Received: 25 March 2021 | Revised: 16 June 2021 | Accepted: 13 July 2021

DOI: 10.1002/humu.24261

REVIEW

Human Mutation HGVS HUMAN GENOME VARIATION SOCIETY WILEY

Pathogenic noncoding variants in the neurofibromatosis and schwannomatosis predisposition genes

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Funding information

U.S. Army Medical Research Acquisition Activity (USAMRAA), Congresionally Directed Medical Research Program (CDMRP), Neurofibromatosis Research Program (NFRP), Grant/Award Number: W81XWH1910334; Manchester National Institute for Health Research (NIHR) Biomedical Research Centre, Grant/Award Number: IS-BRC-1215-20007

Abstract

Neurofibromatosis type 1 (NF1), type 2 (NF2), and schwannomatosis are a group of autosomal dominant disorders that predispose to the development of nerve sheath tumors. Pathogenic variants (PVs) that cause NF1 and NF2 are located in the *NF1* and *NF2* loci, respectively. To date, most variants associated with schwannomatosis have been identified in the *SMARCB1* and *LZTR1* genes, and a missense variant in the *DGCR8* gene was recently reported to predispose to schwannomas. In spite of the high detection rate for PVs in NF1 and NF2 (over 90% of non-mosaic germline variants can be identified by routine genetic screening) underlying PVs for a proportion of clinical cases remain undetected. A higher proportion of non-NF2 schwannomatosis cases have no detected PV, with PVs currently only identified in around 70%–86% of familial cases and 30%–40% of non-NF2 sporadic schwannomatosis cases. A number of variants of uncertain significance have been observed for each disorder, many of them located in noncoding, regulatory, or intergenic regions. Here we summarize noncoding variants in this group of genes and discuss their established or potential role in the pathogenesis of NF1, NF2, and schwannomatosis.

KEYWORDS

intronic, neurofibromatosis, noncoding, pathogenic variants, regulatory, schwannomatosis

1 | INTRODUCTION

Neurofibromatosis type 1, type 2 (NF1, NF2), and non-NF2 schwannomatosis, are a group of autosomal dominant genetic disorders that predispose affected individuals to the development of tumors in the nervous system and specifically on the nerve sheath. Molecular characterization of these disorders has been important for their accurate diagnosis and management, given the considerable clinical phenotypic overlap, particularly between NF2 and non-NF2 schwannomatosis. NF1 causative variants are located within the *NF1* (chr17q11.2) gene (Cawthon et al., 1990; Shen et al., 1996; Viskochil et al., 1990; Wallace et al., 1990) and hallmark features of the disease include predisposition to neurofibromas and the presence of café-au-lait spots (CALS) (McGaughran et al., 1999). NF2 causative variants are located within the *NF2* locus (chr22q12.2) (Rouleau et al., 1993; Trofatter et al., 1993) and predispose to bilateral vestibular schwannomas, which are known to result in imbalance, tinnitus, and loss of hearing (Asthagiri et al., 2009). Other clinical features of NF2 include cutaneous schwannomas, meningiomas, and ependymomas (Evans et al., 1992; Smith et al., 2011). A large proportion of NF2 patients die early as a result of the disease (Hexter et al., 2015). Finally, *SMARCB1*- (chr22q11.23) and

LZTR1- (chr22q11.21) associated schwannomatosis are characterized by the presence of non-vestibular schwannomas that frequently cause intractable pain (Hulsebos et al., 2007; Piotrowski et al., 2014). Indeed, the absence of vestibular schwannomas has previously been used as a diagnostic criterion to differentiate schwannomatosis from NF2 (MacCollin et al., 2005), although there are reports of *LZTR1*-associated schwannomatosis patients with unilateral vestibular schwannomas (Smith, Kulkarni, et al., 2012; Smith et al., 2015). Genetic analysis is therefore useful and in many cases necessary to resolve the diagnosis.

Pathogenic variants (PVs) leading to NF1 and NF2 are loss-of-function (LoF) variants. Like other neoplastic syndromes, NF1 and NF2 follow a two-hit model whereby biallelic inactivation of the *NF1* or *NF2* gene precedes tumorigenesis (Knudson, 2001). However, the full spectrum of mechanisms that lead to partial or total loss of protein is yet to be established, as is the extent to which variation in noncoding regions contributes to disease. Furthermore, the heterogeneity of phenotypes across patients has led to the proposal of a possible role for modifying loci contributing to NF1 and NF2 pathogenesis (Bruder, Ichimura, et al., 1999; Easton et al., 1993; MacCollin et al., 2001; Pemov et al., 2014; Sharafi & Ayter, 2018; Yu et al., 2020), thus increasing the interest in variants potentially affecting regulatory regions.

The genetic profile of non-NF2 schwannomatosis is heterogeneous and although associated variants result in at least partial LoF, they do not always lead to loss of protein. In the case of *SMARCB1*, for example, germline variants leading to schwannomatosis tend to produce a protein with reduced functionality compared to the wild type protein (Boyd et al., 2008; Smith, Walker, et al., 2012; Smith et al., 2014) and loss of SMARCB1 protein in schwannomas (either total or partial) has been shown to happen only in a subset of cells (Patil et al., 2008). In addition, the mechanisms involved in tumorigenesis in schwannomatosis appear to be more complex than the two-hit model described for NF1 and NF2, with somatic biallelic inactivation of *NF2* observed in tumors of patients carrying *SMARCB1* and *LZTR1* germline mutations (Hadfield et al., 2008; Paganini, Sestini, et al., 2015; Piotrowski et al., 2014; Sestini et al., 2008). This seems to be true also for sporadic schwannomatosis cases where inactivating somatic *SMARCB1* and *NF2* variants were identified (Paganini et al., 2018). A recent report adds to this complexity by suggesting a prominent role of microRNAs (miRNAs) in schwannoma formation. Rivera et al. (2020) identified a novel mutation in *DGCR8*, a microprocessor gene, in a family affected by multinodular Goitre and schwannomatosis. This single nucleotide variant (NM_022720.6:c.1552G>A) results in a change in amino acid residue in position 518 of the protein, from glutamate to lysine (p.Glu518Lys).

The continuous development of more sensitive and cost-effective methods to identify rare genetic variants associated with disease has made it possible to identify an increasing number of variants of uncertain significance (VUS). Many of these variants are located in noncoding, regulatory, or intergenic regions and although evidence is still limited, proposed mechanisms are emerging that might explain their role in tumorigenesis. This might be illustrated by newly discovered VUS predicted to affect splicing, which are of interest given the role of aberrant splicing in tumorigenesis and metastasis in NF2-associated tumors (Koga et al., 1998; Luo et al., 2015; Scoles et al., 1998) and the suggestion that alternative splicing may account for at least some of the phenotype variability observed in NF1 (Barron & Lou, 2012).

In addition to splicing variants, variants affecting *cis*-regulatory elements (CREs) have been proposed not only as drivers for disease but also as modulators of penetrance and phenotype, suggesting the possibility of as yet unidentified epistatic interactions between loci. Examples of communication between different regulatory loci include reports of intronic sequences within certain genes that interact with enhancers and regulate transcription of downstream or upstream targets (Stadhouders et al., 2012). Furthermore, intronic regions may contain noncoding RNA transcripts that regulate gene expression and may be relevant to carcinogenesis (Williams & Farzaneh, 2012; Zimta et al., 2020).

The present review aims to summarize the different types of noncoding variants in *NF1*, *NF2*, *SMARCB1*, and *LZTR1* that have been associated with disease and to integrate this data to provide an overview of the implications for future characterization and diagnosis of these disorders.

