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14. ABSTRACT We have previously shown that SPRED1 regulates localization of neurofibromin (the protein product of the NF1 gene) to the plasma membrane where it functions as a negative regulator of RAS signalling. NF1 mutations in neurofibromatosis cause loss of neurofibromin activity, resulting in hyperactivated RAS in patient cells and subsequent manifestation of symptoms. While our understanding of neurofibromin regulation has advanced in recent years, many questions regarding its meticulous control of RAS activity remain to be answered. Our objective is to find ways of increasing neurofibromin's GAP activity, thereby reducing levels of active RAS. NF1 mutations in a hotspot within the N-terminal domain of neurofibromin are known to cause severe phenotype disease. We propose that these mutations confer reduced protein stability. We hypothesize that re-stabilization of the mutant protein provides a therapeutic angle with which to treat severely affected patients, and understanding the pathways that degrade neurofibromin is key to developing this theory. We have identified that neurofibromin exists in an inactive homodimer conformation. Our objective is to understand how these dimers are regulated and how that contributes to neurofibromin activity. In line with this, we propose that activation of the receptor tyrosine kinase c-KIT is an important factor in regulating neurofibromin/SPRED1 complexes: we will determine how activation of c-KIT affects neurofibromin GAP activity.					
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1. Introduction

The overall goal of the project is to find ways of increasing neurofibromin activity, thereby reducing levels of active Ras responsible for pathologies associated with NF1 disease.

2. Keywords

Neurofibromin, SPRED1, Ras GAP, c-KIT

3. Accomplishments

Specific Aim 1

Major Task 1: Identify stability-related posttranslational modifications on neurofibromin protein and relevant enzymes/mechanisms

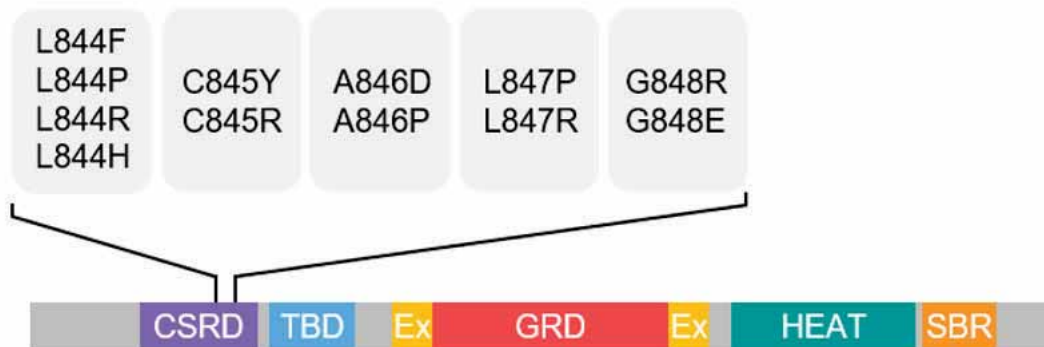


Figure 1. Mutations in neurofibromin that cause severe NF1 phenotypes. CSRD: Cysteine Serine Rich Domain. TBD: Tubulin Binding Domain. EX: Extra domains flanking the GAP Related Domain (GRD). HEAT: Helical Armadillo Repeat Region. SBR: Syndecan Binding Region.

We analyzed biochemical properties of neurofibromin mutants associated with a more severe phenotype (Koczkowska et al, *Am J Hum Genet* 2018). These occur in 5 adjacent codons within the Cysteine/Serine Rich Domain, as shown in Figure 1. We analyzed a representative from each codon.

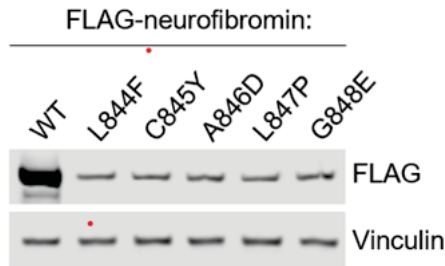


Figure 2. Expression of mutant neurofibromin proteins in 293T cells

We engineered cDNAs for each mutant with an N-terminal FLAG epitope tag, and transiently expressed each in 293T cells. Proteins were analyzed by Western blotting. Figure 2 shows that levels of expression of each mutant was significantly lower than wild type neurofibromin.

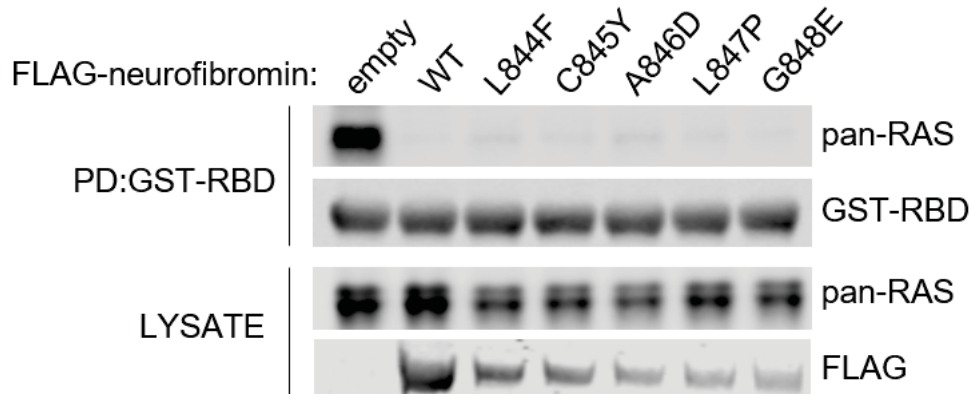


Figure 3. Levels of Ras.GTP in cells transfected with mutant neurofibromin proteins. Lysates were probed with beads coupled to the Ras-binding domain of Raf1 (RBD), beads were washed, eluted and bound Ras proteins detected by Western Blotting.

Despite lower levels of expression, each mutant was able to promote loss of Ras.GTP (Figure 3), showing that they retain GAP activity. We then performed a pulse chase experiment in which cells were treated with cycloheximide to block protein synthesis, and levels of neurofibromin were measured at various times after treatment. Figure 4 shows that one of the mutants tested, L847P, was lost more rapidly than wild type protein, with an estimated half life of about 6 minutes, compared to about 22 minutes for wild type protein (Figure 4).

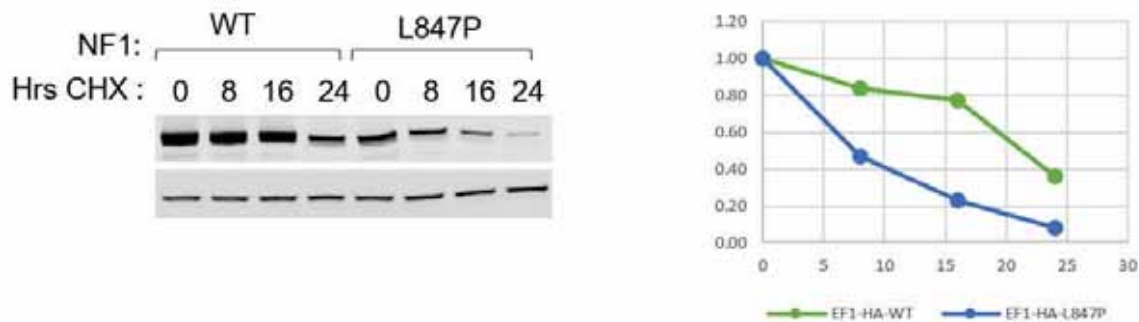


Figure 4. Turnover of wild type neurofibromin and a pathogenic mutant L847P. Cells were treated with cycloheximide (50 ug/ml) at the times shown and levels of proteins determined by Western Blot analysis (left) and digital scanning (right), relative to wild type (1.00).

To address the mechanism by which mutant neurofibromin might be degraded, we analyzed mutant proteins for modification by ubiquitin. We transfected 293T cells with each mutant, pull down the transfected protein through the FLA Tag and probed Western blots with an antibody that recognizes ubiquitin. Figure 5 shows that each mutant is ubiquitylated, each to approximately the same extent.

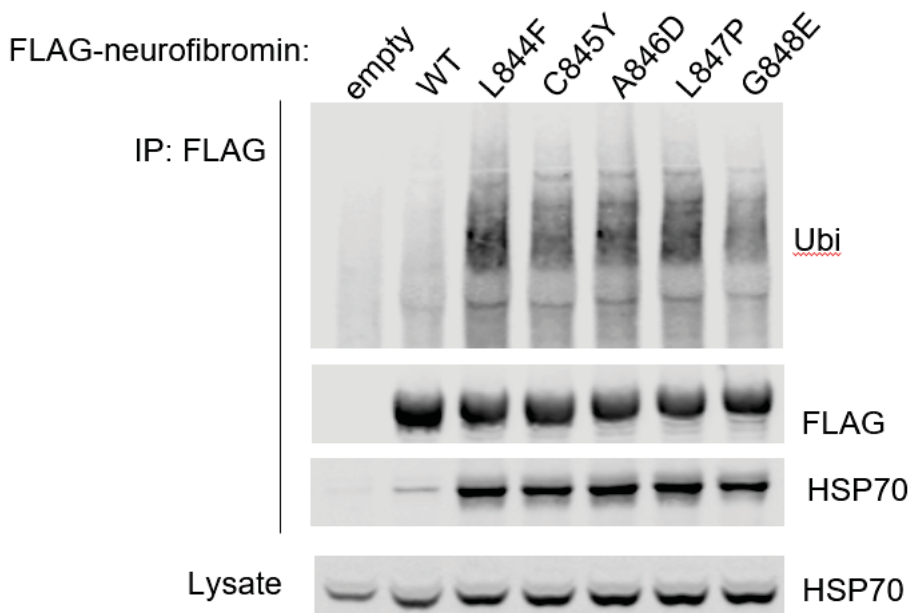


Figure 5. Pathogenic neurofibromin mutants are modified by ubiquitylation.

We also analyzed the G848E mutant protein by mass spectrometry. In comparison to wild type neurofibromin, the mutant protein was associated with components of the proteasome complex, Proteasome 20S Subunit Beta 2-5 (Table 1). In subsequent mass spec analysis, we also identified Hsp70 and Hsp90, and verified these interactions by Western blot analysis (Figure 6).

WT only		mostly WT		G848E only		mostly G848E	
YY2	transcription	HIST1H1T	nucleosome	OTUD4	deubiquitinase	PSMB2	proteolysis
		HIST1H1A	nucleosome	EIF3L	translation	PSMB4	proteolysis
		MYL12B	cell shape	VP52	vesicle trafficking	PSMB5	proteolysis
		CCAR2	genome stability			EIF3M	translation
		MYL12A	cell shape			PABPC3	translation
		MYL9	cell shape			PABPC4	translation
		CDC42EP1	signal transduction			PABPC1L	translation
		AMOT	signal transduction			PSPC1	transcription
		DPY30	cell cycle regulation			ILF3	transcription
		SCAF4	transcription			EFTUD2	splicing
		SARNP	transcription			SF3B1	splicing
		RBM5	splicing			ALDH3A2	metabolism
		ZCCHC6	mRNA decay			PGRMC1	metabolism
		GPATCH4	nucleic acid binding			BABAM2	DSB repair
		DNAJC19	protein import			TAB2	signal transduction
		ATP12A	ion transport				
		BSG	metabolism				
		DCTPP1	metabolism				
		C16orf87	*				

Table 1. Proteins that bind to wild type or mutant neurofibromin, identified by mass spectrometry.

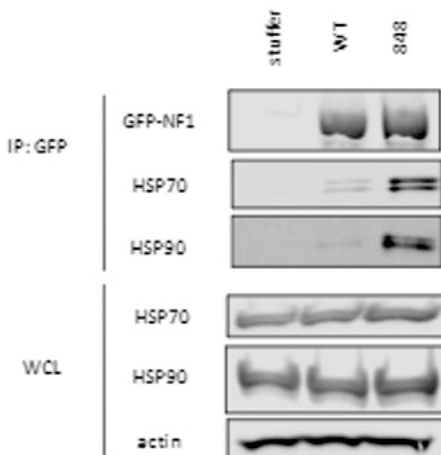


Figure 6. Binding of Hsp70 and Hsp90 proteins to G848E neurofibromin.

Major Task 2: Identify potential targets for therapeutic intervention that stabilize mutant neurofibromin protein

These experiments suggest that pathogenic mutants of neurofibromin such as G848E are degraded by a ubiquitination followed by proteasomal processing. We therefore attempted to stabilize the mutant proteome with proteasome inhibitors such as bortezomib. Figure 7 shows that while the drug was effective at stabilizing beta-catenin, as a positive control, no clear effect was seen on neurofibromin, either wild type or mutant. Similar effects were observed with a different proteasome inhibitor, carfilzomib. We have now tested all available compounds that block proteasomal degradation without a clear outcome. We have developed an assay that detects levels of neurofibromin in cells and plan to test larger libraries in the future.

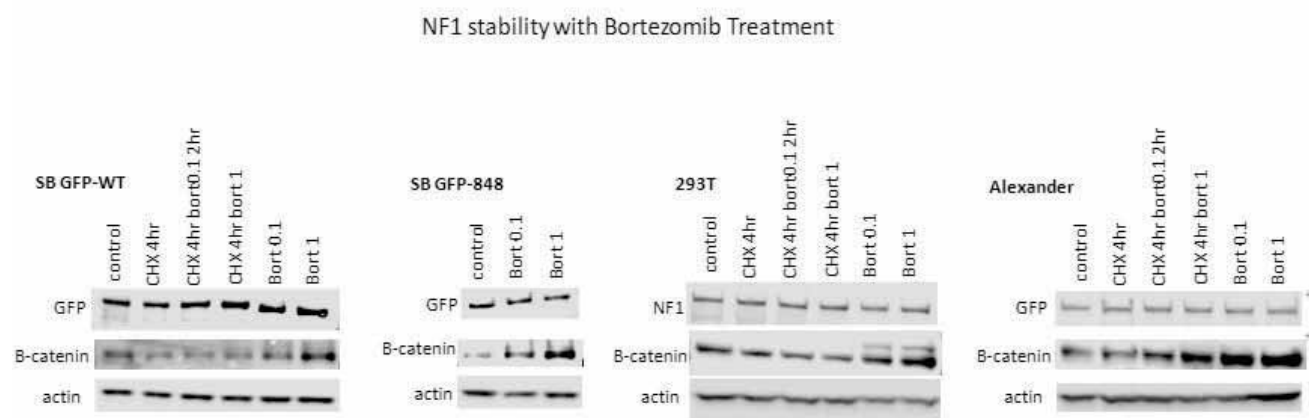


Figure 7. Bortezomib fails to stabilize wild type or mutant neurofibromin. Cells expressing wild type or mutant neurofibromin were exposed to 0.1 μ M or 1 μ M bortezomib in the presence or absence of cycloheximide. Beta-catenin levels were monitored as positive controls for proteasome mediated degradation.

Major Task 3: Examine the effects of wild type neurofibromin on stability of the G848E mutant

Our data showing that mutant neurofibromin is modified by ubiquitylation, associates with proteasome components and is bound to Hsp70 and Hsp90 chaperone proteins (above) indicate this mutant protein is actively degraded. We have also shown the protein retains its ability to dimerize. This suggests the possibility that these high turnover mutants could bind wild type neurofibromin and promote its degradation. To test this, we co-expressed mutant and wild type proteins, each with a different epitope tag, and asked whether increasing doses of the mutant protein caused loss of the wild type. Figure 8 shows that this is indeed the case. Increased expression of either G848E or L847P mutants led to dose-dependent loss of wild type neurofibromin.

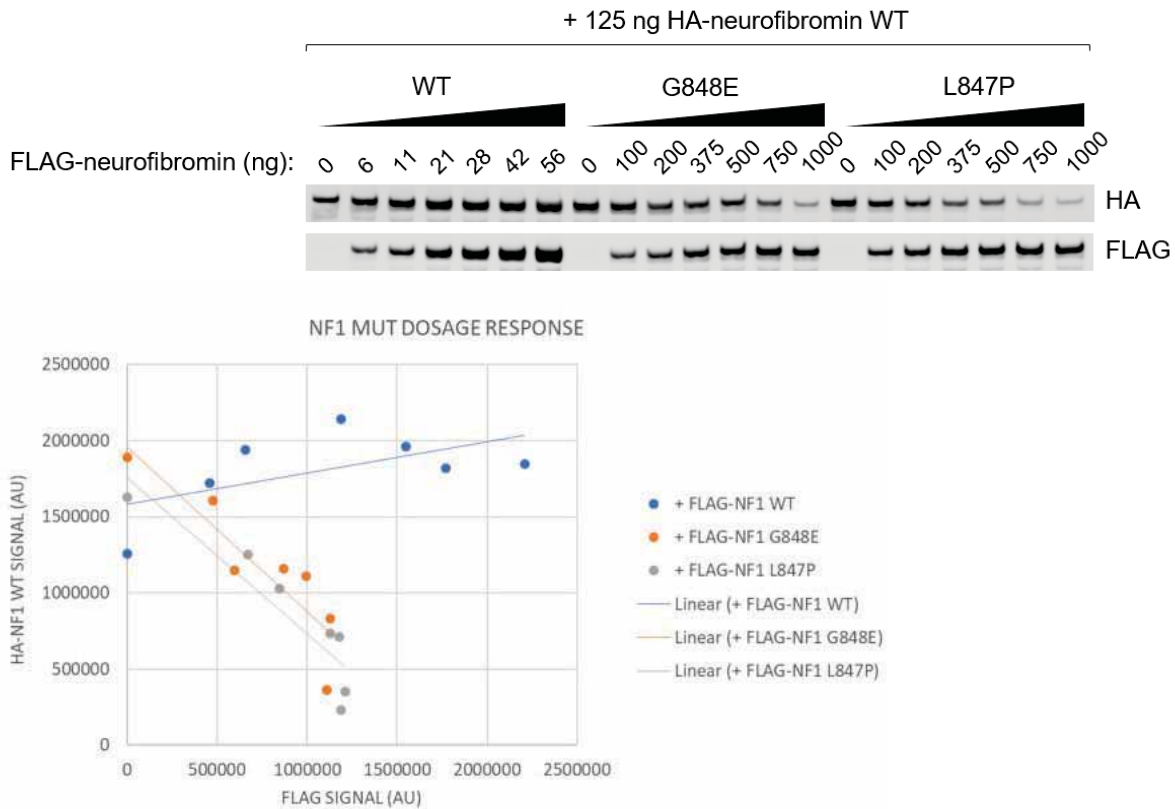


Figure 8. Increased expression of mutant neurofibromin causes loss of wild type protein. 293T cells were transfected with increasing amounts of plasmids expressing wild type or pathogenic HA-tagged mutants, in the presence of fixed amounts of FLAG tagged wild type neurofibromin. Proteins were analyzed by Western blots (top panel) which were analyzed quantitatively in the lower panel.

We sought to provide a structural basis for the severe phenotypic effects of mutations affecting residues 844-848. A recently derived 3.6 Å-resolution cryogenic electron microscopy (cryo-EM) structure of the “short” isoform of human neurofibromin from the Frederick National Lab, shows that ⁸⁴⁴Leu-Cys-Ala-Leu-Gly⁸⁴⁸ form the C-terminal half of α -helix 34 (Figure 9). Except for Gly848, the side chains of the segment line the hydrophobic interfaces of the α 33- α 36 HEAT repeat (Figure 9B, 9C), thus stabilizing the repeat itself and presumably the distal half of the Cysteine-Serine-Rich Domain (CSR) of neurofibromin. The GTPase-activating protein-related domain (GRD) sits atop the α 33- α 36 HEAT repeat, and structural modelling shows that the GRD is inaccessible to RAS binding in the context of full-length neurofibromin dimer due to steric clashes which would form between RAS and the α 33- α 36 HEAT repeat.

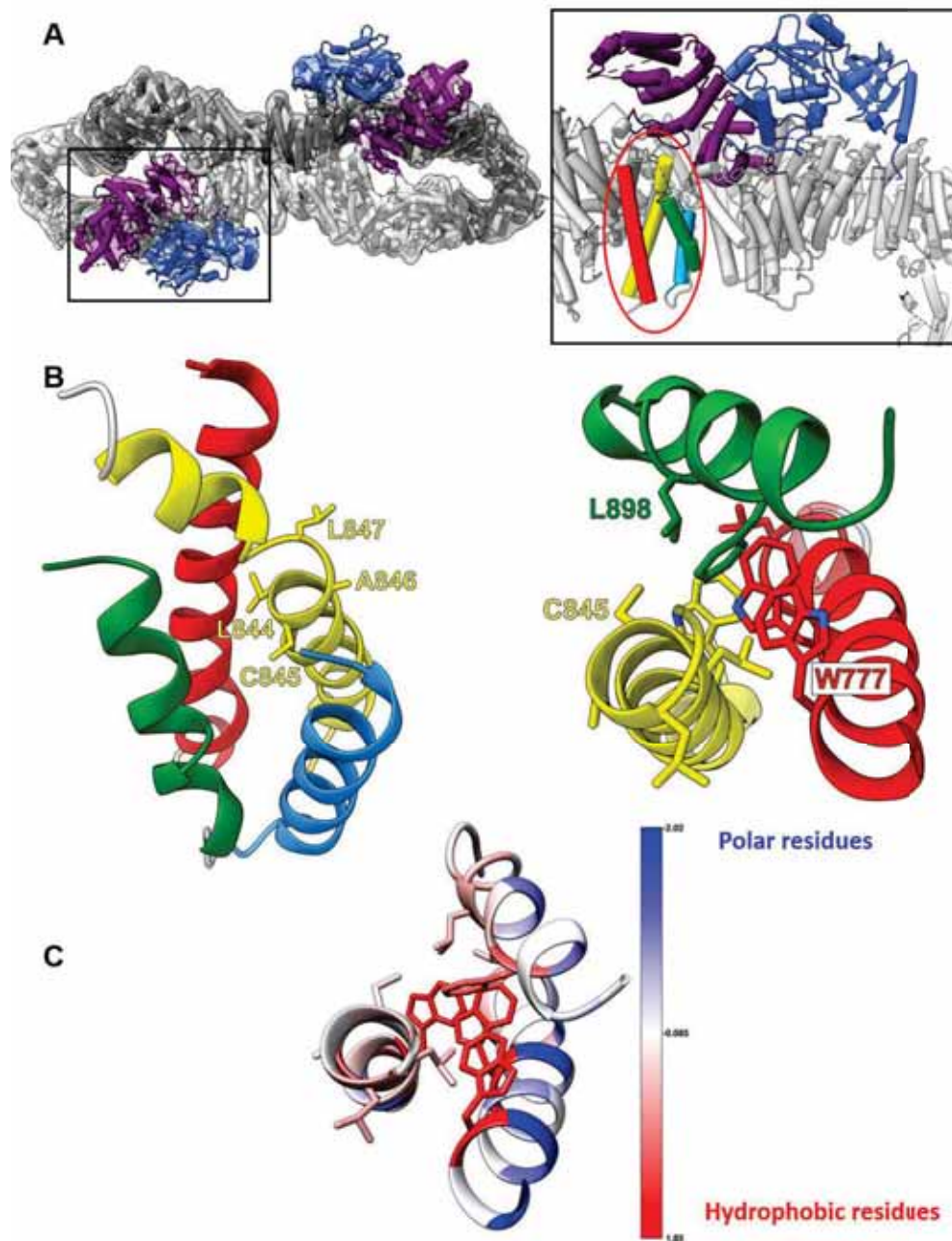


Figure 9. Cryo-EM structure of neurofibromin dimers and detailed view of the 844-848 region.

The above-described mutations localizing within $\alpha 34$ are substitutions of shorter hydrophobic residues with either bulky hydrophobic Phe and Tyr, bulky polar His, Arg and Glu, or a Pro. Introduction of any of the bulkier side chains would necessitate a shift in the position of the backbone atoms which would break the regular pitch of the helix and destabilize the rigid $\alpha 33$ - $\alpha 36$ HEAT interface, whereas introduction of a Pro would directly compromise the fold of α -helix 34 and similarly abolish some of its structural rigidity. Thus, each of these severe phenotype

mutations would disrupt the tight packing of the helices and risk exposing the interfacial hydrophobic segments that are securely buried in the wild-type protein. These uncovered hydrophobic residues would be vulnerable to recognition by members of the protein quality control system, ultimately leading to degradation of the entire neurofibromin dimer and lower levels of protein, both mutant and wild type, in the cell. These structural predictions align with our biochemical analyses and explain why wild type neurofibromin expresses less well in the presence of a destabilizing mutant.

In order to test our structure-driven hypothesis we predicted substitutions in 844-848 residues that would be less disruptive to the α -helix 34 structure. Substitution of Leu847 to Ile and Gly848 to Ala do not cause expression levels to go down like their pathogenic counterparts when expressed in 293T and rescue protein solubility in baculovirus (Figure 10). A similar pattern is observed whereby ‘mild’ substitutions are less ubiquitylated and co-immunoprecipitate with less HSP70 than the pathogenic variants.

We hypothesized that N-terminal pathogenic substitution variants outside of the 844-848 region may be similarly disruptive to neurofibromin structure. We used our cryo-EM model to identify variants W837F, F894S and L898R (present in the ClinVar database) that may cause disruption to the helices, and at the same time predicted ‘less-disruptive’ versions as we had done for L847 and G848 (Figure 10). The helices that contain these variants are conserved between species as for the 844-848. All 3 pathogenic substitutions express less well than wild-type and can be ‘rescued’ by Trp837 to Phe, Phe894 to Ser and Leu898 to Arg in 293T cells (Figure 10 top panel), and change from insoluble to soluble protein in baculovirus (Figure 10 lower panel) and display the same pattern of ubiquitylation and HSP70-binding as for the 847 and 848 pairs. Despite lower expression levels, W837F, F894S and L898R also retain GAP activity and binding to SPRED1. Given the observation the 844-848 substitutions act in a dominant negative manner by destabilizing wild-type protein, we repeated a dose response curve and found that F894S and L898R behave in a similar way, solidifying our observations that variants that disrupt protein structure target wild-type protein for degradation through dimerization (Figure 10).

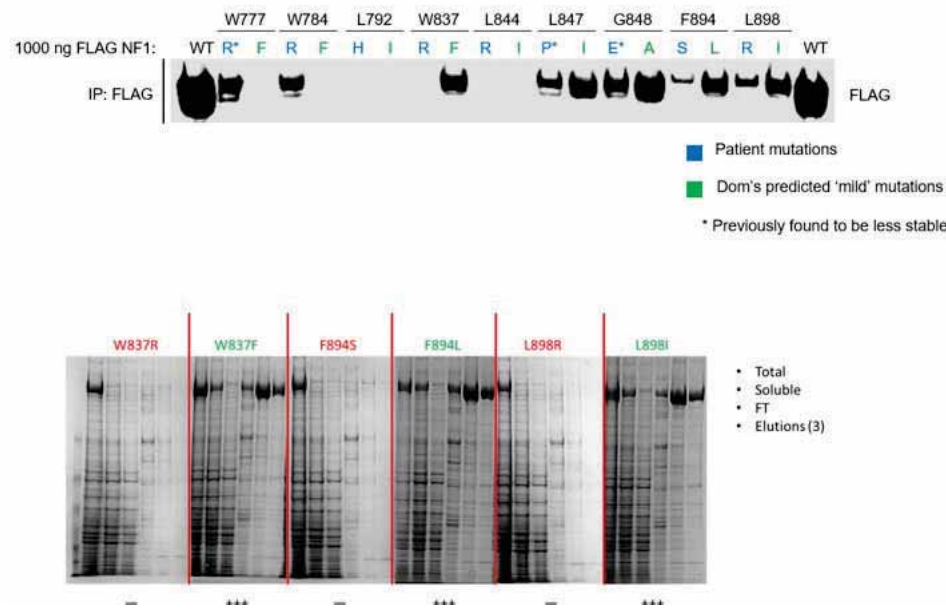


Figure 10. Stability of additional pathogenic mutants expressed in 293T cells (top panel) or in insect cells using baculovirus expression (lower panel).

