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Sphingolipid regulation of ezrin, radixin, and moesin protein families: implications for cell dynamics☆,☆☆

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Abstract

A key but poorly studied domain of sphingolipid functions encompasses endocytosis, exocytosis, cellular trafficking, and cell movement. Recently, the ezrin, radixin and moesin (ERM) family of proteins emerged as novel potent targets regulated by sphingolipids. ERMs are structural proteins linking the actin cytoskeleton to the plasma membrane, also forming a scaffold for signaling pathways that are used for cell proliferation, migration, and invasion, and cell division. Opposing functions of the bioactive sphingolipids ceramide and sphingosine-1-phosphate (S1P), contribute to ERM regulation. S1P robustly activates whereas ceramide potently deactivates ERM via phosphorylation/dephosphorylation, respectively. This recent dimension of cytoskeletal regulation by sphingolipids opens up new avenues to target cell dynamics, and provides further understanding of some of the unexplained biological effects mediated by sphingolipids. In addition, these studies are providing novel inroads into defining basic mechanisms of regulation and action of bioactive sphingolipids. This review describes the current understanding of sphingolipid regulation of the cytoskeleton, it also describes the biologies in which ERM proteins have been involved, and finally how these two large fields have started to converge.

Keywords

Sphingolipids; Ezrin; Radixin; Moesin; Ceramide; Sphingosine-1-phosphate; Cytoskeleton

1. Introduction

Long thought to be chiefly structural and metabolic molecules, sphingolipids have emerged as bioactive constituents of eukaryotic cells. They are involved in many vital cellular processes and functions and are generated in response to many extracellular inducers such as UV light, chemotherapy, growth factors, and cytokines [1]. The bioactive sphingolipid

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ceramide mediates cellular senescence, apoptosis and cell cycle arrest. Its metabolite, S1P, mediates cellular proliferation, mitogenesis, inflammation, angiogenesis, and cancer metastasis [1]. This model of sphingolipid-mediated biologies highlights the opposing roles that can be exerted by the different bioactive sphingolipids[2].

This review focuses on a special area in sphingolipid biology, specifically their pleiotropic effects on the actin cytoskeleton and their contribution to cell shape. Such effects are crucial for mediating cellular changes observed in endocytosis, exocytosis, cellular trafficking, and filopodia and lamellipodia formation, processes required for the maintenance of cell polarity and induction of cell migration [3]. Here we focus on the ERM family and the emerging intimate connections to sphingolipids.

ERM (ezrin, radixin, and moesin) proteins are a group of adaptor molecules linking the cortical actin cytoskeleton to the plasma membrane [4]. They are considered to be core proteins with which different molecules interact to facilitate signal transduction between the cell and the extracellular matrix [5]. ERMs are essential for dictating cellular morphology, a role made apparent by their ability to modulate formation of specialized cellular structures such as filopodia, lamellipodia, and other membrane protrusions in which ERMs exist in abundance. Also, ERMs have important roles in cell biological processes such as cell polarity, adhesion, migration, and invasion. Therefore, ERMs are emerging as critical regulators of cancer metastasis and progression [6-11]. ERM proteins exist in two conformational states, which dictate their function: an inactive or closed state in which the N-terminal domain (FERM) and the C-terminal domain (C-ERMAD) interact and an open or active state in which phosphorylation of the C-terminal threonine residue (ezrin Thr-567, radixin Thr-564, and moesin Thr-558) disrupts this interaction. When in the closed state, ERMs are cytosolic, and when in the open/active state, the FERM domain interacts with the plasma membrane and the C-ERMAD is linked to the actin cytoskeleton [4]. Other phosphorylation sites exist, but these have different functions as will be discussed later.

After providing a brief summary of sphingolipid metabolism and its regulation of cell morphology, this review will address two main areas. First, it will address ERM in-depth to educate the reader about the diverse functions of ERMs. Next, this review it will expand on the connections established between sphingolipid metabolic products and their regulation of ERM functions.

2. The sphingolipid pathway

Sphingolipid synthesis is a complex interconnected set of reactions that yield abundant bioactive intermediate products. *De novo* sphingolipid synthesis that occurs in the endoplasmic reticulum, begins with a condensation reaction between serine and palmitoyl CoA mediated by serine palmitoyl transferase (SPT) to form 3-keto-dihydrosphingosine, which is directly reduced to dihydrosphingosine [12]. This product is acted upon by dihydroceramide synthase (also known as Lass or CerS), to form dihydroceramide which is subsequently desaturated, generating ceramide [13]. Ceramide occupies a central position in this pathway, and it is a target for diverse enzymes to yield hundreds of biosynthetic pathway products. Multiple sugar residues can be added to ceramide to yield complex glycosphingolipids through the initial action of glucosyl- or galactosyl- ceramide synthases, the first steps in glycosphingolipid synthesis [14]. Alternately, sphingomyelin can be generated by the addition of a phosphocholine headgroup, a step catalyzed by sphingomyelin synthases [15]. Ceramide can also be phosphorylated by ceramide kinase to form ceramide-1-phosphate [16].

Sphingolipid catabolism is accomplished by multiple enzymes that drive the production of intermediate bioactive lipids and shift the balance among them. Sphingomyelin is acted upon by 4-6 sphingomyelinases (acid, 1-3 neutral, alkaline, and one that is mitochondrial), which differ in their cellular localization [17]. Complex glycosphingolipids are gradually degraded by hydrolases to form glucosylceramide and galactosylceramide, which serve as substrates for specific glucosidases and galactosidases [18, 19].

Finally, ceramide is metabolized to sphingosine through the action of five known ceramidases (acid, neutral, and three alkaline) that also differ in their cellular localization and tissue distribution [20]. Sphingosine, in turn, participates in the formation of ceramide through the salvage pathway via multiple ceramide synthases, or it can be phosphorylated by two known sphingosine kinase isoforms, SK1 and SK2, to form sphingosoine-1-phosphate (S1P), one of the most studied sphingolipid molecules [21]. S1P can either be dephosphorylated back to sphingosine by S1P phosphatases [22], or it can be cleaved irreversibly to ethanolamine phosphate and hexadecanal by S1P lyase, which represents the only exit point of the sphingolipid pathway [23]. **Figure 1** provides a schematic illustration of the sphingolipid metabolic pathway described above.

