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From the Department of Radiation Sciences, Oncology, University of Umeå, Sweden

The LRIG-family: Identification of novel regulators of ErbB signaling with clinical implications in astrocytoma

Jonas Nilsson

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“Om man inte i det långa loppet kan berätta för var och en vad man gjort blir det man gjort värdelöst.”

Erwin Schrödinger, 1951

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Abstract

Astrocytic tumors are the most common malignancies of the central nervous system, accounting for more than 60% of all primary brain tumors. The prognosis for high grade astrocytoma patients is dismal and there is no curative treatment, today. A molecular hallmark of astrocytic tumors is dysregulated receptor tyrosine kinase signaling, especially of the epidermal growth factor receptor (EGFR, ErbB1). The aim of the present thesis was to identify endogenous human proteins that downregulate the function of the ErbB1 receptor. We identified a human gene family, the *leucine-rich repeats and immunoglobulin-like domains family (LRIG)*, consisting of *LRIG1*, *LRIG2* and *LRIG3*, which might fulfill this criterion.

Two candidates were identified, LRIG1 and LRIG2, which genes were localized to regions frequently deleted in human cancers, chromosome bands 3p14 and 1p13, respectively. *LRIG1* and *LRIG2* mRNA were expressed in all tissues analyzed, with high expression in brain and other organs. The *LRIG* mRNA were predicted to encode integral membrane proteins. Antibodies against LRIG1 and LRIG2 were developed and the protein expression was analyzed. LRIG1 was found to have an apparent molecular weight of 143 kDa and was expressed in most tissues with high expression in glandular tissues of the breast and prostate, and the peptic cells of the stomach. LRIG2 was slightly smaller and had an apparent molecular weight of 132 kDa. The LRIG proteins were localized to the cell surface and to perinuclear structures, where LRIG1 co-localized with the trans-Golgi network and early endosomes.

LRIG1 was found to restrict growth factor signaling by enhancing receptor ubiquitylation and degradation. We showed that LRIG1 interacted with all four members of the ErbB family and induced their downregulation. The interaction with ErbB1 involved both the LRR-domains and the Ig-like domains of LRIG1. LRIG1 enhanced receptor degradation by recruiting the E3 ubiquitin ligase c-Cbl to the LRIG1-ErbB1 complex.

LRIG1, LRIG2, and LRIG3 were expressed in glioma cell lines and tumor tissues. The LRIG expression was analyzed in 404 astrocytic tumor samples. We found that perinuclear LRIG protein expression correlated with increased survival of patients with astrocytic tumors. Especially perinuclear LRIG3 showed strong correlations with patient survival and tumor cell proliferation index.

In summary, this thesis contains the discovery and characterization of human LRIG1 and LRIG2. LRIG1 was found to interact with ErbB receptors and downregulate their function. In a clinical material, expression of LRIG proteins correlated with survival in patients with astrocytic tumors.

Keywords: astrocytic tumors, EGFR, ErbB, *glioblastoma multiforme*, LRIG, negative regulation

List of papers

The results in this thesis are based on the following papers, which are referred to in the text by the corresponding Roman numerals **(I-V)**.

I. **J. Nilsson**, C. Vallbo, D. Guo, I. Golovleva, B. Hallberg, R. Henriksson, H. Hedman (2001) Cloning, characterization, and expression of human LIG1. *Biochem Biophys Res Commun 284:1155-61*

II. **J. Nilsson**, A. Starefeldt, R. Henriksson, H. Hedman (2003) LRIG1 protein in human cells and tissues. *Cell Tissue Res 312:65-71*

III. C. Holmlund, **J. Nilsson**, D. Guo, A. Starefeldt, I. Golovleva, R. Henriksson, H. Hedman (2004) Characterization and tissue-specific expression of human LRIG2. *Gene 332:35-43*

IV. G. Gur, C. Rubin, M. Katz, I. Amit, A. Citri, **J. Nilsson**, N. Amariglio, R. Henriksson, G. Rechav, H. Hedman, R. Wides, Y. Yarden (2004) LRIG1 restricts growth factor signaling by enhancing receptor ubiquitylation and degradation. *EMBO J 23:3270-81*

V. D. Guo, **J. Nilsson**, H. Haapasalo, O. Raheem, T. Bergenheim, H. Hedman, R. Henriksson (2006) Perinuclear leucine-rich repeats and immunoglobulin-like domain proteins (LRIG1-3) as prognostic indicators in astrocytic tumors. *Acta Neuropathol 111:238-46*

Abbreviations

AKT/PKB	*v-akt* murine thymoma viral oncogene homolog 1; protein kinase B
Bad	Bcl2-antagonist of cell death
CDK	Cyclin dependent kinase
CycD	Cyclin D
DER	Drosophila EGF receptor
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
EGFRvIII	EGFR mutant variant III
ErbB	v-ErbB erytroblastosis virus transforming oncogene homolog
GBM	*Glioblastoma multiforme*
GRB2	Growth factor receptor bound protein 2
HB-EGF	Heparin binding-EGF like growth factor
HER	Human epidermal growth factor receptor
kDa	kilo Daltons
LOH	Loss of heterozygosity
LRIG	Leucine-rich repeats and immunoglobulin-like domains
mAb	monoclonal antibody
MAPK	Mitogen activated protein kinase
Mdm-2	Murine double-minute 2
NRG	Neuregulin
NSCLC	Non-small cell lung cancer
p53	tumor suppressor gene
PDGFR	Platelet derived growth factor receptor
PDK1	Phosphoinositide-dependent kinase 1
PI3K	Phosphatidylinositol 3-kinase
PLC-γ	Phospholipase C gamma
PTEN	Phosphatase and tensin homolog
RALT	Receptor associated late transducer
Ras	Rat sarcoma viral oncogene homolog
pRb	Retinoblastoma protein
RTK	Receptor tyrosine kinase
RT-PCR	Reverse transcriptase – polymerase chain reaction
TGF-α	Tumor growth factor-α

1. Introduction

Most, if not all, cancers are derived from a single cell and its progeny. The emerging cancer cells are clonally selected by their growth advantages, and ultimately, a clone gains a phenotype that disregard of normal regulation and a cancer is formed. Both observations of human cancers and animal models support a Darwinsistic evolutionary model, where genetic changes accumulate and progressively convert a normal cell to a cancer cell. During this process the cell acquires the hallmark features of cancer described by Hanahan and Weinberg (Hanahan and Weinberg, 2000); self-sufficiency in growth signals; insensitivity to antigrowth signals; evading apoptosis; limitless replicative potential; sustained angiogenesis; tissue invasion and metastasis. The specific genes altered during tumorigenesis differ between different cancers, but for some cancers the progression from benign neoplasm to a malignant tumor can be described by a linear/unidirectional model of genetic alterations. The prime example is colorectal carcinoma (Kinzler and Vogelstein, 1996), but similar pathways have been described also for malignant glioma (Kleihues and Ohgaki, 1999). In this thesis the involvement of receptor tyrosine kinases (RTK) and especially the ErbB family of RTK in cancer is discussed, together with the findings of a novel ErbB receptor regulatory protein family, the LRIG-family.

2. RTK family and human cancers

All cells survey their environment through different kinds of receptors, which are localized inside the cell and at the cell surface. These receptors and their ligands regulate cellular processes like metabolism, proliferation, and differentiation. The RTK represent one prominent class of cell-surface receptors. Upon binding of extracellular ligands, they transduce signals into the cells that often result in increased cell proliferation, survival, and migration. Accordingly, deregulation of RTK signaling is often seen in cancer (Blume-Jensen and Hunter, 2001). In fact, deregulation of RTK signaling contributes to at least half of the acquired capabilities described by Hanahan and Weinberg as the hallmarks of cancer (Hanahan and Weinberg, 2000). The human RTK family comprises 58 members, divided into 20 subfamilies (Fig. 1).

3. The ErbB RTK family

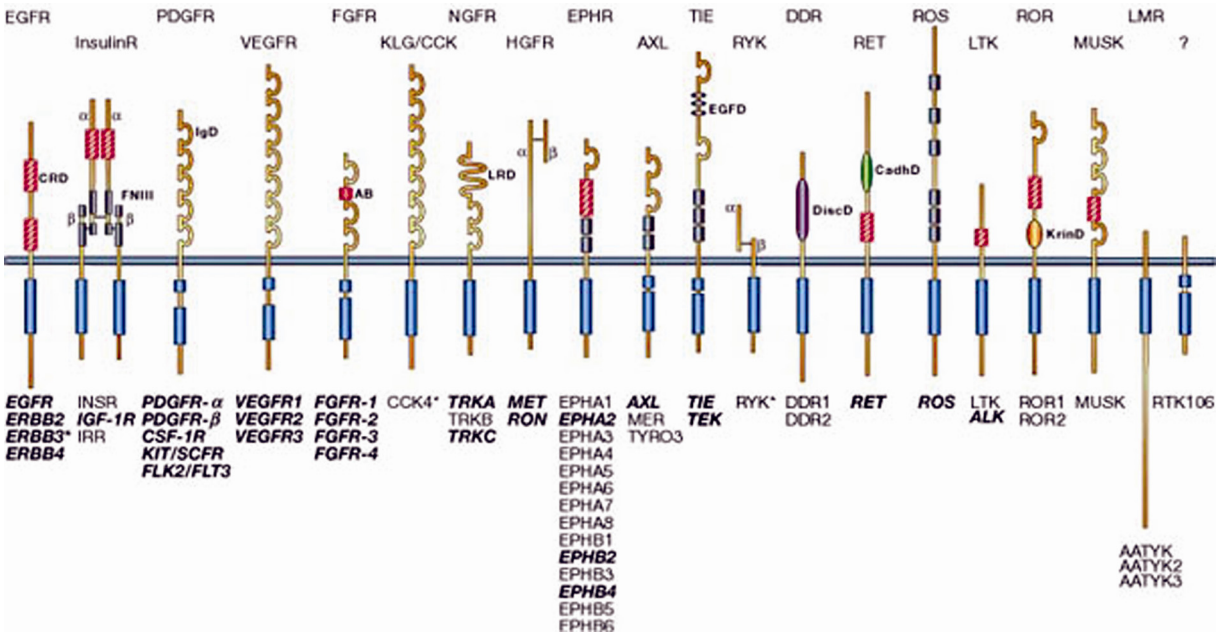

Figure 1: The human receptor tyrosine kinase family. The prototypic receptor for each subfamily is indicated above the respective receptor, and the subfamily members are listed below. Receptors which are listed in bold and italic type have been implicated in human malignancies. An asterisk indicates that the receptor is devoid of intrinsic kinase activity. Figure adopted from (Blume-Jensen and Hunter, 2001) with permission from Macmillan Publishers Ltd.

One of the first proto-oncogenes described in the literature was the epidermal growth factor receptor (EGFR, ErbB1), a member of the ErbB RTK subfamily (Downward et al., 1984). The human ErbB subfamily consists of four receptors, ErbB1 (also called EGFR or HER1), ErbB2 (Neu or HER2), ErbB3 (HER3), and ErbB4 (HER4). All RTK, including the ErbB receptors, consist of an extracellular ligand binding domain, a hydrophobic transmembrane domain, and a cytoplasmic part including the protein kinase domain (Fig. 2). ErbB1 was found as a human homolog of the avian erytroblastosis virus transforming oncogene, *v-ErbB* (Downward et al., 1984). The human cellular gene is located on chromosome 7p12 and encodes a 175 kDa glycoprotein. The human ErbB2 gene is located on chromosome 17q21 and encodes a 185 kDa glycoprotein (Coussens et al., 1985). The human ErbB3 gene is located on chromosome 12q13 and encodes a 160 kDa glycoprotein (Kraus et al., 1989; Plowman et al., 1990). ErbB3 is a kinase-dead RTK. The kinase deficiency makes signaling through ErbB3 dependent on heterodimerization with other ErbB family members. The human ErbB4 gene is located on chromosome 2q34 and encodes a 180 kDa glycoprotein (Plowman et al., 1993; Zimonjic et al., 1995). ErbB4 is unique among the ErbB receptors in

that it has at least four structural isoforms. The ErbB4 isoforms differ at two sites, at an extracellular proteolytic cleavage site and at an intracellular phosphatidylinositol-3 kinase (PI3K) binding site (Junttila et al., 2005; Maatta et al., 2006).

