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Review

Atypical mitogen-activated protein kinases: Structure, regulation and functions

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Abstract

Mitogen-activated protein (MAP) kinases are a family of serine/threonine kinases that play a central role in transducing extracellular cues into a variety of intracellular responses ranging from lineage specification to cell division and adaptation. Fourteen MAP kinase genes have been identified in the human genome, which define 7 distinct MAP kinase signaling pathways. MAP kinases can be classified into conventional or atypical enzymes, based on their ability to get phosphorylated and activated by members of the MAP kinase kinase (MAPKK)/MEK family. Conventional MAP kinases comprise ERK1/ERK2, p38s, JNKs, and ERK5, which are all substrates of MAPKKs. Atypical MAP kinases include ERK3/ERK4, NLK and ERK7. Much less is known about the regulation, substrate specificity and physiological functions of atypical MAP kinases.

Keywords: Protein phosphorylation; MAP kinase; Extracellular signal regulated kinase; Nemo-like kinase; Structure; Substrate specificity

1. Introduction

Mitogen-activated protein (MAP) kinase pathways are ubiquitous signaling modules by which cells transduce extracellular chemical and physical signals into adaptive intracellular responses [1–3]. These modules are classically organized into an architecture of three sequentially acting protein kinases comprising a MAP kinase kinase kinase (MAPKKK), a MAP kinase kinase (MAPKK or MEK), and the effector MAP kinase itself. Once activated, MAP kinases phosphorylate a wide range of substrates present in various subcellular compartments. The efficiency and specificity of signal transmission through MAP kinase pathways is ensured by docking interactions between individual components of the pathway and by scaffolding proteins [4,5].

MAP kinase enzymes are conserved in plants, fungi and animals, and all eukaryotic cells use multiple MAP kinase modules for signal transduction. In mammals, 14 MAP kinase genes have been identified that define 7 distinct MAP kinase pathways (Fig. 1). Pharmacological and genetic studies have revealed that these pathways control numerous cellular processes, such as tissue morphogenesis, cell proliferation and differentiation, cell survival, immune responses, and adaptation [3,6–8]. The archetype MAP kinase pathway is the extracellular signal-regulated kinase 1 (ERK1)/ERK2 module, which is preferentially activated in response to mitogenic factors. Typically, stimulation of growth factor receptors leads to the activation of the small GTPase Ras, which recruits the MAPKKK Raf to the membrane for subsequent activation by phosphorylation. Activated Raf isoforms phosphorylate and activate the dual-specificity MAPKKs MEK1/MEK2, which in turn activate the MAP kinases ERK1 and ERK2 by phosphorylation of the threonine and tyrosine residues within the activation loop motif Thr–Glu–Tyr. ERK1/ERK2 are proline-directed kinases that phosphorylate target substrates on serine and threonine residues followed by a proline [9–11]. More than 150 substrates of ERK1/ERK2 have been identified so far [12],

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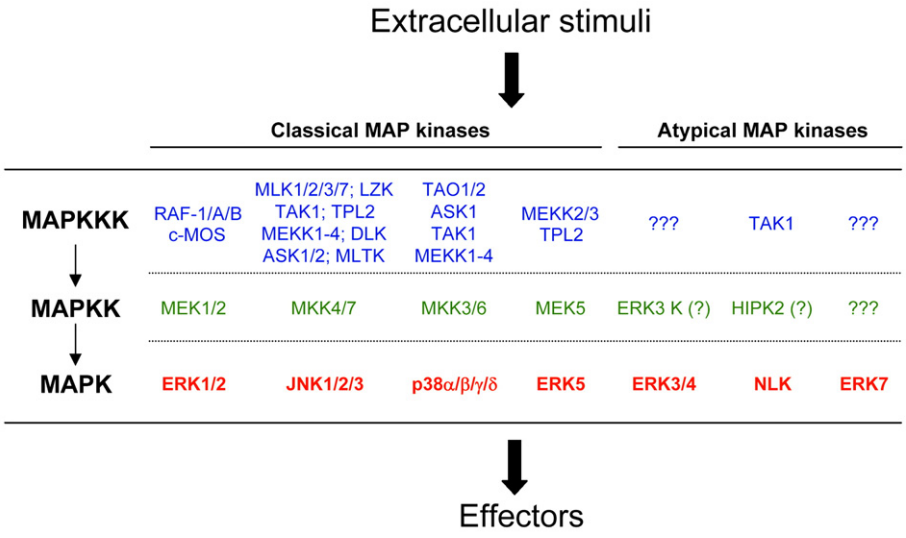

Fig. 1. Human MAP kinase pathways.

although a full validation that these are bona fide targets in vivo has been reported for only a minority of these proteins. The specificity requirements of other MAP kinases are not as well characterized.

2. Conventional and atypical MAP kinase family members

Phylogenetically, MAP kinases belong to the CMGC group of protein kinases, which also includes the cyclin-dependent kinases (CDKs), glycogen synthase kinases, and CDK-like kinases [13]. Members of the MAP kinase branch display more than 40% amino acid identity (60% similarity) to ERK1 across the kinase domain. Individual MAP kinases share a number of common structural and regulatory features, but also have unique characteristics. One distinctive feature of MAP kinases is the presence of a Thr–Xaa–Tyr motif in the activation loop, which is the site of activating phosphorylation by the MAPKK family members MKK1–7. However, not all MAP kinases are regulated in this way. This motif is absent in ERK3, ERK4 and Nemo-like kinase (NLK), where a glycine or glutamic acid residue replaces the tyrosine. ERK7 contains the motif Thr–Glu–Tyr in the activation loop, but there is no evidence that it is a substrate of the MAPKK family. Instead, phosphorylation of this motif appears to be catalyzed by ERK7 itself [14,15]. Thus, the different subfamilies of MAP kinases (Fig. 1) are not always organized into a classical three-tiered MAPKKK–MAPKK–MAP kinase cascade. Here, we propose to classify MAP kinases into conventional or atypical enzymes, based on their ability to get phosphorylated and activated by MAPKK family members. Conventional MAP kinases comprise ERK1/ERK2, p38s, JNKs, and ERK5, which are all substrates of MAPKKs. Atypical MAP kinases include ERK3/ERK4, NLK and ERK7. Much less is known about the regulatory mechanisms and physiological functions of atypical MAP kinases, which are the focus of this review.