3. Sphingolipids and cellular morphology

Sphingolipids are important for membrane and cell shape integrity because of their structural characteristics and their involvement in the activation/inhibition of signaling pathways that alter cellular morphology.

3.1. Sphingolipids in endocytosis and exocytosis

Endocytosis and exocytosis require major cytoskeletal and membranous rearrangements that are initiated by membrane-invagination and conclude with vesicle formation. Endocytosis is dramatically altered in yeast carrying mutations in lcb1p, a component of SPT, which catalyzes the first step of *de novo* lipid synthesis [24]. Such an effect is mediated by a deregulation of actin dynamics [25], an effect that can be reversed to the normal phenotype by overexpression of protein kinase C1 and PKH1 or PKH2 (orthologue of mammalian protein-phosphoinositide dependent protein kinase 1, PDK1) [26, 27]. These studies suggest a possible regulatory role for sphingolipids with these enzymes, modifying cellular structures. In contrast, membrane fusion between a donor vesicle and the plasma membrane during exocytosis requires the presence of membrane-associated proteins, SNAREs. These proteins are encoded by SNC genes, and are altered in the mutant yeast strain *M42A snc2delta* that has defective exocytosis. Interestingly, this defect is restored by overexpression of the sphingoid base phosphate lyase, which diminishes cellular dihydrosphingosine-1-phosphate [28]. In addition, a mutation in S1P lyase in *Drosophila* gives rise to cellular migration defects, specifically affecting F-actin distribution [29, 30]. Also, ceramidase has been reported to be essential in exocytosis in yeast and *Drosophila*. Yeast defective in YPC1, which encodes a ceramidase, have defective vesicular trafficking from the Golgi apparatus to the cell exterior [31]. Moreover, *Drosophila* with a mutated neutral ceramidase, also have defects in neuronal synaptic vesicular fusion and cellular trafficking [32]. Finally, neutral sphingomyelinase has been recently reported to play a crucial role in exosomes secretion [33, 34]. Exosomes are extracellular vesicles derived from the endocytic compartment and carry molecules involved in paracrine signaling. Overexpression of this enzyme increases the secretion of miRNA-loaded exosomes, which causes increased angiogenesis and metastasis [33].

3.2. Sphingolipids and plasma membrane protrusions

S1P is a potent bioactive sphingolipid that exerts its function either through intracellular interactions, or by being transported to cell exterior via the ABC transporter family [35] and/or the spinster-2-transporter [36], where it acts on G-protein coupled receptors (S1P receptors; S1PR). Five S1PRs have been identified (S1P1–5). S1P receptors differ with respect to tissue distribution and with respect to the G-proteins to which they are coupled [37]. S1P is a well-known chemoattractant and a modulator of cell migration, exerting its functions by acting on receptors that, in turn, couple to the Rho and Rac family of small G proteins [3]. Activation of the Rac pathway polarizes the cell and gives rise to the production of lamellipodia along with focal adhesion contacts with the extracellular matrix. Activation of the Rho pathway, in contrast, causes cell detachment and stress-fiber formation. Alternating and concerted activation of these two different pathways ultimately lead to cell migration and forward movement [38]. S1PR1 has been coupled to Rac activation and cell movement whereas S1PR2 has been linked to Rho activation and Rac inhibition, thereby inhibiting cell movement [37]. Detailed effects of S1P receptors on cell movement are beyond the scope of this review and have been adequately reviewed elsewhere [39].

4. Ezrin activation and role in biology

Ezrin activation is a multistep process requiring the phosphorylation of the threonine 567 residue. Multiple cellular stimuli are known to induce ezrin phosphorylation, which has a well-defined biological significance. Table 1 offers a current list of all kinases known to induce ERM phosphorylation with respect to the stimulus applied and the biology involved. The Table also contains details about the specific cell line in which the mechanism has been studied. As previously mentioned, ERM phosphorylation is not limited to the activating threonine 567 residue, but it involves several other residues key for cell signaling (also described in **Table 1**). The Table represents a reference for studying relationships between the ERM family of proteins and sphingolipids, which will be discussed in more detail later. A discussion of different functions affected by ERM phosphorylation is offered below.

4.1. Ezrin in normal physiology

ERM proteins are essential for normal physiologic human body functions. They have been studied in the nervous, digestive, endocrine, and genitourinary system, and they have been studied for their role in cell division.

First, the interaction between ezrin and the actin cytoskeleton is required to maintain a normal cell shape [40]. Phosphorylation of ezrin, or the expression of the phosphomimetic T567D, causes lamellipodia formation along with microvillar projections in LLC-PK1 epithelial cells [41]. Ezrin phosphorylation also produces an elongated cellular morphology and an enrichment of the actin cortical network in HeLa and NIH 3T3 cells [42]. Furthermore, cellular detachment and inhibition of cell adhesion also causes ezrin phosphorylation and induction of microvilli formation in HEK293 cells [43].

Next, ERM protein activation is needed to maintain a healthy reproductive system. Ezrin phosphorylation is needed for sperm capacitation, which is necessary for producing functional sperm [44]. After conception, ERM proteins guide cellular mitosis [45] required for proper blastocyst morphogenesis [46] and germ layer formation during gastrulation [47].

In the nervous system, phosphorylation of ERM proteins is essential for promotion of axonal outgrowth and regeneration. This effect has been related to the activation and recruitment of ERM proteins downstream of neural cell adhesion molecule L1, which creates a highly active cytoskeletal area and a new branch protrusion [48]. ERM have also been reported to

cause this effect through interaction with the deleted-in-colorectal-cancer (DCC) receptor, which subsequently activates a growth cascade [49]. Furthermore, phosphorylation of ERMs has been shown to regulate dopamine transporter activity in the nucleus accumbens [50], and this may be involved in pre-synaptic trafficking [51]. Interestingly, creating an artificial milieu with excessive ERM phosphorylation caused neural growth retardation [52].

In the digestive system, ERM proteins are required for stomach acid secretion through their regulation of acid pump positioning. In fact, expression of the phosphomimetic mutant T567D changes the polarity of gastric parietal cells [53], causing internal cellular membranes to be inserted in the basal vesicles instead of into the apical ones [54]. Cargo proteins such as the H/K-ATPase transporter insert into the basolateral plasma membrane along with long, microvillar projections, effectively halting acid secretion [53]. PKA-mediated ezrin phosphorylation on the serine 66 residue is essential for positioning the acid pump on the apical membrane [55] through its interaction with an adaptor protein—the WW domain-containing oxido-reductase protein [56].

