When cells respond to light

All you need is LOV

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CHAPTER 6

General discussion

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This final chapter will provide a summary of all the previously discussed research and discuss how each part fits into the larger project of reconstituting a minimalistic living membrane *in vitro*. The purpose of such a system is to study the underlying mechanisms of signaling pathways from an easy to understand bottom-up approach. In order to create and control the required minimal signaling system we needed a method to alter specific protein activities with high spatiotemporal precision, i.e. optogenetics. The research in this thesis was therefore dedicated towards developing useful optogenetic tools that enable experimental control over altering microtubule dynamics. The light-induced protein conformational changes have allowed optogenetics to perform previously impossible feats such as regulating a protein's activity on subcellular scales, thereby making a significant impact on empirical research in biology[1]. The wavelength specificity makes the domains perfectly suited for use with laser scanning microscopes, although most domains will respond to a broad range of wavelengths within the visible spectrum, thereby obstructing the amount of other light-sensitive proteins that can be independently used. Currently, only some blue- and (infra)red-responding optogenetic domains, e.g. LOV and phytochrome domains, can be used together[2]. Optogenetics is still a relatively new technique however, and in the future it likely will be figured out how to adjust the wavelength specificity and make it narrower like was done for fluorescent proteins[3]. This could potentially lead to systems with three or more independent light-inducible components, which allows precise spatiotemporal control over an unprecedented amount of interactions and bring us another step closer to understanding complex signaling interactions. We focused on creating a single light regulatable pathway with the asLOV2 domain. We chose to work with LOV-based tools because they perform robustly, they don't require harmful UV light, they are active in all cells due to the omnipresence of its FMN cofactor, and most of all because the LOV domain's structure is well characterized and relatively easy altered through point mutations[4]. This might also make the LOV domain an ideal target for future attempts at modifying the absorption spectrum. Since the cofactor is responsible for absorbing the blue light and subsequently forming a covalent bond with a cysteine[5], this would likely involve generating an artificial cofactor which has its own disadvantages to overcome, but should be possible. The future of optogenetics can go in many directions and it will be interesting to see what possibilities will arise as the technique develops further.

We contributed to the growing optogenetic toolbox during this PhD project, by generating an auxiliary tool to control the diffusion rate of light-induced domains in chapter 2. We found that tandem lipid binding domains are great at lowering diffusion, because they require simultaneous unbinding to become cytosolic, and because the double anchoring points experience more resistance along the crowded plasma membrane itself. Fusing this anchor to the iLID system allowed for more localized responses from recruited proteins as compared to more commonly employed lipid tail-anchors, and will also enable easier purification for *in vitro* studies. When

applying these membrane anchoring domains to the ROPY or PA-CaMKIV constructs made in the following chapters, we discovered that a double C2 lipid binding domain could already sufficiently decrease lateral diffusion *in vivo*. Yet the effect could be increased further by the addition of either an oligomerizing CRY2 domain or a secondary anchor location through a microtubule-binding EB3-domain for example. These might be important for transferring the optogenetic systems into *in vitro* giant unilamellar vesicles, since these are much less crowded than plasma membranes and, therefore they will display much higher diffusion rates of associated domains. The CRY2-2C2 anchor will then likely be the preferred choice because it does not rely on the presence of secondary structures, but on the light-induced formation of big clusters. These large clusters only occur in areas illuminated with blue light to activate the associated LOV-based structures, thereby avoiding untimely clustering effects.

