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Proteomic and genomic integration identifies kinase and differentiation determinants of kinase inhibitor sensitivity in leukemia cells

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Kinase inhibitors are efficient in reducing cancer cell viability in cases where malignant cells present a dependency or addiction to the targeted kinase¹. Genetic alterations can cause constitutive activation of pro-survival and proliferative pathways and often determine the extent by which cancer cells respond to targeted drugs^{2, 3}. However, other biochemical events, not directly linked to genetic mutations may also contribute to the modulation of oncogenic kinase activity and thus influence responses to kinase targeted drugs^{4, 5}. Here, we integrated drug sensitivity, proteomic, phosphoproteomic, immunophenotypic and genomic analyses of primary AML to rationalize responses and identify determinants of sensitivity of AML cells to targeted compounds of clinical and preclinical interest in this disease.

We investigated the effects on cell viability of inhibitors for the kinases FLT3/PKC (midostaurin), PAK (PF-3758309), CK2 (silmitasertib), MEK (trametinib) and P38 (TAK-715). Hereafter named as FLT3/PKCi, PAKi, CK2i, MEKi, and P38i, respectively. Dose response curves for cells obtained from 36 AML patients (Data File S1) showed heterogeneous responses to all compounds (Figure S1). However, samples of the M4 FAB subtype were on average more sensitive than M1 samples to MEKi (Figure 1a).

Based on the surface expression of a set of co-expressed CD markers (Figure S2a), mass cytometry data subdivided our patient samples into two groups (Figure 1b (heat-map)). These analyses could be performed in 30 cases with sufficient number of cells and produced two main groups, which we termed CD^s+ and CD^s-, consisting of 12 and 18 patients, respectively. Untargeted mass spectrometry proteomics (Data File S2) uncovered greater expression in the CD^s+ group relative to CD^s- of a set of proteins linked to differentiation, several kinases and other signal-transduction regulators (Figure S2b-c). Global phosphoproteomics by mass spectrometry showed that CD^s+ cells had an increase in protein phosphorylation relative to the CD^s- cases (Figure S2d, Data File S3) and activated kinases downstream growth factor signaling, as illustrated by Kinase Substrate Enrichment Analysis⁶ (KSEA, Figure 1b (bar-plot)). In addition, individual phosphorylation markers⁷ on ERK1/2 (MAPK3/1), PAK1/2, MEK1

(MAP2K1), and PKC δ (PRKCD) were highly phosphorylated in the CD s^+ group (Figure S2d) and correlated with the surface expression of individual CD markers linked to differentiation (Figure S3a and S3b).

Since CD s^+ cases activated kinase survival pathways to a greater extent than CD s^- cases, we reasoned that cells from these groups would respond differently to kinase inhibitors. Consistently with this hypothesis, cell viability analysis as a function of treatment with kinase inhibitors showed that CD s^+ cases were more sensitive than CD s^- to MEKi (at 10, 100 and 1,000nM), FLT3/PKCi (1 and 10 μ M) and PAKi (1 μ M) (Figure 1c). These concentrations are physiologically relevant for MEKi and FLT3/PKCi^{8,9}. Together, our results suggest that CD s^+ cells had higher expression of proteins associated with myelomonocytic differentiation and kinase-signaling relative to negative cells, and consequently showed high phosphorylation and activation of pro-survival kinases, which was translated into an increased sensitivity to treatments with PAKi, midostaurin and trametinib.

In order to rationalize drug responses with greater detail, we sequenced 25 genes frequently mutated in AML in 27 cases of our cohort (Data File S4, sequencing failed in 3 samples). We found that genes involved in kinase-signaling (*NRAS*, *BRAF* and *FLT3*), were more frequently mutated in CD s^+ cases (Figure S4, $p=0.008$ by hypergeometric test). We performed an integrative and systematic analysis of mutational profiles with the mass spectrometry and cytometry data. Cells positive for *NRAS* mutations, high MAPK1 phosphorylation or the CD s^+ phenotype were more sensitive to MEKi than negative cells (Figure 2a (i-iv)). Cells with the *NRAS/BRAF/FLT3-ITD* genotypes were not more sensitive to MEKi than cells with just either *NRAS* or *BRAF* mutations (Figure 2a (v)). In contrast, cases positive for *NRAS*, *BRAF* mutations or the CD s^+ phenotype (*NRAS/BRAF/CDs+*) were on average more sensitive to MEKi than cells without this molecular signature (Figure 2a (vi-ix)). The p-value assessment for the comparisons showed that the *NRAS/BRAF/CDs+* signature produced the most significant difference followed by the *NRAS/BRAF/p-MAPK1hi/CDs+* signature (Figure 2 (bar-plot)). Our results suggest that, in addition to *NRAS/BRAF* activating mutations, the RAS/MEK/ERK pathway may be activated by other

means in cells with high expression of CD markers. Thus, MEKi treatment was more likely to reduce AML cell viability in cases positive for at least one of these markers (NRAS/BRAF mutations or specific CD pattern expression).

