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Neurofibromin and suppression of tumorigenesis: beyond the GAP

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Abstract

Neurofibromatosis type 1 (NF1) is an autosomal dominant genetic disease and one of the most common inherited tumor predisposition syndromes, affecting 1 in 3000 individuals worldwide. The *NF1* gene encodes neurofibromin, a large protein with RAS GTP-ase activating (RAS-GAP) activity, and loss of *NF1* results in increased RAS signaling. Neurofibromin contains many other domains, and there is considerable evidence that these domains play a role in some manifestations of NF1. Investigating the role of these domains as well as the various signaling pathways that neurofibromin regulates and interacts with will provide a better understanding of how neurofibromin acts to suppress tumor development and potentially open new therapeutic avenues. In this review, we discuss what is known about the structure of neurofibromin, its interactions with other proteins and signaling pathways, its role in development and differentiation, and its function as a tumor suppressor. Finally, we discuss the latest research on potential therapeutics for neurofibromin-deficient neoplasms.

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NEUROFIBROMATOSIS TYPE 1 (NF1) AND NEUROFIBROMIN

Neurofibromatosis type 1 (NF1) is one of the most common inherited tumor predisposition syndromes, affecting 1 in 3000 people worldwide. Affected individuals are prone to the development of various clinical presentations, including glioma, Lisch nodules of the iris, café-au-lait macules (CALMs), autism spectrum disorder, learning difficulties, and neurofibroma [1, 2] (Fig. 1). Neurofibromas are benign peripheral nerve sheath tumors composed of multiple cell types and located in the dermis (cutaneous neurofibroma; cNF) or associated with internal nerve plexus (plexiform neurofibroma; pNF). Most NF1 patients will develop cNF, while more than 50% also develop pNF. Additionally, NF1 patients have an 8–13% lifetime risk of developing malignant peripheral nerve sheath tumors (MPNSTs) [3, 4], which are the major cause of mortality in NF1 patients.

Identified in 1990 by two teams, one led by Dr. Francis S. Collins [5] and another team led by Dr. Frank McCormick [6], the *NF1* gene is located on chromosome 17q11.2. *NF1* is a very large gene that contains 60 exons—57 of which are constitutive and 3 that are alternatively spliced and tissue-specific—and encodes a 2818 amino acid RAS GTP-ase activating protein (GAP) called neurofibromin. Although neurofibromin is ubiquitously expressed, its expression is highest in cells of the nervous system, including neurons, astrocytes, oligodendrocytes, and Schwann cells [7, 8]. In the three decades since the identification of the gene, a great deal has been learned about the neurofibromin protein and its domains, the regulation of *NF1* gene splicing, the molecular interactions of neurofibromin in cells, and its role as a tumor suppressor.

THE STRUCTURE OF NEUROFIBROMIN

Domains of the neurofibromin protein

Although the catalytic RAS-GAP activity of neurofibromin protein is the most studied, neurofibromin contains many other distinct domains. It is possible that mutations within some of these domains are associated with particular clinical manifestations, and thus could offer potential therapeutic targets based on their biological function. Here, we describe known domains of the neurofibromin protein starting at the N-terminus (Fig. 2).

1. The “cysteine and serine rich domain” (CSRD) is contained within amino acids 543–909 [9] (encoded by exons 14–21), and has been shown to increase association of neurofibromin with actin upon phosphorylation [10, 11]. Patients with a mutation in this domain have a higher risk of developing optic glioma [12]. Additionally, a microtubule-associated protein (MAP) domain resides within the CSRD and is thought to regulate association of neurofibromin with microtubules [10, 11, 13].

2. The tubulin-binding domain (TBD), contained within amino acids 1095–1197 [14] (encoded by exons 25–27) [12], interacts with microtubules to impact neurofibromin RAS-GAP activity [15], and also binds with the cytoplasmic Dynein Heavy Chain 1 (DHC) in human melanocytes to regulate melanin expression [16]. The TBD is also a binding partner of the leucine-rich pentatricopeptide repeat motif-containing (LRPPRC) protein, providing clues

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about the contribution of DHC and LRPPRC proteins to NF1 clinical manifestations [17]. Recent studies in cell culture revealed that full-length neurofibromin exists as a dimer and that C-terminal but not N-terminal domains of neurofibromin are capable of forming high-affinity dimers, a process that requires the presence of the TBD [14]. However, the functional relevance of this dimerization has not yet been elucidated.

3. The most studied domain of neurofibromin is the RAS-GAP-related domain (GRD), comprising amino acids 1198–1530 [14] (encoded by exons 27–34 [18]). This domain has RAS-GAP activity, and when mutated, RAS activity is increased affecting multiple downstream pathways including MEK/ERK and PI3K/mTOR [19–24]. Neurofibromin has also been shown to interact with amyloid precursor protein (APP) through the GRD, and to co-localize with APP in the melanosomes of melanocytes, suggesting a potential role in pigment-related manifestations of NF1 [25]. Although the RAS-GAP activity of neurofibromin has been widely studied and is believed to play a prominent role in NF1 disease, in patients with pheochromocytoma *NF1* mutations tend to cluster in the CSRD over the GRD [26]. Additionally, the Sprouty-related EVH1 domain-containing protein 1 (Spred1) binds to the GRD of neurofibromin without interfering with its RAS-GAP catalytic activity [27, 28], demonstrating that the binding site is distant from the RAS-binding site of neurofibromin. This interaction induces plasma membrane location of neurofibromin, with subsequent decrease in Ras-GTP levels [29].

4. Using a yeast two-hybrid screen, Hsueh et al. found that neurofibromin binds to syndecans, a family of transmembrane heparin sulfate proteoglycans. The syndecan-binding regions (SBR) of neurofibromin, contained within amino acids 1357–1473 [30] (exon 30–33 within the GRD region) and amino acids 2619–2719 [31] (encoded by exons 53–56), localizes neurofibromin to specialized regions of the plasma membrane. Although the functional consequences of this localization are not yet known, it is likely involved in cell adhesion and intracellular signaling [31].

5. The Sec14 domain, comprising amino acids 1560–1698 (encoded by exons 35–36), has been shown to bind to various phospholipids [32, 33] but the role of this binding in neurofibromin function/regulation remains unclear.

6. The leucine-rich domain (LRD) is contained within amino acids 1579–1971 (encoded by exons 35–39) and encompasses most of the SEC14-PH domain and a portion of the HEAT-like repeats (HLR). The LRD is reported to be involved in inhibiting tumor metastasis and invasion of human glioblastoma cells, although proliferation is not affected [18, 34]. One study found that 9 out of 18 patients (50%) with mutations in the LRD region of *NF1* had learning disabilities, as well as skeletal problems, including short stature, tibia bone defects, and scoliosis [35].

7. Boyanapalli et al. identified four possible caveolin-binding domains (CBD) within neurofibromin—residues 1606–1613, 1658–1666, 1678–1685, and 2102–

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2109— and showed that neurofibromin and caveolin coimmunoprecipitate in a variety of cell lysates [36]. The two proteins are also found together in caveolin-1 enriched membranes [36]. Additionally, missense mutations are found in 3 of these 4 CBDs in NF1 patients indicating an important role for these domains. The CBDs are encoded by exons 28 and 32 [10], and are involved in the regulation of protein kinase C (PKC)-GPCR-cyclic AMP (cAMP) to maintain neurite length and FAK pathways to affect cytoskeletal organization [36] respectively.

8. Adjacent to the Sec14 domain is the Pleckstrin Homology (PH) domain (amino acids 1715–1816;encoded by exons 36–37). In experiments with mouse models and human cell lines, the PH domain has been shown to regulate constitutive activity of the serotonin 5 hydroxytryptamine 6 (5-HT_6) receptor, a G protein-coupled receptor, to affect cAMP signaling in the prefrontal cortex [37]. As identified in a yeast two-hybrid screen, it also interacts with and inhibits LIM domain kinase 2 (LIMK2), a serine kinase of the Rho/ROCK/LIMK2/cofilin pathway that is involved in actin cytoskeleton dynamics. This interaction demonstrates the crosstalk that occurs between the Ras and Rho signaling pathways and suggests a role for the PH domain in actin cytoskeleton remodeling [38].

9. The HLR is contained in amino acids 1825–2428 (encoded by exons 37–49) and consists of repetitive arrays of short amphiphilic α-helices. The molecular function of this domain is unknown, however, mutations in the HLR domain are associated with a lower risk for optic pathway glioma (OPG) in NF1 patients [12, 39].

10. The C-terminal domain (CTD) of neurofibromin, contained in amino acids 2260–2818 (encoded by exons 45–57), regulates cAMP via G-protein-dependent activation of adenylyl cyclase [40] and the transition from metaphase to anaphase [41]. It has been shown to play a role in learning and immediate memory in a *Drosophila* model system [40]. The CTD also contains the functional NLS (exon 51; amino acids 2534–2550) [42, 43]. Exclusion of this exon abolishes nuclear localization of NF1-fusion proteins in cell culture assays [43]. Neurofibromin has been reported to bind to collapsin response mediator protein 2 (CRMP2), potentially through a domain in the CTD 2260–2818 [44] (encoded by exons 45–57). As shown in a rat model, upon loss of *Nf1*, CRMP2 can then bind to syntaxin 1 A and Cav2.2 resulting in increased release of the pronociceptive neurotransmitter CGRP and higher sensitivity to pain [30]. This increased interaction upon *Nf1* loss may therefore underlie the pain experienced by NF1 patients that occurs independent of tumor burden [45]. In fact, over 50% of patients with NF1 report significant pain and discomfort that greatly affects their quality of life, leading to increased anxiety, depression, stress, and sleep disturbances in both children and adults [46]. This pain is often not associated with a specific lesion and is instead described as a painful sensory neuropathy associated with tingling and weakness in multiple body regions [45]. In addition, patients with NF1 report increased migraines, tension headaches, sciatica, and

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pain as a result of other gastrointestinal and musculoskeletal complications of the disease [45, 47]. This often leads to use of over-the-counter pain medication, prescription opioids, GABA analogues, cigarette, alcohol, and marijuana use in order to manage their chronic pain [45]. Opioid use is particularly concerning as most patients report that opioids are not beneficial in treating their symptoms, even leading to heightened sensitivity to their pain [45], and chronic use of opioids can lead to addiction. Despite being given pain medication, children and adults with NF1 continue to report pain interfering with their daily life [48]. Given the significant burden of pain in neurofibromatosis, this accentuates the need for further study on the origin of this pain, targeted treatments, and the role of pain in biopsychosocial outcomes in these patients.

