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Exploiting receptor tyrosine kinase co-activation for cancer therapy

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Abstract

Studies over the past decade have shown that Receptor Tyrosine Kinase (RTK) co-activation is prevalent in many cancer types. Compelling data demonstrates that cancers are likely to have evolved RTK co-activation as a generic means for driving tumour growth and providing a buffering system to limit the lethal effects of microenvironmental insults including therapy. In this review, we summarise the general principles of RTK co-activation gleaned from key studies over the last decade. We discuss direct and indirect approaches to exploit RTK co-activation for cancer therapy and describe recent developments in computational approaches to predict kinase co-dependencies by integrating drug screening data and kinase inhibitor selectivity profiles. We offer a perspective on the outstanding questions in the field focusing on the implications of RTK co-activation on tumour heterogeneity and cancer evolution and conclude by surveying emerging computational and experimental approaches that will provide further insights into the biology of RTK co-activation and deliver new developments in effective cancer therapies.

Keywords

Receptor tyrosine kinase; signal transduction; kinase inhibitors; computational modelling; cancer

Introduction

Oncogenic signalling by Receptor Tyrosine Kinases (RTKs) are causative drivers in cancer initiation and progression (1). Members of this class of 58 receptors have been studied in detail across multiple scales, from molecules to whole organisms, and there is an increasing consensus that RTKs rarely act in isolation but rather cooperate as networks of multiple receptors that undergo extensive crosstalk - a concept known as “RTK co-activation” (2). It is therefore no longer sufficient to view RTKs as single entities; instead these receptors should be investigated as part of complex networks working in a concerted fashion. In this review, we illustrate the general principles that have emerged from studies in the field of RTK co-activation and their consequences on cancer evolution and drug resistance. We then

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explore the implications of RTK co-activation for cancer therapy and describe computational methodologies for accurately predicting kinase co-dependencies. We conclude by highlighting important outstanding questions in the field and offer a perspective on next-generation strategies to exploit this phenomenon for cancer therapy.

General principles of RTK co-activation

The adoption of phosphoproteomic-based profiling including mass spectrometry, array-based technologies and small molecule profiling strategies such as Multiplexed Inhibitor Beads (MIBs) and Kinobeads, have shown that RTK co-activation is a common event in many cancer types, including lung cancer, soft tissue and bone sarcoma, breast cancer, glioma, gastric cancer, ovarian cancer and mesothelioma among others (2–14). These cancers range from genetically simple tumours originating from single driver events such as the SS18-SSX fusion in synovial sarcomas to cancers that harbour more complex karyotypes such as head and neck cancers (15, 16). Compelling data from many studies show that cancers are likely to have evolved RTK co-activation as a generic means for driving tumour growth and providing a buffering system to limit the lethal effects of microenvironmental insults including therapy. Here we detail the general principles of RTK co-activation gleaned from key studies over the past decade (Figure 1 and 2).

A dynamic and adaptable process

Early profiling studies of human cancer cell lines suggest that RTK co-activation is a static process and that simply identifying the active receptor components in tumours would be sufficient to establish oncogenic dependencies for therapeutic intervention. Examples include the global profiling of tyrosine phosphorylation in lung cancer and sarcoma which revealed a large number of co-activated RTKs present in both cell lines and tumours (5, 6). However, subsequent functional analyses using kinase inhibitors and RNA interference (RNAi) screens show that receptor activation does not always equate to kinase addiction and it is incredibly challenging to *a priori* determine which of the multiple activated RTKs are functionally relevant (5, 7). One reason for the lack of correlation between measured kinase phosphorylation and direct functional effects is that co-activation networks are not static but are highly dynamic events capable of rapidly adapting to RTK blockade. It should be noted that this lack of correlation also implies that within co-activation networks, there may be “dominant” receptors that are responsible for oncogene addiction, a concept that is discussed in greater detail in a subsequent section on hierarchical network topologies.

There are several lines of evidence that shed light on the dynamic nature of this adaptive response. A recent study from Gary Johnson’s group examined the kinome alterations that occur in ERBB2 amplified breast cancer cell lines in response to the ERBB2 targeted agent lapatinib (17). Temporal assessment of cellular levels of activated kinases using the MIBs platform showed that multiple RTKs are upregulated within 48 hours of treatment with lapatinib. Interestingly, this “kinome reprogramming” response was not associated with the expression and activation levels of ERBB2 nor was it dependent on the addiction of these cells to ERBB2 signalling as assessed by genetic depletion of this gene. RTKs that were upregulated by lapatinib included EGFR, ERBB2, IGF1R/INSR, FGFR2 and DDR1 which

ultimately led to the rapid acquisition of acquired drug resistance. In another report using a functional genomics approach, Singleton et al., performed a genome-wide shRNA screen in head and neck squamous cell carcinoma (HNSCC) cell lines in the presence and absence of FGFR tyrosine kinase inhibitors (TKIs) (15). The study identified a synthetic lethal interaction between FGFR TKI with genetic silencing of HER2 and c-MET but not in the vehicle control, suggesting an induced RTK dependency in response to FGFR blockade. Additional cell line specific dependencies were also attributed to the INSR, PTK7 and EPHB2 RTKs. Consistent with the functional screen data, combined inhibition of HER2 or c-MET and FGFR TKIs led to synergistic impairment of HNSCC cell growth. Taken together, these two studies demonstrate that the inhibition of single RTKs results in a rapid rewiring of the kinome which ultimately shifts the dependency from HER2 or FGFR to other RTKs. These findings provide a potential explanation for our current inability to accurately predict kinase dependencies solely by relying on RTK co-activation profiles.

