Frost *et al. J Transl Genet Genom* 2022;6:403-28
DOI: 10.20517/jtgg.2022.14

Journal of Translational Genetics and Genomics

Review

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Rationale for haploinsufficiency correction therapy in neurofibromatosis type 1

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How to cite this article: Frost M, Serra E, Viskochil D, Korf BR, Mattson-Hoss MK, Croston GE, Sarnoff H. Rationale for haploinsufficiency correction therapy in neurofibromatosis type 1. *J Transl Genet Genom* 2022;6:403-28. https://dx.doi.org/10.20517/jtgg.2022.14

Received: 30 Jul 2022 **First Decision:** 3 Aug 2022 **Revised:** 16 Sep 2022 **Accepted:** 19 Oct 2022 **Published:** 25 Oct 2022

Academic Editors: Sanjay Gupta, Bridget Bax, Nathan A. Berger **Copy Editor:** Fangling Lan **Production Editor:** Fangling Lan

Abstract

Neurofibromatosis type 1 (NF1) is a genetic disorder with a wide range of manifestations and severity. Currently, the few available NF1 treatments target specific manifestations, with no available therapies targeted to correct the underlying driver of all NF1 manifestations. Evidence supports that haploinsufficiency in NF1 caused by a decreased amount of wild-type (WT) neurofibromin in all *NF1*$^{+/-}$ cells directly causes or facilitates a range of NF1 manifestations. Consequently, NF1 haploinsufficiency correction therapy (NF1-HCT) represents a potentially effective approach to treat some NF1 manifestations. NF1-HCT would normalize the level of WT neurofibromin in all *NF1*-haploinsufficient cells, including those integral to the NF1 phenotype such as Schwann cells (SCs), melanocytes, neurons, bone cells, and cells of the tumor microenvironment. This would correct altered cellular signaling pathways and, in turn, restore normal function to cells with a retained WT allele. NF1-HCT will not restore WT neurofibromin in *NF1*$^{-/-}$ cells; however, by restoring function in the surrounding *NF1*$^{+/-}$ microenvironment cells, NF1-HCT is predicted to have a beneficial effect on *NF1*$^{-/-}$ cells. NF1-HCT is expected to have a clinical effect in some NF1 manifestations, as follows: (i) prevention, or delay of onset, of potential manifestations; and (ii) reversal, or halting/slowing progression, of established manifestations. This review describes the rationale for NF1-HCT, including specific NF1 considerations (e.g., NF1 clinical phenotype, neurofibromin function/regulation, *NF1* mutational spectrum, genotype-phenotype correlation, and the impact of haploinsufficiency in NF1), HCT in other haploinsufficient diseases, potential NF1-HCT drug treatment strategies, and the potential advantages/challenges of NF1-HCT.

Keywords: Neurofibromatosis type 1, neurofibromin, NF1, haploinsufficiency, treatment

INTRODUCTION

NF1 is a rare autosomal dominant genetic disorder (birth incidence of ~1:2500-3000) whose underlying genetic abnormality is the loss of normal function in one of the two *NF1* alleles[1-3]. In ~50% of cases in North America and Europe, NF1 is inherited, while the remaining half represent a spontaneous new mutation[2]. NF1 impacts about 120,000 Americans (2.5 M worldwide) and is therefore classified by the U.S. Food and Drug Administration (FDA) as an orphan disease.

Typical management comprises careful symptom monitoring, ideally in a specialized center. In some situations, targeted treatment of certain manifestations may be required, including surgical removal of tumors, spine instrumentation for scoliosis, carboplatin/vincristine for optic pathway gliomas (OPGs), MEK inhibitors (MEKi) for a subset of pediatric plexiform neurofibromas (pNFs), and/or stimulant prescriptions for attention deficit hyperactivity disorder (ADHD); however, none of these symptomatic therapies treat the underlying driver of NF1. Given the broad range of NF1 manifestations with significant morbidity, including cognitive and social dysfunction (CD/SD), cutaneous neurofibromas (cNFs) and pNFs, and bone dysplasia, most NF1 individuals experience a decreased quality of life. NF1 individuals also have an increased risk of developing chronic pain, depression and life-threatening complications such as cardiovascular disease and malignancies. For example, the lifetime risk of breast cancer for NF1 individuals is three times that of the general population, and amongst NF1 females under 40 years of age, the risk is eleven times that of age-matched non-NF1 women[4]. Consequently, there is great urgency to develop systemic NF1 treatments that can prevent or significantly delay progression across a range of NF1-related manifestations.

For NF1 individuals (excluding NF1-mosaicism), every cell initially has one normal and one abnormal *NF1* allele ($NF1^{+/-}$ cells). The former encodes WT neurofibromin, while the latter does not. This results in insufficient WT neurofibromin to maintain normal cell function, termed haploinsufficiency. The abnormal cell function resulting from haploinsufficiency is critical in NF1 pathogenesis, with evidence supporting that it causes certain NF1 manifestations and permits or accelerates others[5-17]. Some NF1-associated manifestations arise in cells that lack a normal *NF1* allele ($NF1^{-/-}$ cells), and NF1-HCT will not restore WT neurofibromin levels in these cells. However, because of the close interplay between $NF1^{-/-}$ and $NF1^{+/-}$ cells, restoration of normal function in $NF1^{+/-}$ cells by NF1-HCT is predicted to have a beneficial effect on $NF1^{-/-}$ cells and, in turn, prevent or delay the onset of manifestations that arise in $NF1^{-/-}$ cells. Consequently, NF1-HCT represents a potential NF1 treatment. By normalizing the level of WT neurofibromin in all *NF1*-haploinsufficient cells, NF1-HCT is expected to normalize cell function and consequently reverse, prevent or delay the development of NF1 manifestations [Figure 1]. This review describes in detail the rationale and specific considerations for NF1-HCT, with an emphasis on the potential benefits of a small molecule NF1-HCT approach, and how this compares to (i) currently available NF1 treatments and (ii) *NF1* gene-based therapeutic strategies (e.g., *NF1* gene replacement, and *NF1* gene editing), which represent another potential, albeit more challenging, approach to correct levels of WT neurofibromin.

CONSIDERATIONS OF THE NF1-HCT APPROACH

NF1 clinical phenotype

Successful treatment of NF1 requires understanding its clinical manifestations. In particular, any potential NF1 therapeutic strategy must consider: (i) NF1 has a broad range of manifestations; and (ii) manifestations can vary markedly across NF1 individuals.

While the clinical hallmarks of NF1, namely café au lait macules (CALMs), Lisch nodules (iris hamartoma), and cNFs, occur in almost all NF1 individuals, additional manifestations occur in a significant percentage, including pNFs (35%-50%), malignant peripheral nerve sheath tumors (MPNSTs: 8%-13%), CD/SD (up to 80% of children), bone abnormalities such as scoliosis (true prevalence is unknown - most studies quote figures in the range of 10%-36%), OPGs (15%-20% of pediatric patients), cardiovascular abnormalities, hypertension, and various other malignancies that contribute to a lifetime risk of non-NF1 cancers that is ~2 times the general population[4,18-23]. Disease severity and associated morbidity vary dramatically between NF1 individuals, wherein some may have a few cNFs and CALMs, while others may have thousands of cNFs, large pNFs impinging on normal structures, prominent CD/SD, chronic and severe pain/itchiness, and marked skeletal abnormalities and disfigurement[24-27]. Among family members harboring the same *NF1* mutation, concordance for certain manifestations (e.g., pigmentary changes, numbers of cNFs, and CDs) is high amongst monozygotic twins but decreases with increasing familial separation, indicating that for these manifestations, the germline mutation plays a primary role but that other factors, such as genes unlinked to the *NF1* locus, are important modifiers of the phenotype[28]. Other manifestations show discordance even amongst monozygotic twins (e.g., pNFs and malignancies), supporting the influence of non-heritable factors such as stochastic somatic mutation/deletion of the WT *NF1* allele or environmental factors[29].

Mosaicism in NF1

It is notable that there are no differences in clinical presentation between familial and sporadic cases of NF1. In contrast, those individuals who present with localized manifestations, mosaic NF1, display an attenuated natural history. Mosaic NF1 has been calculated to represent approximately 5% of NF1 cases; however, the true prevalence is unknown[30-32]. Mosaic NF1 arises when the initial *NF1* mutation occurs in the postzygotic state, resulting in a mixture of $NF1^{+/-}$ (low WT neurofibromin level), and $NF1^{+/+}$ (normal WT neurofibromin level) cells. In NF1 mosaics, NF1-HCT would require careful dosing and patient monitoring to correct deficits in $NF1^{+/-}$ cells without adversely affecting $NF1^{+/+}$ cells (i.e., if excess WT neurofibromin were to be detrimental to the cell). If the therapeutic window is too narrow, this milder NF1 category may not be treatable with NF1-HCT.

Normal expression and function of the *NF1* gene product neurofibromin

Crucial to the development of a system-wide NF1 therapy is an understanding of neurofibromin's functions within different cell types and how dysfunction results in various manifestations. The *NF1* gene, located at the 17q11.2 locus, is one of the largest human genes (~280 kb)[30,33,34]. *NF1* contains three embedded genes (*OMGP*, *EVI2B*, *EVI2A*), and fourteen adjacent genes, and all seventeen are co-deleted in the most common form of *NF1* microdeletion (see later section: *NF1* microdeletions)[35]. *NF1* contains 57 constitutively expressed exons and four alternatively included exons: 9a[36,37]; 10a-2[38]; 23a[39,40]; and 48a[41].