Although 15 cases with the *NRAS/BRAF/CDs+* signature were on average more sensitive to MEKi than negative cases, 8 of such cases were resistant (viability>50%) to treatment (Figure 2a (viii)). Within these 15 cases positive for *NRAS/BRAF/CDs+*, cells with *FLT3-ITD* mutations were significantly more resistant to MEKi than cells without this mutation ($p=0.012$, Figure 2b). Several phosphorylation markers were also found to be associated with responses to MEKi within the *NRAS/BRAF/CDs+* cases, including STAT5A^{S780}, STAT5A^{S128}, TOP2A^{S1213}, KDM5C^{S317} and CAMKK1^{S458} (Figure 2b). When the whole cohort of 27 patients was considered, samples positive for *NRAS/BRAF/CDs+* and negative for *FLT3-ITD* or low pSTAT5A or pKDM5C were more sensitive to MEKi than the other cells (Figure 2d, Figure S5a). This higher sensitivity of *NRAS/BRAF/CDs+* cases that were *FLT3-ITD* negative or pKDM5C^{S317} low was consistent across several MEKi concentrations (Figure S5a).

Our results suggest two distinct mechanisms of intrinsic resistance to MEK inhibition. One occurs in cells that are not addicted to the pro-survival actions of MEK because these have low RAS/MEK/ERK pathway activity. The other occurs in cells which, albeit having a highly active RAS/MEK/ERK, bypass MEK inhibition using the *FLT3/STAT5* axis; a pathway known to sustain AML viability and proliferation by acting in parallel to RAS/MEK/ERK signaling^{10, 11}. Pemovska et al. also observed a high response to trametinib in a subgroup of AML primary cells¹². *NRAS* is frequently mutated in AML and in a recent clinical trial ~ 20% of AML patients positive for *NRAS* or *KRAS* mutations responded to trametinib¹³. Our results suggest that selection of patients for therapy based not only on *NRAS/KRAS* mutations but also on direct markers of MEK activity, and STAT5 and KDM5A phosphorylation may increase the proportion of patients that will respond to this treatment.

We also noted that *FLT3*-ITD status was not associated with the responses of cells to FLT3/PKCi (Figure 2c, Figure S5b), an inhibitor recently approved to treat FLT3 mutant AML [ref¹⁴]. In contrast, CD expression and phosphorylation markers on PKC δ and on its substrate GSK3A [Ref¹⁵] were increased in FLT3/PKCi sensitive cells at 10 μ M and 1 μ M (Figure 2c, Figure S5b). Our results suggest that the mode of action of midostaurin may involve the inhibition of PKC δ (a known target of this drug) which we found activated in primary AML (Figure 1b, Figure S4a).

In conclusion, we found that AML cells remodel their kinase-signaling network during differentiation, resulting in a marked increase in the activity of pro-survival pathways regulated by MEK and PKC. Specific combinations of target and parallel kinase-pathway activation (caused by genetic and non-genetic events) determined the extent by which AML cells respond to treatments with trametinib or midostaurin.

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“Supplementary information available at Leukemia’s website”

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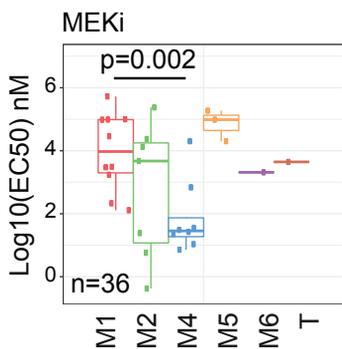
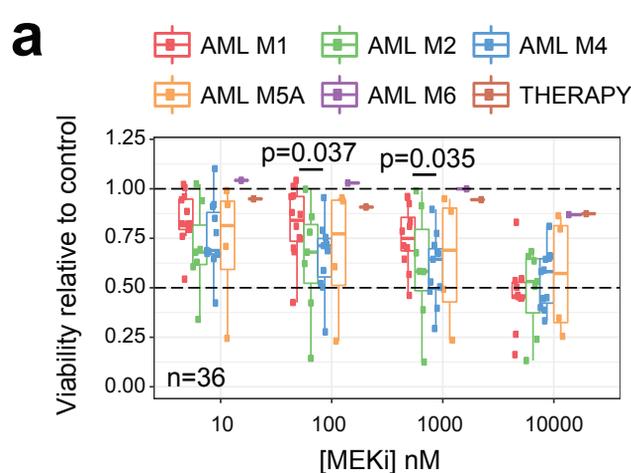
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Figure-Legends

Figure 1: Association of differentiation, kinase activity and sensitivity to kinase inhibitors in primary AML. (a) Sensitivity to MEKi as a function of FAB group. (b) CD expression across 30 cases

