Feedback regulation of EGFR signalling: decision making by early and delayed loops

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Abstract | Human-made information relay systems invariably incorporate central regulatory components, which are mirrored in biological systems by dense feedback and feedforward loops. This type of system control is exemplified by positive and negative feedback loops (for example, receptor endocytosis and dephosphorylation) that enable growth factors and receptor Tyr kinases of the epidermal growth factor receptor (EGFR)/ERBB family to regulate cellular function. Recent studies show that the collection of feedback regulatory loops can perform computational tasks — such as decoding ligand specificity, transforming graded input signals into a digital output and regulating response kinetics. Aberrant signal processing and feedback regulation can lead to defects associated with pathologies such as cancer.

When studying nerve growth factor (NGF) with Rita Levi-Montalcini, Stanley Cohen[1] noted that injecting crude extracts of male mouse salivary gland into newborn mice enlarged their sympathetic ganglia (an activity attributed to NGF) and also, unexpectedly, caused them to open their eyes earlier than normal. Employing precocious eye opening as a tracing assay enabled Cohen to isolate a 53-amino-acid polypeptide that is now known as epidermal growth factor (EGF). Adding EGF to a plasma membrane fraction isolated from A431 epithelial cells allowed Cohen to speculate, two decades later, that the EGF receptor (EGFR) has an intrinsic protein kinase activity[2]. Ullrich and colleagues subsequently confirmed Cohen's prediction by cDNA cloning of EGFR[3] and later discovered a large family of receptor Tyr kinases (RTKs), each of which binds to a specific growth factor (GF) at their extracellular-facing domain and shares a cytoplasmic catalytic function.

The availability of the first GF–RTK pair spearheaded a new research field of signal transduction by Tyr phosphorylation[4]. The identification of phosphotyrosine-binding proteins, which can be adaptors or enzymes[5], revealed that Tyr-phosphorylated EGFR engages at least six biochemical pathways, culminating in cell fate decisions. For example, the adaptors growth factor receptor-bound protein 2 (GRB2) and the SH2 domain-containing family of proteins (hereafter referred to as SHC) recruit a cascade of protein kinases, known as the extracellular signal-regulated kinase pathway (ERK pathway), which regulate entry into the cell cycle. The indirect recruitment of phosphoinositide 3-kinase (PI3K) to Tyr-phosphorylated EGFR instigates PI3K–AKT (in which AKT refers to the AKT protein family) signalling, which regulates cell survival. However, the study of other RTKs soon showed that these and additional EGF-activated pathways are not unique to EGFR, thus raising the question of signal specificity. In addition, the EGFR group of RTKs includes three other receptors, only one of which, ERBB4, is autonomous; ERBB3 is almost devoid of kinase activity, whereas ERBB2 (also known as HER2) does not bind any known EGF-like ligand.

The issue of signalling specificity, along with the existence of non-autonomous RTKs and the broad range of EGF-induced biological outcomes — which exceeds the number of signalling pathways — motivated a paradigm shift a decade ago; the view of simple pathways connecting ERBB proteins to enzymatic processes and biological outcomes has given way to a web of interconnected pipelines, called the EGFR/ERBB network[6]. By increasing the number of possible protein interactions, the layered network allows signal specificity and diverse biological outcomes[7]. Intriguingly, the evolutionary course of the EGFR/ERBB network over the past billion years provides a rare glimpse into the logic and design principles of bio-information relay systems. Starting from a simple cascade comprising one GF and one receptor, the network expanded through gene duplications and sub-functionalization, a genetic process

Extracellular signal-related kinase pathway
A three-tiered kinase module in which the first kinase, RAF, phosphorylates and activates the second kinase, mitogen-activated protein kinase kinase (MEK), which phosphorylates the third kinase, extracellular signal-regulated kinase (ERK), in a two-step, non-processive reaction.

Sub-functionalization
Concurrent partial loss of function of the protein product of duplicated genes, such that collaboration between respective gene products reconstitutes the full set of sub-functions attributed to the original ancestor.

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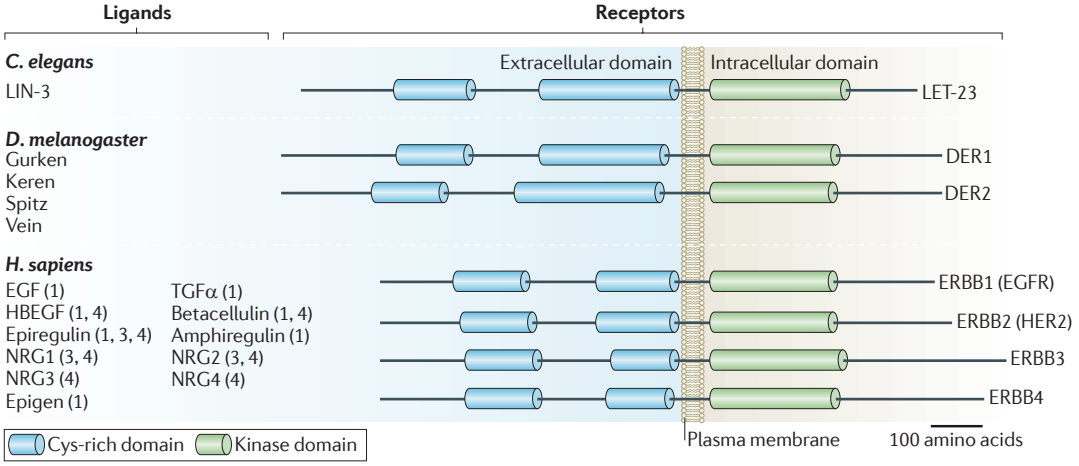
Box 1 | **From a primordial pair of a ligand and a receptor to multiple pairs**



In the course of evolution, the ERBB family expanded from the nematode's single ligand (LIN-3) and receptor (LET-23) to a group of four receptors and 11 ligands in vertebrates (see the figure). All ligands share a conserved epidermal growth factor (EGF)-like structure, maintained by six Cys residues that form three Cys bridges. Similarly, the domain structure of all four receptors is well conserved and includes a ligand-binding extracellular domain that is linked through a single transmembrane region to the cytoplasmic Tyr kinase domain. Note that the two insect receptors represent splicing isoforms of the *Drosophila melanogaster* EGF receptor (DER1 and DER2). Unlike LIN-3, the vertebrate ligands specifically bind to more than one receptor (the numbers of the receptors, 1–4, that they interact with are indicated), but ERBB2 binds no known ligand and ERBB3 cannot signal when present alone, because the respective kinase domain is practically inactive. One interpretation of the existence of two vertebrate receptors, ERBB2 and ERBB3, that cannot form signalling-competent homodimers is that of sub-functionalization. Accordingly, two rounds of whole-genome duplications gave rise to a family of four homologous receptors. Thereafter, protein sequence divergence inactivated sub-functions, such as the enzymatic activity of ERBB3, to promote a collaboration between duplicated gene products. It is notable that after gene duplications, highly connected proteins retain interactions with both gene products, which creates networks enriched in highly connected nodes, or hubs. EGFR, EGF receptor; HBEGF, heparin-binding EGF-like growth factor; NRG, neuregulin; TGFα, transforming growth factor-α.

that differentiates duplicated genes from one another, yet confers redundancy to achieve output reproducibility, specificity and quantitative properties (BOX 1).

The complexity of the EGFR network has been exemplified by integrating all available experimental information into a comprehensive pathway map, which reflects the characteristic bow-tie structure of evolvable networks[8]. The introduction of high-throughput technologies has greatly enhanced the detail of the map (see Supplementary information S1 (box)). For simplicity, the network can be viewed as a 'black box' system, in which a core process receives diverse inputs and reliably integrates them to generate a specific output. A collection of partially redundant feedback loops, both positive and negative, adjusts the output of the core process[9]. These feedback mechanisms (see Supplementary information S2 (table)) enable adjustments to internal and external perturbations, as well as the creation of switches with all-or-none digital output characteristics[10]. Another important virtue of signalling systems is their ability to maintain output reproducibility, despite intrinsic protein variations and inherently stochastic signals[7,11]. This is achieved by the organization of the signalling system into diversified, but redundant, modules. Several recognizable modules, such as homo- and heterodimers of EGFR, ERBB3 and ERBB4, exist within the EGFR/ERBB network[6]. By contrast, the ligand-less receptor ERBB2 is a positive regulator, which amplifies and prolongs signalling by forming functional heterodimers (or higher-order complexes) with ligand-occupied receptors, including the catalytically defective receptor ERBB3.

This Review highlights the identity and mechanisms of activation-dependent regulatory loops, which fall into two temporal domains, immediate and late. The immediate group includes receptor endocytosis, secondary phosphorylation and other covalent protein modifications, and the elimination of microRNAs (miRNAs) that normally repress cellular activation (FIG. 1). The late group encompasses newly synthesized adaptors, transcriptional repressors, RNA-binding proteins and phosphatases of the mitogen-activated protein kinase (MAPK) pathway. Importantly, recent studies attribute the computational tasks of the network, which include the decoding of ligand specificity and the transformation of graded input signals into digital output signals, to the activation-dependent regulatory loops. Collectively, the regulatory loops dictate the duration, amplitude and frequency of signals, which enable threshold setting, binary switching or oscillations, and decoding of input specificity. This remarkably dynamic subfield of signal transduction interfaces with both computational and clinical disciplines. Readers interested in broadening the picture are referred to two recent reviews[12,13].

Redundancy
Coexistence of functionally similar components offering an alternative route for signal propagation if one of the components is inactivated.

Feedback loop
A composite two-arm loop in which a protein, X, activates a downstream protein, Y, or transcriptionally induces a gene encoding Y. On activation, Y regulates X (positively or negatively).

Endocytosis
The intake of material from the extracellular matrix or the membrane into vesicles that arise from the inward folding of the plasma membrane.

Binary switching
When a system's output can transit between two states: 'on' and 'off', with (almost) no intermediate states.

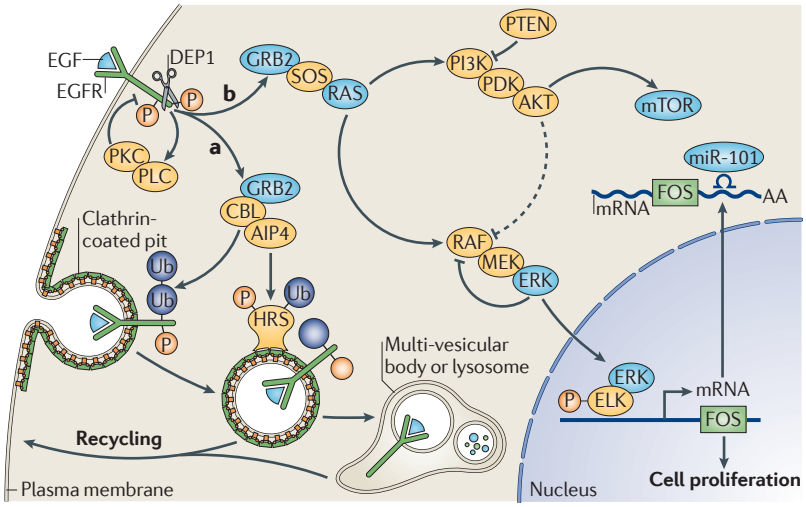


Figure 1 | **Early regulatory loops — the 'all out war'.** Ligand binding to epidermal growth factor receptors (EGFRs) and their subsequent dimerization induces receptor auto-phosphorylation. Receptor Tyr kinase (RTK) phosphorylation is regulated by Tyr-specific phosphatases that shut down signalling — for example, density-enhanced phosphatase (DEP1; also known as RPTPη) dephosphorylates EGFR. Phosphorylated receptors are recognized by adaptors such as growth factor receptor-bound protein 2 (GRB2), which serves as a signalling branching point. **a** | By recruiting an ubiquitin ligase, CBL, GRB2 can sort phosphorylated receptors to endosomes, while another ligase, activation induced phosphatase 4 (AIP4), ubiquitylates clathrin binders such as hepatocyte growth-factor regulated Tyr kinase substrate (HRS). The ubiquitin tags on EGFR recruit HRS and other adaptors, which contain an ubiquitin-interacting motif, and target the receptors to lysosomes for degradation. Deubiquitylating enzymes (DUBs) can abrogate this process and target EGFR to the recycling pathway. **b** | However, GRB2 can initiate downstream signalling by recruiting SOS to RAS, which activates kinase cascades such as the RAF–MEK–ERK (RAF–mitogen-activated protein kinase kinase–extracellular signal-regulated kinase) and PI3K–PDK–AKT (phosphoinositide 3-kinase–pyruvate dehydrogenase kinase–AKT) pathways. The ERK cascade is regulated by intrinsic positive and negative feedback (for example, ERK negatively feeds back to RAF) and extrinsic crosstalk regulation from other kinase cascades (for example, AKT inhibits RAF in most cells, as indicated by the dashed line). Translocation of active ERK to the nucleus results in the removal of DNA-binding repressors or the recruitment of transcription factors (such as ELK) to the promoters of target genes, such as FOS. A small group of pre-existing microRNAs (miRNAs), including miR-101, protects cells against signal-independent leaky transcription. On mitogen activated protein kinase (MAPK) activation these miRNAs undergo degradation to allow the initiation of gene expression programmes. mTOR, mammalian target of rapamycin; PLC, phospholipase C; PKC, protein kinase C; Ub, ubiquitin.

