Overcoming Resistance in Patient-Derived Xenograft Models of BCR-ABL-Rearranged Acute Lymphoblastic Leukemia

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Overcoming Resistance in Patient-Derived Xenograft Models of BCR-ABL-Rearranged Acute Lymphoblastic Leukemia

A Dissertation Presented by

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Table of Contents

Abstract..................................................................................................................................................... 7

Overcoming Resistance in Patient-Derived Xenograft Models of BCR-ABL-
Rearranged Acute Lymphoblastic Leukemia......................................................................................... 8

Introduction.................................................................................................................................................. 8

Highly Characterized Patient-Derived Xenograft Models of BCR-ABL+ ALL .................. 11

Introduction................................................................................................................................................ 11

Methods.................................................................................................................................................. 14

Generation and Use of PDXs............................................................................................................... 14

PDX Characterization........................................................................................................................ 14

Targeted Exome Sequencing............................................................................................................... 15

Whole Transcriptome Sequencing...................................................................................................... 16

Web-Based Data Hosting..................................................................................................................... 17

Results.................................................................................................................................................. 19

Establishment of Patient-Derived Xenografts .................................................................................. 19

Transcriptional, genetic, and phenotypic characterization of PDXs confirms subtype fidelity .................................................................................................................................................. 20

PRoXe: A resource to facilitate studies of leukemia and lymphoma PDXs .................. 22

Discussion............................................................................................................................................ 24

Footnotes.............................................................................................................................................. 28
Precise Measurement of Single Cancer Cell Mass Accumulation Predicts Drug Susceptibility ................................................................................................................................. 29

Introduction ........................................................................................................................................................................... 29

Methods .................................................................................................................................................................................. 32

Cell culture of conventional cell lines .............................................................................................................................. 32

Transgenic mouse model of BCR-ABL B-ALL ....................................................................................................................... 32

Patient sample procurement and processing .................................................................................................................... 34

Measurement and operation of a suspended microchannel resonator (SMR) ................................................................. 34

Results ......................................................................................................................................................................................... 36

Mass accumulation rate (MAR) measurement ................................................................................................................... 36

Single-cell MAR profiles demonstrate heterogeneity within acute leukemias ............................................................... 36

MAR predicts susceptibility to targeted therapeutics in conventional and patient derived cell lines .......................................................... 37

MAR predicts susceptibility to targeted therapeutics in primary leukemia cells ............................................................... 37

MAR predicts susceptibility to targeted therapeutics in primary leukemia cells isolated from circulation .................................................. 39

Patient samples display reduction in MAR when treated either ex vivo or in vivo .......................................................... 39

Discussion ................................................................................................................................................................................. 41

Footnotes .................................................................................................................................................................................... 45

Defining Clonal Phylogeny in BCR-ABL+ ALL by DNA Barcode Labeling ................................................................. 46
Figures ................................................................. 69

Acknowledgments .......................................................... 100
Abstract

The BCR-ABL fusion oncogene defines the most common molecular subtype of acute lymphoblastic leukemia in adults (BCR-ABL+ ALL) and has historically conferred an adverse prognosis. Incorporation of tyrosine kinase inhibitors (TKIs) that bind the ABL kinase domain into frontline regimens induces complete remissions in greater than 90% of patients, but most relapse with resistance mutations in ABL that disfavor drug binding. This suggests that relapses after TKI therapy remain addicted to ABL kinase activity. In contrast to currently approved TKIs, type IV inhibitors bind the spatially distinct myristate site in the ABL kinase domain and allosterically modulate BCR-ABL function. We hypothesize that the combination of catalytic and allosteric inhibitors can prevent the emergence of cross-resistant clones and cure some BCR-ABL+ ALLs. If this hypothesis proves correct, it would revolutionize the treatment of BCR-ABL+ ALL. Even in cases where combined ABL blockade is not curative, we would have the unique opportunity to define mechanisms of in vivo resistance – mutational, transcriptional, or differentiation state-mediated – to what is potentially the future standard therapy for BCR-ABL+ ALL. The studies described herein all relate to our overarching goal of informing curative therapeutic strategies for BCR-ABL ALL by defining the factors that modulate sensitivity and resistance to targeted ABL inhibition.

Keywords: Acute lymphoblastic leukemia, Philadelphia chromosome, BCR-ABL, acquired resistance, patient-derived xenograft models, clonal dynamics, DNA barcoding.
Overcoming Resistance in Patient-Derived Xenograft Models of BCR-ABL-Rearranged Acute Lymphoblastic Leukemia

Introduction.

B-cell acute lymphoblastic leukemia is an aggressive cancer that arises from progenitor B-cells. Nearly one third of adult cases and over one-half of cases in patients older than age 60 harbor the BCR-ABL fusion protein, which promotes leukemogenesis by augmenting cellular proliferation and inhibiting differentiation, DNA damage repair, and apoptosis [1]. BCR-ABL+ ALL has a poor prognosis relative to BCR-ABL-negative ALL [2]. Only 60-80% of patients with the former achieve complete remission (CR) with chemotherapy alone, compared to >90% of patients with the latter [3], translating to a reduction in median overall survival (OS) from >30 months to just eight months [4]. Incorporating targeted BCR-ABL inhibitors into induction regimens improves CR rates to >90% [5], but virtually all patients relapse. Relapses typically harbor ABL point mutations – chiefly T315I – that confer resistance to most or all available TKIs [6, 7]. Allogeneic stem cell transplantation (alloSCT) thus remains the standard curative approach for patients in CR [8], but age, co-morbidities, failure to achieve and maintain a CR, and lack of a suitable donor exclude many patients. Novel immunotherapies such as chimeric antigen receptor T cells (CARTs) are promising but are currently being studied in only the relapse setting. Thus, tolerable front line regimens capable of inducing durable remissions remain urgently needed.
The presence of ABL resistance mutations at relapse argues that the disease remains addicted to BCR-ABL. We therefore hypothesize that cure of BCR-ABL+ ALL may be possible with ABL inhibition alone, but this would require adequate and more sustained inhibition of BCR-ABL than is possible with currently approved agents. The availability of ABL001, a new inhibitor that allosterically binds the ABL myristate site [9], makes it possible to address this hypothesis in vivo. Preliminary data from cell line studies suggest that allosteric and catalytic ABL inhibitors select for largely non-overlapping spectra of ABL point mutations (Figure 1) [10]. Given the low likelihood of multiple high-level resistance mutations co-existing within the same clone at diagnosis, we believe that the combination of ponatinib, which is active against ABL T315I, plus ABL001 may be able to suppress BCR-ABL signaling in all cells within a BCR-ABL+ ALL. This would allow for the first test of whether adequate and sustained kinase inhibition can cure this disease. A precedent exists for curative molecular treatment of acute leukemia, as approximately 90% of patients with acute promyelocytic leukemia are cured with all-trans retinoic acid (ATRA) and arsenic trioxide, which both target the driver oncprotein PML-RARα in a non-cross-resistant fashion [11]. Importantly, BCR-ABL+ chronic myeloid leukemia may actually be cured by BCR-ABL inhibition alone in a subset of patients based on very sustained MRD negativity in patients after TKI withdrawal [12, 13]. In addition, a leukemia stem cell compartment that is insensitive to ABL inhibition has never been conclusively identified in BCR-ABL+ ALL.

We set out to establish an experimental system for defining the activity of combined BCR-ABL inhibition in faithful model systems of BCR-ABL+ ALL as well as mechanisms of resistance thereto. Essential to this effort are technological advances allowing us to: 1) test in vivo combined blockade within controlled and adequately powered pre-clinical trials, 2)
systematically define mechanisms of *in vivo* resistance to combined blockade including clonal dynamics of resistance, and 3) directly target MRD, the reservoir of resistant ALL subclones that drive relapse. The first advance is our repository of >250 patient-derived xenografts (PDXs) of leukemias and lymphomas, which we can use to perform large preclinical trials that assess heterogeneity of response, develop predictive biomarkers, and establish panels of leukemias with acquired *in vivo* resistance [14]. The second advance is a system for labeling individual cells with DNA barcodes to permit longitudinal tracking and clonal deconvolution. The third is a rapid and robust platform to characterize therapeutic sensitivity within individual tumor cells that is amenable to MRD specimens. We can assay drug sensitivity within leukemia specimens by measuring changes in mass accumulation rate (MAR) of individual cells from <25 µL of blood using a device called the suspended microchannel resonator (SMR). This approach has sensitivity in the femtogram range, allowing for precise determination of changes in MAR that occur in response to targeted agents. Cells can be collected downstream of the SMR for single-cell RNA-seq (scRNA-seq) to define transcriptional programs associated with differential response to therapeutics. *Key contributions to these advances were provided by the work of this thesis, as detailed in Methods and Results.*

In future directions, we detail our plan to identify phenotypic and transcriptional biomarkers of early relapse, as well as mechanisms of acquired *in vivo* resistance to combined ABL blockade. We will define the activity of combined catalytic and allosteric ABL inhibition and identify mechanisms of acquired resistance through a large phase II-like trial in BCR-ABL+ ALL PDXs. We will then define mutational, transcriptional, and differentiation-state mediated mechanisms of resistance to combined ABL inhibition, as well as the clonal phylogeny of these resistance lesions. Finally, we will integrate the SMR and scRNA-seq to define the clonal
heterogeneity of inhibitor resistance *within MRD* from both PDXs and primary patient specimens, iteratively test candidate therapeutics, and treat PDXs with agents predicted to be most active based on their effect on MAR. *Through these proposed experiments, we seek to define which patients can be cured with combined ABL blockade, and to elucidate mechanisms of resistance that will guide the development of future therapies for those who cannot.***

**Highly Characterized Patient-Derived Xenograft Models of BCR-ABL+ ALL**

**Introduction.**

Over 90% of drugs with preclinical activity fail to complete the translational journey to regulatory approval, largely due to insufficient efficacy in clinical trials [15-17]. Most early preclinical data are generated in cell lines or genetically engineered mouse models (GEMMs). Cancer cell lines have significant limitations in their ability to model the biology and therapeutic responsiveness of cancers in their native microenvironment [18-20]. Currently available cell lines markedly underrepresent the genomic and transcriptional diversity of primary human cancers, a problem shared by the vast majority of GEMMs. For example, there are over 100 different diagnostic subtypes of hematologic malignancies, and almost every one of these comprises multiple genetic subtypes defined by chromosomal rearrangements, aneuploidies, and/or single-/oligonucleotide sequence alterations [21]. Among the cell lines that do exist, adaptation to in vitro culture and passaging for innumerable generations exert substantial selective pressure not reverted by xenografting [19, 20, 22]. Nearly all cell lines are derived from patients who were previously untreated and/or from sites (e.g. pleural effusions) that are not typical for the disease. GEMMs are also highly homogeneous, typically harboring one genetic driver lesion or at most 2-3 cooperating lesions, without any of the relevant secondary, subclonal
lesions characteristic of human tumors. Finally, the vast majority of cell lines and GEMMs model untreated and not relapsed disease. For these reasons, available cell lines and GEMMs are not representative of either the genetic abnormalities or the treatment status of most patient tumors in which candidate therapies will be tested in early phase clinical trials. Finally, in vivo trials performed by xenografting cell lines subcutaneously into the flanks of recipient mice fail to recapitulate the microenvironmental interactions that can modulate therapeutic efficacy [23].

Patient-derived xenografts (PDXs) established within highly immunocompromised mice overcome many of these shortcomings [24-29]. PDXs are passaged only in vivo and thereby avoid the selective pressures from ex vivo culture. They can be collected from patients with typical presentations of disease, either upfront or in the relapsed/refractory setting. Because engraftment rates are high for a variety of tumor types, very large repositories can be established to more broadly capture the range of human cancer. For example, a bank of over 1,000 solid tumor PDXs (mostly treatment-naïve) was recently reported [30]. Large therapeutic studies of small molecule inhibitors in these PDXs recapitulated population-based response frequencies that were observed in clinical trials. In addition, the synergy identified between IGF1R inhibitors and multiple agents in cell lines was not observed in PDXs [30], a proof-of-principle that in vivo studies with PDXs may challenge results from cell lines.

In some settings, primary cancers can be orthotopically xenografted to recapitulate microenvironmental interactions within patients. The study by Gao et al. [30] utilized subcutaneous flank xenografts of solid tumors, and as such, therapeutic efficacy was based on reduced growth or regression relative to vehicle-treated animals. In contrast, acute leukemias and other bone marrow-resident disorders readily undergo orthotopic engraftment after tail-vein or intra-osseous injection [31]. As a result, therapeutic trials in mice engrafted with these diseases
can utilize endpoints like overall survival or time to disease progression, just as in human trials. Mice can be treated until they progress on therapy, which allows for the development of acquired resistance. Samples can be taken from the peripheral blood or by sacrificing sentinel animals at multiple time points to establish biomarkers of response and resistance. Passaging in adequate numbers of animals also generates essentially unlimited numbers of primary cells for agnostic and targeted discovery efforts.

