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Molecular Pathways: Adaptive Kinome Reprogramming in Response to Targeted Inhibition of the BRAF-MEK-ERK Pathway in Cancer

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Abstract

The central role of the BRAF-MEK-ERK pathway in controlling cell fate has made this pathway a primary target for deregulated activation in cancer. BRaf is activated by Ras proteins allowing Ras oncogenes to constitutively activate the pathway. Activating BRaf mutations are also frequent in several cancers, being the most common oncogenic mutation in thyroid carcinoma and melanoma. There are currently two inhibitors, vemurafenib and dabrafenib, approved for treatment of malignant melanoma having activating BRaf mutations. Concurrent administration of BRAF inhibitor and MEK inhibitor (trametinib) is significantly more active in patients with BRAF mutant melanoma than either single agent alone, but progression to resistance ultimately occurs by different mechanisms that increase the activation of ERK. Such adaptive changes in tumor cell signaling networks allows bypass of targeted oncoprotein inhibition. This is true with targeted inhibitors for BRaf and MEK as well as specific inhibitors for AKT, mTOR and many receptor tyrosine kinases such as EGFR and HER2. It is this adaptive response to targeted kinase inhibitors that contributes to the failure of single agent kinase inhibitors to have durable responses. This failure is seen in virtually all cancers treated with single agent kinase inhibitors, most of which are not as dependent on a single signaling pathway such as BRaf-MEK-ERK in melanoma. Thus, understanding the breadth of adaptive reprogramming responses to specific targeted kinase inhibition will be critical to develop appropriate combination therapies for durable clinical responses.

Background

Two of the major signaling systems controlling proliferation and survival of cells are the mitogen-activated protein kinase (MAPK) and phosphoinositide-3 kinase (PI3K)/AKT signaling networks (1–4). Hence, oncogenic mutations, amplifications and deletions targeting component proteins and regulators of these two pathways are common in many cancers. Development of inhibitors for key enzymes in these two pathways has progressed

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rapidly and several targeting the MAPK network have shown remarkable clinical response in patients with melanoma. Even though these inhibitors can be initially highly effective in eliciting a clinical response, progression to resistance ultimately occurs. This adaptive response involves reprogramming of the kinome to effectively bypass inhibition of the targeted kinases. Cellular mechanisms involving adaptive changes of the kinome in response to inhibitors of the MAPK network is the topic of this Molecular Pathways review.

The prototypical three-tiered mitogen-activated protein kinase (MAPK) pathway is comprised of a MAP3kinase (MAP3K), MAP-extracellular signal-regulated kinase kinase (MEK) and extracellular signal-regulated kinase (ERK) (5, 6). There are multiple MAP3Ks capable of phosphorylating and activating MEK1 and 2 proteins, both of which phosphorylate and activate ERK1 and 2. MAP3Ks that phosphorylate and activate MEK1/2 include Raf1, BRaf, MAP3K1 (MEKK1) and MAP3K8 (Tpl2/COT) (Fig. 1). This occurs on two serines in an identical peptide sequence in the activation loop of both MEK1 and MEK2, making the activation of these kinases indistinguishable by most techniques. In specific cancers, BRaf has been found to be mutated, amplified or have altered splicing leading to increased kinase activity. Raf1, MAP3K1 and MAP3K8 also have been found to be mutated or altered in expression in specific cancers (see The Cancer Genome Atlas Data Portal (7)).

MAPK substrates and cellular functions

Functionally, ERK1 and 2, the MAPKs downstream of these MAP3Ks and MEK 1 and 2, have multiple substrates that control transcription, translation, cell cycle and cell survival (8–10). While a plethora of targets have been reported (9), a much smaller number have been sufficiently validated. Recent proteomics analyses have contributed extensively to our identification of these substrates (11–13). A few representative ERK target substrates relevant to cancer phenotype are shown in Fig. 1.

