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14. ABSTRACT
The overall goal of the project is to determine how neurofibromin is regulated, and to explore the hypothesis that loss of neurofibromin activity leads to up-regulation of specific receptors. We are building on our earlier discovery, that neurofibromin depends on the adapter protein SPRED1, to function, and we are utilizing the latest technical innovations including CRISPR technology to find genes that regulate neurofibromin SPRED function. To date we have demonstrated that oncogenic EGFR signaling disrupts Spred1-NF1 binding. Mass spectrometry was performed on cells overexpressing EGFRL858R to identify potential phosphorylation sites on Spred1 and NF1 that could disrupt Spred1-NF1 binding by steric hindrance. A serine phosphorylation site on Spred1 was identified in which a phosphomimetic and phosphodeficient mutant decreased or increased Spred1-NF1 binding, respectively. Therefore, phosphorylation of Spred1 at this site by a serine kinase downstream of oncogenic EGFR may disrupt Spred1-NF1 binding. Our findings provide one potential mechanism by which oncogenic EGFR signaling disrupts negative feedback to allow for constitutive Ras signaling. Furthermore, this work may elucidate a novel kinase therapeutic target for restoring NF1 mediated inhibition of Ras.

15. SUBJECT TERMS
Neurofibromin, Spred1, Spred2, neurofibromatosis, therapeutic targeting

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Introduction

The overall goal of the project is to determine how neurofibromin is regulated, and to explore the hypothesis that loss of neurofibromin activity leads to up-regulation of specific receptors. We hope that we will be able to find news of up-regulating neurofibromin in cells in which one allele is defective, and that we will be able to suppress hyperactive Ras signaling in cells in which both alleles are lost. We are building on our earlier discovery, that neurofibromin depends on the adapter protein SPRED1, to function, and we are utilizing the latest technical innovations including CRISPR technology to find genes that regulate neurofibromin SPRED function.

Keywords

Neurofibromin, Spred1, Spred2, EGFR, mutant EGFR(L858R), Ras-GTP, Ras-MAPK, RTK, neurofibromatosis, RasGAPs, growth factor signaling, therapeutic targeting.

Major Goals of the Project

Specific Aim 1

Major Task 1: Determine and verify RTKs that interact with Spred1 and Spred2

- Subtask 1: Generation of plasmids and co-IP of exogenous RTKs and Spred1/2
- Subtask 2: Validation of co-IP, endogenous protein co-IP
- Subtask 3: Determine the binding sites and tyrosine phosphorylation pattern on Spred for the RTK/Spred interaction

Major Task 2: Determine effect of NF1/Spred loss on Spred-binding RTK signaling

- Subtask 1: Establish good NF1 and Spred1/2 knockdown protocols for indicated cell lines
- Subtask 2: Establish good biological readout assays for cell lines to be tested in response to specific RTKs
- Subtask 3: Determining Ras-MAPK signaling and biological readouts with NF1 or Spred knockdown in response to RTK activation

Specific Aim 2

Major Task 3: Determine EGFR-mediated changes in Spred and Neurofibromin phosphorylation and interaction partners

- Subtask 1: Purification of Neurofibromin or Spred from cells +/-mutant EGFR
- Subtask 2: Mass spec analysis
- Subtask 3: Mutational analysis of Spred and Neurofibromin phosphorylation sites on Spred/Neurofibromin interaction

- Subtask 4: Knockdown analysis of interacting proteins on Spred/Neurofibromin interaction

Major Task 4: Determine targets for increasing Spred/Neurofibromin interaction and NF1 function in $NF1^{-/+}$ cells

- Subtask 1: Establish good siRNA and/or drug inhibition protocols for the regulators identified above in relevant NF1-/+ cell lines
- Subtask 2: Test the effect of siRNA and/or drugs above in Ras-MAPK signaling and functional assays: proliferation, migration, differentiation

<u>Specific Aim 3</u>

Major Task 5: Determine novel post-translational modifications and interacting proteins with Neurofibromin

- Subtask 1: Generate cells stably expressing Tet-regulatable full length NF1 and NF1 point mutants
- Subtask 2: Purify NF1 from cells with multiple conditions
- Subtask 3: Mass spec analysis, identify proteins that interact differentially between WT and mutant NF1
- Subtask 4: Determination of effect of differentially interacting proteins on NF1 function

Accomplishments

Major Task 1: Determine and verify RTKs that interact with Spred1 and Spred2

Subtask 1: Generation of plasmids and co-IP of exogenous RTKs and Spred1/2

The RTK plasmids that have been generated are shown in table 1. As a pilot experiment, Spred1 has been shown to bind to the activated from of EGFR(L858R) at a higher affinity than wild-type EGFR as shown in figure 1. Interestingly, activated EGFR with EGF does not increase Spred1 binding and EGFR inhibition reduces EGFR(L858R)-Spred1 binding. These results are surprising and important for understanding the role of Spred1 on both wild-type and pathogenic RTKs. Spred1 is deleted across multiple cancer types while Spred2 is not. Therefore, given the clinical importance of Spred1, we have prioritized our studies to thoroughly investigate Spred1 before Spred2. We previously identified phosphorylation sites on Spred1 by mass spectrometry following EGFR(L858R) cotransfection. pSpred1(S105) is particularly interesting due to loss of NF1 binding and so additional RTKs have been tested for ability to phosphorylate Spred1 at S105 as shown in table 1. These samples have been submitted for mass spectrometry analysis and RTK binding is ongoing.

Candidate RTKs	
EGFR	EGFR(L858R)
CKIT	CKIT(D816V)
FLT3	FLT3(ITD) FLT3(D835Y)
PDGFRα	PDGFRα(D842V)
HER2	HER2(V654E)
MET	
BCR-ABL	
CSF1R	

Table 1. RTKs which have been cotransfected with Spred1 to determine ability to phosphorylate Spred1

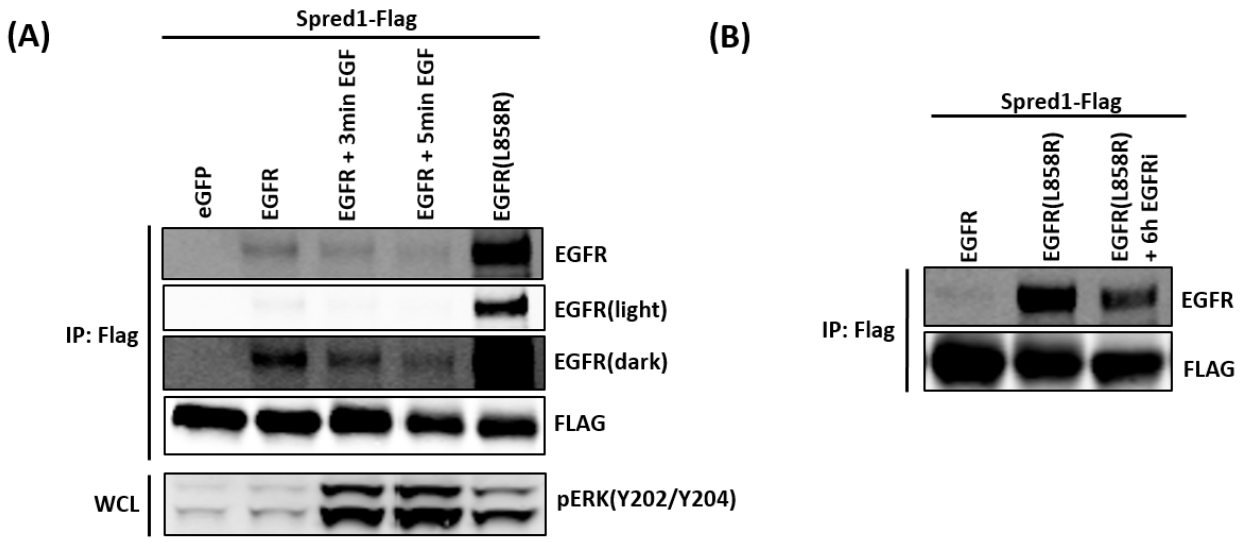


Figure 1. Spred 1 binds mutant EGFR(L858R) with higher affinity than wild-type EGFR. A) HEK 293T cells were transfected, serum starved for 16 hours, and stimulated with 20ng/ml EGF and EGFR binding accessed by Western blot. (B) Cells were transfected and Spred-NF1 binding was assessed by Western blot. EGFR inhibitor AZD9291 used at 1µM for 6 hours.