The central concern over PDXs is that they may fail to capture phenotypic, transcriptional, genetic and other characteristics of the tumors from which they were derived [23, 32]. Despite this concern, multiple entities now offer xenografting of patient tumors followed by in vivo drug testing, which they market as predictive of clinical response. We do not purport that each PDX is directly representative of the clinical sample from which it was derived. Instead, we hypothesize that using PDXs to conduct statistically powered, randomized trials in mice can efficiently define therapeutic activity across a broad range of genetically distinct tumor xenografts and inform the design and execution of human trials. At the same time, these preclinical trials can be used to establish biomarkers predictive of response, to generate models of drug resistance after in vivo exposure, and to interrogate aspects of in vivo biology, including tropism. To this end, we aimed to establish a large repository of well-characterized PDXs of hematologic malignancies, including a significant subset of BCR-ABL+ ALL PDXs.
Methods.

**Generation and Use of PDXs.** Primary bone marrow and blood specimens were collected from patients with leukemia and lymphoma at Dana-Farber Cancer Institute, Brigham and Women’s Hospital, and Boston Children’s Hospital. We also imported and transplanted primary leukemia and lymphoma specimens from 8 additional centers as well as 72 total PDXs that were already established by participating investigators. De-identified patient samples were obtained with informed consent and xenografted under Dana-Farber/Harvard Cancer Center Institutional Review Board (IRB)-approved protocols #13-351. Nod.Cg-PrkdcscidIL2Rγ<tm1Wjl>/SzJ (NSG) mice were purchased from Jackson Laboratories (Bar Harbor, ME) and handled according to Dana-Farber Cancer Institute’s Institutional Animal Care and Use Committee (IACUC)-approved protocol #13-034.

**PDX Characterization.** Upon engraftment, animals were sacrificed via CO2 asphyxiation. Peripheral blood, spleen, bone marrow, and lymph nodes when relevant were harvested. Erythrocytes were eliminated from peripheral blood and spleen samples via chemical lysis (RBC lysis buffer, Qiagen #158902). Tumors were immunophenotyped by multi-parameter flow cytometry for human antigens CD45, CD2/3, CD19, CD33, and CD34. Tumor cells were enriched via fluorescence-activated cell sorting (FACS) or immunomagnetic depletion of murine cells (EasySep Mouse/Human Chimera Isolation Kit, StemCell Technologies #19849). Enriched tumor cell samples were subjected to any or all of the following: (1) banking by viably freezing in fetal bovine serum (FBS) with 10% dimethylsulfoxide (DMSO), (2) further in vivo expansion by re-transplantation into secondary recipient mice, (3) genomic DNA extraction (QIAamp DNA Mini Kit, Qiagen #51304), (4) mRNA extraction (μMACS mRNA Isolation Kit, Miltenyi Biotec #130-090-276), and (5) fixation in formaldehyde-based buffer with 0.1% sodium dodecylsulfate.
In addition, selected tumors were formaldehyde fixed for en bloc for formal histopathologic analysis by the Dana-Farber/Harvard Cancer Center Rodent Histopathology Core.

**Targeted Exome Sequencing.** Targeted exon capture and next-generation sequencing of all coding exons of 205 genes (Table 1) were performed as previously described [33]. A total of 115 PDX models were genotyped. Genomic DNA extracted from enriched PDX tumor cells underwent customized hybrid-capture target enrichment (SureSelect, Agilent) and Illumina sequencing. Non-tumor DNA from remission peripheral blood, remission bone marrow, or saliva samples was also obtained from 45 of the 115 patients whose tumor-derived PDX lines were sequenced so as to facilitate filtering of somatic mutations. A panel-of-normals filter was subsequently generated from all 45 matched non-tumor controls. In addition, genomic DNA from the splenocytes of a normal NSG mouse was sequenced in order to enhance species-specific filtering of human reads. Sequencing reads were filtered using the NSG control and either the matched non-tumor sample, if available, or the panel-of-normals. Variants were rejected as germline artifacts if they were present in the matched non-tumor sample, if available; otherwise, they were rejected if present in two or more of the panel-of-normals. Known germline polymorphisms from the Exome Sequencing Project and dbSNP (build 142) databases were also excluded. We applied the MutSigCV algorithm [34] to identify genes that were altered more often than expected by chance given the background mutation rate. We used the evolutionary conservation of the affected amino acid in protein homologues to predict the functional effect of detected variants. We only considered variants predicted to be non-silent (i.e., missense, nonsense, translation start site, or splice site alterations, or in-frame or frame-shift insertions/deletions) and with variant allele frequencies of 5% or more. For every alteration meeting these criteria, sequencing reads were individually visualized to evaluate mapping quality.
as well as the phase and spatial distribution of alterations within reads. Mutations were then called by cross-referencing candidate variants to ClinVar, COSMIC, and a review of published literature. The final set of curated mutations is shown in Table S1 (provided as a separate excel file).

**Whole Transcriptome Sequencing.** Messenger RNA was extracted from PDX leukemia or lymphoma cells using magnetic microbeads (µMACS mRNA Isolation Kit, Mitenyi Biotec). Non-stranded RNASeq libraries were generated using the True-SeqRNA Sample Prep kit (Illumina) on a SciClone platform (Perkin Elmer). mRNA underwent fragmentation, cDNA synthesis, and next generation library synthesis via end repair, adenylation, adapter ligation and PCR amplification. Libraries were sequenced on a Next-Seq instrument (Illumina) using a paired-end protocol. Analysis of RNA was implemented as a Snakemake workflow [35]. The complete workflow documenting all utilized parameters and tools is available on Bitbucket ([https://bitbucket.org/cfce/weinstock-leukemia](https://bitbucket.org/cfce/weinstock-leukemia)). Paired end RNASeq samples were mapped to the human genome reference assembly (hg19) with STAR 2.4.2a [36]. Transcript expressions were estimated with Cufflinks 2.2.1 without transcript assembly [37]. Gene expressions were calculated as sums of transcript FPKM values. Genes with transcripts smaller than 300 bp were ignored. In order to compare the obtained FPKM values with external datasets such as RNASeq RPKMs from the TCGA AML cohort, RMA-normalized log2-scaled microarray intensities from the CCLE cohort, and RNASeq from NCBI Sequence Read Archive (SRA) study SRP058414 (primary human pre-B ALL whole transcriptome sequencing, [http://www.ncbi.nlm.nih.gov/Traces/study/?acc=SRP058414](http://www.ncbi.nlm.nih.gov/Traces/study/?acc=SRP058414)). All expression values were log2-transformed.
Pre-normalized gene expressions followed similar bimodal distributions in all data sets. Quantile normalization (Limma 3.26.1 [38]) was therefore performed to adjust for library depth and sequence-specific differences (Figure 2). Distribution of gene expressions in log2 scale derived from multiple data sets (CCLE cell lines, PDX models, SRP058414 primary pre-B ALL samples, and TCGA primary AML samples). Data are shown before (Figure 2A) and after (Figure 2B) quantile normalization. The primary sources of variation after quantile normalization were the different batches.

Batch effect correction was performed for normalized gene expression from the various data sets for each primary tumor histology using the ComBat approach from SVA 3.18.0 (Figure 3). Hierarchical clustering with Ward’s linkage and Euclidean distance over the 750 genes with the highest variance to mean ratio was performed separately for each histology (with external datasets) and over all histologies (PDX samples only). Fusion genes were called with the STAR-Fusion 0.4.0 (https://star-fusion.github.io). Evidence for fusions was measured by summing spanning fragments (read pairs that align on both sides of a breakpoint) and junction reads (reads that align over the breakpoint). Fusions with an evidence of at least 10 over all samples were retained, and further filtered manually to conservatively remove putative homology-induced artifacts.

Web-Based Data Hosting. To maximize access to PDX-related data, an internet-based search portal called the Public Repository of Xenografts (PRoXe; www.proxe.org) was created using the R Shiny platform, a web application for R that permits the fluid integration and analysis of heterogeneous data types in a graphical user interface. All underlying code for this application is freely accessible on Bitbucket (https://bitbucket.org/scottkall/proxe). For each PDX for which data were available, an extensive case abstraction for patient- and tumor-level
data was performed using the Dana-Farber Cancer Institute/Brigham and Women’s Hospital electronic medical record. Whenever possible, information was taken from primary laboratory, molecular, or pathology reports. PDX characterization including immunophenotype, targeted exome sequencing, whole transcriptome sequencing, class I HLA types (inferred from RNASeq data), histopathologic data, and kinetics of engraftment are also hosted. Wherever possible, primary data in the form of histopathologic images, flow cytometry plots, and pathology reports are hosted in pdf form and directly accessible through the web site.

PRoXe also incorporates administrative functionality to facilitate repository operations, distribution of PDX lines and material to external investigators, and consultations regarding line selection and PDX methods. Data maintained by other PRoXe team members such as line inventories and distribution restrictions are regularly merged into the R dataframe underlying the app. Data from users in the form of requests for lines, request for consultation, or general inquiries are entered into Google documents linked to from within the app and deposited into password-protected Google spreadsheets. PRoXe team members then respond to requests and document progress in real time on the Google spreadsheet.
Results.

Establishment of Patient-Derived Xenografts. Success rates for engraftment in first passage (i.e., P0) after tail-vein injection varied based on disease subtype: B-cell ALL (B-ALL; 67.5%), T-cell ALL (T-ALL; 46.7%), acute myelogenous leukemia (AML; 23.2%), and all lymphomas (20.3%). Engraftment into P1 recipients also varied by disease, with very high rates of engraftment for B-ALL (95.3%) and lower rates for AML (65.7%), T-ALL (75%), and all lymphomas (76.9%) by eight months after injection. Thirteen (30.2%) of 43 additional lymphomas engrafted in P0 within six months after tumor seeds obtained from tissue biopsies were implanted under the renal capsule.

Table 1 outlines the current repository based on the 2008 World Health Organization classification [21]. In addition to a range of acute leukemia subtypes, we established multiple PDXs representative of lymphomas with no or very few available cell lines or in vivo models, including angioimmunoblastic T-cell lymphoma (AITL), extranodal NK/T-cell lymphoma, breast implant-associated anaplastic large cell lymphoma, peripheral T-cell lymphoma NOS (PTCL-NOS), blastic plasmacytoid dendritic cell neoplasm (BPDCN), and adult T-cell leukemia/lymphoma (ATLL).

Multiple models demonstrated tropism within recipient mice that mimicked the clinical disease. For example, Sézary Syndrome is a form of cutaneous T-cell lymphoma with peripheral blood involvement. We injected peripheral blood from a patient with Sézary Syndrome into the tail-vein of NSG mice and the cells trafficked to and involved the mouse skin, establishing a unique model of human lymphoma epidermotropism. Similarly, a diffuse large B-cell lymphoma (DLBCL) from a patient who did not have central nervous system (CNS) involvement engrafted under the renal capsule in P0. That tumor was dissociated into a single-cell suspension and
injected by tail-vein into P1 mice. All 5 (100%) injected mice succumbed with CNS involvement by DLBCL. Despite the difficulty of developing low-grade lymphoma models in vivo, a marginal zone lymphoma engrafted as a low-grade, CD20⁺ lymphoma after implantation under the renal capsule and a follicular lymphoma engrafted with small BCL2-positive, CD20-positive lymphocytes. Similarly, two different mantle cell lymphomas (MCLs) that involved blood and bone marrow in patients also involved the blood, bone marrow and spleen of xenografted mice. Even more remarkably, MCL obtained from the blood of a patient with blood, nodal and gastrointestinal involvement trafficked to each of these sites after tail vein injection.

In early passage, some PDXs maintained human, non-malignant cell populations that were present in the original tumor. For example, the PTCL-NOS engrafted in P0 with populations of non-malignant CD4⁺ and CD8⁺ T-cells as well as a rare population of EBV-positive B-cells. Some PTCLs harbor a small subset of EBV-positive cells [21], but the role of these cells in disease development and persistence has not been defined.

