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Investigating EGFR Dimerization Dynamics: Analyzing Cancer-Associated Mutants Using DARPins and TOCCSL

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1. Introduction

1.1 Cancer

The term cancer probably derives from the Latin word for crab. The disease persists throughout human history, ancient observants would see similarities between a crab and late-stage cancer reaching into surrounding tissues somewhat resembling a crab's claw. Cancer is therefore usually tied to a malignant growth of cells, meaning that cells display uncontrolled and unwanted proliferation accompanied by loss of typical structural and functional characteristics from their lineage (Stephens and Aigner, 2015). If solid tissue is affected, one generally differentiates between two types of cancer. Carcinoma who exhibit tumors of epithelial origin for example in skin cells, respiratory tract or gland tissue like the pancreas. Sarcomas on the other hand are tied to tumors in connective tissue like bone, muscle, fat or even nerve and blood tissue. Carcinomas are considered more common than sarcomas, which roughly represent 1% of adult solid malignant cancers (Burningham, 2012). The investigation to understand cancer causes began in the 18th century, noting a higher scrotum cancer prevalence among London chimney sweepers. Yamagiwa and Ichikawa's groundbreaking work in the early 20th century established the link between carcinogens and cancer induction, outlining the disease progression from benign stages to metastasis for the first time (Lipsick Joseph, 2021). Cancer's causes are multifactorial, with various elements influencing and altering cell genetics, impacting the expression of tumor suppressor and proto-oncogenic genes. DNA-damaging agents like UV light, radiation, tobacco tar, chemical carcinogens, industrial exposures, prolonged hormone exposure, lifestyle, genetic predispositions, and viruses are recognized contributors to cancer risk (Stephens and Aigner, 2015). Despite advancements in cancer detection and treatment, global variations persist in cancer incidence and survival rates. Some cancers, such as stomach and lung cancers in men show decreasing trends, while others, like pancreatic and lung cancers in women, maintain their incidence rates (Santucci *et al.*, 2020). Cancer is still the first or second leading cause for death before the age of 70 and latest global data cites over 19 million cases a year. Female breast cancer is now the most commonly diagnosed cancer while lung cancer remains the leading cause for cancer deaths (Sung *et al.*, 2021). Therefor any further efforts, without a doubt, to elucidate

cancer processes and improve treatment are much needed to attenuate global cancer burden.

1.2 Lung cancer

Lung cancer is generally divided into two types. One being non-small-cell lung cancer (NSCLC) and the other is defined as small-cell-lung cancer (SCLC). The prevalence is roughly 85% and 15% respectively (Testa, Castelli and Pelosi, 2018). The 5-year survival rate for non-small cell lung cancer patients is very low, at around 16%. The two phenotypical main types of NSCLC are adenocarcinoma (ADC) and squamous cell carcinoma (SCC). Adenocarcinoma often have genetic alterations such as KRAS (kirsten rat sarcoma virus), BRAF and EGFR (epidermal growth factor receptor) mutations, also recurring oncogene mutations and amplifications in ERBB2 (human epidermal growth factor receptor 2), and the FGFR family (fibroblast growth factor receptors) have been shown. LSQCC also frequently display mutations in TP53 (p53), FGFR1 - 3, and genes of the PI3K pathway (Testa, Castelli and Pelosi, 2018). Smoking is considered to be the number one reason for lung cancer, as up to 80% of cases can be related to cigarette consumption. Additionally, studies have clearly shown that risk of dying from lung cancer is significantly increased in smokers when compared to non-smokers (Thun *et al.*, 2013). Other potential risk factors that can contribute to cancer development are air pollution, fuel burning emissions indoors, exposure to radon or asbestos and heavy metals like chromium or cadmium (Raaschou-Nielsen *et al.*, 2013). A summary of established and putative risk factors is shown in Figure 1.

Established and putative risk lung cancer risk factors	
Risk factor	Magnitude of association
Tobacco smoking	20-fold increased risk vs. never smoker
Secondhand smoke	25% to 28% increased risk vs. never smoker
Electronic cigarettes	Presently unknown
Other tobacco use (cigars, pipes, water pipes)	1.9 to 4.6-fold increased risk
Smoked cannabis	Presently no known risk
Radon	14% to 29% increased risk
Asbestos	12% to 24% increased risk
History of COPD, emphysema, or chronic bronchitis	2 to 3-fold increased risk
History of asthma	28% to 44% increased risk
History of pneumonia	30% to 57% increased risk
History of <i>Chlamydia pneumoniae</i>	1.2 to 2.4-fold increased risk
History of tuberculosis	48% to 76% increased risk
HIV	2-fold increased risk

Figure 1: Table listing several lung cancer risk factors. Adapted from Schabath and Cote, 2019.

1.2.1 Small cell lung cancer

SCLC represents the less frequent type of lung cancers. It has its own unique characteristics as well as cellular and molecular mechanisms (Rudin *et al.*, 2021). SCLC belongs to neuroendocrine types of carcinomas, which can arise from cells of the endocrine and nervous system.

It is often characterized by various respiratory symptoms and imaging usually reveals a centrally located lung mass with lymph node involvement. Two thirds of patients have distant metastases at diagnosis, commonly affecting the contralateral lung, brain, liver, adrenal glands, and bone. SCLC displays high levels of circulating tumor cells when compared to other solid tumors (Hou *et al.*, 2012). Despite biological subtypes, the current treatment approach remains consistent. Early-stage patients may undergo surgery and adjuvant platinum-based chemotherapy, while others with more advanced stages may receive concurrent radiation and chemotherapy. Usually,

metastatic patients are treated upfront with systemic chemotherapy with or without immunotherapy (Kalemkerian *et al.*, 2018).

The common risk factor smoking seems to have the strongest epidemiological link to SCLC, with an established dose-response curve (Huang *et al.*, 2015). This is supported by the share of only 2%, of non-smokers or never smokers (less than 100 cigarettes consumed in a lifetime) amongst patients who develop SCLC.

It is still under debate as how much genetic factors contribute to SCLC development, including how genetic variations influence nicotine addiction and therefore indirectly increase the risk for SCLC (Improgo *et al.*, 2010). Interestingly, it has been shown recently that there is a possibility in never smokers, for developing SCLC from lung adenocarcinoma through mutation of the Epidermal Growth Factor Receptor (EGFR) (Quintanal-Villalonga *et al.*, 2020). In the majority of SCLC tumors, tumor suppressor genes, TP53 and RB1 (retinoblastoma protein) exhibit inactivating mutations, which is distinct from NSCLC that seem to rely more on activating oncogenic mutations for tumor formation (George *et al.*, 2015).

Alterations in the lung stroma and immune microenvironment likely contribute to SCLC development as well (Huang *et al.*, 2015). However, the exact impact of these tumor-intrinsic and tumor-extrinsic factors on the cell type of origin, tumor growth, metastasis, and response to therapy in SCLC is not fully understood. Despite other well known players (gene families and their members) in SCLC tumors supported by large data from cohort studies, no obvious defined subtypes of SCLC have been identified. Furthermore, no consistent differences in driver mutations have been identified based on ethnicity or smoking status in SCLC (Rudin *et al.*, 2021).

However, never-smokers with SCLC seem to have a higher prevalence of oncogenic drivers compared to tobacco users.

Some lung adenocarcinomas have furthermore been described to transform into a neuroendocrine phenotype resembling SCLC, particularly in response to EGFR or other tyrosine kinases, inhibitor treatment.

Research efforts to understand the genetic and molecular patterns in SCLC are currently hampered by a lack of patient material. In the future, circulating tumor cells (CTC) may therefore play a greater role in SCLC research (Hodgkinson *et al.*, 2014).

Despite all efforts in screening, prevention and management, SCLC continues to show a poor to moderate five year survival and patients (advanced or late stages) are especially prone to relapse within the first two years of treatment (Faivre-Finn *et al.*, 2017). Therefore, SCLC remains a highly lethal disease, and there are gaps in our understanding that limit the effectiveness of current treatments. Prevention plays a crucial role, considering the strong association between SCLC and tobacco carcinogen exposure.

1.2.2 Non-small cell lung cancer

NSCLC occur comparatively more frequently than SCLC. NSCLC accounts for about 80 to 85% of lung cancers, with adenocarcinoma making up about 40%, squamous cell carcinoma 25 to 30%, and large cell carcinomas 10 to 15% of cases (Schabath and Cote, 2019). The classification of bronchioloalveolar carcinoma (BAC) has been replaced by adenocarcinoma in situ, minimally invasive adenocarcinoma, and invasive adenocarcinoma of the lung. Other less common histological subtypes represent adenosquamous carcinoma, large cell neuroendocrine carcinoma, amongst others.

Although the main subtypes are histologically different, the clinical approach for treatment and management are quite similar for all NSCLC. Similar to SCLC, common symptoms of non-small cell lung cancer are cough, shortness of breath, coughing up blood, chest pain and recurrent chest infections or pneumonia that do not respond to treatment. Spreading to lymph nodes, bones, the brain, or other organs has been observed. Furthermore, systemic effects leading to symptoms such as breast swelling, bone changes (osteopathopathy), clubbing of the fingernails, or nerve-related issues like numbness or tingling sensations (neuropathies) can occur (Stephens and Aigner, 2015).

The outcome and therefore the survival of patients is directly linked to the stage of cancer at the time of diagnosis. The latest classification system stems from the WHO, 2015, that incorporates the evaluation of the degree of progression of the primary tumor, eventual spreading to regional lymph nodes and possible presence of distant metastases (TNM), with stages from 0-4 and a or b for subtype progression. Five year survival rates differ significantly between stage I and IV lung cancer patients, as the former group shows close to 68% and the latter roughly a 6% survival rate (Ganti

et al., 2021). Most NSCLCs are detected in stage IV, which is associated with poor survival.

Early detection in persons at risk, like COPD patients (and/or smokers) by lung cancer screening (low dose CT) have therefore been described to lower mortality rates (de Koning *et al.*, 2020). COPD (chronic obstructive pulmonary disease) and lung cancer are linked through smoking. COPD independently increases lung carcinoma risk, especially squamous cell carcinoma. Smokers with airflow obstruction face up to five times higher lung cancer risk. Excluding factors like overdiagnosis, COPD patients still have twice the lung cancer risk, suggesting potential common mechanisms (premature aging, genetic predispositions) (Young and Hopkins, 2010; Durham and Adcock, 2015).

Surgical resection is the standard treatment for stages I and II, and some stage III, NSCLC. Adjuvant chemotherapy with a cisplatin-based doublet is recommended for completely resected stage IB (tumor > 3 cm) to IIIA NSCLC. However, even with chemotherapy, about half of stage IB and three-quarters of stage IIIA patients experience relapse with metastatic disease (Pignon *et al.*, 2008).

Approximately a fifth of patients is initially diagnosed with stage III cancer which is characterized as either metastasized tumors in the mediastinal area including lymph nodes or tumors with noticeable size that may affect local lymph nodes. Stage III NSCLC is considered to be a heterogeneous disease and its treatment depends on several factors including the patients condition. This leads to treatment that does not necessarily include surgery even though it is possible for carefully selected patients (Mithoowani and Febbraro, 2022). If the patient situation allows it, a multimodal treatment consisting of chemotherapy and radiation therapy should be considered either sequentially or in a concurrent form whereby the concurrent approach seems to increase survivability. With sequential order of treatment, for borderline patients the five year survival lies around 10% (Aupérin *et al.*, 2010). With the advent of immunotherapy an improvement in overall survival can be seen, as trials have successfully demonstrated so when incorporated with conventional therapy (Faivre-Finn *et al.*, 2021). Additionally, neoadjuvant strategies are gaining interest in the treatment of stage III NSCLC. For instance, patients with stage III NSCLC with advanced lymph nodes involvement can receive neoadjuvant monoclonal antibody immunotherapy and chemotherapy, followed by surgery. With this approach a

significant pathological response was observed in which 10% of the cases even achieved a complete pathological response (Rothschild *et al.*, 2021). Durvalumab should be highlighted as the latest analysis of the PACIFIC study confirmed enduring overall survival (OS) and progression-free survival (PFS) advantages with Durvalumab post chemoradiotherapy. Approximately 42.9% of patients receiving Durvalumab survive to the 5-year mark and 33.1% remain both alive and free from disease progression, setting a new standard of care in this context (Spigel *et al.*, 2022). Despite the progress made in lung cancer management, it continues to impose a substantial burden of illness and death worldwide. Preventing people from smoking and screening high-risk individuals are crucial strategies to alleviate this burden.

1.3 The EGFR

The epidermal growth factor receptor belongs to a subclass of tyrosine kinases grouped as receptor tyrosine kinases (RTKs). These RTKs are crucial in mediating several important biological functions such as cell growth, metabolism, differentiation or cell cycle control. Since the description of the first RTK over three decades ago, an overall of 58 additional receptors with 20 subfamilies have been identified in humans (Ullrich and Schlessinger, 1990). RTKs share a common molecular structure, consisting of extracellular ligand-binding domains, a single transmembrane helix, and a cytoplasmic region containing the protein tyrosine kinase domain (TKD). This architecture is conserved across species and indicates their important regulatory functions. Abnormalities in RTKs, including mutations and aberrant activation of their signaling pathways, are associated with various diseases such as cancer, diabetes, inflammation, bone disorders, arteriosclerosis, and angiogenesis (Lemmon and Schlessinger, 2010). An overview of known human RTKs is given in Figure 2. In general, under normal physiological conditions, RTKs are activated by specific ligands, such as growth factors, which bind to their extracellular regions. This ligand binding induces receptor dimerization or oligomerization leading to conformational changes and trans-autophosphorylation of the TKD within the receptor. This autophosphorylation recruits downstream signaling proteins, which bind to phosphotyrosine residues on the receptor. As a result these downstream mediators propagate essential cellular signaling pathways (Du and Lovly, 2018).

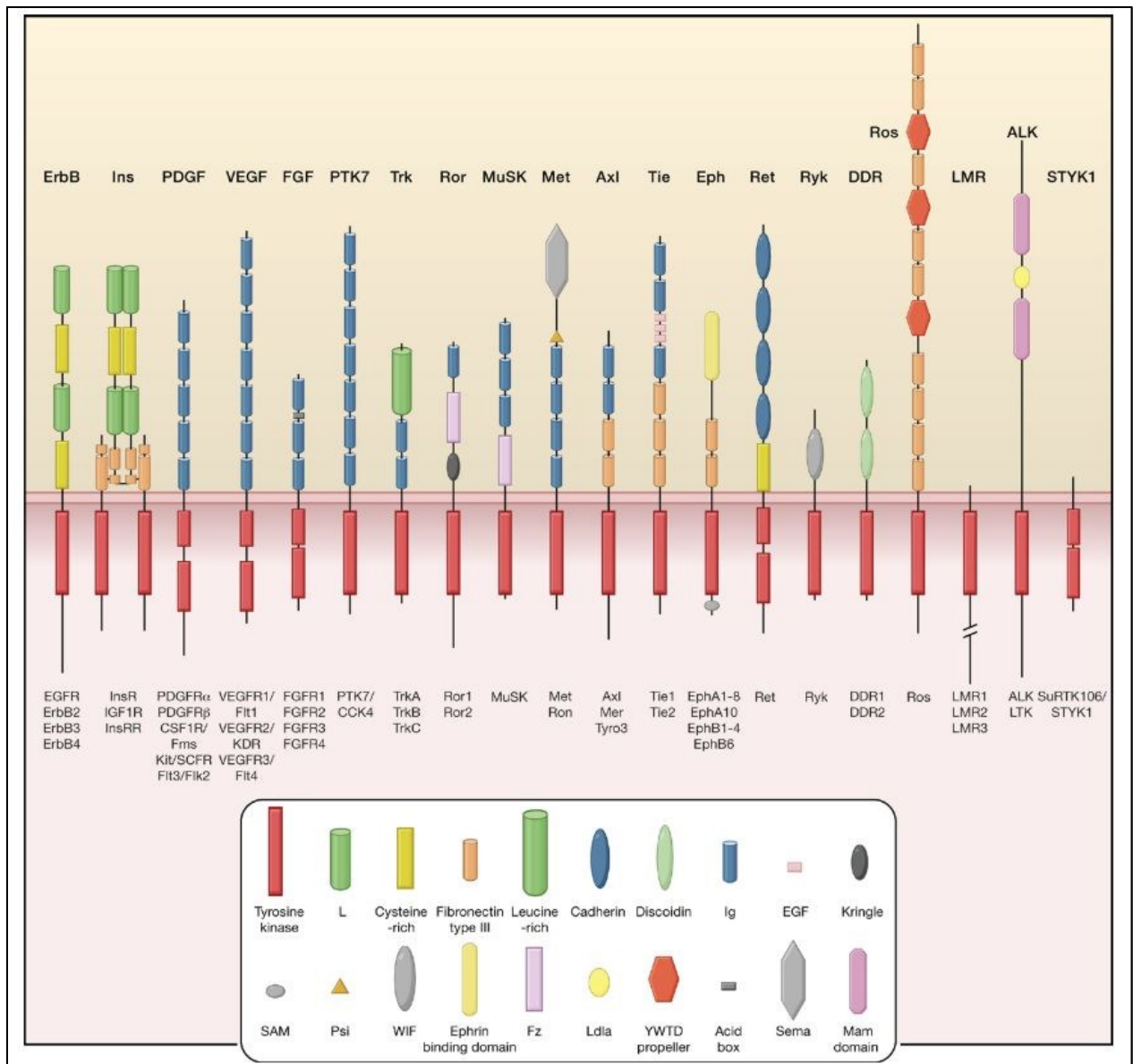


Figure 2: All 20 known human RTKs with family members listed beneath them. Structural domains of the extracellular domain are depicted as keys in the white map below. Red rectangles represent intracellular domains. Adopted from Lemmon and Schlessinger, 2010.

If dimerization is induced by a ligand, four different modes to activate the tyrosine kinase domain have been observed. The first mode involves ligand-mediated dimerization without direct contact between the extracellular regions of the receptors, as seen in TrkA (Tropomyosin receptor kinase A). The second mode relies on receptor-mediated dimerization without physical interaction between activating ligands, as observed in the ErbB (includes the EGFR) family members. The third mode involves ligand homodimers binding to two receptor molecules, which then interact with each other. The fourth mode includes a combination of bivalent ligand binding, direct receptor-receptor contacts, and involvement of accessory molecules like heparin or heparan sulfate in the case of FGFR family of RTKs.

The epidermal growth factor receptor holds historical significance as the prototypical receptor tyrosine kinase. It was the first RTK to be cloned and recognized for the role of ligand-mediated oligomerization in activating the enzyme. Furthermore, EGFR was the first RTK to establish a clear link between abnormal receptor function and cancer development (Schlessinger, 2002; Waterfield, 2007). In normal physiological conditions, EGFR and its homologous members in the EGF receptor family play vital roles in regulating cell growth, differentiation, and migration. EGFR is particularly important in epithelial development, while other family members have well-established functions in cardiac development, mammary glands, and the nervous system. However, when the signaling of these receptors is disrupted or their ligands are dysregulated, it can contribute to various diseases, including nervous system disorders and numerous types of cancer.

