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Review Article

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Alternative splicing of the neurofibromatosis type I pre-mRNA

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Synopsis

NF1 (neurofibromatosis type I) is a common genetic disease that affects one in 3500 individuals. The disease is completely penetrant but shows variable phenotypic expression in patients. *NF1* is a large gene, and its pre-mRNA undergoes alternative splicing. The NF1 protein, neurofibromin, is involved in diverse signalling cascades. One of the best characterized functions of NF1 is its function as a Ras-GAP (GTPase-activating protein). *NF1* exon 23a is an alternative exon that lies within the GAP-related domain of neurofibromin. This exon is predominantly included in most tissues, and it is skipped in CNS (central nervous system) neurons. The isoform in which exon 23a is skipped has 10 times higher Ras-GAP activity than the isoform in which exon 23a is included. Exon 23a inclusion is tightly regulated by at least three different families of RNA-binding proteins: CELF {CUG-BP (cytosine-uridine-guanine-binding protein) and ETR-3 [ELAV (embryonic lethal abnormal vision)-type RNA-binding protein]-like factor}, Hu and TIA-1 (T-cell intracellular antigen 1)/TIAR (T-cell intracellular antigen 1-related protein). The CELF and Hu proteins promote exon 23a skipping, while the TIA-1/TIAR proteins promote its inclusion. The widespread clinical variability that is observed among NF1 patients cannot be explained by *NF1* mutations alone and it is believed that modifier genes may have a role in the variability. We suggest that the regulation of alternative splicing may act as a modifier to contribute to the variable expression in NF1 patients.

Key words: CUG-BP and ETR-3-like factor (CELF), genetic modifier, Hu protein, neurofibromatosis type I (NF1), T-cell intracellular antigen 1 (TIA-1), T-cell intracellular antigen 1-related protein (TIAR)

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ALTERNATIVE SPLICING

With the realization that the human genome contains far fewer genes than initially predicted, it is clear that complex molecular mechanisms must exist to increase the coding capacity of our genome. One of the major mechanisms is alternative splicing. It allows functionally distinct proteins to be generated from a single gene by selectively including or skipping particular exons in mature mRNA messages, which encode proteins. With recent technological advances, such as high-throughput sequencing, which allow large volumes of the genome to be interrogated, it is now accepted that alternative splicing is the general rule in humans with as many as 92–94% of human genes predicted to undergo this process [1,2]. Alternative splicing is important in development, in establishing and maintaining tissue specificity, and in the development and progression of human diseases. Alternative splicing is a highly regulated process in which both *cis*-acting elements, parts of the RNA itself, and *trans*-acting factors, RNA-binding proteins, interact to influence the decision of whether a particular alternative exon will be included or skipped. Alternative splicing regulation and the factors involved in this process have been reviewed extensively by others [3–8].

Alternative splicing is important in the development and progression of human diseases. In fact, current estimates suggest that up to 60% of disease-causing mutations affect splicing [9]. These mutations can affect *cis*-acting elements, or they can lead to the mis-regulation of *trans*-acting splicing factors. Mutations that affect splicing can lead to an alteration in the levels of correctly spliced transcripts, or they can disrupt the normal levels

Abbreviations used: CELF, CUG-BP (cytosine-uridine-guanine-binding protein) and ETR-3 [ELAV (embryonic lethal abnormal vision)-type RNA-binding protein] like factor; CF, cystic fibrosis; CFTR, CF transmembrane conductance regulator; CNS, central nervous system; CSRD, cysteine- and serine-rich domain; ERK, extracellular-signal-regulated kinase; GAP, GTPase-activating protein; GRD, GTPase-activating protein related domain; ISS, intronic splicing silencer; MAP, microtubule-associated protein; NF1, neurofibromatosis type I; PH, pleckstrin homology; Sec14-PH, *Saccharomyces cerevisiae* phosphatidylinositol transfer protein-like domain/PH-like domain; TIA-1, T-cell intracellular antigen 1; TIAR, TIA-1-related protein; $U2AF^{65}$, U2 auxiliary factor large subunit.