Ezrin activation has been linked to the sodium hydrogen exchanger isoforms 1 and 3 (NHE1, NHE3). In fact, ezrin has been reported to bind to NHE1, and, together, these two proteins form a scaffold for AKT activation and cellular proliferation [57]. This signaling role is essential after cardiovascular ischemia for the maintenance of left ventricular function, prompting its hypertrophy [58]. In addition, overexpression of ezrin in kidney cells increases the surface expression of NHE3, which is important for maintenance of body sodium homeostasis [59]. Moreover, ezrin affects phosphate balance through its interaction with the parathyroid hormone receptor and sodium hydrogen exchanger regulatory factor 1 (NHERF-1) that in turn regulates apical expression of the sodium-phosphate co-transporter 2a (NPT2a) [60].

Finally, ERM proteins are needed for a functional endocrine system. Ezrin phosphorylation is essential for insulin secretion from pancreatic cells. In fact, ezrin activation is necessary for insulin granule trafficking and membrane docking [61]. Ezrin deactivation is observed in diabetic mice [61], and in organs affected by diabetic complications such as the kidney [62]. Actually, advanced glycation end products bind the end terminal of ezrin in the kidney, subsequently inhibiting its activation and thus kidney tubule formation [62].

4.2. Ezrin in immunity and inflammation

Increased ezrin phosphorylation and activation is reported to occur in many inflammatory diseases such a rheumatoid arthritis [63, 64], systemic lupus erythematosus [65], pulmonary fibrosis [66] and traumatic brain injury [67]. Ezrin activation in the immune response is thought to be important for inflammation and immunity, either by influencing cellular movement, cell shape, and/or cell survival, which is discussed below.

4.2.1. Leukocyte recruitment—ERM proteins have been described to be needed for all phases of leukocyte recruitment to sites of inflammation. Capture of free circulating leukocytes is the first step in this process, and this is mediated by p-selectin glycoprotein ligand-1 (PSGL-1). The deletion of the ERM binding sequence in the cytoplasmic tail of PSGL-1 dramatically impairs leukocyte tethering, slow rolling on the activated endothelium, and promotes defective MAPK activation [68]. Furthermore, ERM proteins have been shown to link PSGL-1 to Syk kinase [69]. Generation of a moesin mutant that could not attach to Syk abrogated serum response element (SRE) transcriptional activity that is usually induced upon PSGL-1 stimulation [69]. In contrast, transgenic mice expressing ezrin phosphomimetic protein (T567E) had defective T lymphocyte migration across the endothelium but no disturbances with the slow rolling mechanism mentioned above. Such transgenic mice had decreased lymphocytes within lymph nodes along with increased

cellular adhesion and decreased cellular migration rates [70]. White blood cells (WBC) undergo cytoskeletal polarization once outside the blood vessel for proper migration. In B cells, once phosphorylated, ERM proteins translocate to the front of the cell's lamellipodia to participate in forward movement [71]. In concordance with this activity, phosphorylation of ERM proteins in NK cells facilitates their polarization and their migration towards chemoattractants [72]. In T lymphocytes however, overexpression of the phosphomimetic T567D ezrin was reported to widen the uropod on the posterior aspect of the cell, enhancing its migration ability. ERM proteins are hypothesized to establish the “posteriority” of a cell [73]. Other groups reported that ERM proteins, specifically moesin, redistribute adhesion molecules to the posterior of the migrating lymphocytes to increase ligand accessibility [74]. Certainly, ERM proteins are involved in modulating WBC movement, but the mechanism by which this occurs remains unclear.

4.2.2. Endothelial permeability—ERM proteins have variable effects on endothelial permeability and this is dependent on the activation stimulus. While ERM depletion abolished S1P's protective effect on the endothelium that is usually manifested by increased adherens junctions and decreased endothelial permeability [75] (this point will be explained later), individual depletion of ERM proteins markedly attenuated membrane leakiness caused by 2-methoxyestradiol anti-proliferative treatment [76], TNF-alpha treatment [77] and the accumulation of advanced glycation end products in diabetes [78]. Therefore, the effect of ERM proteins on endothelial integrity may be related to different downstream targets with which they associate upon the application of different stimuli.

4.2.3. Infections—ERM phosphorylation is caused by diverse microorganisms such as chlamydia [79], HIV [80, 81], *Listeria monocytogenes* [82], and *E. coli* [83, 84]. This activation appears to be necessary for infectivity or pathogenesis via different mechanisms. Using a dominant -negative ezrin that cannot be phosphorylated, chlamydial infection was attenuated due to diminished entry into host cells [79]. Also, *Listeria's* ability to spread across cells without activating the immune system depends on induction of cellular protrusions, which are compromised upon ERM depletion. [82]. Enteropathogenic and enterohemorrhagic *E. coli* recruit and activate ezrin at their site of attachment on intestinal epithelial cells [84], disrupting the epithelial tight junctions which is manifested by diarrhea and gastric bleeding [83]. Interestingly, this effect is observed specifically in pathogenic strains of *E. coli*; non-pathogenic strains do not activate ezrin [83]. Finally, HIV's effect on ezrin activation depends on the component of HIV studied. Viral protein R (Vpr) decreases ezrin activity that is normally associated with AKT activation, and thus cell survival [80]. In contrast, the envelope glycoprotein gp120 combined with IL-2, causes a sustained ezrin phosphorylation that is associated with increased cell surface expression of CD95 (also named FAS receptor), which causes increased T lymphocyte susceptibility to apoptosis [81].