Early loops: protein modifications

The concept of feedback regulation goes back over 100 years to the inhibition of some metabolic pathways by their end products and the auto-regulation of transcriptional processes[14]. One general role for this type of biological response is the digitalization of analogue signals. An early example refers to a theory by Conrad Hal Waddington[15]; during embryogenesis cells interpret an 'epigenetic landscape' (for example, continuous gradients of GFs) and convert this analogue signal into a stable, all-or-none (digital) fate decision. Accordingly, the early (seconds to minutes) and late (minutes to hours) events of the response to EGF aim at recovery of the resting state, or the establishment of a new cellular state — for example, differentiation into a new cell type. Owing to the immediate nature of the response, the early events cannot rely on newly synthesized molecules. Instead, they comprise both protein translocations and post-translational modifications (FIG. 1).

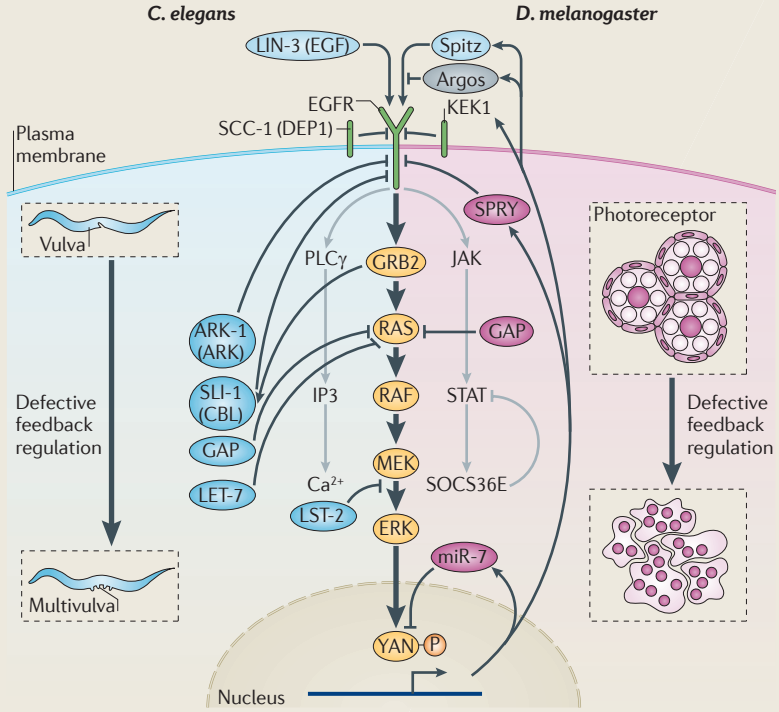
Ligand-induced receptor endocytosis. Using radiolabelled EGF, Carpenter and Cohen[16] established the basis of the process that sorts ligand–receptor complexes for internalization, followed by either recycling or degradation in lysosomes. Although receptor endocytosis is a major negative feedback loop, the internalized receptors remain active while en route for recycling or degradation. Indeed, the receptors for EGF and NGF can generate signals from endosomes — the vesicles that transfer ligand-loaded receptors from the plasma membrane to the pre-lysosomal compartment called the multi-vesicular body (MVB)[17]. The current consensus assumes that EGFR transfer from clathrin-coated pits to endosomes prolongs MAPK signals while terminating PI3K–AKT signalling[18,19]. However, a recent study that used an internalization-defective mutant of EGFR proposed the opposite scenario[20]. Yet another study suggested that the clathrin-mediated route prolongs EGF signalling through EGFR recycling, but at high ligand concentrations clathrin-independent pathways that terminate signalling are favoured[21]. These discrepancies are attributable to the multiplicity of endocytosis mechanisms, and their rapidity and redundancy. Indeed, unlike receptor sorting at the MVB, which depends on receptor ubiquitylation[22,23], sorting at the plasma membrane is executed by ubiquitin-independent, partly redundant mechanisms that involve the activator protein 2 (AP2) clathrin adaptor, GRB2 (REF. 20) and the kinase inhibitor ERBB receptor feedback inhibitor 1 (ERRFI1; also known as MIG6) (REF. 24).

Covalent modifications of EGFR regulate an activation-dependent cascade; auto-phosphorylation of EGFR enables it to recruit GRB2 and the E3 ubiquitin ligase CBL[23,25]. On physical recruitment, CBL initially modifies EGFR with monomers of the ubiquitin-like molecule neural precursor cell expressed developmentally downregulated protein 8 (NEDD8)[26]. Subsequently, CBL modifies multiple Lys residues in the kinase domain of EGFR with monomeric[27,28] and polymeric ubiquitin molecules, as detected by mass spectrometry[22]. The ubiquitins on EGFR are then recognized by ubiquitin-binding proteins of the clathrin coat, such as epidermal growth factor receptor substrate 15 (EPS15), members of the EPS15-interacting protein family (EPSIN1–EPSIN3) and hepatocyte growth factor-regulated Tyr kinase substrate (HRS). These adaptors have ubiquitin- and NEDD8-binding domains, such as the ubiquitin-interacting motif (UIM), and can assemble active receptors at clathrin-coated pits of the plasma membrane, endosomes and the MVB[29]. Although each vesicular compartment of EGFR contains its own adaptors, they all seem to share a mechanism of cargo dissociation and reloading. While at the endosome, E3 ligases of the atrophin-interacting protein 4 (AIP4) and NEDD4 family ubiquitylate the ubiquitin-binding proteins, for example HRS, to promote their intramolecular folding in a way that dissociates the cargo but enables loading of new cargo upon their deubiquitylation[30].

Analogue signal
A continuous signal that changes quantitatively in amplitude or concentration.

E3 ubiquitin ligase
A protein that induces the attachment of ubiquitin, a small, highly conserved regulatory protein, to a Lys on a target protein and thus targets specific protein substrates for degradation.

Box 2 | **Invertebrate versions of the EGFR pathway**



Developmental biologists were able to identify negative regulators of receptor Tyr kinase (RTK) signalling by studying how genetic manipulations cause divergence from the normal phenotype, and many such examples have been applicable to higher organisms. Interestingly, genetic screens primarily identified regulators of the epidermal growth factor receptor–mitogen-activated protein kinase (EGFR–MAPK) pathway, although secondary pathways exist in worms and in insects (see the figure, indicated by grey arrows). In *Caenorhabditis elegans*, LET-23 (EGFR in mammals) signalling can be dissected by examining vulval development (note, the mammalian homologues of the *C. elegans* proteins are shown in brackets in the figure). The vulva is derived from three of six multipotential vulval precursor cells (VPCs), which receive an inductive signal, LIN-3 (EGF in mammals), from the adjacent anchor cell.
Loss-of-function of negative regulators results in more than three VPCs undergoing vulval differentiation (multivulva phenotype). Examples of such negative feedback regulators include the E3 ubiquitin ligase SLI-1 (CBL), the endosomal adaptor LST-2, the miRNA LET-7, the phosphatase SCC-1 (DEP1), and ARK-1 and GAP, enzymes that regulate RAS (see the figure).

The *Drosophila melanogaster* compound eye is a stereotyped array of 800 units (called ommatidia), each unit invariably comprises eight photoreceptor cells. Photoreceptor differentiation is assembled by a sequence of inductive signals mediated by two RTKs, EGFR and Sevenless (not shown). One of the earliest studies to describe negative regulation of EGFR was a loss-of-function mutant of *Argos*, a scavenger of EGF-like ligands, which resulted in excessive recruitment of photoreceptor cells. Interestingly, EGFR signalling transcriptionally induces *Argos*, thus establishing a negative feedback loop (see the figure). In another example, reciprocal negative feedback between miR-7 and a transcriptional repressor, YAN, is induced by EGF. Other examples of transcriptionally induced negative regulators include the scaffold protein CNK (not shown), which regulates RAF activation, the membranal EGFR inhibitor Kekkon 1 (KEK1) and the scaffold protein Sprouty (SPRY). ERK, extracellular signal-regulated kinase; GAP, GTPase activating protein; GRB2, growth factor receptor-bound protein 2; IP3, inositol 3-phosphate; MEK, MAPK kinase; PLC, phospholipase C; STAT, signal transducer and transcription activator.

The RTK family displays a wide range of endocytic programmes, which are determined by the identity of the stimulating ligand[31] and are sensitive to intrinsic conditions, such as oncogenesis, and extrinsic conditions, such as changes in the environment[32]. For example, under hypoxic conditions the rate of EGFR endocytosis is decelerated and the receptor is retained in endosomes[33]. In the ERBB family, EGFR homodimers are effectively sorted for degradation, but heterodimeric complexes evade interactions with CBL and are recycled. Several components and functional features ensure the fail-safe sorting of RTKs for degradation[34]. They include GTP-binding proteins of the RAB family, which coordinate vesicle motility and tethering to target compartments, organelle-specific distribution of phosphoinositol lipids and multi-protein assemblies, such as the four endosomal sorting complexes required for transport (ESCRT-0–ESCRT-III) that reside at the MVB. The multi-component and modular structure of the endocytosis machinery provides multiple interfaces for signal transduction; the actin cytoskeleton[35] and probably also microtubules[36] regulate receptor endocytosis. Although it is currently unclear how the endocytic programme influences quantitative aspects of output signals, the phenotypes of invertebrate animals and manipulated mammalian cells that are defective in components of the endocytic machinery clearly indicate strong relationships between receptor endocytosis and restrained signalling outcomes (BOX 2).

Fine-tuning by deubiquitylation and dephosphorylation. Because activation-dependent internalization and degradation of RTKs crucially determine signalling duration, a wide variety of effectors have evolved the capacity to modulate receptor endocytosis, primarily by intercepting the stability and activity of CBL[37] or by reversing two essential covalent modifications, phosphorylation and ubiquitylation[38]. Two deubiquitylating enzymes that reside in the MVB, STAM-binding protein (STAMBP; also known as AMSH) and ubiquitin isopeptidase Y (UBPY; also known as USP8), regulate EGFR degradation[38]. Both enzymes interact with ESCRT-0, the protein complex involved in the initial sorting of receptors for degradation[39]. Consistent with the involvement of UBPY in inhibiting receptor endocytosis, conditional inactivation of the gene encoding this protein reduced the expression of several RTKs[40].

Multiple protein Tyr-specific phosphatases (PTPs) reverse Tyr phosphorylation on EGFR and other RTKs. The PTP superfamily encodes both cytosplasmic enzymes and transmembrane receptor-like PTPs (RPTPs). Although protein-Tyr phosphatase 1B (PTP1B; also known as PTPN1) resides in the endoplasmic reticulum (ER), it has been implicated in EGFR signalling[41]. According to a recent electron microscopy analysis, expression of an inactive mutant of PTP1B results in the formation of extensive EGFR- and PTP1B-containing contacts between the ER and the outer membrane of the MVB[42]. In addition, expression of the PTP1B mutant reduced the number of MVB internal vesicles, implying that dephosphorylation of EGFR occurs just before inward invagination into the MVB. Alternatively, PTP1B may regulate invagination by dephosphorylating signal transducing adapter molecule 2 (STAM2), a component of ESCRT-0 (REF. 43).

