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Signaling adaptor protein Crk is indispensable for malignant feature of glioblastoma cell line KMG4

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Running title: Role of Crk in glioblastoma cells

Abstract

Signaling adaptor protein Crk has been shown to be involved in pathogenesis of human cancers including brain tumor where Crk was reported to be overexpressed. In this study, we addressed whether Crk is indispensable for malignant phenotype of brain tumor. In 20 surgical specimens of glioma, mRNA of both CrkI and CrkII were found to be elevated in malignant tumor. To define a precise role of Crk, knockdown cell lines for Crk were established by using glioblastoma KMG4 by siRNA, and early phase of cell adhesion to laminin was found to be suppressed. Activation of Rap by FBS was suppressed in Crk knockdown cells. Wound healing assay revealed the decreased cell motility and suppression of both anchorage-dependent and -independent growth were demonstrated in Crk knockdown cells. Furthermore, *in vivo* tumor forming potential was also markedly suppressed. These results suggest that Crk is required for early attachment to laminin, cell motility, and growth of glioblastoma cell line KMG4.

Introduction

Crk belongs to an adaptor family of proteins composed of SH2 and SH3 domains [1]. CrkII consists of an SH2 domain and two SH3 domains (SH2-nSH3-cSH3), and alternative splicing variant composed of an SH2 and an SH3 domains (SH2-SH3) was referred as CrkI [2]. Crk transmits signals from tyrosine-phosphorylated proteins including the components of focal adhesion, growth factor receptors, and signalling scaffold proteins, by binding to them via SH2 domain [1]. Crk associates with GEFs (guanine-nucleotides exchange factors) such as C3G and Dock180 those activate Rap1/R-Ras and Rac, respectively, and controls cell adhesion, proliferation, and motility [3, 4]. Crk has also been reported to regulate Fak tyrosine kinase and PI-3 kinase [5, 6]. Crk is known to be overexpressed in various human cancers [7], and recently, Crk was shown to play an essential role for malignant potentials of human ovarian cancer cells, synovial sarcoma cells, and breast cancer cells [8-10].

Brain tumor, especially malignant glioma, is one of the most aggressive tumors, and in spite of the recent advancement of therapeutic reagents [11], no curative therapy has not yet been established. In WHO classification, gliomas are classified to Grade I to IV, in which Grade I and II are considered as benign, and Grade III tumor and Grade IV as glioblastoma (GB) are malignant [12]. Genetically, EGFR amplification, p16^{INK4A} deletion, and PTEN mutation are shown to be associated with primary glioblastoma which primarily arise as Grade IV tumor, whereas p53 mutation is known to be found in secondary glioblastoma in

which tumor arise initially as benign and recurrence make it being aggressive [13, 14]. In glioma cells, Ras/Erk pathway and PI-3 kinase/Akt pathway are known to contribute to cell proliferation and survival [13].

In addition, characteristic features of malignant glioma are its elevated cell motility and invasion which are currently known to be regulated by various molecules such as extracellular matrix, integrins, matrix metalloproteinases, growth factor receptors, focal adhesion kinases, PI-3 kinase, small GTPase, and transcription factors [15-17].

As Crk is known to localize to focal adhesion, activate PI-3 kinase, interact to growth factor receptors leading to regulation of cell motility, we examined whether Crk is overexpressed in malignant human gliomas and is essential for its malignant potential.

Materials and Methods

Clinical samples and Reverse transcriptase (RT)-PCR Brain tumor specimens were obtained from patients at the Kashiwaba Neurosurgical Hospital (Sapporo, Japan) and the Iwamizawa Municipal General Hospital (Iwamizawa, Japan) under the informed consent and pathological diagnosis was established in our laboratory. Total RNA was isolated with the TRI Reagent (Sigma, St. Louis, MO, USA) and reverse transcribed into cDNA using the oligo-dT primer (Invitrogen, Carlsbad, CA, USA) and the Superscript II (Invitrogen). The levels of *Crkl* and *CrklI* were analyzed by PCR with the *KOD plus* DNA polymerase (Toyobo, Tokyo, Japan). Primers used in this experiment included: 5'- GCA GTG GTG GAA TGC GGA G-3', and 5'-CTG TTG AAC TAT ACT CAG CTG AAG T-3' for human *Crkl* and *CrklI* (The sizes of PCR products were 260 bp for *Crkl* and 429 bp for *CrklI*); 5'-CTC ATG ACC ACA GTC CAT GC-3' and 5'-TTA CTC CTT GGA GGC CAT GT-3' for human *Glyceraldehydes-3-phosphate dehydrogenase (GAPDH)*.