**Transcriptional, genetic, and phenotypic characterization of PDXs confirms subtype fidelity.** We confirmed lineage fidelity in 157 PDXs by flow cytometry for markers including CD3 (T cell), CD19 (B cell), CD33 (early myeloid), CD34 (hematopoietic progenitor), and CD45 (pan-hematopoietic). Whole transcriptome sequencing was performed on 107 PDXs in addition to targeted DNA sequencing of all exons of 205 genes previously reported to be recurrently altered in hematologic malignancies [33] (Table 1). Unsupervised hierarchical clustering based on disease type (AML, T-ALL, B-ALL, BPDCN, or lymphoma (Figure 4, Table S2 (provided as a separate excel file)) correctly clustered 105 (98%) of the 107 PDXs. A broad diversity of genetic alterations is captured within the PDXs, including targetable lesions (e.g. mutations of IDH1, IDH2 or JAK2, rearrangements involving MLL or ABL).
Among B-ALL PDXs, interrogation of RNAseq data for gene fusions rediscovered 28 (96.6%) of 29 fusions identified by clinical cytogenetics analysis of the patient samples, including BCR-ABL1, MLL, ETV6-AML1, and TCF3-PBX1 rearrangements (Figure 9). In addition, previously unrecognized fusions involving PAX5, JAK2, KDM6A, RUNX1, CRLF2 and SPI1 were recovered from the PDXs, with fusion transcripts from both derivative chromosomes identified in multiple PDXs (Figure 9). Interrogation of the transcriptomes from AML PDXs similarly identified both known and unrecognized fusions (Figure 10).

Next, we performed unsupervised clustering of transcriptomes from B-ALL PDXs in combination with transcriptomes of primary B-ALLs and B-ALL cell lines from the Cancer Cell Line Encyclopedia (Figure 5, Table S3 (provided as a separate excel file)) [39]. To permit analysis of the aggregated data, quantile normalization (Limma 3.26.1 [38]) was first performed to adjust for library depth and platform-specific differences. Principle components analysis revealed that the primary source of post-normalization variation derived from batch effects. These batch effects within each general diagnostic category (AML, B-ALL, T-ALL, lymphoma) were successfully removed using the ComBat approach from SVA 3.18.00 [40] agnostic of additional clinical and molecular covariates. Hierarchical clustering with Ward’s linkage and Euclidean distance over the 1000 genes with the greatest variance-to-mean ratio was performed over all diagnostic categories (PDX dataset only) and for each diagnostic category separately (all three datasets).

Compared with cell lines, PDXs spanned a broader diversity of WHO diagnostic categories, patient demographic characteristics, phases of treatment, prior therapies, cytogenetic profiles, and genotypes (Figure 4). At present, there are 28 B-cell leukemia cell lines available through the German cell line repository DSMZ, of which only 10 have RNA expression data
available through the CCLE. In contrast, we have generated 115 B-ALL PDX lines, of which 82 have RNA expression data already available. Unsupervised clustering underscored the biologic influence of canonical fusion genes such as those involving MLL, BCR-ABL1, TEL-AML1, and E2A-PBX1. Although fewer cases were analyzed, separate clustering of AML, T-ALL and lymphomas identified distinct clades in each disease (Figures 6-8). Lymphoma samples segregated by cell lineage (B vs. T), with canonical fusion genes correlating with a number of derivative clades, including CCND1-IGH+ MCL, NPM1-ALK+ ALCL, and DLBCLs with concurrent rearrangements of MYC and BCL2 (Figure 8).

**PRoXe: A resource to facilitate studies of leukemia and lymphoma PDXs.** To massively expand the widespread use of these models and the data derived from them, we established an open source web portal called the Public Repository of Xenografts (PRoXe; www.proxe.org). We created PRoXe using Shiny, a web application library for R that permits the fluid integration and analysis of heterogeneous data types in a graphical user interface. PRoXe provides extensive patient, tumor, PDX, and germline administrative information for each line. Any of the 56 characteristics that are categorical or quantitative can be visualized interactively via histograms, barplots, scatterplots, and boxplots, allowing the user to dynamically interrogate individual variables as well as interactions between these variables. Flow cytometry plots, full-color immunohistochemistry images, detailed pathology reports, and class I HLA alleles inferred from RNAseq [41] are available for a subset of lines. RNA expression in the form of log-transformed RPKM derived from RNAseq can be visualized as bar plots or heat maps for individual genes or panels of genes for selected PDX lines. Curated mutations among panels of genes identified by targeted exon sequencing can be visualized in matrix form using a publically available OncoPrint script (https://gist.github.com/armish/564a65ab874a770e2c26). Whole
transcriptome BAM files are being uploaded to the NCBI Sequence Read Archive (http://www.ncbi.nlm.nih.gov/sra; accession number pending). RPKMs and mutation calls are also being uploaded to the cBio portal (www.cbioportal.org).
Discussion.

Countless previous studies, reviews, and perspectives have discussed the high rate of failure, the long time from discovery to clinical benefit, and the massive cost of developing effective cancer therapeutics. In order to facilitate preclinical drug studies that are more informative, we have established a resource to support randomized PDX trials in mice. We submit that adequately powered studies in PDXs can be used to develop transcriptional, proteomic, and functional biomarkers of response and resistance while defining the efficacy of therapeutics across genetically diverse subtypes of leukemia. Trials of PDXs permit the harvesting of large numbers of biospecimens at multiple time points and also generate large numbers of models with acquired in vivo resistance, a tremendous advantage of this study design (for full details see the full paper upon which this section of the thesis was based [14]).

Leukemias and lymphomas in particular offer several advantages that make them amenable to this experimental approach. First, many subtypes of leukemia and lymphoma readily engraft into NSG mice or similarly immunocompromised strains by tail-vein injection. Second, bone marrow and blood specimens from patients with these diseases are easily obtained in comparison with most solid tumors. Third, tail-vein injection results in orthotopic disease growth, both in the bone marrow and in extramedullary sites that are commonly involved by these diseases (e.g. skin, CNS, lymph nodes, gastrointestinal tract). Finally, studies of orthotopic leukemia PDXs allow for survival or disease progression, rather than tumor shrinkage, to be used as the primary endpoint. Previous discordances between tumor shrinkage in preclinical models and survival in human studies suggest that tumor shrinkage alone can be misleading [42].

It is important to note that direct and adequately powered comparisons for predictive ability between cell lines and PDXs are lacking. This has largely resulted from the inadequate
number of available cell lines for an individual disease subtype. It remains possible that cell lines would be equally predictive as PDXs for many therapeutics if adequate numbers of cell lines were available. Similarly, other types of in vivo models have clear advantages over PDXs. Insertional mutagenesis and chemical carcinogenesis models are highly diverse within an individual mouse and across a population of tumors [43]. Both these models and transgenic models have significant advantages over PDXs for modeling microenvironmental and tumor-immune interactions [23]. Not all human tumors are easily engrafted orthotopically, which further limits the ability to study the effects of tumor microenvironment on therapeutic response [44]. Another clear limitation of PDXs is the bottleneck that tumor cells must navigate to engraft in immunocompromised mice. Although leukemias and lymphomas have relatively high ‘take rates’, certain subsets have proven completely intractable to xenografting, while others only xenograft in a limited fraction of cases [32]. This may introduce an important bias that limits applicability of PDX studies to unselected patient populations.

As a result of these limitations, we make no claims that each individual model in PRoXe is representative of the leukemia or lymphoma from which it was derived. Careful studies of AML PDXs have demonstrated quite clearly that different leukemias can engraft in myriad different ways, as assessed by the relative representation of subclones, even when injected as biologic replicates into congenic recipients [32]. Further complicating the diversity of models are differences in recipient strain, approach to transplantation (e.g. tail-vein injection, intraosseous injection, radiation of recipient mice), timing of injection (e.g. from fresh specimens versus after freezing) and patient demographics between centers that generate PDXs. Our intention was never to analyze the fidelity of individual models to their starting material. Instead, we developed a resource to facilitate the pre-clinical evaluation of therapeutics across a diversity of different
CLONAL RESISTANCE IN BCR-ABL ALL

malignancies. The observation that three different mice engrafted with the same PDX and treated with the same agent have almost exactly the same survival comes as little surprise, as previous studies from our groups [45-47] and many others have shown that treatment of large cohorts of mice injected with the same PDX typically results in survival curves that “fall off a cliff”. Although these curves, in which all mice treated in the same way die within a day or two of each other, provide robust statistical p values, they fail to inform the field about the true activity of a therapeutic across diverse tumors.

To address these limitations, the Weinstock laboratory has gained experience with randomized phase II-like studies of PDXs in mice, which are applicable to a range of therapeutic agents, especially those that act through cell-intrinsic mechanisms. These studies may markedly reduce the time and financial expense of drug development while mitigating the very real human cost of administering toxic and ineffective therapies to patients during the drug development process. Our laboratory has generated data using such approaches highlighting this potential [14], in that subsets of PDXs from a molecularly heterogeneous population were shown to respond or not respond in a manner mirroring that seen in clinical trials of biosimilar drugs in humans. Moreover, we could use the abundance of biospecimens available from these PDXs to define predictive biomarkers that could potentially be applied prospectively to better focus novel therapeutics in patients with the greatest likelihood of clinical benefit. We are engaged in ongoing efforts to apply such methods to define the activity of BCR-ABL-directed kinase inhibitors in PDX models of BCR-ABL^+ ALL, and to use these PDXs as platforms for elucidating leukemia biology.

In order to foster the maximum impact from the PDX repository, we developed PRoXe as an open source website. We are currently in negotiations with multiple different academic centers
to incorporate their PDXs into PRoXe or to develop Shiny-based web portals for their own PRoXe sites. Incorporation of clinical-, pathologic- and PDX-level data from 300 of the solid tumor PDXs recently published by Gao et al. [30] is underway, and we are encouraging others to consider adding their models. Limitations exist in the willingness of centers to share models, based largely on concerns over liability, hopes for financial compensation and confidentiality-related issues germane to unfettered access. Addressing these is an essential next step if the true value of PDXs is ever going to be realized. In addition, academic centers are ill-suited to bear the burden of housing, expanding, archiving, characterizing and disseminating PDXs to investigators (academic and industrial) across the world. As a result, we are licensing a large portion of the models to the Jackson Laboratories, which will allow for industry-scale expansion, quality control, characterization and distribution, much as Addgene has provided this service for DNA plasmids.
Footnotes

A manuscript encompassing the work detailed herein was published during the thesis period as:

Precise Measurement of Single Cancer Cell Mass Accumulation Predicts Drug Susceptibility

Introduction.

Therapeutic decisions in oncology have historically been based on treatment responses observed in large studies across heterogeneous populations, such that most decisions for individual patients remain empiric. The shortcomings of this approach – limited efficacy and avoidable toxicity – have motivated a broad effort to achieve greater precision in cancer therapeutics by basing decisions on the presence or absence of genetic, epigenetic or other molecular biomarkers within an individual’s tumor [48, 49]. While this approach has been successful in some instances (e.g., lung adenocarcinoma with EGFR point mutations or rearrangements involving ALK or ROS1), the vast majority of cancer therapies have no known markers for susceptibility or resistance [50]. Even when marker-based predictions can be made, they do not guarantee patient response, as many are the result of correlations from population-based studies [51, 52].

A second major shortcoming of nearly all available biomarkers is that they are derived from analyses of bulk tumor populations and thus do not predict the emergence of resistant subpopulations. More sensitive approaches of genetic characterization, like single-cell sequencing, are becoming increasingly common as research platforms but are not yet amenable to a clinical setting [53, 54]. These approaches also suffer from the same shortcoming as bulk assays, i.e., the lack of predictive genetic or transcriptional markers.

In contrast with most biomarkers, functional assays can provide phenotype-driven predictors of therapeutic response that represent the integrated output of multi-parameter datasets,
including genetic, epigenetic, environmental and other variables that determine response. Clinical response in a patient following treatment is currently measured by imaging to assess bulk tumor volume or more rarely by direct measurement of tumor burden (e.g. peripheral blast counts). However, these assessments are only useful for making post-hoc treatment decisions. In the ideal scenario, predictive assays would be used to guide selection of the most effective treatment pre facto and thereby maximize efficacy while minimizing toxicity from inefficacious therapies.

Although functional assays are essential clinical tools in assessing the antibiotic susceptibility of microbes, no such approaches have been widely adopted for patients with cancer [55, 56]. Existing platforms to measure cancer cell growth, such as ATP-based assays (CellTiter-Glo), require extended time in culture and a large number of tumor cells [57]. This precludes their use for the large majority of patients, who have limited amounts of cancer tissue available. Furthermore, these bulk approaches are ill-suited for characterizing therapeutic susceptibility of subpopulations that exist within heterogeneous tumors [57].

