We previously identified a region of recurrent amplification on chromosome 22q11.21 in a subset of primary lung adenocarcinomas. Here we show that CRKL, encoding for an adaptor protein, is amplified and overexpressed in non–small cell lung cancer (NSCLC) cells that harbor 22q11.21 amplifications. Overexpression of CRKL in immortalized human airway epithelial cells promoted anchorage-independent growth and tumorigenicity. Oncogenic CRKL activates the SOS1-RAS-RAF-ERK and SRC-C3G-RAP1 pathways. Suppression of CRKL in NSCLC cells that harbor CRKL amplifications induced cell death. Overexpression of CRKL in epidermal growth factor receptor (EGFR)-mutant cells induces resistance to gefitinib by activating extracellular signal–regulated kinase and AKT signaling. We identified CRKL amplification in an EGFR inhibitor–treated lung adenocarcinoma that was not present before treatment. These observations demonstrate that CRKL overexpression induces cell transformation, credential CRKL as an oncogene in a subset of NSCLC. Overexpression of CRKL induces cell transformation and resistance to epidermal growth factor receptor inhibitor treatment and suggest that therapeutic interventions targeting CRKL may confer a clinical benefit in a defined subset of NSCLCs. Cancer Discovery; 1(7); 608–25. ©2011 AACR.

SIGNIFICANCE: These studies credential CRKL as an oncogene in a subset of NSCLC. Overexpression of CRKL induces cell transformation and resistance to epidermal growth factor receptor inhibitor treatment and suggest that therapeutic interventions targeting CRKL may confer a clinical benefit in a defined subset of NSCLCs.
INTRODUCTION

Although the prognosis for patients with non–small cell lung cancer (NSCLC) who present with late-stage disease remains poor, somatic mutations of the epidermal growth factor receptor (EGFR) serve as predictive biomarkers for both clinical response and survival in patients who receive EGFR inhibitors (1–7). Moreover, recent work indicates that mutations or translocations of the ALK tyrosine kinase also occur in a subset of NSCLCs (8, 9) and that tumors that harbor such mutations are sensitive to ALK inhibitors (10, 11). Collectively, these studies suggest that identifying and characterizing genetic alterations in NSCLC will provide new targets for therapeutic strategies.

In previous work, researchers have identified 57 recurrent events of genomic gain or loss in primary NSCLC (12, 13). A small number of these recurrent genomic events has been found to harbor known and novel oncogenes and tumor suppressor genes, including amplification or mutation in EGFR, KRAS, MYC, MDM2, TERT, CCND1, CCNE1, and NKX2-1 and deletions of CDKN2A/B and PTEN (12, 14–17). However, for many of these recurrently amplified regions, the target gene(s) believed to drive the pathogenesis of cancer remains to be identified and validated (12, 14–17). For example, chromosome 22q11.21 is focally amplified in 3% of lung adenocarcinoma samples and the peak region contains 15 genes, including CRKL (v-ck sarcoma virus CT10 oncogene homolog (avian)-like) (12, 16, 17). Recurrent amplifications of 22q11.21 have not been described in squamous cell lung carcinomas (18) or small cell lung carcinomas (19).

CRKL is an adapter protein that participates in signal transduction in response to both extracellular and intracellular stimuli, such as growth factors, cytokines, and the oncogenic BCR-ABL fusion protein (20, 21). CRKL consists of an N-terminal Src homology 2 (SH2) domain followed by two SH3 domains (SH2-SH3N-SH3C) (20). The SH2 domain of CRKL binds to the phosphorylated Y-x-x-P motif present in many docking proteins, such as BCR1 (also known as p130CAS), paxillin, and GAB (20, 21), whereas the SH3N domain binds to proline-rich P-x-x-P-x-K motif-containing proteins, such as Son of Sevenless (SOS), RAPGEF1 (also known as C3G) (22), p85 (23), ABL1, and BCR-ABL (24, 25). Through these interactions, CRKL facilitates the timely and localized formation of protein complexes required for signal transduction in many biological processes, including cell proliferation, survival, adhesion, and migration (20, 21).

CRKL and several proteins that interact with CRKL have been implicated in cancer. Overexpression of CRKL in Rat-1 fibroblast cells has been shown to promote anchorage-independent growth, but the signaling pathways necessary for this phenotype remain undefined (26, 27). Activating mutations of ALK have been shown to activate RAP1 through CRKL-C3G complexes in neuroblastomas (28). Moreover, expression of the oncogenic RET-PTC1 fusion protein leads to increased RAP1 activity, whereas expression of a dominant interfering RAP1N17 inhibits proliferation of papillary thyroid carcinoma cells (29). However, it remains unclear how overexpression of CRKL affects C3G-RAP1 signaling and whether RAP1 signaling plays a role in proliferation, survival, and transformation of NSCLC cells.

In previous work, we and others demonstrated that a subset of NSCLC is dependent on CRKL expression for proliferation (17, 30). Moreover, overexpression of CRKL in immortalized human lung epithelial cells promoted EGFR-independent proliferation (17). Here we credential CRKL as an oncogene in NSCLC that transforms human lung epithelial cells through coordinate activation of the RAS and RAP1 pathways and is involved in resistance to EGFR inhibitors.

RESULTS

Amplification and Overexpression of the CRKL Gene in NSCLC Cells

In previous work, we and others found recurrent focal copy number gain at chromosome 22q11.21 involving CRKL in 3% of 371 primary lung adenocarcinomas, with another 13% of tumors exhibiting broad copy number gain spanning that region (12, 17). To identify other NSCLC cell lines that harbor copy number gain of this region, we examined a panel of 84 NSCLC cell lines that had been characterized by high-density single-nucleotide polymorphism arrays (19, 31) and identified 6 cell lines with high-level focal amplifications of 22q11.21 (Fig. 1A). We confirmed that CRKL was amplified by FISH in 3 of these cell lines: HCC515, H1819, and H1755 (Fig. 1B). In contrast, in the HCC1833 cell line in which we found normal 22q copy number (Fig. 1A), we detected only 2 copies of the CRKL gene (Fig. 1B). To confirm these findings, we also performed quantitative PCR to measure the copy number of CRKL and detected 12 to 18 copies of CRKL in NSCLC cell lines that harbored a 22q11.21 amplification (Fig. 1C).

To determine whether this observed gene amplification correlates with increased CRKL expression, we examined...
expression data from the same panel of NSCLC cell lines (31) and observed that each of the cell lines that harbored 22q11.21 amplification expressed high levels of CRKL (Supplementary Fig. S1). When using quantitative reverse-transcription PCR, we detected a 2- to 7-fold increase in CRKL mRNA levels in NSCLC cells that harbored a 22q11.21 amplification compared with immortalized but nontumorigenic human airway epithelial cells (AALE; Fig. 1D) (32). Correlating with these observed transcript levels, CRKL protein expression was also increased (2.5- to 4.2-fold increase) in NSCLC cells that harbored a 22q11.21 amplification (Fig. 1E and Supplementary Fig. S2A). We note that in addition to CRKL, 4 genes within the amplicon, PI4KA, ZNF74, THAP7, and LZTR1, were also expressed at greater levels in samples that harbored a 22q11.21 amplification (Supplementary Fig. S1). In addition, when we examined gene expression data, we found that CRKL was expressed at high levels in a subset of cell lines that lacked a 22q11.21 amplification (Supplementary Fig. S1C). We confirmed that CRKL protein expression was increased in these cell lines compared with AALE cells, suggesting that mechanisms other than gene amplification may also lead to CRKL overexpression. These observations confirm that CRKL is highly amplified and overexpressed in a subset of NSCLC cells.

**NSCLC Cells That Harbor a 22q11.21 Amplification Are Dependent on CRKL**

We and others showed that a subset of NSCLC is dependent on CRKL expression for proliferation (17, 30).
To examine whether NSCLC cells that harbor the 22q11.21 amplification require CRKL expression for cell proliferation or survival, we tested the effects of suppressing CRKL on the proliferation of several NSCLC cell lines and AALE cells. We found that CRKL suppression by 2 independent short hairpin RNAs (shRNAs) substantially decreased the proliferation of NSCLC cell lines with a 22q11.21 gain (HCC515, HCC1359, H1755, H1819, HCC366, and Colo699; Fig. 2A and Supplementary Fig. S2B) and that exhibited CRKL overexpression but not 22q11.21 gain (H1915, H2009, H28, and H1299; Supplementary Fig. S2A and B). In contrast, suppression of CRKL in 2 NSCLC cell lines (H1833 and H1792) that have normal copy number at 22q11.21 and express lower levels of CRKL were relatively insensitive to CRKL suppression (P = 0.0001 and P = 0.0056 for shCRKL#1 and shCRKL#2, respectively, compared with cell lines that harbored a 22q11.21 gain, t test; Fig. 2A). In addition, the proliferation of immortalized AALE cells, which also exhibit normal copy number at 22q11.21, was unaffected by CRKL suppression (Fig. 2A).

