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Response and Resistance to MEK Inhibition in Leukaemias Initiated by Hyperactive Ras

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Abstract

The Raf/MEK/ERK cascade is a therapeutic target in human cancers with deregulated Ras signaling, which includes tumours that have inactivated the *Nf1* tumour suppressor¹. *Nf1* encodes neurofibromin, a GTPase activating protein that terminates Ras signalling by stimulating hydrolysis of Ras•GTP. We compared the effects of inhibitors of MEK in a myeloproliferative disorder (MPD) initiated by inactivating *Nf1* in mouse bone marrow and in acute myeloid leukaemias (AMLs) in which cooperating mutations were induced by retroviral insertional mutagenesis. Here we show that MEK inhibitors are ineffective in MPD, but induce objective regression of many *Nf1*-deficient AMLs. Drug resistance developed due to outgrowth of AML

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Author Contributions J.O.L and K.M.S. designed and performed experiments, analyzed data and wrote the paper. D.K, D.T.L., M.C., K.K., K.W., and J.M.B designed experiments, conducted studies, analyzed data and provided input to manuscript. K.A. performed bioinformatics analysis of retroviral insertion sequences. Q.L, K.M.C., E. D-F. M.G., M.T. designed and conducted experiments. J.P.R. provided critical reagents, assisted in experimental design and edited the manuscript. N.C., N.J., and L.W. provided retroviral reagents and training in experimental protocols. L.P. provided the mouse strain and input into the manuscript. J. S-L. and S.P. developed protocols for suspending and administering the MEK inhibitor and provided the drug used in all studies.

clones that were present before treatment. We cloned clone-specific retroviral integrations to identify candidate resistance genes including *Rasgrp1*, *Rasgrp4*, and *Mapk14*, which encodes p38 α . Functional analysis implicated increased RasGRP1 levels and reduced p38 kinase activity in resistance to MEK inhibitors. This approach represents a robust strategy for identifying genes and pathways that modulate how primary cancer cells respond to targeted therapeutics and for probing mechanisms of *de novo* and acquired resistance.

Aberrant Ras signalling contributes to the pathogenesis of myeloid malignancies and can result from acquired *RAS* mutations or from alternative genetic mechanisms that include *FLT3* internal tandem duplications, the *BCR-ABL* fusion, *PTPN11* mutations, and *NF1* inactivation (reviewed in²). Children with neurofibromatosis type 1 (NF1) have a 200–500 fold excess incidence of juvenile myelomonocytic leukaemia (JMML), an aggressive MPD characterized by leukocytosis, splenomegaly, and tissue infiltration (reviewed in³). The bone marrows of affected patients frequently show loss of the normal parental *NF1* allele and elevated extracellular signal-related kinase (ERK) activity⁴. Biallelic inactivation of murine *Nf1* causes a MPD in *Mx1-Cre, Nf1^{lox/lox}* mice that closely models JMML⁵.

We injected the MOL4070LTR retrovirus⁶ into *Mx1-Cre, Nf1^{lox/lox}* pups to identify genes and pathways that might cooperate with *Nf1* inactivation to induce progression of MPD to AML². These mice developed acute leukaemia sooner and at a higher rate than control *Mx1-Cre*-negative littermates that retain normal *Nf1* function, (Fig. 1a). *Nf1*-deficient AMLs are highly aggressive (Figs. 1b, 1c); show variable expression of myeloid surface markers; are transplantable into sublethally irradiated recipients; and form cytokine-dependent blast colonies in methylcellulose cultures.

The MEK inhibitor CI-1040⁷ reduced the growth of myeloid progenitor colonies from the bone marrows of *Mx1-Cre, Nf1^{lox/lox}* mice with MPD and WT controls to a similar extent (Fig. 1d). By contrast, blast colony growth from many *Nf1*-deficient AMLs was exquisitely sensitive with abrogation of colony growth at 10–100 fold lower drug concentrations (Fig. 1d). These *in vitro* data suggested that cooperating mutations render *Nf1* mutant AMLs more dependent on Raf/ERK/MEK signalling. To pursue this question, we first determined the maximally tolerated dose (MTD) of CI-1040 to be 100 mg/kg twice daily in WT mice, collected bone marrow at defined time points after a single drug dose, and showed that CI-1040 treatment transiently reduced the ability of granulocyte-macrophage colony stimulating factor (GM-CSF) to increase phosphorylated ERK (pERK) levels (Supplementary Fig. 1a). We then treated control or *Mx1-Cre, Nf1^{lox/lox}* mice with MPD for 28 days (n=5 per group). Consistent with the *in vitro* data, CI-1040 had no beneficial therapeutic index in mice with MPD (Supplementary Figs. 1b, 1c, and data not shown). Biochemical analysis of bone marrow obtained 2–8 hours after the 56th and final dose of CI-1040 revealed reduced ERK phosphorylation that was similar to the responses of WT mice that received a single drug dose (Supplementary Figs. 1a, 1d).

