Cooperative regulation of Ras/MAPK pathway signaling by Spred1 and neurofibromin: Insights into the molecular basis of Legius syndrome

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For the Sprouty family, without whom this work would not have been possible.
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Abstract

Cooperative regulation of Ras/MAPK pathway signaling by Spred1 and neurofibromin: Insights into the molecular basis of Legius syndrome.

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The Ras/Mitogen Activated Protein Kinase (MAPK) plays a critical role in cell signaling downstream of receptor tyrosine kinases. The conserved Sprouty family proteins function as feedback regulators of Ras/MAPK signaling. Loss of function mutations in one family member, SPRED1, cause Legius syndrome, an autosomal dominant human disorder that resembles neurofibromatosis type 1 (NF1). Spred1 functions as a negative regulator of the Ras/MAPK pathway, however, the underlying molecular mechanism is poorly understood. We found Spred1 functioned by reducing Ras-GTP levels; SPRED1 loss-of-function mutations identified in Legius syndrome patients were unable to regulate Ras activity levels. The N-terminal EVH1 domain of Spred1 was found to be crucial for its inhibitory function (point mutants in the EVH1 domain failed to function, and the EVH1 domain alone was sufficient to decrease Ras-GTP levels when localized to the plasma membrane). Thus, we utilized tandem affinity purification of wild-type Spred1 and EVH1 domain mutants found in Legius syndrome to identify novel binding partners of the N-terminus. Analysis of protein complexes by mass spectrometry revealed neurofibromin, the NFI gene product, as a novel Spred1 interacting protein. Here we show that neurofibromin is a Spred1 binding partner that is necessary for Spred1’s inhibitory function. Spred1 requires neurofibromin to down-regulate Ras-GTP levels, and we find that the interaction with Spred1 is required for neurofibromin localization to the
plasma membrane. This novel mechanism for neurofibromin regulation leads to a better understanding of the pathophysiology of these two overlapping developmental disorders.
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Chapter 1: Introduction
Ras Proteins

Ras genes were initially discovered as the transformative factors in the Harvey and Kirsten rat sarcoma retroviruses [1, 2]. Molecular techniques in the late 1970s and early 1980s allowed researchers to identify the ras genes as the initiating genes in experimental transformation and human tumor pathogenesis. The three ras genes were found to encode four highly homologous 21 kD proteins: Hras, Nras, and Kras4B and Kras4A (two proteins that result from alternative splicing) and were subsequently observed to be mutated in many human cancer cell lines [3]. It is now estimated that approximately 30% of all human tumors contain an activating mutation in one of the ras genes [4]. Specific ras oncogenes are preferentially mutated in distinct types of tumors: Kras mutant alleles are commonly found in pancreatic, lung and colon carcinomas, Hras in bladder carcinomas, and Nras in lymphoid malignancies, hematopoietic tumors and malignant melanomas [5]. The Ras proteins are critical regulators of cellular biology, relaying receptor activation to downstream effector pathways that control a diverse array of cellular fates, including proliferation, differentiation, migration, and survival.

Ras Superfamily

The tumor oncoproteins Nras, Hras, and Kras, are founding members of a larger superfamily of small GTPases that function as GDP/GDP-regulated molecular switches [6]. The Ras superfamily is comprised of more than 150 proteins that can be grouped into at least five subfamilies: Ras, Rho, Rab, Arf, and Ran. Beyond the shared biochemical activity of GTP hydrolysis, these proteins also share an ~20 kDa common core G domain which includes a set of conserved GDP/GTP-binding motif elements [7].
While each subfamily has been implicated in specific biological functions, crosstalk can commonly occur between them. In general, the Ras family is thought to be responsible for cell proliferation, Rho for cell morphology, Ran for nuclear transport, and the Arf and Rab families for vesicle transport. The Ras subfamily itself now includes at least twenty-one members: Hras, Kras (A and B), Nras, Rras, TC21-Rras2, Rras3/Mras, Rap1a, Rap1b, Rap2b, Rap2c, Rit, Rin, Rheb, Noey2, DiRas1/Rig, DiRas2, Eras, RalA, RalB, DexRas/RasD1, and RasD2/Rhes. Many of these Ras family GTPases (RFGs) have yet to be fully characterized, however, several appear to also regulate signaling networks [8].

Given the quantity and functional promiscuity of the RFGs, exactly how signaling specificity is achieved remains an important unanswered question. The ability of various RFGs to activate the same effector pathways complicates sorting out individual contributions to human diseases such as cancer. It has been proposed that signaling specificity can be attained by each RFG selectively interacting with distinct sets of effectors in a combinatorial fashion, as well as by selective interactions between isoforms of the same effector families [8].

**Ras lipid modification and membrane targeting**

Signaling specificity is an even bigger issue among the classical p21 Ras isoforms—Hras, Nras, Kras4B, and Kras4A—which are nearly identical over the majority of their sequence. These four Ras proteins share 85% sequence identity and differ primarily in the carboxyl terminus hypervariable region (HVR). The HVR contains a C-terminal CAAX motif (where C stand for cysteine, A for an aliphatic amino acid, and X represents any
amino acid) that can undergo covalent posttranslational modifications by isoprenylation. Modification of this region by lipids controls the affinity of Ras with the plasma membrane [9]. The CAAX motif and residues immediately upstream comprise the membrane-targeting sequences that can dictate interactions with specific membrane compartments and subcellular locations.

Posttranslational farnesylation and carboxylmethylation of Ras proteins is required for directing their localization to the endoplasmic reticulum [10], and secondary targeting motifs in the HVRs stabilize them in distinct plasma membrane microdomains. The Kras4B HVR contains a stretch of polybasic residues and processed Kras primarily localizes to the plasma membrane [11]. In contrast, the Nras and Hras HVRs contain cysteine residues that become palmitoylated and thus constantly shuttle between the Golgi and plasma membrane in a depalmitoylation/repalmitoylation cycle [12, 13]. Therefore, the functional differences among the various Ras proteins may partially be explained by their differential subcellular compartmentalization.

Furthermore, activation of Ras to Ras-GTP has been shown to limit the mobility of Ras within the inner leaflet of the membrane. This confinement results in the sequestration of active Ras to small, densely packed nanoclusters that have isoform specific lipid and protein contents [14-24]. Therefore, the formation of these distinct nanoclusters can also generate signaling specificity among the Ras isoforms. Dissecting the complexity of signaling specificity among the RFGs will further our understanding of how different
RFGs promote differing cellular functions as well as aid in designing strategies for targeting diseases where these pathways are frequently deregulated.

**The GTPase cycle and oncogenic Ras**

Ras proteins act as binary molecular switches that cycle between an active guanosine triphosphate (GTP)-bound state, and an inactive guanosine diphosphate (GDP)-bound state [9]. Ras activation depends on ejection of the GDP nucleotide from the Ras binding site, which promotes passive association with the more abundant cellular GTP nucleotide. In the biologically active GTP bound form, pronounced structural changes occur in the two loop regions of the Ras proteins, Switch I and Switch II. These conformational changes create a high affinity binding surface for downstream effector proteins, which require binding to Ras for their full activation. Hydrolysis of GTP to GDP inactivates Ras due to a lower affinity of the GDP bound form for effector proteins. However, Ras possesses low intrinsic GTP hydrolysis and GDP/GTP exchange activities. Therefore, the on-off switch of Ras GTPases requires further regulation by GTPase activating proteins (GAPs)—which enhance the low intrinsic GTPase activity—and GTPase exchange proteins (GEFs)—which promote the exchange of GDP for GTP [25].

GEFs enhance the rate of GDP dissociation from the nucleotide binding cleft; thus promoting the passive insertion of GTP due its 10 fold increased abundance in the cytosol. The first RasGEF to be identified was the yeast cell-division-cycle-25 (CDC25) protein. Its mammalian counterpart, son of sevenless (SOS)—named for its importance in the development of the *D. melanogaster* compound eye—was identified several years later.
Initial models for SOS activation of Ras suggested that recruitment of SOS to the plasma membrane by RTKs was sufficient to recruit and activate membrane localized Ras [28, 29]. Although it has been found that complex formation can be augmented by increased local concentrations at the membrane, this model did not fully explain SOS mediated Ras activation [30]. Structure-function studies of SOS and Ras revealed that SOS contains an allosteric binding site for Ras, distal to the catalytic site [11, 31]. This second binding site is specific for nucleotide bound Ras, but binds RasGTP with a higher affinity than RasGDP [32-34]. Binding of Ras to the allosteric site relieves SOS from an autoinhibited state, thus inducing low GEF activity that can generate RasGTP. Binding of RasGTP to the same site can further increase SOS activity, providing a positive feedback loop by which to regulate Ras signaling output in response to various stimuli [35].

GAPs perform the opposite reaction as GEFs, accelerating the hydrolysis of GTP to GDP, thereby promoting the allosteric change in Ras to its inactive state. Molecularly, GAP mediated GTP hydrolysis occurs via the RasGAP arginine finger stabilization of a high energy transition state. The first member of the RasGAP family to be identified was p120GAP [36, 37], which was soon followed by the discovery of another RasGAP, neurofibromin [38, 39], a protein found to be disrupted in the genetic disorder Neurofibromatosis-1.

Interestingly, the majority of somatic Ras point mutations found in human cancer occur at residues 12, 13 and 61 [40]. These amino acid substitutions impair the intrinsic GTPase activity and confer resistance to GAPs, locking Ras in a constitutively active GTP bound
state. Oncogenic mutations at positions 12 and 13 prevent the formation of a stable transition state by sterically blocking the proper orientation between the GAP arginine finger and glutamine 61 [41-43]. Mutations at glutamine 61 abolish GAP mediated GTP hydrolysis. Ras activation by these oncogenic mutations leads to a deregulation of important cellular signaling pathways in many human cancers.

**Ras Signaling Pathways**

In response to various extracellular stimuli, such as soluble growth factors and cytokines, Ras proteins function as signaling nodes to help relay extracellular signals to the nucleus (fig. 1.1). Binding of growth factor ligands to their appropriate receptor tyrosine kinase (RTK) induces dimerization and receptor autophosphorylation [11, 28, 44, 45]. Ligand induced conformational changes and phosphorylation creates intracellular docking sites for adaptor molecules such as growth factor receptor-bound protein 2 (GRB2). Grb2 can in turn recruit SOS to Ras, which accelerates the exchange of GDP for GTP on Ras. In addition to recruiting GEFs to the plasma membrane, RTK activation can also recruit p120GAP to downregulate Ras activity [46]. In its active GTP bound form, Ras can productively interact with more than 20 Ras effectors, including Raf, phosphatidylinositol 3-kinase (PI3K), RalGDS [47]. By interacting with multiple, catalytically distinct downstream effectors, Ras can regulate various multiple cellular processes, including gene expression, proliferation, differentiation, and cell survival.

The Ras/Raf/MEK/ERK signaling cascade is the best characterized Ras effector pathway. Also termed the mitogen activated protein kinase (MAPK), this pathway plays a critical
role in relaying extracellular growth and mitogenic signals [4, 5]. The effector domain of activated GTP-bound Ras interacts directly with the serine/threonine Raf kinases (A-Raf, B-Raf, and C-Raf/Raf-1) through the Raf Ras-binding domain (RBD) and forms a secondary interaction via Raf’s cysteine rich domain [48-51]. Binding to Ras recruits the Raf kinase to the plasma membrane and induces the release of inhibitory 14-3-3 proteins from Raf’s N-terminal regulatory region, allowing for Raf activation by a series of positive phosphorylation events [52, 53]. Since Ras has been reported to form dimers [54], Ras activation is also hypothesized to promote Raf homo- and heterodimerization among the various Raf isoforms [55-57]. Physical juxtaposition of Raf proteins creates an allosteric interaction between the two kinase domains that has been shown to be critical for stimulating Raf kinase activity [24, 58].