The EGFR (or HER/ErbB) family consists of four members: EGFR itself, ErbB2 (HER2/Neu in rodents), ErbB3 (HER3) and ErbB4 (HER4). All ErbB receptors possess the typical structural build of a large extracellular region, a single spanning transmembrane (TM) domain, an intracellular juxtamembrane (JM) region, a tyrosine kinase domain and a C-terminal regulatory region. The ligands that regulate ErbB receptors can be categorized into two main groups: the "EGF agonists" that activate EGFR and the neuregulins (NRG) that bind to ErbB3 and ErbB4. There are at least seven different EGF agonists, including EGF, transforming growth factor α (TGF α), amphiregulin (AR), betacellulin (BTC), epigen (EPN), epiregulin (EPR), and heparin binding EGF-like growth factor (HB-EGF). Some of these agonists can also activate ErbB4 and are referred to as bispecific ligands. Each ErbB ligand contains an EGF-like core domain that is biologically active. Unlike the other members, ErbB2 does not have a known soluble ligand and is proposed to activate ErbB receptors by forming heterodimers with other ligand-bound ErbB family members. This also regards to ErbB3 as it does not possess intrinsic kinase activity but interacts with other active family members to transduce signaling. Additionally ErbB2 is structurally distinct and can cause cell transformation independent of ligand activation (Yarden and Sliwkowski, 2001; Citri, Skaria and Yarden, 2003). Several intense structural and crystallographic studies have been conducted in the past to unveil the structure of the extracellular domain of the EGFR and its family. Basically, there are two homologous ligand binding domains designated as domains I and III and two cysteine rich domains designated domains II and IV. Alternatively, these domains are referred to

as L1 and L2 (leucine-rich repeats 1/2) and CR1 and CR2 (cysteine-rich 1/2). It should be noted that domain II harbors a β -hairpin loop which is crucial for receptor function, especially dimer forming. (Wieduwilt M. J. and Moasser M. M., 2011). A simple structural overview is given in Figure 3.

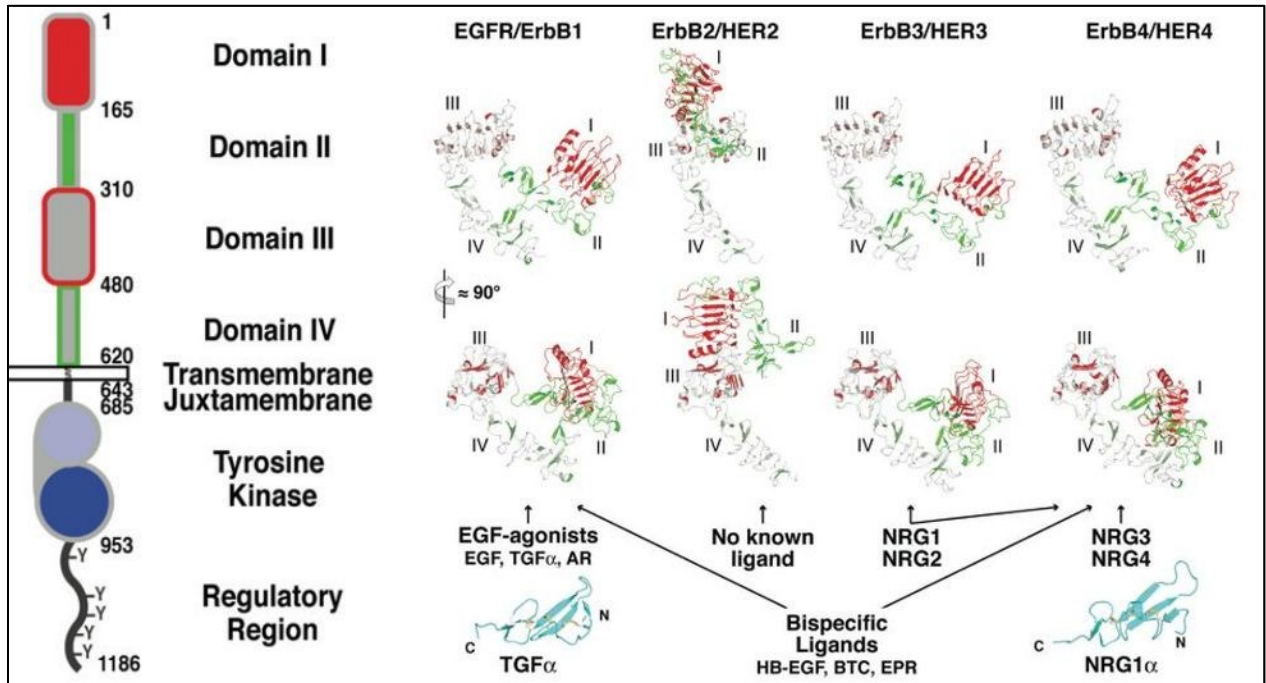


Figure 3: EGFR domains and extracellular regions of the family with their activating ligands. On the left: EGFR and its domains I-IV. Domains I and III share about 37% sequence identity hence the red-grey alike pattern. Domains II and IV are cystine rich and marked as green-grey. Purple: intracellular TKD with its two lobes and a c-terminal tail with key phosphorylation sites implied. On the right: Orthogonal, crystallographic views of extracellular regions in an unliganded state. TGF α and NRG1 α (upscaled) in cyan are representative for the ErbB ligand family. Note the different conformation of ErbB2. Adapted from (Ferguson, 2009)

Certain steps are necessary for proper downstream signaling of activated EGFRs, one being ligand binding. Ligand binding to any of the ErbB ectodomains and receptor dimerization initiate conformational changes in the intracellular tyrosine kinase domain, leading to receptor autophosphorylation. Detailed crystal structure analysis of the intracellular tyrosine kinase domain in a dimeric complex have been providing insights into the underlying mechanism. The tyrosine kinase domain exhibits a bilobed (see Fig.4) structure with ATP binding between the lobes. Activation of the receptor tyrosine kinase necessitates the interaction between the N-lobe of one tyrosine kinase domain and the C-lobe of its partner. After successful activation, which includes tyrosine phosphorylation in the TKD, multiple signaling pathways are activated downstream of the EGFR and its family. These pathways include for example Ras/MAPK, PI(3)K/Akt, PLC γ 1/PKC, STAT, and the Par6-atypical PKC pathway. The Ras/Raf/MAPK pathway promotes cell proliferation. The

PI(3)K/Akt pathway is important for cell survival and is activated by direct binding of a subunit of PI(3)K to activated receptors, particularly ErbB3 and ErbB4. Activation of PLC γ by EGFR regulates cell proliferation also. STAT3 helps regulating gene transcription and maintaining epithelial cell polarity and adhesion (Wieduwilt M. J. and Moasser M. M., 2011).

Now how does ligand binding affect the dimerization or oligomerization process of individual EGF receptors? It is been now widely accepted that the EGFR basically switches between two forms from an unliganded state and after bonding with a ligand.

These forms are referred to as the "tethered" and "extended" state respectively. This mechanistically new view was proven two decades ago and it also established that all contacts between a dimer are all receptor mediated (Garrett *et al.*, 2002; Ogiso *et al.*, 2002). As mentioned before, this is different to other RTK dimers where ligands can contribute to a bond. If a dimeric complex is formed when EGF or TGF α bind to the first three domains of the EGFR, the growth factor binding sites are located away from the dimer interface and do not directly contribute to dimer contacts. Instead, all contacts across the dimer interface are mediated by domain II of the receptor.

Domain II of the receptor contains a beta hairpin known as the "dimerization arm" that makes extensive contacts with the domain II of its binding partner. At the tip of the dimerization arm, it interacts with the opposite domain I. It should be noted that EGF and TGF α are bivalent ligands, meaning that each ligand simultaneously can bind to domains I and III of the same receptor molecule. It is therefore consistent that the exposure of the "dimerization arm" plays a crucial role in regulating receptor dimerization. In the inactive/unliganded state of EGFR, the dimerization arm is hidden due to intramolecular interactions with domain IV. These intramolecular interactions, known as the aforementioned "tethered" conformation, are mutually exclusive with the intermolecular interactions required for dimerization across the dimer interface. Based on these findings, it has been proposed that the tethered structures of EGFR represent an autoinhibited conformation, limiting receptor dimerization and signaling (Ferguson, 2009). Furthermore, the tethered form impedes ligand binding as the two domains involved are placed quite distant from another. Consequently, rearrangement of the domains I and III is necessary for a single EGF molecule to bind. If docking is successful, the rearrangement transfers the EGFR into the extended conformation exposing the dimerization arm on domain II. Figure 4 shows

the transition of conformations after ligand induced dimerization of the extracellular domain.

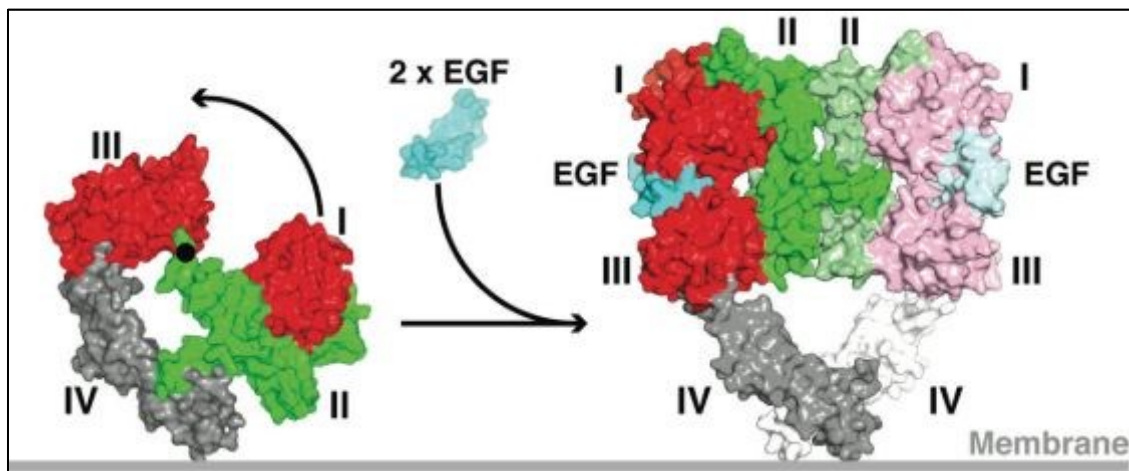


Figure 4: Tethered (left) and extended (right) conformation of an EGFR extracellular domain. Domains I and III in red, IV in gray and II in green. The black dot represents the rotation point in axis. $\sim 130^\circ$ rotation and small translation in plane are needed for transitioning between the two conformational states. EGF molecules in cyan. Adapted from (Ferguson, 2009).

The extended double bound dimer is often referred to as a “heart shape” or back-to-back dimer, but this is just one aspect of many findings out of studies that try to reveal the nature of EGFR monomers, dimers and multimers as well as the interactions that lay beneath the mechanisms by which EGFR is dysregulated.

1.3.1 EGFR dynamics

Although so much effort, methods and techniques have been established and applied, the EGFR is still not fully understood. This is most likely attributed to advantages and drawbacks of said individual methods. For instance, a crystallographic fragment stored online in a protein data bank may give important structural clues but gives no information about the function of a full and flexible protein and its dynamics in a membrane of a living cell. Furthermore, it is an ongoing discussion if selected cell types, the EGFR they are expressing (human EGFR or non-human EGFR), possible expression systems used, labeling agents and their dyes attached, EGF stimulation at various levels, may or may not interfere with the outcome of experiments which in return can lead to different results.

There are a lot of techniques that deliver detailed structures of isolated and purified proteins like the EGFR in solution or crystalline form. The most common ones are nuclear magnetic resonance spectroscopy (NMR), mass spectroscopy (MS) or transmission electron microscopy (TEM). However, it is more desirable to investigate

proteins and their dynamics in biological systems which the aforementioned methods lack to do so. Additionally there is no technique that provides atomic resolution in a cell to date (Martin-Fernandez *et al.*, 2019). Because of this a combination of methods is more likely capable of characterizing the organization of the EGFR on a cell. For further and better understanding I will shortly mention and describe the most common and important methods.

One method used to measure intermolecular distances between proteins, and therefore helps observing oligomeric states, is Förster resonance energy transfer (FRET). FRET involves labeling two proteins with fluorescent markers, one with a donor and the other with an acceptor. The fluorescence emission spectrum of the donor overlaps with the absorption spectrum of the acceptor. When the molecules are within a certain proximity, the donor can transfer its energy to the acceptor. FRET provides precise measurements of intermolecular distances below 8 nm. The fluorescence lifetime of the donor can be used to determine the FRET efficiency, and fluorescence lifetime imaging microscopy (FLIM) allows for investigation of specific areas within cells (Thompson, Larson and Webb, 2002; Chen *et al.*, 2013). For larger protein complexes, single-molecule localization techniques can be employed. One being fluorescence localization imaging with photobleaching (FLImP). It is a method that utilizes the shift in position of fluorescence when one molecule is photobleached. By measuring the distance between a pair of emitters the distribution of distances within protein complexes can be determined. FLImP has been used to study the oligomerization state of EGFR in cells under several distinct conditions, providing insights into the proportion of dimers, trimers, higher-order oligomers and their architecture (Needham *et al.*, 2016; Zanetti-Domingues *et al.*, 2018; Martin-Fernandez *et al.*, 2019). A detailed description and explanation of our method and approach follows in the upcoming chapter.

Looking at ligand bound and unliganded states of the EGFR reveals some interesting suggestions and findings. The back-to-back dimer conformation has been confirmed as a very stable conformation several times with additional support for a recently detected dimerization interface in domain IV, which contributes to the name giving “heart shape” form of the receptor. Beyond that, there has always been an anticipation for higher order oligomers of the EGFR but most methods struggle with resolution and computer based molecular dynamic simulations suffer from immense data points while simulating millions of atoms (Martin-Fernandez *et al.*, 2019).

Nevertheless, the presence of ligand bound multimers has been shown. In one model dimers are stacked side to side and are interfacing with parts from one domain IV with another domain IV from a partner dimer (Huang *et al.*, 2016). Also, recently Needham *et al.* conducted a study using simulation-based methods to create a long-timescale atomic-resolution model of a ligand-bound tetramer of EGFR. The model revealed that the tetramer forms back-to-back dimers that assemble into oligomers through unoccupied ligand-binding sites, resulting in a "face-to-face" interaction. The model is therefore open ended. The lateral and vertical separations between the receptors and ligands predicted by the model were experimentally validated using FLImP and FRET data, respectively. This study suggests that these oligomers could be highly important in organizing kinase-active dimers for optimal auto-phosphorylation.

Since back-to-back dimers first description and the importance of ligand binding for stability an investigation of its effect has been carried out. In this study the removal of one or both ligands from the back-to-back structure caused significant rearrangements. When both ligands were removed (as opposed to only one), the structure became stable, with a filling of the gap between domain I and domain III. These findings supported the assumption of ligand free dimers and provided insights into the link established by ligand-free inactive complexes across the membrane (Arkhipov *et al.*, 2013). Another study expanded that view, where a setup of using various high-resolution imaging techniques (FLImP and FRET) and molecular dynamics simulations to investigate ligand-free inactive dimers of EGFR on the cell surface delivered new possible dimer models. Their findings showed that the kinase domains of ligand free wild-type EGFR complexes do not interact, except for mutant kinase species. They observed an extended dimer architecture in an EGFR mutant, while the binding of a type I tyrosine kinase inhibitor (TKI) or removal of plasma membrane cholesterol induced the formation of a so-called stalk-to-stalk dimer. These results suggested ligand-free dimers on cell surfaces are possible at least for a fraction of a time (Zhang *et al.*, 2006; Zanetti-Domingues *et al.*, 2018). The picture of unbound oligomers has been expanded as well through the years, as investigations of ligand-free oligomers of EGFR using high-resolution imaging methods and molecular dynamics simulations revealed a polymer chain architecture and identified a suitable crystal structure that depicts previously unrecognized oligomer interfaces. Simulations starting from this crystal structure revealed a stable

head-to-head dimer conformation which maintained interactions between specific domains. The head-to-head dimer model exhibited similar features to back-to-back inactive dimers and was validated by experimental FRET results (Clayton *et al.*, 2005; Clayton, Tavarresi and Johns, 2007; Saffarian *et al.*, 2007; Martin-Fernandez *et al.*, 2019). These ligand free pre-formed dimers or potentially oligomers raise the question of if they are capable of signal transduction or if they autoinhibit themselves? A recent study proposes a model and has shed light on the conformational changes that occur during the activation of this pre-formed EGFR dimer. Upon binding of a ligand to the extracellular domain of EGFR, the transmembrane domains undergo rotation or twisting within the cell membrane, causing the intracellular kinase domain dimer to transition from a symmetric inactive state to an asymmetric active state, named as the "rotation model." This model explains the activation of EGFR upon ligand binding but also provides insights into how oncogenic mutations can activate the receptor in the absence of ligands, without requiring the mutations to induce receptor dimerization (Purba, Saita and Maruyama, 2017). A simplistic impression of some of these models from their respective sources is given in Figure 5.

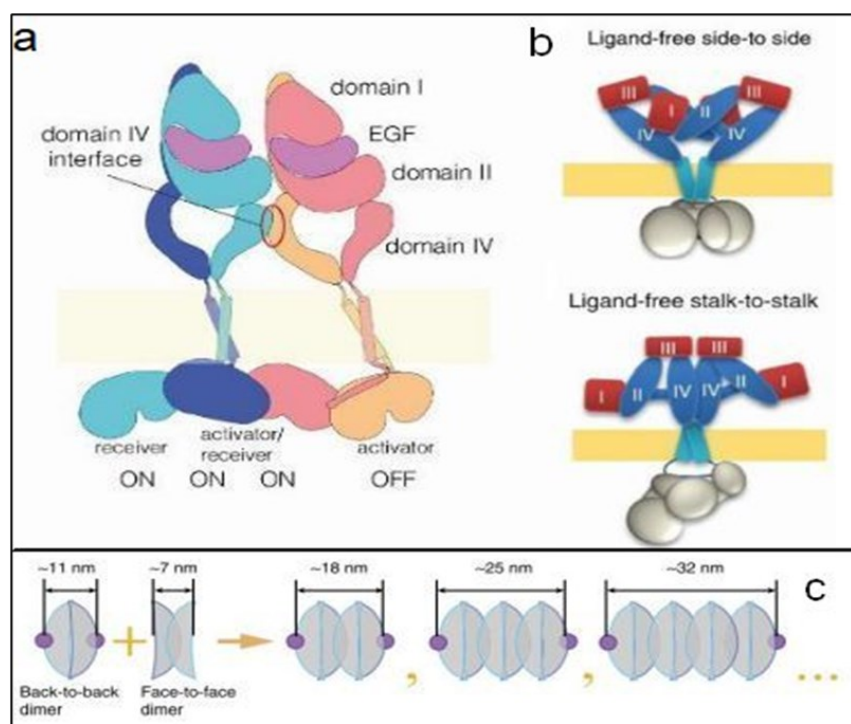


Figure 5: Different structural models of EGFR multimers and oligomers. (a) A model for a ligand bound tetramer. Note the domain IV interface and the kinase chain. (b) Alternative models for ligand free dimers derived from FLImP and FRET imaging experiments and simulations. Note the orientation of the transmembrane helix in cyan. (c) A structural model for EGFR tetramers using FLImP measurements and simulations. The model suggests an open-ended oligomerization pattern involving back-to-back and face-to-face interactions of the extracellular domains. Adapted from (Huang *et al.*, 2016; Needham *et al.*, 2016; Zanetti-Domingues *et al.*, 2018).