1.1 | Coding mutation spectra

1.1.1 | Neurofibromatosis type 1

The *NF1* (neurofibromin 1) gene spans approximately 300 kilobases (kb) of chromosome 17q11.2 and encodes neurofibromin, which negatively regulates the Ras pathway, through its GAP-related domain (GRD). This is thought to be the main mechanism through which neurofibromin exerts its tumor suppressor function (Bollag et al., 1996; Danglot et al., 1995; Dischinger et al., 2018; Y. Li et al., 1995; Marchuk et al., 1991; Martin et al., 1990). Two major isoforms of *NF1* have been identified, containing 57 and 58 exons respectively (Figure 1a). Regions of high homology with other members of GTPase activating protein (GAP) family exist between exons 21 and 27a (Y. Li et al., 1995; G. F. Xu et al., 1990). In addition, three smaller genes are located within intron 26; *OGMP* (oligodendrocyte myelin glycoprotein), *EVI2A* (ecotropic viral integration 2A), and *EVI2B* (ecotropic viral integration 2B) (Cawthon et al., 1990, 1991; Hinks et al., 1995).

Identification of genetic variants leading to NF1 associated phenotypes has been cumbersome, in part due to the large size of the *NF1* locus. Moreover, a large proportion of germline PVs that have been observed in NF1 patients are de novo, consistent with the high de novo mutation rate of the gene (Evans et al., 2010; Messiaen et al., 2000). The most common deleterious variants associated with NF1 are those resulting in premature stop codons (Fahsold et al., 2000). The second most common type of variant affects messenger RNA (mRNA) splicing. Interestingly, these variants also constitute a large proportion of the recurrent PVs observed across NF1 patients

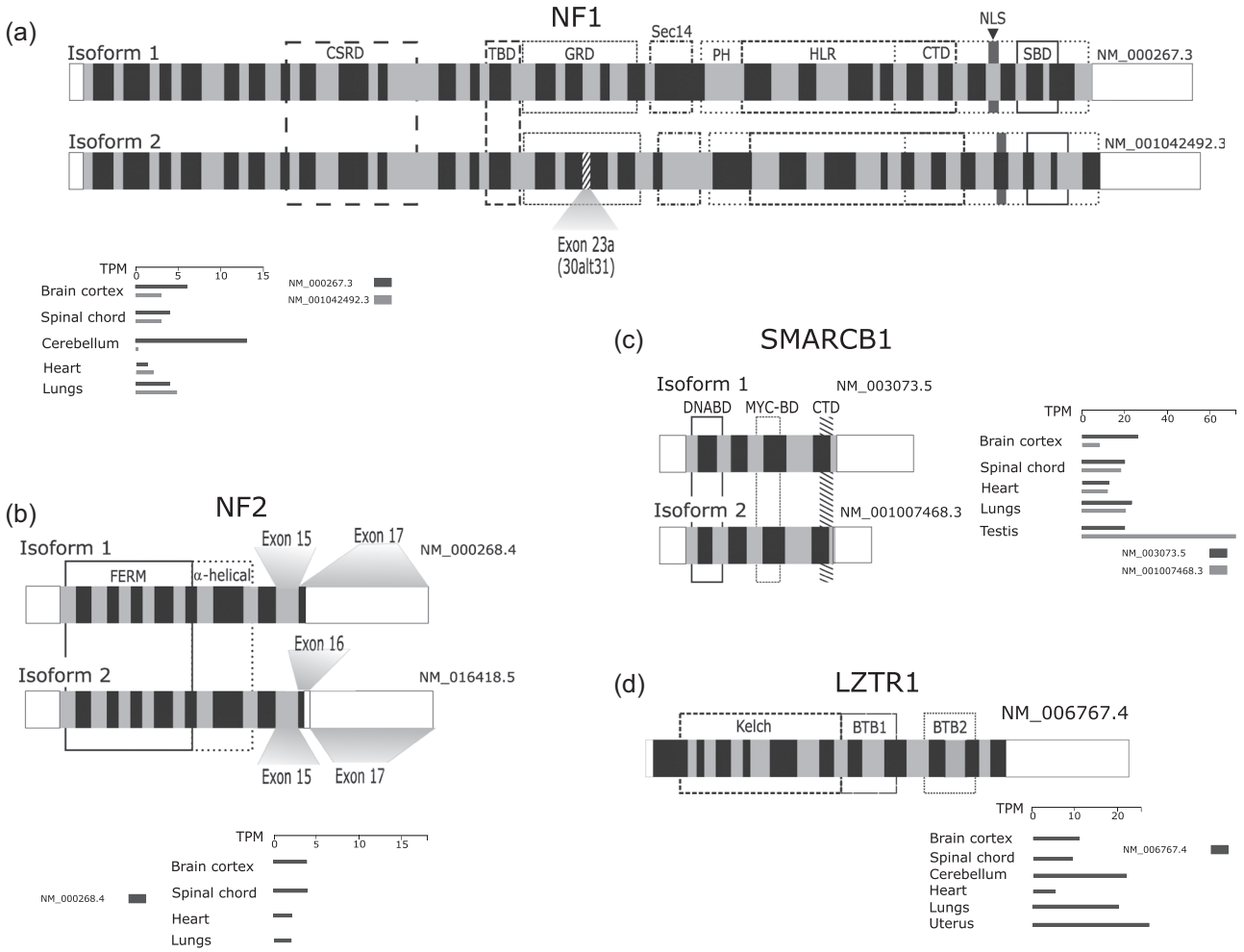

FIGURE 1 Schematic representation of major isoforms for the *NF1*, *NF2*, *SMARCB1*, and *LZTR1* genes. (a) Major isoforms of *NF1* (isoform 1 and isoform 2). While isoform 2 is the predominant transcript expressed in most tissue, isoform 1 is the most abundant form in central nervous system (Andersen et al., 1993). Isoform 2 has been found to be overexpressed in primary brain tumors (Suzuki et al., 1991). Functional domains are indicated as CSRD (cysteine–serine-rich domain), TBD (tubulin-binding domain), GRD (GAP-related domain), Sec. 14 (Sec. 14-like lipid-binding domain), PH (pleckstrin homology domain), HLR (HEAT-like repeat regions), CTD (carboxy-terminal domain), NLS (nuclear localization signal region) and SBD (syndecan binding domain). (b) Two major isoforms of *NF2*; isoform 1, which skips exon 16, and isoform 2, in which inclusion of exon 16 causes a reading frame shift and a slightly shorter protein product. The FERM and α-helical domains are indicated. Both merlin isoforms (1 and 2) are expressed in similar proportions across different cell types, with isoform 2 the slightly more abundant of the two (Chang et al., 2002). (c) Two major isoforms of SMARCB1 (isoform 1 and isoform 2), expressed in almost equal proportion (45% and 50%, respectively) across different tissues (Favre et al., 2003). Functional domains are indicated as DNABD (DNA binding domain), MYCBD (MYC-binding domain) and CTD (c-terminal domain). (d) Main known isoform of LZTR1. Kelch and Broad-complex, tramtrack, and bric-à-brac (BTB) domains are indicated. Tissue-specific expression values from GTEx project (GTEx Consortium, 2020) are included for available transcripts

(Ars et al., 2003). Finally, a number of pathogenic missense variants have been identified in *NF1*, primarily localized within two regions: the GRD domain and a region starting on exon 11 and extending to the cysteine-serine-rich domain (CSRD) of the gene (Assunto et al., 2019; Fahsold et al., 2000; Koczkowska et al., 2018).

Genotype-phenotype correlations of NF1-associated variants are scarce but those that have been established seem to point to mild phenotypes associated with most missense variants, whereas deletions (particularly large ones) tend to produce a more severe phenotype (Kang et al., 2020; Kehrer-Sawatzki, Mautner, et al., 2017; Pinna et al., 2015; Rojnueangnit et al., 2015; Upadhyaya et al., 2007). Notable exceptions to this trend are missense variants affecting particular amino acid residues within mutational hotspots in neurofibromin, namely the CSRD (codons 844-848), the tubulin-binding (TB) domain (codon 1149), and the GRD (codons 1276 and 1423) regions, which have been found to lead to a severe NF1 phenotype (Koczkowska et al., 2018, 2020).

