Hierarchical Lineage Tracing Reveals Diverse Pathways of AML Treatment Resistance

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Abstract

Cancer cells adapt to treatment, leading to the emergence of clones that are more aggressive and resistant to anti-cancer therapies. We have a limited understanding of the development of treatment resistance as we lack technologies to map the evolution of cancer under the selective pressure of treatment. To address this, we developed a hierarchical, dynamic lineage tracing method called FLARE (Following Lineage Adaptation and Resistance Evolution). We use this technique to track the progression of acute myeloid leukemia (AML) cell lines through exposure to Cytarabine (AraC), a front-line treatment in AML, in vitro and in vivo. We map distinct cellular lineages in murine and human AML cell lines predisposed to AraC persistence and/or resistance via the upregulation of cell adhesion and motility pathways. Additionally, we highlight the heritable expression of immunoproteasome 11S regulatory cap subunits as a potential mechanism aiding AML cell survival, proliferation, and immune escape in vivo. Finally, we validate the clinical relevance of these signatures in the TARGET-AML cohort, with a bisected response in blood and bone marrow. Our findings reveal a broad spectrum of resistance signatures attributed to significant cell transcriptional changes. To our knowledge, this is the first application of dynamic lineage tracing to unravel treatment response and resistance in cancer, and we expect FLARE to be a valuable tool in dissecting the evolution of resistance in a wide range of tumor types.

Introduction

Acute myeloid leukemia (AML) is a heterogeneous hematological malignancy that has served as a model of clonal evolution in response to treatment[1–4]. AML subtypes and risk categories have long been primarily defined by cytogenetics, mutation status, and structural genomic variations. However, with recent advances in RNA sequencing, transcriptome profiling has begun to uncover further leukemic disease states, dependencies, and prognostic signatures[5–7]. Upon diagnosis, most patients are treated with induction therapy, including an aggressive cycle of Cytarabine (AraC). While ~80% of patients reach remission following induction, most will relapse[8]. Single-cell RNA sequencing (scRNA-seq) of matched pre- and post-treatment patient bone marrow samples show patients have dramatic disease landscape changes following AraC treatment[2].

It remains unclear how resistance to AraC evolves in many AML patients. Are AraC-resistant subclones pre-existing, or does resistance emerge de novo in rare subpopulations? Does the resistant phenotype pass through multiple intermediate states or emerge in a punctuated evolution? Previous studies have investigated the origin of chemoresistant cell populations in various cancer types[9–12]. Shaffer et al. concluded that while rare subpopulations of cells exhibit pre-resistant-like phenotypes, melanoma cells could transiently shift to a resistant phenotype upon treatment in vitro[11]. In contrast, Yan et al. identified a subpopulation of TP53-mutated AML cells in treatment-naïve samples believed to be the source of future AraC-resistant populations[9]. These studies highlight that treatment failure and resistance are likely driven by diverse disease, drug, and likely, patient-specific mechanisms. While individual patients may display unique lineage developments and gene expression profiles, pathway alterations that arise frequently can be exploited for treatment response prediction, patient stratification, and development of targeted therapies.

We set out to develop a cellular lineage tracing system to record the evolutionary steps of AraC treatment resistance in AML. Here, we present FLARE (Following Lineage Adaptation and Resistance Evolution), a multi-tiered lineage tracing approach, which we use to identify clonal patterns of drug response and expansion in vitro and in vivo. Using advances in CRISPR lineage tracing, we track the evolution of cell fate independently of cell state, recording the relationship between cells in small, integrated recorders, which can be used to recover cellular ‘phylogenetic’ trees[13,14]. This lineage information alone can uncover treatment response

patterns, but when paired with transcriptomic data, it acts as a window into disease evolution. CRISPR lineage recording has been previously used to characterize clonal evolution through tumorigenesis and metastasis, yet the effect of treatment has yet to be explored[14,15].

We use FLARE to uncover multiple heritable and lineage-specific gene modules associated with drug response with matched outcomes in pediatric AML patient survival. FLARE lineage patterns uncovered a highly heritable gene signature in treatment-naive AML cells involved in cell-adhesion-mediated drug resistance (CAM-DR) pathways, the expression of which leads to lineage branch survival and expansion under AraC exposure. In addition, we identified an AML lineage-dependent upregulation of immunoproteasome subunits, with further upregulation leading to improved expansion in vivo. Finally, we validated the clinical relevance of our findings using RNA sequencing data from the pediatric TARGET AML cohort and discover bone marrow and peripheral blood-specific expression profiles significantly associated with patient outcomes.

Results

Hierarchical lineage recording in AML

We set out to develop a dynamic cellular lineage tracing system to model the fitness landscape of AML in vitro and in vivo. Expanding on our previous macsGESTALT lineage tracing system[14], we created a hierarchical system, capturing lineage information at three levels (**Figure 1A-B**). At the top level, FLARE encodes founder populations using a unique combination of static tags accompanying the lineage recorders when integrated into the genome. Within each labeled founder population, individual clones are divided into sub-populations with a second round of tags integrated with the Cas9 cassette. This Cas9 integration also begins the dynamic lineage recording, creating insertions and deletions (indels) within the lineage recorders, capturing sub-clonal lineage information. We can then use computational methods to generate a phylogenetic tree of cell relationships within these founder and clonal populations from these indels. This three-level structure (founder, clone, and subclonal lineage information) allows FLARE to track dynamics across time and to compare sister populations in both the control and treatment arms, in vitro and in vivo.

We first engineered this approach in the C1498 cell line, a syngeneic mouse model of AML compatible with transplantation into immune-competent C57BL/6 mice. Despite being long-passaged, C1498 cells are known to be heterogeneous, making them an optimal model for investigating the diversity of clonal treatment response[16]. We established 11 founder populations from individually isolated cells through successive engineering rounds (**Figure S1A-B)**. Sequencing revealed a unique set of founder tags for each population, varying from 19 to 30 integrations (**Figure S1C**). These isolated founders are heterogeneous, with a wide range of growth kinetics (**Figure S1D**). We found no putative driver mutation differences between individual founders using whole-genome sequencing that would explain this heterogeneity.

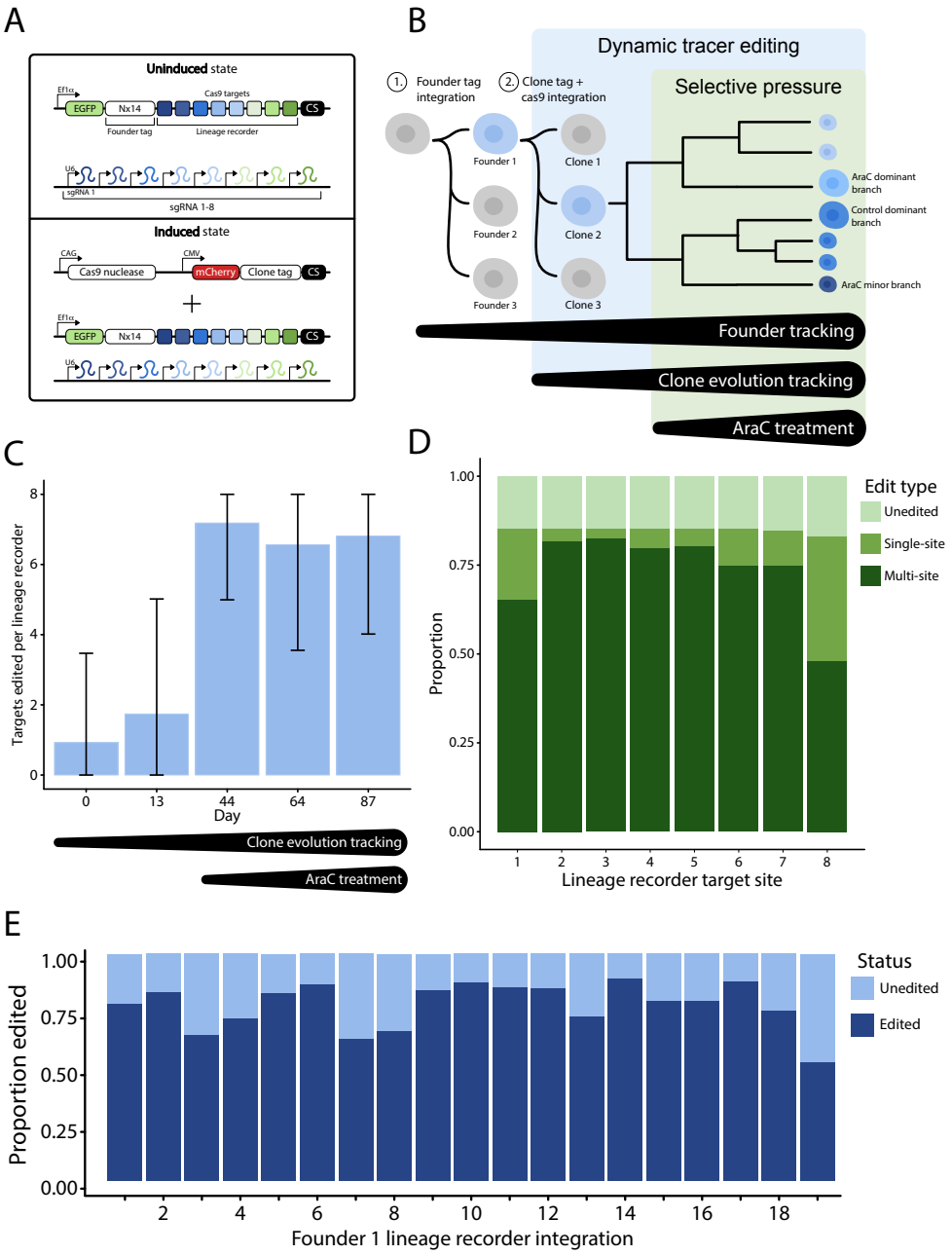

Figure 1: FLARE lineage tracing system allows for hierarchical tracking of cell populations through time. (**A**) Constructs required for FLARE lineage tracing. The top panel includes FLARE-miniV10-10X-UMI (founder tag + lineage recorder) and FLARE-pbV10g-neo (Cas9 guide sequences). We then electroporate the FLARE-pbCas9-tag-mCherry into cells (bottom panel), tagging each clone and activating CRISPR lineage recording. (**B**) The resulting cells record lineage information at 3 levels, (1) founder, (2) clonal, and (3) subclonal (CRISPR recordings). AraC treatment selective pressure is introduced following the initiation of lineage recorder editing. (**C**) Lineage recorders are readily edited following the introduction of Cas9. Editing rates plateau after around 44 days of Cas9 exposure. (**D**) Following 87 days of Cas9 exposure lineage recorder edits are primarily composed of multi-site editing events, with a smaller proportion of edits impacting single sites. Around 15% of lineage recorder sites remain unedited through 87 days of editing. (**E**) Editing rates are consistent across lineage recorder integrations within the C1498-FLARE Founder 1 population.

Characterizing lineage recording

Next, we set out to test the lineage recording capacity of our engineered cells. We activated CRISPR recording by integrating the Cas9 construct into the genome (**Figure 1A-B**). After electroporation and sorting, we sampled Cas9-positive C1498-FLARE cell populations at days 13, 44, 64, and 87 and sequenced the lineage recorders to characterize Cas9 editing (**Figure 1C**). Recording proportions suggest the bulk of lineage recorder editing occurs between day 13 (22.61% of recorders contain >= 1 edit) and 44 (99.97% >= 1 edit). Editing rates remained high through at least day 87 (85.93% >= 1 edit), with continuing fluctuations in editing patterns due to population drift. Editing patterns were comparable between lineage recorder target sites (**Figure 1D)**, and most edits span multiple target sites with a smaller proportion of edits affecting individual targets. Approximately 15% of sequenced lineage recorder targets remained unedited, indicating our ability to trace lineage over at least 87 days without saturation.

We further characterized the response of individual recorder integrations with a single founder group, founder 1 (F1), containing 19 unique integrations. All integrations were active recorders displaying diverse editing outcomes (**Figure 1E**). For all founder populations assessed, individual recording events tended to be large, spanning 3.075 targets on average, and each recorder contained an average of 2.57 individual recording events. While edits consisting of large deletions can lead to loss of lineage information, we could still reliably reconstruct lineage trees with an average depth of 5.566 nodes over relevant timescales. We attribute this to this system's high lineage recording capacity, with engineered cells containing between 152 and 240 CRISPR target sites per cell.

Clonal treatment response and tree generation in vitro and in vivo

Confident in our ability to record and recover cellular lineage information, we set out to characterize C1498-FLARE population dynamics in response to the chemotherapy AraC in vitro and in vivo (**Figure 2A)**. To recreate the heterogeneity observed in leukemia patients, we pooled four C1498 founder groups for our starting population, which was then used for both in vitro and in vivo AraC treatment.

