6 Final discussion and outlook

In the first part of the present studies, semisynthetic HD-Far-MIC-N-Ras 1-181 was immobilized on POPC-SSLB. In this system, dichroic ATR-FTIR spectroscopic experiments proved N-Ras to be uprightly/perpendicular oriented relative to the membrane even at a protein surface loading of approximately 50%. Crucially, these findings contradict the established model that Ras in general lies on membranes being oriented parallel relativ to them.

Additionally, it is under debate that upon switching between its activated and in-activated state Ras changes its orientation. In the present studies, application of dichroic difference spectroscopy could at least not verify major changes in the protein’s orientation further limiting the established model.

In the next step, N-Ras’ surface loading was further decreased to approximately 10%. An innovative and highly customized experimental setup was implemented which for the first time allowed simultaneous data acquisition in concerns of both ATR-FTIR- and time sensitive TCSPC-FRET spectroscopy during the whole experiment duration. Thereby, the setup allowed first, observation of the lipid membrane’s quality as well as quantification of the amount of native, immobilized protein on the membrane (ATR-FTIR) and second, determination of the average inter-protein distance (TCSPC-FRET). The obtained data were analyzed by use of self conceived statistics and data processing routines.

Crucially, the FRET efficiencies determined during these experiments doubtlessly proved HD-Far-MIC-N-Ras 1-181 not to be distributed monomerically on POPC lipid bilayers.

Taken together, these results strongly contradict the established model that Ras is distributed monomerically on lipid bilayers switching between parallel and perpendicular orientation relativ to the membrane depending on its activation state.

In order to overcome the contradiction between these new experimental results and the theoretical model, the former proposal of N-Ras dimerization on POPC-SSLB [20] which has recently been supported by MM-simulations [25] was experimentally verified in the present studies.
Strikingly, the experimentally determined average distance between Ras bound GDPs is in exact accordance with the simulated distance in case of two dimerizing N-Ras proteins. Both values were determined to be approximately 46 Å! Importantly, the found FRET efficiency was shown not to be due to concentration artefacts which indicates an actively driven process. Apart from this, it is noteworthy that Ras dimers were found in 50 out of 71 X-ray structures [69].

In conclusion, the new model of uprightly oriented N-Ras dimers on lipid bilayers was experimentally confirmed. However, it shall be mentioned that the experiments presented in this work can falsify monomeric distribution and parallel orientation of N-Ras on membranes. But, investigation of the FRET efficiencies alone cannot help differentiate between dimers and possible oligomers. In this regard, a dimerization (proved to be stable by MM-simulations) is the least complex model being capable to consider all experimental and computer-simulated results presented here. Therefore, it is preferred to more complex models like higher order oligomers.

In the second part, N-Ras was transferred to a less artificial system by immobilization on giant unilamellar vesicles, GUVs. Once again, TCSPC-FRET experiments revealed average inter protein distances of HD-Far-MIC-N-Ras 1-181 on 5 component anionic, heterogeneous GUVs to be approximately 48 Å. Crucially, this value lies well within the error margin of the simulated value $46 \pm 3$ Å on POPC-SSLBs. Therefore, dimerization of N-Ras on such GUVs is highly probable. Surprisingly, the same protein was found to be distributed significantly different on homogeneous POPC-GUVs rejecting exclusive dimerization of N-Ras on such GUVs.

Further, the N-Ras point mutant HD-Far-Mic-N-Ras 1-181 Y64A was immobilized on anionic, heterogeneous 5 component GUVs in order to verify its dimer-breaking influence which was reported for H-Ras on SSLBs [111], before. Unfortunately, the results do not allow an absolut doubtfree statement whether Y64A completely abolishes dimerization or not. Anyway, the large deviation between the experimentally achieved value for N-Ras Y64A ($\approx 52$ Å) and the value for the wild type ($\approx 48$ Å) on GUVs as well as the immense deviation from the simulated value of 46 Å strongly
suggests considerable differences in the distribution behavior of Y64A compared to the wild type on heterogeneous membranes.