4.3. Ezrin in malignancies

4.3.1. ERM expression and phosphorylation status—ERM proteins have been widely implicated in tumor growth and metastasis; they affect cellular modeling and signaling pathways involved in cell survival and proliferation. Ezrin was found to increase migration and metastasis in dog models of osteosarcoma [85]. Ezrin overexpression in myxofibrosarcoma was associated with a higher histological grade of pathology and cancer stage [86]. In fact, ezrin predicted decreased metastasis-free survival [86]. Its overexpression was also correlated with worse outcomes in gastro-intestinal-stromal-tumors (GIST) [87], esophageal cancers [88] and ovarian epithelial carcinomas [89]. Whether ezrin overexpression alone drives a malignant phenotype, or whether over-activation is the culprit, studies show that overexpression of phospho-mimetic ezrin is sufficient to confer metastatic capacity in mouse xenografts of hepatocellular carcinoma [90]. Also, estrogen has been

shown to increase cell migration and invasion of T47-D breast cancer cells by inducing ezrin phosphorylation [91]. This is also true for dinitrosopiperazine (a known carcinogen) which enhances cell motility, invasion, and metastasis of nasopharyngeal carcinoma through ezrin activation [92]. However other studies suggest that for successful metastatic progression, ezrin must undergo a dynamic recycling between a phosphorylated and dephosphorylated state. For example, ezrin is only found to be phosphorylated just after osteosarcoma seeding in the lung parenchyma and then later during metastatic progression [93]. Stable overexpression of either a phosphomimetic ezrin or a non-phosphorylatable form in osteosarcoma blocks primary tumor growth and metastasis [94]. In fact, cells expressing the T567A mutant undergo significant apoptosis once they arrive within the lung. This apoptosis arises from the cells' inability to handle the metabolic stress of a different milieu. Evidence for this is provided by upregulation of genes involved in cellular metabolism and cellular energy [94]. Thus, a new perspective is emerging relating to the importance of ezrin as an essential molecule allowing cell metabolic flexibility in the face of environmental stressors. Another way by which cell manage these stressors is through ezrin dependent activation of the MAPK pathway [95]. Although an abundance of literature implicates ezrin phosphorylation as a driver of tumor progression and metastasis, other data contradict this. High-grade metastatic osteosarcoma was correlated with dephosphorylated cytoplasmic ezrin rather than an active isoform [96]. In addition, phosphatase of regenerating liver-3 (PRL-3) was shown to exert its function by promoting tumor development via ezrin dephosphorylation on thr567 [97]. Also, Raf-1 oncogene transfection into a mouse hepatic cell line deactivated ezrin [98]. Furthermore, knocking down ezrin in human colonic epithelial cells increased their matrix adhesion and their spreading, thus their invasive capabilities [99]. Finally, knocking down ezrin prevents cisplatin-induced apoptosis in human colon cancer cells [100]. In conclusion, further studies are needed to elucidate the exact role of ERM activation on cancer progression as it might vary depending on the cancer grade and stage.

4.3.2. Downstream effectors of ERM—ERM-promotion of cell invasion and metastasis is mediated by several downstream effectors, depending on which residue of ezrin is phosphorylated. Generally, phosphorylation of tyrosine residues activates a signaling cascade that subsequently activates the PI3K/AKT pathway as seen in breast cancer [101], whereas phosphorylation of threonine 567 promotes cell invasion by modulating cell shape (as seen in melanoma cells that form elongated microvilli) [102]. The tyrosine kinase src can phosphorylate ezrin, and thus promote several outcomes. An ezrin mutant that cannot be phosphorylated by this kinase abolishes src-induced anchorage-independent growth and cell invasion [103]. This has been confirmed in another study which described cooperatively between src and ezrin in destabilizing cell-cell contacts and promoting scattering of mammary carcinoma cells [104]. Furthermore, the pro-metastatic effects of androgen on prostate cancer cells occur through permissive effects on anchorage-independent growth also mediated by ezrin [105]. A possible explanation for how ezrin promotes this type of growth is postulated in a study in which shRNA was used against ezrin in breast cancer [106]. E-cadherin expression was dramatically increased upon ezrin knockdown, implicating this adhesion molecule as a downstream effector of ezrin. Matrix metalloproteinases (MMPs) have also been incriminated as an effector arm for ezrin. Podocalyxin, an anti-adhesive transmembrane protein involved in the development of aggressive forms of breast and prostate cancer, leads to increased MMP expression via interaction with ezrin [107]. Similarly, expression of a dominant-negative form of ezrin reduced MMP expression and invasiveness of human malignant glioma cells [108]. Another downstream effector of ezrin is the NF-KB pathway which is required for colorectal cancer metastasis to the liver [109]. Ezrin is essential for ikb phosphorylation in response to engagement of the L1 cell adhesion molecule (L1CAM) [109].

4.3.3. Upstream effectors of ERM—Many pathways have been shown to regulate ezrin expression and/or phosphorylation. The EGF signaling cascade can increase ezrin expression, and thus cellular invasion and metastasis in MDA-231 [110]. Similarly, fos oncogene-mediated malignant transformation of Rat-1 fibroblasts involves an increase in ezrin expression and ezrin hyperphosphorylation [7]. Inhibiting ezrin function in these transformed cells abolishes fos-mediated membrane ruffling and cell motility [7]. Furthermore, GIST harbors a mutation in the KIT oncogene that activates ezrin at different residues to promote tumor progression [111].

5. Ezrin de-activation

5.1. Deactivators

Little is known about ezrin threonine 567 dephosphorylation. As mentioned before, dephosphorylation of threonine 567 inactivates ezrin. Ezrin has a PIP2 binding site on its N-terminus that is required for its association with the plasma membrane. This site as well as other plasma membrane protein binding sites remain hidden by interacting with the c-terminus site. However, even when dephosphorylated, the two states of ezrin are in equilibrium: interacting with the plasma membrane and resting in the cytosol. Inactive ezrin remains either in a monomeric closed conformation or it forms dimers. Inactivation of ezrin is related to loss of cell attachment, loss of plasma membrane protrusions (filopodia, microvilli, etc) and enhancement of cell invasion, and cell migration. Few ezrin inactivators have been described, and these mainly affect PIP2 at the plasma membrane. Thus, when plasma membrane PIP2 is hydrolyzed, ezrin is quickly dephosphorylated. Another more recent mechanism offered to explain ezrin deactivation involves ceramide production at the plasma membrane, a concept that is expanded in the next section.

