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ERBB4 AS A POTENTIAL THERAPEUTIC TARGET IN CANCER

Veera Ojala

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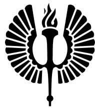
SARJA – SER. D OSA – TOM. 1812 | MEDICA – ODONTOLOGICA | TURKU 2024



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To all of you who have been there for me during this journey

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ABSTRACT

EGFR/ERBB/HER family of receptor tyrosine kinases are frequently mutated, and hence, aberrantly active in cancer. Currently, numerous ERBB-targeted therapies are used to treat various types of cancer, using activating mutations or amplification of EGFR and ERBB2 as predictive biomarkers. Yet, it remains unknown whether cancer patients with activating ERBB4 mutations could benefit from ERBB4-targeted therapy. This is despite previous reports on activating ERBB4 mutations that may drive cancer growth and the availability of clinically used second-generation pan-ERBB inhibitors that potently block also ERBB4. The unclear role of ERBB4 in cancer is largely due to the highly context-dependent and partly opposing functions of the different ERBB4 isoforms, as well as due to the high diversity of cancer-associated ERBB4 mutations lacking obvious mutational hotspots.

The aim of this thesis was to clarify the role of ERBB4 in cancer and to evaluate the potential of using ERBB4 mutations as predictive biomarkers. To elucidate ERBB4 signaling mechanisms in cancer, the interactome of the two cancer-predominant isoforms of ERBB4 was analyzed in breast cancer cells. VAV3 was identified as a novel effector of ERBB4-mediated signaling promoting cancer cell migration. To address the clinical significance of the hundreds of different ERBB4 mutations found in cancer patients, two complementary approaches were employed: an unbiased high-throughput screen and a focused functional characterization of the newly emerging hotspot ERBB4 mutations, of which many are paralogous to known oncogenic mutations in other ERBB genes. These two approaches identified 14 novel gain-of-function ERBB4 mutations of which the five most potent were mechanistically characterized. These most potential activating driver mutations were also targetable with clinically used pan-ERBB inhibitors, and thus, could potentially serve as predictive biomarkers.

Taken together, the results of this thesis have clarified the rationale for targeting ERBB4 in cancer. These findings can facilitate clinical interpretation of ERBB4 mutations and warrant further studies of using activating ERBB4 mutations as predictive biomarkers for pan-ERBB inhibitor therapy.

KEYWORDS: ERBB4, cancer, signaling, activating mutation, screen, tyrosine kinase inhibitor, predictive biomarker

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TIIVISTELMÄ

Syövän kasvua lisäävät EGFR/ERBB/HER-reseptorityrosiinikinaasien toimintaa yliaktivoivat mutaatiot ovat yleisiä syöpätyypeissä. Lukuisia ERBB-reseptoreihin kohdennettuja täsmälääkkeitä onkin tällä hetkellä kliinisessä käytössä eri syöpien hoitoon, missä EGFR:n tai ERBB2:n aktivoivia mutaatioita tai monistumia käytetään ennusteellisina biomarkkereina hoidoille. Myös ERBB4 aktivoivien mutaatioiden on näytetty voivan lisätä syövän kasvua ja ERBB4:n toimintaa estäviä toisen sukupolven pan-ERBB-estäjiä on jo kliinisessä käytössä. Tästä huolimatta ei kuitenkaan vielä tiedetä, voisivatko potilaat, joilla on aktivoiva ERBB4-mutaatio, hyötyä ERBB4-reseptorin kohdennetusta hoidosta. ERBB4:n epäselvä rooli syövässä johtuu suurelta osin siitä, että sen toiminta on hyvin kontekstiriippuvaista ja ERBB4:n eri alamuotojen toiminta on osittain jopa toisiinsa nähden vastakkaista. Lisäksi syövässä esiintyvien erilaisten ERBB4-mutaatioiden määrä on suuri, eivätkä mitkään niistä erotu selvästi erityisen toistuvina, toisin kuin muiden ERBB-reseptoreiden kohdalla.

Tämän tutkimuksen tarkoituksena oli selventää ERBB4:n roolia syövässä sekä arvioida *ERBB4*-geenin aktivoivien mutaatioiden ennusteellisuutta täsmälääkehoidolle. Tutkimuksessa selvitettiin ERBB4:n kahden syövässä eniten esiintyvän alamuodon soluviestinnän mekanismeja. VAV3 löydettiin uutena ERBB4:n soluviestinnän välittäjänä, minkä näytettiin lisäävän syöpäsolujen liikkumista. Lisäksi selvitettiin ERBB4-geenin syövässä esiintyvien mutaatioiden kliinistä merkitystä kahdella toisiaan täydentävällä lähestymistavalla. Näin löydettiin 14 uutta aktivoivaa *ERBB4*-mutaatiota, joista viiden parhaiten solujen kasvua lisäävän mutaation mekanismi selvitettiin. Nämä viisi potentiaalisinta ”ajajamutaatiota” olivat myös herkkiä kliinisessä käytössä oleville pan-ERBB-estäjille ja voisivat siten mahdollisesti toimia ennusteellisina biomarkkereina.

Tutkimuksen tulokset osoittavat, että ERBB4 on potentiaalinen täsmälääkehoidon kohde syövässä. Tulokset auttavat ERBB4-mutaatioiden kliinistä tulkintaa ja tukevat jatkotutkimuksia niiden ennusteellisuudesta pan-ERBB-estäjähoidon teholle potilaissa.

AVAINSANAT: ERBB4, syöpä, soluviestintä, aktivoiva mutaatio, seulonta, tyrosiinikinaasin estäjä, ennusteellinen biomarkkeri

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Abbreviations

AACR-GENIE	American Association for Cancer Research Project Genomics Evidence Neoplasia Information Exchange
ADAM	a disintegrin and metalloprotease
ADC	antibody-drug conjugate
AKT	AKT serine/threonine kinase 1
ALK	anaplastic lymphoma kinase
APC	adenomatous polyposis coli
AREG	amphiregulin
ATP	adenosine triphosphate
BCR-ABL	breakpoint cluster region – Abelson murine leukemia
BRCA1	BRCA1 DNA repair associated (also known as Breast Cancer 1)
BRCA2	BRCA2 DNA repair associated (also known as Breast Cancer 2)
BTC	betacellulin
BRAF	b-raf proto-oncogene, serine/threonine kinase
CBL	casitas B-lineage lymphoma
CML	chronic myeloid leukemia
COSMIC	catalogue of somatic mutations in cancer
CRK	CDC42/RAC1-activated kinase
CRISPR	clustered regularly interspaced palindromic repeats
CDK4/6	cyclin-dependent kinase 4 and 6
CYT	cytoplasmic
DAG	diacylglycerol
ECD	extracellular domain/ectodomain
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
eJM	extracellular juxtamembrane region
EMA	European Medicines Agency
EMT	epithelial-mesenchymal transition
EPGN	epigen (epithelial mitogen)
ERα	estrogen receptor α
ERBB	erythroblastic leukemia viral oncogene

EREG	epiregulin
ERK	mitogen-activated protein kinase 1
ETO2	eight-twenty one oncogene
FDA	United States Food and Drug Administration
GEF	guanine nucleotide exchange factor
GRB2	growth factor receptor-bound protein 2
HB-EGF	heparin-binding EGF-like growth factor
HER	human EGF receptor
HIF	hypoxia inducible factor
HNSCC	head and neck squamous cell carcinoma
ICD	intracellular domain
iJM	intracellular juxtamembrane region
IL3	interleukin-3
iSCREAM	in vitro screen for activating mutations
ITCH	E3 ubiquitin-protein ligase Itchy homolog
JAK	janus kinase
JM	juxtamembrane
JNK	c-JUN N-terminal kinase
KAP1	KRAB-associated protein 1
KRAS	v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog
MAPK	mitogen activated protein kinase
MDM2	mouse double minute 2 homolog
MEK	mitogen activated protein kinase kinase
MLC	myosin light chain
MLH1	MutL homolog 1
MSH2	MutS homolog 2
MSH6	MutS homolog 6
mTOR	mammalian target of rapamycin
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
MYC	v-MYC proto-oncogene protein
NCK	non-catalytic region of tyrosine kinase adaptor protein
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells
NRG	neuregulin (heregulin)
NRAS	neuroblastoma RAS viral oncogene homolog
p85α	p85 subunit α
PAK	p21 (RAC1) activated kinase 1
PDK1	3-phosphoinositide dependent kinase 1
PDX	patient-derived xenograft
PI3K	phosphoinositide-3-kinase
PIP2	phosphatidylinositol 4,5-bisphosphate

PIP3	phosphatidylinositol 3,4,5-trisphosphate
PKC	protein kinase C
PLCγ	phospholipase-C γ
PML	promyelocytic leukemia
PTB	phosphotyrosine-binding
PTEN	phosphatase and tensin homolog
PTM	post-translational modification
pRB	retinoblastoma protein
RAF	rapidly accelerated fibrosarcoma
RIP	regulated intramembrane proteolysis
RTK	receptor tyrosine kinase
SHC	SHC adaptor protein
SOS	son of sevenless
SNV	single nucleotide variant
STAT	signal transducers and activators of transcription
SV	structural variant
TACE	tumor necrosis factor-α-converting enzyme
TGF-α	transforming growth factor α
TKI	tyrosine kinase inhibitor
TM	transmembrane

List of Original Publications

This dissertation is based on the following original publications, which are referred to in the text by their Roman numerals:

I Ojala VK, Knittle AM, Kirjalainen P, Merilahti JAM, Kortesoja M, Tvorogov D, Vaparanta K, Lin S, Kast J, Pulliainen AT, Kurppa KJ, Elenius K. The guanine nucleotide exchange factor VAV3 participates in ERBB4-mediated cancer cell migration. *Journal of Biological Chemistry*, 2020; 295(33): 11559-11571.

II Chakroborty D*, Ojala VK*, Knittle AM, Drexler J, Tamirat MZ, Ruzicka R, Bosch K, Woertl J, Schmittner S, Elo LL, Johnson MS, Kurppa KJ, Solca F, & Elenius K. An unbiased functional genetics screen identifies rare activating ERBB4 mutations. *Cancer Research Communications*, 2022; 2(1): 10–27.

III Ojala VK, Tuohisto-Kokko A, Ahonen S, Jokilammi A, Esparta O, Suominen P, Chakroborty D, Airenne T, Johnson MS, Eli LD, Elenius K*, Kurppa KJ*. Recurrent ERBB4 mutations are transforming and potentially predictive for pan-ERBB inhibitors. Manuscript, 2024.

* These authors contributed equally to the article.

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1 Introduction

ERBB4 is a less-studied member of the EGFR/ERBB/HER-family of receptor tyrosine kinases comprising EGFR/ERBB1/HER1, ERBB2/HER2, ERBB3/HER3 and ERBB4/HER4. These cell surface receptors transduce extracellular signals inside the cell to regulate cell fate in development and adult tissue homeostasis, including cell proliferation, differentiation, migration, apoptosis and survival. Thus, ERBB signaling is often dysregulated in cancer via genetic and epigenetic changes, which can be used as predictive biomarkers for targeted cancer therapy. EGFR and ERBB2 are among the best-defined oncogenes, currently targeted with nearly 40 different clinically approved drugs to treat various types of cancer and multiple ERBB3-targeting drugs are also in clinical development.

Despite i) the high frequency of *ERBB4* mutations in high incidence cancer types, ii) growing evidence of oncogenic ERBB4 functions, and iii) existence of clinically used pan-ERBB inhibitors that potently inhibit ERBB4, the relevance of targeting ERBB4 in cancer remains unknown. This is partly due to lack of obvious mutational hotspots in the *ERBB4* gene, making it difficult to characterize and identify functionally relevant mutations from all the hundreds of clinically occurring mutations. Previous studies have focused on characterizing select *ERBB4* mutations that have occurred in a specific cancer type, and have indeed identified several oncogenic *ERBB4* mutations. However, majority of the cancer-associated *ERBB4* mutations remain of unknown significance.

Another reason why the role of ERBB4 in cancer has remained unclear is that it has highly context-dependent functions. This stems partly from its four different isoforms that are known to have partially opposing functions which are often also cell type-dependent. In cancer tissue, two of the isoforms are predominant and simultaneously expressed but previous studies have not comprehensively analyzed the interaction partners and signaling mechanisms of these ERBB4 isoforms in parallel in a cancer cell context.

This thesis aimed to elucidate ERBB4 signaling mechanisms in cancer by conducting an ERBB4 interactome screen for both the cancer-associated ERBB4 isoforms in breast cancer cells. The used cells are one of the few cancer cell models in which ERBB4 is endogenously expressed in moderate levels, providing a more

cancer relevant biological context than previous larger-scale ERBB4 signaling studies.

Additionally, this thesis addresses the functional significance of the diverse cancer-associated *ERBB4* mutations systematically with two complementary pan-cancer approaches. Firstly, a previously developed unbiased high-throughput screen allowed functional analysis of 7,396 genetic *ERBB4* variants, which is 91.7% of all the theoretically possible *ERBB4* mutations caused by single nucleotide variants (SNV). While this method provided robustness and identified rare oncogenic ERBB4 mutations, the second approach provided more sensitivity by using two very different cell models to functionally screen 18 emerging *ERBB4* hotspot mutations. Finally, the targetability of the identified putatively oncogenic ERBB4 mutations with clinically available pan-ERBB inhibitors was analyzed to assess their potential to serve as predictive biomarkers for targeted therapy.

2 Review of the Literature

2.1 Tumors arise and progress upon genetic alterations

The development of cancer is a multistep process starting from a normal cell acquiring genetic alterations that enable its uncontrolled growth, despite all the mechanisms evolved to protect from cancer growth (Nowell, 1976; Fearon *et al.*, 1990). Later on, further alterations must occur to enable the development of a group of cancer cells into tumor tissue - or rather an organ with multiple different co-operating cell types, its own vasculature, ability to remodel its surrounding tissues and communicate with and spread into distant organs (Egeblad *et al.*, 2010).

Genetic alterations causing cancer arise from i) DNA replication mistakes in dividing cells and ii) from endogenous and environmental carcinogens, such as intracellular processes producing free radicals, radiation, tobacco smoke, certain infectious microbes and chemicals. DNA replication mistakes are rare in humans due to the high-fidelity DNA polymerase and the intricate DNA repair mechanisms, resulting in only 13-47 mutations/genome in a year (Werner *et al.*, 2020; Kakiuchi *et al.*, 2021; Cagan *et al.*, 2022). Together they ensure nearly perfect duplication of the genome prior to cell division but the infrequent alterations enable evolution. Replication mistakes and carcinogen-induced alterations that affect germ cells, i.e. germline alterations can be passed on to progeny and can give rise to hereditary cancers while somatic (non-germ cells) alterations can give rise to sporadic cancers.

Changes that alter cell behavior comprise both genetic alterations that change the DNA sequence and epigenetic alterations that change gene expression via other mechanisms. Genetic alterations include SNVs that are also referred to as point mutations, larger genetic changes including insertions and deletions of nucleotides (indels), structural variants (SV) and changes in chromosome numbers or their structures (Figure 1). For this thesis, SNVs are the most central type of changes, being the most frequent type of ERBB4 alterations in cancer, as will be discussed in section 2.3.4.1. SNVs include silent mutations that do not change the amino acid that the three-nucleotide codon codes for, nonsense mutations that result in an early stop codon, and missense mutations which result in change of the amino acid encoded by the affected codon (Figure 1). Epigenetic alterations include for instance DNA

methylation and chromatin remodeling via histone or nucleosome modifications which affect gene expression patterns. Epigenetic changes arise often from environmental factors, such as dietary components, endocrine disruptors, sedentary lifestyle, alcohol, smoking and pollutants, and are often reversible but can also be transmitted to offspring (Skinner, 2011).

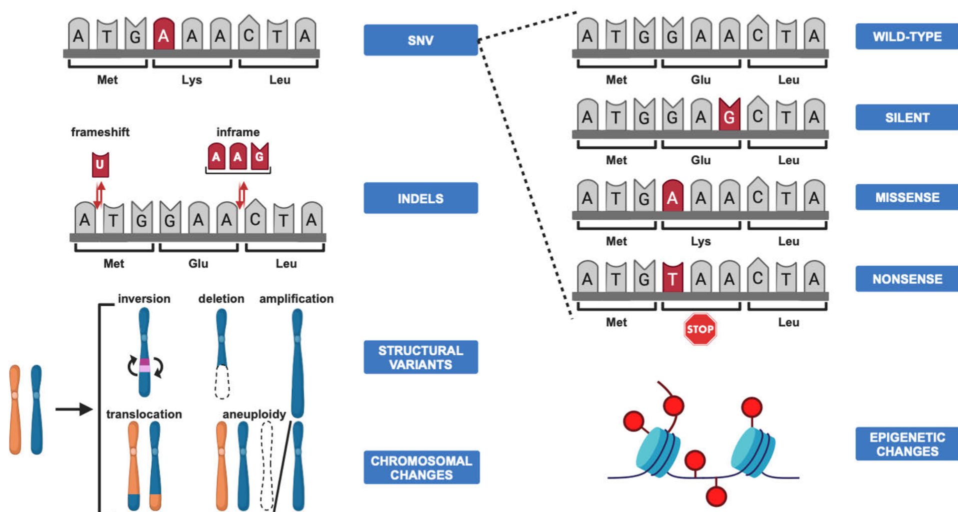


Figure 1. Main types of alterations in cancer. Genetic alterations include single nucleotide variants (SNV), insertions and deletions (causing either frameshift or in-frame mutations), structural variants (SV) including inversions, amplifications and deletions, and chromosomal changes including translocations (such as gene fusions) and reduced or increased numbers of chromosomes. Epigenetic changes include DNA methylation, histone modification, and chromatin remodeling. Created with BioRender.com.

Cancer cells have on average 10^2-10^6 point mutations in addition to bigger genomic rearrangements and copy number alterations (in up to 10% of the genome) (Kandoth *et al.*, 2013; De *et al.*, 2017) but the tumor mutational burden varies considerably between cancer types (Greenman *et al.*, 2007). Cancers with the highest tumor mutational burden (TMB) include those arising from epithelia highly exposed to carcinogens such as smoking-induced lung cancer and UV-exposure-induced cutaneous melanoma, while for instance breast cancers without DNA repair machinery mutations often have lower TMB (Greenman *et al.*, 2007). However, majority of the cancer-associated mutations do not directly propel tumor growth. Such inconsequential mutations are termed “passenger” mutations while those that participate in driving tumor growth are termed “drivers” (Figure 2) (Vogelstein *et al.*, 2013). Driver alterations affect proto-oncogenes and tumor suppressor genes,

which will be discussed in more detail below, but together they regulate important cellular processes involved in cell growth and death.

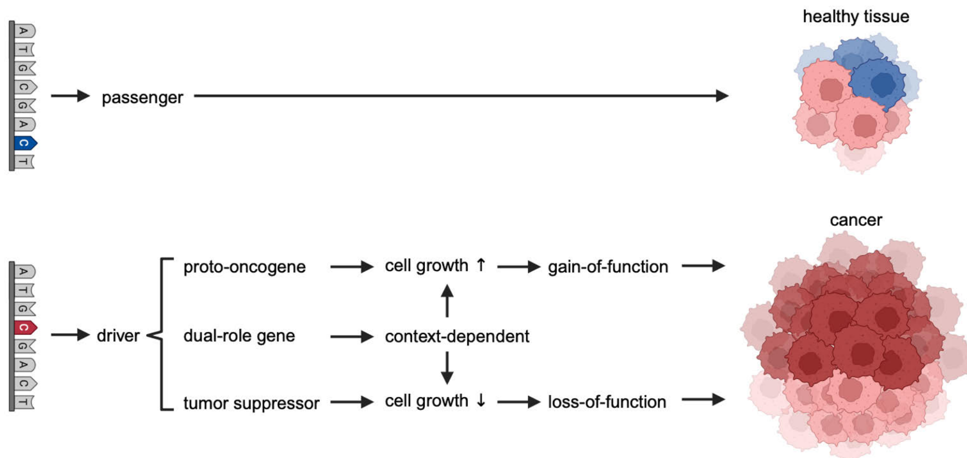


Figure 2. Cancer-associated mutations. Passenger mutations do not provide selective growth advantage for cancer cells, unlike driver mutations. Driver mutations in proto-oncogenes that normally promote cell growth are gain-of-function mutations leading to cancer growth. Driver mutations in tumor suppressor genes that normally limit cell growth are loss-of-function mutations resulting in cancer growth. Driver mutations in dual-role genes can be either gain-of--function or loss-of-function mutations. Adapted from Nourbakhsh et al. 2024. Created with BioRender.com.

The cellular processes whose dysregulation facilitates uncontrolled cellular growth were conceptualized by Hanahan and Weinberg in 2000, by introduction of cancer hallmarks, representing the core elements cancer needs to form and grow. These comprised six hallmark capabilities: self-sufficiency in growth signals, insensitivity to anti-growth signals, evasion of apoptosis, limitless replicative potential, sustained angiogenesis, and tissue invasion and metastasis (Hanahan *et al.*, 2000). Over the years, the concept has been revised as the understanding of cancer biology has improved. First additions to the original hallmarks included energy metabolism adaptations to sustain rapid growth as well as changes that allow cancer cells to evade from the immune surveillance (Hanahan *et al.*, 2011). More recently, two new hallmarks were included: phenotypic plasticity, the ability to evade terminal differentiation into certain developmental lineage, as well as cellular senescence, a non-proliferative state in which cells secrete tumor promoting factors to surrounding cells (Hanahan, 2022). These are crucial abilities for cancer cells to tolerate, survive and regrow upon therapeutic pressure.

In addition to the hallmarks themselves, four features are currently considered to enable the hallmark capabilities of cancer cells: i) genomic instability, ii) tumor-

promoting inflammation, iii) non-mutational epigenetic reprogramming, and iv) polymorphic microbiomes (Hanahan, 2022). The latest update to hallmarks of cancer emphasizes cancer as a systemic disease rather than being a localized tumor, and proposes factoring in environmental and physiological effects on the crosstalk of tumor and host, such as aging, obesity, nervous system and circadian rhythms (Swanton *et al.*, 2024).

Considering the notable range of dysregulated cellular processes in cancer, the number of driver mutations needed for the initial step of malignant transformation of a normal cell into a cancer cell is remarkably low. Malignant transformation has been estimated to require less than ten driver mutations based on findings already in early epidemiological studies (Nordling, 1953; Armitage *et al.*, 1954, 1957). This was later confirmed in *in vitro* studies determining the number of mutations that are required in important pathways to transform cells (Hahn *et al.*, 1999, 2002; Vogelstein *et al.*, 2004). This is now known to be due to a single protein being able to affect multiple hallmarks simultaneously (Vogelstein *et al.*, 2013), which in turn is possible due to very few, albeit intricately regulated, core effectors of cell signaling pathways (Lemmon *et al.*, 2010).

2.1.1 Gain-of-function of oncogenes

Proto-oncogenes encode proteins whose abnormally increased activation can directly provide growth advantage for cells. Genetic or epigenetic alterations leading to their increased activity, i.e. gain-of-function alterations, turn proto-oncogenes into oncogenes - proteins responsible for normal cell proliferation and survival harnessed by cancer to help it grow (Figure 2). Gain-of-function alterations can lead to i) increased abundance or ii) enhanced or prolonged activity of the oncogene, often sufficiently by alteration affecting only one of the two gene alleles. These are often achieved by gene amplifications, activating/stabilizing mutations, and increased transcription via translocations/gene fusions or epigenetic mechanisms. It is noteworthy that activating mutations of oncogenes often occur at a specific amino acid residue, such as KRAS G12C/V/D, and even with a specific amino acid substitution, such as BRAF V600E. These highly recurrent mutations are termed hotspot mutations.

Many proto-oncogenes encode proteins that act as extracellular growth stimuli, transmembrane receptors that receive and confer the signal inside the cell, or as a part of the intracellular signaling network converting the growth signal into functional outputs (Hunter, 1997). Of these, the latter two are frequently kinases; enzymes that transfer a phosphate group from adenosine triphosphate (ATP) to other proteins, or other substrates such as carbohydrates or lipids, in a reaction termed phosphorylation. Phosphorylation cascade of tyrosine, serine or threonine residues

of proteins is an essential means of relaying signals within a cell to control cell behavior and fate in multicellular organisms.

The first identified oncogene was v-SRC, a mutated kinase encoding gene in Rous sarcoma virus (RSV) that caused neoplastic transformation of cultured cells (Martin, 1970). Similar to SRC, many human genes were then linked to cancer by identification of their mutant viral homologs causing cancer, such as RAS family of GTPases, MYC transcription factors and ERBB family of receptor tyrosine kinases (RTK) (discussed in chapter 2.2) (Tsuchida *et al.*, 1982; Downward *et al.*, 1984; Bishop, 1985; Schechter *et al.*, 1985).

Although oncogenes are considered as the driving force of the constant division of cells, cells have developed multiple tumor suppressive mechanisms to limit the uncontrolled growth in case of oncogenic alterations. In fact, oncogene activation in the absence of deactivated tumor suppressors can lead to growth arrest, termed oncogene-induced senescence, or even apoptosis (Chandeck *et al.*, 2010). The antiapoptotic state of senescence is well exemplified by oncogenic *BRAF* mutations in melanocytes forming benign naevi, which can only progress to cancer if additional mutations in tumor suppressor genes, such as phosphatase and tensin homolog (*PTEN)*, overcome the constant antiapoptotic and antiproliferative response (Dhomen *et al.*, 2009).

2.1.2 Loss-of-function of tumor suppressors

Tumor suppressor genes can be divided to i) gatekeepers which limit the proliferation or promote death of cell with damaged DNA and to ii) caretakers that repair damaged DNA to maintain genomic integrity. Thus, their inactivation by loss-of-function alterations allows growth advantage for the cell in which it occurs (Figure 2). Often loss-of-function alterations in both alleles of a tumor suppressor is deleterious while one functional allele can be sufficient to perform its functions, introduced as a two-hit hypothesis (Knudson, 1971). Major loss-of-function alterations of tumor suppressors include transcriptional silencing, deletions and inactivating/destabilizing mutations. The importance of tumor suppressors was initially recognized (Knudson, 1971) and still highlighted by germline mutations in these genes, predisposing to early onset hereditary cancers (Fearon, 1992; Malkin, 1994; Kinzler *et al.*, 1996; Kim *et al.*, 2004).

The best-known tumor suppressor is p53, encoded by *TP53*, which is mutated or the pathways it is involved in are altered in nearly all human cancers due to its key role in halting cell cycle, activating DNA repair, and regulating angiogenesis (Junttila *et al.*, 2009). Although *TP53* is considered as a gatekeeper whose germline mutations are an early event in tumorigenesis in certain cancer types, its mutations often occur at a later stage in other cancer types, indicative of a role in tumor

progression rather than initiation in such cancer types (Kinzler *et al.*, 1996; Levine, 1997). Another key regulator of signaling networks of cell cycle progression is transcription factor encoded by pRB in retinoblastomas (giving rise to its name) as well as in other cancer types (Lipinski *et al.*, 1999). Examples of cell type-specific gatekeepers are *APC* in colon cancer, regulating the transcriptional activity of β-catenin, the master switch of proliferation especially in intestinal cells (Kinzler *et al.*, 1996), whereas von Hippel-Lindau (*VHL*) loss-of-function alterations are known to promote clear-cell renal cell carcinoma, as it negatively regulates hypoxia-inducible factor (HIF) 2 α-mediated angiogenesis and cell proliferation (Mandriota *et al.*, 2002; Kaelin, 2008).

Caretakers are genes involved in different DNA repair mechanisms, such as mismatch repair (MMR) and homologous recombination (HR). Their loss-of-function alterations do not directly promote cell proliferation but rather accelerate the accumulation of genetic alterations, thus increasing the risk of tumorigenesis. Examples of caretaker alterations leading to hypermutated cancers are MMR gene mutations, such as those of *MLH1*, *MSH2*, *MSH6*, and *PMS2,* occurring especially in colorectal cancers (Kinzler *et al.*, 1996), as well as HR involved *BRCA1* and *BRCA2* mutations occurring typically in breast, ovarian and prostate cancers (Lalle *et al.*, 1994; Ashour *et al.*, 2019).

