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Mechanisms of ErbB receptor negative regulation and relevance in cancer

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Abstract

The ErbB family of receptor tyrosine kinases engages a wide variety of signaling pathways that collectively direct transcriptional programs controlling organogenesis during development and tissue maintenance in the adult. These receptors are also frequently found overexpressed or aberrantly activated in various cancers, suggesting that ErbB receptor signaling activity must be very tightly regulated. Sufficient levels of ErbB signaling are necessary to mediate tissue homeostasis, for example, but over-signaling can trigger cellular processes that contribute to cancer initiation or progression. Efforts over the last quarter century have led to a thorough understanding of the signaling pathways that are activated by these receptors and the mechanisms by which ErbB receptors engage these pathways. However, the compensatory negative regulatory mechanisms responsible for attenuating receptor activation have only more recently begun to be explored. Here we review the different known mechanisms of ErbB negative regulation, with particular emphasis on those proteins that exhibit some specificity for the ErbB family. We also describe how loss or suppression of ErbB negative regulators may contribute to tumor development, and discuss how restoration or augmentation of these pathways may represent a novel avenue for the development of ErbB-targeted therapies.

Introduction

Signaling by the ErbB receptor tyrosine kinases (RTKs) is generally initiated through the binding of soluble EGF-like growth factor ligands, and controls a wide array of processes from organogenesis of the developing organism to tissue maintenance and regeneration in the adult (1). The ErbB receptors are the most extensively studied RTKs in biology, largely due to their involvement in cancer initiation and progression. The transforming ability of the ErbBs was first appreciated two dozen years ago with the discovery that an oncogenic chicken virus encodes a constitutively active form (v-erbB) of ErbB1 or EGFR (2). Since that time the three remaining mammalian ErbB receptors have been described, and the signaling pathways initiated upon activation of these receptors have been characterized in detail. It is well documented that ErbB receptor overexpression and concomitant activation is a frequent occurrence in cancer. However, many attempts to reduce signaling through these receptors in tumors using anti-receptor antibodies or small molecule tyrosine kinase inhibitors have been disappointing because tumor cells are often either inherently resistant to ErbB-directed therapies, or develop resistance with treatment. Consequently, we propose that the

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identification and characterization of endogenous negative regulatory pathways present in normal cells and tissues that limit ErbB expression or activation may ultimately help guide the development of novel ErbB-directed therapeutics. Here we describe several mechanisms of ErbB receptor negative regulation, focusing our attention on those negative regulators that show specificity for the ErbB receptors themselves rather than downstream signaling components.

ErbB signaling

Epidermal growth factor receptor (EGFR/ErbB1/HER1) has served as the prototype for the mechanistic study of RTKs for three decades. While new studies continue to refine our understanding of EGFR signal transduction, the general mechanism of ligand-mediated receptor activation and downstream signal amplification are well understood. However, less well understood is how activated receptors are suppressed to limit the amplitude and duration of signaling. Conceptually, it is of critical importance to maintain receptor signaling within a relatively narrow window; sufficient signaling is necessary to ensure the fidelity of necessary tissue development and homoeostatic processes, but over-signaling must be prevented to suppress processes that could lead to neoplasia. In this section we will review the general scheme of ErbB signaling to provide a contextual framework for discussing where negative regulators act to suppress ErbB activity.

Ligand binding and transmembrane signaling

All four ErbB family members share the same basic structural architecture, including an extracellular ligand-binding domain, a single-pass transmembrane region, and an intracellular tyrosine kinase domain. The complexity of the mammalian ErbB signaling network is generated by the thirteen different peptide ligands that bind to the ErbBs, and the different homodimeric and heterodimeric receptor combinations that can be derived from dimerization among the different ErbB family members (3). Binding of a single EGF-like ligand induces a conformational change in the ErbB monomer, rendering it competent to dimerize with another active monomer (4). This ligand-induced dimerization event leads to activation of the receptor's intrinsic tyrosine kinase activity, resulting in trans-autophoshorylation of adjacent receptor monomers and the recruitment of signaling proteins to the newly phosphorylated docking sites. Further adding to the complexity of ErbB signaling, two members of the ErbB family deviate from this general model. First, ErbB2 has no known endogenous diffusible ligand, and structural studies suggest that it is unable to bind ligand because its extracellular ligand-binding domain already assumes the ligand-bound conformation, thereby blocking access of ligand to this region (5). Thus ErbB2 is constitutively poised to dimerize, explaining earlier observations that it is the preferred dimerization partner of the other ErbBs (6). Second, ErbB3 lacks intrinsic kinase activity because several conserved residues critical for kinase activity are altered in this protein. Hence, both ErbB2 and ErbB3 are non-autonomous receptors that must heterodimerize with the other ErbBs to transmit signals. Depending upon the specific activating ligand and ErbB dimer combination, ErbB dimers can stimulate a wide variety of cellular signaling pathways that culminate in the nucleus to drive transcriptional programs controlling cell migration, survival, adhesion, differentiation, and proliferation (7).