Additional RTK–PTP interactions take place at the plasma membrane and in endosomes. For example, attenuation of EGFR signalling by collagen-rich environments might be due to the ability of the collagen-binding integrin, α1β1, to selectively activate the ubiquitously expressed T cell protein Tyr phosphatase (TCPTP)[44]. Another surface enzyme, receptor-type Tyr-protein phosphatase-κ (RPTPκ), dephosphorylates EGFR in human keratinocytes[45]. Interestingly, RPTPκ levels increase in keratinocytes as they reach confluence and establish contact-mediated growth inhibition. Levels of density-enhanced phosphatase (DEP1; also known as RPTPη), a ubiquitously expressed RPTP, also increase in response to growth inhibition. Congruent with a growth inhibition phenotype, it has been reported that the DEP1 orthologue in *Caenorhabditis elegans* negatively regulates EGFR activity[46] and, more recently, these relationships were confirmed in human cells using RNA interference[47]. It is notable that the corresponding gene, *PTPRJ*, is often deleted or mutated in carcinomas[48]. Consistent with a role in tumour suppression, DEP1 dephosphorylates and inhibits EGFR signalling, but remains at the cell surface when EGFR undergoes endocytosis and degradation[47].

Finally, Tyr dephosphorylation regulates another crucial sorting event, namely transport from early to late endosomes and the overall balance between receptor recycling and degradation. A motor protein of the Kinesin-3 family, KIF16B, which is required for the efficient recycling of EGFR[49], anchors PTPD1 (also known as PTPN21) to early endosomes[50]. This complex promotes EGFR recycling by regulating microtubule-dependent motility. In summary, both transmembrane and cytoplasmic PTPs regulate cell growth by fine-tuning EGFR endocytosis at several sites along the endocytic pathway.

Crosstalk and secondary phosphorylation. Several cytokines and environmental conditions trans-modulate EGFR and other RTKs to alter their functions. One of the best-characterized mechanisms of signalling crosstalk enables mitogens such as lysophosphatidic acid, as well as vasoactive peptide ligands of G protein-coupled receptors (GPCRs), to transactivate EGFR. These mitogens stimulate metalloproteinases, which cleave the transmembrane precursor of the heparin-binding EGF-like growth factor (HBEGF) to convert it into a soluble EGFR ligand[51]. Other routes of crosstalk involve trans-phosphorylation of EGFR on Tyr (positive regulation) or Ser and Thr (mostly negative regulation) residues. The cytoplasmic Tyr kinase SRC, which phosphorylates diverse substrates, including cell adhesion proteins, phosphorylates EGFR on Tyr845 and Tyr1101 to positively regulate its function[52]. Many Ser and Thr residues serve as phospho-acceptors that modulate EGFR trafficking or its catalytic activity when phosphorylated under stress conditions — for example, by p38 in response to chemotherapy[53].

Secondary (or backwards) phosphorylation is defined as phosphorylation of upstream components of a signalling cascade. Identification of substrate sites and modifying kinases is experimentally challenging; nevertheless the multiplicity and functional diversity of this type of regulation are increasingly appreciated[12]. As an example, we refer to the uppermost tier of the ERK cascade, namely RAF proteins, which are involved in several cases of crosstalk and secondary phosphorylation. One of the earliest examples was the observation that AKT can phosphorylate RAF1 on Ser259 (REF 54), a major inhibitory site known to recruit the 14-3-3 chaperone. This site is highly phosphorylated in resting cells, but it undergoes transient dephosphorylation by two phosphatases, PP1 and PP2A, after cells are stimulated by GFs[55]. In parallel, phosphorylation of the major stimulatory site of RAF1, Ser338, by GF- or integrin-stimulated p21-activated kinases (PAKs) enhances RAF1's kinase activity and affinity for MAPK kinase (MEK), as well as the translocation of RAF1 to mitochondria. In addition to AKT and PAKs, RAF1 undergoes phosphorylation by SRC, protein kinase A (PKA), PKC and ERKs. ERKs mediate negative feedback regulation by phosphorylating multiple residues in RAF1, thereby desensitizing it to further stimulation[56]. The cryptic nature of secondary phosphorylation unexpectedly translates to the response of patients to drugs. For example, rapamycin, a drug that inhibits mammalian target of rapamycin (mTOR), an effector kinase of AKT, increased AKT activation in a group of patients. This unexpected activation of AKT, which was associated with earlier disease progression after surgery, is presumably due to loss of negative feedback by mTOR[57]. Conceivably, detailed mapping of secondary phosphorylation may help to improve the therapeutic efficacy of rapamycin and other drugs.

Rapid turnover of a select group of miRNAs. miRNAs are a class of small, single-stranded RNA molecules that generally base-pair to the 3′ untranslated region (UTR) of target mRNAs to repress protein expression[58]. Hundreds of miRNAs have been characterized in human cells, and genome-wide networks incorporating miRNAs have emerged as essential features of developmental and pathological processes. For example, EGF-mediated control of insect eye development involves reciprocal negative feedback between the transcription factor YAN and miR-7, with YAN inhibiting miR-7 in progenitor cells and miR-7 inhibiting YAN in photoreceptor cells[59]. EGFR signalling (through ERK) triggers the degradation of YAN, thereby de-repressing miR-7 and promoting a photoreceptor fate. This reciprocal negative regulation shows how signal transduction can generate a stable change in gene expression patterns. In line with the stability of the phenotypes they induce, miRNAs generally have a very long half-life, corresponding to several hours or a few days. Nevertheless, previous research has indicated the dynamic involvement of miRNAs in diverse signal transduction pathways[60]. Likewise, activity-dependent regulation of miRNAs has emerged as an essential component of both the early (transcription-independent) and late regulatory phases stimulated by EGF[61]. Stimulation of mammary cells with EGF leads to the rapid induction of more than 20 miRNAs in parallel with the immediate turnover of a class of ~25 miRNAs, which are known as immediately

14-3-3 chaperone
Adaptor/scaffold proteins that form homo- and heterodimers and bind, through specialized phosphorylated peptide motifs, to various proteins that are involved in signal transduction and in cell-cycle control.

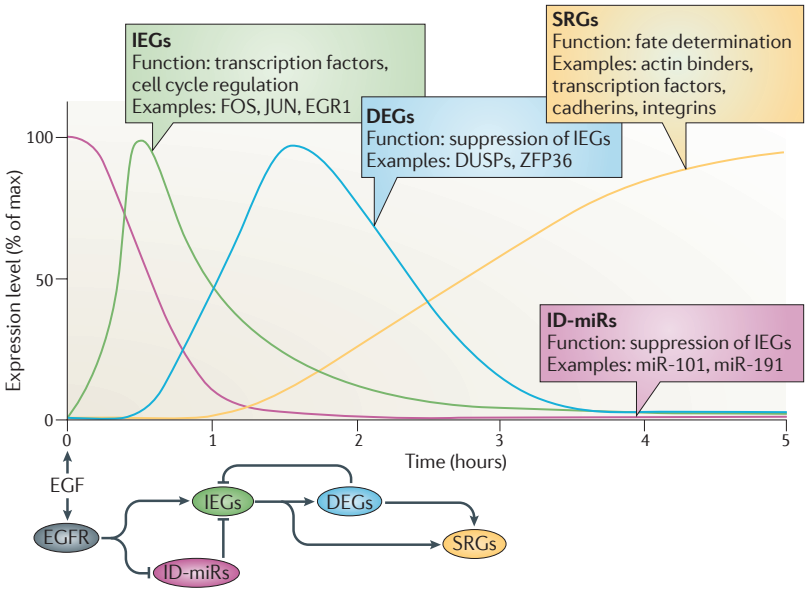


Figure 2 | **Wave-like regulation of mRNAs and microRNAs by EGF.** Cyclic transitions between a resting cellular state and an active state involve several binary switches that are able to control the kinetic profile of immediate early genes (IEGs). The onset of the cycle is induced by an extracellular stimulus, such as epidermal growth factor (EGF). The earliest event involves the immediate turnover of a group of microRNAs (immediately downregulated microRNAs (ID-miRs)), which allows the onset of IEG induction. Subsequently, IEGs induce the transcription of delayed early genes (DEGs, for example, the RNA-binding protein ZFP36, and the dual specific phosphatases (DUSPs)) that dephosphorylate mitogen-activated protein kinases (MAPKs), which shut down the activity of IEGs. The kinetic profiles of the IEGs appear to define the specificity of downstream transcription programmes, including the identity of the secondary response genes (SRGs, for example actin-binding proteins and transcription factors), which define the cellular outcome. The temporal relationships (graph), as well as the topological interactions (schematic) among the groups of gene products are shown. EGFR, EGF receptor; EGR1, early growth response protein 1.

downregulated miRNAs (ID-miRs). Importantly, the targets of ID-miRs are transcripts encoding immediate early genes (IEGs; such as FOS and early growth response protein 1 (EGR1)), which raises the possibility that high expression of ID-miRs in arrested cells silences untimely IEG expression to prevent cell cycle entry (FIG. 2).

Late loops: transcription regulation

In contrast to the early phase, which largely engages pre-existing components involved in receptor endocytosis and EGF-induced phosphorylation events, newly induced RNAs and proteins drive the late regulatory mechanisms. For simplicity, the transcriptional response to EGF may be divided into three temporal phases (FIG. 2). The initial wave, up to 45 minutes from stimulation, consists of a limited set of genes, the IEGs[62], which have primarily positive activities. The group of delayed early genes (DEGs; 45–120 minutes) comprises both positively and negatively acting components[63]. The late, secondary response genes (SRGs; >120 minutes) confer stable phenotypes in a cell context-specific manner. Because IEGs are induced by a large range of stimuli, and oncogenic retroviruses encode aberrant forms of IEGs (for example, viral FOS and JUN), considerable effort has been devoted to characterizing IEG regulation.

Mechanisms underlying immediate transcription. When IEGs were first described, it was speculated that the rapidity of their transcriptional response requires their promoters to be poised for activation[64]. Such permissive mechanisms are only now starting to be described (FIG. 3). One mechanism involves the pre-assembly of RNA polymerase II (Pol II) at the promoters in the basal state, along with histone acetylation and other positive modifications required for transcription. Surprisingly, it was shown that in resting cells, pre-initiation Pol II generates low levels of unspliced and unstable mRNAs, which enables robust, stimulus-dependent transcription[65]. Other mechanisms enabling the rapid induction of IEGs by EGF are removal of negative elongation factor (NELF) from the respective promoter[66], or nucleosome destabilization. As many promoters of inducible genes are free from CpG islands they assemble into stable nucleosomes that can undergo activation-dependent remodelling. By contrast, IEGs induced by both GFs (through the MAPK pathway) and microbial stimuli (through the nuclear factor-κB (NF-κB) pathway) are characterized by GC-rich promoters. Their CpG islands act as nucleosome destabilizers, enabling transcriptional activators such as SP1 to gain access to the promoters and recruit Pol II in the uninduced state, without the need for nucleosome remodelling[67]. The permissive features of IEG promoters might give rise to premature, signal-independent transcription. A putative, pre-emptive regulatory process to counteract this has been described in resting T cells: unlike active genes, the associated histones of which are constitutively acetylated by histone acetyltransferases (HATs), the promoters of a group of primed genes (for example, FOS related antigen 1 (FOSL1)) are characterized by rapid cycles of histone acetylation (by HATs) and deacetylation (by histone deacetylatases (HDACs)), thus preventing Pol II from binding to these genes, but poising them for future activation[68]. Conceivably, rapid HDAC–HAT cycles at the promoters of IEGs, along with ID-miRs[61] and additional mechanisms, are active in growth-arrested cells to repress IEG expression but enable rapid derepression on stimulation by GFs[67].