3. ERK3/ERK4 subfamily

3.1. Discovery of ERK3 and ERK4 enzymes

ERK3 was cloned 15 years ago, alongside the extensively studied MAP kinase ERK2, by homology screening of a rat brain cDNA library using an ERK1-derived probe [16]. Later, the sequences of the human [17,18] and mouse [19] orthologs were reported. Intriguingly, the original rat ERK3 cDNA sequence predicted a protein of 543-amino acids, whereas the human and mouse cDNAs were found to encode a 721/720-amino acid protein with a molecular mass of ~100 kDa. Sequence comparison showed that human/mouse and rat ERK3 sequences were more than 90% identical over their shared length, but the human/mouse proteins contained a C-terminal extension of 178 amino acids. However, close inspection of ERK3 sequences and resequencing of the rat gene revealed that the originally published rat sequence had a missing nucleotide between codons 502 and 503 [19,20]. Addition of a guanosine residue at this position shifts the reading frame of rat ERK3, which now translates into a protein of 720 amino acids with 94% identity to the human protein. In vitro translation of the rat, mouse and human ERK3 cDNAs confirmed that all three genes encode proteins of ~100 kDa. In agreement with these observations, in silico analysis of the human and mouse genome sequence databases demonstrated the presence of a single functional gene encoding ERK3 [21]. Interestingly, database analysis has revealed the presence of six *MAPK6* (official name of the human ERK3 gene) processed pseudogenes localized on four different chromosomes [21]. This likely explains the presence of multiple ERK3-hybridizing bands on Southern blots of human DNA.

A second human kinase with high homology to ERK3, originally designated $p63^{mapk}$, was cloned in 1992 also by virtue of its homology to ERK1 [22]. The published $p63^{mapk}$ sequence was reported to encode a protein of 557 amino acids. We have renamed this kinase ERK4 in accordance with the nomenclature

adopted by the Alliance for Cellular Signaling [23]. As for ERK3, resequencing of the human ERK4 cDNA and analysis of genomic databases revealed that the original p63^{mapk} sequence contains five missing nucleotides (Meloche, S., Keyse, S.M. and Seternes, O.M., unpublished data). Addition of these nucleotides results in the substitution of four amino acids and also in a frameshift within the C-terminus of ERK4, which now translates into a 587-amino acid protein of molecular mass ~70 kDa. ERK4 is most closely related to the protein kinase ERK3 with 73% amino acid identity within the kinase domain. The atypical MAP kinase ERK4 should not be mistaken for a 45-kDa anti-ERK1 immunoreactive band identified in brain and PC12 cell extracts also designated ERK4 [16,24]. This 45-kDa protein may correspond to ERK1b, an alternatively spliced variant of ERK1 with a small insertion at the C-terminus [25].

Comparative genomic analyses revealed that *MAPK6* and *MAPK4* (human ERK4 gene official name) display a similar organization of exon/intron boundaries, which is different from that of genes encoding conventional MAP kinases and related protein kinases [21]. The high amino acid identity between ERK3 and ERK4 in the kinase domain, together with their conserved genomic organization, strongly suggest that the *MAPK6* and *MAPK4* genes arose by duplication of a common ancestor. Contrary to conventional MAP kinases, no gene encoding ERK3 or ERK4 orthologs has been found in invertebrates or in plants. Zebrafish and other teleost fishes encode an ortholog of both ERK3 and ERK4 [26]. Thus, available evidence suggests that ERK3 and ERK4 MAP kinase genes are restricted to chordates or vertebrates.

3.2. Structure of ERK3 and ERK4 proteins

ERK3 and ERK4 contain a kinase domain at their N-terminus followed by a C-terminal extension (Fig. 2). The first 150 residues of the C-terminal extremity of the two proteins are nearly 50% identical, whereas the extreme C-terminus is divergent. However, the C-terminus of ERK3 and ERK4 are conserved throughout vertebrate evolution, suggesting an important function. ERK3 and ERK4 display respectively 45% and 42% amino acid identity to ERK1 in the kinase domain. Despite this similarity, two major features distinguish ERK3/ERK4 from conventional MAP kinases. First, ERK3 and ERK4 contain a single phospho-acceptor site (Ser–Glu–Gly) in their activation loop, instead of the conserved Thr–Xaa–Tyr motif. Predictably, ERK3 was found to be a poor substrate for dual-specificity MAPKK family members [27,28]. Second, all ERK3 and ERK4 orthologs have the sequence Ser–Pro–Arg instead of Ala–Pro–Glu in subdomain VIII of the kinase domain. Intriguingly, ERK3 and ERK4 are the only kinases in the human genome to have an arginine residue at this position [29]. The impact of these features on the regulation and functions of ERK3/ERK4 is currently unknown.

More than 93% of human protein kinases contain a glutamic acid residue in subdomain VIII, a degree of conservation similar to catalytic residues [29]. Crystallographic data indicate, however, that this residue is not involved in catalysis but rather plays a structural role. The Glu residue of the APE motif forms an ion pair with a highly conserved arginine found in subdomain XI, thereby stabilizing the C-terminal lobe of the kinase domain. This interaction is easily seen in the X-ray structure of ERK2 (Fig. 3B) [30]. The C-terminal lobe is involved in substrate binding and this conserved ion pair is proposed to stabilize the interaction of the kinase with its substrate [31]. In support of this idea, mutation of subdomain VIII glutamic acid in protein kinase A markedly decreases its affinity for substrates [32]. Besides arginine, aspartic acid, asparagine and glutamine residues can be found at this position in human kinases [29]. The crystal structure of CDK5

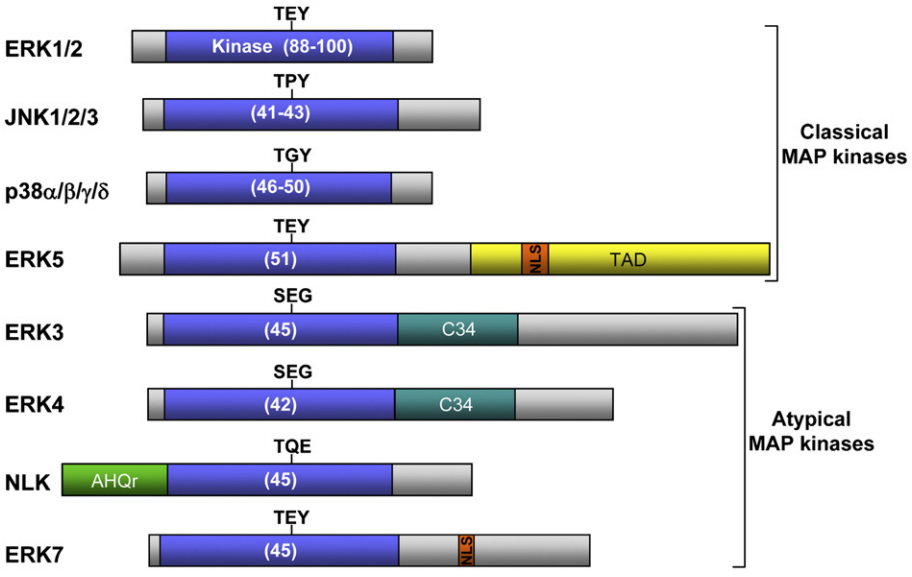

Fig. 2. Schematic representation of human MAP kinases structure. MAP kinases are composed of a kinase domain (in blue) flanked with N- and C-terminus extensions of varying lengths. The identity of the kinase domain with ERK1 is indicated. The activation loop phosphorylation motif is shown for each kinase. TAD, transactivation domain; NLS: nuclear localization sequence; C34: conserved region in ERK3/4; AHQr, alanine (A), histidine (H) and glutamine (Q) rich domain. MAP kinases are shown on scale.

