

Review

Comprehensive Analysis of ERK1/2 Substrates for Potential Combination Immunotherapies

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Recent clinical and therapeutic success with RAF and MEK1/2 inhibitors has revolutionized the existing treatment schemes for previously incurable cancers like melanomas. However, the overall therapeutic efficacies are still largely compromised by the dose-limiting side effects and emerging drug resistance mechanisms. Accumulating evidence has revealed the intricate nature of the RAS-RAF-MEK1/2-ERK1/2 pathway, such as activation mechanisms, kinase–substrate relationships, crosstalk with parallel signaling pathways, feedback regulations, and intimate interplay with immune responses. Limited strategies are currently available to exploit the benefits of combining RAF-MEK1/2-ERK1/2 pathway inhibitors with other targeted therapies or immunotherapies. Here, we compiled the kinase–substrate relationships and analyzed the intricate signaling networks of the renowned pathway, providing an integrated and simplified visualization, to reveal the potentials of RAS-RAF-MEK1/2-ERK1/2-based combination therapies.

The Renowned RAS-RAF-MEK1/2-ERK1/2 Signaling Pathway

The RAS subfamily of small GTPases comprises >150 members [1] and controls almost all essential biological processes. Extracellular signals from various receptor tyrosine kinases, G protein-coupled receptors, N-methyl-D-aspartate receptor, T cell receptor, and C-type lectin receptor, all play a role in converting RAS (HRAS, KRAS, and NRAS) from inactive RAS-GDP to the active RAS-GTP [2]. Cytosolic RAF (ARAF, BRAF, and RAF1/CRAF) is then recruited to the plasma membrane to form the RAF-RAS-GTP complex [3]. Activated RAF initiates a cascade of mitogen-activated protein kinases (MAPKs), from MEK (MAPK kinase, including MEK1/MAP2K1 and MEK2/MAP2K2) to ERK (ERK1/MAPK3 and ERK2/MAPK1), and to MAPK-activated kinases [4] (Figure 1). According to COSMIC v89, KRAS and BRAF mutations have been identified in 17.99% and 17.90% of all tested samples. While not all mutations in the RAS-RAF-MEK1/2-ERK1/2 pathway may ultimately lead to tumorigenesis, multiple mechanisms have been found to promote aberrant ERK1/2 signaling, including but not limited to the: (i) aberrant signaling of the receptor tyrosine kinases, (ii) loss of the neurofibromatosis type 1 tumor suppressor, (iii) driver mutations of RAS, RAF, and MEK1/2, or (iv) overexpression of TPL2/COT [2]. Hyperactivation of the MEK1/2-ERK1/2 pathway has also been highlighted to promote developmental disorders like the RASopathies [5]. Tremendous efforts have been made to reveal how aberrant RAS activation directs abnormal signaling of the RAF-MEK1/2-ERK1/2 pathway, upon which therapeutic strategies are proposed to disrupt or inhibit the: (i) regulator/effector interactions, (ii) membrane association, (iii) downstream RAS effectors, (iv) synthetic lethal interactions, and (v) metabolic pathways [6]. While RAS itself has historically been considered undruggable, three BRAF inhibitors and three MEK1/2 inhibitors have recently been approved by the U.S. Food and Drug Administration to treat cancer patients with $BRAF^{V600E/K}$ mutations [7]. After decades of extensive research and development, highly selective inhibitors targeting the RAS^{G12C} mutations have shown great potentials during clinical trials, of which up to 50% response rates were achieved for KRAS-mutant lung cancer. However, the dose-limiting side effects and the rapid emergence of **drug resistance** (see Glossary) compromise their efficacies, warranting closer scrutiny of the intricacy of this renowned pathway [7]. Since the RAF-MEK1/2-ERK1/2 pathway acts primarily through **phosphorylation**, it would be critical to understand their **kinase–substrate relationships**. This review compiled the *bona fide* substrates of the RAF-MEK1/2-ERK1/2 pathway and analyzed the signaling networks involved, with the aim of providing mechanistic insights towards future **combination therapies**.

From RAF to the MEK1/2 pathway

The paradoxical activation of RAF, especially by the first generation BRAF inhibitors in RAS-mutated cancers, has recentered drug development to inhibitors targeting MEK1/2 [2]. This strategy is

Highlights

The RAS-RAF-MEK1/2-ERK1/2 pathway remains one of the most commonly dysregulated pathways in human cancers, based on which several inhibitors have already been approved by the U.S. Food and Drug Administration for treatment of mutation-positive melanomas since 2011.

Despite the success of RAF-MEK1/2-ERK1/2 inhibitors, their efficacies are compromised by the emerging drug resistance and dose-limiting side effects.

The immunomodulatory roles of oncogenic RAS signaling, the RAF-MEK1/2-ERK1/2 inhibitors, and the TPL2-MEK1/2-ERK1/2 pathway have been progressively recognized, leading to the recent approval of combination immunotherapies with RAF-MEK1/2-ERK1/2 inhibitors and immune checkpoint blockade.

The intricacy of the kinase–substrate relationships in the RAF-MEK1/2-ERK1/2 pathway and the kinase activity-independent physiological roles of RAF, MEK1/2, and ERK1/2 are essential for drug development, but remain unclear.

