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Novel Functions of ErbB4 and NRG-1 in Development and Cancer

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To my family

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Novel functions of ErbB4 and NRG-1 in development and cancer

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ABSTRACT

Cells communicate, or signal, with each other constantly to ensure proper functioning of tissues and organs. Cell signaling is often performed by interplay of receptors and ligands that bind these receptors. ErbB receptors (epidermal growth factor receptors, EGFR, HER) bind extracellular growth factors and transduce these signals inside of cells. ErbB dysfunction promotes carcinogenesis, and also results in numerous defects during normal development. This study focused on the functions of one member of the ErbB receptor family, ErbB4, and growth factor, neuregulin-1 (NRG-1), that can bind and activate ErbB4.

This study aimed to find novel functions of ErbB4 and NRG-1. Hypoxia, or deficiency of oxygen, is common in cancer and ischemic conditions. One of the key findings of the work was the identification and characterization of a cross-talk between ErbB4 and Hypoxia-inducible factor 1α (HIF-1α), the central mediator of hypoxia signaling. ErbB4 activation by NRG-1 was found to increase HIF-1α activity. Interestingly, this regulation occurred in reciprocal manner as HIF-1α was also able to increase protein levels of NRG-1 and ErbB4. Moreover, expression of NRG-1 and ErbB4 was associated with HIF activity in vivo in human clinical samples and in mice. Reduction of functional ErbB4 in developing zebrafish embryos resulted in defects in development of the skeletal muscles. To study ErbB4 functions in pathological situation in humans, clinical samples of serous ovarian carcinoma were analyzed using tissue microarrays and real-time RT-PCR. A specific isoform of ErbB4, CYT-1, was associated with poor survival in serous ovarian cancer and increased anchorage independent growth of ovarian cancer cells in vitro.

These observations demonstrate that ErbB4 and NRG-1 are essential regulators of cellular response to hypoxia, of development, and of ovarian carcinogenesis.

Ilkka Paatero

ErbB4- ja NRG-1 signaloinnin merkitys syövässä ja yksilönkehityksen aikana

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TIIVISTELMÄ

Elimistön solut viestivät keskenään normaalin kehityksen ja aikuisten kudosten normaalin toiminnan aikana. Tämä viestintä varmistaa solujen ympäristöönsä soveltuvan toiminnan. Soluviestintää välittävät reseptorit ja niihin sitoutuvat ligandit. ErbB (epidermaalisen kasvutekijän reseptorit, EGFR, HER) reseptorit sitovat solunulkoisia kasvutekijöitä, ja välittävät viestin solun sisälle. Monissa syövissä ErbB reseptorien toiminnan säätely on häiriintynyt. Tämä tutkimus keskittyi erään ErbB reseptorin, ErbB4:n, ja sen ligandin NRG-1:n toimintaan.

Tutkimuksen päämääränä oli löytää uusia ErbB4:n ja NRG-1:n säätelemiä prosesseja ja niiden toimintamekanismeja. Hapenpuutetta, hypoksiaa, esiintyy monissa syövissä ja iskeemisissä tautitiloissa. Tutkimuksessa löytyi yhteys ErbB4:n välittämän viestinnän ja keskeisen hapen vähyyden viestijän (hypoksian indusoima tekijä 1α, HIF-1α) välillä. NRG-1 ja ErbB4 lisäsivät HIF-1α:n määrää ja aktiivisuutta. Tämä vuorovaikutus oli molemminpuolista, sillä myös HIF-1α:n aktiivisuus johti lisääntyneeseen NRG-1- ja ErbB4-määrään. Lisäksi lisääntynyt HIF-aktiivisuus oli yhteydessä lisääntyneeseen ErbB4:n ja NRG-1:n määrään kliinisissä näytteissä ja hiirimalleissa. ErbB4:n toiminnan estäminen seeprakalan alkioissa johti luurankolihasten kehityksen häiriöihin. ErbB4:n toiminnan tutkimiseksi ihmisissä analysoitiin seröösin munasarjasyövän potilasnäytteitä kudossiruja sekä reaaliaikaista RT-PCR:ää käyttäen. ErbB4:n erityisen CYT-1 isomuodon ilmentyminen oli yhteydessä huonoon ennusteeseen seröösissä munasarjasyövässä sekä lisäsi munasarjasyöpäsolujen kasvua in vitro.

Tutkimuksessa tehdyt havainnot viittaavat siihen, että NRG-1 ja ErbB4 ovat tärkeitä säätelijöitä solujen reagoimisessa hapenpuutteseen, kudosten kehittymisessä sekä seröösissä munasarjasyövässä.

TABLE OF CONTENTS

ABBREVIATIONS

ADAM17	a disintegrin and metalloproteinase domain 17
Akt	v-akt murine thymoma viral oncogene homolog
AP-1	activator protein 1
ATP	adenosine triphosphate
BTC	betacellulin
CAIX	carbonic anhydrase IX
Cbl	cas-br-m murine ecotropic retroviral transforming sequence homolog
cDNA	complementary DNA
Cre	cyclization recombinase
CYT	cytoplasmic
DAG	diacylglycerol
DMOG	dimethyloxalyl glycine
DNA	deoxyribonucleic acid
dpf	days post-fertilization
ECD	extracellular domain
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
EPO	erythropoetin
ErbB	v-erb-a erythroblastic leukemia viral oncogene homolog
Erk	extracellular signal regulated kinase
FAM	6-carboxyfluorescein
Gb	gigabase
GDP	guanosine diphosphate
GLUT-1	glucose transporter 1
Grb2	growth factor receptor-bound protein 2
GST	glutathione-S-transferase
GTP	guanosine triphosphate
HAF	hypoxia associated factor
HB-EGF	heparin-binding epidermal growth factor-like growth factor
HER	human epidermal growth factor receptor
HIF	hypoxia-inducible factor
hpf	hours post-fertilization
HSP90	heat-shock protein 90
ICD	intracellular domain
IP3	inositol 1,3,5 trisphosphate
JM	juxtamembrane
KD	kinase domain
MAPK	mitogen activated protein kinase
Mb	megabase
Mek	mitogen activated protein kinase kinase
MMTV	mouse mammary tumor virus

MO	morpholino
Morphant	morpholino treated embryo
mRNA	messenger RNA
mTor	mammalian target of rapamycin
Myl2	myosin, light chain 2, regulatory, cardiac, slow
n	number of samples
NRG	neuregulin
p	statistical probability
PAX8	paired box gene 8
PCR	polymerase chain reaction
PHD	prolyl 4-hydroxylase
PI3K	phosphoinositol-3 kinase
PIP2	phosphatidylinositol 4,5-diphosphate
PIP3	phosphatidylinositol 3,4,5-trisphosphate
PKC	protein kinase C
PLA	in situ proximity ligation assay
PLC	phospholipase C
PMA	phorbol 13-myristate 12-acetate
ppm	parts per million
PTP	protein tyrosine phosphatase
RACK1	receptor of activated protein kinase C
Raf	v-RAF murine viral oncogene homolog
Ras	ras rat sarcoma viral oncogene homolog
RIP	regulated intramembraneous proteolysis
RNA	ribonucleic acid
RT	room temperature
RT-PCR	reverse transcriptase polymerase chain reaction
RTK	receptor tyrosine kinase
SH2	src homology 2
SH3	src homology 3
Shc	shc transforming protein
siRNA	small-interfering RNA
SNP	single nucleotide polymorphism
Sos	son of sevenless (Drosophila) homolog
SP-1	transcription factor Sp-1
STAT	signal transducer and activator of transcription
TACE	tumor necrosis factor-alpha converting enzyme
TAMRA	tetramethylrhodamine
TGF-α	transforming growth factor α
TM	transmembrane domain
VHL	von Hippel-Lindau tumor suppressor
WAP	whey acidic protein
Xmrk	Xiphophorus melanoma receptor kinase

LIST OF ORIGINAL PUBLICATIONS

The thesis is based on following original publications, which are referred to in the text by the Roman numerals I-V

I Paatero I, Jokilammi A, Heikkinen PT, Iljin K, Kallioniemi OP, Jones FE, Jaakkola PM, Elenius K. 2012. Interaction with ErbB4 promotes hypoxia-inducible factor-1α signaling. J. Biol. Chem. 287(13):9659-71.

II Paatero I, Seagroves TN, Johnson RS and Elenius K. 2013. Hypoxia-inducible factor-1α induces ErbB4 signaling in the differentiating mammary gland. Manuscript.

III Iivanainen E, Paatero I, Heikkinen SM, Junttila TT, Cao R, Klint P, Jaakkola PM, Cao Y, Elenius K. 2007. Intra- and extracellular signaling by endothelial neuregulin-1. Exp. Cell Res. 313: 2896-909.

IV Paatero I, Lassus H, Junttila TT, Kaskinen M, Bützow R, Elenius K. 2013. CYT-1 isoform of ErbB4 is an independent prognostic factor in serous ovarian cancer and selectively promotes ovarian cancer cell growth in vitro. Gynecol, Oncol. in press, electronic publication 9.1.2013.

V Paatero I, Veikkolainen V, Pelliniemi LJ, Elenius K. 2013. ErbB4a is required for skeletal muscle development in zebrafish. Manuscript.

1 INTRODUCTION

Cells communicate with each other constantly. The purpose of this communication, or cell signaling, is to guide cells to respond properly to their context and physiological state to maintain homeostasis or a developmental program. Most prominently cell signaling is perhaps illustrated during development, where clumps of undifferentiated cells differentiate into a variety of different cell types and form beautiful and ordered structures such as the eye or contracting heart (Gilbert 2006; Wolpert 2007). In many diseases, such as cancer, cell signaling is dysregulated resulting in loss of tissue homeostasis and structure (Hanahan and Weinberg 2000; Hanahan and Weinberg 2011).

One of the mechanisms by which cells communicate is by production of soluble growth factors, or ligands, and their specific target molecules, receptors, within the recipient cells. As their name implies, growth factors regulate cellular growth, but they do also regulate many other cellular processes such as metabolism, cell survival and cell migration. Growth factor signaling regulates both development and disease, and it is of significant importance in medicine and developmental biology.

ErbB receptors are receptors for epidermal growth factor like ligands. The focus of this thesis lies in the elucidation of diverse functions of one of the ErbB receptors, ErbB4, and its ligand NRG-1, in both development and disease.

2 REVIEW OF THE LITERATURE

Protein tyrosine kinases are proteins that phosphorylate other proteins on the tyrosine residues. Although the human genome encodes over 90 tyrosine kinases, they are involved in virtually all cellular and developmental processes (Blume-Jensen and Hunter 2001). A subset of the family, receptor tyrosine kinases (RTK), is located on the cell plasma membrane. Their primary function is to bind secreted or membrane-bound growth factors and transduce this information inside the recipient cell (Lemmon and Schlessinger 2010).

2.1 ErbB receptors and ligands

Originally ErbB receptors were discovered as genes homologous to avian erythroblastosis virus oncogene in chicken (Vennstrom and Bishop 1982) and later in human (Jansson et al. 1983) genome. The name of gene family ErbB, *v-erb-a erythroblastic leukemia viral oncogene homolog*, also originates from these discoveries. ErbB receptors are also often called epidermal growth factor receptors (EGFR) after first discovered member EGFR (ErbB1) that binds epidermal growth factor (EGF).

ErbB receptors are transmembrane RTKs with several ligands. They are expressed in all multicellular, metazoan, organisms. The number of receptors and ligands differs between different species (Table 1). Mammals have four different ErbB genes (*ERBB1*, *ERBB2*, *ERBB3* and *ERBB4*) and have at least 11 epidermal-growth factor like ligands that bind ErbB receptors (Stein and Staros 2000; Stein and Staros 2006).

Table 1. Number of predicted ErbB genes and their ligands in selected animal species according to (Stein and Staros 2000; Stein and Staros 2006).

Species	Receptors	Ligands
Caenorhabditis elegans **(soil nematode)**	**1**	**1**
Drosophila melanogaster **(fruit fly)**	**1**	**5**
Danio rerio **(zebrafish)**	**6 (7)**	**14**
Xenopus tropicalis **(african clawed-frog)**	**4**	**14**
Gallus gallus **(chicken)**	**4**	**11**
Mus musculus **(mouse)**	**4**	**11**
Homo sapiens **(man)**	**4**	**11**

The ligands bind ErbB receptors selectively although no high-affinity ligand for ErbB2 has been found (Table 2). Ligands can be divided into four groups based on their capability to directly bind ErbB receptors. The first group can activate only ErbB1. Ligands that belong to this group are amphiregulin (Shoyab et al. 1988), epidermal growth factor (EGF) (Cohen 1962), epigen (Strachan et al. 2001) and transforming growth factor α (TGF-α) (Derynck et al. 1984). The second group consists of ligands that bind ErbB1 and ErbB4. Betacellulin (Sasada et al. 1993), epiregulin (Toyoda et al. 1995) and heparin-binding epidermal growth factor-like growth factor (HB-EGF) (Higashiyama et al. 1991) belong to this group. The third group of ligands can bind both ErbB3 and ErbB4. NRG-1 and NRG-2 form this group, although NRG-2 can also be produced as several isoforms of which alpha type isoforms bind and activate only ErbB3 (Hobbs et al. 2002). The fourth group consists of ligands that bind only ErbB4 and NRG-3 (Zhang et al. 1997) and NRG-4 (Harari et al. 1999) belong to this group. Interestingly, ErbB4 also recruits different signaling proteins depending on which ligand activates the receptors (Sweeney et al. 2000).

Table 2. Binding specificities of human ErbB ligands to human ErbB receptors (Linggi and Carpenter 2006).

	ErbB1	ErbB2	ErbB3	ErbB4
Amphiregulin	X			
EGF	X			
Epigen	X			
TGF-α	X			
Betacellulin	X			X
Epiregulin	X			X
HB-EGF	X			X
NRG-1			X	X
NRG-2			X	X
NRG-3				X
NRG-4				X

2.1.1 Structure & function

ErbB receptors have three distinct domains, which is also the common overall structure of other members of the RTK gene family (Lemmon and Schlessinger 2010). The receptors possess an extracellular domain (ECD), a transmembrane domain (TM) and an intracellular domain (ICD), each having distinct structural and functional properties (Holbro and Hynes 2004).

ECD is the ligand binding domain. The structure of ErbB1 (Garrett et al. 2002), ErbB3 (Cho and Leahy 2002), and ErbB4 (Bouyain et al. 2005) has both an open and a closed conformation, and it is predicted that a ligand can bind and stabilize the open conformation (Ferguson et al. 2003) (Figure 1). However, ErbB2 is constitutively in the open conformation and does not need a ligand to be activated (Garrett et al. 2003). Consistently, no direct activating high-affinity ligands have been found for ErbB2 (Holbro and Hynes 2004). Ligand

binding allows receptors to dimerize and form an active dimer, although the very details of this ligand-induced dimerization are not yet fully understood (Lemmon 2009).

TM domain is the domain that attaches the receptor into the hydrophobic plasma membrane. It composes of a single α helix that spans the membrane once. The α-helical structure contains hydrophobic residues which allow the protein to cross the lipid bilayer. Thus, the TM domain regulates the localization of the receptor (Lemmon and Schlessinger 2010). However, TM domain may also have an active role in ErbB receptor activation (Wides et al. 1990) and dimerization (Mendrola et al. 2002).

ICD possesses the catalytical kinase activity of the receptor. The kinase domain (KD) within the ICD catalyzes transfer of a phosphate group from ATP to a tyrosine residue of a substrate protein (Hubbard and Till 2000). At the non-activated state the autoinhibitory loop prevents the full activity of the KD. As a ligand binds to the receptor, the autoinhibitory loop is phosphorylated by another ErbB receptor in the dimer resulting in full activation of the receptor (Qiu et al. 2008). The KD of ErbB3 is often considered catalytically inactive (Yarden and Sliwkowski 2001; Holbro and Hynes 2004), although it has a low level of autophosphorylation activity (Shi et al. 2010). In addition to the KD the ICD has binding sites for many ErbB substrates and regulators (Schulze et al. 2005).

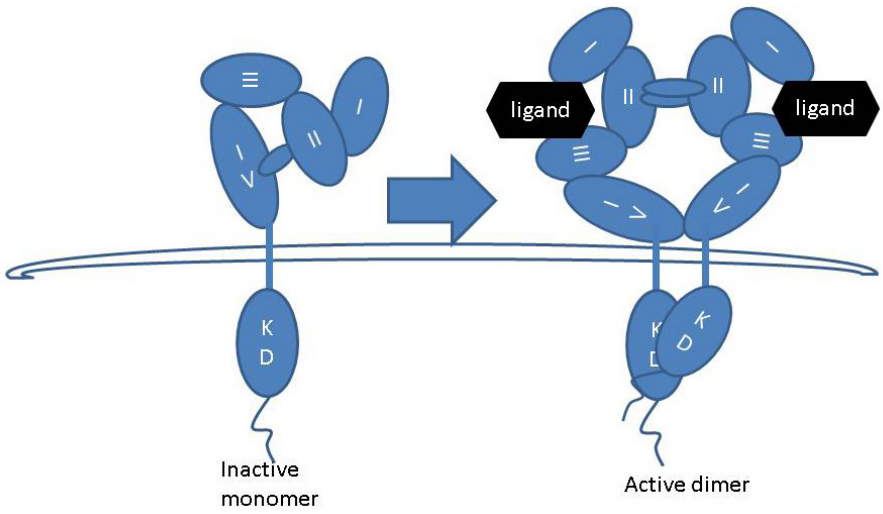

Figure 1. Schematic figure of the structure of the ErbB receptor. The ECD contains four distinct subdomains (roman numerals I-IV). Unoccupied, inactive receptor monomers adopt closed conformation. The ligand binds an open conformation allowing dimerization of two receptor monomers. Upon dimerization, the intracellular KDs transphosphorylate each other resulting in fully activated receptors (Lemmon 2009).

2.1.2 Signaling pathways

ErbB receptors can activate several intracellular signaling pathways such as *phosphoinositol-3 kinase* (PI3K), *phospholipase C* γ (PLC-γ), *signal transducer and activator of transcription*

(STAT) and *mitogen activated protein kinase* (MAPK) pathways (Holbro and Hynes 2004) (Figure 2). The kinase activity of the receptor results in tyrosine phosphorylation of the target proteins, which often leads to altered cellular functions or activities (Lemmon and Schlessinger 2010).

ErbB receptors can activate PI3K pathway by recruiting the regulatory p85 subunit of PI3K via binding to its SH2 domain (Skolnik et al. 1991). Direct binding sites of PI3K have been identified in ErbB4 and ErbB3 (Schulze et al. 2005). Binding of p85 enables activation of the catalytic subunit p110 of PI3K and increased conversion of phosphoinositol-2-phosphate (PIP2) to phosphoinositol-3-phosphate (PIP3). This in turn can activate the Akt/mTor pathway resulting in, depending on the context, increased cell survival and migration (Manning and Cantley 2007).

PLC-γ can also be activated by ErbB receptors (Margolis et al. 1990; Peles et al. 1991). Upon activation it is phosphorylated, which activates the PLCγ. PLCγ cleaves PIP2 to secondary messenger molecules inositol-trisphosphate (IP3) and diacylglycerol (DAG). These secondary messenger molecules activate Protein kinase C (PKC) and increase intracellular calcium levels among other events (Rhee 2001). Both of these signals regulate multiple cellular functions such as migration, cell survival and proliferation (Suh et al. 2008).

ErbB receptors can also activate members of the STAT molecular family. In humans there are seven STAT genes. The ErbB receptors differ in which STAT molecules they activate ErbB1 being capable of activating STAT1, STAT3, STAT5a and STAT5b (Olayioye et al. 1999), ErbB2 can activate STAT1 and 3 and ErbB4 can activate STAT5a and STAT5b (Olayioye et al. 1999). After phosphorylation, the STATs dimerize and translocate into the nucleus where they bind specific target sequences on DNA to regulate gene expression affecting cell growth and survival. Modulation of STAT signaling has potential as a target for cancer therapeutics (Yu and Jove 2004).

Classical mitogenic signaling pathway involving MAPKs can also be activated by all ErbB receptors (Olayioye et al. 2001). All ErbB receptors may bind shc transforming protein (Shc) or growth factor receptor-bound protein 2 (Grb2) adaptor protein (Schulze et al. 2005) to recruit guanine-nucleotide exchange factor Sos, which initiates signaling via MAPK pathway by “catalyzing” the exchange of GDP bound to Ras to GTP. GTP-binding Ras is in active form and interacts with RAF, which in turn phosphorylates MEK. MEK in turn phosphorylates Erk resulting in translocation of Erk into nucleus and activation of several transcription factors such as SP-1 and AP-1 (Mor and Philips 2006).

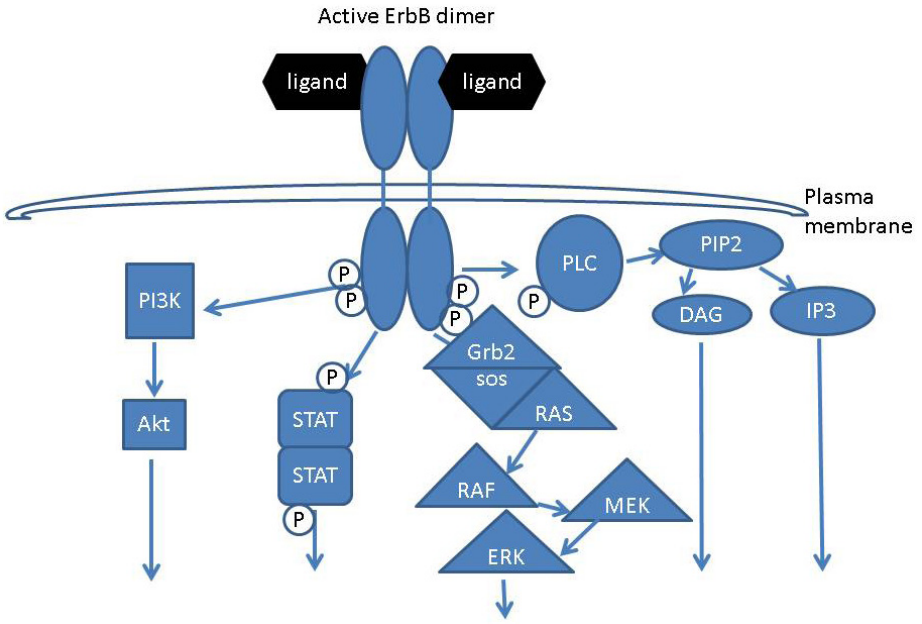

Figure 2. Schematic illustration of signaling cascades activated by the ErbB receptors. P, phosphorylated tyrosine residue.

