Analysis of patient-specific NF1 variants leads to functional insights for Ras signaling that can impact personalized medicine

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Abstract

We have created a panel of twenty-nine NF1 variant cDNAs representing benign missense (MS) variants, pathogenic MS variants, many with clinically relevant phenotypes, in-frame deletions, splice variants, and nonsense (NS) variants. We have determined the functional consequences of the variants, assessing their ability to produce mature neurofibromin and restore Ras signaling activity in NF1 null (-/-) cells. cDNAs demonstrate variant-specific differences in neurofibromin protein levels, suggesting that some variants lead to protein instability or enhanced degradation. When expressed at high levels, some variant proteins are still able to repress Ras activity, indicating that the NF1 phenotype may be due to protein instability. In contrast, other variant proteins are incapable of repressing Ras activity, indicating that some do not functionally engage Ras and stimulate GTP-ase activity. We observed that stability and Ras activity can be mutually exclusive. These assays allow us to categorize variants by functional effects, may help to classify variants of unknown significance, and may have future implications for more directed therapeutics.

INTRODUCTION

Neurofibromatosis type 1 (NF1) is one of the most common autosomal dominant neurological disorders and results in a multifaceted phenotype which includes: bone dysplasia, learning disabilities, benign nerve sheath tumors, and malignant tumors. NF1 is caused by pathogenic variants in the *neurofibromin* (*NF1)* gene, and the clinical phenotypes of individuals with NF1 can vary widely, even among family members with the same genotype. NF1 functions as a GTPase-Activating Protein (GAP), simulating conversion of active Ras-GTP to the inactive form, Ras-GDP (Figure 1). Almost 2,900 pathogenic variants have been reported in the Human Gene Mutation Database (http://www.hgmd.org); most lead to lack of expression of the *NF1* gene product. NF1 does not exhibit a mutagenic hotspot, with variants occurring throughout the gene. A subset (17%) of these variants consists of missense (MS) variants which may result in an unstable or dysfunctional protein (Koczkowska et al., 2019). MS variants in *NF1* are not confined to any specific region of the gene. Some occur in the GAP-related domain (GRD) and might be expected to interfere with GAP function. MS variants occurring outside of this domain may result in other NF1 dysfunction, including protein instability, cellular mis-localization, or the disruption of neurofibromin interaction with other proteins in the cell. Another subset of variants (up to 29% (Kang et al., 2020)) includes nonsense (NS) variants, which result in premature termination of translation, and, in most cases, nonsense-mediated decay of the transcript.

The most critical role of neurofibromin appears to be the regulation of Ras signaling, with loss of neurofi-

bromin function resulting in increased signaling. NF1 tumor cells (*e.g.,* Schwann cells in neurofibromas) exhibit increased Ras signaling as a consequence of loss of function of both *NF1* alleles (one in the germ line and one somatically-acquired (Figure 1; right) (Cichowski & Jacks, 2001). Therapeutic interventions to date have focused on inhibition of upregulated Ras signaling (*e.g.,* MEK inhibition with selumetinib (Figure 1, right)). While MEK inhibitors have demonstrated effectiveness, not all patients benefit, plexiform neurofibromas do not completely disappear, and there can be significant side effects (Baldo, Magnolato, Barbi, & Bruno, 2021). Therefore, additional treatments that can be used in conjunction with MEK inhibitors are needed.

Only a small portion of the NF1 protein, the GRD, directly interacts with Ras. Some of the key NF1 residues involved in the NF1-Ras interaction include R1276 and K1423 (Yan et al., 2020). R1276, a highly conserved arginine residue, also termed the arginine "finger", aids in the stabilization of the GTP/GDP-NF1 complex (Scheffzek et al., 1997). Mutation of this residue has little effect on Ras binding, but results in complete loss of GAP activity (Ahmadian, Stege, Scheffzek, & Wittinghofer, 1997; Sermon, Lowe, Strom, & Eccleston, 1998). NF1 K1423 forms a salt bridge with Ras D38 to stabilize the protein-protein interaction. In addition to dysregulated Ras signaling due to variants located in the GRD, interference with neurofibromin's localization to the cytoplasmic membrane can result in abnormal interaction with Ras (Stowe et al., 2012). MS variants that occur within the SPRED1-binding domain of NF1 can reduce the affinity for SPRED1 such that neurofibromin fails to traffic to the membrane (Dunzendorfer-Matt, Mercado, Maly, McCormick, & Scheffzek, 2016; Hirata et al., 2016; Yan et al., 2020). However, some pathogenic NF1 variants fail to co-immunoprecipiate with SPRED1, do not directly interfere with Ras binding, and do retain GAP activity (Dunzendorfer-Matt et al., 2016).

An additional complexity to NF1 interactions is its dimerization activity (Figure 1, left) (Carnes, Kesterson, Korf, Mobley, & Wallis, 2019; Sherekar et al., 2019). While the mechanistic significance has yet to be determined, dimerization provides a potential explanation for the phenotypes observed with many NF1 disease variants. Often heterozygous NS or frameshift (FS) variants are observed in NF1, where there is still a single copy of wild type (WT) *NF1* allele present. The total amount of full-length neurofibromin in some affected individuals with these variants may be considerably less than the predicted 50% of WT levels (Anastasaki, Woo, Messiaen, & Gutmann, 2015). Neurofibromin levels could be drastically lowered if mutant protein dimerizes with WT and then is targeted to the proteasome for degradation (Figure 1). The ubiquitin-proteasome pathway (UPP) controls NF1 levels and both the amplitude and duration of Ras-mediated signaling (Cichowski, Santiago, Jardim, Johnson, & Jacks, 2003). Excessive proteasomal degradation and genetic loss results in NF1 inactivation in sporadic gliomas (McGillicuddy et al., 2009). Proteasomal degradation of NF1 is partially regulated by the binding of both the SAG-SKP1-CUL1-FBXW7 and RBX1/2-CUL3-KBTBD7 complexes with NF1 (Figure 1; left) (Hollstein & Cichowski, 2013; Tan et al., 2011). The CUL3/KBTBD7 complex has been implicated in the pathogenic destabilization of neurofibromin in glioblastomas (Hollstein & Cichowski, 2013).

