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Sprouty proteins, masterminds of receptor tyrosine kinase signaling

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Abstract Angiogenesis relies on endothelial cells properly processing signals from growth factors provided in both an autocrine and a paracrine manner. These mitogens bind to their cognate receptor tyrosine kinases (RTKs) on the cell surface, thereby activating a myriad of complex intracellular signaling pathways whose outputs include cell growth, migration, and morphogenesis. Understanding how these cascades are precisely controlled will provide insight into physiological and pathological angiogenesis. The Sprouty (Spry) family of proteins is a highly conserved group of negative feedback loop modulators of growth factor-mediated mitogen-activated protein kinase (MAPK) activation originally described in *Drosophila*. There are four mammalian orthologs (Spry1-4) whose modulation of RTK-induced signaling pathways is growth factor – and cell context – dependant. Endothelial cells are a group of highly differentiated cell types necessary for defining the mammalian vasculature. These cells respond to a plethora of growth factors and express all four Spry isoforms, thus highlighting the complexity that is required to form and maintain vessels in mammals. This review describes Spry functions in the context of endothelial biology and angiogenesis, and provides an update on Spry-interacting proteins and Spry mechanisms of action.

Keywords EGF · Endothelial cell · ERK · FGF · Growth factors · MAPK · Receptor tyrosine kinase · Sprouty · Spred · VEGF

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Abbreviations

Ang	Angiopoietin
c-Cbl	Cellular homologue of Casitas B-lineage lymphoma proto-oncogene product
EGF	Epidermal growth factor
EGFR	EGF receptor
eNOS	Endothelial nitric oxide synthase
ERK	Extracellular signal-regulated kinase
FGF	Fibroblast growth factor
FGFR	FGF receptor
GDNF	Glial-derived neurotrophic factor
Grb2	Growth factor receptor-bound protein 2
HMVEC	Human microvascular endothelial cell
Hrs	Hepatocyte growth factor-regulated tyrosine kinase substrate
HUVEC	Human umbilical vein endothelial cell
MAPK	Mitogen-activated protein kinase
MEK	MAPK and ERK kinase
Mnk1	Mitogen-activated protein kinase-interacting kinase 1
PDGF	Platelet-derived growth factor
PKC	Protein kinase C
PLC	Phospholipase C
PP2A	Protein phosphatase 2A
RBD	Raf1-binding domain
RTK	Receptor tyrosine kinase
Shp2	SH2-domain-containing protein tyrosine phosphatase 2
SIAH2	Seven-in-Absentia Homolog 2
SMC	Smooth muscle cell
Sos1	Son of Sevenless 1
Spry	Sprouty
Spred	Spry-related proteins with Enabled/vasodilator-stimulated phosphoprotein homology 1 domain
SPR	Spry-related domain

VEGF Vascular endothelial growth factor
VEGFR VEGF receptor

Introduction

Receptor tyrosine kinases (RTKs) have been at the forefront of cancer research for many years due to their pivotal role in cancer biology as prognostic indicators and therapeutic targets [1, 2]. Thus, the elucidation of RTK signaling cascades and their regulatory networks has been an important objective of many laboratories, especially since angiogenesis and the angiogenic switch necessitate activation of various growth factor receptors [3–5]. The formation of the vasculature by the endothelium and supporting stroma via branching morphogenesis is analogous to what is observed in the development of tubular networks found in other tissues (e.g. lung, kidney, mammary gland, and placenta) [6, 7]. Growth factors, such as the vascular endothelial growth factors (VEGFs), fibroblast growth factors (FGFs), epidermal growth factor (EGF), ephrins, and angiopoietins (Angs), in conjunction with their cognate receptors play a crucial role in blood vessel formation in normal and pathological settings (reviewed in [8, 9]). The majority of RTKs, serving as gatekeepers for these extracellular growth factors, among several signaling pathways, activate the extracellular signal-regulated kinase (ERK)/mitogen-activated protein kinase (MAPK) signaling cascade composed of Raf1, MAPK and ERK kinase (MEK), and MAPK (Fig. 1a). These pathways are precisely controlled spatio-temporally by the activity of various modulatory proteins in a cell context-dependant manner [10–12].