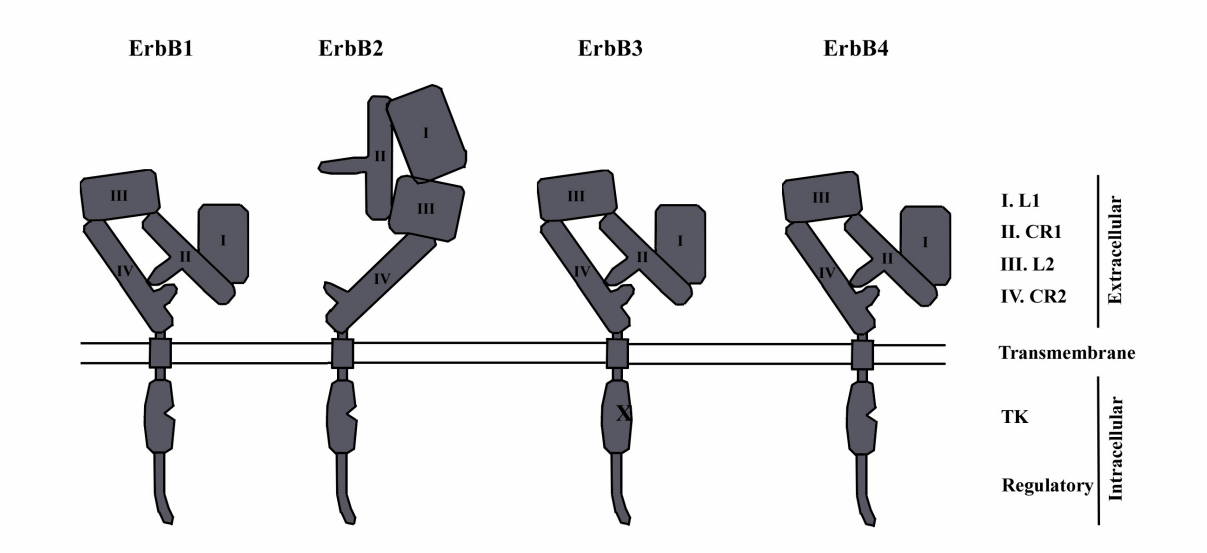

Figure 2: The ErbB family consists of four structurally similar members, ErbB1-4. The extracellular ligand binding domains contain two ligand interacting domains, (L1 and L2), and two cystein-rich dimerization domains (CR1 and CR2). Intramolecular interactions between CR1 and CR2 are responsible for the closed configuration of unstimulated ErbB1, ErbB3, and ErbB4. ErbB2 is defective in the CR1-CR2 interaction, resulting in a constitutively open configuration. The intracellular parts consist of a tyrosin kinase domain (TK) and a regulatory domain. The ErbB3 kinase domain is, however, kinase-dead (**X**). Structures modified from (Burgess et al., 2003).

3.1 Ligands of the ErbB receptors

There are at least ten ErbB receptor ligands (Fig. 3) (Harris et al., 2003; Olayioye et al., 2000). Epidermal growth factor (EGF), amphiregulin, and transforming growth factor-α (TGF-α) bind to ErbB1. Betacellulin, heparin binding EGF-like growth factor (HB-EGF), and epiregulin bind both to ErbB1 and ErbB4. The neuregulins 1-4 (NRG1-4) exist in different splice variants and bind ErbB3 and ErbB4. NRG1-2 bind to both receptors, whereas NRG3-4 bind only to ErbB4. The ErbB proligands are expressed as transmembrane precursors which are proteolytically cleaved to generate soluble and functionally active ErbB ligands (Harris et al., 2003). Genetic knock-out of one of the proteases, TACE/ADAM17 (Peschon et al., 1998), gives a similar phenotype as seen in animals lacking either ErbB1 (Sibilia and Wagner, 1995) or TGF-α (Luetteke et al., 1993), demonstrating the importance of proteolytic processing of the TGF-α proligand. The evolution of several ligands for each ErbB receptor and the

differential expression of ErbB ligands during development and in adult tissues highlights the importance of tight and differential regulation of ErbB receptor signaling.

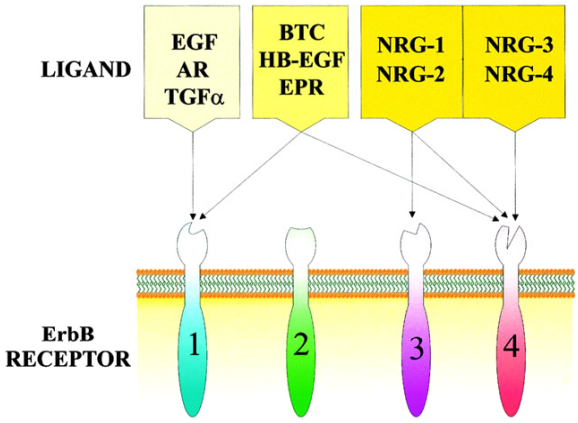

Figure 3: Four categories of ErbB receptor ligands. ErbB1-binding, EGF, AR, and TGF-α; ErbB1- and ErbB4-binding, BTC, HB-EGF, and EPR; ErbB3- and ErbB4-binding, NRG-1, and NRG-2; and ErbB4 binding, NRG-3, and NRG-4. Adopted from (Olayioye et al., 2000) with permission from Macmillan Publishers Ltd.

3.2 Activation of the ErbB receptors

ErbB activation results from receptor homo- or heterodimerization. Receptor dimerization is governed by intermolecular interactions between the extracellular dimerization loops of respective ErbB receptor. In their inactive, ligand-less, states, ErbB1, ErbB3, and ErbB4 have closed configurations, with their dimerization loops intramolecularly sequestered (Fig. 4).

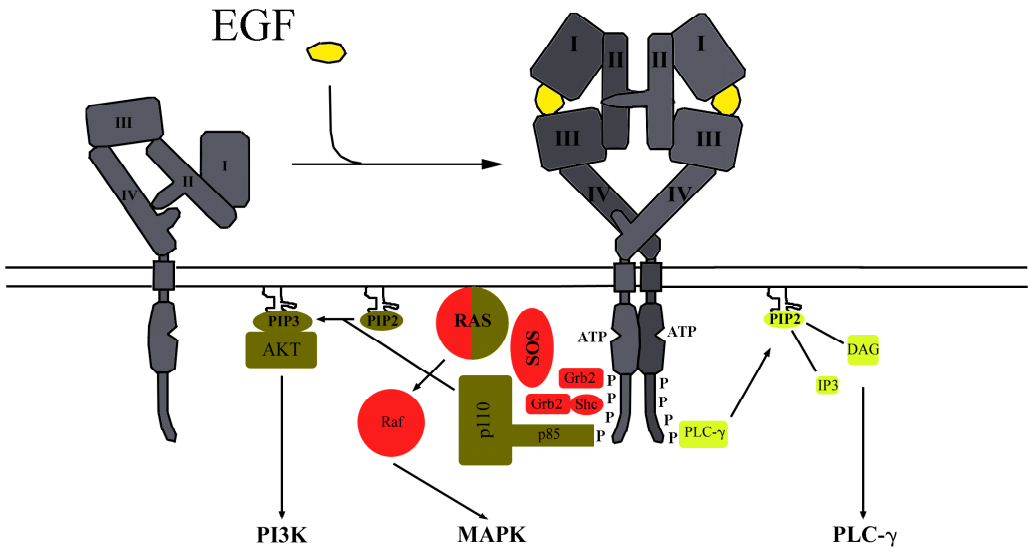

Figure 4: Activation of ErbB receptors. Unstimulated receptors open up after ligand-binding, exposing the dimeriziation arms (II and IV). Dimerization brings the intracellular regions in close proximity, thereby initiating autophosphorylations of the regulatory domains. The phosphorylated tyrosines serve as docking sites for various signaling and adaptor proteins, which initiate downstream signaling pathways (PI3K, MAPK, and PLC-γ pathways).

This prevents the formation of stable homo- or heterodimers in the absence of ligand. ErbB2, in contrast, has a constitutively open configuration with its dimerization loops exposed (Fig. 2). This makes ErbB2 constitutively competent to heterodimerize even though it lacks a ligand of its own. ErbB2 does not normally homodimerize due to electrostatic repulsion between ErbB2 receptors (Garrett et al., 2003). Upon binding of ligand to ErbB1, ErbB3, or ErbB4, the receptors' configuration open up and the dimerization loops get exposed, which enable the formation of receptor homo- and heterodimers (Fig. 4). Formation of receptor dimers bring the intracellular tyrosine kinase domains in close proximity to each other (Burgess et al., 2003), resulting in transphosphorylation of the cytoplasmic regulatory domains of the receptors. The phosphorylated tyrosine groups then serve as docking sites for various adaptor and signaling proteins (Fig. 4). These docking proteins propagate and amplify the extracellular ligand-induced signal into the intracellular space.

3.2.1 ErbB signal transduction

The ErbB receptor subfamily impinge on three prominent signaling pathways, the mitogen-activated protein kinase (MAPK) pathway, the PI3K pathway, and the phospholipase C (PLC) -γ pathway (Fig. 4). In general, the MAPK pathway is involved in cell differentiation and in proliferative responses to receptor activation (Marshall, 1995). The PI3K pathway enhances cell proliferation and resistance to apoptosis through activation of the AKT and PDK1 kinases and regulates the cytoskeleton through Rho kinases (Hall, 1998), The PLC-γ pathway is involved in remodeling of the cytoskeleton and is important for cell motility (Chen et al., 1994; Wells et al., 1998). In addition to these three canonical pathways, ErbB receptors activate other signaling pathways, and hyperactivation of ErbB signaling might even activate non-physiological signaling pathways (Jones et al., 2006). The composition of the receptor-ligand complexes determines the internalization and recycling rate of the receptors (see below), as well as the phosphorylation pattern of the regulatory domains, which influences which docking proteins that are recruited and the downstream cellular response (Fig. 5) (Sweeney and Carraway, 2000; Sweeney et al., 2000).

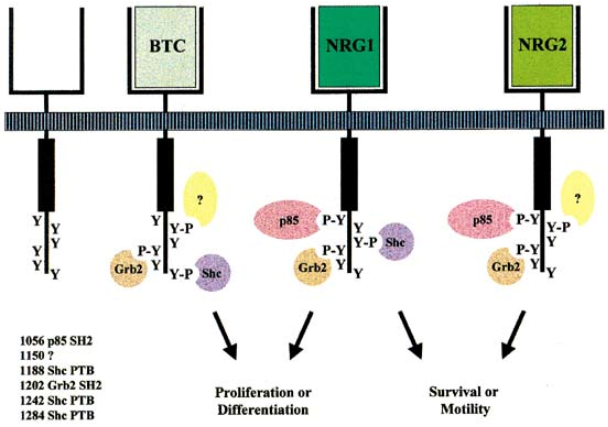

Figure 5: Ligand discrimination. Sweeney and Carraway III have shown that different ErbB4 ligands activate the ErbB4 receptor differently, resulting in differences in phosphorylation pattern, the repertoire of recruited proteins, and cellular outcome. Adopted from (Sweeney and Carraway, 2000) with permission from Macmillan Publishers Ltd.

3.2.2 Internalization of ErbB receptors

Within minutes after binding of ligand and receptor activation, the ErbB1 receptor complexes are internalized by clathrin-coated vesicles and transferred to the endosomal compartment. From the early endosomes, the receptors are either recycled back to the cell surface or targeted for lysosomal degradation. The ligand species and the receptor dimer composition (ErbB1 homodimers or ErbB1 heterodimers with ErbB2, ErbB3, or ErbB4) influence the stability of the receptor complex and how the signal is transmitted inside the cell. Only ErbB1 undergoes ligand-induced internalization. The other ErbB receptors are subject to ligand-induced internalization only when associated as heterodimers with ErbB1. During the maturation of the endosomes, the pH decreases and pH-stable ligand-receptor complexes proceed to the late endosomes and are later degraded in the lysosomes, whereas pH-unstable complexes dissociate and are recycled back to the cell surface (Haigler et al., 1979; Wiley, 2003). The stability of receptor complexes depends on the bound ligand and the receptor-dimer composition. EGF, for example, binds stronger to ErbB1 than TGF-α does. EGF, therefore, preferentially targets ErbB1 for degradation, while TGF-α preferentially targets ErbB1 for recycling. Accordingly TGF-α is more mitogenic than EGF, since TGF-α stimulated receptors are recycled back to the cell surface where they become available for repeated ligand activation (Baass et al., 1995). The composition of the receptor dimers also affect the internalization rate, where homodimers of ErbB1 are more rapidly internalized than heterodimers between ErbB1 and ErbB2, ErbB3, or ErbB4 (Lenferink et al., 1998).

