Identification of genetic targets in acute myeloid leukaemia for designing targeted therapy

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Received 29 June 2020; accepted for publication 4 September 2020

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Summary

Few effective therapies exist for acute myeloid leukaemia (AML), in part due to the molecular heterogeneity of this disease. We sought to identify genes crucial to deregulated AML signal transduction pathways which, if inhibited, could effectively eradicate leukaemia stem cells. Due to difficulties in screening primary cells, most previous studies have performed next-generation sequencing (NGS) library knockdown screens in cell lines. Using carefully considered methods including evaluation at multiple timepoints to ensure equitable gene knockdown, we employed a large NGS short hairpin RNA (shRNA) knockdown screen of nearly 5,000 genes in primary AML cells from six patients to identify genes that are crucial for leukaemic survival. Across various levels of stringency, genome-wide bioinformatic analysis identified a gene in the NOX family, NOX1, to have the most consistent knockdown effectiveness in primary cells (\( P = 5.39 \times 10^{-5} \), Bonferroni-adjusted), impacting leukaemia cell survival as the top-ranked gene for two of the six AML patients and also showing high effectiveness in three of the other four patients. Further investigation of this pathway highlighted NOX2 as the member of the NOX family with clear knockdown efficacy. We conclude that genes in the NOX family are enticing candidates for therapeutic development in AML.

The response to treatment and overall survival of patients with acute myeloid leukaemia (AML) is heterogeneous but remains suboptimal in many disease subtypes. Recent advances in molecular genetic technologies have accelerated insights into some of the critical genetic changes contributing to a leukaemic phenotype. These have included the observation that each leukaemia patient may present with a wide range of mutations across multiple genes — resulting in a new challenge of distinguishing driver from passenger mutations.\(^1,2\) Furthermore, as the development of myeloid leukaemia correlates with ageing, which in itself corresponds with the accumulation of clonal mutations,\(^3,4\) the need to define clonal hierarchy deepens the challenge of target identification. Thus, somatic mutation data may provide too many potential drug targets to be of practical use in individual patient management without corresponding functional analyses.

In theory, genome-wide functional targeting allows for identification of a gene or genes whose activity is essential for the survival of a particular patient’s malignancy\(^5\) regardless of whether the genes themselves are mutated or not. This approach would identify any critical gene deregulated due to, for example, the mutation of a gene upstream in its own signalling pathway, crosstalk from a different pathway or epigenetic mechanisms\(^6\). Previously, we applied short hairpin RNA (shRNA) library screening to detect signalling pathways involved in resistance to tyrosine kinase inhibitors in chronic myeloid leukaemia identifying nucleocytoplasmic transport as a mediator of \( BCR-ABL1 \) kinase-independent resistance, and...
thus a potential therapeutic target. In this study, we innovatively apply a large pooled shRNA library screen to identify genes essential for the survival of primary AML cells (in contrast to most previous shRNA screens performed on cell lines), with the aim of identifying the main pro-survival genes for each AML patient whose singular inhibition would result in a leukaemia-suppressive effect.

Materials and methods

Sample preparation

Samples were assessed from six newly diagnosed AML patients. Samples were collected with Imperial College London Tissue Bank ethical approval. To enrich for leukaemia blast cells, mononuclear cells were separated using lymphoprep (STEMCELL Technologies, Vancouver, BC, Canada).

Pooled shRNA library and cell culturing

The Decipher Human Module 1 shRNA library (Cellecta, Mountain View, CA, USA) targeting 4,974 reliable genes (5,043 total targets) with 27,500 shRNA was used for the screen. AML cells were cultured for 24–48 h in RF10 supplemented with StemSpan CC100 (STEMCELL Technologies) followed by transduction with the pooled shRNA library. The experiment was optimised so that the final number of infected cells was at least 200 to 1,000 times the complexity of the shRNA library. Human bone marrow-derived mesenchymal stromal cells (HS-5) were cultured in six-well plates with 3 × 10⁵ cells per well to prevent possible overgrowth during the co-culture (six days). Twenty-four hours later the transduced AML cells (72 h after transduction) were added to each well in a total volume of 2 ml (10⁶ AML cells per ml). Cells from the six AML patients were co-cultured for six days (nine days from transduction to the end of co-culture); for AML#14, separate aliquots of cells were also co-cultured for three and nine days (six and 12 days from transduction to end of co-culture). To purify the transduced AML cells, puromycin was added during the selection period at 2 µg/ml.

