Final Progress Report for Research Projects Funded by Health Research Grants

Instructions: Please complete all of the items as instructed. Do not delete instructions. Do not leave any items blank; responses must be provided for all items. If your response to an item is "None", please specify "None" as your response. "Not applicable" is not an acceptable response for any of the items. There is no limit to the length of your response to any question. Responses should be single-spaced, no smaller than 12-point type. The report **must be completed using MS Word**. Submitted reports must be Word documents; they should not be converted to pdf format. Questions? Contact Health Research Program staff at 717-783-2548.

1. **Grantee Institution:** The Children's Hospital of Philadelphia

2. **Reporting Period (start and end date of grant award period):** 1/1/2009 – 12/31/2012

3. **Grant Contact Person (First Name, M.I., Last Name, Degrees):** Nicole M. Young, B.S, CRA

4. **Grant Contact Person's Telephone Number:** 267-426-7747

5. **Grant SAP Number:** SAP 4100047628

6. **Project Number and Title of Research Project:** 1 - Role of Crk and CrkL in Normal and Neoplastic Growth

7. **Start and End Date of Research Project:** 1/1/2009 – 12/31/2012

8. **Name of Principal Investigator for the Research Project:** Tom Curran, PhD

9. **Research Project Expenses**.

   9(A) Please provide the total amount of health research grant funds spent on this project for the entire duration of the grant, including indirect costs and any interest earned that was spent:

   $ 2,060,618.00

   9(B) Provide the last names (include first initial if multiple individuals with the same last name are listed) of **all** persons who worked on this research project and were supported with health research funds. Include position titles (Principal Investigator, Graduate Assistant, Post-doctoral Fellow, etc.), percent of effort on project and total health research funds expended for the position. For multiple year projects, if percent of effort varied from year to year, report in the % of Effort column the effort by year 1, 2, 3, etc. of the project (x% Yr 1; z% Yr 2-3).

Last Name	Position Title	% of Effort on Project	Cost
Curran	Principal Investigator	20% Yr 1-4	119,174.86
Park	Research Associate	100% Yr 1-4	226,794.17
Koptyra	Post-doctoral Fellow	100% Yr 3-4	49,791.78
Briggs	Post-doctoral Fellow	100% Yr 2	21,408.80
Brumwell	Senior Research Technician	30% Yr 1	11,167.20
Li	Research Technician	100% Yr 2-4	58,352.51
Finn	Research Technician	100% Yr 3-4	19,776.40
Boyd	Research Technician	100% Yr 1-2	32,742.78
Cheng	Research Technician	50% Yr 2-3	18,046.52
Hsu	Research Technician	50% Yr 2	520.28
Kufahamu	Research Technician	50% Yr 1	500.94
Modest	Research Technician	50% Yr 1	1,167.48

9(C) Provide the names of **all** persons who worked on this research project, but who *were not* supported with health research funds. Include position titles (Research Assistant, Administrative Assistant, etc.) and percent of effort on project. For multiple year projects, if percent of effort varied from year to year, report in the % of Effort column the effort by year 1, 2, 3, etc. of the project (x% Yr 1; z% Yr 2-3).

Last Name	Position Title	% of Effort on Project
None		

9(D) Provide a list of **all** scientific equipment purchased as part of this research grant, a short description of the value (benefit) derived by the institution from this equipment, and the cost of the equipment.

Type of Scientific Equipment	Value Derived	Cost
None		

10. Co-funding of Research Project during Health Research Grant Award Period. Did this research project receive funding from any other source during the project period when it was supported by the health research grant?

Yes_____ No___√_____

If yes, please indicate the source and amount of other funds:

11. Leveraging of Additional Funds

11(A) As a result of the health research funds provided for this research project, were you able to apply for and/or obtain funding from other sources to continue or expand the research?

Yes_____ No____√_____

If yes, please list the applications submitted (column A), the funding agency (National Institutes of Health—NIH, or other source in column B), the month and year when the application was submitted (column C), and the amount of funds requested (column D). If you have received a notice that the grant will be funded, please indicate the amount of funds to be awarded (column E). If the grant was not funded, insert "not funded" in column E.

Do not include funding from your own institution or from CURE (tobacco settlement funds). Do not include grants submitted prior to the start date of the grant as shown in Question 2. If you list grants submitted within 1-6 months of the start date of this grant, add a statement below the table indicating how the data/results from this project were used to secure that grant.

A. Title of research project on grant application	B. Funding agency (check those that apply)	C. Month and Year Submitted	D. Amount of funds requested:	E. Amount of funds to be awarded:
None	☐NIH ☐ Other federal (specify:_____ _____) ☐ Nonfederal source (specify: _____)		$	$

11(B) Are you planning to apply for additional funding in the future to continue or expand the research?

Yes____√____ No_____

If yes, please describe your plans:

The researchers are preparing NIH grant applications including an R01 that will be based on a continuation of the work completed in this project. The grant proposals will use the knowledge and a variety of research resources obtained from this project to investigate defects in normal and aberrant cell growth in the absence of Crk and CrkL. In addition to the substantial cell morphology changes in cells lacking both Crk and CrkL, which is a major finding of Specific Aim 1 of this project, the researchers observed growth arrest of normal fibroblast cells in the absence of Crk and CrkL. Furthermore, cell transformation was inhibited by absence of Crk and CrkL, a key finding of Specific Aim 3 of this project. The proposed projects will address the mechanism of tumor cells' requirement of Crk and CrkL for their proliferation and its application to cancer therapy.

12. Future of Research Project. What are the future plans for this research project?

The researchers plan to continue work related to this project in the following ways:

1. Functions of Crk and CrkL in normal fibroblast growth
The lentiviral infection approaches that were used to address Specific Aim 1 were not efficient enough to deliver genes of interest to highly proliferating fibroblast cell lines. The researchers established an efficient RNA electroporation technique, and it worked very well for fast growing fibroblast cells. By combining knockout and reconstitution of Crk and CrkL, the researchers will address functions of Crk and CrkL in fibroblast cell growth.

2. Dependence of tumor cell growth on Crk and CrkL
The researchers will continue to work on functions of Crk and CrkL in cell transformation and tumor cell proliferation. Specific Aim 3 of this project indicated that Crk and CrkL are required for cell trnasformation. The researchers will test to see whether knockout of both Crk and CrkL has a synergistic effect in blocking cell transformation. Furthermore, the researchers will address whether transformed cells continue to require Crk and CrkL for their malignant proliferation using various in vitro and in vivo approaches.

3. Functions of Crk and CrkL in migration, differentiation and proliferation of neuronal and glial cells
The knowledge and resources obtained from Specific Aim 1 together with mouse models previously established by the reserchers will be used to investigate whether Crk and CrkL are required for neuronal and glial cell differentiation and proliferation. The researchers reported that Crk and CrkL play essential overlapping roles in neuronal cell migration and brain development. However, the underlying cellular mechanism has not yet been clearly defined. Study of primary neurons and glial cells in the absence of Crk and CrkL may provide insights into the mechnism.

13. New Investigator Training and Development. Did students participate in project supported internships or graduate or post-graduate training for at least one semester or one summer?

Yes____√____ No_____

If yes, how many students? Please specify in the tables below:

	Undergraduate	Masters	Pre-doc	Post-doc
Male	1			
Female				
Unknown				
Total				

	Undergraduate	Masters	Pre-doc	Post-doc
Hispanic				
Non-Hispanic	1			
Unknown				
Total				

	Undergraduate	Masters	Pre-doc	Post-doc
White				
Black				
Asian	1			
Other				
Unknown				
Total	**1**			

14. Recruitment of Out-of–State Researchers. Did you bring researchers into Pennsylvania to carry out this research project?

Yes____√____ No_____

If yes, please list the name and degree of each researcher and his/her previous affiliation:

Erin Finn, BS, National Human Genome Research Institute (NHGRI), Maryland
Joseph William Briggs, Ph.D. National Cancer Institute (NCI), Maryland
Mariel Boyd, BS, Fordham University, New York

15. Impact on Research Capacity and Quality. Did the health research project enhance the quality and/or capacity of research at your institution?

Yes____√____ No_____

If yes, describe how improvements in infrastructure, the addition of new investigators, and other resources have led to more and better research.

The health research project helped bring many researchers to the laboratory and perform excellent research. In addition, the project helped bring a very productive institution-wide collaboration with Dr. Janis Burkhardt and her colleagues.

16. Collaboration, business and community involvement.

16(A) Did the health research funds lead to collaboration with research partners outside of your institution (e.g., entire university, entire hospital system)?

Yes___√_____ No_____

If yes, please describe the collaborations:

The researchers actively pursued collaboration with researchers outside of the institution to investigate functions of Crk and CrkL in various cell and tissue types. The collaborations were very productive and helped uncover a variety of functions of Crk and CrkL. Specific Aim 2 included the following collaborations:

1) The researchers collaborated with Dr. Steven Burden and his colleagues at New York University Medical School to investigate roles of Crk and CrkL in neuromuscular synapse formation and found that Crk and CrkL play critical roles in neuromuscular synapse formation.

2) The researchers collaborated with Dr. Scott Oakes and his colleagues at University of California-San Francisco to study functions of Crk in ER stress-induced apoptosis, and found that Crk acts as a pro-apoptotic transducer and is required for efficient ER stress-induced apoptosis.

3) The researchers collaborated with Dr. Lawrence Holzman and his colleagues at University of Pennsylvania to analyze the function of Crk in podocytes in vitro and in vivo, and found that Crk plays critical roles in podocyte foot process spreading induced by protamine sulfate and nephrotoxic serum.

In addition, the researchers are currently collaborating with Dr. Xin Zhang at Indiana University School of Medicine to study functions of Crk and CrkL in eye development, Dr. Bernice Morrow at Albert Einstein College of Medicine to study functions of Crk and CrkL in heart development, and Dr. Jian Zuo at St. Jude Children's Research Hospital to study functions of Crk and CrkL in hair cell development.

16(B) Did the research project result in commercial development of any research products?

Yes_____ No____√_____

If yes, please describe commercial development activities that resulted from the research project:

16(C) Did the research lead to new involvement with the community?

Yes_____ No____√_____

If yes, please describe involvement with community groups that resulted from the research project:

17. Progress in Achieving Research Goals, Objectives and Aims.

List the project goals, objectives and specific aims (as contained in the grant agreement). Summarize the progress made in achieving these goals, objectives and aims for the period that the project was funded (i.e., from project start date through end date). Indicate whether or not each goal/objective/aim was achieved; if something was not achieved, note the reasons why. Describe the methods used. If changes were made to the research goals/objectives/aims, methods, design or timeline since the original grant application was submitted, please describe the changes. Provide detailed results of the project. Include evidence of the data that was generated and analyzed, and provide tables, graphs, and figures of the data. List published abstracts, poster presentations and scientific meeting presentations at the end of the summary of progress; peer-reviewed publications should be listed under item 20.

This response should be a DETAILED report of the methods and findings. It is not sufficient to state that the work was completed. Insufficient information may result in an unfavorable performance review, which may jeopardize future funding. If research findings are pending publication you must still include enough detail for the expert peer reviewers to evaluate the progress during the course of the project.

Health research grants funded under the Tobacco Settlement Act will be evaluated via a performance review by an expert panel of researchers and clinicians who will assess project work using this Final Progress Report, all project Annual Reports and the project's strategic plan. After the final performance review of each project is complete, approximately 12-16 months after the end of the grant, this Final Progress Report, as well as the Final Performance Review Report containing the comments of the expert review panel, and the grantee's written response to the Final Performance Review Report, will be posted on the CURE Web site.

There is no limit to the length of your response. Responses must be single-spaced below, no smaller than 12-point type. If you cut and paste text from a publication, be sure symbols print properly, e.g., the Greek symbol for alpha (α) and beta (ß) should not print as boxes (□) and include the appropriate citation(s). DO NOT DELETE THESE INSTRUCTIONS.

Project Goals and Objectives

Crk and CrkL are adaptor proteins that function in signal transduction processes during normal and neoplastic growth. Understanding the specific biological functions of Crk and CrkL has been challenging because deletion of either gene from the mouse germline results in embryonic or early postnatal lethality, and because both proteins function very similarly in biochemical and molecular assays. Previously, the Curran laboratory generated mutant mice carrying floxed alleles of *Crk* and *CrkL* allowing conditional mutation using the CRE system. Ablation of both Crk and CrkL in neuronal precursor cells demonstrated that they provide specific overlapping functions downstream of Reelin in the control of radial neuronal migration during brain development. The present project represents an expansion and new direction of this study to investigate the role of Crk and CrkL in a range of cell types in vitro and in vivo. Establishment of the $Crk^{fl/fl}/CrkL^{fl/fl}$ strain of mice as well as $Crk^{fl/fl}$ and $CrkL^{fl/fl}$ strains provides a unique and

powerful experimental tool that enables the studies proposed in specific aims 1-3. The overall hypothesis to be addressed is that Crk and CrkL function in signal transduction processes by linking tyrosine phosphorylation signals to alterations in cell growth, adhesion and migration properties. Three specific aims will be addressed that focus on the role of Crk and CrkL in fibroblast growth (Aim 1), in other cell types in vivo and in vivo (Aim 2) and in cell transformation and tumor growth (Aim 3). Comparison of phenotypes obtained from $Crk^{fl/fl}/CrkL^{fl/fl}$ cells and tissues with those from $Crk^{fl/fl}$ and $CrkL^{fl/fl}$ mice will provide detailed understanding of how Crk and CrkL play both overlapping and individually unique functions in various cells and tissues. The initial study of Crk and CrkL functions in fibroblast cells will elucidate their general role in cell biological processes. Understanding both conserved and specific functions of Crk and CrkL in various cell types will enable us to address fundamental questions in birth defects and cancer.

Specific Aim 1. Elucidate the Roles of Crk and CrkL in cultured fibroblasts.

Embryonic fibroblasts from $Crk^{fl/fl}$, $CrkL^{fl/fl}$ and $Crk^{fl/fl}/CrkL^{fl/fl}$ mice will be prepared and infected a viral vector expressing both CRE and green fluorescence protein (GFP) marker. The growth properties of cells lacking either, or both, Crk and CrkL, will be compared with those of control cells. Proliferation rates, density arrest, responses to growth factors, cell death signaling, adhesion and migration properties of the cell populations will be compared.

Achieving Research Goals for Specific Aim 1

The researchers generated embryonic fibroblast cells from $Crk^{fl/fl}$, $CrkL^{fl/fl}$ and $Crk^{fl/fl}/CrkL^{fl/fl}$ mice and established lentiviral vector systems for expressing both CRE and green fluorescence protein (GFP) marker. Loss of both Crk and CrkL from fibroblasts caused substantial changes in cell morphology and motility. The morphological alterations were accompanied by a decrease in focal adhesion sites, reduced actin stress fibers and a collapse of microtubule structures. Therefore, the researchers focused on defining the morphological phenotype and its mechanism. The researchers discovered that Crk and CrkL play critical roles in cell structure and motility by maintaining cytoskeletal integrity through communication with the extracellular environment. The findings from Specific Aim 1 provide the basic and essential groundwork for understanding the functions of Crk and CrkL discovered in Specific Aim 2 and Specific Aim 3. Therefore, the researchers suppose that the findings substantially meet its overall research goals for Specific Aim 1, and will focus more on the cell growth aspect of Crk and CrkL functions in the future.

Background

Crk and CrkL play important roles in signal transduction from receptor tyrosine kinases and nonreceptor tyrosine kinase-coupled receptors to various downstream effectors by interacting with many proteins through their SH2 and SH3 domains. Crk and CrkL are expressed in the vast majority of cell types and they have been implicated in a variety of biological processes including cell growth, migration and adhesion (for review see Birge *et al*., 2009). Crk was originally identified as the cellular counterpart of the viral oncogene v-Crk (Reichman *et al.*, 1992). Expression of v-Crk elevates tyrosine phosphorylation levels in cells although it does not have any catalytic activity itself (Mayer *et al.*, 1988). CrkII contains a single SH2 domain and

two SH3 domains. Alternative splicing generates CrkI and CrkIII, in which the C-terminal SH3 domain is either lacking or modified to be nonfunctional. It is still unclear whether CrkII and its splice variants differ in their expression and function. CrkL is similar to Crk in amino acid composition and overall domain structure, but the gene is located on a different chromosome in mammals. The SH2 domains of Crk and CrkL interact with many phosphotyrosine-containing proteins including paxillin (p70), p130Cas, and c-Cbl, while the N-terminal SH3 domains of Crk and CrkL interact with proline-rich motifs of many proteins including C3G, DOCK180, and the Abl family (reviewed by Feller, 2001).

Since Crk and CrkL are cellular homologues of the viral oncogene v-Crk, their oncogenic properties have been studied extensively. When overexpressed, CrkI induces altered cell morphology, proliferation in soft agar, and tumor growth in nude mice whereas CrkII does not cause transformation (Matsuda *et al.*, 1992). Overexpression of CrkL in fibroblast cells also induces transformation (Senechal *et al.*, 1996). In addition, Crk and CrkL have been reported to be overexpressed in several types of human cancers (for review see Sriram and Birge, 2010). Expression of Crk is significantly elevated in some breast adenocarcinoma (Rodrigues *et al.*, 2005), poorly differentiated lung adenocarcinoma (Miller *et al.*, 2003) and colon cancer (Nishihara *et al.*, 2002). Furthermore, tumor forming potential is significantly inhibited by Crk knockdown in the human ovarian cancer cell line MCAS (Linghu *et al.*, 2006) and in human synovial sarcoma cell lines (Watanabe *et al.*, 2006). Similarly, CrkL is amplified in non-small-cell lung cancers, and knockdown of CrkL in lung cancer cell lines decreases cell proliferation, motility and invasion (Kim *et al.*, 2010). Therefore, it has been suggested that an increase in protein levels of Crk and CrkL abnormally amplifies growth signaling, leading to cell transformation and enhanced tumor cell growth.

Despite elucidation of the oncogenic functions of Crk and CrkL, the normal physiological functions of these proteins are not yet clear. *Crk*-null embryos exhibit defects in cardiovascular and craniofacial development, and they die during embryonic development (Park *et al.*, 2006). *CrkL*-null embryos also die during embryogenesis as a consequence of lethal defects in cardiac and neural crest development (Guris *et al.*, 2001). These studies suggest that Crk and CrkL play essential distinct roles during embryonic development. The generation of floxed alleles of *Crk* and *CrkL* has made it possible to investigate tissue-specific contributions of Crk and CrkL to various biological processes. Using this strategy to remove Crk and CrkL from neuronal precursor cells, the researchers observed profound defects in neuronal migration and positioning, very similar to those described in *reeler* mice (Park and Curran, 2008). In addition, loss of both Crk and CrkL in skeletal muscle led to severe defects in the neuromuscular synapse (Hallock *et al.*, 2010). Since mice lacking either Crk or CrkL alone in neurons or skeletal muscles did not display any defects, it can be concluded that Crk and CrkL play essential overlapping roles in brain development and neuromuscular synapse formation. While these *in vivo* studies point to diverse biological functions of Crk and CrkL, it is important to conduct complementary studies *in vitro* to understand their mechanisms of action at the cellular level. Therefore, the researchers prepared fibroblast cells from mouse embryos carrying floxed alleles of *Crk* and *CrkL*, to examine the consequences of conditional ablation in cell culture.

Materials and Methods

Generation of mouse embryonic fibroblast cells and lentiviral infection
Mouse embryonic fibroblast (MEF) cells were prepared as previously reported (Park *et al.*, 2006). Embryos derived from intercrosses of Crk floxed, CrkL floxed, and Crk/CrkL double floxed mice were harvested at embryonic day 13.5. Heads and viscera were removed and used to extract genomic DNA for PCR genotyping. The remaining embryonic tissues were minced and treated with trypsin-EDTA (Invitrogen), and dissociated cells were cultured in Dulbecco's modified Eagle's medium (Cambrex) supplemented with 10% fetal bovine serum (HyClone), L-glutamine, penicillin, and streptomycin at 37°C under 5% CO_2. MEF cells at their early passages, when cell growth slowed down after the initial phase of massive cell proliferation, were used for lentiviral infection to minimize outgrowth of uninfected cells and to keep percentages of infected cells high during experiments. All the floxed MEF cells as well as wild-type cells grew normally and did not exhibit any significant abnormalities when cultured *in vitro*, which was expected as the floxed MEF cells normally express Crk and CrkL proteins in the absence of Cre expression. They were cryopreserved for future experiments. All mouse studies were carried out according to the protocols approved by the Institutional Animal Care and Use Committee at the Children's Hospital of Philadelphia Research Institute.

Lentiviral infection
Lentiviral vectors expressing GFP alone or GFP plus Cre were either purchased from Lentigen Co. (Baltimore, MD) or kindly provided by Dr. Philip Zoltick (the Children's Hospital of Philadelphia, Philadelphia, PA). For rescue experiments and other quantitative analyses, Cre and various Crk and CrkL constructs were cloned into pLVX-IRES-ZsGreen1 and pLVX-IRES-mCherry vectors (Clontech). Lentiviral particles were generated using Lenti-X HTX packaging system and Lenti-X 293T cell line (Clontech) according to the manufacturer's instructions. For Western blot and immunocytochemistry analyses, MEF cells were harvested, counted, and plated onto 35 mm dishes and 8-well cultureslides (BD), respectively, at a density of 500 cells/cm^2. The next day cells were infected with lentiviral vectors in the presence of 8 μg/ml polybrene. On the following day the medium was replaced with fresh medium and cells were monitored for any changes in morphology and motility.

Live-cell imaging platform
A live-cell imaging platform was set up using Eclipse TE2000-PFS inverted microscope from Nikon (Figure 1). The system combines fully motorized microscope with the Nikon perfect focus system (PFS), which makes constant and accurate focus possible over a period of time. With a Prior motorized stage, multiple points can be revisited during time-lapse acquisition. A big acryl chamber equipped with sensors and controllers was made for regulating temperature and CO_2 to provide optimal cell culture conditions while images are taken over several days.

Immunocytochemistry
For immunostaining, MEF cells cultured on 8-well cultureslides were washed with PBS and fixed with 4% paraformaldehyde plus 4% sucrose in PBS and permeabilized with 0.25% Triton X-100. Then cells were incubated with 100 mM Glycine (Sigma) to reduce auto-fluorescence and incubated in PBS supplemented with 5% bovine serum albumin for blocking nonspecific antibody binding. Primary and secondary antibodies were diluted with the blocking solution and

centrifuged for 2 min at full speed to get rid of precipitates. Cells were incubated with primary antibodies for 1 hour at room temperature, washed in PBS, and incubated with Alexa fluor dye-conjugated secondary antibodies or phalloidin (Invitrogen) for 40 min at room temperature. After extensive wash, well barriers were removed and coverslips were mounted with Vectashield mounting medium containing DAPI (Vector Laboratories). High magnification images of cells were captured using a Nikon 90i microscope equipped with Roper EZ monochrome and DS-Fi1 color cameras or an Olympus IX70 inverted microscope equipped with Deltavision deconvolution software. Images were imported into Adobe Photoshop CS for analysis.

Measurement of cytoplasmic area

To quantitatively assess effects of various Crk and CrkL constructs and chemicals on cell morphology, the researchers developed a fluorescence imaging-based method for calculating the cytoplasmic areas of cells. Fibroblast cells infected with a lentiviral vector expressing green fluorescent protein (GFP) have green fluorescence in the cytoplasm. Using the Object Count function of the Nikon NIS element program, cells with green fluorescence signals stronger than a designated threshold were automatically selected, and areas and other parameters such as equivalent diameter and perimeter of selected objects were calculated. To count only cell-specific fluorescence, DAPI-negative objects were excluded. To obtain representative and nonbiased data, images of fluorescent cells were taken from a minimum of 8 different fields of view throughout the entire area of the well.

Spontaneous motility assay

Three days after infection with lentiviral vectors when green fluorescence signals from infected cells became very strong, green fluorescent cells were selected and time-lapse images were recorded over 48 hours at hourly intervals. For quantitative analysis of spontaneous cell motility, virtual grid lines with 32.5 μm x 32.5 μm for each grid were placed on the images and consecutive images were compared to see whether GFP-positive cells crossed a grid line to move from one grid to another adjacent grid. If the center of a cell moved to a neighboring grid within an hour, it was marked as 1 for the motility. If the center of a cell stayed in the same grid in an hour, it was marked as zero. Forty GFP-positive cells were assessed for each group.

Scratch wound healing assay

MEF cells were plated on cultureslides and infected with lentiviral vectors that expressed either GFP plus Cre or GFP only. Four days after infection, a wound was created using a micropipette tip to scratch through the middle of the cell monolayer, and migration of cells into the gap at the wound site was monitored for three days. Green fluorescent cells either present on both sides or that had migrated into the wound gap were counted using the Object Count function of the Nikon NIS element program, and the percentage of cells migrated to the wound gap was calculated.

Western blot analysis

Lysates of MEF cells were prepared using 1% NP-40 lysis buffer containing 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 5 mM EDTA, 25 mM NaF, 2 mM Na_3VO_4, 0.1% sodium deoxycholate, protease inhibitor cocktail complete mini (Roche), and phosphatase inhibitor cocktail PhosSTOP (Roche). After quantifying the protein content of each cell lysate using Bio-Rad protein assay kit, total cell lysates containing the same amount of protein were loaded, separated by SDS-PAGE, and transferred to nitrocellulose membranes (Invitrogen). Membranes were then

subjected to Western blot analysis using an Odyssey infrared imaging system (Li-Cor Biosciences). For most antibodies, membranes were incubated with Odyssey blocking buffer and incubated with primary antibodies in Odyssey blocking buffer containing 0.2% Tween 20 for an hour at room temperature with a slow rotation. After washing with 1X TBS-T, membranes were incubated with IRDye secondary antibodies, IRDye 680 goat anti-mouse and IRDye 800CW goat anti-rabbit antibodies, for an hour at RT with a slow rotation. Then membranes were extensively washed with TBS-T and PBS and allowed to dry completely on a blotting paper. Membranes were protected from light since the secondary antibody incubation. Infrared images were taken from membranes and quantified using an Odyssey infrared imaging system according to the manufacturer's instructions. Sources of the primary antibodies are as follows: anti-Crk and anti-FAK antibodies are from BD Biosciences; antibodies to CrkL (sc-319) and p-p70 S6 kinase (sc-11759) are from Santa Cruz Biotechnology, Inc.; anti-α-tubulin (T9026) antibody is from Sigma; antibodies against β-Actin (4970), GAPDH (2118), vinculin (4970), Rac (2467), Cdc42 (2462), RhoA (2117), phospho-FAK (3283), Akt (9272), Tor (2972), and phospho-Tor (2971) antibodies are from Cell Signaling Technology; antibodies to phosphorylated forms of Akt (Ser 473) and Src family (Tyr 416) are from BioSource; anti-Src antibody is from Millipore.

Statistical analysis

Statistical analysis of data was carried out using unpaired two-tailed Student's t test for comparison between two experimental groups. Differences were considered to be significant when probability (p) values were <0.05.

Results

Morphological alteration of fibroblast cells in the absence of Crk and CrkL

To investigate the cellular functions of Crk and CrkL, fibroblasts derived from Crk/CrkL double floxed mice were cultured and infected with a lentiviral vector expressing Cre plus GFP. As shown in Figure 2, Crk disappeared from cells at two to three days post infection (DPI). The researchers then compared the morphology of Crk/CrkL double floxed cells expressing Cre plus GFP with that of wild type cells expressing Cre plus GFP. At 1 DPI, a few cells became GFP positive in cultures of both wild-type and double floxed cells without any significant morphological changes (Figure 3A). At 3 and 6 DPI the GFP signal grew progressively stronger. Most GFP/Cre-positive double floxed cells became smaller in size at 3 and 6 DPI, appearing round and refractile under phase-contrast microscopy (white arrows in Figure 3A). In contrast, wild-type and double floxed cells infected with a lentiviral vector expressing GFP alone, did not exhibit any morphological abnormalities (Figure 3B). These results indicate that fibroblasts undergo major morphological alterations in the absence of Crk and CrkL.

Reduced cell motility in the absence of Crk and CrkL

To better understand the morphological alterations that occur in the absence of Crk and CrkL, the researchers captured time-lapse images of live cells starting from 3 DPI. GFP-positive cells were selected and time-lapse images were recorded over 48 hours at hourly intervals. GFP/Cre-positive wild-type cells had well-developed processes and actively moved around. In contrast, GFP/Cre-positive double floxed cells became small and rounded, while displaying decreased motility. For quantitative analysis, virtual grid lines were placed on the images and consecutive

images were compared to determine the number of the times cells crossed a grid line. As shown in Figure 4A, GFP/Cre-positive wild-type cells actively moved away from their initial locations at 3 and 5 DPI. In contrast, many GFP/Cre-positive double floxed cells remained at their initial locations without significantly changing their small rounded shape. The researchers analyzed the hourly movements of forty GFP/Cre-positive cells in each group over a period of two days. GFP-positive double floxed cells exhibited significantly reduced motility, compared to GFP/Cre-positive wild-type cells, during the observation period (Figure 4B).

The scratch wound healing assay was used to examine the contribution of Crk and CrkL to directional cell migration. After double floxed fibroblast cells were infected with lentiviral vectors expressing GFP alone or GFP plus Cre, a wound was created in the cell monolayer and migration of cells into the gap at the wound site was monitored. As shown in Figure 4C and D, while several double floxed cells expressing GFP alone migrated into the wound site this was not the case with double floxed cells expressing GFP plus Cre. Taken together, the results suggest that Crk and CrkL play critical roles in the control of cell motility as well as the maintenance of cell shape.