To investigate the unexpected *in vitro* sensitivity of *Nf1* mutant AMLs to CI-1040, we transplanted 4 independent leukaemias into 23 recipients. Mice with AML blasts in the peripheral blood were assigned to treatment with either vehicle (n = 11) or CI-1040 (n = 12) at the same dose and schedule that was ineffective in the MPD. CI-1040 treatment induced

rapid and extensive reductions in blood leukocyte counts (Fig. 2a) with clearance of blasts and reappearance of normal neutrophils (data not shown). Survival was increased greater than three-fold (Fig. 2b). However, recipients of *Mx1-Cre, Nf1^{flox/flox}* AMLs invariably died with recurrent leukaemia despite ongoing treatment (Fig. 2b). These relapsed AMLs had similar morphologic and immunophenotypic features as the parental leukaemias, but demonstrated *in vitro* resistance to MEK inhibitors and were refractory to treatment in secondary recipients (Fig. 2c and data not shown). Sensitive AMLs showed a greater reduction in 5'-bromodeoxyuridine incorporation following CI-1040 exposure than either WT or resistant leukaemia cells (Supplementary Fig. 2). We also exposed pairs of sensitive and resistant AMLs to CI-1040 *in vitro* to ask if resistance is associated with reactivation of MEK. Importantly, ERK phosphorylation in response to GM-CSF was inhibited at the same concentration of CI-1040 (Figs. 2d, 2e).

PD0325901 is a MEK inhibitor with more favourable pharmacologic characteristics than CI-1040⁸. We defined a MTD of 12.5 mg/kg/day for PD0325901, and found that doses > 5 mg/kg/day reduced ERK activation in response to GM-CSF for 24 hours (Supplementary Fig. 3a). To ask if prolonging the duration of MEK inhibition affects drug response and resistance *in vivo*, we administered PD0325901 to mice that were transplanted with two AMLs from the CI-1040 trial (AMLs #6554 and #6537). Recipients that received PD0325901 demonstrated the same pattern of response, relapse, and overall survival (Supplementary Fig. 3b). We also tested two other *Nf1*-deficient AMLs for response to PD0325901. AML #7723 was less sensitive to MEK inhibition in methylcellulose cultures and administering PD0325901 to recipient mice neither prolonged survival nor changed the IC₅₀ for blast colony formation (Supplementary Fig. 4). AML #7710 showed an *in vitro* response to PD0325901 that was similar to sensitive leukaemias; however, *in vivo* treatment did not prolong survival or select for clones with altered drug responsiveness (Supplementary Fig. 4). Together, these studies define a heterogeneous response of *Nf1*-deficient AMLs to MEK inhibition.

Digesting DNA from AML #6554 with restriction enzymes and Southern blot analysis with a MOL4070LTR-specific probe revealed clonal evolution of the resistant leukemia (Figs. 3a). To identify candidate genes that might influence response to CI-1040, we exploited a shotgun cloning strategy⁹ to characterize MOL4070LTR integrations. Sensitive parental AML #6554 and resistant leukaemias from CI-1040 and PD0325901-treated recipient mice shared some common integrations as well as unique insertions (Fig. 3a and Supplementary Table 1). Importantly, resistant leukaemias from independent recipients that were treated with CI-1040 or PD0325901 invariably showed the same integration patterns (Fig. 3a and Supplementary Table 1). These data and the rapid emergence of resistance infer that resistant subclones that are present within the initial AML are selected *in vivo* during MEK inhibitor treatment.

The patterns of MOL4070LTR integrations in sensitive and resistant AML #6554 suggested that *Rasgrp* genes might contribute to drug resistance (Supplementary Table 1). We developed quantitative real time PCR (RT-PCR) assays to correlate *Rasgrp1* insertion copy number and RNA expression in AML #6554 with the integration data shown in Supplementary Table 1. Whereas analysis of sensitive AML #6554 from 8 independent

