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Quantitative Assessment Of Glioblastoma Cell Phenotypes Establishes Cell Migration As A Robust Readout Of CRK AND CRKL Activity

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QUANTITATIVE ASSESSMENT OF GLIOBLASTOMA CELL PHENOTYPES ESTABLISHES CELL MIGRATION AS A ROBUST READOUT OF CRK AND CRKL ACTIVITY

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ABSTRACT

Background: The expression levels of CT10 regulator of kinase (Crk) and Crk-like (CrkL) are elevated in many human cancers, including glioblastoma (GBM), and are believed to contribute to poor prognosis. Although Crk and CrkL have been proposed as therapeutic targets in these tumors, the lack of a reliable, quantitative assay to measure Crk and CrkL activity has hindered the development of inhibitors.

Methods: Here, we knocked down Crk, CrkL, or both using small interfering RNAs (siRNAs) in a human GBM cell line, U-118MG, to determine the respective, quantitative contributions of Crk and CrkL to cellular phenotypes.

Results: The combined use of specific and potent Crk and CrkL siRNAs induced effective knockdown of CrkII, CrkI, and CrkL. Whereas Crk knockdown did not affect cell morphology, proliferation, adhesion, or invasion, CrkL knockdown caused shrinkage of cells and inhibition of cell proliferation, adhesion, and invasion. Crk/CrkL double knockdown resulted in more pronounced morphological alterations and more robust inhibition of proliferation, adhesion, and invasion. Furthermore, Crk/CrkL double knockdown completely blocked cell migration, and this effect was rescued by transient overexpression of CrkL but not of Crk. Quantification of protein levels indicated that CrkL is expressed more abundantly than CrkII and CrkI in U-118MG cells.

Conclusions/Significances: These results demonstrate both the predominant role of CrkL and the essential overlapping functions of Crk and CrkL in U-118MG cells. Furthermore, our study indicates that migration of U-118MG cells depends entirely on Crk and CrkL. Thus, impedance-based, real-time measurement of tumor cell migration represents a robust assay for monitoring Crk and CrkL activities.

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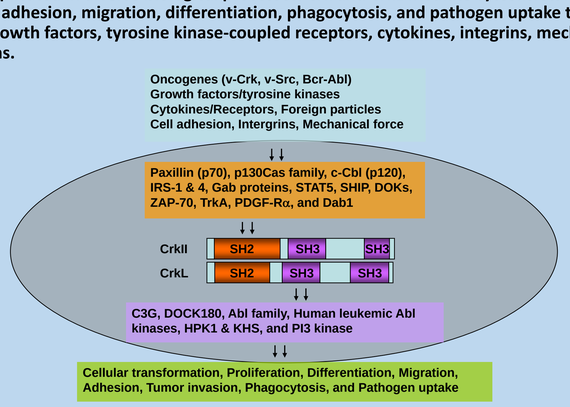
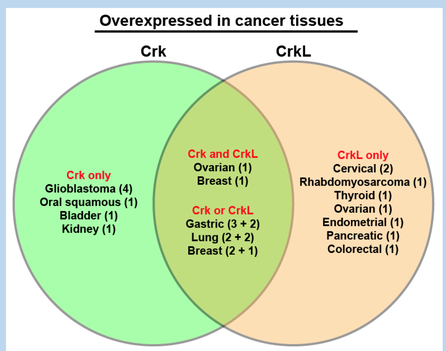
INTRODUCTION

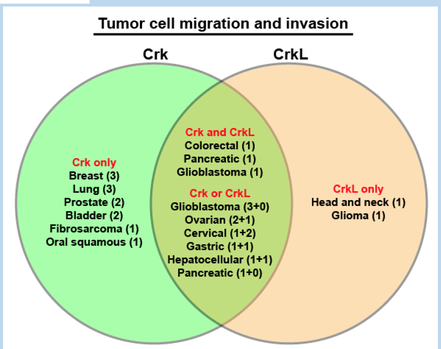
By mediating protein-protein interactions through their SH2 and SH3 domains, Crk and CrkL play essential roles in signal transduction pathways. Crk and CrkL are expressed ubiquitously, and they have been implicated in diverse biological processes. Crk and CrkL mediate cytoskeletal changes, cell proliferation, adhesion, migration, differentiation, phagocytosis, and pathogen uptake that are induced by growth factors, tyrosine kinase-coupled receptors, cytokines, integrins, mechanical force, and pathogens.



