Chapter

Regulation of MAPK ERK1/2 Signaling by Phosphorylation: Implications in Physiological and Pathological Contexts

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Abstract

Protein phosphorylation represents a rapid and reversible post-translational regulation that enables a fast control of protein activation that play key roles in cell signaling. For instance, Mitogen Activated Protein Kinase (MAPK) pathways are activated upon sequential phosphorylations, resulting in phosphorylation of cytosol and nuclear targets. We focus here on MAPK ERK1/2 signaling that accounts for diverse cellular responses such as cell cycle progression, proliferation, differentiation, senescence, migration, formation of GAP junctions, cell adhesion, cell motility, survival and apoptosis. We review the role of protein phosphorylation in MAPK ERK1/2 activation, in its regulation in time and space and how its dysregulation can lead to tumorigenesis.

Keywords: phosphorylation, cell signaling, MAPK, ERK1/2, kinase, phosphatase, cancer, inhibitors

1. Introduction: cell signaling regulation by phosphorylation

Among post-translational modifications, protein phosphorylation is the most common. Vitellin was the first protein which phosphorylation was discovered, by Phoebus Levene in 1906 [1, 2]. In 1954, Burnett and Kennedy reported the process of enzymatic phosphorylation. Then, Edwin Krebs and Edmond Fischer described how phosphorylation and dephosphorylation can take place and they demonstrated how the process is governed by enzymes [3, 4]. In 1992, the Nobel Prize in Physiology or Medicine was awarded jointly to Edmond H. Fischer and Edwin G. Krebs for their discoveries concerning reversible protein phosphorylation as a biological regulatory mechanism.

Phosphorylation is a reversible protein modification and results from the addition of a phosphate group (PO_4) to the polar group of amino acids. The most common amino acids that are phosphorylated are serine (Ser), threonine (Thr) and tyrosine (Tyr). Although phosphorylation of histidine and aspartate residues can also occur, they are less stable than others. Phosphorylation of a protein can change binding to other proteins: because each phosphate group has two negative charges,

phosphorylation can cause a conformational change in the protein by attracting a cluster of positively charged amino acid side chains. This can change the binding of ligands on the protein surface and therefore its activity. On the other hand, the addition of a phosphate group to a protein can be recognized by other proteins having for instance SH2 and PTB domains, that then can attach to phosphorylated proteins such as the cytoplasmatic tail of receptor tyrosine kinases (RTK). Finally, phosphorylation can mask a binding site that otherwise holds two proteins together and then can disrupt this interaction.

Enzymes that catalyze the addition of a phosphate group to a protein are kinases; the reaction is unidirectional because of the large amount of free energy released when the phosphates bonds are broken in ATP to produce ADP. The human genome includes more than 500 protein kinases, and it is estimated that more than one-third of the 10,000 proteins in a typical mammalian cell are phosphorylated at any given time, many with more than one phosphate. Conversely, phosphatases are enzymes that remove a phosphate group from a protein, having the opposite function of kinases. Dephosphorylation has more rapid kinetics than phosphorylation by kinases. The human genome contains more than 200 phosphatases, classified into different families including protein tyrosine phosphatases (PTP), the metal-dependent protein phosphatase PPM, the phosphoprotein phosphatase (PPP) that are pSer/pThr- specific, the dual specificity phosphatase (DUSP) family and the PTEN family of lipid phosphatases [5].

Protein phosphorylation may occur at a single site that primes location for subsequent phosphorylations or directly at multiples sites. Thus, a single protein kinase or multiple kinases may act on the target protein, creating a synchronized cascade of phosphorylations. These events participate in dynamic intracellular signaling that enable cells to respond to extracellular stimuli and to adapt to internal changes. Mitogen-protein activated kinases (MAPK) are conserved kinases in eukaryotes, integrating cell signaling pathways that regulate processes such as cell proliferation, cell differentiation and cell death, from yeast to humans. There are four independent MAPK pathways: MAPK ERK1/2, ERK-5 (also referred to as BMK-1), c-Jun N-terminal kinase (JNK), and p38 signaling families. MAPK modules contain 3-tier kinases that are sequentially activated by phosphorylation. MAPK proteins are designated from upstream to downstream signaling pathway: MAPK kinase kinase (MAPKKK) phosphorylates MAPK kinase (MAPKK); MAPKK phosphorylates and thus activates MAPK. We will focus on MAPK ERK1/2 signaling to illustrate how a particular post-translational modification such as phosphorylation can regulate a signaling pathway and how its dysregulation can be implicated in pathological processes such as tumorigenesis.

2. MAPK ERK1/2 pathway: a cell signaling of sequential phosphorylations

The Extracellular Signal-Regulated Kinases (ERK) have key roles in processes like cell growth, cell proliferation and cell survival. In humans, there are three isoforms of ERK: ERK-1, ERK-2 and ERK-5. Hereon we will concentrate on classical MAPK ERK1/2 to comprehend how this signaling is regulated by phosphorylation.