An ideal functional assay for predicting therapeutic response in patients with cancer would have the following characteristics: 1) accurately measure responses to both single drugs and drugs in combination, 2) require minimal sample input, 3) avoid artifacts that result from long-term, in vitro culture, 4) quantify therapeutic response at the single-cell level, 5) return results within a timeframe conducive to real-time therapeutic decision making, and 6) maintain cell viability to allow for downstream functional and molecular interrogations. An assay with these characteristics could be broadly applied to predict therapeutic susceptibility in multiple contexts, including heterogeneous tumor populations, residual disease following therapy, and circulating tumor cells. To our knowledge, no current assay meets all of these criteria.
In collaboration with the laboratory of Dr. Scott Manalis at MIT, we have advanced an approach that functionally assesses the therapeutic sensitivity of single cancer cells based on mass accumulation; this is a property fundamentally linked to cell growth, as cell division requires the accumulation of biomass. The approach involves weighing individual cells repeatedly over a 15-minute period in a suspended microchannel resonator (SMR) (Fig. 8a) [58-60], either in the presence or absence of cancer therapeutics. Resonator-based approaches have been used to measure an array of cellular physical properties, in some cases in response to therapeutics [61, 62]. Following the incubation of tumor cells with drug, the SMR can detect changes in the growth of single cells to predict therapeutic response without the need for extended culture. To validate this approach, we applied the SMR to traditional cancer cell lines and primary cells.
Methods.

Cell culture of conventional cell lines. L1210, Ba/F3-BCR-ABL, and Ba/F3-BCR-ABL-T315I cells were maintained in suspension in RPMI-1640 media (Invitrogen, Cat#11875-119), supplemented with 10% FBS (Sigma Aldrich, Cat#F4135), Penicillin-Streptomycin (Invitrogen, Cat#15140-122), and kept in a 37°C, 5% CO₂, and humidified incubator. Cells were passaged every 2 days to 5x10⁴ cells/mL, and used for SMR experiments between 24-36 hrs of growth at an approximate cell concentration of 2-4x10⁵ cells/mL. L1210 cells were provided by the Kirschner lab at Harvard, Ba/F3-BCR-ABL, and Ba/F3-BCR-ABL-T315I were created in the Weinstock laboratory from parental Ba/F3 cell lines obtained from the RIKEN BioResource Center. No further cell line validation was performed. All cell lines tested negative for mycoplasma. For drug response experiments, cells in bulk were dosed for the specified interval with 0.1% DMSO, 1 μM imatinib (Santa Cruz Biotechnology #SC-202180), or 100 nM ponatinib (Selleckchem #AP24534). The cells were kept in drugged media during the measurements, and samples sizes were determined by throughput limitations inherent to the single-cantilever SMR.

Transgenic mouse model of BCR-ABL B-ALL. All animal experiments were performed with approval of the DFCI IACUC. A transgenic mouse model of BCR-ABL⁺ ALL was generated by transplantation of lethally irradiated Ts1Rhr mice (B6.129S6-Dp(16Cbr1-ORF9)1Rhr/J; Jackson Laboratory, Bar Harbor, ME, USA; stock #005848) with syngeneic Hardy B cells transduced with an MSCV retrovirus co-expressing GFP and human BCR-ABL cDNA, as previously described [63]. For the present studies, 1x10⁶ bulk splenocytes (P1 generation) were transplanted into lethally irradiated, wild-type, female, C57BL/6 mice at 6-8 weeks of age, which were followed daily for clinical signs of leukemia and sacrificed when
moribund. Splenocytes or blood samples were harvested, subject to erythrocyte lysis (Qiagen #158904), and stained with an antibody targeting murine CD19 (Fisher Scientific #BDB551001). BCR-ABL+ ALL cells were isolated by sorting for CD19/GFP double-positive cells on a FACS Aria II SORP fluorescence activated cell sorter (BD Biosciences).

For drug response experiments, sorted mouse leukemia cells were seeded at a density of 5x10^5/mL and cultured at 37 °C in a humidified 5% CO2 incubator in RPMI (Gibco #11835055) supplemented with 10% FBS, 2 mM L-glutamine (Gibco #25030164), 50 μM 2-mercaptoethanol (Sigma #M3148), 50 IU/ml-50 μg/mL penicillin-streptomycin (Fisher Scientific #ICN1670049), 10 ng/mL recombinant murine IL-3 (PeproTech #213-13), 10 ng/mL recombinant murine IL-7 (PeproTech #217-17), 10 ng/mL recombinant murine stem cell factor (PeproTech #250-03), and 10 ng/mL recombinant murine FLT3-ligand (PeproTech #250-31L). Cells were kept in a 37 °C, 5% CO2, and humidified incubator. Replicate cultures were dosed for 10 hrs with 0.1% DMSO, 1 μM imatinib (Santa Cruz Biotechnology #SC-202180), or 100 nM ponatinib (Selleckchem #AP24534). The cells were kept in drugged media during the measurements, which took place between 10-20 hrs of drug exposure, and samples sizes were determined by practical limitations set by throughput and measurement window length.

For in vivo confirmation of drug efficacy, female C57BL/6 mice were sublethally irradiated and transplanted with 7.5x10^5 murine leukemia cells harboring human BCR-ABL cDNA, of which 95% were BCR-ABL wild type and 5% harbored the T315I allele. Upon engraftment, as defined by the presence of circulating leukemia at a level of 1-3% by peripheral blood flow cytometry, mice were treated with nilotinib 50 mg/kg/day via oral gavage (n=2 mice). Nilotinib serves as a surrogate for imatinib, as both compounds have demonstrated activity against WT but not T315I BCR-ABL. Error bars represent standard deviation. Mice underwent
serial BCR-ABL genotyping via the Sanger method to monitor the allelic frequency of BCR-ABL T315I.

**Patient sample procurement and processing.** Primary samples were obtained from patients with AML at the DFCI and Brigham and Women’s Hospital following informed consent in accordance with IRB-approved protocols. Peripheral blood and bone marrow samples underwent Ficoll density gradient centrifugation to enrich for mononuclear cells, followed by immunomagnetic enrichment of leukemia cells using CD33 MicroBeads (Miltenyi t#130-045-501) if concomitant clinical testing indicated that tumor purity was <80%. Leukemia cells were seeded at 0.5-1.0x10^6/mL in DMEM supplemented with 15% FBS, 2 mM L-glutamine, 50 µM 2-mercaptoethanol, 50 IU/ml-50 µg/mL penicillin-streptomycin, and human cytokines SCF (100 ng/mL, PeproTech #300-07), IL3 (10 ng/mL, PeproTech #200-03), IL6 ((20 ng/mL, PeproTech 200-06), TPO (10 ng/mL, PeproTech #300-18), and FLT3-Ligand (10 ng/mL, PeproTech 300-19), as adapted from published methods [64]. For measurements of *in vivo* response, aliquots of cultured cells were immediately measured in the presence of DMSO or sustained drug pressure with the experimental MDM2 inhibitor. In the case of *ex vivo* treatment, aliquots were treated with an experimental MDM2 inhibitor, cytarabine (1 µM), midostaurin (100 nM), or DMSO (1:1000) and were assessed using the SMR within 10-hour windows centered on 20 and 44 hours.

**Measurement and operation of a suspended microchannel resonator (SMR).** The design and operation of the SMR have been previously described [65, 66]. In short, single cells in suspension are passed through the SMR resulting in a frequency shift that is proportional to cell buoyant mass. The SMR can resolve the instantaneous rate of mass accumulation for a single cell in 15 minutes provided that the cell is weighed approximately every 30 seconds. Mass calculated from frequency shift using a calibration factor derived from measurements of
frequency shifts of polystyrene beads with a known mass (Thermo Scientific #4208A). Mass versus time data of a single cell is then linearly fitted. For measurement in the SMR, cells were suspended in their standard growth media with or without drug. The system and cells were kept in culturing conditions (5% CO\(_2\), 37 °C) for all measurements as previously described [67].
Results.

**Mass accumulation rate (MAR) measurement.** The SMR is a cantilever-based microfluidic mass sensor that measures the buoyant mass (referred to hereafter simply as mass) of live single cells with a resolution near 50 femtograms, which is highly precise given that the average buoyant mass of a hematopoietic cell is ~75 pg [58]. Cells are measured in suspension while under culture conditions, with controlled media temperature and CO$_2$ concentration to maintain cell viability and growth [60]. A series of mass measurements is made on an individual cell every ~30 seconds for ~15 minutes, allowing for determination of the mass accumulation rate (MAR), which is defined as the change in mass over time (Fig. 9a) [59]. A second biomarker that we utilize in this study is single-cell mass, which is determined for each cell during the MAR measurement. By performing the MAR measurement on multiple cells from the same population, the SMR reveals heterogeneity in mass and MAR across the population, rather than an average of the tumor bulk. The degree to which mass and MAR behave as independent biomarkers varies depending on conditions and cell type. Although linear discriminate analysis (LDA) maximizes the predictive capability of these two biomarkers, we have used a simplified metric of MAR normalized by mass for most of the studies in this paper.

**Single-cell MAR profiles demonstrate heterogeneity within acute leukemias.** In order to better characterize the platform’s performance, we applied this method to an in vivo model of acute leukemia, cells of which are known to proliferate in suspension culture. Specifically, we analyzed primary leukemia cells isolated directly from mice with genetically-engineered, BCR-ABL-expressing acute lymphoblastic leukemia (BCR-ABL$^+$ ALL). Consistent with our previous findings [59], the SMR was able to quantify MAR of single cells over ~15 minute intervals with high signal-to-noise ratios (Fig. 9b). During this time, the cells acquired less than a few
picograms of biomass. This is equivalent to an increase in cell diameter on the order of only 10 nm. Almost all primary BCR-ABL+ ALL cells exhibited positive MARs that monotonically increased with cell mass (Fig. 9b).

MAR predicts susceptibility to targeted therapeutics in conventional and patient derived cell lines. To test the ability of MAR measurements to predict drug susceptibility in an established cell line, we utilized Ba/F3 cells engineered to express either wild-type BCR-ABL or the imatinib-resistant mutant BCR-ABL T315I [68, 69]. Treatment of Ba/F3-BCR-ABL cells with the tyrosine kinase inhibitor (TKI) imatinib at a therapeutically achievable concentration of 1 μM for only 2-4 hrs significantly decreased MAR without altering the distribution of mass (Fig. 10). With longer durations of exposure to imatinib, the reduction in MAR became more pronounced (Fig. 10b) and cell mass was reduced (Fig. 10c). When the same conditions were applied to Ba/F3-BCR-ABL T315I cells, no significant change was observed in MAR or mass distributions (Fig. 10). However, exposure of these cells to the third-generation TKI ponatinib, which retains activity against BCR-ABL T315I, recapitulated the same reduction in MAR observed upon imatinib treatment of Ba/F3-BCR-ABL cells (Fig. 10b) [69]. Thus, MAR can distinguish therapeutic susceptibility from resistance within single Ba/F3 cells after only a few hours of drug exposure.

MAR predicts susceptibility to targeted therapeutics in primary leukemia cells. Next, we asked whether MAR measurements could effectively predict therapeutic response in primary tumor cells measured immediately after isolation from the in vivo setting. We harvested transgenic murine ALLs that express BCR-ABL or BCR-ABL T315I from the spleen of mice and flow-sorted to purify leukemia cells [63]. Single-cell MAR data was collected after 10-20 hrs of treatment with 1 μM imatinib or 100 nM ponatinib. Across three independent biological
replicates, we observed a significant reduction in average MAR for leukemias expressing BCR-ABL upon treatment with imatinib or ponatinib, as well as for leukemias expressing BCR-ABL T315I upon treatment with ponatinib (Fig. 11a). In contrast, imatinib had no effect on leukemias expressing BCR-ABL T315I (Fig. 11a). We confirmed that BCR-ABL T315I leukemias are truly resistant to the imatinib analog nilotinib in situ by treatment of mice engrafted with these leukemias. In contrast to the marked effect of imatinib on MAR of wild-type BCR-ABL leukemia cells, exposure to imatinib in vitro for 24 hrs had no effect on the viability of leukemias expressing wild-type BCR-ABL in bulk culture, as determined by flow cytometry for Annexin V and DAPI.