Here, we further validate our heterologous m*Nf1* cDNA expression system and multiple assays for neurofibromin function and use them to evaluate patient-specific variants (Wallis et al., 2018). We have included many MS variants with known genotype-phenotype correlations to assess their functional effects on NF1 levels as well as Ras signaling. We demonstrate that effects on stability and Ras signaling can be mutually exclusive functions. We suggest that such stratification of variant effects will have implications for mutation-targeted therapeutics for NF1, neurofibromin-driven breast cancers, and potentially other phenotypes. These assays may also have utility in classifying newly identified variants of unknown significance. As new Ras-independent functions are discovered for neurofibromin, it will be important to assay variants for their effects in new functional assays.

MATERIALS AND METHODS

Cell culture: HEK293 (WT or *NF1* +/+) cells were obtained from ATCC (CRL-1573) and cultured in DMEM + 10% FBS and 1X Pen/Strep using standard culture procedures. *NF1* -/- or null HEK293 cells were previously created through CRISPR Cas9 targeting *NF1* exon 2 (Wallis et al., 2018) . We have

chosen to evaluate functional NF1 expression and Ras activity in HEK293 cells because this cell line is well characterized, used historically in NF1 research, easily takes up exogenous DNA, and is easy to culture and scale. HEK293s have all three Ras isoforms and recapitulate Ras signaling; hence, HEK293 cells are an appropriate model system for these assays.

Nf1 **cDNA plasmid development:** The wild type (WT)*Nf1* cDNA plasmid was developed by GeneCopoeia and is commercially available. The full-length mouse cDNA (*mNf1)* produces a >250 kDa neurofibromin protein that is capable of modulating Ras signaling (Wallis et al., 2018). We created an empty vector (EV) control plasmid with the same parental backbone that does not contain the *Nf1* cDNA. We also created a panel of mutant cDNAs representing variants found in NF1-affected individuals with different clinically relevant phenotypes and assessed their ability to produce mature neurofibromin and restore *Nf1* activity in*NF1* $^{-/-}$ cells. *mNf1* cDNA is appropriate for study because the full-length cDNA sequences of endogenous*hNF1* and *mNf1* have 92% sequence identity; amino acid sequences share 98% identity and human cDNAs have historically been unstable and toxic. Variants were introduced into shuttle vectors by either site-directed mutagenesis or utilization of synthetic DNA fragments and then cloned into the full-length vector using standard enzymes. Each variant plasmid was confirmed by sequencing the entire*Nf1* cDNA insert and all subsequent DNA preparations were validated by spot checking for the variant of interest. Furthermore, multiple plasmid preps were utilized for each variant to mitigate any variability due to quality of DNA.

Transient transections: For Western blots and GTP-Ras assays, cells were transfected using LipoD293 (SignaGen Lab. Cat# SL100668) with up to 1ug of cDNA per 6-well dish seeded with 500,000 cells per well. Assays were performed 48-72 hours later. For titration experiments, WT cDNA was balanced with EV control such that 1000 ng total DNA was transfected.

Western blotting: Cells were lysed with RIPA buffer supplemented with a protease inhibitor cocktail and phosSTOP, and lysates were cleared by centrifugation at 20,000 RPM for 20 minutes at 4^0C. Protein was quantified with a Bradford assay and 50 ug of protein was loaded per well for NF1 blots and 10 ug of protein was loaded for other blots. 8% SDS-polyacrylamide gels were run at 100 V for 2 hours and transferred at 100 V for 2 hours onto PVDF. Blots were probed overnight at 4^0C with primary antibody, washed, and probed for 1 hour at room temperature with secondary. Primary antibodies include N-Terminal NF1 (Cell Signaling cat# D7R7D 1:1000), tubulin (Abcam cat# ab52866 1:1000), b-actin (Cell Signaling cat# 3700 1:1000), p-ERK (Cell Signaling cat# 9101 1:1000), and total ERK (Cell Signaling cat# 9102 1:1000). Secondary was HRP tagged from Santa Cruz. Chemiluminescent substrate from Bio-Rad was used as per manufacturer's protocols.

RAS-G-LISA Assay: The RAS-G-LISA assay was obtained from Cytoskeleton Inc. and was performed according to the manufacturer's instructions.

Statistical analysi s. All assays were repeated a minimum of three times for each variant. For each experiment wild type *Nf1*cDNA (WT) and empty vector (EV) plasmid control with no cDNA insert were included as controls. Depending on whether we intended to show either 1) presence of WT cDNA repressed Ras activity more than absence of*NF1* cDNA (EV) as in our titration experiments or 2) variant cDNA expression or activity was different from WT cDNA, we normalized expression or activity to WT or EV respectively and made comparisons with EV or WT respectively. Normalization to one control allows us to combine data across independent experiments, and combination of multiple repeat experiments controls for the effects of differential transfection efficiencies. Statistical comparisons using student's t-test were made using Excel software to determine which results were statistically significant.

RESULTS

Assay validation with cDNA Titrations: To further validate our cDNA and assay system (Wallis et al., 2018), we performed titration experiments to show a dose-response effect utilizing 0.25 - 1000ng/well of WT mouse *Nf1* cDNA in *NF1* null HEK293 cells with 500,000 cells balanced with empty vector (EV) control. Neurofibromin/tubulin levels (Figure 2A), GTP-Ras levels (Figure 2B), and pERK/ERK ratios (Figure 2C)

all respond in a dose-response manner to the amount of cDNA transfected into the cells. Neurofibromin levels become detectible via Western blot analysis between 1-4 ng/500,000 cells transfected (Figure 2A). GTP-Ras levels were lowered, beyond that of empty vector (0ng WT cDNA), starting at 1-4 ng/500,000 cells transfected, with statistically significant differences observed at 15 ng and above (Figure 2B). Lowered pERK/ERK ratios, a marker for MAPK signaling activity, were also detected at 1-4 ng/500,000 cells transfected, with statistically significant differences from EV at 250 ng (Figure 2C).

