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# Supplementary information

# RAS-mutant leukaemia stem cells drive clinical resistance to venetoclax

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# RAS-mutant leukaemia stem cells drive clinical resistance to venetoclax

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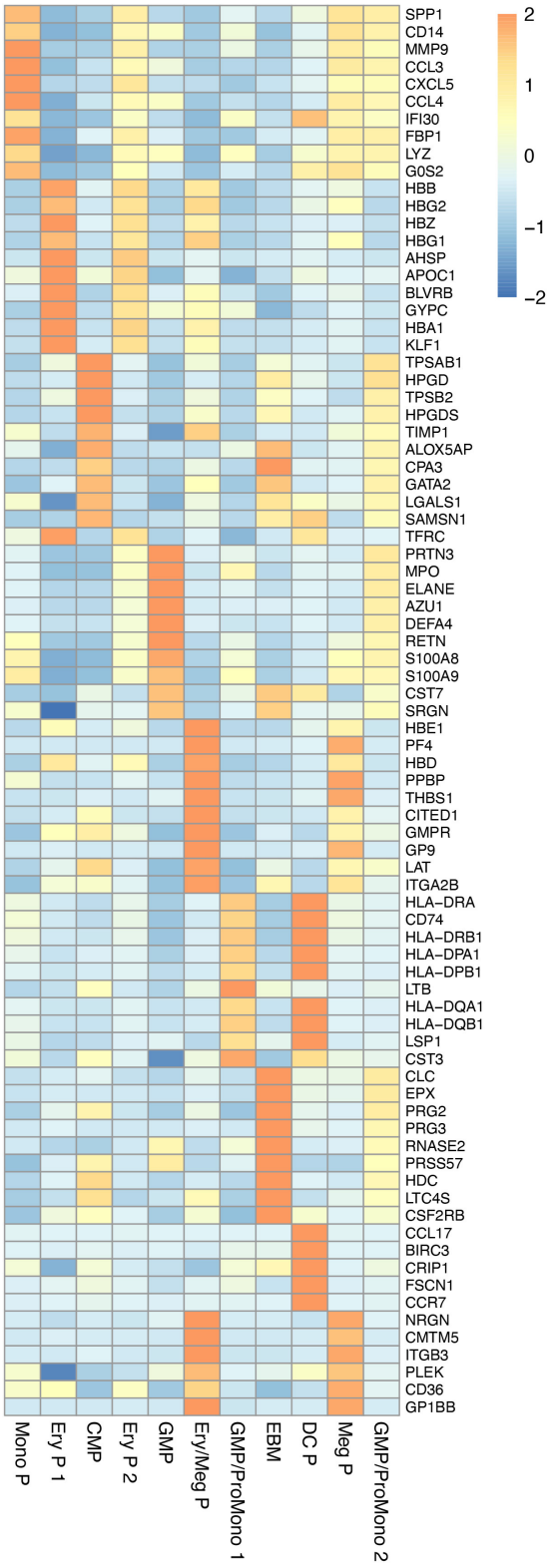
