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Genetic Determinants of Disease Persistence and Overt Off-Target Resistance to TKI Therapy in Chronic Myeloid Leukemia

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Genetic Determinants of Disease Persistence and Overt Off-Target Resistance to TKI therapy in Chronic Myeloid Leukemia

by

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DISSERTATION

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This dissertation is dedicated to my lovely Erin, my family, and dearest friends.

It is in sharing with those that we love most that our accomplishments reveal the greatest emotional reward. This is yours as much as it is mine.

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Contributions to presented work

Chapter 2 of this dissertation contains unpublished material that is currently in preparation:

Reyes G., Cottonham C., DeFilippis R., Braun B., Shah NP. Genetic Determinants of Disease Persistence and Overt Off-Target Resistance to TKI Therapy in Chronic Myeloid Leukemia. (in preparation)

I performed the studies described in chapter 2 under the guidance of Neil Shah (MD, PhD). Charisa Cottonham (PhD) and Rosa-Anna DeFilippis (PhD) under the guidance on Neil Shah and Ben Braun (MD, PhD) aided in the design, experimentation and analysis of the EVI1 mouse BCR-ABL1 transduction experiments.

Genetic Determinants of Disease Persistence and Overt-Off Target resistance to TKI therapy in Chronic Myeloid Leukemia

Gabriel A. Reyes

Abstract

Chronic myeloid leukemia (CML), driven by the fusion protein BCR-ABL1, remains highly responsive to treatment with tyrosine kinase inhibitors (TKIs). Although overt resistance and relapse typically occurs through on-target kinase domain mutations, 25-50% of all resistant cases lack such mutations. Second generation BCR-ABL1 inhibitors, such as dasatinib and nilotinib retain the ability to inhibit a number of kinase domain mutations. Still a number of mutations, notably the T315I gatekeeper mutation, provide continued resistance and only recently with the approval of ponatinib are we able to inhibit all known kinase domain mutations in BCR-ABL1. With effective inhibitors for all known drug-resistant mutations now available, it is likely that poorly understood overt off-target resistance mechanisms will henceforth underlie a greater percentage of overt clinical resistance. Through the use of CML patient samples we sought to define molecular mediators of disease persistence (the occurrence of a hematologic but not cytogenetic response) and overt off-target resistance (a complete loss of cellular response to BCR-ABL1 inhibition), which are two poorly understood mechanisms of resistance in CML. Chronic phase CML patients

exhibiting overexpression of the *EVI1* oncogene displayed disease persistence, while the identification of two blast crisis CML patients exhibiting overt off-target resistance were found to contain *EVI1* over-expression in conjunction with activated *NRAS*. Expressing $NRAS^{Q61K}$ in CML cell lines resulted in maintenance of cell viability despite TKI treatment, and this overt resistance correlated with increased Raf/MEK/ERK signaling. Furthermore, expression of MEK^{DD}, an activated MEK allele, provided a degree of resistance comparable that seen in off-target resistance driven by $NRAS^{Q61K}$ expression. This establishes MAPK signaling as the major mechanism downstream of oncogenic NRAS that mediates the resistant phenotype. This hypothesis was further substantiated by identifying sensitivity to MEK inhibition both alone and to an even greater extent in combination with BCR-ABL1 inhibitors. In all, we show that *EVI1* over-expression can contribute to disease persistence and the presence of activating RAS mutations can lead to overt resistance to BCR-ABL1 tyrosine kinase inhibitors.

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Chapter 1:

Introduction

Part I: Chronic Myeloid Leukemia

Chronic Myeloid Leukemia, CML, is a myeloproliferative neoplasm characterized by the expansion of the myeloid lineage cells that retain the capacity for differentiation[1,2]. The defining molecular lesion and driver of CML is the BCR-ABL1 fusion protein, which results from a 9;22 chromosomal translocation[1–3]. Although typically classified as myeloid expansion with capacity for differentiation; in fact, clinically the disease exhibits progression through three stages[3,4].

The initial stage of the disease, chronic phase CML, routinely lasts years if left untreated and is frequently characterized by mild, vague malaise and weakness. Often, patients diagnosed in chronic phase lack any symptoms at all and can remain in this phase of the disease for long periods of time. Advancement to the accelerated phase of the disease has typically been thought of as being caused by the acquisition of additional genetic mutations and abnormalities[5]. During this phase an increase in weakness and malaise may occur. The third and final stage of the disease, blast crisis, is one in which a block in differentiation results in elevated immature precursor cells[4,5]. As opposed to the chronic phase of the disease in which immature blast counts in the peripheral blood tends to fall under 20% of circulating cells, in blast crisis CML immature blasts in the periphery often rise above the 20-30% mark[6].

Furthermore, the type of cell can be of myeloid or lymphoid origin suggesting the inciting 9;22 translocation and BCR-ABL1 expression is occurring in a stem cell clone primitive enough to give rise to either lineage. Again, this progression occurs as the result of a number of secondary genetic abnormalities. Often these patients exhibit symptoms of splenomegaly, although it is not diagnostic as this can be seen in earlier stages as well. It is in this stage of the disease that despite current therapies patients tend to fare much more poorly.

The diagnosis of CML depends largely on the identification of the Philadelphia chromosome (BCR-ABL1 producing translocation between chromosomes. t(9;22)(q34;q11)) in a patient exhibiting leukocytosis (elevated white blood cell count)[7]. The identification of the Philadelphia chromosome can be accomplished through either karyotype analysis (identification of the translocation using cytogenetic analysis) or fluorescence in-situ hybridization (FISH staining using probes specific to the fusion). A third possible diagnostic test exists, using a molecular diagnostic technique[7,8]. Although FISH analysis is widely trusted as a means for diagnosis false positive results can occur[7]. Alternatively the use of molecular techniques such as PCR and quantitative PCR allow for greater sensitivity in identifying the presence and expression of the BCR-ABL1 fusion[9]. With this increase in sensitivity, detection of BCR-ABL1 transcript can be done not only from bone marrow samples, but from peripheral blood as well.

The 9;22 chromosomal translocation results in the formation of a dysregulated ABL kinase. The constitutive signaling of this kinase results in the activation of a number of pro-survival pathways including RAS/RAF/MEK/ERK (mitogen activated protein or mitogen activated protein kinase; MAPK) pathway, Phospho-inositide 3 kinase (PI3K)/AKT pathway and the JAK/STAT pathway. These have been deemed the canonical pathways vital to BCR-ABL1 pro-survival function[10]. RAS, PI3K and JAK have been known to play a role in a wide range of malignancies, but most often with activation of the RAS/MAPK pathway. This pathway alone has been shown to contain driver mutations in a number of malignancies ranging from pancreatic cancer to colon cancer, lung cancer and melanoma[11]. And while others such as ovarian, breast, colorectal and endometrial cancer have been associated with excessive PI3K signaling, the V617F mutation in JAK2 is a known driver of the other non Philadelphia chromosome myeloproliferative disorders, polycythemia vera (PV), essential thrombocytosis (ET) and primary myelofibrosis (PMF)[12,13]. This only highlights the strength of a protein like BCR-ABL1, one that can signal down such potent pro-survival pathways.

The wide association of RAS with cancer has led to immense study of its effectors and yet the role of RAS downstream of BCR-ABL is but one part of a large signaling cascade. RAS itself is typically found in one of two states, the

inactive GDP-bound state or the active GTP-bound state. This protein regularly switches from the inactive to the active conformation in response to guanine nucleotide exchange factors such as son of sevenless 1 and 2 (SOS-1, SOS-2) as well as CDC25[14–16]. Alternatively, inactivation of RAS through switching from the GTP-bound state to the GDP-bound state is aided by GTPase activating proteins (GAPs) such as neurofibromin-1 (NF-1) and p120[14,17]. The active form of RAS has the capability of binding to and activating a number of signaling partners, the most notable of which are the RAF proteins (A-RAF, B-RAF or C-RAF) within the MAP kinase pathway[18]. In addition to MAP kinase activation, RAS also binds to multiple PI3K isoforms and RAL-GDS family proteins to induce downstream components of these pathways. Further evidence implicating RAS as being a potent cancer causing lesion is that activating mutations in RAS genes are found in as many as 30% of human cancers[14,18]. Further still, activation of the MAP kinase pathway downstream of RAS also plays a role in cancer incidence, as is seen with the activation of B-RAF through a V600E point mutation known to be the driver mutation in melanoma[19,20].

Approximately 99% of all RAS activating mutations occur at either codon 12, 13 or 61[21]. Mutations at these specific codons result in an equilibrium shift of the RAS protein toward the active GTP-bound state. Furthermore, despite the existence of 3 different RAS isoforms (H-RAS, K-RAS and N-RAS), activation via mutation at these particular codons has been seen in every isoform largely due

to the conserved sequence homology between the N-terminus of the isoforms. The differences seen between isoforms largely reside at the C-terminal hypervariable region, which has been shown to direct the trafficking of each isoform to different regions within the cell[21,22]. It is this compartmentalization that is thought to be responsible for the ability of the isoforms to drive different types of cancer. Specifically, HRAS mutations are associated with skin and bladder malignancies. $KRAS^{G12D}$ far outweighs the other mutations and is most commonly encountered in pancreatic, lung and colorectal cancers. And while $NRAS^{G12D}$ has been found in prostate cancer and hematologic malignancies, $NRAS^{Q61K}$ is more common than the G12D mutant and is typically seen in skin and lung cancers[21].

Despite the ability of activated RAS to induce such a potent phenotype in a wide range of tissues, it is known that RAS activation alone is insufficient for inducing chronic myeloid leukemia, as RAS mutations in the absence of the Philadelphia chromosome have never been found to cause CML. While the incidence of NRAS mutations are quite high in other myeloproliferative neoplasms, chronic myelomonocytic leukemia (CMML) and juvenile myelomonocytic leukemia (JMML), they are extremely rare in CML, likely due to the fact that it cannot recapitulate a BCR-ABL1 driven CML as the function of BCR-ABL1 exceeds the reach of activated RAS[23–28]. Still, the function of BCR-ABL1 is in fact dependent on its ability to activate RAS particularly through the

Y177 codon of BCR-ABL1, which interacts with the SH2 domain of Grb-2, of which itself partners with the GEF, SOS-1[29,30]. The importance of codon Y177 in mediating the BCR-ABL1/RAS connection was further demonstrated by $NRAS^{G12D}$ rescue of the CML phenotype in a Y177 mutant BCR-ABL1 murine model[31].

While RAS activation typically garners much interest given its high incidence in cancer and central role in BCR-ABL1 transformative capabilities, in the setting of CML it is not the sole pathway mediating BCR-ABL1 function. This was demonstrated through use of dominant negative alleles of each downstream canonical pathway of BCR-ABL1[10]. Using a patient derived CML cell line, K562, expressing either the dominant negative N17 allele of RAS, the 694F allele of STAT5, or the Δp85 allele of PI3K, it was found that while expression of any one dominant negative allele induced minimal apoptosis of the cells, the co-expression of any two alleles resulted in severe apoptosis. This finding highlights the importance of each pathway as being part of larger signaling machinery guided by BCR-ABL1.

Part II: CML Treatment and Resistance

Treatment of CML can be categorized into two time periods, the time prior to the identification BCR-ABL1 targeted kinase inhibitors and what has come since their discovery and implementation. In the time before, CML treatment was largely dependent on the use of alpha-interferon and while bone marrow transplantation (BMT) was considered curative, not all patients qualified as candidates[2,32,33]. The discovery of imatinib ushered in a new era in cancer treatment, the era of targeted cancer therapy. Not only does the use of a targeted treatment minimize adverse drug effects, in the case of imatinib, it became the poster child for oncogene addiction, the phenomenon that a given cell can develop a dependence on the presence and function of a particular oncogene[34,35]. This has been demonstrated in numerous models of ectopic oncogene expression whereby its introduction into a cell elicits transformation[36–38]. Although cellular transformation following introduction of an oncogene suggests a dependence, it is the subsequent withdrawal and resulting collapse of the cell that is the remarkable feature of oncogene addiction[38]. Although this has been shown through various *in vitro* and *in vivo* models it was CML and the use of the BCR-ABL1 tyrosine kinase inhibitor (TKI) imatinib that confirmed the clinical relevance of oncogene addiction[39].

The discovery of imatinib not only provided insights into oncogene addiction, but also paved the way for targeted cancer therapies. This tyrosine kinase inhibitor (TKI) functions to inhibit BCR-ABL1 through competitive inhibition via binding to the ABL kinase domain and thus preventing ATP binding and hydrolysis[40]. Imatinib is a type II inhibitor, binding predominately to the inactive conformation of BCR-ABL1 during which the activation loop of the ABL kinase domain remains unphosphorylated. This breakthrough in CML treatment resulted in significant improvement in patient outcomes and the achievement of deep and durable remissions in a large percentage of patients[41].

Early studies were done to compare the effectiveness of alpha-interferon versus imatinib in newly diagnosed chronic phase CML patients. While the 3 year survival rates were 81% with interferon and 96% with imatinib, an even greater benefit was seen in the differences between groups in achieving a complete cytogenetic response (27% with interferon, 87% with imatinib)[42]. Most often, newly diagnosed CML patients exhibit leukocytosis (elevated white blood cell counts), and the presence of the Philadelphia chromosome, which can be detected in the bone marrow aspirate. Upon treatment, particularly with imatinib, it is common for the WBC count to return to normal, which is described as achievement of a hematologic response. In addition, a cytogenetic response is one in which imatinib treatment results in the inability to detect the Philadelphia chromosome in the bone marrow. With a drug like imatinib as high as 87% of

patients achieved a complete cytogenetic response versus interferon treatment in which a mere 27% of patients achieved the same response. Although achieving a hematologic response is a goal of treatment, a cytogenetic response is truly a greater benchmark of success merely due to the fact that achieving either complete, no detectable Philadelphia chromosome positive cells in the bone marrow or partial, less than 35% of Philadelphia chromosome positive cells in the bone marrow, is the best prognostic indicator for long-term progression free survival of the patient[41]. This is in contrast to those patients who despite having achieved a hematologic response do not achieve a cytogenetic response thus having a higher likelihood of relapse. This is what is deemed disease persistence, the occurrence of a hematologic response without a cytogenetic response. These patients, despite having a morphologically normal appearing bone marrow, exhibit persistence of Philadelphia chromosome positive cells, which is predictive of a worsened prognosis.

In contrast to disease persistence in which the WBC counts are within normal limits, overt resistance is the setting in which the patient relapses on treatment such that the drug is no longer effectively keeps the leukocyte count from expanding. In CML, this overt resistance is most commonly of the "on-target" variety, meaning despite the presence of imatinib an ABL kinase domain mutation prevent the drug from binding thus restoring BCR-ABL1 function[43,44]. Overt on-target resistance is the most common form of resistance in CML as

most patients (60%-90% varies by study) exhibiting resistance to imatinib contain a kinase domain mutation[45]. Furthermore, a large number of imatinib resistant kinase domain mutations have been identified using the Baf/3 system, and it was subsequently shown that TKI resistance in CML is largely due to either point mutations in BCR-ABL1 or gene amplification[46]. With improved understanding of these imatinib resistant mutations, dasatinib and nilotinib, two second-generation tyrosine kinase inhibitors were designed to circumvent the shortcomings of the first generation TKI[47].

The second-generation inhibitors of BCR-ABL1 not only inhibit many of the imatinib resistant mutations but also exhibit a potency which far exceeded that of imatinib[48]. This allowed for increased effectiveness of BCR-ABL1 inhibition with lower doses. A head to head study of imatinib versus dasatinib showed that dasatinib treated patients not only achieved deeper responses at earlier time-points but also exhibited improved predicted progression free survival[49]. This led to the approval of dasatinib as a first line agent in newly diagnosed CML. Whereas imatinib and nilotinib are type II inhibitors binding only to the inactive conformation of BCR-ABL1, dasatinib binds both the active and inactive form[50]. Furthermore, dasatinib retains the capacity to inhibit BCR-ABL1 despite the presence of many different imatinib resistance-conferring mutations[51]. Still, a number of dasatinib resistant mutations have been discovered, most notable of which is the T315I gatekeeper mutation in BCR-ABL1[52]. This

particular mutation provided resistance to all three of the then approved BCR-ABL1 inhibitors, and remained a burden to treat until the development of a third generation BCR-ABL1 inhibitor.

