Chapter 8

MIG-6 and SPRY2 in the Regulation of Receptor Tyrosine Kinase Signaling: Balancing Act via Negative Feedback Loops

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1. Introduction

Tumor suppressor genes (TSGs) function in concert with diverse parts of the cellular machinery and integrate the signaling networks in a cell [1]. TSGs act to safeguard the networks, to fine-tune signaling outputs, and to maintain tissue homeostasis. The loss of tumor suppressor activity or inactivation of a TSG is often due to genetic alterations such as a loss-of-function mutation or a deletion in the gene; alternatively, epigenetic silencing can result from methylation or histone modification in the TSG's promoter regulatory elements [1-3]. Cells with a loss or a significant reduction of a particular tumor suppressor's activity are prone to develop neoplasia in the tissues/organs where the TSG is expressed [1, 2].

The properties and modes of action of TSGs can be very distinct from one class to another. Most TSGs encode proteins that participate in controlling cell cycle progression, inducing apoptosis, repairing damaged DNAs, or performing other important functions [1]. TSGs can also be a source of microRNAs, a class of small hairpin RNAs that are transcribed in many cells and may act as tumor suppressors by regulating the expression of their targeted genes [4]. In this chapter, we will focus on one class of the TSGs, represented by the mitogen-inducible gene 6 (MIG-6) and Sprouty 2 (SPRY2), whose activities are crucial in regulating receptor tyrosine kinases signaling through negative feedback loops.

Receptor tyrosine kinases (RTKs) are important cellular components, and there are nearly 60 members encoded in the genome [5, 6]. They all possess a single transmembrane domain linking their extracellular ligand-binding region to the cytoplasmic region in which the catalytic kinase domain and the domain for docking of downstream signaling molecules reside. Upon binding of the ligand to its physiologic RTK partner, the receptors dimerize, resulting

in autophosphorylation of key tyrosine residues in the kinase domain. This leads to a conformational change in the receptor and the recruitment of downstream signaling molecules to its docking domain or in close proximity for phosphorylation and activation. In this cellular process, the RTK plays a central role by relaying external stimuli (ligands) to internal signaling cascades such as the RAS-MAPK or PI3K-AKT pathways, translating the signal input into biological actions ranging from mitogenesis, to motility, morphogenesis, metabolism, and many others [5, 6].

RTK signaling is essential in many developmental processes and in normal physiology, and the actions of RTKs must be controlled temporally and spatially [5, 6]. Their actions are tightly regulated at several molecular levels to ensure appropriate cellular responses. Among the mechanisms that keep RTK signaling in "check and balance" are receptor endocytosis/degradation, dephosphorylation by protein tyrosine phosphatases (PTPs), and negative feedback regulation. Signal overactivity caused by inappropriate RTK activation can lead to serious pathological outcomes, particularly cancer. Thus, many RTKs such as epidermal growth factor receptor (EGFR) and the N-methyl-N'-nitroso-guanidine human osteosarcoma (MNNG HOS) transforming gene (MET) have been classified as oncogenes, because activating mutations, amplifications or other anomalies in these receptors have been identified in various human cancers, and their roles in the development and progression of tumor malignancy have been well documented [7, 8]. For example, aberrant activation of MET can result in deregulated cell proliferation, transformation, and promotion of tumor cell invasion and metastasis [8].

Unlike the rapid attenuation resulting from receptor endocytosis/degradation or PTP-mediated dephosphorylation, negative feedback regulation of RTK signaling by MIG-6 and SPRY2 is a delayed event because it requires *de novo* mRNA and protein syntheses. The expression of both MIG-6 and SPRY2 can be induced by ligands of many RTKs including epidermal growth factor (EGF), hepatocyte growth factor (HGF), and fibroblast growth factor (FGF) [9, 10]. In turn, MIG-6 and SPRY2 exerts their inhibitory activities on RTK signaling by either directly affecting the receptor itself or by modulating the signaling molecules downstream of the RTK. In this review, we will highlight the current understanding of how MIG-6 and SPRY2 regulates RTK signaling via negative feedback loops, and shed lights on why the loss of their tumor suppressor activities may affect RTK signaling in cancer cells, as well as their impact on cancer therapy.

2. The features and functions of MIG-6 and SPRY2

2.1. MIG-6

MIG-6 (also known as *gene 33*, *ERRFI1* or *RALT*) is a unique and immediate early response gene that is not present in relatively simple organisms like *Drosophila* and *C. elegans*. It emerges in the more complex, higher-order species [9, 11], underlying its importance in evolution. It encodes a 58 kDa nonkinase protein that resides in the cytoplasm and functions as a scaffolding adaptor for modulating signal transduction. Struc-

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turally, MIG-6 has several functional motifs/domains that are crucial for interaction with other signaling molecules [9, 12]. The Cdc42/Rac-interaction and binding (CRIB) domain of MIG-6 (Figure 1A) shares consensus sequences with many other proteins that associate with Cdc42 or Rac small GTPases, which are important regulators of actin cytoskeleton remodeling and signal transduction [11, 13]. CRIB domain mediates the binding of MIG-6 to active (GTP-bound) Cdc42 and negatively regulates HGF-induced Cdc42 activation and cell migration [12, 14]. This domain has also been shown to play a role in regulating transactivation of nuclear factor κB (NFκB) by sequestering the inhibitor of κBα (IκBα) [15, 16]. Within its middle region, MIG-6 has several proline-rich motifs that likely mediate its binding to various Src homology-3 (SH3] domain-containing proteins such as GRB2, Src, PI3K p85 and PLC-γ [14, 17]. MIG-6 interacts with 14-3-3ζ via the 14-3-3 protein binding motif [12]. MIG-6 also possesses two PEST sequences, and is targeted by ubiquitination and proteasome degradation [18]. The ErbB-binding region (EBR), a large portion of the carboxyl terminus in MIG-6, is required for physical interaction with EGFR family receptors, which resulted in attenuation of EGFR/ErbBs signaling [17, 19, 20]. The EBR domain shares a high homology with the activated Cdc42-associated kinase 1 (ACK1), a non-receptor tyrosine kinase that also interacts with and regulates EGFR [21].