DEGs: superintendents of IEGs. In addition to being rapidly induced in response to EGF, IEGs are often abruptly downregulated. The group of EGF-induced DEGs includes many newly induced phosphatases, as well as DNA- and RNA-binding proteins (see Supplementary information S2 (table)), which robustly shut down IEGs by either inhibiting upstream signal transduction pathways or by promoting transcriptional attenuation[63]. In many cases the EGF-induced attenuator forms a complex with the protein encoded by the stimulating IEG to repress its function. Examples include the repressors inhibitor of DNA binding 2 (ID2) and cyclic AMP-dependent transcription factor 3 (ATF3), which regulate the TCF complex and NF-κB, respectively. Interestingly, many feedback regulators are induced by the pathways that they attenuate. For instance, specific dual-specificity phosphatases (DUSPs; also known as MKPs) are transcriptionally induced by IEGs to inhibit the function of

Immediate early genes (IEGs). Genes that are induced rapidly and do not require new protein synthesis for their transcription.

RNA polymerase II (Pol II). An enzyme that catalyses the transcription of DNA to synthesize precursors of mRNA and most known small RNAs.

Histone acetylation. Addition of an acetyl group to Lys amino acids on histone proteins, which renders DNA more accessible to transcription factors, and thus is linked to transcriptional activation.

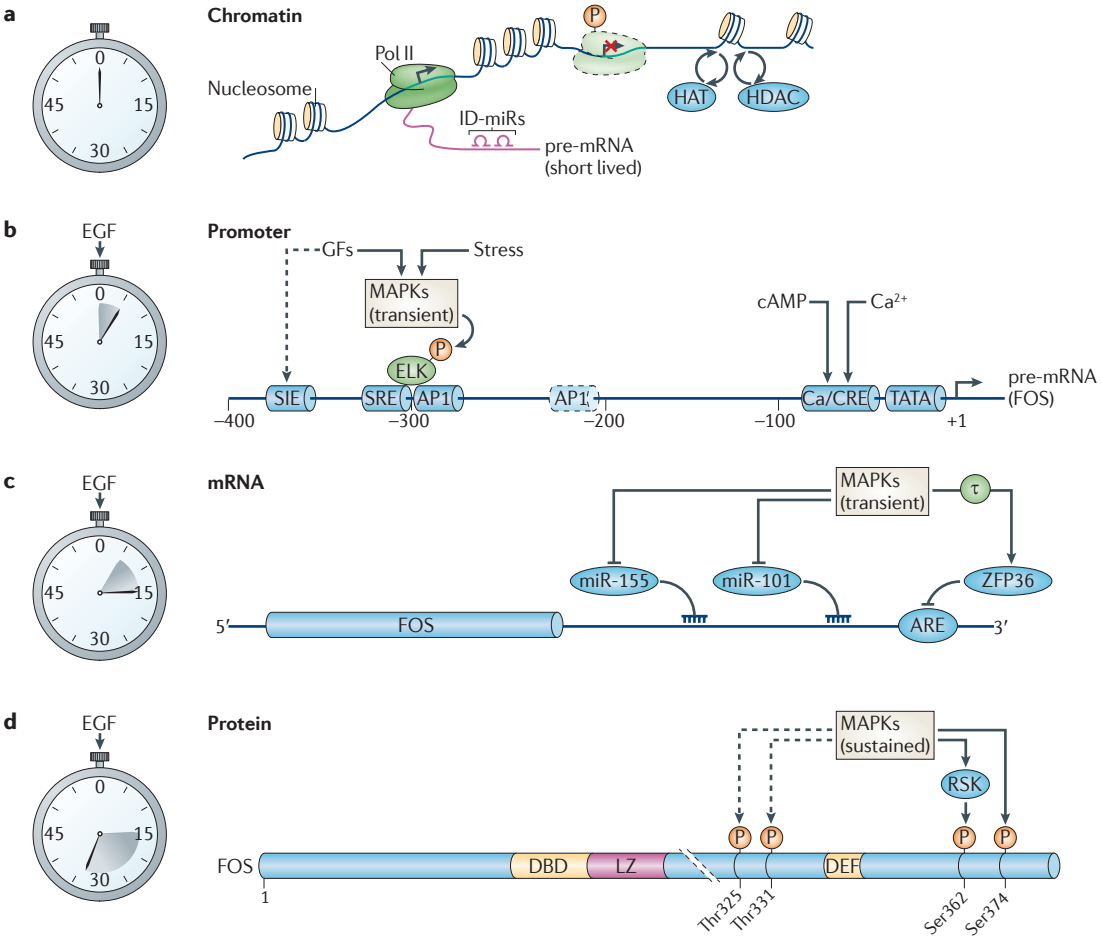


Figure 3 | **Late regulatory loops.** The schemes represent the multiple levels of transcription-dependent feedback regulation of the network. **a** | In resting cells, the promoters of immediate early genes (IEGs) preassemble RNA polymerase II (Pol II). Access of Pol II to the promoters is permitted owing to their nucleosome-free, open chromatin conformation. The transcribed pre-mRNA is short-lived. In addition, Pol II pre-assembly is unstable owing to the rapid association and disassembly cycles of histone acetylases (HATs) and histone deacetylases (HDACs). **b** | Within the first 1–5 minutes after stimulation, extracellular signal-regulated kinase (ERK) translocates to the nucleus, where it mediates IEG transcription by recruiting transcription factors, such as ELK, co-activators (for example, the binding of serum response factor to the serum response element (SRE)) and HATs. Besides growth factors, other inducers of signalling, such as Ca^{2+} and cyclic AMP (cAMP), are able to initiate IEG transcription. **c** | The mRNA of IEGs like FOS is short lived, and regulated via the 3′ untranslated region (UTR). Immediately downregulated miRNAs (ID-miRs) are rapidly degraded after epidermal growth factor (EGF) stimulation, allowing IEG accumulation. However, the delayed induction of RNA-binding proteins such as ZFP36 recruits complexes that degrade the early transcripts. **d** | Phosphorylation of FOS on Ser374 by ERK and on Ser362 by ribosomal protein S6 kinase (RSK) stabilizes the protein. Further docking of ERK on the DEF domain, and phosphorylation on Thr331 and Thr325 enhances the transcriptional activity of FOS. The dashed lines indicate indirect effects that are mediated by several additional steps, which are not shown. AP1, activator protein 1; ARE, AU response element; Ca/CRE, Ca^{2+} and cAMP response element; DBD, DNA-binding domain; LZ, Leu zipper; MAPK, mitogen activated protein kinase; τ, time delay.

CpG island
A genomic region that contains a relatively high content of cytosine (C) and guanine (G) dinucleotides (the 'p' refers to the phosphodiester bond linking the two bases). CpG islands are found in many mammalian promoters and unlike scattered CpGs throughout the genome, which are usually hypermethylated, promoter CpG islands are normally hypomethylated.

Epithelial–mesenchymal transition
(EMT). A phenotypic transformation of a highly polarized epithelial sheet of densely packed cells into sparse, motile cells resembling connective tissue cells. This transition involves a series of molecular switches, which are dependent on newly induced mRNAs and microRNAs.

MAPKs[69], a negative feedback mechanism. An important type of feedback regulation entails RNA-binding proteins that instigate the degradation of specific mRNAs. For example, the RNA-binding protein ZFP36 (also known as tristetraprolin), which is induced by IEGs, recruits mRNA-degrading enzymes to specific IEGs by recognizing AU-rich elements (AREs) in their 3′ UTRs[70]. Interestingly, ZFP36 cooperates with miR-16, a human miRNA containing a sequence complementary to the ARE[71], indicating that miRNAs have essential roles in controlling the duration of active signalling.

SRGs: determining new phenotypes. Along with genes encoding enzymes involved in metabolism and membrane biogenesis, the late-induced wave of transcription comprises sets of mRNAs that collectively confer new phenotypes (leading to differentiation). One example of this late wave of fate-determining transcription is a process called the epithelial–mesenchymal transition (EMT)[72]. GFs such as EGF and transforming growth factor-β (TGFβ) enhance EMT by inducing several transcription factors (for example, SNAI1 and TWIST1), by upregulating the synthesis and secretion of specific matrix

metalloproteinases and by downregulating protease inhibitors. These effectors enable certain GFs to instigate several molecular switches of adhesion (a crucial component of EMT), such as the loss of epithelial cadherin (E-cadherin), which is associated with the downregulation of miR-200, and the gain of neural cadherin (N-cadherin), which is expressed by mesenchymal cells[73]. Another EGF-induced transcriptional switch replaces tensins — linkers of the actin cytoskeleton and the extracellular matrix (ECM) — with CTEN, a member of the tensin family that lacks the actin binding domain, thereby disrupting the physical link between the cytoskeleton and the ECM[74].

Autocrine loops: extracellular positive feedback. The ability of tumour viruses to upregulate the expression of EGFR ligands in infected cells led to the discovery of autocrine stimulation[75]. Activation-dependent auto- and cross-induction of EGF family ligands[32] is mediated by the MAPK pathway and occurs under both physiological (for example, in development) and pathological (for example, in viral infection and cancer) conditions. Autocrine loops constitute a positive feedback mechanism that sustains GF signals and often converts a transient stimulus into a sustained signal. Because EGF-like ligands are synthesized as transmembrane precursors that undergo proteolytic shedding by metalloproteases, autocrine ligands may differ from ligands secreted by neighbouring or distant cells (paracrine stimulation). For example, autocrine ligands stimulate the migration of mammary epithelial cells more efficiently than paracrine GFs[76]. Nevertheless, paracrine ligands also have important roles. For instance, colony-stimulating factor 1 (CSF1), which is produced by mammary epithelial cells, promotes the expression of EGF by macrophages. EGF, in turn, promotes the expression of CSF1 by carcinoma cells, thereby generating a positive-feedback loop[77]. Challenging cells with multiple extracellular signals may induce the release of additional secreted factors, as demonstrated for the pro-inflammatory cytokine tumour necrosis factor (TNF). In addition to a direct response through activated TNF receptors, cells respond to TNF indirectly through the sequential release of TGFα, interleukin-1α (IL-1α) and IL-1 receptor antagonist (IL-1RA), a process known as the 'autocrine cascade'[78]. Beyond its relevance to signal prolongation and digitalization, autocrine stimulation has clinical implications: increased expression of GFs is associated with more aggressive malignant lesions, and identifies a group of ovarian tumours that are 'addicted' to autocrine stimulation[79]. Accordingly, several monoclonal antibodies that block ligand binding to EGFR have been approved for the treatment of colorectal and other types of cancer. In line with antibody-mediated blocking of autocrine loops, patients with tumours that overexpress the EGFR ligands epiregulin and amphiregulin are more likely to benefit from antibody treatment[80]. In summary, autocrine stimulation comprises a pivotal regulatory component of the EGFR/ERBB network, and understanding it could lead to clinical applications.

Computational tasks of feedback loops

The ability of signalling networks to transform incoming signals into stable cell fate decisions is exemplified by the work of Ferrell and collaborators[10] on steroid-induced maturation of *Xenopus laevis* oocytes. The crucial mediators of oocyte maturation are organized into a positive-feedback loop; a signal mediated by MAPK enables a brief exposure of oocytes to hormones to irreversibly trigger maturation. The outcome of such positive-feedback loops often depends on the strength of intrinsic connections between the signalling pathways involved. If strong enough to generate an all-or-none response (bistability), positive feedback may result in hysteresis or the execution of irreversible decisions. This example and the examples below indicate that signalling networks can quantitatively and reproducibly process incoming signals. Before discussing this poorly understood aspect of the EGFR/ERBB network, it is worthwhile reiterating two design principles and one methodological feature that are relevant to the computational competence of the network. First, many negative and positive regulators are induced by the pathway they regulate. Second, components of the network are often assembled into recurring units, called network motifs[81]. From an experimental perspective, the topology of motifs may be derived from measuring changes in their functional outcomes in response to defined perturbations[82].