2.1.3 Nuclear localization and regulated intramembraneous proteolysis

All ErbB receptors have been detected in the cell nucleus in several studies (Marti et al. 1991; Xie and Hung 1994; Lin et al. 2001b; Ni et al. 2001; Offterdinger et al. 2002). The receptors may travel into the nucleus as intact full-length forms (ErbB1) or the ICD can be released from the membrane via intramembraneous proteolysis (ErbB4). For ErbB2 and ErbB3 the mechanisms of nuclear translocation are not clearly understood but probably they use a similar route as ErbB1. All ErbB receptors have a tripartite nuclear localization signal, which allows their trafficking into the nucleus (Hsu and Hung 2007). The receptors may regulate gene expression in the nucleus. Mechanisms for nuclear translocation and functions for ErbB1 and ErbB4 have been studied in detail, and interestingly they seem to differ in their mechanisms and physiological roles (Figure 3).

ErbB1 is first endocytosed from the plasma membrane. After endocytosis, ErbB1 trafficks to the perinuclear area. Then, via interaction with importin b1, ErbB1 is translocated into the nucleoplasm (Lo et al. 2006), or via additional interaction with translocon sec61 first to the inner nuclear membrane and from there into the nucleoplasm (Wang et al. 2010). In the nucleus, full-length ErbB1 can act as a transcriptional co-activator (Lin et al. 2001a). As ErbB2 (Xie and Hung 1994), ErbB3 (Offterdinger et al. 2002) and also ErbB4 (Icli et al. 2012) have been detected in the nucleus as full-length receptors it is plausible that these full-length proteins may be transported via similar route to the nucleus as ErbB1.

ErbB4 ICD can also translocate into nucleus (Ni et al. 2001). The cleavage of the ICD of ErbB4 is enhanced by activation of receptor by a ligand (Ni et al. 2001). Activated receptor can then

be cleaved by tumor-necrosis factor α converting enzyme (TACE) generating a membrane-tethered fragment (m80) and a soluble extracellular fragment of ErbB4 (Elenius et al. 1997; Rio et al. 2000). This m80 fragment is subsequently cleaved by γ-secretase protease complex, which releases the ICD of ErbB4 into the cytosol (Ni et al. 2001). From cytosol the ICD can then translocate into the nucleus where it has been observed to regulate diverse processes such as mammary gland differentiation (Williams et al. 2004; Muraoka-Cook et al. 2006) and differentiation of astrocytes (Sardi et al. 2006).

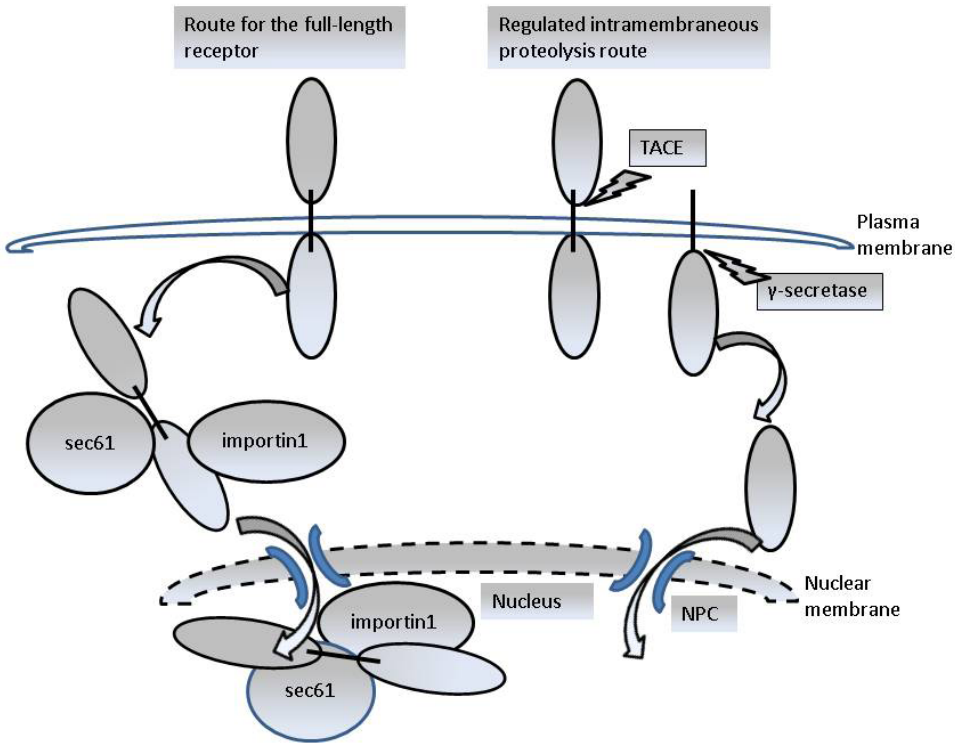

Figure 3: Nuclear transport routes of ErbB receptors. ErbB receptors can traffick to the nucleus either as full-length receptors or as proteolytically released soluble intracellular fragments. NPC=nuclear pore complex.

2.1.4 Negative regulation of ErbB receptors

As signaling of ErbB receptors is initiated by ligand binding or spontaneous activation (Linggi and Carpenter 2006), the signal also needs to be turned off. Three principal mechanisms negatively regulate signaling of ErbB receptors: internalization of the receptors by endocytosis, degradation of the receptors, and dephosphorylation of the receptors by tyrosine phosphatases (Fry et al. 2009).

During endocytosis, receptors are transferred from the cell membrane into intracellular vesicles. In the early endosomes, receptors may still be active and transmit signals (Miaczynska et al. 2004). In the late endosomes, the ligand dissociates from the receptor

and the unoccupied receptor may be translocated back to the cell surface or degraded in lysosomes (Miaczynska et al. 2004). ErbB1 seems to be most efficiently endocytosed (Baulida et al. 1996), although ErbB4 can also be effectively regulated by endocytosis (Sundvall et al. 2008).

Several ubiquitin ligases, that bind ErbB receptors and facilitate their ubiquitination, have been identified (Carraway 2010). Polyubiquitination of a protein typically targets it to degradation in the proteasomes (Pickart 2001), whereas monoubiquitination primarily affects receptor endocytosis and trafficking (Hicke and Dunn 2003). For example, cas-br-m murine ecotropic retroviral transforming sequence homolog (Cbl) binds activated ErbB1 and facilitates its ubiquitination and efficient internalization and degradation (Waterman et al. 1999) thus terminating the ligand-induced signaling.

Protein tyrosine phosphatases (PTP) are enzymes that remove phosphate moieties from phosphorylated tyrosine residues. Human genome harbors approximately 100 PTP genes as compared to approximately 90 tyrosine kinases. This suggests that similar complexity exists both in signal activation and signal attenuation mechanisms in phosphotyrosine signaling (Tonks 2006). Several PTPs have been associated with the regulation of ErbB signaling and affecting receptor endocytosis, receptor levels, and the extent of ErbB signaling (Tarcic et al. 2009; Monast et al. 2012).

2.1.5 ErbB receptors in embryonic development

Several knockout models for ErbB receptors have been generated. Generally, mice carrying homozygous deletion of any *Erbb* gene in all tissues die prematurely during embryonic development or during first three weeks of postnatal development (Gassmann et al. 1995; Miettinen et al. 1995; Sibilia and Wagner 1995; Threadgill et al. 1995). Some mouse strains carrying homozygous deletion of *Erbb1* are viable. However, the mechanisms behind this variation are not fully understood (Miettinen et al. 1995; Sibilia and Wagner 1995; Threadgill et al. 1995).

Consistent with the early observation that EGF is an agent that facilitates opening of eyelids in newborn mice (Cohen 1962), mice lacking functional *Erbb1* gene have defects in the opening of the eyelids (Miettinen et al. 1995; Threadgill et al. 1995). Mice with deleted *Erbb1* have defects in normal development of multiple tissues such as liver (Threadgill et al. 1995), kidney (Threadgill et al. 1995), lungs (Sibilia and Wagner 1995), and colon (Threadgill et al. 1995). Some *Erbb1-/-* mice also have curly or waved hair (Sibilia and Wagner 1995; Threadgill et al. 1995).

Deletion of the *Erbb2* gene in mice has revealed fundamental roles for *Erbb2* in cardiac and neuronal development. Mice deficient of functional *Erbb2* also die at embryonic day 10.5 due to poorly developed ventricular trabeculae (Lee et al. 1995; Erickson et al. 1997; Britsch et al. 1998; Chan et al. 2002). Mice lacking *Erbb2* have defects also in the development of the endocardial cushions, structures that later form the cardiac valves (Lee et al. 1995). During the neural development, the deletion of *Erbb2* affects development of Schwann cells

and myelination of the axons (Erickson et al. 1997; Woldeyesus et al. 1999; Garratt et al. 2000). Moreover, migration of neural crest cells and development of sympathetic nervous system is dependent on *Erbb2* (Britsch et al. 1998).

Erbb3 knock-outs have severe neuropathy involving defective development of Schwann cells and increased neuronal apoptosis (Riethmacher et al. 1997). Similarly to *Erbb2*-deficient mice, *Erbb3*-deficient mice have defects in the development of cardiac valves (Erickson et al. 1997; Qu et al. 2006) leading to heart dysfunction and embryonic lethality.

Erbb4-deficient mice have defects in the development of cardiac trabeculae, nervous system and the mammary gland. Function of ErbB4 during development is discussed in detail in chapter 3.2.2. Taken together, ErbB receptors are necessary for many aspects of normal development, and most striking phenotypes are perhaps observed in the cardiac and neural development.

2.1.6 ErbB receptors in disease

ErbB receptors have been associated with several malignancies, and they have an established role in tumorigenesis (Yarden and Sliwkowski 2001; Hynes and Lane 2005). ErbB receptors may turn oncogenic via several mechanisms such as somatic mutation, gene amplification or overexpression (Hynes and MacDonald 2009). For example, activation of *ERBB1* by somatic mutations has been observed in 19% of non-small cell lung cancers (Han et al. 2005) and *ERBB2* amplification has been reported even in 30% of breast cancers (Slamon et al. 1987). Indeed, ErbB1 and ErbB2 are useful therapeutic targets, and inhibition of ErbB1 in colorectal cancer (Cunningham et al. 2004) or ErbB2 in breast cancer (Slamon et al. 2001; Harris et al. 2011) has clinical anti-tumor activity and tens of thousands of patients are being treated with agents, such as therapeutic antibodies cetuximab and trastuzumab, interfering with ErbB signaling.

Although ErbB-modulating therapies are currently clinically used primarily to treat cancer, preclinical evidence suggests that ErbB-modulation could have therapeutic potential also for several other diseases, such as cardiac myopathia (Bersell et al. 2009), schizophrenia (Stefansson et al. 2002), and polycystic kidney disease (Richards et al. 1998; Wilson et al. 2006)

2.2 ErbB4

2.2.1 *ERBB4* gene and alternative splicing

The human *ERBB4* gene is located on chromosome 2 (2q33.3-q34) and covers about 1.2 Mb of genomic DNA. *ERBB4* cDNA was first cloned in 1993 (Plowman et al. 1993). *ERBB4* gene has 28 exons (Junttila et al. 2003) and at least six different isoforms are generated from *ERBB4* gene via alternative splicing. Variants of juxtamembrane regions (JM) are generated by alternative inclusion of exon 16 (JM-a) or exon 15 (JM-b) in the mature mRNA (Elenius

et al. 1997). Only JM-a isoform is able to undergo regulated intramembranous proteolysis (RIP) conducted by TACE and γ -secretase complex. The RIP process releases soluble ICD of ErbB4 into the cytosol and ICD can also translocate into the nucleus (Vecchi and Carpenter 1997; Ni et al. 2001). Consequently, JM-isoforms differ considerably in their biological functions (Veikkolainen et al. 2011b). Some evidence also exists that both exons 15 and 16 can be omitted (JM-c) or they can also both be present (JM-d) in the final mRNA product (Gilbertson et al. 2001).

Another variable region within the *ERBB4* mRNA is the cytoplasmic region (CYT), wherein exon 26 can be either included (CYT-1) or excluded (CYT-2) in the spliced mRNA product (Elenius et al. 1999; Junttila et al. 2003). Exon 26 encodes for binding sites for PI3K (Elenius et al. 1999) and for WW domain (Sundvall et al. 2008). These sites allow CYT-1 isoform to directly stimulate PI3K signaling and to bind additional WW domain-containing proteins such as Itch and WWOX (Sundvall et al. 2008).

Highest levels of *ERBB4* mRNA are observed in brain, kidney, salivary gland, trachea, thyroid gland, skeletal muscle and heart (Junttila et al. 2005). The different splice variants of *ERBB4* are expressed differently as exemplified by only JM-a being expressed in the kidney and JM-b being dominant in skeletal muscle (Junttila et al. 2005; Veikkolainen et al. 2011b). Cytoplasmic isoforms are both expressed in almost all tissues expressing ErbB4, although not necessarily in similar quantities (Junttila et al. 2005; Veikkolainen et al. 2011b).

2.2.2 ErbB4 in embryonic development

Erbb4 knock-out mice die at embryonic day 9.5 (Gassmann et al. 1995). Mice lacking *Erbb4* show decreased or absent trabeculation that results in defective functioning of the heart (Gassmann et al. 1995). In addition to the malformation of the heart, abnormal neural development is observed in the *Erbb4* deficient mice. *Erbb4* knock-outs that have been rescued for ErbB4 expression in the heart are viable suggesting that early mortality is due to cardiac defects (Tidcombe et al. 2003). These mice have abnormalities in the migration of neural crest cells, development of the nervous system and lung. Several studies have been conducted to analyze tissue-specific roles of ErbB4 using conditional tissue-specific deletions of *Erbb4*. Conditional knock-out mice generated by heart-specific (Myl2-Cre) deletions of *Erbb4* have reduced cardiomyocyte proliferation, absent trabeculae and these mice exhibit dilated cardiomyopathy (García-Rivello et al. 2005; Bersell et al. 2009).

Kidney-specific deletion of *Erbb4* (Pax8-Cre) results in defective polarization of tubular epithelial cells and increased size of the lumen of collecting ducts. Consistently, ErbB4 overexpression under the same promoter results in decreased lumen size and disorganized epithelial polarization (Veikkolainen et al. 2011a).

Mice with deletion of *Erbb4* (WAP-Cre) or expressing dominant-negative truncated form of *Erbb4* (MMTV-Cre) in the mammary gland show lactational failure indicating a critical role for ErbB4 in the development of the mammary gland (Jones et al. 1999; Long et al. 2003).

2.2.3 ErbB4 in disease

Expression of ErbB4 is altered in many cancers. ErbB4 has been reported to be underexpressed in meningioma (Laurendeau et al. 2009), renal cell carcinoma (Thomasson et al. 2004), thyroid cancer (Wiseman et al. 2008), pancreatic cancer (Graber et al. 1999; Thybusch-Bernhardt et al. 2001), glioma (Andersson et al. 2004) and in breast cancer (Srinivasan et al. 1998). On the otherhand, has been reported to be overexpressed in breast cancer (Witton et al. 2003; Junttila et al. 2005), ovarian cancer (Steffensen et al. 2008), medulloblastoma (Gilbertson et al. 1997; Ferretti et al. 2006) and ependymoma (Gilbertson et al. 2002). At least a partial explanation for contradictory reports of ErbB4 expression in breast cancer may be provided by observation that ErbB4 expression in closely associated with estrogen receptor-positive subtype of breast cancer (Junttila et al. 2005), and that in earlier study (Srinivasan et al. 1998) the subtypes of breast cancers were not analyzed.

Expression of ErbB4 has also been shown to have prognostic significance. It has been associated with poor survival in breast cancer (Lodge et al. 2003), medulloblastoma (Gilbertson et al. 1997), gastric cancer (Shi et al. 2012) and colorectal cancer (Lee et al. 2002; Baiocchi et al. 2009). However, expression of ErbB4 has also been linked to favorable prognosis in breast cancer (Pawlowski et al. 2000; Witton et al. 2003; Junttila et al. 2005) and bladder cancer (Memon et al. 2004). Experimental models have also yielded contradictory results as ErbB4 has been suggested to function both as a tumor-promoting and as a tumor-suppressing factor (Naresh et al. 2006; Vidal et al. 2007; Das et al. 2010). This suggests that the role of ErbB4 in tumorigenesis is dependent on the tumor type.

Somatic mutations affecting *ERBB4* have been characterized in melanoma (Prickett et al. 2009; Dutton-Regester et al. 2012), lung cancer (Soung et al. 2006b; Ding et al. 2008), colon cancer (Soung et al. 2006b), gastric cancer (Soung et al. 2006b) and breast cancer (Soung et al. 2006b). At least some mutations affect signaling properties of ErbB4 (Prickett et al. 2009; Tvorogov et al. 2009) and it has been suggested that mutations of *ERBB4* play a role in tumorigenesis in these cancers (Soung et al. 2006b; Prickett et al. 2009). The malignant potential of ErbB4 remains controversial, and differs between cancer types. The reasons for these controversies remain to be elucidated.

ErbB4 has been linked with several neurological disorders. Clearest genetic evidence exist for schizophrenia wherein data from both mouse models (Stefansson et al. 2002) and human patients (Nicodemus et al. 2006; Silberberg et al. 2006; Greenwood et al. 2012) suggest that ErbB4 is associated with schizophrenia. Mice lacking one of the alleles of *Erbb4* or *Nrg1* show symptoms associated with schizophrenia in mice. In humans, single-nucleotide polymorphisms (SNP) of *ERBB4* (Nicodemus et al. 2006; Silberberg et al. 2006; Greenwood et al. 2012) and *NRG1* (Stefansson et al. 2002; Li et al. 2006; Munafo et al. 2006) loci have been statistically associated with likelihood of having schizophrenia.

ErbB4 deficient mice display defects in cardiac development (Gassmann et al. 1995; García-Rivello et al. 2005). Association of ErbB4 with heart failure has been observed also in humans, as hearts of patients suffering from heart failure have significantly reduced expression of

ErbB4 (Rohrbach et al. 2005). Preclinical evidence also implies potential for modulation of ErbB4 function in the treatment of myocardial infarction (Bersell et al. 2009).

2.3 Neuregulin-1

2.3.1 Neuregulin-1 gene and alternative splicing

The human *NRG1* gene is very large (1.4 Mb) and produces at least 15 different NRG-1 isoforms, which differ in their EGF-like domain (α or β), N-terminal ICD (type I,II or III) or being produced initially as soluble or membrane bound forms (NRG1 IIβ3 is the only one produced as a soluble form) (Falls 2003). Membrane-bound forms can be released proteolytically by TACE (Horiuchi et al. 2005). Moreover, an intracellular fragment can be released into the cytosol by the γ-secretase complex (Bao et al. 2003) analogous to ErbB4. NRG-1 can bind its receptors both as soluble and membrane-bound forms and hence is capable of paracrine, autocrine and juxtacrine signaling (Falls 2003).

2.3.2 NRG-1 in embryonic development

Mice lacking *Nrg1* die early in their development around embryonic day 11.5 (Meyer and Birchmeier 1995). These mice have poorly developed ventricular trabeculae (Meyer and Birchmeier 1995) similar to *Erbb4-/-* mice (Gassmann et al. 1995; García-Rivello et al. 2005). Deletion of *Nrg1* has revealed an important role for NRG-1 in the development of Schwann cells (Erickson et al. 1997; Britsch et al. 1998; Brinkmann et al. 2008), heart (Erickson et al. 1997; Britsch et al. 1998), muscle spindle (Hippenmeyer et al. 2002) and testis (Zhang et al. 2011). Besides these phenotypes, also various other defects of nervous system have been observed in the *Nrg1*-deficient mice (Meyer and Birchmeier 1995; Erickson et al. 1997; Liu et al. 1998; Wolpowitz et al. 2000). Interestingly, different NRG-1 isoforms have different roles during the development. For example, type I isoforms are important for development of the neural crest and type III isoforms for the development of Schwann cells (Meyer et al. 1997).

2.3.3 Neuregulin-1 in disease

NRG-1 has been associated with several diseases, such as schizophrenia (Stefansson et al. 2002; Li et al. 2006; Munafo et al. 2006). Mice carrying only one functional copy of *Nrg1* gene display schizophrenia-like phenotypes (Stefansson et al. 2002), resembling the phenotype of mice with *Erbb4* haploinsufficiency (Stefansson et al. 2002).

Consistent with the role of ErbB4 in proper functioning of the heart, also NRG-1 has been associated with heart dysfunction both in mice (Liu et al. 2005) and in humans. Indeed, promising results of using NRG-1 as a therapeutic agent in heart failure have been observed in both mice (Liu et al. 2006; Bersell et al. 2009) and in human clinical trials (Gao et al. 2010; Jabbour et al. 2011).

NRG-1 expression has been observed in many cancers including breast (Dunn et al. 2004), ovarian (Gilmour et al. 2002; Sheng et al. 2010), prostate (Leung et al. 1997; Memon et al. 2004), colorectal (Eschrich et al. 2005), pancreatic (Kolb et al. 2007) and thyroid cancer (Fluge et al. 2000). As with ErbB4, the prognostic role of NRG-1 varies between different cancer types and both associations with poor (Kolb et al. 2007; Sheng et al. 2010) and favorable (de Alava et al. 2007) prognosis have been observed.