In summary one can say that basically two observations are repeatedly reported. The "ligand-induced dimerization model" suggests that EGFR exists as monomers until ligand binding induces dimerization, leading to kinase activation. Another variation suggests an equilibrium between monomers and active dimers with ligands present favoring the formation of active dimers. Opposite of that, some studies provide evidence that EGFR exists in a dimeric, inactive form at the cell surface before ligand binding. Different cross-linking techniques, FRET analysis, fluorescence correlation spectroscopy and single-molecule observations confirm this state prior to ligand binding in living cells. These views on preformed dimers are challenged based on N-terminal tagged FRET analysis, which can be argued as unreliable due to conformational changes upon ligand binding. However, C-terminal tagged FRET analysis supports the presence of preformed EGFR dimers in the absence of ligands. Spectrally distinct quantum dots (Qdots) conjugated to EGF or Fab (fragment of the antigen binding part of an antibody) antibodies have revealed a transition between EGFR monomers and dimers. But, due to its size only one Qdot-conjugated Fab can bind an EGFR dimer. Hence, Qdot-conjugated EGF or Fab molecules may report dimers as monomers (Purba, Saita and Maruyama, 2017 and references therein). Fortunately, certain problems within superresolution microscopy have recently been addressed and new strategies are being developed to differentiate better between true protein clusters and imaging artifacts (Baumgart *et al.*, 2019 and references therein). Nevertheless, the question of how mutations within the EGFR may alter its dynamics have to be considered as well.

A lot of data has accumulated in recent years regarding EGFR mutations within all of the EGFR's domains. These mutations can lead to constitutively active states of the receptor by increasing chances for dimerization of monomers often leading to poorer outcomes in different cancers. Mutations in the extracellular domain of EGFR are frequently observed in glioblastoma (GBM). The most well-observed are three variants. EGFRvI which lacks most of the extracellular domain and is tumorigenic. EGFRvII has a deletion in exons 14 and 15 resulting in ligand-independent activation. EGFRvIII has a deletion of exons 2-7 and exhibits constitutive phosphorylation and high tumorigenic potential (Wong *et al.*, 1992; Frederick *et al.*, 2000; Francis *et al.*, 2014). Other observed missense mutations in the extracellular domain need more examination to proof oncogenic activity. Mutations like V689M and L703F (found in NSCLC) in the intracellular region of the EGFR can lead to its constitutive activation

without ligand binding. These mutations may stabilize an asymmetric active structure of the kinase domain dimer and therefore promoting the transition from an inactive to an active state (Purba, Saita and Maruyama, 2017). The kinase domain harbors several mutations that occur in lung cancer, thyroid cancer or prostate cancer. They often promote active conformations, constant phosphorylation even in the absence of ligands. The L858R mutation belongs to this group of mutations in the EGFR and is commonly found in NSCLC and leads to ligand independent activation. This mutation, like others, is shifting the kinase domain dimer towards an active configuration also referred to as a “superacceptor” conformation. Additionally the extracellular domain structure is also altered, adopting an extended conformation (Shan *et al.*, 2012; Red Brewer *et al.*, 2013). But some of these findings only rely on crystal structure and database analysis alone. Therefore, establishing reliable methods to observe EGFR dynamics on living cell membranes should be strived for.

1.3.2 EGFR and cancer: The L858R and T790M mutations

As briefly touched in chapters before, the EGFR is a key player in cellular processes and its dysregulation is linked to various cancers. Tumors expressing EGFR have been observed in several instances like in colorectal, lung, prostate, breast, glioblastoma and other cancers with different proportions of EGFR expression. The tight control of EGFR signaling can be disrupted through multiple mechanisms, including increased ligand production, excessive EGFR protein production, activating mutations, impaired downregulation and crosstalk with other cell-surface receptors (Grandis and Sok, 2004; Zandi *et al.*, 2007). For example, overexpression (some studies report up to 62% in NSCLC) of wildtype EGFR can lead to ligand independent activation. Additionally, cancer associated mutations in the tyrosine kinase domain or extracellular domain (e.g., EGFRvIII variant or L858R mutant) can inappropriately activate the receptor. Furthermore, some cancers establish autocrine loops (ligands stimulate receptors on their own cell surface) by overexpressing TGF- β . These and other factors may lead to elevated EGFR levels and are associated with poor prognosis in several cancers (Wipt and George, 2008; Purba, Saita and Maruyama, 2017).

When we look closer at lung cancer and especially at non-small cell lung cancer we do identify EGFR as a potential driver gene. As shown in Figure 6, somatic mutations occur frequently in NSCLC and usually studies who look at genetic alterations in

patients repeatedly report a range of 10-30% share of EGFR mutations. At a metastatic stage of NSCLC the focus is directed at quality and improvement of life as well as overall survival. Besides all therapy options it is recommended to test all non-squamous tumors for driver mutations, especially in individuals without a smoking history or with a light smoking history. Targeted therapy is preferred for patients with mutations in EGFR, ALK, or ROS1, as it is more effective and less toxic. The International Association for the Study of Lung Cancer recommends testing for EGFR, ALK, and ROS1 as a minimum, and newer guidelines also suggest testing for several other known players like KRAS, MET (tyrosine-protein kinase Met) or BRAF (proto-oncogene B-Raf) (Lindeman *et al.*, 2018; Mithoowani and Febbraro, 2022).

Concerning adenocarcinoma, its genetic - molecular profile and alterations as well as its incidence and prevalence regarding ethnicities, some interesting findings should be noted. Firstly, referring to the frequency of adenocarcinoma between men and women. The incidence has been increasing among both women and men but among women, adenocarcinoma has been the most frequently diagnosed subtype since the 1970s while among men the incidence rate surpassed squamous cell carcinoma in the mid-1990s. This shift in histological diagnoses is attributed to changes in cigarette design and tobacco composition. The introduction of filtered cigarettes allowed deeper inhalation compared to former mass-produced unfiltered cigarettes. Furthermore, the use of "light" cigarettes and changes in tobacco blends, including higher levels of nitrate and N-Nitrosamines increased the risk of lung cancer by promoting deeper and more frequent inhalation of combusted tobacco smoke (Lewis *et al.*, 2014; Song *et al.*, 2017). Secondly, since advances in genomic profiling have led to a shift in how lung cancers are classified beyond the traditional NSCLC and SCLC categories, tumor biomarkers and genetic alterations, including gene expression, mutations, amplifications and rearrangements have been identified. As they can play a crucial role in tumor development they can be targeted with specific therapies like targeted agents or immune checkpoint blockades. The most common mutated potential driver genes are the EGFR, FGFR1 and KRAS (Levy, Lovly and Pao, 2012). It is important to note that these mutations are mutually exclusive with very few exceptions like the PIK3CA mutation (Testa, Castelli and Pelosi, 2018). As shown in Figure 6, the EGFR usually appears at the top of lists that comprise a molecular profiling of mutated genes in lung adenocarcinoma. This has been

supported through several molecular screening studies with hundreds of samples from patients and dataset comparisons from The Cancer Genome Atlas Program (TCGA) and the Memorial Sloan Kettering Integrated Mutation Profiling of Actionable Cancer Targets (MSK-IMPACT) group (Jordan *et al.*, 2017).

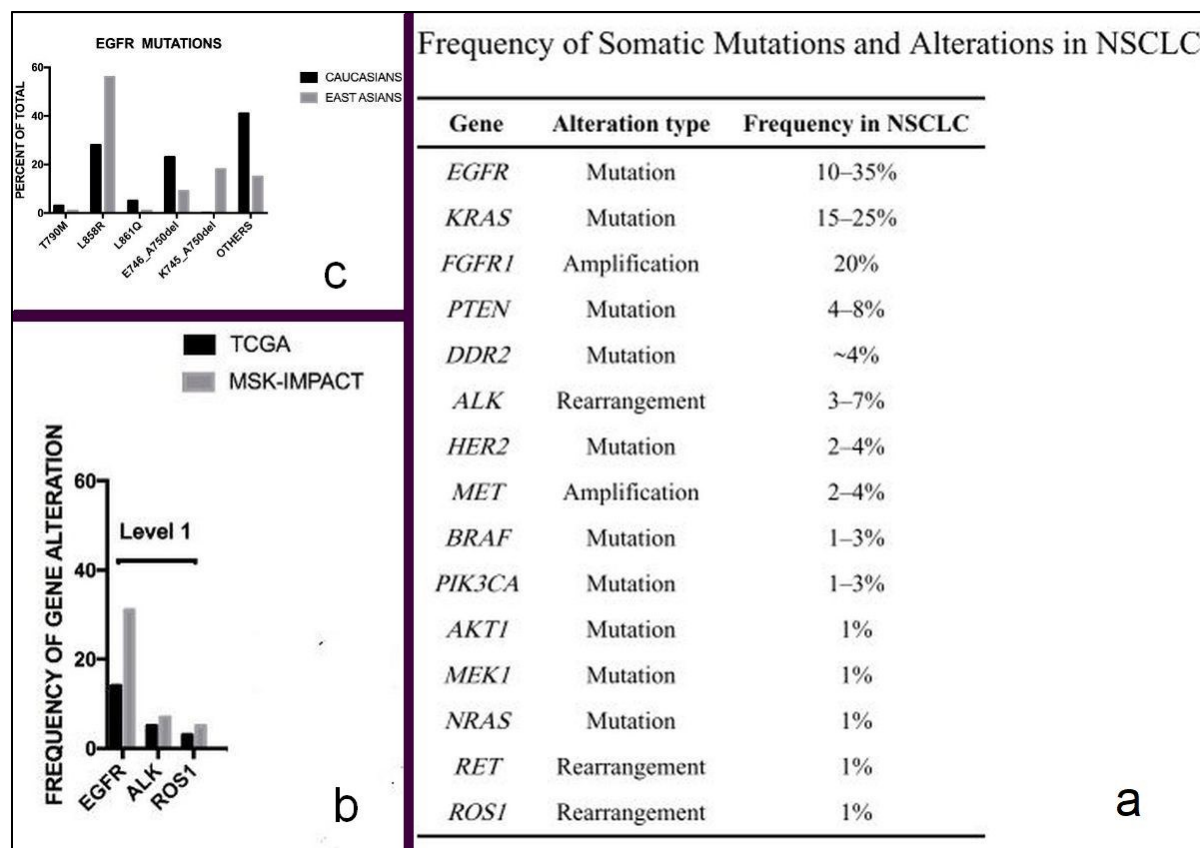


Figure 6: (a) List of the most common driver gene mutations in NSCLC. Adapted from Schabath and Cote, 2019. (b) Observed frequencies of main genetic alterations in lung adenocarcinoma. TCGA data set was based on non-metastatic patients and MSK-IMPACT was data based on recurrent/metastatic lung adenocarcinomas. (c) Various gene mutations of the EGFR gene from two different Ethnicities. Adapted from (Testa, Castelli and Pelosi, 2018).

What makes the EGFR additionally interesting is its difference in frequency and incidence between ethnicities as well as smokers and non-smokers. It has been reported that East Asian patients show higher rates of EGFR mutations when compared to Caucasian populations. Especially in non-smokers of this population a mutated EGFR is involved 80% of the times when lung adenocarcinoma arise (Sun *et al.*, 2010). This is important in terms of cancer management or clinical trial design in different countries.

Likewise, an area of interest is the tyrosine kinase domain of the EGFR as it inhabits key mutation sites that may constitutively activate EGFR and at the same time are interesting targets for therapy by using small molecules with inhibiting function. These

EGFR kinase domain mutations are primarily found in exons 18-21 and arise around the ATP-binding pocket of the enzyme. Mutations in these regions such as L858R (a single amino acid substitution at Leucine-858 to arginine, also EGFR-L834R in the mature protein numbering) and others have been shown to be oncogenic in cell culture and transgenic mouse studies (Prudkin and Wistuba, 2006). The T790M (single substitution at amino acid 790 from threonine to methionine) is a so called second site mutation and arises in exon 20. It can be involved in rendering partially activated mutant EGFRs fully ligand independent and constitutively active. In vitro studies using purified recombinant wild-type and mutant EGFR cytoplasmic domains have shown that these mutants exhibit increased catalytic activity and ATP affinity. Why is that important?

Studies have demonstrated that these mutants can be more sensitive to inhibition by TKIs (Tyrosine kinase inhibitors) or lose sensitivity (competition with ATP for binding to the catalytic site) through second site mutations like T790M (Kobayashi *et al.*, 2005; Sharma *et al.*, 2007).

Tyrosine kinase inhibitors are part of cancer treatment, first line or second line since the introduction of gefitinib and erlotinib in 2004 and EGFR-TKI monotherapy is the standard treatment for advanced EGFR-mutated non-small cell lung cancer ever since. These small molecules are now in their third generation due to cancer adapting to treatment by simply trying to evade death which in return lead to resistance mutations usually within one year of treatment with first generation TKIs. Therefore, several other molecules had to be developed to overcome these resistances. In short, the generations consist of first-generation EGFR TKIs like erlotinib and gefitinib. Second-generation EGFR TKIs, including afatinib, dacomitinib and neratinib who showed potential in preclinical models against T790M but their clinical success was diminished due to dose-limiting toxicity and their side effects. Recently, third-generation inhibitors including osimertinib, rociletinib and olmutinib have been developed, which could more effectively target T790M while sparing wild-type EGFR. It should be noted that TKIs also differ based on their binding characteristics as they can bind reversible or irreversible (Zhang, 2016; Johnson *et al.*, 2022). Nevertheless, despite some drawbacks clinical trials have demonstrated the superior efficacy of EGFR-TKIs compared to standard chemotherapy, with longer progression-free survival (PFS). Osimertinib has shown significant benefits in patients previously treated with other EGFR-TKIs. It demonstrated higher response

rates and longer PFS compared to platinum-based chemotherapy. It has also shown superiority in the first-line setting, outperforming its first-generation counterparts in overall survival (Johnson *et al.*, 2022 and references therein).

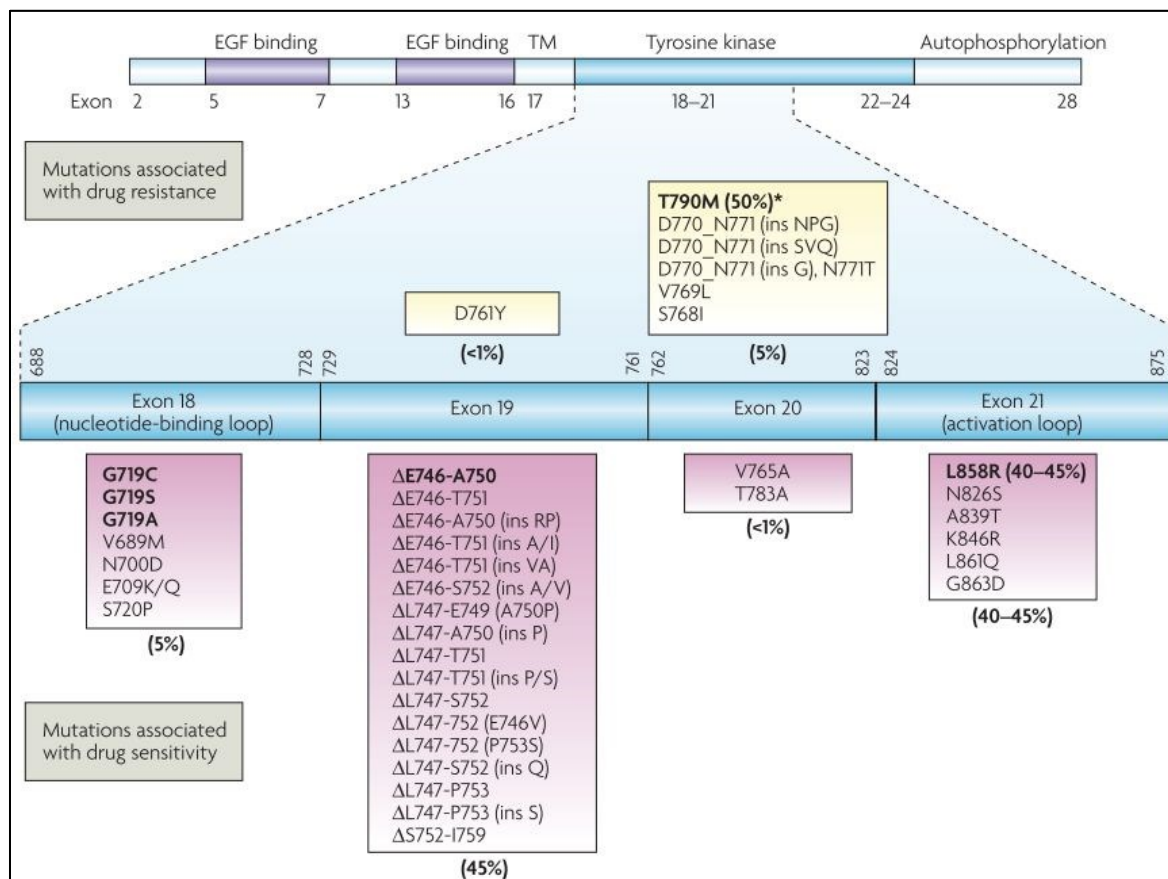


Figure 7: A cartoon summary with distribution of exons over all domains of the EGFR. The tyrosine kinase region of EGFR, specifically exons 18-21 (expanded in cyan) contains mutations associated with sensitivity (magenta boxes) or resistance (cyan boxes) to gefitinib or erlotinib. The most common mutations are in-frame deletions in exon 19 and the L858R substitution in exon 21, comprising a significant percentage of EGFR mutations in NSCLC. Notably, the T790M mutation in exon 20 is clinically significant as it is associated with acquired resistance to gefitinib and erlotinib. Adopted from (Sharma *et al.*, 2007).

