

REVIEW

Evolvable signaling networks of receptor tyrosine kinases: relevance of robustness to malignancy and to cancer therapy

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Robust biological signaling networks evolved, through gene duplications, from simple, relatively fragile cascades. Architectural features such as layered configuration, branching and modularity, as well as functional characteristics (e.g., feedback control circuits), enable fail-safe performance in the face of internal and external perturbations. These universal features are exemplified here using the receptor tyrosine kinase (RTK) family. The RTK module is richly mutated and overexpressed in human malignancies, and pharmaceutical interception of its signaling effectively retards growth of specific tumors. Therapy-induced interception of RTK-signaling pathways and the common involvement of drug resistance are respectively considered here as manifestations of fragility and plasticity of robust networks. The systems perspective we present views pathologies as hijackers of biological robustness and offers ways for identifying fragile hubs, as well as strategies to overcome drug resistance.

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Introduction

The ever-burgeoning amount of information on biological processes and their molecular mechanisms has reached

enormous volumes, especially with the introduction of high-throughput genomic and proteomic platforms. To remain meaningful, the flux of reductionist data must be patterned by novel global concepts, as well as integrated by using computational and other means. Through uncovering engineering principles and deducing the logics of complex networks, systems biology offers some attractive solutions. The aim of this review is to exemplify the systems perspective in the context of information relay networks and their relevance to human malignancies. Interested readers are referred to several recent reviews that analyze complex signaling networks, as well as their pathological malfunction, from a systemic perspective (Csete and Doyle, 2004; Kitano, 2004b; Kolch *et al.*, 2005; Hornberg *et al.*, 2006).

Despite the emerging complexity and rich interconnectivities of signal transduction pathways, *CO-OPTION* (see Box 1) of eight generic signaling pathways dominates embryonic development, normal physiology and many diseases. The list of major pathways includes G protein-coupled receptors, nuclear hormone receptors, transforming growth factor beta, Notch, Janus kinase (JAK), Hedgehog, Wntless-related and receptor tyrosine kinases (RTKs) (Pires-daSilva and Sommer, 2003). For several reasons, the latter are the major focus of this review. First, mutated or overexpressed forms of RTKs are frequently identified in human tumors (Blume-Jensen and Hunter, 2001), and second, pharmaceutical targeting of RTKs, such as the epidermal growth factor receptor (EGFR) and its sibling, HER2, can retard tumor growth, primarily in the case of carcinomas (Yarden and Sliwkowski, 2001).

Signaling by growth factors interacting with RTKs, for example signal transduction by EGFR, is enormously complex (for a recent compilation of available data see Oda *et al.*, 2005). Here we offer two views aimed at simplifying RTK signaling: an evolutionary approach that tracks the gradual build up of signaling complexity and the perspective of viral hijackers, agents that abundantly manipulate network controls for their pathogenic benefit. Following a discussion of biological robustness, with an emphasis on control circuits, we discuss systems vulnerability in the context of cancer therapeutics. Last, we review the involvement of secondary resistance to RTK-targeted cancer drugs, and present acquired resistance to signal transduction pharmaceuticals as a demonstration of systems adaptability.

RTKs: a primer

Shared structural landmarks

To precisely coordinate and integrate cellular decisions such as proliferation, differentiation and apoptosis, metazoans developed a set of information relay systems, including the

Box 1 Glossary

Antibody-dependent cell-mediated cytotoxicity (ADCC):

A mechanism of cell-mediated immunity whereby an effector cell of the immune system (usually a natural killer cell) lyses a target cell that has been bound by specific antibodies.

Autocrine loop: Self-production of a stimulatory activity, resulting in autonomous cellular function. For example, coexpression of a mitogenic growth factor and the cognate RTK in the same cancer cell.

Bistability: The ability of a system to transit between two states, 'On' and 'Off', with no, or little, intermediary states.

Co-option: Reusing existing genetic components, metabolic reactions or signaling modules in diverse biological systems, such as in development of body plans, evolution of transcription factors or construction of signaling networks.

Robustness: An inherent property of a system to maintain normal performance despite external and internal perturbations.

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

Random network: A uniform (democratic) network, where most nodes have roughly the same number of links.

Scale-free network: A non-uniform network whose connectivity (number of nodes with a given degree) follows a power law. Scale-free networks are characterized by coexistence of nodes of widely different degrees (scales) (Barbasi and Alberts, 1999), which means that there are nodes with 1–2 links alongside with major hubs.

Evolvability: The capacity of an organism or a biological system to generate heritable new phenotypes.

Targeted therapy: A relatively new field of pharmacology focusing on drugs tailored to inhibit specific molecules, such as enzymes and receptors, thereby avoiding adverse clinical effects, which are usually associated with the application of broad-specificity regimens.

Network hub: A network node having a relatively large number of links to other nodes within its network.

Network motif: A pattern of interaction that recurs in cellular networks significantly more often than in randomized networks.

Composite feedback loop: A composite two-arm loop in which a protein P, that slowly induces (at the transcriptional level) a gene *g*, which upon translation rapidly regulates protein P at the level of protein–protein interactions.

group of RTKs (Blume-Jensen and Hunter, 2001). There are 58 known RTKs in mammals, and they are distributed in 20 subfamilies. Characteristically, an RTK molecule is divided into two parts by a single transmembrane domain (Figure 1): the extracellular domains of RTKs exhibit remarkable structural variation. On the other hand, the cytoplasmic domain comprises primarily a well-conserved bilobular protein tyrosine kinase region. The N-terminal lobe of the kinase domain comprises beta-strands and a single alpha-helix, whereas the C-terminal lobe is composed mainly of alpha-helices, which nest ATP in a cleft defined by the two lobes (Hubbard and Till, 2000).

Shared functional features

Signaling pathways downstream of RTKs are largely shared, although some pathways, for example IRS activation by the insulin receptor family, are subfamily specific. Nevertheless, each RTK is uniquely coupled to an ensemble of signaling pathways whose identity and relative strength of activation constitute an enormous combinatorial complexity, which can be approached by high-throughput experimental strategies

(Schulze *et al*, 2005; Zhang *et al*, 2005; Jones *et al*, 2006). Both the extracellular and the intracellular domains of RTKs are maintained in autoinhibited, locked conformations, which are released when a ligand binds. Ligand-induced dimerization of RTKs is responsible for instigating these alterations. For example, according to the crystal structures of EGFR/ERBB-1 (Garrett *et al*, 2002; Ogiso *et al*, 2002), prior to ligand binding a dimerization loop imposes an intramolecular 'tethered' conformation. Upon ligand binding, a major conformational change detaches the intramolecular tether and stabilizes the 'active' form in which the unmasked loop projects outwards to mediate dimerization. Ectodomain transition to the ligand-bound, active conformation is relayed across the plasma membrane and culminates in the activation of the kinase domain. According to a recent study, the C-lobe of one kinase domain is juxtaposed next to the N-lobe of the other (Zhang *et al*, 2006). Hence, the C-lobe of one RTK serves as the activator of the other kinase domain.