1.1.2 | Neurofibromatosis type 2

The *NF2* gene (neurofibromin 2), located at chr22q12.2, codes for the protein, merlin (moesin-ezrin-radixin-like protein) (Trofatter et al., 1993). Two main isoforms of merlin have been identified with

both mRNAs sharing the same sequence from exons 1–15 but differing at the last exon (Figure 1b). Isoform 1 contains exon 17 and yields a 595 amino acid protein, whereas isoform 2 contains exon 16 and has a 590 amino acid product (Bianchi et al., 1994). Both of these isoforms contain the N-terminal FERM (4.1 ezrin, radixin, moesin) domain, which is highly conserved among ERM family proteins and has an important role in cell adhesion, motility, and signaling (Chishti et al., 1998). Like other proteins containing a FERM domain, merlin has a well-established role in tumor suppression although the mechanisms involved appear to be different for merlin, compared to other proteins within the ERM family (Cui et al., 2019; Petrilli & Fernandez-Valle, 2016).

A large proportion of germline PVs in *NF2* are truncating variants. These include nonsense, frameshift, and some splice-site variants that have traditionally been associated with a severe NF2 phenotype (higher tumor burden) while PVs resulting in a mutant protein product (missense variants, in-frame deletions, and some splice-site variants), as well as whole-gene deletions, are associated with a milder phenotype (Evans, 2009; Halliday et al., 2017; Ruttledge et al., 1996). The location of the variant also seems to be of importance, with splicing variants affecting exons on the 5′-end of the gene generating a more severe phenotype than those affecting exons closer to the 3′-end of the gene (Baser et al., 2005).

1.1.3 | Schwannomatosis

The considerable clinical overlap between NF2 and non-NF2 schwannomatosis has caused a constant challenge in differentiating these two disorders (Evans et al., 2018). However, a large number of non-NF2 schwannomatosis cases are attributable to two genes, both located in close proximity to the *NF2* gene. The first, *SMARCB1* (SWI/SNF related, matrix associated, actin-dependent regulator of chromatin, subfamily b, member 1), is located in chromosome 22q11.23 (Boyd et al., 2008; Hadfield et al., 2008; Hulsebos et al., 2007; Sestini et al., 2008; Smith, Wallace, et al., 2012). The second, *LZTR1* (leucine zipper-like transcription regulator 1), is found at 22q11.21 and has been linked to a proportion of schwannomatosis cases involving vestibular schwannomas, which led to changes to the recommended diagnostic criteria for NF2 and schwannomatosis (Piotrowski et al., 2014; Smith et al., 2015, 2017). A third schwannomatosis candidate gene, *DGCR8* (DGCR8 microprocessor complex subunit), located in chromosome 22q11.21 has emerged recently in one kindred affected by schwannomatosis and euthyroid multinodular goiter syndrome (Rivera et al., 2020). A germline missense variant NM_022720.6:c.1552G>A (p.Glu518Lys) in the *DGCR8* gene was identified in six members of the family. This variant, designated E518K in the original report, was predicted to reduce affinity of RNA binding to *DGCR8*, resulting in reduced expression of miRNAs, as a consequence of deficient cleavage of primary miRNAs (pri-miRNAs) to produce precursor miRNAs (pre-mRNAs).

The type and location of PVs in the *SMARCB1* gene have a marked effect on phenotype. Genetic variants in *SMARCB1* are associated with several tumor predisposition disorders, including malignant rhabdoid tumors (MRT), a condition that typically presents during infancy and which has a poor prognosis. Although there have been reports of families in which segregation of genetic variants in *SMARCB1* lead to MRT in some family members and schwannomatosis in others, for the most part, these two conditions are distinct both in clinical and genetic terms. While germline PVs associated with schwannomatosis tend to be located at either extreme of the gene and result in a hypomorphic transcript, truncating variants, located around the central part of the gene, mostly lead to the development of malignant rhabdoid tumors (Smith et al., 2014).

LZTR1 coding variants associated with schwannomatosis have been identified across the full length of the gene including truncating and non-truncating variants. Several cases of incomplete penetrance have also been observed for this gene (Hutter et al., 2014; Louvrier et al., 2018; Paganini, Chang, et al., 2015; Paganini, Sestini, et al., 2015; Piotrowski et al., 2014; Smith et al., 2015, 2017).

Together, PVs identified in *SMARCB1* and *LZTR1* account for around 70%–86% of familial and 30%–40% of sporadic non-NF2-associated schwannomatosis cases (Evans et al., 2018; Kehrer-Sawatzki, Farschtschi, et al., 2017). Only one PV in *DGCR8* (NM_022720.6:c.1552G>A) has been discovered so far, pointing to the existence of hitherto undiscovered causative genes and undiscovered variant types within the known genes (Hutter et al., 2014; Min et al., 2020; Rivera et al., 2020; Smith, Wallace, et al., 2012).

1.2 | Variants in the 5′-untranslated region (5′-UTR)

The 5'-UTR is known to harbor a number of elements that play an important role in regulating protein translation (Hinnebusch et al., 2016). Variants disrupting or potentially creating new upstream open reading frames (uORFs) have been reported in both in *NF1* and *NF2* although the mechanisms through which these variants may contribute to disease etiology remain unclear (Evans et al., 2016; Horan et al., 2004; Osborn et al., 2000; Whiffin et al., 2020). uORFs are present in the 5′-UTR of many protein-coding genes and regulate mRNA translation through three main mechanisms: (a) scanning may start at upstream AUG (uAUG) and stop at a uORF stop codon, triggering mRNA decay; (b) the uORF might be fully translated and translation might be reinitiated at the downstream main ORF, although this process is inefficient and usually results in lower protein levels; (c) read-through or *leaky scanning* may occur if the uAUG is present without an in-frame stop codon upstream the main ORF. Regardless of the mechanisms involved, uORFs usually have inhibitory effects on protein translation (Calvo et al., 2009).

Variants in the 5′-UTR of *NF1* were reported in two early studies aiming to identify and characterize variants disrupting regulatory elements (Horan et al., 2004; Osborn et al., 2000). By screening 570 NF1 patients and 105 unaffected controls, these reports identified 10 variants within a 987 bp region upstream of the canonical translational start AUG. Two of these variants NC_000017.11:g.31094851A>C and

NC_000017.11(NM_000267.3):c.-351G>C (originally annotated as A +25C and G+132C, respectively) were located within uORFs, and further characterization of these variants in vitro indicated that they were associated with slightly increased reporter gene activity compared to wildtype constructs. An important caveat to these findings, noted by the authors, is that these variants are rare and unlikely to affect phenotype individually. However, the possibility remains that they collectively reflect disruptions in regulatory mechanisms that might be relevant for disease.

This possibility is supported by more recent evidence. In an RNA analysis study of 361 NF1 patients in Manchester data (Evans et al., 2016) four likely disease-causing variants (NC_000017.11 (NM_000267.3):c.-280C>T, NC_000017.11(NM_000267.3):c.-273A>C, NC_000017.11 (NM_000267.3):c.-272G>C, and NC_000017.11 (NM_000267.3):c.-272G>A) were identified in the *NF1* 5′-UTR region in six affected individuals. Three of these changes (c.-273A>C, c.-272G>C, and c.-272G>A) involved two highly conserved nucleotides. These variants were revisited more recently, by a large-scale study using data from over 15,000 individuals carrying loss-of-function variants aggregated in the gnomAD database (Karczewski et al., 2020) providing insight into the effects of uORF-modifying variants on disease (Whiffin et al., 2020), by demonstrating that two of the previously reported 5'-UTR variants (c.-280C>T and c.-272G>A) in *NF1* create a new upstream uAUG within strong and moderate Kozak consensus sites whilst the other two (c.-273A>C and c.-272G>C) result in removal of the in-frame uORF stop codon.