In the canonical human MAPK ERK1/2 pathway there are three types of MAPKKK (A-Raf, B-Raf and Raf-1 or C-Raf kinases), two MAPKK (MEK1, MEK2) and two MAPK ERK-1, ERK-2. Interestingly, MAPK ERK1/2 signaling is basically regulated by phosphorylations. On the first level, Raf are serine/threonine-protein kinases that phosphorylate human MEK on Ser-218 and Ser-222, producing their activation. The Raf family of kinases includes three isoforms with high homology

and a similar domain organization. On the second level, MEK1/2 are dual specificity protein kinases that phosphorylate a threonine and a tyrosine residue in a Thr-Glu-Tyr sequence located in ERK1/2, rendering them active. While human ERK-1 is phosphorylated on Thr-202 and Tyr-204, ERK-2 is phosphorylated on Thr-185 and Tyr-187 residues for activation. Phosphorylation of ERK1/2 by MEK1/2 leads to the rearrangement of several polar contacts, which results in conformational changes in neighboring structural elements (reviewed on [6]). Finally, ERK1/2 are serine/threonine kinases that phosphorylate a wide variety of substrates in different subcellular compartments including the Golgi apparatus, the mitochondrial membrane, the cytoplasm and the nucleus.

MAPK ERK1/2 phosphorylate substrates in a short Pro-X- Ser/Thr-Pro consensus motif (X representing any residue) and interactions with docking sites are important for specificity. Two motifs have been described, the D- and F-motifs, that can cooperate to enhance the substrate affinity of ERK and to set phosphorylation kinetics [7]. ERK1/2 phosphorylate more than 600 proteins, leading to responses such as cell cycle progression, proliferation, cytokinesis, transcription, differentiation, senescence, cell death, migration, formation of GAP junctions, actin and microtubule networks, neurite extension, cell adhesion and motility, survival and apoptosis [8]. To ensure that these cell responses are adaptive to stimuli in space and time, a fine regulation of MAPK signaling is thus necessary. Remarkably, control of ERK1/2 signaling is in part ensured by phosphorylations and dephosphorylations.

3. Regulation of MAPK ERK1/2 by kinases and phosphatases

The MAPK ERK1/2 has at least 3 tiers of regulation: the control of the phosphorylation and thus of the activity of Raf, MEK1/2 and ERK1/2. Additionally, upstream signals from cell receptors to Raf and downstream targets of ERK1/2 play an active role in regulating the MAPK ERK1/2 pathway. Interestingly, mechanisms of MAPK regulation are based partly on the same mechanisms that activate this pathway: phosphorylation events. In this section we specify how phosphorylation can regulate MAPK ERK1/2 signaling from Raf to ERK1/2 by the activity of kinases in feedback signals, and through dephosphorylation by phosphatases.

When RTK are activated by growth factors, their phosphorylated tyrosines enable the coupling of adaptor proteins such as GRB2. This latter binds with SOS, a guanine exchange factor that promotes the activation of Ras. From this level, MAPK ERK1/2 signaling axis exerts feedback regulations through phosphorylations. Growth factor stimulation (like epidermal growth factor EGF) of the cell induces the phosphorylation of four serine residues in a region encompassing three proline-rich SH3-binding sites in the C-terminal domain of SOS1 [9]. These phosphorylation events are realized by ERK1/2 and constitute a negative feedback regulation that leads to a reduction in Ras activation. Kinetic simulation model using parameters collected in living cells found that possibly more than four phosphorylation sites decisively suppress SOS activity [10]. Indeed, SOS1 is also phosphorylated by the ERK1/2 effector ribosomal S6 kinase 2 (RSK-2) on Ser1134 and Ser1161, leading to the recruitment of 14-3-3 and is thus a negative regulation of ERK1/2 activity [11] (**Figure 1** and **Table 1**).

In platelets and nexus ERK1/2 is also activated downstream of the small GTPase Rap1. RasGRP2 is the predominant guanine exchange factor that specifically activates Rap1. RasGRP2, playing a similar role to SOS for Ras, is phosphorylated by ERK1/2 on Ser394 located in the linker region implicated in its autoinhibition. In this case, RasGRP2 phosphorylation results also in a negative feedback loop that determines the amplitude and duration of active ERK1/2 [12]. Moreover, Rap1 is