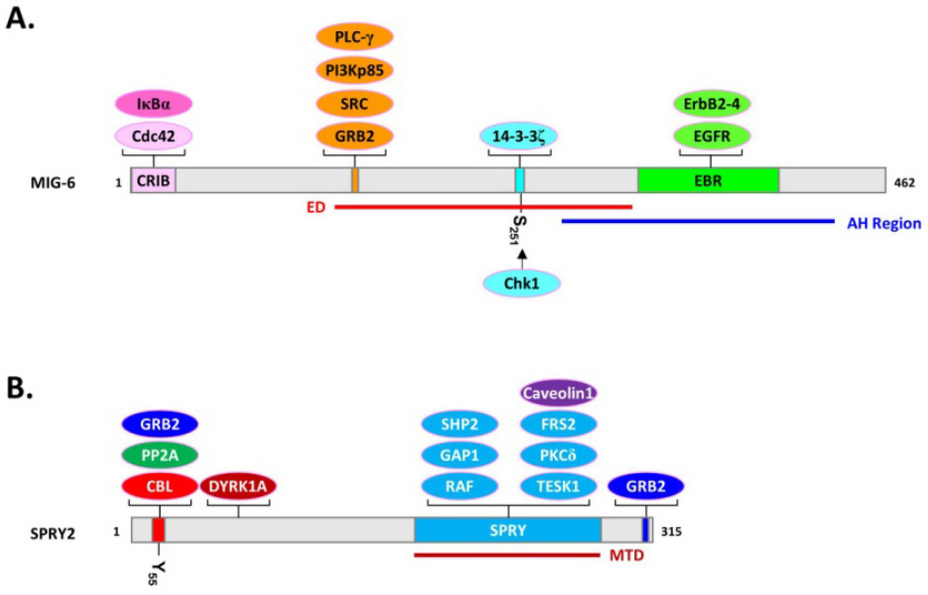

Figure 1. MIG-6 and SPRY2 structures. A. MIG-6 protein structural features and its interacting partners. The CRIB domain (amino acids 1-38) interacts with Cdc42 and IκBα. The orange box indicates the SH3-domain binding motif that likely mediates interactions with SH3-domain-containing proteins such as GRB2 and PI3Kp85. The cyan box (amino acids 246-253) marks the 14-3-3 binding motif in which serine residue 251 can be phosphorylated by Chk1 kinase. The EBR domain (amino acids 337-412) binds to EGFR and other ErbB members. The red bar (ED) indicates the MIG-6 endocytic domain (amino acids 143-323); the blue bar indicates the Ack homology (AH) region (amino acids 264-424). B. Structural features of SPRY2 and its partner molecules. The red box shows the SH2-domain binding motif (amino acids 50-60) that binds to CBL and GRB2; the key tyrosine residue Y_{55} is also indicated. The conserved cysteine-rich SPRY domain (amino acids 178-293) is crucial for its ability to interact with signaling molecules such as FRS2 and SHP2. The SPRY domain is also responsible for membrane translocation (MTD). There is an SH3-domain binding motif (amino acids 303-309) shown in blue at the C-terminal end of SPRY2 that also binds to GRB2.

MIG-6 is expressed in many tissues/organs, with high expression in liver and kidney and low to moderate expression in brain, lung, heart, and other tissues [22, 23]. Its expression can be induced by diverse factors ranging from hormones and growth factors, to chemical agents, to stress stimuli [9]. The induction of MIG-6 expression by growth factors is mainly mediated by the RAS-MEK-MAPK/ERK pathway, while other inducers may involve other pathways such as PI3K [9]. MIG-6 has also been reported to be a G-actin-regulated target gene, because the actin-MAL-serum response factor (SRF) cascade mediates MIG-6 induction by serum or lipid agonists such as lysophosphatidic acid (LPA) or sphingosine 1-phosphate (S1P) [24]. MIG-6 may play a crucial role in patho-physiological conditions such as myocardial ischemic injury and infarction [25], liver regeneration [26-28], joint mechanical injury [29], and diabetic nephropathy and hypertension [12]. Its activity is required for skin morphogenesis [30, 31] and lung development in mice [32], and it plays an important role in the maintenance of tissue homeostasis in joints, the lungs and the uterus [32-35].