Involvement of RTKs in human cancer

The pivotal role of RTKs as regulators of cellular decisions is apparent when acknowledging that these mitogenic receptors are encoded by the largest group of oncogenes sharing structural homology (Blume-Jensen and Hunter, 2001), and as many as ~30% of the RTKs are repeatedly found mutated or overexpressed in different malignancies. Examples of RTKs involved in human cancer (Figure 1) include RET, a coreceptor for glial cell line-derived neurotrophic factors, the subfamily of the hepatocyte growth factor receptors (HGF-R/c-MET), c-KIT, the receptor for the stem cell factor, EGFR and HER2. The most common thyroid tumors are driven by chromosomal rearrangements, which fuse the kinase domain of RET to a variety of protein dimerization domains. In addition to somatic gene fusions, germline mutations are involved in three familial tumor syndromes: multiple endocrine neoplasia 2A and 2B, and familial medullary thyroid carcinoma (FMTC). Most MEN2A and FMTC mutations affect conserved extracellular cysteines, causing constitutive receptor dimerization (Santoro *et al*, 1995). MEN2B is caused by a recurrent mutation replacing a conserved methionine within the kinase domain and altering substrate selectivity of RET (Boccardi *et al*, 1997). Another mechanism of oncogenic activation is exemplified by c-KIT. Several oncogenic mutations found in gastrointestinal stromal tumors affect the catalytic region or the juxtamembrane domain of this RTK, which participate in autophosphorylation and in inhibition of kinase activity (Hirota *et al*, 1998).

Excessive EGFR/ERBB signaling, arising from receptor overexpression, mutations or *AUTOCRINE* stimulation, is a hallmark of a wide variety of solid tumors. Amplification of the *ERBB-2/HER2* gene can be found in 20–30% of metastatic breast lesions (Slamon *et al*, 1987) and high EGFR expression was found in small fractions of several types of carcinoma (e.g., head and neck cancer and brain tumors; Ekstrand *et al*, 1991). Somatic acquired EGFR mutations in lung cancer activate receptor phosphorylation and they predict significant clinical responses to kinase inhibitors (Lynch *et al*, 2004). All mutations are restricted to the tyrosine kinase domain of EGFR. Similar to EGFR mutations, a kinase-mutated HER2/ERBB-2 was shown to be more potent than wild-type HER2

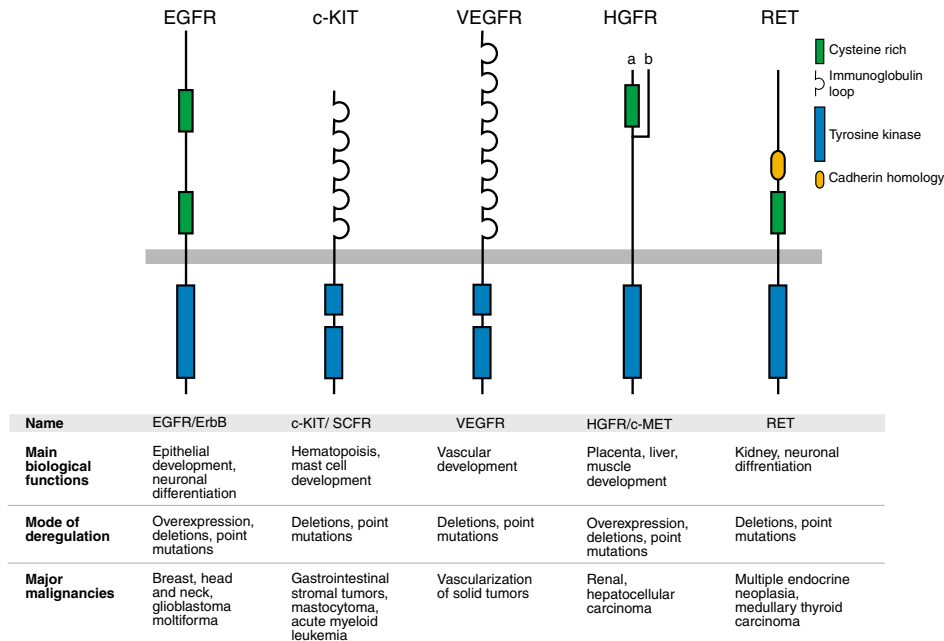


Figure 1 RTKs associated with cancer. The structures of several cancer-relevant RTKs are schematically presented, including conserved domains and information on involvement in malignancies. The horizontal gray bar represents the plasma membrane. VEGFR, vascular endothelial growth factor receptor; HGFR, hepatocyte growth factor receptor; RET, rearranged during transfection.

in the activation of signal transduction pathways and in inducing invasiveness and tumorigenicity (Wang *et al*, 2006).

The enigma of pseudo-RTKs and the ligandless receptor, HER2

Several RTKs (e.g., RYK/VIK and KLG) belong to a group of inactive kinases, which have been termed pseudokinases (Boudeau *et al*, 2006). The best-characterized pseudo-RTK is ERBB-3. Unlike other RTKs, neither ERBB-3 nor HER2 can undergo direct activation by a ligand; whereas ERBB-3's intrinsic kinase activity is impaired (Guy *et al*, 1994), no known soluble ligand binds to and activates HER2 (Klapper *et al*, 1999). Hence, these non-autonomous receptors must heterodimerize with each other, as well as with other RTKs, to generate relatively potent signals for cell growth. All four ERBB proteins evolved from a single precursor RTK represented by the worm's LET-23 (Aroian *et al*, 1990). Why non-autonomous RTKs were preserved in the course of evolution is an open question, which we address below from an evolutionary point of view that highlights the relevance of a systems biology approach to RTK signaling.

The evolution of RTK-signaling networks

According to one interpretation, the HER2-ERBB3 enigma is due to accidental receptor inactivation events that occurred in the course of metazoan evolution. An alternative explanation considers the generation of non-autonomous receptors such as HER2/ERBB-2 and ERBB-3 as a by-product of several evolutionary trends that transformed linear signaling cascades into layered, richly interconnected networks (see Figure 2).

Conceivably, the conversion from linear to network architecture assisted generation of both novel body plans and new cell lineages in metazoans. Furthermore, the evolutionary trends we review below and the ensuing establishment of networks conferred both *ROBUSTNESS* (Stelling *et al*, 2004) and *EVOLVABILITY* (Kirschner and Gerhart, 1998), while the co-option of relatively few pathways avoided inflated expansion of primordial genomes.

Need for increased control and capacity of signaling pathways—from unicellular to multicellular organisms

Unicellular organisms are in close contact with their environment and directly respond to nutrients, vitamins, radiation, radicals and, in some cases, also mating factors. Because these external stimuli often permeate the cell membrane to interact with cytoplasmic or nuclear targets, generic multilayered signaling cascades are rare in unicellular organisms. Such fundamental cascades widely evolved in bilaterians, multicellular organisms presenting a body cavity and bilateral symmetry. However, the more sophisticated RTK cascades are represented almost exclusively in metazoans (Shiu and Li, 2004). One enlightening exception to this observation is seen for the unicellular organism closest to metazoans, a flagella-containing group of protists called Choanoflagellates (Pires-daSilva and Sommer, 2003). These protists are recognized as being closest to prospective unicellular ancestors of metazoans and are 'between' fungi and multicellular animals. Evidence has accumulated that Choanoflagellates already invented generic signaling cascades, raising the possibility that multicellularity evolution necessitated the establishment

