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14. ABSTRACT Juvenile myelomonocytic leukemia (JMML) is a myeloproliferative neoplasm (MPN) that sometimes progresses to acute myeloid leukemia (AML). The <i>NF1</i> gene is frequently inactivated in NF1 patients who develop either JMML or AML. However, AML is a more aggressive malignancy that contains multiple genetic alterations that cooperate with <i>NF1</i> loss. We have characterized MPN and AML in <i>Nf1</i> mutant mice and have investigated mechanisms of drug responses and resistance. Our studies of MEK inhibitors in <i>Nf1</i> mutant mice unexpectedly showed that cooperating mutations that are acquired in AML increase the dependence of these cells on Raf/MEK/ERK signaling. However, resistant AML clones rapidly emerged <i>in vivo</i> . The goal of this project is to deploy mouse models of AML to test the requirement for NF1 GTPase activating protein (GAP) activity in cancer maintenance and to develop a preclinical paradigm for combining conventional and targeted anti-cancer agents <i>in vivo</i> . Major accomplishments of this project include: (1) developing a robust system for modulating gene expression in primary leukemia cells <i>in vivo</i> ; (2) constructing versatile vectors for restoring GAP activity in primary cells; (3) generating data supporting the hypothesis that AML cells remain dependent on <i>Nf1</i> inactivation; (4) implementing protocols for treating mice with CPX-351; and (5) performing <i>in vivo</i> studies of drug combinations in <i>Nf1</i> mutant AML.					
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INTRODUCTION

Malignant peripheral nerve sheath tumor (MPNST), high-grade astrocytoma, and acute myeloid leukemia (AML) are aggressive cancers that cause premature mortality in patients with NF1. Despite recent advances in understanding the molecular genetics and underlying biology, current therapies for these malignancies remain ineffective.

Juvenile myelomonocytic leukemia (JMML) and other myeloproliferative neoplasms (MPNs) progress to acute myeloid leukemia (AML) in a substantial proportion of patients. The *NF1* gene is frequently inactivated in cases of JMML or AML arising in children with NF1. AMLs also contain additional genetic alterations that cooperate with antecedent *NF1* loss. We have extensively characterized MPN and AML in *Nf1* mutant mice and have investigated mechanisms of drug response and resistance. Our studies of MEK inhibitors in *Nf1* mutant mice with MPN and AML showed that cooperating mutations that are acquired as MPN progress to AML unexpectedly increased the dependence of these cells on Raf/MEK/ERK signaling. However, drug resistant AML clones emerged rapidly *in vivo*. We were able to utilize this novel experimental system to validate genes that cause resistance to MEK inhibitors. By contrast, hematopoietic cells from *Nf1* mutant mice with MPN are less dependent on MEK for survival. Interestingly, while treatment with MEK inhibitors do not eliminate *Nf1* mutant cells in mice with MPN, treatment nonetheless induces remarkable hematologic improvement. Our controlled preclinical trials in *Nf1* mutant mice stimulated clinical evaluation of MEK inhibitors for plexiform neurofibroma, and we expect to launch a national clinical trial of the potent and selective MEK inhibitor trametinib in JMML within the next year. Our studies in *Nf1* mutant mice with MPN also informed the correlative biologic studies embedded in this trial. On the other hand, our studies of *Nf1* mutant mice with AML support administering different targeted inhibitors together and/or combining targeted and conventional cytotoxic drugs to treat advanced cancers in NF1 patients.

Our overall goal is to deploy genetically accurate mouse models of NF1-associated AML to develop a preclinical paradigm for combining conventional and targeted anti-cancer agents *in vivo*, and to uncover mechanisms of drug response and resistance.

KEYWORDS

Neurofibromatosis type 1 (NF1), neurofibromin, acute myeloid leukemia, cancer, targeted and conventional anti-cancer drugs, combination treatment, drug resistance, MEK inhibitors

ACCOMPLISHMENTS

Major Goals

The Technical Objectives of this project are:

- (1) To restore neurofibromin GAP activity in murine AMLs to ask if this inhibits the growth of advanced cancers that are initiated by *Nf1* inactivation. We hypothesize that many cancers will remain dependent on hyperactive Ras signaling, but that some will evolve mechanisms that bypass the requirement for neurofibromin expression.
- (2) To develop treatment regimens employing both frontline chemotherapy and MEK inhibitors to treat a heterogeneous collection of primary murine AMLs that were initiated by inactivating the *Nf1* gene. We hypothesize that this will uncover synergistic inhibitory effects and will provide a rationale for testing this general approach in human patients with advanced NF1-associated cancers.

(3) To identify and validate genes and pathways underlying anti-cancer drug sensitivity and resistance in *Nf1* mutant AML. We hypothesize that these experiments will uncover novel mechanisms of drug resistance that will inform the design of clinical trials.

This section opens with a summary of background information and preliminary studies that informed this work, and then concisely summarizes our progress under each objective.

Background and Preliminary Studies

Tumorigenesis in Neurofibromatosis Type 1 (NF1). NF1 is a multi-system dominant familial cancer syndrome caused by germ line mutations in the *NF1* tumor suppressor gene. *NF1* encodes a GTPase activating protein called neurofibromin that negatively regulates Ras signaling by accelerating the hydrolysis of active Ras-GTP to inactive Ras-GDP (1, 2). Clinical manifestations of NF1 include pigmented skin lesions, skeletal dysplasia, learning disabilities, and a propensity to develop benign and malignant tumors. The malignancies seen in NF1 patients include astrocytoma, malignant peripheral nerve sheath tumor (MPNST), pheochromocytoma, and childhood myeloid leukemia. A consistent feature of NF1-associated tumors is somatic loss of the normal *NF1* allele, which is consistent with its role as a tumor suppressor gene and with the biochemical function of neurofibromin as a negative regulator of Ras signaling (1, 2). Patients with NF1 who are cured of a primary cancer are at increased risk of developing treatment-induced secondary malignancies (3-6), and heterozygous *Nf1* mutant mice are predisposed to a spectrum of radiation-induced cancers (7, 8). Together, the benign neoplasms and more aggressive malignancies that develop in NF1 patients are a substantial cause of morbidity and premature mortality. There are currently no effective, mechanism-based therapies for any of the tumors that arise in persons with NF1.

Myeloid Malignancies in NF1. Children with NF1 are at greatly increased risk of developing juvenile myelomonocytic leukemia (JMML), an aggressive myeloproliferative neoplasm (MPN) characterized by over-production of differentiated myeloid lineage cells that show extensive tissue infiltration (9, 10). The median survival of JMML patients is <1 year without hematopoietic stem cell transplantation (HSCT), and the overall cure rate is ~50% after HSCT (11). Children with NF1 who develop JMML show distinct clinical features including older age at diagnosis and worse outcome (11). Our studies of JMML proved that *NF1* functions as a tumor suppressor gene in hematopoietic cells (12, 13), and provided the first direct evidence of deregulated Ras signaling in primary cancer cells from NF1 patients (14). The association of NF1 with JMML also implicated hyperactive Ras in the pathogenesis of this MPN, and our group and other investigators went on to discover germline and somatic mutations in multiple components of Ras signaling networks in JMML patients (15-20). Despite the routine use of HSCT in JMML, up to 30% of patients progress to acute myeloid leukemia (AML). Evolution to AML may be associated with new cytogenetic changes such as monosomy 7 (21). In addition, myeloid malignancies are among the most common treatment-induced cancers diagnosed in children and adults with NF1 (3-5). Somatic *NF1* mutations are also increasingly recognized in patients with AML who do not have neurofibromatosis (22).

