EGF–ERBB signalling: towards the systems level

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Abstract | Signalling through the ERBB/HER receptors is intricately involved in human cancer and already serves as a target for several cancer drugs. Because of its inherent complexity, it is useful to envision ERBB signalling as a bow-tie-configured, evolvable network, which shares modularity, redundancy and control circuits with robust biological and engineered systems. Because network fragility is an inevitable trade-off of robustness, systems-level understanding is expected to generate therapeutic opportunities to intercept aberrant network activation.

Receptor tyrosine kinase
Transmembrane receptor with an intrinsic ability to transfer phosphate groups to tyrosine residues contained in cellular substrates.

Heterodimeric
In contrast to a homodimer, in which two identical receptors bind to form a dimer, heterodimers are formed by two different receptors.

Mitogen activated protein kinase
(MAPK). Parallel kinase cascades lead to the activation of the four serine/threonine MAPKs (ERK, JNK, p38 and ERK5/BMK). Activation of these kinases is critical to cellular signal transduction, driving diverse cell fates.

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The ERBB family of proteins (originally named because of their homology to the erythroblastoma viral gene product, *v-erbB*) comprises four receptors (ERBB1–4, also known as HER1–4) and 13 polypeptide extracellular ligands, which contain a conserved epidermal growth factor (EGF) domain. Although the ERBB family is regarded as the prototypical group of the receptor tyrosine kinase (RTK) family, an important defining feature of the ERBB network is that two members of the family, ERBB2 (also known as HER2/neu) and ERBB3, are non-autonomous. ERBB2 lacks the capacity to interact with a growth-factor ligand[1], whereas the kinase activity of ERBB3 is defective[2]. Despite this lack of autonomy, both ERBB2 and ERBB3 form heterodimeric complexes with other ERBB receptors that are capable of generating potent cellular signals.

The ERBB-receptor network is one of the most extensively studied areas of signal transduction (Supplementary information S1 (figure)), and the one which best exemplifies the pathogenic power of aberrations in biological information transfer. Since our last extensive review[3], the information available on the network has been dramatically enriched, to a point that now demands description in terms of an integrative systems-biology approach. This development and the emerging realization of the tight links of the network to human diseases, will be the highlights of our review.

Following an introduction to ERBB receptors and their physiological functions, we will describe the recent advances in our understanding of the structural basis of ligand-induced receptor activation. On this foundation, we will describe ERBB signalling from the perspective of a systems biologist, with some focus on the compartmentalization of signalling. Many lessons on the organization of the network have been learnt from its evolutionary history (BOX 1); the vertebrate ERBB family evolved from a single ligand–receptor pair in worms through the duplication of ligands and receptors, while the robustness of the developing network was enhanced by using canonical features of complex biological and engineering systems[4,5] (BOX 2).

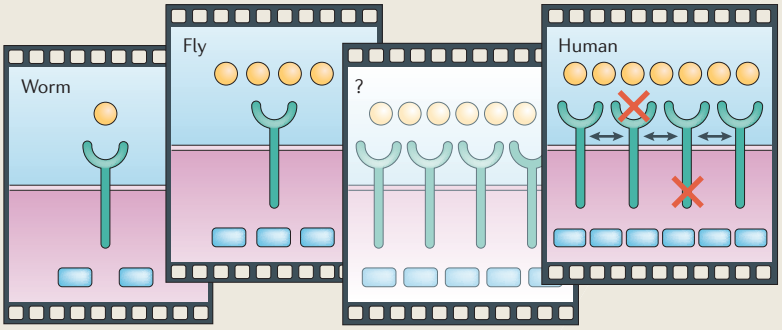
As mathematical modelling constitutes the heart of systems biology, the status of ERBB-network modelling will be described. The fragility of a network is generally considered a trade-off of its increasing robustness. So, our review will culminate with a description of the fragility of the ERBB network, and the opportunities it generates for cancer therapy.

The signalling context of ERBB receptors

The basic functional unit in ERBB signalling is a receptor dimer, to which each partner contributes unique features. Animal and cellular models have uncovered critical features of ERBB signalling such as modularity, redundancy and the role of combinatorial interactions in signal diversification.

ERBB1 and epithelial development. The autonomous receptor ERBB1 binds to multiple ligands and forms homodimers, as well as three functional heterodimers. Several tyrosine-based motifs recruit a number of signal transducers to the phosphorylated form of ERBB1 (REF. 6) such as the adaptor proteins growth-factor-receptor bound-2 (GRB2) and Src-homology-2-containing (Shc), which are responsible for the recruitment of Ras and activation of the mitogen-activated protein kinase (MAPK) cascades. Another direct substrate of ERBB1 is the signal transducer and activator of transcription-5 (STAT5).

Box 1 | **On the evolution of a robust signalling network**



Nematodes and insects express a single ERBB orthologue. A single epidermal-growth factor (EGF)-like ligand, LIN-3, is found in worms, whereas four stimulatory ligands and one inhibitory ligand, Argos, bind to the fly orthologue of EGF receptor (DER; see figure). In worms, EGFR controls vulva development, hermaphrodite sterility, differentiation of the male tail and posterior-ectoderm development[127]. Likewise, DER controls the development of several organs in different stages of embryogenesis[128], and the mammalian ERBB proteins control organogenesis in multiple epithelial tissues. To address the evolution of a complex mammalian network from a simple invertebrate cascade, we propose below a step-wise evolutionary path that is aimed at increasing network robustness.

Gene fusion

The primordial receptor tyrosine kinase (RTK) might have been formed through a gene-fusion event that linked a cytoplasmic tyrosine kinase to a cell-surface receptor.

Gene duplication

Comparison of the amino-acid sequences of the vertebrate ERBB proteins indicates that a gene-duplication event generated two ancestral receptors, the ERBB1/ERBB2 precursor and the ERBB3/ERBB4 precursor[129]. Subsequent gene duplications of these precursors generated the four ERBBs. In parallel to the evolution of the receptors, the vertebrate ligands segregated into ERBB1 ligands and ERBB3/ERBB4 ligands[129].

Partial inactivation of ERBB2 and ERBB3

We postulate that two independent genetic events transformed the linear configuration of four ligand–receptor pathways into a complex network. Accumulated mutations prevented growth-factor binding to ERBB2 (REF. 33), and other mutations inactivated the kinase domain of ERBB3 (REF. 2), denying the autonomy of two of the four receptors. Presumably, the transformation of a collection of linear pathways into a richly interconnected network increased robustness and conferred selective gains[130].

Specialization

Although the modern form of the ERBB network maintains essential features for robustness — such as modularity and redundancy — specialization at the receptor level conferred unique functional features. For example, through redundant connections to Ras and c-Cbl, ERBB1 evolved into a transient stimulator of the Ras–mitogen-activated-protein-kinase pathway, whereas ERBB3 evolved into a strong, but non-autonomous, linker to the phosphatidylinositol-3-kinase–AKT/protein kinase B pathway.

The C terminus of ERBB1 contains a recognition site for the ubiquitin ligase Cbl[7], whereas no site is found that can directly recruit the lipid kinase phosphatidylinositol 3-kinase (PI3K). Consistent with the specificity of its docking sites, ERBB1 cannot directly activate the PI3K–AKT/protein kinase B (PKB) pathway, but it couples to the Ras–MAPK pathway, as well as to the Ras–PI3K–AKT/PKB pathway. ERBB1 signalling is negatively regulated through ubiquitylation by Cbl.