2.2. SPRY2

In term of evolution, the *Spry* gene emerged far earlier than *Mig-6*. *SPRY2* is the mammalian homolog of *Drosophila melanogaster Spry* (*dSpry*), and is one of the four *SPRY* genes in the human genome [10]. The dSpry protein is 63 kDa, while its mammalian counterparts are 32–34 kDa, but they all contain a functional cysteine-rich region in their C-terminus (designated the SPRY domain) and an SH2-binding motif carrying a conserved tyrosine at the N-terminus (Tyr55 residue in human SPRY2) (Figure 1B). These conserved regions are essential for SPRY2 to fully execute its inhibitory function in the regulation of RTK signaling [10, 36, 37]. The SPRY domain is also found in the SPRED (Sprouty-related EVH1 domain-containing protein) family, which like SPRY proteins inhibits RTK signaling upon stimulation by growth factors [10, 38]. The SPRY domain mediates the binding of SPRY2 to many signaling molecules including GAP1, FRS2, SHP2, RAF, PKCδ, TESK1 and caveolin1 [10, 39]. The SPRY domain is also required for translocation of SPRY2 to the membrane during its activation. The Tyr55 residue is essential for the SPRY2 protein's interaction with CBL, PP2A and GRB2 [10, 39]. A cryptic SH3-binding motif (PxxPxR) in the C-terminal end of SPRY2 (but not present in other SPRY members) has been shown to mediate GRB2 interaction as well [39-41]. The dual-specificity tyrosine phosphorylation–regulated kinase (DYRK1A) interacts with and phosphorylates Thr75 on SPRY2 [42]. SPRY2 is targeted for ubiquitination and proteasome degradation by at least two ubiquitin E3 ligases: CBL-mediated Tyr55 phosphorylation-dependent ubiquitination and SIAH2-mediated Tyr55 phosphorylation-independent ubiquitination [43, 44]. On the other hand, SPRY2 protein can be stabilized by phosphorylation of its serines 112 and 121 residues by the mitogen-activated protein kinase-interacting kinase 1 (Mnk1), thereby decreasing growth factor–induced degradation [45].

In *Drosophila*, *dSpry* expression is detected at the tips of branching lung buds and is induced by branchless, the *Drosophila* Fgf. Losing *dSpry* leads to excessive tracheal branching as a result of increased Fgf signaling activity [46]. The *Xenopus* homolog, *xSpry2*, is expressed in a pattern resembling that of *Xenopus* Fgf8 and inhibits Fgf-mediated gastrulation [47]. During mammalian embryogenesis, Spry2 expression tends to localize closest to the sites where Fgf

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activity is needed for organ/tissue development, underlying the importance of this molecule as an intrinsic Fgf signaling regulator in organogenesis [48-50]. Mouse *Spry2* is highly expressed in the terminal buds of peripheral mesenchyme in the embryonic lung, adjacent to that of Fgf10, a key mouse lung-branching morphogen [50]. Ectopic expression of Spry2 in the mouse pulmonary epithelium results in decreased branching; exogenous Fgf10 produces greater lung branching and higher Spry2 expression [50]. Spry2 also plays a role in mouse kidney development; its ectopic expression in the ureteric bud leads to postnatal kidney failure due to deficiency in ureteric branching [51]. Moreover, Spry2 deficiency in mice results in defects of the auditory sensory epithelium development in the inner ear, and leads to enteric neuronal hyperplasia and esophageal achalasia [52, 53]. The lack of phenotypes in other Spry2-deficient tissues is likely due to compensatory roles of other Spry family members, because there are overlapping expressions of Spry1, 2 and 4 in many tissues during the development [54]. In adult mice, Spry2 expression is abundant in the brain, lung, heart, kidney, skeletal muscle and mammary glands [48, 55].

Beyond being an intrinsic inhibitor for Fgf signaling, Spry also regulates signaling driven by other RTKs like Egfr. The *dSpry* gene is required for eye and wing development in *Drosophila*, antagonizing Egfr signaling for neuronal induction in the retina and for vein formation in the wings [56, 57]. Loss of *dSpry* results in excess photoreceptors, cone cells and pigment cells in the retina, while its overexpression leads to phenotypes that mimic loss of Egfr signaling [56, 57].

3. Negative feedback regulation of RTK signaling by MIG-6 and SPRY2

3.1. Regulation of RTK pathways by MIG-6

Many growth factors can induce MIG-6 expression, including EGF, FGF, and HGF [9]. Upon induction, MIG-6 proteins rapidly and transiently accumulate in the cytoplasm, where they feed back to inhibit the activated RTK signaling (Figure 2]. The inhibition of EGFR/ErbB signaling by MIG-6 can occur at two molecular levels: one on the receptor itself, and another on the signaling molecules downstream of the receptor (Figure 2). Through its C-terminal EBR domain, MIG-6 directly binds to EGFR and other ErbB members [17, 19, 20, 58]. The interaction involves the kinase domain of EGFR or ErbB2 and requires their catalytic activities, but does not involve their C-terminal regions in which there are tyrosine residues essential for activating downstream signaling [20, 58]. Crystal structures reveal that the MIG-6 EBR domain binds to the distal surface of the carboxy-terminal lobe (C-lobe) in the kinase domain of EGFR [59]. The C-lobe is crucial in asymmetric EGFR dimer formation [60]; binding of MIG-6 to the C-lobe blocks the dimer interface thereby preventing EGFR activation [59]. MIG-6 coupling also promotes clathrin-mediated endocytosis of EGFR [61], an important mechanism for timely attenuation of ligand-induced EGFR activation [62, 63]. The region responsible for promoting EGFR endocytosis has been mapped to the endocytic domain (ED) of MIG-6 (see Figure 1A) [61], which mediates the binding of MIG-6 to the AP2 adaptor complex, a key component in forming clathrin-coated pits during endocytosis [63]. Interest-

ingly, the non-receptor tyrosine kinase ACK1 also binds to EGFR upon EGF stimulation through a region sharing high homology with the MIG-6 EBR domain [21]. This interaction also regulates clathrin-mediated EGFR endocytosis and degradation [21, 64]. However, it is not clear whether MIG-6 and ACK1 cooperate in regulating EGFR turnover or whether they bind to EGFR in a mutually exclusive way to accomplish individual inhibitory roles under different circumstances. The internalized EGFR is guided to late endosome through the binding of MIG-6 to the endosomal SNARE complex component STX8, en route to degradation in the lysosome [61, 65].