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feasible, especially since MEK1/2 has long been considered the sole substrate of RAF [7]. However, it should be noted that: (i) at least 13 kinases phosphorylate RAF; (ii) other than RAF, MEK1/2 is phosphorylated by a dozen other kinases; and (iii) besides phosphorylating MEK1/2, which is the most well-characterized RAF substrate, RAF also phosphorylates at least 11 other substrates (Figure 1). The activation process of RAF involves an intricate series of events, starting from RAF re-localization, to protein and lipid interactions, and to regulatory phosphorylation/dephosphorylation [3]. In particular, SRC kinases are primarily involved in phosphorylating tyrosine residues in the N-region of RAF, whereas the PAK family (PAK1 and PAK3), PKC family (PKC kinase), and casein kinase 2 (CK2), phosphorylate serine residues of the N-region [3]. New evidence has challenged the long-standing views on CK2 kinase, proposing that it may function as a scaffolding protein in a kinase activity-independent manner [8]. Although some studies have reported how the kinase suppressor of Ras 1 (KSR1) activates RAF1 through phosphorylation [9], the KSR1 is generally considered a scaffolding protein that actively interacts with and assembles the RAF-MEK1/2-ERK1/2 pathway, instead of being a *bona fide* protein kinase [3,10]. During cAMP signaling, the PKA-mediated phosphorylation of serine 259 on RAF directly inhibits BRAF and RAF1, the effects of which are otherwise antagonized by PKA-mediated Rap1 phosphorylation and BRAF/Rap1 binding to the KSR complex [11].

RAF kinases are themselves also phosphorylated by several other kinases (Figure 1). Following RAS-mediated activation, AKT phosphorylates RAF to suppress the RAF-MEK1/2-ERK1/2 pathway and revoke the proliferation of human breast cancer cells [12]. Alternatively, AKT phosphorylates and activates the MST2/Hippo pathway, which initiates the LATS1-dependent feedback phosphorylation of RAF1 and the suppression of MST2 and MEK1/2-ERK1/2 pathways [13]. In contrast, JNK MAPK, which is activated by the RAS-RAF-MEK1/2 pathway, in turn phosphorylates and activates RAF1 to complete the positive feedback loop [14]. The PAK3-mediated RAF1 phosphorylation and activation modulate cell survival and apoptosis by transducing signals from the Rho-family of GTPases, Rac and Cdc42 [15]. Alternatively, CDK1/Cyclin B directly phosphorylates BRAF to activate the MEK1/2 pathway that is critical for mitosis [16]. Surprisingly, the inhibition of AAKP1 blocks the RAF inhibitor-driven paradoxical activation of the ERK1/2 pathway and proliferation of RAS-mutated cancer cells, primarily by targeting AAKP1-mediated RAF1 phosphorylation and formation of RAF1/14-3-3 complex [17]. During crosstalk between the RAF-MEK1/2-ERK1/2 pathway and the extracellular matrix-integrin pathway, fibronectin stimulation activates CaMKII-mediated binding and phosphorylation of RAF, possibly leading to integrin-based cancer cell survival [18]. Activation of RAF by distinct kinases integrates the signaling cues from diverse stimuli before engaging with the MEK1/2 pathway. While MEK1/2 has long been considered the sole substrate of RAF [7], the phosphorylation of at least 12 other substrates by RAF diversifies its regulatory roles through less characterized mechanisms (Table S1 in the supplemental information online).

From TPL2 and Other Kinases to the MEK1/2 Pathway

Besides RAF, TPL2 (also known as COT and MAP3K8) and MOS are the two most commonly implicated kinases that activate the MEK1/2 pathway (Figure 1). The MEK1/2-ERK1/2 pathway is explicitly activated by TPL2 during immune responses by toll-like receptors (TLRs), small peptide sensor NOD2, TNF receptor 1, and IL-1 receptor [19]. TPL2 activation depends on p105 phosphorylation by the IKK complex or NEMO kinase, culminating in physiological outcomes like immune cell development and activation, inflammation, immune defense, and autoimmune diseases [20]. Particularly, the gain-of-function of *Tp12* in inflammatory bowel disease is characterized by increased IL-1 β and IL-18-initiated caspase activation and inflammation [21]. Apart from its proinflammatory roles, *Tp12*-deficiency and MEK1/2 inhibition were reported to elevate the production of IL-12 and type I interferon during TLR9 signaling in macrophages [19]. Several recent reports also highlight the involvement of the TPL2-MEK1/2-ERK1/2 pathway in cancers. In spitzoid and melanomas, truncations and fusions of TPL2 [22] presumably lead to the activation of the MEK1/2-ERK1/2 pathway and imatinib resistance [23]. The predictive values of TPL2 in MEK1/2 inhibitor treatment have been recently recognized in ovarian carcinomas [24], rendering this alternative signaling of the MEK1/2-ERK1/2 pathway as a promising drug target in both immune cells and cancers.

Glossary

Bypass-signaling pathway: a signaling pathway that transduces signals from similar inputs to similar outputs by engaging with a different set of signaling molecules. The existence of a bypass-signaling pathway provides the signaling network necessary redundancy to maintain the robustness and integrity of signal transduction in both homeostasis and disease.

Catalytic activity: a description of the increase in the rate of chemical reaction caused by the presence of a catalyst. In biology, the active site is a region of the catalyst/enzyme where the reactant/substrate binds and undergoes catalyzed/enzymatic reactions. Small inhibitors targeting the catalytic activities of various enzymes have greatly enriched the toolkits for targeted therapies.

Combination therapy: a type of treatment strategy combining more than one therapy to overcome drug resistance or maximize therapeutic effects. Interactions between different therapies, if any, are controlled and favorable.

Drug resistance: a description of unfavorable response to a certain drug treatment. Based on the differences of origins and mechanisms, drug resistance can be classified into innate/primary resistance, adaptive immune resistance, and acquired resistance. Particularly, drug resistance to immunotherapies may also be classified into tumor cell intrinsic and tumor cell extrinsic resistance.

Feedback loop: a closed loop of signaling molecules that link the downstream effectors back to its upstream component, to either positively or negatively adjust the signaling processes. Aberrantly regulated feedback loop often leads to uncontrolled signaling commonly implicated in diverse human diseases like cancer.

