patterns with minimal components. The system contains signaling proteins which can be locally stimulated to activate microtubule effectors that will create a gradient of activity through diffusion. Active transport of signaling proteins along microtubules can enhance a positive feedback loop, as well as any induced membrane protrusions which can increase local concentrations.

Thesis outline

The research in this thesis was performed as part of a larger collaboration, which hoped to reconstitute a minimal membrane system *in vitro*. The aim of this overarching project was to elucidate how spatio-temporal patterns of signaling activity at the plasma membrane are influenced by the interplaying mechanisms of diffusion, cooperativity, active transport, and feedback loops. Several simultaneous research pipelines were started that would be combined in the end to create the artificial membrane system, and this thesis focuses on the signaling proteins. Throughout the thesis, the development of several molecular tools are described, which could prove useful in generating a minimal microtubule regulating system. The choice was made to focus on optogenetic tools, as these provide unparalleled spatio-temporal control over protein signaling, and could therefore limit the required components and subsequently limit the complexity of interplaying mechanisms in a minimal system.

Chapter 2 centers on the diffusion component of cellular signaling. Cytosolic diffusion is not easy to counter, and local gradients are usually achieved through binding interactions with larger structures, potentially at the plasma membrane. Yet membrane tethered components can still display relatively fast diffusion rates, especially in less crowded artificial membranes. Therefore we developed a couple strategies to further decrease lateral diffusion, and showcase their effectiveness through light-induced recruitment of signaling proteins to the plasma membrane.

Chapter 3 addresses the problem of combining blue light responsive domains with FRET sensors. Existing pairs of fluorescent proteins in FRET sensors need to be exchanged for redshifted pairs in order to illuminate the sensors independent from the optogenetic domains. We developed a redshifted stathmin FRET sensor and used it to explore the effect of photoswitchable Rac1 on stathmin and microtubules.

Chapter 4 investigates the possibility of constructing photoswitchable versions of kinases that directly affect stathmin, in order to decrease the intermediates between the stimulus and microtubules. Different designs on different kinases were analyzed until a functioning construct was discovered and characterized.

Chapter 5 presents a way of eliminating the need for intermediates altogether by creating photoswitchable versions of stathmin itself. For the first time, a light-induced reversible sequestering and uncaging tools for tubulin were built. This could be useful to study purely diffusion related effects and introduces a method for regulating microtubule dynamics without activating unwanted signaling pathways.

Chapter 6 finally summarizes and discusses how the tools from previous chapters could be used together in the creation of a minimal membrane regulating system.

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