Alternative splicing of the neurofibromin gene

Alternative splicing of pre-mRNA is a highly regulated process that plays a role in many biological processes including cell differentiation, tissue identity, and homeostasis, as well as in disease [49]. The *NF1* pre-mRNA undergoes alternative splicing involving a number of alternative splice sites. It has been postulated that the various *NF1* isoforms have distinct functional properties, and that alternative splicing may therefore contribute to the phenotypic variability observed in patients who carry the same *NF1* mutation [50]. Here, we briefly summarize known *NF1* alternative splicing events and exon usage.

In the early 1990s following identification of the *NF1* gene, cDNA cloning revealed the existence of several *NF1* transcript variants with predominant patterns of expression [51, 52]. At least five alternatively spliced exons have been reported: (i) An *NF1* isoform that includes exon 9a, which encodes 10 amino acids of unknown function, is expressed predominantly in the forebrain [51]. (ii) Identified in 2002, exon 10a-2 was found to be inserted between exon 10a and 10b, and potentially encodes a transmembrane helix [53]. This isoform is ubiquitously expressed at low levels and thought to be a housekeeping gene. (iii) The 21 amino acids encoded by alternatively spliced exon 30alt31 [18], formerly exon 23a, are inserted within the GRD of neurofibromin and are involved in learning and long-term memory [40, 54]. Mice lacking exon 30alt31 have communication deficits but no social learning deficits [55]. This exon is included in most tissues but, interestingly, is skipped in the central nervous system (CNS). Of note, the isoform that excludes this exon has ten times more Ras-GAP activity than the isoform in which it is included [56–58]. Inclusion of exon 30alt31 is regulated by TIA-1/TIAR protein while skipping of this exon is regulated by CELF and Hu proteins[10] (Fig. 2). Recently, the Serra lab reported that modulation of exon 30alt31 alternative splicing using antisense oligonucleotides impacted neuronal differentiation of PC12 cells [59]. (iv) The *NF1* splice variant lacking exon 51 (exon delta e43; formerly exon 43) lacks the NLS [60, 61], is highly expressed in tissues that do not express *NF1* containing this exon, and is not associated with NF1 disease. It has been postulated that the *NF1* delta e43 may have a unique function in these particular tissues [60]. (v) Alternatively spliced exon 48a, located near the carboxy terminus of neurofibromin, is expressed in and plays a role in the development and differentiation of striated muscle (heart and skeletal) [62, 63], and may play a role in the muscle weakness, reduced muscle weight, and muscle fibrosis experienced by some NF1 patients [64].

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UPSTREAM REGULATION OF THE NEUROFIBROMIN PROTEIN

In addition to splicing regulation of the *NF1* gene, the NF1 protein is also subject to regulation by a variety of other proteins (Fig. 2). Here we discuss some of these upstream regulators.

- ALK: The receptor tyrosine kinase (RTK) Anaplastic Lymphoma Kinase (ALK) was first identified in non-Hodgkins lymphoma, where, due to chromosomal translocation the catalytic domain of ALK is fused to the amino terminus of nucleophosmin (NPM), a conserved nucleolar phosphoprotein [65]. Activating mutations in ALK were subsequently found in a number of different cancers including non-small cell lung cancer, neuroblastoma, and inflammatory myofibroblastic tumors [66]. In 2011, ALK was identified as an upstream activator of neurofibromin-regulated RAS signaling in Drosophila: dALK was shown to extensively co-localize and interact with dNF, and attenuation of *Alk* rescued the body size deficits of *Nf1* mutant flies [67, 68]. It was later reported that pharmacological inhibition of ALK could also rescue cognitive impairment in *Nf1* mutant mice [69].
- SPRED1: As mentioned earlier, SPRED1 was reported to interact with the non-catalytic portion of neurofibromin's GRD, inducing localization of neurofibromin to the plasma membrane and downregulation of RAS signaling [29]. Mutations in the SPRED1 gene have been shown to cause Legius syndrome, a disease with many of the same phenotypic features of NF1 but without development of Lisch nodules, optic glioma, or neurofibroma formation [70, 71]. SPRED1 loss-of-function mutations found in patients with Legius syndrome are often C-terminal truncating mutations. Using human cell lines, Stowe et al. showed that overexpression of SPRED1 harboring loss-of-function mutations found in patients with Legius syndrome cannot inhibit RAS signaling, while artificially localizing a SPRED1 truncated protein to the plasma membrane restores neurofibromin localization there and inhibits RAS, thus highlighting the importance of cellular location for SPRED1 function [29]. Multiple reports have demonstrated that binding of SPRED1 to neurofibromin does not affect its RAS-GAP activity [27–29]. More recently, the crystal structure of the ternary complex formed by fragments of neurofibromin (the GRD domain), SPRED1 (EVH1), and Kras (G domain) was reported [72]. This study showed that most of the missense mutations in the SPRED1 (EVH1) domain are located on the NF1-SPRED1 interface. They also show that phosphorylation of serine 105 on SPRED1 by an oncogenic RTK (EGFRL858R) disrupted the binding of SPRED1 and neurofibromin, and consequently blocked negative regulation of Ras-GTP [72].
- GPCRs: Other upstream regulators of neurofibromin include G protein-coupled receptor (GPCR)-activated G protein Gβγ subunits, which bind to neurofibromin in striatal neurons and prevent its ability to downregulate RAS-AKT-mTOR signaling; this novel mechanism of neurofibromin regulation is involved in opioid addiction [73]. Also, as mentioned earlier, the PH

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domain of neurofibromin physically interacts with the 5-HT$_6$ receptor, a Gs-coupled receptor, to promote constitutive Gs signaling and subsequently cAMP production in prefrontal cortex neurons [37]. Mutations identified in NF1 patients that are located in the PH domain abrogate binding of neurofibromin with 5-HT$_6$ receptor. Abrogation of the neurofibromin-5-HT$_6$ receptor interaction may underlie neuronal defects found in some NF1 patients.

DOWNSTREAM SIGNALING OF NF1: MOLECULAR FUNCTIONS OF NEUROFIBROMIN IN KEY BIOLOGICAL PATHWAYS

The neurofibromin protein also interacts with a number of proteins and pathways (Figs. 2 and 3) to affect a wide variety of cellular processes. Mutation or dysregulation of neurofibromin consequently affects these downstream pathways. Here we discuss some of these key pathways.

- *Neurofibromin-RAS and RAF/MEK/ERK*: The most studied aspect of the neurofibromin protein is its activity as a RAS-GAP, which drives conversion of RAS-GTP to RAS-GDP thus inhibiting RAS signaling. The RAF/MEK/ERK pathway is a key effector downstream of RAS signaling;when neurofibromin is mutated, RAS signaling is hyperactivated, resulting in upregulation of RAF/MEK/ERK signaling which in turn promotes cell growth and survival [74, 75]. Importantly, inhibition of the RAF/MEK/ERK pathway can block neurofibroma progression [19, 76], and in 2020, the MEK inhibitor selumetinib became the first FDA-approved treatment for inoperable pNF [77].
- *Neurofibromin-RAS and PI3K/AKT/mTOR*: The PI3K/AKT/mTOR signaling pathway is another well-studied signaling pathway downstream of RAS signaling. In 2005, Johannessen et al. showed that the mTOR pathway is constitutively activated in human neurofibromas and in *NF1*-deficient primary cells [21], and Dasgupta et al. reported hyperactivation of the mTOR pathway in human and mouse optic nerve glioma [20]. Similar to the MEK signaling pathway, this pathway has been investigated as a potential therapeutic target although with mixed results [19, 78–82].
- *Neurofibromin-cAMP/PKA*: The cAMP-dependent protein kinase/protein kinase A (PKA), first identified in 1956 [83], is a ubiquitously expressed protein that acts as an effector of the second messenger cAMP. In 1996, Izawa et al. reported that neurofibromin was phosphorylated by PKA in human cells in vitro [9]. This was followed by reports that some defects observed in *Nf1* mutant Drosophila and zebrafish could be rescued by PKA overexpression or addition of cAMP demonstrating positive regulation of cAMP signaling by neurofibromin via adenylyl cyclase and indicating RAS-independent functions for neurofibromin [84–87]. Similarly, studies in mammalian cells demonstrated that axon length, growth cone diameter, and survival of CNS neurons depend on neurofibromin upregulation of cAMP levels independent of RAS–MEK or RAS–PI3K signaling [88, 89]. And in a mouse model of NF1, focal reduction of cAMP resulted

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in gliomagenesis, whereas increasing cAMP levels pharmacologically reduced occurrence and tumor size of optic glioma [90].

Conversely, other groups have reported that neurofibromin acts to suppress cAMP. In 2001, Kim et al. reported that *Nf1*-mutant murine Schwann cells have a three-fold higher level of cAMP compared to controls [91]. Similarly, MPNST cell lines derived from NF1 patients were found to have two-fold higher levels of cAMP compared to normal human Schwann cells [92]. More recently, Biayna et al. reported that during differentiation of PC12 cells, alternative splicing of *NF1* from Type 1 to Type II, which includes exon 23, results in increased cAMP activity and decreased MAPK/ERK signaling [59]. Thus, the regulation of cAMP/PKA by neurofibromin is complex and likely cell type-dependent and/or NF1 isoform-dependent.