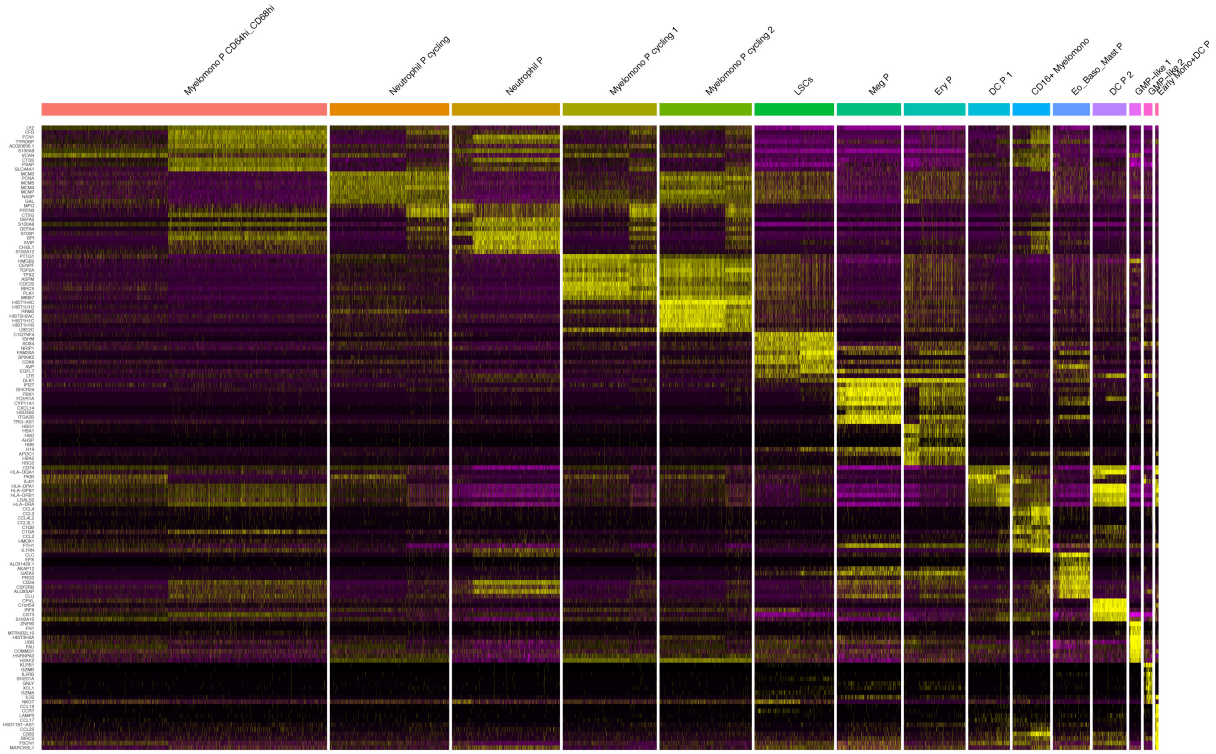
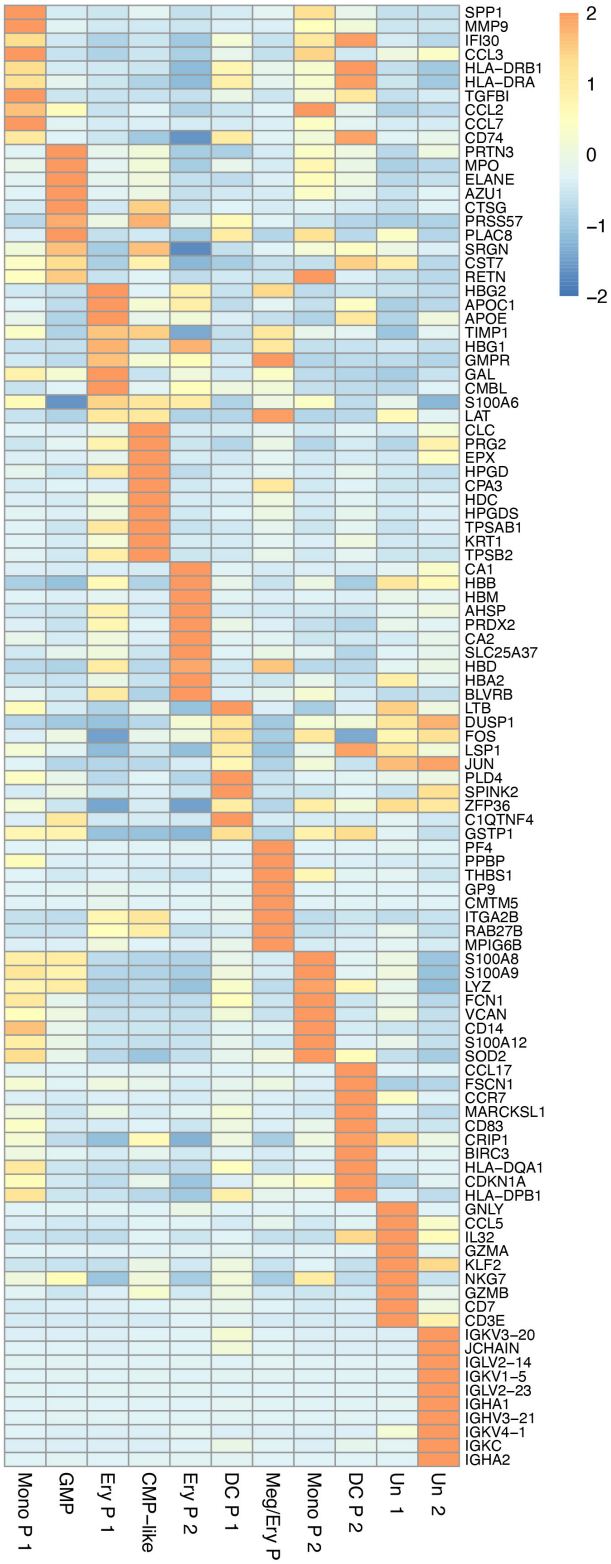



**Supplementary Fig. 1.** Heatmap showing the top 10 differentially expressed genes for each cluster (aggregated cell expression) of the single-cell transcriptome data shown in Fig. 2b in UMAP representation.




