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Oncogene-mediated inhibition of the Spred1 tumor suppressor

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Oncogene-mediated inhibition of the Spred1 tumor suppressor

by

Evan Curtis Markegard

DISSERTATION

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ACKNOWLEDGMENTS

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I'm grateful for the love and support I have received from my parents Gary and Marla Markegard for always encouraging me to pursue my dreams. My sister Erin Di Piero has always been positive and proud of my scientific achievements. I would like to thank my future parents-in-law Linda and Bob Barbera for the home away from home hospitality in the bay area. Finally, I'll like to thank their daughter, Nicole Barbera, my future fiancé. Nicole has provided so much love throughout the last 6 years of my thesis and I can't image completing this journey without her. I cannot wait to take the next step in our lives together.

ABSTRACT

Oncogene-mediated inhibition of the Spred1 tumor suppressor

Evan Curtis Markegard

Spred proteins are tumor suppressors which negatively regulate Ras/MAPK signaling following growth factor stimulation. Inhibition of Ras is thought to primarily occur through the ability of Spred1 to bind and localize Neurofibromin (*NF1*), a RasGAP, to the plasma membrane. *SPRED1* and *NF1* loss-of-function mutations tend to be mutually exclusive and occur across multiple cancer types and developmental diseases. We show oncogenic $EGFR^{L858R}$ signaling leads to the phosphorylation of Spred1 on serine 105 which disrupts the Spred1-Neurofibromin complex. The primary Spred1(S105) kinase was identified as CDK1. Spred1 directly binds CDK1 and enters the nucleus though a newly identified Class I nuclear localization sequence. Additionally, phosphomimetic Spred1 is unable to suppress Ras-GTP following growth factor stimulation and proliferation in the K562 leukemia cell line. Our findings provide one potential mechanism by which oncogenic signaling disrupts Spred1-Neurofibromin negative feedback of the Ras/MAPK pathway.

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CHAPTER 1.

INTRODUCTION

Oncogene and tumor suppressor background

Wild type cells acquire genetic alterations during the transition of becoming a cancer cell. These alterations were described in detail in 2000 as hallmarks of cancer by Hanahan and Weinberg[1]. Two key hallmarks include “Self-sufficiency in growth signals” and “Insensitivity to anti-growth signals”. The growth signals hallmark is important because oncogenes fall into this category. Oncogenes are often a major initiating event in cancer evolution and many have been successfully targeted therapeutically, extending the lives of cancer patients. One prominent example of a targetable driver oncogene is epidermal growth factor receptor (EGFR)[2]. Insensitivity to anti-growth signaling is also critical, illustrated by the frequent loss of tumor suppressors, the most frequent being TP53[2]. Most cancers show multiple hallmarks, for example a gain-of-function oncogenic mutation and a loss-of-function tumor suppressor mutation. However, some cancers have an oncogenic mutation but no tumor suppressor mutation. In these cancers, how is oncogenic signaling maintained in the presence of tumor suppressors? Perhaps oncogenes can inhibit tumor suppressors to sustain proliferation. The interaction between the proto-oncogene EGFR and the tumor suppressor Spred1 is the focus of my thesis.

Spred1 domains and function

Spred1 (Sprouty-related protein with an EVH1 domain) is an ERK-responsive negative regulator of the Ras/MAPK pathway following growth factor, cytokine, and chemokine signaling[3–6]. Spred1 overexpression inhibits the Ras/MAPK pathway[7] which is thought to primarily be through the ability of Spred1 to bind and recruit the Ras-GAP Neurofibromin (NF1) to the plasma membrane[8]. Neurofibromin is essential for terminating the Ras pathways following growth factor stimulation[9]. Furthermore, Neurofibromin-mediated negative feedback terminates growth factor-induced Ras-GTP activation[9]. The region of Spred1-Neurofibromin binding has been identified for both proteins[10,11]. Spred1 binds Neurofibromin in the Enabled/VASP homology 1 (EVH1) domain while Neurofibromin binds Spred1 in the noncatalytic (GAPex) regions which flank the GAP-related domain (GRD). Spred1 also contains a Kit-binding domain (KBD) and a cysteine rich Sprouty (SPR) domain, which is likely palmitoylated and essential for plasma membrane localization[12]. EGF stimulation or Gal-1-mediated induction of Raf dimers has also been shown to translocate Spred1 to the plasma membrane[13]. Other members of the Spred family include Spred2 and Spred3.

Spred protein family

Spred1, Spred2, and Spred3 all share the EVH1 and SPRY domains, but Spred3 does not contain a KBD and does not inhibit the Ras/MAPK pathway to the same extent as Spred1 and Spred2. Spred3 also has a splice variant known as Eve-3. Spred1 and Spred2 are similar and thought to have overlapping roles, with the major difference being tissue specific expression. In mice, Spred1 is primarily expressed in the brain

while Spred2 is more widely expressed across adult tissue[14]. Spred1 and Spred2 are tyrosine phosphorylated in response to multiple factors including Epidermal growth factor (EGF), Stem cell factor (SCF), and Platelet-derived growth factor (PDGF)[3,15]. The Spred proteins are also thought to form homo- and heterodimers, although the functional significance of these dimers remains unknown[7]. While Spred1 has a role in human disease, Spred2 and Spred3 do not.

Biological significance of Spred1

The biological importance of Spred1 has recently begun to be investigated. Spred1-null mice are viable and have impaired synaptic plasticity and hippocampus dependent learning[16] while Spred1 overexpression in the mouse osteosarcoma cell line LM8 reduces proliferation and metastasis in nude mice[4]. Since Spred2 expression may compensate for Spred1 loss, double knockout mice were generated and have myeloproliferative neoplasms (MPN) leading to rapid death[17]. The same group also showed Spred1 negatively influences self-renewal in hematopoietic stem cells (HSCs), which safeguards HSCs homeostasis in respond to a high-fat diet.

The clinical significance of Spred1 alterations have been appreciated in both developmental disorders and human cancer. Spred1 loss-of-function mutations were identified in the RASopathy Legius syndrome (LS), a developmental disorder similar to Neurofibromatosis type 1 (NF1), and are unable to inhibit the Ras/MAPK pathway[18]. LS is typically less severe than NF1 and can include café-au-lait spots, axillary freckling, and learning disabilities, including ADD and ADHD. LS is hypothesized to be less severe than NF1 because of Spred2 compensation. As expected, the clinical

manifestations NF1 and LS has been attributed to hyperactive Ras/MAPK signaling. Genetic studies from The Cancer Genome Atlas (TGCA) show *SPRED1* is altered across many cancer types with deletions and mutations being the most frequent. Additionally, Spred1 expression has been shown to be decreased in hepatocellular carcinoma[19], pediatric acute myelogenous leukemia (AML)[20], and breast cancer[21] further highlighting the importance of Spred1 as a tumor suppressor.

Neurofibromin background

Neurofibromin in a RasGAP and frequently mutated across multiple cancer types and in the genetic disorder Neurofibromatosis type 1. Neurofibromatosis type 1 shares symptoms with Legius syndrome but is more serve and can lead to painful neurofibromas, which are benign tumors. In addition to studying the Neurofibromin effector Spred1, we also sought to identify other effectors of Neurofibromin. Neurofibromin is a large 320 kilodalton protein yet few effectors have been identified. Neurofibromin domains include the central GAP related domain (GRD) and the SEC14 domain and PH-like domain although no cellular lipid–Neurofibromin interaction has been described[22]. Yeast contain the Neurofibromin homologs Ira1 and Ira2, suggesting a conserved importance which is Spred1 independent since yeast do not have Spred proteins. In yeast Ira1 may play a role in nutrient sensing[23], a novel function which could be interrogated further in human cells.

CHAPTER 2.

Oncogenic-Inhibition of Spred1

ABSTRACT

Spred proteins are tumor suppressors which negatively regulate Ras/MAPK signaling following growth factor stimulation. Inhibition of Ras is thought to primarily occur through the ability of Spred1 to bind and localize Neurofibromin (NF1), a RasGAP, to the plasma membrane. *SPRED1* and *NF1* loss-of-function mutations tend to be mutually exclusive and occur across multiple cancer types and developmental diseases. We show oncogenic $EGFR^{L858R}$ signaling leads to the phosphorylation of Spred1 on serine 105 which disrupts the Spred1-Neurofibromin complex. The primary Spred1(S105) kinase was identified as CDK1. Spred1 directly binds CDK1 and enters the nucleus though a newly identified Class I nuclear localization sequence. Additionally, phosphomimetic Spred1 is unable to suppress Ras-GTP following growth factor stimulation and proliferation in the K562 leukemia cell line. Our findings provide one potential mechanism by which oncogenic signaling disrupts Spred1-Neurofibromin negative feedback of the Ras/MAPK pathway.

INTRODUCTION

Despite the emerging importance of Spred1 in both genetic diseases and as a tumor suppressor, post-translational modifications affecting function have not been elucidated. Following the discovery of Neurofibromin as the major Spred1 effector in

2012, little progress has been made on the molecular mechanisms that regulate the complex. Since the activity of Neurofibromin relies on Spred1 binding, understanding complex dysregulation should be of high importance for both cancer and genetic disease research. Pathways which disrupt Spred1-Neurofibromin would be compelling drug targets to restore negative feedback and inhibit Ras in aberrant cells.

Spred1 function has been most extensively studied in response to growth factor signaling through RTKs. Spred1 binds c-kit and from our preliminary work, other receptors including CSF1R, Flt3, and PDGFR. Spreds are tyrosine phosphorylated in response to RTK stimulation although the specific kinase and impact on Neurofibromin bindings is unknown. Spreds also share a sprouty domain with the Sprouty protein which negatively regulate growth factor signaling such as EGF and FGF[24]. Given the numerous links between RTK signaling and Spreds, we sought to investigate the relationship between oncogenic RTKs and Spred1. Additionally, RTK-driven tumors often have wild type Neurofibromin and Spred1, which is essential to study complex regulation.