This study also reported a novel variant, NC_000022.11:g.29603933_29603934insT (NM_000268.3:c.-66_-65insT) in the 5′-UTR of *NF2*, which creates an alternative uAUG with an in-frame stop codon just a few bases downstream. Notably, the insertion also causes the reading frame of an existing uORF to shift so that it overlaps with the canonical coding sequence. Either of these features could cause downregulation of the canonical transcript. Interestingly, the location of the existing uORF in the 5′-UTR of *NF2* seems to coincide with an earlier report of a negative regulatory element in a 100 bp region upstream the start of the gene (Welling et al., 2000).

It is clear from these and other examples of variants disrupting regulatory elements in 5′-UTRs of cancer-associated genes (Kutchko et al., 2015; Somers et al., 2015) that further functional characterization is warranted to improve our understanding of their role in tumor predisposition.

1.3 | Variants in the 3′-UTR

Variants in the 3′-UTR of *NF1* and *NF2* are rare and their relation to pathogenicity is largely unknown (Ruttledge et al., 1996; Upadhyaya et al., 1995). However, the variant, NC_000022.11:g.23834262C>T (NM_003073.4:c.*82C>T), identified in the 3′-UTR of *SMARCB1* is the most common recurrent schwannomatosis-associated variant (Smith et al., 2009; Smith, Wallace, et al., 2012). Further investigation of possible mechanisms for disease associated with this variant revealed reduced expression of the mutant allele ("T") compared to the wildtype allele and a negative effect on mRNA stability (Smith, Walker, et al., 2012).

The role of 3′-UTR regions in regulation of mRNA decay has largely been attributed to the presence of binding sites for *trans*-acting factors, in particular miRNAs. Indeed, the role of miRNAs as regulators of gene expression has long been studied in the context of tumorigenesis and specifically in the context of NF1 (Amirnasr et al., 2020; Sedani et al., 2012). Recently, some evidence of miRNA dysfunction associated with schwannomatosis has also been presented, with the discovery of the missense variant in the *DGCR8* gene NM_022720.6:c.1552G>A (p.Glu518Lys) by Rivera et al. (2020). Further characterization of this variant through analysis of schwannomas and Wilms tumors with a *DGCR8* mutant and loss of alternate allele (p.Glu518Lys)/− genotype or a combined *DGCR8* mutant and wild type genotype (*DGCR8*(p.Glu518Lys)/wt) revealed a defined group of miRNAs whose expression was significantly changed in *DGCR8*(p.Glu518Lys)/− tumors compared to their *DGCR8*(p.Glu518-Lys)/wt counterparts (Rivera et al., 2020). By comparing schwannoma data to that of publicly available Wilms tumor datasets, the authors propose a mechanism through which the *DGCR8* p.Glu518-Lys variant contributes to tumorigenesis, through disruption of miRNA biogenesis mechanisms. However, elucidation of the role of miRNAs in schwannomatosis and neurofibromatosis will likely depend on identification of specific RNA-binding factors and/or motifs that may be relevant, given the large variety of *cis*- and *trans*-acting elements that exists whose function might be cell-type or context-specific (Ray et al., 2013).

1.4 | Intronic and splicing variants

Intronic regions constitute a large proportion of the genome and play an important role in regulation of gene expression by a number of mechanisms (Rigau et al., 2019). While it is increasingly evident that disruption of intronic sequences plays an important role in disease, the identification and characterization of PVs within intronic regions is more challenging than that of coding variants. The majority of characterized PVs within intronic sequences have been identified due to their disruption of splicing mechanisms. Many of these splicing variants do not affect canonical splice sites and at least some of them are deep intronic, located at least 10 bp into an intron (Castellanos et al., 2013; De Klein et al., 1998). Around 3% and 1% of variants in *NF1* and *NF2*, respectively, are deep intronic (Castellanos et al., 2013; Evans et al., 2016; Wallace et al., 2004). Identification and characterization of deep intronic variants is only possible through whole gene sequencing and RNA analysis. This makes them less likely to be detected through current routing screening methods for NF2 and schwannomatosis patients, for whom clinical genetic testing is mainly based on DNA analysis.

Traditionally, transcription and splicing have been regarded as sequential processes but there is evidence that they occur in parallel and with considerable feedback between the processes (Tellier et al., 2020). The implications of co-transcriptional splicing

might prove crucial to our understanding of splicing as an important mechanism for regulating gene expression at different stages of development and in tumorigenesis (Eymin, 2020; Oltean & Bates, 2014).

Alternatively spliced non-pathogenic NF1, NF2, and SMARCB1 transcripts.

The identification of highly conserved alternative transcripts for *NF1* (Andersen et al., 1993; Bernards et al., 1992; Danglot et al., 1994; Gutman et al., 1993; Kyritsis et al., 1992; Nishi et al., 1991; Suzuki et al., 1992), *NF2* (Bianchi et al., 1995; Chang et al., 2002; Haase et al., 1994; Hara et al., 1994; Hitotsumatsu et al., 1994; Lutchman & Rouleau, 1995; Pykett et al., 1994), and *SMARCB1* (Bruder, Dumanski, et al., 1999; Favre et al., 2003) demonstrates the relevance of alternative splicing in these genes (Figure 1). These transcripts may be expressed in a tissue-specific manner. Moreover, in vitro and in vivo studies have shown that expression of some of these isoforms is differentially regulated at distinct developmental stages and that each isoform may have a specific function (Geist & Gutmann, 1996; Gutmann et al., 1994, 1995, 1999; Hinman et al., 2014; Hirvonen et al., 1998; Huynh et al., 1994; Jannatipour et al., 2001; Mantani et al., 1994; Nguyen et al., 2017). This is reflected in the differences observed in the expression profiles of *NF1* isoforms across different tumors (Danglot et al., 1995; Mochizuki et al., 1992; Platten et al., 1996; Suzuki et al., 1991; Takahashi et al., 1994, 1995) and, in the case of *NF2*, in the apparently exclusive role of tumor suppressor for Merlin-isoform 1, which some studies suggest, is not replicated by isoform 2 protein. These changes in function have been adjudicated to differences in sequence at the C-terminal region resulting in an inability to form interdomain associations (Mani et al., 2011; Meng et al., 2000; Neill & Crompton, 2001; Sherman et al., 1997), although this has been contested by more recent studies (Laulajainen et al., 2012; Zoch et al., 2015).

1.4.1 | Pathogenic splicing variants

In *NF1*, *NF2*, *SMARCB1*, and *LZTR1*, PVs leading to splicing defects are common, and many are recurrent (Ars et al., 2000, 2003; Ellis et al., 2011; Messiaen et al., 2000; Piotrowski et al., 2014; Smith, Walker, et al., 2012; Vandenbroucke et al., 2002). They are associated with variable disease severity, a feature that has made their characterization and classification more challenging (Kang et al., 2020; Kluwe et al., 1998).

For the *NF1* gene in particular, given its large size and the large proportion of pathogenic intronic splice variants identified in people with NF1 disease, clinical genetic testing is routinely carried out by RNA analysis (Ars et al., 2000; Evans et al., 2016; Messiaen et al., 2000; Upadhyaya et al., 2009). This is an important distinction in the genetic testing of NF1 patients, compared to other hereditary disorders, because it has allowed for identification of a higher proportion of pathogenic variants that affect splicing, even when they are not located in the canonical splicing sites. Pathogenic intronic variants in *NF1* include changes that disrupt the sequence of splice donor or acceptor sites (Table S1) as well as those resulting in the creation of new splicing sites (Table 1) or disrupting other intronic regions of relevance to splicing. This is the case for variants resulting in the creation of AG dinucleotides in the region between branch point recognition sequences and their corresponding 3′ splice site (3′ss), which have been shown to disrupt splicing by impeding recognition of the AG from the 3′ acceptor site (Ainsworth et al., 1994; Hatta et al., 1995; Wimmer et al., 2020).