Receptor internalization and trafficking

There is constant turnover of ErbB receptors at the plasma membrane as a result of both constitutive (ligand-independent) and ligand-induced endocytosis. Both processes have been studied extensively for EGFR but are less well understood for the other ErbB family members. Kinetic studies of constitutive EGFR endocytosis have shown that while the half-life for receptor internalization is approximately 30 minutes, the half-life for receptor recycling is much faster, approximately 5 minutes (8). The kinetically faster recycling component for

EGFR trafficking then predicts that 80-90% of the receptor should be localized to the plasma membrane, and this is indeed observed in cells not exposed to activating ligand (9). Thus, the default pathway of slow internalization followed by fast recycling localizes the majority of EGFR to the plasma membrane where it is poised for activation.

Upon exposure to the ligand epidermal growth factor (EGF) the internalization rate of EGFR is accelerated 10-fold, resulting in a dramatic relocalization of receptors from the cell surface to internal compartments. This ligand-accelerated internalization is dependent upon EGFR kinase activity (8), which has been proposed to enhance internalization through phosphorylation of the clathrin adaptor protein Eps15 (10). Ligand-induced activation of EGFR kinase activity also recruits the E3 ubiquitin ligase Cbl leading to multiple mono-ubiquitination of EGFR, which has also been proposed to mediate receptor internalization (11,12). However, the importance of receptor ubiquitination in internalization has been challenged (13), and ubiquitination may play a more critical role in subsequent endosomal sorting and degradation steps. Interestingly, the accelerated ligand-induced internalization of EGFR may to be unique to this member of the ErbB family as ErbB2-4 have been reported to be endocytosis impaired (14). However, as outlined below, several negative regulators that influence ErbB trafficking and degradation are commonly absent in tumors and tumor-derived cell lines, complicating interpretations. Currently the constitutive and ligand-dependent endocytic behavior of these receptors is a matter of intense debate (15-19).

Once internalized, either by constitutive or ligand-induced endocytosis, receptors enter the endocytic pathway where they are either rapidly recycled back to the plasma membrane through recycling endosomes, or retained in earlier structures by sorting proteins and trafficked to the lysosome for degradation. Sorting of cargo proteins at the endosome is a highly regulated process, and is a focal point for many of the proteins that regulate the stability of ErbB family members.

Certain cell surface proteins such as the transferrin receptor undergo constant turnover at the plasma membrane, through constitutive internalization that delivers receptors to early and sorting endosomes, followed by re-insertion into the plasma membrane via recycling endosomes (see Figure 1). Internalized ErbB receptors can similarly be recycled back to the plasma membrane through recycling endosomes, or retained in sorting endosomes for trafficking to the lysosome. Current evidence suggests that this sorting decision at the early endosome is determined by the ubiquitination status of the endosomal cargo (Figure 1). Indeed, the importance of ubiquitination for EGFR down-regulation was demonstrated in two studies showing that fusion of a single ubiquitin residue in-frame with EGFR is sufficient to mediate constitutive internalization and enhanced degradation of EGFR (11,12). Ubiquitination of receptors targets them for lysosomal degradation and termination of signaling, while escape from ubiquitination leads to receptor recycling and prolonged signaling, a frequent occurrence in cancer (20). Consequently, proteins that control the ubiquitination status of ErbB receptors help dictate steady-state levels of receptor and initiate downregulation of activated receptors.

Post-transcriptional mechanisms of ErbB overexpression in cancer

Overexpression of ErbB1-3 is a frequent finding in many tumor types, and has been most extensively characterized in breast cancer (21,22). Overexpression of these receptors is associated with elevated kinase activity and signaling *in vitro*, and is often associated with poor clinical outcome in patients (21). Seminal studies (23,24) have demonstrated that a remarkable 25-30% of breast tumors exhibit ErbB2 protein overexpression, and this overexpression correlates with amplification of the *erbB2* gene. However, accumulating evidence suggests that ErbB2 may not act alone. Suppression of ErbB2 signaling in cancer cells reduces cell proliferation in a PI3K-dependent manner (25) and this requires ErbB3 (26), which unlike ErbB2 is able to directly couple to the PI3K pathway. Hence, ErbB2 and ErbB3 might cooperate

to drive oncogenic signaling, an idea confirmed by Holbro et al. (27) who showed that ErbB2 and ErbB3 are co-dependent for oncogenic transformation and form an oncogenic unit. Coupled with clinical studies suggesting that both proteins are often co-overexpressed in breast tumors (28,29), these observations underscore the notion that ErbB2 and ErbB3 may synergistically promote breast tumor progression.