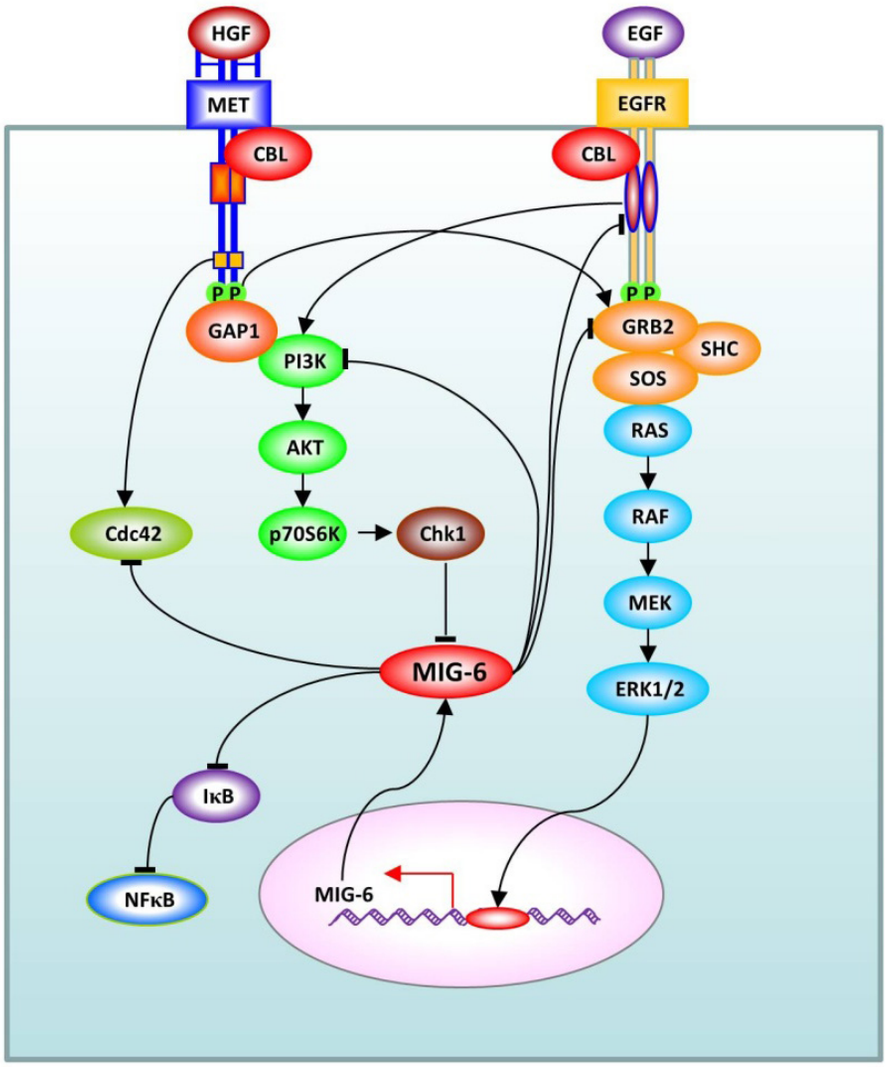

Figure 2. MIG-6 regulates EGFR and MET signaling via a negative feedback loop. Upon ligand stimulation, EGFR and MET activate the RAS-RAF-MEK-ERK pathway and induce *MIG-6* expression. In turn, MIG-6 exerts its inhibitory activity by interacting with signaling molecules to fine-tune RTK signaling and its timely attenuation. The direct MIG-6–EGFR interaction facilitates receptor endocytosis and degradation. This inhibitory activity is unique to the EGFR family and does not extend to other RTKs like MET. The interaction of MIG-6 with signaling molecules such as GRB2 and PI3Kp85 indistinguishably influences the RTK signaling in general, resulting in the inhibition of downstream pathways like RAS-MAPK/ERK and PI3K-AKT.

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The magnitude of MIG-6-mediated inhibition of EGFR signaling is likely maximized and integrated by the second level of molecular regulation, that is, direct inhibition of downstream signaling molecules (Figure 2). MIG-6 binds to the SH3 domain-containing protein GRB2 [14, 17], the key molecule in linking activated RTK to the intracellular signaling cascade; GRB2 brings RAS together with SOS to the phosphorylated receptor for activation [66]. Binding of MIG-6 may block GRB2's interaction with activated EGFR and other RTKs or disrupt GRB2-SOS-RAS complex formation, thereby preventing activation of the RAS-MEK-MAPK pathway. MIG-6 also interacts with Src, the PI3K p85 subunit, PLCγ, and Fyn [17], although those interactions were demonstrated in an artificial system and their biological meaning remains to be determined. More complexity is likely in the dynamics of MIG-6-mediated RTK signaling regulation due to the interaction of MIG-6 with 14-3-3 proteins [12, 67], an adaptor family that may interact with diverse signaling molecules and regulate many biological activities [68]. Nonetheless, most of these direct downstream signaling regulations by MIG-6 remain largely speculation and require further investigation.