This adaptation has also been observed in the clinical setting by comparing pre-treatment and post-treatment patient specimens in TKI trials. Stacchiotti et al., assessed two patients with PDGFRB positive solitary fibrous tumours (SFTs) who were treated with the multi-target TKI sunitinib (18). Assessment of pre-treatment samples using RTK antibody arrays showed a number of co-activated RTKs including PDGFRA, PDGFRB, EGFR, RET, VEGFR1 and VEGFR2. Upon analysis of progressive lesions post-treatment, the authors found that in one patient, the RTK co-activation profile was unchanged from pre-treatment while in a second patient, the post-treatment specimen displayed prominent activation of additional RTKs IGF-1R/INSR and M-CSFR which were negative at presentation. These findings were in contrast to a radiologically stable lesion which showed a downregulation of tyrosine phosphorylation levels in all the RTKs identified in the pre-treatment specimen, presumably due to the action of sunitinib. The authors posit that in some progressive lesions, kinase dependencies may have shifted from PDGFRB pre-treatment to IGF-1R/INSR and M-CSFR post-treatment and a switch to an IGF-1R targeting antibody such as figitumumab may be necessary to effectively treat patients who progress on sunitinib. These findings do not preclude the possibility that the kinase dependency “shift” observed in patients may be the result of clonal selection in a subpopulation of pre-existing sunitinib-resistant cells within a heterogeneous tumour (19), however it does highlight the major clinical challenge of tumour cell adaptation and cancer evolution in driving TKI drug resistance. We anticipate that additional studies comparing pre- and post-treatment patient specimens as well as “window of opportunity” trials that compare a drug’s effect on tumour signalling pathways between a pre-treatment biopsy and subsequent surgical resection post-treatment, would be useful in dissecting the individual contributions of kinome rewiring and clonal selection in the evolution of resistance to RTK targeted therapy (20).

A means to maintain signal robustness and diversification of tumour function

Most RTKs activate common downstream effectors and share the capacity to bind a collection of SH2- and PTB-containing signalling adaptors and proteins (21, 22). In the event of disruption of one or more RTKs within a co-activation network, for instance with TKI therapy, this modular sharing of signalling elements endows other RTKs with the ability to recruit from the same pool of effectors to preserve signalling flux (Figure 3A). The role of

RTK co-activation in maintaining signal robustness is most evident in intrinsic and acquired resistance to targeted therapy. Stommel et al., performed the first study to functionally characterise the effects of RTK co-activation in cancer (10). Using RTK profiling arrays, the authors observed that glioma cell lines displayed high endogenous levels of receptor co-activation which included EGFR, c-MET and PDGFRA. They further showed that in the presence of an EGFR inhibitor, PI3K signalling is maintained in glioma cells expressing an oncogenic form of EGFR (EGFRvIII) by shifting the recruitment of the GAB1-PI3K complex from EGFRvIII to c-MET. This “RTK switching” mechanism ultimately led to EGFR inhibitor resistance (23). Only the combined blockade of EGFRvIII and c-Met was able to disrupt RTK-GAB1-PI3K binding, shutting down oncogenic signalling and leading to cancer cell death.

This compensatory feature of RTK co-activation networks is not limited to receptors which activate similar downstream pathways. Huang et al., showed physical heterotrimeric interactions between ERBB2, ERBB3 and IGF-1R in trastuzumab (an anti-ERBB2 antibody) resistant derivatives of SkBr3 and BT474 ERBB2 amplified breast cancer cell lines (24). This heterotrimer was responsible for conferring drug resistance, however in contrast to the shared activation of PI3K signalling by EGFRvIII and c-MET in glioma, the RTK components in this heterotrimer stimulated distinct survival pathways. The authors find that genetic depletion of ERBB3 reduced AKT signalling while depletion of IGF-1R decreased SRC and ERK phosphorylation. Despite acting on unique pathways, silencing of either receptor alone sensitized the resistant cells to trastuzumab, indicating that RTK co-activation may also function in an additive fashion to activate different survival signalling pathways resulting in acquired drug resistance (Figure 3B).

Despite sharing the ability to bind common effector proteins, distinct RTKs are capable of maintaining specificity and diversity in the activation of downstream signalling events (1). This specificity can manifest in differential intensity, localisation or kinetics of signalling, as exemplified by the classic observation of transient versus sustained MAPK signalling in response to EGF or NGF respectively in PC12 cells (25). It is thought that RTK co-activation is also a means to achieve diversification in signalling outcomes and by extension tumour phenotypes with a limited repertoire of intracellular signalling effectors. The phenotypic contribution of specific RTKs within co-activation networks has not been extensively studied. Rettew et al., performed an siRNA screen of co-activated RTKs identified in two metastatic osteosarcoma cell lines (LM7 and 143B) across four phenotypic assays measuring motility, invasion, colony formation and cell growth (7). They showed that depletion of IGF-1R had a phenotypic effect in all four assays, while EPHA2, FGFR2 and RET silencing was only capable of reducing motility and colony formation with no effects on cell growth and invasion. In contrast, AXL downregulation by siRNA diminished motility, colony formation and invasion but not cell growth. The signalling contribution of each of these RTKs was not investigated in this study, however the functional data would suggest that distinct receptors drive different cancer phenotypes and that RTK co-activation may be a way of increasing the spectrum of tumour-associated functions that can be achieved by any one RTK alone (Figure 3C).

Hierarchical network topology

The idea that RTK co-activation networks are organised in hierarchical topologies was first mooted by Terrance Johns and colleagues (26). Building on the earlier discovery of RTK co-activation driven by the EGFRvIII mutant oncoprotein in glioma (10, 11), they showed that EGFRvIII was the dominant oncogene with the c-MET RTK functioning as a secondary downstream effector. Interestingly, the hierarchy is dynamic and upon blockade of EGFRvIII, c-Met is rapidly elevated to the dominant role in order to maintain oncogenic signalling and ultimately induce resistance to EGFR targeted therapy. This model has implications on combination therapy as it suggests that inhibiting the dominant kinase in combination with multiple secondary RTK effectors may be required for effective tumour cell killing. Indeed, in a recent study by the same group, the authors showed that simultaneous targeting of EGFRvIII in glioma cells using the anti-EGFR antibody panitumumab in combination with a broad spectrum TKI motesanib, which inhibits several of the identified secondary RTKs in glioma cells such as VEGFR1-3, PDGFRA and PDGFRB, c-KIT and RET, was effective in limiting tumour growth in subcutaneous xenograft models (27).

