Drug resistance is a major hurdle in oncology. Responses of acute myeloid leukaemia (AML) patients to cytarabine (Ara-C)–based therapies are often short lived with a median overall survival of months. Therapies are under development to improve outcomes and include targeting the eukaryotic translation initiation factor elf4E with its inhibitor ribavirin. In a Phase II clinical trial in poor prognosis AML, ribavirin monotherapy yielded promising responses including remissions; however, all patients relapsed. Here we identify a novel form of drug resistance to ribavirin and Ara-C. We observe that the sonic hedgehog transcription factor glioma-associated protein 1 (GLI1) and the UDP glucuronosyltransferase (UGT1A) family of enzymes are elevated in resistant cells. UGT1A1s add glucuronic acid to many drugs, modifying their activity in diverse tissues. GLI1 alone is sufficient to drive UGT1A-dependent glucuronidation of ribavirin and Ara-C, and thus drug resistance. Resistance is overcome by genetic or pharmacological inhibition of GLI1, revealing a potential strategy to overcome drug resistance in some patients.

To better understand the molecular basis for relapse in our clinical trial (Fig. 1a and Extended Data Fig. 1), we generated resistant cells using head and neck carcinoma FaDu and AML-M5 THP-1 cells, both of which have highly elevated elf4E and concomitant ribavirin sensitivity. Two forms of drug resistance emerged, characterized by unimpaired growth in clinically achievable ribavirin concentrations and a loss of elf4E targeting (Fig. 1b, c and Extended Data Fig. 2a–c). Type I resistant cells (FRI, THPA, THPB) had severely impaired drug uptake, whereas type II resistant cells (FRII) did not (Fig. 1d and Extended Data Fig. 2d). Type I resistance was characterized by substantial reduction of adenosine kinase (ADK) (Extended Data Fig. 3a, b). ADK acts in cellular retention of ribavirin, as unphosphorylated ribavirin is readily exported by the nucleoside transporter ENT1 (also known as SLC29A1; refs 9, 10). Indeed, ADK reduction alone imparts ribavirin resistance (Extended Data Fig. 3c, d). Analysis of our patient pool indicated that only two patients had features consistent with type I resistance (Extended Data Fig. 3e, f).

Therefore, we investigated type II resistance. Given that elf4E was not mutated and was functional in FRII cells (Extended Data Fig. 2e–g), we examined whether the ribavirin–elf4E interaction was disrupted, by assessing the ability of elf4E to immunoprecipitate 3H-ribavirin (Fig. 1e). Although 3H-ribavirin is enriched ~sixfold in the elf4E-immunoprecipitated fraction in parental cells, this interaction is lost in FRII cells, despite normal uptake and functional elf4E.

RNA sequencing (RNA-seq) analysis revealed that 30 transcripts were differentially expressed in FRII cells, including GLI1 messenger RNA, by 21-fold (Extended Data Table 1). Consistently, GLI1 protein levels were highly elevated, as was GLI1’s target SNAIL (Fig. 1f). We investigated the clinical relevance of this elevation in our patients treated with ribavirin monotherapy. At relapse, leukaemic blasts had elevated GLI1 mRNA levels for 9 out of 9 patients examined (up to tenfold baseline) (Fig. 2a). For instance, GLI1 mRNA and protein levels were elevated at relapse (for example, patients 8 (complete remission (CR)), 11 (partial remission (PR)) and 17 (blast response (BR)); Fig. 2a and Extended Data Fig. 4; confocal microscopy was used owing to limited material at response). Interestingly, GLI1 levels in patient 17 decreased during response and re-emerged at relapse. Patients 9, 13 and 19, who did not respond clinically or molecularly, had highly elevated GLI1 levels before treatment,
relative to healthy individuals or responding patients. Moreover, in our ribavirin and low-dose Ara-C Phase I combination trial, patients A (CR, relapsed at 2 years) and B (CR, relapsed at 9 months) had increased GLI1 mRNA levels at relapse (Fig. 2a and Extended Data Fig. 4); these patients are denoted by letters so as not to be confused with ribavirin monotherapy patients, denoted by numbers. For patient C (PR, off treatment owing to a dose-limiting toxicity), GLI1 mRNA levels were unchanged at end of treatment (EOT), consistent with continued remission at that time. Thus, elevated GLI1 is associated with primary and acquired resistance in both ribavirin clinical trials. We observed that type I and type II resistance coexisted in two patients (Fig. 2 and Extended Data Fig. 3e, f). Whether this occurs in the same cells or whether multiple resistant populations emerge is unknown.

We extended these studies to investigate whether GLI1 levels were elevated in patients who failed more commonly used Ara-C therapies at relapse (R)/diagnosis (D). Roman numerals denote patients treated with standard chemotherapy as opposed to ribavirin monotherapy (numbers) or ribavirin plus Ara-C (letters). c, d, Effects of GLI1 overexpression on drug sensitivity: ribavirin (20 μM), Ara-C (200 nM, clinically achievable). e, siRNA to GLI1 restores drug sensitivity. siLuc, nonspecific control RNA. a, b, RNA results were normalized to ubiquitin. In all panels, error bars denote mean ± s.d. Experiments were completed in triplicate at least three independent times. **P < 0.01, ***P < 0.001 (Student’s t-test). Figure 4a–c shows corresponding western blots. Actin, tubulin and GAPDH provide loading controls.