Single base changes are the most common splicing variants reported by these studies, followed by small deletions or insertions. Interestingly, some of these variants have variable effects on transcription and can lead to different phenotypes in individual carriers with the same variant (Messiaen et al., 2000). An example of this is the report of a variant, NC_000017.11(NM_000267.3): c.5546+1G>C affecting the splice donor site in Exon 37 of *NF1* (previously annotated as Exon 29) and resulting in skipping of either exon 37 or exons 37 and 38 (Faravelli et al., 1999). The variant was observed in DNA from the proband and the affected father, both of whom lacked cutaneous features typical of NF1 so molecular analysis was crucial for accurate diagnosis. In addition, Lisch nodules were observed in the proband, but not in the affected father. A previous study also identified alternative splicing of exons 37 and 38 (originally annotated as exons 29 and 30) in a number of tissue samples from NF1 patients, although no pathogenic variant was reported to account for these isoforms (Park, Kenwright, et al., 1998). Additionally, there are reports of the presence of nonsense (Ars et al., 2000; Buske et al., 1999; Fahsold et al., 2000; Hatta et al., 1995; Hoffmeyer et al., 1998; Messiaen et al., 1997, 2000; Pros et al., 2008; Robinson et al., 1995; Sabbagh et al., 2013; Stella et al., 2018; Upadhyaya et al., 1996; Valero et al., 2011; Wimmer et al., 2000, 2007; Zatkova et al., 2004) and missense (Ars et al., 2000, 2003; Fahsold et al., 2000; Messiaen et al., 1999; Nemethova et al., 2013; Osborn & Upadhyaya, 1999; Park & Pivnick, 1998; Pros et al., 2008; Sabbagh et al., 2013; Stella et al., 2018; Upadhyaya et al., 1996; Valero et al., 2011) variants resulting in exon skipping in the *NF1* gene (Table 1). The cases of nonsense variants leading to splicing defects seem to be rare for NF1, but are a well-documented pathway for alternative splicing. Exonic variants can cause alternative splicing by disrupting exonic splicing enhancer (ESE) or exonic splicing silencer (ESS) sites (Baralle et al., 2006; Colapietro et al., 2003; W. Xu et al., 2014; Zatkova et al., 2004) as well as binding sites for splicing factors (Skoko et al., 2008). Importantly, accurate classification for many of these apparent nonsense and missense variants, as well as a number of variants that would otherwise be designated VUS, as splicing variants has only been possible through RNA analysis (Evans et al., 2016; Messiaen et al., 2000).

Constitutional splice-site variants in the *NF2* gene have been reported by a number of studies (Baser et al., 2004; Bijlsma et al., 1994; Bourn et al., 1995; Dewan et al., 2017; Ellis et al., 2011; Evans et al., 1998, 2005; Faudoa et al., 2000; Hagel et al., 2002; Jacoby et al., 1994, 1996, 1999; Kluwe et al., 1996, 1998, 2000; Louvrier et al., 2018; MacCollin et al., 1994; Mautner et al., 1996, 2002; Mérel et al., 1995; Mohyuddin et al., 2002; Parry et al., 1996; Pemov et al.,

TABLE 1 Germline splicing variants in NF1 not affecting in canonical splice-sites

NF1 NC_000017.11 (NM_000267.3)					
Variant	**ClinVar ID**	**Type of variant**	**Splicing effect**	**Identified in DNA/RNA**	**Reports**
c.910C>T	VCV000187722	Nonsense (R304X)	Skipping of exon 9 (7[a]).	[1,5]DNA, [3,4,8,9]RNA, [2,6,8,10]DNA & RNA	1,2, 3, 4, 5, 6, 7, 8, 9,10
c.943C>T	VCV000547574	Nonsense (Q315X)	Skipping of exon 9 (7[a]). Disruption of ESE	[4,7]RNA	4, 7
c.946G>A		Synonymous (Q315Q)	Skipping of exon 9 (7[a])	RNA	11
c.947C>T		Synonymous (L316L)	Skipping of exon 9 (7[a])		
c.1007G>A	VCV000945440	Nonsense (W336X)	Skipping of exon 9 (7[a]). Disruption of ESE	[4,7,12]RNA	4, 7, 12
c.1466A>G	VCV000000354	Missense (Y489C)	New 5' ss. Skipping of exon 13 (10b[a])	[3,9,13]RNA, [4]DNA, [2,6,10,14,15]DNA & RNA	2, 3, 4, 6, 9, 13, 10, 14,15
c.1513A>G	VCV000839760	Missense (K505E)	Skipping of exon 13 (10b[a]). In frame deletion.	DNA	16
c.1748A>G	VCV000068306	Missense (K583R) Novel splice acceptor	Partial deletion of exon 16	DNA & RNA	17
c.1885G>A	VCV000068308	Missense (G629R)	Creates a new 3' ss. Skipping of exon 17 (12b[a])	[6,10]DNA & RNA, [9]RNA	6, 9, 10
c.3277G>A	VCV000457638	Missense (V1093M)	Creates a new 5' ss in exon 25 (19b[a]) deleting last 40 bp of exon.	[9]RNA, [10]DNA & RNA	9, 10
c.5224C>T		Nonsense (Q1742X)	Skipping of exon 37	DNA & RNA	17
c.5546G>A		Missense (Q1849R)	Skipping of exon 37 (29[a])	[3,9]RNA, [5]DNA, [2,6]DNA & RNA	2, 3, 5, 6, 9
c.6327-4A>G		Novel splice acceptor	Creates a new splice site in Intron 39 (31[a]): Addition of 4bp to exon 40 (32[a]).	[18,19]DNA	18, 19
c.6670C>T		Nonsense (Q2224X)	Skipping of exon 44 (36[a])	[10]DNA & RNA	10
c.6792C>A	VCV000185082	Nonsense (Y2264X)	Skipping exon 45 (37[a]). New branch-point sequence. IF del.	[1,5,20,21,22]DNA, [3,9,23]RNA, [2,6,10]DNA & RNA	1, 2, 3, 5, 6, 9, 10, 20, 21, 22, 23
c.6841C>T	VCV000373973	Nonsense (Q2281X)	Skipping of exon 45 (37[a])	[21]DNA, [10]DNA & RNA	10, 21

[a]Exon notation from original publication. ESE, exonic splicing enhancer. Reports: [1]Hoffmeyer et al. (1998); [2]Ars et al. (2000); [3]Fahsold et al. (2000); [4]Wimmer et al. (2000); [5]Messiaen et al. (2000); [6]Ars et al. (2003); [7]Zatkova et al. (2004); [8]Wimmer et al. (2007); [9]Pros et al. (2008); [10]Sabbagh et al. (2013); [11]Colapietro et al. (2003); [12]Wimmer et al. (2000); [13]Osborn and Upadhyaya (1999); [14]Nemethova et al. (2013); [15]Messiaen et al. (1999); [16]Park and Pivnick (1998); [17]Valero et al. (2011); [18]Ainsworth et al. (1994); [19]Hatta et al. (1995); [20]Robinson et al. (1995); [21]Upadhyaya et al. (1996); [22]Messiaen et al. (1997); [23]Buske et al. (1999).

2020; Rouleau et al., 1993; Ruttledge et al., 1996; Sadler et al., 2020; Sainio et al., 2000; Seong et al., 2010; Sestini et al., 2000; Wallace et al., 2004; Zemmoura et al., 2014). An interesting characteristic of these variants is that they seem to give rise to heterogeneous phenotypes and their position in the gene has been reported to correlate with severity of disease. Early studies appeared to suggest that variants (including splicing variants) which preserved the C-terminus of the protein were associated with a milder phenotype (Mérel et al., 1995). However, later studies have proposed that a severe phenotype is more likely to be associated with an impaired ability of merlin to form self-associations, for which integrity of the N-terminal domain seems to be critical (Giovannini et al., 2000; Gutmann et al., 1998). This led to the prediction that splicing PVs affecting the 3′ end of the gene produce a milder phenotype than those located within the first five exons (Baser et al., 2005). A summary of recurrent splicing variants in *NF2* is presented in Table 2.