Two further computational studies have since demonstrated that this hierarchical topology is not restricted to glioma and is a general means of organising of RTK co-activation networks in other tumour types. In one study, Ciaccio et al., employed microwestern arrays to collect a compendium of signalling data based on 91 phosphosites on 67 proteins across 6 time points after stimulation with EGF in A431 human epidermoid cervical carcinoma cells (28). The data was then used to establish connectivity and directional relationships between different RTKs based on Bayesian network modelling. The authors were able to show that RTK signalling occurred through a hierarchical topology with specific tyrosine phosphorylation sites on EGFR (Y845) and PDGFRB (Y1009) acting as root nodes at the top of the network. This initiated the phosphorylation of a second layer of signalling in EGFR (Y1068 & Y1173), ErbB2 (Y1221/1222) and c-KIT (Y719). The model found that ERBB4 (Y1234), FGFR1 (Y653/654) and EGFR (Y1086) lay downstream of EGFR (Y1068) as the third layer of signalling. Unlike other RTK phosphorylation sites which showed maximum phosphorylation at 1 minute post-EGF stimulation, ERBB4 and FGFR1 phosphorylation profiles were distinct as these two receptors displayed maximum activation at 5 minutes which was sustained for the duration of the 60 minute time course. In these cells, c-MET functioned as both a root node, with its Y1349 site upstream of PDGFR activation, as well as a subsidiary phosphorylation site Y1234/1235 downstream of both ERBB4 and FGFR1.

How these hierarchical topologies are regulated and maintained in cancer cells remain unresolved but a recent computational study by Palacios-Moreno et al., in neuroblastoma suggests that this may be the result of spatial compartmentalisation of different RTKs (29). In this study, the authors first used mass spectrometry-based phosphoproteomics to characterise the tyrosine phosphorylation profiles of 21 neuroblastoma cells lines and found 31 RTKs that were phosphorylated across the panel. Using co-clustering correlation network analysis, they determined that the RTKs clustered into two groups based on their phosphorylation profiles, indicating that RTKs within these clusters were co-activated and may be involved in trans-activating each other. One group included ALK, PDGFRA, FGFR1

and IGF-1R while the second group is composed of EGFR, PDGFRB, EPHA2, EPHB3 and DDR2. Fractionation analysis finds that specific RTKs were enriched in endosomes (ALK, FGFR1, RET, PDGFRA, DDR2, EGFR and IGF-1R) while others (EPHA2 and ROR1) were exclusively in detergent resistant membranes (also known as lipid rafts). Interestingly, stimulation with growth factors or treatment with TKIs revealed that individual tyrosine phosphorylation sites on specific RTKs were differentially regulated and that distinct phospho-specific forms of RTKs were partitioned between the endosomes and detergent resistant membranes. For instance, some ALK and KIT phosphorylated peptides were enriched in endosomes while others were only found in detergent resistant membranes. In contrast, all EGFR and RET phosphopeptides were consistently enriched in the endosomes. Taken together, this data suggests the exciting possibility that spatial compartmentalisation of RTKs and their effectors may be a physical means of maintaining the hierarchical topology of RTK networks. A better understanding of this complex regulation of spatial signalling is necessary to design novel strategies to overcome this hierarchy in RTK networks and disrupt robust oncogenic signals.

Not restricted to kinase amplification and mutations

RTK co-activation is classically associated with kinase amplification or mutational events in cancer. Interestingly, emerging data from functional screens and phosphoproteomic studies show that co-activation may also be induced by alterations in non-kinases including proteins that are widely considered to be “undruggable”, such as phosphatases, chromatin remodelling complexes and transcription factors (4, 16, 30, 31). One example is the protein tyrosine phosphatase PTPN12 which is deleted in 22% of breast cancers and 13% of lung cancers and found to be a tumour suppressor in a mouse model of breast cancer (30, 32). Sun et al., performed an integrated genetic screen coupled with phosphoproteomics in immortalised human mammary epithelial cells (HMECs) and found that PTPN12 functioned as a tumour suppressor by upregulating EGFR, ERBB2 and PDGFRB phosphorylation (30). Combined pharmacological inhibition of these three RTKs was sufficient to impair triple negative breast cancer (TNBC) cell line proliferation both *in vitro* and *in vivo*. The authors further showed that PTPN12 was undetectable in 60% of TNBC patient specimens examined by immunohistochemistry suggesting that rational combinations of TKIs targeting oncogenic RTK addiction conferred by PTPN12 loss may be a general strategy for treatment of this difficult-to-treat breast cancer subtype.

Another example is the SWI/SNF complex which plays an important role in remodeling nucleosomes in chromatin in an ATP-dependent manner, enabling transcriptional regulation (33). Mutations in subunits of the SWI/SNF chromatin remodelling complex are found in ~20% of cancers (34). Examples of SWI/SNF subunit deficiencies with demonstrated tumour suppressive roles include SMARCA4 in non-small cell lung cancer (NSCLC) and pancreatic cancer, SMARCB1 in malignant rhabdoid tumours and ARID1A in ovarian, endometrial and uterine cancers (35–39). Cancers which harbour SWI/SNF subunit deficiencies are almost universally fatal and there are currently no effective treatments for this class of tumours. Several studies have now shown that deficiencies in SWI/SNF subunits upregulate RTK expression. For instance, Papadakis et al., performed a shRNA screen to identify genes whose depletion would confer resistance to c-MET and ALK inhibition in

NSCLC lines (31). The authors found that silencing the SWI/SNF component SMARCE1 was capable of inducing c-MET and ALK TKI resistance and that this was the result of EGFR upregulation. Depletion of additional SWI/SNF components ARID1A and SMARCA4 was similarly able to induce EGFR expression and confer TKI resistance. They further demonstrate that SMARCE1 promotes EGFR transcription via direct interactions with the regulatory elements of the EGFR locus. As expected, EGFR TKIs sensitized SMARCE1-deficient cells to c-MET and ALK inhibitors suggesting that exploiting RTK co-dependencies may have broader utility in treating cancers with SWI/SNF subunit deficiencies.

