Turun yliopisto
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REGULATION OF ERBB4 SIGNALING BY POST-TRANSLATIONAL MODIFICATIONS

Anna M. Knittle

TURUN YLIOPISTON JULKAISUJA – ANNALES UNIVERSITATIS TURKUENSIS
Sarja - ser. D osa - tom. 1265 | Medica - Odontologica | Turku 2017

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The originality of this thesis has been checked in accordance with the University of Turku quality assurance system using the Turnitin OriginalityCheck service.

ISBN 978-951-29-6692-9 (PRINT)
ISBN 978-951-29-6693-6 (PDF)
ISSN 0355-9483 (Print)
ISSN 2343-3213 (Online)
Painosalama Oy - Turku, Finland 2017

To my family

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ABSTRACT

ErbB4 is a member of the epidermal growth receptor family (EGFR/ErbB1, ErbB2, ErbB3, ErbB4) of receptor tyrosine kinases. ErbB receptors are activated by extracellular growth factor ligands, and control the activity of intracellular signaling pathways that regulate fundamental cellular processes such as proliferation, differentiation and survival. ErbB receptors are necessary for the development and homeostasis of several tissues, and aberrant ErbB signaling is a common feature of human pathologies, such as cancer. Accordingly, several regulatory mechanisms are required to ensure the appropriate function of ErbB receptors.

This thesis aimed to characterize novel regulatory mechanisms of ErbB4 signaling, with a focus on ubiquitin and ubiquitin-like post-translational modifications. ErbB4 is expressed as functionally distinct isoforms, and the results of this thesis indicate that ErbB4 is ubiquitinated, endocytosed and degraded in an isoform-specific manner. Ubiquitination, a signal for endocytosis and degradation, was catalyzed by a ubiquitin ligase that specifically interacted with one of the isoforms. This study also demonstrated that ErbB4 intracellular domain (ICD), released by ErbB4 isoforms that undergo regulated intramembrane proteolysis, is modified by small ubiquitin-like modifier (SUMO). SUMOylation promoted the nuclear accumulation of ErbB4 ICD and regulated the nuclear signaling of ErbB4.

Together, these findings represent novel molecular mechanisms that regulate the stability, subcellular localization and activity of ErbB4. Moreover, this study demonstrates for the first time that SUMOylation controls the function of an ICD of a receptor tyrosine kinase in the nucleus. The post-translational regulation of quantitative and qualitative aspects of ErbB4 signaling may have implications for the function of ErbB receptors in both normal tissues and cancer. Finally, the findings of this thesis can potentially be extended to provide new understanding on the mechanisms that regulate the functions of receptor tyrosine kinases other than ErbB4.

Key words: endocytosis, ErbB4, isoform, nuclear signaling, SUMO, ubiquitin

Anna M. Knittle

Translaation jälkeiset modifikaatiot ErbB4-signaloinnin säätelyssä

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TIIVISTELMÄ

ErbB4 on ErbB-reseptoriperheeseen (EGFR/ErbB1, ErbB2, ErbB3, ErbB4) kuuluva tyrosiinikinaasireseptori. ErbB-reseptorit sitovat solun ulkopuolisia kasvutekijöitä ja aktivoivat näin solunsisäisiä signalointireittejä. ErbB-välitteinen signalointi säätelee solujen kasvua, erilaistumista ja selviytymistä, ja on välttämätöntä useiden kudosten kehityksen ja toiminnan säätelyssä. ErbB-reseptorien aktiivisuutta ja toimintaa säädellään tarkasti, ja häiriintynyt ErbB-signalointi on keskeinen tekijä syövän ja eräiden muiden sairauksien synnyssä ja etenemisessä.

Tämän tutkimuksen tavoitteena oli löytää uusia molekyylitason mekanismeja, jotka säätelevät ErbB4-reseptorin toimintaa. Tutkimus keskittyi erityisesti ubikitiinin kaltaisiin translaation jälkeisiin modifikaatioihin. ErbB4 esiintyy erilaisina alamuotoina, ja tutkimuksessa havaittin eroja alamuotojen ubikitinaatiossa, endosytoosissa ja hajotuksessa. Tutkimuksessa löydettiin ubikitiiniligaasi, joka sääteli vain tietyn ErbB4:n alamuodon ubikitinaatiota aiheuttaen ErbB4:n endosytoosin ja hajotuksen. Tutkimuksessa havaittiin myös, että ubikitiinin kaltainen SUMO-modifikaatio (engl. small ubiquitin-like modifier) säätelee ErbB4-reseptorin proteolyyttisesti katkeavista alamuodoista irtoavan solunsisäisen osan toimintaa. SUMO-modifikaatio sääteli ErbB4:n solunsisäisen osan määrää tumassa ja ErbB4:n tumasignaloinnin aktiivisuutta.

Tutkimuksessa tehdyt havainnot osoittavat, että translaation jälkeiset modifikaatiot säätelevät ErbB4-reseptorin hajotusta, solunsisäistä sijaintia ja signalointia. Tutkimuksessa osoitettiin ensimmäistä kertaa, että SUMO-modifikaatio säätelee tyrosiinikinaasireseptorin solunsisäisen osan toimintaa tumassa. Translaation jälkeiset modifikaatiot voivat vaikuttaa ErbB-reseptorien toimintaan sekä terveessä että syöpäkudoksessa. Tutkimuksen tulokset ErbB4-reseptorin uusista säätelymekanismeista saattavat myös olla laajennettavissa muihin tyrosiinikinaasireseptoreihin.

Avainsanat: alamuoto, endosytoosi, ErbB4, SUMO, tumasignalointi, ubikitiini

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ABBREVIATIONS

ADAM	a disintegrin and metalloproteinase
Akt	v-akt murine thymoma viral oncogene homolog
ATP	adenosine triphosphate
Cbl	casitas B-lineage lymphoma
CRM1	chromosomal region maintenance 1
CYT	cytoplasmic
DUB	deubiquitinating enzyme
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
ERα	estrogen receptor α
ErbB	v-erb-b avian erythroblastic leukemia viral oncogene homolog
ERK	extracellular signal-regulated kinase
ETS	v-ets avian erythroblastosis virus E26 oncogene homolog
GRB2	growth factor receptor-bound protein 2
GST	glutathione S-transferase
GTP	guanosine triphosphate
HB-EGF	heparin-binding EGF-like growth factor
HECT	homologous to E6-AP carboxyl terminus
ICD	intracellular domain
Itch	itchy E3 ubiquitin protein ligase
JM	juxtamembrane
LRIG1	leucine-rich repeats and immunoglobulin-like domains-1
MAPK	mitogen activated protein kinase
MEK	mitogen activated protein kinase kinase
MIG6	mitogen-induced gene 6
MVB	multivesicular body
Myc	v-myc avian myelocytomatosis viral oncogene homolog
NEDD4	neural precursor cell expressed, developmentally downregulated 4
NES	nuclear export signal
NLS	nuclear localization signal
NRDP1	neuregulin receptor degradation protein-1
NRG	neuregulin
PDGFR	platelet-derived growth factor receptor
PDK1	3-phosphoinositide dependent kinase 1
PI3K	phosphoinositol-3 kinase

PIAS	protein inhibitor of activated STAT
PLC	phospholipase C
PML	promyelocytic leukemia
PTB	phosphotyrosine-binding
PTEN	phosphatase and tensin homolog
PTM	post-translational modification
PTP	protein tyrosine phosphatase
Rab5	ras-related protein Rab5
Rab7	ras-related protein Rab7
Raf	v-raf murine leukemia viral oncogene homolog
RALT	receptor-associated late inducer
RanBP2	Ran-binding protein 2
RanGAP1	Ran GTPase-activating protein 1
Ras	rat sarcoma
RIP	regulated intramembrane proteolysis
RING	really interesting new gene
RTK	receptor tyrosine kinase
SENP	sentrin-specific protease
SH2	Src homology 2 domain
SHC	Src homology 2 containing
SIM	SUMO interaction motif
SOCS	suppressors of cytokine signaling
SOS	son of sevenless
SP-RING	Siz/PIAS-RING
STAT	signal transduced and activator of transcription
SUMO	small ubiquitin-like modifier
TAB2	TGF-β activated kinase 1/MAP3K7 binding protein 2
NCoR	nuclear receptor corepressor
TACE	tumor-necrosis factor-α converting enzyme
TM	transmembrane domain
TOPORS	topoisomerase I-binding, arginine/serine rich
UBD	ubiquitin-binding domain
UBL	ubiquitin-like modifier
YAP	yes-associated protein

LIST OF ORIGINAL PUBLICATIOS

This thesis by Anna M. Knittle (née Korhonen) is based on the following original publications. The publications will be referred to in the text by their Roman numerals (I-III), and have been reprinted with the permission of the copyright holders.

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

II. Sundvall M*, Korhonen A*, Vaparanta K, Anckar J, Halkilahti K, Salah Z, Aqeilan RI, Palvimo JJ, Sistonen L and Elenius K. Protein inhibitor of activated STAT3 (PIAS3) protein promotes SUMOylation and nuclear sequestration of the intracellular domain of ErbB4 protein. J Biol Chem 2012, 287: 23216-23226.
*Equal contribution

III. Knittle AM, Helkkula M, Sundvall M and Elenius K. SUMOylation regulates nuclear accumulation and function of the soluble intracellular domain of ErbB4. Manuscript.

1 INTRODUCTION

The ErbB family of receptor tyrosine kinases consists of epidermal growth factor receptor (EGFR, ErbB1), ErbB2, ErbB3, and ErbB4. ErbB receptors are activated by the binding of extracellular EGF-like growth factors, and transmit the growth factor signal into the activation of intracellular signaling cascades. Through these signaling cascades ErbB receptors regulate fundamental cellular processes such as proliferation, differentiation and survival, and play essential roles in embryonic development and the homeostasis of adult tissues. Accordingly, the function of ErbB receptors must be tightly controlled, and aberrant ErbB signaling is a common feature of human pathologies, including cancer.

The function of many proteins involved in signal transduction is regulated by post-translational modifications. Post-translational modifications, which include the attachment of ubiquitin and ubiquitin-like polypeptides in addition to small chemical groups, represent a fast and often reversible way to regulate protein function. Chains of ubiquitin molecules often function as proteolytic degradation signals, and the ubiquitination-induced lysosomal degradation of activated EGFR is a well-characterized example of ubiquitination as a negative regulator of cellular signaling. The attachment of ubiquitin-like proteins, however, typically regulates their target proteins by non-proteolytic mechanisms, for example by inducing changes in the activity or subcellular localization.

Although many aspects of ErbB signaling have been extensively studied, the roles of post-translational ubiquitin, and in particular ubiquitin-like modifications in ErbB receptor function are largely unexplored. This thesis focuses on one member of the ErbB family, ErbB4, and elucidates how ErbB4 signaling is regulated by ubiquitin and ubiquitin-like modifications. Identification of the molecular mechanisms that modify the output of ErbB4 signaling will expand the understanding of ErbB4 function in both healthy and diseased tissues.

2 REVIEW OF THE LITERATURE

2.1 Receptor tyrosine kinases

Protein phosphorylation is a post-translational modification that regulates intracellular signaling pathways. Protein kinases are enzymes that catalyze the transfer of the γ-phosphate group from adenoside triphosphate (ATP) to an amino acid residue of a protein substrate. The human genome encodes over 500 protein kinases, of which at least 90 are protein tyrosine kinases. 55 protein tyrosine kinases are transmembrane receptors referred to as receptor tyrosine kinases (RTK), which are further divided in 19 subfamilies. RTKs mediate extracellular growth factor signals across the plasma membrane, and control fundamental cellular processes. The activity of RTKs is tighly controlled during normal embryonic development and homeostasis of adult tissues, but aberrant RTK function is associated with pathologies, especially cancers (Blume-Jensen and Hunter, 2001; Lemmon and Schlessinger, 2010; Wheeler and Yarden, 2015).

2.2 ErbB receptors

The ErbB or epidermal growth factor (EGF) receptor subfamily of RTKs consists of epidermal growth factor receptor (EGFR, or ErbB1) (Ullrich et al., 1984), ErbB2 (Coussens et al., 1985), ErbB3 (Plowman et al., 1990) and ErbB4 (Plowman et al., 1993a) (Figure 1). The receptors are called ErbB for the close similarity of the amino acid sequence of EGFR with the amino acid sequence of avian erythroblastic leukemia retroviral oncoprotein v-erb-B (Downward et al., 1984). ErbB receptors are also called HER receptors, an abbreviation for human EGF receptor.

ErbB receptors are transmembrane type I glycoproteins of approximately 180 kilodaltons. They share a common structure with other receptor tyrosine kinases, with an extracellular domain, a single α-helical transmembrane domain, and an intracellular domain (Figure 1). The ErbB extracellular domain mediates receptor activation *via* ligand binding and receptor dimerization. The transmembrane domain docks ErbB receptors to the plasma membrane, and has minor roles in receptor activation. The ErbB intracellular domain contains tyrosine kinase activity and a carboxy (C)-terminal tail, including phosphorylated tyrosine residues that serve as binding sites for the intracellular signaling molecules upon receptor activation (Lemmon et al., 2014).

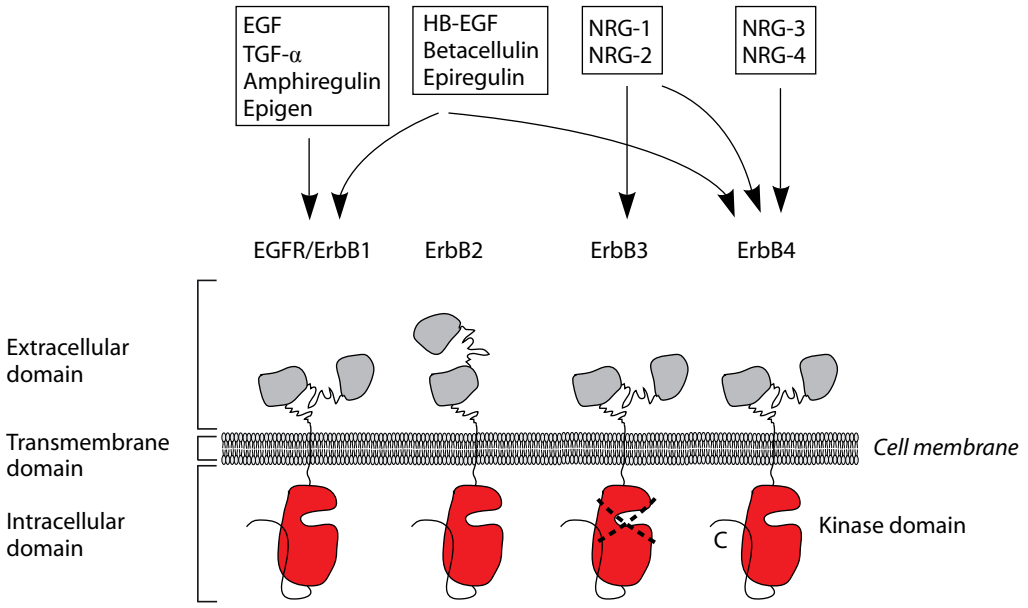

Figure 1. Structures and ligands of ErbB receptors. *Top:* ErbB ligands grouped according to their receptor-binding specificities. EGF, epidermal growth factor; HB-EGF, heparin-binding EGF-like growth factor; NRG, neuregulin; TGFα, transforming growth factor α. *Bottom:* Schematic structures of ErbB receptors in the inactive conformation. ErbB receptors have a ligand-binding extracellular domain, a single α-helical transmembrane domain, and an intracellular domain containing a tyrosine kinase domain (indicated in red) and carboxy-terminal tail (C). The extracellular domain of ErbB2 is constitutively in an active conformation. The kinase domain of ErbB3 is inactive. The receptor structures are modified from Burgess et al., 2003 and Zhang et al., 2006.

2.2.1 Ligands of ErbB receptors

The four ErbB receptors are activated by 11 EGF-like polypeptide ligands that have different receptor-binding preferences (Figure 1). Ligands that bind to and activate EGFR include EGF, heparin-binding EGF-like growth factor (HB-EGF), transforming growth factor α (TGF-α), amphiregulin, betacellulin, epigen, and epiregulin (Riese et al., 1996a; 1996b; 1998; Strachan et al., 2001). In addition to EGFR, HB-EGF, betacellulin and epiregulin also activate ErbB4 (Riese et al., 1996a; Elenius et al., 1997a; Riese et al., 1998). Neuregulins (NRG) are ligands that activate either both ErbB3 and ErbB4 (NRG-1 and NRG-2), or ErbB4 only (NRG-3 and NRG-4) (Plowman et al., 1993b; Carraway et al., 1994; Carraway et al., 1997; Chang et al., 1997; Zhang et al., 1997; Harari et al., 1999). No EGF-like ligands that directly bind to ErbB2 have been identified, an anomaly explained by the crystal structure of the ErbB2 extracellular domain (Cho et al., 2003; Garrett et al., 2003).

The EGF-like ligands are synthesized as transmembrane precursor proteins. Proteolytic processing by extracellular proteases, referred to as shedding, releases the soluble ligand into the extracellular space and controls autocrine and paracrine signaling. Additionally, transmembrane forms of some EGF-like ligands can bind to receptors in neighboring cells, and transmit signals in juxtacrine fashion (Singh and Harris, 2005; Blobel et al., 2009).

2.2.2 Activation of ErbB receptors

Structural studies have illustrated how ligand binding to the extracellular domain of ErbB receptors is converted to the activity of the intracellular tyrosine kinase domain. In the absence of ligand, ErbB receptors exist as monomers, and the extracellular domain of EGFR, ErbB3 and ErbB4 is in a closed, autoinhibited conformation (Cho and Leahy, 2002; Garrett et al., 2002; Bouyain et al., 2005) (Figure 2). Ligand binding to the extracellular subdomains I and III results in a major structural reconfiguration, and the stabilization of an open, active conformation. In this active conformation, the extracellular subdomains II and IV mediate receptor dimerization (Garrett et al., 2002; Ogiso et al., 2002; Burgess et al., 2003). Notably, the extracellular domain of ErbB2 is constitutively in the active conformation, and thus ready to dimerize without ligand binding. The active conformation of ErbB2 is stabilized by an interaction interface of subdomains I and III in a manner that resembles the ligand-induced stabilization of the active conformation of other ErbB receptors. The interaction interface also buries the ligand-binding surface, explaining the inability of ErbB2 to bind to ligands (Cho et al., 2003; Garrett et al., 2003) (Figure 1).

The dimerization of ErbB extracellular domains promotes the formation of an asymmetric dimer of the intracellular tyrosine kinase domains. In the asymmetric dimer, the C-terminal lobe of one kinase molecule (referred to as the activator kinase) makes contacts with the N-terminal lobe of another kinase molecule (referred to as the receiver kinase) (Figure 2). These interactions stabilize the active conformation of the receiver kinase, which in turn phosphorylates the activator kinase in *trans* (Zhang et al., 2006; Qiu et al., 2008). The intracellular juxtamembrane region and the transmembrane domain further stablize the active asymmetric kinase dimer (Bocharov et al., 2008; Brewer et al., 2009; Jura et al., 2009).

The allosteric activation mechanism of ErbB receptors does not require catalytic activity of the activator kinase. Thus, ErbB3, which lacks important catalytic residues and is practically inactive, can function as an activator in the asymmetric kinase dimer of ErbB heterodimers (Guy et al., 1994; Zhang et al., 2006; Shi et al., 2010).

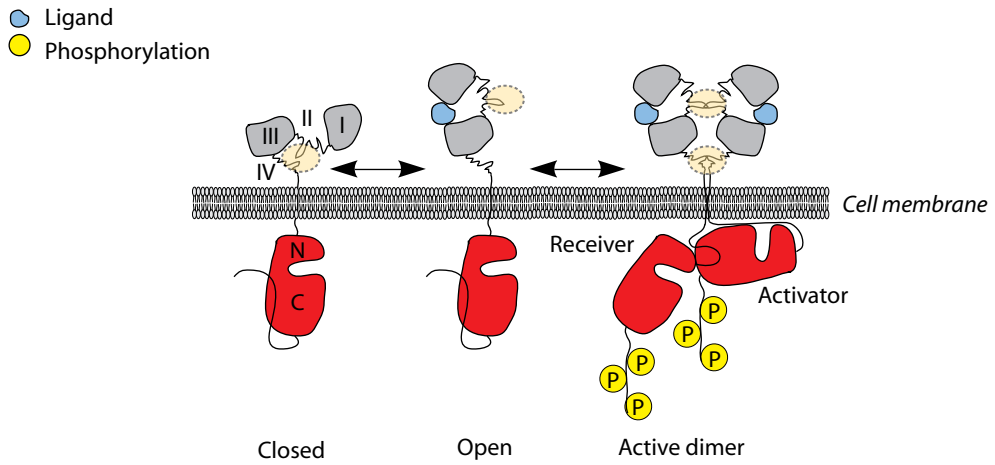

Figure 2. Activation of ErbB receptors. *Left:* Inactive receptor monomers adopt a closed conformation, in which the dimerization interface (indicated by an oval) of the subdomains II and IV is masked. *Middle:* Ligand (indicated in blue) binding to the subdomains I and III induces structural reconfiguration, and receptors adopt an open conformation where the dimerization interface is exposed. *Right:* Dimerization of the extracellular domains, mediated by the subdomains II and IV, promotes the formation of an asymmetric dimer of the tyrosine kinase domains (indicated in red). The C-terminal lobe of the activator kinase makes contacts with the N-terminal lobe of the receiver kinase, resulting in stabilization of the active conformation and autophosphorylation of the receptor dimer. Yellow circles indicate phosphorylated tyrosine residues. Modified from Burgess et al., 2003 and Zhang et al., 2006.