Activated Raf kinases initiate a phosphorylation cascade, beginning with the phosphorylation and activation of the serine/threonine kinase MEK (MAPK extracellular signal regulated kinase kinase), which in turn phosphorylates and activates the mitogen-activated protein kinases (MAPK) ERK1 and ERK2. Once activated, ERK phosphorylates numerous nuclear and cytoplasmic substrates to carry out the cellular response designated by the initial signal [59-62]. Several of the participants in this pathway have been found to be mutated in human disease. ERK is hyperactivated in approximately 30% of human cancers, and activating mutations in one of the three Raf isoforms, Braf have been found in greater than 60% of human malignant melanomas, and in some colon, thyroid and lung tumors [63]. Although Raf-1 mutations are significantly less frequent, Raf-1 has been found to be overexpressed in several human malignancies,
including AML and ovarian cancer. And it has been demonstrated to be required for the initiation and maintenance of squamous cell carcinoma and for the initiation of lung cancer by the oncogenic Kras G12D allele [64, 65]. The MAPK pathway has also been shown to be necessary for Ras induced transformation of the murine NIH3T3 cell line [66-69].

Another well-characterized Ras effector pathway is the PI3’K pathway. RTK activation of Ras leads to activation of the lipid kinase phosphatidyl inositol 3’ kinase (PI3’K) through Ras binding of the catalytically active p110 subunits [70, 71]. In turn, PI3’K generates membrane bound 3’ phosphorylated phosphoinositides that act as secondary messengers and can recruit downstream targets containing pleckstrin homology (PH) domains. One of these proteins, the Akt serine/threonine kinase, has emerged as a critical downstream target of PI3K [72, 73]. Aberrant gain of Akt activation is a common feature of many cancer cells. Upon recruitment to the plasma membrane, Akt becomes activated by two sequential phosphorylations. First, PDK1 phosphorylates the activation loop of Akt, followed by phosphorylation of the carboxyl-terminus hydrophobic motif by the TORC2 complex. The latter phosphorylation on Ser 473 of Akt can be dephosphorylated by PHLPP (PH domain leucine rich repeat containing protein phosphatase). Additionally, Akt activity can be opposed by the tumor suppressor PTEN, which terminates PI3K activity by dephosphorylating phosphoinositides [74]. Like the MAPK pathway, the PI3K pathway has been shown to required for Ras mediated NIH3T3 transformation [75].
Additional Ras effectors

Beyond the well characterized Raf and PI3K effectors, additional Ras effectors exist whose preferential binding to active GTP-bound Ras results in activation. Several of these additional effectors, such as RalGDS, Tiam1, and PLCε, have also been implicated in Ras driven tumorigenesis [76]. Most of the well studied Ras effectors share a region of weak homology, termed the Ras-activating (RA) or Ras binding (RBD) domain, which binds to the effector loop of Ras proteins [52]. Although the RA/RBD domains of Ras effectors have low sequence homology, they share considerable structural similarity and form ubiquitin-like folds. However, some well-defined Ras family effectors lack this motif, such as adenylate cyclase in *S. cerevisiae* and Shoc2 in vertebrates. In yeast, adenylate cyclase is the major downstream effector of Ras1 and Ras2, the functional and biochemical homologues of mammalian Ras [77]. It functions as an activator of the cyclic AMP/PKA pathway. In vertebrates, Shoc2 functions as an adapter protein, recruiting the phosphatase PP1C to dephosphorylate an inhibitory serine on Raf [78]. These two effectors bind Ras family members through their leucine rich repeat (LRR) domains.

Ras in Developmental Disorders

Aberrant Ras signaling has been shown to cause more than oncogenic transformation. Although somatic gain of function mutations in Ras are a well known event in human tumorigenesis, inherited germline mutations that affect the Ras/MAPK pathway have also been identified [4]. Mutations in genes encoding for Ras proteins and Ras regulators have been found to be the underlying cause in several congenital developmental disorders,
suggesting that deregulated Ras signaling can also lead to aberrant development (fig. 1.2). These disorders have been termed the cardio facio cutaneous diseases, or Rasopathies, because they share clinically distinct features such as facial dysmorphism, cardiac defects, reduced growth, learning disabilities, and a predisposition to certain malignancies. They include Neurofibromatosis-1 (NF1), Costello, Noonan, Leopard, CFC, and Legius syndrome, and result from mutations in components of the Ras/MAPK pathway, such as NF1, PTPN, SOS1, Braf, Mek1/2, and even from activating mutations in Ras itself.

Ras and Neurofibromatosis

Neurofibromatosis type 1 (NF1) was first documented as a distinct disorder by Fredrich von Recklinghausen in 1882. NF1 is the most common dominant human autosomal disorder, affecting roughly 1 in 3,500 people worldwide. It is caused by mutations in the NF1 gene, which encodes the RasGAP neurofibromin [38, 39]. Loss of neurofibromin leads to hyperactive Ras signaling, as observed by elevated Ras activities in NF1 patients [79-81]. NF1 disease primarily affects cells of neural crest origin, in particular Schwann cells and melanocytes.

NF1 is characterized by the growth of multiple neurofibromas: benign nerve sheath tumors that arise from Schwann cells, and are comprised of a heterogeneous mix of Schwann cells, endothelial cells, fibroblasts, and inflammatory mast cells [82, 83]. Several types of neurofibromas are found in NF1: cutaneous, subcutaneous, and plexiform neurofibromas. Plexiform neurofibromas affect 25-40% of NF1 patients and
are a major source of morbidity and mortality. Interestingly, sporadic mutation of the remaining wild-type NF1 allele has been detected in the malignant tumors of NF1 patients [82, 83]. Although this loss of heterozygosity (LOH) in Schwann cells is required for neurofibroma formation, a heterozygous NF1 environment in the nearby inflammatory mast cells is also required, as well signaling from the c-Kit tyrosine kinase receptor [84-86]. Studies in NF1+/- mice demonstrate that several aspects of NF1 can arise from constitutive haploinsufficiency of NF1. NF1+/- mouse models show an increase in NF1+/- mast cell activity toward NF1 deficient Schwann cells in an NF1 tumor microenvironment. Heterozygous NF1+/- mouse models have also shown that deficits in c-Kit signaling and an increase in levels of secreted growth factors by mast cells greatly contribute to inflammation and tumor development.

Clinical characteristics of NF1 also include café-au-lait spots, axillary freckling, lisch nodules in the iris of the eyes, optic pathway gliomas, bone deformations, and learning disabilities. In addition, patients with NF1 are also predisposed to a variety of hematopoietic and neuronal malignancies, including malignant peripheral nerve sheath tumors (MPNST), melanoma, leukemia, gliomas and pheochromoytomas. MPNSTs occur in 5 to 10% of NF1 patients, usually arising from a plexiform neurofibroma.

Neurofibromin, the NF1 gene product, is a tumor suppressor that regulates Ras activity by increasing the intrinsic GTPase activity of the wild-type protein. Neurofibromin is a 2818 amino-acid protein, containing a central GAP related domain (GRD) with significant sequence homology to p120GAP [38, 39]. The GRD domain was
characterized twenty years ago, yet it accounts for only 10% of the protein, and little is known about the regulation and function of the other neurofibromin domains. C-terminal to the GRD exists a bipartite lipid-binding region comprised of a Sec14 homologous and pleckstrin homology (PH)-like domain that is thought to mediate binding to membrane phosphotidylinositols [87, 88]. Other neurofibromin domains include the tubulin binding domain (TBD), a cysteine-serine rich domain (CSRD) thought to be important for GAP regulation, and a nuclear localization domain (NLS) [89-91]. Approximately 10% of NF1 derived patient mutations are missense mutations that span the entire neurofibromin protein, suggesting neurofibromin may have other essential functions beyond its GAP activity.

Regulation of neurofibromin activity remains poorly understood. Neurofibromin can be positively regulated by PKC phosphorylation in response to growth factor stimulation, as well as by the cross-linking of surface immunoglobulin [92-94]. In contrast, neurofibromin is negatively regulated by PKA dependent phosphorylations that increase its association with 14-3-3 proteins [95]. It has also been reported to be negatively regulated by proteasomal degradation following growth factor stimulation, potentially by the ubiquitin conjugating protein ETEA [96-98]. Finally, neurofibromin has also been described to interact with the syndecan family of proteins to induce filopodium formation via a PKA mediated pathway [99-101]. Neurofibromin’s interaction with syndecan has been suggested to enable recruitment of neurofibromin to the plasma membrane, yet evidence for the recruitment of neurofibromin to Ras through a syndecan interaction has
yet to be presented. Identifying the unknown functions and regulatory partners of neurofibromin will be critical in defining therapeutic targets for NF patients.

**Legius Syndrome and Spred1**

Legius syndrome was first described as a Neurofibromatosis Type-1 like syndrome [102]. Individuals with Legius syndrome exhibit common NF1 symptoms such as café-au-lait macules, axillary freckling, macrocephaly, Noonan-like facial dysmorphism, and learning disabilities, but fail to display the increased tumor burden observed in NF1. These patients also lack mutations in the NF1 gene. A genome wide linkage analysis scan revealed Legius syndrome to result from causative mutations in the *SPRED1* gene. The protein product, Spred1 (Sprouty related EVH1 domain containing protein 1) (fig. 1.3), had previously been identified as a negative regulator of the Ras/MAPK pathway [103]. Initial experiments revealed that Spred1 mutations were loss of function mutants, incapable of inhibiting the Ras/MAPK pathway. Additional clinical studies now suggest that *SPRED1* mutations account for at least 2% of the pathogenic mutations associated with patients clinically diagnosed with NF1 [104-107].

The Spred1 protein functions as an inhibitor of Ras/MAPK signaling induced by multiple growth factors, cytokines, and chemokines, but its exact mechanism has yet to be elucidated [108-111]. Spred1 is a 444 amino acid, 50 kD protein comprised of a tripartite domain structure containing an N-terminal Ena-Vasp Homology 1 (EVH1) domain, internal c-Kit binding domain (KBD), and C-terminal Sprouty (SPR) domain [110]. Three isoforms have been identified in vertebrates, Spred1, 2, and 3, which all contain
EVH1 and SPR domains [112, 113]. Spred3 lacks an internal KBD, but is still able to inhibit the MAPK pathway, although not as efficiently. Two Spreds are present in Xenopus, and a Drosophila orthologue, AE33, has been described to regulate photoreceptor development [109]. Tissue expression of the three mammalian homologues varies: Spred1 is expressed predominantly in the brain and some fetal tissues, and Spred2 is ubiquitously expressed in adults. Spred3 is expressed primarily in the brain, and a splice variant of Spred3, Eve3 is expressed in the developed liver [114-116]. The Spred family shares its C-terminal SPR domain with the Sprouty family of proteins, also recognized negative regulators of the Ras/MAPK pathway. Sprouty proteins were initially identified in Drosophila as antagonists of fibroblast growth factor (FGF) signaling critical for lung branching morphogenesis [117, 118]. Yet, while the Sprouty proteins have highly variable N-termini, the Spreds have a highly conserved N-terminal EVH1 domain.

The EVH1 domain is a 110 amino acid protein-protein interaction domain that has been shown to bind distinct poly-proline regions [119]. It exists in members of the Ena/Vasp family of proteins, which function actin-cytoskeleton remodeling. The crystal structure of the Xenopus Spred1 EVH1 domain has been solved and reveals that the Spred1 domain adopts a PH domain fold and differing slightly from previously observed EVH1 domains [120]. It is therefore postulated that it may bind less proline rich sequences. A ligand for the EVH1 domain of Spred1 has yet to be identified, although the EVH1 domain has been shown to be critical for Spred1’s inhibitory function. Deletion or replacement of the N-terminus of Spred1 with that of another EVH1 domain containing
protein, WASP, eliminates Spred1’s negative regulatory activity [103]. The Spred3 splice varient, Eve-3, consists of a sole EVH1 domain, and is still able to exhibit inhibitory activity towards the Ras/MAPK pathway [112]. Finally, the majority of the \textit{SPRED1} mutations identified in Legius syndrome are truncation mutations, yet twelve in-frame missense mutations have been detected, nine of which reside in the N-terminal EVH1 domain. Together, this evidence suggests that the N-terminal EVH1 domain is critical for Spred1’s function, and identifying potential ligands for this domain may help elucidate Spred1’s mechanism.