recipients revealed only low levels of a unique integration junction fragment, four independent PD0325901 –resistant leukaemias demonstrated 1,000 fold (range 500–1,500 fold) increases in DNA copy number. Similarly, *Rasgrp1* messenger RNA levels were increased an average of 10 fold (range 6.5–19 fold) in resistant versus sensitive leukaemias (Fig. 3b). RasGRP proteins stimulate guanine nucleotide exchange on Ras, which increases Ras•GTP levels (reviewed in¹⁰). We reasoned that increased *Rasgrp1* expression might result in elevated Ras•GTP levels in resistant AML #6554. Indeed, whereas basal Ras•GTP levels were low in the parental leukaemia and returned to baseline 60 minutes after exposure to GM-CSF, we observed constitutive Ras activation in the resistant AML (Fig. 3c). Resistant AML #6554 also formed cytokine-independent blast colonies in methylcellulose (Fig 3d). This observation is consistent with biochemical and computational analyses in lymphocytes, which predict that Ras signalling becomes independent of receptor input when RasGRP levels are elevated¹¹. To further assess the functional importance of *Rasgrp1* over-expression in resistant AML #6554, we infected the sensitive and resistant leukaemias with a lentiviral vector encoding both a short hairpin RNA that efficiently reduces RasGRP1 protein (Supplementary Fig. 5) and a green fluorescent protein (GFP) marker. GFP-positive cells were isolated by sorting and plated in methylcellulose medium containing GM-CSF and a range of PD0325901 concentrations. Neither the *Rasgrp1* virus nor a control virus altered the sensitivity of parental AML blast colony forming cells to the MEK inhibitor (Fig. 3e). By contrast, infecting resistant AML #6554 with the *Rasgrp1* shRNA lentivirus restored the sensitivity of these cells to PD0325901, with a shift of the IC₅₀ into the same range as in the sensitive parental AML (Fig. 3e).

Multiple recipients that were transplanted with AML #6537 relapsed with the same resistant clone after treatment with either CI-1040 or PD0325901 (Fig. 4a). This resistant leukaemia showed a distinct retroviral integration pattern, including one within *Mapk14*, which encodes p38 α (Supplementary Table 2). RT-PCR of proviral/host DNA junctions confirmed that the inserted allele copy number is increased 1,000 fold (range 1,000–10,000 fold) in the resistant AML. The proviral insertion is in the anti-sense orientation and Southern blot analysis supports inactivation of one *Mapk14* allele (Fig. 4a, 4b). Consistent with this prediction, basal p38 kinase activity is reduced in resistant AML #6537 (Fig. 4c). To further investigate if decreased p38 α activity modulates resistance to MEK inhibitors in primary leukaemia cells, we enumerated blast colonies from AML #6537 in the presence of either CI-1040, SB202190 (a specific inhibitor of p38 α)¹² or both drugs. SB202190 strongly antagonized the inhibitory effects of CI-1040 on the sensitive leukaemia, but had minimal effects on the resistant AML (Fig. 4d).

Cooperating mutations that induce progression of MPD to AML render these more aggressive leukaemias highly dependent upon MEK for proliferation and survival. The transient remissions induced by imatinib in advanced (“blast crisis”) CML¹³ are remarkably similar to the responses of *Mx1-Cre*, *Nf1^{flox/flox}* AMLs to MEK inhibition. Relapse of blast crisis CML and of *Nf1* mutant murine leukaemias is due to outgrowth of a minor population of drug-resistant cells that is present before treatment with targeted agents¹⁴. Similarly, “backtracking” experiments in acute lymphoblastic leukaemias (ALL) showed that the dominant clone at relapse is frequently detectable before treatment¹⁵. Relapsed ALL clones

differ from the predominant clone at diagnosis by a few genetic changes¹⁶. Diverse mutations of genes involved in lymphoid developmental programs, cell cycle control, and DNA damage responses are enriched in drug resistant clones, while alterations in drug import, export, or metabolism are relatively uncommon¹⁶. Understanding how preexisting drug-resistant clones underlie cancer relapse has fundamental implications for developing better therapeutic strategies.

As MEK is one component of a complex network of Ras effectors, it is possible to envision multiple mechanisms of acquired resistance. Our data support this idea. A provocative implication of our studies of AML #6554 showing that *Rasgrp1* over-expression modulates the response to MEK inhibitors is that globally increasing Ras•GTP levels can overcome the effects of inhibiting a major Ras effector in some cancers. We implicated reduced p38 kinase activity as an alternative mechanism of MEK resistance in AML #6537. Interestingly, cancer cell lines that undergo apoptosis in response to oncoprotein inhibition show a rapid decrease in pERK followed by an increase in phosphorylated p38 levels¹⁷. As *Nf1*-deficient AMLs have elevated basal p38 kinase activity, our studies suggest that inhibiting oncogenic Raf/MEK/ERK signalling results in unopposed p38 kinase activity, which contributes to cell death. This general idea is consistent with the observation that p38 inhibitors induce imatinib resistance in cultured CML cells^{17,18}. The AMLs that we have characterized to date showed diverse retroviral integrations (Supplementary Tables 1–3), and comprehensive genetic and preclinical analyses are required to uncover the full spectrum of mutations associated with drug resistance. Despite this limitation, a broad implication of our data is that cell lineage, the nature of cooperating mutations, and the order in which they are acquired in specific cancers will modulate the efficacy of targeted inhibitors of Ras effector pathways. Insertional mutagenesis in genetically accurate mouse cancer models is a powerful tool for dissecting the molecular basis of cellular responses to oncogenic stress and for uncovering genes that contribute to drug sensitivity and resistance.

