REVIEW

SPRED proteins and their roles in signal transduction, development, and malignancy

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The roles of SPRED proteins in signaling, development, and cancer are becoming increasingly recognized. SPRED proteins comprise an N-terminal EVH-1 domain, a central c-Kit-binding domain, and C-terminal SROUTY domain. They negatively regulate signaling from tyrosine kinases to the Ras–MAPK pathway. SPRED1 binds directly to both c-KIT and to the RasGAP, neurofibromin, whose function is completely dependent on this interaction. Loss-of-function mutations in SPRED1 occur in human cancers and cause the developmental disorder, Legius syndrome. Genetic ablation of SPRED genes in mice leads to behavioral problems, dwarfism, and multiple other phenotypes including increased risk of leukemia. In this review, we summarize and discuss biochemical, structural, and biological functions of these proteins including their roles in normal cell growth and differentiation and in human disease.

Ras proteins are binary molecular switches that cycle between GTP-bound on and GDP-bound off states. Accumulation of active Ras-GTP in response to growth factors is understood in some detail where the translocation of guanine exchange factor (GEF), such as SOS, to the plasma membrane stimulates the nucleotide exchange on Ras from GDP to GTP (Buday and Downward 1993). GTP-bound Ras proteins activate the MAPK kinase cascade by binding and recruiting Raf to the membrane (Terrell et al. 2019). This cascade regulates a variety of biological processes such as cell proliferation, differentiation, and survival. Ras proteins also bind activate PI 3′ kinases, RalGDS proteins, and possibly other effector pathways (Simanshu et al. 2017). Conversion of Ras-GTP back to the inactive form, and maintenance of Ras in the inactive form prior to restimulation is mediated by GTPase-activating proteins (GAPs), such as neurofibromin and RASA (p120 RasGAP).

Ras signaling is tightly controlled; hyperactive Ras signaling results in diseases such as cancer, developmental disorders (known collectively as RASopathies) and learning disabilities (Simanshu et al. 2017). To maintain tight control of Ras activity, numerous signals and interactions regulate the duration, magnitude and context of Ras activation. SPRED proteins, and their better-known relatives, proteins of the Sprouty family, are negative regulators of Ras signaling. Although SPRED and Sprouty proteins share a conserved C-terminal Sprouty (SPR) domain, the mechanisms by which they regulate Ras signaling are distinct.

The *SPROUTY* gene was first identified as a negative regulator of FGF signaling and tracheal branching in *Drosophila* (Hacohen et al. 1998). The role of Sprouty proteins as negative regulators of Ras–MAPK signaling is conserved from *Drosophila* to humans (Hacohen et al. 1998; Casci et al. 1999). Sprouty proteins inhibit Ras activation by preventing the membrane localization of SOS, a Ras GEF, by binding and sequestering GRB2, an adaptor protein that links the phosphorylated receptor tyrosine kinase (RTK) with SOS (Fig. 1). Activated RTKs, such as FGFR, EGFR, and VEGFR, phosphorylate a conserved phosphotyrosine in Sprouty, which is recognized by the SH2 domain found on GRB2 and competes for GRB2 binding with SOS (Hanafusa et al. 2002). Sprouty proteins are down-regulated by dephosphorylation of the phosphotyrosine by the phosphatase Shp2 (Hanafusa et al. 2004) or engagement with c-Cbl, an E3 ubiquitin-ligase, which results in polyubiquitination and proteasomal degradation (Hall et al. 2003; Mason et al. 2004). In addition to GRB2 binding, Sprouty may also regulate Ras signaling by binding Raf, which inhibits Raf kinase activity (Fig. 1; Yusoff et al. 2002).

Sprouty-related SPRED proteins were identified in a yeast two-hybrid screen to identify proteins that interact with RTKs c-Kit and c-Fms (Wakioka et al. 2001). This screen identified SPRED1 as a binding partner for the intracellular kinase domain of c-Kit in its inactive state.

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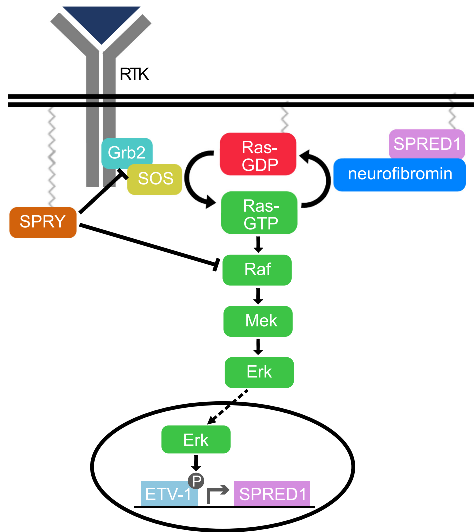

Figure 1. Inhibitory mechanism of Spred and Sprouty proteins on the Ras–MAPK pathway. SPRED proteins down-regulate Ras signaling by recruiting RasGAP, neurofibromin to the membrane. SPRED loss primarily activates Ras–MAPK signaling though Ras activates other pathways such as PI3K, PLC-ε, and RAL-GDS. Sprouty proteins down-regulate Ras signaling by sequestering Grb2 and preventing the membrane recruitment of RasGEF, SOS, and by inhibiting Raf kinase activity.

SPRED2 was identified through searching genomic databases, in the same study, due to its high sequence similarity to *SPRED1*. Similarly, the same group identified *SPRED3*, a few years later (Kato et al. 2003). Like Sprouty, SPRED proteins inhibit growth factor-mediated activation of Ras–MAPK signaling. However, SPRED proteins function through regulation of Ras GAPs rather than Ras GEFs. SPRED1 cooperates with neurofibromin, a Ras GAP, and is essential for neurofibromin function (Stowe et al. 2012; Dunzendorfer-Matt et al. 2016; Hirata et al. 2016). Consistent with this, heterozygous germline loss of function of *SPRED1* results in Legius syndrome (LS), which shares a similar phenotype with neurofibromatosis type 1 (NF1), a syndrome caused by loss-of-function mutations in neurofibromin (Brems et al. 2007). Other reports suggest that SPRED suppresses Ras signaling by preventing the phosphorylation and activation of Raf (Wakioka et al. 2001; Nonami et al. 2005). However, both mechanisms have not been fully characterized.