2.4 Hypoxia-inducible factors

Hypoxia is defined as deficiency of oxygen. Hypoxic conditions are often encountered in pathological conditions such as malignant growth and ischemia. Hypoxia is also found in normal physiological milieu, for example of several cell types within cartilage. Cells sense hypoxia via distinct hypoxia sensing pathways. The key components of this pathway are hypoxia-inducible factors (HIF), which regulate the transcriptional response to hypoxia. HIF-1α was cloned first (Wang et al. 1995), followed soon by HIF-2α (EPAS-1) (Tian et al. 1997) and HIF-3α (Gu et al. 1998). These HIF-α subunits dimerize with the HIF-β subunit also called aryl-hydrocarbon nuclear translocators (ARNT). HIF dimers can bind to DNA at specific hypoxia-response elements (Semenza and Wang 1992), and regulate transcription of specific target genes.

2.4.1 Oxygen-dependent and -independent regulation of HIFs

HIFs are regulated by multiple mechanisms. All HIF-α subunits are regulated by oxygen-dependent degradation, whereas the HIF-β subunit is not. The oxygen-dependent degradation of HIF-α subunit is initiated by post-translational prolyl 4-hydroxylation of the HIF-α subunit (Ivan et al. 2001; Jaakkola et al. 2001). In HIF-1α, prolines 402 and 564 are hydroxylated in response to oxygen. This hydroxylation is mediated by three oxygen-sensitive prolyl 4-hydroxylases (PHDs) (Bruick and McKnight 2001; Epstein et al. 2001). These enzymes need molecular oxygen as a cofactor to function properly and therefore PHDs are fully active only in normal oxygen tension and are inhibited in hypoxic conditions. The hydroxylated proline residues of HIF-1α are detected by von-Hippel Lindau protein (pVHL), which mediates ubiquitination and hence degradation of HIF-1α by the proteasomes. In hypoxic conditions, when prolyl 4-hydroxylation does not occur, pVHL cannot bind and ubiquitinate HIF-1α, ultimately leading into increased protein stability and accumulation of HIF-1α protein in the cells (Pouysségur et al. 2006). In addition to the regulation of the stability of HIF-α subunits, also transcriptional activity of HIF-α subunits is regulated in oxygen-dependent manner by another oxygen-dependent enzyme, factor inhibiting HIF-1α (FIH-1) (Mahon et al. 2001). FIH-1 hydroxylates asparagine residue in the C-terminal transactivation domain of HIF-α subunits thus reducing transcriptional activity HIF-α subunits in normoxic conditions (Lando et al. 2002).

More recently, a few oxygen-independent mechanisms for the regulation of HIF-1α protein stability have been described. These include stabilization of HIF-1α by HSP90 to protect from

RACK1-dependent degradation (Liu et al. 2007) and degradation dependent of hypoxia-associated factor (HAF) (Koh et al. 2008). Also sumoylation (Bae et al. 2004; Berta et al. 2007; Carbia-Nagashima et al. 2007), acetylation (Jeong et al. 2002) and phosphorylation (Richard et al. 1999) of HIF-1α have been described. Moreover, HIF-1α may be regulated via classical signaling pathways such as PI3K (Laughner et al. 2001) and MAPK pathways (Richard et al. 1999). These examples imply that HIF-1α is a subject to a complex regulatory machinery within the cell.

2.4.2 Hypoxia-inducible factors in embryonic development

HIFs are indispensable for normal development of mice. Deletion of *Hif1a* results in embryonic lethality and reduced angiogenesis (Iyer et al. 1998; Ryan et al. 1998). Conditional knock-out of *Hif1a* in mice has implicated a role for HIF-1α also in development and homeostasis of lungs (Saini et al. 2008), heart (Huang et al. 2004), skeletal muscle (Mason et al. 2004), mammary glands (Seagroves et al. 2003) and brain (Tomita et al. 2003). Deletion of *Hif2a* results in embryonic lethality (Tian et al. 1998), altered metabolism (Scortegagna et al. 2003a), impaired development of the heart (Tian et al. 1998) and also in defective hematopoiesis (Scortegagna et al. 2003b). Mice deficient of *Hif3a* are, however, viable, although they exhibit developmental defects in the heart and lungs (Yamashita et al. 2008).

2.4.3 HIFs in disease

The link between increased anaerobic metabolism and cancer has been known for over 50 years (Warburg 1956). HIFs are key regulators of anaerobic metabolism, and indeed HIFs have been characterized as activators of multiple glycolytic enzymes (Semenza et al. 1994). Growth of new vessels, angiogenesis, represents another adaptation mechanism to hypoxia and it has been widely recognized as a highly important component of tumor biology (Folkman 1971). HIFs are important regulators of angiogenesis. Not surprisingly, HIFs are associated with several malignancies and HIF-1α and HIF-2α overexpression has been observed in numerous cancer types (Harris 2002; Pouysségur et al. 2006).

Loss-of-function mutations in *VHL* gene have been linked to von Hippel-Lindau cancer syndrome (Latif et al. 1993) and Chuvash polycythemia (Ang et al. 2002a; Ang et al. 2002b). Similarly, gain-of-function mutations within *HIF2A* locus and loss-of-function mutations in *PHD2* locus have been linked to familial erythrocytosis (Percy et al. 2006; Percy et al. 2008a; Percy et al. 2008b). These observations imply that proper regulation of HIF activity is crucial for correct functioning of human body.

In ischemias of the heart and brain, the blood flow to tissue is suddenly blocked resulting in deficiency in oxygen and nutrients and ultimately to clinical strokes. As HIFs play fundamental roles in oxygen sensing and responses to hypoxia, they have also been implicated in the pathogenesis of ischemias. Interestingly, treatment of animals with HIF-activating compounds seems to reduce tissue damage elicited by ischemic conditions (Bergeron et al. 2000).

2.5 Zebrafish as an in vivo model to study ErbB4 signaling

2.5.1 Zebrafish as a model organism

Zebrafish has become a popular model vertebrate organism because it has several features that make it useful for research. Zebrafish are small vertebrates with a genome size comparable to humans, and the genome has been fully sequenced. Zebrafish lays eggs in large numbers, and they develop rapidly outside of the mother. This allows one to study developmental processes more easily than in mammals (Zon and Peterson 2005).

Many organ systems differ at the macroscopic level, but on microstructural, physiological and molecular level there is a great degree of conservation between zebrafish and mammals such as man and mouse (Zon and Peterson 2005).

Methodologically zebrafish is a highly utilizable and economic model for biomedical research (Zon and Peterson 2005). Several forward genetic screens have yielded mutations in hundreds or thousands of genes and Zebrafish Mutation Project (www.sanger.ac.uk/Projects/D_rerio/zmp/) which aims to mutate every zebrafish gene is currently in process and estimated to be completed in 2015. Moreover, targeted genome editing is achievable through the use of zinc-finger nucleases although homologous recombination techniques do not currently exist for the zebrafish (Doyon et al. 2008; Meng et al. 2008). Protein coding genes can also be easily targeted during early development by using morpholino antisense oligos, which can block translation or correct splicing of the mRNA (Nasevicius and Ekker 2000; Eisen and Smith 2008).

Hundreds of transgenic lines have been generated, which carry tissue-specific fluorescent reporters. These models are especially useful in zebrafish which is practically transparent during embryonic development and even almost transparent adult fish strains have been generated (White et al. 2008). Current transgenesis methods are robust and allow transient transgenic work with founder embryos without time-demanding breeding (Kwan et al. 2007). This allows rapid expression and testing of transgenes.

2.5.2 Biology of zebrafish

The zebrafish is a small fresh water fish that lives naturally in rivers of South East Asia. It is fairly easy to keep and breed in captivity. A single female can produce up to several hundred eggs per week. Lifespan of zebrafish is around 4 years and generation time 2-3 months. The development is rapid. The cleavage period is completed by 2.5 hours post fertilization (hpf), blastula stage by 5 hpf, gastrulation by 10 hpf and segmentation of the embryo is completed by 24 hpf (Figure 5) (Kimmel et al. 1995). The heart starts to beat and first muscle contractions occur one day post fertilization (dpf) and embryos are freely swimming and feeding at 5 dpf (Kimmel et al. 1995).

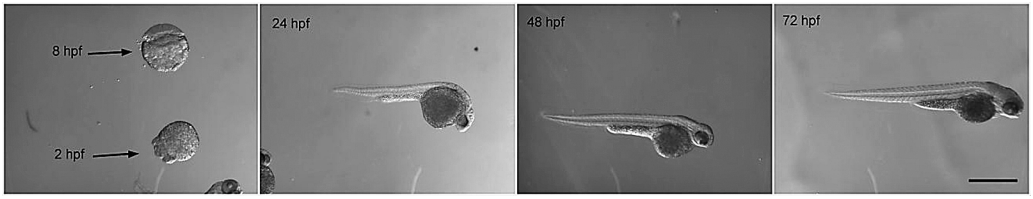

Figure 5. Development of zebrafish embryos at 2, 8, 24, 48 and 72 hours postfertilization (hpf). Embryos at 2 hpf are at cleavage stage, at 8 hpf at gastrulation stage, and at 24 hpf segmentation has been completed. Images have been taken with Zeiss StereoLumar stereomicroscope. Scale bar 0.5 mm.

Zebrafish are teleost (ray-finned) fish and they belong to cyprinid family (carps and minnows). The last common ancestor of teleost and tetrapods lived approximately 400-500 million years ago (Nüsslein-Volhard and Dahm 2002). The size of the zebrafish genome is 1.4 Gb and it consists of 25 autosomal chromosome pairs. It is currently estimated to carry 25,000 protein-coding genes. Zebrafish has no distinct sex chromosomes, but clear genetic component and association of distinct loci with sex determination have been reported (Anderson et al. 2012; Liew et al. 2012). In comparison, human genome is 3.2 Gb, has 23 autosomal chromosome pairs and two sex chromosomes and approximately 23,000 protein-coding genes. In teleosts, and also in the zebrafish, the genome has duplicated partially during the evolution and approximately 30% of genes have duplicated homologues in the zebrafish genome (Nüsslein-Volhard and Dahm 2002).

2.5.3 ErbB receptors in zebrafish

The ErbB family of receptor tyrosine kinase genes has expanded in teleosts and there are two homologues of *ERBB1* (*egfra* and *egfrb*), *ERBB3* (*erbb3a* and *erbb3b*) and *ERBB4* (*erbb4a* and *erbb4b*) in the zerbafish genome (Laisney et al. 2010). Also, some ErbB ligands have been duplicated including homologues of *HBEGF* (*hbegfa* and *hbegfb*)(Laisney et al. 2010) and *NRG2* (*nrg2a* and *nrg2b*)(Honjo et al. 2008).

Table 3. Summary of different phenotypes observed after perturbation of ErbB signaling in zebrafish.

Gene	Mutation / Morpholino	Phenotype	References
egfra	Morpholino	Blood circulation defect.	(Goishi et al. 2003)
erbb2	Mutation	Reduced heart trabeculation, Schwann cell migration and fin regeneration.	(Lyons et al. 2005; Rojas-Muñoz et al. 2009; Liu et al. 2010)
erbb3b	Mutation	Aberrant neural crest migration, pigmentation and fin regeneration.	(Budi et al. 2008; Hultman et al. 2009; Rojas-Muñoz et al. 2009)
nrg1	Mutation, morpholino	Reduced dorsal root ganglion neuron development, oligodendrocyte and neuron specification.	(Honjo et al. 2008; Wood et al. 2009)
nrg2a	Morpholino	Reduced dorsal root ganglion neuron development	(Honjo et al. 2008)
hbegfb	Morpholino	Cardiac defects	(Friedrichs et al. 2009)

The research on ErbB function in zebrafish models has had an emphasis on *erbb2* and *erbb3b* (also known as *picasso*) as loss-of-function mutations of these genes have been isolated (Lyons et al. 2005; Budi et al. 2008). Less work has been done on *egfr* and *erbb4*, although some results from a morpholino experiment to knock-down *egfra* have been published (Goishi et al. 2003). In zebrafish, ErbB receptors seem to be involved in similar developmental processes as in mouse, such as development of the heart and the neuronal system (Table 3). In addition to analysis of ErbB mutants and knock-down experiments, chemical genetic approaches have revealed roles for ErbB receptors in developmental angiogenesis (Tran et al. 2007) and heart valve formation (Scherz et al. 2008). Although tumorigenic potential of ErbB receptors in zebrafish has not been analyzed, in classical melanoma model of related fresh water fish *Xiphophorus* (Gordon 1927; Häussler 1928), an *EGFR* homologue (also called Xmrk) has been characterized as a powerful oncogene (Wittbrodt et al. 1989). In summary, the zebrafish model system seems to recapitulate many of the functions of ErbB receptors in mammals, and therefore can be considered a relevant and useful model system to study ErbB functions during development and disease.

3 AIMS OF THE STUDY

Aim 1) To characterize novel interactions between the ErbB signaling system and HIF-1α.

Aim 2) To determine the biological significance of interactions between ErbB and hypoxia signaling pathways.

Aim 3) To address the clinical significance and signaling mechanisms of ErbB4 in serous ovarian cancer.

Aim 4) To generate a novel in vivo model to study the functions of ErbB4.

4 MATERIALS & METHODS

Materials and methods are described in detail in the original publications. Roman numbers I-V refer to original publication in which the given reagent/method was used.

4.1 Methods

Method	Used in
Affinity purification	I
Cell culture	I,II,III,IV
Cell fractionating	III
Co-immunoprecipitation	I
Confocal microscopy	I,II,V
Electron microscopy	V
Embryo culture	V
Glutathione-S-transferase (GST)-pull down	I
Hypoxia cabinet	I,III
Immunofluorescence	I,II,III,V
Immunohistochemistry	I,II,IV
In silico transcriptomics	I
In situ proximity ligation assay	I
In vitro transcription	V
In vitro translation	I
Ligand stimulations	I,II,III,IV
Luciferase assay	I,II
Microinjections	V
Molecular cloning	I,IV,V
Morpholino antisense oligos	V
Pharmacological modulations	I,II,III,IV,V
Primary cell isolation	III
Production of recombinant proteins in *E.coli*	I
Real-time RT-PCR	I,II,III,IV,V
Retroviral infection	I,IV
RNA extraction and synthesis of cDNA	I,II,III,IV,V
RNA interference with siRNAs	I,II
Soft agar growth assay	IV
Statistical analysis	I,II,III,IV,V
Stereo microscopy	V
Tissue microarray	IV
Transfections	I,II,IV
Transposon mediated transgenesis	V
Video imaging	V
Western blotting	I,II,III,IV,V

4.2 Plasmids

Name	Backbone	Purpose	Used in
β-casein-luc	pGL3	STAT5 activity reporter	II
ErbB4 JM-a CYT-2	pcDNA3.1-	Mammalian expression	I,II
ErbB4 JM-a CYT-2 HA	pcDNA3.1-	Mammalian expression	I,II
ErbB4 JM-b CYT-2	pcDNA3.1-	Mammalian expression	I,II
GST	pGEX-6P1	Bacterial expression	I
HIF1α	pcDNA3	Mammalian expression	I,II
HIF1α-Δ1-174-6xhis	pcDNA3.1+	Mammalian expression	I
HIF1α-Δ1-343-6xhis	pcDNA3.1+	Mammalian expression	I
HIF1α-Δ1-529-6xhis	pcDNA3.1+	Mammalian expression	I
HIF1α-Δ-1-631-6xhis	pcDNA3.1+	Mammalian expression	I
HIF1α(P402A,P564G)	pcDNA3	Mammalian expression	I,II
HIF1α-6xhis	pcDNA3.1+	Mammalian expression	I
HIF1α-GST	pGEX-6P1	Bacterial expression	I
Hygro	pcDNA3.1+	Mammalian expression	I,II
ICD2	pcDNA3.1+	Mammalian expression	I,II
ICD2 ΔC	pcDNA3.1+	Mammalian expression	I
ICD2 ΔN	pcDNA3.1+	Mammalian expression	I
ICD2-GST	pGEX-6P1	Bacterial expression	I
Neo	pcDNA3.1-	Mammalian expression	I,II,
pBabe ErbB4 JM-a CYT-1	pBabe-puro	Mammalian retroviral expression	IV
pBabe ErbB4 JM-b CYT-2	pBabe-puro	Mammalian retroviral expression	I
pBabe-ErbB4 JM-a CYT-2	pBabe-puro	Mammalian retroviral expression	I,IV
pBABE-Puro	pBabe-puro	Mammalian retroviral expression	I,IV
pEGFP	pEGFP-N1	Mammalian expression	II
pPGK6-luc	pGL3	HIF activity reporter	I
pSV40-renilla	pRenilla	Control reporter	I
TK-renilla	pRenilla	Control reporter	I

4.3 Small interfering RNAs

Small interfering RNAs were synthesized as 21 nt double stranded (duplex) RNA. The duplexes were transfected into cells using Lipofectamine 2000 transfection reagent (Invitrogen) following recommendations of the manufacturer.

Name	Species	Company	Target sequence	Used
ErbB4 JM-a	Human	Eurogentec	gucaugacuaguggaccgtt	I
ErbB4 JM-b	Human	Eurogentec	guauugaagacugcaucggtt	I
ErbB4 total	Human	Eurogentec	acugagcucucucucugactt	I
HIF1A#13	Human	Qiagen	aggaagaacuaugaacauaaa	II
HIF1A#5	Human	Qiagen	auggaauauauucugcguuua	II
Negative	Human	Qiagen	aattctccgaacgtgtcacgt	I,II
RACK1#1	Human	Qiagen	aucauguccgggaacugcggg	I
RACK1#5	Human	Qiagen	uaaacuucuagcgugugccuu	I

4.3 Primers and probes

Following real-time RT-PCR primers and probes were used in the studies. Numbered probes are from Universal Probelibrary (Roche), and other probes are standard hydrolyzation probes labeled with 6-carboxyfluorescein (FAM) and tetramethylrhodamine (TAMRA).

Target	Species	Left primer	Right primer	Probe	Used in
BETA-ACTIN	Human	atctggcaccacaccttctacaat	ccgtcaccggagtccatca	tgacccagatcatgtttgagaccttcaacac	II,IV
EF-1α	Human	ccccaggacacagagacttt	gcccattcttggagatacca	#56	I
EPO	Human	tcccagacaccaaagtta atttcta	ccctgccagacttctacgg	#58	I
ERBB4 CYT-1	Human	caacatcccacctcccatctatac	acactccttgttcagcagcaaa	tgaaattggacacagccctcctcctg	IV
ERBB4 CYT-2	Human	caacatcccacctcccatctatac	acactccttgttcagcagcaaa	aattgactcgaataggaaccagtttgtataccgagat	IV
ERBB4 JM-a	Human	ccacccatgccatccaaa	ccaattactccagctgcaatca	catggacgggcaattccactttacca	I,II,IV
ERBB4 JM-b	Human	ccacccatgccatccaaa	ccaattactccagctgcaatca	ctcaagtattgaagactgcatcggcctgat	IV
ERBB4 TOTAL	Human	tcaagcattggataatcccga	agtggctcattcacatactcatcct	tatcacaatgcatccaatggtccaccc	II
GLUT-1	Human	gtgggcatgtgcttccagtc	aagaacagaaccaggagcaca	aactgtgtggtccctacgtcttcatcatct	I,II
HIF-1α	Human	gatagcaagactttcctcagtcg	tggctcatatcccatcaattc	#64	I
PGK-1	Human	tgcaaaggccttggagag	tggatcttgtctgcaact ttagc	#72	I
VEGF-A	Human	tctacctccaccatgccaagt	tgatgattctgccctcctcc	ccaggctgcacccatggcaga	I
cmcl2	Zebrafish	caggagcccagaccaaca	agcagttttccccctcttg	#112	V
erbb4a	Zebrafish	aaaccgcaacttgtctttcc	ccagaggaagatagtcaaactgg	#21	V
erbb4b	Zebrafish	atgtgcatcccctgcact	cgtctgaaggctggcagt	#43	V
myhc4	Zebrafish	caagcagaagcagcgtga	gggtagcacaaagccttcag	#82	V
rpl13a	Zebrafish	gcggaccgattcaataaggg	gaaagacgaccgaggtgaga	#147	V
smyhc1	Zebrafish	tgccaagaccatcagaaatg	cacaccaaagtgaattcggata	#52	V
tpm1	Zebrafish	gaacgccttggacagagc	ttccaactgaattagttcgtcttct	#30	V

4.4 Cell lines

Cell line	Type	Species	Used in
MCF-7	Mammary adenocarcinoma cell	Human	I
T47D	Mammary adenocarcinoma cell	Human	II
COS-7	Fibroblast-like kidney cell	African green monkey	I
HEK-293	Embryonic kidney cell	Human	II
OVCAR-3	Ovarian carcinoma cell	Human	IV
SKOV-3	Ovarian carcinoma cell	Human	IV
HUVEC	Umbilical cord endothelial cell	Human	III
RCC4	Renal cell carcinoma	Human	I
MDA-MB-468	Mammary adenocarcinoma cell	Human	I,II

4.5 Reagents

Name	Target	Source	Used in
AG 1748	ErbB inhibition	Calbiochem	I,II,IV,V
$CoCl_2$	PHD inhibition	Sigma Aldrich	I,II,III
DMOG	PHD inhibition	Cayman Chemicals	II
GSI IX	γ -secretase inhibition	Calbiochem	I,III
LY294002	PI3K inhibition	Calbiochem	I,IV
PMA	Inducer of NRG-1 cleavage	Calbiochem	II

4.6 Antibodies

Antigen	Name	Source	Type	Application	Used in
Akt	sc-1618	Santa Cruz	Goat polyclonal	WB	I,II,IV
Beta-actin	sc-1616	Santa Cruz	Goat polyclonal	WB	I,II,III,IV,V
ErbB1	sc-03	Santa Cruz	Rabbit polyclonal	WB	IV
ErbB2	sc-284	Santa Cruz	Rabbit polyclonal	WB	IV
ErbB3	sc-285	Santa Cruz	Rabbit polyclonal	WB	IV
ErbB4	sc-283	Santa Cruz	Rabbit polyclonal	WB, IHC, IF	I,II
ErbB4	e200	Abcam	Rabbit monoclonal momoclonalmonoclonal	WB, IF	I,II,IV,V
ErbB4	HFR-1	Abcam	Mouse monoclonal	IF,IHC, PLA	IV,IV,V
Erk	9102	CST	Rabbit polyclonal	WB	I,II,IV
GLUT-1	ab14683	Abcam	Rabbit polyclonal	IHC	I,II
GST		Santa Cruz	Goat polyclonal	WB	I
HA	3F10	Roche	Rat monoclonal	WB,IF	I,II
HIF-1α	ab2185	Abcam	Rabbit polyclonal	WB,PLA	I,II
HIF-1α	clone 54	BD	Mouse monoclonal	WB, IP	I,II,III
HIF-1α	H1a67	Abcam	Mouse monoclonal	IHC, PLA	I
Lamin B	sc-6217	Santa Cruz	Goat polyclonal	WB	III
Muscle actin, fast type	F310	DSHB	Mouse monoclonal	WB, IF	V
Muscle, actin, slow type	F59	DSHB	Mouse monoclonal	WB, IF	V
NRG-1	F20	Santa cruz	Rabbit polyclonal	WB	III
p53	DO-1	Santa Cruz	Mouse monoclonal	WB	IV
phospho-Akt	9271	CST	Rabbit monoclonal	WB	I,II,IV
phospho-ErbB4	4757	CST	Rabbit monoclonal	WB	II,IV,V
phosphoErk	9101	CST	Rabbit monoclonal	WB	I,II,IV
RACK-1	ab62735	Abcam	Rabbit polyclonal	WB, PLA	I
Tropomyosin	CH1	DSHB	Mouse monoclonal	WB, IF	V

SC = Santa Cruz Biotechnology, CST = Cell Signaling Technology, DSHB = Developmental studies hybridoma bank, BD = Beckton-Dickinson Biosciences, WB = Western blotting, IF = immunofluorescence, IHC = immunohistochemistry, IP = immunoprecipitation, PLA = in situ proximity ligation assay.