Gene ontology (GO): an up-to-date and comprehensive bioinformatics initiative to annotate genes and gene product attributes across all species. The ontology of defined terms annotates three major domains of gene product properties, including cellular components, molecular

Similar to RAF, the MEK1/2 pathway also integrates signaling cues from diverse stimuli. The stabilization of inactive KSR antagonizes the oncogenic RAF heterodimerization and MEK1/2 activation in RAS-mutated cancer cells, independent of the **catalytic activity** of KSR [25]. As discussed earlier, this evidence further adds to the controversies that KSR may function in kinase activity-dependent [9] or -independent manners [10,25]. A recent study has identified MAST1 as a novel upstream kinase of MEK1 that replaces RAF1 to reactivate the MAPK pathway and confers cisplatin resistance [26]. During treatment with RAF inhibitor, the MLK kinases are upregulated in melanoma patients, leading to the activation of the MEK1/2 pathway through direct phosphorylation [27]. As a novel kinase that phosphorylates both the RAF1 and MEK1/2, PLK1 fine-tunes the activation of the RAF-MEK1/2-ERK1/2 pathway to trigger oncogenic transformation and epithelial-to-mesenchymal transition of prostate epithelial cells [28]. Another kinase known to phosphorylate both the RAF and MEK1/2 is the PAK1. Adhesion to fibronectin of the extracellular matrix induces MEK1 phosphorylation by PAK1 and ensures efficient activation of the MEK1/2 pathway [29]. Interestingly, MEK1 and MEK2 phosphorylation by PDK1 are essential for their activation, but not the prolonged signaling, of the MEK1/2 pathway [30]. While MEKK3 and MEKK4 do not phosphorylate MEK1/2, both the MEKK1 and MEKK2 phosphorylate MEK1/2 but do not activate the ERK1/2 pathway [31]. There is also evidence showing how phosphorylation of MEK1 by ERK1/2, CDK1, or CDK5 reduces its ability to activate the ERK1/2 pathway during neurotransmission in the brain [32]. The abilities of the MEK1/2 pathway to integrate signals from immune responses and other stimuli greatly enable the intricate roles of the RAF-MEK1/2-ERK1/2 pathway.

From MEK1/2 and Other Kinases to the ERK1/2 Pathway

In addition to ERK1/2, there are at least five other substrates known to be phosphorylated by MEK1/2 (Figure 1 and Table S2 in the supplemental information online). To further complicate the signaling of the ERK1/2 pathway, several other kinases, besides MEK1/2, have been shown to phosphorylate and activate ERK1/2, albeit with less clear therapeutic implications. As part of the intricate interplay between the cell cycle and ERK1/2 pathway, CDK1 has been shown to phosphorylate all kinases in the cascade of the RAF-MEK1/2-ERK1/2 pathway (Figure 1). The phosphorylation of an alternatively spliced ERK variant of ERK1c by CDK1 allows ERK1c to bind to a shuttling complex involving 14-3-3 γ , a process essential for Golgi fragmentation during the G2/M transition [33]. In another distinct setting, the phosphorylation of ERK2 by SGK1 enhances ERK-mediated liver regeneration and augments the formation of MEK/ERK complex [34]. Another report identified the lymphocyte-specific protein tyrosine kinase, LCK, as one kinase that phosphorylated but did not activate ERK1 [35]. More mechanistic studies are needed to further address the physiological roles of LCK in ERK1 phosphorylation. RET, a tyrosine kinase receptor implicated in multiple tumor pathogenesis, directly phosphorylates and activates ERK2 [36]. Importantly, RIPK2 mediates a positive feedback loop between RAS-RAF1 and TNF-dependent activation of the ERK1/2, by directly phosphorylating ERK1/2 and being phosphorylated by RAF1 [37]. Similar to the intricate kinase-substrate relationship of RAF, divergent signals converge at MEK1/2 before being transduced primarily to ERK1/2 and further downstream to the ERK1/2 pathway, as detailed below.

The Intricate Relationships between ERK1/2 and Its Substrates

A Compilation of the Direct Substrates of ERK1/2

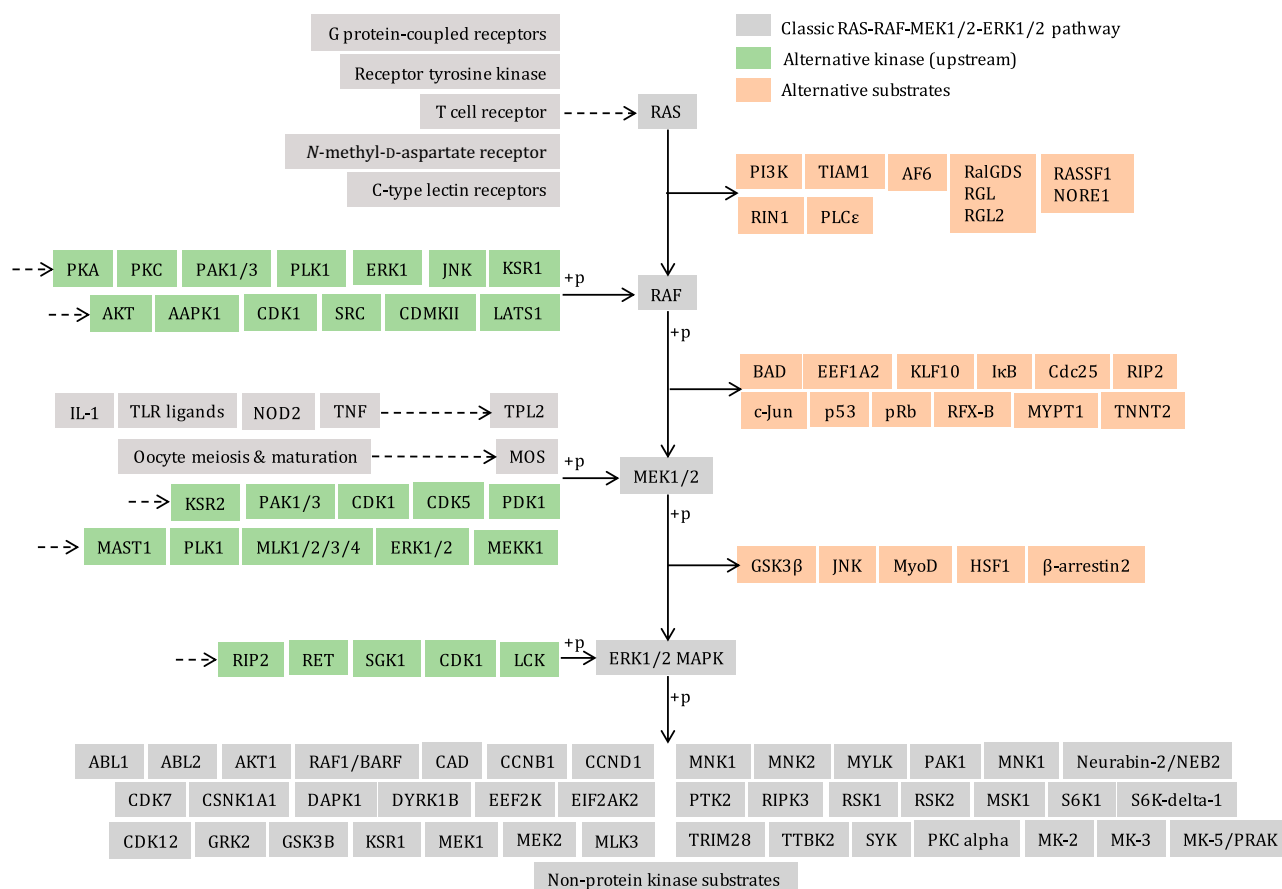
The discoveries of ERK1/2 substrates have increased significantly in the past three decades [38]. However, the kinase-substrate relationships reported using approaches other than purified/recombinant protein-based *in vitro* kinase assays do not necessarily indicate the involvement of a certain kinase, especially when multiple kinases tend to be active when in proximity [38]. Therefore, to better delineate the intricacy of the ERK1/2 pathway, we compiled a total of 497 substrates from literature and several databases (Table S3 in the supplemental information online), using the following criteria: (i) the kinase-substrate relationships were validated *in vitro* using purified or recombinant proteins, (ii) at least one site is phosphorylated, and (iii) physiological relevance is implicated.

