FINAL REPORT: Beyond the dogma: quantitative biophysical analysis of the clustering of receptor tyrosine kinases and its effect on transmembrane signaling

1. Characterization of the effect of the accumulation of sphingolipids in the plasma membrane

We used an *in vitro* model system of Gaucher's disease to induce sphingolipid accumulation in cells. Glucocerebrosidase was inhibited in macrophages by conduritol B epoxide leading to substantial accumulation of sphingolipids not only in lysosomes, but in the plasma membrane as well. We carried out systematic analysis of the consequences of sphingolipid accumulation on membrane biophysical properties. The fluidity and hydration of the membrane was significantly decreased, and the fractional area of compact, liquid-ordered membrane domains significantly increased according to microscopic experiments (Fig. 1). Atomic force microscopy revealed that sphingolipid-enriched membranes were more prone to forming membrane tethers. We attribute this finding to the increased tendency of the membrane to become positively curved, i.e., curved away from the headgroups facilitated by the presence of glycolipids. Ongoing investigations also revealed that sphingolipid-enriched cells also produce more extracellular vesicles whose membrane is also positively curved. The lateral mobility of proteins and lipids preferentially accumulating in lipid rafts was affected only mildly, while non-raft proteins and lipids were restricted significantly according to fluorescence recovery after photobleaching experiments (Fig. 1). This duality of membrane effects was also manifested in the effect of the Gaucher phenotype on endocytosis. The rate of internalization of raft constituents was hardly affected by sphingolipid accumulation, while the internalization of the non-raft constituent transferrin receptor was significantly decreased. Sphingolipid accumulation had further functional consequences on the biological responses of cells. Interferon-gamma-induced STAT phosphorylation and nuclear translocation was inhibited in Gaucher cells. These results not only shed light on the biophysical consequences of sphingolipid accumulation in the cell membrane, but also allow insight into how the physiology of sphingolipid-enriched cells in patients suffering from the lysosomal storage disorder Gaucher's disease may be altered.