SPRED isoforms

SPRED1, SPRED2, and SPRED3 share similar overall structure (Fig. 2A). Their chromosomal localization is summarized in Table 1. Human *SPRED1* gene spans 444 amino acids, while *SPRED2* spans 418 residues with 55% sequence identity. *SPRED3* spans 410 residues and shares 38% sequence identity to *SPRED1*. While both SPRED1 and SPRED2 effectively suppressed growth factor-mediated Erk activation, SPRED3 possessed less inhibitory activity (Kato et al. 2003). This suggests that each member of the SPRED family may have a selective capacity and function for the regulation of Ras–MAPK signaling.

Protein structure

EVH-1 domain

The N-terminal region of SPRED family members is comprised of an enabled/VASP homology-1 (EVH-1) domain (Fig. 2A), one of the four distinct interaction domains among SH3–WW, GYF, and EVH-1—that recognize proline-rich sequences (Peterson and Volkman 2014). The EVH-1 domain has been found in ~630 human proteins (Peterson and Volkman 2014). This domain spans ~115 residues in length and is invariably located at the N terminus. EVH-1 domains are found in multidomain scaffolding proteins that mediate multiprotein complex assembly associated with modulating the actin cytoskeleton or signal transduction in postsynaptic compartments (Ball et al. 2002) Despite having low sequence homology, EVH-1

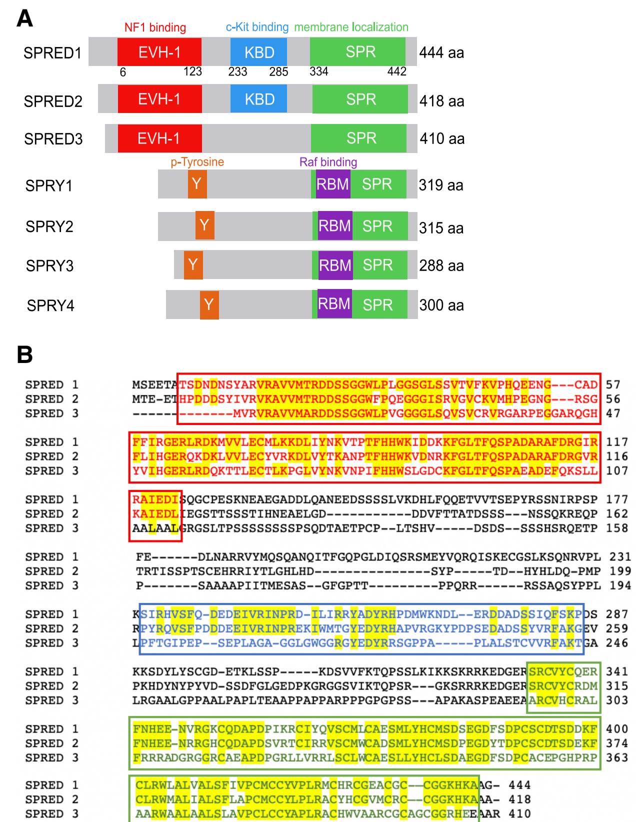

Figure 2. Domain and sequence alignments of human SPRED and Sprouty proteins. (*A*) Domain structure of SPRED and Sprouty proteins. SPRED proteins share a N-terminal EVH-1 domain and C-terminal sprouty domain. SPRED1 and SPRED2 share a central c-Kit-binding domain. Sprouty proteins share a conserved tyrosine and C-terminal sprouty domain. (*B*) Sequence alignment of SPRED proteins. (Red) EVH-1 domain, (blue) KBD, (green) SPR, (highlighted in yellow) conserved residues.

Table 1. *Chromosomal localization*

	Human	Mouse
SPRED1	15q13.2	2E5
SPRED2	2p14	11A3-A4
SPRED3	19q13.13	7A3

Chromosomal localization was identified by an NCBI database search for "human" and Kato et al. 2003 for "mouse."

domains consist of two perpendicular antiparallel β-sheets followed by a C-terminal α-helix (Peterson and Volkman 2014). Structural studies with the SPRED1 EVH-1 domain from *Xenopus tropicalis* found that the polyproline-binding groove is narrower than in other EVH-1 domains (Harmer et al. 2005). The only known interactor for SPRED EVH-1 is neurofibromin, which does not contain proline-rich sequences within the interaction region (Harmer et al. 2005; Stowe et al. 2012). Together, this demonstrates a new binding mechanism for SPRED EVH-1 that does not require proline-rich sequences similar to other EVH-1 interactors (Hirata et al. 2016), and presents a new mode of protein–protein interaction that may exist in other proteins containing an EVH-1 domain. Finally, although the three SPRED proteins bind neurofibromin, the SPRED3 EVH-1 has a much weaker binding affinity compared with SPRED1 and SPRED2 (Hirata et al. 2016). Unlike SPRED1 and SPRED2, sequence alignment of SPRED proteins shows that SPRED3 has a smaller N terminus and contains a three-amino-acid insertion in the β4 strand (Yan et al. 2020); this corresponds to decreased sequence identity between SPRED1 and SPRED3 (57%), compared with SPRED2 (67%). These differences in amino acid sequence likely cause minor changes in tertiary structure of SPRED3 EVH-1 domain and may be responsible for weaker neurofibromin binding and ERK suppression activity of SPRED3. Indeed, several residues in SPRED1 (L32, T86, and T88), which directly interact with neurofibromin via nonbonded contacts and hydrogen bond are not conserved in SPRED3 (Yan et al. 2020).

