MASTERARBEIT / MASTER'S THESIS

Titel der Masterarbeit / Title of the Master's Thesis

Disabled 1, a novel adapter protein
for the epidermal growth factor receptor

verfasst von / submitted by

Maximilian Schwab, B.Sc.

angestrebter akademischer Grad / in partial fulfilment of the requirements for the degree of

Master of Science (MSc)

Wien, 2021 / Vienna, 2021

Studienkennzahl lt. Studienblatt / degree programme code as it appears on the student record sheet:	UA 066 834
Studienrichtung lt. Studienblatt / degree programme as it appears on the student record sheet:	Masterstudium Molekulare Biologie
Betreut von / Supervisor:	ao. Univ.-Prof. Dipl.-Ing. Dr. Johannes Nimpf

Acknowledgement

I hereby would like to thank my PI and supervisor Prof. Dr. Johannes Nimpf for giving me the opportunity to work in his lab, for his guidance and very helpful feedback throughout my time I the lab and during the writing process. I am really grateful for his support which enabled me to take part in additional courses that taught me valuable skills and ultimately lead me to acquiring the FELASA certificate before even being a PhD student. I also would like to express my gratitude for including me and my work in his most recent publication, which gave me a first-hand impression how this essential part of “a scientists life” looks like. Therefore, thank you Johannes for these extremely instructive months in your lab, which laid an excellent foundation for my further scientific life.

I would also like to express my deep gratitude to Dr. Paula Dlugosz. She took over my direct supervision and was always ready to show me methods and help me with advice even long after our shared time together in the lab. In particular, I would like to thank her for her patience and kindness, which always motivated me, but also for her constructive criticism, which helped me grow professionally and personally. For this, my heartfelt thanks, Paula!

Next, I would like to thank Magdalena Teufl, who had already started working in the lab before me and who, together with Dr. Dlugosz, warmly welcomed and showed me the ropes. Working with her was great fun and always created a good atmosphere in the lab. Thank you for being such great partner “in crime” during that time.

I would also like to thank Harald - Harry - Rumpler. He has the technical expertise that so often helped me out of a tight spot whenever I had problems with one of the machines or needed certain chemicals and instruments. Thank you, Harry, for the many sweets and small gifts that always provided nice surprises in the daily lab routine.

Last but not least, I would like to thank my family and friends who accompanied me mentally and physically during this work. In particular, I would like to thank my parents, siblings and especially Elisa, who gave me advice and never-ending support until the end and motivated me at all times to continue to follow my passion for science. Without you people I would not have come this far in the first place! Again, a heartfelt “Thank you” to you!

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Abstract

The epidermal growth factor receptor (EGFR) is part of a larger family of transmembrane receptor tyrosine kinases which regulate survival, proliferation, and growth of cells through the interaction with various adapter proteins. Here, the protein Disabled 1 (Dab1), known for its role in the Reelin signalling pathway, is introduced as a new adapter protein for EGFR. Dab1 has always been described as the signature adapter protein for the Apolipoprotein receptor 2 (ApoER2) which orchestrates the correct cortical layering in the embryonic brain. This work reveals that Dab1 is also able to bind and be phosphorylated by active EGFR. Dab1 co-localises with EGFR and is phosphorylated upon stimulation with EGF. This phosphorylation is dependent on the kinase activity of EGFR and can be inhibited via ATP-binding cassette inhibitors like Gefitinib (Iressa), Neratinib or via genetic inhibition of the kinase domain. Furthermore, presence of ApoER2 seems to inhibit this effect, reducing the phosphorylation of Dab1 to almost background levels. This could indicate that ApoER2 and EGFR are competing for Dab1 binding, pointing to Dab1 being part of a new pathway which is induced upon EGFR stimulation in absence of ApoER2. This pathway might be involved in proliferation in areas which are deprived of ApoER2, such as the crypts of the small intestine, and might play a role in cancer cell proliferation. Interference with this pathway might offer novel cancer treatment strategies by targeting an adapter protein of EGFR instead of the receptor.

Zusammenfassung

Der epidermale Wachstumsfaktor-Rezeptor (EGFR) gehört zu einer großen Familie von Transmembran-Rezeptor-Tyrosin-Kinasen, die das Überleben, die Vermehrung und das Wachstum von Zellen durch die Interaktion mit verschiedenen Adapterproteinen regulieren. Hier stellen wir Disabled 1 (Dab1), ein Protein, das für seine Rolle im Reelin-Signalweg bekannt ist, als neues Adapterprotein für den EGFR vor. Dab1 wurde schon immer als charakteristisches Adapterprotein für den Apolipoproteinrezeptor 2 (ApoER2) beschrieben, der die korrekte Schichtung des Kortex im embryonalen Gehirn steuert. Diese Arbeit zeigt, dass Dab1 auch in der Lage ist, an den aktivierten EGFR zu binden und von diesem phosphoryliert zu werden. Dab1 kolokalisiert mit dem EGFR und wird bei Stimulation mit EGF phosphoryliert. Diese Phosphorylierung ist von der Kinaseaktivität des EGFR abhängig und kann durch ATP-bindende Kassetteninhibitoren wie Gefitinib (Iressa), Neratinib oder durch genetische Hemmung der Kinasedomäne gehemmt werden. Darüber hinaus scheint das Vorhandensein von ApoER2 diesen Effekt zu hemmen, indem es die Phosphorylierung von Dab1 fast auf die Basiswerte reduziert. Dies ist ein Indiz dafür, dass ApoER2 und EGFR um die Bindung von Dab1 konkurrieren, was darauf hinweist, dass Dab1 Teil eines neuen Signalweges ist, der bei EGFR-Stimulation in Abwesenheit von ApoER2 induziert wird. Dieser Signalweg könnte an der Proliferation in Geweben beteiligt sein, denen ApoER2 fehlt, wie z. B. den Krypten des Dünndarms, und könnte eine Rolle im Krebszellwachstum spielen. Inhibierung dieses Signalweges könnte zu neuen Krebsbehandlungsmöglichkeiten beitragen, die auf ein Adapterprotein des EGFR, anstatt auf den Rezeptor selbst abzielen.

1. Introduction

1.1. The epidermal growth factor receptor (EGFR)

1.1.1. Molecular characteristics and activation mechanism

The epidermal growth factor receptor (EGFR) is a 170 kDa sized glycoprotein and the first described member of four transmembrane growth factor receptor proteins which have similar structures and functions. It is an ubiquitously expressed receptor, crucial in initiating and regulating the behaviour of epithelial cells as well as of cells in tumours of epithelial origin, activated among others by its signature ligands, the epidermal growth factor (EGF) and transforming growth factor alpha (TGF-α) (Hackel, Zwick et al. 1999, Singh, Carpenter et al. 2016). EGFR, which is also known as HER-1 or c-ErbB1, forms together with HER-2, HER-3 and HER-4 the c-ErbB family of receptor tyrosine kinases (RTKs) (Yarden and Sliwkowski 2001). They all are single chained transmembrane glycoproteins consisting of an extracellular ligand binding domain, juxta membrane sections, a tyrosine kinase domain, and a tyrosine-containing C-terminal tail (Wieduwilt and Moasser 2008). The ectodomain which binds the ligands consists of four subdomains. First, two leucine-rich domains, L1 and L2, (also called domains I and III), which together bind the ligand and second of two cysteine-rich domains, CR1 and CR2 (also called domains II and IV) mediating the interaction between receptors (Bajaj, Waterfield et al. 1987, Ward, Hoyne et al. 1995). In the unbound form the CR1/2 domains interact with each other , forming a dimerisation loop (Bouyain, Longo et al. 2005). Ligand binding of e.g., EGF to the L1 and L2 domains leads to an extended conformation in which CR1 is exposed, allowing the interaction with other receptors, which have exposed CR1 domains too (Fig.1). Dimerisation can occur in a homo- or heteromeric manner (Bouyain, Longo et al. 2005). In contrast to EGFR, HER-3 and HER-4, HER-2 is permanently fixed in the exposed conformation allowing for interaction with other activated ErbB family members (Garrett, McKern et al. 2003). This dimerisation promotes structural change in, and thus, the activation of the intracellular tyrosine kinase domain (Zhang, Gureasko et al. 2006). Activation of the kinase leads to trans-autophosphorylation of the C-terminus of the receptor monomers. The now phosphorylated tyrosines in the C-terminus then activate, either directly or through adapter proteins, several downstream signalling pathways including the Ras/ mitogen-activated protein kinase (MAPK) pathway, the phospho-lipase C (PLC)-Pathway, the Rac-pathway or the Phosphoinositide 3-kinase (PI3K)/Akt-pathway (Scaltriti and Baselga 2006). Inactivation of EGFR signalling occurs by dephosphorylation and/or internalisation of the receptor dimers in coated pits and their subsequent delivery to the lysosome, where they are degraded - a process called

receptor down-regulation or desensitisation (Wiley, Herbst et al. 1991, Mattila, Pellinen et al. 2005). One of the most prominent downregulating proteins of EGFR is the E3 ubiquitin ligase c-Cbl. It binds to phosphorylated tyrosines, mainly Y1045, and stimulates clathrin mediated endocytosis of the receptor (Grovdal, Stang et al. 2004).

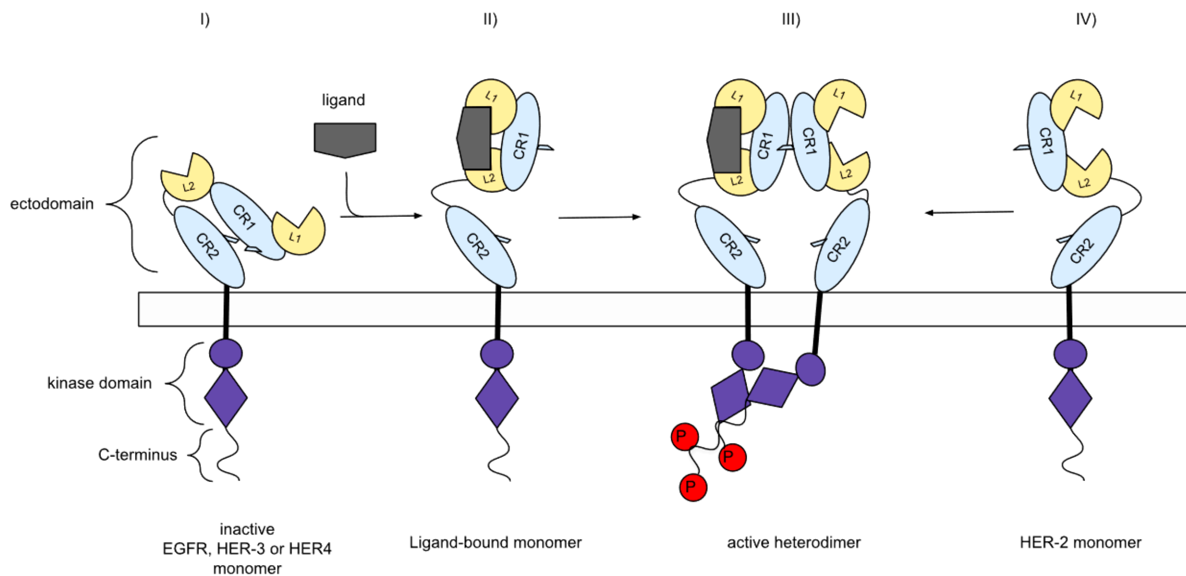

Figure 1: Dimerisation process of c-ErbB family members. **I)** In the unbound state, CR1 and CR2 are folded onto each other, masking the receptor interaction site. **II)** Binding of ligand by L1 and L2 changes the receptor conformation allowing interactions between CR1 domains of different receptors. **III)** Dimerisation of two receptors lead to reciprocal phosphorylation of tyrosines in the C-termini of the receptors creating binding sites for adapters or other downstream signalling molecules. **IV)** In contrast to EGFR, HER-3 and HER-4, HER-2 is permanently fixed in the extended conformation, and thus able to interact immediately with other c-ErbB family members (modified from (Wieduwilt and Moasser 2008).

1.1.2. EGFR adapter proteins

EGFR serves as a binding platform for many different intracellular adapter proteins which start a variety of downstream signals. Most of the binding to EGFR by adapter proteins occurs via Src homology 2 (SH2) or phosphotyrosine-binding domains (PTBs). SH2 domain stands for “Src homology 2” because of its similarity to regions in Src-family and Abelson murine leukaemia (Abl) tyrosine kinases, which are uniquely dedicated to the recognition of phosphotyrosine (pY) sites on molecules (Liu, Jablonowski et al. 2006). These two domains are shared by an otherwise quite diverse family of proteins with different catalytic effects (Songyang, Shoelson et al. 1993). After ligand binding and trans-autophosphorylation of the C-termini of EGFR dimers at residues Y1068, Y1086 and Y1173, these phosphotyrosines serve as binding spots for SH2-domain containing proteins (Batzer, Rotin et al. 1994, Schlessinger 2000). The SH2 binding sites are not random but dependent on the surrounding amino acids

creating an individual and highly specific binding site for the respective SH2 domain. Binding to the negatively charged phosphotyrosines occurs through a positively charged binding pocket within the SH2 domain (Hidaka, Homma et al. 1991, Koch, Anderson et al. 1991). This specificity in turn decides which protein is recruited to a RTK or scaffold protein and thus which downstream pathway is activated. For example, the adapter protein, which activates the Ras/MAPK pathway, growth factor receptor-bound protein 2 (Grb2), predominantly binds to a pYxNx sequence, where x stands for any of the 20 naturally occurring amino acids (Rozakis-Adcock, Fernley et al. 1993). Important for this binding is a tryptophan residue at position 121 (W121) in the SH2 domain and if this residue is mutated, the affinity to this motif will be drastically reduced (Marengere, Songyang et al. 1994). This dependence on a specific sequence is even higher in proteins like PI3K, Src homology region 2 domain-containing phosphatase-2 (SHP2) or Zeta-chain-associated protein kinase 70 (ZAP-70) which contain tandem SH2 domains. Reason for this is that the space between the pY domains on the target must fit perfectly to the SH2 domains to allow simultaneous binding of all the SH2 domains of the adapter proteins (Pluskey, Wandless et al. 1995). pY-binding or PTB domains, other than the name would suggest, do not rely solely on ligand induced phosphorylation of tyrosine on the C-terminus of the receptor, like SH2 domains, but rather bind the acidic heads of phospholipids which enables them to localise to the membrane or the juxta membrane domains of the receptors and thus allows an interaction with the RTK. A common recognition motif of PTB domain substrates is NPxY. PTB containing proteins often lack inherent catalytic activity and therefore contain other domains like SH2 domains to recruit other signalling molecules (Uhlik, Temple et al. 2005). The previously mentioned adapter protein Grb2 is one of the most important adapter proteins of EGFR (among other RTKs) due to its ability to activate two signalling pathways: The Ras/MAPK pathway as well as the PI3K/Akt pathway. Via one of its SH3 domains, which allows interactions with proline rich sites on other proteins, it binds the protein Son-of-Sevenless (SOS) upstream of the Ras/MAPK pathway (Rozakis-Adcock, Fernley et al. 1993). The complex formation of Grb2 with other adapter proteins, like the Src-homology-containing protein (SHC) is possible, however it is not as favoured as the binding to SOS (Sasaoka, Langlois et al. 1994). A second SH3 domain of Grb2 can bind to the scaffold protein Grb2-associated binder (Gab) 1/2, which promotes binding of PI3K to the membrane resulting in downstream activation of the PI3K/Akt pathway (Castellano and Downward 2011). Another important adapter protein of EGFR is PLCγ which binds to EGFR via its SH2 domain (Rotin, Honegger et al. 1992). The kinase domains of EGFR phosphorylate PLCγ mediating its activation. Subsequently, this catalyses the cleavage of phosphatidylinositol 4,5-bisphosphate (PIP_2) into diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP_3) (Berridge and Irvine 1984). A third major group of EGFR

adapter proteins are the signal transducer and activator of transcription proteins (STATs) (Quesnelle, Boehm et al. 2007). STAT proteins are recruited to activated EGFR and bind to the phosphorylated receptor via their SH2 domain. Upon phosphorylation of STAT proteins by the EGFR kinase domain, they dimerize in a homo- or heterodimeric manner, translocate to the nucleus and activate their respective target genes (Darnell 1997). STAT3, for example activates genes involved in increased cell proliferation upon entering the nucleus, whereas STAT1 is able to induce cell cycle arrest and apoptosis via upregulation of caspases and p21 (Bromberg, Fan et al. 1998, Bromberg 2002, Kijima, Niwa et al. 2002). Apart from adapter proteins, which activate downstream signalling pathways involved in cell metabolism and growth, other proteins also bind to the receptor controlling the activity of EGFR and regulating its degradation. One of the most prominent negative EGFR regulators is Casitas B-lineage Lymphoma E3 ubiquitin Ligase (c-Cbl) (de Melker, van der Horst et al. 2001). It is a E3 ubiquitin ligase which associates with Grb2 and subsequently binds to activated EGFRs via its tyrosine kinase binding (TKB) domain, a complex consisting of a SH2 domain variant, a Ca^{2+} binding EF-hand (comprised of two helices E and F) and a four-helix bundle (Meng, Sawasdikosol et al. 1999, Waterman, Katz et al. 2002). Binding of the Grb2-c-Cbl-complex to EGFR induces activation of c-Cbl and mono-ubiquitylation on multiple sites on the receptor leading to its internalisation and subsequent degradation (Haglund, Sigismund et al. 2003, Mosesson, Shtiegman et al. 2003). In summary, EGFR interacts with numerous intracellular proteins, and thus is capable of initiating many different signalling cascades. In the following, some of the pathways mentioned above will be discussed in more detail.