Assessing the effect of MIG-6 on RTK pathways other than those of the EGFR family, however, may provide insightful answers to such speculation, because direct regulation of the RTK itself by MIG-6 appears to be unique to the EGFR family. For instance, MIG-6 can be induced by HGF and function as negative feedback regulator of the MET pathway by inhibiting HGF-induced cell migration and proliferation [14], yet no physical interaction between MIG-6 and MET has been observed. Through its CRIB domain, MIG-6 can bind to and inhibit the activity of the Cdc42 small GTPase [12, 14], and this inhibitory activity is required for blocking of HGF-induced cell migration [14]. The CRIB domain also interacts with IκBα, thereby activating NFκB for transcriptional regulation of its target gene expression [15, 16]. Whether the inhibition of HGF-induced cell proliferation by MIG-6 is mediated by its ability to bind GRB2 or to bind other downstream molecules is still unknown. Negative feedback inhibition of other RTKs (such as FGFR and IGFR) by MIG-6 is also likely to be mediated by its inhibitory activities on the downstream signaling molecules rather than on the receptor itself.

The inhibitory activity of MIG-6 on RTK signaling seems to be modulated by phosphorylation; Liu et al. recently reported that MIG-6 can be phosphorylated by Chk1 upon EGF stimulation [67]. EGF activates Chk1 via the PI3K-AKT-S6K pathway, which in turn phosphorylates Ser251 of MIG-6 and results in inhibition of MIG-6 [67]. Thus, Chk1 counterbalances the EGFR inhibition of MIG-6, positively regulating EGFR signaling. Interestingly, Ser251 resides in the 14-3-3 binding motif of MIG-6 and its phosphorylation is likely involved in the MIG-6 and 14-3-3ζ interaction, because that interaction is abolished by Chk1 depletion [67]. In addition, two tyrosine residues (Tyr394 and Tyr458) in MIG-6 are phosphorylated by EGFR activation [69-71], but the underlying mechanism and the biological significance remain unclear.

3.2. Regulation of RTK pathways by SPRY2

SPRY2 renders another layer of modulation on RTK signaling via a negative feedback loop [10, 36, 37, 72]; its expression is induced by many activated RTKs including EGFR, FGFR,

MET, and VEGFR [10, 72]. As with MIG-6, SPRY2-mediated regulation can occur on two levels: on the RTK itself and on the downstream signaling molecules (Figure 3). However unlike MIG-6, the most prominent inhibitory activity of SPRY2 is derived from its abilities to interact with downstream signaling molecules centering on the RAS-RAF-MAPK pathway, while its effect on the RTK itself seems to be indirect and may provide some signaling specificity for different RTKs [10, 36, 37].