To confirm that the observed effects of these shRNAs on proliferation were specific for CRKL, we constructed a shRNA-resistant CRKL mutant (CRKL_{silent and mutant}) that contained multiple nucleotide substitutions to the targeting sequence of shCRKL#1 that do not change the amino acid sequence of CRKL (Fig. 2B). We then tested whether expression of the CRKL_{silent and mutant} rescued the decreased proliferation induced by shCRKL#1 and 2 other shRNAs that target the 3′ untranslated region (3′UTR) of CRKL mRNA (shCRKL#2 and shCRKL#3). We found that expression of the CRKL_{silent and mutant} permitted H1755 cells expressing each of these CRKL-targeting shRNAs to proliferate, whereas the expression of a control vector failed to rescue such cells (Fig. 2B). These observations extend the findings of previous studies (17) and reveal that NSCLC cells that harbor amplifications of 22q11.21 are particularly dependent on CRKL expression for proliferation, suggesting that, like other oncogenes, amplification of CRKL induces oncogene addiction (33).

To determine whether CRKL suppression induced apoptosis, we performed immunoblotting for PARP and caspase-3. We found that suppression of CRKL in H1755 cells harboring 22q11.21 amplifications resulted in the cleavage of PARP and caspase-3 (Fig. 2C). In contrast, no cleaved forms of PARP and caspase-3 were detected after the suppression of CRKL in HCC1833 cells that did not harbor 22q11.21 gain. These findings show that suppression of CRKL induced apoptotic cell death in NSCLC cells with CRKL amplifications.

We next investigated which domains were essential for CRKL-induced oncogene addiction. Specifically, we generated CRKL mutants with amino acid substitutions predicted to disrupt the function of the SH3N domain (W160L), the SH3C domain (W275L), or Tyr207 phosphorylation (Y207F) (26). We then tested whether expression of these CRKL mutants rescued the decreased cell proliferation induced by CRKL suppression. We first introduced a control vector or each of these CRKL mutants into H1755 cells and then introduced a CRKL-specific shRNA (shCRKL#3) that targets the 3′UTR of CRKL mRNA (Supplementary Fig. S2C). We found that expression of exogenous wild-type CRKL, CRKL_{W275L or Y207F}, or CRKL mutants permitted cells expressing the CRKL-specific shRNA to proliferate (Fig. 2D), whereas the expression of the SH3N mutants (either CRKL_{W160L} or CRKL_{W160L or Y207F}) or a control vector failed to rescue such cells (Fig. 2D). These findings demonstrate that the SH3N domain is essential for CRKL function in NSCLC cell lines with increased 22q11.21 copy number.

Suppression of CRKL in NSCLC Cells Inhibits Tumor Growth

To examine whether CRKL suppression affected tumorigenic growth of NSCLC cells that harbor CRKL amplification, we confirmed that HCC515 cells were able to form tumors after subcutaneous implantation into immunodeficient mice. To enable noninvasive monitoring of tumor growth by luminescent imaging, we generated a polyclonal population of HCC515 cells stably expressing luciferase. We then introduced either a scrambled control shRNA or a validated CRKL-specific shRNA (shCRKL#1) in vectors in which the expression of shRNA was under the control of a doxycycline-inducible promoter. Doxycycline treatment of cells containing the inducible shRNA (shCRKL#1) induced decreased CRKL protein levels compared with cells grown in the absence of doxycycline (Fig. 3A).

In contrast, treatment with doxycycline did not affect CRKL protein levels in cells expressing the scrambled control shRNA. We then injected cells expressing the inducible control shRNA or the CRKL-specific shRNA subcutaneously into opposite flanks of the same immunodeficient mice and allowed tumors to form (65 mm³ in volume, 11 days; Fig. 3B). After 3 weeks, we observed that in mice feeding on doxycycline-containing chow, tumors expressing the control shRNA grew large whereas tumors expressing the CRKL-specific shRNA exhibited growth arrest (Fig. 3C and D). As expected, mice fed with normal chow grew large tumors with comparable size under uninduced condition (Fig. 3C and D). To determine whether CRKL suppression induced apoptotic cell death in tumors, we performed terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL) staining and detected a significantly increased percentage of TUNEL-positive cells in tumors expressing CRKL-specific shRNA compared with tumors expressing a control shRNA (Fig. 3E). Together, these findings demonstrate that CRKL is required for tumorigenic growth of NSCLC cells with CRKL amplifications.

Overexpression of CRKL in Immortalized Human Airway Epithelial Cells Induces Anchorage-Independent Colony Formation

Overexpression of CRKL promotes anchorage-independent growth of Rat-1 fibroblast cells (27) but not NIH3T3 cells (34), demonstrating that transformation induced by CRKL expression is context dependent. To dissect the mechanism and genetic context required for the transformation of NSCLC, we used human airway epithelial cells that can be rendered tumorigenic by the introduction.
We also tested the ability of CRKL mutants that were predicted to disrupt the function of the SH3N domain (W160L), the SH3C domain (W275L), and Tyr207 phosphorylation (Y207F) to induce anchorage-independent growth. We found that the CRKL SH3C and Tyr207 mutants (W275L, Y207F, and Y207F+W275L) induced anchorage-independent colony formation similarly to wild-type CRKL when overexpressed in AALE cells (Fig. 4B). In contrast, the SH3N mutants (W160L, W160L+Y207F, and W160L+W275L) failed to promote anchorage-independent growth (Fig. 4B). These findings of specific combinations of genes (AALE cells) (32) to test whether CRKL expression induces cell transformation.

Specifically, we introduced a retroviral vector encoding CRKL or a control vector into AALE cells and confirmed that CRKL was expressed at levels comparable with those found in NSCLC cells with CRKL amplification and overexpression (Fig. 4A). We found that overexpression of CRKL in AALE cells led to a >4-fold increase in anchorage-independent colony formation as compared with AALE cell lines expressing a control vector (Fig. 4B).
CRKL Induces Transformation and Gefitinib Resistance

Research Article

CRKL Induces Transformation and Gefitinib Resistance through coordinate activation of several signaling pathways. To investigate the mechanism by which CRKL induces cell transformation in human NSCLC, we first determined whether overexpression of CRKL in AALE cells induces cell transformation that is dependent on the integrity of the SH3N domain.

CRKL Induces Cell Transformation by Coordinately Activating Multiple Signaling Pathways

The SH3 domain of CRKL and other CRK family proteins interacts with many proline-rich motif-containing proteins, such as SOS, C3G (35, 36), and p85 (23), implicating CRKL in the regulation of several pathways including those regulated by RAS–mitogen-activated protein kinase (MAPK) (27, 37), C3G-RAP1 (38–42), and phosphoinositide 3-kinase (PI3K)-AKT (23, 43, 44). Thus, we hypothesized that overexpression of CRKL induces cell transformation through coordinate activation of several signaling pathways.