**Supplementary Fig. 2.** Heatmap showing the top 10 differentially expressed genes for each cluster of the single-cell transcriptome data shown in Fig. 3b and Extended Data Fig. 7a in UMAP representation.




**Supplementary Fig. 3.** Heatmap showing the top 10 differentially expressed genes for each cluster (aggregated cell expression) of the single-cell transcriptome data shown in Fig. 5b in UMAP representation.

**Supplementary Fig. 4.** Uncropped Western blots. Samples were run on separate gels.

Fig. 5g AML-47.1

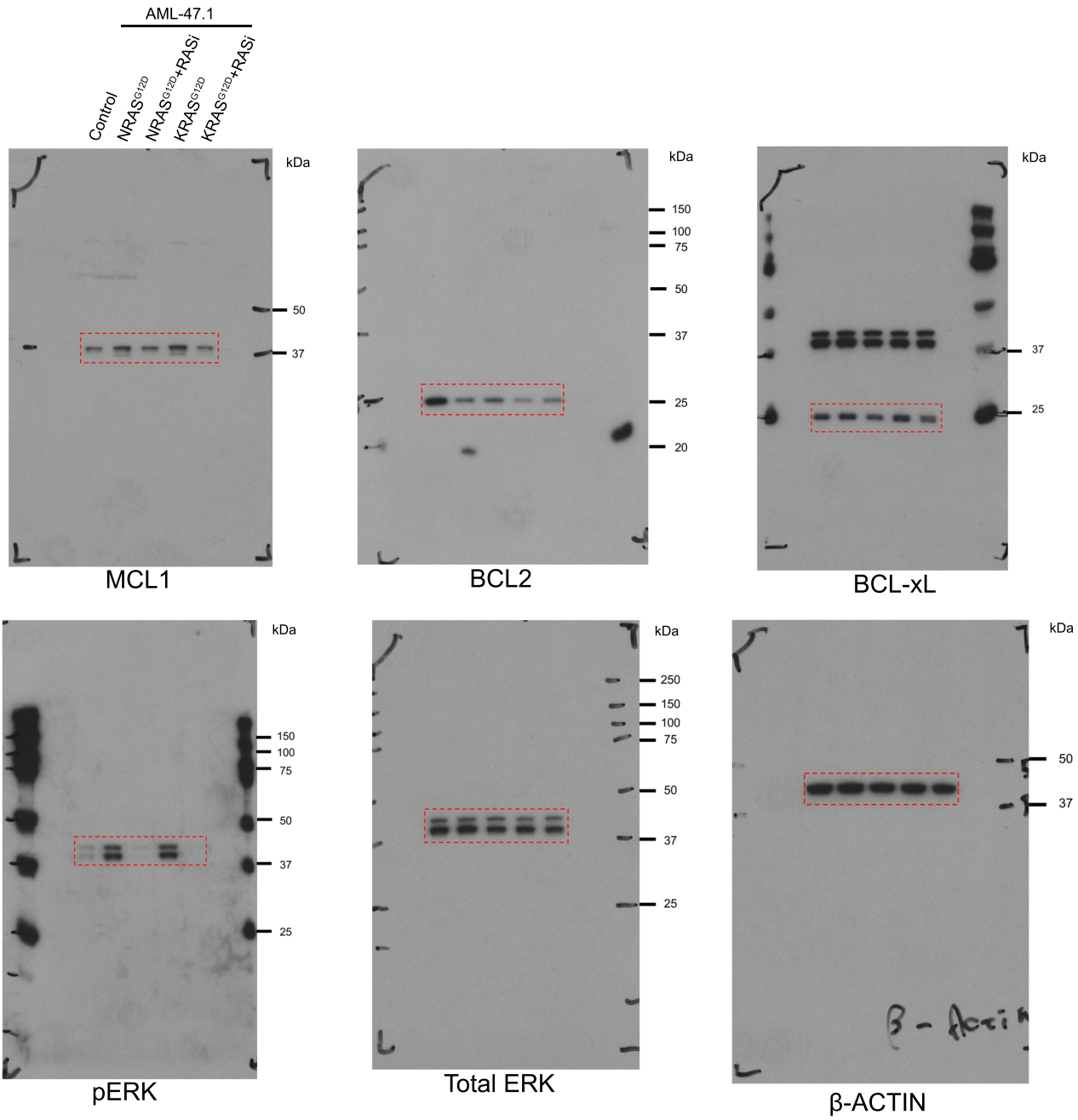
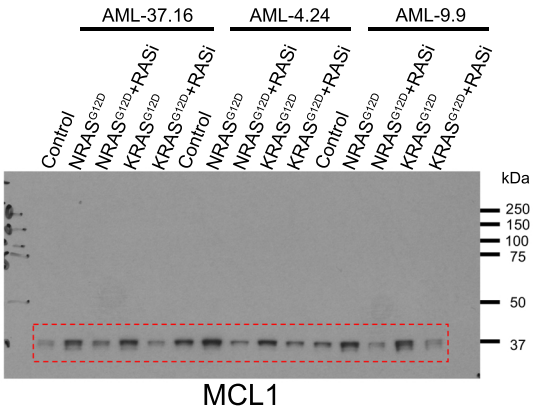
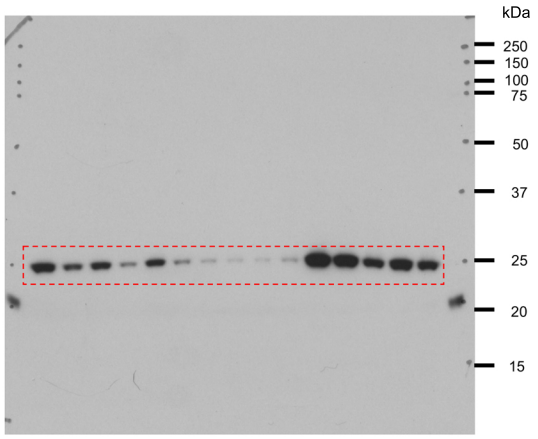