Another level of RTK signal regulation is inherent in the internalization process itself, where different signaling pathways are differently affected by receptor internalization. Vieira et al. (Vieira et al., 1996) showed that prolonged retention of activated receptors at the plasma membrane caused hyperphosphorylation of Shc and PLC-γ, whereas, PI3K and MAPK were hypophosphorylated. This is further supported by Haugh et al. (Haugh et al., 1999a; Haugh et al., 1999b), who found that receptor internalization abrogated PLC-γ signaling, whereas, signaling through Ras continued. So, both the PI3K and MAPK pathways can be activated by Ras after receptor internalization, whereas, PLC-γ is only activated at the plasma membrane.

3.3 Regulators of ErbB signaling

To dampen and terminate ErbB signaling, various regulatory proteins are important. The negative regulators discussed below are activated by receptor signaling; they interact with the active receptors and downregulate their function in negative feedback loops. Such negative feedback loops is a common feature of RTK regulation. The negative regulators can be divided into two groups, early and late attenuators. Early attenuators, such as c-Cbl are activated by receptor signaling and have direct effects on the receptors and the duration of signaling (Rubin et al., 2005). Late attenuators, such as RALT and Sprouty, in contrast, are transcriptionally induced by receptor signaling (Rubin et al., 2005), and fine tune the signals, making the receptors respond to external stimuli in a context dependent manner.

3.3.1 Receptor stability and degradation – Early attenuators

The E3 ubiquitin ligase c-Cbl is a prime example of an early attenuator. c-Cbl interacts with ErbB1 after ligand-induced activation of the receptor and transfers ubiquitin molecules to the receptor (Levkowitz et al., 1999). Ubiquitylated ErbB1 is thereby targeted to the lysosome where it is degraded. The ubiquitylation process takes place in two steps, first at the plasma membrane, where c-Cbl causes mono-ubiquitylation of ErbB1, which functions as protein docking sites in the internalization process; and second, in the endosomal compartment, where ErbB1 is further ubiquitylated and thereby targeted to the lysosome (Marmor and Yarden, 2004; Rubin et al., 2005). In cells with the c-Cbl gene ablated, the internalization process appears functional, but the sorting in the late endosomal compartment to the multivesicular bodies (MVB) and later lysosomal degradation is abrogated (Duan et al., 2003). Thus, c-Cbl is dispensable for receptor internalization, however, c-Cbl is essential for the sorting of ErbB1 in the late endosomes for degradation in the lysosomes (Duan et al.,

2003). ErbB2, ErbB3, and ErbB4 are not ubiquitylated by c-Cbl under physiological conditions, whereas another E3 ubiquitin ligase, Nrdp1, interacts with ErbB3 and ErbB4 and shortens the half-life of ErbB3 (Qiu and Goldberg, 2002; Sweeney and Carraway, 2004). This suggests that Nrdp1 and maybe other E3 ligases can target ErbB receptors for degradation in a c-Cbl independent manner. Ubiquitylation may also influence the degradation of the receptors indirectly by targeting components of the internalization machinery for degradation, since some of the proteins directing the internalization process are themselves subject to ubiquitylation (Katz et al., 2002; van Delft et al., 1997).

3.3.2 Fine tuning of ErbB signaling – Late attenuators

Receptor-associated late transducer (RALT), also named mitogen induced gene-6 (MIG-6) was found as a component of a negative feedback loop that downregulated the mitogenic signals from the ErbB receptors (Hackel et al., 2001; Anastasi et al., 2003). RALT is transcriptionally upregulated by TGF-α, EGF, and NRG1 through the MAPK pathway. RALT controls signal output strength and duration from the ErbB receptors, suppressing the activity of the different ErbB receptors differentially. The effect of RALT on ErbB4 signaling is an abrupt and robust inhibition of both the MAPK and PI3K pathways. ErbB1 signaling is also inhibited by RALT, but not as profoundly as ErbB4 signaling is. ErbB2/ErbB3 heterodimer signaling is inhibited by RALT, but much later than ErbB4 and ErbB1 homodimer signaling are (Anastasi et al., 2003). Thus, RALT functions as a context dependent regulator of signal duration and favor signaling by certain receptor complexes over others.

In mammals, the Sprouty (Spry) family consists of four members, of which Spry2 and Spry4 are transcriptionally upregulated by EGF stimulation. The *Drosophila* dSpry was first described as a negative regulator of the *Drosophila* fibroblast growth factor (dFGF) receptor (Hacohen et al., 1998), and later as a negative regulator of the *Drosophila* EGF receptor (DER) (Casci et al., 1999). Spry2, the mammalian Sprouty protein with most homology to dSpry, seems to have a dual role, being involved both in positive and negative regulation of the ErbB1 receptor (Egan et al., 2002; Rubin et al., 2003). The positive effect is mediated by the N-terminal part of Spry2, which binds to the ring-finger domain of c-Cbl. Spry2, thereby, competes with ErbB1 for binding of c-Cbl, resulting in decreased ubiquitylation and degradation of ErbB1. The C-terminal part of Spry2, in contrast, localizes the protein to the cell membrane and interacts with signal transduction components downstream of ErbB1,

interfering with the MAPK pathway. Spry2, thus, affects the relative signaling output as well as the duration of receptor signaling, thereby functioning to fine tune ErbB1 signaling.

3.4 ErbB regulation in invertebrates

The ErbB signaling unit was present already before the split of the lineages leading to nematodes, insects, and vertebrates. In the nematode *C. elegance*, the ErbB signaling unit consists of one receptor, *Let-23*, and one ligand*, Lin-3*. *Let-23* is important during worm development and in fate determination of several nematode cell types (Chang and Sternberg, 1999). Signaling by Let-23 is mediated by the Ras-MAPK and inositol trisphopsphate pathways (Clandinin et al., 1998; Kariya et al., 2004). The main negative regulator of *Let-23* signaling is *Sli-1*, the nematode ortholog of mammalian *c-Cbl* (Jongeward et al., 1995).

In the insect *Drosophila melanogaster,* there is one ErbB receptor, DER, and four ligands, Spitz, Keren, Gurken, and Vein (Reich and Shilo, 2002; Shilo, 2003). DER is ubiquitously expressed in the fly and a tight regulation of DER signaling is crucial in *Drosophila* development (Fiorini et al., 2001). There are at least four different negative feedback regulators of DER. *Drosophila* Cbl has the same function as mammalian c-Cbl and nematode Sli-1, which is to downregulate DER signaling by inducing receptor ubiquitylation and degradation. Argos is a secreted protein, which is transcriptionally upregulated by DER signaling. Argos acts in an autocrine/paracrine fashion by binding and sequestering the DER-activating ligand Spitz, thereby inhibiting Spitz-induced receptor signaling (Klein et al., 2004). Kekkon-1 is a transmembrane protein, which extracellular domain physically interacts with DER and thereby inhibits ligand binding and receptor dimerization (Ghiglione et al., 1999). Sprouty, finally, is a promiscuous inhibitor of several RTK, which was originally cloned as a negative feedback inhibitor of the dFGF receptor (Hacohen et al., 1998). Sprouty also negatively regulates DER, by inhibiting the Ras/MAPK pathway via its interactions with Drk (Grb-2) and Gap1 (Casci et al., 1999).

Some ErbB regulatory mechanisms are shared between worm, fly, and mammals, e.g., Sli-1/c-Cbl, which is common to all three. Other regulatory proteins, like Sprouty, have orthologs in fly and mammals, whereas Argos and Kekkon-1, do not seem to have any worm or mammalian orthologs. Instead, proteins with similar structure and function seem to have evolved in parallel, like *Drosophila* Kekkon-1 and mammalian LRIG-1.

3.5 ErbB family members in human cancer

The members of the ErbB subfamily are implicated in many forms of human cancer. Their involvement results from enhanced receptor signaling. Enhanced receptor signaling can be due to gene amplifications and protein overexpression, gene mutations resulting in hyper-activated receptors, or activation of autocrine/paracrine growth factor loops by co-expression of receptors and ligands (Yarden and Sliwkowski, 2001).

Table 1. ErbB1 Overexpression in human tumors

Tumor type	**Overexpression of ErbB1 (% of tumors)**
Colon	25-77%
Head and neck	80-100%
Pancreatic	30-50%
Non-small cell lung carcinoma	40-80%
Breast	14-91%
Renal carcinoma	50-90%
Ovarian	35-70%
Glioma	40-63%
Bladder	31-48%

Modified from Herbst and Shin (Herbst and Shin, 2002).

3.5.1 ErbB1 in human cancer

Overexpression of ErbB1 is often the result of gene amplification, resulting in increased cell surface receptor levels (Table 1). Increased receptor levels lowers the ligand concentration needed for receptor activation, and in combination with either growth factor loops or activating receptor mutations, overexpression can contribute to cell transformation (Yarden and Sliwkowski, 2001). ErbB1 mutations are often seen in various cancers. There are at least nine ErbB1 rearrangement-mutants identified, today (Kuan et al., 2001). Several of these ErbB1 mutants lack parts of the extracellular domain, which plausibly prevents formation of the closed configuration, and hence, make the receptors constitutively dimerization competent and active. The most (in)famous of the ErbB1 mutants is EGFRvIII, which lacks most of the ligand binding domain and the first dimerization loop (deletion of amino acids 6-273). EGFRvIII is found in about 20% of *glioblastoma multiforme* and in a small proportion of breast, lung, and ovary tumors (Moscatello et al., 1995; Wikstrand et al., 1995). Many studies have confirmed a role for EGFRvIII in the genesis and progression of human cancers. The tumorigenic potential of EGFRvIII is due to its stimulation of cell proliferation and survival (Ramnarain et al., 2006), with a strong and persistent signaling through the PI3K pathway

(Mellinghoff et al., 2005). Recent findings have shown that the EGFRvIII activate transcription of a different subset of genes than the wild type receptor (Ramnarain et al., 2006). Genes activated by EGFRvIII include the ErbB1 proligands TGF-α and HB-EGF. Since wild-type receptors are co-expressed with EGFRvIII, the expression of the proligands induced by ErbBvIII activate autocrine growth factor loops, thereby enhancing the tumorigenicity of the cell (Ramnarain et al., 2006). This is in accordance with previous findings that co-expression of ErbB1 and its ligands correlate with hyper-activity of the receptor in non-small cell lung cancer (NSCLC) (Hsieh et al., 2000), and prostate cancer (Seth et al., 1999), and with a worse prognosis in invasive breast cancer (Umekita et al., 2000). Another type of ErbB1 mutations occur in NSCLC, with activating mutations around the ATP binding pocket in the kinase domain. It has been suggested that a majority of the NSCLC tumors that respond to anti-EGFR treatment harbors these types of mutations (Lynch et al., 2004; Paez et al., 2004; Taron et al., 2005). This type of gefitinib and erlotinib sensitive ErbB1 mutants seem to be more or less restricted to NSCLC (Lee et al., 2005). Taken together, ErbB1-driven transformation results from enhanced signaling due to receptor overexpression and/or activating receptor mutations.

3.5.2 ErbB2 and ErbB3 in human cancer

Among ductal breast carcinomas, 20-30% show gene amplification of *ErbB2*, and this correlates with aggressive tumor behavior, including increased tumor size, tumor invasiveness, histological grade, and tumor cell proliferation index (Slamon et al., 1987). The more malignant phenotype associated with *ErbB2* amplification may, in part, depend on increased ErbB2 protein expression and the ability of ErbB2 to decrease the internalization and degradation rates of the other ErbB receptors. ErbB2 forms heterodimers with the other ErbB receptors, making them less degradation prone, and ErbB2 heterodimers are retained longer than other dimers at the cell surface. In a model proposed by Harari and Yarden (Harari and Yarden, 2000), ErbB2 “hotwires” the cell cycle via activation of AKT, thereby activating cyclinD/CDK4/6 complexes which drives dissociation of pRb from E2F-1. Free E2F-1 activates target gene transcription, which protein products force the cell to enter the cell cycle (Fig. 8). ErbB2 increases both the proliferation and survival responses, with signaling through AKT as the most important denominator. The ErbB2/ErbB3 heterodimer is the ErbB complex which supports the strongest mitogenic signal. This is attributed to ErbB2’s ability to increase recycling of internalized receptors and to ErbB3’s ability to support robust

PI3K signaling, which results in prolonged and intense AKT signaling (Waterman et al., 1999).