Ezrin is well-studied in lymphocytes, mainly T cells and NK cells. It plays an important role in lymphocyte polarization, immunological synapses, receptor signaling and T-cell activation [112]. In these cells, ezrin function is tightly regulated by tyrosine phosphorylation which is stimulated by ligation of CD3 and CD4 receptors. General activation of ezrin is also stimulated by the CD43 receptor. Other lymphocyte receptors have been reported to play an important role in ezrin biochemistry, such CD95, CD44, CD81 or I-CAMs. A mechanism of ezrin inactivation in lymphocytes is via ezrin cleavage by calpain [113]. The first described deactivator signal for ezrin was a cytokine, stroma derived factor 1-alpha (SDF-1 alpha, or CXCL12). Within seconds after SDF-1 treatment of human peripheral blood T cells, threonine 567 was dephosphorylated, and microvillie were lost. This effect was blocked by the phosphatase inhibitor calyculin A, although no phosphatase was defined in that study [114]. Next, the small GTPase Rac1 was suggested to mediate the SDF-1α effect on microvilli collapse and ezrin dephosphorylation. Accordingly, a constitutively active Rac1 mutant produced ezrin dephosphorylation, and a dominant-negative mutant blocked cytokine-induced ezrin dephosphorylation [115]. Moreover, the SDF-1α effect was blocked by PLC inhibitors, suggesting that hydrolysis of PIP2 was required for SDF-1 effects; the phosphomimetic mutant of ezrin (T567D) was released to the cytosol after PIP2 hydrolysis [116]. In naïve T cells, both chemokines CXCL12 and CCL21 triggered ezrin dephosphorylation, reducing cell attachment and enhancing cell migration.

In other cell lines, dephosphorylation of the ezrin threonine 567 occurred in response to confluence in endothelial cells[77], and in response to chemotherapeutics in breast cancer cell lines [117].

5.2. Phosphatases

Several phosphatases have been reported to be involved in dephosphorylation of ezrin at Threonine 567, and these include myosin phosphatase (PP1β-MYPT1) [118], PP2A, PP2C, PRL-3 (a tyrosine phosphatase reported to have dual activity) [97] and PP1α [119]. This diversity of phosphatase involvement suggests that regulation of ezrin dephosphorylation depends on the cell type and the nature of the stimulus applied.

Different strategies have been developed to study the functional and phenotypic consequences of ezrin dephosphorylation . The most common tool for this is expression of a phospho-incompetent mutant, in which threonine 567 has been substituted with an alanine residue. Knockdown of ezrin is another approach. Recently, two small molecule inhibitors have been reported to induce ezrin dephosphorylation, as well as inhibit cell migration and invasion of osteosarcoma cells [120].

6. Ezrin and sphingolipids

6.1. Ceramide

The first study to describe a connection between ezrin status and sphingolipids was from our group in 2008. We noted that cisplatin caused loss of plasma membrane protrusions in MCF-7 tumor breast cancer cells, which was accompanied by loss of ezrin association to the plasma membrane, ezrin dephosphorylation, and co-localization of ezrin with cortical actin. These cytoskeletal rearrangements coincided with the loss of sphingomyelin and the generation of ceramide. In fact, cisplatin activated the acid sphingomyelinase in a PKC delta-dependent mechanism, causing its translocation to the plasma membrane. Exogenous ceramide, or recombinant bacterial sphingomyelinase from *Bacillus cereus* (bSMase) treatment for 1 h produced the same phenotype as cisplatin treatment, suggesting that ceramide chiefly triggered ezrin inactivation. This study also involved a ceramide activated protein phosphatase (CAPP) as a downstream effector of ceramide that is required for ezrin dephosphorylation [117]. In a follow up study, HeLa cells were shown to undergo ezrin dephosphorylation after bSMase treatment, and this effect was more dramatic. As early as 2 min after treatment (25 mU bSMase), complete ERM dephosphorylation occurred [121]. To elucidate which sphingolipid was required for ezrin dephosphorylation, the sphingolipid pathway from ceramide was dissected using a combination of several recombinant enzymes and SK inhibitors. Thus, treatment with bSMase dephosphorylated ezrin in a dose-dependent manner, localizing ezrin to the cytoplasm. Purified recombinant *Loxosceles* sphingomyelinase D, which generates ceramide 1-phosphate (C1P) instead of ceramide, proved that ceramide, and not decreased sphingomyelin or C1P production, was required for ezrin deactivation [121]. Interestingly, regulation of ezrin dephosphorylation in HeLa cells after ceramide generation proved to be the result of another CAPP phosphatase; pretreatment with okadaic acid, and knockdown of PP2A did not block bSMase effects. In HeLa cells, the only serine/threonine phosphatase responsible for bSMase-induced ezrin dephosphorylation was PP1α [119]. This result was verified using pharmacologic inhibitors, siRNA technology, and PP1 catalytically inactive mutants [119]. Interestingly, bSMase treatment neither altered PIP2 levels nor localization as shown with PLCd-PH domain constructs, which specifically bind PIP2. Those data suggested that ceramide-induced ezrin dephosphorylation did not involve PIP2 loss. In contrast, PIP2 hydrolysis caused ezrin dephosphorylation in a PP1α-independent manner. Activation of PP1α by ceramide seemed necessary for ezrin dephosphorylation, and did not contribute to regulating plasma membrane attachment. This was confirmed by expressing the N-terminal fragment of ezrin, which could bind PIP2 but lacked the C-terminal region containing threonine 567, which could not be dephosphorylated in response to ceramide generation. This “ceramide-PP1α“ axis also suggested a novel mechanism for ERM protein regulation: it not only did

not involve PIP2, but it was independent of PP1β-MYPT1, which has been shown to regulate basal levels of ERM phosphorylation [119].

