Systematic Kinase Inhibitor Profiling Identifies CDK9 as a Synthetic Lethal Target in NUT Midline Carcinoma

Graphical Abstract

Highlights
- Screening 1,505 compounds against 78 cancer cell lines reveals distinct vulnerabilities
- NUT midline carcinoma cells are specifically sensitive to CDK9 inhibition (CDK9i)
- CDK9i perturbs MYC signaling, represses MCL1, and induces apoptosis in NMC cells
- CDK9 may represent a promising therapeutic target in NUT midline carcinoma

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In Brief
By screening 1,505 compounds against 78 cancer cell lines, Brägelmann et al. identify a specific sensitivity of BRD4-NUT-rearranged NUT midline carcinoma (NMC) cells to CDK9 inhibition. CDK9 inhibition affects transcriptional elongation, de-regulates MYC signaling, and induces apoptosis by suppressing anti-apoptotic MCL1. CDK9 may thus be a promising target in NMC.
Systematic Kinase Inhibitor Profiling Identifies CDK9 as a Synthetic Lethal Target in NUT Midline Carcinoma

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SUMMARY

Kinase inhibitors represent the backbone of targeted cancer therapy, yet only a limited number of oncogenic drivers are directly druggable. By interrogating the activity of 1,505 kinase inhibitors, we found that BRD4-NUT-rearranged NUT midline carcinoma (NMC) cells are specifically killed by CDK9 inhibition (CDK9i) and depend on CDK9 and Cyclin-T1 expression. We show that CDK9i leads to robust induction of apoptosis and of markers of DNA damage response in NMC cells. While both CDK9i and bromodomain inhibition over time result in reduced Myc protein expression, only bromodomain inhibition induces cell differentiation and a p21-induced cell-cycle arrest in these cells. Finally, RNA-seq and ChIP-based analyses reveal a BRD4-NUT-specific CDK9i-induced perturbation of transcriptional elongation. Thus, our data provide a mechanistic basis for the genotype-dependent vulnerability of NMC cells to CDK9i that may be of relevance for the development of targeted therapies for NMC patients.

INTRODUCTION

Systematic genomic profiling of tumors, combined with the development of targeted therapeutics, paved the way for a number of breakthroughs in the treatment of cancer patients (Buettner et al., 2013; Clinical Lung Cancer Genome Project (CLCGP) Network Genomic Medicine (NGM), 2013; Hyman et al., 2015; Kandoth et al., 2013). In genetically defined subgroups such as EGFR-mutant lung cancer the therapeutic exploitation of these alterations has already led to dramatic improvements in the clinical care of cancer patients (Flaherty et al., 2012; Rosell et al., 2012; Shaw et al., 2013; Van Cutsem et al., 2011). However, most oncogenic driver lesions are still considered to be undruggable.

Massively parallel interrogation of drug vulnerability across large panels of cancer cell lines has proved to be a valid tool for the identification and validation of genetically defined targets (Barretina et al., 2012; Garnett et al., 2012; Iorio et al., 2016; Mar-tins et al., 2015; Seashore-Ludlow et al., 2015; Sos et al., 2009a, 2009b). Such screens can identify compounds that directly target driver alterations and offer the opportunity to discover additional vulnerabilities in non-mutated genes that only become essential in tumor-specific genetic backgrounds (Chan et al., 2011; Fece de la Cruz et al., 2015). Exploitation of such synthetic lethality has already provided alternative therapeutic approaches to selectively kill cancer cells while sparing normal tissue (McLornan et al., 2014). The cyclin-dependent kinases (CDKs) 1, 2, and 6 have been shown to gain relevance in several MYC-driven tumors and were thus proposed as context-specific synthetic lethal targets (Fece de la Cruz et al., 2015). Moreover, CDK9, which, together with Cyclin-T1, forms the positive transcription elongation factor b (P-TEFb) complex and induces transcriptional activation by hyperphosphorylating RNA polymerase
II (Pol II) (Lu et al., 2015; Morales and Giordano, 2016), was put forth as a potential therapeutic target in hepatocellular carcinoma (Huang et al., 2014), ovarian cancer (Lam et al., 2014), and hematological malignancies (Gregory et al., 2015; Watsby et al., 2014). As a consequence of specificity, several CDK inhibitors have entered clinical trials, but so far, a lack of specificity and resulting toxicity limits the clinical relevance of CDK inhibitors in cancer (Chen et al., 2014; Kumar et al., 2015; Morales and Giordano, 2016).

Here we evaluated the cellular activity of a library of 1,505 kinase inhibitors to systematically uncover genotype-specific vulnerabilities. Our data reveal that CDK9 inhibition specifically modulates transcriptional elongation and effectively impairs viability through induction of apoptosis and DNA damage response of NUT midline carcinoma (NMC) cells.

**RESULTS**

**High-Throughput Cell Line Screening**

In total, 1,505 chemical compounds with a spectrum of common kinase inhibitor motifs were screened against 78 cancer cell lines (Figures 1A–1C) (Barretina et al., 2012; Garnett et al., 2012; Sos et al., 2009b). Overall, 7.5% of all compound-cell line combinations were classified as candidate hits (Z score < −2, corresponding to a residual viability of <25.9% at 10 μM) (Figure S1A). The high number of compounds that elicited only low or no cytotoxic effects across the cell lines is likely attributed to most compounds not having undergone previous target-based chemical or lead optimization (Figure 1A; Figure S1A). Based on the number of hits across cell lines (nhits), compounds showed a range of activity patterns ranging from lack of activity (65.2% of all compounds, termed inactive; nhits < 2) to broad and unselective toxicity (8.0%, termed toxic; nhits > 30% of cell lines) (Figure 1A).

To assess the impact of chemical complexity on compound activity, we calculated extended connectivity fingerprints (ECFP6) (Riniker and Landrum, 2013), whose lengths correspond to the number of distinct chemical features present in a given molecule. Neither biological selectivity nor compound potency depended on chemical complexity, as determined by the ECFP6-fingerprint length (Figure 1B). Inactive, selective, and toxic compounds were distributed at similar frequencies along the fingerprint lengths (Figure 1B, upper panel). However, analyses of compounds grouped by basic chemical scaffold (Hu and Bajorath, 2013) indicated that the number of active compounds varied by core structures (Figure 1C). Specifically, compounds with selective patterns of activity were typically based on common scaffolds of established kinase inhibitors (e.g., amino-pyrimidines, imidazoles, indoles, pyrazoles, pyridines, quinazolines, and thiazoles) (Figure 1C, boxplot). By contrast, compounds based on a pyrazolopyrimidinone scaffold or those with a highly complex core structure (mainly staurosporine and derivatives thereof) were enriched in the group of primarily toxic activity (Figure 1C). Thus, within our dataset core, scaffolds are a major determinant of compound selectivity.

To discover genotype-specific effects of the selective compounds, cell lines were grouped according to the presence or absence of a given genomic alteration, and differences in the viability in those cell lines bearing such alteration and in those lacking it were tested by an ANOVA approach (Barretina et al., 2012; Garnett et al., 2012; Iorio et al., 2016; Sos et al., 2009b). Of all 6,646 possible compound-genotype combinations, 345 (hit rate = 5.2%) showed a significantly decreased viability in altered versus wild-type cell lines (false discovery rate [FDR] ≤ 0.1) with a significant enrichment of EGFR inhibitors scoring in EGFR-mutant cell lines (Figure 1D).

We hypothesized that based on the structural diversity of inhibitors with differential activity against EGFR, we might also be able to predict compound activity by chemical structure alone. To this end, we applied elastic net modeling for regression and classification of activity based on ECFP6 fingerprints using a training subset (90% of compounds), coupled with 10-fold cross-validation and subsequent testing on the remaining 10% of the compounds (Figure 1E) (Zou and Hastie, 2005). We first predicted median residual viability of EGFR-mutant cells as a continuous measure based on the fingerprints of compounds containing thiazoles (n = 398) or quinazolines (n = 172). Overall, a high degree of correlation between predicted and observed median viability was achieved for thiazoles (median Pearson r = 0.74; p = 2.8 × 10^{−3}) (Figure 1F; Figure S1B) and quinazoline-based compounds (median Pearson r = 0.76; p = 2.2 × 10^{−4}) (Figures S1C and S1D). Similarly, when performing binary predictions of compounds as having either high or low anti-EGFR activity in the complete compound set, irrespective of underlying scaffolds, compounds predicted to have high anti-EGFR activity exhibited significantly lower residual viabilities (p = 1.0 × 10^{−8}; area under the curve [AUC] 0.88) (Figure 1G). In an independent validation with data of the GlaxoSmithKline...
Protein Kinase Inhibitor Set (GSK PKIS) compounds, the elastic net model also reliably predicted high versus low activity against L858R-mutated EGFR (p = 1.9 × 10⁻¹⁹; AUC 0.85; sensitivity 77.2%; specificity 81.9%) (Figure S1E).

Thus, our inhibitor screening data capture major genomic dependencies and our elastic net-based algorithm for the systematic deconvolution of genotype-chemotype relationships may be useful for the analysis of similar large-scale screening datasets.