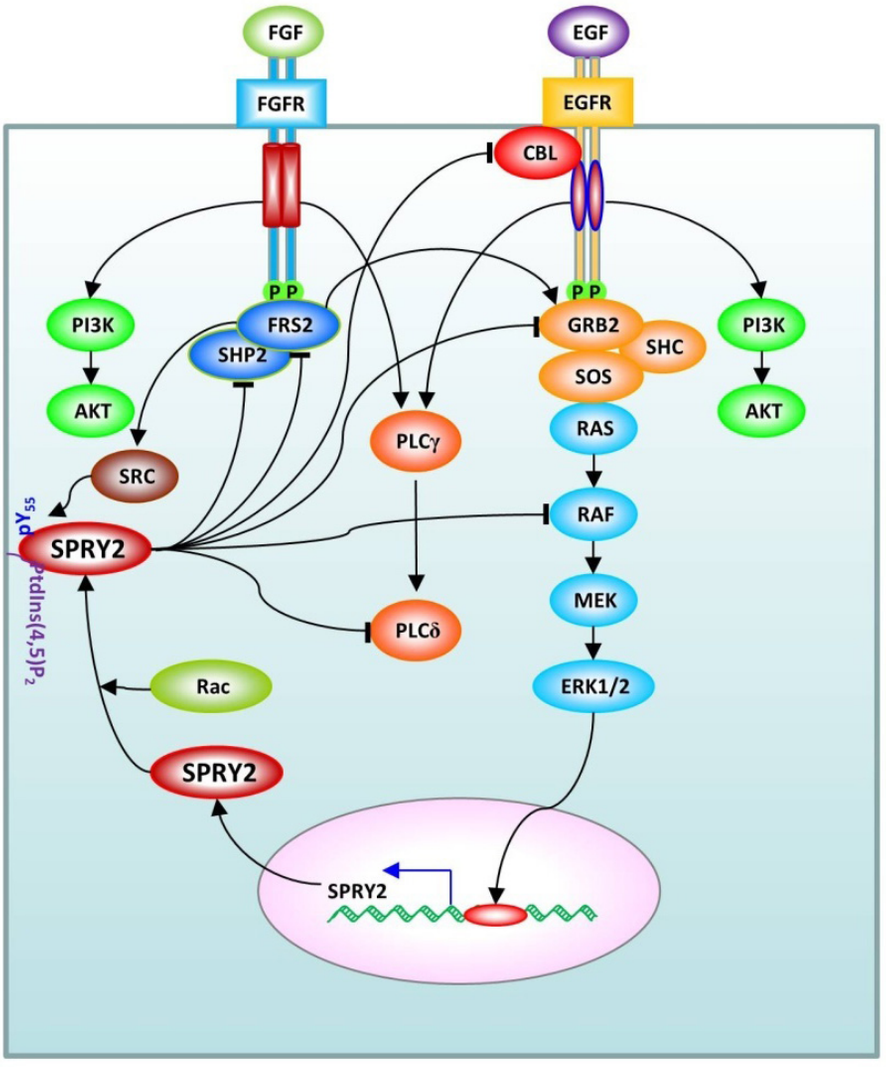

Figure 3. Feedback regulation of EGFR and FGFR signaling by SPRY2. SPRY2, upon induction, translocates to the membrane by binding to PtdIns(4,5)P_2, and is phosphorylated on its Y_{55} residue. This modification is essential for SPRY2's inhibitory activity, which is mediated by interaction with many signaling proteins including FRS2, SHP2, GRB2, RAF and PLCδ. On the other hand, its interaction with CBL E3 ubiquitin ligase may positively regulate EGFR signaling, because such interaction sequesters CBL and prevents CBL-mediated EGFR degradation.

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Upon growth factor stimulation, SPRY2 translocates from the cytosol to the plasma membrane where it binds to phosphatidylinositol-4,5-bisphosphate [PtdIns(4,5)P2] via the conserved SPRY domain [73-75]. Membrane translocation appears to be triggered by the activated Rac small GTPase and is essential for SPRY2 function [76]. The phosphorylation of the Tyr55 residue in SPRY2 is likely regulated by a SRC family kinase, which upon FGF stimulation is recruited to activated FGFR by FRS2 [77]. While Tyr55 phosphorylation enables the binding of SPRY2 to GRB2 via the SH2-binding motif in the N-terminus [78], the SH3-binding motif in its C-terminus may also play a role in the SPRY2-GRB2 interaction [39-41] (Figure 1B). The latter is likely regulated by the protein phosphatase 2A (PP2A), a serine/threonine phosphatase that interacts with Tyr55 and dephosphorylates certain serine residues in SPRY2 for permitting access of GRB2 to the SH3-binding motif on SPRY2 [41, 45]. Consequently, the binding of SPRY2 to GRB2 prevents the recruitment of the GRB2-SOS complex to the FRS2 adaptor or SHP2 phosphatase proximal to the activated RTK, thereby inhibiting the activation of RAS and its downstream molecules [10, 36, 39, 77, 78]. The RTK-RAS-RAF-MAPK/ERK pathway can also be inhibited by direct binding of SPRY2 to RAF [75, 79, 80]. On the other hand, TESK1 negatively regulates SPRY2 inhibitory activity by interfering with the SPRY2 interaction with GRB2 and PP2A [81], while DYRK1A binding results in Thr75 phosphorylation on SPRY2 and suppression of SPRY2 inhibitory activity, thereby enhancing FGF-induced ERK activation [42].

The regulation of EGFR signaling by SPRY2 appears to be more complicated than that of FGFR signaling, because SPRY2 can indirectly influence the turnover of EGFR through its interaction with CBL (Figure 3) [10, 36, 37]. This regulation is mediated by the SH2-binding motif, which includes Tyr55 in the N-terminus of SPRY2. When Tyr55 is phosphorylated, SPRY2 interacts with the SH2-domain on CBL, and prevents CBL from binding to the activated EGFR, thereby interfering with CBL-mediated EGFR endocytosis and degradation [43, 82-84]. This action can prolong the activation of EGFR and the downstream RAS-RAF-MAPK pathway, contrary to the direct inhibitory activity of SPRY2 on the downstream signaling molecules. These two opposite activities render SPRY2 a delicate role in fine-tuning EGFR signaling, negative in some situations and positive in others, depending on the threshold and balance of these two activities.

It is conceivable that RTKs such as MET and PDGFR that are also substrates of CBL might also be affected by SPRY2 like that of EGFR [8, 85], while non-CBL-substrate RTKs like FGFR appears unaffected by SPRY2-CBL interaction [36]. However, it is known that MET protein level is not affected by SPRY2 overexpression that inhibits HGF-induced ERK and AKT activation, indicating that the effect of SPRY2-CBL interaction on EGFR and on MET might not be the same [72, 86]. The sequestration of CBL by SPRY2 can also affect downstream signaling molecules, as it may free proteins like GRB2 from CBL-mediated ubiquitination and degradation [85]. Furthermore, SPRY2 itself can be ubiquitinylated and degraded by the CBL binding, thereby influencing the RTK signaling [83].

4. Tumor suppressor role of MIG-6 and SPRY2 in cancer

4.1. MIG-6 as a tumor suppressor gene

Human chromosome 1p36, a locus frequently associated with many human cancers [87-92], harbors the *MIG-6* gene. In fact, loss or reduction of *MIG-6* expression has been observed in non-small cell lung cancer (NSCLC) [35, 93-95], breast carcinoma [30, 96], melanoma and skin cancer [30, 94], ovarian carcinoma [30], pancreatic cancer [30], endometrial cancer [34], thyroid cancer [97, 98], hepatocellular carcinoma [28], and glioblastoma [91]. Prognostically, low *MIG-6* expression is often associated with poor prognosis or poor patient survival [93, 96, 97].