2.1.3 Dual-role genes and tumor progression

Although increased genomic instability facilitates efficient adaptation to constantly changing environments as tumors grow, metastasize and undergo therapeutic pressure, excessive alterations can be deleterious even for cancer cells. Thus, cancer cells need to maintain a fine balance in not impairing survival and growth mechanisms but having sufficient proficiency for clonal evolution and plasticity. To maintain the balance, cancer cells can for instance harness dual-role genes, whose increased or decreased activity can either promote or limit cell growth, depending on the context (Figure 2). Important for this thesis is that ERBB4 appears to fall in this category, as will be discussed in section 2.3.

Many categorical tumor suppressors have for long known to possess context-dependent roles, such as p53 (Datta *et al.*, 2021). On the other hand, p53 inactivation can allow cell cycle progression despite oncogenic growth signaling and damaged DNA without triggering apoptosis, while on the other hand its overactivation in other contexts can trigger cell cycle arrest and DNA repair to protect cancer cell from damage-induced apoptosis for instance under therapy (Junttila *et al.*, 2009; Soussi *et al.*, 2015; Yue *et al.*, 2017). The dual-role both explains why some tumor suppressors can also have gain-of-function oncogenic alterations (Datta *et al.*, 2021) and why proto-oncogenes can have loss-of-function alterations.

Shen et al. systematically analyzed genes with reported dual-role in cancer in 12 major cancer types and found that such genes are often tumor suppressive in normal tissue but can become oncogenic upon dysregulation (Shen *et al.*, 2018). They found these gene products to be more highly networked than classical oncogenes and tumor suppressors, acting as central nodes in regulating transcription. Thus, it is not surprising that dual-role genes often encode transcription factors and kinases.

Examples of dual-role genes include such that are involved in cellular processes that are known to be advantageous for cancer cells in some contexts or stages of tumor development and disadvantageous in other contexts. These include autophagy (Russell *et al.*, 2022), inflammation (Coussens *et al.*, 2013), as well as epithelial-to-mesenchymal transition (EMT) which is associated with invasion and metastasis but also promotes stemness and therapy resistance (Wilson *et al.*, 2020; Dart, 2023). Loss-of-function of such genes is often due to epigenetic silencing rather than by genetic loss (Kim *et al.*, 2023). In alignment with this, tumor progression especially upon therapy is also enabled by epigenetic rewiring of transcriptional programs instead of only by genetic alterations.

2.1.4 Oncogene-targeted therapies and predictive markers

Targeted therapies have significantly improved cancer therapy over conventional cancer therapies such as chemotherapy and radiation due to more cancer cell-specific mechanisms of action resulting in more tolerable side effects. This is perhaps best demonstrated in oncogene-addicted cancers, a term introduced by Bernard Weinstein. The growth of oncogene-addicted cancer cells is dependent on a single oncoprotein or a pathway it regulates, in spite of harboring and constantly acquiring other genetic alterations which also contribute to the growth of these cancer cells (Weinstein *et al.*, 2006, 2008). Targeted or selective inhibition of such oncoproteins has turned out outstandingly successful in various cancers, including in BCR-ABL fusion gene-driven chronic myelogenous leukemia (CML) targeted with the first clinically approved targeted small-molecule drug imatinib (Druker *et al.*, 2001), as well as in multiple EGFR and ERBB2-driven cancers with EGFR and ERBB2 selective tyrosine kinase inhibitors (TKI) and monoclonal antibodies (mAb) (discussed in more detail in section 2.2.5.2).

As discussed earlier in section 2.1.1, many of the best-known oncogenes are kinases. Their selective inhibition with small-molecule kinase inhibitors competing with or blocking the binding of ATP in the kinase domain has turned out very feasible in the clinics, although resistance usually occurs after initial successful responses to therapy. Another challenge in the use of all targeted therapies is, however, finding predictive markers to facilitate directing the therapies to the right patients that will most likely benefit. The genetic and epigenetic alterations leading

to oncogene-addicted cancers are more often than not very diverse, making it challenging to establish predictive biomarkers for targeted therapies. An example of an ideal predictive marker is a hotspot mutation causing oncogene addiction, such as *BRAF* V600E highly recurrent driver mutation in melanoma, thyroid, colorectal and lung cancer and targetable with BRAF kinase inhibitors (Hauschild *et al.*, 2012; Planchard *et al.*, 2016; Kopetz *et al.*, 2019). Such predictive markers facilitate therapy decisions in large patient population based on a simple mutational analysis of a well-characterized oncogenic SNV. However, vast majority of hotspot mutations occur in less than 5% of all cancers, while less recurrent (non-hotspot), and thus more rarely functionally characterized, mutations may also cause oncogene-addiction (Garraway *et al.*, 2013; Dienstmann *et al.*, 2015; Chang *et al.*, 2016).

2.2 ERBB/HER receptors

The (human) epidermal growth factor receptor (EGFR/HER)-family, is one of the 19 human RTK subfamilies (Lemmon *et al.*, 2010; Wheeler *et al.*, 2015). The majority of all of the 55 human RTKs bind extracellular growth factor ligands and undergo autophosphorylation to start an intracellular signaling cascade in response to ligand binding. RTKs are highly conserved during evolution, as they regulate fundamental cellular functions, such as proliferation, differentiation, cell cycle, apoptosis, migration and metabolism (Ullrich *et al.*, 1990; Blume-Jensen *et al.*, 2001). Thus, perturbations of the intricately regulated RTK functions are linked to many pathologies, particularly to cancer. In fact, RTKs represent a high proportion of dominant oncogenes (Hunter, 1997).

EGFR was the first discovered RTK in 1977 (Das *et al.*, 1977), 15 years after its ligand epidermal growth factor (EGF) was first identified in 1962 (Cohen, 1962). EGFR was also the first RTK identified as oncogenic, as its stimulation was found to result in tyrosine phosphorylation, resembling the effects of the transforming Rous sarcoma virus (Ushiro *et al.*, 1980; Brugge *et al.*, 1981). Moreover, EGFR was found to be an orthologue of a retroviral oncogenic protein kinase, the avian erythroblastic leukemia viral oncogene (v-ERBB; giving rise to the name of ERBB receptors) (Downward *et al.*, 1984). EGFR was cloned in 1984 (Ullrich *et al.*, 1984) and later on, three paralogous genes to human EGFR were identified, ERBB2 (Coussens *et al.*, 1985; Stern *et al.*, 1986), ERBB3 (Plowman *et al.*, 1990) and ERBB4 (Plowman *et al.*, 1993a).

2.2.1 Structure and ligands

ERBB family of RTKs are growth factor receptors consisting of an extracellular/ectodomain (ECD), a single transmembrane helical domain and an

intracellular cytoplasmic domain (ICD) (Figure 3). ERBB receptors are heavily glycosylated transmembrane type I glycoproteins, making the receptors 170-185 kilodaltons (kDa) in molecular weight. The importance of glycosylation for ERBB receptor activity is discussed in more detail in section 2.2.3.

The ECD comprises subdomains I-IV, of which leucine-rich repeat domains I and III (or L1 and L2) mediate ligand binding whereas cysteine-rich II and IV (or C1 and C2) mediate receptor dimerization (Lax *et al.*, 1988; Ward *et al.*, 2001). The extracellular juxtamembrane region (eJM) harbors a proteolytic cleavage site in ERBB2 and ERBB4 which allows non-canonical signaling (discussed in section 2.3.2). The transmembrane domain consists of a single α-helical domain which also forms dimerization interface with another receptor monomer (Endres *et al.*, 2013), known to be enhanced by oncogenic ERBB2 mutations (Bargmann *et al.*, 1988; Weiner *et al.*, 1989). The ICD comprises intracellular JM region (iJM), which participates in regulation of receptor activity, the enzymatic kinase domain and the carboxy (C)-terminal tail harboring the binding sites for interacting proteins (Lemmon *et al.*, 2014).

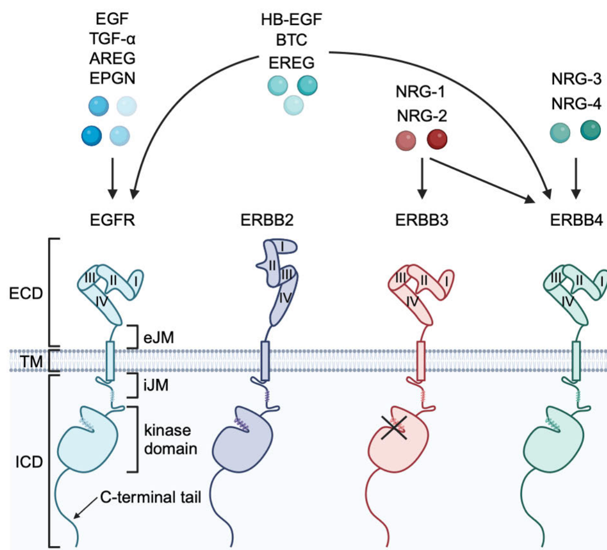


Figure 3. Structures and ligands of ERBB receptors. Ligands are grouped by their receptor specificities. ERBB receptors contain an extracellular/ectodomain (ECD), extracellular juxtamembrane region (eJM), transmembrane domain (TM), intracellular juxtamembrane region (iJM) and intracellular domain (ICD) containing kinase domain and carboxy (C)-terminal tail. Roman numerals indicate the ECD subdomains and the black cross indicates the impaired kinase domain of ERBB3. ERBB receptors are presented in their inactive/tethered/closed conformation, except for ERBB2, which is constitutively in open conformation. Created with BioRender.com.

Despite high level of similarity between the four receptors, ERBB2 is considered an orphan receptor without any known ligands (Cho *et al.*, 2003; Garrett *et al.*, 2003), whereas ERBB3 is a pseudokinase with impaired kinase activity (Guy *et al.*, 1994; Shi *et al.*, 2010). This makes ERBB2 dependent on other ERBB receptors for ligand-mediated activation of receptor dimers and ERBB3 dependent on other ERBB receptors for transphosphorylation of intracellular tyrosine residues (as will be discussed in more detail in section 2.2.2).

Eleven ERBB-binding ligands have been identified, with different binding affinities and specificities for the receptors (Figure 3). Four ligands bind only to EGFR, including EGF, transforming growth factor α (TGF-α), amphiregulin (AREG), and epigen (EPGN) (Cohen, 1962; Riese *et al.*, 1996a; Strachan *et al.*, 2001). Three of the ligands can bind both EGFR and ERBB4, including heparin-binding EGF-like growth factor (HB-EGF), betacellulin (BTC), and epiregulin (EREG) (Riese *et al.*, 1996b; Elenius *et al.*, 1997a; Riese *et al.*, 1998). The remaining four comprise neuregulins 1-4 (NRG), of which NRG-1 and NRG-2 bind to both ERBB3 and ERBB4, whereas NRG-3 and NRG-4 bind exclusively to ERBB4 (Plowman *et al.*, 1993b; Carraway *et al.*, 1994, 1997; Zhang *et al.*, 1997; Harari *et al.*, 1999). No ERBB2-binding ligands have been identified thus far, likely due to missing ligand-binding domain (Cho *et al.*, 2003) and inherent active conformation (Garrett *et al.*, 2003) (discussed in more detail below).

The diverse array of ERBB ligands enable fine-tuning of the signal to be mediated by each ligand via ERBB receptors. Although these ligands share the binding mechanism to ERBB receptors through a conserved EGF-motif (Harris *et al.*, 2003), they are known to activate ERBB receptor signaling with different kinetics, resulting in diversified cellular actions (Beerli *et al.*, 1996; Krall *et al.*, 2011; Macdonald-Obermann *et al.*, 2014; Freed *et al.*, 2017). This has been attributed to their ability to stabilize the receptor in slightly different conformations (discussed in more detail below, section 2.2.2) that in turn affects the signal that is being transduced inside the cell. For instance high-affinity EGFR ligand EGF can produce transient activation of downstream signaling pathways while low-affinity ligand EREG produces more sustained pathway activation, resulting in cell proliferation and differentiation, respectively (Freed *et al.*, 2017). Additionally, different ligands appear to induce selective phosphorylation of specific phosphotyrosines, resulting in recruitment of different interaction partners (Olayioye *et al.*, 1998; Sweeney, Lai, *et al.*, 2000). Ligands with varying receptor specificities also affect which ERBB homo- or heterodimers can be activated (Sweeney and Carraway, 2000; Avraham *et al.*, 2011). Moreover, the differential expression of ligands and receptors across tissues further dictates the output of ligand-mediated ERBB receptor activation.

The precursors of ERBB ligands (proligands) are membrane-bound proteins, which are cleaved by sheddases to release the ligands into the extracellular space, facilitating paracrine and autocrine signaling. These proteolytic sheddases include a disintegrin and metalloprotease (ADAM) family and matrix metalloprotease (MMP) families (Prenzel *et al.*, 1999; Lee *et al.*, 2003; Sanderson *et al.*, 2006), which are known to shed the ectodomains of many RTKs as well, including ERBB receptors (Huang, 2021). Shedding can both stimulate growth factor signaling via releasing soluble ligands to bind receptors but can also create "ligand-traps" by releasing soluble receptor ectodomains that can bind soluble ligands without the ability to elicit cellular responses. Interestingly, also backward signaling has been described, in which soluble ectodomain of ERBB4 binds to membrane-bound proligand NRG-1, eliciting signal transduction in the proligand expressing cell (Bao *et al.*, 2003; Iivanainen *et al.*, 2007; Hancock *et al.*, 2008; Mei *et al.*, 2014). Several ERBB ligands (TGF-α, HB-EGF, AREG and NRG-1) have also been demonstrated to be capable of juxtacrine signaling, in which membrane-bound proligand binds and activates membrane-bound receptor of a neighboring cell (Anklesaria *et al.*, 1990; Bao *et al.*, 2003; Willmarth *et al.*, 2006; Iivanainen *et al.*, 2007; Singh *et al.*, 2007). This enables changes in both cells as a response to the formation of a "synapse" between them.

2.2.2 Mechanism of activation

The mechanism of ERBB receptor activation as a result of ligand binding involves a sequence of outside-in (extracellular to intracellular) conformational changes enabling their autophosphorylation and subsequent downstream signaling pathway activation. Ligand binding mediates changes in extracellular dimer interactions of ERBB receptors, which result in conformational stabilization of the interactions of their transmembrane domains. This, in turn, releases the autoinhibitory conformation of the intracellular kinase domains allowing stabilization of their asymmetric positioning in the dimer required for transactivation, i.e. phosphorylation of receptor C-terminal tail tyrosines in *trans* (Tsai *et al.*, 2019). Many of the mechanisms have been discovered with studies focusing on EGFR but structures affecting the receptor activation-induced conformational changes, including dimerization interfaces, are conserved in other ERBB receptors (Trenker *et al.*, 2024).

Unique features of ERBB receptor activation

Two aspects make ERBB activation unique among RTKs. Firstly, receptor-bound ligands do not reside in the dimerization interface, physically participating in the interaction of two receptor monomers (Lemmon *et al.*, 2010). Instead, activated,

ligand-bound ERBB extracellular dimers are in a back-to-back configuration, in which dimerization arms mediate dimer interactions – as opposed to ligands bridging the monomers together, as ERBB receptor ligands point out from the dimer interface (Figure 4). Secondly, the transactivation of dimerized ERBB receptors occurs via allosteric activation of asymmetrically positioned receptor ICD (Jura *et al.*, 2011). In contrast, for other RTK dimers, autophosphorylation of kinase domain activation loop is a prerequisite for efficient substrate phosphorylation (Lemmon *et al.*, 2010; Maruyama, 2014).

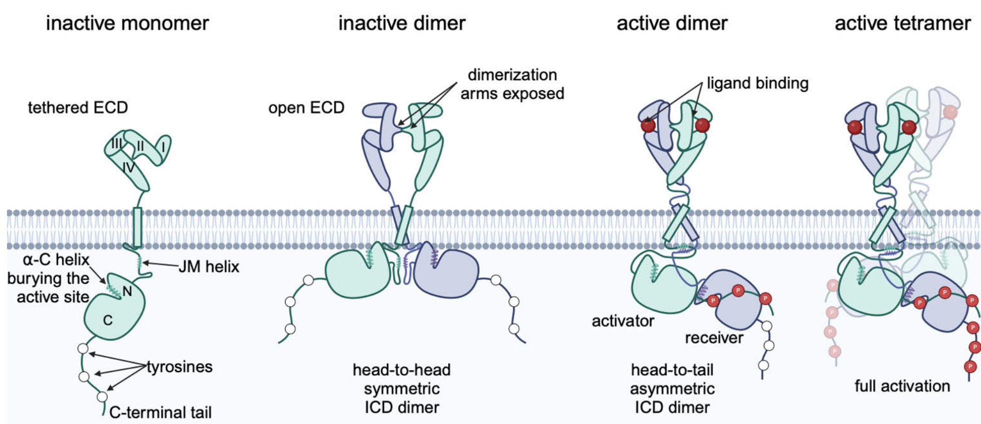


Figure 4. Mechanism of ERBB receptor activation. Inactive ERBB receptors may reside as monomers or predominantly in pre-formed dimers, in which the extracellular/ectodomain may adopt either a tethered/closed conformation (dimerization arm buried) or an open non-liganded conformation (dimerization arm exposed). The intracellular domain (ICD), remains in an autoinhibited state, in which the α-C helix buries the active site of the kinase domain - even in pre-formed dimers, in which the ICDs adopt an autoinhibited head-to-head symmetric juxtapositioning. Upon ligand binding, further conformational changes take place: stabilization of ECD dimerization interfaces of the dimerization arms and subdomain IV region, rotation of transmembrane domains, leading to antiparallel positioning of the ICD JM helices, allowing the ICDs to adopt the asymmetric head-to-tail positioning, resulting in α-C helix uncovering the active site of the kinase and transphosphorylation of the C-terminal tail tyrosine residues of the activator kinase by the receiver kinase. Ligand binding leads to full allosteric activation of ERBB receptors in higher-order oligomers, allowing phosphorylation of all monomers in the complexes. Adapted from Burgess et al. 2003, Zhang et al. 2006, Arkhipov et al. 2013, Walker et al. 2012, Endres et al. 2013, Purba et al. 2017, Huang et al. 2021. Created with BioRender.com.

Apart from the orphan receptor ERBB2, ERBB receptor ECDs remain in inactive/tethered/closed conformation in the absence of a ligand. Ligands bind between the two ECD subdomains I and III, disrupting a tether between subdomains II and IV that are responsible for the autoinhibitory tethered ECD conformation (Cho *et al.*, 2002; Ferguson *et al.*, 2003; Bouyain *et al.*, 2005). Thus, ligand binding stabilizes the open conformation, disclosing the receptor dimerization arm to interact

with another receptor monomer in an open conformation (Burgess *et al.*, 2003). Ligand binding is also essential for further conformational changes through the transmembrane domains, ultimately resulting in ICD kinase activation (Moriki *et al.*, 2001; Maruyama, 2014). This has been demonstrated by mutational disruption of the tethered conformation, resulting in increased ligand affinity but not increased activation in the absence of ligands (Elleman *et al.*, 2001; Ferguson *et al.*, 2003; Mattoon *et al.*, 2004; Walker *et al.*, 2004).

The ERBB receptor kinase domain activation involves the asymmetric head-to-tail configuration of the ICDs in the active dimer, in which the other receptor monomer functions as a receiver kinase and the other as an activator/donor kinase (Figure 4) (Zhang *et al.*, 2006; Qiu *et al.*, 2008; Jura *et al.*, 2009a; Monsey *et al.*, 2010; Aertgeerts *et al.*, 2011; Jura *et al.*, 2011; Endres *et al.*, 2013). Upon activation, the transmembrane domain rotation disrupts the autoinhibitory ICD interactions with the cell membrane (Arkhipov *et al.*, 2013), releasing the iJM of the receiver kinase to come in close contact with the C-terminal lobe (C-lobe) of the activator kinase. This interaction, together with the JM helix interaction of both monomers stabilizes the configuration in which the N-terminal lobe (N-lobe, head) of receiver kinase is juxtaposed with the C-lobe (tail) of the activator kinase. The head-to-tail positioning allows the C-lobe of the activator to induce conformational changes (allosteric activation) in the N-lobe of the receiver (Jura *et al.*, 2009b; Red Brewer *et al.*, 2009). This involves an α-C helix repositioning to uncover the active site (including the activation loop) of the kinase domain of the receiver kinase, sterically allowing the binding of a substrate that can be phosphorylated. The receiver kinase can then phosphorylate the activator kinase in *trans*, which is why the kinase-impaired ERBB3 is efficiently phosphorylated by other ERBB receptors but not vice versa, and hence, ERBB3 acts always as an activator kinase. That is also why ERBB3 is not capable of forming enzymatically active homodimers. Interestingly, also other ERBB receptors have hierarchical preferences for acting as either activator or receiver, depending on the dimerization partner. Ward and Leahy have demonstrated with chimeric ERBB receptors that the preferences are driven by ICDs alone, uncoupled from ECDs, and that EGFR most strongly prefers to act as a receiver in any ERBB heterodimer, followed by ERBB2 and then by ERBB4 (Ward *et al.*, 2015).

Despite the impaired kinase activity of ERBB3, other ERBB receptors have been found to be phosphorylated in dimers with ERBB3. This is currently thought to be due to higher order oligomerization of ERBB receptors, in which kinase active ERBB receptors can transphosphorylate each other (discussed more below). Additionally, ERBB receptors appear to have a weak capacity of autophosphorylation in *cis*, as demonstrated with EGFR (Yun *et al.*, 2007).

The symmetry of ECDs of ERBB receptors in an active dimer correlates with signaling strength (Garrett *et al.*, 2002; Ogiso *et al.*, 2002; Freed *et al.*, 2017). Since ligands stabilize the open conformation of EGFR, ERBB3 and ERBB4, small differences in ligand structures are capable of fine tuning the symmetry in open conformation. High-affinity ligands, such as EGF and TGF-α for EGFR and NRG-1 and BTC for ERBB4 have been shown to form the most symmetric and stable dimers among ERBB receptors (Bessman *et al.*, 2014; Diwanji *et al.*, 2021; Trenker *et al.*, 2024). This is in contrast with the theory that ERBB2 is the preferred dimerization partner for all ERBB receptors due to its constitutively open conformation and early studies on ERBB dimerization hierarchy (reviewed in (Yarden *et al.*, 2012)). However, the recently published cryo-electron microscopy structures of all three ERBB2-containing heterodimeric complexes of ERBB receptors shed light to the mechanisms of ERBB heterodimerization with the orphan receptor ERBB2. These studies show that ERBB2 heterodimers with other ERBB receptors (single liganded) are not as stable as those of liganded EGFR and ERBB4 homodimers because non-liganded ERBB2 does not effectively stabilize dimerization arm of the heterodimerizing receptor (Diwanji *et al.*, 2021; Bai *et al.*, 2023; Trenker *et al.*, 2024). Although the dimerization arm stability is not required for ERBB2 heterodimer activity, the stability enhances signaling output, as demonstrated with an oncogenic ERBB2 S310F mutation that stabilizes ERBB3 dimerization arm in the heterodimer with mutant ERBB2 (Diwanji *et al.*, 2021). Moreover, this could explain why more recent studies have found high-affinity ligand-stimulated EGFR and ERBB4 to highly prefer homodimerization over heterodimerization with ERBB2 (Ferguson *et al.*, 2000; Trenker *et al.*, 2022; Bai *et al.*, 2023).

Paradigm change from ligand-induced dimerization to ligand-induced activation of pre-formed dimers and oligomerization

For long ERBB receptors were thought to be activated via ligand-induced dimerization but studies since early 2000's have accumulated evidence that dimerization and activation are two separate things. ERBB receptors were thought to reside mostly as monomers in tethered, autoinhibited conformation when expressed at physiological levels in the absence of ligands. However, studies utilizing receptor imaging in living cells with fluorescence resonance energy transfer (FRET)-based microscopy, and other advanced microscopy and spectroscopy methods have provided consistent evidence that unliganded ERBB receptors are primarily in pre-formed inactive dimers at the cell surface (Yu *et al.*, 2002; Clayton *et al.*, 2005, 2007; Saffarian *et al.*, 2007; Tao *et al.*, 2008; Nagy *et al.*, 2010; Kozer *et al.*, 2013; Huang *et al.*, 2016; Needham *et al.*, 2016), even at low expression levels

(van Lengerich *et al.*, 2017). Current understanding of ERBB receptor activation involves ligands binding to pre-formed inactive dimers, and inducing a slight conformational change to the already extended inactive dimer conformation, which induces rotation of the transmembrane domains that allows the intracellular kinase domain dimer shift from a symmetric, inactive juxtapositioning to an active asymmetric positioning (Figure 4) (Moriki *et al.*, 2001; Kozer *et al.*, 2011; Walker *et al.*, 2012; Purba *et al.*, 2017).

Moreover, ERBB receptors have been thought to function primarily as dimers but again, ample evidence demonstrates not only the presence but the evident predominance of higher order oligomers over dimers as the active ERBB signaling units (Clayton *et al.*, 2008; Walker *et al.*, 2012; Zhang *et al.*, 2012; Kozer *et al.*, 2013, 2014; Maruyama, 2014; van Lengerich *et al.*, 2017). In fact, it has been proposed that oligomerization could be necessary for full activation of ERBB receptors, and not only in the case of obligate hetero-associating receptors ERBB2 and ERBB3 (Figure 4) (Sliwkowski, 2012; Zhang *et al.*, 2012; Huang *et al.*, 2016; Needham *et al.*, 2016; van Lengerich *et al.*, 2017). For instance, the Jura laboratory reported that effective EGFR phosphorylation is not observed upon ERBB3 stimulation with NRG-1 despite the formation of active EGFR-ERBB3 dimers in which ERBB3 was phosphorylated, as ERBB receptors can be autophosphorylated even in *cis* in a dimer with kinase-impaired ERBB3 (van Lengerich *et al.*, 2017).

2.2.3 Signaling

ERBB receptors transduce the signals they receive by activating intracellular signaling pathways that modify cell behavior often by altering gene transcription (Figure 5). Different ERBB receptors can activate unique and shared signaling networks due to their variable C-terminal tails (Schulze *et al.*, 2005; Roskoski, 2014). The C-terminal tails contain most of the autophosphorylated tyrosines to which the signaling effector proteins can bind *via* their SRC homology (SH) 2 or 3, phosphotyrosine-binding (PTB), or WW domains. Phosphoproteomic screens have unveiled that there are several conserved tyrosines between different ERBB receptors that bind the same effectors. However, each ERBB receptor appears to have a preference for binding certain effectors, as they can contain multiple binding sites for the same effector. EGFR and ERBB4 are the most strongly inclined to binding growth factor receptor –bound protein 2 (GRB2) while ERBB2 is to binding SHC transforming protein (SHC), which both activate the mitogen-activated protein kinase (MAPK) pathway. In contrast, ERBB3 is the most inclined to bind phosphoinositide-3-kinase (PI3K) via its p85 subunit (p85), which activates the AKT pathway. Moreover, especially EGFR and ERBB4 have specific tyrosines that can bind multiple signaling or adaptor proteins (Schulze *et al.*, 2005; Kaushansky *et al.*,

2008; Hause *et al.*, 2012), further expanding the ERBB signaling network. The receptor-intrinsic preferences for coupling certain downstream signaling effectors add to the fine-tuning ability of ERBB receptors in triggering certain cellular responses through activation of specific homo- or heterodimeric complexes.