The regulation of RTK co-activation by SWI/SNF complex may also be important in synovial sarcomas. The SS18-SSX fusion gene is the initiating transforming event in synovial sarcoma and mediates its oncogenic function by competing with wildtype SS18 protein to form a SWI/SNF complex that displaces SMARCB1 (40). Furthermore, reduced SMARCB1 protein expression is observed in a large proportion of synovial sarcomas (41, 42). Synovial sarcomas have been shown to exhibit elevated protein and phosphorylation levels of EGFR, ERBB2, c-MET and PDGFRA (16, 43, 44). Interestingly, Brenca et al., showed that EGFR and c-MET were co-activated in an epithelioid sarcoma cell line which is also characterised by the homozygous gene deletion in SMARCB1 (45). Although a direct link between the loss of SMARCB1 and RTK co-activation in synovial and epithelioid sarcomas has not been definitively established, it is tempting to speculate that exploiting RTK co-dependencies using TKI combinations may be a strategy to overcome SMARCB1 deficiency either arising from biallelic deletion of the SMARCB1 gene or as a result of the SS18-SSX fusion.

Heterogeneity in distinct tumour types and cell lines

Given that RTK co-activation is a common mechanism for resistance to tyrosine kinase targeted therapy, it would be helpful to determine if certain tumour types are enriched in the expression of specific combinations of RTKs. Wagner et al., assessed the gene expression patterns of 6 RTKs (EGFR, c-MET, FGFR1, IGF-1R, PDGFRB and NTRK2) in the Cancer Cell Line Encyclopaedia (CCLE) compendium of 967 cell lines. They showed that a large subset of 196 cell lines co-expressed c-MET, FGFR1 and EGFR. In particular carcinoma, glioma and melanoma cell lines were significantly enriched for these three receptors. This finding is consistent with the preclinical data of the effectiveness of c-Met and EGFR inhibitor combinations in both gliomas and uveal melanoma (10, 11, 46).

While the high-level global analysis performed by Wagner et al., was capable identifying commonalities in RTK co-expression profiles in specific tumour types, there is an underlying heterogeneity in individual cell lines within cancer types. For instance, large-scale phosphoproteomic screens of lung cancer, sarcoma and neuroblastoma cell lines demonstrate that there is heterogeneity in the identities of the individual RTKs that are co-activated in different cell lines from the same tumour type. This heterogeneity was also elegantly demonstrated by Stuhlmiller et al., where MIBs profiling in five breast cancer cell lines showed that the pattern of kinome reprogramming in response to lapatinib was distinct across the cell lines and that different RTKs were utilised by cancer cells to compensate for

the loss of ERBB2 oncogenic signalling in the presence of lapatinib (17). While combinations of TKIs with lapatinib were capable of slowing growth across the cell lines, their efficacy differed depending on the specific TKI combination used and the RTK profile exhibited by that cell line. The mechanistic basis for the observed heterogeneity is still unclear but these studies suggest that it may be challenging to rationally develop cancer type-specific TKI combinations. A personalised approach of individualised tumour profiling to account for the diversity of RTK co-activation patterns may be required in order to translate effective TKI combinations into the clinic (3).

Implications for therapy

Combined inhibition of multiple RTKs

The realisation that RTK co-activation plays a major role in driving tumorigenesis and drug resistance led to the development of direct and indirect therapeutic approaches to target this phenomenon. As described in examples provided above, the most basic strategies involve direct blockade of the multiple RTKs in the co-activation network either through the use of combinations of selective RTK inhibitors or broadly specific TKIs and HSP90 inhibitors that are capable of blocking several RTKs simultaneously (8, 47) (Figure 4). The advantage of these approaches is that one can readily link a RTK co-activation profile of the tumour (measured using arrays or mass spectrometry) with the appropriate TKIs required for effective kinase inhibition, providing a useful biomarker for patient stratification. This can be particularly valuable in the design of next-generation umbrella and basket clinical trials which stratify patient subpopulations to receive targeted therapies matched to their molecularly defined biomarkers (48). However, our inability to *a priori* predict kinome reprogramming effects in response to therapy and to identify the dominant RTKs in co-activation networks makes this approach challenging to implement. This strategy is also prone to the acquisition of drug resistance when patients are subjected to long-term RTK inhibitor therapy which is a major clinical problem (49). Finally, managing the excessive toxicities associated with combining RTK targeted agents remains a significant practical challenge, although optimized multi-drug schedules of these agents may circumvent some of these observed toxicities (50, 51).

Targeting common upstream regulators of RTK co-activation

Another promising strategy for therapy involves the blocking of common upstream regulators of RTK co-expression or activation (Figure 4). One approach is the use epigenetic therapies such as BET bromodomain inhibitors to halt the transcription of multiple RTKs. The BET family of bromodomain-containing proteins are epigenetic “readers” that recognise acetyl-lysine residues on histones. BRD4 is one member of the BET family of proteins which is amplified, overexpressed or mutated in cancers such as multiple myeloma and Nut midline carcinoma (52, 53). In multiple myeloma, the use of competitive inhibitors of BET bromodomains such as JQ1 is capable of displacing BRD4 from chromatin resulting in the downregulation of c-MYC and decreasing cancer cell proliferation (52, 53). Stuhlmiller et al., showed that JQ1 sensitized ERBB2 amplified breast cancer cells that undergo kinome reprogramming in response to lapatinib (17). JQ1 in combination with lapatinib was more effective than the combined use of multiple TKIs in preventing acquired

lapatinib resistance. The authors demonstrated that JQ1 blocked lapatinib-induced RTK co-activation by displacing BRD4 from the promoters of ERBB2, ERBB3, FGFR2 and DDR1. This epigenetic targeting approach addressed three limitations associated with TKI combinations: it overcame the dynamic adaptation in response to TKI therapy, it enabled the blockade of multiple RTKs simultaneously and it removed the need to *a priori* determine the identities of the individual RTKs that are heterogeneously expressed in different cancer cell lines. Interestingly while BRD4 depletion showed a decrease in RTK expression levels, knockdown of two other BET family members BRD2 and BRD3 in ERBB2 amplified breast cancer cells led to an increase in the transcription levels of multiple RTKs in response to lapatinib (17). This unexpected finding indicates that our understanding of role of BET family proteins in kinome reprogramming is still incomplete and more research is required to fully unravel the complex epigenetic regulatory mechanisms of these bromodomain-containing proteins on RTK transcription before BET inhibitors can be effectively utilised in the clinic.