Interestingly, *erbB2* gene amplification alone may not be sufficient to drive ErbB2 protein overexpression in tumors. Gene amplification has been mimicked in mice by transgenic overexpression of the ErbB2 cDNA in the mammary gland using the murine mammary tumor virus (MMTV) promoter/enhancer. These animals develop multifocal metastatic mammary tumors with a long latency relative to other MMTV-expressed oncogenes (30), suggesting that tumor initiation may require other steps in addition to ErbB2 overexpression. Particularly noteworthy, in comparing mammary tumor tissue to adjacent non-tumor tissue derived from MMTV-ErbB2 mice, we have observed that while ErbB2 message is equally overexpressed in both tissue samples, ErbB2 protein is overexpressed only in the tumor samples (31,32). Slamon et al. (24) also observed a significant number of patient breast tumor samples exhibiting ErbB2 protein overexpression in the absence of gene amplification. Furthermore, we and others have observed that ErbB3 message is equally expressed in both tumor and non-tumor MMTV-ErbB2 mammary tissue, while ErbB3 protein is massively overexpressed in tumors (31-34). These observations are consistent with the notion that ErbB2 and ErbB3 protein levels are regulated by powerful post-transcriptional regulatory mechanisms that are lost during the conversion of normal tissue to tumor. Indeed, loss of several of the negative regulatory proteins described below has been observed in breast tumors.

Thus far, clinical interventions aimed at targeting the ErbB family have focused on limiting receptor activation through the use of humanized anti-ErbB receptor antibodies such as cetuximab and trastuzumab or through ErbB specific tyrosine kinase inhibitors (TKIs) such as gefitinib and erlotinib. Trastuzumab is the only ErbB2-targeted therapy approved by the FDA for the treatment of metastatic breast cancer (35), however, most patients who show an initial response generally exhibit disease progression within a year of starting trastuzumab monotherapy. Small molecule tyrosine kinase inhibitors such as gefitinib and erlotinib were developed to target EGFR kinase, and they effectively reduce EGFR phosphorylation in cell-based assays and in tissues from treated patients. However, tumor resistance to the drugs has also restricted their clinical utility. A recent study suggests that TKI-treated cancer cell lines and tumors augment signaling through the kinase-dead ErbB3 receptor, allowing cells to escape the effects of ErbB-directed inhibitors (36). Thus, the loss of ErbB negative regulators by tumor cells may not only facilitate the initiation and progression of tumors, but may also contribute to therapeutic resistance.

ErbB negative regulators

Execution of signaling programs controlling a variety of biological activities by ErbBs requires that the amplitude and duration of receptor activation be exquisitely regulated. The amplitude of ErbB signaling is determined by the local production of diffusible ligand and the abundance of proximal ErbB receptors, while signaling duration is restricted by the engagement of negative regulators. With our expanding knowledge of ErbB negative regulators it is now appreciated that they can target virtually every step of receptor signaling, including ligand-binding, receptor dimerization, and intracellular trafficking (see Figure 1). Clues to the identity of these negative regulators have come from the study of oncogenic animal viruses (37) and through genetic studies in flies of DER (*Drosophila* EGF receptor), the single ErbB ortholog expressed in the fly.

ErbB feedback negative regulators: lessons from flies

By the latter 1990's the study of ErbB receptors in mammalian systems had identified virtually all of the currently known EGF-like polypeptide ligands, all four ErbB family members, and most of the signaling pathways downstream of activated receptors. However, at this time no endogenous negative regulators of ErbB signaling were known in animals. Around this time, using the powerful genetics of *Drosophila*, two groups identified Argos and Kekkon-1 as feedback negative regulators of DER (37,38), paving the way for the later discovery of mammalian ErbB negative regulators.

Argos was originally characterized as a secreted protein that antagonizes DER activation, and it was suggested that direct binding of Argos to the extracellular portion of DER mediates suppression of receptor activity (39-41). More recent studies actually demonstrate that Argos binds directly to the DER activating ligand Spitz, suggesting that Argos antagonizes DER activation through ligand sequestration in a manner reminiscent of that seen for IGFBP-mediated sequestration of IGF1 (42,43). While a human ortholog of Argos has not yet been identified, a recent paper reporting the crystal structure of Argos bound to Spitz shows that they interact through the formation of a three domain clamp resembling the interaction of urokinase-type plasminogen activator (uPA) with its receptor uPAR (44). Bioinformatics-based searches for proteins with similar domain architecture may yet lead to the discovery of an orthologous mammalian protein.

Shortly after the initial discovery of Argos, a second negative regulator of DER activation was discovered in a genetic screen for DER-regulated genes. Kekkon-1 (Kek-1) is a transmembrane protein with an extracellular domain that consists of six leucine rich repeats and an immunoglobulin domain, a single hydrophobic transmembrane domain, and an intracellular domain that lacks any distinguishing features. The leucine- rich repeat domain of Kek1 was found to physically interact with the extracellular region of DER to inhibit receptor signaling (45,46) by either blocking growth factor binding or receptor activation (47). Of the six known Kek family members in the fly, only Kek1 was found to inhibit DER signaling (48).

While Argos and Kek1 antagonize DER activation by different mechanisms, it is interesting to note that both genes are transcriptionally induced by DER activation (39,45). In this way DER activation leads to up-regulation of its own inhibitors to generate a negative feedback loop that serves to limit the amplitude and duration of receptor signaling. The evolutionary conservation of the EGFR signaling module from flies and nematodes through mammals suggests that such negative feedback loops may also be operative in humans. Indeed, guided by this pioneering work in *Drosophila*, research over the past several years has uncovered several novel mammalian negative feedback regulators.