Methods Summary

Inhibitors

CI-1040 and PD0325901 (Pfizer, Ann Arbor, MI) were administered as described in Supplementary Methods. SB202190 (Calbiochem) was diluted in 100% DMSO.

Mouse Strains and RIM

Mice All procedures involving mice were approved by the UCSF Committee on Animal Research. MOL4070LTR stocks were prepared as described⁶. Mice received a single intraperitoneal injection containing 10^5 viral particles in 100 μ L admixed with 500 μ g of pI-pC (to activate *Mx1-Cre* expression) between days 3 and 5 of life. Mice with leukemia were killed and bone marrow was cryopreserved. Leukaemias were classified based on established morphologic and flow cytometric criteria¹⁹.

Adoptive Transfer and Treatment

Cryopreserved AMLs were injected intravenously (10^6 cells/mouse) into 6–8 week old recipient mice that received a single radiation dose of 450 cGy. Recipients with leukaemic

cells in the peripheral blood were randomly assigned to receive either CI-1040/PD0325901 or control vehicle. Mice were weighed weekly to adjust the drug dose, observed daily, and killed when they became moribund or on the 28th day of treatment.

Myeloid Progenitor Growth and Pharmacodynamic Studies

CFU-GM and blast colonies were grown in methylcellulose medium M3231 (Stem Cell Technologies) and scored by indirect microscopy. MEK inhibition was assessed by stimulating bone marrow cells with GM-CSF and then measuring pERK levels by Western blotting (also see Supplementary Methods).

Retroviral Integrations and Quantitative RT-PCR

Junction fragments corresponding to retroviral integrations were identified as described²⁰. PCR primers were designed to detect integration junctions²¹ (also see Supplementary Methods).

p38 Kinase Assay

Kinase activity of mice was quantified in 100 µg lysates in with the Omnia Plate IP kit (BioSource) in which activation of a MAPKAPK2 evokes a change in fluorescent properties due to chelation-enhanced fluorophore. A fluorescent plate reader in real time kinetic mode quantified p38 kinase activities.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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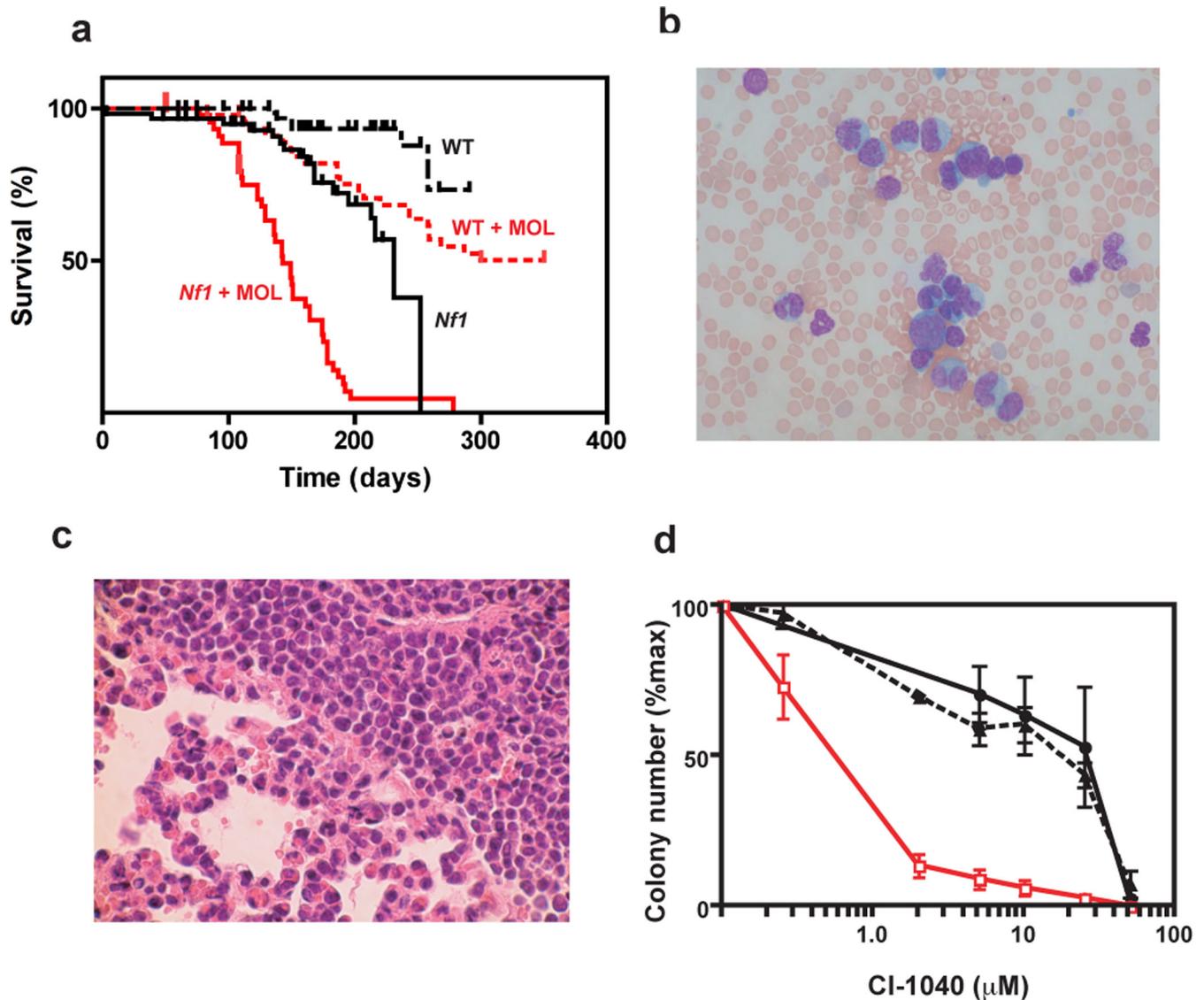