Cell culture The glioblastoma cell line, KMG4 was kindly provided by Dr. Kazuo Tabuchi (Saga University, Japan) [18]. U87MG, U251MG, and T98G cells were obtained from the American Type Culture Collection (Manassas, VA, USA). All cells used were maintained in Dulbecco's modified eagle medium (Seikagaku Co., Tokyo, Japan), supplemented with 10% fetal calf serum. For establishment of Crk knockdown glioma cells, the pSUPER-vector [19] expressing small interference RNA for human Crk [20] was transfected into the KMG4 cells

by means of Fugene 6 (Roche, Indianapolis, IN, USA). Cells were cultured in the presence of 500 ng/ml puromycin (Sigma) and drug-resistant clones were isolated.

Assays for cell adhesion and wound healing Cells were cultured on 96-well plates coated with laminin, collagen, hyaluronic acid, or fibronectin, and adhesion assay was performed as described previously[8]. Cells cultured on cover glasses coated with laminin, collagen, or fibronectin were also subjected to actin cytoskeleton analysis (phalloidin staining) as described previously[9]. The wound healing assay was performed as described previously [21].

Soft-agar colony formation assay and xenograft propagation Soft-agar colony formation assay and xenograft propagation were carried out as described[22, 23].. All animal procedures were performed according to the protocol approved by the institutional Animal Care and Use Committee at Hokkaido University Graduate School of Medicine.

Histological analysis and immunohistochemistry Formalin-fixed paraffin-embedded tissues, including human glioma specimens and the KMG4-derived xenografts, were sectioned and stained with haematoxylin and eosin (H&E) using standard protocol. Immunohistochemistry was performed using anti-Crk (Transduction Laboratories, Lexington, KY, USA) and anti-Ki67 (MIB1; Dako, Glostrup, Denmark) antibodies.

Immunoprecipitation and immunoblotting Protein determination, immunoprecipitation, SDS-PAGE and immunoblotting were carried out as described previously[9]. Antibodies were

obtained from the following sources: anti-phospho-tyrosine (PY20 and Rc20H), anti-p130^{Cas}, anti-Paxillin, anti-Crk and anti-Rap1 antibodies (Transduction Laboratories, Lexington, KY, USA); anti-C3G (C19), anti-DOCK180 (H4) and anti-Crk-L (C20) antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA); an anti-Actin antibody (Chemicon International, Temecula, CA, USA); an anti-Flag (M2) antibody (Sigma).

Results

Increased expression levels of Crk mRNA in malignant gliomas.

In an investigation of Crk functions in human glioma, the levels of *CrkI* and *CrkII* mRNA were analyzed by semi-quantitative RT-PCR using 4 benign (Grade I, n=1; Grade II, n=3) and 16 malignant (Grade III, n=4; Grade IV, n=12) gliomas. In benign gliomas, no increase of either *CrkI* or *CrkII* mRNA was observed compared to those in normal brain tissue (Fig. 1A, lanes 2-5). However, in malignant tumors, 5 cases showed an increase of *CrkI* (Fig. 1A, lanes 7, 8, 12, 15, and 18), and 3 cases showed an increase of both *CrkI* and *CrkII* (Fig. 1A, lanes 19, 20, and 21). The overexpression of Crk in glioblastoma was confirmed by immunostaining (Fig. 1B). In 5 cases of malignant gliomas, *Crk* mRNA was hardly detectable (Fig. 1A, lanes 6, 9, 11, 14, and 16).

Establishment of Crk knockdown KMG4 glioblastoma cell lines by siRNA.