NF1 encodes neurofibromin, which is a GTPase activating protein (GAP) that negatively regulates Ras. Neurofibromin contains the following domains (starting at the N-terminus): cysteine-serine-rich domain, tubulin-binding domain, GAP-related domain (GRD), Sec14-like domain, pleckstrin homology domain, and the C-terminal domain. The three-dimensional structure and the RAS-GAP activity of the GRD have been well-characterized[42-45], whereas the exact role of the other domains is not as well understood. Neurofibromin exists as six main isoforms as a result of alternative splicing. The two most abundant isoforms are (i) isoform 1 (nomenclature of UniProt), which contains 2818 amino acids; has a predicted

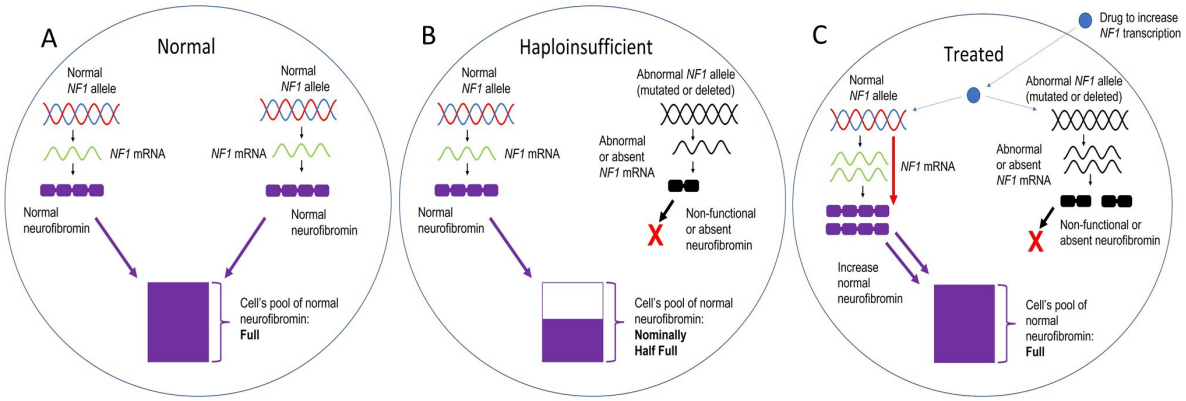

Figure 1. Small molecule therapy to correct *NF1*-haploinsufficiency. In the normal cell (A), two normal *NF1* alleles provide a normal level of WT neurofibromin. By contrast, in the $NF1^{+/-}$ haploinsufficient cell (B), the abnormal *NF1* allele does not contribute any WT neurofibromin, resulting in NF1 protein haploinsufficiency, and a disease state. In the treated $NF1^{+/-}$ cell (C), a drug increases transcription by the normal *NF1* allele (red arrow), resulting in increased WT neurofibromin (purple arrows) and correction of haploinsufficiency. Increasing transcription is just one of several methods to increase neurofibromin expression. NF1: Neurofibromatosis type 1; WT: wild-type.

molecular weight of 327 kDa; and contains no alternatively included exons, and (ii) isoform 2, which contains a 21-amino-acid insertion that is encoded by exon 23a and located within the GRD[46,47]. Notably, the RAS-GAP activity of isoform 1 is 10-fold higher than that of isoform 2[47,48].

Neurofibromin is widely expressed, with varying degrees of expression, and varying isoform ratios, in different tissues and developmental stages[41,49]. From embryonic day 11 onwards, most murine tissues demonstrate high levels of neurofibromin, whereas postnatally neurofibromin levels drop significantly in most terminally differentiated tissues, apart from certain cell types such as Schwann cells, neurons, and adrenal medulla cells[49,50]. In adult rats, isoform 1 is the predominant isoform in CNS neurons, whereas isoform 2 is the predominant form in Schwann cells, adrenal medullary cells and ovary[41]. Neurofibromin is primarily localized in the cytoplasm; however, binding of SPRED1 to the GRD facilitates neurofibromin translocation to the plasma membrane, which is critical as it allows neurofibromin to interact with membrane-bound Ras[51,52].

Neurofibromin exists as an obligate high-affinity, pseudo-symmetric dimer, that is ~620 -kDa, and ~32 nm along the long axis[53]. The dimer exists in closed and open states, with the closed dimer being the predominant form. Dimers in the closed state have both protomers in a self-inhibited, Zn-stabilized state that prevents Ras binding by the GRD. Dimers in the open state have one protomer in a closed conformation and the other in an open conformation that allows Ras binding by the GRD[54].

Neurofibromin plays critical cellular regulatory roles, particularly in neural crest-derived cells, as evidenced by the fact that (i) although $Nf1^{+/-}$ mice do not develop typical NF1 symptoms such as neurofibromas, they have accelerated rates of tumorigenesis (composed of tumors typically seen in older WT mice), and shortened lifespans, compared to $Nf1^{+/+}$ mice, while mice harboring two abnormal *Nf1* alleles die *in utero* by embryonic day 14 due to cardiac abnormalities[55,56], (ii) monoallelic *NF1* abnormalities result in a diverse NF1 phenotype that includes manifestations arising in neural crest-derived cells, and (iii) *NF1* abnormalities are present in various malignancies in non-NF1 individuals, including tumors that arise in neural crest-derived cells (e.g., melanoma) and tumors that arise in non-neural crest-derived cells (e.g., acute myeloid leukemia and various carcinomas)[18].

RAS-dependent functions of neurofibromin

The best characterized neurofibromin function is its critical role as a RAS-GTPase activating protein (RAS-GAP), wherein it negatively regulates RAS by increasing the intrinsic hydrolysis of RAS-bound GTP by a factor of 10^5, resulting in rapid conversion of active RAS-GTP to inactive RAS-GDP[43-45]. RAS is a proto-oncogene and a key regulator of at least eleven intracellular signaling pathways, including those involved in cell differentiation and homeostasis[51,57]. Tight control of RAS activity is essential for normal cellular homeostasis. This is underscored by the fact that oncogenic RAS is present in ~25% of human cancers, where it drives tumor initiation and maintenance[58]. Furthermore, altered RAS function underlies the broad group of developmental disorders known as "RASopathies"[59,60], which have a wide range of phenotypes and include NF1. A variety of NF1 manifestations result from increased RAS signaling secondary to the loss of WT neurofibromin. These include NF1-specific tumors such as pNFs, cNFs, OPGs, and MPNSTs. In addition, RAS plays a neurofibromin-dependent role in cognitive function, as demonstrated in *Nf1*$^{+/-}$ mice displaying spatial learning and attention deficits modeling those seen in NF1 individuals[13,16]. When RAS hyperfunction is normalized in these mice, these deficits are corrected, and deficits in long-term potentiation and GABA-mediated inhibition are reversed[15,16].

Amongst non-NF1 individuals, various sporadic cancers contain *NF1* abnormalities. These include cases confirmed to have increased RAS-GTP without *RAS* mutations, supporting the notion that decreased neurofibromin promotes sporadic tumor development through an upregulated RAS pathway[18,61]. For example, nearly all mammary carcinomas in a breast carcinoma-prone conditional mouse model (CMM) contained *Nf1* gene deletions and increased RAS-GTP, and amongst human breast tumors in The Cancer Genome Atlas, 23% have hemizygous *NF1* deletions and 4% have *NF1* mutations[62]. Sporadic glioblastoma multiforme (GBM) has *NF1* inactivating mutations/deletions in ~23% of cases, while ~14% of cutaneous melanomas have *NF1* mutations[18]. Restoring neurofibromin in neurofibromin-deficient sporadic tumors may represent an effective therapeutic approach, particularly given the efficacy of blocking neurofibromin effectors in these tumors *in vitro* and *in vivo*. For example, MEKi therapy shows efficacy in neurofibromin-deficient sporadic GBM cell lines[63] and *Nf1*-deficient acute myeloid leukemias in cell culture and mouse xenografts[64]. MEKi treatment also restores sensitivity to EGFR inhibitors in neurofibromin-deficient lung cancer cells and mouse xenografts[65]. There are limited reports of MEKi therapy targeting *NF1*-deficient sporadic tumors in clinical practice. In one case report, MEKi (trametinib) administration resulted in a partial response of advanced *NF1*-deficient melanoma[66]. In a separate study assessing the efficacy of genomics-guided treatment in GBM, trametinib was commenced in a patient with progressed *NF1*-deficient GBM, resulting in a time to progression of > 665 days[67]. Two prospective trials are currently investigating MEK-inhibition in sporadic *NF1* mutant tumors (NCT02645149, NCT02465060)[66].