KBD domain

The central region of SPRED contains the c-Kit-binding domain (KBD) (Fig. 2A). This domain spans 50 residues and was initially identified through a yeast two-hybrid screen for tyrosine kinase-binding proteins (Wakioka et al. 2001). This interaction domain is unrelated to any previously identified tyrosine kinase interaction domains such as SH2 and PTB domain (Wakioka et al. 2001). Although SPRED2 KBD only shares 51% identity with SPRED1 KBD, SPRED2 also binds to c-Kit via this region. In contrast, the corresponding domain of SPRED3 shares <20% identity with SPRED1 KBD (Fig. 2A). Arginine 247 of SPRED1 is required for c-Kit binding (Kato et al. 2003), but in SPRED3 the equivalent residue (240) is a glycine. As a result, SPRED3 does not bind or become phosphorylated by c-Kit, unlike SPRED1 and SPRED2 (Kato et al. 2003). Moreover, it is not known whether SPRED proteins can also interact with other RTKs besides c-Kit; perhaps the KBD is a new interaction domain that mediates binding with RTKs, allowing RTKs to regulate SPRED function.

SPR domain

The C-terminal region of SPRED proteins consists of the Sprouty (SPR) domain (Fig. 2A), a conserved cysteine-rich domain analogous to that found in Sprouty proteins (Fig. 2C). Despite attenuation of Ras signaling by apparently distinct mechanisms, membrane localization is consistently important for efficient inhibition by both SPRED and Sprouty. Sprouty proteins translocate to membrane raft/caveolae following growth factor stimulation (Impagnatiello et al. 2001; Lim et al. 2002). Caveolae represent a subset of lipid rafts that are implicated in signal transduction-related events due to the presence of signaling proteins such as RTKs (Bastiani and Parton 2010). Membrane localization of Sprouty proteins may be a consequence of their interaction with a membrane-associated scaffold protein, caveolin-1, and/or through palmitoylation (Impagnatiello et al. 2001). Similar to Sprouty, SPRED proteins localize in lipid rafts/caveolae where they interact with caveolin-1 (Nonami et al. 2005). SPRED1 and SPRED3 also interact with a palmitoyl acetyltransferase, HIP14, and coexpression leads to palmitoylation of SPRED (Butland et al. 2014). Additionally, SPRED1 associates with B-Raf in the cytoplasm, where B-Raf/C-Raf dimerization induces SPRED1 membrane translocation to the plasma membrane (Siljamäki and Abankwa 2016). The SPR domain of SPRED is important for effective Ras inhibition by translocating neurofibromin to the plasma membrane where it can interact with Ras (Stowe et al. 2012). SPRED mutants with deletion of the SPR domain fail to localize to the plasma membrane and fail to inhibit ERK phosphorylation (King et al. 2005). Moreover, pathogenic SPR mutations in SPRED1 bind neurofibromin but fail to recruit it to the plasma membrane (Stowe et al. 2012; Hirata et al. 2016). Altogether, these data show that the SPR domain is necessary to down-regulate Ras signaling by recruiting neurofibromin to the plasma membrane compartment in which it can interact with Ras. RASA1/p120RasGap does not interact with SPRED proteins, as discussed below. This GAP is recruited to the plasma membrane through interactions between its SH2 domains and phospho-tyrosines on activated RTKs, such as PDGFR and EphA2 (Tong et al. 2003). The mechanisms by which other GAPs are recruited to Ras in the plasma membrane remain to be determined.

In addition to membrane localization, the SPR domain plays a role in SPRED and Sprouty dimerization (Hanafusa et al. 2002; King et al. 2005). SPRED1, SPRED2, and SPRED3 have been found to heterodimerize, although SPR deletion mutants cannot (King et al. 2005). However, the affinity, context, and consequences of dimerization are unclear. Furthermore, membrane localization and Ras inhibition in ΔSPR mutants were rescued by the addition of CAAX motif (Stowe et al. 2012), a consensus sequence known to undergo a series of post-translational modifications that allow proteins to associate with membranes (Wright and Philips 2006). It was previously shown that

the CAAX motif induces Ras dimerization (Nan et al. 2015). Interestingly, neurofibromin was recently identified as a high-affinity dimer in cells (Sherekar et al. 2020) and suggests that these signaling complexes are regulated by dimerization, although the precise mechanisms remain to be elucidated.

Protein interactions

Neurofibromin

Neurofibromin interacts with SPRED through the EVH-1 domain (Stowe et al. 2012). Neurofibromin negatively regulates Ras signaling through its function as a GAP to accelerate the hydrolysis of GTP (Martin et al. 1990; Xu et al. 1990). This interaction is necessary for the recruitment of neurofibromin to the plasma membrane where it can down-regulate Ras activity (Stowe et al. 2012) and provides a satisfying explanation for the phenotypic overlap between loss of SPRED1 function in Legius syndrome and loss of function of neurofibromin in NF1, discussed later.

The crystal structure of the neurofibromin GAP-related domain (GRD) (PDB:6V65) consists of a module of two subdomains: the central domain (GAPc), which is necessary and sufficient to bind and inactivate Ras, and the extra domain (GAPex), which is composed of the N-terminal and C-terminal residues flanking the central GAP domain (Fig. 3B; Yan et al. 2020). The neurofibromin-GAPc primarily interacts with RAS. Meanwhile, the neurofibromin-GAPex region forms two crossing α-helix coils at one end outside the GAPc region, and is required for binding to the SPRED1 EVH-1 domain (Fig. 3A,B; Dunzendorfer-Matt et al. 2016). Although the GAPex region has been shown to be dispensable for GAP activity in vitro (Hirata et al. 2016), the GAPex region is the major interaction domain for SPRED1 binding (Dunzendorfer-Matt et al. 2016; Sherekar et al. 2020). The N-terminal residues of neurofibromin GAPex forms a salt bridge and hydrogen bonds with residues in SPRED1 EVH-1 domain. Meanwhile, the C-terminal residues of neurofibromin GAPex have a less direct interaction with SPRED1 and instead act as structure-supporting module to maintain the overall architecture of neurofibromin GAPc and GAPex domains through hydrophobic interactions and a weak hydrogen bond. Besides the GAPex, the GAPc region also interacts with SPRED1 EVH-1 and contributes to complex stabilization mainly through hydrophobic interaction (Yan et al. 2020). Furthermore, the SPRED1 region forming the neurofibromin-SPRED1 interphase in the crystal structure was found to be highly flexible in NMR studies (Führer et al. 2017). LS mutations such as R24Q (Sumner et al. 2011), G30R (Sumner et al. 2011), and T102R (Messiaen et al. 2009) disrupt electrostatic interactions with neurofibromin, and result in decreased or undetectable binding (Führer et al. 2017; Fig. 3A,B; Yan et al. 2020). Likewise, several NF1 patient mutations that map to the GAPex region resulted in reduced binding with SPRED1 EVH-1 domain (Fig. 3D; Hirata et al. 2016). These LS and NF1 mutations are located at the neurofibromin–SPRED1 interface and form direct interactions between neurofibromin and SPRED1 (Fig. 3E; Yan et al. 2020).