The third generation BCR-ABL1 TKI, ponatinib, showed early promise as being the only inhibitor to date with the efficacy against the T315I gatekeeper mutation[53]. Ponatinib was shown to inhibit T315I driven CML in pre-clinical models. Furthermore, in phase II studies 70% of T315I containing chronic phase CML patients achieved a major cytogenetic response[54]. Phase II studies revealed that in all chronic phase patients treated with ponatinib, 94% achieved a hematologic response while 56% achieved a major cytogenetic response. Although this seems promising, patients already suffering from blast crisis had much worse response rates (31% hematologic response, 23% major cytogenetic response). This suggests that although the use of ponatinib shows efficacy against any remaining kinase domain mutations, in advanced stages of the disease, overt off-target resistance mechanisms, of which are currently poorly understood, allow for continued cell survival despite inhibition of BCR-ABL1. Furthermore, as was seen in the chronic phase patients treated with ponatinib, while 94% achieved a hematologic response, only 56% of patients achieved a major cytogenetic response suggesting the existence of a patient cohort that exhibit disease persistence.

Herein lie the two major hurdles in CML treatment, disease persistence and overt-off target resistance. In any given phase of the disease there are patients who achieve a hematologic response and exhibit a morphologically normal bone marrow, yet lack a major cytogenetic response. Persistence of Philadelphia chromosome positive cells in these patients results in a higher likelihood of disease progression and relapse. A central question to be answered is: What cell intrinsic or extrinsic factor(s) may be playing a role in allowing BCR-ABL1 positive cells to persist in the bone marrow despite TKI treatment?

The poorly understood persistence of BCR-ABL1 positive cells despite treatments goes as far back as the pre-TKI era during which interferon was the mainstay of treatment[55]. A number of studies have suggested various underlying causes of disease persistence yet none have been conclusive. *In vitro* studies in which CML cell lines were passaged using imatinib dose escalation revealed increased expression of drug export out of the cell via over-expression of the p-glycoprotein efflux pump, although the amplification and overexpression of BCR-ABL1 was also identified[56]. Drug efflux has not been the only proposed mechanism regarding intracellular drug levels, as it has also been reported that low OCT-1 (organic cation transporter 1) activity may be responsible for diminished intracellular imatinib concentrations in $CD34^+$ CML cells[57]. However this phenomenon was not seen with the second generation TKI nilotinib. In contrast to drug influx/efflux, other work identified the possibility of

autocrine/paracrine signaling as the underlying cause of disease persistence. Specifically, conditioned media from imatinib resistant BCR-ABL1 expressing cells were protective from imatinib and nilotinib induced cell death in primary CML progenitors[58]. Secretion of GM-CSF acting through JAK2/STAT5 pathway mediated this protection. It has even been suggested that disease persistence may merely be the result of increased cellular quiescence and decreased BCR-ABL1 expression level allowing for the insensitivity to imatinib[59,60].

This range of ideas revolves around either the inability of TKI entering the cell, the possibility of extrinsic mitogenic signaling, or the decrease in BCR-ABL1 signaling output. Only one recent study suggests the possibility that disease persistence is due to a cell intrinsic, BCR-ABL1 independent mechanism[61]. Using an *in vivo* approach, these investigators used retroviral insertional mutagenesis in imatinib resistant BCR-ABL1 leukemic cells to identify overexpression of two related transcription factors, RUNX1 and RUNX3. These transcription factors are known to play a role in maintaining the hematopoietic stem cell compartment[62]. A translocation between chromosome 3 and 21 has been shown to form a fusion protein of RUNX1 and the ecotropic viral integration site 1 (EVI1) gene product, which itself is known to play a role in leukemogenesis[63]. Furthermore, 3q26 abnormalities, which lead to EVI1 over-expression, were reported in 12% of blast crisis CML patients without prior TKI treatment but as high as 39% of those patients who had previously been treated.

This is suggestive that EVI1 may play a possible role of mediating TKI resistance in blast crisis progression[64].

EVI1 is a zinc-finger transcription factor that is normally expressed and functions within the bone marrow to maintain the hematopoietic stem cell compartment[65]. While EVI1 knockout mice are not viable past 3 weeks of life due to defects in vascularization, over-expression of EVI1 has been associated with myelodysplastic syndrome and various myeloid leukemias[65]. Over-expression is thought to contribute to leukemogenesis by blocking differentiation, a known phenotype in acute myeloid leukemia. While the mechanism through which EVI1 contributes to myeloid transformation is not known, studies have suggested that EVI1 transcriptional repression of TFG-Beta and/or PTEN may contribute[65–67]. While a mechanism has yet to be clearly elucidated, over-expression of EVI1 in AML patients results in a poor prognosis[68,69]. In addition, EVI1 over-expression has also been shown to induce a myelodysplastic syndrome/phenotype both *in vitro* and *in vivo*[70,71].

In CML, EVI1 over-expression was detected in as high as 71% of blast crisis CML patients and its expression was predictive of survival with use of second generation TKIs in imatinib resistant, chronic phase CML patients[72,73]. Early studies in CML showed that cooperation between BCR-ABL1 and the AML1/MDS/EVI1 fusion was sufficient to induce AML in a murine model, and

more recently, EVI1 overexpression alone was shown not only to cooperate with BCR-ABL1 in producing a blast crisis CML disease but also provide resistance to nilotinib treatment *in vivo*[74,75]. Given that EVI1 is often overexpressed in blast crisis CML, that its expression may contribute to the progression through the stages of CML, and that it has been shown to cooperate with BCR-ABL1 to promote a blast crisis CML phenotype displaying some degree of relative TKI resistance suggests a very possible role for EVI1 in mediating disease persistence in CML.

EVI1 cooperation may not be limited to BCR-ABL1, but might also interact with oncogenic NRAS to induce AML. Furthermore, EVI1 has also not only been shown to be commonly co-expressed with activating mutations in NRAS it was the most common gene overexpressed following a retroviral mutagenesis screen of murine AML driven by G12D activated NRAS[68,76]. While EVI1 may be a very plausible cause of disease persistence in CML, the other major therapeutic roadblock treatment - overt off-target resistance - is also poorly understood. This was described previously in the phase II trial of ponatinib whereby in blast crisis CML only 31% achieved a hematologic response, suggesting a large percentage of patients still exhibit some form of BCR-ABL1 independent mechanism of resistance.

As previously mentioned, kinase domain mutations preventing the binding of BCR-ABL1 inhibitors has largely been the major form of overt resistance encountered clinically to date. With imatinib treatment comes numerous possible mutations many of which can be inhibited with the use of second generation TKIs such as dasatinib and nilotinib, yet those too are not immune to a small group of resistance-conferring kinase domain mutations. Given the recent approval of the pan-BCR-ABL1 inhibitor ponatinib it seems likely that with improved control over BCR-ABL1 function off-target mechanisms will comprise a larger percentage of overt resistance seen in relapsing patients.

Currently, overt off-target resistance in CML is poorly understood. Some studies have suggested a number of possible mechanisms ranging from drug sequestration in the plasma, resistance to apoptosis through epigenetic modification by HDAC proteins and even FGF-2 mediated rescue[52,77]. The other major possible mechanism through which overt off-target resistance may occur is the activation of alternative signaling pathways. As mentioned previously, BCR-ABL1 signals down at least three canonical pathways and the acquisition of an activating mutation in one of those three pathways may provide a potent enough proliferative signal to mediate resistance to tyrosine kinase inhibitor therapy. Most notable of the three pathways, the RAS/MAP kinase pathway has already been shown to provide a mechanism of off-target resistance in other models including FLT3 driven AML as well as $BRAF^{V600E}$ melanoma cell lines[78–81]. The

possibility of RAS-driven overt off-target resistance in CML would not be surprising given its strong proliferative capabilities and previous evidence suggesting such a function in a number of other disease models.

Summary:

With the advancements in the development of BCR-ABL1 tyrosine kinase inhibitors, the ability to inhibit BCR-ABL1 containing any particular kinase domain mutation continues to improve. The latest addition to the repertoire, ponatinib, a pan-BCR-ABL1 inhibitor now inhibits all known resistance mutations. Still, two major hurdles in CML treatment remain. First, disease persistence, as defined by the achievement of a hematologic response in the absence of a cytogenetic response and overt off-target resistance, remains poorly understood. Second, loss of a hematologic response resulting in clinical relapse remains at the nexus of our understanding of CML resistance and treatment. Despite speculation regarding possible underlying factors of disease persistence and overt off-target resistance, no definitive mechanisms have been identified.

Part III: Objective of Thesis

Despite a thorough understanding of the most common type of overt resistance seen in CML (on-target kinase domain mutations), very little is known about the mechanisms underlying either disease persistence or off-target resistance in CML. With the clinical use of ponatinib it is these forms of resistance that will likely become the predominant mechanisms encountered in patients. Thus, it is vital to interrogate and understand the possible underlying mechanisms mediating these forms of resistance.

In Chapter 2 I present work describing the use of CML patient samples exhibiting either disease persistence in the chronic phase of the disease or overt off-target resistance in the blast crisis phase as models to begin interrogating these mechanisms of resistance. I go on to identify potential oncogenic lesions underlying disease persistence and overt-off target resistance in chronic phase and blast crisis CML, respectively. Furthermore, validation using murine models and patient-derived blast crisis CML cell lines allowed for a better understanding of not only these forms of resistance but may help shed light on oncogene addiction, the re-wiring of cellular dependence, and cooperation of oncogenes in CML.

Chapter 2:

Genetic Determinants of Disease Persistence and Overt Off-Target Resistance to TKI Therapy in Chronic Myeloid Leukemia

Abstract

Chronic myeloid leukemia (CML), driven by the fusion protein BCR-ABL1, remains highly responsive to treatment with tyrosine kinase inhibitors (TKIs). Although overt resistance and relapse typically occurs through on-target kinase domain mutations, 25-50% of all resistant cases lack such mutations. With the approval of ponatinib, effective inhibitors for all known drug-resistant mutations are available, suggesting that poorly understood off-target resistance mechanisms will henceforth constitute a greater percentage of overt clinical resistance. Through the use of CML patient samples, we sought to define molecular mediators of disease persistence (the occurrence of a hematologic but no cytogenetic response) and overt off-target resistance (a complete loss of cellular response to BCR-ABL1 inhibition). Chronic phase CML patients exhibiting overexpression of the *EVI1* oncogene displayed disease persistence, while the identification of two blast crisis CML patients exhibiting overt off-target resistance were found to contain EVI1 overexpression in conjunction with somatic *NRAS* mutations. CML cell lines expressing $NRAS^{Q61K}$ exhibited maintenance of cell viability despite TKI treatment and this overt resistance correlated with increased MAPK signaling. In all, we show that EVI1 over-expression can contribute to disease persistence and the presence of activating RAS mutations can lead to overt resistance to BCR-ABL1 tyrosine kinase inhibitors.

Statement of Significance

With the approval and use of the pan BCR-ABL1 inhibitor, ponatinib, the landscape of resistance in CML is poised to change. A shift from on-target resistance mechanisms such as kinase domain mutations to the more poorly understood off-target mechanisms will likely be seen in a higher proportion of relapsing patients. Here we have interrogated two types of off-target resistance, disease persistence and overt off-target resistance, both of which have been encountered in the clinical setting. We have found that EVI1 overexpression in the setting of BCR-ABL1 can provide a degree of TKI resistance that may contribute to the disease persistence identified in two independent chronic phase CML patients. Furthermore, analysis of blast crisis CML patients exhibiting overt off-target resistance to TKI therapy revealed EVI1 overexpression in conjunction with NRAS mutations. It was found that activation of MAP kinase signaling in CML cell lines revealed overt resistance to BCR-ABL1 TKI indicating that in fact this potent proliferative pathway can contribute to off-target resistance in CML. These results predict that in patients for whom ponatinib treatment fails to achieve a deep, durable response, the identification of potential off-target mechanisms of resistance, such as EVI1 overexpression alone or in combination with NRAS activation, may provide a basis for the resistance seen. Furthermore, given that the NRAS-driven TKI resistance CML cells retained sensitivity to combination therapy with MAP kinase inhibition and BCR-ABL1 TKI, these patients may benefit from a dual drug treatment regimen.

Introduction

BCR-ABL1, a fusion protein expressed following a 9;22 chromosomal translocation, is the disease causing oncogene in CML. This disease remains largely responsive to BCR-ABL1 inhibitors such as imatinib, a first generation targeted kinase inhibitor (TKI)[2,40]. Inhibition of BCR-ABL1 leads to very deep and durable remission in patients suffering from the more indolent chronic phase of CML[82]. Although some degree of response is universally achieved (95% complete hematologic response; IRIS trial), a small proportion of chronic phase patients relapse on imatinib[83]. Furthermore, for those suffering from the advanced blast crisis phase of the disease, relapse rates are significantly increased[82,84].

The best long term prognosis is seen in patients exhibiting both a hematologic response (a normalization of WBC count) in addition to a complete cytogenetic response (the inability to detect the BCR-ABL1 fusion)[41]. Alternatively, disease persistence can be defined as achievement of a hematologic response with continued detection of the BCR-ABL1 fusion in bone marrow cells. Overt resistance is a complete lack of response, both hematologic and cytogenetic, either at treatment initiation or as loss of a previously established response despite continuation of therapy. Overt resistance can be sub-categorized as on-target, relying on reactivation of the inciting oncogene, or off-target, the loss of dependence on the original oncogene with newly established reliance on

alternative survival pathways. The only characterized mechanisms of overt resistance in CML to date are of the on-target variety, such as genomic amplification of BCR-ABL1 and BCR-ABL1 kinase domain mutations leading to the inability of TKI binding[45]. At the present time, the two major gaps in our understanding of CML are related to the molecular basis of disease persistence and overt resistance.

Traditionally, the term resistance has lumped together both disease persistence and overt resistance. Given this grouping, ~60% of imatinib-resistant patients contain a kinase domain mutation to explain their phenotype[85]. The remaining ~40% of patients exhibit resistance in the absence of this on-target mechanism. Second-generation tyrosine kinase inhibitors (TKIs) dasatinib and nilotinib address some of the on-target resistance with their ability for continued binding despite kinase domain mutations[86]. Still, the T315I gatekeeper mutation remains resistant to second generation TKIs and only recently has the use of a third generation TKI, ponatinib, shown efficacy against this mutation[53,87]. Despite such promise, a recent look at ponatinib efficacy has shown shortcomings[54]. Specifically, only 31% of blast crisis patients who failed prior TKI achieved a hematologic response on ponatinib and fewer still were those who achieved a cytogenetic response[54]. Although the use of ponatinib will result in improved control over BCR-ABL1, off-target mechanisms of resistance will likely comprise a larger percentage of relapsing patients.

Disease persistence can be seen in patients whose bone marrow remains morphologically normal having achieved a hematologic response, yet the BCR-ABL1 fusion can still be detected in a large percentage of that marrow sample. It is these patients exhibiting a hematologic response in the absence of a cytogenetic response, whose disease persistence indicates a worsened prognosis[41]. Such mechanisms of persistence are largely unknown although speculation includes decreased BCR-ABL1 expression, drug efflux pumps, organic cation transporters, p53 deletion and RUNX1/3 overexpression either alone or in the RUNX1-EVI1 fusion protein[60,61,88–90]. The RUNX1-EVI1 fusion, also known as AML1-EVI1, cooperates with BCR-ABL1 to induce murine AML[74]. EVI1 is a transcription factor known to regulate genes expressed in the hematopoietic stem cell compartment[91]. At normal levels EVI1 functions to maintain this compartment, but its over-expression has been linked to myelodysplastic syndrome and AML[69]. Inversions or translocations involving the 3q26 locus typically result in EVI1 overexpression and, in the setting of CML, EVI1 transcript levels predicted response to second generation TKI following imatinib failure[73,92]. Furthermore, EVI1 activation has been associated with the progression of CML from the chronic phase of the disease to blast crisis, and increased EVI1 expression was seen in cytogenetic non-responders over responders following 12 months of imatinib therapy[64,93,94]. Recently, EVI1 overexpression has revealed a leukemia initiating capacity by cooperating with BCR-ABL1 to not only induce a blast crisis CML phenotype in mice but one

which exhibited relative nilotinib resistance over the non-EVI1 expressing BCR-ABL1 mice[75]. This suggests a possible role for EVI1 in mediating disease persistence in CML. Aside from disease persistence, and given the approval of ponatinib, the other major hurdle in CML treatment is likely to be overt off-target resistance.

Overt off-target resistance has previously been described in models of AML and melanoma in which mitogen-activated protein kinase (MAPK) pathway activation was responsible[80,81]. This pathway has been shown repeatedly to play a key role in resistance mechanisms as further demonstrated by the discovery of activating MEK mutations in melanoma patients resistant to dabrafenib[78]. This observation suggests that a potent proliferative signaling pathway such as MAPK can play a role in overt-off target resistance. In this study we sought to use patient samples to identify and validate novel mechanisms of disease persistence and overt resistance in CML, both of which are currently poorly understood and are likely to emerge as the next major clinical roadblocks in treating this disease.

Results

EVI1 overexpression correlates with relative resistance to BCR-ABL1 inhibitors in chronic phase CML.

