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Review and Update of *SPRED1* Mutations Causing Legius Syndrome

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ABSTRACT: Legius syndrome presents as a mild neurofibromatosis type 1 (NF1) phenotype. Multiple café-au-lait spots and macrocephaly are present with or without axillary or inguinal freckling. Other typical NF1-associated features (Lisch nodules, bone abnormalities, neurofibromas, optic pathway gliomas, and malignant peripheral nerve sheath tumors) are systematically absent. Legius syndrome is caused by germline loss-of-function *SPRED1* mutations, resulting in overactivation of the RAS–MAPK signal transduction cascade. The first families were identified in 2007. Here, we review all identified *SPRED1* mutations and summarize molecular, clinical, and functional data. All mutations have been deposited in a database created using the Leiden Open Variation Database software and accessible at http://www.lovd.nl/SPRED1. At present, the database contains 89 different mutations identified in 146 unrelated probands, including 16 new variants described for the first time. The database contains a spectrum of mutations: 29 missense, 28 frameshift, 19 nonsense, eight copy number changes, two splicing, one silent, one in-frame deletion and a mutation affecting the initiation codon. Sixty-three mutations and deletions are definitely pathogenic or most likely pathogenic, eight *SPRED1* mutations are probably benign rare variants, and 17 *SPRED1* missense mutations are still unclassified and need further family and functional studies to help with the interpretation.

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KEY WORDS: Legius syndrome; SPRED1; NF1; RAS-MAPK; RASopathy

Background

Neurofibromatosis type 1 (NF1; MIM# 162200) or von Recklinghausen's disease is a common autosomal dominant disorder affecting one in 3,000 live births worldwide [Huson et al., 1989]. Typical clinical manifestations include the presence of multiple café-au-lait spots, skin fold freckling, Lisch nodules, optic pathway gliomas, and neurofibromas. Other features associated with NF1 are short stature, macrocephaly, scoliosis and specific bone abnormalities, learning disabilities, and an increased risk for specific malignancies [Brems et al., 2009]. Diagnostic criteria for NF1 were established at the National Institutes of Health (NIH) Consensus Development Conference [Stumpf et al., 1988] and patients must fulfill two or more of the following features: six or more café-au-lait spots (>5 mm in diameter in children, >15 mm in postpubertal individuals), two or more (sub)cutaneous neurofibromas or one plexiform neurofibroma, axillary or inguinal freckling, optic pathway glioma, two or more Lisch nodules, typical bone dysplasia, first-degree relative with NF1. Inactivating mutations in the *NF1* gene were identified in patients with NF1. The *NF1* gene codes for neurofibromin, a protein with rat sarcoma viral oncogene homolog–GTPase activating protein (RAS–GAP) activity, which negatively regulates RAS [Cichowski and Jacks, 2001]. *NF1* was the first gene linked to the RAS–MAPK pathway [Viskochil et al., 1990; Wallace et al., 1990]. More recently, germline mutations in other genes coding for proteins involved in the RAS–MAPK pathway were identified in several human genetic syndromes [Zenker, 2011].

Some of these syndromes have an overlapping phenotype, although there are always unique phenotypic characteristics. The RASopathies, or syndromes of the RAS–MAPK pathway identified so far are as follows: Noonan, cardio-facio-cutaneous, LEOPARD, Costello, capillary malformation–arteriovenous malformation, CBL mutation-associated syndrome, and hereditary gingival fibromatosis. Even multiple genes were identified for some of these syndromes. In 2007, our research group identified a new RASopathy in patients with a mild NF1-like phenotype [Brems et al., 2007]. Inactivating *SPRED1* (MIM# 609291) mutations were detected in these individuals. The disease was initially described as NF1-like syndrome and later renamed as Legius syndrome (MIM# 611431) [Stevenson and Viskochil, 2009; Upadhyaya, 2008]. Patients typically present with multiple café-au-lait spots sometimes associated with skin fold freckling. Fifty percent of individuals with Legius syndrome fulfill the NIH criteria for NF1 [Messiaen et al., 2009], although the phenotype is much milder compared with NF1 and other NF1-associated

Additional Supporting Information may be found in the online version of this article.

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features are not present. SPRED1 is a negative regulator of the RAS–MAPK pathway [Brems et al., 2007; Wakioka et al., 2001] similar to neurofibromin.

SPRED1 Database

A PubMed search was performed using key words SPRED1, Legius syndrome, NF1-like syndrome. The reference list of all papers retrieved this way was hand searched for additional publications. Furthermore, we have been offering *SPRED1* mutation analysis from 2007 and added all *SPRED1* mutations detected since then by our and other research groups. Part of our results has been published elsewhere [Brems et al., 2007; Denayer et al., 2011a; Messiaen et al., 2009; Spencer et al., 2011]. The database describes *SPRED1* mutations found in unrelated individuals. For each proband, the following data are available: the coding DNA change, based on the reference sequence NM_152594.2, the type of alteration (substitution, deletion, insertion, duplication, copy number change [CNC] involving >1 kb), predicted effect at the protein level, exon/intron affected, effect at the RNA level, expected consequence of the alteration (missense, nonsense, frameshift resulting in a premature stop codon, splice, silent, in-frame deletion, CNC, or deletion/duplication of >1kb), a database ID for each individual mutation, a patient ID for each proband, the reference describing a specific proband/family or the submitter of the mutation to the database for unpublished mutations, the tissue in which the *SPRED1* mutation was identified, the reference where a mutation was first described, a synopsis of the phenotype observed in the proband (as described in the published report), and a comment field mentioning whether phenotypic data on relatives of the proband have been published. For missense alterations, it is mentioned whether a known functional domain is involved, whether the amino acid is evolutionary conserved, whether functional studies support pathogenicity.