c-Kit

Both SPRED1 and SPRED2 bind c-Kit and are phosphorylated on tyrosine residues in response to growth factor stimulation with SCF, PDGF, and EGF. The binding site for c-Kit was mapped to a 50-amino-acid region (codon 233–285) in SPRED1 subsequently designated the "c-Kit-binding domain" (Wakioka et al. 2001). Despite this, little is known about the consequence and context of c-Kit binding to SPRED, perhaps interaction with c-Kit contributes to SPRED membrane localization and allows growth factor-dependent regulation of SPRED membrane translocation.

Protein expression pattern

The mild phenotypes of both *SPRED1* and *SPRED2* knockout mice (discussed below) suggest some redundancy between the two proteins. RNA expression profiling shows that *SPRED1* and *SPRED2* have overlapping expressions and are both widely expressed across human tissues (Fig. 4A; The Genotype Tissue Expression Project, https://www.gtexportal.org/home). Protein expression profiling through immunohistochemistry staining of various human tissue sections shows similar expression distribution (Fig. 4B; The Human Protein Atlas, https://www.proteinatlas.org). In contrast, RNA expression profiling shows that, unlike *SPRED1* and *SPRED2*, *SPRED3* expression is limited to the brain, pituitary gland, and breast (Fig. 4A; The Genotype Expression Project, https://www.gtexportal.org/home; Kato et al. 2003).

In addition, expression patterns of SPRED proteins change during development. Comparing protein levels in fetal and adult mouse tissue lysates, SPRED1 is expressed in fetal liver, brain, and heart, whereas SPRED2 is not detected in these fetal tissues (Engelhardt et al. 2004). This suggests that SPRED1 may be more functionally important during fetal development. This is in agreement with the observations that germline mutations in *SPRED1* result in the developmental disorder Legius syndrome (LS) as discussed below, whereas mutations in SPRED2 have never been reported in this disease.

Transcriptional regulation

Ras signaling pathways control gene expression through phosphorylation and regulation of transcription factors, coregulatory proteins, and chromatin proteins (Whitmarsh 2007). Levels of Ras–MAPK activation are tightly controlled by ERK-regulated transcriptional regulation of negative feedback regulators (Pratilas et al. 2009; Dry et al. 2010). For example, *SPROUTY1*, *SPROUTY2*, and *SPROUTY4* expression is known to be positively regulated by ERK signaling resulting from RTK activation (Ozaki et al. 2001; Packer et al. 2009). Recently, *SPRED1* and *SPRED2* expression has been shown to be positively

Figure 3. Model for neurofibromin membrane recruitment through SPRED1 interaction. (*A*) SPRED1 EVH-1 domain directly binds to the GAPex domain of neurofibromin GRD. SPRED1 KBD interacts with c-Kit. SPRED1 SPR is membrane anchored through palmitoylation. Neurofibromin GAPc interacts with Ras. (*B*) Overall structure of the complex formed by GMPPNP-bound KRAS (green), neurofibromin GRD (blue), and SPRED1 EVH-1 (pink) (PDB:6V65). (*C*) Ortholog alignment of SPRED EVH domain with LS mutations that disrupt SPRED1-neurofibromin binding in red. (*D*) Ortholog alignment of neurofibromin GAPex with NF1 mutations that disrupt SPRED1-neurofibromin binding in red. (*E*) Mapping of pathogenic mutations in SPRED1 (pink) and NF1 (green) on the neurofibromin–SPRED1 complex (PDB:6V65).

regulated by Ras–MAPK signaling in melanoma and GIST cell lines (Xie et al. 2018). Pharmacological inhibition of the Ras pathway, either with MEK, c-Kit, or RAF inhibitors, resulted in decreased *SPRED* expression 6 h after treatment and remained down-regulated at 24 h (Packer et al. 2009; Pratilas et al. 2009; Xie et al. 2018). In these cells, *SPRED* expression is regulated by ETV1, a member of the Ets family of transcription factors. ETV1 is overexpressed in melanoma (Jané-Valbuena et al. 2010) and gastrointestinal stromal tumors (Ran et al. 2015). ETV1 transcription activity is stimulated by Ras–MAPK activation where Erk1-mediated phosphorylation regulated ETV1 protein stability (Janknecht 1996; Bosc et al. 2001; Oh et al. 2013). Finally, sequencing of tumor tissues and cancer cell lines demonstrated a correlation between BRAF V600E or KRAS G12 mutations with high SPRED1 mRNA expression (Cerami et al. 2012; Figshare, https://figshare.com/articles/dataset/DepMap_20Q2_Public/12280541).