- *Neurofibromin-syndecan-CASK*: As mentioned above, syndecan-2 was identified in a yeast two-hybrid screen for proteins that interact with neurofibromin; it was then found that neurofibromin interacts with all four members of the syndecan family [31]. This group also reported that the calcium/calmodulin-dependent serine protein kinase (CASK) interacts in a complex with neurofibromin and syndecan, although this interaction most likely requires syndecan, as CASK was not pulled out in the yeast two-hybrid screen for neurofibromin interactors [93]. A subsequent study used cultured hippocampal cells from rat brains to show that neurofibromin mediates the syndecan-2 signal to activate PKA, which is important in dendritic spine formation [94]. Thus, disruption of this interaction due to *NF1* mutation may underlie the learning disabilities associated with NF1.

- *Neurofibromin-caveolin 1*: Caveolin 1 (Cav-1) belongs to a family of integral membrane proteins that make up caveolae, juxtamembrane vesicles involved in a wide range of cellular functions including tumor suppression [95]. Glycophospholipids, cholesterol, and a variety of proteins, including signaling molecules, are localized to caveolar membranes, and interact with the caveolin-1 scaffolding domain directly. Boyanapalli et al. found that neurofibromin also binds to the scaffolding domain of Cav-1, and that cholesterol depletion, which disrupts caveolin-containing membranes, altered *NF1* signaling [36]. They also show using mouse cells that neurofibromin, via its interaction with caveolin, may negatively regulate focal adhesion kinase (FAK) [36]. More recently, Yang et al. reported that Musashi (MSI2), an RNA binding protein that is highly expressed in a number of cancers, interacts with caveolin, increasing caveolin ubiquitylation and degradation, and promoting epithelial-mesenchymal transition (EMT) and metastatic properties of human NF1-MPNST cells [96].

- *Neurofibromin-HIPPO*: The HIPPO pathway is highly conserved and plays a role in a variety of cellular processes including cell proliferation, differentiation, tissue regeneration, and apoptosis. A role for the HIPPO signaling pathway in neurofibroma development was first implicated when whole-genome sequencing of cNFs from NF1 patients revealed mutations in various components of the

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pathway including *SFN*, *RASSF1* (which encodes the protein 14-3-3), and *DLG-4* [97, 98]. Similarly, mutations in HIPPO pathway inhibitory genes as well as activation of YAP/TAZ were observed in NF1-associated MPNSTs [99, 100]. Chen et al. demonstrated that the Hippo pathway can act as a modifier of neurofibromagenesis, showing that cutaneous and pNFs that develop in *Hoxb7;Nf1mut;Lats1*$^{f/+}$*;Lats2*$^{f/+}$ mice are more numerous and larger than those in *Hoxb7;Nf1mut* mice [97].

- *Neurofibromin-ROCK-LIMK-Cofilin*: LIMK2 is a serine threonine kinase component of the Rho/ROCK/LIMK2/cofilin signaling pathway that plays a role in cytoskeleton dynamics [101]. In 2005, Ozawa et al. reported that knockdown of neurofibromin in human cell lines induced changes in cytoskeletal structure and cell morphology and motility via activation of the Rho/ROCK/LIMK2 pathway [102]. In 2010, a proteomics screen by Amano et al. using the active catalytic fragment of the ROCK kinase as bait revealed that neurofibromin and LIMK2 form a complex with ROCK [103]. Subsequently, Vallee et al. identified LIMK2 as a protein that interacts with the secPH domain of neurofibromin in a yeast two-hybrid screen [38]. Loss of neurofibromin results in upregulation of this pathway, thereby affecting actin and microtubule dynamics and altering cell morphology and migration. Studies are underway to determine whether inhibition of the Rho/ROCK/LIMK2/cofilin pathway could be a potential therapeutic avenue for some NF1 manifestations.

- *Neurofibromin-STAT3/JAK2*: Signal transducer and activator of transcription 3 (STAT3) is one member of a family of proteins that regulate gene transcription via relaying signals from activated cytokine and growth factor receptors in the plasma membrane to the nucleus. The Janus Kinases (JAKs) mediate the signal from the cytokine receptors to the STATs and are therefore upstream activators of STATs. Dysregulated JAK-STAT signaling has been implicated in the tumor microenvironment as well as in cancer cells themselves [104]. Wu et al. performed unbiased insertional mutagenesis transposon screening in an *Nf1*$^{fl/fl}$*;Dhh-Cre* mouse model and identified genes involved in the STAT3 signaling pathway [105]. They then tested the effect of deleting STAT3 on neurofibroma development and found that *Stat3*$^{fl/fl}$*;Nf1*$^{fl/fl}$*;Dhh-Cre* mice developed fewer tumors per mouse than their *Stat3*$^{fl/+}$*;Nf1*$^{fl/fl}$*;Dhh-Cre* littermates. These data suggest that inhibition of STAT3 could be a viable therapeutic strategy. Inhibitors of the JAK/STAT pathway are currently being investigated in a number of solid tumor cancers [106]. Interestingly, a recent case report describes the widespread eruption of cNFs in an NF1 patient who was being treated for rheumatoid arthritis with the JAK inhibitor tofacitinib [107]. Upon discontinuation of tofacitinib, these neurofibromas spontaneously regressed. While just a single case report, the role of the JAK-STAT pathway in neurofibroma development needs further investigation.

- *Neurofibromin-estrogen receptor (ER): NF1* mutations have been observed at a low rate (about 2%) in primary ER-positive breast cancer [108] but are more frequently found in ER-positive metastatic breast cancer indicating the

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NF1 mutation is a driver of breast cancer progression [109, 110]. Recently, neurofibromin was reported to act as a co-repressor of estrogen receptor (ER), with depletion of *NF1* in a human breast cancer cell line resulting in enhanced ER transcriptional activity [111]. Interestingly, this repressor activity required the leucine rich domain of neurofibromin and was independent of RAS-GAP activity, further highlighting the importance of neurofibromin domains outside of the GRD.

- *Receptor tyrosine kinases (RTKs)*: RTKs stimulate adapter proteins to activate Ras, thus allowing for the activation of downstream proliferation and survival signaling pathways, including RAF/MEK/ERK and PI3K/AKT/mTOR. There are many RTKs implicated in the pathogenesis of NF1, several of which have recently been targeted as potential treatment strategies [112]. MET, an RTK, and its ligand hepatic growth factor (HGF) were shown to be overexpressed in neurofibromas and MPNSTs, leading to increased tumor growth and metastasis [113, 114]. Interestingly, Wang et al. found that HGF/MET signaling was elevated in MPNST cells that were resistant to MEK inhibitor treatment, and that decreasing HGF/MET restored the sensitivity to the MEK inhibitor [114]. Additionally, when a combination of MEK and MET inhibition was used, it effectively inhibited MPNST cell growth in human cell lines and tumor growth in patient-derived xenograft models [114]. This highlights the need for combination therapy targeting multiple pathways upstream and downstream of Ras in the treatment of MPNST. Cabozantinib, an inhibitor of multiple RTKs, including VEGFR2, c-MET, AXL, RET, was shown to significantly decrease tumor growth, proximal peripheral nerve root volume, and angiogenesis in a mouse model of NF1, while restoring the normal nerve tissue architecture [115]. In a subsequent phase 2 clinical trial, cabozantinib decreased tumor growth, tumor pain intensity, and pain interference in daily life in patients with NF1, demonstrating the significance of multi-RTK inhibition in treating neurofibromatosis [115].

THE ROLE OF NEUROFIBROMIN IN DEVELOPMENT

A role for neurofibromin in the development of a variety of tissues/organs has been demonstrated using mouse knockouts. In 1994, Brannan et al. used gene targeting in embryonic stem cells to generate mice harboring a null mutation in *Nf1* [116, 117]. While they found that *Nf1*-heterozygous mice had no obvious defects, *Nf1* null mice were embryonic lethal, dying before E14.5. Analysis of these embryos revealed heart defects, a delay in hepatic, renal, and skeletal muscle development, and hyperplasia of sympathetic ganglia. Conditional knockout technology using various Cre recombinase drivers circumvented the embryonic lethality of *Nf1*-null mice and refined the role of *Nf1* in development. For example, Zhu et al. used *Synapsin 1-Cre* to study the role of *Nf1* in neuronal development and physiology [118]. While they found that deletion of *Nf1* in this population resulted in defects of the cerebral cortex and massive astrogliosis, no tumors developed. Bajenaru et al. reported that deletion of *Nf1* in mouse astrocytes using *GFAP-Cre* resulted in an increased number of astrocytes in several regions of the brain, however

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astrocytomas did not develop [119]. Deletion of *Nf1* in chondrocytes and adult bone marrow osteoprogenitors (which give rise to osteoblasts) in mice resulted in similar bone defects to those seen in individuals with NF1, including progressive scoliosis and kyphosis, short stature, and tibial bowing [120].

NF1 has also been shown to play a role in stem cell differentiation. Previous studies using mouse models demonstrated that *NF1* loss in the CNS causes aberrant differentiation and increased glial progenitor cell proliferation [121, 122]. More recently, Jecrois et al. showed that migrating glial progenitor cells are overproduced due to *Nf1* loss and are the cells that give rise to optic glioma in their mouse model [123]. Additionally, Mo et al. showed that in human induced pluripotent stem cells (hiPSCs) harboring patient-based *NF1* mutations, *NF1* loss impairs Schwann cell differentiation and promotes stemness, thereby expanding the pool of the tumorigenic cells of origin for neurofibroma [124].