The database can be consulted at http://www.lovd.nl/SPRED1 and will be updated quarterly with data from the literature as well as with variants identified through clinical mutation analysis at the Medical Genomics Laboratory. Furthermore, all scientists/clinicians are invited to submit their unpublished data to the database using an online submission form. Submissions will be checked by the curator (L.M.) and completed if necessary. The unpublished status will be clearly indicated, as well as the identity of the submitter.

Clinical and Diagnostic Relevance

Clinical Features

The clinical manifestations of Legius syndrome were initially based on the description of five families (37 individuals) with a pathogenic *SPRED1* mutation [Brems et al., 2007]. Individuals with Legius syndrome presented with café-au-lait spots with or without axillary or inguinal freckling, macrocephaly. In five individuals a Noonan-like face was present, including hypertelorism, ptosis, short neck with a low posterior hairline, and low-set ears. Learning problems and/or attention deficit were noted in six children and developmental delay in one child. Lipomas were present in 14 individuals and a mild pectus excavatum in three individuals. A childhood renal cancer, lung cancer, and colonadenoma were identified each in one individual.

Seven additional publications confirmed the presence of café-au-lait spots, with or without freckling and the absence of neurofibromas and Lisch nodules in patients with Legius syndrome [Denayer et al., 2011a; Laycock-Van Spyk et al., 2011; Messiaen et al., 2009; Muram-Zborovski et al., 2010; Pasmant et al., 2009a; Spencer et al., 2011; Spurlock et al., 2009]. Macrocephaly (15 of 93) and Noonan-like characteristics (14 of 89) were reported by different research groups (Table 1) [Denayer et al., 2011a; Laycock-Van Spyk et al., 2011; Messiaen et al., 2009; Muram-Zborovski et al., 2010; Pasmant et al., 2009a; Spencer et al., 2011; Spurlock et al., 2009].

Learning disabilities (24 of 115), developmental delay (20/85), or attention deficit (15/87) were noticed in all reports except by Spurlock et al. (2009) (Table 1). One study described observations on intelligence and behavior in 15 patients with Legius syndrome [Denayer et al., 2011b]. The mean full scale IQ in children with Legius syndrome did not differ from the control group but was higher compared with NF1 patients. There were no differences in verbal IQ, although children with Legius syndrome showed a lower performance IQ compared with unaffected family members. Full scale IQ and verbal IQ were significantly lower in patients with NF1 than in those with Legius syndrome. These preliminary data suggested that the cognitive phenotype in children with Legius syndrome is milder compared with NF1.

Additional clinical features reported (Table 1) include pectus deformities (excavatum or carinatum) (12 of 87) [Denayer et al., 2011; Messiaen et al., 2009; Laycock-Van Spyk et al., 2011; Spencer et al., 2011] and unilateral postaxial polydactyly (3 of 159) [Denayer et al., 2011; Messiaen et al., 2009]. In some patients, lipomas were reported [Denayer et al., 2011; Messiaen et al., 2009; Pasmant et al., 2009a; Spencer et al., 2011]. Furthermore, single occurrences of each of the following malignancies have been observed: a monoblastic acute leukemia (same case described twice) [Pasmant et al., 2009a, 2009b], giant cell tumor, dermoid tumor of the ovary, breast cancer [Messiaen et al., 2009], vestibular schwannoma, desmoid tumor [Denayer et al., 2011a]. It is not clear if the childhood leukemia was related to the *SPRED1* mutation as no *SPRED1* second hit mutation was found [Pasmant et al., 2009b]. Batz et al. (2010) found no evidence of *SPRED1* involvement in a small set of juvenile myelomonocytic leukemia cases. Large series will be necessary to show whether there is an increased risk for childhood leukemia in individuals with Legius syndrome. Given the actual still limited number of identified cases, a risk for a specific complication smaller than 1–2% cannot be excluded. For none of the other malignancies, each observed in single cases, tumor analysis was reported and it remains unknown whether these tumors were incidental or *SPRED1* related.

In conclusion, the overall human phenotype in patients with Legius syndrome is milder compared with patients with NF1 (Table 2). None of the individuals with Legius syndrome had discrete cutaneous or plexiform neurofibromas, typical NF1 osseous lesions, or symptomatic optic pathway gliomas. Some other manifestations were reported, although it is not clear yet if these manifestations are part of the clinical phenotype (Table 1). No systematic occurrence of a specific tumor type was detected, except possibly lipomas in adults. However, since the total number of reported cases is relatively small (with a substantial proportion of children), less frequent associations in adults could have been missed. In order to identify rare complications with a prevalence of 1% in adults, at least 250 adults with Legius syndrome have to be investigated [Messiaen et al., 2009]. To date, only 159 cases (all ages) with Legius syndrome have been reported.