We have identified a phosphorylation site on neurofibromin that has not been reported previously. It is at codon 864, adjacent to the 844-848 hot spot regions discussed above. None of the pathogenic mutants characterized here are phosphorylated on this site, as shown in Figure 11. We have made a phosphomimic and a phosphorylation defective mutant at this site and observe that lack of phosphorylation leads to unstable protein. The codon 864 kinase is predicted to be a member of the CDK family, but our tests of effects of inhibitors of CDK1 and CDK4 proved negative.

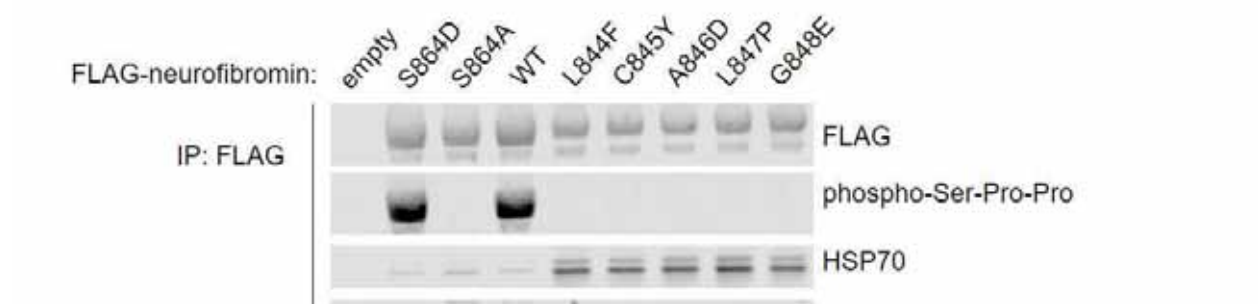


Figure 11. Identification of a novel site of phosphorylation in neurofibromin using a phospho-specific antibody that recognizes the P-Ser-Pro-Pro motif.

Specific Aim 2

Major Task 1: Biochemical analysis of neurofibromin dimers/monomers

Our work on the biochemical analysis of neurofibromin dimers has now been published (Sherekar et al, *J Biol Chem* 2019). The protein appears to be an obligate homodimer, with a dissociation constant of 1 nM, or lower. We have attempted to disrupt the homodimer by co-expressing neurofibromin with oncogenic RAS, and by exposing cells to MEK inhibitors, or AKT inhibitors, but have failed to observe any evidence of dissociation. Our structural analysis of the dimeric structure is nearly complete: this should allow us to test mutations that could affect dimerization and so allow us to assess the functional significance of dimerization on GAP activity. This work is being performed in collaboration with the Cryo-EM facility at the Frederick National Labs in Maryland. As published in Sherekar et al, the full-length dimeric protein is relatively inactive. Cryo-EM structures of the neurofibromin protein structure indicates that RAS proteins are occluded from binding to the baculovirus generated neurofibromin dimer. We conclude that neurofibromin is regulated at the level of RAS binding, and will, in the future, screen for compounds that affect binding using a cell based BRET system that has been developed at Frederick National Labs in collaboration with my lab.

We have previously screened for genes which would affect cells in cells that express wild type neurofibromin, but not in cells lacking the protein. These efforts failed, partly because loss of neurofibromin upregulates Ras activity and makes cells resistant to regulatory proteins that are downstream of Ras, and therefore do not act through neurofibromin itself. We have now taken a different approach: we have searched for compounds that affect the MAPK pathway in the presence of neurofibromin but not in absence using P-ERK as a readout. Using this approach, we have discovered that drugs that inhibit PI 3' kinase-beta cause a decrease in P-ERK in wild type cells, but not in isogenic NF1-knockout cells, or in cells expressing oncogenic RAS. Figure 12 shows that exposure of MEFs expressing wild type KRas 4b to PI 3' kinase inhibitors that block the beta isoform (yellow boxes) inhibits P-ERK (panels 1 and 3, the latter being a repeat experiment identical to panel 1). In contrast, cells in which NF1 has been ablated by CRISPR knock-out are unaffected (panels 2 and 4).

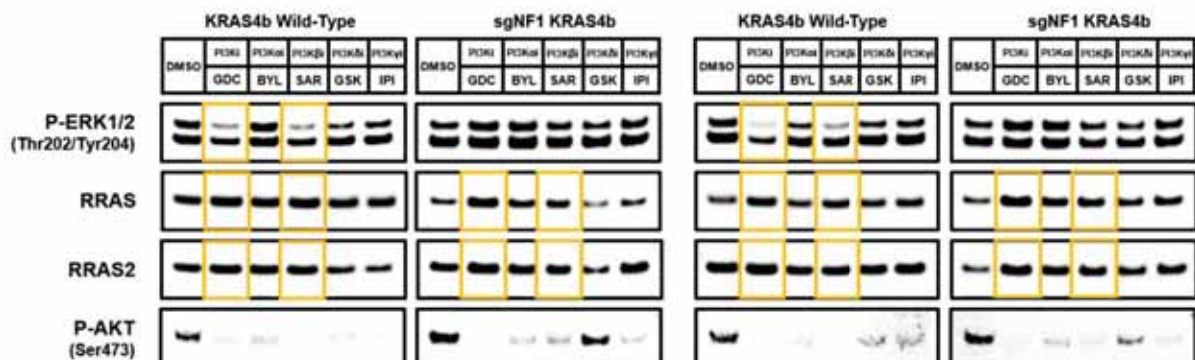
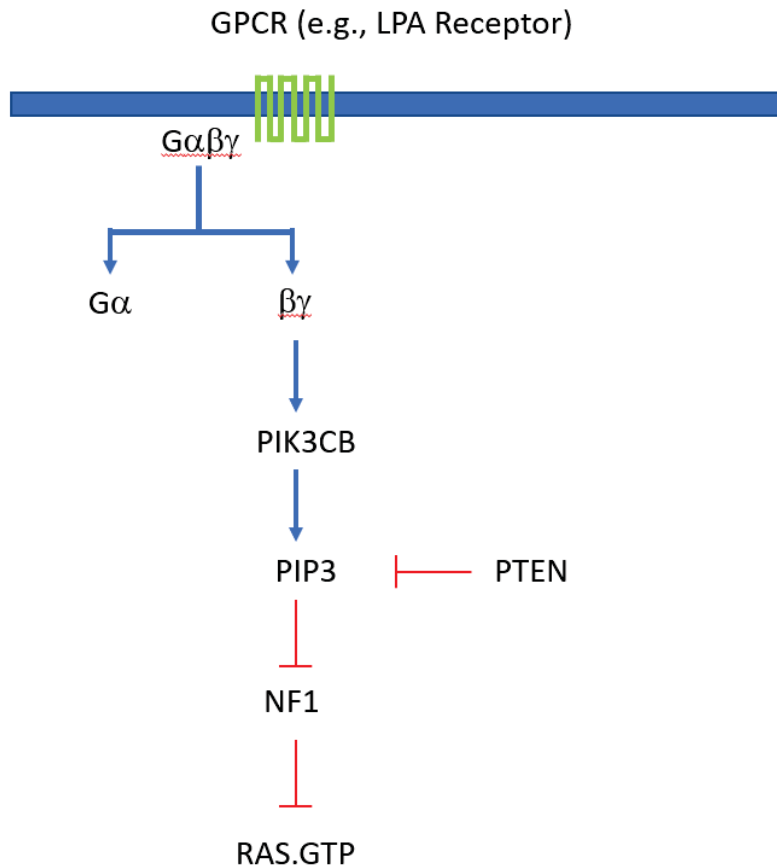


Figure 12. PI3 kinase inhibitors that inhibit the beta-isoform inhibit P-ERK in cells expressing neurofibromin, but not NF1 -/- cells. MEFs expressing KRas4b as the only Ras isoform, or isogenic counterparts in which NF1 has been knocked out by CRISPR were treated with a panel of PI3 kinase inhibitors: GDC: GDC-0941, pan PIK inhibitor. BY: BYL719, PIK-alpha specific inhibitor. SAR: PIK-beta inhibitor. GSK:GSK2126458, PIK/mTOR inhibitor. PIK3K gamma inhibitor. After 4 hours, cells were harvested and analyzed by Western blots.

These results lead to the following model:



In this model, activation of G-proteins known to regulate the MAPK pathway, such as the receptor for lysophatidic acid (LPA) leads to release of G-protein $\beta\gamma$ subunits which activate PI 3' Kinase beta (PIK3CB) and generate PIP3. This inhibits neurofibromin allowing Ras.GTP to accumulate. Inhibition of PI3' kinase beta leads to activation of neurofibromin and suppression of Ras.GTP. We have shown that oncogenic mutants of KRAS (G12C and G12V) are resistant to the effects of PIK2 CA-beta inhibitors, supporting this model. Whether PIP3 acts directly on neurofibromin or via other intermediates remains to be determined. These data were generated in isogenic MEF cell lines and are now being expanded to physiological relevant cell types such as Schwann cells and melanocytes.

We have performed transcriptional profiling of MEFs in which we have knocked out NF1, or SPRED1 and 2, in the background of KRAS G12D. The results are summarized in figure 13. A set of genes associated with EMT was identified as significantly reduced in cells lacking either NF1 or SPRED1 and 2.

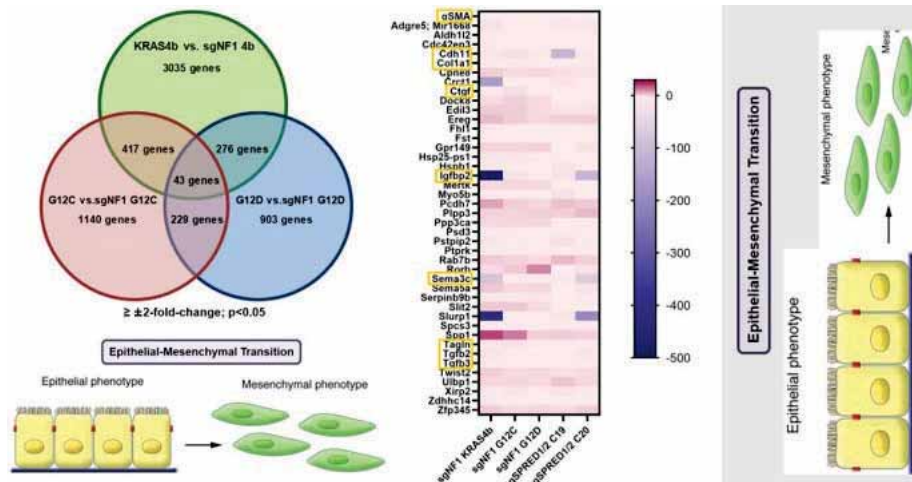


Figure 13. Gene expression analysis from MEFs. Three comparisons are shown: Green circle (left) genes differentially expressed in MEFs expressing only KRAS4b wild type, vs these cells in which NF1 was knocked out by CRISPR. Orange circle; single isoform KRAS G12C MEFs, before and after knockout of NF1. Blue circle, KRASG12D MEFs, before and after NF1 knock out. Shared genes between these treatments are listed in the middle panel. Many are genes known to be involved in EMT, the epithelial to mesenchyme transition.

To verify these data at the protein expression level, we performed Western blot analysis, using antibodies against representative genes down regulated at the level of mRNA expression, namely IGFBP2, COL1A1, CDH11, α -SMA and TAGLN. Of these, IGFBP2, COL1A1 and CDH11 were dramatically reduced following knock-down (Figure 14).

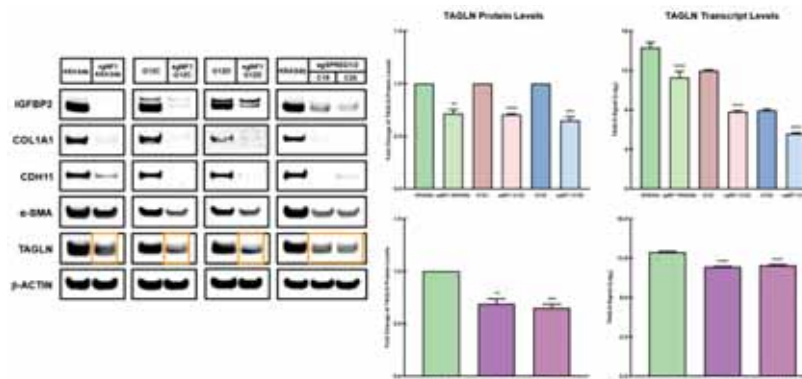


Figure 14. Genes whose expression is significantly reduced following knockout of NF1 or SPRED1 and 2, identified by Western blots from parental MEFs.

We then compared expression of these genes in human Schwann cells from an NF1 patient, and from a neurofibroma from the same patients and another neurofibroma from a second NF1 patient. In both neurofibromas, the NF1 gene has undergone LOH, and no neurofibromin is expressed. Figure 15 shows that, remarkably, the same genes were downregulated in neurofibromas derived from an NF1 patient as those derived from MEFs shown in Figure 14.

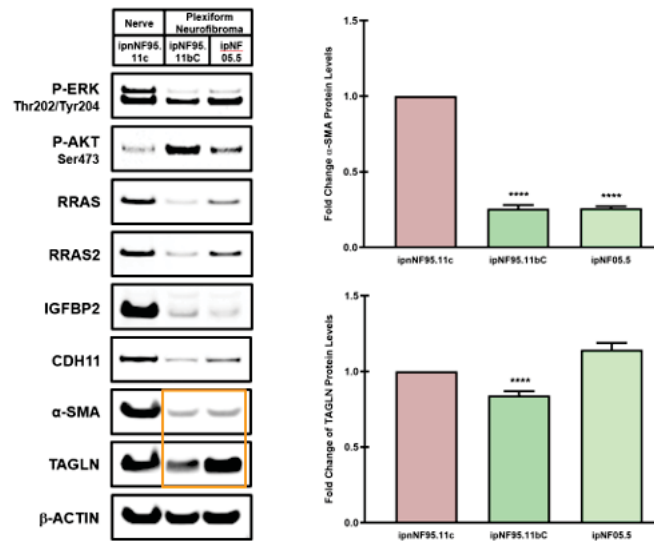


Figure 15. Loss of expression of specific NF1-regulated genes in human schwann cells and neurofibromas derived from an NF1^{+/-} patient

The fact that a set of genes is down regulated following knockout of both NF1 and SPRED1 and 2 suggests these genes are regulated by neurofibromin GAP activity, since this activity is SPRED dependent. More importantly, these genes are affected by NF1 knock down in the presence of oncogenic RAS proteins, which are refractory to GAP regulation by neurofibromin. Loss of expression of these genes these genes is therefore independent of RAS driven MAPK activity and is likely to due to up-regulation of other RAS proteins such as RRAS2 and MRAS, which are expressed in these cells. Figure 16 shows the effects of MEK inhibition on cells following neurofibromin knock out.

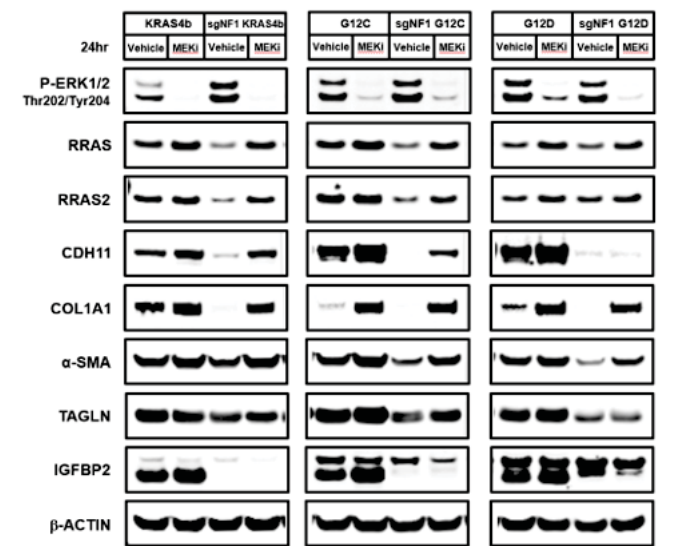


Figure 16. Effects of MEK inhibitors on gene expression following NF1 knock out in G12C or G12D KRAS single isoform MEFs.

Specific Aim 3

Major Task 1: Determine the nature of different neurofibromin and SPRED1 complexes in cells

This task has been pursued in the context of complexes between neurofibromin, SPRED1 and KIT, as described below.

Major task 2: Identify SPRED1 phosphorylation sites

293T cells transfected with FLAG tagged SPRED1 were treated with Stem Cell Factor (SCF) for up to 40 minutes. Cells were lysed, and P-Tyrosine on SPRED1 was detected by Western blot. Figure 17 shows 3 separate biologic replicates in which P-Tyrosine was detected at a maximal level about 30 minutes after stimulation. These replicated samples were analyzed by mass spectrometry.

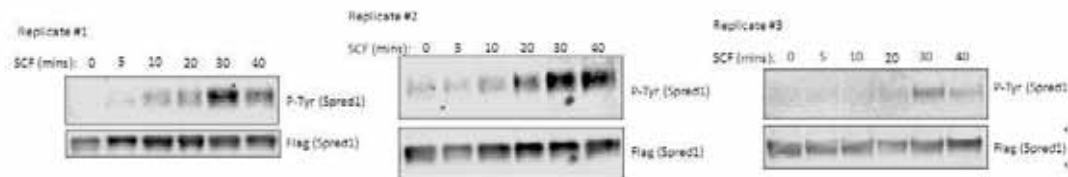


Figure 17. Detection of P-Tyrosine on SPRED1 after treatment with SCF.

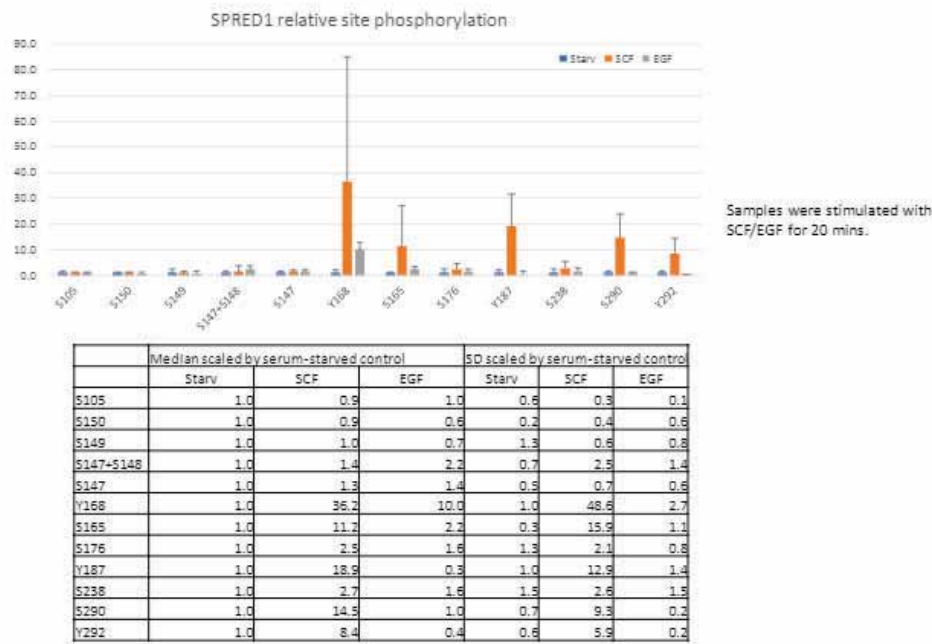


Figure 18. Phosphorylation sites on SPRED1 detected by mass spectrometry following treatment with SCF or EGF.

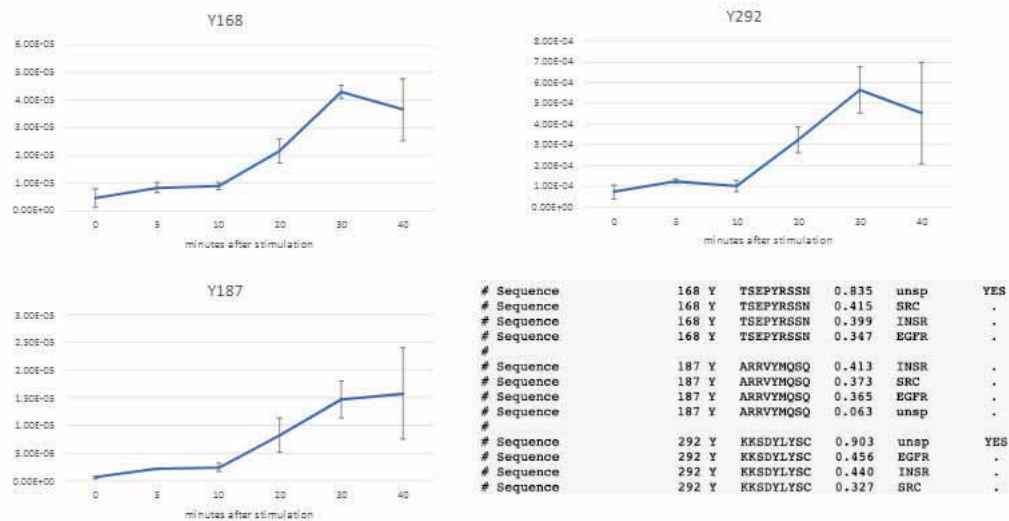


Figure 19. Time course of tyrosine phosphorylation on major sites after treatment with SCF.

Three major phosphorylation sites were identified on SPRED1 on response to SCF stimulation of c-KIT, Y168, Y187 and Y292, shown in Figures 18 and 19. These sites flank the Kit Binding Domain (KBD) on SPRED1 (Figure 20). The EVH1 domain is the site of binding to neurofibromin (Yan et al, *Cell Rep* 2020). SPR is the Sprouty-related Domain, a region thought to be necessary

for plasma membrane binding of the SPRED and SPROUTY family of proteins (Lorenzo and McCormick, *Genes & Dev* 2020).



Figure 20. SPRED1 domains.

We have mapped the region of c-KIT that interacts with SPRED1 using a new technique developed in the McCormick laboratory that uses a modified yeast 2-hybrid technology coupled to next-gen sequencing (Figure 21). This method is described in Castel et al, *J Biol Chem* 2020. Using this novel method, we discovered that SPRED1 binds to the kinase insert region of c-KIT, a region previously identified as the site of binding of the p85 subunit of PIK3CA and the GRB2/SOS complex. We are currently investigating whether SPRED binding affects binding of these key signaling proteins.

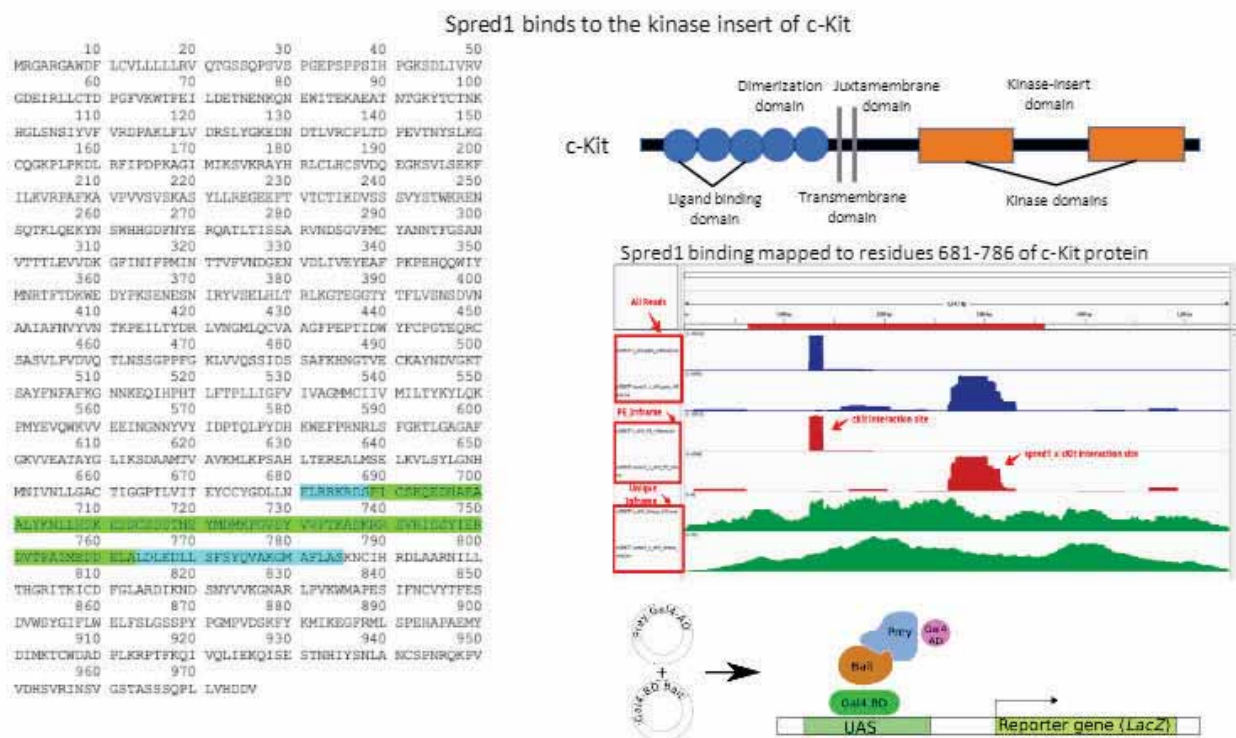


Figure 21. Mapping regions of c-KIT that bind to SPRED1 using Do-My-Seq technology. Fragments of c-KIT were expressed as a library in yeast. Fragments that bind SPRED1 grow in selected medium and are identified by DNA sequencing. The binding region maps to the kinase insert domain of the c-KIT receptor (highlighted as green in the sequence in the left panel).

SPRED1 binds to neurofibromin through the EVH1 domain (Yan et al, *Cell Rep* 2020). Figure 22 shows that a complex forms between neurofibromin, SPRED1 and c-KIT. In this experiment, we transfected SPRED1 null 293T cells with neurofibromin and showed by immunoprecipitation that it associates with c-KIT in a SPRED1-dependent manner. We also knocked out endogenous SPRED2 protein to be sure that the trimeric complex was indeed dependent on SPRED and did not involve a potential heterodimer with SPRED2.

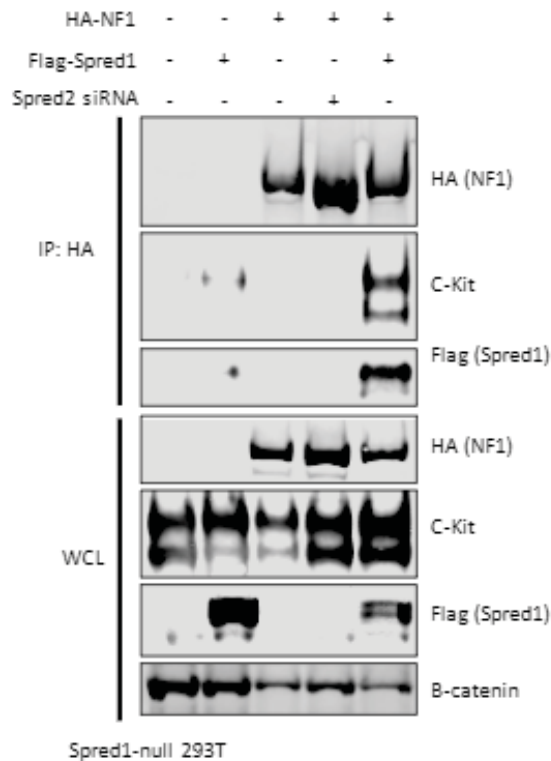


Figure 22. Neurofibromin associates with c-KIT in a SPRED1 dependent manner. Spred1 deficient 293T cells were transfected with neurofibromin and SPRED1, and association with endogenous c-KIT was detected by Western blot analysis.