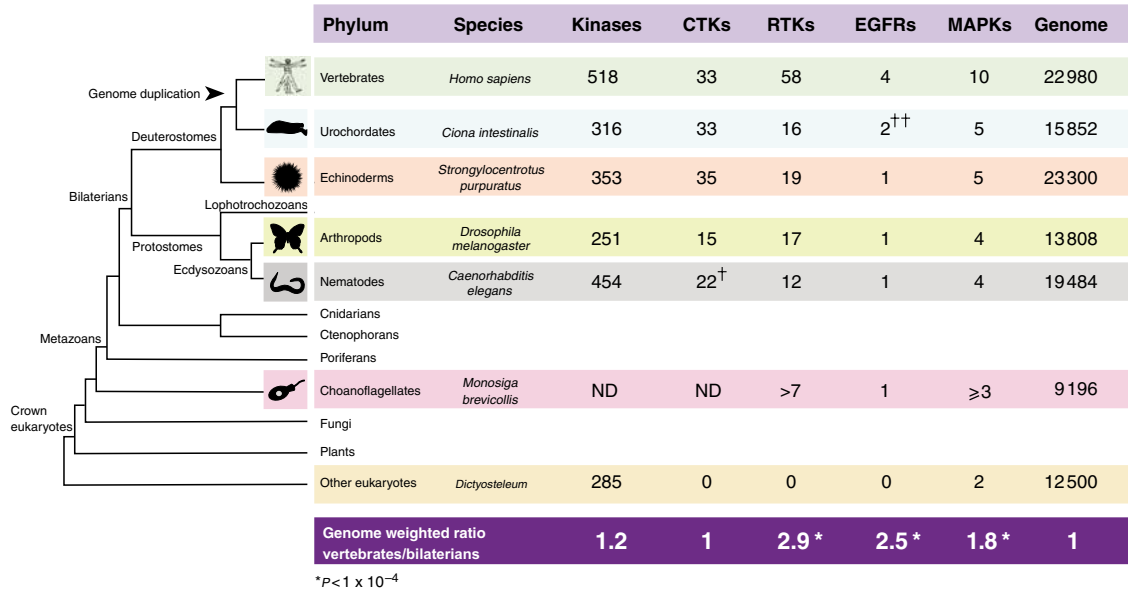


Figure 2 The evolution and expansion of RTK signaling. The number of kinases and MAPKs in several representative species are listed for vertebrates and for key invertebrate phyla, or other eukaryotes with whole-genome data. The number of CTKs (cytoplasmic tyrosine kinases) and RTKs were derived from published total tyrosine kinase counts, except where marked and noted below, as were counts of EGFRs/ErbB (EGFRs) and MAPKs. The ratio of vertebrate to average invertebrate counts of each protein category was calculated, as weighted for total proteome size. Asterisks indicate that the RTK, EGFR and MAPK ratios show the most significant departures from a ratio of one, each with a P -value < 0.0001 . The counts were derived from the respective published genomes and the following references: urochordate (Satou *et al*, 2003); sea urchin (Bradham *et al*, 2006); fruit fly (Morrison *et al*, 2000); slime mold (Goldberg *et al*, 2006); a choanoflagellate [assembly version 1.0 (genome.jgi-psf.org/Monbr1)] and (Shiu and Li, 2004). The notable departures from published counts are as follows: † the 22 *Caenorhabditis elegans* CTKs do not include 37 genes of the expanded 38 gene FER family, nor 19 of the 21 paralogs of the nematode specific expansion of Kin-9 and Kin-16 genes; ††, the two EGFRs in *Ciona intetinalis* reflect a duplication event that took place after urochordates split and diverged from the vertebrate lineage.

of signaling pathways. For example, *Monosiga brevicollis*, a Choanoflagellate, provisionally contains one or more representatives of seven subfamilies of RTKs (genome.jgi-psf.org/Monbr1). In conclusion, tyrosine kinases may be viewed as the providence of metazoans and their immediate ‘unicellular predecessor’.

Gene fusion—from simple proteins to multidomain proteins

The availability of detailed whole-genome sequence data, as well as interspecies comparisons, indicated that vertebrate proteins are characteristically larger and contain more structurally recognizable domains when compared with their orthologs in *Caenorhabditis elegans* and *Drosophila*. A common hypothesis argues that the large modular structures of mammalian proteins involved in information relay systems are the outcome of repeated gene fusion events, combining diverse functions in one protein. RTKs provide an interesting example of gene fusion and ligand–receptor coevolution (for an example of the evolution of the neurotrophin family and TRK receptors see Lanave *et al*, 2007). The origin of their divergent extracellular ligand-binding domains is thought to represent a primordial binding protein, specializing in recognition of extracellular ligands or nutrients. Conceivably, by means of chromosomal rearrangement and gene fusion, this domain likely fused to a transmembrane protein whose hydrophobic domain conferred anchorage to the cell surface, whereas the cytoplasmic tail enabled internalization of the

extracellular ligand. Apparently, a second gene fusion event extended the cytoplasmic domain by adding the catalytic region of a cytoplasmic tyrosine kinase (CTK) similar to the current version of a SRC family kinase. The resulting archetypal RTK acquired an ability to stimulate auto- and *trans*-phosphorylation in response to ligand binding, while harnessing the cargo internalization capability for effective desensitization of signaling.

The need for versatility and control—from nuclear hormone receptors to receptors for polypeptide factors

Figure 2 shows the number of RTKs per phylum throughout evolution. A trend of RTK expansion paralleled evolution of metazoans and peaked in vertebrates. Notably, no comparable trend impacted the superfamily of nuclear hormone receptors (NHRs). Another reflection of the trend leading to expanded signaling by polypeptide growth factors is exhibited by the number of RTK ligands, which increased in the case of the ERBB family from one in worms to 11 ligands and many isoforms in human. Conceivably, the transition from steroids, retinoic acid and other NHR ligands, whose synthesis is cumbersome and transcriptional action direct, to polypeptide ligands, conferred several advantages: polypeptide growth factors are better regulated at the level of synthesis, their multiple binding proteins tightly control availability after synthesis and their actions at the membrane/cytoplasm and at the level of gene expression contrast with the mostly genomic action of NHRs.

Gene duplication and sub-functionalization—from individual genes to gene families

An important feature of the evolution of signaling systems is the occurrence of gene duplications and subsequent protein sequence divergence. Gene duplication events frequently occur in the course of evolution at a background rate of 0.01 duplications per gene in million years (Lynch and Conery, 2000), and in punctuated large-scale events. Although the majority of duplicated genes are either lost or become pseudogenes, in many cases the ensuing genes are retained in the genome. The most notable retaining mechanism is *SUB-FUNCTIONALIZATION*, a process that partially inactivates sub-functions and promotes collaborations between duplicated gene products.

Expanding the tyrosine kinome and the RTK family

The tyrosine kinase protein complement of the vertebrate kinome is larger than that of invertebrate kinomes (Figure 2), and a core increase of RTKs, rather than CTKs, accounts for the larger kinomes of vertebrates (Shiu and Li, 2004). Thus, the kinomes of nematodes, flies, sea urchins and urochordates contain, respectively, 12, 18, 19 and 16 RTKs (Tan and Kim, 1999; Morrison *et al*, 2000; Bradham *et al*, 2006), but the kinomes of mammals contain 58 RTKs. Of these, 14 represent a specific expansion of the Ephrin receptor (EPHR) subfamily, which is presaged by six receptors in the kinome of the basal chordate *Ciona intestinalis* (Leveugle *et al*, 2004). Whether or not the chordate-specific EPHR expansion is discounted, the RTKs represent a two- to fourfold increase from invertebrates to vertebrates, which is consistent with genome duplication at the root of vertebrate evolution. Indeed, the later of the two bursts of gene duplications in metazoans has been dated to 400 million years ago (Miyata and Suga, 2001). Thus, a trend of RTK expansion peaked in vertebrates with duplications or quadruplications, and for nearly every one of the RTK subfamilies, the human paralogs are present in a two- to fourfold excess over their protostome, or invertebrate deuterostome, orthologs.