DNA extraction, shRNA and statistical analyses

Cell pellets collected at baseline and after the selection period were lysed for DNA extraction as previously described. DNA was used for amplification of the barcodes using LNSG-101, NGS Prep Kit for shRNA Libraries in pRSI12 as recommended by the manufacturer. Samples were randomised prior to sequencing on a NextSeq 500 (Illumina, San Diego, CA, USA) to avoid potential biases associated with variable sequencing depths and other NGS-related artefacts. Bioinformatics analyses were performed as previously described.

We determined a statistical calculation to assess the probability of observing a gene highly ranked for knockdown efficacy in multiple samples due to chance alone under the assumption of independence across the samples. As a priori the number of patients for whom a given gene may be important is unknown, we computed the probability of observing a gene of rank ≤ n of N total genes in ≥ m of six total patients for each of six possibilities for m (i.e. for each gene we tested six separate hypotheses, that a gene is important/highly ranked in at least one of six, two of six, three of six, four of six, five of six and in all six patients). This probability for each value of m is:

\[
P = 1 \cdot \left(\frac{N}{N}\right)^6 \quad \text{for } m = 6
\]

\[
+6 \cdot \left(\frac{n}{N}\right)^5 \cdot \left(\frac{N-n}{N}\right)^1 \quad \text{for } m = 5
\]

\[
+15 \cdot \left(\frac{n}{N}\right)^4 \cdot \left(\frac{N-n}{N}\right)^2 \quad \text{for } m = 4
\]

\[
+20 \cdot \left(\frac{n}{N}\right)^3 \cdot \left(\frac{N-n}{N}\right)^3 \quad \text{for } m = 3
\]

\[
+15 \cdot \left(\frac{n}{N}\right)^2 \cdot \left(\frac{N-n}{N}\right)^4 \quad \text{for } m = 2
\]

\[
+6 \cdot \left(\frac{n}{N}\right)^1 \cdot \left(\frac{N-n}{N}\right)^5 \quad \text{for } m = 1
\]

The probability we obtain in the above formula is based solely on the worst rank n of a given gene in m samples out of six, and does not account for the specific ranks of the gene lower than n in the other samples (yet this model provides an upper-bound limit on that probability which is sufficient for our purposes). The above-listed formula is specific for the situation of six total patient samples assessed. Thus, for the scenario of 4,974 total genes (N) that a rank (n) of ≤ 89 is observed in ≥ 5 patient samples (m) out of six total patient samples, the probability of that occurring due to chance alone would be:

\[
P = 1 \cdot \left(\frac{89}{4974}\right)^6 + 6 \cdot \left(\frac{89}{4974}\right)^5 \cdot \left(\frac{4974-89}{4974}\right)^1 = 1.084 \times 10^{-8}
\]

For comparison, the probability of observing a gene of rank ≤ 89 in ≥ 4 of 6 patient samples would be \(P = 1.494 \times 10^{-6}\). To stringently account for multiple testing, Bonferroni adjustment for the 4,974 total genes assessed is performed.

Evaluation of NOX family genes

A NOXI shRNA was cloned into a pRSIT16-U6Tet-sh-CMV-TetR-2A-RFP-2A-Puro construct (Cellecta). The gene expression of NOXI, NOX2 and NOX4 was performed using the TaqMan Gene Expression Assay (Thermo Fisher Scientific). Cells from the screened AML patients, five additional AML patients and two normal controls were evaluated with the NOX2 inhibitor, GSK2795039 (MedChem Express).
Additional details for methods sections are provided in the Supporting Information.

**Results**

**Assessment of primary cell viability**

Primary AML cells were successfully screened by shRNA knockdown in six patients (Table SI; Fig 1). We utilised carefully designed methods to ensure primary cell viability and successful gene knockdown. In addition, continuous cell growth in culture during the selection period was validated by carboxyfluorescein succinimidyl ester (CFSE) assay across the time course of nine days for one patient sample (Figure S1). To assess non-shRNA-related cell death, AML cells were divided into two groups — transduced and non-transduced — and assessed for apoptosis and necrosis across the time course in both. Non-shRNA-related cell death was observed to be significantly reduced by day 3, indicating that cell death from day 3 onward is mainly due to the action of shRNAs (Figure S2).