By day 43 of in vitro AraC treatment we observed two distinct growth phenotypes. Our AraC-resistant flask displayed rapid growth and a 10-fold higher ED50 than control cell populations (**Figure S2A**). We also observed a separate flask phenotype, where cells appeared to be "persistent", displaying minimal proliferation and cell numbers too low for ED50 determination. We sequenced 27,237 single cells from the untreated, resistant, and persistent flasks. Treated cells clustered into distinct groups predominantly separated by flask phenotype (**Figure 2B**). We successfully captured founder tags and lineage recorders from 100% of single cells and clone tags from 31.9%. After filtering, quality control, and lineage assignment, 25,489 single cells were

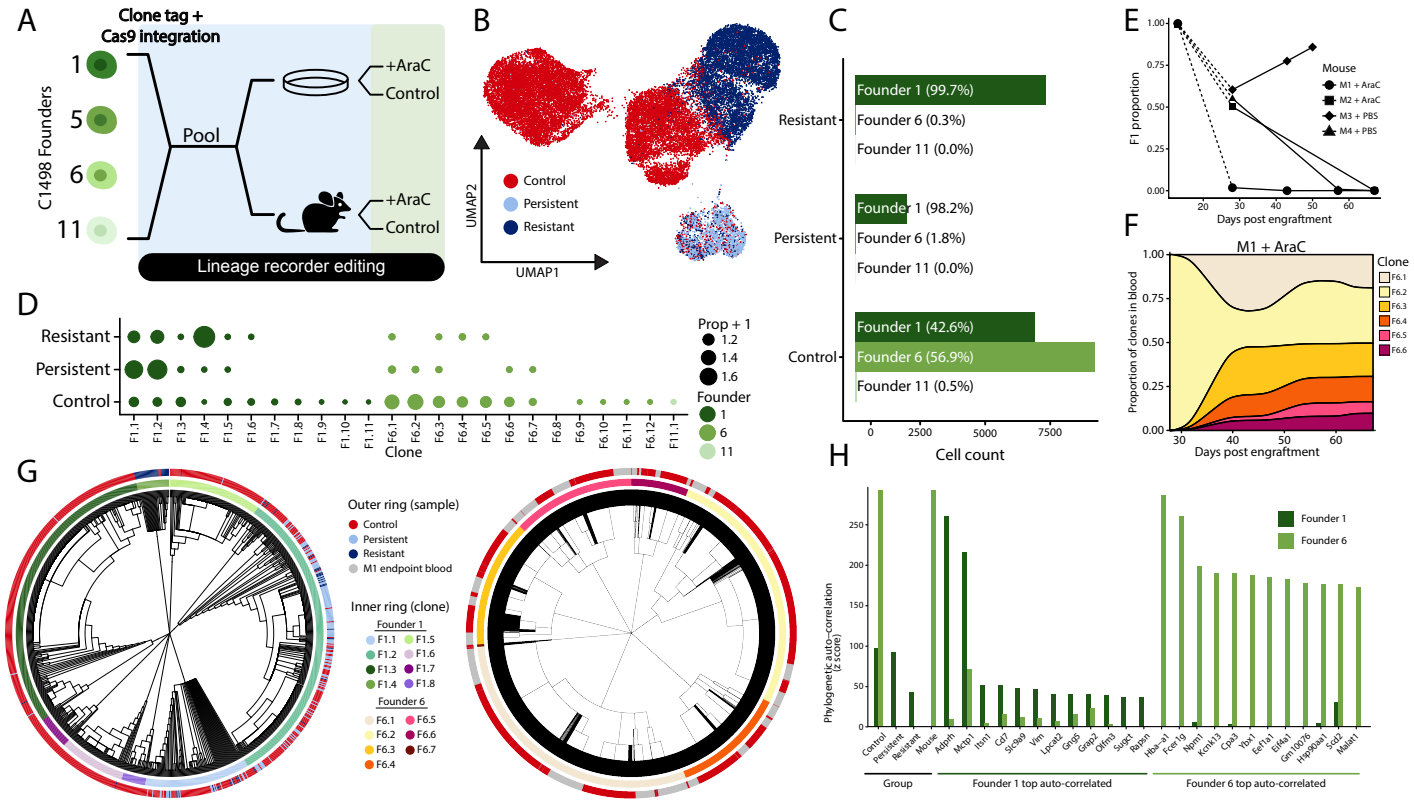

Figure 2: FLARE lineage tracing tracks founder and clone-level evolution in response to AraC treatment in vitro and in vivo. (**A**) Experimental design of lineage tracing C1498-FLARE AML cells in vitro and in vivo. (**B**) Single-cell sequencing of C1498-FLARE cells following 43 days of in vitro AraC treatment. Cells cluster into distinct groups associated with their sample (red - no AraC; dark blue - AraC resistant flask; light blue - AraC persistent flask). (**C**) Founder proportions in each sample display increased survival of F1 cells under AraC treatment in both the resistant and persistent populations. (**D**) Clone populations detected within each sample, ordered by founder and clone size. (**E**) Founder and clone tags detected in blood draws from mice engrafted with C1498-FLARE cells were used to calculate the proportion of circulating leukemic cells belonging to F1 compared to F6. Day 13 proportions were computed using founder tag sequencing and are connected to day 28 with dashed lines. All other timepoint proportions were calculated using clone tag sequencing, normalized for differences in the number of integrations. The F1 proportion was defined as the F1 average tag count divided by the sum of F1 and F6 average tag counts. Untreated mouse M3 was euthanized on day 50 due to disease progression. The remaining mice were euthanized on day 67. (**F**) Bulk sequencing of clone tags detected in blood draws from mouse M1 following AraC treatment on day 13. Clone tag reads were used to determine the clonal proportions of leukemic burden in the blood from day 28 to day 67. (**G**) Phylogenetic reconstruction of founders F1 (left) and F6 (right) generated from single-cell sequencing of in vitro C1498-FLARE lineage tracing and single-cell sequencing of endpoint blood and bone marrow from treated mouse M1. (**H**) Heritability of both sample features and genes for founder 1 and founder 6 computed with PATH.

assigned to founder groups, of which 5,468 were successfully assigned to clones. Founder tag sequencing revealed in vitro fitness differences: in untreated flasks, founder 1 (F1) and founder 6 (F6) dominated (43% and 57% of cells, respectively), and few founder 11 (F11) cells were detected (<0.5% of cells). F1 and F6 control cells clustered separately, suggesting distinct founder-specific transcriptomes (**Figure S2B)**. In contrast, treated populations were primarily composed of F1 with few F6 (<0.7%) and zero F11 cells. F5 was outcompeted in all conditions (**Figure 2C)**.

We performed differential gene expression analysis between founder groups, focusing on F1 and F6 in the control setting. F1 lineages were characterized by high expression of Nkain3, Wnt6, and Chl1 (**Table S1**). In contrast, F6 lineages were characterized by high expression of Sox2ot, Tnni1, and Tnnt1 (**Table S2**). GSEA results indicated enrichment of multiple Hallmark pathways in F6 compared to F1, including MYC targets V1, oxidative phosphorylation, and MTORC1 signaling (**Figure S2C**). Enriching these oncogenic pathways likely contributed to the success of F6 in the absence of treatment. Since these pathways have well-established roles in chemoresistance, we expected F6 to display a survival advantage under AraC treatment[17–20]. Unexpectedly, F6 was almost fully out-competed by F1 in the presence of AraC (**Figure 2C)**.

We then explored founder F1's competitive advantage over F6 and the other founders during treatment. Within F1 populations, there was a distinct and divergent selection of specific clones in AraC-treated and control flasks (**Figure 2D**). The highest proportion of persistent cells belonged to clones F1.1 and F1.2, while the highest proportion of resistant cells belonged to F1.4. F1 cells have a clear survival advantage through in vitro AraC treatment, but the outgrowth of different clones in treated replicates suggests clone-specific responses that contribute to population growth dynamics.

To identify F1 clone-specific treatment responses, we analyzed differentially expressed genes between control, resistant, and persistent-dominant clones (**Table S3**). Our dominant clone in the resistant population, F1.4 (**Figure 2D**), expressed high levels of Igll1 and Evac1 compared to other resistant cells. Clones F1.1 and F1.2 were successful in both persistent and resistant populations; when we compared their control gene expression to all other F1 clones, a transcriptional signature containing Grap2, Trat1, and Ldlrad4 emerged (**Figure S2D**). Trat1 is involved in the regulation of T cell receptors and expression is associated with immune cell infiltration and improved survival in breast, ovarian, and lung cancer [21–23], Ldlrad4 is a negative

196 regulator of TGF-beta signaling [24,25], and expression promotes the proliferation and migration of
197 hepatic cancer cells and is downregulated in CD34+ cells from patients with MDS [26]. Consistent
198 with the upregulation of Ldlrad4 expression in F1.1 and F1.2 control cells, sister cells of both
199 clones in the persistent population display high Ldlrad4. We also identified clones with pre-
200 existing lineage-specific expression patterns. Cells belonging to clone F1.3 tended to colocalize
201 and shared high expression of Adprh and Mctp1 (**Figure S2E-F)**. These genes highlighted by
202 differential gene expression analysis do not have well-established roles in AML.
203
204 In parallel to our in vitro treatment assay, we injected immune-competent C57BL/6 KH2[27] mice
205 with 25k of the pooled founder starting population to induce leukemia with lineage tracing
206 capacity (**Figure 2A)**. Mice were then treated with AraC to a dose comparable to that given to
207 patients in one round of 7+3 induction chemotherapy[9]. We used bulk lineage sequencing to
208 estimate founder and clone dynamics from blood draws of circulating leukemia cells. Contrary to
209 our in vitro results, F6 outcompeted other founder populations in vivo in all but one mouse
210 (**Figure 2E**). This bottlenecking occurred sometime between day 28, when all founders were
211 detectable in control and treated mice (**Figure S3A),** and euthanasia on day 67. A selective
212 sweep was nearly complete in treated mouse M1 (>99.9% F6 by day 43), treated mouse M2
213 (100% F6 by day 67), and untreated mouse M4 (>99.9% F6 by day 57). Control mouse M3's
214 leukemic burden was composed of >77.5% F1 by day 43, and this increased to >85.9% on day
215 50 when M3 was euthanized due to symptomatic disease.
216
217 We then focused on the within-founder clone tag sequences seen in blood draws. Interestingly,
218 M1 displayed sustained clonal diversity split amongst F6.1, F6.2, F6.3, F6.4, and F6.5,
219 indicating these clones survived AraC treatment and had comparable fitness during post-
220 treatment outgrowth. In contrast, blood samples from M3 were primarily dominated by F1's
221 clone F1.2 by day 43 (**Figure S3C)**. Blood samples from M2 and M4 did not contain enough
222 overlap with our known clone tags to infer population dynamics, likely representing diversity not
223 captured in our single-cell sequencing at end-point.
224
225 To further examine the lineage identities and transcriptomes of cells surviving in vivo treatment,
226 we sequenced 10,473 and 6,344 cells from blood and bone marrow, respectively, of treated
227 mouse M1 displaying sustained F6 clonal diversity. Non-leukemic cells clustered by cell type,
228 and leukemic cells were identified by founder and clone tags (**Figure S3D-E)**. Of the leukemic
229 cells, 6 and 6,053 belonged to founders F1 and F6, respectively. Clone groups were assigned to

230 2,002 single cells, 702 belonging to clone F6.1. The remaining clone assignments were split
231 between F6.2, F6.3, F6.4, and F6.5 (**Figures 2F, S3B**). We also detected low frequencies of
232 cells belonging to F6.6 and F6.8. These single-cell sequencing results matched the population
233 detected in bulk sequencing, confirming our ability to reconstruct founder and clone patterns
234 from limited samples, such as blood draws, using FLARE lineage tracing.
235
236 We then created phylogenetic trees for F1 and F6 using FLARE lineage recorder indels
237 captured with our in vitro and in vivo scRNA-seq (**Figure 2G)**. To identify heritable gene
238 expression patterns associated with the development of AraC resistance, we conducted lineage
239 autocorrelation analysis on founder trees using PATH (**Figure 2H**)[28]. PATH highlighted genes
240 previously identified by differential gene expression analysis as highly cross-correlated with
241 resistant and/or persistent cell identities in the F1 tree (**Figure S3F**). These genes include
242 Olfm3, a gene associated with anoikis resistance in AML cells[29], and Cd7, the expression of
243 which in AML is a prognostic risk factor[30–32]; both genes were significantly upregulated in AraC
244 persistent cells. Genes such as Adprh and Mctp1, associated with the F1.3 clone branch, were
245 also strongly heritable but expressed primarily in a subset of control cells.
246
247 These initial experiments with C1498-FLARE cells allowed us to optimize the lineage tracing
248 system and test our ability to track leukemic lineage in immune-competent mice. With FLARE,
249 we successfully identified highly heritable genes selected for through AraC treatment that
250 contribute to drug persistence and resistance.

251 Clonal treatment response and tree generation in human AML cells

252 We next engineered FLARE lineage recording human HL60 cells, a model of FAB-M2 AML[33].
253 We seeded 500 HL60-FLARE founder cells, expanded, and split into parallel cultures (**Figure**
254 **3A**). This starting pool expands on the transcriptional landscape sampled in C1498-FLARE cells
255 while maintaining sister founder populations in the control and treatment arms. Similarly to
256 C1498-FLARE cells, our resulting HL60-FLARE cells clustered in distinct groups based on AraC
257 treatment in vitro (**Figure 3B**). However, unlike C1498-FLARE cells, AraC-treated HL60-FLARE
258 cells in different flasks clustered together despite different regrowth rates (**Figure S4A**). AraC-
259 treated flask A reached cell concentrations comparable to untreated HL60-FLARE cells by day
260 80 of AraC treatment, so we posited these cells would align transcriptionally with C1498-FLARE
261 resistant cells. Surprisingly, differential expression analysis uncovered the upregulation of genes
262 shared with AraC-persistent C1498 F1 cells such as VIM, PSD3, CRIP1, and S100A4 (**Table**

S4). AraC-treated flask B displayed slower growth dynamics but shared flask A's transcriptional profile (**Table S5**).