Genetic models

Mouse models

SPRED1$^{-/-}$ mice were generated by deleting exons encoding for KBD and SPR domains. These mice were generally healthy and fertile despite having lower body weight and a shortened face (Inoue et al. 2005). Mice exhibited increased pigmentation in ears, paw pads, and tails (Tadokoro et al. 2018). Mice lacking SPRED1 demonstrated cognitive impairments in hippocampus-dependent visuo-spatial memory (Denayer et al. 2008). *SPRED1*$^{-/-}$ mice displayed learning deficits in the Morris water maze and T-maze tests (Denayer et al. 2008). In cognitively more demanding stages of the T-maze, *SPRED1*$^{+/-}$ mice performed at an intermediate level between wild-type and *SPRED1*$^{-/-}$ littermates (Denayer et al. 2008). The cognitive and synaptic plasticity phenotype in *SPRED1*$^{-/-}$ mice were remarkedly similar to *NF1*$^{+/-}$ mice, where treatment with statins, which interfere with Ras membrane anchoring and activation, acutely rescued the learning and synaptic plasticity defects (Cui et al. 2008). Similarly, learning difficulties have been reported in Legius syndrome (Brems et al. 2007; Pasmant et al. 2009) and NF1 patients (Costa et al. 2002; Hyman et al. 2005), where deficits visuo–spatial and visual constructive skills are considered hallmarks of NF1. *SPRED1*$^{-/-}$ mice also exhibited allergen-induced airway eosinophilia indicating that SPRED1 may negatively regulate IL-5-mediated eosinophil proliferation, possibly by inhibiting Ras–MAPK signaling (Inoue et al. 2005).

SPRED2$^{-/-}$ mice were generated using a gene trap inserted between exons 4 and 5 of *SPRED2.* These mice appeared healthy and fertile despite having reduced body

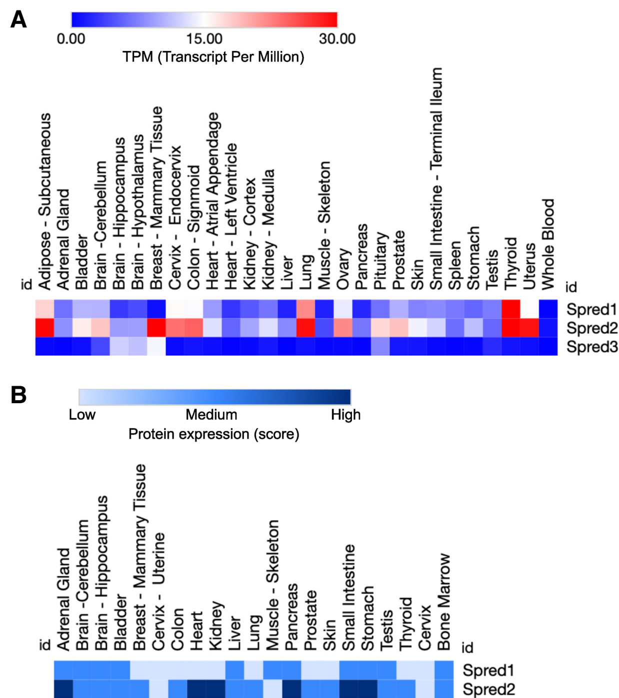

Figure 4. RNA and protein expression of SPRED proteins. (*A*) RNA expression of SPRED1, SPRED2, and SPRED3 across tissues from The Genotype Tissue Expression (GTEX) Project (https://www.gtexportal.org/home). (*B*) Protein expression of SPRED1 and SPRED2 across tissues from The Human Protein Atlas (https://www.proteinatlas.org).

length and weight, and had smaller skeletons compared with wild-type littermates (Tuduce et al. 2010). SPRED2$^{-/-}$ mice were smaller from birth and remained smaller throughout their lifetime, consistent with achondroplasia (dwarfism), a condition caused by activating mutations in FGFR3. FGF and FGFR3 signaling play major roles in the regulation of bone growth in both mice and humans. Indeed, FGFR3-induced Ras–MAPK activity is a negative regulator of bone growth (Colvin et al. 1996). Loss of SPRED2 in mice chondrocytes resulted in increased ERK1/2 phosphorylation in response to FGF, which likely inhibited chondrocyte differentiation and consequently bone growth (Bundschu et al. 2005). In another *SPRED2*$^{-/-}$ mouse model, generated by deleting exons encoding for KBD and SPR domain, adult mice appeared healthy with no apparent abnormalities in most organs (Nobuhisa et al. 2004). However, SPRED2 loss resulted in a significant increase in megakaryocytes, granulocytes, and hematopoietic cells (Nobuhisa et al. 2004). These results suggest that SPRED2 regulates hematopoiesis in the aorta-gonad-mesonephros by regulating c-Kit-mediated Ras–MAPK signaling during hematopoiesis.

The relatively mild phenotype of both SPRED1 and SPRED2 knockout mice may be due to redundancy when they are coexpressed in various organs. Double knockout of *SPRED1* and *SPRED2*, generated by backcrossing *SPRED1*$^{+/-}$ and *SPRED2*$^{+/-}$ animals, resulted in embryonic lethality (Taniguchi et al. 2007a). *SPRED1*$^{+/-}$; *SPRED2*$^{+/-}$ double heterozygotes were healthy and fertile. *SPRED1*$^{+/-}$; *SPRED2*$^{-/-}$ were smaller, consistent with the dwarfism phenotype of SPRED2 knockout mice. *SPRED1*$^{-/-}$; *SPRED2*$^{+/-}$ were born at severely sub-Mendelian frequencies, but several natal mice appeared sick and often died within a few months from uncharacterized causes. Most *SPRED1*$^{-/-}$; *SPRED2*$^{-/-}$ embryos died due to severe subcutaneous hemorrhage and edema between E12.5 to E15.5. This phenotype resembles mouse embryos lacking Syk, SLP-76, or PLCγ–2, which are hematopoietic intracellular signaling proteins important for separation of lymphatic vessels from blood vessels (Abtahian et al. 2003). Together, these suggest that lymphatic vessel development is abnormal in SPRED1/2 double-knockout embryos. In vitro, lymphatic endothelial cells from SPRED1/2 double knockout embryos proliferated more than wild-type cells. These lymphatic endothelial cells also exhibited stronger ERK activation following VEGF-C stimulation compared with wild type (Taniguchi et al. 2007a). Together, these results suggest that SPRED1 and SPRED2 are critical for the separation of lymphatic vessels from the parental vein by regulating VEGFR-3 induced Ras activation. Interestingly, lymphatic abnormalities commonly occur in patients with RASopathies, which further highlights the role of Ras–MAPK signaling in lymphatic vessels (Sevick-Muraca and King 2014).