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of protein isoforms created by alternative splicing. Several human diseases are known to be caused by mutations that lead to aberrant splicing, including DM (myotonic dystrophy), spinal muscular atrophy and certain cancers [9–12]. Recently, it has been proposed that splicing may have a role as a genetic modifier of single-gene human diseases. Splicing can play this role by altering either the normal levels of correctly spliced RNA transcripts or the normal ratios of different isoforms that result from alternative splicing [13,14].

One documented example of a human disease in which splicing acts as a genetic modifier is CF (cystic fibrosis). CF is a relatively common autosomal recessive genetic disorder in which the *CFTR* (CF transmembrane conductance regulator) gene is mutated [15]. So far approximately 1000 CF disease-causing mutations have been identified in the *CFTR* gene [16]. Many CF patients, even from the same family, show differences in the presentation or severity of the CF phenotype, which argues for a role for genetic modifiers in the disease severity. Splicing is believed to act as a genetic modifier of CF severity. Several characterized splicing mutations in the *CFTR* gene itself lead to the complete loss of correctly spliced transcripts, while other known *CFTR* mutations lead to an alteration in the levels of the correctly spliced products [15]. CF patient studies have shown that lower levels of correctly spliced transcripts correlate with a more severe pulmonary system phenotype [15]. Interestingly, patients who carry the same mutations do not have the same levels of correctly spliced transcripts, and therefore modifier genes are implicated in contributing to CF disease severity [15].

NF1 (NEUROFIBROMATOSIS TYPE I) DISEASE

NF1 disease, also known as von Recklinghausen disease, is a prevalent human genetic disease that affects approximately 1 in 3500 individuals without regard to ethnicity or sex [17,18]. NF1 is the result of loss-of-function mutations to the *NF1* gene, and the disease is inherited in an autosomal dominant fashion [19]. The disease is completely penetrant. Certain phenotypic characteristics are considered to be hallmarks of NF1 disease, as they occur in the majority of patients by the onset of adulthood. These phenotypic hallmarks include Lisch nodules of the eye, café au lait spots, auxiliary freckling and the presence of benign neurofibromas [20].

Neurofibromas are one of the defining characteristics of NF1 disease. Neurofibromas are benign tumours that involve Schwann cells, fibroblasts, perineurial cells and mast cells, and they arise from nerve sheath cells [21]. Although being benign tumours, neurofibromas can cause serious complications in NF1 patients, such as spinal cord compression, other neurological problems and cosmetic burden. It is believed that NF1 functions as a tumour suppressor and that neurofibromas develop when both NF1 alleles are disrupted and, thus, functional neurofibromin is not present. Biallelic NF1 inactivation follows Knudsen's Two Hit hypothesis, where one mutated allele is either inherited or is the result of a *de novo* mutation, and the second NF1 allele is then mutated or disrupted in somatic cells in which tumours develop. This idea is supported by the loss of both copies of the gene in NF1-associated tumours which was first shown in a malignant tumour tissue from an NF1 patient [22]. Serra et al. [23], who developed a culture technique that allowed them to look at pure cultures of Schwann cells, showed that the Schwann cells which are present in neurofibromas are the cells that harbour the somatic NF1 mutation in neurofibromas.

Although NF1 shows complete penetrance, there is a high degree of variability in the presentation of the disease. To date more than a 1000 *NF1* gene mutations have been identified [24], yet the mutations alone cannot account for the phenotypic variability that is seen in NF1 patients. A recent study of monozygotic twins with NF1 disease supports the notion that phenotypic differences exist not only in family members with similar mutations but also in individuals who have inherited the same *NF1* germ line mutations [25]. The fact that these twins do show discordant phenotypes for many of the NF1 clinical features suggests that somatic cell mutations affecting other genes could be responsible for the variability that is observed. Even though there are certain aspects of the NF1 phenotype that are common to most patients, there are some characteristics that vary in different individuals. Some of the variable characteristics of the NF1 phenotype are cardiovascular involvement, the development of plexiform neurofibromas and/or malignant peripheral nerve sheath tumours, learning disabilities, skeletal dysplasia, macrocephaly, short stature and optic pathway gliomas [20,26].

Learning disabilities, which vary among NF1 patients, are considered to be among the most difficult aspects of the disease for patients to manage. Current data suggest that as many as 30–65 % of people with NF1 have specific learning deficits [18,27–29]. In general, most NF1 patients with learning difficulties show visuospatial, language and reading deficits, but global cognitive impairments are not common [26,30,31]. Although one study in monozygotic twins by Rieley et al. [25] has shown a high degree of concordance for presentation of IQ (intelligence quotient) scores, speech disorders and learning disabilities in twins with NF1, others have shown that phenotypic differences in learning exist in both human and animal subjects [32,33].

