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ERBB4 MUTATIONS IN CANCER AND AMYOTROPHIC LATERAL SCLEROSIS

Kari J. Kurppa

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I almost wish I hadn't gone down that rabbit hole
– and yet – and yet –
it's rather curious, you know,
this sort of life!

-Alice in Wonderland

Kari J. Kurppa

***ERBB4* mutations in cancer and amyotrophic lateral sclerosis**

Department of Medical Biochemistry and Genetics, and MediCity Research Laboratory, University of Turku, and Turku doctoral programme of Molecular Medicine (TuDMM) and Turku Doctoral Programme of Biomedical Sciences (TuBS), Turku, Finland

ABSTRACT

ErbB receptor tyrosine kinases, epidermal growth factor receptor (EGFR, also known as ErbB1), ErbB2 (HER2 or NEU), ErbB3 (HER3), and ErbB4 (HER4), transduce signals borne by extracellular ligands into central cellular responses such as proliferation, survival, differentiation, and apoptosis. Mutations in *ERBB* genes are frequently detected in human malignant diseases of epithelial and neural origin, making ErbB receptors important drug targets. Targeting EGFR and ErbB2 has been successful in eg. lung and breast cancer, respectively, and mutations in these genes can be used to select patients that are responsive to the targeted treatment.

Although somatic *ERBB4* mutations have been found in many high-incidence cancers such as melanoma, lung cancer, and colorectal cancer and germ-line *ERBB4* mutations have been linked to neuronal disorders and cancer, ErbB4 has generally been neglected as a potential drug target. Thus, the consequences of *ERBB4* mutations on ErbB4 biology are largely unknown. This thesis aimed to elucidate the functional consequences and assess the clinical significance of somatic and germ-line *ERBB4* mutations in the context of cancer and amyotrophic lateral sclerosis.

The results of this study indicated that cancer-associated *ERBB4* mutations can promote aberrant ErbB4 function by activating the receptor or inducing qualitative changes in ErbB4 signaling. *ERBB4* mutations increased survival or decreased differentiation *in vitro*, suggesting that *ERBB4* mutations can be oncogenic. Importantly, the potentially oncogenic mutations were located in various subdomains in ErbB4, possibly providing explanation for the characteristic scattered pattern of mutations in *ERBB4*. This study also demonstrated that hereditary variation in *ERBB4* gene can have a significant effect on the prognosis of breast cancer. In addition, it was shown that hereditary or *de novo* germ-line *ERBB4* mutations that predispose to amyotrophic lateral sclerosis inhibit ErbB4 activity.

Together, these results suggest that ErbB4 should be considered as a novel drug target in cancer and amyotrophic lateral sclerosis.

Kari J. Kurppa

***ERBB4*-geenin mutaatiot syövässä ja amyotrofisessa lateraaliskleroosissa**

Lääketieteellinen biokemia ja genetiikka, MediCity-tutkimuslaboratorio, Turun yliopisto, ja Turun molekyylilääketieteen tohtoriohjelma (TuDMM), sekä Turun biolääketieteellinen tohtoriohjelma (TuBS), Turku

TIIVISTELMÄ

ErbB reseptorit, epidermaalisen kasvutekijän reseptori (EGFR tai ErbB1), ErbB2 (HER2 tai NEU), ErbB3 (HER3) ja ErbB4 (HER4), välittävät solun ulkopuolisten kasvutekijöiden tuomia signaaleja solun sisään, ja siten säätelevät keskeisiä solun toimintoja kuten kasvua, selviytymistä, erilaistumista ja kuolemaa. *ERBB*-geenien mutaatioita havaitaan toistuvasti eri syövissä. ErbB-reseptorit ovat merkittäviä lääkehoidon kohdemolekyylejä. Mutatoituneisiin EGFR- ja ErbB2-reseptoreihin kohdistettuja lääkehoitoja käytetään mm. keuhko- ja rintasyöpäpotilaiden hoidossa.

Somaattisia *ERBB4*-geenin mutaatioita on havaittu useissa yleisissä syövissä kuten melanoomassa, keuhkosyövässä sekä paksu- ja peräsuolisyövässä. *ERBB4*-geenistä on tunnistettu myös monia ituradan mukana periytyviä mutaatioita keskushermoston sairauksissa ja syövissä. ErbB4-reseptorin merkitys lääkehoidon kohteena tunnetaan kuitenkin huonosti. Tässä tutkimuksessa pyrittiin selvittämään, miten *ERBB4*-geenin mutaatiot vaikuttavat reseptorin toimintaan, sekä arvioimaan *ERBB4*-geenin mutaatioiden kliinistä merkitystä syövässä ja amyotrofisessa lateraaliskleroosissa.

Tutkimuksen tulokset osoittavat, että syövässä esiintyvät *ERBB4* geenin mutaatiot voivat johtaa epänormaaliin reseptorin toimintaan joko aktivoimalla reseptorin, tai aiheuttamalla muutoksia reseptorin solunsisäisessä viestinvälityksessä. Nämä muutokset johtivat lisääntyneeseen syöpäsolujen selviytymiseen tai vähentyneeseen solujen erilaistumiseen *in vitro*. Tulosten perusteella ErbB4-reseptorin mutaatiot voivat olla onkogeenisiä. Huomionarvoista on, että nämä mahdollisesti onkogeeniset mutaatiot olivat jakaantuneet useaan ErbB4-reseptorin rakenteelliseen alayksiköön. Tutkimuksessa osoitetiin myös, että *ERBB4*-geenin perinnöllinen variaatio voi vaikuttaa rintasyöpäpotilaan taudin ennusteeseen. Lisäksi tutkimuksessa näytettiin, että *ERBB4* geenin joko perinnölliset tai *de novo* ituradan mutaatiot, jotka altistavat amyotrofiselle lateraaliskleroosille, vähentävät ErbB4-reseptorin aktiivisuutta.

Tulosten perusteella mutatoitunut ErbB4-reseptori voi olla potentiaalinen lääkehoidon kohde syövässä ja amyotrofisessa lateraaliskleroosissa.

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ABBREVIATIONS

ADAM	a disintegrin and metalloprotease
AKT	v-akt murine thymoma viral oncogene homolog
ALS	amyotrophic lateral sclerosis
AR	amphiregulin
BAD	BCL2-associated agonist of cell death
BCR-ABL	breakpoint cluster region - c-abl oncogene
BRCA	breast cancer 1 and 2, early onset predisposing genes
BTC	betacellulin
CDK	cyclin-dependent kinase
CML	chronic myeloid leukemia
CNS	central nervous system
CYT	cytoplasmic
DAG	diacylglycerol
DDFS	distant disease-free survival
DEP1	density-enhanced phosphatase-1
DNA	deoxyribonucleic acid
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
EGFRvIII	EGFR variant III
EPG	epigen
ER	epiregulin
ER	estrogen receptor
ERK	extracellular signal regulated kinase
FALS	familial amyotrophic lateral sclerosis
FDA	United States food and drug administration
FUS	fused in sarcoma
GBM	glioblastoma multiforme
GRB2	growth factor receptor –bound protein 2
HB-EGF	heparin-binding EGF-like growth factor
HIF-1α	hypoxia-inducible factor 1α
HNPCC	hereditary non-polyposis colorectal cancer
ICD	intracellular domain
IL-3	interleukin-3
IP3	inositol triphosphate
ITCH	itchy E3 ubiquitin protein ligase
JM	juxtamembrane
kD	kiloDalton
KRAS	kirsten rat sarcoma viral oncogene homolog
LRIG1	leucine-rich repeats and immunoglobulin-like domains-1

mAb	monoclonal antibody
MAPK	mitogen-activated protein kinase
MEK	mitogen-activated protein kinase kinase
MIG6	mitogen-inducible gene-6
MLH	mutL homolog
MMP	matrix metalloprotease
MSH	mutS homolog
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NRG	neuregulin
NSCLC	non-small cell lung cancer
OPTN	optineurin
PDGFRA	platelet-derived growth factor alpha
PDK1	3-phosphoinositide-dependent kinase 1
PI3K	phosphoinositol-3 kinase
PIAS3	protein inhibitor of activated STAT3
PIP2	phosphoinositol-2-phosphate
PIP3	phosphoinositol-3-phosphate
PKC	protein kinase C
PLC-γ	phospholipase Cγ
PR	progesterone receptor
PTB	phosphotyrosine-binding
PTEN	phosphatase and tensin homologue
PTK	protein tyrosine kinase
PTP	phosphotyrosine phosphatase
RAF	v-RAF murine viral oncogene homolog
RIP	regulated intramembrane proteolysis
RT-PCR	reverse transcription polymerase chain reaction
RTK	receptor tyrosine kinase
SALS	sporadic amyotrophic lateral sclerosis
SH2	SRC-homology 2
SHC	SHC transforming protein
SNP	single nucleotide polymorphism
SOD1	superoxide dismutase 1
SOS	son of sevenless
STAT	signal transducer and activator of transcription
TCGA	the cancer genome atlas
TDP43	TAR DNA binding protein 43
TGF-α	transforming growth factor α
TKI	tyrosine kinase inhibitor
TM	transmembrane
UBQLN2	ubiquilin-2
WWOX	WW domain containing oxidoreductase
YAP	YES-associated protein

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications, which will be referred to by their Roman numerals (I-IV).

I. Tvorogov D, Sundvall M, Kurppa K, Hollmén M, Repo S, Johnson MS, Elenius K. Somatic mutations of *ERBB4*: selective loss-of-function phenotype affecting signal transduction pathways in cancer. J Biol Chem 2009, 284: 5582-5591.

II. Kurppa KJ, Denessiouk K, Johnson MS, Elenius K. Activating somatic *ERBB4* mutations in non-small cell lung cancer. Manuscript.

III. Kurppa KJ, Rokavec M, Sundvall M, Kellokumpu-Lehtinen PL, Joensuu H, Brauch H, Elenius K. *ERBB4* promoter polymorphism is associated with poor distant disease-free survival in high-risk early breast cancer. PLoS One 2014 9:e102388.

IV. Takahashi Y, Fukuda Y, Yoshimura J, Toyoda A, Kurppa K, Moritoyo H, Belzil VV, Dion PA, Higasa K, Doi K, Ishiura H, Mitsui J, Date H, Ahsan B, Matsukawa T, Ichikawa Y, Moritoyo T, Ikoma M, Hashimoto T, Kimura F, Murayama S, Onodera O, Nishizawa M, Yoshida M, Atsuta N, Sobue G; JaCALS, Fifita JA, Williams KL, Blair IP, Nicholson GA, Gonzalez-Perez P, Brown RH Jr, Nomoto M, Elenius K, Rouleau GA, Fujiyama A, Morishita S, Goto J, Tsuji S. *ERBB4* mutations that disrupt the neuregulin-ErbB4 pathway cause amyotrophic lateral sclerosis type 19. Am J Hum Genet. 2013, 93:900-905.

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1. INTRODUCTION

The ErbB family of receptor tyrosine kinases consists of epidermal growth factor receptor (EGFR, ErbB1), ErbB2 (HER2), ErbB3 (HER3) and ErbB4 (HER4). ErbB receptors function as mediators of extracellular signals borne by eleven EGF-like ErbB ligands into activation of a variety of intracellular signaling pathways governing central cellular processes such as proliferation, survival, differentiation and apoptosis. *ERBB* gene alterations resulting in aberrant activity of ErbB receptors are frequently detected in malignancies of epithelial and neural origin. These genetic alterations can serve as biomarkers to select patients that are responsive to drugs specifically designed to inhibit the activity of ErbB receptors.

While *EGFR* and *ERBB2* are well-established human proto-oncogenes that encode specific targets of clinically used ErbB-targeted cancer drugs, *ERBB4* has gained much less attention. However, somatic *ERBB4* mutations have been identified in many high-incidence tumors such as melanoma, lung cancer and colorectal cancer, and genomic germ-line *ERBB4* variants and polymorphisms have been linked to neuronal disorders and cancer. The consequences of *ERBB4* mutations on ErbB4 biology are, however, largely unknown and the question whether mutated ErbB4 receptor could be a drug target remains unanswered.

The aims of this thesis work were to elucidate the functional consequences and analyze the clinical significance of somatic and germ-line *ERBB4* mutations in cancer and amyotrophic lateral sclerosis in order to evaluate the potential role of mutated ErbB4 as a drug target.

2. REVIEW OF THE LITERATURE

2.1. Mutations cause cancer

The genomic sequences in cells are constantly subjected to corruption due to various mechanisms that introduce mistakes, or mutations, into the DNA. While mutations are acquired naturally in every cell division, cells are also exposed to various environmental mutagens such as tobacco smoke, radiation and chemicals. In spite of elaborate repair mechanisms to prevent the formation and propagation of these mutations, some of the mutations go unrepaired and are carried on to cell's progeny as the cell divides. Although acquiring mutations is a natural process, creating genetic and subsequent phenotypic heterogeneity of species and essentially enabling evolution, it can also have pathological consequences.

Cancer is thought to develop when cells over a long period of time gradually acquire somatic mutations that, in one way or another, give the cells a growth advantage over surrounding cells, resulting in a series of clonal expansions. Cells in a typical solid tumor can harbor thousands of mutations (Kandoth et al. 2013). However, a great majority of the mutations are passenger mutations, *i.e.* mutations that do not significantly contribute to tumorigenesis (Vogelstein et al. 2013). Early epidemiological studies suggested that solid tumors needed 5-8 consecutive "hits" in order to develop (Armitage and Doll 1954; Vogelstein et al. 2013). Later studies have shown that these "hits" represent acquired somatic mutations and that cultured human cells can be transformed by disrupting a similar number of cellular regulatory pathways (Hahn et al. 1999; Hahn and Weinberg 2002). This implied that there are certain characteristics normal cells have to acquire through mutations in order to become cancerous. Hanahan and Weinberg proposed six hallmarks of cancer that represent these characteristics: sustaining proliferative signaling, evading growth suppressors, resisting cell death, enabling replicative immortality, inducing angiogenesis, and activating invasion and metastasis (Hanahan and Weinberg 2000). The mutations within tumors that enable the cancer cells to acquire these characteristics and thus directly contribute to tumorigenesis are referred to as driver mutations.

The list of characteristics seems extensive for only a small number of driver mutations to achieve. These cellular processes are, however, governed by a limited number of entwined pathways forming the cellular signaling circuitry (Vogelstein et al. 2013). Thus, a mutation in a gene allowing the cell to sustain proliferative signaling might be in the same pathway or interconnected to a pathway regulating, for example, cell death, explaining at least in part how such a small number of mutations can induce so profound changes in cell behavior. The accumulated knowledge on the nature of the genes involved in tumorigenesis has allowed the division of genes commonly mutated

in cancer into three types: proto-oncogenes, tumor suppressor genes and stability, or "caretaker" genes (Vogelstein et al. 2013).