Crk and CrkL play critical roles in tumor cell migration and invasion in a variety of cancers. In most of these studies, loss of either Crk or CrkL resulted in decreases in tumor cell migration and invasion, suggesting that tumor cell migration and invasion are highly demanding cellular processes that require both Crk and CrkL to reorganize the cellular cytoskeletal network. In particular, both Crk and CrkL have been demonstrated to be important for tumor cell migration and invasion in colorectal cancer, glioblastoma, ovarian cancer, cervical cancer, gastric cancer, and hepatocellular carcinoma



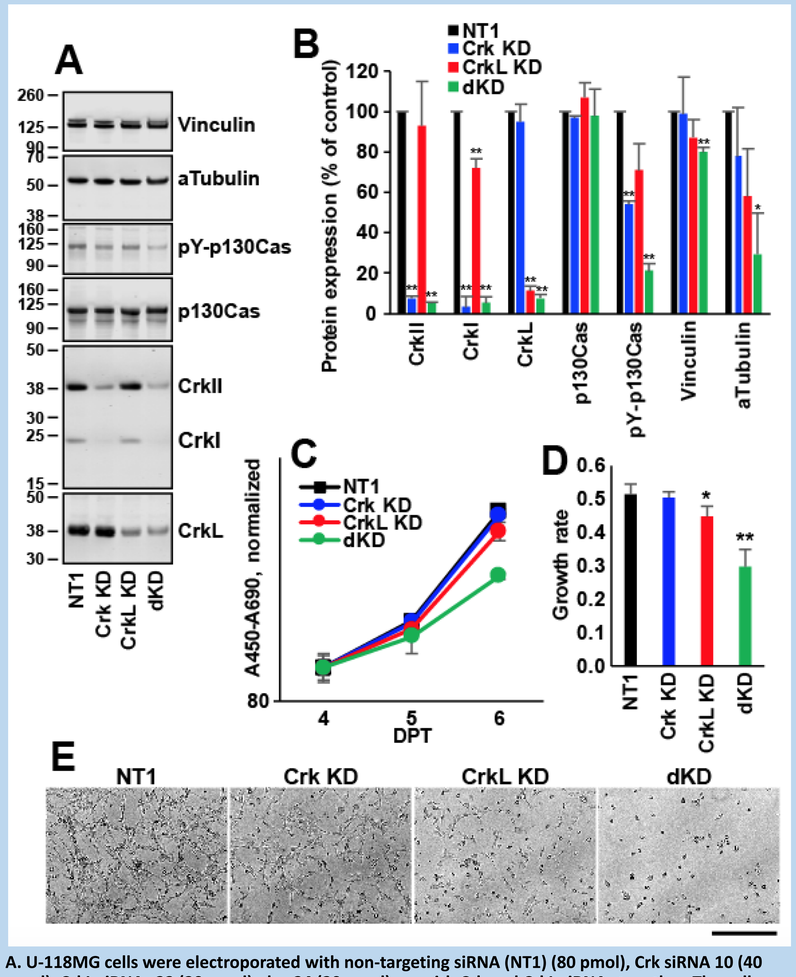
Higher expression of Crk and CrkL in cancer tissues has been reported to contribute to poor prognosis and lower survival of patients in many cancer types.



REFERENCES

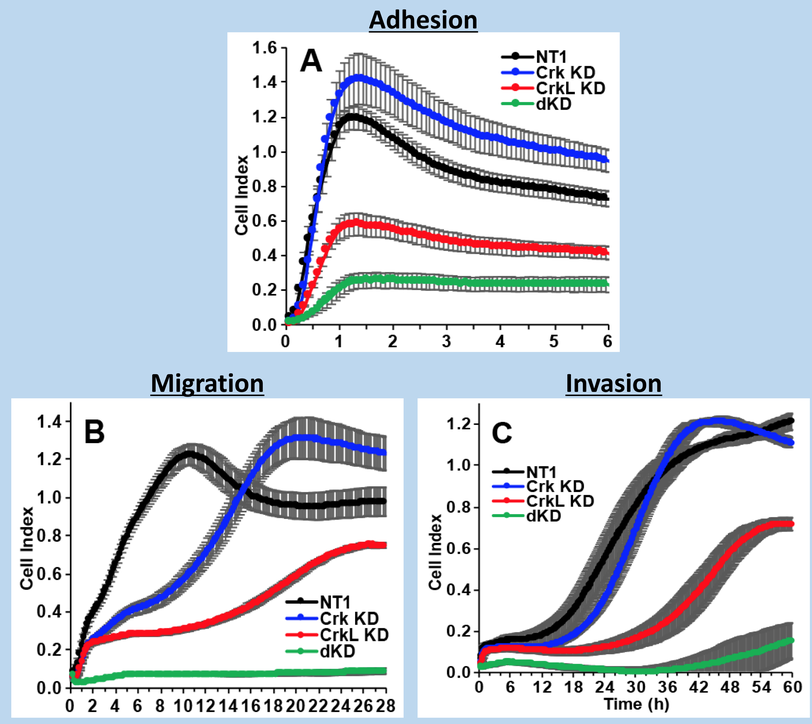
1) Park, T.; Large, N.; Curran, T. Quantitative assessment of glioblastoma phenotypes in vitro establishes cell migration as a robust readout of Crk and CrkL activity. J Biol Chem 2021, 10.1016/j.jbc.2021.100390, 100390, doi:10.1016/j.jbc.2021.100390.
2) Park T. Crk and CrkL as Therapeutic Targets for Cancer Treatment. Cells. 2021 Mar 27;10(4):739. doi: 10.3390/cells10040739.