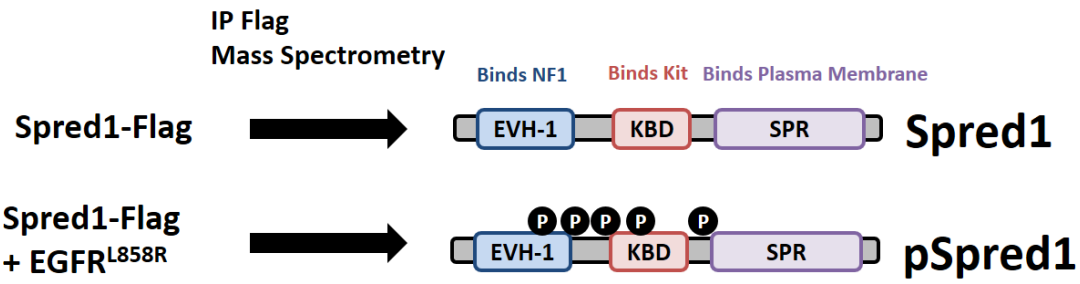
Subtask 2: Validation of co-IP, endogenous protein co-IP

Validation of co-IP and endogenous protein co-IP is ongoing.

Subtask 3: Determine the binding sites and tyrosine phosphorylation pattern on Spred for the RTK/Spred interaction

Flag tagged Spred1 was overexpressed in 293T cells with and without EGFR(L858R). Spred1 was immunoprecipitated and subject to mass spectrometry analysis. Phosphorylation and binding partners with mutant EGFR are shown below in Figure 2.

(A)



(B)

Phospho Site	Domain	Phospho Motif	Kinase Phospho Motif	Human Genetics
S105	EVH-1	FGLTFQpSPADARA	ERK1, ERK2, GSK-3, CDK5, CK1, CK2, GPKR1, PDH	T102R identified in Legius syndrome patient
S148&S149	Near EVH-1	QANEEDSpSpSSLVKD	CK2, BARK	N/A
S238	KBD	KSIRHVpSFQDEDE	CaMKII, PKA, PKC, CK2, AKT, CLK2, PKCe	N/A
Y292	Near KBD	DSKKSDpYLYSCGD	EGFR, PDGFR	Y292F identified in stomach adenocarcinoma patient

(C)

EGFR, ERBB2, ERBB4, CALM1, RPL36, NPM1, HCN3, RPL28, RPL38, NDUFA5, TEX10, NDUFS2, XRCC6, C3orf58, SRP9, FAM91A1, SLIRP, HIGD1A, ZNF444, SUB1, NOB1, EIF3L, PNO1, ASPHD1, MKI67IP, NOP2, TSPYL1, SRSF2, SF3B3, ZNF638, LBR, NSUN2, RRP1, RPUSD4, WDR18, TIMMDC1, FAM132B, ZNF696, ZFPL1, AIFM1, RPL10L, KRT17, KMT2B, SNRPF, DNAJC19, RRP36

Figure 2. Identification of Spred1 phosphorylation and binding partners in the presence of mutant EGFR (L8585R). (A) Schematic for Spred1 mass spectrometry analysis. (B) Identification of Spred1 phosphorylation sites downstream of mutant EGFR. Kinase phosphorylation motifs identified using PhosphoMotif Finder and PhosphoSitePlus. (C) Spred1 interacting proteins identified by mass spectrometry.

Major Task 2: Determine effect of NF1/Spred loss on Spred-binding RTK signaling

Subtask 1: Establish good NF1 and Spred1/2 knockdown protocols for indicated cell lines

NF1-Null and Spred1-Null HEK 293T cells have been generated using CRISPR/Cas9 and single clones have been expanded for biochemical assays (Figure 2). NF1-Null MEFs have also been generated and will be characterized. Spred1-Null HEK 293T and MEFs are currently being generated using CRISPR/Cas9.

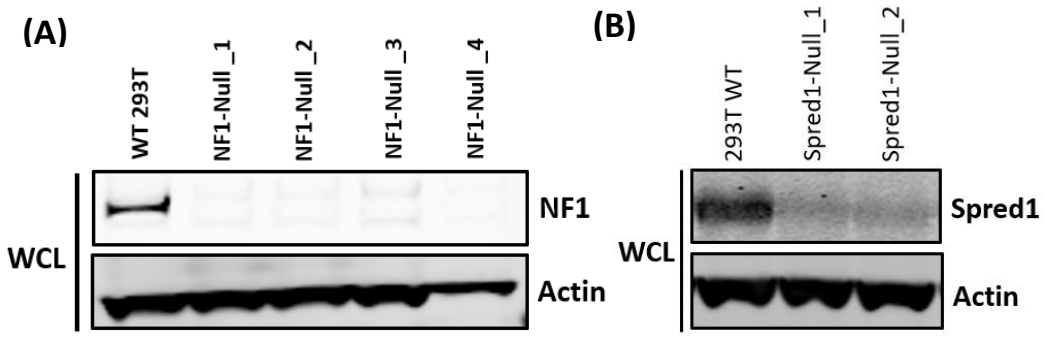


Figure 3. Clonal NF1-Null and Spred1-Null HEK 293T cells lines were generated using CRISPR/Cas9 with a sgRNA targeting exon 2 of NF1 and exon 1 of Spred1. Following antibiotic selection clones were screened by Western blot.

Subtask 2: Establish good biological readout assays for cell lines to be tested in response to specific RTKs

Ras-GTP pulldown assay with GST-RBD, pRAF1, pMEK, and pERK have been optimized as demonstrated in Subtask 3.

Subtask 3: Determining Ras-MAPK signaling and biological readouts with NF1 or Spred knockdown in response to RTK activation

Ras-MAPK signaling in response to EGF has been analyzed in NF1 wild-type and NF1-Null 293T cells (Figure 4). Both the magnitude and duration of Ras-MAPK signaling seems to be enhanced in the NF1-Null 293T cells compared to wild-type. Also, Spred1 overexpression is unable to inhibit Ras-MAPK signaling in NF1-null cells compared to wild-type.

