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Genomic and functional analysis identifies *CRKL* as an oncogene amplified in lung cancer

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Abstract

DNA amplifications, leading to the overexpression of oncogenes, are a cardinal feature of lung cancer and directly contribute to its pathogenesis. To uncover novel such alterations, we performed an array-based comparative genomic hybridization survey of 128 non-small cell lung cancer cell lines and tumors. Prominent among our findings, we identified recurrent high-level amplification at cytoband 22q11.21 in 3% of lung cancer specimens, with another 11% of specimens exhibiting low-level gain spanning that locus. The 22q11.21 amplicon core contained eight named genes, only four of which were overexpressed (by transcript profiling) when amplified. Among these, *CRKL* encodes an adaptor protein functioning in signal transduction, best known as a substrate of the BCR-ABL kinase in chronic myelogenous leukemia. RNA interference-mediated knockdown of *CRKL* in lung cancer cell lines with (but not without) amplification led to significantly decreased cell proliferation, cell-cycle progression, cell survival, and cell motility and invasion. In addition, overexpression of *CRKL* in immortalized human bronchial epithelial cells led to EGF-independent cell growth. Our findings indicate that amplification and resultant overexpression of *CRKL* contributes to diverse oncogenic phenotypes in lung cancer, with implications for targeted therapy, and highlighting a role of adapter proteins as primary genetic drivers of tumorigenesis.

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Keywords

CRKL; lung cancer; DNA amplification; genomic profiling; adapter protein

INTRODUCTION

Lung cancer is the leading cause of cancer death in the United States, accounting for almost 30% of all cancer-related mortality (Jemal *et al.*, 2008). Nearly 80% of lung cancers diagnosed are non-small cell lung cancers (NSCLC), which are classified into three main histological subtypes: adenocarcinoma, squamous-cell carcinoma, and large-cell carcinoma. Despite the advancement of surgical, cytotoxic, and radiological treatment options over the years, lung cancer therapy remains largely ineffective from a clinical standpoint, as evidenced by a low 5-year survival rate (<15%), and underscores the aggressive nature of the disease.

Much effort has been directed towards elucidating the pathogenetic alterations underlying the initiation and progression of NSCLC, with the hope of developing novel therapeutics to selectively target those alterations *in vivo*. Indeed, recent application of epidermal growth factor receptor (EGFR) tyrosine kinase inhibitors has been moderately successful in the treatment of NSCLCs harboring activating point mutations of EGFR (Lynch *et al.*, 2004; Paez *et al.*, 2004). It is likely that other genetic alterations in NSCLC await discovery, and once characterized might provide useful targets for therapy.

Genomic DNA amplifications are a frequent class of aberrations in NSCLC, where increased gene dosage leads to overexpression of key cancer genes. Genomic profiling studies of NSCLC, using cDNA (Kwei *et al.*, 2008; Tonon *et al.*, 2005), BAC (Garnis *et al.*, 2006), oligonucleotide (Kendall *et al.*, 2007; Tonon *et al.*, 2005) and SNP (Weir *et al.*, 2007; Zhao *et al.*, 2005) arrays have revealed focal amplicons harboring known oncogenes such as *KRAS* (12p12.1), *EGFR* (7p12.2), *ERBB2* (17q12), *MET* (7q31.2), *MYC* (8q24.1), *CDK4* (12q14.1), and *CCND1* (11q13.2), and have led to the recent discovery of *TTF1* (14q13) as a lineage-dependent oncogenic transcription factor amplified in lung cancer (Kendall *et al.*, 2007; Kwei *et al.*, 2008; Weir *et al.*, 2007). For other recurrent amplicons, the driver oncogene(s) have not yet been identified, and mapping such loci provides a starting point for cancer gene discovery and characterization. Here, from a cDNA microarray-based genomic profiling analysis of 128 lung cancer specimens, we identify amplification of *CRKL* (22q11) as a recurrent genetic event driving cell proliferation, survival and invasion in lung cancer.

RESULTS

Recurrent 22q11 amplicon in NSCLC spans *CRKL*

To identify recurrent DNA amplifications pinpointing novel oncogenes in NSCLC, we analyzed cDNA array CGH data generated on 52 NSCLC cell lines and 76 NSCLC tumors, the latter comprising 36 adenocarcinomas (including 2 metastases) and 40 squamous cell carcinomas (with 1 metastasis), totaling 128 samples. One of the most frequently amplified loci not associated with a known oncogene occurred at cytoband 22q11.21 (Fig. 1a), where

high-level amplification (fluorescence ratios > 3, corresponding to >5-fold amplification, (Pollack *et al.*, 1999)) was found in 4 of 128 samples analyzed (3%), with low-level gain spanning 22q11.21 present in an additional 14 of 128 samples (11%). There was no significant difference in the frequency of 22q11.21 gain/amplification between adenocarcinoma and other histologies (both for cell lines and for tumors), nor in the NSCLC lines was there a significant association between 22q11.21 gain/amplification and mutation of either *KRAS*, *EGFR* or *TP53* (data not shown). On the other hand, we observed significant (FDR<0.01) association of 22q11.21 gain with concomitant gains elsewhere in the genome, namely at 9q34.3, 11q13.2-q13.3 (*CCND1*), 15q24.1 and 21q22.3 (Supplementary Table 2), suggesting possible cooperative interactions.