In vertebrates, such modulators of RTK signaling include the members of the Sprouty (Spry) family of proteins, comprising various Spry and Spred (Spry-related proteins with

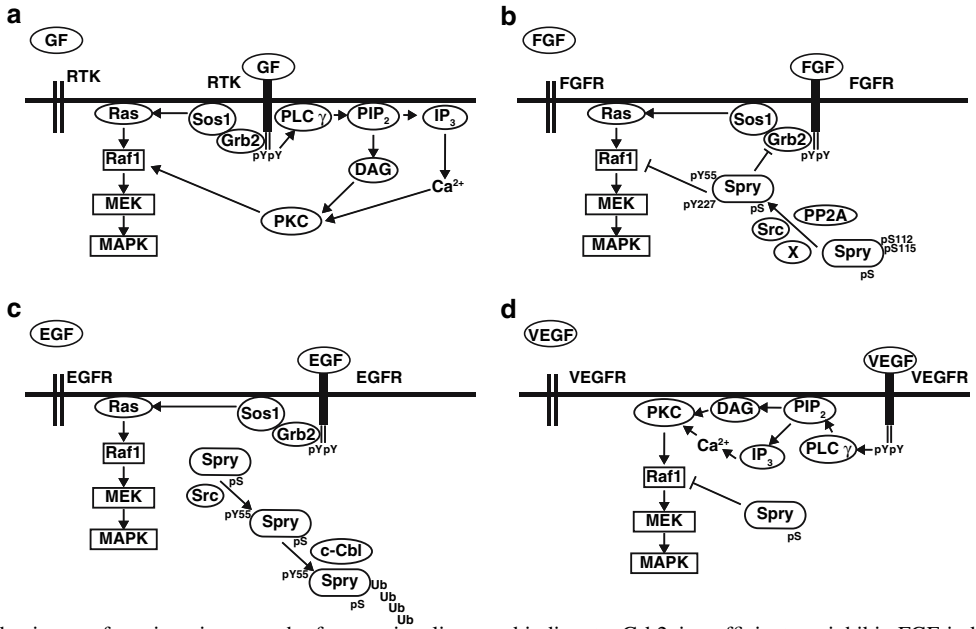

Fig. 1 Spry mechanisms of action in growth factor signaling pathways. (**a**) Canonical signal transduction in the absence of Spry. Upon ligand binding, RTKs can activate MAPK in a Ras-dependent manner via the adapter protein Grb2 and Sos1-mediated activation of Ras, Raf1, MEK, and eventually MAPK. Another conserved RTK pathway that can be activated is that mediated by PLCγ, which converts phosphatidylinositol [4, 5]-bisphosphate (PIP_2) into two second messengers: inositol [1, 4, 5] trisphosphate (IP_3) and diacylglycerol (DAG), leading to calcium (Ca^{2+}) mobilization. Both DAG and Ca^{2+} activate PKC, which in turn phosphorylates Raf1, thus leading to MAPK activation. (**b**) Spry-mediated inhibition of the FGF signaling pathway. Spry is serine phosphorylated on multiple residues in unstimulated cells and at least serines 112 and 115 are dephosphorylated by PP2A upon FGF stimulation [23, 57]. Also, Spry phosphorylation at Y55 by a Src-like kinase and by an unknown kinase (X) at Y227 is required for Spry to inhibit FGF-induced activation of MAPK [24, 48, 52, 56]. Under some conditions, Spry binding to Grb2 is sufficient to inhibit FGF-induced activation of MAPK, while in others Grb2 association is not required [42, 55]. Also, Spry binding to Sos1 and potentially to Raf1 inhibits FGF-stimulated MAPK activity [18, 43]. (**c**) Spry-mediated potentiation of the EGF signaling pathway. Upon EGF binding, Spry is tyrosine phosphorylated and competes with EGFR for binding to c-Cbl. By interacting with c-Cbl, Spry becomes ubiquitinylated and eventually degraded, allowing for sustained EGFR signaling [44–46, 61, 80]. (**d**) Spry-mediated inhibition of the VEGF signaling pathway. The VEGFR signals via the PLCγ/PKC pathway [66]. Upon VEGF activation, Spry interacts with Raf1 via its RBD and inhibits activation of MAPK. Tyrosine phosphorylation of Spry is not required for this activity [67]. pY and pS denote phosphorylated tyrosine and serine residues, respectively. In this schematic, using Spry2 as the representative Spry isoform, pY55 and pY227 represent the N- and C- terminal phospho-tyrosines