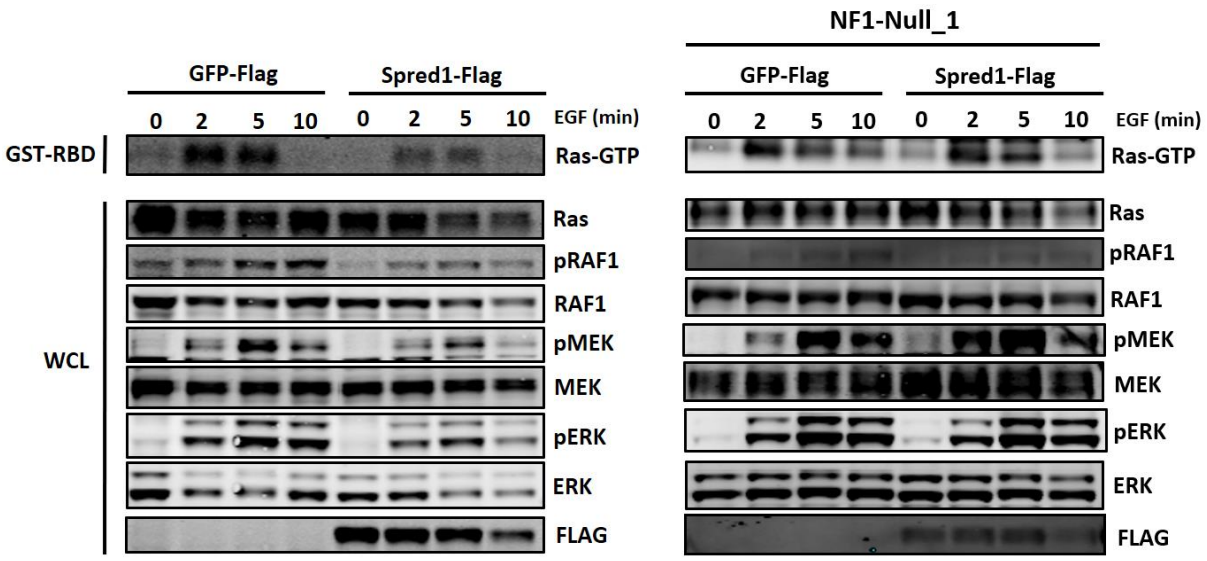


Figure 4. Spred1 overexpression is unable to suppress RasGTP following EFG stimulation in NF1-Null cells. NF1-Null_1 cells were transfected, serum starved for 16 hours, and stimulated with 20ng/ml EGF. Downstream signaling was accessed by Western blot.

Major Task 3: Determine EGFR-mediated changes in Spred and Neurofibromin phosphorylation and interaction partners

Subtask 1: Purification of Neurofibromin or Spred from cells +/-mutant EGFR

See Subtask 2

Subtask 2: Mass spec analysis

Neurofibromin and Spred1 have been successfully purified with and without activating EGFR(L858R) and analyzed by mass spec. For Spred1 see Major Task 1, Subtask 3 and for NF1 see Major Task 5, Subtask 3.

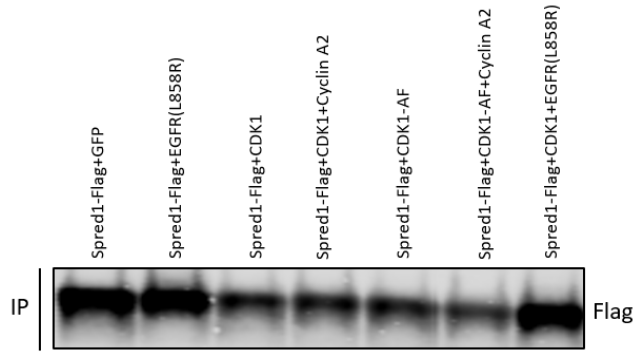


Figure 5. Spred1-flag IP from HERK 293T cells for mass spec analysis.

Subtask 3: Mutational analysis of Spred and Neurofibromin phosphorylation sites on Spred/Neurofibromin interaction

Phosphodeficient and phosphomimic mutants of Spred1 phosphorylation sites identified by mass spectrometry were generated and tested for effects on NF1 binding. Spred1(S105) mutants showed the expected binding (Figure 6). Importantly, phosphomimic Spred1(S105D) is unable to bind NF1 and suppress Ras-GTP following EGF stimulation.

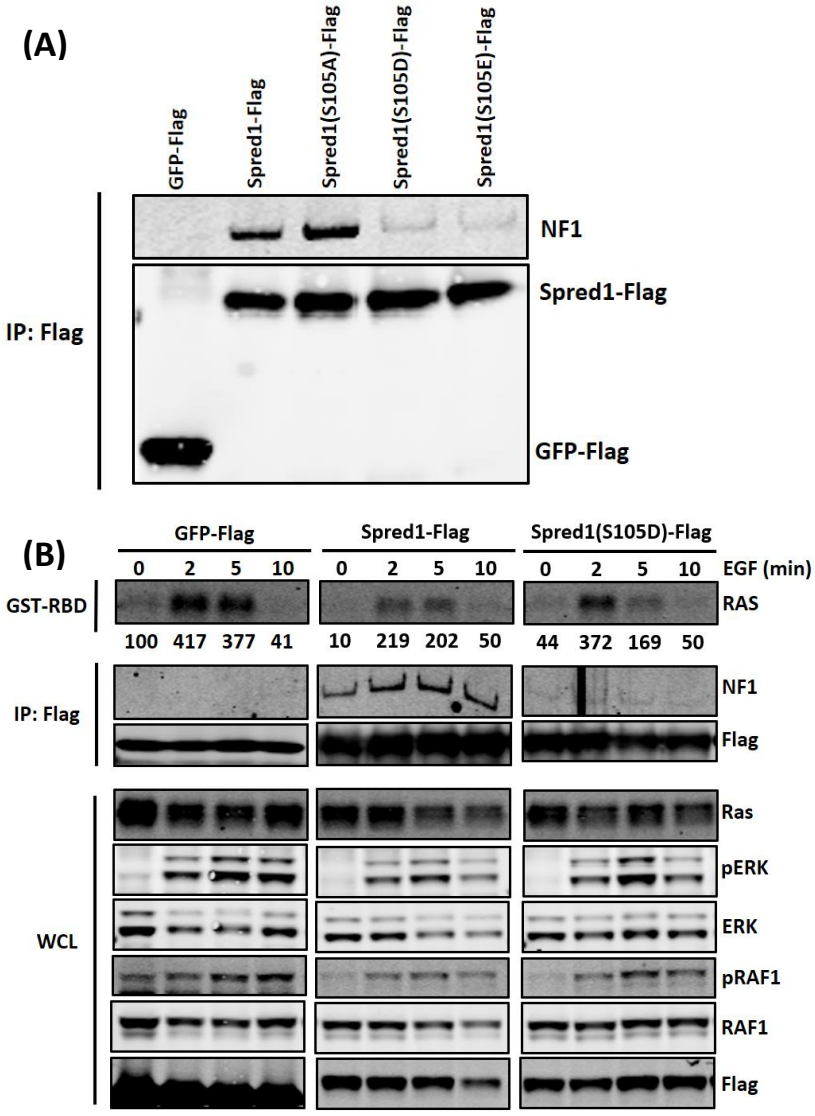


Figure 6. Phosphomimetic Spred1(S105) is unable to bind NF1 and suppress RasGTP following EGF stimulation. (A) Phosphomimetic and phosphodeficient Spred1 mutants at S105 were generated using site directed mutagenesis and transfected into HEK 293T cells to determine NF1 binding by Flag-IP followed by Western blot. (B) HEK 293T cells were transfected, serum starved for 16 hours, and stimulated with 20ng/ml EGF. Downstream signaling was accessed by Western blot.