2.1.1. Mutations in proto-oncogenes

Proto-oncogenes are by definition genes that, once activated by mutations or overexpression, have potential to cause malignant transformation and thus tumors. These proteins are typically constituents of major cellular signaling pathways promoting cell proliferation, growth and survival. The activated proto-oncogenes, oncogenes, often induce the constitutive activation of the signaling pathway they are part of, thus uncoupling the pathway from its normal regulation and promoting cell transformation.

Many of the now classical human proto-oncogenes were initially discovered as human homologs of oncogenes carried by transforming retoviruses that induce tumors in chicken or rodents (Bishop 1985). The viral genes were often altered forms of their normal counterparts, first introducing the concept of oncogenes and proto-oncogenes (Stehelin et al. 1976). For example, *KRAS* (kirsten rat sarcoma viral oncogene homolog), a human homolog of the viral K-*ras* gene identified from Kirsten sarcoma virus, encode a critical signal mediator GTPase in the MAPK (mitogen-activated protein kinase) pathway (see chapter 2.3.4.). The viral gene harbors a subtle point mutation that changes a single glycine residue at codon 12 into serine (Tsuchida et al. 1982), resulting in constitutive activation of the protein product and thus, the pathway.

Although human cells that harbor an activated proto-oncogene gain a growth advantage over the neighboring normal cells, a single oncogene is not able to induce transformation of normal cells on its own. In contrast, it causes cellular senescence and apoptosis (Serrano et al. 1997; Collado and Serrano 2010). This is due to actions of tumor suppressor genes.

2.1.2. Mutations in tumor suppressor genes and caretaker genes

Whereas proto-oncogenes are components of pathways designed to provide signals promoting cell growth and proliferation, the tumor suppressor genes are constituents of pathways whose purpose is the opposite, to monitor cellular signaling and integrity and to counteract by regulating cell cycle progression, inhibiting excessive signaling, or by committing the cell into apoptosis if necessary. Tumor suppressor genes thus represent the main barriers for the formation of cancer. A multitude of tumor suppressor genes have been identified, based on their frequent inactivation in human cancer by various mechanisms, including mutations (Hanahan and Weinberg 2011).

The most well-known human tumor suppressor genes are *RB* and *TP53* (also known as *P53*) (Junttila and Evan 2009; Burkhart and Sage 2008; Sherr and McCormick 2002). The Rb protein functions as gatekeeper of the cell cycle, integrating growth-promoting and -inhibitory signals from various intra- and extracellular pathways, ultimately deciding whether or not the cell should proceed further through the cell cycle. The p53 protein

functions as the guardian of cell integrity and is activated when a cell is challenged by stress or abnormality, such as DNA damage or excessive, oncogenic signaling. Activation of p53 results in rapid cell cycle arrest and, in case the DNA lesions cannot be repaired or the oncogenic signaling persists, p53 commits the cell into apoptosis. Due to their central role in controlling the life and death decisions in cells, the Rb and p53 proteins are inactivated either directly or indirectly in practically all human tumors (Junttila and Evan 2009; Burkhart and Sage 2008; Sherr and McCormick 2002).

Genomic mutations are often acquired in cancer cells in an accelerated pace when compared to normal cells (Kandoth et al. 2013). Normal cells harbor a sophisticated DNA repair machinery, designed to sensor and repair DNA lesions as they occur. In cancer cells, however, mutations in the components of this machinery, the so-called caretaker genes are common (Kandoth et al. 2013), resulting in inappropriate repair of mutations. This leads to rapidly accelerated accumulation of mutations and thus increases the probability of a cancer cell to acquire additional critical mutations.

2.1.3. Oncogenic proteins as targets for cancer therapeutics

Activation of a proto-oncogene is usually an early step in cancer development (Fearon and Vogelstein 1990). Although in the course of cancer evolution the acquisition of genomic mutations and subsequent divergent clonal expansions result in genetically very heterogenous populations of cancer cells, the proliferation of cancer cells in tumors is often still driven by the oncogene that was activated in the initial steps. These cancer cells are dependent, or "addicted", on the growth-promoting signaling provided by the oncogene, a phenomenon referred to as oncogene addiction (Weinstein and Joe 2008). This phenomenon makes oncogenic proteins prime targets for therapeutic cancer drugs. The first clinically used cancer drug specifically targeting an oncogene product was imatinib, a small molecular weight tyrosine kinase inhibitor (TKI) inhibiting the activity of the BCR-ABL (breakpoint cluster region - c-abl oncogene) fusion oncoprotein that is found in more than 95% of patients with chronic myeloid leukemia (CML) (Savage and Antman 2002). Following the success of imatinib, a large number of specifically targeted cancer drugs are now in clinical use, many of which target the ErbB receptor tyrosine kinases, the focus of this thesis (Arteaga and Engelman 2014).

2.1.4. Genomic variants and polymorphisms in cancer

Cancer typically arises due to accumulating somatic mutations, *i.e.* mutations that are not present in the germ-line. However, the natural diversity in genomic constitution between individuals, certain genomic variants and gene polymorphism, can also play a role in the development of cancer. Indeed, it is estimated that various constitutional genomic defects resulting in hereditary susceptibility to cancer underlie 5-10% of all cancer cases.

Germ-line mutations affecting tumor suppressor genes, caretaker genes or proto-oncogenes can give the affected individuals a head-start in tumorigenesis as all cells in these individuals already harbor a cancer-promoting mutation (Vogelstein and Kinzler 2004). These often relatively rare mutations predispose to cancer, but they do not cause cancer *per se*. Rare genomic variants causing the most common forms of hereditary cancer, hereditary breast cancer and hereditary nonpolyposis colorectal cancer (or Lynch syndrome), affect the *BRCA* (*BRCA1* and *BRCA2*) and the *MSH* and *MLH* caretaker genes, respectively (Narod and Foulkes 2004; Kinzler and Vogelstein 1996). Mutations in these genes, involved in DNA repair, result in abnormal accumulation of somatic mutations and dramatically increased susceptibility to cancer (Narod and Foulkes 2004; Kinzler and Vogelstein 1996).

The role of common germ-line polymorphisms and other variants in the outcome of cancer is a less well studied field. Most of these genetic variants reported to associate with cancer prognosis seem to affect the outcome by various indirect mechanisms. Common variants with prognostic significance have been reported mainly in genes involved in drug metabolism, transport and DNA repair which modulate the response to cancer drugs or irradiation (Deenen et al. 2011a, 2011b, 2011c). Some genomic polymorphisms seem to be associated with subtypes of cancer with intrinsically worse prognosis (Sakamoto et al. 2008), and some affect genes modulating immune responses (Kim et al. 2009; Ward et al. 2012).

2.2. Genetics of amyotrophic lateral sclerosis

Amyotrophic lateral sclerosis (ALS) is a devastating neurodegenerative disease affecting the motor neurons. The peak age at onset of ALS is 58-63 years and the patients exhibit progressive paralysis ultimately leading to death by respiratory failure typically 3-5 years after initial symptoms (Kiernan et al. 2011). It has been proposed that more than 20% of the cases have a family history of ALS (Andersen and Al-Chalabi 2011), indicating a strong inherited genetic contribution to the disease. ALS is thus commonly divided into familial ALS (FALS) and sporadic ALS (SALS). However, this segregation may not always be accurate, because in many cases there is not enough available information and DNA samples from the patients´ pedigree to exclude a family history from apparently sporadic cases of ALS. In addition, FALS and SALS are clinically indistinguishable, except for the typically younger age at onset for FALS (47-52 years) (Kiernan et al. 2011; Andersen and Al-Chalabi 2011).

The genetic etiology of ALS is still largely unresolved and much effort has been put into finding genes predisposing to ALS by searching for disease-associated germ-line gene mutations (polymorphisms) in ALS patients. A total of 114 genes have been associated with ALS according to the Amyotrophic Lateral Sclerosis Online Genetics Database (http://alsod.iop.kcl.ac.uk, reviewed 15.8.2014), although a causal link for

the majority of these genes is not clear. However, there are more than twenty genes with accumulating evidence of causal link to ALS pathogenesis. Still, mutations in these genes have been estimated to account for 25-35% of FALS and 5-10% of SALS, at most (Andersen and Al-Chalabi 2011). By mutation prevalence, three genes, *SOD1* (superoxide dismutase 1), *TDP43* (TAR DNA binding protein 43) and *FUS* (fused in sarcoma), are considered to be the most important causative ones (Millecamps et al. 2010; Tsai et al. 2011; Andersen and Al-Chalabi 2011).

The molecular mechanisms by which the ALS-associated genes contribute to the pathogenesis of ALS are still largely elusive. However, the protein products of several ALS-associated genes, such as *TDP43* and *FUS*, as well as emerging ALS-genes *OPTN* (optineurin), *UBQLN2* (ubiquilin-2) and *C9ORF72* are frequently observed in neuronal inclusions which are characteristic for ALS pathology (Blokhuis et al. 2013). Also, ALS-associated *SOD1* mutations have been shown to promote SOD1 aggregation in cells (Karch et al. 2009; Prudencio et al. 2009; Prudencio and Borchelt 2011), and all mutant SOD1-induced mouse models of ALS show neuronal SOD1 aggregates at the end stage of the disease (Wang et al. 2003, 2005, 2006; Johnston et al. 2000; Karch et al. 2009). These observations suggest that protein aggregation may have a similar role in ALS pathogenesis as in other neurogenerative disorders such as Huntington's disease and Alzheimer's disease (Blokhuis et al. 2013; Ross and Tabrizi 2011; Querfurth and Laferla 2010), although this hypothesis still remains to be proven.

2.3. ErbB receptors

Protein phosphorylation is a post-translational modification regulating signal transduction in mammalian cells. The transfer of a phosphate group from ATP (adenosine triphosphate) to a protein substrate is catalyzed by protein kinases. Out of more than 520 known protein kinase genes in the human genome, over 90 encode kinases that catalyze the phosphorylation of specific tyrosine residues in target proteins (protein tyrosine kinases, PTK) (Blume-Jensen and Hunter 2001). Receptor tyrosine kinases (RTKs) are a family of transmembrane PTKs encompassing 58 genes divided into 20 subfamilies. The genes encoding RTKs are very potent proto-oncogenes and frequently deregulated in human cancer by overexpression, autocrine activation, chromosomal translocations or gain-of-function mutations (Lemmon and Schlessinger 2010). The ErbB subfamily of RTKs contains four genes, encoding ErbB1 (epidermal growth factor receptor, EGFR) (Ullrich et al. 1984), ErbB2 (HER2) (Stern et al. 1986), ErbB3 (HER3) (Plowman et al. 1990), and ErbB4 (HER4) (Plowman et al. 1993).

2.3.1. Structure

All ErbB receptors share an identical overall structure with an extracellular domain, a single transmembrane (TM) domain, an intracellular domain containing the tyrosine

kinase enzyme and a C-terminal tail harboring docking sites for signal transduction molecules. The ErbB receptors are heavily glycosylated and have a molecular weight of approximately 180 kD (Figure 1). EGFR are ErbB4 are fully functional receptors, whereas ErbB2 lacks a known ligand (Lemmon et al. 2014) and ErbB3 harbors an inactive kinase domain (Guy et al. 1994).

The extracellular domain can be divided into four distinct subdomains named I, II, III and IV (Lax et al. 1988), or alternatively L1, CR1, L2, CR2 (Ward and Garrett 2001). Ligand binding domains I and III are globular domains of the leucine-rich repeat superfamily and adopt a right-handed beta helix structure. Cysteine-rich domains II and IV are rod-like domains, characterized by multiple disulphide bonds and contain elements involved in receptor autoinhibition and dimerization (Figure 1).

The transmembrane domain is a single hydrophobic alpha helix docking the receptor on the cell membrane. The TM domain can also facilitate receptor dimerization, as emphasized by the cancer-associated point mutation in the ErbB2 TM domain (the *neu* oncogene) that promotes dimerization and activation of the neu oncoprotein (Weiner et al. 1989; Bargmann and Weinberg 1988). EGFR TM domain dimerization has been shown to be mediated by dimerization motifs in the N-terminal region of the TM domain (Endres et al. 2013). However, mutations in this region do not completely abolish EGFR activation, suggesting some degree of plasticity in the dimerization of the TM domains (Lu et al. 2010; Endres et al. 2013).

The intra- and extracellular juxtamembrane (JM) regions flanking the cell membrane have functions related to receptor regulation and activation. The intracellular JM region is important in mediating EGFR downregulation (Sorkin and Goh 2009). In addition, the intracellular JM region is necessary for proper receptor activation by facilitating kinase domain dimer formation (Thiel and Carpenter 2007; Jura et al. 2009a; Red Brewer et al. 2009). The extracellular JM region is involved in the unique ability of ErbB4 to release a signaling-competent intracellular domain (Elenius et al. 1997a). This phenomenon will be discussed in detail later.

The kinase domain of ErbB receptors have a two-lobed structure typical for all previously reported protein kinase domains (Figure 1) (Stamos et al. 2002). It consists of a NH_2-terminal lobe (N-lobe), formed mostly of ß-strands apart from one α-helix (αC), and a mostly α-helical COOH-terminal lobe (C-lobe). The two lobes are separated by a cleft harboring the ATP-binding site. The active site is formed around the ATP-binding cleft with catalytic elements both in N- and C-lobes. (Stamos et al. 2002).

The C-terminal tail following the kinase domain contains several tyrosine residues that are phosphorylated upon receptor activation and serve as docking sites for signaling proteins and regulators (Schulze et al. 2005), some of which are discussed in more detail later.