2.2.3 Signaling pathways activated by ErbB receptors

The ligand-induced activation of the ErbB kinase domain results in the autophosphorylation of several tyrosine residues in the receptor's C-terminal tail. Autophosphorylation sites are determined by the activating ligand and, as ErbB receptors can form homo- and heterodimers, the composition of the receptor dimer (Olayioye et al., 1998; Sweeney et al., 2000). Indeed, the C-terminal tail is the least conserved domain of ErbBs, and each receptor has different preferences for signaling and adaptor proteins that recognize the phosphorylated tyrosines (Schulze et al., 2005). The signaling and adaptor proteins associate with ErbB receptors *via* their Scr homology (SH) or phosphotyrosine-binding (PTB) interaction domains, and link the receptors to downstream signaling cascades including the mitogen-activated protein kinase (MAPK), phosphoinositol 3-kinase (PI3K), phospholipase C (PLC)-γ, and signal transducer and activator of transcription (STAT) pathways (Figure 3). The activity of these signaling processes translates receptor activation to cellular responses including proliferation, differentiation, migration, and survival (Lemmon and Schlessinger, 2010).

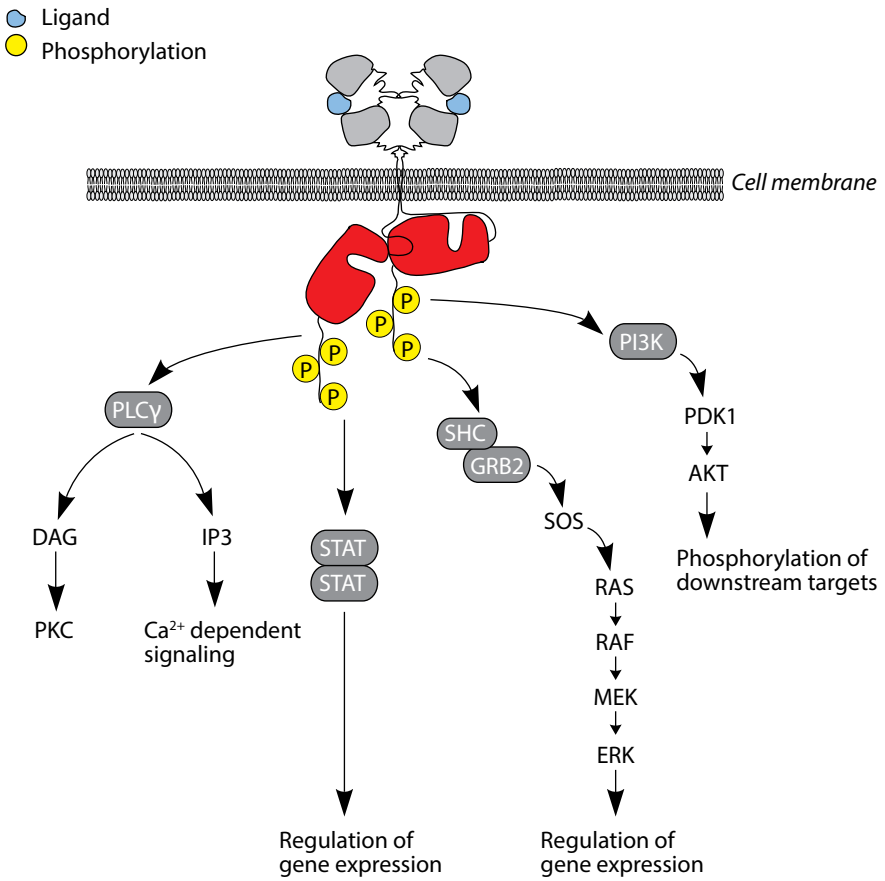

Figure 3. Signaling pathways activated by ErbB receptors. Signaling and adaptor molecules (indicated in grey) are recruited to the phosphorylated tyrosine residues (indicated in yellow) in receptor C-terminal tails, and initiate intracellular signaling pathways that mediate different cellular responses. Akt, v-akt murine thymoma viral oncogene homolog; DAG, diacylglycerol; ERK, extracellular signal-regulated kinase; GRB2, growth factor receptor-bound protein 2; IP3, inositol-1,4,5-triphosphate; MEK, mitogen activated protein kinase kinase; PCK, protein kinase C; PDK1, 3-phosphoinositide dependent kinase 1; PI3K, phosphoinositol 3-kinase; PLCγ, phospholipase C-γ; Raf, v-raf murine leukemia viral oncogene homolog; Ras, rat sarcoma; SHC, Src homology 2 containing; SOS, son of sevenless; STAT, signal transducer and activator of transcription.

The MAPK pathway can be activated by all ErbB receptors. Phosphorylated ErbB receptors harbor docking sites for the SH2 domain containing adaptor protein growth factor receptor-bound protein 2 (GRB2), or the SH2 and PTB domain containing adaptor protein Src homology 2 containing (SHC), which recruit the guanide nucleotide exchange factor son of sevenless (SOS) to the plasma membrane (Schulze et al., 2005; Lemmon and Schlessinger, 2010). SOS activates the small GTPase Ras, which in turn triggers a kinase cascade comprising Raf (MAPK kinase kinase), MEK (MAPK kinase) and ERK (MAPK). Activated ERK translocates into the nucleus where it can activate several transcription factors, including Myc and transcription factors belonging to the

AP-1 and ETS families. The activation of these transcription factors leads to changes in gene expression to promote various cellular responses, most prominently proliferation (Roberts and Der, 2007; Shaul and Seger, 2007).

PI3K pathway is another key signaling pathway activated by ErbB receptors. ErbB3 and ErbB4 have binding sites for the SH2 domain containing PI3K regulatory subunit p85 (Schulze et al., 2005). The PI3K catalytic subunit p110 catalyzes the conversion of phosphatidylinositol-4,5-phosphate to phosphatidylinositol-3,4,5-phosphate, which recruits Akt and 3-phosphoinositide dependent kinase 1 (PDK1). PDK1 phosphorylates Akt, which becomes activated and phosphorylates downstream targets involved in cell survival, metabolism and cytoskeletal rearrangements (Luo et al., 2003). In addition to the direct PI3K activation by ErbB3 and ErbB4, ErbB receptors can activate this pathway indirectly *via* Ras (Rodriguez-Viciana et al., 1994).

Another phosphoinositol signaling pathway is initiated by the recruitment of PLCγ to the activated ErbB receptors by its SH2 domains. EGFR, ErbB2 and ErbB4 phosphorylate PLCγ, leading to its activation (Margolis et al., 1990; Peles et al., 1991; Vecchi et al., 1996). Activated PLCγ hydrolyzes phosphatidylinositol-4,5-phosphate to secondary messengers diacylglycerol and inositol-1,4,5-triphosphate, which in turn stimulate the release of calcium and calcium dependent signaling, as well as the activation of protein kinase C (Rhee, 2001). The PLCγ pathway regulates diverse cellular processes including proliferation, differentiation and migration (Yang et al., 2013).

ErbB receptors also activate STAT proteins, transcription factors that were initially implicated as cytokine signal transducers (Schindler, 2002). Different ErbB receptors activate different STAT proteins. EGFR activates STAT1, STAT3 and STAT5 (Olayioye et al., 1999; Schulze et al., 2005), and ErbB4 activates STAT5 (Jones 1999; Olayioye et al., 1999; Schulze et al., 2005). The inactive STATs reside in the cytosol as monomers, and are activated through phosphorylation-induced dimerization. STAT dimers translocate to the nucleus where they regulate gene expression (Schindler, 2002). In the context of EGFR signaling, STAT-mediated transcriptional responses promote proliferation and survival (Quesnelle et al., 2007). ErbB4, in turn, activates STAT5 to promote differentiation (Long et al., 2003).

2.2.4 Nuclear signaling of ErbB receptors

In addition to the classical signaling pathways activated by ErbB receptors at the cell surface, ligand-activated ErbB receptors can be shuttled to the nucleus where they may function as transcriptional regulators (Chen and Hung, 2015). All ErbB receptors have been observed to localize in the nucleus (Xie and Hung, 1994; Marti et al., 1991; Ni et al., 2001; Offterdinger et al., 2002). Unique among ErbB receptors, ErbB4 can translocate into the nucleus as a soluble intracellular receptor fragment (Ni et al., 2001).

The nuclear translocation mechanism of EGFR has been studied in detail. Ligand-activated receptors are first internalized *via* clathrin mediated endocytosis, and sorted from

endosomes to a retrograde vesicular trafficking route, which transports them to the *trans*-Golgi network and endoplasmic reticulum (Lo et al., 2006; Wang et al., 2010a) (Figure 4). All ErbB receptors contain a tripartite nuclear localization signal (NLS) in the intracellular juxtamembrane region, which allows them to be transported to the nuclei *via* interactions with nuclear transport receptors importin 1α and β (Lo et al., 2006; Hsu and Hung, 2007). Finally, SEC61 translocon activity is required to release transmembrane EGFR from the lipid membrane (Liao and Carpenter, 2007; Wang et al., 2010b). A similar translocation mechanism requiring endocytosis and importin β has been described for ErbB2 and ErbB3 (Giri et al., 2005; Wang et al., 2012; Reif et al., 2016).

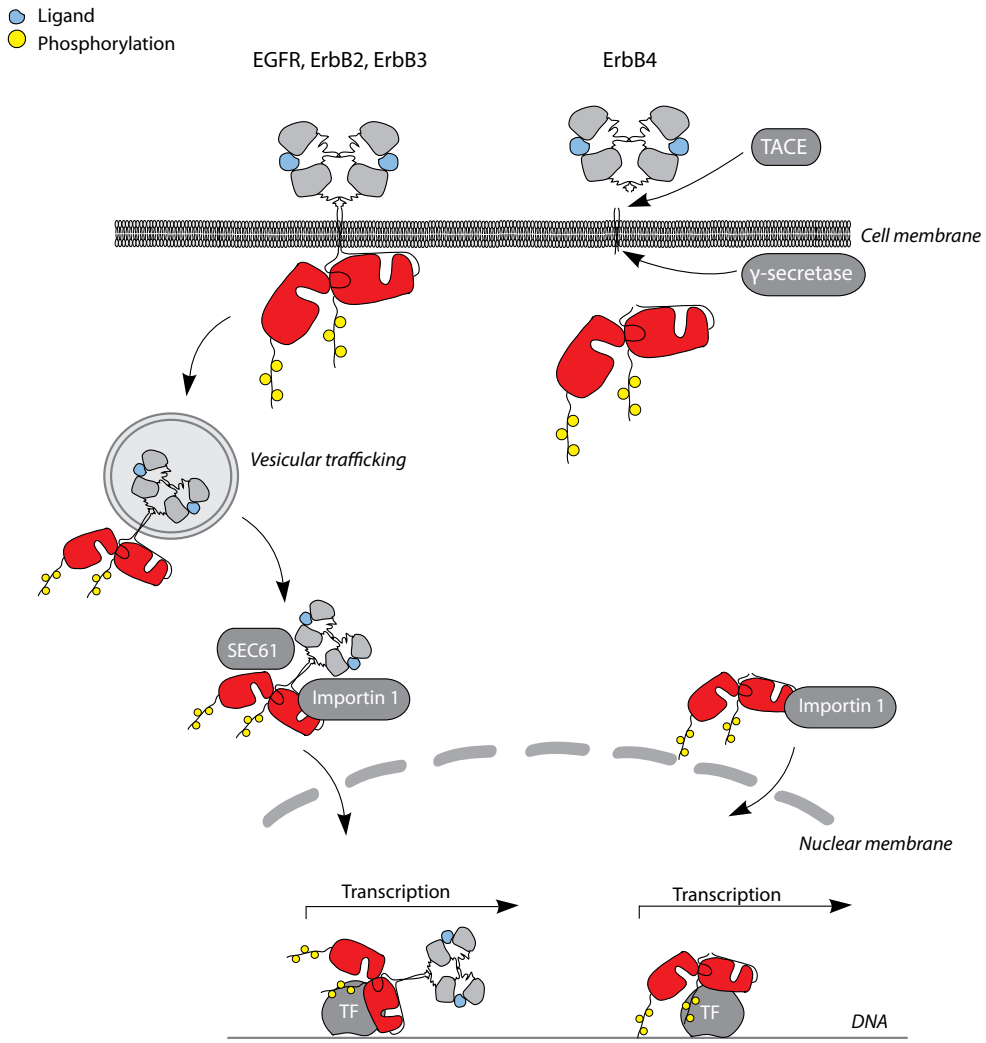

Figure 4. Nuclear translocation and signaling of ErbB receptors. Full-length ErbB receptors are transported to the nucleus via vesicular transport, and interactions with importin 1 and SEC61 translocon. ErbB4 undergoes regulated intramembrane proteolysis mediated by tumor-necrosis factor α converting enzyme (TACE) and γ-secretase, and is translocated into the nucleus as a soluble intracellular fragment. Nuclear ErbB receptors can regulate transcription. TF, transcription factor.

The nuclear translocation of ErbB4 intracellular fragment is initiated by a two-step proteolytic process called regulated intramembrane proteolysis (RIP) (Figure 4). Of note, although ErbB4 was the first RTK described to undergo RIP, many other RTKs have since been identified as RIP substrates (Merilahti et al., unpublished). First, the extracellular domain of ErbB4 is shed by tumor-necrosis factor α converting enzyme (TACE/ADAM17), generating a membrane-tethered receptor fragment (m80) and a soluble extracellular fragment (Rio et al., 2000). This proteolytic event is stimulated by phorbol esters or ligand binding (Vecchi et al., 1996; Zhou and Carpenter, 2000). Next, the m80 fragment is cleaved by γ-secretase complex, releasing the intracellular domain (ICD) into the cytosol (Ni et al., 2001; Lee et al., 2002). The soluble ICD contains a nuclear localization signal that mediates the nuclear translocation (Williams et al., 2004; Hsu and Hung, 2007).

Nuclear ErbB receptors have been demonstrated to promote proliferation by regulation of gene expression (Chen and Hung, 2015). For example, nuclear EGFR associates with cyclin D1 promoter and nuclear ErbB2 associates with cyclooxygenase-2 promoter, promoting their transcription (Lin et al., 2001; Wang et al., 2004). ErbB receptors do not contain DNA-binding domains, but instead regulate gene expression *via* interactions with DNA-binding transcription factors (Chen and Hung, 2015). In addition to transcriptional coregulatory functions, nuclear EGFR has been shown to phosphorylate and regulate the function of proliferating cell nuclear antigen (Wang et al., 2006).

Nuclear ErbB4 ICD regulates transcriptional processes by modifying the activity of transcriptional activators and repressors. The signaling mechanisms and cellular responses of ErbB4 ICD will be discussed in more detail in section 2.3.1.

2.2.5 Negative regulation of ErbB signaling

The signaling of ligand-activated ErbB receptors is attenuated by reversible and irreversible negative regulatory mechanisms. Receptor dephosphorylation and endocytosis-mediated receptor degradation are rapid mechanisms to downregulate signaling. In contrast, the synthesis of protein inhibitors represents a delayed negative regulatory mechanism (Citri and Yarden, 2006).

Protein tyrosine phosphatases (PTP) are enzymes that catalyze the removal of phosphate groups from phosphotyrosines, thus inactivating RTKs. The human genome encodes more than one hundred PTPs (Tonks, 2006). Several of them, for example protein tyrosine phosphatase 1B, have been shown to dephosphorylate and inactivate ErbB receptors (Haj et al., 2003; Monast et al., 2012).

Receptor endocytosis is the major downregulation mechanism of activated RTKs, including EGFR. The receptors are constitutively internalized from the plasma membrane, but ligand binding increases the internalization rate resulting in the rapid

retention of receptors in early endosomes (Goh and Sorkin, 2013). Although clathrin-mediated internalization is the major and fastest internalization pathway for ligand-activated EGFR, clathrin-independent pathways may also be involved (Sigismund et al., 2005; Goh and Sorkin, 2013). Once internalized, the receptors can be recycled back to the plasma membrane in recycling endosomes, or trafficked further into multivesicular bodies and sorted for lysosomal degradation. This sorting event is regulated by post-translational ubiquitin modification, mediated by ubiquitin ligase Cbl (Levkowitz et al., 1999; Goh and Sorkin, 2013) (Figure 5). Unlike EGFR, the ligand-induced endocytosis and degradation of ErbB2, ErbB3 and ErbB4 has been reported to be inefficient (Baulida et al., 1996). However, the stability of ErbB2, ErbB3 and ErbB4 is regulated by other ubiquitin-dependent mechanisms. The role of ubiquitination in the degradation of ErbB receptors will be discussed in more detail in section 2.4.1.

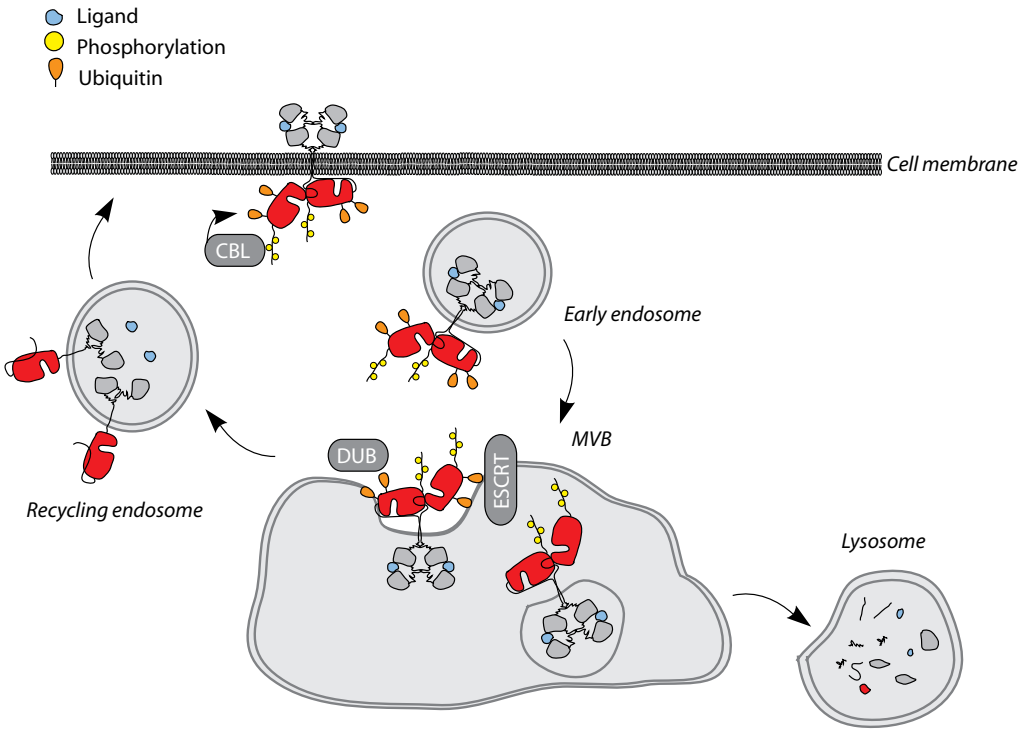

Figure 5. Ubiquitination and the endocytosis of EGFR. Activated EGFR is ubiquitinated (indicated in orange) by Cbl. Ubiquitinated receptors are trafficked from early endosomes to multivesicular bodies (MVB). In MVBs the ubiquitinated receptors are recognized by the endosomal sorting complex required for transport (ESCRT), and sorted for intraluminal vesicles and further to lysosomes. Alternatively, the receptors can be deubiquitinated and recycled back to the cell surface.

In addition to rapid downregulation by dephosphorylation and endocytosis, ErbB signaling is controlled by ligand-inducuble protein inhibitors that form a negative

feedback loop. These protein inhibitors include leucine-rich repeats and immunoglobulin-like domains-1 (LRIG1), receptor-associated late inducer/mitogen-induced gene 6 (RALT/MIG), and suppressors of cytokine signaling (SOCS) (Anastasi et al., 2003; Gur et al., 2004; Laederich, 2004; Kario et al., 2005). While LRIG1 and SOCS enhance ErbB receptor ubiquitination and degradation, RALT/MIG6 binds to the kinase domain and inhibits the formation of the kinase dimer (Gur et al., 2004; Laederich, 2004; Kario et al., 2005; Zhang et al., 2007).

2.2.6 ErbB receptors in embryonic development

The importance of ErbB signaling in normal tissues has been illustrated in genetic knockout studies. *Egfr* deficient mice die before preimplantation or survive until a few weeks after birth, depending on the genetic background (Miettinen et al., 1995; Threadgill et al., 1995). These mice demonstrate defective epithelial and central nervous system development (Miettinen et al., 1995; Threadgill et al., 1995; Sibilia et al., 1998).

Erbb2 null mice die at embryonic day 10.5 due to the lack of ventricular trabeculae, which results in cardiac failure (Lee et al., 1995). ErbB2 is also critical in the development of the nervous system, and *Erbb2* deficient mice demonstrate defects in neural crest-derived tissues such as cranial ganglia and Schwann cells (Lee et al., 1995; Erickson et al., 1997; Woldeyesus et al., 1999).

Erbb3 deletion also results in embryonic lethality. *Erbb3* null mice die at embryonic day 13.5 due to the abnormal development of cardiac cushions, which leads to defective atrioventricular valves and cardiac failure (Erickson et al., 1997; Riethmacher et al., 1997). Inactivation of *Erbb3* also results in severe neuropathies, especially in the peripheral nervous system, due to defective Schwann cell development (Riethmacher et al., 1997). Similar to ErbB2, *Erbb3* is required for the development of cranial ganglia (Erickson et al., 1997; Riethmacher et al., 1997).