RESULTS

Identification of Spred1 phosphorylation which disrupts Spred1-Neurofibromin

We were interested in whether oncogenic receptor tyrosine kinases could inhibit negative feedback by disrupting the Spred1-Neurofibromin complex. Since gain-of-function EGFR mutations tend to be mutually exclusive (log odds ratio <0) with loss-of-function SPRED1 and NF1 mutations in lung adenocarcinoma[25], (Fig. 2-1A), we decided to use oncogenic $EGFR^{L858R}$ as a model system. Expression of $EGFR^{L858R}$

disrupted Spred1-Neurofibromin binding as shown by flag immunoprecipitation of the Neurofibromin GRD domain and western blot for endogenous Spred1 in HEK 293T cells (Fig. 2-1B). To identify phosphorylation sites on Spred1 and Neurofibromin which may disrupt Spred1-Neurofibromin binding we co-expressed oncogenic $EGFR^{L858R}$ and Spred1, immunoprecipitated Spred1 or Neurofibromin, and performed liquid chromatography-tandem mass spectrometry (LC-MS/MS) (Fig. 2-2A-B). Five Spred1 phosphorylation sites were identified, including serine 105 which is in the EVH-1 domain, the domain of Spred1 which binds Neurofibromin. Spectrum and annotated peptides of Spred1(S105) are shown in Fig. 2-2C. Serine 105 is also in close proximity to threonine 102 which is mutated to the charged amino acid arginine in the RASopathy Legius syndrome[26]. $Spred1^{T102R}$ is also unable to bind Neurofibromin and suppress Ras-GTP following EGF stimulation[8]. Furthermore, the *Xenopus tropicalis* EVH-1 domain of Spred1 crystal structure reveals both T102 and S105 are located in a hydrophobic region of Spred1[27]. Therefore, it is likely the negative charge of the phosphate group on the hydrophobic surface of Spred1 sterically repels Neurofibromin. The region flanking human Spred1(S105) is also conserved across multiple species from mouse to fish, further supporting the importance of phosphorylation site (Fig. 2-3A). To interrogate the potential of phosphorylation of Spred1(S105) to disrupt Neurofibromin binding, we generated phosphomimetic and phosphodeficient Spred1(S105) mutants and performed immunoprecipitation experiments. Phosphomimetic (aspartic and glutamic acid) decreases Neurofibromin binding while phosphodeficient (alanine) increases Neurofibromin binding (Fig. 2-3B). These

experiments identify serine 105 as a critical phosphorylation site on Spred1 which disrupts the Spred1-Neurofibromin complex.

CDK1 phosphorylates Spred1(S105)

To determine which serine kinase is primarily responsible for phosphorylation of Spred1(S105), we performed an *in vitro* kinase screen using Spred1 peptide. 93 candidate serine kinases where chosen based on Spred1(S105) phosphorylation motif predictions and kinases downstream of EGFR signaling. Hits which were more than two standard deviations from the mean were CDK1/Cyclin A2, CDK2/Cyclin O, CDK1/Cyclin A1, and CDK9/Cyclin T2 (Fig. 2-4A). Candidate kinases with greater than 10,000 CPM were then screened *in vitro* using full length Spred1 protein followed by LC-MS/MS to confirm Spred1(S105) phosphorylation (Fig. 2-4B). CDK1/Cyclin A2 was the only kinase identified as a hit on both screens. A broad panel of kinase inhibitors were screened for inhibition of phospho-Spred1(S105) with $EGFR^{L858R}$ expression and the CDK1/2/5/9 inhibitor Dinaciclib was the most potent (Fig. 2-4C). We targeted these four CDKs with multiple chemical inhibitors and only CDK1 inhibitors were able to decrease $EGFR^{L858R}$-mediated Spred1(S105) phosphorylation (Fig. 2-D). Since chemical inhibitors could have off-target effects, we also performed genetic knockdown to obtain greater specificity and only CDK1 siRNAs were able to decrease $EGFR^{L858R}$-mediated Spred1(S105) phosphorylation (Fig. 2-5A-B). In addition to kinase inhibition decreasing Spred1(S105) phosphorylation, CDK1 overexpression increased Spred1(S105) phosphorylation (Fig. 2-5C). $EGFR^{L858R}$ expression also increases Spred1-CDK1 binding, supporting the hypothesis that $EGFR^{L858R}$ signaling leads to CDK1 mediated-Spred1(S105) phosphorylation (Fig. 2-5D). Through an initial *in vitro* kinase assay

screen and *in vivo* cell based assay validation we were able to identify CDK1 as the Spred1(S105) kinase.

Spred1 NLS is required for nuclear localization and CDK1 binding

Identification of CDK1 as the Spred1(S105) was surprising since CDK1 is thought to be active in the nucleus while Spred1 function has mostly been described at the plasma membrane. However, experiments from The Human Protein Atlas showed nuclear Spred1 in PC-3 and U-2OS cells by immunofluorescence. We performed nuclear localization sequence (NLS) analysis on Spred1 using cNLS Mapper, NLStradamus, and NucPred which all predicted the same region as an NLS with high confidence. Indeed, Spred1(324KRRK) is a classical NLS Class I sequence near the Sprouty (SPR) domain (Fig. 2-6A). We generated a Spred1 NLS mutant by replacing the four charged amino acids with uncharged glutamines, Spred1(324QQQQ), to test mislocalization. Since $EGFR^{L858R}$ increased phospho-Spred1(S105) we also wondered if $EGFR^{L858R}$ could increase Spred1 nuclear localization. Indeed, our Spred1 NLS mutant showed decreased nuclear localization and increased cytoplasmic localization (Fig. 2-6B). Conversely, $EGFR^{L858R}$ expression increased wild type Spred1 nuclear localization and decreased cytoplasmic localization. Spred1(324QQQQ) was also unable to bind CDK1 with $EGFR^{L858R}$ expression (Fig. 2-6C). These data support a model by which Spred1 enters the nucleus through the NLS, where it binds and is phosphorylated on serine 105 by CDK1.

Phosphomimetic and phosphodeficient Spred1(S105) alter Ras-GTP signaling following EGF stimulation and K562 proliferation

In addition to the biochemical effect of Spred1(S105) phosphorylation on Neurofibromin binding, we were also interested in the biological effects. We have previously demonstrated Spred1 overexpression decreased Ras-GTP following EGF stimulation in HEK 293T cells and Spred1 mutants found in Legius syndrome were unable to decrease Ras-GTP[8]. As expected, phosphomimetic Spred1(S105) is also unable to suppress Ras-GTP following EGF simulation (Fig. 2-6A). To determine if phosphomimetic Spred1(S105) may also alter cancer cell proliferation we used a cancer cell line dependent on Ras/MAPK signaling for proliferation with functional Spred1 and Neurofibromin. Our collaborators Boettcher *et al.* recently discovered, through an unbiased whole genome CRISPRa screen, that SPRED1 and NF1 overexpression inhibit K562 proliferation[28]. K562 is a chronic myeloid leukemia (CML) cell line with the BCR-ABL oncogene, is dependent on Ras-GTP for proliferation and may be unable to phosphorylate and inactivate Spred1 on serine 105. Therefore, we expected the K562 cell line would be an ideal model system to test the biological effects of phosphomimetic Spred1(S105). We infected K562 cells with Spred1-IRES-GFP expressing retrovirus and performed a competition assay between infected (GFP+) and uninfected (GFP-) cells (Fig. 2-7B). Representative flow cytometry GFP histograms show similar infection rates and expression levels (Fig. 2-7C). Spred1 wild type infected GFP positive cells were outcompeted by uninfected GFP negative cells while the empty vector controls were not. Phosphomimetic Spred1(S105) infected cells were unable to inhibit proliferation, along with Spred1 Legius syndrome patient mutants W31C and T102R.

In addition to oncogenic $EGFR^{L858R}$-mediated phosphorylation of Spred1(S105) in HEK 293T overexpression assays, we wondered if Spred1 is phosphorylated in cancer cells with oncogenic EGFR mutations. We tested four EGFR mutant cancer cell lines which all have elevated Spred1(S105) phosphorylation compared to HEK 293T cells, confirming the existence of this critical phosphorylation site in cancer cells (Fig. 2-8A). In addition to oncogenic EGFR, expression of other oncogenic RTKs also increased Spred1(S105) phosphorylation and other phosphorylation sites, broadening the scope of this finding (Fig. 2-8B, Table 2-1). Therefore, in cell lines where the Spred1-Neurofibromin negative feedback machinery is intact, phosphomimetic Spred1(S105) is unable to inhibit Ras-GTP and cancer cell proliferation.

DISCUSSION

This work identifies a critical Spred1 phosphorylation site on serine 105 which disrupts Neurofibromin binding, an important Spred1 effector. Spred1(S105) phosphorylation has been reported before in HeLa cells following synchronization and EGF stimulation as part of a phosphoproteome analysis[29]. Phosphomimetic Spred1(S105), like the Spred1 Legius syndrome patient mutations W31C and T102R, is unable to inhibit K562 cancer cell proliferation. This data supports our previous model showing the inhibitory function of Spred1 is primarily mediated through Neurofibromin binding[8]. Updates to this model include the finding that oncogenic EGFR can inhibit Spred1-Neurofibromin binding through CDK1-mediated Spred1(S105) phosphorylation (Fig. 2-9).

In vitro kinase assays identified Cyclin A2, not Cyclin A1 or Cyclin B1, as the CDK1 cyclin partner necessary for Spred1(S105) phosphorylation. Cyclin A2 is expressed in mitotic cells while Cyclin A1 is restricted to embryogenesis and Cyclin B1 provides unique substrate specificity. Cyclin A2 is expressed during S phase and localizes to the nucleus where it binds CDK1 during the G2 to M phase transition[30]. Therefore, we investigated whether Spred1 could localize to the nucleus as a CDK1/Cyclin A2 substrate and identified a C-terminal class I NLS sequence, Lys-Arg-Arg-Lys. Nuclear localization of Spred1 has not been described before and additional experiments are needed to fully understand the nuclear function of Spred1.

While transient (2 hours) CDK1 inhibition was sufficient to decrease phospho-Spred1(S105) by $EGFR^{L858R}$, transient EGFR inhibition was insufficient. Therefore, longer inhibition of EGFR may be required to decrease phospho-Spred1(S105), indicating the $EGFR^{L858R}$-CDK1 signal may not be immediate. The simplest explanation is chronic $EGFR^{L858R}$ expression leads to an altered cell cycle and CDK1 activity. The direct link between oncogenic RTKs and cell cycle should be investigated further. Additionally, prolonged $EGFR^{L858R}$ expression leads to phospho-Spred1(S105) and decreased Spred1-Neurofibromin binding while transient EGF simulation results in increased Spred1-Neurofibromin binding (data not shown). Therefore, the effect of EGFR signaling on Spred1 phosphorylation is likely distinct between chronic and acute pathway activation.

There are few examples of direct signaling links between oncogenes and tumor suppressors. Our data reveals a novel mechanism by which oncogenic EGFR signaling disrupts the Spred1-Neurofibromin tumor suppressor complex. By dysregulating the

complex, there is likely no additional selective pressure for the cancer cells to lose SPRED1 or NF1, as observed in the lung adenocarcinoma genetics. This finding could be expanded to other cancer types, and potentially to other oncogene-tumor suppressor relationships.

