Review

CellPress

Special Issue: Wiring and Rewiring in Signal Transduction

Feedback and redundancy in receptor tyrosine kinase signaling: relevance to cancer therapies

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Mammalian cells have multiple regulatory mechanisms to deal with perturbations in cellular homeostasis, including feedback loops and crosstalk between the major signaling pathways. While these mechanisms are critically required to help cells survive under dynamic physiological circumstances, they also pose an impediment to the effective treatment of cancer. In this review, we describe what has been learned about interactions between receptor tyrosine kinase-dependent signaling pathways, and how this knowledge can be used to design rational and more effective combination therapies for cancer.

Intrinsic versus acquired resistance to cancer drugs

Activating mutations in the major pathways that impinge on cell proliferation and survival are key events in cancer development. Recent advances in sequencing technology have enabled the generation of large compendia of these so called oncogenic 'driver' events in cancer. A 'driver' refers to a genetic or epigenetic alteration of a molecule that is critically involved in the initiation and/or maintenance of a cancer. Inhibition of these driver events by targeted cancer drugs often elicits impressive clinical responses, because cancer cells are addicted to the cancer-causing signal, a situation referred to as 'oncogene addiction'. This term was initially coined by Dr Bernard Weinstein to describe the phenomenon that proliferation and survival of cancer cells can be dependent on a single oncogene. Inactivation of the oncogenic protein or the pathway it triggers can be detrimental to the tumor, providing a molecular basis for targeted therapy [1]. However, heterogeneity within the tumor (a feature of many tumors) and adaptive mechanisms that help cells survive under changing physiological circumstances, complicate the effective treatment of cancer with targeted therapies.

When advanced cancers are treated with targeted agents that act on an oncogenic driver, resistance emerges almost invariably, either after an initial period of drug response (acquired resistance) or *ab initio* (intrinsic resistance). The acquired drug resistance is often the result of the clonal selection of a (small) pre-existing population of cancer cells that circumvents the targeted agent, often through an additional genetic alteration or adaptive response (rewiring of cell signaling) [2–4]. The intrinsic resistance, as the name implies, results from a rapid adaptive response of the vast majority of cancer cells to inhibition of the oncogenic driver pathway. In this review, we zoom in on drug resistance caused by adaptive cellular responses with a focus on RTK signaling pathways. We discuss how we can take advantage of our insights into these adaptive responses to design more effective rational combination therapies.

Drugging receptor tyrosine kinase signaling

Receptor tyrosine kinases (RTKs) are a family of transmembrane receptors for extracellular signaling molecules, including growth factors and hormones. A typical RTK consists of an extracellular ligand-binding domain, a single-span transmembrane region, and a cytoplasmic kinase domain that becomes phosphorylated on tyrosine residues upon dimerization or oligomerization. Phosphorylated RTKs recruit adaptor proteins to provoke a cascade of protein interactions among intracellular effectors that eventually result in altered gene expression and protein functions. These downstream effectors include small GTPases, such as RAS proteins, members of the mitogen activated protein kinase (MAPK) family, phosphoinositide 3-kinases (PI3K), and Janus kinase/signal transducers and activators of transcription (JAK/STAT) proteins (Figure 1). Together, these proteins govern critical cellular processes such as cell survival, proliferation, and differentiation [5–7]. In addition, other important cellular functions, such as metabolism and cell–cell communication, are also in the custody of RTKs [8].

Given that RTK signaling-mediated cellular processes are vitally involved in cell proliferation and survival, it is not surprising that dysregulation of RTKs, or their downstream effectors, is seen in a wide range of cancers. Mutations, gene rearrangement, or amplification of RTKs themselves or their downstream effectors [e.g., the Kirsten rat sarcoma viral oncogene homolog (*KRAS*) and B-Raf proto-oncogene, serine/threonine kinase (*BRAF*) genes, or

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0968-0004/
 http://dx.doi.org/10.1016/j.tibs.2014.08.010