Despite having a shared SPR domain, SPRED knockout mouse models have a distinct phenotype from SPROUTY knockout mouse models, except shared features with SPRED2 and SPROUTY4 knockout, both of which develop dwarfism (summarized in Table 2).

Drosophila *models*

Only one homolog of SPRED has been identified in *Drosophila*. Habituation deficits were reported in flies with neuron-specific knockdown of SPRED (Fenckova et al. 2019). Habituation is a form of learning that allows an organism's initial response to gradually decline following frequently repeated stimuli (Kimmel 1973). In humans, habituation deficits have been reported in behavioral disorders (Swartz et al. 2013; Ethridge et al. 2016). This phenotype relates to the behavioral deficits observed in SPRED1 knockout mice and Legius syndrome patients. Furthermore, knockdown of neurofibromin or expression of constitutively active Ras mutant recapitulated habituation deficiencies resulting from SPRED1 loss. Additionally, SPRED is involved in photoreceptor cell specification in *Drosophila* eye development (Demille et al. 1996); however, the role of SPRED in *Drosophila* development has not yet been elucidated.

Disease

Developmental disorders

Legius syndrome (LS) is an autosomal dominant condition characterized by multiple café au lait spots, axillary freckling, macrocephaly, and Noonan-like craniofacial

Table 2. *SPRED and Sprouty mouse models*

Genotype	Phenotype	References
SPRED1$^{-/-}$	Shortened face, lower body weight, and allergen-induced airway eosinophilia from IL-5-mediated eosinophil proliferation	Brems et al. 2007
	Spatial learning and memory defects from impaired hippocampal functions	Denayer et al. 2008
	Pigmentary abnormalities and shortened face	Tadokoro et al. 2018
SPRED2$^{-/-}$	Dwarfism from increased FGFR signaling in chondrocytes	Bundschu et al. 2005
	Increase proliferation of megakaryocytes, granulocytes, and hematopoietic cells	Nobuhisa et al. 2004
SPRED1$^{-/-}$ SPRED2$^{-/-}$	Embryonic lethal between E12.5–E15.5 due to severe subcutaneous hemorrhage and edema	Taniguchi et al. 2007a
SPROUTY1$^{-/-}$	Kidney and ureteric bud defects from improper branching morphogenesis	Basson et al. 2005, 2006
SPROUTY2$^{-/-}$	Viable with shortened life span (median survival 3–4 wk) and hearing loss from perturbations in organ of Corti cytoarchitecture	Shim et al. 2005
	Viable with shortened life span and severe gastrointestinal phenotype characterized by enteric nerve dysplasia due to GDNF hyperactivity	Taketomi et al. 2005
SPROUTY1$^{-/-}$ SPROUTY2$^{-/-}$	Highly disorganized palatial rugae from disrupted submandibular gland epithelial development	Economou et al. 2012; Knosp et al. 2015
SPROUTY4$^{-/-}$	Dwarfism and polysyndactyly from hyperactivation of FGF signaling	Taniguchi et al. 2007b
SPROUTY2$^{-/-}$ SPROUTY4$^{-/-}$	Embryonic lethal by E12.5 with craniofacial, limb, and lung morphogenesis abnormalities	Taniguchi et al. 2007b, 2009
SPROUTY2$^{+/-}$ SPROUTY4$^{-/-}$	Tusk-like incisor in the lower jaw due to presence of enamel on lingual surface	Boran et al. 2009; Klein et al. 2008

dysmorphia in some individuals. Other frequently observed features are learning and behavioral problems (Brems et al. 2007; Pasmant et al. 2009). The disorder is caused by loss-of-function mutations in the *SPRED1* gene. The clinical phenotype of LS patients resembles a mild version of neurofibromatosis type I (NF1). NF1 is caused by loss-of-function mutations in the *NF1* gene. Some of the phenotypes associated with NF1 are due to haploinsufficiency, such as developmental problems and learning disorders. Others are caused by loss of the wild-type allele to generate clonal growth of café au lait macules and benign neurofibromas, some of which often progress to malignant tumors of the peripheral and central nervous system (Riccardi 1992). Both NF1 and LS are RASopathies; syndromes caused by germline mutations in genes coding for proteins that regulate the Ras–MAPK pathway resulting in hyperactive signaling (Tidyman and Rauen 2009).

Sequencing of LS syndrome patients identified that most *SPRED1* mutations result in protein truncations, although missense mutations and deletions also occur (Fig. 5A; Brems et al. 2007; Brems and Legius 2013). Biochemical analysis of some EVH-1 missense mutations showed disruption of the interaction with neurofibromin, while SPR mutations fail to localize to the membrane (Stowe et al. 2012; Hirata et al. 2016; Führer et al. 2017). However, missense mutations also map to regions of SPRED1 whose functions in regulating Ras are not yet clear (Fig. 5B; Sumner et al. 2011).

Consistent with pigmentation abnormalities found in knockout mice (Tadokoro et al. 2018), café au lait macules occur in all LS patients. Development of café au lait macules requires a second-hit mutation on the wild-type *SPRED1* allele, such that the melanocytes are devoid of SPRED1 (Brems et al. 2007). These findings are consistent with café au lait macules in NF1 patients that also involve biallelic *NF1* loss (Schepper et al. 2008).