Knockout of the *ErbB1* gene showed that ERBB1 has a pivotal role during epithelial-cell development in several organs. The phenotypes of ERBB1-knockout mice differ depending on their genetic background; mice die at either mid-gestation (SV129 strain), birth (C57BL/6 strain), or postnatal day 20 (CD1, C3H and MF1 strains)[8–10]. Along with brain defects, most of the observed abnormalities involve aberrant proliferation, migration or differentiation of specific epithelial cells (for example, skin, lung, intestine and placenta). Mutant mice that survive after birth develop a strain-independent progressive neurodegeneration[11].

In contrast to the phenotype of the *ErbB1* mutant mice, knockout of specific ERBB1 ligands indicated that there is a significant functional redundancy between the ligands. No significant phenotype was observed in the knockouts of the ERBB1 ligands EGF (the prototypic growth factor, found in human body fluids) and amphiregulin (an important growth factor for keratinocytes)[12], whereas transforming growth factor-α (TGFα)-deficient mice (TGFα is an autocrine growth factor that is found in human malignancies) had only eye abnormalities and derangement of hair follicles[13].

Abnormalities in the small intestine were only observed in the EGF, amphiregulin and TGFα triple knockout[14]. The role of the TGFα–ERBB1 pair in the skin and hair follicles is also shown by the naturally occurring TGFα mutant mice, waved-1 and waved-2 (REFS 15,16), in which a point mutation within the kinase domain of ERBB1 partially inactivates the kinase.

ERBB2: a non-autonomous amplifier. As will be discussed further, we regard ERBB2 as a non-autonomous amplifier of the network, rather than an additional growth-factor receptor (FIG. 1). Although ERBB2 does not bind to EGF-like ligands, it functions as the preferred heterodimeric partner of the other three ERBB members[17]. ERBB2 binds to a much larger subset of phosphotyrosine-binding proteins than the other ligand-binding receptors of the family[18]. Furthermore, ERBB2-containing heterodimers are characterized by a higher affinity and broader specificity for various ligands than the other heterodimeric receptor complexes, owing to slow rates of growth-factor dissociation. Also, ERBB2-containing heterodimers undergo slow endocytosis, and they more frequently recycle back to the cell surface[19–21]. These features translate to potent mitogenic signals[22] owing to the simultaneous and prolonged recruitment of multiple signalling pathways.

ERBB3 activates PI3K. This kinase-defective, non-autonomous receptor binds to four ligands, and forms three functional heterodimers. On heterodimerization, the cytoplasmic domain of ERBB3 undergoes tyrosine phosphorylation and can recruit PI3K to six distinct sites and Shc to one site, although there is no site for GRB2. This segregation enables ERBB3 to evade ligand-induced degradation[19,23], while strongly activating PI3K, especially when it is bound to its preferred heterodimeric partner, ERBB2 (REF. 24).

ERBB4: sharing features with ERBB1. This differentiation-associated[25] autonomous receptor shares recognition and signalling features with ERBB1. They both bind to a large and distinct group of ligands; they both bind to betacellulin and the heparin-binding ligand, HB-EGF, as

Phosphatidylinositol 3-kinase
(PI3K). A lipid kinase, that is the initiating enzyme in a pathway that promotes cell proliferation and survival. A central downstream mediator of the pathway is the serine/threonine kinase AKT/PKB. PI3K phosphorylates the 3′ position of the inositol ring of phosphatidylinositol-4,5-bisphosphate.

Box 2 | **Features that define the robustness of living systems**

The robustness of a biological system is defined as an inherent system property, which enables normal performance despite external and internal perturbations[4]. The establishment of robustness inevitably involves increased complexity and enhances the organism's capacity to generate heritable phenotypic variation (evolvability)[45]. Several attributes are shared by robust systems of eukaryotic and prokaryotic organisms, as well as by engineering systems, and they might collectively function as the framework that underlies robustness[5].

Network architecture
A layered structure that interfaces with diverse sources of input, and fans out into several outputs through a conserved core, characterizes many robust systems (known as a bow-tie or hour-glass structure). Unlike the input and output layers, the core is conserved, and consists of interconnected units that modify signals (or molecules, in the case of metabolic networks) in a highly reproducible manner.

Modularity
Robust systems are configured hierarchically into quasi-autonomous sub-systems (modules). This organization enables a system to locally contain inflicted damage, as well as to promote evolvability.

System controls
Integral and often intertwined negative- and positive-feedback circuits help to maintain appropriate quantitative and dynamic relationships between inputs and outputs within defined limits. This mechanism dampens noise and resists perturbations.

Redundancy
Functional degeneracy of individual components or whole modules, which are non-identical, offers alternative ways to generate an output in the face of severe perturbations.

Buffering
This protective mechanism enables damaged components to maintain, to some extent, their proper functioning. For example, chaperones such as heat-shock protein-90 (HSP90) refold mutated or otherwise perturbed proteins, thereby enabling normal activity while the system accumulates mutations but suppresses their phenotypes[67].

well as to two low-affinity ligands, epiregulin and epigen. Alternative mRNA splicing generates several isoforms of ERBB4, including a juxtamembrane variant (JM-a), which has a 23-amino-acid insertion that enables cleavage by a membrane proteinase resulting in ectodomain shedding[26].

Like ERBB1, ERBB4 recruits GRB2, Shc and STAT5, whereas one isoform of ERBB4 (CYT-1) can activate PI3K[27]. Although ERBB4 might not be able to directly recruit Cbl, and therefore downregulation of this receptor is slow, a proteolytic cleavage product of the cytoplasmic domain of ERBB4 translocates to the nucleus and might possess transcriptional activity[28] (see below).

The physiology of ERBB2, ERBB3 and ERBB4. The phenotypes of ERBB2-, ERBB3-, ERBB4- and neuregulin-1 (NRG1)-deficient mice clearly show that receptor heterodimers function as essential signalling units. NRG1-deficient mice die at around day 10.5 post-fertilization because of aberrant cardiac development[29]. During heart development, NRG1 is expressed in the endocardium, an endothelial ventricular lining, whereas ERBB2 and ERBB4 are expressed in the myocardium, the underlying muscular portion of the atrium and the ventricle. ERBB2- (REF. 30) or ERBB4-mutant (REF. 31) mice share the same embryonic-lethal phenotype as the NRG1-defective mice; trabeculae fail to develop, and the mutant heart is characterized by an irregular beat.

The developmental mechanism most likely involves an inductive signal from NRG1 to the ERBB2/ERBB4-expressing myocardium that initiates ventricular differentiation. Although the process of trabeculation does not require ERBB3, normal cardiac development is dependent on this receptor. In *ErbB3* knockout mice, trabeculation occurs in a delayed, but otherwise normal, fashion. However, their atrioventricular valves are dilated and thinned, and this defect leads to death by embryonic day (E)13.5, thereby indicating that NRG1 and ERBB2 are reused at this developmental stage, now in the context of ERBB3 (REF. 32). In conclusion, it is notable that, although ERBB2-deficient animals share various phenotypic features with mice that lack expression of other ERBB proteins, or their EGF-like ligands, no phenotype unique to ERBB2 has emerged. This observation is consistent with the hypothesis that attributes to ERBB2 a non-autonomous function as a ligand-less, positive regulator of ERBB signalling.