**Assessment of inter- and intra-correlation of shRNA screen knockdown**

While good correlation of shRNA incorporation at baseline across experiments is typically assumed and was confirmed in our data (Figure S3A), we previously observed that not all shRNAs initially get incorporated into the cells and hence their absence at follow-up can be misleading or ambiguous.

Thus, following transduction, baseline samples were obtained for all patients to evaluate successful incorporation of shRNAs. As not all shRNAs exhibit a profound effect on cell viability, similarly good correlation over an entire library screen at follow-up should not be expected. Correspondingly, we observed less correlation though still a generally positive trend in shRNA counts in replicate assessment of shRNA counts after six days of culture (Figure S3B). From the same sample assessed for primary cell viability, we performed next-generation sequencing evaluation at each of three time points. We found that the number of shRNAs exhibiting a fold change depletion in the range of two- to fivefold rose dramatically at day 6 (nine days post transduction), and fell again by day 9 (12 days post transduction), indicating day 6 to be a judicious timepoint for observing the lethal effects of knockdown for many genes (Figure S4). We also noted the number of completely unobserved shRNA (which can indicate the death of all incorporated cells due either to shRNA lethality or to non-shRNA effects) rose considerably at day 9.

**Identification of genes essential for AML survival**

Baseline and follow-up samples were simultaneously sequenced and FASTQ files from the next-generation sequencer deconvoluted to identify the frequency of each shRNA barcode for each sample. The average number of reads obtained per sample was $2 \times 24 \times 10^7$ (range, $2 \times 39 \times 10^6$ to $3 \times 47 \times 10^7$). Fold change depletion was calculated between follow-up and baseline samples as described previously. Due to the potential for outliers caused by extraneous factors,

![Fig 1. Various steps for the pooled short hairpin RNA (shRNA) screening of primary AML cells. The leukaemia cells are represented as yellow circles and the mesenchymal cells are shown in a red colour. * Pooled shRNA plasmids; ** lentiviral particles; *** transduced primary leukaemia cells. (Colour figure can be viewed at wileyonelineibrary.com)](image-url)
evidence of depletion by multiple shRNAs targeting the same gene is required for greatly reducing false positives. Hence, for each gene within each sample we identified the second-highest and third-highest depleting shRNA (Figure S5) and performed separate analyses using the results from each of these evidence levels.

Due to the heterogeneity of AML and the possibility of varying numbers of essential genes, which are marked in this analysis by depletion of a gene’s corresponding shRNAs, we sought to identify genes with consistently high-depleted shRNAs across multiple samples, utilising ranks to allow for variability in the amount of depletion across samples, and also allowing for a gene to show shRNA depletion within only a subset of AML patients (i.e. not requiring that a gene be essential for all patients). Table I shows the 25 genes with the highest shRNA depletion as assessed by the second most depleted shRNA for each gene, for each of the six primary AML samples. In this analysis NOX1 was observed to show the most shRNA depletion of the 4974 genes in two patients, and to be the 24th most depleted in a third AML sample (the gene ranked 79th, 89th and 235th in the other three AML samples respectively). Similar high rankings for NOX1 were observed in the analysis using the third-highest shRNA (Figure S6; Table SII).

To appreciate the likelihood of observing a gene with apparently high ranking across multiple samples, we determined a statistical calculation that would inform the probability of such a scenario (see Materials and methods). For all possibilities of a high rank beyond chance in ≥2 to 5 of the six samples, we observed NOX1 to have the greatest evidence of depletion, showing significance levels far beyond Bonferroni requirements for multiple testing (P values for NOX1 ranged from 2.22 × 10⁻⁶ to 1.08 × 10⁻⁸, Bonferroni-adjusted P₁ = 1.11 × 10⁻² to P₂ = 5.39 × 10⁻⁵, for being so highly ranked across multiple samples; Fig 2, Table SIII). These highly significant findings for NOX1 were also observed when repeating the analyses using the third-highest shRNA (Figure S6; Table SIV).

Only three other genes met Bonferroni significance for any scenario: NEDD8, DISC1 and DNAJC3. These genes were generally ranked higher for depletion in multiple samples, though much less consistently than NOX1. For the analysis of high rank across all six AML patients, DNAJC3 was the only gene observed beyond chance when using the third-highest shRNA, though the degree of depletion was less than the second-most depleted shRNA for each gene and sample (Table SII) for which NOX1 had rankings of 1, 15, 43, 115 and 1235 in the six AML samples respectively.