Loss of focal adhesion in the absence of Crk and CrkL

Focal adhesions are protein complexes that play important roles in cell morphology and migration by connecting the cell cytoskeleton to the extracellular matrix. Previously, v-Crk, CrkL, and p130Cas were shown to interact with proteins important for focal adhesion formation, including paxillin and FAK (Birge *et al.*, 1993; Polte and Hanks, 1995; Salgia *et al.*, 1995). Therefore, the researchers investigated whether focal adhesion formation was affected in the absence of Crk and CrkL. As visualized by anti-vinculin antibody staining in Figure 5, focal adhesions disappeared in GFP/Cre-positive double floxed cells (white arrows), whereas GFP/Cre-negative double floxed cells, as well as GFP/Cre-positive wild-type cells, retained sites of focal adhesion (yellow arrows). The results suggest that focal adhesions are disrupted by the loss of both Crk and CrkL, which in turn leads to morphological alterations and decreased cell motility.

Collapse of cytoskeletal structures in the absence of Crk and CrkL

Since focal adhesions connect stress fibers to extracellular structures, and stress fibers contribute to cell shape changes, the researchers wondered whether stress fiber formation was affected in cells lacking Crk and CrkL. Stress fibers are higher order structures consisting of actin filaments, crosslinking proteins, and myosin motors that can be easily visualized by staining actin with phalloidin. In Figure 6, stress fibers were observed to be present until 2 DPI but then disappeared in strongly GFP/Cre-positive double floxed cells (white arrows). However, GFP/Cre-positive wild-type cells as well as GFP/Cre-negative double floxed cells retained stress fibers (yellow arrows). The results suggest that formation of stress fibers is disrupted by loss of Crk and CrkL. Therefore it appears that Crk and CrkL are required for the maintenance of focal adhesions and stress fibers, two key cellular structures that connect cells to the extracellular matrix.

To further study the overall structural change in the cells lacking Crk and CrkL, the researchers examined the distribution of microtubules. Staining of wild-type cells with an antibody against tubulin demonstrated that microtubules were distributed throughout the cytoplasm, but not in the

nucleus, leaving a cage-like space in the nucleus (Figure 7). In GFP/Cre-positive double floxed cells, microtubules collapsed around the nucleus eventually resembling a poorly ordered and fragmented sheath structure (white arrows in Figure 7). However, GFP/Cre-positive wild-type cells maintained highly ordered microtubule structures. The results suggest that loss of Crk and CrkL in cells leads to collapse of cytoskeletal structures and shrinkage of the cytoplasm, resulting in small, rounded cells consisting mainly of the nucleus enveloped by poorly organized cytoskeletal structures. Therefore, the results demonstrate that Crk and CrkL play essential roles in maintaining the integrity of cytoskeletal structures.

Rapid degradation of actin in the absence of Crk and CrkL

To understand the underlying mechanisms responsible for cytoskeletal collapse in the absence of Crk and CrkL, the researchers attempted to identify proteins that mediate the Crk/CrkL-dependent cytoskeletal connection to the extracellular matrix. The researchers reasoned that such proteins would rapidly disappear or their phosphorylation levels would drastically change once cells lose Crk and CrkL. Therefore, the researchers monitored protein expression and phosphorylation levels of several candidate proteins, proposed to function downstream of Crk and CrkL in many known signaling pathways, to determine if any of these changed their expression or phosphorylation levels following the decrease of Crk and CrkL. As shown in Table 1, double floxed cells expressing both GFP and Cre did not exhibit any significant decreases in Rho GTPases such as Rac, cdc42 and RhoA until 4 DPI. Rac and RhoA decreased modestly only at 5 DPI. On the other hand, the protein and phosphorylation levels of mTor as well as the phosphorylation levels of S6 kinase were not significantly affected by the loss of Crk and CrkL. The expression levels of FAK, Akt, and Src did not decrease at all, while the phosphorylation levels of FAK and Akt decreased modestly but significantly starting from 2 and 3DPI, respectively. Thus, none of the above proteins tested showed a sharp and progressive decline in their expression or phosphorylation levels, such as the protein levels of CrkI, CrkII and CrkL. Interestingly, the protein level of actin decreased substantially over the course of infection while tubulin, GAPDH and vinculin showed either a modest decrease or no change (Figure 6A). Actin showed the most significant decline in its protein level in the absence of Crk and CrkL, although the decrease of actin was not as rapid as the loss of Crk and CrkL. It is unclear how actin decreased in the absence of Crk of CrkL. It has been reported that caspases cleave actin into 15 and 31 kDa fragments under pathological conditions (Du *et al.*, 2004; Kong and Rabkin, 2004; Mashima *et al.*, 1999). Although the researchers do not exclude the possibility of actin cleavage in the absence of Crk and CrkL, the researchers could not detect actin fragments under the experimental conditions (data not shown). On the other hand, the protein levels of actin and vinculin did not change significantly in double floxed cells expressing GFP alone (Figure 8). In contrast, the protein levels of CrkI, CrkII, and CrkL did increase suggesting that the enhanced complexity of cell-to-cell connections results in increased levels of Crk family proteins.

Then the researchers tested whether the decrease in cytoskeletal elements such as actin and tubulin made any contribution to the morphological alteration observed in cells lacking Crk and CrkL. One day after double floxed cells were infected with GFP and Cre, cells were treated with reagents that affect degradation of cytoskeletal elements and cell morphology was examined at 5 DPI. For quantitative analysis, the cytoplasmic area of GFP-positive cells was determined. As shown in Figure 9C, treatment of cells with the proteasome inhibitor MG-132 did not block the

morphological consequences of Crk and CrkL ablation. In addition, paclitaxel, which stabilizes microtubules, did not prevent morphological conversion. However, treatment of cells with jasplakinolide, a reagent that stabilizes polymerized actin, suppressed morphological conversion caused by loss of Crk and CrkL (yellow arrow in Figure 9C). Quantitation of cellular surface area indicated that jasplakinolide partially blocked the morphological alterations caused by loss of Crk and CrkL (Figure 9D). This partial inhibition of the morphological conversion by artificial stabilization of polymerized actin, together with the observation of a substantial decrease in actin protein level in the absence of Crk and CrkL, suggests that actin plays important roles in maintaining the Crk/CrkL-dependent cytoskeletal connection to the extracellular matrix.

Contribution of Crk and CrkL to cytoskeletal integrity

The researchers used quantitative analysis of cytoplasmic area to investigate the individual contributions of CrkI, CrkII and CrkL to the maintenance of cell shape. Crk/CrkL double floxed cells were coinfected with lentiviral vectors expressing GFP/Cre together with vectors expressing various Crk and CrkL proteins. At 5 DPI, images of fluorescent cells were captured and the cytoplasmic areas were determined. As shown in Figures 10A and B, expression of exogenous CrkI or CrkL caused a modest but significant inhibition of the morphological alteration caused by loss of both Crk and CrkL. While some infected cells did show the typical morphological conversion (white arrows in Figure 10A), others retained a larger surface area (yellow arrows in Figure 10A). CrkII was more effective than CrkI or CrkL in preventing morphological alteration. Analyses of other cell size parameters, such as equivalent diameter or perimeter, gave results comparable with the cytoplasmic area findings (Figures 11A and B). These data suggest that CrkI, CrkII and CrkL all contribute to cytoskeletal connection with the extracellular matrix, although CrkII seems to play a dominant role. Mutants of Crk and CrkL containing SH2 domains, but no SH3 domain, failed to rescue morphological conversion. Furthermore, SH2 mutants promoted significant morphological alterations when expressed by themselves, even in the presence of endogenous Crk and CrkL. These findings suggest that Crk and CrkL mediate the connection of the cytoskeleton with the extracellular matrix through both SH2 and SH3 domains, with SH2-only mutants acting as dominant-interfering molecules.

To confirm the individual contributions of Crk family proteins, the researchers infected single floxed cells with a lentiviral vector expressing GFP/Cre to see whether deletion of either Crk or CrkL alone caused any morphological alteration. As shown in Figure 10C, deletion of either Crk or CrkL did not cause the drastic morphological changes seen in cells lacking both Crk and CrkL. However, close examination revealed that some GFP-positive cells lacking either Crk or CrkL showed modest morphological effects (white arrows in Figure 10C). There was also a modest but significant decrease in cytoplasmic area in cells lacking either Crk or CrkL ($68.0 \pm 8.4\%$ and $73.9 \pm 5.7\%$ of the normal size, respectively, in Figures 10D and 11C). The reduction in cell size that occurred after ablation of both Crk and CrkL ($66.5 \pm 2.0\%$) was greater than the combination of the effects caused by individual ablation of Crk and CrkL (49.7% based on $1 - 0.680 \times 0.739 = 0.497$). Analyses of equivalent diameter and perimeter also showed similar reductions in the absence of either Crk or CrkL and greater reductions in the absence of both Crk and CrkL (Figures 11D and E). The greater reduction in the cell size in the absence of both Crk and CrkL is consistent with the drastic morphological alterations observed in cells lacking both Crk and CrkL. These findings suggest that CrkI, CrkII and CrkL play essential, overlapping

roles in the maintenance of cell structure. Taken together, the results indicate that CrkI, CrkII, and CrkL are all required for mediating communication between the intracellular environment and the extracellular matrix.

Figure 1. Front view of the time-lapse imaging platform

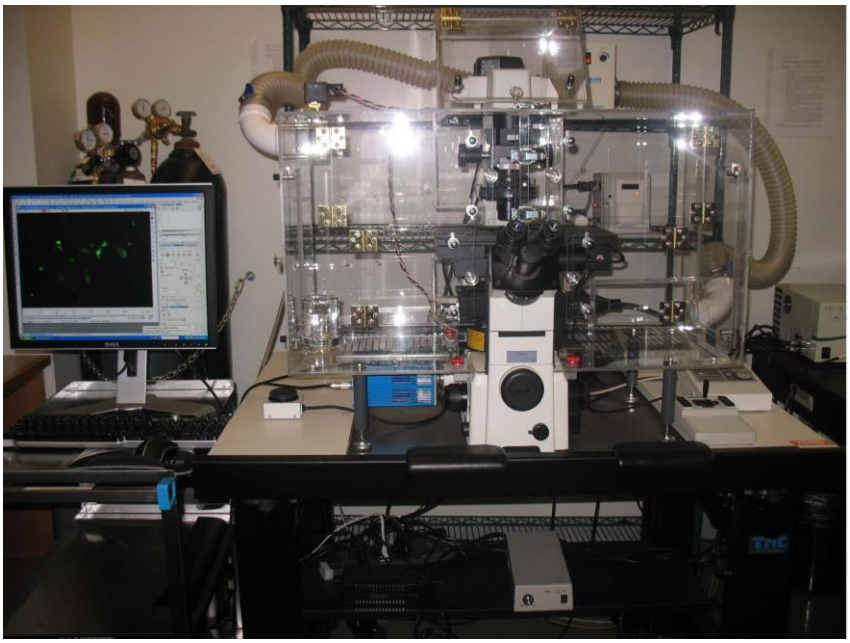
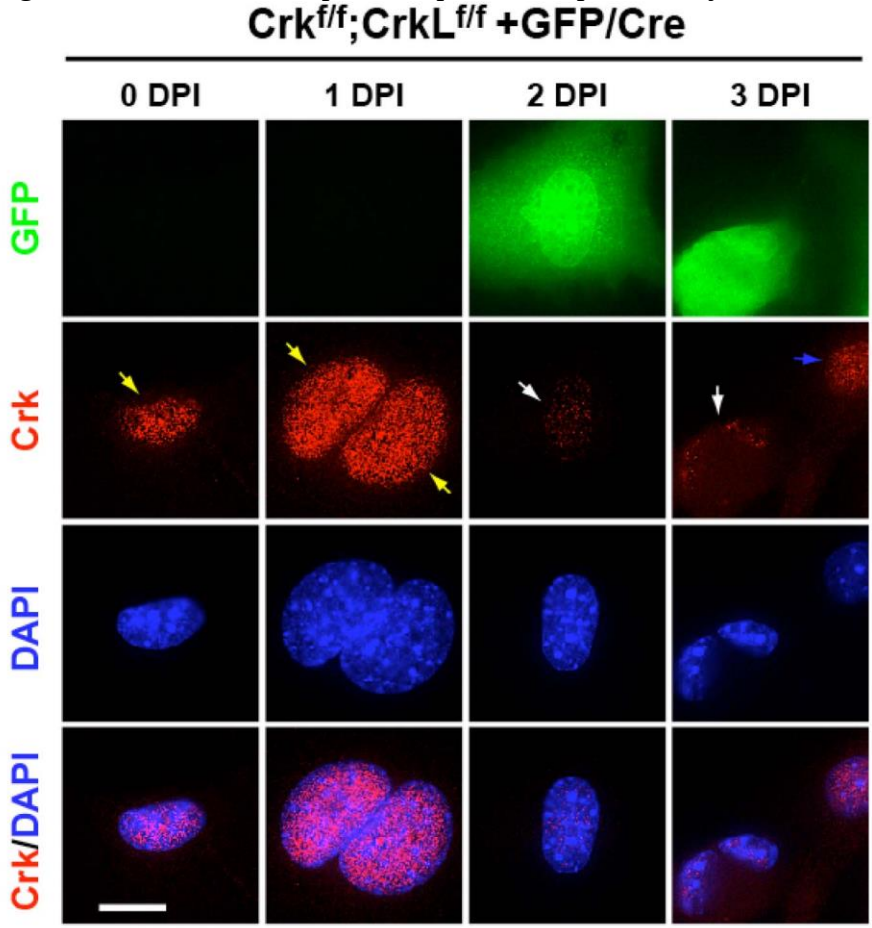
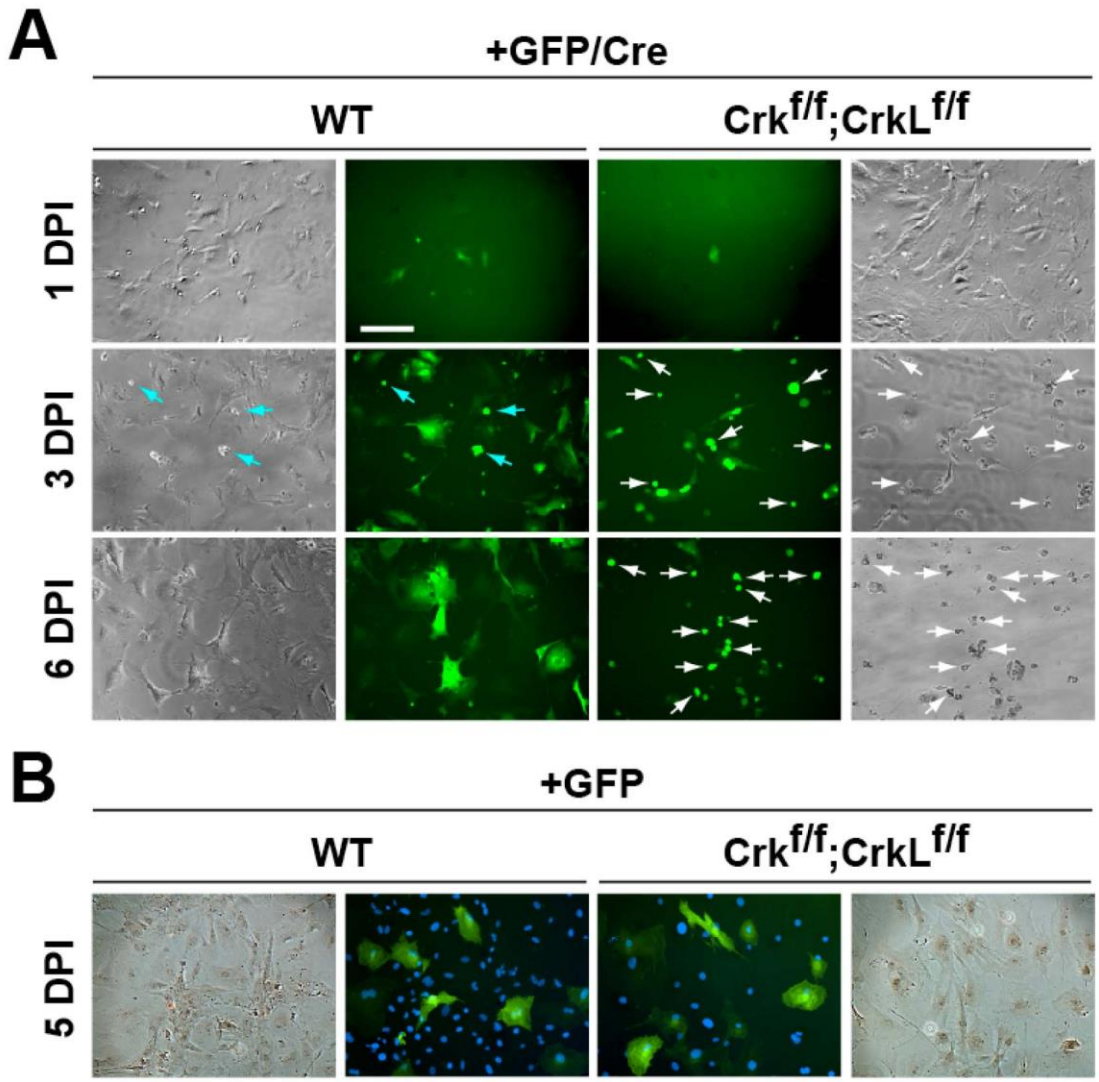


Figure 2. Loss of the Crk protein upon Cre expression by lentiviral infection



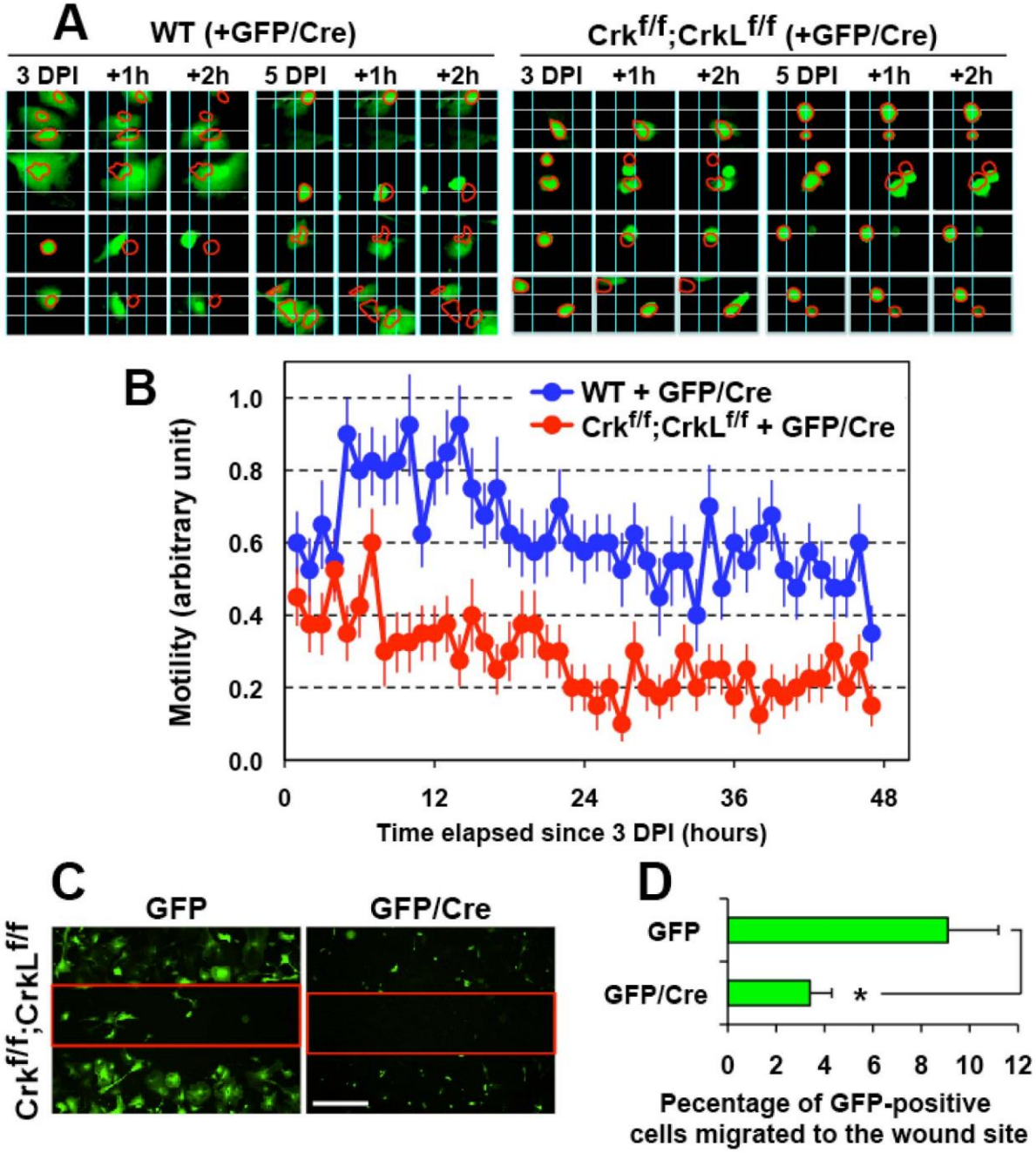
Crk/CrkL double floxed (Crk$^{f/f}$;CrkL$^{f/f}$) cells were infected with a lentiviral vector expressing both GFP and Cre. Cells were fixed at indicated time points and Crk immunostaining was carried out to illustrate Crk protein expression. High levels of Crk expression were observed in cells without infection and cells at 1 DPI (yellow arrows), but Crk expression dropped drastically at 2 and 3 DPI (white arrows). A sustained expression of Crk was observed in a GFP-negative double floxed cell (blue arrow). Scale bar: 20 μm.

Figure 3. Morphological alteration of fibroblast cells in the absence of Crk and CrkL



A, Wild-type (WT) and Crk/CrkL double floxed ($Crk^{f/f}$;$CrkL^{f/f}$) cells were infected with a lentiviral vector expressing both GFP and Cre. Both phase-contrast and fluorescence images of cells were taken at indicated days post infection (DPI). The white arrows indicate GFP-positive double floxed cells with small and round-shaped morphologies. The cyan arrows indicate GFP-positive WT cells with altered morphologies, suggesting that even a few WT cells undergo morphological changes. However, it should be noted that a majority of GFP-positive double floxed cells exhibit morphological alterations whereas many GFP-positive WT cells have well-developed processes at both 3 and 6 DPI. *B*, WT and double floxed cells were infected with a lentiviral vector expressing GFP alone. FITC and DAPI images of fluorescent cells as well as phase-contrast images were taken at 5 DPI. Scale bar: 200 μm.

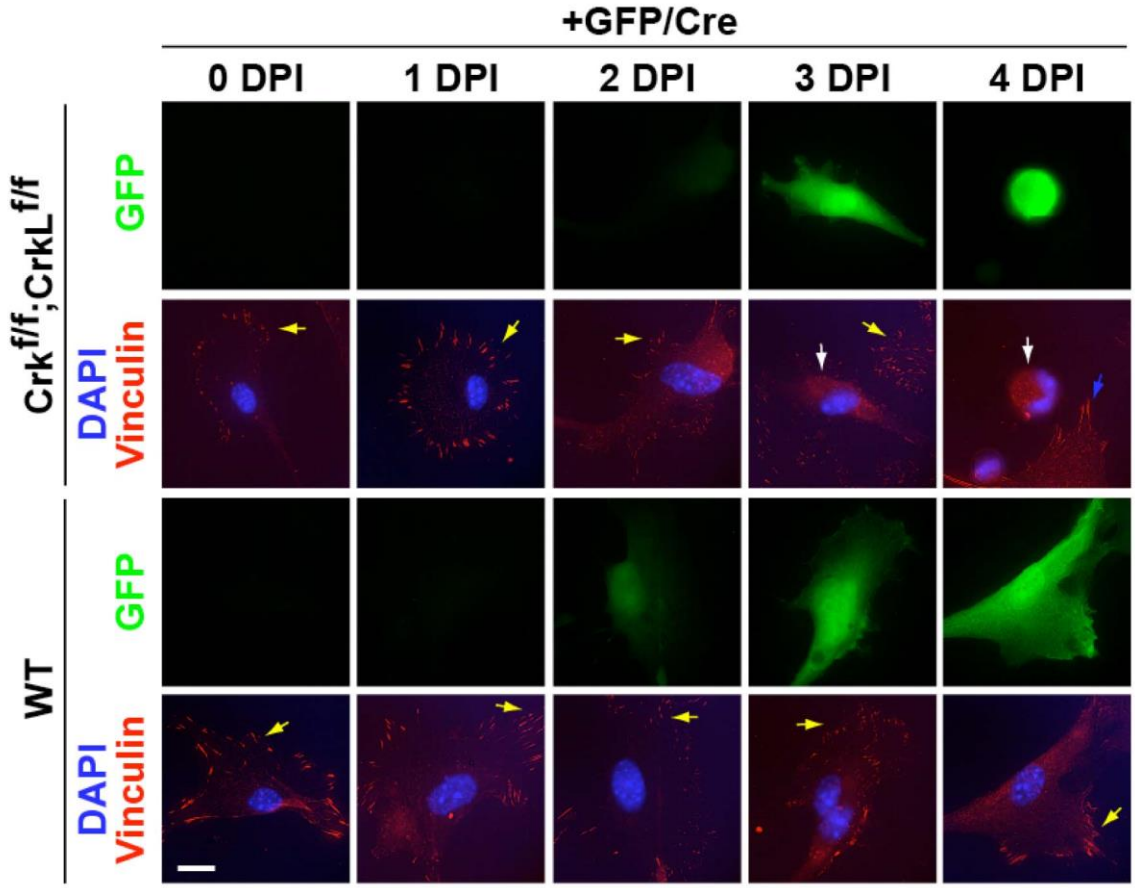
Figure 4. Reduced motility of cells in the absence of Crk and CrkL



Wild-type (WT) and Crk/CrkL double floxed ($Crk^{f/f}$;$CrkL^{f/f}$) cells were infected with a lentiviral vector expressing both GFP and Cre. At 3 DPI, time-lapse images of GFP-positive cells were recorded over 48 hours at hourly intervals. For quantitative analysis of spontaneous cell motility, virtual grid lines were placed on the images using the microscope software and crossing of GFP-positive cells over the grid line was monitored every hour by comparing images obtained from two consecutive time points. *A*, Images of GFP-positive WT and double floxed cells with the virtual grid lines from three consecutive time points at 3 and 5 DPI are shown. Boundaries of core regions with strong GFP fluorescence signals were marked with red lines to track locations of cells. *B*, Forty GFP-positive cells for each group were assessed for their hourly movement

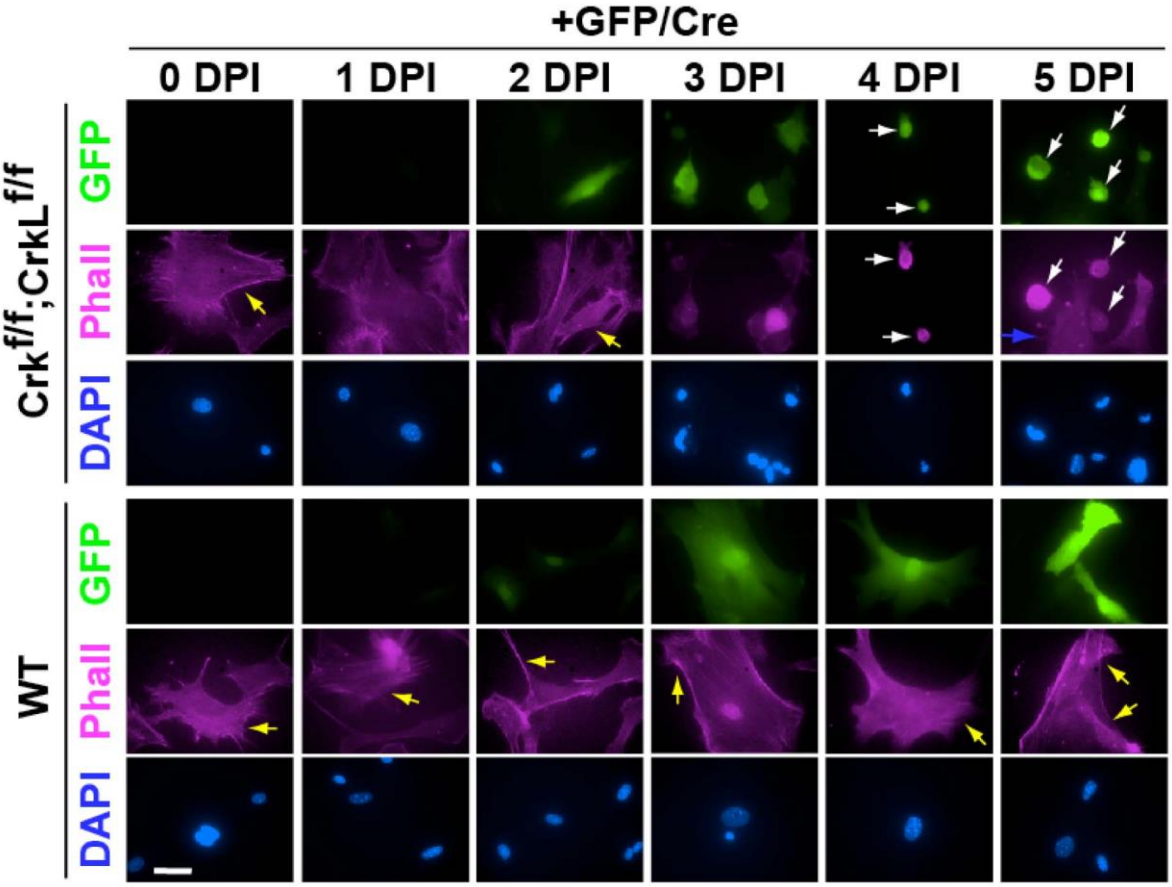
across the grid lines and the cumulative results are shown as the mean ± SEM values in the graph. *C*, Four days after Crk/CrkL double floxed cells were infected with lentiviral vectors expressing either GFP alone or GFP plus Cre, a wound was created by scratching through the cell monolayer with a micropipette tip and migration of GFP-positive cells into the gap at the wound site was monitored for three days. Scale bar: 500 μm. *D*, GFP-positive cells that stayed outside of the wound site and GFP-positive cells that migrated to the wound site were counted and the percentage of GFP-positive cells that migrated to the wound site was calculated. The original wound gaps are marked as the red rectangles. Four separate experiments were done for each group and images were taken from three different areas in each experiment to count cells. Data are shown as the mean ± SEM (bars) values.