We next tested whether SPRED binding to KIT is dependent on KIT tyrosine kinase activity. We transfected HA-tagged SPREDs 1, 2 or 3 and Flag tagged KIT, wither WT, activated or kinase dead mutants, as shown in Figure 23. We pulled down SPREDs through the HA tag and probed Western blots with antibodies against phosphor-tyrosine or FLAG. Association between SPREDs 1 and 2 and KIT was significantly higher in the kinase active KIT mutant (D816V) but was detectable in cells expressing wild type KIT or kinase dead KIT (K623M). SPREDs 1 and 2 were strongly phosphorylated on tyrosine, but SPRED3 was not.

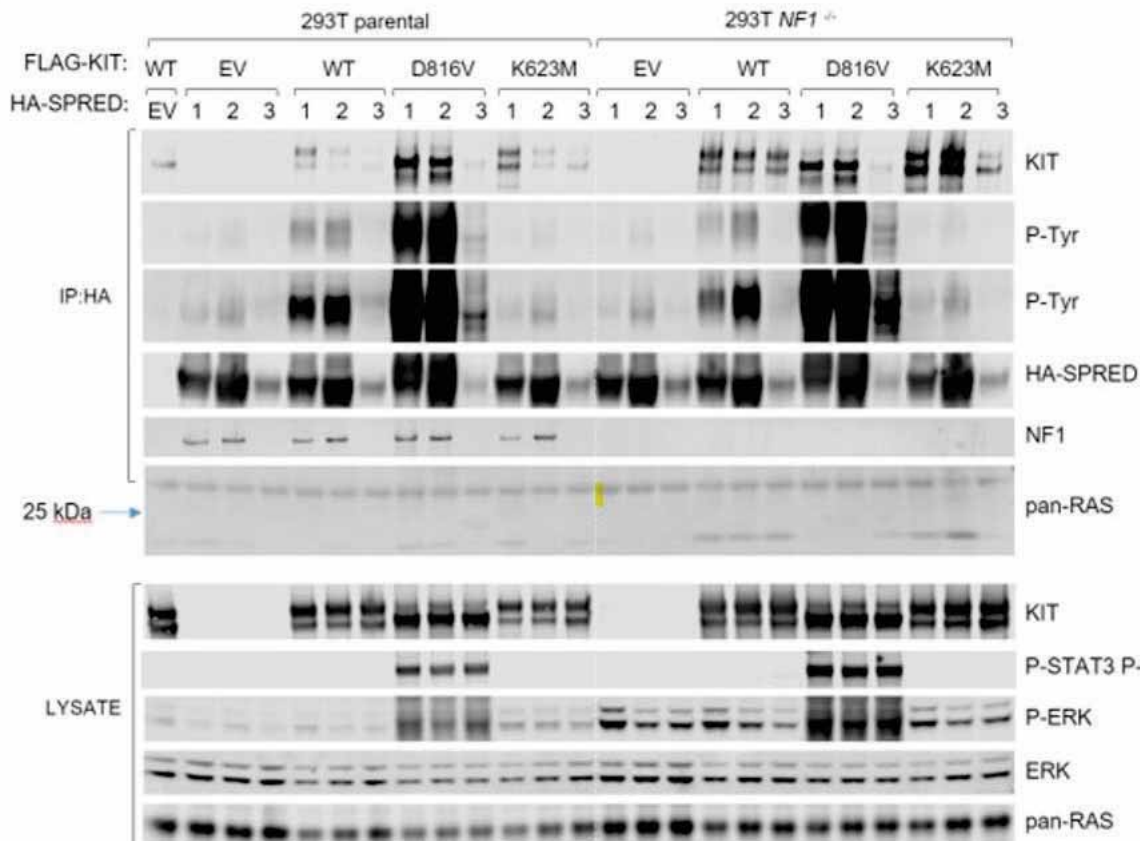


Figure 23. Association between SPREDs and KIT is enhanced by tyrosine phosphorylation.

Recruitment of the SPRED/neurofibromin to KIT in response to SCF, as shown above, could result in repression of signaling from other RTKs, through persistent accumulation of active neurofibromin at the plasma membrane. To test this, we measure the effect of EGF on 293T cells in the presence or absence of SPRED, KIT or neurofibromin. Figure 24 shows that expression of SPRED represses signaling from EGF as expected, but expression of KIT has a similar effect. Furthermore, the repressive effects of KIT are dependent on neurofibromin. Therefore, KIT activation can have different outcomes: it activates the MAPK pathway directly, but can repress activation by other RTKs. This may explain the phenomenon of Piebaldism, in which individuals with defective KIT expression show characteristic loss of pigmentation, but often also exhibit café au lait spots typically seen in NF1 disease through hyperactivation of RAS in melanocytes.

The KIT-SPRED complex regulates signaling from EGF-R in an NF1-dependent manner

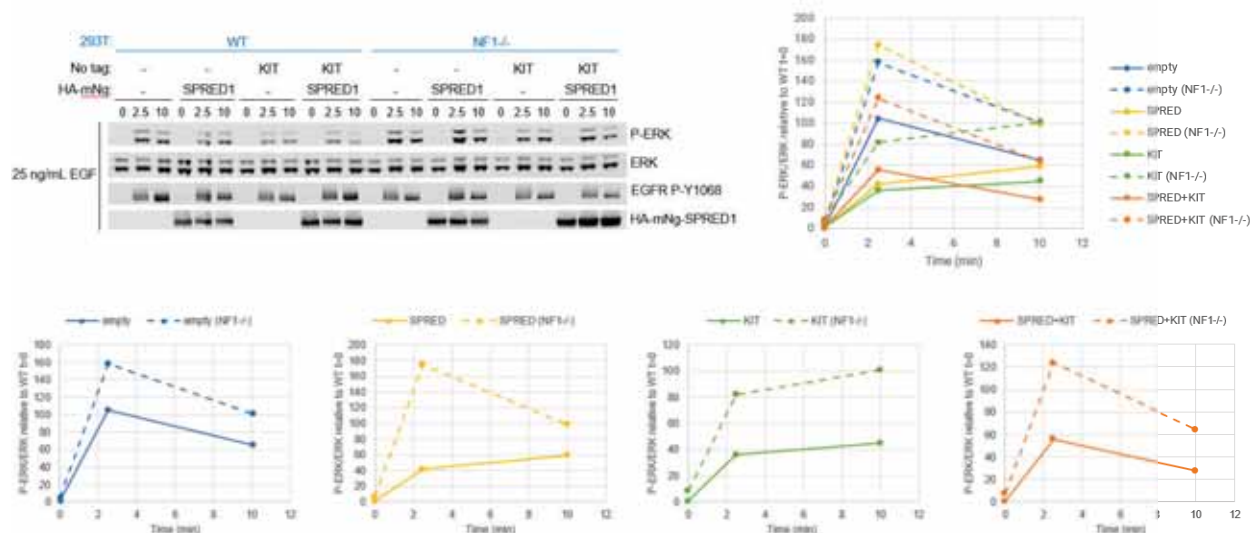


Figure 24. Inhibition of EGF signaling by the KIT/SPRED1/neurofibromin complex

A long-term goal is to elucidate the structure of the KIT/SPRED1/neurofibromin complex by CRYO-electron microscopy. To optimize interactions for this project, we tested a panel of mutations in SPRED1 that we expect to increase the affinity of the interaction, based on our X-Ray structure of the SPRED1 EVH domain bound to the GAP domain for neurofibromin, including S28D, L39E and T102V. The T102V mutant increased this interaction by about 10-fold and enabled us to produce SPRED1/neurofibromin complexes in sufficient quantity to facilitate purification for structural analysis (Figure 25).

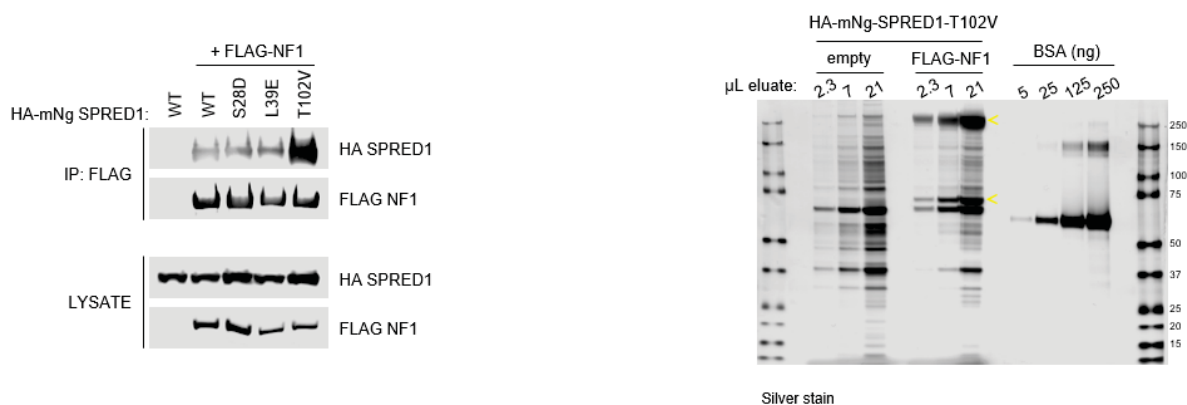


Figure 25. Mutations in SPRED1 that increase binding to neurofibromin and initial purification of complexes utilizing the T106V mutant.

Association between KIT and SPRED1 is increased by exposure of cells to the KIT ligand Stem Cell Growth Factor, as shown in Figure 26.

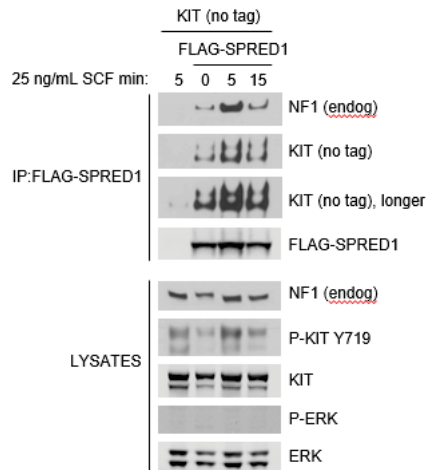


Figure 26. Association of KIT with SPRED1/neurofibromin in response to SCF

In addition to the KIT/SPRED1/neurofibromin complex, we intend to solve the structure of the SPRED1/neurofibromin complex bound to KRAS Q61L, a mutant shown previously to bind to neurofibromin with high affinity. Figure 27 shows that the ternary complex can indeed be produced and purified, and will be further developed to generate sufficient quantities for structural analysis.

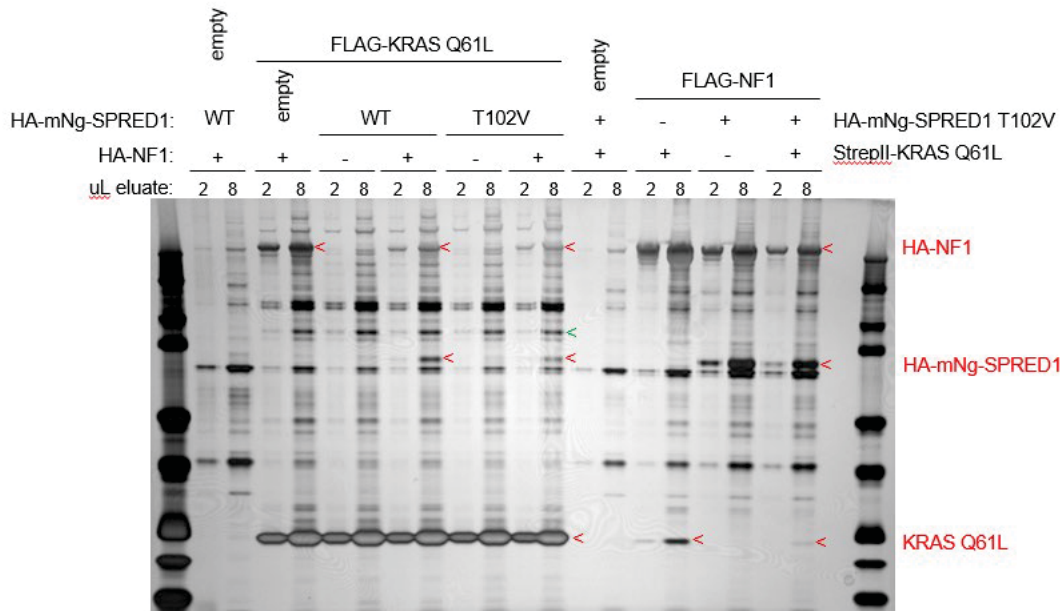


Figure 27. Production of complexes between SPRED1 T102V, neurofibromin and KRAS Q61L

Major task 3: BRET assay and co-IP studies for c-KIT/SPRED1 regulation of neurofibromin

Based on the data shown in Figure 21, we are investigating how the neurofibromin/SPRED1/c-KIT complex is regulated. To facilitate this analysis, we have developed a BRET based assay in which binding of SPRED1 and neurofibromin in cells is measured using energy transfer technology. Figure 28 shows that binding in cells can indeed be detected using BRET technology.

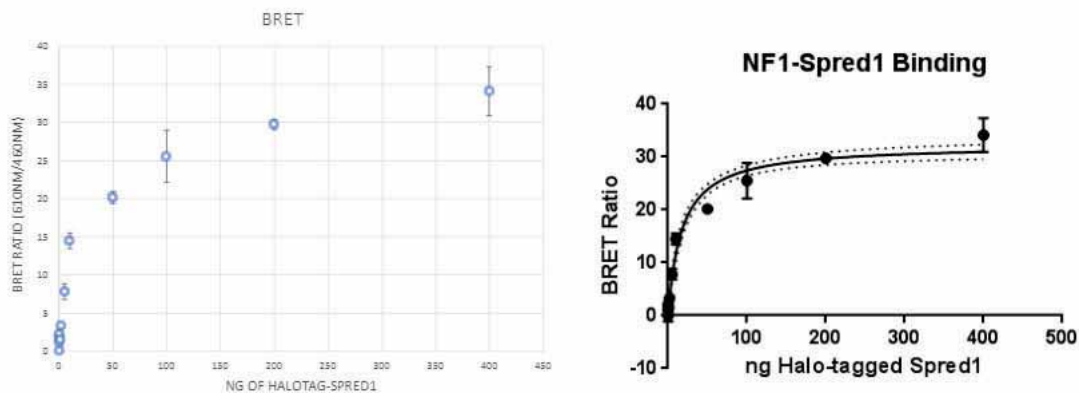


Figure 28. Interaction of SPRED1 and neurofibromin detected in cells using BRET technology.

To identify proteins that bind to different members of the SPRED family, we performed mass spec analysis on epitope tagged SPRED1, 2 and 3 expressed in 293T cells. Surprisingly, SPRED3 was strongly associated with 14-3-3 proteins, indicating this member of the family is highly phosphorylated (Figure 29). However, of more interest, SPRED2 associated specifically with RSK2. Because this kinase plays a central role in signal transduction pathways involving both MAPK and PI 3 kinase pathways, we have pursued this further.

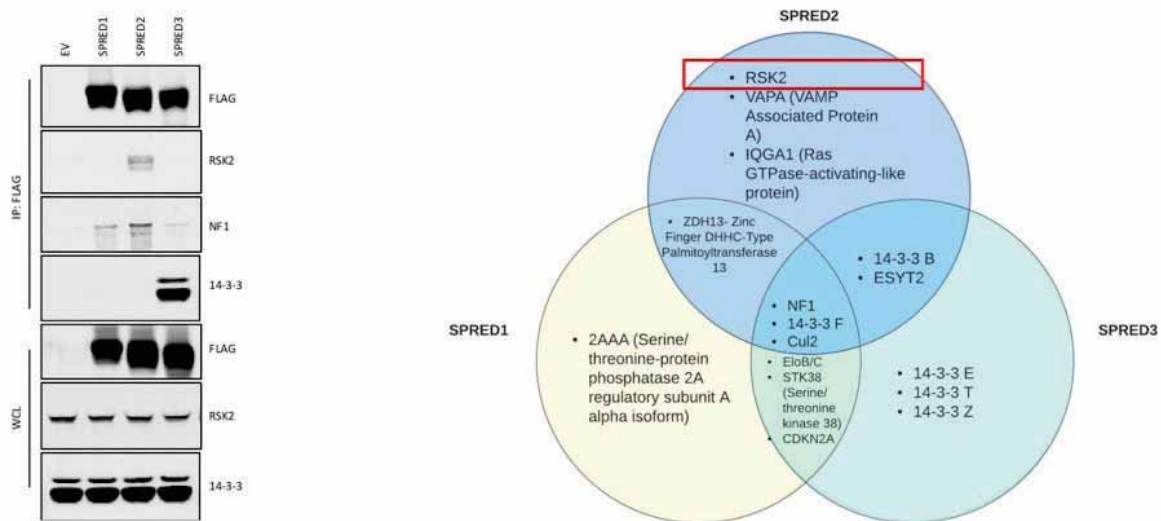


Figure 29. SPRED2, but not SPRED1 or SPRED3, binds to RSK2. Interaction was detected by immunoprecipitation with FLAG-tagged SPRED proteins and probed with antibodies against RSK2 to detect endogenous protein.

Next, we identified the region of SPRED2 to which RSK2 binds, by expressing a panel of deletion mutants in 293T cells, followed by pull-down on these mutants and probing for RSK2 on Western Blots, as shown in Figure 30. Amino acids 123-200 are necessary for binding.

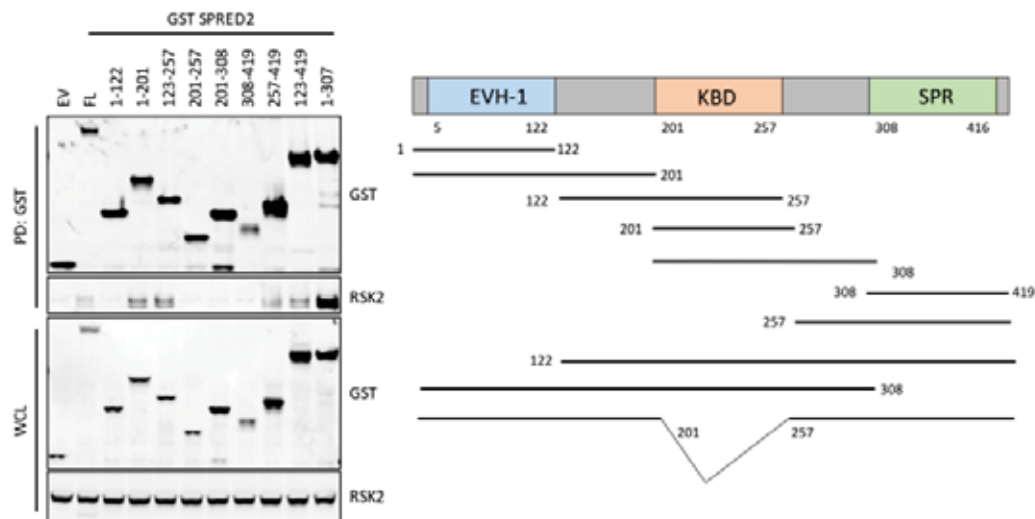


Figure 30. Mapping the region of SPRED2 to which RSK2 binds. A panel of mutants were transfected into 293T cells and endogenous RSK2 binding was detected by Western Blot.

We then repeated this experiment in cells in which the Raf MAPK has been activated by addition of 4-Hydroxy Tamoxifen (4HT). These cells express a RAF:ER fusion protein that is rapidly activated upon addition of 4HT. RSK2 was detected bound to SPRED2 mutants as in Figure 31. Binding is strongly dependent on 5HT. This strongly suggests that RSK2 binding is a feedback loop that is activated when cells activate MAPK signaling.

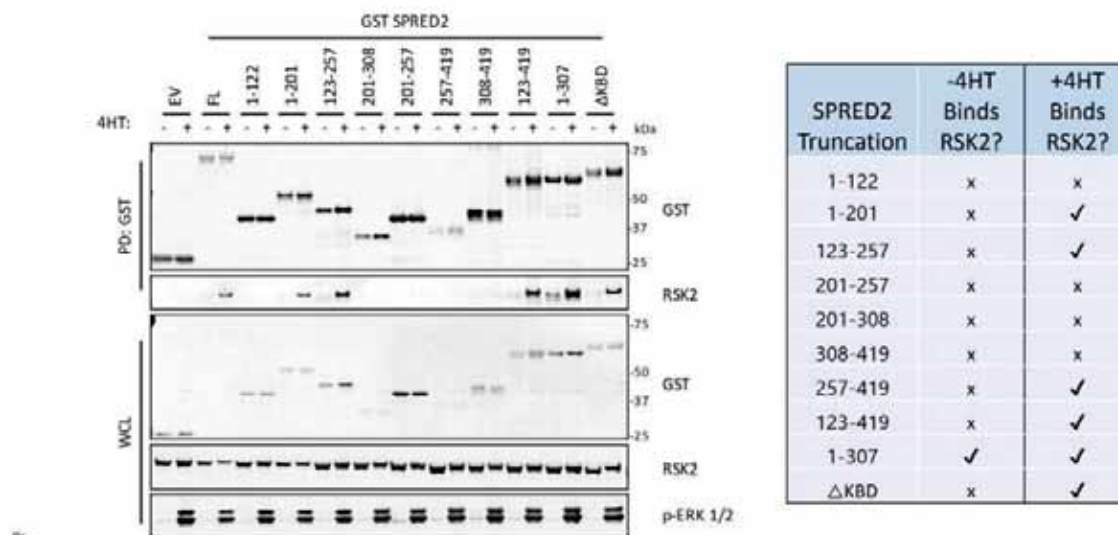


Figure 31. SPRED2 binding to RSK2 is enhanced by activation of the RAF MAPK pathway.

We then tested whether this novel interaction is inhibited by drugs that block MEK (trametinib), ERK (SCH772984) or RSK (LJH685). We found that interaction is completely blocked following inhibition of either MEK or ERK, but not RSK itself.

RSK kinases bind to proteins containing a DDVF motif and protein-protein interactions influence the phosphorylation of RSK substrates. Our biochemical and structural analyses also identified such a motif in SPRED2, and this prompted us to test whether the association with SPRED2 alters the phosphorylation of known RSK substrates. We overexpressed SPRED2 in cells and observed a decrease in the phosphorylation of the canonical RSK substrate, YB1 at S102 (Figure 32 panel A).

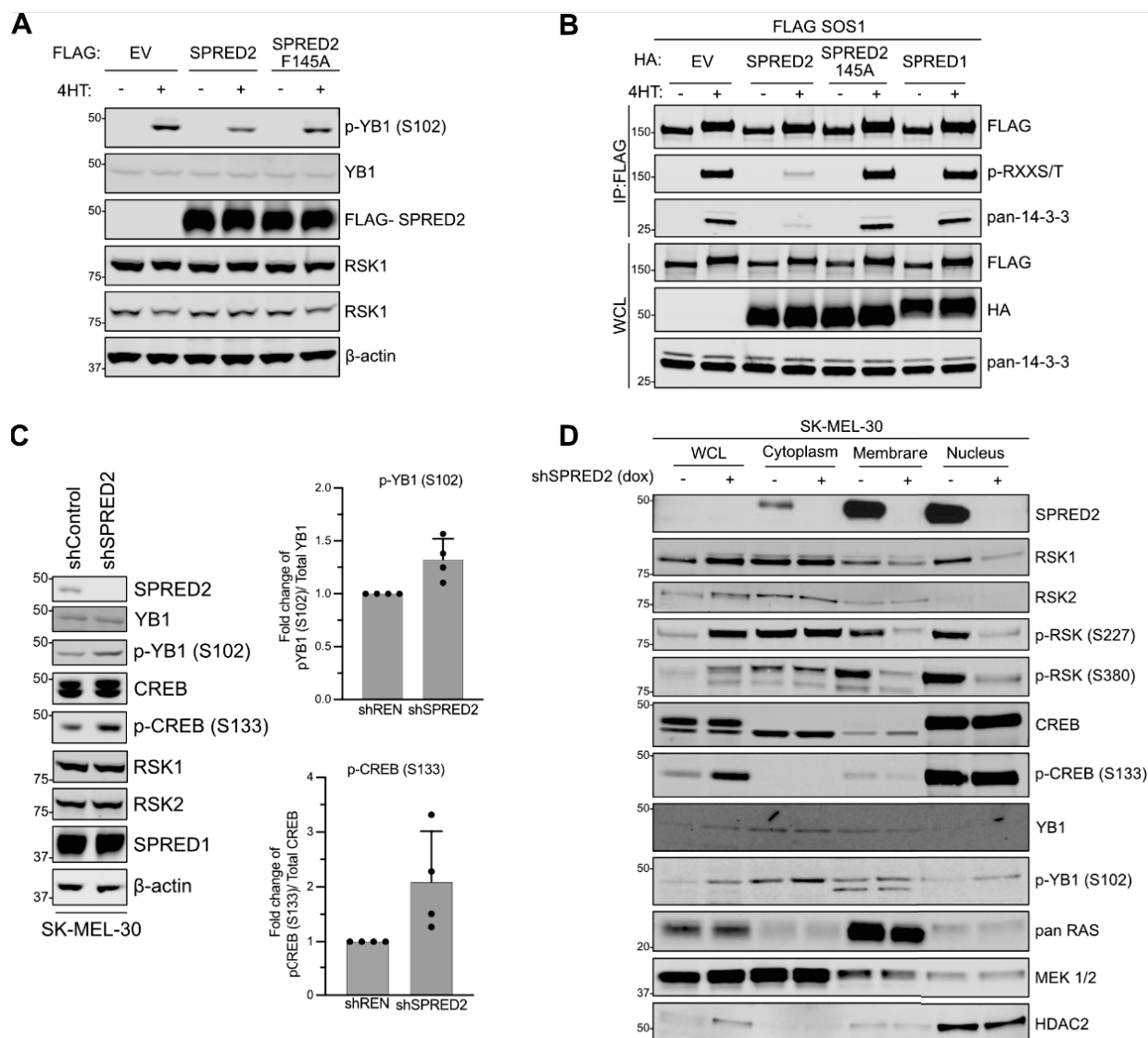


Figure 32. Effects of SPRED2/RSK interaction on RSK substrate phosphorylation

Similarly, we observed a decrease in RSK-mediated phosphorylation of Son of sevenless homolog 1 (SOS1) when SPRED2, but not when the RSK-binding deficient SPRED2 F145A mutant was overexpressed (Figure 32). To further test these findings in an endogenous setting, we knocked down *SPRED2* in several cells (Figure 32C). *SPRED2* knockdown resulted in an increase in phosphorylated CREB (S133) and phosphorylated YB1 (S102), further supporting a model whereby SPRED2 regulates RSK substrate availability. SPRED proteins are known to promote the membrane localization of neurofibromin and thus we hypothesized that SPRED2 may also regulate RSK localization. To test this, we performed subcellular fractionation in cells with *SPRED2* knockdown. We observed a reduction in the phosphorylation of RSK2 at S386 (S380 in RSK1), the phosphorylation site that recruits PDK1, and S227 (S221 in RSK1), the site in the active loop of the NTKD that is phosphorylated by PDK1, at the plasma membrane and nucleus in these cells (Figure 32D). Together, these data indicate that SPRED2 regulates the phosphorylation of RSK substrates, possibly by modulating its subcellular localization.

RSK binding promotes SPRED2 complex formation with neurofibromin and negative feedback of MAPK signaling.