To better understand the molecular basis for resistance, we monitored the ability of eIF4E to immunoprecipitate 3H-ribavirin as a function of GLI1 status (Fig. 3b and Extended Data Fig. 6b–d). Although eIF4E–ribavirin complexes were readily detected in controls, they were absent in GLI1-overexpressing cells (Fig. 3b). Conversely, GDC-0449 treatment followed by ribavirin led to ~60% reduction in growth relative to untreated FRII cells. GDC-0449 treatment alone did not substantially affect growth in either cell line. Importantly, GDC-0449 treatment also restored sensitivity to clinically relevant Ara-C levels (200 nM). Furthermore, GDC-0449 treatment of FaDu-GLI and THP-GLI cells re-sensitized these to ribavirin and Ara-C (Figs 2d and 3a). Finally, a direct inhibitor of GLI1, GANT61 (ref. 14), opposed to ribavirin monotherapy (numbers) or ribavirin plus Ara-C (letters). **

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relapse; that is, patients 11 (CR), 8 (PR) and 17 (BR) in the ribavirin monotherapy trial and in patients A (CR) and B (CR) in the combination trial. Patient C (PR) had no change in UGT1A levels at EOT, consistent with still being in remission. In patients treated with standard Ara-C therapies, UGT1A protein levels were elevated in 6 out of 7 specimens at relapse relative to diagnosis, and this occurred in the patients with concomitant elevated GLI1 (Fig. 2b). There was insufficient material for protein analysis of the remaining two specimens.

Next, we used mass spectrometry (MS) to determine whether ribavirin and Ara-C were glucuronidated in resistant cells (Fig. 4d–i and Extended Data Fig. 7). Metabolites were isolated, subjected to hydrophilic chromatography and detected by electrospray ionization-MS. In parental cells, ribavirin diphosphate (RDP) is the major peak (Fig. 4e, l).

In FRII cells, a new peak emerged with a mass consistent with the ribavirin-glucuronide (Fig. 4d). Using collision-induced ion fragmentation, we observed the triazole moiety of ribavirin as a major fragment supporting this as a site of glucuronidation (Fig. 4j red arrow, and Extended Data Fig. 7a). Relative peak intensities suggest that there is more ribavirin-glucuronide than RDP (Fig. 4d). Notably, GDC-0449 treatments eliminated ribavirin glucuronidation in FRII cells (Fig. 4f). GLI1 overexpression in parental cells led to formation of ribavirin-glucuronides (Fig. 4h). In vitro glucuronidation studies indicated that specific UGT1As are likely to be important to this process, as is ribavirin phosphorylation (Extended Data Fig. 7). Moreover, we observe Ara-C-glucuronides in FRII but not parental cells, and this modification was lost upon GDC-0449 treatment (Extended Data Fig. 7e, f). Thus, Ara-C and ribavirin
glucuronidation were GLI1-dependent, and elimination of the glucuronides by GLI1 inhibition correlated with restored drug sensitivity.

We examined the capacity of ribavirin-glucuronides to bind eIF4E. Ribavirin-glucuronide was isolated by hydrophilic chromatography and confirmed by MS/MS (Extended Data Fig. 7c). Using eIF4E-glutathione S-transferase (GST) immobilized on glutathione agarose, we observed that non-radioactive ribavirin or ribavirin triphosphate (RTP) compete for $^3$H-ribavirin–eIF4E complexes, whereas neither the negative control GTP nor the ribavirin-glucuronide did so (Fig. 4m). Thus, ribavirin glucuronidation impairs its interaction with eIF4E, underpinning resistance.

To further explore the effects of GLI1 inhibition on drug sensitivity, we monitored colony growth of primary AML specimens as a function of GDC-0449 treatment (Fig. 3c). Specimens were selected from patients that had previously failed induction chemotherapy. We observed that although GDC-0449 has little effect on colony growth alone, it strongly potentiated the effects of Ara-C and ribavirin, presumably by elimination of the respective glucuronides. By contrast, we observed little effect in specimens from healthy volunteers, consistent with our results in control cells.

Several factors probably contribute to GLI1 elevation in FRII cells, including reduced patched 1 levels, but not altered promoter methylation or modified hedgehog ligand levels (Extended Data Fig. 8). Glucuronidation is typically perceived as a detoxification pathway but does not always enhance drug excretion\(^{15}\). Similar to our findings with ribavirin and eIF4E, testosterone glucuronidation modifies its targets rather than its efflux\(^{15}\). Our findings reveal a role for GLI1 in drug metabolism and resistance. Here, GLI1 inhibition could restore drug sensitivity and thereby provide therapeutic benefit.