Modeling NF1-Associated Myeloid Malignancies in the Mouse. We collaborated with Dr. Luis Parada to generate *Mx1-Cre, Nf1^{fllox/fllox}* mice, and injected them with polyinosinic-polycytidilic acid (pI-pC) to inactivate *Nf1* in the hematopoietic compartment (23). *Mx1-Cre, Nf1^{fllox/fllox}* mice develop a MPN that closely models JMML between 5 and 6 months of age, which is characterized by hunching, an abnormal gait, and a disheveled appearance. Half of the animals die of MPN by 7.5 months. Importantly, however, this MPN does not spontaneously progress to AML (23).

Retroviral insertional mutagenesis (RIM) is a powerful strategy for generating hematologic cancers in mice and for identifying genes that contribute to leukemogenesis (24-26). We have made extensive use of MOL4070LTR, a replication competent ecotropic murine leukemia virus that induces myeloid leukemia (27). By infecting *Nf1* mutant mice with MOL4070LTR, we generated a diverse collection of primary AMLs that model the multi-step pathogenesis of advanced human cancers. Our ability to transplant primary leukemia cells into irradiated recipient mice provides a powerful system for testing experimental agents and for elucidating mechanisms of drug response and resistance (28). Specific advantages of this approach include: (1) primary cancers are treated in immunocompetent mice; (2) retroviral integration patterns can be used to track the emergence of drug resistant clones; and, (3) relapsed leukaemia cells can be re-transplanted to verify intrinsic resistance and test alternative therapies (28).

Targeted Therapies for Tumors Characterized by Hyperactive Ras Signaling. The adaptive responses of cancer cells to the stress imposed by acquired mutations increases their dependency on aberrant signaling for growth and survival. This process is called “oncogene addiction” (29), and likely explains the *in vivo* therapeutic index of targeted and conventional anti-cancer agents. It is particularly prominent in cancers in which somatic mutations result in the production of activated kinases (29, 30). However, the most common cancer-associated mutations do not encode proteins with aberrant gain-of-function biochemical activities that are as readily “druggable”. For example, *NF1* inactivation abrogates neurofibromin function. The extensive evidence suggesting that elevated levels of Ras-GTP plays a central role in tumorigenesis in *NF1* suggests that inhibiting activated Ras is a logical therapeutic alternative. However, Ras proteins are exceedingly difficult targets for drug discovery because of their very high affinity for guanine nucleotides and structural features of the phosphate-binding (P) loop and Ras/GAP interface (31, 32). Efforts to target post-translational modifications in Ras through the use of farnesyltransferase inhibitors in the 1980s and early 1990s failed due to the existence of alternate processing enzymes for N-Ras and K-Ras (33-35).

Given the inherent difficulties in directly targeting the Ras/GAP molecular switch, small molecule inhibitors of Ras effectors such as Raf, MEK and Akt have been developed and tested in patients with advanced cancers. Of these, the B-Raf inhibitor vemurafenib and the MEK inhibitor trametinib have been approved by the Food and Drug Administration for the treatment of melanomas with *BRAF* mutations. An important limitation of applying this strategy to cancers with mutations in *KRAS*, *NRAS*, or *NF1* is that Ras-GTP activates a complex network of downstream molecules, and it is uncertain which of these effectors contribute to tumor formation and maintenance in different cell types. However, genetic analysis of human cancers demonstrating frequent somatic mutations in components of the Raf/MEK/ERK and phosphatidylinositol 3' kinase/Akt/mammalian target of rapamycin (PI3K/Akt/mTOR) kinase effector cascades as well as studies of tumors from patients with *NF1* and *Nf1* mutant mice showing aberrant activation of these pathways support the potential therapeutic benefit of PI3K, mTOR, and MEK inhibitors (14, 28, 36-39). We have successfully executed preclinical trials in accurate mouse models of early stage and advanced hematologic cancers driven by *Nf1* inactivation or oncogenic *Kras* expression (28, 33, 40-43), and continue to utilize this paradigm to pursue the long-term goal of implementing better therapies for patients with JMML, AML, and other blood cancers. We have utilized funds provided by this award to advance these studies.

Response and Resistance to MEK Inhibition in *Mx1-Cre Nf1^{flox/flox}* Mice with AML. To model the progression of JMML to AML seen in human patients, we previously injected neonatal *Mx1-Cre, Nf1^{flox/flox}* mice and control *Nf1^{flox/flox}* littermates with MOL4070LTR retrovirus and pI-pC (28). In this large-scale experiment, *Mx1-Cre, Nf1^{flox/flox}* mice demonstrated an increased incidence of AML as well as reduced latency. These AMLs are biologically aggressive and are readily transplantable into

recipient mice given a sublethal dose of irradiation (450 cGy).

Our studies of the “first generation” MEK inhibitor CI-1040 showed that 25-50 μ M of this drug abrogated CFU-GM colony formation from *Mx1-Cre*, *Nf1^{flox/flox}* bone marrow. Importantly, however, there was no therapeutic index as CFU-GM growth from wild-type (WT) bone marrow was inhibited at similar concentrations (28). By contrast, blast colony growth from *Mx1-Cre Nf1^{flox/flox}* AML bone marrow was abrogated at much lower drug concentrations of CI-1040. These unexpected data suggested that mutations that are acquired during progression from MPN to AML make leukemic cells more dependent on Raf/MEK/ERK signaling. To test this hypothesis *in vivo*, we transplanted *Nf1* mutant AMLs and treated the recipients with CI-1040 or with PD0325901 (PD901), a MEK inhibitor with enhanced pharmacokinetic properties (28). Both drugs induced clinical remissions and markedly prolonged survival. However, all of the mice eventually relapsed and died of AML despite continued treatment. Resistant leukemias were remarkably less sensitive to MEK inhibitors *in vitro* than the corresponding parental AMLs, and did not respond to treatment in secondary recipients. Drug resistance was not due to acquired *Mek1* mutations, and MEK remained sensitive to biochemical inhibition by CI-1040 or PD901. Importantly, analysis of clinical evolution by Southern blotting revealed recurrent novel retroviral integrations in three resistant clones (6537R, 6554R1, and 6554R2) that emerged in multiple independent recipient mice transplanted with primary AMLs 6537 and 6554. This observation provided compelling evidence that the resistant clones were present at undetectable levels in the primary AML, and is consistent with recent studies of human leukemia (44-47). We exploited a shotgun cloning strategy (48) to identify novel retroviral integrations in AMLs 6537R, 6554R1, and 6554R2. This analysis implicated p38 α and guanine nucleotide exchange factors of the Ras-GRP family in resistance to MEK inhibitors, which we went on to functionally validate. These studies provide “proof of principle” that *in vivo* treatment with targeted agents followed by molecular analysis of paired sensitive/resistant leukemias is a potent and unbiased strategy for monitoring clonal evolution in response to targeted anti-cancer agents and for uncovering genes that underlie “off target” resistance (28). We recently extended this general paradigm from *Nf1* mutant AML to investigate MEK and PI3K inhibitors in mouse models of T lineage acute lymphoblastic leukemia (T-ALL) characterized by aberrant Ras signaling (40, 43). We deployed these established methodologies and our novel panel of well characterized mouse *Nf1* deficient AMLs to pursue the aims of this application.

In contrast to the dramatic (though transient) regression of *Nf1* mutant AMLs upon treatment with CI-1040 or PD901, we observed no hematologic improvement in *Mx1-Cre*, *Nf1^{flox/flox}* mice with a JMML-like MPN that received CI-1040. Importantly, however, a subsequent study revealed dramatic improvement in response to PD0325901, which correlated with sustained *in vivo* Raf/MEK/ERK pathway inhibition (42). Interestingly, treatment did not eradicate *Nf1* mutant cells from the bone marrow, but instead promoted a normal pattern of growth and differentiation *in vivo*. These preclinical studies of *Nf1* mice with MPN and parallel data in *Mx1-Cre*, *Kras^{G12D}* mice (41) provide a strong rationale for testing MEK inhibition in JMML patients with correlative pharmacokinetic analysis and molecular investigation of mutant allele burden to assess mechanisms of response.