Functions of neurofibromin beyond RAS

Neurofibromin also interacts with and regulates other cellular proteins and pathways in a RAS-independent manner, with dysregulation of these likely underlying some NF1 manifestations. Learning deficits in *Nf1*$^{+/-}$ mice result from enhanced neuronal inhibition, which, at least in part, appears to be caused by RAS-independent mechanisms. For example, neurofibromin interacts with hyperpolarization-activated cyclic nucleotide-gated channel 1 (HCN1), and decreased neurofibromin results in attenuation of the HCN1-mediated incoming channel current (Ih) in GABAergic inhibitory interneurons. The resultant hyperexcitability in these interneurons strongly contributes to this neuronal inhibition in neurofibromin-deficient mice. Stimulating the HCN current with lamotrigine corrects the learning deficits in these mice. The HCN1-neurofibromin interaction may be RAS-independent, given that (i) *RAS* knock-in mice do not have altered Ih; (ii) Ih is not affected by MEKi treatment; and (iii) HCN channels lack ERK consensus sites[68].

Neurofibromin positively regulates cAMP in mice[69] and *Drosophila*[70,71]. In the latter, it occurs through two pathways: (1) a Ras-dependent pathway that is associated with long-term memory formation and (2) a Ras-independent pathway that is essential for associative learning and short-term memory formation[69,72,73]. Neurofibromin regulation of cAMP is also essential for somatic growth, which has been reported as Ras-dependent in some studies[74] and Ras-independent in others[75,76]. For example, mice with conditional *Nf1* knockout in the central nervous system (CNS) (BLBP-Cre; $Nf1^{flox/flox}$) are smaller than controls due to reduced growth hormone secretion secondary to disruption of hypothalamic Ras-independent neurofibromin regulation of cAMP[76]. Importantly, these findings provide insight into the mechanisms underlying the reduced stature present in NF1 individuals (20%-30% of NF1 adults have a height below the 3rd centile)[77,78].

In the OPG CMM ($Nf1^{-/-}$ CNS glial cells; all other cells $Nf1^{+/-}$), mice have decreased striatal dopamine, decreased dopaminergic neuron integrity, and deficits in spatial memory, exploratory behavior, and attention. OPG mice are rescued from CDs using treatments that elevate dopamine but not those inhibiting RAS or increasing cAMP. This suggests a RAS-independent role of neurofibromin in regulating CNS dopamine homeostasis, and alteration of this neurofibromin-dopamine axis may contribute to NF1 CDs[79,80]. Neurofibromin has been proposed to regulate neuronal differentiation by (i) direct complex formation with CRMP-2 (Collapsin response mediator protein-2), thus directly blocking access to kinases (Rho, Cdk5 and GSK-3β) that phosphorylate CRMP-2, and (ii) suppressing RAS activation of the same kinases[81]. CRMP-2 is also a key player in NF1-associated pain in rat models through its regulation of CaV2.2 and NaV1.7 channels[82]. In a GBM invasiveness study, the neurofibromin leucine-rich domain inhibited GBM invasion but failed to hydrolyze RAS-GTP, suggesting that its anti-invasive function is RAS-independent[83]. Neurofibromin can promote sensitivity to apoptosis mediated by RAS-dependent, RAS-independent and cAMP-independent pathways[84]. In conclusion, neurofibromin appears to play a RAS-independent regulatory role in a variety of processes, including cognition, axonal growth, pain perception, dopamine homeostasis, tumor invasiveness, apoptosis, and body growth in some models.

NF1 manifestations where neurofibromin's role is unclear

It is not clear how neurofibromin contributes to certain NF1 manifestations. NF1 individuals tend to have larger heads and 24% have macrocephaly (head circumference > 2 SD above the mean). This is suspected to result from increased brain size, although the exact mechanism is unknown[77]. NF1 individuals often experience motor deficits of unknown cause, including difficulties with coordination, decreased muscle tone, strength, and easy fatigability. Originally these were felt to be due to neurocognitive deficits, yet more recent data suggest a possible primary myopathic process[85,86]. NF1 individuals are prone to headaches of unknown causes. Amongst 50 NF1 children-adolescents, 62% experienced headaches (14% in controls), and 54% experienced migraines (12% in controls)[87]. NF1 individuals have an increased incidence of sleep disturbance of uncertain cause[88,89]. In some studies, NF1 individuals have decreased Vitamin D levels of uncertain cause[90-92]; however, Vitamin D levels are not decreased in other studies[93,94].

Implications for NF1-HCT

Given that neurofibromin regulates many unrelated effectors that are associated with a variety of NF1 manifestations, any therapy targeting just one effector is expected to correct only a small subset of manifestations. For example, drugs targeting specific RAS effectors (e.g., MEK) are unlikely to correct RAS-independent CDs mediated by dopamine, cAMP and HCN1, or manifestations arising from other (non-MAPK) RAS pathways. Similarly, by only targeting RAS-independent effectors, ongoing RAS hyperactivity will continue to promote tumor development. However, an NF1-HCT that normalizes neurofibromin levels in all $NF1^{+/-}$ cells is expected to correct all neurofibromin-regulated pathways and benefit the wide range of

NF1 manifestations - including both RAS-dependent and RAS-independent mechanisms. Separately, NF1-HCT may also benefit non-NF1 cancer patients with neurofibromin-deficient tumors.

Regulation of neurofibromin

Development of an NF1-HCT requires understanding the mechanisms regulating neurofibromin levels, as each may be targeted to increase WT neurofibromin. Regulation of *NF1* expression occurs at multiple levels, including transcriptional control, RNA processing, mRNA transport, miRNA regulation, protein targeting and protein degradation[95,96]. Functional sites in the *NF1* promotor bind transcription regulators such as CRE, SP1, and RUNX1, and mutations in these sites significantly decrease *NF1* transcription and neurofibromin levels[97,98]. A CRISPRa *NF1* transcriptional regulator that increases *NF1* RNA expression in immortalized $NF1^{+/-}$ SCs has also been developed (Infixion Bioscience, unpublished data). Also, miRNAs repress *NF1* mRNA in a variety of cell types *in vivo*, including SCs (miR-27)[99] and neurons (miR-128, miR-103, and miR-107)[96]. Finally, proteasomal neurofibromin degradation is a sensitive regulatory process involving protein kinase C and Cullin 3 E3 ligase. Degradation occurs within 5 minutes following growth factor stimulation, resulting in RAS activation. Subsequently, neurofibromin levels normalize within 30 minutes after growth factor removal[100-102].

NF1 mutational spectrum

NF1 individuals have a wide range of *NF1* gene abnormalities that must be considered in any treatment approach. In NF1, the underlying abnormality is within the *NF1* gene on one of the two alleles. In ~95% of cases, this is an intragenic mutation, while in ~5% of cases, there is a complete deletion of *NF1* (microdeletion) and adjacent genes[103]. The result in both cases is a decrease in WT neurofibromin.

NF1 intragenic mutations

The number of documented unique *NF1* pathogenic variants is extensive. As of 2018, the University of Alabama analyzed 8400 unrelated NF1 individuals using a comprehensive *NF1* mutation analysis, of which more than 2800 different germline pathogenic variants were identified, with only 31 present in ≥ 0.5% of unrelated individuals[104]. Most are small genetic changes including single-base substitutions, insertions or deletions[105], that result in splicing (27%), frameshift (26%), nonsense (21%) and missense (16%) pathogenic variants[103]. Approximately 80% of pathogenic intragenic variants predict truncated neurofibromin from generated frameshifts and premature termination codons (PTCs); however, negligible abnormal protein is expected as PTC-containing mRNA typically undergoes nonsense-mediated decay (NMD)[106] [Figure 2]. The remaining ~20% of intragenic mutations result in full-length neurofibromin with decreased function. In either genetic setting, the result is *NF1*-haploinsufficiency.

While decreased WT neurofibromin underlies the NF1 phenotype (i.e. the phenotype results from the lack of WT neurofibromin), it has been suggested that non-truncating mutations may, in rare cases, result in a dominant-negative effect (i.e. certain manifestations may be directly due to the mutant neurofibromin protein). Supporting the latter are reports of NF1 patient subsets that have (i) specific phenotypes, and (ii) rates of non-truncating *NF1* mutations that are higher than the baseline rate of ~20% in the NF1 population. This suggests that the full-length and presumably more stable mutant protein may result in a dominant-negative effect that potentiates the specific phenotype. For example, one group reported that (i) in their literature review, almost all NF1 individuals with Neurofibromatosis-Noonan Syndrome (NFNS) and pulmonary stenosis (PS) had non-truncating mutations (8/9); and (ii) the majority of an additional cohort of NF1 individuals with PS had non-truncating mutations (8/11). Consequently, the authors suggested that amongst the ~1% of NF1 individuals with PS, some may have a non-truncated mutant neurofibromin with a dominant-negative cardiac effect[107]. NF1 individuals with spinal neurofibromatosis represent another NF1 patient subset with a specific phenotype and an increased incidence of non-truncating mutations (43%).