**METHODS SUMMARY**

Ribavirin-resistant cell lines were selected on the basis of prolonged ribavirin exposure, routinely tested for resistance, which was retained even after 6 months in the absence of ribavirin. In the absence of ribavirin, cells grew with indistinguishable doubling times (Extended Data Fig. 2). Drug treatments and cell viability assays were carried out as described\(^{16}\) using Trypan Blue or in parallel, Cell Counting Kit-8. For activity measured by scintillation counting. Colony formation assays in AML specimens from healthy volunteers, consistent with our results in control cells.

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METHODS
Reagents and constructs. Full-length human GLI1 was obtained from Addgene (K12 Plasmid 16419), subcloned into 2-FLAG-pDNA3.1 or pMSCV-GFP (Bioscience vector) and the subsequent clones validated by sequencing. Ribavirin was obtained from Kemprotec (CAS 36791-04-5, cyanosine β-di-arabinofuranoside (C17680) and GANT61 (C9048) from Sigma Aldrich, vismodegib (D449-049, S1082) from Selleckchem, ribavirin 5'-O-β-D-ribofuranoside salt (R414505) from Toronto Research Chemicals.
Antibodies for immunoblotting were as follows: monoclonal antibody (mAb) mouse anti-GFP (BD Pharmingen, 610270), polyclonal antibody (pAb) rabbit anti-eIF4E (BD PharMingen, 610270), polyclonal antibody (pAb) rabbit anti-eIF4G (A-10) (Santa Cruz, sc-13315), mAb mouse anti-GLI1 (C68H3) (Cell Signaling, 2522S), mAb rabbit anti-4E-BP1 (53H11) (Cell Signaling, 2522S), mAb rabbit anti-GLI1 (C68H3) (Cell Signaling, 2522S), mAb rabbit anti-pb-tubulin (Sigma Aldrich, T5168), pAb goat anti-ADK (D-21) (Santa Cruz, sc-23633), pAb goat anti-ENT1 (N-12) (Santa Cruz, sc-45489), pAb rabbit anti-Mcl-1 (S-19) (Santa Cruz, sc-819), mAb mouse anti-c-MyC (9E10) (Santa Cruz, sc-40), mAb mouse anti-HSP90(β-α) (F-8) (Santa Cruz, sc-13119), mAb mouse anti-Ifl4G (A-10) (Santa Cruz, sc-13115), mAb rabbit anti-GLI1 (C68H3) (Cell Signaling, 3588), pAb rabbit anti-UGT1A1 (Cell Signaling, 4371S), mAb rabbit anti-SNAIL (C15D3) (Cell Signaling, 2522S), mAb rabbit anti-eIF-4E (BiP) (S31H1) (Cell Signaling, 9644), mAb rabbit anti-IH (Indian hedgehog; CG5) (Cell Signaling, 22670V), mAb anti- PTCH (H-267) (Santa Cruz, sc-9016), mAb rabbit anti-smo (Smoothed; E-5) (Santa Cruz, sc-166685), UGT2B pan antibody (Santa Cruz, sc-50386). Antibody specificity for UGT1A and GLI1 is shown in Extended Data Fig. 8. Results with UGT1A pan antibody above were confirmed with another pan-UGT1A antibody from Santa Cruz sc-25847. The pan-UGT1A antibodies recognize the common carboxy terminus of UGT1As with UGT1A1 expressing approximately the same molecular weight. Importantly, the effects were specific to UGT1A as UGT2B levels were not changed (Extended Data Fig. 6i).
Cell culture and transfection. FaDu cells (ATCC HTB-43) were maintained in MEM supplemented with 1% MEM non-essential amino acids, 10% heat-inactivated FBS and 1% penicillin-streptomycin (Invitrogen). 2FLAG–GLI1 or 2FLAG vector control FaDu cell lines were generated using TransIT-LT1 transfection reagent (Lonza, LT07-418). All conditions were described previously5,19. Primers were as follows: forward (AGCCAGGGATCGCTCATCAG), UGT1A reverse (AACCGAAGGGTCTGCTTTCC), UGT1A forward (GGAAGCAGATGCGCTTTCC), UGT1A4 forward (GGAAGCAGATGCGCTTTCC), UGT1A4 reverse (CCTGCTGTAGTTTGGGAC), UGT1A9 forward (GGAGGAACATTATTTATGACCCCG), common UGT1A reverse2 (CATAATAAGCTTGCCATTG), UGC forward (TGGACGCGCGGAGTTTGGG), UGC reverse (CAGCGCGCGGAGTTTGGG), control reverse (CAATATATATT) and UGT1A1 forward (GGGAATACCGCTTTCGTTCC), UGT1A1 reverse (CCATATATATT) and UGT1A2 forward (GGGAATACCGCTTTCGTTCC), UGT1A2 reverse (CCATATATATT). Primers were performed using EXPRESS SYBR GreenER QPCR SuperMix (Invitrogen) in an ABI StepOne thermal cycler using the relative standard curve method (Applied Biosystems User Bulletin #2). All conditions were described previously5,19. Primers were used as follows: forward (CACAGAGACACAGGGCTAGT), UGT1A reverse (CCATATATATT) and UGT1A2 forward (GGGAATACCGCTTTCGTTCC), UGT1A2 reverse (CCATATATATT). Primers were performed using EXPRESS SYBR GreenER QPCR SuperMix (Invitrogen) in an ABI StepOne thermal cycler using the relative standard curve method (Applied Biosystems User Bulletin #2). All conditions were described previously5,19.
carried out using a laser-scanning confocal microscope (LSM510 META; Carl Zeiss), exciting 405 and 543 nm or 488 nm with a ×100 objective, ×2 digital zoom (where indicated), and numerical aperture of 1.4. Channels were detected separately, with no crossstalk observed. Confocal micrographs represent single sections through the plane of the cell. Images were obtained from LSM510 software version 3.2 (Carl Zeiss) and displayed using Adobe Photoshop CS2 (Adobe).