1.1.3. The Ras/Mitogen-activated protein kinase (MAPK) cascade

The Ras/MAPK pathway is one of the most important pathways downstream of EGFR activation and is one of the main targets in cancer research due to its role in stimulating cell growth, proliferation, and senescence. Besides, the Ras/MAPK-pathway has been shown to play a role in synaptic plasticity (Orban, Chapman et al. 1999, Lemmon and Schlessinger 2010, Santarpia, Lippman et al. 2012). After binding of a growth factor to EGFR or another RTK, homodimerization occurs, which leads, among others, to cross-phosphorylation of their C-termini by the kinase domains of the respective receptor monomer. The phosphorylated tyrosines on those c-termini now serve as docking sites for adapter molecules such as Grb2 and SHC (Batzer, Rotin et al. 1994, Schlessinger 2000). Grb2 binds the guanine nucleotide exchange factor (GEF) SOS in the cytosol which is recruited to the membrane (Schlessinger 1994). The now membrane-bound

SOS can encounter the membrane bound small G-protein Ras, exchanging the Ras-bound Guanosine diphosphate (GDP) with Guanosine triphosphate (GTP). GTP-bound Ras then recruits Raf-1, a serine/threonine protein kinase, which in turn phosphorylates and activates MAPK/extracellular-signal regulated kinases (Erk) 1 and 2 (MEK1/2). MEK, a tyrosine/threonine kinase recruits, phosphorylates, and thus activates Erk1 and Erk2 (Schlessinger 2000, Morrison 2012). In this context, MEK serves as a cytoplasmic docking site for Erk (Fukuda, Gotoh et al. 1997). Upon phosphorylation, Erk undergoes drastic conformational changes, which do not only heavily increase Erk's catalytic activity but also lead to its dissociation from MEK (Canagarajah, Khokhlatchev et al. 1997). The phosphorylated Erk1/2 proteins then dimerize and translocate to the nucleus. There, they interact and phosphorylate members of the Erythroblast Transformation Specific (Ets) family of transcription factors like Ets-like Protein 1 (Elk1) and thus influence downstream effectors like c-Fos (named after Finkel–Biskis–Jinkins murine osteogenic sarcoma virus), c-Myc (named after Myelocytomatosis virus), cAMP response element-binding protein (CREB) and nuclear factor 'kappa-light-chain-enhancer' of activated B-cells (NF-κB) either directly or indirectly (Leprince, Gegonne et al. 1983, Marais, Wynne et al. 1993, Curtis and Finkbeiner 1999, Kolch 2000, Wang, Ge et al. 2006). As an example, phosphorylated Elk1 binds to the promotor region of the *FOS* gene and enhances its transcription. The produced *FOS* -mRNA and the c-Fos protein then can also be directly phosphorylated by Erk and by this become stabilised (Murphy, Smith et al. 2002). c-Fos is one of the building blocks, which together with c-Jun, a product of the c-Jun N-terminal kinase (JNK) pathway, is making up the AP-1 transcription factor that plays an important role in proliferation, differentiation but also in tumorigenic transformation of cells (Derijard, Hibi et al. 1994, Eferl and Wagner 2003). Given these potentially dangerous functions that might lead to tumour formation, the MAPK pathway must be tightly regulated. In particular, the activity of Erk proteins has to be tightly controlled, as an excess of c-Fos leads to a higher probability of metastasis. This is attributed to the c-Fos-regulated expression of matrix metallo proteases (MMPs) which play a crucial role not only in releasing tumour cells into the blood stream but also in enabling those cells to invade new tissue sites (Hu, Mueller et al. 1994, Itoh and Nagase 2002). This regulation comes into play in the form of phosphatases, which remove the phosphorylation from the Erks and thus reduce their catalytic activity, terminating the signalling cascade. In this context, the MAP kinase phosphatase 3 (MKP3), also known as dual specificity phosphatase (DUSP) 6, is a prominent player. It binds in a MEK-like manner to ERK and removes the phosphates from the regulatory elements of the Erks (Zhou, Wu et al. 2001, Zhang, Zhou et al. 2003). Apart from regulating Erk signalling, there are many other steps along the Ras/MAPK-cascade, which can be regulated and thus ensure the correct signalling process (Hornberg, Binder et al. 2005, Hornberg, Bruggeman et

al. 2005). For example, direct dephosphorylation of EGFR by protein tyrosine phosphatases (PTP) inhibits further binding of adapter proteins and abrogates the signal (Mattila, Pellinen et al. 2005). Another way of inhibiting this pathway is through the protein phosphatase 2A (PP2A) which bind the SHC-domain, which is in complex with Grb2 and prevents its tyrosine phosphorylation, therefore, it inhibits the downstream signal transduction (Ugi, Imamura et al. 2002). Taken together, the Ras/MAPK pathway activated by EGFR is one of the most important and tightly controlled pathways that fulfils multiple functions. Without Ras/MAPK signalling cell survival would not be guaranteed, thus disturbance of the pathway can lead to lethal diseases like cancer.

1.1.4. The PI3K/Akt pathway

In the mid-1980s, PI3-kinases gained a lot of attention in cancer research due to their connection to the transforming ability of the Src kinase or the polyoma virus middle t-antigen (Whitman, Kaplan et al. 1985). Since then, the PI3K induced pathway, which is also called the PI3K/Akt pathway, is taken as the second most important pathway besides the Ras/MAPK-pathway for the regulation of mammalian cell survival and proliferation, which is induced by RTKs like EGFR (Vivanco and Sawyers 2002). The key enzyme of this pathway, PI3K-1A is, like other members of the PI3K-family, a heterodimer consisting of two different subdomains being responsible for regulation or catalytic activity. The regulatory subdomain of PI3K-1A in humans is called p85, whereas the catalytic domain is called p110 (Carpenter, Duckworth et al. 1990). Substrates of p85 are phosphorylated tyrosines on the kinase domains of RTKs, which p85 binds via one of its SH2 domains. A second domain located between the two SH2 domains, the “inter-SH2 domain” leads to a constitutive binding of the catalytic subunit p110 (Vivanco and Sawyers 2002). p110 catalyses the phosphorylation of inositol-containing phospholipids at their C3-position, which are found in the plasma membrane (Falkenburger, Jensen et al. 2010). The main target *in vivo* is phosphatidylinositol- 4,5-diphosphate (PI(4,5)P_2, in the following called PIP_2), which it converts to phosphatidylinositol-3,4,5-triphosphate (PI(3,4,5)P_3, in the following called PIP_3) (Fig. 2) (Leevers, Vanhaesebroeck et al. 1999).

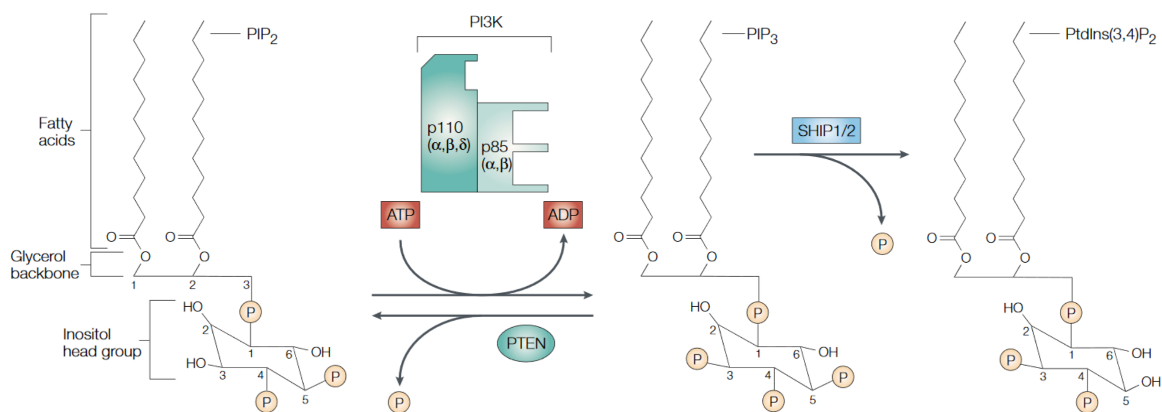

Figure 2: Phosphorylation and dephosphorylation of phosphatidylinositol phosphates. Phosphatidylinositol phosphates consist of fatty acids bound to a glycerol backbone which is bound to an inositol head group. Phosphatidylinositol-3 kinase (PI3K) catalyses the ATP-dependent linkage of a phosphate with the C in position 3 of the inositol head of PI(4,5)P_2, which leads to the formation of PI(3,4,5)P_3. PIP_3 serves as docking station for proteins containing a Pleckstrin Homology (PH)-domain This reaction can be reversed by the Phosphatase and Tensin homolog (PTEN), which removes this phosphate. Alternatively, the SH2-domain-containing inositol polyphosphate 5-phosphatase 1 (SHIP1/2) can remove the phosphate at the C in position 5, resulting in the formation of PI(3,4)P_2. (Taken from (Vivanco and Sawyers 2002)).

The process of converting PIP_2 to PIP_3 is tightly regulated. Upon cross-phosphorylation of the receptor ends, the previously cytosolic p85-p110 complex binds via its SH2 domain to the receptor. This leads to the activation of PI3K due to (i) the close proximity of p110 to the membrane and therefore to its substrates and (ii) due to its interaction of p85 with another RTK like EGFR resulting in a conformational change, which relieves p110 from a possible inhibition caused by p85 (Yu, Zhang et al. 1998). RTKs can also indirectly activate PI3K through activation of Ras (Rodriguez-Viciana, Warne et al. 1994). This shows that the Ras/MAPK-pathway and the PI3K/Akt pathway should not be considered as separate signalling pathways, but rather as connected signalling network. PIP_3, the product of PI3K, serves as a docking site for proteins containing a pleckstrin homology (PH)-domain such as Akt. Three members of the Akt family (Akt-1, 2 and 3) have been described and are expressed ubiquitously throughout the cell. They all have in common that they are serine/threonine kinases and that they play a role in different metabolic processes (Bellacosa, Testa et al. 1991). Akt1 is involved in cellular survival pathways through inhibiting apoptosis and stimulating protein and lipid synthesis, whereas Akt2 is thought to be involved in the insulin metabolism (Chen, Xu et al. 2001, Garofalo, Orena et al. 2003). Akt3's role is not clear at this time point, even though previous studies indicated a function in brain development as Akt3-deficient mice have smaller brains than wild type mice (Yang, Tschopp et al. 2004). Akt activation requires two distinct steps. First, its translocation to the membrane and second phosphorylation on S473 and T308 (Andjelkovic, Alessi et al. 1997). The first step gets induced as soon as PI3K phosphorylates PIP_2 in the plasma membrane which leads to the recruitment of Akt to the membrane via its PH-domain. Another

serine/threonine-kinase, which contains a PH-domain too, named 3-phosphoinositide-dependent protein kinase-1 (PDK1) phosphorylates Akt on T308 (Vanhaesebroeck and Alessi 2000). This alone is already sufficient for Akt activation but as a study from Stokoe, Stephens et al. from 1997 shows, this phosphorylation alone does not exploit the full potential of Akt. Instead, to elicit the maximum activity of Akt, it also needs phosphorylation on S473. (Alessi, James et al. 1997, Stokoe, Stephens et al. 1997). This task is performed by PDK2, an unknown kinase, whose identity is not entirely clear, yet several studies indicate that it could be the mammalian target of the rapamycin complex 2 (mTORC2) (Sarbassov, Guertin et al. 2005, Jacinto, Facchinetti et al. 2006). Upon activation of Akt, it activates Tuberous sclerosis 1 and 2 (TSC1/2) which stimulates Ras homolog enriched in brain (Rheb) GTPase activity to activate mTORC1 (Inoki, Li et al. 2002). The activation of mTORC1 has three consequences: First, it promotes protein synthesis via inhibiting the translational inactivator 4E-BP1 and activating S6-Kinase (S6K) (Ma and Blenis 2009). Secondly, it induces lipid biosynthesis by activating sterol regulatory element-binding protein (SREBP1) and peroxisome proliferator-activated receptor (PPAR) γ transcription factors and third, it inhibits autophagy by blocking Unc-51 like autophagy activating kinase (ULK1) (Kim and Chen 2004, Hosokawa, Hara et al. 2009, Duvel, Yecies et al. 2010). Another protein group regulated by Akt is the group of fork head box O (FOXO) transcription factors (Kops, de Ruiter et al. 1999, Matsuzaki, Ichino et al. 2005). FOXO transcription factors serve as a negative feedback loop on the PI3K-pathway by suppressing the production of growth factor receptors. Akt, in turn, phosphorylates and inactivates FOXOs promoting further growth and survival of the cell (Brunet, Bonni et al. 1999). Other notable downstream effectors are, among many others, the metabolic regulator glycogen synthase kinase-6 (GSK6), cell cycle regulators p21 and p27, the negative NF-κB regulator inhibitor of nuclear factor kappa B (IkB)-kinase (IKK-α) and the p53 inhibitor Mouse double minute 2 homolog (MDM2) (Cross, Alessi et al. 1995, Ozes, Mayo et al. 1999, Rossig, Jadidi et al. 2001, Liang, Zubovitz et al. 2002, Shin, Yakes et al. 2002). All those interactions lead to either stimulation of cell growth or promotion of cell survival (Fig. 3).

Given these diverse effects, Akt activation must be strictly regulated, like in case of the Ras/MAPK pathway. Here the phosphatases come into play. They mainly act on two levels of this pathway (Fig. 3). The activation of PDK1 and the activation of Akt. The former is taken over by the phosphatase PTEN, among others, which carries out a reverse reaction to that of PI3K. It catalyses the dephosphorylation of PIP3 to PIP2, which prevents PDK1 or Akt from binding to the membrane and thus being activated (Maehama and Dixon 1998, Maehama, Taylor et al. 2001). Another way of reversing the PI3K is the production of $PI(3,4)P_2$ by the phosphatases SHIP1/2 (Fig. 2) therefore reducing the amount of PIP_3 in the plasma membrane. However, recent studies

suggest that $PI(3,4)P_2$ is also able to act as proto-oncogene (Fuhler, Brooks et al. 2012). In addition to these reactions, there is also the option of directly preventing the activation of Akt. This is achieved by the two phosphatases PH domain and Leucine rich repeat Protein Phosphatase (PHLPP) and PP2A, which remove the phosphate groups of S473 or T308 respectively (Ugi, Imamura et al. 2004, Gao, Furnari et al. 2005). The consequence of this is that Akt does not become active and thus cannot start any of its downstream pathways.

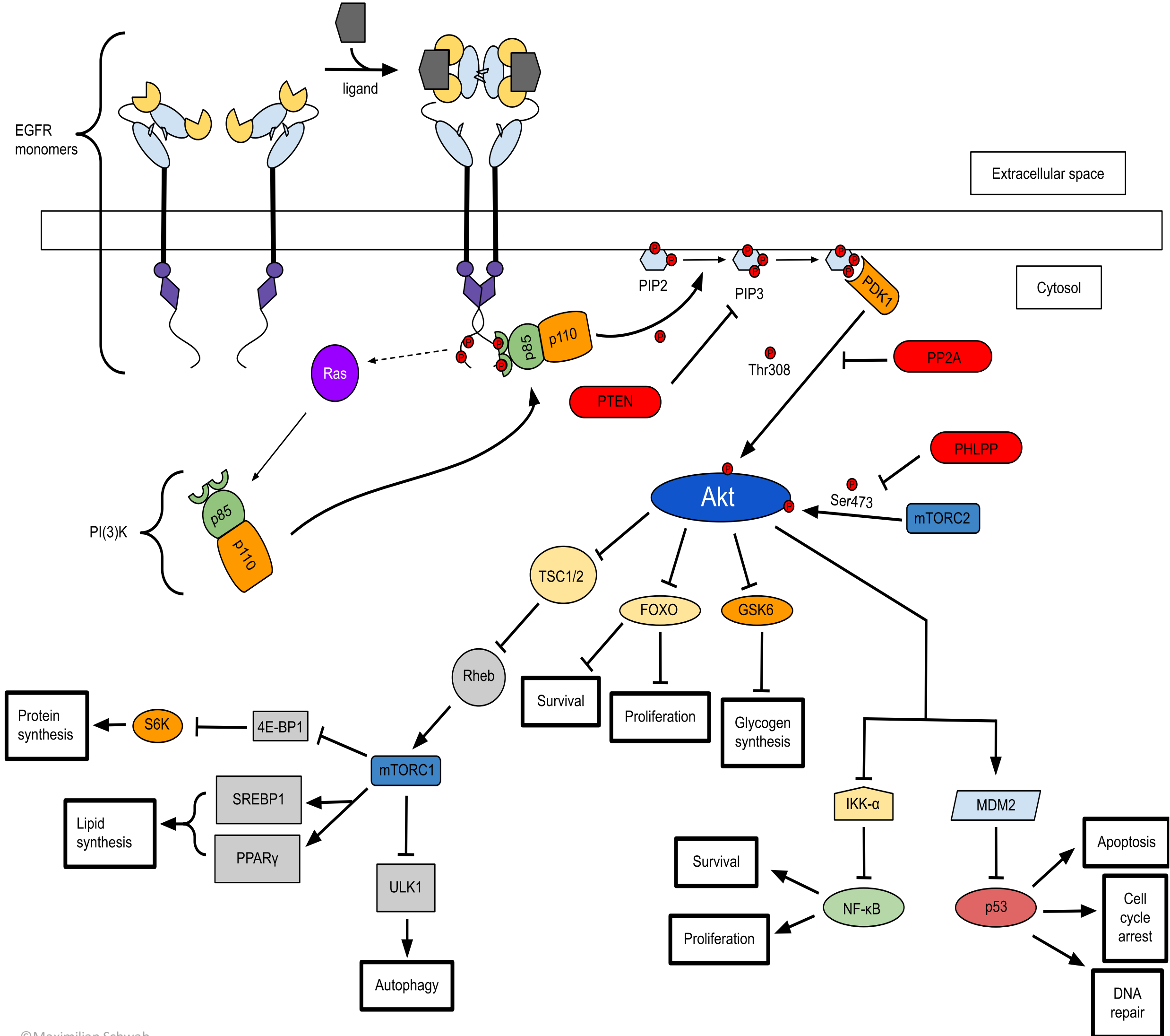

Figure 3: Overview over EGFR induced and Akt mediated pathways. Activation of a RTK like EGFR leads to cross phosphorylation of the C-termini of the receptors followed by either direct interaction of PI3K with the phosphorylated ends of the receptor or its indirect activation via previously activated Ras. PI3K phosphorylates PIP_2 to PIP_3 leading to the binding of PDK1 to the membrane. PDK1 and mTORC2 phosphorylate Akt. Akt activates a variety of pathways which promote cell growth, proliferation, and survival, including activation of mTORC1 and NF-κB as well as inhibition of FOXO, GSK6 and p53. Regulation of this pathway is achieved due to the activity of the phosphatases PTEN, PP2A and PHLPP which dephosphorylate PIP_3 or Akt (modified from (Xu, Na et al. 2020)).