Result 1. Single and double knockdown of Crk and CrkL using siRNAs in human glioblastoma cells



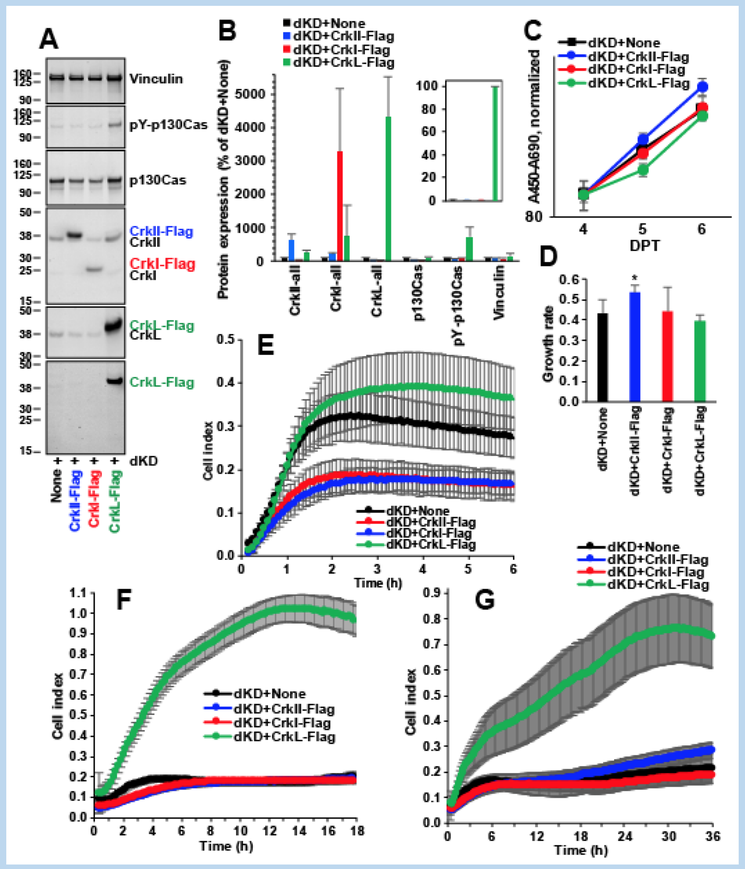
A. U-118MG cells were electroporated with non-targeting siRNA (NT1) (80 pmol), Crk siRNA 10 (40 pmol), CrkL siRNAs 22 (20 pmol) plus 24 (20 pmol), or with Crk and CrkL siRNAs together. The cells were harvested and re-plated at 3 days post transfection (DPT), and total cell lysates were prepared at 4 DPT for Western blot analyses. Protein levels upon siRNA transfection were compared with NT1. Alpha-tubulin and vinculin levels were measured as controls. B. Protein bands were quantified using the Odyssey system, calculated as percentages of the control (NT1), and their mean ± SD values are shown. *p < 0.05, **p < 0.01, compared with NT1. C. Proliferation of U-118MG cells electroporated with Crk and CrkL siRNAs after re-plating at 3 DPT was quantitatively measured using WST-1. The $A_{450-690}$ values are presented in the logarithmic scales. D. Exponential trendlines for the WST-1 assay graphs were drawn and their slopes, the coefficients of x, are presented as the rates for exponential cell growth. E. After cells were transfected with Crk and CrkL siRNAs followed by re-plating at 3 DPT, phase-contrast images of live cells were taken at 4 DPT using the EVOS system. Representative images are shown. Scale bar: 400 µm.

Result 2. Inhibition of cell adhesion, migration, and invasion by Crk and CrkL knockdown