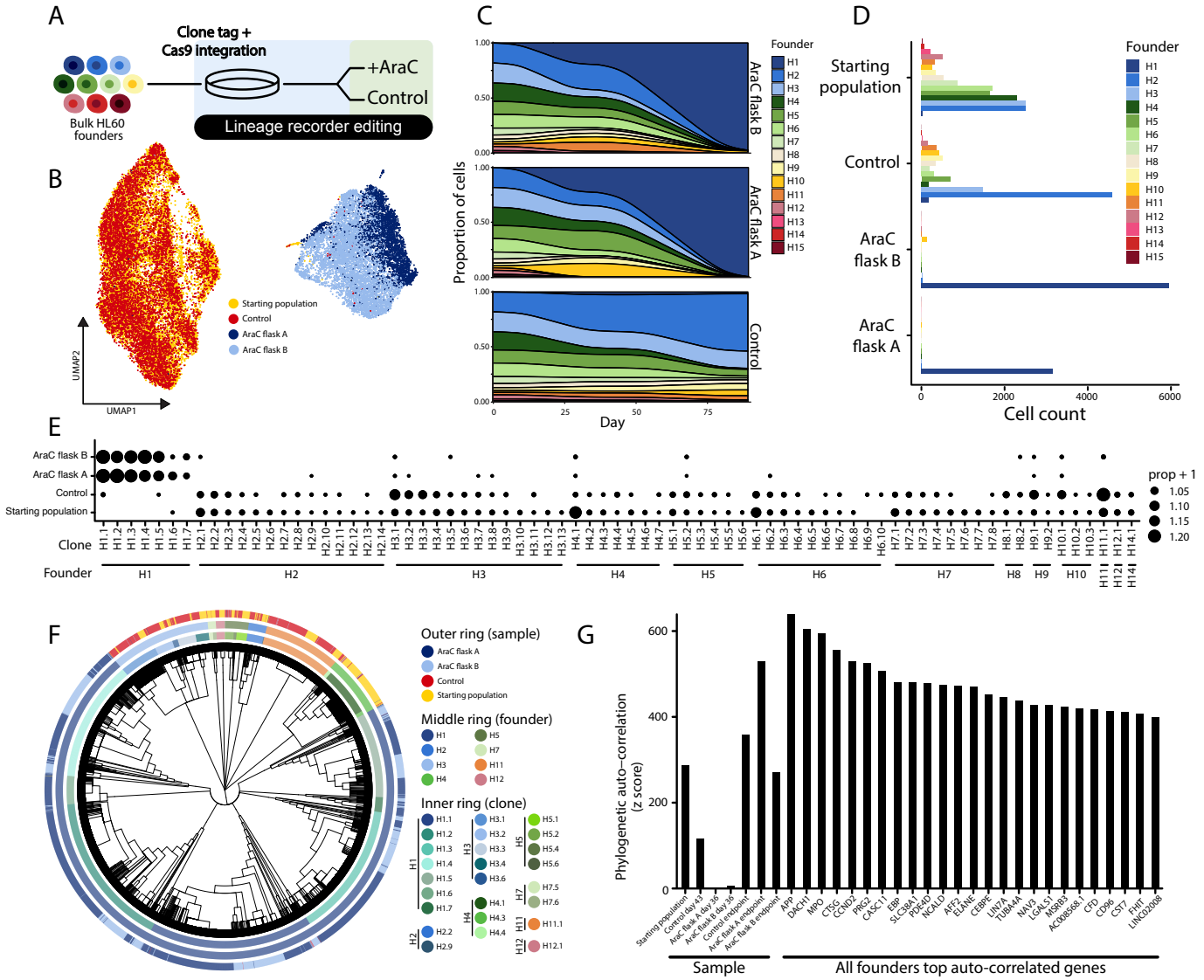

Figure 3: Founder and clone-level selection of founder H1 in response to AraC treatment in human HL60 AML cells. (**A**) Experimental design of HL60-FLARE AML lineage tracing in vitro. (**B**) Single cell sequencing of HL60-FLARE cells following 89 days of in vitro AraC treatment. Cells cluster into distinct groups associated with their sample (yellow - starting population; red - no AraC; dark blue - AraC flask A; light blue - AraC flask B). (**C**) Clone population fluctuations for each flask throughout the experiment. (**D**) Founder proportions in each sample display increased survival of founder H1 cells under AraC treatment in both flask A and B. (**E**) Clone populations for each sample at experimental endpoint, ordered by founder and clone size. (**F**) Phylogenetic reconstruction of HL60-FLARE cells generated from single cell sequencing of in vitro lineage tracing. (**G**) Heritability of both sample features and genes for all HL60-FLARE founders computed via PATH.

266 Single-cell sequencing of founder and clone tags was used to cluster cells into 15 founder
267 populations (H1-H15), individually composed of 1-14 clones. Sequencing from cells collected at
268 the experiment start, midpoint, and endpoint were used to determine founder proportions over
269 time for each flask. All 15 HL60 founders were detected in the experimental starting population
270 and the control flask at endpoint (**Figure 3C-D**). There was an extensive bottleneck in all
271 cultured populations, regardless of treatment. Untreated endpoint cells were most likely to
272 descend from H2, while both AraC-treated flasks consisted primarily of cells belonging to H1.
273 Both treated flasks maintained diversity within H1 on the clonal level, with most cells being
274 evenly split between clones H1.1, H1.2, H1.3, H1.4, and H1.5 (**Figure 3E**).
275
276 The success of H1 in both treated flasks suggests a founder-specific propensity for treatment
277 resistance prior to treatment onset. This is also supported by the comparable fitness of H1
278 clonal populations, indicating the lack of clone-specific resistance evolution. We used differential
279 gene expression analysis to explore H1-specific expression patterns in the presence and
280 absence of treatment to determine whether H1 cells were consistently differentiated from other
281 founders or were primed for reprogramming following the introduction of AraC. Pre-experiment
282 and endpoint control H1 cells shared the upregulation of 104 genes (**Table S6**). Many of these
283 genes were also upregulated in AraC-resistant H1 cells compared to other founders, including
284 MKX, APBA2, LAMP5, CAMK2D, and NAV3.
285
286 We turned to phylogenetic reconstruction and heritability analysis to further dissect the H1's
287 resistance mechanisms. We used lineage recorder indel patterns to reconstruct phylogenetic
288 trees for each clone population. These clonal trees were merged to create founder-level lineage
289 trees, and founder-level trees were merged to create the HL60 population tree (**Figure 3F**).
290 PATH analysis on the population tree revealed many highly heritable genes associated with
291 evolution with and without AraC, including genes previously implicated in AML prognosis and
292 treatment response, such as MPO (**Figure 3G**). This analysis also indicated significant
293 heritability of genes previously identified as upregulated in treatment-naive H1 cells.

294 Identification of pro-leukemic transcriptional programs associated with pre-
295 AraC-resistant lineages

296 To better understand broad transcriptomic changes over AraC-induced clonal evolution, we
297 generated lineage-dependent gene modules for C1498-FLARE F1 and F6 trees, and the HL60-

298 FLARE population tree using Hotspot (**Figure 4A-B, Table S3, Table S7**)[34]. Jaccard indices
299 revealed significant similarity between a number of HL60 and C1498 modules, indicating shared
300 mechanisms across our two experimental lines (**Figure 4C, S4B-C**). Moreover, expression of
301 these modules within the lineage structure showed founder and clone-specific enrichment
302 (**Figure 4D**).
303

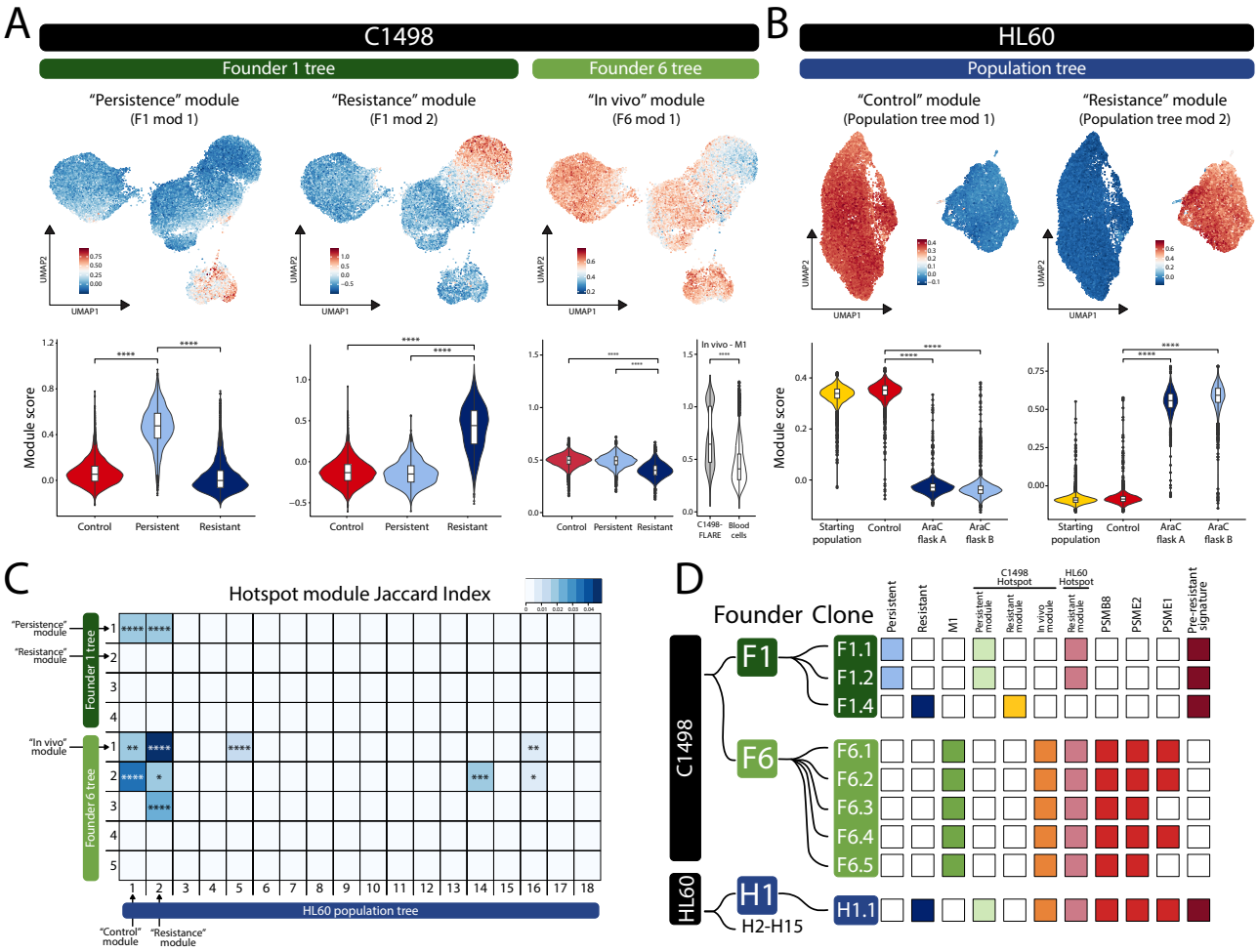

Figure 4: Hotspot gene modules reveal heritable AraC response mechanisms common to mouse and human AML cells. (**A**) Feature and violin plots for selected C1498-FLARE Hotspot modules. Significance determined by wilcoxon rank sum test with Bonferroni correction. (**B**) Feature and violin plots for selected HL60-FLARE Hotspot modules. Significance determined by wilcoxon rank sum test with Bonferroni correction. (**C**) Jaccard Index similarity comparing C1498-FLARE and HL60-FLARE Hotspot modules. Square color indicates Jaccard Index, asterisks represent BH-corrected P-value significance levels. (**D**) Schematic showing the lineage relationships of C1498-FLARE and HL60-FLARE clones. The first three columns to the right of the clone labels indicate the setting in which each clone had a survival advantage, either in the Ara-C persistent population (a growth phenotype we only saw in C1498-FLARE populations), resistant population, or in AraC-treated mouse M1's peripheral blood and bone marrow. The next four columns to the right indicate modules upregulated in each clone. The next three columns indicate whether immunoproteasome gene PSMB8 and 11S regulatory cap genes PSME1 and PSME2 were significantly upregulated in the AraC treated populations. The rightmost column indicates the upregulated expression of the pre-resistant signature in AraC-treated populations.