The poor characterization of disease persistence in CML led us to investigate a potential mechanism through which this phenomenon may occur using two independent patient samples (4057 and 4089)(Figure 1A). Clinically, these patients presented in the chronic phase of CML and, while both demonstrated a complete hematologic response to dasatinib, only patient 4057 achieved a cytogenetic response (Figure 1A). Interestingly, patient 4089 while on treatment developed a 3q26 genetic abnormality that resulted in EVI1 overexpression (Figure 1B). To investigate the colony forming capacity between these samples and identify disease persistence in chronic phase CML, colony forming unit assays were performed from bone marrow of these patients in the absence and presence of dasatinib (Figure 1C). While the bone marrow cells of both formed similar colony numbers in the absence of dasatinib, patient 4089 exhibited increased growth compared to patient 4057 in the presence of dasatinib. A third chronic phase patient sample, 4051, containing a 3q26 abnormality and displaying EVI1 overexpression also exhibited clinical disease persistence despite TKI treatment (Figure 1B). Therefore, EVI1 overexpression appears to correlate with relative resistance to BCR-ABL1 inhibition.

To further investigate the role of EVI1 in mediating relative resistance to TKI treatment, an EVI1 overexpressing murine model was employed (Supplementary Figure 1). Bone marrow PreGM populations from control and EVI1 overexpressing mice were transduced with either empty vector or pMIG+BCR-ABL1, and subsequent 7-day dasatinib exposures were performed in both liquid cultures as well as CFU assays. In both assays, BCR-ABL1 expressing pre-GM cells exhibited sensitivity to dasatinib while the BCR-ABL1/EVI1 expressing population displayed relative resistance to TKI treatment (Figure 1D and Figure 1E).

Blast crisis CML patient samples exhibiting overt TKI resistance display EVI1 overexpression and persistent MAPK signaling despite BCR-ABL1 inhibition.

Like disease persistence, off-target resistance is not only poorly characterized, but will likely emerge as a clinical form of overt resistance. To interrogate this further, two independent blast crisis patient samples, 1688 and 1755, were used. Both patients exhibited overt clinical resistance to BCR-ABL1 inhibitors in the absence of any kinase domain mutations that could explain the phenotype (Supplemental Figure 2). Furthermore, cytogenetic analysis revealed 3q26 abnormalities, and EVI1 overexpression was confirmed by qPCR and western blot analysis (Supplemental Figure 2, Figure 2A and Figure 2B). Following a 2-hour treatment of 1688 cells, K562 cells and KU812 cells (2 patient

derived blast crisis CML cell lines) with 100nM dasatinib, we observed that BCR-ABL1 inhibition was achieved as demonstrated by modulation of phosphorylated BCR-ABL1 and phosphorylated CRKL, a downstream target of BCR-ABL1 (Figure 2B). It has been shown that of the three canonical pathways downstream of BCR-ABL1 (JAK/STAT, MAPK, and PI3K), maintenance of two out of those three pathways is required for BCR-ABL1 driven cell survival[10]. We found that dasatinib treatment modulated the JAK/STAT pathway in 1688 cells, as well as in the cell line controls (K562 and KU812)(Figure 2C). The PI3K pathway, although not modulated by dasatinib treatment, typically requires longer drug exposures to achieve an effect (data not shown). Surprisingly, the MAPK pathway, normally modulated by BCR-ABL1 inhibition, was unchanged in the 1688 sample despite inhibition of BCR-ABL1 (Figure 2C). This finding spurred the gene specific sequencing of NRAS/KRAS, which revealed $NRAS^{G12D}$ and $NRAS^{Q61K}$ mutations in the 1755 and 1688 patient samples, respectively (Figure 2D). Furthermore, these results prompted RAS gene specific sequencing of the chronic phase CML patients that exhibited disease persistence (Figure 1: patient samples 4089 & 4083), although RAS mutations were not identified.

***In vitro* expression of oncogenic NRAS in CML cell lines confers overt TKI resistance.**

Having found NRAS activation in blast crisis CML patients, we recapitulated the TKI resistant phenotype using BCR-ABL1 dependent cell lines.

Two different cell lines - KU812 cells as well as TF1 BCR-ABL1 cells (a human erythroleukemic cell line expressing and dependent on BCR-ABL1[95]) - were modified to express various NRAS alleles (WT, G12C, G12D, Q61K). Dasatinib potently inhibited the cellular viability of the KU812+Empty MIG and KU812+$NRAS^{WT}$, cells while the $NRAS^{G12C}$ and $NRAS^{G12D}$ mutant cells exhibited ~5-fold increase in the IC50 values suggesting a degree of resistance (Figure 3A, *left panel*). The $NRAS^{Q61K}$ expressing KU812 cells exhibited the highest level of dasatinib resistance, with greater than 50-fold increase in its IC50 value relative to $NRAS^{WT}$ cells. TheTF1+BCR-ABL1 cells demonstrated a similar pattern of resistance to the KU812 cells, although the results were more dramatic (Figure 3A, *right panel*). Particularly, the relative resistance of the $NRAS^{G12C}$/$NRAS^{G12D}$ cells was greater compared to the empty vector and $NRAS^{WT}$ cells, while the $NRAS^{Q61K}$ cells displayed complete resistance to dasatinib even at the highest concentrations used (Figure 3A, *right panel*).

Having identified TKI resistance by various NRAS alleles, we next performed RAS immuno-precipitation assays in an effort to identify potential differences in the levels of RAS activation between alleles. In both the KU812 cells and TF1+BCR-ABL1 cells, GTP loaded RAS levels were elevated in the $NRAS^{G12C}$, $NRAS^{G12D}$, and $NRAS^{Q61K}$ cells relative to the empty vector and $NRAS^{WT}$ cells (Figure 3B and Supplemental Figure 3A). In the TF1+BCR-ABL1 cells, the $NRAS^{Q61K}$ expressing cells exhibit the highest degree of resistance to dasatinib

followed by $NRAS^{G12C}$ and then $NRAS^{G12D}$, which correlated with the levels of activated RAS (Figure 3A&B and Supplemental Figure 3B).

To ensure adequate BCR-ABL1 inhibition was achieved by dasatinib treatment, western blot analysis was performed on the various NRAS-expressing KU812 cells and TF1+BCR-ABL1 cells (Figure 3C and Supplemental Figure 3C). BCR-ABL1 inhibition was seen by modulation of phospho-BCR-ABL1 and phospho-CRKL. In contrast, phospho-ERK levels were both elevated and maintained despite the presence of dasatinib in the $NRAS^{G12C}$, $NRAS^{G12D}$, and $NRAS^{Q61K}$ expressing KU812 and TF1+BCR-ABL1 cell lines (Figure 3C and Supplemental Figure 3C). Furthermore, there was a lack of any noticeable change in the PI3K pathway as determined by phospho-AKT. In addition, these cells displayed no sensitivity to the PI3K inhibitor PIK90 following a 48-hour treatment suggesting that in conjunction with the maintenance of the MAPK pathway despite BCR-ABL1 inhibition, the resistant phenotype is likely due to MAPK activation alone (Supplementary Figure 3D, Supplementary Figure 3E and Figure 3C).

To confirm MAPK pathway signaling as mediating overt off-target resistance to BCR-ABL1 inhibition, the TF1+BCR-ABL1cells were transduced with $NRAS^{Q61K}$, MEK^{WT}, and MEK^{DD} (a constitutively active form of the MEK allele)[96]. Although the MEK^{WT} expressing cells do not exhibit resistance, the MEK^{DD} cells mirror the results seen in the $NRAS^{Q61K}$cells, suggesting the resistance downstream of

NRAS is mediated through the MAPK pathway (Figure 3D). Furthermore, the cells expressing these resistance-conferring alleles displayed cross-resistance to the third generation inhibitor ponatinib, validating this mechanism as truly being off-target (Figure 3E).

MEK/BCR-ABL1 combination therapy remains a viable therapeutic option for the treatment of oncogenic NRAS-mediated overt off-target resistance in CML.

To investigate the potential therapeutic option of MAPK inhibition, KU812 cells expressing various NRAS alleles were treated for 48 hours with either 100nM dasatinib, 100nM PD0325901 (a potent MEK inhibitor) or the combination (Figure 4A and Figure 4B). As expected, the G12C, G12D and Q61K NRAS expressing cells exhibited increased viability in comparison to the empty vector and WT NRAS expressing cells in the presence of dasatinib. In contrast, the use of a MEK inhibitor resulted in sensitivity as shown by a decrease in viability compared to WT NRAS cell line (Figure 4A). Interestingly, the combination treatment restored dasatinib sensitivity and elicited inhibition of cell viability in all groups (Figure 4A). Western blot analysis demonstrated inhibition of MEK signaling using this concentration of PD032901 (Figure 3C and Supplementary Figure 3C). Furthermore, with dasatinib treatment alone, the $NRAS^{Q61K}$ cells displayed an increase in the percentage of live cells over the control group as measured by caspase3 activation (Figure 4B). Results from similar experiments

performed with the TF1+BCR-ABL1 cells were comparable to the KU812 line, although the degree of resistance, as assessed by both viability and the percentage of live (caspase3 negative) cells, was more dramatic in the TF1+BCR-ABL1 cells (particularly with the $NRAS^{Q61K}$ cells) (Supplemental Figure 4B and Supplementary Figure 4C). In addition, the TF1+BCR-ABL1 cells expressing $NRAS^{Q61K}$ maintained cell viability even after five days of continued dasatinib treatment (Supplementary Figure 5A and Supplementary Figure 5B). Still, in the TF1+BCR-ABL1 cells, the combination treatment was sufficient to induce apoptosis in a majority of the cells expressing various NRAS alleles (Supplemental Figure 4B and Supplementary Figure 4C).

For patients suffering from this form of overt off-target resistance, we aimed to investigate whether the clinically available MEK inhibitor trametinib could be used as combination therapy. Viability assays were done in the TF1+BCR-ABL1 cells transduced with either empty vector, $NRAS^{Q61K}$, MEK^{WT}, or MEK^{DD} to assess for sensitivity to 100nM dasatinib, the MEK inhibitor trametinib (100nM) or the combination treatment. Much like the $NRAS^{Q61K}$ expressing cells, the MEK^{DD} allele provided overt resistance to dasatinib while combination therapy restored dasatinib sensitivity (Figure 4C). The cellular sensitivity to the trametinib, in combination with dasatinib, suggests that a possible therapeutic option already exists for patients with this form of resistance. To further interrogate the ability of trametinib to restore dasatinib sensitivity the NRAS

expressing TF1+BCR-ABL1 cells were treated with either PD901 or trametinib alone and in combination with dasatinib. Assays revealed similar cellular sensitivities to PD901 alone and trametinib alone (Supplementary Figure 4D, Supplementary Figure 4E, Supplementary Figure 5C and Supplementary Figure 5D). Further, the combination of either MEK inhibitor with dasatinib resulted in decreased cell viability and increased caspase activation (Supplementary Figure 4D and Supplementary Figure 4E).

This TKI resistance and dual-combination therapy sensitivity was not limited to mutant NRAS expressing KU812 and TF1+BCR-ABL1 cells. In fact, a third patient derived, BCR-ABL1 dependent, blast crisis CML cell line, K562, also demonstrated slight dasatinib resistance. While PD901 alone had no effect, combining this MEK inhibitor with dasatinib resulted in decreased cell viability (Supplementary Figure 6A-6C). K562 expressing mutant alleles of NRAS also exhibited increased RAS-GTP levels and persistent MAPK signaling despite BCR-ABL1 inhibition (Supplementary Figure 7A-7B) suggesting a true MAP kinase signaling dependence by these cells. Three independent BCR-ABL1 dependent cell lines all exhibited levels of TKI resistance upon ectopic expression of activated NRAS, although the greatest degree of resistance was seen with the Q61K allele in all three cell lines. More importantly, the dependence on MAP kinase signaling in these cells can be exploited through the use of combination therapy with both BCR-ABL1 TKIs, such as dasatinib, and

MEK inhibitors, such as PD901 or trametinib. This provides evidence for a possible therapeutic intervention for patients suffering from this form of overt off-target resistant CML.

Discussion

Disease persistence and overt TKI resistance represent two of the most pressing currently unmet medical needs in CML. Within the category of overt resistance lies two types; on-target, the most common form seen in CML, and off-target. Although currently less common, off target resistance will likely become the predominant mechanism encountered clinically following improved BCR-ABL1 inhibition with compounds like ponatinib. Moving forward, we must understand these mechanisms and aim to identify future targets of therapy. Here we have shown that chronic phase patients with EVI1 overexpression demonstrate a relative resistance to dasatinib in comparison to control chronic phase patients without a 3q26 abnormality. Resistance was also demonstrated using murine mouse models of EVI1 overexpression. This may suggest a role for EVI1 in mediating disease persistence, a form of resistance commonly seen in CML patients. Further, the function of EVI1 in TKI resistance may contribute to other clinical observations that suggest its role in contributing to the progression from chronic phase to blast crisis[64].

In an attempt to identify overt off-target resistance in CML two independent blast crisis CML patients exhibiting this form of resistance were studied. Genetic findings include the presence of 3q26 abnormalities, suggestive of EVI1 overexpression and activating NRAS mutations. These samples displayed persistent MAPK signaling despite BCR-ABL1 inhibition, which spurred the

validation of this phenotype in CML cell lines. Specifically, we confirmed that the expression of activated NRAS in the setting of BCR-ABL1 provides a mechanism of overt off-target TKI resistance. This mirrors a previously discovered clinical case whereby a patient exhibiting overt off-target resistance was found to contain a *KRAS*^{T58I} mutation, although the contribution of the mutation to the resistance is unclear as the *KRAS*^{T58I} became undetectable upon relapse[97]. That mutation was further shown to provide resistance to imatinib and dasatinib in 32D cells expressing BCR-ABL1 suggesting the importance of MAPK signaling mediating this phenomenon[97]. Validation of MAPK as the crucial signaling pathway for resistance was shown by the use of an activated MEK allele as well as demonstration of dasatinib re-sensitization when used in conjunction with a MEK inhibitor. The therapeutic efficacy of combination therapy was seen with PD901 but, more importantly, with the chemically related and clinically available GSK inhibitor, trametinib. Although RAS has been shown to signal down a number of regulatory pathways, our data strongly implicate the MAPK pathway as mediating its pro-growth/pro-survival phenotype. The importance of this pathway has previously been demonstrated in both melanoma and lung adenocarcinoma but had not, until now, been identified in the setting of BCR-ABL1[98,99].

In regards to EVI1, we must speculate that in accordance with the two-hit hypothesis, and evidence suggesting a role for EVI1 in the progression from chronic phase to blast crisis, that the acquisition of a 3q26 abnormality may just

be one component of the larger picture that is overt off-target resistance. Additionally, MAPK activation cannot be sufficient to supplant BCR-ABL1 function, as activation of this pathway alone has never been seen as a cause of CML. Furthermore, EVI1 overexpression and activated NRAS alleles commonly co-occur and have been shown to play a role in the production of an AML phenotype[68,76]. It may indeed be that EVI1 overexpression enables bypass of BCR-ABL1 addiction in primitive hematopoietic cells. In addition, Evi1 expression may set the stage for a block in differentiation, a known function of EVI1 and common feature of blast crisis CML[2,91]. Although no block in differentiation was seen in our chronic phase patients, the idea that an expression threshold is required for this block in differentiation could hold true. The second oncogenic hit, activation of NRAS, would then be all that is needed for the emergence of a highly proliferative clone indifferent to the presence of BCR-ABL1 inhibition, described here as overt resistance. If the order of mutation acquisition is important, early identification of disease persistence or 3q26 abnormality may provide insight clinically that overt off-target resistance is a mere NRAS mutation away. In the unfortunate event of that second mutation, the transformed disease would no longer be sensitive to BCR-ABL tyrosine kinase inhibitors. Further investigation into the importance of acquisition order could help provide insight into how cells are re-wired during the process of “oncogene addiction”.

Here we have begun to dissect the clinical difference between the poorly understood disease persistence and overt resistance in CML. These data suggest a possible role for EVI1 in maintaining persistent disease in the chronic phase while its cooperation with activated NRAS may be what is needed to allow for overt off-target resistance in CML, a phenomenon with a likely increasing incidence as we become more adept at targeting the currently well understood and more common mechanism of on-target resistance. Furthermore, these data have provided evidence that a clinically available MEK inhibitor, such as trametinib, may be a valuable component of combination therapy for select cases of off-target TKI resistance in CML.