Variant Selection and groupings: We utilized a full gene-encompassing panel of *mNf1* cDNAs transfected into an*NF1* null (-/-) cell line to evaluate the functional effects of unique variants (Figure 3 and Table 1). Variants, in addition to WT and EV, were selected based on the following criteria: controls, genotype-phenotype correlations, occurrences in different NF1 domains, and type of mutation. Benign variants, both within and outside of the GRD, were selected due to lack of pathogenicity and as "controls": E1327G, Q1336R, and P2782L. Variants with published genotype phenotype-correlations were prioritized. Variants associated with "mild" phenotypes include delM992, R1038G, M1149V, and R1809C (Koczkowska et al., 2019; Koczkowska, Callens, et al., 2018; Rojnueangnit et al., 2015; Trevisson et al., 2019; Upadhyaya et al., 2007). Variants associated with "severe" phenotypes include L847P, G848R, R1276Q, and K1423E (Koczkowska et al., 2019; Koczkowska, Chen, et al., 2018; Korf, Henson, & Stemmer-Rachamimov, 2005). We also included variants from multiple domains, despite having "unknown" phenotype associations. C379R falls within the 5' region with no described domain function, W784R falls within the putative CSRD domain, L1490P falls within the SPRED1 interaction domain, D1623 falls within the Sec14 domain, and L1957P, S1997R, and L2317P all fall within the 3' region of the protein but not in well-described domains. Additional variants were selected based on the formation of cryptic "splice" sites: Y489C (Messiaen et al., 1999) and G629R. While splicing is not affected in the cDNA system, assessment of functional effects of the subsequent missense variant is a critical first step in the development of antisense therapeutics that might restore normal splicing but leave the variant intact. Additional "nonsense" variants were prioritized based on incidence as well as location throughout the protein.

NF1 levels First we evaluated neurofibromin levels after transfecting cells with equal plasmid concentrations from each representative cDNA. All cDNAs were assayed for NF1 levels by transfecting a consistent 1 ug cDNA into a 6 well plate with 500,000 cells and harvesting cells 48 hours post transfection. Figure 4 demonstrates quantification of NF1/tubulin ratios for all cDNAs and indicates differential variant-specific effects on neurofibromin levels. For example, while some variants remain stable with levels similar to WT (e.g., R1809C), others show much lower levels of neurofibromin; L1490P and D1623G lead to approximately 20% WT levels. We interpret NF1 levels to reflect protein stability, which may be dependent on mutation-targeted proteasomal degradation. All cDNAs should have similar transfection, transcription, and translation efficiencies. Each experiment included both WT and EV control cDNAs. Samples were normalized to tubulin as a load control and WT/tubulin ratios were set at 1.0 in each experiment. All other cDNA/tubulin ratios were reported relative to WT levels. Normalization allowed comparison across experiments. Western blots of select MS variants show varying full-length NF1 levels (Figure 4B). Western blots of nonsense variants show varying NF1 levels (Figure 4C); note the truncation products run at the anticipated sizes; however, NF1 levels did not correlate with length of prematurely terminated mutant proteins.

Ras Activity: Next we assayed all *Nf1* variant cDNAs for their ability to repress Ras signaling. We evaluated both GTP-Ras levels and pERK/ERK ratios (Figure 5). All cDNAs were assayed by consistently transfecting in 1 ug of cDNA into a 6 well plate with 500,000 cells/well and harvesting protein lysate 48 hours post transfection. Each experiment included both WT and EV control cDNAs. For GTP-Ras levels (blue bars; Figure 5A), each sample was normalized to EV GTP-Ras levels, which were set at 1.0 in each experiment, and all other GTP-Ras levels were reported relative to EV levels. Each cDNA's GTP-Ras level was statistically compared via Student's t-test to the WT cDNA's GTP-Ras level to determine if the variant negatively impacted NF1 ability to repress GTP-Ras levels. Blue asterisks indicate that a variant has statistically significant impaired ability to inhibit GTP-Ras levels; these include: delM992, M1149V, L847P, R1276Q, K1423E, C379R, L1490P, D1623G, S1997R, L2317P, R192X, R461X, R681X, R816X, R1276X, and R1306X.

For pERK/ERK ratios (black bars; Figure 5A), each experiment included both WT and EV control cDNAs, with each sample normalized to the EV cDNA pERK/ERK ratio, which was set at 1.0. Each cDNA's pERK/ERK ratio was statistically compared via Student's t-test to the WT pERK/ERK ratio to determine if the variant negatively impacted its ability to repress pERK activity with black asterisks indicating statistically significant impaired ability; these include M1149V, L847P, R1276Q, K1423E, L1490P, D1623G, S1997R, L2317P, R192X, R461X, R681X, R816X, R1276X, R1306X, and R1947X.

Activity as a function of stability: Neurofibromin function*in vivo* relies on numerous factors, including stability (abundance of protein available), cellular localization, ability to bind interacting proteins (such as Ras), and ability to stimulate Ras GTPase activity. As we measured two of these factors, neurofibromin levels and GTPase activity, we wanted to determine if the combined factors can lead to variant functional insights. To achieve this, we plotted our*Nf1* WT cDNA titration data (derived from Figure 2) such that neurofibromin/tubulin levels at 1000 ng cDNA was set to a maximum of 1 and plotted on the x-axis and corresponding GTP-Ras activity levels were plotted on the y-axis (Figure 6, gray dots). A trend line was generated (Figure 6, blue dotted line). To evaluate this multidimensional concept, we overlaid variant data onto this plot and categorized variants as we had in Figures 4 and 5 as "Control" (green dots), "Splice" (yellow dots), "Mild" (orange dots), "Severe" (pink dots), and "Unknown" (teal dots). The control variants clustered such that NF1/tubulin ratios were > 0.75 and GTP-Ras levels were < 0.52 (Figure 6 green oval). The cryptic splice variants also clustered at NF1 > 0.89 and GTP-Ras < 0.66. While the mild variants didn't cluster together as tightly, three of the four variants had stable protein levels > 0.8 and two had low GTP-Ras levels < 0.66. These loosely clustered mild variants also cluster with the splice variants (Figure 6 orange ovals). Severe and unknown variants did not form a single cluster. Severe variants R1276Q and K1423E that interact with Ras do maintain stability but cannot suppress Ras and are clustered in the top right (Figure 6 pink oval). Outside of those clusters, we find multiple variants that hug the trend line (blue oval): "Severe" variants L847P and G848R and unknown variants L2317P, C379R, W784R, and L1957P (these have been individually labeled in the plot). This suggests that given a certain abundance of neurofibromin, the variants can suppress Ras signaling.