6.2. S1P

Recent experiments indicate that generation of high levels of S1P with the use of purified bacterial recombinant ceramidase (bCDase) after bSMase treatment was accompanied by ezrin hyperphosphorylation. In fact, we reported that S1P production and decreased ceramide was responsible for this dramatic effect. Thus, ceramide and S1P play opposing roles in ezrin regulation [121]. Subsequent studies by our group showed that exogenously added or endogenously produced S1P resulted in a time- and dose-dependent increase in ERM phosphorylation as early as 30 seconds after treatment. S1P induction of ezrin phosphorylation seemed to be a universal effect; it was observed in several cell lines including MDA, MCF7, A549, HEK, and MEF [122]. Dose-response experiments reveal that nanomolar concentrations could trigger the maximum S1P response on ezrin phosphorylation, suggesting a receptor-mediated effect. Indeed, it was shown that this phosphorylation required the activation of S1PR2 exclusively on HeLa cell surfaces, and blocking all other receptors maintained ezrin activation [122]. This was shown using pharmacologic inhibitors, and also siRNA and genetic knockout approaches. S1P-induced ezrin phosphorylation was required for filopodia formation; using dominant-negative nonphosphorylatable ezrin mutant abolished this S1P-mediated effect [122]. The fact that S1P mediated ezrin phosphorylation inhibited cell invasion is contradictory to what is currently known of this bioactive sphingolipid, probably because S1P works on difference receptors. How this system is coordinated is unclear. The significance of this pathway was further explored by Djanybek and colleagues in human pulmonary endothelial cells (EC) in studies of EC barrier function [75]. S1P protected EC barrier integrity by remodeling the actin cytoskeleton, increasing focal adhesion, and adherent junction formation. In this work, S1P was confirmed to activate ezrin and moesin at nanomolar concentrations within a few minutes of treatment. With pharmacological inhibitors, multiple PKC isoforms were shown to be responsible for ezrin and moesin (but not radixin) phosphorylation in response to S1P (in addition to P38 MAPK and Rac1). However, direct interactions with ERMs or other approaches involving these protein kinases were not proposed [75]. Radixin was not phosphorylated after S1P stimulation, but it was shown to be required for S1P's effect on ezrin and moesin activation [75]. A summary of sphingolipids modulation of ERM activity is illustrated in **figure 2**.

7. Conclusion

This review describes novel mechanisms by which different bioactive sphingolipids can differentially modulate the cell cytoskeleton and cell behavior. They can mediate acute changes via activating or deactivating ERM proteins. On one end, ceramide causes acute ERM de-activation by recruiting protein phosphatase 1 alpha; and at the other end, S1P causes acute and potent ERM activation by a yet un-identified kinase that is under investigation in our laboratory.

Until now, the scientific literature reviewed herein described experiments that used sphingolipids exogenously applied to the cell. Although this approach is limited, it does offer discovery of the basic regulation of this novel pathway. Recently, using a known activator of the endogenous SK/S1P pathway, epidermal growth factor (EGF), we found that EGF induced ERM phosphorylation in an SK/S1P-dependent manner. Furthermore, the extracellular S1P generated upon EGF treatment acted on the same previously described receptor (S1PR2) to mediate this activation. This has been confirmed with siRNA technology as well as pharmacologic inhibitors. Interestingly, EGF induced lamellipodia

formation and such invasive behavior was partly dependent on ERM activation through the SK/S1P/S1PR2 pathway [123]. This discovery will open new avenues for novel cancer treatments; EGF-dependent cancers can now be targeted from different angles. Because SK, S1PR2, and ezrin inhibitors are commercially available, they can be tested for cancer prevention or for slowing the progression of established cancers and metastases.

Table 1 lists ERM activators with well-defined biologies. Possibly, these stimuli act through the SK/S1P pathway to mediate this activation and the corresponding biology. A number of these functions have already been described to be sphingolipid-related. These data warrant additional investigations.

Abbreviations

UV	Ultraviolet light
S1P	sphingosine-1-phosphate
SPT	Serine Palmitoyl Transferase
CerS	Ceramide synthase
SK	Sphingosine Kinase
PKH	Phosphoinositide-dependent protein kinase (PDK) Kinase Homolog
SNARE	Soluble NSF attachment protein receptor
S1PR	S1P receptor
PKA	Protein Kinase A
NHE	Sodium Hydrogen Exchanger
NHERF	Sodium Hydrogen Exchanger Regulatory Factor
NPT2a	Sodium Phosphate co-transporter 2a
PSGL-1	P-Selectin Glycoprotein Ligand-1
MAPK	Mitogen Activated Protein Kinase
SRE	Serum Response Element
WBC	White blood cells
NK	Natural Killer Cells
TNF	Tumor Necrosis Factor
HIV	Human Immunodeficiency Virus
GIST	Gastro-Intestinal Stromal Tumors
PI3K	Phospho-Inositide-3-Kinase
MMP	Matrix Metallo-Proteinase
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells
IκB	inhibitor of NF-κB
L1CAM	L1 Cell adhesion molecule
PRL-3	Phosphatase of Regenerating Liver-3
PIP2	Phosphatidylinositol 4,5-bisphosphate
CD	Cluster of Differentiation

SDF-1	Stroma Derived Factor 1 alpha
PP	Protein Phosphatase
bSMase	Bacterial Sphingomyelinase
CAPP	Ceramide Activated Protein Phosphatase
C1P	Ceramide-1-Phosphate
PLC	Phospholipase C
MYPT	Myosin Phosphatase Targeting Protein
bCDase	Bacterial Ceramidase
EC	Endothelial Cells
PKC	Protein Kinase C
EGF	Epithelial Growth Factor

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Highlights

- Sphingolipids are major regulators of the ERM family of Proteins
- ERM family of proteins play essential roles in modulating the cell cytoskeleton
- While ceramide is a major de-activator of these proteins, S1P is a potent inducer
- This regulation reveals a new understanding of many sphingolipid-mediated functions