Experimental Methods

Cell line propagation and generation of cell lines

Cell lines were propagated as previously described[95]. To generate *N-RAS/MEK* expressing cell lines, KU812 cells were first engineered to express the ecotropic receptor (EcoR) through lentiviral transduction (pMOWS-EcoR plasmid). Virus was produced in 293T cells using lipofectamine 2000 (Invitrogen) and transductions were as previously described[95]. Previously engineered TF1-p210 BCR-ABL1 cells[95] and KU812+Eco-R cells underwent transduction with pMSCV-IRES-GFP (pMIG empty vector), pMIG+$NRAS^{WT}$, pMIG+$NRAS^{G12C}$, pMIG+$NRAS^{G12D}$, pMIG+$NRAS^{Q61K}$, pMIG+MEK^{WT}, or pMIG+MEK^{DD} retrovirus. All pMIG+*NRAS* and pMIG+*MEK* constructs were engineered as GFP-fusion proteins to *NRAS/MEK* and GFP expression was normalized between all cells.

Patient Sample Sequencing and Quantitative PCR

Targeted *NRAS* sequencing was performed using patient genomic DNA following amplification of *NRAS* exons 1/2 with the following primers: Exon 1 (For 5' - AGGCCGATATTAATCCGGTG - 3'; Rev 5' - GGACAGGTTTTAGAAACTTCAGC - 3') and Exon 2 (For 5' - TGGGCTTGAATAGTTAGATGC - 3'; Rev 5' - TGTGGTAACCTCATTTCCCC - 3'). Total RNA extraction was performed using the Trizol reagent protocol (Invitrogen) and used for quantitative PCR (qPCR) assays. cDNA generation was done as previously described[95]. qPCR analysis

was performed using TaqMan probes (Life technologies) and TaqMan universal master mix II (Invitrogen): GAPDH (Hs02758991_g1), EVI1 (00602795_m1). Analysis was performed using the Viia 7 Real Time PCR System (Applied Biosystems).

Colony Forming Unit (CFU) Assay

Mononuclear cells were isolated from the bone marrow samples of clinical subjects and were used to perform CFU assays. 1 x 10^5 cells/ml or 9 x 10^5 cells/ml were plated in H4434 Methocult media (Stem Cell technologies) in the absence or presence of dasatinib (5nM or 25nM). Done in triplicate, CFU-GM colony numbers were counted on day 14. For the mouse CFU assays, 500 GFP^+ PreGM cells were plated in methylcellulose (M3231, STEMCELL technologies), supplemented with 0.1mM b-mercaptoethanol, 100-units/ml glutamine and 100 units/ml Pen/Strep. Cells were stimulated with 10ng/ml GMCSF (PeproTech) and treated with Dasatinib or DMSO at the indicated concentrations. Done in triplicate, colony counting was done on day 7.

Mice

Vav-LSL-*Evi1* FVB/n mice were bred with *Mx1-Cre* C57/Bl6 mice to generate animals that ectopically express *Evi1* in hematopoietic cells upon treatment with pIpC (Sigma). Mice were administered pIpC via i.p. injection at 21 days of age

and experiments performed 4 weeks post-injection. The UCSF Committee on Animal Research approved the experimental procedures.

Mouse PreGM cell transduction, liquid culture assay

Nucleated bone marrow cells were stained with an antibody cocktail to detect PreGM cells (Lin-, c-Kit+, Sca1-, CD16/32-, CD34+, CD105-, CD150-). 25,000 PreGM cells were sorted with a BD FACSAriaIII cell sorter (BD Biosciences) and stimulated in MyeloCult M5300 (STEMCELL Technologies) supplemented with 15% FBS, 20ng/ml SCF, 20ng/ml IL-3, 20ng/ml IL-6, 100-units/ml glutamine and 100 units/ml PenStrep for 3 hours. Cells were then mixed with retroviral supernatant (1:1), 5ug/ml polybrene, and 10mM HEPES and centrifuged at 260 x g for 1 hour at 30^0C and incubated at 37^0C, 5% CO_2. GFP expression was determined at 48 hours by flow cytometry. Liquid culture assays were setup using 300 GFP^+ PreGM cells plated into 96-well plates (CoStar) and cultured in Isocove's modified Dulbecco's medium (IMDM), 20% FBS, 0.1mM b-mercaptoethanol, 100 units/ml glutamine and 100 units/ml PenStrep. Cells were stimulated with 10ng/ml GMCSF and treated with dasatinib or DMSO at the indicated concentrations. Cells were counted by flow cytometry on day 7.

Kinase Inhibitors and Drug Treatment

Stock solutions of dasatinib/ponatinib in DMSO were generated at UCSF. The MEK inhibitor PD0325901 was purchased from Selleckchem and GSK1120212

(trametinib) was obtained from the Shannon Lab at UCSF. Drug exposures for western immunoblot assays were done as previously described[95].

Cell lysis, Antibodies, Western Immunoblot, and Immunoprecipitation

Cell lysates were harvested, normalized and separated as previously described[100]. Antibodies for ABL (phospho Y245) (cat. 2861), CRKL (phospho Y207/total) (cat. 3181/cat. 3182), EVI1 (cat. 2593), STAT5A/B (phospho-Y695/Y699 and total) (cat. 9351 and 9363), ERK1/2(phospho-T202/Y204 and total)(cat. 4370 and 9107), AKT (phospho-S473/total) (cat. 4060/9272) were purchased from Cell Signaling Technology. Antibodies against total ABL (cat. OP20) and total RAS (cat. 05-516) were purchased from Millipore. Antibody against Total NRAS (cat. sc-519) and Total GAPDH (cat. sc-25778) were purchased from Santa Cruz. Licor and Odyssey imaging technology was utilized for western blot visualization. Following a 3-hour starve RAS-GTP assays were performed using RAS-IP lysis buffer (50mM Tris pH 7.5, 125mM NaCl, 6.5mM $MgCl_2$, 5% glycerol, 0.2% NP40). Following normalization, cell lysates were tumbled for 1 hour at 4°C with 20μL of RAS assay reagent (Millipore; #14-278).

Flow Cytometry and Cleaved Caspase activation

Apoptosis (Cleaved caspase 3) was measured at 48 hours by flow cytometry using APC-conjugated anti-active caspase-3 antibody purchased from BD biosciences (cat. 560626).

Viability and Apoptosis assays

Cells were plated at 2 x 10^5 cells/ml in 96 well plates (0.1ml total volume) with the appropriate drug type and concentration in triplicate. Viability and apoptosis (caspase 3/7 activation) were assessed at 48-hour by CellTiter-Glo or Caspase-Glo reagent (Promega), respectively, on a Spectramax M3 microplate reader (Molecular Devices). Viabilities and IC50 plots were analyzed using Prism 5 software (GraphPad).

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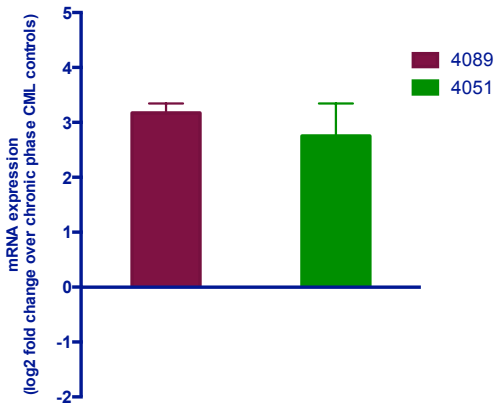
Figure 1. EVI1 overexpression is associated with diminished sensitivity to BCR-ABL1 inhibition in chronic phase CML patients.

(A) Clinical histories of chronic phase CML patients. (B) EVI1 mRNA expression levels, measured by qPCR, in chronic phase CML patients with a 3q26 abnormality relative to control chronic phase CML patients without a 3q26 abnormality (errors bars represent s.d. of triplicates from the same experiment). (C) Colony forming unit (CFU) assay of bone marrow cells from chronic phase CML patients without (4057) and with (4089) an EVI1 overexpressing 3q26 genetic abnormality. Cells were plated in 5nM and 25nM dasatinib and counted on day 14. (D) 7-day viability assay and (E) CFU assay comparing WT and EVI1 overexpressing mice with or without BCR-ABL1 in the presence or absence of (D) 5nM or (E) 250nM dasatinib.

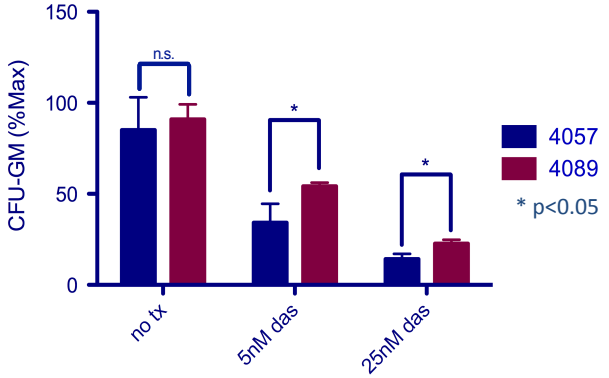
A.

Patient	Disease Phase (at time of diagnosis)	Treatment History	Subsequent Hematologic Response	Cytogenetic (at time of diagnosis)	Cytogenetics (following TKI treatment)
4057	Chronic Phase CML	Imatinib	Complete Hematologic Response	Ph^+	CCR
4089	Chronic Phase CML	Imatinib Dasatinib	Complete Hematologic Response	Ph^+	Ph^+; t(3;7)(q26.2;q21)
4051	Chronic Phase CML	Imatinib	Complete Hematologic Response	Ph^+	Ph^+ 20/20 t(2;3)(p21;q26) 19/20

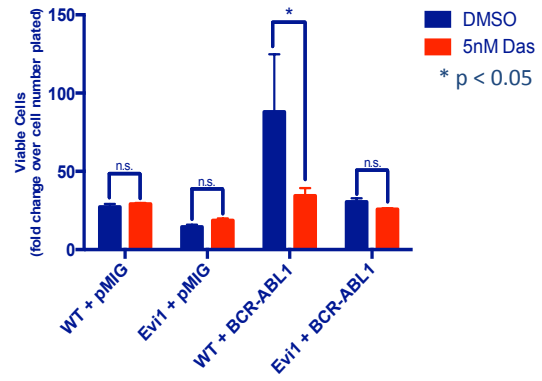
B.



C.



D.



E.

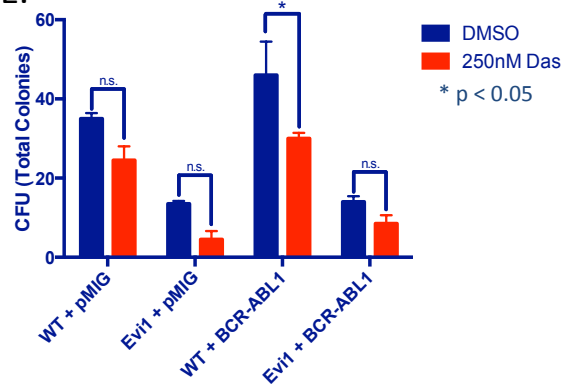
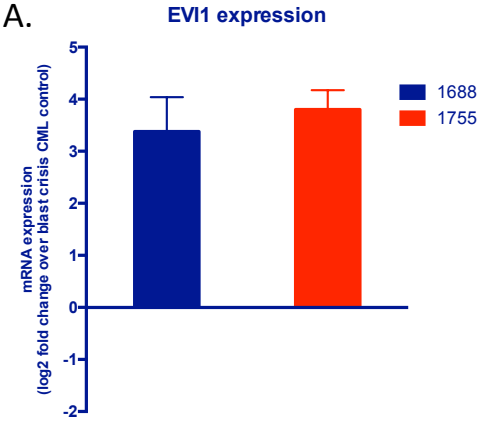


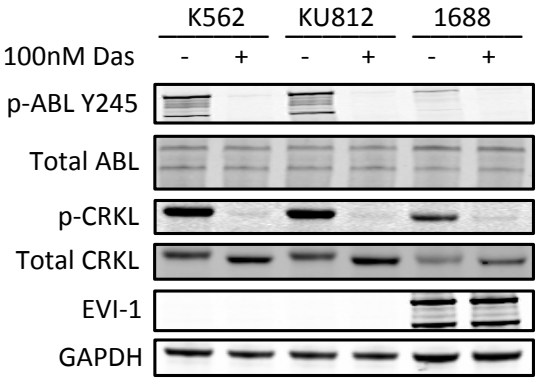
Figure 2. Blast Crisis CML patients exhibiting overt off-target resistance contain EVI1 overexpression, persistent MAPK signaling and *N-RAS* activating mutations

(A) EVI1 mRNA expression levels, measured by qPCR, in blast crisis CML patients with a 3q26 abnormality relative to control blast crisis CML patients without a 3q26 abnormality (error bars represent s.d. of triplicates from the same experiment). (B-C) Western blot analysis on cellular lysates from K562, KU812 and 1688 cells using (B) anti-phospho-ABL, anti-ABL, anti-phospho-CRKL, anti-CRKL, anti-EVI1, (C) anti-phospho-STAT5, anti-STAT5, anti-phosphoERK, anti-ERK, anti-phospho-AKT, and anti-AKT. Cells were exposed to 100nM dasatinib for 90minutes prior to harvesting lysates. (D) *N-RAS* exons 1-2 DNA sequencing results from patient samples 1688 and 1755.

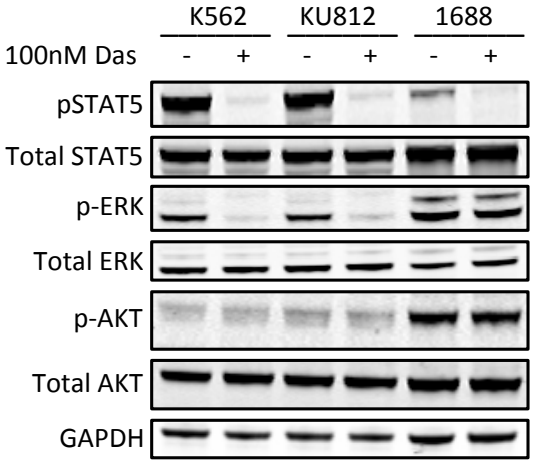
A.


EVI1 expression
mRNA expression
(log2 fold change over blast crisis CML control)
5
4
3
2
1
0
-1
-2
1688
1755

B.


K562
KU812
1688
100nM Das
- + - + - +
p-ABL Y245
Total ABL
p-CRKL
Total CRKL
EVI-1
GAPDH

C.


K562
KU812
1688
100nM Das
- + - + - +
pSTAT5
Total STAT5
p-ERK
Total ERK
p-AKT
Total AKT
GAPDH

D.