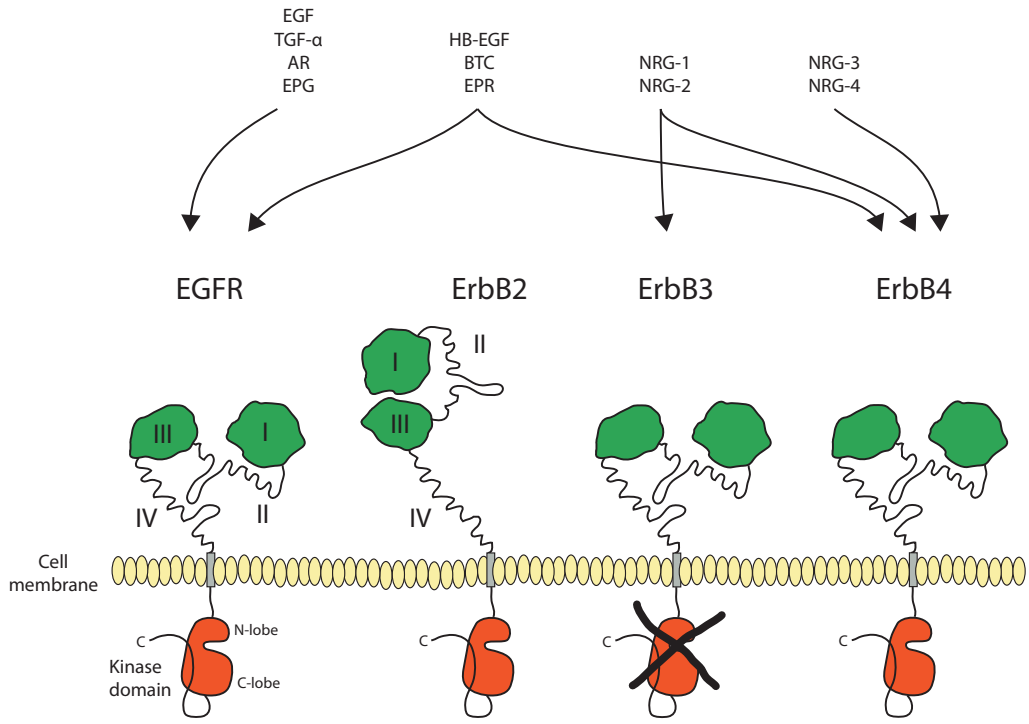

Figure 1: Structures and ligands of ErbB receptors. Top: ErbB ligands grouped according to their specificity towards different ErbB receptors (arrows). Below: schematic representation of protein structures of ErbB receptors in the inactive state. The extracellular domain of ErbB2 does not adopt a closed, inactive conformation. Roman numerals indicate subdomains within the extracellular domain. Black cross indicates the inactive ErbB3 kinase domain.

2.3.2. Ligands

The activity of ErbB receptors is modulated by 11 epidermal growth factor (EGF) -like ligands with different binding specificities to ErbB receptors (Figure 1). EGF, transforming growth factor alpha (TGF-α), amphiregulin (AR), heparin-binding EGF-like growth factor (HB-EGF), betacellulin (BTC), epiregulin (EPR), and epigen (EPG) bind and activate EGFR (Riese II et al. 1996b; Strachan et al. 2001). HB-EGF, BTC, and EPR can also serve as ligands for ErbB4 (Elenius et al. 1997b; Riese II et al. 1996a). Neuregulins (NRG-1, NRG-2, NRG-3, NRG-4) are a family of ligands partially shared by ErbB3 and ErbB4. All neuregulins activate ErbB4, whereas NRG-1 and NRG-2 can also bind and activate ErbB3 (Carraway et al. 1997; Chang et al. 1997; Harari et al. 1999; Zhang et al. 1997). ErbB2 seems to be an orphan receptor, as no high-affinity ligand has been indentified for ErbB2 (Lemmon et al. 2014).

The ErbB ligands are synthesized as membrane-bound precursors and are released from the cell membrane by metalloprotease-mediated cleavage, enabling paracrine and autocrine signaling (Sanderson et al. 2006). The main metalloporteinases responsible for

the release of ErbB ligands belong to the ADAM (a disintegrin and metalloprotease) and MMP (matrix metalloprotease) families (Sanderson et al. 2006). All ErbB ligands share a conserved motif known as the EGF-motif, which is responsible for receptor binding and activation, and consists of six conserved cysteine residues forming three intramolecular disulphide bonds. All ErbB ligands seem to bind their receptors in identical manner, but can elicit a wide range of biological responses, depending on the ligand (Wilson et al. 2009). It has been proposed that different ligands may induce subtly different conformational changes in the ErbB receptor extracellular domains, which may at least partly account for the diverse biological responses (Wilson et al. 2009). However, the divergent binding specificities of ErbB ligands and the subsequent formation of different homo- or heterodimeric ErbB complexes most likely also explain these differences.

2.3.3. Mechanism of activation

Ligand binding induces conformational rearrangements in the ErbB extracellular domain that facilitate receptor dimerization

The extracellular domains of EGFR, ErbB3 and ErbB4 can adopt an open and a closed conformation, representing active and autoinhibited, inactive forms, respectively. In the absence of ligand, the extracellular domain is mostly in the closed conformation, stabilized by intermolecular hydrogen bonding between subdomains II and IV (Figure 2). The closed conformation buries the dimerization arm, an extended ß-hairpin loop from the subdomain II that is critical for receptor dimerization, thus restricting receptor dimerization and activation (Cho and Leahy 2002; Bouyain et al. 2005; Ferguson et al. 2003). Upon activation, the extracellular domain undergoes major structural rearrangement, where subdomains I and II rotate upward around the subdomain II/III "hinge" region, bringing subdomains I and III into close proximity in order to facilitate the binding of a bivalent ligand between subdomains I and III (Figure 2) (Garrett et al. 2002; Ogiso et al. 2002; Liu et al. 2012). Ligand binding stabilizes the open conformation, revealing the dimerization arm and priming the receptor for dimerization (Figure 2) (Garrett et al. 2002; Ogiso et al. 2002; Liu et al. 2012).

Although adopting the open conformation, in order to expose the dimerization arm, is essential for ErbB dimerization (Garrett et al. 2002) it is not sufficient to promote efficient dimerization. Mutating the subdomain II/IV interface in full-length EGFR does not lead to constitutively activated receptor (Mattoon et al. 2004; Walker et al. 2004; Dawson et al. 2005), and EGFR extracellular domains lacking the entire subdomain IV are still dependent on ligand binding for dimerization (Garrett et al. 2002; Ogiso et al. 2002). A bend in subdomain II, induced by ligand binding to subdomains I and III, has been proposed to be the ultimate conformational change regulating ErbB dimerization (Dawson et al. 2005; Alvarado et al. 2010; Liu et al. 2012). This bending enables the optimal juxtaposition of monomeric receptors for dimerization (Alvarado et al. 2010;

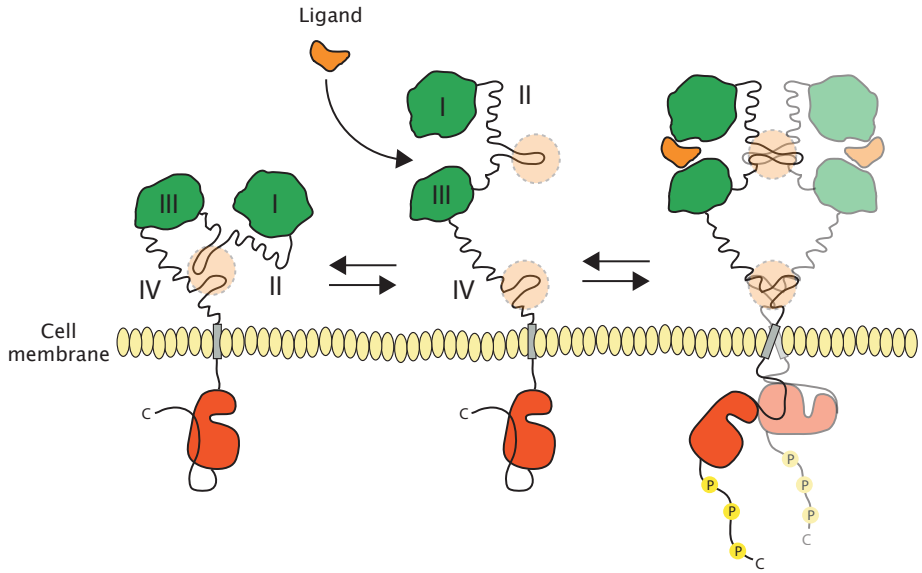

Figure 2: Mechanism of activation of ErbB receptors. Left: in the inactive state, the extracellular domains of ErbB receptors, except for ErbB2, are in closed conformation, masking the dimerization interfaces in subdomain II and IV (indicated by circles). Middle: upon ligand binding, the extracellular domain extends, revealing the dimerization interfaces. Ligand binding stabilizes the extended, open conformation and primes the receptor for dimerization. Right: dimerized receptors make extensive contacts throughout the length of the receptors, leading to the activation of the tyrosine kinase domains and phosphorylation of tyrosine residues in the COOH-terminal tails (indicated by yellow circles).

Liu et al. 2012) and is thought to serve as a barrier preventing the dimerization of spontaneously occurring unligated ErbB dimers (Alvarado et al. 2010; Liu et al. 2012).

Once dimerized, the extracellular domains of ErbB receptors make extensive inter-receptor contacts mediated mainly by subdomains II and IV (Figure 2). However, a mutation analysis has shown that the ErbB extracellular domain dimers are mostly stabilized by subdomain II contacts, subdomain IV contacts providing only a minor contribution to the overall dimerization energy (Dawson et al. 2005). As the dimerized ErbB monomers interact substantially throughout the length of the receptor (discussed in detail later), it is possible that the subdomain IV interactions are more important in optimally positioning the transmembrane and intracellular domains for activation.

Unique structural features of ErbB2

ErbB2 is unique among ErbB receptors as it adopts a constitutively extended conformation, and is thus constantly poised for dimerization (Figure 1) (Cho et al. 2003; Garrett et al. 2003). The amino acid residues responsible for the subdomain II / IV interaction observed in the closed conformation of other ErbBs are not conserved in ErbB2, preventing the formation of the closed conformation in ErbB2 (Cho et al. 2003; Garrett et al. 2003). In

addition, the open conformation of the ErbB2 extracellular domain is stabilized by an interaction between subdomains I and III, mimicking the ligand-mediated stabilization of the open conformation in other ErbB receptors (Figures 1 and 2) (Cho et al. 2003; Garrett et al. 2003). These unique structural features together with amino acid substitutions in the ligand binding site that would inhibit ligand binding in EGFR (Cho et al. 2003; Garrett et al. 2003) rationalize the lack of known ligands for ErbB2.

However, although ligand binding is not needed for the ErbB2 extracellular domain to adopt the open conformation, the formation of ErbB2 homodimers is very weak (Ferguson et al. 2000; Cho et al. 2003; Garrett et al. 2003). The structure of the ErbB2 extracellular domain provides a possible explanation for this apparent discrepancy. In contrast to other ErbB receptors, the dimerization loop and the subdomain II pocket into which the tip of the dimerization loop docks are negatively charged in ErbB2, leading to electrostatic repulsion not favoring the formation of ErbB2 homodimers (Garrett et al. 2003). In addition, the subdomain II of ErbB2 extracellular domain does not adopt the bent conformation seen in ligand-bound EGFR and ErbB4 structures (Garrett et al. 2003; Ogiso et al. 2002; Liu et al. 2012), preventing the optimal juxtaposition of extracellular domains in ErbB2 homodimers (Liu et al. 2012). However, these structural features preventing ErbB2 homodimerization do not prevent ErbB2 heterodimerization with other ErbB receptors. Indeed, ErbB2 has been shown to be the preferred heterodimerizing partner for all ErbB receptors (Tzahar et al. 1996; Graus-Porta et al. 1997) and ErbB2-containing ErbB heteromers are considered to be the most oncogenic of all ErbB receptor homo- or heterodimeric complexes (Zhang et al. 1996; Cohen et al. 1996).

Negative co-operativity of ligand binding

The crystal structures of Drosophila EGFR (dEGFR) have shed light into the puzzling existence of high- and low-affinity ligand binding classes of ErbB receptors (King and Cuatrecasas 1982; Schlessinger 1986). The discovery of open and closed conformations of EGFR and ErbB3 (Garrett et al. 2002; Ogiso et al. 2002; Cho and Leahy 2002; Garrett et al. 2003) suggested a hypothesis that these conformations could be the sources of high- and low affinity ligand binding, respectively. However, that hypothesis was proven wrong soon after (Klein et al. 2004; Walker et al. 2004; Dawson et al. 2005).

Later, a biochemical study suggested that the high- and low affinity EGF binding could result from negative co-operativity of ligand binding to EGFR, i.e. the binding of the first ligand hinders the binding of the second ligand (Macdonald and Pike 2008). The crystal structure of ligand-bound dEGFR dimer revealed the structural mechanisms underlying this phenomenon (Alvarado et al. 2010). The ligand for dEGFR, Spitz, binds to one receptor monomer in weak, preformed dEGFR dimers and induces a conformational change in the subdomain II of the extracellular domain (Alvarado et al. 2010), analogous to the bend observed in human EGFR and ErbB4 (Garrett et al. 2003; Liu et al. 2012). This conformational change allows tight interaction between the subdomains II of the

dimerized dEGFR extracellular domains and results in receptor activation (Alvarado et al. 2010). The second ligand, however, cannot effectively bind to the other monomer without compromizing the tight interaction of the singly-ligated dimer, and therefore has to occupy a compromized binding site with lower affinity (Alvarado et al. 2010). Thus, in dEGFR, the binding of first and second ligand in the preformed dimer represent the observed high- and low-affinity ligand binding classes, rather than two separate populations of receptors (as in the case of open and closed conformations of extracellular domains).

Although singly-ligated human ErbB dimers have not been observed, a detailed analysis of EGFR and ErbB4 extracellular domains and *in vitro* experiments with mutated EGFR and ErbB4 have provided evidence that singly ligated human ErbB dimers could be active (Liu et al. 2012). The physiological implications of the proposed two-step ligand binding are not yet understood, but it has been proposed to be involved in the fine-tuning of ErbB signaling output according to the concentration or affinity of different ErbB ligands (Alvarado et al. 2010; Lemmon et al. 2014).

Allosteric activation mechanism of ErbB kinase domains

The dimerization of the ErbB extracellular domains ultimately leads to activation of the kinase domains of the dimerized receptors (Figure 2). The activation of most protein kinases is dependent on activation loop phosphorylation (Jura et al. 2011). However, the activation of ErbB receptors is not, although all ErbB receptors harbor a conserved phosphorylation site which in EGFR (Tyr845) is robustly phosphorylated following ligand binding (Gotoh et al. 1992).

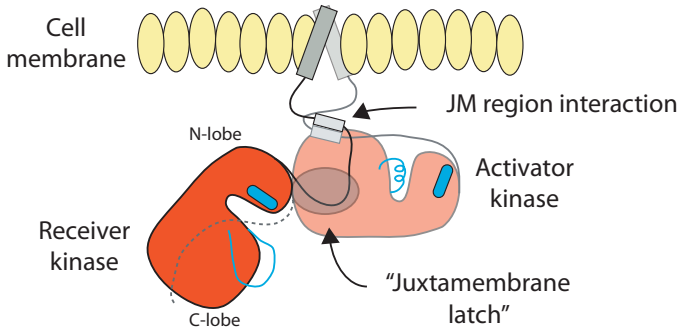

Figure 3: The asymmetric kinase dimer. Upon dimerization, the kinase domains of ErbB receptors interact in head-to-tail manner, where one kinase domain serves as an activator and the other as a receiver. This interaction is stabilized by the juxtamembrane region between the cell membrane and the kinase domains. The juxtamembrane region of the receiver kinase interacts with the C-lobe of the activator kinase, forming a "juxtamembrane latch" that stabilizes the asymmetric dimer. Closer to the cell membrane, the juxtamembrane regions also interact via short helixes, further supporting the kinase dimer. The interaction of kinase domains results in the activation of the activator kinase in *trans*. The dashed line indicates the hypothetical route of the activator kinase C-terminal tail through the active site of the receiver kinase. Blue cylinders and lines indicate the orientations of αC helix and the activation loop, respectively, in active (receiver kinase) and inactive (activator kinase) states. Figure modified from Jura et al. 2009.