Figure 3. Effects of CRKL suppression on NSCLC tumorigenicity. A, generation of NSCLC cell lines expressing a doxycycline (DOX)-inducible CRKL-specific shRNA. A scrambled control shRNA or a validated CRKL-specific shRNA (shCRKL#1) under the control of DOX-inducible promoter were introduced into HCCS15 cells. Immunoblot of CRKL proteins in cells in the absence or presence of 200 ng/mL DOX for 96 hours. B, experimental scheme. Each HCCS15 cell line expressing a DOX-inducible vector encoding either shCRKL#1 or a scrambled control shRNA was implanted subcutaneously into opposite flanks of the same mouse (n = 10). Tumors were allowed to grow for 11 days to attain a volume of ~65 mm3. Mice were then fed on normal chow (n = 5) or DOX-containing chow (n = 5) to induce shRNA expression. Tumor growth was measured by caliper and bioluminescent imaging. C, effect of CRKL suppression on the growth of HCCS15 xenografts. DOX-induced CRKL suppression induced growth arrest of tumors derived from cell lines as described in panels (A) and (B). Data represent mean ± SD. D, representative bioluminescent images of the growth of HCCS15 xenografts. DOX-induced CRKL suppression induced growth arrest of tumors derived from cell lines as described in panels (A) and (B). Images shown are the day 11 images (before DOX) and day 32 images (with or without DOX). E, TUNEL staining of xenografts. Left, DOX-treated tumors expressing a control shRNA or shCRKL#1 were harvested on day 32 as described in (B) and stained for TUNEL (green) and DAPI (blue). Right, quantification of percent of TUNEL-positive cells. Mean ± SD are shown.
Figure 4. The role of SRC-C3G-RAP1 signaling in transformation induced by CRKL. **A**, immunoblot of CRKL in AALE cell lines overexpressing a control vector or CRKL compared with NSCLC cells with CRKL amplifications. The relative intensity of bands is determined. **B**, anchorage-independent growth of AALE cell lines stably overexpressing a control vector, wild-type (WT), or mutant CRKL. Colony number indicates colonies >0.2 mm in diameter 4 weeks after plating. Data represent mean ± SD of 6 replicates from 2 independent experiments. Wild-type CRKL and CRKL mutants that induce anchorage-independent growth are marked in red. **C**, overexpression of CRKL in AALE cells increased the phosphorylation of SRC. Immunoblot of phospho-Y419 SRC proteins in AALE cells overexpressing a control vector, CRKL, or CRKLmutmut. **D**, immunoblot of phospho-T185/Y187 ERK1/2 and phospho-S473 AKT in AALE cell lines overexpressing wild-type (WT) or mutant CRKL. Wild-type CRKL and CRKL mutants that induced anchorage-independent growth in panel (B) are marked in red. **E**, effect of CRKL overexpression on the CRKL-C3G interaction and phosphorylation of C3G in AALE cells. CRKL immune complexes in AALE cells expressing indicated constructs were isolated and immunoblotting for phosphorylated C3G and total C3G protein was performed by the use of specific antibodies. **F**, overexpression of CRKL in AALE cells increased RAP1 activity. A pull-down assay for GTP-bound RAP1 proteins was performed followed by immunoblotting for RAP1 proteins. An immunoblot for RAP1 protein in total lysates before the pull-down assay was used as loading control. The relative intensity of bands is determined. **G**, effect of CRKL suppression on RAP1 activity in NSCLC cells that harbored amplification of CRKL. Pull-down assays were performed to assess the RAP1-GTP levels in NSCLC cells 4 days after infection with a control shRNA targeting GFP or a CRKL-specific shRNA (shCRKL#1) followed by immunoblotting for RAP1. An immunoblot of RAP1 proteins in total lysates shows the relative intensity of bands. Arrow indicates RAP1 protein. **H**, effect of expressing RAP1 on cell proliferation of H1755 cells after CRKL suppression. Each H1755 cell line expressing a control vector, wild-type RAP1A or RAP1B, or constitutively active RAP1A63E or RAP1BV12 mutants was infected with control shRNAs or a CRKL-specific shRNA (shCRKL#3). Cell proliferation was measured 6 days after infection with shRNA. Data represent mean ± SD of 6 replicate measurements. *, P < 0.0001 as compared with cells expressing a control vector and shCRKL#3. **I**, effect of suppression of SRC, C3G, or RAP1 on anchorage-independent growth. Left, immunoblots of SRC, C3G, or RAP1 proteins in CRKL-overexpressing AALE cells expressing the indicated shRNAs. The shRNAs marked in red were validated in panel (J). Right, anchorage-independent growth of AALE cells expressing indicated constructs. Colony number indicates colonies >0.2 mm in diameter 4 weeks after plating. Data represent mean ± SD of 6 replicate determinations from 2 independent experiments. *, P < 0.001 as compared to cells expressing CRKL and shGFP, J, effect of dasatinib treatment on anchorage-independent growth of CRKL-overexpressing AALE cells that expressed wild-type SRC, mutant SRC, or a control vector in the presence of dasatinib at the indicated concentrations. The colony number indicates colonies >0.2 mm in diameter 4 weeks after plating. Mean ± SD are shown. *, P < 0.001 compared with cells expressing a control vector. **K**, effect of dasatinib treatment on phospho-SRC and phospho-ERK1/2 in CRKL-overexpressing AALE cells. AALE cells expressing the indicated constructs were exposed to dasatinib at the indicated concentrations for 6 hours followed by immunoblotting.
CRKL Induces Transformation and Gefitinib Resistance

We confirmed that AALE cells stably overexpressing CRKL exhibited increased levels of phospho-Y419 SRC (Fig. 4C) and phospho-T185/Y187 ERK1/2 (Fig. 4D) compared with cells expressing a control vector by immunoblotting with phospho-specific antibodies. Moreover, CRKL mutants (Y207F, W275L, and Y207F+W275L) that induced anchorage-independent growth when overexpressed in AALE cells also led to increased levels of phospho-ERK1/2, whereas CRKL mutants (W160L, W160L+Y207F, and W160L+W275L) that failed to induce anchorage-independent growth failed to induce similar patterns of tyrosine phosphorylation (Fig. 4D). In contrast, phospho-S473 AKT levels remained unchanged between cells overexpressing CRKL or a control vector (Fig. 4D). These observations indicate that overexpression of CRKL in AALE cells results in specific, constitutive activation of both SRC and ERK1/2 signaling.

We next investigated whether CRKL-induced SRC activation plays a role in transformation of AALE cells. CRKL and SRC have been shown to share many common substrates, such as p130CAS and paxillin. Overexpression of CRKL in AALE cells also resulted in increased phosphorylation levels of p130CAS and paxillin compared with cells expressing a control vector or CRKLW160L mutants (Supplementary Fig. S4A). Moreover, suppression of CRKL resulted in reduced phosphorylation levels of SRC and p130CAS at varied levels in NSCLC cells that harbored CRKL amplifications (Supplementary Fig. S4B). These results suggest that overexpression of CRKL may represent a mechanism for aberrant SRC activation and signaling in NSCLC cells.

Recently, activating point mutations of ALK have been shown to activate RAPI through CRKL-C3G complexes in neuroblastomas (28). C3G, a guanine nucleotide-exchange factor required for RAPI activation, has been shown to form stable complexes with CRKL and other CRK family members (35, 36), and SRC has been implicated in activation of C3G-RAPI through CRK family adaptors (46). We therefore investigated the role of RAPI signaling in CRKL-induced transformation of AALE cells by determining whether overexpression of CRKL in AALE cells affected its interaction with C3G and the phosphorylation status of C3G. We isolated CRKL immune complexes from AALE cells overexpressing CRKL and detected increased C3G bound to CRKL and, to a lesser extent, the CRKLW160L mutant compared with cells expressing a control vector (Fig. 4E). We noted that increased phosphorylation levels of C3G were detected only in CRKL immune complexes isolated from CRKL-overexpressing AALE cells by immunoblotting with a phospho-specific antibody (Fig. 4E). These observations suggest that overexpression of CRKL promotes its interaction with C3G and modulates C3G activity.

We next determined whether overexpression of CRKL in AALE cells also altered RAPI activity by performing pull-down assays to assess the levels of GTP-bound RAPI. Overexpression of CRKL in AALE cells induced substantially increased levels of RAPI-GTP (2.3-fold increase) compared with cells expressing a control vector or CRKLW160L mutant (Fig. 4F). In addition, we found that suppression of CRKL in NSCLC cell lines that harbor CRKL amplifications resulted in reduction of both RAPI-GTP and total RAPI levels (Fig. 4G). Moreover, inhibition of SRC with dasatinib (0.1 μM) led to decreased RAPI-GTP levels in 3 NSCLC cell lines that harbored CRKL amplifications (Supplementary Fig. S4C). These findings indicate that CRKL regulates RAPI activity in a SRC-dependent manner in CRKL-amplified NSCLC cells.

To examine whether CRKL-induced RAPI activation is required for the proliferation of NSCLC cells with CRKL amplifications, we tested the ability of constitutively active RAPI mutants to rescue cells from the cytotoxic effect of CRKL suppression. We transduced H1755 cells with a control lentiviral vector or lentiviral vectors expressing wild-type or constitutively active RAPI mutants and then introduced a CRKL-specific shRNA (shCRKL#3). We found that overexpression of constitutively active forms of RAPIA or RAPIB partially reversed the antiproliferative effect of CRKL suppression (Fig. 4H). In contrast, expression of wild-type RAPIA, RAPIB, or a control vector failed to affect the decreased proliferation induced by CRKL suppression (Fig. 4H). These findings suggest that CRKL-induced RAPI activation in part is required for proliferation and survival of NSCLC cells with CRKL amplification. The partial rescue conferred by active RAPI also suggests that CRKL may activate additional signaling pathways not involving RAPI that are required for proliferation and survival.

Because RAPI has been shown to modulate ERK signaling in a cell context-dependent manner (39–41), we examined whether CRKL-induced RAPI activation and cell transformation requires a signaling pathway involving SRC-C3G-RAPI. Specifically, we introduced SRC-, C3G-, or RAPIA/B-specific shRNAs into AALE cells overexpressing CRKL and then assessed anchorage-independent growth. We found that shRNAs specific for SRC, C3G, or RAPIB, but not RAPIA, substantially decreased the anchorage-independent growth of CRKL-over-expressing AALE cells (Fig. 4I and Supplementary Fig. S5A). Correlating with these observations, suppression of SRC, C3G, or RAPIB in CRKL-overexpressing AALE cells resulted in decreased levels of phospho-ERK1/2 compared with cells expressing a control shRNA targeting GFP (Fig. 4J).