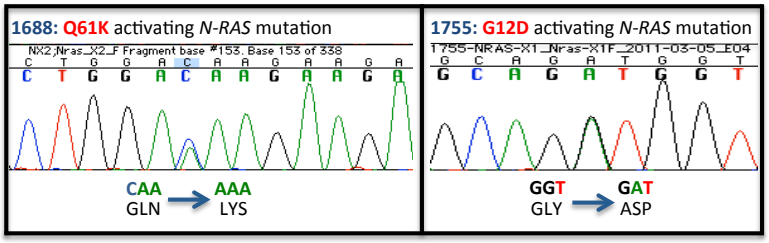
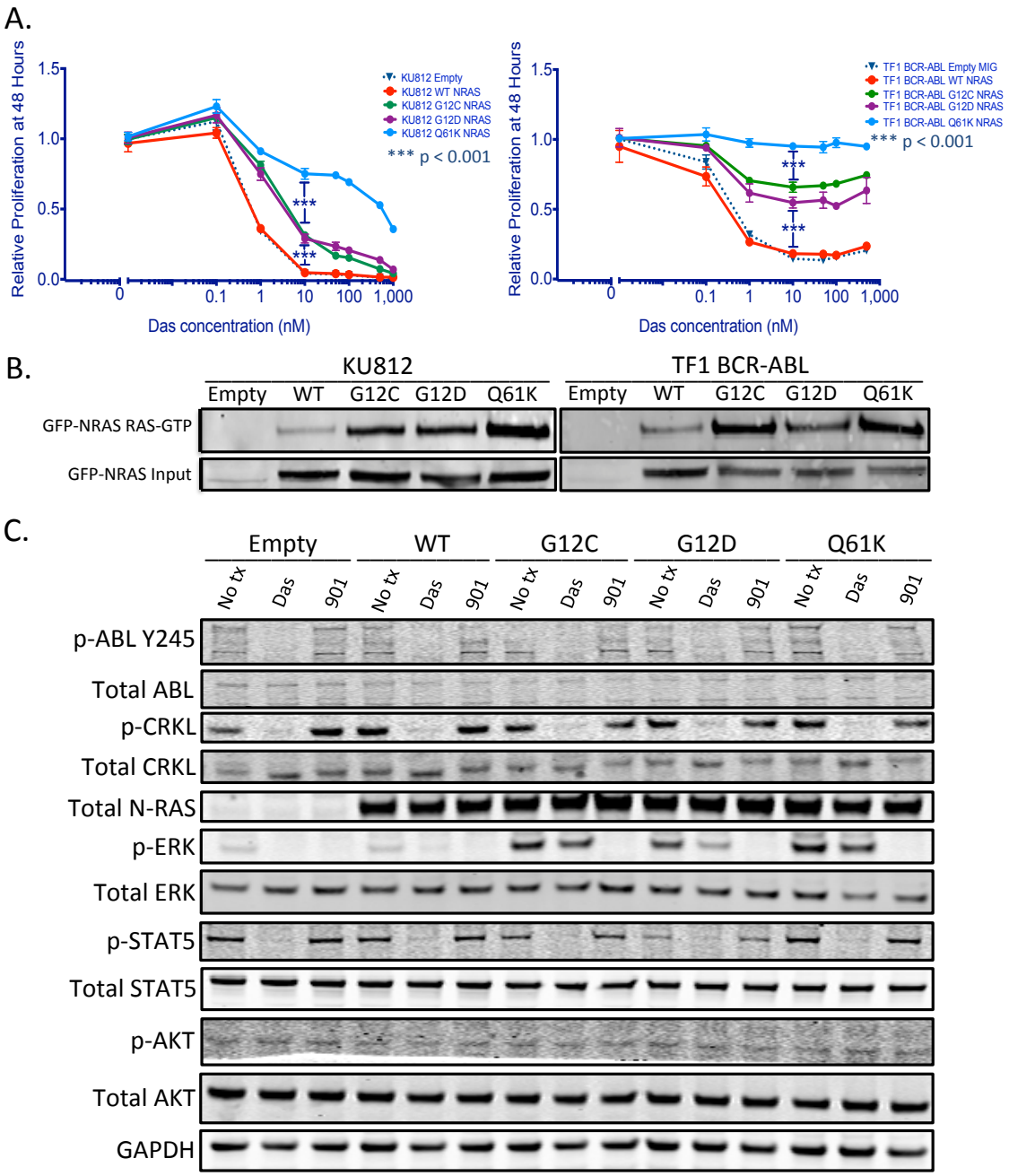
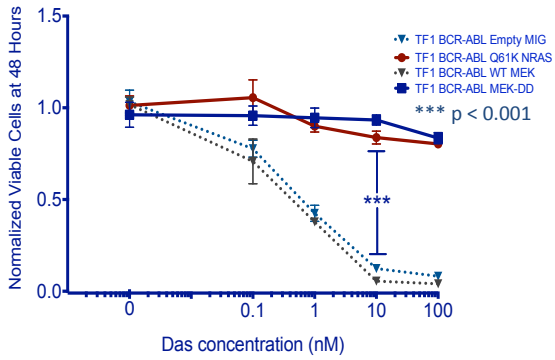

1688: Q61K activating N-RAS mutation
CAA
GLN
AAA
LYS
1755: G12D activating N-RAS mutation
GGT
GLY
GAT
ASP

Figure 3. *N-RAS* activation elicits increased MAPK signaling and provides TKI resistance despite BCR-ABL1 inhibition in CML cell lines.

(A) Viability assay (CellTiter-Glo) of KU812 (*left*) and TF1 BCR-ABL1 (*right*) cells expressing empty vector control, $N\text{-}RAS^{WT}$, $N\text{-}RAS^{G12C}$, $N\text{-}RAS^{G12D}$, or $N\text{-}RAS^{Q61K}$ after 48 hours exposure to various concentrations of dasatinib (error bars represent s.d. of triplicates from the same experiment). (B) RAS-GTP Loading assay of lysates harvested from KU812 (*left)* and TF1 BCR-ABL1 (*right)* cells stably expressing various alleles of *N-RAS* following 3 hour starve conditions. (C) Western blot analysis on cellular lysates from KU812 cells expressing various activated alleles of *N-RAS* using anti-phospho-ABL, anti-ABL, anti-phospho-CRKL, anti-CRKL, anti-NRAS, anti-phospho-ERK, anti-ERK, anti-phospho-STAT5, anti-STAT5, anti-phospho-AKT, and anti-AKT. Cells were exposed to 100nM dasatinib, PD901, or control for 90 minutes prior to harvesting lysates. 48-hour viability assay (CellTiter-Glo) of TF1 BCR-ABL1 cells expressing empty vector, $N\text{-}RAS^{Q61K}$, MEK^{WT}, or MEK^{DD} using various concentrations of (D) dasatinib (E) or ponatinib.


A.
Relative Proliferation at 48 Hours
1.5
1.0
0.5
0.0
0
0.1
1
10
100
1,000
Das concentration (nM)
KU812 Empty
KU812 WT NRAS
KU812 G12C NRAS
KU812 G12D NRAS
KU812 Q61K NRAS
*** p < 0.001
***
***
Relative Proliferation at 48 Hours
1.5
1.0
0.5
0.0
0
0.1
1
10
100
1,000
Das concentration (nM)
TF1 BCR-ABL Empty MIG
TF1 BCR-ABL WT NRAS
TF1 BCR-ABL G12C NRAS
TF1 BCR-ABL G12D NRAS
TF1 BCR-ABL Q61K NRAS
*** p < 0.001
***
***
B.
KU812
Empty
WT
G12C
G12D
Q61K
TF1 BCR-ABL
Empty
WT
G12C
G12D
Q61K
GFP-NRAS RAS-GTP
GFP-NRAS Input
C.
Empty
WT
G12C
G12D
Q61K
No tx
Das
901
p-ABL Y245
Total ABL
p-CRKL
Total CRKL
Total N-RAS
p-ERK
Total ERK
p-STAT5
Total STAT5
p-AKT
Total AKT
GAPDH

D.


Normalized Viable Cells at 48 Hours
1.5
1.0
0.5
0.0
0
0.1
1
10
100
Das concentration (nM)
TF1 BCR-ABL Empty MIG
TF1 BCR-ABL Q61K NRAS
TF1 BCR-ABL WT MEK
TF1 BCR-ABL MEK-DD
*** p < 0.001
***

E.

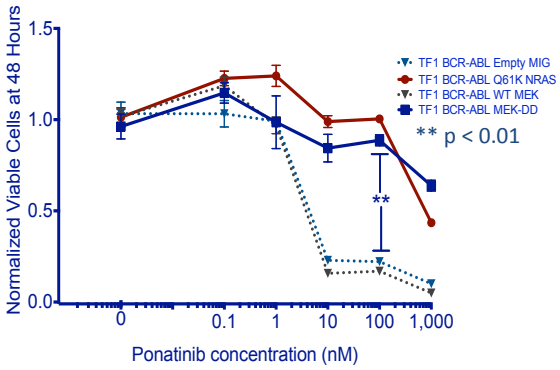
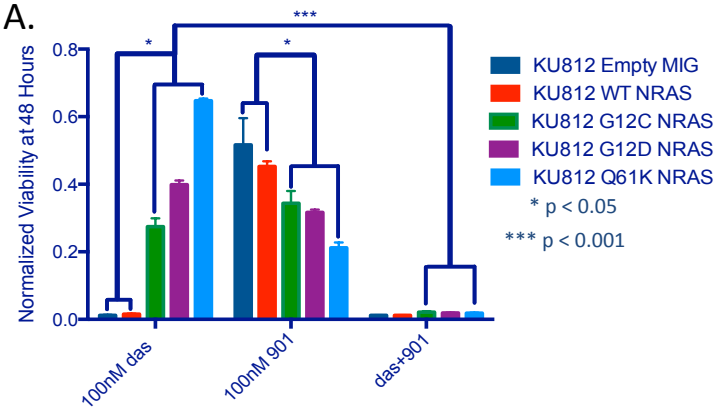

Normalized Viable Cells at 48 Hours
1.5
1.0
0.5
0.0
0
0.1
1
10
100
1,000
Ponatinib concentration (nM)
TF1 BCR-ABL Empty MIG
TF1 BCR-ABL Q61K NRAS
TF1 BCR-ABL WT MEK
TF1 BCR-ABL MEK-DD
** p < 0.01
**

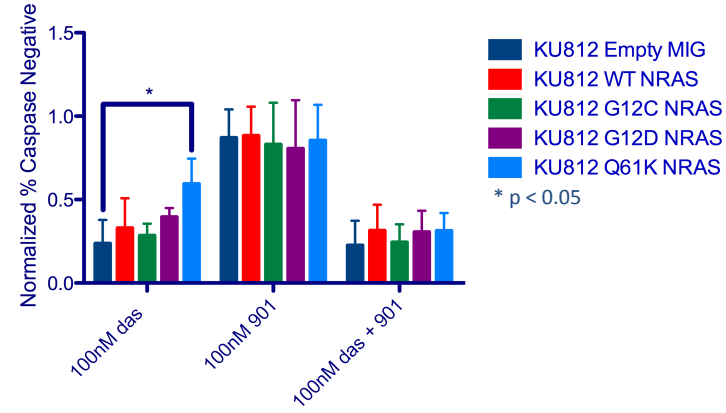
Figure 4. Clinically approved MEK inhibitor trametinib sensitizes overt TKI resistant CML cells *in vitro*

(A) 48 hour viability assay (CellTiter-Glo) or (B) caspase activation assay (showing fraction of cleaved caspase-3 negative population/live cells as measured by flow cytometry) of KU812 cells expressing various activated alleles of *N-RAS* following drug treatment with dasatinib, MEK inhibitor PD901 or combination. (C) 48 hour viability assay (CellTiter-Glo) of TF1 BCR-ABL1 cells expressing empty vector, $N\text{-}RAS^{Q61K}$, MEK^{WT}, or MEK^{DD} following drug treatment with 100nM dasatinib, 100nM trametinib, or combination therapy.

A.


Normalized Viability at 48 Hours
0.0
0.2
0.4
0.6
0.8
*
*
***
100nM das
100nM 901
das+901
KU812 Empty MIG
KU812 WT NRAS
KU812 G12C NRAS
KU812 G12D NRAS
KU812 Q61K NRAS
* p < 0.05
*** p < 0.001

B.


Normalized % Caspase Negative
0.0
0.5
1.0
1.5
*
100nM das
100nM 901
100nM das + 901
KU812 Empty MIG
KU812 WT NRAS
KU812 G12C NRAS
KU812 G12D NRAS
KU812 Q61K NRAS
* p < 0.05

C.

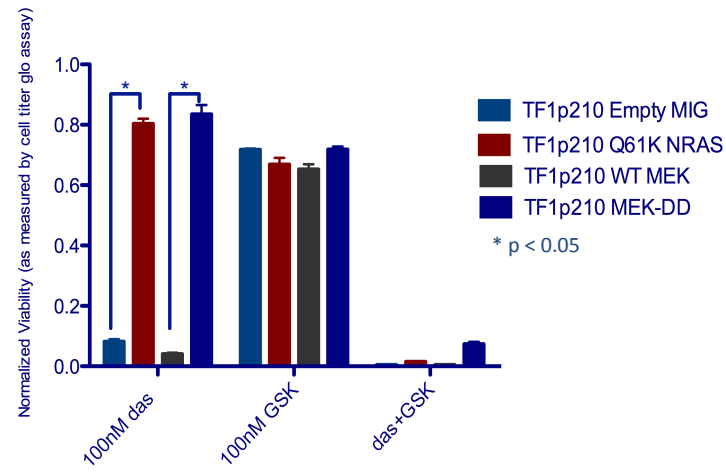
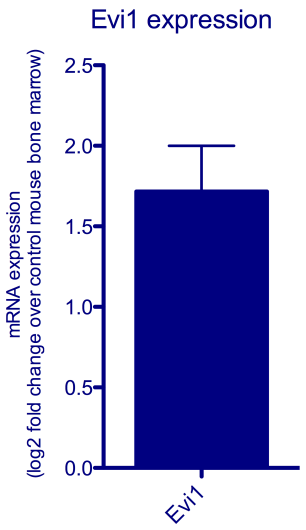

Normalized Viability (as measured by cell titer glo assay)
0.0
0.2
0.4
0.6
0.8
1.0
*
*
100nM das
100nM GSK
das+GSK
TF1p210 Empty MIG
TF1p210 Q61K NRAS
TF1p210 WT MEK
TF1p210 MEK-DD
* p < 0.05

Figure S1. EVI1 Expression is increased following Cre Activation. (A) qPCR analysis of EVI1 transcript levels in LSL-EVI1/MX1-CRE mice following pIpC injection compared to control. (B) Pictorial representation of 7 day liquid culture growth from WT and EVI1 expressing mouse PreGM cells expressing either empty MIG or pMIG+BCR-ABL1 in the presence and absence of 25nM dasatinib.

A.


Evi1 expression
mRNA expression
(log2 fold change over control mouse bone marrow)
2.5
2.0
1.5
1.0
0.5
0.0
Evi1

B.

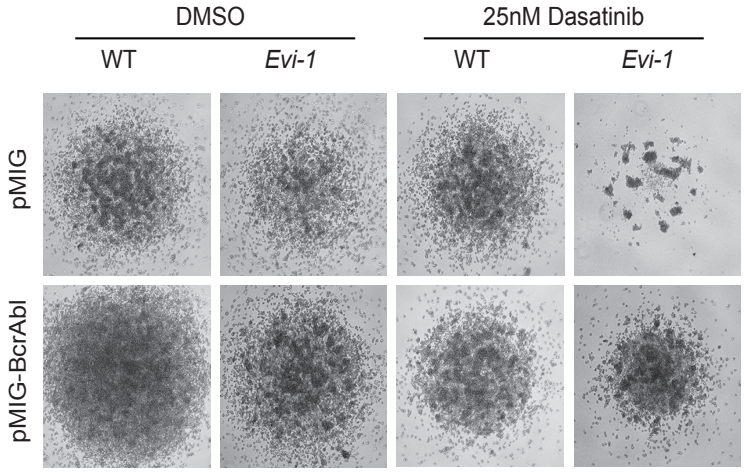

DMSO
25nM Dasatinib
WT
Evi-1
WT
Evi-1
pMIG
pMIG-BcrAbl

Figure S2. Blast crisis CML patients that exhibit overt-off target resistance to BCR-ABL1 inhibitors were found to contain 3q26 abnormalities. (A) Clinical history of blast crisis CML patients.

A.

Patient	Disease Phase (at time of diagnosis)	Treatment History	Cytogenetic (at time of diagnosis)	Cytogenetics (following TKI treatment)
1688	Blast Crisis CML	Imatinib Dasatinib	Ph^+	Ph^+; Inv(3)(q21;q26.2) Monosomy 7 E453K kinase domain mutation
1755	Blast Crisis CML	Imatinib Dasatinib	Ph^+	Ph^+; t(2;3)(p23;q26.2) Absence of kinase domain mutations

Figure S3. Activated NRAS expressing CML cell lines exhibit an elevated level of active GTP-loaded Ras, persistent MAPK signaling despite dasatinib treatment and diminished sensitivity to PI3K pathway inhibition. Normalization and quantification of western blots following the RAS-GTP loading assay from (A) KU812 and (B) TF1 BCR-ABL1 cells stably expressing various alleles of *N-RAS*. (C) Western blot analysis on cellular lysates from TF1+BCR-ABL1 cells expressing various activated alleles of *N-RAS* using anti-phospho-ABL, anti-ABL, anti-phospho-CRKL, anti-CRKL, anti-NRAS, anti-phospho-ERK, anti-ERK, anti-phospho-STAT5, anti-STAT5, anti-phospho-AKT, and anti-AKT. Cells were exposed to 100nM dasatinib, PD901, or control for 90 minutes prior to harvesting lysates. 48 hour viability assay (CellTiter-Glo) of (D) KU812 cells or (E) TF1+BCR-ABL1 cells expressing various activated alleles of *N-RAS* following drug treatment with dasatinib, the PI3K inhibitor PIK90, or combination.