Enabled/vasodilator-stimulated phosphoprotein homology 1 domain) isoforms. Depending on the cell type and physiological conditions, Sprys and Spreds modulate RTK signaling (and sometimes also signaling by G-protein-coupled receptors) by mainly repressing the MAPK pathway (see below). The founding member of the family, *Drosophila* Spry (dSpry), was initially described as a negative feedback loop inhibitor of FGF-mediated branching of the *Drosophila* trachea [13]. There are four mammalian dSpry homologs, Spry1-4 (reviewed in [14–18]) and four Spred proteins, Spred1-3 and EVE-3 (a splice variant of Spred-3) (reviewed in [19]) that all contain the highly conserved cysteine-rich Sprouty-related (SPR) domain. The expression of Spry1, 2, and 4 is widespread in embryos and adults, while that of Spry3 is believed to be more restricted (brain and testes in adult tissues) [20, 21]. Like dSpry, the expression of mammalian Spry proteins is induced by the growth factor cascades that they target [22–24]. Upon growth factor activation, a subset of Spry proteins translocate to the cell membrane where MAPK inhibition presumably occurs [23, 25, 26]. Spry proteins can modulate MAPK activation induced by several stimuli, including EGF, FGF, VEGF, platelet-derived growth factor (PDGF), nerve growth factor, hepatocyte growth factor, insulin, stem cell factor/kit ligand, glial-derived growth factor (GDNF), brain-derived neurotrophic factor, and T-cell receptor activation [17, 18, 22, 24, 27–30]. This review will discuss Spry protein function in the context of endothelial cells and provide an update on their mechanism of action, Spry-interacting partners, and regulation of Spry stability and activity.

Spry expression

Endothelial cells can be cultured in vitro to form capillary-like structures and various molecular approaches have been employed to identify genes required for tube/capillary formation. For example, using subtractive cDNA library hybridization, proliferating human microvascular endothelial cells (HMVECs) were compared to those that differentiate and form tube-like structures on Matrigel [31]. These studies identified several transcripts, including Spry2 mRNA, which were differentially enriched in tube-forming HMVECs versus proliferating HMVECs. Also, in an early DNA microarray study, Spry1 was found highly upregulated in human umbilical vein endothelial cells (HUVECs) during capillary morphogenesis in a three-dimensional collagen matrix [32]. The increase in Spry1 mRNA abundance coincided with a decrease in FGF2 mRNA. Another mRNA following a pattern similar to that of Spry1 was Ang2, an antagonistic ligand of the Tie2 receptor, and one of the most upregulated transcripts detected in this experiment [33]. Similarly, Spry1, along with Ang2 and other mRNAs, was found enriched in microvascular endothelial cells, suggesting a specific role for Spry1 in capillary tube formation [34]. More recently, a comprehensive expression analysis of FGFs, FGF receptors (FGFRs), and various FGF signaling inhibitors was performed in primary human endothelial and vascular smooth muscle cells (SMCs) [35]. This study demonstrated that HUVECs and SMCs express a variety of FGFs (FGF1, 2, 5, 7, 8, 11, 12, 16, 18), FGFRs (FGFR1c, 2c, 3c) and FGF signaling antagonists including all four Spry isoforms. Furthermore, in spheroid co-cultures consisting of HUVECs and SMCs, the repertoire of these various components of the FGF signaling cascade did not significantly change, suggesting that both cell types behaved as an entity rather than as two distinct cell populations. Thus, endothelial cells express all four isoforms of Spry, underlining the need for specialization, yet maintaining redundancy in these highly differentiated cells.

More recently, additional insights into the regulation of Spry expression in endothelial cells have been reported [36]. The highly conserved FoxO genes, FoxO1, FoxO3a, and FoxO4, are expressed in endothelial cells, and null mice for FoxO1 and FoxO3 display vascular phenotypes (reviewed in [37]). Moreover, mice deficient for all three FoxO genes exhibited tissue-specific hemangiomas resulting in premature death [36]. Spry2 was identified by microarray analysis as an important FoxO target gene in affected endothelial cells. The Spry2 promoter contained FoxO-binding elements, and Spry2 expression correlated with FoxO deletion in a tissue-specific manner. Furthermore, in liver endothelial cells, Spry expression was lost upon FoxO deletion, an effect not observed in lung endothelial cells. Phenocopying Spry2 loss by RNA interference technology confirmed that endothelial cells lacking Spry2 were more proliferative and less susceptible to apoptosis. Apparently, both the Fox and the Ets transcription factor families are required for proper angiogenesis, and for endothelial cell function and differentiation [37]. Also, the human Spry2 gene promoter has been shown to harbor a functional binding element for Ets-1, suggesting that it is a target of the Ets transcription factor family [38].