A principal focus has been on characterizing nuclear targets for ERK1/2 because of its well-observed translocation to the nucleus. The list of transcription factors phosphorylated by ERK1/2 is large and includes Myc, Elk1, Ets1, Fos, SP1 and others (9, 14). ERK-mediated phosphorylation appears to stabilize short-lived transcription factors (i.e. Myc, Fos) and to assist in the formation of higher order complexes necessary for transcriptional regulation (i.e., Elk1, Ets1, Fos) (14, 15). Interestingly, recent proteomic experiments, performed in the presence or absence of MEK inhibitors, revealed a role for ERK-mediated phosphorylation in the regulation of JuB (12). Hence activation of the MEK/ERK pathway also contributes to the formation and regulation of AP1 complexes (16). A large-scale analysis of ERK2 substrates also identified an unexpected importance of ERK2 in regulating ETV3, an Ets repressor whose repressive activity was reversed by ERK catalyzed phosphorylation of ETV3 (10).

Not surprisingly the transcriptional targets regulated by MEK/ERK signaling are also broad and includes many genes whose functions are deregulated in cancer. This includes immediate early genes that are activated in response to MEK/ERK signaling (17). Prolonged activation of ERK1/2 or loss of feedback inhibition perturbs the normal transient activation

of these events and contribute to cancer progression. For example, the MAPK phosphatases (DUSPs) and Sprouty (SPRY) are involved in negative feedback regulation of MAPK and growth-factor regulated signaling by receptor tyrosine kinases such as the EGFR (18–20). Their expression is regulated transcriptionally by ERK and is dysregulated with persistent ERK activation.

In addition to the important role in nuclear signaling, the MEK/ERK pathway influences other major cellular events by direct ERK phosphorylation of specific substrates. This includes other kinases that phosphorylate their cognate substrates (Rsk, Msk, Mnk, Pak, KSR1), protein translation (EIF4EBP), RNA splicing/binding (DDX47, Hnrnph2, Bat2), microtubule and cytoskeletal organization (paxillin, cortactin), tight junction formation and cell-cell communication (connexin 43, Tip1) and mitotic spindle assembly (NUMA1). Several proteins involved in chromatin modification have also recently been identified as ERK substrates. This includes specific deacetylases (HDAC6) and bromodomain proteins (BRD1, BRD9) (12, 21).

The ERK-mediated phosphorylation of paxillin regulates an important scaffold for localizing other kinases (FAK) and GTPases (Rac) to focal adhesions (22, 23). The “downstream” phosphorylation and activation of additional kinases (Rsk, Msk, Mnk) allows these kinases to further regulate cell survival and protein translation processes in an ERK-dependent mechanism (24). Negative feedback regulation of “upstream” kinases in the MAPK network (Raf, Pak, KSR, MEK1/2) also plays an essential function in determining signaling magnitude and duration through the ERK pathway (19, 20, 25). Thus, ERK1/2 regulated phosphorylation of these numerous substrates regulates many critical regulatory functions in the cell.

MAPK signaling in cancer

BRaf is a MAP3K coupled strongly with receptor tyrosine kinase-driven MEK-ERK activation. Raf proteins are activated by GTP bound Ras (26, 27), thus oncogenic Ras proteins activate BRAF and the MEK-ERK pathway (26). BRaf is mutated in approximately 50% of metastatic melanomas, 55% of advanced thyroid carcinomas and in a lower proportion of colorectal, ovarian, and lung carcinomas (7, 28–32). The Cancer Genome Atlas (TCGA) is rapidly expanding the tumor genomic landscape database and additional tumor types with lower frequency but still significant numbers of BRaf mutations or amplifications are being defined (7). This is true for cancers where changes in H, K, NRas or BRaf were believed not to have a prominent transforming function because previous sequencing studies did not find activating mutations in these genes. For example, BRaf is amplified in approximately 31% of basal triple negative breast cancers but activating BRaf mutations are uncommon in breast cancer (33, 34). Such amplifications can contribute significantly to the enhanced activation state of the pathway. MEK and ERK are relatively infrequently mutated or amplified in primary tumors compared to BRaf.

In melanoma and thyroid carcinoma, the BRaf-MEK-ERK signaling network is a major oncogenic driver of proliferation and transformation (25, 28, 30, 31). Hence, activating mutations in components of the pathway are common. Activating BRaf mutations are the

most frequent mutations occurring in melanoma and thyroid carcinoma (7). Mutation of valine^{600} to aspartic acid or lysine (BRaf$^{V600E/K}$) is the most common activating mutations found in melanoma, but deep sequencing is uncovering activating mutations in additional residues such as BRaf$^{L597R/S/Q}$ (35, 36), reinforcing the growing realization that whole exome sequencing is required for clinical screening of oncogenic mutations even in well-characterized oncogenes such as BRaf. Infrequently, alternative splicing of the BRaf mRNA has been found to result in the expression of an activated BRaf kinase (37), indicating RNA-seq is required in parallel with whole exome-seq to discover activating BRaf genomic/transcriptomic changes in a patient's tumor. NRas mutation or amplification is found in approximately 30% of melanomas (33), which functionally activates the BRaf-MEK-ERK pathway. In thyroid carcinoma the BRaf^{V600E} mutation is the most common oncogenic mutation with activating NRas^{Q61R} missense mutations found in approximately 8% of thyroid carcinomas (7).