DISCUSSION

cDNA model system. Since discovery of the *NF1* gene in 1990 research efforts have been hindered by the lack of a full-length coding cDNA. This is partially due to the size of the gene and toxicity of the human cDNA construct. We are aware of three potentially available*NF1* cDNAs: mouse *Nf1* (Wallis et al., 2018) (isoform 2 with 2839 amino acids), codon optimized human *NF1* (Bonneau, Lenherr, Pena, Hart, & Scheffzek, 2009) (isoform 1 with 2818 aa), and a human*NF1* with mini-intron 35-36 (Cui & Morrison, 2019) (isoforms 1 and 2). As the *mNf1* cDNA is highly homologous to endogenous human*NF1* , we have developed and validated the mouse *Nf1* cDNA expression system that allows us to examine the biochemical effects of any *Nf1* genetic variant. We have been able to perform dose-response studies to titrate in varying amounts of *mNf1* cDNA and are able to detect a clear dose-response in terms of levels of neurofibromin and repression of GTP-Ras levels and pERK/ERK ratios, giving us confidence in both the cDNA and the functional assays.

Assay Performance: We see similar dynamic ranges between the GTP-Ras and pERK/ERK experiments, with WT cDNA able to repress both GTP-Ras and pERK/ERK ratios by about half that seen with EV control. The Morrison lab reported that inactive variants (R1276P) served as better controls than empty vectors (Cui & Morrison, 2019). While our R1276Q and R1276X both displayed similar GTP-Ras levels as our EV, both showed insignificantly higher pERK/ERK ratios. Indeed, we see significant variability in the pERK/ERK ratios, leading to large error bars. Regardless, each individual cDNA performs similarly between the two assays and across multiple experiments, indicating that the results are reliable. Each variant's ability to affect Ras signaling relative to WT cDNA is consistent between assays; however, we have noted exceptions. delM992 and C379R both have significantly different GTP-Ras levels, whereas pERK/ERK ratios fail to reach significance; and R1748X has a significantly different pERK/ERK ratio, but GTP-Ras doesn't reach significance.

Using our previously published cDNA expression system (Wallis et al., 2018) we can evaluate functional sig-

nificance of variants in individuals with NF1. These data indicate that each variant has a slightly different functional profile in terms of both protein stability and the ability to inhibit Ras signaling. We incorporated non-pathogenic variants in our assays: E1327G, Q1336R, and P2782L. These three cDNAs performed similarly to the WT cDNA in terms of neurofibromin levels and the ability to inhibit Ras signaling, adding confidence to our functional profile characterizations. Some pathogenic variants result in instability of neurofibromin but still retain GRD function. The best examples of this are with the G848R and L1957P variants, which retain the ability to repress Ras activity (Figure 5) in both of our assays, yet are unstable and produce less than 50% of the neurofibromin that is observed with WT cDNA protein (Figure 4). Some variants result in stable protein but have lost GRD function. For example, K1423E and S1997R both produce neurofibromin levels similar to WT cDNA, yet display significantly elevated Ras activity compared with WT cDNA. We also observed that certain variants demonstrated both unstable protein and loss of function; L1490P and D1623G exhibited lowered neurofibromin levels, with inability to repress Ras signaling. Nonsense variants resulting in truncated proteins have variable stability. Nonsense variants with truncations after the GRD may maintain GRD function in these overexpression assays. While both R1947X and R2550X show increased Ras signaling, it is not statistically different from WT cDNA.

Genotype-phenotype Correlations and assay results

Genotype-phenotype correlations indicate allele-specific effects for*NF1* . There are multiple MS and small in-frame deletion variants that are associated with unique NF1 phenotypes that may be utilized for further functional analysis. A mild phenotype is associated with the c.2970-2972 delAAT (delM992) single amino acid deletion, consisting of café-au-lait macules (CALMs) and skinfold freckling and lack of neurofibromas (Koczkowska, Callens, et al., 2018; Upadhyaya et al., 2007). Variants involving R1809 are the most frequent recurrent*NF1* variants and present with multiple CALMs, with or without freckling and Lisch nodules, but externally visible plexiform neurofibromas, symptomatic optic pathway glioma or cutaneous or subdermal neurofibromas are not found (Rojnueangnit et al., 2015). Mild phenotypes are also associated with R1038G (Trevisson et al., 2019) and M1149V (Koczkowska et al., 2019). All of these genotypes are associated with Noonan-like facial features. In fact, 31.1%, 29% and 11.5% of individuals with variation at R1809, M1149, and M992, respectively, show Noonan features, in comparison to only 3.4% of "classic" NF affected individuals (Koczkowska et al., 2019). The R1038G cohort, consisting of two families, is too small to meaningfully compare, but Noonan features are also reported in both families (Trevisson et al., 2019). Overall, NF1 affected individuals with delM992, R1038G, M1149V, and R1809C associated with mild phenotypes lack clinically suspected plexiform, cutaneous, or subcutaneous neurofibromas and are not at risk for malignancy.