Phosphorylated Spred1(S105) has been quantitated and normalized to total Spred1(S105) using transient transfection in 293T cells (Figure 7) and mass spectrometry as previously described. Expression of mutant EGFR(L858R) leads to a 5-fold increase in phospho-Spred1(S105). We will now test candidate kinase inhibitors which reduce phospho-Spred1(S105).

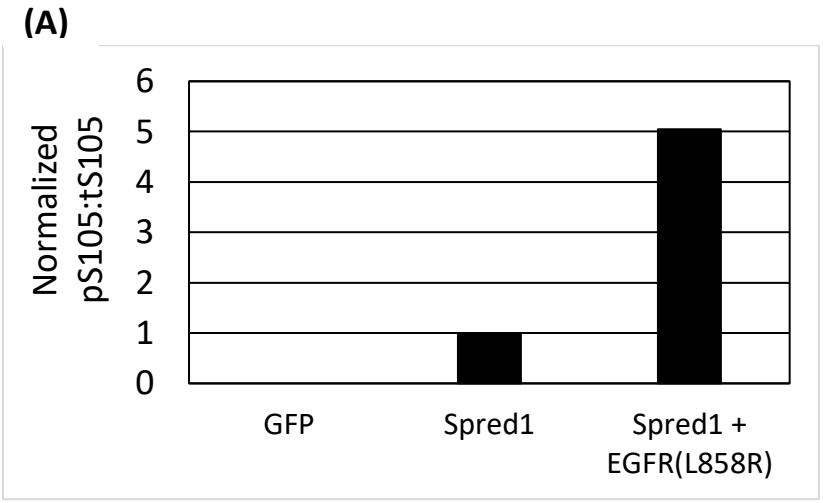


Figure 7. Design of mass spectrometry experiment to identify Spred1(S105) kinase. Quantitation of relative phosphorylation at the S105 site in SPRED1 was carried out in Skyline v3.0 by quantifying MS1 precursor peak areas of the S105-containing peptides and normalizing them by the sum of abundances of all unmodified peptides detected in the same protein. Transient transfection in HEK 293T was carried out as previously described.

In addition to identifying Spred1(S105) as an important phosphorylation site for NF1 binding and Ras signaling we have also identified CDK1 as the Spred1(S105) kinase. Figure 8 shows results from an in vitro kinase assay with Spred1(S105) peptide, an in vitro kinase assay comparing wild-type Spred1 to Spred1(S105A), and a full length Spred1 in vitro kinase assay followed by mass spec for Spred1(S105) phosphorylation. Figure 9 shows Spred1 phosphorylation at S105 decreases in response to CDK1 inhibitors and increases with CDK1 expression.

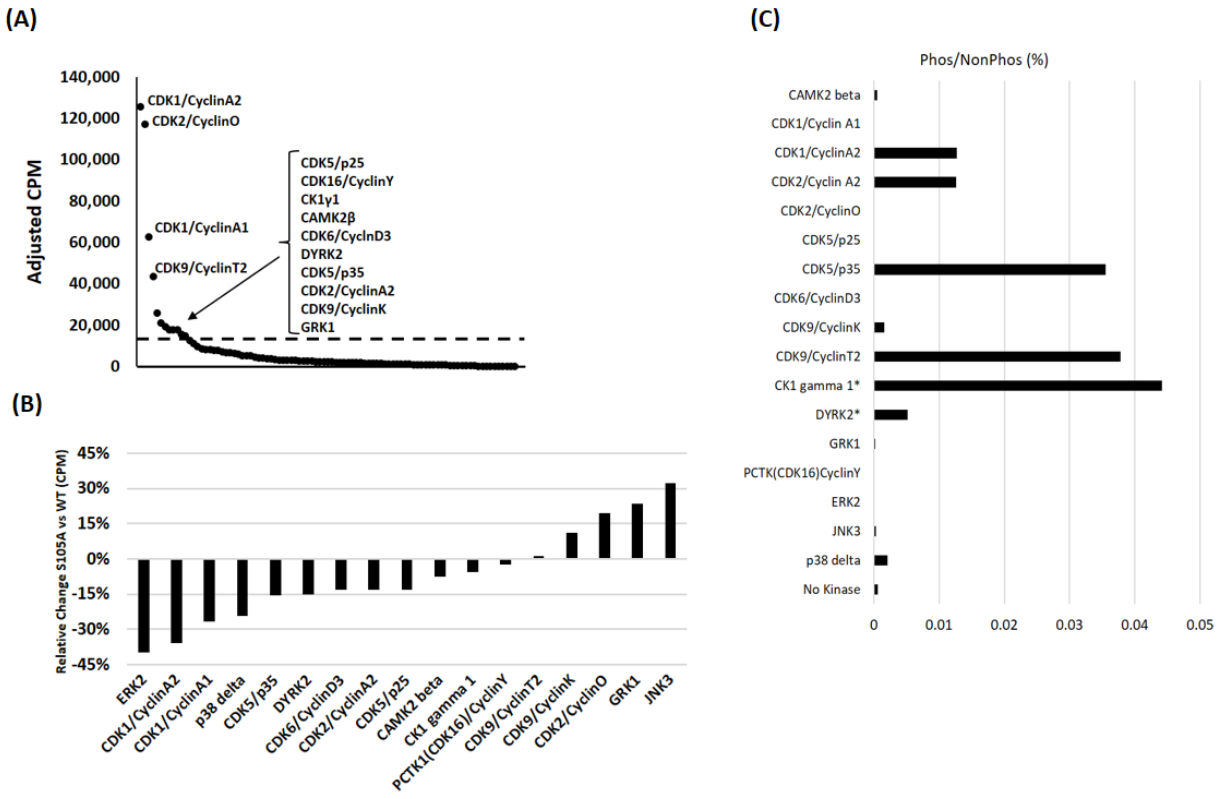


Figure 8. CDK1 phosphorylates Spred1 on S105 in vitro. (A) In vitro kinase assay with Spred1(S105) peptide performed by Kinexus. (B) In vitro kinase assay comparing wild-type Spred1 to Spred1(S105A). (C) In vitro kinase assay with full length Spred1 followed by mass spec for Spred1(S105) phosphorylation.