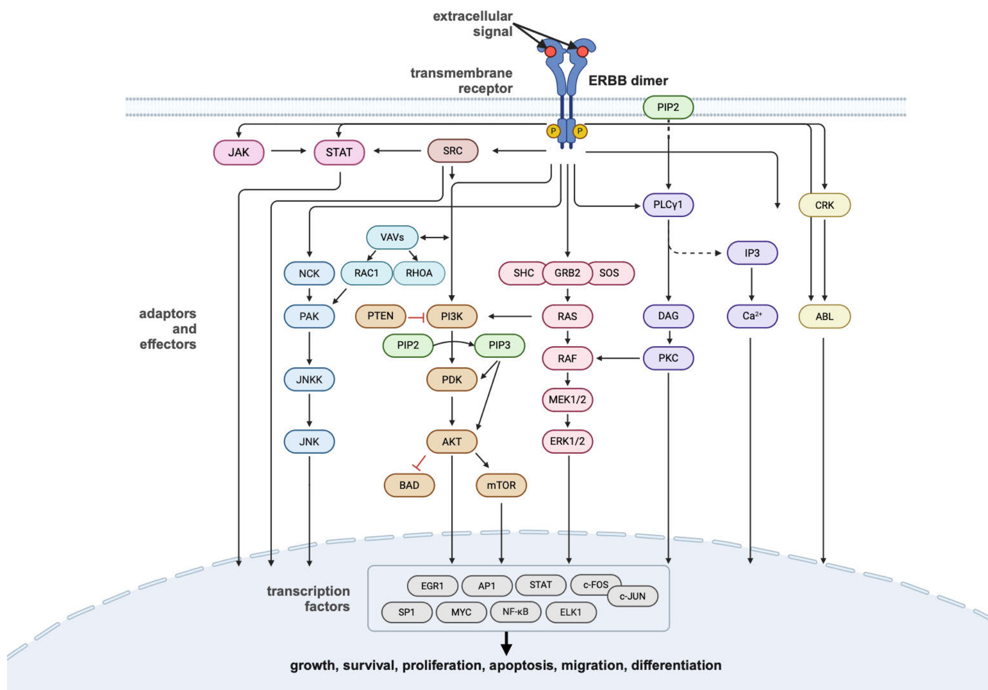


Figure 5. ERBB receptor-induced cell signaling. Ligands act as extracellular stimuli, received by ERBB receptors, which elicit signal inside the cell. ERBB receptor activation leads to recruitment of adaptor and effector proteins that relay the signal resulting in regulation of gene transcription, or direct regulation of for instance apoptosis or cytoskeletal organization. Adapted from Yarden and Sliwkowski 2001. Created with BioRender.com.

MAPK

ERBB receptors activate the MAPK pathway by binding to either GRB2 or SHC which recruit the guanine nucleotide exchange factor (GEF) son of sevenless (SOS) that activates the RAS GTPase (Schlessinger *et al.*, 2003). GTPases serve as molecular switches by alternating between inactive guanine diphosphate (GDP)-bound state and active guanine triphosphate (GTP)-bound state. Activated RAS initiates sequential phosphorylation of RAF (MAPK kinase kinase), MEK (MAPK kinase) and ERK (MAPK). This allows the nuclear translocation of ERK to regulate transcription via transcription factors such as c-FOS or MYC, often promoting cell proliferation. Notably, overactive MAPK pathway signaling is characteristic to

cancer cells, as is well demonstrated by the extraordinarily high prevalence of mutually exclusive oncogenic RAS, RAF and EGFR mutations across cancers (Roberts *et al.*, 2007; Ding *et al.*, 2008).

PI3K-AKT

PI3K-AKT pathway can be activated directly by ERBB3 and ERBB4 by binding to the PI3K regulatory subunit p85 (Schulze *et al.*, 2005). Direct association with EGFR and ERBB2 has not been reported thus far, but all ERBB receptors can also activate PI3K-AKT pathway indirectly, mainly through RAS as well as SRC, a kinase cross-connecting multiple signaling pathways (Rodriguez-Viciana *et al.*, 1994; Stover *et al.*, 1995; Wheeler *et al.*, 2015). The activated PI3K catalytic subunit p110 converts phosphatidylinositol-4,5-biphosphate (PIP2) to phosphatidylinositol-3,4,5-triphosphate (PIP3), recruiting the two serine-threonine kinases of which 3-phosphoinositide-dependent kinase 1 (PDK1) phosphorylates AKT. AKT activation promotes cell survival, growth and regulates metabolism for instance by negatively regulating pro-apoptotic proteins as well as mammalian target of rapamycin (mTOR). AKT can also promote migration, invasion and metastasis (Engelman, 2009). The tumor suppressor function of PTEN is partly due to its negative regulation of this pathway (Zhao *et al.*, 2008).

The heterodimer of ERBB2 and ERBB3 is considered to be the most potent activator of PI3K-AKT pathway among ERBB receptors due to the six p85 binding sites in ERBB3 out of its 11 phosphotyrosines. PI3K-AKT pathway is essential for cancer cell survival especially during anti-cancer therapy, thereby contributing to therapy resistance, especially in ERBB2 and ERBB3 driven cancers (Baselga *et al.*, 2009; Arteaga *et al.*, 2014; Haikala *et al.*, 2021).

PLCγ

PLCγ can be activated by ERBB receptors by direct binding through its SH2 domains, leading to hydrolysis of PIP2 to diacylglycerol (DAG) and inositol-1,4,5-triphosphate (IP3) (Margolis *et al.*, 1990; Peles *et al.*, 1991; Vecchi *et al.*, 1996; Schulze *et al.*, 2005). These in turn activate calcium dependent signaling and protein kinase C. PLCγ pathway activation can result in wide-ranging cellular responses such as proliferation, differentiation and migration (Bunney *et al.*, 2011; Yang *et al.*, 2013).

STAT

The signal transducer and activator of transcription (STAT) pathway can be activated either by direct binding of STATs to ERBB receptors, especially EGFR and ERBB4, or indirectly via ERBB activated SRC or janus kinases (JAK) (David *et al.*, 1996; Leaman *et al.*, 1996; Jones *et al.*, 1999a; Olayioye *et al.*, 1999; Schulze *et al.*, 2005). Upon activation by phosphorylation, STATs dimerize, translocate to nucleus and regulate gene transcription (Schindler *et al.*, 1992). ERBB receptor-mediated STAT signaling results in a diverse array of cellular responses, depending on the activated STAT and on the cellular context, from promoting differentiation, proliferation, survival and apoptosis. The seven different STAT proteins (STAT1-6, including STAT5a and STAT5b) are best known for their cytokine signal transduction in regulating immune cell functions but their activation via ERBB receptors appears to be distinct from that of cytokines (Kloth *et al.*, 2002; Schindler, 2002; Guren *et al.*, 2003; Quesnelle *et al.*, 2007).

VAV

ERBB receptors may also stimulate signaling via VAV family of GEFs that activate RHO family of GTPases, as demonstrated with the direct interaction of EGFR and VAV2 (Pandey *et al.*, 2000). In addition to EGFR, large-scale phosphoproteomic and interaction screens have showed that other ERBB receptors could also bind the three VAV family members *via* their SH2 domains (Kaushansky *et al.*, 2008; Hause *et al.*, 2012), but these interactions are yet to be validated and characterized.

VAV phosphorylation, upon binding to for instance to phosphorylated C-terminal tyrosines of RTKs, is required for their GEF function that activates RHO GTPases but VAVs also have GEF activity-independent functions (Dong *et al.*, 2006; Rodríguez-Fdez *et al.*, 2019). Active RHO GTPases, a subfamily of RAS GTPases, can promote a variety of cellular functions, prominently the reorganization of actin cytoskeleton that facilitates cell migration and invasion (Van Aelst *et al.*, 1997; Rodríguez-Fdez *et al.*, 2019). ERBB-VAV signaling has thus far shown to involve RHO GTPase pathway activation and PI3K-AKT pathway. EGFR can directly phosphorylate VAV2, but the downstream signaling of VAV2 *via* RHO GTPase activation leading to for instance migration also requires PI3K (Moores *et al.*, 2000; Pandey *et al.*, 2000; Tamás *et al.*, 2003). Indeed, VAVs can also activate the PI3K-AKT pathway (Uen *et al.*, 2015), ERBB receptors have been further linked to VAV signaling by studies showing that VAV3 participates in ERBB-induced PI3K-AKT-NF-κB signaling (Dong *et al.*, 2011, 2014). In addition, VAVs can also reciprocally be activated *via* PI3K-AKT pathway, highlighting their closely intertwined functions (Dong *et al.*, 2006; Lee *et al.*, 2008).

The diversity of VAV signaling mechanisms is further demonstrated in cancer, in which VAVs promote migration, invasion, EMT, stemness, proliferation and metastasis also, or perhaps primarily, in RHO- and PI3K-AKT-independent mechanisms (Lee *et al.*, 2008; Citterio *et al.*, 2012; Kwon *et al.*, 2015; Rodríguez-Fdez *et al.*, 2019; Al-Hawary *et al.*, 2023).

Other pathways

In addition to the pathways discussed above, ERBB receptors have been shown to activate several other MAPK pathways, including c-JUN N-terminal kinase (JNK), which they can activate either via binding directly to non-catalytic region of tyrosine kinase adaptor proteins (NCK), or indirectly via VAV GEFs (Olson *et al.*, 1996; Bost *et al.*, 1997; Kaminuma *et al.*, 2001; Poitras *et al.*, 2003; Huang *et al.*, 2004). EGFR and ERBB4 can also activate ABL directly or via CDC42/RAC1-activated kinase (CRK) (Birge *et al.*, 1993; Kaushansky *et al.*, 2008), the dominant oncogene in chronic myeloid leukemia upon fusion with breakpoint cluster region (BCR).

Post-translational regulation of ERBB signaling

ERBB signaling is not only governed by the composition of ligand-receptor complexes and their specific set of phosphotyrosines. Post-translational modifications (PTM) other than tyrosine phosphorylation, also affect ERBB receptor subcellular localization, their accessible interaction partners and the duration of signal firing. Thus, not all PTMs can be categorized in negative or positive regulators but rather as modulators of ERBB signaling. However, regulation mechanisms often associated with terminating ERBB signaling include dephosphorylation by various phosphatases, ubiquitination by various ubiquitin ligases resulting in either lysosomal or proteasomal degradation, promotion of transcription of negative regulatory proteins (Yarden *et al.*, 2001; Wheeler *et al.*, 2015).

One of the major processes regulating ERBB receptor activation and stability is glycosylation, mainly asparagine (N)-linked glycosylation (Cummings *et al.*, 1985; Contessa *et al.*, 2008). It is essential for ERBB receptor trafficking (Duarte *et al.*, 2022), ligand binding (Tsuda *et al.*, 2000; Kaszuba *et al.*, 2015), formation and stabilization of dimers (Tsuda *et al.*, 2000; Takahashi *et al.*, 2008; Motamedi *et al.*, 2022; Trenker *et al.*, 2024), and ability to promote autophosphorylation (Kawashima *et al.*, 2009; Britain *et al.*, 2018). Additionally, lack of glycosylation markedly decreases ERBB protein expression levels (Cummings *et al.*, 1985). Apart from promoting ERBB activity, also autoinhibitory roles for glycosylation have been identified by mutations of certain asparagine residues in ERBB receptors resulting in ligand-independent activation (Tsuda *et al.*, 2000; Takahashi *et al.*, 2008).

Another PTM-regulated process controlling ERBB receptor signaling duration and magnitude is endocytosis and subsequent degradation. EGFR signaling is rapidly attenuated via endocytosis and lysosomal degradation. This is due to ligand stimulated autophosphorylation of EGFR creating a binding site for ubiquitin ligase CBL that results in its sorting to late endosomes and lysosomal degradation (Levkowitz *et al.*, 1998). In contrast, other ERBB receptors are ubiquitinated by other ubiquitin ligases, predominantly controlling their steady-state levels and proteasomal degradation (Baulida *et al.*, 1996; Sundvall, *et al.*, 2008). For this reason, heterodimerization of activated EGFR with other ERBB receptors can block its endocytosis and extending its signaling and lifetime (Waterman *et al.*, 1998; Yarden *et al.*, 2001; Kiuchi *et al.*, 2014). Moreover, heterodimerization with ERBB2 can enhance recycling of all ERBB receptors to the cell membrane, and prolong their signaling in endosomes as a result of slowed ligand dissociation (Worthylake *et al.*, 1997; Lenferink *et al.*, 1998).

As mentioned in section 2.2.1, ERBB ectodomain shedding can create ligand-traps that downregulate ERBB activation (Ancot *et al.*, 2009) but in case the receptor is activated prior to shedding, the membrane-anchored receptor may remain active and keep signaling on its own (Ni *et al.*, 2001a; Liu *et al.*, 2020). In fact, this is a well-known and prevalent signaling mode for ERBB4, as will be further discussed in section 2.3.2. The ectodomain shedding allows a second cleavage that releases the ICD into the cytosol where it can translocate to other subcellular compartments, such as nucleus.

For long ERBB4 was thought to be the only ERBB receptor translocating to nucleus in the form of a soluble cleaved ICD, perhaps due to its widespread occurrence. However, a recent study showed that catecholamine-activated β2-adrenergic receptor signaling stimulates ERBB2 cleavage, resulting in its soluble ICD translocation to nucleus and regulation of transcription (Liu *et al.*, 2020). All ERBB receptors harbor the nuclear localization sequence and endocytosis allows translocation of full-length ERBB receptors to nucleus (Wheeler *et al.*, 2015). Nuclear ERBB receptors have been shown to co-activate or co-repress transcription with other transcription factors as they do not have a DNA-binding domain (Chen *et al.*, 2015).

Taken together, the main factors dictating the choice and magnitude of downstream signaling pathway activation via ERBB receptors are:

- availability of different ligands dictating the
    - activation of their preferred homo- and/or heterodimer complexes
    - intracellular tyrosines that will get autophosphorylated by the activated receptor complexes

- availability of homo- and/or heterodimerization partners
  - dictating the ability to recruit different interacting proteins
- availability of different ERBB receptor interaction partners in the given i) cell type, ii) subcellular location, ii) cellular state, including
  - signaling effector proteins
  - positive and negative regulators of ERBB signaling

2.2.4 ERBB receptors in physiology

ERBB receptor signaling is critical in both embryogenesis and maintenance of adult tissue homeostasis, as demonstrated by early embryonic lethality of *ERBB* knockout in mice and the involvement of dysregulated ERBB signaling in the pathogenesis of various human diseases. EGFR, ERBB2 and ERBB4 knockout mice die at mid-gestation while ERBB3 knockout is lethal at embryonic day 13.5 (Gassmann *et al.*, 1995; Lee *et al.*, 1995; Miettinen *et al.*, 1995; Erickson *et al.*, 1997). EGFR is indispensable in placenta and in epithelium development, such as that of skin, intestine and lung, as well as in central nervous system (Miettinen *et al.*, 1995). Both ERBB2 and ERBB4 are indispensable in heart development (Gassmann *et al.*, 1995; Lee *et al.*, 1995), whereas ERBB3 knockout mouse embryos die 3 days later, with defective atrioventricular valves and defective central and peripheral nervous system (Erickson *et al.*, 1997). The importance of also ERBB2 and ERBB4 in the nervous system development is evident in knockout mice rescued from embryonic lethality with cardiac ERBB2/4 expression resulting in defective central nervous system architecture and neuronal functions (Woldeyesus *et al.*, 1999; Tidcombe *et al.*, 2003). All ERBB receptors play also a major role in mammary gland development. While EGFR and ERBB2 are expressed at all stages across puberty and pregnancy, ERBB4 is clearly upregulated during late pregnancy and lactation, mirroring its importance in differentiation rather than proliferation of the mammary gland epithelium (lobuloalveolar development in the lactating mammary gland (Fowler *et al.*, 1995; Jones *et al.*, 1999a; Jones *et al.*, 1999b; Tidcombe *et al.*, 2003). In adult tissues other than the mammary gland, EGFR and ERBB2 play a significant role in proliferating cells for instance in the skin, lung and intestine, as mirrored in rash, lung interstitial disease and diarrhea being common side effects of ERBB-blocking drugs (Hervent *et al.*, 2012). ERBB4 in turn, has cardioprotective functions together with ERBB2, mirrored with cardiotoxicity of certain ERBB-targeting drugs (Hervent *et al.*, 2012). ERBB3 and ERBB4 are essential also in adult nervous system, such as neurotransmission, and in reproductive tissues (Wheeler *et al.*, 2015).

2.2.5 ERBB receptors in cancer

2.2.5.1 Dysregulation of ERBB signaling in cancer

EGFR was the first RTK linked to human cancer when its overexpression in HNSCC and brain tumors was discovered in 1984, (Hendler *et al.*, 1984; Libermann *et al.*, 1985). Three years later, the identification of ERBB2 amplifications in 15-30% of breast cancers by Slamon et al. was the first ever link of a transforming genetic alteration to poor prognosis in cancer patients (Slamon *et al.*, 1987, 1989). Ever since, oncogenic alterations of all ERBB receptors have been identified across various types of cancers, most prominently in solid tumors of epithelial and neural origin (Yarden *et al.*, 2012; Arteaga *et al.*, 2014; Segers *et al.*, 2020; Haikala *et al.*, 2021). Oncogenic overactivation of ERBB signaling stems mainly from genetic alterations of ERBB receptors or genetic and epigenetic alterations of their ligands. Overactive ERBB signaling promotes tumor growth by providing various hallmark abilities for cancer cells, especially via RAS and SRC-mediated signaling networks that have been demonstrated to be essential for ERBB-mediated cell transformation (Brugge, 1993). Additionally, ERBB signaling via PI3K-AKT pathway activation (direct activation most efficiently by ERBB3 in a heterodimer with ERBB2) is essential especially in ERBB2-addicted cancers that are co-dependent on ERBB3, as well as in ERBB3-mediated therapy resistance (Arteaga *et al.*, 2014; Haikala *et al.*, 2021; Hanker *et al.*, 2021). More recently, the role of ERBB signaling in tumor promoting immune responses has started gaining more attention (Kumagai *et al.*, 2021).

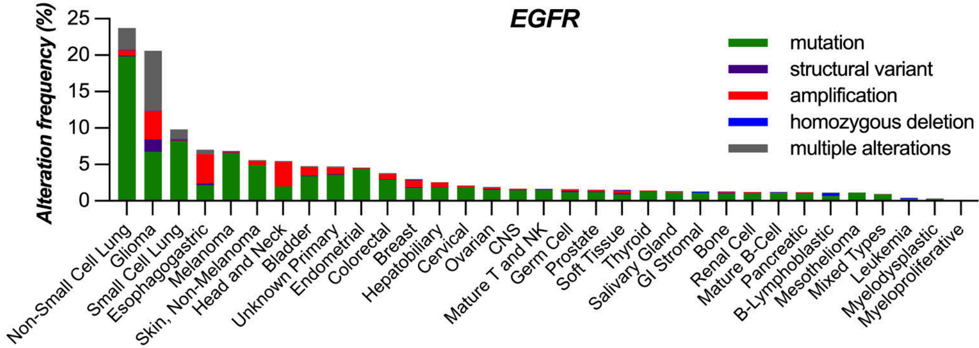
Overactive EGFR is an important driver of tumor growth especially in lung cancer, gliomas, colorectal, head and neck and esophageal cancers. EGFR is altered in 10-40% of lung cancer patients, in which activating kinase domain mutations in exons 18-21, especially L858R and exon 19 deletion mutations, predominate (Jänne *et al.*, 2004; Lynch *et al.*, 2004; Paez, *et al.*, 2004; Pao *et al.*, 2004) (Figure 6). This has led to major efforts in developing EGFR-targeted therapies, notably multiple generations of TKIs aiming to block secondary and tertiary mutations conferring TKI resistance, as will be discussed further in section 2.2.5.2. In the other cancer types, EGFR overactivation is mainly due to its amplification or overexpression via other mechanisms. In gliomas, *EGFR* amplifications (affecting 12-40% of the patients) generally co-occur with in-frame ectodomain mutations causing constitutive ligand-independent activation (Sugawa *et al.*, 1990; Wong *et al.*, 1992; Pedersen *et al.*, 2001) (Figure 6). While activating mutations in the EGFR downstream effectors KRAS, NRAS, and BRAF render cancers unresponsive to EGFR-targeted therapy, BRAF and KRAS-targeted therapy has interestingly shown to induce feedback EGFR activation and (re-)sensitization to EGFR-targeted therapies (Jänne *et al.*,

2005; Amado *et al.*, 2008; Di Nicolantonio *et al.*, 2008; Misale *et al.*, 2012; Prahallad *et al.*, 2012; Amodio *et al.*, 2020; Xue *et al.*, 2020).

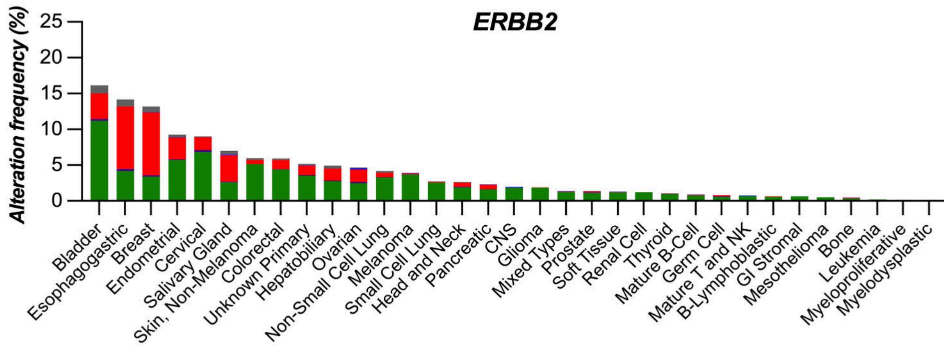
The most frequent type of *ERBB2* alterations in cancer is amplification, which occurs in 10-30% (Slamon *et al.*, 1987; Ross *et al.*, 1998) of breast cancers and also for instance in esophagogastric, colorectal, lung, bladder, endometrial and ovarian cancers (Arteaga *et al.*, 2014) (Figure 6). Additionally, activating *ERBB2* mutations have been identified. Among these, S310F is overall the most recurrent, although the predominant hotspots vary between major cancer types (Robichaux *et al.*, 2019). *ERBB2* mutations are often mutually exclusive with *ERBB2* amplifications, and occur most prominently in breast, lung, colorectal and bladder cancers (Greulich *et al.*, 2012; Bose *et al.*, 2013; Hanker *et al.*, 2017; Hyman *et al.*, 2018). The predictive value of activating *ERBB2* mutations for targeted therapy has not been as high as that of *ERBB2* amplification or activating *EGFR* mutations (Hyman *et al.*, 2018), perhaps partly due to higher co-dependence of ERBB3. While ERBB2-ERBB3 dimers have the strongest transforming potential among ERBB receptor dimers (Pinkas-Kramarski *et al.*, 1996; Tzahar *et al.*, 1996) and the transforming potential of ERBB2 requires ERBB3 in mouse mammary gland (Vaught *et al.*, 2012; Arteaga *et al.*, 2014; Haikala *et al.*, 2021), ERBB2 overexpression also promotes its unnatural homodimerization (Yarden *et al.*, 2001).

While *ERBB3* alterations are less frequent in cancer than those of *EGFR* and *ERBB2*, and despite both ERBB3 and ERBB4 have weaker transforming potential than EGFR or ERBB2 (Zhang *et al.*, 1996), ERBB3 does play an important role in ERBB2-addicted cancers but also in promoting resistance to various cancer therapies (Haikala *et al.*, 2021). The mechanism of ERBB3 activation in cancer is often due to its transcriptional overexpression or due to elevated NRG-1 availability (discussed in more detail below). However, missense mutations are the most common *ERBB3* genetic alterations (Figure 6). Activating *ERBB3* mutations have also been described, mainly in breast and esophagogastric cancers (Jaiswal *et al.*, 2013; Koivu *et al.*, 2024), while *ERBB3* mutations also occur in bladder, endometrial, and colorectal cancers (Figure 6). *ERBB3* mutations have been associated with poor response to pan-ERBB TKI neratinib in *ERBB2*-mutant patients (Hyman *et al.*, 2018; Smyth *et al.*, 2020) and mechanistically shown to enhance dimerization with ERBB2 to promote PI3K-AKT pathway activation (Hanker *et al.*, 2021).

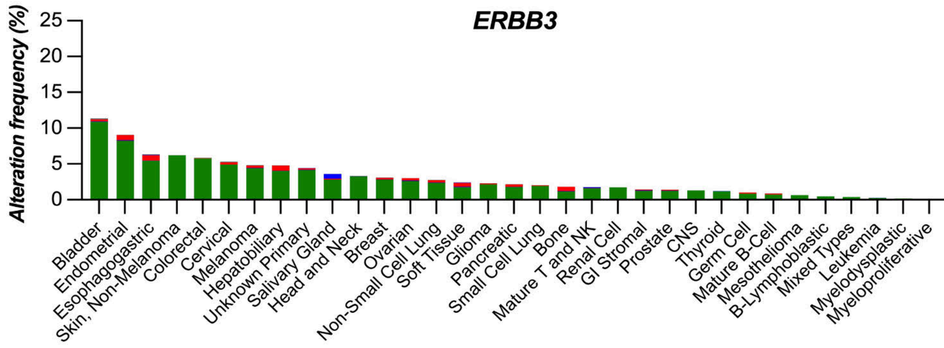
►**Figure 6.** Somatic *ERBB* receptor family gene alterations across cancer types. The Y-axis denotes the percentage of cancer patient samples with an *ERBB* gene alteration and the x-axis denotes the cancer type by histology or tissue of origin. Colors denote different alteration types: small mutations are displayed in green, structural variants in purple, amplifications in red, homozygous deletions in blue and samples with multiple alterations in grey. Original analysis and figure, data from AACR GENIE cancer type datasets in which all samples are profiled for all the shown alteration types and which have in total at least 1000 cases per cancer type (accessed: July 2024).



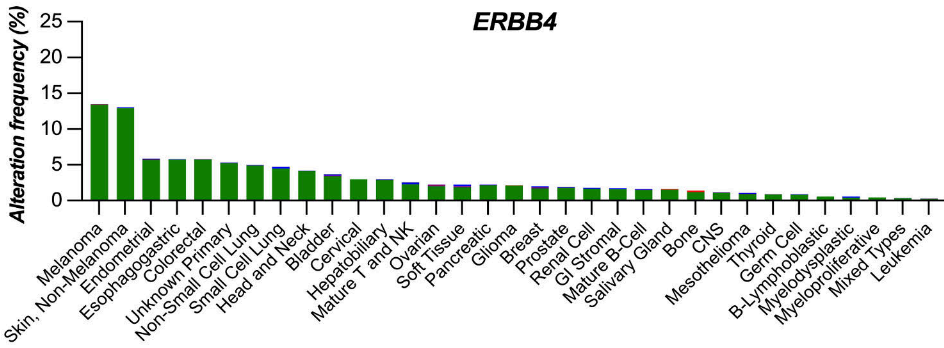
EGFR
Alteration frequency (%)
25
20
15
10
5
0
mutation
structural variant
amplification
homozygous deletion
multiple alterations
Non-Small Cell Lung
Glioma
Small Cell Lung
Esophagogastric
Melanoma
Skin, Non-Melanoma
Head and Neck
Bladder
Unknown Primary
Endometrial
Colorectal
Breast
Hepatobiliary
Cervical
Ovarian
CNS
Mature T and NK
Germ Cell
Prostate
Soft Tissue
Thyroid
Salivary Gland
GI Stromal
Bone
Renal Cell
Mature B-Cell
Pancreatic
B-Lymphoblastic
Mesothelioma
Mixed Types
Leukemia
Myelodysplastic
Myeloproliferative



ERBB2
Alteration frequency (%)
25
20
15
10
5
0
Bladder
Esophagogastric
Breast
Endometrial
Cervical
Salivary Gland
Skin, Non-Melanoma
Colorectal
Unknown Primary
Hepatobiliary
Ovarian
Non-Small Cell Lung
Melanoma
Small Cell Lung
Head and Neck
Pancreatic
CNS
Glioma
Mixed Types
Prostate
Soft Tissue
Renal Cell
Thyroid
Mature B-Cell
Germ Cell
Mature T and NK
B-Lymphoblastic
GI Stromal
Mesothelioma
Bone
Leukemia
Myeloproliferative
Myelodysplastic



ERBB3
Alteration frequency (%)
25
20
15
10
5
0
Bladder
Endometrial
Esophagogastric
Skin, Non-Melanoma
Colorectal
Cervical
Melanoma
Hepatobiliary
Unknown Primary
Salivary Gland
Head and Neck
Breast
Ovarian
Non-Small Cell Lung
Soft Tissue
Glioma
Pancreatic
Small Cell Lung
Bone
Mature T and NK
Renal Cell
GI Stromal
Prostate
CNS
Thyroid
Germ Cell
Mature B-Cell
Mesothelioma
B-Lymphoblastic
Mixed Types
Leukemia
Myelodysplastic
Myeloproliferative



ERBB4
Alteration frequency (%)
25
20
15
10
5
0
Melanoma
Skin, Non-Melanoma
Endometrial
Esophagogastric
Colorectal
Unknown Primary
Non-Small Cell Lung
Small Cell Lung
Head and Neck
Bladder
Cervical
Hepatobiliary
Mature T and NK
Ovarian
Soft Tissue
Pancreatic
Glioma
Breast
Prostate
Renal Cell
GI Stromal
Mature B-Cell
Salivary Gland
Bone
CNS
Mesothelioma
Thyroid
Germ Cell
B-Lymphoblastic
Myelodysplastic
Myeloproliferative
Mixed Types
Leukemia

ERBB4 is also frequently altered in cancer, mainly by missense mutations that occur as frequently as in 13-19% of melanomas and in other high incidence cancers including lung, endometrial, esophagogastric and colorectal cancer (Ding *et al.*, 2008; Prickett *et al.*, 2009; Kandoth *et al.*, 2013) (Figure 6). However, compared to other ERBB receptors, ERBB4 has less recurrent and distinct hotspot mutations; The most recurrent *ERBB4* hotspot mutation R711C has been reported in 64 cancer patients in AACR GENIE database, while the corresponding numbers for other ERBB receptors are: EGFR L858R n=1782, ERBB2 S310F n=487, ERBB3 V104M n=277. The functional significance of ERBB4 dysregulation in cancers will be discussed in section 2.3.4.