To investigate the roles of Crk in malignant gliomas, we employed glioblastoma-derived cell lines, including U251MG, U87MG, KMG4, and T98G cells, and protein levels of Crk and its related molecules including Crk-like (CrkL), C3G, Dock180, paxillin, and p130^{Cas} were analyzed. No significant difference of the protein levels of Crk, CrkL, Dock180, and paxillin were detected, but levels of C3G and p130^{Cas} were increased in T98G cell lines (Fig. 2A). The tyrosine-phosphorylation levels of p130^{Cas} was detected in all cell lines, but the amount of the p130^{Cas} which bound to Crk is almost equal in U251,

T98G, and KMG4 cells and that is lower in U87 cells (Fig.2A and 2B).

As much higher amount of the tyrosine-phosphorylation level of p130^{Cas}, which is closely related to the generation of positive signal for cell growth and motility[24], was observed in KMG4 cells (Fig. 2B), we employ the KMG4 cells for further analyses. Thus, we generated the Crk-knockdown KMG4 cells using siRNA for human *Crk*. As shown in Fig. 2C, both CrkI and CrkII were effectively depleted in three clones, Crki5, Crki17, and Crki21 (Fig. 2C).

Analysis of cell adhesion and motility in Crk knockdown KM-G4 cells.

The Crk-knockdown KMG4 cells were assessed for their ability of spreading and adhesion. Crk-knockdown cells displayed drastically delayed cell spreading on laminin compared with mock-cells. Most of mock-cells spread on laminin-coated plates with polarized extension within 20 min (data not shown), and the actin stress fiber formation was clearly observed (Fig. 3A). In contrast, the Crk-knockdown cells were still round on laminin-coated plates, and only a portion of the cells spread even at 20 min (data not shown). Furthermore, in all Crk-knockdown cells, formation of actin stress fiber was disrupted on laminin-coated plates (Fig. 3A). It should be noted that the majority of Crk-knockdown cells had eventually spread by 30 min (data not shown). The Crk-knockdown KMG4 cells also showed reduced adhesion to laminin at an early time point (Fig. 3B). However, the

knockdown and control cells could not longer be distinguished from one another after 40 min (Fig. 3B), suggesting that cell adhesion was delayed but not completely blocked. The disruption in the ability of spreading and adhesion, observed in the Crk-knockdown KMG4 cells, was specific to laminin, as adhesion to other extracellular matrices, including collagen, hyaluronic acid, and fibronectin, seemed to be normal (Fig. 3C), and as the Crk-knockdown cells clearly formed actin stress fiber on collagen- or fibronectin-coated plate even at early time points (Fig. 3A).

We analyzed the activity of Rap which regulates adhesion. As there was technically limitation to measure Rap activity in re-plated cells, Rap activity was evaluated in cells with or without serum stimulation, and we found that serum-induced fold activation of Rap were lower in Crk knockdown cells compared to mock (Supplementary Figure 1).

Analysis of cell motility and growth of Crk knockdown KMG4 cells.

In addition to the disruption in the ability of cell adhesion, Crk-knockdown resulted in decreased motility of glioblastoma cells, where the recovery of wound was significantly delayed in the Crk-knockdown KMG4 cells (Fig. 4A). Furthermore, Crk-knockdown also resulted in reduced growth properties of the KMG4 cells in the adherent culture (Fig. 4B), soft-agar colony formation assay (Fig. 4C), and xenograft propagation experiment (Fig. 4D). The Crk-knockdown-xenograft (Crki17-xenograft) demonstrated lower cell density as well as lower MIB1 index (Ki67 staining) compared with mock-xenograft by histopathological

analyses (Fig. 4E). These observations clearly indicate that Crk mediates malignant features of glioblastoma cells *in vivo* and *in vitro*.