Mammalian ErbB negative regulators

Our discussion of ErbB receptor activation, internalization and endosomal sorting indicates that there are many steps in the receptor life cycle that could be subject to negative regulation (see Figure 1). While many studies identify proteins that are required for ErbB receptor downregulation, most of these are core components of the endocytic (10) or intracellular trafficking machineries (49-51) and are likely to affect the downregulation of numerous receptor tyrosine kinases. Given the modest therapeutic benefit seen so far with anti-ErbB antibodies and TKI's, exploitation of ErbB negative regulators may eventually be used toward clinical benefit. With this in mind we will focus our discussion of mammalian ErbB negative regulators to those proteins that show some specificity for the ErbB family of receptor tyrosine kinases, and consequently could prove therapeutically useful.

E3 ubiquitin ligases target ErbBs for degradation

The first mammalian ErbB negative regulator was discovered in 1989 in the form of v-cbl, a truncated form of the E3 ubiquitin ligase c-Cbl encoded by a mouse virus (52). However, the biochemical function of c-Cbl was not understood until almost a decade later when it was discovered that Cbl mediates the activation-dependent degradation of EGFR (53). In some ways this example is representative of how our understanding of ErbB negative regulation has lagged behind our much better understanding of ErbB signal transduction. Since the unraveling of Cbl's role in EGFR down-regulation, ubiquitin ligase-mediated degradation has emerged as a recurrent theme in ErbB negative regulation.

As described above, ubiquitin is a key sorting signal at the early endosome, and is required for sorting of endosomal cargoes to the lysosome for degradation. The E3 ubiquitin ligase Cbl is recruited to EGFR in an activation-dependent manner within minutes of ligand binding (53) and binds directly to phosphorylated Y1045 of the activated receptor and indirectly through Grb2 (54,55). Ligand-mediated recruitment of Cbl to the EGFR leads to multiple mono-ubiquitination of the receptor and targeting for lysosomal degradation (11,12). Mutation of EGFR Y1045 impairs Cbl recruitment and leads to reduced receptor ubiquitination and degradation, whereas Cbl overexpression leads to increased receptor ubiquitination and degradation (54). Underscoring the importance of Cbl-mediated receptor downregulation is the finding that two naturally occurring mutants of Cbl (v-cbl and 70Z cbl) act as transforming oncogenes. Mechanistically, it has been found that these oncogenic forms of Cbl increase recycling of internalized EGFR to the plasma membrane and inhibit receptor ubiquitination and degradation (53). This suggests that Cbl-mediated ubiquitination is critical for lysosomal targeting of activated EGFR, and that disruption of this process can lead to cellular transformation.

Recently it was shown that SOCS4 and SOCS5 mediate the Cbl-independent degradation of EGFR in a mechanism that resembles a negative feedback loop (56). The suppressors of cytokine signaling (SOCS) proteins act to negatively regulate cytokine receptors through several different mechanisms, including recruitment of a ubiquitin ligase complex that mediates receptor degradation (57). SOCS proteins have a central SH2 domain that mediates binding to specific phosphorylated substrates, a variable N-terminal region, and a conserved C-terminal SOCS box (SB). The SB has been shown to interact with elongins B and C, which mediate recruitment of Cullin-5 and Rbx-1 to form an E3 ligase complex (58). Kario et al. showed that endogenous SOCS4 and 5 expression was induced by EGF treatment, and further demonstrated that overexpressed SOCS5 was able to mediate EGFR degradation in an SH2 and SB-dependent manner (56). The SB was required for recruitment of the E3-ubiquitin ligase complex, and overexpression of wild-type SOCS5 mediated increased EGFR ubiquitination and degradation in a Cbl-independent manner. These data suggest that while Cbl acts immediately following receptor activation to induce receptor downregulation, SOCS4 and 5 may accumulate in response to receptor activation to ensure feedback negative regulation. Interestingly, these investigators also found that SOCS5 bound to ErbB2 and ErbB4 to mediate their degradation, while effects on ErbB3 were not determined. Clearly SOCS4 and 5 represent interesting new modulators of ErbB signaling. However, because this report relied on overexpression of SOCS4 and 5 it remains to be seen how significant the contribution of endogenous SOCS4 and 5 is to ErbB downregulation.

ErbB2 is unique among the ErbBs as it is the only family member that does not bind a ligand, but instead must heterodimerize with other ligand-bound ErbBs to generate signals. While ligand-induced internalization of EGFR leads to its ubiquitination and degradation, EGFR/ErbB2 heterodimers do not undergo ligand-induced internalization but instead are retained at the plasma membrane (15,60). These observations have led to the conclusion that ErbB2 is endocytosis resistant. However, in the absence of ligand stimulation the metabolic half-life of

ErbB2 is approximately 1-3 hours, compared to almost 10 hours for EGFR (9,60), indicating that there are potent mechanisms in place for the constitutive removal of ErbB2. Given that its overexpression correlates with a negative clinical outcome in breast and other cancers, it is of great interest to identify those proteins that control cellular levels of ErbB2.