**NMC Cells Are Sensitive to CDK9 Inhibition**

The second most abundant genotype-chemotype interaction present in our dataset was identified for a *BRD4-NUT*-rearranged cell line (HCC2429) (Figure 1D) (Yan et al., 2011), which was among the cell lines with the highest degree of sensitivity toward several compounds (Figure S1F). *BRD4-NUT* fusions are a hallmark of NMC, a rare but highly aggressive tumor type associated with poor response to standard chemotherapy (French et al., 2003; Stathis et al., 2016). Among selective compounds with strong activity against HCC2429 cells, we identified LDC67, a known CDK9 inhibitor, as the most genotype-selective inhibitor (Figure 2A) (Albert et al., 2014). The 10 most active compounds shared structural features with LDC67 and known CDK inhibitors (Figure S2A) (Albert et al., 2014; Morales and Giordano, 2016; Rossi et al., 2005), suggesting that these chemotypes may be suited as a backbone for CDK inhibitors. To further validate our findings, we determined half-maximal growth inhibitory concentrations (IC₅₀) values of LDC67 across 64 cell lines, including three NMC cell lines (HCC2429, 143100, and 690100), and found significantly (p < 1 × 10⁻⁴) higher activity in all *BRD4-NUT*-rearranged cells compared to tumor cells lacking the rearrangement (Figure 2B; Figure S2B). We also observed a similar activity profile in the cases of the CDK inhibitor AT7519 (p = 5 × 10⁻⁴) (Squires et al., 2009) and the bromodomain inhibitor JQ1 (p = 1.4 × 10⁻⁴), which was previously shown to be active in NMC cells (Figures S2C and S2D) (Filippakopoulos et al., 2010). In line with these observations, CDK9 inhibition led to significantly reduced cell growth of *BRD4-NUT*-rearranged cells in clonogenic assays (p = 9 × 10⁻⁴) (Figure 2C) and an induction of apoptosis at 24 hr (p = 0.001) and 48 hr (p = 0.005). Similarly, LDC67 treatment led to a significant increase in the sub-G1 fraction (control, 8.0%; LDC67, 57%; p = 0.047) in *BRD4-NUT*-rearranged cells, but not in control cells (Figure S2E). We subsequently sought to determine potential mechanistic links between CDK9 inhibition and apoptosis induction. Because CDK9 has been described as conferring an apoptosis-primed state by repressing anti-apoptotic Mcl-1 (Gregory et al., 2015; Huang et al., 2014; Lemke et al., 2014), we analyzed Mcl-1 expression levels under LDC67 treatment as a function of time. Mcl-1 protein expression was almost abrogated in HCC2429 cells, but not in A549 (KRAS<sup>mut</sup>) cells (Figure 2E). In addition to interfering with global transcription and altering the balance of pro- and anti-apoptotic proteins, CDK9 was shown to be involved in the DNA damage response (Yu et al., 2010; Zhang et al., 2013). We therefore investigated the levels of γH2AX and phospho-Chk2, surrogate markers of DNA damage (Yu et al., 2010), under LDC67 treatment. We observed strong upregulation of γH2AX and phospho-Chk2 in HCC2429, while such induction was considerably lower in A549 cells (Figure 2E). In addition, we were able to confirm a significant increase of γH2AX-positive HCC2429 cells (24 hr) by fluorescence-activated cell sorting (FACS) analysis after co-staining for γH2AX and cleaved caspase-3 (Figure 2F).

Overall, our data suggest that *BRD4-NUT*-rearranged NMC cells may be particularly vulnerable to CDK9 inhibition.

**BRD4-NUT-Driven Cells Display a Distinctive CDK9 Dependency**

To test a specific dependency of NMC cells on CDK9 expression that may explain the observed phenotype in CDK9i-treated cells, we performed short hairpin RNA (shRNA)-mediated knockdown of both components of P-TEFb, CDK9, and Cyclin-T1 (Figures 3A and 3B). Similar to CDK9 inhibition, we observed a significant (p = 2 × 10⁻⁴) reduction in cellular viability of NMC cells (HCC2429), but not of control cells (A549) (Figures 3A and 3B). We next tested the effects of LDC67 in NMC and control cells (A549 and HCC15) on phosphorylation of Pol II and observed a dose-dependent decrease of Ser2 phosphorylation, irrespective of the underlying genotype (Figure 3C). Previously, cellular efficacy of CDK9 inhibitors has been linked with changes in the complex formation of P-TEFb with its negative regulator HEXIM1 (Huang et al., 2014; Itzen et al., 2014; Lu et al., 2015; Morales and Giordano, 2016). To monitor such drug-induced effects, we performed immunoprecipitation assays of endogenous CDK9 in HCC2429 and A549 cells treated with LDC67. We observed a modest but reproducible reduction of HEXIM1-bound CDK9 in both cell lines (Figure 3D), with a more profound disruption of HEXIM1/CDK9 complexes in HCC2429 cells (72.6%) when compared to A549 cells (85.6%) after 4 hr LDC67 treatment (Figure 3D). We were able to validate this CDK9 inhibitor-induced effect when overexpressing FLAG-CDK9 in HCC2429 cells with a HEXIM1/FLAG-CDK9 ratio of 14.6% after LDC67 (4 hr) treatment (Figure S3A).

These data further highlight the relevance of CDK9 expression in NMC and suggest that the effects achieved by LDC67 may be attributable to direct inhibition of CDK9.

**CDK9-Specific Effects in NMC Cells**

Previous reports have implicated MYC expression as a relevant downstream effector of *BRD4-NUT*-driven cells in the context of BRD4 inhibitor treatment (Grayson et al., 2014; Sos et al., 2009b). To test the relevance of MYC expression in *BRD4-NUT*-rearranged cells, we performed shRNA-mediated *MYC* knockdown and observed a significant (p = 8.1 × 10⁻⁴) reduction of viability in HCC2429 cells that did not strongly differ (p = 0.1) from that of A549 control cells (Figure S3B). When monitoring Myc protein levels during drug treatment, bromodomain inhibition with JQ1 led to a constant decrease of Myc protein expression as expected (Figures 4A and 4B; Figure S3C). To our surprise, we observed an initial moderate increase of Myc protein levels and a subsequent reduction after 48 to 72 hr of LDC67 treatment in HCC2429 *BRD4-NUT*-rearranged cells, but not in A549 control cells (Figures 4A and 4B; Figure S3C) (Lu et al., 2015). We observed a similar reduction of Myc protein levels, together with an increase in γH2AX and depletion of Mcl-1 in the 143100 NMC cells but without the initial Myc increase, suggesting that the effect on Myc expression may be cell line specific (Figure S3D). The overlapping effects on Myc expression...
Figure 2. CDK9 Inhibition Exhibits Distinct Effects on BRD4-NUT-Rearranged NMC Cells

(A) Activity of selective compounds against BRD4-NUT-rearranged HCC2429 cells. To obtain the most genotype-selective inhibitor, the percentage of cell lines that were not impacted below the hit threshold was calculated for the ten most potent compounds (inset).

(B) GI50 values from LDC67 dose-response curves (72 hr) across 64 cell lines.

(C) Clonogenic survival assays of HCC2429 and A549 of LDC67 treatment or DMSO control (mean ± SD; n = 3).

(D) Apoptosis measured by Annexin V flow cytometry in BRD4-NUT-rearranged and control cells following treatment with 10 μM LDC67 (mean ± SD; n = 3).

(E) Immunoblot of HCC2429 and A549 cells treated with LDC67 for the indicated periods.

(F) HCC2429 cells treated for 24 hr with 10 μM LDC67, 0.5 μM JQ1, or DMSO were co-stained for cleaved caspase-3 (CC3) and γH2AX and measured by flow cytometry (mean ± SEM; n = 3; p values calculated by two-tailed t tests).
induced by JQ1 and LDC67 may partly explain the additive effects observed for the combination of both compounds and partial cross-resistance of JQ1-persistent HCC2429 clones (GI50 = 11.62 μM JQ1P versus GI50 = 72 nM parental) (Figures S3E–S3H).

We also observed that treatment with both inhibitors led to induction of cleaved caspase-3 within 24–48 hr (Figure 4A). Using a more quantitative approach, we noticed a significantly higher fraction of apoptotic cells under LDC67 compared to JQ1 treatment in flow cytometric analyses (LDC67 61% versus JQ1 31%; p = 0.02) (Figure S3I). By contrast, only bromodomain inhibition, not CDK9 inhibition, led to a dramatic induction of the p53 target gene p21 and a block of S phase entry (Figures 4A–4E; Figure S3J). In parallel, in JQ1-treated, but not LDC67-treated, HCC2429 cells, we observed disassembly of hyperacetylated/p300-positive foci that may lead to restoration of p53 activity, as described in previous reports (Figures 4F and 4G) (Huang et al., 2014; Reynoird et al., 2010).

Thus, our data indicate that in contrast to bromodomain inhibition, CDK9 inhibition does not lead to a cell-cycle arrest and that over time, both perturbations induce a similar reduction of Myc expression.