FIGURE 2.1

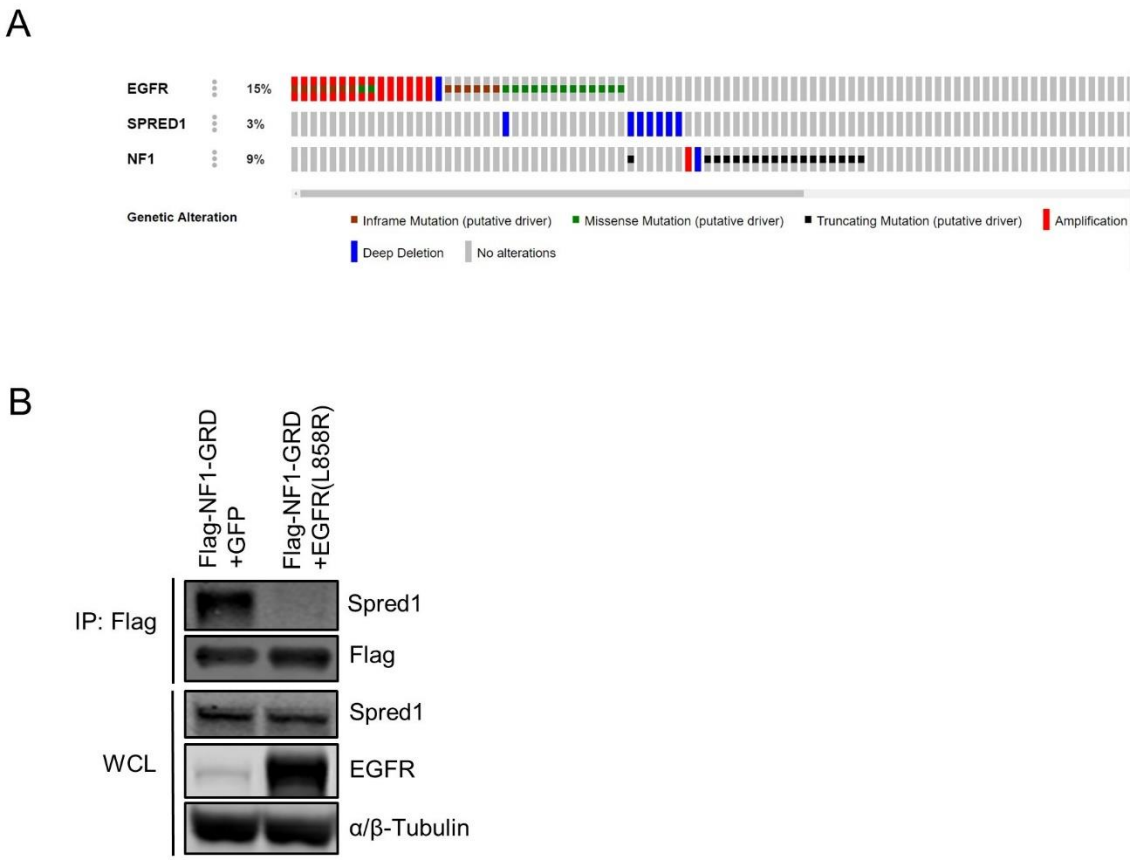

Figure 2-1. Lung adenocarcinoma genetics and disruption of Spred1-Neurofibromin binding.
(A) Lung adenocarcinoma genetics, n=230, with visualization using cBioPortal[31,32]. Variants of unknown significance were excluded.
(B) HEK 293T cells were transfected and Spred1-Neurofibromin binding was accessed by flag-IP and western blot.

FIGURE 2-2

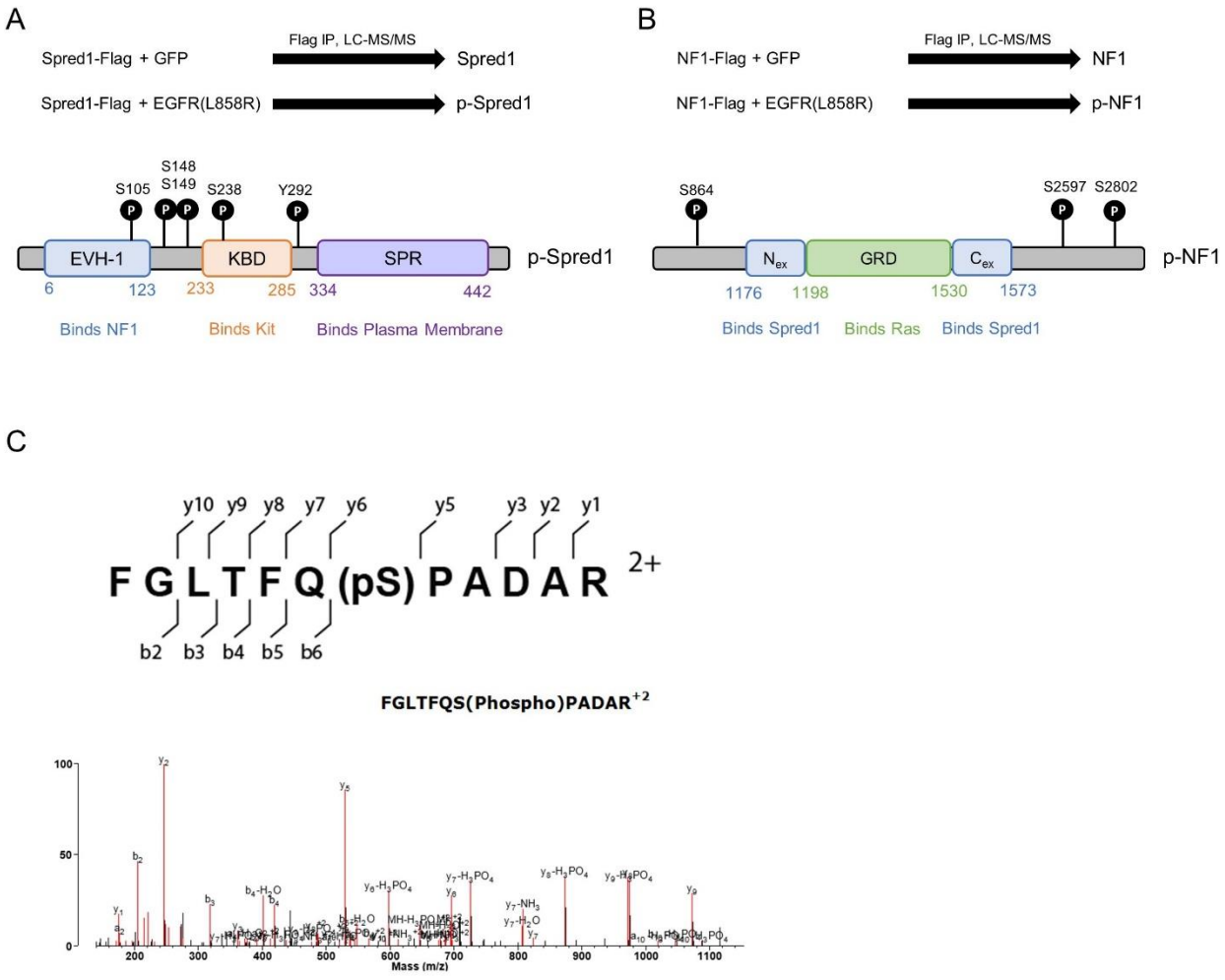

Figure 2-2. Spred1 and Neurofibromin phosphorylation with spectrum.
(A-B) Identification of Spred1 and Neurofibromin phosphorylation sites downstream of $EGFR^{L858R}$ by transfection, flag-IP, and LC-MS/MS.
(C) Spectrum and annotated phospho-Spred1(S105) peptide.

FIGURE 2-3

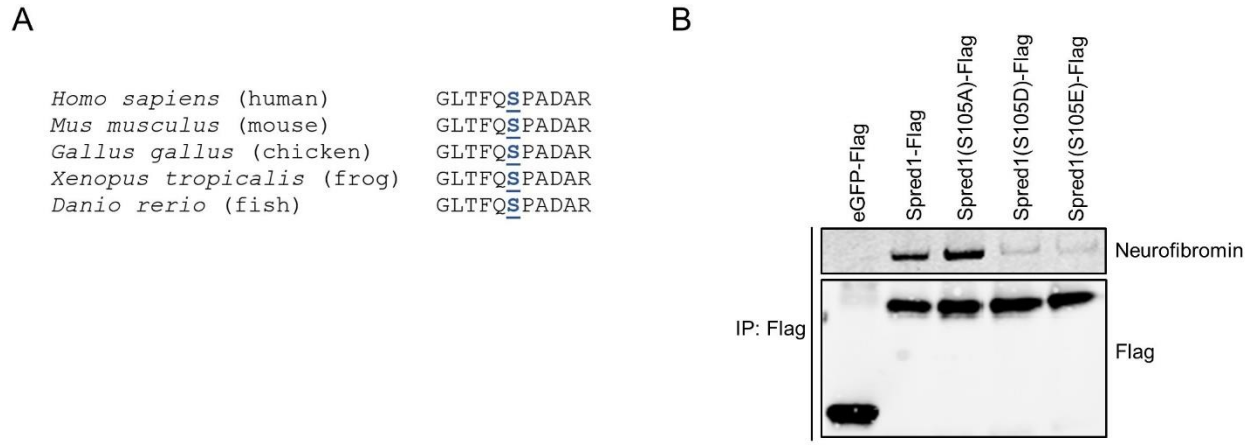

Figure 2-3. Spred1(S105) conservation and disruption of Neurofibromin binding.
(A) Schematic of Spred1(S105) flanking region with DNA alignment across indicated species.
(B) Phosphomimetic and phosphodeficient Spred1(S105) mutants were assessed for Neurofibromin binding as above.