Mature ErbB2 is bound by HSP90, a molecular chaperone that assists in directing the correct folding of nascent polypeptides and in conferring stability to mature proteins. HSP90 stabilizes the mature forms of many client proteins involved in cell signaling, including estrogen receptor, progesterone receptor, ErbB2, and proteins downstream of ErbB2 including Akt, c-src, and Raf (61). Inhibition of HSP90 chaperone activity by geldanamycin or other ansamycin antibiotics leads to the rapid degradation of ErbB2 in a mechanism that involves HSP90 dissociation followed by recruitment of an HSP70/CHIP complex (60). CHIP (carboxyl-terminal HSP70-interacting protein) is an E3-ubiquitin ligase that interacts with both HSP90 and HSP70 through its N-terminal tetratricopeptide repeat (TPR) domain, and its recruitment to ErbB2 with HSP70 mediates the receptor polyubiquitination that precedes ErbB2 proteasomal degradation (61). The potential utility of ansamycin antibiotics in ErbB2 overexpressing tumors is obvious, and preliminary results from phase I clinical trials with the ansamycin analog 17-AAG showed some promise in patients with HER2 positive metastatic breast cancer (62). However, the use of 17-AAG or other ansamycin analogs may be potentially complicated by their pleiotropic effects, including stimulation of the proto-oncogene src (63). Interestingly, ErbB2 stability is dramatically increased in fibroblasts from CHIP-/- mice (61), indicating that CHIP contributes to the normal metabolic turnover of ErbB2 *in vivo*.

A second E3-ubiquitin ligase that may target ErbB2 is the ligand of numb protein X (LNX1). LNX1 was originally identified as an E3 ubiquitin-ligase that targets the membrane-associated cell fate determinant Numb for ubiquitin-dependent degradation (64). More recently LNX1 was shown to directly interact with ErbB2 by yeast-two hybrid screening, and this interaction was confirmed biochemically in mouse brain lysate (65). LNX1 is highly expressed in peri-synaptic Schwann cells (PSCs), with expression levels peaking at postnatal day 14–21 and persisting throughout adulthood. Significantly, this developmental pattern is inversely correlated with ErbB2 expression in PSCs. Furthermore, denervation of muscle fibers, which is known to cause upregulation of ErbB2, results in a loss of LNX1 staining in PSCs (65). These results are consistent with a role for LNX1 in mediating the ubiquitination and degradation of ErbB2 in PSCs, but direct evidence of LNX1 ubiquitin ligase activity towards ErbB2 has not been documented. Remarkably however, in a recent study looking at differential gene expression patterns in a set of 18 gliomas, it was reported that LNX1 was down-regulated in all tumors sampled (66). It will be interesting to see if LNX1 expression is inversely correlated with ErbB2 expression in ErbB2-driven breast tumors.

EGFR trafficking is the yardstick by which trafficking of all other RTKs are judged. In this regard, the other members of the ErbB family have been historically considered endocytosis impaired (14), and mechanisms regulating the cell surface population of ErbB3 and 4 have remained enigmatic. In a screen to identify proteins that are capable of associating with ErbB3 in a ligand-independent manner, we identified neuregulin receptor degradation protein-1 (Nrdp1) as a RING finger domain-containing E3 ubiquitin ligase that regulates ErbB3 stability (67). Nrdp1-mediated ubiquitination and degradation of ErbB3 is ligand-independent (67,68), and overexpression of Nrdp1 in cultured breast cancer cells reduces ErbB3 expression while Nrdp1 shRNA or dominant negative Nrdp1 expression increases receptor levels (33). Importantly, Nrdp1 overexpression and knockdown lead to inhibition and potentiation, respectively, of neuregulin-induced proliferation and motility, further underscoring the role of Nrdp1 in neuregulin-mediated signaling (33). Our studies of Nrdp1 further revealed that it is an intrinsically unstable protein, a property that is likely due to Nrdp1 autoubiquitination (69). In Nrdp1 pull-down experiments we identified USP8 as an Nrdp1-interacting protein,

and found that this ubiquitin isopeptidase acts to stabilize Nrdp1 through its de-ubiquitinating activity (69). Remarkably, Nrdp1 is significantly stabilized in response to neuregulin stimulation, leading to its accumulation to levels compatible with ErbB3 degradation. Mechanistically we have observed that this is due to neuregulin-induced and PI3-K/Akt-dependent phosphorylation of USP8, leading to increased stability of USP8, which in turn promotes the stabilization of Nrdp1, leading to ErbB3 degradation (70). In this scheme, neuregulin-induced stabilization of Nrdp1 represents a novel negative feedback loop with the neuregulin-mediated accumulation of Nrdp1 ultimately leading to degradation of ErbB3 through ubiquitin-mediated degradation.

Importantly, in the MMTV-ErbB2 mouse model of breast cancer, we have observed that Nrdp1 protein levels are significantly suppressed in ErbB2-induced tumors (33), consistent with the dramatic overexpression of ErbB3 in these tumors (31-34). Furthermore, 57% of all patient primary breast tumors that we have analyzed show reduced Nrdp1 protein levels relative to patient-matched normal tissue, and 70% of ErbB3 overexpressing breast tumors show suppressed Nrdp1 (33). The strong correlation between ErbB3 overexpression and Nrdp1 suppression in these tumors suggests that Nrdp1 may play a critical role in suppressing steady-state levels of ErbB3 protein in normal tissue, and that Nrdp1 loss in tumors could contribute to ErbB3 accumulation.