Because of this the EGFR is and remains an important target of cancer research and treatment. Furthermore, trying to illuminate more of its interactions, dimerization dynamics and signaling effects is crucial and should benefit future efforts concerning lung cancer. Additionally, important mutations with high prevalence and interactions that may interfere with treatment should be observed thoroughly. While that has happened on a molecular level and lead to important findings as well as better treatment options, there is barely data if these key mutations themselves influence monomeric or oligomeric states of the EGFR on a native cell membrane.

1.4 The TOCCSL Method

The TOCCSL (Thinning out clusters while conserving stoichiometry of labeling) method is an advanced development of the established FRAP (fluorescence recovery after photobleaching) method. FRAPs introduction can be tracked back to the 1970s when biophysicists studied the properties of fluorescent dyes in a diffusive setting usually using custom built devices. Through high technical advancements in microscopy (laser based confocal microscopes) and the development of viable fluorescent proteins (GFP and others), FRAP transitioned from being capable observing rhodamine dyes in solutions in 1976 into the most important technique for quantifying protein or other cellular dynamics in live cells, especially in the membrane within two decades (Axelrod *et al.*, 1976; Lippincott-Schwartz, Snapp and Phair, 2018). In principle, a high intensity laser is applied to a region of interest (ROI) on the cell. The intense light pulse ideally photobleaches all fluorescent molecules in this area. Due to Brownian motion molecules will diffuse back into the ROI within a certain time. The rate of mobility observed and measured and comparison of fluorescence levels before and after photobleaching give insights into molecule kinetics. It is a very effective non-invasive method.

In our study, we utilize the idea of single molecule microscopy to quantify aggregation at the level of individual dimers in our cell lines. However, when studying the cellular plasma membrane the high density of endogenous proteins makes it challenging to observe individual molecules. Considering a cell with a diameter of 5 μm and a protein expression level of an average 10^4 copies, there would be approximately 130 protein molecules in one square micrometer of the plasma membrane on average. This density exceeds resolution by about 1000-fold (Bramshuber and Schütz, 2012).

TOCCSL can overcome this limitation to accurately quantify molecular aggregation in membranes of live cells.

In a simplified model of a cellular plasma membrane which consists of a fluid lipid bilayer containing fluorescent biomolecules of interest, individual molecules and the spacing between them are often much smaller than the resolution limit of the imaging system. This may result in a homogeneous signal distribution when using conventional fluorescence microscopy. With TOCCSL, a small region of the membrane is selectively photobleached but unlike FRAP, TOCCSL focuses on analyzing individual recovering objects that can be resolved at the early stages of the

recovery process. By adding ultrasensitive microscopy, individual molecules can be visualized as separate diffraction limited spots. To ensure the preservation of the active fluorophores, it is crucial to minimize incomplete bleaching. (Moertelmaier *et al.*, 2005) achieved this by optimizing the photobleaching contrast through the use of a circular field stop focused onto the membrane, combined with high excitation intensity laser light. In summary, the order of events in TOCCSL (given in Figure 8) starts by capturing a control image using a short illumination time. Then, a strong laser pulse is applied for a specific bleaching time to induce photobleaching. The duration of photobleaching has to ensure that no active fluorophores are left within the illuminated region. The surface density of observed molecules in the subsequent postbleach image can be adjusted by selecting an appropriate recovery time. TOCCSL has been used in various occasions to study behavior of molecules in living cell membranes and has proven to be very reliable as well as appropriate for our approach. One example is the direct imaging of small, mobile, long-lived platforms in the plasma membrane in the past. Additionally, in that study the authors found cholesterol-dependent homo-association in the plasma membrane of living CHO and Jurkat T cells in the resting state. They further applied the technology to address structural changes in the plasma membrane during fever type heat shock (Bramshuber *et al.*, 2010). Another example is the description of the coexistence of stable serotonin transporter (SERT) monomers and oligomers in the live cell plasma membrane, where the team proved that the oligomeric state of SERT complexes is stably determined before being integrated into the plasma membrane and that the results reveal the first evidence for kinetic trapping of preformed neurotransmitter transporter oligomers (Anderluh *et al.*, 2014).

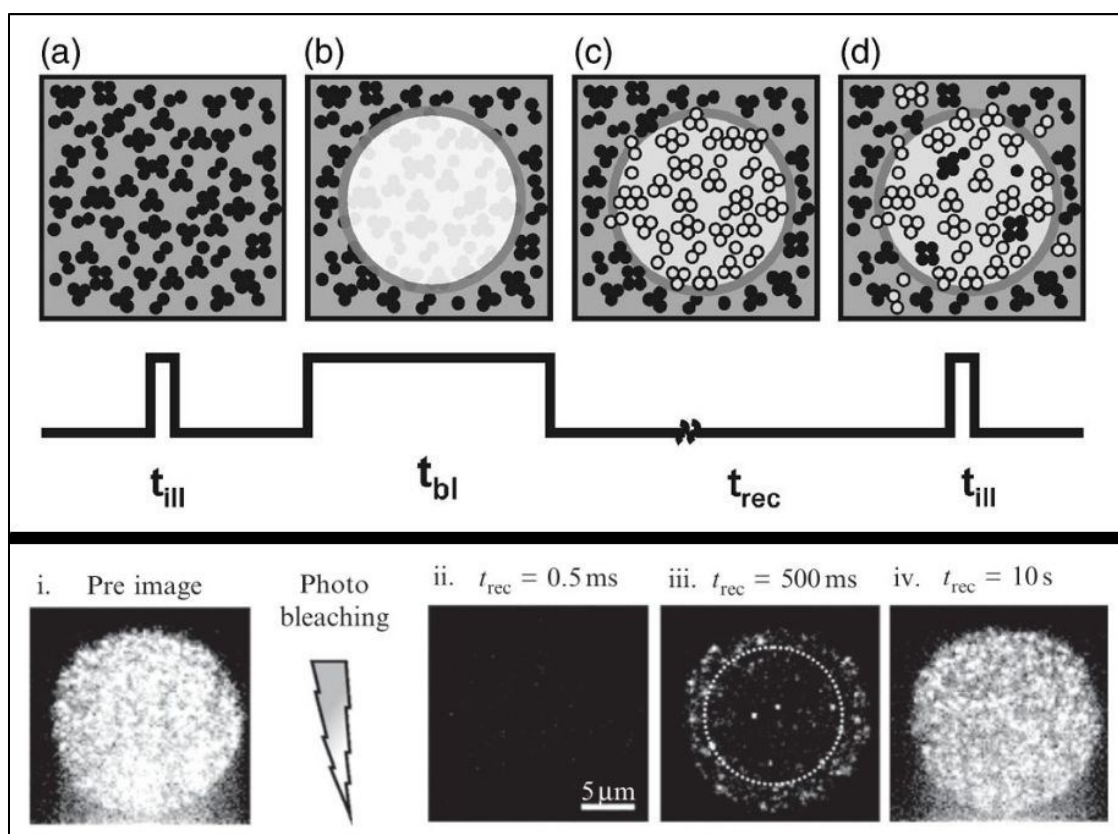


Figure 8: Schematic cartoon and example of the TOCCSL procedure. Squares in the top row represent sketches, the black line in the middle row an imaginary timing protocol. (a) A membrane is densely populated by molecules which cannot be resolved individually. A control picture of that situation is taken with illumination time t_{ill} . (b) Photobleaching of the fluorescent labeled molecules through a field stop confines the excitation to a specific region. This region is adjusted in diameter down to several micrometers and is illuminated by an intensive laser pulse for a specified duration t_{bl} . As a result, all exposed molecules within the illuminated region undergo complete photobleaching, while the molecules outside this region remain unaffected. (c) Situation after photobleaching, region should be exempt from fluorescent activity (white circles). (d) Recovery time starts (t_{rec}), fluorescent molecules diffuse into the field and can now be analyzed as individual spots. Bottom part of the figure depicts an example with real footage and time courses (i-iv). Adapted from (Moertelmaier et al., 2005; Brameshuber and Schütz, 2012).

1.4.1 Two – color TOCCSL

Initially, TOCCSL was introduced to analyze clusters by single molecule brightness analysis. The concept however was extended to a two color – colocalization approach. It works by using spectrally different fluorophores to label a molecule of interest or two distinct molecules with an identical fluorophore in a high density membrane. The principle of the method relies on the standard TOCCSL procedure. After initial imaging, a defined region is photobleached and during recovery, single molecules become resolvable in both color channels allowing colocalization determination. But, thanks to its high precision and the feasible observation of joint molecule movement as an additional step as well as additional data like brightness

or label distance, two-color TOCCSL can enhance the sensitivity of cluster characterization (Ruprecht, Brameshuber and Schütz, 2010). It therefore delivers an option to discern between false positives and true positives which is possible through a given distance threshold between two labels.

The method was successfully applied with equimolar labeled Fab fragments to observe ErbB2/ErbB3 homo - and heterodimerization with or in absence of stimulation (Váradi *et al.*, 2019).

1.4.2 Total internal reflection (TIRF) and requirements

Now considering that fluorescence microscopy is an umbrella that hovers over several techniques, single molecule microscopy is just one method beneath it. A successful execution of single molecule fluorescence microscopy depends on a capable laser, a microscope paired with a ultra-sensitive scientific camera that is capable of fast imaging with high contrast to discriminate against the background. Which leads to the question of the appropriate technique. FRAP and FRET have been mentioned before but recently the visualization of molecules beyond the diffraction limit was successful. This techniques are referred to Photoactivated Localization Microscopy (PALM) and Stochastic Optical Resolution Microscopy (STORM) (Betzig *et al.*, 2006; Hess, Girirajan and Mason, 2006; Ghiran, 2011). These methods, though accessible at the time, were only moderately appropriate, as artefact formation (fixation of cells) and quantitative debatable results are considerable downsides. The complex state of superresolution techniques and their results on cluster detection and potential artefact misinterpretation has been discussed and explained (Rossboth *et al.*, 2018; Baumgart *et al.*, 2019). We therefore favored the use of Total internal reflection fluorescence (TIRF) microscopy. TIRF is an optical sectioning technique that utilizes the reflection of light to image a small area at the interface between a sample and the coverslip. TIRF takes advantage of the evanescent wave formed when the excitation laser is angled to completely reflect off at the buffer-glass interface. This evanescent wave creates a shallow illumination into the sample, exciting fluorescent molecules within a thin (~100 nm) optical section at the surface and in addition selectively through the light wave eliminates out-of-focus signal and improves the signal-to-noise ratio. It is ideal for studying molecules near the sample surface, enhancing the detection of single-molecule fluorescence (Ghiran, 2011). When performing single molecule imaging, the choice of fluorophore

is crucial. Ideal fluorophores should be bright, photostable, small enough to not disrupt biological activity and emit light in the visible spectrum. Additionally, the labeling agents (carriers of fluorophores, usually proteins) or procedure should be taken into account too. Usually, fluorophores themselves are not a huge obstacle when protein labeling is considered. Without a doubt there are differences in photostability, brightness and blinking quality between organic dyes, quantum dots or fluorescent proteins but whether you choose direct or indirect labeling the size of the dyes vary but do usually not impede execution since they reside in the nm range. Depending on the experimental setup and the protein of interest (POI), as well as where it is going to be observed the location of a dye seems more important. Especially when the dye is linked to another protein which targets the POI or a desired specific area the linker protein should ideally not interfere with the activity of the POI. We therefore looked for an innovative, adaptive protein with this desired property that also adds some novelty to single molecule fluorescence microscopy with respect to the three important criteria: size, binding to the target without interfering and the capability of being labeled with an organic dye.

1.5 DARPIs: Designed Ankyrin Repeat Proteins

When live cell surface molecule dynamics are observed one stumbles upon several different proteins, but most likely it's a biotin based, antibody based or some kind of tag based (epitope, HaloTag...) approach. Some of these proteins are even commercially available but do have possible side effects and/or come with more time investment and preparation. Aggregation or clustering is one problem that can occur with biotinylated targets for example. Tags should be designed ideally, to not interfere or react with the functionality of the POI. Additionally, some tags lead to bigger background noise depending on amino acid contents of the POI (Note, Proteins and Imaging, 2018). Looking at maybe the most common used molecule the antibody, some of these drawbacks may arise. Antibodies are immune system proteins that recognize and identify intruders like bacteria and viruses by binding to specific antigen structures. Their specificity comes from recognizing unique epitopes. Labeling antibodies is often done by using a primary antibody to target a protein, followed by a fluorescently labeled secondary antibody. However, this method adds a fluorescent tag about 20 nm away from the protein due to the size of the antibody

structure (150kDa). Of course, this situation can be improved as researchers try to bring the fluorophore closer to the protein of interest by using a labeled primary antibody (direct immunostaining). Alternatively, using a smaller labeled Fab-fragment (50kDa) or Fv-domain (25kDa) offers improved specificity, prevents crosslinking, and facilitates binding to inaccessible protein domains. A combination of techniques is sure also possible. (Chung *et al.*, 2010) for example used a Fab anti EGFR connected to biotin and quantum dots.

The motivation to overcome shortcomings of such proteins mentioned above and to introduce a new scaffold to build upon, lead to the invention of DARPins. These repeat proteins offer potential as versatile binding proteins, similar to antibodies but with distinct advantages. These proteins consist of stacked repeat modules forming compact folded domains, utilizing variable surface residues for extended interaction interfaces. The conserved interfaces between repeats allow manipulation without disrupting the overall structure. Jawless vertebrates have even evolved an adaptive immune system based on leucine-rich repeat (LRR) proteins, expanding the utility of repeat proteins in binding selection. Ankyrin repeat proteins in particular have shown promise in protein engineering for biomedical applications (Pancer *et al.*, 2004; Plückthun, 2015).

Their core properties and design can be summed up as: proteins that are constructed from rather tightly arranged repeats of around 33 amino acids with each repeat comprising a β -turn and two antiparallel α -helices. A single protein can contain up to 29 consecutive repeats, although typical domains consist of four to six. This arrangement results in a right handed solenoid structure with a hydrophobic core and an exposed, solvent accessible surface resulting in a groove-like binding surface (Bork, 1993; Li, Mahajan and Tsai, 2006). It is also possible to establish a library as (Plückthun, 2015) has shown in the past. Through structural analysis followed by randomization excluding certain types, their designed ankyrin repeat protein library includes fixed (for stability and structure) and variable positions who allow potential interactions. A DARPin library can provide considerable diversity, reported between

5.2×10^{15} to 3.8×10^{23} molecules in different binding scenarios.

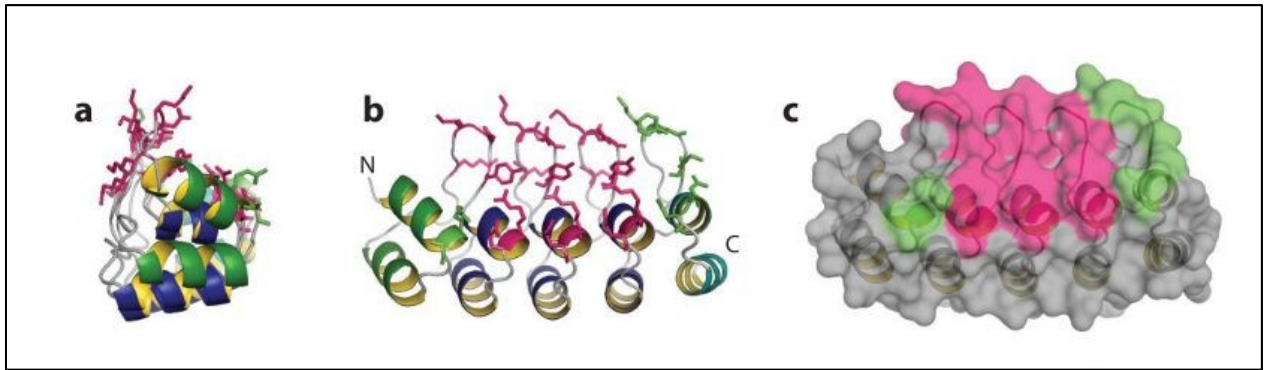


Figure 9: Depiction of a random library DARPIn. Important surface and structural aspects are highlighted. N- and C-cap in green and cyan respectively. Note the exemplary three repeats in between and their interactive residues in magenta. (b) and (c) are 90° rotations of (a) in the y axis, while (c) shows the molecular surface. Adapted from (Plückthun, 2015).

On top of these characteristics, it has been observed that DARPins are quite resistant to heat or denaturants. Furthermore, they can be labeled site specifically and are produced with established methods in high quantity up to grams per liter which is possible due to an important feature of their nature. They do not tend to aggregate even at high concentration levels, neither in storage nor within cytoplasm of their producing bacteria (Kohl *et al.*, 2003; Binz *et al.*, 2004). It is important to emphasize that DARPins are not solely a hypothetically construct. They have been tested on various occasions in different fields and applications. So far, they have shown potential as biosensor for extracellular signal-regulated kinase (ERK) in its non - phosphorylated or phosphorylated active (p-ERK) form. As tool in diagnostics for HER2 and related cancer to HER2 which included tumor targeting and killing in form of a bispecific DARPIn that competed with conventional antibodies. In clinical trials DARPins have been selected with high affinity against human vascular endothelial growth factor (VEGF-A) for treating diabetic macular edema and age-related macular degeneration. These DARPins also showed cross reactivity with VEGF-A in various mammalian species, aiding preclinical and clinical development (Theurillat *et al.*, 2010; Parizek *et al.*, 2012; Campochiaro *et al.*, 2013). It was research like this that lead to a viable candidate DARPIn that is capable of targeting the EGFR without interfering with its activity and is suitable for single molecule fluorescence microscopy.

1.5.1 DARPin E69

Several monoclonal antibodies (mAbs) targeting EGFR, like cetuximab and panitumumab are available and have been explored for inhibiting tumor growth. They hinder EGFR activation by binding to a region that overlaps its ligand-binding site, preventing the receptor from adopting the required open conformation for dimerization and autophosphorylation. Cetuximab's clinical benefits have raised concerns about its cost effectiveness. To improve therapy and offer new innovative proteins and modes of action, a group effort roughly a decade ago designed bispecific DARPins exactly for that purpose (Friedländer *et al.*, 2008; Patel *et al.*, 2009; Boersma *et al.*, 2011). Based on fundamental work before, meaning the creation of four DARPins which were selected against EGFR's ectodomain using phage display (Steiner, Forrer and Plückthun, 2008) and the demonstration of subnanomolar affinities and monomeric behavior in chromatography, these DARPins were approved for further testing. Consequently, their biological impact was tested on A431 cells, a standard anti-EGFR test system. While three DARPins exhibited some activity similar to cetuximab, one DARPin had no effect (Boersma *et al.*, 2011). In that study a combination, through fusion proteins, of these DARPins and their effectiveness against EGFR was tested and compared to the conventional mAb cetuximab. After reassuring binding of the four DARPins (designated E01, E67, E68 and E69) to EGFR, they observed non-interference of E69 with the EGFR binding site and therefore no inhibition of proliferation compared to its counterparts leading to the assumption that its corresponding epitope has to lie outside on the domain. Epitope mapping through yeast display delivered answers for the DARPins (in)activity.