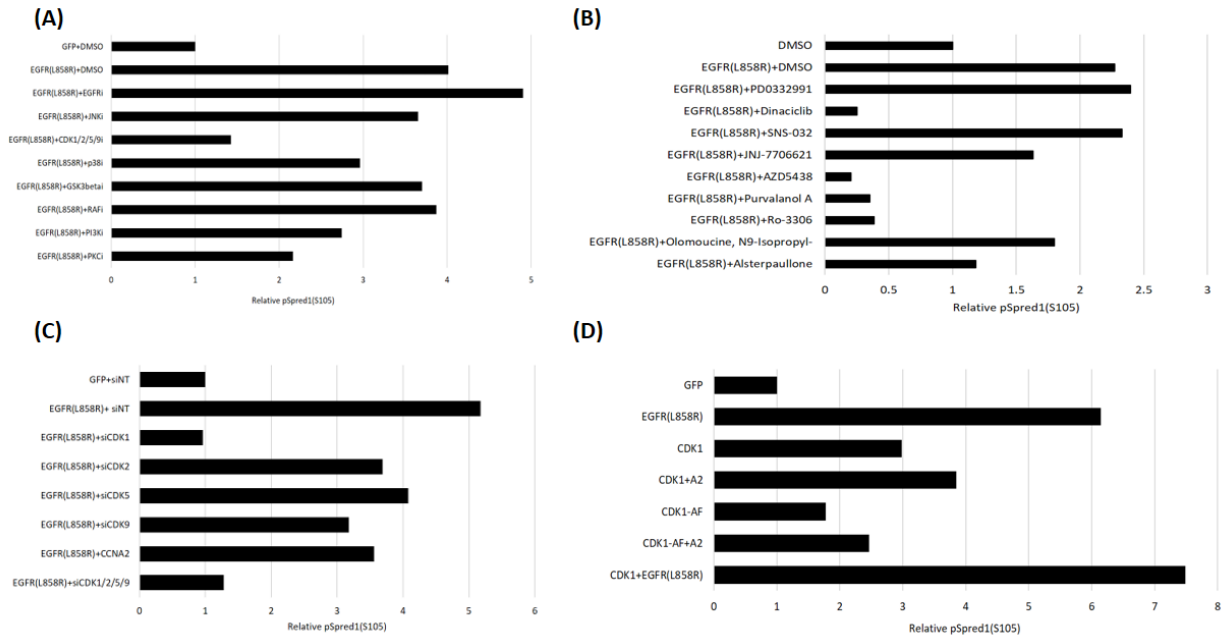


Figure 9. CDK1 phosphorylates Spred1 in vivo. (A) Spred1-Flag was overexpressed in HEK 293T cells, immunoprecipitated, and analyzed for Spred1(S105) phosphorylation by mass spec in the presence of selected pathway inhibitors. All inhibitors were used at 2µM for 2 hours. EGFR inhibitor AZD9291, RAF inhibitor LY3009120, GSK3β inhibitor CHIR-99021, CDK1/2/5/9 inhibitor Dinaciclib, PKC inhibitor Sotrastaurin, JNK inhibitor JNK-IN-8, p38 inhibitor LY2228820, and PI3K inhibition GDC-0941. (B) As shown in (A) but with additional CDK1 inhibitors. (C) As shown in (A) but with siRNA knockdown. (D) Spred1-Flag was overexpressed in HEK 293T cells with the addition of CDK1 constructs.

To identify genes that regulate NF1/SPRED function, including the Spred1(S105) kinase, we will also perform an unbiased CRISPRa screen to activate genes in K562. To ensure hits are specific to NF1/Spred1 function we will counter screen in an isogenic K562 cell line in which we have knocked out NF1 using CRISPR. Recent CRISPRa screening data from our collaborator Dr. Michael Boettcher demonstrated overexpression of NF1, Spred1, and Spred2 all increase K562 sensitivity to imatinib, an approved BCR-ABL inhibitor. Given the robustness of these results, we believe K562 is an ideal cancer cell line system to interrogate regulators of NF1/SPRED function which can then be expanded to other disease models, as described below (“Changes” section). We expect full analysis of the data soon.

Subtask 4: Knockdown analysis of interacting proteins on Spred/Neurofibromin interaction

We plan to knockdown a candidate list of Spred1 interacting proteins and measure Spred-NF1 binding by coimmunoprecipitate and Western blot. Our lab has a particular interest in Calmodulin-1 (CALM1) because it also binds Ras.

Major task 4: Determine targets for increasing Spred/Neurofibromin interaction and NF1 function in NF1-/+ cells

Subtask 1: Establish good siRNA and/or drug inhibition protocols for the regulators identified above in relevant NF1-/+ cell lines

Subtask 1 is ongoing.

Subtask 2: Test the effect of siRNA and/or drugs above in Ras-MAPK signaling and functional assays: proliferation, migration, differentiation

Once subtask 1 is completed we will test whether blocking CDK1 promotes binding, as expected, and whether increased binding reduces Ras.GTP levels and prevents cells from growing. In addition, we will analyze genes that emerge from our functional CRISPR screen that are candidates for enzymes regulating Spred/neurofibromin interaction.

Major task 5: Determine novel post-translational modifications and interacting proteins with Neurofibromin

Subtask 1: Generate cells stably expressing Tet-regulatable full length NF1 and NF1 point mutants

Rather than generate stable cell lines to express NF1, we have optimized transient transfection of NF1-Flag in HEK 293T cells to achieve the same goal as shown in Subtask 2.

Subtask 2: Purify NF1 from cells with multiple conditions

We have successfully purified NF1 from HERK 293T with multiple conditions as shown below (Figure 10) with more details in Figure 12.

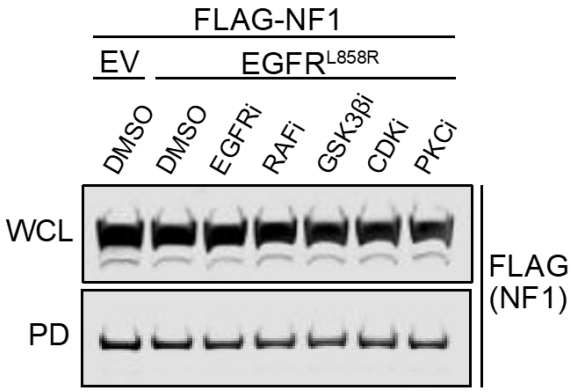
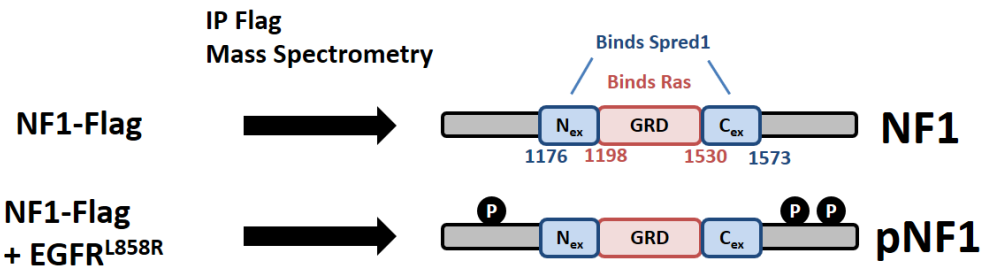


Figure 10. Purification of NF1 from HERK 293T cells under multiple conditions.

Subtask 3: Mass spec analysis, identify proteins that interact differentially between WT and mutant NF1

We have performed mass spectrometry on NF1 as described above with Spred1 to identify phosphorylation sites and binding partners important for function as shown in Figure 11. We have also identified potential NF1 kinases downstream of active EGFR(L858R) by using key pathway inhibitors as shown in Figure 12. The PKC pathway may regulate multiple important phosphorylation sites on NF1 including S665/666, S876/879, S2460, T2510/2514/25115, S2521/2523, S2543, S2597, S2597/2599.