The structures of ligand–ERBB complexes

The last five years have witnessed an avalanche of three-dimensional structures that have helped to unravel a remarkably consistent picture of the first steps of EGF–ERBB signalling[33] (FIG. 2).

Insights from primary structures. Both EGF-family ligands and ERBB receptors are produced as glycosylated transmembrane proteins. At the membrane, the ligands are cleaved by cell-surface proteases to release mature growth factors. The mature ligands contain an EGF-like consensus sequence that consists of six cysteine residues. The receptors are composed of a large extracellular ligand-binding domain, which has four subdomains (I–IV), followed by a transmembrane domain, a small intracellular juxtamembrane domain preceding the kinase domain, and a C-terminal tail, on which the docking sites for phosphotyrosine-binding effector molecules are found. Of the four subdomains in the extracellular region of the ERBB receptors, subdomains I and III are leucine-rich repeats that function in ligand binding (also called L1 and L2), whereas subdomains II and IV are laminin-like, cysteine-rich domains (also called CR1 and CR2; see FIG. 2).

Structures of ligand–receptor complexes. Cumulative evidence has indicated that the ERBB receptors exist in a pre-dimerized state[34]. Ligand binding to this pre-dimerized state forms a 2:2 ligand to receptor configuration[35,36] and induces a rearrangement of each receptor subunit, owing to a rotation of the transmembrane domains[37]. A further dimer–tetramer transition might also take place during receptor activation[38].

The structures of ligand-occupied ERBB1 reveal a back-to-back dimer, in which a dimerization arm from domain II of one partner binds to a docking site at the base of domain II within the second partner[39,40] (FIG. 2). As the ligand-binding surface is on the exterior of the dimer, the mode of dimerization is entirely receptor-mediated. The transition to receptor dimers relieves inhibitory interactions that maintain the inactive form

Autocrine
Secretion of a ligand that stimulates the secreting cell itself.

Waved-1 and waved-2 mice
Naturally occurring mutant mice that have wavy hair. In waved-1 mice, TGFα levels are reduced, whereas waved-2 mice have a partial inactivation of the kinase domain of ERBB1, owing to a point mutation.

Endocytosis
The process of taking in materials from outside a cell in vesicles that arise by the inward folding ('invagination') of the plasma membrane.

Trabeculae
Finger-like extensions of the ventricular myocardium.

of the receptor. This transition also enables ligand binding and levers the dimerization loop, thereby stabilizing the dimer.

ERBB2: ready to tango. ERBB2 does not bind to any ligands[1], but it is the favoured heterodimerization partner of the other ERBB receptors[17]. These characteristics of ERBB2 have been explained by its extracellular domain, which constitutively boasts a structure that is reminiscent of the ligand-bound form of ERBB1 (REF. 41). A strong interaction between domains I and III leads to a constitutively extended dimerization arm and renders ERBB2 incapable of binding to a ligand (FIG. 2). As such, ERBB2 is constantly primed for interactions with ligand-bound receptors of the family. So, the promiscuous behaviour of ERBB2 and its inability to bind to EGF-like ligands, are inherent in its structure.

Circuitry features ensure network robustness

From a systems-biology view, several functional features of the ERBB network contribute to the robustness of signalling, sharing traits with complex biological and engineering systems[4,5] (BOX 2). Furthermore, the systems-biology view of the ERBB network is helpful in light of emerging high-throughput technologies that simultaneously record multiple signalling events. For example, simultaneous analysis of all 89 potential tyrosine-phosphorylation sites of the four ERBB proteins enabled mapping of the phosphotyrosine interactome of the network[6]. Furthermore, analysis of a subset of 61 established phosphorylation sites uncovered many new potential ERBB partners[18]. Likewise, two different quantitative proteomic approaches that used phosphotyrosine antibodies each identified the phosphorylation kinetics of ~80 partners of ERBB1, including many novel substrates[42,43].

In this section, we highlight the landmarks of robust systems to describe the main functional characteristics of the ERBB network. As the systems-biology approach has been influenced by the fields of physics and engineering, the definition of the bow-tie architecture, as well as the other features we will describe, are oversimplifications, making the network more easily accessible to interpretation.

Bow-tie architecture. A bow-tie structure of the ERBB network is presented in FIG. 1. The input of multiple growth factors that function through eight potential receptor hetero- or homodimers activates common signalling cascades collectively defined as a giant strong component network, or core process[44]. This core process results in the specific activation of transcription factors that lead to the selected cell fate. The core process encapsulates a dense array of strongly coupled subnetworks (for example, the phosphoinositide network) that use a remarkably small set of molecular switches and cascades (Supplementary information S2 (figure)).

The input layer. The uppermost layer of the ERBB network consists of 13 growth factors that directly bind to three receptors, ERBB1, ERBB3 and ERBB4. As for other robust systems, the specificity of ligand–receptor interactions displays remarkable redundancy. For example, betacellulin binds to and activates both ERBB1 and ERBB4, whereas epiregulin binds to ERBB1, ERBB3 and ERBB4. The multiplicity of ERBB ligands feeds into the combinatorial nature of the ERBB network, in which homo- or heterodimeric receptors can be formed, thereby establishing a high level of complexity. Biochemical evidence attributes to each ligand-driven receptor dimer distinct functional properties in terms of binding affinity, endocytic routing and effector activation.

Modularity. It is increasingly accepted that complex networks consist of distinct semi-autonomous functional units that show strong internal connections, although they maintain weaker connections with the environment. The modular structure contributes both to the robustness of the system, and to its capacity to generate heritable phenotypic variation (evolvability)[45]. The modularity of the ERBB network is exemplified by the use of different receptor heterodimers during development (as described above).

Redundancy. To secure output generation in the face of recurring perturbations, robust systems often multiply and diversify individual components, degenerate connectivities and duplicate whole modules of protein networks, thereby increasing functional plasticity[46].

The ERBB network displays redundancy at each layer of the bow-tie framework. At the input layers, ligands with overlapping specificities, as well as dimeric receptor complexes, essentially share downstream pathways, therefore displaying degeneracy. Another layer of redundancy is seen at the richly connected core of the network. Enzymes, but also scaffold and adaptor proteins, are present in multiple isoforms, and alternate connectivities co-exist. For example, in the pathway that leads to activation of Raf1 by son of sevenless (SOS), ERBB1 can recruit SOS through either GRB2 or Shc, whereas GRB2 can associate with the receptor either directly[47,48] or through Shc. Likewise, Cbl is recruited to ERBB1 either directly (Tyr1045), or indirectly, through GRB2 (REF. 49). Although these examples show redundancy, the functional outcome of GRB2-mediated recruitment of Cbl to ERBB1 might differ from direct binding of Cbl to the receptor[50], which underscores the non-identical functions of potentially redundant network motifs.

System controls embedded in the network

To dynamically control the amplitude, kinetics and frequency of output signals, the ERBB network evolved positive- and negative-feedback circuits. As this is the main mechanism ensuring robustness, we will devote this section to its description.

Positive-feedback loops. Similar to autocatalysis in enzymatic networks, positive-feedback loops enhance the amplitude and prolong the active state of signalling pathways to convey robustness in the face of variable inputs[51]. In the case of ERBB, the output of the main switch, namely binding of the ligand to the primary receptor, is tuned by

Giant strong component network (or core process)
The largest fully connected part of a network, which functions as the core of the network, and is normally the most complicated part of the network.