Unlike many other tumor suppressor genes whose expression is directly regulated by epigenetic modification of their promoter regulatory elements [3], the silencing of *MIG-6* expression seems otherwise [94]. Although high in CpG contents, the *MIG-6* promoter appears hypomethylated and is not directly affected by either the DNA methyltransferase inhibitor 5-aza-2′-deoxycytidine [5-aza-dC) or the histone deacetylase inhibitor trichostatin A (TSA), indicating indirect regulation [94]. Interestingly, *MIG-6* expression seems to be differently induced by 5-aza-dC and TSA in different cancer types: it is induced by 5-aza-dC in melanoma but not in NSCLC and neuroblastoma, and by TSA in NSCLC and neuroblastoma but not in melanoma, suggesting a possible tissue-specific transcriptional regulation for this gene [90, 94, 96]. However in some papillary thyroid cancer, it is reported that the *MIG-6* promoter is hypermethylated as determined by methylation-specific PCR [98]. It is unclear at this point what cause such differences.

Besides loss or reduction of expression, *MIG-6* can also be inactivated by genetic mutation, even though this occurs rarely [35, 90, 96]. To date, two homozygous mutations (Asp109 to Asn, and Glu83 to a stop codon) in *MIG-6* were identified in human lung cancer cell lines, while heterozygous germline mutations were found in a primary lung cancer (Ala373 to Val) and a neuroblastoma patient (Asn343 to Ser) [35, 90]. Evidence that *MIG-6* is a bona fide tumor suppressor gene also arises from mouse studies. Mice with targeted disruption of *Mig-6* are prone to neoplastic development ranging from epithelial hyperplasia to carcinoma at multiple sites including the lung, gallbladder, bile duct, uterus, gastrointestinal tract and skin [30, 34, 35]. The carcinogen-induced skin cancer seen in Mig-6-deficient mice is likely mediated by EGFR-ERK/MAPK pathway, because inhibiting EGFR kinase activity with gefitinib or replacing it with a kinase-defective EGFR rescues the phenotype [30].

4.2. SPRY2 as a tumor suppressor gene

There is compelling evidence supporting SPRY2 as a tumor suppressor [99]. The *SPRY2* gene is located on human chromosome 13q31, where loss of heterozygosity (LOH) is observed in prostate cancer and hepatocellular carcinoma [86, 100]. Down-regulation of *SPRY2* expression has been reported in breast cancer [55, 101], hepatocellular carcinoma [86, 102, 103], NSCLC [104], prostate cancer [100, 105, 106], endometrial carcinoma [107], gliomas [108], and B-cell lymphomas [109, 110]. However in colon cancer, both downregulation and

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upregulation of *SPRY2* have been reported [111, 112]. Low (or no) *SPRY2* expression is associated with advanced tumor stages and poor survival, and it may be a significant prognostic factor in breast cancer [101], hepatocellular carcinoma [86, 103], prostate cancer [100, 105], gliomas [108], and colon cancer [111]. Further, *SPRY2* expression is inversely correlated with the level of miR-21 microRNA expression in gliomas and colon cancer, indicating that *SPRY2* is a target of miR-21[108, 111].

Downregulation of SPRY2 expression may be attributed to DNA methylation; hypermethylation of its promoter has been observed in prostate cancer [100], hepatocellular carcinoma [86], endometrial carcinoma [107] and B-cell lymphomas [109, 110]. However, controversial results have also been reported, since no methylation in *SPRY2* promoter was found in other studies involving different cancer types [55, 102, 106, 111], indicating that other epigenetic mechanisms might as well be responsible for *SPRY2* down-regulation. Thus far, no mutation has been identified in the *SPRY2* gene in any human cancers, and no neoplastic phenotypes have been observed in any Spry2-deficient mice. This may be due to compensatory roles played by other family members such as SPRY1 or SPRY4.

5. The impacts of MIG-6 or SPRY2 activity on RTK signaling in cancer

The loss of MIG-6 and SPRY2 feedback regulation leads to prolonged RTK signaling activation and may contribute to hallmark activities of cancer [113]. Ectopic expression of MIG-6 results in decreased phosphorylation of EGFR/ErbBs and downstream ERK/MAPK and AKT, and it inhibits cell proliferation in several cancer types [19, 20, 65, 96, 114]. In contrast, down-regulation of *MIG-6* expression by small interference RNA (siRNA)-mediated knockdown leads to prolonged activation of the EGFR or ErbB2 pathway and increases ligand-induced proliferation, cell cycle progression, and cell migration [28, 30, 65, 96, 114]. Likewise, *MIG-6* overexpression inhibits HGF-induced cell migration and proliferation, whereas MIG-6 knockdown by siRNA enhances those activities [14]. Intriguingly, it has been shown that in thyroid cancer, *MIG-6* overexpression suppresses MET phosphorylation along with the inhibition of EGFR, ErbB2, and SRC, while its knockdown does the opposite and enhances cell proliferation and invasion [98]. However, it is unclear how MIG-6 affects MET tyrosine phosphorylation given that no physical interaction between the two is observed [14]. The activity of MIG-6 on apoptosis is unsettled: one group showed that MIG-6 inhibits apoptosis of breast cancer cells [115], while another showed that it promotes the death of cardiomyocytes [25]. This discrepancy might be due to the differences in cellular states (cancer cells versus normal cells) or in tissue types (breast versus cardiac).

Nonetheless, a study of NCI-60 cell lines, which cover a broad spectrum of cancer types, revealed that *MIG-6* expression correlated with EGFR expression, indicating an intrinsic activity for MIG-6 in regulating EGFR signaling [116]. *MIG-6* expression has been shown to have a significant effect on ErbB-targeted cancer therapy [96, 116, 117]. MIG-6 synergizes with the EGFR inhibitor gefitinib to suppress the growth of NSCLC cells carrying gefitinib-sensitive EGFR mutations [116, 117]. Further, the loss of *MIG-6* expression renders ErbB2-amplified

breast cancer cells more resistant to Herceptin (trastuzumab), a neutralizing antibody against ErbB2/HER2 [96]. It will be interesting to see the influence of *MIG-6* expression on cancer therapies targeting other RTKs such as MET as well.