In contrast, other MS variants are associated with more severe phenotypes, including increased risk for malignancy and/or spinal neurofibromas. Constitutional MS variants affecting one of five neighboring NF1 codons—Leu844, Cys845, Ala846, Leu847, and Gly848—located in the cysteine-serine-rich domain (CSRD), typically result in a large number of plexiform and symptomatic spinal neurofibromas, symptomatic optic nerve gliomas, skeletal abnormalities, and malignant neoplasms (Koczkowska, Chen, et al., 2018). R1276Q has been identified in some individuals affected with spinal neurofibromas at all levels and also has been associated with a more severe phenotype (Koczkowska et al., 2019; Korf et al., 2005). K1423E has been associated with a severe phenotype (Koczkowska et al., 2019). Some of these variants (affecting residues G848 and R1276) are observed in individuals with a distinctive phenotype, referred to as "spinal NF." The "spinal NF" phenotype includes few or no cutaneous neurofibromas (cNfs) and a very mild pigmentary phenotype (Burkitt Wright et al., 2013; Ruggieri et al., 2015). These individuals may suffer from a massive internal tumor burden, with neurofibromas at each spinal nerve root and extreme enlargement of most peripheral nerves. These individuals are at great risk of spinal cord compression, pain, and malignant change, and the extreme number of tumors makes surgical treatment difficult or impossible (Koczkowska et al., 2019; Korf et al., 2005). Certain variants correlate with increased incidences of cancer. A greater risk of malignancy for MS variants in codons 844-848 has been reported (Koczkowska, Chen, et al., 2018). Codon 847 is recurrent in NF1 patients with breast cancer (Frayling et al., 2019). A lack of large deletions with an excess of NS and FS variants has been observed with breast cancer (Frayling et al., 2019; Zheng et al., 2020). Thus, the type of*NF1* variant matters when genotype-phenotype correlations can be made and can influence clinical care.

We had hoped that our functional assays might predict which genotypes could result in specific phenotypes, but a simplistic interpretation is not readily available. Our assays indicate that most of the variants associated with mild phenotypes are hypomorphic alleles. All mild variants are relatively stable and produce neurofibromin at levels above 60% of WT levels. It is not surprising that a certain threshold of neurofibromin must be achieved to have a mild phenotype; however, Ras signaling may be altered depending on the variant. Ras signaling for R1038G and R1809 is elevated, but not statistically different than for WT cDNA. The delM992 variant is unable to completely suppress GTP-Ras or pERK activity; this is statistically significant for GTP-Ras. Finally, M1149V has statistically significant increased Ras signaling. These data suggest that delM992, R1038G, and R1809C act as hypomorphs. In contrast, M1149V appears to have lost the ability to inhibit Ras signaling and an explanation for this unanticipated result for this "mild" variant is not available.

Other genotypes are associated with severe phenotypes: L847P, G848R, R1276Q, and K1423E. cDNAs with these genotypes have highly variable NF1 levels, ranging from 38% - 136% that of WT cDNA. L847P and G848R are located in the CSRD; R1276Q and K1423E are located within the GRD and interact directly with Ras. R1276Q and K1423E are completely unable to suppress Ras signaling and are statistically different from WT cDNA, as would be expected for variants that are critical for Ras binding and GTP-hydrolysis. L847P is also unable to repress Ras signaling; however, G848R can repress Ras signaling. Its lack of stability likely explains why it is unable to function properly and causes a phenotype.

Given the interdependency of stability and function, we wanted to evaluate NF1 stability as a function of Ras activity and plotted neurofibromin levels with GTP-Ras levels (given that this assay had less variability than the pERK/ERK assay) and drew a trend line. We noted clustering of controls and cryptic splice variants, and that genotypes associated with mild phenotypes also are loosely clustered (orange circle). The presence of the "unknown" variant S1997R within this cluster suggests that individuals with the genotype may have a mild phenotype. In fact, the Leiden Open Variation Database (LOVD) has classified this variant as a variant of uncertain significance (VUS); however, we have identified an individual that meets NF1 diagnostic criteria with this *de novo* variant. Though still adolescent, no cutaneous or plexiform neurofibromas have been identified and the phenotype is thus-far "mild". In addition, we find multiple variants that hug the trend line: variants L847P and G848R (associated with severe phenotypes) and variants L2317P, C379R, W784R, and L1957P. This suggests that given a certain abundance of neurofibromin, the variants are partially able to repress Ras signaling. If the protein could be stabilized *in vivo* , Ras might be repressed and the phenotype rescued. Thus, we demonstrate two distinct clusters of genotypes associated with severe phenotypes, one (pink oval) indicating loss of GTPase function results in pathogenicity and the other indicating loss of stability leads to pathogenicity.

Six NF1 phenotypic subtypes have recently been proposed, and while genotypic data were inadequate to make statistically significant conclusions, particular variants were noted to be consistent with three of the six clusters (Tabata, Li, Knight, Bakker, & Sarin, 2020). delM992 was consistent with the mild subtype (cluster 1); R1809C was consistent with the freckling-predominant subtype (cluster 2), and L847P and was consistent with the early-onset neural severe (cluster 6) subtype. Ideally, combining genotype and functional data with such phenotypic clustering would be a powerful tool in understanding the phenotypic heterogeneity of NF1. Unfortunately for this cohort (derived from a self-reported registry) only 61 of 2051 participants provided molecular diagnostic data (though ˜50% reported that a molecular diagnosis had been made).

Study Limitations: There are several factors that limit our study. First, HEK293 cells are very different from Schwann cells (one of the cell types primarily affected in individuals with NF1). HEK293 cells are derived from human embryonic kidney cells (not neural crest cells; but they maybe neuronal as they share similarities with embryonic adrenal precursor cells (Lin et al., 2014) transformed by incorporation of 4.5 kb of adenovirus 5 genome into human chromosome 19 and carries a modal chromosome number of 64 in 30% of cells. Notably, this increased chromosome number does not affect any of the RAS or RASGAP genes. HEK293s have all three wild type Ras isoforms. Even though the Ras pathway is remarkably conserved, there could be modifiers in this cell line not present in Schwann cells, melanocytes, neurons, and osteoblasts/osteoclasts. Second, while Ras assays are commonly employed to evaluate *NF1* variants, determination and evaluation

of alternative functions is critical. Little information is available regarding how variants might affect NF1 dimerization, nuclear localization (or cellular localization in general) or even how they might interact with other binding partners. Recently, a new mechanism whereby NF1 binds the estrogen receptor (ER) and acts as a transcriptional corepressor has emerged; this ER activity is functionally independent of GAP activity (Zheng et al., 2020). Thus, NF1 is a dual repressor for both Ras and ER signaling. Defining how variants affect these functions will aid our understanding of neurofibromin structure-function. Finally, our assay is an over-expression assay and hence does not reflect endogenous expression levels.