The widespread clinical variability that presents itself in NF1 patients leads to the hypothesis that other factors must be at play in modulating the NF1 disease phenotype. In fact, a recent family-based association study, by Sabbagh et al. [34], was utilized to investigate the normal *NF1* allele and its potential role in phenotype variability. The study examined 750 French NF1 patients from 275 different families by looking at phenotypic correlations between affected relatives for 12 different NF1 clinical features. The clinical features included the number and size of café au lait spots, the number of cutaneous, subcutaneous and plexiform neurofibromas, the presence of Lisch nodules and blue-red macules, skin-fold freckling, scoliosis, neoplasms and learning disabilities [34]. The findings of Sabbagh et al. [34] demonstrated that there is a strong genetic component for 11 of the 12 traits that

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they examined, and that there was no apparent influence from the identified *NF1* mutation in each family. Additional analysis of the normal *NF1* allele showed that there was not much involvement of this allele in the differences observed in clinical presentation. Overall, these findings support a strong argument for the involvement of genetic modifiers that are not linked to the *NF1* locus in NF1 disease variability [34].

NF1 GENE AND PROTEIN

Human *NF1* is a large gene, spanning approximately 280 kb of genomic DNA, and is located on chromosome 17q11.2 [35,36]. The full-length human *NF1* mRNA transcript contains 57 constitutive exons and three alternative exons (see below). For the purpose of this review, we will use the accepted *NF1* legacy numbering system for exons. For clarity, when introducing an exon we will include the consecutive exon numbering system in parentheses based on the GenBank® reference sequence NM_001042492.2. The major protein product encoded by the *NF1* gene, termed neurofibromin, consists of 2818 amino acids [37]. Neurofibromin is ubiquitously expressed with enrichment in neurons, Schwann cells, oligodendrocytes, astrocytes, leucocytes and adrenal medulla and it is highly conserved among species [38–44].

The neurofibromin protein is essential in mammals. Studies in mice in which the NF1 protein is completely deleted result in embryonic lethality between embryonic days 12.5 and 13.5 due to cardiovascular defects, and they also exhibit endocardial cushion defects and a failure of the neural tube to close properly [45]. Importantly, NF1 and its downstream targets are highly conserved among different species, including mice and humans. Presumably due to its large size, the *NF1* gene is one of the most highly mutated genes in the human genome. Comprehensive mutation analysis showed that 27 % of *NF1* mutations affect pre-mRNA splicing [24].

The large NF1 protein is involved in diverse signalling cascades [46,47]. Several functional domains of the protein have been identified. The CSRD (cysteine- and serine-rich domain) domain of NF1 is enriched with the amino acids cysteine and serine, and it is encoded by exons 11 (15)–17 (22) [47] (Figure 1A). This domain has been shown to increase the association of neurofibromin with actin upon its phosphorylation [47–51]. Located within the CSRD domain is a MAP (microtubule-associated protein) domain [47–49] (Figure 1A). Other domains of the NF1 protein are the caveolin-1 binding domains, which are encoded by exons 28 (37)–33 (42). These domains are believed to be involved in the formation of a complex between neurofibromin and caveolin-1, and the complex is potentially involved in the regulation of several important signalling molecules including PKC (protein kinase C) and p21ras [47,52].

The best-characterized functional domain of neurofibromin is the Ras-GRD (GTPase-activating protein-related domain), which is encoded by exons 21 (27)–27a (35) (Figure 1). The Ras-GTPase domain shares similarity with the catalytic domain of the yeast IRA-1 (inhibitory regulatory protein 1) and IRA-2 (inhibitory regulatory protein 2) proteins as well as the mammalian GAP (GTPase-activating protein), and accounts for approximately 10 % of the entire protein [47]. Several groups have demonstrated that the GRD of neurofibromin has Ras-GAP activity *in vitro* and *in vivo* [53–56]. The NF1 GRD imparts tumour suppressor function to the NF1 protein since it exerts negative controls on the oncogene Ras, resulting in decreased cellular proliferation and growth. GAPs, like neurofibromin, function by accelerating the conversion of active GTP-bound Ras into inactive GDP-bound Ras. Aberrant activation of Ras is associated with many human malignancies [57].