Inactivation of *Erbb4* results in embryonic lethality, and *Erbb4*$^{-/-}$ mice have defects in the development of the heart, central nervous system, mammary gland, kidney and testis. These studies are reviewed in section 2.3.4.

2.2.7 ErbB receptors in cancer

Dysregulation of growth factor signaling is a common feature of cancer (Hanahan and Weinberg, 2000; 2011). Since the identification of EGFR and ErbB2 as homologs of avian and rodent oncogenes, *v-erb-B* (Downward et al., 1984) and *neu* (Schechter et al., 1984), the role of ErbB signaling in cancer has been extensively studied. While *EGFR* and *ERBB2* are well-established oncogenes driving tumors of epithelial tissues and the central nervous system, ErbB3 has been implicated in cancer as a co-receptor able to promote signaling from EGFR and ErbB2 (Arteaga and Engelman, 2014). Compared to other ErbB receptors, the role of ErbB4 in cancer is poorly defined (section 2.3.5).

ErbB signaling in cancer can be abnormally activated by overexpression, somatic mutations, or increased ligand availability (Arteaga and Engelman, 2014). For example, a subset of breast cancers overexpresses *ERBB2* due to genetic amplification (Slamon et al., 1987), and substitutions and deletions of *EGFR* have been found in subsets of non-small cell lung cancers and glioblastomas (Sugawa et al., 1990; Lynch et al., 2004; Paez, 2004; Pao et al., 2004). Additionally, failure to attenuate signaling contributes to the aberrant activity of ErbB receptors in cancer (Avraham and Yarden, 2011; Mellman and Yarden, 2013).

Several drugs have been developed and approved for clinical use to treat cancers with overexpressed or mutated ErbB receptors. Drugs targeting ErbB receptors are either small-molecule tyrosine kinase inhibitors that typically compete with ATP, or monoclonal antibodies that bind to the receptor extracellular domain. For example, trastuzumab, a monoclonal antibody targeting ErbB2, is approved to treat breast cancer patients with *ERBB2* amplification, and erlotinib, a small-molecule tyrosine kinase inhibitor, is used in non-small cell lung cancer patients with *EGFR* mutations (Arteaga and Engelman, 2014).

2.3 ErbB4 receptor

The *ERBB4* gene is expressed as four isoforms that are generated by alternative messenger RNA splicing. Two of the isoforms differ in the extracellular juxtamembrane region (JM-a and JM-b) and two differ in the intracellular cytoplasmic domain (CYT-1 and CYT-2) (Figure 6). Through these structurally and functionally distinct isoforms, ErbB4 can activate both classical RTK-induced signaling pathways (reviewed earlier in section 2.2.3), as well as RIP-mediated signaling.

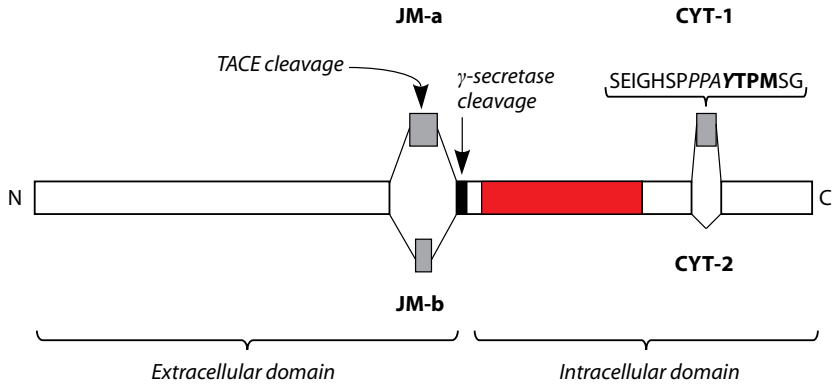

Figure 6. Schematic structure of ErbB4 isoforms. Alternative splicing generates four isoforms that differ at juxtamembrane (JM) or cytoplasmic (CYT) domains. JM-a isoforms, but not JM-b isoforms, include a tumor-necrosis factor α converting enzyme (TACE) cleavage site. Subsequent cleavage by γ-secretase releases a soluble intracellular domain. CYT-1 isoforms, but not CYT-2 isoforms, contain a sequence that has binding sites for PI3K (YTPM; indicated in bold) and WW domain containing proteins (PPAY; indicated in italics). Black indicates transmembrane domain and red indicates tyrosine kinase domain. Modified from Määttä et al., 2006.

2.3.1 Extracellular juxtamembrane isoforms of ErbB4

The JM isoforms of ErbB4, JM-a and JM-b, include either exon 16 or exon 15, respectively, resulting in amino acid and structural differences in the extracellular juxtamembrane region (Figure 6). The JM-a isoform includes a 23 amino acid sequence with a cleavage site for TACE, resulting in the shedding of the JM-a extracellular domain (Elenius et al., 1997b; Rio et al., 2000). In contrast, the JM-b isoform includes a shorter 13 amino acid sequence and lacks the TACE cleavage site (Elenius et al., 1997b). TACE cleavage triggers a second proteolytic cleavage by γ-secretase complex, releasing a soluble ErbB4 ICD (Ni et al., 2001; Lee et al., 2002) (Figure 4).

Soluble ErbB4 ICD can translocate into the nucleus as described in section 2.2.4, but also reside in the cytosol and accumulate in the mitochondria (Ni et al., 2001; Naresh et al., 2006). Nuclear ErbB4 ICD associates with, and either promotes or represses, the activity of transcriptional regulators including yes-associated protein (YAP), STAT5A, estrogen receptor α (ERα), ETO2, the TAB2-NCoR complex, activator protein 2 (AP-2), KRAB associated protein 1 (KAP1), and hypoxia-inducible factor 1 α (HIF-1α) (Komuro et al., 2003; Williams et al., 2004; Linggi and Carpenter, 2006; Sardi et al., 2006; Zhu et al., 2006; Gilmore-Hebert et al., 2010; Sundvall et al., 2010; Paatero et al., 2012). Soluble ErbB4 ICD is an active tyrosine kinase (Linggi et al., 2006), but only a few studies have addressed the role of ErbB4 kinase activity in ICD-mediated signaling (Linggi and Carpenter, 2006; Muraoka-Cook et al., 2006; Naresh et al., 2006). Currently, the only known phosphorylation substrate of ErbB4 ICD is MDM2, a ubiquitin ligase that is critical for regulating p53 protein level (Arasada and Carpenter, 2005).

Cellular responses mediated by RIP-dependent ErbB4 signaling have been elucidated in studies that compare JM-a and JM-b isoforms, or in studies that employ mutagenesis of the γ-secretase cleavage site or ectopic expression of the soluble ICD. Cleavable JM-a CYT-2, but not the non-cleavable JM-b CYT-2, promotes the proliferation and survival of breast cancer and myeloid cells (Määttä et al., 2006) and regulates tubulogenesis of kidney epithelial cells (Zeng et al., 2007). JM-a CYT-2 and JM-b CYT-2 have different target genes and promote opposing cellular effects in serum starved fibroblasts: JM-a promotes survival, whereas JM-b mediates apoptosis (Sundvall et al., 2010). *In vivo*, JM-a, but not JM-b, regulates astrogenesis in the developing brain (Sardi et al., 2006). The function of JM-a CYT-1 in promoting mammary epithelial cell differentiation requires an intact γ-secretase cleavage site, and the ectopically expressed soluble ICD can regulate differentiation *in vitro* and *in vivo* (Muraoka-Cook et al., 2006; 2009).

2.3.2 Cytoplasmic isoforms of ErbB4

The cytoplasmic (CYT) isoforms either include (CYT-1) or exclude (CYT-2) exon 26, encoding a 16 amino acid sequence near the C-terminus (Figure 6). The CYT-1-specific sequence contains a binding site for the SH2 domain of PI3K p85 subunit (YTPM), and

can activate PI3K (Elenius et al., 1999). Moreover, the CYT-1-specific sequence has a PPXY motif that can bind to WW domain containing proteins such as YAP (Komuro et al., 2003; Omerovic et al., 2004).

In studies comparing the cellular responses mediated by different cytoplasmic isoforms, CYT-1, but not CYT-2, was shown to promote survival and chemotaxis of fibroblasts, survival of medulloblastoma cells, and anchorage-independent growth of ovarian cancer cells in a PI3K-dependent manner (Kainulainen et al., 2000; Ferretti et al., 2006; Paatero et al., 2013).

The cytoplasmic isoforms differ in their stability, tyrosine phosphorylation, and subcellular localization (Määttä et al., 2006; Sundvall et al., 2007). Compared to CYT-1, JM-a CYT-2 is more stable and constitutively phosphorylated (Määttä et al., 2006). Consistently, only JM-a CYT-2 promotes the survival of myeloid cells, possibly through its constitutive tyrosine kinase activity (Määttä et al., 2006). JM-a CYT-2 produces more ICD, and ICD of CYT-2 localizes to the nuclei more efficiently than that of CYT-1 (Määttä et al., 2006; Sundvall et al., 2007). Similar to different JM isoforms (2.3.1), CYT isoforms regulate the expression of different target genes (Wali et al., 2014a).

In the context of the mammary gland, inducible expression of ErbB4 ICD of CYT-1 type promotes differentiation both *in vitro* and *in vivo*, while CYT-2 suppresses differentiation and promotes proliferation (Muraoka-Cook et al., 2009). However, a more recent *in vivo* study comparing the mammary gland-specific overexpression of full-length JM-a CYT-1 and JM-a CYT-2 reported that CYT-1 suppresses mammary gland differentiation, and induces neoplasia (Wali et al., 2014b). In contrast, CYT-2 was shown to suppress mammary gland differentiation only mildly, but induce a low incidence of hyperplasia (Wali et al., 2014b). Despite the partially conflicting findings, these studies demonstrate that the cytoplasmic isoforms of ErbB4 have different signaling potentials also *in vivo*.

2.3.3 Expression of ErbB4 isoforms

ErbB4 messenger RNA expression has been analyzed in various mouse and human tissues. The highest ErbB4 expression levels are observed in the nervous system, kidney, salivary and thyroid glands, testis, and heart and skeletal muscle (Elenius et al., 1997b; 1999; Junttila et al., 2005; Veikkolainen et al., 2011).

The alternative splicing of ErbB4 messenger RNA is regulated in a tissue-specific manner, resulting in tissue-specific isoform expression patterns. Epithelial tissues, such as those in the kidney and mammary gland, exclusively express the cleavable JM-a isoform, while mesenchymal tissues such as the heart and skeletal muscle tissue express JM-b either dominantly or together with JM-a (Junttila et al., 2005; Veikkolainen et al., 2011). Both JM-a and JM-b are expressed in nervous tissues such as the brain and the cerebellum, but they have been reported to be present in different cell types (Elenius et

al., 1997b; Junttila et al., 2005; Veikkolainen et al., 2011). In contrast to JM isoforms, both CYT isoforms are usually expressed in the same cell types, although not always in the same quantities (Junttila et al., 2005; Veikkolainen et al., 2011).

2.3.4 ErbB4 in embryonic and adult tissues

Similar to other ErbB receptors, reviewed in section 2.2.6, ErbB4 is essential during embryonic development, and *Erbb4*$^{-/-}$ mice die at embryonic day 10.5. Like *Erbb2*$^{-/-}$ mice, they demonstrate lack of ventricular trabeculae resulting in cardiac failure (Gassmann et al., 1995). A conditional deletion of *Erbb4* in ventricular cardiomyocytes during embryogenesis leads to dilated cardiomyopathy in adult mice (García-Rivello et al., 2005).

ErbB4 is also required for the development of the nervous system. *Erbb4*$^{-/-}$ mice demonstrate defective neural innervation of the hindbrain, migration of the cranial neural crest cells, and abnormalities in the cerebellum (Gassmann et al., 1995; Tidcombe et al., 2003).

The function of ErbB4 in the mammary gland has been revealed in genetic inactivation studies using heart-specific *ERBB4* transgene expression to rescue the embryonic lethality of the *Erbb4* null embryos, or conditional mammary gland-specific *Erbb4* deletion in adult mice (Long et al., 2003; Tidcombe et al., 2003). These mice fail to lactate due to impaired differentiation of the mammary lobuloalveoli and reduced STAT5-mediated milk gene expression (Long et al., 2003; Tidcombe et al., 2003).

Conditional mouse knockout models have also demonstrated roles for ErbB4 in the kidney and the male reproductive system. Kidney-specific deletion of *Erbb4* during embryonic development results in kidney dysfunction with mispolarization in the collecting duct epithelia and larger ductal lumens (Veikkolainen et al., 2012). *Erbb4* inactivation in testis Sertoli cells during embryogenesis reduces testis size and produces aberrant organization of testicular seminiferous tubules, resulting in reduced fertility (Naillat et al., 2014).

2.3.5 ErbB4 in cancer

ErbB4 is expressed in several cancer types, and both increased and decreased expression compared to non-neoplastic control tissue have been reported (Hollmén and Elenius, 2010). For example, overexpression of ErbB4 has been reported in ovarian cancer and central nervous system malignancies, and reduced expression in bladder cancer (Gilbertson et al., 2002; Memon et al., 2004; Ferretti et al., 2006; Steffensen et al., 2008). Both overexpression and reduced expression of ErbB4 have been reported in breast cancer (Srinivasan et al., 1998; Witton et al., 2003; Junttila et al., 2005).

ErbB4 expression can be associated with favorable or poor prognosis (Hollmén and Elenius, 2010). In breast cancer ErbB4 is usually expressed in tumors with estrogen- and progesterone receptor-positive, ErbB2-negative, well-differentiated phenotype, which is a breast cancer subtype that is associated with favorable prognosis (Bacus et al., 1996; Knowlden et al., 1998; Kew et al., 2000; Pawlowski et al., 2000; Sundvall et al., 2008). However, in two breast cancer studies, high ErbB4 expression is associated with poor prognosis (Bièche et al., 2003; Lodge et al., 2003), despite the association of ErbB4 expression with estrogen receptor positivity (Bièche et al., 2003). Another example of the association of ErbB4 expression with favorable prognosis is bladder cancer, and an example of the association of ErbB4 expression with poor prognosis is medulloblastoma (Gilbertson et al., 1997; Memon et al., 2004).

While experimental *in vitro* and *in vivo* models have revealed unique functions for different ErbB4 isoforms (sections 2.3.1 and 2.3.2), only a few studies have specifically analyzed the expression or prognostic significance of ErbB4 isoforms in cancer. Increased expression of CYT-1 isoform is associated with more aggressive medulloblastoma subtype and decreased survival in serous ovarian cancer (Ferretti et al., 2006; Paatero et al., 2013). The expression levels of CYT-1 and CYT-2 isoforms have also been reported to vary in breast cancer (Machleidt et al., 2013; Fujiwara et al., 2014; Kiuchi et al., 2014). Nuclear ErbB4 immunoreactivity, which potentially represents the soluble ICD, is frequently detected in breast cancer irrespective of the breast cancer subtype (Srinivasan et al., 2000). Nuclear ErbB4 epitope is associated with decreased survival when compared to the localization of ErbB4 epitope at the cell surface (Junttila et al., 2005). Conversely, cytoplasmic and membranous ErbB4 staining are associated with increased survival (Aqeilan et al., 2007; Thor et al., 2009).

Cancer sequencing studies collected in the cBioPortal database (www.cbioportal.org) have identified *ERBB4* mutations in varying frequencies in several cancer types including lung cancer, melanoma, gastric cancer and colorectal cancer. Some cancer-associated *ERBB4* mutations have been functionally characterized. *ERBB4* mutations found in melanoma have been shown to be oncogenic, and the lung cancer mutations were found to alter the signaling properties of ErbB4 or promote receptor activation (Prickett et al., 2009; Tvorogov et al., 2009; Kurppa et al., 2016).

Although the role of ErbB4 in cancer is less well characterized than that of other ErbB family members, drugs that target ErbB4 are available. Afatinib, a pan-ErbB tyrosine kinase inhibitor that inhibits ErbB2 and ErbB4 in addition to EGFR, is approved in several countries for the treatment of metastatic non-small cell lung cancer with *EGFR* kinase domain mutations (Solca et al., 2012; Modjtahedi et al., 2014).

2.3.6 ErbB4 in non-neoplastic diseases

Aberrant ErbB4 signaling has been implicated in neurological disorders. Together with *NRG1*, *ERBB4* is a candidate susceptibility gene in schizophrenia (Stefansson et al., 2002; Nicodemus et al., 2006; Norton et al., 2006; Silberberg et al., 2006). Intriguingly, some of the identified genetic *ERBB4* variants are associated with increased expression of the JM-a CYT-1 isoform in postmortem brain studies of schizophrenia patients (Silberberg et al., 2006; Law et al., 2007). In addition to schizophrenia, genetic variants of *ERBB4* have been identified in amyotrophic lateral sclerosis. These include causative germ line and sporadic coding sequence mutations that inhibit ErbB4 activity *in vitro* (Takahashi et al., 2013).

ErbB4 is required for cardiac development during embryogenesis and for the maintenance of adult heart function (Gassmann et al., 1995; García-Rivello et al., 2005). The ErbB4/NRG-1 signaling system has also been shown to regulate the proliferation of cardiomyocytes in response to ischemic heart injury (Bersell et al., 2009). Notably, therapeutic administration of NRG-1 improves cardiac function in experimental models of ischemic heart injury (Bersell et al., 2009; Galindo et al., 2013), and in patients with chronic heart failure (Gao et al., 2010; Jabbour et al., 2011).

2.4 Post-translational modification by ubiquitin and SUMO

Cell signaling is regulated at multiple levels. Compared to altering cell behavior by transcriptional responses, post-translational modification of existing proteins provides a fast and often transient way of controlling the activity of signaling pathways. In addition to small molecules such as phosphate groups, proteins can be modified with much larger polypeptides.

The first example of an entire protein acting as a post-translational modification (PTM) was ubiquitin (Hershko and Ciechanover, 1998). Subsequently, many other ubiquitin-like modifiers (UBL) including small ubiquitin-like modifier (SUMO) have been discovered. UBLs share a similar three-dimensional structure with ubiquitin, and a pathway by which they are covalently conjugated to substrates. First, UBL precursors are proteolytically processed to mature forms. Ubiquitin, which is expressed as a polyubiquitin precursor, is processed to mature ubiquitin monomers, and SUMO is cleaved to expose a carboxy-terminal motif necessary for conjugation. Next, UBLs are activated by an E1 enzyme in an ATP-dependent reaction, and then transferred to an E2 conjugating enzyme. Finally, the composite action of E2 and E3 ligase, or the activity of E3 alone, ligates UBLs to a lysine residue in a target protein by an isopeptide bond, or to an amino-terminal methionine residue by a peptide bond. Attachment of UBLs is reversible, and specific proteases catalyze the cleavage reactions (Hochstrasser, 2009) (Figure 7).

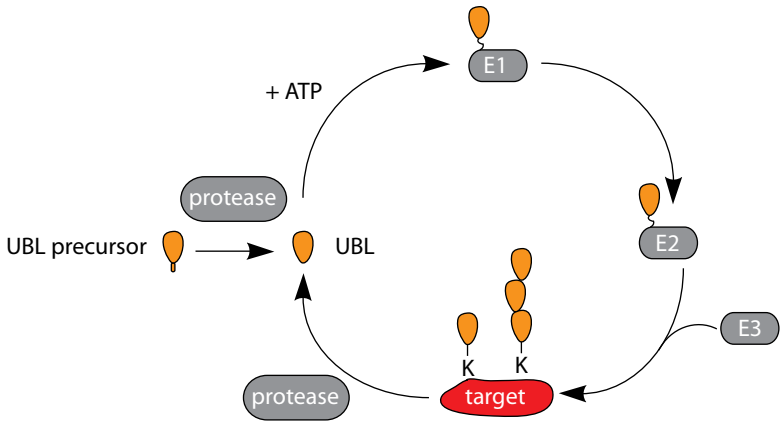

Figure 7. Conjugation of ubiquitin-like modifiers. Ubiquitin-like modifier (UBL; indicated in orange) precursors are proteolytically processed to mature forms, and activated by E1 in an ATP-dependent manner. E2 conjugating and E3 ligating enzymes attach UBLs to a single or multiple lysine (K) residues of target proteins (indicated in red). Some UBLs can also form polymeric chains. Specific proteases catalyze the deconjugation of UBLs.

The consequences of UBL conjugation are diverse. The UBL ”tag” may create or mask interaction surfaces, induce or interfere with other PTMs, or induce conformational changes (Hochstrasser, 2009). Functionally, by altering the stability, activity or localization of proteins, they participate in most signaling events and cellular processes (sections 2.4.1 and 2.4.2). Indeed, the activity of RTKs including ErbB receptors is tighly regulated by ubiquitination (Goh and Sorkin, 2013). Additionally, post-translational modifications of EGFR and insulin-like growth factor receptor 1 (IGF-1R) by SUMO, and EGFR by another UBL, NEDD8, have been described (Oved et al., 2006; Sehat et al., 2010; Packham et al., 2015).