PD901 and trametinib are chemically related allosteric inhibitors of MEK. Despite this, there were compelling theoretical and practical reasons to fully evaluate trametinib in mouse models of NF1-associated cancers. First, whereas PD901 has excellent central nervous system (CNS) penetration in animals (49), trametinib does not accumulate in the brain (50). Since many NF1-associated cancers affect young children, minimizing potential CNS complications is highly desirable. Second, trametinib is regarded as “best in class” among the current generation of MEK inhibitors, and is approved by the FDA for the treatment of advanced melanoma. Finally, GlaxoSmithKline has an active clinical development plan and timeline for trametinib in pediatric patients (including children with NF1), and has a liquid formulation available, which is not true for PD901.

To accelerate clinical translation, we evaluated trametinib in *Mx1-Cre, Nf1^{fllox/fllox}* mice with MPN. These studies are not part of the scientific goals of this project, and were funded by a grant from the Children's Tumor Foundation. We summarized our results in the 2014 Annual Progress Report for this award as they are immediately relevant to the goals of the CDMRP NF Program, and will impact NF1 patients with both JMML and AML. We are pleased to report that we anticipate opening a clinical trial of trametinib in children with JMML, AML, and other advanced hematologic cancers in the next few months.

Accomplishments Related to the Goals of this Project

Technical Objective (Aim 1): Restoring neurofibromin GAP activity in primary AMLs

Primary *Nf1* mutant AMLs are transplantable into sublethally irradiated WT mice, and we exploited this property to perform the preclinical studies of targeted inhibitors described above. As summarized in previous annual Progress Reports, the ability to manipulate AML cells *ex vivo* before transplanting them presents a unique opportunity to ask how restoring neurofibromin GAP activity modulates the proliferation and survival of primary *Nf1*-deficient cancer cells. Pursuing this question required developing efficient methods for expressing exogenous genes in AML cells as well as overcoming potential challenges related to non-physiologic levels of GAP expression from a transgenic promoter. We therefore first focused on developing and validating a lentiviral delivery system for modulating gene expression in human AML cell lines with somatic *NRAS* mutations and in primary murine AMLs that we generated in *Nras* mutant mice (51). Advantages of initially pursuing this strategy include the small size of the *NRAS/Nras* genes and the availability of excellent N-Ras antibodies. In data presented in our 2013 Progress Report, we showed that multiple independent shRNA constructs inhibited the growth of human *NRAS* mutant AML cell lines *ex vivo* and of primary murine *Nras* AML cells *in vivo*, but had no effects on the growth of AML cells with other dominant signaling mutations (52).

In studies presented in the 2014 Progress Report for this CDMRP award, we generated lentiviral vectors expressing either the wild-type neurofibromin GAP related domain (GRD) or a mutant GRD containing a R1276P amino acid substitution under the control of the murine stem cell virus (MSCV) promoter. The R1276P mutation alters a critical arginine that is essential for accelerating Ras GTPase activity (32). Importantly, the substitution was reported in a family with classic NF1 and was previously shown to markedly impair GAP activity (53). We engineered these constructs to encode a N-terminal hemagglutinin (HA) tag that can be used to directly assess protein expression by Western blotting and for immunoprecipitation experiments to measure GAP activity in transduced cells using a ³²P release assay from labeled recombinant Ras-GTP (54, 55). We also cloned a C-terminal "self-cleaving" T2A peptide into this vector followed by genes encoding either green (GFP) or red (mCherry) fluorescent proteins for monitoring expression of the GRD in living cells (**Fig. 1a**). Utilization of the T2A peptide cleavage site establishes an internal reference that insures that the measured fluorescence is directly proportional to the level of GRD expression *in vivo*.

We confirmed expression of the HA-tagged GRD and GRD R1276P proteins by Western blot in NIH 3T3 cells (**Fig. 1b**). Interestingly, multiple experiments performed in different cell contexts suggest that high levels of the wild-type GRD expression are not tolerated presumably due to super-physiologic levels of GAP activity. By contrast, transduced cells express high levels of the mutant HA-GRD R1276P. To test the functional activity of the GAP construct in a disease-relevant context, we overexpressed the HA-GRD and mutant HA-GRD R1276P in a panel of human AML cell lines harboring oncogenic *FLT3* (MOLM-14 and MV-11) or *NRAS* (OCI-AML3 and THP-1) mutations. Following infection, we sorted GFP positive cells to purity, plated cells at equal density, and assessed cell viability after 4 and 7 days of growth in liquid culture (**Fig. 1c**). Human AML cell lines harboring *NRAS* mutations were insensitive to exogenous GRD expression, which is consistent with the known

resistance of oncogenic N-Ras proteins to GAPs. By contrast, AML cells with activating mutations in *FLT3*, which encodes a receptor tyrosine kinase that signals through Ras, showed reduced growth in cells expressing the HA-GRD construct (**Fig. 1c**).

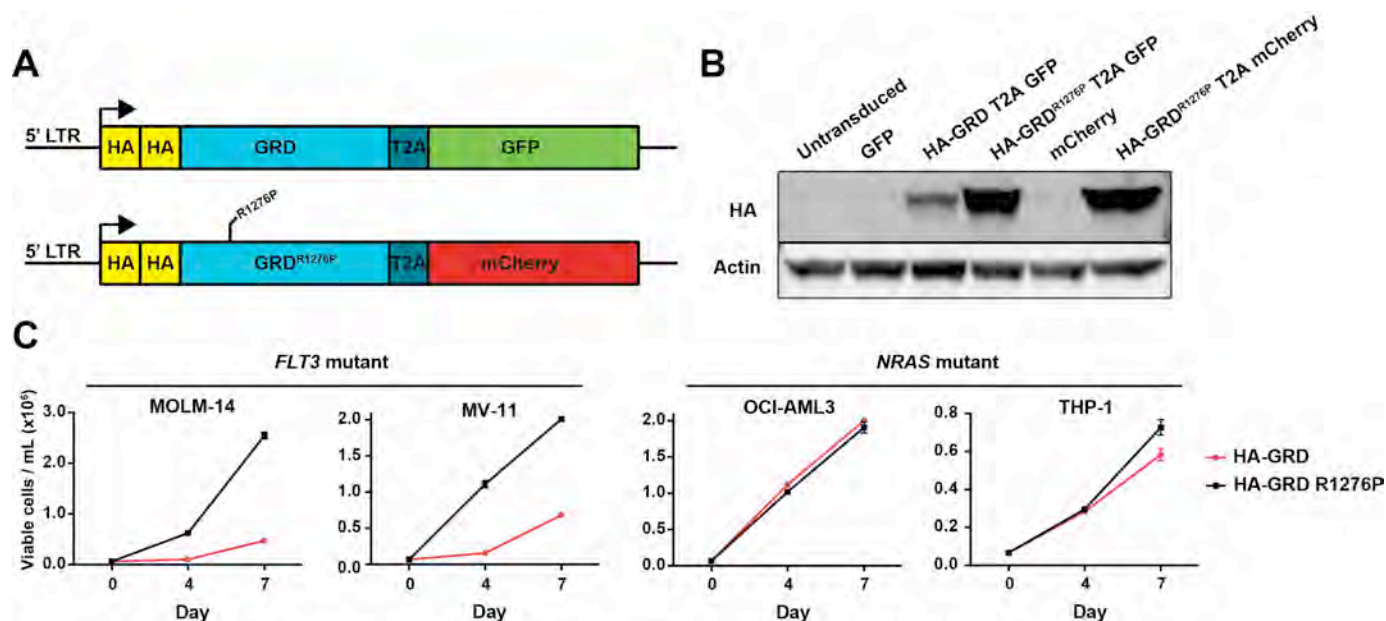


Figure 1. Expression of the neurofibromin GAP related domain (GRD) and “Arg finger” R1276P mutant for assessing dependence on GAP activity. **A.** Lentiviral constructs designed to express the N-terminal HA epitope tagged wild-type GRD or the R1276P mutant with C-terminal self-cleaving T2A peptide followed by a fluorescent protein. **B.** Western blot for HA demonstrating expression of the HA-GRD and R1276P mutant in NIH 3T3 cells. **C.** Overexpression of HA-GRD in *FLT3* mutant human AML cell lines dramatically attenuates growth compared with expression of the control R1276P mutant lacking GAP activity. As expected, control human AML cell lines harboring oncogenic *NRAS* mutations are resistant to increased GAP expression.