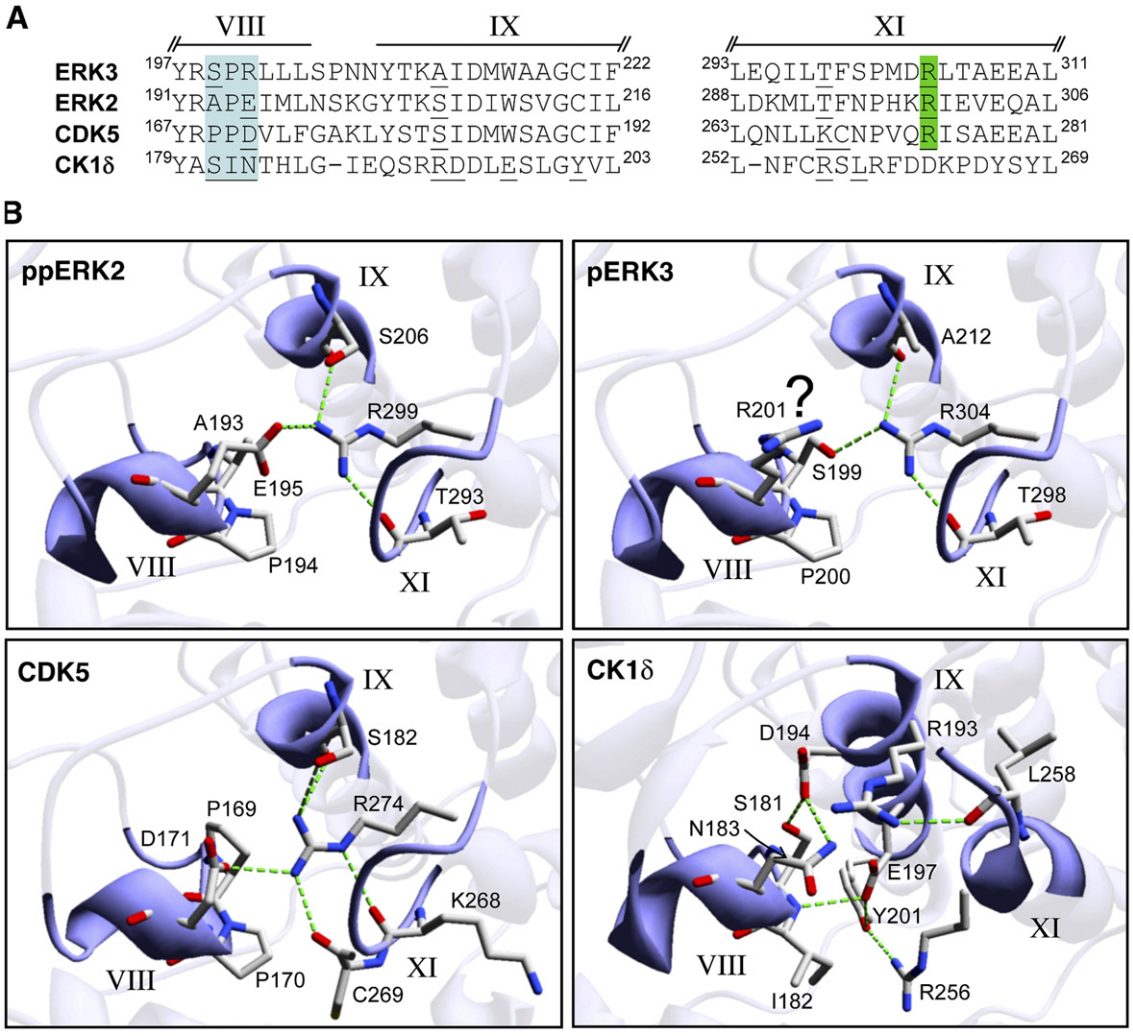

Fig. 3. Subdomain VIII participates in the stabilization of the C-terminal lobe of the kinase domain. (A) Sequence alignment of subdomains VIII, IX, and XI of ERK3, ERK2, CDK5 and CK1delta. SPR motif and subdomain XI-arginine are highlighted in turquoise and green, respectively. Residues contributing to the hydrogen bond network of subdomain VIII are underlined. (B) Details of the interactions involving subdomain VIII. Important residues and polar interactions (broken green lines) are shown. In ERK2, subdomain VIII-Glu 195 interacts with subdomain XI-Arg 299. Similarly in CDK5, subdomain VIII-Asp 171 interacts with subdomain XI-Arg 274. In both cases, the arginine also interacts with residues in subdomains IX and XI. The hydrogen bond network seen in the casein kinase 1 family is different. As shown for CK1delta, subdomain VIII-Asn 183 does not directly interact with subdomain XI. However, these two subdomains are connected via interactions with subdomain IX. Subdomain IX binds both subdomain VIII (via the SIN motif) and subdomain XI (via Arg 256 and Leu 258). ERK3 structure was modeled with the Swiss-Model server using active ERK2 (2ERK) as template [36]. The model predicts that subdomain VIII-Ser 199 interacts with the conserved subdomain XI-Arg 304, which may result in the stabilization of ERK3 C-terminal lobe. The position of the SPR Arg 201 is uncertain. The pdb files used were: ERK2, 2ERK [30]; CDK5, 1H4L [33]; CK1delta, 1CKI [34]. The figure was generated using SPDBV 3.7 [35].

(which has the sequence PPD instead of APE in subdomain VIII) reveals that aspartic acid also forms a salt bridge with subdomain XI Arg residue (Fig. 3B) [33]. Casein kinase 1 isoforms have an asparagine in subdomain VIII and do not possess the subdomain XI arginine residue (Fig. 3A). Nevertheless, structural data show that the C-terminal lobe of CK1 delta is stabilized by an alternative network of polar interactions (Fig. 3B) [34]. These observations clearly indicate that protein kinases can accommodate non-Glu residues in subdomain VIII without compromising enzymatic activity.