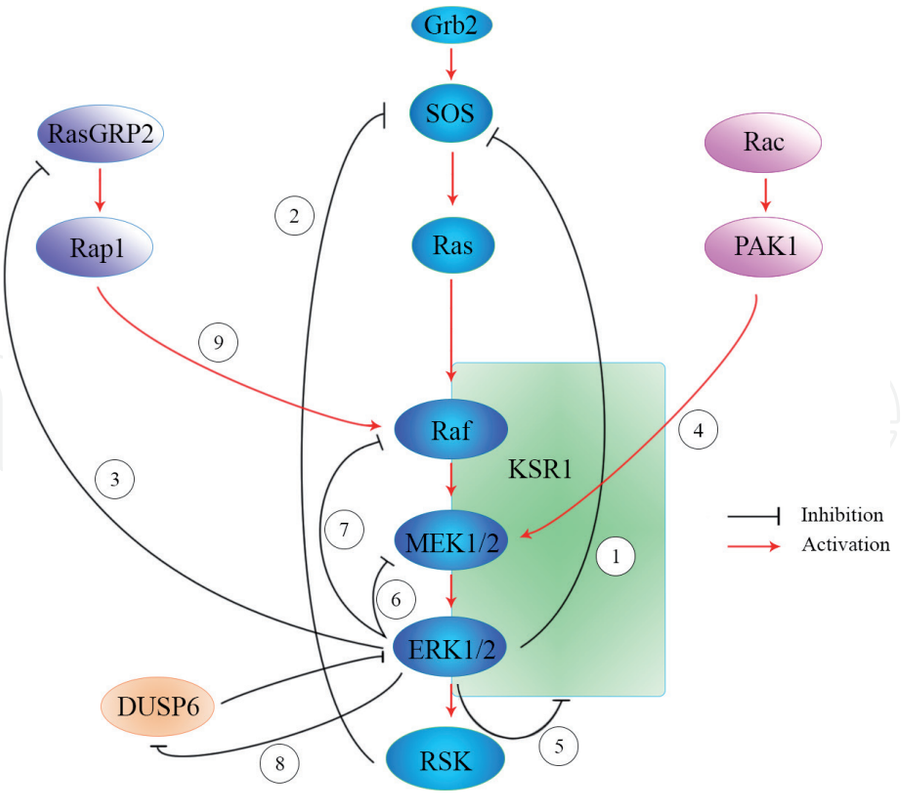

Figure 1.
Representative phosphorylation events leading to activation and feedback signaling in the MAPK ERK1/2 pathway. Phosphorylation constitutes activation (red arrows) or inhibition (black arrows) of proteins of MAPK ERK1/2 signaling. Specific details are provided in ***Table 1****.*

able to phosphorylate and activate B-Raf (but not C-Raf) [23]. Upon cell adhesion and downstream of the small GTPase Rac, the serine/threonine-protein kinase PAK1 phosphorylates the MEK proline-rich sequence (PRS), enhancing its interaction with C-Raf [13] (**Figure 1** and **Table 1**).

Regarding Raf, it has been shown that mitogenic stimuli induce the phosphorylation of C-Raf by ERK1/2 on six residues, needing MEK signaling. Hyperphosphorylation of these sites promotes the subsequent dephosphorylation of C-Raf by PP2A and the return to the inactive state [20]. On the other side, Raf interaction with MEK is also regulated by the inhibitor protein RKIP, which binds to both proteins preventing their physical association. RKIP interferes with the phosphorylation of MEK when bound to C-Raf. Association of RKIP with C-Raf is regulated partly by phosphorylation: phosphorylation of RKIP on serine 153 by PKC or putatively by ERK induces its dissociation from C-Raf [24, 25]. RKIP has then an important role in generating a switch-like behavior of MEK1/2 activity [26].

MEK1/2 is also the target of feedback regulation in the ERK1/2 pathway. Indeed, ERK1/2 phosphorylates MEK1 on Thr292, Thr286 and Thr386, resulting in reduced MEK activity and thus constitutes a negative feedback for MAPK ERK1/2 signaling [18, 19]. Moreover, MEK1 phosphorylation on Thr292 by ERK1/2 interferes with MEK1 binding to ERK2 and reduces MEK1 phosphorylation on S298 by PAK, required for the activation of MEK1 by cell adhesion [13–15] (**Figure 1** and **Table 1**).

Another example of feedback regulation of MAPK ERK1/2 signaling by phosphorylation is the case of the protein scaffold KSR1. In fact, KSR1 can be phosphorylated in Thr256, Thr260, Thr274, Ser320, Ser443, Ser463 by ERK1/2 *in vitro* and depends on MEK1/2 activity. These KSR1 phosphorylations interrupt its association with B-Raf and MEK1/2, drive the release of KSR1 from the plasma membrane, representing then a negative feedback of MAPK ERK1/2 activation [16, 17] (**Figure 1** and **Table 1**).

Number in figure	Protein phosphorylated	Phosphorylation site	Kinase	Type of feedback	Consequence	References
1	SOS1	Ser1132, Ser1167, Ser 1178, Ser 1193	ERK1/2	Negative	Decreased binding affinity of Grb2 to human Sos1	[9]
2	SOS1	Ser1134, Ser1161	RSK	Negative	Facilitates 14-3-3 binding, decreasing MAPK activation	[10]
3	RasGRP2	Ser394	ERK1/2	Negative	Inhibits RasGRP2 ability to activate Rap1, leading to decreased activation of ERK1/2	[12]
4	MEK1/2	Proline-rich sequence (PRS)	PAK1	Positive	Enhances MEK1/2 interaction with C-Raf	[13–15]
5	KSR1	Thr260, Thr274, Ser443	ERK1/2	Negative	Interrupts association of KSR1 with B-Raf and MEK1/2, driving the release of KSR1 from the plasma membrane	[16, 17]
6	MEK1	Thr292	ERK1/2	Negative	Inhibits MEK1 kinase activity towards ERK1/2, interferes with the binding of MEK1 to ERK2 and reduces the ability of PAK to phosphorylate MEK1 on S298 (required for the activation of MEK1 by cell adhesion)	[18, 19]
7	C-Raf	Ser29, Ser43, Ser642, Ser289, Ser296, Ser301	ERK1/2	Negative	Desensitized C-Raf, do not localize to the plasma membrane and do not engage with activated Ras	[20]
8	DUSP6	Ser159, Ser174, Ser197	ERK1/2	Negative	Induces degradation of DUSP6	[21, 22]
9	B-Raf	Ser445	Rap1	Positive	Activation of B-Raf	[23]

Table 1.
Feedback phosphorylation events in MAPK ERK1/2 pathway.