Molecular Genetic Testing

Initially, *Spred1* was discovered in a mouse cDNA osteoclast library [Wakioka et al., 2001]. Sequence homology modeling showed

Table 1. Summary of Clinical Features Observed in Individuals Carrying a SPRED1 Mutation, as Reported in Literature

	Report (number of individuals)							
	Brems (40 = 37+3)	Pasmant (18)	Spurlock (12)	Messiaen (40)	Muram-Zborovski (2)	Denayer (30)	Laycock-Van Spyk (10)	Spencer (7)
Clinical features								
>5 CALs	39	18	12	35	2	28	6	7
Freckling	12/39	14	7/11	17	2	10/27	1	2
Relative or absolute macrocephaly	13/31	1	1/8	7/31	1	3/24	2	
Short stature (< 5th percentile)	3/29			1/30	0	6/26		1
Noonan-like face/characteristics	5		0	1		12		1
Pectus excavatum/carinatum	3			3		7	1	1
Depigmented macules	3			1				
Vascular malformation	1		1	2				
Lipomas	14	2		2 (incl angiolipoma)		2/15 adults		1/2 adults
Learning disabilities	6	5	0	1/38	2	14/25	1	1
(Psychomotor) developmental delay	1		0	6/38		6/18	7	1
AD(H)D/autistic behaviour/concentration problems/hyperactivity	2	1		5/38		6/14	1	2
Headaches		4				2		
Hearing loss				3		1		
Seizures		1		2		1	1	
Polydactyly				1		2		
Clinodactyly 5th finger				2				
Tumors	3 (lung cancer, renal cancer (Wilms tumor), colonadenoma)	1 Monoblastic acute leukemia		3 (giant cell tumor, dermoid tumor ovary, breast cancer)		2 (vestibular schwannoma, desmoid tumor)		
Scoliosis						4	1	
Valve problems	Pulmonic valve stenosis 1			Pulmonic valve stenosis+mitral valve prolapse 1		prolapse mitral+tricuspid valve 1		
Others			Excessive periorbital pigmentation 1, congenital soft tissue swelling scalp	Progressive dystonia 1	Deep iris crypts 1	T2 hyperintense spots subcortical matter 2,Parkinson 1, Chiari malformation with syringomyelia 1, block vertebra 1, arachnoid cyst 1, hypotyroidism 1, Marfan 1	Periorial and ocular hyperpigmentation 1, abnormal connective tissue both hands 1	Deep iris crypts and iris pigmentation 1, cleft palate 1(6.6 Mb deletion, including SPRED1)
	[a] UAB15, 74, 84 included; UAB 31, 48, 88 not included since these are included in clinical cohort by Messiaen							

[a] UAB15, 74, 84 included; UAB 31, 48, 88 not included since these are included in clinical cohort by Messiaen.

Table 2. Overview of Clinical Signs and Features Associated (or Not Associated) with NF1 and Legius Syndrome

NIH diagnostic criteria for NF1: two or more of the following features:	NF1	Legius syndrome
• ≥ 6 café-au-lait spots (> 5 mm diameter in children, > 15 mm in adults)	+	+
• ≥ 2 cutaneous or subcutaneous neurofibromas or 1 plexiform neurofibroma	+	–
• Axillary or inguinal freckling	+	+
• Optic pathway glioma	+	–
• ≥ 2 Lisch nodules	+	–
• Bone dysplasia (pseudarthrosis, tibial bowing, . . .)	+	–
• First-degree relative with clinical diagnosis of NF1, according to these criteria	+	+
Associated features:	**NF1**	**Legius syndrome**
• MPNST	+	–
• Scoliosis	+	–
• Macrocephaly	+	+
• Short stature	+	+
• Specific malignancies	+	– (?)
• Learning disabilities	+	+

human *SPRED1* location on chromosome 15 band q13.2 [Kato et al., 2003]. Linkage analysis on two of the five affected families with mild clinical NF1-like manifestations pointed to the same region on chromosome 15 with inactivating heterozygous *SPRED1* mutations in the affected members [Brems et al., 2007]. Human *SPRED1* is a relatively small gene, spanning approximately 104.4 kb of genomic DNA. Seven exons code for a 7255 bp transcript with an open reading frame of 1,335 nucleotides encoding 444 amino acids.

Molecular genetic testing consists of DNA- or RNA-based sequence analysis of the *SPRED1* coding region [Brems et al., 2007; Messiaen et al., 2009]. Exon–intron boundaries are screened to detect potential splice site defects. Recently, it was shown that CNCs such as multi-exon deletions and whole *SPRED1* gene deletions account for approximately 10% of the 40 detected *SPRED1* mutations in a cohort of 510 *NF1*-negative patients with multiple café-au-lait spots with or without freckling and no other signs of NF1 [Spencer et al., 2011]. These mutations would be missed by Sanger sequencing; therefore, the use of multiplex ligation-dependent probe amplification, arrayCGH, or another technique to detect deletions is advised for comprehensive genetic *SPRED1* evaluation [Spencer et al., 2011].