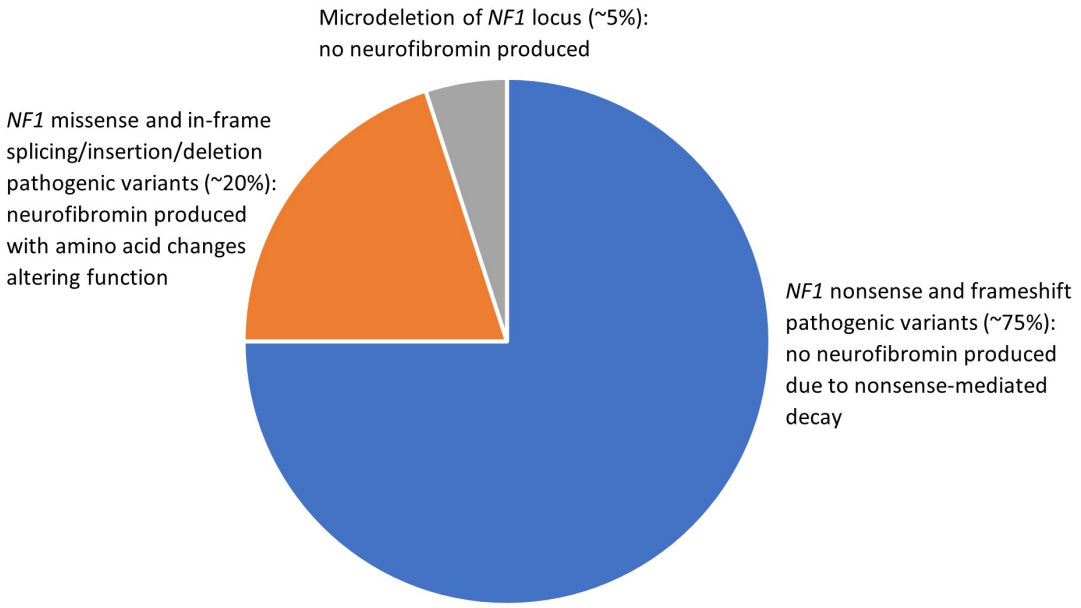

Figure 2. *NF1* abnormalities predict absence or presence of mutant neurofibromin. In NF1-HCT, increased transcription of the abnormal *NF1* allele will result in either (i) no/negligible mutant neurofibromin: *NF1* microdeletion (~5% of cases), and intragenic mutations that result in nonsense-mediated decay (~75% of cases), or (ii) an increase in mutant neurofibromin: intragenic mutations producing full-length neurofibromin (~20% of cases). Note: a small number of *NF1* nonsense and frameshift variants may produce neurofibromin due to escape from nonsense-mediated decay. NF1: Neurofibromatosis type 1; NF1-HCT: NF1 haploinsufficiency correction therapy.

Affected individuals have bilateral neurofibromas involving all spinal roots, but a lower incidence of typical NF1 features: CALMs - 67%; freckling - 18%; cNFs - 31%[108]. The potential benefit of NF1-HCT in these populations will require care and study during any potential future human clinical trials.

NF1 microdeletions

In *NF1* microdeletion cases, there is no neurofibromin produced from the affected allele. Microdeletion cases are separated into four types (type-1, type-2, type-3 and atypical) based on the amount of genetic material deleted along with the *NF1* gene. The most frequent are type-1 (~75% of cases), in which 1.4 Mb of DNA is missing, including *NF1* (~0.35 Mb) and 17 additional genes producing 13 proteins (*CRLF3, ATAD5, TEFM, ADAP2, RNF135, OMG, EVI2B, EVI2A, RAB11FIP4, COPRS, UTP6, SUZ12, LRRC37B*) and four miRNAs (*MIR4733, MIR193A, MIR365B, MIR4725*). Type-1 microdeletions often result in more severe manifestations than intragenic *NF1* mutations[35]. In the remaining 25%, smaller amounts of DNA, and fewer co-deleted genes, are impacted in type-2 and type-3 cases, while atypical-type cases are heterogeneous in terms of size and the number of genes lost, with variable impact on phenotype across these three types. While there is evidence suggesting that co-deleted genes play a key role in disease severity, the exact impact of many co-deleted genes on NF1 manifestations is not well understood[35]. One key co-deleted gene is *SUZ12* which encodes a subunit of the polycomb repressive complex 2. The latter catalyzes histone H3 lysine 27 methylation to mediate the epigenetic silencing of target genes[109].

Implications for NF1-HCT

In all categories of *NF1* germline abnormality (i.e., truncating and non-truncating mutations, and microdeletions), there is decreased WT neurofibromin. Consequently, the NF1-HCT approach of increasing WT neurofibromin from the WT allele is expected to be effective in all cases. However, it is important to consider the effects of upregulating the abnormal allele [Table 1 and Figure 2]. Amongst the

Table 1. Predicted NF1-HCT effect amongst different *NF1* germline abnormalities and mosaic cases

NF1 germline pathogenic variant	Cases (%)	Predicted protein	Level of abnormal neurofibromin		Level of normal neurofibromin		Comment
			Before treatment	After treatment	Before treatment	After treatment	
Frameshift	26	Truncated*	Absent*	Absent*	Insufficient	Adequate	Upregulating alleles that predict truncated proteins are not expected to have a pathogenic effect
Nonsense	21	Truncated	Absent	Absent	Insufficient	Adequate	
Splicing	27	Truncated	Absent*	Absent*	Insufficient	Adequate	
Missense	16	Shorter protein	Present	Increased	Insufficient	Adequate	Increased levels of abnormal shorter or full-length neurofibromin could have untoward effects, but only if the variant causes a pathogenic gain of function. May also have a beneficial effect
		Full-length	Present	Increased	Insufficient	Adequate	
Gene deletion	5	No protein	Absent	Absent	Insufficient	Adequate	Symptoms solely due to co-deleted genes are not expected to be corrected
Mosaicism	See note	Depends on gene abnormality			Insufficient in affected cells. Adequate in normal cells	Adequate in affected cells. Increased in normal cells	The effect of increasing WT neurofibromin in normal cells is not certain

*Virtually all frameshift mutations and most splicing errors result in a PTC and a predicted truncated protein that will not be present due to NMD. *NF1* gene abnormality categories representing < 5% of cases are not represented. Mosaic NF1 has been calculated to represent approximately 5% of NF1 cases; however, the true prevalence is unknown. NF1: Neurofibromatosis type 1; WT: wild-type.

~75% of cases with intragenic mutations predicting a truncated protein, upregulation of the abnormal allele is not expected to have untoward consequences as the mRNA will undergo NMD. Conversely, amongst the ~20% of cases with intragenic non-truncating mutations, NF1-HCT is expected to increase levels of both WT and mutant neurofibromin. If rare mutant proteins have a dominant-negative effect[107], then increasing their levels could have adverse effects. As such, genetic and clinical screening may be required to stratify candidates into optimal NF1-HCT approaches, taking into account potential dominant-negative effects. One potential dominant-negative mechanism represents dimerization of mutant and WT neurofibromin[53,54], with resultant degradation of WT neurofibromin at the faster rate of the mutant[110]. In this scenario, an NF1-HCT approach that decreases neurofibromin degradation may be preferable to one that increases *NF1* transcription. Interestingly, some degree of *benefit* may result from upregulating the mutant *NF1* allele. In particular, when 29 *Nf1* variant cDNAs were transfected into *NF1*$^{-/-}$ HEK293 cells, many corrected RAS activity to at least some degree[111]. Upregulating *NF1* expression in the ~5% of cases caused by microdeletion will not result in any abnormal neurofibromin as the abnormal allele lacks an *NF1* gene. As such, NF1-HCT should correct NF1 manifestations without adverse effects due to a mutant protein. However, as NF1-HCT is not expected to upregulate genes that are co-deleted with *NF1*, those manifestations attributable to co-deleted genes, such as *SUZ12*, are not expected to improve.

NF1 genotype-phenotype correlation

Compounding the challenges associated with developing an NF1 therapy that addresses the wide range of gene abnormalities and manifestations is the absence of a genotype-phenotype correlation in ~90% of cases. Amongst the ~10% of cases where an association has been documented, the majority have been in the ~5% of cases caused by *NF1* microdeletions. In these cases, the phenotype is typically more severe and includes

both new and exaggerated NF1 manifestations such as dysmorphic facial features, hypertelorism, intellectual disability, cardiovascular abnormalities, childhood overgrowth, a greater tumor burden and an increased risk of MPNST[35,112]. It is unknown precisely how much of the microdeletion phenotype is a direct consequence of *NF1*-haploinsufficiency versus that of co-deleted genes. Of the ~95% of NF1 cases caused by intragenic mutations, there are six reported genotype-phenotype correlations, which together represent another 4.8% (383 of 8000 unrelated probands analyzed) of NF1 individuals [Table 2][104,113-117].

The inability to predict phenotype in most (~90%) NF1 individuals makes genetic counseling and tailored clinical management difficult. For example, it is virtually impossible to determine which pre-symptomatic NF1 individuals might benefit from certain targeted therapies. This is further complicated when therapies are largely beneficial when administered pre-symptomatically (e.g., potential therapies to prevent scoliosis) and have possible adverse side effects, thus raising a risk-to-benefit dilemma. By contrast, NF1-HCT is expected to have a preventative or symptom-delaying effect in all NF1 individuals. Consequently, NF1-HCT with a demonstrated safety profile is expected to be indicated in the pre-symptomatic stages of most NF1 individuals, with potential exclusion of certain cases (e.g., mosaicism, and cases with potentially dominant-negative mutant neurofibromin).