Evolvability
The capacity of an organism to generate heritable phenotypic variance.

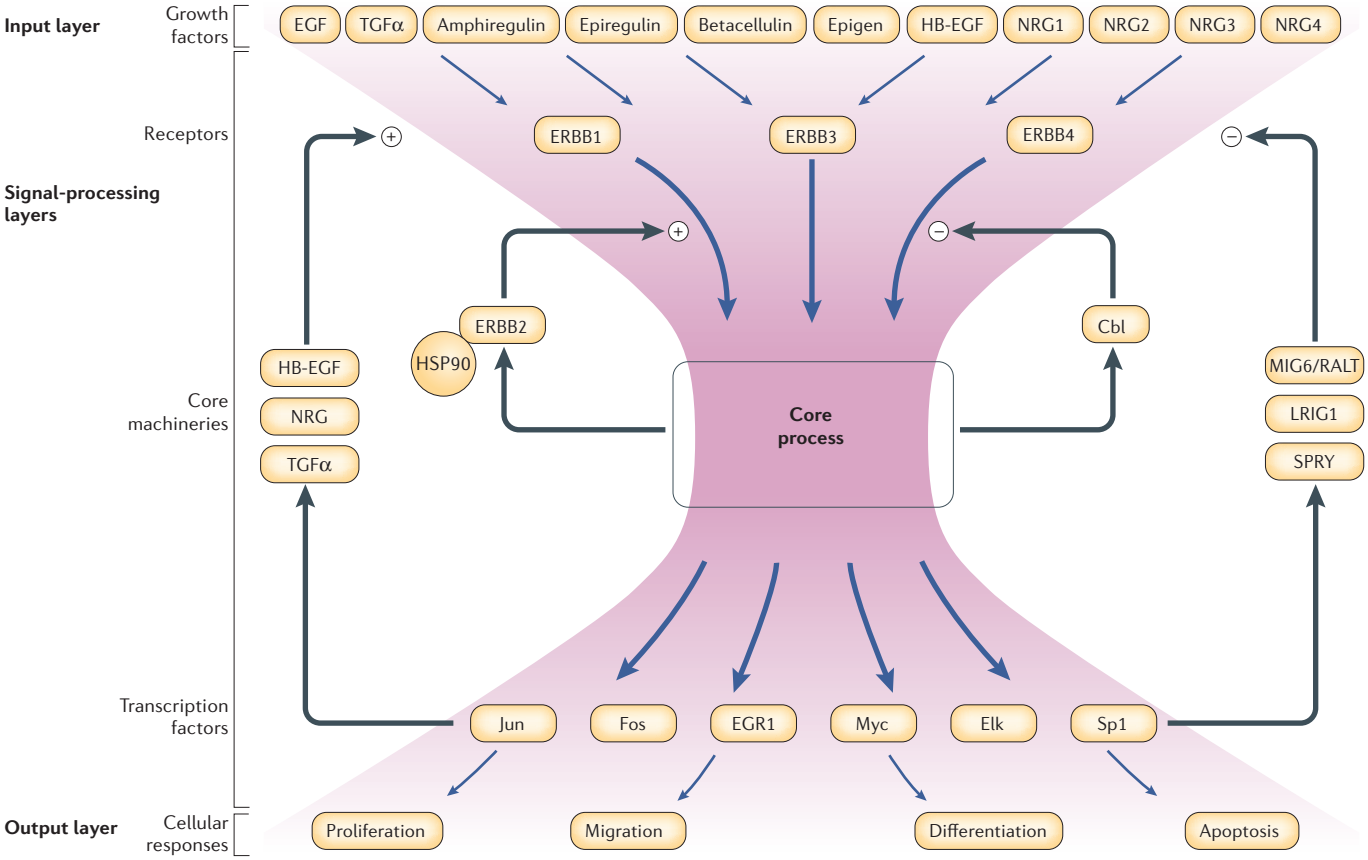


Figure 1 | **A systems perspective of the ERBB network.** A reductionist view of the bow-tie-architectured signalling network is represented. The heart of the system is a core process, a collection of biochemical interactions, which are tightly coupled to each other and interface with two sets of components: three input modules, each comprising an ERBB receptor tyrosine kinase; and a large group of partly redundant ligand growth factors. The output of the core process is translated to gene expression through multiple transcription factors. Depending on the exact combination of transcription factors and the cellular context, the output of the network regulates cell behaviour. The system maintains two steady states (bistability), for which inter-conversions depend on ligand binding. The fail-safe (robustness) action of the system is conferred by structural modularity and functional redundancy, along with rich and stringent system controls. An important positive regulator is ERBB2, a co-receptor. Heterodimerization between ERBB2 and any of the three ERBB input modules enhances and prolongs the respective output. ERBB2 is chaperoned and catalytically suppressed by heat-shock protein-90 (HSP90). On the other hand, a ubiquitin ligase that is involved in receptor degradation, Cbl, controls an important negative-feedback loop. Several activation-dependent control loops fine-tune bistability. These include transcription of ERBB ligands (positive regulation) and newly synthesized negative regulators such as mitogen-inducible gene-6 (MIG6)/receptor-associated late transducer (RALT), sprouty (SPRY) and leucine-rich repeats and immunoglobulin-like domains-1 (LRIG1). EGF, epidermal growth factor; EGR1, early growth response-1; NRG1/2/3/4, neuregulin-1/2/3/4; TGFα, transforming growth factor-α.

the identity of the secondary receptor. ERBB2 can be considered as an important positive regulator; it functions as the preferred secondary receptor, and ERBB2-containing heterodimers evade negative regulation[19–21].

Another important mechanism of positive feedback is based on autocrine and paracrine loops, in which EGF-like ligands, as well as angiogenic factors, are produced following receptor activation. ERBB-mediated activation of the Ras–MAPK pathway strongly induces the transcription of multiple ERBB ligands, including TGFα and HB-EGF[52]. Similarly, transactivation of ERBB1 by G-protein-coupled receptors occurs through the stimulation of surface proteinases, generating mature, active HB-EGF[53].

Negative-feedback loops. It is becoming increasingly apparent that cellular decisions are tuned by mechanisms of signal attenuation, into which a vast cellular effort is invested. Multiple molecular mechanisms, including post-translational modifications, compartmentalization, catalytic inactivation and steric hindrance, participate in signal attenuation. It is useful to distinguish between general attenuation, which functions at the level of the ligand–receptor complex, and pathway-specific inactivation (for example, inactivation of SOS through phosphorylation by the downstream MAPK). Furthermore, negative regulators either pre-exist, or they are newly synthesized following stimulation of ERBBs by their respective ligands.

Paracrine
Activation of a receptor on an adjacent cell by a secreted ligand.