Evolution of subtype I RTKs (ERBB) as an example

The cast of mammalian ERBB proteins fits into the presumed route of RTK evolution. In line with whole-genome quadruplication, the family is represented by a single ligand–receptor pair in *C. elegans*, and the mammalian family includes four members, which were likely preceded by two ancestors, an ERBB-1/2 precursor and an ERBB-3/4 ancestor, as well as two respective groups of ligands: EGF-like growth factors and the neuregulins (Stein and Staros, 2000). Moreover, the family presents an example of sub-functionalization: ERBB-2/HER2 and ERBB-3 are likely the products of a coordinated gene duplication, which complementarily denied a ligand from HER2 and inactivated the kinase function of ERBB-3, thereby promoting receptor collaboration.

From linear cascades to scale-free signaling networks

Similar to the ERBB family, other RTKs and ligands underwent expansion through gene duplications. Beyond the numerical

growth and conversion of individual RTKs to distinct families, gene duplication greatly impacted the topology of evolving RTK-signaling networks: it has been argued that upon duplication highly connected proteins retain interactions with both gene products, which creates networks rich in highly connected nodes (Rzhetsky and Gomez, 2001; Pastor-Satorras *et al*, 2003). As a result, earlier and more conserved nodes evolve into richly linked nodes, namely *NETWORK HUBS*. Further, mathematical analysis of network's growth processes has demonstrated that newly added nodes prefer to connect to nodes that already are well connected (so-called 'preferential attachment'; Barabasi and Oltavi, 2004). This growth process proposes an explanation to yet another trend in the evolution of signaling systems, one that transforms *RANDOM NETWORKS* into the hub-enriched topology called *SCALE-FREE NETWORK*. In conclusion, the trends and growth processes we reviewed gradually transformed simple, relatively fragile, linear arrangements of ligands–RTK–effectors into layered network configurations, which greatly enhance reproducibility and reliability of signal transfer (Figure 3).

How do RTK networks maintain functional robustness?

The above-described collection of evolutionary trends permitted vertebrates to evolve progressively more robust signaling networks, while maintaining the overall gene number of their immediate predecessors. One important advantage of networks of RTKs and other signaling systems is their ability to maintain output reproducibility, despite input variation and inherently stochastic signal processing (Kholodenko, 2006). Several critical design features impart functional robustness (see Box 2). Structurally, robust systems share a bow-tie structure in which a core process receives diverse inputs and reproducibly integrates them to generate a myriad of outputs. Typically, the bow-tie structure comprises several modules, which are partially redundant. Module diversity and redundancy allow compensatory functioning in case of component's failure. Further, modularity enables reutilization of genetic circuits in different biological settings, adaptation to rapidly changing environments (Alon, 2003), as well as the generation of new cell lineages (Tautz, 2000; Alon, 2006). In addition to architectural features, robust networks share functional attributes like dynamic switching of signals into alternative pathways (plasticity), and the ability to transiently accumulate protein aberrations without significantly altering network's outcome (tolerance).

Systems control: the power of feedback loops

Perhaps the main functional feature that accounts for robustness comprises systems control, namely a collection of feedback loops, which quantitatively relate network's output to a varying input (Freeman, 2000). Positive feedback loops enhance the amplitude and prolong the active state to convey robustness. Further, such loops can generate an irreversible biochemical response from a transient growth factor stimulus (Xiong and Ferrell, 2003). One important mechanism of positive feedback is based on autocrine loops in which RTK ligands are produced following receptor activation. Likewise,

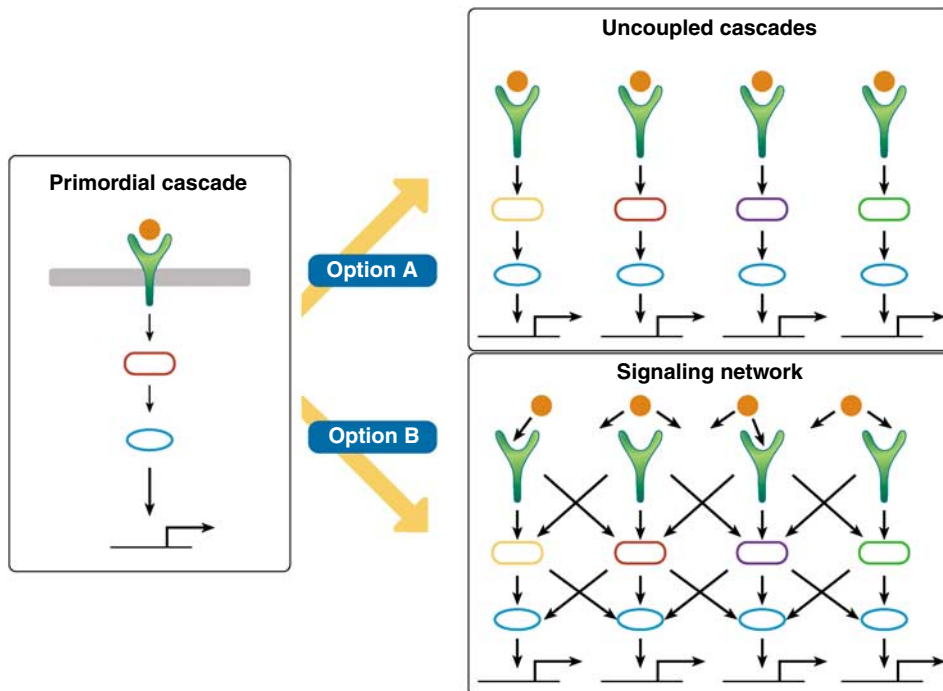


Figure 3 From a vertical RTK cascade to a signaling network. The boxed cascade (left) represents an invertebrate primordial signaling pathway comprising a growth factor, an RTK and a signaling cascade culminating in regulation of gene expression (horizontal arrows). Two RTK expansion scenarios are presented. According to Option A, two events of RTK duplication generate four vertical cascades that gradually diverge, but they remain isolated from each other. According to Option B, the four cascades richly interact and ultimately establish a layered signaling network. The lines of evidence we describe herein propose that the evolution of RTKs in vertebrates preferred Option B, because it imparts robustness and guarantees output reproducibility.

Box 2 Architectural and functional features shared by robust biological and technological systems

Bow-tie structure: An hourglass configuration linking input and output layers through a core circuitry entailing a complex, but reproducible, signal-processing module. In this structure the core circuitry is a point of fragility.

Modularity: Division into partially autonomous sub-systems built to contain damage and diversify signal processing.

Functional overlap (redundancy): Coexistence of functionally similar modules and individual components, thus offering alternate routing of signals in case of component/module inactivation.

System controls: Linked circuitries ensuring dynamic and bidirectional coupling of output to input. Both negative and positive feedback loops exist and they permit amplification of weak signals, restraining of overt signals, as well as noise dampening.

Plasticity (pathway switching): Rich branching and functional flexibility allowing dynamic switching of signals into alternative pathways, to achieve nearly identical outcomes.