2.4.1 Ubiquitination

Ubiquitin is a small, 76 amino acid polypeptide (Figure 8). Ubiquitin is one of the most highly conserved proteins in eukaryotes, with only three conservative amino acid changes from yeast *Saccharomyces Cerevisiae* to *Homo Sapiens*. The human ubiquitin system comprises a vast signaling network, including two ubiquitin E1 enzymes, approximately 35 ubiquitin E2s, and at least 600 E3 ubiquitin ligases in the genome. Conversely, ubiquitination is reversed by approximately 80 deubiquitinating enzymes (DUB) that control the level of ubiquitination along with the E3s (Komander and Rape, 2012; Williamson et al., 2013). The ubiquitin signal is recognized by different ubiquitin binding domains (UBD) that have been identified in over 200 proteins (Husnjak and Dikic, 2012).

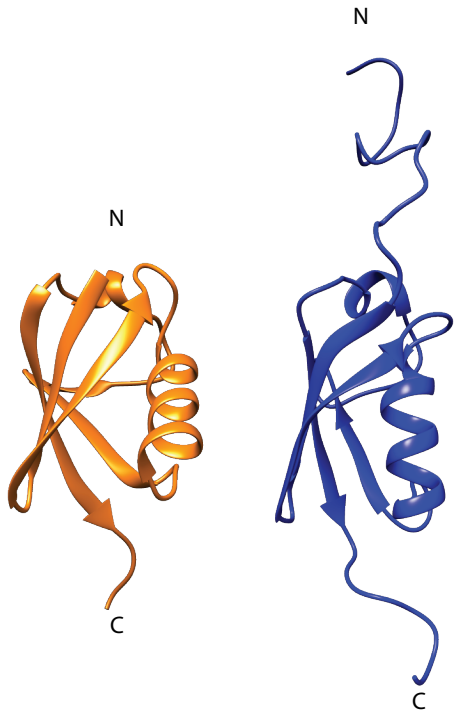

Figure 8. Structures of ubiquitin and SUMO1. Structures of ubiquitin (orange) and SUMO1 (blue) were obtained from Research Collaboratory for Structural Bioinformatics Protein Data Bank (RCSB PDB; accession codes are 1ubq for ubiquitin, and 1a5r for SUMO1) and visualized using UCSF Chimera software. Despite their low amino acid sequence homology, ubiquitin and SUMO share a similar three-dimensional structure.

Types of ubiquitin modification

Proteins can be conjugated with a single ubiquitin molecule, referred to as monoubiquitination, or with a single ubiquitin at multiple lysine residues, referred to as multimonoubiquitination (Komander and Rape, 2012). For example, EGFR has been shown to be multimonoubiquitinated (Haglund et al., 2003; Mosesson et al., 2003). Ubiquitin contains seven lysines, and each of them can be conjugated with ubiquitin molecules leading to the formation of polyubiquitin chains. While all linkages (K6, K11, K29, K33, K48, K63) have been reported, substrates are mainly known for the most commonly used K48 and K63-linked chains (Peng et al., 2003; Komander and Rape, 2012). A polyubiquitin chain can also form in a head-to-tail manner by modification of the amino-termini of ubiquitin molecules (Walczak et al., 2012). The type of ubiquitination can be controlled by an E2, an E3, or a specific E2-E3 complex, and it determines the effect of the modification on substrate function (Kim et al., 2007; Kim and Huibregtse, 2009; Ye and Rape, 2009; Komander and Rape, 2012).

E3 ubiquitin ligases

Members of the large and diverse group of E3 ligases are key regulators of ubiquitination. They directly interact with the target protein and thus determine the specificity of ubiquitination. The ubiquitin E3s can be classified into three major types depending on their characteristic domains, which mediate the ubiquitin transfer to the target protein. Really interesting new gene (RING) type E3s recognize the substrate and function as scaffolds between substrate and E2s, which catalyze the ubiquitin transfer (Deshaies and Joazeiro, 2009). RING E3s are the most abundant type of ubiquitin ligases, comprising more than 95% of human E3s (Deshaies and Joazeiro, 2009). In contrast to RING E3s, much smaller groups of homologous to E6-AP carboxyl terminus (HECT) and RING-between-RING (RBR) E3s ligases are true enzymes. They receive the ubiquitin molecule from the E2, and subsequently catalyze the ubiquitin transfer to the substrate (Rotin and Kumar, 2009; Wenzel and Klevit, 2012).

Functions of ubiquitin modification

Ubiquitination regulates nearly all cellular processes through proteolytic, but also non-proteolytic mechanisms. The proteolytic pathways controlled by ubiquitination include proteasomal degdaration, endocytosis and lysosomal degradation, and autophagy. The non-proteolytic functions are based on the interactions of the ubiquitinated substrate with effector proteins that contain UBDs (Husnjak and Dikic, 2012; Komander and Rape, 2012).

Ubiquitination was first discovered as a signal for proteasomal degradation (Ciechanover, 2015). The proteasome is a large multi-subunit protein complex that recognizes polyubiquitin-tagged proteins with UBDs, and degrades them into peptides in an ATP-dependent manner (Navon and Ciechanover, 2009). All polyubiquitin chains, except those with K63- and amino-terminal linkages, may mediate proteasomal degradation (Xu et al., 2009). The ubiquitin-proteasome system controls the levels, and thus the activity, of many intracellular signaling proteins. For example, ubiquitination regulates the cell cycle by the periodic degradation of cyclins and cyclin-dependent kinase inhibitors (Teixeira and Reed, 2013). Another important function of the ubiquitin-proteasome system is the elimination of misfolded proteins (Navon and Ciechanover, 2009).

Ubiquitination is also a sorting signal in the endocytic pathway, targeting plasma membrane proteins for lysosomal degradation (Piper et al., 2014). The role of ubiquitination in the endocytosis of EGFR has been extensively studied (Goh and Sorkin, 2013; Piper et al., 2014). Upon ligand binding, autophosphorylation of EGFR recruits the RING-type E3 ligase Cbl, which binds to EGFR either directly with its SH2-domains or *via* the SH2-domain containing adaptor protein GRB2 (Levkowitz et al., 1996; 1999; Jiang et al., 2003) (Figure 5). Cbl modifies EGFR with ubiquitin monomers and polyubiquitin

chains that primarily contain K63-linkages (Haglund et al., 2003; Mosesson et al., 2003; Huang et al., 2006). One model suggests that the ubiquitinated receptors are recognized by the UBDs of EGFR pathway substrate 15 (EPS15) and Epsin, which in turn assemble the receptors to clathrin-coated pits through their interactions with clathrin adapter protein complex 2 (Goh and Sorkin, 2013). However, the role of this mechanism in EGFR endocytosis is unclear, as ubiquitination of EGFR is not essential for the internalization step (Huang et al., 2007). Instead, ubiquitination is required for the degradation of EGFR, which is thought to reflect the irreversible ubiquitin-dependent sorting of EGFR from early endosomes into the intraluminal vesicles of multivesicular bodies (MVB) (Huang et al., 2006; Goh and Sorkin, 2013). According to this model, the ubiquitinated receptors are recognized by the UBDs of endosomal sorting complex required for transport (ESCRT)-0 at the limiting membrane of the MVB. Subsequently, the action of ESCRT complexes I-III direct the receptors into the intraluminal vesicles of the MVB, which further targets them into lysosomes where they are degraded (Piper et al., 2014) (Figure 5).

Ubiquitination of ErbB receptors

Along with platelet-derived growth factor (PDGF) β-receptor, EGFR was among the first RTKs reported to be ubiquitinated in response to ligand stimulation (Mori et al., 1992; Galcheva-Gargova et al., 1995). Numerous studies have since establised the key role of ubiquitination and Cbl in ligand-induced degradation of EGFR (Goh and Sorkin, 2013). A few other E3 ligases have also been reported to ubiquitinate EGFR. For example, RING-type E3 ligases RNF126 and Rabring7 were shown to function downstream of Cbl to further promote EGFR ubiquitination and late endosomal sorting (Smith et al., 2013). Furthermore, DUB activity in the endocytic pathway before the ubiquitin-dependent sorting step promotes EGFR recycling to the plasma membrane and delays degradation (Figure 5). These DUBs include ubiquitin specific protease (USP) 2a in early endosomes, associated molecule with the SH3 domain of STAM (AMSH) and ubiquitin specific protease 8 in MVBs, and Cezanne-1 (McCullough et al., 2004; Mizuno et al., 2005; Berlin et al., 2010; Pareja et al., 2011; Liu et al., 2012).

ErbB2, ErbB3 and ErbB4 are also ubiquitinated. However, in contrast to EGFR, they do not interact with Cbl, and are thought to be endocytosed inefficiently (Baulida et al., 1996; Levkowitz et al., 1996). In agreement with this observation, ubiquitin ligases that interact with ErbB2, ErbB3 and ErbB4 are often reported to regulate the steady-state levels and induce proteasomal instead of lysosomal degradation. ErbB2 is ubiquitinated by carboxyl-terminal HSP70-interacting protein (CHIP), an E3 ligase containing a RING-related U-box (Xu et al., 2002; Zhou et al., 2003; Deshaies and Joazeiro, 2009). ErbB3 is ubiquitinated by a RING-type E3 ligase neuregulin receptor degradation protein-1 (NRDP1) and a HECT-type E3 ligase neural precursor cell expressed, developmentally

downregulated-4 (NEDD4) (Diamonti et al., 2002; Qiu and Goldberg, 2002; Huang et al., 2015). In addition to ErbB3, NRDP1 also regulates steady-state levels of ErbB4 (Diamonti et al., 2002). A multisubunit RING-type E3 ligase anaphase-promoting complex/cyclosome (APC/C) induces the proteasomal degradation of soluble ErbB4 ICD (Strunk et al., 2007).

2.4.2 SUMOylation

SUMOs are approximately 100 amino acid polypeptides highly conserved in evolution. Mammalian cells express three SUMO proteins that share only about 20% amino acid sequence identity with ubiquitin, but have a similar three-dimensional structure; the ”ubiquitin fold” (Figure 8). The amino acid sequence of SUMO1 is approximately 50% identical with SUMO2 and SUMO3, which are nearly identical and jointly referred to as SUMO2/3 (Flotho and Melchior, 2013). SUMO1 and SUMO2/3 are conjugated to distinct, but also overlapping substrates (Vertegaal et al., 2006). Unlike SUMO1, SUMO2/3 are able to form polymeric chains through internal lysine residues (Tatham et al., 2001).

The SUMOylation machinery

In contrast to ubiquitination, a considerably smaller set of enzymes regulates SUMOylation. Human cells express a single heterodimeric SUMO E1 enzyme (SUMO activating enzyme 1/2) and a single SUMO E2 enzyme, UBC9 (Flotho and Melchior, 2013). Consequently, UBC9 is required for SUMO conjugation to all substrates, and genetic inactivation of *Ubc9* in mice results in early embryonic lethality due to chromosome condensation and segregation defects (Nacerddine et al., 2005). Even in the abscence of an E3 ligase, UBC9 can directly interact with and modify some target proteins, but the prescence of E3s usually promotes SUMOylation (Flotho and Melchior, 2013).

The currently known human SUMO E3 ligases include the protein inhibitor of activated STAT (PIAS) protein family (PIAS1, PIASxα and β, PIAS3, PIASy) (Kahyo et al., 2001; Sachdev et al., 2001; Kotaja et al., 2002) and Ran-binding protein 2 (RanBP2) (Pichler et al., 2002). PIAS proteins contain a Siz/PIAS(SP)-RING domain similar to the RING of E3 ubiquitin ligases, and they function as scaffolds between the SUMO-loaded E2 and substrates (Figure 9). Despite their well-characterized role as SUMO E3 ligases, PIAS proteins also have SP-RING-independent functions. These functions require their SUMO interaction motif (SIM), or domains that mediate protein-protein or protein-DNA interactions (Figure 9) (Rytinki et al., 2009). RanBP2 is a SUMO E3 ligase that is not RING- or HECT-type (Pichler et al., 2004). Instead, its E3 ligase activity can be attributed to a stable multisubunit complex of RanBP2, UBC9 and SUMO-modified Ran GTPase-activating protein-1 (RanGAP1) (Werner et al., 2012). Other proteins that stimulate SUMOylation of specific targets, but that are not classified as definite SUMO

E3 ligases have been reported. Examples of these include Polycomb 2 and TOPORS (Kagey et al., 2003; Weger et al., 2005).

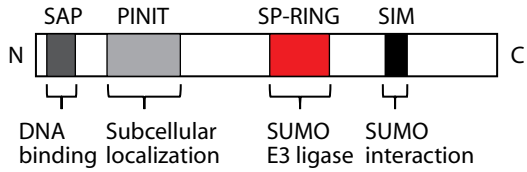

Figure 9. Schematic structure of PIAS proteins. All PIAS proteins have a similar domain architecture with N-terminal SAP (scaffold attachment factor-A/B, acinus and PIAS) and PINIT domains, SP-RING, and SIM. The SAP and PINIT domains mediate DNA binding and subcellular localization, respectively (Rytinki et al., 2009). SP-RING domain interacts with UBC9 and is essential for the E3 ligase activity. SIM interacts with SUMO non-covalently.

SUMOylation is a reversible modification catalyzed by SUMO isopeptidases (SUP). The human sentrin-specifc protease (SENP) family, which deSUMOylates many targets, consists of SENP1, -2, -3, -5, -6 and -7 (Gong et al., 2000; Kim et al., 2000; Nishida et al., 2000; 2001; Gong and Yeh, 2006; Lima and Reverter, 2008). The more recently characterized desumoylating isopeptides 1 and 2 (Shin et al., 2012) and ubiquitin-specific protease-like 1 (Schulz et al., 2012) have a limited number of targets compared to SENPs.

It is still unclear how substrate specificity is achieved with the limited number of SUMO E3 ligases and isopeptidases. Subnuclear compartmentalization of the SUMO machinery may play a role in regulating target selection. Indeed, (de)SUMOylating enzymes are concentrated in promyelocytic leukemia (PML) nuclear bodies and nuclear pores (Saitoh et al., 2006; Palancade and Doye, 2008; Sahin et al., 2014b), and the specificities of SENPs are partially controlled by their localization in nuclear pores, nucleoli or nucleoplasm (Gong et al., 2000; Nishida et al., 2000; Hang and Dasso, 2002; Zhang et al., 2002; Gong and Yeh, 2006). In addition, the SUMOylation machinery has been suggested to simultaneously modify a functionally related group of proteins, rather than individual targets (Jentsch and Psakhye, 2013).

Target lysine selection

Unlike ubiquitination, SUMO is conjugated to a defined lysine residue or residues in a target protein. SUMOylation frequently occurs on a consensus motif ΨKx(E/D) where Ψ is a large hydrophobic residue and x is any amino acid (Rodriguez et al., 2001; Hendriks and Vertegaal, 2016). Notably, UBC9 directly interacts with the consensus motif (Sampson et al., 2001). Several variations of the consensus motif have been identified. For example, the consensus motif can be inverted to E/DxKΨ (Matic et al., 2010), or extended to include a proline-directed serine phosphorylation site (phosphorylation-

dependent SUMOylation motif; ΨKxExxSP) that enhances UBC9 binding and thus SUMOylation (Hietakangas et al., 2006; Mohideen et al., 2009).

Despite the well-defined consensus motif, unbiased proteomic screens have revealed that up to 50 % of the identified SUMO acceptor lysines do not match the consensus (Matic et al., 2010; Hendriks et al., 2014). Selection of a non-consensus site can be dependent on non-covalent interaction of the SUMO-loaded UBC9 with a SIM present in a target protein (Lin et al., 2006). Alternatively, SUMO E3 ligases that interact with both UBC9 and target protein may direct acceptor site selection (Flotho and Melchior, 2013).

Functions of SUMO modification

The consequences of SUMOylation are diverse and depend on the substrate. In contrast to ubiquitination that often results in target proteolysis, SUMOylation is usually a non-proteolytic modification and can thus function as a reversible molecular switch regulating the activity, localization, or stability of its targets. SUMOylation is best characterized in nuclear processes such as transcription and nuclear transport, but also in the regulation of signal transduction pathways (Flotho and Melchior, 2013).

A considerable group of SUMO substrates are transcriptional regulators, whose activity can be modulated, typically repressed, by SUMOylation (Garcia-Dominguez and Reyes, 2009). SUMOylation can repress transcription by recruiting chromatin modifying enzymes such as histone deacetylases, or by inducing the formation of repressor complex *via* SUMO-SIM interactions (Garcia-Dominguez and Reyes, 2009) (Figure 10). SUMOylation can also alter the activity of transcriptional regulators by targeting them into nuclear subdomains, such as promyelocytic leukemia (PML) nuclear bodies (Sachdev et al., 2001; Ross et al., 2002; Bernardi and Pandolfi, 2007).

SUMOylation regulates the subcellular localization of many targets. In fact, SUMO was initially discovered as a post-translational modifier targeting RanGAP1 to the nuclear pore complex (Matunis et al., 1996; Mahajan et al., 1997). SUMOylation can promote either nuclear or cytoplasmic accumulation, depending on the target protein (Huang et al., 2003; Wood et al., 2003) (Figure 10). Although the mechanisms of how SUMO regulates subcellular localization are not known for most targets, SUMOylation has been reported to regulate nuclear export (Du et al., 2008; Bassi et al., 2013; Santiago et al., 2013). SUMOylation adjacent to the nuclear export signal (NES) of Krüppel-like transcription factor 5 inhibits its interaction with a nuclear export receptor, resulting in the inhibition of export and nuclear accumulation (Du et al., 2008). Similarly, SUMOylation is required for the nuclear accumulation of phosphatase and tensin homolog (PTEN), and the nuclear localization of SUMOylation deficient PTEN can be rescued by a chemical inhibitor of nuclear export (Bassi et al., 2013). Conversely, SUMOylation of tumor suppressor protein p53 facilitates its nuclear export by promoting the disassembly of p53-export receptor complex in the cytosol (Santiago et al., 2013).

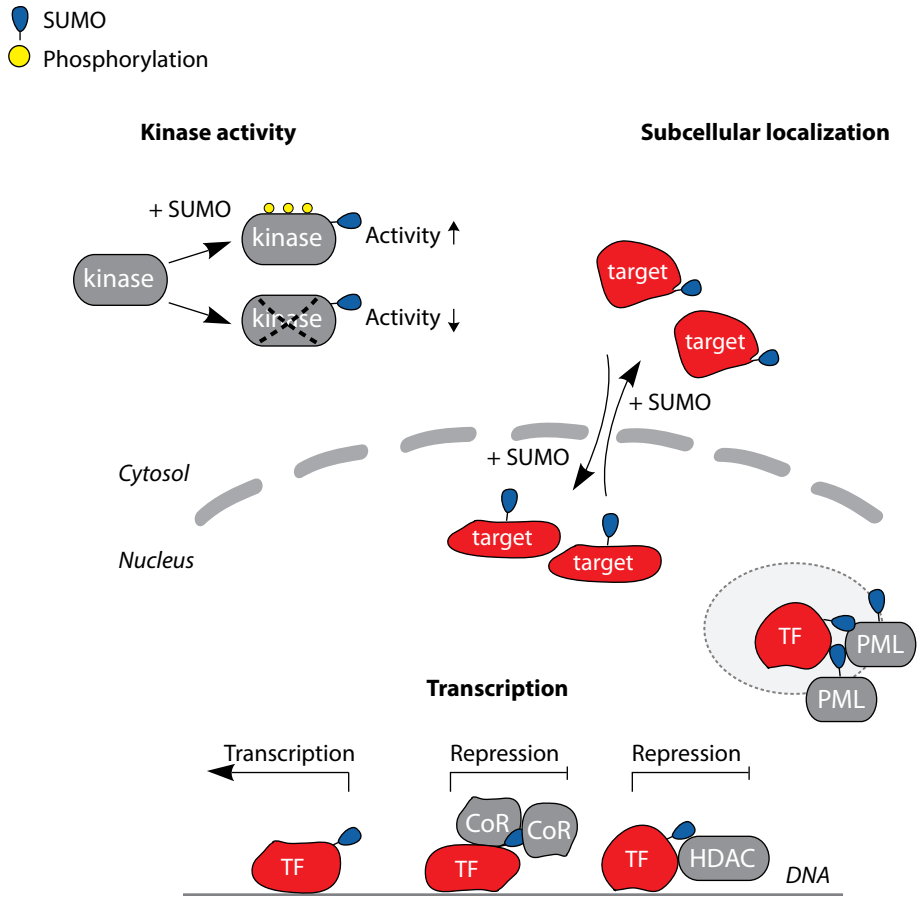

Figure 10. Examples of SUMO functions. SUMOylation can either promote or inhibit kinase activity. SUMOylation regulates the subcellular localization of many target proteins, promoting either nuclear or cytosolic localization. SUMOylated proteins can also accumulate in subnuclear structures such as promyelocytic leukemia (PML) bodies. SUMOylation regulates many transcriptional processes. Transcription factor (TF) SUMOylation can activate or repress transcription. SUMOylation may induce repression *via* the recruitment of corepressor (CoR) complexes or chromatin modifying enzymes (HDAC, histone deacetylase).

More recently, the role of SUMOylation in regulating signal transduction pathways has been elucidated. Kinases are a prominent group of SUMOylated proteins, as indicated by a proteome-wide study (Merbl et al., 2013), and SUMOylation of many kinases has been biochemically characterized. For example, SUMOylation has been shown stimulate the autophosphorylation of focal adhesion kinase, and the activity Akt towards it substrates (Kadare et al., 2003; Li et al., 2013; de la Cruz-Herrera et al., 2014) (Figure 10). Conversely, SUMOylation of MEK inhibited its activity towards ERK by interfering with the MEK-ERK interaction (Kubota et al., 2011).