The central Spred1 c-Kit binding domain (KBD) is a unique receptor tyrosine kinase binding domain by which Spred1 was initially identified. A yeast two-hybrid binding assay utilizing the catalytically active c-Kit and c-Fms tyrosine kinase domains as bait identified Spred1 from an osteoclast cDNA library. Spred1 deletion mutants mapped the c-Kit binding region to 50 amino acids in the center of the Spred1 protein. The KBD is not related to any previously identified tyrosine kinase interaction domains such as SH2, PTB, or the c-Met binding domain, and has yet to be identified in proteins outside the Spred family. Stimulation of cells with the c-Kit ligand, SCF, leads to tyrosine phosphorylation of Spred1 and Spred2, suggesting it may be regulated by the c-Kit receptor. Although the KBD was paramount in discovering the Spreds, it appears to not be necessary for their inhibitory function. When deleted from Spred1 or Spred2, the proteins are still able to inhibit the Ras/MAPK pathway, although not as efficiently. Moreover, both splice isoforms of Spred3 lack a functional KBD, yet are still able to function.
Finally, the Spred proteins also contain a highly conserved, cysteine rich C-terminal SPR domain. This domain is analogous to the Sprouty family C-terminal domain. Therefore, many of the interacting proteins of the Sprouty C-terminal domain have also been shown to interact with the Spred C-terminus. The SPR domain has been implicated in plasma membrane localization of the Spred and Sprouty family of proteins through palmitoylation and interactions with caveolin-1 and phospholipids[121-123]. C-terminal deletion mutants of Spred1 fail to efficiently localize to the plasma membrane, and have been suggested to function as dominant negative forms of Spred1, as their overexpression can augment growth factor induced ERK activation. The SPR domain of Spred1 has also been implicated in binding most of Spred1’s known interaction partners, including Raf1, RhoA, Tesk1, Dyrk1a, and c-Cbl [124-129].

Although Spred family proteins have been implicated in inhibiting the Ras/MAPK pathway, their exact mechanism has yet to be determined. Conflicting reports place Spred1 as functioning both upstream and downstream of Ras [103, 130]. Spred1 can interact with both Ras and Raf, and therefore has been suggested to act between the two proteins, preventing Raf’s activation. Experimental evidence suggests that overexpression of Spred1 could increase Raf’s recruitment to the plasma membrane with Ras, without stimulating Raf activation. Yet, this mechanism was never fully refined, and fails to explain how Spred1 prevents Raf activation. Furthermore, a separate report suggests Spred1 may prevent Ras activation, as evidenced by decreased Ras-GTP levels when
Spred1 is overexpressed [130]. Therefore, determining Spred1’s mechanism will clarify if whether Spred1 can function upstream or downstream of Ras.

The phenotype of Spred1 and Spred2 null mice has implicated the Spred proteins in several physiological processes. Spred1−/− mice are smaller than their wildtype counterparts and display facial dysmorphia, as well as deficits in hippocampal dependent learning [131]. Learning deficits in Spred1 knockout mice are more severe than those in NF1+/− mice. Hematopoietic cell specific Spred1-conditional knockout mice showed increased cutaneous mast cell proliferation, similar to NF1+/− mice. Spred2−/− display signs of dwarfism [132], while the Spred1/2 double knockout result in embryonic lethality, suggesting Spred1 and Spred2 may be able to partially compensate for each other. Multiple studies on the Spred knockout mice have identified the Spred proteins as regulators of bone morphogenesis, hematopoietic processes, and allergen induced airway eosinophilia and hyperresponsiveness [133].

The Spreds have also been implicated in tumorigenesis. When the osteosarcoma cell line LM8 was injected into nude mice, Spred1 expressing cells inhibited tumor growth and metastasis in comparison to control mice [127]. Wound healing and chemotaxis assays also demonstrated that the overexpression of Spred1 could dissolve actin-stress fibers and inhibit chemokine-induced migration. Finally, reduced expression levels of Spred1 and 2 were found in human hepatocellular carcinoma (HCC) tissue samples, and Spred expression levels inversely correlated with tumor aggressiveness [134, 135].
Figure 1.1 Ras signaling pathways. Binding of the appropriate ligand to receptor tyrosine kinases (RTKs) results in activation by dimerization and/or phosphorylation. Tyrosine phosphorylation recruits adapter proteins such as Grb2, which in turn recruits the RasGEF SOS. SOS aids in the exchange of GDP for GTP on Ras, activating the small GTPase. Activated Ras can activate multiple downstream effector pathways dependent on the initiating signal.
Figure 1.2 RASopathies associated with the dysregulation of various components of the Ras/MAPK signaling pathway. RASopathies or neuro cardio facio cutaneous (NCFC) syndromes are caused by defects in genes involved in the Ras/MAPK signal transduction pathway. Individuals with these multi-systemic syndromes present with phenotypes that include craniofacial dysmorphism, congenital heart defects, skin and genital abnormalities, and developmental delay.
Figure 1.3 Spred1 and related proteins. Spred1 and Spred2 are comprised of three conserved domains: an N-terminal EVH-1 domain, internal c-Kit binding domain (KBD), and C-terminal Sprouty (SPR) domain. The SPR domain is conserved in the Sprouty family of proteins, which include Sprouty 1-4 and Spred 1-3. The EVH-1 domain is conserved among Ena/VASP proteins: proteins typically involved in actin and cytoskeleton remodeling.


Chapter 2: Materials and Methods
**Plasmid constructs**

N-terminal tagged full-length Spred1, R325X and M266fsX4 were made by Gateway cloning (Invitrogen) according to the manufacturer’s instructions. Briefly, a Spred1 image clone was obtained from Open Biosystems and PCR cloned into the pENTR vector (Invitrogen). Spred1 truncation mutants were also PCR cloned into separate pENTR vectors. Genes were transferred into Destination vectors using recombination-mediated Gateway technology (Invitrogen, Table 2.1). *SPRED1* point mutants were made by PCR-directed mutagenesis and verified by sequencing. RFG and Raf constructs have been previously described [1, 2]. Full-length NF1 was synthesized by GeneArt. It was transferred to various Gateway Destination vectors as described above (Invitrogen) The N-terminus GS TAP cassette was obtained from the Euroscarf collection [http://web.uni-frankfurt.de/fb15/mikro/euroscarf/index.html](http://web.uni-frankfurt.de/fb15/mikro/euroscarf/index.html). It was moved into an inducible Gateway Destination plasmid (pDEST30) by standard PCR targeting techniques.

**Cell Culture**

Unless otherwise noted, cells were cultured under 7% CO₂ at 37°C in Dulbecco’s modified Eagle’s medium (Gibco/Invitrogen) supplemented with 10% (v/v) fetal bovine serum (FBS). Cell culture media was changed every 2 to 3 days, and cells were passaged when they reached 70 to 90% confluency. Tetracycline-inducible Spred1 cell lines were constructed as follows. Full-length cDNAs encoding human Spred1 were cloned into the pDEST30 or pDEST31 plasmids and transfected into HEK 293 TREX cells (Invitrogen). Stable clones were selected with neomycin and blasticidin, according to the
manufacturer’s protocol. PC3 cells were maintained in RPMI-1640 (Gibco/Invitrogen) supplemented with 10% (v/v) FBS. \( NF1^{-/-} \) 9223 mouse cells, \( NF1^{+/+} \), \( NF1^{+/-} \), and \( NF1^{-/-} \) MEFs were a kind gift from J. Nakamura (UCSF).

**PC12 Cell Differentiation**

PC12 cells were cultured in RPMI-1640 medium supplemented with 10% (v/v) horse serum and 5% (v/v) FBS. Forty-eight hours prior to differentiation, cells were plated at 1 x 10^4 cell/cm² in collagen IV coated cell culture dishes (BD Biosciences). For differentiation, medium was replaced with differentiation medium (RPMI-1640 supplemented with 0.5% (v/v) FBS and 50 ng/mL NGF 2.5S (Invitrogen)). Cells were fed every other day with fresh differentiation medium until ready for harvesting (1-14 days).

**Preparation of whole cell extracts**

For mammalian experiments, 1 x 10^6 cells were seeded in 6 well dishes 24 hours prior to transient transfection with 2 µg total plasmid DNA and 10 µL of Lipofectamine 2000 according to the manufacturer’s instructions (Invitrogen). If necessary, 24 hours post transfection, cells were serum starved (0.5% FBS) overnight. The next day, cells were stimulated with the appropriate growth factor (primarily EGF – 10 ng/mL) and lysed 48 hours post transfection in 350 µL of 1% TX100-TNM (20 mM Tris-HCl, pH 7.5, 500 mM NaCl, 0.1% Triton X-100, 1 mM DTT, phosphatase inhibitor cocktails II and III (Sigma), and a protease inhibitor cocktail (Sigma)) or 1% TX100-TNE ((20 mM Tris-HCl, pH 7.5, 500 mM NaCl, 0.1% Triton X-100, 1 mM DTT, phosphatase inhibitor cocktails II and III (Sigma), and a protease inhibitor cocktail (Sigma)). Lysates were
clarified by centrifugation at 14,000 rpm in a microfuge at 4°C. Protein concentrations were determined by Bradford assay using BSA as a standard. Normalized lysates were used for western blotting and immunoprecipitation.

**siRNA Transfections**

Transfection of siRNA oligonucleotides into cells was performed with RNAiMAX (Invitrogen) according to the manufacturer’s protocol. Briefly, cells were transfected with 4 µL of siRNAs at 20 mM and 2 µL RNAiMAX. Fresh medium was added to the cells 3 to 4 hours post transfection. The following day, the siRNA transfections were repeated. Forty-eight hours after the first transfection, cells were serum starved overnight. Seventy-two hours post the initial transfection, the appropriate growth factor was added and cells were harvested for further analysis.

The following target sequences were used:

Spred1_5: TTCACGTATCATTTCTGCTAAA
Spred1_7: AACGATAATAGTTATGACGA
Spred1_8: TAGGGTCCCTTTGAAATCAAT
Spred2_7: CAGCGGAAATGGGATGTGAGA
NF1_6: CAGGTGGCTTGGGATCAATAA
NF1_7: TACAGTAATAGCAGCTAACC

Non-silencing control siRNAs were from Dharmacon.
Immunoprecipitation

Following the preparation of normalized total cell lysates, approximately 200 to 600 µg of total protein was immunoprecipitated with the indicated antibodies or resins. Protein samples were rotated at 4°C for 2 hours and if appropriate, mixed with protein G-Sepharose 4 Fast Flow beads (GE Healthcare) for 1 hour. The beads were washed 3 times with wash buffer (20 mM Tris-HCl, pH 7.5, 500 mM NaCl, 0.1% Triton X-100) and boiled in 1.5X SDS-PAGE sample buffer for 10 minutes. Samples were separated by pre-cast NuPage gel electrophoresis (Invitrogen) followed by immunoblotting using the indicated antibodies.

Ras Activation Assays

To assay for GTP-bound Ras, normalized whole cell lysates were used for immunoprecipitation with recombinant GST-tagged Raf Ras binding domain (RBD) immobilized on glutathione sepharose. The GST-RBD fusion proteins were prepared as previously described [3, 4]. Affinity precipitation and identification of Ras-GTP levels were performed as described in [3, 5].

Recombinant Protein Purification

Recombinant GST-GFP, GST-RBD, GST-Spred1, and GST-M266fsX4 vectors were expressed in BL21 AI E.coli (Invitrogen) and cultured overnight in 100 mL of LB broth containing 100 µg/mL ampicillin. The following day, 50 mL of the overnight culture was added to 1L of LB broth and grown to a D₆₀₀ of 0.5-0.8. Protein synthesis was induced with the addition of 25 mg of L-arabinose and incubated for 2 hours at 32°C before
harvesting. Cultures were lysed in 1X PBS containing 200 mM NaCl, 1 mM DTT, 1 mM PMSF, 1 mM benzamidine, 1 ug/mL leupeptin, 1 ug/ml pepstatin, 1% Triton X-100 with sonication. Purification was done with Glutathione Sepharose 4B (Amersham Biosciences) according to the manufacturer’s protocol.