To date, NF1 cannot be cured. While MEK inhibitors can block Ras signaling regardless of mutation type, therapeutics that address the underlying cause of the disease by restoring neurofibromin function to a level that leads to a non-pathogenic phenotype do not yet exist. Various gene and mRNA targeting strategies have been proposed and are being evaluated for their therapeutic potential in NF1 (Leier et al., 2020). Compounds such as proteosome inhibitors could be used to stabilize neurofibromin levels if the protein is being targeted for degradation. Potentiators (analogous to those utilized for CFTR) might be used to directly bind NF1 and stabilize it or prevent it from being degraded. NF1 mimetics might be developed to stimulate Ras GTPase activity. Thus, molecular diagnostics and determination of a variant's stability and function are increasingly relevant to guide clinical care for those with known genotype-phenotype correlations and may also have implications for both classification of variants of uncertain significance (particularly those found in breast cancer) and developing therapeutics. Once variants and their effects are established and categorized, new classes of therapeutics become possible.

Figure Legends:

Figure 1: NF1 affects multiple signaling pathways to modulate Ras which can be therapeutically targeted through these various pathways. Left side exhibits NF1 dimerization and regulation through the proteasome. Specific NF1 variants might benefit from ubiquitin proteasome pathway (UPP) inhibitors to prevent excess degradation. Right side illustrates the Ras signaling pathway and the use of MEK inhibitors to slow tumor growth resulting from hyperactive Ras signaling.

Figure 2: *Nf1* assay validations with WT mouse cDNA titrations in *NF1* null HEK293 cells. WT NF1 +/+ HEK293 (293 +/+) cells were used as controls. Varying amounts (0-1000ng) of WT plasmid cDNA was transfected into the null cell line. A. Top panel – Quantitation of neurofibromin levels (n = 3). Bottom panel – Representative western blot of titration experiments. B. GTP-Ras activity levels as determined by Ras-GLISA assay (n = 3). Statistically significant differences were seen at 15ng and greater (red asterisk). C. Top panel – Quantitation of pERK/ERK levels (n = 3). Statistically significant differences were observed at 250ng. Bottom panel – Representative western blot. Error bars represent SEM and red asterisks indicate statistical significance.

Figure 3: Schematic of NF1 protein and putative domains with cDNA variants depicted. Red circles – nonsense mutations, blue squares – missense mutations, green triangles – in-frame deletion, and unfilled black circles – non-pathogenic variants. Amino acid positions are notated above protein domains in black text.

Figure 4: Relative neurofibromin protein levels for each cDNA.A. Quantitation of NF1 normalized to tubulin for all the *Nf1*cDNAs and categorized based on variant type. WT/tubulin ratios were set at 1.0 in each experiment with all other variant ratios relative to WT levels. Graph represents an n > 3 independent experiments for each cDNA. SEM error bars are shown. B. Representative western blot of select missense mutations showing NF1 and tubulin expression. C. Representative western blot of nonsense mutations showing NF1 and tubulin levels. Note the truncation products run at anticipated sizes.

Figure 5: Ras signaling activity assessed via GTP-Ras and pERK/ERK levels for each cDNA. A. Quantitation of GTP-Ras levels (blue bars) and pERK/ERK ratios (black bars) for all cDNAs. EV levels were set 1.0 and each sample was normalized and reported relative to EV levels. Comparisons to WT and statistical significance were determined via a Student's t-test to determine if the variant negatively impacted NF1's ability to repress Ras signaling. Blue asterisks indicate statistically significant impairment in the

ability to inhibit GTP-Ras ($p < 0.05$). Black asterisks indicate statistically significant impaired ability to inhibit pERK/ERK ratios ($p < 0.05$). Graph represents N>3 independent experiments for each cDNA. Bars represent SEM. B. Representative image of one western blot of select missense mutations showing pERK and ERK levels. C. Representative image of one western blot of nonsense mutations showing pERK and ERK levels.

Figure 6: Analysis of protein stability and function to group*Nf1* variants. *Nf1* WT protein concentrations and corresponding GTP-Ras levels were plotted (gray dots) to generate a trend line (blue line). NF1 and GTP-Ras levels for each MS variant were overlaid onto the plot and variants were grouped as controls (green dots), splice (yellow dots), mild (orange dots), severe (pink dots), and unknown (teal dots). Clustering, based on NF1 stability and function is indicated by ovals: control variants – green oval, mild and splice variants – orange oval, select severe variants located in the GRD domain and interacting directly with Ras – pink oval, variants hugging the trend line- blue oval.

REFERENCES:

Ahmadian, M. R., Stege, P., Scheffzek, K., & Wittinghofer, A. (1997). Confirmation of the arginine-finger hypothesis for the GAP-stimulated GTP-hydrolysis reaction of Ras. *Nat Struct Biol, 4* (9), 686-689. Retrieved from https://www.ncbi.nlm.nih.gov/pubmed/9302992

Anastasaki, C., Woo, A. S., Messiaen, L. M., & Gutmann, D. H. (2015). Elucidating the impact of neurofibromatosis-1 germline mutations on neurofibromin function and dopamine-based learning. *Hum Mol Genet, 24* (12), 3518-3528. doi:10.1093/hmg/ddv103

Baldo, F., Magnolato, A., Barbi, E., & Bruno, I. (2021). Selumetinib side effects in children treated for plexiform neurofibromas: first case reports of peripheral edema and hair color change. *BMC Pediatrics, 21* (1), 67. doi:10.1186/s12887-021-02530-5