The smallest region of recurrent amplification (or amplicon core) (Fig. 1b), spanned approximately ~170 Kb within 22q11.21, and contained eight known RefSeq genes, which included *SNAP29* (synaptosomal-associated protein 29), *CRKL* (v-crk avian sarcoma virus CT10 oncogene homolog-like), *AIFM3* (apoptosis-inducing factor, mitochondrion-associated 3), *LZTR1* (leucine-zipper-like transcriptional regulator 1), *THAP7* (THAP domain-containing protein 7), *FLJ39582* (hypothetical protein LOC439931), *P2RXLI* (purinergic receptor P2X-like 1, orphan receptor), and *SLC7A4* (solute carrier family 7 (cationic amino acid transporter, y+ system), member 4). Expression profiling, done in parallel with array CGH, revealed that only four genes (*SNAP29*, *CRKL*, *LZTR1*, *THAP7*) were overexpressed when amplified ($P<0.05$, Student's t-test) (Fig. 2a), thus effectively narrowing the list of candidate "driver" oncogenes. One of these genes, *CRKL*, encodes a Src homology 2 and 3 (SH2/SH3) domain-containing adaptor protein that shares homology with the *CRK* proto-oncogene (ten Hoeve *et al.*, 1993). Best known as a substrate of the BCR-ABL oncogenic kinase in chronic myelogenous leukemia (Sattler & Salgia, 1998), a role of *CRKL* in other cancer types remains largely unexplored. We therefore sought to characterize a possible role of *CRKL* amplification in lung cancer.

***CRKL* amplification promotes cell proliferation and survival**

To assess the functional significance of *CRKL* amplification and overexpression in NSCLC, we utilized siRNAs directed against *CRKL* in two cell lines, HCC515 and H1819, for which *CRKL* amplification had been validated by fluorescence *in situ* hybridization (Fig. 1c), and with increased levels of total and phosphorylated (activated) CRKL protein (Fig. 2b). Transfection of two different siRNAs targeting distinct sequences within CRKL each led to decreased levels of total and p-CRKL protein (Fig. 3a), and to significantly decreased cell proliferation (measured using the WST-1 assay) compared to a negative control siRNA targeting an irrelevant gene, *GFP* (green fluorescent protein) (Fig. 3b). In contrast, knockdown of *CRKL* in H157, a lung cancer cell line without *CRKL* amplification and with comparatively less expression of *CRKL*, led to a more subtle effect on cell proliferation, supporting the specificity of *CRKL* targeting.

The observed decrease in cell proliferation might be attributable to decreased cell-cycle progression, increased apoptosis, or both. To distinguish these possibilities, we assayed cell-cycle progression by measuring BrdU incorporation, and apoptosis by Annexin V staining. Targeted knockdown of *CRKL* in the amplified lines HCC515 and H1819 resulted in

decreased cell-cycle progression, as evidenced by a significant decrease in S phase fraction with G₁ block (Fig. 3c) compared to control siRNA. CRKL knockdown in amplified cell lines also led to increased apoptosis, evidenced by the higher fraction of Annexin-V positive cells with siRNA targeting CRKL versus control (though reaching significance only for HCC515) (Fig. 3d).

CRKL amplification drives cell migration and invasion

Prior studies have implicated a role for CRKL in epithelial cell migration and invasion, with relevance to metastatic potential (Feller, 2001). We therefore also sought to evaluate a possible role of *CRKL* amplification in cell migration/invasion in lung cancer. Targeted knockdown of CRKL in amplified NSCLC cell lines HCC515 and H1819 led to a significant inhibition of both cell migration (Fig. 4a) and invasion (Fig. 4b), compared to control siRNA. Furthermore, no such effect was observed in the lung cancer cell line H157 without amplification (Fig. 4a, b), revealing a specific connection between *CRKL* amplification and cell migration and invasion. Co-transfection of a siRNA-resistant CRKL cDNA (containing silent mutations in the siRNA target site) largely rescued CRKL/p-CRKL levels and invasiveness of H1819 cells (Fig. 4c), further confirming siRNA targeting specificity.

CRKL overexpression promotes growth factor independence

To complement the RNAi knockdown studies, we also sought to determine whether CRKL overexpression might promote oncogenic phenotypes in non-tumorigenic lung epithelial cells. HBEC3 (Ramirez *et al.*, 2004) is a human bronchial epithelial cell line immortalized by hTERT and Cdk4 (the latter bypassing p16-associated growth arrest), and provides a useful model for assessing the contribution of lung cancer genes (Sato *et al.*, 2006). By retroviral transduction, we engineered HBEC3 cells stably overexpressing CRKL, though CRKL levels did not approach those observed in *CRKL*-amplified NSCLC lines (Fig. 5a). Nonetheless, overexpression of CRKL in HBEC3 cells significantly enhanced growth factor (EGF)-independent cell growth (Fig. 5b, c). However, CRKL overexpression in HBEC3 was not sufficient to promote cell invasion or anchorage-independent soft agar colony growth (data not shown).

DISCUSSION

By genomic profiling, we have identified a focal and recurrent amplicon at cytoband 22q11.21 in NSCLC cell lines and tumor samples. Within the amplicon core, *CRKL* is one of only four genes that are overexpressed when amplified. Interestingly, amplification appears even better correlated with p-CRKL levels than with total CRKL protein (Fig. 2b), suggesting that amplification occurs within a genetic or cellular context appropriate for CRKL signaling. RNA interference studies in NSCLC cell lines with 22q11.21 amplification demonstrate a role and functional dependency on CRKL amplification for tumor cell proliferation, survival and motility/invasion. Overexpression studies in non-tumorigenic HBEC3 cells also reveal a function of CRKL in growth-factor independent proliferation, a classic oncogenic phenotype and similar to that previously reported for K-RAS^{V12} expression and p53 knockdown (Sato *et al.*, 2006). However, in contrast to p53

(knockdown) and K-RAS^{V12} (Sato et al., 2006), CRKL alone (at least at the expression levels achieved) appears insufficient to enhance anchorage-independent growth of HBEC3 cells. Further studies may reveal possible cooperative effects with other lung cancer genes.