At this point, the error margins of the different results shall be discussed in more detail. As mentioned before, the relatively high error margins for the experimentally achieved inter protein distances (mostly between 6 Å to 8 Å) are mainly attributable to the high uncertainty of the theoretically determined value of $\kappa^2 \ (0.41 \pm 0.31)$. Crucially, as this value is used for the calculation of $R_0$, which was set to the same value for all experiments performed during the presented studies, its uncertainty can be neglected when comparing different measurements with each other.

As can be taken from Table 14 (previous chapter), the respective error margins significantly decrease when neglecting $\Delta R_0$. In this regard, inspection of the determined average distances $r$ (respectively the corresponding deviations from the simulated value) and taking into account the corresponding error margins allow to propose dimerization of N-Ras on heterogeneous GUVs (as on POPC-SSLBs). In contrast to this, dimerization (of all or at least the majority of the immobilized proteins) can definitively be rejected for N-Ras wild type on homogeneous POPC-GUVs as well as for the point mutant Y64A on heterogeneous GUVs.

Taken together, immobilization of N-Ras wild type on homogeneous GUVs might lead to a shift of an equilibrium between dimers and monomers towards monomers compared to the situation on anionic, heterogeneous GUVs. It is similar for the system of N-Ras Y64A on heterogeneous GUVs.

In this connection, probable reasons for the different behavior of N-Ras on POPC-SSLBs respectively homogeneous POPC-GUVs (deviation of about 6.5 Å in average distance) might be differences in the membrane’s properties as will be discussed in the following. Differences could be distinct protein surface concentrations on both membranes which will not be discussed in more detail as the protein surface concentration on the GUVs could not be approximated exact enough. Apart from this, differences might occur when N-Ras is immobilized on the membrane. This takes place by intruding of the protein’s lipid tail into the membrane. During this process, the lipid tail pushes the membrane’s carbon chains to the side. In case of a
SSLB, this evasive movement might be restricted at least in the edge regions of the supporting crystal. Additionally, SSLBs are planar in contrast to GUVs which are spherical. Again, differences in the arrangement of the membrane’s- respectively the lipid anchor’s carbon chains are probable. In this regard, it is of crucial importance that binding of lipidated proteins on membranes has a decisive influence on the membrane’s condition \[51, 27\].

Next, possible reasons for the effect of Y64A point mutation on the distribution of N-Ras on heterogeneous GUVs will be discussed in more detail. Figure 51 depicts two N-Ras proteins dimerizing by attractive contacts within the dimer interface (in green respectively blue).

Fascinatingly, Tyr 64 (red) is positioned on more or less the opposite side of the protein than the proposed dimer interface (cf. chap. 2). In this regard, it is surprising that point mutation of this residue (tyrosine exchanged by alanine) influences the average interdye distance between N-Ras proteins on heterogeneous GUVs and
therefore, seems to affect the protein’s dimerization behavior. Remarkably, residue 64 is not in close proximity to neither the nucleotide binding region (GDPs in magenta) nor the Ras’ lipid anchorage excluding direct interactions between them.

On the other hand, a dimer interface, distinct from the proposed one, somewhere around tyrosine 64 strongly contradicts all experimentally achieved data of the present studies. This is because in such a case the labelled GDPs would be much closer to each other than for the case of dimerizing via the proposed interface. Consequently, the measured FRET efficiencies would be decisively higher resulting in far lower interdye distances than the simulated one of 46 Å. In fact, not a single experiment yielded such smaller distances strongly rejecting a dimer interface around residue 64. Following this, allosteric, long range interactions via residues between Y64 and the proposed dimer interface are most probable. In fact, such an explanation was suggested elsewhere [111] and was attributed to MD-simulations revealing information transfer across the protein by side chain interaction along isoform specific routes in Ras [116].