1.1.5. The PLC pathways

Between the late 1950s and the early 1980s, the Phosphoinositide-specific phospholipase C (PLC) isoenzymes were recognised as key enzymes in agonist-stimulated phosphoinositide metabolism as well as Ca^{2+} signalling (Hokin and Hokin 1953, Michell 1975). PLCs consist of many subdomains and vary in their molecular weight from 85 kDa up to 150 kDa. They comprise a group of enzymes which hydrolyse the polar head group from phospholipids like $PI(4,5)P_2$ generating inositol 1,4,5-phosphate (IP_3) as well as the second messenger diacylglycerol (DAG) (Rebecchi and Pentyala 2000). PLCγ interacts with EGFR (Xie, Chen et al. 2010). In contrast to other PLC family members like PLC-β or PLC-δ, PLCγ contains 2 SH2, a SH3 and a PH domain, enabling it to interact with lipids and proteins alike (Rebecchi and Pentyala 2000). Via its SH2 domains, it binds to RTKs like EGFR, and is phosphorylated by EGFR's kinase domains (Rotin, Honegger et al. 1992). Subsequently, the active PLCγ translocates to the plasma membrane by binding via its PH-domain to PIP_2 (Kim, Wee et al. 2004). This is also true for PIP_3 creating yet another link between the various pathways of EGFR, as PIP_3 is a known product of the PI3K (Bae, Cantley et al. 1998). Next, PLCγ hydrolyses PIP_2 to form two products, DAG and IP_3, as previously mentioned (Rebecchi and Pentyala 2000). These products function as second messengers inducing different kinds of downstream pathways. DAG, which stays bound to the membrane, binds and activates members of the protein kinase C (PKC) family (Patterson, van Rossum et al. 2005). This binding occurs via the "Conserved 1" (C1) domain on PKC, which is a conserved sequence of 50 amino acids and which is shared among all proteins which bind DAG directly (Yang and Kazanietz 2003). The PKC family includes the "conventional PKCs" (cPKC, PKC-α, β, γ), "new PKCs" (nPKC, PKC- δ, ε, η, and θ) and "atypical PKCs" (aPKC, PKC- ι / λ and protein kinase Mζ). Depending on the family member different factors are needed for their activation. On the one hand, DAG as well as Ca^{2+} is needed for activation of cPKCs. On the other hand, nPKCs require DAG but no Ca^{2+}, and aPKCs do not require any of the afore mentioned molecules (Nishizuka 1995, Halet, Tunwell et al. 2004). Activation of the PKCs lead to phosphorylation of a variety of proteins, having different effects depending on the cell type including regulating growth and cell movement, for example via desensitisation to growth or migration by promoting signals by inducing internalisation of the respective receptors (Gauthier, Torretto et al. 2003, Namkung and Sibley 2004). In addition, they are involved in rearrangement of the cytoskeleton or mediating immune responses by, for example, activating T-cells (Larsson 2006, Gupta, Manicassamy et al. 2008, Smith-Garvin, Koretzky et al. 2009). Besides activation of PKCs, DAG also plays a role in activating Ras in lymphocytes via the "Ras guanyl nucleotide-releasing protein 1" (RasGRP1), a Ras exchange factor (Ebinu, Stang et al.

2000). This again demonstrates that not only the PI3K/Akt pathway is connected to the Ras/MAPK pathway, but also the PLCγ/DAG pathway is interlinked with it. The other product of PLCγ's hydrolytic interaction with PIP_2 is IP_3 (Majerus, Connolly et al. 1986). In contrast to DAG which stays membrane bound, IP_3 is a soluble protein which can bind to IP_3- receptors (IP_3R) in the membrane of the endoplasmic reticulum (ER). There, with the help of Ca^{2+}-ions inside the ER it leads to the release of Ca^{2+} -into the cytosol (Berridge 1984, Patel, Joseph et al. 1999). This process occurs due to a conformational change of IP_3R that happens as soon as all for monomers of the IP_3R tetramer are bound by IP_3 (Alzayady, Wang et al. 2016). Interestingly, the function of the intra-ER Ca^{2+} is bimodal, as it promotes the opening of IP_3R when Ca^{2+} levels in the cytosol are low, whereas it inhibits it, as soon as they reach 300 nM (Berridge 2016). This may act as some sort of regulatory mechanism, controlling the efflux of Ca^{2+}. Efflux of Ca^{2+} out of the ER and therefore depletion of Ca^{2+} ions in the ER triggers a mechanism called "store operated Ca^{2+} entry" (SOCE) which, in turn leads to the influx of extracellular Ca^{2+} (Parekh and Putney 2005). Crucial for SOCE are the stromal interaction molecule (STIM) molecules which are detecting the low Ca^{2+}levels in the ER as well as the Orai proteins, which form a pore in the plasma membrane, leading to the influx of Ca^{2+} (Smyth, Hwang et al. 2010). STIM1 is a transmembrane protein localised in the membrane of the ER, its N-terminus reaching into the ER lumen and its C-terminus in the cytosol. Near the N-terminus, STIM1 contains an EF-hand domain, which can bind Ca^{2+} and a sterile-α motif (SAM), a protein interaction domain that allows self-association. On site of the cytoplasmatic C-terminus it contains a pair of coiled-coil domains, a serine/proline rich region and a poly-basic region. In the coiled-coil domains resides the "STIM1-Orai activating region" (SOAR), which is critical for the interaction and activation of the formed SOCE channels (Dziadek and Johnstone 2007, Yuan, Zeng et al. 2009). The Orai family of proteins consists of three proteins (Orai 1, -2 and -3) each of them have the capacity to oligomerize and thus form Ca^{2+} permeable pores in the plasma membrane with Orai-1 being the most important for SOCE, because of its superior conductance rate of Ca^{2+} (Lis, Peinelt et al. 2007). When Ca^{2+} levels in the ER are high, STIM1 is located all over the ER membrane, but as soon as the ER gets depleted of Ca^{2+} due to activation of the IP_3Rs, Stim1 moves to areas of the ER which are closer to the plasma membrane (Luik, Wu et al. 2006). Here the interaction between STIM1 and the Orai proteins can occur subsequently leading to their activation and the influx of extracellular Ca^{2+} into the cell (Wu, Luik et al. 2007). Ca^{2+} is a powerful second messenger controlling many processes inside the cell ranging from muscle contraction, modulating neuronal excitability up to fertilisation and development of oocytes (Homa, Carroll et al. 1993, Berridge 1998, Baylor and Hollingworth 2011, Gilbert and Barresi 2016). It is also interesting to note that there is a crosstalk within the PLCγ pathways. For instance, the

Ca^{2+} released by IP_3 also activates PKCs, which in turn induce their signalling cascades (Siso-Nadal, Fox et al. 2009). A prime example of the importance of this crosstalk of all the above-mentioned signalling pathways is the activation of T-lymphocytes during an immune response. Upon binding to a suitable antigen, the T-cell receptor activates a signalling pathway leading eventually to the activation of PLCγ and the production of IP_3 and DAG. IP_3 leads to the previously discussed increase in cytosolic Ca^{2+} which subsequently, among other, activates the phosphatase calcineurin (CaN) which goes on to dephosphorylate the cytosolic transcription factor NFAT (Sieber and Baumgrass 2009). NFAT then enters the nucleus and binds to its respective site on the DNA on the promotor of the “Interleukin 2” gene (Shaw, Utz et al. 1988). NFAT alone however is not sufficient for activation of the cell, for this it must work together with NF-κB and AP-1 (Eferl and Wagner 2003). NF-κB is a product of the DAG triggered PKC-θ pathway (Isakov and Altman 2002). AP-1 on the other hand is a result of the Ras/MAPK-pathway which also is activated by DAG via RasGRP1 (Roose, Mollenauer et al. 2005).

1.1.6. EGFR in cancer

Now that the most important pathways triggered by EGFR have been discussed, it is obvious that dysregulation can have serious consequences. These consequences are all based on the fact that the pathways described have three predominant functions: Stimulation of cell growth, proliferation and survival. Failure to control EGFR signalling therefore poses a direct risk for cancer development. In the following, we will discuss EGFRs role in the formation and survival of cancer cells.
The control of EGFR signalling starts at the plasma membrane and revolves around the process of endocytosis, which enables the cell to internalise the receptor and thus terminates signalling (Barbieri, Di Fiore et al. 2016, Bergeron, Di Guglielmo et al. 2016). In the so-called “clathrin-mediated endocytosis” (CME) two players are majorly involved. These are adaptor protein 2 (AP2) and the large GTPase dynamin (McMahon and Boucrot 2011, Kirchhausen, Owen et al. 2014). In CME, the receptors are clustered in clathrin coated pits (CCPs), where AP2 determines the vesicles size and dynamin cleaves off the formed vesicles leading to the internalisation of the receptors. Next, the receptors are either degraded or recycled and reinserted in the plasma membrane (Sorkin and Goh 2009). Both proteins not only regulate the process itself but also its timing (Loerke, Mettlen et al. 2009). However, timing is important as it was shown that, for example, in “non-small-cell lung cancer” (NSCLC) CME is aberrantly accelerated and the maturation of the CCP-vesicles is impaired. Consequently, EGFR is rapidly recycled thus increasing Akt signalling and cell survival (Chen, Bendris et al. 2017). Reason for the high recycling rate is the upregulation of dynamin 1 as well as

of clathrin light chain b (CLCb), which not only has been observed in NSCLC but also in breast cancer (Schmid 2017). In contrast to CME, which is activated at low concentrations of EGF and which predominantly leads to the recycling of EGFR, the "non-clathrin endocytosis" (NCE), gets activated at high concentrations and predominantly works as a signal abrogation through promoting degradation of EGFR (Sigismund, Avanzato et al. 2018). To this date, NCE is not completely understood, but it involves the ER making contact to plasma membrane invaginations, in which ubiquitinylated EGFR is localised, and after Ca^{2+} release leads to the internalisation and eventual degradation of the receptor (Caldieri, Barbieri et al. 2017). Taken this into account, evading NCE is one possibility to sustain EGFR signalling. In cancer types overexpressing ErbB family members, like head and neck as well as brain tumours, this is the case (Hynes and MacDonald 2009). Here, the higher concentration of EGFR in the plasma membrane leads in particular to heterodimerisation with the orphan receptor ErB2 or HER2 which is constitutively active. Heterodimers of these receptors are rarely internalised and even when they are, they are predominantly recycled, therefore evading degradation and leading to constant EGFR signalling (Mellman and Yarden 2013). Other examples for the effects of mutations are the disruption of the recruitment E3 ubiquitin ligases which target EGFR for degradation (Roskoski 2014). Besides the intracellular changes, extracellular changes can also lead to a consecutive and ligand independent activation of the receptor. Interestingly, such mutations result in a de- or hypophosphorylation of tyrosine residues on the site where the negative EGFR regulator c-Cbl would normally bind. This missing phosphorylation inhibits binding of c-Cbl, again, leading to the remaining of the receptor on the cell membrane and thus to sustained signalling (Schmidt, Furnari et al. 2003, Han, Zhang et al. 2006, Grandal, Zandi et al. 2007). Skewed trafficking and subsequent continuous signalling of EGFR also affects the metabolism of the cell. In particular, it influences processes involved in the promotion of proliferation and survival (Sigismund, Avanzato et al. 2018). Promotion of proliferation includes upregulation of fatty acid and pyrimidine biosynthesis as well as changes in glucose catabolism (Guo, Prins et al. 2009, Makinoshima, Takita et al. 2014). Upregulation of these pathways occurs through either direct inhibition of rate-limiting enzymes or indirectly through activation of Akt (Makinoshima, Takita et al. 2015, Lim, Li et al. 2016). One of the most famous metabolic changes is the so-called "Warburg effect," which describes an increase in anaerobic glycolysis and of the production of lactate despite the presence of oxygen. Usually, when oxygen is available to the cell, anaerobic glycolysis leads to the production of pyruvate, which subsequently is further processed in the citrate cycle in the mitochondria, however, this is not the case in cancer cells (Vander Heiden, Cantley et al. 2009). In this special metabolic pathway, EGFR on the one hand, through Akt activation, inhibits the endocytosis of the Glucose receptor GLUT1 (Makinoshima,

Takita et al. 2015). On the other hand, through physical interaction SGLT1, a sodium-glucose co-transporter, is stabilised, again increasing the glucose uptake (Weihua, Tsan et al. 2008). In addition, EGF mediated stimulation of EGFR also controls the expression of hexokinase 1 (HK1) and the phosphorylation pyruvate kinase 2 (PKM2), which catalyse crucial steps in anaerobic glycolysis. In breast cancer cell lines, an upregulation of HK1 and a downregulation of PKM2 has been observed by Lim et al. (Lim, Li et al. 2016). An interesting and notable side effect of anaerobic glycolysis and the high production of lactate in those cells, which in this case is thought to be the consequence of the dysregulation of HK1 and PKM2, is that it can supress the cytotoxic function of T-cells, which further increases survival of the cancer. Furthermore, the inhibition of PKM2 also leads to the production of metabolic intermediates like fructose 1,6 bisphosphate which form a positive feedback loop, enhancing EGFR signalling even more (Lim, Li et al. 2016). Staying in the topic of survival: EGFR does not only increase survival of the cells via the elevated production of lactate but also through other mechanisms, namely autophagy. Autophagy is the process where cells remove, mainly under starving conditions, unnecessary or non-functional components, thus serving as a protective mechanism (Klionsky 2008). Here, EGFR is an essential controller. It can either activate or inhibit autophagy. In the presence of sufficient nutrients, ligand-bound EGFR directly phosphorylates Beclin-1 and inhibits the formation of the autophagy complex, as well as indirectly inhibits autophagy by activating the Akt/mTORC1 axis pathway (Wei, Zou et al. 2013, Tan, Lambert et al. 2016). In serum starved conditions however, EGFR promotes autophagy. Unbound receptors are continuously internalised and accumulate in endosomes. These endosomes interact with the Beclin-1 inhibitor Rubicon, freeing Beclin-1 from it and promoting the assembly of the autophagy complex (Tan, Thapa et al. 2015). In cancer, upregulation of autophagy has been observed as a resistance mechanism to EGFR kinase inhibitors (Han, Pan et al. 2011, White 2012). In addition, autophagy in cancer also reduces apoptosis in isolated cancer cells in-vitro and in-vivo, during metastasis, by regulating the tumour microenvironment (DeNardo, Barreto et al. 2009). In addition, activating mutations in the kinase domain activate anti-apoptotic pathways, again by overactivation of Akt. Luckily, this mutation makes the tumour also susceptible to compounds like Gefitinib. It reversibly blocks the ATP binding pocket of EGFR and thus is blocking the EGFR signal (Sordella, Bell et al. 2004). Besides autophagy or upregulation of anti-apoptotic pathways, another way how EGFR mutations influence the survival of cancerous cells is the induction of the expression of the "programmed death ligand 1" (PD-L1) on cells which leads to apoptosis in engaging T-cells. In NSCLC, this happens due to a L858R mutation in exon 21 or exon-19 deletions (Chen, Fang et al. 2015). However, this mutation, which is present in up to 63% of NSCLC patients again increases the patient's response to EGFR tyrosine kinase inhibitors in

comparison to patients who are PD-L1 negative (Chen, Fang et al. 2015, Jiang, Su et al. 2017). Another important function of EGFR in the formation and survival of cancer is angiogenesis. The generation of new vessels is essential to supply the high oxygen needs of a tumour (Folkman 1971). Here, the vascular endothelial growth factor (VEGF) plays an important role (Shibuya 2014). Under physiological conditions, VEGF is part of a system that is responsible for forming new vessels during embryonal development, after injury or exercise and restoring oxygen supply to tissues under hypoxic conditions (Holmes, Roberts et al. 2007, Palmer and Clegg 2014). In the past it has been showed that VEGF, together with other key mediators of angiogenesis is upregulated in human glioma, prostate and head and neck squamous cell carcinoma (HNSCC) upon activation of EGFR with EGF or TGF-α (Goldman, Kim et al. 1993, P, Rhys-Evans et al. 2000, Ravindranath, Wion et al. 2001, Bancroft, Chen et al. 2002). This observation might be explained by the shown link between Ras and upregulation of VEGF, as Ras is one of the main targets of EGFR signalling (Grugel, Finkenzeller et al. 1995, Rak, Mitsuhashi et al. 1995).