Figure 5. Disruption of focal adhesions in the absence of Crk and CrkL



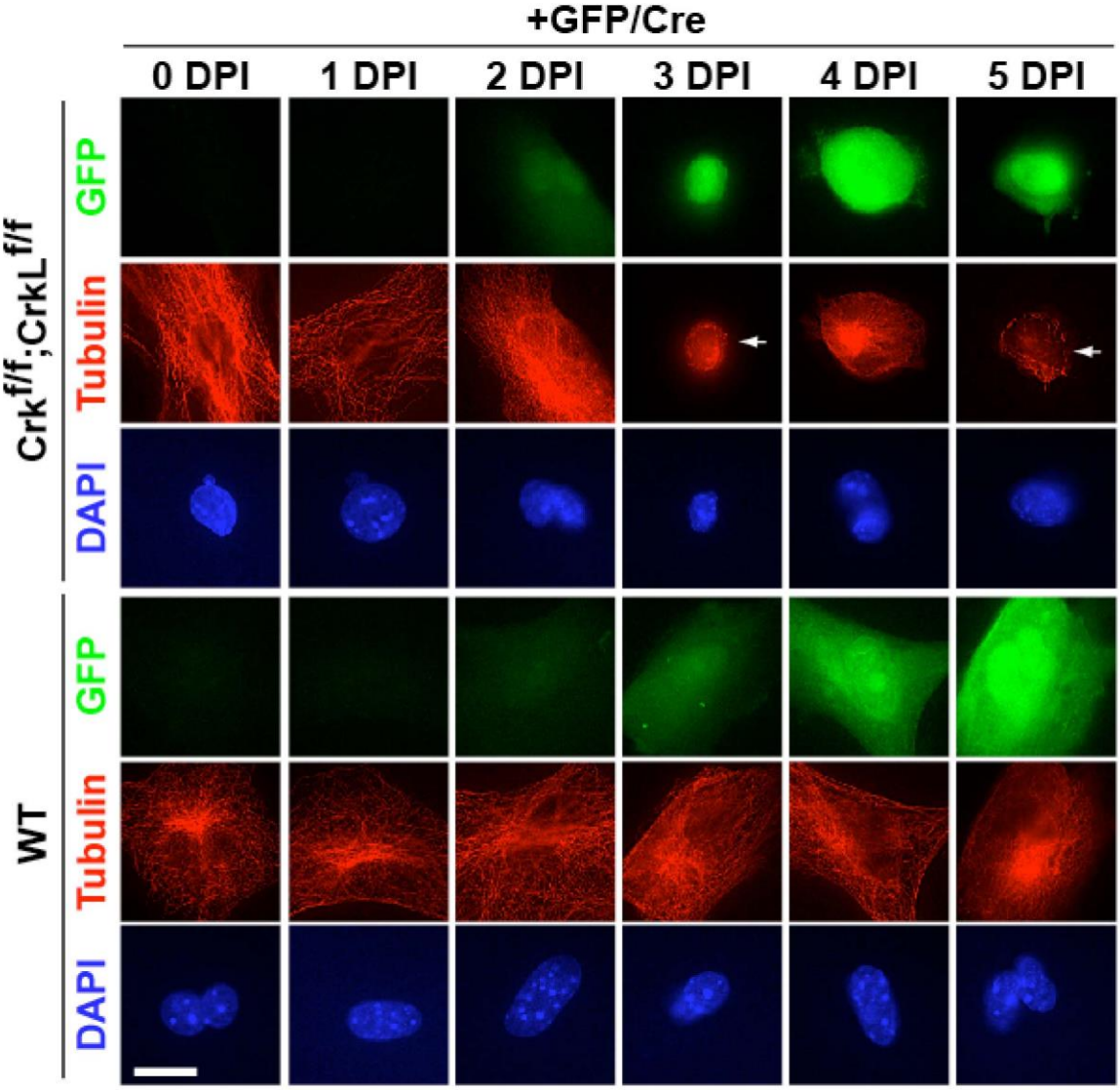
Wild-type (WT) and Crk/CrkL double floxed ($Crk^{f/f}$;$CrkL^{f/f}$) cells were infected with a lentiviral vector expressing both GFP and Cre. Cells were fixed at indicated time points and immunostained with vinculin antibody to illustrate focal adhesions. The yellow arrows indicate focal adhesions observed in GFP-positive cells. The white arrows indicate loss of focal adhesions in GFP/Cre-expressing double floxed cells. The blue arrow indicates focal adhesions in a GFP-negative double floxed cell. Scale bar: 20 μm.

Figure 6. Disorganization of actin stress fibers in the absence of Crk and CrkL



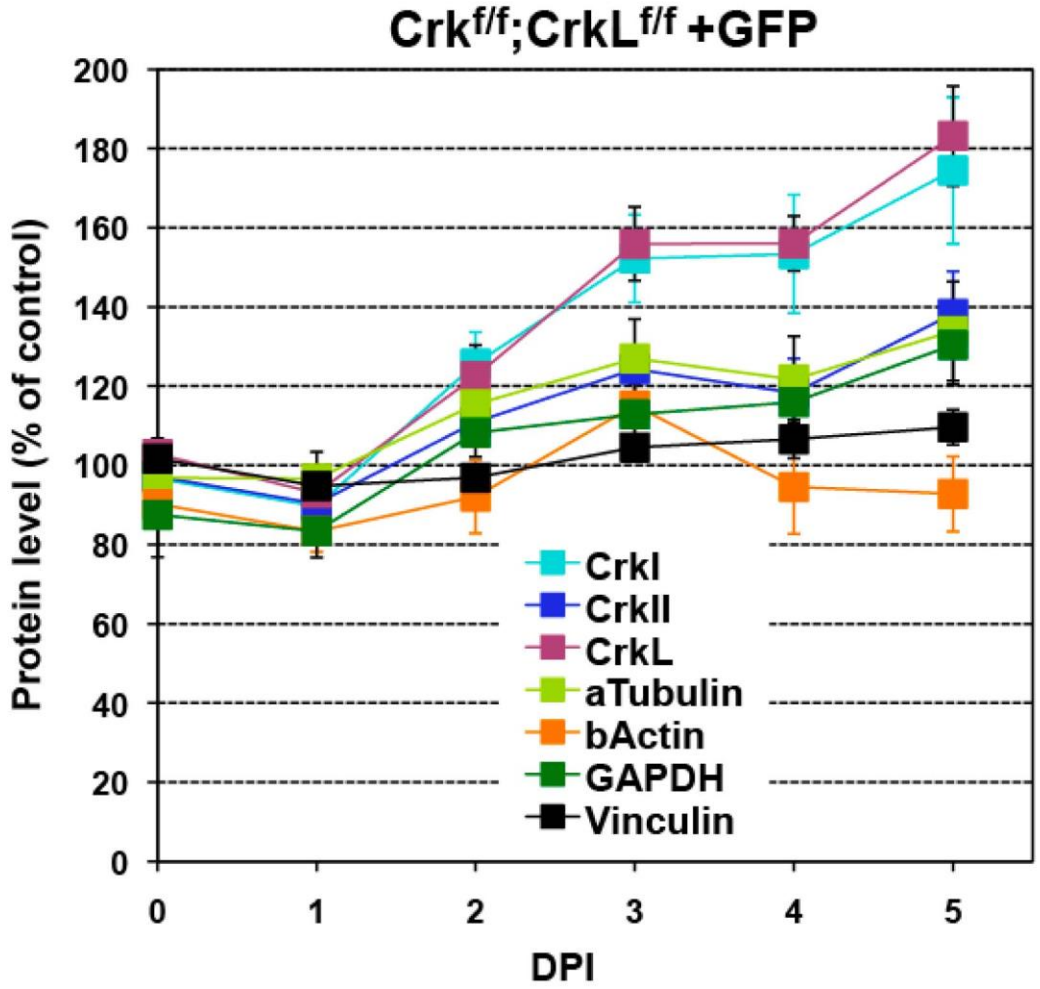
Wild-type (WT) and Crk/CrkL double floxed ($Crk^{f/f}$;$CrkL^{f/f}$) cells were infected with a lentiviral vector expressing both GFP and Cre. Cells were fixed at indicated time points and stained with phalloidin to visualize actin stress fiber formation in cells. The yellow arrows indicate actin stress fibers. The white arrows indicate loss of stress fibers in GFP/Cre-positive double floxed cells. The blue arrow indicates presence of stress fibers in a GFP-negative double floxed cell. Scale bar: 50 μm

Figure 7. Collapse of microtubule structure in the absence of Crk and CrkL



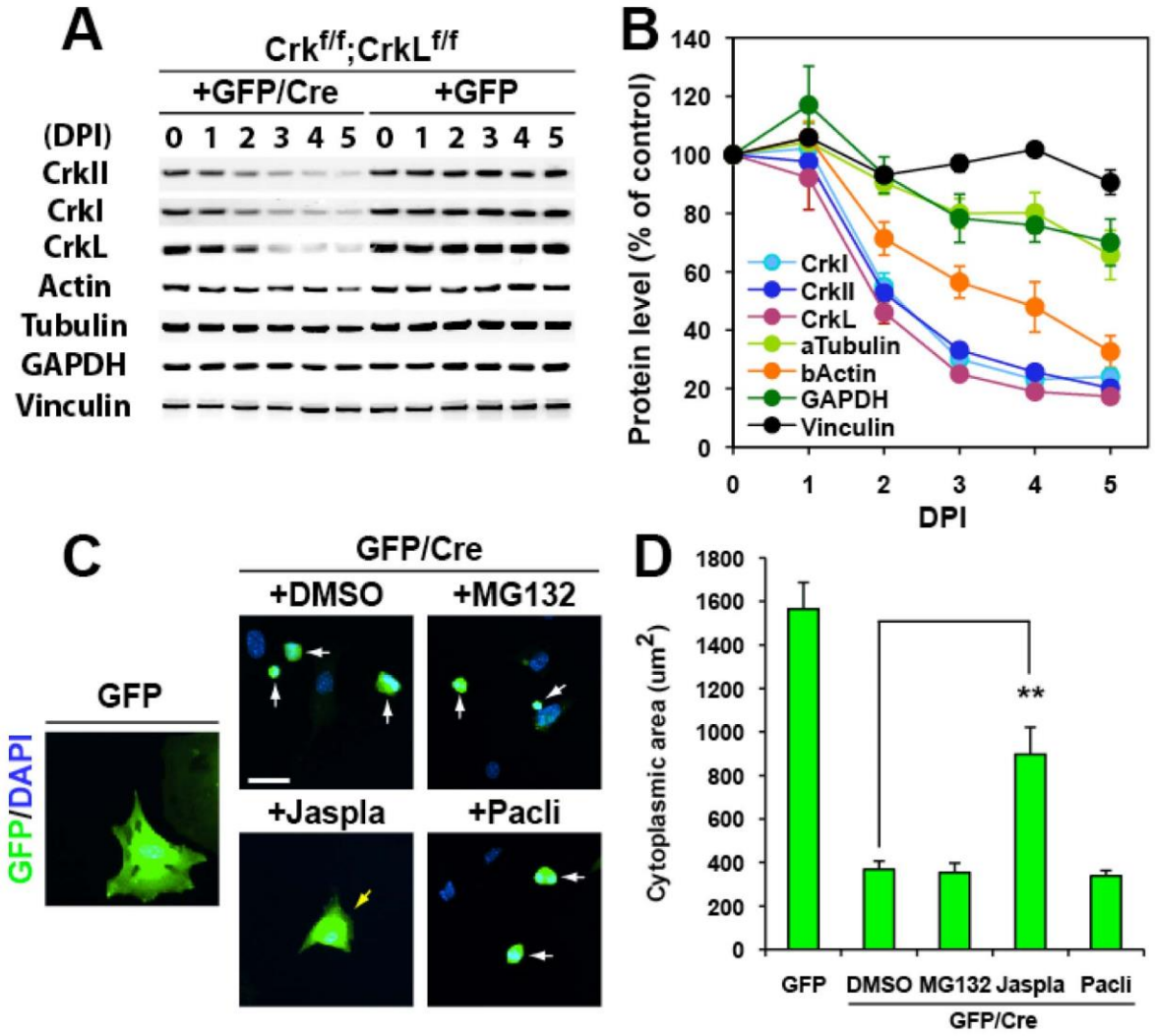
Wild-type (WT) and Crk/CrkL double floxed ($Crk^{f/f}$;$CrkL^{f/f}$) cells were infected with a lentiviral vector expressing both GFP and Cre. Cells were fixed at indicated time points and α-tubulin immunostaining was carried out to visualize microtubules in cells. The white arrows indicate severely deformed microtubule structures in GFP/Cre-expressing double floxed cells. Scale bar: 20 μm.

Figure 8. Changes in protein expression in double floxed cells without Cre expression



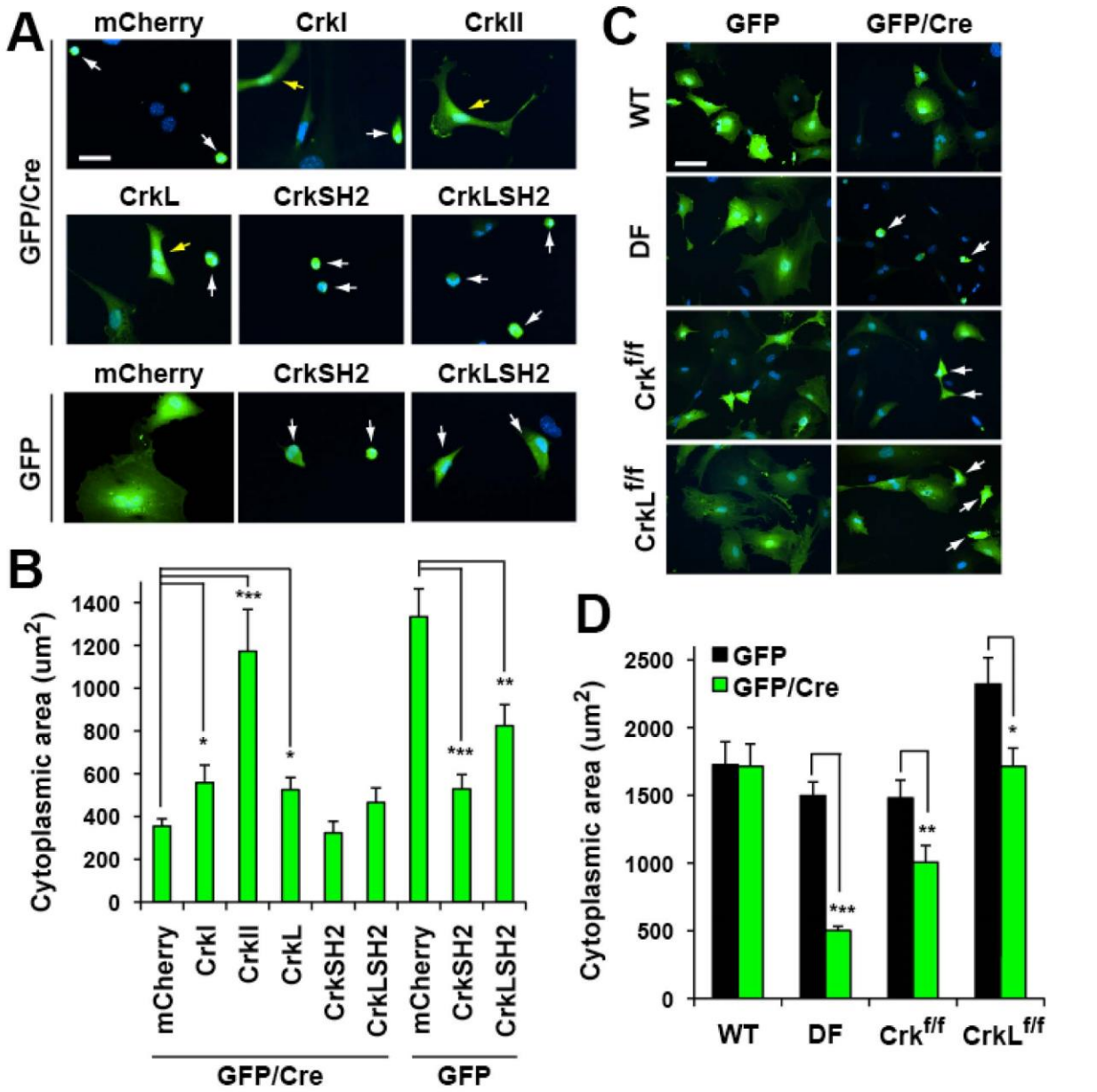
Crk/CrkL double floxed ($Crk^{f/f}$;$CrkL^{f/f}$) cells were infected with a lentiviral vector expressing GFP alone. Total cell lysates were obtained at indicated time points for Western blot analysis. Protein levels from various time points were compared with the control without infection. Protein bands were quantified using the Odyssey system and their mean ± SEM values are shown in the graph.

Figure 9. Decrease of actin following the loss of Crk and CrkL.



Crk/CrkL double floxed ($Crk^{f/f}$;$CrkL^{f/f}$) cells were infected with a lentiviral vector expressing both GFP and Cre or with a lentiviral vector expressing GFP alone. Total cell lysates were obtained at indicated time points for Western blot analyses. Protein levels from various time points were compared with the control levels without infection. *A*, Representative images of three independent Western blot analyses are shown for housekeeping and structural proteins in addition to Crk and CrkL. *B*, Protein bands were quantified using the Odyssey system and their mean ± SEM values are shown in the graph. *C*, One day after Crk/CrkL double floxed ($Crk^{f/f}$;$CrkL^{f/f}$) cells were infected with lentiviral vectors expressing GFP alone or GFP plus Cre, cells were treated with MG-132 (30 nM), jasplakinolide (30 nM), or paclitaxel (100 nM) for four additional days. At these concentrations, the reagents themselves did not cause major changes in cell shape (data not shown). FITC and DAPI images of fluorescent cells were taken at 5 DPI for the cytoplasmic area measurement. Representative images of fluorescent cells are shown. The white arrows indicate GFP-positive cells with altered morphologies. The yellow arrow indicates a GFP-positive cell, which is bigger than the cells lacking both Crk and CrkL. Scale bar: 50 μm. *D*, The cytoplasmic areas of GFP-positive cells were measured as described in the Materials and methods. Data are shown as the mean ± SEM (bars) values. **$p < 0.01$, compared with control.

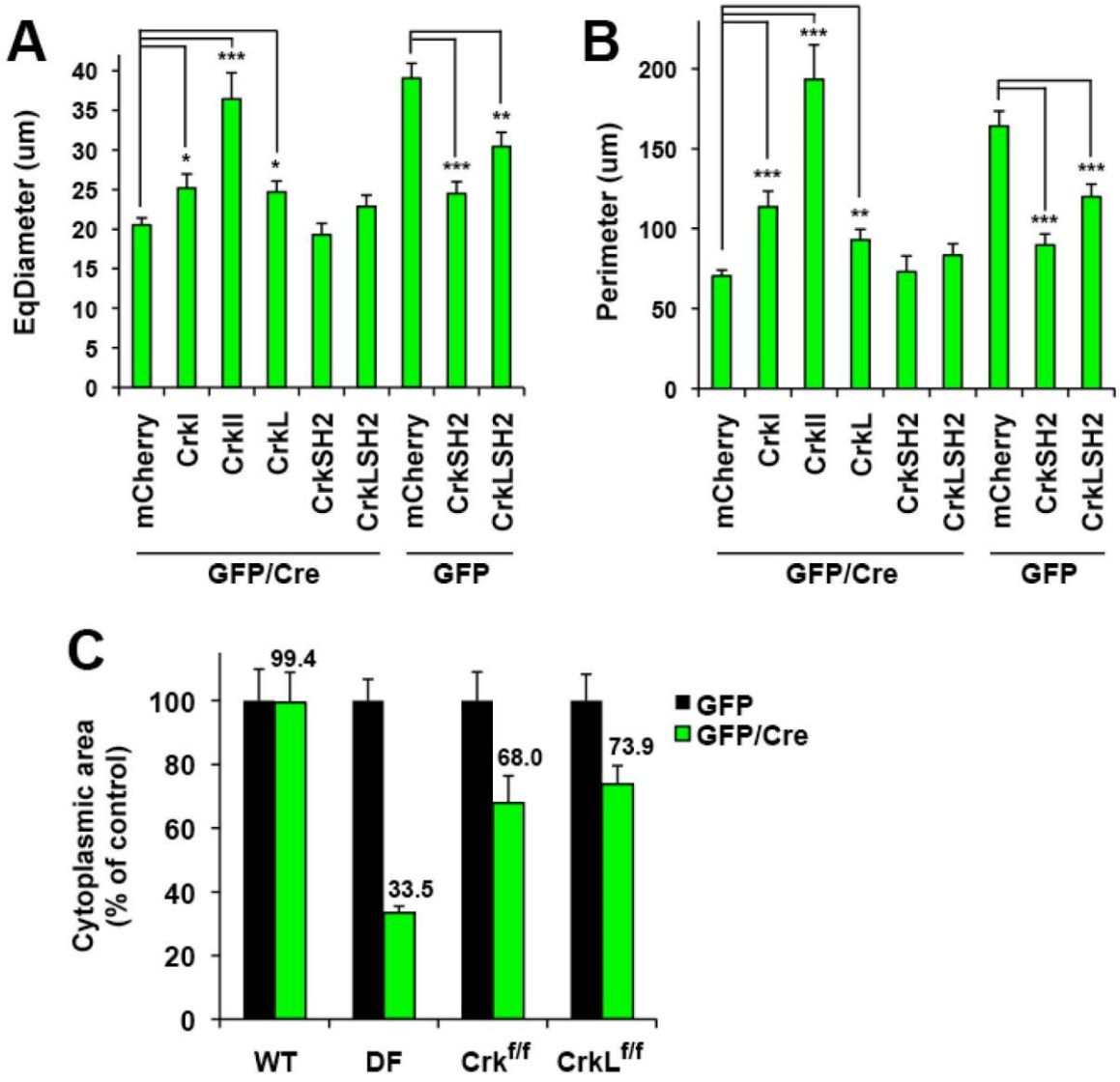
Figure 10. Both Crk and CrkL contribute to the cytoskeletal integrity.

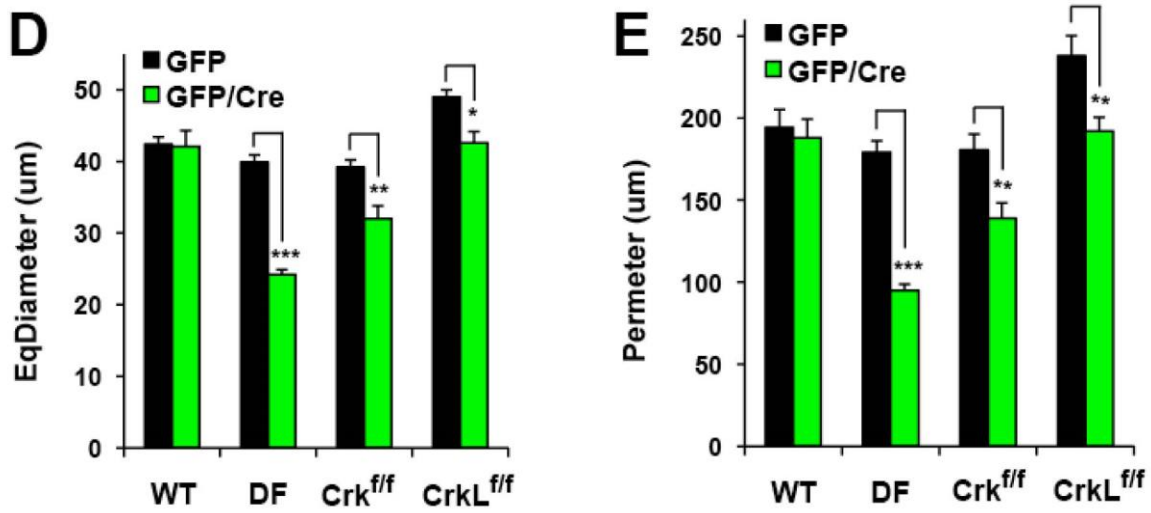


A, Crk/CrkL double floxed ($Crk^{f/f}$;$CrkL^{f/f}$) cells were coinfected with lentiviral vectors expressing GFP and Cre (derived from pLVX-IRES-ZsGreen1) and lentiviral vectors expressing various Crk and CrkL proteins (derived from pLVX-IRES-mCherry). FITC, Texas Red (not shown here), and DAPI images of fluorescent cells were taken at 5 DPI for the cytoplasmic area measurement. Representative images of fluorescent cells are shown. The white arrows indicate GFP-positive and mCherry-positive cells with altered morphologies. The yellow arrows indicate GFP-positive and mCherry-positive cells with increased cell sizes, compared to the cells lacking both Crk and CrkL. Scale bar: 50 μm. *B*, The cytoplasmic areas of both GFP- and mCherry-positive cells were measured according to the Materials and methods. Data are mean ± SEM (bars) values. *$p < 0.05$, **$p < 0.01$, **$p < 0.001$, compared with control. *C*, WT, double floxed

(DF), Crk floxed ($Crk^{f/f}$), or CrkL floxed ($CrkL^{f/f}$) cells were infected with lentiviral vectors expressing GFP alone or GFP plus Cre. FITC and DAPI images of fluorescent cells were taken at 5 DPI for the cytoplasmic area measurement. Representative images of fluorescent cells are shown. The white arrows indicate GFP/Cre-positive cells with altered morphologies. Scale bar: 100 μm. *D*, The cytoplasmic areas of GFP-positive cells were measured as described in the Materials and methods. Data are shown as the mean ± SEM (bars) values. *p < 0.05, **p < 0.01, **p < 0.001, compared with control.

Figure 11. Measurement of the cell size in the absence of Crk and CrkL





A and ***B***. Crk/CrkL double floxed ($Crk^{f/f}$;$CrkL^{f/f}$) cells were coinfected with lentiviral vectors expressing GFP and Cre (derived from pLVX-IRES-ZsGreen1) and lentiviral vectors expressing various Crk and CrkL proteins (derived from pLVX-IRES-mCherry). FITC, Texas Red, and DAPI images of fluorescent cells were taken at 5 DPI for the cell size measurement using equivalent diameter (Eqdiameter) (***A***) or perimeter (***B***). *p < 0.05, **p < 0.01, **p < 0.001, compared with control. ***C***-***E***. WT, double floxed (DF), Crk floxed ($Crk^{f/f}$), or CrkL floxed ($CrkL^{f/f}$) cells were infected with lentiviral vectors expressing GFP alone or GFP plus Cre. FITC and DAPI images of fluorescent cells were taken at 5 DPI for cell size measurements using the cytoplasmic area (***C***), equivalent diameter (***D***), or perimeter (***E***). The cytoplasmic area calculation shown in Figure 7D was converted as percentages of the controls and are shown in the graph in ***C***. Note that the reduction in equivalent diameter that occurred after ablation of both Crk and CrkL (39.3 ± 1.8%) was greater than the combination of the effects caused by individual ablation of Crk and CrkL (29.1% based on 1 - 0.816 x 0.869 = 0.291). Also, the reduction in perimeter that occurred after ablation of both Crk and CrkL (47.0 ± 2.1%) was greater than the combination of the effects caused by individual ablation of Crk and CrkL (37.9% based on 1 - 0.770 x 0.807 = 0.291). *p < 0.05, **p < 0.01, **p < 0.001, compared with control.

Table 1. Changes in protein levels upon lentiviral infection

	$Crk^{f/f}$;$CrkL^{f/f}$ + GFP/Cre					$Crk^{f/f}$;$CrkL^{f/f}$ + GFP				
(DPI)	1	2	3	4	5	1	2	3	4	5
Rac	95±16	109±8	93±6	90±7	69±5**	85±10	118±12	159±10**	168±10**	207±16**
Cdc42	84±6	109±10	94±17	81±10	81±13	76±12	112±21	185±39	140±36	137±30
RhoA	83±3	96±8	95±24	97±12	88±3*	96±4	130±27	169±39	188±24*	192±12*
FAK	88±7	98±5	89±7	96±7	91±7	81±7	97±6	120±11	126±9	149±13*
pY-FAK	94±4	82±4**	74±4***	84±5**	74±5***	80±6	100±6	119±10	122±9	136±8**
Akt	111±14	109±10	122±5	128±4*	115±8	97±11	117±6	155±4*	140±19	160±29
pS-Akt	102±6	102±4	73±4**	76±5**	74±7*	94±12	115±6	107±11	140±14*	145±14*
Src	102±6	117±13	111±13	108±8	93±6	100±11	124±15	138±12	147±11	161±9*
pY-SFK	124±14	86±6	91±7	84±6	78±11	109±10	108±10	149±12*	125±15	146±20
Tor	93±5	104±12	112±9	107±9	99±9	98±10	128±23	145±16	137±9*	152±14*
pS-Tor	121±13	98±9	102±11	111±14	97±8	91±13	114±9	140±21	125±19	142±18
pS-S6k	98±10	93±6	86±3*	97±5	96±4	92±8	86±6	77±5	87±7	83±4

Crk/CrkL double floxed ($Crk^{f/f}$;$CrkL^{f/f}$) cells were infected with a lentiviral vector expressing both GFP and Cre or a lentiviral vector expressing GFP alone. Cell lysates were prepared daily starting from 1 DPI through 5 DPI. Expression levels of proteins were determined using Western blot analysis and presented as percentages of proteins without infection ± SEM. *$p < 0.05$, **$p < 0.01$, **$p < 0.001$, compared with the control levels without infection.