**In vitro interaction assays**

In vitro interaction assays with His-Ras were performed as described [1, 2]. Recombinant Nras and Kras were loaded with either GDP or non-hydrolyzable GTPγS by incubation for 10 minutes at 37°C in loading buffer (20 mM Hepes, pH 7.5, 50 mM NaCl, 5 mM EDTA, 5 mg/mL BSA). Reactions were stopped by placing the tubes on ice and adding MgCl₂ to a final concentration of 20 mM. Ras loaded with either GDP or GTPγS was incubated with 20 µL of packed glutathione beads bound to the appropriate GST-tagged protein, in 300 µL of binding buffer (PBS, 10 mM MgCl₂, and 0.1% Triton X-100). Following rotation for 2 hours at 4°C, samples were washed 3 times with binding buffer, drained, and 1.5X SDS sample buffer was added. After separation with precast NuPage gels (Invitrogen) and western blotting, immunoblots were probed with Ras, His, and GST antibodies.

**Tandem Affinity Purification and Mass Spectrometry**

HEK293 TREX cells (Invitrogen) stably expressing tetracycline inducible TAP-tagged wt Spred1, R325X-CAAX, M266fsX4-CAAX, or other baits were plated in 5 x 15 cm dishes (figure 2.1). Twenty-four hours following induction with doxycycline (1 ug/mL), cells were stimulated with EGF (10 ng/mL). Pooled cell lysates were purified and
analyzed as described [6, 7]. Briefly, cells were lysed in 1% NP-40-TNE lysis buffer and cleared by centrifugation. Lysates were incubated with 300 µL of rabbit-IgG agarose (Sigma) and allowed to rotate at 4°C overnight. Resin beads were washed with lysis buffer 3 times and once with TEV-protease cleavage buffer. Bound complexes were eluted by the addition of 40 µg TEV protease for 2 hours at 37°C. The TEV-protease cleavage product was allowed to elute into a second column by gravity flow. The second round of affinity purification was performed using Ultralink Immobilized Streptavidin Plus (Pierce) at 4°C for 2 hours. Following three washes in lysis buffer, bound proteins were eluted with 200 µL elution buffer containing 1 mM D-biotin (Sigma). Protein complexes were precipitated by TCA precipitation. Briefly, DOC was added at a final concentration of 0.015% and TCA at a final concentration of 10%. The samples were incubated on ice at 4°C overnight. The following day, the precipitated samples were centrifuged at 4°C, washed once with acetone, and the protein pellets were suspended in 50 µL of 1X SDS sample buffer. Individual proteins were separated by pre-cast NuPage gel electrophoresis (Invitrogen) and then stained with SimplyBlue SafeStain coomasie (Invitrogen). Individual protein bands were excised and digested with trypsin as previously described, with the following differences for the MS analysis: Digests were analyzed using a QSTAR Elite mass spectrometer (Applied Biosystems/MDS Sciex). Data were searched against the h UniProtKB.2010.08.10 as of August 10 2010, using in-house ProteinProspector, version 5.2.2 (a public version is available online).
**Immunofluorescence**

For immunofluorescence experiments, cells were grown on coverslips coated with poly-L-lysine. Following transfection, cells were grown in serum free media overnight, washed with PBS and fixed in 4% paraformaldehyde or stimulated with EGF (10ng/ml) prior to fixation. Cells were washed with PBS, followed by extraction and blocking with PBS containing 3% BSA (Sigma-Aldrich), 0.1% Triton X-100 and 0.02% sodium azide (PBS-BT). Coverslips were incubated sequentially with primary antibodies diluted in PBS-BT for 1 h at room temperature or overnight at 4°C. Alexa Fluor dye-conjugated secondary antibodies (Invitrogen) were diluted in PBS-BT 1:250 and incubated sequentially at room temperature for 1 h. Coverslips were mounted using anti-fade mounting media containing PBS, glycerol and P-phenylenediamine. For standard immunofluorescence, images were acquired with Openlab 4.0.4 (PerkinElmer) using an Axiovert 200M (Carl Zeiss, Inc.). Images were processed using Photoshop (Adobe Systems Inc.).

**Immunochemical analysis**

The following antibodies were used for immunoblotting. Kras (F234), Nras (F155), Hras (C20), Myc (A-14), neurofibromin (D), neurofibromin (N), GFP (B-2), SBP (SB19-C4), and GST (B-14) were from Santa Cruz. Total ERK, phospho-ERK (Thr 202/Tyr 204), phospho-Akt (Ser473) and phospho-S338 Raf were from Cell Signaling. Spred1 (rabbit) from Abcam. Spred1 (sheep) from R&D systems. Flag M2 from Sigma. 6X-His from Clonetech. Pan-Ras, and Raf-1 (610151) from BD Transduction Laboratories.
Figure 2.1 Tandem affinity purification scheme. Spred1 proteins were cloned into an N-terminal GS-TAP gateway vector and TAP-Spred1 complexes were purified from 293 TREX cells. The tag consists of an N-terminal Protein G tag that can be precipitated with protein G beads, a Tobacco Etch Virus (TEV) protease cleavage site that can be cleaved with TEV protease, and a streptavidin binding peptide (SBP) tag that can be precipitated with streptavidin beads.


Chapter 3: Investigating the mechanism for Spred1 moderation of Ras/MAPK signaling
3.1 Background

Spred1 functions as a negative regulator of growth factor, cytokine, and chemokine induced ERK activation by specifically inhibiting the Ras/Raf/MEK/ERK pathway [1-4]. Spred1 contains a tripartite domain structure comprised of an N-terminal Ena/Vasp Homology 1 (EVH1) domain, a central c-Kit binding domain (KBD), and a cysteine rich C-terminal Sprouty (SPR) domain. Mammals contain three homologues, Spred 1, 2 and 3, all of which can negatively regulate the Ras/Raf/MAPK pathway [3, 5, 6]. However, the precise mode of action for the Spred family of proteins remains uncertain, with putative sites of action upstream and downstream of Ras [1, 3]. Spred1 has been postulated to act between Ras and Raf, based on observations that mouse Spred1 can associate with Ras and reduce Ras-dependent Raf activation, but does not prevent the membrane translocation of Raf [3]. In a separate study however, a human Spred1 construct was able to reduce Ras GTP-bound levels in a Raf RBD assay, leading the authors to hypothesize Spred1 was functioning upstream of Ras [1]. Determining Spred1’s mechanism of action will initially involve clarifying where along the Ras/MAPK pathway Spred1 functions.
3.2 Objectives

Conflicting reports describe Spred1 as functioning both upstream and downstream of Ras [1, 3]. Since Spred1 has been reported to interact with both Ras and Raf [3, 7], it is unclear if Spred1 might interact with Ras indirectly through Raf. Despite the well-studied interaction between Ras and Raf, the steps leading to Raf activation after its recruitment to the plasma membrane remain uncertain. Therefore, a mechanism by which Spred1 prevented Raf activation was alluring despite a lack of evidence Spred1 could directly interfere with Raf’s activating phosphorylation steps.

To determine where along the Ras/MAPK pathway Spred1 is functioning, we initially chose to study Spred1’s interactions with Ras and Raf. We focused specifically on examining if a Spred1-Ras interaction was direct. This chapter explains the steps taken to determine that Spred1 interacts with the small GTPase Ras in a GTP dependent manner, and functions by decreasing active GTP-bound Ras levels in the cell. Additionally, \textit{SPRED1} mutations identified in Legius syndrome (fig 3.1) were used to interrogate Spred1’s function.
3.3 Results

**Human Spred1 preferentially interacts with the activated forms of Raf**

Mouse Spred1 has been described to be able to interact with Raf [3, 7], and has been hypothesized to prevent Raf activation by potentiating a Ras-Raf interaction and preventing kinases from phosphorylating and activating Raf [3]. To determine if human Spred1 could bind Raf in our assays, GST-Spred1 and flag-Raf1 or flag-Raf1 S259A/S338D, a mutant with higher basal activity, were co-transfected into HEK 293T cells (fig. 3.2a). Phosphorylation of Ser259 mediates Raf binding to 14-3-3 proteins, keeping Raf in a closed conformation and preventing the Raf CRD from interacting with Ras at the plasma membrane [8]. This phosphorylation was reported to play a negative regulatory role as its substitution to alanine results in increased Raf basal activity[9-12]. In contrast, Raf phosphorylation at Ser338 has been implicated in Raf’s activation. Phosphorylation of Ser338 following growth factor stimulation is dependent on Raf’s interaction with Ras and is required for maximal Raf activation [13-15]. Thus, a serine to aspartate substitution at residue 338 also results in an increase in Raf basal activity.

Together, the S259A/S338D mutations result in significant activation of the Raf protein, independently of Ras-GTP binding and thus, is a useful mutant in our experiments.

Pulldown of GST-Spred1 was unable to co-precipitate wild-type Raf1, even after five minutes of EGF stimulation (fig. 3.2a). This suggests that if a Spred1-Raf1 interaction does take place, it might be too transient to observe via coimmunoprecipitation. However, GST-Spred1 was able to co-precipitate the active Raf-1 mutant, which was unexpected due to earlier reports that Spred1 prevents Raf phosphorylation [3]. To
further investigate this, we expanded the forms of Raf used for immunoprecipitation in the inverse experiment (fig. 3.2b). Flag-Raf1 was unable to co-precipitate wild-type Spred1, but forms of Raf with higher basal activity were able to bind Spred1. Thus, B-Raf, and ΔN-Raf-1, a mutant Raf-1 missing the N-terminal regulatory region and unable to bind Ras, were able to co-precipitate Spred1. The N-terminal portion of Raf-1 contains the Ras binding domain (RBD) and the cysteine rich domain (CRD): both of these domains are important for translocation to the plasma membrane and Ras binding [16-18]. The N-terminal regulatory region can also form an intramolecular interaction with the C-terminal kinase domain to inhibit Raf kinase activity [12, 19, 20].

Spred1 can interact with the C-terminal kinase of Raf [7], and thus it was not completely unexpected that Spred1 bound ΔN-Raf-1. This may also help explain why Spred1 interacts with the activated Raf mutant, flag-Raf1 S259A/S338D. Mutant Raf1 is partially activated, and thus is more likely to exist in its open, active conformation, allowing Spred1 to interact with the C-terminal kinase domain. This suggests Spred1 preferentially interacts with Raf bound to Ras. However, we cannot rule out the possibility that activated Raf may be binding Spred1 as part of a negative feedback loop. Future experiments will determine if Spred1 can function downstream of the constitutively active Raf mutants.

**Spred1 binds Ras in a GTP dependent manner**

Cells expressing Spred1 demonstrate decreased Ras/MAPK signaling following acute agonist stimulation by various growth factors, including EGF and FGF (fig. 3.3). After
growth factor stimulation at various time points, modulation of MAPK signaling by Spred1 was evidenced by decreases in phospho-Raf-1 and phospho-ERK when compared to controls. Since Spred1 has previously been reported to interact with small GTPases such as Ras and Rho [1, 3, 21], we first examined if Spred1 bound Ras in a GTP dependent manner. Preferential binding of Spred1 to active GTP-bound Ras in HEK 293T cells was confirmed by performing pulldowns on ectopically expressed proteins (fig. 3.4a). A pulldown of the GST tagged p21 Ras isoforms could co-precipitate myc-Spred1. However, co-expression of Ras and Spred-1 together appeared to modulate Spred1 protein levels, making the results difficult interpret. To circumvent this issue, the inverse experiment was performed (fig. 3.4b). Pulldowns of GST-Spred1 yielded a more uniform quantity of Spred1 protein as observed by western blot. GST-Spred1 was able to co-precipitate myc-tagged Nras and Hras, preferentially binding to the constitutively active G12V Ras mutants versus their wild-type counterparts.