Thus, conditional deletion of *NF1* in particular tissues affects proper development and function. However, while *NF1* clearly plays a role in these various developmental processes, the most prominent function of neurofibromin is as a tumor suppressor.

NEUROFIBROMIN AS A TUMOR SUPPRESSOR

Effects on cell properties

A tumor suppressor is defined as a gene/protein that normally acts to suppress cell proliferation. When inactivated, uncontrolled cell proliferation results leading to the development of tumors. Mutations in tumor suppressors can be inherited or acquired. Tumor suppressors can act via a variety of cell intrinsic mechanisms, for example by inhibiting cell division, regulating DNA repair, or inducing apoptosis.

Neurofibromin is a bona fide tumor suppressor that acts by inhibiting RAS activity. When *NF1* is mutated there is increased RAS signaling which impinges on a wide variety of cellular processes. *NF1* haploinsufficiency has been shown to cause perturbations in cell cycle control, DNA replication and repair, and immune response [125]. *NF1* mutation in one allele may not be able to sustain the normal tumor suppression function and might explain the diverse cellular phenotypes in terms of proliferation, stemness, and cell types affected in individual NF1 patients. For example, neurofibromin activity maintains regular cell cycle and DNA repair pathways, whereas these pathways are upregulated in lymphoblastoid cell lines isolated from NF1 patients; as a result, *NF1* haploinsufficiency itself may serve as an “active state” that ultimately promotes the loss of the other wild-type *NF1* allele [125]. Also, normal expression of neurofibromin in the nucleus is important for preventing aneuploidy: phosphorylation of Ser202 in the CTD of neurofibromin by PKC imports neurofibromin to the nucleus where it interacts with the mitotic spindle to maintain normal chromosome congression. Depletion of neurofibromin has been shown to cause aberrant chromosome congression at the metaphase plate [126].

NF1 can also regulate cell susceptibility to apoptosis. For example, *NF1*-deficient mast cells have reduced FAS antigen expression and are therefore resistant to FAS ligand-mediated apoptosis [127]. *Nf1*-deficient mouse embryonic fibroblasts (MEFs) are resistant

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to apoptosis in a gene dose-dependent manner, with *Nf1*-null cells more resistant to apoptosis than cells heterozygous for *Nf1* [128].

Effects on immune cells

In healthy peripheral nerves, Schwann cells comprise 90% of cells, with immune cells rarely present. However, neurofibromin expression levels affect the immune microenvironment, and neurofibromas are replete with immune cells including mast cells, macrophages, T cells, and dendritic cells. This inflammatory milieu is thought to be critical for neurofibroma formation. It has been shown in mice that *Nf1*-null Schwann cells release stem cell factor (also known as c-Kit ligand), which recruits *Nf1* heterozygous mast cells [129, 130] that in turn secrete excess TGF-β, increasing proliferation of tumor-associated fibroblasts and increased collagen deposition [131]. The infiltrating macrophages observed in neurofibroma are predominantly M1 rather than M2 macrophages [130, 132]. M1 macrophages secrete pro-inflammatory factors that are thought to play a role in preventing the transformation from benign neurofibroma to MPNST. On the other hand, M2 macrophages secrete anti-inflammatory factors and play protumorigenic roles, which might contribute to the establishment and maintenance of MPNST. As such, reprogramming of protumorigenic M2 macrophages to anti-tumorigenic M1 macrophages is emerging as a new anticancer therapy [133–135]. A phase 2 clinical trial using a combination of an RTK inhibitor and sirolimus that was shown to cause a shift from M2 to M1 macrophages in a preclinical model of MPNST [136] is currently ongoing (NCT02584647).

Cxcr3-expressing T cells and dendritic cells have also been reported to play a role in neurofibroma initiation and formation. Global deletion of Cxcr3, the receptor for chemokine CXCL10, inhibited neurofibroma development in a mouse model, and likewise *Dhh*-Cre;*Nf1*$^{fl/fl}$;*Cxcr3*-null mice did not develop pNF or nerve pathology [137]. Another group also found that haploinsufficient effector T cells are increased in low tumor-load NF1 patients and surprisingly reduced in high tumor-load NF1 patients compared to healthy controls, potentially indicating that high tumor-load causes lower T-cell-mediated immune response and consequently higher penetrance of malignancy [138]. This might explain why residual expression of neurofibromin in heterozygous *NF1* cells not only accelerates the incidence of benign tumor development but also intriguingly impairs transformation to malignant tumor [139]. Moreover, neurofibromin has distinct roles in regulating the antitumor activity of type I and II natural killer T (NKT) cells. On the one hand, haploinsufficient *Nf1*$^{+/-}$ Type I NKT cells cause decreased antitumor activity [140]. On the other hand, haploinsufficient *Nf1*$^{+/-}$ Type II NKT cells express lower CD1d, decreasing the inhibition of antitumor immunity by Type II NKT cells and therefore *Nf1*$^{+/-}$ mice have enhanced antitumor immunity compared to wild-type mice [140].

NEUROFIBROMIN IN NF1-ASSOCIATED TUMORIGENESIS

Two-hit theory

Tumorigenesis in NF1 patients is widely accepted to arise following Knudson's "two-hit" theory [141]: the first hit is a germline mutation in the *NF1* gene and is followed by a somatic mutation in the second allele of the *NF1* gene (loss of heterozygosity) in

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different cell lineages, thus causing correspondingly different clinical manifestations [142]. This includes the Schwann cell lineage in neurofibroma [143–145], astroglial lineage in glioma [146], melanocyte lineage in CALMs [147], mesenchymal progenitors in tibial pseudarthrosis [148], etc. Somatic mutations of the second *NF1* allele are believed to be critical drivers of NF1 manifestation/phenotype, however the spatial and temporal aspects of these mutations remain elusive.

Stem cells as cells of origin and tumorigenic maintenance in neurofibroma and MPNST

In accordance with the two-hit theory, evidence supports stem cells as the cells of origin for neurofibroma, meaning the second hit occurs in undifferentiated precursor cells to cause the phenotypic heterogeneity seen in NF1 patients, including the wide range of locations and the differences seen in the size and types of tumors (Fig. 4). The stem cell theory of origin is supported by the fact that pNF in humans develops embryonically when Schwann cell precursors are present suggesting they develop from this stem cell population. This has also been confirmed in mouse models where Cre targeting of this cell population results in the highest incidence of neurofibroma development [149, 150]. Additionally, stem cell- and EMT-related genes are re-expressed in *NF1*-null Schwann cell lineage cells that give rise to neurofibroma, indicating the existence of stem cells in tumors [124]. However, it is still not fully clear at what stage the second hit occurs, which is likely an important determinant in tumor cell fate and pathology, and therefore a key factor involved in engendering the spectrum of disease manifestations seen in NF1 patients.

The MEK inhibitor, selumetinib, was recently approved for the treatment of pNF [77]. Preclinical and clinical findings, however, indicate that treatment with MEK inhibitor, which targets the RAS-MEK-ERK pathway, cannot fully eradicate tumors, and once treatment is halted tumor growth recurs [76, 77, 151]. This suggests that neurofibroma could be seeded and/or maintained by a small population of tumor stem cells (TSCs), which have the capacity for self-renewal, differentiation, and plasticity allowing for aberrant neoplastic progression. Resistance to MEK kinase inhibitor treatment of these remaining cells with stem-like properties could be due to development of gatekeeper mutations [152, 153], by activation of another bypass pathway to restore signaling downstream of the drug [154], or by switching lineages from a MEK-dependent cell type to a MEK-independent cell type [155, 156].

Although NF1-related MPNSTs are thought to arise from benign pNF, it is also possible that MPNST might originate from a subpopulation of neoplastic cells with stem cell properties upon encountering cues from a malignancy-favoring microenvironment. This possibility is supported by two findings: (1) $NF1^{-/-}$;$TP53^{-/-}$ Schwann cell precursors differentiated from hiPSCs, which can form MPNST when implanted into immunocompromised host mice, have a stem-like phenotype [124]. This indicates that additional gene mutations besides *NF1* give the cells malignant properties, and that stem cells are likely the cells that give rise to malignant tumors and therefore the key target to eradicate MPNST; (2) Mutations targeting these stem cells result in higher penetrance of malignancy. For example, mice with expression of a mutant Harvey ras oncogene (*H-ras*) expressed in the hair follicle region where epidermal stem cells reside are predisposed to developing squamous carcinomas with

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higher malignant potential, whereas mice with *H-ras* expressed in interfollicular cells, which have a more differentiated phenotype, develop papilloma with lower malignant potential [157, 158].

The origin of TSCs, however, remains elusive. Recent studies have demonstrated that cells of the microenvironment including immune cells, fibroblasts, adipocytes, and endothelial cells along with their secreted factors and receptors strongly influence stem cells in a number of ways [159–162] and play a significant role in cancer development [163, 164]. These data suggest that TSCs could arise from normal stem cells that are surrounded by a cancer-favoring microenvironment. For example, aggressive prostate cancer cells acquire properties common to normal adult prostate basal stem cells [165]. Evolving evidence supports the notion that TSCs can also arise from differentiated cells that have dedifferentiated, a phenomenon called “bidirectional interconversion” [166, 167]. Glioma, lung, and breast cancer have been reported to exhibit this tumor cell plasticity in some cases [168]. Moreover, dysregulation of epigenetic mechanisms including genomic DNA methylation, histone modification, and microRNA (miRNA) regulation, can also lead to the formation of TSCs [169–175]. Thus, there are a variety of potential mechanisms that can give rise to TSCs, which has important implications for choosing the appropriate therapy.