Another regulation of MAPK activity is accomplished by phosphatases that modulate later phases of ERK1/2 signaling. Ser/Thr phosphatases, protein tyrosine phosphatase and dual-specificity Thr/Tyr phosphatases (DUSP) dephosphorylate and thus inactivate ERK1/2. MAP Kinase Phosphatases (MKP) belong to DUSP and represent specific phosphatases that principally regulate MAPK activity in mammalian cells and tissues. While some DUSP dephosphorylate p38, JNK and ERK1/2, others are specific for p38/JNK or for ERK1/2. In this latter case are found cytoplasmic DUSP that inactivate ERK1/2 in the cytoplasm and include DUSP6/MKP-3, a specific phosphatase that binds to ERK1 and ERK2, inactivating them. This specificity is ensured by the fact that the interaction of DUSP6 with ERK1/2 is a requirement for the catalytic activation of the phosphatase through conformational changes [27, 28]. Interestingly, whilst inactivating ERK1/2, DUSP6 is in turn regulated by ERK1/2. Indeed, stimulation with serum or PDGF-B alone can induce a MEK-dependent phosphorylation of DUSP6 on Ser159, Ser174, and Ser197, which is followed by the degradation of the phosphatase by the proteasome [21, 22]. We have shown that another pathway involved in growth factor signaling, the PI3K/mTOR signaling pathway, accounts for a part of the phosphorylation and degradation of DUSP6 induced by serum growth factors. Furthermore, specific agonists of the mTOR pathway, such as amino acids or insulin/IGF-1 are also able to induce the phosphorylation and degradation of DUSP6. Mutagenesis studies identified Ser159 within DUSP6 as the target of the mTOR pathway [29]. Thus, DUSP6 is a point for double MAPK control: the phosphatase exerts a negative regulation for ERK1/2 activity but at the same time, ERK1/2 is able to phosphorylate DUSP6 and then induces its degradation. DUSP6 appears therefore as a spot for fine ERK1/2 signaling regulation in time. Moreover, DUSP6 is a branch-point for the crosstalk between two major signaling pathways induced by growth factors, the MEK/ERK1/2 pathway and the PI3K/mTOR pathway. Notably, both pathways are frequently overactivated in cancer cells. Thus, a regulation of MAPK ERK1/2 signaling in time and space is necessary to warrant cell physiological responses and to avoid aberrant signaling activation that facilitates pathological conditions.

4. Implications of phosphorylation in MAPK ERK1/2 regulation in time and space

MAPK ERK1/2 signaling can determine excluding cell responses such as proliferation and differentiation. Differences in cell responses upon MAPK ERK signaling depend on the regulation of the pathway through protein interactions by scaffolds and through inhibitory and adaptor proteins that enhance, decrease, or redirect the flow of phosphorylation cascades. In this section, we will describe how phosphorylation can be implicated in this type of MAPK ERK1/2 signaling regulation. Scaffold proteins bind to multiple interacting proteins by interconnecting them into a stable complex. This allows the rapid transmission of the signal. Another role of scaffolds is to sequester sets of interacting proteins to limit interactions with other proteins and minimize crosstalk between pathways that some components may share. Scaffold proteins such as KSR1, β-Arrestin, paxillin and IQGAP1 regulate the kinetics, amplitude, and localization of ERK1/2 signaling [30]. Ras-1 suppressor kinase (KSR1) is one of the best characterized scaffold proteins in the ERK1/2 cascade. It has several different domains through which it can interact with C-Raf, MEK1/2, and ERK1/2. In response to growth factors, KSR1 translocates to the plasma membrane where it promotes the activation of MEK1/2 by presenting it to activated Raf. In the absence of stimulus, the ubiquitin-protein isopeptide ligase family member IMP and the 14.3.3 protein prevent the function of KSR1.

Mitogens induce the dephosphorylation of IMP at S392 by protein phosphatase-2A (PP2A) and the degradation of the protein, which is sufficient to allow KSR1 to translocate to the cell membrane [31]. Activated Ras also induces phosphorylation of KSR1 at residues Thr260, Thr274, and Ser443 [16]. Then, while activated Ras prevents the effects of 14.3.3 and IMP that inhibit KSR1 function, it also induces its phosphorylation at Thr274, preparing KSR1 for degradation. KSR1 can then regulate ERK1/2 activation kinetics and influence the biological fate of the cell. The interaction and in particular the synchronization between these processes generates a combinatorial control to modulate both the amplitude and the duration of ERK1/2 activity.