Many of the SPRED1 mutations identified in Legius syndrome result in C-terminal truncation of the SPR domain of Spred1. To determine if these loss-of-function mutations were still able to bind Ras, we included two of these truncation mutants, R325X and M266fsX4, in the GST-Spred1 pulldown experiment (fig 3.4b). The R325X mutant, which lacks the SPR domain, was not as effective as wild-type at co-precipitating Nras, but its ability to pulldown oncogenic Hras significantly increased. This result was unexpected because R325X is a loss-of-function mutant, and suggested Spred1’s inhibitory activity was not dependent on Ras binding ability. The M266fsX4 mutant, which lacks both the KBD and SPR domains, was also still able to co-precipitate a small
amount of Nras and Hras, but no longer demonstrated a preference for the GTP bound constitutive mutant. This suggests amino acid residues 267-325 of Spred1—which include the c-Kit binding domain—are important for recognizing GTP-bound Ras. However, a caveat of these pulldown experiments is that both Spred1 and Ras were significantly overexpressed. Unfortunately, we were unable to immunoprecipitate either endogenous Ras or Spred1 due to the poor quality of commercially available antibodies and low protein expression levels in 293T cells.

Since Spred1 expression levels varied significantly when co-expressed with various plasmids, we chose to purify recombinant Spred1 from E. coli for future experiments. To determine if recombinant Spred1 could interact with Ras, myc-tagged Nras was co-transfected into 293T cells and the lysates were incubated with GST-Spred1 that been immobilized on glutathione beads (fig. 3.5). Recombinant GST-Spred1 precipitated wild-type Nras from 293T cell lysates in an EGF-stimulation dependent manner, suggesting Spred1 preferentially bound GTP-bound Ras. GST-Spred1 was also able to precipitate the oncogenic Nras mutant, yet in this case, the interaction was not dependent on EGF stimulation. These results indicate Spred1 does indeed interact with active GTP-bound Ras.

**Direct interaction between Ras and Spred1**

Since we had also observed Spred1 binding various forms of partially activated Raf, we next asked whether Spred1 might bind Ras indirectly through Raf. If Spred1 interfered with Raf activation as previously hypothesized [3], it would be plausible that Spred1
might interact with Raf already bound to activated Ras, and therefore appear as though Spred1 bound RasGTP. To determine if Raf was necessary for a Spred1-Ras interaction, we initially expressed Nras and Raf1 in 293T cells, and examined the amount of Nras precipitating with recombinant GST-Spred1 (fig. 3.6a). We hypothesized that if Raf was necessary for the interaction, its addition would increase the amount of Nras binding to GST-Spred1. However, we observed equal amounts of Nras precipitating with GST-Spred1 in the absence or presence of Raf-1, suggesting Raf was not necessary for the interaction. We again observed an increased amount of Nras binding GST-Spred1 following EGF stimulation, confirming Spred1 preferentially bound GTP bound Ras. Although the addition of ectopic Raf-1 did not change the amount of precipitated Nras, we could not exclude the possibility that Spred1 was binding endogenous Raf-1 bound to myc-Nras.

Therefore, we tested for a direct interaction between Spred1 and Ras by performing in vitro binding assays using both recombinant Spred1 and Ras purified from E. coli. In this assay, wild-type, full-length Ras was pre-loaded with either GDP or non-hydrolyzable GTPγS and incubated with GST-Spred1 immobilized on glutathione sepharose beads (fig. 3.7). The wild-type Spred1 protein interacted directly with full length Kras4B and Nras, again, preferentially binding GTP-bound Ras. We also examined if the Legius syndrome truncation mutant, M226fsX4, could interact with Ras in the in vitro binding assay. Our previous overexpression experiments (fig. 3.4) demonstrated a weak interaction between the two proteins, but a loss of GTP dependence. In vitro, the M266fsX4 mutant was unable to interact with either GDP or GTPγS bound Kras4B (fig
3.7a), suggesting that the C-terminal KBD and SPR domains are necessary for a direct interaction between Spred1 and Ras. It is likely that the weak binding demonstrated in the previous experiment was a result of overexpression of the proteins. Together, these data imply that Spred1 preferentially binds GTP-bound Ras both \textit{in vivo} and \textit{in vitro} (figs. 3.4 and 3.7).

**Spred1 overexpression decreases Ras-GTP levels**

Based on the direct interaction between Spred1 and Ras, we examined if Spred1 could affect cellular Ras GTP levels following growth factor stimulation (fig. 3.8). To assay for the amount of GTP-bound Ras we performed pulldowns of Ras-GTP with the minimal Ras-binding domain (RBD) of Raf-1. The Raf RBD has been demonstrated to interact with Ras-GTP three orders of magnitude higher than its interaction with Ras-GDP [22, 23]. The Raf RBD can precipitate Ras-GTP and thus, can function as a specific probe for Ras-GTP levels in the cell. Utilizing this assay, we observed that Ras-GTP levels were significantly reduced after EGF stimulation in the presence of Spred1, suggesting that Spred1 might interfere with Ras regulation.

To test the hypothesis that Spred1 was functioning above the level of Ras, we examined if overexpression of Spred1 could affect the Ras/MAPK pathway in two Ras mutant cell lines (fig 3.9). In both Panc1 (a Kras G12D pancreatic cancer cell line) and in Hs578t (an Hras G12D cell line), overexpression of Spred1 was unable to significantly alter MAPK signaling kinetics, as assayed by changes in phospho-ERK following EGF stimulation. These data demonstrated that Spred1 could not inhibit MAPK signaling in the presence
of oncogenic Ras, and suggested that Spred1 was regulating the pathway by modulating Ras activation.

We next investigated whether *SPRED1* loss-of-function mutations (fig 3.1) found in Legius syndrome might similarly affect Ras activity (fig 3.10). Interestingly, previously identified pathogenic missense mutations in the N-terminal EVH1 domain and C-terminal SPR domain lost the ability to reduce Ras-GTP levels and inhibit ERK activation following growth factor treatment. The truncation mutant M266fsX4, which retains the EVH1 domain, but lacks the SPR and internal Kit-binding domains, was also defective in regulating Ras-GTP and phospho-ERK levels.

**Spred1 depletion increases Ras-GTP levels**

To further investigate the proposal that Spred1 affects Ras regulation, we depleted Spred1 from cancer cell lines and assayed for Ras-GTP levels with Raf RBD pulldowns (fig 3.11). If Spred1 was important in modulating Ras-GTP levels, we expected its depletion to result in increased Ras-GTP following growth factor stimulation. The Spred1 protein is expressed at low levels in most cell lines, and thus, we were limited to cell lines with detectable protein levels by western blot. The PC3 pancreatic cell line had previously been reported to have high Spred1 protein levels, and was thus ideal for our experiment [24]. Depletion of Spred1 alone or the related Spred2 protein with multiple siRNA oligos was unable to significantly increase Ras-GTP or phospho-ERK levels. One oligo, siRNA Spred1_5, did cause a slight augmentation of Ras-GTP, however, since other Spred1 specific oligos did not, this may have been due to non-specific targeting of
Spred2 or Spred3. This scenario seems likely, because knockdown of both Spred1 and Spred2 caused a significant increase in Ras-GTP and phospho-ERK following EGF stimulation. Thus, it appears as if the Spred family member, Spred2, can compensate for the absence of Spred1, because reduction of either one alone did not considerably alter Ras/MAPK signaling.
3.4 Discussion

In this study, we report that Spred1 interacts directly with Ras in a GTP dependent manner. This interaction is independent of a Spred1-Raf interaction and does not require the presence of Raf. Furthermore, Spred1 can modulate Ras activation following growth factor stimulation. Our data challenges the current model of Spred1 function, in which Spred1 acts downstream of Ras negatively regulate the Ras/MAPK pathway by preventing Raf activation. Overexpression of Spred1 decreases Ras-GTP levels, and its depletion increases Ras-GTP levels, suggesting it functions upstream of Ras activation.

Spred1 has no known catalytic domains and is comprised primarily of protein-protein interaction domains. We show here that Spred1 can interact directly with Ras, and therefore, postulate Spred1 may be functioning as a scaffold protein to bridge the interaction between Ras and a Ras modulator. The M266fsX4 Spred1 truncation mutant is unable to bind Ras in an in vitro binding assay, leading us to hypothesize the C-terminal SPR domain is necessary for a Spred1-Ras interaction. This remains to be tested with in vitro binding assays between recombinant Ras and the recombinant SPR domain alone. Yet, since the N-terminal EVH1 domain present in the M266fsX4 mutant has previously been reported to be critical for Spred1’s function, it is possible that an EVH1 domain binding partner is regulating Ras activity. Chapter 4 describes the identification of a novel Spred1 EVH1 domain interacting partner critical for Ras regulation.

Previous reports have hypothesized that Spred1 prevents Raf activation by Raf kinases because overexpression of Spred1 results in increased Raf recruitment to the plasma
membrane and increased Ras-Raf binding [3]. Surprisingly though, an increase in Raf binding to Ras does not increase Raf activation but rather decreases the amount of phospho-S338 Raf. Initially, these data appears to contradict our findings that Spred1 decreases Ras-GTP levels because Ras preferentially interacts with Raf in a GTP bound state, and thus lower amounts of GTP-bound Ras would be expected to bind less Raf molecules. Yet, given recent reports that Ras dimerization is critical for maximal Raf activation [25], it is possible that decreased quantities of Ras-GTP result in decreased Ras dimerization. Thus, Raf can bind the low levels of Ras-GTP, but cannot be fully activated. High resolution immunofluorescence techniques would be useful in determining if Spred1 expression could modulate the levels of Ras dimers. Alternatively, the levels of Ras bound Raf could be examined after the overexpression of a bona fide Ras-GTP regulator, such as p120GAP or neurofibromin.

Interestingly, Spred1 also interacts with the C-terminal kinase domain of Raf1 and a partially activated mutant of Raf1. These results suggest that Spred1 preferentially interacts with Ras-bound Raf, since the C-terminal domain only becomes exposed after Raf translocates to the plasma membrane and interacts with Ras-GTP. If Spred1 specifically targets Raf-bound Ras, this suggests a mechanism by which Spred1 could directly target the MAPK pathway and not other Ras mediated signaling pathways.