In order to gauge how robustly MAR measurements can predict primary ALL single-cell drug sensitivity, we generated a receiver-operating characteristic (ROC) after performing linear discriminate analysis (LDA) on each replicate’s dataset. So far we have used the single metric of MAR per mass, however we also considered MAR and mass as independently variable biomarkers. LDA projects the populations of the two-dimensional MAR versus mass data onto a single axis that provides the best ability to distinguish two populations, and then defines the ideal threshold for this classification (Fig. 11b). Subsequent ROC curve analysis is performed and its area under the curve (AUC) is a metric of the ability to properly identify a single cell’s classification as sensitive or resistant to therapy [70]. A random classifier has an AUC equal to 0.5, and a perfect classifier has an AUC of 1. The average AUC of non-selective conditions (DMSO-treated compared to imatinib-treated T315I-mutant leukemia) was 0.57, consistent with the expectation that resistant cells are indistinguishable from untreated cells (Fig. 11a, c). Under selective conditions, the ROC curves for MAR versus mass showed excellent resolution of sensitive and resistant populations, with an average AUC of 0.85 (Fig. 11a, c). ROC curves using
mass or MAR as single parameters had significant power to classify single cells, but the single parameters were less consistent between replicates and on average less accurate than using both parameters for classification (Fig. 11c).

**MAR predicts susceptibility to targeted therapeutics in primary leukemia cells isolated from circulation.** Although murine spleens provide access to an unlimited number of tumor cells, we wanted to measure MARs from small samples, simulating the limited tissue available with patient biopsies. To this end, we isolated tumor cells from the peripheral blood of mice by lancing the submandibular vein (cheek bleed), which resulted in only 25 µL of total volume and does not compromise mouse survival. We performed these bleeds when circulating disease was as low as 4% of circulating mononuclear cells. This approach typically provided on the order of $10^3$ total tumor cells for measurement following purification by flow sorting. In order to measure samples of low cell count and volume, we implemented a next-generation SMR array device that greatly simplifies fluidic handling, increasing throughput by 20-fold and enables the use of low-volume samples [71].

Single-cell MAR data was then collected on both cheek bleed samples (~25 µL) and cardiac bleeds (~500 µL) exposed to either DMSO or 100 nM ponatinib for 14-20 hrs in vitro. Classification of single-cell drug response was similar using cheek bleeds (AUC = 0.85) compared with measurements from splenocytes (AUC = 0.85) or cardiac bleeds (AUC = 0.80) (Fig. 11d). The ability of MAR measurements to assay drug sensitivity of single cells isolated from very small amounts of blood makes it feasible to longitudinally screen for phenotypic resistance within individual patients through iterative sampling.

**Patient samples display reduction in MAR when treated either ex vivo or in vivo.** To define MAR assay compatibility with clinical samples, we ran two separate experiments using
primary patient samples. First, we assayed Ficoll gradient-separated peripheral blood samples from a patient with relapsed acute myeloid leukemia (AML). A sample obtained while the patient was not receiving treatment was composed largely of slightly positive MARs (Fig. 12a). After the patient had received 48 hrs of treatment with an experimental MDM2 inhibitor, a second peripheral blood sample was taken. This demonstrated a large population of cells with negative MARs of less than -1 pg/hr, indicating a shift in population MAR dynamics in vivo among the leukemia present in the peripheral blood (Fig. 12a). This shift towards negative MARs following treatment is consistent with our observations from ex vivo treatment of susceptible murine primary cells (Fig. 11).

Finally, we performed ex vivo treatment of a patient sample analogous to the approach applied to primary murine samples. Bone marrow leukemia cells from a patient presenting with newly diagnosed FLT3-mutated, complex karyotype AML was treated in media with a range of therapies, including DMSO, 1μM cytarabine (cytotoxic chemotherapy), 100nM midostaurin (multikinase inhibitor), and an experimental MDM2 inhibitor. These cultures were incubated, and MARs were measured on three next-generation SMR array devices in parallel during 10-hour windows that centered on 20 and 44 hrs. Cells in midostaurin showed no significant change in their distribution of MAR as compared to a DMSO control, consistent with the limited activity of single-agent midostaurin in FLT3-mutated AML [72]. In comparison, cytarabine did result in a reduction in MAR that was highly significant at 48hrs as compared to the control. Finally, cells treated with the experimental MDM2 inhibitor showed reduced MAR at 24 hrs, which rebounded by 48 hrs, potentially indicating a brief period of p53 target induction followed by the rapid induction of adaptive resistance.
Discussion.

Here we have presented a novel functional assay for assessing single cancer cell therapeutic sensitivity based on measurements of MAR and mass of individual cells, where both biomarkers offer power in classifying single-cell response but together offer greater precision for classifying single-cell response. We validated this capability by confirming susceptibility or resistance of genetically-defined cell lines and primary murine ALLs in response to targeted therapeutics. Additionally, by measuring mass accumulation rates of single cells across populations of cell lines, we have shown for the first time the extent to which single-cell growth heterogeneity can vary both within and across these populations. A substantial body of experiments need to be performed to prove utility of MAR measurements with primary patient samples. However, our initial data with primary patient leukemia samples, treated either \textit{ex vivo} or \textit{in vivo}, demonstrated response characteristics that were consistent with our more tractable disease models.

MAR measurement within the SMR is not a terminal assay, as cells are kept viable throughout the measurement and thereby remain compatible with downstream analyses. Thus, a key advantage of MAR measurements is that cells can be studied downstream of the SMR using other single-cell assays. This ability will ultimately allow for correlations between single-cell changes in MAR, other functional outcomes and non-functional biomarkers (e.g. genetics, gene expression, chromatin modifications). Further studies are needed to assess the effects of passage through the SMR on aspects of tumor cell biology, including changes in the transcriptome, genome, and proteome. Importantly, previous studies have found that cellular and genomic properties of single cells can be measured using techniques such as RNA-sequencing and are well-preserved following exposure to microfluidic environments [73].
An essential feature of MAR measurements is that they allow for application to minimal residual disease, circulating tumor cells, or selected subpopulations (e.g. cancer stem cells). As we showed for primary ALL cells isolated directly from the circulation of mice, flow sorting of an exceedingly small number of cells can be performed prior to measurement of mass and MAR.

Recent studies have used other functional approaches to predict therapeutic sensitivity of individual cancers. For example, Crystal et al. applied standard proliferation-based assays to quantify therapeutic susceptibilities of bulk cultures of patient-derived cell lines (PDCLs) [57]. Proliferation assays, a longstanding functional method of screening drug response in vitro, do not reveal cell-to-cell growth heterogeneity. The authors identified multiple combinations that could overcome therapeutic resistance to targeted agents by establishing and screening PDCLs from patients who had already developed resistance to these agents. The results from their ex vivo screening predicted the response of in vivo xenografts to combination therapies. However, the utility of this strategy for clinical decision making is limited by the months of prolonged culture required for PDCL creation. Additional constraints in PDCL production (i.e. bulk population-based approach, incomplete success in PDCL generation) further limit clinical application.

Montero et al. recently reported a functional approach called ‘dynamic BH3 profiling’, in which therapies are applied to cell lines or clinical tumor isolates, and then the percentage of cells that undergo mitochondrial outer membrane permeabilization (MOMP) is measured after introduction of a pro-apoptotic BH3 peptide [74]. An increase in the percentage of cells that undergo MOMP upon pre-incubation with a single agent or combination of agents is used as a marker of “apoptotic priming”. Dynamic BH3 profiling robustly predicted which patients would respond to a given therapy across multiple cancer types. However, this approach requires cell
permeabilization, which complicates the application of both downstream assays and phenotype validation, and does not clearly distinguish subsets of cells with phenotypic heterogeneity.

MAR measurement addresses many of these limitations by assessing therapeutic susceptibility in single, live cells without the need for PDCL generation, but is subject to its own set of constraints. Most notably, in vitro culture is still necessary for a length of time adequate to elicit a growth response to applied therapies. In Ba/F3 cells this occurred within 2-4 hrs, but primary leukemia cells required longer culture to appreciably change MAR in the presence of targeted inhibitors. Thus, the MAR measurement can reduce, but does not completely eliminate, the time within in vitro culture. Additionally, the SMR currently requires cells to be in a single-cell suspension or small clumps for mass and MAR measurement. While this is not an issue for leukemias, future studies will explore the utility in solid tumor systems, where the extent of dissociation required may perturb cellular viability and/or response. MAR measurements also initially suffered from low throughput. However, single-cell mass and MAR can now be obtained with a throughput exceeding 60 cells/hr/device without sacrificing precision [71].

Perhaps the most important shortcoming of our approach, and the vast majority of functional assays, is a potential bias towards assessing only cell intrinsic drug susceptibility. Microenvironmental interactions are known to influence in vivo drug response, but cellular ‘memory’ of these interactions may degrade during the course of ex vivo treatment [56]. There have been recent advancements in this arena involving implantable devices, but these approaches are currently only compatible with solid tumors, and require tumors of a minimum size that are easily accessible [75]. To address the role of microenvironmental interactions, future studies using MAR measurements should explore whether alternative culture conditions can help address the contribution of cell extrinsic factors under controlled conditions; e.g., tumor cells
could be maintained \textit{in vitro} in the presence of both drug and co-culture with stromal and/or immune cells prior to measurement. Alternatively, patient drug response \textit{in situ} could be monitored. For example, patient tumor cells could be analyzed with MAR measurements immediately before and hours to days following treatment to help inform pharmacokinetics and pharmacodynamics. In fact, we applied this approach to a patient with AML (Figure 14) to assess overall feasibility for clinical scenarios that could be explored in the future.

Significant future work needs to be performed to define the utility of mass and MAR as biomarkers for treatment response across disease types, in comparison to alternative functional assays, for drugs in combination, and across a wider range of drug mechanisms, where response may differ based on the mechanism of cell death. However, given the scarcity of functional assays with the necessary characteristics to merit widespread application, MAR measurements in the SMR hold great potential as both a biological tool and a clinical platform.
Footnotes

A manuscript encompassing the work detailed herein was drafted and submitted for publication during this thesis period as: Stevens, M.M.,* Maire, C.L.,* Chou, N.,* Murakami, M.A,* Knoff, D., Kikuchi, Y., Kimmerling, R.J., Liu, H., Haidar, S., Calistri, N.L., Cermak, N., Olcum, S., Cordero, N., Ibdaih, A., Wen, P.Y., Weinstock, D.M., Ligon, K.L., and S.R. Manalis. Precise Measurement of Single Cancer Cell Mass Accumulation Predicts Drug Susceptibility. At the time of thesis submission, this manuscript was under editorial consideration at Nature Biotechnology. *Drs. Stevens, Maire, Chou, and Murakami are listed as co-first authors on this manuscript, a copy of which has been included in this thesis submission.

Defining Clonal Phylogeny in BCR-ABL+ ALL by DNA Barcode Labeling

Introduction.

For targeted agents to cure a leukemia, it is intuitive that they must control any clones that are either present when treatment is initiated or that develop during treatment. Other groups have reported that ABL mutations driving clinical relapse can be found retrospectively at very low frequencies in banked pre-treatment samples [76, 77], suggesting that “acquired resistance” can be predicted if this genetic information is available pre factum. However, existing next-generation sequencing platforms are limited to detecting variant alleles at frequencies of 1-5%, which is clearly inadequate to detect very rare subclones [78]. Even ultra-deep sequencing, which the Weinstock laboratory previously utilized to identify variants at <10^{-4} [79], may still be inadequate. Cellular barcoding with DNA oligonucleotide sequences can overcome these limitations and more precisely delineate intratumoral clonal architecture and evolution.

We speculate, based on our preliminary data, that BCR-ABL-mediated resistance to the combination of ponatinib and ABL001 will require compound and/or novel mutations in ABL kinase. While such mutations have not to our knowledge been reported in de novo leukemias, even with modern sequencing techniques [77], we cannot exclude the possibility that such lesions exist at allelic frequencies below the threshold of detection. The presence or absence of such clones at diagnosis would have strong implications for the curative potential of combined BCR-ABL inhibition and the design of alternative therapeutic approaches to overcome this resistance.

We are utilizing a published high-complexity barcode library of >70 million unique oligonucleotides cloned within a lentiviral delivery vector [10] to label PDXs of BCR-ABL^+
ALL. By transplanting and treating barcoded leukemias in parallel within a cohort of mice and comparing barcode sequences that emerge in different animals during treatment, we can powerfully discriminate pre-existing resistance from resistance that develops during therapeutic selection in vivo. Of note, the Stegmeier laboratory at Novartis transduced one of our BCR-ABL+ ALL PDX lines with this barcode library and transplanted labeled cells into a cohort of NSG mice (personal communication). At engraftment, library integration was validated by next-generation sequencing (NGS) of barcodes recovered from genomic DNA. High-level library complexity was preserved ($\geq 10^5$ barcodes recovered per mouse), and recovered barcodes were detected at low copy numbers per cell. Validation studies using pre-defined barcode libraries demonstrated no evidence of PCR amplification bias. Taken together, these findings demonstrated the feasibility of efficient, low copy-number barcode labeling of PDXs with this construct.