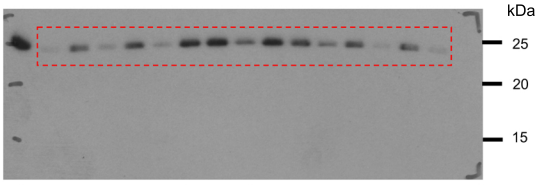
Fig. 5g AML-37.16, AML-4.24, AML-9.9



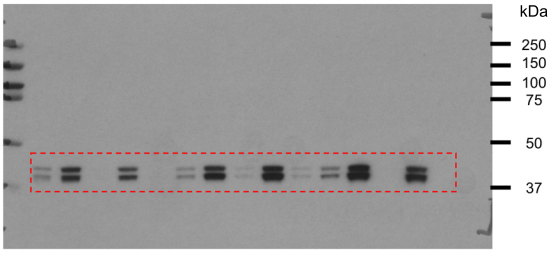
MCL1



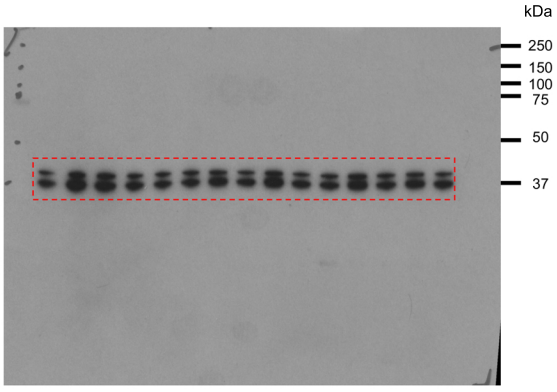
BCL2



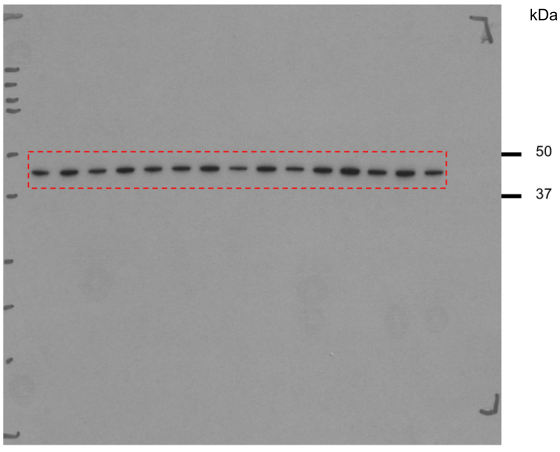
BCL-xL



pERK

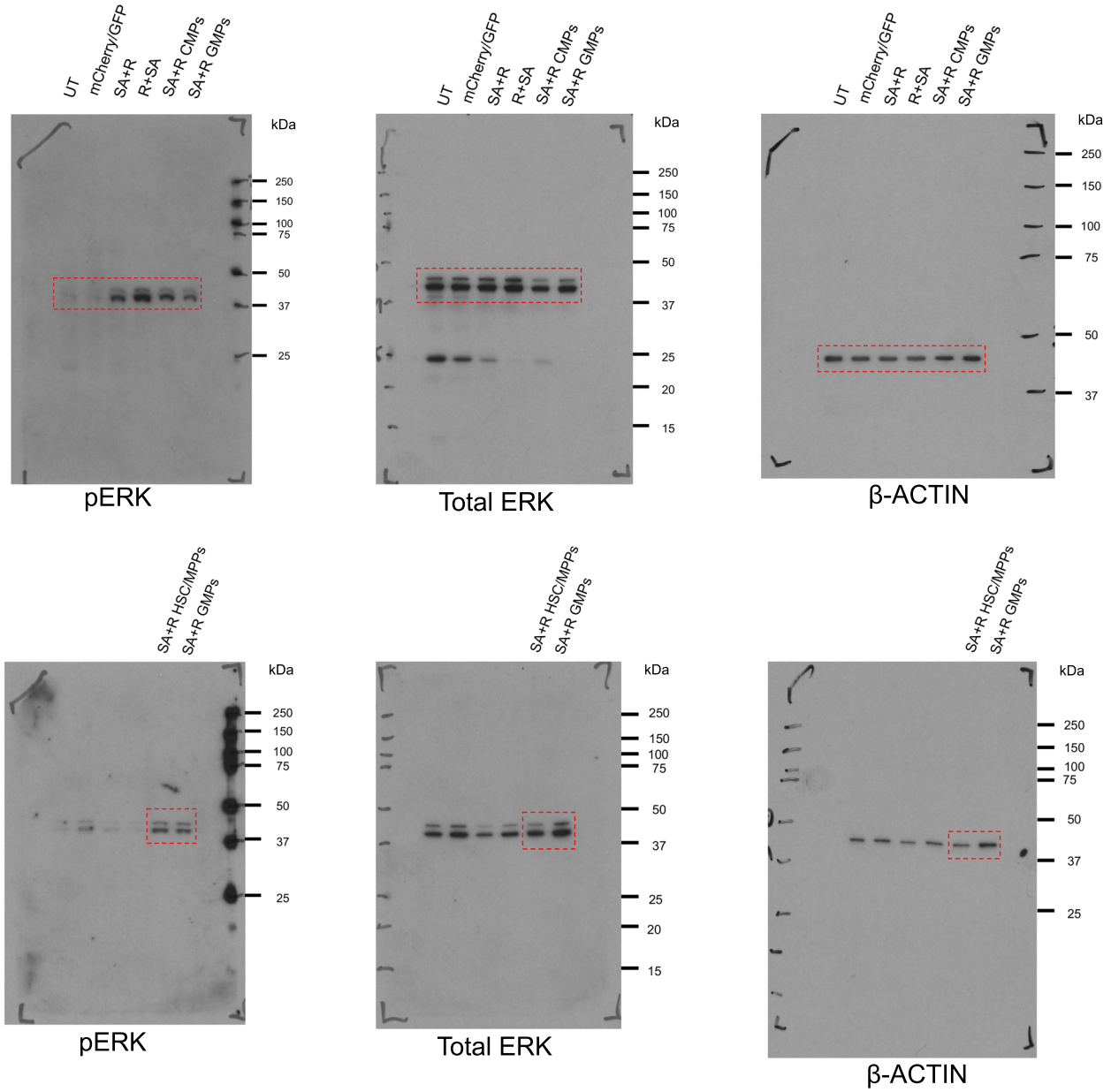


Total ERK



β-ACTIN

Extended Data Fig. 6b



| | Model | Figures in manuscript | Leukemic features | | | |
|---|---|---|---|---|---|---|
| | | | Required | | Additional | |
| | | | Engraftment of myeloid-restricted immature human cells | Engrafted cells harbor AML driver mutations | Engraftment is initiated by an HSPC population that should not normally show durable engraftment | Lethal disease |
| #1 | **CD34+ HSPCs derived from genetically engineered iPSCs** | Fig. 1a, b and Extended Data Fig. 1 and 2 | Up to ~10% hCD45+ BM engraftment 13-15 weeks post-transplantation of exclusively CD33+ cells with immature (blast) morphology (Fig. 1b, Extended Data Fig. 1h-j, Extended Data Fig. 2e, i and previously reported in PMID: 33571445) | HSPCs derived from clonal triple mutant (SAR) iPSC lines (Extended Data Fig. 1a, Extended Data Fig. 2c, d) and/or flow cytometric demontration of transduction with mutant transgenes (Extended Data Fig. 2i) | Yes (HSPCs derived from genetically unmodified normal iPSCs through the in vitro differentiation protocol used in the study are not engraftable.) | No |