Since the clinical phenotype of Legius syndrome and NF1 partially overlap, it is essential to make a correct diagnosis. The identification of a pathogenic *SPRED1* mutation allows establishing the diagnosis of Legius syndrome, which cannot be decided solely based on the clinical pigmentary features alone. We recommend analyzing the *NF1* gene first, and if negative, considering *SPRED1* testing in patients with café-au-lait spots with or without freckling and no other NF1-specific diagnostic features. This recommendation is based on the finding that a pathogenic *NF1* mutation was identified in 43% of sporadic cases (café-au-lait spots only with or without freckling and no other diagnostic criteria) and only in 1.3% a pathogenic *SPRED1* mutation was identified [Messiaen et al., 2009]. In the cohort of familial cases with the same phenotypic criteria, 73% carried a *NF1* mutation and 19% a *SPRED1* mutation.

SPRED1 Mutations and Polymorphisms

Legius syndrome is caused by heterozygous inactivating germline mutations in the *SPRED1* gene. Familial as well as sporadic individuals have been identified [Brems et al., 2007; Denayer et al., 2011a; Laycock-van Spyk et al., 2011; Messiaen et al., 2009; Muram-Zborovski et al., 2010; Pasmant et al., 2009a; Spencer et al., 2011; Spurlock et al., 2009]. In the largest study of *SPRED1* mutations published so far, 39% of the individuals with *SPRED1* mutations were sporadic cases (13 of 33) [Messiaen et al., 2009]. Pathogenic *SPRED1* mutations were only identified in the *NF1*-negative individuals with café-au-lait spots with or without freckling and no other NF1 diagnostic criterion.

As of January 2012, 146 probands with a *SPRED1* mutation were registered in the SPRED1-Leiden Open Variation Database. Mutations are named according to the HGVS nomenclature guidelines (www.HGVS.org) and numbered with respect to the *SPRED1* cDNA reference sequence (NM_152594.2) with the A of the start codon marked as +1. Twenty-three probands with mutations in the *SPRED1* gene are described in this report here for the first time and 16 of these mutations were never reported before (Supp. Table S1). These 16 unreported sequence variants consist of five frameshift, four CNCs, four nonsense, one splice-site, and nine missense mutations. Eight missense mutations are unclassified variants and one (p.Ser149Asn) was previously identified as a likely benign rare variant.

Eighty-nine different variants were identified so far and the mutations are distributed over the entire *SPRED1* gene (Fig. 1A and B). The majority of mutations are either truncating or result in absence of the protein, that is, 28 frameshift mutations, 19 nonsense mutations, eight CNCs, two splicing defects, and one mutation that affects the initiating codon (Fig. 1A and B; Supp. Table S1). Twenty-nine missense mutations were identified until now: eight are probably benign rare variants, three pathogenic, one suggested to be pathogenic (based on familial segregation and amino acid conservation), and 17 are still unclassified and need further family and functional studies to help clarify their significance. Only a minority of identified *SPRED1* missense mutations is pathogenic (Fig. 1B, Supp. Table S1).

Furthermore, the public dbSNP135 database reports three validated missense variants (p.Asp274Gly, p.Val309Ala, and p.Pro315Leu): p.Asp274Gly and p.Pro315Leu have not yet been identified in individuals with suspected Legius syndrome (L.M., personal communication) and are probably benign rare variants. Functional data confirm the neutral role of the missense change p.Val309Ala (H.B., personal communication).

There is only limited data available regarding somatic mutations in *SPRED1*. Only one somatic mutation has been published [Brems et al., 2007]. Melanocytes cultured from a café-au-lait spot from an individual with Legius syndrome showed in addition to the germline mutation (p.Arg24*) a somatic frameshift *SPRED1* mutation (c.304dupA; p.Thr102Asnfs*7), resulting in complete loss-of-function.