4.7 Microinjected materials

Reagent	Type	Function	Used in
Control MO	Morpholino	Negative control	V
erbb4a MO	Morpholino	ErbB4a knock-down, prevents translation of ErbB4a mRNA.	V
EGFP	RNA	Negative control mRNA	V
ERBB4-EGFP	RNA	Rescue mRNA for *erbb4a* morpholino.	V
Tol2 transposon	RNA	Facilitate integration of transgenes into genome.	V
pDESTTol2A2-acta1-*EGFPpA*	DNA	Zebrafish muscle cell actin promoter driven muscle-cell specific expression of EGFP. Tol2 sites for genome integration by Tol2 transposase.	V
pDESTTol2A2-acta1-*ERBB4-P2A-EGFPpA*	DNA	Zebrafish muscle cell actin promoter driven muscle-cell specific expression. of human ErbB4 (JM-a CYT-1) and co-expression of EGFP with self-cleaving P2A-sequence. Tol2 sites for genome integration by Tol2 transposase.	V
pDESTTol2A2-*βactin-EGFPpA*	DNA	Zebrafish beta-actin promoter driven expression of EGFP. Tol2 sites for genome integration by Tol2 transposase.	V
pDESTTol2A2-*βactin-ERBB4-P2A-EGFPpA*	DNA	Zebrafish beta-actin promoter driven expression of human ErbB4 (JM-a CYT-1) and co-expression of EGFP with self-cleaving P2A-sequence. Tol2 sites for genome integration by Tol2 transposase.	V

4.8 Zebrafish methods

4.8.1 Maintenance

Adult zebrafish were maintained at 26 °C in glass aquaria equipped with in-tank filters and aeration using standard procedures (Westerfield 2000). The system water was active charcoal filtered tap water and it was allowed to warm to 26°C overnight prior to addition to the aquaria. Fish were fed with flakes, frozen blood worms and live *Artemia nauplii* once or twice per day. Dried *Artemia* cysts were hatched at 26 °C (in medium containing 3% sea salt in system water) in inverted plastic bottles with vigorous aeration (Nüsslein-Volhard and Dahm 2002).

4.8.2 Breeding and embryo culture

Eggs were obtained by natural spawning by using self-made mesh-bottomed mating boxes to separate eggs from adult fish. The mating boxes were placed into the bottom of the fish aquaria in the afternoon. Zebrafish mate at the morning right after the lights have been

turned on, and hence the fertilized eggs could be collected from the mating boxes during the next morning. The eggs were collected by pouring the water from mating boxes into a tea strainer and washed several times with system water.

The fertilized eggs and developing embryos were cultured in E3-medium (5 mM NaCl, 0.17 mM KCl, 0.33 mM $CaCl_2$, 0.33 mM $MgSO_4$) at 28 °C (Nüsslein-Volhard and Dahm 2002). E3-medium was supplemented with methylene blue (100 ppm), penicillin and streptomycin to reduce microbial growth. When needed, pigmentation of embryos was inhibited by adding phenyl-thio-urea (0.2 mM) into the E3-medium.

4.8.3 Microinjection

Embryos at 1-8 cell stage were microinjected using Femtotip glass capillary needles (Eppendorf), Femtojet pressure system (Eppendorf) and Injectman2 micromanipulator attached to SteReoLumarV.12 stereomicroscope (Zeiss). A microscope glass slide was placed on a bacterial culture plate and embryos were aligned along the side of the slide. Excess water was aspirated and surface tension of water prevented the embryos from moving during the injections. Injection volume was calculated by injecting a small droplet into hydrocarbon oil (Sigma). The diameter of the droplet was measured and the volume calculated using following equation: $V = \frac{4}{3} \times \pi \times r^3$. Morpholinos, in vitro transcribed mRNAs and DNA constructs were injected into yolk (3 nl injection volume) near the animal pole of the embryo.

4.8.4 Immunofluorescence

Prior to fixation embryos were dechorionated by forceps or by incubation with 2 mg/ml pronase (Roche). Embryos were fixed with 4% paraformaldehyde in PBS overnight at 4 °C, or with 100% methanol for at least 20 min at –20 °C. Paraformaldehyde fixation was followed by permeabilization with methanol at –20 °C. To rehydrate the samples, they were placed for 5 min periods in 100%, 75%, 50%, 25% and 0% of methanol in PBST (0.1% Tween-20 in PBS). Embryos were blocked with 5% normal goat serum in PBST for 3 hours at +4°C. Primary antibodies were added to the solutions and samples were incubated overnight at 4 °C. Unbound antibodies were washed off with four washes with PBST (15 min, room temperature). Subsequently, Alexa -488 and Alexa 555 goat anti-mouse secondary antibodies were applied in 5% blocking solution, and incubated 3 hours at room temperature with nuclear stain DAPI. Unbound secondary antibodies were washed off with four 15 min washes with PBST at room temperature. Next, the embryos were cleared and mounted in 87% glycerol. Images were taken with LSM510 META confocal microscope (Zeiss) or with StereoLumar V.12 stereomicroscope (Zeiss).

4.8.5 RNA extraction

First, the embryos were collected into fresh microcentrifuge tubes. Medium was aspirated from the tubes and embryos were homogenized with a motorized pellet pestle (Sigma-

Aldrich) into 500 µl of Trisure RNA extraction reagent (Bioline) supplemented with 2 µg/ml glycogen (Roche) as an RNA carrier. Five hunded microliters of Trisure and 250 µl of chloroform was added and samples were mixed thoroughly and incubated on ice for 15min. The samples were centrifuged at 12000 g for 15 min at 4 °C, and upper solution phase containing RNA was transferred into new tube. Equal volume of isoporanol was added, samples vortexed and centrifuged at 12000 g for 15 min at 4°C. The RNA was precipitated and supernatant discarded. The pellet was washed with 75% ice-cold ethanol, dried and resuspended in water. The protocol was used for sample sizes of 1-8 embryos resulting in total RNA yield of 1.2-2.4 µg/embryo.

4.8.6 Touch-response assay and digital video motion analysis

The motility of zebrafish embryos was measured using a touch-response assay (Granato et al. 1996). Dechorionated 48 hpf embryos acclimated in room temperature for at least 15 min in E3-medium were used in the assay. The tail of the embryo was gently touched by using a plastic inoculation loop. This resulted in rapid escape-response, or burst-swim, of the embryo. To quantitate the response the assays were video recorded, and the length and average speed of the burst was analyzed by ImageJ and ParticleTracker plug-in (written by Fabrice Cordelieres, Institute Curie, Orsay, France; http://rsb.info.nih.gov/ij/plugins/track/track.html). Embryos that did not respond to touch at all were discarded and not used in statistical analyses.

5 RESULTS

5.1 *Erbb4* and *Hif1α*-deficent mice have similar phenotypes in lactating mammary gland (I)

During literary searches to find novel genes associated with ErbB4 function I observed that mice specifically deficient of *Erbb4* (Jones et al. 1999; Long et al. 2003) and *Hif1α* (Seagroves et al. 2003) in their mammary glands had highly similar phenotypes during lactation. In both mice, the lobuloalveoli were condensed and production of milk proteins was reduced, indicating that secretory differentiation of mammary gland epithelium was affected. These observations suggested that some kind of interplay between ErbB4 and HIF-1α might exist.

5.2 ErbB4 and HIF-1α target genes are co-expressed in humans (I)

To further study the potential interplay of ErbB4 and HIF-1α an *in silico* gene expression analysis was carried out. *In silico* analysis (Kilpinen et al. 2008) of mRNA expression in 975 normal and 3577 cancer tissue samples revealed that expression of *ERBB4* and *NRG1* had clear positive association with expression of surrogate markers of HIF-1α activity, eryhtropoetin (*EPO)*, carbonic anhydrase IX (*CAIX)* and glucose transporter 1 (*GLUT1*) (I, Fig. 2, Table S1 and S2). This association was observed in both normal and malignant tissues. In normal tissue types, a statistically significant positive association was observed 32 tissue types and only one negative association was observed between *ERBB4* or *NRG1* and *EPO*, *GLUT1* or *CAIX* ($p<0.001$). Similar situation was observed for malignant tissues where 37 positive and 2 negative statistically significant associations were found ($p<0.001$). Statistically significant associations between the expression of *ERBB4* and *EPO* ($p=0.002$), *CAIX* ($p=0.003$) or *GLUT1* ($p<0.001$) implied that these genes are indeed co-expressed in human tissues in vivo. Similarly, co-expression of *NRG1* and *EPO* ($p<0.001$), *CAIX* ($p=0.002$) or *GLUT1* ($p=0.002$) was observed.

5.3 ErbB4 promotes HIF-1α activity (I)

The similarity of phenotypes in *Erbb4* and *Hif1α*-deficient mice, and clear association of the expression of ERBB4 with the expression of HIF-1α target genes, supported a hypothesis that these two signaling pathways were connected. To address this hypothesis, series of in vitro experiments were carried out. Indeed, ErbB4 activity increased transcription of HIF-1α target genes EPO, GLUT1, vascular endothelial growth factor A (VEGF-A) and phosphoglycerate kinase 1 (PGK1) (I, Fig 1B). Moreover, ErbB4 and NRG-1 increased HIF-1α transcriptional activity in luciferase reporter assays (I, Fig. 4A, B and G). Only JM-a isoforms of ErbB4 that are able to undergo γ-secretase mediated RIP, accumulated HIF-1α protein and induced HIF-1α activity (I, Fig. 4C and D). Moreover, treatment of cells with GSI IX, a γ

-secretase inhibitor, inhibited ErbB4-mediated HIF-1α accumulation and HIF-activity (I, Fig. 4E and F).

5.4 Physical interaction between ErbB4 and HIF-1α (I)

The observation that induction of HIF-1α protein expression and transcriptional activity was dependent on the cleavage of ErbB4 raised the possibility that ErbB4 and HIF-1α could directly interact in the nucleus. Indeed, physical interaction between ErbB4 and HIF-1α was observed in co-immunoprecipitation (I, Fig. 5D and S4B), GST-pull down (I, Fig. 5F and G) and in situ proximity ligation (PLA) assays (I, Fig. 5B and C). GST pull down experiments using in vitro translated and bacterially expressed recombinant ErbB4 and HIF-1α suggested that the physical interaction between ErbB4 and HIF-1α was direct, and did not need any scaffold protein (I, Fig. S4A). GST-pull down analysis of in vitro translated deletion constructs of HIF-1α and ErbB4 suggested that residues of 1-343 of HIF-1α and residues 676-996 of ErbB4 were necessary for the physical interaction between HIF-1α and ErbB4 (I, Fig. 4F and G). Moreover, using PLA assay, this interaction was observed in the nucleus of MCF-7 cells (I, Fig. 5B and C), where the colocalization of HIF-1α and ErbB4 was also observed (I, Fig. 5A).

5.5 ErbB4 attenuates RACK1-mediated degradation of HIF-1α (I)

To further characterize the role of ErbB4 in the regulation of HIF-1α, the mechanism of accumulation of HIF-1α by ErbB4 was investigated. Stimulation of cells with the ErbB4 ligand NRG-1 reduced HIF-1α degradation as indicated by increased HIF-1α protein levels after inhibition of protein synthesis by cycloheximide (I, Fig. 3E and F). Interestingly, this effect was neither dependent on prolyl 4-hyroxylation (I, Fig. 3G) nor the presence of pVHL (I, Fig. 3D). To find potential regulators of HIF-1α stability, I performed a thorough literary screen. Indeed, RACK1 (GNB2L1) had been characterized as a protein that regulated proteasomal degradation of HIF-1α in an oxygen-independent manner (Liu et al. 2007). As the deletion mapping of interaction between ErbB4 and HIF-1α revealed a similar binding region for ErbB4 and RACK1 within the HIF-1α protein (I, Fig. 5G and (Liu et al. 2007)), RACK1 represented a strong candidate for being involved in the ErbB4-mediated regulation of HIF-1α. RNA interference of RACK1 increased basal level of HIF-1α, and reduced the relative effect of NRG-1 in accumulating HIF-1α (I, Fig. 6A). Moreover, stimulation of cells with NRG-1 led to reduced binding of HIF-1α and RACK1 (I, Fig. 6B and C). Taken together, these results imply that ErbB4 stabilizes HIF-1α by interfering with RACK1-HIF-1α interaction and thus reduces ubiquitination and proteasomal degradation of HIF-1α.

5.6 HIF-1α promotes accumulation of ErbB4 protein (II)

To further assess the interplay of ErbB4 and HIF-1α we turned our attention to the HIF-1α deficient mice. Interestingly, mice deficient for HIF-1α showed reduced ErbB4 expression

in their lactating mammary glands (II, Fig 1). RNA interference of HIF-1α also resulted in reduced levels of ErbB4 in T47D breast cancer cells (II, Fig. 2B) and HEK293 human embryonic kidney cells (II, Fig. 3A). Consistently, increasing HIF-1α levels by stabilization of HIF-1α by hypoxia mimicking compounds cobalt chloride and dimethyloxalyl glycine (DMOG) , as well as, transfection of HIF-encoding plasmids, resulted in increased protein levels of ErbB4 (Fig. 2A , 2B and 3A). This mechanism was associated with reduced degradation of ErbB4, as after cobalt chloride treatment ErbB4 was degraded less rapidly after blocking protein synthesis by cycloheximide treatment (II, Fig 3D). Accumulation of ErbB4 also resulted in prolonged ErbB4 phosphorylation by NRG-1 and increased activity of ErbB4 to induce transcription from β-casein promoter construct in a luciferase assay (Fig. 4A and B).

5.7 ErbB4 and HIF-1α interplay enhances differentiation of mammary epithelial cells (II)

An in vitro model of ErbB4-mediated mammary gland differentiation (3D matrigel cultures of MDA-MB-468 mammary adenocarcinoma cells (Tvorogov et al. 2009)) was used to study functional interplay of ErbB4 and HIF-1α in vitro. In this model, the differentiation is seen by formation of organized ball-like structures called acini, whereas the undifferentiated cells form disorganized colonies (II, Fig 4C). In the studies, chemical induction of HIF-1α (DMOG) increased ErbB4-mediated differentiation of MDA-MB-468 cells in vitro by 51% (II, Fig 4D). Consistently, targeting endogenous HIF-1α by siRNAs inhibited ErbB4-mediated differentiation of MDA-MB-468 cells by 62% (II, Fig 4E).

5.8 HIF signaling regulates bidirectional signaling of the ErbB4 ligand NRG-1 (III)

As HIFs are main regulators of angiogenic responses both during development and disease (Pouysségur et al. 2006), we analyzed the role and function of ErbB signaling in endothelial cells. In primary human vein endothelial cells isolated from umbilical cord (HUVEC), high-levels of NRG-1 was observed to be expressed in membrane bound form (III, Fig. 1A and B). ErbB1 and ErbB2 were expressed but ErbB3 and Erbb4 were not expressed in HUVECs (III, Fig. 3A and B). A soluble intracellular fragment of NRG-1 was generated in response to binding of ErbB4 ECD to membrane bound NRG-1. The ICD of NRG-1 was observed in the nucleus of endothelial cells (III, Fig. 2B and C). Interestingly, the mechanism of NRG-1 cleavage occurred in a similar manner to the regulated intramembranous proteolysis of ErbB4 and Notch. The membrane bound domain of NRG-1 was accumulated by γ-secretase inhibitor and the cleavage process was enhanced by phorbol 13-myristate 12-acetate (PMA) (III, Fig. 2A).

The hypoxia-mimick cobalt chloride and hypoxia increased NRG-1 protein level indicating potentiated NRG-1 signaling under these conditions (III, Fig. 2A and data not shown, respectively). The effect of bidirectional NRG-1 signaling was analyzed by measuring

chemotactic migration of endothelial cells. Interestingly, induction of NRG-1 ICD cleavage suppressed endothelial migration, and inhibition of ICD release by using γ-secretase inhibitor restored migration back to control level (III, Fig. 2D).

Consistent with the observation of absence of NRG receptors, addition of NRG-1 did not increase proliferation, migration or differentiation of HUVECs in vitro (III, Fig.4 A-C). However, NRG-1 induced angiogenesis in vivo in the mouse corneal micropocket angiogenesis assay (III, Fig. 5) and in the chicken chorioallantoic membrane (CAM) assay (III, Fig. 7D). NRG-1 induced VEGF-A production of HACAT cells (III, Fig. 6) and VEGFR inhibition by SU1498 in CAM assay inhibited pro-angiogenic effect of NRG-1 (III, Fig. 7D). This indicated that NRG-1 induces angiogenesis in vivo by increasing VEGF-A production in paracrine manner.

5.9 The expression of CYT-1 isoform of ErbB4 is associated with poor prognosis in ovarian cancer (IV)

Functions of ErbB4 had been well studied in breast cancer, but only few studies on samples of ovarian cancer had been conducted and the role of ErbB4 in this disease was largely unknown. To analyze the potential role of ErbB4 in serous ovarian cancer, a tissue microarray analysis of ErbB4 protein (n=482) and real-time RT-PCR analysis of ErbB4 isoforms (n=198) were carried out. 90% of samples were detected positive for an intracellular epitope of ErbB4 protein by immunohistochemistry and strong expression was observed in 76% of cases (IV, Fig. 1 and Table 1). Total immunoreactivity of ErbB4 did not associate with any of the clinico-pathological markers used (IV, Fig. 1). Cytoplasmic expression of ErbB4 was associated with low grade and stage (IV, Table 1), but was not significantly associated with survival (p=0.11, data not shown).

When real-time RT-PCR analysis of the samples was conducted, it was observed that ErbB4 was expressed in 87% of samples (IV, Fig. 2B). Expression of JM-a isoform did not yield any significant associations and JM-b variants were not expressed in the sample material (IV, Fig. 2B). Interestingly, expression of the CYT-1 isoform was associated with poor survival of patients (p=0.0028, n=121), whereas expression of CYT-2 was not (IV, Fig. 2C). When the CYT-1:CYT-2 ratio was used in statistical analyses, an even stronger association with poor survival was observed (p<0.0001, n=113) (IV, Fig. 2C). Moreover, in multivariate analysis of survival performed by using Cox proportional hazards model, CYT-1 was identified as an independent prognostic indicator (IV, Hazard ratio 1.79, p= 0.021) in serous ovarian cancer.

5.10 ErbB4 CYT-1 promotes anchorage-independent growth of ovarian cancer cells in a PI3K dependent mechanism (IV)

To solve the apparent controversy between immunohistochemical and RT-PCR analysis of ErbB4 in serous ovarian cancer, a series of in vitro experiments were performed. ErbB4 variants were retrovirally introduced into naturally ErbB4-positive OVCAR-3 and ErbB4-negative SKOV-3 ovarian cancer cell lines (IV, Fig. 3B and C), and these cells were analyzed

in soft agar anchorage-independent growth assay to assess their transformed phenotype. ErbB4 JM-a CYT-1 isoform was able to increase growth of OVCAR-3 and SKOV-3 cells in soft agar, whereas JM-a CYT-2 variant was not (IV, Fig. 3D and E) suggesting that CYT-1 overexpression promoted transformation. CYT-1 isoforms possess a binding site for a subunit of PI3K and hence can directly activate PI3K, while CYT-2 isoforms cannot (Elenius et al. 1999). The signaling mechanism of the ErbB4 JM-a CYT-1 variant was pharmacologically analyzed by using chemical ErbB and PI3K inhibitors. Indeed, the increased growth in soft agar induced by CYT-1 was blocked by both ErbB kinase inhibitor and PI3K inhibitor (IV, Fig 4A). These results imply that ErbB4 JM-a CYT-1 can act as tumor growth promoting factor in ovarian cancer, and that this effect is dependent on ErbB kinase and PI3K activity.