Instead of activation loop phosphorylation, the activity of ErbB receptor kinase domains is regulated allosterically, through interaction between the two kinase domains of dimerized receptors (Zhang et al. 2006). The allosteric activation mechanism was first described for EGFR (Zhang et al. 2006), and in later studies also shown to mediate the activation of other ErbB receptors in homo- and heterodimers (Jura et al. 2009b; Monsey et al. 2010; Qiu et al. 2008). Upon dimerization, the kinase domains interact in head-to-tail manner, where the bottom of the C-lobe of one kinase (the activator kinase) contacts the top of the N-lobe of the other (the receiver kinase) (Figures 2 and 3) (Zhang et al. 2006). The interaction stabilizes the active conformation of the receiver kinase and results in phosphorylation of the activator receptor monomer in *trans*. This mechanism of regulating kinase activity closely resembles the mechanism of how the cyclin-dependent kinases (CDK) are regulated by cyclins, and is unique among RTKs (Bae and Schlessinger 2010).

The asymmetric kinase dimer is weak, however, and isolated EGFR kinase domains remain mostly monomeric and inactive in solution, even in relatively high concentrations (Jura et al. 2009a; Zhang et al. 2006). The interaction between kinase monomers in the asymmetric dimer, and thus the proper activation of the kinases, has been shown to be mediated by the intracellular juxtamembrane domain (Thiel and Carpenter 2007; Jura et al. 2009a; Red Brewer et al. 2009; Macdonald-Obermann and Pike 2009). Crystal structures of EGFR and ErbB4 kinase domains demonstrated that JM region above the N-terminus of the receiver kinase packs against the C-lobe of the activator kinase, forming a "juxtamembrane latch", thus docking the two kinase domains in place (Figure 3) (Jura et al. 2009a; Red Brewer et al. 2009). In addition, the N-terminal parts of the JM regions form short interacting helixes, which further support the asymmetric dimer (Figure 3) (Jura et al. 2009a). Interestingly, the juxtamembrane latch is occupied by the C-terminal tail of the kinase in the absence of asymmetric dimer, putatively providing an autoinhibitory mechanism restricting spontaneous dimerization of the kinase domains (Figure 2) (Jura et al. 2009a).

In crystal structures of asymmetric EGFR kinase domains including the JM regions, the very N-termini of the JM-regions align very well with the C-termini of transmembrane domain dimer NMR structures, suggesting that the juxtaposition of dimerized ErbB extracellular domains is structurally directly coupled to asymmetric kinase formation by the transmembrane domains (Jura et al. 2009a). This also provides a model of how the information provided by ligand binding to extracellular domains of ErbB receptors is relayed into the activation of intracellular signaling pathways by series of structural arrangements throughout the length of the receptors.

Allosteric regulation of ErbB transactivation rationalizes ErbB3 signaling activity

Studies revealing the allosteric mechanism of ErbB receptor kinase domain activation have also helped to understand how ligand binding to kinase-inactive ErbB3 can

result in ErbB3 phosphorylation and signaling. The ErbB3 kinase domain lacks two important catalytic residues in the active site, a conserved glutamate in αC helix, and the aspartate serving as catalytic base (Guy et al. 1994). In the kinase dimer, however, the activator kinase does not have to be active (Zhang et al. 2006). The activator kinase C-lobe interface is conserved in ErbB3 (Zhang et al. 2006; Jura et al. 2009b) and ErbB3 can thus readily serve as the activator kinase for other members of the ErbB family in heterodimers (Jura et al. 2009b). In contrast, the receiver interface in the N-lobe of the ErbB3 kinase is not conserved (Zhang et al. 2006). This means that the ErbB3 kinase domain cannot serve as the receiver kinase, which is understandable considering the lack of ErbB3 kinase activity.

The asymmetric kinase dimer explains the phosphorylation of ErbB3 in a heterodimer, but it does not explain how the heterodimerization partner of ErbB3 is phosphorylated. A recent study demonstrated that NRG-1-induced ErbB3/ErbB2 heterodimers form tetrameric complexes, where the ErbB2 receptors phosphorylate each other in *trans* (Zhang et al. 2012). An RNA aptamer specifically binding to the putative tetramer interface in ErbB3 did not affect NRG-1-induced ErbB3 dimerization with ErbB2 or ErbB3 phosphorylation by ErbB2, but greatly reduced the phosphorylation and downstream signaling of ErbB2 (Zhang et al. 2012). These results suggest that higher-order ErbB complexes not only exist, but are crucial for normal ErbB signaling (Zhang et al. 2012; Sliwkowski 2012).

Although ErbB3 has been generally accepted as an inactive receptor, a kinase activity about 1000 times lower than that of the EGFR kinase domain has been reported for ErbB3 *in vitro* (Shi et al. 2010). The physiological relevance of this low activity, however, is still to be elucidated.

2.3.4. Signaling pathways

ErbB receptor activation upon ligand binding leads to the phosphorylation of tyrosine residues in the C-terminal tail of the receptor. These phosphorylated tyrosines serve as docking sites for signaling and adaptor proteins, which bind to the receptors *via* their SH2 (SRC-homology 2) or PTB (phosphotyrosine-binding) domains. The recruited signaling/adaptor proteins are typically subsequently phosphorylated either by the ErbB kinase or by their intrinsic kinase activity, leading to activation of intracellular signaling pathways (Schlessinger 2000). All ErbB receptors have a characteristic pattern of signaling/adaptor molecule binding sites (Schulze et al. 2005), resulting in qualitative as well as quantitative differences in the capability to promote intracellular signaling. ErbB receptor signaling is further complicated by the different homo- and heterodimeric complexes stimulated by ligands with different binding specificities to individual ErbB receptors, making ErbB signaling output highly context-dependent (Olayioye et al. 1998).

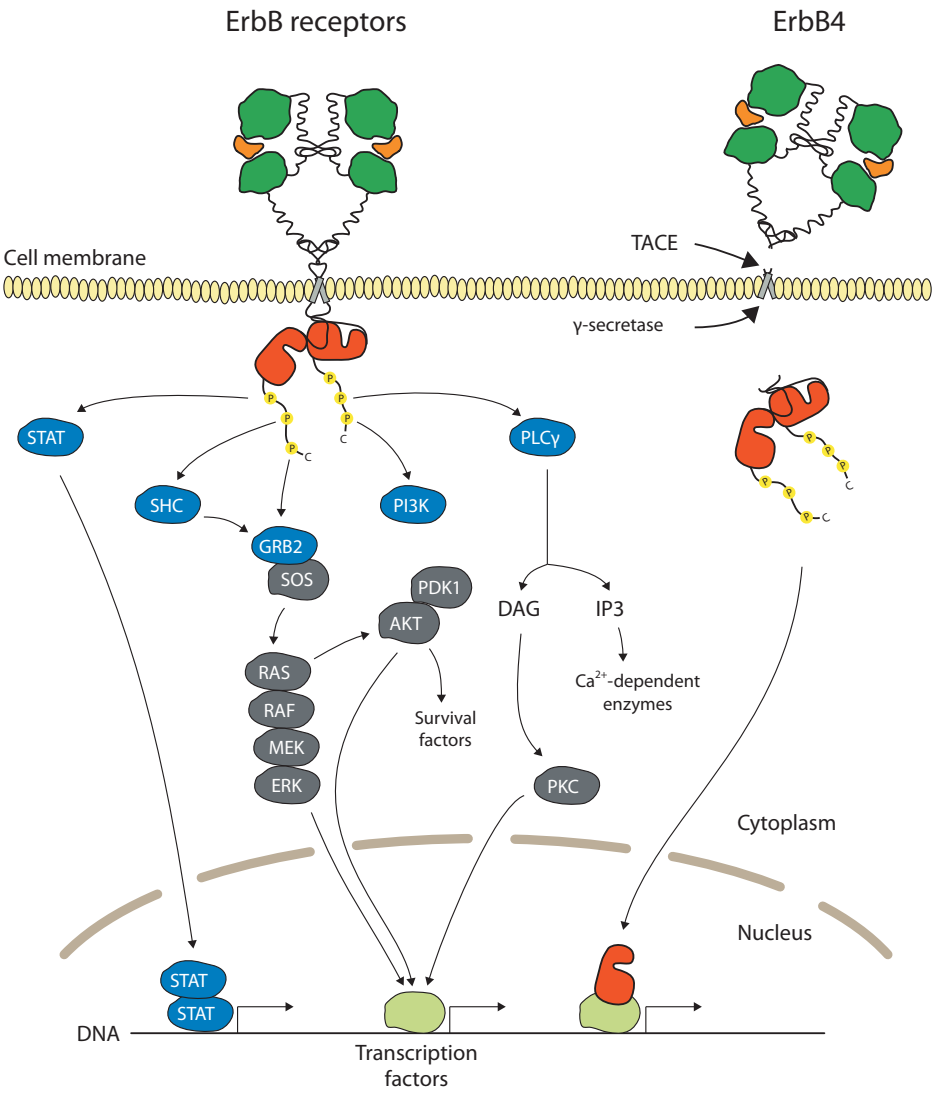

Figure 4: Cell signaling by ErbB receptors. The binding of signal transduction molecules to phosphorylated tyrosine residues in C-terminal tails of ErbB receptors initiates the activation of intracellular signaling cascades. The main pathways activated by the ErbB receptors are shown. ErbB4 is only ErbB receptor to undergo proteolytic processing on the cell membrane. The released soluble intracellular domain can translocate to nucleus where it can bind and regulate transcription factors. Blue color indicates molecules directly interacting with ErbB receptors. Gray color indicates other molecules in signaling pathways. Light green color indicates transcription factors.

Mitogen-activated protein kinase pathway

All ErbB receptors can activate the MAPK pathway by recruiting the GRB2 (growth factor receptor –bound protein 2) – SOS (son of sevenless) complex directly or *via* SHC (SHC transforming protein) (Figure 4) (Yarden and Sliwkowski 2001; Schulze et al. 2005). The recruitment of GRB2-SOS complex brings it close to the plasma membrane,

where the guanidine-nucleotide exchange factor SOS can activate RAS. The activation of RAS in turn triggers a cascade where subsequent phosphorylations of RAF, MEK and ERK result in translocation of ERK to the nucleus where it can activate a number of transcription factors. MAPK signaling can mediate various cellular processes but above all the MAPK signaling pathway is a potent mediator of cell proliferation and commonly targeted by oncogenic mutations in RAS, RAF or EGFR (Roberts and Der 2007).

Phosphoinositol-3 kinase pathway

Phosphoinositol-3 kinase (PI3K) pathway (Figure 4) is also an important mediator of ErbB receptor signaling. ErbB3 and ErbB4 harbor direct binding sites for the PI3K regulatory subunit p85, whereas EGFR and ErbB2 can activate PI3K indirectly, for example *via* activation of Ras (Schulze et al. 2005; Rodriguez-Viciana et al. 1994). PI3K catalyzes the conversion of PIP2 (phosphoinositol-2-phosphate) into PIP3 (phosphoinositol-3-phosphate), which recruits Akt kinase and its activator kinase PDK1 (3-phosphoinositide-dependent kinase 1) to the cell membrane. Activation of Akt initiates several signaling cascades which can affect a variety of cellular responses including migration, metabolism, and survival (Zhao and Vogt 2008). Akt activation contributes to cell survival by for example directly phosphorylating pro-apoptotic proteins such as BAD (Bcl-2 associated death domain) and caspase-9 and by suppressing the expression of pro-apoptotic genes (Datta et al. 1999). PI3K pathway activation can also inhibit the transcription of cell cycle control proteins p21 and p27 and increase the levels of oncogenic transcription factors Myc and Jun (Zhao and Vogt 2008). Akt activation is negatively regulated by PTEN (phosphatase and tensin homologue), which dephosphorylates the Akt-recruiting PIP3. The PI3K pathway is frequently activated in cancer by mutations as well as loss of PTEN. The PI3K pathway is a major contributor to tumor growth and acquired cancer drug resistance (Zhao and Vogt 2008).

Phospholipase C gamma pathway

ErbB receptors are also able to activate another pathway involving phosphoinositol metabolism, mediated by phospholipase C gamma (PLC-γ) (Figure 4). PLC-γ is phosphorylated by EGFR, ErbB2 and ErbB4 (Margolis et al. 1989; Peles et al. 1991; Vecchi et al. 1996), leading to PLC-γ activation. Activated PLC-γ generates secondary messengers IP3 (inositol-triphosphate) and DAG (diacylglycerol) by catalyzing the hydrolysis of PIP2. IP3 stimulates the increase in intracellular calcium levels and thus can affect the activity of Ca^{2+}/calmodulin –dependent enzymes. In addition, both calcium and DAG can activate PKC (protein kinase C) (Rhee 2001). PLC-γ activation and the subsequent actions of second messengers can regulate various transformation-promoting cellular functions such as migration, proliferation and survival (Carpenter and Ji 1999; Griner and Kazanietz 2007).

STAT pathway

STATs (signal transducer and activator of transcription), encoded by seven genes in humans, were initially discovered as intracellular signal transducers of cytokines (Schindler and Darnell 1995). Upon activation by cytokines or growth factors, the STATs are phosphorylated, which leads to STAT dimerization and translocation into the nucleus, where the STATs act as transcription factors regulating the expression of genes involved in cell growth, survival and differentiation (Figure 4) (Yu et al. 2009). ErbB receptors can activate different members of the STAT family, EGFR activating STAT1, STAT3, STAT5a, and STAT5b, and ErbB4 activating STAT5a, and STAT5b (Olayioye et al. 1999). ErbB2 and ErbB3 have not been shown to directly activate STATs (Olayioye et al. 1999).

In addition to canonical RTK signaling, ErbB4 can also signal *via* its intracellular domain, which is released from the membrane by a two-step proteolytic cleavage (Figure 4). The intracellular domain can translocate into the nucleus and regulate gene transcription. This unique feature of ErbB4 will be discussed later in further detail.

2.3.5. Negative regulation of ErbB signaling

Because ErbB receptor-mediated signaling regulates fundamental cellular processes, it is vital for cells to be able to regulate their ErbB signaling output. Protein tyrosine phosphatases (PTP) are important regulators of RTK activity that counteract the actions of protein tyrosine kinases by dephosphorylating phosphotyrosines (Schlessinger 2000). A number of protein phosphatases have been identified that regulate ErbB signaling, such as DEP1 (density-enhanced phosphatase-1) and protein tyrosine phosphatase-1B (Citri and Yarden 2006).