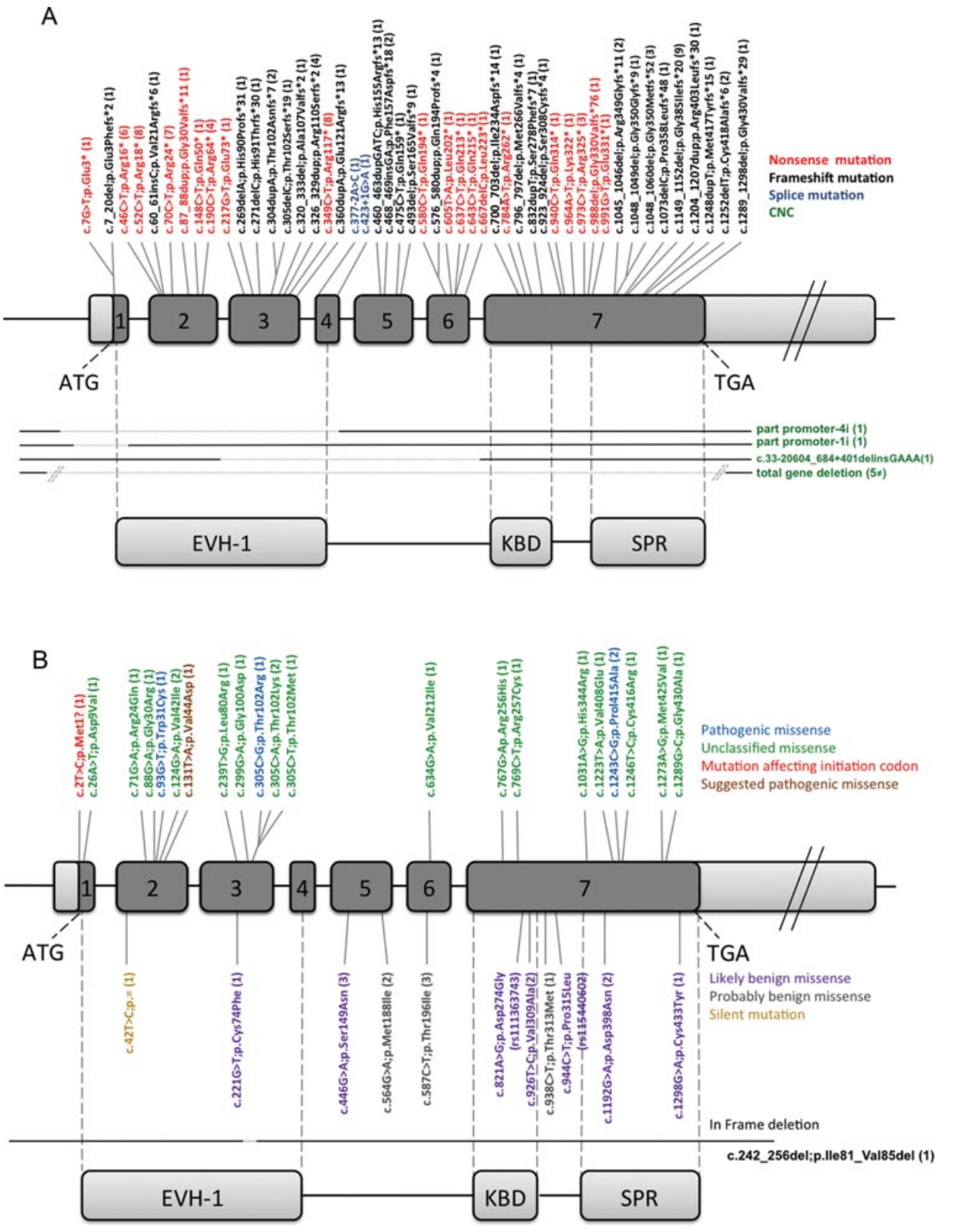

Figure 1. *SPRED1* mutation spectrum. Nucleotide numbering reflects the cDNA transcript with +1 corresponding to the A of the ATG translation initiation codon in the reference sequence NM_152594.2. The reference protein sequence for missense mutations is NP_689807.1. The location of mutations within the functional domains of the *SPRED1* gene is indicated: EVH-1, KBD, and SPR. Mutations listed in Figure 1A would be expected to result in absence (entire gene deletions) or significant truncation of the *SPRED1* mRNA (e.g., nonsense mutations, splice mutations, intragenic deletions, or frameshift mutations). Mutations that would not be predicted to result in loss or significant truncation (e.g., missense mutations, in-frame deletions) are listed in Figure 1B. In addition, missense mutations that are likely benign are also listed in Figure 1B.

Genotype–Phenotype Correlation

Different pathogenic mutations (including frameshift, nonsense, missense, in-frame deletion, CNCs, and splicing) were already identified distributed over the *SPRED1* gene, but no genotype–phenotype correlation could be identified yet. A recent study by Spencer et al. (2011) reported for the first time seven individuals carrying multi-exon to whole *SPRED1* deletions. Surprisingly, the phenotype did not differ from Legius syndrome patients with a pathogenic point mutation in *SPRED1*, even in the patient where two other genes were codeleted (*TMCO5A*, *FAM98B*). Nevertheless, isolated cleft palate was noticed in one patient with a large deletion (6.6 Mb) spanning *SPRED1* as well as 29 additional annotated reference genes. Large deletions in this region (not including *SPRED1*) were previously linked with cleft lip/palate and developmental delay [Chen et al., 2008; Erdogan et al., 2007; Schwartz et al., 1985].

To draw conclusions on genotype–phenotype correlations, clinical and mutational data from more individuals with Legius syndrome will need to be collected in the near future.

Biological Relevance

Protein Structure and Function

The human SPRED1 protein has a molecular mass of 50 kDa, contains 444 amino acids, and belongs to the Sprouty/SPRED family. The SPRED family contains three members: SPRED1, SPRED2, and SPRED3. Four isoforms of the mammalian Sprouty protein are known (SPROUTY1-4). Three functional domains are known in SPRED1, an N-terminal Ena/Vasp Homology 1 domain (EVH-1), a central KIT-binding domain (KBD), and a C-terminal SPROUTY-related domain (SPR), reviewed by Bundschu et al. (2007) (Fig. 1A and B).