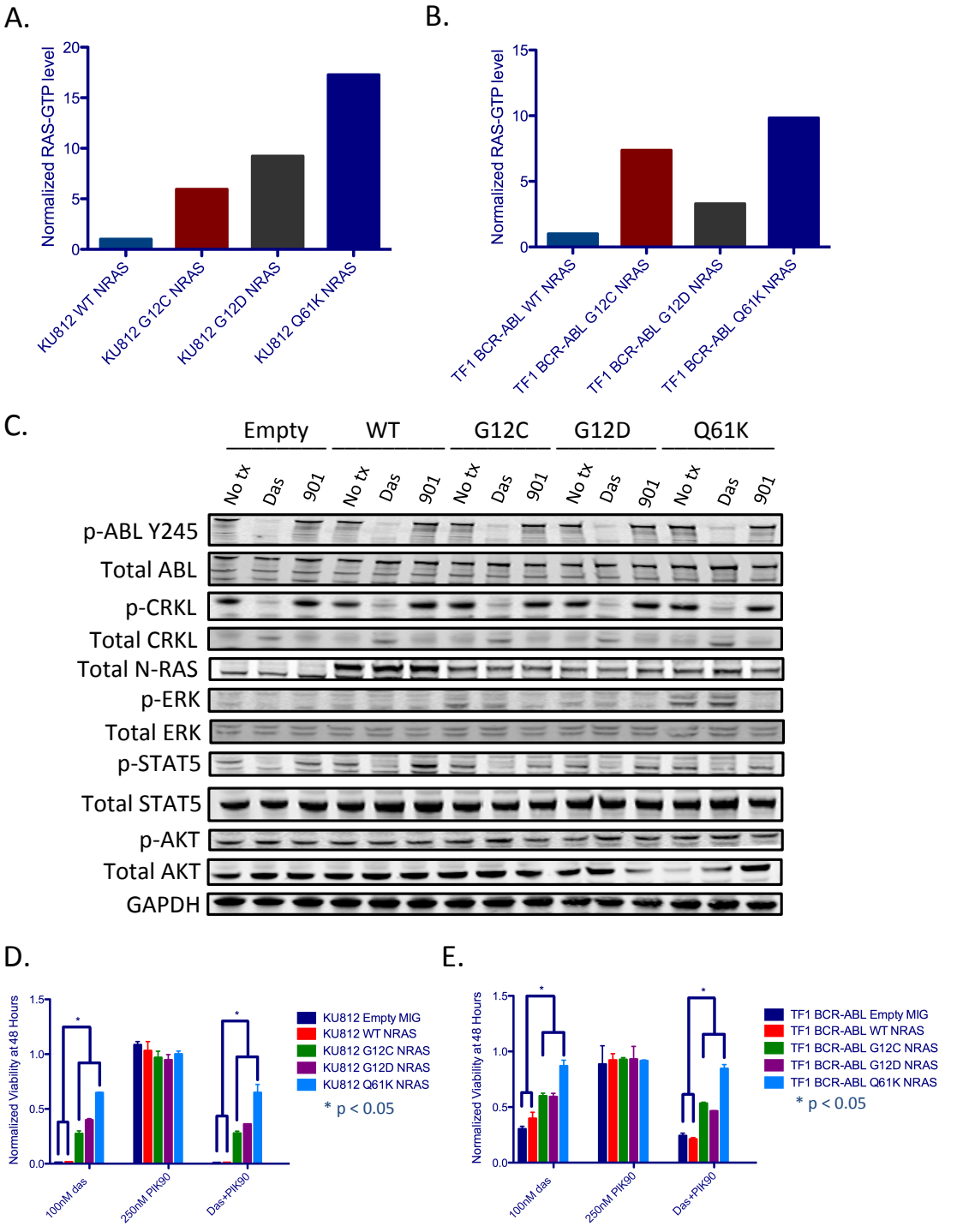
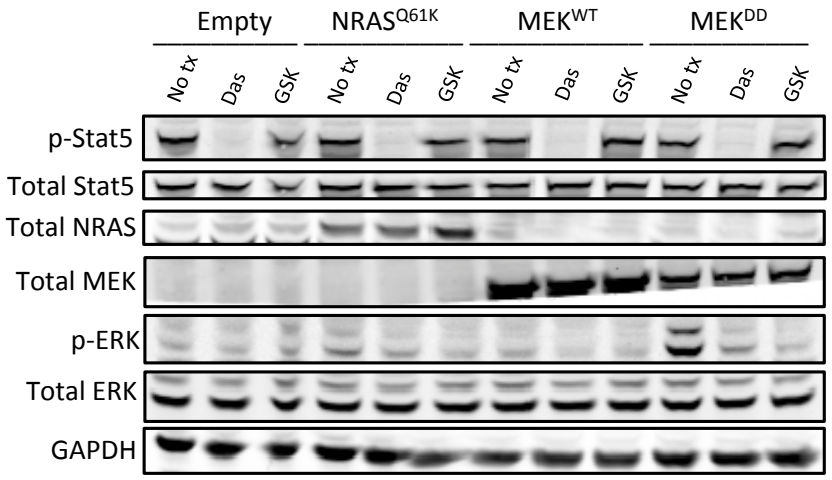
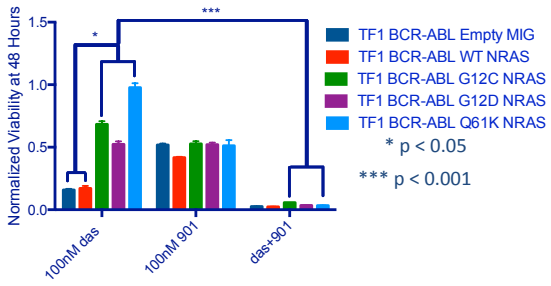

A.
Normalized RAS-GTP level
20
15
10
5
0
KU812 WT NRAS
KU812 G12C NRAS
KU812 G12D NRAS
KU812 Q61K NRAS
B.
Normalized RAS-GTP level
15
10
5
0
TF1 BCR-ABL WT NRAS
TF1 BCR-ABL G12C NRAS
TF1 BCR-ABL G12D NRAS
TF1 BCR-ABL Q61K NRAS
C.
Empty
WT
G12C
G12D
Q61K
No tx
Das
901
p-ABL Y245
Total ABL
p-CRKL
Total CRKL
Total N-RAS
p-ERK
Total ERK
p-STAT5
Total STAT5
p-AKT
Total AKT
GAPDH
D.
Normalized Viability at 48 Hours
1.5
1.0
0.5
0.0
100nM das
250nM PIK90
Das+PIK90
KU812 Empty MIG
KU812 WT NRAS
KU812 G12C NRAS
KU812 G12D NRAS
KU812 Q61K NRAS
* p < 0.05
E.
Normalized Viability at 48 Hours
1.5
1.0
0.5
0.0
100nM das
250nM PIK90
Das+PIK90
TF1 BCR-ABL Empty MIG
TF1 BCR-ABL WT NRAS
TF1 BCR-ABL G12C NRAS
TF1 BCR-ABL G12D NRAS
TF1 BCR-ABL Q61K NRAS
* p < 0.05

Figure S4. Mutant NRAS expressing TF1+BCR-ABL1 cells exhibit sensitivity to combination therapy of dasatinib and MEK inhibition. (A) Western blot analysis on cellular lysates from TF1+BCR-ABL1 cells expressing empty vector, $NRAS^{Q61K}$, MEK^{WT} or MEK^{DD}, using anti-phospho-STAT5, anti-STAT5, anti-NRAS, anti-MEK, anti-phospho-ERK, anti-ERK, and anti-GAPDH. Cells were exposed to 100nM dasatinib, trametinib, or control for 90 minutes prior to harvesting lysates. (B) 48 hour viability assay (CellTiter-Glo) or (C) caspase activation assay (showing fraction of cleaved caspase-3 negative population/live cells as measured by flow cytometry) of TF1+BCR-ABL1 cells expressing various activated alleles of *N-RAS* following drug treatment with dasatinib, MEK inhibitor PD901 or combination. (D) 48 hour viability assay (CellTiter-Glo) or (E) caspase activation assay (showing % caspase activation as measured by Caspase-Glo assay) of TF1+BCR-ABL1 cells expressing various activated alleles of *N-RAS* following drug treatment with control treatment, dasatinib, MEK inhibitor PD901, MEK inhibitor trametinib or combination of either MEK inhibitor with dasatinib.

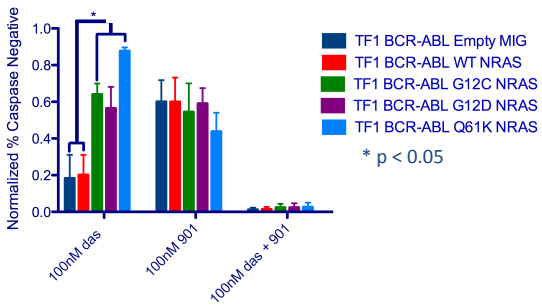
A.


Empty
NRAS^Q61K
MEK^WT
MEK^DD
No tx
Das
GSK
p-Stat5
Total Stat5
Total NRAS
Total MEK
p-ERK
Total ERK
GAPDH

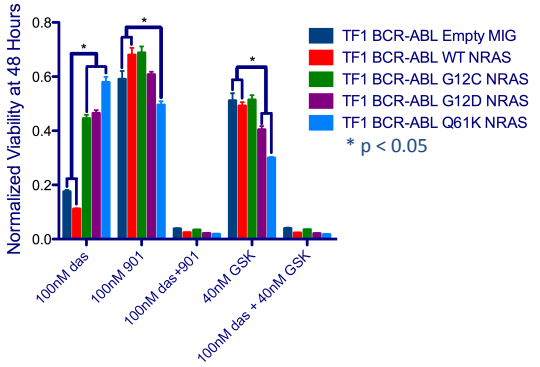
B.


Normalized Viability at 48 Hours
100nM das
100nM 901
das+901
TF1 BCR-ABL Empty MIG
TF1 BCR-ABL WT NRAS
TF1 BCR-ABL G12C NRAS
TF1 BCR-ABL G12D NRAS
TF1 BCR-ABL Q61K NRAS
* p < 0.05
*** p < 0.001

C.


Normalized % Caspase Negative
100nM das
100nM 901
100nM das + 901
TF1 BCR-ABL Empty MIG
TF1 BCR-ABL WT NRAS
TF1 BCR-ABL G12C NRAS
TF1 BCR-ABL G12D NRAS
TF1 BCR-ABL Q61K NRAS
* p < 0.05

D.


Normalized Viability at 48 Hours
100nM das
100nM 901
100nM das+901
40nM GSK
100nM das + 40nM GSK
TF1 BCR-ABL Empty MIG
TF1 BCR-ABL WT NRAS
TF1 BCR-ABL G12C NRAS
TF1 BCR-ABL G12D NRAS
TF1 BCR-ABL Q61K NRAS
* p < 0.05

E.

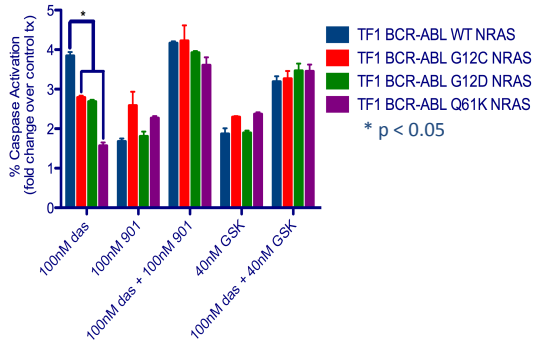
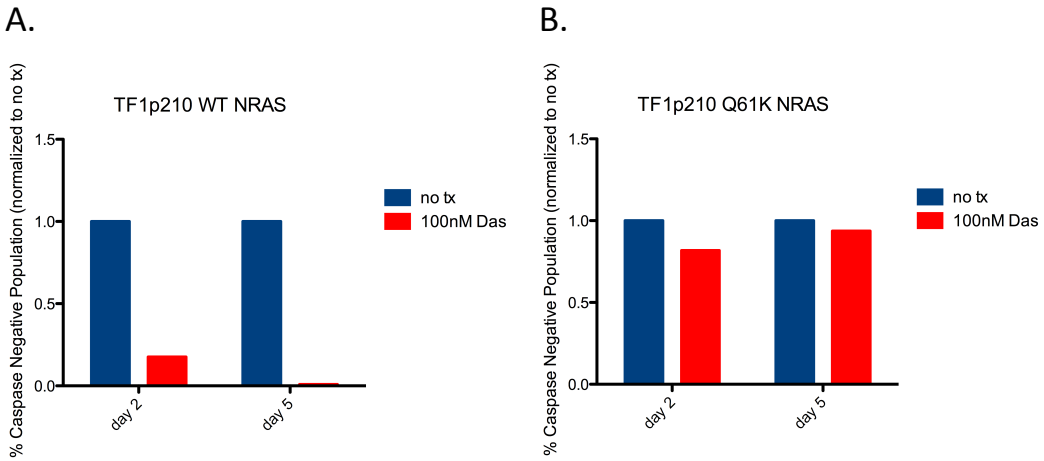
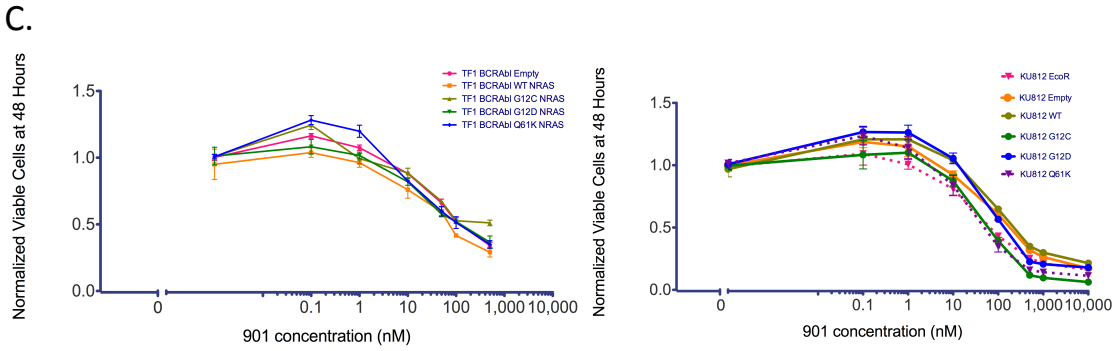

% Caspase Activation
(fold change over control tx)
100nM das
100nM 901
100nM das + 100nM 901
40nM GSK
100nM das + 40nM GSK
TF1 BCR-ABL WT NRAS
TF1 BCR-ABL G12C NRAS
TF1 BCR-ABL G12D NRAS
TF1 BCR-ABL Q61K NRAS
* p < 0.05

Figure S5. TF1+BCR-ABL1 expressing $NRAS^{Q61K}$ exhibits TKI resistance despite five days continued inhibition. Caspase activation (shown as % Caspase negative population) of (A) TF1+BCR-ABL1 expressing $NRAS^{WT}$ cells or (B) TF1+BCR-ABL1 expressing $NRAS^{Q61K}$ cells following 2 or 5 day continued exposure to control or 100nM dasatinib treatment. (C) Viability assay (CellTiter-Glo) of (left) TF1+BCR-ABL1 cells or (right) KU812 cells expressing empty vector control, $N\text{-}RAS^{WT}$, $N\text{-}RAS^{G12C}$, $N\text{-}RAS^{G12D}$, or $N\text{-}RAS^{Q61K}$ after 48 hours exposure to various concentrations of the MEK inhibitor PD901 (error bars represent s.d. of triplicates from the same experiment). (D) Viability assay (CellTiter-Glo) of TF1+BCR-ABL1 cells expressing empty vector control, $N\text{-}RAS^{WT}$, $N\text{-}RAS^{G12C}$, $N\text{-}RAS^{G12D}$, or $N\text{-}RAS^{Q61K}$ after 48 hours exposure to various concentrations of trametinib (error bars represent s.d. of triplicates from the same experiment).