304 We first focused on the overlap between HL60-FLARE “resistance” module 2 and C1498-
305 FLARE “persistence” F1 module 1 (**Table S9**), the most heritable modules enriched in treatment
306 conditions. The HL60-FLARE “resistance” module was distinctly enriched in all AraC-treated
307 cells (**Figure 4B**), while the C1498-FLARE “persistence” module was associated with AraC-
308 persistent populations (**Figure 4A**). The HL60 resistance module was primarily expressed in the
309 H1 founder population and contained many genes found to be distinctly upregulated in
310 treatment-naive H1 cells (**Table S6-7**). This suggests that the “resistance” module describes a
311 lineage-specific profile that can identify cells most likely to become refractory to AraC treatment.
312
313 The HL60-FLARE “resistance” module shared 9 genes with the C1498 “persistence” module:
314 ITSN1, VIM, CRIP1, S100A11, ANXA2, MYL6, S100A4, PSD3, and DOCK10. This set of genes
315 is highly active in cytoskeleton maintenance and remodeling. Such pathways are implicated in
316 the epithelial-to-mesenchymal transition (EMT), a type of transdifferentiation involved in cancer
317 cell migration, invasion, and drug resistance[35]. While EMT is less well studied in hematological
318 malignancies than solid tumors, there is increasing evidence of its relevance in diseases like
319 AML[36–40]. There was no significant correlation of module expression with any tested EMT gene
320 sets, suggesting that F1 module 1 and HL60 module 2 describe cell adhesion and motility
321 processes largely independent from canonical EMT (**Figure S4D**). The co-upregulation and
322 significant heritability of these genes in both cell lines under AraC treatment supports the pro-
323 survival role for cytoskeletal dynamics separate from EMT processes, potentially constituting a
324 form of CAM-DR. This is further supported by cell adhesion molecule genes present in the HL60
325 “resistance” module, including ITGB1, a primary mediator of CAM-DR[41] and LGALS1, implicated
326 in CAM-DR in multiple myeloma [42].
327
328 To identify whether heritable upregulation of CAM-DR genes in leukemic lineages prior to
329 treatment was associated with the generation of AraC resistance, we examined related
330 pathways in pre-experiment and control H1 cells. Sixty-five genes significantly upregulated in
331 treatment-naive H1 cells were also found in the HL60 population tree “resistance” module,
332 constituting an H1-specific pre-resistant state (**Figure S4E**).The majority of these genes are
333 also involved in cell motility and adhesion, such as NCAM1[43], PRICKLE1[44], BMP2[45], CPNE8[46],
334 and NAV3[47]. Also included are ANXA2 and PSD3, genes from the C1498 “persistence” module.
335 While H1 cells uniquely express this pre-resistant signature prior to AraC exposure, treatment
336 increased H1 population expression in a time-dependent manner (**Figure S4F**). In the control

337 setting, this signature does not clearly contribute to cell fitness, but under AraC, it confers a
338 large survival benefit.

339 Identification of pro-leukemic transcriptional programs associated with in 340 vivo immune escape

341 The mouse M1 “in vivo” module was the most heritable module over the F6 phylogenetic tree; it
342 showed significant enrichment in cells collected from M1 and may represent an in vivo
343 mechanism of survival through AraC treatment (**Figure 4A, S4G**). To determine shared
344 mechanisms with our HL60-FLARE modules, we again compared modules and found a strong
345 Jaccard index score between the M1 “in vivo” and our HL60-FLARE “resistance” modules
346 (**Table S10**, **Figures 4C, S4B-C**). These two modules share immunoproteasome genes
347 PSME1, PSME2, PSMB8, and PSMB9, immunoproteasome components involved in antigen
348 processing and presentation for MHC class I-mediated immune response. PSMB8 and PSMB9
349 encode two of the three beta core subunits of the 20S immunoproteasome core complex[48]. In a
350 variation of the immunoproteasome, the 20S core can complex with the 11S (or PA28ab)
351 regulatory cap, the primary subunits of which are encoded by the PSME1 (PA28a) and PSME2
352 (PA28b) genes[49]. There is evidence that peptide fragments produced by the 20S/11S
353 immunoproteasome may be less conducive to MHC class I presentation and lead to tumor cell
354 immune escape[50–52].
355
356 We investigated Psme1 and Psme2 expression patterns in F6 cells that survived engraftment
357 and treatment in our in vivo model. Differential gene expression analysis comparing F1 and F6
358 in vitro revealed universal F6-specific upregulation of 11S regulatory cap genes Psme1 and
359 Psme2 (**Table S2**). Additionally, Psmb8, Psme1, and Psme2 were significantly upregulated in
360 F6 clones in vivo compared to their in vitro counterparts proportional to clone success in vivo
361 (**Figures 4D, S4H**). F6 clones 1-5 upregulated Psme2 and Psmb8 in the in vivo setting,
362 representing a founder-specific enrichment. Expression of Psme1 differentiated F6 clones
363 further; significant in vivo upregulation was unique to clones F6.1, F6.2, and F6.4, the three
364 most successful clones in the blood (**Figures 4D, S3B**). While Psmb8 was significantly heritable
365 for F6 clones 1-5, Psme2 was only heritable in clones 1-3. In contrast, Psme1 expression was
366 only heritable over the lineage tree within clone F6.5. The evolutionary plasticity of Psme1
367 expression in the F6.1, F6.2, and F6.4 lineages may provide a competitive advantage within the
368 broader F6 immunoproteasome resistance mechanism.

Patient analysis

Phylogenetic reconstruction of in vitro and in vivo AraC-treated C1498-FLARE and HL60-FLARE cells revealed heritable gene modules associated with generating and maintaining AraC resistance. To validate their clinical relevance, we used gene expression data from the TARGET AML cohort (2,007 patients, ages 0-29), the most extensive, publicly available AML patient dataset[53]. The prognostic impact of module expression in bone marrow samples, collected at initial diagnosis, showed significantly increased hazard ratios associated with the expression of HL60-FLARE "resistance" module (HR 4.99), C1498-FLARE "persistence" module (HR 2.69), and "in vivo" module (HR 3.18) (**Figure 5A**). Kaplan Meier curves confirmed that heightened expression of these modules was associated with shorter overall survival (**Figure 5B-D**). These results support the ability of FLARE lineage tracing to generate heritability patterns relevant to AML patient survival.

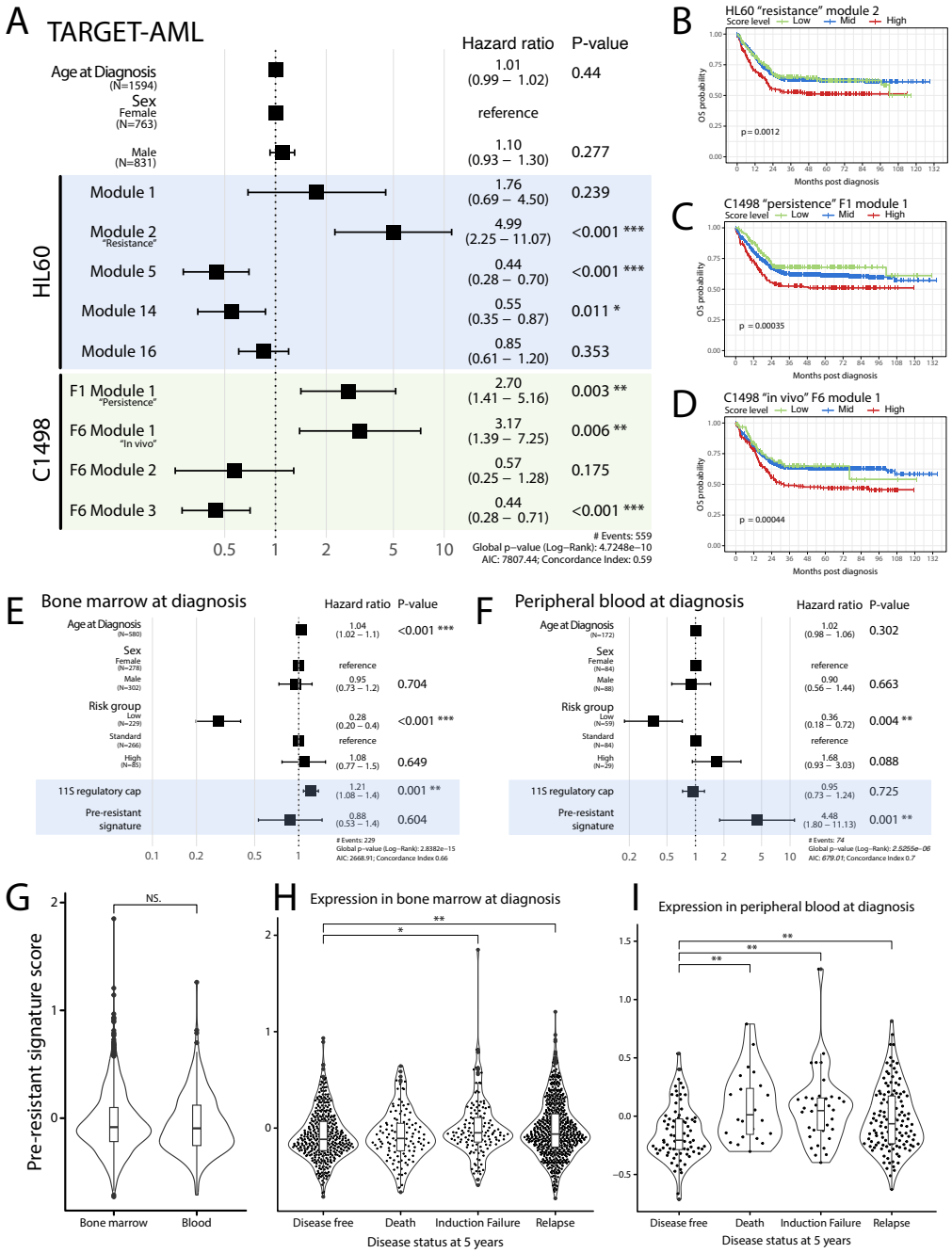

Figure 5: Expression levels of HL60-FLARE modules, C1498-FLARE modules, 11S regulatory cap genes, and pre-resistant signature genes are significantly associated with survival in pediatric AML patients. (**A**) Cox proportional hazard ratios for HL60-FLARE and C1498-FLARE Hotspot module scores calculated from bone marrow samples taken at diagnosis in the TARGET-AML cohort. (**B**) Kaplan Meier survival plots for the TARGET-AML cohort stratified into low, mid, and high expression of HL60-FLARE "resistance" module 2. Corresponding risk table can be found in supplementary table S11. (**C**) Kaplan Meier survival plots for the TARGET-AML cohort stratified into low, mid, and high expression of C1498-FLARE "persistence" F1 module 1. Corresponding risk table can be found in supplementary table S11. (**D**) Kaplan Meier survival plots for the TARGET-AML cohort stratified into low, mid, and high expression of C1498-FLARE "in vivo" F6 module 1. Corresponding risk table can be found in supplementary table S11. (**E**) Cox proportional hazard ratios for 11S regulatory cap and pre-resistant scores calculated from bone marrow samples taken at diagnosis in the TARGET-AML cohort. (**F**) Cox proportional hazard ratios for 11S regulatory cap and pre-resistant scores calculated from peripheral blood samples taken at diagnosis in the TARGET-AML cohort. (**G**) Violin plot showing pre-resistant signature scores calculated from samples taken at diagnosis. Bone marrow and blood samples do not significantly differ in signature scores. (**H**) Violin plot showing pre-resistant signature scores calculated from bone marrow samples taken at diagnosis. Mean scores for patients who experience induction failure or relapse within 5 years post diagnosis are minimally but significantly higher than patients who are disease free 5 years post diagnosis. Significance was calculated with Wilcoxon rank sum tests and Bonferroni corrected. (**I**) Violin plot showing pre-resistant signature scores calculated from peripheral blood samples taken at diagnosis. Mean scores for patients who experience death, induction failure, or relapse within 5 years post diagnosis are significantly higher than patients who are disease free 5 years post diagnosis. Significance was calculated with Wilcoxon rank sum tests and Bonferroni corrected.

We then focused on our pre-resistant/CAM-DR sub-module and 11S cap subunits of the 20S/11S immunoproteasome variant to explore their roles in treatment response and survival. We generated Cox proportional hazard ratios, including patient risk groups as covariates to account for interactions between risk group and signature expression. Expression levels of both signatures were associated with the risk of death independent of predefined risk groups. Interestingly, signature-associated risk of death differed drastically when patient expression scores were calculated from bone marrow or peripheral blood taken at diagnosis. In bone marrow samples, expression of 11S regulatory cap genes, but not the pre-resistant signature, was significantly associated with the risk of death (HR 1.21, p-value < 0.001, **Figure 5E**). Conversely, in peripheral blood, expression of the pre-resistant signature, but not the 11S regulatory cap genes, was significantly associated with a greater risk of death (HR 4.48, p-value < 0.001, **Figure 5F**). While 11S regulatory cap genes were expressed slightly more in bone marrow samples, potentially explaining why PSME1 and PSME2 expression have higher prognostic impact in bone marrow, there was no significant difference in expression of the pre-resistant sub-module between sample types (**Figure S5A, 5G**).

We then assessed the relationship between signature expression in pre-treatment diagnosis samples and patient status at 5 years post-inclusion. Specifically, we compared expression levels between patients who experienced death, induction failure, or relapse within 5 years compared to patients who remained disease-free for 5 years. Patients who experienced induction failure or relapse, but not death, tended to have higher pre-resistant sub-module scores in their blood and bone marrow samples at diagnosis (**Figure 5H-I**). Importantly, these

425 trends were more dramatic when comparing blood samples at diagnosis; patients experiencing
426 induction failure, relapse, and death expressed significantly higher levels of the pre-resistant
427 signature in blood compared to patients disease-free at 5 years. There was a slight negative
428 relationship between pre-resistant sub-module score and peripheral blast percentage at
429 diagnosis, suggesting this pattern does not reflect peripheral leukemic burden (**Figure S5B-C**).