Suppressors of receptor activity

A second class of ErbB negative regulatory proteins interacts directly with the receptors to modulate receptor activation in response to ligand binding. Several ErbB splice variants encoding extracellular domain fragments have this activity. Herstatin is a secreted protein of approximately 68 kDa that encodes the first 340 amino acids of the ErbB2 extracellular domain, followed by a novel C-terminus derived from exon 8 of the ErbB2 gene (71). This splice variant binds to the full-length ErbB2 receptor with high affinity and blocks ErbB2 dimerization with itself and other ErbBs, thus inhibiting their activation (72). It has also been observed that Herstatin overexpression leads to retention of full-length ErbB2 in the endoplasmic reticulum, suggesting that Herstatin may also suppress ErbB2 function through sequestration (73). Herstatin blocks both EGF- (74) and NRG- (75) induced cell proliferation, and its expression is suppressed in ErbB2-overexpressing tumors, suggesting that decreased Herstatin levels may give ErbB2-overexpressing tumors a selective advantage (71). Conversely, transfection of Herstatin into glioblastoma cells leads to growth inhibition both *in vitro* and *in vivo* (76).

A similar EGFR variant named ERRP (for EGFR-related peptide) has been described (77) and encodes a 55 kDa protein that shares a high degree of homology with the EGFR extracellular domain. Similar to Herstatin, ERRP is able to inhibit the proliferation of ErbB overexpressing cancer cells (78). However, unlike Herstatin, which is produced by alternative splicing of the ErbB2 transcript, ERRP was originally proposed to be a unique gene product (77). The existence of an endogenous ERRP gene has recently been challenged however (78), and it remains to be determined if ERRP is a true endogenous regulator of the ErbB family.

A splice variant of the ErbB3 receptor has also been described (80). P85-s-ErbB3 arises from alternative splicing of the ErbB3 gene to produce an 85 kDa secreted protein capable of binding and sequestering neuregulin. Functionally, this leads to inhibition of NRG-induced activation of Erbb2-4, reduced downstream signaling through MAPK and Akt, and potent inhibition of NRG-stimulated growth. The potential for exploiting these endogenous inhibitors for therapeutic use seems very promising.

Receptor-associated late transducer (RALT/gene 33/Mig6) is a negative regulator of EGFR and ErbB2 that was originally identified in a yeast two-hybrid screen for proteins interacting with the ErbB2 kinase domain (81). Fiorentino et al. reported that RALT binds directly to

activated ErbB2, and that RALT expression is induced by both serum and neuregulin. Importantly, they also observed that RALT overexpression inhibited ErbB2-driven cell proliferation and transformation, and inhibited Erk activation downstream of ErbB2 but not serum stimulation. These observations suggested that RALT is a negative feedback regulator that controls signaling output through the ErbB2 receptor. More recent studies have extended the effects of RALT to all four members of the ErbB family, making RALT a pan-ErbB inhibitor (82). Structural analysis of the RALT-EGFR interaction revealed that RALT binding to the EGFR kinase domain leads to a dual mechanism of receptor inhibition involving RALT-mediated disruption of asymmetric dimer formation and kinase activity (83). Targeted overexpression of RALT in mouse skin leads to the same “waved” phenotype observed in the skin of mice with targeted EGFR disruption (84), whereas genetic knockout of RALT leads to EGFR hyperactivation and increased sensitivity of these mice to mutagen-induced tumors (85). Suppression of RALT expression and RALT missense and nonsense mutations have all been detected in breast cancer cell lines (86) suggesting that loss of RALT contributes to oncogenic transformation.

Leucine rich repeat proteins target ErbBs for degradation

Two different leucine-rich repeat (LRR) domain-containing proteins negatively regulate ErbB signaling. LRIG-1, the presumptive human ortholog of the fly Kekkon-1 discussed earlier, and decorin both interact directly with ErbB extracellular domains to suppress ligand-dependent receptor activation and reduce cellular receptor levels. Decorin is a secreted leucine-rich proteoglycan that is expressed at high levels in quiescent and post-confluent cells but is virtually absent in most transformed cells (87), suggesting a role in the negative regulation of cell proliferation. Decorin binds to EGFR in a region that is distinct from, but overlaps with, the EGF binding region of the receptor (87). Overexpression of decorin antagonizes the formation of ligand-dependent and independent dimers, and leads to ligand-independent receptor degradation through caveolar-mediated endocytic trafficking (88). Significantly, in an orthotopic mammary carcinoma model, decorin protein core decreased the growth of primary tumors by 70%, and blocked metastases from the primary tumor (89). Moreover, decorin expression in this model led to reduced ErbB2 levels, suggesting that ectopically expressed decorin may reduce tumor cell growth and metastases through suppression of ErbBs.