(A)



(B)

Phospho Site	Domain	Phospho Motif	Kinase Phospho Motif
S864	N/A	SGLATYpSPPMGPV	GRK1, GSK-3, ERK1, ERK2, CDK5
S2597	N/A	PHLRKVpSVSESNV	CaMKII, PKA, PKC, PKCe, CK2, GSK-3, Phosphorylase, MAPKAPK2, GRK1
S2802	N/A	KENVELpSPTTGHC	GSK-3, ERK1, ERK2, CDK5, CK1, CK2

(C)

NONO, RSP16, HNRNPH2, TUBAL3, RPS8, C22orf28, SMARCA4, EEF1A1, EEF1A1P5, ATP5B, TUBB3, TUBB6, HSPA4, RPL6, NDUFA13, EEF1B2, LEPRE1, HSPA4L, HSPH1, RPS5, ZADH2, NDUFA4, DDX53, DHRS7B, RCN2, SMARCA2, DYNC1H1, MRPS27, DDX3Y, XRN2, DSP, CALM1, KHSRP, HNRNPF, RPLPO, RPLPOP6, ATAD3C, ATAD3A, CAD, FKBP8, PPM1B, COX20, HERC2P3, DDX6, KHDRBS1, ALDH3A2, RPS19, NPM1, FAM98A, FXR1,

Figure 11. Identification of NF1 phosphorylation and binding partners in the presence of mutant EGFR(L8585R). (A) Schematic for NF1 mass spectrometry analysis. (B) Identification of NF1 phosphorylation sites downstream of mutant EGFR. Kinase phosphorylation motifs identified using PhosphoMotif Finder. (C) NF1 interacting proteins identified by mass spectrometry.
To determine which pathways influence NF1 phosphorylation we used a panel of chemical inhibitors and identified NF1 phosphorylation. Multiple inhibitors were found to decrease NF1 phosphorylation sites.

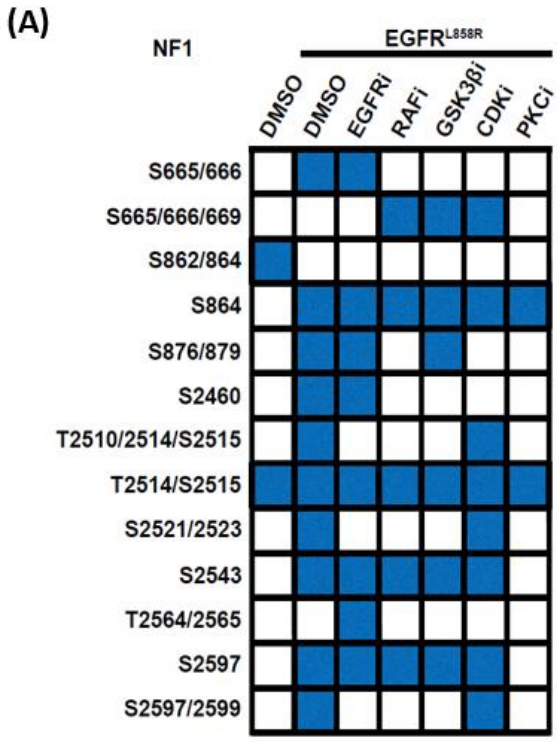


Figure 12. Identification of additional NF1 phosphorylation sites and potential kinases induced by mutant EGFR(L858R). All inhibitors were used at 2.5µM for 4 hours. EGFR inhibitor AZD9291, RAF inhibitor LY3009120, GSK3β inhibitor CHIR-99021, CDK1/2/5/9 inhibitor Dinaciclib, PKC inhibitor Sotrastaurin.

We have also published our findings of the Spred1-binding domain of NF1, which flank the Gap Related Domain (GRD) domain (N_{ex} and C_{ex}) "The neurofibromin recruitment factor Spred1 binds to the GAP related domain without affecting Ras inactivation". Theresia Dunzendorfer-Matt, Ellen L. Mercado, Karl Maly, Frank McCormick, and Klaus Scheffzek, PNAS, 113, 7497-7502, 2016.

Subtask 4: Determination of effect of differentially interacting proteins on NF1 function

We plan to knockdown a candidate list of NF1 interacting proteins and measure NF1 function by Ras-GTP assay. Our lab has a particular interest in Calmodulin-1 (CALM1) because it also binds Ras.

Opportunities for training and professional Development

Graduate student Evan Markegard (funded by the project) is given one-on-one mentorship by Dr. McCormick which includes at least bi-weekly meetings either face-to-face or by skype to discuss his progress and goals. Evan presents his research at lab group meetings approximately four times per year, as well as at the UCSF Helen Diller Comprehensive Cancer Center "Research in Progress" seminar series and he presents at the Cancer Center annual retreat. This year he also presented a research poster at the American Association of Cancer Research 108th Annual Meeting in Washington D.C (April 1-5, 2017). He will have the opportunity to present at a National or International Conference in each year of his graduate studies.

Dissemination of Results

1) Evan Markegard - Poster Presentation at the 108th Annual meeting of American Association of Cancer Research (April 1-5, 2017).

Abstract 1370: EGFR-mediated Spred1 phosphorylation inhibits NF1 to sustain constitutive Ras/MAPK signaling Evan Markegard, Ellen L. Mercado, Jillian M. Silva, Jacqueline Galeas, Marena I. Trinidad, Anatoly Urisman and Frank McCormick. DOI: 10.1158/1538-7445.AM2017-1370 Published July 2017

2) Evan Markegard - Poster Presentation at the 107th Annual meeting of American Association of Cancer Research (April 16-20, 2016).

Abstract 1874: Oncogenic EGFR signaling inhibits the Spred1-NF1 interaction to sustain constitutive Ras signaling. Evan Markegard, Ellen L. Mercado, Jacqueline Galeas, Marena I. Trinidad, Anatoly Urisman and Frank McCormick. DOI: 10.1158/1538-7445.AM2016-1874 Published 15 July 2016

2) Frank McCormick - Helen Diller Comprehensive Cancer Center Seminar Series (February 12, 2016)

3) Presentations and discussions at joint lab meetings with the labs of Ophir Klein (Department of Orofacial Sciences, UCSF), Natalia Jura (Department of Cellular and Molecular Pharmacology UCSF) and Kevin Shannon (Department of Pediatrics, UCSF).

4) Frank McCormick – Presentation at CTF Meeting, Austin TX June 2016

5) “The neurofibromin recruitment factor Spred1 binds to the GAP related domain without affecting Ras inactivation”, Theresia Dunzendorfer-Matt, Ellen L. Mercado, Karl Maly, Frank McCormick, and Klaus Scheffzek, *PNAS*, 113, 7497-7502, 2016.

Impact

Nothing to report

Changes/Problems

While the Aims of the project remain unchanged, we will use new technologies to address these Aims. To identify proteins that regulate Neurofibromin and/or Spred, we will use functional screens in place of biochemical, mass spectrometry-based approaches. The latter approach has generated lists of proteins that appear to bind to Neurofibromin when the protein is expressed in cells in culture, but validation of these is challenging. We have been working closely with our UCSF colleagues Drs. Luke Gilbert, Michael Boettcher and Michael McManus to use CRISPRa and CRISPRi systems to identify regulatory proteins (Gilbert et al, *CELL*, 159, 647-661, 2014). We will use biochemical approaches to validate these hits, but strongly feel the primary screen should be functional. As described above, our colleagues performed a full genomic screen for genes that affect how K562 cells depend on imatinib. These cells were used because they grow in suspension, making full genome screens possible at high coverage. One of the strongest hits from the screen was Neurofibromin: over expression of this gene by CRISPRa mediated activation of the promoter, sensitized cells to the drug, whereas suppression made cells drug resistant. KRAS had exactly the opposite effect, as predicted. SPRED genes also score strongly in this system, behaving exactly as Neurofibromin (over-expression caused sensitivity, deletion caused resistance). In addition, we found that loss of Neurofibromin negated the toxic effects of SPRED over-expression, confirming that SPRED proteins (both SPRED1 and SPRED2) act through Neurofibromin. These results show that this system is ideal for screening for genes or drugs that regulate Neurofibromin or SPRED, as the cells are extremely sensitive to the activity of this pathway, and retain wild type, functional genes for the entire Ras pathway downstream of RTK signaling. We therefore expect this system to allow us to obtain candidate regulators, and will use biochemical screens in physiologically relevant cell systems to follow up on these hits, as described in the original proposal. An example of the power of this technology is shown in Figure 13.