In contrast, suppression of p130CAS, one of the substrate proteins of CRKL and SRC, failed to inhibit CRKL-induced ERK activation and anchorage-independent growth in AALE cells (Supplementary Fig. S4D). To confirm that SRC is required for CRKL-induced cell transformation, we assessed the anchorage-independent growth of AALE cells overexpressing CRKL in the presence of the SRC inhibitor dasatinib. SRC inhibition significantly decreased the anchorage-independent growth and phospho-ERK levels in CRKL-overexpressing AALE cells (Fig. 4K and L). Expression of the gatekeeper SRCT184I mutant, but not wild-type SRC, conferred resistance to dasatinib and allowed CRKL-overexpressing AALE cells to grow in an anchorage-independent manner in the presence of dasatinib (Fig. 4K and L). Taken together, these findings suggest that the SRC-C3G-RAPI signaling pathway is required for sustaining CRKL-induced ERK activation and cell transformation.
CRKL-Induced Cell Transformation Requires Activation of SOS1-RAS-RAF Signaling

Since prior work has linked CRKL function and RAS signaling (27, 37), we determined whether overexpression of CRKL in AALE cells altered RAS activity. We found that overexpression of CRKL in AALE cells resulted in increased levels of GTP-bound, active RAS (Fig. 5A). The CRKL-induced RAS activity in turn activates MAPK pathways as indicated by increased in vitro kinase activity of BRAF (Fig. 5B) and increased phosphorylation levels of RAF1 (Fig. 5C). These observations suggest that CRKL augments the activation of the RAS-RAF-MAPK pathway.

Previous investigators also have shown that CRK family proteins interact with SOS1, a guanine nucleotide-exchange factor that is a well-known RAS activator (22, 38, 47). We therefore determined whether overexpression of CRKL affects its interaction with SOS1 proteins in AALE cells. We isolated CRKL immune complexes and detected the presence of SOS1 proteins in AALE cells overexpressing CRKL but not in cells expressing a control vector or CRKLW160L mutant (Fig. 5D). The observation that CRKL promoted the interaction with SOS1 proteins via the SH3 domain suggests that CRKL works as a RAS activator (22, 38, 47). We therefore determined whether overexpression of CRKL activates SOS1 proteins in AALE cells overexpressing CRKL but not in cells expressing a control vector or CRKLW160L mutant (Fig. 5D). The observation that CRKL promoted the interaction with SOS1 proteins via the SH3 domain suggests that CRKL augments the activation of the RAS-RAS-RAF-MAPK pathway.

CRKL Cooperates with Loss of NF1 to Induce Tumorigenicity in Human Lung Epithelial Cells

Expression of oncogenic alleles of H- or K-RAS confers the ability to form anchorage-independent colonies and tumors to AALE cells (Fig. 6A) (32). In contrast, overexpression of CRKL alone in AALE cells induced anchorage-independent growth but failed to allow tumor formation in AALE cells. In previous work, we and others found that the coexpression of activated alleles of members of known RAS effector pathways, such as mitogen-activated protein/extracellular signal–regulated kinase (MEK) and AKT or MEK and IKBKE, substitute for RAS and induce tumorigenicity (48-51). We therefore searched for potential candidates that would cooperate with CRKL to promote tumorigenicity in the context of lung epithelial cells.

We introduced constitutively active alleles or shRNAs into AALE cells that activated receptor tyrosine kinase signaling (EGFRL858R), MAPK signaling (BRAFV600E), PI3K-AKT signaling (myristoylated AKT1, PIK3CAH1047R), or PTEN-shRNA, c-MYC, or NF1-shRNA. We then introduced CRKL or a control vector into these cell lines and tested their ability to grow in anchorage-independent manner. We observed that overexpression of CRKL in combination with each of these alleles promoted anchorage-independent growth beyond that induced by CRKL alone (Fig. 6A). In particular, overexpression of CRKL together with NF1 suppression showed a pronounced synergy in promotion of anchorage-independent growth ($P = 0.0006$ and $P = 0.0003$ compared with cells overexpressing CRKL- or NF1-shRNA alone, respectively, t-test; Fig. 6A). These results suggest that overexpression of CRKL promotes anchorage-independent growth in many different contexts.

Because coexpression of CRKL and EGFRL858R, BRAFV600E, or NF1-shRNA led to the most significant increase in anchorage-independent growth among all combinations tested, we examined whether overexpression of CRKL together with these alleles promotes tumorigenicity in immunodeficient mice. We found that the AALE cells overexpressing EGFRL858R or BRAFV600E were tumorogenic (Fig. 6B), suggesting that activation of EGFR or MAPK signaling suffices to induce tumorigenicity. Overexpression of CRKL in the setting of EGFR or BRAF expression failure increased the rate of tumor formation. Interestingly, shRNA-mediated suppression of NF1 in AALE cells failed to induce subcutaneous tumors in immunodeficient mice (Fig. 6B). In contrast, overexpression of CRKL together with NF1 suppression conferred the ability to form tumors in 4 of 12 injection sites (33%) in AALE cells ($P = 0.006$, the Fisher exact test; Fig. 6B).

In consonance with these findings, the RAS-GTP levels (Fig. 6C) and in vitro BRAF kinase activity (Fig. 5B) were found to be highly increased in AALE cells coexpressing CRKL and NF1-shRNA, indicating a robust activation of RAS-RAS signaling. These observations further demonstrate the transforming capability of CRKL overexpression, which participates in promoting tumor formation in a context-dependent manner.

To investigate whether CRKL amplification and NF1 loss co-occur in primary NSCLC tumors and cell lines, we reanalyzed mutation and copy number data generated in the Tumor Sequencing Project (12, 52). Among 188 primary lung adenocarcinomas, 1 of the 3 samples harboring focal amplification of CRKL was found to also harbor an inactivating mutation of NF1. No mutations were detected in the other oncogenes that were analyzed by sequencing in the remaining 2 CRKL-amplified tumor samples. In cell lines, we examined the expression of NF1 protein in 4 CRKL-amplified cell lines and found that HCC1359 cells failed to express NF1 compared with immortalized AALE cells (data not shown). These observations suggest that CRKL amplification and NF1 loss co-occur in a subset of NSCLCs and thus may cooperate in cell transformation.

Overexpression of CRKL Decreases Sensitivity of NSCLC to EGFR Inhibitor Treatment

We noticed mutual exclusivity between amplifications of CRKL and amplifications and mutations of EGFR in the 371 primary lung adenocarcinomas (Supplementary Fig. S6A) (12, 52). Specifically, we found that tumors with focal high-level CRKL amplifications did not exhibit amplifications of the EGFR gene ($P < 0.0001$, Fisher exact test; Supplementary Fig. S6A). Similarly, when we analyzed a panel of NSCLC cell lines (31, 53) by co-occurrence of genome alterations, we observed that the 6 CRKL-amplified cell lines appeared to form a subgroup distinct from the 12 cell lines with mutations...
CRKL Induces Transformation and Gefitinib Resistance

**Figure 5.** CRKL-induced cell transformation requires SOS1-RAS-RAF. **A**, overexpression of CRKL increased RAS activity. The levels of GTP-bound RAS in AALE cells overexpressing a control vector or CRKL were measured by a pull-down assay followed by immunoblotting for RAS. Total RAS levels in total lysates were used as loading control. Positive and negative technical controls were obtained by incubating the total lysates with a nonhydrolyzable analog of GTP (GTPγS) or GDP, respectively, before pull-down assays. **B**, overexpression of CRKL increased in vitro BRAF kinase activity. The BRAF proteins in AALE cells expressing the indicated constructs were isolated by immunoprecipitation. The kinase activity was assessed by incubation with substrate proteins (MEK1). Immunoblots of phospho-MEK1 and BRAF proteins in the isolated BRAF immune complexes after kinase activity assay are shown. Arrow indicates phospho-MEK protein. **C**, immunoblot of phospho-S338–RAF1 in AALE cell lines overexpressing wild-type or mutant CRKL. **D**, interaction between CRKL and SOS1 in AALE cells overexpressing CRKL. CRKL immune complexes were isolated followed by immunoblotting for SOS1 or CRKL proteins in AALE cells expressing the indicated constructs. **E**, CRKL-induced anchorage-independent growth requires SOS1-RAS-BRAF/RAF1 signaling. Left, immunoblots of SOS1, KRAS, BRAF, RAF1, or ARAF proteins in CRKL-overexpressing AALE cell lines expressing a control shRNA targeting GFP or each gene-specific shRNA. shRNAs that suppressed more than 50% of target protein levels are marked in red. Right, anchorage-independent growth of AALE cells expressing the indicated constructs. The colony number indicates colonies >0.2 mm in diameter 4 weeks after plating. Data represent mean ± SD of 6 replicates from 2 independent experiments. *, P < 0.0001 as compared with cells expressing CRKL and shGFP.

and/or high-level amplifications of EGFR (P < 0.0001, Fisher exact test; Supplementary Fig. S6B). These observations suggest that amplifications of CRKL and EGFR may share some proliferation/survival pathways.