Figure 1. Retroviral mutagenesis induces AML in *Mx1-Cre, Nf1^{lox/lox}* mice and alters response to MEK inhibition

a, *Mx1-Cre, Nf1^{lox/lox}* mice that were infected with MOL4070LTR (*Nf1* + MOL; n=47) had markedly reduced survival compared to control littermates that received this virus (WT + MOL; n=49; $p < 0.0001$). Survival curves for *Mx1-Cre, Nf1^{lox/lox}* (*Nf1*) and WT mice that were not injected with MOL4070LTR are also shown. **b and c**, Myeloblasts in the peripheral blood (**b**) and infiltrating into lung tissues (**c**) of *Mx1-Cre, Nf1^{lox/lox}* mice with AML. **d**, Myeloid colony growth from the bone marrows of WT mice (closed circles, n=12) *Mx1-Cre, Nf1^{lox/lox}* mice with MPD (closed triangles, n=6), and *Mx1-Cre, Nf1^{lox/lox}* mice with AML (open squares, n=8) over a range of CI-1040 concentrations (log scale). Colony growth was assayed in the presence of a saturating concentration of GM-CSF. Error bars represent s.e.m.

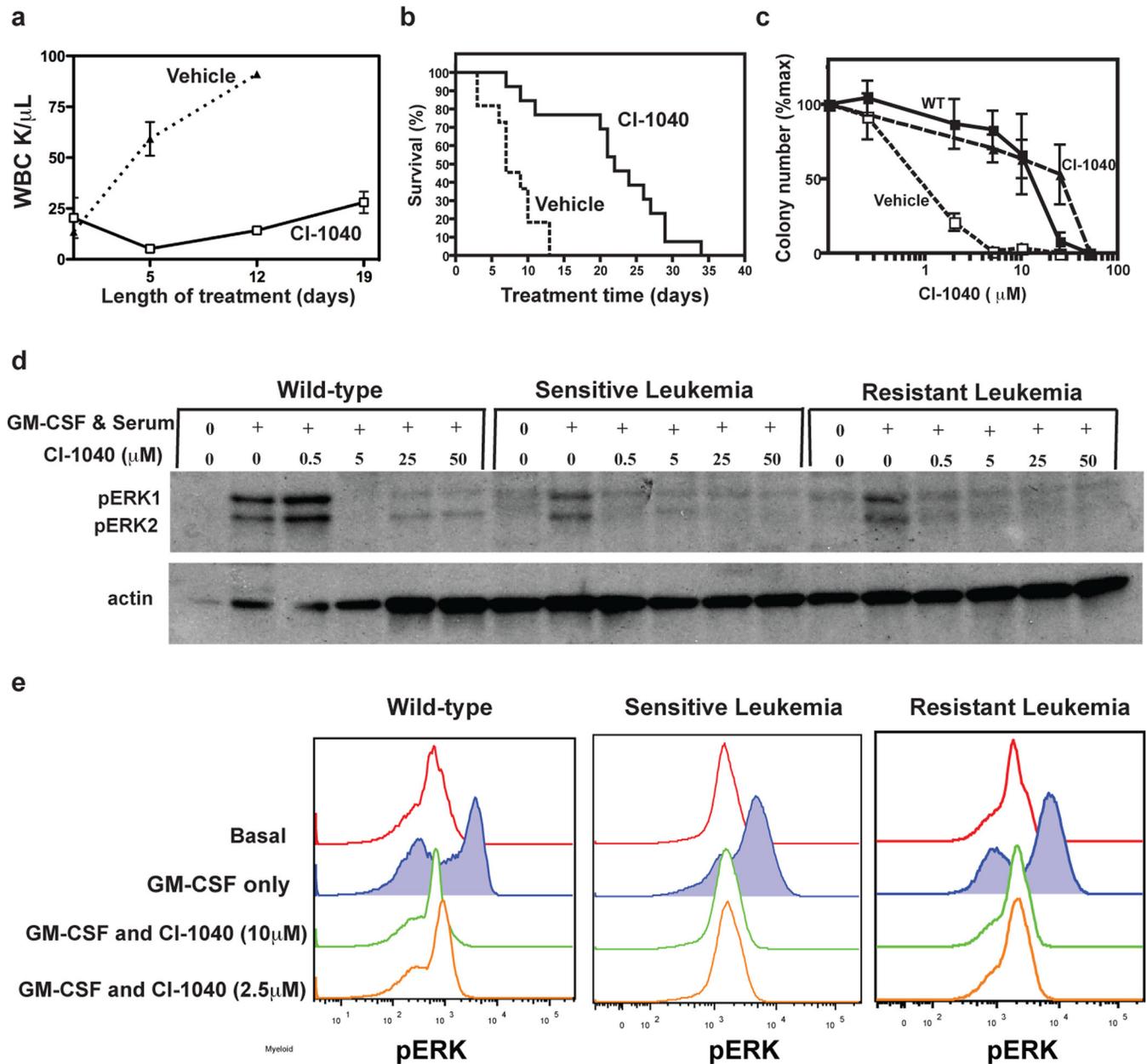


Figure 2. Response and resistance to CI-1040 in *Mx1-Cre, Nf1^{fllox/fllox}* mice with AML
a, Leukocyte counts were markedly decreased in mice treated with CI-1040 (n=13) compared to the vehicle (n=12). Error bars represent s.e.m. **b**, Survival was prolonged by approximately three-fold in mice that received CI-1040 (OR 3.1; CI 2.7–3.6; $p < 0.0001$). **c**, Myeloid colony growth from the bone marrows of mice with recurrent leukaemia are less sensitive to CI-1040 inhibition than the parental AMLs. Colony growth in methylcellulose is compared for WT bone marrow (closed squares, n=2), for primary AML cells harvested from vehicle-treated mice (open squares, n=4), and for AML cells obtained after relapse in recipients that were treated with CI-1040 (closed triangles, n=3). Error bars represent s.e.m. **d and e**, CI-1040 abrogates the ability of GM-CSF to stimulate ERK phosphorylation in

both sensitive and resistant AMLs as assessed by Western blotting (**d**) or phospho-flow cytometry (**e**).