**CDK9 Inhibition Perturbs Defined Transcriptional Programs in NMC Cells**

To further investigate the signaling patterns induced by CDK9 inhibition, we performed transcriptome profiling (RNA sequencing [RNA-seq]) in NMC cells. RNA-seq indicated that LDC67 treatment induces an initial increase (8 hr), followed by downregulation (48 hr) of transcripts involved in RNA binding and translation, of ribosomal subunits in gene set enrichment analyses (GSEAs) and included known surrogate markers of P-TEFb complex activity, such as FOS (Figures 5A–5C; Figure S4A; Tables S1 and S2) (Lu et al., 2015; Stathis et al., 2016; Yan et al., 2011). We were also able to confirm that LDC67 and JQ1 treatment was associated with a perturbation of cellular processes linked with Myc activity (Figure 5C; Tables S3 and S4). The changes induced by LDC67 were most prevalent in genes regulated by promoters with high affinity for Myc (Figure S4B) (Lorenzin et al., 2016; P.J. O’Dwyer et al.,...
As expected, the timing of the transcriptional changes and the individual gene sets in HCC2429 cells treated with the bromodomain inhibitor JQ1 strongly differed from those treated with the CDK9 inhibitor LDC67 (Figure 5C; Figures S4A–S4D). We also observed a robust enrichment of genes involved in cytoskeletal regulation in JQ1-treated cells and a reduction of cell-cycle gene sets (Figure 5C; Figure S4A; Tables S1 and S2), which may correspond to the morphological changes induced by JQ1 (Figure S4E) (Alekseyenko et al., 2015; Filippakopoulos et al., 2010; Grayson et al., 2014; Stathis et al., 2016).

To validate our RNA-seq results and to assess the impact of CDK9i on de novo transcription, we performed qRT-PCR of mature mRNA and of unspliced pre-mRNA for a set of upregulated genes (FOS, JUNB, and MYC) and downregulated genes (FOXO6 and KLHL23) after LDC67 treatment. We chose 18S rRNA for qPCR normalization that remained stable under inhibitor treatment while RNA-seq normalizes expression relative to the complete transcriptome. Overall, the RNA-seq results validated well for mature and pre-mRNA (Figures 5B and 5E). Increased pre-mRNA levels of FOS and JUNB suggest that these genes are actively transcribed despite CDK9i. However, the strong decrease of FOXO6 and KLHL23 pre-mRNA indicates CDK9i-mediated elongation defects and abrogation of de novo transcription. MYC mRNA did not increase but instead stayed constant at 8 hr of LDC67 treatment, followed by a delayed reduction at 48 hr (Figure 5B). The apparent difference to the RNA-seq results is most likely due to the aforementioned differences in normalization. Furthermore, MYC pre-mRNA levels were decreased after 8 and 48 hr of LDC67 treatment (Figure 5B). As expected, bromodomain inhibition with JQ1 led to a constant decrease of MYC pre-mRNA, mature mRNA, and protein expression (Figure 4A; Figures 5D and 5E). Depletion of CDK9 or Cyclin-T1 in these cells led to a similar reduction of premature and mature MYC mRNA corresponding to the respective knockdown efficacies (Figure 5F; Figure S4F).
Thus, CDK9 inhibition may lead to defined genotype-specific transcriptional changes but may also interfere with MYC mRNA stability and increase MYC translation rather than de novo transcription.

**CDK9 Inhibition Has a Major Effect on Transcriptional Elongation in NMC Cells**

We next sought to directly evaluate the effect of CDK9 inhibition on the process of transcriptional elongation. To this end, we performed chromatin immunoprecipitation (ChIP) experiments after short-term CDK9 inhibition and measured Pol II occupancy for genes in which expression was increased (MYC, FOS, JUNB, and SF3B4) or did not increase (FOXO6, KLHL23, BRG1, and NPM1) relative to the global transcriptome in RNA-seq analyses after short-term CDK9 inhibition (Figures 6A–6C; Figure S5). When assessing Pol II distribution with an antibody raised against the unphosphorylated C-terminal domain (CTD), Pol II occupancy in the gene body (GB) remained constant or was even increased in upregulated genes, while the GB signal was decreased in the other genes (Figures 6A–6C; Figure S5). The signal at the transcription start site (TSS) was more variable. Overall, this translated into decreased pausing indices (PIs, or the ratio of TSS-bound Pol II to GB-bound Pol II) for the upregulated genes and constant or increased PI for the other genes (Figure 6D). These findings are compatible with higher transcription rates in the upregulated genes and correspond to significantly lower RNA expression of the genes with increased

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**Figure 5. Transcriptional Dynamics after CDK9 Inhibition in NMC Cells**

(A) Time course of RNA-seq log2 fold changes for all genes (gray lines) between LDC67-treated and control (cont.) HCC2429 cells. Two gene ontology (GO) gene sets (yellow and green) and MYC and FOS (red) are indicated. Error bars represent median and 10% or 90% quantiles of all log2 fold changes at respective time points.

(B) qRT-PCR (normalized to 18S rRNA) of selected genes following LDC67 (10 μM) or DMSO control (cont.) for mature mRNA (left) and for unspliced pre-mRNA (right) (mean ± SD; n = 3).

(C) GO terms enriched in gene set enrichment analysis (GSEA, C5) of RNA-seq data from HCC2429 treated (48 hr) with JQ1 (gray) or LDC67 (blue) compared to controls (x axis, normalized enrichment score; FDR-corrected q values < 0.1 are considered significant).

(D) Time course of RNA-seq log2 fold changes for all genes (gray lines) between JQ1-treated and control (cont.) HCC2429 cells. Color codes as in (A).

(E) qRT-PCR (normalized to 18S rRNA) of selected genes following JQ1 (500 nM) treatment for mature mRNA (left) and for unspliced pre-mRNA (right) (mean ± SEM; n = 3).

(F) qRT-PCR time course of MYC mRNA normalized to 18S rRNA after CDK9 (black) or Cyclin-T1 (gray) knockdown compared to shGFP controls (mean ± SEM; n = 3).
pausing (p = 0.01 for PI > 1 versus PI < 1) (Figure 6E) (Huang et al., 2014; P.J. O’Dwyer et al., 2016, Cancer Res., abstract). By contrast, A549 cells showed reduced Pol II occupancy in all genes on the GB and at varying degrees at the TSS (Figures 6B and 6C; Figure S5).

To further investigate CDK9i-induced Pol II distribution, we performed ChIP analyses for Pol II p-Ser5 and Pol II p-Ser2, which indicate poised Pol II and elongating Pol II, respectively. As expected, the signal for p-Ser5 Pol II corresponded well to the total CTD-Pol II across the TSS and GB (r = 0.98, Figure S5).

**Figure 6. Effects of CDK9 Inhibition on Pol II Occupancy**

(A and B) ChIP qPCR with an antibody raised against the unphosphorylated CTD assessing Pol II occupancy (displayed as a percentage of input DNA) at the transcription start site (TSS) and the gene body (GB) of FOS and FOXO6 in HCC2429 (A) and A549 (B) after 4 hr LDC67 (10 μM) treatment compared to DMSO controls (mean ± SD, n = 3).

(C) Relative changes of unphosphorylated CTD Pol II signal at TSS and GB between LDC67 and control cells.

(D) Pausing index (Pol II at TSS to Pol II in GB) for the selected genes normalized to DMSO control (mean ± SEM; n = 3).

(E) Log2 fold changes in RNA-seq of HCC2429 cells treated with LDC67 (8 hr and 10 μM) for the genes used for ChIP experiments. The p value between genes with high versus low PI was calculated by a Welch t test.

(F) Proposed model of the differential effects of CDK9 and JQ1 inhibition in NMC cells.
Pol II pause release and productive elongation at 2842 genes (Figure S5). Surprisingly, we noticed a more pronounced KLHL23 et al., 2016) vulnerabilities and potential therapeutic targets (Barretina et al., 2015; Seashore-Ludlow et al., 2015; Sos et al., 2009b, 2009a). Overall, differential effects were observed not only between HCC2429 and A549 but also among the genes investigated in HCC2429. Altogether, these data underline the distinctive role of CDK9 for transcriptional control in NMC cells, which may be linked with their specific vulnerability to CDK9 inhibition.

DISCUSSION

Systematic screening of genetically annotated cancer cell lines has proved to be a suitable tool for the identification of genetic vulnerabilities and potential therapeutic targets (Barretina et al., 2012; Garnett et al., 2012; Iorio et al., 2016; Martins et al., 2015; Seashore-Ludlow et al., 2015; Sos et al., 2009b, 2009a). Our scoring approach involving 1,505 kinase inhibitors coupled with a systematic deconvolution and prediction of geno-type-chemotype relationships enabled a structure-based prediction of biological activity in silico and may thus be of value to focus future screening projects on the most promising candidate compounds.

To our surprise, one of the most striking genotype-specific vulnerabilities in our screen was the exquisite activity of LDC67, a known CDK9 inhibitor in NMC cells (Albert et al., 2014). Our chemical genomics approach uncovered a role of CDK9 as a non-oncogenic driver for tumorigenesis in BRD4-NUT-dependent cells mediated by regulation of transcription and Myc protein levels in NMC. CDK9 has also been identified as a key regulator of transcriptional regulation in MYC-overexpressing hepatocellular carcinoma (Huang et al., 2014). However, the evident CDK9i-induced differences on the level of Pol II-mediated transcriptional elongation observed in NMC and hepatocellular carcinoma indicate that these processes may by distinct for individual lineages.