3.5.3 ErbB4 in human cancer

The involvement of ErbB4 in human cancer is controversial (Gullick, 2003). For example, some studies have shown that overexpression correlates with a good prognosis in breast cancer (Witton et al., 2003), while other studies have found the opposite (Lodge et al., 2003). A study from our group (Thomasson et al., 2004) suggests that ErbB4 might function as a tumor suppressor in renal cell carcinoma. Junttila et al. recently showed that ErbB4 is expressed as four different isoforms, which differ in their cellular functions and transformation potential (Junttila et al., 2000; Junttila et al., 2005; Maatta et al., 2006). Therefore, some previous reports, with conflicting findings regarding the role of ErbB4 in human cancer, must be re-evaluated due to lack of isoform information, since isoform type and their sub-cellular protein localization seems to be important determinants for the transforming effects of ErbB4 (Junttila et al., 2005; Maatta et al., 2006).

3.6 ErbB family members as therapeutic targets in cancer

Conventional treatments using radiotherapy or chemotherapy are more or less toxic for the patients, and the administered doses must therefore usually be limited, in order to avoid severe or life threatening adverse effects. Thus, alternative approaches directed against relevant molecular targets are of great importance to find; to be used alone or to be used to selectively enhance the radio- and chemotherapeutic killing of tumor cells and minimize the side-effects. The RTK family, especially the ErbB subfamily of receptors, has attracted a lot of interest due to the frequent dysregulation of ErbB receptor signaling in human cancers. Dysregulation of ErbB signaling usually correlates with tumor resistance against radio- and chemotherapy. Moreover, both radio- and chemotherapeutics have been shown to activate the ErbB receptors in a ligand-independent manner, thereby enhancing the resistance even in tumors without initial alterations of ErbB activity (Benhar et al., 2002; Dent et al., 1999; Schmidt-Ullrich et al., 2003). Breaking the resistance is crucial in sensitizing the tumor for conventional therapeutic regimes, so that effective treatments can be obtained. Numerous approaches have been devised to target ErbB family members, including anti-receptor monoclonal antibodies (mAbs), small molecule kinase inhibitors, ligand conjugates, immunoconjugates, and antisense oligonucleotides (Fig. 6). There are to date, four clinically

approved drugs that specifically target ErbB family members, however, many more are expected to be introduced in the clinic in the next few years.

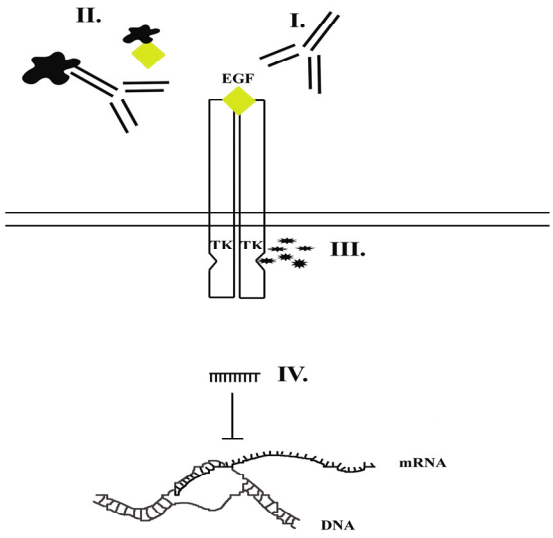

Figure 6: Anti-ErbB-targeted approaches. **I.** Monoclonal antibodies (mAb) against the extracellular domain, **II.** Conjugates of ErbB mAb or ErbB receptor ligand and cytotoxic compounds, **III.** Small molecule inhibitors of the tyrosine kinase, and **IV.** Antisense based therapies.

3.6.1 Anti-ErbB-mAbs

Several ErbB mAbs have completed phase-III trials and are now commercially available. The ErbB2 mAb, trastuzumab (Herceptin®), is used in the treatment of breast cancer (Plosker and Keam, 2006). The ErbB1 mAb, cetuximab (Erbitux®), is approved for treatment of colorectal cancer in combination with chemotherapy, and in head and neck squamous cell carcinoma in combination with radiotherapy (Bonner et al., 2006). ABX-EGF (Panitumumab), another ErbB1 mAb, is undergoing phase-II trials for first line therapy in various tumor types, including colorectal carcinoma, NSCLC, and renal cell carcinoma. Although intravenous delivery is needed, the anti-ErbB mAb treatments are usually well-tolerated without severe side effects. Thus, in contrast to its essential functions during development (Sibilia and Wagner, 1995; Miettinen et al., 1995; Sibilia et al., 1998), ErbB1 function seems dispensable for most aspects of adult life. The major side-effects associated with ErbB1-directed therapies are associated with the function of skin and mucosa, such as the frequently reported acne-like skin rashes.

3.6.2 Small molecule kinase inhibitors

Small inhibitory molecules targeting the ATP binding site in the kinase domain of the ErbB receptors have recently been introduced in the clinic. These compounds prevent transphosphorylations in the receptor dimers and, thus, transduction of the downstream signals. Among these compounds, the ErbB1 specific inhibitors erlotinib (Tarceva®) and gefitinib (Iressa®) are used in NSCLC (Cascone et al., 2006; Cohen et al., 2004; Frampton and Easthope, 2005), and causes significant therapeutic responses in a subgroup of patients. Although significant clinical responses are seen in various ethnicities, patients with south-east Asian ethnicity display in general a high sensitivity to erlotinib and gefitinib compared to other populations. Moreover, non-smoking patients seem to respond better than smokers (Taron et al., 2005). Recently, it was found that several of the responding patients had mutations around the ATP binding pocket of the ErbB1 tyrosine kinase domain, which most likely, at least in part, explains the good therapeutic effects in this subgroup of patients. There are also other factors influencing the responses to treatment with ErbB inhibitors, such as of ErbB1 gene amplifications, which correlate with therapy responsiveness (Cappuzzo et al., 2005). It has also been proposed that a subgroup of GBM patients, with mutant EGFRvIII together with normal PTEN activity, respond favorably to gefitinib or erlotinib treatment, whereas patients with mutated PTEN responded poorly (Mellinghoff et al., 2005). These heterogeneous responses to tyrosine kinase inhibitors point toward the need of sensitive tools characterizing individual tumors, which distinguish responsive subgroups from the large unresponsive population in order to optimize the treatment for each individual patient.

3.7 Molecular pathology of astrocytic tumors

Astrocytic tumors are the most common malignancies of the central nervous system, accounting for more than 60% of all brain tumors (Kleihues and Cavenee, 2000). In its most severe form, g*lioblastoma multiforme* (GBM), or grade IV astrocytoma, it has a dismal prognosis with an average survival time of about one year. Dysregulation of RTK signaling is strongly associated with GBM and the tumor aggressiveness correlates with hyperactivity of the ErbB1 receptor. GBM are, thus, good candidates for trials with ErbB directed therapies. Molecular characterization of GBM has revealed two distinct tumor transformation pathways, a primary, *de novo*, and a secondary, progressive pathway (Fig. 7) (Kleihues and Ohgaki, 1999; Ichimura et al., 2004).

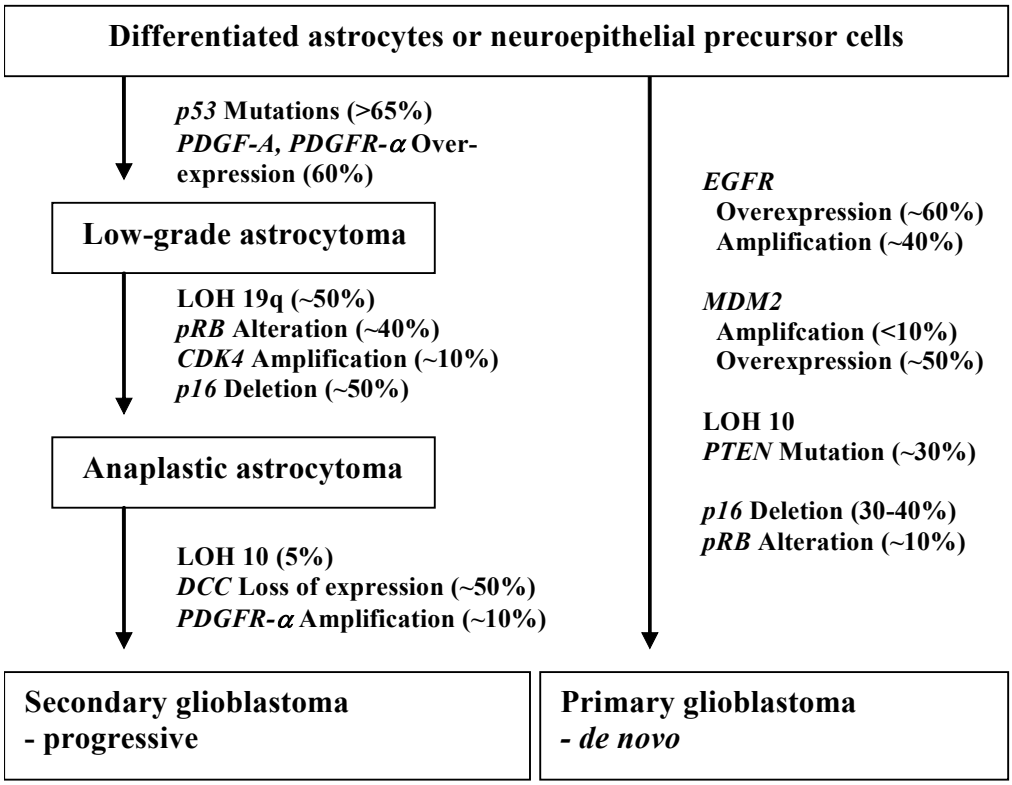

Figure 7: Molecular pathways of glioblastoma transformation. Molecular characterization of astrocytic tumors and GBM has reveled two subgroups of GBM with distinct molecular pathologies. Modified from (Kleihues and Ohgaki, 1999) and (Benjamin et al., 2003).

Primary glioblastoma, arises *de novo*, without any past clinical history. These tumors are often seen in older patients (mean age, 55 years). Secondary glioblastoma originates from low-grade astrocytomas (grade II) that stepwise progress to a grade IV tumor (GBM). These tumors usually affect younger people (mean age, 40 years). In both of the GBM subtypes, genetic alterations in at least three signaling pathways give rise to the malignant phenotype (Fig. 8). The dysregulated pathways are: the RTK pathway, the cell cycle entry retinoblastoma (Rb) pathway, and the DNA damage control p53 pathway (Kleihues and Ohgaki, 1999).

The two most studied RTK pathways in glioblastoma tumor transformation are the ErbB1 pathway and the platelet derived growth factor receptor (PDGFR) pathway. In a large proportion of *de novo* GBM, the *ErbB1* gene is amplified (40%) and/or over expressed (60%) (Fig. 8). In progressive GBM, the PDGFR-α is overexpressed together with its ligand PDGF-A in the early stages (60%) and the *PDGFR-α* gene is amplified in the late stages (10%) (Kleihues and Ohgaki, 1999). Dysregulation of these RTK pathways desensitize the cell to

regulatory control, by hyper-activation of downstream pathways, such as the MAPK- and the PI3K pathways. The latter pathway is a major determinant in protecting the cell against apoptosis, through its activation of AKT, which inhibits the function of the proapoptotic protein Bad (She et al., 2005). Activation of AKT and MAPK pathways via Ras also stimulate cell proliferation, mainly by influencing the expression, stability, and subcellular localization of proteins that are involved in cell cycle regulation (Jones and Kazlauskas, 2001), such as Myc (Sears et al., 1999) (Nasi et al., 2001), Mdm2 (Roth et al., 1998; Zhou et al., 2001b), CyclinD (Diehl et al., 1998; Jones and Kazlauskas, 2001), $p21^{cip1}$ (Zhou et al., 2001a), and AFX/FKHR (Kops et al., 1999; Medema et al., 2000) (Fig. 8).