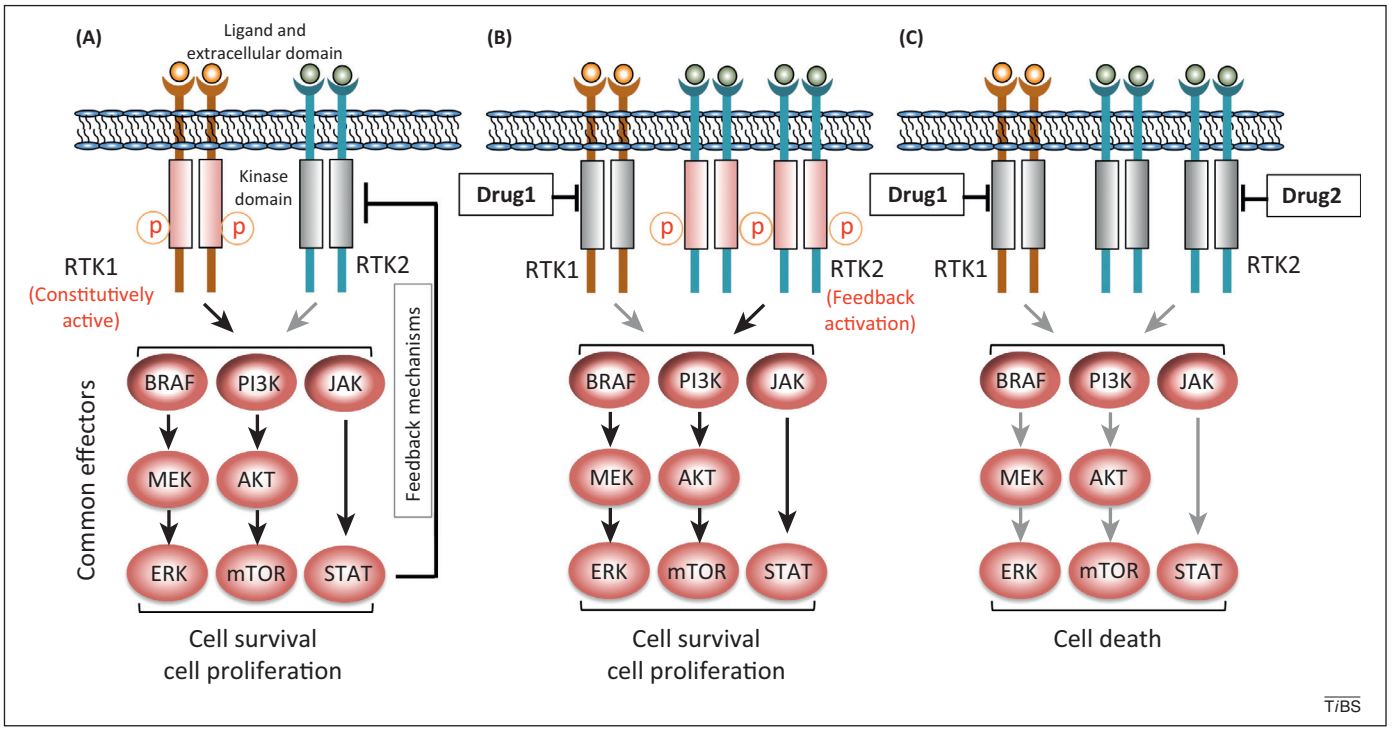

Figure 1. Crosstalk between receptor tyrosine kinases (RTKs) can cause intrinsic resistance to RTK-targeted therapies. **(A)** Constitutively active RTKs (RTK1 in the figure) result from gene mutation, translocation, or amplification, and are usually the oncogenic drivers in cancer. Red boxes represent active kinase domain of RTKs, while gray represents inactivity; Phosphate groups are shown as circled 'p' symbols. Black arrows represent active signal transduction, while grey arrows represent quiescent signal. RTKs share many downstream effectors that control many cellular activities, including cell survival and proliferation. These effectors are also part of homeostatic feedback regulation of RTK signaling (indicated by the inhibitory bar connecting to RTK2). **(B)** Cancer cells respond adaptively to RTK inhibitors. Inhibition of an oncogenic RTK decreases the signal to effector pathways; this relieves feedback suppression of a second RTK (two RTK2 dimers represent upregulation of RTK2). Activation of the second RTK compensates the loss of activation of effector pathways by the oncogenic RTK, and thereby supports cell viability while the oncogenic RTK is inhibited. **(C)** When combinatorial treatment (Drug 1 and Drug 2) targeting the primary oncogenic RTK1 and feedback-activated RTK2 is applied, the common downstream pathways shared by RTKs are shut down; therefore, cell death is seen. Abbreviations: *BRAF*, B-Raf proto-oncogene, serine/threonine kinase; MEK, mitogen-activated protein kinase kinase; ERK, mitogen-activated protein kinase; PI3K, phosphoinositide 3-kinases; AKT, v-akt murine thymoma viral oncogene homolog; mTOR, mammalian target of rapamycin; JAK, Janus kinase; STAT, signal transducers and activators of transcription.

the phosphatidylinositol-4,5-bisphosphate 3-kinase, catalytic subunit alpha (*PIK3CA*)] often results in constitutively active oncoproteins. Moreover, epigenetic alterations that silence suppressors of RTK signaling pathways [i.e., phosphatase and tensin homolog (PTEN)] or upregulate key components of RTKs can also contribute to oncogenic signaling. Cancer cells often depend on these constitutively active RTKs or downstream effectors. This dependency on oncogenic signals forms the basis for molecularly targeted cancer therapies that abruptly deprive cancer cells of their addiction. The high prevalence of alterations and the 'druggable' nature of the components of RTK signaling pathways have attracted considerable attention from the pharmaceutical industry. Indeed, most of the approved targeted therapy drugs are inhibitors of RTK signaling pathways. Table 1 summarizes the major oncogenic driver events in RTK signaling pathways that can be inhibited by targeted cancer drugs.

The success of imatinib, which targets the fusion RTK, BCR-ABL, in chronic myelogenous leukemia (CML), served as proof-of-concept that genotype-directed cancer therapy can be highly effective while having modest toxicity [9,10]. More recent clinical examples exploiting RTKs as therapeutic targets include the use of crizotinib for anaplastic lymphoma receptor tyrosine kinase (ALK)-positive non-small-cell lung cancer (NSCLC) [11]; gefitinib, erlotinib, or afatinib for epidermal growth factor receptor (EGFR)-mutated NSCLC [12–15] and trastuzumab or lapatinib for v-erb-b2 avian erythroblastic leukemia viral oncogene homolog 2 (*ERBB2*)-positive breast cancer [16,17]. EGFR and ERBB2 are members of the same family comprising four closely related RTKs: EGFR, ERBB2, ERBB3, and ERBB4. Dimerization between the members initiates RTK signaling. Moreover, downstream components of RTKs are also appealing therapeutic targets. A successful example is the use of vemurafenib for *BRAF* mutant melanoma [18]. The rapid development of targeted agents against a variety of signaling pathways, together with advances in genomic technologies that enable robust tumor profiling, have reshaped the landscape of cancer therapy towards more genotype-directed medicine. However, a considerable fraction of patients do not respond to therapies tailored to their oncogenic lesion. This can be exemplified by the data from clinical trials showing that 26% of *EGFR*-mutant NSCLCs, 35% of ALK-positive NSCLCs, and 52% of *BRAF*-mutant melanomas failed to achieve a clinically meaningful response to inhibitors targeting the respective oncogenic lesions [11,12,18]. Moreover, drugs currently in development also showed disappointing outcomes in preclinical assays or in clinical trials. For instance, fibroblast growth factor receptor (FGFR) inhibitor treatment appeared ineffective for FGFR-positive bladder cancer cell lines [19]. Similarly, inhibitors targeting the mitogen activated protein kinase