In addition to studying the functional consequences of restoring wild-type GRD expression in primary mouse leukemias, we have extended this system in the current fund year to investigate non-enzymatic mechanisms of neurofibromin in growth control. Elegant work performed by Frank McCormick’s lab showed that Spred1 (Sprouty-related protein with an EVH1 domain) functions as a negative regulator of the Ras/Raf/MEK/ERK pathway by binding to neurofibromin and mediating translocation to the plasma membrane where it can function as a Ras GAP in regulating growth factor receptor signaling (56). A deletion of GRD amino acid M1215 abrogates a neurofibromin-Spred1 while retaining full biochemical Ras GAP activity. In work performed during the current fund year, we generated and validated a HA-GRD delM1215 T2A GFP vector to ask if membrane recruitment by Spred1 is required for efficient AML growth suppression by neurofibromin.

The 2014 Progress Report for this award presented results of experiments in which we infected mouse *Nf1*-deficient AML 6537 (28) with lentiviral vectors encoding GFP only, HA-GRD T2A GFP, or HA-GRD R1276P T2A GFP. We sorted transduced, GFP-positive (GFP⁺) cells to purity after 2 days of growth in liquid culture. Exogenous expression of the wild-type GRD, but not the R1276P mutant GRD, attenuated Raf/MEK/ERK pathway activation and profoundly reduced AML blast colony formation by *Nf1*^{-/-} AML 6537 cells. While these studies suggested that *Nf1* deficient AML cells remain dependent on loss of this tumor suppressor gene, we became concerned that non-physiologic levels of GRD expression in this system have strong growth-suppressive properties. In studies performed during the current fund year, we set out to rigorously explore this hypothesis. We first replaced the GFP cassette in the MSCV HA-GRD T2A GFP backbone with a mCherry fluorescent marker, which is brighter and thus allows us to isolate transduced cells with greater precision. To test these new constructs, we infected the human *NRAS* mutant AML cell line OCI-AML3 with all three

MSCV-based vectors and performed flow cytometry on cells that were grown in culture for 2 days. As shown in Figure 2a, transduction efficiencies and mCherry expression levels were similar in OCI-AML3 cells transduced with all three GRD constructs (WT, R1276P, delM1215). This experiment verified equivalent protein expression from all three vectors in a cell context where neurofibromin GRD expression is not expected to affect growth because N-Ras onco-proteins are insensitive to GAP stimulation (32). By contrast, we observed dramatic dropout of *Nf1* deficient AML 6537 cells engineered to express either WT GRD or the delM1215 mutant GRD after 1-3 days in culture compared to cells transduced with the catalytically inactive R1276P mutant GRD (**Fig. 2b**). These data provided compelling evidence that WT GRD and the delM1215 mutant GRD strongly suppress the growth of primary *Nf1* deficient AML cells *ex vivo*. Unfortunately, this result was also a “deal breaker” with respect to using these vectors to achieve our goal of investigating how restoring neurofibromin GAP activity affects AML growth *in vivo*.

We considered two potential strategies to address this fundamental bottleneck. First, we thought of working to establish a tetracycline-controlled transcriptional activation system by utilizing one lentiviral construct engineered to express the Tet-On transactivator (rtTA) with a blue fluorescence protein (BFP) and a second construct that expresses HA-GRD T2A GFP under the control of a Tet-On promoter with constitutive mCherry expression from the PGK promoter. Although we successfully piloted this system in mouse hematopoietic cell lines where we observed robust induction of GFP after 36 hours of doxycycline treatment with no background, it is challenging to infect primary AML cells with two independent constructs, and regulating protein expression is problematic with Tet-On systems, particularly *in vivo*. We therefore sought a promoter that would drive a lower level of constitutive gene expression in primary AML cells.

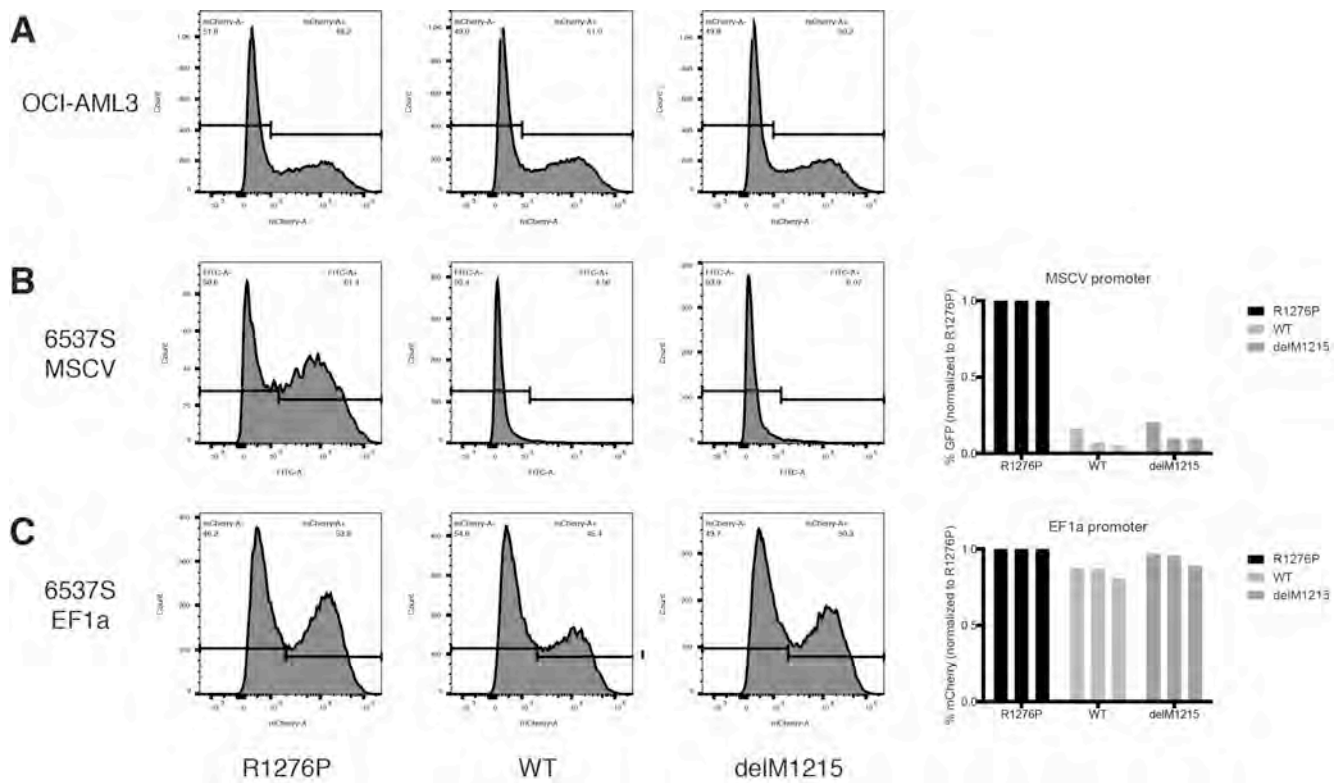


Figure 2. WT GRD and delM1215 mutant GRD suppress the growth of primary *Nf1* deficient AML cells in a dose-dependent manner. **A.** Transduction efficiencies of lentiviral constructs R1276P, WT, and delM1215 GRDs in the NRAS mutant AML cell line OCI-AML3 demonstrating similar GFP protein expression from all three GRD T2A GFP constructs. **B.** The same experiment performed in *Nf1* deficient AML 6537 cells *ex vivo* results in dramatic negative selection of cells transduced with WT GRD and delM1215 mutant GRD constructs compared to the R1276P mutant GRD construct. **C.** Replacing the MSCV promoter with the EF1a promoter greatly attenuates negative selection for cells transduced with the WT GRD and delM1215 mutant GRD constructs. Robust GFP expression over 3 days in culture allowed us to isolate sufficient cell numbers from all three populations for transplantation into mice.