Spry functions

In initial experiments to characterize the function of Spry1 and Spry2, HUVECs were employed to examine their role in VEGF, FGF, and EGF signaling [23]. Forced expression of Spry1 and Spry2 in endothelial cells inhibited VEGF-, FGF-, and EGF-stimulated growth and sprouting. Interestingly, these Spry isoforms only repressed FGF- and VEGF-induced phosphorylation of MAPK and not that stimulated by EGF, demonstrating that various RTK cascades were differentially affected by Spry proteins. In another study,

Spry4 function was examined in endothelial cells using a combination of in vitro and in vivo experiments [39]. Endothelial cell proliferation, migration, and MAPK activation induced by either FGF2 or VEGF were inhibited by Spry4 expression, and the Spry4-expressing cells arrested in the G_1/G_2 phase of the cell cycle. Notably, Spry4 inhibition could be overcome by a constitutively active form of Ras, suggesting that Spry4 exerted its functions upstream of Ras. Moreover, the cell cycle inhibitor p21 was induced and phosphorylation of the cell cycle control protein Rb was decreased, which correlated with the presence of Spry4 in these cells. Lastly, gene transfer-mediated expression of Spry4 in the vascular endothelium of the mouse embryo resulted in marked repression of sprouting and branch formation of the yolk sac vasculature [39]. Thus, Spry proteins are important regulators of RTK function (i.e. growth and differentiation) in endothelial cells and inhibit angiogenesis in in vitro and in vivo model systems.

Spry mechanisms of action

The exact mode of action for Spry proteins has been difficult to ascertain, as noted by early reports examining the functional role of the various Spry isoforms in different growth factor pathways in diverse cell types and species [15–18, 40]. Vertebrate Spry proteins are now viewed as general modulators of growth factor signaling, and for most RTK signaling cascades, Spry proteins repress MAPK activation. For FGF signaling, all mammalian Spry proteins inhibit FGF-induced MAPK activation to varying degrees [41–43]. However, in the case of EGF stimulation, Spry2 can potentiate MAPK activation in some cell types [44–46]. This context-specific dual function of Spry has also been illustrated for T-cell receptor signaling where Spry1 acts either as an inhibitor or as an enhancer depending on the differentiation state of the T-cell [30]. In short, Spry modulation of RTK signaling is very pliable and highly cell- and context-dependent.

All Spry isoforms possess a highly conserved N-terminal tyrosine residue, which in the case of Spry1 and Spry2 is phosphorylated upon EGF and FGF signaling and in the case of Spry4 is phosphorylated upon FGF and insulin signaling [24, 42, 47, 48]. Mutation of this tyrosine generates a dominant-negative Spry unable to inhibit FGF-stimulated MAPK activation [24, 48, 49]. The Src family of kinases is likely responsible for phosphorylation of Spry at this conserved tyrosine, while Shp2 phosphatase is involved in dephosphorylating this critical residue [48, 50–52].

FGF signaling

The FGF/FGFR signaling axis is important for endothelial growth, migration, and morphogenesis and is critical for angiogenesis in both normal and disease states [53]. To elucidate the Spry mechanism of action, most studies have focused on Spry2, the most highly conserved isoform across vertebrates. Initially, both Spry1 and Spry2 have been proposed to bind Grb2 via the N-terminal conserved tyrosine residue, thus interrupting the signaling cascade before MAPK can be activated [22, 43, 45, 49, 54] (Fig. 1b). The role of Grb2 in Spry-mediated inhibition of FGF-induced MAPK pathway has remained controversial. Lao and co-workers discovered, using an FGFR overexpression system (i.e. long-term MAPK signaling), that Spry2 requires an FGFR-induced conformational change involving a cryptic PXXPXR motif on its C-terminus to bind Grb2 via its N-terminal SH3 domain [42]. However, a study involving acute FGF signaling (i.e. short-term MAPK activation without FGFR overexpression) demonstrated an uncoupling of Spry2–Grb2 binding from Spry-mediated inhibition of MAPK activation [55]. This work revealed that Spry2 harbors two Grb2-binding sites and that a mutant hSpry2 lacking both sites still antagonized FGF signaling. Thus, how FGF signaling is antagonized by Spry may depend on the type and/or duration of the original stimulus.