Haploinsufficiency and complete loss of function in NF1

Amongst the NF1 manifestations with known molecular pathogenesis, there are two primary mechanisms by which an *NF1* gene abnormality results in the clinical phenotype: (1) complete loss of neurofibromin function (cLOF) and (2) haploinsufficiency. In order to develop a global NF1 therapy, a fundamental understanding of both, including the interaction between them, is required.

Impact of complete loss of function in NF1

cLOF, occurring when both *NF1* alleles are abnormal, is a key initiator of certain NF1 manifestations. *NF1* is a tumor suppressor gene (TSG); however, its effects extend well beyond tumor suppression. As a TSG, it follows the Knudson two-hit hypothesis for tumor development[118]. The "first hit" represents the germline abnormality present in one *NF1* gene allele from conception. While this first hit is insufficient to cause certain NF1 manifestations (e.g., tumors), it results in haploinsufficiency which can directly cause other NF1 manifestations (e.g., cognitive deficits). The "second hit" refers to a pathogenic change in the remaining WT allele, thus resulting in cLOF. When certain cells acquire a second-hit, manifestations such as cNFs and pNFs (cLOF in SCs), CALMs (cLOF in melanocytes), pseudarthroses (cLOF in mesenchymal precursor cells), and gastrointestinal stromal tumors can develop[32]. Importantly, the development of certain cLOF-associated NF1 manifestations in mouse models either requires or is facilitated by a microenvironment of haploinsufficient cells[7,8,17].

Evidence supporting stand-alone effect of haploinsufficiency in NF1

Despite the multitude of different germline *NF1* pathogenic variants, the ultimate result is the same: all cells in NF1 individuals, excluding mosaics, begin with one abnormal *NF1* allele[119]. The other allele produces WT neurofibromin, but this is insufficient to maintain normal cellular function [Figure 1]. This is termed haploinsufficiency, and evidence supports that this alone (i.e., without a 'second hit') can result in certain NF1 manifestations, such as CD/SD, osteopenia, osteoporosis, short stature, macrocephaly, and vasculopathy[5,10,11,32,78,120-122].

CD/SD occurs in most NF1 individuals (65%-80%) and represents a source of morbidity starting in childhood. The high prevalence and generalized nature support that *NF1*-haploinsufficiency alone is the driver. This is further supported by $Nf1^{+/-}$ haploinsufficient mice demonstrating a range of correctable

Table 2. Genotype-phenotype associations in NF1 patients bearing small pathogenic variants

#	Genotype	Mutation type	% of NF1 cases	Total in 8000 unrelated probands	Associated Phenotype (key features listed)	Publication
1	p.Met992del	Single aa loss	0.9	74	Mild. CALMs, SFF. No evNFs	[115,116]
2	p.Arg1809	Missense	1.2	99	Mild. CALMs, Lisch nodules. No evNFs. 25% NLF. 50% CDs/learning disabilities	[117]
3	codons 844-848	Missense	0.8	67	More severe. Major superficial pNFs, OPGs, sNFs, skeletal abnormalities, malignancies	[104]
4	p.Met1149	Missense	0.4	34	Mild. CALMs, SFF, CDs, NLF, low prevalence PS	[114]
5	p.Arg1276	Missense	0.7	57	More severe. sNFs, NLF, hpCCA	[114]
6	p.Lys1423	Missense	0.7	52	More severe. NLF, hpCCA. No evpNFs	[114]

SFF: Skin fold freckling, sNFs: spinal NFs, NLF: Noonan-like features, hpCCA: high prevalence of cardiac and cardiovascular abnormalities, evNFs/evpNFs: externally visible NFs/pNFs.

cognitive and social deficits mirroring those seen in NF1 individuals [Table 3]. The correctability of these deficits in adult mice underscores their labile nature and further supports NF1-HCT as a strategy to prevent *and reverse* NF1-related CD/SD.

Decreased bone mineral density, which underlies osteopenia and osteoporosis, is detected in up to 50% of NF1 individuals at an early age[78]. An increased incidence of fractures has also been reported[78]. The frequent occurrence and generalized (i.e., non-focal) involvement of the skeleton by NF1-related osteopenia and osteoporosis supports the underlying driver being *NF1*-haploinsufficiency[121]. Moreover, $Nf1^{+/-}$ mice have increased numbers of osteoclasts (bone resorptive cells)[5], and $Nf1^{+/-}$ osteoprogenitors form fewer osteoblasts (bone-forming cells)[123]. $Nf1^{+/-}$ mice show a trend towards lower bone formation[123], whereas $Nf1^{+/-}$ mice, and mice with $Nf1^{+/-}$ restricted to myeloid progenitor cells (MPCs), lose twice as much bone mass as WT mice following ovariectomy (a pro-resorptive model of osteoporosis)[5,6] [Table 3]. These findings demonstrate an abnormal phenotype in *Nf1*-haploinsufficient bone remodeling cells, resulting in net increased bone resorption, which correlates with increased bone resorption markers in NF1 individuals[91,124]. $Nf1^{+/-}$ mice also display significantly impaired distal tibial fracture healing, which is improved by coadministration of pro-anabolic and anti-catabolic agents[125,126]. Collectively, these studies lend strong support for NF1-HCT as a strategy to prevent/improve NF1-related skeletal manifestations such as osteopenia, osteoporosis, and impaired fracture healing.

NF1-related vasculopathy, affecting up to 6.4% of NF1 individuals[122], is associated with excess mortality, especially in younger NF1 individuals[127-129]. The most common lesions include aneurysms or stenoses of aortic, renal, carotid and cerebral arteries[128,129]. Several mouse models provide compelling evidence that haploinsufficiency alone causes NF1-related vasculopathy. In $Nf1^{+/-}$ mice; in WT mice transplanted with $Nf1^{+/-}$ bone marrow (BM)[120]; and in mice with $Nf1^{+/-}$ restricted to MPCs[10], carotid artery endothelial damage leads to an exaggerated neointimal proliferation of vascular smooth muscle cells (VSMCs), and a 6-fold increase in luminal stenosis compared to similarly injured WT mice[11]. However, this does not occur in $Nf1^{+/-}$ mice transplanted with WT BM[120]. As such, *Nf1*-haploinsufficiency in BM cells is necessary, and *Nf1*-haploinsufficiency in MPCs is sufficient for exaggerated neointimal proliferation following endothelial injury, whereas correction of BM haploinsufficiency in $Nf1^{+/-}$ mice is preventative. In $Nf1^{+/-}$ mice and mice with $Nf1^{+/-}$ restricted to MPCs, angiotensin II infusion results in excess aortic aneurysm formation, increased vessel wall macrophages, and VSMC proliferation in the vessel wall media[12]. Treatment of $Nf1^{+/-}$ mice (pre- and post-induction) is preventative in both the neointima (Gleevec & rosuvastatin)[10,11] and aneurysm (simvastatin & apocynin) models [Table 3][12]. Collectively, these findings suggest that blood vessel wall *NF1*-haploinsufficient macrophages in NF1 individuals may promote excess VSMC proliferation,

Table 3. *Nf1* mouse models demonstrating manifestations caused directly by *Nf1*-haploinsufficiency

System modeled	Mouse genotype	*Nf1*$^{+/-}$ cells	Induction	Manifestation	Treatment	Publication
Bone mineral density	*Nf1*$^{+/-}$	All	Ovariectomy	Excess bone mass loss	Not performed	[5,6]
	LysMCre;Nf1$^{flox/+}$	MPCs only				
Bone - impaired fracture healing	*Nf1*$^{+/-}$	All	Distal tibial open fracture	Significantly higher proportion with non-union	Combined rhBMP and bisphosphonates decreased non-union rate	[125,126]
Blood vessels - stenosis	*Nf1*$^{+/-}$	All	Endothelial injury	Excess neointimal proliferation and stenosis	Gleevec & rosuvastatin were preventative	[10,11]
	LysMCre;Nf1$^{flox/+}$	MPCs only				
	Nf1$^{+/-}$ with WT BM	All cells except BM		No excess neointimal proliferation	WT BM was preventative	[11]
Blood vessels - aneurysm	*Nf1*$^{+/-}$	All	Angiotensin II infusion	Excess aortic aneurysm formation	Simvastatin & apocynin were preventative	[12]
	LysMCre;Nf1$^{flox/+}$	MPCs only				
Spatial learning	*Nf1*$^{+/-}$	All	None	Spatial learning deficits	Reversed with additional training, Picrotoxin, farnesyl transferase inhibitor & Lovastatin	[13-16]
Attention deficit	*Nf1*$^{+/-}$	All	None	Attention deficits and ADHD-type behavior	Reversed with Lovastatin	[16]
Social learning	*Nf1*$^{+/-}$	All	None	Selective social behavioral deficits	Reversed with Pak1 inhibitor	[133]

In all listed models, there is a single *Nf1* allele mutated in either all cells in the body (*Nf1*$^{+/-}$) or in a subset of cells (e.g. *LysMCre;Nf1*$^{flox/+}$ model has an *Nf1* allele missing in MPCs only, with all other cells being *Nf1*$^{+/+}$). None of the models are designed to have any *Nf1*$^{-/-}$ cells. NF1: Neurofibromatosis type 1.

which, in the intima, results in luminal narrowing and distal ischemia, and in the media, results in aneurysm formation and rupture. The ability to prevent these changes in *Nf1*$^{+/-}$ mice, using small molecules and transplanted WT BM, strongly supports NF1-HCT as a strategy to prevent NF1-related vasculopathy.