Comparative studies in transduced leukemia cell lines showed that lentiviral constructs utilizing an EF1a promoter consistently exhibited lower levels of protein expression than MSCV vectors (**Fig. 3**). We therefore constructed a series of EF1a HA-GRD T2A mCherry vectors and obtained promising preliminary results using these reagents. Specifically, there was only modest selection against AML 6537 cells expressing either WT GRD or the delM1215 mutant GRD after 1-3 days in culture compared to catalytically dead R1276P GRD (**Fig. 2c**). We also observed a modest difference between the WT and delM1215 constructs, which suggests that the later has reduced biological activity. We transplanted the three populations of transduced AML 6537 cells shown in Figure 2c into recipients on July 31, 2015 and are monitoring these mice for evidence of leukemia. Mice will be euthanized when they become moribund and we will measure the relative percentages of mCherry positive to mCherry negative cells at that time point. We hypothesize that the catalytically dead R1276P GRD will have no effect on AML fitness, and that cells expressing WT GRD will be markedly under-represented relative to the input population. We also postulate that the delM1215 GRD will impair the repopulating potential of primary AML cells to a lesser extent than the WT GRD. We will sort mCherry-positive bone marrow cells from mice that die from AML to assess GRD protein expression by Western blotting.

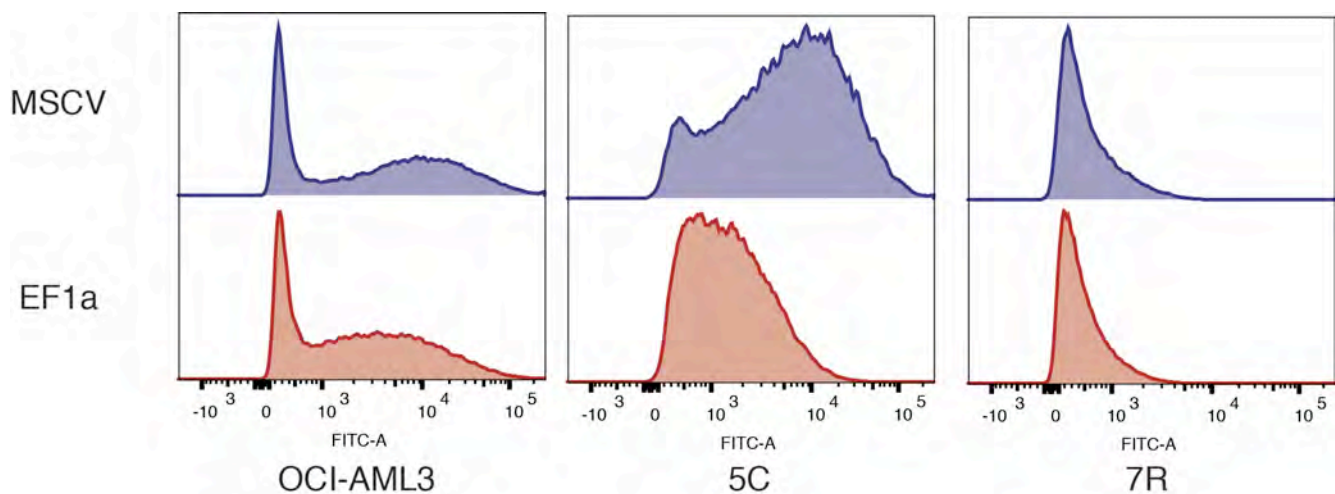


Figure 3. Lentiviral constructs utilizing the EF1a promoter exhibit lower protein expression in cell lines derived from human and murine leukemias. Human AML cell line (OCI-AML3) and two murine acute lymphoblastic leukemia (ALL) cell lines (5C and 7R) were transduced with lentiviral constructs expressing GFP from either an MSCV promoter or an EF1a promoter. The two vectors contain the same backbone and inserts and only differ in their respective promoters. Lentiviral production for both vectors was performed in parallel under identical experimental conditions. The MSCV promoter results in a brighter GFP signal compared to the EF1a promoter as assessed by mean fluorescence intensity. The transduction efficiency (percentage of cells scored positive by showing a GFP signal over background) did not differ between the MSCV and EF1a vectors.

In summary, we have established experimental conditions for efficiently transducing primary *Nf1* deficient AMLs and for assaying the effects of restoring neurofibromin GAP activity on the growth of these cells. Studies using lentiviral vectors containing a strong MSCV promoter that drives neurofibromin GRD expression support the hypothesis that these primary leukemias are dependent on persistent *Nf1* inactivation for *in vitro* growth. However, high levels of GRD expression in this system raised questions about the physiologic relevance of these data, particularly as cells expressing the WT GRD were rapidly depleted in culture, which precluded performing *in vivo* studies. We recently overcame this problem by generating a new series of retroviral vectors in which an EF1a promoter drives GRD expression, and transplanted recipient mice with transduced AML 6537 cells. We are currently isolating, infecting, and transplanting a resistant subclone of AML 6537 that emerged during *in vivo* treatment with MEK inhibitors, and will perform similar studies using AML 6554 and two

resistant subclones of this leukemia. We execute these experiments by the end of 2015 and expect to submit a paper soon thereafter.

Technical Objective (Aim 2): Preclinical studies combining cytotoxic chemotherapy with MEK inhibitors in primary murine AMLs.

Our preclinical studies in *Nf1* mutant mice and observations in patients treated with small molecule inhibitors of signaling molecules showed that many advanced cancers respond transiently before becoming resistant (28, 57). Emerging data also suggests that combining targeted and conventional chemotherapeutic agents increases clinical efficacy (58). Our novel collections of genetically diverse transplantable *Nf1* mutant AMLs provide a robust system for applying this principle to NF1-associated cancers. Figure 4 presents an overview of our general approach to performing controlled *in vivo* preclinical trials of experimental agents in mice transplanted with primary leukemias, which is also described in our published work (28, 43, 52).

The standard treatment for AML involves administering high doses of cytarabine in combination with an anthracycline agent such as daunorubicin. These aggressive regimens induce remissions in ~80% of patients who are <60 years old; however, over half ultimately relapse despite receiving consolidation chemotherapy and/or hematopoietic stem cell transplantation (HSCT). CPX-351 is a liposomal formulation that contains cytarabine and daunorubicin at an optimized ratio to enhance AML killing without added toxicity (59). CPX-351 (Celator, Inc) is being investigated in human clinical trials, and dosing schedules were developed for treating immunodeficient mice engrafted with human leukemia cell lines (60, 61). Based on the “front line” role of cytarabine:daunorubicin in human AML treatment protocols and our data demonstrating murine AMLs that have inactivated the *Nf1* gene are sensitive to MEK inhibitors *in vitro* and *in vivo*, we used funds provided by this CDMRP award to undertake preclinical trials with the goal of combining CPX-351 with rational targeted anti-leukemia agents such as PD901 to ascertain if this might improve efficacy and inform clinical translation.