functions, and biological processes.

Kinase-substrate relationship: a description of phosphorylation-based regulatory mechanism involving a pair of kinase and substrate. The specificity and substrate preference of a kinase determine whether the relationship is one-to-one or one-to-many.

Pathogen-recognition receptor: a type of receptor that recognizes the conserved pathogen-associated molecular patterns of invading pathogens or danger-associated molecular patterns (from self), to mount tailored immune responses that may engage with diverse cell types in both innate and adaptive immunity. Agonists designed to activate the immunomodulatory activities of these receptors represent one of the most promising cancer-immunotherapy strategies.

Phosphorylation: a common form of post-translational modification where a substrate acquires a phosphate group from high-energy phosphate-donating molecules like ATP in the presence of a kinase. Protein phosphorylation occurs mostly at the serine, tyrosine, and threonine residues of a substrate and can be reversed by another type of enzyme called phosphatase. Phosphorylation of a protein alters its structural conformation, resulting in its activation, deactivation, inhibition, degradation, relocalization, or other regulatory outputs.



Trends in Pharmacological Sciences

Figure 1. The Kinase–Substrate Relationships in the Renowned RAS-RAF-MEK1/2-ERK1/2 Pathway.

The classic RAS-RAF-MEK1/2-ERK1/2 pathway transduces extracellular signals from G protein-coupled receptors, through receptor tyrosine kinase, T cell receptor, N-methyl-D-aspartate receptor, and the C-type lectin receptors, to unlock the inactive form of RAS-GDP and recruit RAF to the plasma membrane. Activation of RAF by phosphorylation initiates the MEK1/2-ERK1/2-substrate pathway through direct phosphorylation of MEK1 and MEK2 kinases. Besides RAF, the RAS effectors also collaborate with at least eight alternative proteins or complexes, including PI3K, TIAM1, AF6, RIN1, PLCε, RASSF1/NORE1, and RalGDS/RGL/RGL2. Apart from the most well-characterized kinase–substrate relationships in the cascade of RAF-MEK1/2-ERK1/2 MAPK, the past three decades of research have unveiled a variety of protein kinases and non-protein kinase substrates, which have also been shown to either phosphorylate (+p) RAF, MEK1/2, and ERK1/2 kinases or be phosphorylated (+p) by RAF, MEK1/2, and ERK1/2 kinases. Feedback phosphorylation mediated by ERK1/2 further raises the intricacy of this signaling network and has been implicated in diverse physiological conditions. The dashed-line arrows indicate indirect or unspecified relationships involving additional signaling molecules. See also compiled lists in Tables S1 and S2 in the supplemental information online.

The Diverse Roles and Functions of ERK1/2 Substrates

Amongst the compiled substrates, 495 are curated by the Uniprotⁱⁱ and Gene Ontology Consortiumⁱⁱⁱ databases. Gene ontology (GO) terms for the cellular components, molecular functions, and biological processes of annotated ERK substrates were retrieved and analyzed. ERK1/2 substrates either directly control essential processes like RNA polymerase II-mediated gene transcription, or indirectly through catalytic activities exhibited by at least 149 ERK1/2 substrates (Table S3 in the supplemental information online). Empowered by the complex but tiered signaling network, the RAF-MEK1/2-ERK1/2 pathway not only integrates but also diversifies the signaling pathways, which are intertwined with feedback loops [39,40] and crosstalks with parallel pathways, as detailed below.

Common Substrates of ERK1/2 and Parallel Pathways

In line with the essential roles of the RAS-RAF-MEK1/2-ERK1/2 pathway in cancer [2,41], 63 ERK1/2 substrates are involved in the pathways in cancer (hsa05200). Over 20% of genes annotated within the ErbB, VEGF, and thyroid hormone signaling pathways were identified as ERK1/2 substrates. Notably, 49 ERK1/2 substrates are involved in the MAPK signaling pathway, the second highest amongst all retrieved 54 KEGG^{iv} signaling pathways (Table S4 in the supplemental information online), thus providing the basis for ERK1/2-based feedback regulations and drug resistance mechanisms, as elaborated in the next section. Substrates involved in the PI3K-AKT, JAK-STAT [42], Wnt [43], chemokine, and **pathogen-recognition receptor** (e.g., TLR, NOD-like receptor)-mediated signaling pathways are also observed.