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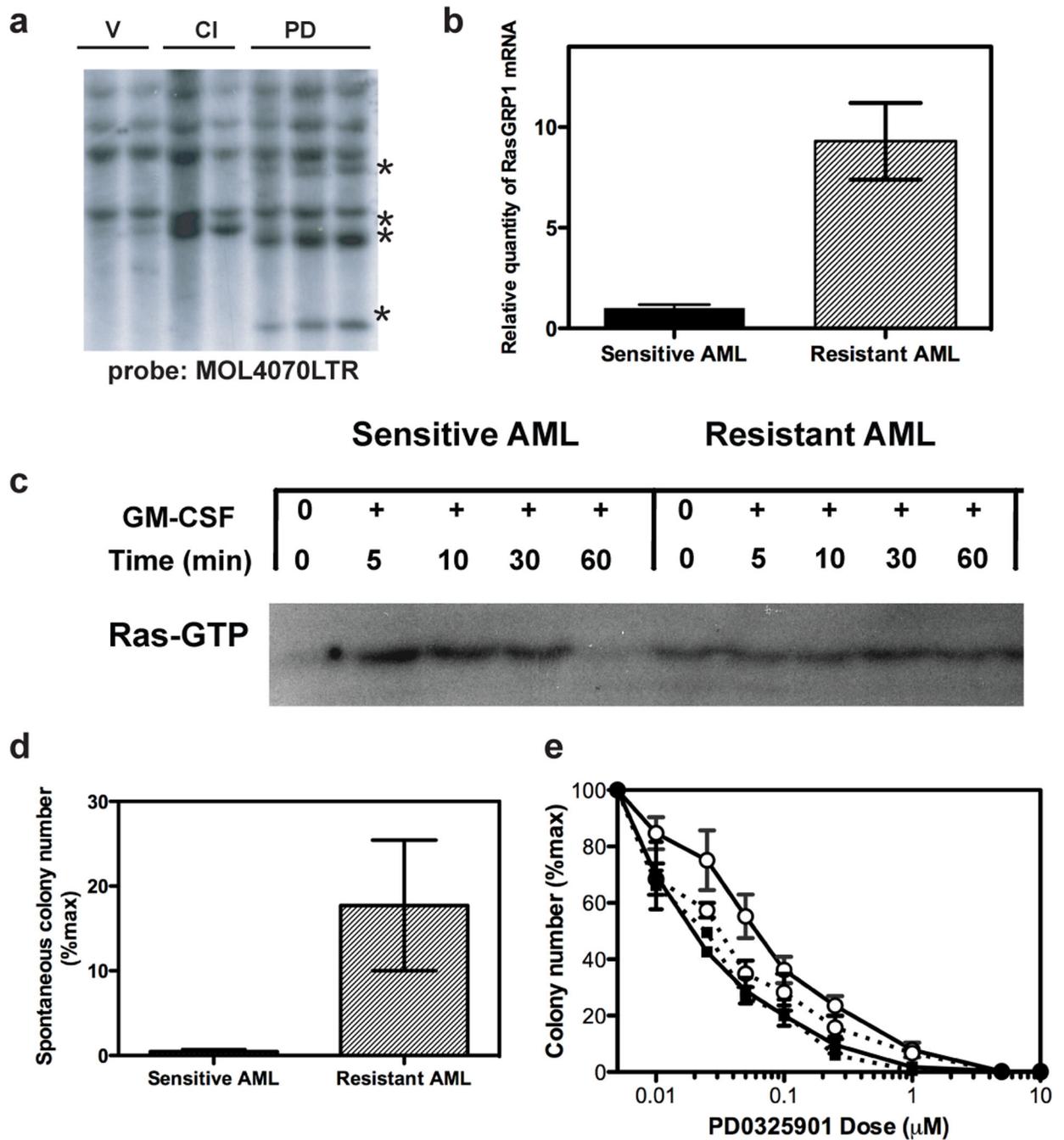


Figure 3. Genetic and functional analysis implicate *Rasgrp1* overexpression as a resistance mechanism in AML #6554

a, Retroviral integrations in DNA samples from AML #6554 in independent mice following treatment with vehicle (V), CI-1040 (CI) or PD0325901 (PD). Asterisks denote restriction fragments that are present in resistant AMLs, but are not seen in the sensitive parental leukaemia. **b**, Quantitative PCR analysis of *Rasgrp1* expression in sensitive and resistant AML #6554 (n=3 in each group, mean with SD, $p < 0.002$ student's t-test). **c**, Ras-GTP levels in AML #6554 show constitutive activation in the resistant leukemia. **d**, Resistant

AML #6554 demonstrates substantial cytokine-independent blast colony growth (n=5), which is not seen in the sensitive leukaemia (n=6) (p=0.0350, t-test). **e**, Resistant AML #6554 that is infected with a *RasGRP1* shRNA (open circles dotted line, n=7) demonstrates an $IC_{50}=0.03 \mu M$ (95% CI 0.02–0.03 μM), which is significantly lower than the IC_{50} of resistant cells infected with the control vector ($IC_{50}=0.06 \mu M$, 95% CI 0.05–0.07 μM ; open circles, solid, n=7). The IC_{50} of parental sensitive AML infected with shRNA vector to *RasGRP1* ($IC_{50}=0.02 \mu M$, 95% CI 0.017–0.024 μM ; closed squares, dotted line, n=3) or control vector ($IC_{50}=0.02 \mu M$, 95% CI 0.017–0.028 μM ; closed squares, solid line n=3) are not statistically significantly different than Resistant AML #6554 infected with *RasGRP1* shRNA. Error bars represent s.e.m. in **d** and **e**.