Using the HR1 cell system, we observed that MAPK activation also promoted interaction between SPRED2 and neurofibromin, in a similar manner as SPRED2-RSK2. These findings led us to hypothesize that RSK2 promotes SPRED2 complex formation with neurofibromin. To test this, we stably expressed SPRED2 or the RSK2 binding deficient mutant F145A in HEK293T cells, and tested SPRED2 interaction with endogenous neurofibromin (Figure 33). We noted a decrease in neurofibromin binding with the SPRED2 F145A mutant compared to WT. Furthermore, we observed that inserting the RID from SPRED2 into SPRED1 (SPRED1 chimera), which confers RSK binding capacity to SPRED1, also enhanced binding to neurofibromin compared to SPRED1. These differences in binding were observed both in normal serum media and Epidermal Growth Factor (EGF) stimulated conditions, further suggesting that RSK2 promotes SPRED2-neurofibromin complex formation. Since neurofibromin is a negative regulator of the RAS-MAPK pathway, we hypothesized that this deficiency in SPRED2 binding has functional consequences on MAPK signaling. To test this, we performed an EGF time course stimulation and observed that cells expressing SPRED2 F145A had higher RAS activation (as assessed by RAS GTP loading) and elevated ERK1/2 phosphorylation, compared to cells expressing SPRED2 WT (Figure 33C). Given the importance of SPREDs in neurofibromin membrane localization, we hypothesized that this difference in MAPK activation may be due to the inability of SPRED2 F145A to membrane localize neurofibromin. However, when fractionating cells, we did not observe a quantitative difference in neurofibromin membrane localization between SPRED2 F145A and SPRED2 expressing cells.

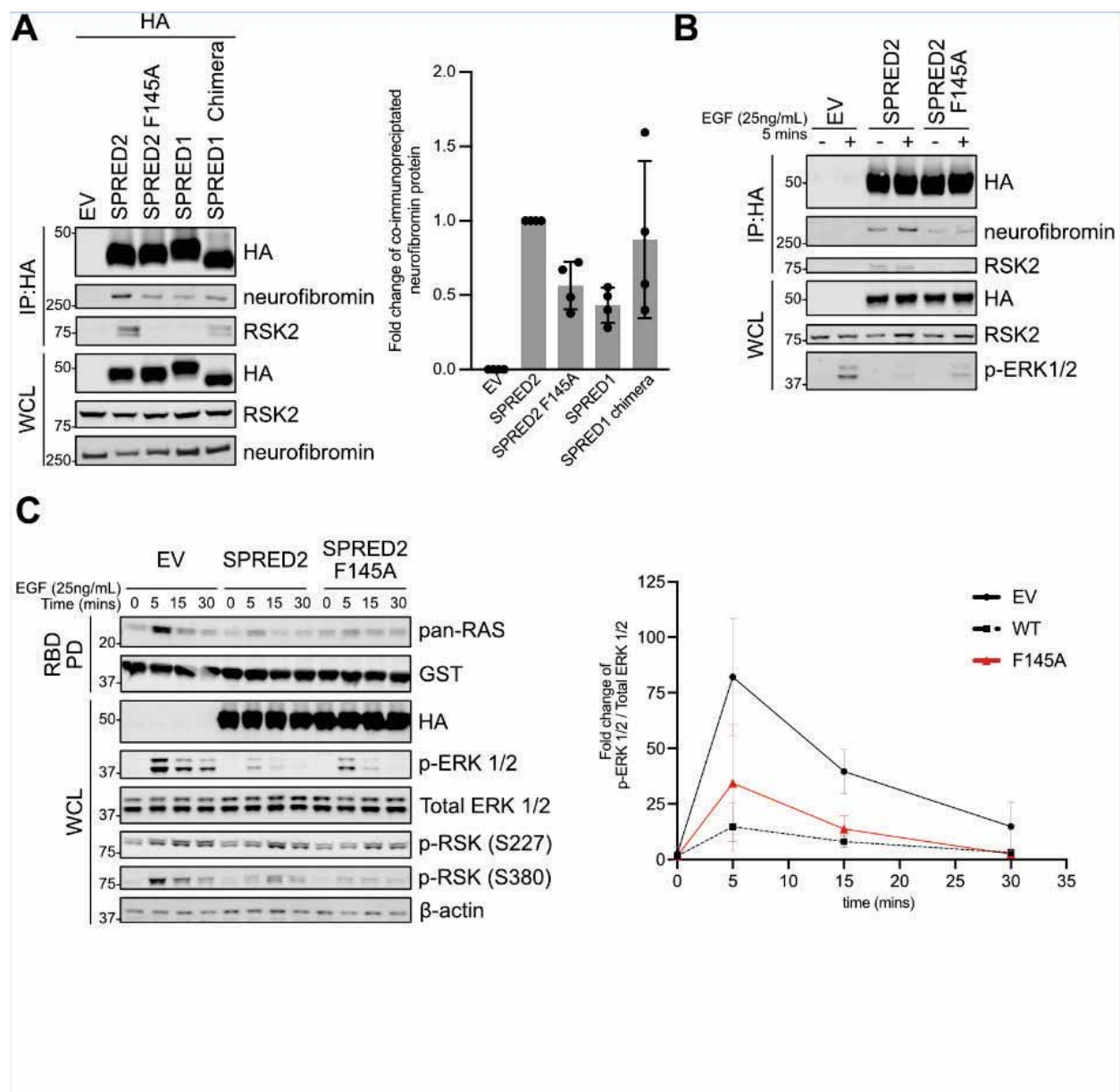


Figure 33. Effects of RSK2 binding on neurofibromin activity

These data suggest a model in which the SPRED2-RSK complex formation is regulated by MAPK activation. Upon MAPK stimulation, active ERK1/2 phosphorylates RSK2 at T369, S365, and T5tt (T363, S359, and T573 in RSK1). This activates the RSK-CTKD and results in autophosphorylation at S386. PDK1 binds at the S386 (S380 in RSK1) phosphorylation site, and then phosphorylates S227 (S221 in RSK1) on the RSK NTKD, allowing RSK to activate and phosphorylate substrates. We suspect that phosphorylation by ERK1/2 relieves autoinhibition of RSK and causes a conformational change to promote SPRED2 binding. Our study highlights the crucial role of the interaction between SPRED2 and RSK in the negative regulation of MAPK signaling. Disruption of this interaction leads to hindered interaction with neurofibromin, which in turns results in elevated MAPK signaling (Figure 34).

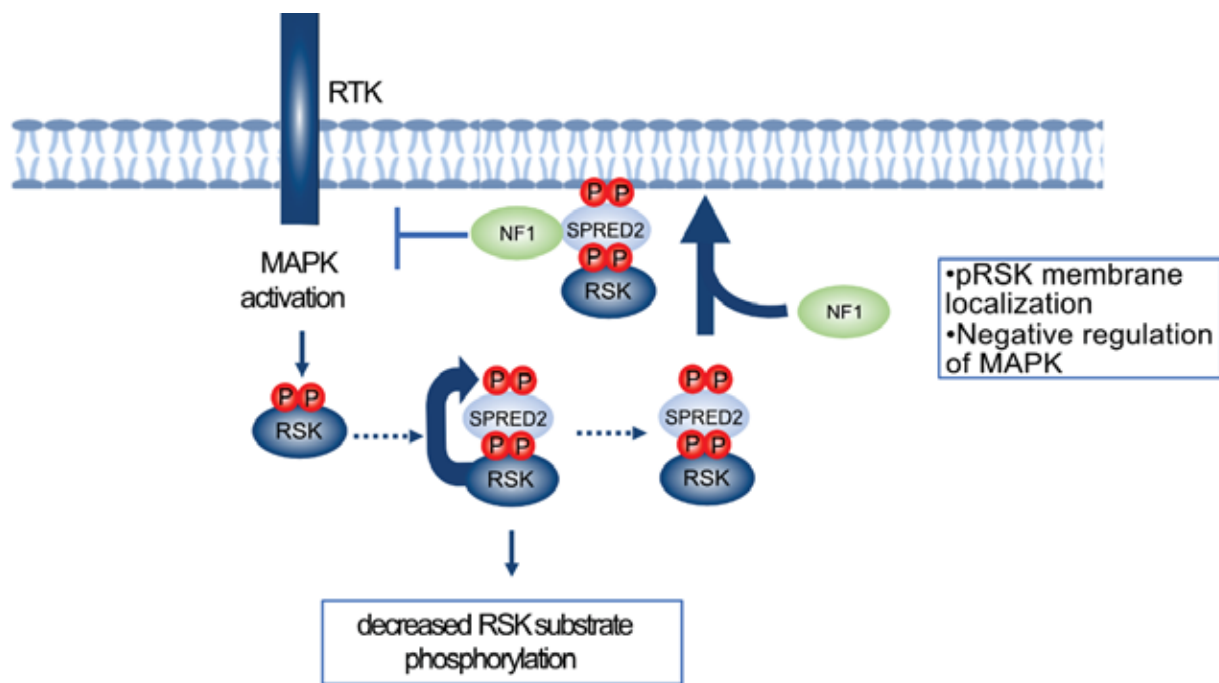


Figure 34 A model for the regulation of MAPK signaling by the RSK/SPRED2 complex

Opportunities for training and professional Development

Since the last reporting period, post-doctoral scholar Dr. Lucy Young was promoted to Specialist. Dr. Young continued to meet with Dr. McCormick on a weekly to discuss project and career progress and goals. Dr. Young presented her research at lab group meetings and at the 2022: Fourth RAS Initiative Symposium held at the Frederick National Laboratory for Cancer Research and her talk was well received. Dr. Young has presented on genotype-phenotype relationships that may point to particularly interesting regions of the protein that are critical for its function, as well as post-translational modifications identified by mass spectrometry. Dr. Young has been a major contributor to these discussions and ongoing collaborations. Additionally, Dr. Young has been co-mentoring and supervising the graduate trainees in my Lab which entails daily and weekly supervision of their research projects. She continues her lab training education by attending training courses at UCSF and elsewhere as opportunities become available.

Dr. McCormick continues to run the NF1 Monthly Meetings between scientists and collaborators at Frederick National Laboratory for Cancer Research and the McCormick Lab. These meetings have offered the opportunity for trainees to participate in scientific discussions and collaborations between both groups. Topics have included updates on CRYO-EM analysis, progression of biochemical analysis of neurofibromin and SPRED proteins and on biological consequences of neurofibromin loss. NF1 Monthly Meetings include Drs. Young, Sale, Vasudevan, Francois, Assistant Specialists, Jr Specialists, Clinical Fellows and graduate students from the UCSF TETRAD Graduate Program: Armando Martinez and Miranda Cabanski-Dunning. One of our

dedicated Jr Specialist that worked on our DOD grant project, Ms. Ruby Goldstein de Salazar applied and was accepted at UCLA and will be attending graduate school in the Fall 2023.

Dissemination of Results

a. Lucy Young|Conferences Participation & Presentations:

1. Vasudevan, H.N., Payne, E., Sale, M.J., **Young, L.C.**, Salazar, R.G., Forget, A., **McCormick, F.** “*NF1 loss in immortalized peripheral nerve (iPN) cells drives cell growth and alters responses to the MEK inhibitor selumetinib.*” 2022 Children’s Tumor Foundation Neurofibromatosis Conference, Philadelphia, PA.
2. ***What have we learned about regulation of RAS and NF1? Session:*** **Presentation:** “*Neurofibromin cryo-EM structure can be used to predict missense variants that disrupt protein stability,*” 4th NCI RAS Initiative Symposium, October 17-19, 2022|Frederick National Laboratory for Cancer Research, Frederick, MD.
3. **Meet the Experts Sessions (Panelist):** 4th NCI RAS Initiative Symposium, October 17-19, 2022|Frederick National Laboratory for Cancer Research, Frederick, MD.

b. Frank McCormick|Conferences Participation & Presentations:

1. **2022 ASCO Annual Meeting: Education Session Chair & Presenter:** Development of Novel Inhibitors of the RAS Pathway, June 3-7, 2022| Chicago, IL.
2. **The 10th Symposium of Genome Drug Discovery and Emergence Forum, Session:** “*Drug Potentials of Targeted Protein Degradation*”, **Invited Speaker**, July 12, 2022|Virtual.
3. **4th RAS Targeted Drug Development Summit 2022, Invited Speaker:** “*Critical Updates from the RAS Community,*” September 26-29, 2022|Virtual.
4. **4th NCI RAS Initiative Symposium (Co-Chair)**, October 17-19, 2022| Frederick National Laboratory for Cancer Research, Frederick, MD.
 - i. “*What is the progress targeting RAS directly in the clinic (and what are emerging resistance mechanisms)?: Overview of Targeting RAS in the clinic*”(Frank McCormick, Chair & Presenter)
5. **AACR Translational Cancer Research for Basic Scientists Workshop, Invited Faculty Member & Presenter:** *Translating Differently: Innovative and Emerging Approaches: “Hitting KRAS Directly,”* November 13 – 18, 2022|Boston, MA.

6. **AACR Special Conference: Targeting RAS (Co-Chair)/Session Chair: Plenary Session 1: Biochemistry, Function, and Structure**, March 5-8, 2023| Philadelphia, PA.
7. **2023 AACR Annual Conference: Plenary Session: Beating Kras: A 30-year Overnight Sensation, Invited Speaker**, April 14-19, 2023| Orlando, FL.
8. **Annual Francis Miller Endowed Lecture in Cancer Research at South Dakota State University, Invited Speaker**, May 1, 2023| Brookings, SD.
9. **2023 The Children's Tumor Foundation's (CTF) annual Neurofibromatosis (NF) Conference, Keynote Speaker: Drug Discover: "Drugs That Target RAS and Their Relevance to NF1,"** June 24-27, 2023|Scottsdale, AZ.
10. **Ivy Brain Tumor Center at the Barrow Neurological Center, The Ivy Center Seminar Series, Invited Speaker: "Progress in Treating KRAS Cancers,"** June 26, 2023| Phoenix, AZ.
11. **5th RAS-Targeted Drug Development Summit 2023, Invited Speaker**, September 26-28|Boston, MA.
12. **Centro de Investigacion del Cancer: Targeting RAS, New Avenues and Challenges Conference, Invited Speaker**, September 13-15, 2023| Salamanca, Spain

c. **Abstracts: 2022-2023**

- 1) **"Progress in Targeting KRAS and Future Prospects," Frank McCormick.** The 10th Symposium of Genome Drug Discovery and Emergence Forum, Session: "Drug Potentials of Targeted Protein Degradation", Invited Speaker, Virtual, July 12, 2022.
- 2) **"Drugs that target RAS and their relevance to NF1," Frank McCormick.** 2023 The Children's Tumor Foundation's (CTF) annual Neurofibromatosis (NF) Conference, Keynote Speaker: Drug Discover: "Drugs That Target RAS and Their Relevance to NF1," June 24-27, 2023|Scottsdale, AZ.
- 3) **"The End of KRAS cancers?," Frank McCormick.** Centro de Investigacion del Cancer: Targeting RAS, New Avenues and Challenges Conference, Invited Speaker, September 13-15, 2023| Salamanca, Spain.

- 4) *DDDR-06. Neurofibromatosis Tumor Suppressors Cooperatively Drive Tumor De-Differentiation and MEK Inhibitor Resistance in Peripheral Nervous System Tumors.* Harish Vasudevan, Emily Payne, Cyrille Delley, Matthew Sale, S. John Liu¹, Sydney Lastella, Calixto-Hope Lucas, Charlotte Eaton, Tim Casey-Clyde, Stephen Magill, William Chen, Alyssa Reddy, Steve Braunstein, Arie Perry, Line Jacques, Melike Pekmezci, Adam Abate, **Frank McCormick**, and David Raleigh. *Neuro-Oncology*, Volume 24, Issue Supplement_7, November 2022, Page vii99, <https://doi.org/10.1093/neuonc/noac209.371> [abstract]

- 5) William E. Burgan, Nicole Fer, Katie Powell, Bingfang Xu, Mackenzie Meyer, Scott Eury, Abigail Neish, Daniel Soppet, Dominic Esposito, **Frank McCormick**, Dwight V. Nissley. *An isogenic H/N/KRAS-less mouse embryonic fibroblast cell line panel derived from a size sorted diploid clonal parent* [abstract]. In: Proceedings of the AACR Special Conference: Targeting RAS; 2023 Mar 5-8; Philadelphia, PA. Philadelphia (PA): AACR; *Mol Cancer Res* 2023;21(5_Suppl):Abstract nr A028.

- 6) DRUG RESISTANCE AND NOVEL THERAPEUTICS| MAY 01 2023
Megan Rigby, John Columbus, Vanessa Wall, Dominic Esposito, Thomas Turbyville, Dharendra Simanshu, Dwight Nissley, **Frank McCormick**, Anna Maciag. *Bioluminescence resonance energy transfer (BRET) as a tool for assessing mutant KRAS-effector affinity and drug efficacy* [abstract]. In: Proceedings of the AACR Special Conference: Targeting RAS; 2023 Mar 5-8; Philadelphia, PA. Philadelphia (PA): AACR; *Mol Cancer Res* 2023;21(5_Suppl):Abstract nr B027.

- 7) Alok K. Sharma, Marcin Dyba, Dominic Esposito, Bin Wang, Pedro J. Beltran, Eli Wallace, Dwight Nissley, **Frank McCormick**, Anna E. Maciag. *Structural plasticity of KRAS oncogenic mutants – A case of misleading conclusions from GTP analogues* [abstract]. In: Proceedings of the AACR Special Conference: Targeting RAS; 2023 Mar 5-8; Philadelphia, PA. Philadelphia (PA): AACR; *Mol Cancer Res* 2023;21(5_Suppl):Abstract nr PR01.

- 8) Robert A. D'Ippolito, Caroline J. DeHart, Dana Rabara, Maria Abreu Blanco, Emily Alberico, Nitya Ramakrishnan, Stephanie Widmeyer, Simon Messing, David Turner, Michelle Arkin, Anna Maciag, Andrew Stephen, Dominic Esposito, **Frank McCormick**, Dwight V. Nissley. *A top-down proteomic assay to evaluate KRAS4B-compound engagement* [abstract]. In: Proceedings of the AACR Special Conference: Targeting RAS; 2023 Mar 5-8; Philadelphia, PA. Philadelphia (PA): AACR; *Mol Cancer Res* 2023;21(5_Suppl):Abstract nr A018.

- 9) Brian Smith, Alok K. Sharma, Roger Ma, Megan Rigby, Vandana Kumari, Dominic Esposito, Dwight Nissley, **Frank McCormick**, Anna E. Maciag. *KRAS codon 12 oncogenic mutations modulate protein conformation within the Switch II/Helix3 pocket* [abstract]. In: Proceedings of the AACR Special Conference: Targeting RAS; 2023 Mar 5-8; Philadelphia, PA. Philadelphia (PA): AACR; *Mol Cancer Res* 2023;21(5_Suppl):Abstract nr A033.

- 10) Caroline J. DeHart, Robert A. D'Ippolito, Kanika Sharma, Nicole Fer, Brian Smith, Mackenzie Meyer, Scott Eury, Abigail Neish, Katie Powell, Vanessa Wall, William Burgan, Dominic Esposito, Anna E. Maciag, **Frank McCormick**, Dwight V. Nissley. *Expanded RAS proteoform landscape in malignant cell lines revealed by top-down mass spectrometry [abstract]*. In: Proceedings of the AACR Special Conference: Targeting RAS; 2023 Mar 5-8; Philadelphia, PA. Philadelphia (PA): AACR; *Mol Cancer Res* 2023;21(5_Suppl):Abstract nr A016.
- 11) HN Vasudevan, E Payne, C Delley, MJ Sale, SJ Liu, S Lastella, CHG Lucas, CD Eaton, T Casey-Clyde, ST Magill, WC Chen, AT Reddy, SE Braunstein, A Perry, L Jacques, M Pekmezci, AR Abate, **F McCormick**, DR Raleigh. “*Neurofibromatosis tumor suppressors cooperatively drive tumor de-differentiation and MEK inhibitor resistance in peripheral nervous system tumors.*” 2022 Society for Neuro-Oncology Annual Meeting, Tampa Bay, FL.
- 12) Vasudevan, H.N., Payne, E., Sale, M.J., **Young, L.C., Salazar, R.G.,** Forget, A., **McCormick, F.** “*NF1 loss in immortalized peripheral nerve (iPN) cells drives cell growth and alters responses to the MEK inhibitor selumetinib.*” 2022 Children’s Tumor Foundation Neurofibromatosis Conference, Philadelphia, PA.
- 13) HN Vasudevan, M Sacconi, E Payne, M Sale, A Forget, **F McCormick.** “*A functional genomic Ras GAP atlas reveals distinct neurofibromin functions and druggable dependencies in the peripheral nervous system.*” 2023 Children’s Tumor Foundation Neurofibromatosis Conference, Scottsdale, AZ.

d. Publications: 2022-2023

1. **Young, L.C.,** Goldstein de Salazar, R., Han, S.W., Huang, ZY.S., Merk, A., Drew, M., Darling, J., Wall, V., Grisshammer, R., Cheng, A., Allison, M.R., Sale, M.J., Nissley, D.V., Esposito, D., Ognjenovic, J., **McCormick, F.** 2023. Destabilizing NF1 variants act in a dominant negative manner through neurofibromin dimerization. *Proc Natl Acad Sci U S A.* 120 (5). <https://doi.org/10.1073/pnas.220896012>
2. Blakeley JO, Le LQ, Lee SY, Ly I, Rhodes SD, Romo CG, Sarin KY, Staedtke V, Steensma MR, Wolkenstein P; 2022 **NTAP Cutaneous Neurofibroma Symposium Participants.** (2023) A Call for Discovery and Therapeutic Development for Cutaneous Neurofibromas. *J Invest Dermatol*: S0022-202X(23)01983-8. doi: 10.1016/j.jid.2022.11.027. Epub ahead of print. PMID: 37354152.

3. Raleigh, D., Vasudevan, H., Payne, E., Delley, C., Sale, M., Liu, S., Lastella, S., Calixto-Hope, L., Eaton, C., Casey-Clyde, T., Magill, S., Chen, W., Reddy, A., Braunstein, S., Perry, A., Jacques, L., Pekmezci, M., Abate, A., **McCormick, F.** (2023). Functional interactions between neurofibromatosis tumor suppressors drive Schwann cell tumor de-differentiation and treatment resistance. Preprint is Under Review at Nature Portfolio.
<https://www.researchsquare.com/article/rs-1933793/v1>

Reported May 2021:

1. **Lorenzo, C.** and **McCormick, F.** 2020. SPRED proteins and their roles in signal transduction, development, and malignancy. *Genes & Dev.* 34: 1410-1421. doi: 10.1101/gad.341222.120.
2. **Castel, P.**, Holtz-Morris, A., Kwon, Y., Suter, B.P., **McCormick, F.**, 2020. DoMY-Seq: A yeast two-hybrid-based technique for precision mapping of protein-protein interaction motifs. *J Biol Chem* 23:296:100023. doi: 10.1074/jbc.RA120.014284.
3. Yan, W., **Markegard, E.**, Dharmaiah, S., Urisman, A., Drew, M., Esposito, D., Scheffzek, K., Nissley, D.V., **McCormick, F.**, Simanshu, D.K. 2020. Structural Insights into the SPRED1-Neurofibromin-KRAS Complex and Disruption of SPRED1-Neurofibromin Interaction by Oncogenic EGFR. *Cell Rep.* Jul 21;32(3):107909. doi: 10.1016/j.celrep.2020.107909.
4. Sherekar, M., **Han, SW.**, Ghirland, R., Messing, S., Drew, M., Rabara, D., Waybright, T., Juneja, P., O'Neill, H., Stanley, CB., Bhowmik, D., Ramanathan, Ar., Subramaniam, S., Nissley, DV., Gillette, W., **McCormick, F.**, 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

4. Impact

My group discovered the interaction between neurofibromin and SPRED1, and so explained why loss of SPRED1 in Legius Syndrome results in a phenotype that resembles NF1. Here we show the importance of this interaction in cell signaling, with a focus on pathways regulated by the KIT receptor and its ligand, stem cell factor. We show that activation of KIT recruits a complex consisting of SPRED1 and neurofibromin to the plasma membrane, and this complex can turn off signaling to RAS from other RTKs and

this inhibit proliferation. Activation of KIT leads to multiple phosphorylation sites on SPRED1, many of which have been mapped and analyzed. We have also identified the region of KIT to which the SPRED/neurofibromin complex binds, and suggested how this complex can regulate other signaling effectors that bind at the same region. NF1 disease can be caused by a wide range of mutations and deletions. The most informative are missense mutations, of which many have been reported but very few characterized. We chose to examine a cluster of missense mutations that result in a very severe phenotype, and showed that these mutations create dominant negative proteins that are unstable, and de-stabilize wild type neurofibromin with which they heterodimerize. These studies suggest a therapeutic approach in which degradation of the mutant neurofibromin protein is prevented, sparing the negative effects on the wild type allele. We also present a structural analysis of the mutant proteins that accounts for instability.

5. Changes/Problems

Specialist, Dr. Lucy Young, has registered with the Synapse platform and will continue to work with Sage Bionetworks to meet the requirements for the Data and Resources Sharing Plan. She is currently working on the mass spectrometry data to share with Sage for processing.

6. Products:

- a) **Young, L.C., Goldstein de Salazar, R.,** Han, SW., Huang, ZY.S., Merk, A., Drew, M., Darling, J., Wall, V., Grisshammer, R., Cheng, A., Allison, M.R., Sale, M.J., Nissley, D.V., Esposito, D., Ognjenovic, J., **McCormick, F.** 2023. Destabilizing NF1 variants act in a dominant negative manner through neurofibromin dimerization. *Proc Natl Acad Sci U S A.* 120 (5). <https://doi.org/10.1073/pnas.2208960120>
- b) Blakeley J.O., Le LQ, Lee SY, Ly I, Rhodes SD, Romo CG, Sarin KY, Staedtke V, Steensma MR, Wolkenstein P; 2022 **NTAP Cutaneous Neurofibroma Symposium Participants.** (2023) A Call for Discovery and Therapeutic Development for Cutaneous Neurofibromas. *J Invest Dermatol*: S0022-202X(23)01983-8. doi: 10.1016/j.jid.2022.11.027. Epub ahead of print. PMID: 37354152.
- c) Raleigh, D., Vasudevan, H., Payne, E., Delley, C., Sale, M., Liu, S., Lastella, S., Calixto-Hope, L., Eaton, C., Casey-Clyde, T., Magill, S., Chen, W., Reddy, A., Braunstein, S., Perry, A., Jacques, L., Pekmezci, M., Abate, A., **McCormick, F.** (2023). Functional interactions between neurofibromatosis tumor suppressors drive Schwann cell tumor de-

7. Participants & Other Collaborating Organizations

Participants: *No change for key personnel. As reported last year, Graduate student Claire Lorenzo graduated March 2022 with a Master's Degree and is now a Research Scientist with BMS.*

Name:	Frank McCormick
Project Role:	PI

Name:	Lucy Young
Project Role:	Post-doctoral scholar

Name:	Claire Lorenzo
Project Role:	Graduate Student
Nearest Person Month Worked:	3.0
Contribution to Project:	<i>Graduated, Master's Degree (March 2022)</i>
Funding Support:	This award

Other collaborating organizations

We are collaborating with scientists at the Frederick National Laboratories for Cancer Research, Frederick, MD to solve the structure of neurofibromin using Cryo-EM and (for small domains) by conventional crystallography.

Changes in PI and Key Personnel support since Award Made:

FRANK MCCORMICK

ACTIVE

W81XWH2010129

McCormick (PI)

04/01/2020 – 03/31/2023

DOD/CDMRP

Regulating neurofibromin through degradation, dimerization and binding to SPRED1
Our objective is to identify ways of increasing neurofibromin's GAP activity that could lead to new therapeutic strategies for treating Neurofibromatosis Type 1, and potentially other syndromes caused by elevated levels of Ras.GTP, such as Noonan Syndrome and specific types of autism.