If scaffold proteins play a key role in regulating ERK1/2 signaling in subcellular locations, different factors modulate the strength and the duration of ERK signaling in time: the density of cell surface receptor and its different internalization patterns, the surrounding extracellular matrix and the interaction between kinases and phosphatases. The duration of the signal is critical in determining cell response to ERK1/2 signaling. For instance, long-term ERK1/2 activation can cause differentiation while short-term ERK1/2 activation can lead to cell division. This was initially demonstrated in rat pheochromocytoma PC-12 cells, in which transient activation of ERK1/2 by epidermal growth factor (EGF) or insulin peaks at 5 min and fells back to near-background levels within 15 minutes, and results in cell proliferation. On the other hand, sustained activation of ERK1/2 by nerve growth factor (NGF) persists for more than 60 minutes and induces cell differentiation [32]. This type of cell response according to duration of ERK1/2 signaling has been also reported in fibroblasts, macrophages and T lymphocytes [33–35]. As this type of studies has been made using mainly immunoblotting techniques to monitor ERK1/2 activation dynamics, the use of new approaches gaining spatio-temporal resolution will be of great interest to advance in the understanding of ERK1/2 signaling in time and in subcellular localizations. For example, using Förster Resonance Energy Transfer (FRET)-based ERK biosensors, Keyes et al. showed that EGF induces sustained ERK1/2 activity near the plasma membrane in contrast to the transient activity observed in the cytoplasm and in the nucleus. This supports the concept that the spatial and temporal regulation of ERK1/2 activity is integrated by the cell to control the specificity of signaling [36].

Studies on RTK receptors have shown that their activation kinetics and regulatory mechanisms also play a key role in the activation of the MAPK ERK1/2 pathway. For example, PC-12 cells that express few NGF receptors do not undergo differentiation in response to NGF [37]. Moreover, changing the amount of receptor occupation by decreasing the concentration of agonists alters the duration of ERK1/2 signaling. The rate and degree of receptor internalization also contribute to ERK signaling, not only as a checkpoint for signal termination, but may exhibit additional signaling by the receptor-ligand complex from an internalized cellular location [38].

5. Dysregulation of MAPK ERK1/2 signaling in human cancer

The MAPK/ERK signaling module is considered the most important oncogenic driver of human malignancies [39]. Mutational oncogenic activation of the Ras/Raf/MEK/ERK pathway occurs in a wide variety of cancers concerning approximately 34% of all human cancers. Activation of the ERK1/2 signaling pathway promotes proliferation and has anti-apoptotic effects, increasing tumor invasion and metastasis. The overexpression of the pathway can lead to cell transformation, tumor proliferation, invasion, metastasis, extracellular matrix degradation and

tumor angiogenesis. VEGF is an important pro-angiogenic factor and the most powerful pro-vascular endothelial growth cytokine that promotes cell division and vascular construction. The MAPK ERK1/2 signaling pathway can activate transcription factors to enhance the transcription of VEGF, promoting the formation of blood vessels and tumor angiogenesis [40, 41].

Aberrant activation of the Ras/Raf/MEK/ERK pathway may be driven by abnormal receptor kinase activation or by oncogenic mutations of pathway components, leading to tumorigenesis. Overactivation of Ras is observed in approximately 30% of all human cancers but can be higher in some cancers like pancreas cancer (90%), colon cancer (50%) and thyroid cancer (50%) [42, 43]. Mutations in Ras occurs in codons 12, 13, 59 and 61, leading to its constitutive activation. Indeed, mutant oncogenic Ras proteins are insensitive to GTP-catalyzed GTPase hydrolysis activator protein, resulting in a constitutively active GTP-bound Ras. K-Ras and N-Ras are the most common mutated isoforms in human cancer, although H-Ras can also be involved. K-Ras is involved in up to 96% of pancreatic ductal adenocarcinomas, 52% of colorectal carcinomas and 32% of lung adenocarcinomas [44].

Downstream of Ras, Raf can be activated by mutations that mainly affect B-Raf isoform, the most potent activator of MEK1/2 compared with the other Raf isoforms (A-Raf and C-Raf). B-Raf can be mutated in 70% of melanomas, in 36-53% of papillary thyroid cancer, in 30% of ovarian cancer and in 22% of colorectal cancer [45]. The most common mutation of B-Raf is the change of a valine to a glutamic acid in position 600 (V600E). Other B-Raf mutations in cancer are mainly clustered in the activation segment or the so-called glycine-rich loop in B-Raf [46]. Oncogenic mutations of B-Raf lead to hyperactivity of its downstream effectors MEK1/2 and ERK1/2. For cellular transformation to occur, two mutations in Ras/Raf/MEK/ERK1/2 pathway can be needed: for instance, B-Raf and Ras mutations can drive tumorigenesis for colorectal cancer (K-ras G13D; B-Raf G463V), for ovarian cancer (K-ras G13D; B-Raf G463E), and for non-small cell lung cancer (N-Ras Q61K; B-Raf L596V) [45].