Post-translational modification of Spry likely plays a central role in determining its function and activation status. In unstimulated cells, Spry1 and Spry2 have been found phosphorylated on serine residues [23]. Spry2 inhibition of FGF signaling requires phosphorylation of tyrosine 227 (Y227), which is dispensable for Spry2 potentiation of EGF signaling [56]. Recently, Lao and co-workers reported that a change in Spry2 phosphorylation status was required for the inhibition of FGFR-induced MAPK activation [57]. Upon FGFR activation, Spry2 is dephosphorylated by the protein phosphatase 2A (PP2A) at highly conserved serine residues (S112 and S115) in the Spry N-terminus, thus activating Spry [57]. PP2A competes with the E3 ubiquitin ligase, c-Cbl, for binding to Spry2, thus establishing two pools of Spry2-containing complexes and striking a balance between Spry2 activation and degradation. Hence, both tyrosine phosphorylation and serine dephosphorylation are required for Spry2-mediated inhibition of FGFR signaling (Fig. 1b).

Another characteristic of Spry proteins is their ability to form homo- and hetero-complexes [24, 41, 48, 49]. In the context of FGF signaling all four Spry isoforms have been reported to interact with each other via their cysteine-rich domains [43]. In a heterodimer of Spry1 and Spry4, Spry1 can bind to Grb2, while Spry4 interacts with Sos1. These studies also revealed that the interaction between Spry1 and Spry4 was the most potent combination for inhibiting FGF2-induced MAPK activation. Thus, the combinatorial set of Spry isoforms within a cell determines the extent of FGF signaling inhibition.

EGF signaling

The role and components of the EGF-induced MAPK cascade in normal endothelium are still under debate; however, there is consensus that EGF signaling plays an important role in tumor endothelium [58–60]. The function of Spry in the EGF signaling cascade has been the most controversial due to the different cell types that have been examined. The general consensus is that Spry can potentiate EGF-induced MAPK activation in certain cellular contexts. The EGF receptor (EGFR) is normally rapidly downregulated due to its interaction with the E3 ubiquitin ligase, c-Cbl. However, Spry2 interferes with this interaction and competes with the EGFR for c-Cbl-binding preventing receptor internalization, thus sustaining EGFR-mediated signaling [44, 46, 61, 62] (Fig. 1c). Moreover, Spry2 binds CIN85, an endocytic adapter, thus regulating the clustering of c-Cbl that is required for EGFR endocytosis and degradation [63]. Other RTKs, such as the FGFR, are also degraded by c-Cbl, and it is still not clear how Spry discriminates between EGF and FGF signaling. In a recent study, where Spry2 levels were reduced by RNA interference, FGF signaling was increased, while EGF signaling was reduced [56]. These experiments led to the discovery that phosphorylation at a conserved tyrosine (Y227) discriminates between the EGF and FGF signaling pathways, suggesting that unidentified proteins bind to this C-terminal region depending on the signaling context. More recently, Kim et al. [64] described that Spry2 interfered with activated EGFR trafficking to late endosomes. Spry2 interacts with hepatocyte growth factor-regulated tyrosine kinase substrate (Hrs) in early endosomes. The Spry2–Hrs complex disrupts the Hrs association with tumor susceptibility gene 101, an interaction required for EGFR movement from early to late endosomes, resulting in delayed EGFR trafficking and perturbation of phospho-MAPK accumulation in late endosomes. Thus, potentiation of EGF signaling by Spry relies on interactions with proteins that are involved in the endocytosis and degradation of EGFR.

VEGF signaling

VEGF-induced signal transduction is the most critical and best studied signaling pathway in endothelial cells and plays a central role in vascular development and in physiological and pathological angiogenesis [65]. In endothelial cells, VEGF binds to VEGF receptor (VEGFR) and activates MAPK via the phospholipase C gamma (PLC γ) and protein kinase C (PKC) pathway where PKC phosphorylates Raf1, thereby activating ERK/MAPK in a Ras-independent manner [66] (Fig. 1a). Here, Spry-mediated inhibition of VEGF signaling occurs via a different mechanism as compared to FGF and EGF signaling [67] (Fig. 1d). Spry4 binds to Raf1 via a highly conserved C-terminal domain, thus ablating VEGF signaling by segregating Raf1 away from the cascade. All Spry isoforms carry this Raf1-binding domain (RBD) in their highly conserved C-terminal domain suggesting that all Spry isoforms may be capable of inhibiting VEGF signaling in a similar manner.