Other recently published genomic profiling studies using various microarray platforms have also reported amplifications spanning 22q11.21 in NSCLC. Using sub-megabase resolution tiling (SMRT) BAC arrays, Garnis *et al.* identified high-level amplification at 22q11.21 in 2/28 NSCLC cell lines analyzed (Garnis et al., 2006), both of which were verified in our study. Using 115K SNP arrays, Zhao *et al.* described 22q11.21 amplification in a panel of lung cancer cell lines (including HCC515, HCC1359 and H1819) and primary lung tumors (Zhao et al., 2005). They were able to localize the amplicon to a 1Mb region in 22q11.21, and suggested *CRKL* and *PIK4CA* (catalytic subunit of phosphatidylinositol 4-kinase α) (centromeric to *CRKL*, and in our dataset mapping outside the amplicon core), as possible driver genes. Interestingly, they went on to exclude *CRKL* as the likely driver because its protein levels were not increased in cell lines with amplification (their data not shown), a finding clearly discordant with ours. Most recently, Weir *et al.* used 250K SNP arrays to survey 371 lung adenocarcinomas, reporting high-level amplification at 22q11.21 in 2.4% of tumors, a frequency consistent with our findings (Weir et al., 2007). However, from the specimens surveyed they were only able to narrow down the amplicon to a ~1 Mb interval containing about 15 genes. Our own studies define a ~140 Kb amplicon core, including four genes that are overexpressed when amplified. Though our functional studies implicate *CRKL* as the driver, we cannot exclude the possibility that one or more of the other three genes (*SNAP29*, *LZTR1*, *THAP7*) contributes, though we note that their known functions do not relate in obvious ways to carcinogenesis. Of interest, very recently, Luo *et al.* identified *CRKL* through a short hairpin RNA (shRNA) screen as essential for cell proliferation in a subset of NSCLC cell lines studied (Luo et al., 2008).

Our findings define a role of *CRKL* amplification in NSCLC pathogenesis. CRKL (Crk-Like) (ten Hoeve et al., 1993) is a member of the human Crk adaptor protein family, which also includes two alternatively spliced isoforms (CRKI/II) of *CRK*, the cellular homologue of the avian retroviral v-crk oncogene (Feller, 2001). CRKL contains SH2 and SH3 domains that mediate protein-protein interactions connecting tyrosine-phosphorylated upstream signaling components (e.g. p130CAS, paxillin, CBL, GAB1) to downstream effectors (e.g. C3G, DOCK180), regulating diverse cellular processes like cell adhesion, migration and immune cell responses (Feller, 2001). Early studies identified CRKL as a key substrate and effector of the BCR-ABL oncogenic tyrosine kinase in chronic myelogenous leukemia (Hemmerlyckx et al., 2001; Senechal et al., 1996; ten Hoeve et al., 1994). CRKL overexpression was also shown to activate Ras and JUN kinase (JNK) signaling pathways, and to transform Rat-1 (Senechal et al., 1996), though not NIH-3T3 (de Jong et al., 1997), rodent fibroblasts.

In epithelial cells, CRKL has been shown to potentiate hepatocyte growth factor (HGF; scatter factor)-induced cell motility through protein complexes connecting the MET receptor tyrosine kinase to downstream activation of effector proteins like Rap1 and Rac (Feller, 2001; Furge et al., 2000). The connection to MET is particularly intriguing given that MET is activated by mutation or amplification in some lung cancers (Ma et al., 2003; Zhao et al.,

2005). In our NSCLC cell line panel, amplification of *MET* and *CRKL* was mutually exclusive (data not shown), consistent with their acting in the same pathway (with *CRKL* downstream of *MET*). However, in preliminary studies, while knockdown of *CRKL* in a NSCLC cell line with *MET* amplification (H1648) reduced cell proliferation and survival, knockdown of *MET* in that same cell line reduced proliferation/survival (consistent with a recent report (Lutterbach *et al.*, 2007)) but not phospho-*CRKL* levels (unpublished findings). Further studies are required to clarify the connection between *MET* and *CRKL* in NSCLCs with *MET* amplification.

While we report here the amplification of *CRKL* in lung cancer, *CRKL* may play a pathogenic role in other epithelial cancers as well. Recently, Singer *et al.* described overexpression of phospho-*CRKL*, measured by immunohistochemistry, in breast, ovarian and colon cancer, as well as lung cancer, in comparison to the corresponding normal tissues (Singer *et al.*, 2006). Our own array CGH studies have identified amplifications spanning 22q11.21 in breast and pancreatic cancers (Bashyam *et al.*, 2005; Bergamaschi *et al.*, 2006). Further studies are needed to establish a functional role of *CRKL* amplification/overexpression in these cancers. We also note that the related adapter *CRK* (at 17p13.3), while occasionally found within broad low-level gains in lung cancer, is not focally amplified as we have found *CRKL*.