Discussion

In this study, we showed that Crk is essential for enhanced growth and invasion of glioblastoma cell line KMG4, especially for early cell attachment to laminin. Laminin is heterotrimer protein containing α , β , and γ chains, and formerly, lamin 1 to 15 were identified but currently they are re-named by using the chain number[25, 26]. Laminin 5 (currently laminin 332 as $\alpha 3$, $\beta 3$, and $\gamma 2$) is known to associated with various cancers, and especially it is involved in human glioblastomas through integrin $\alpha 3 \beta 1$ as cellular receptor[27]. Laminin 8 ($\alpha 3 \beta 1 \gamma 1$) is also known to be essential for invasion of glioblastoma[28-30]. Previously, Crk is reported to function downstream of laminin 10/11 to regulate Rho and Rac regulating cell motility[31]. Considering that Crk localized in focal adhesion forming by integrin $\beta 1$ stimulation[32] and integrin $\beta 1$ regulates PI-3 kinase through p130^{Cas}[33], Crk may function downstream of laminin involving in cell survival of glioblastoma. Furthermore, implication of Crk in glioblastoma cell growth is also supported by the data that HEF1 which is Cas family docking protein for Crk, was reported to be essential for tumor growth[34]. Alternatively, as growth of glioblastoma is suppressed by anti-c-Met Ab[35] and Crk functions downstream of Met[9], Crk may mediate Met-dependent signals for growth of glioblastoma.

In this study, we demonstrated that overexpression of Crk is correlated with malignancy of glioma, and this is consistent with previous report by Takino et al[36].

Although Crk is known to play an important role for various human cancers, the differential expression of CrkI and CrkII is controversial. Recently, we demonstrated by structural analysis that CrkI is active form and CrkII can be negatively regulated by Y221 tyrosine-phosphorylation of CrkII itself [37]. In fact, CrkI exhibits transforming activity in fibroblast 3Y1 cells, but CrkII does not. In addition, Crk-null mice were embryonic lethal[38] but mice only express CrkI not CrkII, by insertional mutation, are born [39], thus CrkI is necessary for embryonic development.

The differential expression of *CrkI* and *CrkII* is not extensively analyzed and there is one report that overexpression of *CrkI* is related to glioblastoma[36]. To confirm this evidence, we have analyzed 20 glioma cases and found that *CrkI* was overexpressed in 5 cases of malignant glioma, and elevation of both mRNAs of *CrkI* and *CrkII* was observed in three cases. Thus, Crk, especially CrkI, may play an important role for malignant glioma as predicted by the structural analysis, and Crk may be a therapeutic target in future.

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Figure Legends

Fig. 1 Expression of Crk in human gliomas (A) The mRNA levels of *CrkI* and *CrkII* in human glioma specimens were analyzed by semi-quantitative RT-PCR. Twenty gliomas including WHO Grade I (G1, n=1), Grade II (G2, n=3), Grade III (G3, n=4), and Grade IV tumors (G4, n=12) were tested. N, normal brain. (B) Immunohistochemical staining was done with an anti-Crk antibody on archival tissue sections. The representative images of Crk positive (left) and negative tumors (right). Scale bar, 100 μ m.

Fig. 2 Generation of Crk-knockdown glioblastoma cells (A) Protein extracts from four glioblastoma cell lines were analyzed by immunoblotting using indicated antibodies. (B) The cell extracts immunoprecipitated with an anti-p130 antibody were analyzed by immunoblotting using an anti-PY antibody (upper panel). The amount of p130^{Cas} was also analyzed by immunoblotting (with an anti-p130 antibody) using the lysates immunoprecipitated with anti-Crk antibody (lower panel). (C) The Crk-knockdown KMG4 cells were established. The decreased expression of Crk protein in each clone (Crki5, Crki17, and Crki21) was confirmed by immunoblotting using an anti-Crk antibody. IB, immunoblotting; IP, immunoprecipitation.

Fig. 3 Crk-knockdown results in disruption of adhesion to Laminin (A) Cells cultured on cover glasses coated with laminin (Lam), collagen (Col), or fibronectin (FN), were subjected to the phalloidin staining. Scale bar, 10 μ m. (B) The cells were plated on culture

plates coated with Lam, Col, hyaluronic acid (HA), or FN, as indicated. After 10 or 20 min incubation, the number of attached cells was quantified as described in Materials and Methods. The results were represented by mean $\pm$ SE of three independent experiments. (C). The cells were plated on laminin (Lam)-coated dishes and the number of attached cells was quantified at 0, 10, 20 30 and 60 min after re-plating. The results were represented by mean $\pm$ SE of three independent experiments.