****Final reporting in process****

(no grant #) McCormick (PI) 10/10/2020-02/01/2024
Boehringer Ingelheim RCV GmbH & Co KG
Direct targeting of SOS1 in SOS1-mutant cancer
The goal of the project is to identify potential drug targets in SOS1-mutant cancers.
Award continuation; no effort to report; no overlap with DOD grant

(no grant #) McCormick (PI) 07/01/23-06/30/2025
F. Hoffmann-La Roche Ltd.
The role of NRAS and SHOC2 in the regulation of RAF activity in NRAS mutant Melanoma.
The goal of the project is to show evidence for SHOC2 as a therapeutic target in NRAS-mutant melanoma.
Award for post-doctoral scholar Rony François, MD PhD; Dr. McCormick is supervising PI

PREVIOUS

15R35CA197709 McCormick (PI) 04/01/2016-03/31/2023
NIH/NCI
New Ways of Targeting K-Ras
The overall goal is to find new ways of suppressing the function of hyper-active Ras proteins through a better understanding of how they function and how they are regulated.

no grant #) McCormick (PI) 01/01/2021-12/31/2021
Oncogenuity Inc.
Oncogenic inhibition using peptide nucleic acids
Our objective is to identify ways of increasing neurofibromin's GAP activity that could lead to new therapeutic strategies for treating Neurofibromatosis Type 1, and potentially other syndromes caused by elevated levels of Ras.GTP, such as Noonan Syndrome and specific types of autism.

Key Personnel:

LUCY YOUNG

ACTIVE

W81XWH2010129 McCormick (PI) 04/01/2020 – 03/31/2023
DOD/CDMRP
Regulating neurofibromin through degradation, dimerization and binding to SPRED1
Our objective is to identify ways of increasing neurofibromin's GAP activity that could lead to new therapeutic strategies for treating Neurofibromatosis Type 1, and potentially other syndromes caused by elevated levels of Ras.GTP, such as Noonan Syndrome and specific types of autism.
Final reporting in process

8. Special Reporting Requirements

Specialist, Dr. Lucy Young, has registered with the Synapse platform and will continue to work with Sage Bionetworks to meet the requirements for the Data and Resources Sharing Plan. She is currently working on the mass spectrometry data to share with Sage for processing.

9. Appendices

A. Products and Publications



Destabilizing NF1 variants act in a dominant negative manner through neurofibromin dimerization

Lucy C. Young^a, Ruby Goldstein de Salazar^a, Sae-Won Han^b, Zi Yi Stephanie Huang^a, Alan Merk^c, Matthew Drew^d, Joseph Darling^c, Vanessa Wall^d, Reinhard Grisshammer^c, Alice Cheng^a, Madeline R. Allison^a, Matthew J. Sale^a, Dwight V. Nissley^d, Dominic Esposito^d, Jana Ognjenovic^{c,1}, and Frank McCormick^{a,1}

Edited by Joan Brugge, Harvard Medical School, Boston, MA; received May 24, 2022; accepted December 15, 2022

The majority of pathogenic mutations in the neurofibromatosis type I (*NF1*) gene reduce total neurofibromin protein expression through premature truncation or microdeletion, but it is less well understood how loss-of-function missense variants drive NF1 disease. We have found that patient variants in codons 844 to 848, which correlate with a severe phenotype, cause protein instability and exert an additional dominant-negative action whereby wild-type neurofibromin also becomes destabilized through protein dimerization. We have used our neurofibromin cryogenic electron microscopy structure to predict and validate other patient variants that act through a similar mechanism. This provides a foundation for understanding genotype–phenotype correlations and has important implications for patient counseling, disease management, and therapeutics.

NF1 | cryo-EM | neurofibromatosis type I

Neurofibromin is a GTPase activating protein (GAP) that accelerates the intrinsic GTPase activity of RAS proteins, converting them from an active GTP (guanosine-5'-triphosphate)-bound state to an inactive GDP (guanosine diphosphate)-bound one (1). It is targeted to the membrane through interaction with SPRED (Sprouty-related, EVH1 domain-containing) proteins (2). In the absence of this negative regulation, levels of RAS-GTP and activity of downstream pathways are elevated, leading to profound effects on cell proliferation, differentiation state, and cell survival. Dysregulation of RAS signaling through *NF1* loss is commonly found in a range of malignancies including those of skin, lung, and myeloid origin (3).

NF1 is part of the RASopathies group of developmental syndromes which are driven by hyperactivation of the RAS-ERK (extracellular signal-related kinase) pathway (4). NF1 patients exhibit a range of symptoms which usually begin during infancy and develop over time into adulthood (5). Common features include café au lait modules (CALMs), cutaneous neurofibromas, and Lisch nodules. Some patients develop more severe manifestations including intellectual disability, skeletal abnormalities, cardiac defects, plexiform neurofibromas, optic pathway gliomas, and other malignancies. There are over 2,800 individual mutations identified in NF1 patients but relatively few reports have correlated genotype with phenotype. More notably, patients with p.Met992del exhibit a mild phenotype, (6, 7) those with p.Arg1276, p.Lys1423, and p.Arg1809 substitutions present Noonan syndrome-like features including cardiovascular anomalies (8, 9), while patients carrying *NF1* microdeletions exhibit a more severe phenotype including increased prevalence of neurofibromas and skeletal abnormalities (10). Substitution mutations clustering at codons 844 to 848 are linked to a particularly severe phenotype and cause an increased incidence of major external and spinal plexiform neurofibromas, skeletal abnormalities, and Noonan syndrome features amongst other symptoms (11).

Results

In our efforts to better understand how neurofibromin is regulated, we discovered that neurofibromin exists as a high-affinity dimer in cells (12); however, the biological implications of this complex were more difficult to explain. Hypothesizing that neurofibromin dimers may have a role in disease, we tested several pathogenic point mutations in *NF1* but found that they were all capable of binding to wild-type protein. We noticed that missense mutations in residues 844 to 848 (Fig. 1*A*) reduce protein expression when compared to wild type (Fig. 1*B*) in *NF1* knockout 293T cells. This is consistent with previous reports of lower mutant protein expression observed in a G848R mouse model of NF1 (13, 14) and in cells transiently expressing mouse *Nf1* cDNA (complementary DNA) with L847P and G848R (15). Despite lower levels of expression, these mutants are still able to form heterodimers with wild-type protein (Fig. 1*C*) and maintain their

Significance

This study describes a model for the severe phenotype of patients with Neurofibromatosis Type 1 (NF1) caused by specific missense mutations. We show, that these mutations act as dominant negative mutants, through dimerization with wild-type neurofibromin, and use our newly solved cryo-EM structure of the neurofibromin dimer to explain why these mutations disrupt protein structure and to predict and validate other patient variants. Until now, there have been very few genotype–phenotype relationships for the NF1 disease, despite the identification of more than two thousand pathogenic variants. Mutations at codons 844 to 848 exhibit a severe phenotype, but the mechanism for their action was not known. This work has important implications for clinical management and understanding neurofibromin loss-of-function in disease.

Author contributions: L.C.Y., D.E., J.O., and F.M. designed research; L.C.Y., R.G.d.S., S.-W.H., Z.H., A.M., M.D., J.D., V.W., R.G., A.C., M.R.A., M.J.S., and J.O. performed research; L.C.Y., R.G.d.S., S.-W.H., Z.H., A.M., M.D., J.D., D.E., J.O., and F.M. analyzed data; F.M. and D.V.N. supervised study; and L.C.Y., D.E., J.O., and F.M. wrote the paper.

The authors declare no competing interest.

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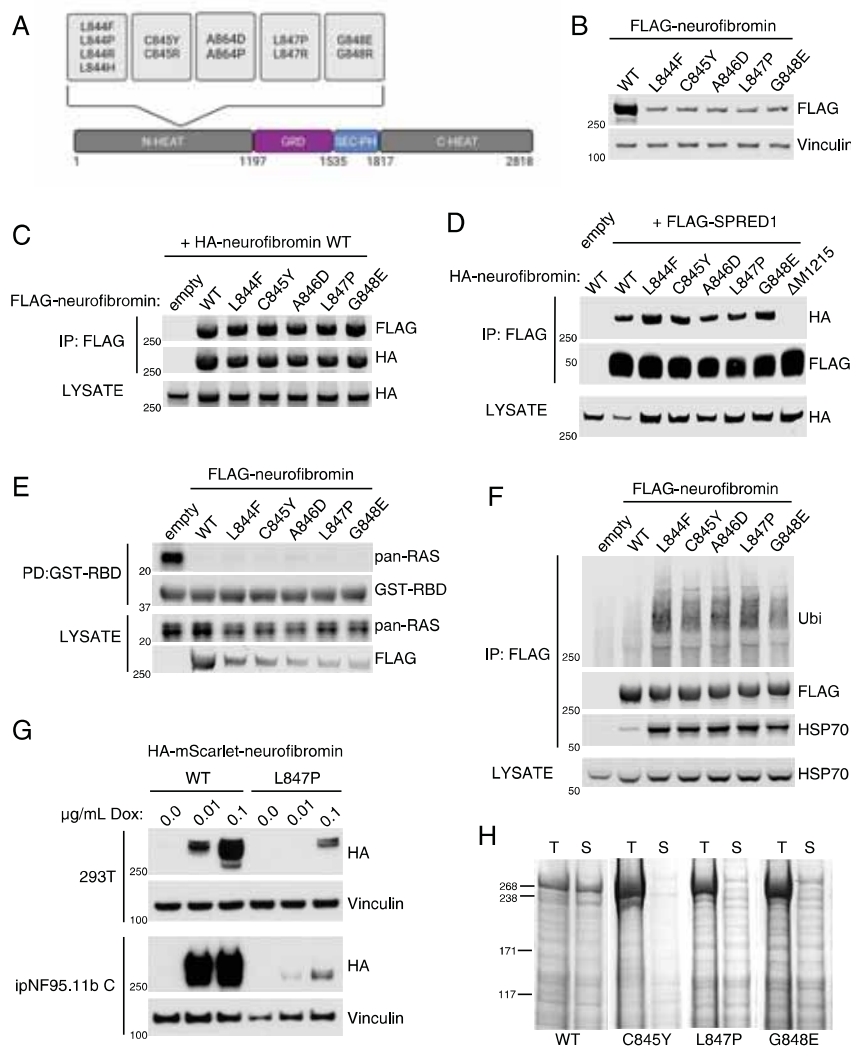


Fig. 1. 844 to 848 neurofibromin mutants are less stable than wild-type protein. (A) Domain structure of neurofibromin showing the location of mutation hotspot. (B) Lysates from 293T *NF1*^{-/-} cells expressing FLAG-tagged neurofibromin. Expression was analyzed via western blot. (C) FLAG-WT and mutant neurofibromin immunoprecipitates (IPs) from 293T *NF1*^{-/-} were tested for dimerization with cotransfected HA-WT neurofibromin. The amount of mutant *NF1* plasmid was increased to even levels of expression with wild type. (D) HA-tagged WT and mutant neurofibromin coimmunoprecipitated with FLAG-SPRED1. *NF1* Δ M1215 is used as a control for the absence of SPRED1 binding. (E) Levels of RAS-GTP by GST-RAF1-RBD (RAS Binding Domain) pull-down from lysates indicate the level of neurofibromin GAP activity in 293T *NF1*^{-/-}. (F) Ubiquitin (Ubi) and HSP70 were detected on FLAG immunoprecipitates of mutant neurofibromin. (G) Lysates from HA-mScarlet-NF1-transduced 293T and ipNF95.11b C Schwann cells after 48 h Dox treatment at the indicated concentrations. (H) Recombinant mutant neurofibromin proteins are highly insoluble in baculovirus-infected insect cells. Images of Coomassie-stained SDS-PAGE. T = Total, S = Soluble.

interaction with SPRED1 (Fig. 1D). Levels of RAS-GTP in *NF1*^{-/-} cells were reduced upon expression of these mutants indicating that they retain GAP activity (Fig. 1E). We wondered whether the mechanism behind low protein expression was due to targeting for degradation. We observed that 844 to 848 mutants are highly ubiquitinated compared to wild-type neurofibromin and coimmunoprecipitate with increased amounts of HSP70 (Fig. 1F), an indicator that the cell is responding to protein misfolding (16). To verify that the effects on expression levels were not an artifact of transient transfection, we generated a doxycycline (Dox)-inducible expression system in the *NF1* knockout 293T cells. We previously thought that it was not possible to efficiently transduce full-length *NF1* lentivirus due to its size. However, we successfully introduced a fluorescent epitope-tagged (HA-mScarlet) WT and L847P *NF1* (SI Appendix, Fig. S1) which was expressed in a Dox dose-dependent manner (Fig. 1G). We observed that the L847P mutant expressed considerably less than the WT under the same conditions, confirming that the previous response was not transient-transfection-specific. To rule out the possibility that

NF1 mutant effects on stability were 293T-specific and to examine the phenomenon in an *NF1* disease-relevant biological system, we transduced a previously characterized immortalized peripheral neurofibroma-derived Schwann cell line (ipNF95.11b C) (17) in which we had knocked out *NF1*. We saw the same phenotype as in the 293T, and given the corroborating data from the aforementioned mouse model, we concluded that 844 to 848 mutants are less stable in a variety of biological scenarios. We also found that these mutations completely abolish soluble recombinant protein expression from baculovirus-infected insect cells (Fig. 1H). We concluded that these hotspot mutations primarily function through protein instability rather than impaired GAP activity or disruption of well-established protein–protein interactions (i.e., SPRED1).

We noticed that, when coexpressed with 844 to 848 mutants, wild-type neurofibromin expression levels were also reduced. To examine whether the mutant was causing lower expression of wild type, we transfected increasing amounts of either FLAG-tagged wild-type, G848E or L847P with constant amounts of HA-tagged

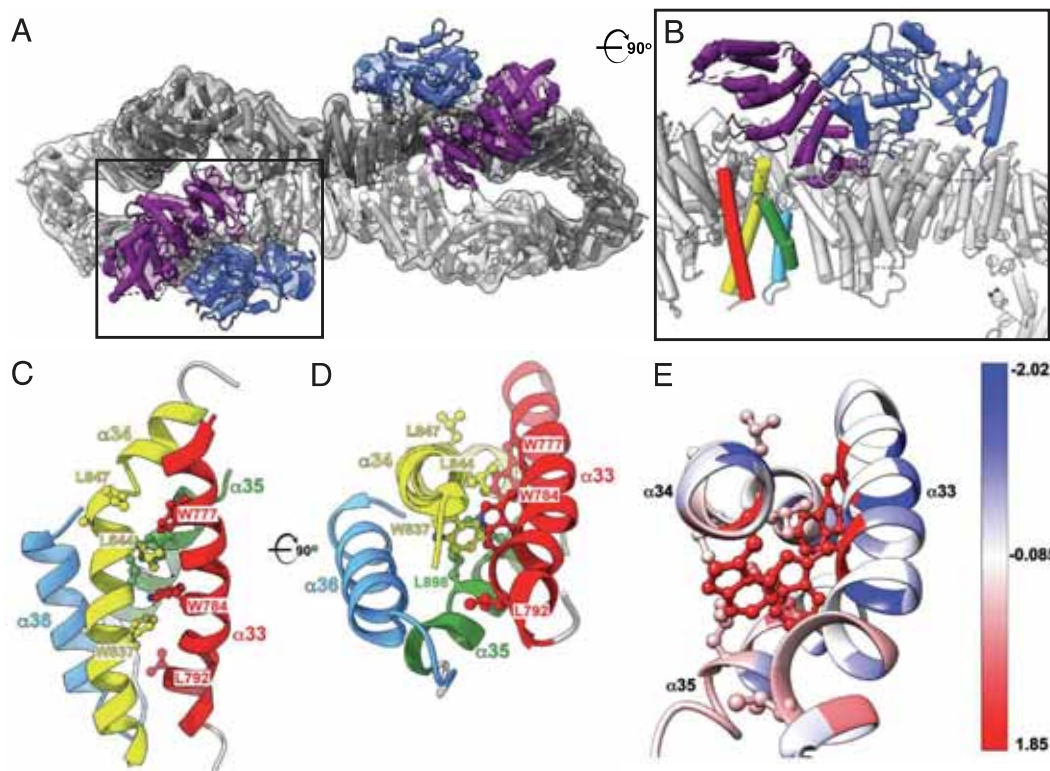


Fig. 3. Cryo-EM structure of the intact short isoform of human neurofibromin. (A) Top view of the final model of NF1 dimer (cartoon) and the corresponding 3D cryo-EM density (transparent grey surface) showing NF1 protomers in dark and light gray, GRD in purple, and Sec-PH (pleckstrin homology) domain in blue. Boxed area indicates the location of the GRD (purple) and Sec-PH (blue) domains in one of the NF1 protomers. PDB: 8E20, EMDB: EMD-27826. (B) A close-up and rotated view of the boxed area from A shows that helices $\alpha 33$ to $\alpha 36$ (red, yellow, green, and light blue) are located directly below the GRD domain (purple). The view is rotated $\sim 90^\circ$ clockwise around the horizontal axis when compared to A. (C) Side view of the $\alpha 33$ to $\alpha 36$ helical bundle (ribbon cartoon colored red, yellow, green, and light blue). Severe phenotype mutations map to the region encompassing residues 844 to 847 (side chains shown in ball-and-stick representation). (D) Top view of the same bundle. For clarity, helix $\alpha 33$ is not shown. The view is rotated $\sim 90^\circ$ clockwise around the horizontal axis relative to C. (E) Residues affected by pathogenic mutations are colored using Kyte-Doolittle amino acid hydrophobicity scale where hydrophobic and polar residues are red and blue, respectively.

whether it was indicated in the molecular mechanisms of NF1 disease. Our results have provided a basis for rationalizing why missense mutations in residues 844 to 848 of neurofibromin cause severe phenotypes in patients, including an increased incidence of major plexiform and spinal neurofibromas (11). These mutations

also reduce the level of protein expression through targeted protein degradation and exert a dominant-negative effect on wild-type protein through the heterodimerization. This is expected to cause a reduction in total neurofibromin levels in haploinsufficient cells, below a threshold observed for other genotypes and subsequently,

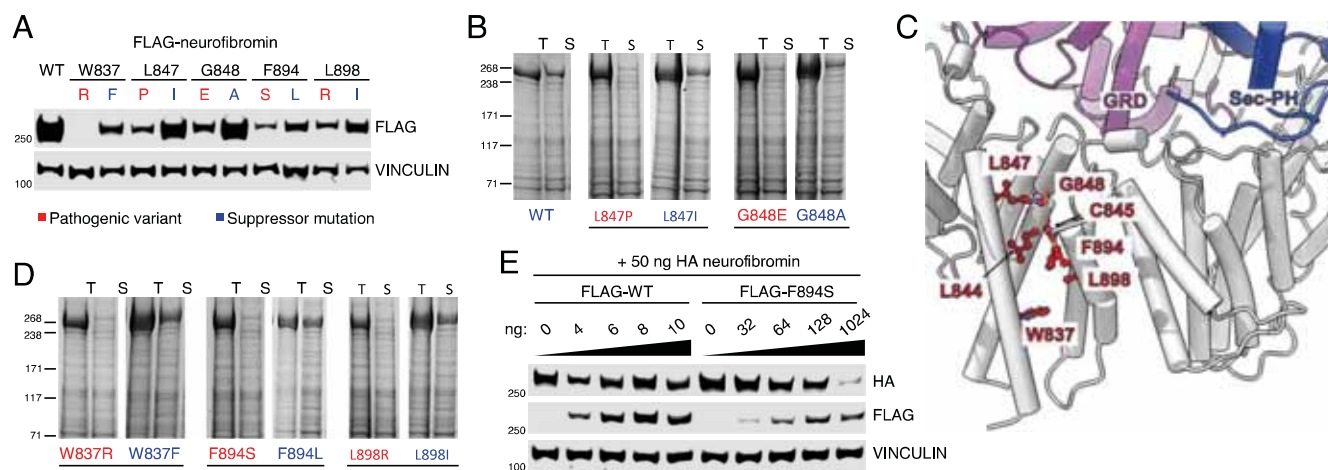


Fig. 4. Loss of stability in α -helix 34 of the N-terminal HEAT domain can be predicted and rescued with hydrophobic residue substitution. (A) Lysates from 293T *NF1*^{-/-} cells expressing FLAG-tagged neurofibromin wild type or with pathogenic and alternative 'suppressor' residues. (B) Solubility and purification of His6-tagged WT, L847P or L847I and G848E or G848A neurofibromin mutants from baculovirus lysates. T = Total, S = Soluble. (C) Cryo-EM structure reveals the location of additional residues mutated in human patients. These residues (red ball-and-stick) map to the $\alpha 33$ - $\alpha 36$ HEAT repeat (gray cartoon) and are juxtaposed to the functionally pivotal HEAT:GRD:Sec-PH interface. GRD is colored purple and Sec-PH is blue. (D) Solubility and purification of W837R, W837F, F894S, F894L, L898R, and L898I mutants as in B. (E) Lysates from 293T *NF1*^{-/-} where HA-neurofibromin WT was cotransfected with increasing levels of either wild type or mutant (FLAG-tagged).

a larger increase in RAS signaling (Fig. 5A). As an example, plexiform neurofibromas initiate when Schwann cells lose *NF1* heterozygosity through the loss of the wild-type allele by either acquired deletion or mutation. Studies have demonstrated the importance of the heterozygous neurofibroma cells (e.g., mast cells, fibroblasts, macrophages) in creating and sustaining a supportive inflammatory microenvironment for Schwann cell survival and proliferation (25). In patients with 844 to 848 mutations, haploinsufficient cells may have increased RAS signaling and would therefore contribute to disease progression (26) (Fig. 5B). This may include enhanced responsiveness to signaling by other cells, secretion of SCF (stem cell factor) by *NF1*^{-/-} Schwann cells that recruits and supports *NF1*^{+/-} mast cells, and increased cytokine and growth factor secretion that would stimulate other cells within the tumour (5). It is likely that there are other pathogenic mutations in *NF1* that affect protein stability and we have been able to use structural information to identify some of those that behave in the same way as 844 to 848 residues. There is little to no phenotypic information for

variants at W837, F894, and L898. Independent studies describe symptoms of CALMs (27), CALMs and bone abnormalities (28), neurofibromas and CALMs (29), and cutaneous neurofibromas (30) in different patients. It may be possible to use this technique to further characterize genotype–phenotype correlations which are not only important for understanding the mechanisms of disease but also to be able to offer patient counselling or more informed therapeutic decisions.

Operable plexiform neurofibromas are given surgical intervention, and although the MEK inhibitor Selumetinib is approved for the treatment of inoperable tumours (31), MEK inhibition is not without side effects or the potential for acquired resistance. There are also no approved therapies for many of the other symptoms of NF1 such as behavioral or bone and cardiac defects. Here, we provide a mechanism of neurofibromin protein homeostasis in disease. Once we understand the mechanisms of protein turnover, we may be able to design therapeutic approaches that restore neurofibromin function through the stabilization of the protein. This could be either by preventing posttranslational modifications and degradation or through modulation of neurofibromin dimerization. We have also developed a methodology for the transduction of inducible tagged full-length *NF1* into cell lines, including *NF1*^{-/-} 293T and immortalized Schwann cell lines. Our prior attempts to stably reexpress NF1 were hindered by apparent TRE3G promoter silencing and loss of inducibility of expression. We have overcome this by redesigning the expression vectors to select for cells that remain inducible, perhaps due to their integration in regions of chromatin resistant to epigenetic silencing. This system has numerous applications for the study of NF1 disease and is particularly relevant in the field of gene therapy.

Materials and Methods

Cloning and Mutagenesis. Gateway LR recombination according to the manufacturer's protocol to insert *NF1*, *SPRED1*, and *RAS* entry clones into transient mammalian expression vectors pCAN-FLAG-DEST and pCAN-HA-DEST. Point mutations in *NF1* isoform-2 were generated by PCR mutagenesis of Addgene plasmid 70423.

Mammalian Cell Culture, Immunoprecipitation, and Western Blot. Generation of *NF1*-null 293T cells has been previously described (12). ipNF95.11b C was purchased from ATCC, and CRISPR technology was used to generate a homozygous *NF1* knockout clone (methodology to be published elsewhere). All cells were maintained in DMEM with 10% fetal bovine serum and penicillin-streptomycin (10 U/mL) and were tested regularly for mycoplasma contamination with MycoAlert (Lonza). Plasmids were transfected using JetOPTIMUS reagent (Polyplus) according to the manufacturer's protocol. Cells were lysed 48 h later in a lysis buffer (50 mM Tris pH 7.5, 150 mM NaCl, 1 mM EDTA (or 5 mM MgCl₂ where RAS activity was concerned), 1% Triton-X-100, 1 mM DTT, EDTA-free protease inhibitors (Roche), and phosphatase inhibitor cocktails (Sigma)). Supernatants from cleared lysates were incubated with Anti-FLAG M2 affinity gel (Sigma) to immunoprecipitate FLAG-tagged protein complexes, or recombinant GST-RAF1 RBD beads to pull down GTP-bound RAS, for 2 h at 4 °C followed by 3 washes with lysis buffer (without protease and phosphatase inhibitors). Beads and lysates were mixed with NuPAGE LDS (ThermoFisher) sample buffer and heated at 70 °C for 10 min prior to loading on either 3 to 8% Tris-Acetate or 4 to 12% Bis-Tris NuPAGE gels. Standard Western Blotting procedures were followed and visualized using 680- and 800-conjugated secondary antibodies (Pierce, Jackson Laboratories) on an Odyssey Li-COR scanner or HRP-conjugated antibodies and development on film using Crescendo substrate (Millipore). Primary antibodies used were FLAG-M2 and KRAS (Sigma), HA, HSP70, and Ubiquitin (Cell Signaling), pan-RAS (Abcam), GST and Vinculin (Bethyl Laboratories), and RASA1 (Santa Cruz Biotechnology). Quantification of Western Blot bands was performed using Image-Studio (Li-COR) and visualized in Prism (GraphPad).

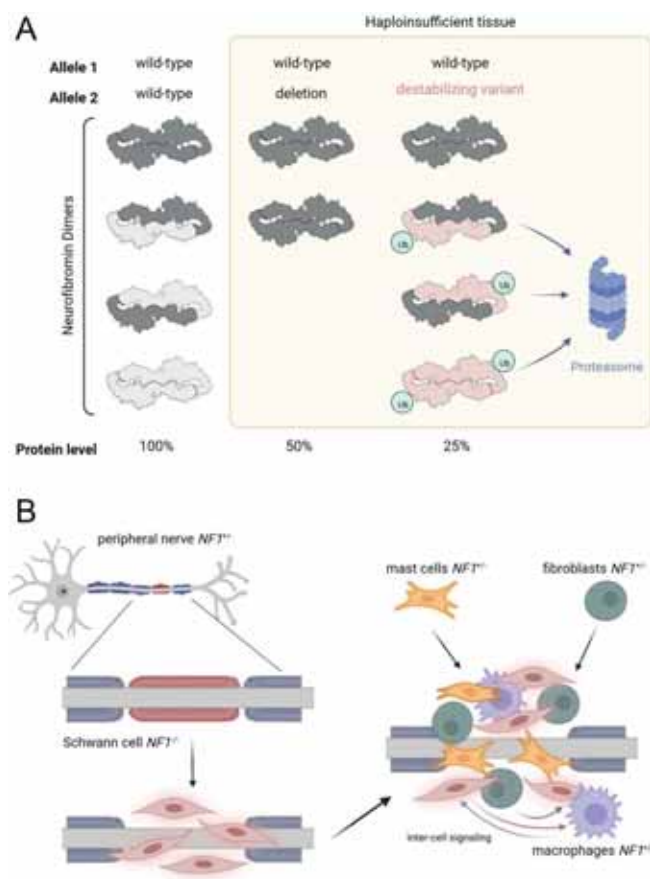


Fig. 5. Reduced levels of neurofibromin in patients with 844 to 848/destabilizing variants may cause severe phenotype through enhanced RAS pathway signalling in haploinsufficient tissues. (A) Schematic to demonstrate the predicted effect of different *NF1* allele combinations in NF1 disease and unaffected cells. Loss of expression by premature truncation or microdeletion of 1 allele of *NF1* reduces total protein expression by half. In the presence of 844 to 848 codon variants or other destabilizing substitutions, the total level of neurofibromin is predicted to be lower due to the dominant negative effect of the mutant on the wild type, through dimerization and targeting for degradation. (B) Hypothetical consequences of a destabilizing-variant microenvironment. Using plexiform neurofibroma as an example, loss of heterozygosity in a Schwann cell or precursor initiates recruitment of other cell types. Haploinsufficient cells of the microenvironment, such as fibroblasts, macrophages and mast cells, are predicted to be hyperresponsive to signals from the cells of origin (Schwann cells), such as cytokines and growth factors, and may create an enhanced tumor-supportive environment/inflammatory response.