Reduced neurofibromin level in hypomorphic mutations of NF1

For some gene mutations, 50% of gene function is sufficient for normal function and no phenotype is observed when one allele of the gene is deleted or mutated [176]. However, in some cases, having just one wild-type copy of a gene is insufficient for normal gene function and results in a phenotype, a situation known as haploinsufficiency [177, 178]. In some cases, severe hypomorphic alleles fail to rescue null lethality if the gene is critical for embryo development; while in other cases, the intermediate hypomorphic allele overcomes lethality and yields a variety of abnormalities affecting body weight, size, and tissue development [179]. It is worth noting that certain mutations in the *NF1* allele result in much more than a 50% reduction in protein and potentially function as hypomorphs [180].

Genotype-phenotype correlations

More than 2600 unique inherited *NF1* mutations have been identified in NF1 patients [181], and this large variety of *NF1* mutations along with the clinical heterogeneity of symptoms has led to the hypothesis that there may be a genotype-phenotype correlation. While data linking specific *NF1* mutations with particular clinical manifestations has remained elusive, some emerging data has begun to reveal that mutations in certain regions are associated with particular disease features [182, 183] (Fig. 5). We discuss some of these here.

- *NF1* microdeletion syndrome: Patients harboring a 1.5 Mb heterozygous *NF1* deletion display what has been termed “NF1 microdeletion syndrome” or “17q11 microdeletion syndrome”. This syndrome includes earlier onset of neurofibroma development and a higher number of tumors, increased risk for MPNST development, dysmorphic facial features, and intellectual disabilities [184–186]. Recently, Wegscheid et al. used hiPSCs harboring the 17q11.2 microdeletion to identify cytokine receptor-like 3 (*CRLF3*) as a critical gene in this region that

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normally plays a role neuronal maturation and dendritic outgrowth, and mutation of which is associated with increased autistic burden in NF1 patients [187].

- *5′-end mutation clustering*: Studies looking at the correlation of 5′-end mutations and risk of OPG have provided mixed results, with some studies showing that 5′-end mutation clustering is significantly higher in OPG than in that of non-OPG [188–191], while others have found no significant correlation [192]. Further studies are required to definitively support this correlation.
- *Missense mutations affecting NGF1 codons 844–848:* Koczkowska et al. reported the identification of mutational hotspots affecting one of five neighboring codons, 844–848, located in the CSRD of neurofibromin that correlate with a more severe phenotype, including higher prevalence of pNF and optic glioma, as well as skeletal abnormalities [193].
- *p.Met1149 variant*: In a study of 281 individuals, Koczkowsaka et al. identified the p.Met1149 variant as a non-truncating mutational hotspot [183]. NF1 patients with this mutation have a Noonan-like phenotype that is milder with predominantly pigmentary manifestations.
- *c.3721 C > T; p.(Arg1241*) variant:* Scala et al. performed a retrospective study on 583 individuals with NF1 and found the p.Arg1241* *variant* mutation had a positive correlation with alterations in brain structure [194].
- *p.Lys1423 variant*: This variant was also identified by Koczkowska et al. and correlates with a mild form of NF1, with decreased incidence of neurofibroma and other neoplasms [183].
- *c.6855 C > A; p.(Y2285*) variant:* This variant was also identified in the Scala et al. study and was found to correlate with increased presence of Lisch nodules and endocrine disorders [194].
- *p.Arg1809 mutation*: Patients with a missense mutation affecting p.Arg1809 have a high incidence of Noonan syndrome features but, interestingly, do not develop neurofibroma [177, 195, 196]. Analyses performed on an isogenic series of hiPSCs engineered with CRISPR/Cas9 to harbor various patient-based mutations showed that the Arg1809Cys mutation (c.5425 C > T) mutation resulted in different cell properties (increased proliferation and apoptosis) in a cerebral organoid system compared to hiPSCs harboring other mutations [197]. These data indicate that different *NF1* mutations result in phenotypic variability, supporting the genotype-phenotype theory.
- *c.2970-2972 delAAT deletion*: This 3-base pair in-frame deletion in exon 17 of the *NF1* gene (also referred to as p.Met992del) leads to the loss of the methionine residue at 992 of the neurofibromin protein. This deletion has been reported to result in a much milder presentation of the disease and a lack of cutaneous and plexiform neurofibromas [178, 198].

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Other mechanisms underlying NF1 tumorigenesis

Recent studies have shown that short, dysfunctional telomeres can be subject to large-scale genomic rearrangements that confer a poor prognosis in many tumor types [199]. Interestingly, the telomere length of MPNST cells is shorter than that observed in neurofibromas [200], and therefore may drive genomic instability and facilitate progression to malignancy. However, in another study, overall survival was found to be increased for NF1-MPNST patients with short telomeres, intermediate for those with normal telomeres, and decreased for those whose tumors were positive for alternative lengthening of telomeres, a telomerase-independent mechanism for maintenance of telomere length [201]. Thus, further analyses are required to determine the role of telomere length in MPNST development.

miRNAs are a class of non-coding RNAs that are involved in post-transcriptional regulation of gene expression. A number of studies have identified particular miRNAs that play a role in NF1-related malignancy, including several that function as tumor suppressors (miR-34a [202], miR-29c [203], miR-204 [204], miR-193A, miR-365b [184], let-7b-5p, miR-143-3p, and miR-145-5p [205]) and several that function as oncogenes (miR-214 [202], miR-10b [206], miRNA-21 [207], miR135b-5p, and miR-889-3p [205]). These miRNAs regulate target genes and processes that are critical for tumor malignancy and metastasis. This area of research is evolving and holds promise for the identification of novel druggable targets.

NF1 MUTATIONS IN SPORADIC TUMORS

With advances in whole genome sequencing technology and the availability of publicly accessible databases, acquired somatic mutations in the *NF1* gene have been identified in a variety of sporadic cancers that are not typically associated with NF1 Type 1, including breast, colon, lung, ovarian, skin and other cancers [108, 208–222] (Fig. 6). While beyond the scope of this review, a recent review by Philpott et al. summarizes the various non-NF1-related cancers for which NF1 mutations have been observed [181]. They report that in most of these cases, the *NF1* mutation is a driver mutation. *NF1* mutations may also contribute to drug resistance as observed in the treatment of retinoic acid for neuroblastoma [211], RAF inhibitor for melanoma [223], EGFR inhibitor for lung cancer [224] and endocrine therapy to breast cancers [225]. Next-generation sequencing technologies and single-cell/single-nucleotide technologies will provide additional insights into the molecular underpinnings of these sporadic tumors.

TRANSLATING CURRENT RESEARCH INTO FUTURE THERAPIES FOR NF1-DEFICIENT NEOPLASMS

Gene therapy

The use of gene therapy for disease treatment continues to expand, with clinical successes reported for a number of single-gene diseases including inherited retinal dystrophy (RPE) [226], X-linked retinitis pigmentosa [227], severe combined immunodeficiency (SCID-IX) [228], and hemophilia [229]. In gene therapy, a wild-type copy of the affected gene is introduced into patients, typically using replication-defective retroviral, adeno-associated

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viral, or lentiviral vectors which are efficient delivery tools for gene therapy [230–232]. Gene therapy as a potential treatment for NF1 is also being explored, however, the large size of the neurofibromin cDNA is a technical challenge due to the size constraints of viral vector cargos. Cell toxicity is also observed when overexpressing full-length neurofibromin, although Cui and Morrison were able to overcome the toxicity issue by introducing a miniintron sequence into their *NF1* minigene construct [233].

Another potential strategy would be to rescue *NF1* loss of function by introducing just the GRD sequence of the *NF1* gene into cells [232], as loss of RAS-GAP activity is thought to be the critical mechanism in many NF1 manifestations. However, studies using an inducible NF1-GRD in a murine model have shown this was not able to rescue the $Nf1^{-/-}$ developmental phenotype in neural crest derivatives [234], strongly supporting the important role of neurofibromin domains outside the GRD.

An alternative to transduction of the *NF1* gene using viral vectors is to perform genome editing using CRISPR/Cas9 technology, a strategy that has some important advantages: (1) CRISPR/Cas9 can be performed either ex vivo or in situ for both gene addition and gene correction; (2) The more precise genome editing not only overcomes many disadvantages that random genomic insertion has when employing viral vectors, such as genotoxicity, or disruption of nearby normal gene expression, but also reflects more physiologically regulated gene expression under endogenous promoter control [235, 236]; (3) There are no packaging size limitations as with viral vector delivery. The Cas9 and guide RNA (gRNA) are typically delivered to cells using mRNA or nanoparticles containing protein-gRNA complexes, and in an organ-specific manner [237, 238]. While delivery of the donor DNA to host cells that is required by homology-directed repair for gene correction is the bottleneck of this technology [239], a new generation of nanoparticles is making it more feasible and promising [238]. However, other issues such as avoiding off-target effects, limiting immunogenicity of the Cas9 nuclease, and improving the targeting of gene delivery to particular organs/tissues are limitations with the CRISPR/Cas9 system that are currently being investigated.

Transcription factor therapy

Transcription factors and other proteins that regulate transcription by binding to a specific DNA sequence play a vital role in controlling normal cellular processes including cell proliferation, differentiation, metabolism, and immune response [240]. Aberrant transcription factor activity is frequently observed in numerous cancer types, for example GABP in glioblastoma [241], RUNX2 in breast cancer [242], STAT1 and RUNX2 in melanoma [243, 244], and MYC in lymphoma [245] by regulating stem cell properties [246], EMT [247], replicative immortality [241], and immune evasion [248]. Considering the many signaling pathways that transcription factors in the *NF1-Ras* axis interact with such as SNAIL, SLUG, TWIST, ZEB [249, 250], RUNX1/3 [251], and HIPPO pathway [97, 99], targeting transcription factors could be an effective strategy to treat NF1-associated tumors.