Table I. Genes with the highest short hairpin RNA (shRNA) depletion in primary cells from each AML sample.

| Gene rank | AML-7     | AML-8 | AML-9 | AML-11 | AML-14 | AML-18 |
|-----------|-----------|-------|-------|--------|--------|--------|
| 1         | ARHGEF6   | NOX1  | ABLIM3| GIPR   | COL4A3BP| NOX1   |
| 2         | IRAK3     | PTPRF | PTGD8 | COL4A2 | NTS51B | NME2   |
| 3         | CD74      | RAD51D|RPL13A | H19    | FASLG  | GRK1   |
| 4         | HAO1      | HSPE1 | RBBP7 | NEDD8  | SSTR5  | PRTN3  |
| 5         | Rnf19A    | GATM  | PDPK1 | HPN    | BLK    | SHMT2  |
| 6         | IFIT1     | ZBTB32|RARA   | EME1   | ASIC5  | B4GALT1|
| 7         | SYT1      | NR5A1 | MAP3K14| MYH6   | DAR52  | FSHR   |
| 8         | ZFP36     | CCNA1 | EMP1  | CCNB3  | H19    | SLC30A3|
| 9         | BAD       | CD274 | CLK3  | BMP4   | HOXD11 | COASY  |
| 10        | FOXA1     | YARS  | SEM1  | ATP6V0E1| GN5S   | ILIR2  |
| 11        | ENTPD5    | SAT1  | TUR3C | GRK3   | MAD1L1 | TECR   |
| 12        | TP73      | PIN1  | MMP14 | LEPR   | GALNT2 | MCFD2  |
| 13        | DGKZ      | ITS1N | TOP2B | VPREB1 | I6R6   | TNGFSF13B|
| 14        | FSCN3     | ACAT2 | KAT6A | ARG1   | ICAM5  | CD52   |
| 15        | GTF2H1    | GSK3A | CD207 | RPL13A | G052   | CCR3   |
| 16        | RPA2      | ALB   | JAM2  | HES3   | EXTL3  | MMAB   |
| 17        | HIBCH     | ABCB4 | CACNA1D| PRAK2G | ET7V   | TPH1   |
| 18        | SLP1      | PENK  | BGAT1 | S100A6 | STAT1  | SLC9A3R1|
| 19        | PDE7A     | LCAT  | MSLN  | PAFAH1B1| PSENE   | SP1    |
| 20        | AHR       | HNF4A | NFE2L2| PRF1   | CARD14 | AKRIC3 |
| 21        | FZD4      | NFAT5 | LHN   | SCN1B  | GABRA4 | ATG3   |
| 22        | LPAR2     | NOTCH4| ST3GAL6| CAPNS2 | LMO2   | IFT57  |
| 23        | BLK       | PPMID | CCL13 | CES1P1 | UB2D2  | GPRC5D |
| 24        | GRN       | SALL1 | KRXG  | NOX1   | DCTD   | C12orf57|
| 25        | MYLK2     | TCIRG1| NEDD8 | CAMKK2 | GALNT5 | IL10RA |

Shown are the top 25 most depleted genes (out of 4974 assessed in the library screen) in the primary AML cells from each patient as assessed by the second-most depleted shRNA for each gene and sample. Table S2 shows the results using the third-most depleted shRNA for each gene and sample.
Fig 2. Genes most essential for AML survival in multiple patients. Using the second-highest short hairpin RNA (shRNA) for each of the targeted 4974 genes, the probability of observing a given gene ranked consistently high in at least \( m \) of six samples due to chance alone was calculated. As a priori the number of patients for whom the shRNA for a particular gene may show a large effect is unknown, six unique hypotheses (corresponding to each value of \( m \)) were tested. The \( y \)-axis shows the negative of the base-10 logarithm of the probabilities. The \( x \)-axis shows the chromosome and position for each gene. Shown are the probabilities for each gene for the hypotheses that out of six samples the shRNA for a gene is highly ranked in at least \( m = 1 \) sample (A), two samples (B), three samples (C), four samples (D), five samples (E) and in all six samples (F). Red horizontal lines indicate the Bonferroni significance threshold. [Colour figure can be viewed at wileyonlinelibrary.com]
that observed in many other genes for any given sample. Across our various analytical assessments, no gene came close to showing the consistency and magnitude of depletion across multiple AML samples that was observed for NOX1.