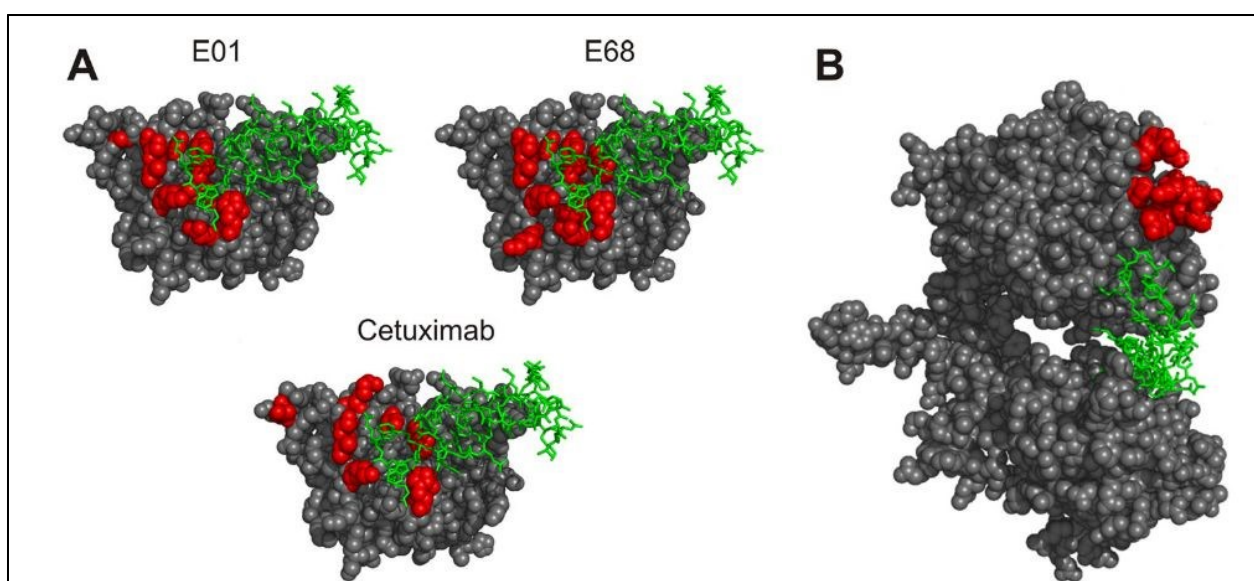


Figure 10: DARPin E69 binding site compared to E01, E68 and Cetuximab. (A) epitopes of E01, E68 and cetuximab on Domain III of the EGFR. Surface areas involved in binding are shown in red, green sticks represent EGF molecule. Note the overlap of areas, hence the inhibitory effect of all three molecules as well as their competition with EGF binding. (B) binding area of E69, in red, outside the binding pocket compared to EGF in green sticks, located on Domain II of the EGFR. Adapted from (Boersma *et al.*, 2011)

As it turned out, the three inhibitory active DARPins displayed a binding behavior similar to cetuximab as shown in Figure 10. Their binding area overlaps with the EGF binding pocket, it was therefore clarified why they would show similar or better performance compared to cetuximab as individual or bispecific proteins. E69 individually would show no effect on cell viability, though it was viable as bispecific partner with either of the other three DARPins. Due to its open C-terminal end it was a perfect option for a maleimide dye thioester reaction. The bond is considered as stable and varieties of excitable, photostable fluorophores attached to the maleimide molecule are widely available. Additionally, the position and the distance to the EGFR ectodomains and the size of E69 would give a good setup for further FRAP and FRET analysis. At the timepoint of writing this thesis and also at the time of experiments, DARPins were not used for the specific task we set out to. In terms of microscopy related approaches, just a few studies can be mentioned. In these two occasions DARPins were used to observe interactions with membrane proteins, either connected to gold nanoparticles or tagged with mCherry fluorescent protein. In both studies EGFR was the target surface protein and confocal microscopy was one method applied (Mironova *et al.*, 2014; Deyev *et al.*, 2017).

2. Materials&Methods

2.1 Cell Culture and Conditions

Mouse Embryonic Fibroblasts (MEF) were received from the in-house cell bank, cells were cultured in DMEM (Dulbeccos Modified Eagle Medium) with 10% Fetal Calf Serum, 1% Penicillin/Streptomycin and 1% L-Glutamin. Cells were maintained at 37°C and 5% CO₂ in a humidified incubator. All MEF cell lines were checked for mycoplasma.

A431 cells were acquired from the house own cell bank. Cells were cultured in DMEM (Dulbeccos Modified Eagle Medium) with 10% Fetal Calf Serum at 37°C and 5% CO₂ in partial air pressure.

2.2 Western Blot

SDS gels (10% and 7% for total proteins and phosphorylated proteins respectively) were loaded with 15µg of protein. Separated proteins were then transferred to a nitrocellulose membrane at 250mA for 2 hours at 4°C. Membranes were washed and subsequently blocked for 1 hour at room temperature (5% BSA in TBS-T). Incubation with primary antibodies was carried out at 4°C over night followed by washing and incubating with secondary HRP conjugated antibodies (1hr at RT). Enhanced chemiluminescent (ECL) agent was applied to develop the membrane. Pictures were analyzed and obtained with a Chemidoc by BioRad (Bio-Rad Laboratories, Inc. USA).

2.2.1 PEGFR

For phosphorylated EGFR blots the cell lines were split and seeded with their corresponding controls into 6 well dishes each. Cells were then starved in starvation medium (DMEM without FCS) for 16 hours. After several washing steps the cells were incubated with EGF (50ng/µl) at 37 °C. As soon as the time course was complete cells were washed again and put on 4°C. For further analysis cells were

scratched of the plate with proteinase K inhibitors and phosphatase inhibitors and subsequently frozen.

2.3 Plasmids and cloning

EGFR WT plasmid was a gift from Matthew Meyerson (Addgene plasmid # 11011). EGFR L858R plasmid was also a gift from Matthew Meyerson (Addgene plasmid # 11012). The EGFR L858R+T790M mutant was created with the **Q5® Site-Directed Mutagenesis Kit** from New England Biolabs (New England Biolabs, Inc. Ipswich, MA, USA) following its protocol. Mutagenic primers were created with the company's NEBBaseChanger tool.

E.Coli carrying desired plasmids were plated onto selective agarplates (Ampicillin 100µg/ml). Positive colonies were picked with a pipette tip and afterwards used for inoculation of 3-5 ml LB Medium (Ampicillin 100µg/ml or Carbenizilin 50µg/ml). Vials were kept at 37°C or 30°C respectively and shaking over night before further processing. Plasmids were extracted with a Qiagen plasmid preparation kit following the company's protocol. DNA yield was measured with a Nanodrop.

Test digests were performed on 2% agarose gel and afterwards analyzed on a Chemidoc (Bio-Rad Laboratories, Inc. USA).

2.4 Transfection of Mouse Embryonic Fibroblasts

GP-E 86 cells containing packaging and envelope vector for retroviral production were a gift from Agnes Csiszar from the IKF (Institut für Krebsforschung).

In short, GP-E 86 cells were plated the day before ($4,5 \times 10^6$) and incubated overnight at 37°C in DMEM + 10% FBS. Cells were then split to attain 50% confluence and were afterwards allowed to attach for at least 3 hours. DNA transfection mix consisted of 10µg DNA of interest, 61µl CaCl_2 and dH₂O complemented up to 500µl. The DNA mix was then added dropwise to 2x HBS buffer (pH=7,06) while vortexing the polypropylene tube. HBS/DNA solution was immediately added onto target cells (Mouse Embryonic Fibroblasts EGFR Knockout, 50% confluence). GPE medium was changed the next day and reduced to 5ml. On the 3rd day the first infection, the

supernatant of transfected cells was filtered through a 0,45µm PVDF filter to remove dead cells. Viral supernatant was then gently added to target cells containing 0,5-0,8µg/ml polybrene. After 4 hours, medium on target cells was changed and the infection cycle repeated 20 hours later. 24-48 hours post infection, cells were biochemically assayed by selecting with the appropriate antibiotic (Puromycin, c=1µg/ml). Drug selection was prolonged for 5-7 days with media changes every 48hrs. Elimination of negative cells was assumed finished, as soon as MEF canaries were dead. Positive cells were split and cultured as usual.

HBS 2x: 50mM HEPES, KCl 10mM, Dextrose 12mM, NaCl 280mM, Na₂HPO₄ 1,5mM,

2.5 Immunofluorescence

2.5.1 Immunofluorescence with conjugated EGF

Target cell lines were kept in their respective medium (see cell culture and conditions) and seeded into 8 well chamber slides (ibidi GmbH Germany, glass bottom removable). 4 hours prior to the assay the medium was changed to pure DMEM (starvation). Ahead of stimulation with EGF cells were washed with PBS or PBS with 0,025 M CaCl₂ and 0,025 M MgCl₂. After incubation with EGF- AF 488 or 647 (Thermofisher Scientific, final concentration 1µg/ml) cells were washed again and fixed with 4% PFA in PBS at RT for 15 minutes or when needed on ice for 15 minutes followed again by 15 minutes at RT. Blocking was performed with 1% BSA, 5% horse serum and 0,2% Triton in PBS for 1 hour at RT. Incubation time for Phalloidin conjugates (Santa Cruz Biotechnology, Inc. USA, 488 and 594 diluted 1:2000) and Hoechst dye (1µg/ml, diluted 1:2000) were set to 1 hour and 10-15 minutes at RT respectively. Afterwards samples were washed and immediately mounted with a fluorescence preserving mounting medium (Fluoroshield Sigma-Aldrich, USA) for final examination with a confocal microscope (Zeiss AG, Germany).

2.5.2 Immunofluorescence of EGFR

Cell lines were grown in 8 well removable chambers (ibidi GmbH, Germany) until they reached 60-70% confluency. After removal of growth medium followed by several washing steps, samples were fixed with 4% PFA in PBS for 15 minutes at RT. Subsequent blocking was carried out for 1 hour in a blocking solution of 1% BSA, 5% horse serum and 0,2% Triton in PBS at room temperature. Samples were then washed again at least three times and then incubated with an anti EGFR antibody (Cell signaling #4267, diluted 1:50) at 4°C over night. The following day, samples were washed and incubated with a secondary species-specific antibody (AF 594, Abcam UK, diluted 1:400) for one hour. Cells were then washed again and shortly after stained with Hoechst dye (1µg/ml, 1:2000) for 10 -15 minutes. Finally, samples were washed thoroughly and mounted with Fluoroshield (Sigma-Aldrich, USA) before examination and imaging.

2.6 TOCCSL Experiments

Method is described in detail in the introduction.

Cells were split and seeded to reach 50% confluency at least 4 hours before the experiment. The required TIRF ready two well slides were obtained by ibidi (ibidi GmbH, Germany) and treated with fibronectin (10µg/ml) beforehand. DARPins (produced and gifted from ETH Zürich; Plückthun Group) were diluted 1:3000 and 1:4500 (AF 647 and AF488 respectively) in HBSS and 5% BSA. The A431 cell line and the MEF cell lines were both incubated with the DARPIn solution for 15-20 minutes and subsequently washed with PBS (with MgCl₂) at least 5 times. Following the preparations, 16-20 cells were randomly chosen and observed each for TOCCSL and FRET.

Experimental settings for TOCCSL were $t_{\text{ill}}=20\text{ms}$, $t_{\text{bl}}=750\text{ms}$ and a variable $t_{\text{rec}}=2000\text{-}5000\text{ms}$ followed by $t_{\text{del}}=20\text{ms}$ in between subsequent images. Samples were illuminated with a typical intensity of 400-650mW. The microscopic setup in short: Zeiss Axiovert 200 equipped with a APOCHROMAT objective (100x NA= 1.46 alpha Plan, Zeiss-Oberkochen, Germany). Samples were excited with 488 nm light from an Ar⁺ Laser (Model 2017-05AR, Spectra Physics, CA, USA) and 647 nm light from a Kr⁺ -Laser (Stabilite 2017-KR, Spectra Physics) and put in an TIR configuration on the epiport. A slit aperture (width= $\sim 8\mu\text{m}$) served as a field stop for field of view confinement. After appropriate filtering, the emitted signals were split into

two channels (according to color) and then imaged on a liquid nitrogen cooled CCD camera (Micro Max 1300-PB, Roper Scientific, Trenton, NJ).

Single molecule data analysis was carried out with house intern algorithms within MATLAB (MathWorks, Natick, MA), see in Brameshuber et al., 2005.

2.6.1 Correction of Chromatic aberration

To compensate for chromatic aberrations, we conducted a calibration step before each experiment using identical conditions. To achieve this, we immobilized multi-color beads (TetraSpeck, Invitrogen) onto a glass slide and identified the positions of these beads in both color channels by applying a Gaussian function fitting method (TU).

2.6.2 Maleimide Dye Staining Protocol

We used Alexa Fluor® Succinimidyl Esters (Thermo Fisher Scientific Inc. USA, NHS-Alexa Fluor 647 and NHS-Alexa Fluor 488 (Thermofischer, 2012)) and conjugated them to DARPIN E69. We followed the guidelines of the manufacturer. In short: DARPIs were prepared in reaction buffer: 0.1 M sodium phosphate with a pH 7.2 to 7.5 and 150 mM NaCl. Proteins were then transferred into the reaction vial containing the dye, immediately mixed and incubated for one hour at room temperature. Un-conjugated dyes were removed over a short gel filtration column and finally stored at 2-6 °C in the dark.

2.7 Flow Cytometry

Cell lines of interest were harvested aiming for a final concentration of $2-5 \times 10^6$ cells/ml. Cells were handled on ice in PBS with 10% FCS. Before further treatment, cells were washed then centrifuged and resuspended in blocking solution (3%BSA in PBS) adding the desired conjugated antibodies (concentration of 10µg/ml, DARPin E69AF647 and BD PE #555997-BD Biosciences, USA). Staining was performed in the dark (1,5ml Eppendorf tubes), on ice for 30 minutes. Cells were then washed by 3x centrifugation (400g) and resuspension in 500µl PBS with 10% FCS, followed by

immediate analyzation on the flow cytometer (FLOW CYTOMETRY Calibur BD Biosciences, USA). Collected Data, and graphical data presentation, was processed in FlowJo (FlowJo, LLC, Ashland-Oregon, USA).

2.8 QPCR and RNA Isolation

Cells were homogenized and treated under RNase free conditions.

1mL Trizol was added to 1×10^6 cell suspension followed by adding 240 μ L H₂O and 200 μ L chloroform. After incubating for ten minutes at room temperature the suspension was vortexed and spun for 15 minutes at 12.000g at 4°C. The aqueous phase was transferred to a fresh tube and 15 μ L glycoblue was added. Following 600 μ L of cold isopropanol, cells were at least incubated for ten minutes on ice and spun again for 10 minutes at 12.000g at 4°C. The resulting pellet was washed and resuspended with Ethanol (75%) and subsequently spun down at 7500g and 4°C. For further processing, samples were dissolved in H₂O and shortly incubated at 37°C. Backup samples were stored at -80°C.

For QPCR analysis, samples were aliquoted according to their concentration within two master mixes to create the desired cDNA on a Flexcycler. Resulting data was analyzed and plotted in Microsoft Excel and Graphpad (Graphpad Prism Inc.).

2.9 Statistical Analysis

Data from QPCR, FLOW CYTOMETRY or Western Blot signals (2-way ANOVA) were blotted and analyzed with Graphpad Prism 7 (Graphpad Prism Inc.).

3. Results

3.1 MEF cell lines are positive for EGFR

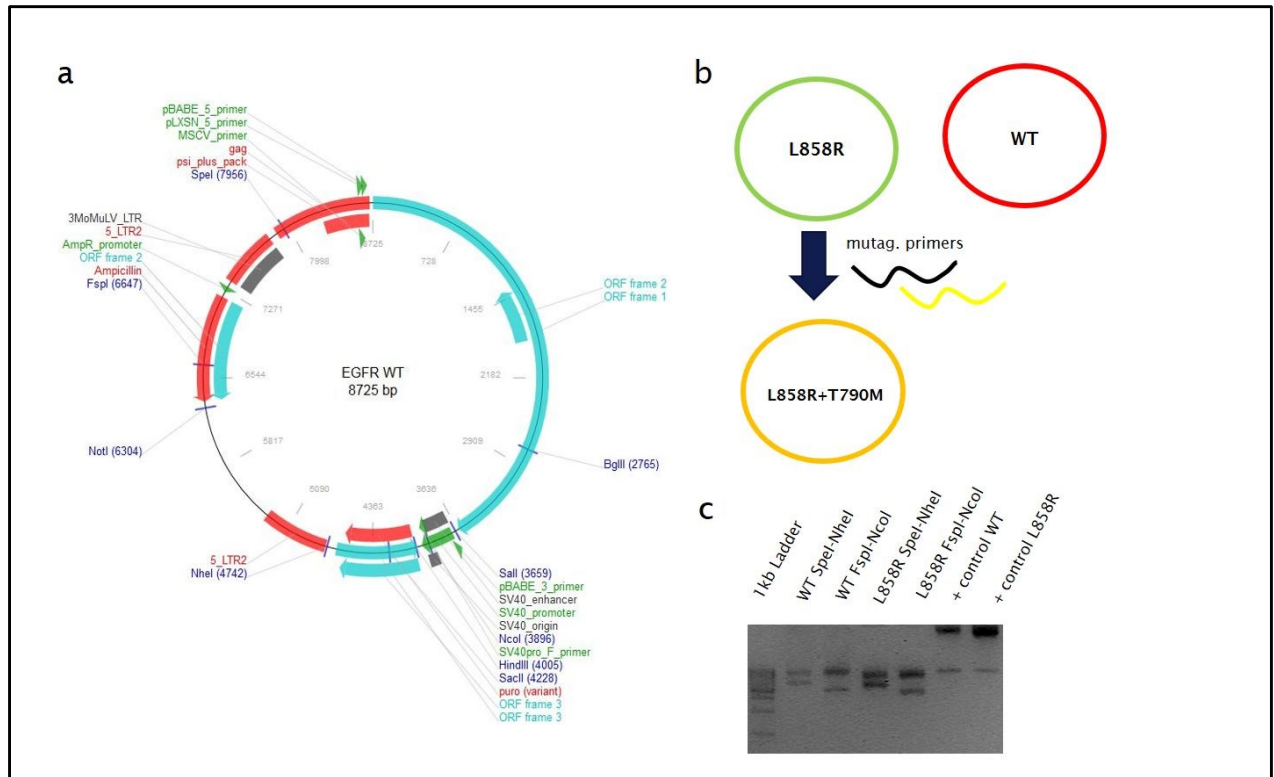


Figure 11: Schematic of the generation of variant EGFR cell lines. (a) pBAGE puromycin vector which served as backbone for the approach. (b) mutant variant L858R+T790M was created with mutagenic primers in a PCR. (c) test digest of the received pBAGE EGFR WT and L858R vector at different restriction sites.