While typically considered “undruggable”, various strategies are now being used to target transcription factors: (1) Compounds that bind to the DNA minor groove of target gene

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promoter sequences and disrupt transcription factor-DNA binding have been developed [115, 252]. (2) Protein–protein interactions between transcription factors and co-activators/co-repressors affect gene expression, therefore, small-molecule inhibitors that disrupt these interactions have been developed [253]. For example, the interaction between transcription factors and proteins involved in ubiquitylation and deubiquitylation is modulated via regulation of E3 ligase binding to transcription factors [254, 255], or small-molecule inhibition of deubiquitinases for transcription factors [256]. Currently, combination of LXS196 (a PKC inhibitor) and HDM201 (an inhibitor of HDM2 (an E3 ubiquitin ligase) that blocks degradation of p53) is being tested in a phase 1 clinical trial for uveal melanoma (NCT02601378). (3) Similarly, ligand-receptor binding could be targeted as well. Once hormone binds to a nuclear hormone receptor via the ligand-binding domain, the nuclear hormone receptor regulates gene expression via its own DNA-binding domain [257]. Therefore, designing small molecules that competitively bind to nuclear hormone receptors is a plausible method [258]. (4) Proteins involved in epigenetic signaling including histone deacetylases, acetyl transferases, methyltransferases, and demethylases that function as regulators of expression of transcription factors could also be druggable targets. Small inhibitors of BRD4, a protein belonging to the Bromodomain and extra-terminal (BET) family whose bromodomains bind to acetylated lysine residues on histones and transcription factors, decreased the levels of c-MYC, a transcription factor regulated by the BET family that is upregulated in numerous cancers [259]. Currently, multiple clinical trials targeting epigenetic modifications are underway [260]. (5) Knocking down protein levels in cells by proteolysis-targeting chimera (PROTAC) is another potential method. PROTAC has been used successfully to degrade BRD4, a histone acetyltransferase and transcriptional coactivator, in a mouse model [261], and this method can also be applied to transcription factors. For example, PROTAC-based degradation of androgen receptors for the treatment of castration-resistant prostate cancer, and degradation of ERs for the treatment of ER-positive breast cancer are currently in phase 1/2 clinical trials (NCT03888612).

Synthetic lethality

In cancer, the concept of "synthetic lethality" refers to when the combination of one gene harboring a cancer-specific mutation and the pharmacological inhibition of another gene leads to the death of cancer cells whereas normal cells are spared [262]. While loss of a tumor suppressor gene is not druggable, a synthetic lethality strategy allows one to target the synthetic lethal "interactor", thereby making it druggable, and specific for the mutation-harboring cancer cells. An archetypal example of this concept was the discovery of PARP inhibition in cancers associated with BRCA mutation [263–265]. PARP inhibitors cause BRCA mutant cancer cells to die while having minimal effect on normal cells that have at least one wild-type allele, thus extending the median duration of progression-free survival of cancer patients with BRCA mutation [265, 266]. Large-scale target studies [267, 268] have also identified other candidates for synthetic lethality, including Werner syndrome ATP-dependent helicase (WRN) [269]. Deletion of WRN using CRISPR knockout or RNA interference induces double-stranded DNA breaks and apoptosis in cancers with microsatellite instability [269].

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The genetic basis of NF1 disease makes it amenable to synthetic lethal approaches, and chemical screens targeting *NF1*-mutant cells are being actively pursued. The loss of *NF1* could render Schwann cells, which have homozygous loss of *NF1* in NF1-associated tumors, sensitive to pharmacologic inhibition of another gene. Efforts have been made to discover novel therapeutic drugs that block or slow *NF1*-mutant cell growth. For example, a recent study reported a screen of 472 known bioactive compounds against *Nf1* wild-type MEFs or MEFs with *Nf1* loss in both alleles [270]. The screen identified the protein phosphatase 2A inhibitor cantharidin and the L-type calcium channel blocker nifedipine as potential candidates and showed that these drugs could inhibit MPNST cell growth and tumor growth in an MPNST xenograft mouse model.

Appropriate cell types to use for synthetic lethal screening include immortalized *NF1*-null Schwann cells from patients and differentiated hiPSCs [271]. Using cells from patients provides the ability to identify a personalized therapy strategy. CRISPR/sgRNA libraries could be applied to those cell lines in genome-scale loss-of-function screens to interrogate dependency and essentiality of genes that are required for cell fitness and to identify synthetic lethal drug targets [272]. *NF1* is a "marked" tumor suppressor gene with specific genetic mutations; however *NF1* loss might activate other "unmarked" oncogenes that are not activated by genetic mutation, translocation, or amplification but rather are activated in specific genetic contexts [262]. In such a scenario, CRISPR libraries can be used for synthetic lethal screening to identify novel drug-targeting context-dependent unmarked oncogenes [273]. For example, *NF1*-null Schwann cell lines could be infected with CRISPR/sgRNA library and then treated with or without MEK inhibitor. The knockout of context-dependent unmarked oncogenes will sensitize the cells to MEK inhibitor treatment, and they will be depleted. Therefore, inhibitors of such context-dependent "unmarked" oncogenes can be combined with MEK inhibitor as novel combination treatments.

CONCLUSION AND FUTURE PERSPECTIVES

Hundreds of years after the first formal clinical reports and documentation of NF1 in the 1880s, there is still no cure for NF1, and surgical removal remains the mainstay treatment for neurofibromas, the hallmark tumors of NF1. However, since the identification of the *NF1* gene in the 1990s, great strides have been made in understanding this large and complex protein. Decades of research have yielded important insights into the different functional domains of the protein, and it has become clear that while the RAS-GAP activity of neurofibromin is the key molecular feature, other domains also play a role in engendering clinical symptoms. It is also clear that neurofibromin interacts with a variety of signaling pathways, thus offering the potential for new therapeutic targeting opportunities. For example, while MEK inhibitor treatment alone may not be able to completely eradicate neurofibromas, it is possible that combination treatment using MEK inhibitor together with drugs that target other pathways could be more effective. Also, the tyrosine kinase inhibitor cabozantinib was recently shown to be effective in a phase 2 clinical trial for inoperable pNF, reducing tumor volume and pain [274]. The study found that cabozantinib appears to act on both the *NF1*-null Schwann cells and cells of the tumor microenvironment, highlighting the potential of more effective combination therapy that target both. Furthermore, drug treatments are also needed for the many other manifestations

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of NF1. The hope is that a more thorough understanding of the neurofibromin protein, and its interactions with key signaling pathways, will offer actionable targets for drug development. Expanding the treatment options—not just for neurofibromagenesis, but for all the various clinical manifestations of NF1—is an ambitious but hopefully achievable goal that will greatly improve the quality of life for NF1 patients.

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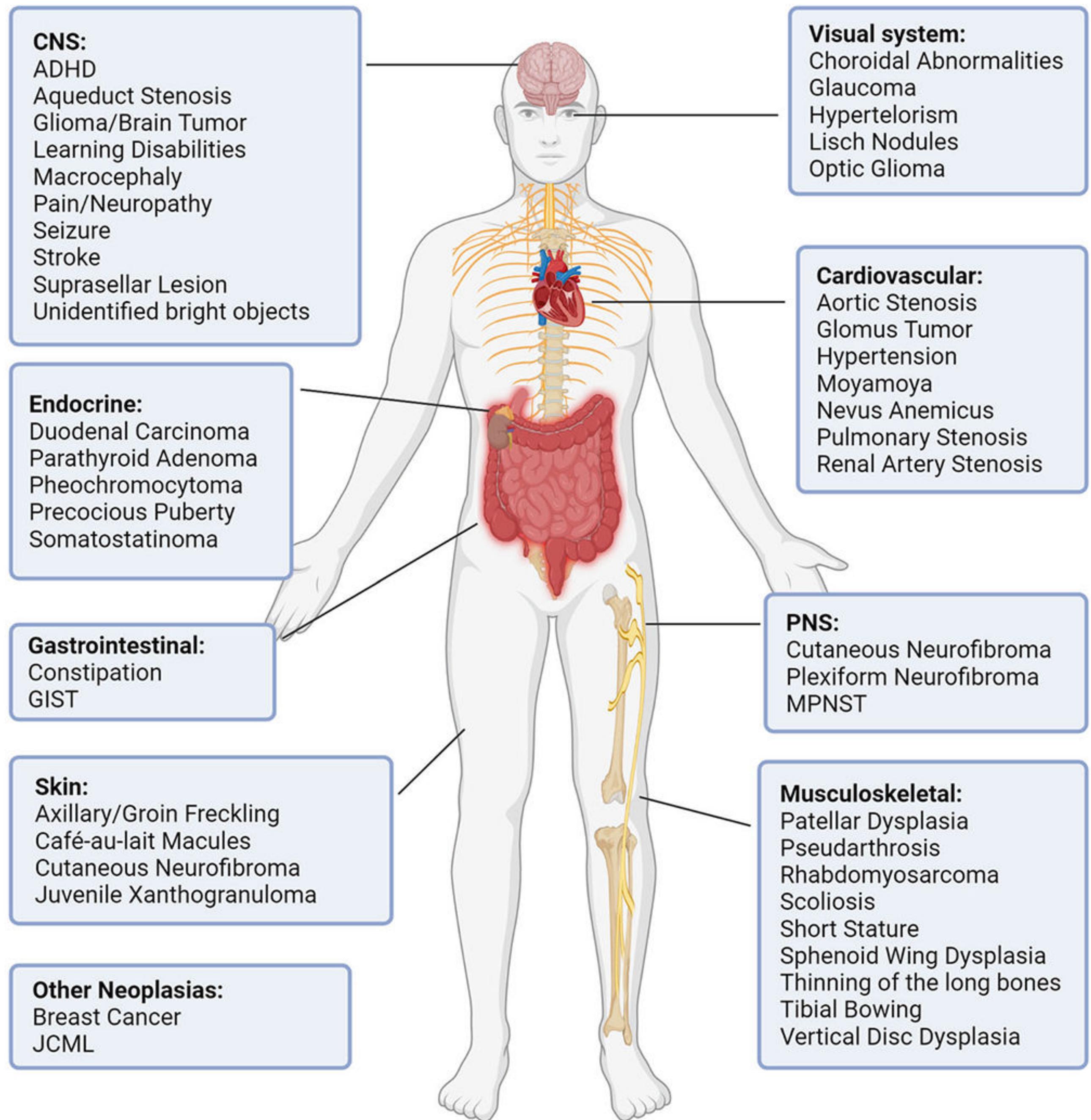

Fig. 1. Diagram of the clinical manifestations of NF1.