In addition to dysregulation of ERBB receptors, their ligands have been observed to become more abundant in diverse array of cancer types, especially as a mechanism of therapy resistance via epigenetic regulation (Ritter *et al.*, 2007; Trusolino *et al.*, 2012; Nafi *et al.*, 2014; Arribas *et al.*, 2023; Debets *et al.*, 2023). While tumors and cancer cells resistant to chemotherapy or targeted therapies, including but not limited to ERBB-targeted therapies, have often increased levels of multiple ERBB ligands (Trusolino *et al.*, 2012; Schwarz *et al.*, 2017; Arribas *et al.*, 2023), the most abundant and widespread evidence is for NRG-1 (Ritter *et al.*, 2007; Wang *et al.*, 2008; Bertotti *et al.*, 2011; Carrión-Salip *et al.*, 2012; Trusolino *et al.*, 2012; Wilson *et al.*, 2012; Hegde *et al.*, 2013; Nafi *et al.*, 2014; Yonesaka *et al.*, 2015; Schwarz *et al.*, 2017; Ogier *et al.*, 2018; Miyake *et al.*, 2020; Iida *et al.*, 2022; Debets *et al.*, 2023). In addition to increased expression levels of NRG-1, increased release of ERBB ligands has been observed as a result of increased activity of the pro-ligand sheddase ADAM17 (Wang *et al.*, 2008). Interestingly, MEK inhibitor resistance appears to act in the opposite direction, as it impairs ADAM17/ADAM10 sheddase activity, increasing the abundance of their substrate RTKs (such as AXL, MET, ERBB2, ERBB4) on the cell membrane, and thus, increasing cancer cell survival signaling via other pathways, such as PI3K-AKT (Miller *et al.*, 2016). In addition to epigenetic dysregulation, structural variants creating fusion proteins of NRG-1 and NRG-2 have been observed in cancer, especially in invasive mucinous lung adenocarcinoma (Trombetta *et al.*, 2017; Laskin *et al.*, 2020; Nagasaka *et al.*, 2022). These cancers are often highly sensitive to pan-ERBB inhibition by afatinib (Jones *et al.*, 2017; Laskin *et al.*, 2020; Cadranel *et al.*, 2021; Chen *et al.*, 2022), or to dual targeting of ERBB2 and ERBB3 (Schram *et al.*, 2022), indicative of its oncogene-addiction driving potential.

It is not completely understood why ERBB dysregulation mechanisms vary between cancer types, some having overexpression by amplifications or epigenetic regulation or increased ligand availability while others have activating mutations (Yarden *et al.*, 2001; Arteaga *et al.*, 2014). However, specific mutational signatures of carcinogens, epigenetic mechanisms such as chromatin accessibility, as well as

tissue specific availability of ligands, heterodimerization partners and downstream signaling interaction partners likely contribute to the differences between cancer types. Interestingly, while *EGFR, ERBB2* and *ERBB3* have distinct hotspots among mutations observed across all cancer types, also the hotspots vary and their predictive potential for ERBB-targeted therapies vary depending on the cancer type (Paez *et al.*, 2004; Pao *et al.*, 2004; Lee *et al.*, 2006; Vivanco *et al.*, 2012; Jaiswal *et al.*, 2013; Ma *et al.*, 2017; An *et al.*, 2018; Hyman *et al.*, 2018; Robichaux *et al.*, 2019; Smyth *et al.*, 2020).

2.2.5.2 ERBB-targeted therapies

Since the discovery of the transforming potential of EGFR and ERBB2, monoclonal antibodies and small molecule TKIs have been developed to block their functions. This led to the approval of ERBB2-targeting mAb trastuzumab for ERBB2-amplified metastatic breast cancer in 1998 as the first antibody-based targeted cancer therapy. The currently clinically used ERBB-targeted mAb-based and small molecule TKI drugs are listed with their predictive biomarkers and cancer type indications in Table 1. ERBB-targeted therapy indications currently comprise only cancers with EGFR or ERBB2 alterations. While both mAbs and TKIs are used to treat ERBB2-amplified cancers, EGFR-targeting mAbs are primarily used in cancer types in which EGFR is overexpressed whereas EGFR-targeting TKIs are used in cancer types with activating *EGFR* kinase domain mutations. This is mainly because EGFR-targeting mAbs can effectively block receptor overexpression-induced ligand-dependent EGFR signaling (Arteaga *et al.*, 2014) while the first-generation EGFR TKIs turned out to have higher affinity for the ligand-independently activating kinase domain hotspot mutants (exon 21-mutant L858R and exon 19 deletion mutants) over wild-type EGFR (Lynch *et al.*, 2004; Paez *et al.*, 2004), hence showing superior benefit in the treatment of non-small cell lung cancer, in which these mutations are highly prevalent (Maemondo *et al.*, 2010; Rosell *et al.*, 2012).

ERBB-targeting mAbs act either by disrupting ligand binding or dimers, inhibiting either ligand-dependent or ligand-independent ERBB signaling. EGFR-targeting antibodies block ligand binding, and thus, ligand-dependent EGFR signaling in EGFR-overexpressing cancer types in tissues naturally expressing EGFR ligands (Van Cutsem *et al.*, 2007, 2009). Yet, trastuzumab acts by inhibiting mostly ligand-independent signaling of the orphan receptor ERBB2 whose overexpression leads to aberrant activation of ERBB2 homodimers and heterodimers with ligand-free ERBB3 (Junttila *et al.*, 2009; Ghosh *et al.*, 2011). This is due to its binding to ERBB2 domain IV, which also blocks ERBB2 ectodomain shedding that would otherwise allow constitutive signaling of a

truncated protein that cannot be blocked with an antibody (Molina *et al.*, 2001). However, trastuzumab cannot effectively block ligand-induced activation of ERBB2 heterodimers with other ERBB receptors (Agus *et al.*, 2002), explaining its partial agonist effects on proliferative cancer cell signaling that were reported already in 1991 (Sarup *et al.*, 1991), whereas ERBB2 domain II-binding pertuzumab is more effective in blocking ligand-induced ERBB2-ERBB3 heterodimers (Agus *et al.*, 2002; Junttila *et al.*, 2009). The efficacy of trastuzumab and other ERBB-targeted mAbs (to varying degrees depending on the isotype of the mAb (Trivedi *et al.*, 2016)) *in vivo* stems strongly from their ability to bind Fc receptor expressing immune cells, hence inducing both innate and adaptive immunity through antibody-dependent cellular cytotoxicity and generation and recruitment of effector T cells (Clynes *et al.*, 2000; Scheuer *et al.*, 2009; Park *et al.*, 2010; Stagg *et al.*, 2011; Rugo *et al.*, 2021). The clinically approved ERBB2-targeting antibody-drug conjugates (ADC) have the mechanism of action of the mAb itself combined to the cytotoxic effect of the conjugated chemotherapeutic agent (Junttila *et al.*, 2011; Ogitani *et al.*, 2016).

The ERBB-targeting TKIs compete with ATP at the catalytic site in the kinase domain of ERBB-receptors, and thus can act by inhibiting the kinase activity of EGFR, ERBB2, and ERBB4 but not directly the pseudokinase ERBB3. The first-generation ERBB TKIs were developed as reversible inhibitors of EGFR, except for the EGFR/ERBB2-selective lapatinib that is also considered as a pan-ERBB inhibitor due to its ERBB4-blocking activity at higher concentrations (Rusnak *et al.*, 2001). The emergence of acquired resistance to the first-generation EGFR TKIs erlotinib and gefitinib was found to be conferred most prevalently by the secondary, gatekeeper mutation T790M that increases affinity for ATP (Kobayashi *et al.*, 2005; Pao *et al.*, 2005; Yun *et al.*, 2008). To overcome the resistance, second-generation TKIs were developed to irreversibly bind to the ATP-binding site at cysteine 773. They can indeed block T790M-mutant EGFR but at concentrations that block also wild-type EGFR, thus limiting their use in patients with secondary T790M mutations (Solca *et al.*, 2012). Yet, the second-generation EGFR TKIs afatinib, dacomitinib and neratinib, that are also termed as pan-ERBB inhibitors due to their high activity against ERBB2 and ERBB4 (Rabindran *et al.*, 2004; Li *et al.*, 2008; Solca *et al.*, 2012), are currently in clinical use (Table 1, and in Europe: afatinib and dacomitinib for treating advanced non-small cell lung cancer with activating EGFR mutations and neratinib as an extended adjuvant in early-stage hormone receptor-positive and ERBB2-positive breast cancer (ema.europa.eu)). The more recently approved third-generation irreversible ERBB TKIs include highly EGFR-mutant-selective osimertinib and mobocertinib, as well as the highly ERBB2-selective tucatinib that all spare wild-type EGFR (Cross *et al.*, 2014; Criscitiello *et al.*, 2023; Kobayashi *et al.*, 2024), resulting in more tolerable side-effect profiles.

Targeting the pseudokinase ERBB3 has been challenging. Although pan-ERBB inhibitors and mAb-based therapies have been clinically tested, none have thus far shown sufficient benefit for clinical approval, excluding the indirect ERBB2-targeting mAb pertuzumab (Haikala *et al.*, 2021). Pertuzumab binds ERBB2 domain II dimerization arm, blocking ligand-dependent ERBB2-ERBB3 signaling (Agus *et al.*, 2002; Franklin *et al.*, 2004) which is especially relevant in cancers with increased ERBB3 ligand availability, as discussed above in section 2.2.5.1. Recently however, ERBB3-targeting ADC patritumab-deruxtecan has showed significant benefit in clinical trials, especially in lung cancer (Jänne *et al.*, 2022), and is currently (since December 2023) under accelerated review for potential approval by FDA.

Acquired therapy resistance remains as the main challenge of all targeted therapies. Main mechanisms of resistance to ERBB-targeted therapies include mutations blocking drug binding (notably exon 20 insertions in *EGFR* and *ERBB2* and various ectodomain alterations), bypass signaling via other ERBB receptors or other RTKs, downstream pathway activating mutations, as well as *TP53* alterations that facilitate acquisition of genetic alterations (Garrett *et al.*, 2011; Wilson *et al.*, 2012; Misale *et al.*, 2012; Trusolino *et al.*, 2012; Bardelli *et al.*, 2012; Arteaga *et al.*, 2014; Smyth *et al.*, 2020; Sudhan *et al.*, 2020; Haikala *et al.*, 2021; Hanker *et al.*, 2021; Vokes *et al.*, 2022). Consequently, various strategies are being explored to overcome acquired resistance to ERBB-targeted drugs. These include new second-generation pan-ERBB inhibitors (developed against resistance mutations in EGFR/ERBB2 exon 20), fourth-generation allosteric EGFR TKIs that hold promise to block a wider range of resistance mutations while sparing wild-type EGFR (Gero *et al.*, 2022; To *et al.*, 2022), combinations with inhibitors of downstream effectors (such as KRAS and PI3K) (Amodio *et al.*, 2020; Hanker *et al.*, 2021) and covalent ligands inducing receptor degradation (Xie *et al.*, 2014; Yao *et al.*, 2020). Of particular interest, pan-ERBB inhibition strategies with already clinically used second-generation ERBB TKIs or combinations of multiple ERBB-targeting drugs have shown potential to overcoming resistance, also in sequential use (Kobayashi *et al.*, 2015; Yonesaka *et al.*, 2015; Schwarz *et al.*, 2017; Falk *et al.*, 2023; Udagawa, Nilsson, *et al.*, 2023; Frenel *et al.*, 2024).

Table 1. Table of currently FDA-approved ERBB-targeted therapies, indications and first-approval years. Adapted from Arteaga & Engelman 2014. +, amplification/overexpression; gen, generation; ins, insertion; mAb, monoclonal antibody; wt, wild-type.

Drug	Target	Cancer	Biomarker	Drug class	Year
Cetuximab	EGFR	colorectal, head and neck	EGFR+, wt KRAS (colorectal), BRAF V600E (colorectal)	mAb	2004
Panitumumab	EGFR	colorectal	wt KRAS, wt NRAS	mAb	2006
Necitumumab	EGFR	lung	N/A	mAb	2015
Amivantamab	EGFR, MET	lung	EGFR exon 20 ins	bispecific mAb	2021
Erlotinib	EGFR	lung, pancreas	EGFR exon 19 del or L858R	1st gen TKI	2004
Gefitinib	EGFR	lung	EGFR exon 19 del or L858R	1st gen TKI	2015
Dacomitinib	EGFR, ERBB2, ERBB4	lung	EGFR exon 19 del or L858R	2nd gen TKI	2018
Afatinib	EGFR, ERBB2, ERBB4	lung	EGFR exon 19 del or L858R /non-resistant EGFR mutation	2nd gen TKI	2013
Mobocertinib	EGFR (exon 20 ins)	lung	EGFR exon 20 insertions	3rd gen TKI	2021
Osimertinib	EGFR (T790M)	lung	EGFR T790M, exon 19 del or L858R	3rd gen TKI	2015
Pertuzumab	ERBB2	breast	ERBB2+	mAb	2012
Margetuximab	ERBB2	breast	ERBB2+	mAb	2020
Trastuzumab	ERBB2	breast, gastric	ERBB2+	mAb	1998
Trastuzumab emtansine	ERBB2	breast	ERBB2+	antibody-drug conjugate	2013
Trastuzumab deruxtecan	ERBB2	breast, gastric, lung	ERBB2+ (breast and gastric), ERBB2low (breast cancer), activating ERBB2 mutation (lung)	antibody-drug conjugate	2019
Lapatinib	ERBB2, EGFR	breast	ERBB2+	1st gen TKI	2007
Neratinib	ERBB2, EGFR, ERBB4	breast	ERBB2+	2nd gen TKI	2017
Tucatinib	ERBB2, (ERBB3)	breast, colorectal	ERBB2+ (breast), ERBB2+/wt RAS (colorectal)	3rd gen TKI	2020

2.3 ERBB4

2.3.1 ERBB4 isoforms

Unlike other ERBB receptors, ERBB4 has four naturally occurring alternative splice variants, encoding different isoforms of the receptor (Figure 7). These isoforms are expressed at different ratios in different tissues and have shared and unique functions.

Juxtamembrane isoforms

ERBB4 has two eJM splice variants, of which JM-a is produced by inclusion of exon 16 whereas JM-b includes exon 15. Exon 16 present in JM-a isoforms encodes a 23-amino acid region harboring a cleavage site for ADAM17 (a disintegrin and metalloprotease 17, also known as TACE, (tumor necrosis factor-α converting enzyme)), which is absent in the 13-amino acid region in JM-b (Elenius *et al.*, 1997b) (Figure 7). The ADAM17 cleavage site renders JM-a isoform prone to a two-step cleavage process involving ectodomain shedding (Rio *et al.*, 2000), which allows subsequent cleavage of the intracellular domain by γ-secretase, termed regulated intramembrane proteolysis (RIP) (Ni *et al.*, 2001). The cleaved ectodomain can serve for instance as a ligand-trap, diminishing the available ligands that could activate full-length ERBB4 receptors. RIP, in turn, releases the ERBB4 ICD in the cytosol to act as an independent signaling unit that can translocate for instance into nucleus or mitochondria (Ni *et al.*, 2001; Naresh *et al.*, 2006). Thus, ERBB4 cleavage via RIP facilitates diversified non-canonical signaling and an additional level of regulation of its functions (reviewed in more detail below) that differentiate JM-a and JM-b isoforms from each other.

Cytoplasmic isoforms

ERBB4 has also two different cytoplasmic splice variants, CYT-1 containing exon 26 and CYT-2 that lacks it (Elenius *et al.*, 1999). This 16-amino acid region encoded by exon 26 contains the only ERBB4 binding site for PI3K regulatory subunit p85, rendering the direct PI3K activation specific to CYT-1 isoform (Elenius *et al.*, 1999) (Figure 7). The CYT-1-specific region includes also an extra PPXY motif, additional to the two other PPXY domains shared between both CYT isoforms. PPXY serves as a binding site for WW domain-containing proteins such as YAP (yes-associated protein) (Komuro *et al.*, 2003) and ubiquitin ligases ITCH (E3 ubiquitin-protein ligase Itchy homolog) (Sundvall, *et al.*, 2008), and WWP1 (WW domain-containing E3 ubiquitin protein ligase 1) (Feng *et al.*, 2009). Despite two other PPXY domains

are shared between both CYT isoforms, the additional PPXY motif in CYT-1 has been shown to be essential for ubiquitination, endocytosis, and degradation of CYT-1, rendering CYT-2 notably less prone to negative regulation and thus, more biochemically active (Määttä *et al.*, 2006; Sundvall *et al.*, 2007; Sundvall, *et al.*, 2008). This more stable activity of CYT-2 is also the reason why ERBB4 CYT-2 translocates more efficiently into the nucleus (discussed more in section 2.3.2) (Sundvall, *et al.*, 2008).

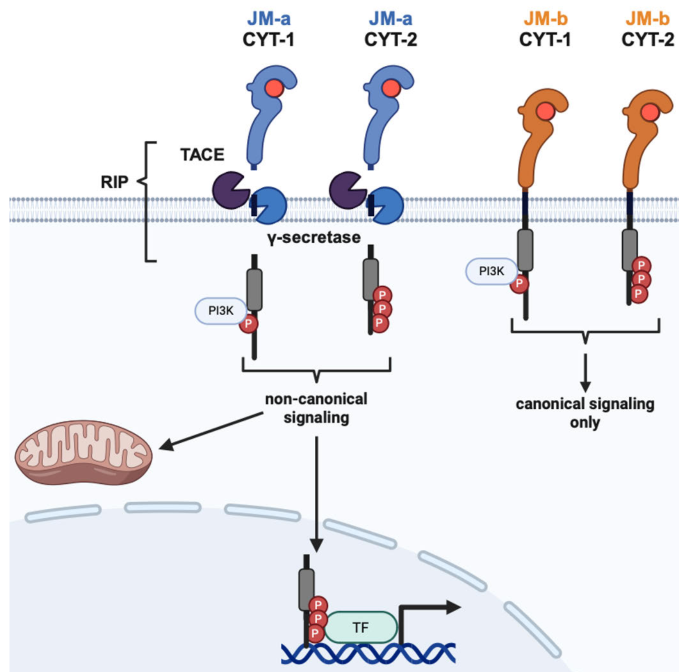


Figure 7. ERBB4 isoforms and non-canonical signaling via regulated intramembrane proteolysis (RIP). The four alternatively spliced consist of juxtamembrane (JM) a and b and cytoplasmic (CYT) 1 and 2 isoforms. JM-a isoforms harbor an extracellular cleavage site for tumor necrosis α-converting enzyme (TACE), which allows transmembrane site cleavage by γ-secretase. The RIP cleavages allow non-canonical ERBB4 signaling via soluble intracellular domain (ICD), which is an active kinase that can translocate to nucleus to regulate gene transcription or to mitochondria to regulate apoptosis. JM-b isoforms cannot be cleaved, rendering them only capable of canonical signaling. CYT-1 isoform contains a unique binding site for phosphatidylinositol 3-kinase (PI3K) subunit p85α, as well as an extra PPXY motif, both lacking from CYT-2. This makes CYT-1 but not CYT-2 prone to activation-induced degradation while CYT-2 isoforms are more stable upon activation and hence, more phosphorylated (as indicated with red circled P). Created with BioRender.com.

2.3.2 Non-canonical ERBB4 signaling via RIP

ERBB4 was the first RTK shown to undergo the two-step cleavage process RIP, including the ectodomain shedding by TACE/ADAM17 (Rio *et al.*, 2000) and subsequent cleavage by γ-secretase (Ni *et al.*, 2001). RIP is known for producing β-amyloid in Alzheimer's disease and for NOTCH signaling (Wolfe, 2020). ERBB4 RIP studies have then paved the way to understanding the role of RIP in RTK regulation and the discovery that at least half of all human RTKs can undergo RIP (Merilahti *et al.*, 2017). Now RIP is recognized as a significant post-translational regulation mechanism allowing vast diversification of RTK functions by enabling interaction with other signaling partners than the full-length membrane-bound RTKs (Ancot *et al.*, 2009; Merilahti *et al.*, 2017). Yet, among ERBB receptors, RIP-mediated signaling was for long thought to be unique to ERBB4 (JM-a isoforms) but ERBB2 was recently shown to undergo RIP, upon activation β2-adenergic receptors with catecholamines, and (Liu et al., 2020).

Ligand binding stimulates ERBB4 RIP, releasing the ICD containing an activated kinase into the cytosol, where it can signal on its own prior to its proteasomal degradation (Figure 7) (Linggi *et al.*, 2006a; Strunk *et al.*, 2007). The soluble ERBB4 ICD can either remain in the cytosol, translocate into nucleus to co-regulate gene transcription (Hollmén *et al.*, 2010), or into mitochondria to promote apoptosis (Naresh *et al.*, 2006). ERBB4 ICD-mediated transcriptional co-regulation has been demonstrated by its association with estrogen receptor (ER) α and β, ETO2, HIF-1α, AP2, KAP1, STAT5a, TAB2-NCoR, YAP, (Komuro *et al.*, 2003; Hoek *et al.*, 2004; Linggi *et al.*, 2006b; Sardi *et al.*, 2006; Zhu *et al.*, 2006; Gilmore-Hebert *et al.*, 2010; Sundvall *et al.*, 2010; Zscheppang *et al.*, 2011a; Zscheppang *et al.*, 2011b; Paatero *et al.*, 2013). Additionally, ERBB4 ICD has been shown to regulate p53 levels via phosphorylating its negative regulator MDM2 (Arasada *et al.*, 2005).

The nuclear ERBB4 functions are dependent on ERBB4 kinase activity, as that is required for its nuclear localization (Linggi *et al.*, 2006b; Määttä *et al.*, 2006; Muraoka-Cook, *et al.*, 2006a; Naresh *et al.*, 2006). Thus, the higher stability of CYT-2 (mentioned above) explains why it is the predominant ERBB4 isoform in the nucleus (Sundvall *et al.*, 2007; Sundvall *et al.*, 2008) and why CYT-2 promotes YAP target gene expression more strongly than CYT-1 (Haskins *et al.*, 2014), despite having one less YAP binding sites than CYT-1. The kinase activity of ERBB4 ICD is further enhanced by SUMOylation of ERBB4 ICD which in turn is required for nuclear accumulation of ERBB4 ICD (Knittle *et al.*, 2017). SUMOylation alters the nuclear functions of ERBB4 likely due to sequestering it in promyelocytic leukemia (PML) nuclear bodies in which various transcriptional co-regulators of ERBB4 also reside (Sundvall *et al.*, 2012; Knittle *et al.*, 2017).

As reviewed in section 2.2.4, ERBB4 plays a role in embryogenesis and homeostasis of adult tissues, and several of these functions have been attributed to

ICD-mediated ERBB4 signaling with *in vivo* models, including mammary gland development (Muraoka-Cook, *et al.*, 2006a; Paatero *et al.*, 2014), astrocyte differentiation in brain development (Sardi *et al.*, 2006), and fetal lung development (Hoeing *et al.*, 2011; Zscheppang *et al.*, 2011a; Zscheppang *et al.*, 2011).

2.3.3 Context-dependency of ERBB4 functions

The two JM isoforms are differentially expressed in tissues and cell types. Transcriptional analyses of human and mouse tissues have shown that JM-a predominates in many epithelial tissues (such as mammary gland, bladder, kidney, prostate, testis, uterus), while JM-b predominates mesenchymal tissues (such as heart and skeletal muscle) (Elenius *et al.*, 1997b; Junttila *et al.*, 2005; Veikkolainen *et al.*, 2011). Although both isoforms appear to be expressed in tissues of the nervous system, cell type level analyses have shown that JM isoforms are mutually exclusively expressed (Elenius *et al.*, 1997b; Veikkolainen *et al.*, 2011). To date, there are no reports of endogenous expression of both JM isoforms in a same cell type. In contrast to JM isoforms, both CYT isoforms are generally expressed at similar levels across tissues (Elenius *et al.*, 1999; Junttila *et al.*, 2005; Veikkolainen *et al.*, 2011). However, only CYT-2 has been detected in placental tissue (Veikkolainen *et al.*, 2011).

While ERBB4 signaling is known to regulate cell growth, differentiation, cell death and migration, these functions appear to be highly dependent on the isoform in question as well as on the cell context. In fact, previous studies have shown even contradictory functions for the different isoforms when expressed in a certain cell context while the same isoform can have opposing functions depending on cellular context. However, many of the ERBB4-related functional studies have relied on overexpressing a single ERBB4 isoform and not comparing side-by-side the different isoforms – even the isoforms that are known to be co-expressed physiologically or pathologically in tissues/cell types corresponding the used cell models. This has led to considerable bias in how ERBB4 functions are interpreted.

Direct comparisons of different overexpressed ERBB4 isoforms in non-cancerous cell lines have shown that ERBB4 JM-a CYT-2 promotes survival and proliferation of for instance fibroblasts and myeloid cells while JM-b CYT-2 promotes cell death (Määttä *et al.*, 2006; Sundvall *et al.*, 2010). In contrast, JM-b CYT-1 can sustain NRG-1-dependent myeloid cell growth whereas JM-a CYT-1 does not (Määttä *et al.*, 2006). Another study reported that in JM-b context CYT-1 promotes fibroblast survival and migration while CYT-2 promotes fibroblast proliferation instead (Kainulainen *et al.*, 2000). Contradictorily, CYT-2 isoforms (both JM-a and JM-b) were shown to promote kidney cell migration better than CYT-1 isoforms (Zeng *et al.*, 2007).

A more recently generated ERBB4 JM-a-specific knockout mouse model demonstrated that ERBB4 JM-a isoforms are indispensable for brain cortical development (Doherty *et al.*, 2022), as predicted earlier with the combination of ERBB4 knockout mouse model and isoform-specific *in vitro* models (Sardi *et al.*, 2006). In contrast, ERBB4 JM-a isoforms are not necessary for heart, mammary gland nor sensory ganglia development even though non-canonical nuclear ERBB4 (JM-a) signaling has previously been demonstrated to participate in mammary gland development (Williams *et al.*, 2004; Muraoka-Cook *et al.*, 2006a; Muraoka-Cook *et al.*, 2009b). Together, these raise a question whether ERBB4 JM-b can compensate for the loss of non-canonical ERBB4 JM-a signaling during mammary gland development. Doherty et al. also created an ERBB4 shedding-site-mutant mouse model but it failed to impair ERBB4 shedding and non-canonical RIP-mediated ERBB4 signaling, highlighting the challenges in modeling and deconvoluting isoform specific ERBB4 functions.