Schwannomatosis-associated variants disrupting consensus splice sites in *SMARCB1* (Boyd et al., 2008; Ding et al., 2019; Hadfield et al.,

TABLE 2 Recurrent germline splicing variants in patients with neurofibromatosis type 2

NF2 NC_000022.11 (NM_000268.3)				
Variant	**Type of variant**	**Splicing effect**	**Identified in DNA/RNA**	**Reports**
c.115-2A>G	Splice acceptor site	Skipping of exon 2	[1]DNA, [2]RNA	1, 2
c.240+1G>T	Splice donor site	Predicted splice defect (exon 2)	[3]DNA & RNA, [1,4,5]DNA,	1, 3, 4, 5
c.240+1G>C	Splice donor site	Predicted splice defect (exon 2)	[5,6,7]DNA	5,6, 7
c.241-2A>G	Splice acceptor site	Predicted splice defect (exon 3)	[5,7,8]DNA	5, 7, 8
c.363+1G>A	Splice donor site	Predicted splice defect (exon 3)	[9]DNA, [10]DNA & RNA	9, 10
c.447+1G>A	Splice donor site	Predicted splice defect (exon 4)	[5,7,11]DNA	5, 7, 11
c.447+2T>C	Splice donor site	Predicted splice defect (exon 4)	[5,7]DNA	5, 7
c.448-1G>T	Splice acceptor site	Predicted splice defect (exon 5)	[3,10]DNA & RNA, [1,5,12,13]DNA	1, 3, 5, 10, 12, 13
c.448-1G>A	Splice acceptor site	Predicted splice defect (exon 5)	[5,8,12,14]DNA, [10]DNA & RNA	5, 8, 10, 12, 14
c.448-2A>G	Splice acceptor site	Predicted splice defect (exon 5)	[5,6]DNA, [10,15]DNA & RNA	5, 6, 10, 15
c.448-2A>T	Splice acceptor site	Predicted splice defect (exon 5)	[6]DNA, [15]DNA & RNA	6, 15
c.516+1G>A	Splice donor site	Predicted splice defect (exon 5)	[3,15]DNA & RNA, [1,5,7]DNA	1, 3, 5, 7, 15
c.600-3C>G	Splice acceptor site	Predicted splice defect (exon 7)	[5,14]DNA	5, 14
c.600-2A>G	Splice acceptor site	Predicted splice defect (exon 7)	[5,7,14,16]DNA	5, 7, 14, 16
c.675+5G>A	Splice donor site	Predicted splice defect (exon 7)	[10,17]DNA & RNA, [7,18]DNA	7, 10, 17, 18
c.810G>A	Splice donor site and synonymous (E270E)	Predicted splice defect (exon 8)	[4,19]DNA	4, 19
c.1123-2A>G	Splice acceptor site	Predicted splice defect (exon 12)	[5,20]DNA	5, 20
c.1340+1G>A	Splice donor site	Predicted splice defect (exon 12)	[3]DNA & RNA, [1,5]DNA	1, 3, 5
c.1341-1G>A	Splice acceptor site	Predicted splice defect (exon 13)	[15]DNA & RNA, 1[9]DNA	15, 19
c.1341-2A>C	Splice acceptor site	Del. 8 bp of 5′ side of exon 13	[2]RNA, [21]DNA	2, 21
c.1447-2A>G	Splice acceptor site	Skipping of exon 14	[12,22]DNA, [10]DNA & RNA, [2]RNA	2, 10, 12, 22
c.1574+2T>C	Splice donor site	Skipping of exon 15	[5,7]DNA, [2]RNA	2, 5, 7
c.1575-1G>A	Splice acceptor site	Skipping of exon 15	[10,15,23]DNA & RNA, [2]RNA	2, 10, 15, 23
c.1737G>T	Missense (K579N)	Skipping exon 15	[12]DNA, [10]DNA & RNA, [2]RNA	2, 10, 12
c.1737+1G>C	Splice donor site	Predicted splice defect (exon 15)	[6,24]DNA	6, 24
c.1737+3A>T	Splice donor site	Skipping of exon 15 and most of exon 16	[25]DNA & RNA, [5]DNA	5, 25

Reports: [1]Merel et al. (1995); [2]Ellis et al. (2011); [3]Rouleau et al. (1993); [4]Mohyuddin et al. (2002); [5]Baser et al. (2004); [6]MacCollin et al. (1994); [7]Wallace et al. (2004); [8]Evans et al. (2005); [9]Bijlsma et al. (1994); [10]Kluwe et al. (1998); [11]Sadler et al. (2020); [12]Kluwe et al. (1996); [13]Mautner et al. (2002); [14]Evans et al. (1998); [15]Parry et al. (1996); [16]Kluwe et al. (2000); [17]Jacoby et al. (1996); [18]Hagel et al. (2002); [19]Ruttledge et al. (1996); [20]Bourn et al. (1995); [21]Dewan et al. (2017); [22]Mautner et al. (1996); [23]Faudoa et al. (2000); [24]Sestini et al. (2000); [25]Sainio et al. (2000).

2008; Paganini, Sestini, et al., 2015; Smith, Walker, et al., 2012) lead to a much less severe phenotype than *SMARCB1* splice variants associated with rhabdoid tumors. In general, schwannomatosis-associated splice variants lead to an in-frame transcript with an exon, or part of an exon, deleted. In contrast, rhabdoid tumor-associated splice variants lead to out-of-frame transcripts that are degraded by nonsense-mediated decay. However, a study of 14 schwannomatosis families provided some evidence that splicing variants located towards the 3′-end of *SMARCB1* may be more likely to produce out-of-frame variants that can escape nonsense-mediated decay to some degree (Smith, Walker, et al., 2012).

Pathogenic splice variants have also been identified in *LZTR1* in schwannomatosis patients (Piotrowski et al., 2014; Smith et al., 2015). Recurrent pathogenic splicing variants in *LZTR1* have not been reported so far, although the number of splice-site variants and potentially splice-affecting variants continues to increase, especially in recent years with studies re-assessing previous diagnoses. An example of this is the variant in the splice donor site of intron 8, NC_000022.11 (NM_006767.3):c.791+1G>A, was recently detected in peripheral lymphocytes and tumor tissue of a patient presenting solely with unilateral vestibular schwannoma at age 14 (Gripp et al., 2017). A similar, more recent study in patients with unilateral vestibular schwannoma reported two VUS in *LZTR1*, NM_006767.3:c.1230C>T and NM_006767.3:c.1687G>C, with the potential of affecting splicing, as predicted by in silico tools (Evans et al., 2019).

1.4.2 | Somatic splicing variants in schwannomas

Splicing variants in *NF2* for NF2-associated tumors and sporadic schwannomas have been reported by several studies (Table S2). These include a study that resulted in reclassification of patients diagnosed with schwannomatosis who were more accurately diagnosed as having mosaic NF2 (3/8 of cases without germline variants in *NF2*, *LZTR1*, or *SMARCB1*) or *LZTR1*-associated schwannomatosis (5/15 cases) after molecular testing further highlighting the importance of genetic screening for accurate diagnosis (Kehrer-Sawatzki et al., 2018). Similarly, a more recent study was able to classify a subset of patients with sporadic vestibular schwannomas as mosaic NF2 (7/394) or schwannomatosis (5/394) cases (Sadler et al., 2020). A notable finding from this study was the proportion of somatic-splicing variants in *NF2* accounting for a "first hit" (38%). Similarly, in their comprehensive approach for identification of germline mutations and molecular characterization of NF1-, NF2- and schwannomatosis-associated tumors Louvrier et al. (2018) have highlighted the importance of genetic testing and variant classification in accurate diagnosis and clinical management of patients, particularly in cases where clinical presentation overlaps two conditions, as is sometimes the case for the neurofibromatoses.