The retinoblastoma (Rb) pathway regulates entry into the cell cycle. Cyclin/CDK (cyclin dependent kinase) complexes phosphorylate pRb, which thereby is released from the transcription factor E2F-1. Free E2F-1 then activates transcription of genes that drives the cell into the cell cycle. Perturbations of the Rb pathway in GBM involve the pRb protein itself, the CDK4 protein, or the Cyclin-CDK complex inhibitor, p16 protein (Fig. 8). In *de novo* GBM, both *pRb* alterations (10%) (Benjamin et al., 2003) and *p16* deletions (30-40%) are seen, but these aberrancies are mutually exclusive (Kleihues and Ohgaki, 1999). In progressive GBM, *pRb* alterations (40%) (Benjamin et al., 2003), *CDK4* amplifications (10%) (Benjamin et al., 2003), and loss of *p16* (50%) (Benjamin et al., 2003) are seen. These changes take place during transformation from low grade astrocytomas (II) to anaplastic astrocytoma (grade III).

The p53 pathway constitutes a surveillance mechanism that responds to intrinsic or extrinsic stress signals (Harris and Levine, 2005). p53, thus, activates genes which products can arrest the cell in the cell cycle, induce apoptosis, or are involved in DNA repair. Therefore, loss of p53 function results in increased genomic instability which facilitates transformation of neoplastic cells (Harris and Levine, 2005). Perturbation of the p53 pathway is one of the first steps in progressive GBM, where more than 65% of tumors show *p53* mutations. *De novo* GBM do not show *p53* mutations, instead they show amplification (10%) or overexpression (50%) of the p53 inhibitor Mdm2, an ubiquitin E3 ligase that causes degradation of the p53 protein (Fig. 8) (Kleihues and Ohgaki, 1999).

The genetic abnormalities described above are not the only alterations occurring in GBM, but they represent general genetic alterations found in a large proportion of astrocytic tumors. A summary of the discussed genetic alterations in *de novo* and progressive GBM is shown in Fig. 8. The recognition of apparent subtypes of GBM can, in the future, be important for the treatment of these tumors and in the development of new targeted approaches.

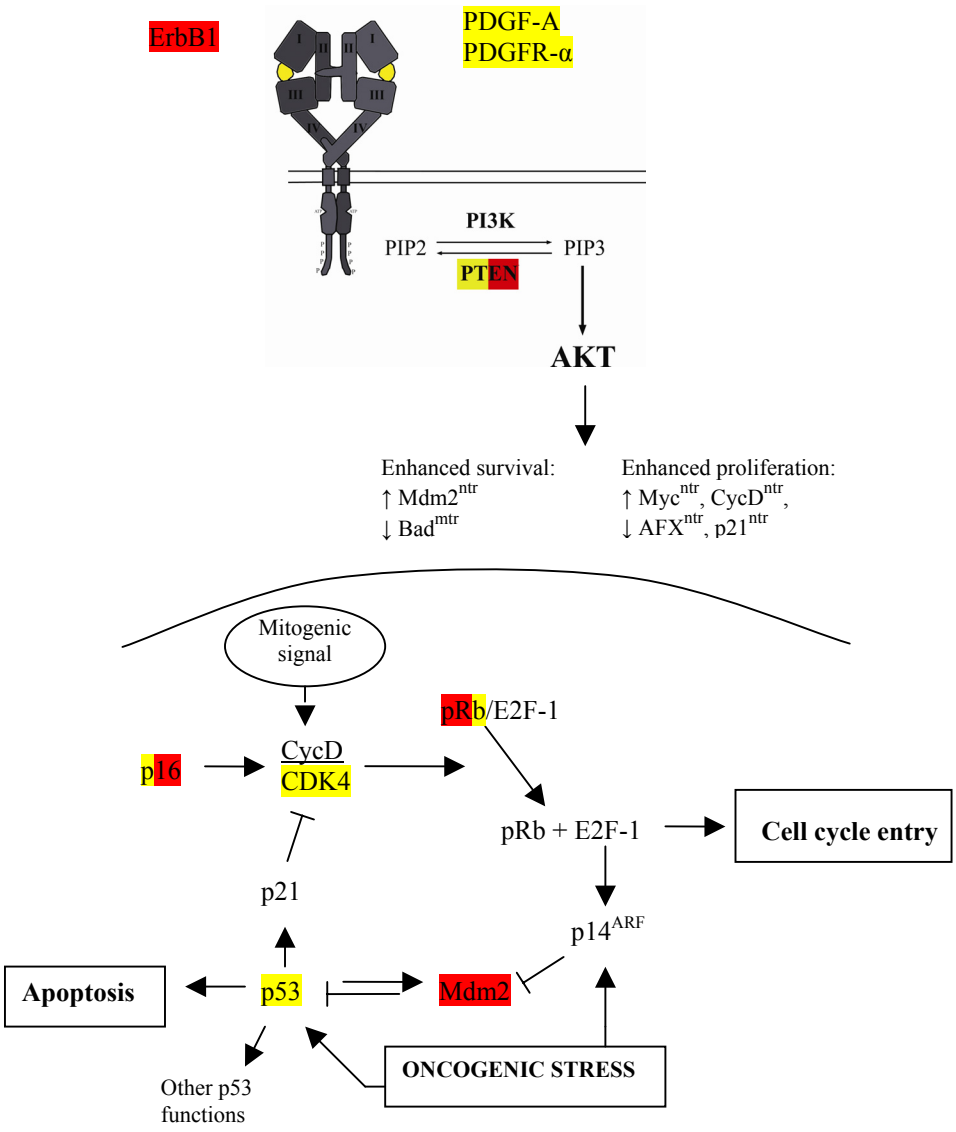

Figure 8: Molecular pathways in GBM transformation. A schematic representation of pathways involved in the transformation of GBM and how they are interlinked. Yellow boxes indicate alterations caused in the progressive GBM and red boxes by alterations in *de novo* GBM. Lines with arrowheads and crossed strokes represent activating and inhibiting actions, respectively. ^{ntr} and ^{mtr} represent nuclear and mitochondrial translocations, respectively.

4. The rational for the present study

The present study was initiated as an effort to identify endogenous human suppressors of ErbB receptor signaling. Initially we searched for a human ortholog of the *Drosophila* inhibitor of DER, Kekkon-1. We did not find a human Kekkon-1 ortholog; however, we noticed in the literature a mouse gene, *Lrig1* (formerly Lig-1), which was expressed in glial cells and showed structural similarities to Kekkon-1. Now, with the sequencing of the human and *Drosophila* genomes, it does not appear to exist a true ortholog of Kekkon-1 in man. Nevertheless, we identified the human LRIG proteins and found that LRIG1 suppresses the human ErbB receptors.

5. Aims of the thesis

The aims of this thesis were to:

- Identify and characterize the human LRIG family members.
- Elucidate the molecular functions of LRIG1.
- Investigate the expression pattern of the LRIG proteins in astrocytic tumors and their correlation to clinical parameters.

6. Results and Discussion

6.1 Cloning and identification of human *LRIG1* (I) and *LRIG2* (III)

Because of the structural similarities between *Drosophila* Kekkon-1 and mouse Lrig1 (previously LIG-1) we cloned and analyzed human LRIG1. By using BLAST, a human expressed sequence tag (EST) (AA293029) was identified, with high similarity to a region of the mouse *Lrig1* mRNA sequence. From the human EST sequence, primers and probe for real-time PCR were designed. Real-time PCR was then used to screen an arrayed human brain cDNA library for LRIG1 cDNAs. One of the isolated cDNA clones showed high homology with mouse *Lrig1*, but it lacked the 5'end. The missing 5' sequence was obtained by RT-PCR from a combination of brain and glioma (U-105 MG) total RNA. The cloned human *LRIG1* cDNA consisted of 4763 nucleotides with a 3279 nucleotide coding sequence and a long 3'

untranslated region. The sequence was deposited in GeneBank with the accession no. AF381545 (**I**).

LRIG2 was identified in a BLAST search for human *LRIG1* homologs in GeneBank. A deposited mRNA sequence, KIAA0806 (AB018349), was identified, which showed high homology with *LRIG1*. Based on the KIAA0806 sequence a LRIG2 cDNA was cloned from a human brain cDNA library. Due to the high homology with the *LRIG1* gene, the gene was named *LRIG2* (**III**). The third human *LRIG* gene family member, *LRIG3,* was identified in our laboratory by Guo Dongsheng et al. in 2004 (Guo et al., 2004).

6.2 Gene structure and genomic localization (I and III)

The *LRIG1* and *LRIG2* genes consist of 19 exons spanning 121 kb and 50 kb, respectively. In paper **I** we indicated that *LRIG1* spanned 200 kb, however, this was based on a draft sequence of the human genome which subsequently has been extensively revised in the *LRIG1* region. The size of the *LRIG1* gene is, thus, most likely around 121 kb. Fluorescence in-situ hybridization (FISH) revealed that *LRIG1* and *LRIG2* were localized at chromosome bands 3p14 and 1p13, respectively. Both of these genomic regions are frequently deleted in human cancers (Mitelman et al., 2006). Genetic instability around 3p14 has been associated with the fragile site FRA3B (Becker et al., 2002) and loss of heterozygosity (LOH) is commonly found in various cancers, including carcinomas of the breast, kidney, and lung (Maitra et al., 2001; Mitelman et al., 2006). The instability of the 3p14 region is further emphasized by the recent finding of a common amplification of this region in breast cancer (Ljuslinder et al., 2005). Similar to the *LRIG1* locus, the genomic region around the *LRIG2* locus at 1p13, is frequently deleted in human cancers. Deletions of the short arm of chromosome 1 (1p) and the long arm of chromosome 19 (19q) are the most common genetic alterations found in oligodendroglioma (Kleihues and Cavenee, 2000). LOH of 1p is also often observed in meningioma, head and neck squamous cell carcinoma, NSCLC, renal cell carcinoma, and breast cancer (Knuutila et al., 1999). Further studies are needed to elucidate how the chromosomal instabilities at 3p14 and 1p relate to tumor transformation.

6.3 LRIG protein structure and sub-cellular distribution (I, II, III, IV, and V)

The LRIG protein structures were predicted to be type I transmembrane glycoproteins consisting of a signal peptide, followed by 15 leucine-rich repeats with cysteine-rich N- and

C-flanking regions, three immunoglobulin (Ig)-like domains, a transmembrane domain, and a cytoplasmic domain (**I, III**). Amino acid sequence stretches in the extracellular, transmembrane, and intracellular juxtamembrane parts were highly homologous between the LRIG family members (**III**). Less similarity was found in the membrane-distal cytoplasmic parts (**III**). LRIG antibodies were generated against intracellular peptides (**II, III**) and used in immunohistochemical and Western blot analyses. The glycoproteinic nature of LRIG1 and LRIG2 was investigated by digesting the proteins with N-glycosidase F, which specifically cleaves off asparagine-linked oligosaccharides. N-glycosidase F treatment resulted in reduced apparent weights of the proteins, showing that LRIG1 and LRIG2 were glycoproteins with asparagine-linked carbohydrates (unpublished results, **III**). The subcellular distribution of the LRIG proteins was investigated by cell surface biotinylation experiments, confocal fluorescence laser microscopy, and immunohistochemistry (**II, III, IV,** and **V**). The LRIG proteins were mainly localized to the plasma membrane and to the perinuclear area (**II, III, IV,** and **V**). In the polarized epithelial Madin-Darby canine kidney (MDCK) cell line, ErbB proteins localize to the basolateral surface, and a similar distribution was observed for the LRIG1 protein (**IV**). The perinuclear localization of LRIG1 was further analyzed with confocal fluorescence laser microscopy and there LRIG1 showed co-localization with markers for early endosomes (EEA-1) and the trans-Golgi network (AP-1) (**IV**). The LRIG1 protein has potential intracellular and extracellular proteolytical cleavage sites. Accordingly, we have detected apparent cleavage fragments of both ectopically overexpressed and endogenous LRIG1 proteins (unpublished results, **II**). The biological significance of LRIG protein cleavage and differential subcellular protein distribution is not known, however, the subcellular LRIG protein localization seems important for the outcome of patients with astrocytic tumors (**V**, see below). Protein sorting and localization to different subcellular compartments is fundamental to eukaryotic cellular function, allowing compartmentalization of biochemical processes. The questions of how the subcellular localization of LRIG proteins is regulated, and the consequences thereof, are open, and further studies are needed.