Assessment of NOX family genes shows possible functional effect from off-target NOX2 inhibition

Within the NOX family, NOX1, NOX2 and NOX4 have been reported to be expressed in haematopoietic cells. Previous investigation has shown NOX2 to be the most consistently highly expressed in AML cells, while NOX1 has been shown to be expressed in smaller numbers of primary AML samples. Given the importance of multiple NOX family members to haematopoiesis, we investigated the specificity of the depleted NOX1 shRNA and the possibility of an off-target effect on an alternative NOX gene. The NOX1-specific shRNA which had shown the highest level of depletion was used to transduce the HL60 and K562 cell lines followed by measurement of NOX1, NOX2 and NOX4 gene expression.

The expression of NOX1 and NOX4 was low even prior to shRNA activation; however, the expression of NOX2 was initially high and following activation of shRNA after 72 h of doxycycline treatment a 40–50% reduction in expression was observed (Fig 3A). The latter observation indicates that an anti-leukaemic effect could be due to targeting of NOX2 rather than NOX1. Of the six screened AML samples, RNA was available for four patients prior to shRNA transfection. Expression of NOX1 and NOX4 was detected in none of these samples but AML#11, AML#14 and AML#18 showed relatively high expression of NOX2, correlating with significant shRNA depletion. In contrast NOX2 expression in AML#7, the only sample for which the NOX1 shRNA was not notably depleted, was detectable but significantly lower (Fig 3B).

The chemical inhibition of NOX2 using GSK2795039 reduced the cell viability of HL60 cells, with an IC50 of 22 µM at 72 h (Figure S7A). GSK2795039 reduced partial cell viability/proliferation in primary cells from six AML patients with different subtypes (including AML#11, AML#14 and AML#18 as well as three additional patients not assessed in the shRNA library screen: AML#21, AML#22 and AML#28) at a higher dose (>25 µM) in the conditioned medium (derived from HS-5 cell culture) at 72 h (Figure S7B). There was a small reduction of viability or proliferation (<20%) at 25 µM for all except one sample and 40 to 50% reduction at 50 µM for all after 72 h. CD34+ cells from two normal donors were treated with 12±5, 25 and 50 µM of GSK2795039 using the same conditioned medium. The normal cells did not show sensitivity to GSK2795039 at 25 µM; however, 30% to 40% reduction in viability or proliferation was observed at 50 µM, demonstrating the relatively higher sensitivity of HL60 and primary AML cells to GSK2795039 in comparison to normal CD34+ cells (Figure S7C). The relatively modest degree of sensitivity of the AML cells might be due to short exposure and/or the difference in measuring sensitivity from the original co-culture method. To address this difference, mononuclear cells from an additional two newly diagnosed AML patients (AML#31 and AML#32) were co-cultured with HS-5 cells in trans-well plates where the HS-5 and leukaemia cells were separated using a membrane and the cells were treated with 0.5, 1 and 5 µM GSK2795039. The cell counts for AML#31 and AML#32 were determined after 7 and 12 days (Figure S7D, E). A differential sensitivity

![Fig 3](image-url)
to GSK2795039 was observed between the two, as at day 7 significant reduction was observed only for AML#31 at 0.5 µM while AML#32 showed reduction after a longer exposure (12 days) and at higher dose (5 µM). These observations suggest heterogeneity among the AML cells in their reliance on NOX2 for viability and proliferation and the contribution of the microenvironment to this reliance. The apparent heterogeneity in the role of NOX2 in the pathogenesis of AML warrants a larger study of AML patients having various genomics drivers in a bone marrow (BM)-mimicking microenvironment to fully characterise the subset of patients that may benefit most from NOX2 inhibition.

Discussion

Improved characterisation of AML subtypes has not yet revolutionised the treatment of AML except in a few having very clear and dominant driver abnormalities. The existence of various driver mutations makes it very difficult to develop a therapeutic strategy with broad applicability. The outcome of genetic and epigenetic mutations is higher activity of genes involved in cell proliferation that might not be mutated themselves and therefore not detected through sequencing, leading to the popularity of functional methods to identify targets for AML.