430 Discussion

431 Chemotherapy resistance likely arises from the selection of pre-existing resistant populations
432 and the reprogramming of previously sensitive cells. There is an unmet need for tools to dissect
433 this evolution over time. We developed FLARE, a CRISPR-based dynamic lineage tracing
434 system capable of tracking founder, clone, and subclone lineage in tandem with single-cell
435 transcriptomics in response to chemotherapy. We demonstrated how our hierarchical approach
436 can record cancer transcriptional evolution over an extended treatment time course, capturing
437 both clonal evolution and selection of pre-existing cellular states. We used this technology to
438 dissect the transcriptional evolution of AML, leveraging both in vitro and vivo systems and
439 mouse and human models.
440
441 Using FLARE we uncovered the (1) heritable patterns of cell adhesion-associated AraC-
442 tolerance and the (2) upregulation of immunoproteasome 11S regulatory cap subunits
443 contributing to clone-specific in vivo fitness. The expression of both signatures in AML patients
444 is significantly associated with shorter overall survival. High expression of PSME1 and PSME2,
445 the major subunits of the 11S regulatory cap, in bone marrow but not peripheral blood, is
446 associated with poorer prognosis. In contrast, high expression of our pre-resistant signature,
447 which consists of various genes involved in cytoskeletal and cell adhesion pathways, is more
448 informative when measured in blood samples taken at diagnosis. Both the 11S regulatory cap
449 and pre-resistant signatures are composed of many genes not previously implicated in AML.
450
451 Our pre-resistant signature comprises many cell adhesion-mediated drug resistance (CAM-DR)
452 genes. This mechanism has long been studied in the context of solid tumors, but its importance
453 in hematological malignancies is growing[54,55]. Leukemic CAM-DR is thought to result from
454 interactions between AML blasts and bone marrow stromal cells via various integrin
455 molecules[56]. These interactions may protect leukemia cells, leading to minimum residual
456 disease persistence and treatment failure upon disease recurrence[55]. This increased survival is

457 thought to result from anoikis resistance, in which cells avoid apoptosis in response to becoming
458 detached from the extracellular matrix[57]. Interestingly, the emergence of CAM-DR in our work
459 was primarily seen in cells treated with AraC in standard in vitro cell culture. This may indicate
460 that treatment alone can lead to such resistance outside of the bone marrow niche.
461 Furthermore, survival analysis of the TARGET AML cohort revealed that expression of our
462 potential CAM-DR-associated pre-resistant signature had a significantly higher prognostic
463 impact when measured from peripheral blood samples taken at diagnosis. Our survival analysis
464 also suggests that the presence of leukemic blasts with enrichment of cytoskeletal, cell
465 adhesion, and cell motility processes in the blood before induction therapy is associated with
466 induction failure, death and relapse within 5 years of diagnosis. Therefore, patients with "pre-
467 resistant" lineages prior to treatment are less likely to benefit from induction chemotherapy, with
468 cells already displaying CAM-DR that can survive treatment and expand to outcompete other
469 leukemic lineages. This mechanism needs further exploration, but it suggests a secondary role
470 of CAM-DR in AML outside of the bone marrow niche.
471
472 While the exact pro-leukemic mechanism of our 11S signature cap is still unclear, there are data
473 to suggest the 11S regulatory cap leads to the generation of fewer peptides conducive to MHC
474 class I presentation and can interfere with tumor-specific epitope production[51,52,58]. 11S subunit
475 expression levels in pediatric AML patient bone marrow samples taken at diagnosis are
476 significantly associated with an increased risk of death and shorter overall survival. PSME1 and
477 PSME2 expression levels in bone marrow samples differed negligibly between patients with
478 adverse outcomes within 5 years compared to those that remained disease-free. This indicates
479 that while PSME1 and PSME2 expression in bone marrow is associated with a higher risk of
480 death, it is not highly predictive of induction failure or relapse within 5 years of diagnosis. It may
481 instead indicate the presence of leukemic lineages that are better able to evade immune
482 detection, resulting in a more aggressive and "colder" immune microenvironment.
483
484 In the future, the FLARE approach could be used to dissect the transcriptional evolution of other
485 tumor types under selective treatment pressure. Given the ability to sample tumor evolution
486 from tagged cell populations in circulating blood, this can be multiplexed to explore many more
487 initial cell states and treatment spaces. Moreover, this approach can be coupled to mouse
488 lineage-tracing models such as DARLIN[59] or the MARC1[60] mouse to capture the interplay
489 between engrafted tumor and immune cell populations in the context of checkpoint inhibitors
490 and other immunotherapies.

Methods

Cell Lines and drugs

The murine C1498 cell line (Cat# TIB-49) was purchased from ATCC in 2021. The HL60 AML cell line was a gift from Dr. Bonnie Lau's lab. C1498 cells were cultured in DMEM, supplemented with 10% FBS, 100 u/mL penicillin, and 100 ug/mL streptomycin. HL60 cells were cultured in Iscove's Modified Dulbecco's Medium, supplemented with 20% FBS, 100u/mL penicillin, and 100ug/mL streptomycin. All cell lines were expanded, aliquoted, and cryopreserved in culture media plus 5% DMSO and stored in liquid nitrogen upon receival. Active cell cultures were stored at 37°C with 5% CO_2. All cultured lines were regularly checked for the presence of mycoplasma using the Universal Mycoplasma Detection Kit (ATCC, 30-1012K). Cytarabine (AraC) powder was purchased from Selleck Chemicals (Cat # S1648). AraC was resuspended in PBS fresh for each in vitro and in vivo treatment.

Mice

KH2/iCas9 mice (Jackson Laboratory strain #029415) were a gift of the Huang lab at Dartmouth College. All mice were maintained in pathogen-free housing at the Center for Comparative Medicine and Research in Dartmouth Hitchcock Medical Center. All experimental protocols were approved by and performed in accordance with the guidelines of the Institutional Animal Care and Use Committee at Dartmouth College. To attenuate the immune response to Cas9, all injected KH2/iCas9 were exposed to doxycycline during in utero development through irradiated feed containing 625ppm doxycycline (ScottPharma, Inc. Marlborough, MA).

Molecular cloning

GESTALT lineage tracing system engineering

All digested vectors and fragments and PCR products were purified with the Monarch Gel Purification Kit (Cat# T1120S) or the Zymo Clean and Concentrate-5 Kit (Cat# D4003). All Gibson assembly reactions were conducted with NEB HiFi DNA Assembly 2x Master Mix (Cat# E2621S), following the ssDNA HiFi assembly protocol. Golden Gate assemblies were conducted following the Rice University Esp3I protocol. Chemical bacterial transformations were done with DH5alpha competent cells following the NEB Transformation Protocol with a 2-hour recovery at 37°C. Electroporated transformations were conducted with NEB Electrocompetent E. coli and were electroporated with the following conditions: 2kV, 200Ohms, 25uFd. All

521 bacterial cultures were grown in a 37°C shaker at 220-290rpm overnight. Vectors were purified
522 using either the Qiagen Miniprep Kit or the Zymo Midiprep II Kit. Correct cloning of all final
523 constructs was confirmed via Circuit-seq[61].

524 **FLARE-miniV10-10x-UMI**

525 Eight Cas9 target sequences were designed for high efficiency (>69% Doench) and low off
526 target scores. The eight-target array was purchased as a gene block from IDT, with upstream
527 Esp3I sites for founder tag insertion and NdeI + BamHI sites for cloning purposes. The target
528 gBlock and pLARRY-EGFP vector (Addgene #140025) were digested with NdeI+BamHI and
529 ligated together with Quick Ligase (Cat# M2200s). Resulting construct is referred to as
530 pLARRY-targets.
531
532 The PB-CMV-MCS-EF1-puro vector (Systems Bio cat #PB510B-1) was digested with SpeI and
533 HincII, removing the puromycin resistance gene, and then circularized with a Gibson assembly
534 reaction with a short single stranded joining oligo. The resulting construct is referred to as
535 miniPB.
536
537 The FLARE-miniV10-10x vector was then constructed by amplifying EGFP and the target array
538 from pLARRY-targets. The PCR primers used KpnI and EcoRI sites. The amplified
539 EGFP+target array and miniPB were digested with KpnI and EcoRI, gel purified, and ligated
540 together using T7 ligase. The 10X capture sequence 1 was incorporated via Gibson assembly.
541 The FLARE-miniV10-10x construct is available at Addgene (Plasmid #234492).
542
543 Founder tag oligos consisting of 14 degenerate bases (the founder tag) and Esp3I sites were
544 purchased from IDT. One cycle of PCR was used to anneal and extend the oligos. Founder tag
545 oligos were then inserted into FLARE-miniV10 via a Golden Gate Assembly reaction; the
546 resulting product is FLARE-miniV10-10X-UMI.

547 **FLARE-pbV10g-neo**

548 Individual Cas9 guides complementary to the target sequences in FLARE-miniV10 were
549 designed and cloned into the pX330-mCherry (Addgene #98750) vector using the Zheng Lab
550 pX330 cloning protocol. Guides, along with their individual U6 promoters, were amplified from
551 pX330-mCherry vectors. A secondary PCR reaction added flanking Esp3I sites to each U6-
552 guide fragment. Esp3I sites were also added to the PB-CMV-MCS-EF1-puro vector, while the
553 puro resistance gene was removed. U6/guide sequences were joined and inserted into PB-

554 CMV-MCS-EF1-puro via a 9-part Golden Gate Assembly reaction. The neomycin resistance
555 gene was amplified from the hCas9 vector and inserted into the U6/guide array vector via
556 Golden Gate Assembly. The FLARE-pbV10g-neo construct is available at Addgene (Plasmid
557 #234493).
558
559 In earlier versions of FLARE constructs, Cas9 guides were encoded into a single construct
560 along with the lineage recorder Cas9 target array. For reasons not yet fully understood, splitting
561 this into two vectors (FLARE-miniV10-10X and FLARE-pbV10g-neo) led to significantly higher
562 editing rates across targets.

563 **FLARE-pbCas9-tag-mCherry**
564 The miniPB construct was digested with KpnI for a Gibson Assembly insertion of a landing pad
565 containing a NotI and a new KpnI site. The resulting vector is referred to as miniPB-LP. The
566 pX330 construct was digested with KpnI and NotI to isolate the Cas9 transgene, which was then
567 ligated into the landing pad miniPB-LP vector. The resulting construct is referred to as miniPB-
568 Cas9.

569 The pX330-mCherry construct was digested with BamHI and HindIII for the insertion of a clone
570 tag landing pad containing type IIs restriction sites in the 3' UTR of the mCherry transgene using
571 Gibson Assembly. The mCherry transgene, including the clone tag landing pad, was amplified
572 and extended to include Gibson Assembly adaptors complementary to the insertion region in
573 miniPB-Cas9. The miniPB-Cas9 construct was then digested with NotI and the mCherry and
574 clone tag landing pad were inserted using Quick Ligase. The resulting construct is referred to as
575 FLARE-pbCas9-mCherry, and is available at Addgene (Plasmid #234494).

576 Lastly, clone tag oligos with Esp3I sites were purchased from IDT. For C1498-FLARE cells, the
577 clone tag consisted of 14 degenerate bases followed by 10x capture sequence 2. For HL60-
578 FLARE cells, the clone tag consisted of 38 patterned bases
579 (NNNWSNNNWSNNNWSNNNWSNNNWSNNNWSNNNWSNNNWSNNN, pattern from Johnson et al [62])
580 followed by 10x capture sequence 1. One cycle of PCR was used to anneal and extend the
581 oligos, and the resulting product was inserted into FLARE-pbCas9-mCherry via Golden Gate
582 Assembly. Capture sequence 2 was replaced by capture sequence 1 for HL60-FLARE cells due
583 to the planned discontinuation of support for capture sequence 2 announced by 10x.

584 Cell engineering and lineage tracing system testing

585 We used the Neon Electroporation System to integrate all piggyBac constructs into cells to
586 generate stable cell lines. Electroporations were carried out with the following protocols: C1498
587 cells were resuspended at 2x10^7 cells/mL and electroporated with 1080V, 50ms, and 1 pulse;
588 HL-60 cells were resuspended at 2x10^7 cells/mL and electroporated with 1450V, 35ms, and 1
589 pulse. All electroporations were done with the Neon 100ul tip system. Transposase:transposon
590 ratios for piggyBac integration ranged from 1:1 to 1:8. All FACS was conducted on a BD
591 FACSAria II or BD FACSAria Fusion. For antibiotic selection cells were incubated with selection
592 media for 1-3 weeks, and dead cells were removed from culture 2-3 times/week using a Ficoll
593 density gradient.
594
595 GESTALT constructs were introduced to cell lines in the following order: first low passage cells
596 were electroporated with FLARE-miniV10-10X-UMI and FLARE-pbV10g-neo. EGFP-positive
597 and neomycin resistant cells were isolated via FACS and 1ug/mL G418 selection. This step was
598 repeated two to three more times, gating for higher EGFP expression during FACS in each
599 subsequent round. For the third round of FACS for the C1498 cell line, individual cells were
600 sorted into wells of a 96-well plate to initiate individual clonal populations.
601
602 Prior to lineage tracing experiments, engineered lineage tracing cells were electroporated with
603 FLARE-pbCas9-tag-mCherry and mCherry+ cells were isolated via FACS. Cells were sorted
604 into bulk populations and 96-well plates at 100 cells/well.
605
606 Lineage recorder editing levels in C1498-FLARE cells over time were calculated using single
607 cell and bulk lineage barcode data. Day 0 editing was calculated from bulk (not a subset of
608 founders) C1498-FLARE cells prior to FLARE-pbCas9-tag-mCherry integration; day 13 editing
609 was calculated from this same population 13 days following integration post FACS. The
610 remaining editing rate timepoints were measured using the cell population used in C1498-
611 FLARE founder pool AraC treatment experiment and lineage recorder reads were subset for
612 reads containing valid founder tags associated with founders 1, 5, 6, and 11. Day 44 editing was
613 calculated from single cell sequencing of the founder pool subset for F1 and F6 prior to AraC
614 treatment. Day 64 editing was calculated from single cell sequencing of one untreated flask
615 cultured from the same population as used in day 44, and was subset for valid cells identified
616 via GEX analysis. Day 87 was also measured from one control flask from this population -
617 editing was calculated from single cell lineage endpoint data.