Table 1. Major oncogenic driver events in RTK signaling pathways that can be inhibited by targeted cancer drugs

Oncogene	Common genetic alteration	Examples of targeted agent	Common tumor type[a]
ALK	Translocation	Crizotinib, ceritinib	NSCLC
ABL	Translocation	Imatinib, nilotinib, dasatinib, bosutinib, ponatinib	CML
EGFR	Mutation or amplification	Gefitinib, erlotinib, cetuximab, panitumumab, lapatinib, afatinib, dacomitinib, neratinib, AZD8931	NSCLC, CRC, GBM
ERBB2	Amplification or mutation	Trastuzumab, pertuzumab, lapatinib, neratinib, afatinib, dacometinib, AZD8931	Breast cancer, gastric cancer
ERBB3	Mutation	MM-121, AV-203, and other anti-ERBB drugs	CRC, gastric cancer
ERBB4	Mutation or translocation	Dacomitinib	Melanoma
MET	Amplification or mutation	Crizotinib, tivantinib, foretinib, volitinib, MK-8033, INC280	Gastric cancer, NSCLC
KIT	Mutation	Imatinib, sunitinib, dasatinib, nilotinib	GIST, melanoma, AML
FGFR1	Amplification	Dovitinib, PD173074, AZD4547, E-3810, BGJ398, lucitanib, BAY1163877, ponatinib	NSCLC, breast cancer
FGFR2	Amplification or mutation	Dovitinib, AZD4547, E-3810, BGJ398, BAY1163877, ponatinib	Gastric cancer, breast cancer, endometrial cancer
FGFR3	Translocation or mutation	Dovitinib, AZD4547, PD173074, BGJ398, BAY1163877, ponatinib	Bladder cancer, NSCLC,
FGFR4	Mutation or amplification	Ponatinib	RMS
FLT3	Mutation	Lestaurtinib, midostaurin crenolanib, quizartinib, sunitinib, sorafenib	AML
JAK2	Mutation	Ruxolitinib, fedratinib, AZD1480, SB1578	Myelofibrosis
KRAS	Mutation	None	Pancreatic cancer, CRC, NSCLC
NRAS	Mutation	None	Melanoma
BRAF	Mutation	Vemurafenib, dabrafenib, encorafenib, sorafenib	Melanoma, NSCLC, thyroid cancer, Hairy-cell leukemia
MEK1/2	Upstream genetic alteration, i.e., RAF or RAS mutation	Trametinib, selumetinib, PD-0325901, MEK162, pimasertib, refametinib, TAK-733, RO5126766	Melanoma, NSCLC, CRC, pancreatic cancer
PIK3CA	Mutation, amplification, or PTEN loss	Dactolisib, GDC-0941, XL147, BKM120, BEZ235, XL765, GDC-0890, GSK1059615, CAL-101, INK1117, BYL719	Breast cancer, endometrial cancer, CRC, ovarian cancer, GBM, NSCLC
AKT1/2/3	Amplification, mutation, or translocation	Perifosine, MK-2206, AZD5363, GDC-0068, GSK690693	Breast cancer, pancreatic cancer, gastric cancer, ovarian cancer
mTORC1/2	Upstream genetic alteration, i.e., PI3K mutation or PTEN loss	Sirolimus, everolimus, temsirolimus, ridaforolimus, INK128, AZD8055, OSI-027	Breast cancer, renal cell carcinoma, liver cancer

[a]Abbreviations: NSCLC, non small cell lung cancer; CML, chronic myeloid leukemia; CRC, colorectal cancer; GBM, glioblastoma; RMS, rhabdomyosarcoma.

kinase–extracellular signal related kinase (MEK–ERK) or phosphoinositide 3-kinase–v-akt murine thymoma viral oncogene homolog (PI3K–AKT) pathways, which are key effector pathways downstream of RAS, exert only modest effects on *RAS*-mutant cancer [20,21]. Perhaps most surprisingly, vemurafenib, a BRAF inhibitor highly effective for *BRAF(V600)*-mutant melanoma, is close to useless for colon cancer that harbors the very same oncogenic mutation [22]. These unfavorable preclinical and clinical experiences threaten to erode confidence in genotype-guided therapies. However, what these discrepant results point at is that RTK signaling pathways should not be viewed in isolation.

Cross talk between RTKs

RTK signaling involves elaborate mechanisms that diminish RTK signaling when it becomes hyperactive and increase the signal when required. Regulatory machineries ubiquitously exist along the entire path of RTK signaling from cell membrane to the nucleus. Key components of these machineries that maintain equilibrium in RTK signaling include spatial and temporal control of ligand binding, tyrosine phosphorylation and dephosphorylation, interactions between effectors, and gene expression control. These connections provide compensatory mechanisms in RTK signaling, allowing cells to respond dynamically and adaptively to perturbations. Indeed, cross talk among these effector pathways through feedback mechanisms has been reported widely [23–26]. Many intrinsic resistance mechanisms against drugs that act on RTK signaling have been identified. Understanding these resistance mechanisms provides an opportunity to design effective therapeutic interventions to overcome resistance to single agent therapies.