It was important for our approach to establish cell lines that would stably express the epidermal growth factor, avoiding transient systems in which more likely overexpressing vectors are used. We therefore chose a pBAGE backbone which is not a highly efficient system but since we were looking for low to mid-level expression of the EGFR it fit well in to our experimental setup. Additionally, two of our desired EGFR variants were already established and available in the Addgene database. Figure 11 shows the map of the EGFR WT with all restriction sites and sequences partially harboring resistance cassettes. The L858R mutant variant vector of the epidermal growth factor is identical except for a base pair switch which yields the mutagenic properties. We therefore took this variant to create the L858R+T790M mutant by additionally inserting another base pair switch with mutagenic primers within a PCR.

This goal was achieved as seen in Figure 12. We continued by transfecting a cell line that already contained the packaging and envelope vector compatible to our pBAGE

vector system. The produced lentiviral particles were successfully used to infect mouse embryonic fibroblasts (an EGFR knock out cell line). The three independent cell lines were tested for EGFR expression with a western blot. All cell lines were positive though intensity levels of the signals hinted at varying numbers of positive cells expressing the EGFR protein within a cell line.

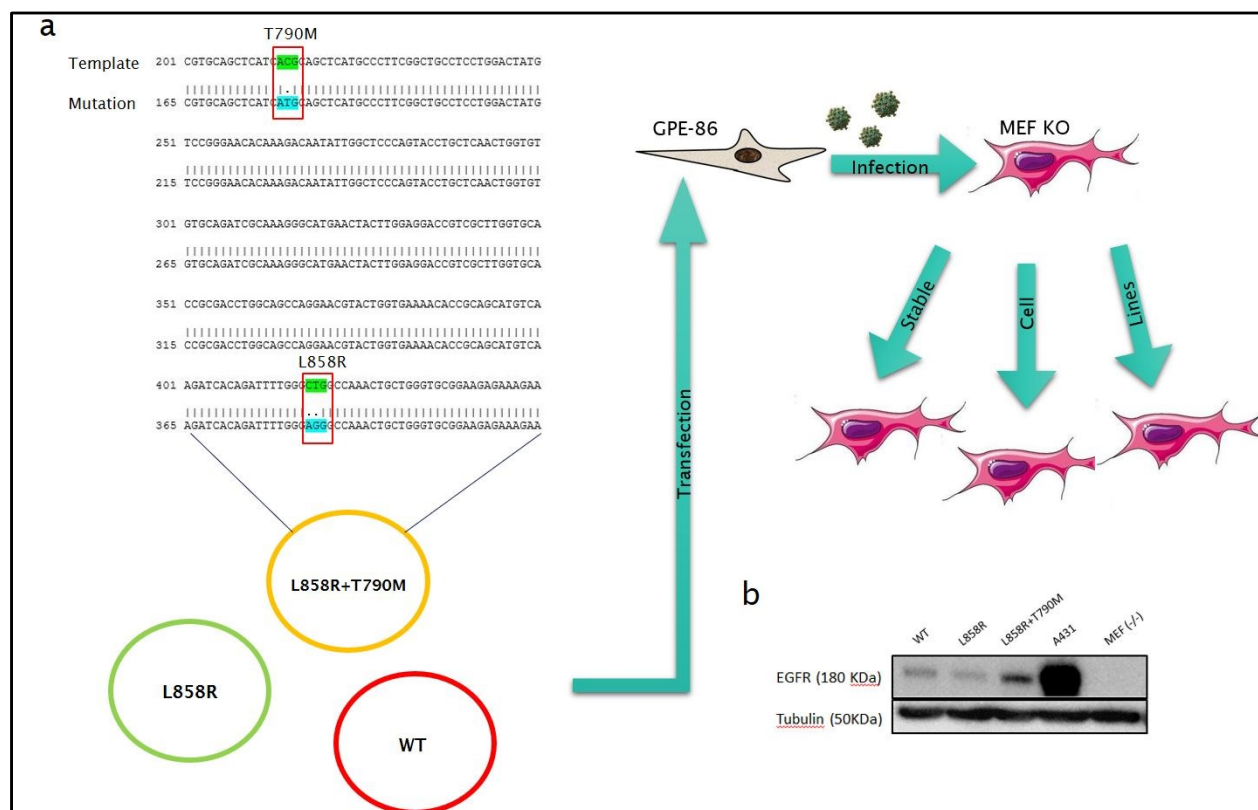


Figure 12: EGFR cell lines generation. (a) Sequence alignment of the original template (L858R DNA) and the mutated strand. Red boxes highlight mutation sites. GPE-86 cell lines were used as packaging cells to produce lentiviral particles containing the desired DNA, which were used to infect target cells. (b) shows a western blot confirming successful transformation of mouse embryonic fibroblasts. Tubulin was chosen as control protein, note the signal intensity difference between the established MEF cell lines.

This was later confirmed with an immunofluorescence assay. The positive control (A431 cell line) and negative control behaved as expected, displaying a strong and no signal respectively.

3.2 Immunofluorescence assay of hEGFR MEF cells

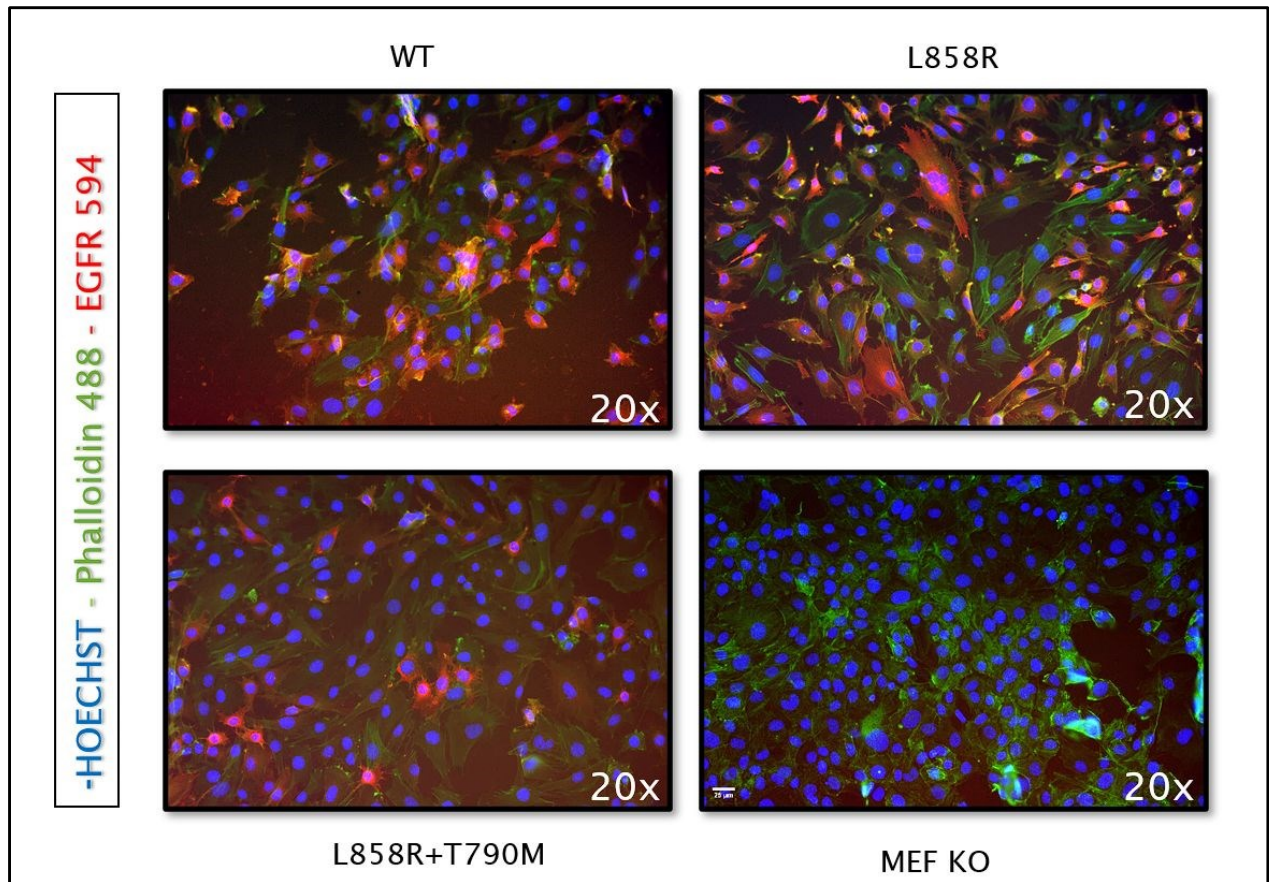


Figure 13: Immunofluorescence of the EGFR on MEF cell lines. Cells that display red fluorescence are positive for the epidermal growth factor receptor. Pictures show a representative field of view out of a twenty times magnification. Note the variability of positive cells between the different cell lines. WT = wild type, KO = knock out, MEF = mouse embryonic fibroblasts. Green and blue fluorescence signals for counterstaining belong to actin and nuclei of cells respectively. Scale bar represents 25 μ m.

Ahead of all the other experiments we aimed to determine the proportion at which our cell lines express EGFR. The immunofluorescence assay revealed varying amounts of positive cells within the cell lines as shown in Figure 13. This is not unexpected since the lentiviral infection can lead to integration of the target gene into silenced regions within the host genome. Overall, the number of positive cells was sufficient to continue and to conduct further experiments. Furthermore, the western blot results had been explained and validated.

3.3 Immunofluorescence of labeled EGF on MEF cells

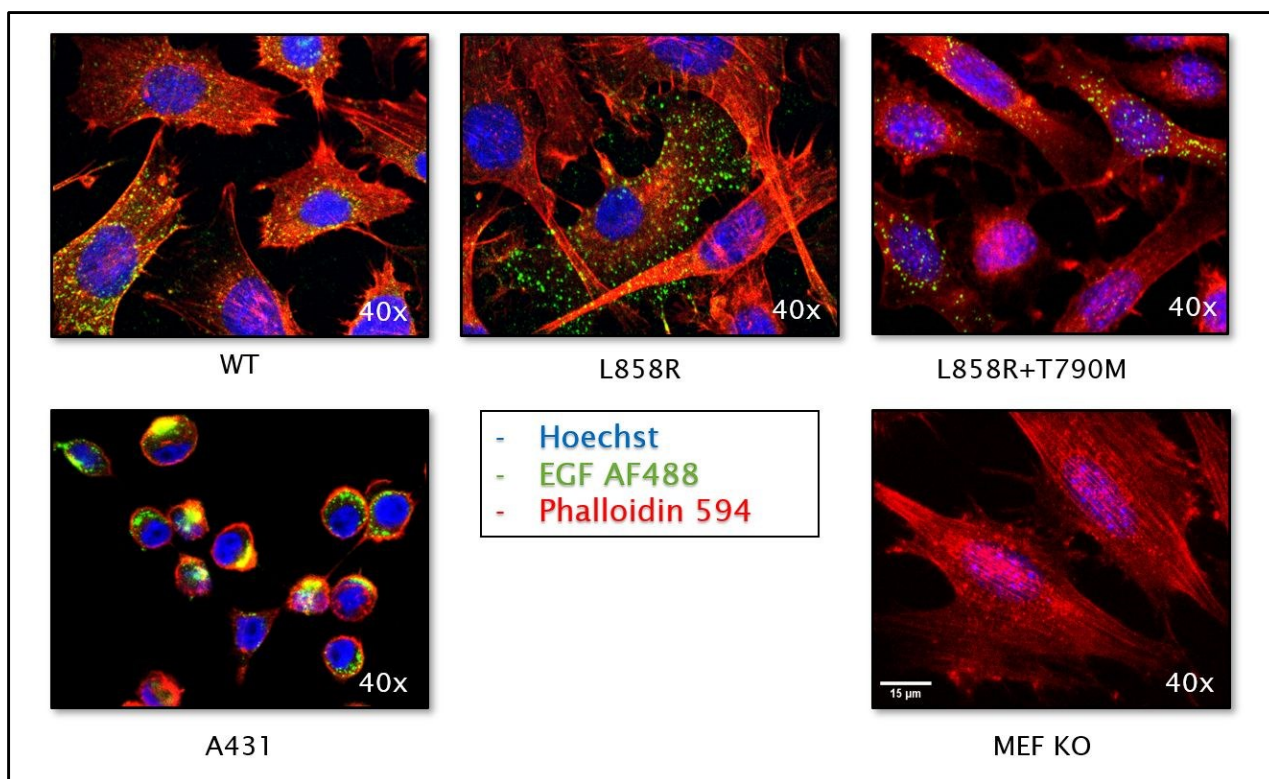


Figure 14: Immunofluorescence with labeled EGF: Established cell lines and controls are shown. Pictures are representative sections from a group of samples taken after one minute of stimulation with EGF (50ng/μl). Green signals belong to EGF (Alexa Fluor 488), blue signals to Hoechst and red signals to Phalloidin (Alexa Fluor 598). All pictures were taken on a confocal microscope at 40 times magnification with the same settings. Pictures display a representative part out of a sample. Scale bar represents 15 μm. WT = wild type, KO = knock out, MEF = mouse embryonic fibroblasts.

After we had confirmed successful EGFR expression, we were interested if we could quantitatively see a difference in internalization of the receptor between the different variants. We therefore starved the cells for at least four hours and then subsequently exposed them to a saturating concentration of labeled EGF (50 ng/μl) for 30s up to 1 minute of time. Short timepoints deemed to be more conclusive as EGF is likely to be internalized fast. Figure 14 displays a one-minute snapshot of treated cell lines. As receptor internalization can be clathrin mediated, we conclude that our strong EGF signals derive from such clathrin vesicles which harbor several receptors and therefore enhance the signal leading to rather easy tracking of the process. Although we went for a short stimulation period, we could not see a difference in internalization speed and progress between the wild type and the mutants. Over all samples, except negative controls we could find a high variability of the internalization progress ranging from membrane region positions to perinuclear regions or migrating through

cytosolic space at the same time. Compared to the positive control, signals were highly distinguishable. This is attributable to a very high amount of EGFR on the cell surface of A431 cells which makes a distinctive statement difficult.

3.4 Pronounced Akt/ERK stimulation in L858R expressing cells

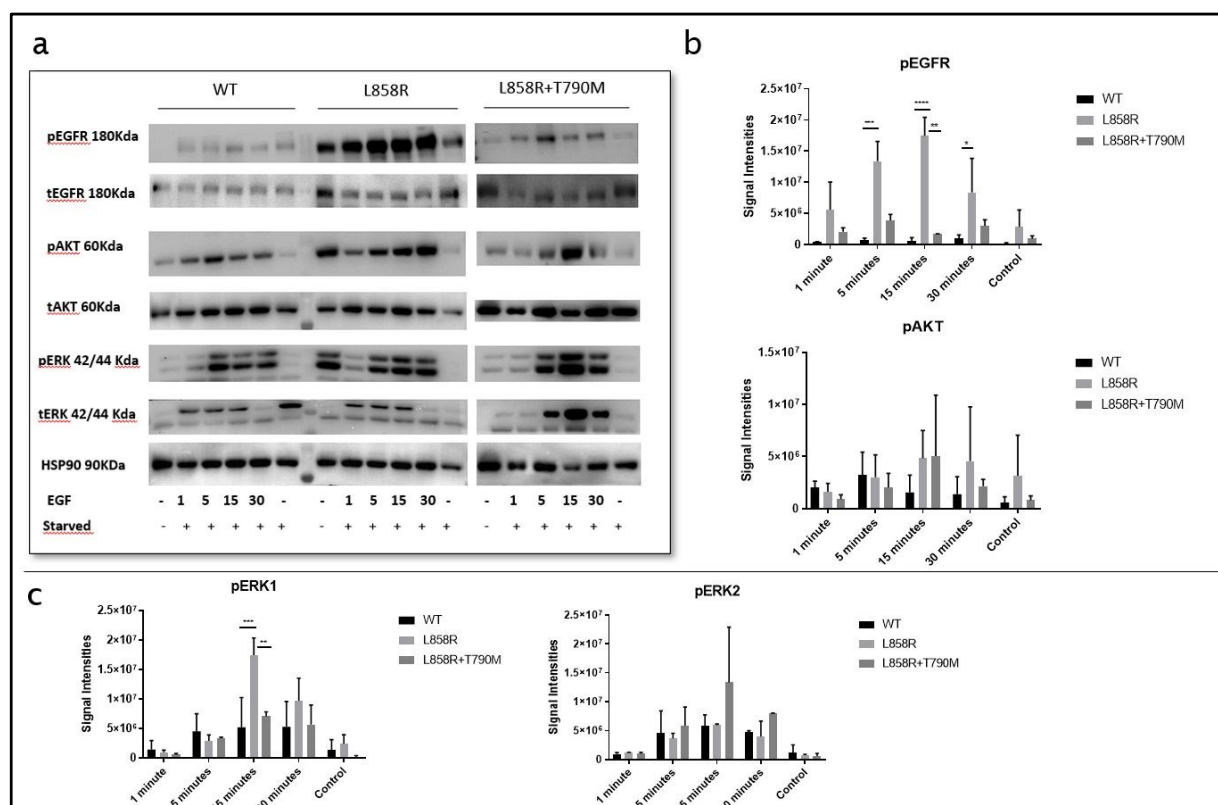


Figure 15: Western Blot analysis of cell lines: Qualitative and quantitative western blot analysis of the wild type EGFR cell line and its distinct mutants L858R and L858R+T790M from two individual immunoblots. Cells were starved for several hours and then treated with 50ng/μl hEGF for 1,5,15 or 30 minutes. (a) shows the blot including all our downstream signaling proteins of interest (pAKT and pERK) as well as both controls. (b) quantitative analysis of pEGFR and pAKT. Mean signal intensities for each timepoint and cell line are shown. (c) same as b but showing pERK1/2 mean intensity levels.

Furthermore, we wanted to investigate the downstream signals of activated EGFR in L858R and L858R/T790M overexpressing cell lines. Similar experiments have been conducted before but to our knowledge not with this setup, containing the WT and these two mutants together with a time course stretching from one minute to thirty minutes. We starved the different cell lines and then stimulated them with hEGF for the respected time period. Figure 15 (a) shows the outcome. As expected, both mutants show more pronounced EGFR phosphorylation upon receptor stimulation compared to the WT, in which the L858R mutant displays stronger differences in signal strength. Accordingly, also activation of signaling pathways downstream of the

EGFR are more pronounced in cells expressing the L858R and L858R+T790M mutant EGFR, demonstrated by slightly increased Akt phosphorylation and more prominently increased ERK1 activation, especially for the L858R mutant.