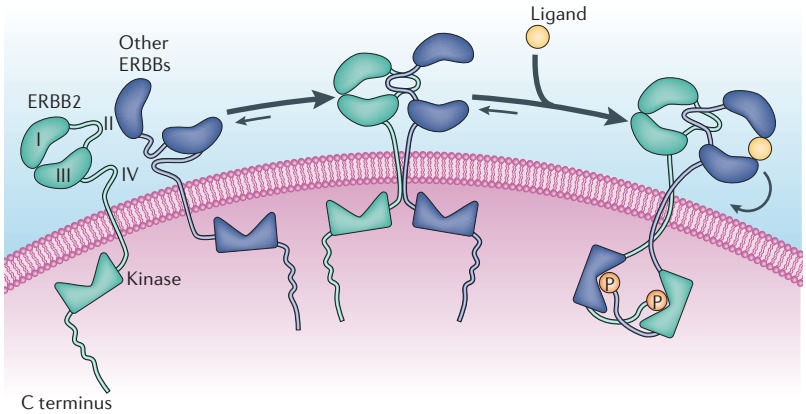


Figure 2 | **Structural basis for ERBB-receptor dimerization and activation.** We depict a steady-state equilibrium between receptor monomers and ERBB2-containing heterodimers, in which dimerization is driven by ligand binding and by the constitutively active conformation of ERBB2. By stabilizing a dimer and forcing a rotation in the vicinity of the membrane, ERBB ligands activate the kinase activity of the receptor. The prototypic ERBB in its monomeric state is autoinhibited through an interaction of domain II with domain IV. This interaction keeps subdomains I and III at a distance that does not allow the simultaneous binding of a ligand to both subdomains, and at the same time sequesters the dimerization loop. In the dimeric, ligand-bound form, domains I and III are brought together, driving a separation of the inhibitory domain II–IV interaction, which promotes the accessibility of the dimerization loop within domain II for interaction with the docking site on the dimerization partner. By contrast, the structure of ERBB2 is consistent with its role as a preferred dimerization partner of the other ERBB receptors: the dimerization loop of ERBB2 is constitutively extended, even in the monomeric state, and a strong interaction of domains I and III closes the binding pocket, which abolishes accessibility to ligands. P, phosphate.

Pre-existing attenuators primarily control receptor dephosphorylation and degradation. Receptor internalization coupled to degradation is considered the most effective, irreversible process that robustly attenuates signalling by targeting surface receptors for degradation in lysosomes (see below, and REF. 54). Another general mechanism of signal attenuation that functions at the receptor level is instigated by tyrosine phosphatases such as density-enhanced phosphatase-1 (DEP1), which dephosphorylates ERBB1 as well as other RTKs[55], and protein tyrosine phosphatase-1B (PTP1B), which dephosphorylates RTKs in endosomes[56].

Unlike pre-existing attenuators, the level of expression of newly synthesized attenuators rises after stimulation, reaching a peak within an hour of the initial stimulation and thereby defining the window of active signalling. Transcriptional up-regulation of this group of attenuators affects multiple processes. For example, EGF treatment induces expression of the suppressor of cytokine signalling-5 (SOCS5) that leads to a marked reduction in the levels of the receptor by promoting ERBB1 degradation, possibly by the 26S proteasome[57].

Two other newly induced proteins (albeit with slower induction kinetics) that participate in ERBB homeostasis through Cbl are the adaptor protein sprouty (SPRY) and leucine-rich repeats and immunoglobulin-like domains-1 (LRIG1), an adhesion molecule that is related to Kekkon of insects. SPRY2 regulates the Ras–MAPK pathway through several regulatory mechanisms, including inhibition at the levels of GRB2 (REF. 58), Ras-GTPase activating protein (Ras-GAP), or the Raf1 kinase[59,60]. The phosphorylated form of SPRY2 binds to and sequesters Cbl[61]. Similarly, up-regulation of LRIG1 is followed by enhanced ubiquitylation and degradation of ERBB1 through a mechanism that involves recruitment of Cbl and simultaneous ubiquitylation of both ERBB1 and LRIG1 (REFS 62,63).

Mitogen-inducible gene-6 (MIG6)/receptor-associated late transducer (RALT) is another newly synthesized negative regulator that functions directly on the receptor[64,65] through a domain that is homologous to both RALT and activated CDC42-associated kinase-1 (ACK1)[66]. This implies a novel mechanism of catalytic attenuation that is shared by the pre-existing (ACK1) and newly synthesized (MIG6/RALT) proteins. A good example of a negative-feedback loop that functions downstream of the receptor and is common to many mitogens, including ERBB ligands, involves the inducible expression of dual-specificity phosphatases of MAPKs, which thereby defines the time frame of MAPK activation.

Buffering. The ability to maintain proper function in the face of significant damage to individual components is a protective mechanism that is characteristic of robust systems[5,67]. Similar to other signalling networks, heat-shock protein-90 (HSP90) is the main chaperone that protects against damage inflicted on the ERBB system[68,69]. By contrast to the other ERBB receptors, which are mostly HSP90-independent, ERBB2 is one of the most prominent kinase targets of HSP90. A ternary complex of HSP90, ERBB2 and a co-chaperone, CDC37, stabilizes ERBB2 at the plasma membrane; inhibition of HSP90 by specific drugs induces marked degradation of ERBB2 by the 26S proteasome[70].

Moreover, the buffering role of HSP90 in modulating the ERBB network extends beyond the regulation of protein stability. Binding of a HSP90–CDC37 complex to the αC–β4 loop[71–73] within the kinase domain of ERBB2 restrains kinase activity and limits the capacity of ERBB2 to recruit ligand-bound receptors, such as ERBB3, into active heterodimers[71]. Therefore, by selecting ERBB2, the master positive regulator of the ERBB network, HSP90 acquired the ability to function as a molecular switch that regulates heterodimer formation and catalytic function, as well as protein stability.

Subcellular compartmentalization

Compartmentalization is a central mechanism that controls output from the ERBB network (FIG. 3). By enabling nuclear translocations of receptors and allowing signal generation at endosomal compartments, the ERBB system gains diversity and robustness.

Endocytosis of ERBB1. The mechanism used for sorting active ERBB1 molecules for lysosomal degradation is a prototype of that used in receptor trafficking. In many cells, both ERBB1 and ERBB2 are found in caveolae. Ligand-induced internalization of ERBB1 might follow the migration of active receptors out of caveolae, and subsequent receptor clustering over clathrin-coated

Heat shock protein-90
(HSP90). A molecular chaperone that buffers the conformation and activity of a distinct subset of cellular molecules that are involved in signal transduction. HSP90 is one of the most abundant cellular proteins.

Caveolae
Cholesterol-rich membrane microdomains that are stabilized by the protein caveolin.