C1498-FLARE founder isolation and characterization

To screen individual C1498-FLARE founder populations for copies of FLARE-miniV10-10X-UMI integrations gDNA was collected and founder tags were amplified and prepared into sequencing libraries. Founder tag libraries for individual populations were multiplexed and sequenced on an Illumina MiniSeq with a Mid-Ouput 300-cycle kit. Founder tags for each clone were determined as having > 4,300 reads. Cells from each clone were expanded, aliquoted, and cryopreserved.

Growth curves

To begin growth curves for individual founders, cells were resuspended at 50k cells/mL and plated in T25 flasks in triplicate. From day 0-7, two 10ul aliquots from each flask were mixed with 10ul Trypan Blue and counted on the Luna II Cell Counter daily. Growth curve data were analyzed and plotting using the Growthcurver R package [63].

AraC dose-response curves

Cells were plated at 15,000 cells/well in a 96-well plate in media containing various AraC dosages in triplicate (0.0001mg/ml-10mg/ml). 100ul of CellTiter 96 AQueous One Solution (Cat# G3582) was added to each well and after incubation for 1-3 hours absorbance was measured at 490nm on a SpectraMax i3x. Absorbance from wells containing media without cells or drug were averaged to calculate background absorbance. Corrected absorbance for each well was calculated as (well absorbance - background absorbance). Corrected absorbances were then loaded into the drc R package for generating dose-response curves, ED50 values, and EC50 values[64].

Whole genome sequencing

High molecular weight (HMW) gDNA was isolated from parental C1498 cells and individually engineered clones using the Monarch High Molecular Weight gDNA Kit for Cells (Cat# T3050S). HMW gDNA samples were multiplexed and prepared into a sequencing library following the Oxford Nanopore SQK-NBD114.24 kit. The library was then sequenced on a PromethION flow cell with R10.4.1 chemistry. Sequencing data were basecalled and demultiplexed using Guppy. Variants were called using PEPPER-Margin-DeepVariant, SNPs were isolated and filtered for a minimum quality of 20 and mean depth between 10 and 80. To remove population variants, the GRCm39 dbSNP was annotated using Ensembl VEP and variants with moderate or high impact were removed. The resulting dbSNP was subtracted from filtered SNPs using Bedtools [65]. Remaining variants were annotated with Ensembl VEP and intersected with datasets of known AML mutations.

650 ## AraC response and resistance experiments

651 **C1498-FLARE founder pool treatment response and resistance**

652 C1498-FLARE founders F1, F5, F6, and F11 were sorted by FACS on day 13 following
653 electroporation with the FLARE-pbCas9-tag-mCherry vector. Cells were sorted into 96-well
654 plates at 100 cells/well. In vitro AraC treatment began on day 44 (31 days post FACS). Cells
655 were allowed to grow for a longer period following sorting due to growth rate differences
656 between founders. On day 44, 1 well of sorted cells for each clone was pooled at equal
657 numbers and split into four flasks for in vitro treatment with an aliquot set aside for mouse
658 injections.

659 *In vitro*

660 Two flasks were maintained in normal culture media, the remaining two flasks were maintained
661 in media containing 0.05ug/ml AraC. Flasks were maintained for 43 days to allow for the
662 development of AraC resistance. Media was replaced 2-3 times/week and split when needed.
663 Dose-response curves were calculated for each flask on day 15 of treatment, and a final dose-
664 response curve was calculated for flask displaying resistance on day 36 of treatment. On day 43
665 of treatment, cells were prepared for MULTIseq tagging and single cell capture following
666 MULTIseq and 10x protocols.

667 *In vivo*

668 Pooled C1498 clone cells were washed twice with PBS, resuspended at 250,000 cells/mL and
669 filtered through a 40uM filter. Mice were injected IV with 100ul of cell suspension (25,000
670 cells/mouse). On day 14 mice were randomly assigned to treatment groups; on days 14-16
671 treatment mice received 100mg/kg AraC IP and control mice received an equal volume of PBS.
672 Blood was collected via the retro-orbital sinus on days 13, 28, 43, 50, and 57. Prior to blood
673 draws mice were anesthetized with Isoflurane. Microhematocrit capillary tubes were placed in
674 1.7mL tubes containing 50mM EDTA and then used to collect 50-200ul of blood from the retro-
675 orbital sinus into EDTA coated tubes. Blood samples were then kept on ice until further
676 processing. Mice displaying symptoms of high AML burden were euthanized. All surviving mice
677 were euthanized on day 67 post cell injections. Upon euthanasia whole blood was collected via
678 cardiac puncture and femoral bone marrow was harvested. gDNA was isolated from 20ul of
679 whole blood, and remaining blood was separated using a Ficoll-paque density gradient to
680 isolate PBMCs. To isolate bone marrow, 18g needles were used to puncture the bottom of
681 0.5mL tubes, femur bones were placed in the 0.5mL tubes and nested in 1.7mL tubes, and

682 tubes were spun at > 10,000g for 20 seconds. Bone marrow pellets and PBMCs were methanol
683 fixed and permeabilized following 10x protocols.

684 **Human AML cell line in vitro treatment response**

685 Engineered lineage tracing HL60-FLARE cells were sorted into 96-well plates at 100 cells/well
686 following electroporation with FLARE-pbCas9-tag-mCherry to establish GESTALT lineage
687 tracing populations, as described above. Sorted well populations were expanded for 23 days.
688 On day 23 post FACS, five expanded wells were combined into an initial cell pool. The pool was
689 then split for 10x scRNA-seq preparation, methanol fixation, cryopreservation, and in vitro AraC
690 treatment. For in vitro AraC treatment cell pools were split into control (no AraC) and AraC
691 conditions (0.01ug/mL AraC). All treatment wells were cultured in duplicates. AraC solutions and
692 media were made fresh for media changes twice per week, and wells were split as needed. Cell
693 concentration for each well was measured once per week in duplicates using the Luna II Cell
694 Counter. Cells were regularly cryopreserved through the experiment for later analysis. In vitro
695 treatment continued for 89 days, after which cells were prepared for 10x scRNA-seq preparation
696 with MULTIseq multiplexing.

697 Lineage library preparation and sequencing

698 All PCR reactions were conducted using Q5 HotStart Polymerase (Cat # M0494L) and Sybr
699 Green dye. DNA was purified from PCR reactions using Omega TotalPure NGS magnetic
700 beads with 0.8-1.5x ratios based on amplicon size.

701 **Bulk DNA library preparation and sequencing**

702 gDNA was collected from cultured cells, PBMCs, and bone marrow samples using the Qiagen
703 DNeasy Blood and Tissue Kit (Cat# 69504). For library preparation, lineage recorders (including
704 founder tags and clone tags) were first tagged with sequencing UMIs prior to amplification to
705 control for PCR bias, with the exception of libraries constructed from blood collections. Blood
706 collection libraries were treated as sensitive samples, as C1498-FLARE cells were sparse in the
707 blood. As a result, these libraries were amplified without sequencing UMIs and initial PCRs were
708 set up in a PCR hood to prevent contamination. For all libraries, TruSeq/Nextera indices and
709 Illumina adaptors were subsequently attached with PCR. Final sequencing libraries were
710 quantified with the Qubit fluorometer and the NEB NextQuant Library Quant kit (Cat # E7630L).
711 Libraries were then diluted to the appropriate loading concentration for sequencing.

712 **Single cell library preparation and sequencing**

713 Cells were captured for single cell sequencing following 10x guidelines for 3' scRNA-seq with
714 feature barcoding. For multiplexed single cell experiments, cells were first tagged following
715 MULTIseq protocols (Millipore Sigma, LMO001)[66]. Gene expression and MULTIseq libraries
716 were constructed and quantified following 10x and MULTIseq guidelines with one alteration - a
717 portion of cDNA was set aside following initial cDNA amplification but prior to SPRIselect
718 cleanup to be used for FLARE lineage library construction. This was done to avoid splitting
719 FLARE transcripts between GEX and feature barcoding fractions.

720 The previously set aside cDNA was purified using a 2x bead cleanup. For each sample, cDNA
721 was split to generate a library of dynamic lineage recorders (which includes founder tags) and a
722 library of clone tags. All single cell FLARE libraries were initially amplified in 9-16 replicate wells
723 and then combined into 3-4 separate sub-libraries. TruSeq/Nextera indices and Illumina
724 adaptors were subsequently attached with PCR. Final sequencing libraries were quantified with
725 the Qubit fluorometer and the NEB NextQuant Library Quant kit (Cat # E7630L). Libraries were
726 then diluted to the appropriate loading concentration for sequencing.

727 Computational analysis

728 **Sequencing data pre-processing**

729 For bulk DNA sequencing raw data were converted to fastq format using bcl2fastq2 or BCL
730 Convert (Illumina). For single cell RNA sequencing raw data were converted to fastq format
731 using CellRanger mkfastq. Cells were then called using CellRanger count for non-multiplexed
732 10x runs or CellRanger multi for MULTIseq multiplexed 10x runs[67].

733 **Single cell RNA-seq analysis**

734 Cellranger output filtered matrices were analyzed with Seurat 5.1.0[68]. Low quality cells were
735 filtered out based on the number of RNA features and percentage of mitochondrial reads. For
736 non-multiplexed samples doublets were identified and removed using the scDblFinder R
737 package[69]. For multiplexed samples, MULTIseq barcode filtered matrices were added as Assay
738 Objects to Seurat objects. Cells were demultiplexed and doublets were identified and removed
739 with the *MULTIseqDemux()* function. For PBMC and bone marrow samples cell type
740 annotations were added using the SingleR R package with the MouseRNAseqData reference
741 set from the celldex R package[70]. All single cell Seurat objects were normalized with the
742 SCTransform R package for data visualization and integration with other objects[71,72]. Differential

743 gene expression analysis was conducted using the *FindMarkers()* and *FindAllMarkers()* Seurat
744 functions.

745 **Gene set enrichment analysis**

746 C1498-FLARE founder-specific gene set enrichment analysis was conducted with the fGSEA R
747 package[73]. Differentially expressed genes were identified for each founder by Wilcoxon rank
748 sum test and input into the fGSEA function with Mus musculus Hallmark gene sets from
749 mSigDB.

750 **Single cell lineage analysis and tree reconstruction**

751 *Founder and clone tag clustering*

752 To map founder tags to founder tag groups (or founders) and clone tags to clone tag groups (or
753 clones), we used a clustering strategy based on per cell tag set similarity. Briefly, given a
754 mapping of cells to a set of tags, a graph was constructed with cells as nodes and Jaccard
755 similarity as edges. Jaccard similarity was calculated pairwise as the intersection between per
756 cell tag sets divided by the union of per cell tag sets. Any pairs of cells with a Jaccard similarity
757 of 0 (indicating no overlapping tags) shared no edges. Cells were assigned to clusters using the
758 Louvain algorithm for community detection which returned the partition that maximized
759 modularity and with resolution = 1.

760 *Founder assignment*

761 To extract lineage information from scRNA-seq-captured dynamic lineage recorders, we first
762 used SingleCellLineage, as previously described[13,14]. Briefly, sequences are collapsed by 10x
763 UMI and aligned to the reference to obtain a pattern of insertions and deletions across a target
764 array per cell. Founder tags, located immediately upstream of the lineage recorders, were
765 extracted per collapsed merged read. We filtered cell barcodes against the 10x feature
766 barcoding Cell ID whitelist. Merged reads with less than 85% identity to the reference sequence
767 were removed from the dataset. To ensure we were using high quality barcodes to build single-
768 cell lineage trees, we leveraged tools from the Cassiopeia pipeline[74] to perform additional data
769 cleaning. Founder tags were corrected for PCR errors via Starcode[75] with Levenshtein distance
770 = 1. UMIs were error corrected using Cassiopeia function *error_correct_umi()*. Finally, we
771 filtered our error corrected founder tags and target arrays using *filter_molecule_table()* to
772 remove low read count UMIs and cells with too few UMIs. Additionally, this step ensures there is
773 only one target array per 10x cell ID-founder tag combination.
774

775 Founders were assigned by the combination of founder tag integrations in cells. For C1498-
776 FLARE founder populations, founder tag assignments were previously determined via sub-
777 founder culturing and bulk gDNA sequencing. Therefore, if cells had reads mapping to more
778 than one founder based on the founder tags found in its lineage data, it was assigned to a
779 founder based on the total sum of reads and UMIs associated with a certain founder group.
780 Only cells with 90% identity to a certain founder were kept.
781
782 Unlike the C1498-FLARE founders, HL60-FLARE founders had no ground truth mapping of
783 founder tags to founder groups. Subsequently, we took additional steps to ensure we had a
784 confident list of founder tags per cell and group. To remove within-cell background noise, we
785 removed integrations that made up less than 1.5% of reads per cell. This threshold was based
786 on qualitative inspection of the distribution of integration read counts per integration per cell.
787 The founder tags were further filtered by removing tags found in fewer than 5 cells across the
788 dataset. The cleaned set of tags were clustered into founders as described previously (*Founder*
789 *and clone tag clustering*). Finally, to refine the final set of founder tags defining a founder, we
790 filtered for tags with greater than 50% identity to a single founder group. This threshold was
791 based on visual inspection of the distribution of tag identity to founders, determined by the total
792 number of cells with a tag in a founder divided by the total number of cells with a tag. This
793 additionally ensures that each founder tag is found in one founder.