The crosstalk between the RAF-MEK1/2-ERK1/2 pathway and PI3K/AKT/mTOR pathway has long been recognized to occur through multiple mechanisms [44], drawing considerable interest in both preclinical and clinical studies [45]. Since the RAF-MEK1/2-ERK1/2 pathway and PI3K-AKT pathway are two of the most essential pathways involved in the signaling of RAS and ErbB, VEGF, and thyroid hormone, it is unsurprising that some ERK1/2 substrates are also phosphorylated by AKT. Apart from the most well-known cross-inhibitions between these two pathways [44], information on how they crosstalk through downstream kinase-substrate relationships remains elusive. Therefore, this review primarily highlights the substrate-level crosstalk between them. There are a total of 197 unique proteins phosphorylated by AKT *in vitro* [46], of which 48 are shared with ERK1/2 (Table S5 in the supplemental information online). Based on GO molecular functions, estrogen receptor genes (*ESR1* and *ESR2*, encoding estrogen receptors, ER α and ER β , respectively) in the list of shared substrates show more than 100-fold enrichment for estrogen response element binding (GO:0034056) and ER activities (GO:0030284) (Figure 2A). This is of particular significance, considering that ERs are crucial in the development and progression of hormone-related breast cancers and endocrine therapy resistance [47]. Notably, ERK1/2 phosphorylates multiple sites on ER α , many of which act as positive feedback loops of ER signaling and thus confer acquired resistance to breast cancer drug, tamoxifen [47]. Similarly AKT phosphorylates serine 167 of ER α and enhances ER α -dependent transcription [47]. It should be noted that crosstalk between AKT and ERK1/2 also occurs through ER-related transcription factors, as reviewed before [44].

Other substrates shared by AKT and ERK1/2 are generally involved in kinase and phosphatase binding, or DNA and transcription factor binding during biological processes like immune cell development (Figure 2B). For example, GATA1 and GATA2 are two shared substrates essential for the differentiation of eosinophil and primitive erythrocyte, as well as the fate-commitment of eosinophil. Besides, GATA1 also controls the differentiation of basophil (GO:0030222), together with GAL1. Another example of AKT-ERK1/2 crosstalk occurs during T cell differentiation. Similar to prolonged ERK1/2 signaling that dictates the alternative $\alpha\beta$ and $\gamma\delta$ T cell lineage fates [48], the inhibition of AKT uncouples T cell differentiation from expansion [49], possibly through the AKT-FOXO1/FOXO3 axis in IL-2 signaling [50]. Despite the roles of ERK1/2-FOXO1/FOXO3 in T cell development, AKT inhibition does not impair ERK1/2 pathway activation [49], indicating that these two pathways are distinctly wired. Given that 13 and 30 out of 48 shared substrates exhibit at least one kinase activity and catalytic activity, respectively (Table S5 in the supplemental information online), they may engage with additional substrates and further expand the regulatory networks. Therefore, the crosstalk between these two renowned pathways appears far more complicated than commonly recognized. Future investigations into their division of functions in cancer, especially the kinase-substrate relationships, are needed to delineate the signaling complexity and reveal unexplored but highly promising therapeutic opportunities.

Current and Promising Strategies for Combination Therapies

Inhibitors targeting the RAF-MEK1/2-ERK1/2 pathway have revolutionized the treatment schemes for presumably incurable advanced-stage melanoma patients and have spurred ample interest in novel combination therapies [41]. However, the intricate nature of the RAF-MEK1/2-ERK1/2 pathway, as detailed above, and its complex interplay with parallel pathways are purported in various drug



Figure 2. Crosstalk between AKT Pathway and ERK Pathway.

AKT substrates with both *in vitro* validation and cellular implications were retrieved from the PhosphoSitePlus database [46]. Substrates shared between the AKT and ERK were shortlisted and subjected to analysis of (A) Gene ontology (GO) molecular function and (B) GO biological process, using PANTHER v14.1 GO enrichment analysis [88,89]. Fisher's exact test was performed by default and corrected using the recommended Bonferroni correction for multiple testing. (A) The shared substrates are most highly enriched and clustered for their activities in enzyme binding, nucleic acid binding, receptor activity,

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resistance mechanisms. In the era of immune checkpoint blockade-based immunotherapies, the immunomodulatory roles of RAS oncogenic signaling [51] and the pervasive activities of the RAF-MEK1/2-ERK1/2 pathway [52,53] are becoming widely recognized. To explore the therapeutic opportunities on the kinase–substrate relationships in this pathway, the following strategies have been proposed and explored for future development of novel combination therapies.

RAF/MEK/ERK and the Bypass-Signaling Pathways

Based on our compiled kinase–substrate relationships in the RAF-MEK1/2-ERK1/2 pathway, we proposed that this pathway may be bypassed by parallel pathways which engage: (i) other RAS effectors; (ii) alternative kinases that activate ERK substrates through RAF-, MEK1/2-, and/or ERK1/2-independent mechanisms; or (iii) alternative substrates of RAF and MEK1/2 (Figure 3). It is, therefore, possible that targeting both the RAF-MEK1/2-ERK1/2 pathway and these **bypass-signaling pathways** may largely circumvent the emergence of drug resistance mechanisms. One well-characterized example is the PI3K/AKT/mTOR pathway, which is another important RAS effector and bypasses the RAF-MEK1/2-ERK1/2 pathway through multiple mechanisms [54]. Hitherto, there are 16 distinct combinations under clinical evaluation with inhibitors targeting both the PI3K/AKT/mTOR pathway and the RAF-MEK1/2-ERK1/2 pathway (Figure 4 and Table S6 in the supplemental information online). The clinical results have been promising in patients with advanced cancers, albeit at the expense of greater toxicity [55,56]. However, it is interesting but still unclear how the shared AKT and ERK1/2 substrates such as the GSK3 β [57] and PAK1 [58] may be targeted to avoid dose-limiting side effects from occurring after concurrent blockades of these two essential pathways.