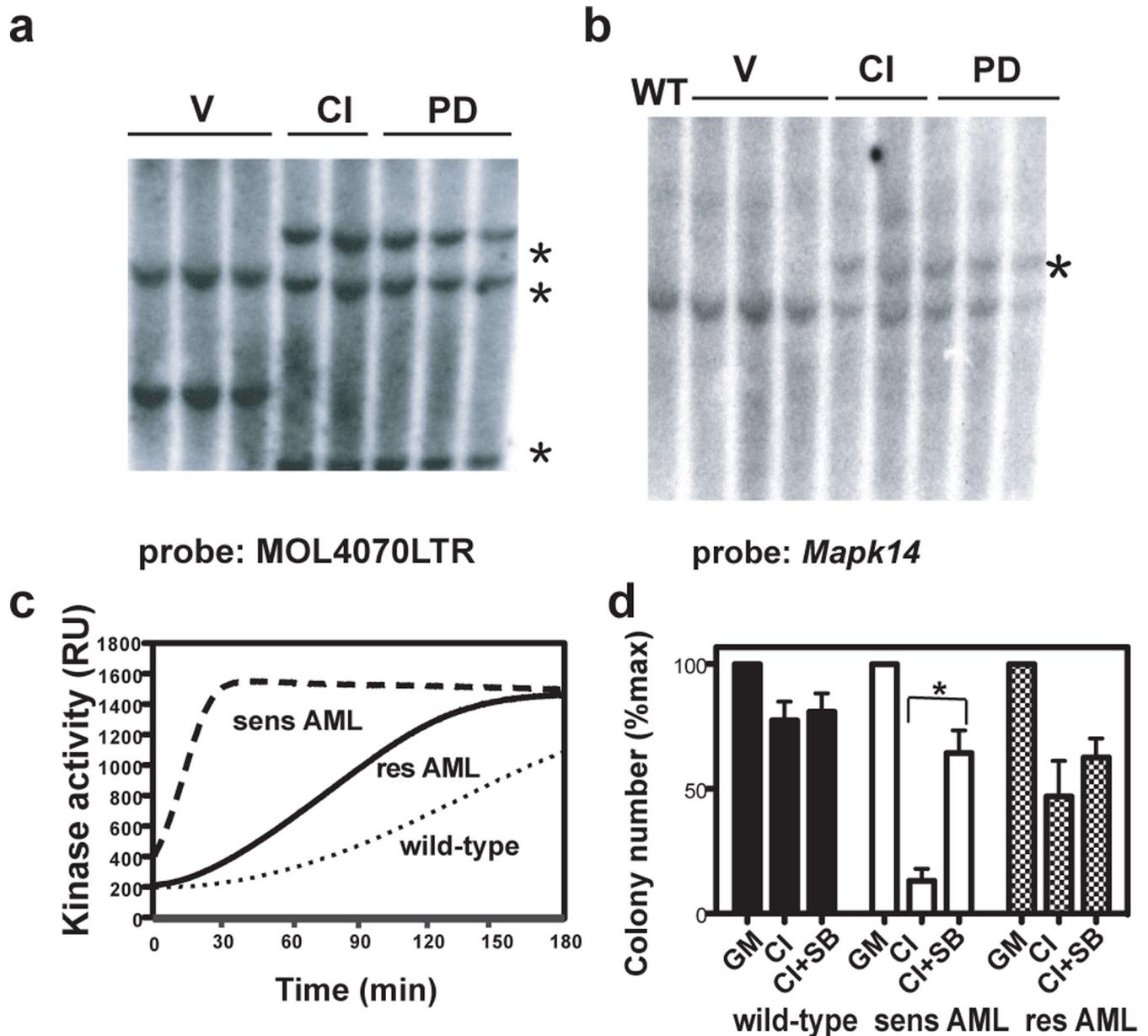


Figure 4. Genetic and functional analysis of AML #6537 associate reduced p38 α kinase activity with resistance to MEK inhibition

a. Southern blot analysis of MOL4070LTR integrations of AML #6537 following treatment with vehicle (V), CI-1040 (CI) or PD0325901 (PD). Asterisks denote restriction fragments that are present in all the resistant AMLs, but are not seen in the sensitive leukaemia. **b.** Southern blot analysis of paired sensitive and resistant AML #6537 with a *Mapk14* probe. The resistant leukaemias shown in lanes CI and PD show a *Mapk14* hybridization fragment, which overlaps with one of the MOL4070LTR bands in the resistant leukaemias shown in panel **a**. **c.** Basal p38 kinase activity of resistant AML #6537 cells (solid line) is reduced in comparison to the parental leukaemia (heavy dashed line), but remains elevated above WT bone marrow cells (dotted line). This is a representative example of 3 independent

experiments. **d**, SB202190 (SB), a p38 α inhibitor, antagonizes the ability of CI-1040 (CI) to reduce blast colony inhibition in parental AML #6537 (solid white bars, n=7) in the presence of GM-CSF (GM). Blast colony growth of sensitive AML #6537 is significantly increased by the addition of SB to CI-1040 (*p= 0.0043; unpaired t-test). By contrast, CI-1040 (2.5 μ M) has a inhibitory effect on WT CFU-GM colony (solid black bars, n=8) and resistant AML #6537 (chequered bars, n=5) blast colony growth, which is not affected by 2.5 μ M SB202190. Error bars represent the s.e.m.

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