Most functional investigations were performed on mouse Spred1 and only limited functional data are available on human SPRED1. Mouse Spred1 is predominantly expressed in brain, some fetal tissues, and is highly enriched in the central nervous system germinal zones during neurogenesis. Spred1 is critical for normal cortical development, modulates progenitor self-renewal/proliferation, and helps to maintain the integrity and organization of the germinal zones [Phoenix and Temple, 2010].

Mouse Spred1 plays an important role in Ras/Raf signaling [Wakioka et al., 2001]. Spred1 inhibits Ras–MAPK activation after growth factor induction, while there is no effect on Akt or Rac activation. The SPR domain is necessary for membrane localization and colocalization with Ras. Spred1 inhibits MAPK activity by suppressing Raf activation by Ras–GTP. The KBD is involved but not essential in ERK suppression [Nonami et al., 2004; Wakioka et al., 2001]. Spred1 localizes in the lipid raft/caveolar fraction by virtue of its C-terminal part (SPR) and interacts with caveolin [Nonami et al., 2005]. Spred1 expression has an effect on the actin cytoskeleton via its N-terminal EVH-1 and C-terminal SPR domain. Disruption of stress fiber formation requires both an N-terminal EVH-1 domain and a C-terminal SPR domain of Spred1 [Miyoshi et al., 2004].

New interaction partners of Spred1 were recently identified. A direct interaction was demonstrated between the microtubule-associated protein/microtubule affinity-regulating kinase-activating kinase, Spred1, and the testis-specific protein kinase (TESK1) [Johne et al., 2008]. Spred1 interacts with the two other proteins through its C-terminal SPR domain and it has been suggested that the three proteins affect F-actin and the microtubular cellular network. The inhibition of TESK1 by Spred1 makes F-actin fibers dynamic. Fibroblast growth factor receptor like-1 (FGFRL1) is another binding partner of the SPR domain; Spred1 increased the retention time of FGFRL1 at the plasma membrane [Zhuang et al., 2011]. The SPR domain of Spred1 interacts with the kinase domain of a novel kinase, DYRK1A, by inhibiting the ability of DYRK1A to phosphorylate its substrates, Tau and STAT3 [Li et al., 2010].

Mouse Spred1 is a substrate for the tyrosine phosphatase SHP2. Tyrosine dephosphorylation increases the inhibitory action of Spred1 in the Ras/Erk pathway [Quintanar-Audelo et al., 2011].

Recently, two microRNAs were linked to Spred1, miR-126, and miR-212. MicroRNA-126 enhances VEGF signaling during angiogenesis and promotes blood vessel formation by suppressing Spred1 [Wang et al., 2008]. MicroRNA-126 positively regulates mast cell proliferation and cytokine production by suppressing Spred1 [Ishizaki et al., 2011].

Increased striatal miR-212 activity plays an important role in the addiction to cocaine by amplifying the stimulatory effects of cocaine on cAMP response element binding protein signaling [Hollander et al., 2010]. This occurs through miR-212-enhanced Raf1 activity via Spred1 downregulation. There is a binding site for miR-212 in the Spred1 promoter region.

Human wild-type SPRED1 also has the ability to inhibit growth factor-induced RAS–MAPK signaling. The EVH-1 as well as the SPR domain seem to be necessary for inhibition [Brems et al., 2007]. Other functions of human SPRED1 or ligands of the EVH-1 domain of human SPRED1 have not yet been identified.

Human hepatocellular carcinoma (27 of 32) showed decreased expression levels of SPRED1 and SPRED2 [Yoshida et al., 2006]. SPRED1 expression inversely correlated with the tumor incidence of invasion and metastasis. Spred1 overexpression inhibits cell proliferation of this carcinoma in vitro and in vivo and inhibits the RAS–MAPK pathway activation. The secretion of matrix metalloproteinases, important in tumor invasion and metastasis, was reduced by Spred1 overexpression.

No obvious SPRED1 immunohistochemical staining with specific Spred1 antibodies could be shown in human adult tissue samples [Engelhardt et al., 2004], although GeneNote shows that *SPRED1* mRNA is expressed in whole human genome expression profiles in normal tissues [Shmueli et al., 2003; Yanai et al., 2005]. *SPRED1* is expressed in lung, brain, spinal cord, and spleen and lower expression profiles are seen in liver, pancreas, prostate, kidney, heart, thymus, muscle, and bone marrow.

It has been demonstrated that *SPRED1* is also expressed in human melanocytes [Brems et al., 2007]. To study the potential role of *SPRED1* in the pathogenesis of café-au-lait spots, melanocyte cultures from control skin ($SPRED1^{+/+}$), from normal skin ($SPRED1^{+/-}$), and a café-au-lait spot ($SPRED1^{-/-}$) from the same patient with Legius syndrome were compared [Brems et al., 2007]. After stimulation, the functional effect was studied on the RAS–MAPK pathway with higher levels of activated MEK and ERK in the $SPRED1^{-/-}$ melanocytes and lowest levels in the $SPRED1^{+/+}$ melanocytes, with intermediate levels in the $SPRED1^{+/-}$ melanocytes.