3.5 Flow Cytometry of DARPIn E69 proves binding specificity

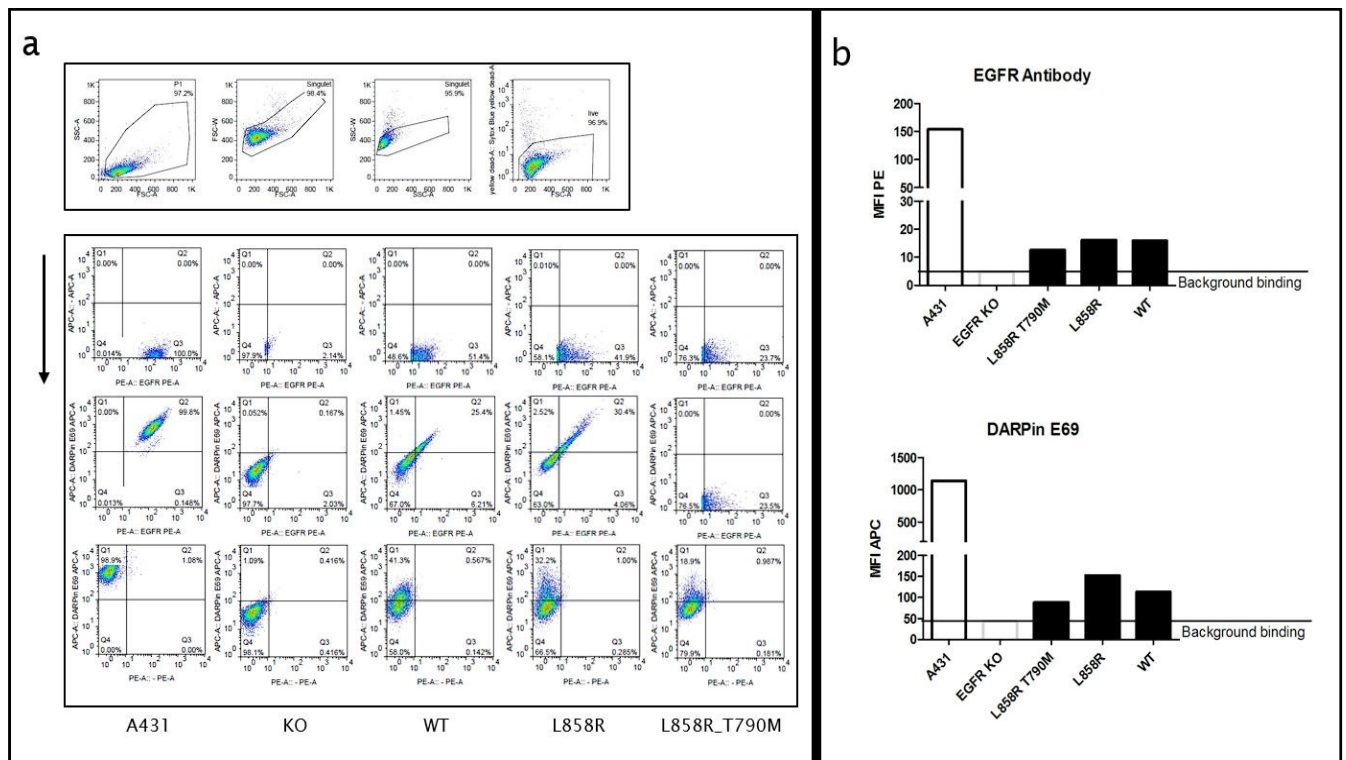


Figure 16: Flow cytometry analysis with DARPIn E69AF647: In advance of ensuing TOCCSL experiments we successfully labeled the created cell lines with an EGFR antibody and a DARPIn (E69) alike and tested their binding properties. Additionally, we anticipated a clear look for positively transfected cells and their population size within their respective cell lines. (a) Pseudo color dot plot and analysis of all cell lines including positive and negative control. Top box shows gating strategy throughout the experiments. Bottom box: each column (arrow direction) represents one cell line with following label top to bottom: x-axes APCA – APCA, APCA-DARPIn E69-APCA, APCA-DARPIn E69-APCA and y-axes PEA- EGFR-PEA, PEA- EGFR-PEA, PEA-PEA. A431 cells served as positive control and KO = Knock Out, as negative control. WT = Wild type. Note the odd double stain in the L858R_T790M mutant. (b) Depicts the MFI, as median, (mean fluorescence intensity) of all samples for EGFR antibody and DARPIn E69.

To visualize DARPins, we labeled them with a C2 (AF 488) and a C5 (AF 647) maleimide dye. We confirmed correct labelling by flow cytometry using a conventional EGFR antibody to ensure specificity. As Figure 16 shows, DARPIn E69 performs well in flow cytometry applications and seems to show a more pronounced signal than the conventional EGFR antibody (Fig. 16b). Next to validating positive binding events, we wanted to make sure that the population sizes of our transfected cell lines are sufficient to carry out single molecule fluorescence microscopy. The data from flow cytometry analysis shows that population sizes vary between cell lines. The wild type population harbored 41,3% and 51,4% (E69 and EGFR antibody

respectively) positive cells. These numbers decline when we look at the mutant cell lines. While the L858R cell line still possesses at least one third positive cells (32,2% E69 and 41,9% EGFR antibody), the L858R_T790M transfected cells account for even less EGFR positive cells (18,9% with E69 and 23,5% with EGFR antibody) to only around a fifth of the total population. This matches our observations in our immunofluorescence assays where the mutant cell lines tended to show fewer positive signals as well. Despite these partly low percentages we deemed the population sizes to be sufficient for TOCCSL experiments.

3.6 TOCCSL on A431 cell line

We selected all created MEF cell lines and the A431 cell line as positive control for our two-color TOCCSL experiments. The cell lines were seeded with at least 50% confluency on fibronectin coated chamber slides and subsequently left alone for several hours to adhere. Right before TIRF and TOCCSL utilization the cells were equimolar labeled with DARPin E69 AF488 and AF647. No EGF stimulation was applied since we wanted to investigate native states of the EGFR. A431 cells displayed good staining and their high EGFR density on the cell surface made a direct inspection of oligomerization states impossible. Because of that we employed the TOCCSL method and applied a high intensity laser pulse as shown in Figure 17 to bleach the region of interest of a cell facing the coverslip. A post bleach image was taken directly after photobleaching to check for correct and full bleaching. Afterwards, single molecule signals outside the photobleached area diffused back into the observation field and were now visible as single, separated signals. These signals could be tracked in both channels and signals in proximity were analyzed for colocalized events by taking several images in sequence with a 20 ms delay (t_{del}) between individual color channel recordings. Eventual homoassociation was determined by co-localization analysis and regarded as associated when within a radius of 100-200nm (see supplementary Figures and (Váradi *et al.*, 2019)). The procedure was repeated several times over different areas of the coverslip to locate and gather data of different cells.

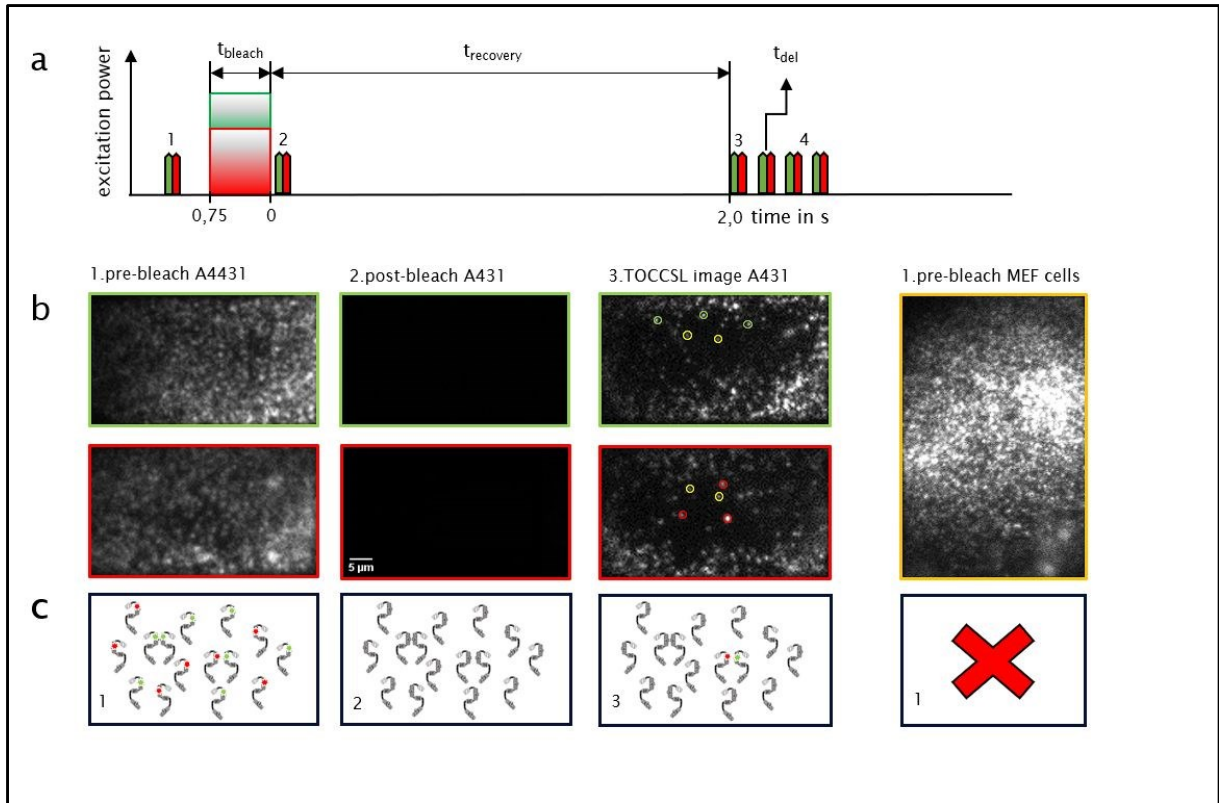


Figure 17: TOCCSL strategy for EGFR oligomerization detection: (a) The protocol for our two-color TOCCSL approach, given as schematic cartoon with indication of laser power and time schedule. (b) Images of a defined region of A431 cells after equimolar labeling of EGFR with DARPin E69AF488 (green framed) and DARPin E69AF647 (red framed). Pictures were taken in TIRF mode, split into two color channels and immediately imaged with the same CCD camera. In the pre bleach image (1) it is evident that the density of molecules is too high to single out events. Therefore, only after bleaching with a laser pulse (750ms) controlled by a post bleach image (2) we were able to observe diffraction limited signals in both channels as expected (3). Exemplary signals that entered the region of interest are circled in the corresponding color (red and green) while yellow circles represent possible co-localized events. After TOCCSL a series of images was recorded with a delay of 20ms (4) to track potential individual and colocalized signals. Note that the MEF population on either mutant cell lines failed to deliver proper staining, as mainly unspecific binding of the DARPINS was observed. An example out of several sequences is given (yellow framed image). (c) Simple sketch of the TOCCSL procedure following the protocol. EGFR domain cartoon was adapted and altered from (Nair, Bonner and Bredel, 2022)

As proof of principle, we were able to carry out a successful TOCCSL run on our positive control A431 cell line. Unfortunately, we were not able to apply two-color TOCCSL on our MEF cell lines. We hardly observed cells in TIR mode and therefore even less EGFR positive cells were available for a TOCCSL run. As shown in (b) Figure 17, yellow framed image we only captured bright fluorescence of DARPins non-specifically bound to the bottom of the cover slip with no clear cell surface staining. Therefore, in this work solely our observations in the A431 cells are described.

3.7 Signal intensity distribution on A431 cells

The TOCCSL data of A431 cells were viable for further analysis, e.g. the probability distribution of detected photons (intensity) from a single dye molecule and how it relates to the intensity distribution of multiple colocalized dye molecules which in turn may reveal oligomeric states. The results are given as probability density function denoted as pdf, based on the number of photons detected from a single dye molecule. Through a series of convolution integrals with software from the TU lab our data was used to analyze intensity distribution in both color channels. Intensities can vary when multiple dye molecules are present and colocalized in an area.

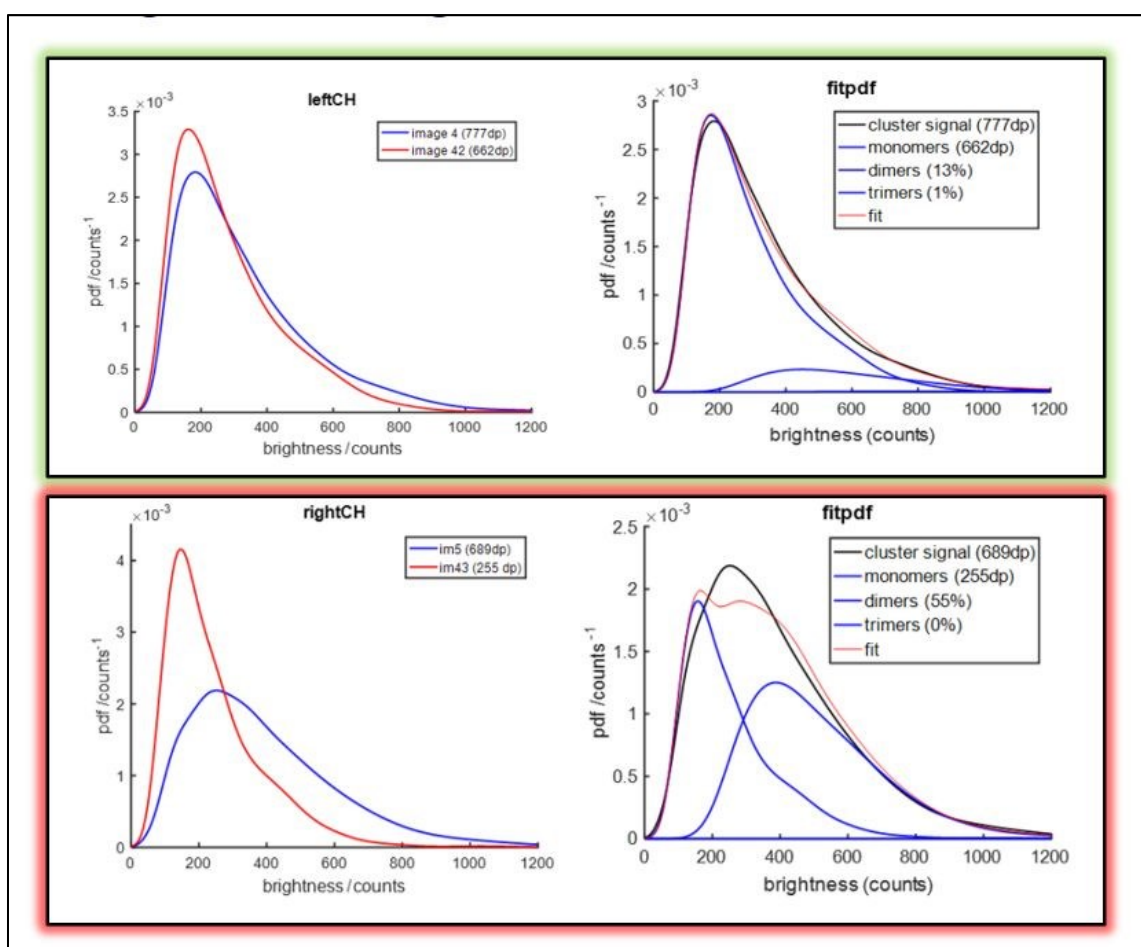


Figure 18: Single molecule brightness distribution of A431 cells: Results of both channels are shown: Red and green frames refer to the appropriate fluorophore color (channel) used. Graphs on the left display brightness distribution data from post bleach to the last sequence image recorded, whereas graphs on the right show the brightness distribution of observed localized spots via TOCCSL in their respective channels. The signals were fitted (fitpdf) and oligomeric states are given as individual slopes. Note that monomers seem to be the dominant population.

The observation and analysis of hundreds of recovering single molecule signals in both channels (median values AF 488: 4531.0313 counts and AF647: 3991.1185 counts) indicates the existence of a proper population of dimeric EGFR states. Dimers in the red channel take up ~55% while 45% represent a monomeric state. The green channel shows a wider disparity with ~13% dimers and 87% monomers respectively. Over both channels this yields a hypothetical averaged ratio of 35:65 dimers to monomers, but these data points mainly represent red-red and green-green dimers as green-red and red-green inputs were not analyzed. A software-based co-localization analysis across the channels would provide more insight as we noticed a surplus of signals through likely unequimolar labeling in the red channel. False positives cannot be completely excluded, although two-color TOCCSL minimizes such data points. Additionally, analysis of over 2700 trajectories (see methods of (Wieser and Schütz, 2008)) yielded a Diffusion constant (D) of 0.044 +/- 0.0036 $\mu\text{m}^2/\text{s}$ which confirms the movement of EGFR molecules can be explained by free Brownian motion. (See supplementary Figures 1 and 2 for search radius and msd respectively).

4. Discussion

Cell Lines and setup

We chose mouse embryonic fibroblast cells as target cell line for a few reasons. Firstly, a MEF EGFR knock-out cell line was already established at the institute which granted easier access to create the desired mutant cell lines. Secondly, MEF cells are frequently used in research. Due to their functional importance and the ease of obtaining them, mouse embryonic fibroblasts are widely utilized in life science research to identify and understand diverse phenomena. And without a doubt their generation, preparation and culture has been well established over the years (Xu, 2005; Tan and Lei, 2019). MEF cells are not an unlimited cell line but our starting material was far enough off of going senescent and passage numbers were in a reasonable range even with lentiviral preparation steps taken into account. Autofluorescence can occur in MEF cells, as has been shown for Flow cytometry but we could not observe experiment disturbing levels in our case (Çelik-Uzuner and O'Neill, 2021).

A431 cells serve as a valuable positive control for investigating the cell cycle and signaling pathways associated with cancer due to their overexpression of Epidermal Growth Factor Receptor (EGFR). Hence, we employed them as a benchmark in comparison to our mutant EGFR cell lines. They have been used for single molecule fluorescence microscopy as well before, successfully (Yanagida, Sako and Minoghchi, 2000). Concerning the establishment of mutant harboring cell lines, we relied on a retroviral packaging system. They are considered a reliable source for introducing genes into a target cell and at the same time secure that the introduced gene is passed on to daughter cells (Morgenstern and Land, 1990; Salmons and Günzburg, 2000). While other approaches often rely on transient (vector) systems with success, but usually accompanied with higher protein expression we expected and aimed at a different outcome in which a rather moderate expression level was desirable. Through application of the pBabe vector system we managed to establish three cell lines stably expressing EGFR. As Figure 12 shows, the amount of EGFR expressed varied and hinted at low positive cell numbers but with reversed roles as the T790M mutant would show later on.