5.11 Zebrafish model of ErbB4 signaling (V)

As expression of ErbB4 and HIF-1α target genes was associated in many human tissues, I hypothesized that interplay of ErbB4 and HIF-1α could be needed for development of several other tissues besides mammary gland. To explore the biology of ErbB4, I established a zebrafish model. Zebrafish genome harbors two *ERBB4* homologues, *erbb4a* and *erbb4b*. The *erbb4a* gene was the primary gene expressed during organogenesis, whereas *erbb4b* was mainly expressed during very early development (V, Fig 1A). Thus the further analyses were focused on *erbb4a*.

Activity of zebrafish ErbB4 was reduced by using the ErbB kinase inhibitor AG1478 (50% reduction in ErbB4 phosphorylation) and by blocking translation of ErbB4 protein by using anti-sense morpholino RNA interference oligos (99% reduction in ErbB4 protein expression) (V, Fig. 2A and 2E). Morpholino oligos were designed to block translation of mRNA into protein, an approach previously shown to be highly effective in the zebrafish model (Nasevicius and Ekker 2000).

Knock-down or pharmacological inhibition of the zebrafish homologue of *ERBB4*, caused defects in the developing skeletal and cardiac muscles. Slow-type muscle fibers were more condensed as, although they were thinner (V, Fig. 4A and B), fibers contained similar levels of slow-type myosin heavy chain protein as control fibers (V, Fig. 6C). Electron microscopic analysis of embryos revealed signs of myofibril degeneration in the *erbb4a* morphants (V, Fig. 5).

Zebrafish embryos exhibit an early escape response when touched gently on the tail already at day 2 of development (Naganawa and Hirata 2011), although actual swimming capability is achieved on day 5 of development when swim bladder inflates. To assess functional consequences of *erbb4a* knock-down, touch-response assays were performed. The speed and distance during early escape response was measured and quantified using video recordings. Interestingly, embryos treated with the ErbB kinase inhibitor or the *erbb4a* targeting morpholino showed both reduced average speed and distance of burst swimming (V, Fig. 2A-D). Swimming behavior of *erbb4a* morphants co-injected with *ERBB4* mRNA was

normal indicating that human *ERBB4* can functionally replace zebrafish *erbb4a*, and that defects associated with *erbb4a* morpholino injection were indeed caused by deficiency of *erbb4a* (V, Fig. 3A-D). Moreover, mosaic expression of human *ERBB4* under a muscle-specific promoter partially restored swimming ability of *erbb4a* morphants (V, Fig. 7B-D), indicating that *erbb4a* expression in the muscle cells was sufficient for restoring proper swimming ability.

6 DISCUSSION

6.1 Cross-talk between HIF-1α and ErbB signaling pathways

ErbB4 was found to exhibit a close interplay with HIF-1α. This interplay may function bidirectionally as ErbB4 can induce HIF-1α, but HIF-1α can also induce ErbB4 signaling. These observations suggest that ErbB4 and HIF may form a positive feed-back loop (Figure 6). In this type of a signaling pathway, the net outcome is a sharpened and intensified response of both pathways to activating stimulus (Alon 2007; Avraham and Yarden 2011). The conceptual model suggests that once the signaling loop is activated by an ErbB4 ligand or HIF-activation, the activation is more rapid, and intense and longer as compared to signaling without such a feed-back loop. Moreover, NRG-1 signaling may be induced by HIF-activation by cobalt chloride or hypoxia, in the endothelial cells. Hypoxia/HIF regulation of other ErbB4 ligands , such as HB-EGF (Xia et al. 2003; Munk et al. 2012) and NRG-2 (Munk et al. 2012), has also been described. These ligands may also potentially participate in the ErbB4-HIF cross-talk.

However, as HIF seems to reside both upstream and downstream of ErbB4 in the signaling networks, it is possible that both modes of signaling exist in vivo. To date several independent reports of either HIF regulation of the ErbB pathway or ErbB regulation of the HIF pathway exists (Zhong et al. 2000; Laughner et al. 2001; Munk et al. 2012). The full understanding of the effects of ErbB-HIF cross-talk in both pathways may require detailed kinetic and quantitative analysis of all involved proteins followed by mathematical modeling.

Currently it is not known if cross-talk of the ErbB and HIF pathways is a fundamental property of these pathways, or merely a fine tuning mechanism existing in mammals. In support of ErbB4-HIF cross-talk also in other organisms, knock-down of zebrafish homologue of HIF-1α (HIF1ab) by HIF1ab targeting translation blocking morpholino oligos resulted in reduction of ErbB4 level and knock-down of ErbB4 resulted in reduced amount of HIF-1α (Paatero et al, unpublished observations). These data imply that reciprocal regulation of ErbB4 and HIF-1α may also exist in zebrafish.

As both ErbB receptor and HIF genes are co-expressed in almost all multicellular animals (Stein and Staros 2000; Srivastava et al. 2010; Rytkonen et al. 2011), it is possible that the cross-talk between these signaling pathways has evolved early during evolution of the animals. Nematode *C. elegans* has only one ErbB receptor (*let-23*), one ErbB ligand (*lin-3*), and one HIF (*hif*) gene, suggesting that it would be a good candidate model for future research to elucidate the question of conservation of potential HIF-ErbB cross-talk across different phyla.

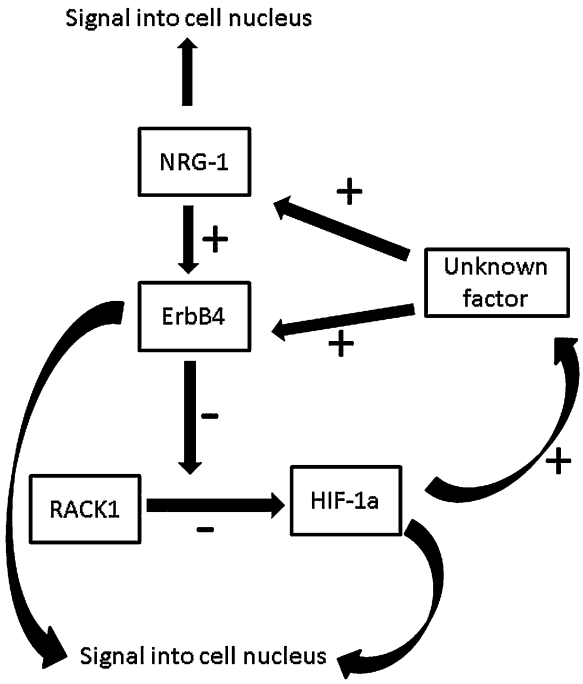

Figure 6. Schematic figure of ErbB4-HIF-1α cross-talk. Arrow denotes for a functional interaction, plus sign (+) is for positive regulation and minus sign (-) for negative regulation.

6.2 Molecular insights into regulation of ErbB4 and HIF

My studies have uncovered the molecular mechanism by which ErbB4 regulates HIF-1α. ErbB4 reduced proteasomal degradation of HIF-1α in a process that was dependent on ErbB4 cleavage, proteasome function, kinase activity of ErbB4, binding of ErbB4 to HIF-1α, and the presence of RACK1. On this basis I postulate that ErbB4 increased HIF-protein stability by interfering with binding of RACK1 to HIF-1α. This mechanism is distinct from the earlier observation that the ErbB receptors are capable of activating HIF-1α via the PI3K pathway (Zhong et al. 2000; Laughner et al. 2001) and most probably also via the MAPK pathway (Richard et al. 1999; Olayioye et al. 2001). These pathways, however, are generic signaling pathways activating numerous genes and proteins.

The molecular mechanism by which HIF-1α regulates ErbB4 remains more elusive. ErbB4 protein degradation was reduced by induction of HIF-1α but long over-night incubations were needed for this effect. This implies that some HIF-1α regulated genes may be responsible for the reduced degradation of ErbB4. As HIF almost exclusively activates transcription of genes (Schödel et al. 2011), it seems plausible that this unknown factor is a HIF-1α-induced repressor of ErbB4 degradation.

6.3 Bidirectional signaling of NRG-1 and ErbB4

Endothelial cells are a vital part of the organismal response to hypoxia, as HIFs induce production of many angiogenic factors. Therefore, we analyzed the role of ErbB signaling in endothelial cells. Interestingly, the ErbB4 ligand NRG-1 was expressed and processed by RIP in endothelial cells. In the process, soluble ICD was generated from NRG-1, and this ICD was capable of translocating into the nucleus. Interestingly, the generation of nuclear ICD reduced migration of endothelial cells, whereas paracrine signaling via the released soluble ectodomain of NRG-1 did not. These observations suggest that the generation of NRG-1 ICD produces a signal within the endothelial cell. In neurons, generation of NRG-1 ICD has been associated with neuronal survival (Bao et al. 2003).

In the RIP process active form of NRG-1 ECD is also released into the extracellular space. In vivo in the mouse corneal micropocket angiogenesis assay and in the chicken chorioallantoic membrane assay, NRG-1 ectodomain induced angiogenesis. Moreover, NRG-1 ectodomain induced VEGF-A production in the HACAT (III, Fig.6) and MCF-7 (I, Fig. 1B) cells indicating that endothelial NRG-1 may transmit angiogenic signals in paracrine manner. This suggests that RIP-mechanism may regulate both retrograde signaling of NRG-1 and also paracrine activation of NRG-1 receptors.

Interestingly, also other ErbB4 ligands BTC and HB-EGF have been observed to be processed by RIP. The BTC-ICD potentially transmits antiproliferative signals (Stoeck et al. 2010) whereas HB-EGF-ICD is associated with invasiveness of gastric cancer cells (Shimura et al. 2012). Similarly, bidirectional signaling mediated by RIP has been observed with for example Dll1/NOTCH (De Strooper et al. 1999; Six et al. 2003) and within the receptor tyrosine kinase family with Ephrins and their receptors (Tomita et al. 2006; Haapasalo and Kovacs 2011). Taken together, bidirectional signaling of membrane-bound ligands and their receptors is clearly a phenomenon extending beyond the RTK family and their ligands, and is potentially regulating many yet unidentified processes in development and disease.

6.4 Developmental implications of ErbB-HIF cross-talk

Analysis of normal tissues by in silico transcriptomics implied that ErbB-HIF co-regulation may also exist in normal tissues. Mammary gland specific deletion of *Erbb4* in mice showed reduced HIF signaling and mammary gland specific deletion of *Hif1a* reduced ErbB4 protein levels. This supports the hypothesis that ErbB4 and HIF pathways interact during normal development of the mammary gland. However, NRG-1/ErbB4 and HIF co-expression was identified also in several other tissues such as heart, muscle, pancreas, prostate and kidney, evoking hypothesis that co-regulation may occur also in these tissues. As knock-down of *erbb4a* in zebrafish embryos reduced hif1ab protein level (Paatero et al, unpublished data), it is plausible that ErbB4-HIF-1α cross-talk occurs during development of other tissues besides the mammary gland.

6.5 Clinical implications of ErbB HIF regulation

The modulation of ErbB-HIF cross-talk could provide an opportunity to treat cancer. Both ErbB and HIF pathways are highly active in many solid tumors (Harris 2002; Holbro and Hynes 2004), implying that the cross-talk of these pathways might have a role in the biology of the tumors. The in silico transciptomics analysis indicated that *ERBB4*/*NRG1* and HIF-1α-regulated genes are co-expressed in many different cancer types. Moreover, ErbB4 overexpression in xenograft mouse tumor model increased HIF-signaling (Paatero et al, unpublished data). Several potential ways to inhibit ErbB-HIF cross-talk could be designed. Inhibition of ErbB receptors by kinase inhibitors and therapeutic antibodies already in use in the clinic could be used to reduce HIF-signaling in tumors. The interaction of ErbB4 and HIF-1α could also be targeted, although inhibition of intracellular protein-protein interactions is currently technically challenging. Increasing ErbB activity by growth factor administration could increase HIF signaling and yield therapeutic effects for example in ischemic conditions.

6.6 Role of ErbB4 in biology of ovarian cancer

Despite of some earlier studies, the role of ErbB4 in the ovarian cancer has remained unclear. Increased total ErbB4 expression has been observed with increased stage of ovarian cancer (Gilmour et al. 2001; Steffensen et al. 2008; Pejovic et al. 2009). However no significant associations with survival have been found (Pejovic et al. 2009). Our analyses were focused on samples of serous ovarian cancer. Ovarian cancer can be divided into four subgroups and serous ovarian cancer comprises of about 40% of ovarian cancers (Kobel et al. 2008). Serous ovarian cancer originates from epithelial cells of fallopian tube (Bowtell 2010). Despite decades of research, the survival of patients with ovarian cancer has not prolonged since the 1970s (Vaughan et al. 2011).

Here I show that a specific isoform of ErbB4 CYT-1 was selectively associated with poor survival, and it increased the anchorage-independent growth of ovarian cancer cells. This indicates a role for a specific ErbB4 isoform in the biology of the serous ovarian cancer and may partially explain why significant correlations between total ErbB4 protein and survival in ovarian cancer had not been observed in earlier studies.

6.7 Targeting ErbB4 to treat ovarian cancer

My results support the idea that targeting of ErbB4 may provide a new approach to develop therapeutics for serous ovarian cancer. Targeting of other ErbB receptors such as ErbB1 and ErbB2 has shown a modest clinical effect in treatment of ovarian cancer (Gordon et al. 2006; Palayekar and Herzog 2007; Makhija et al. 2010). As ErbB receptors extensively heterodimerize and thus form a diverse signaling network (Yarden and Pines 2012), it may be necessary to block multiple, if not even all four, ErbB receptors to achieve a robust therapeutic response in the clinic. As ovarian cancer frequently harbors genetic lesions

activating signaling pathway components down-stream of the ErbB receptors (Network CGAR 2011), inhibiting these downstream targets, such as the PI3K-pathway, together with ErbB receptors may be necessary to achieve significant responses.

As CYT-1 and CYT-2 isoforms of ErbB4 demonstrated different role in serous ovarian cancer, it might be beneficial to selectively target the CYT-1 isoform. However, neither kinase inhibitors nor therapeutic antibodies can differentiate between the two ICDs. Therefore, more advanced approaches are needed to for selective targeting of CYT-1. Such potential methods would be RNA interference (Burnett et al. 2011), genetic inactivation of CYT-1 exon within the tumor by targeted nucleases (Kim et al. 2009) or homologous recombination (Russell and Hirata 1998), or by expressing CYT-1 targeting single-chain intracellular antibodies, intrabodies (Alvarez et al. 2000). However, all of these methodologies are still at the experimental stage.

6.8 Utilizing ErbB4 as a prognostic marker in ovarian cancer

Based on the results of this thesis, ErbB4 CYT-1 could be evaluated as a prognostic marker to predict the outcome of serous ovarian cancer. Both CYT-1 and the ratio of the expression levels of CYT-1 and CYT-2 were strongly associated with poor survival (IV, Fig. 2C). However, independent studies with additional patient cohorts are needed to confirm this association and to make it a clinically utilizable marker. As single markers often do not provide strong enough associations to make firm prognostic predictions (Zhang et al. 2007), the ErbB4 CYT-1, or CYT-1/CYT-2 ratio, could be combined with other molecular markers to strengthen the prognostic value and clinical usability. Currently, the prognostic significance of expression of any ErbB receptor in ovarian cancer is unclear, as many conflicting studies have been published (Lafky et al. 2008). This situation may be resolved by using larger patient cohorts and quantitative methods, such as real-time RT-PCR, in order to obtain robust statistical associations.

6.9 Modeling of ErbB4 signaling in zebrafish

To gain deeper insight into the role of ErbB4 during development, a zebrafish embryo model of ErbB4 signaling was developed. Treatment with morpholino antisense oligonucleotides or with an ErbB4 kinase inhibitor resulted in effective inhibition of ErbB4 function in the developing zebrafish embryos. Moreover, rescue experiments with human ErbB4 encoding mRNA and transgenes showed both specificity of the morpholino treatment and functional redundancy between human and zebrafish ErbB4.

Using the morpholino antisense oligonucleotides and the ErbB4 kinase inhibitor, ErbB4 (*erbb4a*) was found necessary for normal development of skeletal muscles in developing zebrafish embryos. In skeletal muscle specific conditional knockout mice deficient of both *Erbb2* and *Erbb4* (*HSA-Cre+/-;Erbb4*$^{flox/flox}$*;Erbb2*$^{flox/flox}$) muscles have been reported to be normal and no strong muscle phenotype was reported (Escher et al. 2005), although some

defects in acethylcholine receptor recycling and signal transmission in the neuromuscular junction may occur (Schmidt et al. 2011). Nevertheless, these mice have significantly reduced body weight consistent with a defect in muscle growth (Escher et al. 2005). Heterozygous *Erbb4* null (*Erbb4* $^{flox/-}$) mice also showed reduced body weight during post-natal period and homozygous nervous-system specific *Erbb4* null mice (*Nestin-CRE+/-; Erbb4* $^{flox/-}$) reduced muscle strength and reduced physical activity (Golub et al. 2004). As these mice show multiple neurological defects (Stefansson et al. 2002; Golub et al. 2004), the direct effects on the muscle development are difficult to interpret from these studies.

Pathological relevance of the NRG-1/ErbB4 signaling in the human skeletal muscle is unclear. Both NRG-1 and ErbB4 are upregulated in the rat skeletal muscle after muscle denervation (Nicolino et al. 2009). Moreover, in a mouse model of muscular dystrophy (mdx mouse) NRG-1 injections ameliorate the muscular loss and dysfunction (Krag et al. 2004). Observations made with *erbb4a* morphant zebrafish suggest a critical role for ErbB4 in the development of skeletal muscles and imply that the therapeutic effect of NRG-1 in the mouse model of muscular dystrophy may be transduced via ErbB4.

When compared to results of a large zebrafish mutagenesis screen, *erbb4a* knock-down phenotype resembles the phenotype of mutants having reduced motility, reduced muscle striation, and heart defects (group A3) or mutants having reduced striation and muscle degeneration (group A4) (Granato et al. 1996). Genes affected in the mutant lines *slop*, *jam* and *slinky* in the A3 category have not been isolated. Mutations in the A4 category are located or linked to *dystrophin (dmd, sapje)* (Bassett et al. 2003), *laminin B2 (lamb2, softy)* (Jacoby et al. 2009) or *titin (tttnb, runzel*) (Steffen et al. 2007) genes. These genes are all associated with different subtypes of muscular dystrophy in humans (Steffen et al. 2007; Lin 2012). Interestingly, ultrastructural analysis by electron microscopy have revealed that the *erbb4a* morphants have similar degenerative changes in sarcomeres as do embryos with *titin* mutation (Steffen et al. 2007).

Skeletal muscle differentiation and development has been well-characterized in zebrafish. Somitogenesis begins around 10 hpf and ends around 24 hpf, when all the embryonic somites have been formed (Kimmel et al. 1995). By 48 hpf the skeletal muscles have been innervated and are able to contract. Slow-type muscle cells develop earlier, and characteristic coiling behavior mediated by slow muscle contraction can be observed already at 24 hpf (Naganawa and Hirata 2011). Fast-type muscle cells develop later and burst swimming behavior characteristic to fast muscle cells can be first observed around 48 hpf (Naganawa and Hirata 2011). In the burst swim assays inhibition of ErbB4 either by morpholino oligos or with the kinase inhibitor reduced burst swimming speed and length, suggesting defects in functioning of fast-type muscle cells.

The zebrafish model of ErbB4 signaling could reveal other new phenotypes associated with ErbB4 function. As ErbB4 is strongly expressed in the brain of the zebrafish embryo, it seems plausible that future research will find neurological phenotypes of ErbB4-deficient embryos. The zebrafish model of ErbB4 signaling could also be used as a tool to functional

analysis of the increasing number of ErbB4 genetic variations and mutations associated with schizophrenia (Nicodemus et al. 2006; Silberberg et al. 2006; Greenwood et al. 2012) and cancer (Soung et al. 2006a; Ding et al. 2008; Prickett et al. 2009; Dutton-Regester et al. 2012).

7 CONCLUSIONS

In this study, functions of NRG-1 and ErbB4 were studied in vitro and in vivo. The following key conclusions can be made.

1) NRG-1/ErbB4 signaling stabilizes HIF-1α and increases HIF-1 signaling.

2) Expression of NRG-1/ErbB4 is associated with increased HIF-1 activity in vivo in mice and in humans.

3) Reciprocally, HIF-1 activity can increase signaling via NRG-1/ErbB4 pathway by increasing NRG-1 and ErbB4 protein levels.

4) NRG-1 signal is transmitted both within the NRG-1 expressing endothelial cells as well as to adjacent cell expressing ErbB receptors in paracrine manner.

5) Expression of the specific ErbB4 isoform CYT-1 is associated with poor prognosis in serous ovarian cancer.

6) ErbB4 is needed for normal development of skeletal muscles in zebrafish embryos.

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aukeaa tuntematon.

– uusi erämaa,

taas kutsuu kulkijaansa.

9 REFERENCES

Alon U. 2007. *An introduction to systems biology: design principles of biological circuits*. Chapman & Hall/CRC, Boca Raton, FL.

Alvarez RD, Barnes MN, Gomez-Navarro J, Wang M, Strong TV, Arafat W, Arani RB, Johnson MR, Roberts BL, Siegal GP et al. 2000. A cancer gene therapy approach utilizing an anti-erbB-2 single-chain antibody-encoding adenovirus (AD21): a phase I trial. *Clin Cancer Res* **6**: 3081-3087.

Anderson JL, Rodriguez Mari A, Braasch I, Amores A, Hohenlohe P, Batzel P, Postlethwait JH. 2012. Multiple sex-associated regions and a putative sex chromosome in zebrafish revealed by RAD mapping and population genomics. *PLoS One* **7**: e40701.