To investigate whether CRKL overexpression promoted the proliferation and survival of NSCLC cells in response to EGFR inhibition, we determined whether overexpression of CRKL in a gefitinib-sensitive cell line (HCC827) exhibiting the EGFR<sup>E746-A750del</sup> mutation (54) but a normal copy number of 22q11.22 (Fig. 1A) and low expression levels of CRKL (Fig. 1C) affected gefitinib sensitivity. We observed that HCC827 cells overexpressing CRKL became resistant to gefitinib (IC<sub>50</sub> = 2.2 μM) compared with cells expressing a control vector (IC<sub>50</sub> = 0.01 μM; Fig. 7A).

We next examined whether CRKL-induced ERK and SRC activation observed in the transformation of AALE cells...
Moreover, activation of SRC or RAP1 by overexpression of SRC or constitutively active RAP1 alleles in HCC827 cells failed to induce resistance to gefitinib (Supplementary Fig. S8). Together, these results suggest that SRC activation is not required for CRKL-induced gefitinib resistance.

To investigate whether overexpression of CRKL affects its interaction with SOS1, we isolated CRKL immune complexes from HCC827 cells treated with or without an EGFR inhibitor and examined SOS1 interactions. We detected an increase in the abundance of SOS1 associated with CRKL in HCC827 cells overexpressing CRKL (Fig. 7C), and this interaction was further increased upon gefitinib treatment. These results suggest that overexpression of CRKL activates MAPK pathways by interacting with SOS1 in response to gefitinib.

To determine whether SOS1 was required for CRKL-induced EGFR inhibitor resistance, we introduced two SOS1-specific shRNAs or a control shRNA into CRKL-overexpressing HCC827 cells and measured the gefitinib sensitivity. Although suppression of SOS1 alone led to a reduction in proliferation (Supplementary Fig. S9A), suppression of SOS1 in combination with gefitinib resulted in a significant reduction in the relative proliferation of CRKL-overexpressing cells compared with cells treated with gefitinib alone (Fig. 7D). In contrast, expression of the control shRNA targeting GFP failed to alter gefitinib resistance in HCC827 cells induced by CRKL overexpression. We observed that, upon gefitinib treatment, the phosphorylation of EGFR was completely suppressed in HCC827 cells either overexpressing CRKL or a control vector (Fig. 7B). In contrast, phosphorylation of ERK1/2 persisted at substantially greater levels in the presence of gefitinib in HCC827 cells overexpressing CRKL compared with cells expressing a control vector (Fig. 7B). Overexpression of CRKL in HCC827 cells also markedly elevated phosphorylation levels of SRC compared with control vector-expressing cells that were unaffected by gefitinib (Fig. 7B). These results demonstrate that overexpression of CRKL in HCC827 cells activates persistent ERK and SRC signaling upon gefitinib treatment.

To determine whether SRC activation contributes to CRKL-induced EGFR inhibitor resistance, we examined whether SRC inhibition suppressed growth of CRKL-overexpressing HCC827 cells. Cells were exposed to dasatinib alone or in combination with gefitinib. Interestingly, we observed that cells overexpressing CRKL also exhibited resistance to dasatinib treatment alone compared to cells expressing a control vector (Supplementary Fig. S7A). In addition, combined treatment failed to decrease the relative proliferation of CRKL-overexpressing cells compared with gefitinib treatment alone (Supplementary Fig. S7B and S7C).
Figure 7. Overexpression of CRKL induces EGFR inhibitor resistance in NSCLC cells with EGFR mutations. A, effect of overexpressing CRKL on gefitinib sensitivity in HCC827 cells harboring an EGFR mutation. Cell proliferation was measured 72 hours after treatment with gefitinib. Data represent mean ± SD of triplicate measurements relative to untreated cells. B, effect of overexpressing CRKL on phosphorylation levels of EGFR, ERK, AKT, and SRC in response to gefitinib. HCC827 cells expressing a control vector or CRKL were exposed to gefitinib at the indicated concentrations for 6 hours and cell lysates were collected for immunoblotting with the indicated specific antibodies. C, effect of overexpressing CRKL on the interaction with SOS1 proteins. HCC827 cells expressing a control vector or CRKL were exposed to 1 μM gefitinib for 6 hours. After isolating CRKL immune complexes from cell extracts, immunoblotting for SOS1 and CRKL proteins was performed. D, effect of SOS1 suppression on CRKL-induced gefitinib resistance. Cell proliferation of each HCC827 cell line expressing the indicated constructs was measured 3 days after treatment with gefitinib. Data represent mean ± SD of 6 replicate determinations relative to untreated cells for each cell line. E, effect of SOS1 suppression on phospho-ERK levels in CRKL-expressing HCC827 cells in response to gefitinib. Cells expressing the indicated constructs were exposed to 1 μM gefitinib for 6 hours followed by immunoblotting with the indicated specific antibodies. F, effect of overexpressing CRKL on the interaction of p85 with CRKL and ERBB3 proteins. HCC827 cells expressing a control vector or CRKL were either untreated or treated with 1 μM gefitinib for 6 hours. After isolating p85 immune complexes, immunoblotting for CRKL, phospho-Y1289 ERBB3, total ERBB3, and p85 proteins was performed. G, effect of GDC-0941 on CRKL-induced gefitinib resistance. Cell proliferation of CRKL-expressing HCC827 cells was measured 3 days after treatment with gefitinib alone or in combination with GDC-0941 at the indicated concentrations. Data represent mean ± SD of triplicate measurements. H, effect of GDC-0941 on phospho-AKT levels in CRKL-expressing HCC827 cells in response to gefitinib. Cells were exposed to 1 μM gefitinib alone or in combination with GDC-0941 at indicated concentrations for 6 hours followed by immunoblotting with specific antibodies. I, acquired amplification of CRKL in an EGFR inhibitor-resistant lung tumor. FISH analysis of a NSCLC obtained from a patient prior to EGFR inhibitor therapy (left) or after acquiring drug resistance (right) using a CRKL-specific probe (green) and chromosome 22 telomere-specific probe (red) was performed. Nuclei were stained with DAPI (blue).
resistance (Fig. 7E). In consonance with these observations, suppression of SOS1 in CRKL-overexpressing HCC827 cells led to reduced phospho-ERK1/2 levels compared with cells expressing shGFP upon treatment with gefitinib (Fig. 7E). These results suggest that SOS1-dependent MAPK signaling contributes in part to CRKL-induced gefitinib resistance.

Because MET amplification has been shown to induce gefitinib resistance in EGFR-mutant tumors by activating ERBB3-dependent PI3K-AKT signaling (54), we examined whether overexpression of CRKL in HCC827 cells affects phosphorylation of AKT in response to gefitinib. We observed that, upon treatment with gefitinib, phosphorylation of AKT persisted at substantially greater levels in HCC827 cells overexpressing CRKL compared with cells expressing a control vector (Fig. 7B). These results suggest that like MET, overexpression of CRKL in HCC827 cells leads to persistent AKT signaling in response to gefitinib.

Since CRKL has been shown to interact with the p85 regulatory subunit of PI3K (23), we hypothesized that overexpression of CRKL promotes its interaction with p85 proteins to induce persistent PI3K/AKT signaling that is resistant to gefitinib treatment. We isolated CRKL immune complexes from HCC827 cells that overexpressed CRKL or a control vector and detected a markedly increased interaction with p85 proteins in cells over-expressing CRKL (Fig. 7C). Consistently, when we isolated endogenous p85 immune complexes, we also detected increased interaction between p85 and CRKL proteins in cells that overexpressed CRKL (Fig. 7F). Treatment with gefitinib did not affect this enhanced interaction between CRKL and p85 proteins (Fig. 7C and F). In contrast, overexpression of CRKL in HCC827 cells reduced the interaction between p85 and phospho-ERBB3 or ERBB3 proteins under normal culture conditions (Fig. 7F). Upon treatment with gefitinib, the interaction between p85 and ERBB3 was completely disrupted in cells that either overexpressed CRKL or a control vector (Fig. 7F). Together, these results suggest that overexpression of CRKL promotes its interaction with p85 proteins to induce persistent ERBB3-independent PI3K/AKT signaling in the presence of an EGFR inhibitor.