As summarized in our 2013 and 2014 Progress Reports, we first established the maximally tolerated dose (MTD) of CPX-351 in recipient mice that received sublethal irradiation and were transplanted with primary congenic AMLs. These experiments proved challenging due to substantial drug-induced mortality at multiple dose levels. After extensive consultation with the Celator clinical team, we fixed the CPX-351 dose at a fixed cytarabine:daunorubicin ratio of 5 mg:2.2 mg. This dose is clinically relevant dose and maintains anti-leukemic activity in humans. To evaluate the efficacy of CPX-351 in primary murine AMLs, we transplanted recipient mice with *Nf1* mutant (n=1), *Kras*^{G12D} (n=1), and *Nras*^{G12D} (n=3) AML cells and treated them with CPX-351 using the schedule outlined above. Data presented in our 2014 Progress Report revealed a modest improvement in overall survival that did not reach statistical significance. Interestingly, recipients transplanted with *Nf1* mutant AML 6537 were particularly sensitive to CPX-351, and we went on to test the combined effects of cytotoxic therapy with CPX-351 followed by PD901 consolidation. Unfortunately this dosing schedule proved

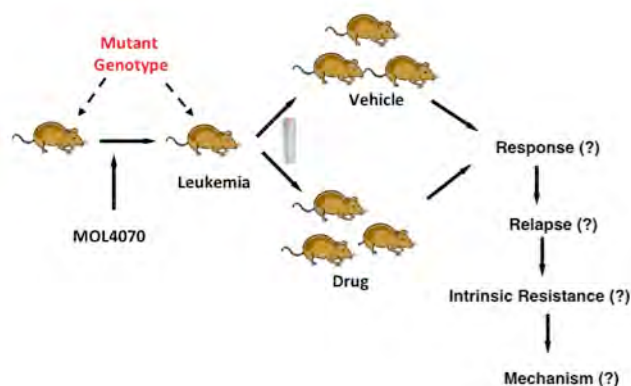


Figure 4. In Vivo Strategy for Characterizing Drug Response and Resistance in Primary Leukemias. The MOL4070 retrovirus induces leukemia by insertional mutagenesis, which can be performed on a sensitized genetic background or by inducing cooperating mutations after viral injection using Mx1-Cre. Preclinical efficacy is assessed by transplanting primary leukemias into cohorts of congenic recipient mice and treating them. Secondary transplantation and retreatment is used to test leukemias that relapse after an initial response to treatment for intrinsic drug resistance. A variety of molecular techniques are then deployed to characterize resistance mechanisms.

too toxic, as the majority of animals succumbed with bone marrow failure shortly after starting the MEK inhibitor. The observed toxicity is perhaps not unexpected as irradiation, leukemic marrow infiltration, cytotoxic drugs, and PD901 all contribute to bone marrow suppression. In particular, conventional anti-leukemia drugs strongly suppress proliferation, and we directly demonstrated anti-proliferative effects of PD901 in murine AMLs (52). Despite multiple attempts to adjust the dose and timing of PD901 administration after CPX-351 induction during the current funding year, these combination regimens proved too toxic and we decided it would be more productive to explore other rational drug combinations.

The differentiation and self-renewal of hematopoietic stem cells is tightly regulated by epigenetic mechanisms that are frequently altered in AML by mutations in genes controlling DNA methylation (e.g. *DNMT3A*, *TET2*, *IDH1/2*) and histone modification (e.g. *EZH2*, *ASXL1*). The BET bromodomain proteins are epigenetic 'reader' molecules that bind histones at acetylated lysine residues to promote transcription of nearby genes. Broadly speaking, the activating BET proteins antagonize the inhibitory effects of the polycomb repressor complex 2 (PRC2). Experimental data broadly support the hypothesis that loss of PRC2 function promotes an epigenetic state that is both permissive for AML development and is essential for leukemia maintenance (Fig. 5). Recent studies have identified BET bromodomain proteins as a particularly promising class of therapeutic targets in this disease (62, 63). Importantly, BET bromodomain proteins are viable biochemical targets for drug discovery, and a number of small molecule inhibitors are currently in development or undergoing clinical evaluation.

We negotiated a Materials Transfer Agreement (MTA) with Plexxikon in late 2014 to obtain PLX51107, a potent and selective BET bromodomain inhibitor. The company previously established 20 mg/kg/day as the MTD in immunodeficient mice due to severe gastrointestinal toxicity at higher dose levels. This dose was fatal in immunocompetent mice on our strain background that received sublethal irradiation; however, we observed no toxicity at the 10 and 15 mg/kg/day dose levels. Pharmacokinetic analysis performed at Plexxikon revealed excellent drug exposures at both doses. We next considered whether to investigate PD901 or CPX-351 in combination with PLX51107, and selected PD901 based on extensive prior experience and superior efficacy as a single agent compared to CPX-351. We selected *Nras*^{G12D} AML #6695, which is highly aggressive and showed a ~2 fold increase in survival in response to PD901, for our initial

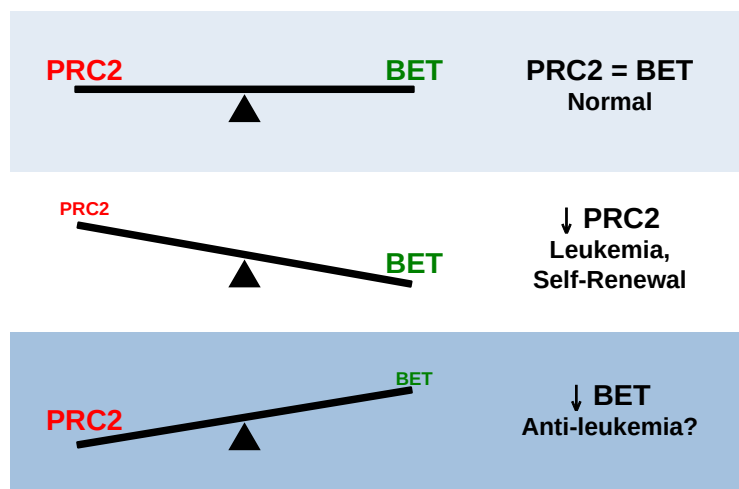


Figure 5. Rationale for targeting BET bromodomain proteins in AML. Normal hematopoietic differentiation requires balancing the competing activities of the PRC2 complex and BET proteins. In AML, aberrant BET activation antagonizes the PRC2 function; thereby impairing the differentiation of AML cells and promoting their self-renewal. This model predicts that inhibiting BET activity will be efficacious against AML cells, particularly when combined with signal transduction inhibitors.

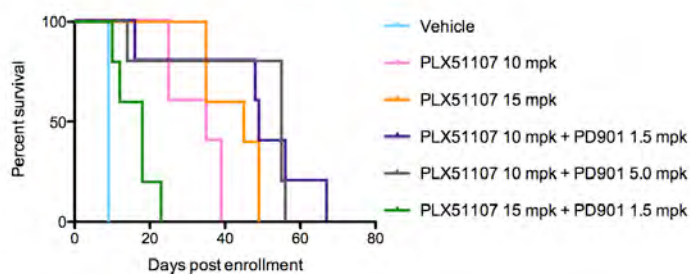


Figure 6. Preclinical evaluation of PLX51107 +/- PD901 in AML #6695. Recipients transplanted with AML #6695 ($n = 29$) were randomly assigned to receive control vehicle or to one of the 5 treatment groups shown above. All mice succumbed with AML except for mice given PLX51107 + PD901 (green line), which died from drug-induced toxicity (bone marrow failure).

evaluation of PLX51107 as a single agent and in combination with PD901 (52). Remarkably, PLX51107 markedly extended the survival of mice when administered either as a single agent or with PD901 (**Fig. 6**). While the 15 mg/kg/day dose of PLX51107 was well tolerated, mice receiving this dose in combination with PD901 died from bone marrow failure (**Fig. 6**). By contrast, recipients of AML #6695 treated with 10 mg/kg/day appeared well and exhibited a remarkable improvement in overall survival, which was particularly evident when PLX51107 was administered with PD901.