The functionally related protein LRIG-1 differs from decorin in that it is anchored to the plasma membrane through its single-pass transmembrane region. Otherwise LRIG-1 has an extracellular region consisting of 15 LRRs, three immunoglobulin (Ig) domains, and a short cytoplasmic tail lacking any distinguishing features. LRIG-1 is expressed in most epithelial tissues, as well as heart, endothelium, and smooth and striated muscle (90), and is highly expressed in secretory epithelia, including breast and prostate (91). We have observed that LRIG-1 expression is significantly lower in breast tumors compared to matched normal breast tissue, and that LRIG-1 expression correlates inversely with both tumor grade and ErbB2 expression level (31). Moreover, 3p14.3, the LRIG1 chromosomal locus, is frequently deleted in many tumor types, and LRIG1 expression is suppressed in tumor cell lines derived from lung, prostate and colon in comparison to normal tissue (92). LRIG-1 function has also been studied in mouse genetic models. LRIG-1 knockout mice are viable, but have a psoriasis-like phenotype due to epithelial hyperplasia (93). Interestingly, transgenic mice overexpressing ErbB ligands also display this psoriatic phenotype (94,95), suggesting that LRIG1 knockdown leads to hyperactivation of the ErbB receptors, and consistent with a role for LRIG1 as a negative regulator of ErbB signaling.

In fact, LRIG-1 interacts with all four of the ErbB family members. The LRRs and Ig domains of the LRIG-1 ectodomain are each sufficient to mediate binding to ErbBs (96), and this interaction increases the ligand-dependent and independent ubiquitination of the receptors,

leading to increased receptor degradation (96,97). Ligand-dependent increases in ErbB ubiquitination by LRIG-1 have been reported to be due to increased recruitment of the E3-ligase Cbl to activated receptors (96). However, LRIG-1 also increases the ligand-independent ubiquitination of ErbBs in a Cbl-independent manner (96,97). Despite our incomplete understanding of how LRIG-1 suppresses ErbB receptor levels, it is clear that LRIG-1 is able to significantly reduce the steady-state level of ErbB receptors, a property that could prove useful in the treatment of ErbB-overexpressing cancers. In fact a recent article reported that the LRRs of the LRIG-1 ectodomain were sufficient to mediate growth inhibition of ErbB-overexpressing cancer cell lines (98).

Perspectives

ErbB2 overexpression is observed in 25-30% of breast tumors, and cooperates with EGFR and ErbB3 to drive oncogenic transformation. Notably, ErbB2 and ErbB3 act as an oncogenic unit to synergistically contribute to the proliferation breast cancer cells and the malignancy of breast tumors. In contrast with the widely accepted notion that ErbB2 protein overexpression arises from erbB2 gene amplification, data from a transgenic mouse model suggest that amplification of the *erbB2* gene may not be sufficient to give rise to ErbB2 protein overexpression. Studies with matched normal and tumor tissue indicate that tumors markedly overexpress ErbB2 and ErbB3 proteins despite containing similar ErbB message levels as normal tissue. These observations suggest that very potent post-transcriptional mechanisms control ErbB receptor levels in normal tissue, and these mechanisms are lost as the mammary gland becomes transformed. We propose that the suppression of negative regulator proteins by tumors is a common mechanism contributing to the ErbB overexpression and aberrant activation commonly observed in breast and other tumors. For example, we have observed that normal expression of the ErbB3-directed E3 ubiquitin ligase Nrdp1 is lost in mouse mammary tumors and patient primary breast tumors. We suspect that in a similar manner ubiquitin ligases controlling ErbB2 expression may also be suppressed in breast tumors.

Given the relatively modest therapeutic benefits observed so far with TKIs and antibodies directed against ErbB family members, it seems that we would be well served by learning how normal cells suppress signaling through these receptors to thwart oncogenic processes. Research over the past decade has identified some of the key proteins that attenuate signaling through the ErbB receptors. These negative regulators can act in a reversible manner to sequester growth factor or block receptor dimerization, or they can act irreversibly by ubiquitin-mediated receptor degradation (see Figure 1). While the clinical utility of ErbB negative regulators has not yet been explored, it seems likely that these proteins or their derivatives could be rather easily exploited for therapeutic use. Indeed, the soluble ErbB negative regulators Decorin, Herstatin, and p-85-sErbB3, as well as a soluble recombinant version of LRIG1, all act on the extracellular domain of the ErbBs and inhibit tumor cell growth. Because ubiquitin ligases act on the receptor's cytoplasmic region, therapeutic delivery of these ligases, or augmentation of the endogenous proteins, is more challenging but could prove extremely useful in lowering receptor levels in ErbB over-expressing cells. While characterization of ErbB negative regulators is still in its infancy, current research suggests the potential usefulness of these proteins in a clinical setting. It will be exciting to see in the years to come if this potential can be harnessed.