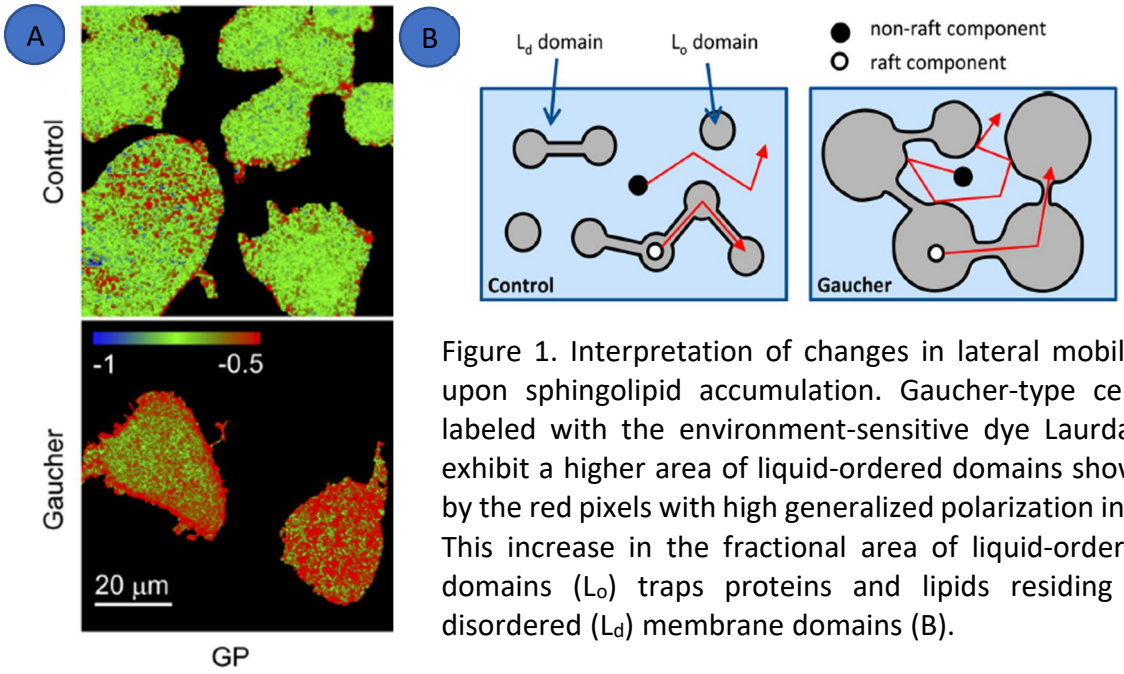

Figure 1. Interpretation of changes in lateral mobility upon sphingolipid accumulation. Gaucher-type cells, labeled with the environment-sensitive dye Laurdan, exhibit a higher area of liquid-ordered domains shown by the red pixels with high generalized polarization in A. This increase in the fractional area of liquid-ordered domains (L_o) traps proteins and lipids residing in disordered (L_d) membrane domains (B).

2. Measurement and modeling of equilibrium EGF binding to EGF receptor

While the fact that the extracellular domain of EGF receptor assumes a closed and an extended conformation with only the latter binding the growth factor significantly has been known for about two decades, several aspects of the receptor-ligand binding, e.g., the appearance of negative and positive cooperative binding depending on experimental conditions, and the effect of the intracellular kinase domain on ligand binding, have not been investigated in detail. We used cells stably transfected with GFP-tagged EGF receptor and measured the binding of fluorescently-labeled EGF to different subpopulations of these cells exhibiting different receptor expression levels by flow cytometry. These subpopulations were identified based on their GFP fluorescence intensity. Equilibrium binding of EGF was measured in the presence and absence of two kinase inhibitors stabilizing the EGF receptor kinase domain in its active and inactive conformation, enabling us to decipher the role of the kinase domain. A model was generated in which two distinct dimerization pathways are present (Fig. 2). The model was solved to yield the amount of cell-bound EGF as a function of receptor expression level and ligand concentration, and this model was fitted to the experimental data. Fitting revealed that dimers harboring inactive kinase domains are characterized by low affinity and positive cooperativity, while dimers with active kinase domains have high affinity and negative cooperativity. Positive cooperative EGF binding was abolished by inhibiting receptor glycosylation and by disrupting actin filaments. The model

provides a comprehensive view on the molecular transitions taking place upon EGF binding to its receptor.

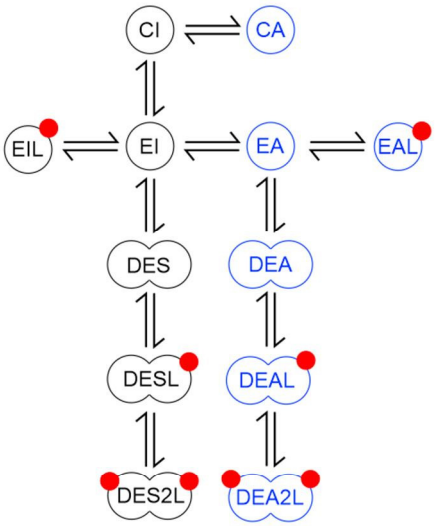

Figure 2. Model of EGF binding. The extracellular part of the receptor is present in either a closed (C) or extended (E) conformation with both of these states coupled to either an active (A) or inactive (I) kinase domain generating four possible monomers (CI,CA – closed extracellular domain with inactive and active kinase domain, respectively; EI, EA – extended extracellular domain with inactive and active kinase domain, respectively). Liganded species are designated by an 'L' at the end of their names (red circles – EGF). Dimers are designated by a D at the beginning of their names. Two distinct dimerization pathways beginning from monomers with inactive and active kinase domains are present. The former is the beginning of the pathway with dimers with symmetric kinase domain dimers (DES, DESL, DES2L), while asymmetric kinase domain dimers (DEA, DEAL, DEA2L) are present in the latter pathway.