Efforts have also been made to target other less-characterized kinases that bypass the RAF-MEK1/2-ERK1/2 pathway. One recent illustration of an alternative kinase-mediated bypass is the PAK kinases that activate ERK1/2 independent of RAF, a mechanism distinct from its most well-recognized catalytic roles in the phosphorylation of CRAF [59] and MEK1 [29]. Alternatively, it regulates the JNK and mTOR pathway to bypass the entire RAF-MEK1/2-ERK1/2 pathway, resulting in drug resistance to RAF-MEK1/2-ERK1/2 inhibitors [58]. Consistent with MEK1/2 activation by MLKs that bypass the RAF and confer drug resistance to RAF inhibitors (Figure 3), the gain-of-function *MLK* mutations and upregulation of *MLK* expression are identified in multiple melanoma patients [27]. Importantly PDK1 bypasses RAF by directly phosphorylating MEK1/2 or indirectly through the AKT-PAK-MEK1/2 axis. It may also bypass both RAF and MEK1/2 by PDK1-SGK1-mediated ERK1/2 phosphorylation (Figure 3). Inhibition of PDK1 suppresses the growth of *BRAF*-mutant and *BRAF* inhibitor-resistant melanoma cells by rewiring reactive oxygen species-mediated inactivation of pyruvate dehydrogenase PDE-1 α [60]. Furthermore, the roles of SGK1, as one of the kinases that may bypass both the RAF and MEK1/2, inhibit apoptosis and promote proliferation of intestinal epithelial cells by engaging with the ERK1/2-p53 pathway, as shown in colitis [61].

Strikingly, the alternative substrates of RAF and MEK1/2 may also bypass the oncogenic signaling of the RAF-MEK1/2-ERK1/2 pathway, rendering them novel targets to circumvent drug resistance to single or combined RAF and MEK inhibition. Of note, *BRAF* phosphorylation of I κ B activates the NF- κ B pathway, which presumably triggers the invasiveness of thyroid cancer cells. The concurrent inhibition of MEK1/2 and NF- κ B significantly reduces tumor growth [62]. The phosphorylation of HSF1, a newly identified MEK1/2 substrate, deactivates its pro-oncogenic roles and provokes proteomic chaos leading to amyloidogenesis [63]. MEK1/2 also phosphorylates JNK to phosphorylate and unlocks the autoinhibitory conformation of a MEK1/2 scaffolding protein, BPGAP1, driving proliferation and tumorigenic transformation of breast cancer cells [64]. Since the functions of ERK1/2 substrates overlap significantly with effectors from multiple signaling pathways (Figure 3),

Figure 2. Continued

and kinase activity. Selected substrates involved in each gene set are shown in different colors corresponding to the *P* values of fold enrichment. (B) The shared substrates are essential for diverse biological processes, such as immune cell development and tissue regeneration, based on their regulatory roles in catalytic activities, metabolic processes, and others. Only gene sets enriched with larger than 100 folds, FDR (false-discovery rate) < 0.05, and *P* values < 0.001 were shortlisted and presented. See also Table S5 in the supplemental information online.