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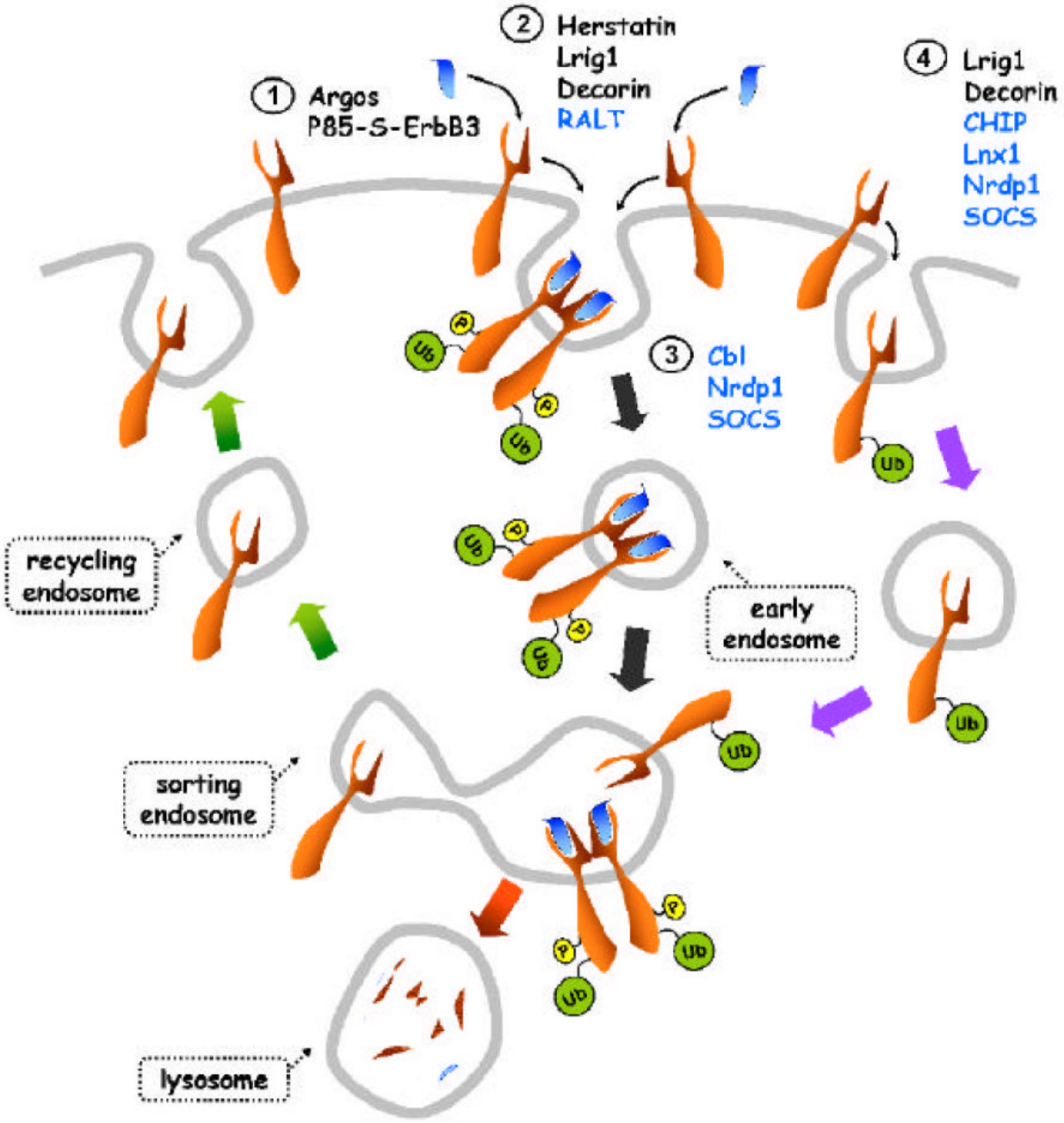

Figure 1.

Negative regulators suppress ErbB activation and alter trafficking to attenuate receptor activity. Depicted are ErbB receptors (orange) undergoing ligand-dependent (black arrows) and -independent (purple arrows) trafficking to the sorting endosome from the plasma membrane. At the sorting endosome receptors are trafficked to the lysosome (red arrow) if ubiquitinated, or returned to the cell surface via recycling endosomes (green arrows) if not. The negative regulators characterized thus far act at four general steps in the ErbB life cycle. 1) Argos and p85-s-ErbB3 both sequester activating ligand away from receptors, consequently reducing receptor activation, while 2) Herstatin, Lrig1, Decorin and RALT all directly bind to receptors to suppress their stimulation. 3) Ligand-dependent internalization of receptors into early

endosomes leads to the direct recruitment of Cbl to phosphorylated EGFR, followed by Cbl-mediated receptor ubiquitination, while Nrdp1 and SOCS4/5 protein levels are increased in response to ligand binding to mediate the ubiquitination of ErbB3/4 and ErbB1/2/4 respectively. 4) Lrig1, Decorin, Chip, Lnx1, Nrdp1, and SOCS4/5 mediate the ligand-independent degradation of receptors (see text for receptor specificity), with Chip, Lnx1, Nrdp1, and SOCS directly mediating receptor ubiquitination. Negative regulator proteins listed in black act extracellularly, while those listed in blue act cytosolically. Overall, reversible negative regulators such as p-85-s-ErbB3 and Herstatin act early in the signaling pathway to control receptor activation, while ubiquitin ligases such as Cbl and Nrdp1 direct receptors away from the default recycling pathway to the lysosome for the irreversible process of ubiquitin-mediated degradation.