3. The effect of cancer-associated fibroblasts on the clustering and signaling of ErbB proteins

In order to shed light on the effect of cancer-associated fibroblasts (CAF) on cells expressing ErbB proteins (EGF receptor or ErbB2), we kept two different breast cancer cell lines, JIMT-1 and SKBR-3, overexpressing ErbB2, and another epithelial carcinoma line, A431, overexpressing EGF receptor, in five different conditions: (i) alone; (ii)-(iii) culture with medium conditioned with CAFs or normal fibroblasts (iv)-(v) coculture with CAFs and normal fibroblasts with the cancer cells and the fibroblasts separated from each other by a filter to prevent direct cell-cell contact. Normal fibroblasts and CAFs were obtained as cell lines from ATCC and Asterand. According to our measurements, the proliferation of the three cancer cell lines was enhanced when they were cultured in the presence of medium conditioned with either CAFs or normal fibroblasts. This change in proliferation was accompanied by a decrease in the cell surface expression of EGF receptor without significant effect on the total cellular expression level of the protein implying internalization. Under the same experimental conditions, the activation level of EGFR and ErbB2, assessed by their tyrosine phosphorylation, was significantly enhanced. Furthermore, the heterodimerization of EGF receptor and ErbB2 was slightly, but significantly enhanced by the same experimental

conditions without significant effect on the homodimerization of any of the proteins. Although we undertook efforts to identify the soluble mediator responsible for the aforementioned effects, these experiments have not yet led to unambiguous results.

4. The effect of the labeling ratio on the affinity and fluorescence quantum yield of monoclonal antibodies

Since most experiments in my workgroup are carried out by fluorescence techniques, understanding the behavior of antibodies coupled to fluorescent dyes is of great practical importance. Therefore, we carried out a systematic analysis of how fluorescence labeling influences antibody affinity and the fluorescence quantum yield of dyes. Using several different approaches, we established that conjugation of fluorescent dyes decreases the affinity of monoclonal antibodies in a dye-dependent way. We developed a method capable of determining the degree of labeling (DOL) of the cell-bound antibody fraction, and explicitly showed that the aforementioned phenomenon leads to an underrepresentation of antibodies with a high DOL in the cell-bound fraction (Fig. 3). In the case of certain antibodies, this effect is so profound that the DOL of the bound fraction is practically independent of the DOL of the stock. Furthermore, the fluorescence quantum yield of certain kinds of dyes was significantly decreased by labeling. These results are essential for quantitative evaluation of fluorescence measurements (e.g., intensity-based FRET measurements). Although the results have already been published, ongoing efforts are still underway to incorporate these findings into analysis protocols.

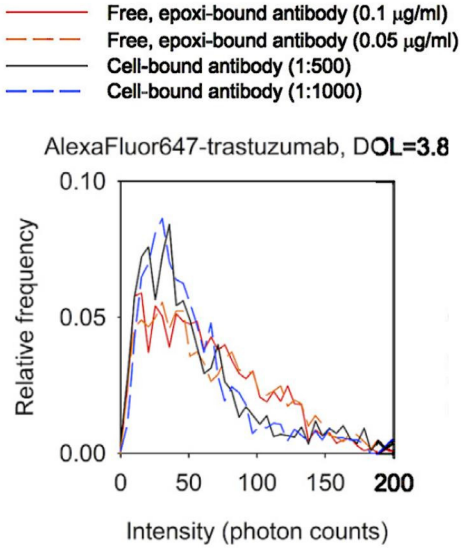

Figure 3. Antibodies with a high degree of labeling are underrepresented in the cell-bound fraction. AlexaFluor647-conjugated trastuzumab Mab was coupled to epoxy-functionalized coverslips at a low enough density to allow single molecule detection by confocal microscopy. The intensity distribution of individual fluorescent spots at two different antibody dilutions is shown by the continuous and dashed red lines. SKBR-3 cells were labeled with the same stock of fluorescent antibody mixed with unlabeled trastuzumab at two different molar ratios. The intensity distribution of individual fluorescent spots reveals an underrepresentation of high intensity spots corresponding to antibodies with a high degree of labeling (black and blue lines).