In summary, the EGFR is one of the most important players in the development and survival of cells through its numerous downstream signalling pathways. In cancer, many of these pathways are unfortunately hijacked and become uncontrolled, leading to immune evasion, angiogenesis, and uncontrolled growth. However, there is hope, because although some mutations in EGFR can increase the aggressiveness of tumours, they also often make them more susceptible to drugs such as Gefitinib, which can be exploited to tackle this disease. Nevertheless, we are still far from finding a universal solution, because not only EGFR plays a role in the development of cancer.

1.2. The epidermal growth factor (EGF)

The epidermal growth factor (EGF) is the signature ligand of EGFR and is known as one of the most thoroughly studied and biologically potent growth factors (Carpenter and Cohen 1979, Harris, Chung et al. 2003). Its existence has been discovered in the early 1960s during studies on the nerve growth factor (NGF). The experimenters discovered that when they injected new-born mice with extracts of the submaxillary gland the pups precociously opened their eyes as well as erupted their incisors. This was due to a direct stimulation of epidermal growth and keratinisation (Cohen 1962). Isolation of this factor revealed that it is a protein which is not only found in mice but also in humans (Starkey, Cohen et al. 1975). Human EGF is a small, single chained polypeptide which consists of 53 amino acids and has a molecular weight of around 6 kDa (Carpenter and Cohen 1990, Harris, Chung et al. 2003). It also comprises six cysteine residues which form three disulphide bonds, and which are essential for its

biological function, as it was shown that reduction of these bonds render the protein non-functional. The authors explained their findings by arguing that a lack of disulphide bridges leads to a conformational change, inactivating its native function, such as binding to its receptor (Taylor, Mitchell et al. 1972). Even though EGF is the signature ligand of EGFR, there are several other members of the EGF-family of proteins, namely: Transforming growth factor alpha (TGF-α), heparin-binding EGF (HB-EGF), amphiregulin (AREG), betacellulin (BTC), epiregulin (EREG) and epigen (EPGN) (Harris, Chung et al. 2003, Schneider and Wolf 2009). All members of this family are synthesized in a similar manner, as a type one transmembrane protein. They all contain an N-terminal extension, the EGF module, a short juxta membrane stalk, a hydrophobic transmembrane region and a C-terminal, cytoplasmic tail. EGF, being the largest protein of the family comprises nine EGF motifs (Schneider and Wolf 2009). After proteolytic cleavage of the precursor proteins from the cell membrane, the soluble growth factors are released (Harris, Chung et al. 2003). This cleavage is performed by ADAM (a disintegrin and metalloprotease) proteins which are membrane anchored metalloproteases (Sahin, Weskamp et al. 2004). After their release, the growth factors may bind and activate receptors on neighbouring but also on distant cells (Schneider and Wolf 2009). Studies have shown that the interaction between EGF and other members of its family and their cognate receptor happens within the amino-terminal extracellular domain, leading to activation of the receptor (Bajaj, Waterfield et al. 1987, Brown, Debanne et al. 1994, Lemmon, Bu et al. 1997). The C-terminus seems to be responsible for the membrane trafficking, i.e. the insertion of the precursor ligand into the cell membrane and is also involved strengthening pro-proliferation signals by inhibiting cell-cycle regulators like Bcl6 (Fernandez-Larrea, Merlos-Suarez et al. 1999, Higashiyama, Iwabuki et al. 2008). Three different mechanisms of EGF binding and subsequent receptor dimerisation have been proposed in the past (Fig. 4). The first proposed mechanism, the so-called “ligand dimerisation” is a direct interaction between two EGF molecules which form a dimer in an NGF-like manner and bind to the receptor (Fig. 4A) (Wiesmann, Ultsch et al. 1999). “Ligand bivalency”, i.e., the 1:1 interaction between each one ligand molecule with one receptor is the second proposed mechanism (Fig. 4B) (Lemmon, Bu et al. 1997). Here, the bound ligands work as allosteric connectors between the two receptors leading to their dimerisation. This type of dimerisation has already been described for the fibroblast growth factor (FGF) and its receptor FGFR (Plotnikov, Schlessinger et al. 1999). The third proposed mechanism, the so-called “receptor mediated dimerisation,” is the mechanism, which is majorly accepted, and which has been confirmed by crystallographic studies in 2002 (Ogiso, Ishitani et al. 2002). It suggests that the ligand leads to a conformational change in the receptors, exposing their dimerisation sites and eventually to their dimerisation in a 2:2 complex (Fig. 4C) (Ogiso, Ishitani et al. 2002). EGF can be found

in many body fluids like human urine, saliva, milk, plasma, intestinal fluids and several more (Carpenter and Cohen 1979, Read, Upton et al. 1984, Fisher, Salido et al. 1989, Fisher and Lakshmanan 1990, Nair, Warner et al. 2008). Depending on the location of the EGF secretion, EGF fulfils different functions. For example, salivary EGF is responsible for maintaining oro-esophageal and gastric integrity. Its physiological functions include healing of ulcers, inhibition of gastric acid secretion and protection from injurious factors in the gut lumen such as chemicals or bacteria (Venturi and Venturi 2009). In addition to this, EGF taken up via milk is involved in the correct development of the intestinal mucosa (Dvorak 2010). On the other hand, EGF in the blood was shown to be released during the process of blood coagulation and might play a role in vessel repair (Oka and Orth 1983). Taken together EGF and its other family members take on important roles in the development and maintenance of the whole body. For this reason, it is not surprising that several diseases are caused by mutations in this protein family. For example: Already in the 1980s it has been shown that EGF overexpression and thus overstimulation of EGFR can lead to cancer (Stoscheck and King 1986). However, more recent studies indicated that loss-of function mutations in the pro-EGF gene can lead to “Isolated autosomal recessive hypomagnesemia” (IRH), a disease which is characterized by magnesium loss because of defects in renal reuptake (Knoers 2009). Deficiencies in TGF-α, the second most prominent ligand for EGFR, can lead to hair follicle and eyelid closure defects and the absence of HBEGF even leads to postnatal death due to heart valve malformations and hypoplastic lungs (Iwamoto, Yamazaki et al. 2003, Jackson, Qiu et al. 2003). All in all, understanding the “modus operandi” of growth factors such as EGF is essential for broadening our knowledge of developmental and proliferative diseases.

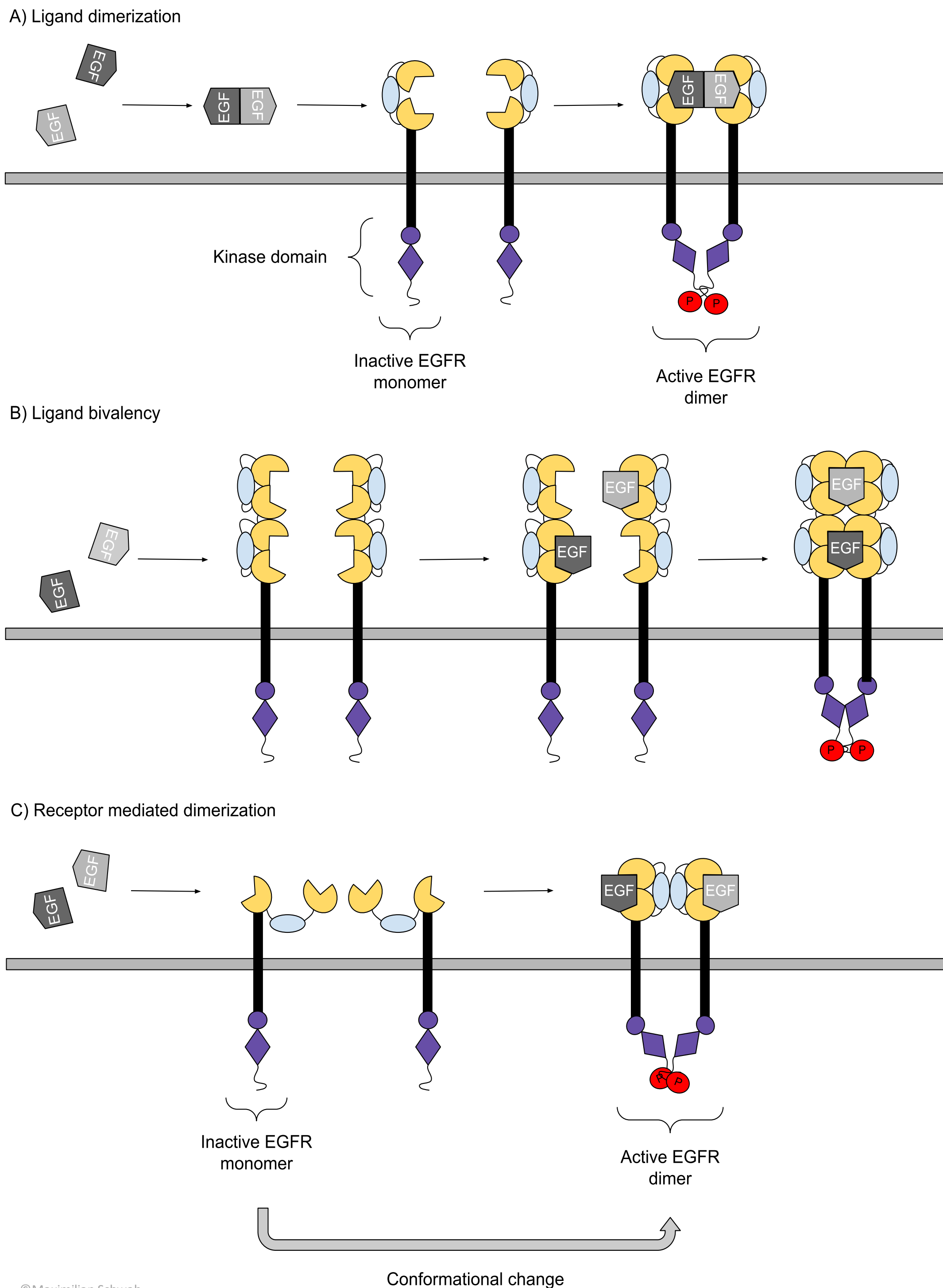

Figure 4: Proposed EGF-to-EGFR binding mechanisms. **A:** Ligand dimerisation. EGF monomers dimerize and bind to inactive EGFR monomers. Subsequent dimerisation of EGFR activates downstream signals. **B:** Ligand bivalency: One EGF monomer binds to one EGFR molecule. Two 1:1 paired complexes interact and form an active dimer. **C:** Receptor mediated dimerisation. EGF molecules bind to inactive folded EGFR molecules and elicit a conformational change in the receptors. The two ligand bound receptors now interact via receptor-receptor binding (Based on (Ogiso, Ishitani et al. 2002)).

1.3. Disabled homolog 1 (Dab1)

Disabled homolog 1 or Dab1 is a 82 kDa large cytosolic glycoprotein first discovered in mice and is mainly expressed in the brain and gastrointestinal tract (Garcia-Miranda, Peral et al. 2010, Vazquez-Carretero, Garcia-Miranda et al. 2014). It functions as an intracellular adapterprotein for receptor tyrosine kinases and is most famously known for its role in the Reelin signalling pathway, where it interacts with the “Apolipoprotein E receptor 2” (ApoER2) and the “Very Low Density Lipoprotein receptor” (VLDLR). This pathway is crucial for the correct layering of the cortex. Without a properly functioning Dab1, the so-called “Reeler-phenotype” is observed which is characterized by severe motor deficits, mental retardation and abnormal layering of many laminated structures in the brain (Lambert de Rouvroit and Goffinet 1998, Tissir and Goffinet 2003, Frotscher 2010). Dab1 binds to NPxY motifs on receptor tyrosine kinases (RTKs) and other non-RTK transmembrane proteins like ApoER2 and VLDLR, which leads to its phosphorylation by Src-family kinases such as Fyn and Src on specifc tyrosine residues (Arnaud, Ballif et al. 2003, Bock and Herz 2003). The phosphorylated and now active Dab1 then recruits other donwstream proteins, such as CrK-Ligand (CrKL) of the proto-oncogene c-Crk (Crk) family or PI3K. Subsequently, signalling cascades including the PKB/Akt and the CrkL/Rap1 pathways are activated (Beffert, Morfini et al. 2002, Ballif, Arnaud et al. 2004). Activation of the CrkL/Rap1 pathway plays a crucial role in the proper positioning of neurons during embryonic development directly by the CrkL as well as the CrkL mediated activation of C3G, an guanine nucleotide exchange factor (GEF) for Rap1 (Gotoh, Hattori et al. 1995). Apart from the reeler phenotype two other neuonal conditions have been shown to stand in relation with mutations in the *DAB1* gene, being schizophrenia (SCZ) and autism spectrum disorder (ASD), where two point mutations lead to an impaired Dab1 (Nawa, Kimura et al. 2020). Since the development of a healthy brain requires not only proper layering of the neurons but also a correct vascular system, it is intriguing that Dab1, which is already involved in the former, also plays a role in the latter. In a recent study by Marta Segarra et al., it was shown that the Reelin/Dab1 signalling, this time mediated by ApoER2 and the vascular endothelial growth factor receptor 2 (VEGFR2) which is also present in endothelial cells, ensures this (Segarra, Aburto et al. 2018). The elicited signal due to the interaction of Dab1 with the two receptors exerts strong postangiogenic effects and ensures proper development of the CNS vasculature. This is achieved through controlling endothelial proliferation and regulating active filopodia extension of the forming vasculature (Segarra, Aburto et al. 2018). Dab1 is mostly expressed in the brain, but over the last years more and more evidence evolved indicating that it is also present in peripheral tissues (Howell, Gertler et al. 1997, McAvoy, Zhu et al. 2008, Khialeeva and Carpenter 2017). One of those tissues is the mammary gland. Here,

Dab1 is expressed by the luminal cells in the adult mammary ducts. In Dab1 knockout mice, the mammary ducts show many ductal tree abnormalities, such as chaotic branching patterns, disorganized cellular walls and increased proliferation of ductal cells (Khialeeva, Lane et al. 2011). Another important tissue where Dab1 is found is the small intestine. The intestinal epithelial tissue consists of two compartments: The crypts and the villi. The crypt cells contain highly proliferating cells, while cells that are no longer proliferating are mainly found in the villi. At the crypt villus junction, the proliferation of the cells stops and cells either move to the villi, differntiating into enterocytes, endocrine and goblet cells or downward to the base of the crypts differentiating into paneth cells (Garcia-Miranda, Vazquez-Carretero et al. 2012). Dab1, but not Reelin, is expressed in crypt cells, whereas ApoER2, together with the VLDLR, are only present at the tip of the villi (Garcia-Miranda, Vazquez-Carretero et al. 2012). Interestingly, the expression levels of Dab1 in the intestine are at the same level as in the brain (Sato, Fukushima et al. 2006, Garcia-Miranda, Peral et al. 2010). Yet, the Dab1 present in the intestine has a lower relative molecular mass, weighing only 63 kDa compared to the 83 kDa of the variant expressed in the brain (Vazquez-Carretero, Garcia-Miranda et al. 2014). Since crypt cells are the driving force of proliferation in the intestine, they are thought to be responsible for the cellular homeostastis and high turnover rate of intestinal epithelial cells (Garcia-Miranda, Vazquez-Carretero et al. 2012). Along this line, it has been shown in the past that in Dab1 knockout mice the turnover time of epithelial cells is significantly reduced, which also affects the morphology of the vili. This, taken together with the high Dab1 levels in the intestine, leads to the assumption that Dab1 has a central role in the proliferation of small intestinal epithelial cells (Vazquez-Carretero, Garcia-Miranda et al. 2014). The conclusion that Dab1 takes on functions that lead to increased proliferation is also supported by the fact that it has been shown to be involved in the growth and metastasis of tumours (Khialeeva, Chou et al. 2017). Of particular interest in this respect is Dab1's role in the Notch-Dab1-Abl axis. Studies have shown that the Notch pathway is hyperactive in patients with colorectal cancer (Pandurangan, Divya et al. 2018). This pathway is essential in determining cell fate and is essential for the survival of intestinal stem cells (Ghorbaninejad, Heydari et al. 2019). Sonoshita and others were able to show that Dab1 is also part of the Notch pathway in the so-called Notch-Dab1-Abl axis (Sonoshita, Itatani et al. 2015). After Notch receptors become active in intestinal crypt cells, Dab1 is phosphorylated by the Abl tyrosine kinase, which leads to a positive feedback loop resulting in increased Abl activity. Abl in turn promotes cell migration and proliferation, which in case of dysregulation leads to colorectal tumours or even metastases (Sossey-Alaoui, Li et al. 2007, Sonoshita, Itatani et al. 2015). In line, knockout of Dab1 and inhibition of Abl led to suppression of cancer invasiveness and prevented the formation of metastases. Yet, Dab1 is not only involved in colorectal

cancer (Sonoshita, Itatani et al. 2015). Recently a study was published on Dab1 in relation to pediatric acute T-lymphoblastic leukaemia (T-ALL) (Erarslan-Uysal, Kunz et al. 2020). The study found that T-ALL cells resemble in their gene expression pattern that of of immature T-lymphocytes and that Dab1 is not only present but also overexpressed in those cells. In conclusion, Dab1 is a versatile protein that is involved in the development of tissues such as the brain, breast and vascular system, as well as playing an important role in neuronal and oncological diseases, as described in the examples of colorectal cancer.