Small molecule inhibitors and humanized monoclonal antibodies directed against RTKs are among the most effective targeted therapies in the clinic. In spite of the fact that the tumors bearing constitutively active RTKs are usually addicted to RTK signaling, the therapeutic responses to these drugs are rarely homogeneous and many tumors demonstrate intrinsic drug resistance despite genotype-directed drug selection. Because RTKs share many common downstream effectors [8], a common strategy by which tumors can ignore RTK inhibition is parallel activation of a second RTK that is not targeted by the drug (Figure 1). For instance, in some breast cancers with *ERBB2* amplification, unresponsiveness to ERBB2 inhibition can be attributed to transcriptional upregulation of *ERBB3*. This process is mediated by forkhead box O 3A (FOXO3A), a transcription factor suppressed by AKT.

ERBB2 inhibitors transiently abrogate PI3K–AKT signaling, thereby releasing FOXO3A to activate *ERBB3* transcription. Upregulated ERBB3 provides compensatory input to PI3K–AKT signaling that rescues cancer cells from ERBB2 targeted therapies [27]. Besides, computational modeling of the ERBB signaling network also identified ERBB3 as a driver of resistance to agents targeting ERBB2 in ERBB2-amplified breast cancer [28,29]. Likewise, in *EGFR* mutant glioblastomas (GBM) that are resistant to EGFR inhibitors, the EGFR variant III (EGFRvIII) transcriptionally suppresses the gene encoding platelet-derived growth factor receptor, beta polypeptide (PDGFRB), in a mammalian target of rapamycin complex 1 (mTORC1)- and ERK-dependent manner. Upon pharmacological inhibition of EGFR, derepressed *PDGFRB* provides alternative RTK signaling that confers drug resistance [30].

The adaptive signaling rewiring between different RTKs can be bidirectional. Bladder cancer cell lines that exhibit activating mutation or translocation of the gene encoding FGFR3 (*FGFR3*) show partial or no response to FGFR3 inhibition. Two distinct mechanisms were identified as responsible for the modest drug efficacy. In the cells partially responsive to FGFR inhibition, FGFR3 inhibition leads to feedback upregulation and activation of EGFR. In contrast, in the cells that are fully resistant to FGFR3 inhibition, EGFR suppresses *FGFR3* expression and dominates the signal input to the downstream pathways. Consequently, EGFR inhibition leads to upregulation of *FGFR3*. In both cases, dual treatment with EGFR and FGFR3 inhibitors overcomes these resistance mechanisms [19].

Small numbers of drug-tolerant cells can pre-exist in tumors. During drug treatment, these cells are positively selected and become the major component of the tumor. Frequently, drug tolerance in these cells is driven by overexpression of, or acquisition of mutations in redundant RTKs. For example, *MET* amplification emerged in tumors that acquired resistance to anti-EGFR therapies in both NSCLC and colorectal cancer (CRC). The presence of *MET* amplification at a low level in pre-treatment tumor tissues indicates that drug resistance clones driven by MET signaling were preexistent and enriched by the selective pressure of EGFR inhibitors [31,32]. Similarly, activation of EGFR was shown to be responsible for compromised crizotinib efficacy in ALK-positive NSCLC. The presence of mutant *EGFR* at a low level in ALK-positive NSCLC prior to crizotinib therapy again supports the preexistence of drug resistant subclones [33,34]. In several cases of intrinsic resistance, co-expression of RTKs was found in the major population of tumor cells before the initiation of treatment, which correlates with a poor response to initial treatment with inhibitors of one of the RTKs. For instance, *MET* expression is associated with a poor response to EGFR inhibitors in *EGFR* mutant NSCLC [35]. Similarly, in some *ERBB2*-amplified breast cancer cells activation of MET can be responsible for trastuzumab resistance [36], and alternatively, a high level *EGFR* expression compromises treatments exclusively focusing on ERBB2 [37–39]. In triple-negative breast cancer cells, *AXL* is transactivated by EGFR, which limits the response to EGFR-targeted inhibitors [40]. Furthermore, in pediatric glioblastomas that express high levels of insulin-like growth factor receptor (IGF1R) and IGF2, an IGF1R inhibitor only achieved modest therapeutic effect because of co-activation of platelet-derived growth factor receptors A and B (PDGFRA and PDGFRB) [41]. In all of these cases, combinatorial treatment using inhibitors targeting the redundant RTKs can increase the anti-tumor effect.

Besides the pre-existence of amplification or mutational activation of a second RTK, resistance can also be attributed to adaptive epigenetic regulation of RTK expression. In a cell culture model derived from *EGFR* mutant NSCLC (PC9) that is sensitive to EGFR inhibitors, a subset of cells stayed viable while the inhibitors killed the vast majority of the cells. This subpopulation exhibited an altered chromatin state and increased IGF1R activity, which was proven to be the driver of EGFR inhibitor-resistance. However, after termination of the treatment with EGFR inhibitors, the drug-tolerant cells became sensitive to EGFR inhibition again. This process implicates a dynamic rewiring of RTK signaling at an epigenetic level [4].

Feedback response to RAF–MEK–ERK pathway inhibition

The RAF–MEK–ERK pathway

The RAF–MEK–ERK pathway is among the most commonly dysregulated cell signaling route in human cancer. The druggable properties of the kinase components of the pathway make them attractive therapeutic targets for cancer [42,43]. Attempts to inhibit a single component of the RAF–MEK–ERK pathway revealed elaborate feedback and compensatory mechanisms that redirect cell signaling back to RTKs and other effector pathways. These regulatory mechanisms allow cancer cells to adapt to pharmacological pathway perturbations. Under physiological conditions, RAF–MEK–ERK signaling starts with RTK-mediated RAS activation. Activated RAS GTPases recruit RAF kinases from cytosol to plasma membrane and promote dimerization and activation of RAF kinases. Active RAF signals through MEK–ERK by a series of phosphorylation events, in which RAF phosphorylates and activates MEK, which in turn phosphorylates and activates ERK. ERK can regulate cytosolic targets and also enter the nucleus where it phosphorylates transcription factors involved in many cellular processes (Figure 2) [44,45].