To determine whether CRKL-induced EGFR inhibitor resistance involves PI3K/AKT activation, we examined whether treatment with the PI3K inhibitor GDC-0941 suppressed growth of CRKL-overexpressing HCC827 cells in response to gefitinib. Cells were exposed to GDC-0941 alone or in combination with gefitinib. Combined treatment with GDC-0941 and gefitinib resulted in a substantial decrease in the relative proliferation of CRKL-overexpressing HCC827 cells compared with gefitinib treatment alone (Fig. 7G). The observed decrease in proliferation correlates with decreased phosphorylation levels of AKT in CRKL-overexpressing HCC827 cells after combined treatment with GDC-0941 and gefitinib (Fig. 7H). Similar findings were also observed with another PI3K inhibitor, LY294002 (Supplementary Fig. S9B-D). In contrast, combined treatment with the MET inhibitor PHA665752 and gefitinib failed to alter the relative proliferation of CRKL-overexpressing HCC827 cells (Supplementary Fig. S9E). These observations show that activation of PI3K-AKT signaling contributes to CRKL-induced EGFR inhibitor resistance.

CRKL Amplification in EGFR Inhibitor-Resistant Lung Cancer

We next examined whether amplification of CRKL occurs in EGFR-mutant tumors that develop acquired resistance after EGFR inhibitor treatment by performing FISH on 11 EGFR inhibitor-resistant tumors. We identified copy number gain of CRKL (with an average of 5.7 ± 0.3 copies per cell; Fig. 7I) in one EGFR inhibitor-resistant sample that was not present in the sample obtained before EGFR inhibitor treatment (average of 2.2 ± 0.2 copies of CRKL per cell). Since we also detected increased signal by using a chromosome 22 telomere-specific probe (5.7 ± 0.3 copies per cell), we concluded that the amplification event likely involves the gain of a large fraction of chromosome 22. This patient had shown a prolonged response to erlotinib for 2 years but showed signs of tumor regrowth while still receiving the treatment.

Although 8 of 11 tumors had acquired the known resistance-inducing EGFR T790M mutation, several laboratories have reported that other known resistance mechanisms may occur concomitantly with the EGFR T790M mutation, including MET amplification (54) and CTNNB1 (β-catenin) mutations (55). These observations indicate that the copy number gain of CRKL detected in the EGFR inhibitor-resistant tumor represents a new genetic event after treatment and implicates amplification of CRKL as a mechanism of acquired resistance to EGFR inhibitor treatment in a subset of NSCLCs.

DISCUSSION

Amplification of CRKL Defines a New Subset of NSCLCs

In previous work, we and others identified a recurrent high-level and focal amplification peak on chromosome 22q11.21 in 3% of primary lung adenocarcinomas (n = 371) (12). An additional 13% of tumors exhibited broad copy number gain spanning that region (12, 17). We also observed a mutually exclusive relationship between amplification of CRKL and EGFR in both primary lung adenocarcinomas and the DFCI-84 NSCLC cell line collection. Herein, we extend previous work that showed that proliferation of NSCLC cells harboring a 22q11.21 amplification containing the CRKL gene depends on CRKL expression to a larger panel of NSCLC cell lines and show that suppression of CRKL induces apoptosis. Suppression of CRKL in established tumors derived from NSCLC cells with amplification of CRKL induced tumor regression in vivo. Overexpression of CRKL in immortalized human airway epithelial cells induced anchorage-independent growth and cooperated with suppression of NF1 to induce tumorigenicity in vivo. Furthermore, we demonstrated that amplification of CRKL and loss of NF1 co-occur in a subset of NSCLC. These observations implicate CRKL as a NSCLC oncogene. Although CRKL amplifications occur in a relatively small fraction of NSCLC, the finding that a similar fraction of NSCLC with translocations involving ALK respond to
CRKL Induces Transformation and Gefitinib Resistance

**Amplification of CRKL Induces Cell Transformation by Activating RAS and RAP1 Signaling**

By using both biochemical and genetic approaches, we have found that malignant transformation induced by CRKL involves both RAS and RAP1 signaling. By using a multiplex Luminex assay, we identified ERK and SRC as the downstream targets of CRKL. We further confirmed that CRKL activates RAS-RAF-ERK and SRC-RAP1 signaling by forming complexes with SOS1 and C3G, respectively. Our findings that expression of constitutively active RAP1 partially rescued NSCLC cells from proliferation inhibition induced by CRKL suppression define an important role of RAP1 signaling in proliferation and survival of NSCLCs with CRKL amplifications.

In a previous report in which the authors used a cell-free assay system, they showed that recombiant RAP1 stimulates BRAF kinase activity at a comparable level to KRAS (42). RAP1 was also able to enhance KRAS-stimulated BRAF kinase activity in an additive manner (42). In agreement with these findings, we found that RAP1 activation contributes to CRKL-induced MAPK activation and anchorage-independent growth in AALEE cells. These findings suggest that CRKL induces transformation of human airway epithelial cells through its ability to coordinate activation of both RAS and RAP1 pathways, resulting in robust activation of MAPK signaling.

**Overexpression of CRKL Induces Resistance to Small-Molecule EGFR Inhibitors**

Although most NSCLCs with EGFR mutations initially respond to treatment with an EGFR inhibitor, the majority of these tumors ultimately develop resistance to the treatment. In approximately one-half of these cases, resistance is attributable to the occurrence of a secondary mutation (T790M) in EGFR. Prior study showed that other genetic events, such as amplification of MET or mutation of PIK3CA, may contribute to drug resistance in some of the remaining cases and also co-occur with secondary EGFR mutations, possibly because of the selection of drug-resistant clones using different resistance mechanisms (54, 55). These alterations promote cell growth by activating PI3K-AKT signaling in the presence of an EGFR inhibitor. Here we report that overexpression of CRKL in EGFR-mutant cells induces resistance to an EGFR inhibitor by activating both SOS1-dependent MAPK and p85-dependent PI3K-AKT signaling.

When we examined samples derived from tumors resistant to EGFR inhibitor treatment, we found CRKL amplifications in a patient whose tumors exhibited a clinical response to EGFR inhibitor therapy but subsequently developed acquired resistance. Because CRKL amplification was not identified in a pretreatment sample, these observations implicate CRKL amplifications as another mechanism of resistance to EGFR inhibitor therapy beyond secondary EGFR mutations, MET amplifications, and PIK3CA mutations (55).

**CRKL as a Novel Therapeutic Target in NSCLCs Harboring CRKL Amplifications**

We found that suppression of CRKL has no effect on proliferation of AALE cells but selectively induced apoptotic cell death in NSCLC cells with CRKL amplifications, suggesting that targeting CRKL may have a high therapeutic index. By using both RNAi and a complementation approach, we further confirmed that disruption of the SH3N domain abolished the ability of CRKL to rescue NSCLC cells from shCRKL-induced apoptosis. Our findings not only validate CRKL as a therapeutic target but also demonstrate that blocking the function of the SH3N domain is sufficient to modulate CRKL activity in NSCLCs harboring CRKL amplifications.

The statistical analysis of copy number alterations in 3,131 cancer specimens derived from 27 histologic types identified the 22q11.21 amplicon as one of the top 12 most commonly amplified regions in multiple cancer types (GISTIC q-value = 3.71 × 10^-19), including NSCLCs, melanoma, ovarian, and colorectal cancers (19). CRKL is located at the center of the minimal region of amplification at 22q11.21 (19). These findings suggest that CRKL acts as an oncogene in other cancers harboring CRKL amplifications and that strategies to inhibit CRKL signaling may be useful in several cancer types.

**METHODS**

**Plasmids**

Human CRKL (obtained from the CCSB ORFeome collection) was cloned into pWZl-blast and pLenti6.3-blast-C-terminal VS epitope-tagged vectors. The CRKL mutants (CRKLY207F, CRKLW160L, CRKLY207F+W160L, CRKLY207F+W275L, CRKLW160L+W275L, CRKLW160L+W275L, CRKL W275L, CRKL Y207F, CRKL W160L, CRKL Y207F+W275L, CRKL Y207F+W160L) were generated by use of the Quikchange Site-Directed Mutagenesis kit (Stratagene). Human RAP1A, RAP1A Q63E (provided by Gromoslaw Smolen, Massachusetts General Hospital), RAP1B (Origene), and RAP1BG12V were cloned into the pLenti6.2-blast-LacZ control vector (30).