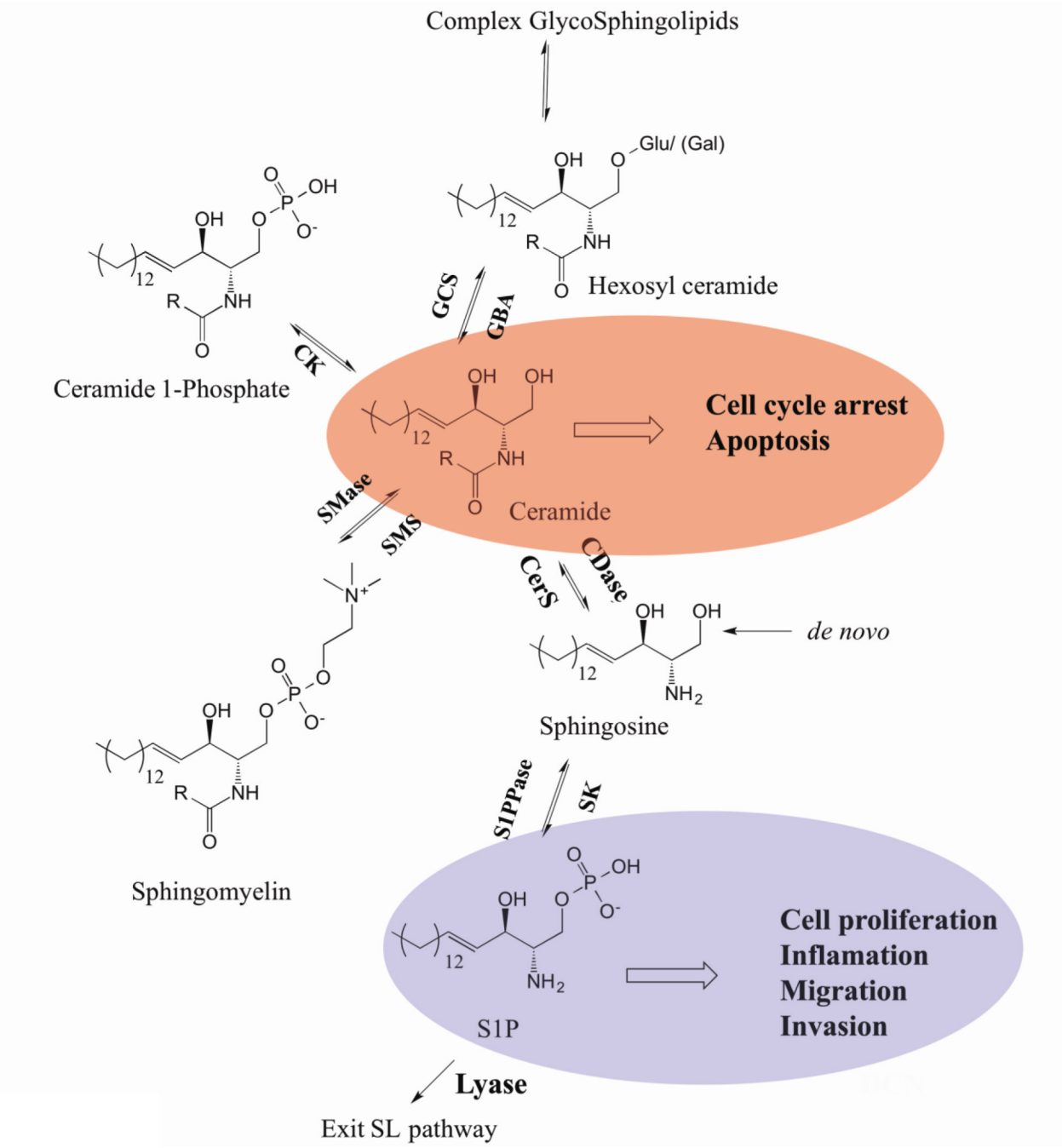

Figure 1. Sphingolipid metabolic pathway

This figure illustrates the sphingolipid metabolic pathway described in the text along with the structure of each product. It emphasizes the opposing roles played by two major bioactive products of sphingolipid metabolism, ceramide and sphingosine-1-phosphate. While ceramide is known to play a role in cell cycle arrest and apoptosis, S1P is known to be involved in promoting cell proliferation, inflammation, migration, and invasion. Glu (Glucose), Gal (Galactose), GCS (Glucosylceramide Synthase), GBA (Glucocerebrosidase), CK (ceramide kinase), SMS (sphingomyelin synthase), SMase (sphingomyelinase), CDase (Ceramidase), CerS (Ceramide Synthase), SK (sphingosine kinase), S1PPase (S1P Phosphatase).