RAF–MEK–ERK signaling-dependent post-transcriptional responses

In human cancer, oncogenic RTKs, mutated RAS GTPases, or the BRAF kinase are common drivers of RAF–MEK–ERK signaling. Pharmacological inhibition of RAS proteins in the clinic remains challenging, despite encouraging progress that has been made recently[46,47]. An alternative approach to target *RAS* mutant cancer is using small molecule inhibitors to block the effector pathways downstream of RAS. Multiple inhibitors against MEK, ERK, or BRAF kinases have been developed. However, in contrast to the remarkable clinical response in patients with *BRAF*-mutant melanoma [18], these inhibitors deliver only marginal therapeutic effect in other types of cancer

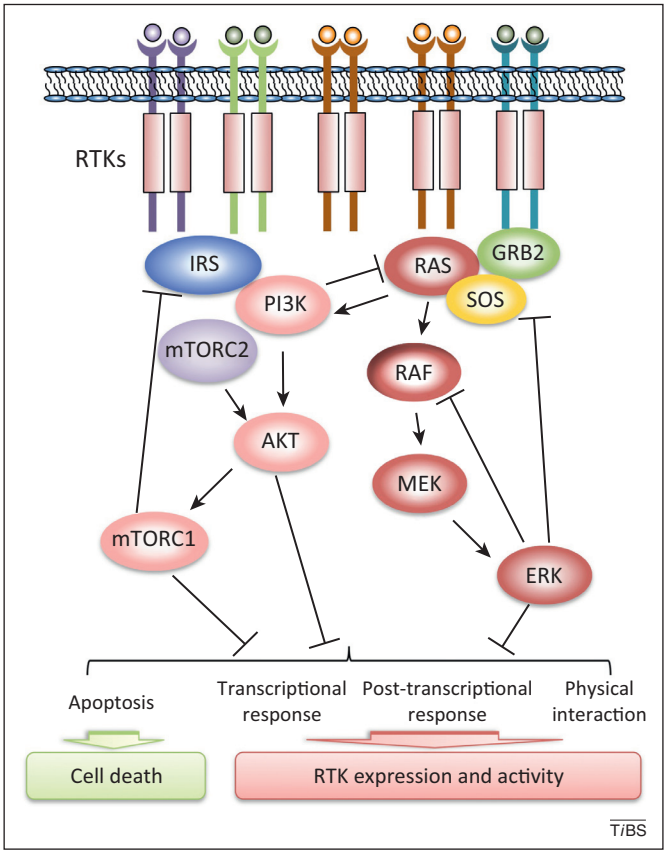

Figure 2. Schematic depiction of signaling circuits among RAF–MEK–ERK and PI3K–AKT–mTOR pathways and receptor tyrosine kinases (RTKs). Many RTKs (marked with different colors) share common downstream effector pathways. RAF–MEK–ERK and PI3K–AKT–mTOR pathways are the most widely studied ones. Under physiological conditions, RAF–MEK–ERK signaling starts with RTK-mediated RAS activation. Activated RAS GTPases recruit RAF kinases from cytosol to the plasma membrane, and promote dimerization and activation of RAF kinases. Active RAF signals through MEK–ERK by a series of phosphorylation events, in which RAF phosphorylates and activates MEK, which in turn phosphorylates and activates ERK. ERK can inhibit RAF and be responsible for dissociation of GRB2 and SOS complex thus reduced RAS activation. ERK also regulates activity of RTKs by physical interaction (i.e., inhibitory phosphorylation) and expression level of RTKs through transcriptional or post-transcriptional processes. ERK is also a negative regulator of apoptosis signaling. For PI3K–AKT–mTOR pathway, RTKs activate the signaling cascade through direct recruitment of PI3K to the plasma membrane or indirect recruitment that involves adaptor proteins IRS or GTP-bound RAS. PI3K phosphorylates Phosphatidylinositol (4,5)-bisphosphate to generate phosphatidylinositol (3,4,5) trisphosphate (not shown), which subsequently recruits AKT to the plasma membrane where mTORC2 phosphorylates it. Active AKT can phosphorylate mTORC1, which in turn activates transcriptional/post-transcriptional responses downstream of PI3K–AKT. mTORC1 can inhibit IRS through inhibitory phosphorylation. Both mTORC1 and *AKT* can inhibit apoptosis by blocking pro-apoptosis proteins and/or activating anti-apoptosis proteins. Components in the RAF–MEK–ERK and PI3K–AKT–mTOR pathways are inter-and intra-connected. Targeting one of the signaling nodes in the network can initiate feedback signaling to the upstream or paralleled components, which often compensates the signal loss caused by the drug treatment. Abbreviations: RAF–MEK–ERK, RAF proto-oncogene, serine/threonine kinase–mitogen activated protein kinase kinase–extracellular signal related kinase; (PI3K–*AKT*–mTOR), phosphoinositide 3-kinase–v-akt murine thymoma viral oncogene homolog–mammalian target of rapamycin; mTORC1, mammalian target of rapamycin complex 1; mTORC2, mammalian target of rapamycin complex 2; IRS, insulin receptor substrate; GRB2, growth factor receptor-bound protein 2; SOS, son of sevenless homolog.

bearing a *BRAF* mutation, including CRC and thyroid cancer [22,48,49]. Intrinsic resistance of *BRAF* mutant colon cancer to BRAF inhibitors arises as a result of feedback activation of EGFR. BRAF inhibition inactivates ERK, which decreases activation of CDC25C, a phosphatase that negatively regulates EGFR [50]. Enhanced EGFR activity not only counteracts BRAF inhibition by activating RAS that promotes heterodimerization of RAF proteins, but also activates the PI3K–AKT pathway in parallel [49–51]. As expected, concomitant inhibition of EGFR and BRAF synergistically induces apoptosis and suppresses tumor growth in *BRAF* mutant CRC [49,50]. The remarkable differential responses of *BRAF* mutant CRC and melanoma to BRAF inhibitors can be attributed to the notion that EGFR is broadly expressed in CRC, but rarely expressed in melanoma. *BRAF* mutant thyroid cancer cells that express a high level of EGFR are also sensitive to the combinatorial treatment consisting of EGFR and BRAF inhibitors, but not to either one of the inhibitors alone [49].