Nevertheless, many of the functional differences of JM-a and JM-b functions can likely be attributed to soluble ICD-mediated signaling versus membrane-bound full-length ERBB4 signaling (discussed below), as well as to the two JM isoforms being expressed in different tissue/cell types. Additionally, it was recently shown that the JM isoforms have unique motifs dictating their location at cell surface and affecting their downstream signaling functions (Vaparanta *et al.*, 2022). Since CYT-1 and CYT-2 are generally expressed simultaneously, studies taking into account their interplay and possible predominance in pathological conditions could further improve our understanding of ERBB4 functions.

Taken together, the highly variable and context-dependent functions of ERBB4 are understandable considering the following differences of ERBB4 isoforms:

- tissue/cell type expression patterns – affecting availability of interaction partners
- binding sites for interacting proteins (signaling partners and regulatory proteins) – affecting signaling qualitatively and quantitatively
- subcellular localization patterns – affecting availability of interaction partners

2.3.4 ERBB4 in cancer

The role of ERBB4 in cancer has remained ambiguous due to conflicting results providing evidence for both a tumor suppressive and an oncogenic role (Arteaga *et al.*, 2014; Mota *et al.*, 2017; Segers *et al.*, 2020; Lucas *et al.*, 2022). Main challenges in understanding the role of ERBB4 in cancer include:

- context-dependency of the role of ERBB4 regarding molecular subtype of cancer, stage, and other clinical characteristics
- complexity of the role of different ERBB4 isoforms
- complexity of ERBB4 functions in different subcellular compartments
- low endogenous expression levels in 2D cell cultures of majority of cancer cell lines
- rarity of specific cancer-associated *ERBB4* mutations despite overall high frequency of *ERBB4* mutations across various cancers

2.3.4.1 ERBB4 dysregulation in cancer

Altered ERBB4 expression and prognostic value in cancer

ERBB4 expression is frequently dysregulated in various cancer types but its clinical significance remains ambiguous due to conflicting reports of its prognostic value. Both increased and decreased ERBB4 expression levels compared to non-cancerous tissue have been reported but ERBB4 expression has been shown to positively correlate with more advanced tumor stage in colorectal cancer (Williams *et al.*, 2015), whereas negative correlation has been shown in hepatocellular cancer (Lee *et al.*, 2004). Genetic alterations affecting ERBB4 expression levels include copy number alterations, and both gains/amplifications and homozygous deletions even in same cancer types have been reported, without a consensus of their functional significance (cBioPortal and AACR GENIE). Epigenetic dysregulation of ERBB4 in cancer is less studied but the reports to date about miRNA-mediated regulation demonstrate predominantly ERBB4 upregulation in cancer. These argue for an oncogenic role of ERBB4 in gastric, esophageal, ovarian, thyroid, and lung cancers and gliomas (Zhang *et al.*, 2014; Liang *et al.*, 2015; Song *et al.*, 2017; Chen *et al.*, 2018; Nie *et al.*, 2020; Liu *et al.*, 2021; Zhou *et al.*, 2021).

Studies reporting high ERBB4 expression associating with more favorable prognosis, such as in hormone receptor-positive breast cancer, hepatobiliary cancers, prostate cancer and bladder cancer, suggest that ERBB4 may function as a tumor suppressor in these contexts. In contrast, studies reporting high ERBB4 expression associating with worse prognosis, such as triple negative breast cancer, hormone-independent prostate cancer, sarcomas, lung, esophagogastric, colorectal, ovarian, and head and neck cancers are indicative of ERBB4 functioning as an oncogene (Segers *et al.*, 2020; Lucas *et al.*, 2022). Notably, there are inconsistent results within same cancer types about ERBB4 expression association with prognosis. However, more detailed studies, especially in breast cancer, in which ERBB4 has been most

widely studied, have provided explanations for the conflicting results. Thus, the current understanding is that prognostic significance of ERBB4 can be affected by cancer molecular subtype, ERBB4 isoform, subcellular localization, hormone-dependency and treatment history (Chuu *et al.*, 2008; Nafi *et al.*, 2014; Kalita-de Croft *et al.*, 2020; Segers *et al.*, 2020; Brockhoff, 2022; Lucas *et al.*, 2022).

JM-a isoforms are considered as cancer-associated ERBB4 isoforms due to being the predominant ERBB4 isoforms expressed across various cancer types (Junttila *et al.*, 2003, 2005; Veikkolainen *et al.*, 2011). Even in the cancer types in which JM-b is more abundant than JM-a, such as in brain cancers, the abundance of JM-a relative to JM-b has been shown to increase compared to corresponding non-cancerous tissue (Gilbertson *et al.*, 2001, 2002; Zeng *et al.*, 2009; Donoghue *et al.*, 2018). For instance in gliomas, increased JM-a expression has been associated with increased ERBB4 phosphorylation and worse prognosis despite overall reduction in ERBB4 expression levels (due to JM-b downregulation) (Donoghue *et al.*, 2018). Although both CYT isoforms are virtually always co-expressed in healthy and cancerous tissues (Veikkolainen *et al.*, 2011), high JM-a CYT-1 expression has been found to associate with worse prognosis in ovarian cancer while JM-a CYT-2 not (Paatero *et al.*, 2013), again highlighting the divergent functions of ERBB4 isoforms.

Since the JM-a isoforms are prone to RIP, they can also localize to nucleus and nuclear localization of ERBB4 has been associated with poor prognosis and therapy resistance for instance in different subtypes of breast cancer and in esophageal cancer (Junttila *et al.*, 2005; Tovey *et al.*, 2006; Xu *et al.*, 2008; Nafi *et al.*, 2014; Kalita-de Croft *et al.*, 2020). These patient data suggesting a more aggressive oncogenic role for the nuclear signaling of ERBB4 have also been corroborated by functional studies (discussed in section 2.3.4.2.). Apart from nuclear ERBB4, there is also a constantly growing body of clinical evidence that overactivation, overexpression and mutations of ERBB4 associate with resistance to both targeted and chemotherapy in various cancer types; Higher ERBB4 expression and phosphorylation associates with resistance to ERBB2-targeted therapy in breast cancer regardless of ERBB2 expression status (Nafi *et al.*, 2014; Debets *et al.*, 2023), to PI3K inhibitor idelalisib in B cell lymphomas (Arribas *et al.*, 2023), and to antiestrogen therapies and CDK4/6 inhibitor abemaciclib in ER-positive breast cancer (Wege *et al.*, 2018; Albert *et al.*, 2024). Higher ERBB4 expression associates also with resistance to chemotherapy in ovarian cancer (Saglam *et al.*, 2017), sarcomas (Merimsky *et al.*, 2002; Mendoza-Naranjo *et al.*, 2013) and non-small cell lung cancer (Merimsky *et al.*, 2001). *ERBB4* mutations, in turn, have been associated with shorter progression free survival (PFS) in *EGFR*-mutant non-small cell lung cancer patients treated with second or later-line osimertinib after progression during first-generation EGFR TKIs (Vokes *et al.*, 2022), as well as in gastric cancer patients treated with adjuvant chemotherapy (Yuan *et al.*, 2023) and in ERBB2-amplified gastric cancer patients treated with

trastuzumab (Wang *et al.*, 2019; Zhang *et al.*, 2023). However, the mutations identified in the relapsing patients were not functionally analyzed in these studies. Notably, preclinical data showing that ERBB4 promotes therapy resistance even in cancer types in which it is strongly believed to function as a tumor suppressor, such as in hepatocellular and prostate cancer (Vexler *et al.*, 2008; Carrión-Salip *et al.*, 2012; Breitenecker *et al.*, 2023), argues against categorizing ERBB4 strictly as either oncogenic or tumor suppressive based on cancer type. Instead, it further supports the role of ERBB4 as an important oncogene in promoting cell survival and proliferation under therapy pressure, regardless its role in tumorigenesis in a given tissue.

Taken together, mere expression levels of ERBB4 are often not indicative of its oncogenic activity in cancer tissue. Instead, ERBB4 phosphorylation could be a more useful clinical measure of its role as an oncogenic driver, disregarding the mechanism of overactivation - whether it is genetic or epigenetic alteration of ERBB4 expression levels or activating genetic alterations. Yet, the clinical contexts in which ERBB4 activation could have predictive value warrant further elucidation.

Cancer-associated ERBB4 mutations

Reports of high frequency of somatic *ERBB4* mutations in cancer started to emerge after targeted sequencing efforts in major cancer types by Soung et al. in 2006, and by Kandoth et al. reporting *ERBB4* to be among the four most mutated RTKs in major cancer types (Soung *et al.*, 2006; Kandoth *et al.*, 2013). However, despite the high frequency of cancer-associated *ERBB4* missense mutations (Figure 6), there are no distinct *ERBB4* hotspots (II, Supplementary Fig. S1B), unlike in other ERBB genes (discussed in section 2.2.5.1). Consequently, the functional data of these mutations has remained very limited.

Four studies, apart from this thesis, have functionally characterized a set of *ERBB4* mutations in several major cancer types; melanoma (Prickett *et al.*, 2009), head and neck (Nakamura *et al.*, 2016), breast, gastric, colorectal and lung cancer (Tvorogov *et al.*, 2009; Kurppa *et al.*, 2016). Together, 11 gain-of-function mutations and two loss-of-function mutations were identified in these studies to be potentially oncogenic. Seven melanoma-associated mutations were found to be transforming, activating, promoting PI3K-AKT signaling, and their growth promoting effects were sensitive to first-generation pan-ERBB inhibitor lapatinib (Prickett *et al.*, 2009). Kurppa et al. found four lung cancer-associated mutations to be activating and to enhance dimer interactions also with ERBB2, whereas three of these mutations were shown to promote cell survival under serum-starvation via RIP-mediated signaling (Kurppa *et al.*, 2016). A head and neck cancer patient mutation was found to be transforming, activating and targetable *in vivo* with second-generation pan-ERBB afatinib (Nakamura *et al.*, 2016). Another *in vivo* evidence for

second-generation pan-ERBB inhibitor efficacy against an activating ERBB4 mutation, E317K (first identified as a gain-of-function mutation by Prickett et al. 2009), was shown in a GBM xenograft model (Donoghue *et al.*, 2018).

In contrast, two loss-of-function mutations, lung cancer-associated G802dup and colon cancer-associated D861Y were found to be virtually kinase-dead, and to suppress differentiation of triple negative breast cancer cells upon NRG-1 stimulation (Soung *et al.*, 2006; Tvorogov *et al.*, 2009). Interestingly, while these kinase-dead mutations impaired STAT5-mediated differentiation signaling, they did not affect ERBB2-heterodimer-mediated MAPK and PI3K-AKT pathway activation (Tvorogov *et al.*, 2009), suggesting that they are able to mediate proliferative and survival signaling when transphosphorylated by a kinase-competent dimerization partner. Additionally, a gastric cancer patient mutation S774G has been suggested to sensitize ERBB2-amplified gastric cancer cells to trastuzumab but the experiment was not controlled with alike overexpression of wild-type *ERBB4*, and thus cannot be distinguished from the effect of wild-type ERBB4 (Wang *et al.*, 2019).

Together, the current evidence suggests that driver *ERBB4* mutations are predominantly gain-of-function mutations, and thus, could potentially be targeted with ERBB4-blocking drugs. Yet, the clinical significance of the majority of cancer-associated *ERBB4* mutations remains poorly understood.

2.3.4.2 ERBB4 functions in cancer

Oncogenic ERBB4 signaling

The oncogenic potential of ERBB4 was discovered upon its overexpression being able to transform fibroblasts (Cohen *et al.*, 1996; Zhang *et al.*, 1996). Since then, overactive ERBB4 signaling has been demonstrated to promote growth, cell survival, migration and EMT *in vitro* and *in vivo* models of various cellular backgrounds (Arteaga *et al.*, 2014; Mota *et al.*, 2017; Segers *et al.*, 2020; Lucas *et al.*, 2022). The oncogenic functions have been mainly attributed to the cancer-associated ERBB4 isoforms, JM-a CYT-1 and JM-a CYT-2 (Veikkolainen *et al.*, 2011).

ERBB4 oncogenicity has been demonstrated in several *in vivo* models. A mammary gland-specific overexpression of ERBB4 JM-a CYT-1 and JM-a CYT-2 resulted in neoplastic lesions in mice, more often with JM-a CYT-1 than JM-a CYT-2, and in a solid tumor in one out of 12 JM-a CYT-1 overexpressing mice (Wali *et al.*, 2014a). However, mammary gland-specific overexpression of soluble ERBB4 ICDs (mimicking non-canonical ERBB4 signaling) has demonstrated neoplastic growth promoting potential for CYT-2 but not for CYT-1, suggesting that soluble ERBB4 ICDs have divergent functions from full-length ERBB4 *in vivo* (Wali *et al.*,

2014b). The oncogenic role of ERBB4 soluble ICDs *in vivo* was also demonstrated with patient-derived xenografts (PDX) of ALK-negative anaplastic large-cell lymphoma, expressing truncated ERBB4, the constitutively active soluble ICD of ERBB4. Moreover, the PDX tumors were sensitive to second-generation pan-ERBB inhibitor neratinib (Scarfò *et al.*, 2016). Also other studies using xenograft models of cancer cells of various lineages have shown that downregulation or pharmacological inhibition of ERBB4 compromises tumor growth (Nakamura *et al.*, 2016; Donoghue *et al.*, 2018; Wang *et al.*, 2018; Xu *et al.*, 2018).

Growth stimulatory and survival promoting functions of ERBB4 have been mainly attributed to activating MAPK, PI3K-AKT and WNT5a pathways (Segers *et al.*, 2020; Lucas *et al.*, 2022). Additionally, ERBB4 has been shown to promote more aggressive cancer cell phenotypes through EMT, chemotaxis, migration and invasion by interacting with YAP in the nucleus (Komuro *et al.*, 2003; Haskins *et al.*, 2014; Shi *et al.*, 2018; Kalita-de Croft *et al.*, 2020), and via PI3K-AKT pathway (Kainulainen *et al.*, 2000; Kang *et al.*, 2007; Mendoza-Naranjo *et al.*, 2013; Li *et al.*, 2024), as well as via uncharacterized mechanisms (Mill *et al.*, 2011; Kiuchi *et al.*, 2014). The oncogenic functions of nuclear ERBB4 ICD, particularly those of the CYT-2 isoform (due to its higher stability, kinase activity and nuclear retention, as discussed in section 2.3.2), also include inhibition of differentiation and promoting the growth of undifferentiated structures in mammary epithelial models *in vitro* and *in vivo* (Muraoka-Cook *et al.*, 2009; Sundvall *et al.*, 2012; Knittle *et al.*, 2017), Together, these studies provide mechanistic evidence for the reported clinical associations of nuclear ERBB4 with poor survival, as discussed in section 2.3.4.1.

Importantly, ERBB4 has also been implicated in resistance to chemotherapy and EGFR/ERBB2-targeted therapy in various cancer types (as discussed above). Mechanistically, nuclear signaling of ERBB4 ICD, such as via YAP and PI3K-AKT, could be responsible for ERBB4-mediated therapy resistance for instance to ERBB2-targeted therapies in breast and gastric cancer (Nafi *et al.*, 2014; Shi *et al.*, 2018). This is supported by the high nuclear ERBB4 staining in trastuzumab treated ERBB2-amplified breast cancer patients and xenografts while the effect can be reversed in mice with pan-ERBB inhibitor neratinib (Nafi *et al.*, 2014). Intriguingly, despite the ability to promote trastuzumab resistance, ERBB4 co-expression in ERBB2-amplified breast cancer has been shown to sensitize to trastuzumab therapy (Sassen *et al.*, 2009). This could be due to trastuzumab being able to block oncogenic ERBB2-ERBB4 heterodimer signaling perhaps via canonical signaling pathways, whereas nuclear ERBB4 ICD signaling could promote survival upon prolonged therapy pressure.

Tumor suppressive ERBB4 signaling

Growth inhibition and differentiation promoted by overactive ERBB4 signaling have been mechanistically linked to STAT5a and JNK pathway activation, promoting p53 and BRCA1 activity, as well as stimulation of apoptosis via soluble ICD localization to mitochondria (Segers *et al.*, 2020; Lucas *et al.*, 2022). Interestingly, while many of the oncogenic ERBB4 mutations are attributed to non-canonical signaling via soluble ICD, especially to its nuclear transcription co-regulatory functions, many of the growth inhibitory functions are associated with ERBB4 soluble ICD as well (Williams *et al.*, 2004; Vidal *et al.*, 2005; Naresh *et al.*, 2006). However, these ERBB4 RIP-dependent growth inhibitory roles have been demonstrated with ERBB4 JM-a CYT-1 isoform mainly in mammary epithelial or breast cancer cell culture contexts, an example of experimental settings in which JM-a CYT-1 is growth inhibitory whereas JM-a CYT-2 is growth promoting (Sartor *et al.*, 2001; Junttila *et al.*, 2005; Vidal *et al.*, 2007; Muraoka-Cook *et al.*, 2009b). Thus, it is not surprising that the constitutively activating synthetic ERBB4 mutations in JM-a CYT-1 isoform (not reported in cancer patients thus far) created by the Jones and Riese laboratories were also shown to be growth inhibitory or pro-apoptotic while those of JM-a CYT-2 were not (Pitfield *et al.*, 2006; Lucas *et al.*, 2022). Importantly, patient data and *in vivo* experimental data align with the theory that the soluble (nuclear) CYT-2 ICD is more oncogenic. Firstly, CYT-2 is more oncogenic than CYT-1 in a mouse model of mammary gland-specific overexpression of soluble ERBB4 ICDs (Wali *et al.*, 2014b). Secondly, the association of nuclear ERBB4 with poor prognosis and therapy resistance especially in breast cancer (discussed above), a context in which both CYT isoforms are present, is logical considering that CYT-2 localizes more efficiently in the nucleus due to its enhanced stability and higher autophosphorylation level (Määttä *et al.*, 2006; Sundvall *et al.*, 2007; Sundvall *et al.*, 2008).

In vivo studies demonstrating tumor suppressive functions of ERBB4 are mainly from hepatocellular cancer models. Epigenetic silencing of *ERBB4 via* miR-93-5p has been shown to be tumor suppressive in hepatocellular cancer xenograft mouse model (Li *et al.*, 2021). A liver-specific *ERBB4* knockout resulted in spontaneous tumor formation in 11% of the mice, accompanied with increased inflammation and reduced p53 function mechanistically linked to ERBB4 regulated gene transcription (Liu *et al.*, 2017). Additionally, in mouse models of liver injury and chemically induced inflammation, ERBB4 deficient mice formed more tumors compared to controls, further supporting an anti-inflammatory and anti-fibrotic role for ERBB4. Since these findings were from mice with *ERBB4* knockout in all tissues except for heart, they support the emerging anti-inflammatory role of ERBB4 previously recognized in macrophages (Schumacher *et al.*, 2017; Vermeulen *et al.*, 2017; Segers *et al.*, 2020). The anti-inflammatory role of ERBB4 could possibly promote crosstalk

between cancer cells and immune effector cells, perhaps allowing immune evasion of the cancer once its established.

The theory of ERBB4 becoming tumor promoting at a later stage in some contexts (such as immune evasion and therapy resistance), while playing a tumor suppressive role in the initiation stage of certain cancer types, fits well with the role of ERBB4 co-operating with tumor suppressors p53 (Arasada *et al.*, 2005) and BRCA1 (Muraoka-Cook *et al.*, 2006b). As mentioned in section 2.1.2, these both maintain genome integrity by promoting growth arrest and DNA repair. Since ERBB4 promotes survival across various cancer types upon DNA damaging chemotherapy as discussed above, it is possible that ERBB4 could contribute to protecting cancer cells from damage-induced cell death, by allowing the cell to repair lethal damage, and thus, survive re-and re-enter proliferative state. Nevertheless, the observed growth inhibitory and growth promoting functions together with the highly interconnected and transcriptional co-regulatory functions of ERBB4 align with those of dual-role genes (discussed in section 2.1.3).

3 Aims

The overarching aim of this thesis was to evaluate the potential of ERBB4 as a therapeutic target in cancer. The complex ERBB4 signaling in cancer and the clinical significance of the hundreds of cancer-associated *ERBB4* mutations remain largely uncharacterized and call for more comprehensive analyses.

Therefore, the specific aims of this thesis were to:

- Elucidate ERBB4 signaling mechanisms in cancer by analyzing the protein interactome of both cancer-predominant ERBB4 isoforms in cancer cells
- Distinguish potential driver *ERBB4* mutations from passenger mutations by an unbiased high-throughput functional screen and by focused characterization of the emerging *ERBB4* hotspot mutations
- Evaluate the potential of using activating *ERBB4* mutations as biomarkers predicting sensitivity to clinically used pan-ERBB inhibitors

4 Materials and Methods

4.1 Plasmids (I-III)

4.1.1 Cloning

The previously described expression plasmids used in this study are listed in Table 2, and their references are listed in I-III. The expression plasmids generated in this study are listed in Table 3. In brief, expression plasmids were generated using standard molecular cloning procedures, either by restriction enzymes and ligation (I-III) or by Gateway cloning using BP or LR clonase II mix (Invitrogen) for recombination reactions to generate either pDONR221 donor vectors or pBABE-gateway retroviral expression plasmids, respectively (II-III). Site-directed mutagenesis was used to create point mutations either directly to pcDNA3.1hygro(+)-ERBB4JM-aCYT-2-HA and pEF4myc/His-VAV3 (I), or to pDONR221 vectors with primers listed in the supplementary material of I and II. All generated constructs were verified by sequencing the insert.

Table 2. Previously described expression plasmids used in this study.

Insert	Isoform	Backbone	Purpose	Used in
ERBB4-HA	JM-a CYT-2	pcDNA3.1hygro(+)	mammalian expression	I
ERBB4-MYC	JM-a CYT-2	pcDNA3.1hygro(+)	mammalian expression	I
ERBB4 ICD2-HA	ICD2	pcDNA3.1hygro(+)	mammalian expression	I
ERBB4 K751R-HA	JM-a CYT-2	pcDNA3.1hygro(+)	mammalian expression	I
VAV3	canonical	pEF4myc/His	mammalian expression	I
VAV3-R697L	canonical	pEF4myc/His	mammalian expression	I
VAV3-DC1-SH3B	canonical	pEF4myc/His	mammalian expression	I
VAV1	canonical	pC.HA	mammalian expression	I
VAV2	canonical	pC.HA	mammalian expression	I
VAV3	canonical	pC.HA	mammalian expression	I
ERBB3-HA	canonical	pcDNA3.1hygro(+)	mammalian expression	II

Insert	Isoform	Backbone	Purpose	Used in
ERBB4 K751R-HA	JM-a CYT-2	pcDNA3.1hygro(+)	mammalian expression	II
ERBB4 V956R-HA	JM-a CYT-2	pcDNA3.1hygro(+)	mammalian expression	II
-	-	pMSCV-PGK-Puro-IRES-GFP	retroviral expression	II
EGFR	canonical	pBABE-puro-gateway	retroviral expression	III
ERBB2	canonical	pBABE-puro-gateway	retroviral expression	III
ERBB3	canonical	pBABE-puro-gateway	retroviral expression	III
HIV-1 GAG/POL	-	pMLDg/pRRE	lentivirus packaging, mammalian expression	I-III
VSV-G	-	pMD2.G	lentivirus packaging, mammalian expression	I-III
REV	-	pRSV-Rev	lentivirus packaging, mammalian expression	I-III

Table 3. Expression plasmids generated in this study.

Insert	Isoform	Backbone	Purpose	Used in
ERBB4-STREP-TAG	JM-a CYT-1	pcDNA3.1neo(-)	mammalian expression	I
ERBB4-STREP-TAG	JM-a CYT-2	pcDNA3.1neo(-)	mammalian expression	I
ERBB4 ICD2-STREP-TAG	CYT-2	pcDNA3.1neo(-)	mammalian expression	I
ERBB4 Y1022F	JM-a CYT-2	pcDNA3.1hygro(+)-ERBB4JM-aCYT-2-HA	mammalian expression	I
ERBB4 Y1162F	JM-a CYT-2	pcDNA3.1hygro(+)-ERBB4JM-aCYT-2-HA	mammalian expression	I
ERBB4 Y1202F	JM-a CYT-2	pcDNA3.1hygro(+)-ERBB4JM-aCYT-2-HA	mammalian expression	I
ERBB4 Y1208F	JM-a CYT-2	pcDNA3.1hygro(+)-ERBB4JM-aCYT-2-HA	mammalian expression	I
ERBB4 Y1258F	JM-a CYT-2	pcDNA3.1hygro(+)-ERBB4JM-aCYT-2-HA	mammalian expression	I
VAV3 DN	canonical	pEF4myc/His	mammalian expression	I
eGFP	canonical	pBABEpuro-gateway	retroviral expression	II-III
ERBB4	JM-a CYT-1	pBABEpuro-gateway	retroviral expression	II
ERBB4	JM-a CYT-2	pBABEpuro-gateway	retroviral expression	II-III
ERBB4 Y52C	JM-a CYT-2	pBABEpuro-gateway	retroviral expression	II
ERBB4 R124K	JM-a CYT-2	pBABEpuro-gateway	retroviral expression	II

Insert	Isoform	Backbone	Purpose	Used in
ERBB4 R687K	JM-a CYT-2	pBABEpuro-gateway	retroviral expression	II
ERBB4 E715K	JM-a CYT-2	pBABEpuro-gateway	retroviral expression	II
ERBB4 G741R	JM-a CYT-2	pBABEpuro-gateway	retroviral expression	II
ERBB4 G802D	JM-a CYT-2	pBABEpuro-gateway	retroviral expression	II
ERBB4 M993I	JM-a CYT-2	pBABEpuro-gateway	retroviral expression	II
ERBB4 V1172F	JM-a CYT-2	pBABEpuro-gateway	retroviral expression	II
ERBB4 G1217R	JM-a CYT-2	pBABEpuro-gateway	retroviral expression	II
ERBB4 K1218N	JM-a CYT-2	pBABEpuro-gateway	retroviral expression	II
ERBB4 E715K, V954R-HA	JM-a CYT-2	pcDNA3.1hygro(+)	mammalian expression	II
EGFR K721R-HA	canonical	pcDNA3.1hygro(+)	mammalian expression	II
ERBB2 K753M-HA	canonical	pcDNA3.1hygro(+)	mammalian expression	II
ERBB4 R124K	JM-a CYT-1	pMSCV-PGK-Puro-IRES-GFP	retroviral expression	II
ERBB4 R687K	JM-a CYT-1	pMSCV-PGK-Puro-IRES-GFP	retroviral expression	II
ERBB4 E715K	JM-a CYT-1	pMSCV-PGK-Puro-IRES-GFP	retroviral expression	II
ERBB4 E715K	JM-a CYT-2	pMSCV-PGK-Puro-IRES-GFP	retroviral expression	II
ERBB4 G741R	JM-a CYT-1	pMSCV-PGK-Puro-IRES-GFP	retroviral expression	II
ERBB4 K935I	JM-a CYT-1	pMSCV-PGK-Puro-IRES-GFP	retroviral expression	II
ERBB4 R106C	JM-a CYT-2	pBABEpuro-gateway	retroviral expression	III
ERBB4 S303F	JM-a CYT-2	pBABEpuro-gateway	retroviral expression	III
ERBB4 R393W	JM-a CYT-2	pBABEpuro-gateway	retroviral expression	III
ERBB4 E452K	JM-a CYT-2	pBABEpuro-gateway	retroviral expression	III
ERBB4 R524C	JM-a CYT-2	pBABEpuro-gateway	retroviral expression	III
ERBB4 R544W	JM-a CYT-2	pBABEpuro-gateway	retroviral expression	III
ERBB4 R711C	JM-a CYT-2	pBABEpuro-gateway	retroviral expression	III
ERBB4 G741E	JM-a CYT-2	pBABEpuro-gateway	retroviral expression	III
ERBB4 S774G	JM-a CYT-2	pBABEpuro-gateway	retroviral expression	III
ERBB4 L798R	JM-a CYT-2	pBABEpuro-gateway	retroviral expression	III
ERBB4 V840I	JM-a CYT-2	pBABEpuro-gateway	retroviral expression	III
ERBB4 R847H	JM-a CYT-2	pBABEpuro-gateway	retroviral expression	III
ERBB4 G870R	JM-a CYT-2	pBABEpuro-gateway	retroviral expression	III
ERBB4 G907E	JM-a CYT-2	pBABEpuro-gateway	retroviral expression	III
ERBB4 R992C	JM-a CYT-2	pBABEpuro-gateway	retroviral expression	III
ERBB4 K1223T	JM-a CYT-2	pBABEpuro-gateway	retroviral expression	III
ERBB4 S1289A	JM-a CYT-2	pBABEpuro-gateway	retroviral expression	III
ERBB4 R1304W	JM-a CYT-2	pBABEpuro-gateway	retroviral expression	III
shRNA Erbb3	-	Tet-pLKO-neo vector	lentiviral expression	III

4.1.2 Short hairpin RNA

Short-hairpin RNA used in this study for gene silencing are listed in Table 4.