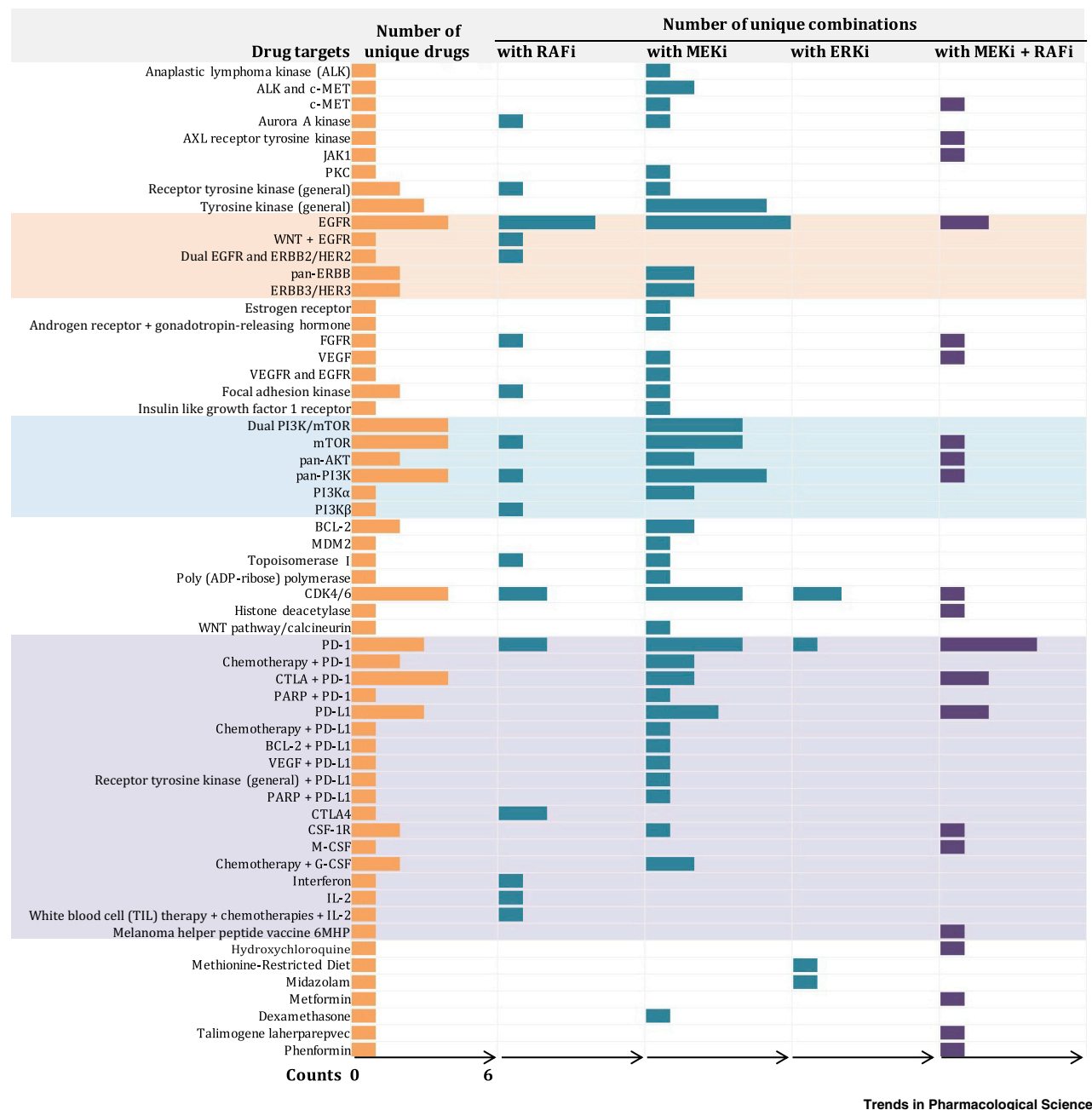


Figure 4. The Number of Clinical Trials Testing Combinations with RAF-MEK-ERK Inhibitors and Other Therapies.

Clinical trials involving combinations using at least one inhibitor selectively targeting the RAF-MEK1/2-ERK1/2 pathway and another agent (excluding chemotherapy and radiotherapy) were reviewed and retrieved from the [ClinicalTrials.gov](https://clinicaltrials.gov) registry⁹. Combination therapies using different agents or aimed at different drug targets are considered as different combinations and counted under corresponding categories. Clinical trials testing the same combination using the same drugs were not counted. Triple combinations with RAF-MEK-ERK inhibitors and another two targeted therapies or immunotherapies are also highlighted. Nonspecific RAF inhibitor, sorafenib tosylate, was not counted towards RAF-based combination therapies. In general, 131 distinct combinations have been or are currently evaluated in clinical trials, amongst which 58% involve combinations with MEK inhibitors. Combinations involving both MEK inhibitors and RAF inhibitors constitute 21% as compared to 17% with only RAF inhibitors. Besides the RAF-MEK-ERK pathway, a great majority of these combinations target the EGFR/ERBB signaling axis, the PI3K/AKT/mTOR, and the immune checkpoint molecules, representing the trending strategies of drug development. See also [Table S6](#) in the supplemental information online.

RAF/MEK/ERK inhibitors, as reviewed before [39,40]. Therefore, it would be interesting to closely examine how ERK1/2 substrates may be targeted to harness the regulatory roles of feedback loops. One emerging example is the protein tyrosine phosphatase, PTPRR. Negative feedback mediated by PTPRR results in deactivation of the ERK1/2 pathway, a mechanism that clearly influences androgen receptor-activated ERK1/2 pathway and progression of prostate cancer [65]. Besides the inhibitory roles in RAF activation, RKIP has been shown to modulate multiple intracellular signaling, such as the TRAIL-induced apoptosis [66] and E-cadherin-based epithelial-mesenchymal transition [67]. Remarkably, these mechanisms are complementary to ERK1/2-mediated transcriptional regulation of genes like DUSPs that exhibit inhibitory functions in various tumorigenesis [68].

Amongst the 49 ERK1/2 substrates that are phosphorylated by ERK1/2-mediated feedback signaling, the most well-characterized drug targets are EGFR [69], RAF1 [70], and MEK1 [71], of which their phosphorylation by ERK1/2 contravenes their physiological functions by constraining further activation of the ERK1/2 pathway. One most recently characterized drug target is the protein tyrosine phosphatase SHP2, which is inhibited by ERK1/2-mediated phosphorylation (Table S3 in the supplemental information online). The combination of SHP2 inhibition and MEK1/2 inhibitor, trametinib, is highly efficacious in multiple cancer models, regardless of *KRAS* mutations [72]. This is of substantial therapeutic significance as *KRAS*-mutated cancers are generally insensitive to RAF or MEK1/2 inhibitions [2]. Since SHP2 controls multiple signaling axes, including the PI3K-AKT pathway, it is unclear whether SHP2 inhibitors could be combined with PI3K-AKT inhibitors. Another example of negative feedback-mediated drug sensitivity is that of ERK2-FRS2, as its loss confers drug resistance to MEK1/2 inhibition. Since FRS2 phosphorylation is essential for FGFR1 activation, the combination of FGFR1/FRS2 inhibitor and trametinib abrogates the adaptive resistance to trametinib in *KRAS*-mutated lung cancer [73]. A recent integrative study shows that mutations of two ERK1/2 substrates, capicua and dystrophin, are associated with activation of the RAS-RAF-MEK1/2 pathway in neuroblastoma [74], suggesting that RAF/MEK/ERK substrate-mediated negative feedback loops are more prevalent than currently recognized [40].

RAF/MEK/ERK and the Immunomodulatory Agents

Apart from the essential roles of the TPL2-MEK1/2-ERK1/2 pathway in immune response, there is increasing evidence showing how RAS oncogenic signaling [51] and RAF-MEK1/2-ERK1/2 inhibitors [53] might modulate immune cells in the tumor microenvironment. The immunomodulatory effects of the RAF-MEK1/2-ERK1/2 pathway can be explained, at least in part, by its roles in B and T cell activation, interleukin signaling pathway, and inflammation mediated by chemokine and cytokine signaling pathways (Table S7 in the supplemental information online). However, RAF-MEK1/2-ERK1/2 inhibitors do not favorably modulate the activation and polarization of all the tumor-associated immune cells. It is therefore essential to closely examine how the immunomodulatory roles of RAF-MEK1/2-ERK1/2 inhibitors may synergize with various strategies of immunotherapies. The ongoing clinical trials registered in the [ClinicalTrials.gov](https://clinicaltrials.gov) registry^v will soon provide clearer evidence to instruct future designs of **combination therapy** (Figure 4).