Functional Analysis

Functional assays are used to evaluate the effect of the identified human *SPRED1* mutations with regard to the RAS–MAPK pathway. In the initial paper on Legius syndrome, three different assays were described, a biochemical analysis using HEK293T cells, a luciferase assay, and an in vitro Raf1 kinase assay [Brems et al., 2007].

The nature of most identified mutations suggested loss-of-function. The ability to inhibit ERK activation was investigated in

a biochemical assay of HEK293T cells. HEK293T cells were transiently transfected with wild type and mutated SPRED1 constructs and cotransfected with Erk2. Cells were stimulated with bFGF or EGF, lysed and analyzed via immunoblot. Wild-type SPRED1 was able to inhibit ERK activation, whereas mutant constructs (for example, p.Ile81_Val85del, p.Met266fsVal*4, p.Arg325*) were unable to reduce ERK activation. Some of the mutant SPRED1 proteins were not stably expressed and were immediately degraded by the proteasome. They could not be used for further analysis.

The luciferase assay was the second assay used by Brems et al. (2007) and Messiaen et al. (2009). In this sensitive assay, FGF- or EGF-induced ERK activation was measured by an Elk-1 responsive reporter. Elk-1, a transcription factor, is a substrate for phosphorylation if the MAPK (ERK) pathway is activated. Elk-1 phosphorylation induces binding to the promoter region of target genes and initiates transcription. Elk-1 activation was measured by a GAL4 DNA binding domain/Elk-1 fusion system. Overexpression of cotransfected wild-type SPRED1 constructs efficiently inhibited the activation of the MAPK pathway after stimulation, as measured by low luciferase activity. No decrease in luciferase activity was seen after overexpression of inactivating SPRED1 mutants.

In the third assay, an in vitro Raf1 kinase assay, 293T cells were transiently transfected with Flag-Raf1 and SPRED1 constructs, stimulated with FGF or EGF and cells were lysed, and immunoprecipitated with an anti-Flag antibody. An additional incubation step was carried out with ATP, $MgCl_2$, and inactive recombinant MEK1 as substrate. Immunoblots measured phosphorylated MEK and phosphorylated Raf1 levels. Decreased Raf1 kinase activity was observed with wild-type SPRED1 overexpression, in contrast to mutant SPRED1.

A fourth and sensitive assay was described by Messiaen et al (2009). In vitro neurite outgrowth of rat pheochromocytoma cells (PC12 cells) is highly dependent on MAPK activity after nerve growth factor (NGF) stimulation. Wild-type SPRED1 overexpression in PC12 cells inhibited the MAPK pathway activation after NGF stimulation and decreased the number of cells showing neurite outgrowth. SPRED1 overexpression of inactivating mutants did not inhibit neurite outgrowth after NGF stimulation.

Missense Mutations

Twenty-six percent of all *SPRED1* mutations identified are missense mutations: in 39 of 146 unrelated *SPRED1*-positive individuals, one of 29 different missenses has been found. Only a minority of missense mutations was confidently classified as pathogenic and many are still unclassified. It is difficult to draw final conclusions regarding pathogenicity of certain *SPRED1* missense mutations.

We suggest that at least three different criteria are used to predict pathogenicity [Brems et al., 2007; Denayer et al., 2011a; Messiaen et al., 2009;]: family data, amino acid evolutionary conservation, and two independent sensitive functional MAPK pathway assays.

A missense mutation is classified as pathogenic if the identified *SPRED1* mutation is de novo or if the mutation segregates with the Legius syndrome phenotype in a large family. This means that only the affected proband and affected relatives who have the clinical signs of Legius syndrome carry the mutation. A missense mutation is probably a rare benign variant, if it is found in an unaffected relative.

An amino acid in the human SPRED1 protein sequence is considered to be not evolutionary conserved if another amino acid is found in at least two of the following species *Callitrix jacchus* (XP_002753607.1), *Mus musculus* (NP_277059.1), *Rattus norvegicus* (NP_001040554.1), *Oryctolagus cuniculus* (XP_002717848.1), *Bos Taurus* (XP_598113.3), *Xenopus tropicalis* Q66JG9.1), *Danio rerio* (NP_998397.1), and *Drosophila melanogaster* (NP_610988.1). Additional supportive data can be derived from either PolyPhen (http://genetics.bwh.harvard.edu/pph) or SIFT (http://sift.jcvi.org). In addition, in silico analysis of the identified *SPRED1* missense mutation can be carried out by homology modeling, which will provide additional information about the putative consequences of the respective missense mutation. However, the crystal structure of *X. tropicalis* only provides information of the EVH-1 domain and no crystal structure of the KBD or SPR domain is available yet [Harmer et al., 2005].

Finally, functional studies demonstrating that the missense mutation lacks the potential to downregulate the RAS–MAPK pathway provide the ultimate proof of pathogenicity.

We cannot exclude that some mutations that do not affect the MAPK pathway (as evidenced by functional studies) might be pathogenic by a different mechanism. However, there is no evidence for this at the moment: all de novo mutations as well as all known familial *SPRED1* mutations segregating with the phenotype are deficient in downregulating the MAPK pathway.