A finding of particular interest from our study is that the amplification of an adapter protein, functioning solely to assemble other proteins, drives strong and pleiotropic oncogenic phenotypes. Prototypic oncogenic amplifications include tyrosine kinases (*ERBB2*, *EGFR*, *MET*), ras proteins (*KRAS*), cell-cycle modulators (*CCND1*, *CDK4*), and transcription factors (*MYC*). While overexpression of SH2/SH3 adapter proteins such as *GRB2* (Daly *et al.*, 1994), *GRB7* (Stein *et al.*, 1994) and *GAB2* (Daly *et al.*, 2002) have been implicated in oncogenesis, and v-Crk itself was discovered as an avian oncogene, our findings now place amplification of an adapter protein gene (*CRKL*) as a primary genetic event driving human cancer.

Our findings also underscore the potential of novel therapeutics targeting adapter protein interactions, and targeting *CRKL* in lung cancer in particular. Indeed, a small molecule inhibitor disrupting interactions of the *GRB2* SH2 domain has been described (Gay *et al.*, 1999), and more recently a peptide inhibitor selectively targeting the *CRKL* SH3 domain, disrupting its interaction with *BCR-ABL* (Kardinal *et al.*, 2000). In summary, our combined genomic and functional analysis defines a novel role of *CRKL* amplification in lung carcinogenesis, potentiating cell proliferation, growth-factor independence, survival and migration/invasion, highlighting the oncogenic role of adapter proteins and suggesting a new point for therapeutic intervention.

MATERIALS AND METHODS

Lung cancer cell lines and tumors

“NCI-H” series NSCLC cell lines, established at the National Cancer Institute, and “HCC” series cell lines, established at the Hamon Center for Therapeutic Oncology Research, UT Southwestern Medical Center (Dallas, TX), together totaling 52 cell lines, were obtained

from the latter's tissue culture repository (most lines are currently available through the American Type Culture Collection). For functional studies, cells were cultured in RPMI-1640 (Invitrogen, Carlsbad, CA) with 10% fetal bovine serum (Hyclone, Fisher Scientific, Pittsburgh, PA). HBEC3 cells (Ramirez et al., 2004), obtained from the same repository, were grown in K-SFM medium (Invitrogen) containing 50 µg/mL bovine pituitary extract (Invitrogen) with 5 ng/mL EGF (Invitrogen). Seventy-six freshly-frozen lung tumors were banked at the University Hospital Charité, Berlin, Germany, with Institutional Review Board approval. Specimens were verified by H&E staining to contain at least 70% tumor cells.

Genomic and gene-expression profiling

cDNA microarray-based genomic profiling by comparative genomic hybridization (CGH), and mRNA transcript profiling, of 128 lung cancer cell lines and tumors was described in a preliminary report of our study, focused on the identification of *TITF1* amplification (Kwei et al., 2008). cDNA microarrays contained 39,632 human cDNAs, representing 22,279 mapped human genes plus 4,230 additional mapped expressed sequence tags (ESTs). The complete microarray datasets are available at Stanford Microarray Database (<http://smd.stanford.edu>) and at the Gene Expression Omnibus (accession GSE9995).

Microarray data analysis

Background-subtracted fluorescence ratios were normalized by mean-centering genes for each array. For array CGH analysis, we selected only those genes whose Cy3 reference-channel fluorescence signal intensity was at least 40% above background in at least 50% of samples. Map positions for arrayed cDNA clones were assigned using the NCBI genome assembly (Build 36), accessed through the UCSC genome database (Kent *et al.*, 2002). We utilized the method cghFLasso (R package) to identify DNA gains and losses (Tibshirani & Wang, 2008). High-level DNA amplifications were defined as contiguous regions called by cghFLasso where at least 50% of genes display fluorescence ratios ≥ 3 . To detect associations between DNA copy number alterations at distinct loci, a Pearson's correlation was computed between the mean copy number of a given cytoband and that of all other cytobands. Statistically significant correlations were determined by randomly permuting cytoband labels and recalculating correlations 100 times; a false discovery rate (FDR) of 1% was used to establish a significance threshold. For expression profiling, fluorescence ratios were normalized for each array, and then well-measured genes (fluorescence intensities for the Cy5 or Cy3 channel at least 50% above background) were subsequently mean-centered (i.e. reported for each gene relative to the mean ratio across all samples).

Fluorescence *in situ* hybridization (FISH)

Probe labeling and FISH were performed using Vysis (Downers Grove, Illinois; now Abbott Molecular) reagents according to the manufacturer's protocols. A locus-specific BAC probe targeting *CRKL* at 22q11.21 (RP11-1058B20) (BACPAC Resources Centre, Oakland, CA) was labeled with SpectrumGreen-dUTP, and co-hybridized with SpectrumOrange-dUTP labeled chromosome 22 telomeric probe (TelVysion 22q; Vysis). Chromosomal locations of labeled BAC probes were validated on metaphase slides prepared from normal donors.

Slides were counterstained with DAPI, and imaged using an Olympus BX51 fluorescence microscope with Applied Imaging (San Jose, CA) Cytovision 3.0 software.

Small interfering RNA (siRNA) transfections

Two different siRNAs targeting *CRKL*, along with a negative control siRNA targeting an irrelevant gene, *GFP*, were obtained from Qiagen. Complete siRNA sequences are provided (Supplementary Table 1). Cell lines were maintained at 37°C in complete media of RPMI-1640 with 10% FBS prior to transfection. For transfection, 80,000-175,000 cells were seeded per 6-well plate well, and transfected using Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer's protocol. Cells were transfected with a final concentration of 50nM siRNA for 6 hrs, subsequently replaced with complete growth media.