A novel approach to overcome the activation of multiple RTKs is to target common scaffolding proteins which are essential for the maintenance of RTK co-activation (Figure 4). FAK is a ubiquitously expressed intracellular tyrosine kinase scaffold that facilitates protein-protein interactions critical for propagating RTK and integrin signalling (54–56). FAK is highly activated in ERBB2 amplified breast cancer cell lines subjected to lapatinib treatment and a combination of FAK inhibitions with lapatinib displayed synergy in overcoming lapatinib resistance (17). Greenall et al., showed that the EGFRvIII-MET heterodimer in glioma cells is maintained by FAK which acts as a scaffold that co-localises in actin-rich membrane ruffles (27). Chemical inhibition of FAK reduced EGFRvIII-MET heterodimers and suppressed EGFRvIII-mediated transactivation of MET without affecting the phosphorylation of EGFRvIII itself. While FAK inhibitors induced cell death and reduced cell proliferation and migration in vitro, these cellular findings did not translate into reduced tumour volume in murine xenograft models. Further investigation showed that the transactivation of other RTKs such as AXL by EGFRvIII were unperturbed by FAK inhibition, suggesting that while the FAK scaffold was required for EGFRvIII-MET heterodimers, it is dispensable for the activation of other RTKs in the co-activation network, limiting its therapeutic efficacy in vivo. In addition to FAK, other scaffold proteins are likely to be required for maintaining RTK co-activation in cancer cells. One example is the tetraspanin class of membrane proteins which similarly associate with EGFR and MET and is required for ligand-induced receptor activation (57–59). The biology of scaffolding proteins are generally understudied and an in-depth characterisation of the interactions between RTKs and their cognate scaffolds is necessary to better exploit this strategy as a therapeutic means for overcoming RTK co-activation.

Targeting shared downstream targets of RTK co-activation – a focus on AKT and MEK inhibitors

Given the heterogeneity associated with RTK co-activation in cancer cells, one intuitive strategy for disrupting oncogenic signalling is to suppress downstream signalling elements which are shared among the different RTKs. This approach is also likely to overcome acquired TKI resistance as bypass mechanisms of resistance largely act to re-activate these

shared downstream components (49). Much of this effort in the field has focused on the use of inhibitors of the RAS-RAF-MEK and PI3K-AKT-mTOR pathways (Figure 4). These two major pathways act downstream of multiple RTKs and regulate a variety of cancer phenotypes including cell proliferation, survival and dysregulated cancer metabolism (60–62). However, the efficacy of these inhibitors is limited as the RAS-RAF-MEK and PI3K-AKT-mTOR pathways also actively suppress feedback loops that control RTK expression and phosphorylation. In two landmark studies, Chandarlapaty et al., and Duncan et al., showed that chemical inhibition of AKT or MEK respectively induce the transcription and activation of multiple RTKs in cancer cells through the alleviation of feedback inhibition (4, 63).

Chandarlapaty et al., employed the ERBB2 amplified BT474 breast cancer cell line model to demonstrate that treatment with an allosteric inhibitor of AKT increased both the expression and phosphorylation of multiple members of the EGFR receptor family, most prominently ERBB3 (63), blunting the efficacy of AKT inhibition. This upregulation was specific to AKT blockade as inhibition of the ERK pathway did not modulate ERBB3 expression or phosphorylation. Using RTK antibody arrays, the authors further showed that ERBB3, IGF-1R and INSR were recurrently upregulated in response to AKT inhibition across a panel of cell lines representing different tumour types. Mechanistically, the increase in RTK expression and phosphorylation was mediated by separate molecular events. RTK expression is controlled by the activation of the FOXO family of transcription factors. Upon AKT inhibition, FOXO proteins are recruited to the ERBB3 promoter resulting in an increase in the mRNA and protein levels of this RTK. In contrast, the increase in ERBB3, IGF-1R and INSR phosphorylation was due to the inhibition of the mTORC1 complex downstream of AKT which relieves p70S6K-dependent feedback inhibition (64). Employing a combination of lapatinib (to inhibit ERBB2–ERBB3 signalling) and the AKT inhibitor repressed ERBB3, IGF-1R and INSR expression and phosphorylation levels which was accompanied by effective tumour regression in subcutaneous xenograft models in vivo (63). In a related study, the same group also showed that ATP-competitive mTOR kinase inhibitors that block both mTORC1 and mTORC2 complexes similarly promote RTK co-activation, maintaining AKT signalling and limiting the effectiveness of this class of drugs (65). Treatment of BT474 cells with mTOR inhibitors suppressed AKT S473 phosphorylation in a sustained fashion but only transiently inhibits T308 phosphorylation which is sufficient to maintain AKT activity. In this system, transient AKT phosphorylation is the result of mTOR inhibition relieving the feedback inhibition of multiple RTKs (EGFR family, IGF-1R, INSR and FGFR family). RTK co-activation promotes PI3K activation and subsequent downstream phosphorylation of AKT T308. Correspondingly, a combination of mTOR kinase inhibition together with lapatinib showed enhanced anti-tumour effects in BT474 xenografts.