FIGURE 2-4

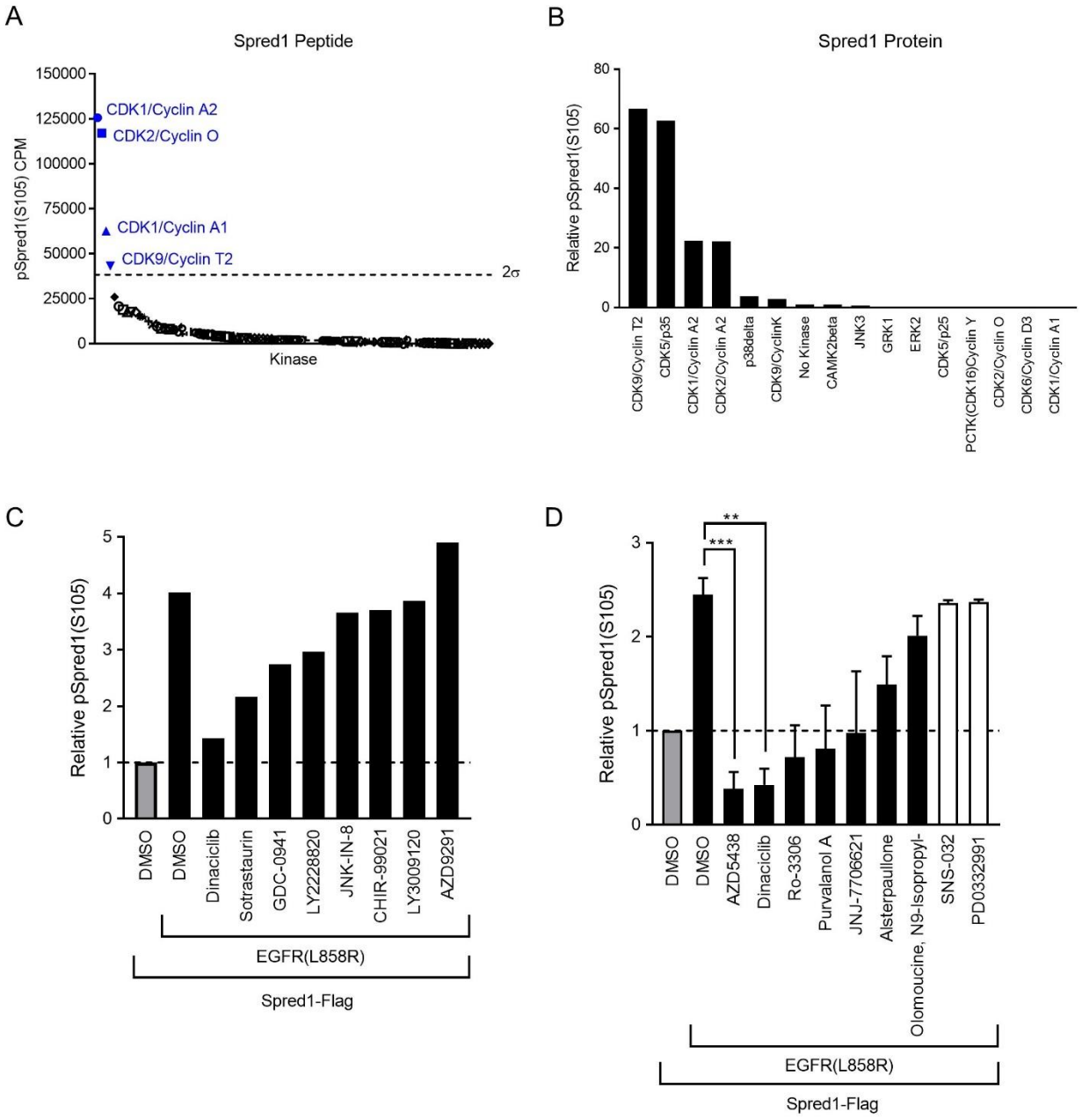

Figure 2-4. *In vitro* and chemical screen to identify Spred1(S105) kinase.
(A) *In vitro* kinase assay screen using Spred1 peptide and radiolabeled ATP.
(B) *In vitro* kinase assay using full length Spred1 protein followed by LC-MS/MS.
(C) HEK 293T cells were transfected as before and treated with kinase inhibitors for 2 hours at 10 µM before IP and LC-MS/MS. Relative Spred1(S105) phosphorylation was determine by normalization to total peptide and untreated normalized to 1.
(D) As above, except HEK 293T were treated with CDK1 inhibitors for 4 hours at 2 µM.

FIGURE 2-5

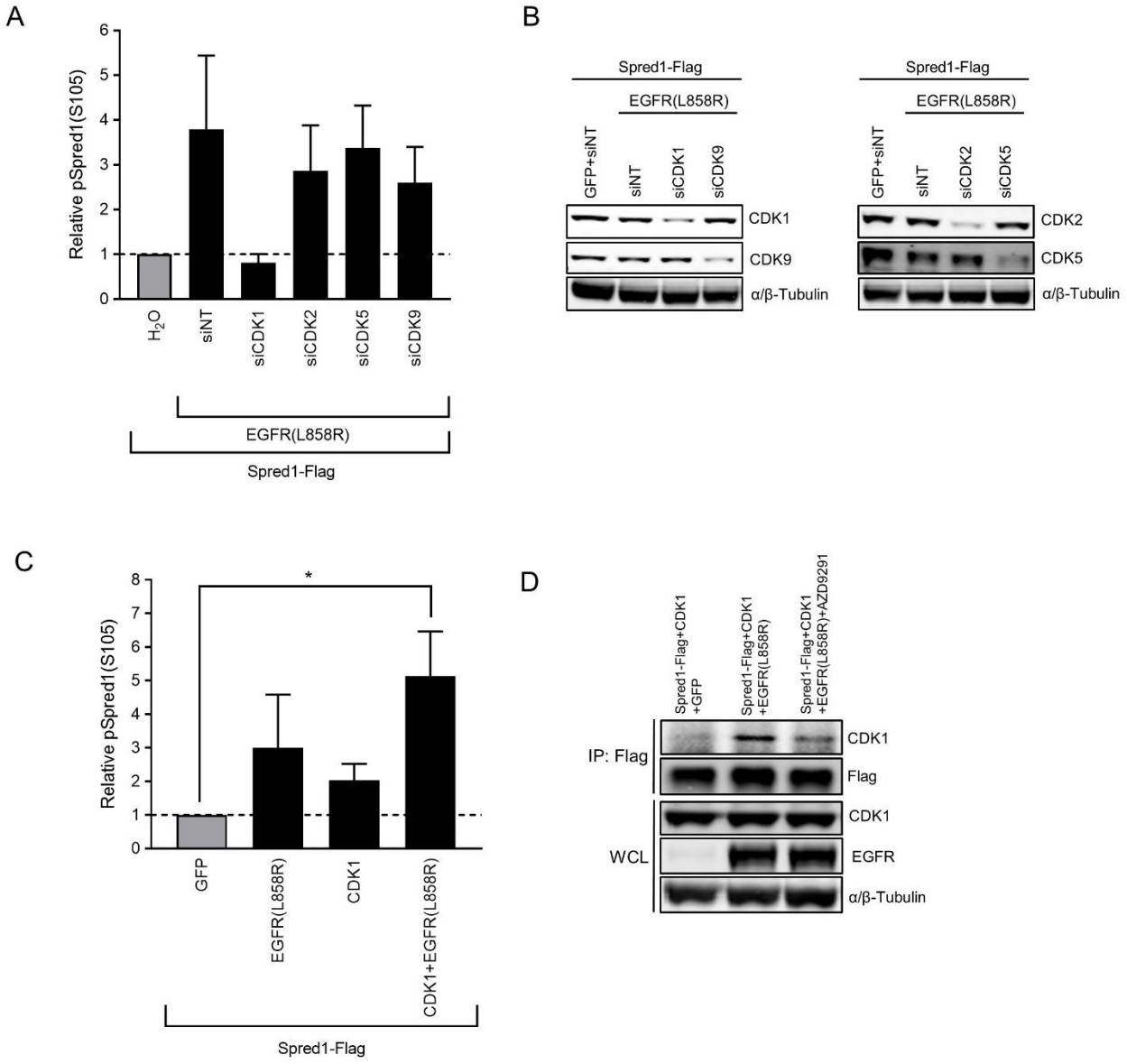

Figure 2-5. siRNA screen and candidate overexpression to identify Spred1(S105) kinase.

(A) Candidate CDK knockdown transfected with 400 pmol of siRNA for 48 hours and pSpred1(S105) as before.

(B) Western blots for CDK knockdown efficiency.

(C) Candidate gene transfection, IP, and LC-MS/MS as above. The statistical significance of the difference between indicated samples was determined using a two-tailed t test. *$P < 0.05$.

(D) Transfection and IP followed by western blot for Spred1-CDK1 binding.

FIGURE 2-6

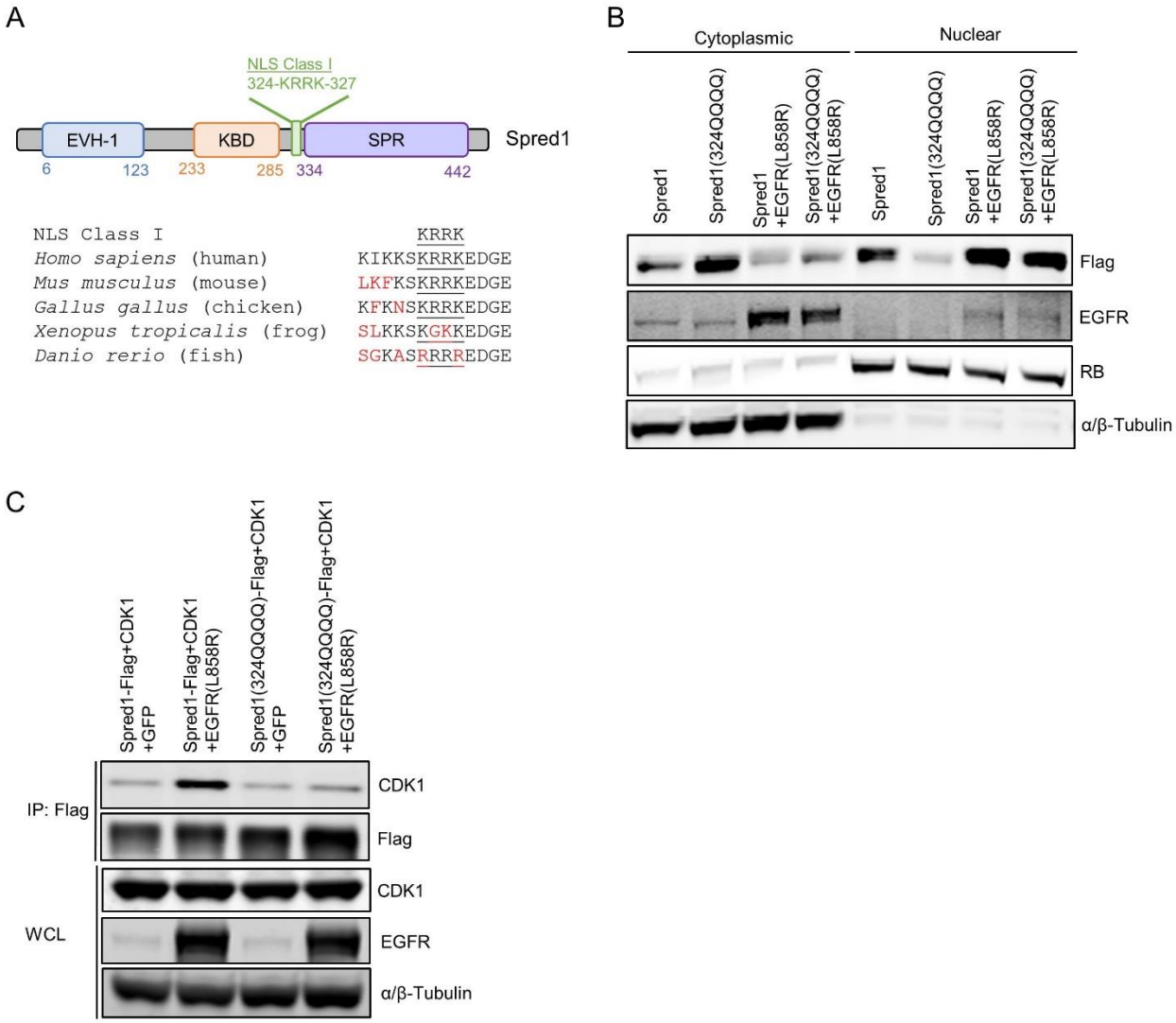

Figure 2-6 Spred1 NLS is required for nuclear localization and CDK1 binding.
(A) Schematic of the Spred1 NLS and flanking region with DNA alignment across indicated species.
(B) Spred1 localization by cytoplasmic and nuclear fractionation followed by western blot.
(C) Spred1 NLS mutant transfection, IP, and western blot as above.