Table 4. shRNA used in this study.

Target	TRC number	Company	Species	Used in
Scramble control	-	Sigma	-	I-II
VAV3	TRCN0000047702	Sigma	human	I
VAV3	TRCN0000047699	Sigma	human	I
ERBB4	TRCN0000001411	Sigma	human	I-II
ERBB4	TRCN0000039688	Sigma	human	I
Erbb3	TRCN0000023432	-	mouse	III

4.2 Cell culture

Cell lines and their culture conditions used in this study are listed in Table 5.

Table 5. Cell lines used in this study.

Cell line	Cell type	Species	Culture medium	Used in
Ba/F3	lymphoid pro-B cells	mouse	RPMI 1640, 10% FCS, GPS, 5% WEHI-conditioned medium (source of IL3)	II, III
BEAS-2B	bronchial epithelium	human	RPMI 1640, 10% FCS, GPS	II
COS-7	kidney fibroblast-like	monkey	DMEM, 10% FCS, GPS	I-III
HEK293T	embryonic kidney	human	DMEM, 10% FCS, GPS	I-III
MCF7	breast cancer	human	RPMI 1640, 10% FCS, GPS, 1 nM 17-β-estradiol, 10 µg/ml insulin	I
MCF10A	mammary epithelium	human	DMEM/F-12, 10% FCS, GPS, 20 ng/ml EGF, 0.5 µg/ml hydrocortisone, 100 ng/ml cholera toxin, 10 µg/ml insulin	III
NIH-3T3	fibroblast	mouse	DMEM, 10% FCS, GPS	II
NIH-3T3-7d	fibroblast	mouse	DMEM, 10% FCS, GPS	I
Phoenix-AMPHO	embryonic kidney	human	RPMI 1640, 10% FCS, GPS	II, III
Platinum E	embryonic kidney	human	DMEM, 10% FCS, GPS	II

GPS (2 mM L-glutamine, 50 U/ml penicillin and streptomycin)

4.2.1 Transient transfection (I-III)

COS-7, HEK293T, NIH 3T3-7d, MCF7, Phoenix-Ampho and Platinum-E cells were transiently transfected using either Lipofectamine 2000 (Invitrogen), FuGENE 6 (Promega), or X-tremeGENE 9 transfection reagent (Roche) according to manufacturers' instructions.

4.2.2 Generation of stable cell lines (I-III)

To generate stable cell lines retroviral or lentiviral transductions were carried out. For retrovirus production, Phoenix-AMPHO or Platinum-E packaging cells were transfected with retroviral expression constructs listed in Table 2 and Table 3. For lentivirus production, HEK293T cells were transfected with lentiviral packaging plasmids (pMLDg/pRRE, pMD2.G, and pRSV-Rev) and plasmids encoding shRNA listed in Table 4. The viral supernatants were harvested 24-48 hours after transfection and used for transduction of the recipient cells with or without 0.8-8 µg/ml hexadimethrine bromide (Polybrene, Sigma-Aldrich), as described in I-III. Stable cell pools were selected with either puromycin (Life Technologies or Gibco) or neomycin (Geneticin, Gibco), as described in more detail in I-III.

4.3 Reagents for cell treatments (I-III)

Reagents, including growth factors and inhibitors that were used to treat cells in this study are listed in Table 6. The concentrations and incubation times in each experiment are indicated in I-III.

Table 6. Reagents used in this study. Abbreviations: SBCT, Santa Cruz Biotechnology; TKI, tyrosine kinase inhibitor.

Reagent	Type	Company	Used in
neuregulin-1β (NRG-1)	ERBB4 stimulation	R&D	I-III
bis(sulfosuccinimidyl)suberate (BS_3)	crosslinker	Thermo Fisher	III
afatinib	2nd gen pan-ERBB TKI	Boehringer Ingelheim	II-III
dacomitinib	2nd gen pan-ERBB TKI	Cayman Chemicals	II-III
neratinib	2nd gen pan-ERBB TKI	SCBT, Puma Biotechnology	II-III
poziotinib	2nd gen pan-ERBB TKI	Selleck Chemicals	II
ibrutinib	BTK TKI	Selleck Chemicals	II
lapatinib	1st gen pan-ERBB TKI	SCBT	II
erlotinib	EGFR TKI	SCBT	II

4.4 Primary antibodies (I-III)

Primary antibodies that were used for western blot and immunoprecipitation are listed in Table 7.

Table 7. Primary antibodies used in this study. Abbreviations: CST, Cell Signaling Technology; SBCT, Santa Cruz Biotechnology; IP, immunoprecipitation; WB, western blotting.

Antigen	Cat#/clone	Company	Type	Application	Used in
Actin	A5441	Sigma-Aldrich	goat polyclonal	WB	I-III
AKT	2920	CST	mouse monoclonal	WB	II-III
c-MYC	9E10; 2272	Invitrogen; CST	mouse monoclonal	IP, WB	I
EGFR	4726	CST	rabbit monoclonal	WB	II
EGFR	2232	CST	rabbit monoclonal	IP, WB	III
ERBB2	MA5-14057	Invitrogen	mouse monoclonal	IP, WB	II-III
ERBB3	4754	CST	rabbit monoclonal	WB	II
ERBB3	12708	CST	rabbit monoclonal	IP, WB	III
ERBB4	sc-283	SCBT	rabbit polyclonal	IP	I
ERBB4	HFR-1	Abcam	mouse monoclonal	IP, WB	I, III
ERBB4	E200	Abcam	rabbit monoclonal	IP, WB	I-III
ERK	9102	CST	rabbit polyclonal	WB	III
GFP	ab183734	Abcam	rabbit monoclonal	WB	II
HA	3F10	Roche Applied Science	rat monoclonal	IF, WB	I
HIS	H1029	Sigma-Aldrich	mouse monoclonal	WB	I
phospho-MLC	ab2480	Abcam	Rabbit polyclonal	WB	I
phospho-STAT5	9351	CST	Rabbit polyclonal	WB	III
phospho-AKT	4060	CST	rabbit monoclonal	WB	III
phospho-EGFR	2220	CST	rabbit polyclonal	WB	III
phospho-ERBB2	2247	CST	rabbit polyclonal	WB	III
phospho-ERBB3	4791	CST	rabbit monoclonal	WB	III
phospho-ERBB4	4757	CST	rabbit monoclonal	WB	I-III
phospho-ERBB4	3790	CST	rabbit polyclonal	WB	III
phospho-ERBB4	PAB0486	Abnova	rabbit polyclonal	WB	III
phospho-ERK	9101	CST	rabbit polyclonal	WB	III
phospho-MLC	ab2480	Abcam	Rabbit polyclonal	WB	I
phospho-STAT5	9351	CST	rabbit polyclonal	WB	III
phospho-tyrosine	4G10	Upstate; produced in house	mouse monoclonal	WB	I, III
STAT5a	sc-271542	SCBT	mouse monoclonal	WB	III

4.5 Cell lysis, immunoprecipitation and western blotting (I-III)

Cells were lysed in lysis buffer as described in I-III. In brief, protein concentrations of the lysate supernatants were measured using Bradford protein assay (Bio-Rad). For immunoprecipitations, lysates containing 400-1000 µg protein were precleared with protein G Sepharose beads (GE Healthcare or Cytiva) for 2 hours at +4 °C and subjected to immunoprecipitation by overnight incubation with antibodies recognizing proteins of interest described in more detail in I and III, and subsequently incubated for 2 hours with protein G Sepharose beads. Beads were washed four times with 1 ml lysis buffer to remove non-specific binding. All lysates were boiled in SDS-PAGE loading buffer to denature the proteins prior to loading to gels. Proteins were analyzed by western blotting by transferring proteins from SDS-PAGE gels onto nitrocellulose membranes and incubating with primary antibodies listed in Table 7 and described in more detail in I-III. Protein signals were imaged either by enhanced chemiluminescence (Advansta) using horseradish peroxidase (HRP)-conjugated secondary antibodies or using the Odyssey CLx imaging system (LI-COR) with fluorophore-conjugated secondary antibodies.

4.6 Mass spectrometry (I)

For the identification interaction partners of ERBB4 JM-a isoforms in cancer cells, ERBB4 co-immunoprecipitating proteins were analyzed by non-targeted mass spectrometry using MCF7 breast cancer cells stably expressing Strep-tagged JM-a CYT-1 or JM-a CYT-2, as described in detail in I. For the validation of ERBB4-VAV3 interaction, MCF7 cells endogenously expressing ERBB4 JM-a isoforms were subjected to targeted mass spectrometry analysis, as described in I.

4.7 Cell migration (I)

The migration of MCF7 human breast cancer cells upon knockdown of their endogenous ERBB4 or VAV3, or overexpression of activation domain-mutant VAV3 constructs, or pharmacological inhibition of ERBB4 by afatinib, and the migration of NIH-3T3-7d fibroblasts (stably expressing ERBB4 JM-b CYT-1) upon overexpression of dominant-negative VAV3 were analyzed in the presence and absence of NRG-1. Cells were seeded on Transwell inserts (8 µm pore size; BD Falcon) as described in I, let migrate for 8 hours, fixed with 4 % paraformaldehyde and stained with 0.2% crystal violet. The migrated cells in the lower chamber side of Transwell membrane were imaged and cells were counted from six randomly selected fields.

4.8 *In vitro* screen for activating mutations (II)

For a high-throughput functional screen of *ERBB4* mutations the previously published method, *in vitro* screen for activating mutations (iSCREAM) (Chakroborty *et al.*, 2019) was adapted as described in detail in II. Briefly, the expression libraries of *ERBB4* mutations were generated by error-prone PCR (ep-PCR) with Genemorph II (Agilent Technologies, Cat #200550), using *ERBB4* JM-a CYT-1 or *ERBB4* JM-a CYT-2 constructs as a template. PCR amplicons were gel-purified and cloned into retroviral expression vectors (pBABEpuro-gateway) by Gateway cloning.

Expression libraries were retrovirally transduced into Ba/F3 murine pro-B lymphoid cells as described in 4.2.2 and puromycin-selected stable cell pools were used for conducting the cell transformation assay of these normally IL3-dependent cells (Warmuth *et al.*, 2007). Ba/F3 cells expressing wild-type *ERBB4* JM-a CYT-1 or *ERBB4* JM-a CYT-2 mutation libraries were subjected to IL3-deprivation in the presence or absence of 10 ng/ml NRG-1 and their growth was monitored with the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) cell viability assay (as described in section 4.9). Genomic DNA was extracted and deep-sequenced from the IL3-independently growing Ba/F3 cells expressing *ERBB4* mutation library and from the original pool of Ba/F3 cells expressing the transduced mutation library. *ERBB4* cDNA was amplified from the genomic DNA for 150 bp paired-end sequencing with the Illumina MiSeq instrument. Two biological replicates of the screen were performed with cells transduced with independently generated *ERBB4* JM-a CYT-2 mutation libraries.

4.9 Ba/F3 IL3-independent growth (II-III)

Ba/F3 transformation assays were conducted by washing cell pools twice with PBS and seeding in i) IL3-containing medium as a control, ii) IL3-free medium supplemented with NRG-1 as described in II and III, iii) IL3-free medium. Cell viability was monitored at time of seeding and then at indicated time points to create growth curves. Quadruplicate (II) or triplicate (III) technical replicates were used for performing the MTT assay (CellTiter 96 nonradioactive cell proliferation assay, Promega) according to manufacturer's instructions. Ba/F3 doubling times were calculated and statistical analyses were made as described in II and III.

4.10 BEAS-2B three-dimensional growth (II)

To validate the transforming potential of activating *ERBB4* mutations identified in the iSCREAM, BEAS-2B non-cancerous human lung bronchial epithelial cells were selected as a more relevant model of epithelial tumorigenesis. BEAS-2B cells are

devoid of endogenous ERBB4 but express other ERBB receptors. 3D growth of BEAS-2B cells was analyzed by seeding the cells in quintuplicates on poly(2-hydroxyethyl methacrylate) (poly-HEMA) (Sigma-Aldrich) -coated 96-well plates in 2% Growth Factor Reduced Matrigel (Corning), in medium containing 2% FCS and 50 ng/ml NRG-1. The growth of GFP-expressing cells was monitored by fluorescence intensity at day seven compared to day of seeding. The growth assay data was generated by collaborators at Boehringer Ingelheim, Vienna, Austria, after which data analysis and statistics of biological replicates were conducted in Turku, as described in II.

4.11 NIH-3T3 anchorage-independent growth (II)

As an additional model of validating the transforming potential of activating *ERBB4* mutations, the widely used NIH-3T3 murine fibroblasts, devoid of endogenous ERBB4, was selected. Anchorage-independent growth of NIH-3T3 in the presence of 50 ng/ml NRG-1 and 10% FCS was assessed by a 96-well plate-format soft-agar assay adapted from (Ke *et al.*, 2004), as described in II. After 11-day culture, cell viability was evaluated using AlamarBlue (Invitrogen) according to manufacturer's instructions, and statistical analyses of biological replicates were performed as described in more detail in II.

4.12 MCF10a EGF-independent growth (III)

To screen the transforming potential of recurrent *ERBB4* mutations in parallel with IL3-independent growth of murine lymphoid Ba/F3 cells described above, non-cancerous human mammary epithelial MCF10a cells were selected as another model. MCF10a cells provide a cellular context complementary to that of Ba/F3, as MCF10a are epithelial cells resembling more of a solid tissue and they lack endogenous ERBB4 while expressing EGFR, ERBB2 and ERBB3, enabling heterodimerization of ectopically expressed ERBB4 variants with other ERBB receptors. MCF10a transformation assay was conducted by eight-day EGF-deprivation in the presence and absence of 50 ng/ml NRG-1. MCF10a cells were seeded in triplicates and cell viability was measured by the MTT assay (as described above) on the day of seeding and after eight-day culture, and statistical analyses of biological replicates were performed as described in more detail in III.

4.13 Drug sensitivity assay (II-III)

To assess the effects of *ERBB4* mutations on TKI sensitivity, Ba/F3 cells expressing *ERBB4* variants or empty vector were treated with concentration series of indicated

(II, III) TKIs for 72 hours. Cell viability of quadruplicate technical replicates (II) was assessed with the MTT assay. Cells expressing empty vector were treated in the presence of IL3, and NRG-1 dependent cells in the presence of 10 ng/ml NRG-1 but absence of IL3 and NRG-1-independent cells in the absence of both (II, III). Dose-response curves of mean ± standard deviation were created and IC50 values were calculated from indicated numbers of biological replicates using either R or GraphPad Prism 9 or 10, as described in II and III.

4.14 *In vivo* allograft tumor growth assay (II)

The tumorigenic potential of *ERBB4* variants was analyzed in an allograft model by inoculating 5 x 10^6 Ba/F3 cells into both flanks of 6-8 week old female NMRI nude mice (BomTac:NMRI-Foxn1nu). Tumors were measured three times a week by caliper measurements. Tumor volumes were calculated with the following formula: $V = \text{length} \times \text{width}^2 \times \pi/6$, and the data were plotted by GraphPad Prism 9 using the mean + SEM. The animal studies were conducted by collaborators at Boehringer Ingelheim in Vienna, Austria under the approval of Austrian authorities, in accordance with EU legislation and in an animal facility accredited by the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) International.

4.15 *In silico* protein structural analysis (II-III)

To assess potential structural effects of ERBB4 mutations structural analyses, including molecular dynamics simulations (II), were performed by collaborators based on crystal structures of ERBB receptors as described in more detail in II and III.

4.16 Real-time RT-qPCR (I, III)

Real-time reverse transcription PCR was used to analyze human and mouse VAV mRNA expression levels in MCF and NIH-3T3 cells, respectively, and ERBB4 (CYT-2 isoform) mRNA expression levels in allogenic Ba/F3 tumors. RNA was extracted with either TRIsure (Bioline) (I) or NucleoSpin TriPrep kit (Macherey-Nagel) (II), and reverse transcription was made with SensiFAST cDNA Synthesis Kit (Bioline) according to manufacturers' instructions. Primers and probes are listed in Table 8, and either 7900HT Fast Real-Time PCR System (Applied Biosystems) (I) or The QuantStudio 12K Flex Real-Time PCR System (Thermo Fisher Scientific) (II) was used for RT-qPCR. mRNA expression levels of interest were quantified with the $2^{-\Delta CT}$ method using mouse or human β-actin as reference genes.

Table 8. Primers and probes used in this study for RT-qPCR.

Target	Sequence	Company	Species	Used in
Vav1	forward 5'-tcctcttgtgccaattgct-3', reverse 5'-cgaatgttcttaagacaaaggaact-3', probe #38	Roche	mouse	I
Vav2	forward 5'-tccagtgctccatagaaaacc-3', reverse 5'-agctccccgtcaatctttg-3', probe #78	Roche	mouse	I
Vav3	forward 5'-cgcgtgctgaagtatcacc-3', reverse 5'-ctccatagggtcatgggtgt-3', probe #17	Roche	mouse	I
VAV1	forward 5'-gacggaggagaagtacactgaca-3', reverse 5'-tctcaatgtcttgaggtttcagg-3', probe #42	Roche	human	I
VAV2	forward 5'-gaggacatcatcaaggtggag-3', reverse 5'-cttcagtcatgcccattttct-3', probe #66	Roche	human	I
VAV3	forward 5'-tgtggttcctatgcaacgtg-3', reverse 5'-tcggatcagtggtatgtttgac-3', probe #25	Roche	mouse	I
ERBB4 CYT-2	forward 5'-caacatcccacctcccatctatac-3', reverse 5'-acactccttgttcagcagcaaa-3', 5'-fam-aattgactcgaataggaaccagtttgtataccgagat-tamra-3'	Pharmacia, Eurogentec	human	II
β-actin	forward 5'-ctaaggccaaccgtgaaaag-3', reverse 5'-accagaggcatacagggaca-3', probe 5'-tgacccagatcatgtttgagaccttcaacac-3', probe #64	Eurofins Genomics, Roche	mouse	II
β-actin	forward 5'-atctggcaccacaccttctacaat-3', reverse 5'-ccgtcaccggagtccatca-3', probe 5'-tgacccagatcatgtttgagaccttcaacac-3'	Roche	human	I

4.17 Transactivation assay (II)

To assess the transactivation efficiency of ERBB4 E715K mutant compared to wild-type ERBB4, COS-7 kidney fibroblasts of African green monkey were selected as a model due to their efficiency as transfection hosts and lack of endogenous ERBB4 expression. COS-7 were transiently co-transfected with pcDNA3.1 constructs encoding HA-tagged ERBB receptors as follows: ERBB4 E715K or wild-type forced by V956R point mutation to act as a receiver kinase + kinase-dead EGFR/ERBB2/ERBB3 (naturally kinase dead)/ERBB4 to act as an activator kinase. Basal ERBB4 phosphorylation was analyzed by western 24 hours after transfection and pairwise comparisons of log-transformed data were made by one-sample t tests.

4.18 Analysis of active ERBB4 homodimers (III)

Homodimerization of ERBB4 variants was assessed by crosslinking cell surface proteins of ERBB4 variant of vector-transfected COS-7 cells with membrane impermeable BS_3 crosslinker. Cells were serum-starved for four-days in the presence or absence of 50 ng/ml NRG-1 and subsequently washed with ice-cold PBS and incubated with 2 mM BS_3 in PBS for 1 hour on ice. The reaction was terminated with 15-minute incubation on ice in 50 mM Tris-HCl, pH 7.4, 150 mM NaCl prior to lysis. The active ERBB4 homodimers were analyzed by running the samples on 6 % SDS-PAGE gels and detecting phosphorylated high-molecular weight ERBB4 dimers by western blot.

4.19 Patient data (II-III)

The clinical occurrence of cancer-associated *ERBB4* alterations, patients' cancer types and co-occurring alterations were sourced from cBioPortal (https://cbioportal.org) (Cerami *et al.*, 2012; Gao *et al.*, 2013), AACR GENIE (https://genie.cbioportal.org) (Sweeney *et al.*, 2017), and COSMIC (https://cancer.sanger.ac.uk/cosmic) (Forbes *et al.*, 2017; Tate *et al.*, 2019).

Clinical data of neratinib efficacy as a single agent (240 mg/day) in patients harboring *ERBB4* alteration and enrolled in PUMA-NER-5201 the SUMMIT trial (NCT01953926) were obtained from Puma Biotechnology and cBioPortal.

4.20 Statistical analyses (I-III)

All statistical analyses were performed using either parametric tests for normally distributed data or non-parametric tests for non-normally distributed data, corrections for multiple comparisons were performed with the indicated tests as described in the original publications I-III.

5 Results

5.1 Proteomics identifies VAV3 as a novel signaling partner of ERBB4 in cancer

Understanding ERBB4 functions in cancer is particularly complex, since its different isoforms have partially convergent and divergent signaling outputs. This is attributed to their differential regulation of stability, subcellular localization and ability to bind different interaction partners. The expression of the cleavable JM-a isoforms are rather consistently upregulated across various cancer types, with most abundant evidence in breast cancer (Gilbertson *et al.*, 2001; Junttila *et al.*, 2003, 2005; Määttä *et al.*, 2006; Veikkolainen *et al.*, 2011; Hollmén *et al.*, 2012; Donoghue *et al.*, 2018). Yet, ERBB4 signaling in cancer is poorly understood, since the majority of ERBB4 signaling studies have been conducted in non-tumorigenic cell lines with ectopic ERBB4 expression and many have analyzed only one (JM-a CYT-1) of these two cancer-predominant ERBB4 isoforms.

To study the signaling mechanisms of ERBB4 in a cancer cell context, the interactome of both JM-a CYT-1 and JM-a CYT-2 isoforms was investigated in parallel in MCF7 breast cancer cells known to endogenously express these cancer-associated ERBB4 isoforms (Määttä *et al.*, 2006). The ER-positive, non-ERBB2-amplified MCF7 cells are a model of hormone receptor-dependent breast cancer growth to which ERBB4 has been reported to contribute to by co-regulating transcription with ER (Tang *et al.*, 1998, 1999; Junttila *et al.*, 2005; Zhu *et al.*, 2006). MCF7 cells also represent a subtype of breast cancer in which ERBB4 is overexpressed (Sundvall *et al.*, 2008). Additionally, estradiol is known to enhance ERBB4 cleavage in MCF7 cells and their growth is suppressed by an investigational ERBB4 JM-a isoform-specific antibody mAb1479 that blocks both ERBB4 activation and cleavage, and thus, non-canonical ERBB4 signaling for instance in the nucleus (Hollmén *et al.*, 2009, 2012).

To be able to differentiate the interactomes of the two isoforms in mass spectrometric (MS) analysis, constructs encoding Strep-tagged versions of the JM-a CYT-1 or JM-a CYT-2 isoform were stably expressed in MCF7 breast cancer cells and cell lysates were purified with Strep-tactin. Proteins co-precipitating with Strep-tagged ERBB4 cleavable JM-a isoforms were separated by SDS-PAGE, silver-

stained and analyzed by MS. Ten of the identified proteins were shared between the two ERBB4 JM-a isoforms, seven were unique to CYT-1 (e.g. p85α subunit of PI3K), and 11 were unique to CYT-2 specific (e.g. VAV3) (I, Fig. 1C). As the p85α was found to be unique to CYT-1, and it is known to be able to interact with CYT-1 only because CYT-2 lacks its binding site (Kainulainen *et al.*, 2000), this validated the feasibility of the screen. Interestingly, ERBB2 and SOS1 appeared to interact with CYT-2 only, although both are known to be able to interact with ERBB receptors (Schulze *et al.*, 2005; Lemmon *et al.*, 2010), suggesting that CYT-2 may interact with these proteins more than CYT-1 in breast cancer cells. Among the novel interaction partners was VAV3, a guanine nucleotide exchange factor (GEF) for RHO GTPases that regulate the cytoskeleton (Rodríguez-Fdez *et al.*, 2019). Since VAV3 is known to interact with other RTKs, including EGFR (Pandey *et al.*, 2000; Tamás *et al.*, 2003), the novel ERBB4-VAV3 interaction was selected for further analyses.

Targeted MS validated the ERBB4-VAV3 interaction in the context of endogenous expression levels in non-transduced MCF7 breast cancer cells that were stimulated with NRG-1 (I, Fig. S1A). Additionally, co-immunoprecipitation studies with overexpression models showed that VAV3 can also interact with the CYT-1 isoform, although to a lesser extent than with CYT-2 (I, Fig. 2), as suggested by MS (I, Fig. 1C). ERBB4 was also found to be able to interact with other members of the VAV family (I, Fig. 4).

Mechanistic characterizations demonstrated that ERBB4-VAV3 interaction was dependent on i) ERBB4 kinase activity (I, Fig. 3C), ii) phosphorylation of ERBB4 tyrosines Y1022 and Y1162 (I, Fig. 3D), and iii) VAV3 phosphotyrosine-binding SH2-domain (I, Fig. 3A-B), as demonstrated by the loss of interaction when these sites were mutated. ERBB4 stimulated VAV3 phosphorylation and downstream signaling resulting in myosin light chain activation (I, Fig. 5), predominantly via phosphorylation of VAV3 activation domain tyrosines (Y160F, Y164F, Y170F), which are critical for its GEF activity (Bustelo *et al.* 2014). Interestingly, the soluble ICD of ERBB4 was able to interact and activate VAV3 (I, Fig. 3A, 5A).

5.2 ERBB4 mediates cancer cell migration via VAV3

To investigate the functional effects of ERBB4-VAV3 interaction, the role of VAV3 in ERBB4-mediated cell migration was analyzed. ERBB4 is known to mediate cancer cell migration in response to NRG-1 stimulation, for instance in MCF7 breast cancer cells (Mill *et al.*, 2011; Haskins 2014; Kiuchi *et al.*, 2014), and VAV proteins can activate RHO GTPases that result in changes of the cytoskeleton, allowing for instance cell migration (Rodríguez-Fdez *et al.*, 2019). Indeed, both knockdown of

VAV3 and mutation of the VAV3 activation domain tyrosines (Y160, Y164, Y170) impaired the NRG-1-stimulated migration of MCF7 cells compared to MCF7 cells expressing wild-type VAV3 (I, Fig. 6D-G). Similarly, ERBB4 knockdown and its pharmacological inhibition by second-generation pan-ERBB inhibitor afatinib impaired migration of MCF7 cells (I, Fig. 6D-F, S4). Interestingly, NRG-1-stimulated migration was also reduced by introduction of dominant-negative VAV3 in NIH-3T3-7d fibroblasts that endogenously express VAV2 and VAV3 and were transduced to stably express ERBB4 JM-b CYT-1 (JM-b being the predominant ERBB4 isoform in mesenchymal cells) (I, Fig. 6A-C). Taken together, these results demonstrate that different isoforms of ERBB4 can interact with VAV3 to promote cell migration while the primary VAV3-interacting ERBB4 isoform in MCF7 breast cancer cells appears to be JM-a CYT-2.