Plasmid constructs

A full-length human *CRKL* cDNA expression vector, pCMV6/XL4-CRKL, and the parent vector pCMV6/XL4, were purchased from OriGene (Rockville, MD). A *CRKL* siRNA#1-resistant *CRKL* cDNA was engineered using the QuickChange XL II Site-Directed Mutagenesis Kit from Stratagene (La Jolla, CA), with the following mutational primers: 5'-GGTTGGTGACATCGTGAAGGTGACCCGGATGAACATTAATGGCCAGTGGGA AG-3' (degenerate, mutated bases denoted by bold text) and 5'-CTTCCCACTGGCCATTAATGTTCATCCGGGTCACCTTACGATGTCACCAACC-3'. To generate HBEC3 cells stably overexpressing *CRKL*, a retroviral expression vector was created by PCR-amplifying full-length *CRKL* cDNA and subcloning the product into the *HpaI* and *XhoI* restriction sites of the vector pMSCV-Hyg (Clontech, Mountain View, CA).

Viral transduction and stable selection

293T cells were transfected with either pMSCV-Hyg or pMSCV-Hyg-CRKL together with pVpack-VSVG and pVpack-GP vectors (Stratagene) to generate replication-defective retrovirus. Viral supernatants were collected 48 hrs post-transfection, and used to infect HBEC3 cells, which were subsequently treated with 10 µg/mL hygromycin B (Invitrogen) for 14 days for stable selection.

Western blot analysis

Cells were lysed in 1X RIPA Lysis buffer (Upstate/Chemicon, San Francisco, CA) supplemented with 1X Complete Protease Inhibitor (Roche, Indianapolis, IN), 0.1mM sodium orthovanadate, 1mM sodium fluoride and 1mM PMSF, and protein quantified using the BCA assay (Pierce, Rockford, IL). For Western blot, 20-30µg of protein lysate was electrophoresed on a 4-15% Criterion Tris-HCl polyacrylamide gradient gel (Biorad, Hercules, CA) and transferred overnight to a PVDF membrane (Biorad). After blocking in TBS-T buffer (20mM Tris-HCl pH 7.4, 0.15M NaCl, 0.1% Tween 20) with 5% dry milk for 45min, blots were incubated with primary antibody overnight (for phospho-specific) at 4°C or 90min (for native) at room temperature. After sequential washing steps, blots were incubated with HRP-conjugated secondary antibody for 45min at room temperature in TBS-T buffer. The following antibodies were used: phospho-CRKL Y207 (1:500; #3181, Cell

Signaling, Danvers, MA); anti-CRKL rabbit polyclonal antibody (1:1000; sc-319, Santa Cruz Biotechnology, Santa Cruz, CA); GAPDH (1:5000; for loading control; Santa Cruz Biotechnology); HRP-conjugated anti-rabbit IgG (1:20,000, Pierce).

Proliferation assay

Cell proliferation was quantified by WST-1 assay (Roche), a colorimetric assay based on the metabolic cleavage of the tetrazolium salt WST-1 in viable cells, according to the manufacturer's protocol. 100 μ l of WST-1 reagent was added to 1ml of culture volume in 6-well plates and incubated at 37°C for 30min. Absorbance was then measured at 450nm with reference to 650nm using a Spectra Max 190 plate reader (Molecular Devices, Sunnyvale, CA). Transfections were performed in triplicate and average (\pm 1 SD) OD reported.

Cell-cycle analysis

Cell-cycle distribution analysis was performed by flow cytometry using the BrdU-FITC Flow kit (BD Biosciences, San Jose, CA) per the manufacturer's instructions. Cells were incubated with 10 μ M BrdU at 37°C for 5hrs, then fixed and permeabilized with Cytofix/Cytoperm buffer (BD Biosciences). Cellular DNA was treated with DNase at 37° C for 1hr to expose incorporated BrdU, then cells were stained with anti-BrdU FITC antibody to quantify incorporated BrdU and 7-aminoactinomycin D (7-AAD) to quantify total DNA content. 15,000 events were scored by FACSCalibur (BD Biosciences) flow cytometer and analyzed using CellQuest software (BD Biosciences). Transfections were performed in triplicate and average (\pm 1 SD) cell-cycle fractions reported.

Apoptosis assay

Apoptosis levels were assayed by annexin V staining, quantified by flow cytometry using the Vybrant Apoptosis Assay kit #2 (Invitrogen) per manufacturer's recommendations. Briefly, floating and trypsinized adherent cells were pooled and resuspended in 200 μ l 1X annexin binding buffer. 1 μ l Alexa Fluor 488 annexin V and 1 μ l of 100 μ g/ml propidium iodide (PI) solution were added and cells incubated for 15min at room temperature. Cells were then resuspended in equal volume of 1X annexin binding buffer and analyzed immediately by flow cytometry. 15,000 events were scored by FACSCalibur and analyzed using CellQuest software. Transfections were performed in triplicate, and average (\pm 1 SD) percent apoptosis reported.