NMC is a rare but highly aggressive tumor with a median survival of 6.7 months for which no approved therapies exist (Stathis et al., 2016). An initial report from a BET inhibitor phase I/II trial (GSK525762 and NCT01587703) described partial responses in 2 of 10 NMC patients (P.J. O’Dwyer et al., 2016, Cancer Res., abstract), while another preliminary analysis reported a partial response in 3 of 4 NMC patients after BET inhibitor OX015/MK-8628 with relapse within a few months (Stathis et al., 2016). Of 10 NMC cases treated with GSK525762, four patients responded with stable disease (P.J. O’Dwyer et al., 2016, Cancer Res., abstract). This is in line with the previous observations and our results, indicating that BRD4 inhibition leads to dissolution of hyperacetylated nuclear foci, release of p53 with induction of p21, cell-cycle arrest, and differentiation (Figures 4, 5C, 5D, and 6F; Figure S4E) (Alekseyenko et al., 2015; Grayson et al., 2014; Reynoird et al., 2010; Yan et al., 2011). By contrast, our data reveal that CDK9i may lead to robust Mcl-1 suppression, induction of DNA damage response and apoptosis in these cells (Figure 6F). For several genes, including FOS, transcription is increased following CDK9 inhibition. This has partly been attributed to CDK9i-induced release of P-TEFb from its inhibitory complex with HEXIM1 by a CD99 inhibitor (Lu et al., 2015), an effect we also observed in NMC cells (Figure 3D and 6F; Figure S3A). We speculate that this phenomenon may be related to structural changes of P-TEFb induced by CDK9 inhibitor binding (Baumli et al., 2008). Although CDK9i-induced perturbation of MYC expression partially overlaps with the effects of bromodomain inhibition, it remains to be seen how these effects contribute to the overall cellular phenotype observed for these types of inhibitors. Overall, our findings uncover major molecular differences between the mode of action of bromodomain and that of CDK9 inhibitors in NMC and suggest that CDK9 may be an attractive drug target in NMC patients.

In the past, clinical studies investigating spectrum CDK inhibitors such as dinaciclib or flavopiridol reported high rates of side effects and dose-limiting toxicities (Kumar et al., 2015; Morales and Giordano, 2016), but more selective compounds such as ribociclib (CDK4 and CDK6) demonstrated the feasibility of CDK inhibition even as first-line cancer treatment (Hortobagyi et al., 2016). For this reason, several CDK9 inhibitors with improved selectivity profiles were developed and hold promise for future development in clinical applications (Albert et al., 2014; Lam et al., 2014; Lu et al., 2015; Morales and Giordano, 2016). Our findings may therefore be of relevance for the future development of these drugs and the stratification of patients receiving these types of selective CDK9 inhibitors.

In conclusion, our study provides a framework for the deconvolution and prediction of genotype-chemotype relationships in a large-scale kinase inhibitor screen and identifies CDK9 as a druggable target in NMC. Our results also provide insight into CDK9 exerted control of transcriptional elongation and its geno-type-specific effects in BRD4-NUT-rearranged tumors.

EXPERIMENTAL PROCEDURES

High-Throughput Screening

For high-throughput screening 78 genomically annotated patient-derived lung cancer cell lines were assayed against 1,505 small-molecular compounds predominantly consisting of compounds before lead or target-based optimization across a range of chemical scaffolds and a number of established reference kinase inhibitors. For screening, cell lines were treated at a single-dose concentration, which was determined during a preliminary screen. Residual viability was assessed after 72 hr by CellTiter-Glo (CTG, Promega). Chemical
information was captured by simplified molecular input line entry specification (SMILES) codes and by manual annotation of scaffolds (Figure S8). A number of compounds and cell lines were screened in duplicate to assess reproducibility. Moreover, external validity was assessed in a subset of compounds and cell lines by testing compound activity in dilution series to assess GI50 after 72 hr by CTG (Promega). Genotype-specific compound activity was assessed using an ANOVA approach similar to previous studies (Barretina et al., 2012; Garnett et al., 2012; Seashore-Ludlow et al., 2015), incorporating genotype and histological subtype in a random effects model. Activity predictions were done with elastic net regression models using ECFP6 fingerprints of the compounds as the predictor and residual viability or compound activity as the response. Models were trained on a subset of compounds with 10-fold cross-validation and were evaluated on the compounds not involved in model building and on an external validation dataset (Elkins et al., 2016).

**Apoposis, Proliferation, and Survival Assays**

Apoposis was measured by flow cytometry following Annexin V and propidium iodide staining on a FACSS Gallos Flow Cytometer and the corresponding Kaluza analysis software (Beckman Coulter, USA). Cell-cycle analyses were performed by flow cytometry on methanol-fixed cells after propidium iodide staining. For FACs analysis of cleaved caspase-3 (CC3) and γH2AX, cells were treated for indicated times, harvested by trypsinization, and fixed in 80% methanol. Fixed cells were permeabilized and blocked with PBS/1% BSA before they were incubated with primary antibodies at 4°C overnight. The following day, cells were washed, incubated with Alexa Fluor secondary antibodies (Thermo Scientific), and measured on a Gallos Flow Cytometer (Beckman Coulter, USA).

For clonogenic survival assays, cells were seeded in 6-well plates, treated for indicated times, fixed with 4% formaldehyde, and stained with crystal violet solution. For quantification, a 1% SDS solution was added to the wells for 30 min and absorption was measured at 590 nm in the supernatant.

**Immunoblot, Immunoprecipitation, and Immunofluorescence Assays**

Cellular signaling following LDC67 or JO1 treatment was assessed by protein gel electrophoresis. Equal amounts of protein lysates were separated on 4%–20% Novex Tris-glycine gels (Invitrogen), transferred to polyvinylidene fluoride (PVDF) membrane, and incubated with indicated primary antibodies. Proteins were detected with the Odyssey CLx imaging system (LI-COR Biosciences). For immunoprecipitation, antibodies directed against endogenous CDK9 or transiently transfected FLAG-CDK9 were used for precipitation at 4°C overnight, followed by immunoblotting. FLAG-CDK9 plasmids were a gift of Prot. Qiang Zhou (University of California, Berkeley, USA) (Lu et al., 2019) and were transiently transfected before immunoprecipitation.

For immunofluorescence, cells were grown on coverslips and treated for 24 hr before fixation with 4% formaldehyde, followed by staining with the indicated primary antibodies at 4°C overnight. Samples were incubated with Alexa Fluor secondary antibodies (Thermo Scientific) for 2 hr and mounted with DAPI before imaging (Zeiss Meta 510 or Zeiss Meta 710).

**shRNA Knockdowns**

For knockdowns, respective shRNAs or shRNA against GFP (shGFP) were generated with pLKO.1-puro vectors. Replication-deficient lentiviruses were produced in HEK293T cells by co-transfection of pLKO.1-puro vectors and helper plasmids. Supernatant collected 48 hr after transfection of HEK293T cells was used to transfect HCC2429 and A549 cells. Knockdown efficiency and effects on cell viability were validated by immunoblotting and CTG (Promega) 4–6 days after transfection, as described previously (Sos et al., 2009a).

**ChIP**

For ChIP experiments, cells were cross-linked with formaldehyde before chromatin was extracted, sonicated, and incubated with primary antibodies (Poi II, pSer2-Poi II or pSer5-Poi II) or mouse immunoglobulin G (IgG) overnight. Antibody complexes were then captured with protein G beads, and DNA was eluted, decrosslinked, and purified. ChIP signals were calculated by qPCR (Table S5) relative to input levels after (IgG) background subtraction.

**RNA Analysis**

For RNA-seq and qPCR (Table S5) analyses, total RNA was isolated following LDC67 or JO1 treatment. 3′ RNA-seq libraries were prepared with the QuantSeq FWD 3′ mRNA-Seq Kit (Lexogen, Austria), sequenced on an Illumina HiSeq 4000, and quantified after alignment to the human genome reference hg38. Data processing and statistical analyses were performed using Microsoft Excel (Microsoft, USA), GraphPad (Prism, USA), and R (R Development Core Team, 2011). Half-maximal growth inhibitory (GI50) concentrations of cell viability were inferred by fitting sigmoidal dose-response curves. Data are represented as mean ± SEM, and significance was calculated by unpaired Student’s t tests or Mann-Whitney tests unless indicated otherwise. The p values are always two-sided. For details, see Supplemental Information.

**ACCESSION NUMBERS**

The accession number for the RNA-seq data sets reported in this paper is European Genome-Phenome Archive: EGA50001002588.

**SUPPLEMENTAL INFORMATION**

Supplemental Information includes Supplemental Experimental Procedures, six figures, and five tables and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2017.08.082.

**AUTHOR CONTRIBUTIONS**

J.B., M.A.D., F.D., J.M.H., A.C., S.B., C.L., V.T., A.R., D.B., Z.F., S.O.-C., M.G., M.T., M.S., and H.B.-W. performed experiments. A.C., J.E., U.K., F.D., Z.F., P.H., M.G., M.T., and M.S. performed drug screening. J.B., F.L., and M.P. analyzed RNA-seq data. C.A.F. provided NMC cell lines. Z.G., Z.V., L.O., G.K., and S.A. helped in design and provided compounds and chemical structural information. J.B., M.A.D., S.B., A.C., Z.F., C.A.F., Z.V., L.O., G.K., H.C.R., S.A., L.C.H., R.B., D.R., B.M.K., R.K.T., and M.L.S. conceived experiments and wrote the manuscript. R.K.T. and M.L.S. conceived the study.