3 AIMS OF THE STUDY

The general aim of this study was to identify and characterize novel post-translational modifications that regulate ErbB4 function. The signaling mechanisms and regulation of ErbB receptors have been extensively studied. However, the roles of post-translational ubiquitin, and in particular ubiquitin-like modifications in ErbB signaling are less well characterized. Identification of the molecular mechanisms that modify the output of ErbB signaling will expand the understanding of ErbB function in healthy and diseased tissues.

CYT-1 and CYT-2 isoforms of ErbB4 have different degradation rates, suggesting that an isoform-specific, post-translational mechanism such as ubiquitination differentially regulates their stability. As the cytoplasmic isoforms represent functionally distinct ErbB4 proteins, it is important to understand the molecular mechanisms regulating the abundance of each. Moreover, little is known about the mechanisms that control the subcellular localization and nuclear functions of ErbB4 ICD. Since SUMOylation is involved in many nuclear signaling processes, it could represent a novel mechanism to regulate the signaling of an ICD of a receptor tyrosine kinase.

To understand the roles of ubiquitin and ubiquitin-like modifications in ErbB4 signaling, the specific aims of this thesis were:

1) To functionally characterize the ubiquitination of ErbB4 isoforms.

2) To characterize the SUMO modification of ErbB4 intracellular domain.

3) To analyze the role of the SUMO system in ErbB4 function.

4 MATERIALS AND METHODS

4.1 Cell culture (I-III)

The cell lines used in the study are listed in Table 1. MCF-7, OVCAR-3, and WM-266-4 are human cancer cell lines that express ErbB4 endogenously. COS-7, HEK293, and HEK293T cells were used for transient expression of proteins from expression plasmids (4.2). Stable NIH 3T3-7d cell lines (Zhang et al., 1996; Elenius et al., 1999) were used for internalization analyses (4.8). Phoenix-Ampho HEK293T cells were used to produce retroviruses (4.1.2). HC11 and MDA-MB-468 cells were used to study differentiation in three-dimensional cultures (4.15).

COS-7, HEK293, MDA-MB-468, NIH 3T3-7d, Phoenix-Ampho HEK293T, and WM-266-4 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) (Lonza). HC11, MCF-7 and OVCAR-3 cells were cultured in Roswell Park Memorial Institute (RPMI) 1640 (Lonza). Both culture media were supplemented with 10% fetal calf serum (Biowest), 2 mM L-glutamine (Lonza), and 50 units/ml penicillin-streptomycin solution (Lonza). The culture medium of HC11 cells was further supplemented with 5 μg/ml insulin (Sigma-Aldrich) and 10 ng/ml EGF (Sigma-Aldrich or R&D), the medium of MCF-7 cells with 10 μg/ml insulin and 1 nM 17-β-estradiol (Sigma-Aldrich), and the medium of OVCAR-3 cells with 10 μg/ml insulin.

Table 1. Cell lines used in the study.

Cell line	Type	Species	Used in
COS-7	Kidney fibroblast-like cell	African green monkey	I, II, III
HC11	Mammary epithelial cell	Mouse	II, III
HEK293 and HEK293T	Embryonic kidney cell	Human	II
MCF-7	Mammary adenocarcinoma cell	Human	II, III
MDA-MB-468	Mammary adenocarcinoma cell	Human	II
NIH 3T3-7d	Fibroblast	Mouse	I
OVCAR-3	Ovarian carcinoma cell	Human	I
Phoenix-Ampho HEK293T	Embryonic kidney cell	Human	II, III
WM-266-4	Melanoma cell	Human	II

4.1.1 Transient transfection (I-III)

COS-7, HEK293, HEK293T, and Phoenix-Ampho HEK293T cells were transiently transfected with expression plasmids (4.2) with FuGENE6 transfection reagent (Promega). MCF-7 and WM-266-4 cells were transiently transfected with Lipofectamine 2000 (ThermoFischer Scientific). Transfections were carried out according to the manufacturers' protocols.

4.1.2 Generation of stable cell lines with retroviral infection (II, III)

Stable HC11 and MDA-MB-468 cell lines expressing different ErbB4 constructs were generated by retroviral infection. For retrovirus production, retroviral expression plasmids (4.2) encoding wild-type or mutant ErbB4 were transfected into Phoenix-Ampho HEK293T virus packaging cells. Retrovirus-containing media were harvested 36 hours after transfection, and incubated on recipient cells for 8 hours in the presence of 8 μg/ml polybrene (Sigma-Aldrich). To generate stable cell lines, infected cells were selected with 2 μg/ml puromycin (Sigma-Aldrich).

4.2 Expression plasmids (I-III)

Mammalian and retroviral expression plasmids were used to express the indicated wild-type and mutant proteins in cell lines and to produce retroviruses, respectively. Bacterial expression plasmids were used to express recombinant proteins in *Escherichia coli*.

The expression plasmids listed in Table 2 were generated in this study using standard molecular cloning procedures. Amino acid substitutions were introduced to expression plasmids by site-directed mutagenesis. Retroviral pBABE-ErbB4JM-aCYT-2 and pBABE-ErbB4JM-bCYT-2 plasmids were generated using restriction enzymes and ligation as described in (II). All generated constructs were verified by sequencing.

The expression plasmids listed in Table 3 have been previously described. The references of these plasmids are described in (I-III).

Table 2. Expression plasmids generated in this study.

Insert	Backbone	Purpose	Used in
ErbB4 ICD2-K1002/1143/1181/1202R-HA	pcDNA3.1(+)	Mammalian expression	III
ErbB4 ICD2-K1143/1181/1202R-HA	pcDNA3.1(+)	Mammalian expression	III
ErbB4 ICD2-K1202R-HA	pcDNA3.1(+)	Mammalian expression	III
ErbB4 ICD2-K714/719/722R-HA	pcDNA3.1(+)	Mammalian expression	III
ErbB4 ICD2-K714R-HA	pcDNA3.1(+)	Mammalian expression	III
ErbB4 ICD2-K719R-HA	pcDNA3.1(+)	Mammalian expression	III
ErbB4 ICD2-K722R-HA	pcDNA3.1(+)	Mammalian expression	III
ErbB4 ICD2-V721/V723/L724A-HA	pcDNA3.1(+)	Mammalian expression	III
ErbB4 JM-aCYT-2-K714R-HA	pcDNA3.1(+)	Mammalian expression	III
ErbB4 JM-aCYT-1-P1054A-HA	pcDNA3.1(+)	Mammalian expression	I
ErbB4 JM-aCYT-1-Y1056F-HA	pcDNA3.1(+)	Mammalian expression	I
ErbB4 JM-aCYT-2-K714R-HA	pBABE-puro	Retroviral	III
ErbB4 JM-aCYT-2-V675A-HA	pcDNA3.1(+)	Mammalian expression	II
ErbB4 JM-aCYT-2-ΔNLSI/II-HA	pcDNA3.1(+)	Mammalian expression	II
ErbB4 JM-bCYT-1-P1054A-HA	pcDNA3.1(+)	Mammalian expression	I
ErbB4 JM-bCYT-1-Y1056F-HA	pcDNA3.1(+)	Mammalian expression	I
ErbB4 JM-aCYT-2	pBABE-puro	Retroviral	II
ErbB4 JM-bCYT-2	pBABE-puro	Retroviral	II
FLAG-PIAS3-C299S	pFLAG	Mammalian expression	II, III

Table 3. Previously described expression plasmids.

Insert	Backbone	Purpose	Used in
6xHis-SUMO1	pSG5	Mammalian expression	II, III
6xHis-SUMO3	pcDNA3.1(-)	Mammalian expression	II, III
ErbB4 ICD1-HA	pcDNA3.1(+)	Mammalian expression	II
ErbB4 ICD2-GAL4	pSG424	Mammalian expression	II
ErbB4 ICD2-GST	pGEX-6P-1	Bacterial expression	II
ErbB4 ICD2-HA	pcDNA3.1(+)	Mammalian expression	II, III
ErbB4 ICD2-K751R-HA	pcDNA3.1(+)	Mammalian expression	III
ErbB4 ICD2-ΔC-GST	pGEX-6P-1	Bacterial expression	II
ErbB4 ICD2-ΔC-HA	pcDNA3.1(+)	Mammalian expression	III
ErbB4 ICD2-ΔN-GST	pGEX-6P-1	Bacterial expression	II
ErbB4 ICD2-ΔN-HA	pcDNA3.1(+)	Mammalian expression	III
ErbB4 JM-aCYT-1-HA	pcDNA3.1(+)	Mammalian expression	I
ErbB4 JM-aCYT-1-Myc	pcDNA3.1(+)	Mammalian expression	I
ErbB4 JM-aCYT-2-HA	pcDNA3.1(+)	Mammalian expression	I, II, III
ErbB4 JM-aCYT-2-HA	pBABE-puro	Retroviral	III
ErbB4 JM-aCYT-2-Myc	pcDNA3.1(+)	Mammalian expression	I
ErbB4 JM-bCYT-1-HA	pcDNA3.1(+)	Mammalian expression	I
ErbB4 JM-bCYT-2-HA	pcDNA3.1(+)	Mammalian expression	I
FLAG-CRM1	p3xFLAG-CMV-10	Mammalian expression	III
FLAG-Itch	pCMV5-FLAG1	Mammalian expression	I
FLAG-Itch-C830A	pCMV5-FLAG1	Mammalian expression	I
FLAG-PIAS1	pCMV5-FLAG	Mammalian expression	II
FLAG-PIAS3	pFLAG	Mammalian expression	II, III
FLAG-PIASxα	pFLAG	Mammalian expression	II
FLAG-PIASy	pFLAG	Mammalian expression	II
FLAG-SENP1	pcDNA3.1(-)	Mammalian expression	III
FLAG-SENP1-C603A	pcDNA3.1(-)	Mammalian expression	III
FLAG-SENP2	pFLAG-CMV	Mammalian expression	III
FLAG-SENP6	pFLAG-CMV	Mammalian expression	III
FLAG-SENP7	p3xFLAG-CMV-10	Mammalian expression	III
FLAG-Ubiquitin	pEF	Mammalian expression	I
GAL4-driven Firefly luciferase	pFR-Luc	GAL4 activity reporter	II
GFP-Rab5a	pEGFP-C3	Mammalian expression	I
GFP-Rab7	unknown	Mammalian expression	I
GFP-SENP3	pEGFP-C3	Mammalian expression	III
GFP-SENP5	pEGFP-C3	Mammalian expression	III
GFP-SUMO1	pEGFP	Mammalian expression	II
GST-Itch-WW	pGEX-KT	Bacterial expression	I
GST-Itch-WW1	pGEX-KT	Bacterial expression	I
GST-Itch-WW2	pGEX-KT	Bacterial expression	I
GST-Itch-WW3	pGEX-KT	Bacterial expression	I
GST-Itch-WW4	pGEX-KT	Bacterial expression	I
HA-Ubiquitin	pMT123	Mammalian expression	I
HA-Ubiquitin-K48R	pMT123	Mammalian expression	I
HA-Ubiquitin-K63R	pMT123	Mammalian expression	I
Myc-Itch	pRK	Mammalian expression	I
Myc-Itch-C830A	pRK	Mammalian expression	I
Omni-YAP2	pcDNA4-HisMaxB	Mammalian expression	II
PML-3	unknown	Mammalian expression	II, III
Renilla luciferase	pTK-RL	Control reporter	II
STAT5a	pME18S	Mammalian expression	III
WWOX-Myc	pCMV-Myc	Mammalian expression	II

4.3 Primary antibodies (I-III)

Primary antibodies (Table 4) were used to detect proteins of interest or their epitope tags by immunofluorescence, immunoprecipitation, *in situ* proximity ligation assay (PLA), and Western blotting.

Table 4. Primary antibodies used in the study. Application abbreviations: IF, immunofluorescence; IP, immunoprecipitation; PLA, *in situ* proximity ligation assay; WB, Western blotting. Company abbreviations: CST, Cell Signaling Technology; SBCT, Santa Cruz Biotechnology. * FK1 antibody recognizes polyubiquitinated conjugates; FK2 antibody recognizes mono- and polyubiquitinated conjugates.

Antigen	Cat#/ Clone	Company	Type	Application	Used in
Actin	sc-1616	SCBT	Goat polyclonal	WB	I, II, III
Akt	sc-1618	SCBT	Goat polyclonal	WB	III
c-Myc	9E10	Zymed	Mouse monoclonal	IF, WB	I, II
EGFR	sc-03	SCBT	Rabbit polyclonal	IP	I
ErbB4	sc-283	SCBT	Rabbit polyclonal	WB	I, II
ErbB4	HFR-1	Abcam	Mouse monoclonal	IF, IP, PLA	II, III
ErbB4	HFR-1	Neomarkers	Mouse monoclonal	IP	I
ErbB4	E200	Abcam	Rabbit monoclonal	WB, IF	I, II, III
Erk	9102	CST	Rabbit polyclonal	WB	III
FLAG	M2	Sigma-Aldrich	Mouse monoclonal	IP, WB	I, II, III
GFP	sc-9996	SCBT	Mouse monoclonal	WB	III
GST		GE Heathcare	Goat polyclonal	WB	I, II
HA	3F10	Roche	Rat monoclonal	IF, WB	I, II, III
HA	HA-7	Sigma-Aldrich	Mouse monoclonal	WB	III
HA	6E2	CST	Mouse monoclonal	IP, WB	III
HA	ab18181	Abcam	Mouse monoclonal	WB	II
HSP90	AC88	Calbiochem	Mouse monoclonal	WB	II
Itch	611198	BD Biosciences	Mouse monoclonal	WB	I
Lamin B	sc-6217	SCBT	Goat polyclonal	WB	II, III
MEK1/2	4694	CST	Mouse monoclonal	WB	II, III
mono- and poly-Ub*	FK2	Enzo	Mouse monoclonal	WB	I
Omni	sc-7270	SCBT	Mouse monoclonal	WB	II
PCNA	sc-56	SCBT	Mouse monoclonal	IP, WB	I
phospho-Akt	9271	CST	Rabbit polyclonal	WB	III
phospho-ErbB4	4757	CST	Rabbit monoclonal	WB	III
phospho-Erk1/2	9101	CST	Rabbit polyclonal	WB	III
phospho-STAT5	9351	CST	Rabbit polyclonal	WB	III
phospho-tyrosine	4G10	produced in house	Mouse monoclonal	WB	III
PIAS3	sc-46682	SCBT	Mouse monoclonal	IF, WB	II
PIAS3	ab22856	Abcam	Rabbit polyclonal	IF, PLA	II
PML	sc-966	SCBT	Mouse monoclonal	IF	II
PML	sc-5621	SCBT	Rabbit polyclonal	WB	II
PML	36.1-104	Millipore	Mouse monoclonal	IF	II
poly-Ub*	FK1	Enzo	Mouse monoclonal	WB	I
STAT5	sc-835	SCBT	Rabbit polyclonal	WB	II, III
SUMO1	ab1172	Abcam	Rabbit polyclonal	IF	II
Ubiquitin	P4D1	SCBT	Mouse monoclonal	WB	I

4.4 Growth factors and inhibitors (I-III)

The growth factors and chemical inhibitors used in the study are listed in Table 5. The concentrations and incubation times in each experiment are indicated in the sections describing the experimental procedures.

Table 5. Growth factors and inhibitors used in the study.

Reagent	Application	Company	Used in
EGF	EGFR stimulation in internalization analysis and HC11 culture	Sigma-Aldrich or R&D	I, II, III
HB-EGF	EGFR stimulation in internalization analysis	Sigma-Aldrich	I
NRG-1-β1 (referred to as NRG-1)	ErbB4 stimulation	R&D	I, II, III
ALLN (N-Acetyl-Leu-Leu-Nle-CHO)	Inhibition of proteasomes	Calbiochem	I
Cycloheximide	Inhibition of translation	Sigma-Aldrich	I, III
GSI IX	Inhibition of γ-secretase	Calbiochem	II
Leptomycin B	Inhibition of nuclear export	Sigma-Aldrich	II, III
LY294002	Inhibition of PI3K	Calbiochem	I
N-ethylmaleimide	Inhibition of SUMO isopeptidases	Sigma-Aldrich	II
PMA (Phorbol 12-myristate 13-acetate)	Stimulation of ErbB4 cleavage	Sigma-Aldrich	I

4.5 Small interfering RNAs (II)

Small interfering RNAs (siRNA) were used to downregulate the expression of PIAS3 and PML (4.9.3, 4.12, 4.15). The sequences of siRNA oligonucleotides are listed in Table 6. Cells were transfected with siRNAs at final concentrations of 20-50 nM using Lipofectamine 2000 (ThermoFischer Scientific) according to the manufacturer's protocol. Knockdown efficacy was confirmed using Western blotting or immunofluorescence analysis.

Table 6. siRNA oligonucleotides.

Target	Sequence	Company	Species	Used in
Non-silencing control	Cat#SI03650318	Qiagen	-	II
Non-silencing control	Cat#AM4611	Ambion	-	II
PIAS3	GGUCGAAGUUAUUGACUUGTT	Ambion	Human	II
PIAS3	GGUGCAGCUAAGGUUCUGUTT	Ambion	Human	II
PIAS3	GGGACCCUUCUACAAAAACTT	Ambion	Mouse	II
PIAS3	GGUUAAUGGGAAACUCUGCTT	Ambion	Mouse	II
PML	CCAAGAUCUAAACCGAGAATT	Qiagen	Human	II
PML	GGAGCAGGAUAGUGCCUUUTT	Qiagen	Human	II

4.6 Cell lysis, immunoprecipitation and Western blotting (I-III)

To prepare cell lysates for immunoprecipitation and Western blotting, cells were washed with PBS, lysed in lysis buffer (1% Triton X-100, 10 mM Tris-Cl pH 7.4, 150 mM NaCl, and 1 mM EDTA) supplemented with protease and phosphatase inhibitors (5 mM NaF, 10 µg/ml aprotinin, 10 µg/ml leupeptin, 1 mM Na_3VO_4, 2 mM PMSF, and 10 mM $Na_4P_2O_7$), and centrifuged at 16 000 x *g* for 10 minutes. Protein concentration of the supernatants was measured by Bradford protein assay (Bio-Rad).

Immunoprecipitation was used in degradation, interaction and receptor activation studies, and in post-translational modification analyses (4.7-4.11, 4.13). Cells lysates were pre-cleared with 30 µl protein G agarose (GE Healthcare or Santa Cruz Biotechnology) at 4 °C for 1 hour, and subjected to immunoprecipitation with antibodies recoginizing the protein of interest and 30 µl protein G agarose at 4 °C overnight. Beads were washed four times with 1 ml of lysis buffer to remove non-specific binding, and heated at 95 °C for 5 minutes in Laemmli loading buffer to elute and denature the precipitated proteins.

Western blotting was used to determine the abundance and phosphorylation of ectopic and endogenous proteins in cell lysates, and to analyze immuno- and pull-down precipitates (4.7-4.15). Cell lysates were denatured by heating at 95 °C for 5 minutes in Laemmli loading buffer. Samples were separated by SDS-PAGE and transferred to nitrocellulose membranes. Membranes were incubated with primary antibodies as indicated in original publications, followed by horseradish peroxidase (HRP)-conjugated anti-goat, anti-mouse, anti-rabbit and anti-rat secondary antibodies (Santa Cruz Biotechnology). Signals were detected using enchanced chemiluminescence (ThermoFischer Scientific).

4.7 Internalization of cell surface receptors (I)

The internalization rates of ErbB4 isoforms from the cell surface were analyzed by measuring the uptake of iodinated NRG-1 into the cytosol. As a control, the internalization rates of EGFR upon EGF and HB-EGF stimulus were measured. All growth factors were iodinated as previously described (Elenius et al., 1997a). NIH 3T3-7d transfectants stably expressing ErbB4 isoforms or EGFR were treated with 20 ng/ml ^{125}I-NRG-1, ^{125}I-EGF, or ^{125}I-HB-EGF for 1, 5, 10 or 15 minutes. Cells were washed with an acidic buffer (pH 2.8) to remove cell surface-bound growth factors, and lysed with NaOH to release internalized growth factors. Radioactivity in acidic washes (containing cell surface-bound growth factors) and cell lysates (containing internalized growth factors) were measured with a γ-counter to determine the ratio of internalized *versus* cell surface growth factors. Representative data of reproduced experiments are shown.

The abundance of membrane-associated ErbB4 upon ligand stimulus was analyzed using biotin-labeling of cell surface proteins. COS-7 transfectants were starved without serum,

and stimulated with 50 ng/ml NRG-1 for 0, 5, 15 or 30 minutes. Cells were washed with DMEM, incubated with 0.5 mg/ml EZ-linked Sulfo-NHS-LC-Biotin (ThermoFischer Scientific) for 45 minutes to biotinylate cell surface proteins, and lysed. Cell lysates were immunoprecipitated with anti-ErbB4 antibody, and the precipitates were analyzed by SDS-PAGE and Vectastain ACB HRP-kit (Vector Laboratories) to detect the abundance of biotinylated ErbB4. Signal intensities were quantified using MCID M5+ software. Representative data of reproduced experiments are shown.

4.8 Degradation of ErbB4 (I, III)

4.8.1 Analysis of ErbB4 half-life using metabolic labeling (I)

Metabolic labeling with [^{35}S]methionine was used to determine the degradation rates of ErbB4 isoforms. As a control, the degradation rate of EGFR was measured. COS-7 cells were transfected with ErbB4 isoforms or EGFR, and metabolic labeling was carried out by incubating the cells first with methionine-free medium for 2 hours, followed by incubation with methionine-free medium containing 20 µCi/ml [^{35}S]methionine (MP Biomedicals) for 1 hour. After metabolic labelling, cells were incubated with methionine-free medium for additional 0, 2, 4 or 6 hours, and lysed. The abundances of ErbB4 and EGFR in cell lysates were analyzed by immunoprecipitation, followed by SDS-PAGE and autoradiography to detect the abundances of [^{35}S]methionine-labeled receptors. Signal intensities were quantified using MCID M5+ software. Representative data of reproduced experiments are shown.