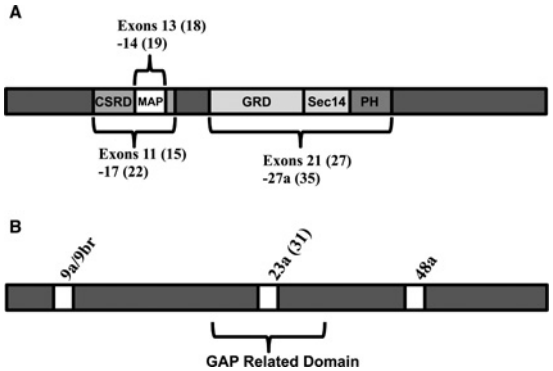

Figure 1 Schematic representations of neurofibromin protein domains and alternative exons

(**A**) The neurofibromin protein with important domains and the exons that encode them highlighted. Protein domains are shown as grey or white boxes. The MAP domain is found within the CSRD. (**B**) Neurofibromin protein with naturally occurring alternative exons shown as white boxes. Exon 23a is found within the GAP-related domain.

Another NF1 protein domain that has recently been characterized is the Sec14-PH [*Saccharomyces cerevisiae* phosphatidylinositol transfer protein-like domain/PH (pleckstrin homology)-like domain] module, as shown in Figure 1(A). This module, first identified by D'Angelo et al. [58] using structural proteomics, features a Sec14p-like domain that is located at the C-terminal end of the NF1 GRD and a PH-like domain that is in close association with the Sec14p-like portion of the domain. In other proteins, Sec14-like domains help to shuttle lipids between different cellular compartments within the aqueous environment; thus it is hypothesized that the NF1 Sec14-like domain performs a similar task [46,59–61]. The other portion of this NF1 module, the PH domain, is believed to communicate closely with the Sec14p-like domain through a linker region. In other proteins, PH domains provide a structural scaffold that enables different kinds of ligands to bind to the host protein, and in NF1 it is believed that the interaction between the Sec14 and PH domains regulates ligand binding to the Sec14 portion of the module [46,58,62–64]. Recently, research effort has been put forth to determine the binding partners of the NF1 Sec14-PH module. This work has revealed that the NF1 Sec14-PH module binds to glycerophospholipids, specifically (3-*sn*-phosphatidyl)-ethanolamine and 1-(3-*sn*-phosphatidyl)-*sn*-glycerol [46,62].

NF1 ALTERNATIVE SPLICING

The *NF1* pre-mRNA undergoes alternative splicing. Although some alternative splicing events are the results of mutations, the focus of this review will be on naturally occurring splice variants.

Three alternative exons that do not alter the reading frame of *NF1* have been identified in humans and rodents, including 9a/9br, 23a and 48a (Figure 1B) [65–68]. Alternative exon 9a/9br adds 10 amino acids to the transcript, and its inclusion seems to be restricted to the CNS (central nervous system) [42,65,69]. The inclusion of the 9a/9br exon does not appear to affect the function of the GRD. The alternative exon 48a adds 18 amino acids to the transcript. The expression of *NF1* exon 48a is highest in both fetal and adult cardiac and skeletal muscle tissues [68]. Although its function is still under investigation, it is believed that exon 48a might have a role in the development and differentiation of heart and skeletal muscles [66,70]. Additionally, the inclusion of exon 48a does not appear to affect the GRD. The main focus of this review will be on *NF1* exon 23a, which will be more thoroughly discussed later in the text.

Exon 23b is a rodent-specific alternative exon, which when included causes a frameshift that inserts a premature stop codon [72]. The introduction of the stop codon causes the truncated protein to lack much of its GRD. The *Nf1* isoform that includes exon 23b but lacks exon 23a (31) is enriched in the testes of both rats and mice [70,72].