Tolerance: Network's ability to transiently accumulate structural and functional aberrations without significantly altering the outcome of incoming signals. The molecular basis of tolerance includes chaperone proteins that refold mildly mutated client proteins (Sangster *et al*, 2004).

negative feedback loops constitute a central mechanism by which systems attain robustness, as they comprise a major stabilizing role in complex circuits (Smolen *et al*, 2000). From an engineering point of view, denser feedback circuitries characterize rapidly responding elements such as the immediate-early genes (IEGs) regulating AP-1 activity (Sassone-Corsi *et al*, 1988), and nuclear or cytoplasmic hubs, such as c-Myc,

MAPK and p53. The large spectrum of RTK's feedback control mechanisms may be divided into two types: feedback loops comprising pre-existing components, which undergo post-translational modifications to enable immediate tuning of the output (Dikic and Giordano, 2003; Santos *et al*, 2007), as well as feedback loops relying on newly synthesized components, a collection of IEGs and delayed-early genes (DEGs; Table I), which control response time and increase network's robustness. Because mRNA synthesis and subsequent protein synthesis and post-translational modifications/translocations may take 15–90 min, this time window defines the major temporal domain of RTK signaling (Figure 4).

Pre-existing negative feedback regulators of RTKs

Ubiquitin ligases, protein kinases and phosphatases, as well as adaptor proteins, play major roles in immediate regulation of RTK signals (Dikic and Giordano, 2003). c-CBL is a phosphotyrosine-activated mammalian E3 ubiquitin ligase that critically instigates signal attenuation by conjugating ubiquitin to activated RTKs, thereby promoting receptor endocytosis and lysosomal degradation (Marmor and Yarden, 2004). A second example, which seems to be a recurrent circuit (*NETWORK MOTIF*) in signaling pathways, includes an inhibitory phosphorylation connecting a downstream signaling component with its upstream activating enzyme, as in the case of the ERK/MPAK-signaling cascade where both MAPKKK (RAF) and MAPKK (MEK) are feedback regulated by negative edges from the downstream MAPK (ERK) (Santos *et al*, 2007).

Table I IEGs and DEGs downstream of RTKs

Gene symbol	Time of peak expression ~ (mRNA, min)	Transcription complex/signaling pathway
<i>IEG</i>		
FOS ^a	10–20	AP1
JUN ^a	10–20	AP1
EGR1	30	EGR
<i>DEG</i>		
KLF2	40–60	AP1, NFKB
KLF6	40–60	AP1, NFKB
ZFP36	40	AP1, NFKBs stability
JUNB	40–60	AP1
FOSL1	60–120	AP1
FOSL2	60	AP1
NAB2	120	EGR
MAFF	120	AP1, NF-E2
EGR3	40–60	EGR
DUSPs	30–120	MAPKs
ATF3	40–60	NFKB, AP1
CREM	60–120	CREB
NFKBIA (IKB α)	30	NFKB
NFKBIE (IKB ϵ)	90–120	NFKB

Abbreviations: DEG, delayed-early gene; IEG, immediate-early gene; RTK, receptor tyrosine kinase.

^aHomologous to a retroviral oncogene.

Newly synthesized feedback regulators of RTKs

Transcriptional negative feedback regulation of RTKs first emerged from genetic screens of lower organisms (Casici and Freeman, 1999). For instance, growth factor activation of the ERK/MAPK-signaling pathway in mammalian cells culminates in ERK translocation to the nucleus, to activate transcriptional complexes. Along with transcriptional repressors, and other proteins (Table I), a broad group of dual-specificity phosphatases (DUSPs, also known as MKPs) are transcriptionally induced by MAPK activity to feedback inhibit the function of MAPKs (Amit *et al.*, 2007). A similar example entails Sprouty proteins, which are newly induced by growth factors and antagonize RTK signaling. Similarly, the fibroblasts-derived growth factor receptor (FGFR) inhibitor, SEF, is newly synthesized in response to FGF (Tsang and Dawid, 2004). In another example, cytokine signaling through the JAK/STAT-signaling pathway is feedback inhibited by the suppressor of cytokine signaling (SOCS1), which targets for proteasomal degradation several proteins of the JAK/STAT pathway. Interestingly, the inducible adaptor protein ERRFI1/MIG6/RALT specifically inhibits ERBB proteins by reducing their autophosphorylation (Ferby *et al.*, 2006), whereas NFKB signaling is feedback inhibited by the combined ubiquitinylation and deubiquitinylation activity of the TNFAIP3/A20 protein (Wertz *et al.*, 2004).

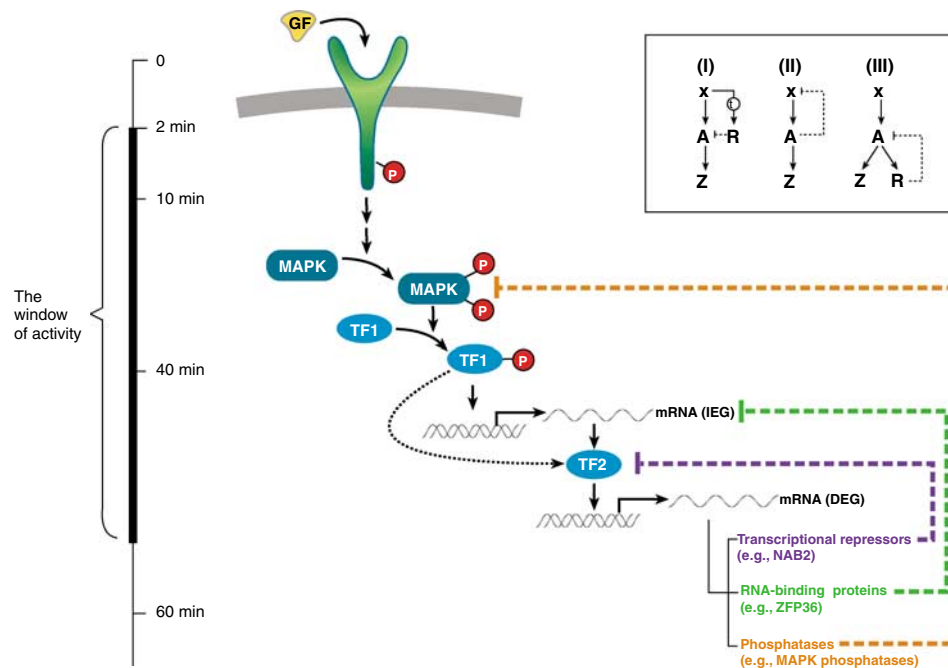


Figure 4 Dense feedback circuits define the window of RTK activity. The timeline (left) indicates the window of growth factor (GF) activity following binding to an RTK. Receptor phosphorylation (denoted by P) is followed by sequential activation of a kinase cascade culminating in MAPK activation through double phosphorylation. MAPK translocation to the nucleus enables direct phosphorylation of transcription factors (TF1), which activate transcription of IEGs (e.g., the AP-1 components JUN and FOS). IEGs regulate a second wave of transcription. The DEGs encode a broad range of proteins, including negative regulators. The signaling arm (orange line) is regulated at the tier of MAPKs by the group of DUSPs, whereas transcription is regulated by the induction of transcriptional repressors (blue line; e.g. KLF2 and ATF3) and RNA-binding proteins (green line; e.g. ZFP-36), which regulate mRNA stability. Collectively, these feedback loops shut the window of RTK signaling. Boxed are three diagrams of mixed feedback circuits potentially forming stable expression profiles of late-induced genes (e.g., gene Z; solid lines represent transcriptional edges, and a dashed line indicates protein–protein interactions). **I:** Transcription factor x immediately activates a transcriptional activator A and slowly activates, after an intrinsic delay τ , a transcriptional repressor R. This module enables pulsed induction of a target gene Z. **II:** A negative feedback loop comprising a transcription activator x, whose activity is attenuated by its target gene A, resulting in a defined window of expression of gene Z. **III:** A negative feedback loop comprising a repressor R, which is connected by a transcriptional edge to the immediate-early transcriptional activator A, thus defining the temporal activity of A by the transcription of both R and the output gene Z.