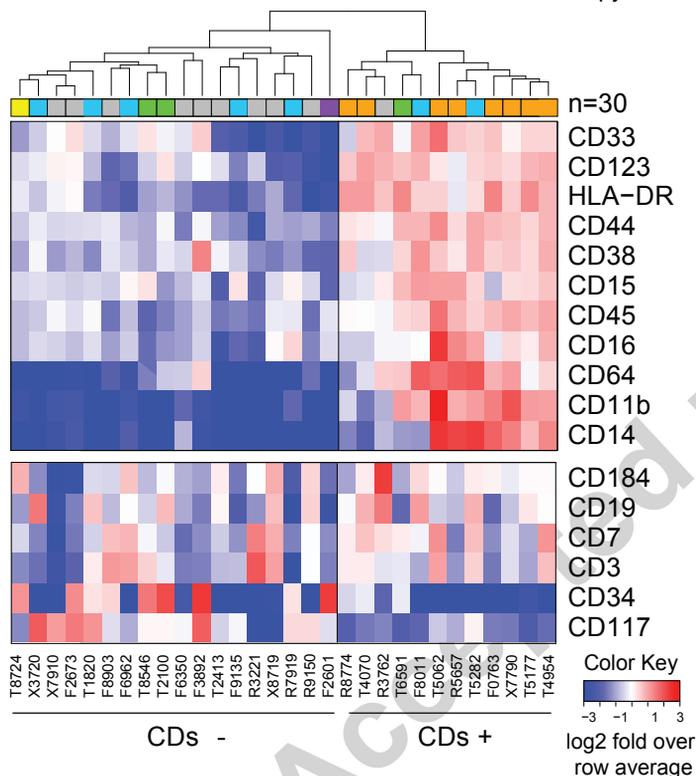
and estimation of individual kinase activities in CD^{s+} and CD^{s-} groups. (c) Sensitivity to kinase inhibitors as a function of CD pattern expression. Significance was assessed by Mann-Whitney test in (a, c) and with a z-test in (b).

Figure 2: Integration of genomic, phosphoproteomics and mass cytometry data to rationalize kinase inhibitors sensitivity. (a) Viability of AML cells within the indicated genotype/phenotype groups after treatment with MEKi. (b) Sensitivity of NRAS/BRAF/CD^{s+} positive cells to MEKi as a function of the indicated factors. (c) FLT3/PKCi sensitivity of AML cells with the indicated phenotype/genotype. Phosphorylations are denoted as (hi) and (lo) based on a greater or lower phosphorylation than the median across all cases. Significance was assessed by Mann-Whitney test.