Consistent with the essential roles of ERK1/2 in immune cell development [75], prolonged MEK1/2 inhibition greatly impairs the development of embryonic stem cells [76] while prolonged ERK1/2 inhibition leads to the degradation of MYC and senescence-like growth suppression [77]. Notably, tolerable doses of RAF-MEK1/2-ERK1/2 inhibitors have distinct roles on anticancer effector cells and immunosuppressive cells. Of significance are the RAF-MEK1/2-ERK1/2 inhibitors, which exert mild or even stimulatory effects on dendritic cells, CD4⁺ T cells, and tumor antigen-specific CD8⁺ T cells [78], but inhibitory effects on infiltrations of regulatory T cells and monocyte/macrophages [79,80]. The immunomodulatory roles of targeted therapies like RAF-MEK1/2-ERK1/2 inhibitors greatly complement the currently trending immunotherapies that focus primarily on how the: (i) activities of tumor-killing effector cells, such as the tumor antigen-specific CD8⁺ T cells and natural killer cells, can be enhanced; and (2) immunosuppressive cell types in the tumor microenvironment can be eliminated or subverted [81].

It is now well-recognized that oncogenic RAS signaling promotes immune tolerance to tumors by stabilizing gene expression of immune checkpoint, PD-L1, in a MEK1/2-ERK1/2-dependent manner [82]. As recently proposed, the combination of RAF-MEK1/2-ERK1/2 inhibitors and immune checkpoint blockades represents one of the most promising combination strategies to treat advanced cancers [78]. To exploit the potential synergistic effects between cancer cells and tumor-associated immune cells, immune adjuvants/agonists (e.g., TLR and STING agonists) may be used to engage with pathogen-recognition receptors to activate the alternative TPL2-MEK1/2-ERK1/2 pathway. This strategy may be incorporated into the above combination to unlock TPL2-MEK1/2-ERK1/2-mediated inhibition of the pleiotropic interferon responses [20]. It would also be interesting to examine the synergistic anticancer efficacies of a triple combination of immune adjuvants/agonists-based stimulants with RAF-MEK1/2-ERK1/2 inhibitors and immune checkpoint blockades.

Concluding Remarks

The vast numbers of druggable kinases and kinase–substrate relationships represent one of the most exciting areas of drug development, especially for combination therapy. Although a majority of approved targeted therapy constitutes small inhibitors aiming at membrane-bound receptors or kinases [83], whether and how they may be combined with other therapies remains challenging (see Outstanding Questions). The unexplored opportunities lying in the human genome remind us that combination therapy is still in its infancy. Despite the therapeutic success and full potentials of RAF-MEK1/2-ERK1/2 inhibitors, the hyperactivation of ERK1/2 is, otherwise, deleterious to RAS/RAF-mutated melanoma [84] and lung adenocarcinoma cells [68]. The kinase activity-independent mechanism of RAF1 in DNA repair [59] and RAS-driven oncogenesis [85], the kinase activity-independent role of BRAF in the activation of MEK1/2 pathway [86], and the genome-binding activities of ERK2 [87] have also been revealed, calling for a full appreciation of the intricacy of RAF-MEK1/2-ERK1/2 pathway. High-throughput analysis of tumor heterogeneity [81] and preclinical screening using patient-derived xenograft models [88] are enabling more predictive powers. These efforts are increasingly pointing to underexplored pathways and targets for potential drug combination designs. Remarkably, recent efforts have combined RAF-MEK1/2-ERK1/2 inhibitors with highly promising drugs like the autophagy inhibitor chloroquine or hydroxychloroquine and identified synergistic anticancer effects in RAS-driven cancers [89,90] and metastatic melanoma [91]. With the fast-growing understanding of the intricate signaling of the RAS-RAF-MEK1/2-ERK1/2 pathway, even limited kinase–substrate relationships have been explored in the settings of combination therapy, as discussed above. Future studies may benefit directly from our compiled kinase–substrate relationships and the proposed bypass-signaling pathways, which will contribute to more actionable opportunities for novel drug combinations targeting the RAF-MEK1/2-ERK1/2 pathway.

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Supplemental Information

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Resources

ⁱ<https://cancer.sanger.ac.uk/cosmic>

ⁱⁱwww.uniprot.org/

ⁱⁱⁱ<http://geneontology.org/>

^{iv}www.genome.jp/kegg/pathway.html

^v<https://clinicaltrials.gov>

Outstanding Questions

How would underexplored kinase–substrate relationships, reviewed here, be targeted to enhance anticancer efficacies of RAF-MEK1/2-ERK1/2 inhibitors?

What determines whether and how certain substrates of RAF, MEK1/2, and ERK1/2 are to be phosphorylated?

How would the substrate preferences of aberrantly activated RAF, MEK1/2, and ERK1/2 differ from the normal conditions?

What are the physiological roles of the alternative kinases that phosphorylate RAF and MEK1/2, and the alternative substrates that are phosphorylated by RAF and MEK1/2, especially in human cancers?

What are the ERK substrates that mediate the most essential oncogenic signaling in human diseases that can be targeted as alternatives to RAF-MEK1/2-ERK1/2 pathway inhibitors?

How could the kinase activity-independent roles of RAF, MEK1/2, and ERK1/2 be targeted to improve existing targeted therapies in human cancers, given that agents targeting the synthetic lethal interactions of RAS have emerged as one of the most promising areas of drug development?

What are the upstream cues that determine the activation of certain bypass-signaling and drive drug resistance to the RAF-MEK1/2-ERK1/2 pathway inhibitors?

If more ERK1/2 substrates that are directly involved in the MAPK pathway are to be targeted, how would they influence the anticancer efficacies of and drug resistance to RAF-MEK1/2-ERK1/2 inhibitors?

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