Bonneau, F., Lenherr, E. D., Pena, V., Hart, D. J., & Scheffzek, K. (2009). Solubility survey of fragments of the neurofibromatosis type 1 protein neurofibromin. *Protein Expr Purif, 65* (1), 30-37. doi:10.1016/j.pep.2008.12.001

Burkitt Wright, E. M., Sach, E., Sharif, S., Quarrell, O., Carroll, T., Whitehouse, R. W., . . . Evans, D. G. (2013). Can the diagnosis of NF1 be excluded clinically? A lack of pigmentary findings in families with spinal neurofibromatosis demonstrates a limitation of clinical diagnosis. *J Med Genet, 50* (9), 606-613. doi:10.1136/jmedgenet-2013-101648

Carnes, R. M., Kesterson, R. A., Korf, B. R., Mobley, J. A., & Wallis, D. (2019). Affinity purification of NF1 protein-protein interactors identifies keratins and neurofibromin itself as binding partners. *Genes, in press* .

Cichowski, K., & Jacks, T. (2001). NF1 tumor suppressor gene function: narrowing the GAP. *Cell, 104* (11239415), 593-604. Retrieved from http://www.hubmed.org/display.cgi?uids=11239415

Cichowski, K., Santiago, S., Jardim, M., Johnson, B. W., & Jacks, T. (2003). Dynamic regulation of the Ras pathway via proteolysis of the NF1 tumor suppressor. *Genes Dev, 17* (4), 449-454. doi:10.1101/gad.1054703

Cui, Y., & Morrison, H. (2019). Construction of cloning-friendly minigenes for mammalian expression of full-length human NF1 isoforms. *Hum Mutat, 40* (2), 187-192. doi:10.1002/humu.23681

Dunzendorfer-Matt, T., Mercado, E. L., Maly, K., McCormick, F., & Scheffzek, K. (2016). The neurofibromin recruitment factor Spred1 binds to the GAP related domain without affecting Ras inactivation. *Proc Natl Acad Sci U S A, 113* (27), 7497-7502. doi:10.1073/pnas.1607298113

Frayling, I. M., Mautner, V. F., van Minkelen, R., Kallionpaa, R. A., Aktas, S., Baralle, D., . . . Upadhyaya, M. (2019). Breast cancer risk in neurofibromatosis type 1 is a function of the type of NF1 gene mutation: a new genotype-phenotype correlation. *J Med Genet, 56* (4), 209-219. doi:10.1136/jmedgenet-2018-105599

Hirata, Y., Brems, H., Suzuki, M., Kanamori, M., Okada, M., Morita, R., . . . Yoshimura, A. (2016). Interaction between a Domain of the Negative Regulator of the Ras-ERK Pathway, SPRED1 Protein, and the GTPase-activating Protein-related Domain of Neurofibromin Is Implicated in Legius Syndrome and Neurofibromatosis Type 1. *J Biol Chem, 291* (7), 3124-3134. doi:10.1074/jbc.M115.703710

Hollstein, P. E., & Cichowski, K. (2013). Identifying the Ubiquitin Ligase complex that regulates the NF1 tumor suppressor and Ras. *Cancer Discov, 3* (8), 880-893. doi:10.1158/2159-8290.CD-13-0146

Kang, E., Kim, Y. M., Seo, G. H., Oh, A., Yoon, H. M., Ra, Y. S., . . . Lee, B. H. (2020). Phenotype categorization of neurofibromatosis type I and correlation to NF1 mutation types. *J Hum Genet, 65* (2), 79-89. doi:10.1038/s10038-019-0695-0

Koczkowska, M., Callens, T., Chen, Y., Gomes, A., Hicks, A. D., Sharp, A., . . . Messiaen, L. M. (2019). Clinical spectrum of individuals with pathogenic NF1 missense variants affecting p.Met1149, p.Arg1276 and p.Lys1423: genotype-phenotype study in neurofibromatosis type 1. *Hum Mutat* . doi:10.1002/humu.23929

Koczkowska, M., Callens, T., Gomes, A., Sharp, A., Chen, Y., Hicks, A. D., . . . Messiaen, L. M. (2018). Expanding the clinical phenotype of individuals with a 3-bp in-frame deletion of the NF1 gene (c.2970_2972del): an update of genotype-phenotype correlation. *Genet Med* . doi:10.1038/s41436-018-0269-0

Koczkowska, M., Chen, Y., Callens, T., Gomes, A., Sharp, A., Johnson, S., . . . Messiaen, L. M. (2018). Genotype-Phenotype Correlation in NF1: Evidence for a More Severe Phenotype Associated with Missense Mutations Affecting NF1 Codons 844-848. *Am J Hum Genet, 102* (1), 69-87. doi:10.1016/j.ajhg.2017.12.001

Korf, B. R., Henson, J. W., & Stemmer-Rachamimov, A. (2005). Case records of the Massachusetts General Hospital. Case 13-2005. A 48-year-old man with weakness of the limbs and multiple tumors of spinal nerves. *N Engl J Med, 352* (17), 1800-1808. doi:10.1056/NEJMcpc059008

Leier, A., Bedwell, D. M., Chen, A. T., Dickson, G., Keeling, K. M., Kesterson, R. A., . . . Wallis, D. (2020). Mutation-Directed Therapeutics for Neurofibromatosis Type I. *Mol Ther Nucleic Acids, 20* , 739-753. doi:10.1016/j.omtn.2020.04.012

Lin, Y. C., Boone, M., Meuris, L., Lemmens, I., Van Roy, N., Soete, A., . . . Callewaert, N. (2014). Genome dynamics of the human embryonic kidney 293 lineage in response to cell biology manipulations. *Nat Commun, 5* , 4767. doi:10.1038/ncomms5767

McGillicuddy, L. T., Fromm, J. A., Hollstein, P. E., Kubek, S., Beroukhim, R., De Raedt, T., . . . Cichowski, K. (2009). Proteasomal and genetic inactivation of the NF1 tumor suppressor in gliomagenesis. *Cancer Cell, 16* (1), 44-54. doi:10.1016/j.ccr.2009.05.009