| #2 | **CB CD34+ HSPCs after extended in vitro culture** | Fig. 1c-h, 2a-c, 4g-i and Extended Data Fig. 3 | 20-65% engraftment 6-8 weeks post-transplantation (end point determined by lethality) of CD33+ immature blasts (Fig. 1d-h and Extended Data Fig. 3b, d-f) | Triple-transduced SA+R (Extended Data Fig. 3a,c) | Yes (CB HSPCs are minimally engraftable under the experimental conditions used in the study, i.e. 11 days of in vitro culture in standard serum-free media.) | Yes (Fig. 1e, 4h and Extended Data Fig. 3e) |
| #3 | **Sorted CMPs and GMPs from CB CD34+ cells** | Fig. 2g-i and Extended Data Fig. 5 | Up to 50% engraftment 6-13 weeks post-transplantation (end point determined by lethality) of CD33+ immature blasts (Extended Data Fig. 5f, i-k) and secondary engraftment (Extended Data Fig. 5g, h) | Triple-transduced SA+R or SAR (Extended Data Fig. 5d, e, l) | Yes (Sorted GMPs and CMPs are committed progenitors with limited short-term engraftment potential. See also Extended Data Fig. 5i,j) | Yes (Fig. 2g, h) |
| #4 | **CD34+ HSPCs from patient-derived AML-iPSC lines** (AML-4.24, AML-4.10, AML-47.1, AML-37.16, AML-9.9) | Figs 3a-d, 4e,f 5f,g and Extended Data Fig. 7a,e, 8c-f, 9a-e and 10g | Up to 99% engraftment 3-16 weeks post-transplantation of CD33+ immature blasts (Fig. 3a-d, Extended Data Fig. 7a and previously reported in PMID: 28215825, PMID: 32492433 and PMID: 37067914) | HSPCs derived from clonal iPSC lines with AML driver mutations (PMID: 28215825, PMID: 32492433 and PMID: 37067914) | Yes (HSPCs derived from genetically unmodified normal iPSCs through the in vitro differentiation protocol used in the study are not engraftable.) | Yes (PMID: 28215825, PMID: 32492433 and PMID: 37067914) |