1.4. The Apolipoprotein E receptor 2 (ApoER2)

The Apolipoprotein E receptor 2 (ApoER2) or Low-density lipoprotein receptor-related protein 8 (LRP8) belongs to the low-density lipoprotein (LDL) receptor family together with the Low- and very-low-density lipoprotein receptors (LDLR and VLDLR) which all share a common ancestor gene (Fig. 5) (Kim, Iijima et al. 1996, Dlugosz and Nimpf 2018). It has a size of 105 kDa, consists of 963 amino acids and is most preferently expressed in the CNS However, ApoER2 can be found in non-neuronal tissues like placenta, testis, ovary, platelets and in the vili of the small intestine as well (Kim, Iijima et al. 1996, Lutters, Derksen et al. 2003, Garcia-Miranda, Vazquez-Carretero et al. 2012, Dlugosz and Nimpf 2018). One of its most prominent roles is to ensure the correct layering of the cortex, where it mediates proper positioning of the neurons during development (Dlugosz and Nimpf 2018). The structure of all LDLR family proteins comprises six modules: A ligand binding domain at the N-terminus which contains a variable number of LDLR type A repeats, a constant amount of three EGF-precursor-like repeats (type B), a YWTD-β-propeller, an O-linked sugar domain, a transmembrane domain, and most importantly a cytoplasmic domain containing an NPXY-motif. This motif is responsible for correct trafficking and the mediation of endocytosis of the receptor as well as offering a binding site for adapters (Tissir and Goffinet 2003). Extracellular ligands of ApoER2 include among others the Apolipoprotein E (ApoE), the glycoprotein Reelin, Clusterin and Selenoprotein P (Sepp1) (Burk, Hill et al. 2014). ApoE being involved in the uptake of cholesterol in astrocytes and neurons, whereas Reelin, as discussed above, is important for the layering of the Cortex (Wang, Kulas et al. 2021). Clusterin which has also been implacted to play a role in Alzheimer’s disease and cancer also signals through the Reelin pathway and stimulates the proliferation of neurons in the subventricular zone of the cortex and enables neuronal outgrowth (Leeb, Eresheim et al. 2014, Dlugosz and Nimpf 2018). Sepp1 is mediating uptake of the important micronutrient selenium in different tissues (Hill, Zhou et al. 2007). Deficieny of selenium leads to severe

neurological impairments and in male indiviuals to hypofertility and structural defects in sperm (Burk, Hill et al. 2007, Olson, Winfrey et al. 2007). In this context, ApoER2 as the major receptor of Sepp1 facillitates its endocytosis, and thus ensures the supply of selenium to the cells (Hill, Zhou et al. 2003). Looking on the inside of the cell, the intracellular adapters of ApoER2 are just as versatile. They include JNK-interacting Proteins (JIP) 1 and 2, the postsynaptic density protein 95 (PSD-95) and of course Dab1 (Gotthardt, Trommsdorff et al. 2000, Stockinger, Brandes et al. 2000, Divekar, Burrell et al. 2014). JIP1 and 2 serve as a molecular scaffold for the Janus kinase (JNK) which siganal cascade is essential for cell proliferation, differentiation, migration and apoptosis (Stockinger, Brandes et al. 2000). With the help of PSD-95, ApoER2 can intercact with N-Methyl-D-Aspartate (NMDA) receptors and enables to cluster and thus stabilises them (Divekar, Burrell et al. 2014). Along these lines, active Dab1 leads to an enhanced phosphorylation of the NMDAR subunits NR2A and NR2B in turn increasing its conductivity. In the follwing, activation of the cAMP response element-binding (CREB) protein, a transcription factor, mediates the transcription of memory and learning associated genes, eventually leading to long term potentiation (LTP) (Weeber, Beffert et al. 2002, Chen, Beffert et al. 2005).

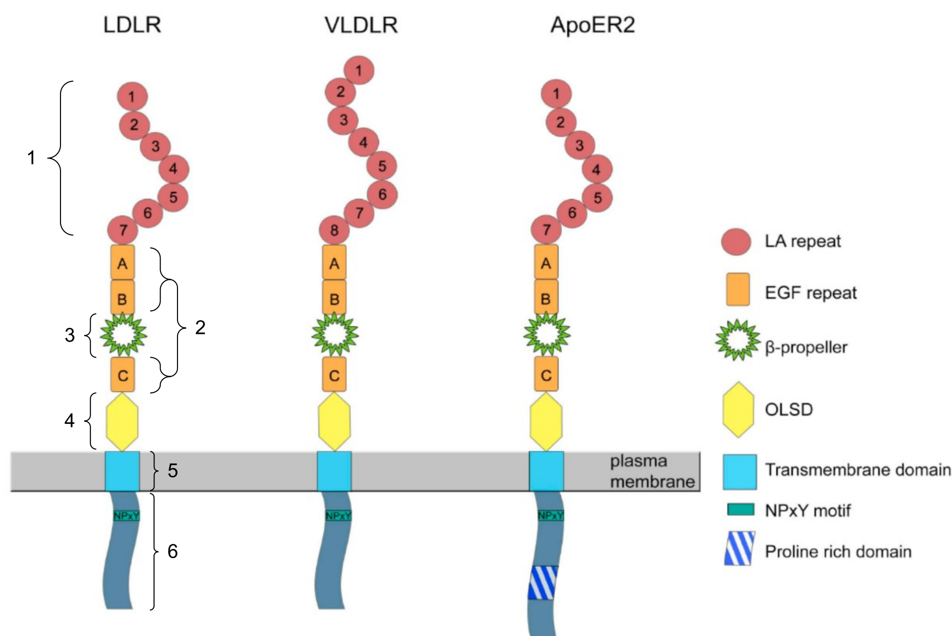

Figure 5: Receptors of the Low-density lipoprotein receptor-related protein family. Display of the molecular structures of LDLR (l),VLDLR (m) and ApoER2 (r). All of the receptors consist of six structural modules. **1)** The ligand binding domain consisting of a variable amount of LDL receptor type A repeats (LA repeat). **2)** Epidermal growth factor precursor-like repeats A, B and C (EGF repeat). **3)** YWTD or β-propeller domain. **4)** *O*-linked sugar domain. **5)** Transmembrane domain. **6)** Cytplasmic domain, varying in size containing the NPxY motif and may or may not contain a proline rich domain. Modified from (Dlugosz and Nimpf 2018).

1.5. Aims of this study

EGFR is one of the most prominent receptors involved in proliferation and survival of cells and often hyperactive or overexpressed in various cancer types ranging from NSCLC, to breast cancer, to colorectal cancer (Markman, Javier Ramos et al. 2010). It has therefore long been the focus of many research groups worldwide. On the other hand, Dab1 has not yet received as much attention in this field because it is mainly associated with the Reelin pathway and thus neuronal developmental biology. However, as discussed above, Dab1 serves also functions independently of Reelin, so our interest was raised. In particular, we became aware of Dab1's role in proliferation and cell survival, features often involving EGFR. The fact that ApoER2, the signature receptor of Dab1, has already been shown to interact with other receptors such as VEGFR2 further strengthened this interest.

For that reason, we addressed the question of whether Dab1 can also serve as an adaptor protein for receptors other than ApoER2, with a particular focus on the epidermal growth factor receptor, EGFR, due to its involvement in apparently similar processes. Both Dab1 and EGFR are over-represented in cancer, play a role in cell migration and are known to have many binding partners. Furthermore, we wanted to determine whether Dab1 can be activated by EGFR. In this way we want to determine possible interactions between these two highly interesting proteins since this could mean the discovery of completely new pathways.

2. Materials and Methods

Parts of the Materials and Methods used in this study have been published already in (Dlugosz, Teufl et al. 2021).

2.1. Cell lines and transfection

HEK293 (Human embryonic kidney cells 293, ATCC) and HEK293T (Human embryonic kidney cells 293, ATCC CRL-3216) were cultivated in Dulbecco's modified Eagle's medium (DMEM; Gibco) supplemented with 10% foetal calf serum (FCS) (Sigma) at 37 °C and 5% CO2. After 24 h of cultivation, HEK293 cells were transfected using PEI (polyethyleneimine) and the indicated constructs as described in (Tom, Bisson et al. 2008) whereas HEK293T cells were transfected utilizing the jetPRIME® transfection kit from Polyplus transfection® accordingly to the manufacturer's protocol.

Table 1: Used constructs for transfection of HEK293 or HEK293T cells

Name of the plasmid	Vector	Content
pCIneo_humanEGFR	pCIneo	Human EGFR
pmCherry_N1_humanEGFR	pmCherry_N1	pmCherry C-terminal tagged human EGFR
pmCherry_N1_mmApoER2	pmCherry_N1	pmCherry C-terminal tagged murine ApoER2
pCIneo_mmApoER2	pCIneo	Murine ApoER2
pHom_1_mmDab1_mGFP	pHomMem1	mGFP C-terminal tagged murine Dab1
pDSred_Dab1_555	pDSred	Human Dab1
pBABE_puro_EGFR_WT	pBABE_puro	Human EGFR (Used for HEK293T)
pBABE_puro_ K721A-EGFR	pBABE_puro	Kinase-dead Human EGFR (Used for HEK293T)
pmGFP	pHomMem1	mGFP
pmCherry_N1	pmCherry_N1	mCherry

pBABE_puro_EGFR_WT and pBABE_puro_ K721A-EGFR were a kind gift of Maria Sibilia (Medical University of Vienna) and pDSred_Dab1_555 (with stop codon before RFP ORF) was a kind gift of Brian W. Howell (Upstate Medical University).

2.2. Preparation of Reelin conditioned medium (RCM)

Reelin-expressing HEK293 cells were cultivated and used for production of Reelin conditioned medium (RCM) as explained in (Brandes, Kahr et al. 2001). The HEK293 cells stably carrying the full-length mouse Reelin expression construct pCrl (a kind gift of Tom Curran, Perelman School of Medicine at the University of Pennsylvania, Philadelphia, PA, USA) were cultivated in DMEM + GlutaMax (Thermo Scientific) supplemented with 10% foetal calf serum (Sigma) and 0.5 mg/mL G418 (Roth) at 37 °C and 5% CO2. As soon as the cells reached 70% confluency the culture medium was replaced by serum-free medium (OptiMEM, Gibco). After 48h the conditioned medium was collected, sterile filtered and used for the experiments.

2.3. EGF, RCM and inhibitor treatment

Cells were washed and incubated in starvation medium (referred to in the following as imaging medium or IM) consisting of 4 mM Glutamine in Hank's Balanced Salt solution (HBSS) for 30 min at 37 °C + 5% CO2. Subsequently, the dishes were inoculated with 10 ng/mL of human EGF (PHG0315, Thermo Scientific, Waltham, MA, USA) and were incubated for 3, 10, or 20 min at 37 °C + 5% CO2. One dish was left untreated and served as negative control. In case of the EGFR inhibitor treatment, 1 µM Neratinib (MCE, Cat. No.: HY-32721) or 1 µM Gefitinib/Iressa (MCE, Cat. No.: HY-50895) was added 2 h preceding starvation. During starvation and EGF treatment, again 1 µM of the inhibitors was added and the cells incubated at 37 °C + 5% CO2. Then, EGF treatment was conducted as described above. In case of the inhibition of Src-family kinases, cells were incubated with 9 µM of PP2 (Cat. Number: AG-CR1-3563, Lot No. A00105, AdipoGen, Switzerland) 30 min prior and during the 30 min of starvation. For control conditions the same amount of DMSO was added at the same time points. After the starvation, plates where either incubated with IM - as a negative control - or 10 ng/ml EGF for 3 min or 2 mL of RCM for 20 min. All media were either supplemented with 9 µM of PP2 or DMSO.

2.4. Lysis, SDS-PAGE, Western Blotting, Immunoprecipitation (IP)

Cell extracts were prepared in NP-40 lysis buffer (150 mM NaCl, 1.0% Nonidet P-40, 10% glycerol, 20 mM Tris, pH 7.4) containing complete™ EDTA-free protease inhibitor cocktail (Roche), 0.05 mM NaF, 1 mM Na_3VO_4 and 1 mM EDTA and were immediately used for SDS-PAGE or for immunoprecipitation. Extracts containing 400–500 µg of

protein were incubated for 1 h at 4 °C with anti-Dab1 (Ab 54), anti-EGFR, anti-ApoER2 (Ab 186) or an unspecific anti-mouse/anti-rabbit IgG antibody. Subsequently they were incubated for 1 h with Protein A Sepharose 4B (Invitrogen, Carlsbad, CA, USA). Beads were centrifuged at 500× g for 1 min at 4 °C, collected and washed three times using NP-40 lysis buffer supplemented with protease inhibitor cocktail, 1 mM EDTA, 0.05 mM NaF and 1 mM Na_3VO_4. Bound proteins were eluted with 60 µL of 4× SDS protein sample buffer, followed by boiling of the samples for 3 min at 96 °C. The samples were centrifuged for 5 min at 17.000× g at room temperature and the supernatant was collected. Proteins were separated by reducing SDS-PAGE and transferred onto Amersham™ Protran nitrocellulose membrane (GE Healthcare, Chicago, IL, USA) by wet blotting. Membranes were blocked in PBS or TBS containing 0.1% Tween-20 and 5% bovine serum albumin (BSA) and incubated with primary antibody over night at 4 °C. Membranes were washed and subsequently incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies (Jackson ImmunoResearch Laboratories, West Grove, PA, USA). For analysis, enhanced chemiluminescence solutions, NOVA 2.0 and Supernova (Cyanogen, Bologna, Italy), were used. Blots were stripped by inoculating the blots for 30 min in a stripping buffer consisting of 25 mM Glycine (pH = 2) with 2% SDS. After three 3 washing steps in PBS or TBS + 0.1% Tween-20, blocked in 5% BSA, PBS/TBS+ 0.1% Tween the blots were incubated with the primary antibody over night at 4 °C. After washing membranes again 3 times with PBS or TBS + 0.1% Tween-20 the blots were incubated with HRP-conjugated secondary antibodies (Jackson ImmunoResearch Laboratories, West Grove, PA, USA).

2.5. Microscopy

HEK293 WT cells were cultivated on a 24 well plate with 12 mm sterile glass coverslips added and coated with 50 µg/mL collagen I (A10483-01, Gibco). After 24 h, when cells were 50–70% confluent, they were transfected with, ApoER2_mCherry, EGFR_mCherry, Dab1_mGFP, mGFP or pmCherry by using PEI reagent. After 24 h cells were incubated for 30 min with IM. Cells were washed with PBS and fixed with 4% formaldehyde for 15 min at RT in darkness after which the cells were washed three times with PBS and stained with DAPI (4′,6-diamidino-2-phenylindole, 5 µg/mL) for 5 min. Subsequent to another washing step with PBS the cells were quenched with glycine (100 mM) for 15 min. They were washed twice with H_2O and mounted with 3 µL DAKO fluorescent mounting medium (DAKO S3023, Lot 10067534) and sealed with nail polish. Pictures were obtained with a Laser Scanning Confocal microscope (LSM 700, ZEISS) using ZEN software.

2.6. Antibodies

In this study, the following antibodies have been used at the indicated concentrations (Table 2).

Table 2: Used antibodies, catalogue numbers, companies of origin, dilutions, and type of application. WB: Western blotting. IP: immunoprecipitation

Epitope	Catalogue number	Company	Dilution/Use
pEGFR	sc-12351	Santa Cruz Biotechnology (Dallas, TX, USA)	WB 1:1000
EGFR	sc-373746	Santa Cruz Biotechnology (Dallas, TX, USA)	WB 1:1000, IP
ApoER2	Ab 20	Produced in the lab	WB 1:5000
p-Tyr (PY99)	sc-7020	Santa Cruz Biotechnology (Dallas, TX, USA)	WB 1:500
D4 Dab1	N/A	Kind gift from André Goffinet (UCLouvain)	WB 1:8000
D4 Dab1	Ab54	Produced in the lab	IP
GAPDH	G8795	Sigma-Aldrich (St. Louis, MO, USA)	WB 1:10,000
Normal mouse IgG	12-370	Merck Millipore (Burlington, MA, USA)	IP
Normal rabbit IgG	sc-2025	Santa Cruz Biotechnology (Dallas, TX, USA)	IP

2.7. Statistical analysis

All statistical tests were performed using GraphPad Prism version 8. Preceding the analysis, normal distribution of the data was checked by the Shapiro–Wilk normality test. To compare two unpaired sample groups, two-tailed Student t-test was performed. To compare more than two sample groups one way ANOVA multiple comparison test was used. Results were considered significant when * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$ and **** $p \leq 0.0001$. Graphs are presented as mean ± standard deviation. ImageLab software (Bio-Rad) was used to quantify the signal intensity of Western blot bands.

3. Results

In the following I will present the results of this thesis. Parts of these results have been published already in (Dlugosz, Teufl et al. 2021).

3.1. Sequence alignment of EGFR, ApoER2 and VLDLR

In order to test our hypothesis that Dab1 is able to bind other receptors like EGFR, we first investigated by sequence alignments whether such a theoretical possibility exists. From the literature it is known that Dab1 uses NPxY sites on ApoER2 or VLDLR to bind to them. If EGFR also has these binding sites, this will provide a theoretical basis for our hypothesis. Thus, we performed a sequence alignment analysis. We aligned the amino acid sequence of the intracellular domain of human and murine EGFR, ApoER2 and VLDLR and specifically searched for NPxY sites. Our analysis revealed three NPxY sites in the sequence of EGFR, one of which is shown in Fig. 6. The site indicated in Fig. 6 is located in the human EGFR at position 501-504, while the other two sites are located at the positions 439-402 and 466-469. In the murine EGFR, the site shown in Fig. 6, is located at position 499-502 and the other two are located at the positions 437-440 and 464-467.