Using this technology, we now seek to establish a large stock of this DNA barcode library in an efficient lentiviral vector, a robust protocol for DNA barcode labeling of cell lines and B-ALL PDXs, and a reproducible pipeline for identification, quantification, and clonal deconvolution of DNA barcodes recovered from studies involving labeled cells. While borrowing heavily from existing analysis programs developed by other groups, we seek to augment their utility for studies of clonal dynamics in PDXs through the following means: (1) refining the handling of similar barcodes according to Hamming distance, (2) adding a module for calculating barcode enrichment between samples, (3) integrating barcode data with variant allele frequencies for BCR-ABL mutations to improve precision of clonal deconvolution, and (4) implementing the entire analytic pipeline as a Snakemake workflow [80]. We seek to first establish these methods in BCR-ABL-dependent cell lines Ba/F3 and SUPB15, and then to
deploy them in BCR-ABL\textsuperscript{+} PDX models. The deliverable result of this work will be a robust package for calculating barcode frequencies, enrichment, and intersection and enabling basic reconstruction of clonal evolution in labeled tumor samples treated with antineoplastic agents. It should be accessible to and useful for laboratory researchers with modest computational ability.
Methods.

Lentivirus generation. The pRS19-barcode lentivirus was a gift of the Stegmeier laboratory at the Novartis Institutes for Biomedical Research (NIBR), which had cloned the barcode library into the pRS19-U6-(sh)-UbiC-TagRFP-2A-Puro plasmid (Addgene #28289). Human 293T cell lines confirmed to be free of mycoplasma contamination were seeded at a density of $2 \times 10^6$ in 10 mL of Dulbecco’s modified Eagle medium (DMEM) with 10% fetal bovine serum and 1% penicillin/streptomycin. After 24 hours, cells were transfected with the pRS19-barcode lentivirus (2.4 µg), the pCMV-VSV-G envelope plasmid (0.6 µg, Addgene #8454), and the psPAX2 2nd generation packaging plasmid (2.4 µg, Addgene #12260) in the presence of polyethyleneimine (PEI) at a ratio of 3 µL per 1 µg of RNA. Viral supernatant was harvested at 48 and 72 hours thereafter and concentrated approximately 100-fold using PEG-it virus concentration solution (System Biosciences #LV810A-1).

Cell lines and PDX models. BCR-ABL-WT-transformed Ba/F3 cell lines and BCR-ABL-T315I-transformed Ba/F3 cell lines were created in the Weinstock laboratory from parental Ba/F3 cell lines obtained from the RIKEN BioResource Center. No further cell line validation was performed. All cell lines tested negative for mycoplasma. For PDX generation, primary bone marrow and blood specimens were obtained with informed consent from patients with leukemia and lymphoma at Dana-Farber Cancer Institute, Brigham and Women’s Hospital, and Boston Children’s Hospital and xenografted under Dana-Farber/Harvard Cancer Center Institutional Review Board (IRB)-approved protocols #13-351. Nod.Cg-Prkdc<sup>Scid</sup>Il2rg<sup>-tm1Wj1</sup>/SzJ (NSG) mice were purchased from Jackson Laboratories (Bar Harbor, ME) and handled according to Dana-Farber Cancer Institute’s Institutional Animal Care and Use Committee (IACUC)-approved protocol #13-034. Ba/F3 cell lines were maintained in RPMI with 10% FCS, 4 mM L-
glutamine, 1% penicillin-streptomycin, and no IL3 at a density of $1 \times 10^5$ to $1 \times 10^6$ cells/mL. SUPB15 cells were maintained in Iscove’s modified Dulbecco’s medium with 20% FCS, 4 mM L-glutamine, 1% penicillin-streptomycin, 1.5 g/dL sodium bicarbonate, and 0.05 mM 2-mercaptoethanol at a density of $1 \times 10^6$ to $3 \times 10^6$ cells/mL. B-ALL PDX cells were seeded in RPMI with 10% FCS, 4 mM L-glutamine, 1% penicillin-streptomycin, 0.05 mM 2-mercaptoethanol, and human cytokines IL3 10 ng/mL, IL7 10 ng/mL, SCF 10 ng/mL, and FLT3-ligand 10 ng/mL.

**Lentiviral transduction and MOI determination.** Concentrated lentivirus was added to cells in a dilution series (1:1, 1:5, 1:10, 1:100) under centrifugation at 1,000 x g for 90 minutes. Cells were returned to culture at 37°C with 5% CO2; medium was replaced after 16 hours. Lentiviral transduction efficiency was assessed at 48-120 hours post-transduction by fluorescence-activated cell sorting for RFP expression. Rates of RFP positivity among cells transduced with dilution series of concentrated viral supernatant were used to calculate infectious titers using published methods [81]. Infectious titers were calculated separately for BCR-ABL-dependent cell lines Ba/F3 and SUPB15, as well as B-ALL PDXs.

**Barcode library validation.** To directly assess barcode copy number per cell, cells labeled at each MOI (1:1, 1:5, 1:10, 1:100) were sorted individually into wells of 96 well plates and expanded in long-term culture. At the time of this writing, these colonies are expanding but are not yet large enough for genomic DNA extraction. Among SUPB15 cells, each well is visually estimated to hold approximately 1,000 cells. Ba/F3 colonies are visually estimated to be approximately 5,000-10,000 cells (cells are approaching confluence). Colonies will continue to be expanded until they reach a size of approximately 100,000, at which point genomic DNA will be extracted for PCR retrieval of barcodes.
PCR retrieval of DNA barcodes. Barcodes were retrieved from labeled cells by PCR using published forward primer WS FWD (ATTTGGAATCACACGACCTGGATG) and reverse primer 1309 RVS (GTACATGAAAGCTGGTAGCCAGGTGT) primers, and Sanger sequenced using forward primer CACCATTATCGTTTCAGACCCACC.

Drug treatment of BCR-ABL-dependent cell lines and PDX models. Ba/F3 and SUPB15 cell lines were seeded at 3x10^5 cells/mL and 1x10^6 cells/mL, respectively, in the presence of serial dilutions of DMSO, ponatinib (Selleckchem #AP24534), and ABL001 (Novartis, per MTA). Cellular viability was measured by Cell Titer-Glo chemiluminescence (Promega #G7572) using an EnVision Multilabel Plate Reader (Perkin-Elmer) at 0 hrs and 48 hrs. Each cell line and drug concentration combination was tested in 14 technical replicates. Luminescence values were normalized to that of the vehicle (DMSO) wells in the same plate column (n=4 wells with DMSO, n=14 wells with drug per column).

Analytic pipeline. A number of publicly available DNA sequencing analysis programs were identified for quantifying, comparing, and visualizing DNA barcodes reads retrieved from cellular genomic DNA. ClonTracer v1.0, a Python-based program for identifying and quantifying barcode sequenced [10], was obtained from the Stegmeier laboratory (NIBR); a subsequent version is now available through Addgene (https://www.addgene.org/pooled-library/clontracer). The eulerAPE v3, an automatic area-proportional Euler diagram-drawing tool [82], was accessed at www.wulerdiagrams.org/eulerAPE. UpSetR, an R package for scalable visualization of data set intersection [83], is available at https://cran.r-project.org/web/packages/UpSetR/index.html. Finally, packages for subclonal composition analysis from DNA sequencing data include PhyloWGS [84] (Python/C++ based, available at https://github.com/morrislab/phylowgs) and ClonEvol [85] (R-based, available at
https://github.com/hdng/clonevol). These packages are being integrated in a Snakemake workflow [35] to facilitate processing of fastq files and generate barcode sequences, read counts, enrichment scores, and visualizations of barcode intersection and inferred clonal evolutionary dynamics.
Results.

Validation of plasmid insert. The presence of the 30mer oligonucleotide library flanked by the expected index sequences was confirmed by PCR and Sanger sequencing of the plasmid upon receipt from the Stegmeier laboratory.

Generation of DNA barcode lentiviral stocks. Lentiviral stocks were generated by transfection of 293T cell lines with the pRS19-barcode lentiviral plasmid, the pCMV-VSV-G envelope plasmid, and the 2nd generation packaging plasmid psPAX2. Viral supernatant was harvested at 48 hrs and 72 hrs and pooled. Infectious titers were calculated by spinfection of Ba/F3 and SUPB15 cell lines and B-ALL PDX cells by serial dilutions of virus, followed by flow cytometry for RFP expression at 48-120 hours. Based on published algorithms [81], we generated a stock of concentrated lentivirus with an infectious titer of 3.52x10^6 IU/mL for Ba/F3 BCR-ABL WT (Figure 15a, c), 11.1x10^6 IU/mL for SUPB15 cells (Figure 15b, c), and 44.7x10^6 for B-ALL PDX cells (Figure 16). Data shown are the means of biologic duplicates for each cell line and condition. Data for B-ALL PDXs are based on the results of a single experiment and are being repeated.

Determination of barcode copy per cell. Labeled Ba/F3 and SUPB15 cell lines were sorted individually into wells of 96 well plates for clonal expansion (one plate per cell line per dilution; 1:1, 1:5, 1:10, 1:100). At the time of this writing, colonies are actively expanding, with most wells approaching confluence with Ba/F3 cells, and colonies of SUPB15 cells estimated at ~1,000 cells. These SUPB15 colony sizes are roughly consistent with projections based on published doubling times (60 hrs per DSMZ), although the Ba/F3 cells appear to be growing at around half the rate of the published doubling time (20 hrs per DSMZ). Genomic DNA will be harvested when colonies reach ~10^5 cells for PCR retrieval of barcodes and sequencing to
determine barcode copy per cell. Ultimately, the highest infectious titer for which the mode barcode copy per cell remains 1 will be utilized in future studies with that cell line. The MOI for PDX cells will be extrapolated from the cell line data, in that we will use the dilution of viral stock that labels the same proportion of PDX cells with RFP at 48 hours as that which is identified in cell lines as the highest infectious titer for which the mode barcode number per RFP+ cell remains 1. For example, hypothetically, if a 1:100 dilution of virus labels 10% of SUPB15 cells with RFP at 48 hrs, and this is the highest titer of virus for which the colony assay demonstrates a mode of 1 barcode per RFP+ cell by DNA sequencing, then we will infect PDX cells with the titer of virus associated with 10% RFP positivity at 48 hrs.

**IC50 determinations for Ba/F3 and SUPB15 cell lines.** Cell lines have been tested for sensitivity to ponatinib and ABL001 by the Cell TiterGlo luminescent viability assay. Data are currently being processed. Based on published data from the KCL-22 cell line treated with BCR-ABL inhibitors [10], we plan to treat cells at doses approximating five times the IC50 in order to eliminate the vast majority of cells but permit the outgrowth of resistant subclones.

**Analysis pipeline.** In lieu of primary sequencing data from Ba/F3 and SUPB15 cell lines treated with BCR-ABL inhibitors ponatinib and ABL001, we obtained external data sets thanks to the generosity of collaborators. Dr. Carrie Bhang of the Stegmeier lab (NIBR) provided raw sequencing data from barcode-labeled KCL-22 cell lines that had been treated in vitro with the second-generation ABL catalytic inhibitor nilotinib or ABL001 through response and to outgrowth of resistant clones. In addition, Drs. Daniel Stover, Johan Kuiken, and Laura Selfors of the Brugge lab (Harvard Medical School) provided raw sequencing data from barcode-labeled PDX models of estrogen receptor negative, progesterone receptor negative, HER2 negative (“triple negative”) breast cancer that had been treated in vivo with cytotoxic chemotherapy
through initial response until progression. In both cases, genomic DNA was harvested from resistant populations, the DNA barcodes were PCR retrieved, and barcodes were identified by next generation Illumina sequencing. These data sets, consisting of raw fastq files, have been run through the ClonTracer package for exploratory analyses while the proposed pipeline is being established, as depicted schematically in Figure 17. A timeline of these ongoing aspects of the project appears in the Concluding Remarks on pp. 57-58.
Discussion.

These investigations of the clonal phylogeny of resistance to targeted inhibitors in BCR-ABL+ leukemia models have the potential to illuminate the origins of clinically relevant molecular lesions with a degree of precision unattainable with existing technologies. As such, they represent a high priority in our research portfolio. However, these studies demand considerable technical sophistication in both the laboratory techniques required as well as the statistical and computational tools for clonal deconvolution based on barcode sequencing data. Efforts to develop these tools remain in progress at this time.