794 *Clone assignment*
795 Clone tags were extracted from sequencing reads using the SingleCellLineage pipeline. For
796 HL60-FLARE clone tag reads, only tags matching the expected 38bp IUPAC pattern were kept.
797 Clone tag reads with more than one missing base within the tag sequence window were
798 removed from the dataset, then clone tags were error corrected using Starcode with
799 Levenshtein distance = 1 for 14 bp tags or Levenshtein distance = 2 for 38 bp tags. These clone
800 tags were then filtered for read count > 1 and number of UMIs > 1. Tags with nucleotide repeats
801 of 5 or longer were also removed (i.e. AAAAA, TTTTT, GGGGG, CCCCC). Background tags,
802 defined as contributing to fewer than 10% of all reads in the cell, were filtered out. Finally, clone
803 tags were discretized to founders by keeping tags with 90% identity (C1498s, 2 founders) or
804 50% identity (HL60s, 15 founders) to a given founder based on the total proportion of reads per
805 clone tags. C1498-FLARE and HL60-FLARE cells were assigned to clones as previously
806 described (*Founder and clone tag clustering*) given the final set of clone tags.

807 *Building Trees*

808 Trees were reconstructed per clone using Cassiopeia's[74] greedy vanilla solver from the pattern
809 of indels per target array. The method was chosen for its scalability given the size of our trees
810 ranges from tens to thousands of cells. Briefly, a tree is built in a top-down fashion by splitting
811 cells into two groups based on the most frequent mutation in the population. Cell subsets are
812 repeatedly split until only one cell remains per subset. Clones belonging to the same founder
813 population were combined into founder trees using a custom python script to attach trees at the
814 root, due to the hierarchical nature of the lineage tracing tag system. All founder trees were
815 combined with the same method to generate a population tree. Cells with unedited/wildtype
816 target arrays were removed from the analysis prior to building the trees.

817 *PATH analysis*
818 To identify genes that were significantly heritable in our clonal population we used the recently
819 published tool, PATH [28]. PATH takes as input a phylogenetic tree along with either discrete
820 annotations like cluster assignments or continuous data like gene expression or module scores.
821 We considered the top 3000 highly variable features per tree to identify genes with strong
822 autocorrelations, suggesting high heritability.

823 *Hotspot module generation*
824 To identify informative gene expression modules based on our single-cell lineage trees and
825 gene expression data, we leveraged the tool Hotspot[34] as described in the following vignette
826 https://hotspot.readthedocs.io/en/latest/Lineage_Tutorial.html. Briefly, we ran Hotspot on our
827 log-normalized data via the 'normal' model filtered for genes in a minimum of 10 cells. Then we
828 generated the KNN graph with 10 neighbors. Autocorrelation scores are calculated per gene to
829 perform feature selection. These genes are then correlated to generate a gene-by-gene matrix,
830 which is used to identify modules using a hierarchical clustering approach with
831 min_gene_threshold = 10 to control the size of modules being joined.

832 *Module overlap analysis*
833 C1498-FLARE module mouse genes were converted to human orthologs for analysis. C1498-
834 FLARE and HL60-FLARE modules were assessed for significant overlap as measured by
835 Jaccard indices and odds ratios compared to genomic background using the GeneOverlap R
836 package[76].

837 **Survival analysis**

838 TARGET-AML RNAseq, clinical, and sample data were accessed through the cBioPortal.
839 Expression datasets were split by sample type where applicable. Sample expression scores for
840 modules and gene sets were calculated by averaging the FPKM or RPKM scores. Cox
841 proportional hazard ratios were generated using the survival R package[77]. To stratify patients for
842 Kaplan-Meier analysis, we first determined the expression score mean and standard deviation
843 within the samples of interest. Patients with scores within one standard deviation of the mean
844 were assigned to the Mid-expression group. Patients with scores above and below one standard
845 deviation of the mean were assigned to the High and Low expression groups, respectively. We
846 excluded patient samples missing information for tissue type.

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865 Data availability

866 All sequencing data associated with this publication have been deposited in NCBI's Gene
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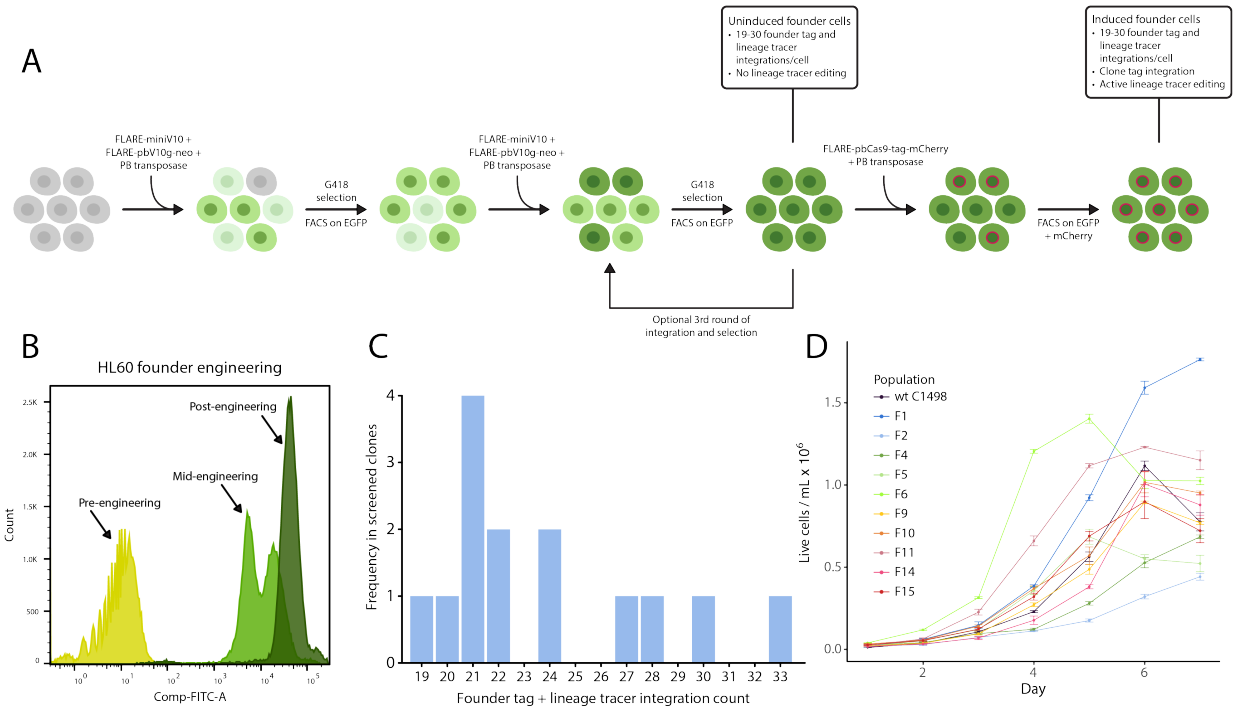
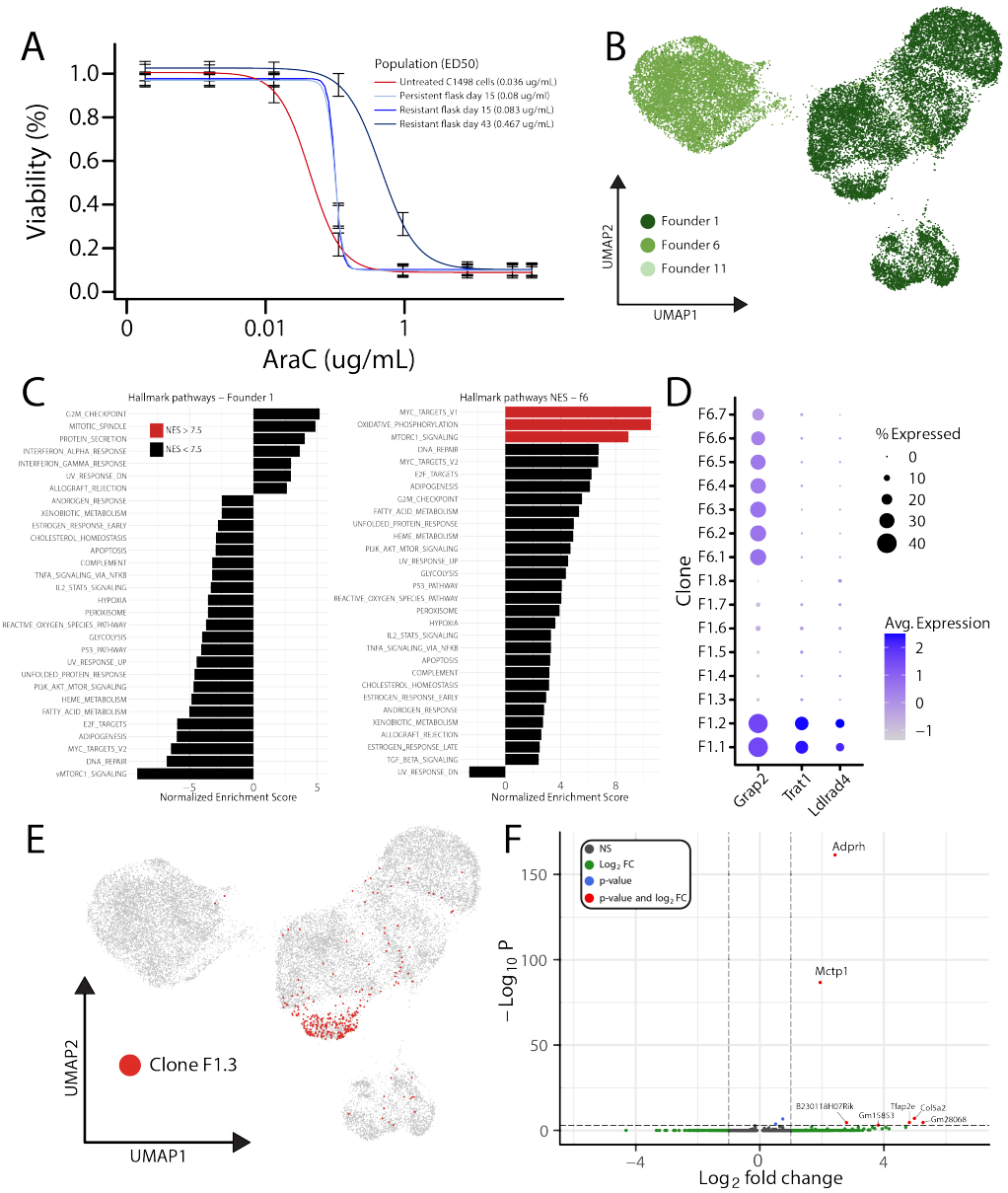
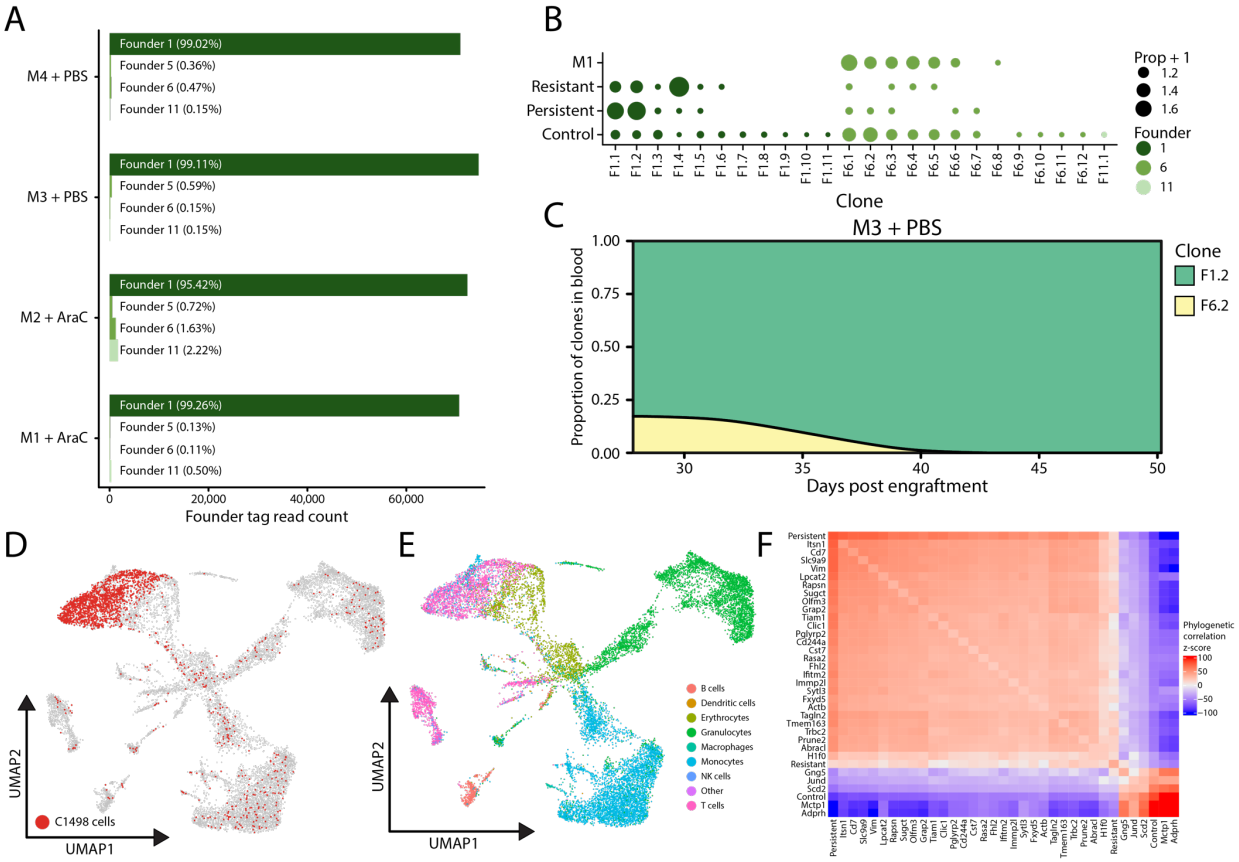