Mass spectroscopy. FaDu, FRIL, 2FLAG, 2FLAG–GLI1 or GDC-0449-treated cells grown in culture were trypsinized and put in to suspension at a density of 10^6 cells per ml media. For isolation of ribavirin-Glu mix (1/2 ribavirin + 1/2 C2 ribavin in a 50:50 ratio) were added in duplicate per condition and samples were incubated in a 37° C shaker. A 100 µl aliquot was taken at different time points (0, 10, 30, 60, 120, 180, 240, 300 and 360 min) mixed with equal volume of 100% methanol (Fisher Scientific, HPLC grade, A12P-4) and flash frozen in liquid nitrogen. Samples were stored overnight at −80°C. At the time of analysis, samples were thawed, centrifuged at 10,000 r.p.m. for 10 min and subjected to hydrophilic chromatography in line with mass spectrometer. The system used was an Agilent 1100 HPLC coupled to an Agilent MSD Trap SL, with an ESI source. The autosampler system was kept at 4°C. The HPLC column was an InertSil HLIC, 150 × 4.6 mm, 5 µm and the chromatography was obtained using solvent A (2 mM ammonium formate in water, pH 3.2) and solvent B (100% acetonitrile). The injection volume was 50 µl and the flow rate 1 ml per min. The column compartment was heated at 30°C. The initial gradient was 95% B and 5% A, which changed during a 30-min course to 5% B and 95% A followed by 6 min equilibration at 95% B and 5% A. The total run time of the gradient was 36 min. The ESI source of the coupled MS ion-trap was set in positive ion mode, the nitrogen drying gas flow was 12 ml per min, the nebulizer pressure at 55 PSI and the temperature of the capillary at 350°C with a voltage of 4,500 V. The mass analyser was set to scan from 50 to 1,500 m/z. For Ara-C glucuronidation assay, the same protocol was applied; however, the initial gradient was ran over a 20 min time course rather than 30 min.

Glucuronidation assay. Glucuronidation of ribavirin by human liver microsomes expressing UGT1A1, UGT1A4, UGT1A6 and UGT1A9 was performed as described previously. In brief, 50 µl of pooled human liver microsomes (BD Biosciences, 452116) were incubated with 25 µg alamethicin (Sigma-Aldrich, A4665) and 50 mM Tris-HCl, pH 7.4, containing 10 mM MgCl2, for 15 min on ice to allow formation of channels in the micromass membrane thus enabling access to the UGT active sites. Following incubation, 8.5 mM saccharic acid 1,4-lactone (β-glucuronidase inhibitor, Sigma-Aldrich, S0375) and 1 mM ribavirin 5′-triphosphate were added and the mixture was incubated at 37°C for 5 min. Finally, to activate the reaction, 100 µM uridine 5′-diphosphoglucuronic acid (UDPGA, Sigma Aldrich, U6751) was combined and incubations were performed at 37°C for 6 h. Note that no glucuronides were observed in the absence of UDPGA addition (data not shown). All reactions were made in 100 µl final volume and were terminated by the addition of 100 µl cold methanol (100%). The mixtures were kept at ~80°C for at least 3 h, then thawed and centrifuged for 10 min at 10,000 r.p.m. and the resulting supernatants were analysed by hydropilic chromatography in line with the mass spectrometer, as described above. We note that RTP was clearly glucuronidated in these microsomes whereas ribavirin was not, suggesting that ribavirin needs to be phosphorylated in order to be efficiently or stably glucuronidated. However, this step would be before glucuronidation and glucuronides could be neutralized by the reducing environment of the ribavirin-glucuronide. Furthermore, we did not observe glucuronidation of RTP in supernatant that express only UGT1A1, suggesting that other UGT1A1s must be present. Finally, although we observed glucuronidation of Ara-C efficiently in FRII and FaDu-GLI1 cells, we did not observe it in the microsomes, suggesting that some other UGT1A (than UGT1A1, UGT1A4, UGT1A6 and UGT1A9) needs to be present for efficient glucuronidation. Clearly this family member is well expressed in the FRII and FaDu-GLI1 cells.