A different mechanistic insight in response to BRAF–MEK pathway inhibition was revealed by a study using EGFR- or ERBB2-addicted cell line models. In these systems, MEK inhibition induces posttranscriptional upregulation and activation of ERBB3, which impedes the efficacy of MEK inhibitors. The feedback loop is dominated by ERK-mediated phosphorylation of inhibitory phosphosites on EGFR(T669) and ERBB2(T677) respectively [52,53]. MEK inhibition reduces ERK activation, which in turn activates EGFR or ERBB2 through decreased inhibitory phosphorylation. ERBB3 activation is secondary to EGFR or ERBB2 activation, in line with the established notion that activation of kinase-impaired ERBB3 is substantially dependent on heterodimerization with other ERBB family members [8,53].

Moreover, the RTK IGF1R was found to be critically involved in MEK inhibitor resistance in some *KRAS* mutant NSCLC and CRC cells. MEK inhibition leads to feedback activation of IGF1R and increased interaction between PI3K and insulin receptor substrate (IRS) proteins. Concomitant inhibition of IGF1R and MEK reduces AKT phosphorylation to a level much lower than that in basal conditions, whereas IGF1R inhibition does not further inhibit ERK phosphorylation in the presence of a MEK inhibitor, indicating IGF1R signaling drives MEK inhibitor resistance dominantly through PI3K–AKT pathway [54–56].

Notably, feedback response to mutant-specific targeted agents can cause unwanted effects in cells that do not have an oncogenic lesion. All RAF inhibitors to date inhibit ERK signaling only in tumors with *BRAF* mutations. In cells that express wild type *BRAF*, treatment with these inhibitors paradoxically promotes and stabilizes RAF dimerization that consequently activates ERK signaling. Two mechanistic explanations have been described. First, physical binding of a RAF inhibitor changes the conformation of wild type BRAF and Raf-1 proto-oncogene, serine/threonine kinase (RAF1), which drives dimer formation between inhibitor-bound RAF protein and uninhibited CRAF. Second, wild type BRAF remains in the cytosol in an autoinhibited state under physiological conditions; however, the RAF inhibitor causes release of BRAF autoinhibition and RAS-dependent recruitment to the plasma membrane, where it heterodimerizes with RAF1. Both mechanisms are dependent on RAS activity. As a consequence, either RTK activation or oncogenic *RAS* mutations (both of which stimulate RAS signaling) cause undesired effects of RAF inhibitors, including development of benign skin tumors

and enhanced progression and metastasis of tumors with *NRAS* mutations [57–63].

RAF–MEK–ERK signaling-dependent transcriptional responses

A recent study found that transcriptional upregulation of *ERBB3* is responsible for intrinsic resistance to MEK inhibitors in *KRAS* mutant NSCLC and CRC. MYC, a transcriptional suppressor of *ERBB2* and *ERBB3*, is stabilized by ERK-mediated phosphorylation. MEK inhibition reduces ERK activity, leads to dephosphorylation and degradation of MYC leading to de-repression of *ERBB2* and *ERBB3* expression. Increased ERBB signaling activates the PI3K–AKT pathway, which compensates for inhibited MEK–ERK signaling [64]. Simultaneous inhibition of ERBBs and MEK effectively suppresses AKT and also reduces ERK phosphorylation compared with mono-treatment with the MEK inhibitor [64]. These findings [54,55,64,65] suggest that PI3K–AKT and MEK–ERK pathways can compensate for each other in promoting cell survival and proliferation. Consistently, combining inhibitors of both MEK–ERK and PI3K–AKT pathways can suppress tumor growth in a murine model of *KRAS* mutant lung cancer, patient-derived xenograft (PDX) of *RAS* mutant CRC, and xenograft model of *NRAS* mutant melanoma cells [20,66,67]. Nevertheless, severe toxicity of this combination may limit their clinical use [68,69].

Transcriptional upregulation of RTKs upon MEK inhibition was also seen in triple negative breast cancer (TNBC). MEK inhibition causes upregulation of multiple RTKs including PDGFRB, kinase insert domain receptor (KDR), AXL receptor tyrosine kinase (AXL), and discoidin domain receptor tyrosine kinases (DDR1/DDR2), but not ERBB3, in a MYC-dependent manner. Activation of these RTKs is accompanied by increases in AKT and ERK signaling. Although the role of AKT signaling in drug resistance was not investigated further in the study, the authors showed that a MEK inhibitor plus an RTK inhibitor or RAF inhibitor both caused a further reduction in ERK phosphorylation and greater anti-proliferation effect versus MEK inhibitor alone [70]. This suggests that combinatorial strategies that enhance suppression of ERK signaling are sufficient to overcome resistance to MEK inhibitor [70]. This notion was further supported in *KRAS* mutant cancer models [64,65].