Downstream of Raf, MEK1/2 can be highly phosphorylated in colorectal cancer, gliomas, prostate cancer, breast cancer and head and neck cancer [47–51]. Constitutively active mutants of MEK-1 have higher basal activity than the wild-type unphosphorylated MEK. Expression of these mutants in mammalian cells lead to ERK1/2 activation in growth factor-deprived cells, cellular transformation and solid tumor growth in nude mice [33, 52, 53]. If mutant MEK can act as oncogene, its frequency in human cancers appears to be rare [54]. Finally, MAPK ERK1/2 are not frequently mutated. However, some mutations in ERK have been described: ERK2 mutants were identified as rare cancer-associated gain- and loss-of-function gene products: ERK2 D321N, ERK2 E322K, ERK2 L73P, ERK2 S151D and ERK2 D319N [55–60]. While ERK2 D319N has not an increased basal kinase activity, it shows an elevated sensitivity to low levels of signaling *in vivo* [55]. In human cancer cell lines, ERK2 E322K has constitutive phosphorylation [61]. Finally, ERK2 L73P and S151D mutations increase by 8-to-12-fold ERK2 activity alone, and both mutations have a synergetic action that increases by 50-fold ERK2 activity [57]. Moreover, overexpression of ERK2-L73P/S151D can induce growth arrest in prostate cancer cell lines [62]. Although ERK1/2 mutations are rare, mutations that lead to overactivation of RTK, Ras, Raf and MEK can lead to increased ERK1/2 signaling in cancer cells. Downstream of ERK1/2, both cytoplasmic and nuclear targets can be upregulated in tumoral contexts. One of the main cancer-associated ERK substrates is c-Myc, a transcriptional factor that participates in cell cycle progression, becoming an oncogene. Phosphorylation of c-Myc by ERK1/2 due to Ras activation keeps overexpressed this transcriptional factor in various cancers [63]. Other important targets of ERK are the

transcriptional factor Elk1, c-Fos and Jun. These two latter were identified as viral oncoproteins and can play a role in tumorigenesis. Mutations that affect MAPK ERK1/2 proteins can then promote protein hyperactivation that induces the cascade of phosphorylation downstream events, favoring cell proliferation, cell transformation and the emergence and progression of tumors. Currently, MAPK inhibitors represent specific target treatments for cancers with overactivation of this cell signaling pathway.

6. MAPK ERK1/2 inhibitors: possibility to regulate cell signaling overactivation

Hyperactivation of Ras/Raf/MEK/ERK signaling pathway in human cancers prompted the development of small molecule inhibitors that target its components for use in cancer therapeutics (**Table 2**). Pharmacological inhibition of Ras has been a major challenge. For instance, the affinity of Ras protein for GTP is extraordinarily high and it is then very difficult to develop a competitive binding strategy. Over the past few years, several groups discovered and developed small molecule Ras modulators using protein structure-guided design approaches [80–82] and exploring SOS as a target for Ras activation [83]. Cysteine-reactive inhibitors that bind to the mutant K-Ras G12C, which is commonly found in cancer, have been developed: SML-8-73-1 and SML-10-70-1 can selectively inhibit K-Ras G12C, changing the nucleotide preference to favor GDP over GTP and thus blocking Ras signaling [69, 84]. These compounds may be used in the future for additional K-Ras mutations.

Sorafenib is an orally available compound that was initially developed as a C-Raf inhibitor and was then identified as a multikinase inhibitor for B-Raf, VEGFR1/2/3, Kit, PDGFR, RET, and Flt3. Sorafenib is currently approved by the FDA for renal and hepatocellular carcinoma for its anti-angiogenic effects [84, 85]. For other cancers like melanoma, sorafenib produced favorable responses in less than 5% of patients in clinical trials [85, 86]. This low response rate can be due to the fact that its activity against B-Raf V600E mutants and wild-type enzymes is low. Subsequent efforts have focused on targeting B-Raf for the treatment of B-Raf mutant melanoma. Vemurafenib and dabrafenib, two B-Raf V600E inhibitors, have achieved benefits in clinical trials [87, 88]. Currently, vemurafenib is approved by the FDA for metastatic and unresectable melanoma with B-Raf V600K mutation [89] and dabrafenib for metastatic melanoma with B-Raf V600K-mutated [84, 88]. Although B-Raf inhibitors have achieved clinical benefit in the treatment of cancer, all ATP-competitive Raf inhibitors including vemurafenib, dabrabenib, and sorafenib can lead to paradoxical activation of the MAPK pathway in wild-type B-Raf cells [90, 91]. Some reports suggest that insensitivity to Raf inhibitors might be due to EGFR-mediated reactivation of MAPK signaling in B-Raf mutant colorectal cancer [92]. Indeed, the combination of EGFR and B-Raf inhibitors block the reactivation of MAPK signaling of B-Raf mutant in colorectal cancer cells and *in vivo* [93]. LGX818, TAK-632 and MLN2480 are other selective B-Raf V600E inhibitors with a very slow inactivation rate, and thus may be beneficial for the treatment of tumors that are resistant to other Raf inhibitors or for the treatment of tumors with Ras mutations [66, 94].