The emphasis over the next six months will be to build on the data science, statistical, and computational experiences gained through the totality of the work presented here in order to establish the proposed analytic pipeline for barcode analysis, enrichment calculation, and clonal deconvolution. Upon successful deployment within in vitro studies using Ba/F3 and SUPB15 cell lines, we will proceed to labeling BCR-ABL+ PDXs in order to conduct the in vivo PDX studies in which our laboratory specializes. As depicted in schematic form in Figure 18, we seek to label a group of our most frequently utilized BCR-ABL+ PDX lines, which will then serve as subjects in treatment studies of combination BCR-ABL inhibition with ponatinib and ABL001. Such studies will not only provide additional data into the activity of combination therapy and generate models of in vivo resistance for mechanistic interrogation, they will also permit us to infer the temporal origins of the lesions driving relapse, which can then guide future diagnostic and therapeutic efforts.
Footnotes

The work detailed in this chapter will continue according to the plan laid out in the discussion and further elaborated in the Concluding Remarks. Upon completion of these studies, we will draft a manuscript describing the biological data as well as the analytic pipeline and pursue publication in a bioinformatics journal such as *PLoS One*.
Concluding Remarks

To fully develop the DNA barcode labeling system and its associated analytic pipeline and thereby fulfill the aims of this thesis, we have developed the following plan: A simplified version of the analytic pipeline will be implemented as a Snakemake workflow and used to analyze the two external data sets to which we have access (the KCL-22 chronic myelogenous leukemia blast crisis cell line model treated with nilotinib and ABL001, and the triple-negative breast cancer PDX model treated with cytotoxic chemotherapy). To this will be subsequently added several features. First, we will incorporate an algorithm for calculating enrichment scores based on barcode counts pre- and post-therapy, with a specific focus on adjudicating barcodes that are only detected post-therapy, using a hypergeometric distribution or other statistical approach. Second, we will develop a method for handling highly similar barcodes (Hamming distance ≤2) by defining a relative barcode abundance threshold that would justify merging a less abundant barcode with a more abundant barcode, on the basis of putative DNA replication and/or sequencing error.

We anticipate that this pipeline will be primarily used in studies of targeted therapy for genetically-defined malignancies, such as BCR-ABL^+ ALL, which harbors a driver oncogene in which point mutations undergo Darwinian selected by targeted kinase inhibitors. We will therefore work to optimize the utility of our pipeline by incorporating a parallel analytic process for point mutation data based on variant allele frequencies (VAF). The VAF data will be integrated with the barcode abundances to enhance the precision of clonal dynamics modeling. A final aim, which is regarded as aspirational, would be to develop a visualization module for generating so-called whale plots that depict the relative sizes of tumor subclones over time.
The biologic data for chapter three will be generated in parallel with the computational developments, which will have been trained on the pre-existing external data sets, with a plan to analyze our novel biological data using the completed pipeline. To this end, we will first sequence the barcodes from the Ba/F3 and SUPB15 colonies derived from single labeled cells to directly assess the distributions of barcode copy numbers per cell across a range of MOI (1:1, 1:5, 1:10, 1:100). The highest MOI for which the mode barcode copy number per cell remains <2 will used in the subsequent drug treatment studies. 2.5x10^7 Ba/F3 and SUPB15 cells lines will be transduced with the pRS19-barcode lentivirus at the selected MOI, and RFP^+ cells isolated by FACS. Based on preliminary data this is likely to be a 1:100 MOI for SUPB15 cells and a lower MOI for Ba/F3 cells, with 10-15% of cells being labeled by the virus. The initial labeled population of 2-4x10^6 cells will then be expanded for 5 population doublings prior to generation of 5x10^6 cell aliquots, which will then undergo treatment with ponatinib, ABL001, and the combination (n=5 replicates per condition) at 5.5 times the IC50 values for each drug. Cultures will be maintained through therapeutic contraction until outgrowth of resistant subclones. Genomic DNA for barcode library complexity will be obtained from the initial labeled cell line populations, the expanding bulk population after each doubling, and the treatment replicates before therapy and after expansion of resistant subclones. After PCR retrieval of barcodes, they will undergo NGS using Illumina PE50 chemistry. Targeted amplicon sequencing of the ABL1 kinase domain will be performed in parallel. The resulting barcode and ABL1 mutation data will be used to reconstruct the phylogeny of resistant clones using the analytic pipeline. These methods and data will then be summarized in a manuscript to be submitted for publication in the bioinformatics literature.
Taken together, the three advances described here will create a powerful yet tractable experimental system for translational investigation in oncology. This system will significantly enhance multiple aspects of biological discovery and preclinical drug development in oncology:

1. Definition of the in vivo activity of novel anti-cancer agents in populations of well-characterized, primary human tumor xenografts that capture the heterogeneity of typical patient populations;
2. Identification of molecular signatures of response and resistance, some of which may have clinical potential as predictive biomarkers;
3. Targeted interrogation of minimal residual disease for evidence of resistant subclones destined to drive relapse, with the opportunity to evaluate susceptibility to alternative therapies before clinical progression;
4. Systematic evaluation of mechanisms of resistance – mutational, transcriptional, phosphoproteomic, differentiation state-mediated, and other – owing to the abundance of tissue available from PDXs; and finally
5. Identification of the origins of subclonal resistance, the knowledge of which we can exploit by regenerating PDXs from the original tumor material so as to recapitulate the period of occult subclonal resistance (in essence, re-setting the clock in a particular PDX model) and then testing novel diagnostic or therapeutic approaches based on the evaluation of the untreated PDX model.

Historically, processing and analyzing the numerous streams of high-throughput data generated by our basic and translational research have served as rate-limiting steps in our investigative efforts, as is the case with many wet labs. Now, however, we are hopeful that the bioinformatic capacity we are building will alleviate some of these restrictions and enable us to perform that much more high-impact science. Importantly, all of the tools and technologies
described in this thesis can be applied to studies of the many other hematologic malignancies for which we have PDX models, and in fact they are intended for wide dissemination. This is in keeping with the philosophy of the Weinstock laboratory to aggressively share resources that will facilitate translational research and expedite preclinical drug development in oncology. In conclusion, through this thesis work we have laid a solid foundation for our attempts to define mechanisms of resistance to targeted inhibitors in BCR-ABL+ ALL and to overcome this resistance with novel therapeutic approaches. We look forward to accelerating our efforts through the use of key bioinformatics resources now under development.
References


**Tables**

*Table 1.* 205 genes that were sequenced using an established next-generation sequencing platform [33] in PDXs.

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**Table 2.** PDXs established in first (P0) or later passages classified according to the 2008 World Health Organization Classification [21].

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<tr>
<td>AML NOS</td>
<td>16</td>
<td>10</td>
</tr>
<tr>
<td><strong>Myelodysplastic syndrome</strong></td>
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<td>1</td>
</tr>
<tr>
<td>Refractory cytopenia with multilineage dysplasia</td>
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<td>1</td>
</tr>
<tr>
<td><strong>Acute lymphoid leukemia</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B-ALL NOS</td>
<td>53</td>
<td>28</td>
</tr>
<tr>
<td>B-ALL with t(12;21) <em>TEL-AML1</em> (<em>ETV6-RUNX1</em>)</td>
<td>17</td>
<td>3</td>
</tr>
<tr>
<td>B-ALL with t(9;22) <em>BCR-ABL1</em></td>
<td>16</td>
<td>6</td>
</tr>
<tr>
<td>B-ALL with t(v;11q23) <em>MLL</em> rearranged</td>
<td>12</td>
<td>7</td>
</tr>
<tr>
<td>B-ALL with t(1;19) <em>E2A-PBX1</em> (<em>TCF3-PBX1</em>)</td>
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<tr>
<td>B-ALL with hypodiploidy</td>
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<tr>
<td>B-ALL with hyperdiploidy</td>
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<tr>
<td>T-ALL</td>
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<td>28</td>
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<tr>
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<td>0</td>
</tr>
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<td>4</td>
<td>3</td>
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<tr>
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<td>2</td>
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<tr>
<td>Mixed phenotype acute leukemia with t(9;22) <em>BCR-ABL1</em></td>
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<td>0</td>
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<td>B/myeloid acute leukemia</td>
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<tr>
<td><strong>Mature B-cell neoplasms</strong></td>
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<td>13</td>
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<tr>
<td>Mantle cell lymphoma</td>
<td>6</td>
<td>4</td>
</tr>
<tr>
<td>DLBCL NOS</td>
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<td>4</td>
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<tr>
<td>Follicular lymphoma</td>
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<td>2</td>
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<tr>
<td>B-cell lymphoma, unclassifiable, with features intermediate between DLBCL and BL</td>
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<td>Extranodal marginal zone lymphoma</td>
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<tr>
<td>Subclassification pending annotation</td>
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<td>0</td>
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<tr>
<td><strong>Mature T- and NK-cell neoplasms</strong></td>
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<td>Angioimmunoblastic T-cell lymphoma</td>
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<tr>
<td>Anaplastic large cell lymphoma, <em>ALK</em> positive</td>
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</tr>
<tr>
<td>Anaplastic large cell lymphoma, <em>ALK</em> negative</td>
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</tr>
<tr>
<td>Subclassification pending annotation</td>
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<td>0</td>
</tr>
<tr>
<td><strong>All Diagnoses</strong></td>
<td>248</td>
<td>138</td>
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**Figure 1.** IC50 values for the catalytic inhibitor nilotinib and the allosteric inhibitor ABL001 against a panel of ABL point mutants in Ba/F3 cell lines. Courtesy of Dr. Andrew Wylie (NIBR).
Figure 2. Distribution of gene expressions in log2 scale derived from multiple data sets (CCLE cell lines, PDX models, SRP058414 primary pre-B ALL samples, and TCGA primary AML samples). Data are shown before (a) and after (b) quantile normalization.
Figure 3. Batch effect correction of RNA expression data from PRoXe and external data sets. (a) Principle components analysis of quantile-normalized gene expressions from multiple data sets (CCLE, PDX, SRP058414, and TCGA). The factor explaining the greatest amount of variation in the data as plotted against the top eigenvectors is the source of data (i.e. batch effects). For the histology-specific analysis, we successfully removed batch effects within each tumor type using the ComBat approach from SVA 3.18.0 (http://doi.org/10.1038/nbt.1621) without specifying additional covariates. Batch effect correction was performed separately for each primary tumor histology (b – B-ALL, c – AML, d – T-ALL, e – lymphoma).
Figure 4. Integrative analysis of leukemia and lymphoma PDXs. (A) Unsupervised hierarchical
clustering over expression of 1000 genes with the greatest variance-to-mean ratios among 107 PDXs representing all WHO diagnostic categories encompassed by our repository. (B) Key clinical characteristics of patients and their tumors from which PDXs were derived. Patient age in years reflects the time when the xenografted tumor specimen was obtained. Phases of treatment are defined as: Untreated, prior to therapy directed at the xenografted tumor (n.b., does not include therapy directed at prior malignancies); Primary refractory, failed to respond to all tumor-directed therapy to date; Relapse, recurred by standard disease-specific criteria after achievement of a complete remission; Refractory, disease that is progressing during or shortly after the administration of salvage therapy for relapsed disease; Progression, specific to lymphomas progressing by clinical or radiographic criteria after a period of stable disease or partial remission. (C) Binary matrix of prior therapies to which patient was exposed prior to sampling of the referenced tumor, if known. (D) Selected cytogenetic features of patient tumors from which PDXs were derived. (E) OncoPrint of selected mutations detected in PDXs by targeted exon sequencing of a panel of 205 genes [33] (see Tables S1-S3, provided as separate excel files, for details).
**Figure 5.** Integrative analysis of B-ALL PDXs, primary samples and cell lines. (A) Unsupervised hierarchical clustering of RNA expression profiles among 60 B-ALL PDXs (PDX cohort), 19 primary pre-B ALL samples (SRP058414), and 10 B-cell leukemia cell lines (CCLE), using the same methods as for Figure 4. (B) Key clinical characteristics of cell lines and primary samples, including those from which PDXs were derived. (C) Binary matrix of prior therapies to which patient was exposed prior to sampling of the referenced tumor, if known. (D) Selected cytogenetic features of cell lines and primary samples, including those from which PDXs were derived. (E) OncoPrint of selected mutations detected in PDXs by targeted exon sequencing, or reported in cell lines by ATCC or DSMZ. Mutation data for the primary samples (SRP058414) were not available (see Tables S1-S3, provided as separate excel files, for details).
CLONAL RESISTANCE IN BCR-ABL ALL