Eventually, we are interested in studying the effects of mechanisms like diffusion on minimalistic microtubule regulating systems based around stathmin. Therefore, we needed a way to assess the phosphorylation state of stathmin. FRET sensors are ideal reporters for this, and a stathmin sensor with a cyan and yellow fluorescent protein pair was already available[6]. Most FRET sensors make use of optimized cyan and yellow fluorescent proteins, and unfortunately they share a spectral overlap with most of the optogenetic domains. The popular blue light responsive optogenetic domains can be excited with wavelengths of up to around 500nm, thus a yellow and red fluorescent FRET pair would be compatible. We identified the most suitable FRET pair in this excitation range to ensure the FRET signal would be optimal even in sensors with lower dynamic ranges, such as the stathmin COPY sensor. This is especially important in combination with optogenetic tools, which usually still display some dark state activity that can affect the FRET sensor's basal state. One point mutation in mScarlet-I significantly boosted the dynamic range, the R125I mutation, which leads to dimerization in coral-derived fluorescent proteins and in our case specifically increased the intramolecular affinity between the FRET pair. This observation could be of importance in the generation of other optogenetically compatible FRET sensors, of which there is a serious lack at the moment. While the speed at which new light-inducible systems are produced is incredible, without compatible reporters their usefulness is limited. A surge in optogenetic compatible tools and readouts is therefore required in the coming years in order to fully utilize the potential of the technique. Thanks to the ROPY sensor we were able to observe how local stathmin stimulation resulted in mostly localized microtubule extensions, even though the resulting FRET activity of cytosolic sensors was diffusing across most of the cell. This suggests that even a small gradient of activity is sufficient to polarize the concentration levels of free tubulin, and that the tubulin incorporation rate into microtubules must outweigh the cytosolic diffusion rate.

While this system already provides a shortcut to control microtubules as compared to the endogenous regulation in cells, it still consists of three diffusible components with PAK1 as intermediate between the inducible PA-Rac1 and stathmin. We believed we could reduce the amount of components further by developing a photoswitchable kinase that could directly phosphorylate stathmin and thereby eliminating one diffusible component (the upstream kinase-activating component). PAK1's activation mechanism is rather complex and turned out to be difficult to control via light-inducible domains. CaMKIV on the other hand is a kinase that can displace its auto-inhibitory mechanism via a single structural loop serving as a sort of hinge[7]. In the wildtype version this is triggered by calcium-bound calmodulin interacting with the auto-inhibitory domain. Here we were able to induce this change directly through light-induced unfolding of an asLOV2 domain inserted in the structural loop flanking the auto-inhibitory domain. The addition of the large LOV domain also prevents the endogenous regulation by calmodulin, making it only responsive to blue light and not to calcium fluxes. The fact that it does not depend on calcium for activation is also beneficial for future *in vitro* studies. We continued by combining the membrane anchors from chapter 1, the ROPY sensor from chapter 2, and PA-CaMKIV from chapter 3 to enable contained and light-induced kinase activity locally in an *in vivo* setting. Perhaps with even slower diffusing anchors we can achieve the same result *in vitro* and have a way to create and test the effect of various gradients of free αβ-tubulin dimer concentrations. The induced gradients will be highly dependent on the amount of phosphatases present in the system, rendering complete control over the gradients slightly more difficult. Therefore, if we desire to have full control over stathmin-induced tubulin gradients, we need a single regulatable component, which is why we looked into creating a photoswitchable stathmin.

Stathmin's conformational change from an elongated tubulin-bound α-helix to an intrinsically disordered structure upon phosphorylation gave us the idea of controlling its structure and function by forcing it into the disordered state through light-inducible dimers. Upon passive release of any bound tubulin dimers, a blue light pulse will prevent reassociation to PI-OP18 and subsequently result in an increased concentration of free tubulin. We also attempted the reverse effect, where optogenetic dissociation domains would allow for the induced capturing of free αβ-tubulin dimers. While FRET experiments indicate that this PA-OP18 version is altering its conformation in response to light, no effect on microtubules was observed. Still this variant could be very useful, because of its inert dark state, but more research will be required to figure out the discrepancy. PI-OP18 on the other hand is effective at inducing microtubule rescues and extending microtubules both locally and cell-wide. We observed how microtubules can grow into preformed membrane protrusions upon PI-OP18 triggered tubulin release, but are unable to generate enough force to induce protrusions themselves. Instead, when an excess of tubulin is released without room to grow straight for the microtubules, they will start to buckle. Of course the stiffness of membranes in *in vitro* experiments will differ greatly from plasma membranes and there we might observe different effects from PI-OP18.