Even though MEK1/2 mutations are rare in human cancers, MEK1/2 have become an attractive drug target because these proteins are downstream of Ras and Raf in the signaling pathway [95]. The first MEK1/2 inhibitor, PD098059, is an allosteric inhibitor that acts on the not-phosphorylated form of MEK1 and mutant MEK1 S217 and S221E [96]. The allosteric MEK inhibitor CI-1040 was

Protein	Mutation	Cancer	Inhibitor	Test/effect/approval	Reference
B-Raf	V600E V600K V226M	Melanoma (66%) Ovarian cancer (35-70%) Thyroid cancer (70%)	Vemurafenib	Approved by the FDA for metastatic and unresectable melanoma with B-Raf V600K mutation	FDA
			Dabrafenib and Trametinib	Approved by the FDA and EMA for melanoma cancer, anaplastic thyroid cancer, NSCLC	FDA, EMA [64]
			LGX818	Approved by the FDA for the treatment of patients with unresectable or metastatic melanoma with B-Raf mutations	FDA [65]
			TAK-632	TAK-632 demonstrates potent antiproliferative effects both on NRAS-mutated melanoma cells and B-Raf-mutated melanoma cells; the combination of TAK-632 and the MAPK kinase (MEK) inhibitor TAK-733 exhibits synergistic antiproliferative effects on these cells	[66]
			MLN2480	In vitro analysis of MLN2480 and TAK-733 (allosteric MEK kinase inhibitor) demonstrates synergistic activity in cell proliferation. In vivo, MLN2480 shows antitumor activity in melanoma, colon, lung, and pancreatic cancer xenograft models	[67, 68]
			Sorafenib	Approved by the FDA for renal and hepatocellular carcinoma	FDA
N-Ras	Q61R Q61L G12D	Melanoma (15- 20%) Myeloid leukemia (30%) Lung cancer (35%)	Ribociclib and Binimetinib	Phase Ib/II trials in patients with locally advanced or metastatic N-Ras mutant melanoma	Clinical trial NCT01781572
	G12V	Thyroid carcinoma (27%)	Trametinib and Palbociclib	Phase I/II trial in patients with solid tumors and with a specific cohort for N-Ras-mutant melanoma	Clinical trial NCT02065063

Protein	Mutation	Cancer	Inhibitor	Test/effect/approval	Reference
K-Ras	G12D G12C G13D	Lung cancer (30%) Colorectal cancer (45%) Pancreatic cancer (90%) Blandder cancer (50%)	SML-8-73-1, SML-10-70-1	SML-10-70-1, a prodrug of SML-8-73-1, inhibits lung cancer A549, H23, and H358 cells	[69]
MEK	S217E S221E	Melanoma (3-8%) Breast cancer (7-9%)	Selumetinib	Approved by the FDA for treatment of pediatric patients aged 2 years and older with neurofibromatosis type 1 (NF1) who have symptomatic, inoperable plexiform neurofibromas; approved by the EMA for the treatment of neurofibromatosis	FDA, EMA
			Trametinib	Approved by the FDA and EMA for treatment of patients with unresectable or metastatic melanoma with B-Raf V600E or V600K mutations	FDA, EMA
			Pimasertib	Has demonstrated potent antitumour activity in human lung, colorectal, melanoma cancer cells and xenograft models; phase I/II clinical trial in patients with locally advanced or metastatic solid tumors	[70–72]
			Cobimetinib	Approved by the FDA and EMA for use in combination with vemurafenib for the treatment of metastatic melanoma	FDA, EMA
			G-573, GDC-0623	In vitro GDC-0623 inhibits cellular proliferation of mutant cancer cells A375 (B-Raf V600E), HCT116 (KRAS G13D), COLO 205 (BRAF V600E), HT- 29 (BRAF V600E), and HCT116 (KRAS G13D). In vivo GDC-0623 causes potent tumor growth inhibition in mouse MiaPaCa-2, A375 and HCT116 xenografts	[73]
			TAK-733	In vitro TAK-733 demonstrates broad activity in most melanoma cell lines; in vivo TAK-733 demonstrates broad antitumor activity in mouse xenograft models of human cancer including melanoma, colorectal, NSCLC, pancreatic and breast cancer	[74]
			Binimetinib(Mek 162)	Approved by the FDA for treatment in combination with LGX818 for patients with unresectable or metastatic melanoma with a B-Raf V600E or V600K mutation; approved by the EMA for the treatment of colorectal carcinoma	FDA, EMA

Protein	Mutation	Cancer	Inhibitor	Test/effect/approval	Reference
ERK 1/2	NA	Melanoma (67%)	Ulixertinib (BVD-523)	In vitro combined Ulixertinib (BVD-523) and VS-5584 treatment causes significant induction of cell death in human pancreatic cancer (HPAC) cells, in pancreatic ductal adenocarcinoma cell lines BxPC-3, MIAPaCa-2, and CFPAC-1. Clinical trials in phase I for tumor advanced pancreatic and other solid tumors cancer and phase II for advanced malignancies harboring MEK or atypical B-Raf alterations.	[75] Clinical trial NCT03454035 and NCT04488003
			GDC-0994	In vitro Ravoxertinib (GDC0994) decreases the viability of lung adenocarcinoma cell lines (A549, HCC827, HCC4006). In vivo GDC0994 results in significant single-agent activity in multiple cancer models, including K-Ras-mutant and B-Raf-mutant human xenograft tumors in mice. Clinical trials phase I for locally advanced or metastatic solid tumors, NSCLC, metastatic colorectal cancer, metastatic NSCLC, metastatic cancers and melanoma	[76] Clinical trials NCT01875705 and NCT02457793
			SCH772984	In vitro SCH772984 results in a G1 arrest in SCH772984-sensitive melanoma cells. In vivo antitumor activity is observed in the K-Ras-mutant pancreatic MiaPaCa model	[77]
			AEZS-134	Synergistic effect of triptorelin, ERK inhibitor AEZS-134 and dual PI3K/ERK inhibitor AEZS-136 in MDA-MB-231 triple-negative breast cancer cells	[78]
			(S)-14 K	In vivo (S)-14 k inhibited tumor growth in mouse xenograft models	[79]