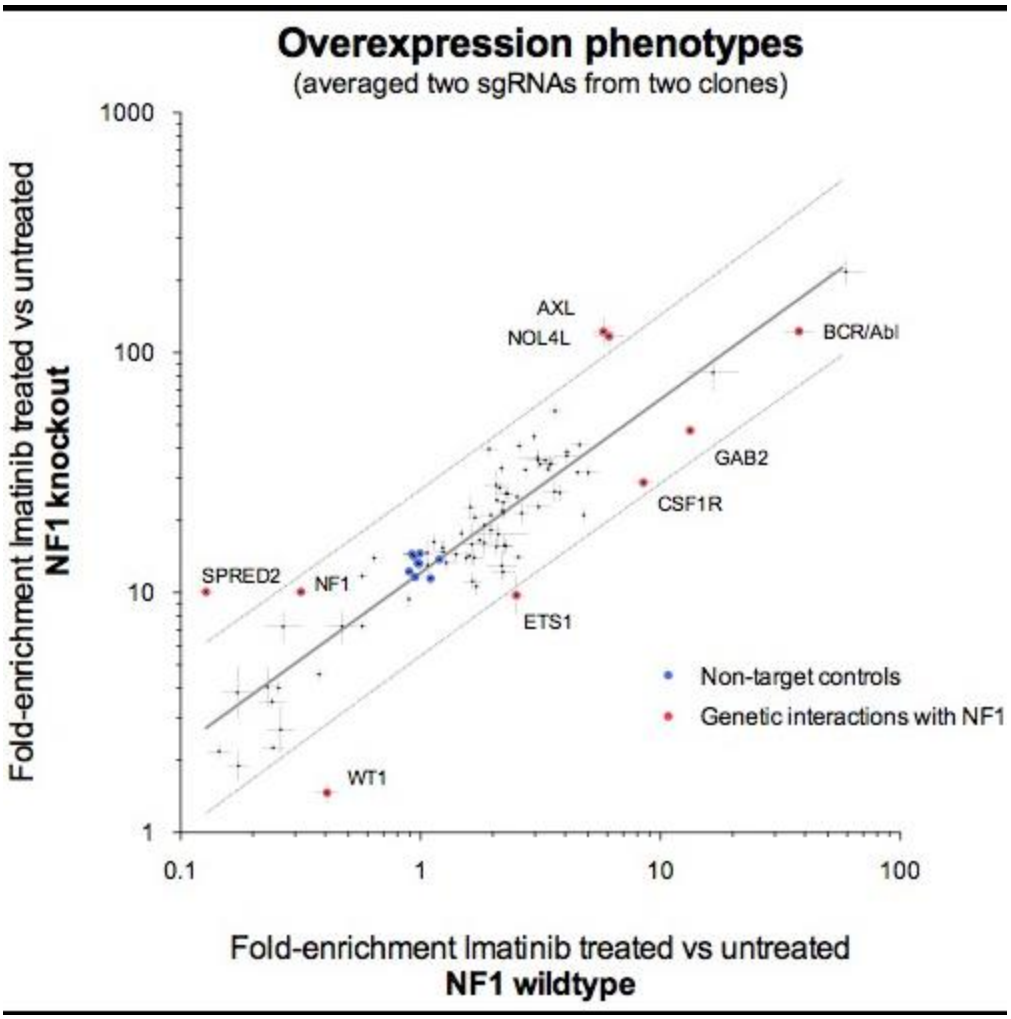


Figure 13. Screen for genes that differentially affect NF knockout cells vs wild type cells. A small set of genes were over-expressed using CRISPRa technology, in either background, and viability measured by deep sequencing.

In this screen, over-expression of SPRED2 or NF1 had no effect on viability of NF1 deficient cells (score of 10 represents no effect in NF1 KO cells), but both reduced viability of wild type cells (score of 1 represents no effect in wild type cells), as expected. However, over-expression of the receptor tyrosine kinase AXL and the AML fusion gene partner NOL4L greatly increased viability in the NF1 knockout cells, suggesting that these genes normally suppress growth through interaction with neurofibromin. Our efforts to identify tyrosine kinase receptors that are regulated by neurofibromin (described above) will now be expanded to include AXL. We will also use these preliminary data as the basis of a full genome wide screen to identify regulators of Neurofibromin and Spred signaling.

Products

Poster Presentation at the 107th Annual meeting of American Association of Cancer Research (April 16-20, 2016).
Abstract 1874: **Oncogenic EGFR signaling inhibits the Spred1-NF1 interaction to sustain constitutive Ras signaling.** Evan Markegard, Ellen L. Mercado, Jacqueline Galeas, Marena I. Trinidad, Anatoly Urisman and Frank McCormick. **DOI:** 10.1158/1538-7445.AM2016-1874 Published 15 July 2016

Poster Presentation at the 108th Annual meeting of American Association of Cancer Research (April 1-5, 2017).
Abstract 1370: **EGFR-mediated Spred1 phosphorylation inhibits NF1 to sustain constitutive Ras/MAPK signaling**. Evan Markegard, Ellen L. Mercado, Jillian M. Silva, Jacqueline Galeas, Marena I. Trinidad, Anatoly Urisman and Frank McCormick. DOI: 10.1158/1538-7445.AM2017-1370 Published July 2017

Participants and other collaborating organizations

We are collaborating with scientists at the Frederick National Labs to solve the structure of Neurofibromin, alone or in complexes with RAS and/or Spred proteins. Full length Neurofibromin made in baculovirus vectors has been extensively purified and shown to be an obligate dimer, using size-exclusion chromatography, sedimentation analysis and electron microscopy, as shown in Figure 14.

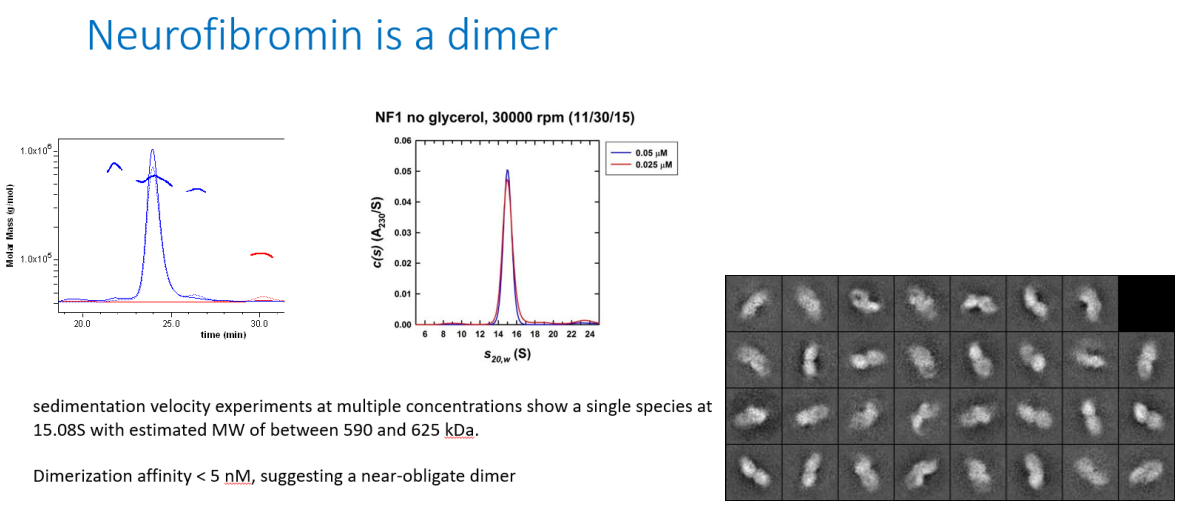


Figure 14. Biophysical characterization of recombinant, full length neurofibromin. Left panel: size exclusion chromatography. Middle panel, sedimentation analysis. Right panel, Negative-stain electron microscopy.