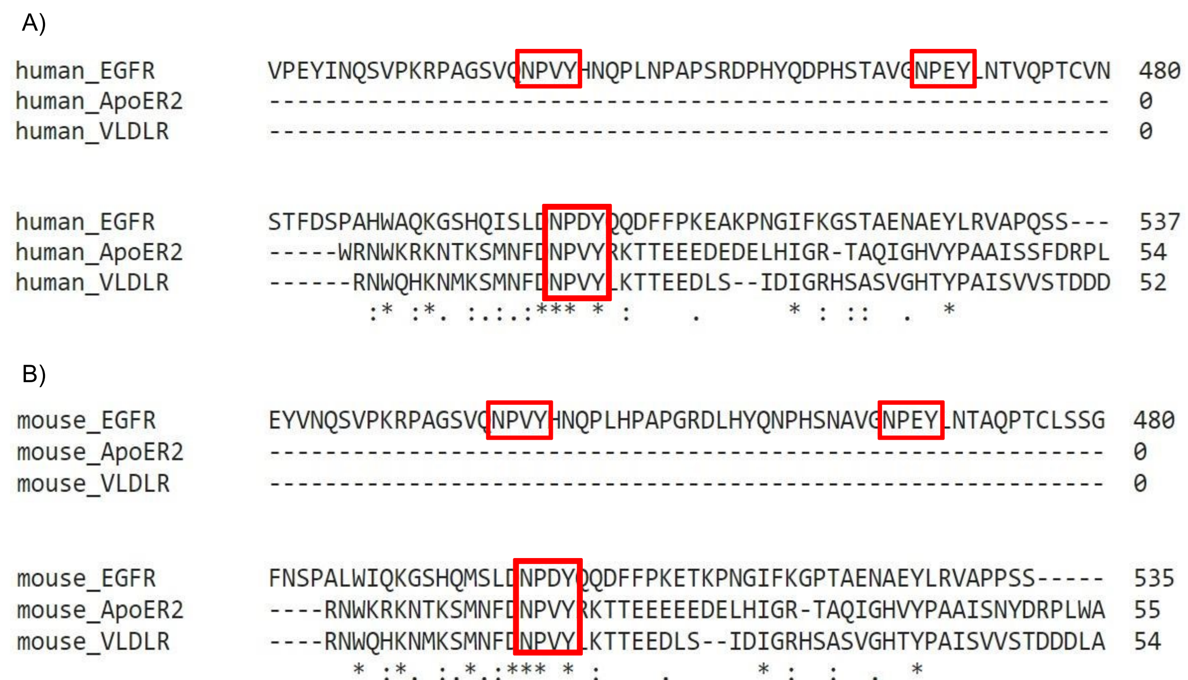

Figure 6: Amino acid sequence alignment of human (A) and murine (B) epidermal growth factor receptor (EGFR), apolipoprotein E receptor 2 (ApoER2) and very low-density lipoprotein receptor (VLDLR). The shared NPxY motif is indicated by the red frame. Sequence alignment was created using Clustal Omega ® tools. Residues which are identical in all alignments are indicated by an asterisk, conserved substitutions are represented by a colon and semi-conserved substitutions are indicated by a dot.

3.2. Co-localisation of EGFR and Dab1

Now that we determined that there are potential binding sites for Dab1 in EGFR, we wanted to verify this with experimental data. For this purpose, we performed a co-immunoprecipitation (co-IP) on HEK293 cell extracts derived from cells transfected with EGFR and Dab1. After pulldown using a specific EGFR antibody, we were able to detect Dab1 in the precipitate by immunoblotting using an antibody specific for Dab1 (Fig. 7, upper panel, lane 2) as well as EGFR after the blot was stripped and re-probed with the same specific antibody for the receptor, serving as a pulldown control (Fig. 7, lower panel, lane 2). In the control lane depicting a pulldown with a non-specific antibody, neither Dab1 nor EGFR were present, which indicates the specificity of the co-IP (Fig. 7, lane 1). The second band visible above the EGFR signal (Fig. 7, lower panel, lane 2) represents a different glycosylation form of EGFR. Yet, the identity of the band above the Dab1 signal in the upper panel is not clear, since it does not appear in the input lane (Fig. 7, lane 3).

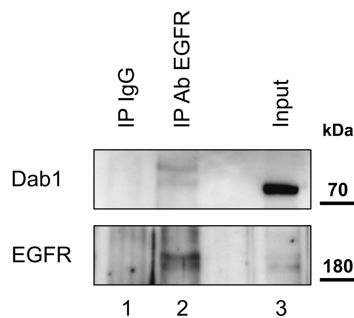

Figure 7: Co-immunoprecipitation of EGFR and Dab1. Immunoblot analysis of total protein extracts of HEK293 cells expressing EGFR and Dab1 (Lane 3, input), which were subjected to immunoprecipitation using a specific antibody against EGFR (Lane 2) or an unrelated mouse IgG antibody (Lane 1). The upper panel shows the Dab1 levels detected by a specific Dab1 antibody (Ab D4). The blot was stripped and re-probed with a specific antibody for EGFR (lower panel) as a pulldown control.

To assure that the observed signal indeed indicates an interaction between the two proteins, we checked the possible co-localisation of EGFR and Dab1 in a second experiment. For this purpose, we again employed HEK293 cells that expressed both proteins tagged with fluorophores (EGFR-mCherry and Dab1-mGFP) and analysed their expression patterns via confocal microscopy. If the two proteins indeed interact with each other as suggested by the results of the Co-IP experiment, we should be able to see a co-localisation of the two proteins, represented by a yellow or orange signal. As EGFR is a transmembrane protein, we expected it to be predominantly expressed at the cell membrane, whereas Dab1 should not only be visible at the cell membrane, but also in the cytoplasm. These expectations were confirmed as demonstrated in Fig. 8 (first row). EGFR indeed is clearly visible at the cell membrane and the signal derived from the fluorescently tagged Dab1 is distributed among the interior of the cell (Fig. 8A and B). In addition, the overlay image of the red EGFR and the green Dab1 staining suggests co-localisation of EGFR and Dab1 at the cell membrane (Fig. 8C). Due to the co-localisation of the soluble fluorescent tags alone in the cytoplasm (Fig 8M/N), the co-localisation of EGFR and Dab1 at the membrane is suggested to be specific and independent of the fluorescent proteins (Fig. 8C). Cells

expressing EGFR-Cherry and mGFP do not show any co-localisation signals at the cell membrane or in the filopodia-like extensions (Fig 8D/E/F). In line, cells expressing Dab1-mGFP and mCherry do not indicate any fluorescent protein dependent co-localisation signals either. Dab1-GFP is evenly distributed inside the cytoplasm, while not being accumulated at the cell membrane (Fig. 8H). To further elucidate the mechanism of co-localisation, we analysed the signal distribution in cells expressing ApoER2 and Dab1 (Fig 8J, K and L). Here we see the co-localisation of Dab1 and ApoER2 described in the literature, which is similar to the one observed in cells expressing EGFR and Dab1 (Fig 8C). Therefore, we can conclude that Dab1 seems to interact with EGFR in a similar manner to its interaction with ApoER2.

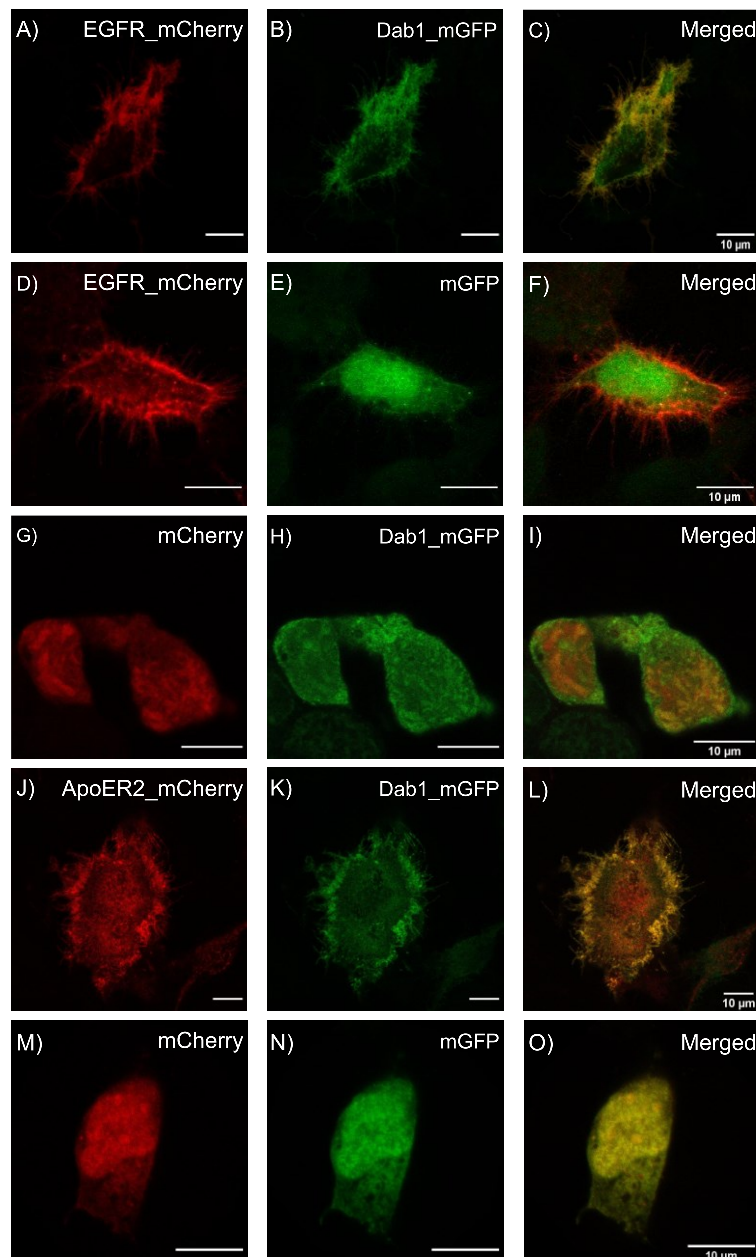

Figure 8: **Confocal microscopy of HEK293 cells** expressing fluorescently tagged versions of EGFR (A/D), ApoER2 (J) and Dab1 (B/H/K). As controls, cells expressing the fluorophores, mCherry or mGFP alone were analysed (E/G/M/N). Merged Images (C/F/I/L/O). Red: mCherry or mCherry tagged proteins. Green: mGFP or GFP tagged proteins. Yellow: Overlaying structures. Scale bar represents 10 µm.

3.3. Phosphorylation of Dab1 upon EGFR activation

Having demonstrated that EGFR and Dab1 interact at the cell membrane, we asked ourselves whether this interaction might be part a new signalling pathway where activation of EGFR would result in Dab1-phosphorylation. Therefore, we transfected HEK293 cells again with EGFR and Dab1 and treated the cells with EGF, the ligand activating EGFR. The cells were lysed and subjected to immunoprecipitation (IP) using an antibody specific for Dab1 (Ab 54). Phosphorylation of Dab1 was tested by Western blotting using an antibody specific for phosphorylated tyrosine residues (Ab PY99).

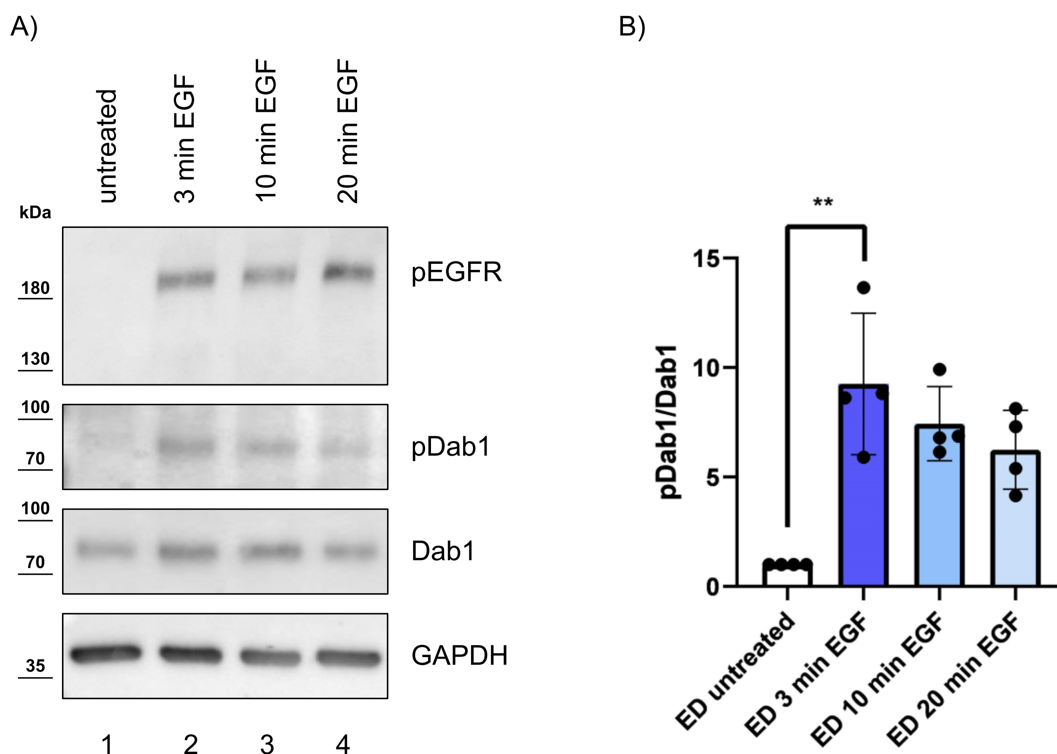

Figure 9: EGF induced Dab1 phosphorylation. A) Immunoblot analysis of HEK293 cells expressing EGFR (E) and Dab1 (D). Cells were either left untreated (Lane 1) or treated with [10 ng/ml] human EGF for 3 (Lane 2), 10 (Lane 3) or 20 min (Lane 4) and subsequently lysed and the resulting cell extracts subjected to immunoprecipitation using a specific antibody for Dab1 (Ab 54). Phosphorylation levels of EGFR (panel 1) and Dab1 (panel 2) were detected by using antibodies specific for EGFR (Ab Y1173) or phosphotyrosines (Ab PY99). Blots were re-probed using an antibody specific for Dab1 to determine Dab1 levels. As a loading control glycerinaldehyd-3-phosphat-dehydrogenase (GAPDH) was used. **B)** Relative phosphorylation levels of Dab1 of four independent experiments. Signal intensity of anti-phosphotyrosine was normalised to the signal of anti-Dab1 and relative phosphorylation levels of the untreated control set to 1. Data were analysed using an unpaired, two-tailed t-test. * $p \leq 0.05$, ** $p \leq 0.01$, ns, not significant; dotes, number of experiments. Error bars represent standard deviation.

Indeed, phosphorylation of Dab1 was observable after a 3 min treatment with EGF as well as after 10 and 20 min (Fig. 9A, second panel). The phosphorylation of EGFR (9A, first panel) also indicated that EGFR had been activated. In order to semi-quantify these data, the blot was stripped and re-probed for Dab1 to determine the Dab1 levels (Ab D4). The ratio of the signal intensity from phosphorylated Dab1 (Fig. 9A, second panel) to total Dab1 (Fig. 9A, third panel) was subsequently calculated and the results blotted (Fig. 9B). EGF stimulation of the HEK293 cells results already after 3 min in a significant increase in phosphorylated Dab1 (Fig. 9B). After 10 and 20 min, phosphorylated Dab1 is still detectable, yet to a lower extent compared to the 3 min time point after stimulation, suggesting that the peak phosphorylation occurred 3 min after the initial stimulus.

3.4. Chemical and genetic inhibition of EGFR abolishes Dab1 phosphorylation

Due to the fact that EGF stimulation did not only activate EGFR, but was also accompagnied by phosphorylation of Dab1, the next step was to unravel this phosphorylation mechanism. Therefore, EGFR inhibition was considered in order to evaluate whether Dab1 phosphorylation was dependent upon EGFR phosphorylation. For that, HEK293 cells were transfected with plasmids coding for EGFR and Dab1. In contrast to the previous experiment, one hour prior transfection, the cells were treated with two different EGFR inhibitors, Gefitinib and Neratinib. Both inhibitors block the ATP-binding pocket of EGFR and inhibit the auto-phosphorylation of the receptor upon EGF-induced activation, and thus, the signaling pathways downstream of EGFR activation. The expected inhibition is demonstrated in Fig. 10A, third panel, lanes 3 and 4 , as no phosphorylated EGFR was detectable upon treatment of the cells with EGF. Interestingly, also the Dab1-specific anti-phosphotyrosine signal which correlates to the amount of phosphorylated Dab1 was also absent, (Fig. 10A, first panel). Even though Dab1 levels were stable in all conditions (Fig. 10A, second panel), only the untreated cells show Dab1 phosphorylation. This indicates that the inhibited kinase domain of the receptor is necessary for Dab1 phosphorylation. Since we could not exclude that these observed effects seen in Fig. 10A might be influenced by possible off-target effects of the compounds, and to confirm these results, we expressed Dab1 together with a mutated EGFR variant containing a non-functional kinase domain (referred to as kinase-dead mutant or KD) in HEK293T cells. The cells were treated with EGF and subjected to IP using the same specific anti-Dab1 antibody following analysis via immunoblotting. Cells expressing the wild type EGFR variant (EGFR WT) show again clear EGFR activation (Fig 10B, third panel) together with Dab1 phosphorylation (Fig. 10B, first panel), whereas the KD-mutant did not indicate any

signs of receptor activation (Fig. 10B, third panel, lane 3 and 4). Quantification of Dab1 phosphorylation normalised to the untreated controls revealed significantly reduced Dab1 phosphorylation levels in cells expressing KD-EGFR compared to WT-EGFR expressing cells (Fig. 10B, quantification indicated above panel 1). Altogether, we can conclude that the kinase activity of EGFR is the decisive factor for phosphorylation of Dab1.