**siRNAs and CRKL shRNAs**

A pool of 85 control shRNAs targeting reporter genes (GFP, RFP, Luciferase, and LacZ) was used to generate control lentiviruses (Control shRNAs) (30). Human R-containing CRKL shRNAs were generated. shCRKL#1 (TRCN0000036378), shCRKL#2 (TRCN0000036380), and shCRKL#3 (TRCN0000036381) were cloned into the pWZl-blast and pLenti6.3-blast-SRC and SRC T351I were generated by use of the Quikchange Site-Directed Mutagenesis kit (Stratagene). Human RAP1A, RAP1A Q63E (provided by Gromoslaw Smolen, Massachusetts General Hospital), RAP1B (Origene), and RAP1B T351I were cloned into the pWZl-blast and pLenti6.3-blast-SRC and SRC T351I have been described (45). The pLenti6.2-blast-LacZ control vector was provided by Guo Wei, Dana-Farber Cancer Institute. pMKO.1-puro-shNF1, pBabe-puro-HRAS (29), and myristoylated AKT1 have been described (51). pKO.1-puro-shRNA constructs were obtained from The RNAi Consortium. The sequences targeted by CRKL-specific shRNAs are as follows: shCRKL#1 (TRCN0000036378), 5′-GCTCTGCTCTACCATGTTTAA-3′, shCRKL#2 (TRCN0000036380) 5′-CGTGAAGTCAGAGGAAGCTA-3′, shCRKL#3 (TRCN0000036381), 5′-GCCTACTGAGTAGCTTTCAT-3′, shCRKL#4 (TRCN0000036382), 5′-GCTCTGCTCTACCATGTTTAA-3′. The sequences targeted by shNF1 and shHRAS were as follows: shNF1 (TRCN000010984), 5′-GCTCTGCTCTACCATGTTTAA-3′, 5′-GCTCTGCTCTACCATGTTTAA-3′, 5′-GCTCTGCTCTACCATGTTTAA-3′. The sequences targeted by shNF1 and shHRAS were as follows: shNF1 (TRCN000010984), 5′-GCTCTGCTCTACCATGTTTAA-3′, 5′-GCTCTGCTCTACCATGTTTAA-3′, 5′-GCTCTGCTCTACCATGTTTAA-3′.
Cell Culture and Virus Production

HCC515, H1819, HCC1833, H2087, and HCC827 cells were maintained in DMEM (Mediatech) supplemented with 10% FBS (Sigma-Aldrich). HCC1359, H1755, H1437, and H1792 cells were maintained in RPMI1640 (Mediatech) supplemented with 10% FBS. Immortalized human lung airway epithelial (AALE) cells (32) were maintained in SABM supplemented with SAGM SingleQuots (Lonza). Retroviruses were produced by transfecting 293T packaging cells with pBabe/pWzl/pMKO and pCL-Ampho plasmids (51). Lentiviruses were produced by transfecting 293T packaging cells with a 3-plasmid system (30). To generate stable cell lines, cells were selected in media containing 2 μg/mL puromycin for 2 days or 10 μg/mL blasticidin for 4 days.

Chemicals

Gefitinib, dasatinib, and GDC-0941 were purchased from Selleck Chemicals. PHA665752 was purchased from Tocris Biosciences. LY294002 was purchased from EMD Biosciences.

Cell Proliferation Assay

Cells were seeded into 96-well plates for 24 hours. Six replicate infections were performed for control shRNAs or each gene-specific shRNA in the presence of 4 μg/mL polybrene for 24 hours followed by selection in media containing 2 μg/mL puromycin. The ATP content was measured at 5 days by use of the CellTiter-Glo luminescent cell viability assay (Promega). Data represent mean ± SD of 6 replicate measurements.

For the rescue experiments, H1775 (1.8 × 106) cells were incubated with lentiviruses expressing Lacz control, CRKL, RAP1, RAP1A 63E, RAP1B, or RAP1B1212 in 24-well plates in the presence of 4 μg/mL polybrene and spin-infected at 1,000 × g for 2 hours at 37°C. Cells were then trypsinized and replated at a density of 1,500 cells/well in 96-well plates for 24 hours. For measuring IC50, HCC827 cells (1.5 × 105) expressing CRKL or a control vector were seeded into 96-well plates for 24 hours and then incubated with gefitinib for 72 hours. The ATP content was measured by use of the CellTiter-Glo luminescent cell viability assay (Promega). Data represent mean ± SD relative to untreated cells for each cell line. For the RNAi experiments, HCC827 cells overexpressing CRKL or a control vector were infected with lentiviruses encoding indicated shRNAs for 24 hours, and then selected with 2 μg/mL puromycin for 2 days. Cells were replated into 96-well plates for 24 hours before treatment with gefitinib for 72 hours.

Luminex Immunosandwich Assay

AALE (1.5 × 105) cells were plated onto each well of 6-well plates for 24 hours. Triplicate transfections with 3 μg of pLenti-LacZ, CRKL, or CRKL1212 were performed with FuGENE 6 (Roche) for 16 hours. After the incubation, medium was replaced with fresh medium and cells were cultured for another 32 hours before selecting cell lysates. The Luminex immunoassay was performed as described (45).

Anchorage-Independent Growth Assay

Growth in soft agar was determined by plating 5 × 103 cells in triplicate in 0.4% Noble agar (51). Colonies greater than 0.2 mm in diameter were counted 4 weeks after plating. Data represent mean ± SD of 6 replicate measurements from 2 independent experiments.

Tumorigenicity Assay

Tumor xenograft experiments were performed as described (51). AALE cells expressing the indicated constructs were trypsinized and collected in DMEM supplemented with 10% FBS. Cells (8 × 106) were resuspended in 400 μL of 1× PBS and mixed with 400 μL of Matrigel-Basement Membrane Matrix, LDEV-free (BD Biosciences). The cell mixture (200 μL, containing 2 × 106 cells) was injected subcutaneously into 6-week-old male BALB/c nude mice (Charles River). Tumor injection sites were monitored for 5 months.

For tumor growth studies, HCC515 cells were infected with lentiviruses expressing luciferase followed by selection in media containing 10 μg/mL blasticidin for 5 days. Luciferized HCC515 cells were transduced with lentiviruses encoding pLKO-Tet-On-scrambled control shRNA or pLKO-Tet-On-shCRKL#1 and selected in 2 μg/mL puromycin to generate stable cell lines. Cells (5 × 105) were resuspended in 200 μL of PBS and injected subcutaneously into 6-week-old male BALB/c nude mice (Charles River). Tumor size was measured twice weekly by a caliper. Noninvasive bioluminescent imaging was performed at 11 and 32 days after implantation. Mice were given a single intraperitoneal injection of a mixture of luciferin (50 mg/kg), ketamine (150 mg/kg), and xylazine (12 mg/kg) in sterile water. After 5 minutes, mice were placed in a chamber and photons were captured for a time period of 120 seconds with an IVIS imaging camera (Xenogen). Images were generated by the use of LIVING IMAGE 2.60.1 software.

Immunoblotting

Cell lysates were prepared by scraping cells in lysis buffer (50 mM Tris, pH 8; 150 mM NaCl; 1% Nonidet P40; 0.5% sodium deoxycholate; and 0.1% sodium dodecyl sulfate) containing complete protease inhibitors (Roche) and phosphatase inhibitors (10 mM sodium fluoride and 5 mM sodium orthovanadate). Protein concentration was measured by use of the BCA Protein Assay kit (Pierce). An equal amount of protein (30 μg) was separated by NuPAGE Novex Bis-Tris 4%–12% gradient gels (Invitrogen) and then transferred onto a polyvinylidene difluoride membrane (Amersham). The membrane was then incubated with primary antibodies for 1 hour at room temperature. Phospho-specific antibodies against phospho-S473 AKT (#9271), phospho-Y1068 EGFR (#3777), phospho-Y1289 ERBB3 (#4791), phospho-T202/Y204 ERK1/2 (#4370), phospho-Y118 Paxillin (#2541), phospho-S338 RAF1 (#9427), and phospho-Y416 SRC (#2113) were purchased from Cell Signaling. Antibodies against ERK1/2 (#4695), ARAF (#4432), RAF1 (#9422), Caspase-3 (#9662), and PARP (#9532) were purchased from Cell Signaling Technology. Antibodies against BRAF (sc-5284), phospho-Y504 C3G (sc-12926), C3G (sc-869), CRKL (sc-319), KRAS (sc-30), SOS1 (sc-256), and SOS2 (sc-19) were from Santa Cruz Biotechnology, Inc. Antibodies against phospho-Y249 p130CAS (#558401) and p130CAS (#610272) were from BD Biosciences. Antibodies against p85 (sc-0088), RAS (sc-005), RAF1 (sc-07-161), phospho-MEK1/2 (#9126), and phospho-tyrosine (4G10) were from Millipore. Antibody specific for NF1 was provided by Karen Chichowski, Brigham and Women’s Hospital.

After incubation with the appropriate horseradish peroxidase-linked secondary antibodies (Bio-Rad), signals were visualized by enhanced chemiluminescence plus Western blotting detection reagents (Amersham). Expression of β-actin was also assessed as an internal loading control by the use of a specific antibody (sc-8432-HRP; Santa Cruz Biotechnology, Inc.). Intensities of bands were quantified by LabWorks image analysis software (UVP).