1.4.3 | Deep intronic variants

Intronic variants that do not disrupt canonical splice sites have also been identified in *NF1*, *NF2*, and *SMARCB1*. In most cases, they affect splicing by activation of cryptic splice sites. This was shown to be the case for the deep intronic variant, NC_000017.11(NM_000267.3):c.5750-279A>G, in intron 38 of *NF1* (originally designated c.31-279A>G in intron 30). The variant created a cryptic exon by generating a new 3′ splice site (ss) and activating a weak 5′ ss, 172 nucleotides downstream of the variant (Raponi et al., 2006). A similar mechanism was observed for NC_000017.11(NM_000267.3):c.3198-314G>A, reported in another NF1 patient (Fernández-Rodríguez et al., 2011). This variant, located in intron 24 (previously 19a) of the *NF1* gene, creates a new acceptor site that activates two cryptic donor sites, resulting in two mutant transcripts predicted to produce the same truncated protein (p.Asp1067Trpfs*7). Interestingly, wildtype transcripts were also produced by the mutant allele, presumably due to leaky scanning and leading to a mild phenotype for the disease. Other deep intronic variants in *NF1* have been identified from large screening studies, although many of them remain uncharacterized (Ars et al., 2003; Evans et al., 2016; Fahsold et al., 2000; Osborn & Upadhyaya, 1999; Perrin et al., 1996; Pros et al., 2008). Three deep intronic variants that have been characterized were originally discovered in a cohort of 374 unrelated NF1 patients (Pros et al., 2008). These defects were subsequently reported to be corrected by antisense morpholino oligomers (Pros et al., 2009). Overall, deep intronic splice variants account for around 3% of all *NF1* pathogenic germline variants (Abernathy et al., 1997; De Luca et al., 2004; Evans et al., 2016; Hatta et al., 1995; Messiaen et al., 2000; Nemethova et al., 2013; Upadhyaya et al., 2009).

There is another less common, but notable, example of a deep intronic variant in *NF1*, leading to aberrant splicing. This is a de novo introduction of an *Alu* repeat element 44bp upstream of exon 41 of the *NF1* gene (NM_000267.3) that results in disruption of branch-point recognition and leads to skipping of the exon and loss of 771 amino acids on the C terminus of the protein. The authors compared the repeat to other known members of the *Alu* family and found it to be most similar to the most recently inserted human-specific *Alu* subfamily, whose members are reported to be polymorphic across human populations, as well as transcriptionally active (Wallace et al., 1991). *Alu* elements, along with other short interspersed nuclear elements (SINE), are known to be important drivers of structural variation and alternative splicing in the human genome (Huang et al., 2010; Iskow et al., 2010). Previous studies have sought to determine the extent to which SINE contribute to genetic variation in the *NF1* gene and potentially to pathogenesis. One such study reports a polymorphism associated with the presence of a L1 element in intron 30 of *NF1* (Bleyl et al., 1994). Further, 18 L1 insertions, 14 of which were *Alu* elements, have been identified in *NF1*, as part of a study that provided evidence or intergenic regions susceptible to L1-endonuclease mediated retrotransposition (comprising exon 21–23 of the gene) and confirmed the role of *Alu* elements in alternative splicing mechanisms in *NF1* (Wimmer et al., 2011).

Deep intronic variants have also been found in the *NF2* gene (Castellanos et al., 2013; De Klein et al., 1998; Ellis et al., 2011; Hagel et al., 2002; Jacoby et al., 1994, 1996; Kluwe et al., 1998; Ruttledge et al., 1996; Wallace et al., 2004). De Klein et al. reported a single nucleotide change, NC_000022.11(NM_000268.3):c.516+232G>A in

intron 5, that results in generation of a consensus branch point sequence that, in turn, activates a cryptic exon (De Klein et al., 1998). Interestingly, the authors report higher than expected amounts of wildtype RNA in tumor tissue suggesting that the variant does not fully impair normal splicing or perhaps that it is compensated by the wild-type allele. This observation is consistent with the mild phenotype observed in the affected family. More recently a study reported a deep intronic variant in intron 13 of *NF2* (NC_000022.11(NM_000268.3):c.1447-240T>A), which leads to the inclusion of a 167bp cryptic exon and produces a truncated merlin protein (p.Pro482Profs*39) that results in a severe phenotype (Castellanos et al., 2013). An assessment of the proportion of PVs that are deep intronic is not possible outside of familial disease due to the high rate of mosaicism in de novo cases. With a detection rate of 96% for 165 s generation NF2 families only one of the seven families with unfound variants after tests for large rearrangements, chromosome translocations and 5′-UTR variants had a deep intronic variant (NC_000022.11 (NM_000268.3):c.516+232G>A) representing only 1/165 (0.6%) (unpublished data from Manchester Centre for Genomic Medicine).

A deep intronic variant (NC_000022.11(NM_003073.4):c.795+1498C>T) in intron 6 of *SMARCB1* was also reported recently in all affected members of a large schwannomatosis family. This variant causes insertion of 94 nucleotides from intron 6, resulting in premature termination of the transcript (Smith et al., 2020). The association of this variant with schwannomatosis, rather than rhabdoid tumors, lends further support to the theory that some residual expression of this type of variant occurs or that enough normal transcript is expressed to keep the effect hypomorphic.

It is important to note that identification and accurate characterization of deep intronic variants, as well as splice-altering synonymous and nonsense variants, has only been possible through RNA analysis, since many of these variants are located at least 10bp away from exon-intron boundaries and would not be assessed by exon screening techniques. This issue is illustrated by a large mRNA study of NF1 in neurofibromatosis type 1 cases, for which ~12% of variants (41/348) were deemed to be only classifiable through RNA transcript analysis (Evans et al., 2016).

1.5 | Further role of CREs in neurofibromatosis and schwannomatosis

The emerging knowledge of the mechanisms involved in regulation of gene expression by CREs has been valuable for characterization of noncoding variants associated with different types of cancer (Bailey et al., 2016; Hayward et al., 2017; Hoang et al., 2018; K. Li et al., 2020; Rheinbay et al., 2017; Vinagre et al., 2013; Zhou et al., 2020). Conserved regions have provided clues to the identification of relevant CREs as well as binding sites for *trans*-acting elements (Kelly et al., 2015). An example of this is the highly homologous regions of the *NF1* GRD that have been observed in Ras proteins across species (Bourne et al., 1990; Buchberg et al., 1990). Furthermore, comparison of the full human and murine neurofibromin sequences revealed a high degree of similarity (>98%) and high conservation levels across 5′- and 3′-UTRs (Bernards et al., 1993; Hajra et al., 1994). A subsequent *in silico* study compared the 5′ upstream region and intron 1 of *NF1* and homologous genes in human, mouse, rat, and puffer fish (*Fugu rubripes*). The authors found high homology segments throughout the region across all species, including two exact matches and one near exact match in the 5′-UTR, as well as one exact match in intron 1. Along with these findings, they predict two transcription start sites for *NF1* and corresponding promoter regions, despite not being able to identify a TATA box or any other core promoter element (Lee & Friedman, 2005). Interestingly, an independent study found that TATA-box binding protein like 1 (*TBPL1*), interacts with the TATA-less promoter region of *NF1* and drives its expression, both in humans and in mice (Chong et al., 2005). This points to a mechanism of regulation distinct to that involved in translation of TATA containing transcripts.