Triple negative breast cancer (TNBC) provides a different example of changes in the BRaf-MEK-ERK pathway from melanoma and thyroid carcinoma. In basal-like TNBC gene amplification of BRaf or upstream regulators of the MAPK pathway is frequent in the absence of activating mutations (7, 34). TCGA analysis of basal-like TNBC has determined that approximately 80% of basal-like breast cancers have some degree of genomic amplification of members of the EGFR-KRas-BRaf signaling network. EGFR, KRas and BRaf were amplified approximately 22, 32 and 31%, respectively, in basal-like tumors. The BRaf-MEK-ERK pathway is commonly activated in basal-like breast cancers consistent with the gene amplification discovery of key regulators of the pathway (7, 34, 37). Only one KRas^{G12V} and one BRaf^{V600E} mutation were discovered in the TCGA analysis. In addition, approximately 90% of basal-like TNBC tumors that were sequenced had a genomic event that would enhance activity of the PI3K/AKT pathway (7), consistent with both MAPK and PI3K/AKT being critical signaling networks in basal-like TNBC (38). In cancers such as pancreatic and ovarian carcinomas where the kinome is often silent in terms of activating oncogenic mutations, it will be important to define amplifications such as that found in TNBC. Alternative RNA splicing and altered transcript expression, possibly resulting from deregulated noncoding RNAs, must be defined using next-generation sequencing technologies for understanding deregulation of signaling networks that can be therapeutically targeted with the expanding list of kinase inhibitors.

Clinical-Translational Advances

Activated ERK has a complex feedback regulation of several components in the MAPK signaling network (Fig. 1). This feedback regulation involves ERK phosphorylation of specific receptor tyrosine kinases such as EGFR, Son of Sevenless 1 (SOS), which is a Ras guanine nucleotide exchange factor, Raf1, BRaf and MEK1. ERK phosphorylation of each of the proteins decreases their activity, effectively suppressing the activation of ERK. NF1, a Ras GTPase activating protein, is phosphorylated by ERK and this modification is thought to stabilize the protein, which could contribute to regulating Ras and control of BRaf and Raf1 activation. This complex feedback regulation of upstream members of the ERK signaling network was recently reviewed in detail (25). Of clinical significance, the mutation of BRaf^{V600} to E/K activates its kinase activity and makes it insensitive to ERK-mediated

inhibition of upstream signaling components, effectively circumventing the feedback control of this upstream activation network.

There are currently two BRaf inhibitors approved for treatment of malignant melanoma, vemurafenib and dabrafenib (39–41). Both are ATP competitive inhibitors and inhibit $BRaf^{V600E/K}$ as well as wild type BRaf and Raf1. Vemurafenib and dabrafenib have IC50s of 10 nM and 0.8 nM for purified $BRaf^{V600E}$, respectively, being 4–8 fold less potent towards wild type Raf proteins. Both vemurafenib and dabrafenib have significant clinical response in patients with $BRaf^{V600E/K}$ melanoma (39, 40, 42). In contrast, patients with wild type BRaf do not respond to these inhibitors because of a paradoxical Raf activation leading to significant ERK activity (43–45). MEK inhibitors such as trametinib inhibit ERK activation in melanoma and have shown positive clinical responses with $BRaf^{V600V/E}$ melanoma but at a lower response rate than BRaf inhibitors (45). Clinical studies have shown that concurrent administration of BRAF and MEK inhibitors is significantly more active in patients with BRAF mutant melanoma than either single agent alone (39). The concurrent administration of dabrafenib and trametinib was associated with a higher incidence of complete response (9% vs. 4%) and longer progression-free survival (9.4 months vs. 5.8 months) compared to dabrafenib alone. Even though the combination of BRAF/MEK inhibitors is initially highly effective in treating melanoma, progression to resistance ultimately occurs (45). Several mechanisms have been defined for melanoma progression to BRaf inhibitor resistance that increase the activation of ERK including: mutation of NRas (46), loss of NF1 (47, 48), overexpression of BRaf or Raf1 (49, 50), splice variants of BRaf that dimerize independent of Ras (36), increased expression of Tpl2/COT (51), activating MEK mutations (52, 53) and the induction of specific receptor tyrosine kinases (54, 55). Increased activity of the AKT/mTOR pathway has also been defined as a resistance mechanism in melanoma (56). Solit, Rosen and their co-workers have referred to such resistance mechanisms as “adaptive resistance” (25). The end result of these resistance mechanisms is the tumor cell is less dependent on the targeted oncoprotein.