Multitasking by ERK: distinct pathway outcomes. Although the ERK cascade assembles a simple, three-tier architecture, it is involved in different GF-induced cellular processes, such as proliferation, migration and differentiation. Because rat pheochromocytoma PC-12 cells differentially respond to EGF and NGF, and both stimuli activate ERK, these cells are an experimental model for studying how the activation of a downstream signalling cascade by different receptors leads to diverse outcomes. Treatment of PC-12 cells with NGF leads to neurite outgrowth (differentiation), whereas treatment with EGF leads to cell proliferation[83]. Both outcomes involve ERK, but with notable kinetic differences: ERK activation persists for several hours following NGF stimulation, but it is only transiently induced by EGF[84]. This early observation highlighted the importance of activation duration, and was later extended to other cellular models. For example, stimulation of mammary cells with the ERBB3- and ERBB4-specific ligand neuregulin 1 induces prolonged ERK activation and differentiation, whereas EGF enhances cell proliferation through transient ERK activation[85]. As indicated in FIG. 4, several biochemical mechanisms account for the differential kinetics of ERK activation in response to different ligands. Using PC-12 cells to validate mathematical modelling, it was shown that stimulation of ERK by NGF activates the upstream kinase RAF to cause positive feedback, whereas ERK activation by EGF inhibits RAF through a negative feedback loop that results in transient ERK signalling[86]. These differences, along with differences in the stability of IEG products induced by EGF- or NGF-induced ERK activation[87] and

Hysteresis
A mode in which it is easier to maintain the system in its 'on' state than to toggle the system between 'on' and 'off'.

Network motifs
A pattern of interactions that recurs in cellular networks significantly more often than in randomized networks.

Pheochromocytoma
An adrenal gland tumour that originates from neural crest cells.

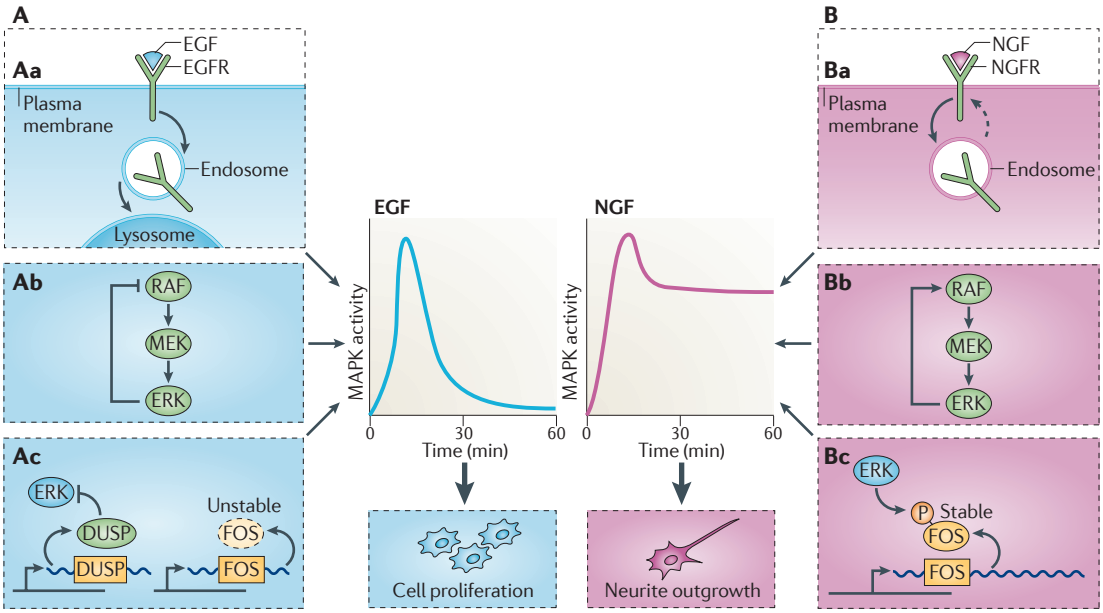


Figure 4 | **Decision making by PC-12 cells.** The temporal specification of active extracellular signal-regulated kinase (ERK) in PC-12 cells is the basis for the phenotypic distinction between stimulation by epidermal growth factor (EGF) (cell proliferation) and neural growth factor (NGF) (cell differentiation and neurite outgrowth). The differential activation kinetics of extracellular signal-related kinase (ERK) by the two stimuli are shown in the graphs, alongside the signalling mechanisms that can lead to transient activation (which is induced by EGF) and sustained activation (which is induced by NGF). **A** | Transient and adaptive ERK activation by EGF is determined by rapid receptor degradation in lysosomes (**Aa**). In addition, transient ERK signals trigger a negative-feedback loop between ERK and RAF, which ensures rapid signal termination (**Ab**). ERK-mediated transcriptional induction of several mitogen-activated protein kinase phosphatases (dual-specificity phosphatases (DUSPs; also known as MKPs)) finally terminates mitogen-activated protein kinase (MAPK) activity (**Ac**). This transient ERK activity results in a short-lived FOS protein and culminates in a specific gene expression programme, which leads to proliferation. **B** | NGF-induced ERK activation is accompanied by receptor recycling (**Ba**), secondary phosphorylation that induces a positive-feedback loop between ERK and RAF (**Bb**), and phosphorylation-mediated stabilization of FOS (**Bc**). Sustained FOS activity results in induction of gene expression programmes that lead to differentiation and neurite outgrowth. Recent studies implicate more subtle, multifaceted mechanisms involving transcriptional repressors, a GTPase-activating protein, crosstalk to AKT and >30 other proteins, which are not presented here. EFGR, EGF receptor; MEK, MAPK kinase; NGFR, NGF receptor.

the engagement of RAP1A[88], a GTP-binding protein homologous to RAS, may only be the tip of the iceberg of a multifaceted mechanism; a recent proteomic analysis of proteins physically associated with ERK during differentiation identified 60 proteins that changed their interaction with ERK on stimulation with NGF[89]. Characterization of a subset of these proteins uncovered diverse mechanisms, including not only feedback and signal duration changes but also alterations in ERK localization, changes in crosstalk with the AKT pathway and the differential phosphorylation of transcription factors.

Multitasking by ERK: the mechanisms. Multiple membrane, cytoplasmic and nuclear mechanisms underlie the differential activation of positive- and negative-feedback loops in PC-12 cells. The internalization of membrane receptors and their differential routing to the recycling or degradation pathways emerges from recent computational models as a mechanism that not only differentiates between EGF and NGF but also precisely measures ligand concentrations and enhances signalling accuracy[90,91]. One cytoplasmic mechanism involves crosstalk between ERK and PKC. The ERK module is negatively regulated by the adaptor protein RAF kinase inhibitory protein (RKIP), which binds to RAF1 and MEK to disrupt their interaction[92]. Unlike EGF signals, NGF-induced signalling is accomplished by the simultaneous activation of ERK and PKC. PKC-mediated phosphorylation of RKIP releases it from RAF1 (REF. 93), thereby enabling direct RAF1 phosphorylation by ERK and positive feedback. Another cytoplasmic mechanism that contributes to distinct outcomes of ERK signalling involves MAPK phosphatases. Accordingly, when this family of phosphatases is present at low abundance the system exhibits bistable behaviour, such that a brief stimulus results in sustained ERK activation[94]. However, a subsequent activation-induced increase in the abundance of the phosphatase eliminates the prolonged response capability.

Nuclear mechanisms are equally important. FOS, an IEG product, functions as a sensor of ERK signal duration[87]. When ERK activation is transient, the activity of FOS decreases as FOS is rapidly degraded. However, when ERK signalling is sustained, FOS is phosphorylated by the persistent activity of ERK and the ribosomal protein S6 kinase 2 (RSK2). Phosphorylation of FOS at its carboxyl terminus stabilizes it and primes it for

additional phosphorylation by exposing a docking site for ERK. From a topological perspective, sustained induction of FOS depends on activation of both ERK and its downstream target RSK, which enhances the expression and stabilization of FOS. This type of three-node module (ERK, RSK and FOS), termed a 'coherent feedforward loop' (FFL), is a recurring motif in signalling and transcriptional networks[81]. In summary, an overwhelmingly rich and diversified list of mechanisms accounts for the differential kinetics of MAPK activation in response to EGF, NGF and probably additional GFs, and the consequent cellular outcomes.

Distinct pathway outcomes: output oscillations. The frequency of oscillatory outputs provides yet another way to encode signal specificity. However, owing to asynchronous oscillations, assays that average output over large groups of cells often overlook this important mechanism. For example, pulsed responses of p53 to radiation became evident only when analysed at single-cell resolution[95]. Early studies predicted MAPK oscillations on the basis of both the multi-layer configuration of the cascade, which confers ultrasensitivity, and inherent feedback loops[96–98]. Because of the latter, transient EGF stimulation affects only the signal's duration, but chronic stimulation generates an oscillatory behaviour of ERK[99]. Only recently did single-cell imaging confirm EGF-induced, sustained and persistent oscillations of ERK between the nucleus and cytoplasm[100,101]. Still, how these oscillations mediate the response to EGF remains poorly understood. However, the fact that the frequency of oscillatory outputs can encode signal specificity is well illustrated by the oscillatory system that underlies regulation of NF-κB by TNF[102]. Similarly to other oscillatory pathways, a negative-feedback loop comprising an inhibitory subunit, inhibitor of NF-κB (IκB), is essential (FIG. 5). Predictably, similar mechanisms govern EGF-induced oscillations, but their identification awaits further analyses of individual or synchronized cells.

Complex quantitative tasks of feedback regulation. We propose that the EGFR system gained computational competence in the course of evolution by undergoing two important transitions. The first entailed gene duplications (BOX 1) and the acquisition of a configuration characteristic of scale-free networks[103]. Although the scale-free design is robust against accidental failures, the multiplicity of components and reliance on network hubs confers vulnerability — the removal of a few key hubs splinters the system into small clusters. The other transition is likely to entail the gradual development of sophisticated quantitative abilities by means of integrating a limited number of generic network motifs[81]. Although the full scope of the computational competence remains unknown, it is likely to exceed the aforementioned multitasking and oscillatory mechanisms. One remarkable example refers to adaptation, namely the ability to reset after responding to a stimulus, which relates to maintaining homeostasis in the face of perturbations[104]. A recent study mathematically examined the architectural designs of all three-node protein circuits that can achieve adaptation, and identified only two competent topologies: a negative-feedback loop with a buffering node and an incoherent feedforward loop[105] (FIG. 5). In this vein, recent studies involving activation of ERK2 by EGF in epithelial cells[101], as well as the control of β-catenin abundance in colorectal cells[106], raised the interesting possibility that the response in these systems is proportional to the fold-change in the stimulus relative to the background, rather than to the absolute level. Both negative-feedback loops and incoherent feedforward loops can detect fold-change and achieve adaptation. However, as-yet-unknown additional mechanisms are likely to allow the EGFR/ERBB network to filter noise and buffer stochastic variations in protein expression levels.

Defective feedback regulation in cancer

Subtle manipulations of the EGFR/ERBB network are harnessed by several hyper-proliferative and inflammatory diseases, including atherosclerosis, psoriasis and several types of cancer. Overexpressed or mutant forms of ERBB4, EGFR and ERBB2 drive subtypes of melanoma, brain and breast cancer, respectively[107–109], among several other tumour types. In addition, other aberrations, such as mutant forms of RAF or PI3K, manipulate downstream signalling in cancer. As we discuss below, these hub-targeting aberrations are often accompanied by weakening of negative-feedback loops. Alternatively, they may enhance positive regulation in a way that prolongs the activation of EGFR/ERBB signalling. These principles have been adopted by oncogenic viruses. For example, RAS proteins encoded by murine sarcoma viruses induce the synthesis of multiple EGF-like ligands to enhance positive feedback, whereas a short form of CBL encoded by the Cas NS-1 murine leukaemia virus inhibits EGFR degradation to weaken negative feedback.

Oncogenic mechanisms evading feedback control. One way to manipulate the EGFR network entails setting the level of activity just below the threshold required for the mobilization of control machineries. For example, mutant forms of EGFR frequently detected in lung cancer are characterized by a basal, ligand-independent function, which is sufficient to weakly activate downstream signals but insufficient to recruit CBL to trigger receptor degradation[110,111]. This feature is shared by deletion mutants of EGFR, which are frequently expressed in brain tumours. When present in very high numbers at the cell surface, the wild-type form of EGFR similarly exhibits basal activity, which is uncoupled from ubiquitylation. A large group of EGF-like ligands, including epiregulin, amphiregulin and epigen, along with a large group of ligands encoded by poxviruses, stimulate EGFR for a prolonged period by enabling it to evade desensitization mechanisms[112]. Owing to a low affinity for the cognate ERBB receptor, these broad-specificity GFs weakly activate receptor phosphorylation and ubiquitylation, and their clearance from the medium is ineffective, a mechanism that is also harnessed by a highly mitogenic, recombinant mutant of EGF[113].