Generation of Inducible Expression Constructs and Transduction. To generate constructs for the Tet-inducible expression of NF1 WT and mutants, the lentiviral attR1-attR2 Gateway destination vector pLIX403 (Addgene 41395) was modified to attR4-attR2 by Gibson assembly. Gateway multisite reactions into pLIX403 attR4-attR2 were then performed with entry clones for the TRE3G promoter (attL4-attL5), HA(hemagglutinin)-m(monomeric)Scarlet (attR5-attR1), and NF1 WT or mutants (attL1-attL2) using LR Clonase II (Thermo Fisher Scientific). Lentiviral particles were then generated by equimolar cotransfection of these NF1 expression vectors with psPAX2 (Addgene 12260) and pMD2.G (Addgene 12259) packaging plasmids in HEK293T cells. Forty-eight hours later, the supernatant viral titer was measured using Lenti-X GoStix Plus (Takara Bio) following the manufacturer's instructions. Normalized viral titers for NF1 WT and mutants were then mixed with 5 μ g/mL hexadimethrine bromide (Polybrene, MedChemExpress), 0.45 μ m filtered, and applied to target NF1-null HEK293T and ipNF95.11b C cells with centrifugation at $1,000 \times g$ for 45 min. Forty-eight hours after infection, cells were selected and then subsequently maintained in a medium containing 0.4 μ g/mL puromycin. Expression of HA-mScarlet-NF1 WT and mutants was induced with 1 μ g/mL Dox for 48 h, and cells exhibiting robust expression were sorted by flow cytometry using a Sony SH800 Cell Sorter (Sony). Following cell sorting, Dox was withdrawn for at least 10 d before the commencement of experiments.

Baculovirus. Constructs for recombinant production of full-length neurofibromin in insect cells were previously described (12). Point mutants were generated using the Agilent QuickChange site-directed mutagenesis kit per the manufacturer's instructions, and constructs were completely sequence verified to ensure that no additional mutations were introduced. Baculovirus production and insect cell expression in Tni-FNL cells were done as previously described (12).

Purification of Full-Length Human NF1 Isoform 2. Full-length NF1 (short isoform), preceded by a hexa-histidine tag and a Tev protease recognition site, was produced in Tni-FNL cells and purified as described (12). Briefly, 250 mL of frozen cells were thawed, resuspended, and homogenized using a Dounce homogenizer. Following centrifugation at $235,000 \times g$, the supernatant was supplemented with 40 mM imidazole and loaded onto a 5 mL HisTrap HP Ni-Sepharose column (Cytiva). After rinsing the column with 50 mM Tris-HCl pH 8.0, 300 mM NaCl, 4 mM TCEP, 40 mM imidazole, and Sigma protease inhibitors in DMSO at a dilution of 1 in 500, the protein was eluted with the same buffer supplemented with 500 mM imidazole. Fractions containing the neurofibromin dimer were further purified on a preparative HiLoad 16/600 Superose 6 pg column (Cytiva) in 50 mM Tris-HCl pH 8.0, 300 mM NaCl, and 5 mM TCEP, supplemented with Sigma protease inhibitors in DMSO at a dilution of 1:500. The neurofibromin dimer fractions were combined, concentrated, and repurified on a Superose 6 10/300 GL column (Cytiva) in 20 mM Tris-HCl pH 8.0, 300 mM NaCl, 5 mM TCEP. The neurofibromin-dimer peak fraction was cleared by centrifugation at $21,000 \times g$ for 10 min at $+10^\circ\text{C}$. The pure sample was diluted to ~ 0.3 mg/mL and used for grid preparation.

Cryo-EM Sample Preparation and Data Acquisition. UltraAuFoil grids (300 mesh, Electron Microscopy Sciences) were plasma cleaned using a SOLARUS plasma cleaner (Gatan Inc.). Then, 3 mL of purified neurofibromin was subsequently deposited onto the grids, which were then plunge frozen in liquid ethane using a Leica EM GP plunge freezer (Leica Microsystems Inc.). Grid screening and data collection were carried out on a CryoARM200 microscope (JEOL Inc.) equipped with a K3 camera (Gatan) operated in super-resolution mode and correlated double sampling mode. A total of 42,049 movies were collected over five separate data collection sessions. Each image was fractioned into 60 frames with

an exposure time of 6.232 s and a dose rate of 9.6 electrons/ $\text{\AA}^2$. All data collection was performed with SerialEM.

Cryo-EM Data Processing. Data processing was done using the RELION (32) software package. Collected movie frames were motion-corrected and dose-weighted using MotionCorr (33) followed by contrast transfer function estimation using CTFFIND4 (34). Initial particles were picked using Laplacian-of-Gaussian-based auto-picker. Obtained representative 2D classes were used for another round of template-based auto-picking that yielded 692,875 particles. Following several rounds of 2D classifications, a cleaned particle stack was used to generate an initial model without imposing symmetry. Subsequent processing steps involved Refine3D and CTF Refine and particle polishing, resulting in a cryo-EM map at 3.6 $\text{\AA}$ overall resolution with C2 symmetry. Particle subtraction with a mask around one Neurofibromin lobe, followed by multibody refinement using four masks (*SI Appendix, Fig. S3A*), yielded the map at the overall resolution of 3.3 $\text{\AA}$ with improved density for the most of the Neurofibromin core domains.

Model Building and Refinement. Published models of GRD and Sec-PH (PDB: 1nf1 and 2d4q, respectively) were rigid-body fitted in the density using Chimera. The core of the NF1 dimer was completely manually modeled in Coot (35) and then compared with the AlphaFold2 (36) predictions of separate domains. Structure refinement was done in Phenix (37). Iterative manual inspection and rebuilding of the 3D model in Coot relied on real-space difference maps calculated in Servalcat (38).

PDB and EMDB Accession Codes. Neurofibromin full dimer pdb: [8E20](#), EMDB: [EMD-27826](#)

Body1 pdb: [8EDO](#), EMDB: [EMD-28038](#)

Body2 pdb: [8EDL](#), EMDB: [EMD-28035](#)

Body3 pdb: [8EDM](#), EMDB: [EMD-28036](#)

Body4 pdb: [8EDN](#), EMDB: [EMD-28037](#)

Illustrations. Figs. 1A and 5 and *SI Appendix, Fig. S1* were created with BioRender. com. Fig. 2 B and C and *SI Appendix, Fig. S4D* were made in GraphPad Prism.

Data, Materials, and Software Availability. Coordinates and maps have been deposited in PDB [www.rcsb.org](#) and EMDB [www.ebi.ac.uk/emdb/](#) (see accession codes above), respectively, and will be released upon publication. All study data are included in the article and/or *SI Appendix*.

ACKNOWLEDGMENTS. We thank Hannah Ambrose and Ashley Mitchell for their technical expertise in insect cell production work and Jennifer Mehalko for assistance in the production of neurofibromin proteins. We thank Antonio Cuevas-Navarro and Richard Van for their technical assistance. This project was funded in part with federal funds from the National Cancer Institute, the NIH, contract no. 75N91019D00024, The Department of Defence CDMRP grant number W81XWH2010129, and the Neurofibromatosis Therapeutic Acceleration Program (NTAP).

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A Call for Discovery and Therapeutic Development for Cutaneous Neurofibromas

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Skin is the calling card of humanity—the body's first line of defense against external threats and a major part of a person's identity. Diseases of the skin are common and a leading cause of nonfatal disease burden in humans (Hay et al., 2014). Effectively addressing skin diseases not only halts or prevents physical illness, it often treats or prevents the common comorbidities of skin disease: depression, anxiety, and suicidal ideation (Hay et al., 2014; Misery et al., 2018). Such mental health diagnoses carry their own substantial disease burden and dramatically impact QOL for both the person and the community affected by chronic skin diseases (Dalgard et al., 2015). Accordingly, an important aspect of effectively treating or preventing skin disease is improving mental health comorbidities and QOL through therapeutic discovery (Kaundinya et al., 2022; Renert-Yuval et al., 2022). Of course, to achieve that critical step, we must first understand disease pathophysiology, develop therapies on the basis of established biologic mechanisms, know the natural history of the disease, and validate meaningful outcome measures for therapeutic interventions. Successful generation of this enormous dataset can result in incredible benefits for people living with skin disease. Indeed, there has been a recent explosion of new drug therapies (including a raft of new

biologic agents targeting specific mediators of disease pathobiology) for conditions such as atopic dermatitis, psoriasis, and vitiligo (Arkwright and Koplin, 2023; Chovatiya and Paller, 2021; Malik and Guttman-Yassky, 2018). Such developments give inspiration and direction for another skin condition that has been without therapeutic advance: cutaneous neurofibromas (cNFs).

cNFs are the most common tumor in people with the autosomal dominant neurocutaneous condition, neurofibromatosis type 1 (NF1) (Guiraud et al., 2019). Nearly all adults with NF1 are afflicted with cNFs ranging in number from a handful to several thousand tumors involving the skin of all regions of the body (Cannon et al., 2018; Ehara et al., 2018). cNFs are intermittently painful and itchy and can compromise skin integrity. In all cases, cNFs are disfiguring, negatively impacting mental health and QOL (Guiraud et al., 2019; Maguiness et al., 2021).

NF1 is the most common autosomal dominant neurocutaneous disease (estimated incidence of 1:3,000), and nearly 100% (roughly 2.5 million people worldwide) develop cNFs. Indeed, people living with NF1 report that cNFs are the manifestation that they find most distressing (Guiraud et al., 2019; Maguiness et al., 2021). Yet, historically, there has been a lack of dedicated effort to understand the pathophysiology, relevant preclinical and clinical endpoints, and potential therapies to prevent and treat cNFs. A committed effort to understanding and effectively treating NF1-associated cNFs will benefit not only people with NF1 but all people with dermal lesions that are associated with neuropathic irritation, itch, and disfigurement because the core elements of cNFs (Schwann cells, fibroblasts, mast cells, and macrophages) are shared across many skin conditions. The time is optimal for multidisciplinary investment in unraveling the elemental factors that contribute to cNF formation, their associated symptoms, and the many opportunities to prevent and treat these tumors and their comorbidities.

The articles presented in this "Spotlight on Cutaneous Neurofibroma" reflect the work undertaken by a community of invested stakeholders (patients and caregivers, basic scientists, clinical scientists, dermatologists, neuro-oncologists, and regulatory experts) to explore key questions pertaining to developing effective therapeutics for cNFs in people with NF1. Individuals with expertise in various areas participated in the 2022 cNF symposium organized by the Neurofibromatosis Therapeutic Acceleration Program. Four teams

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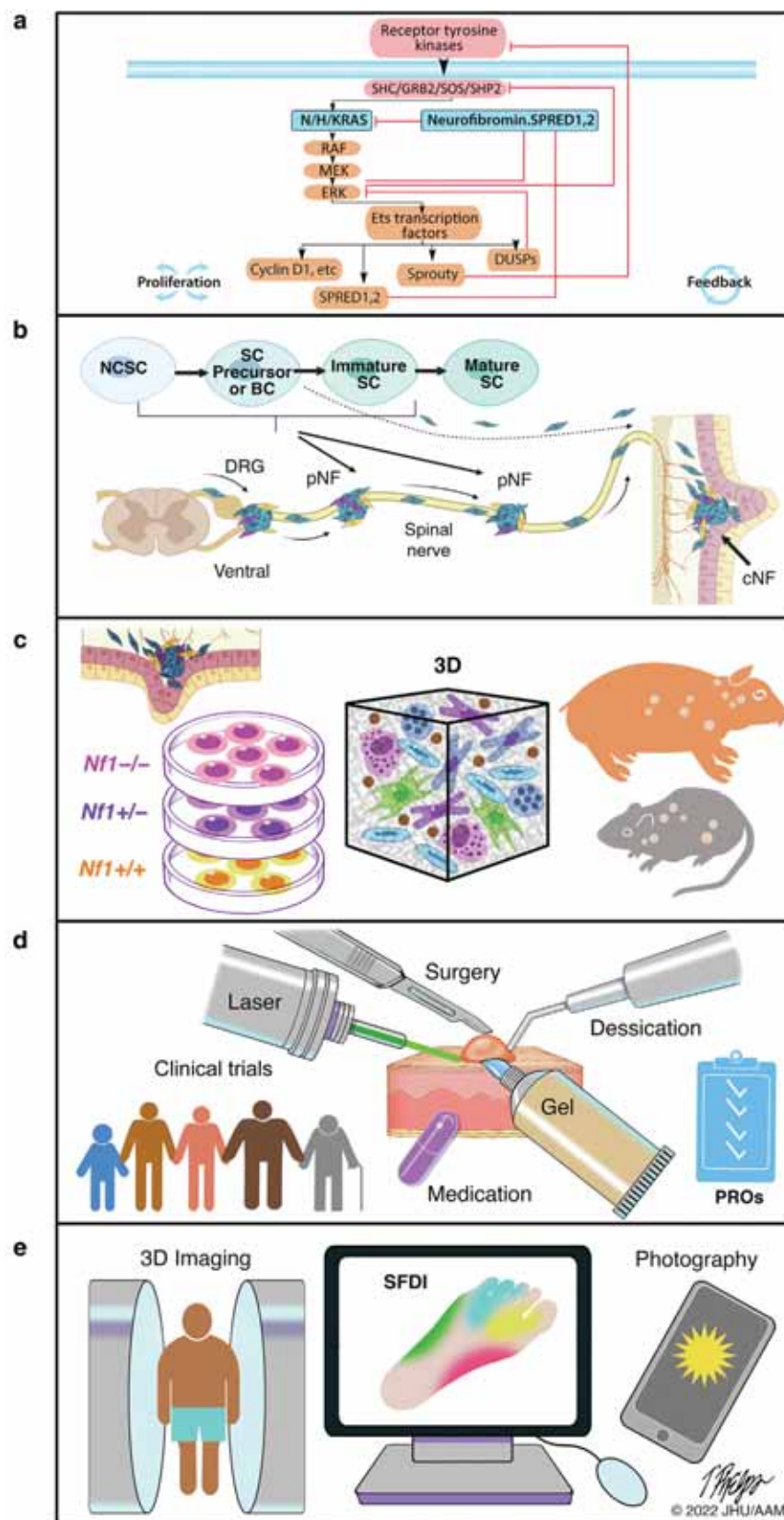


Figure 1. Cellular pathways, cell types, therapeutic approaches, and diagnostic imaging for human cNFs. In this edition, the *Journal of Investigative Dermatology* shines a spotlight on cNFs in the context of neurofibromatosis type 1, the most common neurocutaneous tumor–predisposition syndrome and a field that has seen remarkable scientific progress in the last two decades. With the hope to inform and accelerate research, five articles delve into (a) the current state of knowledge of RAS signaling in cNF pathogenesis and therapeutic development for cNF as well as the molecular mechanisms that remain to be elucidated. (b) The concept of cNF multiplicity, which is the vast heterogeneity of cNF presentation and evolution, and the growing evidence that a myriad of factors are implicated in cNF development. Understanding the underpinnings of this variability is critical in the development of innovative and targeted

focused on addressing core concepts in: cNF Pathology and Biology, Therapeutic Targets for cNF Treatment, Preclinical Testing and Assays, and Human Clinical Trials: Therapeutic Strategy and Development. Through a review of published and unpublished data and leveraging the recent therapeutic advances for other NF1-associated tumors such as plexiform neurofibromas (pNFs) and optic pathway gliomas (Fangusaro et al., 2021; Gross et al., 2020) and positive outcomes for other skin conditions such as vitiligo and alopecia areata that were previously without meaningful therapeutic advance (Chovatiya and Paller, 2021; Sheikh et al., 2022), the teams addressed what is known as well as the knowledge gaps needing to be filled to support meaningful therapeutic development for NF1-associated cNFs.

cNF biology and pathophysiology

NF1 results from inactivating pathogenic variants in the *NF1* gene, which is located on chromosome 17q11.2 (Brosseau et al., 2018; Carey et al., 1986; Upadhyaya et al., 1997). The gene product, neurofibromin, is widely expressed in almost all tissues but is most abundant in the central and peripheral nervous systems (Brosseau et al., 2018; Daston and Ratner, 1992; Upadhyaya et al., 1997). NF1 can result from a de novo or familial pathogenic variant and is transmitted in an autosomal dominant inheritance pattern. Roughly 50% of patients with NF1 have a spontaneous pathogenic variant since the *NF1* gene has one of the highest rates of spontaneous variation (Carey et al., 1986).

Absence of functional neurofibromin contributes to the constitutive activation of RAS (Bollag et al., 1996). RAS is a commonly mutated oncogene that is implicated in most human cancers. Altered RAS regulation is also associated with a range of conditions affecting multiple organs termed the RASopathies (Matthews et al., 2022). NF1 is one such Rasopathy because variants in the gene *NF1* lead to reduced function of neurofibromin and dysregulation of RAS. This results in highly variable clinical manifestations ranging from the nearly universal cNFs (including the various forms of the most common, discrete cNFs or the rarer diffusely infiltrating dermal neurofibromas (Ortonne et al., 2018) to the uncommon but potentially fatal sarcomas and gliomas in people with NF1. The contribution of altered regulation of RAS to cNF formation, growth, and maintenance is explored in detail in the article “RAS Signaling Gone Awry in the Skin: The Complex Role of RAS in Cutaneous Neurofibroma Pathogenesis, Emerging Biological Insights” (Rhodes et al., 2023). Although the specific RAS isoforms activated in cNF development have yet to be defined, RAS and its down and upstream effectors are leading targets for cNF therapeutic

development (Figure 1a). Furthermore, there are compelling developments highlighting the relatively protective role of germline loss of *NF1* against malignancy versus the causative role of somatic *NF1* alterations in common cancers, including melanoma (Brosseau et al., 2018). These observations indicate that there may be a complex balance in RAS regulation between benign and malignant tumors that, once understood, can be therapeutically harnessed (Figure 1a).

Key challenges in the treatment or prevention of cNFs are their multiplicity and variability in clinical and molecular features across people with NF1 and even across tumors in a single individual. There are many factors that contribute to the regulation of cNF multiplicity addressed in the article “Cutaneous Neurofibroma Heterogeneity Factors that Influence Tumor Burden in Neurofibromatosis Type 1” (Jiang et al., 2023). First, biallelic inactivation of the *NF1* gene in Schwann cells is critical for cNF formation (and indeed, the formation of all peripheral nerve tumors in people with NF1) (Brosseau et al., 2018; Chen et al., 2019; Radomska et al., 2019; Staser et al., 2012). The cell of origin of cNFs is defined as a boundary cap cell (Radomska et al., 2019) or a skin-derived precursor cell (Chen et al., 2019) derived from the embryonic dorsal nerve root and the dorsal root ganglion (DRG) (Figure 1b). These recent discoveries link *NF1* loss and RAS regulation with neural and skin development and homeostasis; both helping to identify new targets and experimental endpoints for cNFs and highlighting the important interaction between skin and the peripheral nervous system (Figure 1b). Work is ongoing to define the spatial and temporal loss of somatic *NF1* and how *NF1* variants influence cNF behavior in skin and pNF in deep nerves.

Another hallmark of cNFs is the infiltration of inflammatory cells. Recent efforts revealed that cNF development in murine models is a multistep process, involving a number of intersecting cellular and inflammatory processes (Chen et al., 2019; Radomska et al., 2019). Mast cells play a key role in the development of pNFs (infiltrating tumors of the peripheral nerve) where they are recruited by the chemoattractant stem cell factor secreted by *NF1* null Schwann cells (Liao et al., 2018; Staser et al., 2012). Early studies indicate that there is also increased mast cell density in cNFs versus in uninvolved skin (with relatively higher density in small cNFs, cNFs removed from people in early adulthood, and symptomatic cNFs), but their role in tumorigenesis is still unclear (Kallionpää et al., 2022). Macrophages are also abundant in cNFs compared to unaffected skin, and there is a proposed relationship between the recruitment and proliferation of macrophages in human cNFs and the expression of YAP and TAZ, core effectors in the Hippo pathway (Jia et al., 2020).

therapeutics. (c) A review of the currently available preclinical models that have allowed progress in our understanding of cNF pathogenesis; this article highlights the uses and limitations of these models as well as future avenues for discovery. (d) The current landscape of therapeutic options for cNFs, which includes a review of clinical trials completed thus far and others that are actively enrolling. In addition, this article explores regulatory considerations specific to cNF therapeutic development and strategies to improve cNF clinical trial design. (e) The current techniques available in dermatology to identify and monitor cNFs, with a focus on novel imaging techniques under investigation for better characterization and prospective tracking of cNFs. This article also describes emerging technologies that enable the detection of early cNFs that are not yet clinically apparent to enable prevention and early-intervention strategies. Illustration was provided by Tim Phelps courtesy of 2022 JHU AAM Department of Art as Applied to Medicine The Johns Hopkins University School of Medicine. 3D, three-dimensional; BC, boundary cap; cNF, cutaneous neurofibroma; DRG, dorsal root ganglion; ERK, extracellular signal-regulated kinase; MEK, MAPK/extracellular signal-regulated kinase kinase; NC, neural crest; pNF, plexiform neurofibromas; SC, stem cell.

Chen et al. (2019) showed that the HOXB7 lineage—derived cells were the source cells for both cNFs and pNFs in a *Hoxb7-Cre;Nf1^{fl/fl}* genetically engineered mouse model and that the Hippo pathway is a key modulator of both the (more common) discrete and (rarer) diffuse cNFs. They also showed that Hippo and MAPK pathway dysregulation interact in the setting of *Nf1* loss to support both cNF and pNF development (Chen et al., 2019). Finally, in 36 cNF samples from 10 patients, genetic variation was observed in genes within the Hippo pathway (Faden et al., 2017; Gosline et al., 2017). The specific relationship between *NF1*, RAS, cNF formation, and alterations in the Hippo pathway is yet to be elucidated. This is of particular interest for cNFs because there are several therapeutics (systemic and topical) that are effective for regulation of altered Jak—signal transducer and activator of transcription (STAT) signaling in skin conditions such as atopic dermatitis and psoriasis (Chovatiya and Paller, 2021; Papp et al., 2021). The Jak—STAT pathway interacts with the Hippo pathway and may be a promising area of investigation for cNFs (Guo et al., 2021; Rosmarin et al., 2020; Wang et al., 2018).

Finally, there are emerging data about the relationship between neuronal tissue and other cell components in cNFs such as Schwann cells and the cells of the microenvironment. This includes potential contributions of neurturin and artemin signaling between aberrant C-fiber terminals and Schwann cells in the dermis contributing to both the initiation and propagation of early-stage cNFs (Rice et al., 2019). This finding is reminiscent of the critical role of cells derived from the DRG in cNF development in preclinical models of cNF (Chen et al., 2019; Radomska et al., 2019) (Figure 1b). As addressed by Jiang et al. (2023), an improved understanding of the factors that contribute to cNF heterogeneity (cell specific, tissue intrinsic, and systemic) will enable therapeutic development for both prevention and treatment of cNFs.

An important element in a functional pipeline for therapeutic discovery and development for cNFs is the availability of feasible and informative model systems relative to human disease. Fortunately, tremendous progress has been made in the development and validation of preclinical models and their use for therapeutic assessment. These models range from two-dimensional semi-immortalized cell cultures to three-dimensional cocultures, human induced pluripotent stem cells harboring *NF1* gene sequence variations, and multiple in vivo models (fly, mouse, pig) (Figure 1c) (Chen et al., 2019; King et al., 2020; Li et al., 2016; Mazuelas et al., 2020; Mo et al., 2021; Radomska et al., 2019; Stemmer-Rachamimov et al., 2004). The applicability of each of the models to specific questions pertinent to cNF therapeutic development is addressed in the article by Staedtke et al. (2023), “Existing and Developing Preclinical Models for Neurofibromatosis Type 1—related Cutaneous Neurofibromas.” One important role of these models is to enable the investigation of the temporal and spatial influence of *Nf1* variants on the behavior of the various cells that make up cNFs. As such, these models enable therapeutic discovery for the condition NF1 (cNFs and other manifestations) as well as skin conditions influenced by the RAS—MAP kinase pathways.

cNF epidemiology

Despite the nearly universal presence of cNFs in people with NF1, the natural history of cNFs is not fully elucidated. It is well-documented that cNFs become apparent later in life than pNFs and increase in number and perhaps size over decades (Cannon et al., 2018; Ehara et al., 2018). Specific patterns have also been observed: cNFs have a greater propensity to develop and grow on the trunk than on the head and neck or extremities, with tumors typically being apparent in late childhood (Ehara et al., 2018). However, some may appear and progress as late as the third or fourth decade of life. The evolution of cNFs over time, across different age groups and sex and in people with different skin types, has not been adequately studied. A prospective natural history study of 22 adults with NF1 and cNFs monitored a region of the back over an 8-year period to quantify cNF size and number (Cannon et al., 2018). The investigators utilized calipers to measure cNFs at baseline and then every 4 months for 2 years and finally, in 14 of 22 participants, at 8 years. The data showed that both the size and the number of cNFs increased slowly over time.

Others groups have attempted to elucidate the natural history of cNFs and identify prognostic features. For example, the role of systemic hormones has been addressed in a series of studies. In in vitro studies, the proliferation of *NF1* nullizygous Schwann cells (but not *NF1* heterozygous cells) was significantly increased by estradiol, testosterone, and human chorionic gonadotropin (Pennanen et al., 2018). However, these findings have not been confirmed in clinical studies, and data in humans are conflicting. In a study of 80 participants with NF1, over 92% of cNFs expressed progesterone receptor and GPER-1, but an association with clinical behavior was not assessed (Rozza-de-Menezes et al., 2021). In a retrospective study, 60% of 105 women with NF1 recalled new cNFs during pregnancy, and 52% recalled enlargement of existing cNFs (Dugoff and Sujansky, 1996). However, a recent study of 13 women examined during pregnancy found no difference in cNF growth between pregnant and nulliparous women, although pregnant women were noted to have an increase in the size of individual cNFs during pregnancy (Well et al., 2020).

Current landscape of treatments for cNFs

The current standard of care for cNFs is either observation (with symptomatic management of pain or itch), surgical resection, or destruction through instrumental dermatology (Chamseddin and Le, 2020; Verma et al., 2018) (Figure 1d). However, both surgery and device-based treatments are limited in efficacy owing to the number of cNFs needing treatment, associated scarring, and the limited access to cNF skin experts for many people with NF1 around the world. Various forms of laser therapies are also used for cNF destruction, but there is an absence of prospective data specific to cNFs and concern about scarring and pigmentary changes when treating large swaths of skin (Kriechbaumer et al., 2014; Verma et al., 2018). In addition, standardized therapeutic guidelines have not been established, and selection of treatment varies widely between providers. A better understanding of the natural history of cNFs as well as a systematic and prospective assessment of outcomes of

current treatment options across different cNF subtypes, skin phototypes, and age groups are required to improve therapeutic discovery and assessment of therapeutic efficacy. The article "Target Product Profile for Cutaneous Neurofibromas: Clinical Trials to Prevent, Arrest, or Regress Cutaneous Neurofibromas" (Ly et al., 2023) provides an outline of treatment options ranging from excision and localized treatments to systemic options that are in current clinical use or under investigation (Figure 1d). The authors highlight development opportunities for each of these approaches and provide recommendations for the minimum set of criteria required to advance experimental treatments into efficacy clinical trials. In addition, the paper offers insights into the regulatory considerations for cNF clinical trials such as development and selection of appropriate clinical trial endpoints. This includes further investigation and incorporation of professional, caregiver, and patient perspectives into clinical trial measures.