It is now realized that many tumors respond to targeted inhibitors with rapid adaptive changes in signaling networks that allow bypass of targeted oncoprotein inhibition. This is true with targeted kinase inhibitors such as dabrafenib and trametinib as described above as well as specific inhibitors for AKT (57), mTOR (58) and many receptor tyrosine kinases such as EGFR and HER2 (59–61). It is this adaptive response to targeted kinase inhibitors that contributes to the failure of single agent kinase inhibitors to have durable responses. This failure is dramatic in melanoma but is seen in virtually all cancers treated with single agent kinase inhibitors, most of which are not as dependent on a single signaling pathway such as BRaf-MEK-ERK in melanoma.

Recently, chemical proteomic methods have been developed that allow assay *en masse* of the activation state of 75–80% of the expressed kinome in tumor cells (37). Study of the kinome response in TNBC to MEK inhibition by selumetinib or trametinib demonstrated a rapid upregulation of multiple receptor tyrosine kinases due to loss of ERK activation (37). In both cell lines and genetically engineered mouse models (GEMMs) of TNBC, MEK inhibition induced the upregulation of Axl, DDR1/2, KDR, PDGFRβ and additional receptor tyrosine kinases. The upregulation of the receptors was accompanied by increased

expression of the cytokines for these receptors effectively establishing autocrine/paracrine loops that activate the receptor kinase activity. Measurement of kinome activation dynamics demonstrated that the upregulated and activated tyrosine kinases in MEK inhibitor treated tumor cells stimulated the activity of additional tyrosine kinases including many Src family kinases and serine/threonine kinases represented in each of the kinase subfamilies of the kinome (37). The findings demonstrate a resiliency of the kinome that readily allows bypass of targeted kinase inhibition. We have referred to this dynamic response as “kinome reprogramming” because the response is broad and involves kinases in each of the seven subfamilies of the kinome.

It was also evident in these studies that the induction and activation of receptor tyrosine kinases was driving escape from MEK inhibition (37, 62). The chemical proteomic methods used to analyze kinome reprogramming identifies kinases using mass spectrometry, which allows identification of specific phosphorylated tyrosines, serines and threonines in closely related proteins such as the phosphosites in the activation loops of MEK1 and MEK2 that are not distinguished by available antibodies. It was found that the activity of both MEK1 and MEK2 was initially inhibited by selumetinib treatment of TNBC cells and tumors, but with continued administration of selumetinib MEK1 remained inhibited but MEK2 escaped inhibition allowing reactivation of ERK. Thus, MEK2 selectively escapes inhibition by allosteric MEK inhibitors.

The mechanism for selective MEK2 escape from inhibition has significant clinical implications for the use of MEK inhibitors as single agents. MEK inhibitors such as trametinib and selumetinib bind to an allosteric regulatory site conserved in MEK1 and MEK2 (8). Binding of trametinib, selumetinib and other MEK inhibitors to this allosteric regulatory site inhibits MEK1 and MEK2 kinase activity towards ERK1/2. In response to MEK inhibition the adaptive response leads to upregulated receptor tyrosine kinases that stimulate the formation of GTP-bound Ras leading to Ras-induced dimerization and activation of Raf (Fig. 2). Activated Raf kinases phosphorylate two conserved serines on the activation loops of MEK1 and MEK2. This dual serine phosphorylation is required for MEK1 and MEK2 activation leading to phosphorylation of ERK1/2 (8). As the MEK activation loop serines become phosphorylated by Raf, the affinity of MEK inhibitors decreases significantly for the allosteric regulatory site that they bind. When both activation loop serines are phosphorylated the affinity of MEK inhibitors for binding to the allosteric regulatory site is decreased by 20-fold (63, 64), effectively diminishing the potency of the inhibitors (Fig. 2). In addition, MEK1 encodes a threonine at position 292 ($MEK1^{T292}$) that is absent in MEK2 (8, 65). $MEK1^{T292}$ is phosphorylated by ERK and functions as a negative feedback regulatory site functionally inhibiting MEK1 (65). This site is not conserved in MEK2, so that when MEK1 and MEK2 are phosphorylated on their activation loops and regain activity due to diminished potency of the allosteric inhibitor, ERK can be at least partially reactivated. MEK1 would be subject to feedback inhibition but the activated MEK2 would escape.