Messiaen, L. M., Callens, T., Roux, K., Mortier, G. R., Paepe, A. D., Abramowicz, M., . . . Wallace, M. R. (1999). Exon 10b of the NF1 gene represented a mutational hotspot and harbors a recurrent missense mutation y489c associated with aberrant splicing. *Genetics In Medicine, 1* , 248. doi:10.1097/00125817-199909000-00002

Rojnueangnit, K., Xie, J., Gomes, A., Sharp, A., Callens, T., Chen, Y., . . . Messiaen, L. (2015). High Incidence of Noonan Syndrome Features Including Short Stature and Pulmonic Stenosis in Patients carrying NF1 Missense Mutations Affecting p.Arg1809: Genotype-Phenotype Correlation. *Hum Mutat, 36* (11), 1052-1063. doi:10.1002/humu.22832

Ruggieri, M., Polizzi, A., Spalice, A., Salpietro, V., Caltabiano, R., D'Orazi, V., . . . Nicita, F. (2015). The natural history of spinal neurofibromatosis: a critical review of clinical and genetic features. *Clin Genet, 87* (5), 401-410. doi:10.1111/cge.12498

Scheffzek, K., Ahmadian, M. R., Kabsch, W., Wiesmuller, L., Lautwein, A., Schmitz, F., & Wittinghofer, A. (1997). The Ras-RasGAP complex: structural basis for GTPase activation and its loss in oncogenic Ras mutants. *Science, 277* (5324), 333-338. Retrieved from https://www.ncbi.nlm.nih.gov/pubmed/9219684

Sermon, B. A., Lowe, P. N., Strom, M., & Eccleston, J. F. (1998). The importance of two conserved arginine residues for catalysis by the ras GTPase-activating protein, neurofibromin. *J Biol Chem, 273* (16), 9480-9485. Retrieved from https://www.ncbi.nlm.nih.gov/pubmed/9545275

Sherekar, M., Han, S. W., Ghirlando, R., Messing, S., Drew, M., Rabara, D., . . . Esposito, D. (2019). Biochemical and structural analyses reveal that the tumor suppressor neurofibromin (NF1) forms a high-affinity dimer. *J Biol Chem* . doi:10.1074/jbc.RA119.010934

Stowe, I. B., Mercado, E. L., Stowe, T. R., Bell, E. L., Oses-Prieto, J. A., Hernandez, H., . . . McCormick, F. (2012). A shared molecular mechanism underlies the human rasopathies Legius syndrome and Neurofibromatosis-1. *Genes Dev, 26* (13), 1421-1426. doi:10.1101/gad.190876.112

Tabata, M. M., Li, S., Knight, P., Bakker, A., & Sarin, K. Y. (2020). Phenotypic heterogeneity of neurofibromatosis type 1 in a large international registry. *JCI Insight, 5* (16). doi:10.1172/jci.insight.136262

Tan, M., Zhao, Y., Kim, S. J., Liu, M., Jia, L., Saunders, T. L., . . . Sun, Y. (2011). SAG/RBX2/ROC2 E3 ubiquitin ligase is essential for vascular and neural development by targeting NF1 for degradation.*Dev Cell, 21* (6), 1062-1076. doi:10.1016/j.devcel.2011.09.014

Trevisson, E., Morbidoni, V., Forzan, M., Daolio, C., Fumini, V., Parrozzani, R., . . . Clementi, M. (2019). The Arg1038Gly missense variant in the NF1 gene causes a mild phenotype without neurofibromas.*Mol Genet Genomic Med, 7* (5), e616. doi:10.1002/mgg3.616

Upadhyaya, M., Huson, S. M., Davies, M., Thomas, N., Chuzhanova, N., Giovannini, S., . . . Messiaen, L. (2007). An absence of cutaneous neurofibromas associated with a 3-bp inframe deletion in exon 17 of the NF1 gene (c.2970-2972 delAAT): evidence of a clinically significant NF1 genotype-phenotype correlation. *Am J Hum Genet, 80* (1), 140-151. doi:10.1086/510781

Wallis, D., Li, K., Lui, H., Hu, K., Chen, M. J., Li, J., . . . Kesterson, R. A. (2018). Neurofibromin (NF1) genetic variant structure-function analyses using a full-length mouse cDNA. *Hum Mutat, 39* , 816-821. doi:10.1002/humu.23421

Yan, W., Markegard, E., Dharmaiah, S., Urisman, A., Drew, M., Esposito, D., . . . Simanshu, D. K. (2020). Structural Insights into the SPRED1-Neurofibromin-KRAS Complex and Disruption of SPRED1-Neurofibromin Interaction by Oncogenic EGFR. *Cell Rep, 32* (3), 107909. doi:10.1016/j.celrep.2020.107909

Zheng, Z. Y., Anurag, M., Lei, J. T., Cao, J., Singh, P., Peng, J., . . . Chang, E. C. (2020). Neurofibromin Is an Estrogen Receptor-alpha Transcriptional Co-repressor in Breast Cancer. *Cancer Cell, 37* (3), 387-402 e387. doi:10.1016/j.ccell.2020.02.003

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Conflict of Interest:

Conflicts of Interest are as follows; the remaining co-authors do not have any conflicts of interest.

Bruce Korf:

- Chair, Children's Tumor Foundation Medical Advisory Committee
- Chair, External Advisory Committee for NTAP and also for NF Research Initiative
- Member, advisory committees for AstraZeneca and Springworks

Robert Kesterson is a lead advisor for

Infixion

David Bedwell is consultant for

PTC Therapeutics, Inc.

Deeann Wallis, Robert Kesterson, and Bruce Korf are inventors on US Provisional Patent Application No. 62/903,521 - Exon skipping to treat Neurofibromatosis Type 1.

Data Sharing: Data Availability Statement: Data available on request from the authors**.**

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`Table 1.docx` available at https://authorea.com/users/423838/articles/529065-analysis-of-patient-specific-nf1-variants-leads-to-functional-insights-for-ras-signaling-that-can-impact-personalized-medicine