4.8.2 Analysis of ErbB4 half-life using cycloheximide (I, III)

Degradation analysis utilizing a translation inhibitor cycloheximide was used to study the stability of ErbB4 isoforms and the role of Itch in ErbB4 degradation (I), and to determine the stability of SUMOylation deficient ErbB4 (III). In all analyses, COS-7 transfectants were starved without serum, and treated with 100 µg/ml cycloheximide for 0, 2, 4 or 6 hours. The abundances of ErbB4 protein in cell lysates, and when indicated, Itch, were analyzed by Western blotting. The data were quantified as ErbB4 signal intensities relative to Actin abundance using ImageJ software. Representative results of at least three independent experiments are shown.

4.9 Interaction analyses (I-III)

4.9.1 Coimmunoprecipitation (I-III)

The interactions of ErbB4 with Itch (I), PIAS3 (II), and CRM1 (III) were characterized by coimmunoprecipitation. To study the interactions of ErbB4 isoforms and PPXY-

domain mutants with Itch (I), COS-7 transfectants were lysed and subjected to immunoprecipitation with antibodies recognizing ErbB4 or epitope-tagged Itch. Coprecipitating proteins were analyzed by Western blotting with antibodies recognizing epitope-tagged Itch or ErbB4. To study the interaction of endogenous ErbB4 and Itch, OVCAR-3 cells were treated with 50 ng/ml NRG-1 for 10 minutes to stimulate ErbB4 phosphorylation, and lysed. Lysates were subjected to immunoprecipitation with anti-ErbB4 antibody, followed by Western blot analysis with anti-Itch antibody. The interaction of ErbB4 and PIAS3 (II) was studied by using COS-7 transfectants. Cell lysates were subjected to immunoprecipitation with antibodies recognizing ErbB4 or epitope-tagged PIAS3, and precipitates were analyzed by Western blotting with antibodies recognizing epitope-tagged PIAS3 or ErbB4. The interaction of ErbB4 and CRM-1 (III) was studied similarly in COS-7 transfectants, by using antibodies recognizing epitope-tagged ErbB4 and epitope-tagged CRM1. All coimmunoprecipitation experiments were reproduced at least twice.

4.9.2 Glutathione-S-transferase pull-down assay (I, II, III)

Glutathione-S-transferase (GST) pull-down assay was used to study the interactions of ErbB4 with WW domains of Itch (I), ErbB4 with PIAS proteins (II), and ErbB4 with SUMO1 (III). For all experiments, GST fusion proteins were expressed in BL-21 DE3 strain of *Escherichia coli* (Invitrogen), affinity-purified using Glutathione Sepharose 4B (GE Healthcare), and eluted with a buffer containing 20 mM glutathione, 100 mM NaCl, 0.5% Triton X-100, and 1 mM dithiothreitol.

To study the interactions of ErbB4 with WW domains of Itch (I), GST fusion proteins including each, or all four of the WW-domains of Itch, were incubated with lysates of COS-7 cells expressing epitope-tagged ErbB4. COS-7 cells were lysed (0.5% Triton X-100, 150 mM NaCl, 50 mM Tris-HCl, pH 7.5), and aliquots of the lysates corresponding to 1000 µg of total protein were incubated with 10 µg of GST fusion proteins and 25 µl of Glutathione Sepharose 4B at 4°C for 6 hours. Beads were washed four times with 1 ml of lysis buffer to remove non-specific binding, and heated at 95 °C for 5 minutes in Laemmli loading buffer to elute and denature precipitated proteins. Ppull-down precipitates were separated with SDS-PAGE, and analyzed by Western blotting with antibodies recognizing epitope-tagged ErbB4 and GST fusion proteins. Representative data of three independent experiments are shown.

To characterize the interactions of ErbB4 with PIAS proteins (II), GST fusion proteins including C- or N-terminal deletion of ErbB4 ICD were incubated with lysates of COS-7 cells expressing epitope-tagged PIAS proteins. GST pull-down was performed as described above. Pull-down precipitates were analyzed by Western blotting with antibodies recognizing epitope-tagged PIAS proteins and GST fusion proteins. Representative data of at least three independent experiments are shown.

To study the non-covalent interaction of ErbB4 with SUMO1 (III), GST-SUMO1 fusion protein was incubated with COS-7 lysate expressing epitope-tagged ErbB4 ICD or PIASy (a positive control for non-covalent SUMO1-binding). GST pull-down assay was performed as described above. Pull-down precipitates were analyzed by Western blotting with antibodies recognizing epitope-tagged ErbB4, PIASy, and GST fusion proteins.

4.9.3 In situ proximity ligation assay (II)

The interaction between endogenous ErbB4 and PIAS3 was analyzed using PLA, which allows for detection of endogenous protein complexes *in situ* (Söderberg et al., 2008). MCF-7 cells were starved without serum, treated with 50 ng/ml NRG-1 for 15 minutes to stimulate ErbB4 phosphorylation, and fixed with methanol. Fixed cells were incubated with anti-ErbB4 and anti-PIAS3 antibodies, and proximity ligation was carried out with Duolink II *in situ* PLA kit (Olink Biosciences) following the manufacturer's protocol. PLA signals were detected by confocal microscopy, and classified as cytosolic or nuclear depending on their colocalization with DAPI. The specificity of the interactions was controlled using siRNAs targeting PIAS3 (4.5). The PLA experiment was carried out twice. Differences between two groups were examined using *t* tests.

4.10 Ubiquitination of ErbB4 (I)

The ubiquitination of ErbB4 was studied by using COS-7 transfectants expressing ErbB4 and ubiquitin constructs, and OVCAR-3 cells, which express ErbB4 endogenously. To study the role of Itch in ErbB4 ubiquitination, wild-type or mutant Itch was cotransfected. Lysine-to-arginine ubiquitin mutants were used to analyze the polyubiquitination of ErbB4.

Cells were starved without serum, and when indicated, treated with 50 ng/ml NRG-1 for 10 or 30 minutes to stimulate ErbB4 phosphorylation, 100 ng/ml PMA for 30 minutes to stimulate ErbB4 cleavage, or with 150 μM ALLN for 2 hours to inhibit proteasomes. Cells were lysed, and the lysates were subjected to immunoprecipitation with anti-ErbB4 antibody. Precipitates were analyzed for ErbB4 ubiquitination by Western blotting using antibodies recognizing epitope-tagged ubiquitin, endogenous ubiquitin, or specific ubiquitin conjugates. The expression of Itch was analyzed using antibodies recognizing epitope-tagged Itch. All experiments were reproduced at least twice.

4.11 SUMOylation of ErbB4 (II, III)

The SUMOylation of ErbB4 ICD was studied using COS-7, HEK293, MCF-7, and WM-266-4 transfectants expressing ErbB4 ICD and epitope-tagged SUMO constructs. To study the role of SUMO E3 ligases and isopeptidases in ErbB4 SUMOylation, wild-type

or mutant PIAS3 (II) or SENP proteins (III) were cotransfected. The role of PIAS3 was further studied using RNA interference (II) (4.5). C- or N-terminal deletion and lysine-to-arginine amino acid substitution constructs of ErbB4 ICD were used to identify the SUMO acceptor lysine (III). Representative data of at least two independent experiments are shown.

The SUMOylation of ErbB4 was analyzed by Western blotting with ErbB4 antibody, or immunoprecipitation with ErbB4 antibody followed by Western blotting with ErbB4 antibody (II). SUMO-modified ErbB4 was observed as higher molecular weight species compared to unmodified ErbB4. To inhibit the activity of SUMO isopeptidases, 20 mM *N*-ethylmaleimide was added in lysis and immunoprecipitation buffers. Alternatively, cells were lysed in a denaturing buffer (8 M urea, 0.1 M Na_2HPO_4/NaH_2PO_4, 10 mM Tris-HCl pH 7.0, 10 mM imidazole, 10 mM β-mercaptoethanol), and His-tagged SUMO conjugates were precipitated using Ni^{2+}-NTA agarose (Qiagen) (II, III). After extensive washing, the His-SUMO conjugates were eluted with elution buffer (200 mM imidazole, 5 % SDS, 150 mM Tris-HCl pH 6.8, 30 % glycerol, 720 mM β-mercaptoethanol), separated by SDS-PAGE, and analyzed for ErbB4 by Western blotting. When indicated, the expression of epitope-tagged PIAS3 and SENP proteins was analyzed by Western blotting.

4.12 Subcellular localization of ErbB4 (I-III)

4.12.1 Immunofluorescence analyses of ErbB4 in cytoplasmic vesicles (I)

The subcellular localization of ErbB4 isoforms and PPXY domain mutants in cytoplasmic vesicles was analyzed by immunofluorescence using COS-7 transfectants. Cells were cultured on coverslips, and fixed and permeabilized with methanol at -20°C for 15 minutes. After washing with PBS, fixed cells were incubated with primary antibodies for 2 hours, and Alexa Fluor conjugated fluorescent secondary antibodies (ThermoFischer Scientific) for 45 minutes. Both primary and secondary antibodies were diluted in PBS containing 3% bovine serum albumin. Nuclei were visualized with DAPI (a fluorescent DNA stain; Sigma-Aldrich). Coverslips were mounted with Vectashield (Vector Laboratories) or Mowiol 4-88 (Sigma-Aldrich) mounting medium, and the images were acquired with confocal microscopy. When indicated, cells were treated with 20 μM LY29400 for 4 hours to inhibit PI3K, or with 50 ng/ml NRG-1 for 0, 5 or 30 minutes to stimulate ErbB4 phosphorylation. The role of Itch in the subcellular localization of ErbB4 isoforms was studied by cotransfection of wild-type or mutant Itch. The identity of cytoplasmic vesicles was determined by coexpressing fluorescent-protein-tagged Rab-proteins. Representative data of experiments reproduced several times are shown.

4.12.2 Immunofluorescence analyses of ErbB4 in cell nuclei (II, III)

The roles of PIAS proteins in the localization of ErbB4 in cell nuclei *versus* cytosol were determined using overexpression and RNA interference of PIAS proteins, followed by immunofluorescence analysis as described (4.12.1) (II). Depending on the experimental setup, COS-7 transfectants, HC11 retroviral cell lines, and MCF-7 cells were used. When indicated, cells were treated with 5 μM GSI IX for 4 hours to inhibit γ-secretase, 25 ng/ml leptomycin B for 3 hours to inhibit nuclear export, or 50 ng/ml NRG-1 for 0 or 45 minutes to stimulate ErbB4 phosphorylation. Quantification of nuclear ErbB4 in HC11 cells was performed using ImageJ software. Differences between two groups were examined using *t* tests. (II).

The localization of ErbB4 mutant constructs with or without PIAS3 overexpression was analyzed to characterize the roles of γ-secretase cleavage site (II), and nuclear import (II) and export signals (III) in determining the subcellular localization. In these experiments, COS-7 cells expressing wild-type or mutant constructs of ErbB4 were scored for predominantly cytosolic (more signal in the cytosol than in the nucleus) or nuclear (equal signal in the nucleus and in the cytosol or more signal in the nucleus than in the cytosol) staining in at least 200 randomly selected cells. Differences between two groups were examined using *t* tests in (II). Frequences of nuclear staining intensities were examined using the Chi-squared test in (III).

The colocalization of ErbB4 with SUMO1, PIAS3, and PML was analyzed in COS-7 transfectants, and the colocalization of endogenous ErbB4 with SUMO1 and PML in leptomycin B- and NRG-1-treated WM-266-4 cells (II). Representative data of reproduced experiments are shown. The colocalization of ErbB4 with PIAS3 and PML was analyzed quantitatively in HC11 cells (n=15 for PIAS3 and n=10 for PML) retrovirally expressing ErbB4 using BioimageXD software (Kankaanpää et al., 2012) (II).

4.12.3 Subcellular fractionation (II, III)

The localization ErbB4 in cell nuclei *versus* cytosol was also analyzed by subcellular fractionation with a NE-PER kit (ThermoFischer Scientific), followed by Western blotting detection of the proteins of interest. Antibodies against Lamin B (a nuclear marker) and MEK1/2 (a cytosolic marker) were used to control the fractionation.

The subcellular localization of overexpressed ErbB4 was studied in COS-7 cells upon PIAS3 overexpression, and that of endogenous ErbB4 in MCF-7 cells upon RNA interference of PIAS3 (II). The role of SUMOylation in nuclear localization of ErbB4 was characterized in MCF-7 transfectants expressing wild-type or SUMOylation deficient ErbB4, with or without overexpression of PIAS3 (III). Representative data of at least two independent experiments are shown. To study the role of nuclear export in

subcellular localization of ErbB4, MCF-7 cells were treated with 0, 5, 10 or 20 ng/ml leptomycin B for 3 hours to inhibit nuclear export (III).

4.13 ErbB4 tyrosine phosphorylation and activity of signaling pathways (III)

The basal tyrosine phosphorylation of wild-type and SUMOylation deficient ErbB4 mutant was studied by using COS-7 transfectants. Cells were serum starved and lysed, and lysates were subjected to immunoprecipitation with anti-ErbB4 antibody. Precipitates were analyzed by Western blotting for phosphotyrosine content. To analyze the activity of signaling pathways downstream of wild-type and SUMOylation deficient ErbB4, COS-7 transfectants were serum starved and stimulated with 50 ng/ml NRG-1 for 10 minutes. Cell lysates were analyzed by Western blotting for the phosphorylation of Akt, ERK and STAT5 with phospho-specific antibodies. Representative data of three independent experiments are shown.

4.14 ErbB4-mediated transactivation (II)

A previously described transactivation assay (Komuro et al., 2003), where ErbB4 ICD fused to the GAL4 DNA-binding domain and YAP coactivate the transcription of a Firefly luciferase reporter gene driven by GAL4 binding sites, was used to study the transcriptional coregulatory activity of ErbB4. PIAS proteins were cotransfected to analyze their role in ErbB4-mediated transactivation, and a cotransfected *Renilla* luciferase construct was used as an internal control. Firefly and *Renilla* luciferase activities were measured with Dual-Luciferase Assay Reporter System (Promega). The expression of PIAS proteins and YAP were controlled by Western blotting. The experiment was repeated three times, and differences between two groups were examined using *t* tests.

4.15 Three-dimensional Matrigel cultures (II, III)

When cultured in a reconstituted basement membrane matrix such as Matrigel, normal breast epithelial cells are able to form structurally and functionally differentiated mammary acini (Petersen et al., 1992). In contrast, transformed breast carcinoma cells grow in disorganized colonies (Petersen et al., 1992). The function of ErbB4 in this system was characterized by using MDA-MB-468 human breast cancer cells (II) and HC11 mouse mammary epithelial cells (II, III), both retrovirally expressing ErbB4 (4.1.2). Single cell suspensions were suspended into Matrigel (MDA-MB-468) or Growth Factor Reduced Matrigel (HC11) in triplicates on 96-well plates, supplemented with 50 ng/ml NRG-1, and maintained cell culture incubator for 6-20 days as indicated (II, III). Both Matrigels were obtained from Corning. Colonies were counted from three

to four independent views through the whole thickness of the Matrigel, and classified as undifferentiated colonies or differentiated acini on the basis of their morphology as described (Tvorogov et al., 2009). To study the function of the soluble ErbB4 ICD in MDA-MB-468 cells, ErbB4 RIP was inhibited with 5 μM GSI IX (II). The function of the soluble ICD in HC11 cells was analyzed by comparing the effects of the cleavable and non-cleavable ErbB4 isoforms on the three-dimensional growth (II). For RNA interference experiments, cells were transfected with siRNAs targeting PIAS3 or PML (4.5), or with negative control siRNAs, and suspended into Matrigel 24 hours after siRNA transfection (II). The role of ErbB4 SUMOylation was determined by comparing retroviral HC11 cell lines expressing wild-type and SUMOylation deficient ErbB4 (III). All experiments were reproduced two to four times. Differences between two groups were examined using *t* tests (II), or one-way analysis of variance followed by pairwise comparisons using *t*-tests (III). Correction for multiple testing was performed using the Benjamini-Hochberg procedure (III).

5 RESULTS

5.1 ErbB4 isoforms are differentially endocytosed (I)

Ligand-stimulated endocytosis followed by lysosomal degradation is a well-characterized mechanism to downregulate RTK signaling. While EGFR undergoes efficient endocytosis, other ErbB receptors including ErbB4 have been previously considered endocytosis-impaired (Baulida et al., 1996). However, ErbB4 CYT-1 isoform has been shown to localize in cytoplasmic vesicles (Määttä et al., 2006). Moreover, CYT-1 and CYT-2 isoforms have been reported to have different degradation rates, suggesting that isoform-specific mechanisms regulate their stability (Sundvall et al., 2007).

Immunofluorescence microscopy of COS-7 cells expressing different ErbB4 isoforms demonstrated that CYT-1 isoforms localized in cytoplasmic vesicles more frequently than CYT-2 isoforms (I, Fig 1B and C). To address whether the localization of ErbB4 CYT-1 in cytoplasmic vesicles was associated with faster internalization, the uptake of ^{125}I-labelled NRG-1 was measured. The ligand-stimulated internalization of CYT-1 was significantly faster than that of CYT-2 (I, Fig 1D, left) and had kinetics similar to EGFR, which is efficiently internalized (I, Fig 1D, right). To analyze the identity of the CYT-1-positive cytoplasmic vesicles, ErbB4 isoforms were coexpressed with Rab5 or Rab7, markers for early and late endosomes, respectively (Zerial and McBride, 2001). Confocal microscopy demonstrated that CYT-1, but not CYT-2, clearly colocalized with Rab5 and Rab7 (I, SI Fig 5). As these results indicated that ErbB4 CYT-1 was endocytosed, the degradation rates of the cytoplasmic isoforms were analyzed. Indeed, the half-life of CYT-1 was shorter than that of CYT-2 (I, Fig 4C, lanes 1-4, and SI Fig 6B). Taken together, these data demonstrate that ErbB4 CYT-1 isoform is endocytosed and degraded more efficiently than CYT-2.

5.2 ErbB4 CYT-1 functionally interacts with Itch E3 ubiquitin ligase (I)

The ErbB4 CYT-1 isoform contains a 16 amino acid sequence that is absent from CYT-2. This sequence harbors a PI3K binding motif (Elenius et al., 1999). However, a chemical inhibitor of PI3K activity did not prevent the targeting of CYT-1 into the endocytic vesicles, indicating that CYT-1 endocytosis was not PI3K-dependent (I, Fig 2C). The CYT-1-specific sequence also has a PPXY motif that has been shown to interact with WW-domain containing proteins (Komuro et al., 2003). Analysis of the PPXY motif mutants Y1056F and P1054A demonstrated that this motif was indeed necessary for the targeting of CYT-1 into the endocytic vesicles (I, Fig 2B and D).

Ubiquitination is a key regulator of receptor endocytosis (Goh and Sorkin, 2013). In accordance with the previously reported difference in the ubiquitination of the membrane-tethered m80 fragments produced by different cytoplasmic isoforms (Sundvall et al., 2007), ErbB4 CYT-1 was ubiquitinated more efficiently compared to CYT-2 (I, Fig 2E). Interestingly, similar to endocytosis, efficient ubiquitination of ErbB4 CYT-1 required the intact CYT-1-specific PPXY motif (I, Fig 2G and H).

The requirement of the PPXY motif in the ubiquitination and endocytosis of CYT-1 suggested that this motif could function as a binding site for WW-domain containing ubiquitin ligases of the NEDD4 family. They contain a C2 domain that mediates membrane targeting, a catalytic HECT domain, and two to four WW domains that interact with PPXY motifs (Rotin and Kumar, 2009) (Figure 11). To test for an interaction between ErbB4 and two candidate NEDD4 family proteins, coimmunoprecipitation of ErbB4 with either NEDD4 or Itch was analyzed. While no interaction between ErbB4 and NEDD4 was detected (data not shown), ErbB4 associated with Itch (I, Fig 3A and B, and SI Fig 7). The efficient interaction between ErbB4 CYT-1 and Itch required an intact PPXY motif (I, Fig 3C), as well as Itch WW domains 1 and 2 (I, Fig 4D and E). A weak interaction of ErbB4 CYT-2 with Itch was also detected, possibly mediated by the two PPXY domains shared between ErbB4 cytoplasmic isoforms (I, Fig 3A) (Komuro et al., 2003).

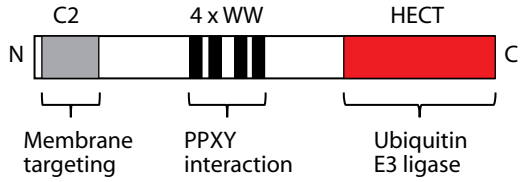

Figure 11. Schematic structure of NEDD4 family proteins. All NEDD4 family proteins have a similar domain architecture with an N-terminal C2 domain, two to four WW domains, and a C-terminal HECT domain. The C2 domain mediates membrane targeting, WW domains interact with substrate PPXY motifs, and HECT is a catalytic domain.