FIGURE 2-7

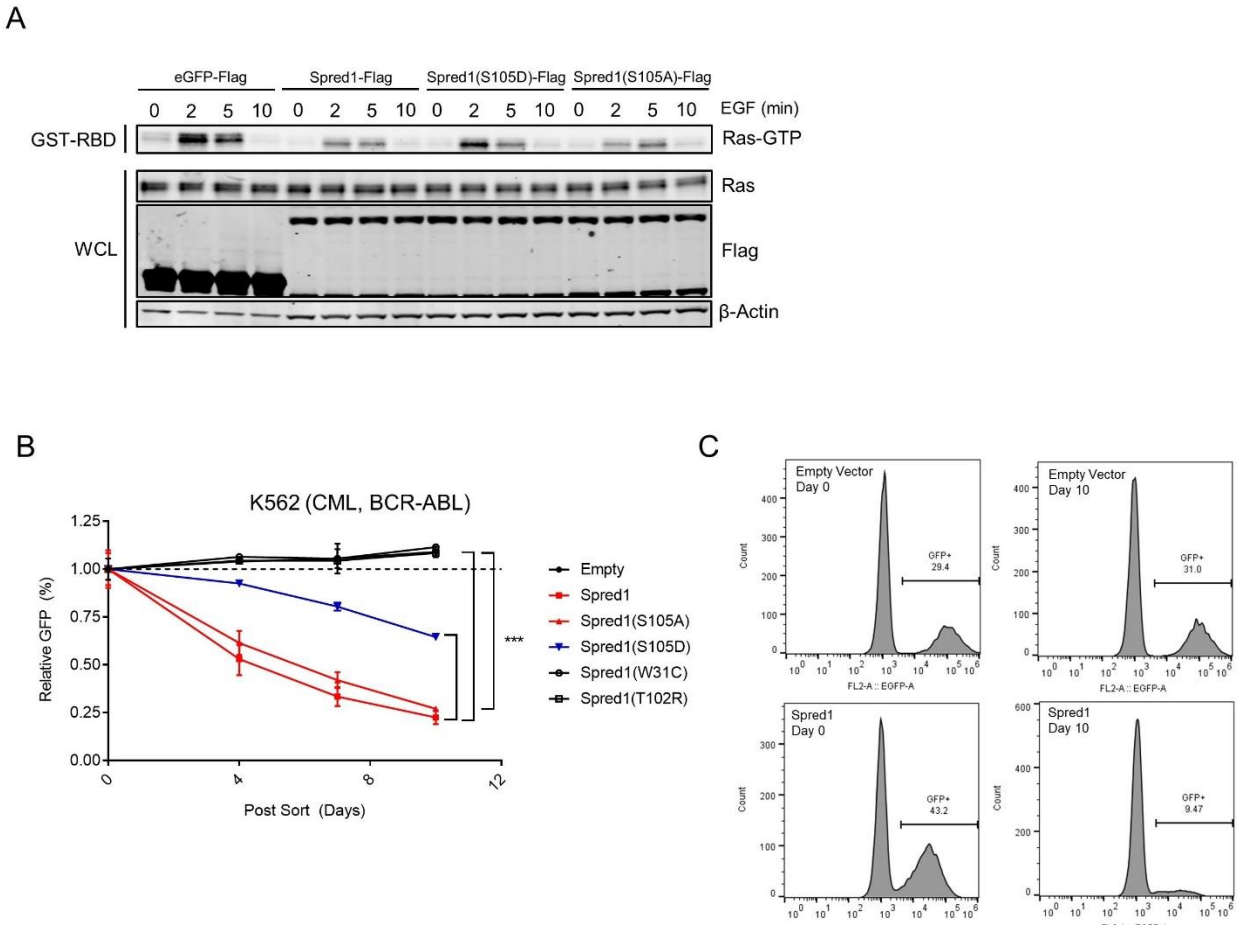

Figure 2-7 Phosphomimetic Spred1(S105) alters Ras-GTP signaling following EGF stimulation and K562 proliferation.

(A) HEK 293T cells were transfected, serum starved for 16 hours, and stimulated with 10 ng/ml EGF. Downstream signaling was accessed by western blot and Ras-GTP pulldown assay.

(B) K562 cells were infected with Spred1-IRES-GFP expressing retrovirus. Three days after infection baseline GFP-positive cells were measured by flow cytometry and normalized to 1. GFP positive cells were monitored over time to measure the effect of Spred1 expression on proliferation. The statistical significance of the difference between indicated samples was determined using a two-way ANOVA. ***P < 0.001.

(C) Representative flow cytometry GFP histograms.

FIGURE 2-8

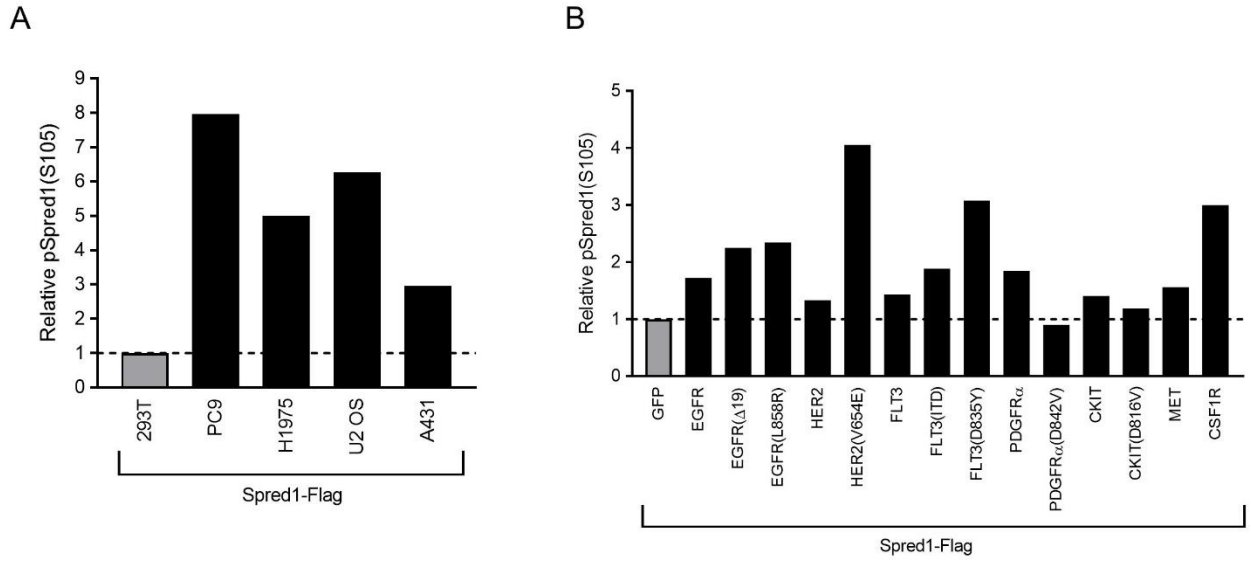

Figure 2-8. Spred1(S105) phosphorylation in cancer cell lines and across RTKs.
(A) Cancer cells lines were infected with Spred1-Flag expressing retrovirus, selected with 1 µg/ml puromycin, Spred1-flag IP, and LC-MS/MS as above.
(B) RTK panel screen for phosphorylated Spred1(S105).

FIGURE 2-9

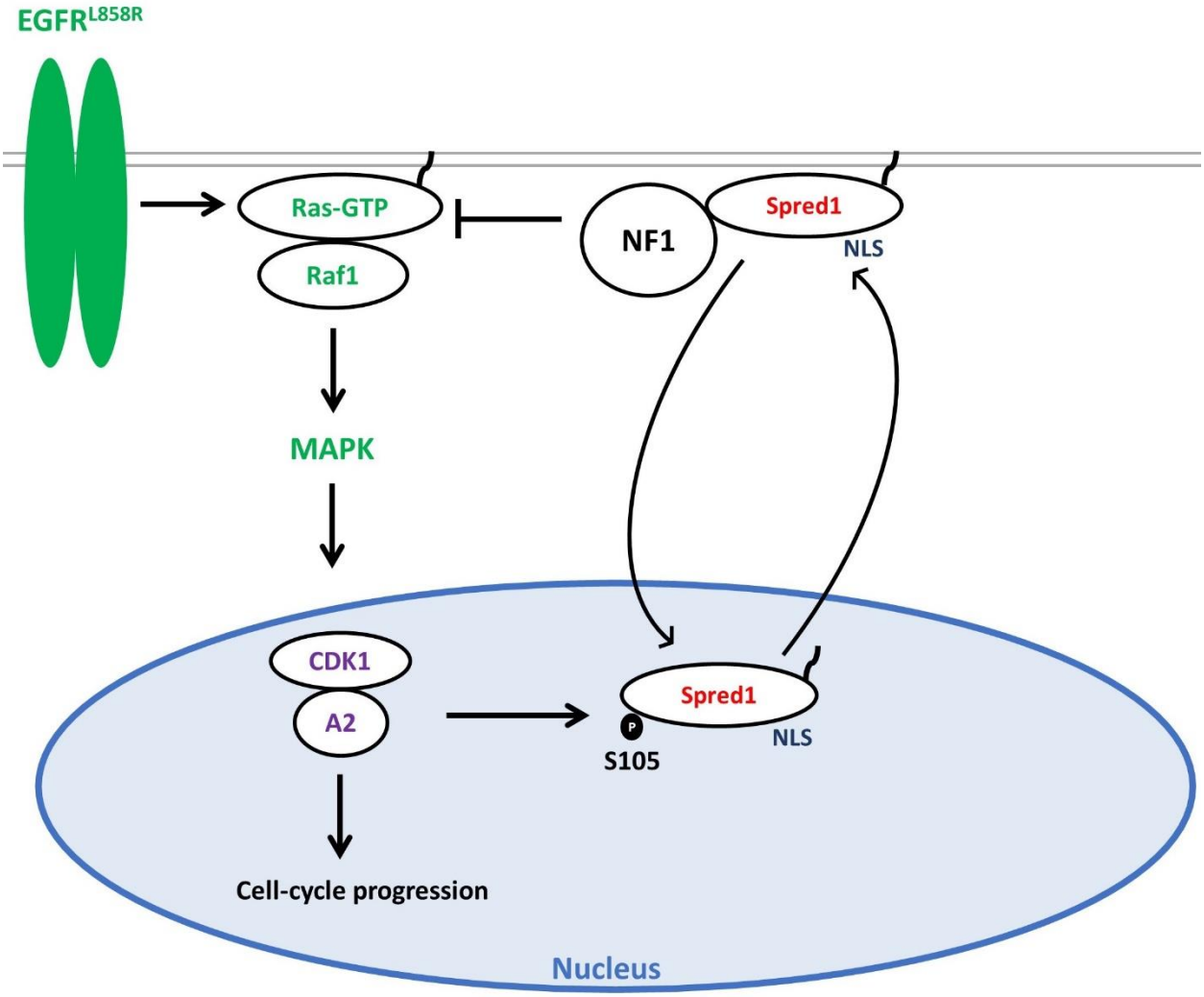

Figure 2-9 Model of Spred1(S105) phosphorylation.
Oncogenic EGFR leads to CDK1-mediated Spred1(S105) phosphorylation. Nuclear import of Spred1 likely occurs through the NLS. Phosphorylated Spred1(S105) is unable to bind Neurofibromin and inhibit Ras-GTP signaling.