Concluding Remarks

The observation that MEK2 can escape from inhibition by allosteric site inhibitors suggests that MEK inhibitors will need to be used in combination with other inhibitors. But in tumors where BRaf is wild type, paradoxical Raf activation will result from treatment with Raf inhibitors and would result in an even greater adaptive response. ERK inhibitors are currently in preclinical development and have been shown in cell lines to inhibit the emergence of MEK inhibitor resistance as well as overcome acquired resistance to MEK inhibitor (66, 67). Thus, cotargeting MEK and ERK may provide significantly more durable responses than either agent alone. It must be noted that both MEK and ERK inhibitors will cause loss of ERK activity and initiate adaptive responses involving upregulation of receptor tyrosine kinases that will not only activate the Ras-Raf network but alternative pathways such as PI3K/AKT. This has led to the proposed use of intermittent BRaf inhibitor treatments (68) or combination therapies that includes an inhibitor of the Raf-MEK-ERK pathway and receptor tyrosine kinase inhibitor (54, 55). An example of the latter is lapatinib in combination with vemurafemib in thyroid carcinoma having $BRaf^{V600E}$ mutation (69).

Given the heterogeneity of adaptive responses to targeted kinase inhibitors and the resiliency of the kinome to effectively bypass targeted inhibition, it seems that combinations of specific kinase inhibitors can prolong clinical response but resistance and disease progression will ultimately occur. For targeted kinase inhibitors to have truly durable responses novel therapeutic strategies will need to be developed. We propose that it is necessary to prevent the upregulation of receptor tyrosine kinases and the adaptive kinome reprogramming that is seen with targeted kinase inhibition. A more complete understanding of the molecular mechanisms of adaptive kinome reprogramming will be required to effectively develop therapeutic approaches to arrest and prevent the progression to resistance seen with kinase inhibitors.