Immunofluorescence assays

Contrary to the primary western blot signals for positive EGFR MEFs, the immunofluorescence data revealed reverse roles in quantity when all three distinct MEF cell lines were compared. In most cases within a representative field of view the L858R-T790M mutant cell line would tend to yield the lowest number of positive cells. This is contrary to the stronger western blot signal but could be explained through some influential conditions. Firstly, the lentiviral transduction does not guarantee a fully infected population of cells and as mentioned before, an integration of the target gene into the host genome can coincidentally occur in a silenced region which may show up as false negative event. Secondly, random picks out of a cell culture for experiments may lead to slightly altered variations when observed visually. Therefore, a Southern blot analysis would have proved highly beneficial for identifying significant target gene alterations within our MEF cells such as amplifications, deletions or structural rearrangements. Since it is a time intensive method and we anyways aimed for Flow cytometry analysis we did not carry out said method. However, the established cell lines performed well despite some difficulty in the form of abundance.

Looking at internalization of the WT EGFR and its mutant counterparts it seems to follow conventional consensus. The EGFR is typically internalized and transported from early to late endosomes and lysosomes for degradation. It may also be recycled back to the plasma membrane. These two processes are delicately balanced and regulated by post-translational modifications and various proteins. A disruptions in these endocytic processes can impact EGFR activity in cancers (Sapmaz and Erson-Bensan, 2023). We likely observed clathrin-mediated endocytosis (CME). CME provides a rapid internalization route, but slower clathrin-independent processes exist as well. It has been reported that ligand concentration influences the EGFRs degradation pathway. Higher EGF levels (20 ng/ml and above) can enhance clathrin-independent endocytosis (CIE), possibly due to clathrin pathway saturation (Tomas, Futter and Eden, 2014). However, a visual assay through images will only reveal proper binding and internalization of the RTKs. EGF signals after one minute of stimulation were observed from the plasma membrane throughout the plasma until the perinuclear region, indicating no unexpected behavior. Signal strength increase is highly likely derived from clathrin pits, early endosomes or multivesicular bodies wherein several degraded EGF labeled extracellular residues may accumulate.

Therefore, the IF assays fulfilled their purpose in showing that the EGFR-MEF cell lines were apparently ready for further studies.

Downstream Signaling

The EGFR signaling network has been researched extensively over the years. Its ligands and most important pathways including phosphorylation targets and downstream proteins are mostly understood. Surely, the complexity of the whole signaling network can't be overstated. The ERBB signaling network includes numerous proteins and interactions and studies have revealed that EGF can activate EGFR and lead to the phosphorylation of thousands of proteins (Oda *et al.*, 2005; Olsen *et al.*, 2006). However, two pathways and proteins residing in there are commonly observed and targeted in immunoblot assays. The PI3K-AKT-mTOR pathway regulates various cellular processes including metabolism, proliferation, survival and motility. Its hyperactivation in cancer is associated with mutations in EGFR family members, PI3K, AKT and reduced PTEN expression (Wee and Wang, 2017). The RAS-RAF-MEK-ERK MAPK pathway is central to the EGFR's biological response and a significant target for cancer treatment. It involves the key players RAS and RAF and ERK MAPK with their interactions having impact on growth, proliferation, differentiation, migration and apoptosis (Lewis, Shapiro and Ahn, 1998). We therefore looked at altered pEGFR, pAKT and pERK levels to possibly find differences based on the mutant EGFR carrying cell lines.

Based on literature, kinase mutant EGFRs can show more autophosphorylation and be constitutively active. Interestingly, our L858R mutant does exhibit this behavior in absence of EGF treatment while the T790M mutant shows no significant difference to the wild type. The L858R tends to keep higher signaling levels overall and especially around the fifteen-minute mark represented by pERK1 signaling values. However, the T790M mutant imitates similar behavior only around fifteen minutes. This is contrary to a very recent study, where the L858R-T790M mutant variant was highly autophosphorylated. Additionally, the team observed that upon EGF exposure pAkt and pERK levels increased in cells expressing WT, T790M, or L858R. Notably, cells with the T790M/L858R double mutant exhibited elevated pAkt levels but not pERK. In both cases, exposure to EGF did not further raise the activation level (Shao and Wells, 2023). We cannot confirm this outcome with our data. It appears that L858R is capable of downstream signaling without EGF stimulation as its pAKT and pERK levels indicate. Even the dip at the one-minute mark may not be a counterargument,

as pre-EGF states of the cells may already internalized a significant number of receptors leading to a short vacant time until EGF stimulation propels levels up again between one to five minutes. Although some error bars seem skewed (Fig.15 b and c), the overall impression is that both mutant cell lines clearly elevate their downstream signaling compared to their wild type pendant. After 30 minutes, levels appear to be attenuated globally maybe due to unknown autoinhibitory processes or at least moderately consumed EGF molecules. A final verdict would need more testing.

DARPin in single molecule microscopy

As flow cytometry has shown DARPins are arguably a suitable tool for research lab methods. They behaved as expected and delivered comparable binding specificity and robustness on par or even better in comparison to their famous antibody counterparts which made their handling effortless as has been proven before (Boersma *et al.*, 2011; Plückthun, 2015). Despite the odd staining in the double mutant (Fig.16 a) probably due to a technical error during handling and/or analysis in Flow Jow, we did get valuable information. In general, an MFI score could tempt to assert that a higher score means a higher expression of protein compared to a lower score. Since we can exclude a disparate experiment setting and environment (fluctuating laser power, calibration, compensation) as both targeting proteins and fluorophores were observed in one session, it is fair to argue that the MFI obtained shows a slightly more sensitive DARPin outcome and that A431 cells prove their overexpressing nature compared to the MEF cell lines. Comparing said cell lines, their MFI scores are similar as only the DARPin L858R sample within its test group shows a distinguishable signal. However, positive EGFR cell population sizes within in the mutant cell lines were on the lower end. This was unfortunately a difficulty but no general hurdle in TOCCSL experiments to come.

DARPins combined with TOCCSL

The existing structural model for EGFR activation proposes ligand-induced dimerization of the ectodomain, propagating across the plasma membrane and juxtamembrane domains ultimately leading to activation of the kinase domain via asymmetric dimerization (Zhang *et al.*, 2006; Arkhipov *et al.*, 2013; Endres *et al.*, 2013). However, this early model relied on crystal structures and biochemical data protein fragments. Additionally several other aspects of this model, including ligand

occupancy, receptor structure and oligomerization are subjects of ongoing debate (Clayton *et al.*, 2005; Alvarado, Klein and Lemmon, 2010; Needham *et al.*, 2016; Zanetti-Domingues *et al.*, 2018). Consequently, it was a main spur for us to further investigate the receptor's behavior with special attention to its oligomeric states. We successfully applied DARPins in single molecule microscopy (TOCCSL) to observe and record EGFR dimerization dynamics on a living cell membrane. The only setback was the MEF cell lines availability. Unfortunately, we could not retrieve data from either mutant cell line or the wild type therefore a substantial comparison was not possible. The main reasons can be summed up as: firstly, cell populations of both mutant cell lines had low to mid shares of EGFR+ cells and secondly, their connection to the chamber slide surface was significantly more fragile compared to A431 cells. The latter could potentially be avoided by much higher fibronectin concentrations and the other problem should be possibly circumvented by fluorescence activated cell sorting (FACS). It should be noted that experience with the double mutant in the past within the lab has been observed as partially difficult as it did not behave as stable and reliable as other mutants. Interestingly, even Shao *et.al.* failed at producing a L858R-T790M melanoma cell line (WM983A and WM983B) for one of their experiments.

Based on our data gathered from A431 cells we can assume that EGFR can exist in a dimeric state on the cell membrane in an unstimulated environment. Even though a co-localization analysis of red – green events was not performed we could show that dimers are present in both channels (Fig.17 and Fig.18 red channel and green channel results) and demonstrate substantial subpopulations, while trimers or higher oligomeric states could not be observed as was reported by Clayton *et al.*. The share of dimers could hypothetically be even higher, as monomer signals within their respective channels could at the same time also be part of a dimer signal across red – green channels. These results are in accordance to previous findings, where dimers in a native state (though short lived) have been detected on living cells (Kozier *et al.*, 2013). Since A431 cells are overexpressing EGFR and EGFR is capable of clustering (ligand bound) on their membrane surface it is not far-fetched to assume that even in a non-ligand bound situation EGFR forms dimers as our data presents (Clayton *et al.*, 2005; Chung *et al.*, 2010). Highly crowded surface areas (see Fig.17 b) can create higher probabilities for transient co-confined areas which enhance repeated encounters and dimer formation (Low-Nam *et al.*, 2011). There is also

evidence that pre-formed dimers occur on lower and medium expressing cell lines as well. These studies report ranges down to 20.000 copies of EGFR per cell and still observed dimerization compared to findings based on 600.000 receptors per cell (Nagy *et al.*, 2010; Yavas, Macháň and Wohland, 2016).

Conclusion and Outlook

Here, using DARPins and TOCCSL we show that dimers and monomers coexist in varying shares on the membrane surface of living A431 cells. We established a novel approach at single molecule fluorescence microscopy by combining robust, cheap, target specific designed and sensitive ankyrin repeat proteins combined with an established and precise fluorescence microscopy method. All in all, these preliminary results encourage further application of this approach to achieve the initial goal of examining the behavior of NSCLC related EGFR mutants on living cells. Alongside FACS, strengthening the amount of EGFR+ MEF cells and improved slide coating for mutant cell lines as well as the potential individual adaption as needed fueled by DARPins, the original goal is attainable. Ongoing discussions regarding the EGFRs state on living cell membranes with and without EGF stimulation could be expanded in this way.

5. References

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II Supplementary Figures

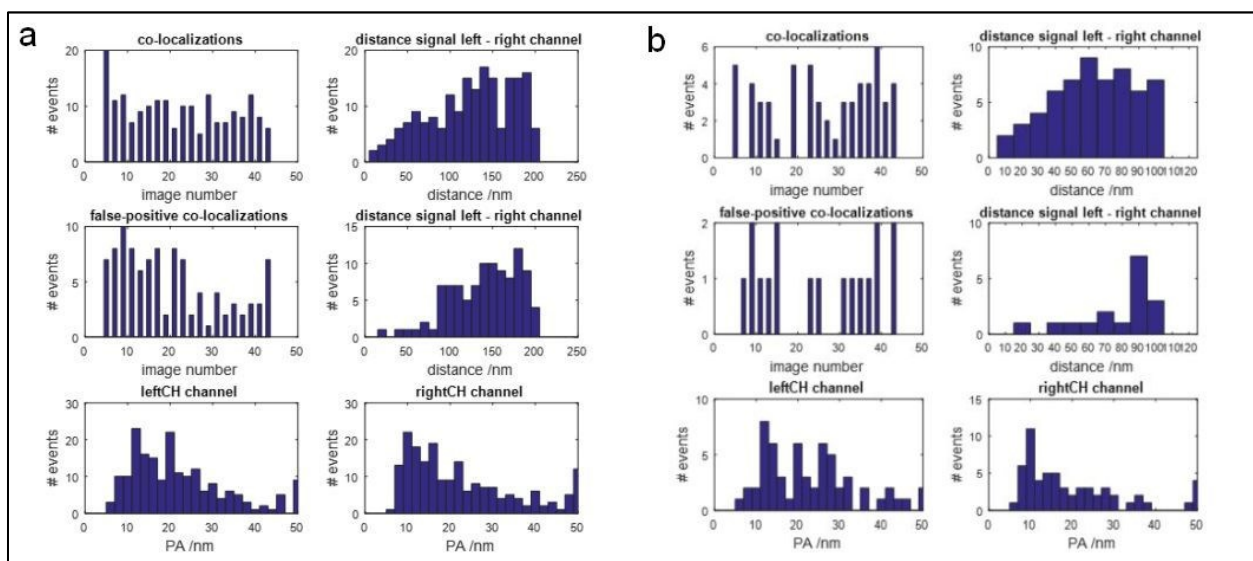


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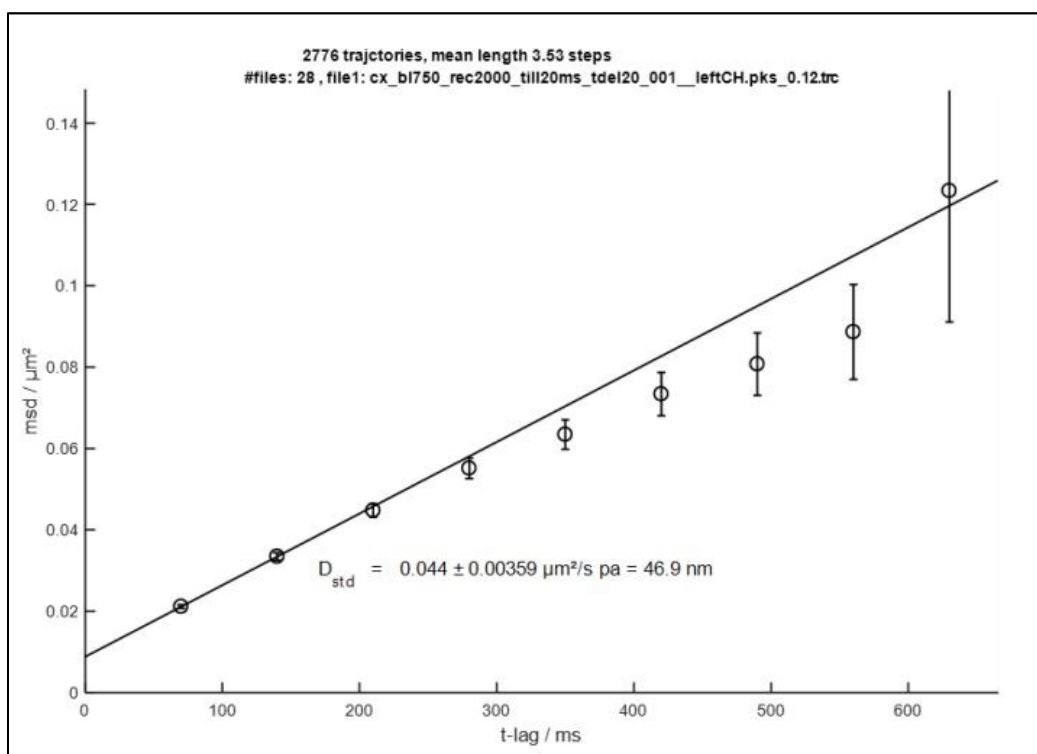


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III Abstract

Cancer persists as a significant global burden with millions of deaths every year and the effort to counteract its actions cannot be ever enough. The Epidermal Growth Factor Receptor (EGFR), also HER1/ErbB1, is a highly important cell surface receptor-tyrosine kinase crucial for various cellular processes, including differentiation, metabolism, and growth. Dysregulation of EGFR, along with its family members, has been well-established as a significant contributor to the development of various types of cancer. We therefore set out to elucidate the mechanisms and dimerization dynamics of the EGFR as questions still persist regarding its oligomeric states especially in a native state. Therefore, we focused on comparing EGFR variants bearing mutations found in human cancers with the wild-type receptor. Notably, the L858R and L858R+T790M mutants are frequently observed in non-small-cell lung cancer patients, making them particularly interesting. We successfully cloned and altered plasmids with a mutagenesis step, wherein through a base pair switch in a L858R plasmid a L858R+T790M construct was established. These human EGFR variants were subsequently transfected into mouse embryonic fibroblasts (MEF) derived from EGFR knockout mice through a lentiviral delivery system. We obtained data, for verification, from MEF cell lines with immunofluorescence assays as well as western blot and Flow Cytometry. To visualize the oligomerization of EGFR effectively, we established a precisely balanced fluorophore system. By employing DARPins (Designed Ankyrin Repeat Proteins) labeled with a maleimide dye we successfully observed EGFR dynamics on a cell surface via single molecule microscopy and additionally tested their potential as labeling protein. With the application of TOCCSL (developed at TU Wien) we obtained data from A431 cells revealing monomeric and dimeric states of EGFR on the cell surface. In summary, the acquired data encourages further studies backed by proof of principle in using DARPins and TOCCSL coupled as tools.

IV Zusammenfassung

Krebs ist nach wie vor eine erhebliche globale gesundheitliche Belastung mit Millionen von Todesfällen pro Jahr. Jegliche Bemühungen seinen Auswirkungen entgegenzuwirken sind immens wichtig. Der Epidermal Growth Factor Receptor (EGFR), auch HER1/ErbB1, ist eine äußerst wichtige Zelloberflächenrezeptor-Tyrosinkinase, die für verschiedene zelluläre Prozesse, einschließlich Differenzierung, Stoffwechsel und Wachstum, entscheidend ist. Eine Fehlregulation von EGFR und seinen Familienmitgliedern gilt nachweislich als wesentlicher Faktor für die Entstehung verschiedener Krebsarten. Wir haben uns daher für diese Arbeit vorgenommen, die Mechanismen und die Dimerisierungsdynamik des EGFR aufzuklären, da immer noch Fragen zu seinen oligomeren Zuständen bestehen, insbesondere im nativen Zustand. Der Fokus liegt auf den Vergleich von EGFR-Varianten, die Mutationen tragen die bei Krebserkrankungen beim Menschen vorkommen, mit dem Wildtyp-Rezeptor. Besonderes Augenmerk galt den Mutanten L858R und L858R+T790M die häufig bei Patienten mit nicht-kleinzelligem Lungenkrebs beobachtet werden und dadurch interessant sind.

Wir haben erfolgreich Plasmide mit einem Mutageneseschritt geklont und verändert, wobei durch einen Basenpaarwechsel in einem L858R-Plasmid ein L858R+T790M-Konstrukt etabliert wurde. Diese menschlichen EGFR-Varianten wurden anschließend über ein lentivirales Transduktionssystem in embryonale Mausfibroblasten (MEF) transfiziert, die von EGFR-Knockout-Mäusen stammten. Zur Verifizierung haben wir die MEF-Zelllinien mit Immunfluoreszenztests sowie Western Blot und Durchflusszytometrie geprüft. Um die Oligomerisierung von EGFR effektiv zu visualisieren, haben wir ein präzise ausgewogenes Fluorophorsystem etabliert. Durch den Einsatz von DARPins (Designed Ankyrin Repeat Proteins), die mit einem Maleimidfarbstoff markiert sind, konnten wir die EGFR-Dynamik auf Zelloberflächen mittels Einzelmolekülmikroskopie erfolgreich beobachten und zusätzlich ihr Potenzial als Markierungsprotein testen. Mit der Anwendung von TOCCSL (entwickelt an der TU Wien) haben wir Daten von A431-Zellen erhalten, die den Monomer- und Dimerzustand von EGFR auf der Zelloberfläche aufzeigen. Zusammenfassend lässt sich sagen, dass die erfassten Daten weitere Studien anregen, die durch die grundlegende Funktion von DARPins und TOCCSL als gekoppelte Werkzeuge gestützt werden.