The strategy in this study was to identify genes whose activity is increased as a result of the genetic and epigenetic alterations and interactions of leukaemia cells with the microenvironment. To mimic the in vivo condition of the BM microenvironment, the screen was performed in the presence of HS-5 cells. HS-5 cells secrete a wide range of cytokines which protect myeloid leukaemia cells against the inhibitory effect of many anti-leukaemic agents or spontaneous apoptosis.

The pooled shRNA library screen of leukaemic stem cells (LSC)/blast cells from six AML patients identified various targets across the samples, with NOX1 out ranking all others for effectiveness in two patients and showing considerable efficacy in three others. The NOX1 gene encodes a member of the NADPH oxidase family of enzymes. Although the screen identified NOX1 as a top target for five of the six patients, its expression was very low in the AML samples and as reported by others. Due to the homology between NOX family members, and the expression of NOX1/2/4 in haematopoietic cells, we measured their transcription levels following the transduction of HL60 and K562 cells by the NOX1 shRNA, showing the NOX1 shRNA to have a particularly striking effect on NOX2. These observations suggest that depletion of NOX1 shRNA in the screen may have been due to NOX2 targeting. Absence of shRNAs targeting NOX2 in this library module prevented initial detection of NOX2 as a candidate in our library screen. Interestingly, the only AML patient cells for which NOX1 shRNA was not depleted (AML#7), had very low expression of NOX2.

A recent study demonstrated the role of NOX2 in regulating self-renewal and differentiation in AML stem cells. Elevated levels of reactive oxygen species (ROS) have not been limited to particular developmental subtypes of AML and may explain the apparent importance of NOX in a heterogeneous group of samples in our study.

The detection of several genes associated with the pathogenesis of AML, such as NEDD8, H19, MSLN, RAD51D and GSK3A is encouraging and underscores the power of this approach for better understanding the deregulated genes and signalling pathways in AML. Further technical developments would help to refine this tool. First, the cell population for the screen should preferentially be LSCs as this is the population responsible for relapse. The ideal number of cells for the library screen should be between 200 and 1,000 times the complexity of the library. For a library of 27,500 shRNAs, more than 5x4 million cells should be transduced. To achieve a multiplicity of infection (MOI) of 1, during the transduction, three times more cells are required (i.e. >16 million cells are needed). Obtaining this number of LSCs from an AML sample is not easy due to the low number of LSCs and variation among AML patients for the frequency of LSCs within the mononuclear cells (estimated from 1 in 1x10^6 to 1 in 1x10^6). Development of a smaller library which can be applied to smaller numbers of LSCs enriched from AML samples would be a solution to this challenge.

Second, microenvironment conditions under which the cells are grown have a significant impact on protecting leukaemia cells against therapeutic agents. Leukaemia cells alter the function of stromal cells through the secretion of various molecules including ROS product, reshaping the BM microenvironment. In return the altered microenvironment promotes the survival of AML cells through the secretion of various cytokines. To identify genes essential for the survival of leukaemic cells, an environment similar to BM is required. One of the challenges in this regard is the absence of a standard culturing system with all of the major components of the BM, though there have been extensive activities in this field.

In conclusion, through a successful shRNA screen of primary AML cells, we have identified genes which may be essential for AML survival in a substantial proportion of patients. An improved BM-mimicking culture and focused shRNA library screens may identify other genes necessary for AML survival.

Acknowledgements

We thank the participants in the study. Funding for this study was provided by Leuka and Friends of Hammersmith Hospital. CCM was funded by the Pediatric Cancer Program which is supported by the Intermountain Healthcare and Primary Children’s Hospital Foundations and the Department of Pediatrics at the University of Utah. Infrastructure support for this research was provided by the NIHR Imperial Biomedical Research Centre (BRC) and the Imperial College Biomedical Research Centre (BRC) and the Imperial College
Healthcare Tissue Bank (ICHTB). The Imperial BRC Genomics Facility has provided resources and support that have contributed to the research results reported within this paper. The Imperial BRC Genomics Facility is supported by NIHR funding to the Imperial BRC. The support and resources from the Center for High Performance Computing at the University of Utah are gratefully acknowledged.

Author contributions

JSK and CCM wrote the manuscript; AGR, MJB and JFA edited the manuscript; JSK, CRF, LB, AC and MA performed experiments; SC, EY-F and EN-M provided samples, JSK, CRF, MJB and CCM performed data analyses.