MEK inhibitors have shown efficacy in a number of different cancer types and are currently being evaluated in advanced clinical trials (61). Duncan et al., found that MEK inhibition in TNBC cells reduced c-MYC phosphorylation at S62, promoting c-MYC degradation by the proteasome (4). Repression of c-MYC expression led to the transcriptional de-repression of multiple RTKs, including PDGFRB, DDR1 and VEGFR2. Treatment of TNBC cells with the proteasome inhibitor bortezomib maintained c-MYC expression levels in cells despite

MEK inhibition, preventing the upregulation of RTKs. The RTK co-activation profile induced by MEK inhibition was distinct from that activated by the dual PI3K/mTOR inhibition. Exploiting this new knowledge, the authors combined MEK inhibition with the broadly-selective TKI sorafenib which targeted a number of the observed upregulated RTKs and showed that this combination had synergistic effects in TNBC cell line in vitro and in the C3tag mouse model of TNBC in vivo. Interestingly, Miller et al., recently demonstrated that post-translational shedding of multiple RTKs is also a mechanism for MEK inhibitor resistance (66). Treatment of a panel of cell lines with MEK inhibitors led to a downregulation of RTK shedding, in particular MET and ALK shedding was reduced in the supernatant by 50%. This reduction resulted in an increase in the overall levels of total and phosphorylated AXL on the surface of cancer cells inducing tumour cell resistance to MEK inhibitors. Combinations of MEK and AXL inhibitors were capable of overcoming resistance in vitro and in xenograft models. The authors elegantly demonstrated that AXL was cleaved by the sheddases ADAM10 and ADAM17 and that MEK inhibition promoted the interaction of ADAM10 with its inhibitor TIMP1 preserving AXL function at the cell surface. In line with this mechanism, neutralising TIMP-1 with an antibody (T1-NAB) overcame MEK inhibitor resistance.

Collectively, these four studies demonstrate that while the inhibition of AKT and MEK pathways are conceptually attractive, the practical application of shared pathway inhibition is challenging to implement as these pathways actively suppress feedback mechanisms that regulate RTK expression and activation. Current strategies still largely rely on combinatorial inhibition of RTKs with AKT or MEK to prevent the reactivation of feedback pathways, shut down oncogenic signalling and induce cancer cell death.

Computational approaches to assess kinase co-dependencies

A strategy to interrogate kinase dependencies in cancer cells is to perform high-throughput pharmacological screens and integrate this data using computational methods (67). As the majority of small molecule kinase inhibitors interact with multiple members, within or across protein kinase families, this cross-reactivity or “off-target” effect provides another approach to dissect RTK co-activation and kinase co-dependencies. Integrating the pharmacological screening data with kinase selectivity profiles of inhibitor panels represents an attractive computational strategy to deconvolute kinase co-dependencies in cancer cells. For example, Pal and Berlow developed Kinase Inhibition Map (KIM), an algorithm that utilises set theory to predict the cancer cell line sensitivity of new drugs or drug combinations based on kinase inhibition profiles of the drugs (68). The algorithm employs the following two superset and subset rules to generate circuit representations of KIM: a) drugs that inhibit a superset of an effective set of inhibited kinases will also be successful in inhibiting tumour cell growth, and b) drugs that inhibit subsets of ineffective sets of inhibited kinases will also be unsuccessful in blocking tumour cell growth. The KIM algorithm identifies kinase co-dependencies by determining a minimal set of kinases that is most predictive for drug sensitivity and in doing so reveals the kinase combinations that have to be inhibited in order to prevent tumour growth. This method is predictive of tumour cell sensitivity to targeted therapy based on experimental data in four canine osteosarcoma cell lines treated with 60 small molecule kinase inhibitors (69). In another study that further

expands on the KIM approach, Tang et al., developed Target Inhibition inference using Maximization and Minimization Averaging (TIMMA), which utilizes maximization and minimization rules and a model selection algorithm based on sequential forward floating search for drug sensitivity and combination prediction in cancer cells (70, 71). Using this strategy, the authors demonstrated that the TIMMA program is capable of improving the computational speed and prediction accuracy of the original KIM program.

To translate these high-throughput pharmacological screens into clinical practice, Tyner et al., developed an experimental methodology to assess the sensitivity of 151 primary leukaemia patient samples to a panel of 66 small molecule kinase inhibitors (72). They built a computational algorithm that can predict kinase dependencies based on the analysis of inhibitor sensitivity patterns. Employing this approach, the authors identified kinase dependencies in leukaemia patients with known dysregulated tyrosine kinase pathways, including a rare FLT3 extracellular oncogenic mutation (S451F) that is not covered by standard molecular diagnostic tests. Similarly, Pemovska et al., developed an Individualized Systems Medicine (ISM) approach that can optimize cancer drug therapies for individual patients based on the integration of molecular profiling and *ex vivo* Drug Sensitivity and Resistance Testing (DSRT) of patient cancer cells to 187 oncology drugs (73). They applied the ISM approach to 28 acute myeloid leukaemia (AML) patients and identified five major taxonomic drug-response subtypes based on DSRT profiles. The authors also demonstrated that the ISM approach can be employed to delineate the evolution of resistance mechanisms by analysing sequential samples from treated patients who have developed acquired resistance to multiple lines of conventional and kinase inhibitor therapy, and potentially uncover new dependencies such as sensitivity to kinase inhibitors that were previously ineffective as first line therapy.

Motivated by these studies, Ryall et al., developed the Kinase Addiction Ranker (KAR), an algorithm that integrates high-throughput drug screening data, comprehensive kinase inhibition data, and gene expression profiles to determine kinase dependency in cancer cells (74). As a proof of concept, they applied KAR to rank the kinase dependencies in 21 lung cancer cell lines using publicly available pharmacological screening data from the Genomics of Drug Sensitivity in Cancer (GDSC). For example, KAR predicted FGFR1 and MTOR codependency in the lung large cell carcinoma cell line H1581 which was experimentally validated by testing drug combinations. Along the same lines, Szwajda et al., developed the kinase inhibition sensitivity score (KISS) that maps kinase inhibitor sensitivity and selectivity profiles onto a drug-target network, and ranks the individual kinases according to their likelihood of being essential for the growth of a particular cancer cell (75). They applied this computational approach to 21 breast cancer cell lines perturbed by 40 kinase inhibitors and identified subtype-specific addiction patterns which clustered in agreement with patient subtypes. They further experimentally validated the top predictions of co-dependencies in these cell lines, and revealed novel combinations such as dasatinib and axitinib TKIs in the triple-negative HCC1937 cell line.