Sequence alignment suggests that ERK3 and ERK4 possess the subdomain XI arginine, much like APE-containing kinases (Fig. 3A). In the absence of crystal structure, it is possible to predict the structure of ERK3 by homology modeling using the crystal structure of phosphorylated ERK2 as template [35,36]. The modeled ERK3 kinase domain is predicted to fold with a topology similar to other MAP kinases [37]. The model structure shows that subdomain XI Arg 304 and subdomain VIII Arg 201 cannot interact because of electrostatic repulsion. However, Arg 304 is predicted to form a hydrogen bond with Ser 199 of the SPR motif, which may contribute to the stabilization of the C-terminal lobe of the kinase (Fig. 3B). The exact position of Arg 201 is uncertain and it does not interact with other residues in the model. The elucidation of the X-ray structure of ERK3/ERK4 will be necessary to understand the molecular basis of their catalytic activity and substrate specificity.

3.3. Regulation of ERK3 and ERK4 expression and localization

ERK3 mRNA is expressed ubiquitously in adult mammalian tissues [UniGene]. The highest expression is found in brain, skeletal muscle and gastrointestinal tract. In the mouse, the expression of ERK3 increases markedly between embryonic days 9 and 11 of development, coincident with the period of early organogenesis [19]. In vitro studies have showed that *ERK3* mRNA is up-regulated upon differentiation of P19 embryonal carcinoma cells to the neuronal or muscle lineage [16]. These observations point to a possible role for ERK3 in vertebrate embryogenesis. ERK3 also accumulates during differentiation of model cell lines into neurons and myotubes [37]. The mechanistic details of ERK3 gene regulation remain largely unexplored. A transcriptional profiling study identified *ERK3* as one of the genes that are significantly up-regulated in response to proteasome inhibitor treatment [38]. These authors further suggested that ERK3 induction by proteasome inhibition is dependent on p38 MAP kinase activity. However, the generality of this observation needs to be examined further. Analysis of EST databases indicates that *ERK4* mRNA expression is restricted to the brain, colon, eye, heart, kidney, lung, ovary, pancreas, placenta, prostate, and skin. The highest expression is found in the brain [22,39]. Nothing is known about the regulation of ERK4 gene expression.

ERK3 was found to be a highly unstable protein, with a half-life of 30–45 min in exponentially proliferating cells [37,40]. This provided the first documented example of a MAP kinase family member whose activity is acutely regulated by protein turnover. The proteolysis of ERK3 is executed by the proteasome and is dependent on the polyubiquitination of the protein. Treatment with proteasome inhibitors or inactivation of the ubiquitin-activating enzyme (E1) results in the accumulation of endogenous or ectopically-expressed ERK3 protein. The turnover of ERK3 is independent of its activation loop phosphorylation, enzymatic activity or C-terminal extension. Analysis of a series of chimeras made between the stable ERK1 kinase and ERK3 delimited two degrons in the N-terminal lobe of ERK3 kinase domain that are both necessary and sufficient to target ERK3 for proteasomal degradation [37]. Importantly, the stability of ERK3 is also regulated by the cellular context. For example, accumulation of ERK3 during differentiation of C2C12 myoblasts into muscle cells is associated with the time-dependent stabilization of the protein [37]. This suggests that protein turnover may play a key role in the regulation of ERK3 biological functions. In contrast, ERK4 is a highly stable protein (Rousseau, J. and Meloche, S., unpublished data), thereby revealing a major difference between the two kinases of this subfamily.

Most substrates of the ubiquitin-proteasome pathway are conjugated to ubiquitin via an internal lysine residue [41,42]. Interestingly, recent evidence suggests that ERK3 is targeted for degradation via an alternative mode of ubiquitination, involving the conjugation of a polyubiquitin chain to the free NH_2 terminus of the protein [43]. Surprisingly, a lysine-less mutant of ERK3 (ERK3-0K) was shown to be ubiquitinated and degraded by the proteasome with kinetics comparable to the wild type protein. Genetic analysis confirmed that proteolysis of ERK3-0K is dependent on its ubiquitination. The N-terminal ubiquitination of ectopically expressed ERK3 was directly documented by mass spectrometry. Thus, ERK3 adds to a growing list of protein substrates that are conjugated to the first ubiquitin moiety through the α-NH_2 group of their N-terminal residue [44]. The ubiquitin ligase (E3) implicated in ERK3 N-terminal ubiquitination is unknown.

At the subcellular level, ERK3 is found in both the cytoplasmic and nuclear compartments of a variety of exponentially proliferating cells [20,45–47]. Unlike conventional MAP kinases, exposure of cells to common mitogenic stimuli or chemical stresses does not cause the relocalization of ERK3 to a distinct subcellular compartment [45]. The cellular distribution of ERK3 is also independent of activation loop phosphorylation or enzymatic activity. ERK3 is imported into the nucleus by an active temperature-dependent mechanism, and is exported to the cytoplasm in part by a CRM1-dependent active mechanism [45]. Biochemical studies have shown that recombinant ERK3 directly binds to the exportin CRM1 in vitro. ERK3 was also reported to localize to the endoplasmic reticulum Golgi intermediate compartment (ERGIC)/*cis*-Golgi, and it has been suggested that translocation of the kinase from the Golgi to the nucleus is contingent upon proteolytic removal of a C-terminal fragment [20]. Preliminary studies indicate that the subcellular distribution of ERK4 differs from that of ERK3. In many cell types examined, ERK4 is predominantly localized in the cytoplasm (unpublished data).

Two recent studies have shown that ERK3 interacts with the MAP kinase-activated protein kinase MK5 [46,47]. Interestingly, co-expression of ERK3 and MK5 causes the redistribution of both proteins from the nucleus to the cytoplasm. On the other hand, downregulation of MK5 expression leads to a dramatic reduction in ERK3 protein levels, suggesting that MK5 acts as a chaperone for ERK3. The physiological significance of these observations is not clear.

3.4. Mechanism of activation of ERK3 and ERK4

Little is known about the regulation of ERK3 and ERK4 enzymatic activity. Metabolic labeling experiments and immunoblotting analysis with a phospho-specific antibody have shown that ERK3 is phosphorylated on activation loop Ser 189 in intact cells [37,43,48]. A catalytically inactive mutant of ERK3 is phosphorylated to the same extent as the wild-type protein, indicating that phosphorylation of Ser 189 is executed in trans by a cellular ERK3 kinase [43]. A protein kinase capable of binding ERK3 and phosphorylating it on Ser189 has been identified and partially purified from rabbit muscle [49]. The ERK3 kinase was found to be highly specific as demonstrated by its inability to phosphorylate ERK1, ERK2, or ERK2 mutants that mimic ERK3 in the activation loop.

In contrast with conventional MAP kinases, the phosphorylation of ERK3 in the activation loop is constitutive in serum-starved cells and is not further increased by exposure to growth factors or chemical stresses (Coulombe, P. and Meloche, S., manuscript in preparation). An important question that remains

to be addressed is whether activation loop phosphorylation of ERK3 correlates with enzymatic activity.