Invasion and migration assay

Invasion and migration assays were carried out using BD Biocoat (BD Biosciences) modified Boyden chambers and control inserts with polyethylene membrane, respectively. For invasion assays, precoated filters (8 μ m pore size, Matrigel 100 μ g/cm²) were rehydrated with 500 μ l of complete growth media (RPMI-1640, 10% FBS), then 1-5 \times 10⁴ cells resuspended in RPMI-1640 media with 2% FBS were seeded into the upper chamber. Following incubation for 16-72hrs at 37°C, cells were fixed in 10% buffered formalin and then stained with crystal violet. Migration assays were performed similarly to invasion assays, with the exception of using control inserts (w/o Matrigel coating) and using fewer cells (1-2.5 \times 10⁴ cells) and shorter time points (16-48hrs). Non-invaded (or migrated) cells in

the upper membrane were removed by swabbing, and the amount of invasion (or migration) was quantified by counting stained cells on the underside of the membrane. Assays were performed in triplicate and the average (\pm 1 SD) cell count reported.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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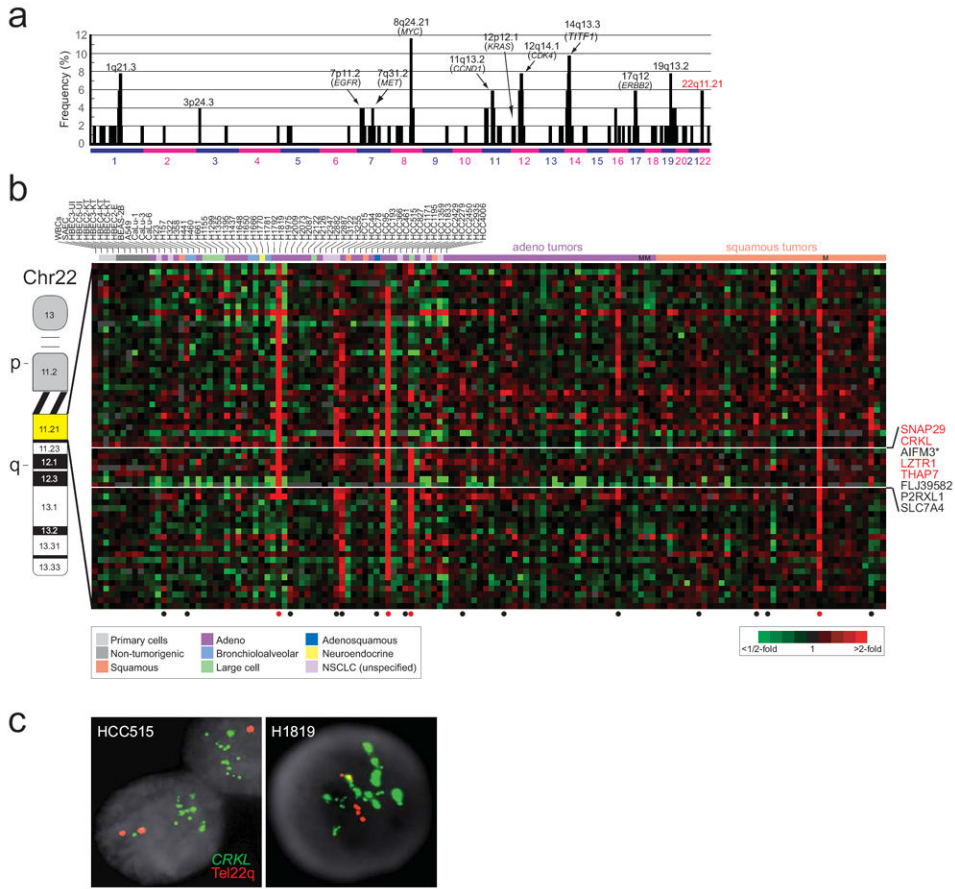


Figure 1. Recurrent 22q11 amplicon in NSCLC spans *CRKL*

(a) Frequency plot of cytobands harboring high-level DNA amplification in NSCLC cell lines. Cytobands containing known oncogenes in lung cancer are indicated, with 22q11.21 highlighted in red. (b) Heatmap representation of array CGH profiles of NSCLC cell lines and tumors representing a segment of 22q11.21. Each column represents a different sample (histologies indicated, M=metastasis) and each row represents a different gene ordered by chromosome position. Red indicates positive tumor/normal array CGH ratios (scale shown), and samples called gained (by cghFLasso; ref. (11)) or highly-amplified at 22q11.21 are marked below by closed black or red circles, respectively. Genes residing within the smallest common region of gain (amplicon core) are indicated; those with increased expression when amplified (see Fig. 2a) are highlighted in red. *AIFM3* (asterisk) was not present on the array but resides where shown. (c) FISH validation of *CRKL* amplification in NSCLC cell lines HCC515 and H1819. DNA amplification is indicated by increased *CRKL* (green) to telomere-22q (red) signals or signal clusters.