Composite feedback loops

An important machinery of feedback control is condensed at the level of mRNA regulation, a network layer enriched with hub elements (Figure 5). Early observations reported on ‘super-induction’ of IEG products, upon cell treatment with growth factors, especially in the presence of protein synthesis inhibitors (Lau and Nathans, 1987). This phenomenon has been attributed to inducible proteins that regulate the rates of both mRNA synthesis and mRNA degradation. Consistent with ‘super-induction’, large-scale analysis of regulatory pathways in yeast identified a *COMPOSITE FEEDBACK LOOP* as a recurring motif (Yeager-Lotem *et al*, 2004). This two-arm loop comprises a protein P that slowly induces (at the transcription level) several target genes, including a gene whose translation product rapidly regulates protein P through protein–protein interactions. The alternative, a combination of two slow arms, would cause oscillations, which explains the stabilizing role for composite feedback loops in RTK networks. Three previously described general scenarios of composite feedback loops (Alon, 2006) are exemplified below in the context of RTKs (Table I; Figure 4).

Type I

Rapid induction of a transcription activator and slow transcription of a transcription repressor, allowing a temporally defined window of activity. Examples include FOSL1 and JUNB, which are induced in a delayed manner compared with the rapid induction of the AP1 components FOS and JUN.

Type II

This autoregulatory loop utilizes the lag between transcription and translation. For example, newly synthesized FOS binds to

elements within its own promoter to inhibit transcription of the FOS mRNA (Sassone-Corsi *et al*, 1988).

Type III

This feedback loop comprises a transcriptional activator regulating its own transcription repressor. For example, the TCF transcription factors are feedback regulated by their own transcriptional products, namely the Id proteins (Yates *et al*, 1999).

Viruses and diseases are master manipulators of robust RTK networks

Although many robust cellular programs maintain stable and regulated function under a broad range of perturbations, as will be described below, certain pathologies and various viruses selected vulnerable network’s nodes, as well as features of systems control, thereby taking advantage of the intrinsic robustness of the cellular program for their own purposes.

DNA and RNA viruses

Certain types of oncogenic DNA and RNA viruses devised strategies to harness cellular programs. Interestingly, while retroviral oncogenes are the products of transduction of cellular genes, oncogenes of DNA viruses represent primarily novel designs. Nevertheless, DNA and RNA viruses share some cellular targets. One example relates to the CTK SRC, a truncated form of which is encoded by the Rous sarcoma virus, and the cellular form of which is bound and activated by the middle T antigen of a DNA virus, Polyoma (Courtneidge and

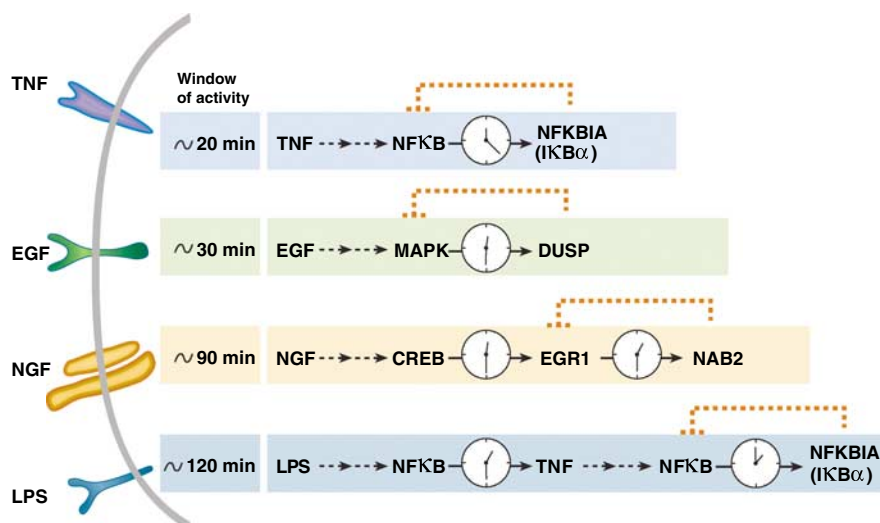


Figure 5 Examples of composite feedback loops defining windows of activity of various extracellular ligands. Ligand–receptor interactions (left) are followed by rapid activation of a signaling protein (e.g., MAPK), which induces in a delayed manner (see clock) an inhibitory protein (e.g., DUSP; solid lines define transcriptional edges and dashed lines represent protein–protein interactions). The coupling of a slow transcriptional arm and a rapid protein interaction arm sets the interval of window opening (~30 min in the case for EGF and MAPK). A longer (~90 min) window of transcriptional activity is achieved when a relay of two transcriptional processes (e.g., EGR1 and NAB2) is needed to produce the negative regulator (NAB2) downstream of the nerve growth factor (NGF). NFκB serves as a target of several growth factors and cytokines. Two examples of pulsed NFκB activation are presented: a short window of activity is generated by the circuit of the negative regulator IκBα, which inhibits NFκB activity downstream of the TNFR. A relatively long (~120 min) activation of NFκB is induced by lipopolysaccharide (LPS): an initial weak activation of NFκB produces a feed-forward element (TNF) and the second is a strong activation of NFκB by TNF, which activates the transcription of the inhibitor IκBα.

Smith, 1984). Yet another example relates to EGFR: the avian erythroblastosis virus encodes a truncated version of EGFR (Downward *et al*, 1984), whereas many ligands of this receptor, and of other ERBB family members, are encoded by pox viruses (Tzahar *et al*, 1998). By encoding (or by inducing) ligands, or active forms of either RTKs or their downstream targets, pathogenic viruses manipulate an important feature of systems control. RTKs, transcription factors and other control modules, are able to oscillate between two or more states. State transitions are normally controlled by switch-like mechanisms involving both positive and negative feedback loops, but viruses often lock such systems in the active state (Hunter, 2000). It is worth noting that oncogenic viruses disproportionately lock specific signaling hubs, such as the RAS-RAF and the PI3K-AKT nodes (Rapp *et al*, 2006), and these very same hubs are frequently mutated in human tumors (Hanahan and Weinberg, 2000). Conceivably, this viral preference reflects the scale-free nature of cellular signaling networks, as well as the inherent vulnerability of major hubs developed along the transition from uniform to scale-free systems (Barabasi and Oltavi, 2004). Along this vein, hubs independently identified by both viruses and cancer mutations may serve as targets for effective therapeutic interventions (see below).

Cancer tactics that circumvent systems control

Malignant growth associates with several traits common to most types of human tumors (Hanahan and Weinberg, 2000). Some traits, for example those related to apoptosis, angiogenesis and metastasis, are directly regulated by RTKs. For example, self-sufficiency in growth signals often results from autocrine loops such as those involving the transforming growth factor alpha and HB-EGF, whose synthesis and cleavage at the cell surface require activation of MAPK and proteinases of the ADAM family (Schafer *et al*, 2004), respectively. Acquisition of growth autonomy by tumor cells may be imparted by mutationally activated RTKs (Blume-Jensen and Hunter, 2001), as well as by mutations affecting a relatively small group of signaling molecules. For instance, oncogenic mutations impinge on components regulating either the RAS-MAPK pathway, primarily mutations in RAS (Bos, 1989) and B-RAF (Davies *et al*, 2002), or the PI3K-AKT/PKB pathway, including mutations in the catalytic subunit of PI3K (Samuels *et al*, 2004), as well as loss of the PTEN tumor suppressor.