To analyze the functional consequences of ErbB4-Itch interaction, the role of Itch in the ubiquitination of ErbB4 was studied. Coexpression of wild-type Itch, but not the catalytically inactive mutant (that interacted with ErbB4; I, Fig 3A), stimulated ErbB4 ubiquitination (I, Fig 4A). Itch-induced ubiquitination was detected with an antibody that recognizes both mono- and polyubiquitinated proteins (FK2), but not with a polyubiquitin-specific antibody (FK1) (I, Fig 4B). Itch also induced the ubiquitination of ErbB4 when ubiquitin mutants unable to form K48- or K63-linkages were overexpressed (I, SI Fig 8). These results suggest that Itch catalyzes mono- or multimonoubiquitination of ErbB4. In addition to ubiquitination, overexpression of wild-type, but not catalytically inactive Itch also promoted the targeting of CYT-1 into endocytic vesicles (I, Fig 4D, and

SI 4B), and enhanced the degradation of CYT-1 (I, Fig 4C). In keeping with the weaker association of ErbB4 CYT-2 with Itch, CYT-2 stability and subcellular localization were only weakly affected by Itch overexpression (I, Fig 4C and D). Taken together, these data indicate that ErbB4 CYT-1 contains a binding site for WW-domain containing Itch E3 ubiquitin ligase, which regulates ubiquitination, endocytosis and degradation of ErbB4 CYT-1 isoform.

5.3 ErbB4 ICD is SUMOylated (II)

Ligand-induced RIP generates a soluble ErbB4 intracellular domain, which can translocate into the nucleus and coregulate transcription (section 2.3.1). However, little is known about the mechanisms that regulate the subcellular localization and functions of ErbB4 ICD. Many nuclear proteins are post-translationally modified by SUMO, and SUMOylation regulates processes such as nucleo-cytoplasmic transport and transcription (section 2.4.2). Therefore, we aimed to determine whether ErbB4 ICD is SUMOylated.

To examine the presumed SUMOylation, COS-7 cells expressing ErbB4 ICD and His- or GFP-tagged SUMO1 were lysed in the prescence of a SUMO isopeptidase inhibitor, and analyzed by Western blotting. In addition to the unmodified ErbB4 ICD (~80 kDa), Western blot analyses revealed higher molecular weight ErbB4 species, corresponding to the size of ErbB4 ICD modified with His- or GFP-tagged SUMO (~90 kDa and ~120 kDa, respectively) (II, Fig 1G). Higher molecular weight ErbB4 species were also detected when cells expressing ErbB4 ICD and His-SUMO were lysed and subjected to Ni^{2+}-NTA agarose pull-down to purify SUMOylated proteins in denaturing buffer (II, Fig S2A-D). Similar results of ErbB4 ICD SUMOylation were obtained using different cell lines, as well as with His-tagged SUMO3 (II, Fig S2A-D). These data demonstrate that ErbB4 ICD can be covalently conjugated to SUMO1 and SUMO3 (II, Fig S2A-D).

5.4 PIAS3 promotes and SENPs reverse ErbB4 ICD SUMOylation (II, III)

To characterize the interactions of ErbB4 ICD with SUMO E3 ligases, GST-tagged ErbB4 deletion constructs were produced in bacteria, and incubated with cell lysates expressing PIAS proteins (II, Fig 1A-B). PIAS3 and PIASy interacted with a construct containing the kinase domain of ErbB4 (II, Fig 1C), but no interactions between ErbB4 and PIAS1 or PIASxα were detected. The physical interaction of ErbB4 and PIAS3 was further validated using coimmunoprecipitation (II, Fig 1D) and *in situ* proximity ligation (II, Fig 1E-F) assays. Coexpression of wild-type PIAS3, but not the SP-RING disrupted PIAS3 mutant, promoted the SUMOylation of ErbB4 ICD (II, Fig 1H). Furthermore, RNA interference of PIAS3 inhibited ErbB4 SUMOylation (II, Fig S2E). These results demonstrate that PIAS3 functions as a SUMO E3 ligase for ErbB4.

To identify factors negatively regulating ErbB4 SUMOylation, the activities of the SENP family of SUMO isopeptidases towards SUMOylated ErbB4 ICD were analyzed. Both SENP1 and SENP2 could efficiently deconjugate SUMO1 and SUMO3 from ErbB4 ICD (III, Fig 2A and B). DeSUMOylation was dependent on the catalytic activity of SENP1 (III, Fig 2A). SUMO2/3-specific SENPs were less potent in reducing the level of SUMO modified ErbB4 ICD, but SENP5 overexpression reduced ErbB4 SUMOylation (III, Fig 2C). These results indicate that ErbB4 ICD SUMOylation is a reversible process, catalyzed by SENP1, SENP2 and SENP5.

5.5 Identification of the ErbB4 SUMOylation site (III)

The amino acid sequence analysis of ErbB4 ICD revealed two ΨKxE SUMOylation consensus motifs, a shorter KxE consensus motif, and an inverted consensus motif DxKΨ. To examine whether these lysines could function as ErbB4 SUMOylation sites, they were replaced with arginines by site-directed mutagenesis (III, Fig 1A), and the mutant constructs were compared with wild-type ErbB4 ICD for their SUMOylation. However, consensus site mutations had no effect in the pattern of ErbB4 SUMOylation (III, Fig 1B and S1). A possibility of SIM-directed SUMOylation was also excluded, as ErbB4 ICD did not interact non-covalently with SUMO1 in a GST pull-down assay (III, Fig S2). These results indicate that the SUMOylation of ErbB4 is not consensus motif- nor SIM-directed.

The analysis of ErbB4 deletion constructs indicated that only a construct containing the kinase domain was SUMOylated (III, Fig S4). Of note, ErbB4 kinase domain also interacted with PIAS3 (II, Fig 1C). Intriguinly, three lysines (K714, K719 and K722) were located within a previously suggested functional domain, a leucine rich putative nuclear export signal (Ni et al., 2001) (III, Fig 1C). Site-directed mutagenesis and Western blot analyses of these non-consensus sites revealed that the replacement of lysine 714 with an arginine was sufficient to disrupt the formation of the ~90 kD SUMO modified ErbB4 ICD (III, Fig 1D and S1). A structural model of active ErbB4 kinase domain indicated that K714 is accessible to the SUMOylation machinery (III, Fig 1G). Finally, the analysis of ErbB4 amino acid sequences from different species showed that the SUMO modification site is conserved in vertebrates (III, Fig 1H), suggesting functional importance. Taken together, these data demonstrate that the non-consensus lysine 714 is the major SUMO acceptor site in ErbB4 ICD.

5.6 SUMOylation promotes nuclear accumulation of ErbB4 ICD (II, III)

Nuclear ErbB4 immunoreactivity is frequently detected in breast cancer (Srinivasan et al., 2000). However, in cultured cells that express cleavable ErbB4 JM-a isoform either endogenously or ectopically, nuclear localization of ErbB4 epitope is barely detectable

(II, Fig 2A, 3 and 4). Interestingly, endogenous ErbB4-PIAS3 complexes were detected in the nucleus (II, Fig 1E and F). When full-length cleavable ErbB4 JM-a isoform was visualized in cells coexpressing PIAS3 or PIASy, strong nuclear ErbB4 signal was detected (II, Fig 2A). In accordance with these data, RNA interference of PIAS3 reduced nuclear ErbB4 immunoreactivity (II, 2F, 6G and H) and ErbB4 ICD protein level in the nuclear fraction (II, 2G). Furthermore, ErbB4, SUMO1, and PIAS3 were found to colocalize in subnuclear structures identified as PML nuclear bodies (II, Fig 3 and 4). The PIAS3-stimulated nuclear accumulation of ErbB4 required intact γ-secretase cleavage and SUMOylation sites (II, Fig 2D and E; III, Fig 3), as well as a functional NLS (II, Fig 2E). These results indicate that PIAS3-stimulated SUMOylation promotes the nuclear accumulation of soluble ErbB4 ICD, and that NLS-mediated nuclear translocation is required for the accumulation.

Because the identified SUMO modification site was located within a sequence resembling NES (III, Fig 1C), the role of nuclear export in ErbB4 subcellular localization was further examined. Interference of nuclear export with a chemical inhibitor promoted the nuclear accumulation of ErbB4 ICD (II, Fig S4; III, Fig 5A). ErbB4 ICD was also found to interact with chromosomal region maintenance 1 (CRM1), a major nuclear export receptor for proteins (III, Fig 5B). Replacement of the hydrophobic NES residues with alanines resulted in an increased nuclear localization of ErbB4 ICD, indicating that these residues were critical for the nuclear export (III, Fig 5D and E). These results imply that nuclear export regulates the nuclear accumulation of ErbB4 ICD. Taken together, these data are consistent with a model in which ErbB4 SUMOylation at K714 promotes the nuclear accumulation by interfering with the nuclear export.

5.7 SUMOylation is required for nuclear signaling of ErbB4 ICD (II, III)

To assess whether the major SUMOylation site is required for basic functions of ErbB4, wild-type and K714R ErbB4 were compared for their phosphorylation, and ability to activate signaling cascades. Wild-type and SUMOylation deficient mutant ErbB4 demonstrated efficient constitutive tyrosine phosphorylation in the absence of ligand, both in the context of soluble ICD and full-length cleavable JM-a CYT-2 isoform (III, Fig 7A). Upon ligand stimulation, both wild-type and K714R ErbB4 were equally efficient to activate Akt, ERK1/2 (III, Fig 7B), and STAT5A (III, Fig 7C). These data indicate that the major SUMOylation site does not regulate NRG-induced activation of signaling pathways downstream of ErbB4 at the cell surface.

As soluble ErbB4 ICD is a constitutively active tyrosine kinase, the relationship between ErbB4 phosphorylation status and SUMOylation was examined. Kinase-dead ErbB4 ICD was efficiently SUMOylated, indicating that SUMOylation was independent of the intrinsic catalytic activity of ErbB4 (III, Fig 6). The analysis of tyrosine autophosphorylation of SUMOylated ErbB4 ICD revealed that the SUMO-modified

ErbB4 ICD was phosphorylated, in fact to a higher extend than the unmodified ErbB4 (III, Fig 6).

Nuclear ErbB4 ICD can function as a transcriptional coregulator (section 2.3.1). As the SUMO system often regulates transcriptional processes, the role of PIAS3 in the transcriptional coregulatory activity of ErbB4 ICD was analyzed. Overexpression of PIAS3 repressed transcription in a model system where ErbB4 ICD and YAP coactivate expression of a luciferase gene (II, Fig 5). The repressive effect of PIAS3 was dependent on its SP-RING domain, an indication that the SUMO E3 ligase activity of PIAS3 was required (II, Fig 5).

To characterize the role of SUMOylation in cellular responses regulated by ErbB4, an *in vitro* model of mammary gland differentiation was used. Stable transfectants of human breast cancer (MDA-MB-468) and mouse mammary epithelial (HC11) cells expressing empty vector or ErbB4 JM-a CYT-2 were generated, and analyzed for three-dimensional growth in Matrigel. A high proportion of vector control cells grew in spherical or acinar structures, and were thus classified as differentiated. However, a significantly smaller proportion of ErbB4 expressing cells formed spheres but grew in undifferentiated, disorganized colonies instead (II, Fig 6B and F). Inhibition of ErbB4 RIP by a γ-secretase inhibitor (MDA-MB-468 cells; II, Fig 6A and B), or by expression of the non-cleavable JM-b isoform (HC11 cells; II, Fig 6E and F), increased the formation of mammary acini, indicating that ErbB4-mediated inhibition of differentiation was ICD-dependent. PIAS3 and PML were required for the inhibitory function of ErbB4 ICD, as demonstrated by the partial rescue of the phenotype upon RNA interference of PIAS3 or PML (II, Fig 6C, D and G). Finally, cells expressing the SUMOylation deficient K714R mutant of ErbB4 were comparable to vector control cells in their ability to differentiate, demonstrating that ErbB4 SUMOylation was required for the function of ErbB4 ICD in this model system (III, Fig 8B). Taken together, these data indicate that PIAS3-induced SUMOylation regulates the nuclear signaling of ErbB4 ICD.

6 DISCUSSION

6.1 NEDD4 family ligases as regulators of ErbB4 ubiquitination

Cbl is the major regulator of ubiquitination and degradation of activated RTKs, such as EGFR, PDGFRα and β, and hepatocyte growth factor receptor (Levkowitz et al., 1999; Miyake et al., 1999; Peschard et al., 2001; Goh and Sorkin, 2013). However, ErbB4 does not interact with Cbl, and ErbB4 has been reported to be endocytosis-impaired (Baulida et al., 1996; Levkowitz et al., 1996). This study revealed that the ubiquitination of ErbB4 is regulated in an isoform-specific manner by Itch, a NEDD4-family E3 ubiquitin ligase (I). In contrast to the central role of Cbl in mediating RTK ubiquitination, TrkA receptor, fibroblast growth factor receptor 1, and more recently ErbB3 have all been reported to be ubiquitinated by NEDD4-like ligases (Arévalo et al., 2006; Persaud et al., 2011; Huang et al., 2015). The specificity of Itch-mediated ErbB4 CYT-1 ubiquitination was achieved by the interaction of CYT-1-specific PPXY motif with the WW-domains of Itch (I, Fig 3). ErbB4-Itch interaction, as well as the ubiquitination and internalization of ErbB4 were stimulated by NRG-1, suggesting ligand-induced endocytosis and lysosomal degradation similar to that of EGFR (I, Fig 1, 2F, and SI Fig 7). Thus, the function of Itch is different from the previously identified steady-state ubiquitination and degradation of ErbB4 by NRDP1 (Diamonti et al., 2002). In contrast to Cbl, the interaction of NEDD4-like ligases with RTKs is not dependent on the RTK autophosphorylation. However, ligand-induced RTK activation has been shown to enhance the ubiquitin ligase activity of NEDD4 by inducing its tyrosine phosphorylation (Persaud et al., 2014).

The type of EGFR ubiquitination has been studied by comparing the signals obtained with ubiquitin antibodies that recognize both mono- and polyubiquitinated proteins, or polyubiquitinated proteins only. Based on these analyses, EGFR has been suggested to be monoubiquitinated (Haglund et al., 2003). Instead of a defined band representing monoubiquitinated EGFR, the signal detected with ubiquitin antibodies is smeary. This pattern has been proposed to arise from multimonoubiquitination of EGFR, as the attachment of multiple ubiquitin monomers, together with receptor phosphorylation and glycosylation, would result in receptor species of different molecular weights (Haglund et al., 2003). However, proteomic studies have shown that EGFR is mainly conjugated with K63-linked polyubiquitin chains (Huang et al., 2006; 2013). Although experimental conditions may affect the type of ubiquitination detected, multiple monoubiquitination and K63-linked polyubiquitin chains are both considered as important sorting signals in the endocytic pathway (Piper et al., 2014).

In this study, the analyses of ErbB4 ubiquitination type were in part based on the same mono- and polyubiquitin-specific antibodies that have been used to study the

ubiquitination of EGFR (Haglund et al., 2003) (I, Fig 4B). In alignment with EGFR, the data suggested mono- or multimonoubiquitination of ErbB4. Additionally, lysine-to-arginine ubiquitin mutants unable to form K48- or K63-linked polyubiquitin chains were utilized (I, SI Fig 8). The ubiquitin signal was also detected upon the overexpression of ubiquitin mutant constructs, suggesting that ErbB4 is not modified with K48- or K63-linked polyubiquitin chains.

It should be noted that analytical methods may affect the type of ubiquitination detected, and that the methodology to study ubiquitination has improved since the experiments for this study were carried out. Ubiquitin is a highly abundant protein, and overexpression of ubiquitin mutants does not overcome the endogenous ubiquitin pool. Thus, the detected polyubiquitin chains may also contain K48- and K63-linkages, and the ubiquitin mutant constructs may function as chain-terminating mutants, rather than as tools to prevent the formation of a specific polyubiquitin chain. In fact, Meijer *et al*. reported that the Itch binding site mediates K63-linked polyubiquitination of ErbB4 CYT-1 (Meijer et al., 2013). K63-linked polyubiquitination is plausible, as NEDD4 and Itch have been shown to catalyze the formation these linkages (Kim et al., 2007; Scialpi et al., 2008). At present, ubiquitin chain linkages can be analyzed with linkage-specific antibodies, or by using a recently developed Ubiquitin Chain Restriction (UbiCRest) method, which utilizes the linkage-specificity of DUBs (Hospenthal et al., 2015). Finally, to refine the experimental setup, ubiquitination of ErbB4 could be studied in ubiquitin pull-down experiments using denaturing conditions. A denaturing system would confirm the covalent ubiquitin modification of ErbB4, and formally exclude the possibility of the detected ubiquitin signal arising from an ErbB4-interacting, ubiquitinated protein.

Notably, the Itch-induced ubiquitination and degradation of ErbB4 CYT-1 has been confirmed by other researchers. Omerovic *et al*. showed that Itch interacted with and ubiquitinated ErbB4 CYT-1 (Omerovic et al., 2007). Moreover, two other NEDD4-family ligases, NEDD4 and WWP1, have been shown to be functionally similar to Itch in their ability to interact with, ubiquitinate, and promote the degradation of ErbB4 CYT-1 (Feng et al., 2009; Li et al., 2009; Zeng et al., 2009). Together, the results of this thesis and the above-mentioned studies indicate a key role for the NEDD4 family of E3 ubiquitin ligases as regulators of ErbB4 CYT-1 ubiquitination and stability. The NEDD4-like ligases may be functionally redundant, or alternatively regulate the ubiquitination of ErbB4 CYT-1 in a physiologically relevant manner in different tissue contexts. For example, while both Itch and WWP1 are expressed in breast cancer, including the estrogen receptor-positive breast cancer subtype where ErbB4 is typically expressed, WWP1 is frequently overexpressed due to amplification (Chen et al., 2007; 2009; Salah et al., 2014). WWP1 could thus regulate the ubiquitination of ErbB4 CYT-1 in this context.

6.2 Isoform-specific ubiquitination may induce qualitative and quantitative changes in ErbB4 signaling

Failure to attenuate ErbB signaling by endocytosis and degradation results in enhanced signaling. Indeed, defective RTK endocytosis can lead to malignant transformation (Wells et al., 1990). Different mechanisms may contribute to deficient downregulation and thus ongogenic ErbB signaling in cancer. EGFR overexpression may prolong signaling due to the limited capacity of the endocytic machinery (French et al., 1994). ErbB2 overexpression, on the other hand, enhances the recycling of EGFR/ErbB2 heterodimers, partially due to reduced receptor ubiquitination (Levkowitz et al., 1996; Lenferink et al., 1998). Additionally, cancer-associated deletion and kinase domain mutants escape efficient Cbl-mediated ubiquitination and degradation, resulting in prolonged signaling that contributes to the oncogenic properties of the mutant receptors (Huang et al., 1997; Han et al., 2006; Grandal et al., 2007; Shtiegman et al., 2007).

The finding that the abundance of ErbB4 cytoplasmic isoforms is differentially regulated at post-translational level may explain some of the functional differences of ErbB4 isoforms. While CYT-1 isoforms were efficiently downregulated in the presence of active NEDD4-like E3 ligases (I, Fig 4C and SI Fig 9), CYT-2 isoforms were degradation-resistant and could thus remain active. Accordingly, ErbB4 CYT-2 isoform, but not CYT-1, is capable of promoting ligand-independent cell survival and proliferation *in vitro* (Määttä et al., 2006). ErbB4 CYT-2 also produces more ICD, perhaps due to its increased stability compared to CYT-1 (Määttä et al., 2006). The difference in stability could thus allow enhanced signaling of CYT-2 type ICD in the nucleus.

Although EGFR is efficiently endocytosed upon ligand stimulus, heterodimerization with endocytosis-impaired ErbB2 or ErbB3 attenuates EGFR degradation (Lenferink et al., 1998). Interestingly, endocytosis-resistant ErbB4 CYT-2, but not CYT-1, has a related function in providing stability to EGFR/ErbB4 heterodimers (Kiuchi et al., 2014). As ErbB2 and ErbB3 also heterodimerize with ErbB4, isoform-specific ubiquitination and degradation of ErbB4 may regulate the stability and signaling output of all ErbB receptors in tissues where they are coexpressed. Moreover, as the cytoplasmic isoforms of ErbB4 are expressed together in the same tissues, including malignant tissues, CYT-2 could also provide stability to CYT-1/CYT-2 dimers in addition to ErbB heterodimers. Such dimers would allow for stronger CYT-1-mediated signaling, for example through PI3K, while retaining CYT-2 function. Thus, CYT-1/CYT-2 dimers could represent a more potent ErbB4 signaling unit compared to the function of either isoform alone.

Finally, ErbB4 CYT-1 isoform is overexpressed relative to CYT-2 in medulloblastoma, ovarian cancer and schizophrenia (Ferretti et al., 2006; Silberberg et al., 2006; Law et al., 2007; Paatero et al., 2013). These findings suggest that stoichiometry of ErbB4 isoforms is relevant in tissue homeostasis and pathology. While increased CYT-1 signaling can be

achieved through mechanisms that promote CYT-1 expression, insufficient activity of NEDD4-like E3 ubiquitin ligases that control CYT-1 levels post-translationally may also contribute to the malignant role of CYT-1 in these tissues.

6.3 SUMOylation of ErbB4 ICD

SUMOylation is an inducible, reversible, and typically non-proteolytic post-translational modification that acts as a molecular switch to alter the activity, localization or stability of its target proteins (Flotho and Melchior, 2013). SUMOylation is a predominantly nuclear modification, and the majority of SUMO-modified proteins are involved in nuclear processes (Hendriks and Vertegaal, 2016). However, reversible SUMOylation cycles also control the activity of many critical signal transduction proteins (Kubota et al., 2011; de la Cruz-Herrera et al., 2014).