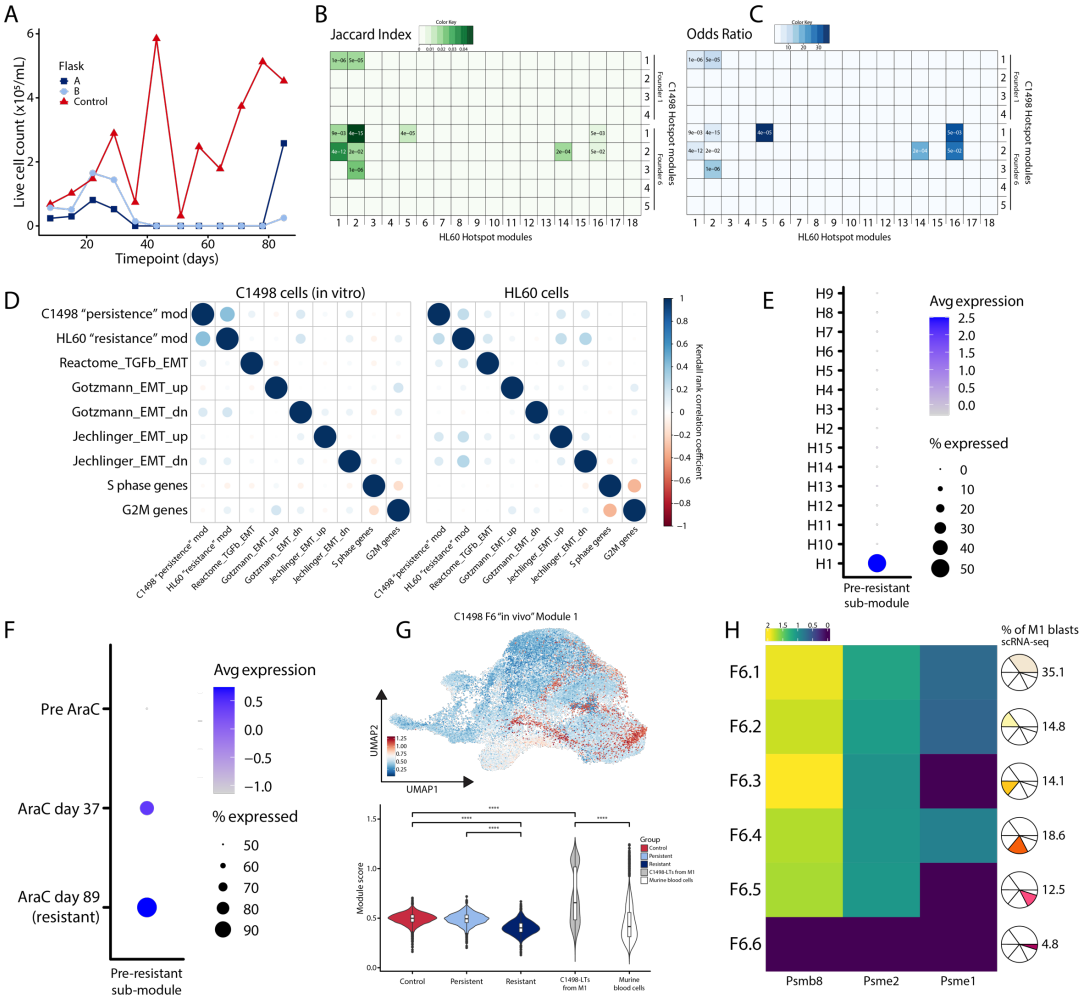
Supplemental figure 1: FLARE cell line engineering allows for the generation of founder populations maintaining intra-founder heterogeneity. (**A**) Schematic of engineering lineage tracing AML cells. Wildtype cells are first electroporated with FLARE-miniV10-10X-UMI + FLARE-pbV10g-neo + PB-transposase. Successfully integrated cells are selected via G418 treatment (FLARE-pbV10g-neo) and FACS for EGFP (FLARE-miniV10-10X-UMI). This process is repeated 1-2 more times, successively selecting for cells with high numbers of integrations. Once the desired number of integrations is reached, cells are considered uninduced founder cells. Prior to the start of a lineage tracing experiment, uninduced founder cells are electroporated with FLARE-pbCas9-tag-mCherry and PB-transposase and sorted on EGFP (FLARE-miniV10-10X-UMI) and mCherry (FLARE-pbCas9-tag-mCherry). Resulting cells are referred to as induced founder cells and contain all the necessary integrations for founder and clonal tracking. (**B**) FACS plots showing the increase in EGFP expression in HL60 cells through 2 rounds of electroporation with FLARE-miniV10-10X-UMI + FLARE-pbV10g-neo + PB transposase. Post-engineering uninduced HL60-FLARE founder cells uniformly express high levels of EGFP. (**C**) Uninduced C1498-FLARE founder populations were subcultured to determine founder tag and lineage recorder integration counts. Founders contained between 19 and 33 integrations. (**D**) Subcultured founder populations display a range of growth rates and carrying capacities, indicating sustained heterogeneity following the engineering process.



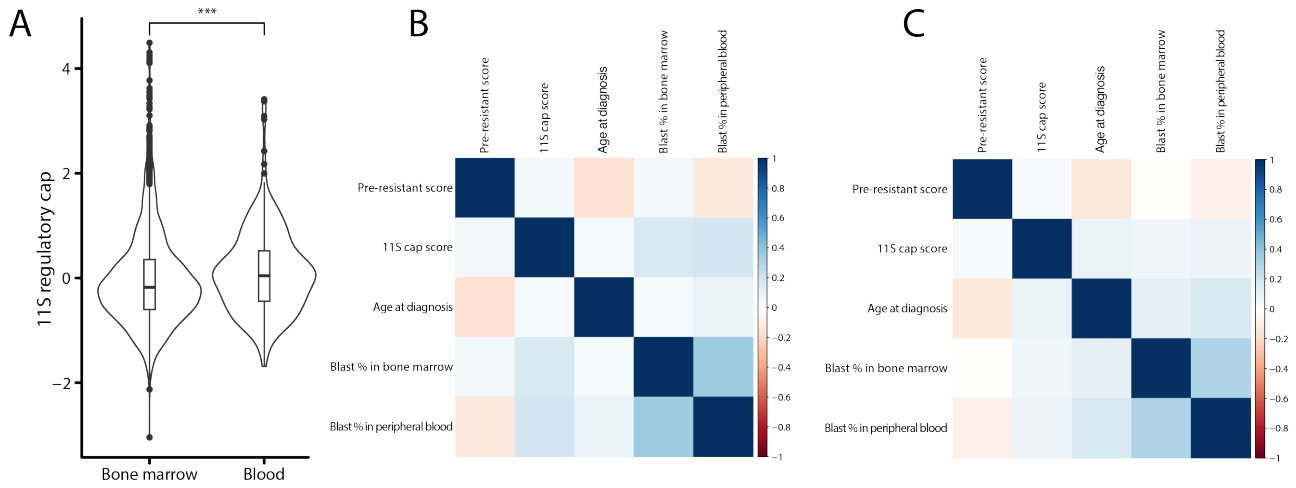
Supplemental figure 2: AraC treatment of C1498-FLARE cells in vitro leads to the generation of AraC-insensitive populations. FLARE recorder analysis reveals founder and clone-level transcriptomic differences. (**A**) AraC dose-response curves for C1498-FLARE cells through in vitro treatment. Cells in the resistant flask displayed an increase in ED50 values throughout treatment, indicating the emergence of an AraC-resistant population. Cells in the persistent flask also displayed an increase in ED50 values on day 15 of AraC treatment, but did not recover enough for another dose-response curve on day 43, suggesting higher AraC sensitivity compared to the resistant flask. (**B**) Captured C1498-FLARE cells colored by founder population. (**C**) GSEA analysis on founder 1 and founder 6 control cells to identify basal founder-specific differences. Analysis was conducted using the fGSEA R package and Hallmark gene sets from the mSigDB. (**D**) Dot plot showing expression of Grap2, Trat1, and Ldlrad4 in C1498-FLARE clone populations. (**E**) UMAP of C1498-FLARE cells with clone F1.3 cells highlighted in red. (**F**) Differential gene expression analysis on clone F1.3 cells compared to all founder 1 control cells uncovers clone-specific increased expression of Adprh and Mctp1.



Supplemental figure 3: AraC treatment of C1498-FLARE cells in vivo leads to outgrowth of founder 6 cells. (**A**) Founder tag sequencing of blood draws taken 28 days after mice were injected IV with C1498-FLARE cells. (**B**) Clone populations for each sample, including in vivo sequencing from M1, first ordered by founder then by clone size. (**C**) Bulk sequencing of clone tags detected in blood draws from mouse M3. Clone tag reads were used to determine the clonal proportions of leukemic burden in the blood from day 30 to euthanasia on day 50. Founder proportions differ from figure 2E due to subsetting for clone tags detected in single cell data. (**D**) Captured single cells from endpoint blood and bone marrow collected from mouse M1. (**E**) Captured single cells from endpoint blood and bone marrow collected from mouse M1. Predicted C1498-FLARE cells were defined as having sufficient founder and clone tag reads to assign lineage identities, highlighted in red. (**F**) Cross-correlation heatmap of the top 35 auto-correlated genes over the Founder 1 tree.



Supplemental figure 4: AraC treatment of HL60-FLARE cells in vitro leads to the generation of chemoresistance. Resistance-associated heritable Hotspot modules in C1498-FLARE and HL60-FLARE cells suggest the existence of drug response pathways common to murine and human AML cells. (**A**) Live cell counts for HL60-FLARE cells undergoing in vitro AraC treatment. Each flask was counted in duplicates at each timepoint, and counts were averaged. Control flask counts vary due to culture splitting in the days prior to counting. Treated flasks A and B did not have enough cells to be detected between day 40 and 80 and were not split during that time frame. (**B**) Jaccard Index similarity comparing C1498-FLARE and HL60-FLARE Hotspot modules. Square color is the Jaccard Index; values indicate BH-corrected P-values. (**C**) The odds ratio of gene overlaps comparing C1498-FLARE and HL60-FLARE Hotspot modules. Square color determined by odds ratio; values indicate BH-corrected P-values. (**D**) Expression scores for C1498 "persistence" Founder 1 module 1, HL60 "resistance" module 2, various EMT gene sets, and cell cycle gene sets are minimally correlated as measured by Kendall ranked correlation coefficient in C1498-FLARE and HL60-FLARE cells in vitro. (**E**) Dot plot showing expression of the pre-resistant sub-module in pre-experiment HL60-FLARE cells by founder. Founder H1 shows distinct upregulation of the signature. (**F**) Dot plot showing expression of the pre-resistant sub-module in AraC-treated HL60-FLARE H1 cells pre-experiment, at midpoint, and at endpoint. Pre-resistant signature expression increases through AraC treatment. (**G**) UMAP of founder 6 module 1 expression in the in vitro C1498-FLARE cells integrated with M1 endpoint sequencing. Below is expression quantification by cell group and shows module expression enrichment in cells collected from M1. (**H**) Log2 fold change values of Psmb8, Psme1, Psme2 genes for C1498-FLARE founder 6 clones comparing in vitro to in vivo clone cells.



Supplemental figure 5: Expression of the 11S cap and pre-resistant signature are not strongly correlated with bone marrow or peripheral blood blast percentage at diagnosis. (**A**) Violin plot showing 11S regulatory cap gene set scores calculated from TARGET-AML patient samples taken at diagnosis. Mean expression in blood is significantly higher than in bone marrow. (**B**) TARGET-AML expression scores for 11S regulatory cap and pre-resistant signature in the bone marrow at diagnosis, age at diagnosis, and percentage of leukemic blasts in bone marrow and peripheral blood are minimally correlated as measured by Kendall ranked correlation coefficient. (**C**) TARGET-AML expression scores for 11S regulatory cap and pre-resistant signature in the peripheral blood at diagnosis, age at diagnosis, and percentage of leukemic blasts in bone marrow and peripheral blood are minimally correlated as measured by Kendall ranked correlation coefficient.