Comparing data from serial preclinical trials of AML #6695 showed that PLX51107 is superior to either CPX-351 or PD901 when administered as a single agent (**Fig. 7**). Recipient mice given PLX51107 invariably died with progressive AML despite continuous treatment. Importantly, we observed clonal evolution in some of these mice by the criterion of detecting novel retroviral integrations by Southern blotting (**Fig. 8**). These molecular data indicate that drug treatment exerted sufficient *in vivo* selective pressure to suppress the dominant AML clone. Consistent with our previous observations in this robust preclinical system (28, 43, 52), a marked increase in survival that was also associated with clonal evolution at the time of death indicated the emergence of a drug resistant subclone, which we confirmed by transplanting these AMLs into secondary recipients and retreating them *in vivo* (**Fig. 9**).

Based on the compelling response of AML #6695, we recently transplanted multiple independent *Nf1* deficient and *Nras* mutant AMLs cohorts, and began treating the recipients with PLX51107 and the PLX51107/PD901 combination. We have recently observed some variability in toxicity, which is related to the intrinsic biologic aggressiveness of each AML. Reducing the number of transplanted AML cells from 5×10^6 to 2×10^6 has largely overcome this problem, and we anticipate generating substantial new data in the next few months.

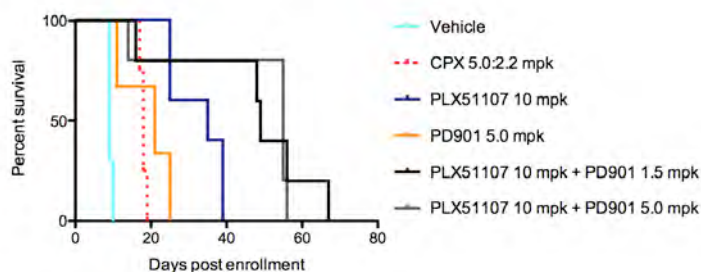
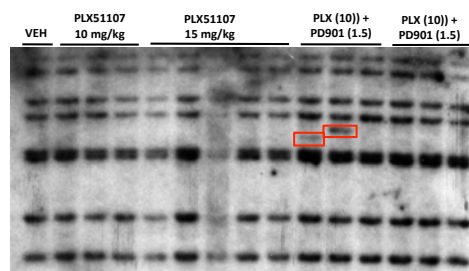


Figure 7. Comparative efficacy of PD901, CPX-351, and PLX51107 in AML #6695. Recipients transplanted with AML #6695 ($n = 47$) that were treated on a series of preclinical trials with various drugs. Note that PLX51107 (dark blue line) is the most effective single agent. Combining PLX51107 with PD901 further improved survival. All of the mice enrolled of these studies ultimately died from AML.



30 32

Figure 8. Clonal evolution of AML #6695 after treatment with PLX51107 + PD901. Unique retroviral integrations identified in AML cells isolated from recipients #30 and #32 are indicated in the red boxes. Shared integrations among all leukemias indicate that they evolved from the same “founder” clone.

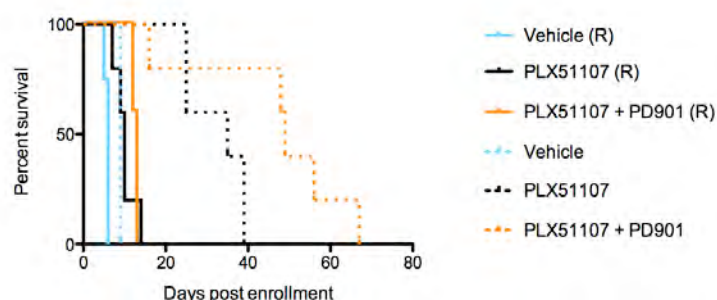


Figure 9. Intrinsic drug resistance of AML #6695 after treatment with PLX51107 + PD901. AML cells isolated from mouse #32 in Figure 8 were transferred into secondary recipients and retreated. Solid lines indicate survival of these secondary recipients, and dotted lines show the survival of the initial (primary) recipients of this leukemia.

Technical Objective (Aim 3): Identify and validate resistance genes in Nf1 mutant AML

Initiating these studies are dependent on generating AMLs that exhibit robust responses to single agents and/or drug combinations before relapsing. We failed to isolate any leukemias fulfilling these criteria during the first 2.5 years of this project, but have now succeeded in isolating at least two resistant subclones of AML #6695. Based on our previous experience, we are optimistic that our ongoing studies of PLX51107 will yield multiple resistant AML subclones. As *Nf1* mutant AMLs #6537 and #6554 are highly responsive to MEK inhibition, we are particularly interested in: (1) determining if PLX51107 treatment of these parental leukemias selects for novel resistant clones; (2) if we isolate such clones, probing the mechanisms underlying the resistant phenotype; and, (3) asking if the PLX51107/PD901 combination cures any of the recipient mice. Depending on the progress of these studies, we will also consider follow-on studies to investigate the combination of PLX51107 + CPX-351 as well as replacing PD901 with trametinib.

Summary of Key Research Accomplishments

- (a) We developed a series of lentiviral vectors encoding the wild-type and mutant neurofibromin GAP related domain (GRD), and deployed these novel reagents to investigate how restoring GAP activity modulates the growth of primary *Nf1* mutant AMLs. Our studies to date support the hypothesis that *Nf1* inactivation remains essential for the growth of these advanced cancers despite the presence of multiple additional cooperating mutations. We are currently extending this work to investigate: (1) if loss of Spred1 binding modulates the ability of the neurofibromin GRD to suppress AML growth *in vitro* and *in vivo*; and; (2) if *Nf1* deficient AMLs that have acquired resistance to MEK inhibition resistant have bypassed this requirement for neurofibromin GAP inactivation. We expect to submit a research paper reporting these studies at the end of 2015 or early in 2016.
- (b) We developed a regimen for administering CPX-351 to recipients of primary murine AMLs at a dose levels that is comparable to exposures achieved in human patients. Treating a panel of primary murine AMLs characterized by hyperactive Ras signaling (*Nf1*, *Kras*, and *Nras* mutant) demonstrated a modest, but significant, increase in overall survival. However, we did not observe clonal evolution at the time of death, and efforts to combine CPX-351 and PD901 failed due to toxicity that is likely due cumulative anti-proliferative effects of both drugs. Our studies of CPX-351 establish a “backbone” based on clinical AML induction protocols that will be useful for future studies combining this drug with other targeted agents.
- (c) We tested a novel BET bromodomain inhibitor called PLX51107 in our AML models and observed impressive efficacy. Combining this novel drug with the MEK inhibitor PD901 further extended survival. Importantly, drug resistant clones emerged during treatment that also showed clonal evolution. These exciting studies have translational implications for developing combinations of targeted inhibitors to treat myeloid malignancies in NF1 patients and in the general population, and also provide a robust forward genetic system for characterizing mechanisms of drug resistance.

Training Opportunities Provided By This Award

This award provided partial salary support to the Principal Investigator (Kevin Shannon, MD) and to three permanent members of the Shannon lab (Jasmine Wong, PhD, Pilar Alzamora, and Eugene Hwang). In addition, three trainees who received salary support from other sources (Michael Burgess, MD, PhD, Benjamin Huang, MD, and Willa Li) utilized resources provided by this awards. The impact on four of these individuals is briefly summarized below.