Feedforward loop:
A regulatory pattern in which a stimulus (X) feeds into a response (Y) via more than one route: directly into Y or indirectly via Z (which interacts with Y). In a coherent feedforward loop the sign of the leg from X to Y equals the summation of the alternative leg (X-to-Z-to-Y). In any other case, the design is referred to as an incoherent feedforward loop.

Scale-free network
A non-uniform network whose connectivity (the number of edges of each of the nodes) follows a power law. Scale-free biological networks are mostly comprised of nodes with one to two edges, and several highly linked hubs with six or more edges.

Network hubs
Richly linked nodes of the network that account for most of the vulnerability of scale-free networks, as damage to one of the hubs of a network can break it up into segregated sub-graphs.

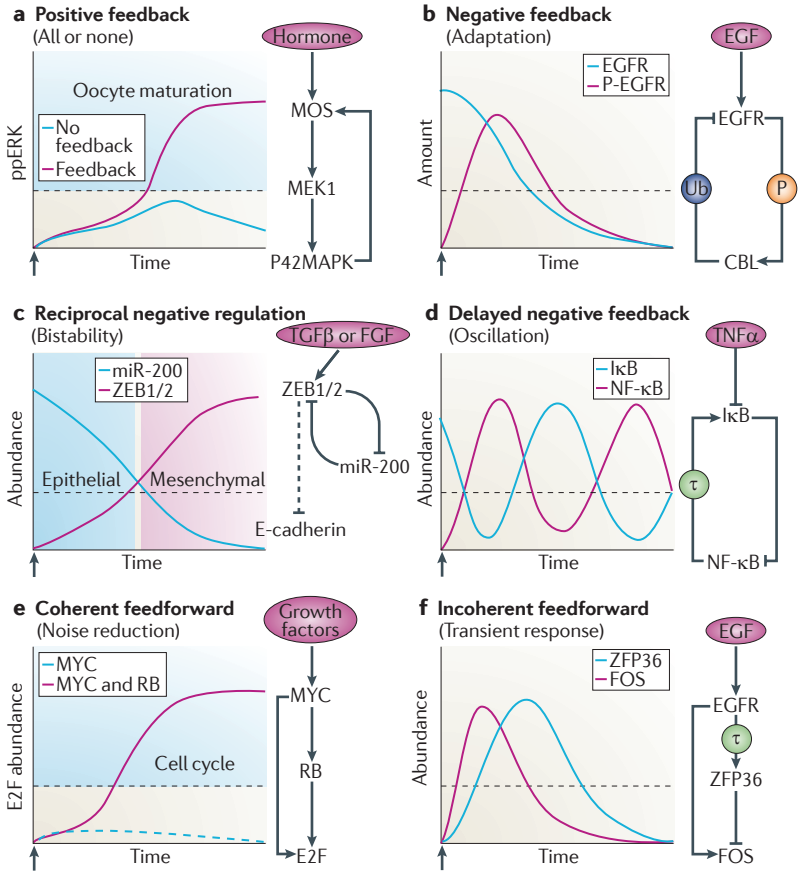


Figure 5 | **Examples of network motifs in mammalian signalling systems. a** | Positive feedback: this design comprises the linear kinase cascade MOS–MEK1–P42MAPK where MOS is a mitogen-activated kinase (MAPK) kinase kinase, MEK1 is a MAPK kinase and P42MAPK is a MAPK. This pathway is stimulated by a steroid hormone to promote oocyte maturation in Xenopus laevis. Positive feedback transforms the reversible hormonal signal into a self-sustained loop. **b** | Negative feedback: activated epidermal growth factor receptor (EGFR) phosphorylates CBL, which in turn tags EGFR with ubiquitin, leading to signal termination. **c** | Reciprocal negative regulation: an epithelial phenotype is maintained by high levels of miR-200, which inhibits ZEB family repressor proteins to upregulate epidermal cadherin (E-cadherin). The switch to a mesenchymal state can be induced by transforming growth factor-β (TGFβ), which upregulates ZEB. This in turn maintains a mesenchymal state through ZEB binding to the promoters of E-cadherin and miR-200 to repress them. **d** | Delayed negative feedback: the combination of a slower transcription arm (NF-κB–IκB (nuclear factor-κB–inhibitor of nuclear factor-κB)) and a faster protein-interaction arm (IκB–NF-κB) helps to stabilize a composite loop. **e** | Coherent feedforward: E2F induction relies on the binding of both RB and MYC to its promoter. Thus, a critical duration of strong serum stimulation allows phosphorylation of RB, and the subsequent binding of both transcription factors to the E2F promoter. **f** | Incoherent feedforward: EGFR rapidly activates FOS, but also represses FOS by activating the RNA-binding protein ZFP36. As a result, when EGFR is stimulated, FOS is rapidly produced. Later on, however, ZFP36 levels accumulate to reach the repression threshold. The dashed horizontal line in all of the panels indicates a threshold. FGF, fibroblast growth factor; ppERK, bi-phosphorylated ERK; P-EGFR, phosphorylated EGFR; τ, time delay; TNFα, tumour necrosis factor-α; Ub, ubiquitin.

Enhanced positive feedback control in cancer. As mentioned above, following receptor activation, the MAPK pathway strongly induces the transcription and secretion of multiple ERBB ligands[114]. This mode of autocrine positive feedback characterizes a large fraction of human tumours of epithelial origin. For example, expression of TGFα in colorectal tumours is associated with increased risk of developing liver metastases[115]. Autocrine loops necessitate co-expression of a ligand and one of its receptors. However, enhanced expression of the co-receptor shared by all three ERBB members, ERBB2, further enhances signalling. Recruitment of ERBB2 into ligand-induced heterodimers or higher oligomers is considered a positive regulatory mechanism that shapes the signalling output by multiple mechanisms, such as stronger and more permissive ligand binding, decreased coupling to CBL and the engagement of a broader range of signalling proteins. Accordingly, the proliferation and invasion of several types of human tumours is enhanced through the amplification of a portion of chromosome 17 that encodes ERBB2. For example, amplification of *ERBB2* occurs in ~20% of invasive mammary tumours, which exhibit poor prognosis and high resistance to certain therapies[108].

Weakened negative regulation of the network in tumours. Both transcription-mediated and transcription-independent mechanisms underlie negative-feedback regulation, and both are weakened in tumours. DEGs normally undergo ligand-induced upregulation to gradually shut down EGFR signalling. However, transcriptome-wide analyses of a large range of tumours revealed low expression of most DEGs in tumours, relative to the corresponding normal tissues. Furthermore, low DEG expression correlates with a shorter survival time of patients with prostate and ovarian cancer[63], in line with DEG growth-inhibitory functions. ID-miRs act similarly to DEGs in EGFR-driven breast and brain tumours[61]. Other negative regulators of EGFR signalling are usually lost in tumours. Cytoplasmic localization or reduced expression of a group of transmembrane suppressors of EGFR signalling, called LRIGs, is related to aggressiveness of human oligodendroglioma and ependymoma[116], and the chromosomal region 1p36 encoding ERRFI1, a negative regulator of EGFR, is frequently deleted in glioblastomas[117]. Another example relates to CBL, which displays inactivating mutations in myeloid leukaemia[118]. Yet more examples highlight defects in RTK endocytosis and the involvement of the actin cytoskeleton[32]. The huntingtin-interacting proteins (HIP1 and HIP1R) uncouple endocytic vesicles from actin assembly, thereby inhibiting receptor degradation. Accordingly, HIP1 overexpression has been observed in various tumours, including lymphoid and prostate lesions, and correlated with relapse after prostatectomy[119].

Concluding remarks

Stanley Cohen's early work on EGF signalling laid a solid basis for an elaborate understanding of signalling principles, which is extending now to other GFs and cytokines. Studies of the past 3–5 years have revealed a broader than expected common basis between signalling mediated by EGF and other GFs, dictated by the principles of biological robustness[11] and network design[7]. In the context of robustness, an important shared attribute is the striking wealth of activity-dependent negative and positive modules, which comprise recognizable network motifs[63,81]. These may be classified into

early, transcription-independent mechanisms — such as receptor endocytosis, secondary phosphorylation[86] and loops involving miRNAs[61] — and late mechanisms regulated by newly synthesized proteins and miRNAs. Along with their function as signal attenuators and guardians of steady states, feedback and feedforward loops have emerged as computational circuits that decode ligand specificity, read ligand concentrations along gradients[120], ensure reproducibility and shape the overall output (FIG. 5).

It is pertinent to note that the combination of covalent modifications, subcellular translocations and the recurring patterns of transcription regulation and protein–protein interactions[121] confer a specific time constant to each regulatory module. This Review offers the possibility that mutational, viral and other perturbations that occur under pathological conditions can harness their host's networks by manipulating the time constants of crucial regulatory modules. For example, overexpression of EGFR, a frequently diagnosed aberration of brain tumours, alters the time constants of EGFR–MAPK signalling by overwhelming the endocytic machinery[122] or by prolonging MAPK signalling[123], thereby favouring cell proliferation. From a pathological perspective, feedback regulation is likely to take centre stage as a determinant of the resistance of diseases to new drugs and a guide for selecting specific drug combinations, as exemplified by a recent Phase I clinical trial of rapamycin in brain tumours[57]. Another future avenue relates to the ability of biological systems to execute signal consolidation — that is, to integrate the milieu of multiple, simultaneous extracellular signals into a digital decision. We propose that signal integration into additive, synergistic or null responses is yet another computational task of activation-dependent systems, such as the EGFR/ERBB network.