Receptor endocytosis is a major regulatory mechanism of EGFR. Activation of EGFR is followed by rapid ubiquitination and endocytosis to early endosomes. The internalized receptors can then be either recycled back to the plasma membrane or targeted for degradation in lysosomes (Sorkin and Goh 2009). With the exception of ErbB4 CYT-1 isoforms, other ErbB receptors than EGFR are considered endocytosis-impaired (Sundvall et al. 2008; Baulida et al. 1996), undergoing endocytosis with much slower kinetics than EGFR and/or exhibiting higher rates of recycling (Baulida et al. 1996; Sorkin and Goh 2009).

The activity of ErbB receptors can also be regulated by protein inhibitors such as MIG6 (mitogen-inducible gene-6) and LRIG1 (leucine-rich repeats and immunoglobulin-like domains-1), whose expression is typically induced upon EGFR activation (Citri and Yarden 2006). MIG6 directly binds to the EGFR kinase domain inhibiting the formation of kinase dimer and thus activation (Zhang et al. 2007). LRIG1 binds to all ErbB receptors and increases ubiquitin-mediated receptor downregulation and degradation (Laederich et al. 2004; Gur et al. 2004).

2.3.6. ErbB receptors in cancer

The early findings that human EGFR and ErbB2 are homologs of chicken and rodent oncogenes *v*-erbB (Downward et al. 1984) and *neu* (Schechter et al. 1984), respectively, already implicated a potential role for ErbB receptors in tumorigenesis. Since then, numerous studies analyzing ErbB biology in human tumors have established *ERBBs* as very potent proto-oncogenes whose functions are perturbed in various human epithelial and central nervous system malignancies (Arteaga and Engelman 2014). Overexpression with or without gene amplification and somatic mutations are the most common mechanisms of oncogenic ErbB receptor activation and these alterations can serve as predictive markers for ErbB-targeted cancer therapy in specific types of cancer (Arteaga and Engelman 2014; Slamon et al. 2001; Lynch et al. 2004).

EGFR and *ERBB2* are the most well-established oncogenes among ErbBs and these receptors have long been the focus of ErbB-related cancer research. ErbB3 and ErbB4 have gained less attention, and their contribution to tumorigenesis is not well known. In studies assessing the transforming potential of ErbB receptors, both ErbB3 and ErbB4 were shown to be able to transform NIH 3T3 cells when co-expressed with EGFR or ErbB2 and stimulated with ligand (Zhang et al. 1996; Cohen et al. 1996). ErbB3 overexpression is frequently observed in human malignancies including lung, breast, colorectal and ovarian cancers and melanoma, often together with expression of other members of the ErbB family (Gullick 1996; Aurisicchio et al. 2012). Indeed, being the most potent activator of the PI3K pathway among ErbB receptors, ErbB3 can be a major contributor to tumorigenesis as a co-receptor for other ErbBs (Arteaga and Engelman 2014).

ErbB4 is also expressed and mutated in cancer, as will be discussed in detail later.

Mutant ErbB receptors as cancer drug targets

The discovery that the tumorigenic viral *v*-erbB oncogene resembles truncated EGFR lacking the extracellular ligand binding domain (Downward et al. 1984) and the *neu* oncogene encodes the ErbB2 receptor with a single point mutation in the transmembrane domain (Bargmann et al. 1986) already demonstrated that mutated variants of ErbB receptors can promote tumor formation. Another early observation that the proliferation of A431 cancer cells, harboring amplified EGFR gene, is inhibited by anti-EGFR monoclonal antibody (Kawamoto et al. 1983) provided evidence that growth of cancer cells can be driven by aberrant ErbB activity and laid the foundation for the development of cancer drugs specifically targeting the ErbB receptors. Subsequent studies have shown that ErbB receptors are frequently activated by oncogenic point mutations, deletions, insertions and gene amplifications in human cancer.

Activating EGFR kinase domain mutations are detected in 8-10% of Caucasian lung cancer patients and in a higher percentage of patients of East Asian origin (Lynch et

al. 2004; Paez et al. 2004; Pao et al. 2004; Pao and Chmielecki 2010). The mutations typically target two hot spots in the kinase domain, the L858R point mutation and small overlapping insertions in exon 19 being the most common mutations (Pao and Chmielecki 2010). EGFR kinase domain mutations have been shown to sensitize lung cancer cells to EGFR tyrosine kinase inhibitors (TKI) erlotinib and gefitinib *in vitro*, and predict treatment response in patients (Lynch et al. 2004; Pao et al. 2004; Paez et al. 2004). After the first reports, a number of clinical trials have shown the efficacy of EGFR TKIs for lung cancer patients harboring EGFR kinase domain mutations (Pao and Chmielecki 2010). Several EGFR TKIs are now routinely used in the clinic and lung cancer patients are systematically screened for EGFR kinase domain mutations to select the patients suitable for EGFR TKI treatment.

EGFR mutations targeting the extracellular domain are very common in glioblastoma multiforme (GBM) and are almost exclusively accompanied by *EGFR* gene amplification (Sugawa et al. 1990; Frederick et al. 2000; Lee et al. 2006). The most frequent alteration (~40% of patients) is the deletion of exons 2-7, resulting in a truncated receptor lacking 276 amino acids from the extracellular domain, thus resembling the protein product of the viral v-*erbB* gene (Sugawa et al. 1990). The mutant receptor, EGFRvIII, displays aberrant signaling and reduced rate of downregulation and is highly tumorigenic both *in vitro* and *in vivo* (Gan et al. 2013). Other types of *EGFR* deletion mutants are also frequent in GBM (Frederick et al. 2000). In addition, several recurrent point mutations targeting the extracellular domain of EGFR have been reported from GBM. Most frequently targeted amino acids are A289 (A289V/D/T) and G598 (G598V) (Lee et al. 2006). The point mutations enhance EGFR sensitivity to ligand stimulation and are transforming in Ba/F3 and NIH 3T3 cells (Lee 09). In spite of the apparent driver role of mutant EGFR in GBM, these tumors have proven highly resistant to treatment with EGFR TKIs in clinical trials (Rich et al. 2004; van den Bent et al. 2009; Franceschi et al. 2007).

Gene amplification is the most common genetic alteration of *ERBB2* and *ERBB2* amplification has been detected in breast, ovarian, gastric and bladder cancer (Lofts and Gullick 1992). Most research regarding the cancer biology of *ERBB2* amplification has been concentrated on breast cancer, where the frequency of gene amplification is high (15-25%) and the amplification-positive patients typically have poor prognosis (Slamon et al. 1987, 2001). Targeting ErbB2 in amplification-positive breast cancer cells with a ErbB2-targeted monoclonal antibody (mAb) trastuzumab was shown to be effective *in vitro* (Hudziak et al. 1989) and subsequently also in clinical trials (Slamon et al. 2001). This led to the FDA (United States food and drug administration) approval of trastuzumab for the treatment *ERBB2* amplification-positive breast cancer. Several other ErbB2-targeted drugs have since entered the clinic and trastuzumab has also been approved for the treatment of *ERBB2* amplification-positive advanced gastric cancer (Bang et al. 2010).

Besides gene amplification, other types of somatic oncogenic mutations in *ERBB2* have also been reported in human cancer, typically targeting the subdomain II of the extracellular domain and the kinase domain (Greulich et al. 2012; Bose et al. 2013). These additional oncogenic *ERBB2* mutations are transforming *in vitro* and tumorigenic *in vivo*, and cells expressing the oncogenic *ERBB2* mutants are sensitive to ErbB2 inhibition *in vitro* (Greulich et al. 2012; Bose et al. 2013). However, these types of somatic *ERBB2* mutations are rare (Greulich et al. 2012; Bose et al. 2013), and the clinical significance of these mutations is yet to be demonstrated.

Although somatic *ERBB3* mutations have been reported from various human cancers, the assessment of the functional consequences of these mutations is currently limited to a one study (Jaiswal et al. 2013). This study reported highest *ERBB3* mutation frequencies in gastric (12%) and colon cancers (11%) (Jaiswal et al. 2013). *ERBB3* mutations affected all functional subdomains of the receptor with putative mutation hot spots in the extracellular domain and the kinase domain. The *ERBB3* hot spot mutants were transforming *in vitro* and tumorigenic *in vivo* when co-expressed with ErbB2, but were not able to transform cells when expressed alone (Jaiswal et al. 2013). Interestingly, mAbs targeting ErbB3 or ErbB2 could block the leukemia-like disease caused in mice by implanting Ba/F3 cells expressing ErbB3 mutants together with wild-type ErbB2. This indicates that targeting of mutant ErbB3 should be tested for therapeutic potential also in a clinical setting (Jaiswal et al. 2013).

ERBB4 is also frequently mutated in cancer and the potential of mutant ErbB4 as a drug target will be discussed in detail later.

2.4. ErbB4

2.4.1. ErbB4 isoforms

Juxtamembrane isoforms and regulated intramembrane proteolysis

ErbB4 is the only ErbB receptor to undergo alternative splicing to yield different receptor isoforms. Alternative inclusion of either exon 16 or exon 15 results in JM-a or JM-b isoforms, respectively (Elenius et al. 1997a; Junttila et al. 2003). The exons 16 (23 amino acids) and 15 (13 amino acids) affect the ErbB4 amino acid sequence at the extracellular juxtamembrane segment with the consequence that the JM-a isoforms can be proteolytically cleaved from the cell membrane, whereas the JM-b isoforms cannot (Elenius et al. 1997a). The proteolytic process, regulated intramembrane proteolysis (RIP), involves a two-step cleavage of ErbB4. The first step is performed by ADAM17/TACE (tumor necrosis factor-α converting enzyme) at the JM-a region and the second step by gamma-secretace at a cleavage site within the transmembrane domain (Figure 4) (Rio et al. 2000; Ni et al. 2001). The RIP of ErbB4 results in the release of a soluble intracellular fragment (ICD), which may translocate to the nucleus and regulate gene

transcription (Figure 4) (Ni et al. 2001) (Ni et al. 2001; Lee et al. 2002a; Komuro et al. 2003; Williams et al. 2004; Määttä et al. 2006; Sardi et al. 2006; Sundvall et al. 2010; Paatero et al. 2012). Thus, the JM-a isoforms harbor a unique signaling entity the JM-b isoforms lack, rendering the JM isoforms also functionally distinct (see later and Veikkolainen et al. 2011).

Cytoplasmic isoforms

The cytoplasmic (CYT) isoforms of ErbB4 rise from the inclusion (CYT-1) or exclusion (CYT-2) of exon 26 (16 amino acids) (Junttila et al. 2003). The CYT-1 –specific region, located at the C-terminal tail of ErbB4, harbors a unique binding site for PI3K and thus enables the ErbB4 CYT-1 isoform to directly activate the PI3K pathway (Elenius et al. 1999). The CYT-1 region also contains an additional PPXY motif, which serves as the binding site for the ubiquitin ligase Itch (itchy E3 ubiquitin protein ligase) (Sundvall et al. 2008). Through the actions of Itch, ErbB4 CYT-1 isoforms are ubiquitinated and efficiently endocytosed, whereas the CYT-2 isoforms, lacking the Itch binding site, are not (Sundvall et al. 2008). Thus, also the ErbB4 CYT isoforms differ in their potency to elicit downstream signaling.

Expression of ErbB4 isoforms

In addition to their functional differences, the ErbB4 isoforms are also differentially expressed in normal and malignant tissues. JM-a isoforms are the predominant JM isoforms in kidney, salivary gland and testis, whereas skeletal muscle and heart predominantly express the JM-b isoform (Junttila et al. 2005; Veikkolainen et al. 2011). Both CYT-1 isoforms are typically expressed in all tissues where ErbB4 is expressed, although not always at similar relative levels (Junttila et al. 2005; Veikkolainen et al. 2011). In both malignant and benign tumors, JM-a isoforms typically dominate over JM-b isoforms, whereas the CYT isoforms are usually both expressed at roughly similar levels (Junttila et al. 2005; Veikkolainen et al. 2011; Kurppa et al. 2014), with a few exceptions (see chapter 2.4.4.).

2.4.2. ErbB4 signaling

ErbB4 is able to mediate ligand-borne extracellular signals into cellular responses *via* two alternative, independent routes: *via* canonical RTK signaling from the cell membrane (see chapter 2.3.4.), or *via* RIP-mediated signaling.

Following the two-step proteolytic cleavage, the soluble ErbB4 ICD can translocate to the nucleus, where it associates with and regulates transcription factors including YAP (YES-associated protein), STAT5, ER (estrogen receptor), ETO2, TAB2-N-CoR, AP-2, and HIF-1α (hypoxia-inducible factor 1α) (Komuro et al. 2003; Williams et al. 2004; Zhu et al. 2006; Linggi and Carpenter 2006; Sardi et al. 2006; Sundvall et al. 2010; Paatero et al. 2012). ErbB4 ICD-mediated signaling has been shown to regulate complex

cellular processes *in vivo*, such as differentiation of astrocytes in developing brain and epithelial cells in developing mammary gland (Sardi et al. 2006; Muraoka-Cook et al. 2009). Moreover, the overexpression of ErbB4 JM-a CYT-2, but not ErbB4 JM-b CYT-2 has been shown to promote IL-3-independent growth of 32D cells and promote survival of NR6 fibroblasts in the absence of serum (Määttä et al. 2006; Sundvall et al. 2010). These finding indicate that ErbB4 ICD-mediated signaling can also be oncogenic.

The actions of ErbB4 ICD are negatively regulated by SUMOylation by PIAS3 (protein inhibitor of activated STAT3), which sequesters ErbB4 ICD in PML (promyelocytic leukemia) bodies in the nucleus and represses ErbB4 ICD-mediated transcriptional activity (Sundvall et al. 2012). Also, the binding of WWOX (WW domain containing oxidoreductase) to ErbB4 inhibits ErbB4 ICD signaling by both stabilizing the full-length, uncleaved ErbB4, and by preventing the nuclear translocation of ErbB4 ICD (Aqeilan et al. 2007).

2.4.3. ErbB4 in neurological disorders

NRG-ErbB4 signaling is indispensable for the development of the central nervous system (CNS) (Gassmann et al. 1995). Loss of function of NRG-1 or ErbB4 or disrupting NRG-1 signaling can disturb neuronal migration, the outgrowth of neurites, axon projection, axon myelination, and the formation of synapses (Mei and Xiong 2008). In addition to effects on the anatomical development of the CNS, ErbB4 also has a role in neurotransmission in the adult brain, as both glutamaterigic and GABAergic pathways are affected by NRG-1-ErbB4 signaling (Huang et al. 2000; Woo et al. 2007). Moreover, NRG-1 has neurotrophic and neuroprotective effects *in vitro* (Mei and Xiong 2008).