While all *NF1*$^{+/-}$ cells have reduced WT neurofibromin, the degree of reduction can be highly variable across NF1 individuals. For example, analysis of neurofibromin levels in fibroblasts from 11 NF1 individuals with different germline *NF1* mutations identified two distinct groups: one with < 25% of reductions in neurofibromin and the other with > 70%[130]. The findings were suspected to reflect *NF1* allelic imbalance[131,132]. All cases had high RAS activity, indicating that even small decreases in neurofibromin can significantly dysregulate RAS. However, dopamine levels in neural progenitor cells derived from these fibroblasts were variably decreased and linearly correlated with neurofibromin levels[130]. The findings suggest that near-complete restoration of neurofibromin may be required to correct RAS-dependent manifestations, while small increases in neurofibromin may improve some RAS-independent manifestations (e.g. CDs secondary to lowered dopamine).

In summary, the high prevalence, generalized involvement, and mouse study findings support that *NF1*-haploinsufficiency alone is responsible for a significant portion of NF1-related morbidity (CD/SD, osteopenia and osteoporosis), mortality (vasculopathy) and altered growth (short stature and macrocephaly). Moreover, in mouse models, many of these manifestations are indeed preventable or reversible [Table 3]. The findings support NF1-HCT as a strategy to prevent/reverse many NF1-related manifestations.

Haploinsufficient background cells facilitate a cLOF phenotype in NF1

Haploinsufficiency ($NF1^{+/-}$) and cLOF ($NF1^{-/-}$) are interrelated in NF1, with both required for the development of certain manifestations. In the *Krox20-cre* CMM, $Nf1^{-/-}$ SCs only develop into pNFs if they are within a haploinsufficient microenvironment[7], of which $Nf1^{+/-}$ mast cells are a key component[134]. In the same CMM, correction of the haploinsufficient background by transplantation of WT BM prevents pNF development[135]. In the *GFAP-cre* CMM, where astrocytes have cLOF, OPGs develop in all mice with a haploinsufficient background, but in no mice with a WT background[136]. In the *Prss56-cre*, *Plp-cre*, and *Hoxb7-cre* CMMs, mice with $Nf1^{-/-}$ SCs develop neurofibromas in both haploinsufficient and WT backgrounds; however, symptoms develop sooner and/or survival times are shorter in mice with haploinsufficient backgrounds [Table 4][8,9,137]. The variable reliance on a haploinsufficient background seen in these neural crest (NC)-SC axis *Nf1* CMMs likely stems from the fact that they knockout *Nf1* within different NC-derived cell populations[137]. Nonetheless, all CMMs show that a haploinsufficient background promotes tumor formation by $Nf1^{-/-}$ SCs. Interestingly, when *Plp-cre* CMM mice with pNFs were allowed to age, MPNSTs developed in 10% of mice with an $Nf1^{+/+}$ (normal) microenvironment but in none of the mice with an $Nf1^{+/-}$ (haploinsufficient) microenvironment. The authors proposed a model wherein some *NF1* haploinsufficient immune cells in the tumor microenvironment (i.e., $NF1^{+/-}$ mast cells, macrophages) promote pNF development, whereas others (i.e., $NF1^{+/-}$ T cells) delay pNF progression to MPNST[138].

Implications for NF1-HCT

The essential role of *NF1*-haploinsufficiency in the disease phenotype, whether acting singlehandedly or in concert with cLOF, makes NF1-HCT a promising strategy, with the potential to prevent, reverse or delay virtually all NF1 manifestations. Moreover, as the relative number of $NF1^{-/-}$ cells is expected to be much less in NF1 individuals (accumulate throughout a lifetime) than in the CMMs outlined here (all produced during embryogenesis), haploinsufficiency correction may show a greater protective effect in NF1 individuals than in CMMs. Also, because baseline neurofibromin levels are highly variable across NF1 individuals, clinical monitoring with a pharmacodynamic biomarker may be valuable to guide dosing to achieve optimal neurofibromin levels. Given that one small study showed increased pNF progression to MPNST in aged mice with WT backgrounds, NF1-HCT animal studies and clinical trials will need to further study if NF1-HCT risks inducing malignant transformation of NF1-related tumors.

Increased *NF1* gene copy phenotype

While NF1 is caused by a loss of function in *NF1*, there are 29 reported individuals possessing three *NF1* alleles due to microduplication of *NF1* and additional genes within and surrounding the *NF1* locus[35]. Developmental delay and intellectual disability are frequently present in affected individuals, whereas NF1-specific manifestations are typically absent. This raises the possibility of an adverse effect of excess WT neurofibromin; however, given that elevated neurofibromin levels have not been confirmed and other genes are also duplicated, the role each element plays in this rare phenotype is unknown[35,139-141]. In the absence of regulatory feedback loops, an extra *NF1* allele would be expected to decrease RAS-GTP levels, the exact significance of which is difficult to predict. However, mouse models with germline *Ras* deletions (in contrast to oncogenic mutations) and normal phenotypes may offer some insight. In particular, mice with the following *Ras* isoform deletions are viable and develop normally without phenotypic manifestations: $N\text{-}ras^{+/-}$, $N\text{-}ras^{-/-}$[142], $H\text{-}ras^{+/-}$, $H\text{-}ras^{-/-}$[143], and $K\text{-}ras^{+/-}$[144]. Mice with certain combinations of *Ras* isoform deletions are also viable: $N\text{-}ras^{-/-}/H\text{-}ras^{-/-}$ and $N\text{-}ras^{+/-}/K\text{-}ras^{+/-}$[145]. The findings imply significant functional overlap amongst RAS isoforms, suggesting that decreased RAS-GTP secondary to excess neurofibromin may be functionally tolerated to some degree. However, given the potential for adverse effects secondary to neurofibromin overcorrection, the incorporation of a biomarker to guide NF1-HCT dosing should prove beneficial, if not essential.

Table 4. Beneficial effect of WT background in Nf1 CMMs

Cre-linked gene promoter	*Nf1*$^{-/-}$cell	Tumor type	Results based on *Nf1* in background cells		Beneficial effect of WT (+/+) background	Publication
			+/-	+/+		
GFAP	Astrocyte	OPG	Tumors in all mice	No tumors	Prevented tumors	[17]
Krox20	NC-SC axis	pNF	Tumors in all mice	No tumors	Prevented tumors	[7]
Plp	NC-SC axis	pNF	50% survival (months)		Increased median survival by 6 months (1.8-fold increase)	[8]
			8	14		
Hoxb7	NC-SC axis	pNF & cNF	50% survival (months)		Increased median survival by 11.5 months (1.7-fold increase)	[9]
			17.5	29		
Prss56	Boundary cap cells	pNF & cNF	Symptom onset (months)		Increased mean time of symptom onset by 3 months (1.3-fold increase)	[137]
			10	13		

Homozygous *Nf1* knockout occurs only in cells expressing the Cre-linked gene and their progeny. In each CMM, mice were stratified into groups with either a haploinsufficient (*Nf1*$^{+/-}$) or WT (*Nf1*$^{+/+}$) background. NC-SC axis: Neural crest (NC)-SC axis. NF1: Neurofibromatosis type 1; CMM: conditional mouse model.

HAPLOINSUFFICIENCY IN OTHER DISEASES

Haploinsufficiency is not unique to NF1, with over 660 genes known to cause a broad range of human diseases due to haploinsufficiency[146,147]. These include cancer and tumorigenesis (e.g. familial adenomatous polyposis), mental retardation (e.g. Deletion 22q11.2 syndrome), neurological disorders (e.g. epilepsy in tuberous sclerosis complex and Dravet syndrome), growth retardation, and immunodeficiency[147]. Haploinsufficiency is also an important contributor to certain sporadic cancers[148].

Two haploinsufficiency syndromes demonstrate similarities with NF1, including the potential for HCT. Glucose transporter-1 deficiency syndrome (Glut1DS), caused by monoallelic *SLC2A1* gene mutations, results in decreased Glut1 protein levels and impaired brain glucose transport. Patients present with infantile drug-resistant seizures and developmental delay. Early intervention with a ketogenic diet, which provides a non-glucose source of brain fuel, reduces disease severity; however, some symptoms persist and long-term treatment is challenging[149,150]. Next, monoallelic *SCN1A* mutations result in decreased voltage-gated sodium channel 1.1 (NaV1.1) levels. Individuals present with epilepsy syndromes ranging from mild to severe (e.g. Dravet syndrome)[151]. Similar to *NF1*, *SLC2A1* and *SCN1A* undergo multiple distinct pathogenic mutations, and affected individuals display a wide range of severity. Importantly, HCT improves the phenotype in *Slc2a1*$^{+/-}$ and *Scn1a*$^{+/-}$ CMMs (see next section).