These computational approaches exploit the vast amount of high-throughput pharmacological screening data, comprehensive kinase inhibitor selectivity data and cancer cell line genomic and transcriptomic data. Newer methodologies that incorporate high-

throughput quantitative phosphoproteomics data which depict signalling alterations in response to drug perturbations are also emerging (76, 77). Each data type provides a different piece of information in relation to kinase dependencies in tumours which together generates a complete portrait of kinase co-dependencies in cancer cells. By integrating these disparate data sources, new computational tools have the potential to harness the power of big data in biomedical sciences and provide new strategies to assess RTK co-activation and kinase co-dependencies for exploitation in cancer therapy (Figure 5). Novel computational tools coupled with other next generation high-throughput profiling technologies will undoubtedly advance the discovery of kinase dependencies and lead to the translation of rational drug combinations for personalized medicine in the clinical setting.

Future perspectives - Tackling intratumoural heterogeneity

It is clear that intratumoural heterogeneity and cancer evolution play major roles in drug resistance and tumour recurrence (78). Intratumoural heterogeneity in RTK expression was first demonstrated in glioblastoma (GBM) by three groups independently (79–81). These studies found that while bulk measurements such as array comparative genomic hybridisation (aCGH) analysis of GBM specimens in The Cancer Genome Atlas (TCGA) dataset showed that a fraction (4–8%) of tumours displayed co-amplification of multiple RTKs, single cell analysis by multi-colour fluorescence in situ hybridisation (FISH) indicated that the vast majority of tumour cells exhibited mutually exclusive amplification of EGFR, PDGFRA or c-MET. Only a small fraction of cells in a tumour harboured the co-amplification of two or more RTKs within the same cell. Snuderl et al., determined that these different subpopulations shared common genetic alterations such as homozygous deletion of CDKN2A or TP53 mutations which suggests that they arose from the same precursor and that RTK amplification is likely a late event in tumour evolution (79). Intriguingly, Szerlip et al., showed that the subpopulation distribution was plastic and could be readily modulated by growth factor stimulation or TKI treatment (80). Using tumoursphere lines derived from primary tumours, they found that that growth in PDGFB containing media led to an enrichment of cells with the co-amplification of both PDGFRA and EGFR while cells grown in EGF showed a stable persistent population of co-amplified cells (25% of total cells). In addition to GBM, intratumoural heterogeneity in RTK expression has also been observed in breast cancer. In contrast to GBM where co-amplified cells are in the minority, ERBB2 amplified breast cancers had variable levels of tumour cells (ranging from 0–52%) that co-expressed both ERBB2 and c-MET (82).

These studies raise important questions about the role of RTK co-activation in the context of intratumoural heterogeneity and its implications for targeted therapy in cancer. For instance, we do not fully understand the effects of kinase inhibitor therapy on the dynamics of distinct RTK subpopulations. Szerlip et al., found that the addition of the EGFR inhibitor gefitinib to PDGFB treated glioma tumoursphere cells resulted in a further enrichment of co-amplified cells, while administering imatinib (a PDGFRA inhibitor) to cells grown in EGF led to a dramatic decrease in PDGFRA/EGFR co-amplified cells (80). Given that the bulk of our knowledge of RTK co-activation is derived from population levels measurements in cell culture, there is a need to complement current array-based and mass spectrometry measurements with new tools capable of single cell analysis to dissect the heterogeneity of

RTK co-amplification events in tumours (Figure 5) (83). Advances in mass cytometry, in particular its application to in situ analysis of signalling proteins in tumour tissue at the resolution of single cells, will undoubtedly shed light on how targeted therapy impacts the evolution of heterogeneous populations of RTK amplified cells within a tumour (84, 85). Additionally, the development of single cell phosphoproteomics approaches such as the single cell barcode chip (SCBC) platform and single cell western blotting capable of measuring a limited number of phosphosites in single cells with small amounts of input material will enable the translation of mass spectrometry bulk measurements into assays amenable for clinical trial stratification and monitoring of tumour evolution in response to targeted therapies (Figure 5) (86–88).

Another important question that remains unanswered is what are the mechanisms that facilitate the co-existence of multiple RTK amplified subpopulations within a tumour. Snuderl et al., suggests that one possibility is a cooperative model where the co-existence of different RTK amplified populations confers a survival benefit (79). It is likely that the selective pressure to maintain each RTK amplified sub-clone is the result of complex cell-cell interactions. New mass spectrometry approaches to analyse signalling networks between distinct cell populations in co-culture, such as cell type-specific labelling using amino acid precursors (CTAP) will enable the systematic characterisation of the complex interactions between different tumour cell populations as well as tumour-stromal interactions that are necessary for maintaining intratumoural heterogeneity (89, 90). An understanding of these dynamic cell-cell interactions may provide options for disrupting critical interactions that maintain the coexistence of different RTK subpopulations and inhibit overall tumour growth (Figure 5).

More recently, the Hemann and Lauffenburger research groups developed a systems biology approach to predict the most effective drug combinations for tackling intratumoural heterogeneity (91–93). Using shRNAs genetic silencing, they first developed “RNAi signatures” that are characteristic of specific classes of compounds (either drug sensitive or resistant). Next, they developed a multi-objective linear optimization algorithm that simultaneously incorporates efficacy and toxicity of drug combination effects on many heterogeneous tumour compositions (91, 93). Here, their objective is to maximize tumour cell death and minimize the outgrowth of tumour subpopulations. They tested and validated this systems biology approach in mouse lymphoma cells, and discovered that the optimal treatment for a heterogeneous tumour may not necessarily incorporate drugs that most efficiently kill in individual subpopulations (92). This study challenges the current dogma of combining drugs that would most effectively treat particular predominant subpopulations in a heterogeneous tumour.