**ACKNOWLEDGMENTS**

We thank Prof. Oliver Gautschi (Cantonal Hospital Lucerne, Switzerland) for insightful discussions, Dr. Graziai Bosco (University of Cologne, Germany) for help with RNA-seq data processing, and Prof. Nicolas Friedrich (University of Cologne, Germany) for immunohistological staining. We thank Prof. Qiang Zhou (University of California, Berkeley, USA) for providing FLAG-CDK9 constructs. B.M.K. dedicates this work to the memory of Dr. György Keri, CEO of Vichem and professor at the Semmelweis University, Budapest, Hungary. György recently lost his fight against cancer. He was a great scientist and a wonderful friend. We are also sad to announce that Z.G. passed away during the course of this project. With him we lost a productive researcher and much-valued colleague. This work was supported by the German federal state North Rhine Westphalia (NRW) as part of the FIT program (grant 314-4000-1209 to B.M.K. and LDC), by the European Union (European Regional Development Fund: Investing In Your Future) as part of the PerMedNERW initiative (grant 005-1111-0025 to R.K.T., D.R., R.B., and LDC) and the EFRE initiative (grant EFFRE-0800397 to B.M.K., H.C.R., D.R., R.K.T., M.L.S., and LDC), by the German Ministry of Science and Education and BMBF as part of the eMed program (grants 01ZX1303 and 01ZX1603 to R.K.T., H.C.R., R.B., M.P., and D.R. and grant 01ZX1406 to M.L.S. and M.P.), by the German Consortium for Translational Cancer Research (DKTK) Joint Funding program (to R.K.T.), and by the BMBF as part of the NGFNplus program (grant 01GSO8100 to R.K.T.). F.D. was supported by the Mildred-Scheel-Doktorandenprogramm of the Max-Planck Society (grant 110770 to F.D. and R.K.T.). V.T. is the recipient of a joint ERS/EMBO Long-Term Research Fellowship (LTRF 2014-2951) and a Swiss Cancer League postdoctoral research fellowship (BI5 KFS-3402-02-2014). R.K.T. is a consultant of NEO New Oncology GmbH and received honoraria from AstraZeneca, Bayer, NEO New Oncology GmbH, Boehringer Ingelheim, Clovis Oncology, Daiichi-Sankyo, Eli Lilly, and Cell Reports 20, 2833–2845, September 19, 2017 2843
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Supplemental Information

Systematic Kinase Inhibitor Profiling Identifies CDK9 as a Synthetic Lethal Target in NUT Midline Carcinoma

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Figure S1

Performance measures of the high-throughput cell line screen and structural activity modeling (Related to Fig. 1). a) Residual viabilities in the high-throughput screen of 1505 compounds against 78 NSCLC cell lines. b) Distribution of differences between predicted and observed residual viabilities in EGFRmut cell lines in the validation sets of 100 elastic net models (histogram) and p-values of respective correlations between predicted and observed/randomly permuted viabilities of thiazole compounds (boxplot). c) Pearson correlation coefficients, residuals and correlation p-values for 100 elastic net models predicting residual viability for quinazoline based compounds in EGFRmut cell lines. d) GSK PKIS compounds ordered by median activity against EGFRmut cell lines colored according to high (yellow) vs. low (grey) predicted activity based on the threshold determined from the LDC compound training set. Mann-Whitney p-value based on ordering by observed EGFR L858R activity. Inset shows ROC analysis across different prediction cut-offs with corresponding AUC and 95% Confidence Interval. f) Number of compounds with hits per cell line.
Figure S2
Compound and target specific phenotypic effects on NMC cells (Related to Fig. 2). a) Top ten most active compounds against HCC2429 cell line. b) Dose response curves (72h) as assessed for CDK9 inhibitor LDC67. c)+d) GI₅₀ values from dose response curves across NSCLC cell lines (n=13) with multiple genotypes for multi-CDK inhibitor AT7519 and BRD4 inhibitor JQ1 (mean±SEM from n≥2 experiments in triplicates each; two-tailed t-test). e) Distribution of average cell cycle states in BRD4-NUT-rearranged (HCC2429, 690100 and 143100) and control cells (A549, HCC15) measured via FACS analysis of propidium-iodide stained cells after LDC67 (10µM) treatment for indicated time periods.
**Figure S3**

**Differential effects of bromodomain and CDK9 inhibition (Related to Fig. 4).**

a) Immunoprecipitation (IP) of FLAG-CDK9 after transient overexpression in HCC2429 cells show lower amounts of CDK9-bound HEXIM1 after LDC67 treatment (n=2). In transfected input controls both FLAG-CDK9 (1) and CDK9 wt (2) are expressed.

b) Viability measured with CTG 96h after infection with shRNA directed against MYC or eGFP in HCC2429 and A549 (left panel mean±SEM, n=3; two-tailed t-test). Knock-down efficiency was validated by immunoblot (bottom).

c) Immunoblot of A549 (KRAS mut) treated with JQ1 or LDC67 for indicated times.

d) Immunoblot of 143100 (BRD4-NUT) and HCC15 (NRAS mut) cells after LDC67 treatment to assess impact on Myc, Mcl-1, and yH2AX levels.

e) Combination index (CI, circle) values were calculated for every data point of the combined JQ1 and LDC67 treatment matrix. Trend line (blue) indicates CIs interpolated at every effect level.

f) Isobologram analysis of combinatorial treatment with LDC67 and JQ1 in HCC2429 cells to achieve 75% growth inhibition.

**Figure S3**

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Figure S4
Expression changes induced by CDK9 or bromodomain inhibition in NMC cells (Related to Fig. 5). a) Gene ontology terms enriched in HCC2429 by gene set enrichment analysis against the MSigDB C5 collection after 8h of treatment with LDC67 or JQ1 by RNAseq (x-axis: normalized enrichment score; FDR-adjusted q-values indicate significance). b) RNAseq expression changes of MYC-binding genes with high or low MYC affinity (as defined by Lorenzin et al. (Lorenzin et al., 2016)) after LDC67 treatment (FDR-adjusted p-values of Wilcoxon signed rank tests above gene sets; x-axis: GO sets as given by Lorenzin et al. with Str const rib = Structural constituent of ribosome; Cell bios process = Cellular biosynthetic process; Subst spec transp activity = Substrate specific transporter activity; G-prot coupled recept activity = G-protein coupled receptor activity) c) Overlap of significantly differentially expressed genes (p<0.01) of JQ1 (8h) vs. DMSO and LDC67 vs. DMSO in RNA-Seq. d) Heatmap representation of significant genes (columns) from S4c) with individual replicates (rows) being displayed. Color code in columns indicate whether a gene was significant after JQ1 or LDC67 or both treatments. Red box marks the small set of genes regulated by both inhibitors. d) Brightfield microscopic images (10x microscope objective) of HCC2429 cells after 72h treatment with JQ1 or LDC67 compared to DMSO control cells indicate the morphological changes after JQ1. e) Relative mRNA levels of CDK9 and Cyclin T1 (CCNT1) as assessed by qRT-PCR normalized to 18S rRNA following knock-down of CDK9 (left) or CCNT1 (right) (mean±SEM, n=3).
Figure S5
ChIP experiments in NMC and control cells following CDK9 inhibition (Related to Fig. 6). ChIP qPCR assessing Pol II, Pol II p-Ser2, and Pol II p-Ser5 occupancy (displayed as percentage of input DNA) at the transcription start site (TSS) and the gene body (GB) of indicated genes in HCC2429 and A549 after 4h LDC67 treatment compared to DMSO controls. ChIPs were performed with antibodies against phosphorylated Serine-2 of the CTD (pSer2, light grey), against phosphorylated Serine-5 of the CTD (pSer5, dark grey) or an antibody raised against the unphosphorylated CTD (Pol II, black). Each experiment was performed with three independent replicates, values represent mean±SD).
Figure S6

Characteristics of the compound libraries used for screening and for validation of activity modeling (Related to Fig. 1). a) Distribution of Z-factors for the subset of compounds (n=197) pre-screened at 1µM and 10µM to determine optimal screening concentration. Significance was determined via a two-tailed paired Wilcoxon signed rank test. b) Correspondence between residual viability determined in high-throughput screen and GI50 values determined in dilution series experiment for a subset of compounds (n=228) and cell lines (n=62, significance determined by Kruskal-Wallis test between groups). c) Distribution of inhibitory activity against L858R mutated EGFR in in vitro kinase assay across GSK PKIS compounds, displayed as residual kinase activity at a 1µM concentration. d) Pairwise Tanimoto coefficients based on ECFP6 fingerprints within GSK (red) and LDC (blue) library and between compound libraries (yellow), indicating a slightly higher chemical similarity within each library. e) Unsupervised hierarchical clustering (complete linkage, Euclidean distance) based on Tanimoto coefficients between GSK PKIS (green) and LDC (red) compounds.
Supplemental Methods

High-throughput cell line screening
To systematically evaluate vulnerabilities in non-small cell lung cancer (NSCLC) we utilized a set of 78 genomically annotated, patient derived NSCLC cell lines that capture an array of genetic alterations and histological subtypes and had been compiled during a previous study (Sos et al., 2009). Identity of cell lines was validated by short tandem repeat (STR) profiling or by SNP arrays. We are aware the NCI-H157 was shown to be derived from the same individual as NCI-H1264 and therefore did not include NCI-H1264. Cell lines were cultured at 37°C in a humidified incubator and routinely passaged using 0.25% trypsin in culture medium supplemented with 10% fetal calf serum (FCS) and 1% Penicillin/Streptomycin according to manufacturer instructions and were tested for Mycoplasma infection at regular intervals.