**Supplementary Table 1. Summary of in vivo leukemic features of all models used in this study.**

**a. Monocytic/Non-monocytic AML cohort**

| Patient characteristics | Monocytic AML (N=31) | Non-monocytic AML (N=87) | p |
|---|---|---|---|
| Age, years | 72 (64-85) | 72 (49-89) | .319 |
| Male sex | 15 (48) | 49 (56) | .446 |
| ECOG PS ≥2 | 12 (39) | 20 (23) | .091 |
| PB blasts, % | 3 (0-59) | 11 (0-91) | .012 |
| BM blasts, % | 40 (8-83) | 36 (2-90) | .195 |
| Diagnosis | | | |
| De novo AML | 17 (55) | 40 (46) | .397 |
| sAML with AHD | 8 (26) | 36 (41) | .124 |
| Untreated AHD | 5 (16) | 11 (13) | |
| Therapy for AHD | 3 (10) | 25 (29) | .089 |
| t-AML | 6 (19) | 15 (17) | .069 |
| FAB type | | | |
| M0 | | 11 (13) | |
| M1 | | 18 (21) | |
| M2 | | 31 (36) | |
| M4 | 18 (58) | | |
| M5 | 8 (26) | | NA |
| M6 | | 3 (3) | |
| M7 | | 2 (2) | |
| RAEB-T | 5 (16) | 22 (25) | |
| ELN 2017 CG risk | | | |
| Favorable | 0 (0) | 0 (0) | |
| Intermediate | 19 (61) | 41 (47) | |
| Adverse | 11 (35) | 46 (53) | .291 |
| Complex CG | 8 (26) | 36 (41) | |
| Mutations | | | |
| *NPM1* | 14 (45) | 14 (16) | .001 |
| *FLT3-ITD/TKD* | 8 (26) | 10 (11) | .057 |
| *IDH1/2* | 8 (26) | 16 (18) | .378 |
| *TP53* | 6 (19) | 30 (34) | .116 |
| *RUNX1* | 6 (19) | 15 (17) | .792 |
| *ASXL1* | 4 (13) | 16 (18) | .484 |
| *K/NRAS* | 6 (19) | 20 (23) | .675 |
| ELN 2017 risk group | | | |
| Favorable | 12 (39) | 14 (16) | |
| Intermediate | 4 (13) | 12 (14) | .030 |
| Adverse | 15 (48) | 61 (70) | |