Apparently, Spry mechanisms of action are as varied as their target RTKs. For each signaling cascade studied in depth so far, Spry proteins have evolved an inhibitory strategy unique to each pathway.

Spry-interacting proteins and Spry activity

Spry proteins have accumulated a long list of interacting and associated proteins that regulate Spry function, stability, and localization, many of which interact with the C-terminal SPR domain (reviewed in [17, 18]). Included in this list is Caveolin-1, a transmembrane protein and the major architectural component of caveolae, flask-shaped pits on the cell membrane [68]. Caveolin-1 promotes capillary tube formation, and its role in regulating angiogenesis has been underlined in transgenic overexpression and knockout experiments in mice [69–72]. Caveolin-1 also inhibits RTK signaling, yet in a cell confluency-dependent manner [73]. Caveolin-1 co-localizes and immunoprecipitates with Spry1 and Spry2 [23]. Upon further analysis, all Spry isoforms interact with Caveolin-1 and Spry binds to Caveolin-1 via its highly conserved cysteine-rich C-terminal domain [41]. Caveolin-1 in turn binds to the various Spry isoforms via two domains, a N-terminal and a C-terminal domain. Spry proteins do not require Caveolin-1 for their function nor for their membrane targeting; however, Caveolin-1 acts cooperatively with some Spry isoforms depending on whether the EGF or FGF pathways are being stimulated. In summary, Caveolin-1 modulates Spry activity in an isoform-, cell density-, and growth factor-specific manner, suggesting that together these two proteins fine tune growth factor signaling.

Recently, Necl-5, also known as poliovirus receptor/CD155/Tage4, has been identified as a novel Spry2-interacting protein [74]. Necl-5 is a mediator of contact inhibition that forms a complex with integrin $\alpha_v\beta_3$, is upregulated by PDGF and FGF, and is also found on endothelial cells [74–76]. Necl-5 interacts with Spry2 in a cell confluency-dependent manner and controls Spry2 tyrosine phosphorylation, thus modulating the PDGF-activated MAPK signaling pathway. This novel binding partner demonstrates once again the importance of cell–cell contacts in the fine-tuning of Spry-mediated inhibition of RTKs.

Another previously described Spry-interaction partner, TESK1, a cofilin kinase, has now been shown to interact with all Spry proteins [77]. Initially, TESK1 was described as a Spry4-binding protein that co-localizes with Spry4 in endosomes [20]. Spry4 binds TESK1 via its C-terminal cysteine-rich domain and inhibits TESK1 activity resulting in decreased cell spreading [78]. TESK1 itself inhibits Spry2 activity by interfering with its subcellular localization so as to prevent serine dephosphorylation and also binding to Grb2 [77].

Regulation of Spry protein stability and activity

Spry protein stability and turnover are important determinants of how this family of negative feedback inhibitors affects endothelial cell homeostasis. While growth factor stimulation does induce Spry gene transcription [23, 24, 79], it also results in proteolytic degradation of Spry proteins [45, 46, 80]. This negative feedback loop system is thought to regulate Spry function temporally as a consequence of RTK stimulation [17, 18, 81]. Three recent studies have demonstrated that Spry stability is regulated in a complex manner [82–84]. As mentioned above, Spry proteins are phosphorylated on serine residues in unstimulated cells and are further phosphorylated on serine residues upon EGF and FGF stimulation [23, 82]. Phosphorylation of Spry2 at S112 and S121 is mediated by the MAPK-interacting kinase 1, Mnk1, which interferes with Spry2 tyrosine phosphorylation and thus stabilizes Spry2. Degradation of Spry2 is linked to phosphorylation of the conserved N-terminal tyrosine residue. A mutant version of Spry2 lacking all serine and tyrosine phosphorylation sites prevents Spry2 degradation, and a mutant version of Spry2 lacking the serine phosphorylation sites is no longer able to inhibit FGF-induced MAPK activation [82]. Together these data reveal that Spry stability and function require both tyrosine and serine phosphorylation.