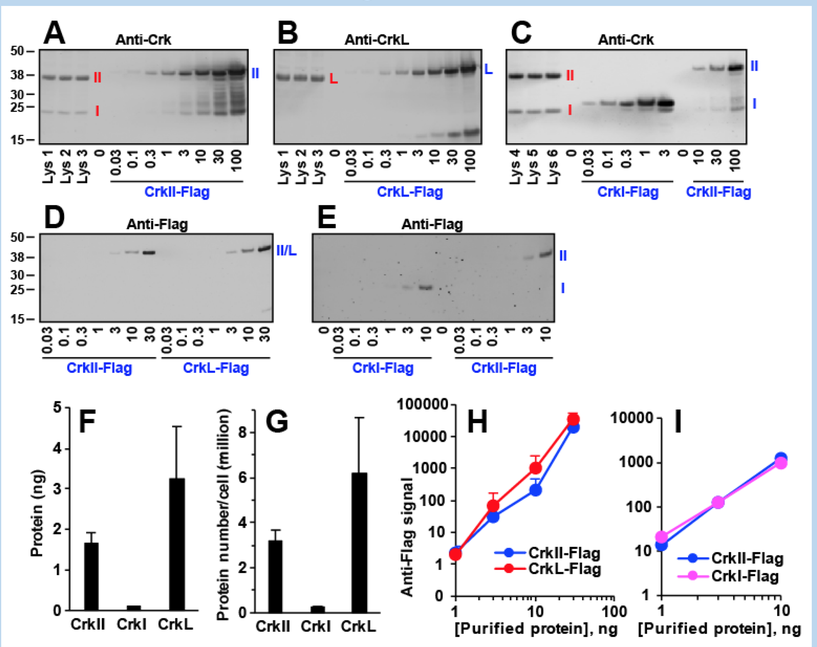
U-118MG cells were electroporated with NT1 (80 pmol), Crk siRNA 10 (40 pmol), CrkL siRNAs 22 (20 pmol) plus 24 (20 pmol), or with Crk and CrkL siRNAs together. At 3 DPT, the cells were harvested and plated onto an E-Plate 16, and two CIM-Plates without and with Matrigel coating for cell adhesion (A), migration (B), and invasion (C) using the xCELLigence system according to the Experimental Procedures. Cell index values were obtained from 4 wells for each sample, and their mean ± SD values are shown. Three independent experiments were carried out, and the results were reproducible.

Result 3. CrkL overexpression rescues the inhibitory effects induced by Crk/CrkL knockdown



U-118MG cells were first electroporated with Crk siRNA 10 (40 pmol) and CrkL siRNAs 22 (20 pmol) plus 24 (20 pmol) together. At 3 DPT, the cells were harvested, electroporated again with synRNA of Flag-tagged CrkII, CrkI, or CrkL, and re-plated for the various assays. A. At 4 DPT, total cell lysates were obtained for Western blot analyses. Vinculin levels were measured as controls. B. Protein bands were quantified using the Odyssey system, calculated as percentages of the control (None) signal, and their mean ± SD values are shown. Inset. Protein bands detected with anti-Flag antibody were quantified, calculated as percentages of the maximal signal (dKD + CrkL-Flag), and their mean ± SD values are shown. C. Proliferation of U-118MG cells electroporated first with siRNAs and then again with synRNA was quantitatively measured using WST-1. The $A_{450-690}$ values are presented in the logarithmic scales. D. Exponential trendlines for the WST-1 assay graphs were drawn and their slopes, the coefficients of x, are presented as the rates for exponential cell growth. *p < 0.05, compared with double knockdown only. E-G. U-118MG cells electroporated first with siRNAs and then again with synRNA were plated onto an E-Plate 16, and two CIM-Plate 16 without and with Matrigel coating for cell adhesion (E), migration (F), and invasion (G) using the xCELLigence system. Cell index values were obtained from 4 wells for each sample, and their mean ± SD values are shown.

Result 4. More abundant expression of CrkL in human glioblastoma cells



A-C. Crk and CrkL proteins were detected using anti-Crk (A and C) or anti-CrkL (B) antibodies from three different lysates prepared from U-118MG cells without any transfection (5 µg per lane). The indicated concentrations (ng) of purified proteins (A: CrkII-Flag, B: CrkL-Flag, and C: CrkI-Flag) were loaded together as the standard proteins. Endogenous Crk and CrkL proteins are marked in red. Purified proteins are marked in blue. F. For each Western blot image, protein bands were quantified using the Odyssey system, a standard curve was drawn for the purified protein, and a fourth-order polynomial trendline was obtained using the Microsoft Excel program. The trendline was used to determine the protein concentrations in the cell lysates. G. U-118MG cells were plated onto 35 mm dishes with similar densities to those for Western blot experiments. One day later, both the cell numbers and the total protein amounts were determined, and the mean of the total protein per cell was calculated to be 536 pg. This number was used to estimate the cell numbers for the U-118MG cell lysates that were prepared without counting. Protein molecular weights were calculated on http://web.expasy.org/protparam/. Then, the numbers of the protein molecules per cell were calculated. D-E. The indicated concentrations (ng) of purified Flag-tagged Crk and CrkL proteins were detected using an anti-Flag antibody. H-I. Protein bands for Flag-tagged Crk and CrkL proteins were quantified using the Odyssey system, and their mean ± SD values are shown in logarithmic scales.