**b. Extended cohort**

| Patient characteristics | N (%) |
|---|---|
| Age, years | 72 (49-89) |
| Male sex | 78 (57) |
| ECOG PS ≥2 | 43 (31) |
| PB blasts, % | 8 (0-91) |
| BM blasts, % | 24 (20-81) |
| Diagnosis | |
| De novo AML | 63 (46) |
| sAML with AHD | 51 (37) |
| Untreated AHD | 20 (15) |
| Therapy for AHD | 31 (23) |
| t-AML | 23 (17) |
| ELN 2022 CG risk | |
| Favorable | 0 (0) |
| Intermediate | 74 (54) |
| Adverse | 63 (46) |
| Mutations | |
| *NPM1* | 29 (21) |
| *FLT3-ITD/TKD* | 11 (8) |
| *IDH1/2* | 29 (21) |
| *TP53* | 42 (31) |
| *RUNX1* | 23 (17) |
| *ASXL1* | 29 (21) |
| *K/NRAS* | 31 (23) |
| ELN 2022 risk group | |
| Favorable | 11 (8) |
| Intermediate | 17 (12) |
| Adverse | 109 (80) |

All results expressed as number (%) or median (range). ECOG PS: Eastern Cooperative Oncology Group performance status; ELN: European LeukemiaNet; AML: acute myeloid leukemia; PB: peripheral blood; BM: bone marrow; sAML: secondary AML; AHD: antecedent hematologic disorder; AML; t-AML: therapy-related AML; FAB: French American British; RAEB-T: refractory anemia with excess blasts in transformation; CG: cytogenetics

**Supplementary Table 5.** Baseline characteristics of patients with newly diagnosed monocytic and non-monocytic AML, shown in Fig. 4a,b, treated with 10-day DEC and VEN (NCT03404193) (a) and of an extended cohort including the same patients and additional 19 patients enrolled since prior data cut-off with longer follow-up, shown in Fig. 4c,d and Extended Data Fig. 8a,b. (Information on monocytic differentiation status is not available for the additional patients enrolled after the prior data cut-off.)

| Outcomes | Monocytic AML (N=31) | Non-monocytic AML (N=87) | p | Extended cohort (N=137) |
|---|---|---|---|---|
| Overall response rate | 27 (87) | 70 (80) | .407 | 110 (80) |
| CR | 16 (52) | 43 (49) | .650 | 74 (54) |
| CRi | 8 (26) | 16 (18) | | 22 (16) |
| MLFS | 3 (10) | 11 (13) | | 14 (10) |
| No response | 3 (10) | 16 (18) | | 23 (17) |
| Inevaluable / Aplasia | 1 (3) | 1 (1) | | 4 (3) |
| MRD negative by FCM | 16/27 (59) | 36/67 (54) | .626 | 63 (46) |
| Relapse | 10/27 (37) | 29/70 (41) | .693 | 56 (41) |
| 30-day mortality | 0 (0) | 2 (2) | 1.00 | 5 (4) |
| 60-day mortality | 3 (10) | 9 (10) | .916 | 18 (13) |
| No of cycles to response | 1 (1-4) | 1 (1-4) | .535 | 1 (1-5) |
| SCT after response | 3 (10) | 13 (15) | .375 | 24 (18) |

All results expressed as number (%) or median (range). CR=complete remission, CRi = CR with incomplete hematologic recovery; MLFS: morphologic leukemia-free state; MRD = measurable residual disease; FCM = flow cytometry; SCT = allogeneic stem cell transplantation

**Supplementary Table 6.** Outcomes of patients with newly diagnosed AML treated with 10-day DEC and VEN.

**Supplementary Discussion (**Extended discussion)

Here we provide evidence that the LSCs sustaining RAS-MT subclones in AML originate from GMPs. Previous mouse modeling studies have shown that committed progenitors can be or need to be the target cells of transformation in AML[1,2]. Previous studies have also shown the existence of GMP-like LSCs in human AML[3]. While our studies do not exclude that RAS mutations may also originate in a more primitive HSC/MPP that can give rise to GMP LSCs in some AML cases, they firmly establish that GMPs can be the target cell from which RAS-MT LSCs originate. We thus demonstrate that the subclonal RAS-mutated AML LSC can emerge from a different and more mature cell type than the cell-of-origin of the major AML clone, which in most cases is a primitive HSC/MPP (Extended Data Fig. 10h and Extended Data Fig. 11a). Importantly, this has far-reaching implications in the clinic.