Individuals with NF1 have a wide spectrum of clinical manifestations, from café-au-lait macules to benign neurofibroma to malignant peripheral nerve sheath tumor (MPNST) as well as cognitive symptoms including learning disabilities and attention deficit disorder [1, 2]. ADHD attention-deficit/hyperactivity disorder, CNS central nervous system, GIST gastrointestinal stromal tumor, JCML juvenile chronic myelogenous leukemia, PNS peripheral nervous system. Created with BioRender.com.

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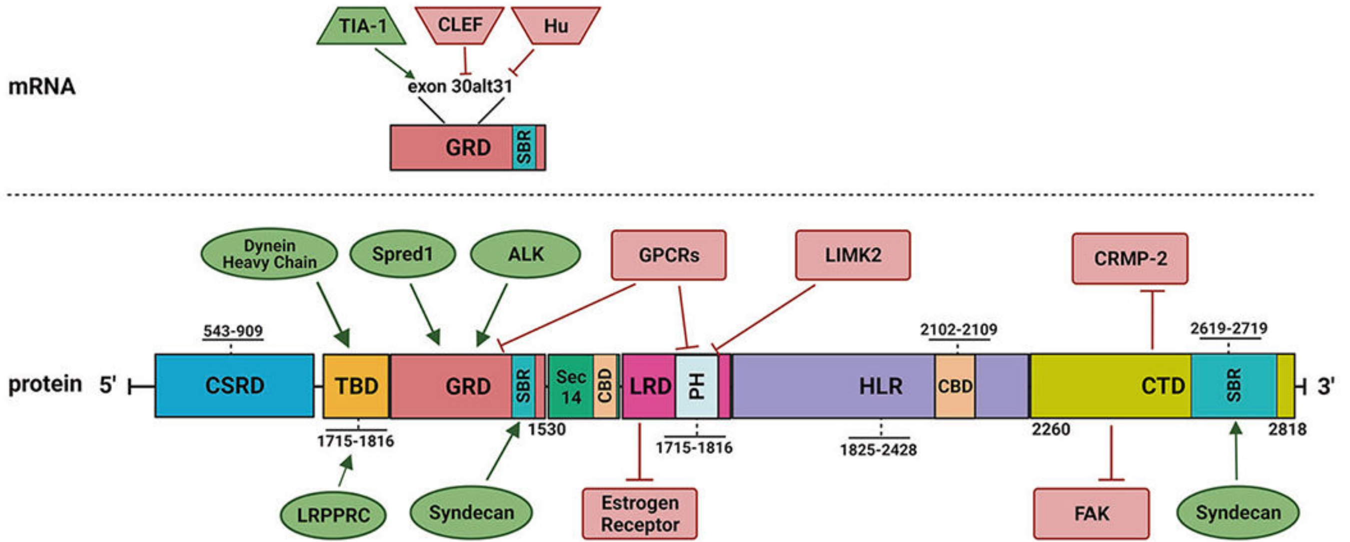

Fig. 2. Domains of the neurofibromin protein and proteins that directly interact with those domains.

Schematic presentation of known domains of NF1. Numbers indicate the amino acid residues comprising the domains. (Top) Diagram shows neurofibromin regulation at the level of mRNA splicing. Proteins shown in red result regulate splicing that excludes exon 30alt31, while green indicates inclusion of that exon. (Bottom) Proteins that directly interact with neurofibromin are shown in green for positive regulation and red for negative regulation [16, 29, 31, 36–38, 44, 67, 68, 73, 111]. Domains: CBD caveolin-binding domains, CSRD cysteine and serinerich domain, CTD C-terminal domain, GRD RAS-GAP-related domain, HLR HEAT-like repeats, LRD leucine-rich domain, PH pleckstrin homology, SBR syndecan-binding region, TBD tubulin-binding domain. Proteins: ALK anaplastic lymphoma kinase, CELF CUG-BP1 and ETR-3-like factors, CRMP-2 collapsin response mediator protein 2, FAK focal adhesion kinase, GPCRs G-Protein-Coupled Receptors, LIMK2 LIM Domain Kinase 2, LRPPRC leucine-rich pentatricopeptide repeat motif-containing protein, Spred1 Sprouty-related, EVH1 domain-containing protein 1. TIA-1 T-cell intracellular antigen 1. Created with BioRender.com.

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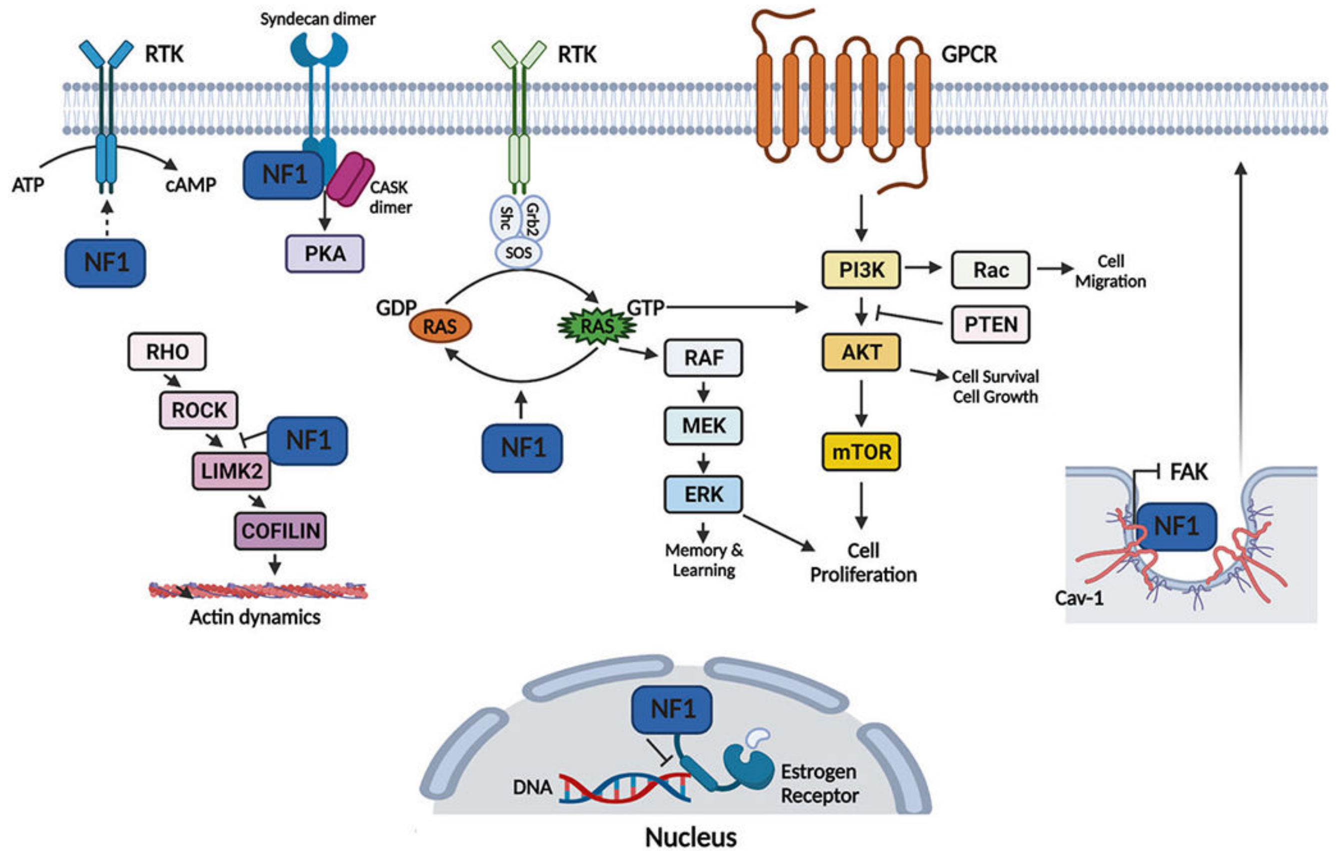

Fig. 3. Downstream signaling of NF1: molecular functions of neurofibromin in key biological signaling pathways.

Known signaling pathways downstream of NF1 are shown [9, 19–21, 31, 36, 38, 74–76, 88–90, 103, 111] Created with BioRender.com.