3.5. Substrates and physiological functions of ERK3 and ERK4

The recombinant ERK3 protein purified from *E. coli* is capable of autophosphorylating on Ser 189 *in vitro*, demonstrating that ERK3 has intrinsic phosphotransferase activity ([48]; unpublished data). Moreover, a chimeric kinase containing the N-terminal domain of ERK3 (subdomains I–V) and the C-terminal half of the ERK2 catalytic core displays relatively high specific activity towards the ERK2 substrate myelin basic protein (MBP), further indicating that ERK3 binds ATP in a manner appropriate for catalysis [50]. However, ERK3, either expressed in bacteria or immunoprecipitated from cultured cells, did not phosphorylate a panel of generic protein kinase substrates in vitro ([48]; unpublished data). More recently, ERK3 was reported to bind to and phosphorylate MK5 in the activation loop, leading to the activation of the kinase [46]. However, another group suggested that ERK3 promotes MK5 autophosphorylation instead [47]. Additional work is required to resolve this discrepancy and clarify whether MK5 is a bona fide ERK3 substrate.

Why does ERK3 kinase activity has been so difficult to demonstrate experimentally? Sequence analysis and structural modeling may help understand the distinct substrate specificity of ERK3. Crystallographic data reveal that phosphorylated residues in the activation loop of kinases interact in an intramolecular fashion with phosphate binding sites located near the loop [51]. The phosphorylated Thr–Xaa–Tyr motif of conventional MAP kinases interacts with so-called primary and secondary phosphate binding sites. The primary site, also known as the RD pocket, is defined by three highly conserved Arg residues that coordinate the phosphate of the threonine, as seen in the structure of di-phosphorylated ERK2 (Fig. 4) [30]. Similarly, the phospho-tyrosine interacts with the secondary phosphate binding site composed of two conserved arginine (Fig. 4). Sequence alignment indicates that ERK3 possesses the three arginine of the primary site (Fig. 4A). Intriguingly, ERK3 also has the two positively charged residues of the secondary phosphate binding site (Fig. 4A). Because ERK3 only has the primary phosphorylation site (Ser 189), the function of the putative secondary binding site is unclear. Other protein kinases also have "extra" phosphate binding sites. For example, GSK3β only has the second tyrosine phosphorylation site in its activation loop (Fig. 4A). Sequence analysis indicates, however, the presence of both the primary and secondary phosphate binding sites in GSK3β (Fig. 4A). The crystal structure of GSK3β shows that the secondary site is occupied by the phospho-tyrosine as in the case of ERK2 (Fig. 4B) [52]. Notably, the primary site is filled with a sulfate anion, indicating that it is functional (Fig. 4B). What is then the role of the primary site? GSK3β is known to require phosphate priming of its substrates at the P+4 position for efficient recognition [53]. It is thought that the primary site of GSK3β interacts directly with the primed phosphorylated substrate, thus positioning it at the active site. In agreement with this idea, mutation of one of the three positively charged residues of the primary site impairs GSK3β activity towards phosphorylated substrates, but has no impact on non-primed substrates [53]. The yeast SR-kinase Sky1p also has a secondary phosphate binding site, even though this enzyme does not require activation loop phosphorylation for activity (Fig. 4A) [54]. Like GSK3β, biochemical data suggest that Sky1p acts in a processive manner on pre-phosphorylated substrates [51,55]. These examples of CMGC kinases show that phosphate binding sites may dictate substrate specificity. These enzymes are sometimes referred to as phosphate-directed kinases.

Similarly, the secondary phosphate binding site of ERK3 could enable the kinase to act on primed phosphorylated substrates. To further test this possibility, we generated a model of ERK3 phosphorylated on Ser 189 based on di-phosphorylated ERK2 (Fig. 4B). The model clearly shows that ERK3 primary site interacts with phospho-Ser 189, arguing that this phosphorylation event leads to ERK3 activation. The predicted structure also suggests that the secondary site of ERK3 is functional and could chelate an anion, like the phosphate ion modeled in Fig. 4B. If ERK3 has phosphate-directed specificity, this would explain the lack of kinase activity detected in vitro towards non-phosphorylated substrates. Future biochemical and structural studies will clarify these issues.

The phenotype of ERK3 and ERK4 deficient mice has not been reported and the physiological functions of these kinases remain to be defined. Several lines of evidence suggest a role for ERK3 in the control of cell differentiation. For example, ERK3 mRNA and protein levels are up-regulated during terminal differentiation of P19 [16], PC12 [37] and C2C12 [37] cell lines in vitro. Differentiation of these model cell lines is associated with G1 arrest of the cell cycle. ERK3 expression is also induced following treatment of Raji lymphoma cells with inhibitory gangliosides [56] or after plating of squamous cell carcinoma lines on type IV collagen [57], two conditions associated with inhibition of cell proliferation. These observations suggest that ERK3 may act as a negative regulator of cell cycle progression in certain cellular contexts, thereby facilitating cell differentiation. In agreement with this idea, overexpression of ERK3 was found to inhibit S phase progression of various cell types [37,45,57]. However, the putative effects of ERK3 on cell cycle progression and cellular differentiation need to be validated by loss-of-function studies. It has been recently suggested that ERK3 is involved in glucose-induced insulin secretion [58]. Treatment with prolactin was shown to induce ERK3 mRNA and protein levels in isolated pancreatic islets. Downregulation of ERK3 expression by antisense oligonucleotides abolished the insulin secretion stimulated by glucose in rat islets. Measurement of insulin and glucose levels in ERK3 deficient animals will provide rigorous testing of the involvement of ERK3 in glucose metabolism.

4. NLK subfamily

4.1. Discovery of NLK enzyme

Drosophila nemo was discovered in 1994 as a gene required for proper photoreceptor cells rotation during ommatidia