In accordance with the apparent role of ErbB4 in neuronal function, ErbB4 has been linked to various neuronal disorders. ErbB4 and NRG-1 protein levels are increased in patients with Alzheimer's disease and Parkinson's disease, in which NRG-1-ErbB4 mediated signaling has been proposed to have neuroprotective effects (Chaudhury et al. 2003; Depboylu et al. 2012; Carlsson et al. 2011; Min et al. 2011). The strongest evidence comes from the involvement of ErbB4 in the pathogenesis of schizophrenia, where a number of genomic variants in *ERBB4* and *NRG-1* have been associated with the disease (Stefansson et al. 2002, 2003; Williams et al. 2003; Law et al. 2007; Norton et al. 2006; Nicodemus et al. 2006). Furthermore, *ERBB4*- or *NRG-1*-hypomorphic and conditional knock-out mice show symptoms associated with schizophrenia (Gerlai et al. 2000; Golub et al. 2004).

2.4.4. ErbB4 in cancer

ErbB4 expression in cancer

Studies analyzing ErbB4 expression in cancer have reported both under- and overexpression with varying associations to clinical characteristics and prognosis.

In breast cancer where ErbB4 has been most intensively studied, ErbB4 expression is typically associated with ER- and PR (progesterone receptor)-positivity, ErbB2-negativity, well-differentiated phenotype and favorable outcome (Bacus et al. 1996; Kew et al. 2000; Sassen et al. 2008; Koutras et al. 2008). On the other hand, ErbB4 overexpression has been associated with shorter relapse-free survival in early, node-negative tumors and with decreased survival in patients with node-positive tumors (Bièche et al. 2003; Lodge et al. 2003). In addition to breast cancer, ErbB4 expression has been associated with favorable prognosis in bladder cancer (Memon et al. 2004), but with poor prognosis in medulloblastoma, colorectal cancer, esophageal squamous cell carcinoma, and gastric cancer (Gilbertson et al. 1997; Baiocchi et al. 2009; Lee et al. 2002b; Xu et al. 2008; Shi et al. 2012).

Interestingly, ErbB4 expression has also been associated with resistance to chemotherapy in non-small cell lung cancer (NSCLC) and in osteo- as well as soft tissue sarcomas (Merimsky et al. 2001, 2002, 2003). In accordance with these observations, a recent study demonstrated that ErbB4-mediated signaling plays an important role in tumor recurrence after chemotherapy in various mouse models of NSCLC (Hegde et al. 2013).

While most expression studies have not taken into account the complex biology of ErbB4 with functionally distinct isoforms, few studies have addressed this issue. In medulloblastoma, increased CYT-1:CYT-2 ratio was associated with more aggressive disease (Ferretti et al. 2006). In serous ovarian cancer, CYT-1 isoform expression was shown to be an independent prognostic factor for poor survival (Paatero et al. 2013). The CYT-1 and CYT-2 isoforms were also shown to be expressed at different ratios in triple-negative breast cancer, but the observed favorable prognostic impact of *ERBB4* expression on overall survival was not affected by the isoform expression ratio (Machleidt et al. 2013).

ERBB4 mutations in cancer

Cancer-associated *ERBB4* mutations have been poorly characterized, both in terms of mutation frequency in different cancers as well as of functional consequences of *ERBB4* mutations. Studies using targeted sequencing of tumors have rarely included *ERBB4*, and as a consequence very few studies have reported *ERBB4* mutations in cancer (Soung et al. 2006). The major advances in genomic sequencing technology combined with the recent efforts to comprehensively characterize the mutational landscapes in all major human malignancies have, however, also unraveled the frequency of *ERBB4* mutations in various types of cancer (Figure 5). The cancer types with the highest somatic *ERBB4* mutation frequency include melanoma (11-19%), various subtypes of lung cancer (5-21%), gastric cancer (13%), colorectal cancer (8-11%) and esophageal cancer (10%), whereas the lowest frequencies are detected in glioblastoma (0.3%), low-grade glioma (0.3%), prostate cancer (0.4%), multiple myeloma (0.5%), and thyroid cancer (0.5%)

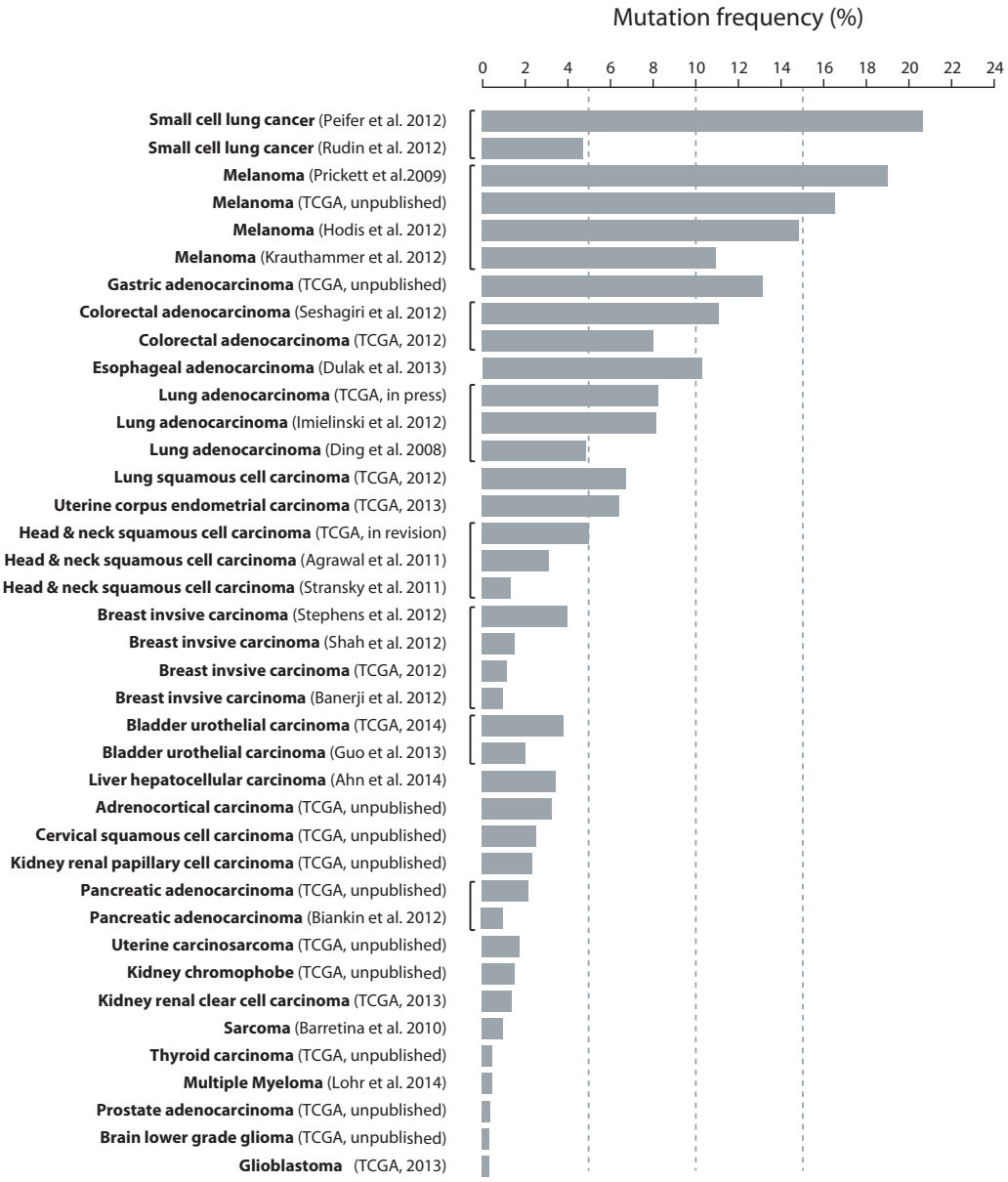

Figure 5: Frequency of ERBB4 mutations in different cancer types. The figure indicates the frequency of nonsynonymous *ERBB4* mutations (horizontal axis) in different cancer types (vertical axis). The data represents 55 studies across 30 different cancer types that are available in the cBioportal database (www.cbioportal.org, reviewed 26.8.2014). Only studies reporting *ERBB4* mutations are shown. Studies on same cancer types are emphasized by brackets. Figure modified from cBioportal data output. TCGA, The Cancer Genome Atlas research network.

(Figure 5). However, as can be seen from figure 5, the *ERBB4* mutation frequencies reported by different studies analyzing the same tumor type seem to vary considerably. Of the tumor types where there is more than one independent study, melanoma, colorectal

cancer and lung adenocarcinoma have consistently indicated high prevalence of *ERBB4* mutations (Figure 5). A characteristic feature of *ERBB4* mutations across all studies is the lack of obvious mutation hot spots, *i.e.* mutations recurrently occurring at the same site. Possibly due to this feature, and also the rather inconsistent literature regarding the role of ErbB4 in cancer (see above), *ERBB4* mutations are not regarded as driver mutations in most of the published studies listed in figure 5.

In spite of high incidence of *ERBB4* mutations in some tumor types, the functional effects of these mutations remain poorly characterized as only one such study exists outside this thesis (Prickett et al. 2009). In the study by Prickett and others, reporting *ERBB4* mutations in 19% of clinical metastatic melanoma samples, the authors showed that some of the mutations are oncogenic and drive the proliferation of melanoma cells (Prickett et al. 2009). Interestingly, melanoma cells harboring endogenous *ERBB4* mutations were also shown to be more sensitive to the EGFR/ErbB2-targeting TKI lapatinib, suggesting that mutant *ERBB4* could be a potential drug target in metastatic melanoma (Prickett et al. 2009).

3. AIMS OF THE STUDY

1. To functionally characterize cancer-associated *ERBB4* mutations.

2. To assess the clinical significance of *ERBB4* promoter region polymorphisms and kinase domain mutations in breast cancer.

3. To study the significance of *ERBB4* polymorphisms in amyotrophic lateral sclerosis.

4. MATERIALS AND METHODS

A more detailed description of the materials and methods used in this thesis can be found in the original publications, referred to with their roman numerals.

4.1. Methods

Method	Used in
Bioinformatics	*IV**
Cell Culture	I, II, IV
Cross-linking	II
Direct nucleotide sequencing	*I,* II*, IV*
Genotyping	*III*, *IV*
Immunohistochemistry	III
Immunoprecipitation and co-immunoprecipitation	*I*, II
In vitro kinase assay	*I*
Ligand stimulation	I, II, IV
Linkage analysis	*IV*
Molecular cloning	I, II, IV
Molecular modeling of ErbB4	*I*, *II*
MTT cell viability/proliferation assay	I, II
Real-time RT-PCR	II
Retroviral infection to generate stable gene expression	I, II
RNA extraction and synthesis of cDNA	II
Site-directed mutagenesis	I, II, IV
Statistical analysis	I, II, *III*, *IV*
Structural analysis	I, II
Three-dimensional cultures	I
Transfection	I, II, IV
Western blotting	I, II, IV
Whole genome sequencing	*IV*

*Methods used in the original publications, but not by the author of this thesis are indicated in italics.

4.2. Plasmids

Name	Backbone	Insert	Backbone type	Used in
pcDNA neo(-)	pcDNA3.1.Neo(-)		mammalian expression	I
pcDNA A2	pcDNA3.1.Neo(-)	ErbB4 JM-a CYT-2	mammalian expression	I
pcDNA A2 V721I	pcDNA3.1.Neo(-)	ErbB4 JM-a CYT-2	mammalian expression	I
pcDNA A2 A773S	pcDNA3.1.Neo(-)	ErbB4 JM-a CYT-2	mammalian expression	I
pcDNA A2 R782Q	pcDNA3.1.Neo(-)	ErbB4 JM-a CYT-2	mammalian expression	I
pcDNA A2 G802dup	pcDNA3.1.Neo(-)	ErbB4 JM-a CYT-2	mammalian expression	I
pcDNA A2 E810K	pcDNA3.1.Neo(-)	ErbB4 JM-a CYT-2	mammalian expression	I
pcDNA A2 P854Q	pcDNA3.1.Neo(-)	ErbB4 JM-a CYT-2	mammalian expression	I
pcDNA A2 D861Y	pcDNA3.1.Neo(-)	ErbB4 JM-a CYT-2	mammalian expression	I
pcDNA A2 E872K	pcDNA3.1.Neo(-)	ErbB4 JM-a CYT-2	mammalian expression	I
pcDNA A2 T926M	pcDNA3.1.Neo(-)	ErbB4 JM-a CYT-2	mammalian expression	I
pcDNA A2 I1033M	pcDNA3.1.Neo(-)	ErbB4 JM-a CYT-2	mammalian expression	I
pBabe	pBABE-puro		retroviral	I, II, IV
pBABE A2	pBABE-puro	ErbB4 JM-a CYT-2	retroviral	I
pBABE A2 G802dup	pBABE-puro	ErbB4 JM-a CYT-2	retroviral	I
pBABE A2 D861Y	pBABE-puro	ErbB4 JM-a CYT-2	retroviral	I
pcDNA ErbB2	pcDNA3.1.Neo(-)	ErbB2	mammalian expression	I
pcDNA hyg(+)	pcDNA3.1.Hyg(+)		mammalian expression	II
pcDNA A2HA	pcDNA3.1.Hyg(+)	ErbB4 JM-a CYT-2-HA	mammalian expression	II
pcDNA A2HA K751R	pcDNA3.1.Hyg(+)	ErbB4 JM-a CYT-2-HA	mammalian expression	II
pcDNA A2HA K751R, D931Y	pcDNA3.1.Hyg(+)	ErbB4 JM-a CYT-2-HA	mammalian expression	II
pcDNA A2HA K751R, K935I	pcDNA3.1.Hyg(+)	ErbB4 JM-a CYT-2-HA	mammalian expression	II
pcDNA A2HA V954R	pcDNA3.1.Hyg(+)	ErbB4 JM-a CYT-2-HA	mammalian expression	II
pBABE A2HA	pBABE-puro	ErbB4 JM-a CYT-2-HA	retroviral	II, IV
pBABE A2HA N181S	pBABE-puro	ErbB4 JM-a CYT-2-HA	retroviral	II
pBABE A2HA T244R	pBABE-puro	ErbB4 JM-a CYT-2-HA	retroviral	II
pBABE A2HA Y285C	pBABE-puro	ErbB4 JM-a CYT-2-HA	retroviral	II
pBABE A2HA R306S	pBABE-puro	ErbB4 JM-a CYT-2-HA	retroviral	II
pBABE A2HA V348L	pBABE-puro	ErbB4 JM-a CYT-2-HA	retroviral	II
pBABE A2HA D595V	pBABE-puro	ErbB4 JM-a CYT-2-HA	retroviral	II
pBABE A2HA H618P	pBABE-puro	ErbB4 JM-a CYT-2-HA	retroviral	II
pBABE A2HA D931Y	pBABE-puro	ErbB4 JM-a CYT-2-HA	retroviral	II
pBABE A2HA K935I	pBABE-puro	ErbB4 JM-a CYT-2-HA	retroviral	II
pBABE A2HA K592I	pBABE-puro	ErbB4 JM-a CYT-2-HA	retroviral	II
pBABE A2HA R114Q	pBABE-puro	ErbB4 JM-a CYT-2-HA	retroviral	IV
pBABE A2HA A158E	pBABE-puro	ErbB4 JM-a CYT-2-HA	retroviral	IV
pBABE A2HA H374Q	pBABE-puro	ErbB4 JM-a CYT-2-HA	retroviral	IV
pBABE A2HA R927Q	pBABE-puro	ErbB4 JM-a CYT-2-HA	retroviral	IV
pBABE A2HA R1275W	pBABE-puro	ErbB4 JM-a CYT-2-HA	retroviral	IV