The goal of creating these light-regulatable microtubule signaling effectors with FRET readout was to use them in a minimal bottom-up approach to understand the mechanisms underlying complex signaling systems. However, generating these functional constructs took a longer amount of time than anticipated and therefore the *in vitro* experiments have yet to be performed. The components from partner labs who have been working on generating stable microtubule asters in synthetic droplets or vesicles, *in vitro* gene expression, membrane splicing, and more could now be combined into an artificial living membrane system to study a range of different mechanisms and interactions. One key determining factor in signaling regulation is diffusion, and we generated three light-inducible systems that control the same protein with varying amounts of diffusible components. This allows for an in-depth look at the influence of diffusion in a controlled environment, ranging from three cytosolic components in the PA-Rac1/PAK1/ROPY combination, the two component PA-CaMKIV/ROPY system, and the single cytosolic PI-OP18 variant, to the membrane-tethered PI-OP18 version which will negate diffusion entirely. By comparing the effect of these systems on microtubules from similar local stimulation with light, we should be able to empirically determine the influence that each extra diffusible intermediate has. However, such a simple system is not only dependent on diffusion but also one other key factor: concentration ratios. One aspect we encountered during every part of the project was the importance of expression levels. If the ROPY sensor was overexpressed it would interact with so many tubulin dimers that the integrity of the microtubule network within cells became compromised. In addition, severe overexpression of PA-CaMKIV would increase its dark state activity to levels that could not be effectively countered by phosphatases. Basically, the right balance between all components is necessary for their proper functioning which we also need to take into account in the *in vitro* system. Of course, thanks to optogenetics it would also be possible to quantify the influence of concentration levels. For example in a system with a fixed phosphatase activity, tubulin dimers, the ROPY sensor, and a PA-CaMKIV variant with a V416L point mutation that shifts its dark reversion halftime to over one hour[8]. By slowly increasing confined blue pulses to such a setup, the total kinase activity can be controlled until a threshold can be detected via the ROPY sensor. Depending on the property to be studied, adjustments to the LOV domain kinetics can be an extra tool for creating new experimental setups and should therefore not be overlooked.

Once the characteristics of the most basic diffusion-governed microtubule regulating systems are understood, we could expand the complexity to include other processes such as active transport through motor proteins along the microtubules. One possibility would be to compare the FRET gradients of the ROPY sensor and directional microtubule growth from local stimulation of only cytosolic PA-CaMKIV versus a system where motor proteins also interact with (active) PA-CaMKIV to transport it back to the area of simulation. It would also be possible to explore the impact of feedback loops, and we would not necessarily need to add extra components in the case of the PA-Rac1/PAK1/ROPY system. Since PAK1 displays autophosphorylation activity upon Rac1

stimulation to increase its own activity[9], this counts as a positive feedback loop. Thanks to the versatility of optogenetically controlled proteins, we could even simulate the effects of feedback loops without actually requiring protein interactions. For example, a negative feedback loop which will quench its own activity to create short bursts of activity, which can be mimicked by activating a photoswitchable protein with a single light pulse and then letting it revert back to its dark state. Or we could imitate the effect from a positive feedback loop by steadily increasing the amount of light-activated proteins. It is of course also possible to extend the features of this system in the future to beyond just microtubule regulation, by including other regulatable pathways such as an actin network and observe the interplay between both systems. The bottom-up approach allows for studying complex interactions in a more manageable manner than the *in vivo* setting, hopefully leading to new insights. In combination with the versatile optogenetics technique, these systems can create highly specific situations which cannot be studied in isolation otherwise, and will therefore likely have a big impact on future research.

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