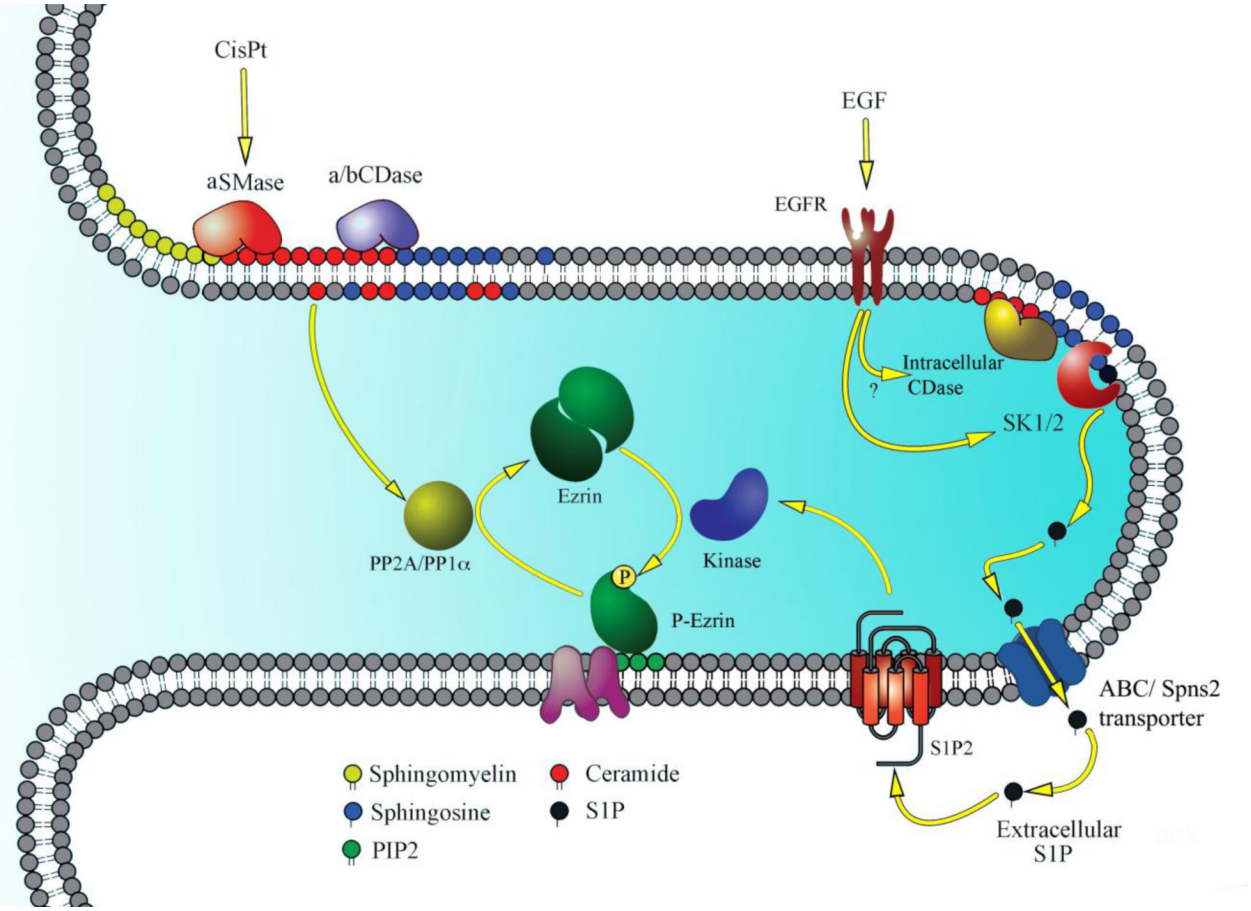

Figure 2. Sphingolipid regulation of ERM activation

This figure illustrates the mechanism of ERM regulation by sphingolipids on the tip of a cellular extension. Cisplatin causes activation of the acid sphingomyelinase (aSMase), which causes ceramide production. Ceramide activates protein phosphatase 2A (PP2A) to cause ezrin dephosphorylation and detachment from the cell membrane. Alternatively, the use of bacterial sphingomyelinase causes activation of protein phosphatase 1 alpha (PP1a) to cause ezrin dephosphorylation. In contrast, S1P, generated after bacterial ceramidase use (bCDase) or after EGF treatment translocates to the extracellular space using one of the described transporters such as the ABC transporter to activate sphingosine-1-phosphate receptor 2 (S1P2). S1P2 signaling activates a yet unknown kinase to cause ezrin phosphorylation and binding to the plasma membrane using phosphatidylinositol biphosphate (PIP2).

Table 1

The kinases known to phosphorylate ERM proteins and their functional consequences. (N.B. 1- The ERM column refers to the specific ERM member that is phosphorylated by the corresponding kinase, 2- the numbering of the phosphorylation sites reflects their position in the original ERM sequence; this might slightly differ in the original manuscripts as some authors use their tagged constructs for numbering).

P-site	Kinase	ERM	Stimulus	Cell line	Function	Ref
Ezrin^{Thr567}, Radixin^{Thr564}, Moesin^{Thr558}	**PKC**	ERM	Amphetamine	PC12 cells	Synaptic plasticity	[50]
		E	Dinitrosopiperazin	NPC cells	Motility, invasion, metastasis	[92]
		E	N/A	Oteoscarcoma cells	Migration and invasion	[85, 93]
		ERM	2-methoxyestradiol	HPAEC cells	Hyperpermeability	[76]
		ERM	Glutamate	Hippocampal cells	Filopodia and synaptic boutons formation	[51]
		E	Latent membrane protein-1 expression	NPC cells	Motility and invasion	[6]
		E	N/A	Intestinal epithelial cells	Epithelial differentiation	[124]
		M	Hypotonicity	Collecting duct epithelial cells	Cytoskeletal remodeling	[125]
		E	Androgen	LNCaP-FGC	Invasion	[126]
		E	Parathyroid Hormone	Oposum Kidney cells	Endocytosis	[127]
		E	N/A	2C4 Fibrosarcoma	Migration	[128]
		ERM	Netrin	Cortical Neurones	Axonal growth	[49]
		E	Estrogen	T47-D Breast cancer cells	Migration and invasion	[91]
		ERM	LPA	Ovarian Cancer	Migration and metastasis	[129]
		E	N/A	Hepatoma cells	Invasion	[90]
	Rho/ROCK	E	Cisplatin	HT29 cells	Apoptosis	[100]
		E	N/A	NPC cells	Metastasis	[130]
		EM	Fas receptor activation	Jurkat T cells	Apoptosis	[131]
		E	TNF	Endothelial cells	Inhibition of proliferation	[132]
		E	Net and Dbl overexpression	NIH3T3 fibroblasts	Malignant transformation	[133]
	P38 MAPK	ERM	TNF	Synoviocytes	Proliferation	[63]
		M	Advanced glycation end products	Endothelial cells	Hyperpermeability	[78]
		R	Wound closure	MDCK cells	Migration	[134]
	GRK-2	ERM	Angiotensin-II	Kidney Podocytes	Actin turnover and migration	[135]
		E	Acetylcholine	Hep2 and HEK293 cells	Membrane ruffles formation and internalization	[136]
	NIK	ERM	EGF, PDGF	MTLn3 cells	Lamellipodia formation	[137]

P-site	Kinase	ERM	Stimulus	Cell line	Function	Ref
	AKT2	E	Glucose	Caco-2BBe cells	Translocation of NHE-3 to plasma membrane	[138]
	LRRK2	ERM	N/A	Primary Neurons	Retardation of growth and increased filopodia	[52]
	Mst-4	E	N/A	W4 cell line	Apical brush border formation	[139]
	LOK	ERM	N/A	Lymphocytes	Decreased migration and polarization	[140]
	Slik	M	Mitosis initiation	Drosophila S2 cells	Cortical microtubular stability	[45]
	MRCK	ERM	Cdc42 overexpression	NIH3T3 fibroblasts	Filopodia Formation	[141]
Tyr146 and/or 354 and/or 478	**Syk**	E	CD81 engagement	B lymphocytes	Cell reorganization and signaling	[142]
	Flt3	E	Kit overexpression	Proerythroblasts	Apoptosis resistance and proliferation	[143]
	Src	E	EGF	A431 cells	Spreading and proliferation	[144]
		E	ICAM1 ligation	Endothelial cells	N/A	[145]
		E	N/A	Hek239	Association with KBTBD2	[146]
	Lck	E	N/A	Jurkat T cells	Signaling during T cell activation	[147]
Thr235	**Cdk5**	E	Overexpression of Retinoblastoma	Osteosarcoma	Cell senescence and flat cell morphology	[148-150]
Ser66	**PKA**	E	Histamine	Gastric Parietal cells	Acid secretion	[55, 56]