4.3. Cell lines

Cell line	Type	Species	Used in
COS-7	Fibroblast-like kidney cell	African green monkey	I, II, IV
MCF7	Breast cancer cell	Human	I
MDA-MB-468	Breast cancer cell	Human	I
NIH 3T3	Fibroblast	Mouse	II

4.4. Reagents

Reagent	Type	Company	Used in
AZ10398863	ErbB2 TKI	AstraZeneca	I
bis(sulfosuccinimidyl)suberate (BS_3)	Cross-linker	Pierce	II
gefitinib	ErbB TKI	AstraZeneca	I
neuregulin-1	ErbB4 ligand	R&D systems	I, II, IV
Fugene6	Transfection reagent	Roche	I, II, IV

4.5. Antibodies

Antigen	Clone/cat. #	Company	Type	Application	Used in
Actin	sc-1616	Santa Cruz	Rabbit polyclonal	WB	I, II, IV
Akt	sc-1618	Santa Cruz	Rabbit polyclonal	WB	I, II
ErbB2	sc-284	Santa Cruz	Rabbit polyclonal	WB, IP	I
ErbB2	MA5-14057	Thermo Scientific	Mouse monoclonal	WB, IP	II
ErbB4	sc-283	Santa Cruz	Rabbit polyclonal	WB, IP	I
ErbB4	E200	Abcam	Rabbit monoclonal	WB, IP	II, IV
ErbB4	HFR-1	Abcam	Mouse monoclonal	IHC	III
Erk	9102	Cell Signaling	Rabbit polyclonal	WB	I, II
HA	3F10	Roche	Rat monoclonal	WB	II
PCNA	sc-56	Santa Cruz	Mouse monoclonal	WB	II
PDGFR-alpha	07-276	Millipore	Mouse polyclonal	WB	II
phospho-Akt	9271	Cell Signaling	Rabbit polyclonal	WB	I, II
phospho-ErbB4	4757	Cell Signaling	Rabbit polyclonal	WB	I, II, IV
phospho-Erk	9101	Cell Signaling	Rabbit polyclonal	WB	I, II
phospho-tyrosine	4G10	produced in house	Mouse monoclonal	WB	I, II
STAT5	sc-835	Santa Cruz	Rabbit polyclonal	WB	I

5. RESULTS

5.1. Cancer-associated *ERBB4* mutations can induce a qualitative shift in ErbB4 signaling (I)

The first study screening *ERBB4* mutations in cancer was published by Soung and others in 2006 (Soung et al. 2006). The mutation screen was restricted to *ERBB4* kinase domain. A total of twelve somatic mutations were detected in 180 gastric carcinoma, 94 breast ductal carcinoma, 104 colorectal carcinoma and 217 NSCLC samples, and nine of the observerd mutations changed an amino acid in ErbB4 (Soung et al. 2006). Another study identified an *ERBB4* mutation affecting the C-terminus of ErbB4 in one out of 146 colorectal cancer samples (Parsons et al. 2005). The effects of these mutations (I; Fig. 1) were analyzed in transiently transfected MCF-7 and COS-7 cells. While none of the mutations increased the activity of ErbB4, two mutants, G802dup and D861Y, demonstrated highly reduced both basal and NRG-1-stimulated phosphorylation (I; Fig. 2A). Subsequent *in vitro* kinase assay analysis showed that the two mutants completely lacked kinase activity (I; Fig. 2B).

Interestingly, despite of impaired kinase activity, the two ErbB4 mutants were found as effective as the wild-type receptor in activating ErbB2 in response to NRG-1 stimulation, as well as in activating ErbB2-mediated MAPK and PI3K signaling (I; Fig. 4, 7C). In contrast, the kinase-dead ErbB4 mutants were not able to activate STAT5, whose phosphorylation upon NRG-1 stimulation was ErbB4 kinase-dependent (I; Fig. 6, 7B). Moreover, overexpression of ErbB2 together with the kinase-dead ErbB4 mutants completely rescued the NRG-1-stimulated ErbB4 phosphorylation of the mutants (I; Fig. 5). These results indicated that the G802dup and D861Y mutations promoted an "ErbB3-like" phenotype in ErbB4 where signaling potency was strictly dependent on heterodimerization with kinase-competent ErbB receptor, and promoted a qualitative shift in ErbB4 signaling (I; Fig. 9).

STAT5 activation has previously been shown to mediate ErbB4-induced differentiation of mammary carcinoma cells (Muraoka-cook et al. 2006). Therefore, a three-dimensional MDA-MB-468 breast cancer cell differentiation model was used to analyze the functional effects of the G802dup and D861Y mutants. While NRG-1 stimulation increased the amount of differentiated acinar structures in cells stably transfected with wild-type ErbB4, in cells expressing the G802dup and D861Y mutants the NRG-1 stimulation decreased the number of acini, as the cells mostly grew as disorganized colonies (I; Fig. 8). These results demostrated that the mutants had not only lost their ability to promote differentiation but had gained an ability to actively suppress differentiation.

Taken together, these results suggest that mutations leading to loss of kinase activity in ErbB4 may lead to a qualitative shift in the balance between tumorigenic and suppressive pathways, favoring cancer cell proliferation and survival over differentiation.

5.2. Mutations in various functional subdomains of ErbB4 can lead to receptor activation (II)

A study analyzing the exons of 623 potential cancer genes in 188 clinical lung adenocarcinoma samples identified *ERBB4* as a highly mutated gene in lung adenocarcinoma (Ding et al. 2008). The nine identified mutations (4.8% of all samples) affected all functional subdomains of the ErbB4 receptor, with seven mutations in the extracellular domain and two mutations in the kinase domain (II; Fig. 1). The *ERBB4* mutations were mutually exclusive with activating *EGFR* kinase mutations (Ding et al. 2008) and neither of the *ERBB4* kinase domain mutations corresponded to mutations already analyzed in (I). In contrast to the previously analyzed kinase domain mutations, four mutations (Y285C, D595V, D931Y and K935I) demonstrated increased basal and NRG-1-induced phosphorylation when expressed in COS-7 cells, whereas the other five did not affect ErbB4 activation (II; Fig. 2).

Interestingly, although the four activating ErbB4 mutations targeted three different functional subdomains of ErbB4 (II; Fig. 1), all mutations were structurally mapped in the dimerization interfaces in ErbB4 dimers (II; Fig. 3D, E, 4E). This suggested that the observed increase in activation may result from enhanced interaction of receptor monomers in ErbB4 homo- or heterodimers. Indeed, Y285C and D595V mutations demonstrated markedly increased ligand-induced (homo)dimerization in cross-linking experiments (II; Fig. 3A). In addition, as observed by co-immunoprecipitation, the two mutations also demonstrated increased heterodimerization with ErbB2 in response to ligand stimulation (II; Fig. 3B).

Because the D931Y and K935I mutations are located in the activator kinase in the asymmetric kinase dimer interface (see chapter 2.3.3.), the ability of the mutants to serve as activator kinases was analyzed (II; Fig 4A, B). While K935I mutation enhanced the activation of ErbB4 homodimers in *trans*, the activation was decreased by D931Y mutation (II; Fig. 4C). However, when NRG-1-stimulated activation of ErbB2 was assessed, both mutants were more potent than wild-type ErbB4 in activating ErbB2, with no difference between the mutants in transactivating capability (II; Fig. 4D). This suggests that the ErbB4 D931Y mutation induced a qualitative shift in the activity of ErbB4 dimers by increasing the activity of ErbB4-ErbB2 heterodimers as compared to wild-type ErbB4, while decreasing the activity of ErbB4 homodimers. This effect resembles the selective loss-of-function phenotype promoted by the kinase-dead ErbB4 mutants (see previous chapter). The ErbB4 K935I mutation, on the other hand, was a

more potent activator of both ErbB2 heterodimers and ErbB4 homodimers than wild-type ErbB4.

Taken together, these results demonstrate that mutations in various functional subdomains of ErbB4 can lead to activation of the receptor, possibly providing some explanation for the dispersed mutation pattern observed for *ERBB4* in cancer.

5.3. Activating ErbB4 mutations promote survival in NIH 3T3 cells and increase RIP-mediated ErbB4 signaling (II)

The functional consequences of the activating *ERBB4* mutations were analyzed in NIH 3T3 cells stably transfected with wild-type *ERBB4* or *ERBB4* mutants Y285C, D595V, or K935I. While wild-type or mutant *ERBB4* expression did not have an effect on the proliferation of NIH 3T3 cells in full (10%) serum (data not shown), the cells expressing the *ERBB4* mutants were significantly more resistant to serum starvation than cells expressing wild-type *ERBB4* or vector control cells (II; Fig. 5A). This suggested that the activating *ERBB4* mutations are oncogenic.

The effects of serum starvation on ErbB4 phosphorylation and intracellular signaling pathway activation was analyzed by Western blotting. While all three ErbB4 mutants demonstrated more prominent ErbB4 phosphorylation both in full serum and upon serum starvation than the wild-type ErbB4, the effects on MAPK and Akt pathway activation were negligible (II, Fig. 5B). However, the mutants demonstrated increased ErbB4 cleavage basally as well as upon serum starvation (II; Fig. 5B). The signaling activity of the soluble ErbB4 ICD was addressed by analyzing the expression of *PDGFRA* (platelet-derived growth factor receptor-alpha), as ErbB4 ICD has previously been reported to associate with the *PDGFRA* promoter and stimulate *PDGFRA* transcripton in response to serum starvation (Sundvall et al. 2010). *PDGFRA* mRNA and protein levels were more robustly increased in response to serum starvation in NIH 3T3 cells expressing the *ERBB4* mutants than in vector control cells or cells expressing wild-type *ERBB4* (II; Fig. 5C, D), indicating that the soluble ErbB4 ICD produced from the ErbB4 mutants was actively translocated to the nucleus where the ErbB4 ICD regulated gene expression. These results suggest that the activating ErbB4 mutants predominantly activated RIP-mediated ErbB4 signaling as opposed to canonical RTK signaling in response to serum starvation.

5.4. Oncogenic *ERBB4* kinase domain mutations are rare but present in breast cancer (III)

To investigate the frequency and clinical significance of the *ERBB4* kinase domain mutations reported by Soung et al. (Soung et al. 2006) in breast cancer, the mutations

were genotyped from the 1010 patients who participated in the adjuvant phase III FinHer trial (see above and Joensuu et al. 2006). The frequency of the *ERBB4* mutations was found to be low as only two mutations, G802dup and E872K, were observed (0.2% of all genotyped patients). However, both mutations have been shown to functional and promote cancer cell/tumor growth *in vitro* (see above and Prickett et al. 2009), indicating the rare presence of potentially oncogenic *ERBB4* mutations in breast cancer.

5.5. ERBB4 polymorphism has prognostic significance in breast cancer (III)

While *ERBB4* polymorphisms have previously been associated with risk of breast cancer (Rokavec et al. 2007; Kim et al. 2012; Murabito et al. 2007), no studies have assessed the possible predictive or prognostic value of *ERBB4* gene variants. In order to investigate the prognostic significance of *ERBB4* polymorphisms in breast cancer, 1010 patients with high-risk early breast cancer who participated in the adjuvant phase III FinHer trial (Joensuu et al. 2006) were genotyped for two *ERBB4* promoter region polymorphisms (SNPs), -782G>T (rs62626348) and -815A>T (rs62626347) (Rokavec et al. 2007), and the association of these *ERBB4* polymorphisms with distant disease-free survival (DDFS) of the patients was analyzed.

The frequencies of the *ERBB4* variants in the patient cohort were 2.5% (23 out of 936 patients) and 1.3% (12 out of 932 patients) for -782G>T and -815A>T, respectively. When the *ERBB4* promoter region SNP status was compared with clinicopathological characteristics, the -782G>T variant was associated with well-differentiated cancer (P = 0.018) (III, Table 1). No other associations with clinicopathological features were observed for either variant. The -815A>T variant was significantly associated with poor prognosis (HR = 2.86 [95% CI 1.15-6.67], P = 0.017, III; Fig. 1B), whereas -782G>T variant did not show association with survival (III, Fig. 1A).

The -782G>T variant has been shown to decrease, and -815A>T to increase *ERBB4* promoter activity in an *in vitro* reporter assay (Rokavec 07). Therefore, paraffin-embedded tumor sections from primary tumors of all 1010 patients were analyzed for ErbB4 expression by immunohistochemistry using HFR-1 monoclonal antibody recognizing the C-terminus of ErbB4. However, no significant associations were found between *ERBB4* SNP status and ErbB4 staining intensity (III, Table 1). ErbB4 protein expression also did not associate with DDFS of the patients, but with ER-positivity. This is consistent with earlier reports associating ErbB4 protein expression with markers of favorable prognosis (Bacus 96, Kew, 00, Sassen 08, Koutras).

These results suggest that the heterozygous genotype *ERBB4* -815A/T could be a prognostic marker in high-risk early breast cancer. This is also the first indication of prognostic significance for a genetic variant of *ERBB4* in cancer.

5.6. Causative germ-line *ERBB4* mutations in amyotrophic lateral sclerosis inhibit *ERBB4* activity (IV)

A team of researchers led by professor Shoji Tsuji from the University of Tokyo indentified two causative germ-line *ERBB4* mutations in ALS patients, R927Q from Japanese and Caucasian patients with familial ALS, and a *de novo* R1275W mutation from a Japanese patient with sporadic ALS (IV; Fig. 1). We collaborated with the Japanese team to functionally characterize the ALS-associated *ERBB4* mutations. The mutations were cloned into an expression vector and transiently transfected into COS-7 cells, where the phosphorylation of wild-type and mutant ErbB4 in response to NRG-1 stimulation was analyzed by Western blotting. Intriguingly, both R927Q and R1275W mutations reduced ErbB4 phosphorylation upon NRG-1 stimulation (IV, Fig. 2). Three other *ERBB4* mutations that were indentified from ALS patients, but were assessed as not being causative mutations, did not alter ErbB4 phosphorylation upon NRG-1 stimulation (IV, Fig. 2). This suggests that causative *ERBB4* mutations in ALS reduce NRG-1-ErbB4 signaling.