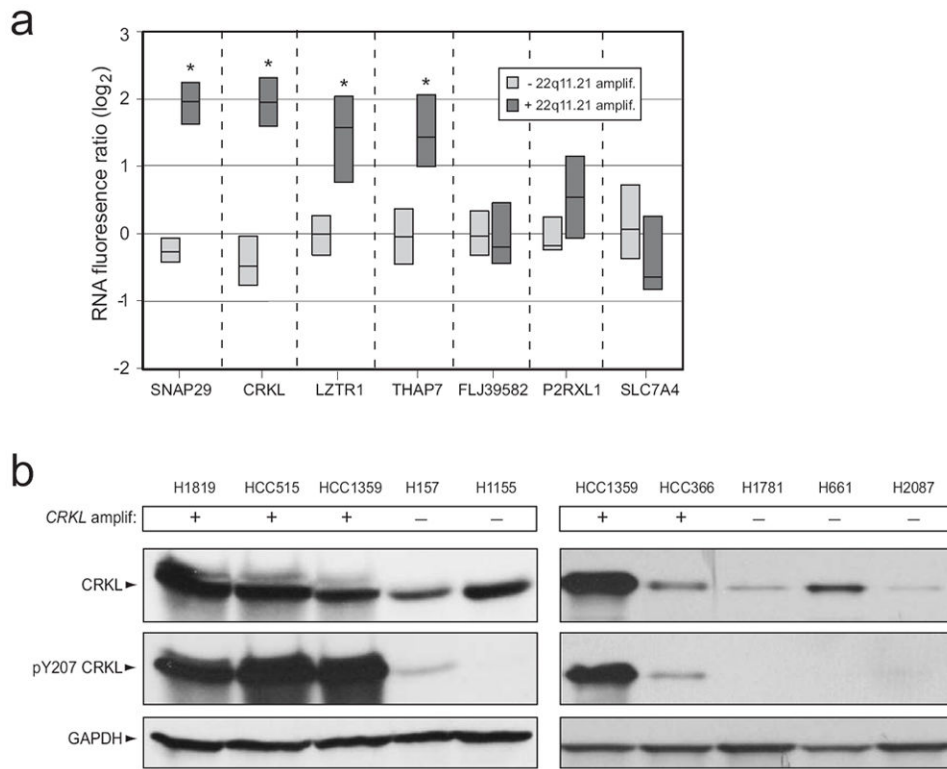


Figure 2. *CRKL* is overexpressed when amplified

(a) mRNA transcript levels (centered \log_2 ratios, measured by microarray) of genes residing within the 22q11.21 amplicon core, plotted separately for specimens with and without 22q11.21 amplification. Box plots show 25th, 50th (median) and 75th percentiles of expression for samples; genes whose expression is significantly (Student's t-test, $P < 0.05$) elevated with DNA amplification are indicated (*). (b) Western blot analysis of CRKL protein in representative NSCLC cell lines confirming overexpression in NSCLC cell lines with (compared to without) 22q11.21 amplification. Total and phosphorylated (active) CRKL (pY207) levels are shown. GAPDH serves as a loading control. Note, *CRKL* amplification appears better correlated with p-CRKL than total CRKL (see Discussion).