In conclusion, we have found that Spred1 functions by decreasing Ras-GTP levels. In Chapter 4 we will address the mechanism by which Spred1 can modulate Ras activity.
Figure 3.1 Spred1 domain structure and Legius syndrome mutations. Spred1 is comprised of three conserved domains: an N-terminal EVH-1 domain, internal c-Kit binding domain (KBD), and C-terminal Sprouty (SPR) domain. The mutations at the top of the figure are used in this study.
Figure 3.2 Spred1 preferentially interacts with activated forms of Raf. a, HEK 293T cells were transfected with GST-Spred1 and either wild-type Raf-1 or a Raf-1 mutant with higher basal activity, S259A/S33D Raf-1. Cells were serum starved overnight and stimulated with 10 ng/mL EGF for 5 minutes. GST-Spred1 was precipitated from cell lysates with a glutathione sepharose 4B resin, and pulldowns were analyzed by western blotting with the indicated antibodies. b, HEK 293T cells were transiently transfected with GST-Spred1 and the indicated isoform or mutant version of Raf. Raf was immunoprecipitated from cell lysates with an anti-Flag M2 antibody, and samples were analyzed by immunoblotting.
Figure 3.3 Ectopic expression of Spred1 results in inhibition of the Ras/MAPK signaling pathway. HEK 293 TREX cells stably expressing TET inducible 6X-His eGFPf or Spred1 were stimulated with 10 ng/mL EGF for the times indicated. Cell lysates were subject to immunoblot analysis by the antibodies indicated on the left.
Figure 3.4 Spred1 interacts with multiple isoforms of Ras. a, HEK 293T cells were transiently transfected with myc-Spred1 and the indicated wild-type or oncogenic form of Ras. Twenty-four hours following transfection, cells were serum starved overnight. GST-tagged Ras was precipitated with glutathione sepharose 4B beads and immunoblotted with anti-myc antibodies. b, Cells transfected with GST-Spred1 and the indicated Ras protein. Following serum starvation, GST-Spred1 was precipitated with glutathione sepharose 4B affinity resin and western blots were probed with the antibodies indicated on the left.
Figure 3.5 Spred1 interacts with Nras in a GTP dependent manner. Recombinant GST-Spred1 was purified from *E.coli* and immobilized on glutathione sepharose 4B affinity resin. HEK 293T cells were transiently transfected with either wild-type or oncogenic G12V myc-tagged Nras. Cells were serum starved overnight and stimulated with 10 ng/mL EGF at the indicated timepoints. Cell lysates were incubated with either glutathione sepharose beads alone or GST-Spred1 beads. Samples were analyzed by western blot with the antibodies indicated on the left.
**Figure 3.6** Spred1 differentially binds Nras versus Hras. **a, b**, HEK 293T cells were transiently transfected with either wild-type or oncogenic G12V Nras (**a**) or Hras (**b**). Following treatment, whole cell lysates were incubated with immobilized GST-eGFP, GST-Spred1, or GST-Raf RBD (**b**). Samples were analyzed by immunoblot with myc and GST antibodies. **a**, Twenty four hours following transfection, cells were serum starved overnight and stimulated with 10 ng/mL EGF for 5 minutes before harvesting. **b**, Hras transfected cells were serum starved overnight before harvesting. Cell lysates were pre-incubated with either GDP or non-hydrolyzable GTPγS before incubation with recombinant GST proteins.
Figure 3.7 Spred1 interacts directly with Ras. **a, b**, HIS-tagged full-length wt Kras4B (a) or Nras (b) purified from *E. coli* was pre-loaded with either GDP or GTPγS and incubated with immobilized recombinant GST-GFP, GST-Raf Ras binding domain (RBD), GST-Spred1, or GST-M266fsX4, and subject to immunoblot analysis using Kras, Nras, or GST antibodies. Full-length wild-type Spred1 interacted preferentially with GTP-bound Kras4B and Nras, whereas a C-terminal truncated Spred1 mutant (M266fsX4) failed to bind Kras. In **b**, the levels of recombinant GST-M266fsX4 were significantly higher than GST-GFP or GST-Spred1, making it hard to interpret if M266fsX4 can truly interact with Nras.
**Figure 3.8** Spred1 suppresses Ras activity. Tetracycline inducible 6X-HIS eGFPf or Spred1 expressing 293 cell lines were stimulated with EGF (10 ng/mL) for the indicated times, and whole-cell lysates were affinity purified using GST-Raf1 RBD to precipitate active Ras. Immunoblot analysis was performed with the antibodies indicated on the left. The expression of Spred1 decreased the levels of GTP-bound Ras and phospho ERK following EGF stimulation.
Figure 3.9 Overexpression of Spred1 in Ras mutant cell lines does not inhibit Ras/MAPK signaling. Two cancer cell lines, Panc1 (a) and Hs578t (b) were transfected with Flag-tagged GFP or Spred1. Following 16 hours of serum starvation, cells were stimulated with 50 ng/mL EGF at the indicated timepoints. Harvested samples were analyzed by immunoblot.
Figure 3.10 Legius syndrome *SPRED1* mutants are unable to suppress Ras activity.

HEK 293T cells were transiently transfected with Flag-tagged eGFPf, Spred1, or the indicated Spred1 mutants were stimulated with 10 ng/mL EGF for the indicated times. Active Ras was precipitated with GST-RBD beads. Immunoblot analysis was performed with the antibodies indicated on the left.
Figure 3.11 Spred siRNA depletion increases Ras-GTP and phospho ERK levels following EGF stimulation. Glioblastoma U87 cells (a) or prostate cancer PC3 cells (b) were transfected with the indicated siRNA oligonucleotides. Forty-eight hours post transfection, cells were serum starved; seventy-two hours post transfection cells were stimulated with EGF (20 ng/mL) for 5 minutes before harvesting. GTP-bound Ras was immunoprecipitated with Raf RBD beads and samples were analyzed by western blotting.


Chapter 4: Identification of a novel Spred1 interacting partner
4.1 Background

Multiple gain-of-function germline mutations that affect components of the Ras/MAPK pathway have been identified in a series of disorders termed the cardiofacio cutaneous syndromes, or Rasopathies. These autosomal dominant growth and developmental disorders result from dysregulated Ras signaling due to mutations in various components involved in the MAPK signaling module, including \textit{BRAF}, \textit{RAF1}, \textit{SOS1}, \textit{PTPN11}, \textit{SHOC2}, \textit{MEK1}, and the \textit{RAS} genes themselves. NF1, an autosomal dominant, multisystem disorder that affects approximately 1 in 3,500 individuals, was the first disorder found to originate from a component of the Ras/Raf/MAPK pathway. The \textit{NF1} gene product, neurofibromin, negatively regulates Ras signaling by functioning as a Ras GTPase activating protein (RasGAP) and accelerating the hydrolysis of active Ras-GTP to inactive Ras-GDP \cite{2,3}. Heterozygous germline loss-of-function mutations have been identified in \textit{SPRED1} in Legius syndrome, a developmental disorder that shares a number of phenotypes with NF1 \cite{1}.

Legius syndrome has been characterized as a milder form of NF1, with individuals displaying multiple café-au-lait spots, axillary freckling, and macrocephaly, but lacking other common NF1 manifestations such as Lisch nodules, neurofibromas, osseous lesions, or optic pathway gliomas \cite{1,4-11}. Similar to loss-of-function mutations in \textit{NF1}, germline mutations in \textit{SPRED1} result in dysregulated activation of Ras/MAPK pathway and its downstream effectors. It is now hypothesized that \textit{SPRED1} mutations account for approximately 2% of the pathogenic mutations associated with patients clinically diagnosed with NF1 \cite{6}. Since Legius syndrome is a recently identified disorder, the
number of patients identified remains low, and thus the full mutational and clinical spectrum remains unknown. Most of the mutations discovered are point mutations that result in C-terminally truncated proteins, although approximately 12 missense mutations have been identified (table 4.1). More recently, a more comprehensive analysis of SPRED1 copy number changes uncovered four different deletions in the SPRED1 gene, indicating a need for dosage analysis to uncover further causative mutations in SPRED1 [10]. Thus far, attempts to uncover mutations in SPRED2-3 or the family members, SPRY1-4, have been unsuccessful and only mutations in SPRED1 have been identified in a Rasopathy [7, 11].
4.2 Objectives

Legius syndrome very closely phenocopies NF1, yet no defined link has been identified between Spred1 and neurofibromin beyond them both being negative regulators of Ras/MAPK signaling. Spred1 is able to suppress the Ras/MAPK pathway, yet had previously been suggested to act between Ras and Raf [12-14]. In Chapter 3, we found that Spred1 was able to modulate Ras-GTP levels, and thus hypothesized that Spred1 acted at the level of Ras, similar to neurofibromin. However, since Spred1 has no known catalytic domains, we questioned how Spred1 was able to downregulate Ras-GTP. We hypothesized Spred1 might be functioning through a yet unidentified binding partner. Thus, we performed tandem affinity purification to identify novel Spred1 interacting proteins. This chapter describes the steps taken to better determine Spred1’s molecular mechanism and our identification of neurofibromin as a novel Spred1 interacting protein.

During the course of this investigation, it became clear that the majority of Legius syndrome mutations were truncating mutations and therefore we also examined the characteristics of the Spred1 C-terminus. We determined that the Sprouty domain of Spred1 was critical for recruiting Spred1 to the plasma membrane, and by artificially localizing the truncation mutants at the membrane, Spred’s inhibitory function was restored.
4.3 Results

The majority of reported Spred1 mutations often produce a premature stop codon, likely resulting in C-terminally truncated protein products [1, 5-11, 15, 16]. As the SPR domain has previously been reported to target Spred1 to the plasma membrane via interactions with phospholipids and caveolin-1 [17-19], we surmised that the truncated mutants might be unable to function as a result of mislocalization. To test this hypothesis, we artificially localized the EVH-1 domain Spred1 mutant (M266fsX4) to the membrane by fusing it to the membrane targeting CAAX motif of K-Ras4B (fig. 4.1). The K-Ras4B C-terminus is a well-characterized membrane targeting motif that contains a C-terminal polybasic domain and a farnesylation motif whereas the C-termini of H-Ras, N-Ras, and K-Ras4A contain cysteine residues that are palmitoylated [20-22]. Thus, unlike the other Ras family members, processed K-Ras4B is confined primarily to the plasma membrane, and its C-terminus is ideal for plasma membrane targeting.

Immunofluorescence microscopy revealed that the mutant Spred1 truncated protein localized primarily to the cytoplasm, whereas the mutant CAAX fusion protein was localized to the plasma membrane. The mutant CAAX protein also showed a restored ability to inhibit EGF induced ERK activation (fig. 4.2a), indicating that the inhibitory activity of Spred1 resides at the N-terminus. Intriguingly, this also suggests that defective membrane targeting of Spred1 is responsible for the loss-of-function of Spred1 in Legius syndrome-associated C-terminal truncation mutants.
Thus far, approximately twelve missense mutations in \textit{SPRED1} have been characterized as pathogenic in Legius syndrome: eleven point mutations and one four amino acid in-frame deletion [1, 6, 8, 10, 11, 16] (table 4.1). Of these twelve missense mutations, nine localize to the EVH1 domain. When we introduced two of these mutations into the mutant CAAX fusion protein, both prevented the ability of the CAAX motif to rescue Spred1 function (fig. 4.2b). Together, these results underscore the importance of the EVH1 domain of Spred1 in inhibiting ERK activation, and the SPR domain in directing the plasma membrane localization of Spred1.

EVH1 domains are protein-protein interaction modules that belong to the pleckstrin homology (PH) domain [23]. The Enabled/Vasp homology 1 (EVH1) domain is an approximately 115 amino acid domain present in proteins associated with actin-based structures and involved in actin and cytoskeleton remodeling [23-27]. Typically, physiological ligands for the EVH1 domains are proline-rich sequences. However, the crystal structure of the \textit{Xenopus tropicalis} Spred1 EVH1 domain suggests a distinct peptide binding mechanism compared to other identified EVH1 domains [28]. Its peptide-binding groove shows increased structural flexibility and is somewhat narrower than homologous structures. This suggests a less proline-rich peptide may bind and may induce conformational changes following binding. Ligands that lack a proline-rich sequence have been previously described as atypical EVH1 binding partners, specifically in the case of Tes, which despite lacking the classical FPPPP sequence motif, can still bind to the EVH1 domain of Mena [29]. Together, these data suggest that the Spred1 EVH1 domain binding partner may not contain a typical proline-rich sequence, and thus
an unbiased approach would be the most useful in determining specific interacting proteins.

Proteins that specifically interact with the Spred1 EVH1 domain have yet to be determined, although an interacting protein for the Spred2 EVH1 domain has been recently reported [30]. Utilizing a yeast two-hybrid approach, Mardakheh et al. identified Neighbor of BRCA1 (NBR1) as interacting and co-localizing with Spred2. They postulate that Spred2’s interaction with NBR1 was critical for directing activated receptors to the lysosomal degradation pathway and thus attenuating RTK signaling. However, they also observe that NBR1 is a specific late endosomal protein, and whereas Spred2 has previously been found to be associated with endosomal markers, the same observation has not been made for Spred1 [31]. Attempts to co-precipitate NBR1 with Flag-Spred1 were unsuccessful (data not shown), and thus we were unable to determine if NBR1 was a bona fide interactor of the Spred proteins.