Andersson U, Guo D, Malmer B, Bergenheim AT, Brannstrom T, Hedman H, Henriksson R. 2004. Epidermal growth factor receptor family (EGFR, ErbB2-4) in gliomas and meningiomas. *Acta Neuropathol* **108**: 135-142.

Ang SO, Chen H, Gordeuk VR, Sergueeva AI, Polyakova LA, Miasnikova GY, Kralovics R, Stockton DW, Prchal JT. 2002a. Endemic polycythemia in Russia: mutation in the VHL gene. *Blood Cells Mol Dis* **28**: 57-62.

Ang SO, Chen H, Hirota K, Gordeuk VR, Jelinek J, Guan Y, Liu E, Sergueeva AI, Miasnikova GY, Mole D et al. 2002b. Disruption of oxygen homeostasis underlies congenital Chuvash polycythemia. *Nat Genet* **32**: 614-621.

Avraham R, Yarden Y. 2011. Feedback regulation of EGFR signalling: decision making by early and delayed loops. *Nat Rev Mol Cell Biol* **12**: 104-117.

Bae SH, Jeong JW, Park JA, Kim SH, Bae MK, Choi SJ, Kim KW. 2004. Sumoylation increases HIF-1alpha stability and its transcriptional activity. *Biochem Biophys Res Commun* **324**: 394-400.

Baiocchi G, Lopes A, Coudry RA, Rossi BM, Soares FA, Aguiar S, Guimaraes GC, Ferreira FO, Nakagawa WT. 2009. ErbB family immunohistochemical expression in colorectal cancer patients with higher risk of recurrence after radical surgery. *Int J Colorectal Dis* **24**: 1059-1068.

Bao J, Wolpowitz D, Role LW, Talmage DA. 2003. Back signaling by the Nrg-1 intracellular domain. *J Cell Biol* **161**: 1133-1141.

Bassett DI, Bryson-Richardson RJ, Daggett DF, Gautier P, Keenan DG, Currie PD. 2003. Dystrophin is required for the formation of stable muscle attachments in the zebrafish embryo. *Development* **130**: 5851-5860.

Baulida J, Kraus MH, Alimandi M, Di Fiore PP, Carpenter G. 1996. All ErbB receptors other than the epidermal growth factor receptor are endocytosis impaired. *J Biol Chem* **271**: 5251-5257.

Bergeron M, Gidday JM, Yu AY, Semenza GL, Ferriero DM, Sharp FR. 2000. Role of hypoxia-inducible factor-1 in hypoxia-induced ischemic tolerance in neonatal rat brain. *Ann Neurol* **48**: 285-296.

Bersell K, Arab S, Haring B, Kühn B. 2009. Neuregulin1/ErbB4 signaling induces cardiomyocyte proliferation and repair of heart injury. *Cell* **138**: 257-270.

Berta MA, Mazure N, Hattab M, Pouysségur J, Brahimi-Horn MC. 2007. SUMOylation of hypoxia-inducible factor-1alpha reduces its transcriptional activity. *Biochem Biophys Res Commun* **360**: 646-652.

Blume-Jensen P, Hunter T. 2001. Oncogenic kinase signalling. *Nature* **411**: 355-365.

Bouyain S, Longo PA, Li S, Ferguson KM, Leahy DJ. 2005. The extracellular region of ErbB4 adopts a tethered conformation in the absence of ligand. *Proc Natl Acad Sci U S A* **102**: 15024-15029.

Bowtell DD. 2010. The genesis and evolution of high-grade serous ovarian cancer. *Nat Rev Cancer* **10**: 803-808.

Brinkmann BG, Agarwal A, Sereda MW, Garratt AN, Muller T, Wende H, Stassart RM, Nawaz S, Humml C, Velanac V et al. 2008. Neuregulin-1/ErbB signaling serves distinct functions in myelination of the peripheral and central nervous system. *Neuron* **59**: 581-595.

Britsch S, Li L, Kirchhoff S, Theuring F, Brinkmann V, Birchmeier C, Riethmacher D. 1998. The ErbB2 and ErbB3 receptors and their ligand, neuregulin-1, are essential for devel-

opment of the sympathetic nervous system. *Genes Dev* **12**: 1825-1836.

Bruick RK, McKnight SL. 2001. A conserved family of prolyl-4-hydroxylases that modify HIF. *Science* **294**: 1337-1340.

Budi EH, Patterson LB, Parichy DM. 2008. Embryonic requirements for ErbB signaling in neural crest development and adult pigment pattern formation. *Development* **135**: 2603-2614.

Burnett JC, Rossi JJ, Tiemann K. 2011. Current progress of siRNA/shRNA therapeutics in clinical trials. *Biotechnol J* **6**: 1130-1146.

Carbia-Nagashima A, Gerez J, Perez-Castro C, Paez-Pereda M, Silberstein S, Stalla GK, Holsboer F, Arzt E. 2007. RSUME, a small RWD-containing protein, enhances SUMO conjugation and stabilizes HIF-1alpha during hypoxia. *Cell* **131**: 309-323.

Carraway KL, 3rd. 2010. E3 ubiquitin ligases in ErbB receptor quantity control. *Semin Cell Dev Biol* **21**: 936-943.

Chan R, Hardy WR, Laing MA, Hardy SE, Muller WJ. 2002. The catalytic activity of the ErbB-2 receptor tyrosine kinase is essential for embryonic development. *Mol Cell Biol* **22**: 1073-1078.

Cho HS, Leahy DJ. 2002. Structure of the extracellular region of HER3 reveals an interdomain tether. *Science* **297**: 1330-1333.

Cohen S. 1962. Isolation of a mouse submaxillary gland protein accelerating incisor eruption and eyelid opening in the new-born animal. *J Biol Chem* **237**: 1555-1562.

Cunningham D, Humblet Y, Siena S, Khayat D, Bleiberg H, Santoro A, Bets D, Mueser M, Harstrick A, Verslype C et al. 2004. Cetuximab monotherapy and cetuximab plus irinotecan in irinotecan-refractory metastatic colorectal cancer. *N Engl J Med* **351**: 337-345.

Das PM, Thor AD, Edgerton SM, Barry SK, Chen DF, Jones FE. 2010. Reactivation of epigenetically silenced HER4/ERBB4 results in apoptosis of breast tumor cells. *Oncogene* **29**: 5214-5219.

de Alava E, Ocana A, Abad M, Montero JC, Esparis-Ogando A, Rodriguez CA, Otero AP, Hernandez T, Cruz JJ, Pandiella A. 2007. Neuregulin expression modulates clinical response to trastuzumab in patients with metastatic breast cancer. *J Clin Oncol* **25**: 2656-2663.

De Strooper B, Annaert W, Cupers P, Saftig P, Craessaerts K, Mumm JS, Schroeter EH, Schrijvers V, Wolfe MS, Ray WJ et al. 1999. A presenilin-1-dependent gamma-secretase-like protease mediates release of Notch intracellular domain. *Nature* **398**: 518-522.

Derynck R, Roberts AB, Winkler ME, Chen EY, Goeddel DV. 1984. Human transforming growth factor-alpha: precursor structure and expression in E. coli. *Cell* **38**: 287-297.

Ding L, Getz G, Wheeler DA, Mardis ER, McLellan MD, Cibulskis K, Sougnez C, Greulich H, Muzny DM, Morgan MB et al. 2008. Somatic mutations affect key pathways in lung adenocarcinoma. *Nature* **455**: 1069-1075.

Doyon Y, McCammon JM, Miller JC, Faraji F, Ngo C, Katibah GE, Amora R, Hocking TD, Zhang L, Rebar EJ et al. 2008. Heritable targeted gene disruption in zebrafish using designed zinc-finger nucleases. *Nat Biotechnol* **26**: 702-708.

Dunn M, Sinha P, Campbell R, Blackburn E, Levinson N, Rampaul R, Bates T, Humphreys S, Gullick WJ. 2004. Co-expression of neuregulins 1, 2, 3 and 4 in human breast cancer. *J Pathol* **203**: 672-680.

Dutton-Regester K, Irwin D, Hunt P, Aoude LG, Tembe V, Pupo GM, Lanagan C, Carter CD, O'Connor L, O'Rourke M et al. 2012. A high-throughput panel for identifying clinically relevant mutation profiles in melanoma. *Mol Cancer Ther* **11**: 888-897.

Eisen JS, Smith JC. 2008. Controlling morpholino experiments: don't stop making antisense. *Development* **135**: 1735-1743.

Elenius K, Choi C, Paul S, Santiestevan E, Nishi E, Klagsbrun M. 1999. Characterization of a naturally occurring ErbB4 isoform that does not bind or activate phosphatidyl inositol 3-kinase. *Oncogene* **18**: 2607-2615.

Elenius K, Corfas G, Paul S, Choi C, Rio C, Plowman G, Klagsbrun M. 1997. A novel juxtamembrane domain isoform of HER4/ErbB4. Isoform-specific tissue distribution and differential processing in response to phorbol ester. *J Biol Chem* **272**: 26761-26768.

Epstein AC, Gleadle JM, McNeill LA, Hewitson KS, O'Rourke J, Mole DR, Mukherji M, Metzen E, Wilson MI, Dhanda A et al. 2001. C. elegans EGL-9 and mammalian homologs define a family of dioxygenases that regulate HIF by prolyl hydroxylation. *Cell* **107**: 43-54.

Erickson SL, O'Shea KS, Ghaboosi N, Loverro L, Frantz G, Bauer M, Lu LH, Moore MW. 1997. ErbB3 is required for normal cerebellar and cardiac development: a comparison with ErbB2-and heregulin-deficient mice. *Development* **124**: 4999-5011.

Escher P, Lacazette E, Courtet M, Blindenbacher A, Landmann L, Bezakova G, Lloyd KC, Mueller U, Brenner HR. 2005. Synapses form in skeletal muscles lacking neuregulin receptors. *Science* **308**: 1920-1923.

Eschrich S, Yang I, Bloom G, Kwong KY, Boulware D, Cantor A, Coppola D, Kruhoffer M, Aaltonen L, Orntoft TF et al. 2005. Molecular staging for survival prediction of colorectal cancer patients. in *J Clin Oncol*, pp. 3526-3535, United States.

Falls DL. 2003. Neuregulins: functions, forms, and signaling strategies. *Exp Cell Res* **284**: 14-30.

Ferguson KM, Berger MB, Mendrola JM, Cho HS, Leahy DJ, Lemmon MA. 2003. EGF activates its receptor by removing interactions that autoinhibit ectodomain dimerization. *Mol Cell* **11**: 507-517.

Ferretti E, Di Marcotullio L, Gessi M, Mattei T, Greco A, Po A, De Smaele E, Giangaspero F, Riccardi R, Di Rocco C et al. 2006. Alternative splicing of the ErbB-4 cytoplasmic domain and its regulation by hedgehog signaling identify distinct medulloblastoma subsets. *Oncogene* **25**: 7267-7273.

Fluge O, Akslen LA, Haugen DR, Varhaug JE, Lillehaug JR. 2000. Expression of heregulins and associations with the ErbB family of tyrosine kinase receptors in papillary thyroid carcinomas. *Int J Cancer* **87**: 763-770.

Folkman J. 1971. Tumor angiogenesis: therapeutic implications. *N Engl J Med* **285**: 1182-1186.

Friedrichs F, Zugck C, Rauch GJ, Ivandic B, Weichenhan D, Müller-Bardorff M, Meder B, El Mokhtari NE, Regitz-Zagrosek V, Hetzer R et al. 2009. HBEGF, SRA1, and IK: Three cosegregating genes as determinants of cardiomyopathy. *Genome Res* **19**: 395-403.

Fry WHD, Kotelawala L, Sweeney C, III KLC. 2009. Mechanisms of ErbB receptor negative regulation and relevance in cancer. **315**: 697–706.

Gao R, Zhang J, Cheng L, Wu X, Dong W, Yang X, Li T, Liu X, Xu Y, Li X et al. 2010. A Phase II, randomized, double-blind, multicenter, based on standard therapy, placebo-controlled study of the efficacy and safety of recombinant human neuregulin-1 in patients with chronic heart failure. *J Am Coll Cardiol* **55**: 1907-1914.

García-Rivello H, Taranda J, Said M, Cabeza-Meckert P, Vila-Petroff M, Scaglione J, Ghio S, Chen J, Lai C, Laguens RP et al. 2005. Dilated cardiomyopathy in Erb-b4-deficient ventricular muscle. *Am J Physiol Heart Circ Physiol* **289**: H1153-1160.

Garratt AN, Voiculescu O, Topilko P, Charnay P, Birchmeier C. 2000. A dual role of erbB2 in myelination and in expansion of the schwann cell precursor pool. *J Cell Biol* **148**: 1035-1046.

Garrett TP, McKern NM, Lou M, Elleman TC, Adams TE, Lovrecz GO, Kofler M, Jorissen RN, Nice EC, Burgess AW et al. 2003. The crystal structure of a truncated ErbB2 ectodomain reveals an active conformation, poised to interact with other ErbB receptors. *Mol Cell* **11**: 495-505.

Garrett TP, McKern NM, Lou M, Elleman TC, Adams TE, Lovrecz GO, Zhu HJ, Walker F, Frenkel MJ, Hoyne PA et al. 2002. Crystal structure of a truncated epidermal growth factor receptor extracellular domain bound to transforming growth factor alpha. *Cell* **110**: 763-773.

Gassmann M, Casagranda F, Orioli D, Simon H, Lai C, Klein R, Lemke G. 1995. Aberrant neural and cardiac development in mice lacking the ErbB4 neuregulin receptor. *Nature* **378**: 390-394.

Gilbert SF. 2006. *Developmental biology*. Sinauer Associates, Inc. Publishers, Sunderland, Mass.

Gilbertson R, Hernan R, Pietsch T, Pinto L, Scotting P, Allibone R, Ellison D, Perry R, Pearson A, Lunec J. 2001. Novel ERBB4 juxtamembrane splice variants are frequently expressed in childhood medulloblastoma. *Genes Chromosomes Cancer* **31**: 288-294.

Gilbertson RJ, Bentley L, Hernan R, Junttila TT, Frank AJ, Haapasalo H, Connelly M, Wetmore C, Curran T, Elenius K et al. 2002. ERBB receptor signaling promotes ependymoma cell proliferation and represents a potential novel therapeutic target for this disease. *Clin Cancer Res* **8**: 3054-3064.

Gilbertson RJ, Perry RH, Kelly PJ, Pearson AD, Lunec J. 1997. Prognostic significance of HER2 and HER4 coexpression in childhood medulloblastoma. *Cancer Res* **57**: 3272-3280.

Gilmour L, Macleod K, McCaig A, Gullick W, Smyth J, Langdon S. 2001. Expression of erbB-4/HER-4 growth factor receptor isoforms in ovarian cancer. *Cancer Res* **61**: 2169-2176.

Gilmour L, Macleod K, McCaig A, Sewell J, Gullick W, Smyth J, Langdon S. 2002. Neuregulin expression, function, and signaling in human ovarian cancer cells. *Clin Cancer Res* **8**: 3933-3942.

Goishi K, Lee P, Davidson AJ, Nishi E, Zon LI, Klagsbrun M. 2003. Inhibition of zebrafish epidermal growth factor receptor activity results in cardiovascular defects. *Mech Dev* **120**: 811-822.

Golub MS, Germann SL, Lloyd KC. 2004. Behavioral characteristics of a nervous system-specific erbB4 knock-out mouse. *Behav Brain Res* **153**: 159-170.

Gordon M. 1927. The Genetics of a Viviparous Top-Minnow Platypoecilus; the Inheritance of Two Kinds of Melanophores. *Genetics* **12**: 253-283.

Gordon M, Matei D, Aghajanian C, Matulonis U, Brewer M, Fleming G, Hainsworth J, Garcia A, Pegram M, Schilder R et al. 2006. Clinical activity of pertuzumab (rhuMAb 2C4), a HER dimerization inhibitor, in advanced ovarian cancer: potential predictive relationship with tumor HER2 activation status. *J Clin Oncol* **24**: 4324-4332.

Graber HU, Friess H, Kaufmann B, Willi D, Zimmermann A, Korc M, Buchler MW. 1999. ErbB-4 mRNA expression is decreased in non-metastatic pancreatic cancer. *Int J Cancer* **84**: 24-27.

Granato M, van Eeden FJ, Schach U, Trowe T, Brand M, Furutani-Seiki M, Haffter P, Hammerschmidt M, Heisenberg CP, Jiang YJ et al. 1996. Genes controlling and mediating locomotion behavior of the zebrafish embryo and larva. *Development* **123**: 399-413.

Greenwood TA, Light GA, Swerdlow NR, Radant AD, Braff DL. 2012. Association analysis of 94 candidate genes and schizophrenia-related endophenotypes. *PLoS One* **7**: e29630.

Gu YZ, Moran SM, Hogenesch JB, Wartman L, Bradfield CA. 1998. Molecular characterization and chromosomal localization of a third alpha-class hypoxia inducible factor subunit, HIF3alpha. *Gene Expr* **7**: 205-213.

Haapasalo A, Kovacs DM. 2011. The many substrates of presenilin/gamma-secretase. *J Alzheimers Dis* **25**: 3-28.

Han SW, Kim TY, Hwang PG, Jeong S, Kim J, Choi IS, Oh DY, Kim JH, Kim DW, Chung DH et al. 2005. Predictive and prognostic impact of epidermal growth factor receptor mutation in non-small-cell lung cancer patients treated with gefitinib. *J Clin Oncol* **23**: 2493-2501.

Hanahan D, Weinberg RA. 2000. The hallmarks of cancer. *Cell* **100**: 57-70.

-. 2011. Hallmarks of cancer: the next generation. *Cell* **144**: 646-674.

Harari D, Tzahar E, Romano J, Shelly M, Pierce J, Andrews G, Yarden Y. 1999. Neuregulin-4: a novel growth factor that acts through the ErbB-4 receptor tyrosine kinase. *Oncogene* **18**: 2681-2689.

Harris A. 2002. Hypoxia--a key regulatory factor in tumour growth. *Nat Rev Cancer* **2**: 38-47.

Harris CA, Ward RL, Dobbins TA, Drew AK, Pearson S. 2011. The efficacy of HER2-targeted agents in metastatic breast cancer: a meta-analysis. *Ann Oncol* **22**: 1308-1317.

Hicke L, Dunn R. 2003. Regulation of membrane protein transport by ubiquitin and ubiquitin-binding. *Annu Rev Cell Dev Biol* **19**: 141-172.

Higashiyama S, Abraham JA, Miller J, Fiddes JC, Klagsbrun M. 1991. A heparin-binding growth factor secreted by macrophage-like cells that is related to EGF. *Science* **251**: 936-939.

Hippenmeyer S, Shneider NA, Birchmeier C, Burden SJ, Jessell TM, Arber S. 2002. A role for neuregulin1 signaling in muscle spindle differentiation. *Neuron* **36**: 1035-1049.

Hobbs SS, Coffing SL, Le AT, Cameron EM, Williams EE, Andrew M, Blommel EN, Hammer RP, Chang H, Riese DJ, 2nd. 2002. Neuregulin isoforms exhibit distinct patterns of ErbB family receptor activation. *Oncogene* **21**: 8442-8452.

Holbro T, Hynes N. 2004. ErbB receptors: directing key signaling networks throughout life. *Annu Rev Pharmacol Toxicol* **44**: 195-217.

Honjo Y, Kniss J, Eisen JS. 2008. Neuregulin-mediated ErbB3 signaling is required for formation of zebrafish dorsal root ganglion neurons. *Development* **135**: 2615-2625.

Horiuchi K, Zhou HM, Kelly K, Manova K, Blobel CP. 2005. Evaluation of the contributions of

ADAMs 9, 12, 15, 17, and 19 to heart development and ectodomain shedding of neuregulins beta1 and beta2. *Dev Biol* **283**: 459-471.

Hsu SC, Hung MC. 2007. Characterization of a novel tripartite nuclear localization sequence in the EGFR family. *J Biol Chem* **282**: 10432-10440.

Huang Y, Hickey RP, Yeh JL, Liu D, Dadak A, Young LH, Johnson RS, Giordano FJ. 2004. Cardiac myocyte-specific HIF-1alpha deletion alters vascularization, energy availability, calcium flux, and contractility in the normoxic heart. *FASEB J* **18**: 1138-1140.

Hubbard SR, Till JH. 2000. Protein tyrosine kinase structure and function. *Annu Rev Biochem* **69**: 373-398.

Hultman KA, Budi EH, Teasley DC, Gottlieb AY, Parichy DM, Johnson SL. 2009. Defects in ErbB-dependent establishment of adult melanocyte stem cells reveal independent origins for embryonic and regeneration melanocytes. *PLoS Genet* **5**: e1000544.

Hynes N, Lane H. 2005. ERBB receptors and cancer: the complexity of targeted inhibitors. *Nat Rev Cancer* **5**: 341-354.

Hynes N, MacDonald G. 2009. ErbB receptors and signaling pathways in cancer. *Curr Opin Cell Biol* **21**: 177-184.

Häussler G. 1928. Über Melanomblidungen bei Bastarden von Xiphophorus helleri und Platypoecilus maculatus var. rubra. *Klinische Wochenschrift* **7**: 1561-1562.

Icli B, Bharti A, Pentassuglia L, Peng X, Sawyer DB. 2012. ErbB4 localization to cardiac myocyte nuclei, and its role in myocyte DNA damage response. *Biochem Biophys Res Commun* **418**: 116-121.

Ivan M, Kondo K, Yang H, Kim W, Valiando J, Ohh M, Salic A, Asara J, Lane W, Kaelin WJ. 2001. HIFalpha targeted for VHL-mediated destruction by proline hydroxylation: implications for O2 sensing. *Science* **292**: 464-468.