Purification of ribavirin-glucuronides. Ribavirin-glucuronide was isolated from our microsomal preparation using the same liquid chromatography method described above with a few changes for initial gradient conditions to obtain a better separation of the different metabolites. In brief, given that the Rib- Glu peak elutes around 8.9 min, three 500-µl fractions were collected bracketing this time and lyophilised by centrifugation using Sarvand SpeedVac High Capacity Concentrator (Thermo Scientific, SC210A-115). The materials obtained from lyophilisation were re-suspended in a small volume of water: methanol (50:50 v/v) and an aliquot was reanalysed by HPLC-MS to verify the isolation and purity of the metabolite. To estimate the concentration of the purified Rib-Glu, a standard curve with the metabolite dilute ribavirin-5C2 (used as an internal standard) was generated. The concentration of the external standard curve was from 10 ng to 1 µg/ml. The curve was constructed by plotting the concentration of the internal standard against the area of the analyte.

1H-ribavirin pulldown. To determine whether Rib-Glu binds eIF4E, we performed an in vitro binding assay. In brief, 10 nM purified recombinant eIF4E-GST protein was coupled to 40 µl glutathione sepharose beads (GE Healthcare Life Sciences, 17-5132-01) for 30 min at room temperature in buffer containing 50 mM Tris-HCl, pH 7.4, 300 mM NaCl, 2.5 mM MgCl2, 0.5 mM DTT, 0.015% NP40, 0.5% protease-free BSA, and protease inhibitors. Washed beads were then incubated with 250 nM 1H-ribavirin and an equivalent concentration of either pure rib-Glu, 1H-ribavirin, RTP, GTP or blank for 30 min at room temperature in a buffer containing 100 mM sodium phosphate, pH 7.5, 300 mM NaCl, 0.015% NP40, 10 µM BSA and protease inhibitors. Washed beads then were eluted in 1X Laemmli sample buffer (containing β-mercaptoethanol) and radioactivity in supernatants was measured by scintillation counting (note that for washing the beads, 100 µM of GTP was added). The eluted Glu pulldown was concentrated to 5 µl and the reaction was loaded on an EpiTYPER primers.

To determine whether GLI1 regulates UGT1A protein’s half-life, we investigated the effect of MG132 proteasome inhibitor (Sigma Aldrich) on UGT1A’s half-life. Both FRII and wild-type cells were seeded at 70% confluency in 10-cm plates 24 h before treatment. Cells were then treated with 10 µM MG132 or an equivalent volume of methanol as a control. Ten hours post-treatment, cells were harvested and lysed in phosphoryl buffer and UGT1A protein stability was assessed by western analysis using a pan-UGT1A antibody.

Primary AML specimens and healthy volunteers. A total of 19 patients were included in the study in three participating centres: Jewish General Hospital, Hamilton Health Sciences, and Hôpital Maisonneuve Rosemont. In total, 15 patients were eligible for evaluation. Written informed consent was obtained as per the Helsinki Protocol. This study received IRB (all sites) and Health Canada approval. ClinicalTrials.gov registry is NCT00559091. Clinical response was assessed using the Cheson criteria. Patients had to receive a diagnosis of primary or secondary AML, French–American–British (FAB) subtypes M4 or M5 only, relapsed or refractory after at least one cycle of conventional chemotherapy; or newly diagnosed but not be candidates for induction chemotherapy. Patients must also have at least 18 years of age and must have had an Eastern Cooperative Oncology Group (ECOG) performance status lower than 3 and a life expectancy of at least 12 weeks. Other requirements were as outlined in ref 5. The ribavirin Ara-C combination Phase I trial (ClinicalTrials.gov NCT01056523) is recently completed, and patients with remissions were analysed for GLI1 and UGT1A1 levels. Criteria were the same as for the monotherapy trial described above. Written informed consent according to the Declaration of Helsinki was obtained from all patients as for the monotherapy trial. For analysis of primary specimens in colony growth assays, specimens were obtained from the Leukaemia Cell Bank of Quebec (BCLQ), with no identifying information. Leukaemic blasts were isolated by flow cytometry as described in ref 5. For tissue-matched controls at diagnosis and during the relapse, patient specimens were collected with written informed consent from either University of Rochester Medical Center RSRB approval and ClinicalTrials.gov NCT01311258 or from the BCLQ. For controls, normal bone marrow, peripheral blood mononuclear cells or normal CD34+ cells were used as indicated, and were obtained from StemCell Technologies. Protein and RNA were isolated as described (see below).

Bone marrow staining. Squash preparations of bone marrow aspirates and touch prints of bone marrow biopsies were air-dried and stained with Wright–Giems. To examine the specimens Leica DML5 microscope with the ×50 objective oil immersion lens was used. The Infinity 1-2C-184976 camera was used to capture images of the slides. The analysis were performed on the Infinity Analyze Image acquisition software (release 5.0.2, 2002-2009 Infinity corporation). Lysosome staining was as described previously. Images for lysosome staining were captured Leica DM LB2 microscope, and Leica DFC 350X camera and displayed with Adobe Photoshop 7.0 software.