Using a yeast two-hybrid screen, Nadeau and co-workers recently reported that Spry proteins interact with and are targets of the E3 ubiquitin ligase, "seven-in-absentia homolog 2" (SIAH2) [84]. Notably, SIAH2-mediated proteasomal degradation of Spry is independent of its tyrosine phosphorylation of the N-terminal tyrosine. These results uncover another mechanism for regulating Spry protein levels and function.

Finally, transforming growth factor-beta 1 (TGF-β1) has been shown to regulate Spry2 at both the mRNA and protein levels [83]. TGF-β1 plays a critical role in vessel maturation and in the initial stages of angiogenesis [85]. TGF-β1-mediated degradation of endogenous Spry2 protein is independent of MAPK activation, cannot be rescued by addition of EGF, and occurs in the lysosome. Furthermore, TGF-β1 induces a transcriptional downregulation of Spry2 that is due to diminished mRNA production and requires de novo protein synthesis and histone deactylase activity. Lastly, Spry2's modulation of FGF and EGF signaling declined in cells pretreated with TGF-β1, suggesting that TGF-β signaling, possibly via the Smad proteins, can regulate Spry2 function by altering its mRNA and protein levels. In summary, Spry protein and mRNA turnover can be regulated by various signals, some of which are RTK-dependent while others are not (summarized in Fig. 2].

Spry and nitric oxide

Recently, it has been reported that Spry2 can assemble into large, spherical 24-mers with an iron–sulfur complex suggesting that they can function as redox sensors as these structures exist in oxidized, reduced, or nitrosylated forms

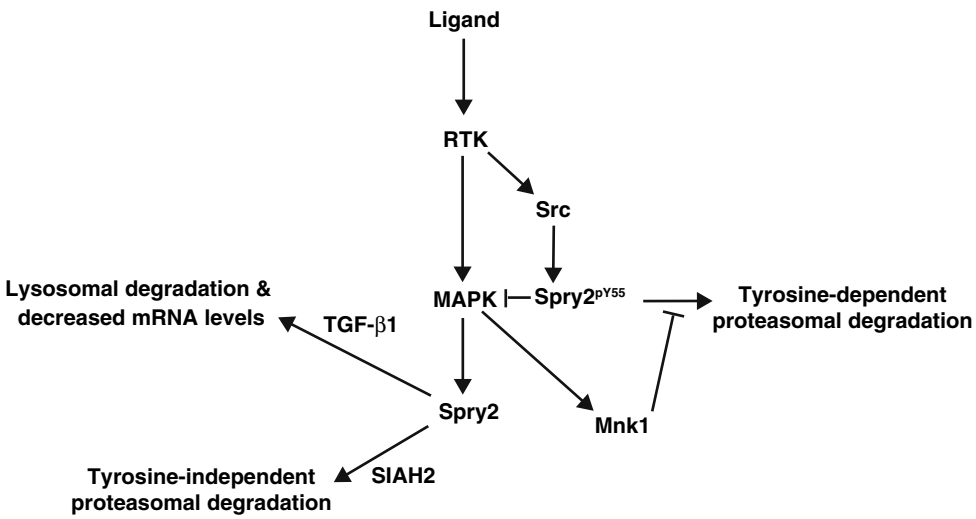

Fig. 2 Regulation of Spry2 stability. A diagram depicting the RTK-dependent and RTK-independent pathways that control Spry2 protein turnover. There are at least five signaling pathways that regulate Spry2 accumulation in the cell: transcriptional upregulation by the MAPK signaling pathway, serine phosphorylation by Mnk1, tyrosine phosphorylation by Src, ubiquitinylation by SIAH2, and TGF-β1-mediated transcriptional downregulation and lysosomal degradation [23, 24, 79, 82–84]. pY55 denotes the N-terminal tyrosine phosphorylation on Spry2

[86]. Notably, based on these observations it was proposed that these Spry2 complexes can hold an electrical charge and exhibit properties akin to an intracellular battery. Curiously, ectopic expression of nitric oxide synthase (NOS) in *Drosophila* mimics the dSpry mutation of increased tracheal branching [87]. In mammals, endothelial NOS (eNOS) is essential for endothelial cell proliferation and is required for vessel morphogenesis, branching, and stabilization (reviewed in [88]). Furthermore, the Spry-interacting protein, Caveolin-1, binds to and inhibits eNOS activity [89]. Thus, it is tempting to speculate that, in endothelial cells, Spry2 has additional functions that involve changes to its redox state and eNOS. To what extent, if any, these modifications affect Sprys' MAPK inhibitory activities has yet to be determined.