In this matter, the dimer breaking influence of Y64A on H-Ras dimers on SSLBs, postulated by Lin and Iversen [111], is not confirmed by the present data. Instead, as mentioned above, a shift in an equilibrium distribution between dimers and monomers towards monomers (compared to wild type) is proposed. Noteworthy, Jay Groves, supervisor of Lin and Iversen, supported this alternative proposal when he was confronted with the presented results in a recent personal communication [115].

Finally, the relative protein surface concentrations on homogeneous- respectively heterogeneous GUVs will be considered. In fact, N-Ras exhibited considerably higher protein surface concentrations (3 to 10 fold) on POPC-GUVs than on heterogeneous ones. This is most probably due to a higher binding affinity of the protein for the POPC bilayer. Counterintuitively, N-Ras was proven to be spread further apart (≈54.5 Å) on POPC-GUVs than on heterogeneous ones (≈48 Å). Therefore, the measured FRET efficiency on heterogeneous GUVs was not evoked by concentration artefacts because a lower $E_{FRET}$ was measured on homogeneous POPC-GUVs (at higher protein surface concentrations). These results strongly support
an actively driven dimerization process of N-Ras on heterogeneous GUVs which is independent from the protein surface concentration.

In conclusion, the established model that membrane bound N-Ras is monomerically distributed and oriented parallel relative to the membrane needs to be revised. From now on, dimerization of N-Ras should seriously be considered. It is noteworthy, that membrane-driven dimerization of Ras was suggested before [93]. The authors investigated the activation of Raf-1 kinase by Ras-GTP and revealed additional presence of liposomes to be essential. Surprisingly, they found Ras-homo-dimers at those liposomes in cross-linking experiments. This led them to investigate whether artificially synthesized Ras-GTP dimers could activate Raf-1 kinase in solution. As it did, they proposed membrane-driven dimerization of Ras.

Importantly, rare nanoclustering of N-Ras on membranes [69], depending on the membrane’s composition as well as condition was discussed before [27, 37]. Fascinatingly, N-Ras nanoclustering was rarely observed within the experiments of the present studies (cf. Fig. 42 - round protein accumulation on the GUV’s downside). However, a quantification of such nanoclusters was not performed during the presented studies.

In this regard, a dynamic distribution of protein mono-, di- and oligomers seems probable with the balance being shifted towards specific types depending on parameters as the membrane, protein anchorage and of course mutations. Crucially, the increased average inter protein distances between N-Ras on POPC-GUVs or between the N-Ras point mutants Y64A on heterogeneous GUVs are in line with such a model.

As a consequence, in vivo dimers and monomers might coexist on membranes with transient dimerization possibly catalyzing nano-clustering. These nano-clusters enable strong signal bursts that lead to high-fidelity signal transmission [94].

Further, in the context of cancer therapy, exact knowledge of N-Ras’ membrane environment could be very valuable for blocking the protein and thereby interrupting oncogenic downstream signalling. In this connection, it makes a major difference whether Ras is distributed as monomers, dimers or some sort of a mixture of both.
Finally, taking dimerization of N-Ras into account, these new findings could be of great value in concerns of cancer drug development. Future studies should concentrate on the exact geometry of Ras dimerization (MM-simulations and mutant experiments), the different cell-physiological parameters influencing the balance between dimers and monomers (experiment series changing lipid composition, temperature, surrounding salt concentration, etc.), and the exact interactions between the membrane and the protein’s lipid anchorage (MM-simulations and mutant experiments). Further, great efforts should be made to covalently bind suitable (best non invasive, stable, high quantum yield) fluorophores (FRET-pair) to Ras in order to overcome the limitations due to the nucleotide exchange during the FRET experiments. Besides this, experiments using N-Ras with point mutations in the proposed dimer interface need to be performed in order to get to know more about the exact mechanism. Of course, great efforts should be made in order to perform similar experiments as the ones described in the present studies using different Ras isoforms in order to achieve further general information about the groundlying role of Ras in the development and progression of cancer.