Along with mutational activation of RTK signaling, high-throughput analyses reveal that loss of negative feedback loops characterizes solid tumors (Amit *et al*, 2007). For example, EGF upregulates a delayed burst of negative regulators, including MAPK phosphatases (e.g., DUSP6 and DUSP7), transcription repressors (e.g., KLF2 and FOSL1) and RNA-binding proteins (e.g., ZFP-36 and TIAL1), which act upon MAPK and components of the AP1 complex to down-regulate their proliferation-promoting activity. Particularly interesting are late-induced proteins, like ZFP-36, able to bind AU-rich elements (AREs) in 3' untranslated regions of mRNAs (Carballo *et al*, 1998). A large number of the RTK-induced genes, including *c-FOS*, contain AU-rich sequences within their 3' untranslated regions. Interestingly ZFP-36 cooperates with a micro-RNA (miR16) in mRNA degradation (Jing *et al*, 2005),

raising the possibility that micro-RNAs play essential roles in the feedback regulation of RTK signaling. Interestingly, a subset of the late-induced RNA- and DNA-binding proteins is constitutively downregulated in a large variety of solid tumors, and diminished expression predicts shorter survival of ovarian and prostate cancer patients (Amit *et al*, 2007). In striking similarity, a partially overlapping set of negative feedback loops is upregulated upon treatment of thyrocytes with the thyroid-stimulating hormone (TSH; van Staveren *et al*, 2006). Moreover, some regulators were found to be downregulated in adenomas, suggesting a loss of negative feedback control in the tumors. In conclusion, in similarity to oncogenic viruses, cancer-promoting mutations lock RTK signaling in the active state by elevating forward processes, as well as by inhibiting negative feedback loops.

Network's fragility: the basis of RTK-targeted cancer therapy

According to a widely accepted model, originally applied to colorectal cancer (Vogelstein and Kinzler, 2004), stepwise accumulation of mutations in proto-oncogenes, tumor suppressor genes, as well as genes encoding DNA-repair proteins, drives cancer progression from a hyperplastic, benign lesion to a metastasizing tumor. Cataloging the set of oncogenic mutations of specific carcinomas already permits clinicians to intercept tumorigenic mechanisms by using novel targeted therapies. Unlike cytotoxic strategies, which are relatively non-selective and inadvertently increase intratumoral heterogeneity, *TARGETED THERAPY* addresses homogeneously distributed lesions. From a systemic perspective, successful application of cancer therapy necessitates identification of fragile aspects of tumors' robustness, an emergent property acquired throughout cancer progression. The Highly Optimized Theory (HOT), originally applied to technological systems, argues that evolvable systems are robust against common perturbations, but they show fragility against unusual ones (Carlson and Doyle, 2000). When applied to RTK networks, HOT predicts resistance to interceptions of individual components (single-agent therapy), but fragility in the face of simultaneous perturbations (combination therapy), a rarely occurring event for evolution-trained networks. Another type of fragility derives from the exaggerated reliance of scale-free systems on very few hubs. Indeed, the tradeoff of tumors' robustness is 'addiction' to specific oncogenes (Weinstein, 2002), as well as to nutrients and blood supply. Hence, drug-mediated blockade of specific oncogenes, as well as deprivation of blood supply, may retard tumor growth. Frequent genetic aberrations in epithelial tumors, as well as roles in cell proliferation, metastasis and angiogenesis, make RTK signaling one of the most attractive target for anticancer therapies (Baselga, 2006). In the following sections we review clinically approved and experimental RTK-targeting drugs (see Table II) from a systems biology perspective.

Monoclonal antibodies

Humanized, chimerized or completely human antibodies to RTKs are already in clinical use. They include an antibody to

HER2/ERBB-2 (trastuzumab), approved for breast cancer treatment, and two anti-EGFR/ERBB-1 antibodies (cetuximab and panitumumab), approved for treatment of colorectal cancer and head and neck cancer. Likewise, an antibody to the vascular endothelial growth factor (VEGF), Bevacizumab, has been approved for treatment of colorectal cancer (Ferrara, 2005), raising the possibility that more anti-ligand and anti-receptor monoclonal antibodies (mAbs) will show clinical efficacy. Indeed, mAbs to VEGFR-2, insulin-like growth factor 1 receptor (IGF1-R) and c-MET/HGF-R may enter clinical tests in the near future (Ben-Kasus *et al*, 2007). Apparently, two classes of molecular mechanisms enable mAbs to inhibit cancer cell growth: immune mechanisms involving *ANTI-BODY-DEPENDENT CELL-MEDIATED CYTOTOXICITY* (ADCC; Clynes *et al*, 2000) and a variety of non-immune mechanisms that intercept tumorigenesis, including triggering of mitochondria-mediated apoptosis, blocking angiogenesis, inhibiting cell cycle progression, interfering with signaling cascades and accelerating receptor internalization (Ben-Kasus *et al*, 2007). Presumably, the combination of immune and other mechanisms presents uncommon perturbations that fail tumor robustness. Remarkably, mAbs to RTKs are clinically used primarily in combination with cytotoxic regimens. Thus, combining trastuzumab with anthracyclins or taxanes increases the average time to breast cancer progression, both clinically approved antibodies to EGFR, cetuximab and panitumumab, improve cytotoxicity of chemotherapy, and the combination of cetuximab and radiotherapy reduces mortality of patients with head and neck cancer (Bonner *et al*, 2006). Conceivably, the ability of mAbs to sensitize cancer cells to cytotoxic drugs is a manifestation of the ability of double interceptions to overcome network's modularity.

Tyrosine kinase inhibitors

Low-molecular-weight compounds that block the ATP-binding sites of RTKs present surprisingly high selectivity. Several

reversible inhibitors have already been approved (Table II), and irreversible (covalent) inhibitors are in clinical development. Among the approved drugs are Imatinib (Gleevec), an inhibitor of BCR-ABL and c-KIT, approved for treatment of leukemia and gastrointestinal spindle tumors, as well as two EGFR inhibitors, gefitinib and erlotinib, approved for non-small cell lung cancer. Likewise, sorafenib and sunitinib are broader specificity compounds acting at VEGF receptors and approved for advanced renal cell carcinoma (Carmeliet and Jain, 2000). From a system's perspective, the efficacy of TKIs targeting two or more RTKs (e.g., lapatinib) might be greater than that of mono-specific drugs. Indeed, lapatinib, a dual specificity inhibitor of EGFR and HER2, shows promising results in clinical studies, in line with system's fragility against simultaneous double hits, a rare event in evolution.

Acquired resistance to cancer therapy: systems plasticity at work

In similarity to chemotherapy, the main challenge of targeted therapy is drug resistance. For example, only one-third of HER2-overexpressing mammary tumors respond to trastuzumab (primary resistance), and patients who initially respond to mAbs or to TKIs often relapse due to evolution of secondary resistance (Pao *et al*, 2005). The mechanisms underlying resistance are poorly understood and they differ between mAbs and TKIs (Hynes and Lane, 2005). The studies we review below attribute acquired (secondary) drug resistance to the remarkable ability of RTK networks to dynamically switch their signaling circuitries.