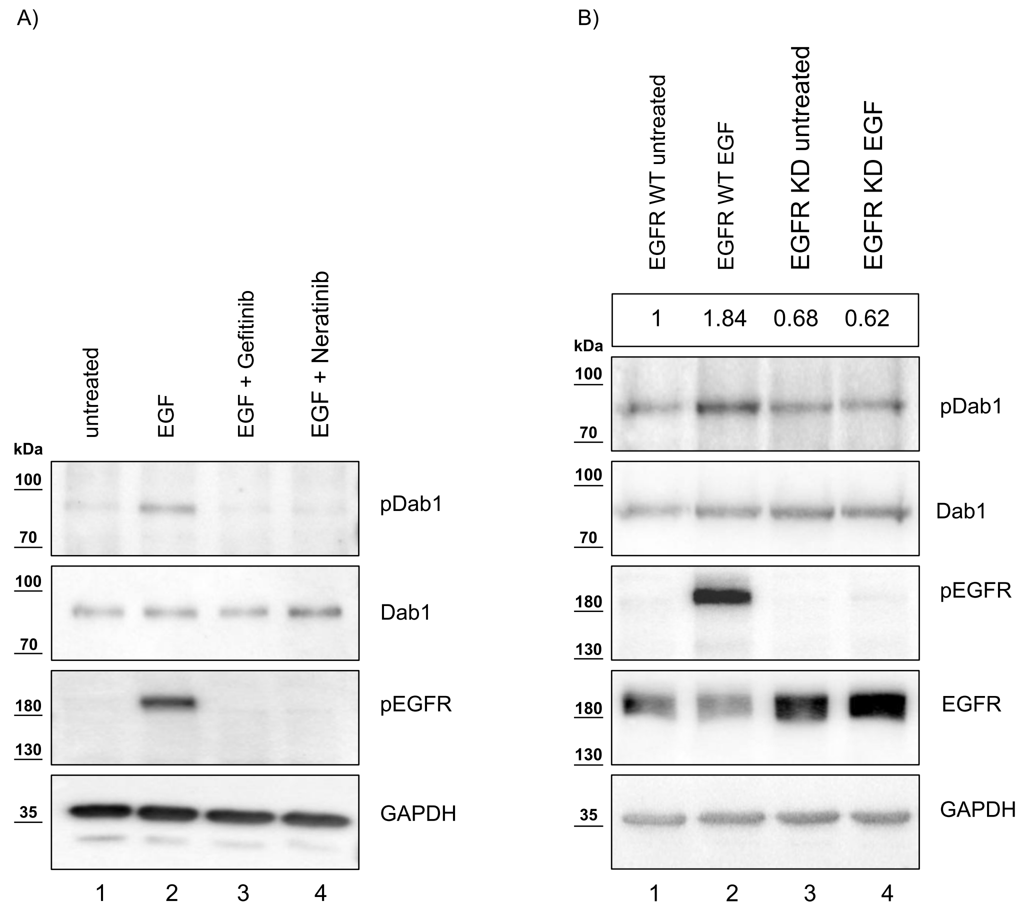

Figure 10: Chemical and genetic inhibition of EGFR abolishes Dab1 phosphorylation. A) Western blot analysis of HEK293 cell extracts expressing EGFR and Dab1. Two hours before EGF treatment (3 min) EGFR kinase inhibitors Gefitinib (Lane 3) and Neratinib (Lane 4) were added to the cells. Immunoprecipitation with an specific antibody for Dab1 was performed. Lane 1 represents the untreated control, lane 2 represents cells treated with [10 ng/ml] of human EGF for 3 minutes, lane 3 and 4 represent the cells which were treated with the kinase inhibitors. Cell exctracts were analysed for the presence of phosphorylated EGFR using a specific antibody (Ab Y1173). As a loading control GAPDH was used. **B)** Western blot analysis of HEK293T cell extracts expressing Dab1 and either a wild-type (WT) or kinase-dead (KD) mutant of EGFR. Immunoprecipitation was performed using a specific antibody for Dab1. Lanes 1 and 3 represent the untreated controls, whereas lanes 2 and 4 show extracts colleceted from cells that underwent EGF treatment. Phosphorylation levels of Dab1 (Panel 1) wer detected using an antibody against phosphotyrosines (Ab PY99). Phosphorylation levels of EGFR (Panel 3) in the cell extracts were analysed using an antibody specific for phosphorylated EGFR (Ab Y1173). The blots were reprobed either for Dab1 (Ab D4, Panel 2) or EGFR (Panel 3). Using the ChemiDoc Touch Imaging System (BioRad, Hercules, CA, USA) the signal intensity of the anti-phosphotyrosine bands (panel 1) was scanned and normalised to the anti-Dab1 (panel 2) signal and the intensity of WT EGFR (panel 1, lane 1) was set to 1. Quantification of the signal is shown in the box above panel 1. As a loading control GAPDH was used.

3.5. PP2 inhibits Dab1 phosphorylation by both ApoER2 and EGFR

As Dab1 phosphorylation depends on EGFR activation, the next question we addressed was whether the phosphorylation of Dab1 was directly associated with activation of the EGFR kinase domain or, comparably to ApoER2, whether there were other players involved. In case of ApoER2, Dab1 phosphorylation is the result of Src-family kinase activation induced by ligand induce receptor oligomerisation (Dlugosz and Nimpf 2018). To investigate whether the same mechanism is true for the EGFR mediated Dab1 phosphorylation we employed the chemical compound pyrazolopyrimidine (PP2), a specific Src-kinase inhibitor, according to the literature (Bain, Plater et al. 2007). In the respective experiment, we used HEK293 cells expressing either EGFR and Dab1 (ED) or ApoER2, EGFR and Dab1 (AED) and pre-treated the cells for 30 min either with DMSO or PP2. The cells were either left untreated (IM), treated with EGF in case of the ED expressing cells and in case of the triple transfected cells (AED) with a “Reelin conditioned medium” (RCM). The cell lysates were subjected to IP with a specific antibody for Dab1 (Ab 54). Subsequently, the samples were analysed via Western blotting (Fig. 11) using specific antibodies for the phosphorylation of EGFR (Fig. 11, first panel) and phosphotyrosines to detect phosphorylated Dab1 (Fig. 11, third panel). The blots were re-probed with antibodies detecting EGFR (Fig. 11, second panel) or Dab1 (Fig. 11, fourth panel 4). EGFR phosphorylation in both EGF treated conditions was induce as expected, as EGF leads to activation of EGFR. Furthermore, we expected to see Dab1 phosphorylation in cells expressing ApoER2, which were treated with RCM, because Reelin elicits the well-established Reelin pathway leading to Dab1 phosphorylation by Src-kinases. We also expected to see an abolished Dab1 phosphorylation in the same cells when PP2 is present, since the Reelin pathway is dependent on the kinases which are inhibited by PP2. Remarkably, we were not able to detect phosphorylation of EGFR in any of the conditions except the one where EGFR and Dab1 expressing HEK cells were treated with DMSO and EGF (Fig. 11, lane 3). However, we were able to find phosphorylated Dab1 in all DMSO (control) samples apart from the band in lane 1 which is probably due to background phosphorylation. In summary, our results show that EGFR phosphorylation is also abolished when PP2 is applied even in the presence of EGF (Fig. 11, lane 4). These data suggest that PP2 is not only an inhibitor for Src family kinases but is also able to inhibit EGF-induced EGFR activation and thus abolishes Dab1 phosphorylation by EGFR and ApoER2. Consequently, no further conclusions can be drawn whether Dab1 phosphorylation is directly achieved through EGFR’s kinase domain or whether Src-kinases are involved in this pathway.

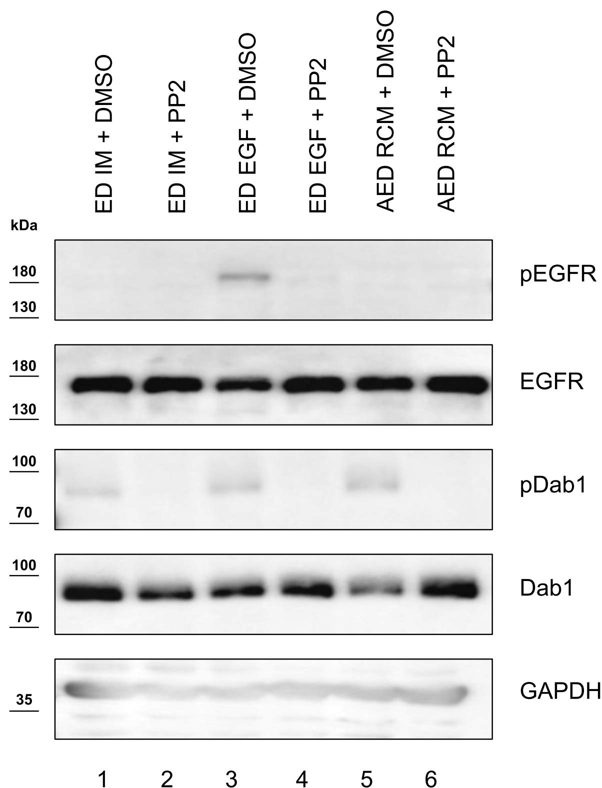

Figure 11: Abolishment of Dab1 and EGFR phosphorylation by the Src-family kinase inhibitor pyrazolopyrimidine compound PP2. Immunoblot analysis of HEK293 cell extracts expressing either EGFR and Dab1 (ED) or ApoER2, EGFR and Dab1 (AED). 30 minutes before treatment cells were either treated with DMSO (lane 1, 3 and 5) or PP2 (lane 2, 4 and 6). Cells then were either left untreated (IM), treated with EGF (10 ng/ml, 3 min) or Reelin conditioned medium (RCM, 20 min). Immunoprecipitation using a specific antibody for Dab1 (Ab 54) was performed. Phosphorylation levels of EGFR (panel 1) and Dab1 (panel 3) were detected by using antibodies specific for EGFR (Ab Tyr 1173) or phosphotyrosines (Ab PY99). Blots were stripped and re-probed using an antibody specific for Dab1 to determine Dab1 and EGFR levels (panel 2 and 4). As a loading control GAPDH was used.

3.6. ApoER2 and EGFR competition

Since both ApoER2 and EGFR seem to be able to induce Dab1 phosphorylation either depending on their kinase domain or on additional enzymes like Src-kinase, we investigated whether there was any regulation or a competition between these two receptors. To address this question, we used HEK293 cells expressing either EGFR and Dab1 or ApoER2, EGFR and Dab1 and stimulated them with EGF in line with the previous experiments. Phosphorylation of Dab1 and EGFR in the cell lysates were analysed by Western blotting as above. The blot subsequently was re-probed and Dab1 levels were determined. As expected, cells expressing EGFR and Dab1 showed phosphorylated EGFR as well as phosphorylated Dab1 (Fig. 12A, lane 2, panels 1 and 2). Comparing the cells expressing both ApoER2 and EGFR together with Dab1, on the one hand, still a signal was detectable indicating the presence of phosphorylated EGFR (Fig. 12A, lane 4, panel 1). On the other hand, barely any signal representing phosphorylated Dab1 was apparent (Fig. 12A, lane 4, panel 2). Indeed, quantification of the signal intensity revealed that co-expression of ApoER2 leads to a dramatic decrease in relative Dab1 phosphorylation induced by EGF as shown in Fig. 12B (compare bar 2 and 4). Statistical analysis of the two groups revealed that this decrease is significant indicating that the presence of ApoER2 indeed diminishes EGF/EGFR mediated Dab1 phosphorylation, even though EGFR is phosphorylated and thus active in both cell lines.

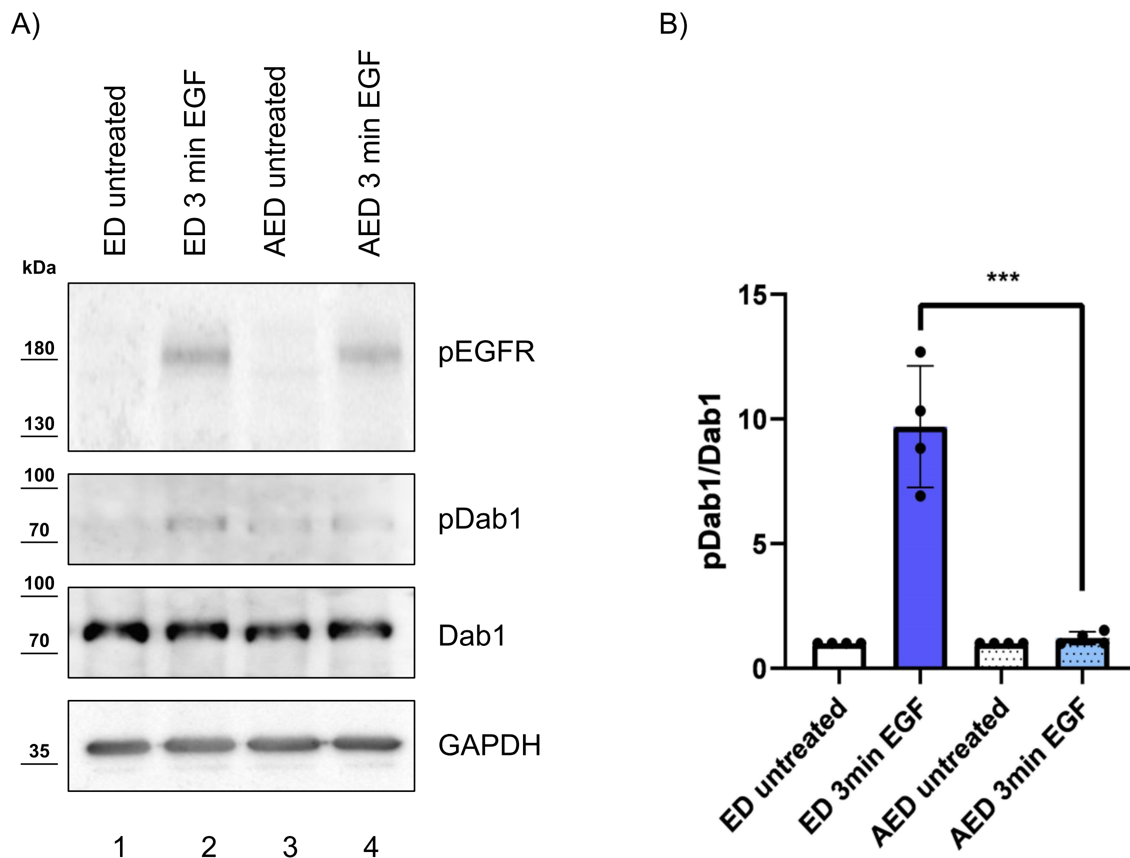

Fig 12: ApoER2 diminishes EGFR-mediated Dab1 phosphorylation. A) Western blot analysis of HEK293 cells expressing either EGFR and Dab1 (ED, lanes 1 and 2) or ApoER2, EGFR and Dab1 (AED, lanes 3 and 4). Cells were treated with EGF (10 ng/ml, 3 min, lanes 2 and 4) or left untreated (lanes 1 and 3). Immunoprecipitation was performed using a Dab1 specific antibody (Ab 54) and phosphorylated Dab1 levels were blotted using an anti-phospho-tyrosine antibody (Ab PY99, panel 2). The presence of phosphorylated EGFR in the extracts was determined using an antibody specific for phosphorylated EGFR (Ab Y1173, panel 1). Subsequently, the blots were stripped and Dab1 levels detected by re-probing the blot with and Dab1 specific antibody (Ab D4, panel 3). As a loading control GAPDH was used (panel 4). **B)** Quantification of the ration between phosphorylated Dab1 and total Dab1 levels. Using the ChemiDoc Touch Imaging System (BioRad, Hercules, CA, USA) the signal intensity of the anti-phosphotyrosine bands (Fig.12A, panel 2) was scanned and normalised to the anti-Dab1 (Fig. 12A, panel 3) signal. The levels of phosphorylation of ED untreated (Fig. 12A panel 2, lane 1) was set to 1. Data was analysed using an unpaired, two-tailed t-test. * $p \le 0.05$, ** $p \le 0.01$, ns, not significant; dotes, number of experiments. Error bars represent standard deviation.