The compound panel containing 1505 small molecule kinase inhibitors was purchased from ViChem Chemie (Budapest, Hungary). To detect novel compound-genotype interactions, primarily compounds that had thus far been uncharacterized regarding biological activity and had not undergone any significant target-based lead optimization were included following selection by ViChem and the LDC (LDC Dortmund, Germany). Moreover they were chosen so as to cover a range of chemical scaffolds with known activity in small molecular inhibitors. In addition we included the established kinase inhibitors erlotinib (Pao et al., 2004), gefitinib (Ciardiello et al., 2001), afatinib (Li et al., 2008), PD318088 (Ohren et al., 2004), PD334581 (Ohren et al., 2004), selumetinib (Yeh et al., 2007), enzastaurin (LDC041740:01) (Graff et al., 2005), 17-AAG (LDC041741:01) (Hostein et al., 2004), tofacitinib (LDC041833:01), Dovitinib (LDC041934:01) (Trudel et al., 2005), fasudil (LDC001436:01) (Shirotani et al., 1991), wortmannin (LDC041556:01) (Arcaro and Wymann, 1993), tozasertib (LDC000454:01) (Harrington et al., 2004), SB216763 (LDC042081:01) (Coghlan et al., 2000), GSK269662A (LDC042082:01) (Do et al., 2007), PD157655 (Sartor et al., 1997), BX912 (LDC042098:01) (Feldman et al., 2005), and staurosporine (Andrejauskas-Buchdunger and Regenass, 1992). Structure information was available for all compounds encoded as SMILES codes (Weininger and Weininger, 1989) and main chemical core structures/scaffolds were annotated manually. An additional representation of chemical structure can be achieved by molecular fingerprints that encode presence of chemical motifs as binary bit strings (Maggiora et al., 2014). The length of a molecular fingerprint thus represents the number of distinct chemical features present in a given molecule. To accurately capture the chemical complexity of compounds we therefore calculated ECFP6 fingerprints (Riniker and Landrum, 2013; Rogers and Hahn, 2010) for all compounds using the R package reddk (Guha, 2007).

Compounds were dissolved in DMSO at 10mM concentrations and stored in aliquots at -20°C or -80°C, following manufacturer’s instructions. For high-throughput compound activity profiling cell lines were plated in 384 well plates 24h before compounds were added at a single dose using a robotic pin tool. Residual viability between treated wells and untreated controls (8 wells per cell line) was determined 72h later with cell titer glo (CTG, Promega, USA). To determine the optimal screening concentration a pilot experiment with 197 compounds screened at 1µM and 10µM against the cell lines was performed. To assess the drugs’ abilities to exert differential effects among cell lines, for each compound and concentration the z’-factor (Zhang et al., 1999) was calculated. Overall, screening at 10µM achieved higher z’-factors than 1µM (median 0.5 vs. 0.1, p = 3.6x10^-22) (Fig. S6a). The superior discriminatory power of the higher concentration may be rationalized by the large number of premature compounds and screening of the remaining compounds was therefore performed at 10µM for a total of 1.2 x 10^5 compound-cell line combinations. Candidate hits were defined as z-scores of residual viability below -2 and the number of hits (n_hits) of a substance across all cell lines was used to categorize compounds as inactive, selective or toxic.

Assessment of internal and external validity
Reproducibility of the screening set-up was determined by duplicate screening of 7 cell lines (H2087, H1437, H3122, H322, HCC2429, H661 and DV90) against all compounds (median Pearson r = 0.93) and of 15 compounds screened against all cell lines, revealing a high reproducibility for selective compounds (median Pearson r = 0.90) and toxic compounds (Pearson r = 0.99). To assess the external validity of the screening approach 62 cell lines were subjected to dilution series experiments in 96-well plates with 8 different concentrations of 228 compounds. Viability was measured after 72h with CTG (Promega, USA). Half-maximal growth inhibitory concentrations (GI50) were inferred after fitting sigmoidal dose response curves with the R package drc (Ritz and Streibig, 2005). Compounds with strong (z-score < -2), intermediate (z-score -1 to -2) or no activity (z-score > -1) against a specific cell line in the initial screen showed a reasonable concordance with GI50 (p < 2.2x10^-16) (Fig. S6b). Moreover, residual viability obtained in the initial 384-well screen and residual viability at 10µM in the 96-well dilution series experiments showed good agreement with higher correlation for compounds with strong effects and more noise in inactive cell line-compound combinations (overall r = 0.6, p < 2.2x10^-16).
Genotype-specific compound activity
The cell line panel used for our study has been genomically characterized by others and us (Barretina et al., 2012; Garnett et al., 2012; Sos et al., 2009) and we employed known driver-alterations to assess genotype-specific compound activities. Following published approaches such as in (Barretina et al., 2012), (Garnett et al., 2012), (Stransky et al., 2015) and (Iorio et al., 2016) we employed a two-way ANOVA model on the log-transformed residual viabilities of selective compounds with one factor representing presence/absence of a genetic alteration and a second factor adjusting for differences in histological subtypes modeled as a random effect. To increase the detection power of candidate compounds for further follow-up and validation, both residual viability vectors from the duplicate screen of the BRD4-NUT positive cell line HCC2429 were included in this analysis. The overall false discovery rate (FDR) was adjusted at 10%.

Prediction of anti-EGFR activity based on chemical structure
Based on the observation that chemical scaffolds can act as a major determinant of compound potency, but are insufficient to fully determine biological activity we aimed to develop a computational model that integrates scaffold information and ECFP6-fingerprints to predict compound activity based on chemical structure alone. Due to the large number of compounds selectively inhibiting EGFR mutated cell lines PC9, HCC827 and H3255 and the well-established molecular mechanism of EGFR mutations, EGFR inhibition was chosen for prediction with elastic net regression and classification models based on the ECFP6 fingerprints (Jang et al., 2014; Zou and Hastie, 2005). In general, compounds were randomly split in a “training set” (90% of compounds) used for model training and feature selection applying 10x cross-validation. The models were evaluated on the remaining 10% of compounds not included in model building (“validation set”). In a first step we aimed to continuously predict the median residual viability of the three EGFR
mut cell lines following treatment. To this end an elastic net regression model (10x CV, \( \alpha = 0.1 \), minimizing mean squared error) was trained on 90% of all quinazolines or thiazoles using the R package glmnet (Friedman et al., 2010) and residual viability was predicted in the remaining compounds. This was repeated 100 times and concordance between predicted and observed values was recorded as residuals and correlation coefficients with corresponding p-values. To minimize the risk of structural biases in the compounds or in the model building influencing the results, we also applied a random permutation approach to obtain a null-distribution of expected correlation coefficients. To this end, predictions of the same models were also correlated after the viability vector in the respective validation set had been randomly shuffled (Rücker et al., 2007).

Additionally we performed an elastic net-based classification approach of all compounds irrespective of the underlying scaffold as having high or low anti-EGFR activity with high activity defined as a median residual viability in the EGFR
mut cell lines below the hit threshold (i.e. <25.9% residual viability). Model training was performed as described above except that for optimization of misclassification rate was used to tune model parameters. Discriminatory capability of the classification model was evaluated using the area under the curve (AUC) of receiver operating characteristics (ROC) in the validation set. The optimal threshold from a receiver operating curve (ROC) analysis of the training set was used to bin compounds in the validation set into high or low anti-EGFR activity prediction categories and to calculate sensitivity and specificity compared to observed activity.

External validation data set
To assess the generalizability of our model and to avoid confounding by the underlying chemical structure composition of the compound library or the biological screening methodology results from the Glaxo-Smith-Kline Protein-Kinase-Inhibitor-Set (GSK PKIS, a collection of 366 small-molecule inhibitors) (Drewry et al., 2014) were obtained. The GSK PKIS compounds have been tested at 1\( \mu \)M concentrations in in vitro kinase assays against several kinases including EGFR containing the activating L858R mutation. Results from this EGFR L858R kinase inhibition assay and chemical structures of all compounds were made publicly available (Fig. S6c) (Drewry et al., 2014; Elkins et al., 2015). Chemical similarity between both inhibitor libraries was assessed by ECFP6-fingerprint based pairwise Tanimoto coefficients (\( T_{CmpA,CmpB} = \frac{\# \text{shared features in A and B}}{\# \text{all features in A and/or B}} \)) (Maggiora et al., 2014) within and between both compound sets and by an unsupervised hierarchical clustering (complete linkage) by TCs based on ECFP6 fingerprints. Neither method indicated relevant systematic chemical differences between both libraries (Fig. S6d,e). Compounds with a TC > 0.9 (\( n = 10 \)) were excluded from the GSK PKIS set to avoid identical compounds in both libraries. Training of the classification model was performed on the complete LDC compound set after exclusion of unselective toxic compounds prior to prediction. Observed kinase inhibition activity between GSK PKIS compounds predicted as having high vs. low anti-EGFR activity was based on the cut-off determined in the training set and compared using a Mann-Whitney-U test. Discriminatory capacity was determined by the AUC.