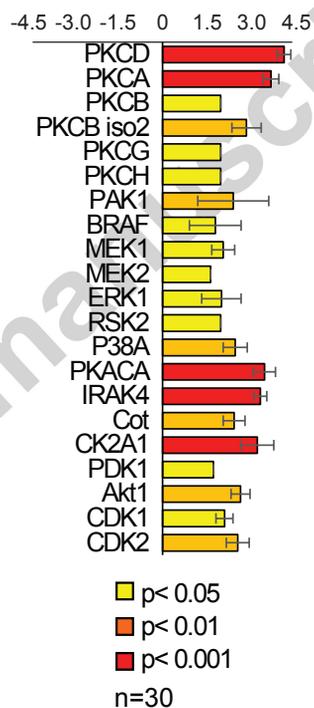


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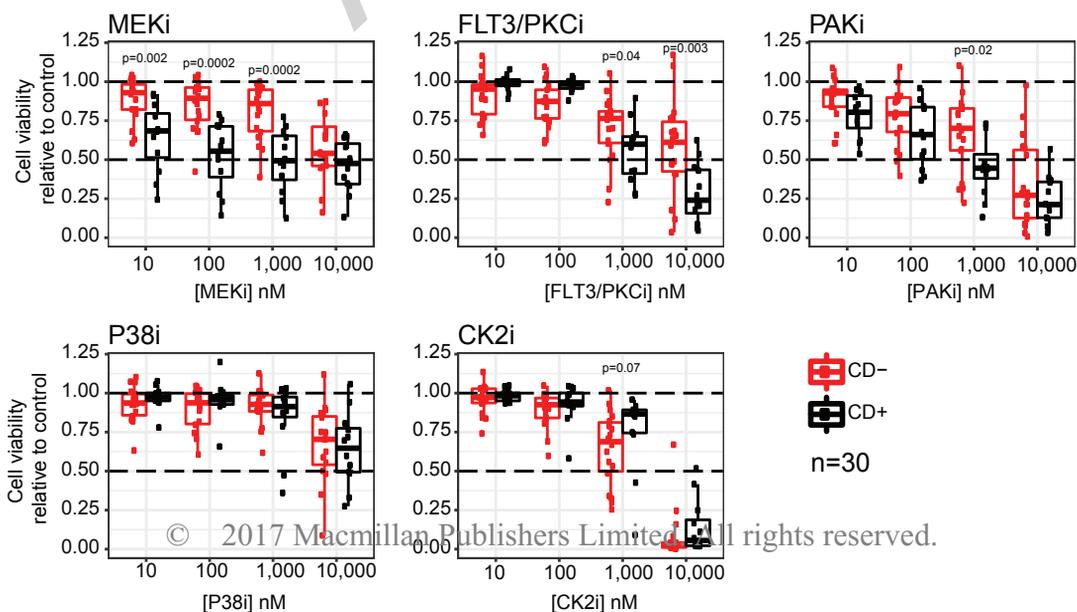
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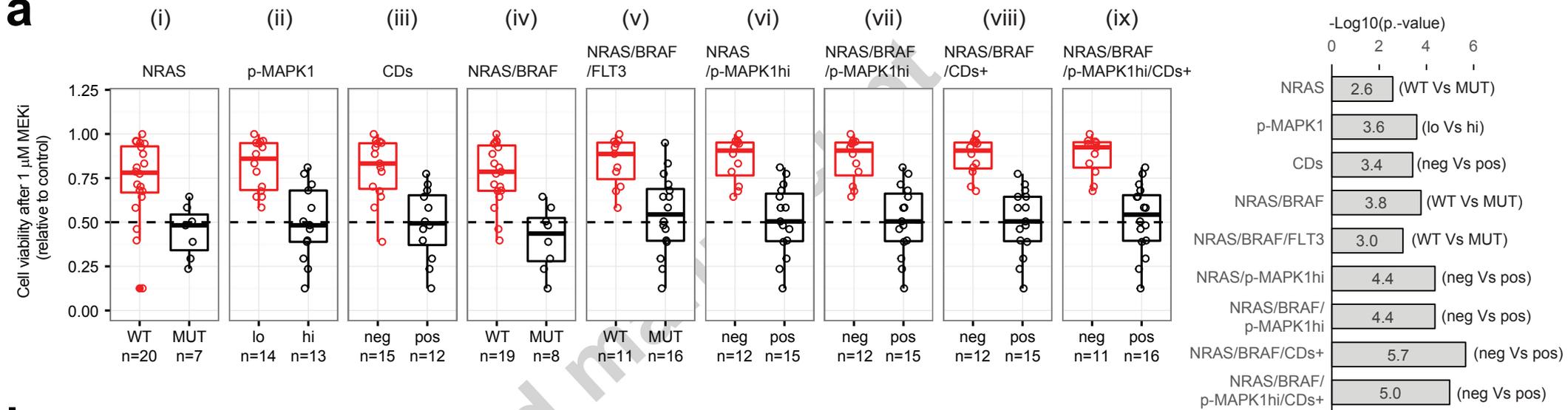
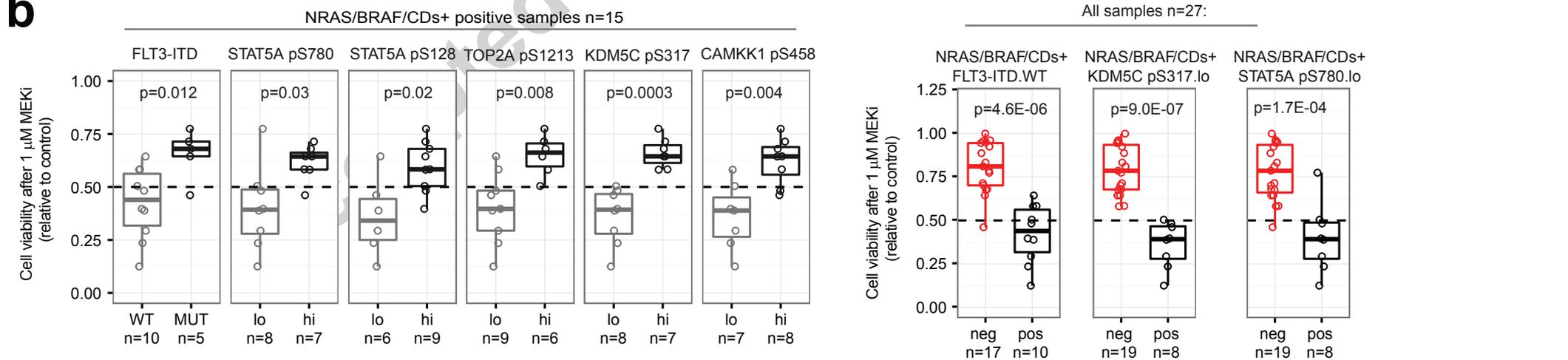


KSEA score CD_s+ / CD_s-



c



a**b****c**