Murine Spry knockout studies

The generation of Spry null mice has yielded exciting new information about Spry function as well as confirming the central role of Sprys in modulating branching morphogenesis and growth factor signaling. Mice lacking Spry1 exhibit kidney and ureteric bud defects arising from improper branching morphogenesis [27, 90]. These malformations arise due to de-regulated signaling of GDNF by its cognate receptor c-Ret, which is essential for proper kidney development. Spry2 null mice have been generated independently in two laboratories. In one report, Spry2 null mice are viable, have a shortened life span, and exhibit hearing loss due to aberrant FGF8 signaling that results in malformation of the organ of Corti [91]. The other Spry2 knockout mouse line also exhibits a reduced lifespan and, in addition, a severe gastrointestinal phenotype characterized by enteric nerve dysplasia presumably due to GDNF hyperactivity [92].

More recently, two independent Spry4 null mouse lines have been reported [93, 94]. In a first report, these mice were used to show that Spry2 and Spry4 control tooth development by antagonizing FGF signaling from different tissues; while Spry2 was expressed in the epithelium, Spry4 was found in the mesenchyme [93]. In a second report, Spry4 null mice showed reduced viability due to mandible defects, while the surviving mice exhibited growth retardation and polysyndactyly (i.e. fusion and duplication of forelimb digits) [94]. Also, Spry2/Spry4 double knockout mice were embryonic lethal with the majority of these embryos dying at E12.5 with apparent morphogenetic abnormalities of the head, lung and limbs, including cyclopia. Together, these data suggest that Spry2 and Spry4 have redundant activities during embryonic development. Although Spry overexpression markedly affects endothelial cell proliferation, tube formation, and angiogenesis in experimental models (see above), none of the Spry knockouts described thus far exhibits an overt vascular phenotype.

Sprouty-related proteins, Spreds

Spreds are related structurally and functionally to Spry proteins and have recently been reviewed [19]. Like Spry proteins, Spreds inhibit the MAPK pathway induced by various mitogens and share with Sprys the highly conserved cysteine-rich C-terminal SPR domain. Studies in *Xenopus* have highlighted the functional differences between Spry and Spred proteins. While in *Xenopus* Spry proteins inhibit PKC δ and calcium signaling, Spred proteins interfere with Ras–MAPK signaling [95]. In mammals, there seems to be more overlap in the signaling pathways that can be targeted by both Spry and Spreds, but Spred function tends to be more focused on MAPK inactivation [19]. A recent study examining the overlapping roles of Spred1 and Spred2 in double knockout mice demonstrates that Spreds are essential for development of the lymphatic system [96]. These mice are embryonic lethal at E12.5–E15.5 and display hemorrhages, edemas, and dilated lymphatic vessels, suggesting incomplete partitioning of lymphatic vessels from blood vessels. Expression of Spred1 and Spred2 is restricted to lymphatic endothelial cells, while Spry2 and Spry4 are found in both blood and lymphatic endothelial cells, suggesting that Spry function cannot replace Spred function in lymphatic endothelial cells. Apparently, Spred1 and Spred2 are able to repress the VEGF-C/VEGFR3-mediated signaling pathway. In contrast, Spry4 exerts only a very moderate inhibitory effect on this pathway. Curiously, both Spred proteins and Spry4 repress VEGF-A-induced MAPK activation via VEGFR2 [96]. In summary, while the Spred and Spry families share common features and activities, they have evolved to exert distinct and specific roles in modulating RTK signaling cascades.

Summary and conclusions

The critical contribution of Spry proteins in the control of signal transduction pathways in a variety of cell types, including endothelial cells, is well established. Nearly a decade has passed since they were first identified as antagonists of the FGF-induced signaling pathway. Ever since, their role in biology has expanded to encompass many more signaling pathways and biological processes, mostly involving an inhibitory function in RTK-induced MAPK signaling. While there are many open questions regarding Sprys' mechanisms of action in individual signaling pathways, it is now clear that Spry proteins are

multi-functional and themselves highly regulated in their expression and function. Continuing studies will shed more light on the complexity of Spry function in controlling the multiple outputs of mitogen-induced cascades in physiological and pathological processes in general and in angiogenesis in particular. Novel insights about their molecular function may set the stage for the development of innovative therapeutic approaches to interfere with RTK-mediated pathological processes.

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