Discussion

Crk and CrkL fulfill diverse biological functions in cell growth, migration and adhesion by binding to many proteins through SH2 and SH3 domain interactions. These interactions are often triggered by activation of receptor, or nonreceptor, tyrosine kinase-mediated signal transduction pathways to ensure that cells respond appropriately to a variety of external stimuli. One of the major questions now facing the field is to understand how these multiple interactions are coordinated, spatially and temporally, in response to changes in the extracellular environment. It has been assumed that Crk and CrkL provide a platform for the assembly of arrays of signaling proteins that coordinate complex cellular responses. However, it is still unclear which cellular changes Crk and CrkL induce in response to stimuli and their biological significance. Previously, reduced focal adhesions and altered cell morphology were observed in NIH3T3 fibroblast cells in which Crk and CrkL were suppressed using shRNA approaches (Antoku and Mayer, 2009). In addition, human tumor cell lines in which Crk levels were reduced showed decreases in cell motility, cell spreading, and formation of actin stress fibers and focal adhesions (Rodrigues *et al.*, 2005). These studies linked the effects of Crk and CrkL to changes in cell cytoskeleton. The researchers used Cre/loxP recombination technology to induce individual and collective ablation of CrkI, CrkII and CrkL from cultured fibroblasts. The results suggest that CrkI, CrkII and CrkL all participate in mediating cytoskeletal communication with the extracellular matrix. In the absence of CrkI, CrkII and CrkL, formation of both focal

adhesions and actin stress fibers, the two key cellular structures that connect the cell cytoskeleton to the extracellular matrix, are disrupted. Once cells lose their connection with the extracellular matrix, they fail to maintain cytoskeletal integrity. Taken together with previous studies, the results provide compelling evidence that Crk and CrkL mediate changes in cell structure in response to alterations in the extracellular environment. As suggested previously (Park and Curran, 2008), the researchers propose that once the specific components of a signaling pathway are activated in response to a change in the extracellular environment, Crk and CrkL are then recruited by specific phosphotyrosine containing proteins. Here the researchers suggest that Crk and CrkL then mobilize general signaling proteins to link cytoskeletal components to the extracellular environment, thereby enabling appropriate structural changes. In other words, Crk and CrkL translate changes in the extracellular environment into alterations in cell structure. The results also demonstrate that, unless the connection with the extracellular matrix is maintained by Crk and CrkL, the cell cytoskeleton becomes disorganized. Therefore these findings imply that cytoskeletal structures inside cells actively communicate with the extracellular environment through protein-protein interactions mediated by Crk and CrkL.

Although Crk and CrkL are structurally very similar, most of the previous functional studies focused on the role of either Crk or CrkL individually, but not both, partly because only one of the proteins was initially identified as a binding partner of signaling molecules in a given pathway (George *et al.*, 2012; Hallock *et al.*, 2010). However, *in vivo* studies using mouse knockout models have clearly demonstrated that Crk and CrkL exhibit overlapping functions (Hallock *et al.*, 2010; Park and Curran, 2008). To better understand the underlying mechanism responsible for the *in vivo* functions of Crk family proteins, the researchers took an *in vitro* approach to investigate the effects of Crk and CrkL ablation at the cellular level. While fibroblasts lacking both Crk and CrkL display prominent morphological alterations, fibroblasts lacking either Crk or CrkL exhibit only modest changes in cell morphology. Previous investigations (Antoku and Mayer, 2009; Birge *et al.*, 2009; Fathers *et al.*, 2012) using siRNAs or shRNAs to suppress *Crk* and *CrkL* expression, did report alterations in cell behavior and morphology, however, these were much less substantial than the absolute requirement for Crk and CrkL in maintaining cell shape that the researchers show here. This difference in morphological phenotypes likely reflects incomplete suppression of Crk and CrkL in the previous siRNA and shRNA suppression studies. In addition, some Crk shRNAs also caused partial suppression of CrkL (Fathers *et al.*, 2012), making it difficult to distinguish Crk-mediated functions from CrkL-mediated functions.

Double floxed cells also allowed us to assess the individual contributions of CrkI, CrkII, and CrkL to the maintenance of cell structure. While exogenous expression of CrkI and CrkL modestly inhibited morphological conversion in the absence of endogenous Crk and CrkL, exogenous CrkII was much more effective at maintaining cell shape. These results imply that all three proteins contribute to the cytoskeletal connection with the extracellular matrix. The more potent effect of CrkII was rather unexpected. CrkI is more effective than CrkII in inducing cell transformation (Matsuda *et al.*, 1992), and most of the Crk-binding proteins interact with the common SH2 domain and the N-terminal SH3 domain. It has been generally accepted that the C-terminal SH3 domain, which is present only in CrkII, plays an autoinhibitory role through intramolecular domain-domain interactions (Ota *et al.*, 1998; Zvara *et al.*, 2001). The study by the researchers, however, indicates that the C-terminal SH3 domain does not act as an inhibitory

element. Instead, it appears to promote the ability of Crk to maintain the cytoskeleton, raising an interesting possibility that Crk and CrkL may influence the connection to the extracellular matrix via a different mechanism than that responsible for cell transformation. This positive functional contribution of the C-terminal SH3 domain of CrkII was also observed in NIH3T3 fibroblast cells, in which either CrkI or CrkII mutants lacking the C-terminal SH3 domain failed to rescue the PDGF-induced Rac1 activation (Antoku and Mayer, 2009). It may be interesting to examine whether a compact structure and/or cis-trans isomerization of CrkII (Kobashigawa *et al.*, 2007; Sarkar *et al.*, 2007) are responsible for these activities. Further analyses will be required to understand why CrkII is much more effective than CrkI and CrkL in rescuing the morphological conversion. Nevertheless, the failure of the SH2-only mutants of Crk and CrkL in rescuing morphological alterations suggests that both the SH2 and SH3 domains are required for this function. The study underscores the notion that certain key biological functions of Crk and CrkL can be uncovered only by deleting both Crk and CrkL from cells or tissues. Thus, floxed mice for Crk and CrkL provide a useful tool for future studies.

Based on these findings, the researchers hypothesize that overexpression of Crk and CrkL could strengthen the cytoskeletal connection with the extracellular matrix by creating proximities among signaling proteins. Under this condition, the number and/or strength of the cellular connection points with the extracellular matrix such as focal adhesions would increase, and signals from the extracellular environment could have a greater impact than in normal cells, leading to enhanced cellular responses such as increased proliferation and motility. This notion is supported by cell transformation induced by overexpression of Crk and CrkL and by observations of increased expression of Crk and CrkL in several human cancers (Sriram and Birge, 2010). Therefore, Crk and CrkL might serve as therapeutic targets for suppressing tumor cell growth and metastasis.

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Specific Aim 2. Investigate the Function of Crk and CrkL in other cell types.

Single and double mutations in Crk and CrkL will be generated by targeted disruption using transgenes that express CRE in a tissue-specific and temporally-restricted manner. Deletions will be targeted to neural stem cells, glial cells, kidney cells, spinal cord neurons and immune system cells among others. The role of Crk family proteins in the development and function of these tissues will be examined.

Achieving Research Goals for Specific Aim 2

In order to take advantage of the researchers' unique research resources such as genetically engineered mice and cells and to broaden the knowledge of Crk and CrkL functions, the researchers actively pursued collaborations with many researchers in different research areas. The collaborations have been very productive and led to many important findings in different tissues, which are described in detail below. The collaborative research efforts helped provide wider and deeper perspectives of Crk and CrkL functions. Therefore, the researchers are assured that the research goals for Specific Aim 2 were fully met.

2-1. Functions of Crk and CrkL in Neuromuscular Synapse Formation

The following text and figures are reproduced and adapted from a previously published research article (Hallock PT, Xu CF, Park TJ, Neubert TA, Curran T, Burden SJ. Dok-7 regulates neuromuscular synapse formation by recruiting Crk and Crk-L. Genes and Development 2010 Nov 1;24(21):2451-61. doi: 10.1101/gad.1977710). Cold Spring Harbor Laboratory Press holds the copyright.

Background

Neuromuscular synapse formation requires a complex exchange of signals between motor neurons and skeletal myofibers (Sanes and Lichtman, 2001). Agrin, supplied by motor neurons, binds to the low-density lipoprotein receptor-related protein 4 (Lrp4) and stimulates tyrosine phosphorylation of MuSK, a receptor tyrosine kinase expressed in skeletal muscle (Jennings et al., 1993; Kim et al., 2008; Valenzuela et al., 1995; Zhang et al., 2008). Once phosphorylated,

MuSK recruits docking protein-7 (Dok-7), an adaptor protein containing N-terminal pleckstrin homology (PH) and phosphotyrosine binding (PTB) domains, which are required to bind tyrosine phosphorylated MuSK, and a C-terminal domain (Okada et al., 2006). Neuromuscular synapse formation requires Dok-7, as neuromuscular synapses fail to form in mice and humans lacking *Dok*-7 (Okada et al., 2006; Vogt et al., 2009). Moreover, recessive mutations in human *Dok-7* are responsible for one form of congenital myasthenia (CMS), characterized by simplified and degenerating neuromuscular synapses, leading to muscle weakness and fatigue (Beeson et al., 2006; Muller et al., 2007; Selcen et al., 2008).

Following recruitment of Dok-7 to tyrosine phosphorylated MuSK, Dok-7 itself becomes tyrosine phosphorylated (Okada et al., 2006). The role of Dok-7 phosphorylation is poorly understood, but mutations that result in loss of the C-terminal domain cause congenital myasthenia (Beeson et al., 2006). These findings raise the possibility that phosphorylation of tyrosine residues in the C-terminal domain of Dok-7 has an important role in signal transduction downstream from Dok-7. The researchers collaborated with Dr. Steven Burden and his colleagues at New York University Medical School to investigate roles of Crk and CrkL in neuromuscular synapse formation.

Results

Phosphorylated Dok-7 peptides purify Crk/CrkL from muscle

The researchers sought to identify the proteins that are recruited to pY396 and pY406 in Dok-7, using a biochemical assay to isolate proteins that bind these phosphorylation sites. The researchers synthesized two biotinylated 15mer phosphopeptides, one centered on pY396 and a second centered on pY406, as well as two control biotinylated peptides, centered on Y396F or Y406F, and the researchers used the phosphopeptides to isolate binding proteins in lysates from C2 myotubes and 293 cells (Figure 12A). The phosphopeptides were attached to streptavidin-agarose beads, which were incubated with cell lysates from cultured myotubes. The bound proteins were resolved by SDS-PAGE and detected by silver staining. A doublet of proteins, which migrated at ~37 kD, bound specifically to the pY406 (Figure 12B) and pY396 peptides (data not shown) but neither control peptide. The proteins contained within this doublet were excised and analyzed by Q-TOF MS, which identified the proteins as CrkL and CrkII (Feller, 2001; Matsuda and Kurata, 1996).

In addition, the researchers probed Western blots of the proteins that bound the phosphopeptide-affinity columns with antibodies specific for CrkI or CrkII, Crk isoforms that contain one or two SH3 domains, respectively, as well as with antibodies against the related gene, CrkL. Figure 12C shows that CrkI, CrkII and CrkL bound to pY396 and pY406 peptides but not to non-phosphorylated control peptides. These data confirm that CrkII and CrkL as well as CrkI bind to both phosphopeptides. Thus, Crk and CrkL bind directly to Dok-7 phosphorylated at Y396 or Y406.

Agrin stimulates recruitment of CrkL and Crk to Dok-7

The researchers asked whether Crk, CrkL and Nck are recruited to Dok-7 following Agrin stimulation. To determine whether Agrin stimulates association of CrkL and Nck proteins with Dok-7, the researchers stimulated muscle cells with Agrin, isolated CrkL or Nck by

immunoprecipitation and probed Western blots for Dok-7. Figure 13A shows that CrkL associated with Dok-7 following Agrin-stimulation. In contrast, Nck1/2 failed to associate with Dok-7 under the same conditions (Figure 13B). To determine whether CrkI and/or CrkII associates with Dok-7 in muscle, the researchers treated muscle cells with Agrin, isolated Dok-7 by immunoprecipitation and probed Western blots with antibodies that recognize both forms of Crk. Figure 13C shows that CrkII associated with Dok-7 in an Agrin-dependent manner. Substantially lower levels of CrkI co-isolated with Dok-7, likely due to the lower expression level of CrkI in muscle cells. Thus, Agrin stimulation leads to the association of CrkL and Crk with Dok-7 in skeletal muscle.

Loss of CrkL does not affect neuromuscular synapse formation
Association of Crk and CrkL with tyrosine phosphorylated Dok-7 suggest that these adapters may have a role in postsynaptic differentiation. To determine whether these adapter proteins are concentrated at neuromuscular synapses, the researchers stained frozen sections of adult muscle with antibodies to CrkL and with antibodies to both forms of Crk. Figure 14A shows that CrkL is concentrated at synapses. CrkL staining persisted at denervated synapses, showing that staining is not within nerve terminals (Figure 14A). In contrast, the researchers failed to detect an accumulation of Crk at neuromuscular synapses (Figure 14A).

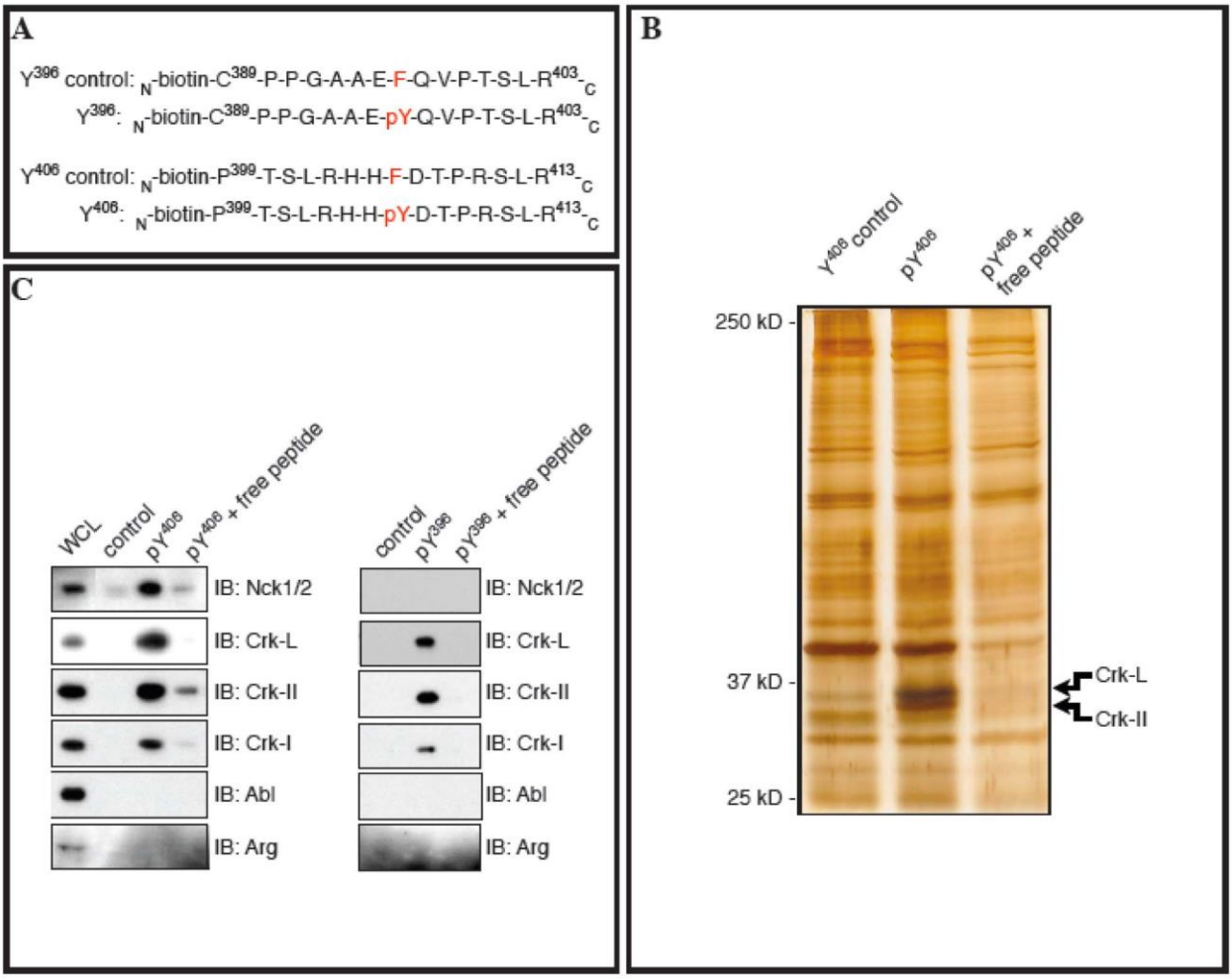
Because Crk can bind to tyrosine phosphorylated Dok-7, the researchers sought to determine whether a loss of CrkL would lead to accumulation of Crk at synapses. The researchers inactivated CrkL in skeletal muscle by generating mice that carry floxed alleles of CrkL and a human skeletal actin (HSA)::cre transgene and stained whole mounts of skeletal muscle for Crk. The researchers found that Crk indeed accumulated at synaptic sites in $CrkL^{f/-}$; HSA::cre mice (Figure 14B), indicating that Crk might substitute for CrkL and function redundantly with CrkL at neuromuscular synapses.

Crk and CrkL play critical roles in neuromuscular synapse formation
The researchers sought to determine the role of Crk and CrkL at neuromuscular synapses. The researchers inactivated Crk and CrkL selectively in skeletal muscle by crossing $myf5^{cre}$ mice, which express Cre recombinase in myoblasts and myotubes (Tallquist et al., 2000), with mice carrying floxed alleles of CrkL and Crk. The researchers failed to recover $Crk^{f/f}$;$CrkL^{f/f}$;$myf5^{cre}$ mice postnatally, although all other allele combinations were recovered at the expected Mendelian frequency (Table 2). Because neuromuscular synapses are dispensable during embryonic development but required for survival at birth, these data raised the possibility that a loss of skeletal muscle Crk and CrkL led to neonatal lethality. Indeed, at E18.5, one day prior to birth, the researchers recovered $Crk^{f/f}$;$Crk\text{-}L^{f/f}$;$myf5^{cre}$ mice at the frequency expected for Mendelian inheritance (data not shown).

The researchers analyzed neuromuscular synapses in E18.5 mice by staining whole mounts of muscle with probes that label motor axons, nerve terminals and AChRs. In wild-type mice, motor axons branch and terminate in a band of synapses, adjacent to the main intramuscular nerve, in the middle of the muscle. Each muscle fiber is innervated at a single synaptic site, marked by an accumulation of synaptic vesicles in the nerve terminal and a high density of AChRs in the postsynaptic membrane (Burden, 1998; Sanes and Lichtman, 2001).

Figure 15 shows that mice deficient in skeletal muscle Crk and CrkL displayed severe defects in presynaptic and postsynaptic differentiation (Figure 15). First, motor axons were not confined to a narrow endplate band but were distributed in a broader region of Crk/CrkL deficient muscle (Figure 15A). Second, the number and size of synapses were reduced by two-fold (Figure 14A-C). Third, the density of synaptic AChRs was reduced by 25% (Figure 15D). Thus, Crk/CrkL mutant synapses contain ~three-fold fewer AChRs than normal synapses. Because the safety factor for synaptic transmission at mouse neuromuscular synapses is 2.5 to 4 (Harris and Ribchester, 1976; Wood and Slater, 2001), this reduction in AChR number may compromise the reliability of synaptic transmission and result in neonatal lethality of mice deficient in muscle Crk and CrkL. Finally, motor axons often failed to terminate at synapses and instead continued to grow beyond AChR clusters (Figures 15E), resembling terminal sprouts found in adult muscle after inhibiting synaptic transmission or following reinnervation (Betz et al., 1980; Brown and Ironton, 1977; Son and Thompson, 1995). Thus, muscle-derived Crk/CrkL fulfills a critical role in the clustering of AChRs at synapses, the regulation of synaptic size and the control of motor axon growth.

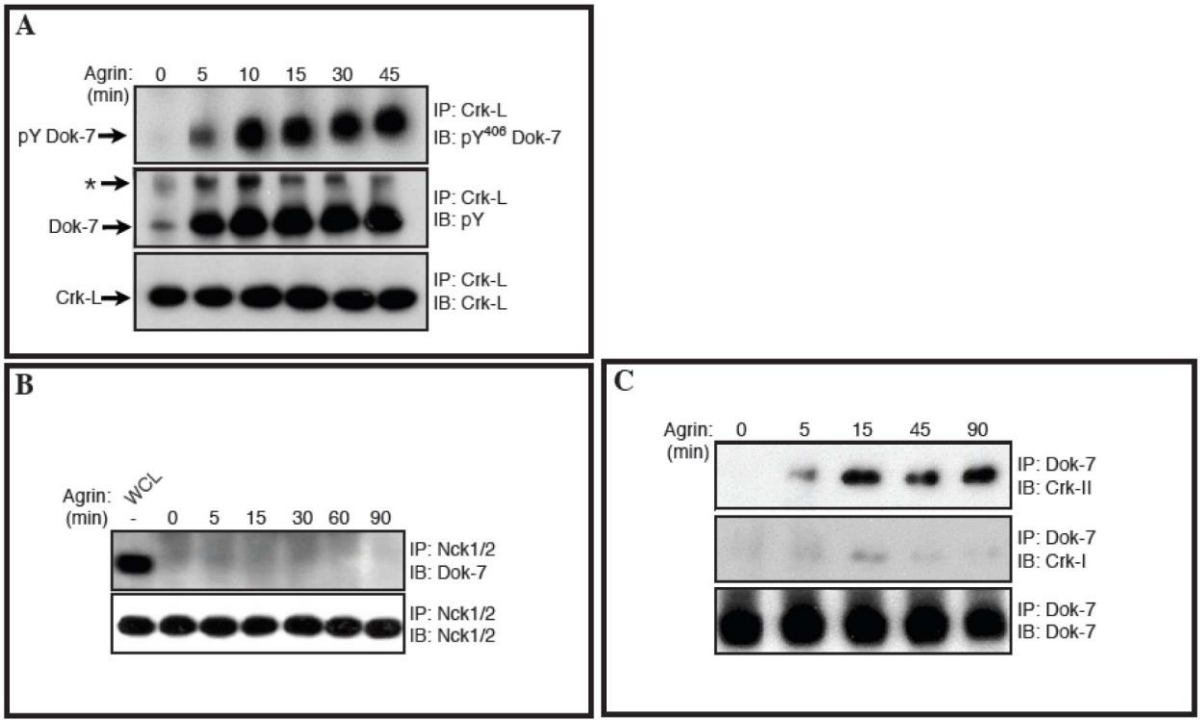
Figure 12. Dok-7 Y396 and Y406 phosphopeptides bind to Crk-L and Crk in myotube lysates



(A) The amino acid sequences of the biotinylated Dok-7 phosphopeptides. (B) The Dok-7 biotin-pY406 phosphopeptide purified a ~37 kD doublet. The doublet failed to bind the non-phosphorylated biotin-Y406 peptide, and binding to the biotin-pY406 phosphopeptide was

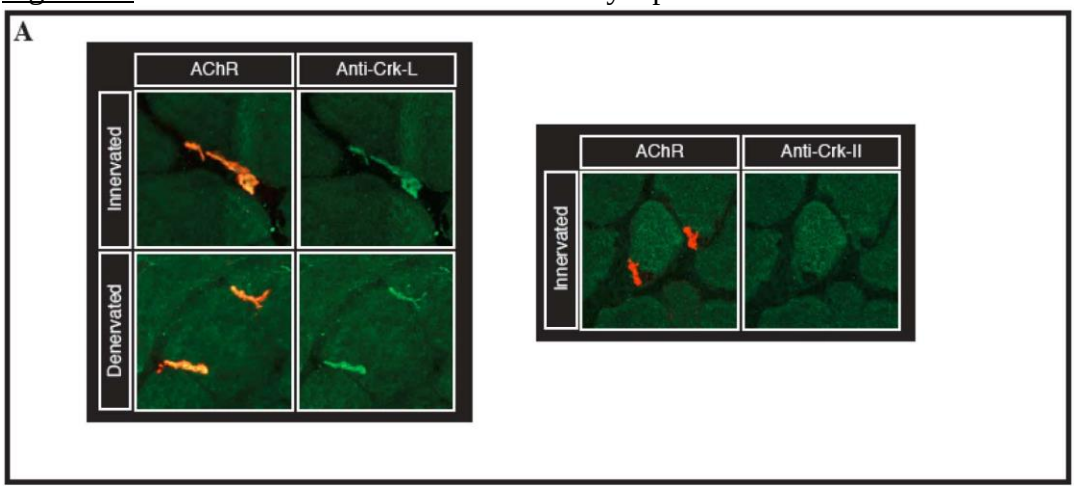
inhibited by addition of excess phosphopeptide. The ~37 kD doublet was identified as Crk-II and CrkL by Q-TOF MS. (C) Western blots of myotube proteins isolated by binding to pY396 or pY406 phosphopeptides were probed with antibodies to Nck1/2, CrkI, CrkII, CrkL Abl or Arg. Both phosphopeptides bound CrkI, CrkII and CrkL; in addition, the pY406 phosphopeptide bound Nck1/2. Neither phosphopeptide bound Abl or Arg.

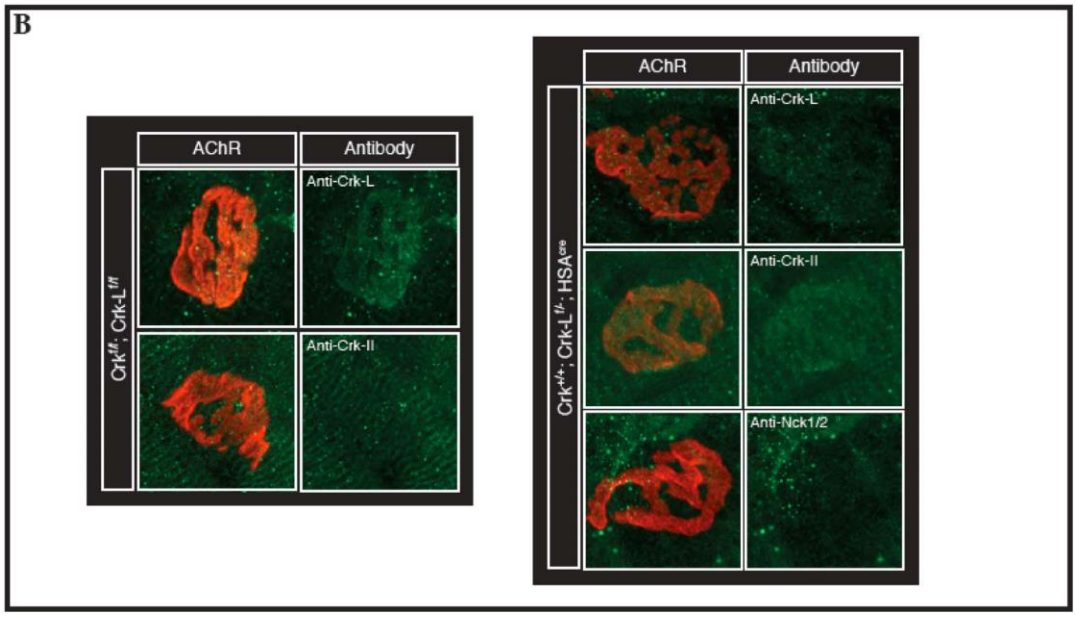
Figure 13. Agrin stimulates recruitment of CrkL and Crk to a MuSK/Dok-7 signaling complex



(A) Agrin stimulation of C2 myotubes led to the formation of a signaling complex between phosphorylated Dok-7 and CrkL (top panel). Further, two tyrosine phosphorylated proteins, Dok-7, and a 70kd protein (*, middle panel), coimmunoprecipitated with CrkL. (B) Nck1/2 and Dok-7 do not coimmunoprecipitate. (C) Agrin stimulated the formation of a complex containing Dok-7, CrkI and CrkII.