A.
TF1p210 WT NRAS
% Caspase Negative Population (normalized to no tx)
1.5
1.0
0.5
0.0
day 2
day 5
no tx
100nM Das
B.
TF1p210 Q61K NRAS
% Caspase Negative Population (normalized to no tx)
1.5
1.0
0.5
0.0
day 2
day 5
no tx
100nM Das


C.
Normalized Viable Cells at 48 Hours
1.5
1.0
0.5
0.0
0
0.1
1
10
100
1,000
10,000
901 concentration (nM)
TF1 BCRAbl Empty
TF1 BCRAbl WT NRAS
TF1 BCRAbl G12C NRAS
TF1 BCRAbl G12D NRAS
TF1 BCRAbl Q61K NRAS
Normalized Viable Cells at 48 Hours
1.5
1.0
0.5
0.0
0
0.1
1
10
100
1,000
10,000
901 concentration (nM)
KU812 EcoR
KU812 Empty
KU812 WT
KU812 G12C
KU812 G12D
KU812 Q61K

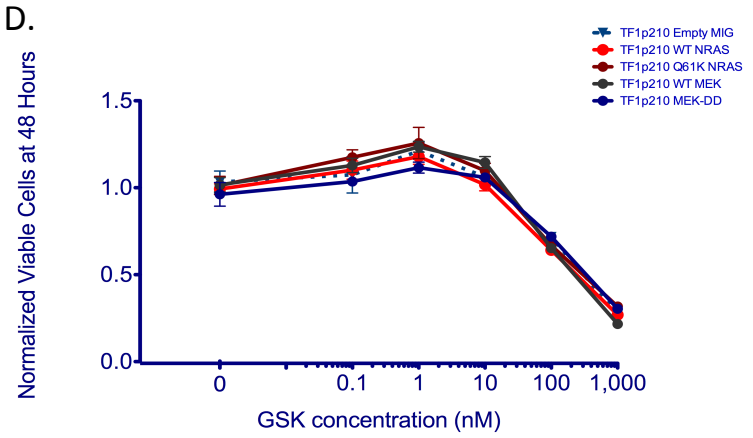
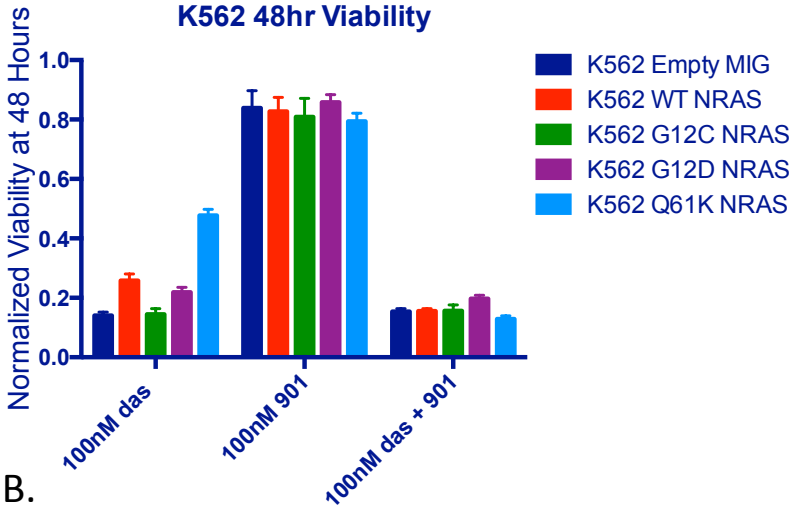
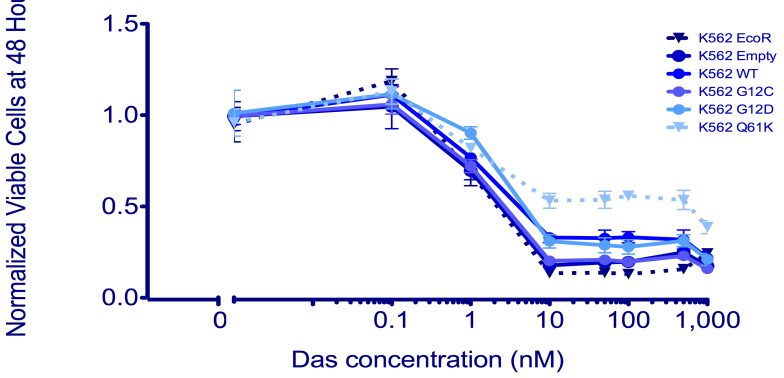

D.
Normalized Viable Cells at 48 Hours
1.5
1.0
0.5
0.0
0
0.1
1
10
100
1,000
GSK concentration (nM)
TF1p210 Empty MIG
TF1p210 WT NRAS
TF1p210 Q61K NRAS
TF1p210 WT MEK
TF1p210 MEK-DD

Figure S6. CML cell line, K562, displays TKI resistance upon ectopic expression of NRAS^{Q61K}. (A) K562 cells expressing various activated alleles of *N-RAS* following drug treatment with dasatinib, the MEK inhibitor PD901, or combination. (B) Viability assay (CellTiter-Glo) of K562 cells expressing empty vector control, *N-RAS^{WT}, N-RAS^{G12C}, N-RAS^{G12D},* or *N-RAS^{Q61K}* after 48 hours exposure to various concentrations of dasatinib (error bars represent s.d. of triplicates from the same experiment). (C) Viability assay (CellTiter-Glo) of K562 cells expressing empty vector control, *N-RAS^{WT}, N-RAS^{G12C}, N-RAS^{G12D},* or *N-RAS^{Q61K}* after 48 hours exposure to various concentrations of the MEK inhibitor PD901 (error bars represent s.d. of triplicates from the same experiment).

A.


K562 48hr Viability
Normalized Viability at 48 Hours
1.0
0.8
0.6
0.4
0.2
0.0
100nM das
100nM 901
100nM das + 901
K562 Empty MIG
K562 WT NRAS
K562 G12C NRAS
K562 G12D NRAS
K562 Q61K NRAS

B.


Normalized Viable Cells at 48 Hours
1.5
1.0
0.5
0.0
0
0.1
1
10
100
1,000
Das concentration (nM)
K562 EcoR
K562 Empty
K562 WT
K562 G12C
K562 G12D
K562 Q61K

C.

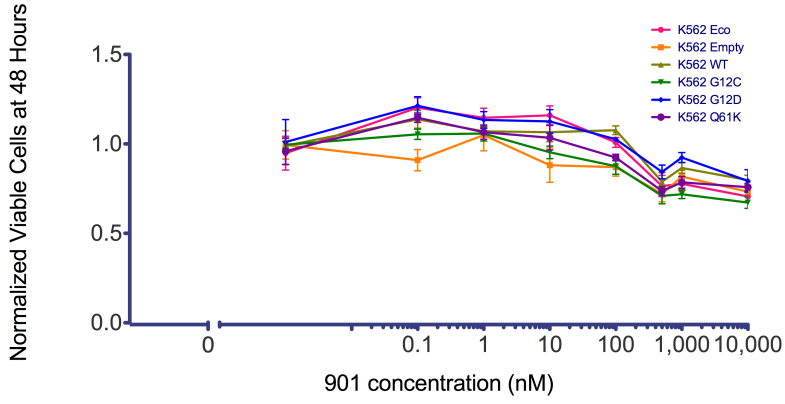
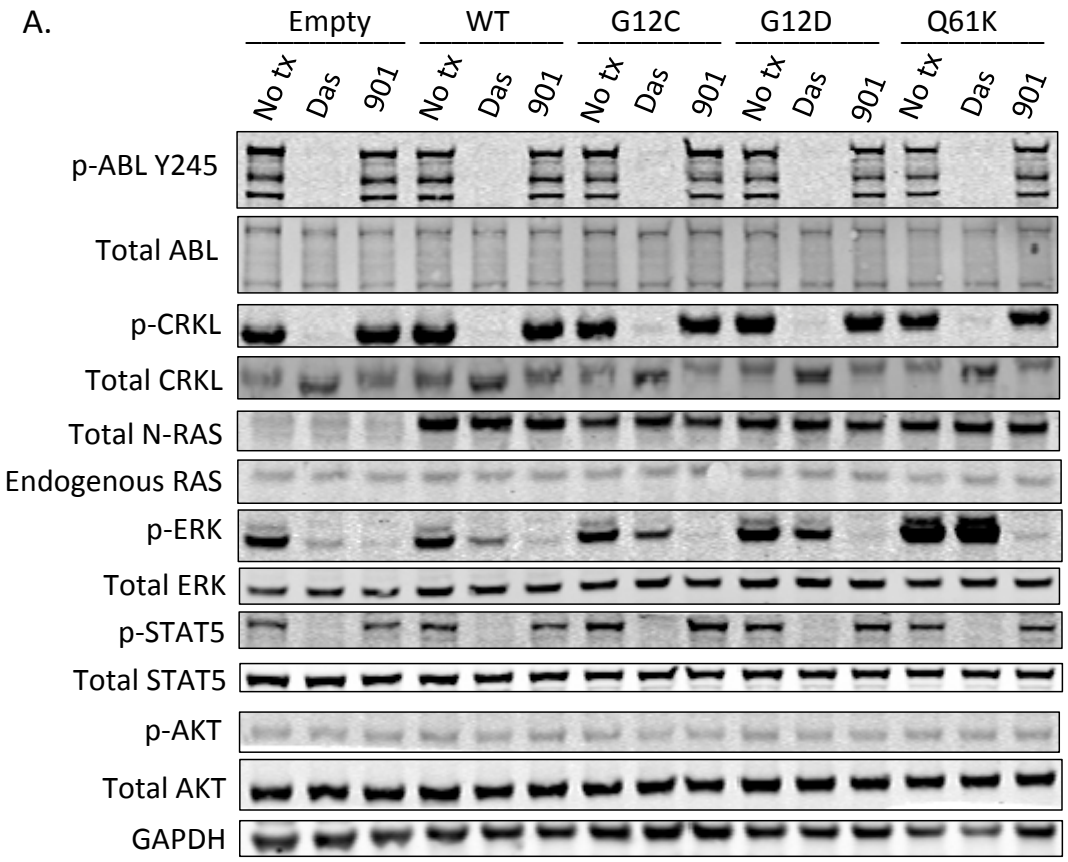
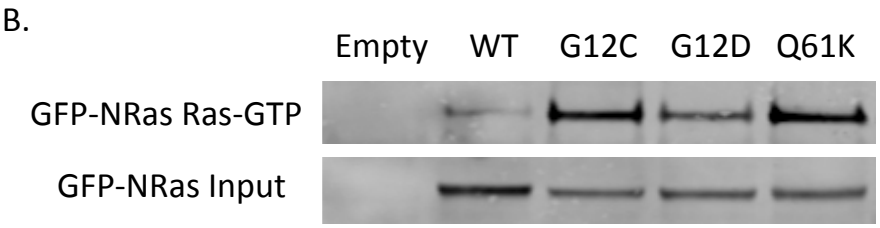

Normalized Viable Cells at 48 Hours
1.5
1.0
0.5
0.0
0
0.1
1
10
100
1,000
10,000
901 concentration (nM)
K562 Eco
K562 Empty
K562 WT
K562 G12C
K562 G12D
K562 Q61K

Figure S7. K562 cells expressing activated alleles of NRAS display persistent MAPK signaling despite BCR-ABL1 inhibition. (A) Western blot analysis on cellular lysates from K562 cells expressing various activated alleles of *N-RAS* using anti-phospho-ABL, anti-ABL, anti-phospho-CRKL, anti-CRKL, anti-NRAS, anti-phospho-ERK, anti-ERK, anti-phospho-STAT5, anti-STAT5, anti-phospho-AKT, and anti-AKT. Cells were exposed to 100nM dasatinib, PD901, or control for 90 minutes prior to harvesting lysates. (B) RAS-GTP Loading assay of lysates harvested from K562 cells stably expressing various alleles of *N-RAS* following 3 hour starve conditions.


A.
Empty
WT
G12C
G12D
Q61K
No tx
Das
901
p-ABL Y245
Total ABL
p-CRKL
Total CRKL
Total N-RAS
Endogenous RAS
p-ERK
Total ERK
p-STAT5
Total STAT5
p-AKT
Total AKT
GAPDH


B.
Empty
WT
G12C
G12D
Q61K
GFP-NRas Ras-GTP
GFP-NRas Input

Chapter 3:

Discussion and Concluding Remarks

Conclusions:

The first clinical trial of imatinib in June 1998 ushered in a new era of cancer treatment. The inhibition of the BCR-ABL1 fusion protein opened the door for the identification and targeting of driver mutations in diseases as widespread as lung cancer (EGFR driven) and melanoma ($BRAF^{V600E}$ driven). Despite the deep durable remissions patients achieved on imatinib, resistance to treatment became an ongoing problem. In fact the mechanism of imatinib resistance revolved around the re-constitution of BCR-ABL1 function. This was seen via the acquisition of mutations in the kinase domain resulting in the inability for drug binding. With the understanding of this mechanism came the second generation tyrosine kinase inhibitors, dasatinib and nilotinib, drugs which were not only more potent but exhibited the power to inhibit a majority of the resistance-conferring mutations in BCR-ABL1. Still, some kinase domain mutations, such as the gatekeeper mutation T315I provided resistance to all three of the then approved TKIs. Only with discovery and approval of the third generation TKI ponatinib, are have we found a compound capable of inhibiting all known BCR-ABL1 mutations.

This pan-BCR-ABL1 inhibitor will allow for control over any on-target resistance mechanisms that has been seen in CML. Although off-target mechanisms have been the predominate form of resistance seen in this disease, given that we possess agents to combat on-target resistance, we can now

speculate that it is increasingly likely that off-target resistance mechanisms through which cellular survival is independent of BCR-ABL1 function, will become the major form of resistance seen clinically.

The work presented in this dissertation has focused on identifying genetic determinants underlying the two major roadblocks for CML treatment. The first of which, disease persistence, is the case in which a patient, upon TKI treatment, exhibits a hematologic response in the absence of achieving a cytogenetic response. The morphologically normal appearing bone marrow seems like a worthy achievement yet the continued presence of the Philadelphia chromosome in bone marrow cells is predictive of a poor prognosis and high likelihood of relapse. Through the use of two chronic phase CML patient samples exhibiting disease persistence we identified 3q26 genetic abnormalities and validated the resultant EVI1 overexpression. CFU growth assays revealed that EVI1 overexpression provides a degree of dasatinib resistance compared to non-EVI1 overexpressing patients (Chapter 2). This dasatinib resistant phenotype was further validated through the use of EVI1 overexpressing primary murine bone marrow transduced with BCR-ABL (Chapter 2).

The second major roadblock in CML treatment will likely be the emergence of overt off-target resistance. In relapsing patients, this loss of hematologic response and disease recurrence has always been accomplished

through on-target kinase domain mutations. With the diverse binding capabilities of currently available TKIs the likelihood of on-target resistance mechanisms will become a thing of the past and newer previously unseen and poorly understood off-target mechanisms will predominate. Here we present a novel mechanism through which overt off-target resistance may occur in CML. Through the use of two independent blast crisis CML patient samples that exhibited overt resistance in the absence of any kinase domain mutation that could this phenotype, we identified two oncogenic lesions common to both samples: 3q26 abnormalities suggestive of EVI1 overexpression and NRAS activating mutations (Chapter 2).

The ectopic expression NRAS activating mutations in both patient derived blast crisis CML cell lines as well as BCR-ABL1 addicted TF1 cell lines displayed dasatinib resistance by viability as well as lack of caspase activation (Chapter 2). It was found that expression of $NRAS^{Q61K}$ in particular resulted in the greatest degree of dasatinib resistance suggesting a possible fundamental difference in NRAS signaling capability between RAS alleles. Analysis of intracellular signaling revealed that NRAS activation resulted largely in increased MAPK pathway signaling of which could not be modulated by TKI treatment. Given the absence of PI3K pathway change with the presence of activated NRAS it was concluded that the pertinent signaling pathway mediating resistance downstream of NRAS was the MAP kinase pathway (Chapter 2). This was validated through the introduction of MEK^{DD}, a constitutively activated allele of MEK, into TF1+BCR-

ABL1 cells. Expression of activated MEK recapitulated the dasatinib resistant phenotype seen with $NRAS^{Q61K}$ in TF1+BCR-ABL1 cells (Chapter 2).

The identification of MAPK signaling as mediating overt off-target resistance to dasatinib led to the interrogation of a possible therapeutic intervention in this model. Indeed, MEK inhibition alone resulted in modulation of MAPK signaling and led to a slight but significant decrease in cell viability, particularly in the activated NRAS expressing cells. The combination of MEK inhibition with dasatinib resulted in decreased viability and marked apoptosis and cell death. The efficacy of dasatinib combination therapy was not only seen with the MEK inhibitor PD901 but more importantly with the GSK compound trametinib, a clinically available MEK inhibitor suggesting that for patients suffering from this form of resistance two already clinically approved drugs when used in combination could provide great benefit (Chapter 2).

In summary, the work presented here has focused on two distinct emerging problems on the horizon in CML relapse and response. We provide evidence that EVI1 overexpression provides a relative resistance to TKI treatment in the setting of BCR-ABL and the finding that this oncogenic lesion was found to be overexpressed in two independent chronic phase CML patients exhibiting disease persistence. We found that blast crisis CML patients displaying overt off-target resistance to BCR-ABL1 tyrosine kinase inhibitors

contained EVI1 overexpression and NRAS activation. Additional sequencing of NRAS in the chronic phase CML patients exhibiting disease persistence did not result in any findings. MAP kinase signaling downstream of activated NRAS was then validated as being capable of providing overt resistance in blast crisis CML cell lines. Furthermore, dasatinib combination therapy with MEK inhibitors was sufficient to induce apoptosis in these overt off-target resistant CML cell lines. Still, the blast crisis CML patients contained not only the activation of NRAS but also the overexpression of EVI1. It may in fact be the case that clinical overt off-target resistance is the product of both genetic lesions, EVI1 overexpression providing a block in differentiation while activation of MAP kinase signaling provides a proliferative signal. If that is indeed the case, given the clinical samples interrogated here, it may be that the occurrence of EVI1 overexpression is the first inciting event permitting or causing disease persistence long enough for the acquisition of NRAS activation after which disease transformation and overt off-target resistance can materialize. Future directions include the interrogation of the potential cooperation between EVI1 overexpression and NRAS activation and the possible mechanism through which resistance is mediated. This work has contributed to the understanding of disease persistence and overt off-target resistance in CML through the identification of genetic lesions that potentiate these phenotypes in murine models and CML cell lines (Figure 1).