6.4 Expression of LRIG1 and LRIG2 in normal tissues (I, II, III, and V)

The *LRIG* mRNA in tissues were analyzed by both Northern blotting and quantitative real-time RT-PCR. From the Northern blot analyzes, the transcript sizes for *LRIG1* and *LRIG2* were determined to 5.5 kb and 4.8 kb, respectively (**I, III**). The LRIG2 transcript in heart was slightly smaller (4.6 kb) than in the other tissues (4.8 kb). We found no other evidence for alternative splicing of the *LRIG1* or *LRIG2* transcripts. *LRIG* mRNA expression was seen in

all tissues analyzed, but with a wide range of expression levels. *LRIG1* and *LRIG2* showed unique, but overlapping expression patterns. The highest mRNA expression levels for *LRIG1* were in brain, kidney, lung, and mammary glands (**I**), whereas the highest levels for *LRIG2* were in skin, uterus, and ovary (**III**). Notably, in mammary gland, *LRIG1* was highly expressed and *LRIG2* almost undetectable, whereas in placenta, *LRIG1* was almost undetectable and *LRIG2* was highly expressed.

LRIG protein expression was analyzed by Western blotting and immunohistochemistry. There was a good correlation between mRNA and protein expression, especially in tissues with high expression (**II** and **III**). By immunohistochemistry, LRIG1 showed highest protein expression in peptic cells of the stomach, glandular cells in prostate, bronchus tissue, and epithelial cells of breast tissues. LRIG2 was not analyzed by immunohistochemistry. The ubiquitous expression of the LRIG-family proteins indicates that they may be involved in fundamental functions of mammalian cells. Regulation of growth factor signaling may be such a fundamental function, which is shared by most, if not all, mammalian cells (see below).

6.5 LRIG1 driven degradation of ErbB family proteins (IV)

In paper **IV**, we found that LRIG1 participated in an EGF-driven negative feedback loop, where EGF-stimulated ErbB1 signaling resulted in LRIG1 expression and LRIG1-directed ubiquitylation and degradation of the ErbB1 receptor. *LRIG1* mRNA expression was induced 2-4 fold, four hours after EGF-stimulation of HeLa cells. Similarly, LRIG1 protein increased in a time-dependent manner following EGF-stimulation of these cells. The LRIG1-ErbB interactions were examined by co-immunoprecipitation experiments and LRIG1 was, thus, found to physically interact with all four members of the ErbB subfamily. The ErbB1 interacting domains of LRIG1 were determined by LRIG1 deletion constructs. Both the LRR and the Ig-like domains recognized and bound to ErbB1.

Intriguingly, co-expression of LRIG1 and the ErbB receptors resulted in reduced ErbB receptor levels. Puls-chase experiments showed that this was due to reduced ErbB receptor half-life. Since the main mechanism for ligand activated ErbB1 degradation is ubiquitylation by c-Cbl, followed by lysosomal degradation, the effects of LRIG1 overexpression on ErbB1 ubiquitylation of ErbB1 were examined. LRIG1 was found to enhance both ligand-induced and ligand-independent ubiquitylation of ErbB1 by recruiting the E3 ubiquitin ligase c-Cbl to the receptor complex. The sites of interaction between LRIG1 and c-Cbl were mapped by co-

immunoprecipitation experiments to the amino-terminal part of c-Cbl and to amino acids 900-939 in the cytoplasmic part of LRIG1.

LRIG1 could, thus, downregulate the ErbB1 receptor levels; concordantly, LRIG1 inhibited EGF induced proliferation in HEK-293 cells, EGF induced transcription of *c-fos* in COS-7 cells, and EGF induced apoptosis in A431 cells. Therefore, deregulation of LRIG1 could have profound effects on tumor progression, since it affects ErbB signaling. Indeed, LRIG1 is downregulated in renal cell carcinoma (Thomasson et al., 2003) and in high grade cutaneous squamous cell carcinoma (Tanemura et al., 2005).

6.6 LRIG in astrocytic tumors (V)

To evaluate the *LRIG* mRNA expression levels in astrocytoma, six glioma cell lines and six astrocytic tumor samples with corresponding non-neoplastic normal brain controls were analyzed. In the cell lines, mRNA was detected for all the *LRIG* in various amounts, with the highest apparent expression of *LRIG1*. The tumors showed considerable heterogeneity in *LRIG* expression levels and there was no consistent up or downregulation in the tumors versus the control samples. To evaluate possible associations between LRIG1 expression and clinical parameters, a tissue micro array with 404 astrocytic tumors was analyzed by immunohistochemistry. Most tumors showed immunoreactivity for LRIG proteins; only 4% of the tumors were completely negative. There was a granular and diffuse immunoreactivity in positive tumor cells in the nuclear, perinuclear, and cytoplasmic compartments. Statistically significant compartment specific correlations were observed for both patient survival and WHO-malignancy grade. A most striking finding was that perinuclear LRIG expression correlated with patient survival, where perinuclear LRIG proteins were associated with better survival (LRIG3, $p<0.0001$; LRIG2, $p<0.01$; LRIG1, $p=0.10$). Furthermore, in a Cox forward stepwise regression analysis, the perinuclear localization of LRIG3 was in addition to histological grade an independent prognostic factor. The perinuclear staining of the LRIG proteins was highest in grade II tumors, intermediate in grade III tumors, and almost absent in grade IV (GBM) tumors. Hence, it is plausible to assume that the LRIG-family members can be of importance in tumor progression. However, these significant clinical findings must be combined with a better molecular understanding before the role of LRIG proteins in tumor progression can be established.

7. Concluding remarks

We have identified a novel ErbB regulatory protein family, the LRIG-family. LRIG1 was shown to negatively regulate the human ErbB family members, by recruiting the E3 ubiquitin ligase c-Cbl to the receptor complex, thereby enhancing receptor ubiquitylation and degradation. Due to the structural similarities within the LRIG family, it will be interesting to see if LRIG2 and LRIG3 have similar ErbB suppressing functions as LRIG1. LRIG1 was found to be proteolytically processed and the functions of the various LRIG1 fragments remain to be elucidated. Perinuclear localization of LRIG proteins correlated with increased patient survival in high-grade astrocytoma. Strikingly, localization of LRIG3 was in addition to malignancy grade an independent prognostic factor in astrocytic tumors. It will be interesting to investigate how the subcellular localization is regulated and whether the various fragments localize differently in the cell. In conclusion, we have opened up a new research field, by identifying the LRIG-family and showing that LRIG1 interacts with the ErbB receptors. The potential biological and clinical implications of our novel findings need to be further elucidated.

Populärvetenskaplig sammanfattning på svenska

I takt med en ökad kunskap inom onkologi har nya angreppssätt inom tumörbehandling utvecklats. Bl.a. har behandlingar riktade mot specifika mål på cancercellen utvecklats, varav flera redan används i kliniken, t.ex. vid behandling av bröstcancer med Herceptin®. Dessa behandlingar är selektiva och söker upp sitt mål på cancercellen och förhindrar den från att växa. En typ av mål som de selektiva behandlingarna riktar sig mot är receptorer på cellytan (mottagare av tillväxtfaktorer). Dessa är ofta överaktiverade i tumörer, vilket leder till en ökad tålighet mot cellgifts- och strålbehandling, samt till en ökad tumörtillväxt och spridning. Därför vill man stänga av alternativt bromsa de överaktiverade receptorerna och på så sätt bekämpa tumören. ErbB-receptorerna tillhör en klass av cellytereceptorer och när de är överaktiverade, vilket är ofta förekommande i olika tumörer, är de associerade med en sämre överlevnad.

Jag har i denna avhandling sökt efter naturligt förekommande bromsmekanismer av ErbB-receptorerna. Vi utgick från ett bananflugeprotein, Kekkon-1, som fungerar som en broms av flugans motsvarighet till ErbB-receptorn. Vi klonade och karaktäriserade ett liknande protein i människa som döptes till, leucin-rikt och immunoglobulin-likt protein 1 (Lrig1). Vi fann att LRIG1 tillhör en egen protein familj med tre medlemmar, LRIG1, LRIG2 och LRIG3, varav LRIG1 binder till ErbB-receptorerna och stänger av deras funktion genom att öka nedbrytning av dessa. Vi har visat i studier av hjärntumörer att uttrycket av LRIG-proteinerna har ett samband med en bättre överlevnad hos patienterna och att uttryck av LRIG3 är en oberoende prognostisk faktor i astrocytiska tumörer (elakartade hjärntumörer).

Sammanfattningsvis innehåller avhandlingen identifieringen och karaktäriseringen av LRIG-familjen och dess inverkan på ErbB-receptoraktivering. Vidare har vi i ett kliniskt material, visat att uttrycket av LRIG-proteinerna har ett samband med en ökad överlevnad hos patienter med elakartade hjärntumörer.

Acknowledgements

With this book my work here has reached the end. It has been a wonderful journey, over the years, seeing the LRIG-family blossom. Before I leave, I would like to thank some of the people that have helped me through these years.

Först och främst vill jag TACKA mina två handledare **Håkan Hedman** och **Roger Henriksson** som tog hand om mig och hjälpte mig bli den forskare jag är idag.
Det har varit ett privilegium att få jobba med er.

Sen vill jag rikta ett tack till alla mina medarbetare på labbet där några har kommit och andra gått…. **Anna, Annika, Camilla, Calle, Carin, Guo, Kerstin, Lotta, Marcus, Micke, Tina, Terese, Ulrika, Yvonne, Yi Wei**…. för många roliga utflykter och upplevelser som vi delat genom åren.

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Ett tack till alla i fika rummet för många trevliga fika stunder och goda bakverk.

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Utanför portarna finns andra som underlättat och förgyllt tillvaron under min doktorand tid.

Lunchgänget eller som de vill kalla sig, **Fantastiska fyran** med **Captain America, Dr Black, Grinchen och Nitesh Mistry**…. vi har hunnit med att kläcka många vilda teorier under våra luncher.

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Sen sist men inte minst min älskade familj, **Sara och småttingarna**… för att ni står ut med mig.

References

Anastasi, S., Fiorentino, L., Fiorini, M., Fraioli, R., Sala, G., Castellani, L., Alema, S., Alimandi, M., and Segatto, O. (2003). Feedback inhibition by RALT controls signal output by the ErbB network. Oncogene *22*, 4221-4234.

Baass, P. C., Di Guglielmo, G. M., Authier, F., Posner, B. I., and Bergeron, J. J. (1995). Compartmentalized signal transduction by receptor tyrosine kinases. Trends Cell Biol *5*, 465-470.

Becker, N. A., Thorland, E. C., Denison, S. R., Phillips, L. A., and Smith, D. I. (2002). Evidence that instability within the FRA3B region extends four megabases. Oncogene *21*, 8713-8722.

Benhar, M., Engelberg, D., and Levitzki, A. (2002). Cisplatin-induced activation of the EGF receptor. Oncogene *21*, 8723-8731.

Benjamin, R., Capparella, J., and Brown, A. (2003). Classification of glioblastoma multiforme in adults by molecular genetics. Cancer J *9*, 82-90.

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

Bonner, J. A., Harari, P. M., Giralt, J., Azarnia, N., Shin, D. M., Cohen, R. B., Jones, C. U., Sur, R., Raben, D., Jassem, J.*, et al.* (2006). Radiotherapy plus cetuximab for squamous-cell carcinoma of the head and neck. N Engl J Med *354*, 567-578.

Burgess, A. W., Cho, H. S., Eigenbrot, C., Ferguson, K. M., Garrett, T. P., Leahy, D. J., Lemmon, M. A., Sliwkowski, M. X., Ward, C. W., and Yokoyama, S. (2003). An open-and-shut case? Recent insights into the activation of EGF/ErbB receptors. Mol Cell *12*, 541-552.