Since the EVH1 domain appears to be essential for Spred’s function, we hypothesized that an unidentified binding partner of the EVH1 domain might be required for Spred1’s inhibitory activity. To identify novel Spred1 EVH1 interacting proteins, we performed tandem affinity purification [32], using wild-type Spred1 and the EVH1 CAAX fusion protein as bait (fig. 4.3). Following mass spectrometry analysis, we selected various candidates as potential Spred1 interacting proteins (table 4.2). One novel Spred1 EVH1 interacting partner we identified was the NF1 gene product, neurofibromin. Neurofibromin is a tumor suppressor and a RasGAP that regulates Ras GTPase activity
by catalyzing the hydrolysis of active GTP-bound Ras to inactive GDP-bound Ras. Loss-of-function of neurofibromin removes this regulation and leads to uncontrolled cell growth and proliferation. The identification of this RasGAP as a binding partner of Spred1 was consistent with our biochemical results that Spred1 suppresses Ras/Raf/MEK/ERK activation and reduces RasGTP levels, and was particularly intriguing given the overlapping phenotypes observed in Legius syndrome and NF1.

To confirm this interaction, we performed co-immunoprecipitation experiments in mammalian cells. Ectopically expressed wild-type Spred1 co-immunoprecipitated with endogenous neurofibromin in human embryonic kidney 293T cells, but not with p120 GAP, another RasGAP (fig. 4.4). Similarly, endogenous neurofibromin could coprecipitate stably expressed full length Spred1 (fig. 4.6). We also found that the other Spred1 family members, Spred2 and Spred3, could also precipitate neurofibromin (fig 4.5). However, Sprouty proteins, which lack the N-terminal EVH1 domain, did not coprecipitate neurofibromin. Moreover, pathogenic point mutations in the EVH1 domain of Spred1 prevented association with neurofibromin, whereas constructs encoding the EVH1 domain alone, or with a CAAX motif, were able to interact with neurofibromin (fig 4.4). Finally, a pathogenic point mutation in the C-terminal SPR domain, P415A, did not prevent Spred1 from binding neurofibromin. These results indicated that the ability of Spred1 to associate with neurofibromin was dependent on its EVH1 domain. These data support the idea that missense EVH1 mutations are pathogenic due to loss of interaction with neurofibromin.
To determine if neurofibromin is necessary for Spred1’s inhibitory function, we depleted neurofibromin by siRNA in HEK 293 cells stably expressing Spred1 (fig. 4.7). Reducing neurofibromin expression suppressed Spred1’s ability to reduce Ras-GTP levels and decrease ERK phosphorylation, indicating that neurofibromin is necessary for Spred1 function. However, the siRNA depletion of neurofibromin in the cells stably expressing Spred1 was not as complete as in control eGFPf expressing cells, despite the fact that Spred1 was not induced in these cells until 48 hours following the first siRNA transfection. This effect was consistently observed in multiple experiments, suggesting the presence of Spred1 might stabilize the neurofibromin protein or increase its translation.

Because neurofibromin depletion was less efficient in Spred1 expressing cells compared to control cells, we also examined Spred1’s activity in NF1-/- cells. Initial experiments were conducted in NF1-/- 9223 mouse cells due to their high transfection efficiency. This cell line was created from a breast cancer in a heterozygous NF1+/- mouse that lost the wild-type NF1 allele following radiation treatment [33]. Surprisingly, expressing full-length neurofibromin in NF1-/- 9223 mouse cells did not significantly alter ERK activation following EGF stimulation (fig. 4.8). Yet, since this tumor cell line had been induced by radiation, it is possible de novo mutations in other genes prevented neurofibromin expression from rescuing the altered MAPK signaling.

Also surprisingly, Spred1 overexpression slightly decreased phosphorylated ERK levels in NF1-/- 9223 mouse cells. However, since Spred1 can interact with Ras in a GTP-
dependent manner, it is possible that Spred1 overexpression can interfere with Ras effector binding to a small degree. The decrease in ERK activation was considerably magnified by co-expression of neurofibromin, indicating Spred1 and neurofibromin can synergize to inhibit the Ras/MAPK pathway (fig. 4.8). We are currently repeating this experiment in NF1 null MEFs to circumvent the effects of any additional mutations in the radiation induced 9223 cell line. Taken together, these results show that neurofibromin is a novel Spred1 binding partner necessary for Spred1’s inhibitory activity. Importantly, this provides the first molecular evidence linking NF1 and Legius syndrome.

Because Spred1 is also able to interact with Ras directly (Ch. 3), and re-localizing the Spred1 EVH1 domain to the plasma membrane restored its function (fig. 4.1), we reasoned that Spred1 might recruit neurofibromin to active signaling complexes. A precedent for GAP recruitment to Ras via adapter proteins exists with p120GAP, as p120 translocation to the plasma membrane is dependent on RTK binding [34]. To test this hypothesis, we examined the effect of Spred1 on neurofibromin localization (fig. 4.9). Immunofluorescence microscopy revealed that full-length neurofibromin localized primarily to the cytoplasm, whereas Spred1 was in the plasma membrane. When co-expressed, however, Spred1 and neurofibromin co-localized to the membrane, indicating Spred1 expression was sufficient to recruit neurofibromin to the plasma membrane. The co-localization of these two proteins was slightly enhanced by EGF stimulation (fig. 4.10, bottom panel). We also examined the effect of the Spred1 point mutations on neurofibromin localization and observed that the T102R mutation was still able to localize to the plasma membrane, but failed to recruit neurofibromin to the membrane.
(fig. 4.9). In contrast, the P415A Spred1 mutant co-localized with neurofibromin at the cytoplasm rather than the plasma membrane. These data were consistent with our observations that the T102R mutant failed to interact with neurofibromin by co-IP, whereas the pathogenic P415A mutant could still bind neurofibromin.

To confirm that neurofibromin recruitment was dependent on the EVH1 domain, we also examined neurofibromin localization after co-expression of the M266fsX4-CAAX mutant (fig. 4.9). Consistent with our biochemical interaction data, we observed that neurofibromin translocated to the membrane in the presence of the membrane targeted (CAAX) Spred1 mutant. However, when the T102R point mutation was introduced into the M266fsX4-CAAX construct, mutant Spred1 still localized to the membrane, but failed to recruit neurofibromin, which remained cytoplasmic (fig. 4.9, bottom panel). In sum, Spred1’s inhibitory activity on the Ras/MAPK pathway is likely due to its recruitment of neurofibromin to the plasma membrane.
4.4 Discussion

On the basis of the data described here, we propose a new model for Spred1 inhibition of the Ras/Raf/MEK/ERK pathway. We show that the N-terminal EVH1 domain of Spred1 interacts with neurofibromin and mediates NF1 translocation to the plasma membrane, where neurofibromin can perform its function as a RasGAP. This model is consistent with the ability of Spred1 to reduce cellular Ras-GTP levels in the absence of any known enzymatic activity (Ch. 3). Given the overlapping clinical phenotypes of Legius syndrome and NF1, our findings provide a molecular basis for these similarities and support a role for Spred1 as a novel regulator of neurofibromin function. Furthermore, since Spred2 can also interact with neurofibromin, these two isoforms may compensate for loss of Spred1, and thus may help explain the milder phenotype associated with Legius syndrome in comparison to NF1.
Figure 4.1 Plasma membrane localization is necessary for Spred1’s function. HeLa cells were transfected with myc-Spred1, myc-M266fsX4, or myc-M266fsX4. Cells were serum starved or serum starved followed by 5 min of EGF stimulation. Cells were fixed and immunostained with antibodies against myc (green), and DNA was stained with DAPI. Scale bars, 5 µm.
Figure 4.2 Spred1’s inhibitory activity resides in the EVH1 domain.

a, b, 293 cells stably expressing Flag TET inducible NTAP-tagged eGFPf, Spred1, or the indicated Spred1 mutants were stimulated with 10 ng/mL EGF for the indicated times. Immunoblot analysis was performed with the antibodies indicated on the left. a, NTAP-Spred1 mutants with a C-terminal CAAX motif rescued suppression of ERK phosphorylation. b, Introducing known pathogenic missense mutations into NTAP-M266fsX4-CAAX, abolished its restored activity.
Figure 4.3 Tandem affinity purification of Spred1 and Spred1 truncation mutants. HEK 293 TREX stable cell lines were made that expressed TET inducible GS-TAP tagged constructs. Five 10 cm dishes per construct were serum starved for 16 hours and stimulated with 20 ng/mL EGF for 2 minutes. Following two steps of affinity purification, samples were TCA precipitated and separated by pre-cast NuPage gel electrophoresis. Gels were stained with SimplyBlue SafeStain Coomassie.
Figure 4.4 Neurofibromin is a novel Spred1 binding partner. Flag-tagged eGFPf, RKIP, wild-type or the indicated Spred1 mutant constructs were expressed in HEK 293T cells, serum starved for 16 hours, and lysed 24 hours post transfection. Anti-Flag immunoprecipitates were blotted for co-precipitating endogenous neurofibromin or p120 GAP.
**Figure 4.5** Multiple Spred isoforms interact with neurofibromin. HEK 293T cells were transiently transfected with the indicated Flag-tagged construct. Twenty-four hours post transfection, Flag-tagged Spred isoforms or Spry2 or 4 were immunoprecipitated with a flag antibody and subject to western blotting for co-precipitating endogenous neurofibromin or p120 GAP.
Figure 4.6 Ectopic and endogenous neurofibromin can co-precipitate Spred1.

a, Full length Flag-NF1 was transfected into HEK 293 cells stably expressing TET inducible 6X-HIS eGFPf or Spred1. Following 5 minutes of EGF stimulation, Flag-NF1 was immunoprecipitated from whole cell lysates, and blotted for 6X-HIS. b, HEK 293 TREX cells stably expressing TET inducible 6X-His Spred1 were induced with 10 ng/mL doxycycline for 16 hours before harvesting. Lysates were incubated with either control Rabbit IgGs or a neurofibromin antibody for 1 hour, followed by incubation with Protein G sepharose. Immunoprecipitates were analyzed by western blot.
Figure 4.7 Neurofibromin is necessary for Spred1’s inhibitory function. Neurofibromin was siRNA depleted in TET-inducible 6X-HIS eGFPf and Spred1 cell lines, serum starved, and stimulated with EGF for the times indicated. Active Ras was precipitated from whole cell lysates using GST-Raf RBD beads. Western blotting analysis indicated neurofibromin depletion (RNAi) expression suppressed the ability of Spred1 to reduce Ras-GTP levels and decrease ERK phosphorylation.
Figure 4.8 Neurofibromin is necessary for Spred1’s inhibitory function in NF1\(^{-/-}\) cells.

GFP-NF1, Flag-GFPf, Flag-Spred1, or GFP-NF1 and Flag-Spred1 constructs were transfected into NF1\(^{-/-}\) 9223 mouse breast cancer cells. Following serum starvation, cells were treated with EGF for the indicated times, lysed, and analyzed by immunoblot.
Figure 4.9 Spred1 recruits neurofibromin to the plasma membrane. HeLa cells were transfected with either GFP-NF1 alone, myc-Spred1 alone, GFP-NF1 and myc-Spred1, or GFP-NF1 and the the indicated myc-Spred1 mutant. Cells were serum starved and stimulated with EGF for 10 minutes. Fixed cells were immunostained for myc (red), GFP (green), and DNA (DAPI). When co-expressed, Spred1 and neurofibromin co-localize at the plasma membrane, suggesting Spred1 is necessary for neurofibromin translocation to the plasma membrane. The Spred1 T102R mutant is also able to localize to the plasma membrane, but fails to recruit neurofibromin to the membrane. Scale bars, 5 µm.
Figure 4.10  EGF stimulation increases Spred1 and neurofibromin localization to the plasma membrane. HeLa cells were transfected with either GFP-NF1 alone, myc-Spred1 alone, or GFP-NF1 and myc-Spred1, and either serum starved or serum starved and stimulated with 15 ng/mL EGF for 5 minutes. Fixed cells were immunostained for myc (red), GFP (green), and DNA (DAPI). Scale bars, 5 µm.
Table 4.1  *SPRED1* mutations identified in Legius syndrome used in this study. Most *SPRED1* germline mutations truncate the protein, but eleven missense mutations and one small in-frame deletion have been reported. The four missense mutations, in-frame deletion, and two truncation mutations utilized in this report are described above.