Jasmine Wong, PhD. Dr. Wong assisted with multiple aspects of this work and was recently proposed for promotion from a Specialist in the Professional Series to the faculty as an Assistant Professor of Pediatrics. Ms. Alzamora performed experiments under her supervision in year 1.

Michael Burgess, MD, PhD. Dr. Burgess joined the lab as a Fellow in the Division of Hematology/Oncology, UCSF Department of Medicine. Dr. Burgess was initially supported by an institutional T32 grant from the National Cancer Institute (Principal Investigator: Zena Werb, PhD), and subsequently received an American Cancer Society Research Fellowship. Mr. Hwang provided expert technical support to Dr. Burgess. Dr. Burgess was promoted to the rank of Instructor in Medicine before leaving UCSF to take a position at Celgene, where he is working to bring new drugs to patients with AML and other hematologic cancers.

Benjamin Huang, MD. Dr. Huang is a Fellow in the Division of Pediatric Hematology/Oncology who joined the lab in July 2014. He assumed a leading role in conducting the research studies supported by this CDMRP-funded project after Dr. Burgess left UCSF, and is receiving extensive training from Drs. Shannon and Wong. Dr. Huang's salary is supported by an institutional T32 grant from the National Cancer Institute (Principal Investigator: Kevin Shannon, MD). Dr. Huang is continuing to pursue the goals of this project, and anticipates submitting two research papers in the coming year.

Willa Li. Ms. Li is a high school student who received extensive training during two summers in the lab when she worked on this project through a joint program between the UCSF Medical Scientist Training Program and Lowell High School. Based on her contributions to this project, she was named a Semi-finalist in the Siemens National Science Talent Search. Ms. Li will enter Harvard University this fall and is currently planning to pursue a career as a physician/scientist.

Dissemination of Results and Reagents

Dr. Shannon presented the initial results of this research during a Keynote Talk at the 2014 Neurofibromatosis Research Conference, which was held in Washington, DC. The laboratory participates in the Neurofibromatosis Preclinical Trials Consortium, which is sponsored by the Children's Tumor Foundation. Dr. Shannon shares unpublished research results at these meetings. In addition, the lab has provided the vectors shown in Figure 1 to multiple investigators in the NF research community.

Goals for Next Reporting Period

Not applicable, though we continue to pursue the goals of this research project through a small grant from the Rally Foundation and expect to publish 2 research papers in the next year.

IMPACT

Primary Discipline

This project has developed novel "proof of concept" data that advanced cancers arising in NF1 patients remain dependent on loss of neurofibromin. This finding supports testing small molecules to target aberrant Ras signaling in these NF1-deficient cancers and, ultimately, pursuing the goal of restoring neurofibromin GAP activity as the field of genome editing/gene therapy advances. The novel vectors developed through this research project are facilitating studies in other laboratories.

Other Disciplines

Technologies and approaches developed through this project are broadly applicable to the leukemia research field.

Technology Transfer

As above, we have provided lentiviral vectors developed through this project to other researchers.

Impact to Society

None to date

CHANGES/PROBLEMS

We did not encounter problems during the course of this project that required that we change the overall experimental approach. There was no involvement of human subjects in this project, and there were no significant changes in either the scope or focus of research involving vertebrate animals.

PRODUCTS

(a) Journal Publication

Burgess MR, Hwang E, Firestone AJ, Xu J, Bohin N, Wen T, Haigis KM, Shannon K*, Li Q*. Preclinical efficacy of MEK inhibition in *Nras* mutant acute myeloid leukemia. *Blood* 2014; **124**: 3947-55.* - Corresponding authors.

(b) Abstract/Conference Presentation

Kevin Shannon, MD. Keynote Talk at the 2014 Neurofibromatosis Research Conference, Washington, DC.

Burgess MR, Hwang E, Firestone AJ, Xu J, Bohin N, Wen T, Haigis KM, Shannon K, Li Q. Preclinical efficacy of MEK inhibition in *Nras* mutant acute myeloid leukemia. *Blood* 2014; 124 (Suppl 1):3753. Presented at the 2014 annual meeting of the American Society of Hematology, San Francisco, CA.

(c) Funding applied for based on work supported by this award

Research Grant and Consortium Grant in Pediatric Cancer Research: Bear Necessities and Rally Foundation (Kevin Shannon, MD)- awarded in 2014; renewed in 2015.

American Cancer Society Postdoctoral Fellowship (Michael Burgess, MD, PhD) – awarded in 2014.

National Cancer Institute Specialized Center of Research Excellence (SPOR) grant (Principal Investigators: D. Wade Clapp and Kevin Shannon). – awarded August 2015.

(d) Technologies/Techniques

Not applicable

(e) Inventions/patent Applications

Not applicable

(f) Other Products

Lentiviral vectors encoding the neurofibromin GRD described above.

PARTICIPANTS AND COLLABORATING ORGANIZATIONS

Individuals Who Worked on This Project

Name	Kevin Shannon, MD
Project Role	PI
Nearest Person Month	4 (total for 3 year project period)
Contribution	Designed and supervised all aspects of this project
Funding Support	NCI grants P01 CA40046, R37 CA72614, R01 CA180037, and R01 CA193994; American Cancer Society Professorship; St. Baldrick's Foundation Research Grant; Rally Foundation; Children's Tumor Foundation
Name	Jasmine Wong, PhD
Project Role	Research Specialist, Shannon Lab
Nearest Person Month	6 (total for 3 year project period)
Contribution	Assisted in designing and conducting experiments
Funding Support	No other support at PI or Co-PI; additional salary support from awards to PI listed above
Name	Pilar Alzamora
Project Role	Research Technician
Nearest Person Month	6 (total for 3 year project period)
Contribution	Assisted with animal care and worked under Dr. Wong's supervision to perform experiments
Funding Support	No other support at PI or Co-PI; additional salary support from awards to PI listed above
Name	Eugene Hwang
Project Role	Research Technician
Nearest Person Month	13 (total for 3 year project period)
Contribution	Assisted PI and other lab members in executing experiments. Tabulated and summarized data. Maintained mouse colony and performed preclinical trials of investigational agents.
Funding Support	No other support at PI or Co-PI; additional salary support from awards to PI listed above

Changes in Support for PI Since Last Report (June 1, 2014)

New Awards

R01 CA193994 (Shannon) NIH/NCI <i>Selectively Targeting Oncogenic NRAS in Cancer</i>	04/01/15 – 03/31/20	1.8 CM
No Number (Shannon) Rally Fdn for Cancer Research/The Truth 365 <i>Response & Resistance to Targeted & Conventional Agents in Myeloid Leukemia</i>	04/01/15 – 03/31/16 0.3 CM	
P50 CA196519-01 (Clapp/Shannon) NIH/NCI <i>Developmental and Hyperactive Ras SPORE</i>	08/01/15-06/30/20	2.4 CM
No Number (Shannon) Edward P. Evans Foundation <i>In Vivo Functional Analysis of Chromosome 7q22 Deletions in MDS</i>	09/1/2015-08/31/17	1.2 CM

Projects Completed

U54 CA143874 (van Oudenaarden) NIH/NCI (Subcontract from MIT) <i>Complementary Studies to Deconvolute Ras Signaling Networks in T Cell Lymphoma</i>	10/01/09 – 09/30/14 (Project 2 for Roose/Shannon/Braun)	0.6 CM
P01 CA40046 (Le Beau) NIH/NCI Subcontract from University of Chicago <i>Etiology of Treatment-Induced Secondary Leukemia</i>	9/01/09 – 8/30/14 (Project 4 only)	1.5 CM
No Number (Shannon) St. Baldrick's Foundation <i>Chromosome 7 Deletions in Refractory Pediatric Leukemia</i>	07/1/2014-06/30/15	0.6 CM

Other Organizations

Not applicable - all work on this project was performed at UCSF.

SPECIAL REPORTING REQUIREMENTS

Not applicable

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