Cappuzzo, F., Hirsch, F. R., Rossi, E., Bartolini, S., Ceresoli, G. L., Bemis, L., Haney, J., Witta, S., Danenberg, K., Domenichini, I.*, et al.* (2005). Epidermal growth factor receptor gene and protein and gefitinib sensitivity in non-small-cell lung cancer. J Natl Cancer Inst *97*, 643-655.

Casci, T., Vinos, J., and Freeman, M. (1999). Sprouty, an intracellular inhibitor of Ras signaling. Cell *96*, 655-665.

Cascone, T., Morelli, M. P., and Ciardiello, F. (2006). Small molecule epidermal growth factor receptor (EGFR) tyrosine kinase inhibitors in non-small cell lung cancer. Ann Oncol *17*, ii46-ii48.

Chang, C., and Sternberg, P. W. (1999). C. elegans vulval development as a model system to study the cancer biology of EGFR signaling. Cancer Metastasis Rev *18*, 203-213.

Chen, P., Xie, H., Sekar, M. C., Gupta, K., and Wells, A. (1994). Epidermal growth factor receptor-mediated cell motility: phospholipase C activity is required, but mitogen-activated protein kinase activity is not sufficient for induced cell movement. J Cell Biol *127*, 847-857.

Clandinin, T. R., DeModena, J. A., and Sternberg, P. W. (1998). Inositol trisphosphate mediates a RAS-independent response to LET-23 receptor tyrosine kinase activation in C. elegans. Cell *92*, 523-533.

Cohen, M. H., Williams, G. A., Sridhara, R., Chen, G., McGuinn, W. D., Jr., Morse, D., Abraham, S., Rahman, A., Liang, C., Lostritto, R.*, et al.* (2004). United States Food and Drug Administration Drug Approval summary: Gefitinib (ZD1839; Iressa) tablets. Clin Cancer Res *10*, 1212-1218.

Coussens, L., Yang-Feng, T. L., Liao, Y. C., Chen, E., Gray, A., McGrath, J., Seeburg, P. H., Libermann, T. A., Schlessinger, J., Francke, U., and et al. (1985). Tyrosine kinase receptor with extensive homology to EGF receptor shares chromosomal location with neu oncogene. Science *230*, 1132-1139.

Dent, P., Reardon, D. B., Park, J. S., Bowers, G., Logsdon, C., Valerie, K., and Schmidt-Ullrich, R. (1999). Radiation-induced release of transforming growth factor alpha activates the epidermal growth factor receptor and mitogen-activated protein kinase pathway in carcinoma cells, leading to increased proliferation and protection from radiation-induced cell death. Mol Biol Cell *10*, 2493-2506.

Diehl, J. A., Cheng, M., Roussel, M. F., and Sherr, C. J. (1998). Glycogen synthase kinase-3beta regulates cyclin D1 proteolysis and subcellular localization. Genes Dev *12*, 3499-3511.

Downward, J., Yarden, Y., Mayes, E., Scrace, G., Totty, N., Stockwell, P., Ullrich, A., Schlessinger, J., and Waterfield, M. D. (1984). Close similarity of epidermal growth factor receptor and v-erb-B oncogene protein sequences. Nature *307*, 521-527.

Duan, L., Miura, Y., Dimri, M., Majumder, B., Dodge, I. L., Reddi, A. L., Ghosh, A., Fernandes, N., Zhou, P., Mullane-Robinson, K.*, et al.* (2003). Cbl-mediated ubiquitinylation is required for lysosomal sorting of epidermal growth factor receptor but is dispensable for endocytosis. J Biol Chem *278*, 28950-28960.

Egan, J. E., Hall, A. B., Yatsula, B. A., and Bar-Sagi, D. (2002). The bimodal regulation of epidermal growth factor signaling by human Sprouty proteins. Proc Natl Acad Sci U S A *99*, 6041-6046.

Fiorini, M., Alimandi, M., Fiorentino, L., Sala, G., and Segatto, O. (2001). Negative regulation of receptor tyrosine kinase signals. FEBS Lett *490*, 132-141.

Frampton, J. E., and Easthope, S. E. (2005). Spotlight on gefitinib in non-small-cell lung cancer. Am J Pharmacogenomics *5*, 133-136.

Garrett, T. P., McKern, N. M., Lou, M., Elleman, T. C., Adams, T. E., Lovrecz, G. O., Kofler, M., Jorissen, R. N., Nice, E. C., Burgess, A. W., and Ward, C. W. (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.

Ghiglione, C., Carraway, K. L., 3rd, Amundadottir, L. T., Boswell, R. E., Perrimon, N., and Duffy, J. B. (1999). The transmembrane molecule kekkon 1 acts in a feedback loop to negatively regulate the activity of the Drosophila EGF receptor during oogenesis. Cell *96*, 847-856.

Gullick, W. J. (2003). c-erbB-4/HER4: friend or foe? J Pathol *200*, 279-281.

Guo, D., Holmlund, C., Henriksson, R., and Hedman, H. (2004). The LRIG gene family has three vertebrate paralogs widely expressed in human and mouse tissues and a homolog in Ascidiacea. Genomics *84*, 157-165.

Hackel, P. O., Gishizky, M., and Ullrich, A. (2001). Mig-6 is a negative regulator of the epidermal growth factor receptor signal. Biol Chem *382*, 1649-1662.

Hacohen, N., Kramer, S., Sutherland, D., Hiromi, Y., and Krasnow, M. A. (1998). sprouty encodes a novel antagonist of FGF signaling that patterns apical branching of the Drosophila airways. Cell *92*, 253-263.

Haigler, H. T., McKanna, J. A., and Cohen, S. (1979). Direct visualization of the binding and internalization of a ferritin conjugate of epidermal growth factor in human carcinoma cells A-431. J Cell Biol *81*, 382-395.

Hall, A. (1998). Rho GTPases and the actin cytoskeleton. Science *279*, 509-514.

Hanahan, D., and Weinberg, R. A. (2000). The hallmarks of cancer. Cell *100*, 57-70.

Harari, D., and Yarden, Y. (2000). Molecular mechanisms underlying ErbB2/HER2 action in breast cancer. Oncogene *19*, 6102-6114.

Harris, R. C., Chung, E., and Coffey, R. J. (2003). EGF receptor ligands. Exp Cell Res *284*, 2-13.

Harris, S. L., and Levine, A. J. (2005). The p53 pathway: positive and negative feedback loops. Oncogene *24*, 2899-2908.

Haugh, J. M., Huang, A. C., Wiley, H. S., Wells, A., and Lauffenburger, D. A. (1999a). Internalized epidermal growth factor receptors participate in the activation of p21(ras) in fibroblasts. J Biol Chem *274*, 34350-34360.

Haugh, J. M., Schooler, K., Wells, A., Wiley, H. S., and Lauffenburger, D. A. (1999b). Effect of epidermal growth factor receptor internalization on regulation of the phospholipase C-gamma1 signaling pathway. J Biol Chem *274*, 8958-8965.

Hsieh, E. T., Shepherd, F. A., and Tsao, M. S. (2000). Co-expression of epidermal growth factor receptor and transforming growth factor-alpha is independent of ras mutations in lung adenocarcinoma. Lung Cancer *29*, 151-157.

Ichimura, K., Ohgaki, H., Kleihues, P., and Collins, V. P. (2004). Molecular pathogenesis of astrocytic tumours. J Neurooncol *70*, 137-160.

Jones, R. B., Gordus, A., Krall, J. A., and MacBeath, G. (2006). A quantitative protein interaction network for the ErbB receptors using protein microarrays. Nature *439*, 168-174.

Jones, S. M., and Kazlauskas, A. (2001). Growth factor-dependent signaling and cell cycle progression. FEBS Lett *490*, 110-116.

Jongeward, G. D., Clandinin, T. R., and Sternberg, P. W. (1995). sli-1, a negative regulator of let-23-mediated signaling in C. elegans. Genetics *139*, 1553-1566.

Junttila, T. T., Sundvall, M., Maatta, J. A., and Elenius, K. (2000). Erbb4 and its isoforms: selective regulation of growth factor responses by naturally occurring receptor variants. Trends Cardiovasc Med *10*, 304-310.

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

Kariya, K., Kim Bui, Y., Gao, X., Sternberg, P. W., and Kataoka, T. (2004). Phospholipase Cepsilon regulates ovulation in Caenorhabditis elegans. Dev Biol *274*, 201-210.

Katz, M., Shtiegman, K., Tal-Or, P., Yakir, L., Mosesson, Y., Harari, D., Machluf, Y., Asao, H., Jovin, T., Sugamura, K., and Yarden, Y. (2002). Ligand-independent degradation of epidermal growth factor receptor involves receptor ubiquitylation and Hgs, an adaptor whose ubiquitin-interacting motif targets ubiquitylation by Nedd4. Traffic *3*, 740-751.

Kinzler, K. W., and Vogelstein, B. (1996). Lessons from hereditary colorectal cancer. Cell *87*, 159-170.

Kleihues, P., and Cavenee, W. K., eds. (2000). WHO classification of tumours: Tumours of the nervous system (Lyon: IARC).

Kleihues, P., and Ohgaki, H. (1999). Primary and secondary glioblastomas: from concept to clinical diagnosis. Neuro-oncol *1*, 44-51.

Klein, D. E., Nappi, V. M., Reeves, G. T., Shvartsman, S. Y., and Lemmon, M. A. (2004). Argos inhibits epidermal growth factor receptor signalling by ligand sequestration. Nature *430*, 1040-1044.

Knuutila, S., Aalto, Y., Autio, K., Bjorkqvist, A. M., El-Rifai, W., Hemmer, S., Huhta, T., Kettunen, E., Kiuru-Kuhlefelt, S., Larramendy, M. L*., et al.* (1999). DNA copy number losses in human neoplasms. Am J Pathol *155*, 683-694.

Kops, G. J., de Ruiter, N. D., De Vries-Smits, A. M., Powell, D. R., Bos, J. L., and Burgering, B. M. (1999). Direct control of the Forkhead transcription factor AFX by protein kinase B. Nature *398*, 630-634.

Kraus, M. H., Issing, W., Miki, T., Popescu, N. C., and Aaronson, S. A. (1989). Isolation and characterization of ERBB3, a third member of the ERBB/epidermal growth factor receptor family: evidence for overexpression in a subset of human mammary tumors. Proc Natl Acad Sci U S A *86*, 9193-9197.

Kuan, C. T., Wikstrand, C. J., and Bigner, D. D. (2001). EGF mutant receptor vIII as a molecular target in cancer therapy. Endocr Relat Cancer *8*, 83-96.

Lee, J. W., Soung, Y. H., Kim, S. Y., Park, W. S., Nam, S. W., Lee, J. Y., Yoo, N. J., and Lee, S. H. (2005). Absence of EGFR mutation in the kinase domain in common human cancers besides non-small cell lung cancer. Int J Cancer *113*, 510-511.

Lenferink, A. E., Pinkas-Kramarski, R., van de Poll, M. L., van Vugt, M. J., Klapper, L. N., Tzahar, E., Waterman, H., Sela, M., van Zoelen, E. J., and Yarden, Y. (1998). Differential endocytic routing of homo- and hetero-dimeric ErbB tyrosine kinases confers signaling superiority to receptor heterodimers. Embo J *17*, 3385-3397.

Levkowitz, G., Waterman, H., Ettenberg, S. A., Katz, M., Tsygankov, A. Y., Alroy, I., Lavi, S., Iwai, K., Reiss, Y., Ciechanover, A*., et al.* (1999). Ubiquitin ligase activity and tyrosine phosphorylation underlie suppression of growth factor signaling by c-Cbl/Sli-1. Mol Cell *4*, 1029-1040.

Ljuslinder, I., Malmer, B., Golovleva, I., Thomasson, M., Grankvist, K., Hockenstrom, T., Emdin, S., Jonsson, Y., Hedman, H., and Henriksson, R. (2005). Increased copy number at 3p14 in breast cancer. Breast Cancer Res *7*, R719-727.

Lodge, A. J., Anderson, J. J., Gullick, W. J., Haugk, B., Leonard, R. C., and 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.

Luetteke, N. C., Qiu, T. H., Peiffer, R. L., Oliver, P., Smithies, O., and Lee, D. C. (1993). TGF alpha deficiency results in hair follicle and eye abnormalities in targeted and waved-1 mice. Cell *73*, 263-278.