<table>
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<th>Protein Change</th>
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<tr>
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<tr>
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Table 4.2  Mass spectrometry data for Spred1 and EVH1 protein binding complexes. Proteins were identified from two independent TAP/mass spectrometry experiments using GS-TAP tagged fusion proteins. Candidate proteins were selected based on their presence in both experiments and their absence in control TAP pulldowns of unrelated baits.

<table>
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<th>M266fsX4 CAAX</th>
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<td>HUWE1</td>
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<td>87</td>
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<tr>
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Chapter 5: Future Directions
The NF1 tumor suppressor gene product, neurofibromin, an important negative regulator of Ras, is known to be regulated by both protein kinases and ubiquitin-related enzymes. However, many of the proteins that interact with neurofibromin to either positively or negatively regulate its function remain unknown. In this report, we identify a novel interaction between Spred1 and neurofibromin and propose a novel mechanism for neurofibromin regulation that helps explain the pathophysiology of the two clinically related developmental disorders: NF1 and Legius syndrome. With this study, we identify the first component of the Ras/MAPK pathway able to modulate neurofibromin’s function. To better understand the dynamics of a Spred1-neurofibromin interaction, we propose here future studies to map the region on NF1 which Spred1 interacts with, and to determine which signals are required for the interaction.

5.1 The region of neurofibromin necessary for a Spred interaction

Despite neurofibromin’s implicated role in the Ras/MAPK pathway via its GTPase-activating protein related domain (GRD), much of the functional role of this 2818 amino acid protein remains poorly understood. Beyond the central 350 amino acid GRD, only the small Sec14p domain and pleckstrin homology (PH)-like domain have been found to comprise portions of this large tumor suppressor protein. The flanking N and C-terminal fractions of neurofibromin are largely evolutionarily conserved, but have yet to be ascribed a function. To map the region critical for Spred binding, co-immunoprecipitations will be performed in mammalian cells between transiently transfected Spred and various NF1 fragments. Since preliminary data in the lab suggests neurofibromin may be able to dimerize, co-IP experiments will be performed in either NF1−/− MEFs or 9223 mouse breast cancer cells, a NF1−/− cell line kindly provided by J. Nakamura at UCSF. Previous attempts to identify binding partners of NF1 have utilized
arbitrarily divided neurofibromin fragments that are not necessarily properly folded or functional [1]. In a more recent study, however, bait regions were created utilizing the position of evolutionary insertions and deletions, as well as conserved exon boundaries, to make segments more likely to retain their native structure [2]. To determine which region of neurofibromin is critical for the interaction, these same segments will be utilized in co-IP experiments with Spred.

Although using these rationally segmented constructs will be the most effective in determining which region interacts with Spred, it is possible two or more sites are necessary for Spred1 binding, or there is a requirement for post-translational modifications not occurring on the fragments. Post-translational modifications necessary for this interaction should also be determined. If we are unable to identify the region of Spred1 binding via these NF1 fragments, we can alternatively perform co-IP experiments with point mutations in the full length NF1 construct.

Until recently, the full length NF1 cDNA did not exist due to poison sequences in the gene that prevented the gene’s propagation in bacteria. By optimizing the nucleotide base pair code for growth in bacteria, but preserving the amino acid sequence, our lab was able to employ GeneArt to synthesize a full-length NF1 cDNA vector. Point mutations will be introduced into full length NF1 via PCR mutagenesis, and the resultant NF1 point mutants will be transiently transfected into mammalian cells and examined for their ability to interact with Spred1 by co-IP. If we identify the region of neurofibromin critical for binding via a fragment approach, we will then evaluate the ability pathogenic mutations in neurofibromin in the same region to bind Spred1. Approximately 5 to 10% of pathogenic mutations identified in neurofibromatosis type 1 are
classified as missense mutations [3]. While most of these missense mutations disrupt
neurofibromin’s GRD function, point mutations exist outside of this domain that have yet to be
characterized.

If the fragment approach does not identify the region of Spred interaction, NF1 pathogenic
missense mutations can be made to tile the whole length of the gene. These point mutants will be
tested for their ability to bind Spred1 as previously described. Additionally, several mutations
have been described in exon 17 of the NF1 gene that result in a similar phenotype as Legius
syndrome [4, 5]. Patients with a 3 base-pair inframe deletion (c.2970-2972 del AAT) still
display café-au-lait spots and axillary freckling, but lack the cutaneous or plexiform
neurofibromas found in more severe forms of neurofibromatosis type 1 [5]. Pathogenic point
mutations in exon 17 with therefore be among the first mutants tested for loss of Spred1 binding.
Identifying why specific small mutations in NF1 correlate with the expression of a particular
clinical phenotype will be invaluable in understanding the pathiophysiology of the disease.

Following the identification of NF1 point mutants unable to bind Spred, these mutants will be
examined for their ability to localize to the membrane and associate with Ras. Spred1 point
mutants that fail to interact with neurofibromin also fail to relocalize neurofibromin to the
plasma membrane (Chapter 4). Therefore, we will perform immunofluorescence microscopy on
the NF1 point mutants alone, or in the presence of Spred to determine if NF1 point mutants
unable to bind Spred1 also mis localize. We will also examine the ability of these mutants to
associate with Ras by co-transfecting them with tagged wild-type and constitutively active Ras
and assessing their ability to co-precipitate with Ras. Finally, we will test their ability to decrease
RasGTP levels by performing Raf RBD pulldown assays after their transient transfection into mammalian cells. As controls we will compare the above results to full-length wild-type neurofibromin as well as pathogenic point mutants that retain Spred1 binding ability.

5.2 Binding of Spred to the various NF1 isoforms

Additionally, we will also examine if the Spred proteins show a preference for any specific NF1 isoform. An alternatively spliced NF1 exon, 23a, encodes for a 63 bp region in the GRD of NF1. Inclusion of this exon results in the type II isoform, whereas exclusion results in the type I isoform. The type II isoform has an increased affinity for binding to Ras, but a decreased ability to inactivate Ras by 10-fold [6, 7]. Mice lacking exon 23a of NF1 are viable and physically normal, but show defects in hippocampal dependent learning, similar to Spred1 -/- mice [8]. Exon 23a is included in most tissues, but is skipped in neurons. A decrease in inclusion of the exon correlates with neuronal differentiation and a decrease in cellular proliferation. As our full length NF1 cDNA clone currently includes the extra 63 bp, the 63 bp will first be excised by PCR amplifying the flanking N and C terminal regions, and performing PCR fusion to anneal the two fragments together. To determine if exon 23a plays a role in binding Spred1, the full length neurofibromin proteins excluding and including exon 23a will be expressed in mammalian cells lacking endogenous NF1, and examined for their ability to co-precipitate the three Spred isoforms. Fragments comprising the GRD portions of the two isoforms will also be tested for their ability to interact with the various Spreds. Determining if the three Spreds show variable binding to the various NF1 isoforms may help explain why Spred1, but not 2 or 3, is found mutated in Legius syndrome.
5.3 Requirement of Post-Translational Modifications on a Spred-neurofibromin interaction

Post translational modifications have been shown to play a vital role in intracellular signal transduction. Both Spred proteins and neurofibromin have previously been demonstrated to become phosphorylated following acute agonist stimulation. Specifically, Spred1 has been shown to become serine and tyrosine phosphorylated upon serum stimulation, and has been reported to be a substrate for the Shp2 phosphatase, a tyrosine phosphatase involved in the activation of the Ras/MAPK pathway [9, 10]. Neurofibromin has been reported to be a substrate of Protein Kinase C following EGF stimulation, leading to phosphorylation events necessary for proper signaling dynamics [11, 12]. Given the importance of phosphorylation events in signal transduction, we surmise several post-translational modifications may be important for the interaction between Spred and neurofibromin. Also, previous attempts to identify Spred1 EVH1 binding partners such as yeast two hybrid assays or peptide arrays, have been unsuccessful, most likely due to their failure to incorporate the proper post-translational modifications.

To determine the post-translational modifications present on Spred and neurofibromin during their interaction, tandem affinity purification followed by mass spectrometry analysis will be performed. Mammalian cells stably expressing inducible Protein G tagged Spred1 will be transiently transfected with NF1 tagged with Calmodulin Binding Peptide (CBP). Following cell lysis, Protein G-tagged Spred1 will be immobilized on IgG-agarose beads via the Protein G moiety of the TAP tag. Protein complexes associated with Spred are eluted from the beads by TEV protease cleavage. To isolated only Spred-neurofibromin complexes, a second affinity step is performed, where the CBP tag of neurofibromin is bound to calmodulin coated beads. Purified components are then analyzed by mass spectrometry to detect any phosphorylation events.
Alternatively, Spred1 can be first be immobilized to beads and then discarded, followed by precipitation of neurofibromin from the supernatant, in an attempt to isolate only non-Spred interacting neurofibromin. In order to determine phosphorylation events dependent on growth factor stimulation, TAP experiments will be performed with samples that have been serum starved or serum starved and then stimulated at various time points.

To confirm that the observed modifications are necessary for Spred-neurofibromin binding, the detected amino acid residues in either protein will be mutagenized to either block or mimic serine/threonine phosphorylation, or block tyrosine phosphorylation. These mutants will be examined for proper binding by co-IP. Alternatively, we will also mutate the tyrosine residues of Spred1 described to be de-phosphorylated by Shp2 in the literature [10], as well as the serine/threonine residues implicated in neurofibromin signaling. These mutants will also be tested for their ability to interact. Identifying the post-translational modifications necessary on Spred and neurofibromin for their interaction will be a critical first step in determining the dynamic regulation of this complex.

### 5.4 Ras dependence of a Spred-neurofibromin interaction

We have observed Spred1 can interact with Kras both *in vitro* and *in vivo* in a GTP dependent manner (Chapter 3). Since Spred1 preferentially binds activated Ras, we hypothesize GTP-bound Ras is critical for recruiting a Spred-neurofibromin complex to its site of action. Yet, given the similarity of the Ras family GTPases, it is possible Spred1 physiologically interacts with another small Ras family GTPase (RFG). To initially determine which small GTPase Spred1 is interacting with, a panel of wild-type and mutant constitutively active Ras family members will
be tested for their ability to co-precipitate Spred1. We have demonstrated that Kras can precipitate Spred1, yet it remains to be seen if another Ras family member has a higher affinity for Spred. Following these initial overexpression experiments, we will test the ability of recombinant Spred1 and candidate RFGs to interact *in vitro*, as has already been tested with Spred1 and Kras (Chapter 3). Finally, we will examine the ability of Spred1 to co-precipitate the endogenous candidate RFG from mammalian cells. Since we expect a Spred1-RFG interaction to be transient, non-hydrolyzable GTP can be added to the cell lysate to keep the RFG in its GTP bound form. Alternatively, cross-linking reagents can also be added to the cell lysate to keep transient interactions intact during the co-IP process.

Once the relevant RFG has been determined, we can examine its importance in recruiting Spred1 and neurofibromin to the plasma membrane by immunofluorescence microscopy. We demonstrate here that co-expression of Spred1 and neurofibromin in HeLa cells increases neurofibromin’s localization at the plasma membrane. The recruitment of the two complexes is moderately enhanced by growth factor stimulation. Co-expression of a constitutively active RFG along with Spred and neurofibromin should further enhance the presence of these two proteins at the plasma membrane. Alternatively, the endogenous RFG can be siRNA depleted and then the localization of Spred and neurofibromin can be examined by IF. If the RFG is critical for the membrane localization of these two proteins, we would expect their presence at the membrane to decrease in RFG depleted cells. Determining the signals necessary for plasma membrane recruitment will give valuable insight into the regulation of this critical interaction.
In conclusion, this study identifies Spred1 as a regulator of neurofibromin, and provides a molecular link between NF1 and Legius syndrome. It also provides the framework for future studies to further understand how neurofibromin and Spred1 co-regulate the Ras/MAPK pathway. A Spred/neurofibromin interaction not only helps explain the phenotypic similarities between NF1 and Legius syndrome, but also significantly broadens our basic knowledge of how Ras is regulated in the context of neurofibromin.


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