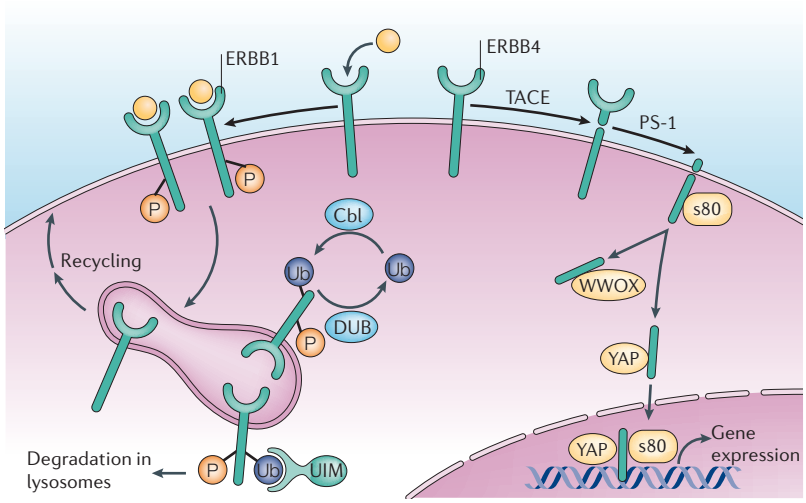


Figure 3 | **Endocytosis and nuclear translocation of ERBB proteins.** Ligand binding to ERBB1 receptors and their subsequent dimerization induces receptor internalization into endosomes, which is followed by recycling back to the cell surface in a kinase-independent manner. In the endosomes, autophosphorylated receptors might recruit the E3 ubiquitin ligase Cbl and undergo ubiquitylation. The appended ubiquitins then recruit adaptors that contain a ubiquitin-interacting motif (UIM) and target internalized receptors to lysosomes for degradation. De-ubiquitylating enzymes (DUBs) might abrogate this process and target ERBB1 molecules to the default recycling pathway. On the other hand, ERBB4 molecules are processed by TACE, a protease that is activated by protein kinase C and that releases a soluble ectodomain of ERBB4. Subsequently, an intramembrane-proteolysis event that is mediated by γ-secretase (PS-1) releases a cytosolic soluble ERBB4 fragment (s80), which functions in the nucleus. s80 functions as a chaperone that facilitates the translocation of the YES-associated protein (YAP) to the nucleus, where the complex regulates gene expression. By contrast, the WW-containing oxidoreductase (WWOX) retains s80 in the cytoplasm. P, phosphate; Ub, ubiquitin.

regions of the plasma membrane. Although, according to several reports, receptor internalization is driven by the intrinsic kinase activity, a recent study concluded that internalization at the plasma membrane is largely a kinase-independent process[74], which is followed by efficient recycling[75,76]. To evade the recycling route, which is characteristic to unoccupied receptors[77], and to commit to the alternative degradatory fate, ERBB1 must recruit ubiquitin ligases, along with a group of monoubiquitin-binding proteins (reviewed in REF. 78).

Cbl is the primary E3 ubiquitin ligase that is recruited to ERBB1 in a kinase-activity-dependent fashion. Binding of Cbl to phosphorylated Tyr1045 of ERBB1 promotes receptor ubiquitylation[7], and recruits several protein partners, including GRB2 and CIN85. GRB2 seems essential for receptor endocytosis[79], and its knockdown indicates an important function in the initial steps of receptor internalization[50]. CIN85 is a scaffold molecule that recruits endophilins, molecules that assist in curving the planar plasma membrane[80,81]. Once sorted to clathrin-coated vesicles, internalized receptors are delivered within 2–5 minutes to a tubular–vesicular network that is located at the cell periphery.

This endosomal trafficking is controlled by a group of GTPases and E3 ubiquitin ligases of the NEDD4 family that mediate the ubiquitylation of several adaptor proteins, which contain a ubiquitin-interacting motif (UIM)[82,83]. These steps might enable the loading of monoubiquitylated receptors onto a ubiquitin-bound multimolecular complex that sorts ERBB1 in early endosomes. Further processing of ERBB1 to luminal vesicles of the pre-lysosomal compartment (which is known as the multivesicular body) is mediated by a complex of the tumour-susceptibility gene-101 (TSG101, a ubiquitin-binding protein) with TSG101-associated ligase (TAL, an E3 ubiquitin ligase)[84,85].

The endocytosis/signalling interface. In general, ligand-induced receptor endocytosis downregulates growth-factor signalling. Nevertheless, accumulating evidence indicates that internalized receptors might couple to effectors in pre-degradative intracellular compartments, and thereby activate signalling pathways distinct from those that are activated at the cell surface[54]. For example, internalized ERBB1 molecules are enzymatically active, hyperphosphorylated and associated with Shc, GRB2 and SOS[86]. Moreover, endosomal ERBB1 signalling is sufficient to activate the main signalling pathways that lead to cell proliferation and survival[87]. The formation and fusion of ERBB1-containing endocytic vesicles are controlled by a family of GTPases, but mainly by RAB5. APPL1 and APPL2, two new interacting partners of RAB5 have been recently identified[88]. In response to EGF, APPL1 translocates to the nucleus, where it interacts with a histone deacetylase to control gene expression.

Translocation of ERBB proteins to the nucleus. Although ERBB4 is inefficiently targeted to the endocytic pathway, the cleavage of this receptor is stimulated either by ligand binding or by activation of protein kinase C[89]. The shedding activity is attributed to a transmembrane member of the ADAM metalloprotease family, ADAM17/TACE[26]. ADAM17/TACE cleaves at the extracellular domain of ERBB4, whereas a γ-secretase activity cleaves within the transmembrane domain. The second cleavage generates a soluble 80 kDa protein (s80), which translocates to the nucleus[28].

Recent studies indicate that s80 is a chaperone that facilitates the nuclear entry of the transcription factors STAT5 (REF. 90) and YES-associated protein-1 (YAP1)[91–93]. Displacement of YAP1 by another WW-domain protein, WWOX, retains s80 in the cytoplasm and presumably inhibits transactivation[93]. These studies are relevant to the frequent presence of ERBB4 in the nucleus of tumour cells and they attribute to ERBB4 a spectrum of activities that are distinct from the plasma-membrane signalling processes.

Although less well characterized, a similar case might apply to translocation of intact, rather than fragmented, ERBB1 and ERBB2 (REF. 94). According to the scenario proposed by Hung and collaborators, the transactivational function of ERBB1 is due to its physical association with STAT3 in the nucleus, which leads to activation of transcription[95], whereas ERBB2 directly binds to the COX2 promoter. Clearly, substantiation of the nuclear activities of ERBB proteins will add a new dimension to our current view of the ERBB network.

Clathrin-coated vesicles
Specialized vehicles of internalization from the plasma membrane, coated with a polyhedral lattice of the protein clathrin.