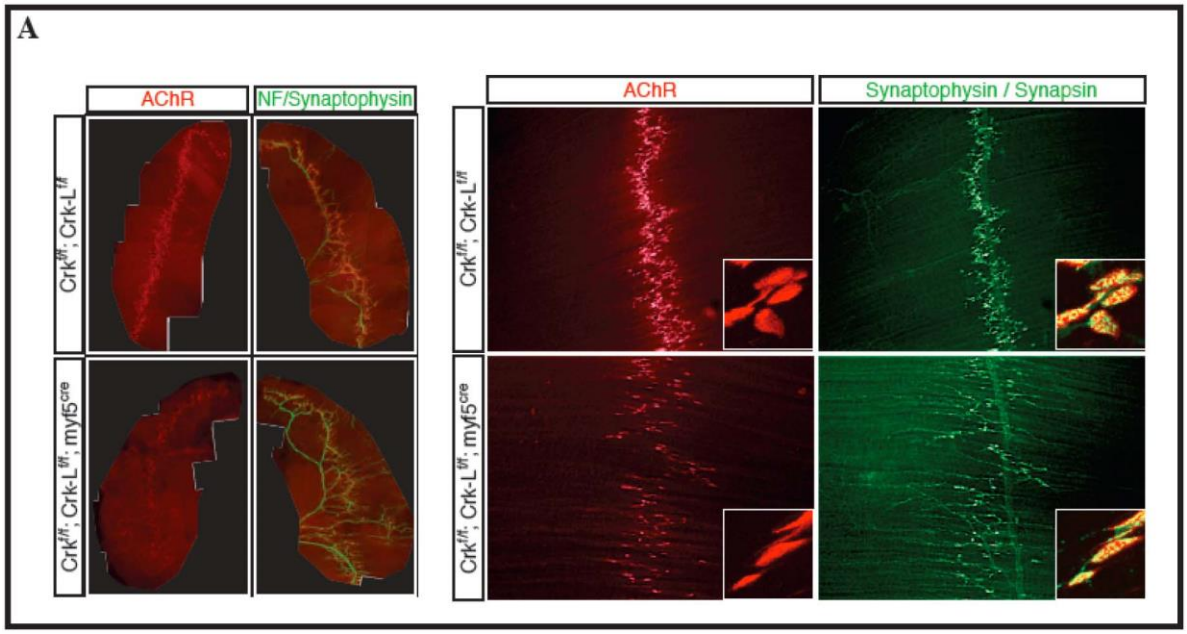
Figure 14. CrkL is enriched at neuromuscular synapses

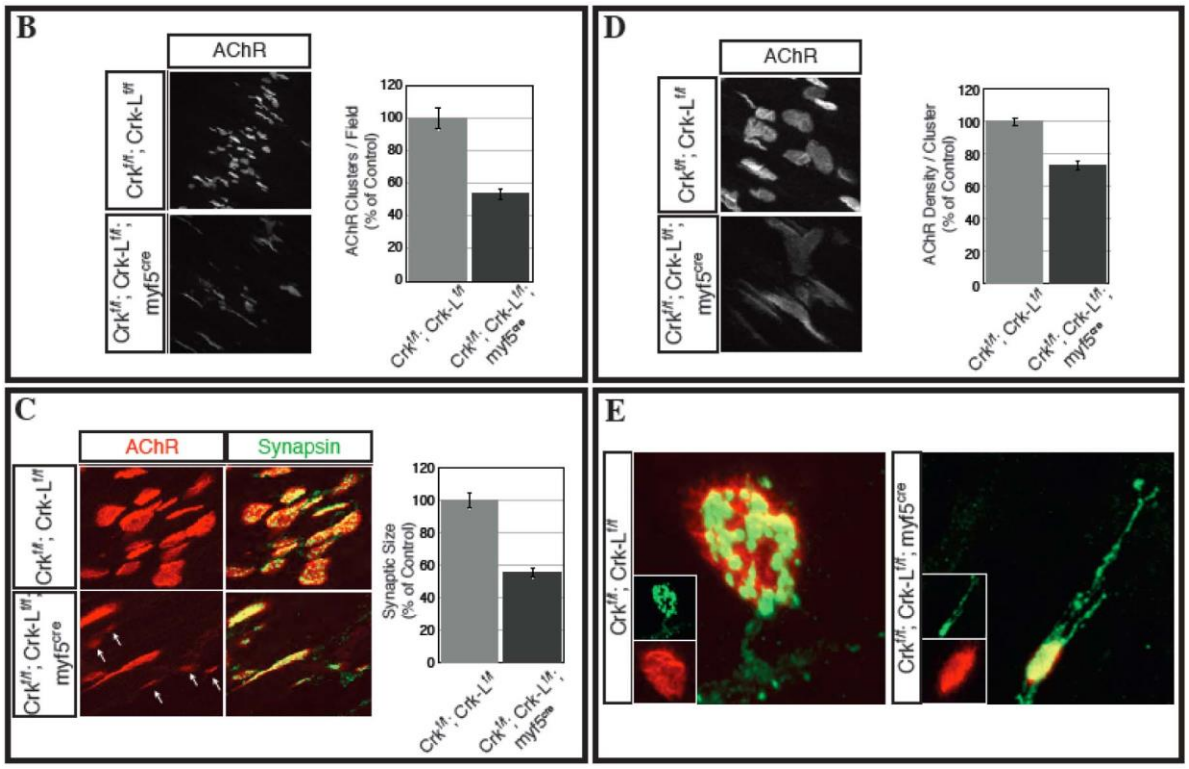




(A) Cross sections of innervated and denervated adult rat gastrocnemius muscles were stained with Alexa-594 a-BGT, which labels synapses (red), and antibodies to CrkL (green, left panels). CrkL staining was enriched at synaptic sites (yellow, merge). CrkL was concentrated in the postsynaptic membrane, since staining remained at denervated synaptic sites. In contrast, CrkI/II staining was evident within myofibers but was not enriched at synaptic sites (right panels). (C) Whole mounts of P30 diaphragm muscles from *Crk-L$^{f/-}$; HSA^{cre}* mutant mice were stained with Alexa-594 a-BGT and antibodies to CrkL, CrkI/II, and Nck1/2; in contrast to wild-type synapses, CrkI/II was enriched at CrkL deficient synapses. Nck1/2 was not enriched at synapses.

Figure 15. Crk and CrkL play critical roles in neuromuscular synapse formation





(A, B, C) Muscles from E18.5 mice deficient in muscle Crk/CrkL contained fewer synapses, which were smaller and more broadly distributed, than in control mice. Moreover, the density of synaptic AChRs was reduced (A, D). (E) In Crk/CrkL-deficient muscle, motor axons contacted AChR clusters but failed to stop growing. n=3, error bars indicate standard errors of the means.

Table 2. Mice that are deficient in muscle-derived Crk/CrkL die at birth

$Crk^{f/f}$; $Crk\text{-}L^{f/f}$ x $Crk^{f/+}$; $Crk\text{-}L^{f/+}$; $myf5^{Cre}$

	Observed	Expected	Chi-square
$myf5^{Cre}$ negative	28	24.5	0.500
$Crk^{f/+}$; $Crk\text{-}L^{f/+}$; $myf5^{Cre}$	7	6.13	0.123
$Crk^{f/f}$; $Crk\text{-}L^{f/+}$; $myf5^{Cre}$	6	6.13	0.002
$Crk^{f/+}$; $Crk\text{-}L^{f/f}$; $myf5^{Cre}$	8	6.13	0.569
$Crk^{f/f}$; $Crk\text{-}L^{f/f}$; $myf5^{Cre}$	0	6.13	6.13
Total	49		

(A) $Crk^{f/f}$; $Crk\text{-}L^{f/f}$ mice were crossed with $Crk^{f/+}$; $Crk\text{-}L^{f/+}$ mice, which carried the $myf5^{cre}$ transgene, and surviving progeny were genotyped at P0 or P1. χ-square analysis shows that the distribution of genotypes is unlikely (P>0.05) to occur by chance, indicating that mice deficient in muscle-derived Crk and CrkL die at birth.

Discussion

Agrin stimulates tyrosine phosphorylation of MuSK, leading to the recruitment and tyrosine phosphorylation of Dok-7 (Okada et al., 2006). The steps following Dok-7 tyrosine phosphorylation are poorly understood. Here, the researchers show that Agrin stimulates phosphorylation of two tyrosine residues in the C-terminal domain of Dok-7, which leads to the recruitment of two adaptor proteins, Crk and CrkL. Furthermore, the researchers demonstrate that Dok-7 tyrosine phosphorylation regulates the number and size of AChR clusters that form in cultured muscle cells, and the researchers provide genetic evidence supporting a redundant role for Crk and CrkL in the regulation of both presynaptic and postsynaptic differentiation in vivo. These data indicate that Crk and CrkL are key components in the Agrin/MuSK signaling pathway that function downstream from Dok-7. These experiments demonstrate a second instance in which Crk and CrkL function in a redundant manner downstream from a Lrp. Reelin, a ligand that binds two Lrps, Lrp8 and VLDR, stimulates tyrosine phosphorylation of Disabled, a PTB domain-containing adapter protein, leading to recruitment of Crk and CrkL (Ayala et al., 2007; Rice and Curran, 2001; Park and Curran, 2003).

The SH3 domains in Crk/CrkL can also bind Abl1 and Abl2 (Feller et al., 1994), non-receptor tyrosine kinases that have been implicated in clustering AChRs (Finn et al., 2003). Because Abl also regulates actin dynamics (Lanier and Gertler, 2000; Zandy et al., 2007), Crk/CrkL may serve as an integrator for multiple pathways, including a Rac/Rho/Pak pathway and an Abl/Ena/Vasp pathway, to regulate cytoskeletal reorganization and AChR clustering following Agrin stimulation.

Mutations in human Dok-7 cause congenital myasthenia, a heterogeneous group of neuromuscular disorders typified by muscle weakness and fatigue (Beeson et al., 2006; Muller et al., 2007; Selcen et al., 2008). In patients with mutations that truncate Dok-7 and lead to a loss of Y396 and Y406, neuromuscular synapses are small, and motor axons fail to reliably terminate, resembling defects found in mice deficient in muscle-derived Crk/CrkL. Understanding how Dok-7 and Crk/Crk-L work to stimulate synaptic differentiation will hopefully lead to insights for enhancing synaptic differentiation and partially restoring neuromuscular function in patients with congenital myasthenia.

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2-2. Function of Crk in Irremediable Endoplasmic Reticulum Stress

The following text and figures are reproduced and adapted from a previously published research article (Austgen K, Johnson ET, Park TJ, Curran T, Oakes SA. The adaptor protein CRK is a pro-apoptotic transducer of endoplasmic reticulum stress. Nature Cell Biology 2011 Dec 18;14(1):87-92. doi: 10.1038/ncb2395).

Background

Excessive demands on the protein folding capacity of the endoplasmic reticulum (ER) cause irremediable ER stress and contribute to cell loss in a number of cell degenerative diseases. If unable to resolve ER stress through its homeostatic signaling outputs, the unfolded protein response activates the intrinsic mitochondrial apoptotic pathway. Irremediable ER stress represents a form of intrinsic cell damage that culminates in activation of the BAX/BAK-dependent mitochondrial apoptotic pathway (Oakes et al., 2006). Homo-oligomerization of BAX and/or BAK consequently results in outer mitochondrial membrane permeabilization and release of pro-death mitochondrial proteins (e.g., cytochrome *c*) into the cytosol, causing activation of effector caspases (Du et al., 2000; Susin et al., 1999; Liu et al., 1996). For many forms of cell injury, including ER stress, there is a limited understanding of the cellular transducers that relay the information of upstream damage to BAX/BAK oligomerization at mitochondria. To date, the only known BAX and/or BAK activators are members of the pro-apoptotic BH3-only family, a diverse class of polypeptides containing a loosely conserved ~9-12 amino acid BH3 death domain (Chipuk and Green, 2009; Strasser, 2005; Lomonosova and Chinnadurai, 2008). The BH3-only proteins BID, BIM, NOXA and PUMA have been previously implicated in ER stress-induced death Upton et al., 2008); however, cells deficient in one or more of these proteins are not completely resistant to this form of apoptosis (Lindsten et al., 2000; Wei et al., 2001). Therefore, it is conceivable that additional proteins that communicate ER stress to the mitochondrial apoptotic machinery remain to be discovered. The researchers collaborated with Dr. Scott Oakes and his colleagues at University of California-San Francisco to study functions of Crk in ER stress-induced apoptosis.

Results

Crk is the principal component of ER stress-induced cytosolic apoptotic activity
Given their strong resistance to ER stress-induced apoptosis (Lindsten et al., 2000; Wei et al., 2001), the researchers reasoned the *Bax*$^{-/-}$*Bak*$^{-/-}$ mouse embryonic fibroblasts (MEFs) present a unique and powerful tool to trap and identify pre-mitochondrial apoptotic signals activated by ER stress. Therefore, the researchers challenged SV40-transformed *Bax*$^{-/-}$*Bak*$^{-/-}$ MEFs with the pharmacological agent Brefeldin A (BFA), which blocks ER-golgi protein transport, to induce ER stress and initiate the pre-mitochondrial apoptotic program. The researchers prepared cytosolic extracts (S100) from untreated or BFA-treated *Bax*$^{-/-}$*Bak*$^{-/-}$ MEFs, incubated isolated Jurkat mitochondria with these extracts, and measured the amount of mitochondrial cytochrome *c* released as a readout for pro-apoptotic activity. The S100 of BFA-treated *Bax*$^{-/-}$*Bak*$^{-/-}$ cells triggers the release of ~90% of cytochrome *c* from isolated mitochondria in a BAX/BAK-dependent manner (Upton et al., 2008), while the S100 fraction from untreated *Bax*$^{-/-}$*Bak*$^{-/-}$ MEFs

induces negligible (<5%) cytochrome *c* release (Figure 16A). Thus, ER stress induces a cytosolic activity capable of releasing mitochondrial cytochrome *c*, which the researchers have termed *C*ytochrome *c* *R*eleasing *A*ctivity (CcRA). The researchers designed and performed a biochemical purification strategy to isolate additional CcRA factors in the BFA-treated *Bax*$^{-/-}$ *Bak*$^{-/-}$ S100 (Figure 16B). The CcRA-containing fractions from the final step (MonoQ gradient) of the purification scheme were analyzed by MALDI mass spectrometry. Crk was the highest confidence protein identified by mass spectrometry in the active fractions, with approximately 25% of the total sequence represented in 6 tryptic peptides.

Crk is required for efficient ER stress-induced apoptosis

To test if Crk plays a role in ER stress-induced apoptosis in cells, the researchers challenged *Crk*$^{-/-}$ and wild-type (WT) matched MEFs with BFA or tunicamycin (TUN), an ER stress agent that specifically inhibits *N*-linked glycosylation. Notably, S100 from BFA-treated *Crk*$^{-/-}$ MEFs has significantly decreased CcRA compared to that from BFA-treated WT MEFs (Figure 17A), indicating that Crk is required for the majority of the ER stress-induced apoptotic signal. In addition, *Crk*$^{-/-}$ MEFs are strikingly resistant to ER stress-induced apoptosis, but as sensitive as WT MEFs to staurosporine (STS), a pan-kinase inhibitor known to activate the mitochondrial apoptotic pathway independently of ER stress, thereby confirming the pro-apoptotic role of CRK in the ER stress-induced apoptotic pathway (Figure 17C, D).

Proteolytic cleavage converts Crk into a pro-apoptotic protein

The researchers found that Since ER stress caused depletion of full-length CrkII. To determine if CrkII is reduced in a specific subcellular compartment, the researchers probed subcellular fractions from *Bax*$^{-/-}$*Bak*$^{-/-}$ MEFs and found that CrkII is partially localized to the ER, mitochondria, and cytosol (Figure 18A). Moreover, upon ER stress, 38kD ER- and cytosol-localized CrkII is reduced, while mitochondrion-localized full-length CrkII changes little (Figure 18A). Interestingly, the researchers found that upon ER stress, CrkII appears to be sequentially cleaved at least twice, resulting in several distinct fragments (Figure 18A). Proteolytic cleavage is a post-translational modification recognized to activate other known pro-apoptotic proteins, such as BID, but has not been described previously for Crk (Li et al., 1998; Luo et al., 1998; Wang et al., 1996; Wei et al., 2000).

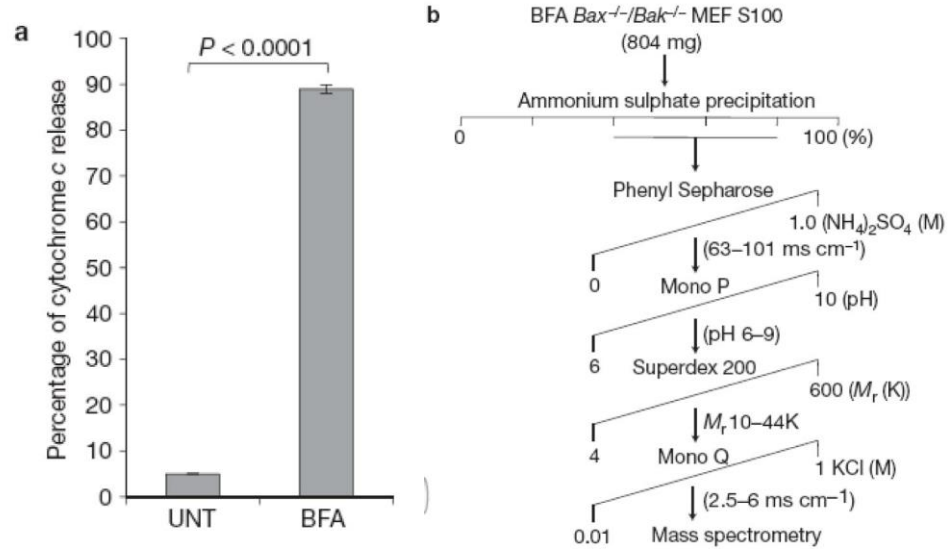
The researchers tested if CrkI also undergoes cleavage events upon ER stress. Following ER stress, at least one CrkI-specific cleavage product is readily observed (Figure 18B). In addition, the researchers observed depletion of full-length endogenous CrkI and CrkII in both WT and *Bax*$^{-/-}$*Bak*$^{-/-}$ MEFs upon BFA treatment (Figure 18C), indicating that their cleavage occurs upstream of the mitochondrial apoptotic pathway.

In an attempt to determine the role of Crk cleavage for its apoptotic activity, the researchers tested a small panel of protease inhibitors for their ability to block this event (data not shown). The researchers found that the pan-cysteine protease inhibitor ZVAD-FMK prevents ER stress-induced loss of full-length CrkI and CrkII (Figure 19B) and protects *Bax*$^{-/-}$*Bak*$^{-/-}$ MEFs from the cytopathic effects of ER stress (Figure 19A). These data suggest that an ER stress-activated cysteine protease is responsible for Crk cleavage.

To determine if the observed ER stress-induced cleavage of Crk is critical for its apoptotic

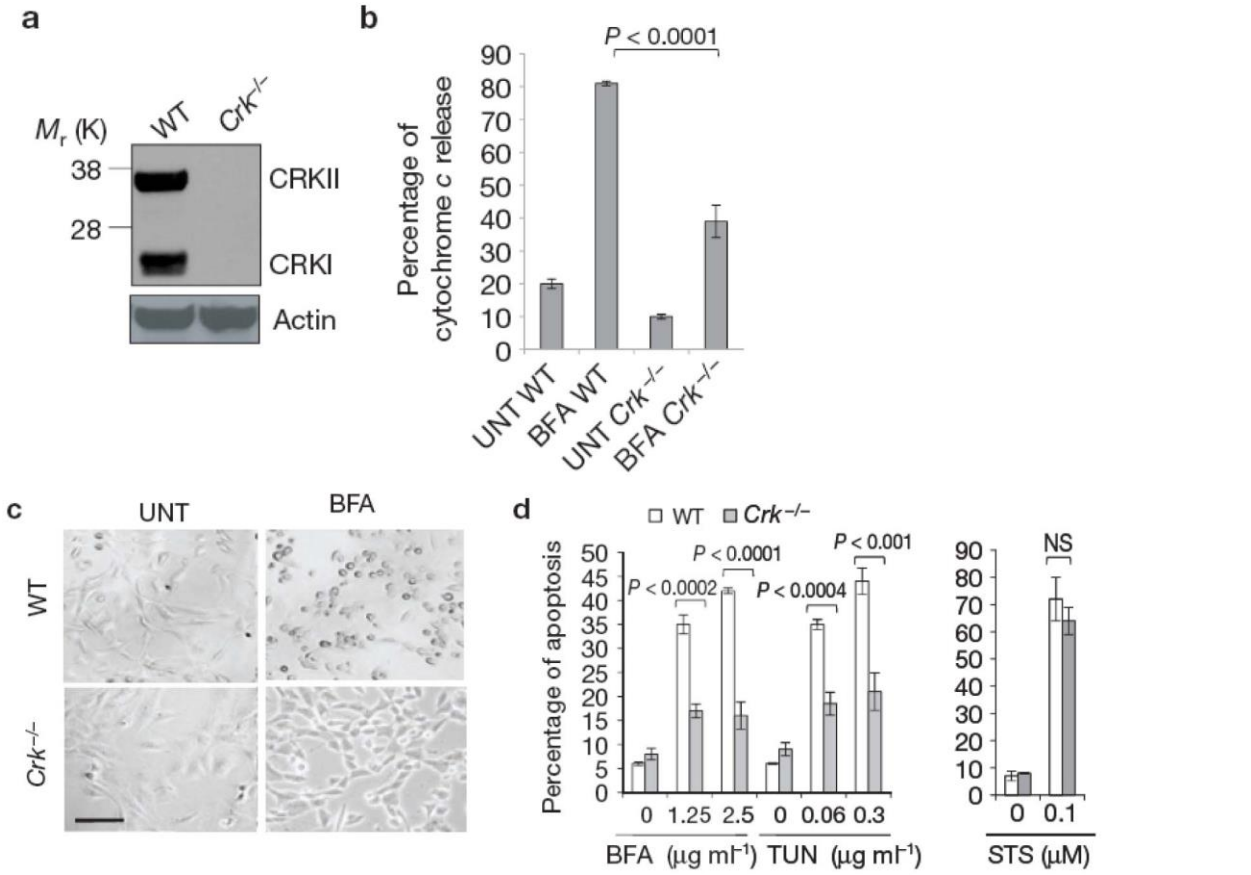
activity, the researchers individually mutated each potential cysteine protease cleavage site (aspartic acid) in the Crk sequence. When stably reconstituted into *Crk*$^{-/-}$ MEFs, CrkII ΔD110A (*Crk*$^{-/-}$ *+ CrkII (ΔD110A))* was the only aspartic acid mutant unable to be cleaved in response to ER stress (Figure 18D). Furthermore, non-cleavable CrkII ΔD110A is defective in restoring *Crk*$^{-/-}$ MEF sensitivity to ER stress-induced apoptosis (Figure 18E, F), arguing that this cleavage event is critical for its apoptotic activity. Cleavage at D110 is predicted to produce one fragment of approximately 25kD, which can be detected by a C-terminal-specific antibody (Figure 18D), and a second N-terminal fragment of ~14kD, which is undetectable using available antibodies. As both CrkI and CrkII restore *Crk*$^{-/-}$ sensitivity to ER stress, it is likely the shared N-terminal fragment (~14kd) contains the critical domain for its apoptotic function. To test this prediction, the researchers transiently expressed the N-terminally FLAG-tagged fragment (NF110) in the absence of ER stress (Figure 18G) and measured apoptosis. As predicted, NF110 was able to potently induce cell death independently of ER stress (Figure 18H, I). From these data, the researchers conclude that Crk is cleaved upon ER stress at D110, to produce a novel pro-apoptotic fragment.

Figure 16. Biochemical purification of ER stress apoptotic activity identifies Crk



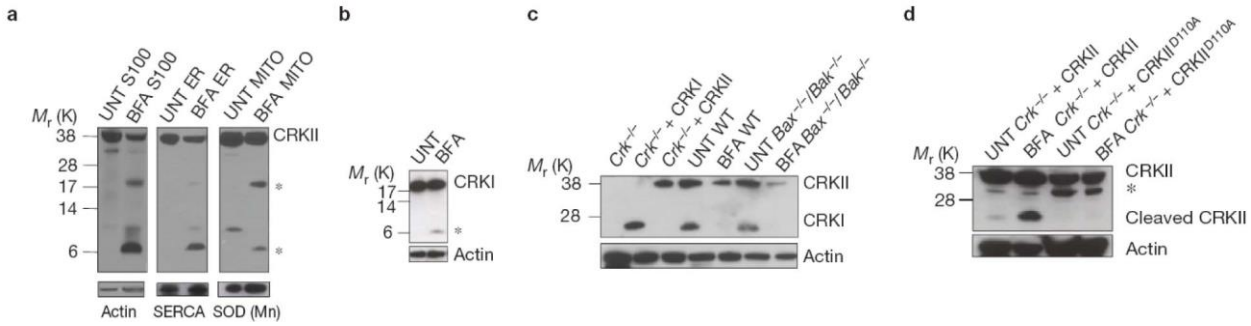
(a) Induction of cytochrome *c* release from isolated Jurkat mitochondria by cytosolic extracts (S100) from untreated (UNT) and 24h Brefeldin A (BFA) 2.5 μg/ml treated *Bax*$^{-/-}$*Bak*$^{-/-}$ MEFs. (b) FPLC purification scheme of cytochrome *c* releasing activity (C*c*RA) present in BFA *Bax*$^{-/-}$ *Bak*$^{-/-}$ S100. Active fractions from each purification step are indicated.

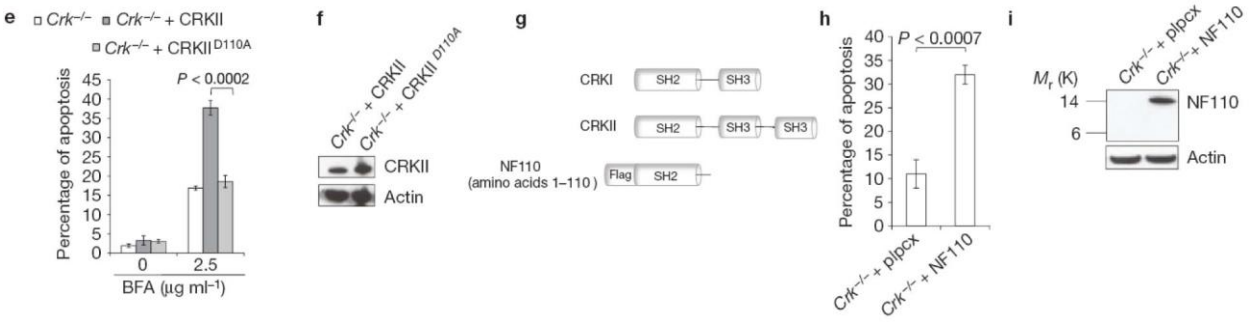
Figure 17. *Crk*$^{-/-}$ MEFs are significantly resistant to ER stress-induced apoptosis



(a, b) 18h BFA (2.5 μg/ml)-treated *Crk*$^{-/-}$ MEF S100 contains significantly less C*c*RA in comparison to 18h BFA-treated (2.5 μg/ml) wild-type MEF S100. (c) *Crk*$^{-/-}$ MEFs are visually resistant (phase contrast) to ER stress-induced apoptosis (BFA 2.5μg/ml). (d) *Crk*$^{-/-}$ MEFs are strongly resistant to BFA and TUN-induced apoptosis, but equally sensitive to staurosporine (STS), in comparison to wild-type MEFs.

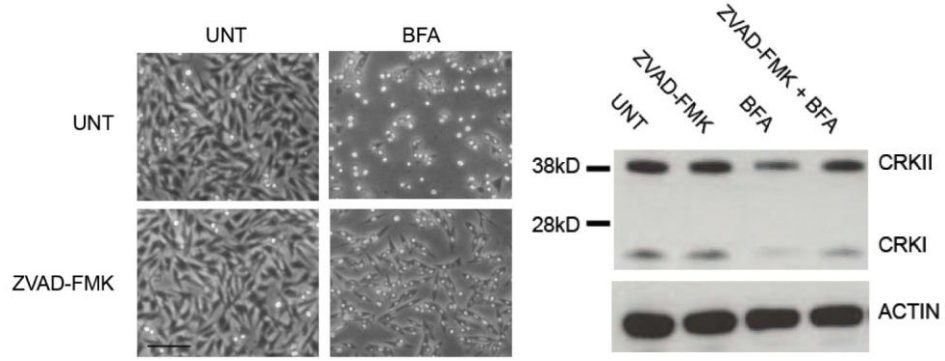
Figure 18. Crk is proteolytically cleaved into an apoptotic signal upon irremediable ER stress





(a) Upon 24h BFA (2.5 μg/ml) treatment of *Bax*$^{-/-}$*Bak*$^{-/-}$ MEFs, full-length CrkII is depleted in the cytosol and at the ER. CrkII-specific fragments (*) appear in the cytosol, ER, and mitochondria. (b) Transiently expressed CrkI is also cleaved upon 24h BFA (2.5 μg/ml) treatment in *Crk*$^{-/-}$ MEFs. * = CrkI-specific fragment. (c) Loss of full-length, endogenous CrkI and CrkII observed upon 18h BFA treatment of WT and *Bax*$^{-/-}$*Bak*$^{-/-}$ MEFs. (d) Upon ER stress, Crk is cleaved at D110. Mutation of this site (D110A) in CrkII prevents cleavage following 24h 2.5 μg/ml BFA treatment in stably reconstituted in *Crk*$^{-/-}$ MEFs. (e, f) CrkII (D110A) is not able to rescue *Crk*$^{-/-}$ MEF sensitivity to ER stress-induced apoptosis induced by 24h 2.5 μg/ml BFA, in contrast to *Crk*$^{-/-}$ MEFs stably expressing wild-type (WT) CrkII. (g) Diagram of Crk (1-110a.a.) cleavage fragment (NF110) produced upon ER stress. (h, i) Transient expression of NF110 induces apoptosis independent of ER stress.

Figure 19:



(a) Crk is cleaved by a cysteine protease upstream of the mitochondria. *Bax*$^{-/-}$*Bak*$^{-/-}$ MEFs treated 24h with 100 μM pan-cysteine protease inhibitor (ZVAD-FMK) followed by 18h BFA (1.25 μg/ml) show visibly decreased cytopathic effects of ER stress-induced apoptosis. (b) Cleavage of CrkI and CrkII in *Bax*$^{-/-}$*Bak*$^{-/-}$ MEFs treated 24h with 100 μM pan-cysteine protease inhibitor (ZVAD-FMK) followed by 18h BFA (1.25 μg/ml) is significantly decreased in comparison to *Bax*$^{-/-}$*Bak*$^{-/-}$ MEFs treated with BFA alone.

Discussion

The researchers have identified a previously unknown pro-apoptotic function common to both Crk isoforms. A role for Crk in apoptosis has been suggested across species. For example, the *C. elegans* Crk-homologue CED-2 regulates apoptotic engulfment (Tosello-Trampont et al., 2007, 2001). Mammalian CrkII has been reported to induce death in some transformed cell types upon overexpression (Parrizas et al., 1997) and be required for a cell-free apoptotic activity that can be detected in *Xenopus* egg extracts (Evans et al., 1997). However, the role of

endogenous Crk in mammalian apoptosis has remained elusive. These results obtained from fibroblast cells identify Crk as a major pro-apoptotic signal required for the execution of ER stress-induced cell death. During ER stress, CrkI and CrkII are cleaved by a yet to be identified cysteine protease to generate an N-terminal fragment with potent apoptotic activity. Furthermore, Crk interacts with anti-apoptotic BCL-XL and its apoptotic activity is upstream of the BAX/BAK-dependent mitochondrial pathway. Both Crk isoforms contain a putative BH3 domain, which sensitizes isolated mitochondria to tBID-induced cytochrome *c* release and when mutated diminishes apoptotic activity in cells. These data argue that Crk is a novel BH3-only-like protein, which upon ER stress is proteolytically processed into a pro-death signal. These findings suggest that Crk may be a valuable therapeutic target in diseases where ER stress-induced cell loss is implicated, including some forms of neurodegeneration and diabetes (Marciniak and Ron, 2006; Lin et al., 2008).