Conflicts of Interest

The authors have no conflict of interest to declare.

Supporting Information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Figure S1. Cell proliferation assessment with carboxyfluorescein succinimidyl ester (CFSE) assay. Histograms for the CFSE experiment for the sample AML#14. The shift from the right to left during the follow-up period indicates cell division. The mean fluorescent intensity was used to calculate the level of fluorescent reduction and to estimate the number of cell divisions.

Figure S2. shRNA-related and unrelated death in AML cells during the selection period. Non-shRNA-associated apoptosis/death is observed within the first 48–72 h. The red and blue lines represent the shRNA transduced and non-transduced cells respectively. (A) Percentage of apoptotic/necrotic cells within the first five days of culture following transduction. (B) The number of live cells is shown for the same experiment as measured by cell counting using an automated cell counter.

Figure S3. Replicate assessments. (A) Scatterplot of total sequencing reads observed for each of the 27 500 shRNA barcodes at baseline and in a replicate baseline measurement for the same sample (AML#18). (B) Scatterplot of total sequencing reads observed for each of the 27 500 shRNA barcodes after six days of culture following baseline transduction, and in a replicate measure at the same timepoint and sample (AML#18). Black lines indicate best linear regression fit, dotted lines indicate 95% confidence intervals, and r indicates the Pearson correlation coefficient.

Figure S4. Temporal changes in shRNA depletion. Distribution of fold changes of barcode reads for the 27 500 shRNA from baseline to time of culture at (A) three days, (B) six days and (C) nine days for a single patient (AML#14).

Figure S5. shRNA depletion in six AML patients. Depletion fold changes from baseline to six days of culture for shRNA targeting 4 974 genes for each of the six AML patients’ primary cells. Five or six shRNAs targeted each of the genes; shown are the fold changes for (A) the highest depleting (greatest fold change) shRNA for each gene, (B) the second-highest depleting shRNA for each gene and (C) the third-highest depleting shRNA for each gene.

Figure S6. Genes most essential for AML survival in multiple patients. Using the third-highest shRNA for each of the targeted 4 974 genes, the probability of observing a given gene ranked consistently high in at least ‘m’ of six samples due to chance alone was calculated. As a priori the number of patients for whom the shRNA for a particular gene may show a large effect is unknown, six unique hypotheses (corresponding to each value of ‘m’) were tested. The y-axis shows the negative of the base-10 logarithm of the probabilities. The x-axis shows the chromosome and position for each gene. Shown are the probabilities for each gene for the hypotheses that out of six samples the shRNA for a gene is highly ranked in at least m = 1 sample (A), two samples (B), three samples (C), four samples (D), five samples (E) and in all six samples (F). Red horizontal lines indicate the Bonferroni significance threshold.

Figure S7. Effects of NOX2 inhibitor GSK2795039 on cells from AML patients and normal controls. (A) Cell viability of HL60 cells following GSK2795039 treatment for 72 h, as determined by MTS assay. IC50 value was determined as 22 µM. Data are represented as mean ± SEM for three independent experiments. (B) Cell viability of AML cells from six patients following GSK2795039 treatment for 72 h, determined by MTS assay. Cells from patients AML#18, AML#11 and AML#14 are from samples used for the pooled shRNA library screen; AML#21, AML#22 and AML#28 are samples from additional AML patients. The data for each condition are an average of triplicates. (C) Cell viability of CD34 + cells from two normal donors following GSK2795039 treatment for 72 h, determined by MTS assay. The data for each condition are an average of triplicates. (D, E) Normalised viable cell counts for patients AML#31 and AML#32 following seven and 12 days, respectively, are shown. The y-axis (for B, C, D and E) shows the per cent absorbance and cell counts in the treated to untreated control for each sample.

Table S1. Characteristics of AML patients screened by pooled shRNA library and/or for NOX2 inhibition.

Table SII. Genes with the highest shRNA depletion in primary cells from each AML sample using the third-highest shRNA for each gene and sample.

Table SIII. Probabilities of genes being highly ranked for shRNA depletion across various numbers of samples due to chance alone using the second-highest shRNA per gene and sample.

Table SIV. Probabilities of genes being highly ranked for shRNA depletion across various numbers of samples due to chance alone using the third-highest shRNA per gene and sample.
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