SPRY2 overexpression inhibits MET-mediated ERK and AKT activation in leiomyosarcoma and hepatocellular carcinoma cells, and it suppresses cell proliferation, migration and invasion induced by HGF [72, 86, 102]. In NSCLC, SPRY2 suppresses ERK but not AKT activation, and it inhibits the migration of the cells expressing wild-type but not constitutively activated K-RAS; however, proliferation and tumorigenesis of cells with either wild-type or mutant K-RAS can be inhibited by *SPRY2* overexpression [104]. SPRY2 has different effects on wild-type and V599E mutant B-RAF in melanoma cells: its downregulation enhances ERK phosphorylation only in melanoma cells carrying wild-type B-RAF, likely because of its ability to interact with wild-type, but not mutant, B-RAF [118]. Overexpression of *SPRY2* suppresses ERK activation in osteosarcoma and B-cell lymphoma, and it inhibits tumor growth and metastasis *in vivo* [109, 119], while suppression of SPRY2 activity by its dominant negative mutant $SPRY2^{Y55F}$ enhances the proliferation and tumorigenesis of breast cancer cells [55]. Surprisingly, SPRY2 has also been reported to enhance cell proliferation and HGF-induced ERK and AKT activation, migration and invasion of colon cancer cells [112], quite opposite to another report showing that in the same cell line, SPRY2 negatively regulates ERK and AKT phosphorylation and inhibits proliferation, migration and tumorigenesis [111]. The discrepancy is quite puzzling, and it is unclear whether it is due to differences in the experimental approaches or to other factors such as clonal effects originating from tumor cell heterogeneity. Using an inducible system in the same cell line might be able to solve the puzzle of whether the effects in those two reports were truly the results of *SPRY2* overexpression.

There is limited evidence that *SPRY2* expression, like that of *MIG-6*, influences ErbB-targeted therapy in human cancers [101, 120]. Breast cancer patients with low *SPRY2* expression show poorer response to trastuzumab treatment, and have a significant lower survival rate relative to those with high *SPRY2* expression [101]. Low *SPRY2* expression is usually associated with high ErbB2/HER2 in breast cancer, while reconstituting SPRY2 may enhance the sensitivity of breast cancer cells *in vitro* to trastuzumab treatment [101]. In colon cancer cells, low *SPRY2* expression is associated with less sensitivity to gefitinib, whereas its ectopic overexpression can enhance gefitinib responsiveness [120].

6. Conclusion and perspective

The negative feedback loops to receptor tyrosine kinases of MIG-6 and of SPRY2 provide crucial intersecting points for tumor suppressor genes and oncogenes, placing them in the same signaling networks for regulating physiologic and oncogenic activity. This sophisticated regulatory mechanism allows timely attenuation of RTK signaling by those TSGs, and ensures a proper cellular response following growth factor stimulation. MIG-6 and SPRY2 are no more than the representatives for the class of TSGs involved in RTK

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signaling regulation, and we believe there are more such TSGs in the human genome either remain to be discovered or have already been revealed (such as other SPRY family members). To date, most of the studies on MIG-6 and SPRY2 have focused on their activity in regulating selected RTKs such as EGFR, MET and FGFR. Their roles in regulating most other RTKs and the clinical relevance of such regulation remain largely unknown. Also, there are conflicting results on how MIG-6 and SPRY2 may regulate the RTK signaling, on both the receptor and the downstream signaling levels, and further studies are required to address those issues. Although EGFR and other RTKs like MET appear to be regulated slightly differently by MIG-6 and SPRY2, it remains to be determined to what extent these TSGs may provide signaling specificity to different RTKs. Beyond all aforementioned issues, a broader question might be why an RTK network needs multiple negative feedback regulators to fine-tune its signaling; might the regulators function differently for each RTK in a temporal and spatial manner i.e. at right place, on right time and for right target?

While conventional mechanisms such as mutation or promoter methylation may contribute to the inactivation of MIG-6 or SPRY2 tumor suppressor roles, their activities are also likely silenced by unconventional means in cancer. For example, in most cancer types investigated so far, *MIG-6* expression is not down-regulated by direct methylation or histone deacetylation in its promoter, but rather by an indirect mechanism involving other unidentified transcriptional factor(s) or transcriptional co-regulator(s). It is also striking to see that different promoter methylation status in *MIG-6* or *SPRY2* gene is observed in different cancer types: methylated in some cancers, but unmethylated in others. The cause of such difference is a curiosity, but if only for TSG down-modulation, genome instability in the cancer cell environment could provide many mechanisms.

Clinically, it is important to understand how tumor suppressor genes may affect the therapeutic outcome of RTK-targeted therapies, which can be effective in treating certain human cancers. In a limited number of studies, low expression of *MIG-6* or *SPRY2* is associated with poorer patient responses to ErbB-targeted therapies (i.e., the EGFR inhibitor gefitinib and the ErbB2/HER2 inhibitor Herceptin). The question is, can *MIG-6* or *SPRY2* expression be used in conjunction with RTK status to select patients for RTK-targeted "personalized" cancer therapy? The approach sounds plausible, given that those TSGs negatively regulate the RTK signaling activities, but extensive studies will certainly be required before implementing such measures.

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