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2-3. Function of Crk in Kidney Podocyte Foot Process Spreading

The following text and figures are reproduced and adapted from a previously published research article (George B, Verma R, Soofi AA, Garg P, Zhang J, Park TJ, Giardino L, Ryzhova L, Johnstone DB, Wong H, Nihalani D, Salant DJ, Hanks SK, Curran T, Rastaldi MP, Holzman LB. Crk1/2-dependent signaling is necessary for podocyte foot process spreading in mouse models of glomerular disease. Journal of Clinical Investigation 2012 Feb 1;122(2):674-92. doi: 10.1172/JCI60070).

Background

When functioning properly in health, the kidney filtration barrier selectively prevents the passage of macromolecules from the blood compartment into the urinary space. Differentiated podocytes form a remarkable octopus-like morphology, extending numerous interdigitating foot processes defined by a unique 3-dimensional actin cytoskeletal architecture and requiring formation of a specialized intercellular junction. These foot processes adhere to and cover an extracellular matrix interposed between podocytes and an endothelium that creates the glomerular capillary wall. Podocytes undergo cytoskeletal remodeling to alter their morphology in nearly all forms of human glomerular disease, exhibiting what has been described as foot process spreading and retraction or as foot process effacement. This process by which podocytes change their cytoskeletal architecture appears to be a component of a common response of the podocyte to cellular injury, correlating with loss of normal filtration barrier selectivity and predicting the development of proteinuria in human disease and in experimental models (Johnstone and Holzman, 2006; Faul et al., 2007)

Nephrin is a transmembrane protein of the Ig superfamily located at the differentiated podocyte intercellular junction (Ruotsalainen et al., 1999). Mutations in the gene encoding nephrin lead to congenital nephrotic syndrome of the Finnish type, which is characterized by developmental failure of foot process morphogenesis and podocyte intercellular junction formation and heavy

proteinuria at birth, or delayed-onset disease, presenting as steroid-resistant nephrotic syndrome with variable glomerular histopathology (Kestila et al., 1998; Philippe et al., 2008). Nephrin and the related Ig superfamily protein Neph1 form hetero-oligomeric receptor complexes that associate via cis- and trans-interactions (Barletta et al., 2003; Gerke et al., 2003). The activated nephrin-Neph1 receptor complex induces actin filament nucleation and elongation at the plasma membrane by recruiting (in a tyrosine phosphorylation–dependent manner) Src homology domain 2 adaptor proteins, including Nck1/2, the regulatory p85 subunit of PI3K, phospholipase Cγ, and Grb2, and subsequently an actin polymerization complex (Jones et al., 2006; Verma et al., 2006; Verma et al., 2003; Zhu et al., 2008; Garg et al., 2010; Harita et al., 2009; Garg et al., 2007). Because genetic deletion of nephrin or Neph1 results in abnormal podocyte process development, it has been hypothesized that beyond solely inducing actin polymerization, nephrin and Neph1 — likely in association with other membrane-associated proteins — participate in determining actin architecture. Nephrin may regulate enzymes that drive actin cytoskeletal remodeling during podocyte process development or during morphological changes that occur after podocyte injury or recovery. Podocyte process formation during glomerular development or remodeling events that occur after podocyte injury or in human glomerular disease all require directed cytoskeletal rearrangement. Since it has been reported that Crk associate with nephrin in a tyrosine phosphorylation–dependent fashion (Verma et al., 2006), the researchers collaborated with Dr. Lawrence Holzman and his colleagues at University of Pennsylvania to analyze the function of Crk in podocytes in vitro and in vivo by selectively deleting Crk from kidney podocytes in mice.

Results

Crk associates with nephrin in a tyrosine phosphorylation–dependent manner
It has been reported that an interaction between Crk and nephrin appears to require nephrin tyrosine phosphorylation (Verma et al., 2006). This interaction was identified by pulldown of Crk from isolated glomerular lysate with recombinant tyrosine-phosphorylated glutathione S-transferase–tagged nephrin cytoplasmic domain (GST-nephrinCD; Figure 20A). To confirm this interaction in an in vivo system, the researchers used a single-dose rat puromycin aminonucleoside (PAN) model of podocyte injury. Rats injected with PAN develop foot process effacement and proteinuria within 3 days of injection (Garg et al., 2007). Within the same time period, nephrin became tyrosine phosphorylated, and Crk-nephrin complex formation increased compared with vehicle control–injected rats (Figure 20B). The nephrin-Crk interaction was also examined in an in vitro model, which induces nephrin tyrosine phosphorylation and allows for investigation of nephrin activation–mediated signaling events in a simplified model system (Verma et al., 2006; Garg et al., 2010; Garg et al., 2007). Plasmid constructs were generated in which the CD16 extracellular domain and the CD7 transmembrane domain are fused either with nephrinCD or with an HA tag as negative control (referred to herein as CD16/7-nephrinCD or CD16/7-HA, respectively). These constructs were individually expressed with myc-tagged CrkII in a human podocyte cell line. As described previously, addition of mouse anti-CD16 antibody and a fluorescent secondary anti-mouse IgG antibody to the culture media of live cells resulted in "clustering" and tyrosine phosphorylation of CD16/7-nephrinCD fusion proteins in the apical membrane of cultured podocytes (Figure 20C; Verma et al., 2006; Garg et al., 2007). Concomitantly, this activated CD16/7-nephrinCD recruited and colocalized with CrkII, whereas CrkII was not recruited to CD16/7-HA.

CrkII is necessary for nephrin-induced lamellipodia formation

To determine whether nephrin-induced lamellipodial protrusive activity requires CrkI/II, human podocyte cell lines with stable knockdown of CrkI/II were generated by lentiviral infection using 5 different shRNA templates. Expression of Crk shRNA3 resulted in efficient knockdown of CrkI/II (Figure 21A). Upon transient CD16/7-nephrinCD expression and engagement, CrkI/II-deficient podocytes exhibited attenuated induction of lamellipodial activity relative to controls (Figure 21B). Indeed, the extent to which lamellipodial activity was induced after CD16/7-nephrinCD clustering appeared proportional to CrkI/II abundance. The Crk knockdown phenotype was rescued by expressing mouse CrkII in Crk-knockdown human podocytes (Figure 21C and D). Expression of mouse CrkI partially rescued nephrin-induced lamellipodial protrusion activation. Taken together, these results demonstrate that Crk is necessary for induction of nephrin-mediated lamellipodia dynamics in cultured podocytes.

Podocyte-specific deletion of CrkI/II in the mouse blocks protamine sulfate–induced foot process spreading

The researchers speculated that Crk-dependent lamellipodia protrusion in culture might be a surrogate for foot process spreading observed after podocyte injury in situ. To analyze the function of Crk in podocytes in vivo, the researchers used a strategy in which Crk was selectively deleted from kidney podocytes in mice (referred to herein as $Crk^{fl/fl}$;podocin-CreTg/+ mice, homozygous for the floxed Crk allele and heterozygous for podocin-Cre; Figure 22A and Moeller et al., 2002; Moeller et al., 2003). Podocyte-specific knockout of Crk proteins was confirmed by indirect immunofluorescence staining (Figure 22B). At birth, mice were obtained at the expected Mendelian frequency, and $Crk^{fl/fl}$;podocin-CreTg/+ mice behaved and aged normally. Relative to control mice, mutants did not have increased proteinuria over a 20-month observational period (data not shown).

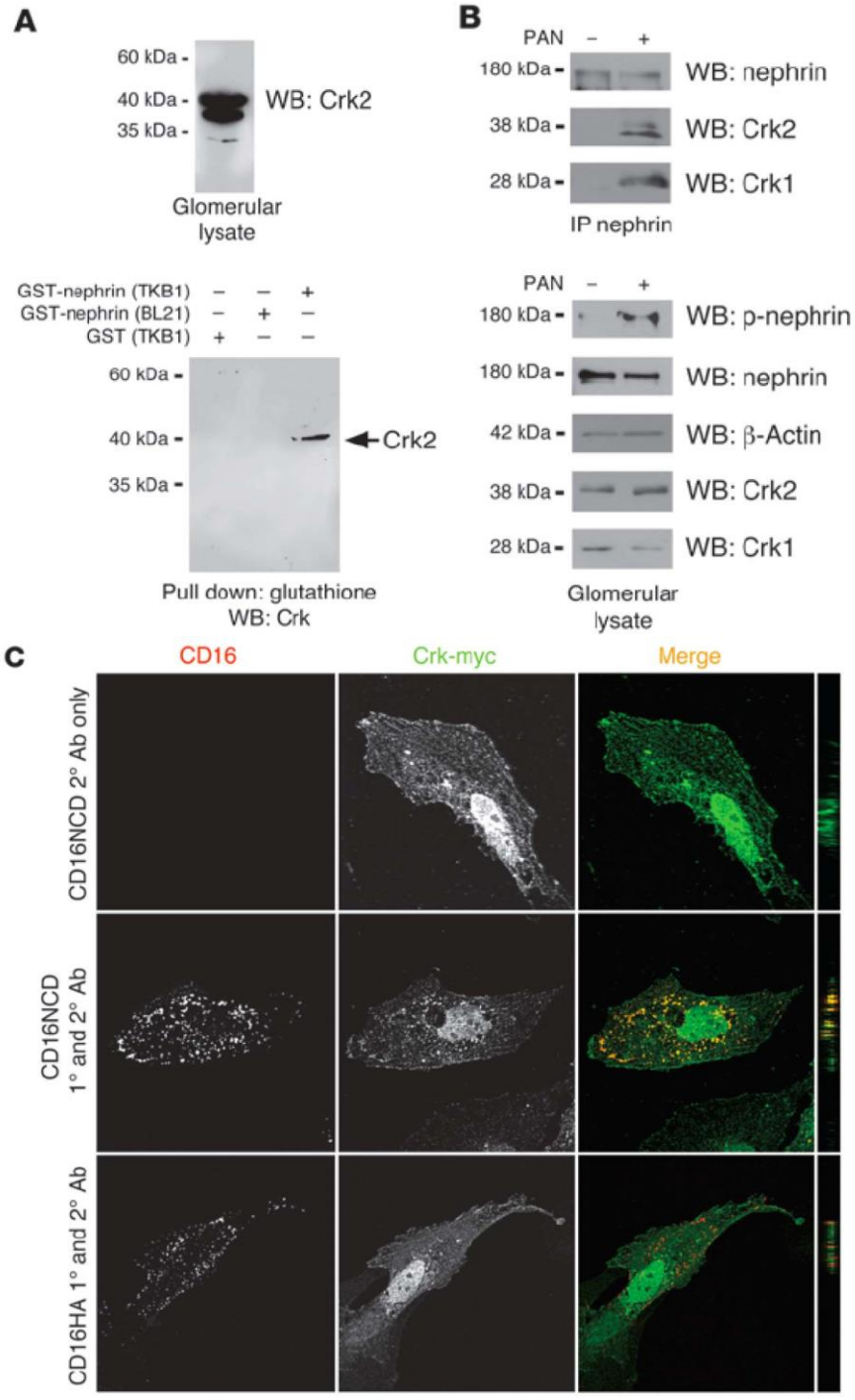
Because Crk is necessary for cell spreading in culture, the researchers speculated that functional loss of Crk might become evident when challenging these mice using the protamine sulfate model of podocyte injury. $Crk^{fl/fl}$;podocin-CreTg/+ mice and their control littermates (2–3 months old) were perfused via the renal artery with either protamine sulfate or control buffer. Kidneys were fixed and analyzed by scanning and transmission EM. Whereas perfusion of kidneys of control littermates with protamine sulfate resulted in foot process spreading, foot process morphology was remarkably preserved in protamine sulfate–perfused $Crk^{fl/fl}$;podocin-CreTg/+ mice (Figure 23A and B). Evaluation of podocyte intercellular junction frequency by transmission EM confirmed that control mice had a significantly reduced junction frequency after injury, whereas foot process architecture of protamine sulfate–perfused $Crk^{fl/fl}$;podocin-CreTg/+ mice remained similar to that of buffer-perfused control mice (Figure 23C). The results are consistent with the conclusion that Crk is necessary for protamine sulfate–induced foot process spreading.

Podocyte-specific deletion of Crk attenuates the glomerular phenotype induced by nephrotoxic serum

To test whether the observations made in the protamine sulfate model could be extended to another mouse model of podocyte foot process spreading, the researchers used the previously described nephrotoxic serum (NTS) model, in which mice are injected with sheep anti-rat

glomerular lysate antiserum (Clement et al., 2011; Lin et al., 2004, 2002; Quigg et al., 1998; Hebert et al., 1998; Yanagita et al., 2002). Intravenous injection of NTS into WT mice resulted in a dramatic increase in proteinuria within 24 hours that persisted for more than 48 hours, whereas injection of control sheep IgG had no apparent effect. This was accompanied by severe foot process effacement, as evaluated by both scanning and transmission EM (Figure 24). Remarkably, in $Crk^{fl/fl}$;podocin-CreTg/+ mice, while proteinuria of similar magnitude was obtained at 24 hours after NTS injection, proteinuria appeared to decline rapidly by 48 hours. In parallel with these results, there was no significant difference in podocyte morphology at 24 hours between NTS-treated mice on WT and $Crk^{fl/fl}$;podocin-CreTg/+ backgrounds, whereas there was partial recovery of normal podocyte morphology by 72 hours in $Crk^{fl/fl}$;podocin-CreTg/+ mice, but not WT mice.

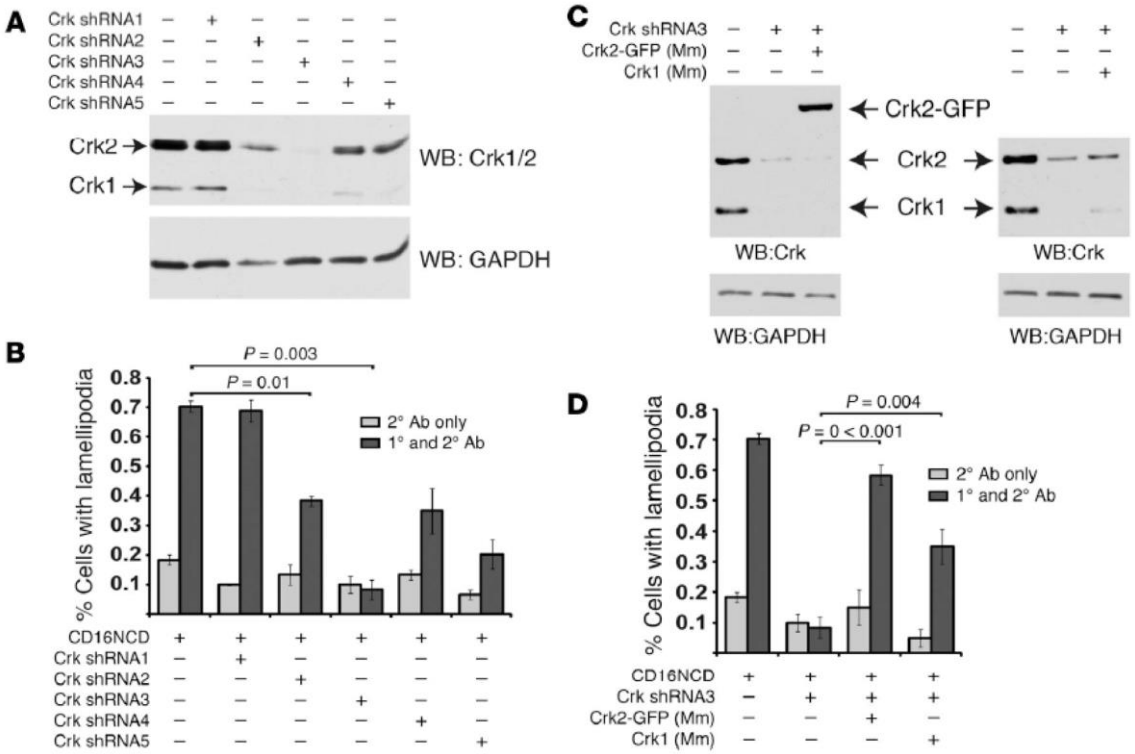
Figure 20. Crk interacts with nephrin in a tyrosine phosphorylation–dependent fashion



(A) Purified recombinant GST-nephrinCD expressed in BL21 or TKB1 E. coli (to produce nonphosphorylated or tyrosine-phosphorylated nephrin, respectively) or purified GST alone was mixed with rat glomerular lysate, pulled down with glutathione agarose, and immunoblotted with monoclonal anti-Crk antibody. (B) Glomerular lysates from rats injected with PBS (control) or PAN were immunoprecipitated and/or immunoblotted using the indicated antibodies. (C) Cultured human podocytes expressing CD16/7-nephrinCD (CD16NCD) or CD16/7-HA (CD16HA) and CrkII-myc were activated by clustering: namely, addition of monoclonal anti-

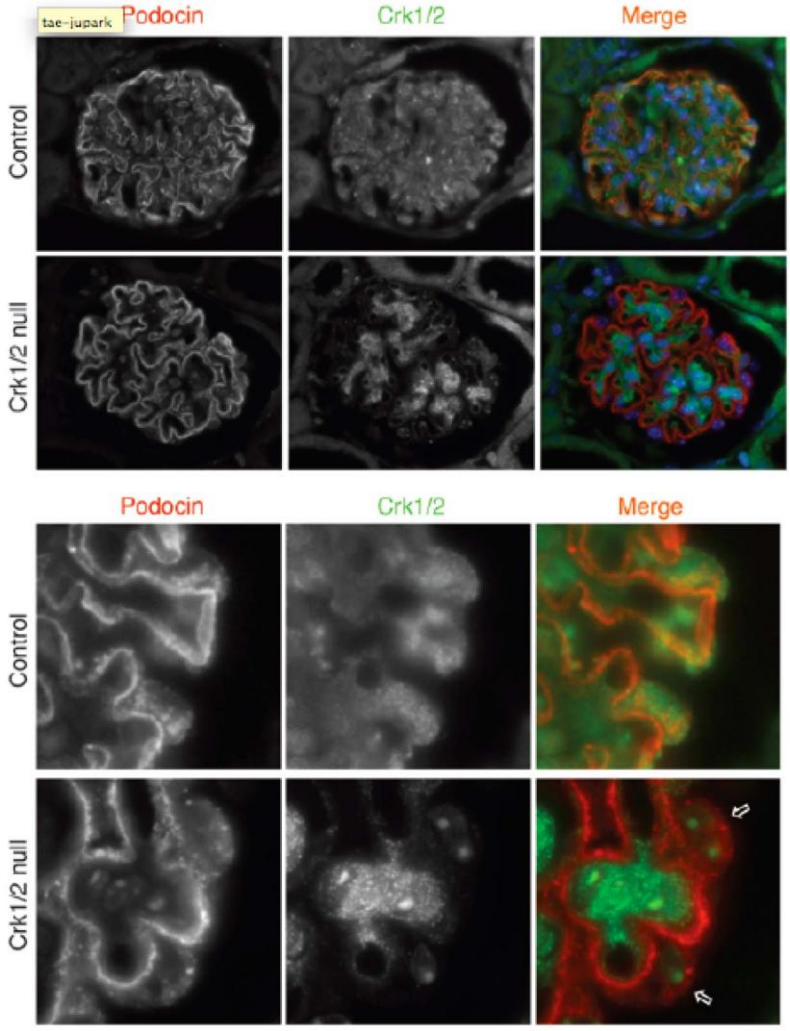
CD16 primary antibody (1°) and/or goat anti-mouse IgG Texas Red–conjugated secondary antibody (2°), as indicated, to the media of live cells. CrkII-myc was detected with rabbit polyclonal anti-myc primary antibody and Alexa Fluor 488–labeled secondary antibody. Cells were analyzed by confocal microscopy. CD16/7-nephrinCD (red) and CrkII-myc (green) colocalized in the plane of the plasma membrane.

Figure 21. Crk knockdown attenuates nephrin-induced lamellipodial protrusive Activity.



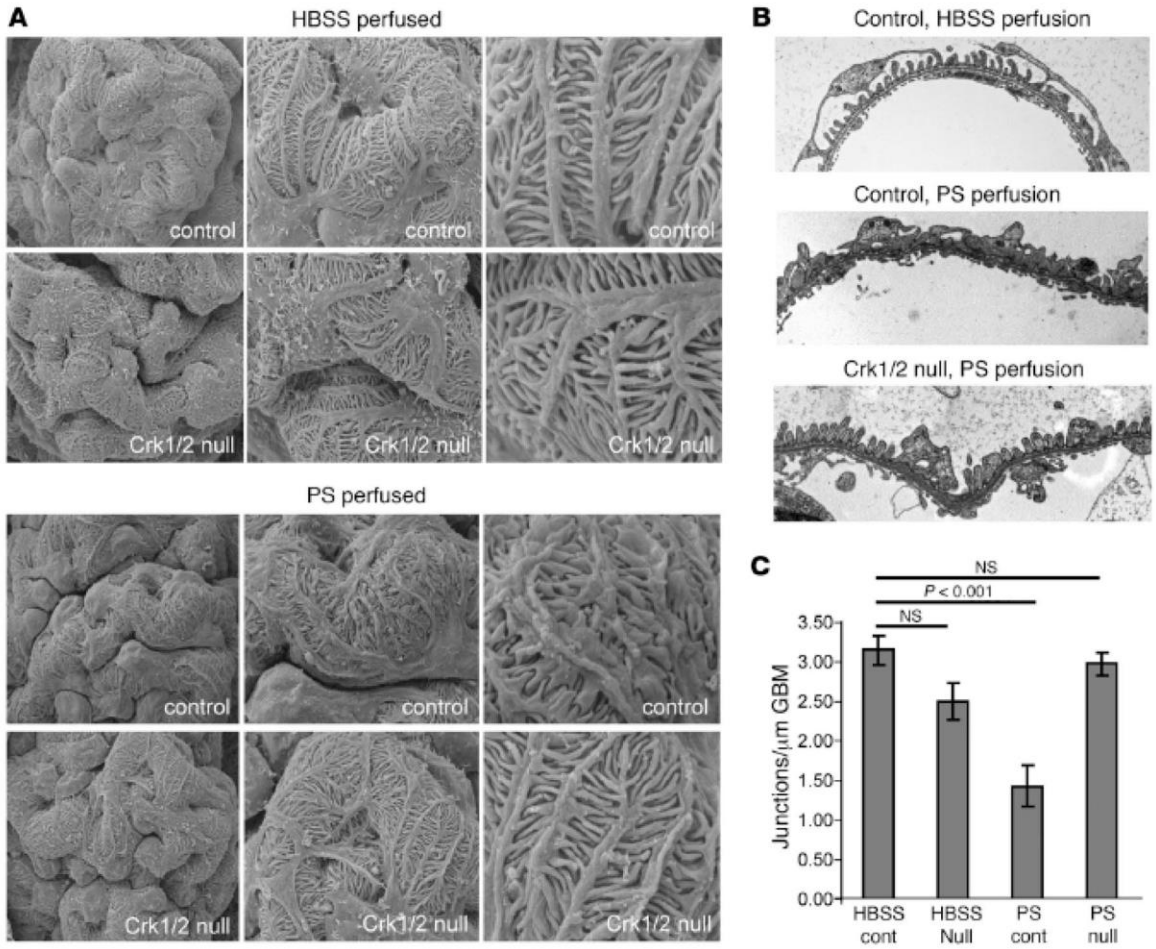
(**A**) Immunoblot demonstrating attenuation of CrkI/II expression in human podocyte cell lines stably expressing 1 of 5 different shRNA constructs targeting the Crk gene. GAPDH protein expression served as loading control. (**B**) Podocytes stably expressing the indicated Crk shRNA constructs were transfected with CD16/7-nephrinCD and actin-GFP and were activated by clustering as in Figure 1. The fraction of cells exhibiting lamellipodial protrusions was evaluated after fixation. (**C**) Immunoblot showing that CrkII-GFP or mouse CrkI were reexpressed in stably knocked down human Crk shRNA3 podocytes. Mm, *Mus musculus*. (**D**) Crk shRNA3 podocytes were transfected with CD16/7-nephrinCD and actin-GFP and activated by clustering. After fixation, the fraction of cells exhibiting lamellipodial protrusions was determined. Results (mean ± SEM) are representative of 3 independent experiments.

Figure 22. Selective deletion of Crk in mouse podocytes



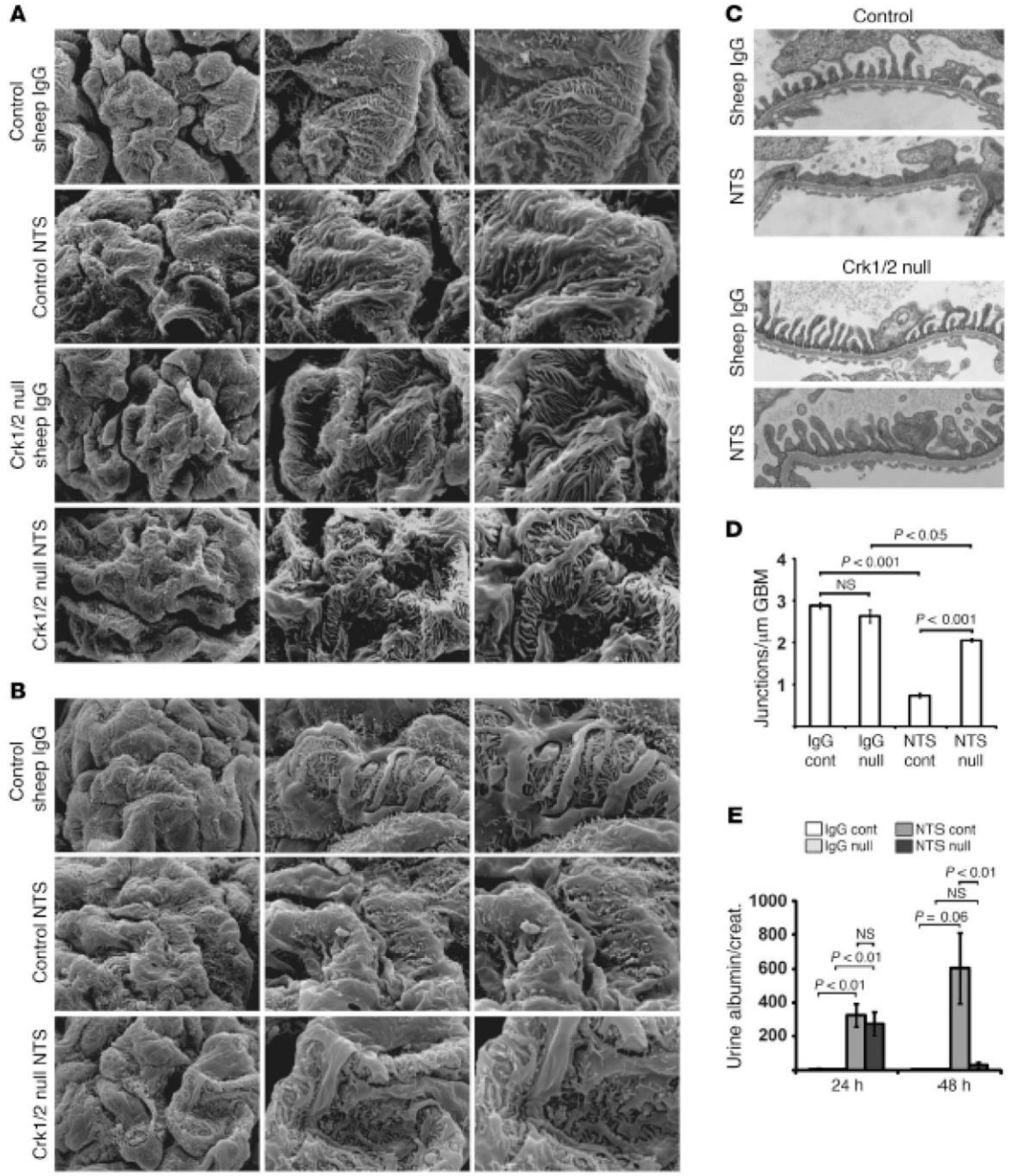
Paraffin-embedded mouse kidney sections of $Crk^{fl/fl}$ (control) and $Crk^{fl/fl}$;podocin-CreTg/+ (Crk null) mice were double stained for CrkI/II (green) and podocin as a podocyte marker (red). Higher-magnification images of portions of a glomerulus are shown below. Note the absence of Crk staining in podocytes (arrows) of $Crk^{fl/fl}$;podocin-CreTg/+, whereas Crk was still expressed in mesangial cells. Original magnification, ×600 (top); ×4,200 (bottom).

Figure 23. Foot process spreading in Crk$^{fl/fl}$;podocin-CreTg/+ mice is prevented by protamine sulfate perfusion



(A and B) Scanning EM (A) and transmission EM (B) of Crk$^{fl/fl}$ and Crk$^{fl/fl}$;podocin-CreTg/+ mice perfused with HBSS or protamine sulfate. Results are representative of 3–5 mice per group. Original magnification, ×3,000 (A, left); ×7,000 (A, middle); ×20,000 (A, right); ×15,000 (B). (C) Number of junctions per micron glomerular basement membrane (GBM), as seen by transmission EM. Data are mean ± SEM.

Figure 24. Podocyte-specific Crk deletion attenuates the glomerular phenotype induced by NTS.