4. Discussion

In this study, we showed that Dab1 functions as a novel adapter protein for EGFR, which can be phosphorylated independently of Reelin upon EGF stimulation, ergo being part of a novel signalling pathway. Even though Dab1 was originally discovered in the context of neurodevelopmental processes, in recent years, more and more evidence has emerged that Dab1 also acts in other tissues than the central nervous system independent of its signature signalling pathway in the brain (Ware, Fox et al. 1997, Khialeeva and Carpenter 2017). In particular, the fact that defective or mutated Dab1 has often been found in connection with uncontrolled proliferation of tissues and thus in the context of cancer, aroused our interest (Sonoshita, Itatani et al. 2015, Pandurangan, Divya et al. 2018). Receptor tyrosine kinases (RTKs) are particularly interesting in this context, as an interaction of Dab1 with VEGFR2, one of the representatives of this VEGFR family of RTKs, has already been demonstrated. Furthermore, RTKs are known for containing a NPxY motif in their intracellular domain (ICD) just like Dab1's native receptors ApoER2 and VLDLR (Howell, Hoda et al. 2013, Wagner, Stacey et al. 2013). This special motif is the binding site for adapter proteins of RTKs containing a PTB domain that recognises phosphorylated tyrosines on the receptor tails (Uhlik, Temple et al. 2005). However, for this motif to be accessible to the adapter proteins, the RTK must first be activated. This occurs as soon as an appropriate ligand binds to the RTK, triggering dimerisation or oligomerisation and subsequent autophosphorylation of the ICDs of the receptors (Wagner, Stacey et al. 2013). As soon as the NPxY motif is phosphorylated, the PTB domain of an adapter protein can bind. Keeping this in mind, our hypothesis that Dab1 might interact with RTKs seems reasonable since Dab1 harbours a PTB domain, which is even independent of the phosphorylation status of the NPxY motif and binds to the ICD of receptors via hydrogen bonds or hydrophobic bonds (Stolt, Jeon et al. 2003). EGFR is known for its potent ability to stimulate cell growth and proliferation, it is also expressed to a comparable high degree in a tissue together with Dab1: the small intestine (Vazquez-Carretero, Garcia-Miranda et al. 2014, Tang, Liu et al. 2016). Due to those reasons, we investigated whether Dab1 is able to bind to EGFR. The first step in this process was to examine whether EGFR indeed contains the NPxY motifs which are the known binding sites for Dab1. For this we aligned the intracellular domain sequences of EGFR with ApoER2 and VLDLR of both human and murine origin, to the two known binding partners of Dab1 (Fig. 6). Indeed, we were able to identify 3 distinct NPxY sites on EGFR which might be binding sites for Dab1. Thus, we performed a Co-IP on HEK293 cells which expressed both EGFR and Dab1 (Fig. 7). We were able to precipitate Dab1 and EGFR together, indicating that there is an interaction between the receptor and the adapter protein. In addition to the immunoblot analyses, we

analysed the expression patterns of fluorescently tagged Dab1 and EGFR in transfected cells (Fig. 8). Here a co-localisation of the two proteins at the cell membrane became evident (Fig. 8C). Interestingly, Dab1 alone is not predominantly found at the membrane but rather spread throughout the cytosol (Fig. 8I), suggesting a recruitment of Dab1 to EGFR and thus to the membrane in presence of the receptor. This co-localisation and recruitment are similar to the one observed in the presence of ApoER2 and Dab1 (Fig. 8L) representing a similarity in Dab1's behaviour towards ApoER2 and EGFR. The results of those two experiments taken together make a strong case for Dab1 binding to EGFR. Next, we examined whether this novel interaction might be part of a newly identified pathway. For that, HEK293 cells expressing both components were treated with EGF in expectation to see an activated receptor and phosphorylated Dab1. The activity of Dab1 is dependent of its phosphorylation status, ergo a phosphorylated Dab1 (pDab1) represents an active protein (Long, Bock et al. 2011). Interestingly, we found that both, EGFR and Dab1, are phosphorylated after 3 min of EGF treatment (Fig. 9A). Phosphorylation of EGFR was expected due to its inherent ability to phosphorylate itself after being activated (Carpenter and Cohen 1979). The phosphorylation of Dab1 in this context, however, has not been shown until now. After the 3 min, which also mark the peak of the Dab1 phosphorylation, the pDab1 levels decrease due to polyubiquitination by E3 ubiquitin ligases and subsequent proteasomal degradation of Dab1, a process which also serves as abrogation of the neuronal migration signal during cortical development (Kerjan and Gleeson 2007). Consecutively, our next step was to analyse whether this process is still occurring when EGFR is inhibited. To inhibit EGFR, we used two different approaches, the first being a chemical inhibition of the ATP binding cassette of EGFR's intracellular domain via the two compounds Gefitinib and Neratinib. As already mentioned, both inhibit the ICD of EGFR, thus preventing autophosphorylation of the C-termini and thus preventing EGFR signalling. The difference between the two compounds is that Gefitinib is a reversible antagonist, whereas Neratinib binds irreversibly to EGFR (Yasuda, Kobayashi et al. 2012). In our setting, both compounds where not only able to inhibit phosphorylation of EGFR, as expected, but also to prevent Dab1 phosphorylation completely (Fig. 10A). In the second approach, EGFR's function was impaired by employing a kinase-dead mutant of EGFR. This mutant is characterised by a point mutation (K721A) which renders its kinase domain non-functional (Fig. 10B). In that manner transfected HEK293T cells show no phosphorylation of Dab1 nor EGFR, as the semi-quantitative analysis of pDab1 levels revealed (Fig.10B, box above panel 1). These experiments lead to the conclusion that a functional kinase domain in EGFR is essential for the phosphorylation of Dab1, since both, the chemical inhibition of the kinase domain as well as using a KD-mutant of EGFR, prevented Dab1 phosphorylation in this setting. In contrast to EGFR, ApoER2

does not contain a kinase domain on its own, but Dab1 phosphorylation depends on clustering of the receptors ApoER2 and VLDLR by Reelin, leading to clustering of Dab1 and consecutive phosphorylation by recruited secondary kinases from the Src-kinase family (Dlugosz and Nimpf 2018). In line, EGFR has already been shown to interact with Src-kinases too (Irwin, Bohin et al. 2011). For that reason, we investigated whether Src-family kinases might also be involved in the propagation of this EGFR-Dab1 signal. To address this question, we used a specific Src-kinase inhibitor, pyrazolopyrimidine (PP2) on cells expressing either EGFR and Dab1 (ED) or ApoER2, EGFR and Dab1 (AED). Interestingly, even when stimulated with EGF in the ED case or Reelin conditioned medium (RCM) in the AED case, phosphorylation of Dab1 was inhibited (Fig. 11, third panel). This was expected for the AED condition since the RCM should trigger the Reelin pathway which is dependent on active Src. In cells only expressing EGFR and Dab1, however, this came as a surprise, especially because we were also not able to detect EGFR phosphorylation anymore. This poses the question whether PP2 is actually specific for Src-kinases or also able to inhibit RTKs like EGFR or whether the concentration of PP2 was too high so that EGFR also was inhibited as an off-target effect. Src and EGFR interact as Src phosphorylates the receptor's kinase domain on Y845, which is thought to enhance the binding ability of EGFR to ATP. However, it is still controversial whether this interaction is important for the general activation of the receptor (Biscardi, Maa et al. 1999, Burgess, Cho et al. 2003). Because of this discrepancy between our results and the claims of many studies, further literature investigation revealed that there already have been reports of PP2 being able to inhibit the phosphorylation ErbB2 and ErbB3 and was even claimed to be even less selective for Src than the well-studied and very promiscuous inhibitor Dasatinib (Brandvold, Steffey et al. 2012). This stands in contrast to other studies that claim PP2 to be specific and thus recommended for inhibition of Src-family kinases which we used as an inspiration for the experimental setup (Bain, Plater et al. 2007). Keeping PP2's apparent lack of specificity in mind, it might be possible that not only the two previously named ErbB- receptor family members are inhibited by PP2 but also EGFR. If this is the case, no statement can be made whether the non-existing phosphorylation of Dab1 in this experiment is due to the dependence on Src-kinases as mediators between EGFR and Dab1 or whether it is caused by the fact that the inhibition of EGFR was prevented and thus no signal could be induced. Since our results in the previous experiments (Fig. 6-10) show that Dab1 is an adapter protein for EGFR and is phosphorylated after EGF stimulation, we investigated the effect of expression of ApoER2 together with EGFR in HEK cells. Therefore, ApoER2 together with EGFR were expressed and the cells were stimulated with EGF (Fig. 12A). As expected, EGFR is phosphorylated after treatment. Surprisingly, as soon as the cells co-expressed ApoER2, Dab1 phosphorylation vanished to almost background levels

(Fig 12B). This suggests that ApoER2 and EGFR compete for Dab1 binding. This competition apparently resulted in the favour of ApoER2 since its sole co-expression even without its activation by Reelin abrogates Dab1 phosphorylation. This is most probably due to the fact that Dab1 binding to EGFR is reduced in the presence of ApoER2. This conclusion is supported by the fact that from a biochemical view ApoER2 is more suited for Dab1 binding since the ideal binding site for Dab1 is ΦxNPxY (where Φ stands for F or Y) (Howell, Lanier et al. 1999). Even though EGFR harbours three NPxY sites which can be bound by Dab1's PTB-domain, none of these matches this amino acid pattern exactly. This might result in a hierarchy between the here described EGFR-mediated pathway and the Reelin pathway, which takes the route through ApoER2 and VLDLR (Dlugosz and Nimpf 2018). The competition between these pathways was also supported by further investigation of our group which found that EGF stimulation of primary murine neurons which express high amounts of ApoER2 did not lead to Dab1 phosphorylation even though EGFR is again phosphorylated (Dlugosz, Teufl et al. 2021). Taken together, these results presented here, left us with the assumption that the EGFR mediated Dab1 phosphorylation is probably not occurring in neuronal tissues since ApoER2 is present. Because of that, our group turned to further experiments, studying the small intestine. However, these studies are beyond the scope of my thesis. Indeed, Dab1 phosphorylation was induced in murine intestinal epithelial cells which were shown to express EGFR but do not contain ApoER2 or VLDLR. In addition to those experiments our group was also able to find that HEK293 cells lacking Dab1 have a way lower proliferation rate than wild-type cells (Dlugosz, Teufl et al. 2021). All this can be put into a greater context when keeping in mind that the intestinal epithelium is characterized by a short turnover time of its cells and that Dab1 has already been shown in the past to be involved in cell proliferation and plays a role in invasion and metastasis of colorectal cancer cells (Khialeeva, Lane et al. 2011, Garcia-Miranda, Vazquez-Carretero et al. 2012). In intestinal epithelial cells, for example previous studies found that when Dab1 was knocked out, the turnover time of the cells was slowed down. Along this line, the so-called scrambler mutation, which is a homozygous knockout of Dab1, leads to a drastic reduction of Paneth cells in the intestinal crypts further stressing Dab1's importance for the homeostasis of the small intestine (Garcia-Miranda, Vazquez-Carretero et al. 2013). On the one hand, this is of particular interest when keeping in mind that EGFR is also expressed in the crypts, where it performs many tasks including development of the intestine, maintaining the intestinal barrier function and regulating the height of the villi and depth of the crypts (Tang, Liu et al. 2016). On the other hand, Dab1, when knocked out does not only decrease cell proliferation but when overexpressed can cause colorectal cancer via the Notch-Dab1-Abl-RhoGEF protein Trio pathway (Sonoshita, Itatani et al. 2015).

In summary, we were able to show that Dab1 does not only bind to EGFR but can also be phosphorylated in the presence of EGF. This pathway is different from the Reelin pathway since in this case, Dab1 phosphorylation is depending on EGFR's kinase domain. However, the nature of the connection between EGFR activation and Dab1 phosphorylation is not completely clear. Is it a direct interaction or are other players involved? Thus, further experiments are required to investigate the possible involvement of Src-family kinases by applying a more specific inhibitor or genetic knockdowns. Also, analysis of the possible downstream players of Dab1, like PI3K, could reveal further interesting mechanistic insights into this novel pathway. When aiming for a translational application of this knowledge, a promising approach would be the screening of intestinal cancer patients derived tumour cells for hyperphosphorylated Dab1 and/or EGFR.

In conclusion, our results taken together with additional work of our group suggest that the discovered EGFR-Dab1 axis might play an important role in the proliferation and cell homeostasis of the intestinal epithelium and might be a missing link for understanding why skewed or missing Dab1 in the intestine has such an impact on the integrity of the tissue. On top of that, it could also function as a second pathway in the intestine besides the famous Wnt-pathway, which is considered to be the major player to this date, in intestinal cell proliferation (Dlugosz, Teufl et al. 2021). Everything considered, the discovery of this pathway may lay the groundwork for future advances in the understanding of EGFR's and Dab1's roles in homeostasis of tissues like the small intestine and might present a new target in the development of an alternative treatment or drugs for intestinal cancer.

5. Abbreviations

Abl	Abelson murine leukaemia tyrosine kinase
ADAM	A disintegrin and metalloprotease
AP2	Adapter protein 2
aPKC	atypical protein kinase C
ApoE	Apolipo protein E
AREG	Amphiregulin
ASD	Autism spectrum disorder
ATP	Adenosine triphosphate
BSA	Bovine serum albumin
BTC	Betacellulin
C1 (Domain)	Conserved 1
cAMP	Cyclic adenosine monophosphate
CaN	Calcineurin
c-Cbl	Casitas B-lineage Lymphoma E3 ubiquitin Ligase
CCP	Clathrin coated pits
CLCb	Clathrin light chain b
CME	Clathrin mediated endocytosis
Co-IP	Co-immunoprecipitation
cPKC	Conventional protein kinase C
CREB	cAMP response element-binding protein
CrkL	Protooncogene c-Crk Ligand
Dab1	Disabled 1
DAG	Diacylglycerol
DMEM	Dulbecco’s modified Eagle’s medium
DUSP	Dual specificity phosphatase
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
Elk	Ets-like Protein 1
ER	Endoplasmic reticulum
EREG	Epiregulin
Erk 1/2	Extracellular-signal regulated kinases 1 and 2
Ets	Erythroblast Transformation Specific
FCS	Foetal calf serum
FOXO	Fork head box O transcription factor
Gab	Grb2-associated binder
GAPDH	Glycerinaldehyd-3-phosphat-Dehydrogenase
GDP	Guanosine diphosphate
GEF	Guanine nucleotide exchange factor
GLUT1	Facilitated glucose transporter member 1
Grb2	Growth factor receptor-bound protein 2
GSK6	Glycogen synthase kinase 3
GTP	Guanosine triphosphate
HB-EGF	Heparin binding- epidermal growth factor
HEK293	Human embryonic kidney cells
HEK293T	Human embryonic kidney cells (T-Antigen expressing)

HK1	Hexokinase 1
HNSCC	Head and neck squamous cell carcinoma
ICD	Intracellular domain
IgG	Immunoglobulin G
IkB	Inhibitor of nuclear factor kappa B
IKK-α	Inhibitor of nuclear factor kappa B kinase
IM	Imaging medium
IP	Immunoprecipitation
IP_3	Inositol triphosphate
IP_3R	Inositol triphosphate receptor
IRH	Isolated autosomal recessive hypomagnesemia
JIP	JNK-interacting Protein
JNK	c-Jun-N-terminal kinase
LDL	Low density lipoprotein
LDLR	Low density lipoprotein receptor
LRP8	Low-density lipoprotein receptor-related protein 8
LTP	Long term potentiation
MAPK	Mitogen activated protein kinase
MDM2	Mouse double minute 2 homolog
MEK 1/2	MAPK/extracellular-signal regulated kinases 1 and 2
MMP	Matrix metallo protease
mTORC 1/2	Mammalian target of the rapamycin complex 1 and 2
NCE	Non-clathrin mediated endocytosis
NFAT	Nuclear factor of activated T-cells
NF-κB	Nuclear factor kappa B
NGF	Nerve growth factor
nPKC	New protein kinase
NSCLC	Non-small cell lung cancer
Orai	Calcium release-activated calcium channel protein
PBS	Phosphate buffered saline
PDK1/2	3-phosphoinositide-dependent protein kinase-1
PD-L1	Programmed cell death 1 ligand 1
PEI	Polyethyleneimine
PH-Domain	Pleckstrin homology domain
PHLPP	PH domain and Leucine rich repeat Protein Phosphatase
PI(3)K	Phosphoinositide 3 Kinase
PIP_2	Phosphatidylinositol-4,5-bisphosphat
PIP_3	Phosphatidylinositol-3,4,5-bisphosphat
PKC	Protein kinase C
PKM2	Pyruvate kinase 2
PLC	Phospholipase C
PP2A	Protein phosphatase 2A
PPAR	Peroxisome proliferator-activated receptor
PSD-95	Postsynaptic density protein 95
PTB	Phosphotyrosine binding (domain)
PTEN	Phosphatase Tensin homolog
PTP	Protein tyrosine phosphatases
pY	Phosphor tyrosine
RasGRP	Ras guanyl nucleotide-releasing protein 1

RCM	Reelin conditioned medium
RTK	Receptor tyrosine kinase
S6K	S6-Kinase
SAM	sterile-α motif
SCZ	Schizophrenia
SDS	Sodium dodecyl sulphate
Sepp1	glycoprotein Reelin, Clusterin and Selenoprotein P 1
SGLT1	Sodium-glucose co-transporter 1
SH2	Src-homology 2
SHC	Src-homology-containing protein
SHIP 1/2	SH2-domain-containing inositol polyphosphate 5-phosphatase 1
SHP2	Src homology region 2 domain-containing phosphatase-2
SOAR	STIM1-Orai activating region
SOCE	store operated Ca2+ entry
SOS	Son of Sevenless
c-Src	Cellular sarcoma
SREBP1	Sterol regulatory element-binding protein
STAT	Signal transducer and activator of transcription proteins
STIM1	Stromal interaction molecule 1
T-ALL	T-acute lymphoblastic leukaemia
TBS	Tris buffered saline
TGF-α	Transforming growth factor-α
TSC 1/2	Tuberous sclerosis 1 and 2
ULK1	Unc-51 like autophagy activating kinase 1
VEGF	Vascular endothelial growth factor
VEGFR	Vascular endothelial growth factor receptor
VLDLR	Very low-density lipoprotein receptor
ZAP70	Zeta-chain-associated protein kinase 70

6. References

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