5.3 Functional screens identify potential driver *ERBB4* mutations

5.3.1 Unbiased screen using the iSCREAM method

Cancer-associated *ERBB4* mutations are not accumulated in as obvious mutational hotspots as in genes encoding other ERBB receptors (II, Supplementary Fig. S1B), making it difficult to select mutations for functional analyses. To identify potential activating driver *ERBB4* mutations, the previously developed high-throughput iSCREAM method was utilized to functionally screen virtually all possible *ERBB4* missense and nonsense mutations in an unbiased manner (Chakroborty *et al.*, 2019). The screen relies on the ability of Ba/F3 murine lymphoid cells to transform to grow IL3-independently upon introduction of an oncogenic kinase (Warmuth *et al.*, 2007), and the enrichment of growth promoting variants of the introduced gene from a library of randomly generated mutations (Chakroborty *et al.*, 2019) (Figure 8). Of the two cancer-associated ERBB4 isoforms, JM-a CYT-1 and JM-a CYT-2, only the mutant library of the latter was able to render Ba/F3 cells IL3-independent although not in the absence of ERBB4 ligand NRG-1 (II, Supplementary Fig. S2B). The screen identified ten mutations highly enriched (> 50-fold, $q < 0.00001$) in the IL3-independently growing Ba/F3 cells, in which 7,396 *ERBB4* mutations were originally present, covering 91.7% of all the 8,065 theoretically possible *ERBB4* mutations caused by SNVs and 98.1% of the original cDNA mutation library used for Ba/F3 cell transduction (II, Fig. 1, Fig. S4).

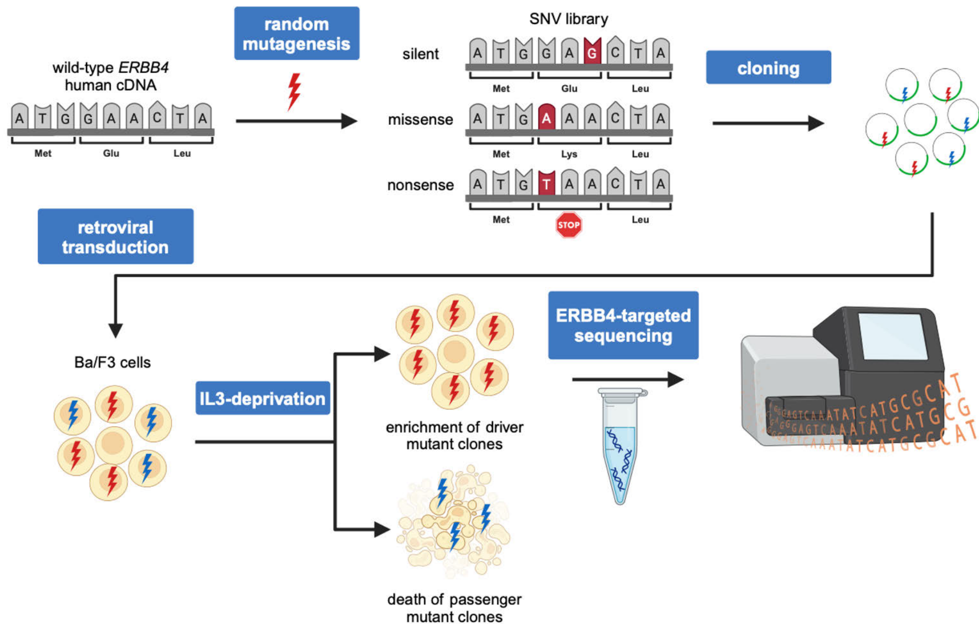


Figure 8. *In vitro* screen for activating mutations (iSCREAM). The workflow and schematic were adapted from Chakroborty et al. 2019. Human ERBB4 cDNA was subjected to random mutagenesis by error-prone PCR, to generate a library of nearly all possible ERBB4 single nucleotide variants (SNV). Mutation library was cloned into retroviral expression vectors to transduce Ba/F3 cells. Ba/F3 transformation assay by interleukin-3 (IL3) deprivation was used to functionally screen activating driver ERBB4 mutations from passenger mutations. Genomic DNA was extracted from the clones that survived and proliferated upon IL3-deprivation, and processed for ERBB4-targeted next-generation sequencing to identify enriched mutations that provided selective growth advantage. Created with BioRender.com.

Validations of the ten candidate driver mutations were carried out by separate transductions of the select mutant *ERBB4* expression constructs in Ba/F3 lymphoid cells and NIH-3T3 fibroblasts. In the presence of NRG-1, five of the ten candidates (R687K, E715K, G741R, G802D, M993I) provided significant growth advantage over wild-type ERBB4 in both cell models, apart from G741R, which did not reach statistical significance in promoting anchorage-independent growth of NIH-3T3 cells (II, Fig. 2A, S5A, S7A). Strikingly, the E715K-mutant was found to promote NRG-1-independent growth of Ba/F3 cells despite none of the cells transduced with the mutant library survived in the same conditions (II, Fig. 5A, S2A). This highlights the limited sensitivity of the iSCREAM method that is tailored for high throughput screening instead.

To further validate the screen, it was reproduced with a new mutation library, and intriguingly, four of the new candidate mutants overlapped with those of the first screen (R124K, R687K, E715K, G741R) (II, Fig. 2D) indicative of the robustness

of the screen. Of these four mutations, R687K and E715K promoted also 3D-growth of non-tumorigenic BEAS-2B lung epithelial cells, even more potently than the previously characterized oncogenic *ERBB4* mutation K935I (Kurppa *et al.*, 2016) found from a lung cancer patient (Ding *et al.*, 2008) (II, Fig. 3A). Moreover, E715K and K935I promoted tumor growth *in vivo* in a Ba/F3 allograft model significantly better than wild-type ERBB4 (II, Fig. 3C-D).

5.3.2 Screen of recurrent *ERBB4* mutations

The most strongly oncogenic *ERBB4* mutations identified by the high-throughput iSCREAM method, were found to be rare in patients (II, Fig. S4B), limiting the possibilities to test them as predictive markers for targeted therapy. Intriguingly, the rapidly accumulating tumor sequencing data has started to reveal emerging hotspot mutations and hotspot regions in *ERBB4*, indicative of potential functional significance in cancer. Moreover, many of these recurrent *ERBB4* mutations are located at sites important for dimer interactions as well as at amino acid residues paralogous to known oncogenic mutations in other ERBB receptors (III, Fig. 1A). These observations and knowing the limitations in the sensitivity of the high-throughput iSCREAM method, it was hypothesized that the emerging *ERBB4* hotspot mutations could be oncogenic drivers as well, despite not being identified by the iSCREAM method. Therefore, a more sensitive approach was employed for functional screening of 18 emerging *ERBB4* hotspot mutations. For this, two different non-tumorigenic cell models, the Ba/F3 murine lymphoid cells and the MCF10a human breast epithelial cells were used to assess the transforming potential of the mutants in the presence and absence of NRG-1 (III, Fig. 2A).

Eleven out of 18 recurrent *ERBB4* missense mutations were transforming in either of the two cell models (III, Fig. 2B-D). Three of the mutants (S303F, E452K, L798R) potently transformed both cell types, especially S303F, which also promoted NRG-1-independent transformation of Ba/F3 cells, similar to the most potent mutation identified with iSCREAM (II, Fig. 2-3, S7). Two of the 11 transforming mutants (R992C and S1289A) were transforming only in Ba/F3 cells, which are devoid of other ERBB receptors, apart from low levels of endogenous murine ERBB3 (Riese*et al.*, 1995) In turn, six of the mutants (R393W, R544W, R711C, S774G, V840I, G870R) were transforming only in MCF10a cells, which endogenously express other ERBB receptors (III, Fig. 4A). These data suggest strong oncogenic potential for S303F, E452K and L798R, and that the mutants providing growth advantage only in MCF10a cells, may benefit from ERBB heterodimers to exert their transforming potential.

5.4 Oncogenic *ERBB4* mutations are activating

Of the oncogenic *ERBB4* mutations, E715K was constitutively activating also in basal cell culture conditions in which the growth of the cells was not dependent on ERBB4 (II, Fig. 2B). E715K rendered ERBB4 highly phosphorylated compared to wild-type ERBB4 in all tested cell models (see above), also in the absence of NRG-1 (II, Fig. 2B-C, 3B, 5B, F, S7B, D). While S303F was also strongly transforming in all tested cell models and it was the only other mutant in addition to the potent E715K mutation able to promote NRG-independent transformation of Ba/F3 cells, it did not appear constitutively activating in basal culture conditions (III, Supplementary Fig. S2A, S4A). Instead, transformation assay culture conditions (III, Fig. 4A-B) as well as prolonged serum starvation (III, Supplementary Fig. S4B) revealed its markedly increased biochemical activity compared to wild-type ERBB4, even in the absence of NRG-1. The level of ERBB4 phosphorylation under transformation pressure and/or prolonged serum starvation mirrored the growth promoting potential of the analyzed ERBB4-mutants (II, Fig. 5B, Supplementary Fig. S7D; III, 4A-B, Supplementary Fig. S4B).

To investigate whether the oncogenic *ERBB4* mutations could enhance heterodimer activation, the phosphorylation of endogenously expressed ERBB receptors in MCF10a (human EGFR, ERBB2 and ERBB3) and in Ba/F3 cells (murine ERBB3) were analyzed. The three most strongly transforming recurrent *ERBB4* mutations enhanced activation of all other ERBB receptors in MCF10a cells upon transformation in comparison to cells expressing vector or wild-type ERBB4 (III, Fig. 4A). In Ba/F3 cells, ERBB3 was also more phosphorylated in IL3-independently growing S303F-mutant cells compared to the negligible activity in IL3-dependent cells (III, Fig. 4B). Interestingly, the receptor expression levels of endogenous murine ERBB3 were also upregulated upon IL3-deprivation, suggesting a functional role for ERBB3 in IL3-independent growth.

To address the potential functional relevance of ERBB3 in ERBB4-mediated cell transformation, endogenous ERBB3 was knocked down in Ba/F3 cells stably expressing ERBB4 variants, thus making it devoid of ERBB heterodimers. ERBB3 knockdown did not impair ERBB4 wild-type or S303F-mediated cell transformation in the presence of NRG-1 (III, Fig. S3B-C). However, in the absence of NRG-1, ERBB3 knockdown slowed the IL3-independent growth of Ba/F3 cells expressing ERBB4 S303F (III, Fig. S3B-C). These data suggest that ERBB3 contributes to ligand-independent ERBB4 S303F-mediated transformation of Ba/F3 cells, although ERBB3 is likely not indispensable for ERBB4-mediated cell transformation.

5.5 Oncogenic *ERBB4* mutations enhance dimer interactions

To shed light on the detailed mechanisms of enhanced activity of the most potent oncogenic *ERBB4* mutations characterized in this work (II, III) (Fig. 8), structural modeling was conducted. The activation mechanisms of the most potent mutations, E715K and S303F, were validated in more detail and are described below. Of the three other activating mutations, the extracellular E452K mutation was suggested to potentially stabilize the interactions in the open dimerized conformation of the receptor (III, Fig. 3C), R687K was shown to stabilize the interactions of the antiparallel JM-helices of the two dimerized ERBB4 monomers near the intracellular transmembrane domain (II, Fig. 6B-D), and the intracellular kinase domain mutation L798R was suggested to be able to stabilize interactions with ATP at the catalytic site (III, Fig. 3E). Overall, the common theme in the predicted mechanisms of activation was that the structural effects of these mutations may result in enhanced dimer interactions and/or stabilization of the active state of the receptor, similar to four previously characterized activating and potentially oncogenic *ERBB4* mutations (Kurppa *et al.*, 2016).

E715K is located near the C-lobe of the kinase domain, at the dimerization interface when positioned as the receiver kinase in the asymmetric dimer (Figure 9). E715K was predicted to form strong ionic interaction with the ERBB4 E934-residue of the activator kinase or the paralogous conserved residue in other ERBB-receptors (II, Fig. 5D-E). This *in silico*-proposed mechanism was confirmed with an *in vitro* transactivation assay, utilizing mutant expression constructs that force ERBB4 E715K to act as a receiver kinase and the co-expressed ERBB receptor to act as an activator kinase (schematic in II, Supplementary Fig. S10B). Indeed, ERBB4 E715K enhanced transactivation of ERBB4 homodimers and heterodimers with EGFR, ERBB3 and ERRB2 (II, Fig. 5F, Supplementary Fig. S10C).

The ectodomain mutation S303F was in turn predicted to stabilize hydrophobic dimerization arm interactions with other ERBB receptors, highly comparable to the paralogous ERBB2 S310F oncogenic hotspot mutation (Diwanji *et al.*, 2021). ERBB4 S303F-stabilized homodimer and heterodimer interactions were validated *in vitro* by crosslinking and co-immunoprecipitations, respectively (III, Fig. 4C-D).

Some of the ERBB4 ECD mutations could also impact ERBB4 glycosylation/glycan structures, which can, as discussed in section 2.2.2, affect receptor activity for instance by stabilizing dimers. Yet, no direct ERBB4 glycosylation site-mutants at its 11 known glycosylated asparagine residues (N138, N174, N181, N253, N358, N410, N473, N495, N548, N576, and N620 (Trenker *et al.*, 2024)) have been identified as oncogenic in this or previous studies, nor being recurrent in patients thus far. Yet, the possibility of indirect effects on ERBB4 glycan structures by mutations adjacent to its glycosylation sites cannot be excluded.

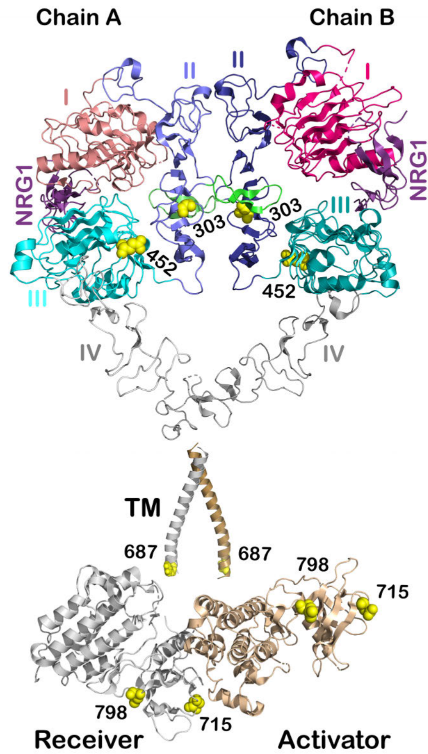


Figure 9. Sites of the most potent activating ERBB4 mutations identified in this work, highlighted by yellow spheres in the crystal structure of the ligand-bound ERBB4 dimer. The figure was created with PyMOL (The PyMOL Molecular Graphics System, Version 2.4, Schrödinger, LLC), using PDB:3U7U for the extracellular domains of an active ERBB4 dimer, PDB:3BCE for the active ERBB4 asymmetric kinase dimer and the dimeric TM-helix of ERBB4 was created with Alphafold2 (Jumper et al., 2021) using the COSMIC²-gateway (Cianfrocco et al., 2017). Figure created by and used here with the permission of Tomi T. Airenne (co-author in III).

5.6 Oncogenic *ERBB4* mutations are targetable with clinically available pan-ERBB inhibitors

Since all the oncogenic *ERBB4* mutations characterized in this study were activating, their functions could be therapeutically targeted with ERBB4-blocking drugs. Therefore, the sensitivity of the identified activating mutants to clinically used second-generation pan-ERBB TKIs was evaluated. All of the five most oncogenic ERBB4-mutants rendered NRG-dependently growing Ba/F3 cells sensitive to afatinib, dacomitinib and neratinib at low nanomolar concentrations, comparable to wild-type ERBB4 expressing cells (II, Fig. 4A-C; III, Fig. 5A). The two mutants potent enough to promote NRG-independent Ba/F3 cell growth (S303F, E715K) were found to be even more sensitive to these inhibitors than their NRG-dependent

counterparts (II, Fig. 5C; III, Fig. 5B). EGFR-selective TKI erlotinib was used as a negative control. Indeed, erlotinib IC50 values for cells expressing ERBB4 variants were similar to those of pan-ERBB inhibitors in IL3-dependent vector cells, representing off-target toxicity (II, Fig. 4D).

The SUMMIT basket trial that evaluated the predictive potential of *ERBB2* and *ERBB3* mutations for neratinib, had also enrolled seven patients harboring *ERBB4* mutations. Three of these patients were enrolled for the trial for their *ERBB4* mutation and four were enrolled primarily for their *ERBB2* mutation but none of the seven patients benefited from neratinib (III, Table 1). While four of the patients had an *ERBB4* mutation identified as potentially oncogenic in this study (R544W, V840I, R711C, L798R), all these patients had a co-occurring *TP53* mutation which is associated with a lack of response to neratinib (Hyman *et al.*, 2018). Furthermore, the patients harboring ERBB4 V840I or L798R mutation had co-occurring *ERBB2* amplification which is predictive for neratinib sensitivity in breast cancer (Chan *et al.*, 2016), but not the in cancer types of these particular patients. Nevertheless, the *in vitro* drug response assays confirmed that the potentially oncogenic *ERBB4* mutations found in these patients were not intrinsically resistant to neratinib, nor to afatinib or dacomitinib (III, Fig. 5B). On the contrary, R544W and R711C appeared to be sensitizing to these TKIs.

These results indicate that the activating, oncogenic *ERBB4* mutations are targetable with clinically available second-generation pan-ERBB inhibitors, and that further investigations of their potential utility as predictive markers is justified.

5.7 Summary of activating *ERBB4* mutations

The findings of functional effects and targetability of the *ERBB4* mutations characterized in this study are summarized in Table 9.

Table 9. Summary of the functional effects of the ERBB4 mutations characterized in this study. Strong: mutations that were significantly transforming in all tested cellular backgrounds; moderate: mutations that were significantly transforming in at least one of the tested cellular backgrounds; *in vivo*: Ba/F3 allograft model; N/A: not applicable; sensitizing: significantly more sensitive than wild-type ERBB4.

RANK	MUTATION	GAIN-OF-FUNCTION EFFECT?	LIGAND-INDEPENDENT?	SENSITIVE TO PAN-ERBB INHIBITORS?
1	E715K	strong (+*in vivo*)	yes	yes
2	S303F	strong	yes	yes
3	E452K*	strong	yes (MCF10a), no (Ba/F3)	yes
4	L798R	strong	no	yes
5	R687K	strong (not *in vivo*)	no	yes
6	K935I*	strong (+*in vivo*)	no	yes (data not shown)
1	G802D	moderate	no	yes (data not shown)
2	M993I	moderate	no	yes (data not shown)
3	R544W*	moderate	yes	sensitizing
4	R711C	moderate	no	sensitizing (afatinib)
5	S774G	moderate	yes	N/A
6	R393W	moderate	yes	N/A
7	V840I	moderate	no	yes
8	G870R	moderate	no	N/A
9	R992C	moderate	no	yes (data not shown)
10	S1289A	moderate	no	yes (data not shown)
11	G741R	moderate (not *in vivo*)	no	yes
1	R124K	no	no	yes (data not shown)
2	Y52C	no	no	yes (data not shown)
3	R106C	no	no	yes (data not shown)
4	R524C	no	no	N/A
5	G741E	no	no	yes (data not shown)
6	R847H	no	no	N/A
7	G907E	no	no	N/A
8	V1172F	no	no	N/A
9	G1217R	no	no	N/A
10	K1218N	no	no	N/A
11	K1223T	no	no	N/A
12	R1304W	no	no	N/A

* First characterized as gain-of-function in other models than in this study (E452K: *in vitro*, NIH-3T3 mouse fibroblasts and human melanoma cell lines (Prickett et al. 2009); K935I: *in vitro* NIH-3T3 (Kurppa et al. 2016); R544W: *in vitro* NIH-3T3 (Prickett et al. 2009)).

6 Discussion

6.1 Should ERBB4 be targeted in cancer?

6.1.1 ERBB4-mediated tumor aggressiveness - VAV3 as a part of the puzzle

Although the role of ERBB4 in cancer has for long remained ambiguous, there is a recurring theme in the literature about higher ERBB4 activity in aggressive cancers (metastatic and therapy-resistant) (Mendoza-Naranjo *et al.*, 2013; Saunus *et al.*, 2015; Donoghue *et al.*, 2018; Kalita-de Croft *et al.*, 2020; Arribas *et al.*, 2023; Yuan *et al.*, 2023). This appears to be the case even in cancer types in which ERBB4 is believed to act categorically as a tumor suppressor, such as hepatocellular carcinoma (Padthaisong *et al.*, 2020; Breitenecker *et al.*, 2023) and prostate cancer (Vexler *et al.*, 2008; Carrión-Salip *et al.*, 2012). Yet, ERBB4 signaling mechanisms in cancer have remained poorly characterized, in part due to the intricate regulation of ERBB4 functions *via* different isoforms and subcellular localization.

This thesis work elucidated ERBB4 signaling mechanisms in cancer cells by analyzing both of the cancer-associated ERBB4 isoforms JM-a CYT-1 and JM-a CYT-2 in parallel (I). This work was the first to corroborate the previously predicted interactions of ERBB4 with all three VAV proteins (Hause *et al.*, 2012). This was also the first description of VAV signaling involvement in ERBB4-mediated cell migration, aligning with previous reports of ERBB4 mediating cell migration in response to NRG-1 (Kainulainen *et al.*, 2000; Haskins *et al.*, 2014). It is possible that ERBB4-mediated cancer cell migration promoted by its other ligands, including NRG-2 (Mill *et al.*, 2011) and HB-EGF (Kiuchi *et al.*, 2014) may also utilize VAVs.

While both the cancer-predominant ERBB4 isoforms were able to stimulate migration via direct interaction with VAV3, and ERBB4 JM-a CYT-2 was able to interact with all three VAVs, the mass spectrometry data suggest that ERBB4 JM-a CYT-2 and VAV3 are the prominent interactors in ER-positive MCF7 breast cancer cells. This is further supported by clinical data showing that both VAV3 and ERBB4 are upregulated in ER-positive breast cancer (Lee *et al.*, 2008; Sundvall *et al.*, 2008; Citterio *et al.*, 2012) (and IST Online analyses in I). This could in turn be explained by both ERBB4 and VAV3 being co-activators of ER by interacting and

translocating to nucleus with ER, which results in a positive feedback loop that increases the transcription of both ERBB4 and VAV3, as well as breast cancer cell proliferation (Junttila *et al.*, 2005; Dong *et al.*, 2006; Zhu *et al.*, 2006; Lee *et al.*, 2008; Han *et al.*, 2014). In agreement, overexpression of ERBB4 and VAV3 promotes resistance to the ER antagonists in breast cancer (Lee *et al.*, 2008; Hutcheson *et al.*, 2011; Aguilar *et al.*, 2014; Wege *et al.*, 2018).

The potential co-operative role of ERBB4 and VAV3 in therapy resistance, plasticity and metastasis may encompass also other cancer types: there are reports of ERBB4 associating with or directly promoting tumor aggressiveness and metastasis, regardless of the breast cancer subtype and also in other cancer types (Bièche *et al.*, 2003; Lodge *et al.*, 2003; Junttila *et al.*, 2005; Hollmén and Elenius, 2010; Mendoza-Naranjo *et al.*, 2013; Mohd Nafi *et al.*, 2014; Saunus *et al.*, 2015; Miller *et al.*, 2016; Donoghue *et al.*, 2018; Yuan *et al.*, 2019; Padthaisong *et al.*, 2020; Jia *et al.*, 2020; Kalita-de Croft *et al.*, 2020; Debets *et al.*, 2023) – similar to VAV3 (reviewed in (Al-Hawary *et al.*, 2023)). The direct link between ERBB4 and VAV3 signaling provided by this thesis unveils novel VAV-mediated mechanisms how ERBB4 may promote tumor progression that go beyond the ability to promote cell migration. It is possible that the previously characterized mechanisms of ERBB4-mediated EMT, metastasis and therapy resistance, such as co-regulation of transcription in a complex with YAP/TAZ (Haskins *et al.*, 2014; Sudol, 2014; Shi *et al.*, 2018), non-canonical YAP activation (Kalita-de Croft *et al.*, 2020), PI3K-AKT-FAK-RAC1 pathway activation (Mendoza-Naranjo *et al.*, 2013) and AKT-lamin A/C pathway activation (Li *et al.*, 2024) also involve VAV3 signaling. Alternatively, they could provide VAV3-independent mechanisms for ERBB4 to promote EMT and metastasis. VAVs can activate PI3K-AKT pathway *via* its GEF catalytic activity but possibly also independently of it (Uen *et al.*, 2015), whereas VAV-mediated activation of YAP appears to require GEF activity (Lorenzo-Martín *et al.*, 2020; Fernández-Parejo *et al.*, 2024). Nevertheless, through VAV3 activation, ERBB4 may promote different steps of tumor progression, especially metastasizing cancer cell extravasation and colonization of distant tissues (Citterio *et al.*, 2012). Again in line with this, both VAV3 (Chen *et al.*, 2015) and ERBB4 (Lodge *et al.*, 2003) associate with poor prognosis in node-positive breast cancers, further supporting their potential co-operation in promoting tumor aggressiveness.

6.1.2 List of activating driver *ERBB4* mutations grows

The 14 novel potential driver *ERBB4* mutations identified in this study (II, III; summarized in Table 9) add to the previous number of 11 activating gain-of-function *ERBB4* mutations found in cancer patients (Figure 10A) (Prickett *et al.*, 2009; Kurppa *et al.*, 2016; Nakamura *et al.*, 2016; Donoghue *et al.*, 2018; Kawahara *et al.*,

2022). This thesis improves our understanding of the clinical significance of the myriad cancer-associated *ERBB4* mutations (Figure 10B; also: II, Supplementary Fig. S1B; III, Fig. 1B-C and Supplementary Fig. S1C) with comprehensive, pan-cancer approaches that aimed to distinguish potential driver mutations from passenger mutations. Specifically, out of 3753 unique amino acid substitutions and 231 nonsense mutations reported in cancer patients in cBioPortal, AACR GENIE, and COSMIC databases (Figure 10B), this thesis work analyzed virtually all of them with the unbiased functional high-throughput screen that covered 7,396 (91.7%) of all 8,065 theoretically possible non-synonymous *ERBB4* SNVs. Additionally, 28 mutants that have all been identified in cancer patients (II, Supplementary Fig. S4B and Table S2; III, Fig. 1C) were characterized individually.

The unbiased functional screen (II) robustly identified novel rare but strongly activating potential driver *ERBB4* mutations among virtually all possible *ERBB4* missense and nonsense mutations. This came at the expense of sensitivity, as none of the previously characterized activating *ERBB4* mutations were identified by the screen. To overcome this limitation (discussed further in section 6.3.) in the focused functional screen of the emerging *ERBB4* hotspot mutations (III), all the selected 18 mutants were i) analyzed individually and ii) an additional cell model of different lineage and with co-expressed ERBB receptors was included to identify more context-dependent mutations. Indeed, this approach demonstrated that majority (11/18) of the analyzed emerging *ERBB4* hotspot mutations had transforming potential in either of the two cell models. The potential reason for some mutations being transforming in certain cell types but not in others could be related to differences in the availability of dimerization partners, ligands or other interaction partners. This phenomenon is also observed with other oncogenes (Jaiswal *et al.*, 2013; Kohsaka *et al.*, 2017; Koivu *et al.*, 2024). For instance, the most recurrent ERBB3 hotspot mutation V104M is transforming in cell models of the same lineage in which it occurs in cancer patients (such as colonocytes) while it is not capable of transforming Ba/F3 lymphoid cells (Jaiswal *et al.*, 2013; Koivu *et al.*, 2024).

While the majority of the individually characterized ERBB4-mutants (6/10 of the unbiased screen hits and 11/18 hotspot mutations) turned out as gain-of-function mutations, there were also in total three mutations (R106C, R847H and G907E – all among the recurrent mutations) that had a loss-of-function effect across the analyzed cell models. However, validation of the unbiased screen hits showed loss-of-function phenotype for three mutants (Y52C, R124K and G1217R) in Ba/F3 cells in which the screen was originally performed, indicative of false positives. However, performing biological replicates of the screen enabled dropping out two of these. Yet, none of the three mutants had a loss-of-function phenotype in other tested cell models, again highlighting lineage-dependent differences. Since many of the *ERBB4* mutations were found to be tissue-specific (III, Supplementary Fig. S5A-B; II,

Supplementary Table S2), it would be interesting to test whether the recurrent mutations that did not appear transforming or had loss-of-function phenotype in the models used in this work would have gain-of-function phenotype in lineages/models better recapitulating the cancer tissues in which they occur in patients.