5. The effect of fluorophore saturation on intensity-based FRET measurements

Besides increasing the degree of labeling of antibodies, another way for increasing the fluorescence signal is to enhance the excitation intensity. However, this approach leads to fluorophore saturation that not only limits the extent of signal increase achieved by the higher laser power, but can also lead to artifacts in the evaluation of intensity-based FRET measurements. In our experiments, we showed that commonly used excitation laser intensities result in significant underestimation of the FRET efficiency in confocal microscopy (Fig. 4). We developed several approaches for correcting FRET calculations for the presence of fluorophore saturation. The simplest formula involves the apparent FRET efficiency calculated using the conventional formalism corrected for the presence of fluorophore saturation. The most complicated formalism not only takes fluorophore saturation into consideration, but also accounts for FRET frustration, i.e. the lack of FRET if acceptors are excited. The formalisms developed add significantly to the device independence and rigor of intensity-based FRET calculations.

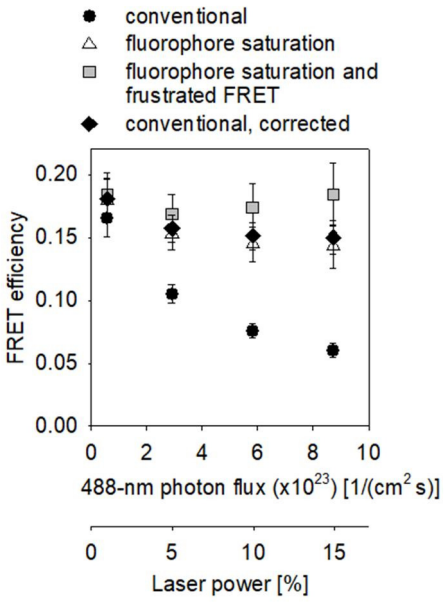

Figure 4. Evaluation of intensity-based FRET measurements in the presence of fluorophore saturation. FRET between AlexaFluor488-tagged and AlexaFluor546-tagged antibodies was measured by confocal microscopy as a function of donor excitation laser power. The conventional evaluation strategy led to significant underestimation of the FRET efficiency at high excitation intensities (●). Correcting the conventional formalism for fluorophore saturation (◆) and explicitly taking fluorophore saturation into account during the evaluation itself (△) significantly reduced the dependence of the apparent FRET efficiency on excitation intensity. Taking fluorophore saturation and FRET frustration into account essentially eliminated the intensity-dependent underestimation of the FRET efficiency (■).

6. The effect of membrane biophysical properties on the cellular uptake of cell penetrating peptides

Since the biophysical properties of the cell membrane are expected to influence the uptake of positively-charged cell penetrating peptides (e.g., penetratin), we extended our investigations to studying whether it is possible to enhance penetratin uptake by altering membrane biophysical characteristics. Penetratin was labeled with a pH sensitive

(naphthofluorescein) and a pH insensitive (AFDye532) fluorophore enabling the distinction between endosomal and cytosolic localizations. First, we showed that (i) the positive intramembrane dipole potential inhibits penetratin uptake; and (ii) uptake is mainly mediated by endocytosis followed by membrane crossing in endosomes. Having established the role of the dipole potential in regulating penetratin uptake, we searched for ways to decrease it in a way that is compatible with potential future medical applications. We found that ω-3 fatty acids and statins (e.g., atorvastatin), used for lowering blood cholesterol level in hypercholesteremic patients, not only decrease the membrane dipole potential, but also boost the cellular uptake of penetratin (Fig. 5). Ongoing investigations are aimed at investigating the applicability of the aforementioned principle in animal experiments.

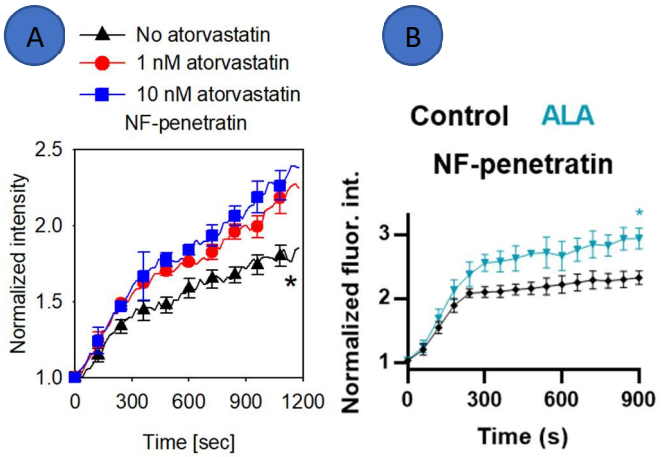

Figure 5. The cellular uptake of penetratin is enhanced at decreased dipole potentials. The uptake of naphthofluorescein (NF)-tagged penetratin, reporting on the amount of penetratin in non-acidic compartments mainly corresponding to the cytosol, was measured by flow cytometry. The uptake was significantly enhanced by atorvastatin (A) and by α-linolenic acid (ALA), an ω-3 fatty acid (B).