The results of this study demonstrated that the intracellular domain of ErbB4 is SUMOylated (II, Fig 1G). Although this conclusion is based on experiments where ErbB4 ICD was ectopically expressed, it is supported by other data presented in this study. First, endogenous ErbB4 was shown to interact with a SUMO E3 ligase PIAS3, and colocalize with SUMO1 (II, Fig 1E and 4). Second, the PIAS3-ErbB4-interaction was functionally relevant, demonstrated by the role of PIAS3 as a regulator of the subcellular localization of ErbB4 in breast cancer cells (II, Fig 2F and G). Finally, mutation of a single lysine residue resulted in the loss of SUMO-modified ErbB4 ICD (III, Fig 1).

Like the majority of SUMO-modified proteins, only a minor proportion of ErbB4 ICD was SUMOylated without overexpressing SUMO. Indeed, many proteins are thought to be quantitatively SUMOylated only in response to a stimulus, such as cellular stress or activation of a specific signaling pathway (Flotho and Melchior, 2013). Even upon specific stimuli SUMOylation is a transient modification due to the activity of SUMO isopeptidases, and only a small fraction of a target protein is modified at a given time. This complicates the detection of SUMO modification without overexpression of SUMO, or SUMO together with a target protein.

SUMOylation status of many proteins changes upon DNA damage, or oxidative or proteotoxic stress (Saitoh and Hinchey, 2000; Tempé et al., 2008; Golebiowski et al., 2009; Sahin et al., 2014a; Hendriks et al., 2015). Intriguingly, the soluble ErbB4 ICD, which is detected at low abundance in normal cell culture conditions, accumulates upon cellular stress (Knittle et al., unpublished observations). This observation raises the possibility that RIP-mediated ErbB4 signaling plays a role in cellular stress conditions, and raises the question of whether SUMOylation regulates ErbB4 ICD function in such signaling contexts. Identification of a stress stimulus that induces the SUMOylation of endogenous ErbB4 ICD should be a focus of future studies.

Although ErbB4 contains SUMOylation consensus motifs, a single non-consensus lysine residue 714 in the kinase region was shown to serve as the major SUMOylation site (III, Fig 1). Indeed, not all lysine residues that perfectly fit the consensus motif are modified (Matic et al., 2010; Hendriks et al., 2014). Other factors, such as non-covalent interactions of the target protein with SUMO or a SUMO E3 ligase can influence the lysine selection (Flotho and Melchior, 2013). In the case of ErbB4 ICD, the selection of SUMOylation site may be directed by PIAS3, which interacted with the ErbB4 kinase region.

In addition to ErbB4, many other RTKs produce soluble ICDs *via* RIP (Merilahti et al., unpublished). Although the possible nuclear functions of these RTK ICDs are yet to be discovered, SUMOylation could represent a more general mechanism to regulate RTK ICDs. At present, two other RTKs, IGF-1R and EGFR, have been shown to be SUMOylated (Sehat et al., 2010; Packham et al., 2015). However, even though IGF-1R is a RIP substrate releasing a soluble ICD, these reports suggest SUMOylated of the full-length receptors (McElroy et al., 2007).

6.4 Mechanisms of SUMOylation-induced nuclear accumulation of ErbB4

Despite the accumulating evidence of nuclear localization and functions of ErbB4 ICD, the factors determining its subcellular distribution have remained poorly characterized. Previous studies have characterized a functional NLS, and suggested that nuclear export contributes to the subcellular localization of ErbB4 (Ni et al., 2001; Williams et al., 2004; Määttä et al., 2006; Hsu and Hung, 2007). However, in cultured cells that express full-length cleavable ErbB4 JM-a isoform, only low levels of nuclear ErbB4 epitope are detected, even when ErbB4 is overexpressed. This study demonstrated that PIAS3-induced SUMOylation promotes the nuclear accumulation of ErbB4 ICD. Thus, the results of this study expand the understanding of the molecular mechanisms that regulate the subcellular localization of ErbB4 ICD.

Both wild-type and SUMOylation deficient ErbB4 ICD were detected in the nucleus (III, Fig 3). Moreover, PIAS3, a SUMO E3 ligase that stimulated the SUMOylation, promoted the nuclear accumulation of ErbB4 ICD only when ErbB4 NLS was intact (III, Fig 3). These results indicate that SUMOylation is not necessary for the nuclear translocation *per se*. Intriguingly, the ErbB4 SUMO modification site resided within a functional nuclear export signal (III, Fig 1 and 5). Du *et al*. showed that SUMOylation of Krüppel-like factor 5 to a lysine adjacent to NES inactivated its nuclear export, and presented a number of proteins whose nuclear accumulation is promoted by SUMOylation and that have a NES in close proximity to the modification site (Du et al., 2008). While further experiments are needed to define the mechanism, the results presented in this thesis suggest a model in which SUMO conjugation at lysine 714 interferes with the interaction of ErbB4 NES with nuclear export receptors, resulting in nuclear accumulation.

6.5 Insights into the role of SUMOylation in ErbB4 signaling

ErbB4 has an established role in mammary gland biology regulating the lobuloalveolar differentiation during lactation (Jones et al., 1999; Long et al., 2003; Tidcombe et al., 2003). Previous studies have suggested a differentiation promoting role for the soluble ICD of CYT-1 type, but a proliferation promoting role for the CYT-2 ICD (Muraoka-Cook et al., 2006; 2009). Accordingly, the results of this study showed that RIP-mediated signaling of ErbB4 CYT-2 inhibited the differentiation of mouse mammary epithelial cells, and instead promoted growth in undifferentiated structures (II, Fig 6F). A similar ErbB4 RIP-dependent conversion toward a more malignant phenotype was induced in human breast cancer cells (II, Fig 6B). Significantly, SUMOylation was required for the differentiation-inhibiting function of ErbB4 ICD, as demonstrated by RNA interference of PIAS3 and mutagenesis of the modification site (II, Fig 6C and G; III, Fig 8B). Similar results produced by the two approaches indicate that although PIAS proteins sometimes regulate their interaction partners in a manner that is independent of their SUMOylation (Rytinki et al., 2009), the SUMO modification of ErbB4 is mechanistically involved. Since the mutation of the modification site did not disrupt the kinase activity or classical RTK-activated signaling pathways downstream of the full-length ErbB4, or influence its stability, it is likely that the loss-of-function phenotype upon inhibition of SUMOylation is due to decreased nuclear ErbB4 signaling.

ErbB4 ICD regulates many transcriptional events in mammary epithelial cells, including the activation of STAT5A-mediated transcription (Jones et al., 1999; Wali et al., 2014a). SUMOylation could thus regulate ErbB4 function by altering its activity in transcriptional processes. Indeed, PIAS3 overexpression repressed the transcriptional coregulatory activity of ErbB4 ICD (II, Fig 5). The mechanism of altered transcriptional activity could in turn involve the sequestration of ErbB4 in PML nuclear bodies (II, Fig 3 and 4). PML bodies contain ErbB4-interacting transcriptional regulators, and may thus provide a site for formation of transcriptional regulatory complexes (Khan et al., 2001; Fleischer et al., 2006; Lapi et al., 2008). Like PIAS3 and ErbB4 SUMOylation site, PML was also required for the ErbB4 ICD-mediated inhibition of differentiation (II, Fig 6D). Finally, although SUMO E3 ligase activity of PIAS3 was not necessary in promoting colocalization of ErbB4 with PML (II, Fig 3), the fraction of ErbB4 in PML bodies may still be SUMOylated, as most proteins in these nuclear bodies are (Bernardi and Pandolfi, 2007).

In addition to repressive effects on ErbB4 ICD-dependent transcription, SUMOylation enhanced the autophosphorylation of ErbB4 (III, Fig 6). Intriguingly, SUMOylation has been shown to stimulate the activity of both tyrosine and serine/threonine kinases and modulate the phosphorylation status of many proteins (Kadare et al., 2003; Yao et al., 2011; de la Cruz-Herrera et al., 2014). As the nuclear localization of ErbB4 ICD is kinase activity-dependent (Muraoka-Cook et al., 2006; Sundvall et al., 2007), SUMOylation

could contribute to the nuclear accumulation through increased tyrosine phosphorylation. One can also speculate that SUMOylated ErbB4 ICD could be more active towards its nuclear phosphorylation substrates. Currently, the molecular mechanism by which SUMOylation promotes kinase activity of ErbB4 or other kinases is unknown. Hypothetically, SUMOylation could induce a conformational change resulting in more efficient activation of a kinase domain, or make the activated kinases poorer substrates for specific phosphatases.

6.6 SUMOylation may regulate ErbB4 function in breast cancer

The expression of PIAS3 has been shown to be increased in breast cancer (Wang and Banerjee, 2004; McHale et al., 2008). The relatively highest expression is detected in the estrogen receptor-positive, lobular subtype (In Silico Trancriptomics database; ist. medisapiens.com, (Kilpinen et al., 2008)). Intriguingly, the overexpression of PIAS3 has been reported to promote the proliferation of estrogen receptor-positive breast cancer cells, but conversely to inhibit the proliferation of estrogen receptor-negative breast cancer cells (Yang et al., 2016). The overexpression of PIAS3 also induced resistance to anti-estrogen hormone therapy in estrogen receptor-positive breast cancer cells (Yang et al., 2016). At the molecular level, PIAS3 promotes ERα expression as well as its SUMOylation, which activates ERα-mediated transcription (Sentis et al., 2005; Park et al., 2011). Together, these studies suggest an oncogenic role for PIAS3 in estrogen receptor-positive breast cancer, a subtype in which ErbB4 is also expressed.

In breast cancer the localization of ErbB4 epitope in different cellular compartments is associated with different clinical outcomes (Junttila et al., 2005; Aqeilan et al., 2007; Thor et al., 2009). In particular, nuclear ErbB4 staining is associated with poor prognosis, as compared to the localization of ErbB4 epitope at the cell surface (Junttila et al., 2005). It is interesting to speculate whether ErbB4 SUMOylation could be induced in PIAS3-overexpressing, estrogen receptor-positive breast cancers. Indeed, SUMOylation could represent a molecular mechanism controlling the nuclear accumulation and potentially oncogenic activity of ErbB4 ICD in breast cancer.

To address this hypothesis, the SUMOylation status of ErbB4 should be studied in breast cancer tissue samples. Although the analysis of SUMOylation in tissue samples is complicated compared to cell culture models, SUMOylation of some target proteins has been detected in mouse tissue using denaturing lysis and immunoprecipitation with SUMO antibodies (Becker et al., 2013). PLA can also be applied to analyze protein SUMOylation in tissue sections, allowing the detection of SUMOylated proteins *in situ* (Sahin et al., 2016). The PLA results should be interpreted with caution, however, as the detection PLA signal does not require covalent SUMO modification, but rather the proximity of SUMO with the target protein. Lastly, the potential correlation between

the abundance of PIAS3 and nuclear ErbB4 epitope could be analyzed in breast cancer tissue sections by immunohistochemistry.

The results of this study demonstrated that ErbB4 ICD inhibits the differentiation of normal and malignant breast epithelial cells, and that SUMOylation was required for this function (II, Fig 6; III, Fig 8). While the molecular mechanism remains elusive, it potentially involves altered transcriptional or kinase activity upon ErbB4 SUMOylation, or merely the increased abundance of nuclear ErbB4 ICD (II, Fig 2 and 5; III, Fig 3 and 6). These results give an indication that SUMOylation may promote oncogenic signaling ErbB4 ICD, but additional research is required to determine the relevance of SUMOylation on ErbB4 function in the context of breast cancer. Future experimentation should involve *in vitro* and *in vivo* models of breast cancer comparing the activities of wild-type and SUMOylation-deficient ErbB4. For example, the function of SUMOylation-deficient ErbB4 could be characterized using a xenograft mouse model of breast cancer, in which tumor growth is dependent on ErbB4 RIP (Hollmén et al., 2012). The recently developed clustered regularly interspaced short palindromic repeats-CRISPR associated (CRISPR-Cas)-system enables genome editing (Wright et al., 2016), and provides a valuable tool to characterize the role of a modification site without ectopic expression of a mutated protein. As such, CRISPR-Cas-engineered breast cancer cell lines expressing SUMOylation-deficient ErbB4 could be useful models for future studies.

7 CONCLUSIONS

This thesis aimed to characterize novel regulatory mechanisms of ErbB4 signaling, with a focus on two post-translational modifications: ubiquitination and SUMOylation.

Based on the results of this study (summarized in Figure 12), the following key conclusions can be made:

1) Ubiquitination, endocytosis and degradation of ErbB4 are regulated in an isoform-specific manner through the action of Itch E3 ubiquitin ligase. Targeting of CYT-1, but not CYT-2 isoforms to degradation may result in quantitative and qualitative changes in ErbB signaling.

2) The intracellular domain released by the cleavable ErbB4 isoforms is modified by SUMO. SUMOylation is induced by PIAS3 SUMO E3 ligase, and regulates the nuclear localization and differentiation-inhibiting function of the soluble ErbB4 ICD. Thus, SUMOylation is a novel mechanism controlling the signaling of an ICD of a receptor tyrosine kinase in the nucleus.

Together, these findings offer new insights into the molecular mechanisms that regulate the stability, subcellular localization, and transcriptional and kinase activities of ErbB4. The regulation of quantitative and qualitative aspects of ErbB4 signaling may have implications for the ErbB-regulated biological processes in both healthy tissues and cancer. Thus, further studies are warranted to explore the role of post-translational regulation of ErbB4 signaling in these contexts. Finally, the findings of this thesis can potentially be extended to provide new understanding on the mechanisms that regulate the functions of receptor tyrosine kinases other than ErbB4.

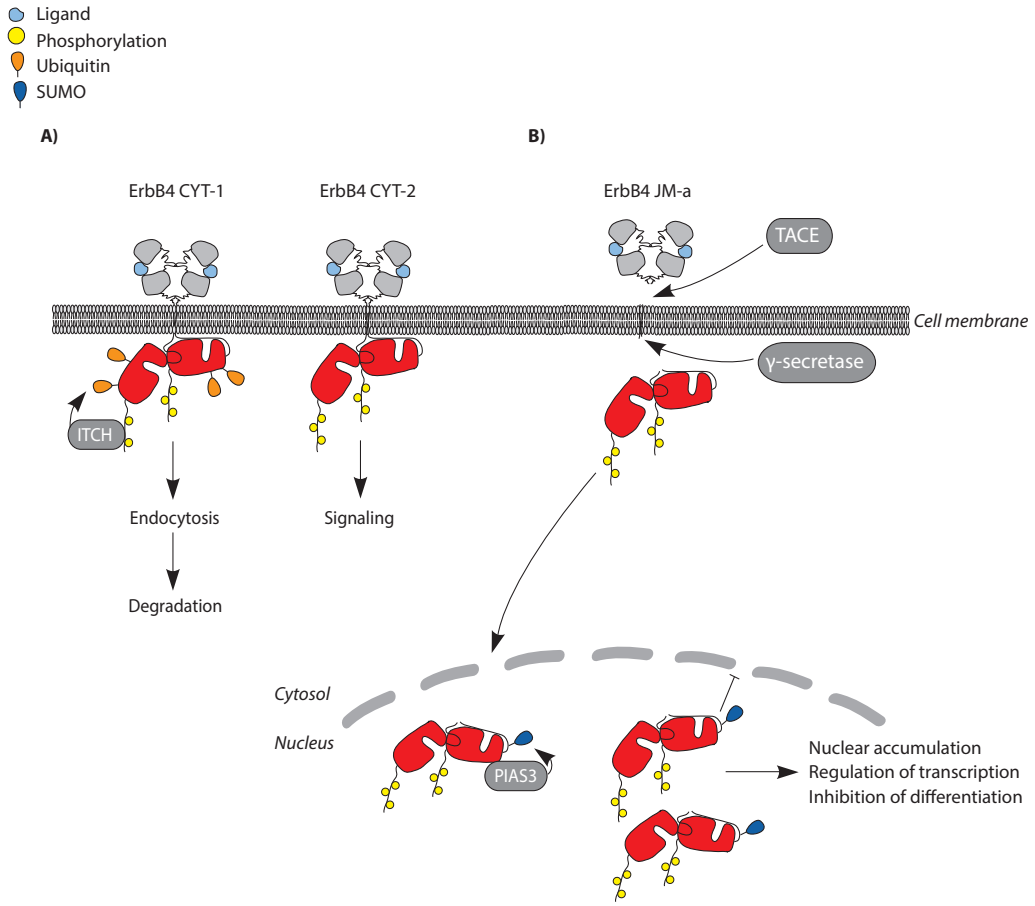

Figure 12. A model of post-translational modifications in ErbB4 signaling. A) The interaction of Itch E3 ubiquitin ligase results in isoform-specific ubiquitination, endocytosis and degradation of CYT-1 isoforms, while CYT-2 isoforms are resistant to Itch-induced degradation. CYT-2 isoforms can thus mediate prolonged signaling of ErbB4 homo- and ErbB heterodimers. B) Regulated intramembrane proteolysis of the ErbB4 JM-a isoforms, mediated by tumor-necrosis factor α converting enzyme (TACE) and γ-secretase, releases a soluble ErbB4 intracellular domain (ICD) into the cytosol. The ICD translocates into the nucleus and is SUMOylated by PIAS3 SUMO E3 ligase. SUMOylation promotes the nuclear accumulation and alters the nuclear signaling of ErbB4 ICD. CYT, cytoplasmic; JM, juxtamembrane; TACE, tumor-necrosis factor α converting enzyme.

8 ACKNOWLEDGEMENTS

This study was carried out at the Department of Medical Biochemistry and Genetics, and in MediCity Research Laboratory, University of Turku. I wish to acknowledge professors Klaus Elenius and Sirpa Jalkanen for providing truly excellent research facilities.

I wish to express my gratitude to my supervisors, professor Klaus Elenius and docent Maria Sundvall. I want to thank Klaus for all the guidance and mentoring I have received over the years, and for the opportunity to explore science in a positive and creative atmosphere. I am grateful to Maria for her vast contribution and countless ideas, and for sharing her passion for science.

I want to warmly acknowledge the members of my thesis committee, professor Lea Sistonen and assistant professor Noora Kotaja, for their interest to my research and valuable advice during my doctoral training.

I would like to thank Turku Doctoral Programme of Molecular Medicine and Turku Doctoral Programme of Biomedical Sciences, and their directors, professors Kati Elima and Olli Lassila, for providing indispensable financial and educational support, and many enjoyable social events. Financial support from the Cancer Society of Southwestern Finland, Finnish Cancer Organizations, Finnish Concordia Fund, Finnish Cultural Foundation, Finnish Cultural Foundation Varsinais-Suomi Regional Fund, Ida Montin Foundation, Orion-Farmos Research Foundation, and Turku University Foundation is also acknowledged.

Dr. Annika Meinander and docent Pipsa Saharinen are warmly acknowledged for carefully reviewing the thesis manuscript, and for giving insightful and valuable comments.

I would like to thank my co-authors Drs. Julius Anckar, Rami I. Aqeilan, Carlo M. Croce, Eugenio Gaudio, Kalle Halkilahti, Maria Helkkula, Gerry Melino, Ilkka Paatero, Jorma J. Palvimo, Zaidoun Salah, Lea Sistonen, and Katri Vaparanta for significantly contributing to this study.

The present and former members of the Elenius lab, Erika Alanne, Deepankar Chakroborty, Marika Hakanen, Juho Heliste, Maria Helkkula, Maija Hollmén, Anne Jokilammi, Anna Khudayarov, Peppi Kirjalainen, Maarit Kortesoja, Kari Kurppa, Johannes Merilahti, Matias Mäenpää, Elli Narvi, Janne Nordberg, Veera Ojala, Ilkka Paatero, Arto Pulliainen, Fred Saarinen, Minna Santanen, Mika Savisalo, Jori Torkkila, Maria Tuominen, Denis Tvorogov, Katri Vaparanta, and Ville Veikkolainen, as well as the members of the Heino group, are all warmly thanked for making the lab a sociable, relaxed, and collaborative research environment, where both successes and struggles are shared. I also wish to thank all my colleagues at the Department of Medical Biochemistry. The friendly and helpful atmosphere has made it a very pleasant place to work.

I am grateful to professor Peter ten Dijke for the opportunity to join his lab at the Department of Molecular Cell Biology in Leiden University Medical Center. The years in Leiden provided me an exciting introduction to the world of TGF-β signaling, and greatly contributed to my scientific training. I also wish to acknowledge all my LUMC colleagues for collaboration and friendship.

Minna Santanen, Raili Salonen, Mika Savisalo, and Maria Tuominen are deeply acknowledged for their skillful technical assistance. I also want to thank Rakel Mattsson, Pia Tahvanainen, Hanna Tuominen, Eeva Valve, and Nina Widberg for excellent secretarial help.

I have been lucky to have many good friends, and I want to warmly thank all of them for friendship and the joyful moments we have shared. My special thanks go to Angela, Jemiina, and the two Marias Salmela&Tuominen, who have closely accompanied me in my academic path. Over the years, we have shared the ups and downs of life in the lab and outside of it, after-work runs and the great outdoors, and a healthy number of (sparkling) wine bottles. Your support has been indispensable!

Finally, I wish to express my deepest gratitude to my family. I want to thank my parents Eeva and Antero for the unconditional love and support that continues to encourage me in everything I do. Keegan, thank you for your endless love and bringing happiness to my life. You are an invaluable companion to me, and I would not have made it without you.

Turku, December 2016

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