NA: not available; FDA: food and drug administration of USA; EMA: European medicines agency; NSCLC: non-small cell lung cancer.

Table 2.
Available inhibitors for MAPK ERK1/2 proteins.

the first small molecule to enter clinical trials. Although it had antitumor effects, the development of this compound was stopped due to poor bioavailability and lack of efficacy in phase II clinical trials [97]. Other highly selective inhibitors of MEK1 and MEK2 include selumetinib and trametinib [98–100]. This latter prevents Raf-dependent MEK phosphorylation and activation. Other MEK inhibitors in development include pimasertib [101], cobimetinib [102], rafametinib [103], G-573, GDC-0623 [73], TAK-733 [104], RO5126766, RO4987655 [105, 106] and MEK162 [107].

Because there are few ERK1/2 mutations in human cancers, this MAPK has been only considered as a target in 35 clinical trials, compared with more than 300 clinical trials for the inhibition of Raf and MEK. Nonetheless, due to drug resistance resulting from Raf and MEK1/2 inhibitors, ERK1/2 have become an interesting target for inhibiting MAPK ERK1/2 signaling in cancer [46]. ERK1/2 inhibitors can reverse overactivation of the MAPK pathway induced by upstream mutations, including Ras mutations [84, 92, 108]. For instance, MAPK inhibition in B-Raf V600E mutant metastatic melanoma provokes drug resistance and recovery of ERK activity [109, 110]. Interestingly, selective removal of ERK1 or ERK2 *in vitro* can induce melanoma cell death and enhances the action of B-Raf inhibitor [111].

One of the challenges in cancer treatment is developing drug resistance. The mechanisms involved in resistance are complicated and include genetic mutations that occur in target proteins like in MAPK signaling, loss of functions in the control of MAPK signaling feedback, and abnormal tumor suppressor gene alterations [112]. Yet, MAPK inhibitors represent good options for targeting cancer cells with MAPK overactivation or MAPK ERK1/2 mutations. In the future, cell-specific deliverance of MAPK inhibitors to tumoral cells should enhance their efficiency and decrease side effects in patients.

7. Conclusions

MAPK are conserved kinases in eukaryotes, containing 3-tier kinases that are sequentially activated by phosphorylation. This post-translational modification plays an essential role in MAPK ERK1/2 signaling. Not only the activation but also the regulation of this pathway is achieved through the actions of kinases and phosphatases, establishing positive and negative signaling feedbacks. Control of MAPK ERK1/2 signaling in time and space is ensured by proteins such as scaffolds that are themselves regulated by phosphorylation events. Changes in duration of ERK1/2 phosphorylation and thus activity, can result in different cell responses, can result in different cell responses. Thus, a tight regulation of MAPK ERK1/2 signaling is needed to guarantee adaptive cell responses. Aberrant activation of Ras/Raf/MEK/ERK pathway can lead to tumorigenesis and MAPK inhibitors, already in clinical use, represent good options for targeting cancer cells with MAPK overactivation or MAPK ERK1/2 mutations.

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Conflict of interest

The authors declare no conflict of interest.

Appendices and nomenclature

DUSP	dual specificity phosphatase
EGF	epidermal growth factor
EMA	European medicines agency
ERK	extracellular signal-regulated kinases
FDA	food and drug administration
FRET	Förster resonance energy transfer
GRB2	growth factor receptor-bound protein 2
KSR1	Ras-1 suppressor kinase
MAPK	mitogen-protein activated kinases
MAPKK	MAPK kinase
MAPKKK	MAPK kinase kinase
MKP	map kinase phosphatase
mTOR	mechanistic target of rapamycin
NGF	nerve growth factor
NSCLC	non-small cell lung cancer
PDGF-B	platelet-derived growth factor subunit B
PDGFR	platelet-derived growth factor
PI3K	phosphatidylinositol 4,5-bisphosphate 3-kinase
PPM	metal-dependent protein phosphatase
PPP	phosphoprotein phosphatase
PRS	proline-rich sequence
PTEN	phosphatidylinositol 3,4,5-trisphosphate 3-phosphatase and dual-specificity protein phosphatase
PTP	protein tyrosine phosphatases
RTK	receptor tyrosine kinase
RSK-2	ribosomal S6 kinase 2
SOS1	son of sevenless homolog 1

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