Lynch, T. J., Bell, D. W., Sordella, R., Gurubhagavatula, S., Okimoto, R. A., Brannigan, B. W., Harris, P. L., Haserlat, S. M., Supko, J. G., Haluska, F. G*., et al.* (2004). Activating mutations in the epidermal growth factor receptor underlying responsiveness of non-small-cell lung cancer to gefitinib. N Engl J Med *350*, 2129-2139.

Maatta, J. A., Sundvall, M., Junttila, T. T., Peri, L., Laine, V. J., Isola, J., Egeblad, M., and Elenius, K. (2006). Proteolytic cleavage and phosphorylation of a tumor-associated ErbB4 isoform promote ligand-independent survival and cancer cell growth. Mol Biol Cell *17*, 67-79.

Maitra, A., Wistuba, II, Washington, C., Virmani, A. K., Ashfaq, R., Milchgrub, S., Gazdar, A. F., and Minna, J. D. (2001). High-resolution chromosome 3p allelotyping of breast carcinomas and precursor lesions demonstrates frequent loss of heterozygosity and a discontinuous pattern of allele loss. Am J Pathol *159*, 119-130.

Marmor, M. D., and Yarden, Y. (2004). Role of protein ubiquitylation in regulating endocytosis of receptor tyrosine kinases. Oncogene *23*, 2057-2070.

Marshall, C. J. (1995). Specificity of receptor tyrosine kinase signaling: transient versus sustained extracellular signal-regulated kinase activation. Cell *80*, 179-185.

Medema, R. H., Kops, G. J., Bos, J. L., and Burgering, B. M. (2000). AFX-like Forkhead transcription factors mediate cell-cycle regulation by Ras and PKB through p27kip1. Nature *404*, 782-787.

Mellinghoff, I. K., Wang, M. Y., Vivanco, I., Haas-Kogan, D. A., Zhu, S., Dia, E. Q., Lu, K. V., Yoshimoto, K., Huang, J. H., Chute, D. J.*, et al.* (2005). Molecular determinants of the response of glioblastomas to EGFR kinase inhibitors. N Engl J Med *353*, 2012-2024.

Miettinen, P. J., Berger, J. E., Meneses, J., Phung, Y., Pedersen, R. A., Werb, Z., and Derynck, R. (1995). Epithelial immaturity and multiorgan failure in mice lacking epidermal growth factor receptor. Nature *376*, 337-341.

Mitelman, F., Johansson, B., and Mertens, F., eds. (2006). Mitelman Database of Chromosome Aberrations in Cancer (http://cgap.nci.nih.gov/Chromosomes/Mitelman).

Moscatello, D. K., Holgado-Madruga, M., Godwin, A. K., Ramirez, G., Gunn, G., Zoltick, P. W., Biegel, J. A., Hayes, R. L., and Wong, A. J. (1995). Frequent expression of a mutant epidermal growth factor receptor in multiple human tumors. Cancer Res *55*, 5536-5539.

Nasi, S., Ciarapica, R., Jucker, R., Rosati, J., and Soucek, L. (2001). Making decisions through Myc. FEBS Lett *490*, 153-162.

Olayioye, M. A., Neve, R. M., Lane, H. A., and Hynes, N. E. (2000). The ErbB signaling network: receptor heterodimerization in development and cancer. Embo J *19*, 3159-3167.

Paez, J. G., Janne, P. A., Lee, J. C., Tracy, S., Greulich, H., Gabriel, S., Herman, P., Kaye, F. J., Lindeman, N., Boggon, T. J.*, et al.* (2004). EGFR mutations in lung cancer: correlation with clinical response to gefitinib therapy. Science *304*, 1497-1500.

Peschon, J. J., Slack, J. L., Reddy, P., Stocking, K. L., Sunnarborg, S. W., Lee, D. C., Russell, W. E., Castner, B. J., Johnson, R. S., Fitzner, J. N.*, et al.* (1998). An essential role for ectodomain shedding in mammalian development. Science *282*, 1281-1284.

Plosker, G. L., and Keam, S. J. (2006). Trastuzumab: A Review of its Use in the Management of HER2-Positive Metastatic and Early-Stage Breast Cancer. Drugs *66*, 449-475.

Plowman, G. D., Culouscou, J. M., Whitney, G. S., Green, J. M., Carlton, G. W., Foy, L., Neubauer, M. G., and 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.

Plowman, G. D., Whitney, G. S., Neubauer, M. G., Green, J. M., McDonald, V. L., Todaro, G. J., and Shoyab, M. (1990). Molecular cloning and expression of an additional epidermal growth factor receptor-related gene. Proc Natl Acad Sci U S A *87*, 4905-4909.

Qiu, X. B., and Goldberg, A. L. (2002). Nrdp1/FLRF is a ubiquitin ligase promoting ubiquitination and degradation of the epidermal growth factor receptor family member, ErbB3. Proc Natl Acad Sci U S A *99*, 14843-14848.

Ramnarain, D. B., Park, S., Lee, D. Y., Hatanpaa, K. J., Scoggin, S. O., Otu, H., Libermann, T. A., Raisanen, J. M., Ashfaq, R., Wong, E. T.*, et al.* (2006). Differential gene expression analysis reveals generation of an autocrine loop by a mutant epidermal growth factor receptor in glioma cells. Cancer Res *66*, 867-874.

Reich, A., and Shilo, B. Z. (2002). Keren, a new ligand of the Drosophila epidermal growth factor receptor, undergoes two modes of cleavage. Embo J *21*, 4287-4296.

Roth, J., Dobbelstein, M., Freedman, D. A., Shenk, T., and Levine, A. J. (1998). Nucleo-cytoplasmic shuttling of the hdm2 oncoprotein regulates the levels of the p53 protein via a pathway used by the human immunodeficiency virus rev protein. Embo J *17*, 554-564.

Rubin, C., Litvak, V., Medvedovsky, H., Zwang, Y., Lev, S., and Yarden, Y. (2003). Sprouty fine-tunes EGF signaling through interlinked positive and negative feedback loops. Curr Biol *13*, 297-307.

Rubin, C., Gur, G., and Yarden, Y. (2005). Negative regulation of receptor tyrosine kinases: unexpected links to c-Cbl and receptor ubiquitylation. Cell Res *15*, 66-71.

Schmidt-Ullrich, R. K., Contessa, J. N., Lammering, G., Amorino, G., and Lin, P. S. (2003). ERBB receptor tyrosine kinases and cellular radiation responses. Oncogene *22*, 5855-5865.

Sears, R., Leone, G., DeGregori, J., and Nevins, J. R. (1999). Ras enhances Myc protein stability. Mol Cell *3*, 169-179.

Seth, D., Shaw, K., Jazayeri, J., and Leedman, P. J. (1999). Complex post-transcriptional regulation of EGF-receptor expression by EGF and TGF-alpha in human prostate cancer cells. Br J Cancer *80*, 657-669.

She, Q. B., Solit, D. B., Ye, Q., O'Reilly, K. E., Lobo, J., and Rosen, N. (2005). The BAD protein integrates survival signaling by EGFR/MAPK and PI3K/Akt kinase pathways in PTEN-deficient tumor cells. Cancer Cell *8*, 287-297.

Shilo, B. Z. (2003). Signaling by the Drosophila epidermal growth factor receptor pathway during development. Exp Cell Res *284*, 140-149.

Sibilia, M., and Wagner, E. F. (1995). Strain-dependent epithelial defects in mice lacking the EGF receptor. Science *269*, 234-238.

Sibilia, M., Steinbach, J. P., Stingl, L., Aguzzi, A., and Wagner, E. F. (1998). A strain-independent postnatal neurodegeneration in mice lacking the EGF receptor. Embo J *17*, 719-731.

Slamon, D. J., Clark, G. M., Wong, S. G., Levin, W. J., Ullrich, A., and McGuire, W. L. (1987). Human breast cancer: correlation of relapse and survival with amplification of the HER-2/neu oncogene. Science *235*, 177-182.

Sweeney, C., and Carraway, K. L., 3rd (2000). Ligand discrimination by ErbB receptors: differential signaling through differential phosphorylation site usage. Oncogene *19*, 5568-5573.

Sweeney, C., Lai, C., Riese, D. J., 2nd, Diamonti, A. J., Cantley, L. C., and Carraway, K. L., 3rd (2000). Ligand discrimination in signaling through an ErbB4 receptor homodimer. J Biol Chem *275*, 19803-19807.

Sweeney, C., and Carraway, K. L., 3rd (2004). Negative regulation of ErbB family receptor tyrosine kinases. Br J Cancer *90*, 289-293.

Tanemura, A., Nagasawa, T., Inui, S., and Itami, S. (2005). LRIG-1 provides a novel prognostic predictor in squamous cell carcinoma of the skin: immunohistochemical analysis for 38 cases. Dermatol Surg *31*, 423-430.

Taron, M., Ichinose, Y., Rosell, R., Mok, T., Massuti, B., Zamora, L., Mate, J. L., Manegold, C., Ono, M., Queralt, C.*, et al.* (2005). Activating mutations in the tyrosine kinase domain of the epidermal growth factor receptor are associated with improved survival in gefitinib-treated chemorefractory lung adenocarcinomas. Clin Cancer Res *11*, 5878-5885.

Thomasson, M., Hedman, H., Guo, D., Ljungberg, B., and Henriksson, R. (2003). LRIG1 and epidermal growth factor receptor in renal cell carcinoma: a quantitative RT--PCR and immunohistochemical analysis. Br J Cancer *89*, 1285-1289.

Thomasson, M., Hedman, H., Junttila, T. T., Elenius, K., Ljungberg, B., and 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.

Umekita, Y., Ohi, Y., Sagara, Y., and Yoshida, H. (2000). Co-expression of epidermal growth factor receptor and transforming growth factor-alpha predicts worse prognosis in breast-cancer patients. Int J Cancer *89*, 484-487.

van Delft, S., Govers, R., Strous, G. J., Verkleij, A. J., and van Bergen en Henegouwen, P. M. (1997). Epidermal growth factor induces ubiquitination of Eps15. J Biol Chem *272*, 14013-14016.

Waterman, H., Alroy, I., Strano, S., Seger, R., and Yarden, Y. (1999). The C-terminus of the kinase-defective neuregulin receptor ErbB-3 confers mitogenic superiority and dictates endocytic routing. Embo J *18*, 3348-3358.

Wells, A., Gupta, K., Chang, P., Swindle, S., Glading, A., and Shiraha, H. (1998). Epidermal growth factor receptor-mediated motility in fibroblasts. Microsc Res Tech *43*, 395-411.

Vieira, A. V., Lamaze, C., and Schmid, S. L. (1996). Control of EGF receptor signaling by clathrin-mediated endocytosis. Science *274*, 2086-2089.

Wikstrand, C. J., Hale, L. P., Batra, S. K., Hill, M. L., Humphrey, P. A., Kurpad, S. N., McLendon, R. E., Moscatello, D., Pegram, C. N., Reist, C. J., and et al. (1995). Monoclonal antibodies against EGFRvIII are tumor specific and react with breast and lung carcinomas and malignant gliomas. Cancer Res *55*, 3140-3148.

Wiley, H. S. (2003). Trafficking of the ErbB receptors and its influence on signaling. Exp Cell Res *284*, 78-88.

Witton, C. J., Reeves, J. R., Going, J. J., Cooke, T. G., and Bartlett, J. M. (2003). Expression of the HER1-4 family of receptor tyrosine kinases in breast cancer. J Pathol *200*, 290-297.

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

Zhou, B. P., Liao, Y., Xia, W., Spohn, B., Lee, M. H., and Hung, M. C. (2001a). Cytoplasmic localization of p21Cip1/WAF1 by Akt-induced phosphorylation in HER-2/neu-overexpressing cells. Nat Cell Biol *3*, 245-252.

Zhou, B. P., Liao, Y., Xia, W., Zou, Y., Spohn, B., and Hung, M. C. (2001b). HER-2/neu induces p53 ubiquitination via Akt-mediated MDM2 phosphorylation. Nat Cell Biol *3*, 973-982.

Zimonjic, D. B., Alimandi, M., Miki, T., Popescu, N. C., and Kraus, M. H. (1995). Localization of the human HER4/erbB-4 gene to chromosome 2. Oncogene *10*, 1235-1237.