7. The role of the cell membrane in the protective role of protein phosphatase Z against oxidative damage

Protein phosphatase Z (PPZ) is a fungus-specific phosphatase whose deletion has been shown to decrease the competitive fitness of *C. albicans* cells and make them more sensitive to oxidative damage. Since oxidizing agents often primarily damage the membrane, we investigated if the cell membrane is the target of the protective role of PPZ against oxidative damage. Mild oxidative damage induced by tert-butyl-hydroperoxide (tBOOH) led to a decrease in membrane hydration, shrinkage of ordered membrane domains and a retardation of lateral diffusion. These changes were significantly exacerbated in the PPZ-KO strain. In particular, the lateral diffusion of membrane components practically ceased in tBOOH-challenged PPZ-KO cells according to fluorescence recovery after photobleaching (FRAP) measurements (Fig. 6). A lower level of the aforementioned changes in the biophysical properties of the cell membrane were also present in oxidatively unchallenged, PPZ-KO cells

implying the presence of latent oxidative damage. The results underline the important role of the cell membrane in the protective role of PPZ against oxidative damage in *C. albicans*.

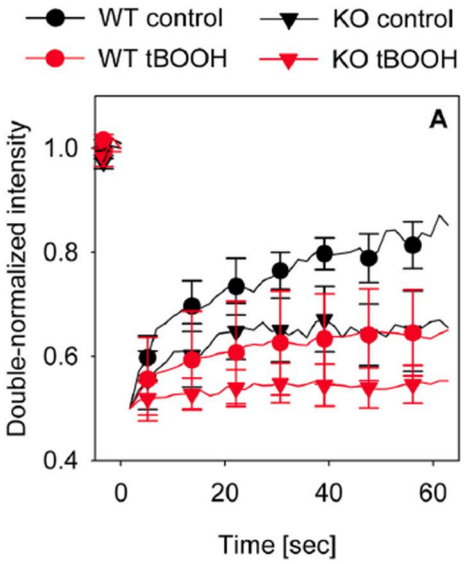

Figure 6. FRAP measurements reveal inhibited lateral diffusion upon oxidative damage. Wild-type (WT) and PPZ-KO *C. albicans* cells were left untreated ("control") or treated with tBOOH followed by labeling their membrane with the fluorescent marker FAST-DiI. Lateral diffusion was measured by FRAP. A membrane area with an approximate diameter of 1.5 μm was bleached resulting in the sudden loss of fluorescence at 0 sec. Return of the fluorescence to the bleached spot due to lateral diffusion was monitored.

8. Measurement and modeling of the spread of local activation

Contradictory results have been published about whether local stimulation of the EGF receptor leads to lateral spreading of receptor activation in the plasma membrane. A systematic analysis of the phenomenon as a function of ligand concentration and receptor expression level has not been carried out. Therefore, in collaboration with the Technical University of Vienna, we attempted to establish a system in which a micropattern of streptavidin was printed on coverslips and biotinylated anti-EGF receptor antibodies were used to recruit EGF receptor to the pattern. Since the overwhelming majority of EGF receptors were recruited to the pattern, stimulation of cells with soluble EGF led to local stimulation. The system enabled us to investigate a large number of local stimulation events. This feature is an advantage compared to stimulation with EGF-coated microspheres making the stimulation of only few cells possible. We detected an increased tyrosine phosphorylation of the EGF receptor upon growth factor stimulation, however, the spread of the signal from the micropatterns was very limited. We assumed that the high local density of EGF receptors above the printed pattern may be the reason for the lack of signal spreading. Attempts at reducing the density of printed streptavidins was not successful due to technical problems with the technique. We are currently in the process of solving this problem before proceeding to a systematic analysis of the effect of local receptor density on signal spreading.