Iyer N, Kotch L, Agani F, Leung S, Laughner E, Wenger R, Gassmann M, Gearhart J, Lawler A, Yu A et al. 1998. Cellular and developmental control of O2 homeostasis by hypoxia-inducible factor 1 alpha. *Genes Dev* **12**: 149-162.

Jaakkola P, Mole D, Tian Y, Wilson M, Gielbert J, Gaskell S, von Kriegsheim A, Heberstreit HF, Mukherji M, Schofield CJ, Maxwell PH, Pugh CW, Ratcliffe PJ. 2001. Targeting of HIF-alpha to the von Hippel-Lindau ubiquitylation complex by O2-regulated prolyl hydroxylation. *Science* **292**: 468-472.

Jabbour A, Hayward CS, Keogh AM, Kotlyar E, McCrohon JA, England JF, Amor R, Liu X, Li XY, Zhou MD et al. 2011. Parenteral administration of recombinant human neuregulin-1 to patients with stable chronic heart failure produces favourable acute and chronic haemodynamic responses. *Eur J Heart Fail* **13**: 83-92.

Jacoby AS, Busch-Nentwich E, Bryson-Richardson RJ, Hall TE, Berger J, Berger S, Sonntag C, Sachs C, Geisler R, Stemple DL et al. 2009. The zebrafish dystrophic mutant softy maintains muscle fibre viability despite basement membrane rupture and muscle detachment. *Development* **136**: 3367-3376.

Jansson M, Philipson L, Vennstrom B. 1983. Isolation and characterization of multiple human genes homologous to the oncogenes of avian erythroblastosis virus. *EMBO J* **2**: 561-565.

Jeong JW, Bae MK, Ahn MY, Kim SH, Sohn TK, Bae MH, Yoo MA, Song EJ, Lee KJ, Kim KW. 2002. Regulation and destabilization of HIF-1alpha by ARD1-mediated acetylation. *Cell* **111**: 709-720.

Jones F, Welte T, Fu X, Stern D. 1999. ErbB4 signaling in the mammary gland is required for lobuloalveolar development and Stat5 activation during lactation. *J Cell Biol* **147**: 77-88.

Junttila T, Laato M, Vahlberg T, Söderström K, Visakorpi T, Isola J, Elenius K. 2003. Identification of patients with transitional cell carcinoma of the bladder overexpressing ErbB2, ErbB3, or specific ErbB4 isoforms: real-time reverse transcription-PCR analysis in estimation of ErbB receptor status from cancer patients. *Clin Cancer Res* **9**: 5346-5357.

Junttila T, Sundvall M, Lundin M, Lundin J, Tanner M, Härkönen P, Joensuu H, Isola J, Elenius K. 2005. Cleavable ErbB4 isoform in estrogen receptor-regulated growth of breast cancer cells. *Cancer Res* **65**: 1384-1393.

Kilpinen S, Autio R, Ojala K, Iljin K, Bucher E, Sara H, Pisto T, Saarela M, Skotheim R, Bjorkman M et al. 2008. Systematic bioinformatic analysis of expression levels of 17,330 human genes across 9,783 samples from 175 types of healthy and pathological tissues. *Genome Biol* **9**: R139.

Kim HJ, Lee HJ, Kim H, Cho SW, Kim JS. 2009. Targeted genome editing in human cells with zinc

finger nucleases constructed via modular assembly. *Genome Res* **19**: 1279-1288.

Kimmel CB, Ballard WW, Kimmel SR, Ullmann B, Schilling TF. 1995. Stages of embryonic development of the zebrafish. *Dev Dyn* **203**: 253-310.

Kobel M, Kalloger SE, Boyd N, McKinney S, Mehl E, Palmer C, Leung S, Bowen NJ, Ionescu DN, Rajput A et al. 2008. Ovarian carcinoma subtypes are different diseases: implications for biomarker. *PLoS Med* **5**: e232.

Koh M, Darnay B, Powis G. 2008. Hypoxia-associated factor, a novel E3-ubiquitin ligase, binds and ubiquitinates hypoxia-inducible factor 1alpha, leading to its oxygen-independent degradation. *Mol Cell Biol* **28**: 7081-7095.

Kolb A, Kleeff J, Arnold N, Giese NA, Giese T, Korc M, Friess H. 2007. Expression and differential signaling of heregulins in pancreatic cancer cells. *Int J Cancer* **120**: 514-523.

Krag TO, Bogdanovich S, Jensen CJ, Fischer MD, Hansen-Schwartz J, Javazon EH, Flake AW, Edvinsson L, Khurana TS. 2004. Heregulin ameliorates the dystrophic phenotype in mdx mice. *Proc Natl Acad Sci U S A* **101**: 13856-13860.

Kwan KM, Fujimoto E, Grabher C, Mangum BD, Hardy ME, Campbell DS, Parant JM, Yost HJ, Kanki JP, Chien CB. 2007. The Tol2kit: a multisite gateway-based construction kit for Tol2 transposon transgenesis constructs. *Dev Dyn* **236**: 3088-3099.

Lafky J, Wilken J, Baron A, Maihle N. 2008. Clinical implications of the ErbB/epidermal growth factor (EGF) receptor family and its ligands in ovarian cancer. *Biochim Biophys Acta* **1785**: 232-265.

Laisney JA, Braasch I, Walter RB, Meierjohann S, Schartl M. 2010. Lineage-specific co-evolution of the Egf receptor/ligand signaling system. *BMC Evol Biol* **10**: 27.

Lando D, Peet DJ, Gorman JJ, Whelan DA, Whitelaw ML, Bruick RK. 2002. FIH-1 is an asparaginyl hydroxylase enzyme that regulates the transcriptional activity of hypoxia-inducible factor. *Genes Dev* **16**: 1466-1471.

Latif F, Tory K, Gnarra J, Yao M, Duh FM, Orcutt ML, Stackhouse T, Kuzmin I, Modi W, Geil L et al. 1993. Identification of the von Hippel-Lindau disease tumor suppressor gene. *Science* **260**: 1317-1320.

Laughner E, Taghavi P, Chiles K, Mahon P, Semenza G. 2001. HER2 (neu) signaling increases the rate of hypoxia-inducible factor 1alpha (HIF-1alpha) synthesis: novel mechanism for HIF-1-mediated vascular endothelial growth factor expression. *Mol Cell Biol* **21**: 3995-4004.

Laurendeau I, Ferrer M, Garrido D, D'Haene N, Ciavarelli P, Basso A, Vidaud M, Bieche I, Salmon I, Szijan I. 2009. Gene expression profiling of ErbB receptors and ligands in human meningiomas. *Cancer Invest* **27**: 691-698.

Lee JC, Wang ST, Chow NH, Yang HB. 2002. Investigation of the prognostic value of coexpressed erbB family members for the survival of colorectal cancer patients after curative surgery. *Eur J Cancer* **38**: 1065-1071.

Lee KF, Simon H, Chen H, Bates B, Hung MC, Hauser C. 1995. Requirement for neuregulin receptor erbB2 in neural and cardiac development. *Nature* **378**: 394-398.

Lemmon MA. 2009. Ligand-induced ErbB receptor dimerization. *Exp Cell Res* **315**: 638-648.

Lemmon MA, Schlessinger J. 2010. Cell signaling by receptor tyrosine kinases. *Cell* **141**: 1117-1134.

Leung HY, Weston J, Gullick WJ, Williams G. 1997. A potential autocrine loop between heregulin-alpha and erbB-3 receptor in human prostatic adenocarcinoma. *Br J Urol* **79**: 212-216.

Li D, Collier DA, He L. 2006. Meta-analysis shows strong positive association of the neuregulin 1 (NRG1) gene with schizophrenia. *Hum Mol Genet* **15**: 1995-2002.

Liew WC, Bartfai R, Lim Z, Sreenivasan R, Siegfried KR, Orban L. 2012. Polygenic sex determination system in zebrafish. *PLoS One* **7**: e34397.

Lin S, Makino K, Xia W, Matin A, Wen Y, Kwong K, Bourguignon L, Hung M. 2001a. Nuclear localization of EGF receptor and its potential new role as a transcription factor. *Nat Cell Biol* **3**: 802-808.

Lin SY, Makino K, Xia W, Matin A, Wen Y, Kwong KY, Bourguignon L, Hung MC. 2001b. Nuclear localization of EGF receptor and its potential new role as a transcription factor. *Nat Cell Biol* **3**: 802-808.

Lin YY. 2012. Muscle diseases in the zebrafish. *Neuromuscul Disord* **22**: 673-684.

Linggi B, Carpenter G. 2006. ErbB receptors: new insights on mechanisms and biology. *Trends Cell Biol* **16**: 649-656.

Liu FF, Stone JR, Schuldt AJ, Okoshi K, Okoshi MP, Nakayama M, Ho KK, Manning WJ, Marchionni MA, Lorell BH et al. 2005. Heterozygous knockout of neuregulin-1 gene in mice exacerbates doxorubicin-induced heart failure. *Am J Physiol Heart Circ Physiol* **289**: H660-666.

Liu J, Bressan M, Hassel D, Huisken J, Staudt D, Kikuchi K, Poss KD, Mikawa T, Stainier DY. 2010. A dual role for ErbB2 signaling in cardiac trabeculation. *Development* **137**: 3867-3875.

Liu X, Gu X, Li Z, Li X, Li H, Chang J, Chen P, Jin J, Xi B, Chen D et al. 2006. Neuregulin-1/erbB-activation improves cardiac function and survival in models of ischemic, dilated, and viral cardiomyopathy. *J Am Coll Cardiol* **48**: 1438-1447.

Liu X, Hwang H, Cao L, Buckland M, Cunningham A, Chen J, Chien KR, Graham RM, Zhou M. 1998. Domain-specific gene disruption reveals critical regulation of neuregulinsignaling by its cytoplasmic tail. *Proc Natl Acad Sci U S A* **95**: 13024-13029.

Liu Y, Baek J, Zhang H, Diez R, Cole R, Semenza G. 2007. RACK1 competes with HSP90 for binding to HIF-1alpha and is required for O(2)-independent and HSP90 inhibitor-induced degradation of HIF-1alpha. *Mol Cell* **25**: 207-217.

Lo HW, Ali-Seyed M, Wu Y, Bartholomeusz G, Hsu SC, Hung MC. 2006. Nuclear-cytoplasmic transport of EGFR involves receptor endocytosis, importin beta1 and CRM1. *J Cell Biochem* **98**: 1570-1583.

Lodge AJ, Anderson JJ, Gullick WJ, Haugk B, Leonard RC, Angus B. 2003. Type 1 growth factor receptor expression in node positive breast cancer: adverse prognostic significance of c-erbB-4. *J Clin Pathol* **56**: 300-304.

Long W, Wagner K, Lloyd K, Binart N, Shillingford J, Hennighausen L, Jones F. 2003. Impaired differentiation and lactational failure of Erbb4-deficient mammary glands identify ERBB4 as an obligate mediator of STAT5. *Development* **130**: 5257-5268.

Lyons DA, Pogoda HM, Voas MG, Woods IG, Diamond B, Nix R, Arana N, Jacobs J, Talbot WS. 2005. erbb3 and erbb2 are essential for schwann cell migration and myelination in zebrafish. *Curr Biol* **15**: 513-524.

Mahon PC, Hirota K, Semenza GL. 2001. FIH-1: a novel protein that interacts with HIF-1alpha and VHL to mediate repression of HIF-1 transcriptional activity. *Genes Dev* **15**: 2675-2686.

Makhija S, Amler L, Glenn D, Ueland F, Gold M, Dizon D, Paton V, Lin C, Januario T, Ng K et al. 2010. Clinical activity of gemcitabine plus pertuzumab in platinum-resistant ovarian cancer, fallopian tube cancer, or primary peritoneal cancer. *J Clin Oncol* **28**: 1215-1223.

Manning BD, Cantley LC. 2007. AKT/PKB signaling: navigating downstream. *Cell* **129**: 1261-1274.

Margolis B, Li N, Koch A, Mohammadi M, Hurwitz DR, Zilberstein A, Ullrich A, Pawson T, Schlessinger J. 1990. The tyrosine phosphorylated carboxyterminus of the EGF receptor is a binding site for GAP and PLC-gamma. *EMBO J* **9**: 4375-4380.

Marti U, Burwen SJ, Wells A, Barker ME, Huling S, Feren AM, Jones AL. 1991. Localization of epidermal growth factor receptor in hepatocyte nuclei. *Hepatology* **13**: 15-20.

Mason SD, Howlett RA, Kim MJ, Olfert IM, Hogan MC, McNulty W, Hickey RP, Wagner PD, Kahn CR, Giordano FJ et al. 2004. Loss of skeletal muscle HIF-1alpha results in altered exercise endurance. *PLoS Biol* **2**: e288.

Memon AA, Sorensen BS, Melgard P, Fokdal L, Thykjaer T, Nexo E. 2004. Expression of HER3, HER4 and their ligand heregulin-4 is associated with better survival in bladder cancer patients. *Br J Cancer* **91**: 2034-2041.

Mendrola JM, Berger MB, King MC, Lemmon MA. 2002. The single transmembrane domains of ErbB receptors self-associate in cell membranes. *J Biol Chem* **277**: 4704-4712.

Meng X, Noyes MB, Zhu LJ, Lawson ND, Wolfe SA. 2008. Targeted gene inactivation in zebrafish using engineered zinc-finger nucleases. *Nat Biotechnol* **26**: 695-701.

Meyer D, Birchmeier C. 1995. Multiple essential functions of neuregulin in development. *Nature* **378**: 386-390.

Meyer D, Yamaai T, Garratt A, Riethmacher-Sonnenberg E, Kane D, Theill LE, Birchmeier C. 1997. Isoform-specific expression and function of neuregulin. *Development* **124**: 3575-3586.

Miaczynska M, Pelkmans L, Zerial M. 2004. Not just a sink: endosomes in control of signal transduction. *Curr Opin Cell Biol* **16**: 400-406.

Miettinen PJ, Berger JE, Meneses J, Phung Y, Pedersen RA, Werb Z, Derynck R. 1995. Epithelial immaturity and multiorgan failure in mice lacking epidermal growth factor receptor. *Nature* **376**: 337-341.

Monast CS, Furcht CM, Lazzara MJ. 2012. Computational Analysis of the Regulation of EGFR by Protein Tyrosine Phosphatases. *Biophys J* **102**: 2012-2021.

Mor A, Philips MR. 2006. Compartmentalized Ras/MAPK signaling. *Annu Rev Immunol* **24**: 771-800.

Munafo MR, Thiselton DL, Clark TG, Flint J. 2006. Association of the NRG1 gene and schizophrenia: a meta-analysis. *Mol Psychiatry* **11**: 539-546.

Munk M, Memon AA, Goetze JP, Nielsen LB, Nexo E, Sorensen BS. 2012. Hypoxia changes the expression of the epidermal growth factor (EGF) system in human hearts and cultured cardiomyocytes. *PLoS One* **7**: e40243.

Muraoka-Cook R, Sandahl M, Husted C, Hunter D, Miraglia L, Feng S, Elenius K, Earp Hr. 2006. The intracellular domain of ErbB4 induces differentiation of mammary epithelial cells. *Mol Biol Cell* **17**: 4118-4129.

Naganawa Y, Hirata H. 2011. Developmental transition of touch response from slow muscle-mediated coilings to fast muscle-mediated burst swimming in zebrafish. *Dev Biol* **355**: 194-204.

Naresh A, Long W, Vidal GA, Wimley WC, Marrero L, Sartor CI, Tovey S, Cooke TG, Bartlett JM, Jones FE. 2006. The ERBB4/HER4 intracellular domain 4ICD is a BH3-only protein promoting apoptosis of breast cancer cells. *Cancer Res* **66**: 6412-6420.

Nasevicius A, Ekker SC. 2000. Effective targeted gene 'knockdown' in zebrafish. *Nat Genet* **26**: 216-220.

Network CGAR. 2011. Integrated genomic analyses of ovarian carcinoma. *Nature* **474**: 609-615.

Ni C, Murphy M, Golde T, Carpenter G. 2001. gamma -Secretase cleavage and nuclear localization of ErbB-4 receptor tyrosine kinase. *Science* **294**: 2179-2181.

Nicodemus KK, Luna A, Vakkalanka R, Goldberg T, Egan M, Straub RE, Weinberger DR. 2006. Further evidence for association between ErbB4 and schizophrenia and influence on cognitive intermediate phenotypes in healthy controls. *Mol Psychiatry* **11**: 1062-1065.

Nicolino S, Panetto A, Raimondo S, Gambarotta G, Guzzini M, Fornaro M, Battiston B, Tos P, Geuna S, Perroteau I. 2009. Denervation and reinnervation of adult skeletal muscle modulate mRNA expression of neuregulin-1 and ErbB receptors. *Microsurgery* **29**: 464-472.

Nüsslein-Volhard C, Dahm R. 2002. *Zebrafish : a practical approach*. Oxford University Press, Oxford.

Offterdinger M, Schofer C, Weipoltshammer K, Grunt TW. 2002. c-erbB-3: a nuclear protein in mammary epithelial cells. *J Cell Biol* **157**: 929-939.

Olayioye MA, Badache A, Daly JM, Hynes NE. 2001. An essential role for Src kinase in ErbB receptor signaling through the MAPK pathway. *Exp Cell Res* **267**: 81-87.

Olayioye MA, Beuvink I, Horsch K, Daly JM, Hynes NE. 1999. ErbB receptor-induced activation of stat transcription factors is mediated by Src tyrosine kinases. *J Biol Chem* **274**: 17209-17218.

Palayekar M, Herzog T. 2007. The emerging role of epidermal growth factor receptor inhibitors in ovarian cancer. *Int J Gynecol Cancer*.

Pawlowski V, Revillion F, Hebbar M, Hornez L, Peyrat JP. 2000. Prognostic value of the type I growth factor receptors in a large series of human primary breast cancers quantified with a real-time reverse transcription-polymerase chain reaction assay. *Clin Cancer Res* **6**: 4217-4225.

Pejovic T, Pande N, Mori M, Mhawech-Fauceglia P, Harrington C, Mongoue-Tchokote S, Dim D, Andrews C, Beck A, Tarumi Y et al. 2009. Expression profiling of the ovarian surface kinome reveals candidate genes for early neoplastic changes. *Transl Oncol* **2**: 341-349.

Peles E, Levy RB, Or E, Ullrich A, Yarden Y. 1991. Oncogenic forms of the neu/HER2 tyrosine kinase are permanently coupled to phospholipase C gamma. *EMBO J* **10**: 2077-2086.

Percy MJ, Beer PA, Campbell G, Dekker AW, Green AR, Oscier D, Rainey MG, van Wijk R, Wood M, Lappin TR et al. 2008a. Novel exon 12 muta-

tions in the HIF2A gene associated with erythrocytosis. *Blood* **111**: 5400-5402.

Percy MJ, Furlow PW, Lucas GS, Li X, Lappin TR, McMullin MF, Lee FS. 2008b. A gain-of-function mutation in the HIF2A gene in familial erythrocytosis. *N Engl J Med* **358**: 162-168.

Percy MJ, Zhao Q, Flores A, Harrison C, Lappin TR, Maxwell PH, McMullin MF, Lee FS. 2006. A family with erythrocytosis establishes a role for prolyl hydroxylase domain protein 2 in oxygen homeostasis. *Proc Natl Acad Sci U S A* **103**: 654-659.

Pickart CM. 2001. Mechanisms underlying ubiquitination. *Annu Rev Biochem* **70**: 503-533.

Plowman GD, Culouscou JM, Whitney GS, Green JM, Carlton GW, Foy L, Neubauer MG, Shoyab M. 1993. Ligand-specific activation of HER4/p180erbB4, a fourth member of the epidermal growth factor receptor family. *Proc Natl Acad Sci U S A* **90**: 1746-1750.

Pouysségur J, Dayan F, Mazure N. 2006. Hypoxia signalling in cancer and approaches to enforce tumour regression. *Nature* **441**: 437-443.

Prickett TD, Agrawal NS, Wei X, Yates KE, Lin JC, Wunderlich JR, Cronin JC, Cruz P, Rosenberg SA, Samuels Y. 2009. Analysis of the tyrosine kinome in melanoma reveals recurrent mutations in ERBB4. *Nat Genet* **41**: 1127-1132.

Qiu C, Tarrant MK, Choi SH, Sathyamurthy A, Bose R, Banjade S, Pal A, Bornmann WG, Lemmon MA, Cole PA et al. 2008. Mechanism of activation and inhibition of the HER4/ErbB4 kinase. *Structure* **16**: 460-467.

Qu S, Rinehart C, Wu HH, Wang SE, Carter B, Xin H, Kotlikoff M, Arteaga CL. 2006. Gene targeting of ErbB3 using a Cre-mediated unidirectional DNA inversion strategy. *Genesis* **44**: 477-486.

Rhee SG. 2001. Regulation of phosphoinositide-specific phospholipase C. *Annu Rev Biochem* **70**: 281-312.

Richard DE, Berra E, Gothié E, Roux D, Pouysségur J. 1999. p42/p44 mitogen-activated protein kinases phosphorylate hypoxia-inducible factor 1alpha (HIF-1alpha) and enhance the transcriptional activity of HIF-1. *J Biol Chem* **274**: 32631-32637.

Richards WG, Sweeney WE, Yoder BK, Wilkinson JE, Woychik RP, Avner ED. 1998. Epidermal growth factor receptor activity mediates renal cyst formation in polycystic kidney disease. *J Clin Invest* **101**: 935-939.

Riethmacher D, Sonnenberg-Riethmacher E, Brinkmann V, Yamaai T, Lewin GR, Birchmeier C. 1997. Severe neuropathies in mice with targeted mutations in the ErbB3 receptor. *Nature* **389**: 725-730.

Rio C, Buxbaum J, Peschon J, Corfas G. 2000. Tumor necrosis factor-alpha-converting enzyme is required for cleavage of erbB4/HER4. *J Biol Chem* **275**: 10379-10387.