Individuals working on the project at UCSF

Name:	Evan Markegard
Project Role:	Graduate Student
Researcher Identifier:	
Nearest Person Month Worked:	12
Contribution to Project:	Evan performed many of the experiments in major tasks 1-5.
Funding Support:	This award

Name:	Frank McCormick
Project Role:	PI
Researcher Identifier:	
Nearest Person Month Worked:	1 (0.24)
Contribution to Project:	Oversight of all research
Funding Support:	This award

Name:	Osamu Tetsu
Project Role:	Senior investigator
Researcher Identifier:	
Nearest Person Month Worked:	2 (1.6)
Contribution to Project:	Dr. Tetsu investigated RAS activation via NF1 loss in EGFR mutant cancer cells and the effect of c-Src on Spred1/2 expression. He has acted as an invaluable mentor for the postdoc and graduate student.
Funding Support:	This award

Name:	Ellen Mercado
Project Role:	Post Doc
Researcher Identifier:	
Nearest Person Month Worked:	None this year
Contribution to Project:	Dr. Mercado carried out the mass spectrometry analysis in major tasks 3 and 5.
Funding Support:	McCormick UCSF discretionary funds

Changes in PI support since Award Made:

Please see below for the Dr. Frank McCormick's (PI) active and pending other support:

ACTIVE

Funding Number: 1R35CA197709 PI: McCormick, Frank

Project Title: *New Ways of Targeting K-Ras*

Performance Period: 04/01/2016-03/31/2023

Time Commitment: 6.00 calendar months

Supporting Agency: NIH/NCI

Contracting Officer: Joanna Watson PhD., watsonjo@mail.nih.gov, (240) 276-6230

Level of Funding: $531,651 direct/yr 02

Project Goals: To identify ways of suppressing the function of hyper-active Ras proteins for cancer therapy.

Specific Aims: i) Analysis of GTPase Activating Proteins (GAPs) that regulate Ras; ii) analysis of distinct biochemical functions of different K-Ras mutants; iii) analysis of a novel, unique function of K-Ras 4B.

Overlap: None

Funding Number: U01 CA168370 PI: McManus, Michael; McCormick, Frank; Weissman, Jonathan

Project Title: Bay Area Cancer Target Discovery and Development Network

Performance Period: 05/01/12-04/30/2017

Time Commitment: 0.24 calendar months

Supporting Agency: NIH/NCI

Contracting Officer: Daniela Gerhard PhD., gerhardd@mail.nih.gov, (301) 451-8027

Level of Funding: $99,228 direct/yr 04

Project Goals: To bridge the gap between the enormous volumes of data generated by the comprehensive molecular characterization of a number of cancer types and the ability to use these data for the development of human cancer therapeutics. Our end goal is to generate game-changing reagents, and data valuable for the development of cancer therapeutics

Specific Aims: i) Develop next generation EXPAND libraries targeting cancer specific genetic alterations; ii) Identify recurrently mutated genes that regulate oncogenic pathways and drug responses; iii) Produce genetic interaction maps to uncover pathway relationships between candidate drivers.

Overlap: None

Funding Number: N/A PI: McCormick, Frank

Project Title: Preclinical Evaluation of Signaling Pathways Involved in Cancer Malignancy

Performance Period: 03/22/2015 – 03/21/2017

Time Commitment: 0.12 calendar

Supporting Agency: Daiichi-Sankyo Company, Limited

Contracting Officer: Masashi Aonuma, PhD, VP, Oncology Research Laboratories, aonuma.masahsi.jt@daiichisankyo.co.jp, +81-3-5696-8777

Level of Funding: $189,274 direct/yr 02

Project Goals: Analysis of the Ras pathway in epithelial and mesenchymal cells.

Specific Aims: i) Determine how EMT affects MAPK and P13K pathways downstream of oncogenic Ras; ii) identify genes required for survival in epithelial and mesenchymal cells.

Overlap: None

Funding Number: N/A PI: McCormick, Frank

Project Title: Target identification of Ras-related drug resistance

Performance Period: 04/01/2017 – 03/31/2019

Time Commitment: 0.12 calendar

Supporting Agency: Daiichi-Sankyo Company, Limited

Contracting Officer: Kosaku Fujiwara, PhD, VP, Oncology Research Laboratories, fujiwara.kosaku.t2@daiichisankyo.co.jp, +81-80-3529-4061

Level of Funding: $126,183 direct/yr 01

Project Goals: Identification of Ras-related drug resistance and identification of Ras-binding compounds using Second Harmonic Generation (SHG) screening techniques.

Specific Aims: i) Investigate Ras-related drug resistance; ii) identify Ras-binding compounds using SHG screening techniques.

Overlap: None

Funding Number: N/A PI: McCormick, Frank

Project Title: New ways of Treating Pancreatic Cancer Based on Reversing K-Ras-Mediated Stemness

Performance Period: 01/01/2015 – 12/31/2017

Time Commitment: 1.2 calendar months

Supporting Agency: Lustgarten Foundation

Contracting Officer: Kerri Kaplan., kkaplan@lustgarten.org, (516) 737-1550

Level of Funding: $418,436 direct/yr 03

Project Goals: Preclinical analysis of humanized anti-LIF monoclonal antibodies, using several models of pancreatic cancer, with the intention of moving these antibodies into clinical testing based on the results of these experiments

Specific Aims: i) generate human and mouse pancreatic cancer cells with LIF stably knocked down and elucidate the effects of this knock down; ii) validate LIF as a target of intervention for mouse pancreatic cancer in a syngenic mouse model; iii) compare the functions of LIF and IL6 in pancreatic cancer cells; iv) evaluate the therapeutic effects of LIF neutralizing antibody; v) elucidate the signaling pathways specifically driven by LIF and not other IL6 cytokine family members in K-Ras driven pancreatic cancers; vi) pre-clinically evaluate prostratin pro-drug in mouse models of pancreatic cancer; vii) investigate the mechanism of prostratin's tumor suppressing action on pancreatic cancer cell lines.

Overlap: None

Funding Number: N/A PI: McCormick, Frank

Project Title: Targeting KRAS Mutant Lung Cancers

Performance Period: 08/01/2015—07/31/2018

Time Commitment: 1.2 calendar months

Supporting Agency: Subcontract through Massachusetts General Hospital

Contracting Officer: Maida Broudo, mbroudo@partners.org, (617) 816-7789

Level of Funding: $384,750 direct/yr 03

Project Goals: Preclinical evaluation of prostratin and LIF for treating lung adenocarcinoma.

Specific Aims: i) Test the specific therapeutic effects of prostratin or its analogs on K-Ras driven lung cancers; 2) Examine the expression patterns of LIF in tumors and stromal cells.

Overlap: None

PENDING

None

Special Reporting Requirements - None

Appendices - None