TABLE 2-1

pSite	GFP	EGFR	EGFR(Δ19)	EGFR(L858R)	HER2	HER2(V654E)	FLT3	FLT3(ITD)	FLT3(D835Y)	PDGFRα	PDGFRα(D842V)	CKIT	CKIT(D816V)	MET	CSF1R
pS38	1.00	1.07	0.78	0.83	0.52	0.54	0.96	0.64	0.71	0.93	1.11	1.26	2.40	1.27	3.14
pS105	1.00	1.72	2.25	2.34	1.33	4.06	1.44	1.89	3.08	1.84	0.90	1.40	1.19	1.56	3.00
pS147	1.00	2.82	9.09	8.60	2.38	3.61	2.64	1.19	1.52	0.74	0.81	0.59	9.03	1.30	0.04
pY168	1.00	5.72	0.69	3.72	4.04	7.10	19.91	7.84	15.52	2.06	39.03	0.16	143.21	0.67	3.13
pY187	1.00	1.62	0.25	1.47	1.77	1.36	7.30	3.07	6.50	3.07	15.10	0.78	71.69	0.60	6.47
pS238	1.00	1.07	0.69	1.06	0.64	0.93	1.51	1.08	1.36	1.00	1.32	0.88	1.54	1.00	0.97
pY292	1.00	44.44	22.99	57.45	57.24	68.66	54.37	22.43	40.86	16.87	109.80	1.30	332.89	4.92	6.70
pY294	1.00	290.16	119.17	490.90	328.34	472.33	427.93	108.56	298.78	48.18	279.01	9.19	2581.67	53.88	0.00

Table 2-1. Heatmap of Spred1 phosphorylation with RTK expression.

CHAPTER 3.

Screen for Novel NF1 Effectors

ABSTRACT

Whole genome CRISPRa screening offers a new way to interrogate the contribution of gain-of-function genetic changes on a pathway of interest. We performed a CRISPRa screen to identify novel regulators of Neurofibromin in the Ras-dependent leukemia cell line K562. To ensure Neurofibromin and/or Ras dependent effects, we compared wild type to NF1-null K562 cell lines with low levels of the ABL kin inhibitor imatinib for selective pressure. Our screen identified many negative regulators of the Ras pathway which when overexpressed decreased proliferation in the wild type cells but not the NF1-null cells including DUSP9, RASA3, CBL, PTPN1, RASA2, and SPRED1. Conversely, hits which had the opposite effect include Ras pathway positive regulators BCR, SOS1, GAB2, and SHOC2. Ras pathway hits validate our screen, which also generated many additional hits which have not been previously linked to Ras signaling. Validation of these hits and further mechanism interrogation will identify novel regulators of Neurofibromin and/or the Ras pathway.

INTRODUCTION

CRISPRa is a relatively new and efficient screening technique to identify the effect of gene overexpression[33]. Our colleagues Boettcher *et al*., performed a full genome CRISPRa screen in the BCR-ABL fusion leukemia cell line K562 with imatinib,

an ABL inhibitor to apply selective pressure, to identify genes that effect proliferation[28]. These cells are an ideal model system since they grow in suspension, allowing for high coverage of a whole genome screen. Genes which when overexpressed inhibited proliferation included NF1, SPRED1, and SPRED2. Importantly, KRAS overexpression increased proliferation, confirming Ras dependence. Further validation screening revealed that Neurofibromin loss negated the toxic effects of SPRED2 overexpression, confirming Spred proteins act through Neurofibromin.

These results show the K562 cell line is an ideal model system for screening genes which regulate Neurofibromin since proliferation is dependent on activity of the fully functional Ras pathway. Additional hits from the Neurofibromin loss candidate screen include the RTK AXL and the AML fusion gene partner NOL4L. When overexpressed in NF1-null cells, but not wild type, proliferation increased, suggesting these genes may suppress proliferation through Neurofibromin. To expand this exciting preliminary data, we performed a whole genome CRISPRa screen comparing wild type to NF1-null K562 cells in the presence of imatinib. We identified many novel genes which may promote or inhibit proliferation through Neurofibromin.

RESULTS

Our CRISPRa screen generated many compelling hits from the Ras pathway, as expected (Table 3-1). The top hit from the CRISPRa screen, which when overexpressed inhibited proliferation in the wild type but not NF1-null cells, was Transforming growth factor beta receptor 2 (TGFBR2). TGF-β signaling inhibits proliferation and stimulates erythroid differentiation. The TGFBR2 ligand TGF-β1 inhibits proliferation of K562 cells,

potentially through differentiation into red blood cells (RBCs)[34]. Additionally, ABL and MEK inhibition differentiate K562 cells into RBCs, supporting the role of MAPK signaling to prevent differentiation and maintain proliferation[35,36].

Importantly, in this whole genome, unbiased screen Spred1 was a top hit, further validating the inhibitory function of Spred1 is dependent on Neurofibromin. Other notable hits were DUSP9, an ERK1/2 phosphatase. In wild type cells, increasing expression of DUSP9 is expected to decrease phosphorylated ERK1/2 and inhibit proliferation. However, NF1-null cells are expected to have elevated Ras-GTP, which may increase phosphorylated ERK1/2 to counterbalance DUSP9 overexpression. Other hits include CBL, a known E3 ubiquitin-protein ligase which degrades BCR-ABL, and tyrosine-protein phosphatase non-receptor type 1 (PTPN1 also known as PTP1B) an ABL phosphatase. Both CBL and PTPN1 inhibit BCR-ABL signaling in K562 wild type cells, inhibiting proliferation, while NF1 deletion rescues proliferation, likely through elevated Ras-GTP signaling downstream of BCR-ABL. RASA2 and RASA3 are both hits and RasGAPs, suggesting these GAPs may be not be functional without Neurofibromin or levels of Ras-GTP are too high from Neurofibromin loss to be reduced. The hit FOXO4 encodes the transcription factor Forkhead box protein O4 which inhibitors proliferation, but this inhibition may be dependent on Neurofibromin.

Our CRISPRa screen also identified gene which when overexpressed increased proliferation in wild type K562 cells but not NF1-null. As expected, our top hit was BCR, which leads to increase BCR-ABL expression, a previously validated resistance mechanism to imatinib[37]. However, NF1-null K562 proliferation does not increase with BCR overexpression, suggesting NF1 deletion is sufficient to maximize proliferation,

likely through increased Ras-GTP signaling. SOS1, a RasGEF, is also an expected top hit. Additional hits in the Ras pathway include SHOC2, which facilitates Ras/Raf1 binding, and GAB2, a scaffolding protein that links receptor signaling to effectors.

Gene Set Enrichment Analysis (GSEA) was performed to identify pathways of interest (Table 3-2). Example enrichment plots for WNT β-Catenin Signaling and KRAS Signaling are shown in Fig. 3-1. Of special interest, TGF-β signaling genes were enriched when proliferation was inhibited in wild type cells but not NF1-null cells, supporting the potential role of Neurofibromin in TGF-β signaling.

DISCUSSION

Our CRISPRa screen identified many potentially novel regulators of Neurofibromin which must be validated in additional cell lines. One of the most promising hits was Transforming growth factor beta receptor 2 (TGFBR2) which has not been directly linked to Neurofibromin. Additional insight into erythropoiesis could be gained by studying TGFBR2 and MAPK signaling. The TGF-β pathway could also be validated through treatment of wild type and NF1-null K562 cells with the TGFBR2 ligand TGF-β1. Wild type cells may differentiate while NF1-null cells continue to proliferate, with the caveat that receptor expression may be limiting. To circumvent this limitation, K562 cells overexpressing TGFBR2 which have previously been generated could be used[34].

The potential of certain GAPs, such as RASA2 and RASA3, to depend on the GAP Neurofibromin for function is a compelling hypothesis that should be investigated further. Ras-GTP levels should be measured with RASA2 and RASA3 overexpression

in the wild type and NF1-null K562 cells to confirm a decrease and no change, respectively. Different Ras isoforms should be tested individually to determine if RasGAPs may prefer K-, N-, or H-Ras. Due to feedback, NF1 loss could suppress RASA2 and/or RASA3 expression, so real-time PCR could be performed for expression in wild type and NF1-null K562 cells.

As expected for a Ras-dependent cell line, BCR and SOS1 overexpression increases proliferation in wild type K562 cells but decreases proliferation in NF1-null K562 cells. This data supports the hypothesis of oncogene overdose. NF1-null cells likely have elevated Ras-GTP and were selected for optimal proliferation. But the overexpression of BCR and SOS1 likely leads to super elevated Ras-GTP, which is detrimental to proliferation. This hypothesis should be validated with Ras-GTP assays in multiple cell lines. Additional hits should be validated to identify novel potential Ras pathway effectors and imatinib bypass mechanisms.

Our CRISPRa screen comparing wild type to NF1-null K562 cell lines generated many expected hits, validating the screen. Many of the hits were unexpected, which could lead to the discovery of novel Ras and/or Neurofibromin effectors. Additional follow-up is needed to validate hits with individual sgRNA in the K562 cell line and move beyond leukemia to other solid cancer cell lines. To strength the hits, a separate CRISPRi screen could be performed using the same library and protocol to generate a list of hits to cross reference as previously demonstrated[38]. We have already deleted NF1 in the K562 cell line containing the stable CRISPRi machinery in anticipation of this screen.

FIGURE 3-1

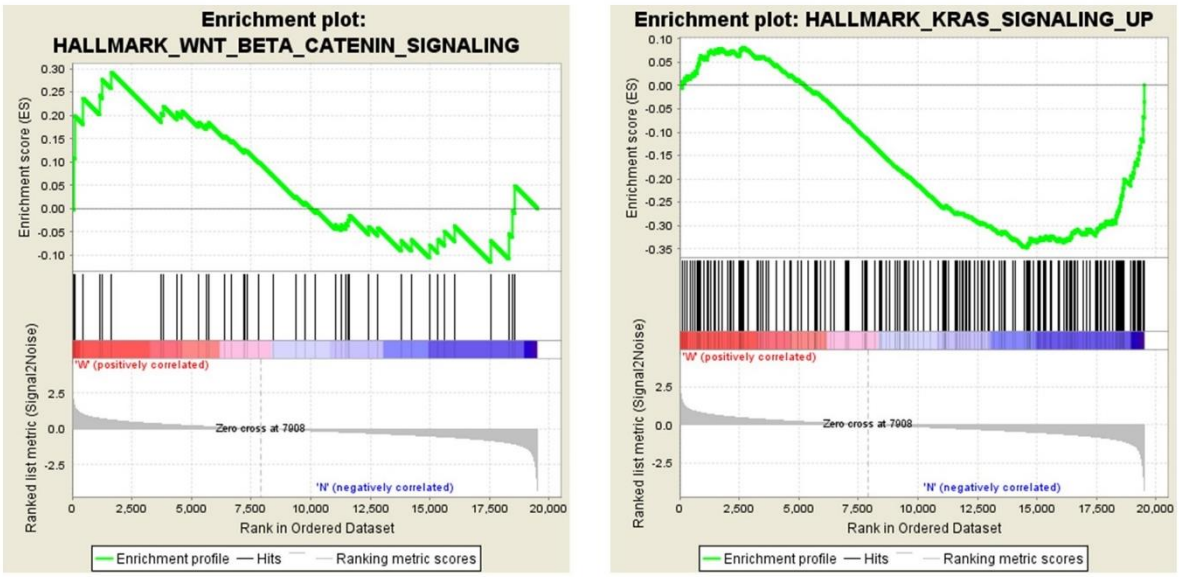

Figure 3-1. Representative GSEA plots from the CRISPRa screen.