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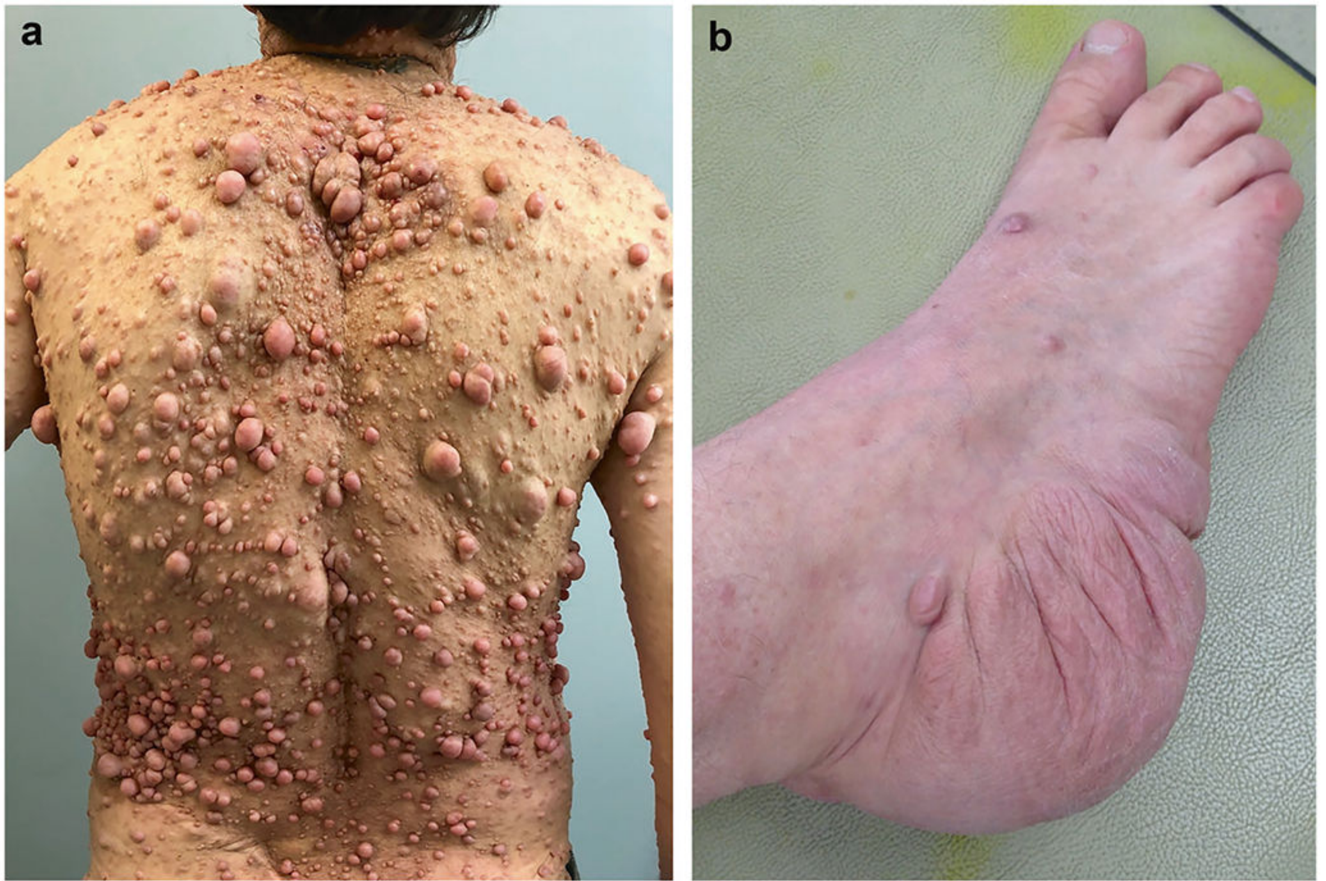

Fig. 4. Clinical manifestations of NF1.

a Cutaneous neurofibroma on the back. **b** Plexiform neurofibroma on the right ankle.

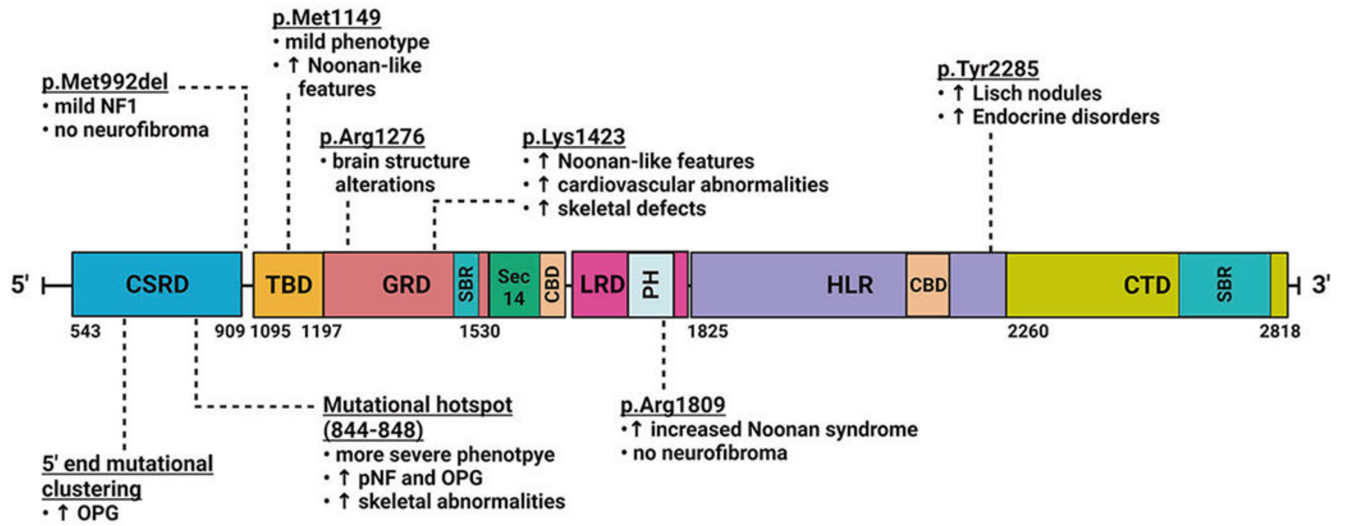

Fig. 5. NF1 genotype-phenotype correlations.

NF1 mutations reported to correlate with particular phenotypes are indicated [177, 178, 183–191, 193–198]. Created with BioRender.com.

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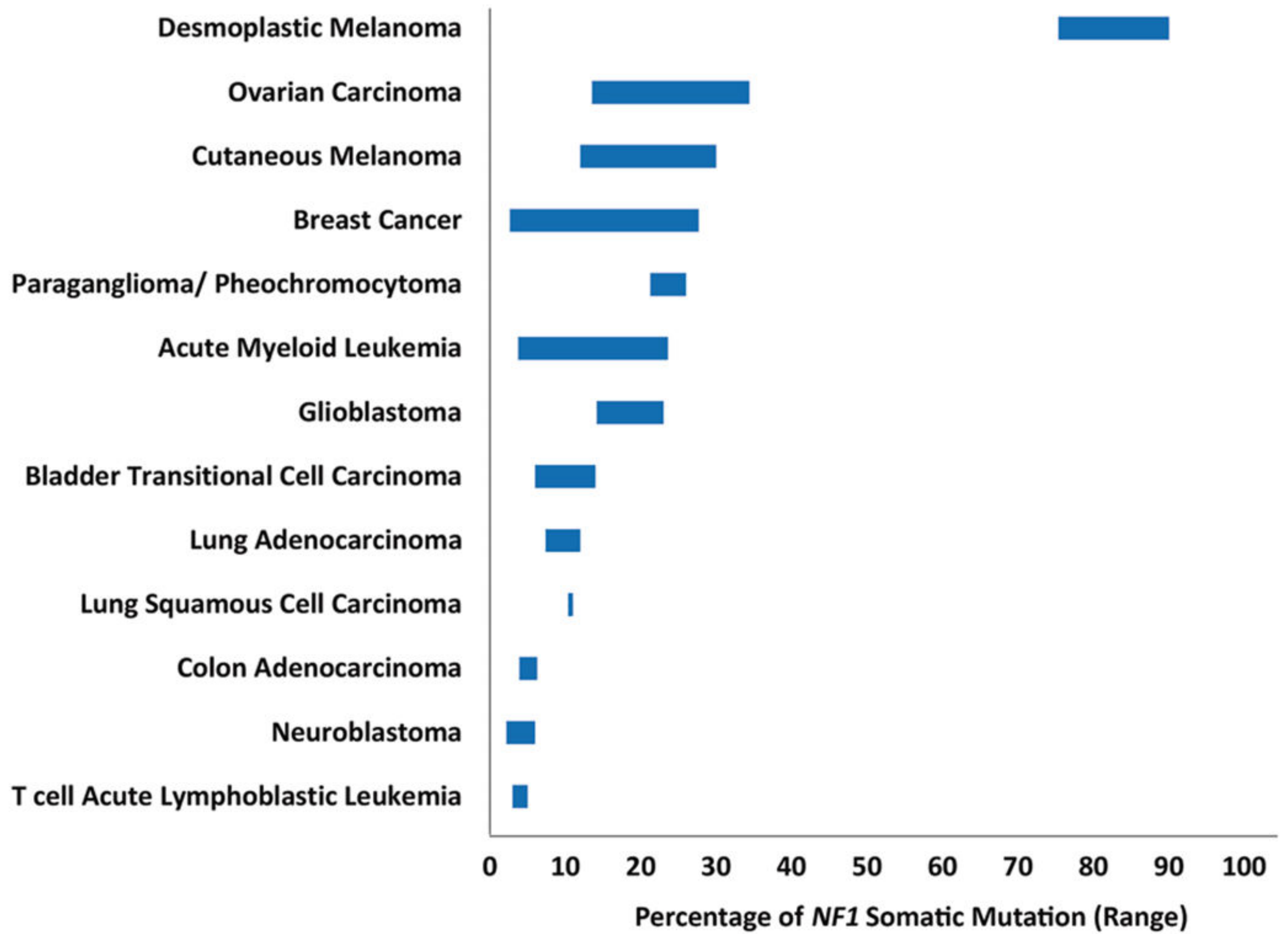

Fig. 6. List of sporadic tumors with NF1 mutation.

The range of frequency (%) of NF1 mutations reported in other cancer types is shown [178, 208, 213–222, 275–286].