Single-focus DNA methylation assays. Total genomic DNA was extracted from 2 × 10^6 FaDu and FRIL cells using the Gentra Puregene cell kit (Qiagen) and eluted in RNase-free water. EpiTYPER assays (Sequenom) were performed on bisulphite-converted DNA. Bisulphite conversion was performed using EZ DNA Methylation kit from Zymo Research. EpiTYPER primers were designed to cover 29 GLI1 CpGs (25 of them in CpG islands) using Sequenom Epipdesigner beta software (http://www.epidesigner.com/). EpiTYPER primers. GLI1 forward 1 GGTTTTTGGGTTGTAAATAGG, GLI1 reverse 1 CCTCTAAAAACATCAACCTCCC, GLI1 forward 2 TTGGGAT GAGTTTTTAAAGGTTG, GLI1 reverse 2 CCTAAATATCCTAATAATCA ATAACC.
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Extended Data Figure 1 | Ribavirin resistance in some ribavirin monotherapy clinical trial patients. Bone marrow biopsies for responding (patient 8 achieved a PR; patient 11, a CR, is shown in Fig. 1a) and non-responding patients (patient 9 was a PD and patient 13 a SD). Note abundance of blasts before treatment and at EOT and reduction in blasts and restoration of haematopoiesis during response. For Wright–Giemsa-stained samples magnification was ×50 with oil (patients 8 and 13), and for lysozyme staining ×20 (patient 9).
Extended Data Figure 2 | Characterization of FaDu- and THP-1-derived resistant cell lines. **a**, Detailed growth curves for FRI and FRII resistant cells. All cell lines have indistinguishable doubling times in the absence of ribavirin (far right panel). **b**, THP-1 resistant (TR) cell lines are not sensitive to treatment with ribavirin at the doses and times used. Ribavirin no longer targets eIF4E activity (that is, Mcl-1) in resistant cells (far right panel). There were no changes in eIF4E levels between resistant and parental cell lines (and Fig. 1c). Actin provides a loading control. **c**, Resistance is retained after 6 months of growth in the absence of ribavirin. **d**, Incubation of live cells with 3H-ribavirin indicates that THP-1 resistant cells have impaired uptake of ribavirin similar to FRII cells. **e**, eIF4E cap binding and eIF4G binding activity are retained in FRII cells. **f**, FRII cells are sensitive to eIF4E knockdown measured by cell growth. **g**, Effects of RNAi-mediated knockdown of GLI1 or eIF4E on UGT1A levels. Western blots were probed as indicated. RNAi-mediated knockdown of GLI1 led to reduced levels of UGT1A whereas knockdown of eIF4E did not. For UGT1A, a pan-UGT1A antibody was used. Antibody controls for UGT1A and GLI1 are shown in Extended Data Fig. 8c. Results are representative of at least three independent experiments. Average values are reported and error bars indicate ± standard deviations. Experiments were carried out in triplicate, three independent times. Western blots are representative of at least three independent experiments.
Extended Data Figure 3 | Pro-drug metabolism is impaired in type I resistance. a, b, Western analysis reveals that ADK levels were reduced in FRI cells (a) and THP1 resistant cells (b). Treatments were 48 h at 20 μM ribavirin. c, d, Knockdown of ADK leads to ribavirin resistance as shown by cell growth. Western blot confirms knockdown of ADK. Hsp90 provides a loading control. e, f, ENT1 and ADK mRNA levels for patients’ specimens. Patient 11 (CR) was responding clinically at (and before) 84 and 112 days and relapsed around day 252, when both ADK and ENT1 mRNA levels decreased. Analysis of RNA samples isolated before and at the end of the first 28-day cycle for patient 9 (who did not respond to ribavirin) compared to a healthy volunteer. Averaged values for ADK and ENT1 RNAs were normalized to glucose 6-phosphate dehydrogenase (G6PDH). Error bars indicate ± s.d.; centre values are averages. All experiments were performed in triplicate at least three independent times. **P < 0.01, ***P < 0.001 (two-tailed Student’s t-test). Results are representative of at least three independent experiments.
Extended Data Figure 4 | Confocal micrographs of leukaemic blasts isolated from bone marrows of responding and non-responding patients before treatment, at response or at EOT. Immunostaining for GLI1 and UGT1A are shown. DAPI is in blue. Note nuclear accumulation of GLI1 in non-responding patients, indicating elevated GLI1 activity. High levels of GLI1 and UGT1A suggest primary resistance. All confocal settings were identical between specimens and thus lower signal is indicative of less protein. A ×100 objective with no digital zoom was used for patients 9, 11, 13, 17 and 19. The same objective but a digital zoom of ×2 was used for patients 8, A, B and C. Note patient C was still in remission at EOT (see main text). For each patient, staining was carried out three independent experiments. Controls for specificity of GLI1 and UGT1A antibodies are provided in Extended Data Fig. 8c.
Extended Data Figure 5 | Higher GLI1 expression is found in poor cytogenetic risk group and predicts a trend of worse survival outcome in AML. To study the prognostic value of GLI1 gene expression in AML, we mined the publicly available AML data set published by The Cancer Genome Atlas Research Network\(^1\). a, GLI1 gene expression in 176 de novo AML patients grouped by cytogenetic risks. The expression level is represented by RPKM value (reads per kilobase of transcript per million mapped reads in RNA-seq). Each patient is represented by a symbol. Error bars represent median ± IQR (interquartile range) of each group. Nonparametric Mann–Whitney U-test was used to analyse the differences between groups. A total of 176 de novo AML patients with complete mRNA-seq and cytogenetic risk classification data are included in this analysis. b, Kaplan–Meier plots of events-free survival (EFS) and overall survival (OS) of 168 de novo AML patients segregated by median GLI1 expression (RPKM = 0.8596) (b) or high GLI1 expression (RPKM greater than or equal to 2) (c). Each tick on the survival curve represents a censored event because the patient is still alive at the end of the TCGA study. A total of 168 de novo AML patients with complete mRNA-seq, and reliable EFS and OS data are included in this analysis (patient information details are described in the Supplementary Table 1 of the NEJM study\(^1\). Mantel–Cox test was performed to calculate log-rank P values. We also observed that abnormally low levels of GLI1 were also correlated with poor outcome (data not shown), suggesting that GLI1 levels must be in a ‘Goldilocks’ zone.
Extended Data Figure 6 | Effects of modulation of GLI1 levels on UGT1A.