Legend

RNA Expression Z-score
-3
-2
-1
0
1
2
3

Sample Cohort
CCLE
PCIX
TCGA

Disease WHO-classification
AML, t(8;21)
AML Therapy-Related
AML with inv(16) CSF3R-MYH11
AML with MDS-related changes
AML with MLL rearrangement
AML with variant MLL translocation
Bcr-Abl1 acute lymphoblastic leukemia
BRCAness
Insufficient information to classify
LAA with t(11;22) MLL rearranged
MDS refractory anemia with multilineage dysplasia

Disease Phase of Treatment
Data not available
Primary relapse
Refractory
Relapse
Untreated

Patient Gender
F
M

Patient Age (Years)
<9
10-17
18-30
31-64
65-79
80+

AML Cyrogentic Risk Category
Adverse
Favorable
Intermediate
Not available
Not available
Poor
Unable to assess

AML FAB Classification
M0
M1
M2
M3
M4
M5
M6
M7
M7
Not Classified

PDX Mutations
Dominant-negative mutation
Frame Shift Deletion
Frame Shift Inserion
RTD
Misserse
Nonsense
Not specified
Structural variant
Type A
Figure 6. Integrative analysis of AML PDX models. (a) Unsupervised hierarchical clustering of RNA expression profiles among 20 AML PDX models (PDX cohort), 104 external primary AML samples (TCGA cohort), and 20 AML cell lines (CCLE cohort), using the same methods as for figure 4. (b) Key clinical characteristics of patients from whose tumors PDX models (PDX cohort) or cell lines (CCLE cohort) were derived, or whose tumors were sequenced directly (TCGA cohort), including specific diagnosis (by WHO 2008 classification), phase of treatment (as previously defined in Figure 4 legend), patient gender, patient age when the referenced tumor specimen was obtained, FAB classification (for historical reference), and cytogenetic risk category (using criteria of Grimwade et al. [86]). (c) Binary matrix of prior therapies to which patient was exposed prior to sampling of the reference tumor, if known. (d) Selected cytogenetic features of patient tumors from which PDX models (PDX cohort) or cell lines (CCLE cohort) were derived, or of the tumors themselves (TCGA cohort). Shown are cytogenetic features or groups of related features reported in at least three samples within this data set. (e) OncoPrint of selected mutations detected in PDX models by targeted exome sequencing, reported in cell lines by ATCC or DSMZ or reported in primary samples within the clinical annotation available from TCGA. Overall, AML clustering tended to reflect cytogenetic changes, with t(15;17) M3 AMLs forming a distinct clade, as with pediatric t(8;21) M1/M2 AMLs and AMLs with MDS-related cytogenetic changes. Of note, our repository includes nine blastic plasmacytoid dendritic cell neoplasms (BPDCN), of which seven underwent whole transcriptome sequencing; these, too, formed a tight cluster.
CLONAL RESISTANCE IN BCR-ABL ALL

Legend

RNA Expression z-score
- 4
- 2
- 0
- 2

Sample Cohort
- CCLE
- PDX

Disease WHO-classification
- T-ALL

Disease Phase of Treatment
- Refractory
- Relapse
- Untreated

Patient Gender
- F
- M

Patient Age (Years)
- 0-9
- 10-17
- 18-39
- 65-79

PDX Mutations
- Frame Shift Deletion
- Frame Shift Insertion
- In Frame Deletion
- Missense
- Not specified
- Structural variant
**Figure 7.** Integrative analysis of T-ALL PDX models and cell lines. (a) Unsupervised hierarchical clustering of RNA expression profiles among 19 T-ALL PDX models (PDX cohort) and 16 T-ALL cell lines (CCLE cohort), using the same methods as for figure 4. (b) Key clinical characteristics of patients from whose tumors PDX models (PDX cohort) or cell lines (CCLE cohort) were derived, including specific diagnosis (by WHO 2008 classification), phase of treatment (as previously defined in Figure 4 legend), patient gender, and patient age when the referenced tumor specimen was obtained. (c) Binary matrix of prior therapies to which patient was exposed prior to sampling of the referenced tumor, if known. (d) Selected cytogenetic features or groups of related features reported in at least three samples within this data set. (e) OncoPrint of selected mutations detected in PDX models by targeted exome sequencing of or reported in cell lines by ATCC or DSMZ. Overall, T-ALLs comprised two primary clusters, one enriched in samples with isolated *NOTCH1* mutations, and the other with mutations in various other genes, including *FBXW7*. 
Figure 8. Integrative analysis of non-Hodgkin lymphoma PDX models and cell lines. (a) Unsupervised hierarchical clustering of RNA expression profiles among 8 non-Hodgkin lymphoma PDX models (PDX cohort) and 29 non-Hodgkin lymphoma cell lines (CCLE cohort), using the same methods as for figure 4. (b) Key clinical characteristics of patients from whose tumors PDX models (PDX cohort) or cell lines (CCLE cohort) were derived, including specific diagnosis (by WHO 2008 classification), phase of treatment (as previously defined in Figure 4 legend), patient gender, and patient age when the referenced tumor specimen was obtained. (c) Binary matrix of prior therapies to which patient was exposed prior to sampling of the referenced tumor, if known. (d) Selected cytogenetic features or groups of related features reported in at least three samples within this data set. (e) OncoPrint of selected mutations detected in PDX models by targeted exome sequencing of or reported in cell lines by ATCC or DSMZ.
Figure 9. Gene fusions identified in B-ALL PDX models based on transcriptome sequencing. Candidate fusions were called from RNA-Seq data using the STAR-Fusion 0.4.0 algorithm (https://star-fusion.github.io) and quantitatively evaluated by summing spanning fragments and junction reads. Those with sums of ≥10 over all samples were retained and manually filtered to remove likely homology-induced artifacts. Fusions detected in B-ALL PDX models are depicted here in heatmap form, with color coding for strength of evidence supporting fusion calls. Both canonical and reciprocal fusions (e.g., BCR-ABL1 and ABL1-BCR) are shown, when detected. PDX samples are ordered alphanumerically to facilitate sample identification.
**Figure 10.** Gene fusions identified in AML PDX models based on transcriptome sequencing.

Candidate fusions were called from RNA-Seq data and filtered as described. Fusions detected in AML PDX models are depicted here in heatmap form, with color coding for strength of evidence supporting fusion calls. Both canonical and reciprocal fusions (e.g., KMT2A-AFF1 and AFF1-KMT2A) are shown, when detected. PDX samples are ordered alphanumerically.
**Figure 11:** Mass accumulation rate (MAR) measurements characterize single-cell heterogeneity in growth across conventional cell lines. (a) Schematic of workflow. Single cells are weighed repeatedly over a 15-minute interval by iterative passage through the SMR device. A linear fit is applied to those measurements and the resulting data is plotted as MAR versus buoyant cell mass. (b) MAR measurements over ~15 minutes for single cells from primary BCR-ABL+ ALLs FACS-enriched from mouse spleens. The specific single-cell plots shown in the middle column
are represented as red open-circles along with other single cells (black dots) plotted as a function of mass.
Figure 12: Murine Ba/F3 lymphoblastoid cells rapidly reduce MAR upon exposure to active kinase inhibitors. (a) MAR versus cell mass of imatinib-sensitive Ba/F3-BCR-ABL or imatinib-
resistant Ba/F3-BCR-ABL T315I cells exposed to 1 μM imatinib (imat.). (b, c) Same data as in (a) shown as MAR per mass (b) or mass box-plot (c), also including Ba/F3-BCR-ABL T315I cells treated with 100 nM ponatinib. Boxes represent the inter-quartile range and white squares the average of all measurements. p-values were calculated using the non-parametric Mann-Whitney U test, comparing treatment groups to DMSO for the same treatment duration. **** p<0.0001 in highlighted segments. Time points were taken on at least three biological replicate cultures on different days. From left to right, n = 46, 20, 48, 37, 36, 42, 15, 41, 27, 41, 41
**Figure 13:** MAR distributions predict drug sensitivity of primary murine ALL cells to targeted therapy. (a) MAR per mass distributions of paired measurements from primary murine B-ALL cells dependent on BCR-ABL or BCR-ABL T315I and treated with 1 μM imatinib, 100 nM ponatinib, or DMSO. Biological replicates from individual mice are separated by a vertical dotted line. n indicates the number of cells for each measurement. AUC values for ROC curve of each paired dataset are listed below the x-axis. Boxes represent the inter-quartile range and white squares the average of all measurements. p-values were calculated using the non-parametric Mann-Whitney U test, comparing treated cells to the DMSO control. (b) Representative MAR versus mass plot with overlay of an orthogonal vector (dotted line) designating the threshold
resulting from LDA. (c) ROC curves of paired control and treatment data for each treatment replicate. Cells treated with therapy to which they are sensitive or resistant are shown with blue solid lines or red dotted lines, respectively. (d) MAR per mass distributions of paired measurements from primary murine B-ALL cells that are dependent on BCR-ABL. Cells isolated from the bloodstream of mice by either cardiac or cheek bleed and treated with 100 nM ponatinib or DMSO for the specified interval. p-values were calculated using the Mann-Whitney U test, comparing treated cells to the DMSO control.
**Figure 14:** Patient samples treated *in vivo* or *ex vivo* show consistent reduction in MAR. (a) The fraction of cells with MAR of less than -1 pg/hr from pre-treatment patient sample (n = 86), and from sample obtained after the patient received 48 hrs of treatment with an experimental MDM2 inhibitor (n = 95). MAR versus mass data for same sample set shown in Supplementary Figure 10. (b) Boxplots of MAR per mass of bone marrow leukemia cells treated with either DMSO, 100 nM midostaurin, 1 μM cytarabine, or an experimental MDM2 inhibitor, and measured during 10 hour windows centered around 20 and 44 hrs. Boxes represent the inter-quartile range and white squares the average of all measurements. p-values were calculated using the non-parametric Mann-Whitney U test, comparing treated cells to the DMSO control. From left to right, n = 137, 102, 111, 78, 119, 103, 67, 48.
CLONAL RESISTANCE IN BCR-ABL ALL

A

B

C

Viral Titer Determination

- Black: Ba/F3 (BCR-ABL WT)
- Grey: SUPB15 (BCR-ABL WT)

Viral Supernatant Dilution Factor

RFP+ at 48 hrs (%)

0 20 40 60 80 100

1 5 10 100
Figure 15. Flow cytometry-based determination of MOI for pRS-19-barcode lentivirus in WT BCR-ABL-dependent cell lines Ba/F3 and SUPB15. Representative flow cytometry plots for Ba/F3 cells (a) and SUPB15 cells (b) 48 hours after viral infection with progressive dilutions of pRS19-barcode lentivirus (stock, 1:5, 1:10, 1:10). (c) Summary of flow cytometry data. Infectious titer of viral stock was calculated to be $3.52 \times 10^6$ for Ba/F3 BCR-ABL WT cells and $11.1 \times 10^6$ for SUPB15 cells according to published methods [81]. Data are based on results of biologic duplicates (n=2 for each cell line and condition).
Figure 16. Flow cytometry-based determination of MOI for a B-ALL PDX. (a) Representative flow cytometry plots for B-ALL PDX cells 120 hours after viral infection with progressive dilutions of pRS19-barcode lentivirus (stock, 1:5, 1:10, 1:10). (b) Summary of flow cytometry data. Infectious titer of viral stock was calculated to be $44.7 \times 10^6$ for B-ALL PDX cells according
to published methods [81]. Data are based on results of a single experiment and are currently being repeated.
Figure 17. Analytic pipeline for clonal deconvolution based on DNA barcode sequencing data. Primary sequencing data in the form of fastq files for the input of the ClonTracer Python program, which performs quality assessment, read trimming, and read merging. Output in the
form of text files containing unique barcodes and associated read counts are processed through publicly available packages for visualization of intersection among treatment replicates (eulerAPE, UpSetR) or visualization of clonal architecture within individual treatment replicates (ClonEvol, PhyloWGS). The entire analysis is implemented as a Snakemake workflow [35].
Figure 18. Schematic of planned in vivo study of subclonal resistance phylogeny through the use of DNA barcode labeling. Briefly, four BCR-ABL+ ALL PDX lines will be labeled with the barcode library, expanded in vivo in absence of therapeutic selective pressure, and transplanted into secondary recipient NSG mice. Animals will be treated with BCR-ABL inhibitors as monotherapy or combination therapy through response until the development of resistance. Animals progressing on monotherapy will crossover to combination therapy. Animals will be phlebotomized biweekly to permit barcode recovery and sequencing. At the end of study, DNA barcode sequencing, quantification, and analysis using the pipeline under development in this
thesis will permit inference of clonal origins and reconstruction of clonal dynamics. N=26 mice per line, 8 mice per treatment arm.
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