TABLE 3-1

Overexpression inhibits Proliferation of K562 WT but not NF1-Null					Overexpression promotes proliferation of K562 WT but not NF1-Null				
Hit	Gene	WT	NF1-Null	Difference	Hit	Gene	WT	NF1-Null	Difference
1	TGFBR2	-3.72	0.08	3.81	1	BCR	2.97	-1.30	-4.28
2	DUSP9	-3.61	-0.31	3.30	2	SOS1	3.51	-0.42	-3.92
3	HNF4A	-4.03	-0.78	3.25	3	ABCG2	3.39	0.35	-3.04
4	UBASH3A	-3.46	-0.25	3.20	4	GAB2	2.28	-0.40	-2.68
5	TFAP2A	-3.94	-0.85	3.08	5	RTDR1	1.08	-1.13	-2.20
6	PRDM1	-3.74	-0.70	3.04	6	SLC6A14	2.49	0.34	-2.14
7	IKZF3	-3.85	-0.83	3.02	7	EP300	1.83	-0.31	-2.14
8	TP73	-3.86	-0.90	2.96	8	SLC1A3	1.55	-0.54	-2.09
9	BHLHE40	-2.99	-0.11	2.88	9	SLC4A7	2.31	0.32	-1.99
10	SAMD1	-3.73	-0.86	2.88	10	MAP2K3	2.94	1.00	-1.95
11	DBN1	-3.05	-0.22	2.82	11	GAREM	1.58	-0.30	-1.88
12	UBASH3B	-3.25	-0.52	2.74	12	SLC16A1	1.29	-0.48	-1.78
13	RASA3	-2.71	0.02	2.73	13	SLC38A3	2.19	0.50	-1.69
14	CBL	-2.94	-0.23	2.70	14	PDGFRB	1.62	-0.07	-1.69
15	DUSP9	-3.26	-0.62	2.64	15	PTP4A2	1.27	-0.41	-1.68
16	PTPN1	-2.79	-0.17	2.62	16	PDGFRA	1.37	-0.30	-1.67
17	PTPN9	-3.62	-1.02	2.60	17	SEPT5	0.50	-1.11	-1.61
18	RASA2	-2.88	-0.28	2.60	18	MAP2K6	1.12	-0.41	-1.53
19	POU3F2	-3.76	-1.17	2.59	19	SLC7A2	2.09	0.57	-1.53
20	WT1	-3.75	-1.17	2.58	20	MAML3	1.91	0.39	-1.52
21	CNN1	-3.50	-0.92	2.58	21	RCOR1	1.53	0.04	-1.49
22	PITX1	-2.97	-0.41	2.56	22	MAP3K4	1.37	-0.11	-1.48
23	TFAP2C	-2.61	-0.07	2.54	23	SP3	1.20	-0.28	-1.48
24	FOXO4	-3.11	-0.59	2.52	24	SRM	0.88	-0.59	-1.47
25	MSN	-2.89	-0.40	2.50	25	BCL9L	1.46	-0.01	-1.46
26	EOMES	-2.46	-0.01	2.45	26	BBIP1	1.27	-0.19	-1.46
27	CDKN2C	-3.15	-0.71	2.45	27	MYB	0.81	-0.64	-1.45
28	SPRED1	-2.78	-0.35	2.43	28	GP1BB	0.26	-1.16	-1.43
29	E2F7	-3.24	-0.81	2.42	29	CCNE1	1.66	0.23	-1.43
30	ZBTB1	-3.36	-0.97	2.40	30	SHOC2	1.09	-0.33	-1.41

Table 3-1. Hits from CRISPRa screen for novel Neurofibromin effectors.
All values are log2.

TABLE 3-2

Overexpressed promotes proliferation of K562 WT but not NF1-Null	Overexpression inhibits proliferation of K562 WT but not NF1-Null
HALLMARK_NOTCH_SIGNALING	HALLMARK_UV_RESPONSE_DN
HALLMARK_WNT_BETA_CATENIN_SIGNALING	HALLMARK_PANCREAS_BETA_CELLS
HALLMARK_DNA_REPAIR	HALLMARK_TGF_BETA_SIGNALING
	HALLMARK_HYPOXIA
	HALLMARK_MYOGENESIS
	HALLMARK_IL6_JAK_STAT3_SIGNALING
	HALLMARK_APICAL_JUNCTION
	HALLMARK_PI3K_AKT_MTOR_SIGNALING
	HALLMARK_ESTROGEN_RESPONSE_LATE
	HALLMARK_MTORC1_SIGNALING
	HALLMARK_TNFA_SIGNALING_VIA_NFKB
	HALLMARK_CHOLESTEROL_HOMEOSTASIS
	HALLMARK_KRAS_SIGNALING_UP
	HALLMARK_COMPLEMENT
	HALLMARK_G2M_CHECKPOINT
	HALLMARK_MITOTIC_SPINDLE
	HALLMARK_ESTROGEN_RESPONSE_EARLY
	HALLMARK_APOPTOSIS
	HALLMARK_P53_PATHWAY
	HALLMARK_PEROXISOME
	HALLMARK_KRAS_SIGNALING_DN
	HALLMARK_UV_RESPONSE_UP
	HALLMARK_E2F_TARGETS

Table 3-2. Gene Set Enrichment Analysis (GSEA) from the CRISPRa screen.

CHAPTER 4.

MATERIALS AND METHODS

Cell culture

HEK 293T cells were cultured in DMEM, high glucose (Thermo Fisher Scientific, 11965-084) and K562 were cultured in RPMI 1640 Medium (Thermo Fisher Scientific 118775-093). Media was supplemented with 10% FBS (Atlanta Biologicals S11550H) and Penicillin-Streptomycin (Thermo Fisher Scientific, 15140-122).

Plasmids and transient transfections

Spred1 plasmids were generated as previously described[8]. Additional mutants were generated by PCR-directed mutagenesis and confirmed by sequencing. Transient transfection in HEK 293T cells was performed with Lipofectamine 2000 Transfection Reagent (Thermo Fisher Scientific, 11668019) and Opti-MEM Reduced Serum Medium, with GlutaMAX Supplement Thermo Fisher Scientific, 51985091) following manufacturer's recommendation. Fresh media was added 16 hours after transfection and cells were lysed the follow day in lysis buffer containing 20mM Tris (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1% Triton-X100, 1 mM DTT, protease (Sigma Aldrich, P8340) and phosphatase inhibitors (Sigma Aldrich, P0044 and P5726). Immunoprecipitations were performed with 20 µl of EZview Red Anti-Flag M2 Affinity Gel clone M2 (Sigma-Aldrich, F2426).

Ras GTP and Fractionation Assays

Ras-GTP assays were performed with the Ras Pull-down Activation Assay Biochem Kit (Cytoskeleton, BK008). Cells were serum starved for 16 hours and stimulated with 10 ng/ul EGF (Thermo Fisher Scientific, PHG0311). Fractionation was performed using NE-PER Nuclear and Cytoplasmic Extraction Reagents (Thermo Fisher Scientific, 78835)

Chemical Inhibitors and siRNA

CDK inhibitors AZD5438 (Selleckchem, S2621), Dinaciclib (SCH727965) (Selleckchem, S2768), Ro-3306 (Enzo Life Sciences, ALX-270-463-M001), Purvalanol A (Abcam S7747), JNJ-7706621 (Selleckchem, S1249), Olomoucine, N9-Isopropyl- (ALX-270-397-M001), Alsterpaullone (Enzo Life Sciences, ALX-270-275-M001), PD0332991 (Selleckchem, S1116), SNS-032 (BMS-387032) (Selleckchem, S1145). EGFR inhibitor AZD9291 (Selleckchem, S7297), PKC inhibitor, Sotrastaurin (Selleckchem, S2791), PI3K inhibitor GDC-0941 (Selleckchem, S1065), p38 inhibitor LY2228820 (Selleckchem, S1494), JNK inhibitor JNK-IN-8 (Selleckchem, S4901), GSK3β inhibitor CHIR-99021 (Selleckchem, S2924), RAF inhibtor LY3009120 (Selleckchem, S7842). All siRNA are Human SMARTpool: ON-TARGETplus from GE Dharmacon. CDK1 (L-003224-00-0005), CDK2 (L-003236-00-0005), CDK5 (L-003239-00-0005), CDK9 (L-003243-00-0005) Non-targeting (D-001810-10-05). Lipofectamine 3000 and Opti-MEM were used for transfection as above.

Antibodies

Flag 1:1,000 (Sigma, V8137), Spred1 1:1,000 (Cell Signaling, 94063), α/β-Tubulin (Cell Signaling 2148), EGFR 1:200 (Santa Cruz Biotech, 1005, SC-03), Neurofibromin (Santa Cruz Biotech, sc-67), CDK1 1:200 (Santa Cruz Biotech, sc-54), CDK2 1:200 (Santa Cruz Biotech sc-6248), CDK5 1:1,000 (Cell Signaling, 2506), CDK9 1:1,000 (Cell Signaling 2316), RB 1:1,000 (Cell Signaling 9309), Ras 1:200 (Cytoskeleton, AESA02), β-Actin 1:10,000 (Sigma-Aldrich, A5441).

K562 Competition

Spred1 was cloned into the pMIG (Addgene, #9044) MSCV-IRES-GFP vector. Retrovirus was generated using the VSV-G envelope expressing plasmid pMD2.G (Addgene #12259) and the packaging plasmid gag/pol (Addgene #14887). HEK 293T cells were transfected with Lipofectamine 3000 Transfection Reagent (Thermo Fisher Scientific, L3000015) as described above. 16 hours after transfection fresh media was added containing ViralBoost Reagent (Alstem, #VB1000). 24 hours later virus was filtered (0.45 µM), polybrene (Sigma Aldrich, H9268) at 4 µg/ml. was added, and K562 cells were infected by spinfection at 2,000 RPM for 1 hour. GFP positive cells were analyzed on the Sony Cell Sorter SH900Z.

Spred1 Mass Spectrometry

One 10 cm plate of HEK 293T cells per condition was transfected with plasmids as above. Immunoprecipitations were carried out using 60 µl of anti-FLAG antibodies coupled to magnetic beads (Anti-FLAG M2 Magnetic Beads, Sigma, M8823). Rotating

at 4°C for 2 hours and washed three times with TME. Two final washes with ice-cold 20 mM TrisHCl pH8 + 2 mM CaCl2 were carried out on ice. The beads were then resuspended in 9 µL of 20 mM Tris-HCl pH 8.0. The proteins were reduced by adding 0.4 µL of 100 mM DTT and incubating at room temperature for 30 min with agitation and alkylated by adding 0.6 µL of 100 mM iodoacetamide and incubating at room temperature for 10 min with agitation. Digestion with 500 ng of trypsin (Sigma Trypsin Singles, T7575) was carried out at 37°C overnight with mild agitation. The digest was stopped by adding formic acid to a final concentration of 2%. The samples were desalted using ZipTip u-C18 pipette tips (Millipore) according to the manufacturer's protocol and reconstituted in 12 µL of 0.1% formic acid.