HAPLOINSUFFICIENCY CORRECTION AS A TREATMENT APPROACH

Evidence supporting HCT has been reported in NF1 and other haploinsufficiency disease models, as illustrated in the following examples: (1) Introducing WT neurofibromin into *NF1*$^{+/-}$ fibroblasts restores function and normalizes pERK without signs of toxicity when neurofibromin levels far exceed normal[119], (2) *PAX6*-related aniridia is a haploinsufficient panocular condition with substantial visual impairment. In a representative CMM (*Pax6*$^{Sey\text{-}Neu/+}$), small molecule administration (MEKi) increased *PAK6* expression, corrected *PAK6*-haploinsufficiency, and significantly improved the phenotype, including increased retinal function and vision[152], (3) In a Glut1DS CMM (*Slc2a1*$^{+/-}$), HCT (systemic AAV9-associated *Glut1* cDNA) given pre-symptomatically raised cerebral Glut1 and CSF glucose levels, decreased seizure activity, and improved motor performance. Similarly-treated adult mice showed no benefit, indicating the importance of early intervention[153]. A small molecule approach to Glut1DS-HCT has also been suggested[150], (4) In Dravet syndrome, a sodium channelopathy caused in most cases by *SCN1A*-haploinsufficiency, patients present in

infancy with therapy-resistant severe epilepsy[151,154]. Delivery of a dCas9-based system that increases *Scn1a* transcription resulted in increased NaV1.1 channel protein in *Scn1a*$^{+/-}$ (but not *Scn1a*$^{+/+}$) neurons and rescue of membrane excitability and action potential firing. AAV9-associated intraventricular delivery of this system in *Scn1a*$^{+/-}$ mice attenuated hyperthermia-induced seizures[154], (5) CRISPR activation of the normal allele of *Sim1*- and *Mc4r*-haploinsufficient neurons in mice corrected haploinsufficiency and the obesity phenotype[146], (6) Angelman Syndrome is a severe developmental disorder caused by an abnormal maternally-derived *UBE3A* gene. Using a high-content screen, a small molecule (Topotecan) was identified that could 'unsilence' the paternally-derived *Ube3a* gene (purportedly by reducing transcription of a regulatory antisense RNA), resulting in increased paternally-derived *Ube3a* transcription and restoration of UBE3A brain levels in mice[155], and (7) A separate high-throughput screen identified diverse small molecule classes, including epigenetic agents, that upregulate transcription of *PARK2*, which is linked to haploinsufficient familial forms of Parkinson's disease[156].

NF1-HCT USING SMALL MOLECULES

Small-molecule drugs are organic compounds with low molecular weight (most are < 500 Daltons)[157,158] that are capable of modulating biochemical processes to treat or prevent diseases[159,160]. Small molecule drugs represent approximately 80%-90% of the marketed therapeutics[161] and include common therapeutics such as aspirin (180 Daltons). Small molecule drugs are distinct from the category of biological products that include vaccines, blood products, tissues, cells, gene therapies (e.g., gene replacement, CRISPR gene editing), and recombinant proteins (enzymes and antibodies)[161].

The benefits of small molecules in drug discovery include their well-defined structures, relative ease of manufacturing, oral administration, mostly non-immunogenic profiles, and potential to cross the blood-brain barrier. Candidate small molecules may be discovered through screening compound libraries, including repurposing drug libraries where drug toxicity and safety are already established, allowing for expedited clinical trial evaluation[161] and an easier regulatory approval path.

The known NF1 pathobiology allows for target-based drug discovery, wherein small molecules may be screened based on their ability to raise neurofibromin levels via different mechanisms (e.g., increased *NF1* transcription, decreased miRNA repression of *NF1* mRNA, and decreased neurofibromin degradation by the proteasome). This approach enables lower development cost, greater ease of structure-activity relationship development, and a faster pathway to clinical trials compared to phenotypic-based approaches[162].

Small molecules have previously been utilized to treat genetic diseases. Cystic fibrosis (CF) is a lethal inherited genetic disease caused by mutations in the gene encoding the CF transmembrane conductance regulator (CFTR) protein. Using combinations of small molecules that decrease abnormal CFTR degradation and increase its effectiveness can confer significant clinical benefits for CF patients[163]. The integrated stress response is activated in the brains of Down syndrome patients and mice, resulting in translation reprogramming. Small molecule inhibition of a branch of the integrated stress response reverses the translation changes, and rescues deficits in long-term memory and synaptic plasticity in Down syndrome mice[164].

Small molecules that alter gene expression indirectly are widely utilized to treat disease. For instance, 10% of FDA-approved cancer drugs target nuclear hormone receptors, which function as transcription factors. For example, prostate cancer, which is hormone-driven and mediated by androgen receptors, responds to various small molecules that downregulate androgen receptor signaling and in turn alter target gene

transcription[165]. The anti-rejection drug Tacrolimus causes pronounced immunosuppression by indirectly decreasing *IL2* transcription in T-cells. This occurs via binding to immunophilin, which results in calcineurin inhibition and subsequent inactivation of the *IL2* transcription factor[166].

TIMING OF NF1-HCT

The primary aim of NF1-HCT is to prevent and delay the onset/progression of NF1 manifestations by normalizing neurofibromin levels, ideally pre-symptomatically, and continuing this protective therapy throughout the NF1 person's life. To determine the optimum age for NF1-HCT commencement, two main factors must be considered: (i) the temporal and spatial distribution of neurofibromin, and (ii) the onset period of various NF1 manifestations. The importance of establishing the temporal and spatial requirements of HCT, and replenishing protein at an early age, has been noted in other haploinsufficiency disorders[150].

Widespread neurofibromin expression begins during embryogenesis

Whole-body mouse *Nf1* mRNA levels are low-undetectable on embryonic days E8-E10 (levels not measured prior to E8) and rise five-fold on E11 to a level that is maintained until birth (E11-E16)[167,168]. Neurofibromin is essentially expressed by all tissues from E11-E16, with fluctuations in some tissues[49,167]. Neurofibromin drops to low-undetectable levels in most tissues upon reaching terminal development, except for the nervous system and adrenal medulla, where it remains enriched[169].

Timeline of NF1 manifestation presentation

NF1 manifestations can be broadly grouped into three age ranges during which they present[1,23,170], although it is not clear if and when somatic inactivation of the normal allele occurs with respect to key manifestations associated with neural crest cells:

1. Gestation-adolescence: pNFs (believed to be congenital), CALMs, juvenile myelomonocytic leukemia (JMML), OPG (most develop before age 6), and skeletal abnormalities (includes congenital lesions such as sphenoid wing and/or long bone dysplasia).

2. Early childhood-adulthood: speech, language and learning issues, and CD/SD.

3. Late childhood-adulthood: cNFs (become detectable in teenage years and increase in number/size with age), MPNST (typically after age 30 but can occur in teenage years), cardiovascular disease, and non-NF1 cancers.

Summary of timing of NF1-HCT

Given that some NF1 manifestations are congenital and widespread neurofibromin expression begins in mice on E11, NF1-HCT would ideally commence at the corresponding period in human gestation (week 4-5)[171]. If intrauterine diagnosis and drug delivery are not possible, then NF1-HCT should start promptly following diagnosis, as significant manifestations begin early in life. Nonetheless, starting NF1-HCT at later ages is still expected to be beneficial in delaying the progression of established manifestations and preventing those that typically occur later. Moreover, NF1-HCT may ameliorate/reverse established manifestations that have an element of plasticity (e.g. CDs) [Figure 3].

SMALL MOLECULE NF1-HCT VERSUS OTHER NF1 TREATMENTS

While the potential exists for *NF1* gene replacement therapy by cDNA delivery, this currently has significant challenges. Traditional gene replacement therapy would require full-length *NF1* cDNA (8.5 kb) delivery; however, this far exceeds the maximum size deliverable by standard adeno-associated virus (AAV) vector

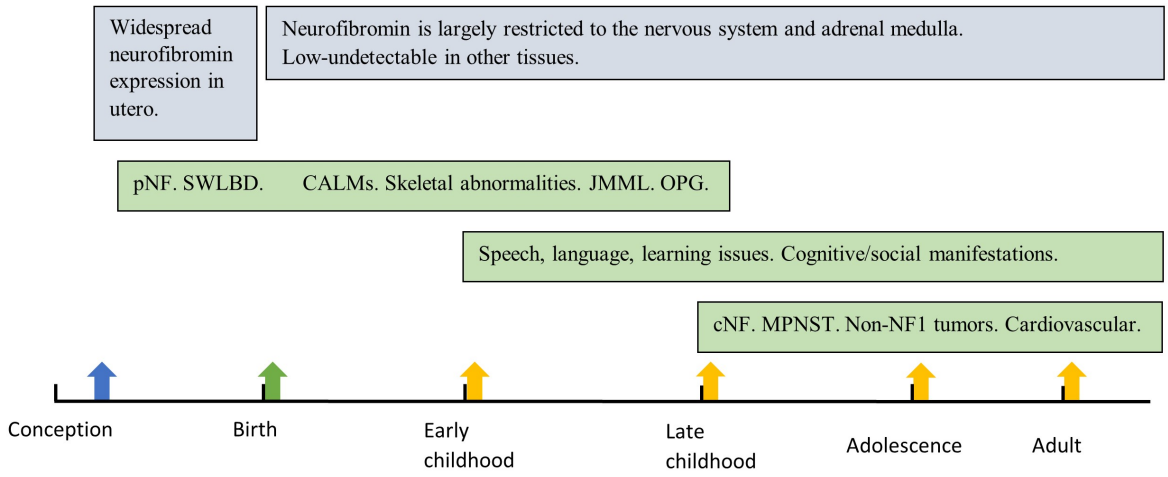

Figure 3. Timeline of neurofibromin expression, NF1 manifestations, and timing of NF1-HCT. Blue boxes: neurofibromin expression. Green boxes: three NF1-manifestation groups based on the age of occurrence. NF1-HCT would ideally commence at weeks 4-5 of gestation when neurofibromin is widely expressed at high levels (blue arrow). Otherwise, NF1-HCT should begin as early as possible after birth (green arrow). Even if started later in life (yellow arrows), a benefit is expected given the lifelong increase in NF1 manifestations and non-NF1 tumors, and the potential to reverse established manifestations that have an element of plasticity. SWLBD: Sphenoid wing and/or long bone dysplasia. NF1: Neurofibromatosis type 1; NF1-HCT: NF1 haploinsufficiency correction therapy.