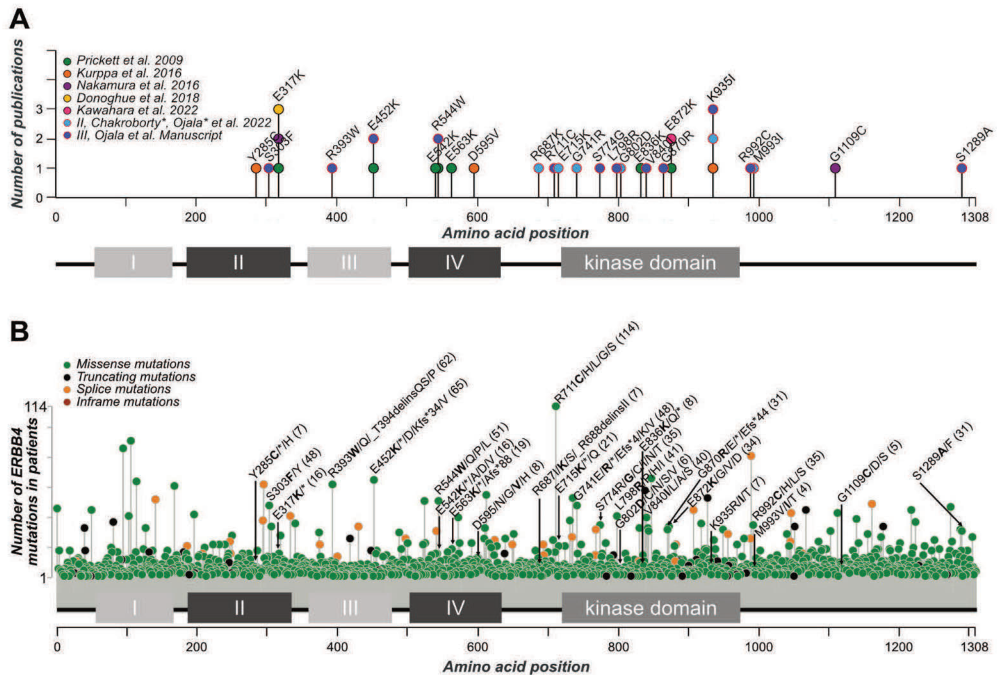


Figure 10. Potential driver mutations among somatic cancer-associated *ERBB4* mutations. **A)** The y-axis denotes the number of publications (PubMed-indexed international peer-reviewed publications and articles included in this thesis) that have demonstrated the indicated *ERBB4* mutations to be activating gain-of-function (potential driver) mutations. The lollipops outlined with red indicate the potential drivers identified in this thesis. The x-axis denotes the amino acid position **B)** Number of cancer patient samples with the potential driver *ERBB4* mutations shown in A, and other mutations of the same amino acid residue: the Y-axis (and parentheses) denote the total number of cases with a mutation at the indicated *ERBB4* residue, bolded one-letter amino acid codes denote the exact amino acid substitutions with functional evidence (as shown in A). Different mutations at the same residue are listed in the order of higher recurrence. Original analyses and figures visualized as lollipop diagrams generated with and edited from the MutationMapper tool in cBioPortal. Data for B from cBioPortal, AACR GENIE and COSMIC, January 2024 (overlapping data removed).

Surprisingly, the most potential *ERBB4* driver mutations identified by the unbiased screen (E715K and R687K) are not among the most recurrent hotspot ERBB4 mutations (Figure 10, also: II, Supplementary Fig. S1B or III, Figure 1B versus II, Fig. S4 and Table S2). In fact, they are less recurrent in patients than the most strongly transforming hotspot mutations (source: cBioPortal, AACR GENIE,

and COSMIC databases - data from the same analysis as in Figure 10B, accessed in January 2024, overlapping data removed): E715K n=10, R687K n=2 versus E452K n=61, S303F n=36, and L798R n=32. Inversely, out of the most recurrent *ERBB4* hotspot mutations, only G741 mutation was identified in the unbiased screen (II), albeit the exact substitution was G741R instead of the more recurrent variant G741E that also trended towards a gain-of-function effect (III). The potential reasons underlying the minimal overlap between the unbiased screen hits and the gain-of-function ERBB4 hotspot mutations include the abovementioned low sensitivity of the unbiased screen with Ba/F3 cells and context-dependent activation of some mutants. In support of the latter, both E715K and R687K showed enhanced activation across tested all cell models and culture conditions – even when cell growth was not dependent on ERBB4, which is likely why the mutants were consistently identified by the stringent unbiased high-throughput screen (II). In contrast, the most potent hotspot mutations showed greatly enhanced activation only when the cells became dependent on ERBB4, i.e. in the transformation assay culture conditions and upon prolonged serum starvation (III, Fig 4C-D, Supplementary Fig. S4B); another condition in which ERBB4 JM-a CYT-2 activity is enhanced to promote cell survival (Sundvall *et al.*, 2010; Kurppa *et al.*, 2016). Notably, even the unbiased screen hits that were considered as false positives in Ba/F3 cells became more phosphorylated than wild-type ERBB4 upon prolonged serum starvation in NIH-3T3 cells (II, Supplementary Fig. S7C). Together, these data may imply that it is more common for cancers to utilize *ERBB4* mutations whose enhanced biochemical activity is context-dependent, which warrants further investigation.

Clinical studies have recently demonstrated associations between *ERBB4* mutations and shorter progression free survival time in trastuzumab-treated (Wang *et al.*, 2019; Zhang *et al.*, 2023) or adjuvant chemotherapy-treated gastric cancer patients (Yuan *et al.*, 2023) and in third-generation EGFR TKI osimertinib-treated lung cancer patients (Vokes *et al.*, 2022), suggesting a functional role for mutant ERBB4 in tumor relapse. Furthermore, several other studies have reported *ERBB4* mutations in patients whose cancer relapsed during EGFR-targeted therapy in the contexts of lung cancer (Jänne *et al.*, 2022), and colorectal cancer (Cremolini *et al.*, 2019; Yaeger *et al.*, 2023), including a patient treated with the combination of mutant KRAS- and EGFR-targeted therapies. Additionally, *ERBB4* mutations have been identified in ERBB2-amplified breast and gastric cancer patients not responding to ERBB2-targeted therapy (Boulbes *et al.*, 2015; Wang *et al.*, 2019). Of these mutations, only S303F has been functionally characterized (III), and L798I affects the same amino acid residue as the functionally characterized L798R mutation (III), both indeed being activating and growth promoting. In the light of the previous evidence of a causal role of overactive ERBB4 in resistance to EGFR-targeted therapy (Carrión-Salip *et al.*, 2012; Donoghue *et al.*, 2018; Breitenecker *et al.*, 2023),

it is possible that these *ERBB4* mutations contributed to the tumor relapse during treatment. The increasingly available pre- and post-treatment clinical sequencing data should be exploited in future studies to shed more light to the role of activating *ERBB4* mutations as a mechanism of acquired resistance to targeted therapy.

6.2 How to target ERBB4 in cancer?

6.2.1 Pan-ERBB inhibition as the most potential strategy

The currently most potential means of targeting ERBB4 in cancer include the clinically available second-generation pan-ERBB inhibitors afatinib, neratinib and dacomitinib (www.fda.gov and www.ema.europa.eu) that are potent inhibitors of ERBB4 (Davis *et al.*, 2011). Of note, all the potential driver ERBB4 mutants identified in this study were sensitive to these three drugs (II, III). Also the newer experimental second-generation pan-ERBB inhibitors that were developed against *EGFR* and *ERBB2* exon 20 insertion mutants, notably poziotinib, tarloxotinib and pyrotinib, have high potency against ERBB4 (II, Fig. S9) (Udagawa, *et al.*, 2023; Popović *et al.*, 2024). In addition, several ERBB4-selective drugs have been preclinically developed, including mAb1479, which specifically targets the cancer-associated cleavable ERBB4 JM-a isoforms and shows tumor growth blocking activity *in vivo* (Hollmén *et al.*, 2009, 2012). ERBB4 JM-a isoform selectivity could mitigate toxicities in normal tissues, especially in heart and nervous system, which predominantly express the JM-b isoforms (Veikkolainen *et al.*, 2011). This could be particularly beneficial in the scenario of drug combinations targeting both ERBB2 and ERBB4.

Pan-ERBB-targeting strategy with either second-generation pan-ERBB-blocking TKIs or in a combination with mAbs, would likely be more beneficial than ERBB4-selective for several reasons. Firstly, the clinical settings, in which ERBB4 has been shown to be overactive, often involve an ERBB ligand (such as NRG-1, NRG-2 or HB-EGF) enriched TME, leading to activation of multiple ERBB receptors simultaneously. Such settings include acquired therapy resistance (to chemotherapy, EGFR or ERBB2-targeted or other targeted therapies) (Wilson *et al.*, 2012; Hegde *et al.*, 2013; Yonesaka *et al.*, 2015; Schwarz *et al.*, 2017; Iida *et al.*, 2022; Arribas *et al.*, 2023; Debets *et al.*, 2023), and NRG-fusion cancers (Kohsaka *et al.*, 2020; Udagawa *et al.*, 2023), brain cancers (Gilbertson *et al.*, 2002; Donoghue *et al.*, 2018) and brain metastases (Saunus *et al.*, 2015; Kalita-de Croft *et al.*, 2020). The pan-ERBB inhibition strategy is further supported by the clinical findings that trastuzumab-treated ERBB2-amplified breast cancer patients (in which ERBB4 is known to promote therapy resistance (Nafi *et al.*, 2014; Canfield *et al.*, 2015; Debets *et al.*, 2023)) benefit from combination to pan-ERBB inhibitor neratinib or pyrotinib

(Blackwell *et al.*, 2019; Ma *et al.*, 2023). Notably, a mixture of EGFR, ERBB2 and ERBB3 antibodies has been shown to be highly effective in eradicating ERBB ligand overexpressing ERBB2 ADC-resistant xenograft tumors (Schwarz *et al.*, 2017), highlighting the importance of the whole ERBB network in mediating resistance. Also NRG-fusion cancers relying predominantly on either ERBB4 or EGFR-ERBB3 mediated signaling benefit more from a combination of mAbs and pan-ERBB inhibitors (Udagawa, *et al.*, 2023).

Secondly, the putative importance of ERBB receptor co-operation also in *ERBB4*-mutant cancers is supported by the findings of this thesis and Kurppa *et al*; The potential driver *ERBB4* mutations mechanistically characterized thus far enhance dimer interactions and appear to readily activate other ERBB receptors as well (II, III) (Kurppa *et al.*, 2016). This mechanism of activation is also common for *ERBB2* and *ERBB3* hotspot mutations (Greulich *et al.*, 2012; Jaiswal *et al.*, 2013; Hanker *et al.*, 2021), as opposed to driver *EGFR* mutations often being directly kinase-activating. It is not fully understood why the recurrent activating mutations of *ERBB2/ERBB3/ERBB4* are different from those of *EGFR*, and why the mutational landscape of each gene also differs between cancer types – a common but poorly characterized feature for oncogenes (Chang *et al.*, 2016; Haigis *et al.*, 2019). Yet, one possible explanation for the enrichment of dimer interface mutations in *ERBB2/ERBB3/ERBB4* is that they are markedly more potent drivers when they can heterodimerize more effectively. In support of this theory, EGFR mutations that confer resistance to EGFR-selective TKIs have in fact also been shown to stabilize dimer interfaces (Hayes *et al.*, 2024; Iyer *et al.*, 2024), which could potentially enhance heterodimerization with other kinase-competent ERBB receptors, enabling the EGFR-blockade bypass. This theory is of course in line with the physiological role of ERBB2 and ERBB3 as obligate heterodimerizing receptors but also with the context-dependent roles of ERBB4 and its otherwise strong tendency to homodimerize (Trenker *et al.*, 2024) (discussed in section 2.2.2). Perhaps the mutations that increase ERBB4 heterodimerization could result in a shift in the balance from some of the opposing functions of different ERBB4 isoforms towards more oncogenic signaling. The lower frequency of *ERBB4* "hotspots" compared to those of *ERBB2* and *ERBB3,* together with their high tissue-specificity could indeed reflect the mutational fine-tuning needed for oncogenic ERBB4 gain-of-function phenotypes, which varies across tissue types.

Furthermore, high co-occurrence of multiple *ERBB* mutations in cancer patients has been reported, with *ERBB2/ERBB3/ERBB4* mutations often being co-mutated with any of the *ERBB* genes while *EGFR* mutations co-occur more often with other *EGFR* mutations (Saito *et al.*, 2020; Hanker *et al.*, 2021) (III, Supplementary Fig. S5D). This supports the concept that EGFR homodimers are strong oncogenic drivers while other ERBBs can benefit from heterodimerization. The co-occurring

ERBB mutations have been demonstrated to co-operate to enhance oncogenic signaling (Skoulidis *et al.*, 2019; Saito *et al.*, 2020; Smyth *et al.*, 2020; Hanker *et al.*, 2021; Koivu *et al.*, 2024) which further suggests that combination therapy approaches could yield more durable responses in patients.

6.2.2 Prospects of evaluating *ERBB4* mutations as predictive markers

Activating *ERBB4* mutations identified in this thesis and in previous studies could potentially serve as biomarkers for ERBB4-targeted therapy. The potential driver *ERBB4* mutations characterized in this thesis were all found to be sensitive to clinically used second-generation pan-ERBB inhibitors (II, III; Table 9). Additionally, two other activating *ERBB4* mutations have previously been demonstrated to be targetable in xenograft mouse models with second-generation pan-ERBB inhibitors afatinib and dacomitinib (Nakamura *et al.*, 2016; Donoghue *et al.*, 2018). Although several activating *ERBB4* mutations have been found to sensitize primary melanoma cells to the first-generation reversible pan-ERBB inhibitor lapatinib, the IC50 values were notably high (Prickett *et al.*, 2009) - especially compared to those reported for second-generation irreversible pan-ERBB inhibitors in ERBB4 wild-type- or mutant-dependent cells (II, III)(Rusnak *et al.*, 2001; Davis *et al.*, 2011; Solca *et al.*, 2012; Popović *et al.*, 2024). Since both *in vitro* and *in vivo* data demonstrate that overactive ERBB4 can mediate resistance to lapatinib (Canfield *et al.*, 2015), it could also be one of the reasons why two out of two *ERBB4*-mutant melanoma patients treated with lapatinib in a clinical trial NCT01264081 (www.clinicaltrials.gov), did not respond to the therapy.

The only other completed clinical trial that has specifically enrolled *ERBB4*-mutant patients to be treated with ERBB4-targeting therapies to date is the neratinib basket trial, SUMMIT (NCT01953926). Only three *ERBB4*-mutant patients were enrolled alongside *ERBB2*- and *ERBB3*-mutant patients to receive second-generation pan-ERBB inhibitor neratinib. None of the *ERBB4*-mutant patients responded (III, Table 1), although two out of the three patients harbored an *ERBB4* mutation characterized as transforming in this thesis (III). However, the patients had co-occurring *TP53* mutations that are associated with a lack of response to neratinib in patients harboring *ERBB2* or *ERBB3* mutations (Hyman *et al.*, 2018). While it is also possible that these transforming mutations were not strong enough drivers, it would be instrumental to see whether patients harboring the most potent activating ERBB4 mutations, such as S303F and E715K, would benefit from second-generation pan-ERBB inhibitors.

Yet, perhaps one of the most challenging steps in clinical evaluation of the predictive potential of *ERBB4* mutations is the low frequency of specific mutations,

limiting the possibility of enrolling patients with characterized oncogenic *ERBB4* mutations for clinical trials – even in basket trial settings, as demonstrated in the SUMMIT trial that managed to recruit only seven patients with *ERBB4* alterations. Therefore, molecular tumor board-driven off-label use of second-generation pan-ERBB inhibitors in *ERBB4*-mutant patients could give invaluable insights into the targetability and predictive potential of *ERBB4* mutations. The preclinical evidence in this thesis together with previous findings (summarized in Figure 10, and Table 9) will help molecular tumor boards to select patients that would most likely benefit from ERBB4-targeted therapy. Positive results in individual patients with known activating *ERBB4* mutations could then guide optimal design of future clinical trials. They should aim to prioritize cancer types with the most convincing evidence of a functional role of overactive ERBB4, such as EGFR-mutant lung cancer and ERBB2-amplified breast/gastric cancer upon resistance to EGFR- and ERBB2-targeted therapy, and consider rational combinations with other therapies, as mentioned above and further discussed below. Of note, even though the overall frequency of the identified activating *ERBB4* mutations is low, it is now known, through the increased availability of genome-wide sequencing efforts, that specific driver mutations, even hotspots, are in fact a rare event in cancer, with a few exceptions such as BRAF V600E (Chang *et al.*, 2016; Chang *et al.*, 2018).

Multiomic clinical data would help to comprehensively understand the contexts of cancers driven by ERBB4 dysregulation via different mechanisms, such as activating mutations, overexpression of ERBB4 or its ligand(s) and availability of different heterodimerization partners. This will be imperative to know what to strive for in creating better preclinical models, considering the technical challenges which have likely biased previous results and limited the interest in studying ERBB4. Such challenges include but are likely not limited to the low endogenous ERBB4 expression levels in 2D cell culture (Segers *et al.*, 2020), potential need for creating deprived culture conditions to reveal ERBB4-dependency (II, III) (Kang *et al.*, 2007; Sundvall *et al.*, 2010; Mendoza-Naranjo *et al.*, 2013; Kurppa *et al.*, 2016; Wang *et al.*, 2018) and lack of potentially crucial crosstalk within the TME as demonstrated with brain metastases that reside in NRG-1 enriched microenvironment (Saunus *et al.*, 2015; Kalita-de Croft *et al.*, 2020).

Since *ERBB4*-mutant cancers would most likely benefit from combination therapies, as discussed above, and as appears to be the case with cancers harboring activating *ERBB2* mutations due to their weaker transforming potential than that of *ERBB2* amplification (Hyman *et al.*, 2018), preclinical studies should also focus on identifying the best combination treatment strategies. For instance, combinations of ERBB-targeting antibodies and TKIs can efficiently block activating mutations with various mechanisms of activation (Arteaga *et al.*, 2014; Schwarz *et al.*, 2017; Smyth *et al.*, 2020). Given the importance of ERBB4 in promoting cell survival in various

contexts, PI3K inhibitors could also be potential candidates for combinations with pan-ERBB inhibitors – as demonstrated with preclinical models of therapy-resistant ERBB2-amplified breast cancer driven by ERBB4 activation (Canfield *et al.*, 2015). and in ERBB2/ERBB3-mutant-driven breast cancer (Hanker *et al.*, 2021). This is further supported by this thesis work showing the AKT pathway being highly activated upon cellular transformation by activating ERBB4-mutants compared to wild-type ERBB4-transformed cells (III, Figure 4A). Finding the rational combinations for ERBB4-targeted therapy is especially important considering the evidence that ERBB4-addiction may emerge in later stages of cancer, which are more difficult to treat and often require combination therapies.

6.3 Limitations of the study and future perspectives

The first aim of this study was to elucidate ERBB4-mediated signaling mechanisms of the two cancer-associated isoforms in cancer. This study did identify novel and known interaction partners of ERBB4 and focused on detailed characterization of the interaction with VAV3, its ERBB4-mediated activation, showed MLC as one of the downstream signaling effectors and migration as a functional output. However, the full picture of the complex ERBB4-mediated signaling networks in cancer cells remains still to be deciphered. To get a more comprehensive view, integration of data from different modern omics techniques could shed light on the regulators, effectors and targets of ERBB4 activated signaling pathways. For instance, a recently published, first prior data-independent multiomics data integration pipeline (Vaparanta *et al.*, 2024) could be utilized to reliably predict even completely novel signaling networks and to further dissect the differences of the two cancer-associated ERBB4 isoforms by expressing them both alone and together.

In the quest to distinguish potential driver *ERBB4* mutations from passenger mutations among the highly diverse clinically observed *ERBB4* missense mutations (the predominant type of *ERBB4* mutations in cancer), the iSCREAM method (Chakroborty *et al.*, 2019), provided a particularly suitable platform to functionally screen nearly all of them in one go (II). However, the error-prone PCR-generated mutation library constitutes SNVs of *ERBB4* coding sequence, thus excluding other than SNV-encoded small mutations, structural variants, amplifications and deletions, as well as epigenetic changes. Technical improvements in generation of completely synthetic large mutant libraries of large genes now allow even more comprehensive functional screens. This was recently demonstrated by the Meyerson laboratory by screening nearly all possible EGFR amino acid substitutions for their erlotinib resistance conferring potential (Hayes *et al.*, 2024).

While the iSCREAM reproducibly identified novel potentially oncogenic activating *ERBB4* mutations, it failed to identify any of the previously discovered

activating *ERBB4* mutations, as well as the hotspot *ERBB4* mutations that were subsequently found to have a gain-of-function phenotype in the same Ba/F3 cell model (III). This apparently high false negative rate could be in part due to the pooling of all 7,396 *ERBB4* SNVs together, thus creating perhaps a too competitive setting to categorize non-hits as passengers. Based on similar functional screens with smaller mutation libraries, particularly with the method by Mano laboratory (Kohsaka *et al.*, 2017; Nagano *et al.*, 2018), inclusion of multiple time points could have indeed revealed additional mutations that may dominate in the cell pool at an earlier or later stage of the clonal evolution, such as the ERBB4 S303F. Moreover, the use of additional cell lineages (as done in III and discussed earlier in 6.1.2) and *in vivo* setting (Melnikov *et al.*, 2014; Berger *et al.*, 2016; Kohsaka *et al.*, 2017; Nagano *et al.*, 2018) could have helped to identify different activating mutations that are perhaps more potent in the presence of other interaction partners, a tumor microenvironment, or nutrient and oxygen deprivation, all of which may indeed affect ERBB4 biology (Sundvall *et al.*, 2010; Hollmén *et al.*, 2012; Paatero *et al.*, 2014).

The murine lymphoid (pro-B) Ba/F3 cells that were widely used in this study from transformation assays to *in vivo* allograft growth and drug sensitivity assays do not recapitulate the most typical solid tumor settings in which ERBB4 may drive tumor growth, although ERBB4-driven therapy resistance has also been identified in mature B cell neoplasms (Arribas *et al.*, 2023). However, in addition to being a widely used model feasible for high-throughput screening of oncogenic variants of kinases, including ERBBs (Jiang *et al.*, 2005; Greulich *et al.*, 2012; Jaiswal *et al.*, 2013; Kohsaka *et al.*, 2017; Nagano *et al.*, 2018; Chakroborty *et al.*, 2019; Robichaux *et al.*, 2021; Udagawa *et al.*, 2023; Koivu *et al.*, 2024), they are also one of the rare well-established models with negligible endogenous expression of ERBB receptors which could confound the results. Thus, Ba/F3 were used as a reductionist model to help dissecting ERBB4 homodimer functions from those of the heterodimers. However, it was found in this work that the normally very low endogenous ERBB3 expression levels (Riese *et al.*, 1995) were upregulated upon ERBB4-mediated, and notably also upon EGFR-mediated, Ba/F3 cell transformation. Therefore, *Erbb3* knockout Ba/F3 cells could serve as a *bona fide* ERBB receptor-devoid model in future studies.

The use of various non-cancerous cell models with or without co-expressed endogenous ERBB receptors was suitable for answering the questions whether activating *ERBB4* mutations can transform cells and whether they affect sensitivity to pan-ERBB inhibitors. However, in the light of the more established role of ERBB4 in promoting tumor progression, metastasis and therapy resistance, a perhaps more informative setting to study the oncogenicity of *ERBB4* mutations would be to use cancer cell models that are addicted to ERBB4. While such models have for long

been thought to be scarce, as discussed earlier, a greater body of evidence, in addition to this work, has emerged suggesting that 3D cell culture, *in vivo* settings, nutrient deprived- and therapy resistant-states demonstrate ERBB4 addiction (Kang *et al.*, 2007; Sundvall *et al.*, 2010; Hutcheson *et al.*, 2011; Hollmén *et al.*, 2012; Mendoza-Naranjo *et al.*, 2013; Canfield *et al.*, 2015; Kurppa *et al.*, 2016; Wang *et al.*, 2018; Li *et al.*, 2024). For instance, the oncogenic functions of ERBB4 (Canfield *et al.*, 2015), including gain-of-function effects of activating *ERBB4* mutations (Elster *et al.*, 2018), cannot be detected in cancer cells addicted to strong oncogenes, such as overexpressed ERBB2, without using a setting in which the cells become dependent on ERBB4; For instance, ERBB4 (and ERBB4-mediated activation of PI3K-AKT pathway) is indispensable for the survival of ERBB2-amplified breast cancer cell models resistant to lapatinib, trastuzumab, and especially to their combination, but not for their parental drug sensitive counterparts (Canfield *et al.*, 2015).

The functional analyses of *ERBB4* mutations in this study were conducted with models ectopically expressing only one ERBB4 isoform at a time, like other studies in the past aiming to differentiate the functions of the different isoforms. However, since (as discussed in section 2.3) i) both cancerous and non-cancerous cells typically express both CYT isoforms simultaneously, ii) the two CYT isoforms have differential functions *in vitro* and *in vivo*, also depending on cell type, and iii) the ratio of CYT-1 and CYT-2 expression can change during tumor progression, the findings in this work may not reflect the situation with naturally spliced ERBB4 isoforms. Interestingly, the direct comparison of cells expressing ERBB4 variants in either JM-a CYT-1 or JM-a CYT-2 isoforms showed that the phenotypes can either be very different, as in Ba/F3 cells (II, Supplementary Fig. S2), or very similar, as in BEAS-2B lung epithelial cells (II, Fig. 3A). Therefore, a more physiological and unbiased model to study the functional impact of *ERBB4* mutations could entail individual mutations generated by CRISPR-Cas9 or base-editing methods, also to avoid confounding effects from overexpression. While this is currently not feasible for very high-throughput screens, monitoring whether the expression ratio of the different ERBB4 isoforms in the presence or absence of an activating mutation changes *in vivo* during tumor progression could give valuable, more relevant insights into the complex ERBB4 cancer biology.

In conclusion, a logical next step after this study would be to evaluate the therapy resistance-promoting potential of the strong activating ERBB4 mutations, S303F and E715K, introduced into cancer cell genome with CRISPR-Cas9. For this, perhaps the most relevant settings based on clinical evidence discussed in this thesis could be *in vivo* models of *ERBB2*-amplified breast cancer or *EGFR*-mutant lung cancer treated with ERBB2/EGFR-targeted therapies. The potential targetability of ERBB4 could be addressed by analyzing whether second-generation pan-ERBB inhibitors can overcome or delay the expected ERBB4-mediated resistance in these models.

7 Summary/Conclusions

While other ERBB receptors are therapeutic targets in cancer, ERBB4 has for long been disregarded due to its ambiguous role in cancer and context-dependent functions, despite its notably high mutation rate in various cancers. This thesis aimed to evaluate the therapeutic potential of ERBB4 in cancer by elucidating its signaling mechanisms in cancer cells and by identifying potential driver mutations among the hundreds of cancer-associated *ERBB4* mutations.

The following conclusions can be made based on the results of this thesis:

1. VAV3 is a novel effector of ERBB4-mediated cancer cell signaling and cell migration, which may contribute to cancer cell metastasis
2. An unbiased high-throughput screen of activating ERBB4 mutations identified rare potential driver mutations that are targetable with clinically used second-generation pan-ERBB inhibitors
3. Many of the emerging hotspot ERBB4 mutations are transforming and the most potent recurrent activating mutations may serve as predictive biomarkers for clinically used second-generation pan-ERBB inhibitors

Together, these results strengthen the rationale for clinical evaluation of ERBB4 as a therapeutic target in cancer. The findings of this thesis can facilitate clinical interpretation of cancer-associated *ERBB4* mutations and guide the selection of patients for further clinical assessment of their targetability with clinically approved second-generation pan-ERBB inhibitors. Given the low prevalence of specific known activating *ERBB4* mutations in cancer patients, molecular tumor boards will be instrumental in facilitating this. As highlighted by this work, it will also be crucial to refine preclinical models to better recapitulate the contexts in which ERBB4 participates in driving cancer maintenance and progression. Finally, the methods used in this thesis may be exploited to understand other frequently mutated genes with a highly context-dependent role in cancer.

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