Five µL of each digest were analyzed by liquid chromatography tandem mass spectrometry (LC-MS/MS) on a Q Exactive Plus instrument (Thermo Fisher Scientific) online with Waters NanoAcquity UPLC system (Waters). Reversed phase chromatography was performed on a 15 cm silica-C18 EasySpray column (Thermo Fisher Scientific) at 45°C with a binary buffer system (Buffer A = 0.1% formic acid in water; Buffer B = 0.1% formic acid in acetonitrile) and a flow rate of 400 nL/min. The sample was loaded at 2% B for 20 min followed by a 2-60% B gradient over 60 min, followed by a brief wash at 80% B and equilibration at 2% B. The Q Exactive Plus instrument was operated in Full-MS/ddMS2 mode with one survey scan (350-1500 m/z, R=70,000 at 200 m/z, AGC target of 3e6), followed by up to 10 data-dependent HCD MS2 scans (AGC target of 5e4, max IT 120 ms, R=17,500 at 200 m/z, isolation window 4.0 m/z, NCE 25%, 4% underfill ratio, and 10 s dynamic exclusion). Raw data files were converted to peak list files using Proteome Discoverer v. 1.4 (Thermo Fisher Scientific)

and searched using Protein Prospector[39,40] version 5.14.0 against human SwissProt database[41] downloaded on 07/29/2013 and corresponding random concatenated decoy database with default “ESI-Q-high-res” parameters, including up to two allowed missed cleavage sites, Carbamidomethyl-C constant modification, default variable modifications plus phosphorylation at STY, up to 3 modifications per peptide, and 20 ppm precursor mass and fragment mass accuracy. False discovery rate of <1% was used as the cutoff for peptide expectation values. Quantitation of relative phosphorylation at the S105 site in SPRED1/2 was carried out in Skyline v 3.0[42] 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.

In vitro kinase assays

Kinexus Bioinformatics performed the *in vitro* kinase assay using Spred1(S105) peptide (KKGLAFQSPADAR) at 500 μM with 93 candidate kinases. JNK1, JNK2, JNK3, P38alpha, P38beta, P38delta, P38gamma, GSK3alpha, GSK3beta, CK1alpha1, CK1alpha1L, CK1delta, CK1epsilon, CK1gamma1, CK1gamma2, CK gamma3, CK2alpha1, CK2alpha2, CDK1/CyclinA1, CDK1/CyclinA2, CDK1/CyclinB1, CDK2/CyclinA1, CDK2/CyclinA2, CDK2/CyclinE1, CDK2/CyclinO, CDK3/CyclinE1, CDK4/CyclinD1, CDK4/CyclinD3, CDK5/p25, CDK5/p35, CDK6/Cyclin D1, CDK6/CyclinD3, CDK7/CyclinH1/MNAT1, CDK9/CyclinK, CDK9/CyclinT2, MAK, ICK, PCTK1, (CDK16)/Cyclin Y, PCTK (CDK17)/CyclinY, PFTK (CDK14)/Cyclin Y, NLK, CHK1, CHK2, AMKK2, SRPK2, RSK1, RSK2, RSK3, RSK4, COT, DYRK2, GRK1, RAF1, BRAF, ERK1, ERK2, ERK5, MEK1, MEK2, MEK5, MEK6, AKT1, AKT2, AKT3,

ALK1, ALK2, PKCalpha, PKCbeta I, PKCbeta II, PKCdelta, PKCepsion, PKCeta, PKCgamma, PKCiota, PKCmu, PKCnu, PKCtheta, PKCzeta, IKKalpha, IKKbeta, IKKepsilon, CAMK1, CAMK1beta, CAMK1delta, CAMK1gamma, CAMK2alpha, CAMK2beta, CAMK2delta, CAMK2gamma, CAMK4, CAMKK1, CAMKK2, and CASK. A radioisotope assay format was used for profiling evaluation of the kinase activities towards their substrates and all assays are performed in a designated radioactive working area. Protein kinase assays were performed at ambient temperature for 20-40 minutes (depending on the kinase) in a final volume of 25 µl according to the following assay reaction recipe:

5 µl of diluted active protein kinase (~10-50 nM final protein concentration in the assay)

5 µl of assay solution of peptide substrate

10 µl of kinase assay buffer

5 µl of [g-33P] ATP (250 µM stock solution, 0.8 µCi)

The assay was initiated by the addition of [g-33P] ATP and the reaction mixture incubated at ambient temperature for 20-40 minutes, depending on the protein kinase tested. After the incubation period, the assay was terminated by spotting 10 µl of the reaction mixture onto a multiscreen phosphocellulose P81 plate. The multiscreen phosphocellulose P81 plate was washed 3 times for approximately 15 minutes each in a 1% phosphoric acid solution. The radioactivity on the P81 plate was counted in the presence of scintillation fluid in a Trilux scintillation counter.

For the full length Spred1 in vitro kinase assay Spred1 (MyBioSource, MBS1321540) was purchased and resuspended to 25 µM in MOPS, pH 7.4. The kinase

assay was performed as above except cold ATP was used. Phosphorylation of Spred1 was analyzed by LC-MS/MS as above.

CRISPRa Screen

Our CRISPRa screen was performed in K562 cells stability expressing SunTag-VP64 constructs[33]. We generated NF1-null clones with CRISPR, single cell cloned, and screened by PCR. Wild type K562 and K562 NF1-null were infected with the whole genome hCRISPRi-v2 library[33] which contains 10 guides per gene. Library was packaged into lentivirus using dR8.91 and pMD2.G as above. Following infection cells were expanded for 48 hours, selected with puromycin (2 µg/mL) for 72 hours, time 0 cell pellets were frozen, and cells were split into duplicates for a total of 4 spinner flasks (Bellco, 1965-83005) with Dura-Mag 5 Position Magnetic Stirrer (PAW Bioscience, CLS-4100-06) at 25 RPM. Cells were maintained in 2 liters of RPMI 1640 Medium with 10% FBS and Penicillin and Streptomycin at less than $1x10^6$/mL in increasing concentration imatinib to increase selective pressure, from 25-100nM, for approximately 12 cell doublings. During all stages of the screen >1000x coverage was maintained. Cellecta (Mountain View, CA) performed the DNA library prep, NGS with >100M reads, and analysis. Gene Set Enrichment Analysis (GSEA) was performed with software from the Broad Institute.

CHAPTER 5.

CONCLUSION AND FUTURE PERSPECTIVES

The primary goal of this dissertation was to understand how cancer cells sustain Ras signaling in the presence of negative feedback. Neurofibromin is a major negative regulator of Ras signaling mediated by increased Spred1 expression and localization to the plasma membrane. Despite the importance of the Spred1-Neurofibromin complex, it's regulation is unknown. We hypothesized oncogenic RTKs could modulate Spred1-Neurofibromn binding and identified serine 105 on Spred1 which when phosphorylated disrupts Neurofibromin binding. Serine 105 is located on the hydrophobic surface of Spred1, which may be the Neurofibromin binding interface.

Identification of CDK1 as the Spred1 serine 105 kinase provides an exciting therapeutic target. CDK inhibitors have shown success in the clinic, exemplified by approval of the CDK4/6 inhibitor Palbociclib in breast cancer in 2017. CDK1 inhibitor development has been limited due to potential toxicity concerns, but most inhibitors are not selective for CDK1. Therefore, generation of a more selective CDK1 inhibitor could be effective at treating $EGFR^{L858R}$-driven cancers. Indeed, preliminary data shows $EGFR^{L858R}$ cancer cell lines are sensitive to CDK1 inhibitors. To ensure the expected mechanism of action in response to CDK1 inhibition Spred1-Neurofibromin binding and Ras-GTP levels need to be measured. Phospho-Spred1(S105) could be measured as a potential biomarker for cancer cells likely to respond to CDK1 inhibition. LC-MS/MS is a low throughout method for screening, so a phospho-Spred1(S105) antibody would be ideal. We have tried twice to generate a rabbit polyclonal antibody with Kinexus

Bioinformatics and Eurogentec using different Spred1(S105) peptides but both antibodies are not specific to phospho-Spred1(S105).

Spred1 was also shown to bind EGFR and HER2 by mass spectrometry, indicating the potential for receptor specific recruitment of Neurofibromin and Ras-GTP inhibition. Indeed, such local Ras-GTP inhibition could occur at c-kit, a previously described RTK Spred1 binding partner[43]. It is unknown if Spred1 binds these receptors through the Kit-binding domain (KBD) or another domain. Future experiments should identify specific receptors, wild-type and oncogenic, which Spred1 may bind and direct Neurofibromin to for local Ras-GTP inhibition. Our lab has generated the following RTKs to test for Spred1 binding by co-IP and western blot: EGFR, $EGFR^{L858R}$, CKIT $CKIT^{D816V}$, FLT3, $FLT3^{ITD}$, $FLT3^{D835Y}$, PDGFRα, $PDGFR\alpha^{D842V}$, HER2 $HER2^{V654E}$, MET, BCR-ABL, and CSF1R.

Oncogenic RTK-driven cancer cells may disrupt Spred1-Neurofibromin negative feedback through phosphorylation of Spred1(S105). However, oncogenic RTK expression leads to Spred1 phosphorylation on multiple sites with unknown consequence as shown in the phospho-Spred1 heatmap, adding an additional layer of complexity. Phosphorylation on some sites may alter localization and function, negating the Neurofibromin binding effects of Spred1(S105) phosphorylation. Phospho-mimetic and -deficient mutants should be generated at these sites to see if Neurofibromin binding and Ras-GTP levels are altered.

Using an unbiased, whole genome CRISPRa screen we identified novel potential regulators of Neurofibromin. The screen will be validated with individual guide RNAs and in additional cell lines, including expansion to solid tumor cell lines. Expected hits

like Spred1 and BCR give us a high degree of confidence in the robustness of our hits. In addition to identifying potential Neurofibromin effectors, all hits are expected to modulate the Ras pathway. Of particular interest is TGFBR2 of the TGF-β pathway, the number one hit. Understanding more about the Neurofibromin tumor suppressor beyond GAP activity could help lead to novel cancer and Neurofibromatosis type 1 therapeutic targets.

Our findings into Spred1 tumor suppressor biology provide one potential mechanism by which oncogenic signaling disrupts negative feedback to sustain constitutive Ras/MAPK signaling. Furthermore, this work identifies CDK1 as a novel anticancer drug target for restoring Neurofibromin-mediated inhibition of Ras-GTP. Our unbiased, whole genome CRISPRa screen has potentially identified novel effectors in Neurofibromin which will increase our understanding of this important tumor suppressor and Spred1 effector.

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