6. DISCUSSION

6.1. The pattern of cancer-associated *ERBB4* mutations may not be random after all

Cancer-associated *ERBB4* mutations are characterized by the lack of mutational hot spots. Indeed, even when all *ERBB4* mutations in 12386 samples from 55 studies across 30 different cancer types available at cBioPortal for cancer genomics (www.cbioportal.org) are overlaid, no obvious hot spots can be detected (Figure 6). R711 is the most frequently targeted amino acid in ErbB4 with five individual samples harboring a mutation at this position. Several amino acids are mutated in two or three different samples, but these positions show no particular pattern. In contrast, in EGFR, ErbB2 and ErbB3, distinct hot spots can be readily recognized, with 9-34 samples harboring mutations targeting the same amino acid (Figure 6).

This somewhat peculiar pattern of cancer-associated mutations has caused ErbB4 to be generally neglected as a potential driver oncogene and drug target in cancer. However, the results of this study demonstrated that mutations targeting various sites in the extracellular and kinase domains of ErbB4 can promote oncogenic changes in the functions of ErbB4. This suggests that the apparently random pattern of mutations in *ERBB4* may not be random after all.

An image of randomness can potentially arise from the typical way of illustrating mutations in proteins as schematic figures similar to those in figure 6. This type of illustration does not take into account the complex three-dimensional structure of ErbB receptors. The present study serves as a good example. The activating ErbB4 mutations characterized in (II), while random-looking in a schematic figure (II; Fig. 2), were all located in critical sites in dimerization interfaces in the three-dimensional structures of the receptor (II; Fig. 3D, E, 4E).

Scattered and concentrated mutation patterns have been classically associated with tumor suppressor genes and oncogenes, respectively, following a rationale that a protein function can be broken by several mechanisms but activated only by few. The ErbB receptors however, are subjected to several layers of structural negative regulation both in the extracellular and intracellular domain (see chapter 2.3.3. and Lemmon et al. 2014). Thus, using the same rationale as above, these regulatory mechanisms could be compromized by mutations in several different sites. Also, the dimerization-mediated activation of ErbB receptors involves multiple interactions between the receptor monomers throughout the length of the receptors (see chapter 2.3.3.). Mutations in any of these interaction sites could potentially modulate the activity of the receptor dimer, quantitatively or qualitatively.

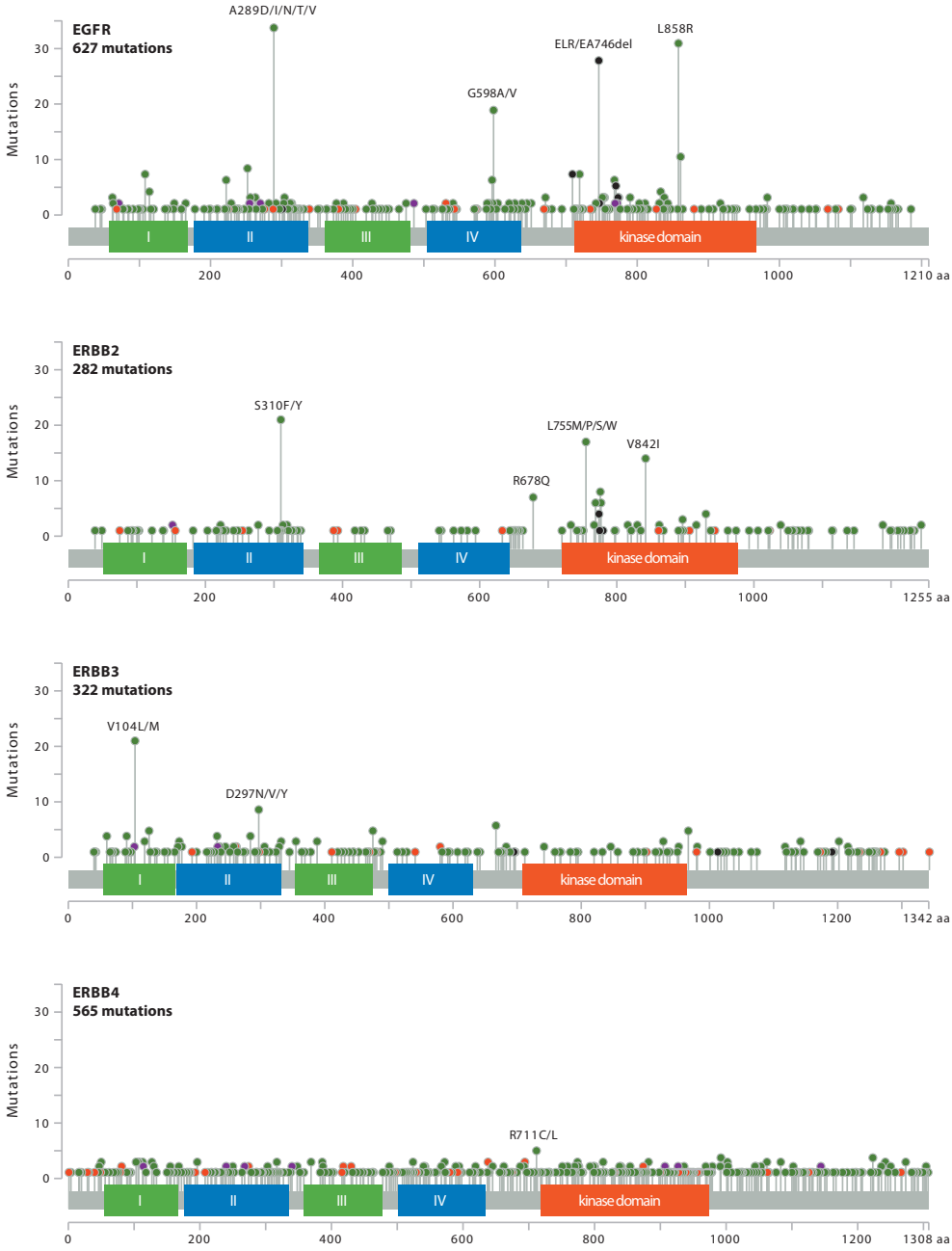

Figure 6: The distribution of cancer-associated mutations in ErbB receptors. The figure shows the overlay of all nonsynonymous mutations in individual ErbB receptors from 12386 clinical cancer samples or cell lines. The data represents 55 studies across 30 different cancer types that are incorporated in the cBioportal database (www.cbioportal.org, reviewed 3.9.2014). Mutations are indicated as pins. The height of the pin indicates the number of mutations at the particular site. Colors indicate mutation type: green - missense, red - truncating, black - inframe deletion or insertion, purple - multiple types of mutations at the same site. Most recurrent mutations are labeled. Figure modified from cBioportal data output (www.cbioportal.org).

Consistently, activating ErbB4 Y285C mutation was shown in this thesis to disrupt a subdomain I-II interaction that normally stabilizes the dimerization-inhibiting conformation of subdomain II. Also, the D595V mutation led to increased dimerization most likely due to more tight interaction of subdomains IV in ErbB4 dimers. Moreover, the kinase domain mutations G802dup, D861Y, D931Y and K935I affected the activation of ErbB4 homo- and heterodimers both quantitatively and qualitatively. All these mutants were shown to promote oncogenic changes in ErbB4 functions (I, II). Thus, the complex regulatory and activation mechanisms of ErbB receptors may well rationalize the scattered pattern of mutations in *ERBB4* as a source of oncogenic mutations.

6.2. Role of ErbB4 in amyotrophic lateral sclerosis

Very few studies have addressed the role of NRG-1-ErbB4 signaling in ALS. It is known that ErbB4 is expressed in motor neurons (Ricart et al. 2006; Pearson and Carroll 2004) and ErbB4 is involved in the development of the central nervous system and in the maintenance of neuronal functions in adults (Mei and Xiong 2008). NRG-1 has been shown to have neurotrophic effects in motor neurons *in vitro* (Ricart et al. 2006), and mice lacking the cysteine rich domain-containing NRG-1 isoforms demonstrate severe loss of motor neurons and die perinatally due to respiratory failure (Wolpowitz et al. 2000). In ALS patients and transgenic SOD1 ALS mouse model, the level of membrane-bound type III NRG-1 is decreased in parallel with neuronal loss, but in contrast the level of secreted form of NRG-1 is increased at late stage of the disease (Song et al. 2012). While the authors proposed that the downregulation of type III NRG-1 reflected the motor neuron loss, the pathological implications of increased expression of soluble NRG-1 were not clear (Song et al. 2012). It was proposed that increased NRG-1 expression could contribute to ALS pathogenesis *via* activated glial cells (Song et al. 2012), but considering the neurotrophic effects of NRG-1 on motor neurons *in vitro* (Ricart et al. 2006) and *in vivo* (Wolpowitz et al. 2000), it could be argued that NRG-1 levels could be also increased in ALS as an attempt to support the survival of degenerating motor neurons expressing ErbB4.

The neurotrophic role of NRG-1 also suggests that the disruption of NRG-1-ErbB4 signaling by germ-line *ERBB4* mutations (IV) could contribute to the pathogenesis of ALS by compromizing the survival of motor neurons, subsequently leading to neuronal degeneration, which is the hallmark of ALS (Kiernan et al. 2011). However, additional studies are needed in order to elucidate the detailed mechanisms by which aberrant NRG-1-ErbB4 signaling is involved in the pathogenesis of ALS.

6.3. Mutated ErbB4 as a potential drug target

ERBB4 is frequently mutated in cancer (Figure 5) and has been identified as one of 127 significantly mutated genes that putatively represent driver genes in 12 major human cancer types (Kandath et al. 2013). *ERBB4* has also been found to be significantly mutated in both adenocarcinoma as well as squamous cell carcinoma of the lung (Ding et al. 2008; Kan et al. 2010). These results, although solely based on statistical analyses, implicate mutated *ERBB4* as an important contributor to tumorigenesis and thus a potential drug target.

The results of this study support a hypothesis that cancer-associated *ERBB4* mutations are oncogenic (I, II). Moreover, *ERBB4* mutations have previously been shown to sensitize metastatic melanoma cells to the ErbB TKI lapatinib (Prickett et al. 2009), suggesting that *ERBB4* mutations could be used as predictive markers for targeted therapy. Consistently, this study also demonstrated that an *ERBB4* polymorphism is associated with poor distant disease-free survival in breast cancer (III). This serves the hypothesis that 1% of breast cancer patients that harbor this *ERBB4* variant (III; Table 1; Rokavec et al. 2007) have intrinsically poor prognosis and could potentially benefit from ErbB4-targeted therapy in the adjuvant setting.

Therapeutic options to target ErbB4 are already available. The FDA-approved pan-ErbB TKI afatinib, specifically designed to inhibit ErbB4 in addition to EGFR and ErbB2, could be used clinically to target ErbB4. In addition, pan-ErbB TKIs canertinib and neratinib are currently in phase II clinical trials (www.clinicaltrials.gov). Moreover, clinically used EGFR- and ErbB2 –targeted TKIs erlotinib, gefitinib, and lapatinib could be used as anti-ErbB4 drugs as they also inhibit ErbB4 activity (Solca et al. 2012), albeit with higher concentrations.

However, the scattered pattern of cancer-associated *ERBB4* mutations gives no clear consensus on which mutations are oncogenic and which represent passenger mutations. As only gain-of-function oncogenic mutations would serve as targets for therapeutic ErbB4 antagonists, the lack of hot spot mutations as guides hinders the selection of cell lines or patients in which the benefit of targeting ErbB4 could be evaluated. Future studies concentrating on separating oncogenic *ERBB4* mutations from passenger mutations are thus warranted.

Mutated ErbB4 is emerging as a novel drug target also in ALS. The finding that germ-line mutations reducing ErbB4 activity predispose to ALS (IV) could have therapeutic implications. Supporting ErbB4 signaling by ErbB4 agonists such as recombinant NRG-1 could potentially have beneficial effects in patients. This approach has been shown in clinical trials to be successful in treating chronic heart failure (Gao et al. 2010; Jabbour et al. 2011). Also, several polymorphisms in *NRG-1* and *ERBB4* demonstrate a causative link to schizophrenia (Mei and Xiong 2008), suggesting that targeted therapeutics modulating ErbB4 activity could be beneficial also in this disease. However, as the

mechanistic details of how disrupted NRG-1-ErbB4 signaling contributes to the pathogenesis of ALS are still largely unknown, further studies are needed to rationalize possible ErbB4-targeted treatment strategies in this disease.

7. CONCLUSIONS

ErbB receptors represent important drug targets. *EGFR* and *ERBB2* are well-characterized human proto-oncogenes and several drugs targeting EGFR and ErbB2 receptors are already in use in the clinic. ErbB4 has gained less attention as a potential drug target, although somatic *ERBB4* mutations have been identified in various high-incidence cancers and germ-line *ERBB4* mutations have been linked to neuronal disorders and cancer. As a consequence, the functional effects of *ERBB4* mutations are largely unknown. This thesis work aimed to elucidate the functional consequences and assess the clinical significance of somatic and germ-line *ERBB4* mutations in the context of cancer and amyotrophic lateral sclerosis.

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

1) Cancer-associated *ERBB4* mutations can be oncogenic *in vitro*, and oncogenic changes in ErbB4 functions can be promoted by mutations at various sites in the ErbB4 receptor. This is most likely due to complex regulatory and activation mechanisms of ErbB4 and can possibly explain the scattered pattern of mutations that is characteristic for *ERBB4*.

2) Approximately 1% of breast cancer patients harbor a specific polymorphism in *ERBB4* that can intrinsically be associated with poor prognosis.

3) Causative germ-line *ERBB4* mutations occur in ALS and reduce ErbB4 activity.

Together, the results of this study support a hypothesis that therapeutic modulation of ErbB4 activity could be beneficial both in cancer and ALS patients harboring *ERBB4* mutations. However, in the context of cancer, the lack of mutational hot spots in *ERBB4*, although not indicating the lack of oncogenic mutations, hinders the identification of gain-of-function *ERBB4* mutations that would serve as targets for therapeutic ErbB4 antagonists. The main challenge for future studies is to be able to separate oncogenic ErbB4 mutations from passenger mutations. Achieving this would enable a comprehensive evaluation of potential benefit of targeting mutated ErbB4 in cancer.

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