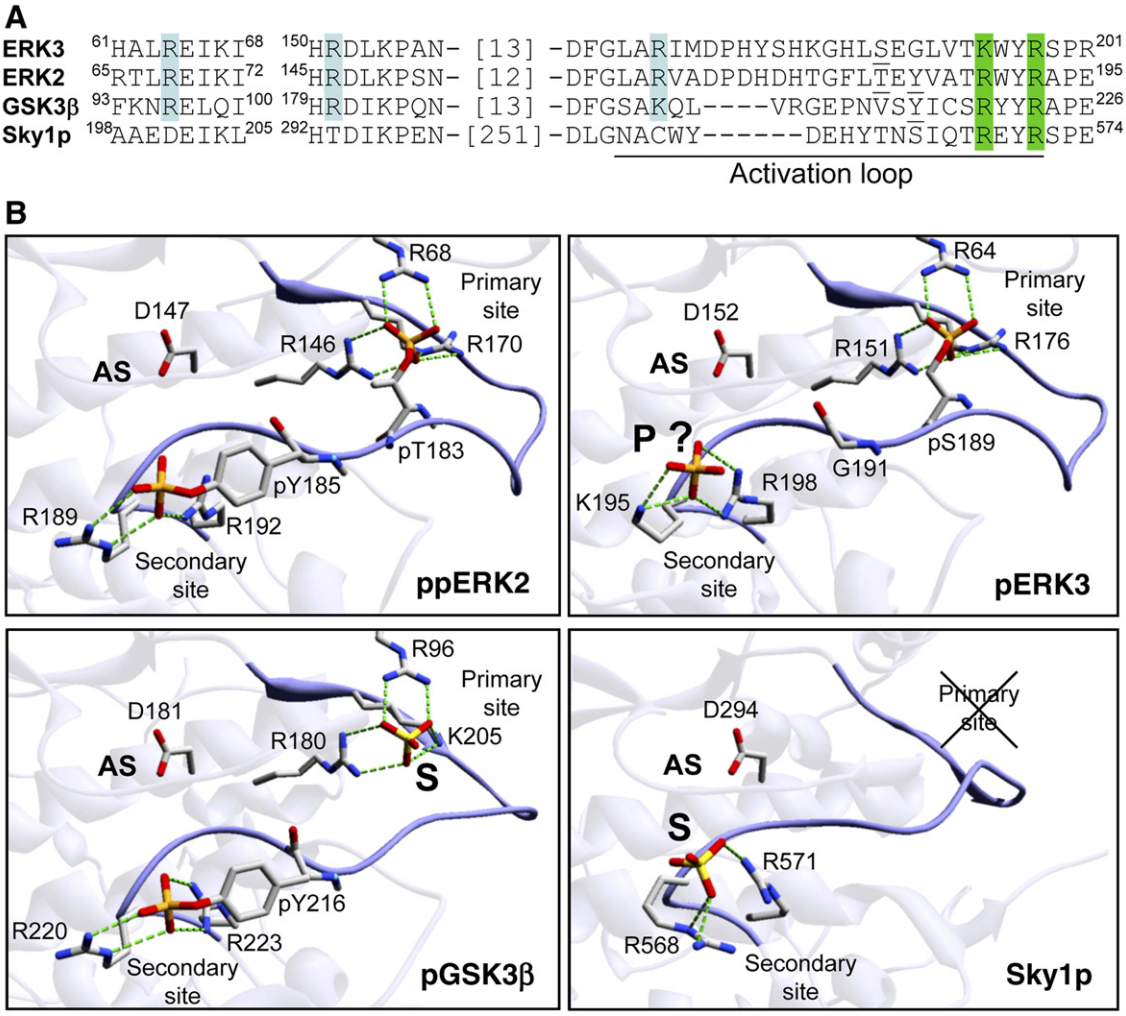

Fig. 4. Structural modeling suggests that ERK3 might be a phosphate-directed kinase. (A) Sequence alignment of the residues involved in the formation of the phosphate binding sites of ERK3, ERK2, GSK3β and Sky1p. Residues forming the primary and the secondary phosphate binding sites are highlighted in turquoise and in green, respectively. Note that ERK3 is predicted to have both sites. One arginine of the putative secondary site is substituted with a lysine in ERK3. The activation loop is underlined. (B) Structural details of the phosphate binding sites. Important residues forming the primary and the secondary sites are shown. Hydrogen bonds between amino acid residues and anions are depicted by green broken lines. The position of the active site (AS) located near the catalytic aspartic acid is indicated. In GSK3β and Sky1p, the structure contains a sulfate anion (S) located in the primary and secondary site, respectively. The tertiary structure of activated ERK3 was modeled with the Swiss-Model server using di-phosphorylated ERK2 (2ERK) as template. Phosphate ions were introduced in both phosphate binding sites, using active ERK2 as a guide. The predicted secondary site of ERK3 can form four hydrogen bonds with the phosphate (P ?). The pdb files used were: ERK2, 2ERK [30]; GSK3β, 1GNG [52]; Sky1p, 1HOW [54]. The figure was made using SPDBV 3.7 [35].

morphogenesis in the eye [59]. The *nemo* gene was found to encode a serine/threonine kinase with homology to MAP kinases. Four years later, the murine ortholog of Nemo was isolated by PCR cloning using degenerate primers based on MAP kinase family sequences [60]. The murine gene product was denoted NLK, because of its similarity to *Drosophila* Nemo. An ortholog of NLK, known as LIT-1, was also identified in *C. elegans* as a gene product that interacts genetically with the Wnt signaling pathway [61,62].

4.2. Structure of NLK protein

Human NLK is a 515-amino acid protein with a central kinase domain bordered by N- and C-terminal extensions of ~100 residues (Fig. 2A). The extended N-terminus has a unique sequence that is highly enriched in alanine, glutamine, and histidine residues. However, this region of the kinase is poorly conserved among NLK orthologs and its function remains obscure. The C-terminal extension is conserved from worm to human (45% identity between LIT-1 and human NLK) and may contribute to the interaction of the kinase with specific substrates or targets [63–65]. The kinase domain of NLK displays more sequence similarity to the MAP kinases (45% identity with ERK2) than to CDKs (38% identity with CDK2). Like ERK3 and ERK4, NLK lacks the tyrosine phosphorylation site in its activation loop. The TQE motif found in NLK resemble the THE sequence of CDK2.

4.3. Regulation of NLK expression and localization

In the mouse, *NLK* mRNA is expressed in most adult tissues, with the highest levels seen in brain and lymphoid organs ([60];

UniGene). The subcellular localization of NLK is not well characterized. Ectopically expressed NLK is found predominantly in the nucleus with some diffuse expression in the cytoplasm [60,66]. It is not known whether activation of NLK influences its cellular distribution.

4.4. Mechanism of activation of NLK

Based on its homology to MAP kinases and cyclin-dependent kinases, NLK is predicted to be regulated by phosphorylation of its activation loop Thr 286. Surprisingly, there is no published biochemical data confirming that this site is phosphorylated in vivo. However, mutation of Thr 286 abolishes the ability of NLK to autophosphorylate and to induce the degradation of c-Myb, indicating that activation loop phosphorylation is necessary for its activity [60,66].