Our data strongly point to monocytic differentiation and VEN resistance being two independent effects with a common cause, RAS mutations. This can explain the observed associations between monocytic AML and VEN resistance in some studies but not others. In addition, we show here that leukemic transformation by RAS mutations is dependent on the cellular milieu and chromatin landscape, whereas VEN resistance is conferred more broadly in all HSPC types by RAS mutations, again pointing to different mechanistic underpinnings of these processes.

Our data thus shed new light on recent observations related to clinical responses to VEN in AML. While we confirm that mature monocytes are resistant to VEN, in agreement with previous findings[4], we show that this resistance at the cellular level has no impact on the clinical outcome, which is instead determined by the response of LSCs (Extended Data Fig. 11a,b). This is in line with a large body of evidence showing that the targeting of LSCs is essential to achieving lasting therapeutic responses in AML, while the sensitivity or lack thereof of the more mature AML cells to therapeutic agents is not consequential to the long-term clinical outcome[5-7]. In addition, in view of our data, the monocytic subclones that have been observed to outgrow and be selected upon VEN treatment in patients are likely to, at least in many of the cases, correspond to RAS-MT subclones[4,8,9]. Since RAS-MT AML subclones contain a higher fraction of monocytic cells than antecedent RAS-WT clones, selection of the RAS-MT subclone at the LSC level can give the appearance of an expansion of cells with monocytic features upon relapse or resistance, with the latter being an epiphenomenon and not causative to the relapse or resistance (Extended Data Fig. 11c). Recently identified “monocytic LSCs” shown to generate monocytic AML, to have a distinct transcriptome and to downregulate BCL2, may also conceivably correspond, at least in a fraction of the cases, to RAS-MT LSCs[4,9]. The association of monocytic features with poor outcomes after VEN therapy in some cohorts[4,8] vs lack of association in others[10,11], including the one we present here, may be explained by variable degrees of enrichment of the respective monocytic cohorts in AML cases with RAS mutations. Thus our findings can synthesize and reconcile previous seemingly contradictory observations into a coherent model.

Our findings have important implications for clinical practice. The resistance of *N/KRAS*-MT LSCs to VEN implies that combination therapy with VEN may have limited benefit for patients with preexisting *N/KRAS* mutations and may even accelerate disease progression by promoting the growth of the *N/KRAS*-MT subclone (Fig. 4g-i and Extended Data Fig. 11c). Consistent with this, we recently reported rapid selection of RAS-MT subclones in AML patients treated with VEN[12]. Our findings also provide strong rationale for combining VEN with MCL1 inhibitors and potentially BCL-xL inhibitors, as frontline therapy in patients with detectable RAS mutations or all eligible patients[13,14]. However, the development of MCL1 and BCL-xL inhibitors has been hampered by on-target dose-limiting toxicities.

The emergence of VEN resistance has also been associated with other progression mutations in AML, such as FLT3 and TP53 mutations[15,16]. FLT3-ITD has been shown to induce higher expression of MCL1 and BCL-xL[17,18]. It would be interesting to investigate whether LSCs harboring other progression mutations are, similarly to RAS-MT LSCs, resistant to VEN and have altered BCL2 family expression profiles. Consistent with our proposition that VEN resistance and monocytic differentiation are independent processes, FLT3-ITD mutations are not associated with monocytic differentiation, and, on the contrary, appear to give rise to more primitive leukemic blasts (Extended Data Fig. 7k)[19-21].

The malignant cells in AML are the product of two orthogonal processes: one that stems from the clonal evolution of genetic clones and subclones through the sequential acquisition of driver genetic lesions; and one that arises from a differentiation hierarchy within each genetic clone and subclone wherein AML LSCs give rise to more differentiated progeny[22]. Our study shows how these intersect and impact each other, specifically how a specific cell differentiation state along the myeloid lineage is selected by mutant RAS as the target cell of transformation and how this, in turn, changes the hierarchical structure of the resulting leukemia to one more biased towards mature monocytic output. It has long been debated whether the phenotype of leukemic blasts is determined by the degree of differentiation of the LSC cell-of-origin or, alternatively, by the transforming event and its effects on the developmental program of the LSCs[23]. Our results propose a new paradigm, whereby the oncogenic event (i.e. RAS mutation) selects for a specific differentiation state of a progenitor cell (i.e. a GMP) that is the target cell of transformation, with the resulting blast phenotype (i.e. monocytic differentiation) being the result of the interaction between both the target cell type and the mutational event. Furthermore, importantly, we show that this interaction between the genetic and developmental AML hierarchy determines not only the phenotype, but also critical properties of the disease with far-reaching implications for its treatment. In view of our findings, more such dependencies between genetic and non-genetic determinants of AML pathogenesis are likely to exist that await discovery and can potentially inform clinical practice.

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