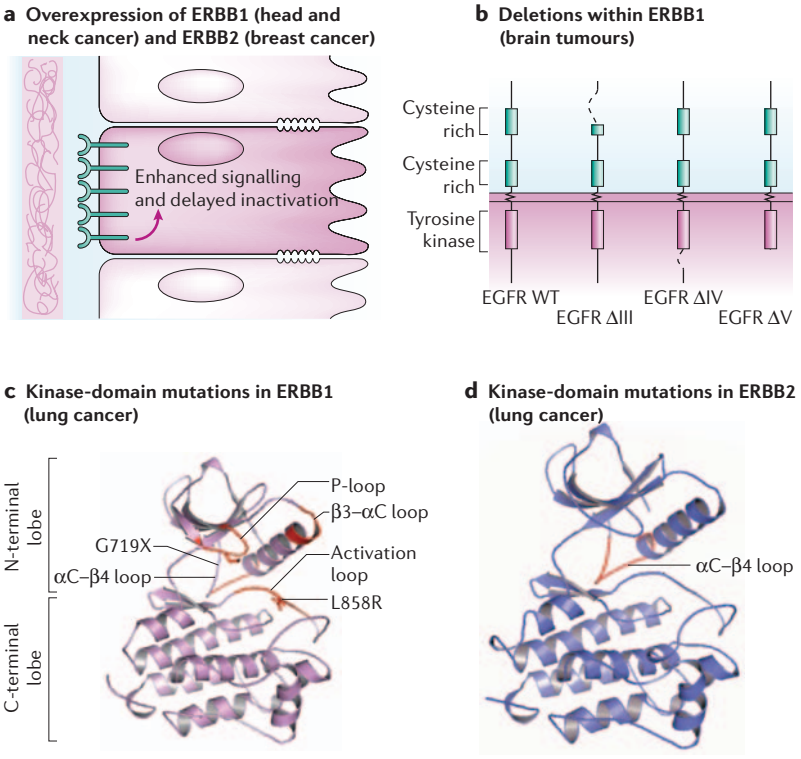


Figure 4 | **Multiple pathways to oncogenesis. a** | Overexpression of ERBB receptors, owing primarily to gene amplification, results in the exaggerated activation of signalling pathways and delayed endocytosis. ERBB1 overexpression has been observed in 80% of head and neck tumours[121]; it correlates with poor prognosis and resistance to therapy. ERBB2 overexpression has been found in breast, lung, pancreas, colon, endometrial and ovarian cancer and it has been frequently associated with an adverse prognostic value[122,123]. **b** | Large deletions within *ERBB1* that are commonly found in brain tumours create constitutively active protein products that lack parts of the ligand-binding domain, or the C-terminal tail[115,116]. **c** | Multiple short deletions within the β3–αC loop of the ERBB1 kinase domain, as well as within the activation loop, P-loop and αC–β4 loop (all marked in red) have been observed in lung cancer. Likewise, mutations of Gly719 and Leu858 are common. These aberrations affect regulatory regions and result in enhanced kinase activity, or altered substrate specificity. The presence of these diverse mutations predicts significant clinical responses to specific kinase inhibitors[109,110]. **d** | Small duplications within the kinase domain of ERBB2 have been detected in lung cancer and other malignancies. This is an infrequent event of unclear functionality[115]. However, all the mutations that have been identified reside within the αC–β4 loop, the region that is implicated in the interaction of ERBB2 with heat-shock protein-90 (REF. 71).

Modelling unravels hidden system attributes

The dynamic and complex nature of signalling networks, along with the co-existence of multiple molecular species, make the description of well studied cellular systems incredibly difficult. *In silico* replicas of cellular networks are therefore becoming an increasingly useful tool for network description[96]. Signalling systems like the ERBB network, which are both well understood and sufficiently complex, often feature emergent behaviour, the identification of which is one of the key motivations of computational modelling. Indeed, mathematical models of ERBB signalling are beginning to uncover unexpected functional features. Recent coherent reviews describe the process of modelling biological systems[96,97].

Emergent behaviour
Complex behaviour that cannot be predicted from the properties of system components in isolation, but only emerges when the components are put together in a functional whole.

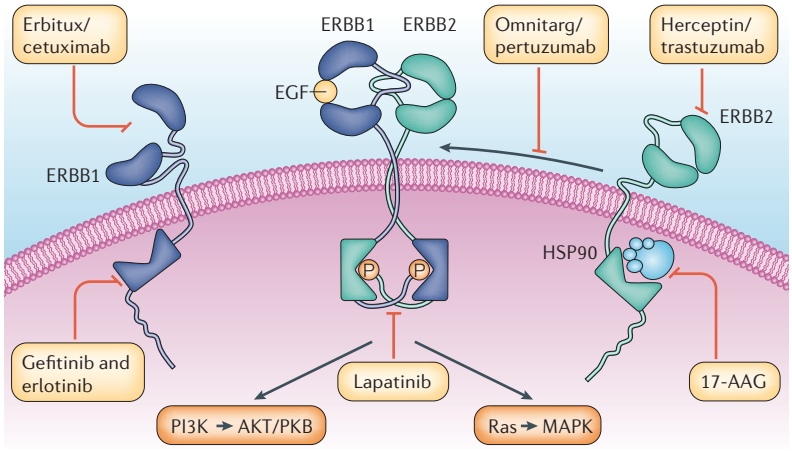
Models of the ERBB network. Mathematical models of the ERBB network have evolved to deal with the increasing numbers of molecular species and cellular compartments that it encompasses. The original models addressed questions of EGF-receptor binding and internalization, for which quantitative data were most readily accessible[98]. These models were then expanded to address the relationship of receptor–ligand interactions to cell proliferation[99], as well as to the early steps of receptor trafficking[100]. Although models that address signalling events have recently been developed, they are evolving rapidly[101–104]. A recently structured comprehensive pathway map of the ERBB signalling network might function as a platform for generating models of higher complexity[105].

Dynamic models of ERBB signalling. One of the first models to incorporate ERBB1 with its associated adaptor proteins[101] predicted that the kinetics of ERBB1 phosphorylation are defined by interactions of the receptor with adaptor proteins, which mask receptor phosphotyrosines from dephosphorylation. The second step in the evolution of the modelling of ERBB signalling was the formation of a most influential large-scale dynamic model of the basic ERBB pathway. This model contains a two-compartment implementation of receptor internalization, and models both Shc-dependent and Shc-independent signal transduction to MAPK activation[102]. An important conclusion that arises from this model is that the binding affinity of the ligand defines signal efficacy, by governing the initial velocity of receptor activation, which potentially explains the utility of EGF-ligand multiplicity. Models for receptor trafficking have also been extensively developed by attempting a realistic simulation through modelling 275 compartments and 13,000 reactions[103], or through the use of partial differential equations to create a continuous transition between states, which abolishes the need for handling a huge number of distinct compartments[104].

An interesting example of a non-intuitive observation, which is supported by the mathematical modelling of ERBB1 endocytosis, is the high biological potency of a low-affinity mutant of EGF[106]. This mutant has a high mitogenic potential because of increased receptor recycling, a mechanism that is harnessed by EGF-like ligands that are encoded by pox viruses[107]. Another emergent feature of EGF signalling attributes to receptor endocytosis a protective effect at high ligand concentrations, but attributes a signal-amplification effect at low concentrations of EGF[102,108].

The horizons of modelling the ERBB network. Along with the significant limitations of spatiality, the bottlenecks of mathematical ERBB modelling are incomplete biological knowledge, lack of quantitative kinetic data, oversimplified molecular descriptions and the weight of the bias with which the modeller enters the system. This situation will probably change in the near future because of technological advances in high-throughput, quantitative analysis of parameters in signal transduction and the increasing popularity of cDNA arrays, as well as

Box 3 | **Network fragility — the pharmacist's opportunity**



Complex diseases, such as cancer, are thought to hijack aspects of the robust physiological systems they perturb[131]. Because a necessary trade-off of robustness is network fragility in the face of unexpected perturbations[132], the fragile hubs that are inherited by tumours might function as potential targets for cancer therapy (see figure).

Monoclonal antibodies

Therapeutic antibodies function by recruiting cytotoxic lymphocytes[133], as well as through direct effects on signalling in the target tumour cell[134]. Antibodies in use at present are trastuzumab (Herceptin; Genentech; which targets ERBB2) for treatment of breast tumours, and cetuximab (Erbitux; Bristol Myers Squibb (BMS)/ImClone; which targets ERBB1) for treatment of colorectal cancer. Novel experimental therapeutic approaches target the heterodimerization of ERBB2 (Omnitarg/pertuzumab), or use antibody combinations[135] to promote receptor degradation[136].