Rohrbach S, Niemann B, Silber RE, Holtz J. 2005. Neuregulin receptors erbB2 and erbB4 in failing human myocardium -- depressed expression and attenuated activation. *Basic Res Cardiol* **100**: 240-249.

Rojas-Muñoz A, Rajadhyksha S, Gilmour D, van Bebber F, Antos C, Rodríguez Esteban C, Nüsslein-Volhard C, Izpisúa Belmonte JC. 2009. ErbB2 and ErbB3 regulate amputation-induced proliferation and migration during vertebrate regeneration. *Dev Biol* **327**: 177-190.

Russell DW, Hirata RK. 1998. Human gene targeting by viral vectors. *Nat Genet* **18**: 325-330.

Ryan H, Lo J, Johnson R. 1998. HIF-1 alpha is required for solid tumor formation and embryonic vascularization. *EMBO J* **17**: 3005-3015.

Rytkönen KT, Williams TA, Renshaw GM, Primmer CR, Nikinmaa M. 2011. Molecular evolution of the metazoan PHD-HIF oxygen-sensing system. *Mol Biol Evol* **28**: 1913-1926.

Saini Y, Harkema JR, LaPres JJ. 2008. HIF1alpha is essential for normal intrauterine differentiation of alveolar epithelium and surfactant production in the newborn lung of mice. *J Biol Chem* **283**: 33650-33657.

Sardi S, Murtie J, Koirala S, Patten B, Corfas G. 2006. Presenilin-dependent ErbB4 nuclear signaling regulates the timing of astrogenesis in the developing brain. *Cell* **127**: 185-197.

Sasada R, Ono Y, Taniyama Y, Shing Y, Folkman J, Igarashi K. 1993. Cloning and expression of cDNA encoding human betacellulin, a new member of the EGF family. *Biochem Biophys Res Commun* **190**: 1173-1179.

Scherz PJ, Huisken J, Sahai-Hernandez P, Stainier DY. 2008. High-speed imaging of developing heart valves reveals interplay of morphogenesis and function. *Development* **135**: 1179-1187.

Schmidt N, Akaaboune M, Gajendran N, Martinez-Pena y Valenzuela I, Wakefield S, Thurnheer R, Brenner HR. 2011. Neuregulin/ErbB regulate neuromuscular junction development by phosphorylation of alpha-dystrobrevin. *J Cell Biol* **195**: 1171-1184.

Schulze WX, Deng L, Mann M. 2005. Phosphotyrosine interactome of the ErbB-receptor kinase family. *Mol Syst Biol* **1**: 2005 0008.

Schödel J, Oikonomopoulos S, Ragoussis J, Pugh CW, Ratcliffe PJ, Mole DR. 2011. High-resolution genome-wide mapping of HIF-binding sites by ChIP-seq. *Blood* **117**: e207-217.

Scortegagna M, Ding K, Oktay Y, Gaur A, Thurmond F, Yan LJ, Marck BT, Matsumoto AM, Shelton JM, Richardson JA et al. 2003a. Multiple organ pathology, metabolic abnormalities and impaired homeostasis of reactive oxygen species in Epas1-/- mice. *Nat Genet* **35**: 331-340.

Scortegagna M, Morris MA, Oktay Y, Bennett M, Garcia JA. 2003b. The HIF family member EPAS1/HIF-2alpha is required for normal hematopoiesis in mice. *Blood* **102**: 1634-1640.

Seagroves T, Hadsell D, McManaman J, Palmer C, Liao D, McNulty W, Welm B, Wagner K, Neville M, Johnson R. 2003. HIF1alpha is a critical regulator of secretory differentiation and activation, but not vascular expansion, in the mouse mammary gland. *Development* **130**: 1713-1724.

Semenza G, Wang G. 1992. A nuclear factor induced by hypoxia via de novo protein synthesis binds to the human erythropoietin gene enhancer at a site required for transcriptional activation. *Mol Cell Biol* **12**: 5447-5454.

Semenza GL, Roth PH, Fang HM, Wang GL. 1994. Transcriptional regulation of genes encoding glycolytic enzymes by hypoxia-inducible factor 1. *J Biol Chem* **269**: 23757-23763.

Sheng Q, Liu X, Fleming E, Yuan K, Piao H, Chen J, Moustafa Z, Thomas RK, Greulich H, Schinzel A et al. 2010. An activated ErbB3/NRG1 autocrine loop supports in vivo proliferation in ovarian cancer cells. *Cancer Cell* **17**: 298-310.

Shi F, Telesco SE, Liu Y, Radhakrishnan R, Lemmon MA. 2010. ErbB3/HER3 intracellular domain is competent to bind ATP and catalyze autophosphorylation. *Proc Natl Acad Sci U S A* **107**: 7692-7697.

Shi J, Yao D, Liu W, Wang N, Lv H, He N, Shi B, Hou P, Ji M. 2012. Frequent gene amplification predicts poor prognosis in gastric cancer. *Int J Mol Sci* **13**: 4714-4726.

Shimura T, Yoshida M, Fukuda S, Ebi M, Hirata Y, Mizoshita T, Tanida S, Kataoka H, Kamiya T, Higashiyama S et al. 2012. Nuclear translocation of the cytoplasmic domain of HB-EGF induces gastric cancer invasion. *BMC Cancer* **12**: 205.

Shoyab M, McDonald VL, Bradley JG, Todaro GJ. 1988. Amphiregulin: a bifunctional growth-modulating glycoprotein produced by the phorbol 12-myristate 13-acetate-treated human breast adenocarcinoma cell line MCF-7. *Proc Natl Acad Sci U S A* **85**: 6528-6532.

Sibilia M, Wagner EF. 1995. Strain-dependent epithelial defects in mice lacking the EGF receptor. *Science* **269**: 234-238.

Silberberg G, Darvasi A, Pinkas-Kramarski R, Navon R. 2006. The involvement of ErbB4 with schizophrenia: association and expression studies. *Am J Med Genet B Neuropsychiatr Genet* **141B**: 142-148.

Six E, Ndiaye D, Laabi Y, Brou C, Gupta-Rossi N, Israel A, Logeat F. 2003. The Notch ligand Delta1 is sequentially cleaved by an ADAM protease and gamma-secretase. *Proc Natl Acad Sci U S A* **100**: 7638-7643.

Skolnik EY, Margolis B, Mohammadi M, Lowenstein E, Fischer R, Drepps A, Ullrich A, Schlessinger J. 1991. Cloning of PI3 kinase-associated p85 utilizing a novel method for expression/cloning of target proteins for receptor tyrosine kinases. *Cell* **65**: 83-90.

Slamon DJ, Clark GM, Wong SG, Levin WJ, Ullrich A, McGuire WL. 1987. Human breast cancer: correlation of relapse and survival with amplification of the HER-2/neu oncogene. *Science* **235**: 177-182.

Slamon DJ, Leyland-Jones B, Shak S, Fuchs H, Paton V, Bajamonde A, Fleming T, Eiermann W, Wolter J, Pegram M et al. 2001. Use of chemotherapy plus a monoclonal antibody against HER2 for metastatic breast cancer that overexpresses HER2. *N Engl J Med* **344**: 783-792.

Soung Y, Lee J, Kim S, Wang Y, Jo K, Moon S, Park W, Nam S, Lee J, Yoo N et al. 2006a. Somatic mutations of the ERBB4 kinase domain in human cancers. *Int J Cancer* **118**: 1426-1429.

Soung YH, Lee JW, Kim SY, Wang YP, Jo KH, Moon SW, Park WS, Nam SW, Lee JY, Yoo NJ et al.

2006b. Somatic mutations of the ERBB4 kinase domain in human cancers. *Int J Cancer* **118**: 1426-1429.

Srinivasan R, Poulsom R, Hurst HC, Gullick WJ. 1998. Expression of the c-erbB-4/HER4 protein and mRNA in normal human fetal and adult. *J Pathol* **185**: 236-245.

Srivastava M, Simakov O, Chapman J, Fahey B, Gauthier ME, Mitros T, Richards GS, Conaco C, Dacre M, Hellsten U et al. 2010. The Amphimedon queenslandica genome and the evolution of animal complexity. *Nature* **466**: 720-726.

Stefansson H, Sigurdsson E, Steinthorsdottir V, Bjornsdottir S, Sigmundsson T, Ghosh S, Brynjolfsson J, Gunnarsdottir S, Ivarsson O, Chou TT et al. 2002. Neuregulin 1 and susceptibility to schizophrenia. *Am J Hum Genet* **71**: 877-892.

Steffen LS, Guyon JR, Vogel ED, Howell MH, Zhou Y, Weber GJ, Zon LI, Kunkel LM. 2007. The zebrafish runzel muscular dystrophy is linked to the titin gene. *Dev Biol* **309**: 180-192.

Steffensen K, Waldstrøm M, Andersen R, Olsen D, Jeppesen U, Knudsen H, Brandslund I, Jakobsen A. 2008. Protein levels and gene expressions of the epidermal growth factor receptors, HER1, HER2, HER3 and HER4 in benign and malignant ovarian tumors. *Int J Oncol* **33**: 195-204.

Stein RA, Staros JV. 2000. Evolutionary analysis of the ErbB receptor and ligand families. *J Mol Evol* **50**: 397-412.

-. 2006. Insights into the evolution of the ErbB receptor family and their ligands from sequence analysis. *BMC Evol Biol* **6**: 79.

Stoeck A, Shang L, Dempsey PJ. 2010. Sequential and gamma-secretase-dependent processing of the betacellulin precursor generates a palmitoylated intracellular-domain fragment that inhibits cell growth. *J Cell Sci* **123**: 2319-2331.

Strachan L, Murison JG, Prestidge RL, Sleeman MA, Watson JD, Kumble KD. 2001. Cloning and biological activity of epigen, a novel member of the epidermal growth factor superfamily. *J Biol Chem* **276**: 18265-18271.

Suh PG, Park JI, Manzoli L, Cocco L, Peak JC, Katan M, Fukami K, Kataoka T, Yun S, Ryu SH. 2008. Multiple roles of phosphoinositide-specific phospholipase C isozymes. *BMB Rep* **41**: 415-434.

Sundvall M, Korhonen A, Paatero I, Gaudio E, Melino G, Croce C, Aqeilan R, Elenius K. 2008. Isoform-specific monoubiquitination, endocytosis, and degradation of alternatively spliced ErbB4 isoforms. *Proc Natl Acad Sci U S A* **105**: 4162-4167.

Sweeney C, Lai C, Riese DJ, 2nd, Diamonti AJ, Cantley LC, Carraway KL, 3rd. 2000. Ligand discrimination in signaling through an ErbB4 receptor homodimer. *J Biol Chem* **275**: 19803-19807.

Tarcic G, Boguslavsky SK, Wakim J, Kiuchi T, Liu A, Reinitz F, Nathanson D, Takahashi T, Mischel PS, Ng T et al. 2009. An unbiased screen identifies DEP-1 tumor suppressor as a phosphatase controlling EGFR endocytosis. *Curr Biol* **19**: 1788-1798.

Thomasson M, Hedman H, Junttila TT, Elenius K, Ljungberg B, Henriksson R. 2004. ErbB4 is downregulated in renal cell carcinoma--a quantitative RT-PCR and immunohistochemical analysis of the epidermal growth factor receptor family. *Acta Oncol* **43**: 453-459.

Threadgill DW, Dlugosz AA, Hansen LA, Tennenbaum T, Lichti U, Yee D, LaMantia C, Mourton T, Herrup K, Harris RC et al. 1995. Targeted disruption of mouse EGF receptor: effect of genetic background on mutant phenotype. *Science* **269**: 230-234.

Thybusch-Bernhardt A, Beckmann S, Juhl H. 2001. Comparative analysis of the EGF-receptor family in pancreatic cancer: expression of HER-4 correlates with a favourable tumor stage. *Int J Surg Investig* **2**: 393-400.

Tian H, Hammer RE, Matsumoto AM, Russell DW, McKnight SL. 1998. The hypoxia-responsive transcription factor EPAS1 is essential for catecholamine homeostasis and protection against heart failure during embryonic development. *Genes Dev* **12**: 3320-3324.

Tian H, McKnight SL, Russell DW. 1997. Endothelial PAS domain protein 1 (EPAS1), a transcription factor selectively expressed in endothelial cells. *Genes Dev* **11**: 72-82.

Tidcombe H, Jackson-Fisher A, Mathers K, Stern D, Gassmann M, Golding J. 2003. Neural and mammary gland defects in ErbB4 knockout mice genetically rescued from embryonic lethality. *Proc Natl Acad Sci U S A* **100**: 8281-8286.

Tomita S, Ueno M, Sakamoto M, Kitahama Y, Ueki M, Maekawa N, Sakamoto H, Gassmann

M, Kageyama R, Ueda N et al. 2003. Defective brain development in mice lacking the Hif-1alpha gene in neural cells. *Mol Cell Biol* **23**: 6739-6749.

Tomita T, Tanaka S, Morohashi Y, Iwatsubo T. 2006. Presenilin-dependent intramembrane cleavage of ephrin-B1. *Mol Neurodegener* **1**: 2.

Tonks NK. 2006. Protein tyrosine phosphatases: from genes, to function, to disease. *Nat Rev Mol Cell Biol* **7**: 833-846.

Toyoda H, Komurasaki T, Uchida D, Takayama Y, Isobe T, Okuyama T, Hanada K. 1995. Epiregulin. A novel epidermal growth factor with mitogenic activity for rat primary hepatocytes. *J Biol Chem* **270**: 7495-7500.

Tran TC, Sneed B, Haider J, Blavo D, White A, Aiyejorun T, Baranowski TC, Rubinstein AL, Doan TN, Dingledine R et al. 2007. Automated, quantitative screening assay for antiangiogenic compounds using transgenic zebrafish. *Cancer Res* **67**: 11386-11392.

Tvorogov D, Sundvall M, Kurppa K, Hollmén M, Repo S, Johnson M, Elenius K. 2009. Somatic mutations of ErbB4: selective loss-of-function phenotype affecting signal transduction pathways in cancer. *J Biol Chem* **284**: 5582-5591.

Vaughan S, Coward JI, Bast RC, Jr., Berchuck A, Berek JS, Brenton JD, Coukos G, Crum CC, Drapkin R, Etemadmoghadam D et al. 2011. Rethinking ovarian cancer: recommendations for improving outcomes. *Nat Rev Cancer* **11**: 719-725.

Vecchi M, Carpenter G. 1997. Constitutive proteolysis of the ErbB-4 receptor tyrosine kinase by a unique, sequential mechanism. *J Cell Biol* **139**: 995-1003.

Veikkolainen V, Naillat F, Railo A, Chi L, Manninen A, Hohenstein P, Hastie N, Vainio S, Elenius K. 2011a. ErbB4 Modulates Tubular Cell Polarity and Lumen Diameter during Kidney Development. *J Am Soc Nephrol*.

Veikkolainen V, Vaparanta K, Halkilahti K, Iljin K, Sundvall M, Elenius K. 2011b. Function of ERBB4 is determined by alternative splicing. *Cell Cycle* **10**: 2647-2657.

Vennstrom B, Bishop JM. 1982. Isolation and characterization of chicken DNA homologous to the two putative oncogenes of avian erythroblastosis virus. *Cell* **28**: 135-143.

Vidal GA, Clark DE, Marrero L, Jones FE. 2007. A constitutively active ERBB4/HER4 allele with enhanced transcriptional coactivation and cell-killing activities. *Oncogene* **26**: 462-466.

Wang GL, Jiang BH, Rue EA, Semenza GL. 1995. Hypoxia-inducible factor 1 is a basic-helix-loop-helix-PAS heterodimer regulated by cellular O2 tension. *Proc Natl Acad Sci U S A* **92**: 5510-5514.

Wang YN, Yamaguchi H, Huo L, Du Y, Lee HJ, Lee HH, Wang H, Hsu JM, Hung MC. 2010. The translocon Sec61beta localized in the inner nuclear membrane transports membrane-embedded EGF receptor to the nucleus. *J Biol Chem* **285**: 38720-38729.

Warburg O. 1956. On the origin of cancer cells. *Science* **123**: 309-314.

Waterman H, Levkowitz G, Alroy I, Yarden Y. 1999. The RING finger of c-Cbl mediates desensitization of the epidermal growth factor receptor. *J Biol Chem* **274**: 22151-22154.

Westerfield M. 2000. *The zebrafish book. A guide for the laboratory use of zebrafish (Danio rerio)*. University of Oregon Press, Eugene, USA.

White RM, Sessa A, Burke C, Bowman T, LeBlanc J, Ceol C, Bourque C, Dovey M, Goessling W, Burns CE et al. 2008. Transparent adult zebrafish as a tool for in vivo transplantation analysis. *Cell Stem Cell* **2**: 183-189.

Wides RJ, Zak NB, Shilo BZ. 1990. Enhancement of tyrosine kinase activity of the Drosophila epidermal growth factor receptor homolog by alterations of the transmembrane domain. *Eur J Biochem* **189**: 637-645.

Williams C, Allison J, Vidal G, Burow M, Beckman B, Marrero L, Jones F. 2004. The ERBB4/HER4 receptor tyrosine kinase regulates gene expression by functioning as a STAT5A nuclear chaperone. *J Cell Biol* **167**: 469-478.

Wilson SJ, Amsler K, Hyink DP, Li X, Lu W, Zhou J, Burrow CR, Wilson PD. 2006. Inhibition of HER-2(neu/ErbB2) restores normal function and structure to polycystic kidney disease (PKD) epithelia. *Biochim Biophys Acta* **1762**: 647-655.

Wiseman SM, Griffith OL, Melck A, Masoudi H, Gown A, Nabi IR, Jones SJ. 2008. Evaluation of type 1 growth factor receptor family expression in benign and malignant thyroid lesions. *Am J Surg* **195**: 667-673; discussion 673.

Wittbrodt J, Adam D, Malitschek B, Maueler W, Raulf F, Telling A, Robertson SM, Schartl M. 1989. Novel putative receptor tyrosine kinase

encoded by the melanoma-inducing Tu locus in Xiphophorus. *Nature* **341**: 415-421.

Witton CJ, Reeves JR, Going JJ, Cooke TG, Bartlett JM. 2003. Expression of the HER1-4 family of receptor tyrosine kinases in breast cancer. *J Pathol* **200**: 290-297.

Woldeyesus MT, Britsch S, Riethmacher D, Xu L, Sonnenberg-Riethmacher E, Abou-Rebyeh F, Harvey R, Caroni P, Birchmeier C. 1999. Peripheral nervous system defects in erbB2 mutants following genetic rescue of heart development. *Genes Dev* **13**: 2538-2548.

Wolpert L. 2007. *Principles of development*. Oxford University Press, Oxford ; New York.

Wolpowitz D, Mason TB, Dietrich P, Mendelsohn M, Talmage DA, Role LW. 2000. Cysteine-rich domain isoforms of the neuregulin-1 gene are required formaintenance of peripheral synapses. *Neuron* **25**: 79-91.

Wood JD, Bonath F, Kumar S, Ross CA, Cunliffe VT. 2009. Disrupted-in-schizophrenia 1 and neuregulin 1 are required for the specification of oligodendrocytes and neurones in the zebrafish brain. *Hum Mol Genet* **18**: 391-404.

Xia G, Rachfal AW, Martin AE, Besner GE. 2003. Upregulation of endogenous heparin-binding EGF-like growth factor (HB-EGF) expression after intestinal ischemia/reperfusion injury. *J Invest Surg* **16**: 57-63.

Xie Y, Hung MC. 1994. Nuclear localization of p185neu tyrosine kinase and its association with transcriptional transactivation. *Biochem Biophys Res Commun* **203**: 1589-1598.

Yamashita T, Ohneda O, Nagano M, Iemitsu M, Makino Y, Tanaka H, Miyauchi T, Goto K, Ohneda K, Fujii-Kuriyama Y et al. 2008. Abnormal heart development and lung remodeling in mice lacking the hypoxia-inducible factor-related basic helix-loop-helix PAS protein NEPAS. *Mol Cell Biol* **28**: 1285-1297.

Yarden Y, Pines G. 2012. The ERBB network: at last, cancer therapy meets systems biology. *Nat Rev Cancer*.

Yarden Y, Sliwkowski M. 2001. Untangling the ErbB signalling network. *Nat Rev Mol Cell Biol* **2**: 127-137.

Yu H, Jove R. 2004. The STATs of cancer--new molecular targets come of age. *Nat Rev Cancer* **4**: 97-105.

Zhang D, Sliwkowski MX, Mark M, Frantz G, Akita R, Sun Y, Hillan K, Crowley C, Brush J, Godowski PJ. 1997. Neuregulin-3 (NRG3): a novel neural tissue-enriched protein that binds and activates ErbB4. *Proc Natl Acad Sci U S A* **94**: 9562-9567.

Zhang J, Eto K, Honmyou A, Nakao K, Kiyonari H, Abe S. 2011. Neuregulins are essential for spermatogonial proliferation and meiotic initiation in neonatal mouse testis. *Development* **138**: 3159-3168.

Zhang Z, Yu Y, Xu F, Berchuck A, van Haaften-Day C, Havrilesky LJ, de Bruijn HW, van der Zee AG, Woolas RP, Jacobs IJ et al. 2007. Combining multiple serum tumor markers improves detection of stage I epithelial ovarian cancer. *Gynecol Oncol* **107**: 526-531.

Zhong H, Chiles K, Feldser D, Laughner E, Hanrahan C, Georgescu M, Simons J, Semenza G. 2000. Modulation of hypoxia-inducible factor 1alpha expression by the epidermal growth factor/phosphatidylinositol 3-kinase/PTEN/AKT/FRAP pathway in human prostate cancer cells: implications for tumor angiogenesis and therapeutics. *Cancer Res* **60**: 1541-1545.

Zon LI, Peterson RT. 2005. In vivo drug discovery in the zebrafish. *Nat Rev Drug Discov* **4**: 35-44.