1. Levi-Montalcini, R. & Cohen, S. Effects of the extract of the mouse submaxillary salivary glands on the sympathetic system of mammals. *Ann. N. Y. Acad. Sci.* **85**, 324–341 (1960).
2. Cohen, S., Carpenter, G. & King, L. Jr. Epidermal growth factor-receptor-protein kinase interactions. Co-purification of receptor and epidermal growth factor-enhanced phosphorylation activity. *J. Biol. Chem.* **255**, 4834–4842 (1980).
3. Ullrich, A. *et al.* Human epidermal growth factor receptor cDNA sequence and aberrant expression of the amplified gene in A431 epidermoid carcinoma cells. *Nature* **309**, 418–425 (1984).
4. Hunter, T. Tyrosine phosphorylation: thirty years and counting. *Curr. Opin. Cell Biol.* **21**, 140–146 (2009).
5. Pawson, T. & Scott, J. D. Signaling through scaffold, anchoring, and adaptor proteins. *Science* **278**, 2075–2080 (1997).
6. Citri, A. & Yarden, Y. EGF-ERBB signalling: towards the systems level. *Nature Rev. Mol. Cell Biol.* **7**, 505–516 (2006).
7. Kholodenko, B. N. Cell-signalling dynamics in time and space. *Nature Rev. Mol. Cell Biol.* **7**, 165–176 (2006).
8. Oda, K., Matsuoka, Y., Funahashi, A. & Kitano, H. A comprehensive pathway map of epidermal growth factor receptor signaling. *Mol. Syst. Biol.* **1**, 20050010 (2005).
Presents a comprehensive pathway map of EGFR signalling. The map reveals that the overall architecture of the pathway is a bow-tie structure with several feedback loops.
9. Freeman, M. Feedback control of intercellular signalling in development. *Nature* **408**, 313–319 (2000).
10. Xiong, W. & Ferrell, J. E. Jr. A positive-feedback-based bistable 'memory module' that governs a cell fate decision. *Nature* **426**, 460–465 (2003).
Shows that a brief exposure of frog oocytes to progesterone irreversibly triggers their maturation through a positive-feedback loop involving the MAPK cascade. However, the response becomes transient when positive feedback is blocked.
11. Kitano, H. Biological robustness. *Nature Rev. Genet.* **5**, 826–837 (2004).
12. Kholodenko, B. N., Hancock, J. F. & Kolch, W. Signalling ballet in space and time. *Nature Rev. Mol. Cell Biol.* **11**, 414–426 (2010).
13. Lemmon, M. A. & Schlessinger, J. Cell signaling by receptor tyrosine kinases. *Cell* **141**, 1117–1134 (2010).
14. Jacob, F. & Monod, J. Genetic regulatory mechanisms in the synthesis of proteins. *J. Mol. Biol.* **3**, 318–356 (1961).
15. Waddington, C. H. Canalization of development and the inheritance of acquired characters. *Nature* **150**, 563–565 (1942).
16. Carpenter, G. & Cohen, S. 125I-labeled human epidermal growth factor. Binding, internalization, and degradation in human fibroblasts. *J. Cell Biol.* **71**, 159–171 (1976).
17. Grimes, M. L., Beattie, E. & Mobley, W. C. A signaling organelle containing the nerve growth factor-activated receptor tyrosine kinase, TrkA. *Proc. Natl Acad. Sci. USA* **94**, 9909–9914 (1997).
18. Vieira, A. V., Lamaze, C. & Schmid, S. L. Control of EGF receptor signaling by clathrin-mediated endocytosis. *Science* **274**, 2086–2089 (1996).
19. Fehrenbacher, N., Bar-Sagi, D. & Philips, M. Ras/MAPK signaling from endomembranes. *Mol. Oncol.* **3**, 297–307 (2009).
20. Goh, L. K., Huang, F., Kim, W., Gygi, S. & Sorkin, A. Multiple mechanisms collectively regulate clathrin-mediated endocytosis of the epidermal growth factor receptor. *J. Cell Biol.* **189**, 871–883 (2010).
Demonstrates, using receptor mutagenesis, that clathrin-dependent internalization of EGFR is regulated by four mechanisms: ubiquitylation of the receptor kinase domain, the clathrin adaptor complex AP2, the GRB2 adaptor protein and acetylation of three C-terminal Lys residues.
21. Sigismund, S. *et al.* Clathrin-mediated internalization is essential for sustained EGFR signaling but dispensable for degradation. *Dev. Cell* **15**, 209–219 (2008).
Reports that EGFRs internalized via clathrin-mediated endocytosis are recycled to the cell surface and their signalling is prolonged. By contrast, clathrin-independent internalization preferentially commits the receptor to degradation.
22. Huang, F., Kirkpatrick, D., Jiang, X., Gygi, S. & Sorkin, A. Differential regulation of EGF receptor internalization and degradation by multiubiquitination within the kinase domain. *Mol. Cell* **21**, 737–748 (2006).
23. Levkowitz, G. *et al.* Ubiquitin ligase activity and tyrosine phosphorylation underlie suppression of growth factor signaling by c-Cbl/Sli-1. *Mol. Cell* **4**, 1029–1040 (1999).
24. Frosi, Y. *et al.* A two-tiered mechanism of EGFR inhibition by RALT/MIG6 via kinase suppression and receptor degradation. *J. Cell Biol.* **189**, 557–571 (2010).
25. Joazeiro, C. A. *et al.* The tyrosine kinase negative regulator c-Cbl as a RING-type, E2-dependent ubiquitin-protein ligase. *Science* **286**, 309–312 (1999).
26. Oved, S. *et al.* Conjugation to Nedd8 instigates ubiquitylation and down-regulation of activated receptor tyrosine kinases. *J. Biol. Chem.* **281**, 21640–21651 (2006).
27. Mosesson, Y. *et al.* Endocytosis of receptor tyrosine kinases is driven by monoubiquitylation, not polyubiquitylation. *J. Biol. Chem.* **278**, 21323–21326 (2003).
28. Haglund, K. *et al.* Multiple monoubiquitination of RTKs is sufficient for their endocytosis and degradation. *Nature Cell Biol.* **5**, 461–466 (2003).
29. Katzmann, D. J., Odorizzi, G. & Emr, S. D. Receptor downregulation and multivesicular-body sorting. *Nature Rev. Mol. Cell Biol.* **3**, 893–905 (2002).
30. Polo, S. *et al.* A single motif responsible for ubiquitin recognition and monoubiquitination in endocytic proteins. *Nature* **416**, 451–455 (2002).
31. Roepstorff, K. *et al.* Differential effects of EGFR ligands on endocytic sorting of the receptor. *Traffic* **10**, 1115–1127 (2009).
32. Abella, J. V. & Park, M. Breakdown of endocytosis in the oncogenic activation of receptor tyrosine kinases. *Am. J. Physiol. Endocrinol. Metab.* **296**, E973–E984 (2009).
33. Wang, Y. *et al.* Regulation of endocytosis via the oxygen-sensing pathway. *Nature Med.* **15**, 319–324 (2009).
34. McMahon, H. T. & Gallop, J. L. Membrane curvature and mechanisms of dynamic cell membrane remodelling. *Nature* **438**, 590–596 (2005).
35. Merrifield, C. J., Feldman, M. E., Wan, L. & Almers, W. Imaging actin and dynamin recruitment during invagination of single clathrin-coated pits. *Nature Cell Biol.* **4**, 691–698 (2002).
36. Deribe, Y. L. *et al.* Regulation of epidermal growth factor receptor trafficking by lysine deacetylase HDAC6. *Sci. Signal.* **2**, ra84 (2009).
Reports that HDAC6 negatively regulates EGFR endocytosis and degradation by controlling the acetylation of α-tubulin. A negative-feedback loop consisting of EGFR-mediated phosphorylation of HDAC6 resulted in reduced deacetylase activity.
37. Jozic, D. *et al.* Cbl promotes clustering of endocytic adaptor proteins. *Nature Struct. Mol. Biol.* **12**, 972–979 (2005).
38. Clague, M. J. & Urbe, S. Endocytosis: the DUB version. *Trends Cell Biol.* **16**, 551–559 (2006).
39. McCullough, J. *et al.* Activation of the endosome-associated ubiquitin isopeptidase AMSH by STAM, a component of the multivesicular body-sorting machinery. *Curr. Biol.* **16**, 160–165 (2006).
40. Niendorf, S. *et al.* Essential role of ubiquitin-specific protease 8 for receptor tyrosine kinase stability and endocytic trafficking *in vivo*. *Mol. Cell Biol.* **27**, 5029–5039 (2007).
41. Haj, F. G., Verveer, P. J., Squire, A., Neel, B. G. & Bastiaens, P. I. Imaging sites of receptor dephosphorylation by PTP1B on the surface of the endoplasmic reticulum. *Science* **295**, 1708–1711 (2002).
42. Eden, E. R., White, I. J., Tsapara, A. & Futter, C. E. Membrane contacts between endosomes and ER provide sites for PTP1B-epidermal growth factor receptor interaction. *Nature Cell Biol.* **12**, 267–272 (2010).
Shows that the EGFR–PTP1B interaction occurs at sites of direct contact between the perimeter membrane of multivesicular bodies and the ER. This population of EGFR undergoes ESCRT-mediated sorting within MVBs, and PTP1B activity promotes EGFR sequestration into internal vesicles of the MVB.