Mouse Model

The Spred1 protein is well conserved during evolution with a 93.5% protein identity between human and mouse. The *Spred1* gene is located on mouse chromosome 2 and it is expressed in fetal brain, heart, lung, liver, and bone [Engelhardt et al., 2004]. In adult tissue, *Spred1* is predominantly expressed in brain [Engelhardt et al., 2004; Kato et al., 2003]. A conventional Spred1 knockout mouse model is available and these mice were generated via homologous recombination with the KBD and SPR domain deleted [Inoue et al., 2005]. Spred1 knockout mice are fertile although they have a lower weight and a shortened face [Brems et al., 2007; Inoue et al., 2005]. In an earlier study, $Spred1^{-/-}$ bone marrow-derived mast cells showed enhanced proliferation and Erk activation after stimulation [Nonami et al., 2004]. The Spred1 knockout mouse model was initially studied as an ovalbumin-induced allergic asthma model [Inoue et al., 2005].

Since learning problems were reported in several children with Legius syndrome [Brems et al., 2007], the Spred1 mouse model was investigated for hippocampus-dependent learning and memory in two different learning tasks [Denayer et al., 2008]. In the hidden version of the Morris water maze, a spatial discrimination task, $Spred1^{-/-}$ mice showed decreased performance in learning and memory. This defect could not be explained by a difference in neuromotor performance or sensory perception. In a second learning task, the visual discrimination task (T-maze), where little motor proficiency is required, $Spred1^{-/-}$ mice performed significantly worse in nearly all stages of visual discrimination training. During the final mixed trial presentations, $Spred1^{-/-}$ mice performed poorly, with the $Spred1^{+/-}$ mice at an intermediate level between the $Spred1^{-/-}$ and $Spred1^{+/+}$ mice.

Electrophysiological recordings on brain slices from Spred1 knockout mice identified short- and long-term synaptic hippocampal plasticity defects [Denayer et al., 2008], including a deficiency in long-term potentiation (LTP) and long-term depression in the CA1 region. Biochemical analysis, after LTP induction, demonstrated increased Erk activation in $Spred1^{-/-}$ slices, compared with control brain slices.

These findings are similar to the learning and synaptic plasticity defects in the $Nf1^{+/-}$ mice [Cui et al., 2008], and stress the importance

of the RAS–MAPK pathway in learning and memory. The learning and synaptic plasticity in adult *Nf1*$^{+/-}$ mice can be acutely rescued with lovastatin treatment [Cui et al., 2008]. Similar experiments have not yet been reported in Spred1 mice.

Management and Future Prospects

At present, a less intense medical follow-up has been recommended for individuals with Legius syndrome compared with NF1. However, clinical data are only available from 159 individuals with Legius syndrome. Therefore, it is essential to collect information on more patients and to offer long-term clinical surveillance for patients with Legius syndrome. Only if clinical and molecular data from several hundred individuals are collected, we will be able to draw conclusions regarding rare complications and potential tumor prevalence associated with Legius syndrome. We hope that the data will confirm the low frequency of malignancies as observed so far in patients with Legius syndrome. A correct diagnosis has important implications for prognosis, counseling, and potential prenatal diagnosis. The diagnosis of Legius syndrome may relieve the psychological burden in families who are otherwise anxious of serious NF1-related complications. We expect that specific therapies might become available in the future for the treatment of cognitive problems in children with NF1 and with Legius syndrome.

Only a minority of *SPRED1* missense mutations were classified as pathogenic and some newly identified missense mutations have not yet been fully studied (family and functional studies). It is crucial to continue studying these mutations for pathogenicity. The combination of family data (de novo or linked to the typical phenotype in many relatives), amino acid conservation, and functional effects on the RAS–MAPK pathway will allow us to classify most of these mutations with respect to their pathogenicity. However, it is possible that some *SPRED1* missense mutations result in a normal activity to the MAPK pathway, but they still may have an abnormal activity to another pathway. Consequently, we must remain conservative and classify such mutations as "probably benign rare variant." Nevertheless, present data show that all *SPRED1* missense mutations segregate with the clinical pathogenic picture and are deficient in the RAS–MAPK pathway, similar as the typical inactivating mutations.

There are still clinical cases with multiple café-au-lait spots, familial and sporadic, in cohorts negative for a pathogenic *NF1* and *SPRED1* mutation. We know that in some sporadic cases, mosaicism for a *NF1* mutation is present, explaining the NF1 features in the absence of a detectable *NF1* mutation in white blood cells [Maertens et al., 2007]. It is possible that other patients with multiple café-au-lait spots without a *NF1* or *SPRED1* mutation might be the result of mutations in other genes with a direct or indirect link to the RAS–MAPK pathway, as was recently shown in CBL mutation-associated syndrome [Niemeyer et al., 2010; Pérez et al., 2010].

Note added in Proof: At the time when the mutation database was initiated and this paper was submitted, a total of 146 unrelated individuals carrying a SPRED1 mutation were entered. With the first quarterly update we have added another 44 unrelated individuals carrying a SPRED1 mutation, increasing the number of entries with ~30%.

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