a, Effects of the direct GLI1 inhibitor GANT61 on restoring ribavirin sensitivity (20 μM) in FRII cells. Effects are dependent on GANT61 dose. b–d, Controls for eIF4E–ribavirin immunoprecipitations (IP) shown in Figs 1e and 3b. Inputs, supernatants (Sn) and IP controls for 3H ribavirin anti-eIF4E IPs are shown for GDC-0449-treated cells (b), FRII and FRII cells (c), and RNAi-mediated knockdown of GLI1 (d). e–g, qPCR analysis of GLI1 (e) and UGT1A (f) using a pan-UGT1A primer or primers for specific UGT1As (g). mRNA levels were normalized to RNA polymerase IIa. These findings are consistent with Extended Data Table 1, which indicates lower levels of UGT1A mRNA levels. Further, UGT1A3 and UGT1A8 decreased similarly (data not shown). Experiments were carried out in triplicate, at least three independent times. Average values are reported and error bars indicate standard deviations. These findings, that GLI1 elevation leads to reduced mRNA levels but increased protein levels, are counterintuitive. We propose that GLI1 elevation increases protein stability of UGT1As (see below) and this leads to some sort of feedback mechanism leading to reduced UGT1As. Other scenarios are possible but the main point that GLI1 elevation leads to increased UGT1A protein production is clear. h, GLI1 increases UGT1A protein stability as shown by studies with the proteasomal inhibitor MG132 (MG) and a pan-UGT1A antibody. Here, MG132 addition stabilizes levels of UGT1A in parental cells, but in FRII cells where levels are already increased, there is no further increase with MG132. This indicates that UGT1A proteins are already stabilized in the FRII cells. All results are representative of three independent experiments. i, Western blot analysis with a pan-UGT2B antibody indicates that UGT2B levels are unchanged in FRII relative to FaDu cells, suggesting the glucuronidation effects are mediated mostly through the UGT1A family. 293T cells are shown for comparison. Tubulin provides a loading control.
Extended Data Figure 7 | MS analysis of ribavirin and Ara-C glucuronidation. a, MS/MS collision-induced fragmentation analysis indicates that a breakdown product of the ribavirin glucuronide missing the ribose ring (exact mass 288.07) was further fragmented into a fragment of this glucuronide (exact mass 244.08, red asterisk) and to the triazole ring, the key moiety of ribavirin (exact mass 112.04). No ribose-glucuronide or ribose fragment was detected in our experiments, suggesting that this is not a major glucuronidation site in these cells. However, we cannot rule out that this exists and could not be detected. b, Microsomes expressing UGT1A1, UGT1A4, UGT1A6 and UGT1A9 were treated with RTP, underwent hydrophilic interaction liquid chromatography (HILIC) and the resulting extracted ion chromatogram (EIC) is shown. The Rib-Glu peak is clearly present and fragmentation analysis as in a confirms that this is glucuronidated ribavirin. We note that microsomes only expressing UGT1A1 do not glucuronidate RTP; and that RTP, but not ribavirin, is glucuronidated in microsomes. These studies suggest that UGT1A4, UGT1A6 and/or UGT1A9 are required for glucuronidation, as is some phosphorylation event before glucuronidation. c, Using HILIC chromatography, we isolated the fraction containing the Rib-Glu peak in b. A portion of this was re-assessed by MS/MS to be sure that the correct peak was isolated. This material was used in the 3H-ribavirin competition assay in Fig. 4m. Material was quantified using a standard curve of ribavirin (see Methods). d, Western blot demonstrating equal loading of eIF4E–GST in the 3H-ribavirin pulldown assay shown in Fig. 4m. All results are representative of at least three independent experiments. e, AraC is glucuronidated (AraC-Glu) in FRII cells but not parental FaDu cells where AraC-TP (triphosphate) is observed. AraC-TP is also observed in FRII cells, but at much lower levels than AraC-Glu. Treatment of FRII cells with GDC-0449 results in the loss of the AraC-Glu peak and causes no alteration to the parental FaDu cells. Fragmentation strongly suggests that the cytosine is the major site of glucuronidation (data not shown). We did not observe masses consistent with an arabinose breakdown product or an arabinose-glucuronide but cannot rule out that they are present at low levels or that our isolation procedure precluded their detection. f, Structures of AraC and AraC-TP are shown. The red arrow indicating the most likely glucuronidation site, as per our mass spectrometry data. Note that no glucuronides were observed when reactions were incubated in the absence of UDP-glucuronic acid (data not shown).
Extended Data Figure 8 | Investigations into why GLI1 levels are elevated in FRII cells. a, Analysis of expression of a subset of hedgehog signalling pathway proteins. Western blots are probed as indicated and are representative of three independent experiments. Tubulin and Hsp90 provided loading controls. Patched 1 (PTCH1) was the most significant change. PTCH1 is 210 kDa, with an often observed degradation product at 170 kDa. IHH, Indian hedgehog; SHH, sonic hedgehog; Smo, smoothened. b, GLI1 DNA methylation. CpG methylation was interrogated on bisulphite-converted DNA from GLI1 promoter region and first exon. The amplicon covered 29 CpGs, 25 of them located within a CpG island. DNA CpG methylation is shown as per cent methylation for FaDu (top) and FRII (bottom) cells. There was no difference observed between the cell lines. c, Antibody controls. Analysis of GLI1 and pan-UGT1A antibodies as a function of RNAi-mediated knockdown of these proteins as indicated. Note that UGT1A family members have approximately the same molecular weight. Results are representative of at least three independent experiments.
Extended Data Table 1 | RNA-seq results of genes with different expressions in FRII versus parental and FRI cells