43. Stuible, M. *et al.* PTP1B targets the endosomal sorting machinery: dephosphorylation of regulatory sites on the endosomal sorting complex required for transport component STAM2. *J. Biol. Chem.* **285**, 23899–23907 (2010).
44. Mattila, E. *et al.* Negative regulation of EGFR signalling through integrin-α1β1-mediated activation of protein tyrosine phosphatase TCPTP. *Nature Cell Biol.* **7**, 78–85 (2005).
45. Xu, Y., Tan, L. J., Grachtchouk, V., Voorhees, J. J. & Fisher, G. J. Receptor-type protein-tyrosine phosphatase-κ regulates epidermal growth factor receptor function. *J. Biol. Chem.* **280**, 42694–42700 (2005).
46. Berset, T. A., Hoier, E. F. & Hajnal, A. The *C. elegans* homolog of the mammalian tumor suppressor *Dep*-1/Scc1 inhibits EGFR signaling to regulate binary cell fate decisions. *Genes Dev.* **19**, 1328–1340 (2005).
47. Tarcic, G. *et al.* An unbiased screen identifies DEP-1 tumor suppressor as a phosphatase controlling EGFR endocytosis. *Curr. Biol.* **19**, 1788–1798 (2009).
48. Ruivenkamp, C. A. *et al.* Ptprj is a candidate for the mouse colon-cancer susceptibility locus Scc1 and is frequently deleted in human cancers. *Nature Genet.* **31**, 295–300 (2002).
49. Hoepfner, S. *et al.* Modulation of receptor recycling and degradation by the endosomal kinesin KIF16B. *Cell* **121**, 437–50 (2005).
50. Carlucci, A. *et al.* PTPD1 supports receptor stability and mitogenic signaling in bladder cancer cells. *J. Biol. Chem.* **285**, 39260–39270 (2010).
51. Prenzel, N. *et al.* EGF receptor transactivation by G-protein-coupled receptors requires metalloproteinase cleavage of proHB-EGF. *Nature* **402**, 884–888 (1999).
52. Biscardi, J. S. *et al.* c-Src-mediated phosphorylation of the epidermal growth factor receptor on Tyr845 and Tyr1101 is associated with modulation of receptor function. *J. Biol. Chem.* **274**, 8335–8343 (1999).
53. Zwang, Y. & Yarden, Y. p38 MAP kinase mediates stress-induced internalization of EGFR: implications for cancer chemotherapy. *EMBO J.* **25**, 4195–4206 (2006).
54. Zimmermann, S. & Moelling, K. Phosphorylation and regulation of Raf by Akt (protein kinase B). *Science* **286**, 1741–1744 (1999).
55. Dhillon, A. S., Meikle, S., Yazici, Z., Eulitz, M. & Kolch, W. Regulation of Raf-1 activation and signalling by dephosphorylation. *EMBO J.* **21**, 64–71 (2002).
56. Dougherty, M. K. *et al.* Regulation of Raf-1 by direct feedback phosphorylation. *Mol. Cell* **17**, 215–224 (2005).
57. Cloughesy, T. F. *et al.* Antitumor activity of rapamycin in a Phase I trial for patients with recurrent PTEN-deficient glioblastoma. *PLoS Med.* **5**, e8 (2008).
Results of a Phase I clinical trial of rapamycin, an inhibitor of mTOR, in patients with recurrent glioblastoma. Rapamycin treatment led to AKT activation in seven out of 14 patients, presumably owing to loss of negative feedback.
58. Bartel, D. P. MicroRNAs: genomics, biogenesis, mechanism, and function. *Cell* **116**, 281–297 (2004).
59. Li, X. & Carthew, R. W. A microRNA mediates EGF receptor signaling and promotes photoreceptor differentiation in the *Drosophila* eye. *Cell* **123**, 1267–1277 (2005).
60. Davis, B. N., Hilyard, A. C., Lagna, G. & Hata, A. SMAD proteins control DROSHA-mediated microRNA maturation. *Nature* **454**, 56–61 (2008).
61. Avraham, R. *et al.* EGF decreases the abundance of microRNAs that restrain oncogenic transcription factors. *Sci. Signal.* **3**, ra43 (2010).
Reported EGF-induced rapid turnover of a group of microRNAs, termed ID-miRs, which regulate transcription of oncogenic transcription factors.
62. Herschman, H. R. Primary response genes induced by growth factors and tumor promoters. *Annu. Rev. Biochem.* **60**, 281–319 (1991).
63. Amit, I. *et al.* A module of negative feedback regulators defines growth factor signaling. *Nature Genet.* **39**, 503–512 (2007).
Uncovered transcription-dependent feedback loops, which inactivate the early response genes which are collectively upregulated in response to growth factors.
64. Lau, L. F. & Nathans, D. Expression of a set of growth-related immediate early genes in BALB/c 3T3 cells: coordinate regulation with c-fos or c-myc. *Proc. Natl Acad. Sci. USA* **84**, 1182–1186 (1987).
65. Hargreaves, D. C., Horng, T. & Medzhitov, R. Control of inducible gene expression by signal-dependent transcriptional elongation. *Cell* **138**, 129–145 (2009).
66. Fujita, T., Piuz, I. & Schlegel, W. Negative elongation factor NELF controls transcription of immediate early genes in a stimulus-specific manner. *Exp. Cell Res.* **315**, 274–284 (2009).
67. Ramirez-Carrozzi, V. R. *et al.* A unifying model for the selective regulation of inducible transcription by CpG islands and nucleosome remodeling. *Cell* **138**, 114–128 (2009).
68. Wang, Z. *et al.* Genome-wide mapping of HATs and HDACs reveals distinct functions in active and inactive genes. *Cell* **138**, 1019–1031 (2009).
References 65–68 demonstrated permissive structures of the promoters of IEGs, which enable rapid induction of transcription, and also uncovered novel mechanisms that suppress IEG expression at the basal state.
69. Bluthgen, N. *et al.* A systems biological approach suggests that transcriptional feedback regulation by dual-specificity phosphatase 6 shapes extracellular signal-related kinase activity in RAS-transformed fibroblasts. *FEBS J.* **276**, 1024–1035 (2009).
70. Carballo, E., Lai, W. S. & Blackshear, P. J. Feedback inhibition of macrophage tumor necrosis factor-α production by tristetraprolin. *Science* **281**, 1001–1005 (1998).
71. Jing, Q. *et al.* Involvement of microRNA in AU-rich element-mediated mRNA instability. *Cell* **120**, 623–634 (2005).
72. Yilmaz, M. & Christofori, G. EMT, the cytoskeleton, and cancer cell invasion. *Cancer Metastasis Rev.* **28**, 15–33 (2009).
73. Nieman, M. T., Prudoff, R. S., Johnson, K. R. & Wheelock, M. J. N-cadherin promotes motility in human breast cancer cells regardless of their E-cadherin expression. *J. Cell Biol.* **147**, 631–644 (1999).
74. Katz, M. *et al.* A reciprocal tensin-3-cten switch mediates EGF-driven mammary cell migration. *Nature Cell Biol.* **9**, 961–969 (2007).
75. Sporn, M. B. & Todaro, G. J. Autocrine secretion and malignant transformation of cells. *N. Engl. J. Med.* **303**, 878–880 (1980).
76. Joslin, E. J., Opresko, L. K., Wells, A., Wiley, H. S. & Lauffenburger, D. A. EGF-receptor-mediated mammary epithelial cell migration is driven by sustained ERK signaling from autocrine stimulation. *J. Cell Sci.* **120**, 3688–3699 (2007).
77. Goswami, S. *et al.* Macrophages promote the invasion of breast carcinoma cells via a colony-stimulating factor-1/epidermal growth factor paracrine loop. *Cancer Res.* **65**, 5278–5283 (2005).
78. Janes, K. A. *et al.* The response of human epithelial cells to TNF involves an inducible autocrine cascade. *Cell* **124**, 1225–1239 (2006).
Shows that challenging cells with multiple extracellular signals induces an autocrine cascade involving the release of, and response to, additional, newly secreted factors.
79. Sheng, Q. *et al.* An activated ErbB3/NRG1 autocrine loop supports *in vivo* proliferation in ovarian cancer cells. *Cancer Cell* **17**, 298–310 (2010).
80. Khambata-Ford, S. *et al.* Expression of epiregulin and amphiregulin and K-ras mutation status predict disease control in metastatic colorectal cancer patients treated with cetuximab. *J. Clin. Oncol.* **25**, 3230–3237 (2007).
81. Alon, U. Network motifs: theory and experimental approaches. *Nature Rev. Genet.* **8**, 450–461 (2007).
82. Bruggeman, F. J., Westerhoff, H. V., Hoek, J. B. & Kholodenko, B. N. Modular response analysis of cellular regulatory networks. *J. Theor. Biol.* **218**, 507–520 (2002).
83. Marshall, C. J. Specificity of receptor tyrosine kinase signaling: transient versus sustained extracellular signal-regulated kinase activation. *Cell* **80**, 179–185 (1995).
84. Heasley, L. E. & Johnson, G. L. The β-PDGF receptor induces neuronal differentiation of PC12 cells. *Mol. Biol. Cell* **3**, 545–553 (1992).
85. Nagashima, T. *et al.* Quantitative transcriptional control of ErbB receptor signaling undergoes graded to biphasic response for cell differentiation. *J. Biol. Chem.* **282**, 4045–4056 (2007).
86. Santos, S. D., Verveer, P. J. & Bastiaens, P. I. Growth factor-induced MAPK network topology shapes Erk response determining PC-12 cell fate. *Nature Cell Biol.* **9**, 324–330 (2007).
Modular-response analysis was applied to PC-12 cells activated with EGF or NGF. On EGF stimulation, the network exhibited negative feedback, whereas a positive feedback was observed on NGF stimulation.
87. Murphy, L. O., Smith, S., Chen, R. H., Fingar, D. C. & Blenis, J. Molecular interpretation of ERK signal duration by immediate early gene products. *Nature Cell Biol.* **4**, 556–564 (2002).
88. Sasagawa, S., Ozaki, Y., Fujita, K. & Kuroda, S. Prediction and validation of the distinct dynamics of transient and sustained ERK activation. *Nature Cell Biol.* **7**, 365–373 (2005).
89. von Kriegsheim, A. *et al.* Cell fate decisions are specified by the dynamic ERK interactome. *Nature Cell Biol.* **11**, 1458–1464 (2009).
Using proteomics and GF-stimulated PC-12 cells, this report identified 60 proteins that changed their binding to ERK during differentiation, including effectors of signal duration, ERK localization and crosstalk with the AKT pathway.
90. Shankaran, H., Wiley, H. S. & Resat, H. Receptor downregulation and desensitization enhance the information processing ability of signalling receptors. *BMC Syst. Biol.* **1**, 48 (2007).
91. Aquino, G. & Endres, R. G. Increased accuracy of ligand sensing by receptor internalization. *Phys. Rev. E Stat. Nonlin. Soft Matter Phys.* **81**, 021909 (2010).
92. Yeung, K. *et al.* Suppression of Raf-1 kinase activity and MAP kinase signalling by RKIP. *Nature* **401**, 173–177 (1999).
93. Corbit, K. C. *et al.* Activation of Raf-1 signaling by protein kinase C through a mechanism involving Raf kinase inhibitory protein. *J. Biol. Chem.* **278**, 13061–13068 (2003).
94. Bhalla, U. S., Ram, P. T. & Iyengar, R. MAP kinase phosphatase as a locus of flexibility in a mitogen-activated protein kinase signaling network. *Science* **297**, 1018–1023 (2002).
95. Lahav, G. *et al.* Dynamics of the p53-Mdm2 feedback loop in individual cells. *Nature Genet.* **36**, 147–150 (2004).
96. Kholodenko, B. N. Negative feedback and ultrasensitivity can bring about oscillations in the mitogen-activated protein kinase cascades. *Eur. J. Biochem.* **267**, 1583–1588 (2000).
97. Dong, C., Waters, S. B., Holt, K. H. & Pessin, J. E. SOS phosphorylation and disassociation of the Grb2-SOS complex by the ERK and JNK signaling pathways. *J. Biol. Chem.* **271**, 6328–6332 (1996).
98. Waters, S. B. *et al.* Insulin and epidermal growth factor receptors regulate distinct pools of Grb2-SOS in the control of Ras activation. *J. Biol. Chem.* **271**, 18224–18230 (1996).
99. Shin, S. Y. *et al.* Positive- and negative-feedback regulations coordinate the dynamic behavior of the Ras-Raf-MEK-ERK signal transduction pathway. *J. Cell Sci.* **122**, 425–435 (2009).
100. Shankaran, H. *et al.* Rapid and sustained nuclear-cytoplasmic ERK oscillations induced by epidermal growth factor. *Mol. Syst. Biol.* **5**, 332 (2009).
101. Cohen-Saidon, C., Cohen, A. A., Sigal, A., Liron, Y. & Alon, U. Dynamics and variability of ERK2 response to EGF in individual living cells. *Mol. Cell* **36**, 885–893 (2009).
References 99–101 observed oscillatory nucleo-cytoplasmic translocations of ERK in EGF-treated cells, and combined the data with mathematical models. A fold-change response, along with negative-feedback loops (for example, from ERK to SOS) and other mechanisms were found to have crucial roles in generating oscillatory behaviour.
102. Ashall, L. *et al.* Pulsatile stimulation determines timing and specificity of NF-κB-dependent transcription. *Science* **324**, 242–246 (2009).
103. Barabasi, A. L. & Oltvai, Z. N. Network biology: understanding the cell's functional organization. *Nature Rev. Genet.* **5**, 101–113 (2004).
104. Behar, M., Hao, N., Dohlman, H. G. & Elston, T. C. Mathematical and computational analysis of adaptation via feedback inhibition in signal transduction pathways. *Biophys. J.* **93**, 806–821 (2007).
105. Ma, W., Trusina, A., El-Samad, H., Lim, W. A. & Tang, C. Defining network topologies that can achieve biochemical adaptation. *Cell* **138**, 760–773 (2009).
The authors searched all possible three-node enzyme topologies to identify those that could perform adaptation. Only two core topologies emerged: a negative-feedback loop with a buffering node and an incoherent feedforward loop with a proportioner node.

106. Goentoro, L. & Kirschner, M. W. Evidence that fold-change, and not absolute level, of β-catenin dictates Wnt signaling. *Mol. Cell* **36**, 872–884 (2009).
107. Ekstrand, A. J. *et al.* Genes for epidermal growth factor receptor, transforming growth factor α, and epidermal growth factor and their expression in human gliomas *in vivo*. *Cancer Res.* **51**, 2164–2172 (1991).
108. Slamon, D. J. *et al.* Studies of the HER-2/neu proto-oncogene in human breast and ovarian cancer. *Science* **244**, 707–712 (1989).
109. Prickett, T. D. *et al.* Analysis of the tyrosine kinome in melanoma reveals recurrent mutations in ERBB4. *Nature Genet.* **41**, 1127–1132 (2009).
110. Lynch, T. J. *et al.* Activating mutations in the epidermal growth factor receptor underlying responsiveness of non-small-cell lung cancer to gefitinib. *N. Engl. J. Med.* **350**, 2129–2139 (2004).
111. Shtiegman, K. *et al.* Defective ubiquitinylation of EGFR mutants of lung cancer confers prolonged signaling. *Oncogene* **26**, 6968–6978 (2007).
112. Tzahar, E. *et al.* Pathogenic poxviruses reveal viral strategies to exploit the ErbB signaling network. *EMBO J.* **17**, 5948–5963 (1998).
113. Reddy, C. C., Niyogi, S. K., Wells, A., Wiley, H. S. & Lauffenburger, D. A. Engineering epidermal growth factor for enhanced mitogenic potency. *Nature Biotech.* **14**, 1696–1699 (1996).
114. Schulze, A., Lehmann, K., Jefferies, H. B., McMahon, M. & Downward, J. Analysis of the transcriptional program induced by Raf in epithelial cells. *Genes Dev.* **15**, 981–994 (2001).
115. Barozzi, C. *et al.* Relevance of biologic markers in colorectal carcinoma: a comparative study of a broad panel. *Cancer* **94**, 647–657 (2002).
116. Yi, W. *et al.* Expression of leucine-rich repeats and immunoglobulin-like domains (LRIG) proteins in human ependymoma relates to tumor location, WHO grade, and patient age. *Clin. Neuropathol.* **28**, 21–27 (2009).
117. Ying, H. *et al.* Mig-6 controls EGFR trafficking and suppresses gliomagenesis. *Proc. Natl Acad. Sci. USA* **107**, 6912–6917 (2010).
118. Sargin, B. *et al.* Flt3-dependent transformation by inactivating c-Cbl mutations in AML. *Blood* **110**, 1004–1012 (2007).
119. Rao, D. S. *et al.* Huntingtin-interacting protein 1 is overexpressed in prostate and colon cancer and is critical for cellular survival. *J. Clin. Invest.* **110**, 351–360 (2002).
120. Goentoro, L. A. *et al.* Quantifying the Gurken morphogen gradient in *Drosophila* oogenesis. *Dev. Cell* **11**, 263–272 (2006).
121. Yeger-Lotem, E. *et al.* Network motifs in integrated cellular networks of transcription-regulation and protein-protein interaction. *Proc. Natl Acad. Sci. USA* **101**, 5934–5939 (2004).
122. French, A. R., Sudlow, G. P., Wiley, H. S. & Lauffenburger, D. A. Postendocytic trafficking of epidermal growth factor-receptor complexes is mediated through saturable and specific endosomal interactions. *J. Biol. Chem.* **269**, 15749–15755 (1994).
123. Traverse, S. *et al.* EGF triggers neuronal differentiation of PC12 cells that overexpress the EGF receptor. *Curr. Biol.* **4**, 694–701 (1994).

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