NF1 EXON 23A AND ITS BIOLOGICAL IMPORTANCE

The first two NF1 isoforms that were identified, types I and II, have the alternative exon 23a (31) skipped and included respectively. *NF1* exon 23a (31) is an alternative exon that lies within the GRD of neurofibromin. Exon 23a is considered to be one of the most interesting *NF1* alternative exons, due to both its location within the GRD and the observation that the two protein isoforms differ in their abilities to control Ras signalling. The inclusion of exon 23a inserts 21 amino acids between constitutive exons 23-II (30) and 24 (32). Exon 23a is differentially spliced in a tissue-specific manner (Figure 2A). Exon 23a is predominantly included in most tissues and predominantly skipped in CNS neurons. Although the NF1 isoform that includes exon 23a still functions as a Ras GAP, *in vitro* studies have shown that it is approximately 10 times weaker in regulating Ras signalling than the isoform in which exon 23a is skipped. Exon 23a adds basic amino acids, which significantly change the structure of the NF1 GAP-related domain, and thus weaken its ability to regulate Ras [67,73].

In 2001, Costa et al. [74] sought to better understand the biological importance of *NF1* exon 23a, so they generated a mouse model in which exon 23a is deleted in all tissues. Unlike the mouse model in which *Nf1* is completely deleted, the

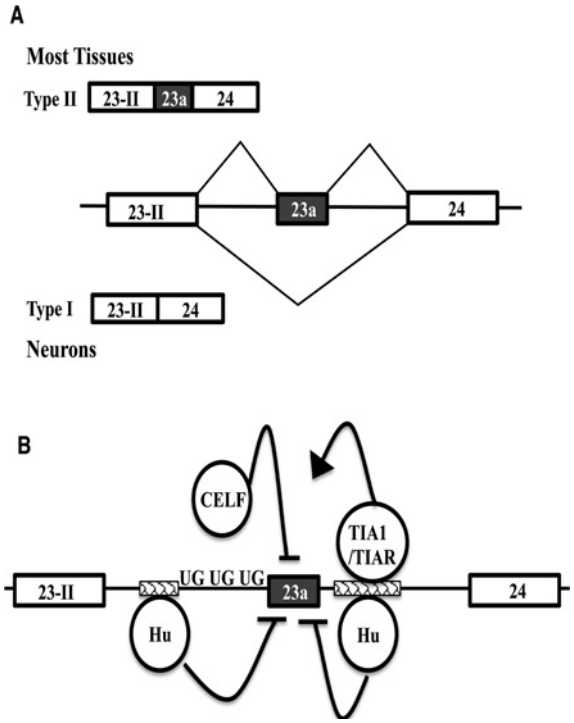

Figure 2 Schematic representations of *NF1* exon 23a endogenous splicing patterns and exon 23a regulation

(**A**) Exon 23a is included in most tissues and skipped in the CNS. Constitutive exons 23-II and 24 are shown as white boxes and the alternative exon 23a is shown as a grey box. Introns are shown as straight black lines. (**B**) Exon 23a splicing regulation. Constitutive exons 23-II and 24 are shown as white boxes and the alternative exon 23a is shown as a grey box. Introns are shown as straight black lines. Proteins that act as splicing factors are shown as circles. Hu proteins promote exon 23a skipping by binding to ISS elements located upstream and downstream of exon 23a indicated by a textured rectangle. TIA-1 and TIAR proteins promote exon 23a inclusion by competing with Hu proteins for binding of the downstream sequence element. CELF proteins promote skipping of exon 23a by binding UG-rich elements upstream.

$Nf1^{23a-/23a-}$ mice are viable and develop normally. Although these mice do not exhibit a tumour phenotype, they do show differences in spatial learning and memory when tested by the Morris water maze and another hippocampal-dependent assay [74]. These findings suggest that it is necessary to have a balance between the two NF1 isoforms which either contain or skip exon 23a.

REGULATION OF *NF1* EXON 23A INCLUSION

Our laboratory has put a great deal of research effort into elucidating the regulatory mechanisms that govern *NF1* exon 23a inclusion. It is common for alternative exons to be under tight regulation by a combination of different protein factors. The inclusion of *NF1* exon 23a is tightly regulated both

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developmentally and in specific tissues, and it has been determined that this alternative splicing event is under complex control. To date two families of proteins that act as negative regulators of this system by promoting exon 23a skipping and another family of proteins, which promotes inclusion, have been identified. There are at least two families of proteins, Hu proteins and the CELF {CUG-BP (cytosine-uridine-guanine-binding protein) and ETR-3 [ELAV (embryonic lethal abnormal vision)-type RNA-binding protein] like factors}, which promote exon 23a skipping [75,76] (Figure 2B). Both of these protein families are highly expressed in the brain, where exon 23a is predominantly skipped [75,77–80]. TIA-1 (T-cell intracellular antigen 1) and TIAR (TIA-1-related protein) are positive regulators, which promote *NF1* exon 23a inclusion [76].