(A–C) Scanning EM (A and B) and transmission EM (C) of $Crk^{fl/fl}$ and $Crk^{fl/fl}$;podocin-CreTg/+ mouse glomeruli, either 72 (A and C) or 24 (B) hours after injection of NTS or control sheep IgG. Original magnification, ×3,000 (A and B, left); ×7,000 (A and B, middle); ×10,000 (A and B, right); ×25,000 (C). (D) Number of junctions per micron glomerular basement membrane, as seen by transmission EM 72 hours after injection (mean ± SEM). (E) Albumin/creatinine ratios of $Crk^{fl/fl}$ and $Crk^{fl/fl}$;podocin-CreTg/+ mouse urines 24 and 48 hours after injection with NTS or sheep IgG. Data (mean ± SEM) are representative of 3 (A–D) or 3–9 (E) mice per group.

Discussion

There is little contention that Nephrin functions during podocyte development since Nephrin mutations in humans or genetic deletion of Nephrin in mice result in abnormal formation of podocyte tertiary processes and intercellular junctions. Therefore, given the relationship established in culture between Nephrin and Crk, it was surprising that podocytes lacking Crk in vivo developed normally and did not display a primary phenotype even with aging. Although this result might suggest that Crk is not necessary for podocyte differentiation and morphogenesis, one cannot exclude the possibility that normal podocyte development occurred due to functional genetic complementation of Crk by the paralogue CrkL, which shows an amino acid composition and domain structure similar to CrkII. In the Reelin pathway, Crk and CrkL fulfill essential overlapping functions to control neuronal positioning in the developing brain as mutation of either gene by itself in mice did not compromise Reelin signaling (Park and Curran, 2008). To determine whether CrkL can functionally complement Crk in the podocyte will require additional study.

Protamine sulfate perfusion of the kidney induces foot process spreading, which occurs within minutes and can be reversed within minutes by further perfusion with heparin sulfate. It had been argued that protamine sulfate causes alteration in podocyte structure by dissipating the repulsive negative charges on the apical surface of podocytes thought to maintain separation between processes (Seiler et al., 1975). The rapid alteration in podocyte cytoskeletal architecture observed in this model argues that protamine sulfate induces changes that are dependent on regulation of podocyte cytoskeletal dynamics. The observation by the researchers that genetic deletion of Crk prevented detectable foot process spreading following perfusion of protamine sulfate provides first evidence that protamine sulfate induces a signal that results in regulated podocyte cytoskeleton remodeling.

The observation that deletion of Crk prevents foot process effacement in the protamine sulfate model suggests that disrupting Crk-dependent signaling attenuates foot process spreading in human glomerular disease. This conclusion is likely too simplistic given the results obtained investigating the NTS nephritis model or investigating several human glomerular diseases. Unlike attenuation of foot process effacement observed in the Crk null mouse following treatment with protamine sulfate, treatment of null mice with NTS resulted in initial foot process effacement and proteinuria at 24 h followed by dramatic attenuation in proteinuria and effacement by 48 h relative to control. These results suggest that podocyte injury by NTS is mediated by at least two independent mechanisms: one mechanism that is Crk-independent and occurs early and transiently and another delayed mechanism that relies on Crk. These results also suggest that Crk-dependent foot process effacement induced by protamine sulfate occurs by a mechanism distinct from effacement observed early after NTS-induced podocyte injury.

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2-4. Functions of Crk and CrkL in T Cell Migration

Transendothelial migration of T cells occurs through three steps: selectin-mediated rolling, chemokine triggered activation and integrin-dependent arrest. Each step is tightly regulated by different membrane receptors on the migrating T cell and the interacting endothelial cell. Among these, chemokines and their receptors play a pivotal role, triggering rapid changes in T cell adhesion and actin cytoskeleton remodeling. Abl family kinases are known to be required for T cell actin responses and T cell migration. However, little is known about Abl kinase substrates in T cells. Abl family kinases are known to phosphorylate Y221 on CrkII and Y207 on CrkL, but it is unclear if Crk proteins are involved in c-Abl dependent actin regulation and migration in T cells. The researchers collaborated with Dr. Janis Burkhardt and her colleagues at the Children's Hospital of Philadelphia to generate mice that are conditionally deficient for Crk and CrkL in the mature T cell compartment and study functions of Crk and CrkL in T cell migration.

Generation and characterization of T cell-specific Crk/CrkL deficient mice.
c-Abl family kinases are required for T cell actin responses and T cell migration. Abl family kinases are known to phosphorylate Y221 in CrkII and Y207 in CrkL, but little is known about the identity of Abl kinase substrates in T cells in vivo. To study the *in vivo* function of Crk and CrkL in T cells, the researchers in collaboration with other researchers at the same institute generated mice that are conditionally deficient for Crk and CrkL in the mature T cell compartment by breeding mice bearing loxP-flanked *Crk* and *CrkL* alleles with mice carrying the Cre recombinase gene under the transcriptional control of the endogenous CD4 promoter. T cell-specific Crk/CrkL knockout mice were born in expected mendelian ratios, and the spleen, thymus and lymph nodes exhibited normal cellularity. In addition, T cell developed normally.

Crk/CrkL-deficient T cells show defects in Rap1 activation, adhesion and migration.
To test whether Crk and CrkL are required for activation of Rap1 and Rac1 in chemokine signaling pathways, the researchers stimulated CD4+ T cell blasts with CCL21, the ligand for CCR7 that regulates T cell trafficking in vivo. Normal T cells exhibited Rac1 activation, and neither the magnitude nor the kinetics of Rac1 activation was affected by deficiency of Crk and

CrkL. However, Rap1 activation was attenuated in Crk/CrkL deficient T cells, demonstrating that upon chemokine stimulation Rap1 activation depends on Crk and CrkL. Since one of the important biological effects of Rap1 signaling is the acute regulation of cell adhesion mediated by the activation of integrins, the researchers tested if the impaired Rap1 activation affects T cell adhesion in response to chemokine stimulation. Anti-CD3 treatment increased T cell adhesion to ICAM-1, the ligand that binds to the β2 integrin LFA-1 by 50%. However, Crk/CrkL-deficient T cells showed 2-fold reduction in adhesion to ICAM-1 under both unstimulated and anti-CD3 stimulated conditions. This phenotype was even more dramatic when T cells were activated by chemokines such as CXCL12 and CCL21. The results show that Crk and CrkL are required for integrin-dependent T cell adhesion. The researchers also asked whether Crk and CrkL are functionally important for T cell migration. CCL21, a CCR7 ligand that regulates T cell trafficking to lymphoid organs *in vivo*, and CXCL10, a CXCR3 ligand that preferentially regulates Th1 cell trafficking, induced wild-type T cell migration in a dose-dependent manner. In contrast, Crk/CrkL-deficient T cells displayed 20-50% reductions in migration in response to both chemokines. Taken together, these results demonstrate that Crk and CrkL are required for adhesion and migration of T cells.

2-5. Functions of Crk and CrkL in Other Types of Tissues

In addition, the researchers are currently collaborating with Dr. Xin Zhang at Indiana University School of Medicine to study functions of Crk and CrkL in eye development, Dr. Bernice Morrow at Albert Einstein College of Medicine to study functions of Crk and CrkL in heart development, and Dr. Jian Zuo at St. Jude Children's Research Hospital to study functions of Crk and CrkL in hair cell development.

Specific Aim 3. Determine the Effect of Crk and CrkL on tumor cell growth.

Crk and CrkL were first identified as cellular counterparts of the *v-Crk* oncogene. Both have oncogenic potential when they are overexpressed. The potential for fibroblasts to be transformed by oncogenes such as *Ras* and *Src* will be investigated using the fibroblast lines isolated in Aim 1. The ability of transformed cells to form tumors will be assessed in allograft transplantation models. Finally, $Crk^{fl/fl}/CrkL^{fl/fl}$ mice will be crossed with tumor prone strains of mice, such as *p53* null and $Ptch1^{+/-}$, and the tumor cells will be transduced with vectors expressing CRE to determine the role of Crk and CrkL in tumor formation and maintenance.

Achieving Research Goals for Specific Aim 3

The researchers established fibroblast cell lines from various mouse models of Crk and CrkL. The researchers found that cell transformation by oncogenes is severely impaired in the absence of either Crk or CrkL. The findings lay the basic foundation for future studies. Although the researchers could not address the role of Crk and CrkL in tumor formation and maintenance using in vivo approaches, the results obtained from Specific Aim 1 and 3 studies will help the researchers continue to investigate functions of Crk and CrkL in tumors and to pursue Crk and CrkL as targets for cancer therapy. The researchers believe that the research goals for Specific Aim 3 were significantly met.

Background

Crk and CrkL are known to exhibit oncogenic potential and they can induce anchorage independent growth of fibroblast cells (Matsuda et al., 1992; Senechal et al., 1996). v-Crk increases total intracellular tyrosine phosphorylation levels in transformed cells, even though it doesn't have a kinase domain itself (Mayer et al., 1988). Moreover, overexpression of Crk and CrkL was described in several types of human cancers (Sriram et al., 2010)). In particular, expression of Crk was found to be significantly elevated in poorly differentiated lung adenocarcinoma (Miller et al., 2003), primary breast tumors (Rodrigues et al., 2005), glioblastomas (Wang et al., 2007) and colon cancers (Nishihara et al., 2002). Moreover, inhibition of Crk expression in a human ovarian cancer cell line and a human synovial sarcoma cell line suppressed tumor formation (Linghu et al., 2006; Watanabe et al., 2009). CrkL has also been observed to be overexpressed in non-small cell lung cancer and CrkL knockdown decreased tumor cell invasiveness (Kim et al., 2010). Finally, CrkL is an indicator of Bcr/Abl1 activity and serves as a prognostic factor for remission of chronic myeloid leukemia following imatinib targeted therapy (Oda et al., 1994).

Tumorigenesis is associated with a breakdown in the regulation of gene expression. Gene expression changes may result from mutation of transcription factors (Salomoni et al., 2002) or from accumulation of defects in many signaling pathways (Scandura et al., 2002). The researchers used an *in vitro* model to determine whether Crk or CrkL play a role in cell transformation. The Fos and Ras oncogenes function in signal transduction pathways whose constant activity is sufficient to induce cell transformation and tumorigenesis (Miao et al., 1994; Bakin et al., 1999; DeFeo et al., 1981; Ellis et al., 1981). Fyn and Src belong to the Src family of tyrosine kinases and they both have oncognenic potential (Kawakami et al., 1988; Johnson et al., 1985; Collett et al., 1979; Wang et al., 1976). Using MEF fibroblast cells and MEF-derived cell lines, the researchers determined the effect of Crk and/or CrkL ablation on cellular transformation induce by each of the oncogenes Fos, Ras, Src and Fyn. The Researchers used cell morphology, saturation density and anchorage independent growth in soft agar as indicators of transformation.

Materials and methods

Cells, retroviral and lentiviral infection

Mouse embryonic fibroblast (MEF) wild type (WT), Crk knockout ($Crk^{-/-}$), CrkL knockout ($CrkL^{-/-}$), Crk floxed conditional knockout ($Crk^{f/f}$), CrkL floxed conditional knockout ($CrkL^{f/f}$) or double floxed Crk and CrkL conditional knockout ($Crk^{f/f}$;$CrkL^{f/f}$) cells were prepared as previously reported (Park et al., 2006). MEF derived cell lines were generated in the researchers laboratory using SV40 large T antigen. Briefly, early passage MEF cells seeded at low density were transfected with pSG5 Large T vector (Addgene) using FuGENE 6 transection reagent (Roche) according to the manufacturer's protocol. Transfected cells were cultured and passaged at least 8 times until cell lines were developed. Rat 208F cells were described previously (Miller et al., 1985). Cells were cultured in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% fetal bovine serum (HyClone), L-glutamine, penicillin, and streptomycin at 37°C under 5% CO_2. MEF cells were used at early passages. The Platinum-E

(Plat-E) cell line (Cell Biolabs, INC) was obtained from Dr. A. Resnick, the Children's Hospital of Philadelphia, and it was cultured according to the manufacturer's instructions.

The delivery of v-fos, h-ras-V12, k-ras-V12, src and src-527F was performed by retroviral infection using viruess produced with the following constructs: pFBJ/R (Miller et al., 1985), pBABE-puro-H-Ras-V12 (Addgene), pBABE-puro-K-Ras-V12 (Addgene), pLNCX-Src or pLNX-Src-527F (kindly obtained from Dr. Joan Burge from Harvard Medical School), respectively. Retroviral particles were generated by transient transfection of retroviral vectors into the Plat-E cell line. Briefly, 80% confluent cells were transfected using FuGENE 6 (Roche) transfection reagent according to the manufacturer's instructions. Virus particles were harvested 48 h later, filtered by 0.45 μm filter and stored at -80°C. Virus titration was performed using the rat fibroblast 208F cell line. The delivery of fyn or fynΔ525 was performed by transient transfection of pCMV5-fyn or pCMV5-fynΔ525 (kindly obtained from Dr. Marilyn D. Resh from Memorial Sloan-Kettering Cancer Center) respectively into MEF fibroblast cells or T antigen immortalized cell lines by FuGENE 6 (Roche) according to the manufacturer's protocol.

Cre delivery was performed by lentiviral infection using the construct pLVX-IRES-ZsGreen1. Lentiviral particles were generated using the Lenti-X HTX packaging system and Lenti-X 293T cell line (Clontech) according to the manufacturer's instructions. MEF cells were harvested, counted, and plated onto 60 mm dishes (BD) at a density of 800 cells/cm^2. The next day cells were infected with retroviral v-fos or h-ras-V12 particles in the presence of 8 μg/ml polybrene. A day after retroviral infection, Cre lentiviral particles were delivered in the presence of 8 μg/ml polybrene. On the following day the medium was replaced with fresh medium and cells were monitored for any changes in morphology.

Focus assay

MEF fibroblast cells or MEF derived cell lines were seeded in triplicate in 60 cm dish at the density of 800 cells/cm^2 or 200 cells/cm^2 respectively and maintained for 24 hrs. Designed samples were infected with the oncogene carrying viral particles. Six (for MEF derived cell lines) or ten (for MEF cells) days post infection cells were stained with methylene blue for 5 minutes (0.4% in 50% deionized water, 50% Methanol), washed three times with water and dried. Colonies were counted using an inverted microscope.

Soft agar colony forming assay

The soft-agar method of Macpherson and Montagnier (Macpherson and Montagnier, 1964) was used to culture transformed cells. Briefly, a 1.5-ml lower layer of 0.6% agar in DMEM/High glucose (GIBCO) containing 10% fetal calf serum (Hyclone), was placed in a 35 mm plastic Petri dish and permitted to solidify at room temperature. For colony formation assays, cells were suspended in a plating layer (0.5 ml) of 0.4% agar in DMEM/High glucose with 10% fetal calf serum. The agar was permitted to solidify at room temperature and was inspected for cell clumps. Cultures were set up in triplicates and incubated at 37C° in a humidified incubator in an atmosphere of 5% CO2 and 95% air. Cultures were examined with an inverted phase microscope (lens 4x). Final colony counts were made between 2 and 3 weeks after plating. Groups of 30 or more cells were considered as colonies.

Statistical analysis
Statistical analysis of data was carried out using unpaired two-tailed Student's t test for comparison between two experimental groups. Differences were considered to be significant when probability (p) values were <0.05.

Results

Expression of Fos or Ras induces cell transformation but not in the absence of Crk or CrkL
To investigate the cellular requirement of Crk or CrkL in Fos driven transformation the researchers used fibroblast (MEF) cells derived from WT or $Crk^{-/-}$ or $CrkL^{-/-}$ mice. The researchers infected the cells with the retroviral particles carrying the v-fos oncogene and monitored for morphological changes. Two weeks after infection WT cells presented an obvious transformation phenotype with noticeable changes in cell morphology and density. Cells that underwent transformation were smaller, they presented a "spindle like" shape, and they grew to higher densities. $CrkL^{-/-}$ cells did not present any obvious morphological changes characteristic of transformation (Figure 25A). Similarly, $Crk^{-/-}$ cells also did not present any evidence of morphological changes related to transformation (Figure 25B). Further analyses were performed using MEF derived T antigen immortalized cell lines. The researchers transformed the cell lines with v-fos. Six to eight days later it was noticed that WT cell lines presented an obvious transformation phenotype whereas $Crk^{-/-}$ or $CrkL^{-/-}$ knockout cell lines exhibited a much reduced or absent transformation phenotype (Figure 25C).

Next, the researchers used the Ras oncogene in the transformation assays. To evaluate the possible role of Crk or CrkL in ras transformation WT, $Crk^{-/-}$ or $CrkL^{-/-}$ cell lines were infected with h-ras-V12 and observed for morphological changes as in the previous experiments. Six days post infection it was observed that WT cells presented a transformation phenotype with higher density and more compact morphological shape. However, $Crk^{-/-}$ or $CrkL^{-/-}$ cells did not show any obvious morphological changes related to transformation (Figure 25D). Using the same cellular model, the researchers were not able to obtain a clear transformation phenotype induced by fyn-wt, fynΔ526 (constitutively active Fyn mutant), src-wt or src-527F mutant (constitutively active Src mutant) (data not shown).

To compare transformation potencies quantitatively, the researchers performed focus-formation assays. First WT, $Crk^{-/-}$ or $CrkL^{-/-}$ MEF fibroblast cells were infected with retrovirus carrying v-fos. Ten days post infection, dishes with cells were stained with methylene blue and the number of foci of transformed cells was counted. High levels of transformed foci were obtained in WT cells, however, only a a few transformed foci were detected in $Crk^{-/-}$ cells (Figure 26A) and significantly reduced numbers of foci were seen in $CrkL^{-/-}$ fibroblasts (Figure 26B). Similar experiments were performed using establish cell lines. Cells were infected with a v-fos retrovirus, and 6 days later dishes with cells were stained. High numbers of transformed foci were detected in the WT cell lines, whereas both $Crk^{-/-}$ and $CrkL^{-/-}$ cells presented significantly lower numbers of foci (Figure 26C). Taken together these results suggest that cell transformation driven by the Fos or Ras oncogenes requires Crk or CrkL.

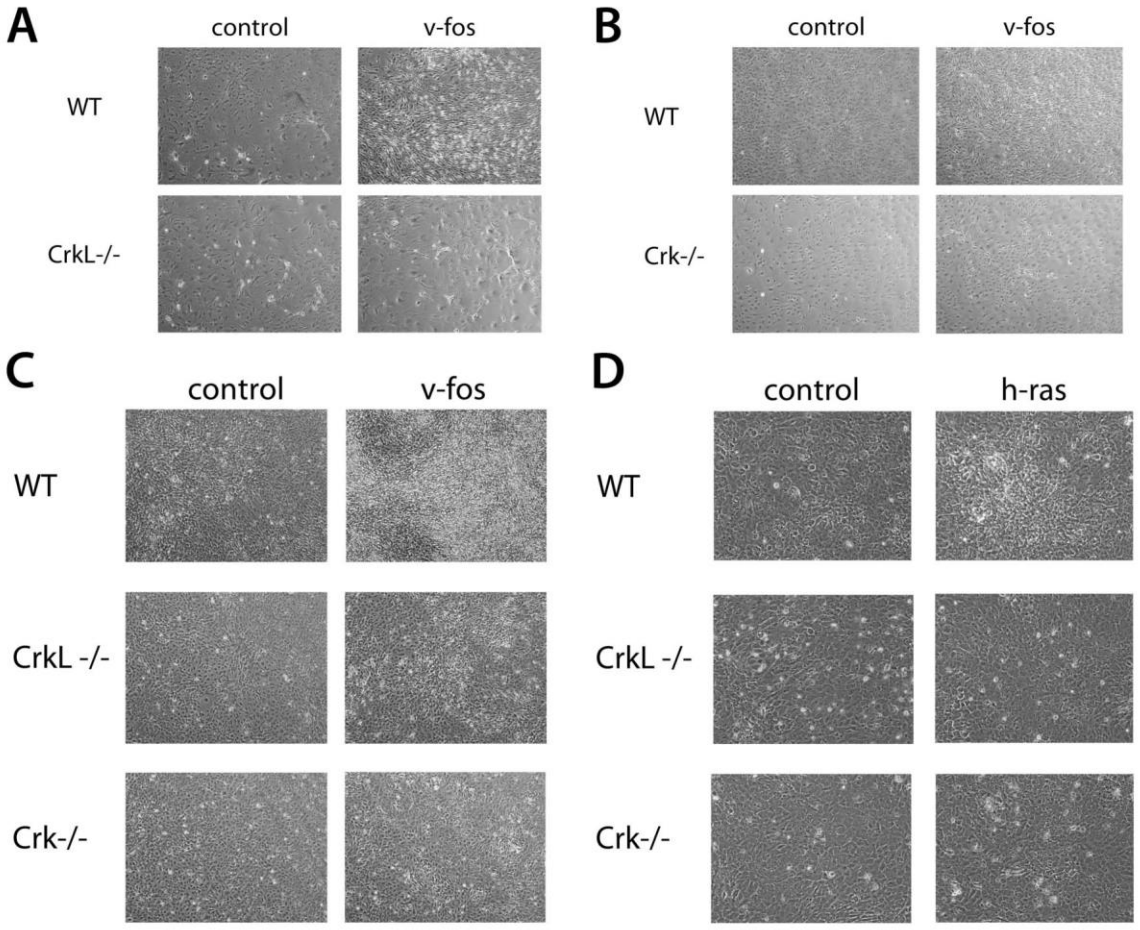
$Crk^{-/-}$ or $CrkL^{-/-}$ cells form less colonies in soft agar assay

Since oncogenes like Fos and Ras can induce anchorage independent colony formation, the researchers tested whether the colony forming abilities of Fos or Ras are affected by the loss of Crk or CrkL protein. Cells were infected with vectors expressing v-fos, h-ras-V12 or k-ras-V12. At 48 hours post infection cells were detached, plated in soft agar medium mixture and observed for colony formation. No colonies in soft agar were obtained using MEF fibroblast cells (data not shown). However, when cell lines were used, potent colony formation was observed in WT cells expressing Fos or Ras two weeks after infection. Interestingly, in each of the experiments, regardless of the oncogene, $Crk^{-/-}$ and $CrkL^{-/-}$ cells presented significantly reduced numbers of soft agar colonies (Figure 26D). These results indicate that the absence of Crk or CrkL reduces the ability of Fos and Ras to induce anchorage-independent growth.

Maintenance of transformation is disrupted by removal of Crk or CrkL

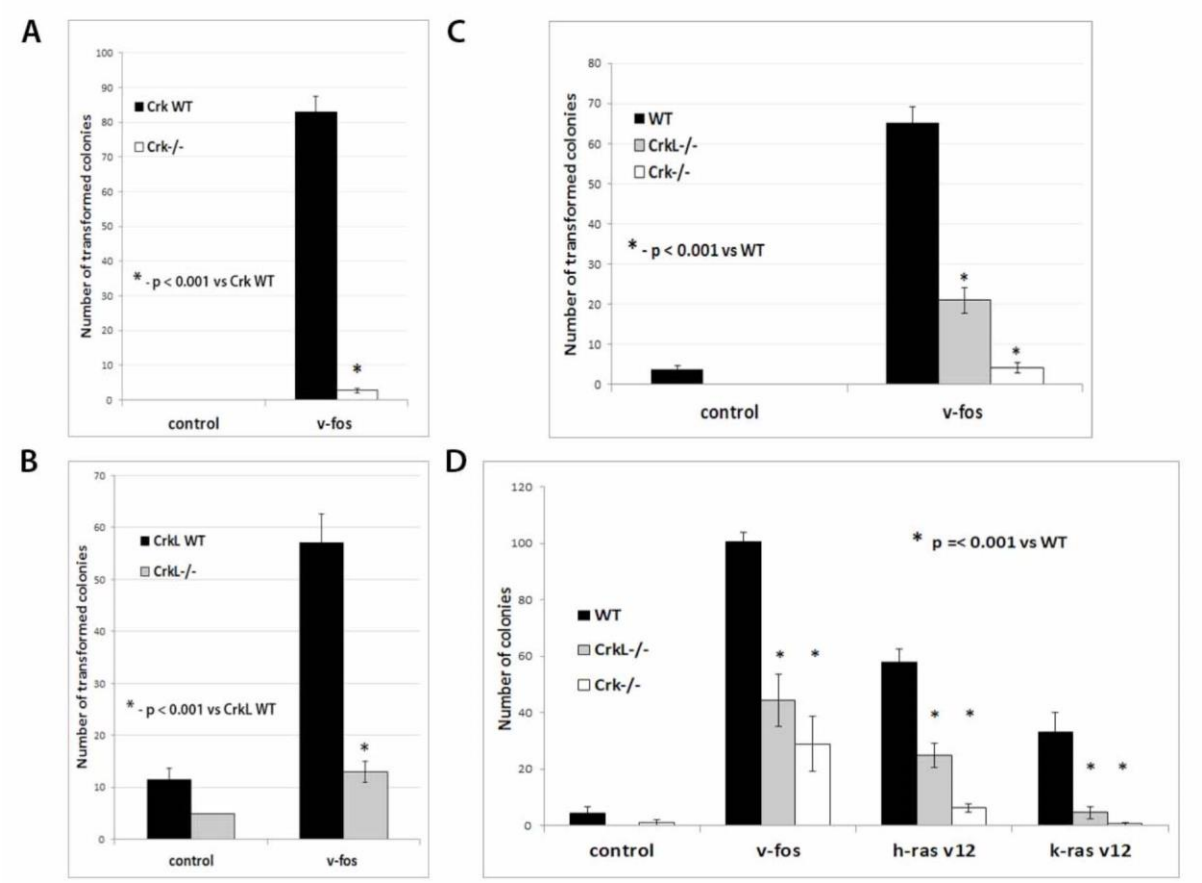
The previous experiment tested the role of Crk or CrkL in the transformation process from the point of initiation. The researchers also determined whether the established transformation phenotype can be disrupted by removal of Crk or/and CrkL. Fibroblasts derived from single floxed Crk ($Crk^{f/f}$) or CrkL ($CrkL^{f/f}$) mice or Crk/CrkL double floxed mice were used for the experiments. Fibroblast cells were cultured and infected with a retroviral vector expressing v-fos or h-ras-V12 to induce transformation and 24 h after initiation of transformation cells were infected with a lentiviral vector expressing Cre plus GFP and observed for morphological changes. Five days after the initial infection researchers observed morphological changes in WT cells transformed with v-fos or h-ras-V12 (Figure 27). At the same time it was found that removal of CrkL noticeably reduced the transformation phenotype of v-fos or h-ras-V12 while compared to WT cells. Moreover, independent removal of Crk also visibly diminished the transformation phenotype in cells undergoing v-fos or h-ras-V12 induced alterations. Removal of both Crk and CrkL at the same time from the cells undergoing transformation caused a reduction in the number of cells. However, this result can't be interpreted since removal of both Crk and CrkL from the WT cells alters the morphology of the cells, making them smaller, round and retractile. Taken together, the results indicate that Crk and CrkL play essential roles in maintaining the transformation phenotype.

Figure 25. Expression of Fos or Ras induces cell transformation but not in the absence of Crk or CrkL



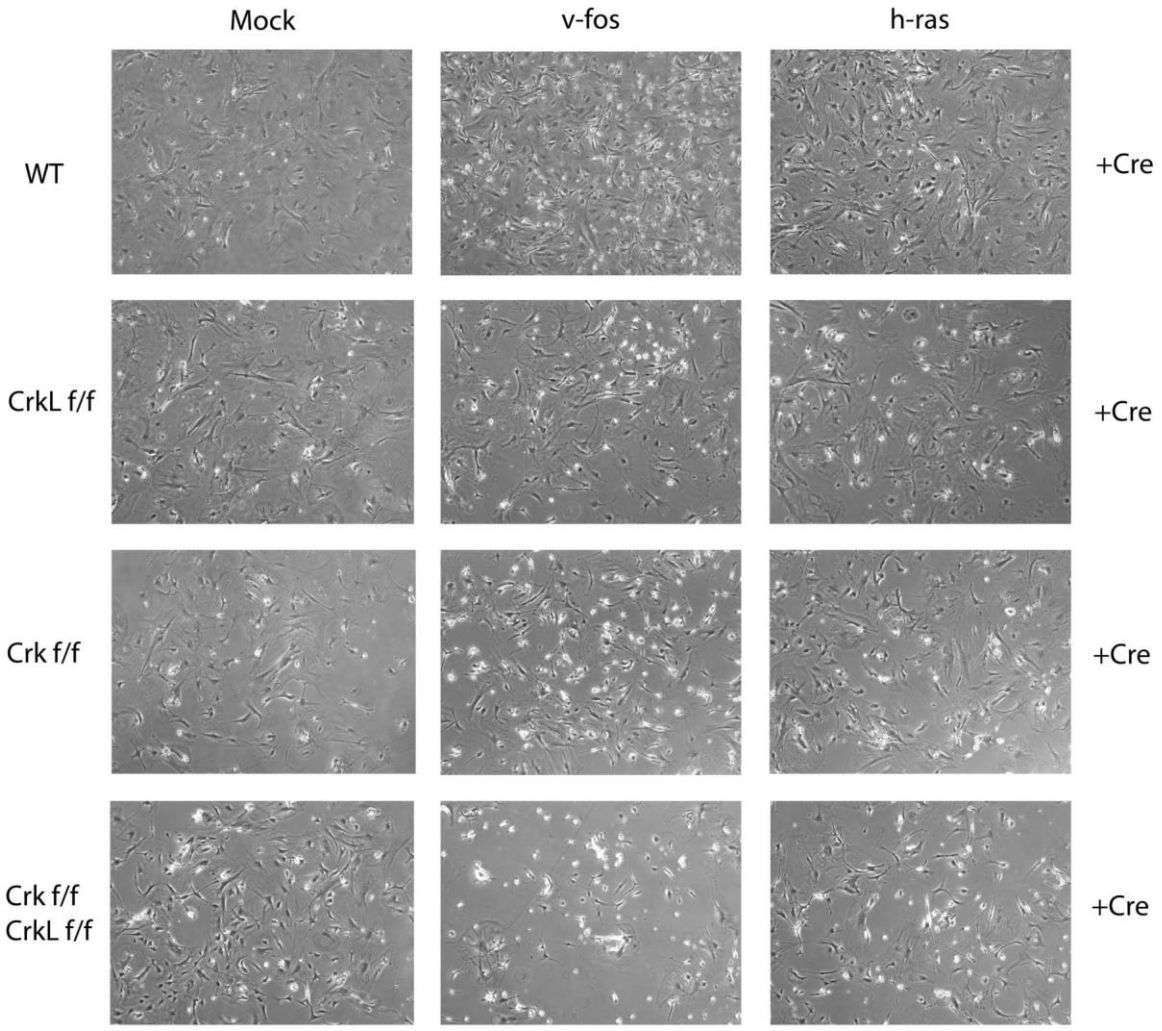
WT and CrkL$^{-/-}$ MEF cells (A), WT and Crk$^{-/-}$ MEF cells (B), or T antigen transformed WT, CrkL$^{-/-}$ or Crk$^{-/-}$ cell lines (C, D) were infected with a retroviral vector expressing v-fos (C) or h-ras-V12 (D). Phase-contrast images of cells were taken at 14 day post infection (A, B) or 6 days post infection (C, D).