Evaluation and monitoring of cNFs

The final article in this series, "Current and Emerging Imaging Techniques for Neurofibromatosis Type 1-Associated Cutaneous Neurofibromas" (Li et al., 2023), explores another important aspect in therapeutic development: the need for a consistent set of measurement techniques and endpoints to reliably and objectively document the biologic or clinical benefit of treatment. Through a comprehensive search of the literature and the presentation of early-stage results from ongoing evaluations of new techniques, including optical coherence tomography, spatial frequency domain imaging, and application of hand-held and whole-body stereoscopic optical imaging devices to cNFs, the authors present the available sensitivity, specificity, and applicability of various measures for assessing response to therapy in cNFs.

Published measurement approaches include calipers, digital photography, and high-frequency ultrasound sonography (Figure 1e). These tools enable a more accurate and nuanced understanding of the natural history of cNFs and enable quantification of change with treatment. In addition, they aid in identifying nascent or latent tumors not visible to the eye. Detecting these incipient cNFs opens the possibility for prevention and early therapeutic interventions. However, ongoing optimization of these techniques and evaluation of new modalities is required to accurately identify cNFs at different developmental stages, differentiate them from other skin lesions, establish reliability and reproducibility, and establish the clinically significant change to fuel therapeutic trials.

Conclusions

The convergence of an incredible period of development of effective therapies for a range of common and rare skin diseases, the maturation and expansion of data explaining foundational elements underlying NF1-associated cNF formation, and the existence of tools and resources that enable (even encourage) multidisciplinary collaboration to solve clinical problems that were once deemed unsolvable highlight the opportunity to accelerate meaningful discovery and therapies for NF1-associated cNFs. Now is the ideal time for

the specialists in skin, nerve, and therapeutic development to bring forward the next revolution in therapeutics for skin disease, this time for NF1-associated cNFs.

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CONFLICT OF INTEREST

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AUTHOR CONTRIBUTIONS

Conceptualization: JOB; Writing - Original Draft Preparation: JOB, CGR; Writing - Review and Editing: JOB, LQL, SYL, IL, SDR, CGR, KYS, VS, MRS, PW

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Appendix

The 2022 NTAP Cutaneous Neurofibroma Symposium Participants include: David Largaespada, Eduard Serra, Muzlifah Haniffa, Annette Bakker, Frank McCormick, Ross L. Cagan, William Ju, Anat Stemmer-Rachamimov, Kevin Grimes, Piotr Topilko, Deanna Kornacki, Kristen M. Kelly, Sally Gottesman, Zachary York, Roselyn Epps.

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Functional interactions between neurofibromatosis tumor suppressors drive Schwann cell tumor de-differentiation and treatment resistance

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Abstract



Schwann cell tumors are the most common cancers of the peripheral nervous system and can arise sporadically or in patients with neurofibromatosis type-1 (NF-1) or type-2 (NF-2). NF-1 is caused by loss of NF1, a negative regulator of Ras signaling. NF-2 is caused by loss of NF2, a pleiotropic tumor suppressor that inhibits PAK signaling. Functional interactions between the NF1 and NF2 tumor suppressors and broader mechanisms underlying malignant transformation of the Schwann lineage are unclear. Here, we integrate DNA methylation profiling, whole exome sequencing, bulk and single-cell RNA sequencing, biochemistry, and pharmacology across human samples, patient-derived cell lines, and mouse xenografts to identify cellular de-differentiation mechanisms driving malignant transformation and treatment resistance in Schwann cell tumors. Our data show molecular groups of Schwann cell tumors are distinguished by de-differentiation trajectories that drive resistance to MEK inhibition, the only approved molecular therapy for patients with NF-1. Functional genomic screening for mediators of MEK inhibitor responses in NF1-deficient tumor cells reveals NF2 loss and PAK activation underlie Schwann cell tumor de-differentiation and MEK inhibitor resistance. In support of these findings, we identify a group of de-differentiated Schwann cell tumors with concurrent loss of NF1 and NF2, and find combination molecular therapy inhibiting MEK and PAK is an effective treatment for de-differentiated

suppressors that sheds light on a novel therapeutic vulnerability.

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Functional interactions between neurofibromatosis tumor suppressors drive Schwann cell tumor de-differentiation and treatment resistance

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Abstract

Schwann cell tumors are the most common cancers of the peripheral nervous system and can arise sporadically or in patients with neurofibromatosis type-1 (NF-1) or type-2 (NF-2). NF-1 is caused by loss of NF1, a negative regulator of Ras signaling. NF-2 is caused by loss of NF2, a pleiotropic tumor suppressor that inhibits PAK signaling. Functional interactions between the NF1 and NF2 tumor suppressors and broader mechanisms underlying malignant transformation of the Schwann lineage are unclear. Here, we integrate DNA methylation profiling, whole exome sequencing, bulk and single-cell RNA sequencing, biochemistry, and pharmacology across human samples, patient-derived cell lines, and mouse xenografts to identify cellular de-differentiation mechanisms driving malignant transformation and treatment resistance in Schwann cell tumors. Our data show molecular groups of Schwann cell tumors are distinguished by de-differentiation trajectories that drive resistance to MEK inhibition, the only approved molecular therapy for patients with NF-1. Functional genomic screening for mediators of MEK inhibitor responses in NF1-deficient tumor cells reveals NF2 loss and PAK activation underlie Schwann cell tumor de-differentiation and MEK inhibitor resistance. In support of these findings, we identify a group of de-differentiated Schwann cell tumors with concurrent loss of NF1 and NF2, and find combination molecular therapy inhibiting MEK and PAK is an effective treatment for de-differentiated Schwann cell tumor xenografts. In sum, we elucidate a paradigm of de-differentiation driving malignant transformation and treatment resistance, uncovering a functional link between the NF1 and NF2 tumor suppressors that sheds light on a novel therapeutic vulnerability.

Main Text

The neural crest derived Schwann cell lineage gives rise to schwannomas, neurofibromas, and malignant peripheral nerve sheath tumors (MPNSTs), which comprise the most common cancers of the peripheral nervous system¹. Despite their shared embryonic origin, the clinical course and molecular drivers of Schwann cell tumors are distinct. Neurofibromas and schwannomas are benign tumors that can be cured with surgery or radiotherapy, while MPNSTs metastasize and are often incurable². Neurofibromas and MPNSTs are associated with loss of *NF1*, a tumor suppressor that inhibits Ras/Raf/MEK/ERK signaling. Schwannomas are associated with loss of *NF2*, a pleiotropic tumor suppressor that modulates numerous downstream effectors including PAK signaling, the Hippo pathway, apoptosis, contact inhibition, and the proteasome³. Germline loss of *NF1* causes neurofibromatosis type-1 (NF-1)⁴, and germline loss of *NF2* causes neurofibromatosis type-2 (NF-2)⁵, which are among the most common cancer predisposition syndromes.

MPNSTs are the most aggressive Schwann cell tumors and can arise from *NF1*-deficient plexiform neurofibromas in the setting of second hits disrupting epigenetic differentiation^{6–8}. Although rare, *NF2*-deficient schwannomas can also undergo malignant transformation⁹, but it remains unclear if *NF1* and *NF2* interact during Schwann cell tumorigenesis or treatment response. Moreover, despite the recent approval of the MEK inhibitor selumetinib to treat neurofibromas in patients with NF-1^{10,11}, there are

currently no effective molecular therapies for patients with MPNSTs¹². To address these gaps in our understanding of Schwann cell biology and the unmet translational need for new therapies to treat Schwann cell tumors, we performed multiplatform bulk and single cell molecular profiling combined with biochemical, pharmacologic, and functional genomic interrogation of human Schwann cell tumors, patient-derived cell lines, and mouse xenografts. Our results demonstrate Schwann cell de-differentiation and functional interactions between *NF1* and *NF2* underlie malignant transformation and treatment resistance, elucidating a novel druggable dependency in Schwann cell tumors.

Multiplatform bulk and single-cell molecular profiling reveals de-differentiation underlies malignant transformation of Schwann cell tumors.

DNA methylation profiling provides robust classification of central nervous system tumors, but how this approach applies to peripheral nervous system tumors is incompletely understood¹³. To elucidate the epigenetic landscape of Schwann cell tumors, DNA methylation profiling was performed on schwannomas (n = 66), plexiform neurofibromas (n = 11), or MPNSTs (n = 42) from patients who were treated at a single institution from 1991 to 2021. Neuropathology review using the most recent World Health Organization criteria validated all histologic diagnoses¹⁴. Unsupervised hierarchical clustering revealed 3 DNA methylation groups (Fig. 1a, Extended Data Fig. 1a, and Supplementary Table 1). Group 1 tumors were enriched for MPNSTs and were distinguished by CNVs deleting *SUZ12* or *EED*, obligate members of the PRC2 epigenetic complex that is recurrently lost in MPNSTs^{6–8} (Fig. 1a and Extended Data Fig. 1b-e). Both Group 1 and 2 tumors harbored CNVs deleting *CDKN2A/B*, a tumor suppressor implicated in Ras-induced senescence that can be lost during neurofibroma transformation to MPNST including in the transitory premalignant stage defined as atypical neurofibromatous neoplasm of uncertain biologic potential (ANNUBP)^{6,8,15–17} (Fig. 1a and Extended Data Fig. 1b-e). Group 3 tumors were enriched for schwannomas and distinguished by minimal CNVs, with the exception of recurrent CNVs deleting chromosome 22q, which harbors *NF2* (Fig. 1a and Extended Data Fig. 1d-e). Chromosome 22q loss was also enriched Group 1 compared to 2 tumors (60% *NF2* loss Group 1 versus 24% *NF2* loss Group 2, p = 0.02, Chi-squared test), typically in combination with *NF1* or PRC2 alterations (Fig. 1a and Extended Data Fig. 1e). Differential DNA methylation and principal component analysis across Schwann cell tumor groups showed hypermethylated sites in Group 2 tumors were enriched for PRC2 target genes while hypermethylated sites in Group 1 tumors were enriched for cell differentiation transcription factors (Extended Data Fig. 1f-h). In sum, these data suggest loss of PRC2 or loss of *NF2* drive cellular de-differentiation and distinguish DNA methylation groups of differentiated (Group 2) or de-differentiated (Group 1) Schwann cell tumors.

To understand genetic and gene expression features distinguishing DNA methylation groups of Schwann cell tumors, whole exome sequencing (n = 34), RNA sequencing (n = 17), or immunohistochemistry (n = 36) were performed on Group 1 or Group 2 tumors. Whole exome sequencing identified recurrent short somatic variants in the core PRC2 components *SUZ12* or *EED* in Group 1 but not Group 2 tumors (Fig. 1a, Extended Data Fig. 2, and Supplementary Table 2). RNA sequencing revealed transcriptomic signatures

separated according to DNA methylation groups (Extended Data Fig. 3a and Supplementary Table 3), and differential expression analysis showed enrichment of Schwann cell differentiation genes (*S100B*, *SOX10*) and SUZ12 target genes in Group 2 compared to Group 1 tumors (Fig. 1a and Extended Data Fig. 3b-d). Immunohistochemistry for H3K27 trimethylation, an epigenetic marker of PRC2 activity, or immunohistochemistry for the Schwann cell differentiation markers S100B or SOX10, demonstrated loss of all three in Group 1 tumors compared to Group 2 tumors (Fig. 1a, b and Extended Data Fig. 3e).

Intratumor heterogeneity represents a challenge for histologic diagnosis of neurofibroma or MPNST¹⁴ (Extended Data Fig. 3f). To define the cellular architecture across groups of *NF1*-deficient Schwann cell tumors, single nuclear RNA sequencing was performed on 19,276 nuclei from Group 1 (n = 3) or Group 2 (n = 3) tumors (Supplementary Table 4). Uniform manifold approximation and projection (UMAP) revealed a total of 13 cell clusters that were defined using cell signature gene sets or differentially expressed cluster marker genes¹⁸. Five clusters were shared across Group 1 and Group 2 tumors, and the remaining 7 clusters were distinct to Group 1 or Group 2 tumors (Fig. 1c and Extended Data Fig. 4a). All shared clusters corresponded to non-tumor cell populations such as smooth muscle, pericytes, macrophages, lymphocytes, or endothelia (Fig. 1c-d and Extended Data Fig. 4b-c). Group 1 tumor single-cell clusters were distinguished by enrichment of de-differentiation markers (*MEST*, *POSTN*, *DLK1*, *SOX4*) or proliferation markers (*KI67*, *TOP2A*) (Fig. 1d and Extended Data Fig. 4c). Group 2 tumor single-cell clusters were distinguished by enrichment of differentiation markers (*SRPX2*, *TNXB*) or suppression of proliferation markers (Fig. 1d and Extended Data Fig. 4c). Assignment of cell cycle phases based on consensus gene signatures demonstrated enrichment of G2/M phase cells in Group 1 tumors compared to Group 2 tumors (Fig. 1e and Extended Data Fig. 4c). Among non-tumor single-cell clusters, the Schwann cell cluster was comprised almost entirely of cells from Group 2 tumors (Fig. 1d and Extended Data Fig. 4b-c), consistent with enrichment of Schwann cell differentiation markers in Group 2 compared to Group 1 tumors from bulk RNA sequencing or immunohistochemistry (Fig. 1a, b). Moreover, Schwann cell differentiation markers were suppressed and PRC2 target genes were enriched in single-nuclear RNA sequencing of Group 1 tumors compared to Group 2 tumors (Fig. 1f).

Schwann cell de-differentiation underlies MEK inhibitor resistance

To determine if PRC2 drives Schwann cell tumor differentiation, we analyzed a panel of patient-derived neurofibroma or MPNST cells with *NF1* and PRC2 mutations (Extended Data Fig. 5a). RNA sequencing showed enrichment of PRC2 target genes and suppression of differentiation genes in MPNST cells compared to neurofibroma cells (Extended Data Fig. 5b and Supplementary Table 5). Integration of RNA sequencing data from CRISPR PRC2 knockout neurofibroma cells¹⁹ revealed both MPNST and PRC2-deficient neurofibroma cells exhibited suppression of Schwann cell differentiation markers (*S100B*, *SOX10*), enrichment of de-differentiated early neural crest markers (*EN1*, *SOX9*, *FOXF1*), and enrichment of Ras/Raf/MEK/ERK target genes (*DUSP6*, *SPRY2*, *ETV4*) (Extended Data Fig. 5c-e). Hierarchical clustering of all 16 patient-derived neurofibroma or MPNST cell lines based on a consensus PRC2 target

gene set comprised of 24 differentiation, early neural crest, or Ras/Raf/MEK/ERK target genes segregated PRC2-intact from PRC2-deficient cells (Fig. 2a). Moreover, analysis of published H3K27 trimethylation ChIP-seq data¹⁹ revealed epigenetic de-repression of early neural crest markers (*EN1*, *SOX9*, *FOXF1*) but not Ras/Raf/MEK/ERK target genes (*DUSP4*, *SPRY2*, *ETV4*) in PRC2-deficient cells, suggesting direct epigenetic mechanisms underlie Schwann cell tumor de-differentiation (Extended Data Fig. 5f).

The MEK inhibitor selumetinib is effective for neurofibromas but shows mixed results for MPNSTs^{10–12}. Thus, we examined the relationship between cell differentiation and selumetinib response across patient-derived neurofibroma or MPNST cell lines. PRC2-deficient MPNST cells demonstrated resistance to selumetinib compared to PRC2-intact cells (Extended Data Fig. 5g, h). To determine if PRC2 inactivation was sufficient for selumetinib resistance, we used CRISPR interference (CRISPRi) to suppress *SUZ12* or *EED* in *NF1*-deficient NF95.11b neurofibroma cells (Fig. 2b). Consistent with CRISPR PRC2 knockout data (Fig. 2a and Extended Data Fig. 5c-e), CRISPRi suppression of PRC2 inhibited Schwann cell differentiation markers and was sufficient for selumetinib resistance (Fig. 2b).

To interrogate cellular mechanisms underlying response to MEK inhibition, we performed biochemical or transcriptomic analyses of *NF1*-deficient NF95.11b neurofibroma cells after treatment with selumetinib (Supplementary Table 6). Bulk RNA sequencing confirmed repression of Ras/Raf/MEK/ERK target genes (*DUSP4*, *SPRY2*) and revealed loss of Schwann cell differentiation markers such as *S100B* after selumetinib treatment compared to vehicle treatment (Extended Data Fig. 6). Immunoblotting of neurofibroma cell lysates after selumetinib treatment showed an initial repression of ERK phosphorylation (pERK) and early induction of apoptosis (cleaved Caspase-3, cleaved Caspase-7), followed by recovery of pERK in cells that persisted despite continued selumetinib treatment (Fig. 2c). To determine if de-differentiation underlies MEK inhibitor resistance *in vivo*, we performed single-cell RNA sequencing on 65,314 cells from JW23.3 male MPNST xenografts²⁰ grown in female recipient mice that were treated with selumetinib or vehicle control (Supplementary Table 7). UMAP analysis revealed 13 clusters of cells (Fig. 2d and Extended Data Fig. 7a). Tumor microenvironment cells in female recipient mice were distinguished from male MPNST xenograft cells using *Xist* expression from the X chromosome (Extended Data Fig. 7b), leading to identification of 9 tumor microenvironment clusters that were defined using cell signature gene sets or differentially expressed cluster marker genes (Extended Data Fig. 7c-d). Analysis of the remaining 4 tumor cell clusters revealed suppression of Schwann cell differentiation markers in the one cluster that was enriched after selumetinib treatment compared to vehicle treatment (Fig. 2e, f). In sum, these data demonstrate Schwann cell de-differentiation underlies MEK inhibitor resistance *in vitro* and *in vivo*.

NF2 inactivation drives de-differentiation and MEK inhibitor resistance in NF1-deficient Schwann cell tumors

To identify druggable mechanisms underlying MEK inhibitor resistance, we performed triplicate genome-wide CRISPRi screens in *NF1*-deficient NF95.11b neurofibroma cells treated with selumetinib or vehicle control (Extended Data Fig. 8a and Supplementary Table 8). As expected, sgRNAs targeting tumor

suppressor genes such as *TP53* or negative regulators of the Ras pathway (*RASA2*, *SPRY2*) were enriched following selumetinib treatment, consistent with selumetinib resistance (Fig. 3a and Extended Data Fig. 8b-c). In contrast, sgRNAs targeting the cell cycle (*CCNE1*, *E2F4*) or differentiation (*CDH2*, *SETD9*, *KDM1B*) were depleted following selumetinib treatment, consistent with selumetinib sensitization (Fig. 3a and Extended Data Fig. 8b-c). sgRNAs suppressing *NF2* were among the strongest drivers of selumetinib resistance (Fig. 3a), and *Nf2* expression was suppressed in de-differentiated, selumetinib-resistant single-cells following selumetinib treatment of MPNST xenografts (Fig. 3b).

To validate *NF2* as a driver of MEK inhibitor resistance and elucidate functional consequences of combined *NF1* and *NF2* loss in the Schwann cell lineage, we used CRISPRi to suppress *NF2* in *NF1*-deficient NF95.11b neurofibroma cells (Fig. 3c and Supplementary Table 9). RNA sequencing validated *NF2* suppression and revealed loss of Schwann cell differentiation markers, *SUZ12*, and *SUZ12* target genes upon *NF2* suppression (Fig. 3c and Extended Data Fig. 9a-b). Moreover, *NF1*/*NF2*-deficient neurofibroma cells were resistant to selumetinib compared to *NF1*-deficient, *NF2*-intact neurofibroma cells (Fig. 3c).

Epigenetic mechanisms regulating cell differentiation remain challenging pharmacologic targets²¹. In contrast, PAK activation following *NF2* loss²² represents a druggable dependency²³ that can be inhibited by small molecules under clinical development²⁴. In support of this hypothesis, CRISPRi suppression of *NF2* in *NF1*-deficient NF95.11b neurofibroma cells induced PAK phosphorylation (pPAK) (Fig. 3d). High pPAK was observed in *NF1*/*SUZ12*-deficient ST88-14 MPNST cells independent of CRISPRi suppression of *NF2* (Extended Data Fig. 9c-d and Supplementary Table 10), suggesting PAK activation occurs upon de-differentiation of Schwann cell tumors. *NF1*/*NF2*-deficient NF95.11b neurofibroma cells maintained pERK activity (Fig. 3e) and cell viability (Fig. 3c) in response to selumetinib compared to *NF2*-intact neurofibroma cells, suggesting loss of *NF2* is sufficient to drive selumetinib resistance. In contrast, the PAK1 inhibitor NVS-PAK1-1 repressed pPAK1 and pERK in both *NF2*-intact and *NF2*-deficient NF95.11b neurofibroma cells (Fig. 3e). To determine if PAK inhibition was effective *in vivo*, we treated MPNST xenografts with vehicle, selumetinib, NVS-PAK1-1, or selumetinib plus NVS-PAK1-1 and found combination molecular therapy additively inhibited xenograft growth compared to vehicle or either selumetinib or NVS-PAK1-1 monotherapy (Fig. 3f).

Discussion

Neurofibromatosis was first reported over 140 years ago²⁵, and the *NF1* gene product's function as a Ras GAP was determined over 30 years ago^{26,27}. Molecular therapies have only recently become standard of care for patients with neurofibromatosis^{10,11,28}, but with recent reports of the NF1 protein structure^{29–31} additional therapeutic strategies are on the horizon. Given the fact that components of growth factor signaling and the Ras pathway, such as *NF1*, are mutated in nearly half of human cancers³², such translational insights may be transformative for clinical oncology. Here, we identify 3 DNA methylation groups of neurofibromatosis-associated peripheral nervous system tumors distinguished by cellular de-

differentiation mechanisms underlying malignant transformation and treatment resistance. Loss of the epigenetic regulator PRC2 is sufficient to drive de-differentiation, linking Schwann cell development and treatment resistance. *NF2* inactivation and pPAK induction drive de-differentiation and selumetinib resistance in *NF1*-deficient tumors, shedding light on a novel druggable dependency underlying the most common tumors of the peripheral nervous system. In sum, our data provide a functional link between the *NF1* and *NF2* tumor suppressor genes.

Despite the inarguable clinical success of selumetinib, additional therapies for patients with MPNSTs or recurrent neurofibromas or schwannomas are needed. Thus, our identification of PAK as a target for combination molecular therapy to treat Schwann cell tumors has potential clinical implications, particularly given the challenges of directly targeting epigenetic or de-differentiation mechanisms in patients. Beyond PAK inhibition, other signaling mechanisms downstream of *NF2* loss such as the Hippo Pathway³³, the Rho/Rac/Cdc42 family of small GTPases³⁴, or IRF-mediated apoptosis³⁵ may also be candidates for future combination molecular therapies to treat neurofibromatosis-associated tumors³⁴.

Several key questions remain regarding de-differentiation and treatment resistance in neurofibromatosis-associated tumors. First, does cellular de-differentiation underlie MEK inhibitor resistance in human patients with NF-1, and how does the single cell architecture of tumor cell states or non-tumor cell types change in response to MEK inhibition in humans? As MEK inhibition becomes more common for patients with NF-1, serial molecular analyses will be critical to unravel mechanisms of treatment response or resistance to optimize combination or sequential molecular therapies. Second, is de-differentiation mediated malignant transformation or treatment resistance of neurofibromatosis-associated peripheral nervous system tumors a relevant mechanism for neurofibromatosis-associated central nervous system tumors, such as gliomas? Ongoing clinical trials are evaluating the efficacy of MEK inhibition for gliomas arising in patients with NF-1 (ClinicalTrials.gov NCT03871257), and multiplatform molecular analysis of these precious samples will no doubt help determine if the relationships between activation of the Ras family of small GTPases following *NF1* loss, and induction of the Rho/Rac/Cdc42 family of small GTPases following *NF2* loss, are conserved across oncologic contexts. If so, the strategy of dual Ras/Raf/MEK/ERK and Rho/Rac/Cdc42/PAK inhibition may be broadly useful across other Ras-mutated tumors, which comprise the most common molecular alterations in human cancers³².

Declarations

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Author contributions statement

All authors made substantial contributions to the conception or design of the study; the acquisition, analysis, or interpretation of data; or drafting or revising the manuscript. All authors approved the manuscript. All authors agree to be personally accountable for individual contributions and to ensure that questions related to the accuracy or integrity of any part of the work are appropriately investigated, resolved, and the resolution documented in the literature. H.N.V. designed, performed, and analyzed all experiments and bioinformatic analyses. E.P. performed biochemistry, qPCR, cell viability assays, and mouse xenograft experiments. C.D. performed single-cell RNA sequencing and assisted with bioinformatic analysis. M.J.S. performed biochemistry, pharmacology, and helped with experimental design. S.J.L. assisted with bioinformatic analysis, CRISPRi cell line generation, and genome-wide screening. S.L. performed qPCR analysis and provided critical mouse support. C.H.L. performed pathology review and assisted with processing human tumor samples for genomic analyses. C.D.E. performed biochemistry and assisted with mouse xenograft experiments. T.C.C. performed qPCR, immunofluorescence, and assisted with mouse xenograft experiments. S.T.M. extracted nucleic acids from tumor specimens and guided experimental design from patient samples. W.C.C. helped assemble human tumor resection specimens and assisted with bioinformatic analysis. A.T.R., S.E.B., and L.J. provided key insight into study design and provided clinical data. A.P. and M.P. assembled tumor resection specimens, provided clinical data, supervised C.H.L. for pathologic review, and assisted with study design. A.R.A. supervised H.N.V. and C.D. for single cell sequencing experiments and aided with genomic analysis. F.M. and D.R.R. conceived, designed, and supervised the study.

Competing interests statement

The authors declare no competing interests.

Tables

Not applicable

Methods

This study complied with all relevant ethical regulations and was approved by the UCSF Institutional Review Board (13-12587, 17-22324, 17-23196, 18-24633). As part of routine clinical practice at UCSF, all

patients included in this study signed an informed consent waiver to contribute de-identified data to scientific research projects.

Nucleic acid extraction for DNA methylation profiling, whole exome sequencing, or bulk RNA sequencing

DNA and RNA were isolated from cell lines, human samples, or mouse xenografts using the All-Prep Universal Kit (#80224, QIAGEN). For fresh frozen human samples or mouse xenografts, specimens were thawed in RLT Plus Buffer with beta-mercaptoethanol. Formalin fixed paraffin embedded tissue was de-paraffinized. All tumor or tissue samples were mechanically lysed using a TissueLyser II (QIAGEN) with stainless steel beads at 30 Hz for 90 seconds. QiaCubes were used for standardized automated nucleic acid extraction per the manufacturer's protocol. For cell line samples, pellets were directly lysed in RLT Plus buffer with beta-mercaptoethanol. RNA quality was assessed by chip-based electrophoresis on a BioAnalyzer 2100 using the RNA 6000 Nano Kit (#5067-1511, Agilent Technologies), and clean-up was performed as needed using the RNeasy kit (QIAGEN). DNA quality was assessed by spectrophotometry, and clean-up was performed as needed using DNA precipitation. Only samples with high-quality DNA ($A_{260}/A_{280} > 1.8$, $A_{260}/A_{230} > 1.6$) and/or RNA ($RIN > 8$) were used for DNA methylation profiling, whole exome sequencing, or bulk RNA sequencing.

DNA methylation profiling and analysis

Genomic DNA from human tumors were processed for methylation analysis using the Illumina Methylation EPIC Beadchip (#WG-317-1003, Illumina) according to the manufacturer's instructions. Preprocessing and normalization were performed in R using the minfi Bioconductor package^{36,37}. Only probes with detection $p < 0.05$ in all samples were included for further analysis. Data were normalized using functional normalization³⁷. Probes were filtered based on the following criteria: (i) removal of probes mapping to the X or Y chromosomes, (ii) removal of probes containing a common single nucleotide polymorphism (SNP) within the targeted CpG site or on an adjacent basepair, and (iii) removal of probes not mapping uniquely to the hg19 human reference genome. DNA methylation-based molecular neuropathology brain tumor classification¹³ or CNV estimation¹³ were performed as previously described.