NLK is activated by various extracellular ligands, notably the Wnt ligand Wnt-1, the cytokines interleukin-6 and granulocyte colony-stimulating factor, and transforming growth factor-β (TGF-β) family members [66–69]. In certain cell types, elevation of intracellular Ca^{2+} is sufficient to stimulate NLK enzymatic activity, by a mechanism dependent on Ca^{2+}/calmodulin-dependent protein kinase II activation [70]. Genetic and biochemical evidence have implicated the MAPKKK TGF-β-activated kinase 1 (TAK1) and its regulatory subunit TAK1-binding protein 1 (TAB1) in NLK activation [62,63,67,68,71]. However, it remained unclear whether an intermediate kinase is functioning between the MAPKKK TAK1 and the MAP kinase NLK. More recently, homeodomain-interacting protein kinase (HIPK2), a member of the CMGC group of protein kinases, was shown to interact with and phosphorylate NLK in vitro [66]. HIPK2 is a large nuclear protein kinase that was originally identified as a co-repressor for homeodomain transcription factors [72]. The phosphorylation of NLK by HIPK2 is associated with an increase in phosphotransferase activity. However, the phosphorylation site(s) of NLK has not been characterized and it is presently unclear whether HIPK2 directly phosphorylates NLK in the activation loop or induces its autophosphorylation. In addition, TAK1 was shown to phosphorylate HIPK2 in vitro, although phosphorylation did not translate into enzymatic activation [66]. These findings suggest that HIPK2 may represent the missing MAP kinase kinase (distinct from MAPKK/MEK family members) in the TAK1-NLK signaling pathway (Fig. 1).

4.5. Substrates and physiological functions of NLK

Immunoprecipitated NLK does not phosphorylate generic MAP kinase or CDK substrates in vitro, suggesting that it has different or more restricted substrate specificity [60]. In the past few years, a number of transcription factors were shown to be regulated by NLK phosphorylation. The first NLK substrates to be identified are members of the T-cell factor (TCF)/lymphoid enhancer factor (LEF) family, which are ultimate mediators of the Wnt signaling pathway [73]. Genetic analyses of endoderm specification in *C. elegans* have revealed that *lit-1* negatively regulates *pop-1* (which encodes a TCF/LEF related protein) activity and that mutations in *pop-1* are epistatic to mutations in *lit-1* [61,62,74]. A biochemical explanation for these observations came with the finding that LIT-1/NLK associate with and phosphorylate TCF/LEF proteins in vitro [61,63]. The functional consequence of this phosphorylation is to relocalize TCF to the cytoplasm and/or prevent its binding to target gene promoters. Further biochemical characterization indicated that NLK phosphorylates LEF-1 and TCF-4 on two conserved serine and threonine residues that are followed by a proline [70]. Phosphorylation sites of other NLK substrates (see below) were also found to be followed by a proline, demonstrating that NLK has proline-directed specificity like conventional MAP kinases. Recently, the RING finger protein NARF was isolated from a yeast two-hybrid screen as an NLK-interacting protein [65]. NARF catalyzes the ubiquitination of TCF/LEF members in vitro and it has been suggested that ubiquitination and degradation of TCF/LEF is contingent upon their phosphorylation by NLK.

The NLK pathway plays a critical role in TGF-β-induced mesodermal development in *Xenopus* embryos [67]. Biochemical studies have shown that NLK associates with and phosphorylate the transcriptional regulator STAT3 on its C-terminal Ser 727 residue [67,69]. In vivo depletion studies in *Xenopus* embryos indicated that NLK is involved in serine phosphorylation of endogenous STAT3 and that both serine and tyrosine phosphorylation of STAT3 C-terminus are necessary for mesoderm induction [67]. NLK was also shown to phosphorylate the Myb family members c-Myb and A-Myb at multiple sites [66,75]. In the case of c-Myb, phosphorylation by NLK promotes its ubiquitination and proteasome-dependent degradation in vivo, resulting in inhibition of c-Myb-dependent transcription [66].

The gene encoding NLK has been disrupted in mice by insertion of a *lacZ* cassette into the kinase domain [7]. The phenotype of $Nlk^{-/-}$ mice is modulated by the genetic background; whereas NLK-null mice die during the third semester of pregnancy in a C57BL/6 background, mutant mice bred to a 129/Sv background survive for up to 4–6 weeks. The latter $Nlk^{-/-}$ mice are growth retarded and suffer from neurological abnormalities. Histopathological examination revealed that NLK deficient mice have a compromised hematopoietic system, characterized by lymphopenia and abnormal differentiation of bone marrow stromal cells. Surprisingly, no intrinsic defect of lymphocyte development was documented in these mice. Given the roles of the Wnt and STAT3 signaling pathways in development, the phenotype of NLK mutant mice is milder than expected. This suggests the existence of functional redundancy in the NLK signaling pathway that can genetically compensate for the loss of the kinase.

5. ERK7 subfamily

5.1. Discovery of ERK7 enzyme

ERK7 was cloned in 1999 by PCR amplification of a rat brain cDNA library using degenerate primers based on

conserved MAP kinase sequences [76]. Three years later, the same group reported the cloning of a human kinase with high similarity to ERK7, which they named ERK8 [77]. The rat ERK7 and human ERK8 sequences share 69% amino acid identity overall (82% in the kinase domain), which is lower than the homology typically observed between other rodent and human MAP kinase orthologs. However, analysis of the human kinome did not reveal the presence of another kinase more similar to ERK7 [13]. Furthermore, phylogenetic analyses indicated that a single gene encoding ERK7 is present in metazoan and also in the social amoeba *Dictyostelium discoideum* (unpublished data). Intriguingly, this kinase is significantly less conserved across evolution as compared with other MAP kinases. For example, zebrafish ERK7 is 51% identical to the human kinase, whereas ERK1 shows more than 90% similarity between the two species. The evidence indicates that all these kinases are true ERK7 orthologs. Hence, we will refer to the human kinase as ERK7 in this review.

5.2. Structure of ERK7 protein

Human ERK7 is a 544-amino acid protein with an N-terminal kinase domain similar to MAP kinases and a C-terminal extension of 204 residues (Fig. 2). The kinase domain of ERK7 is 45% identical to ERK1. The C-terminal region does not show any significant similarity to other proteins. Notably, it contains two proline-rich regions that may represent SH3 domain ligands, and a putative nuclear localization sequence (NLS). Like ERK1/ERK2 and ERK5, ERK7 contains the TEY motif in its activation loop (Fig. 2).

5.3. Regulation of ERK7 expression and localization

Human *ERK7* mRNA is widely expressed in adult tissues, with predominant expression seen in lung and kidney ([77]; UniGene). Similar to ERK3, the activity of ERK7 appears to be controlled by protein turnover [78]. Ectopically expressed ERK7 is rapidly ubiquitinated and degraded by the proteasome in proliferating cells, with a half-life of ~2 h. Analysis of a series of chimeras between ERK2 and ERK7 revealed that the N-terminal 20 amino acids of the kinase domain (subdomain I) are necessary and sufficient to signal ERK7 degradation. ERK7 is stabilized by overexpression of an N-terminal mutant of Cullin-1, suggesting the involvement of an SCF family E3 ligase in mediating ERK7 ubiquitination.