Immunoprecipitation

Cell lysates were collected by scraping cells in immunoprecipitation buffer (20 mM Tris, pH 8; 100 mM NaCl, 2 mM EDTA; and 0.5% NP-40) containing protease and phosphatase inhibitors. Protein extracts (1 mg) were incubated with 2 μg of anti-CRKL antibody (sc-319; Santa Cruz Biotechnology, Inc.), 5 μg of anti-p85 antibody (sc-106-496; Millipore), or normal Rabbit IgG (sc-2070; Santa Cruz Biotechnology, Inc.) for 2 hours at 4°C followed by incubation with 30 μL of Protein A/G-PLUS agarose beads (sc-2033; Santa Cruz Biotechnology, Inc.) for 1 hour at 4°C. Beads were collected by centrifugation at 2,000 × g for 3 min at 4°C and resuspended in 1 mL of immunoprecipitation buffer.
for washing. After 4 repeats of washing and centrifugation, beads were boiled for 5 minutes in 1× NuPAGE LDS sample buffer (Invitrogen).

The pull-down assay for GTP-bound RAS or RAP1 was performed by the use of RAS and RAP1 activation assay kits (Millipore), respectively. For measuring in vitro BRAF kinase activity, 500 µg of protein extracts was incubated with 2 µg of anti-BRAF antibody (sc-5284; Santa Cruz Biotechnology, Inc.) or normal mouse IgG (sc-2025; Santa Cruz Biotechnology, Inc.) for 2 hours at 4°C followed by incubation with 20 µL of Protein A/G-Plus agarose beads for 1 hour at 4°C. After 5 washes with immunoprecipitation buffer, the beads were incubated with 1.34 µg of MEK substrate proteins (#17-359; Millipore) in the presence of 37.5 mM MgCl2 and 250 µM ATP for 30 minutes at 30°C. The beads were boiled in sample buffer for 5 minutes, and the supernatants were resolved for immunoblotting for phospho-MEK1 (#07-461; Millipore).

**Real-Time Quantitative Reverse-Transcription PCR**

Total RNA was extracted with TRIzol reagent (Invitrogen) and 1 µg of total RNA was used to synthesize the first-strand cDNA using Oligo(dT)20/random hexamer primer cocktails and Superscript III reverse transcriptase (Invitrogen). Quantitative PCRs were performed with the use of SYBR green PCR Master Mix (Applied Biosystems). The primer sequences used are as follows: CRKL-primer set 1 (forward: 5'-CTTAGCTGTTCCAGAATCT-3' and reverse: 5'-CAGGCCCTCAAGTTGACCTG-3'), CRKL-primer set 2 (forward: 5'-AACGACCACAATGGTGTCAGT-3' and reverse: 5'-ACTCCACCCTGTTCCAGG-3'), and GAPDH (forward: 5'-CCTGTTCGACAGTCA-3' and reverse: 5'-AAAGCCGCTCAACTACATGG-3'). Triplicate reactions were performed separately on the same cDNA samples by use of the ABI 7900HT real-time PCR instrument (Applied Biosystems). The mean cycle threshold was used for the comparative cycle threshold analysis method (ABI User Bulletin #2; http://www3.appliedbiosystems.com/cms/groups/mcb_support/documents/generaldocuments/cms_040980.pdf).

**Quantitative PCR for Gene Copy Number**

The standard curve method was used to determine the copy number of CRKL in NSCLC and AALE cells. Genomic DNA was extracted by use of the DNeasy blood and tissue kit (QIAGEN). The primer sequences for detecting CRKL were 5'-TTGACAGGCACTGCGTTAGA-3' and 5'-GGACCTCACCACGTTCTT-3'. The primer sequences for detecting LINE-1 were 5'-AAACGGCTCAACTACATGG-3' and 5'-TCTGATTGAGCGAGGTCC-3'. The standard curve was generated by PCR of serially diluted genomic DNA of AALE cells (50, 10, 2, 0.4, and 0.08 ng). Triplicate reactions were performed by the use of 2 ng of genomic DNA extracted from NSCLC cells. The gene copy number was normalized to LINE-1 and normal reference DNA of AALE cells.

**Fluorescence In Situ Hybridization**

The BAC RP11-505B16 clone containing CRKL (Invitrogen) was labeled with digoxigenin (Roche) and the BAC RP11-47N6 clone containing 22q13.2 as a reference (Invitrogen) was labeled with digoxigenin (Roche) and the BAC RP11-47N6 clone (Invitrogen). Hybridization was performed for 18 hours at 37°C in a dark, humid chamber. After hybridization, slides were washed in 0.5× SSC at 72°C for 5 minutes and rinsed in PBS containing 0.025% Tween-20 at room temperature. Slides were blocked with CAS-Block containing 10% normal goat serum (Invitrogen) and incubated with FITC-anti-digoxigenin (Roche) and Alexa Fluor 594 Streptavidin (Invitrogen). Slides were washed in PBS containing 0.025% Tween-20 and counterstained with DAPI (Invitrogen). Images were captured with the use of a Zeiss Axio Observer Z1 microscope and AxioVision imaging software (Zeiss).

Tumor specimens from erlotinib-treated patients were obtained from Dana-Farber Cancer Institute, Brigham and Women’s Hospital, and Massachusetts General Hospital. All samples were analyzed under Institutional Review Board-approved protocols. The presence of EGFR mutations in each specimen was confirmed as described (54, 55). FISH analysis of tumor specimens with a CRKL-specific probe (BAC RP11-505B16) and a chromosome 22 telomere-specific probe (Abbott Molecular) were performed as described previously (54).

**Disclosure of Potential Conflicts of Interest**

W.C. Hahn and M. Meyerson are consultants for Novartis Pharmaceuticals; P.A. Jäne is a consultant for Roche, Genentech, Astra-Zeneca, Boehringer Ingelheim, and Pfizer. No other potential conflicts of interest were disclosed.

**Acknowledgments**

We thank Karen Cichowski, Heidi Greulich, Guo Wei, and Gnomoslaw Smolen for reagents and cell lines; Rosanne Drapkin, Lynette Sholl, Craig Mermel, Rameen Beroukhim, Milan Chheda, Susan Moody, and David Barbie for helpful discussions; and Qi Wang, Laura Johnson, and Sapana Thomas for technical assistance.

**Grant Support**

This work was supported in part by grants from the National Institutes of Health (R33 CA128625, U54 CA112962, R01 CA135257, R01 CA114465, P50 CA090578).

Received March 4, 2011; revised September 23, 2011; accepted October 6, 2011; published OnlineFirst October 17, 2011.

**REFERENCES**


CRKL Induces Transformation and Gefitinib Resistance


Amplification of **CRKL** Induces Transformation and Epidermal Growth Factor Receptor Inhibitor Resistance in Human Non–Small Cell Lung Cancers

Hiu Wing Cheung, Jinyan Du, Jesse S. Boehm, et al.

*Cancer Discovery* 2011;1:608-625. Published OnlineFirst October 17, 2011.

<table>
<thead>
<tr>
<th>Updated version</th>
<th>Access the most recent version of this article at: doi:10.1158/2159-8290.CD-11-0046</th>
</tr>
</thead>
<tbody>
<tr>
<td>Supplementary Material</td>
<td>Access the most recent supplemental material at: <a href="http://cancerdiscovery.aacrjournals.org/content/suppl/2011/10/17/2159-8290.CD-11-0046.DC1">http://cancerdiscovery.aacrjournals.org/content/suppl/2011/10/17/2159-8290.CD-11-0046.DC1</a></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Cited articles</th>
<th>This article cites 55 articles, 28 of which you can access for free at: <a href="http://cancerdiscovery.aacrjournals.org/content/1/7/608.full.html#ref-list-1">http://cancerdiscovery.aacrjournals.org/content/1/7/608.full.html#ref-list-1</a></th>
</tr>
</thead>
<tbody>
<tr>
<td>Citing articles</td>
<td>This article has been cited by 17 HighWire-hosted articles. Access the articles at: <a href="http://cancerdiscovery.aacrjournals.org/content/1/7/608.full.html#related-urls">http://cancerdiscovery.aacrjournals.org/content/1/7/608.full.html#related-urls</a></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>E-mail alerts</th>
<th>Sign up to receive free email-alerts related to this article or journal.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reprints and Subscriptions</td>
<td>To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at <a href="mailto:pubs@aacr.org">pubs@aacr.org</a>.</td>
</tr>
<tr>
<td>Permissions</td>
<td>To request permission to re-use all or part of this article, contact the AACR Publications Department at <a href="mailto:permissions@aacr.org">permissions@aacr.org</a>.</td>
</tr>
</tbody>
</table>