BRD4-NUT positive midline carcinoma cell lines
Cell lines HCC2429 (included in the primary screen) (Haruki et al., 2005), 143100 and 690100 (used for validation of screening results) were a kind gift of Dr. Christopher French (Boston, USA). Cells were cultivated with DMEM medium, supplemented with 10% FCS and 1% penicillin/streptomycin. Presence of the BRD4-NUT gene fusion was validated using RNA sequencing as described previously (Fernández-Cuesta et al., 2015). In brief, cDNA libraries
were prepared by reverse transcription from PolyA-selected RNA extracted from the cell lines using the Illumina TruSeq protocol for mRNA. The libraries were sequenced with a paired-end 2x100bp protocol on an Illumina HiSeq 2000 (Illumina, USA). Detection of fusion reads was performed using the TRUP pipeline (Fernández-Cuesta et al., 2015), which performs an alignment to the human genome (hg19) followed by split-pair analysis with de novo assembly to detect chimeric transcripts. The detected fusion transcripts are listed below (breakpoint = ||, underlined = NUTM1).

HCC2429:
CAGTGGAGTCCTCTCTGACAGCGGACACTCCGAAAACAG||CATCTGCAATGGCCGGACCGGATAGTGCAGATGACCTAGTGCCCGCCT

143100:
CAGCACCCACCATTACCCCGGACCACCAAGTCGGACCCTACTCAACCG||CATCTGCAATGGCCGGACCGGATAGTGCAGATGACCTAGTGCCCGCCT

690100:
AGCACAGCACTCCACCACCACCAAGTCGGATCTCTCCTCTCAACCG||GATCTGCAATGGCCGGACCGGATAGTGCAGATGACCTAGTGCCCGCCT

**Apoptosis analysis by flow-cytometry**
For detection of apoptosis cells were seeded in 6cm dishes at 50-70% confluence and were left to adhere for 24h prior to compound treatment. At the indicated time points cells and supernatant were harvested, centrifuged and washed twice with cold PBS (Sigma-Aldrich, USA) before further processing with the Annexin V-FITC Apoptosis Detection Kit I (BD Biosciences). In brief, cells were resuspended in 1x binding buffer before staining with propidium iodide (PI) and FITC-labeled Annexin V antibody. After 20min incubation in the dark samples were adjusted with binding buffer to the required amounts and were analyzed with a FACS Gallios Flow Cytometer and the corresponding FACS Kaluza analysis software (Beckman Coulter, USA).

**Cell cycle analysis**
For cell cycle analysis cells were seeded at 50-70% confluence, were left to adhere for 24h before being treatment with compounds for indicated time periods. Subsequently cells were harvested, washed twice with cold PBS and fixed in 70% methanol at -20°C over night. The next day cells were washed twice with ice-cold PBS and were incubated with RNase A (Qiagen, Germany) before addition of PI at a final concentration of 0.05mg/ml. The mixture was incubated in the dark at room temperature for 20min prior to analysis using a FACS Gallios Flow Cytometer and the corresponding FACS Kaluza analysis software (Beckman Coulter, USA).

**Screening of JQ1 and AT7519**
For the screening of the BRD4-inhibitor JQ1 and the CDK-inhibitor AT7519 (both purchased at Selleckchem, USA) in a subset of cell lines, cell were plated in 96-well plates in triplicates and compounds were added at 8 decreasing compound concentrations one day after seeding. 72h later cell viability was measured via CTG (Promega, USA) and was normalized to DMSO treated controls. GI50 values were calculated using a sigmoidal dose response curve as described above and were averaged from at least two repetitions.

**Knock-down Experiments**
shRNAs targeting c-Myc (CCTGAGACAGATCAGCAACAA), CDK9 (CCGCTGCAAGGGTAGTATA), CCNT1 (CyclinT1) (AAGAGTCATCCAGCAACTTA), and eGFP (control, GAAGCAGCACGACTTCTTC) were cloned into a pLKO.1-puro vector and cotransfected with pMD.2 and pCMV.d.8.9 helper plasmids into HEK 293T cells using TransIT-LT1 reagent (Mirus). 48h post transfection, replication-incompetent lentiviruses were collected from the supernatant for infection of HCC2429 and A549 cells in the presence of 8µg/ml polybrene. 24h after infection, medium was replenished with growth medium containing puromycin (0.8µg/ml for HCC2429 and 3 µg/ml for A549) to select for transduced cell clones. Depending on onset and duration of efficient knock-down as evaluated by immunoblotting, cell viability was measured 4-6d after infection by CTG-Assay (Promega) and normalized to the average signal of shGFP controls.

**Reverse transcription-quantitative PCR (RT-qPCR)**
Total RNA was isolated using the RNeasy Mini Kit (Qiagen) according to the manufacturer’s instructions. 1.5µg of total RNA subjected to DNaseI digestion followed by reverse transcription using SuperscriptIII (Life Technologies) with random hexamer primers. Quantitative real-time PCR (qPCR) was performed using 7500 Real-Time PCR System (Applied Biosystems) and the Power SYBR Green PCR Master Mix (Applied Biosystems). The qPCR primers are listed in Table S5. 18S rRNA was used for normalization of qPCR data.
Chromatin Immunoprecipitation (ChIP) and ChIP qPCR
ChIP experiments were carried out as described before (Bierhoff et al., 2014) with minor adjustments. Briefly, cells were crosslinked with 1% formaldehyde and subsequently quenched with 0.125 M glycine. Chromatin was prepared and sonicated to an average length of 300-500 bp using a Diagenode Bioruptor UCD200. Similar amounts of chromatin were incubated over night with 4 µg of Pol II antibody (8WG16 - sc-56767, Santa Cruz Biotechnology), 5 µg of Pol II pSer2 antibody (ab5095, Abcam), 2.5 µg of Pol pSer5 antibody (ab5131, Abcam) and 5 µg of mouse IgG (sc-2025, Santa Cruz Biotechnology), respectively. Antibody complexes were captured with Protein G Dynabeads (Thermo Fisher) and washed 5 times with LiCl-Wash buffer (100 mM Tris [pH 8], 500 mM LiCl, 1% NP-40, 1% Na-DOC) followed by one wash with 1xTE (10 mM Tris [pH 7.5], 0.1 mM EDTA). DNA was eluted from the beads by incubation with elution buffer (100 mM NaHCO3, 1% SDS) for 1h at 65°C, followed by decrosslinking at 65°C over night, RNaseA and ProteinaseK digestion. DNA was purified using the ChIP DNA Clean & Concentrator kit (Zymo Research). Pol II occupancy at the transcription start site (TSS) and the gene body (GB) of the indicated genes was determined by qPCR using the indicated primers, listed in Table S5. ChIP signals were calculated relative to Input levels after (IgG) background subtraction. The Pausing Index (ratio between TSS-bound Pol II / GB-bound Pol II) was calculated as described before (Huang et al., 2014).

Western blotting
For Western Blot analysis, cells were harvested and lysed in cold RIPA buffer (150 mM NaCl, 50 mM Tris/HCl [pH 7.5], 1% Triton-X 100, 0.5% Na-Deoxycholate, 0.1% SDS) in the presence of protease inhibitors (Roche) and phosphatase inhibitors (Calbiochem). Equal amounts of protein were separated on 4-20% Novex Tris-Glycine gels (Invitrogen), blotted on PVDF membranes and incubated with specific primary antibodies and fluorescently labeled secondary antibodies (IRDye, LI-COR). Proteins were detected with the Odyssey CLx imaging system (LI-COR). Protein levels were quantified with ImageJ (Abramoff et al., 2004) and normalized to loading control. Antibodies: Actin (C-2) sc-8432 (Santa Cruz), c-Myc #9402 (Cell Signaling), RNA Pol II (8WG16) sc-56767 (Santa Cruz), RNA Pol II pospho CTD Ser 2 (04-1571, Millipore), CDK9 (D-7) sc-13130 (Santa Cruz) and CDK9 ab38840 (Abcam), caspase 3 #9662 (Cell Signaling), cleaved caspase 3 #9664 (Cell Signaling), p300 (N-15) sc-584 (Santa Cruz), p-Chk2 (T68) #2661 (Cell Signaling), p21 #2947 (Cell Signaling), γH2AX (Ser-139) 05-636 (clone JBW301, Millipore) and ab22551 (Abcam), Mcl-1 (S19) sc-819 (Santa Cruz), HEXIM1 (D5YSK) #12604 (Cell Signaling), BRD4 A310-985 (Bethyl Laboratories), Hsp90 (16F1) ADI-SPA-835 (Enzo), Cyclin-T1 (C-6) sc-271575 (Santa Cruz).