There are four members in the Hu protein family, HuR, HuB, HuC and HuD. HuR is expressed ubiquitously, while HuB, HuC and HuD are brain-specific family members [80]. Hu proteins are RNA-binding proteins with diverse molecular functions, including roles in mRNA stability, cellular stress response and alternative splicing [81]. Hu proteins bind to AU- or U-rich sequences on RNA. Currently, there are four documented Hu protein-mediated alternative splicing events, which include *Fas* exon 6, *HuD* exon 6, the *Ikaros* isoforms and *NF1* exon 23a [82–86]. Hu proteins exert their effects as negative regulators by binding to AU-rich sequences, which function as ISS (intronic splicing silencers), upstream and downstream of *NF1* exon 23a. *In vitro* analyses have shown that, at the downstream site, Hu proteins block the binding of the critical splicing machinery components, U1 and U6 to the 5′ splice site [76]. Hu proteins also inhibit the binding of critical splicing components upstream of this regulated exon. Zhu et al. [76] have shown that Hu proteins decrease the binding of the splicing factor, U2AF^{65} (U2 auxiliary factor large subunit) at the 3′ splice site of the upstream intron.

There are six CELF protein family members: CUG-BP1, ETR-3/CUG-BP2, CELF3, CELF4, CELF5 and CELF6. CELF family members 3–6 are considered to be brain-specific, while CUG-BP1 and ETR-3 are widespread with enrichment in the brain [77–79]. The CELF proteins are well-established regulators of alternative splicing [78,79,87]. CELF proteins bind to UG-rich *cis*-sequences on RNA [87,88]. The CELF proteins are closely related to the Hu proteins, as all of the proteins share the common structure of three RNA recognition motifs and a hinge domain. In our laboratory, we have shown that the CELF proteins promote exon 23a skipping by binding to the UG-rich elements upstream of *NF1* exon 23a. Although the specific *cis*-elements responsible for CELF protein-mediated splicing regulation have not been elucidated, *in vitro* binding assays suggest that the binding sites are located upstream of exon 23a [75]. Although all of these family members promote exon 23a skipping, CELF6 is a weaker regulator [75], presumably due to differences in its amino acid composition and/or cellular localization, which have been characterized by others [79]. *In vitro* UV cross-linking/immunoprecipitation experiments have shown that recombinant CUG-BP1 competes with endogenous U2AF^{65}, a critical component of the splicing machinery, in HeLa cell nuclear extracts [75].

TIA-1 and TIAR proteins have been shown to promote exon 23a inclusion. The Hu proteins and the TIA-1 and TIAR proteins engage in a competition for binding of a U-rich sequence downstream of *NF1* exon 23a, and the competition is decided depending on which factors are present at higher levels in a particular tissue. *In vitro* studies using nuclear extracts have shown that TIA-1 and TIAR promote exon 23a inclusion, by binding to the downstream U-rich site and increasing the binding of the U1 and U6 snRNPs (small nuclear ribonucleoproteins) at the 3′ splice site [76].

A ROLE FOR ALTERNATIVE SPLICING IN NF1 PHENOTYPIC VARIABILITY?

As discussed above, the phenotypic variability seen in NF1 patients cannot be explained solely by *NF1* gene mutations. Many researchers have hinted at the idea of genetic modifiers for this disease, particularly since *Nf1*$^{+/-}$ mice from different genetic backgrounds show differences in phenotype severity with regards to the learning and behavioural aspects of the phenotype, as well as the differences in the susceptibility to form astrocytomas [89,90]. An intriguing hypothesis is that a change in the levels of protein isoforms generated via pre-mRNA alternative splicing could act as a genetic modifier in NF1 as it does in other diseases such as CF.