Interestingly, the RTK rewiring in response to RAF–MEK–ERK pathway inhibition does not necessarily cause drug resistance. In *BRAF* mutant melanoma, RAF inhibitors potently inhibit ERK, and thereby cause a relief of ERK-mediated suppression of the upstream signaling [71,72]. Similar to the above mentioned cases, feedback activation of RTKs and RAS also leads to a modest increase of ERK signaling in melanoma. However, the signal intensity is insufficient to confer resistance to RAF inhibitors, which is in line with the fact that melanoma generally exhibits low basal RTK activity [50]. However, any further increase in RTK signaling by ligand stimulation (particularly HGF), or upregulated RTK expression, confers resistance to BRAF inhibitors in *BRAF* mutant melanoma [72–74].

A recent study found that melanomas can upregulate expression of multiple RTKs through suppression of the transcription factor SRY (sex determining region Y)-box 10 (SOX10) in response to BRAF or MEK inhibitor treatment. However, this increased RTK signaling is detrimental to the cells in the absence of drug because it leads to hyperactivation of BRAF and MEK. The supra-physiological level of BRAF–MEK signaling induces a state of oncogene-induced senescence, which explains why these drug-tolerant cells exist as a minor population in the first place [75]. This study supports a model in which a small population of cells carries a 'burden' that impairs cell proliferation, but confers an advantage in the presence of the drug. These cells are positively selected by drug treatment, but counter-selected upon treatment termination. Pre-clinical and clinical reports demonstrate that certain tumors that have developed drug resistance can regain sensitivity to the same drug after a 'drug holiday' [76–79]. The reversible and adaptive transcription response to targeted agents in cancer cell models may provide a molecular explanation for these observations. Moreover, increased RTK expression in drug-tolerant tumors is a potential biomarker to identify patients that may benefit from retreatment after a drug holiday. It is also worth testing whether targeting these emergent RTK(s) in addition to the primary targets would provide a durable drug response.

Feedback response to PI3K–AKT–mTOR pathway inhibition

PI3K–AKT–mTOR pathway

The PI3K–AKT–mTOR pathway regulates several cellular functions critical for cancer, including cell survival, proliferation, metabolism, and motility [43]. Its inappropriate activation has been found in a broad range of human cancers. Dysregulation of this pathway can be attributed to mutational activation of the key components of the pathway, PI3KCA or AKT; loss of the AKT inhibitor, PTEN; or constitutive upstream oncogenic signals fueled by RTKs or RAS. Normally, RTKs activate the signaling cascade through direct recruitment of PI3K to the plasma membrane or indirect recruitment that involves adaptor proteins IRS, GRB2-associated binding protein (GAB) or GTP-bound RAS. PI3K phosphorylates phosphatidylinositol-(4,5)-bisphosphate [PtdIns(4,5)P_2; also known as PIP2] to generate phosphatidylinositol-(3,4,5)-trisphosphate [PtdIns(3,4,5)P_3; also known as PIP3], which subsequently recruits AKT to the plasma membrane where PDK1 and mTORC2 phosphorylate it. Active AKT can phosphorylate mTORC1, which in turn activates S6-Kinase, a mediator of many transcriptional responses downstream of PI3K-AKT (Figure 2). Alternatively, AKT can directly regulate transcription factors including the FOXO family. AKT suppresses the FOXOs by direct phosphorylation to provide docking sites for 14-3-3 binding, which sequesters the transcription factors in the cytosol [43]. Similar to the RAF–MEK–ERK pathway, feedback mechanisms involved in the regulation of PI3K–AKT–mTOR signaling impede clinical effectiveness of single-agent therapies targeting this pathway. Recent findings have indicated an important role for RTKs in promoting resistance to this class of drugs.

PI3K–AKT–mTOR signaling-dependent feedback mechanism

Analogs of rapamycin, which target mTORC1, were initially explored as inhibitors of the PI3K pathway, but yielded limited clinical benefits as single agents [80]. Adaptor proteins physically bridge RTKs and their downstream effectors participate in the feedback activities that underlie unresponsiveness. For instance, growth factor receptor-bound protein 10 (GRB10) is an adaptor protein that functions as a negative regulator of insulin receptor (IR) and IGF1R; its binding to RTKs blocks signal transduction to downstream effector pathways. mTORC1-dependent phosphorylation of GRB10 increases its stability. Therefore, mTORC1 inhibition can initiate feedback signaling by degradation of GRB10, which relieves the block of insulin receptor (IR) and IGF1R [81–83]. Moreover, in breast cancer cell line models, mTORC1 inhibition inactivates S6K, which is a suppressor of the adaptor protein IRS1 through inhibitory phosphorylation. Relieving inhibition of IRS1 can augment IGF1R signaling, which enhances mTORC2-mediated AKT activation and activates mTORC1-independent targets such as the FOXO transcription factors. As expected, IGF1R inhibition can prevent feedback activation of AKT and sensitize tumor cells to mTORC1 inhibitors [84]. To prevent mTORC2-mediated AKT activation caused by mTORC1 inhibition, dual mTORC1–mTORC2 inhibitors, AKT inhibitors, and dual PI3K–mTOR inhibitors were developed. However, despite the initial dephosphorylation of AKT, feedback-mediated induction of RTK signaling leads to reactivation of AKT and/or ERK signaling which compromise therapeutic efficacy during prolonged treatment [85–89]. Further investigations showed that increased RTK signaling can be a consequence of FOXO-dependent transcriptional upregulation of RTKs, mTORC1-mediated activation of RTKs and cap-independent translation of RTKs upon direct or indirect inactivation of AKT [85–88]. Combinatorial treatment targeting both RTKs and the PI3K–AKT–mTOR pathway represent a promising approach to overcome resistance to single-agent therapies [84,85,87,88].