In another study, the Hemann and Lauffenburger groups developed a novel approach to exploit cancer vulnerabilities by incorporating the dynamics of clonal evolution in the presence of drug treatment (94). They termed this approach as “temporal collateral sensitivity” in intratumoural heterogeneity, a phenomenon where some clones may develop resistance toward a drug or drugs which comes at the expense of sensitivity to other drugs, and this selection process is highly dynamic. By integrating pharmacological screens and drug resistance selection in a BCR-ABL1 acute lymphoblastic leukaemia murine model,

they identified tumour subpopulations that showed “temporal collateral sensitivity” to non-classical BCR-ABL1 TKIs (e.g. crizotinib, foretinib, cabozantinib and vandetanib) during the evolution of resistance toward initial treatment with BCR-ABL1 TKIs. They also demonstrated that sequential drug switching between BCR-ABL1 TKIs and non-classical BCR-ABL1 TKIs changes the clonal trajectories in this model, suggesting that understanding and predicting clonal trajectories toward resistance may improve cancer therapy (94).

These studies highlight the potential power of integrating functional and computational approaches in unravelling new insights about design principles for combination therapy in the context of intratumoural heterogeneity, and provide new directions in developing new models for predicting drug combinations in cancer cells (Figure 5).

Conclusions

A decade has passed since the discovery of RTK co-activation in cancer and we are just starting to develop effective strategies to exploit this phenomenon for cancer therapy. Emerging data suggests that a better understanding of the nature and role of intratumoural heterogeneity in response to targeted tyrosine kinase therapy will be key to overcoming acquired and intrinsic resistance. There is still a significant amount of knowledge that remains to be uncovered. Much of what we know of RTK co-activation biology has focused on a number of well-studied RTK families like EGFR, PDGFR, MET and FGFR. Several RTKs (such as the Eph receptors, Discoidin Domain Receptors and pseudokinases like PTK7) that have been identified in tumour phosphoproteomic screens are poorly characterised and there is a need to build on existing foundations and establish the role of these RTKs within co-activation networks in tumour biology (95–100). The effects of autocrine and paracrine ligands on RTK co-activation are also largely unexplored. Studies have shown that exogenous addition of growth factor ligands is capable of bypassing RTK co-activation dependencies through the activation of alternative RTKs, but the importance of these resistance mechanisms *in vivo* is unknown and requires further investigation (101, 102). Moving forward, we anticipate that advances in measurement technologies and computational approaches to dissect intratumoural heterogeneity will provide further insights into the biology of RTK co-activation and deliver exciting new developments in effective cancer therapies.

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Gloss

Over the past decade, emerging evidence has demonstrated that receptor tyrosine kinases (RTKs) do not function as isolated oncogenic drivers. Instead, cancer cells employ multiple RTKs which are co-activated in dynamic and hierarchical signalling networks that fuel tumour growth, maintain tumour heterogeneity and adapt in response to cancer therapeutics. In this Review, which contains 5 figures and 102 references, we summarise the key studies which have defined the general principles of RTK co-activation, describe computational methods to explore kinase dependencies and discuss the implications of this phenomenon on cancer therapy.

Figure 1. Timeline of the key studies highlighting the importance of RTK co-activation in cancer
 These studies illustrate the important general principles of RTK co-activation and strategies for targeting this phenomenon for cancer therapy.

Figure 2. General principles of RTK co-activation

Depiction of the five key principles of RTK co-activation which have been established over the past decade of research in the field.

Figure 3. Mechanisms of RTK co-activation contributing to cancer development

Mechanisms underlying RTK co-activation can drive tumour growth and promote survival in response to microenvironmental stresses such as cancer therapeutics. (A) Many RTKs can bind to a shared pool of common SH2- and PTB-containing adaptor proteins, maintaining downstream oncogenic signal transduction despite inhibition of single RTKs. (B) Individual RTKs can contribute to distinct survival pathways. In the event that one receptor is disrupted leading to the suppression of its downstream effector signalling, effectors activated by other RTKs robustly maintain alternative survival pathways. (C) Differential signalling from individual RTKs can play roles in distinct cancer phenotypes, therefore RTK co-activation can broaden the spectrum of tumorigenic capabilities including invasion and metastatic potential.

Figure 4. Approaches to exploiting RTK co-activation in cancer therapy

RTK co-activation can be targeted using several strategies. (1) The activity of multiple RTKs can be blocked simultaneously using broad-spectrum TKI, HSP90 inhibitors or combinations of selective TKIs. Drawbacks to this approach include the dynamic kinome reprogramming response which overcomes specific RTK dependencies. Additional disadvantages include the development of acquired drug resistance after prolonged exposure to TKIs and the potential for excessive toxicity from combination therapy. (2) Targeting upstream regulators of RTK co-activation including scaffolding proteins such as FAK and epigenetic “reader” BET bromodomain proteins is a novel and promising approach that can disrupt RTK co-activation. (3) Shared downstream signalling components of multiple RTKs such as AKT and MEK can be targeted, although this strategy may be limited by paradoxical increases in RTK co-activation due to the alleviation of feedback inhibition and downregulation of RTK shedding from the cell surface.

Figure 5. Current and future approaches for measuring and integrating datasets for exploiting RTK co-activation in cancer therapy

Tumour profiling at both the global population and single cell levels are required to build a more complete picture of RTK co-activation in the context of intratumoural heterogeneity. Integrating these different datasets using computational approaches will facilitate the identification of RTK co-dependencies in critical tumour subpopulations. Leveraging on this integrated approach, strategies can be developed to utilise single cell profiling assays for patient stratification in umbrella and basket clinical trials, design combination therapies to overcome cancer evolution and prevent dynamic RTK rewiring in heterogeneous tumour sub-populations.