Whole exome sequencing and analysis

Library preparation, exome capture, and sequencing were performed at the Institute for Human Genetics at UCSF. Sequencing libraries were prepared using the Kapa Hyper Prep Kit (#07962312001, Roche) and exome capture was performed using the Nimblegen SeqCap EZ Human Exome Kit v3.0 (Roche). Paired end sequencing with read length 100 base pairs was performed on an Illumina HiSeq4000. Whole exome data were analyzed following the Genome Analysis Toolkit (GATK) best practices^{38,39}. Raw FASTQ files

were aligned to the reference genome with Bowtie2⁴⁰. Only uniquely aligned reads were included for further processing using the Genome Analysis Toolkit to carry out de-duplication, local realignment, and base quality score recalibration. Alignment quality metrics and header information were determined using the Picard suite (<http://broadinstitute.github.io/picard/>). Somatic variants (point mutations, small indels) were identified from matched tumor-normal samples (n=15) and using a panel of normal (PoN) samples with Mutect2, per GATK best practices, when a matched normal sample was not available (n=19). Variants were annotated using Snpeff⁴¹ and were further filtered to include only those marked as high/moderate/low priority, only those occurring in protein coding or splice site locations, and only those meeting the following hard filters: (i) >5 reads in tumor compared to normal samples, (ii) >10% variant reads in tumor, and (iii) >90% reference reads in normal. The full list of parameters and filters can be found in the headers of the VCF files that will be deposited in GEO.

RNA sequencing

Library preparation was performed using the TruSeq RNA Library Prep Kit v2 (#RS-122-2001, Illumina) and 50 bp single end reads were sequenced on an Illumina HiSeq 2500 or NovaSeq to a minimum depth of 25M reads per sample at Medgenome, Inc. Quality control of FASTQ files was performed with FASTQC, and after trimming of adapter sequences, reads were filtered to remove bases that did not have an average quality score of 20 within a sliding window across 4 bases (<http://www.bioinformatics.babraham.ac.uk/projects/fastqc/>). Reads were mapped to the appropriate reference genome (hg19 or mm10) using HISAT2 with default parameters⁴². Transcript abundance estimation in transcripts per million (TPM) and differential expression analysis were performed using DESeq2⁴³. Differentially expressed transcripts with an adjusted p-value<0.1 were identified and filtered based on an expression cutoff (TPM>1) and a fold change threshold (log2FC>1) to prioritize biologically relevant gene sets. Clustering dendrograms and heatmaps were generated in R using TPM values and plotted as normalized row expression values with the heatmaps.2 function.

Immunohistochemistry (IHC)

IHC was performed as previously described⁴⁴ using formalin-fixed, paraffin-embedded tissue sections from tumor resection specimens on a combination of whole slide sections or tissue microarrays using the following primary antibodies: H3K27me3 (Cell Signaling Technology, #9733, clone C36B11, 1:50 dilution), SOX10 (Cell Marque, #383R-1, clone EP268, 1:50 dilution), S100B (Ventana, #760-2523, 1:2 dilution), or p53 (Dako, #GA61661-2, clone DO-7, 1:50 dilution). All IHC was performed on a Ventana Benchmark XT automated stainer (Roche) using standard techniques. IHC studies that were previously performed as part of clinical diagnostic workup, or stains obtained as part of prior research studies were reviewed for protein expression concordance⁴⁴. For quantitative analysis, percent staining for H3K27me3, SOX10, or S100B was estimated as the percentage of positive tumor cells on available stained tissue.

Single-nuclear or single-cell RNA sequencing and analysis

Frozen human neurofibroma or MPNST resection specimens were thawed on ice, minced with sterile razor blades, and mechanically dounced on ice in cell lysis nuclei extraction buffer until all macroscopically visible tissue dissolved into suspension. Cell suspensions were filtered through a 50 mm filter, centrifuged at 500g for 5 minutes at 4 degrees Celsius, and resuspended in 0.1% BSA in PBS. Nuclei were stained using DAPI (#D3571, Thermo Fisher Scientific) and counted. A total of 10,000 nuclei were loaded per single-nuclei RNA sequencing sample. For MPNST mouse xenograft single-cell RNA sequencing, tumors were minced with sterile razor blades and enzymatically dissociated with papain (#LS003, Worthington) at 37 degrees Celsius for 45 minutes. Samples were centrifuged at 500g for 5 minutes, resuspended in RBC lysis buffer (#00-4300-54, eBioscience), incubated for 10 minutes at room temperature, and resuspended in 5% FBS in PBS. Cell suspensions were serially filtered through 70 mm and 40 mm filters before being resuspended again in 5% FBS in PBS for manual cell counting using a hemacytometer. For cell culture single-cell RNA sequencing, cells were trypsinized and resuspended in 0.04% BSA in PBS before manual cell counting using a hemacytometer. A total of 10,000 cells were loaded per single-cell RNA sequencing sample.

Single-nuclei or single-cell RNA sequencing was performed using the Chromium Single Cell 3' Library & Gel Bead Kit v3.1 on a 10x Chromium controller (10X Genomics) using the manufacturer recommended default protocol and settings. Samples were sequenced on an Illumina NovaSeq at the UCSF Center for Advanced Technology, and the resulting FASTQ files were processed using the CellRanger analysis suite (<https://github.com/10XGenomics/cellranger>) for alignment to the hg38 reference genome, identification of empty droplets, and determination of a count threshold. All downstream analyses were performed in Seurat (<https://satijalab.org/seurat/>) using the default pipeline. In brief, data were empirically filtered on a per sample basis to remove outliers with regard to gene count, UMI count, or mitochondrial genes followed by cluster identification, UMAP generation, and marker gene list generation using computed highly variable features and the top ten principal component dimensions as previously described^{35,45}. The Schwann cell differentiation gene module (GO 0014037) and PRC2 target gene module (M8448) were downloaded from www.gsea-msigdb.org.

Cell culture, cell viability assays, and in vitro pharmacology

Patient-derived neurofibroma (NF95.11b, NF95.6) or MPNST (SNF02.2, SNF94.3, SNF96.2, ST88-14) cell lines⁴⁶ were obtained from the Neurofibromatosis Therapeutic Acceleration Program or American Type Culture Collection. Cell lines were grown in Dulbecco's Modified Eagle Medium (#11960069, Life Technologies) with 10% FBS and 1X Pen-Strep (#15140122, Life Technologies). Cell lines were regularly tested and verified to be mycoplasma negative (#LT07-218, Lonza). Viability assays were carried out with the CellTiter 96 Non-Radioactive Cell Proliferation Assay (#G410, Promega) and a Glomax Discovery

Multimode Microplate Reader (Promega). For pharmacologic assays, cells were seeded at a density of 5,000 cells per well in a 96 well plate the night prior to treatment, after which cells were treated with drugs at the indicated concentrations for the indicated periods (or 48 hours if not indicated) prior to experimentation.

Immunoblotting

Whole cell lysates were harvested using RIPA buffer (50 mM Tris-HCl at pH 8.0, 150 mM NaCl, 0.5% Deoxycholate, 0.1% SDS, 1% IGEPAL CA-630) with fresh protease (#P8340, Sigma) and phosphatase inhibitor (#P2850, Sigma) cocktails. A total of 10-20 mg of protein was loaded into pre-cast NuPAGE electrophoresis gels (Life Technologies). Samples were separated by SDS-PAGE, transferred to nitrocellulose or PVDF membranes, and blocked in either 5% bovine serum albumin or 5% skim milk in TBS buffer for 1 hour at room temperature. Primary antibodies were incubated overnight at the indicated dilution at 4 degrees Celsius and HRP conjugated secondary antibodies were incubated for 1 hour at room temperature followed by ECL based detection on film. A minimum of 2 biologic replicates were performed for all experiments. The following antibodies were used: pERK (Cell Signaling Technologies, #4370, 1:1,000 dilution), beta tubulin (Developmental Hybridoma Studies Bank, #E7, 1:10,000 dilution), pAkt (Cell Signaling Technologies, #4060, 1:1,000 dilution), pMEK (Cell Signaling Technologies, #9121, 1:1,000 dilution), pPAK (Cell Signaling Technologies, #2601, 1:1,000 dilution), Caspase-3 (Cell Signaling Technologies, #9662, 1:1,000 dilution), Caspase-7 (Cell Signaling Technologies, #9492, 1:1,000 dilution), or NF2 (Abcam, #ab88957, clone AF1G4, 1:2,000 dilution).

Quantitative reverse transcription polymerase chain reaction (qRT-PCR)

RNA was extracted from cell lines using the RNeasy Mini Kit (#74106, QIAGEN) according to manufacturer's instructions, and cDNA was synthesized from RNA using iScript cDNA Synthesis kit (#1708891, Bio-Rad). Real-time qPCR was performed using PowerUp SYBR Green Master Mix (#A25918, Thermo Fisher Scientific) on a QuantStudio 6 Flex Real Time PCR system (Life Technologies). The following qRT-PCR primers were used: *GAPDH*-F (5'-GTCTCCTCTGACTTCAACAGCG-3'), *GAPDH*-R (5'-ACCACCCTGTTGCTGTAGCCAA-3'), *SUZ12*-F (5'-AGGCTGACCACGAGCTTTTC-3'), *SUZ12*-R (5'-GGTGCTATGAGATTCCGAGTTC-3'), *EED*-F (5'-GTGACGAGAACAGCAATCCAG-3'), *EED*-R (5'-TATCAGGGCGTTCAGTGTTTG-3'), *NF2*-F (5'-TTGCGAGATGAAGTGGAAGG-3'), *NF2*-R (5'-CAAGAAGTGAAAGGTGACTGGTT-3'), *S100B*-F (5'-TGGCCCTCATCGACGTTTTTC-3'), or *S100B*-R (5'-ATGTTCAAAGAACTCGTGGCA-3').

CRISPRi cell line generation and genome-wide screening

Lentivirus containing pMH0001 (UCOE-SFFV-dCas9-BFP-KRAB, #85969, Addgene) was produced from transfected HEK293T cells with packaging vectors (pMD2.G #12259, Addgene, and pCMV-dR8.91, Trono Lab) following the manufacturers protocol (#MIR6605, Mirus). Neurofibroma NF95.11b cells were stably transduced to generate parental NF95.11b^{dCas9-KRAB-BFP} cells and selected by flow cytometry using a SH800 sorter (Sony). Subsequent gene specific knockdowns were achieved by individually cloning single-guide RNA (sgRNA) protospacer sequences into the pCRISPRi-v2 vector (#84832, Addgene) between BstXI and BlnI restriction sites. All constructs were validated by Sanger sequencing of the protospacer region. The following protospacers were used: sgNTC (GTGCACCCGGCTAGGACCGG), sgSUZ12-1 (GCTGAAACGTCTTTGGAAGG), sgSUZ12-2 (GGCAGCGGGTCGGAGATCGA), sgEED-1 (GAGTCTAGAGCCACCGTCCA), sgEED-2 (GCAGGGAGCAGGTAGCTGCT), sgNF2-1 (GTCGGGACGGGACCCCTAGA), or sgNF2-2 (GGACTCCGCGCGCCTCTCAG). Lentivirus was generated as described above and cells were selected to purity using 1 mg/mL puromycin for at least 5 days.

For genome-wide CRISPRi screening, a genome-wide dual sgRNA library was constructed by cloning the top 2 on-target sgRNAs for 23,483 genes as described previously⁴⁷ into the library expression vector pU6-sgRNA Ef1alpha Puro-T2A-GFP derived from pJR85 (#140095, Addgene) and modified to express a second sgRNA using the human U6 promoter. 1137 non-targeting sgRNA pairs were also included as negative controls in the screen. To generate lentiviral pools, HEK293T cells were transfected with the sgRNA library along with packaging plasmids as described, and viral supernatant was collected 72 hours following transfection. Lentiviral libraries were infected into NF95.11b^{dCas9-KRAB-BFP} cells, cultured for 2 days following infection, selected in 1 mg/mL puromycin for 2 days, and then allowed to recover in 10% FBS in DMEM for 1 day. Infection efficiency was evaluated by measuring BFP positivity on flow cytometry, and cell pellets were subsequently frozen down at this “T0” timepoint. The screen was subsequently carried out in biologic triplicate, with cells cultured in either 1 mM selumetinib or vehicle (DMSO) control for 10 days. Cell pellets were frozen down at this “T10” timepoint and processed for sgRNA abundance library preparation using Q5 High-Fidelity DNA Polymerase (NEB) and sequenced on an Illumina NextSeq-500 as previously described⁴⁸. Enrichment or depletion of sgRNA abundances were determined by down sampling trimmed sequencing reads to equivalent amounts across all samples, and then calculating the log2 ratio of sgRNA abundance in experimental conditions to sgRNA abundance in control conditions at T10, or between sequencing reads from T10 and T0 timepoints within experimental or control conditions. Statistical significance was calculated using Student's t-test comparing replicates across conditions. sgRNAs with fewer than 100 reads at T0 were removed from subsequent analysis. Hits were selected by normalizing log2 ratios by the standard deviations of the non-targeting control sgRNAs, and genes were considered significant at a 'threshold' >5 calculated as the |normalized log2 ratios| * -log10(p-value) to prioritize biologically meaningful hits for mechanistic or functional investigation.

Mouse tumor xenografts and in vivo pharmacology

The study was approved by the UCSF Institutional Animal Care and Use Committee (AN174769) and all experiments were conducted in compliance with institutional and governmental regulations. Subcutaneous xenografts were performed by implanting 5 million JW23.3 MPNST xenograft cells into the flanks of 5-6 week old female NU/NU mice (Harlan Sprague Dawley). For pharmacologic experiments, mice were treated with 25 mg/kg selumetinib twice-daily by oral gavage in 0.5% methylcellulose solution with 0.2% v/v Tween-80, 100 mg/kg 1-ABT followed by 10mg/kg NVS-PAK1-1 2 hours later in 60% PEG400/40% water, or vehicle control gavaged once daily. Tumors were measured using calipers 3 times per week.

Statistical Analysis

All experiments were performed as repeated, independent biologic replicates, and statistics were derived from biologic replicates. The number of biologic replicates is indicated in each panel or figure legend. No statistical methods were used to predetermine sample sizes. Considering the rarity of MPNSTs and accounting for the number of genomic approaches used in this study, our total cohort size is similar to prior publications^{6–8}. The clinical samples used were retrospective and non-randomized, and all samples were equally interrogated within the constraints of sufficient tissue for each analytical method. Cells and animals were randomized to experimental conditions, and no clinical, molecular, cellular, or animal data points were excluded from analysis. Unless otherwise specified, data are plotted as mean with error bars representing the standard error of the mean. The statistical tests of choice were selected based on the input data and are noted in the methods and figure legends. All statistical tests were one-sided. Where appropriate, multiple hypothesis testing corrections were performed. Statistical significance thresholds are indicated in each figure legend and exact p-values are provided when possible.

Data availability

Human tumor DNA methylation (n=119), RNA sequencing (n=41), whole exome sequencing (n=34), or single-cell RNA sequencing data (n=6) reported in this manuscript will be deposited in the NCBI Gene Expression Omnibus. Cell line RNA-sequencing (n=6), selumetinib-treated cell line RNA-sequencing (n=10), CRISPRi *NF2*-deficient cell line RNA-sequencing (n=4), or single-cell RNA sequencing of mouse xenograft data (n=5) reported in this manuscript will similarly be deposited in the NCBI Gene Expression Omnibus. Additional RNA-sequencing and H3K27 trimethylation ChIP sequencing data from previously reported PRC2-intact or PRC2-deficient neurofibroma cell lines is available under GSE 118185 (<https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE118185>) or GSE118183 (<https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE118183>), respectively¹⁹. The publicly available GRCh37 (hg19, https://www.ncbi.nlm.nih.gov/assembly/GCF_000001405.13/) and GRCm38 datasets (mm10, https://www.ncbi.nlm.nih.gov/assembly/GCF_000001635.20/) were used in this study.

Code availability

The open-source software, tools, and packages used for data analysis in this study, as well as the version of each program, were ImageJ (v2.1.0), R (v3.5.3 and v3.6.1), FASTQC (v0.11.9), HISAT2 (v2.1.0), featureCounts (v2.0.1), Bowtie2 (v2.3), snpEff (v5.1), Mutect2 (v4.0), picard (v2.2), cellranger (v6.1.2), Seurat R package (v3.0.1), DESeq2 (Bioconductor v3.10), minfi (Bioconductor v3.10), ConsensusClusterPlus (Bioconductor v3.10), Heatmap.2 R package (gplots v3.13), and ggplot2 (v3.3.6). No custom software, tools, or packages were used.

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Figures

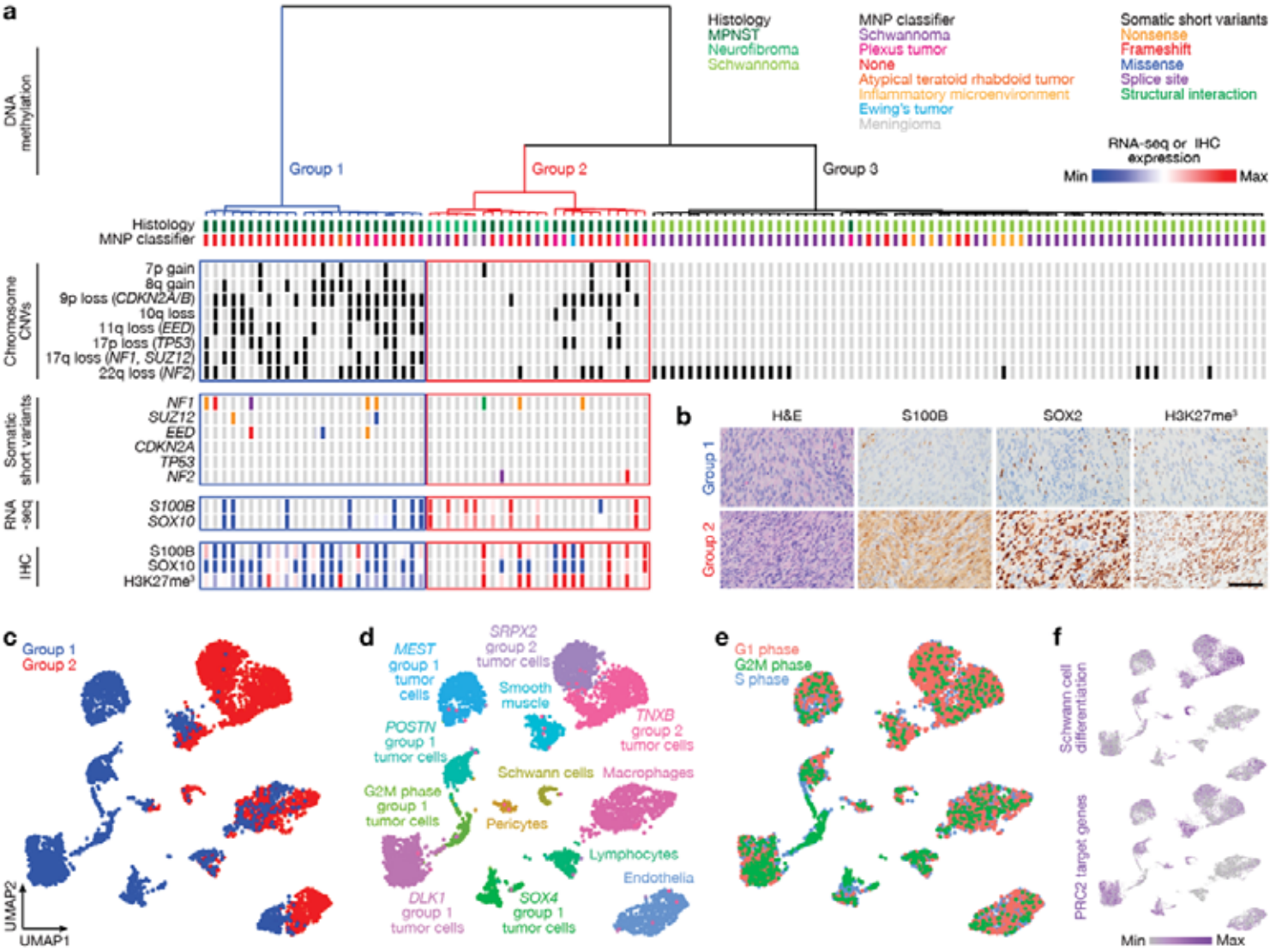


Figure 1

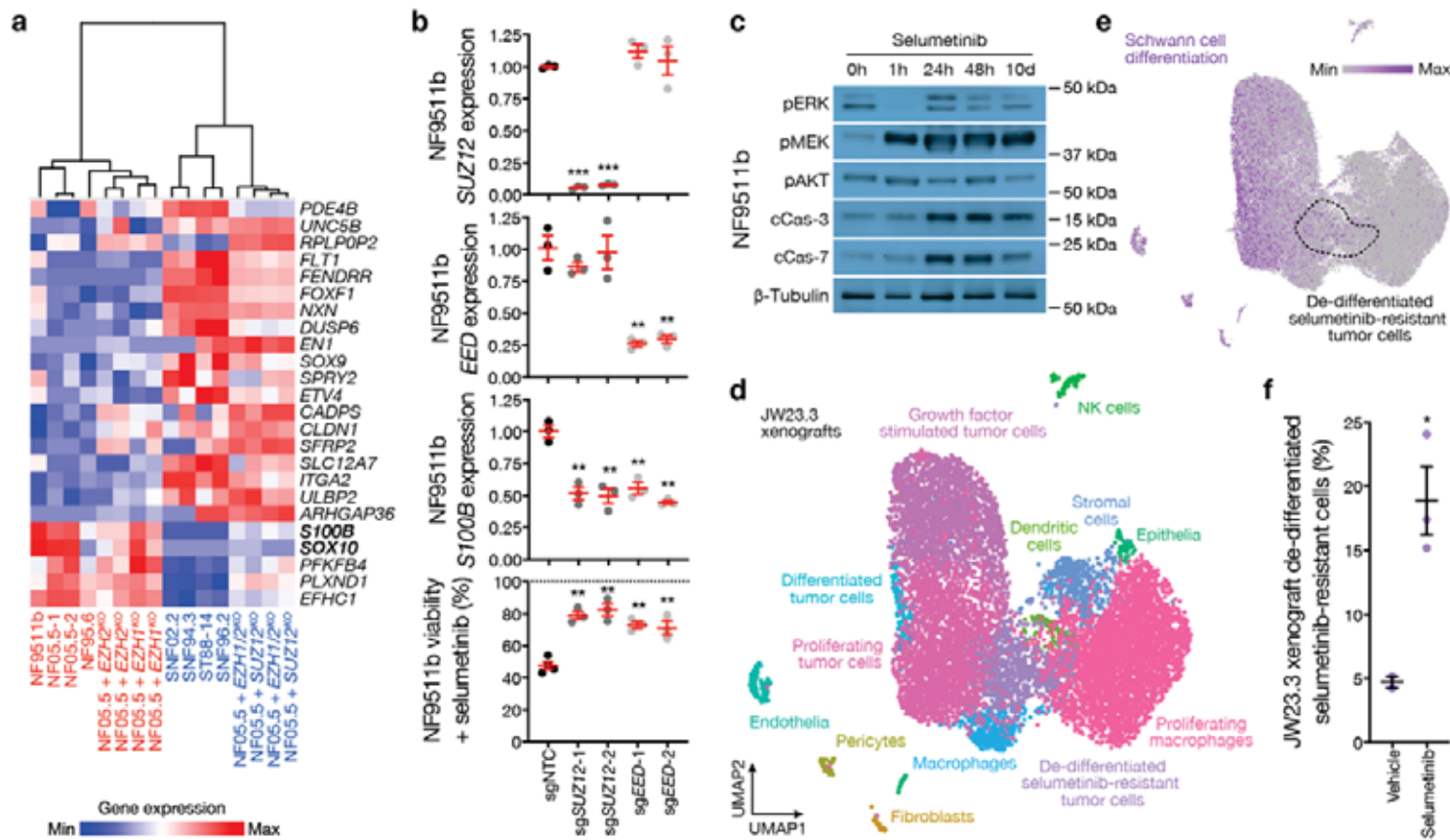


Figure 2

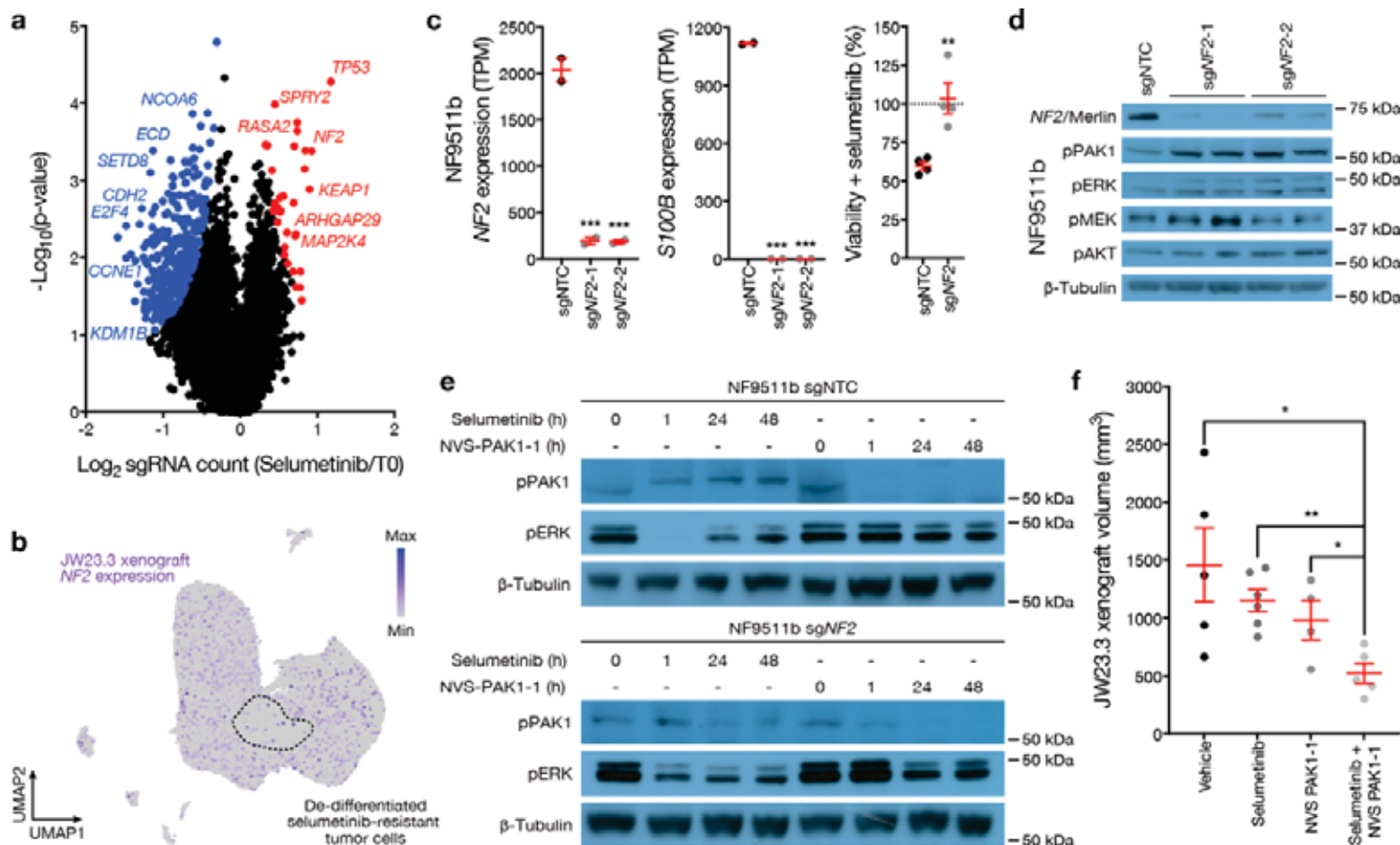


Figure 3

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