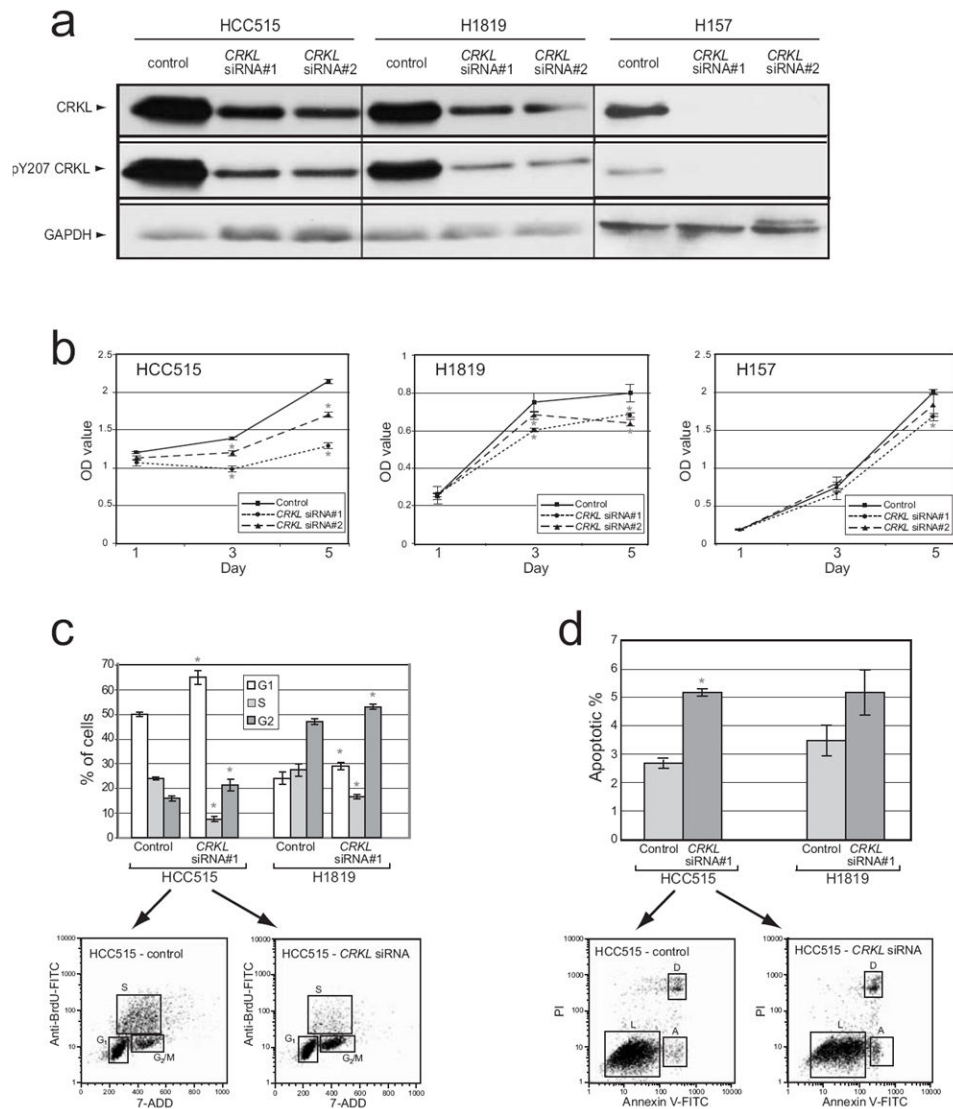


Figure 3. CRKL amplification contributes to cell proliferation and survival

(a) Confirmation of siRNA-mediated knockdown of CRKL protein by western blot analysis. Two different siRNA constructs targeting CRKL reduce total and phosphorylated protein levels in *CRKL*-amplified cell lines (HCC515 and H1819) and a cell line without amplification (H157), compared to a negative control siRNA targeting an irrelevant gene, *GFP*. GAPDH serves as a loading control. (b) siRNA-mediated knockdown of CRKL results in decreased cell proliferation, compared to control siRNA, as measured by WST-1 assay in *CRKL*-amplified cells (HCC515, H1819), with less pronounced effects in a non-amplified line (H157) expressing lower levels of CRKL (*, $P < 0.05$, Student's t-test, CRKL siRNA compared to control). (c) Knockdown of CRKL reduces cell-cycle progression as measured 72-hours post-transfection by BrdU incorporation, indicated by a decrease in S-phase fraction with G₁ block compared to control siRNA (*, $P < 0.05$, Student's t-test). Representative flow cytometry plots are also shown. (d) Knockdown of CRKL leads to increased apoptosis levels 72-hours post-transfection, as quantified by flow cytometry-based

Annexin V staining, compared to control siRNA (*, $P < 0.05$, Student's t-test). Representative flow cytometry plots are also shown; L, live; A, apoptotic; D, dead.

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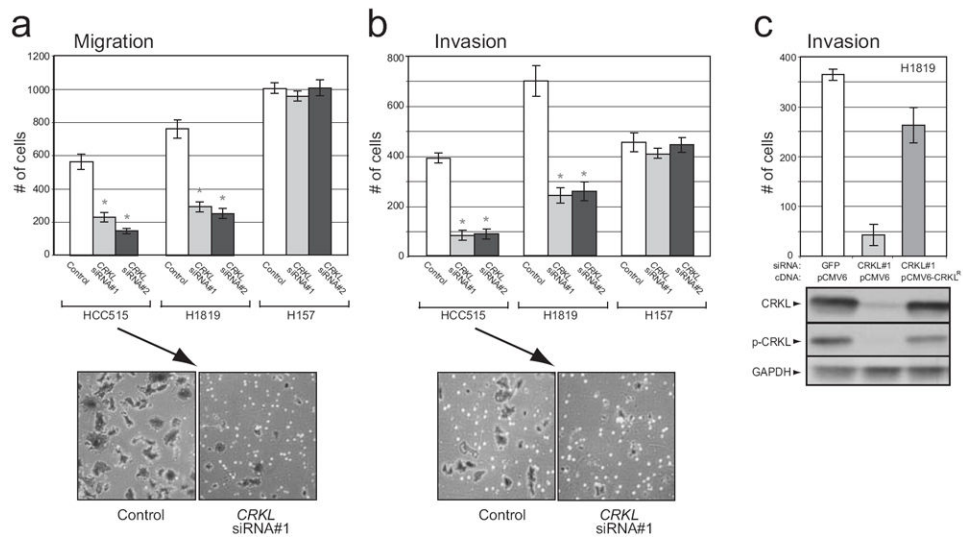


Figure 4. *CRKL* amplification potentiates cell motility and invasion
 siRNA-mediated knockdown of *CRKL*, compared to control siRNA, results in significantly decreased cell counts for (a) migration and (b) invasion in cell lines with *CRKL* amplification (HCC515 and H1819) (*, $P < 0.05$, Student's t-test). No significant effects are seen in H157, a cell line without *CRKL* amplification. Representative microphotographs depicting cell migration and invasion (CRKL siRNA compared to control) are shown. (c) Rescue of siRNA knockdown confirms targeting specificity. H1819 cells were co-transfected with siRNAs and cDNA expression vectors as indicated, and invasion assayed. pCMV6-*CRKL*^R contains silent mutations within the siRNA#1 target site, creating an siRNA-resistant transcript (see Methods section). Western blot (below) confirms knockdown and rescue of *CRKL* and p-*CRKL* levels; GAPDH serves as loading control.

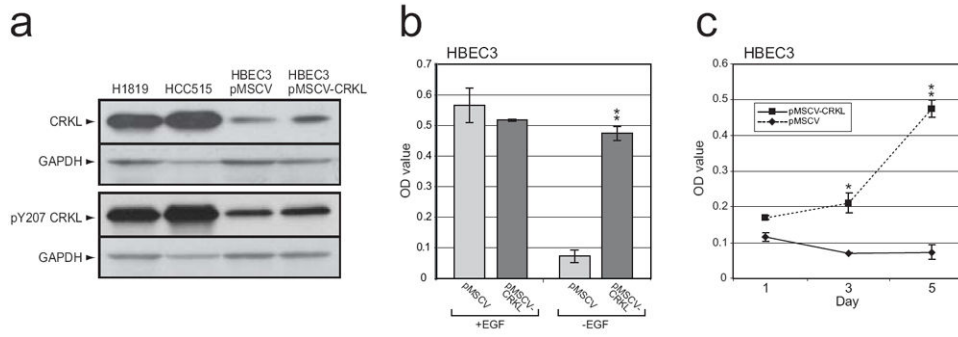


Figure 5. CRKL overexpression promotes growth-factor independent proliferation

(a) Western blot confirmation of CRKL and p-CRKL overexpression in stably-transduced HBEC3 cells. H1819 and HCC515 lines provide a comparison for *CRKL*-amplified levels. GAPDH serves as a loading control. (b) CRKL overexpression leads to EGF-independent cell growth, measured by WST assay. (c) Time course confirmation of CRKL-driven EGF-independent cell growth (*, $P < 0.05$; **, $P < 0.01$, Student's t-test; pMSCV-CRKL compared to pMSCV control).