The cutoff was set at a $P_{adj}$ value of $<0.005$.

| ID     | Fold Change | log2FoldChange | padj  |
|--------|-------------|----------------|-------|
| NM_005389 | GLI1        | 2.108551186   | 4.369586065  | 4.55763E-19 |
| NM_019076 | UGT1A8      | 0.071858361   | -3.798158182 | 5.14486E-10 |
| NM_021027 | UGT1A9      | 0.071858361   | -3.798158182 | 5.14486E-10 |
| NM_019075 | UGT1A10     | 0.071858361   | -3.798158182 | 5.14486E-10 |
| NM_019077 | UGT1A7      | 0.071858361   | -3.798158182 | 5.14486E-10 |
| NM_019083 | UGT1A3      | 0.075269208   | -3.731793399 | 1.03655E-09 |
| NM_0205862 | UGT1A6     | 0.071407143   | -3.807787779 | 1.03956E-09 |
| NM_019078 | UGT1A5      | 0.076934805   | -3.730219774 | 1.05656E-09 |
| NM_000463 | UGT1A1      | 0.081523375   | -3.618642411 | 1.75766E-09 |
| NM_0010772 | UGT1A6     | 0.078970619   | -3.644389132 | 1.95545E-09 |
| NM_007120 | UGT1A4      | 0.082083144   | -3.606770194 | 1.95854E-09 |
| NM_031479 | INHBE       | 5.23391175    | 2.541895632  | 1.36343E-07 |
| NM_003256 | TIMP4       | 12.36630007   | 3.627174907  | 2.27089E-07 |
| NM_000728 | CALCB       | 10.86981890   | 3.441757277  | 9.14295E-07 |
| NM_006098 | NDRG1       | 4.095681837   | 2.034061377  | 1.21428E-05 |
| NM_144717 | IL20RB      | 5.043343881   | 2.334380599  | 1.5497E-05  |
| NR_026572 | PDE2A       | 12.80113496   | 3.678198321  | 1.70545E-05 |
| NM_005980 | S100P       | 4.549241234   | 2.185625939  | 1.94272E-05 |
| NM_002462 | MX1         | 4.548672811   | 2.185445664  | 1.95955E-05 |
| NM_018818 | ABCG1       | 7.009493444   | 2.808692575  | 2.18839E-05 |
| NM_001144025 | MX1 | 4.464277528 | 2.158426717 | 2.78929E-05 |
| NM_001005340 | GPNMB  | 4.317327762   | 2.110138623  | 2.78929E-05 |
| NM_138420 | AHNK2       | 4.477547206   | 2.162708642  | 3.45273E-05 |
| NM_002272 | KRT4        | 0.141121624   | -2.824989024 | 3.48607E-05 |
| NM_001795 | CDH5        | 0.13455534    | -2.893728445 | 3.48607E-05 |
| NM_001901 | CTGF        | 0.11659076    | 3.099146095  | 0.030029063 |
| NM_001561 | TNFRSF9     | 10.39142803   | 3.377322023  | 0.000410797 |
| NM_018980 | NGEF        | 6.809341444   | 2.785203477  | 0.000500865 |
| NM_020318 | PAPPA2      | 0.0439344     | 2.595486002  | 0.000763335 |
| NM_016408 | MUC4        | 0.080049648   | -3.642808111 | 0.000785474 |
| NM_002408 | AGTR2       | 0.030727143   | -5.024325503  | 0.004798889 |