Overexpressed ERK7 protein is predominantly found in the nucleus [76]. The subcellular localization of the kinase is independent of its catalytic activity. Deletion of the C-terminal extension of ERK7 results in some redistribution of the protein to the cytoplasm, consistent with the presence of a putative NLS in that region. The localization of the endogenous protein is unknown.

5.4. Mechanism of activation of ERK7

The regulation of ERK7 enzymatic activity is unique among MAP kinases. Ectopically expressed rat ERK7 is constitutively phosphorylated on the activation loop Thr–Glu–Tyr motif in vivo, and displays significant kinase activity against various MAP kinase substrates [14,76]. Notably, the catalytically-inactive K43R mutant of ERK7 is not phosphorylated on the TEY motif in cells, suggesting that activation loop phosphorylation results from an autophosphorylation event [76]. In strong support of this idea, it was shown that recombinant ERK7 expressed in *E. coli* is phosphorylated on both threonine and tyrosine residues of the TEY motif and exhibits high specific activity towards exogenous substrates [14,15]. In contrast, catalytically-inactive mutants of ERK7 were not phosphorylated on these sites. Surprisingly, the activity of bacterially expressed human ERK7 is decreased by 95% after treatment with the serine/threonine-specific phosphatase PP2A, but only by 15% after treatment with a tyrosine-specific phosphatase [15]. This suggests that phosphorylation of the threonine residue is the major determinant of ERK7 activity. Indeed, when overexpressed in 293 cells, human ERK7 displays 100-fold less activity than the recombinant purified kinase and is found monophosphorylated on tyrosine [15]. Treatment with the serine/threonine phosphatase inhibitor okadaic acid induces threonine phosphorylation of the kinase and stimulates its enzymatic activity. These findings suggest that the activity of ERK7 is determined by the relative rates of ERK7 autophosphorylation and its dephosphorylation on the activation loop threonine. Although not limiting, tyrosine autophosphorylation is nevertheless required for ERK7 activity based on the observation that the TEF mutant is inactive. Additionally, mutagenesis studies have suggested that the C-terminal extension of ERK7 is required for its full activation [76].

How physiological agonists regulate ERK7 activity is unclear. It was originally reported that the extent of phosphorylation and activity of the kinase are not increased following stimulation with growth factors, phorbol ester or chemical stresses [76]. Subsequent studies showed however that oxidative stress and mitogens stimulate the activation loop phosphorylation of ERK7 [15,77]. Activated Src and RET tyrosine kinases were also found to induce ERK7 phosphorylation and activation [77,79]. Notably, ERK7 was found to physically associate with Src in cells. It is unclear whether tyrosine kinase signaling stimulates a MAP kinase kinase or instead inhibits a phosphatase dedicated to ERK7.

5.5. Substrates and physiological functions of ERK7

ERK7 phosphorylates the classical ERK1/2 substrate MBP in vitro [76,77]. Interestingly, the major residues in MBP phosphorylated by ERK7 were identified as Ser 126 and Thr 94, two residues followed by a proline, which are distinct from the site phosphorylated by ERK2, namely Thr 97 [9,15]. This indicates that ERK7 is a proline-directed kinase, but with a distinct substrate specificity than ERK1/2. The physiological substrates of ERK7 remain to be identified.

The physiological functions of ERK7 are unknown. The phenotype of ERK7 deficient mice has not been reported yet. Published studies suggest that ERK7 may play a role in the regulation of cell proliferation, chloride transport and nuclear

receptor signaling. Overexpression of ERK7 significantly inhibits S-phase entry in CV-1 cells [76]. Intriguingly, this effect was found to be independent of ERK7 kinase activity but to require an intact C-terminus. Loss-of-function experiments are necessary to confirm that endogenous ERK7 exerts anti-proliferative actions. The transmembrane protein chloride intracellular channel 3 (CLIC3) was isolated in a yeast two-hybrid screen using the C-terminal extension of ERK7 as bait [80]. CLIC3 is a member of a family of small proteins proposed to function as chloride channels [81]. Biochemical data indicated that ERK7 is not able to phosphorylate CLIC3 in vitro, although the situation may be different in the in vivo context. It is not known whether ERK7 influences CLIC3 chloride transport activity.

In recent years, evidence has accumulated that ERK7 may regulate nuclear receptor signaling. Ectopic expression of ERK7 was shown to decrease the steady-state levels of estrogen receptor alpha (ERα) by promoting its degradation by the proteasome [82]. This effect of ERK7 is dependent on its catalytic activity, although direct phosphorylation of ERα was not demonstrated. Intriguingly, immunoblot analysis of a small panel of normal and tumor breast tissues suggested that breast cancer progression is correlated with loss of ERK7 expression [82]. These observations will need to be validated on larger cohorts of patients using immunohistochemistry techniques. More recently, hydrogen peroxide inducible clone-5 (Hic-5)/androgen receptor activator-55 (ARA55) was identified as an ERK7 interacting protein in a yeast two-hybrid screen [83]. Hic-5 is a LIM domain containing co-activator of the androgen receptor (AR), glucocorticoid receptor alpha (GRα), progesterone receptor (PR) and peroxisome proliferator-activated receptor gamma (PPARγ) nuclear receptors [84–86]. Gene reporter assays showed that overexpression of ERK7 negatively regulates the transcriptional co-activation of AR and GRα by Hic-5 in heterologous cells. This effect is independent of the kinase activity of ERK7. Reciprocally, silencing of endogenous ERK7 in airway epithelial cells enhanced dexamethasone-stimulated transcriptional activity of GRα.

6. Concluding remarks

The MAP kinases ERK3 and ERK4 were identified contemporary to the archetype MAP kinase ERK2, but have received much less attention by the signaling community. Similarly, less progress has been made towards understanding the regulation and functions of the more recently identified MAP kinases NLK and ERK7. However, recent studies have started to shed some light on the biological properties of these enzymes. The observation that ERK3 and ERK7 are constitutively degraded by the ubiquitin-proteasome pathway has highlighted a novel mode of regulation of these MAP kinases. NLK and ERK7 have been shown to have proline-directed specificity and physiological substrates of NLK have been identified in different species. Biochemical studies have demonstrated the unique mechanism of ERK7 activation by autophosphorylation. However, several important questions remain unanswered. For instance, the substrate specificity of ERK3 and ERK4 is unknown. No substrate of these enzymes has yet been identified, which severely hampers the study of their regulation and biological functions. Another aspect of the regulation of ERK3 and ERK4 that is currently unclear is how phosphorylation controls enzymatic activity. The identification of the kinase responsible for activation loop phosphorylation of ERK3 and ERK4 would also help understand the regulation of their activity. Finally, little is known about the physiological functions of atypical MAP kinases. Gene targeting studies in the mouse and RNA interference experiments will be instrumental in this regard.

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