An early study comparing the open reading frame from human *NF2* with its mouse homologue found a high level of identity between the two complementary DNA sequences including the 5′-UTR and promoter regions (Claudio et al., 1994). A later comparison of the full *NF2* gene across five species (human, baboon, mouse, rat, and puffer fish) found highly conserved elements in exonic and extra-exonic sequences, including the promoter region. Notably, one such element, *inter 1*, is conserved in all five organisms, and is located between the promoters of *NF2* and *NIPSNAP1* (Hansson et al., 2003). In a follow up study the authors used DNA from 100 meningioma samples and sequenced the same well conserved regions, including non-exonic regions. The study reported 39 alterations, one of them located in the *inter 1* region (NC_000022.11:g.29589068C>A), approximately 15 kb upstream of the *NF2* promoter. However, the relevance of this variant remains unclear since the meningioma in which it was identified also contained a pathogenic frame-shift variant in exon 13 and presented monosomy of chromosome 22 (Hansson et al., 2007).

It has become increasingly evident that enhancers are an important component of the transcription regulatory machinery. These elements interact with their targets in a conformation-dependent way and their function has an effect on, and is affected by, DNA topology (Furlong & Levine, 2018; Rickels & Shilatifard, 2018). The availability of large amounts of data on regulatory elements throughout the genome, integrated through initiatives like the ENCODE project database (ENCODE Project Consortium, 2012), has made it possible to examine in more detail the role of transcription enhancers in the pathophysiology of NF1. An in silico analysis of long-range DNA-DNA interactions predicted a number of DNA regions interacting with the region containing *NF1*, defined in the study as 17:29000000-29999999 (GRCh37). Most interactions were observed between the *NF1* containing region and the adjacent region spanning 17:28000000-28999999. The combination of these findings with ENCODE data also revealed each of these DNA fragments contained a region enriched for H3K27ac, a histone recognized as a marker for active enhancers. Sequencing of these H3K27ac-rich

regions in 47 unrelated NF1 patients identified three variants. One of these novel single nucleotide changes, NC_000017.11:g.30519775 C>T, was present in two individuals. Although the effects of these variants are still uncertain, the authors note that at least one of these variants (NC_000017.11:g.30519865C>T) is located within overlapping transcription factor binding sites (Hamby et al., 2013).

No link between *SMARCB1* or *LZTR1* variants and disruption of cis-regulatory elements has been established so far. However, normal function of the SWI/SNF complex is intrinsically relevant to promoter activation and is important for a wide range of signaling mechanisms in numerous tissues. Complete loss of SMARCB1 protein has been mainly associated with the development of rhabdoid tumors and has been shown to lead to aberrant SWI/SNF complex formation in yeast (Sen et al., 2017). This has been supported by a study which characterized the role of the SWI/SNF complex in enhancer function in rhabdoid tumor cell lines (Wang et al., 2017). This study found that SMARCB1 was essential for stability of the SWI/SNF complex and required for enrichment of the protein complex at enhancers, thus mediating gene expression. Gene Ontology (GO) analysis revealed that genes affected by loss of SMARCB1 were mainly cell-type specific, although those that were observed to be dysregulated in all cell lines tend to be involved in regulation of cell differentiation. Interestingly, SMARCB1 was not necessary for enrichment of the SWI/SNF complex to super-enhancers that may be required for tumor growth.

2 | CONCLUDING REMARKS

Noncoding variants make up a significant proportion of pathogenic variants identified in neurofibromatosis and schwannomatosis-associated genes. The great majority of these variants exert their deleterious effect by producing changes in splicing, in many cases through mechanisms that do not involve disruption of canonical splice sites (Raponi et al., 2009; Wimmer et al., 2020).

In addition to splicing variants, the effect of CREs (and mutations within them) on NF1, NF2, and non-NF2 schwannomatosis genes have been proposed not only as drivers for disease but also as modulators of penetrance and phenotype, suggesting the possibility of as yet unidentified epistatic interactions between loci (Kehrer-Sawatzki, Farschtschi, et al., 2017; Sabbagh et al., 2009). Communication between different regulatory loci might be relevant for intronic regions as well. Stadhouders et al. present several examples of intronic sequences within certain genes, that interact with enhancers and regulate transcription of downstream or upstream targets (Stadhouders et al., 2012). Intronic regions are also of interest because they may contain noncoding RNA transcripts that have a role in regulation of gene expression that may be relevant to carcinogenesis (Williams & Farzaneh, 2012; Zimta et al., 2020). This is a particularly intriguing possibility in the context of the established role of schwannomatosis-associated genes, such as *SMARCB1* and *DGCR8*, in epigenetic mechanisms (Jung et al., 2020; Weissmiller et al., 2019).

The relevance of noncoding variants to disease has been laid bare by the fact that a large proportion of loci associated with common disease, that have been identified by large GWAS studies, are not contained within the coding sequence (Albert & Kruglyak, 2015). This points to the relevance of noncoding regions as genetic factors for disease. An increasing number of studies have reported variants located in intronic and regulatory regions as causative of rare disorders (Johnston et al., 2019; Vuckovic et al., 2020; Whiffin et al., 2020). Moreover, disruption of regulatory mechanisms of gene expression is recognized as an important factor in carcinogenesis (K. Li et al., 2020; Lowdon & Wang, 2017; Orlando et al., 2018; Weinhold et al., 2014). Furthermore, some studies have been able to define tumor subtypes by the recurrent noncoding variants they carry, suggesting the possibility of the use of epigenetic signatures in diagnosis and treatment (Hayward et al., 2017; Torchia et al., 2016; Wellenreuther et al., 1995)

The continuous development of tools for genomic analysis, including long-read sequencing, chromosome conformation capture (Hi-C), and clustered regularly interspaced short palindromic repeats (CRISPR) are rapidly increasing our understanding of the mechanisms by which noncoding variants cause or modify disease (Cumming et al., 2018; Hamby et al., 2013; Kremer et al., 2017; Sanjana et al., 2016). In addition, large databases of genetic variants, gene expression, and transcriptional regulation, such as gnomAD (Karczewski et al., 2020), GTEX (GTEx Consortium, 2020), and ENCODE (ENCODE Project et al., 2020), will continue to support the creation of better bioinformatics tools for appropriate clinical classification of VUS, particularly those located in noncoding regions, and may also aid identification of the missing heritability in neurofibromatosis and schwannomatosis (Evans et al., 2010, 2018). Indeed, the availability of large whole-exome and whole-genome sequencing datasets has made it possible to calculate more accurate frequency estimates for rare variants that can be used for prediction of their pathogenicity (Whiffin et al., 2017). ACMG guidelines, used in interpretation and classification of genetic variants, define five categories of variants as "pathogenic," "likely pathogenic," "uncertain significance," "likely benign," or "benign." Variants are in turn assigned to each category on the basis of specific criteria for which evidence is considered and classified as very strong, strong, moderate, or supporting (Richards et al., 2015). These guidelines also provide a framework for integration of in silico predictive tools to use as evidence of pathogenicity and this framework is continuously updated and improved to produce a more robust methodology that provides support for cascade testing of relatives (Ellard et al., 2020).

Better understanding of the mechanisms through which noncoding variants lead to NF1, NF2, or schwannomatosis might prove crucial for identification of novel causative variants and possible new genes and loci associated with these disorders. This in turn might contribute to more accurate diagnosis, clinical management, and ultimately future therapeutics for these disorders.

ACKNOWLEDGMENTS

This study was supported by a USAMRAA CDMRP Neurofibromatosis Research Program, Investigator-Initiated Research Award (W81XWH1910334). DGE and MJS are also supported by the Manchester National Institute for Health Research (NIHR) Biomedical Research Centre (IS-BRC-1215-20007).

CONFLICT OF INTERESTS

The authors declare that there are no conflict of interests.

WEB RESOURCES

https://gtexportal.org/home/

https://variantvalidator.org/service/validate/

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How to cite this article: Perez-Becerril, C., Evans, D. G., & Smith, M. J. (2021). Pathogenic non-coding variants in the neurofibromatosis and schwannomatosis predisposition genes. *Human Mutation*, 1–21. https://doi.org/10.1002/humu.24261