Figure 1. Model of disease persistence and overt off-target resistance in a murine model and CML cell lines respectively.

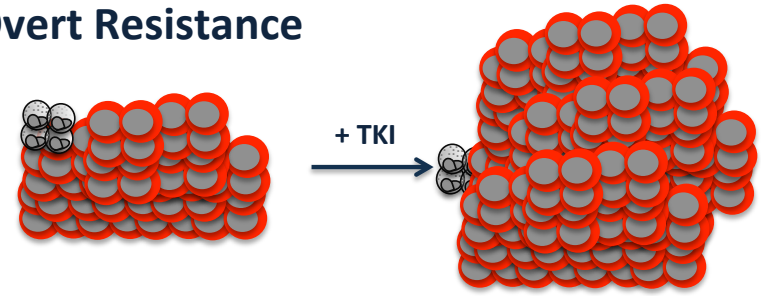
Normal TKI response

BCR-ABL^{+} PreGM cells

Disease Persistence

BCR-ABL^{+}/EVI1 PreGM cells

Overt Resistance



BCR-ABL^{+}/NRAS^{Q61K}
CML cell lines

- Normal
- BCR-ABL^{+}
- BCR-ABL^{+}/EVI1
- BCR-ABL^{+}/NRAS^{Q61K}

Appendix: Selected Protocols

RNA Isolation/cDNA Preparation/qPCR:

Cell Lysis and RNA extraction:

1.) Collect 5x10^6 cells pelleted in centrifuge at 400xg at 20 degrees Celsius for 5 minutes.

2.) Aspirate media and resuspend pellet in 1ml TRIzol reagent (do not wash prior to addition of TRIzol). Lyse cells by pipetting up and down.

3.) Incubate at room temperature (RT) for 5 minutes.

4.) Add 0.2ml chloroform/ml TRIzol reagent used. Shake by hand for 15 seconds. Incubate at RT for 2 minutes.

5.) Centrifuge at 12,000xg for 15 minutes at 4 degrees Celsius.

6.) Remove upper (aqueous) phase of the sample carefully being sure not to pipet up the lower phenol phase. Place aqueous phase in a new tube.

7.) Add 0.5ml 100% isopropanol to the aqueous phase to precipitate RNA

8.) Incubate at RT for 10 minutes. Centrifuge at 12,000xg for 10 minutest at 4 degrees Celsius

9.) Remove supernatant from tube leaving behind the small white RNA pellet. Wash the pellet with 1ml of 75% ethanol, vortex and spin at 7,500xg for 5 minutes at 4 degrees Celsius. Discard the supernatant. Let pellet dry and resuspend RNA in 0.020ml RNase free water. Incubate tube at 55 degrees Celsius and store at -20 degrees Celsius.

cDNA Synthesis using Invitrogen SuperScript II kit:

1.) Add the following components to a nuclease free eppendorf tube:

   a. 2ug RNA

   b. 1ul Oligo$(dT)_{12\text{-}18}$

   c. 1ul dNTP mix (10mM)

   d. Sterile, distilled water up to 12ul total volume

2.) Heat mixture to 65 degrees Celsius for 5 minutes and quickly chill on ice. Briefly centrifuge to collect contents then add:

   a. 4ul 5x First-Strand Buffer

   b. 2ul 0.1M DTT

3.) Mix contents briefly, incubate at 42 degrees Celsius for 2min. Add:

   a. 1ul (200 units) of SuperScript II RT and mix.

4.) Incubate at 42 degrees Celsius for 50 minutes

5.) Inactivate the reaction by heating at 70 degrees Celsius for 15 minutes.

qPCR/Taqman analysis for RNA quantification:

1.) Make a master mix for each triplicate by adding the following to a sterile PCR tube:

   a. 16.65ul 2x Taqman universal master mix II (No UNG)
   b. 0.549ul cDNA
   c. 1.665ul 20x probe (validated taqman probes purchased from life technologies)
   d. 14.418ul sterile distilled water
   e. Total = 33.3ul; pipet 10ul into 3 different wells of a 96-well plate (this is 1 triplicate; do triplicate for each primer/RNA condition)

2.) Applied Biosystems Viia 7 real time PCR setup:

   a. Highlight the following menu setup:
      i. 96-well
      ii. Delta-Delta CT
      iii. Fast Block
      iv. Standard Method
      v. FAM Reporter, no Quencher

Cell proliferation/Viability Assay by CellTiter Glo assay (Promega):

1.) Cells were counted using Vi-Cell counter with appropriate cell specification settings
2.) Spin down appropriate number of total cells at 400xg for 5minutes at 20 degrees Celsius. Cells are resuspended at a concentration of 4x10^5 cells/ml
3.) Pipet 50ul of cells into triplicate (for each drug concentration/condition) wells of a CellTiter-Glo compatible white walled 96-well plate.
4.) Drug solutions (control, dasatinib, PD901, trametinib etc) are to be made up in a 2x stock solution in 10%FBS in RPMI +P/S +L-Glut media. (ie. for 100nM das make a 200nM stock solution).
5.) Pipet 50ul of drug to appropriate wells for a total final volume of 100ul in each well.
6.) Incubate at 37 degrees Celsius incubator for 48 hours prior to plate reading (using CellTiter-Glo reagent/protocol and Spectramax M3 microplate reader)

Caspase Activation Assay by flow cytometry (BD biosciences kit):

1.) Cells were counted using Vi-Cell counter with appropriate cell specification settings
2.) Spin down appropriate number of total cells at 400xg for 5minutes at 20 degrees Celsius. Cells are resuspended at a concentration of 4x10^5 cells/ml
3.) Pipet 1ml of cells into a 12-well flat-bottomed tissue culture plate
4.) Drug solutions (control, dasatinib, PD901, trametinib etc) are to be made up in a 2x stock solution in 10%FBS in RPMI +P/S +L-Glut media. (ie. for 100nM das make a 200nM stock solution).
5.) Pipet 1ml of drug to appropriate wells for a total final volume of 2mls in each well.
6.) Incubate at 37 degrees Celsius incubator for 48 hours (if using Caspase-Glo reagent/kit follow protocol and read on Spectramax M3 microplate reader). If using active-caspase 3/7 antibody and flow cytometry follow the following steps:
    a. Pipet 2mls of cells into 5ml FACS tubes.
    b. Spin cells down at 400xg for 5 minutes at 4 degrees Celsius
    c. Wash 2x with 1ml cold PBS (spin cells down between washes)
    d. Spin cells down, aspirate off PBS and add 500ul cytofix/perm buffer (BD biosciences active caspase 3/7 kit). Incubate on ice for 20 minutes.

e. Spin cells down, aspirate off cytofix/perm and add 500ul of perm/wash buffer. Can let sit overnight at 4 degrees if needed.
f. Spin cells down, aspirate off perm/wash buffer and block cells for 15 minutes at RT using 0.25ul Rat anti-mouse CD16/CD32 Mouse Fc block in 20ul perm/wash buffer for each tube
g. Add 5ul anti-active-caspase 3/7 antibody (FIT-C conjugated antibody included in kit; can also use APC-conjugated antibody if using cells that simultaneously express GFP on the FIT-C channel) in 25ul perm/wash buffer for each tube (total volume is 50ul). Incubate at RT for 30 minutes.
h. Wash with 100ul PBS, spin cells, aspirate and resuspend in 500ul PBS. Vortex, cover with foil and run on LSRII flow cytometer.

Caspase Activation Assay by Caspase-Glo (Promega):

1.) Cells were counted using Vi-Cell counter with appropriate cell specification settings

2.) Spin down appropriate number of total cells at 400xg for 5minutes at 20 degrees Celsius. Cells are resuspended at a concentration of 4x10^5 cells/ml

3.) Pipet 50ul of cells into triplicate (for each drug concentration/condition) wells of a Caspase Glo compatible white walled 96-well plate.

4.) Drug solutions (control, dasatinib, PD901, trametinib etc) are to be made up in a 2x stock solution in 10%FBS in RPMI +P/S +L-Glut media. (ie. for 100nM das make a 200nM stock solution).

5.) Pipet 50ul of drug to appropriate wells for a total final volume of 100ul in each well.

6.) Incubate at 37 degrees Celsius incubator for 48 hours prior to plate reading (using Caspase Glo reagent/protocol and Spectramax M3 microplate reader)

Cell Lysis, Western Immunoblot and RAS Immunoprecipitation Assay:

1.) Cells were counted using Vi-Cell counter with appropriate cell specification settings

2.) Spin down appropriate number of total cells at 400xg for 5minutes at 20 degrees Celsius. Cells are resuspended at a concentration of 1x10^6 cells/ml

3.) Add 5mls of cells (5x10^6 total number of cells) to a 15ml falcon tube.

4.) Drug solutions (control, dasatinib, PD901, trametinib etc) are to be made up in a 2x stock solution in 10%FBS in RPMI +P/S +L-Glut media. (ie. for 100nM das make a 200nM stock solution).

5.) Pipet 5mls of drug to appropriate wells for a total final volume of 10mls in each tube.

6.) Incubate at RT for 90 minutes prior to cell lysis. At time of lysis, spin cells down at 400xg for 5 minutes at 4 degrees Celsius.

7.) Wash cells 2x with 5mls cold PBS (spin down and aspirate supernatant in between washes) transferring pellet to 1.5ml eppendorf tube on last wash step. To cell pellet in 1.5ml eppendorf tube add the following cell lysis solution:

    a. 100ul Hepes Lysis buffer supplemented with 1% phosphatase inhibitor and 1% protease inhibitor.

    b. Mix by pipetting and incubate on ice for 15 minutes. Spin down at 12,000xg for 10 minutes at 4 degrees Celsius

c. Proceed to cell lysate normalization using BCA protein assay kit (thermo-scientific)
d. Normalized cell lysates were resolved on 10% Bis-Tris SDS PAGE gels (Life Technologies) and transferred to nitrocellulose for antibody incubation and detection.
e. Antibodies for ABL (phospho Y245) (cat. 2861), CRKL (phospho Y207/total) (cat. 3181/cat. 3182), EVI1 (cat. 2593), STAT5A/B (phospho-Y695/Y699 and total) (cat. 9351 and 9363), ERK1/2(phospho-T202/Y204 and total)(cat. 4370 and 9107), AKT (phospho-S473/total) (cat. 4060/9272) were purchased from Cell Signaling Technology. Antibodies against total ABL (cat. OP20) and total RAS (cat. 05-516) were purchased from Millipore. Antibody against Total NRAS (cat. sc-519) and Total GAPDH (cat. sc-25778) were purchased from Santa Cruz. Licor and Odyssey imaging technology was utilized for western blot visualization.

8.) RAS Immunoprecipitation Assay (Keep all cells/buffers cold throughout the process):

a. Following steps 1-3 above, cells are incubated in 0.1%FBS in RPMI +P/S +L-Glut starve media for 3 hours prior to cell lysis (drug treatment and cytokine stimulation can be done during this time prior to cell lysis if desired).
b. Spin cells down at 400xg for 5 minutes at 4 degrees Celsius.

c. Wash cells 2x with 10ml cold PBS

d. Aspirate off PBS and lyse cells using RAS IP lysis buffer:

    i. Tris-Lysis buffer supplemented with 5mM $MgCl_2$:

        1. 50mM Tris pH 7.5
        2. 125mM NaCl
        3. 1.5mM $MgCl_2$
        4. 5% glycerol
        5. 0.2% NP40
        6. Supplemented with 5mM $MgCl_2$ (add 100ul of 0.5M $MgCl_2$ to 10mls of RAS IP buffer)
        7. Supplemented with 1% protease inhibitor and 1% phosphatase inhibitor

e. Incubate for 30 minutes on ice.

f. Normalize lysates (using BCA protein assay kit) to 1ug/ul protein concentration (total 300ug protein in 300ul)

g. Remove 25ul (25ug) and set aside for use as input control. To this 25ul add 4ul reducing agent and 11ul loading dye = 40ul total; boil at 95 degrees Celsius for 10 minutes and use when running western blot

h. To the remaining 275ug/275ul protein lysates add 20ul Ras assay reagent (Millipore #14-278) mixing well when pipetting up ras assay reagent.

i. Tumble tubes for 60 minutes at 4 degrees Celsius (cold room)
j. Spin down lysates/beads at 1400xg 5-10 seconds. Pipet off supernatant being careful to leave beads behind.
k. Add 200ul cold Ras IP buffer and incubate for 2 minutes. Spin down and repeat wash 2x
l. Remove final wash supernatant leaving beads only
m. Resuspend beads in the following 2x loading buffer:
    i. 8ul 10x reducing agent
    ii. 20ul 4x loading dye
    iii. 12ul Ras IP buffer
    iv. Total of 40ul but only add 30ul to beads for a total volume + beads of 40ul.
    v. Boil beads at 95 degrees Celsius for 10 minutes.
    vi. Mix well prior to gel loading (load all 40ul into 12-well gel)
    vii. Load 20ul (half) of input lysates into their own lanes of gel as well.
    viii. Run gel in 4 degrees Celsius setting (cold room) with 170V maximum. Transfer to nitrocellulose and incubate with anti-Ras antibody for detection of RAS-GTP levels.

Mouse background, isolation and transduction of bone marrow PreGM cells:

Mice:

1.) *Vav*-LSL-*Evi1* FVB/n mice were bred with *Mx1-Cre* C57/Bl6 mice to generate animals that ectopically express *Evi1* in hematopoietic cells upon treatment with pIpC (Sigma).

2.) Mice were administered pIpC via i.p. injection at 21 days of age.

Isolation and transduction of PreGM cells:

1.) Nucleated bone marrow cells were stained with a cocktail of antibodies (see Table below) to detect progenitor populations. PreGM cells (Lin-, c-Kit+, Sca1-, CD16/32-, CD34+, CD105-, CD150-) were sorted with a BD FACSAriaIII cell sorter (BD Biosciences).

Antibodies used for flow cytometry.			
Antibody	Fluorophore	Dilution	Company
CD3e	PE-Cy7	1:200	BioLegend
CD4	PE-Cy7	1:400	BioLegend
CD5	PE-Cy7	1:800	BioLegend
CD8a	PE-Cy7	1:800	BioLegend
B220	PE-Cy7	1:200	BioLegend
CD11b	PE-Cy7	1:1600	BioLegend
Gr1 (Ly6/Ly6C)	PE-Cy7	1:800	BioLegend
Ter119	PE-Cy7	1:100	BioLegend
CD117	PerCP/Cy5.5	1:400	BioLegend
Sca1 (Ly6A/E)	Brilliant Violet 510	1:400	BioLegend
CD16/32	Alexa 647	1:100	BioLegend
CD34	FITC	1:30	BioLegend
CD105	Pacific Blue	1:25	BioLegend
CD150	PE	1:200	BioLegend

2.) Approximately 25,000 PreGM cells were incubated in MyeloCult M5300 (STEMCELL Technologies) supplemented with 15% FBS, 20ng/ml SCF, 20ng/ml IL-3, 20ng/ml IL-6, 100-units/ml glutamine and 100 units/ml PenStrep for 3 hours.

3.) Cells were mixed with retroviral supernatant (1:1), 5ug/ml polybrene, and 10mM HEPES.

4.) Cells were centrifuged at 260 x g for 1 hour at 30^0C and then incubated at 37^0C, 5% CO_2. GFP expression was determined 48 hours later by flow cytometry.

Liquid culture assay:

1.) A total of 300 GFP+ PreGM cells were plated into U-bottom 96-well plates (CoStar) and cultured in Isocove's modified Dulbecco's medium (IMDM), 20% FBS, 0.1mM b-mercaptoethanol, 100 units/ml glutamine and 100 units/ml PenStrep.

2.) Cells were stimulated with 10ng/ml GMCSF or treated with Dasatinib or DMSO at the indicated concentrations.

3.) After 7 days of culture, images were captured and cells were counted using an Accuri C6 flow cytometer (BD Biosciences).

CFU-GM assay:

1.) A total of 500 GFP+ PreGM cells were suspended in methylcellulose medium (M3231, STEMCELL technologies), supplemented with 0.1mM b-mercaptoethanol, 100-units/ml glutamine and 100 units/ml PenStrep.

2.) Cells were stimulated with 10ng/ml GMCSF (PeproTech) or treated with Dasatinib or DMSO at the indicated concentrations.

3.) Colonies were counted 7 days later.

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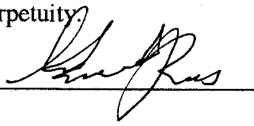
Author Signature  Date 06/10/14