Figure 26. Crk$^{-/-}$ or CrkL$^{-/-}$ cells form fewer colonies in focus assays and in soft agar assays



(A, B, C) MEF fibroblast cells (A, B) or cell lines (C) were plated and infected with a retroviral vector expressing v-fos. 10 days (A, B) or 6 days post infection (C) cells were stained with methylene blue and colonies were counted. (D) Cell lines were infected with a retroviral vector expressing v-fos, h-ras-V12 or k-ras-V12. At 48 h post infection, the soft agar colony forming assay was performed. Colonies were counted between two to three weeks post infection. Data are shown as the mean ± SEM (bars) values; p value was calculated by the student t-test.

Figure 27. Transformation is disrupted by sudden removal of Crk or CrkL



Wild-type (WT), Crk conditional knockout ($Crk^{f/f}$), and Crk/CrkL double floxed ($Crk^{f/f}$;$CrkL^{f/f}$) cells were infected with a retroviral vector expressing v-fos or h-ras-v12. At 24 h post infection samples were infected with a lentiviral vector expressing only Cre and GFP. Presented phase-contrast and fluorescence (not shown) images of cells were taken at 5 days post initial infection.

Discussion

Crk and CrkL are integral parts of a network of essential signaling pathways regulating diverse biological functions in cell growth, migration and adhesion. Such a complex role is due to internal SH2 and SH3 domains by which Crk and CrkL interact in a highly organized manner with proteins involved in many physiological pathways. In this report, Crk or CrkL single knockout cell lines as well as Cre/loxP recombination technology were used to investigate the possible role of Crk or/and CrkL in transformation. Using the oncogenes Fos and Ras, the researchers showed that, in the absence of Crk or CrkL, cells can't fully undergo transformation and they form significantly fewer colonies in soft agar compared with their WT counterparts. Several previous reports underscore the importance of Crk or CrkL expression in established and

fully transformed cancer cell lines (Miller et al., 2003; Rodrigues et al., 2005; Wang et al., 2007; Nishihara et al., 2002). The researchers extended these observations using conditional knockout of Crk or CrkL at 24 h post induction of transformation. The researchers discovered that removal of either Crk or CrkL disrupts the process of transformation. Since the removal of Crk or CrkL was performed at 24 h post induction of transformation, cells had already passed the initial stage of transformation. Therefore, not only presence of Crk or CrkL is essential for induction of transformation, but they are necessary for maintenance of the transformation phenotype. These findings are in agreement with previous reports showing decreased invasiveness and cell mobility in cancer cells after Crk or CrkL siRNA mediated downregulation invasiveness (Miller et al., 2003; Rodrigues et al., 2005; Wang et al., 2007; Nishihara et al., 2002). The researchers' findings suggest that Crk and CrkL are independently engaged in the transformation process, indicating that their roles may be overlapping. The results show that Crk or CrkL expression is necessary during the initiation and early stage of transformation.

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Published Abstracts, Posters, Presentations at Meetings

1) Park TJ and Curran T (2012) Essential roles of Crk and CrkL in cell structure and motility in fibroblast cells. Am. Soc. Cell Biol. 2012 Annual Meeting, Dec. 15-19, San Francisco, CA
2) Park TJ and Curran T (2012) Crk and CrkL Are Required to Maintain Cytoskeletal Integrity in Fibroblast Cells. CHOP Research Poster Day, Feb. 22, Philadelphia, PA
3) Park TJ and Curran T (2011) Essential and overlapping roles of Crk and CrkL in cytoskeletal organization in fibroblast cells. CHOP Research Poster Day, Feb. 23, Philadelphia, PA
4) Park TJ and Curran T (2010) Essential and overlapping roles of Crk and CrkL in cytoskeletal organization in fibroblast cells. Am. Soc. Cell Biol. 50th Annual Meeting, Dec. 11-15, Philadelphia, PA
5) Park TJ and Curran T (2010) Crk and CrkL Play Essential and Overlapping Roles in Cell Survival and Cytoskeletal Organization in Fibroblast Cells. CHOP Research Poster Day, Feb. 24, Philadelphia, PA

18. Extent of Clinical Activities Initiated and Completed. Items 18(A) and 18(B) should be completed for all research projects. If the project was restricted to secondary analysis of clinical data or data analysis of clinical research, then responses to 18(A) and 18(B) should be "No."

18(A) Did you initiate a study that involved the testing of treatment, prevention or diagnostic procedures on human subjects?

_____Yes
___√__No

18(B) Did you complete a study that involved the testing of treatment, prevention or diagnostic procedures on human subjects?

_____Yes
__√___No

If "Yes" to either 18(A) or 18(B), items 18(C) – (F) must also be completed. (Do NOT complete 18(C-F) if 18(A) and 18(B) are both "No.")

18(C) How many hospital and health care professionals were involved in the research project?

_____Number of hospital and health care professionals involved in the research project

18(D) How many subjects were included in the study compared to targeted goals?

_____Number of subjects originally targeted to be included in the study
_____Number of subjects enrolled in the study

Note: Studies that fall dramatically short on recruitment are encouraged to provide the details of their recruitment efforts in Item 17, Progress in Achieving Research Goals, Objectives and Aims. For example, the number of eligible subjects approached, the number that refused to participate and the reasons for refusal. Without this information it is difficult to discern whether eligibility criteria were too restrictive or the study simply did not appeal to subjects.

18(E) How many subjects were enrolled in the study by gender, ethnicity and race?

Gender:
_____Males
_____Females
_____Unknown

Ethnicity:
_____Latinos or Hispanics
_____Not Latinos or Hispanics

_____Unknown

Race:
_____American Indian or Alaska Native
_____Asian
_____Blacks or African American
_____Native Hawaiian or Other Pacific Islander
_____White
_____Other, specify:_____
_____Unknown

18(F) Where was the research study conducted? (List the county where the research study was conducted. If the treatment, prevention and diagnostic tests were offered in more than one county, list all of the counties where the research study was conducted.)

19. Human Embryonic Stem Cell Research. Item 19(A) should be completed for all research projects. If the research project involved human embryonic stem cells, items 19(B) and 19(C) must also be completed.

19(A) Did this project involve, in any capacity, human embryonic stem cells?
_____Yes
___√__ No

19(B) Were these stem cell lines NIH-approved lines that were derived outside of Pennsylvania?
_____Yes
_____ No

19(C) Please describe how this project involved human embryonic stem cells:

20. Articles Submitted to Peer-Reviewed Publications.

20(A) Identify all publications that resulted from the research performed during the funding period and that have been submitted to peer-reviewed publications. Do not list journal abstracts or presentations at professional meetings; abstract and meeting presentations should be listed at the end of item 17. **Include only those publications that acknowledge the Pennsylvania Department of Health as a funding source** (as required in the grant agreement). List the title of the journal article, the authors, the name of the peer-reviewed publication, the month and year when it was submitted, and the status of publication (submitted for publication, accepted for publication or published.). Submit an electronic copy of each publication or paper submitted for publication, listed in the table, in a PDF version 5.0.5 (or greater) format, 1,200 dpi. Filenames for each publication should include the number of the research project, the last name of the PI, the number of the publication and

an abbreviated research project title. For example, if you submit two publications for PI Smith for the "Cognition and MRI in Older Adults" research project (Project 1), and two publications for PI Zhang for the "Lung Cancer" research project (Project 3), the filenames should be:

Project 1 – Smith – Publication 1 – Cognition and MRI
Project 1 – Smith – Publication 2 – Cognition and MRI
Project 3 – Zhang – Publication 1 – Lung Cancer
Project 3 – Zhang – Publication 2 – Lung Cancer

If the publication is not available electronically, provide 5 paper copies of the publication.

Note: The grant agreement requires that recipients acknowledge the Pennsylvania Department of Health funding in all publications. Please ensure that all publications listed acknowledge the Department of Health funding. If a publication does not acknowledge the funding from the Commonwealth, do not list the publication.

Title of Journal Article:	Authors:	Name of Peer-reviewed Publication:	Month and Year Submitted:	Publication Status (check appropriate box below):
1. Dok-7 regulates neuromuscular synapse formation by recruiting Crk and Crk-L.	Hallock P., Xu C. F., Park T. J., Neubert T. A., Curran T., and Burden S. J.	Genes and Development	2010, 24(21), 2451-2461	☐Submitted ☐Accepted **X** Published
2. The adaptor protein CRK is a pro-apoptotic transducer of endoplasmic reticulum stress.	Austgen K., Johnson E. T., Park T. J., Curran T., and Oakes S. A.	Nature Cell Biology	2011, 14(1), 87-92	☐Submitted ☐Accepted **X** Published
3. Crk1/2-dependent signaling is necessary for podocyte foot process spreading in mouse models of glomerular disease.	George B, Verma R, Soofi A. A, Garg P., Zhang J., Park T. J., Giardino L., Ryzhova L., Johnstone D. B., Wong H., Nihalani D., Salant D. J., Hanks S. K., Curran T., Rastaldi M. P., and Holzman L. B.	The Journal of Clinical Investigation	2011, 122(2), 674-692	☐Submitted ☐Accepted **X** Published

4. The adaptor proteins Crk and CrkL are required for T cell migration.	Huang Y., Clarke F., Chen E., Park, T. J., Debes, G. F., Curran T., and Burkhardt J. K.	The Journal of Immunology	May2012	**X** Submitted ☐Accepted ☐Published
5. Essential roles of Crk and CrkL in fibroblast structure and motility.	Park T. J. and Curran T.	Oncogene	October 2012	**X** Submitted ☐Accepted ☐Published

20(B) Based on this project, are you planning to submit articles to peer-reviewed publications in the future?

Yes____√____ No_____

If yes, please describe your plans:

Based on the results obtained from Specific Aim 3, the researchers are writing a manuscript on the requirement of Crk and CrkL in cell transformation and will submit it to a peer-reviewed journal.

21. Changes in Outcome, Impact and Effectiveness Attributable to the Research Project. Describe the outcome, impact, and effectiveness of the research project by summarizing its impact on the incidence of disease, death from disease, stage of disease at time of diagnosis, or other relevant measures of outcome, impact or effectiveness of the research project. If there were no changes, insert "None"; do not use "Not applicable." Responses must be single-spaced below, and no smaller than 12-point type. DO NOT DELETE THESE INSTRUCTIONS. There is no limit to the length of your response.

None

22. Major Discoveries, New Drugs, and New Approaches for Prevention Diagnosis and Treatment. Describe major discoveries, new drugs, and new approaches for prevention, diagnosis and treatment that are attributable to the completed research project. If there were no major discoveries, drugs or approaches, insert "None"; do not use "Not applicable." Responses must be single-spaced below, and no smaller than 12-point type. DO NOT DELETE THESE INSTRUCTIONS. There is no limit to the length of your response.

The biggest discovery in this project is the demonstration that Crk and CrkL play essential overlapping roles in various signaling pathways. Their overlapping and distinct functions can be uncovered only by simultaneously knocking out both Crk and CrkL together and comparing the results with those obtained by knocking out either Crk or CrkL alone. The

mice and cells generated by the researchers are unique and valuable resources for this approach.

In addition, this project helped discover several key functions of Crk and CrkL as summarized below:

1) Requirement of Crk and CrkL in tissue development

In the absence of both Crk and CrkL, neuromuscular synapse formation was defective, which is reminesent of defective brain development in the absence of both Crk and CrkL. Although the upstream signaling molecules are different between Reelin/Dab1-dependent neuronal migration and MuSK/Dok-7-dependent neuromuscular synapse formation, Crk and CrkL are common signaling molecules that link specific molecules in the upstream to generic molecules in the downstream in both pathways. It will be very interesting to investigate whether such a mechanism is shared by other signaling pathways that utilize Crk and CrkL.

2) Requirement of Crk and CrkL in cell motility

This project discovered that Crk and CrkL are required for cell migration for fibroblast and T cells. Further studies are required to see whether Crk and CrkL also play key roles in motility of other types of cells.

3) Requirement of Crk and CrkL in stress-induced cell death

Another striking discovery from this project is that Crk is required for stress-induced cell death in vitro and in vivo as described in Specific Aim 2-2 and 2-3. Although it is unclear whether ER stress-induced cell death triggered by the N-terminal fragment of Crk is responsible for the podocyte foot processing induced by protamine sulfate or nephrotoxic serum, the results provide a novel insight to the mechanism for stress-induced cell death. It is possible that those stress signals may act as ligands and stimulate certain membrane or cytoplasmic proteins, leading to activation of a very specific cell death pathway which requires Crk. It will be important to examine whether CrkL plays similar roles under such conditions. Further defining the roles of Crk and CrkL in stress-induced cell death can help better understand many neurodegenerative diseases such as Parkinson's disease and Alzheimer's disease that are known to be associated with ER stress.

4) Requirement of Crk and CrkL in cell transformation

Overexpression of Crk and CrkL are observed in several types of human cancer. This observation may have an interesting association with the discovery in Specific Aim 3 that Crk and CrkL are required for cell transformation. Further studies will be required to determine whether Crk and CrkL play critical roles in cell transformation and tumor cell proliferation in vivo before the researchers pursue Crk and CrkL as targets for cancer therapy.

23. Inventions, Patents and Commercial Development Opportunities.

23(A) Were any inventions, which may be patentable or otherwise protectable under Title 35 of the United States Code, conceived or first actually reduced to practice in the performance of work under this health research grant? Yes_____ No___√_____

If "Yes" to 23(A), complete items a – g below for each invention. (Do NOT complete items a - g if 23(A) is "No.")

a. Title of Invention:

b. Name of Inventor(s):

c. Technical Description of Invention (describe nature, purpose, operation and physical, chemical, biological or electrical characteristics of the invention):

d. Was a patent filed for the invention conceived or first actually reduced to practice in the performance of work under this health research grant?
Yes_____ No____

If yes, indicate date patent was filed:

e. Was a patent issued for the invention conceived or first actually reduced to practice in the performance of work under this health research grant?
Yes_____ No____
If yes, indicate number of patent, title and date issued:
Patent number:
Title of patent:
Date issued:

f. Were any licenses granted for the patent obtained as a result of work performed under this health research grant? Yes_____ No____

If yes, how many licenses were granted?_____

g. Were any commercial development activities taken to develop the invention into a commercial product or service for manufacture or sale? Yes___ No____

If yes, describe the commercial development activities:

23(B) Based on the results of this project, are you planning to file for any licenses or patents, or undertake any commercial development opportunities in the future?

Yes_____ No_____√____

If yes, please describe your plans:

24. Key Investigator Qualifications. Briefly describe the education, research interests and experience and professional commitments of the Principal Investigator and all other key investigators. In place of narrative you may insert the NIH biosketch form here; however, please limit each biosketch to 1-2 pages. *For Nonformula grants only – include information for only those key investigators whose biosketches were not included in the original grant application.*

Biosketch forms for Tom Curran (PI), Tae-Ju Park (Research Associate), and Mateusz Koptyra (Postdoctoral Fellow) are included on the following pages.

BIOGRAPHICAL SKETCH

Provide the following information for the Senior/key personnel and other significant contributors in the order listed on Form Page 2.
Follow this format for each person. **DO NOT EXCEED FOUR PAGES.**

NAME	POSITION TITLE
CURRAN, Tom	Deputy Scientific Director, CHOP Research Institute Professor of Pathology and Laboratory Medicine
eRA COMMONS USER NAME (credential, e.g., agency login) TCURRAN	

EDUCATION/TRAINING *(Begin with baccalaureate or other initial professional education, such as nursing, include postdoctoral training and residency training if applicable.)*

INSTITUTION AND LOCATION	DEGREE *(if applicable)*	MM/YY	FIELD OF STUDY
University of Edinburgh, Edinburgh, Scotland	B.Sc.(Hons.)	1978	Biological Sciences
Imperial Cancer Research Fund Laboratories and University College, London, United Kingdom	Ph.D.	1982	Zoology and Anatomy
Salk Institute, San Diego	Postdoctoral	1982-84	Molecular Oncology

Positions and Employment

1984-1995 Sen. Scientist, Asst. Member, Assoc. Member, Full Member, Assoc. Dir., Roche Inst. of Molecular Biology, Nutley, NJ
1989-1995 Adjunct Professor, Columbia University, New York, NY
1995-2006 Affiliated Professor, The University of Tennessee, College of Medicine, Memphis, TN
1995-2006 Member and Founding Chairman, Dept. of Developmental Neurobiology, St. Jude Children's Research Hospital, Memphis, TN
2006-present Professor, Pathology and Laboratory Medicine, University of Pennsylvania School of Medicine, Philadelphia, PA
2006-present Deputy Scientific Director, Joseph Stokes Jr. Research Center, Children's Hospital of Philadelphia, Philadelphia, PA
2006-present Associate Director, Translational Genomics Penn Genome Frontiers Institute (PGFI), University of Pennsylvania School of Medicine
2008-present Professor of Cell and Developmental Biology, University of Pennsylvania School of Medicine, Philadelphia, PA

Other Experience and Professional Memberships

1991-1994 Member, NIH Study Section Cellular Biology and Physiology 2
1992-1994 Member, Scientific Review Board Hoffmann La Roche Inc
1996-2000 Member, NCI Initial Review Group Committee Subcommittee C
2000-2001 President, American Association for Cancer Research
2000-2005 National Cancer Institute Board of Scientific Advisors

Selected Honors

1992, Passano Foundation Young Scientist Award, Rita Levi Montalcini Award in Neurosciences, Tenovus-Scotland Medal, Glasgow University, Scotland: 1993,American Association for Cancer Research, for Outstanding Achievement in Cancer Research Award: 1994, Golgi Award, Italian Academy of Neuroscience and the Camillo Golgi Foundation,

Brescia, Italy, Fellow of the American Association for the Advancement of Science, Fellow of the American Society of Microbiology: 2000 Highly Cited Scientist by Institute for Scientific Information (ISI) in three categories; Neuroscience, Molecular Biology & Genetics, and Microbiology: 2001, Javitz Neuroscience Investigator Award, National Institute of Neurological Disorders and Stroke, NIH: 2002, Peter M. Steck Memorial Award for Brain Tumor research, Houston, Texas: 2004 LIMA International Award for Excellence in Pediatric Brain Tumor Research, Pediatric Brain Tumor FD, NY, NY: 2005, Elected as a Fellow of The Royal Society: 2009, Elected as a Member of the Institute of Medicine of The National Academies (IOM), Washington, DC, Elected to the Academy of Arts and Sciences 2012.

C. Selected Peer-reviewed Publications (from a total of 270, H-index 100)

Romer JT, Kimura H, Magdaleno SM, Sasai K, Fuller C, Baines H, Connelly M, Stewart CF, Gould S, Rubin LL, Curran T. Suppression of the Shh pathway using a small molecule inhibitor eliminates medulloblastoma in $Ptc1^{+/-}p53^{-/-}$ mice. Cancer Cell 3:229-40, 2004.

Park TJ., Boyd K., Curran T.: Cardiovascular and craniofacial defects in Crk-null mice. Molecular & Cellular Biology 26(16): 6272-82, Aug 2006.

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BIOGRAPHICAL SKETCH

Provide the following information for the key personnel and other significant contributors in the order listed on Form Page 2.
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NAME	POSITION TITLE
PARK, TAE-JU	Research Associate
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EDUCATION/TRAINING *(Begin with baccalaureate or other initial professional education, such as nursing, and include postdoctoral training.)*

INSTITUTION AND LOCATION	DEGREE *(if applicable)*	YEAR(s)	FIELD OF STUDY
Pohang University of Science & Technology/Pohang, Korea	Ph.D.	1995-1999	Neurophysiology
Pohang University of Science & Technology/Pohang, Korea	M.S.	1993-1995	Neurophysiology
Konkuk University/Choongju, Korea	B.S.	1989-1992	Biochemistry

Positions and Employment

1999-2002	PostDoc Fellow	Pohang University of Science and Technology, Korea
2000-2002	PostDoc Fellow	RIKEN Brain Science Institute, Japan
2002-2006	PostDoc Fellow	St. Jude Children's Research Hospital, Memphis, USA
2006-present	Research Associate	Children's Hospital of Philadelphia, Philadelphia, USA

Honors

2008 Alavi-Dabiri postdoctoral fellowship award from Mental Retardation and Developmental Disabilities Research Center, Children's Hospital of Philadelphia
1999 Award of Excellent Thesis from the Korean Society of Molecular Biology
1995 Research Funding for Young Scientist from the Korea Research Foundation

Selected Publications

1) George B, Verma R, Soofi A. A, Garg P., Zhang J., Park T. J., Giardino L., Ryzhova L., Johnstone D. B., Wong H., Nihalani D., Salant D. J., Hanks S. K., Curran T., Rastaldi M. P., and Holzman L. B. (2012) Crk1/2-dependent signaling is necessary for podocyte foot process spreading in mouse models of glomerular disease. *J. Clin. Invest.* 122(2), 674-692.
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3) Hallock P., Xu C. F., Park T. J., Neubert T. A., Curran T., and Burden S. J. (2010) Dok-7 regulates neuromuscular synapse formation by recruiting Crk and Crk-L. Genes Dev. 24(21), 2451-2461.
4) Park T. J. and Curran T. (2008) Crk and CrkL play essential overlapping roles downstream of Dab1 in the Reelin pathway. *J. Neurosci.* 28(50), 13551-13562.
5) Park T. J., Boyd K., and Curran T. (2006) Cardiovascular and craniofacial defects in *Crk*-null mice. *Mol. Cell Biol.* 26(16), 6272-6282.

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7) Park T. J. and Kim K. T. (2003) Activation of B2 bradykinin receptors by neurotensin. *Cell Signal.* 15(5), 519-527.
8) Park T. J., Park Y. S., Lee T. G., Ha H., and Kim K. T. (2003) Inhibition of acetylcholine-mediated effects by borneol. *Biochem. Pharmacol.* 65(1), 83-90.
9) Park T. J., Bae S. I., Choi S. Y., Kang B. J., and Kim K. T. (2001) Inhibition of nicotinic acetylcholine receptors and calcium channels by clozapine in bovine adrenal chromaffin cells. *Biochemical. Pharmacol.* 61(8), 1011-1019.
10) Park T. J., Seo H. K., Kang B. J., and Kim K. T. (2001) Noncompetitive inhibition by camphor of nicotinic acetylcholine receptors. *Biochemical. Pharmacol.* 61(7), 787-793.
11) Park T. J., Lee I. S., Ha H., and Kim K. T. (1999) Temperature sensitivity of catecholamine secretion and ion fluxes in bovine adrenal chromaffin cells. *Mol. Cells* 9, 67-71.
12) Park T. J., Chung S., Han M. K., Kim U. H., and Kim K. T. (1998) Inhibition of voltage-sensitive calcium channels by the A_{2A} adenosine receptor in PC12 cells. *J. Neurochem.* 71, 1251-1260.
13) Park T. J., Shin S. Y., Suh B. C., Suh E. K., Lee I. S., Kim Y. S., and Kim K. T. (1998) Differential inhibition of catecholamine secretion by amitriptyline through blockage of nicotinic receptors, sodium channels, and calcium channels in bovine adrenal chromaffin cells. *Synapse* 29(3), 248-25.
14) Park T. J., Song S. K., and Kim K. T. (1997) A_{2A} adenosine receptors inhibit ATP-induced Ca^{2+} influx in PC12 cells by involving protein kinase A. *J. Neurochem.* 68, 2177-2185.
15) Park T. J. and Kim K. T. (1996) Cyclic AMP-independent inhibition of voltage-sensitive calcium channels by forskolin in PC12 cells. *J. Neurochem.* 66, 83-88.

BIOGRAPHICAL SKETCH

NAME	POSITION TITLE
KOPTYRA, MATEUSZ PAWEL	Postdoctoral Research Fellow

EDUCATION/TRAINING *(Begin with baccalaureate or other initial professional education, such as nursing, and include postdoctoral training.)*

INSTITUTION AND LOCATION	DEGREE *(if applicable)*	YEAR(s)	FIELD OF STUDY
Warsaw University, Poland	M.S.	2001	Molecular Biology
The Medical University, Warsaw, Poland	Ph.D.	2008	Medical Sciences - Molecular Carcinogenesis
Thomas Jefferson University, Kimmel Cancer Center, Department of Cancer Biology, Philadelphia, PA,USA	Postdoctoral training	2009 - present	Cancer Biology – Signaling Transduction
Children's Hospital of Philadelphia Research Institute, Department of Pathology and Laboratory Medicine, Philadelphia, PA	Postdoctoral training	2011 - present	Cancer Biology, Genomics

Professional Experience:

01/2001-08/2001 Research Assistant, Department of Molecular Biology, Oncology Center - Institute, Warsaw, Poland.

08/2001-06/2005 Research Fellow, Center for Biotechnology, College of Science and Technology, Temple University, Philadelphia, PA.

07/2005-07/2009 Research Assistant, Department of Microbiology and Immunology, School of Medicine, Temple University, Philadelphia, PA.

08/2009-08/2011 Postdoctoral Research Fellow, Thomas Jefferson University, Kimmel Cancer Center Department of Cancer Biology, Philadelphia, PA.

09/2011-present Postdoctoral Research Fellow, Children's Hospital of Philadelphia Research Institute, Department of Pathology and Laboratory Medicine, Philadelphia, PA.

Awards and Fellowships:

2005 Award form the Minister of Health (Poland) for: "Mechanisms of proteasome inhibitor antitumor effect and grounds for genomic instability in cells transformed by oncogene - BCR/ABL". Criteria: Project merit, Scientific and Educational Achievements; The Medical University of Warsaw, Poland 2005.

2004 Travel Award from the American Society of Hematology (ASH), ASH annual meeting, San Diego, CA.

2001 Third place award at II Conference of Polish Science Society of Oncology "Diagnostic and Treatment of Breast Cancer" for poster, "Generation of RT-PCR based two marker test to detect breast cancer cells in peripheral blood" Warsaw, Poland, April 2001.

Publications:

1. Nieborowska-Skorska M, Kopinski PK, Ray R, Hoser G, Ngaba D, Flis S, Cramer K, Reddy MM, **Koptyra M**, Penserga T, Glodkowska-Mrowka E, Bolton E, Holyoake TL,

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3. **Koptyra** M, Stoklosa T, Hoser G, Glodkowska-Mrowka E, Seferynska I, Klejman A, Blasiak J, Skorski T.Monoubiquitinated Fanconi anemia D2 (FANCD2-Ub) is required for BCR-ABL1 kinase-induced leukemogenesis. Leukemia. 2011 Aug;25(8):1259-67
4. Cramer K, Nieborowska-Skorska M, **Koptyra M**, Slupianek A, Penserga ET, Eaves CJ, Aulitzky W, Skorski T., BCR/ABL and Other Kinases from Chronic Myeloproliferative Disorders Stimulate Single-Strand Annealing, an Unfaithful DNA Double-Strand Break Repair. *Cancer Res*, 68: 6884-8, 2008.
5. **Koptyra, M.**, Cramer, K., Slupianek, A., Richardson, C., Skorski, T. BCR/ABL promotes accumulation of chromosomal aberrations induced by oxidative and genotoxic stress. *Leukemia*, 22: 1969-72, 2008.
6. Stoklosa, T., Poplawski, T., **Koptyra, M.**, Nieborowska-Skorska, M., Basak, G., Slupianek, A., Rayevskaya, M., Seferynska, I., Herrera, L., Blasiak, J., Skorski, T. BCR/ABL kinase inhibits mismatch repair to protect from apoptosis and induce point mutations resulting in genomic instability. *Cancer Res*., 68: 2576-2580, 2008.
7. **Koptyra, M.,** Falinski, R., Nowicki, M.O., Stoklosa, T., Majsterek, I., Nieborowska-Skorska, M., Blasiak, J., Skorski, T. BCR/ABL kinase induces self-mutagenesis via reactive oxygen species to encode imatinib resistance. *Blood*, 108: 319-327, 2006.
8. Trojanek J, Ho T, Croul S, Wang JY, Chintapalli J, **Koptyra M**, Giordano A, Khalili K, Reiss K, "IRS-1-Rad51 nuclear interaction sensitizes JCV T-antigen positive medulloblastoma cells to genotoxic treatment", *Int J Cancer*, 119: 539-48, 2006.
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13. Stoklosa T, Slupianek A, Datta M, Nieborowska-Skorska M, Nowicki MO, **Koptyra M**, Skorski T. BCR/ABL recruits p53 tumor suppressor protein to induce drug resistance. *Cell Cycle*, 11: 1463-72, 2004.