Mutations in *SPRED2* and *SPRED3* have not been reported to cause developmental disorders in humans. Due to the overlapping functions of SPRED family proteins, it is likely that loss of function of either SPRED2 or SPRED3 does not cause a developmental phenotype because the other family members compensate for this loss. However, the emerging use of high-throughput sequencing in clinical genetics could reveal syndromes caused by these genes. Perhaps SPRED2 mutations could be associated with mild

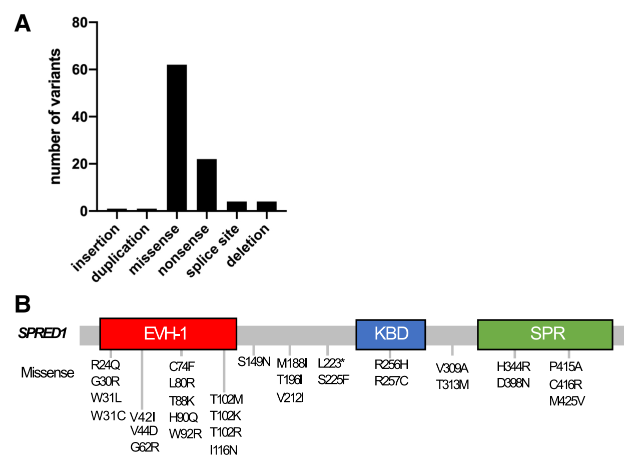

Figure 5. SPRED1 mutations found in Legius syndrome. (*A*) Frequency of each SPRED1 mutation variants found in Legius syndrome patients (http://arup.utah.edu/database/SPRED1/SPRED1_welcome.php). (*B*) Missense mutations identified in patients with Legius syndrome.

achondroplasia since loss of *SPRED2* in mice results in dwarfism (Bundschu et al. 2005).

Cancer

Hyperactivation of Ras–MAPK signaling contributes to the proliferation, survival, metastasis, and many other traits characteristic of tumor cells. Therefore, negative regulators of this pathway have been observed to protect against tumorigenesis. SPRED and Sprouty proteins are putative tumor suppressors due to their ability to down-regulate Ras–MAPK signaling (Kawazoe and Taniguchi 2019). By binding and translocating neurofibromin to the plasma membrane, SPRED may play a critical role in regulating cellular levels of Ras-GTP in many contexts (Stowe et al. 2012). Hence, loss of SPRED in tumor tissue likely permits hyperactivation of Ras signaling, which contributes to driving tumor growth and invasion.

SPRED1 mutations occur in ∼2% of cancers (TCGA). The most common alterations are deletions, truncations, and missense mutations (Fig. 6A). Currently, there are no known mutational hotspots for *SPRED1* (Fig. 6B); however, biochemical studies have shown that some EVH-1 and SPR missense mutations result in defective neurofibromin binding or membrane localization (Dunzendorfer-Matt et al. 2016; Hirata et al. 2016). Furthermore, some of the *SPRED1* missense mutations in cancer have also been found in Legius syndrome. Similarly, there are no known mutational hotspots for *SPRED2* (Fig. 6C). Further biochemical characterization is necessary to determine the biological significance of these mutations. Finally, the lower frequency of *SPRED3* mutations in cancer may relate to its restricted tissue expression (Figs. 4A, 6D).

Individuals with Legius syndrome have a predisposition for pediatric leukemia resulting from loss of heterozygosity (Pasmant et al. 2009). Likewise, NF1 is associated with a predisposition to juvenile myelomonocytic leukemia (JMML) and acute lymphoblastic leukemia (ALL) (Stiller et al. 1994), since loss of SPRED1 binding partner, neurofibromin, also results in a similar hyperactivation of Ras signaling. Furthermore, *SPRED1* somatic mutations occurred in 2% of pediatric acute leukemias, and *SPRED1* expression was significantly decreased in pediatric AML and T-ALL (Pasmant et al. 2015). In those samples, decreased SPRED1 protein and RNA levels correlated to high ERK phosphorylation (Pasmant et al. 2015).

SPRED1 loss was also found as a driver of mucosal melanoma where 37% of mucosal melanomas examined