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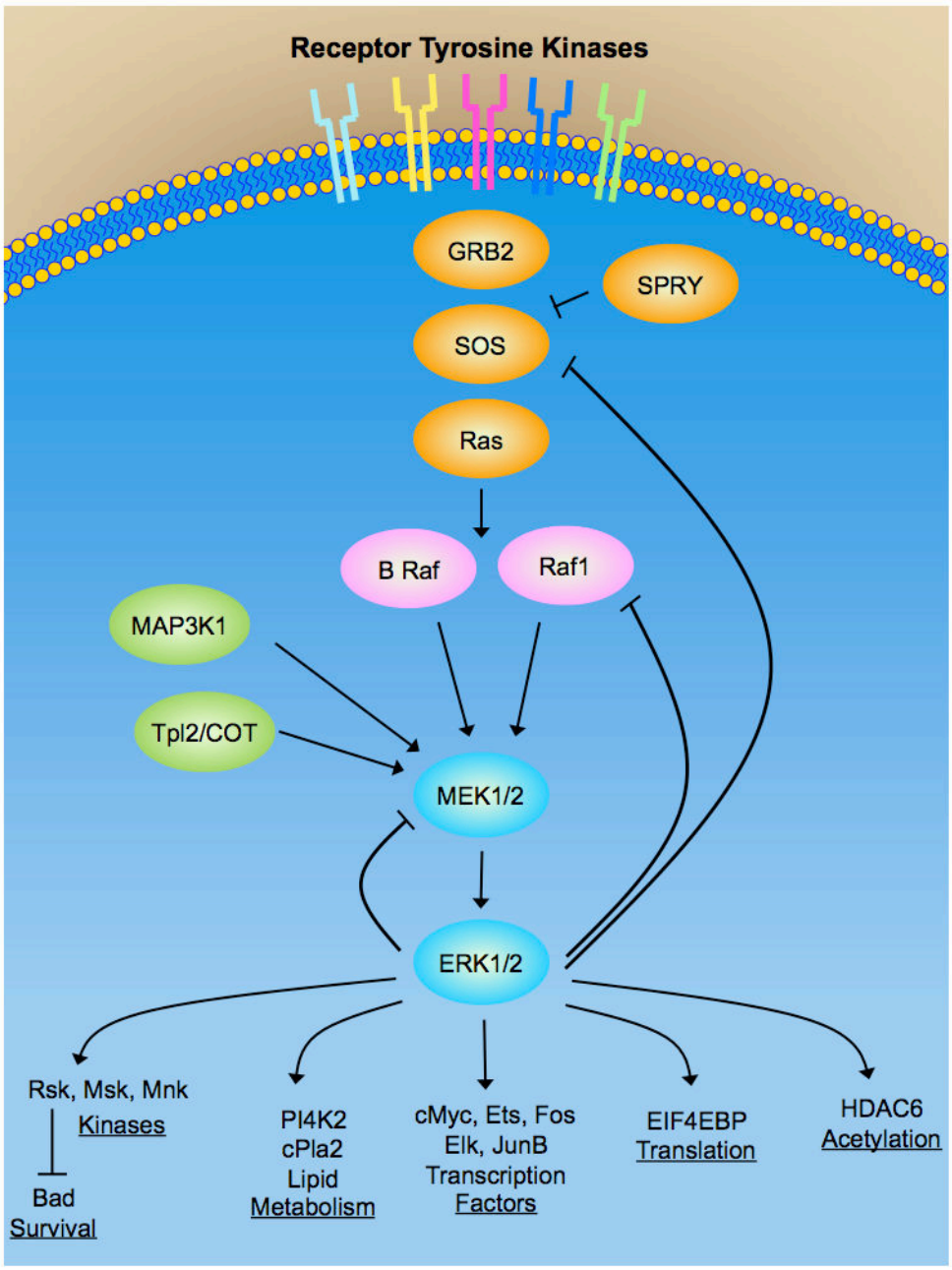

Figure 1.

Model of the ERK1/2 MAPK signaling network controlled by receptor tyrosine kinases and Ras. ERK1/2 is part of a three kinase cascade involving BRaf/Raf1 and MEK1/2. MAP3K1 (also known as MEKK1) and Tpl2/COT (also known as MAP3K8) function as MAP3Ks that can also phosphorylate and activate MEK1/2 and regulate and bypass Raf inhibition. ERK1/2 phosphorylate upstream kinases including BRaf/Raf1 and MEK1 to feedback inhibit their activity. ERK1/2 also phosphorylates and inhibits the Ras guanine nucleotide exchange activity of SOS.

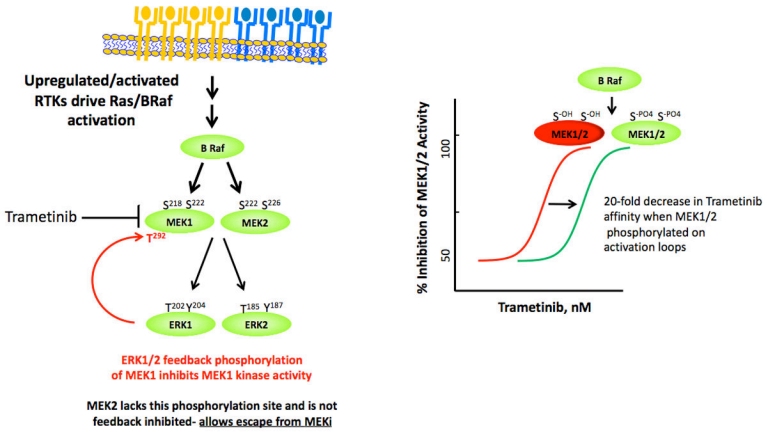

Figure 2.

Phosphorylation of the activation loop serines on MEK1 and 2 decreases affinity for allosteric inhibitors such as trametinib and selumetinib. MEK1 T^{292} is a phosphorylation site for ERK1/2 that negatively regulates MEK1 kinase activity. MEK2 lacks this negative feedback regulatory site and is not feedback inhibited by ERK1/2 dependent phosphorylation.