PI3K was also described as an upstream regulator of both RAS and AKT. PI3K inhibitors not only suppress PI3K, but also transiently block ERK activity through inactivation of RAS [90,91]. Simultaneous loss of AKT and ERK activation through intermittent administration of PI3K inhibitors induces apoptosis in RAS wild type cells [91].

Similar to dual inhibition of RAF and MEK, some cases of combinatorial treatment targeting two components of PI3K–AKT–mTOR cascade that completely block the pathway are also effective. Combinatorial strategies, such as an mTOR inhibitor plus an AKT inhibitor, and a PI3K–mTOR dual inhibitor plus an mTOR inhibitor, both abolish mTOR inhibition-induced AKT activation, thereby synergistically inducing tumor cell death [85,92,93]. In this context, it is important to point out that ERK and AKT are both negative regulators of pro-apoptotic proteins such as BAD and BIM [94]. Apoptosis commences only when sufficient pro-apoptotic signaling is present. Combination therapies that adequately inactivate ERK and/or AKT can, therefore, synergize in induction of pro-apoptotic signaling, thereby suppressing tumor growth.

Concluding remarks

Toxicity matters

Understanding cross talk between signaling pathways is essential for the development of powerful drug combinations. Sometimes, several potential combination strategies can be identified for a given cancer. For instance, MEK inhibitors in combination with AKT, IGF1R, pan-ERBB, RAF, or B-cell lymphoma – extra large (BCL-XL) inhibitors, were each shown to be more effective than each drug alone and were found to be well-tolerated in *KRAS* mutant mouse cancer models [54,64,65,95–97]. This gives physicians the option to select those combinations that have the least toxicity. However, mouse cancer models are poor predictors of toxicity in patients. Indeed, in a clinical trial, severe toxicity of the combination of MEK and AKT inhibitors precluded adequate dual pathway inhibition [69], whereas this was possible in the animal model [96]. Whether other combinatorial regimens will be successful in the clinic will depend to a large extent on how well the patients tolerate the combined toxicities.

In some cases, combinatorial regimens can reduce some side effects caused by each drug alone. For instance, BRAF inhibitors induce benign tumors in healthy skin tissues because of the 'paradoxical' MEK activation [61], while MEK inhibitor causes acneiform rash, which is associated with MEK inhibition. When patients received MEK and BRAF inhibitors in combination, they developed less hyperproliferative skin lesions than that expected with BRAF inhibitor monotherapy, and the frequency and severity of acneiform rash seemed to decrease [98]. It is not clear at this point whether a full dose of each drug is required for a given drug combination. Synergy may already be obtained at less than the maximum tolerated dose. In such cases, toxicity may be reduced through a lower dose of each of the two drugs in the combination. However, there is little experience with this in the clinic to date.

Find the right drug combination for the right patient

One important lesson has been learned from studying RTK signaling is that genotype-directed therapies have to take into account the complexity of the signaling network and the heterogeneity of tumors. Information on pathway activation can be extracted from genomics, quantitative transcriptomics, and proteomics analysis of pre- and post-treatment cancer cells or tumor samples. However, perturbation of the same pathway can have different consequences depending on the cell type, tissue type, and specific genetic alterations [99,100]. The most notable example is that colon cancers and melanomas harboring exactly the same mutation in *BRAF* respond remarkably differently to BRAF inhibition [18,22]. In terms of rewiring cell signaling pathways, the evidence shows that drug treatment-induced pathway activation does not necessarily contribute to drug resistance. Scrutinizing the MEK inhibitor-induced RTKs in TNBC using small interfering RNA (siRNA) revealed that not all of the upregulated kinases contributed to cell survival [70]. A similar

Box 1. Outstanding questions

- How do we find the best combination therapy for each individual patient?
- How to manage the toxicities of combination therapies?
- How can we predict emergence of acquired resistance to cancer therapies?

conclusion was also reached for *BRAF* mutant CRC treated with BRAF inhibitors. IGF1R is notably activated by vemurafenib treatment in these cells, but EGFR is the only genuine driver of vemurafenib resistance [50].

Functional screens excel in their ability to distinguish 'driver' from 'passenger' events in feedback regulation. RNA interference (RNAi) or clustered regularly interspaced short palindromic repeats (CRISPR)-based loss-of-function genetic screens can identify synthetic lethal interactions between signaling pathways while gain-of-function genetic screen by ectopic expression of cDNAs can identify genes sufficient to confer drug resistance. An imperfection of these functional approaches is the heavy reliance on the model system used in the screening. Moreover, the tools used in functional screening are far from perfect. This makes it impossible to interpret a negative result (false negatives are rampant), and should make one cautious in interpreting a positive result (false positives are frequent). Joint endeavors that assess genetic and epigenetic alterations in tumors and functional interrogation of the regulatory mechanisms involved in signal transduction will help to elucidate clinically-relevant resistance mechanisms and effective drug combinations.

Outlook

We are just beginning to understand how signaling networks are interconnected within cancer cells. One of the big open questions is how heterogeneous cancers are in their cross talk between signaling pathways (Box 1). Moreover, how do additional mutations in cancers affect the response to combination therapies? These questions seem daunting. In spite of this, there are already encouraging results from clinical trials testing combination therapies that were based on novel insights in cross talk between signaling pathways [101]. It will be a long time before we can deduce from a cancer genome sequence which combination therapy will work best for the individual patient. However, as the Chinese philosopher Laozi has said: 'a journey of a thousand miles begins with a single step'.

Acknowledgments

We apologize to the many authors whose work we could not cite directly because of space limitations. The research of the authors was supported by grants from the Dutch Cancer Society (KWF), a grant to the CancerGenomics.nl consortium, and the European Research Council, grant #250043.

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