approaches (4.7 kb)[161]. Alternative large gene delivery approaches have been explored (e.g., oversized gene, and multiple vector systems); however, these have limitations and require careful design and consideration of the target tissues[172,173]. Another potential approach is to deliver only the GRD portion of the *NF1* cDNA (~1.1 kb)[174]; however, non-GRD neurofibromin functions are not expected to be restored. The varied distribution and function of neurofibromin isoforms pose another challenge[175], as this complexity will be difficult to replicate with a single cDNA. In the case of gene editing strategies, a personalized approach would be required to specifically edit each of the thousands of unique *NF1* mutations[32]. Also, system-wide delivery of any gene therapy to multiple organs, including the brain, would be challenging. Finally, stopping gene therapy would be difficult in the event of adverse effects. By contrast, a small molecule NF1-HCT would (i) be easy to administer, (ii) have systemic distribution, (iii) cross the blood-brain barrier, (iv) utilize the normal *NF1* allele, thus ensuring correct neurofibromin isoform ratios and full function, and (v) can be quickly stopped in the event of adverse effects.

NF1 therapies blocking RAS signaling pathways, such as the MEKi Selumetinib, have shown partial shrinkage of NF1 plexiform tumors[176]. However, as neurofibromin's regulatory role is not limited to one signaling pathway, any therapy targeting a specific downstream effector will likely result in only partial NF1-phenotype resolution, as opposed to therapies that restore neurofibromin levels and thus correct all dysregulated pathways. This necessity for a system-wide therapy to correct haploinsufficiency in NF1 and in neurofibromin-deficient tumor microenvironments has been echoed by other authors[18,119].

Despite the limitations associated with drugs targeting single downstream effectors, these solutions do hold the potential for treating at least a subset of manifestations. As such, a tailored multitherapeutic approach that includes these approaches and NF1-HCT may be beneficial. One scenario includes lifelong NF1-HCT to correct haploinsufficiency in all $NF1^{+/-}$ cells, with supplemental therapies targeting specific manifestations driven by $NF1^{-/-}$ cells (e.g., pNF), as required. Such an approach is expected to provide a lifelong baseline protective state by correcting haploinsufficiency; a broader range of effectiveness due to targeted treatment of specific manifestations that may not be as amenable to NF1-HCT alone; and fewer side effects due to the

potential use of smaller drug doses.

ADVANTAGES OF SMALL MOLECULE NF1-HCT

The potential advantages of a small-molecule NF1-HCT approach have been described in detail previously in this review and are summarized here.

1. Treats the underlying cause of NF1 - decreased neurofibromin - using the body's innate ability to make this protein.

2. Prevention, delay and/or reversion of at least a subset, and potentially the majority, of NF1 manifestations.

3. Affects manifestations caused by haploinsufficiency alone, and those initiated by cLOF that require a haploinsufficient background.

4. Benefit not restricted to a single pathway.

5. Unaffected by lack of genotype-phenotype correlations.

6. Benefit seen regardless of the *NF1* gene abnormality, avoiding the complexity of thousands of unique *NF1* mutations.

7. Small molecule drugs have an established drug development path compared to traditional gene therapies.

8. Avoids challenges of gene editing/replacement therapy.

9. Dosage can be tailored using a biomarker, and therapy can be halted in the event of adverse effects.

10. Proof of concept for NF1-HCT shown in NF1 and other haploinsufficient genetic disorders.

11. Possible extension to non-NF1 individuals with *NF1*-haploinsufficient tumors.

12. May provide a drug development platform for 660+ haploinsufficient disorders.

CHALLENGES FOR NF1-HCT

Identifying challenges associated with NF1-HCT is important to optimize and adapt research efforts, as well as prepare for clinical trial approaches. These challenges, along with strategies to overcome them, are listed here.

Establishing a safe and effective level of cellular neurofibromin

As with any novel therapy, a primary challenge is to establish a safe therapeutic window resulting in a beneficial effect while avoiding under- and over-treatment. To achieve this, the use of a pharmacodynamic biomarker will be helpful, and monitoring the clinical response will also be important to assess the impact on phenotype. Once an appropriate dosage is determined for an NF1 individual, this will ideally be consistent and require infrequent adjustment.

Possible reversion of *NF1*-haploinsufficiency potentially protective features

NF1-haploinsufficiency may be associated with protection from certain processes as follows:

Diabetes: NF1 individuals have a reduced rate of type 2 diabetes (HR: 0.27) and a statistically non-significant reduction in the rate of type 1 diabetes (HR: 0.58)[177]. As NF1-HCT may remove this protective effect, fasting glucose and HbA1c should be monitored to determine if NF1-HCT increases glucose levels.

Progression to MPNST: Given the proposed model wherein the $NF1^{+/-}$ microenvironment may have a *protective* effect in delaying malignant transformation of pNFs[138], careful evaluation, first in animal models and then in clinical trials, will be required to determine if NF1-HCT potentiates malignant transformation in benign tumors.

Drug delivery to a wide variety of NF1-relevant cell types (e.g., SCs, myeloid cells, neurons)

Resolution is similar to comparable small molecule drug treatment development efforts. This will require an understanding of the *NF1* regulatory process differences across key cell types and validation of any potential therapy accordingly.

Possible incomplete resolution in microdeletion cases

Manifestations arising from codeleted genes are not expected to be corrected by NF1-HCT. However, NF1-HCT is still expected to correct the typical NF1 manifestations in this group. Careful follow-up of this subgroup will be instructive to determine the extent of benefit from NF1-HCT.

Adverse effects from upregulation of the abnormal *NF1* allele

In cases of missense variants with dominant-negative effects, upregulation of the mutant allele could theoretically worsen clinical manifestations. Therefore, genetic and clinical screening will be required and clinical trials should exclude cases with potential dominant-negative variants. Additionally, careful monitoring of all NF1 individuals receiving therapy will be required to assess for untoward effects.

Off-target effects

Any potential small molecule NF1-HCT may have off-target effects that will need to be evaluated. This is not unique to NF1-HCT, but is applicable to any new small molecule therapy. Given that the desired neurofibromin increase is minimal (nominal 2X increase) and the required drug dose is therefore not expected to be substantial to achieve benefit, this alone may minimize significant off-target effects. The following strategies can also be employed to minimize off-target effects: (i) medicinal chemistry to improve drug specificity; (ii) drug combinations to reduce dosage; and (iii) preference for repurposed drugs with limited off-target effects.

NF1 mosaicism

In NF1 mosaics, careful drug dosage will be required to obtain a beneficial neurofibromin increase in affected cells while not reaching potentially harmful neurofibromin levels in unaffected cells. This may be challenging and may limit the use of NF1-HCT in this group.

CONCLUSION

NF1 has a broad range of manifestations and severity, resulting in decreased quality of life and increased mortality in most patients. Despite the need for a system-wide curative NF1 therapy, none is currently available. Haploinsufficiency is integral to NF1 manifestation development, and HCT has been shown to be potentially useful in NF1 and other haploinsufficient genetic disorders. Consequently, it is predicted that systemic small molecule NF1-HCT has the potential to prevent, delay and/or alleviate a range of NF1

manifestations. In addition, NF1-HCT potentially may be extended to non-NF1 patients with tumors harboring decreased neurofibromin levels, and to demonstrate a drug development platform model for identifying small molecules to treat other haploinsufficient conditions.

DECLARATIONS

Acknowledgments

We thank Dr. Robert Kesterson PhD (UAB) and Dr. Kaleb Yohay MD PhD (NYU) for their careful review of the manuscript.

Authors' contributions

Concept and design of manuscript: Sarnoff H, Croston GE, Frost M
Wrote the manuscript: Frost M
Substantial contribution to the manuscript: Serra E, Viskochil D, Korf BR, Croston GE, Mattson-Hoss MK, Sarnoff H

Availability of data and materials

Not applicable.

Financial support and sponsorship

None.

Conflicts of interest

Sarnoff H, Frost M, Croston GE, and Mattson-Hoss MK are core members of Infixion Bioscience Inc.
Viskochil D and Korf BR are scientific advisors/consultants for Infixion Bioscience Inc.
Serra E is a scientific advisor to and wet-lab collaborator with Infixion Bioscience Inc.
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Korf BR is a medical advisor to SpringWorks Therapeutics and GenomeMedical.

Ethical approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

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