Resistance to therapeutic mAbs

In contrast to patient response to Rituximab, an anti-CD20 mAb, which is affected by polymorphisms of Fc receptors expressed on the surface of natural killer T cells, resistance to anti-RTK antibodies has not been associated with defects in

Table II Novel drugs targeting RTKs in human cancer

Molecular target	Name of drug	Description	Cancer indication	Approval status (year of first approval)
EGFR	Cetuximab (Erbix)	Chimeric mAb	CRC (with CT) Head and neck (with RT)	Approved (2004)
	Panitumumab (ABX-EGF)	Human mAb	CRC (with CT)	Approved (2006)
	Matuzumab (EMD 72000)	Humanized mAb	Lung cancer	Phase II
	Gefitinib (Iressa)	TKI	NSCLC	Approved (2005)
EGFR and HER2	Erlotinib (Tarceva)	TKI	NSCLC Pancreatic cancer (with CT)	Approved (2004)
	Lapatinib (Tykerb)	TKI	Breast cancer (with CT)	Approved (2007)
HER2	Trastuzumab (Herceptin)	Humanized mAb	Breast cancer (with CT)	Approved (1998)
	Pertuzumab (Omnitarg)	Humanized mAb	Ovarian cancer, breast cancer	Phase II
	MDX210	Bispecific antibody	Ovarian cancer	Phase III
Pan-HER/ERBB	CI-1033	TKI	NSCLC	Phase II
EGFR, HER2, VEGFR	AEE-788	TKI	Glioblastoma	Phase I
	VEGFA	Bevacizumab (Avastin)	Humanized mAb	CRC, NSCLC (with CT)
VEGFR2	CDP791	Human F(ab) ₂	Lung cancer	Phase II
VEGFR1-3, KIT, RET, PDGFR α	Sunitinib (Sutent)	TKI	Renal cell carcinoma, GIST	Approved (2006)
VEGFR2, KIT, PDGFR β , (RAF)	Sorafenib (Nexavar)	TKI	Renal cell carcinoma	Approved (2005)
HGFR	XL880	TKI	Solid tumors	Phase I
KIT, PDGFR, (ABL)	Imatinib (Glivec, Gleevec)	TKI	GIST	Approved (2006)

Abbreviations: CT, chemotherapy; EGFR, epidermal growth factor receptor; HER2, human EGFR 2; GIST, gastrointestinal stromal tumor; mAb, monoclonal antibody; NSCLC, non-small cell lung cancer; PDGFR, platelet-derived growth factor; RT, radiotherapy; TKI, tyrosine kinase inhibitor; VEGFR, vascular endothelial growth factor receptor.

ADCC. Instead, high expression of a soluble form of HER2, or steric hindrance of antigen–antibody interactions by a surface glycoprotein called MUC-4 (Nagy *et al*, 2005), seem to underlie part of patient resistance to trastuzumab, the most extensively studied mAb (reviewed in Nahta and Esteva, 2006). Alternatively, the existence, or emergence, of compensatory signaling pathways has been proposed, including paracrine or autocrine loops involving an ERBB ligand capable of stimulating alternative ERBB dimers (Valabrega *et al*, 2005). Likewise, activation of a downstream target of HER2 signaling, such as mutational activation of RAS, B-RAF or the AKT-PI3K pathway, which frequently occurs in solid tumor, may circumvent pharmaceutical blocking of HER2. In this vein, analysis of a small panel of HER2-overexpressing primary breast tumors reported a correlation between patient response to trastuzumab and expression levels of PTEN (Nagata *et al*, 2004). The IGF1-R shares with HER2 and EGFR the ability to stimulate AKT and MAPK, and increased expression of this receptor was shown to reduce trastuzumab-mediated growth arrest of HER2-overexpressing breast cancer cells. Collectively, these studies suggest that the PI3K–AKT pathway, stimulated by either IGF1 signaling or through PTEN loss, offers an escape route under treatment with mAbs.

Resistance to tyrosine kinase inhibitors

Two types of mechanisms appear to underlie resistance to TKIs. The major one involves avoidance of drug–target interactions and the other likely relates to compensatory signaling pathways (reviewed in Burgess and Sawyers, 2006). The first reported clinical resistance mutation, T351I, has been identified in BCR–ABL (Gore *et al*, 2001). Overexpression of the wild-type target kinase was found in a smaller fraction of resistant patients. Along with the identification of additional resistance-conferring mutations in BCR–ABL, similar alterations in EGFR, c-KIT and the PDGF-receptor were associated with clinical resistance of non-small-cell lung cancer, gastrointestinal stromal tumors and the hypereosinophilic syndrome to the respective TKIs. Active efflux of imatinib, the most extensively studied TKI, as well as compensatory activation of SRC family kinases, well characterized downstream effectors of BCR–ABL, have been implicated in the resistance of the minority of imatinib-resistant patients, who carry no kinase mutations. Last, according to a recent study, resistance of breast cancer patients to lapatinib involves a switch from HER2 and EGFR signaling to dependency on steroid hormone signaling (Xia *et al*, 2006), which reinforces the primary role played by network adaptability in the acquisition of drug resistance.

Conclusions and future directions

Universal laws govern a surprisingly broad spectrum of complex systems, ranging from engineering and communication to business and society (Barabasi and Oltavi, 2004). Complex biological systems, such as metabolism and signal transduction obey these general laws as means that impart fail-safe functioning (robustness; Stelling *et al*, 2004; Kitano, 2004a). Along with robustness and common design principles, all complex systems evolved from significantly simpler

modules (Kirschner and Gerhart, 1998). Our review has put forward the notion that the evolutionary process of systems growth and training discloses fundamental information, which is not only vital for understanding systems' logic, but can also guide pharmaceutical intervention. Interestingly, although the numbers of genes in the human and the nematode genomes are comparable, the RTK family of mammals is fourfold larger (Figure 2). Further, by following the evolution of a single group of RTKs, the ERBB/HER family, we infer a growth process that utilized gene duplication and sub-functionalization to transform a linear signaling cascade into a modular, richly interconnected network.

In mammals, RTKs complement their predecessors, nuclear hormone receptors, in multiple events of inductive cell lineage determination. Several functional and architectural attributes underlie the robust nature of RTK signaling, including an intricate array of cytoplasmic and nuclear negative feedback loops. Here we have concentrated on a recurring systems control module, namely a composite feedback loop comprising a slow transcriptional arm coupled to a rapid protein–protein interaction arm (Yeager-Lotem *et al*, 2004; Alon, 2006). In similarity to other complex systems, the growth of the RTK network necessitated the establishment of several highly connected nodes (hubs), for example, the RTKs themselves, RAS, RAF and PI3K. These hubs expose vulnerable points of intervention: cancer-driving mutations, as well as pathogenic viruses, frequently target the hubs, thereby locking the 'ON' state of the bistable system. An alternative locking device occurring in human cancer comprises partial disabling of multiple negative feedback loops (van Staveren *et al*, 2006; Amit *et al*, 2007).

In-depth understanding of complex signaling systems, as well as the tradeoffs of their robustness, will likely translate to better management of diseases. In addition to identifying critical hubs amenable for pharmacological interception, systems level approaches predict that uncommon interventions would collapse the emergent robustness of oncogenic RTK signaling (Carlson and Doyle, 2000). The relatively high clinical efficacy of anti-receptor antibodies (Baselga, 2006) may be regarded a successful uncommon perturbation involving directed mobilization of the immune system. Likewise, the broadly applicable replacement of the single-drug paradigm by multi-component therapy is another reflection of systems fragility against uncommon perturbations. *In silico* replicas of RTK signaling, along with sophisticated high-throughput drug discovery, are expected to identify points of fragility and reduce drug toxicity. Moreover, because resistance to drugs is an inevitable outcome of the ability of robust networks to switch to compensatory signaling pathways, systems-inspired dynamic RTK modeling will enable selection of drug combinations that can overcome secondary resistance in patients.

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