Tyrosine-kinase inhibitors

These small molecules block the nucleotide-binding pocket of ERBB proteins. Two ERBB1-specific drugs, gefitinib (Iressa; AstraZeneca) and erlotinib (Tarceva; Genentech/OSI), were recently approved for the treatment of non-small-cell lung cancer, and have been shown to be effective against tumours that express catalytically hyperactive ERBB1 mutants[109,110]. A more effective strategy would be to use dual-specificity inhibitors, such as Lapatinib (GlaxoSmithKline), CI-1033 (Pfizer) and EKB-569 (Wyeth-Ayerst Research), thereby targeting both ERBB1 and ERBB2 (REF. 137).

Inhibitors of heat-shock protein-90 (HSP90)

ERBB2 is strictly dependent on the HSP90 chaperone complex for maintenance of its stability, identifying an Achilles heel of the ERBB system[69]. The clinical efficacy of HSP90 inhibitors, such as 17-*N*-allylamino-17-demethoxygeldanamycin (17-AAG), is under evaluation. However, a novel approach for the specific targeting of ERBB2 for proteasomal degradation involves the use of kinase inhibitors that dissociate HSP90 from ERBB2 (REF. 138).

Drug combination

Genetic heterogeneity and cellular dynamics are thought to underlie tumour resistance to drugs, but combinations of non-cross-resistant treatment regimens might prevent its recurrence[139]. Therapeutic strategies that target multiple components within the ERBB network might be beneficial[140]. Integration of anti-ERBB drugs with conventional anti-cancer chemotherapy and radiotherapy have been shown to improve outcome[141] and overcome drug resistance[142].

AKT/PKB, AKT/protein kinase B; EGF, epidermal growth factor; MAPK, mitogen-activated protein kinase; P, phosphate; PI3K, phosphatidylinositol 3-kinase.

Psoriasis
A chronic skin disorder of genetic origin that is caused by inflammation-driven hyperproliferation of epidermal cells. Appears as red, scaly elevated plaques, specifically on joints.

the development of quantitative proteomics and other sophisticated analytical techniques.

Furthermore, the definition of common experimental conditions, as carried out by the Alliance for Cell Signaling, and proposed by the RTK consortium, the introduction of a standard representation of biological models (for example, the Systems Biology Markup Language), and the establishment of shared modelling databases (for example, the BioModels database), will allow rapid advances in the acquisition of standardized quantitative datasets, which could be fed into common ERBB models.

When system controls go awry

Several pathological conditions (for example, cancer, psoriasis and atherosclerosis) harness the ERBB network by dysregulating its essential hubs. Gain-of-function perturbations exploit network bistability and incapacitate system controls in diverse malignancies (see below and FIG. 4; BOX 3). Several clinically approved drugs target ERBB signalling, thereby highlighting points of network fragility.

Kinase-domain mutations and deletions. Certain heterozygous, somatically acquired ERBB1 mutations in lung cancer correlate with clinical responses to ERBB1-specific kinase inhibitors[109–111]. These aberrations, which include deletions, insertions and missense point mutations that stabilize interactions with ATP and kinase inhibitors, concentrate in regulatory regions that surround the ATP-binding cleft of ERBB1.

ERBB1 mutants have been associated with enhanced autophosphorylation and cell survival; this finding provides an explanation as to why kinase inhibitors selectively induce the apoptosis of mutant-expressing cells[112,113]. Along with pathway-selective activation, ligand-induced downregulation of ERBB1 mutants seems impaired[109], and the association with ERBB3 seems enhanced[114], which is in line with multiple mechanisms of network dysregulation. Although significantly less frequent, cancer-associated kinase-domain mutations have been reported in ERBB2 (REF. 115). Consistent with the importance of receptor heterodimers, mutations in ERBB1 and ERBB2 have not been detected in the same tumours.

Large deletions in ERBB1. Brain tumours, especially gliomas, display multiple rearrangements within the *ERBB1* gene, including large deletions, point mutations and insertional mutations[116]. Up to 20% of glioblastomas show *ERBB1* rearrangements and up to 40% of glioma tumours overexpress the ERBB1 receptor[117,118]. The most frequent mutant, EGFRvIII, lacks the dimerization arm and an essential part of the ligand-binding domain[116], yet this mutant is constitutively active at the plasma membrane and evades downregulation[119], thereby bypassing system controls.

Receptor overexpression. ERBB1 overexpression due to gene amplification or increased translation has been reported in diverse tumours, such as lung, pancreas and breast lesions[120]. In head and neck cancer, ERBB1 overexpression is observed in at least 80% of tumours[121], and correlates with a reduction in patient survival rates. Likewise, overexpression of ERBB2 has been reported in breast, lung, pancreas, colon, endometrium and ovarian cancer[122], and it has been associated with a poor prognosis for breast and ovarian cancer patients[123].

Atherosclerosis
A progressive disease of the arterial blood vessel. It is caused by the formation of plaques that cause narrowing and hardening of the arteries, reducing blood flow to the heart.

Bistability
Defines that the system will transit between two states, 'on' and 'off', with no, or little, intermediary states.

Network fragility
As a network evolves robustness to particular changes, this necessarily entails an increase in its vulnerability to perturbations from unexpected sources, defining its points of fragility.

Along with high basal autophosphorylation, receptor overexpression delays ligand-induced degradation because of the limited capacity of clathrin-mediated endocytosis.

Autocrine mechanisms. Co-expression of an ERBB protein and one or more of its ligands might establish an autocrine loop that drives uncontrolled cell growth. In cancer patients, the autocrine production of TGFα or EGF is associated with reduced survival[124,125]. A similar mechanism has been proposed in the case of NRG-producing human vestibular schwannoma cells[126]. In accordance with these observations, the parallel analysis of both ERBB1 and its cognate ligands provides a strong predictive tool for patient survival in several types of human cancer (reviewed in REF. 120). Therefore, bistability, an important aspect of network control, is the focus of manipulations leading to oncogenic transformation by genetic aberrations.

Future directions

The described emergence of a systems-level view of EGF–ERBB signalling, along with the first large computational models of the system, are natural sequels of the post-genomic phase of ERBB research: most components of the signalling network are well characterized, and their connectivities are rapidly becoming apparent. The systems-level perspective provides a useful framework that accommodates the increasingly voluminous amounts of incoming experimental data. The popularity and spread of high-throughput methodologies, such as gene arrays and quantitative proteomics, are expected to increase the challenge.

Nevertheless, facing the challenge is worthwhile for several reasons: the impact on less well characterized RTK systems will probably be broad and will reveal common network principles and motifs. Second, it will function as a prelude to the immense mission of embedding the ERBB system in a mosaic network of networks, which includes G-protein coupled receptors, cell-adhesion machineries, nuclear responses and other networks that interface with ERBB. Even more importantly, the development of a comprehensive understanding of the system, and perfecting *in silico* replicas, will contribute to the ultimate goal of predicting system behaviour, as well as expose the fragility of network hubs — the trade-off of network robustness.

Because ERBB signalling is so pivotal to some of the most virulent human malignancies, reliable quantitative modelling will probably identify new targets for cancer therapy, as well as predict the consequences of combining specific drugs and clinical procedures. Although the future medical promise is truly great, fulfilling this promise will require a transformation in the way biologists conduct experiments, analyse data and share datasets.

A common experimental ground is required for a computational model to make use of available data and to be useful to a large audience. The ongoing efforts to form compendia that make use of defined cell types under defined conditions of growth and manipulation might produce the 'common language' that is required to promote data sharing among different laboratories. Potentially, this could lead to a better interaction between experimentalists and theoreticians, and result in exponential advances in this field, which is extremely relevant to human health.

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