Although it is unlikely that the NF1 patient tumour phenotype, which results from the loss of both copies of *NF1*, is strongly affected by the action of alternative splicing regulation as a genetic modifier, such a mechanism could become important for aspects of the NF1 phenotype that are due to the haploinsufficiency of neurofibromin. Two interesting examples of aspects of the NF1 phenotype which are thought to be due to neurofibromin haploinsufficiency are vascular disease and cognitive impairment.

An important variable aspect of the NF1 phenotype, which has come under closer scrutiny in recent studies, is vascular disease. It has been suggested that vascular disease is a major cause of mortality in young NF1 patients, but even so very few patients are routinely screened for this aspect of the disease [91,92]. One group has reported that juvenile NF1 patients who underwent brain MRIs showed a number of cerebrovascular system abnormalities including narrowing of vessels and aneurysms [93]. Much has been learned about vasculopathy using *Nf1*$^{+/-}$ mice as models of *Nf1* haploinsufficiency. These heterozygous mice have an increased risk of developing tumours, and they exhibit specific learning deficits [33,94]. One study by Lasater et al. [95] focused on neointima formation in *Nf1*$^{+/-}$ and wild-type controls with mechanical carotid artery injuries. Neointimas are newly formed or thickened inner linings of blood vessels, which commonly occur after tissue injury. The study showed that there was increased neointima formation in response to the injuries in the heterozygous mice compared with the wild-type controls [95]. More recently, the same group has determined that heterozygous inactivation of *Nf1* in mouse BMDCs (bone

marrow-derived cells) is necessary and sufficient to bring about neointima formation [96]. In addition to being prone to neointima formation upon arterial injury, there is evidence of vascular inflammation in *Nf1*$^{+/-}$ mice. Interestingly, peripheral blood samples from human NF1 patients also show evidence of chronic inflammation with increased levels of circulating monocyte cells compared with unaffected controls [96]. It has been established that VSMCs (vascular smooth muscle cells) that lack neurofibromin proliferate and migrate more readily than control cells in response to PDGF (platelet-derived growth factor), and this is believed to be due to the hyperactivation of the Ras-ERK (extracellular-signal-regulated kinase) pathway [97].

The learning disability phenotype for NF1 patients has profound implications for the management of the disease. The *Nf1*$^{+/-}$ mouse has learning disabilities, which appropriately mimic the human learning and behaviour phenotypes, suggesting that these aspects of the phenotype are due to neurofibromin haploinsufficiency. The mouse model fails at tasks that parallel attention deficits and visual–spatial deficits in NF1 patients [31]. Another model of the learning disabilities phenotype is the *Nf1* exon 23a$^{-/-}$ mouse. The mouse model shows that it is important to have the right balance of the two isoforms (types I and II) during development, since this animal lacks exon 23a and has a learning phenotype.

It is possible that alteration of alternative splicing could have a role as a genetic modifier of the severity of vascular disease and learning in NF1 patients. Specifically the regulation of *NF1* exon 23a could play an important role. Like other alternative exons, exon 23a is characterized by the presence of weak consensus sequences, surrounding the exon, which are not readily recognized by the splicing machinery, and, as mentioned above, there are many regulatory factors involved in the regulation of exon 23a inclusion or skipping. Although they have not yet been identified, it is possible that mutations that affect the *cis*-acting elements around exon 23a could result in the failure of important *trans*-acting factors to bind, and this could in turn lead to an altered ratio of the two isoforms. Conversely, it is also possible that mutations could exist in the genes that encode the regulatory factors that bind to the *NF1 cis*-acting elements. These effects could increase inclusion of this exon to a level that would allow hyperactivation of the Ras-ERK pathway in specific cells involved in the formation of neointima in the case of the vascular aspects of the disease. Likewise, the mutations could cause an imbalance in the distribution of the types I and II isoforms and lead to phenotypic variance in learning in patients.

With these concepts in mind, it is crucial to identify any additional *cis*-acting and *trans*-acting factors that regulate *NF1* exon 23a inclusion. It is also of interest to further mechanistically dissect and understand the biological importance of all of the naturally occurring *NF1* splice variants. An intriguing approach to better understand the biological importance of these splice variants is to use genetic techniques to generate mouse models in which the ratio of protein isoforms is altered. Manipulation of the alternative transcript levels in different tissues will most likely provide new insights into why these alternative exons are so tightly regulated *in vivo*.

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