Immunoprecipitation
For immunoprecipitation of endogenous CDK9 HCC2429 and A549 cells were seeded at 50-70% density in 10cm dishes and were left to adhere overnight. Treatment was done with 10µM LDC67 or DMSO for 4h before harvesting. Cell were washed 2x in PBS and lysed in lysis buffer containing 20mM HEPES-KOH, 15% glycerol, 150mM KCl, 1.5mM MgCl2, 0.2% NP40 and protease inhibitors. Lysates were incubated on ice and sonicated with a Sonopuls HD3100 (Bendelin). After removal of cell detritus by centrifugation, protein concentrations were measured and equilibrated in samples before taking input control. Samples were split and incubated overnight at 4°C with either anti-CDK9 (ab38840, Abcam) or normal mouse IgG (sc-2025, Santa Cruz) as unspecific control. Next day samples were incubated with Protein G sepharose beads (p3296, Sigma) for 2.5h at 4°C and spun at 1000g before taking unbound control. Elution from beads was done with 3xSDS. For immunoblotting samples were loaded on 4-20% Novex Tris-Glycine gels (Invitrogen) and processed as described in Western blotting.

For FLAG-CDK9 immunoprecipitation cells were seeded in 10cm dishes and left to adhere overnight. The next day Opti-MEM medium (gibco, Thermo Scientific) was mixed with Lipofectamine 2000 (Invitrogen). Opti-MEM/Lipofectamine was mixed with FLAG-CDK9 plasmids and added to cells with fresh penicillin/streptomycin free medium. For negative control Lipofectamine without FLAG-CDK9 was used. After incubation for 48h, cells were treated for 4h with 10µM LDC67 or DMSO before harvest and lysates were prepared as described above. Incubation with anti-FLAG M2 beads (Sigma) was done for 2h at 4°C. Unbound control was taken after centrifugation before elution of protein from the beads with 3xSDS. Samples were analyzed by immunoblotting as described above. Plasmids were a kind gift of Prof. Zhou (University of Berkeley, USA) (Lu et al., 2015).

Immunofluorescence
For immunofluorescence HCC2429 cells were grown at 50-70% confluency on cover slips in 6-well dishes for 24h before treatment with 10µM LDC67 or 500nM JQ1. After 24h treatment cells were washed 2x with PBS and fixed in 4% formaldehyde for 30min on ice. Cells were washed 2x with PBS and permeabilized with PBS/0.2% Triton and washed 2x with PBS/0.5% Tween-20 before blocking in TBS+1% fish gelatin blocking buffer for 1h.
Incubation with primary antibody was done at 1:500 dilutions in blocking buffer at 4°C overnight (for primary antibodies see Western blotting). The next day samples were washed 2x with blocking buffer and 2x with TBST before being incubated with secondary antibodies (Alexa Fluor anti-mouse 647nm and anti-rabbit 488nm, both Thermo Scientific) for 2h. Cover slips were mounted on slides with DAPI containing mounting medium (P36931, Thermo Scientific) and imaged using a Zeiss Meta510 or Zeiss Meta710.

Clonogenic survival assays
For clonogenic survival assays cells were plated at 80% confluency in 6-well plates, left to adhere overnight and were subsequently treated with 10µM LDC67 or DMSO for indicates times. Subsequently, cells were washed twice with PBS, fixed in PBS with 4% paraformaldehyde for 15min at room temperature, washed twice with PBS and stained with 0.1% crystal violet solution for at least 15min at room temperature. After removing the staining solution, wells were rinsed twice with PBS. For quantification 1% SDS solution was added to the wells for 30min, the supernatant was diluted 1:5 before measuring absorption at 590nm.

FACS analysis of γH2AX and cleaved caspase 3
HCC2429 or A549 cells were grown in 6cm dishes for 24h before treatment with 10µM LDC67 or 500nM JQ1 or DMSO for indicated times. Cells including supernatant were harvested by trypsination, washed in PBS and mixed with 80% ice cold methanol and stored >24h at -20°C for fixation. Fixated cells were washed, permeabilized by incubation with PBS/0.25% TritonX-100 for 15min on ice and blocked with PBS/1%BSA blocking buffer. Primary antibodies for cleaved caspase 3 (#9664, Cell Signaling) and γH2AX (JW301, Millipore) were diluted in PBS+1% BSA and incubated overnight at 4°C. The next day cells were washed 2x with blocking buffer and secondary antibodies (Alexa Fluor anti-mouse 647nm and anti-rabbit 488nm, both Thermo Scientific) diluted in blocking buffer were incubated with the cells 1h in the dark. Finally, cells were incubated with propidium iodide and RNase A (Thermo Scientific) for 15min prior to analysis using a FACS Gallios Flow Cytometer and the corresponding Kaluza analysis software (Beckman Coulter, USA).

Time-dependent gene expression dynamics in treated HCC2429
HCC2429 we plated at equal amounts in 10 cm dishes as triplicates and were left to adhere for 24h before treating with ~4-5x the GI50 concentrations of JQ1 or LDC67 (0.5µM and 10µM, respectively) or with DMSO. Cells were harvested 8h and 48h after treatment, washed and lysed before proceeding to total RNA extraction (RNeasy mini kit, Qiagen, Germany). RNA quality and integrity was evaluated on a TapeStation (Agilent, USA) and libraries were prepared using the Lexogen QUANT SEQ FWD 3’ mRNA-Seq Library Prep Kit (Lexogen, Austria) following the manufacturer’s protocol as described previously(Moll et al., 2014). Starting with 500ng total RNA, library generation is initiated by oligoDT primers already containing Illumina-compatible linker sequences. After first strand synthesis the RNA is removed and second strand synthesis is initiated by random primers also containing Illumina-compatible linker sequences. Second strand synthesis is followed by a magnetic bead-based purification step. The library is then amplified (12 PCR cycles), introducing the sequences required for cluster generation. After library validation and quantification (Agilent 2100 Bioanalyzer), equimolar amounts of library were pooled. The pool was quantified by using the Peqlab KAPA Library Quantification Kit and the Applied Biosystems 7900HT Sequence Detection System and sequenced by using an Illumina HiSeq 4000 SR Cluster Kit and an Illumina HiSeq 4000 SBS Kit on an Illumina HiSeq 4000 sequencer with a single read (1x 51 cycles) protocol.

Raw sequencing reads were mapped to the human reference genome Hg38 with the STAR aligner software(Dobin et al., 2013) and gene expression levels were quantified as counts using RSEM(Li and Dewey, 2011). Differential gene expression and adjusted log2 gene expression fold-changes between DMSO controls and treated cells were calculated from count level data using DESeq2 with standard parameters(Love et al., 2014). Resulting p-values were adjusted using Benjamini-Hochberg correction(Benjamini and Hochberg, 2000). To investigate the enrichment of biological functions among up- or downregulated genes we performed gene set enrichment analysis (GSEA)(Mootha et al., 2003; Subramanian et al., 2005) with gene ontology (GO) terms of the , the Hallmark collection and the C2 Canonical pathways of the MSigDb v5.1(Liberzon et al., 2011). Moreover, a set of MYC specific signatures was selected from the MSigDB to assess the impact on MYC signaling following inhibitor treatment. To incorporate the magnitude and direction of alteration and the variation among replicates all genes were ranked based on their test statistic of the differentially expression analysis before performing unweighted GSEA with the preranked tool. q-values were derived from FDR-corrected p-values.

JQ1-persistent HCC2429 cells
JQ1-persistent HCC2429 (JQ1P) cells were generated by continuous exposure of HCC2429 cells to 500 nM JQ1 for 2 weeks. Growth rates were determined by cell-counting of JQ1P and parental HCC2429 cells at indicated time points. For determination of JQ1 and LDC67 sensitivity JQ1P. HCC2429 were seeded in triplicates in 96-well
plates, treated for 72h before determination of cell viability with CTG (Promega, USA) and calculation of GI<sub>50</sub> values as described above.

**Combinatorial treatment with JQ1 and LDC67**
To assess the joint effect of JQ1 and LDC67 HCC2429 cells were seeded in a 6x6 matrix format on a 96-well plate and were treated at increasing concentrations of LDC67 (0, 0.12, 0.37, 1.11, 3.33,10 µM) across columns and of JQ1 (0, 0.012, 0.037, 0.11, 0.33, 1 µM) across rows to obtain multiple compound concentration combinations. The experiment was performed three times. To quantify the combined effect we used the combination index (CI) method proposed by Chou et al. derived from Loewe additivity models in which CI =1 means additivity and CI <1 or >1 imply synergy or antagonism, respectively (Chou, 2010; Zhao et al., 2010). CI values were calculated with the following equation: 

\[
CI = \frac{C_{JQ1,x}}{C_{JQ1,x}^{LDC67,x}} + \frac{C_{LDC67,x}}{C_{LDC67,x}^{LDC67}}
\]

where C<sub>JQ1,x</sub> and C<sub>LDC67,x</sub> are the concentrations necessary to achieve x% growth inhibition in combination and GI<sub>x</sub> are the single compound concentrations reaching the same effect. In addition we performed an isobologram analysis at the 75% effect level. Required values were derived from non-linear regression using log-logistic growth curves as described above.
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