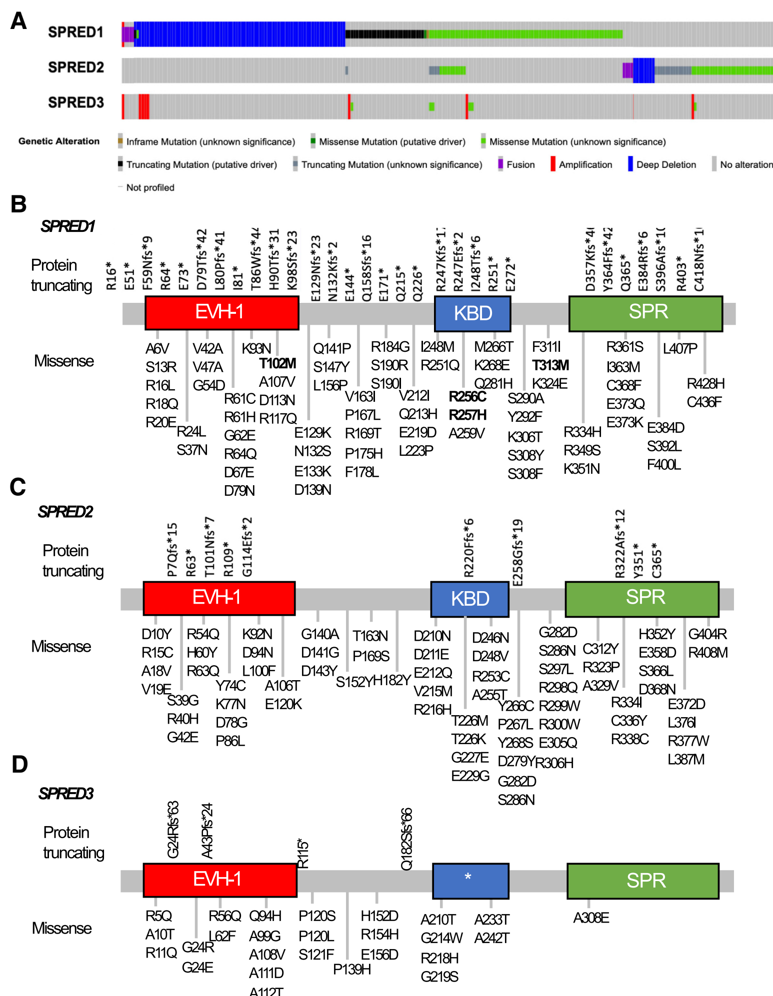

Figure 6. Mutations in SPRED proteins in human cancers. (*A*) Genetic alterations in SPREDs found in cancer (adapted from data from cBioPortal, https://www.cbioportal.org). (*B*) Truncating (*top*) and missense (*bottom*) mutations in SPRED1 found in cancer (TCGA; https://portal.gdc.cancer.gov). (*C*) Truncating (*top*) and missense (*bottom*) mutations in SPRED2 found in cancer (TCGA). (*D*) Truncating (*top*) and missense (*bottom*) mutations in SPRED3 found in cancer (TCGA).

showed S*PRED1* loss of function (Ablain et al. 2018). In most cases, melanomas were triple wild type for *BRAF*, *RAS*, and *NF1*. Notably, *c-KIT* activating alterations co-occurred in 30% of cases with *SPRED1* loss (Ablain et al. 2018). This suggests that *SPRED1* loss may cooperate with oncogenic events that activate the Ras–MAPK pathway.

In other cancers, although *SPRED1* is not mutated, SPRED1 mRNA and/or protein levels are reduced (Yoshida et al. 2006; Kachroo et al. 2013; Wang et al. 2017; Jiang et al. 2018). In oral squamous cell carcinoma tumor tissues, SPRED1 is significantly down-regulated due to the up-regulation of miR182 in malignant tissues that directly targets SPRED1 (Wang et al. 2017). Likewise, SPRED1 is significantly down-regulated in ER^+ breast cancer due to the up-regulation of miR-196a that directly targets SPRED1 (Jiang et al. 2018). In hepatocellular carcinoma, SPRED1 and SPRED2 were simultaneously down-regulated tumor tissue compared with noncancerous tissue (Yoshida et al. 2006). In prostate cancer, SPRED2 expression levels were significantly reduced in tumor tissue relative to benign glands, and SPRED2 expression was significantly down-regulated in grades 4 and 5 tumors (Kachroo et al. 2013). In all cases, down-regulation of SPRED correlated with increased ERK phosphorylation.

SPRED1 and *NF1* mutations tend to be mutually exclusive with gain-of-function *EGFR* mutations in lung adenocarcinoma (Collisson et al. 2014). In a model system expressing oncogenic EGFR L858R, SPRED1 was highly phosphorylated at S105 (Yan et al. 2020), which is in close proximity to T102, which is mutated to arginine in LS (Messiaen et al. 2009). Mutation of SPRED1 S105 resulted in disruption in neurofibromin-SPRED1 binding and augmented cell proliferation in K562 cells (Yan et al. 2020). Furthermore, SPRED1 S105 phosphorylation was found to be elevated in four additional EGFR-mutant cancer cell lines (PC9, U2OS, A431, and H1975) (Yan et al. 2020). To date, this is the only known mechanism by which RTK signaling directly regulates neurofibromin-SPRED1 interaction. Thus, it raises the possibility that SPRED1 function is normally regulated in response to signals to modulate Ras-GTP levels and downstream signal transduction.

Summary and future directions

SPRED proteins are crucial regulators of Ras–MAPK signaling. Although SPRED and Sprouty proteins share an SPR domain, each family regulates Ras through distinct yet complementary mechanisms. Sprouty limits GTP loading of Ras, while SPRED promotes Ras-GTP hydrolysis. Despite these findings, numerous unresolved questions remain regarding the function, regulation, interactions, and signaling control of SPRED proteins.

While the role of the EVH-1 and SPR domains in recruitment of neurofibromin to the membrane are clear, the role of the KBD in SPRED is not well understood. Specifically, what is the context of c-Kit binding to SPRED? Can SPRED bind other RTKs besides c-Kit? Is c-Kit binding necessary for efficient Ras inhibition? Does SPRED binding inhibit c-Kit signaling, especially Ras activation? Furthermore, c-Kit signaling is important for melanocyte development. Café au lait macules and axillary freckling are features of Legius syndrome and NF1 that affect melanocytes. Thus, is the interaction between SPRED and c-Kit what limits Legius syndrome phenotype to melanocytes, compared with other tissues, as in NF1?

The regulation of SPRED and neurofibromin binding needs to be further defined. Signals that regulate SPRED binding to the plasma membrane are not yet known. Sprouty proteins are regulated by phosphorylation and dephosphorylation of a conserved tyrosine. Hence, it seems likely that SPRED activity is regulated by phosphorylation, as well as dimerization and association with other protein partners. At present, little is known about the physiological functions of SPRED3. SPRED3 has the least sequence similarity to other SPRED proteins and exhibits reduced binding to neurofibromin and does not bind c-Kit (Kato et al. 2003; Hirata et al. 2016).

Generation of SPRED3 knockout mice is important to understand its biological function and further inform shared mechanisms of regulation for SPRED proteins. Understanding how SPRED proteins are regulated and in what signaling context will help the development of new therapies for RASopathies and cancers resulting from hyperactive Ras signaling.

Competing interest statement

F.M. is a consultant for the following companies: Aduro Biotech; Amgen; Daiichi Ltd.; Ideaya Biosciences; Kura Oncology; Leidos Biomedical Research, Inc.; PellePharm; Pfizer, Inc.; PMV Pharma; Portola Pharmaceuticals; and Quanta Therapeutics. F.M. is a consultant and cofounder for the following companies (with ownership interest, including stock options): BridgeBio; DNAtrix, Inc.; Olema Pharmaceuticals, Inc.; and Quartz. F.M. is Scientific Director of the NCI Ras Initiative at Frederick National Laboratory for Cancer Research/Leidos Biomedical Research, Inc.

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