Fig. 4 Crk contributes to motility and growth properties of glioblastoma cells (A) The recovery of wound formation was examined 0, 6 and 12 h after wound formation, and results (percentage of recovery), represented by mean $\pm$ SE of 3 independent experiments, were displayed as a graph (left). The typical microscopic images of this experiment were also shown (right). (B) Anchorage-dependent cell proliferation of Crk-knockdown cells. Cell numbers were counted every day and results were expressed in the mean $\pm$ SE of 3 independent experiments. (C) The cells (1×10^5) were plated in soft-agar and incubated for 21 days. The results were represented by mean $\pm$ SE from two independent triplicate experiments. (D) For xenograft propagation, 1×10^7 cells were injected s.c. into nude mice (mock, n=3; Crki17, n=3). An example of mouse bearing xenografts was shown (left). *Arrows*, tumor masses. Macroscopical appearance of tumors was also shown (right). (E) Formalin-fixed paraffin-embedded sections of Crki17- (right) and mock- (left) xenografts were subjected to histological analyses. H&E staining (top; scale bar, 100 μ m) and immunohistochemistry using

an anti-Ki67 antibody (MIB1; bottom) are shown. *Arrow heads*, mitoses.

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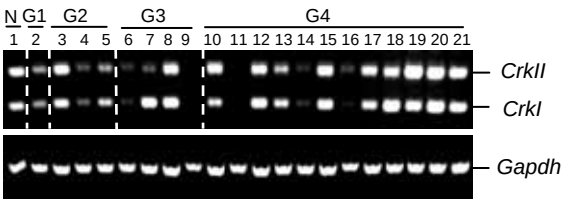
Legend for Supplementary Figure1

Pulldown assay of Rap-1 in Crk knockdown cells with or without serum stimulation.

Fold activation of Rap-1 of Representative result of three independent experiments were shown as bar graph. Representative results of immunoblotting for Rap-1 is displayed; active Rap (upper panels) and total amount of Rap (lower panels). Protein extracts, lysed in the buffer described previously (Linghu, H., et al., Oncogene, 25, 3547-3556, 2006), were centrifuged. The supernatants were incubated with 10 µg of purified GST-RalGDS-RBD and glutathione-Sepharose 4B beads, and the resulting precipitants were analyzed by immunoblotting with an anti-Rap1 antibody.

Fig. 1

A



B

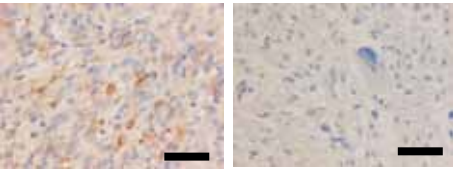


Fig. 2

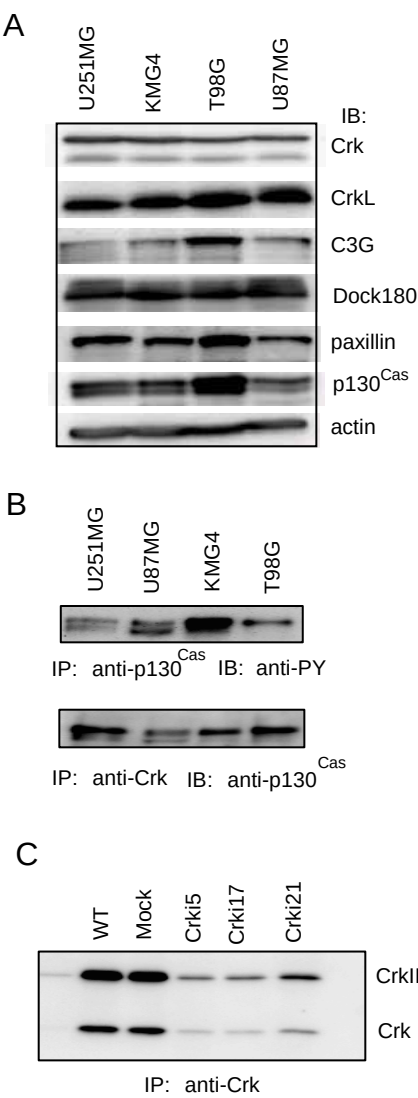


Figure3

Fig. 3

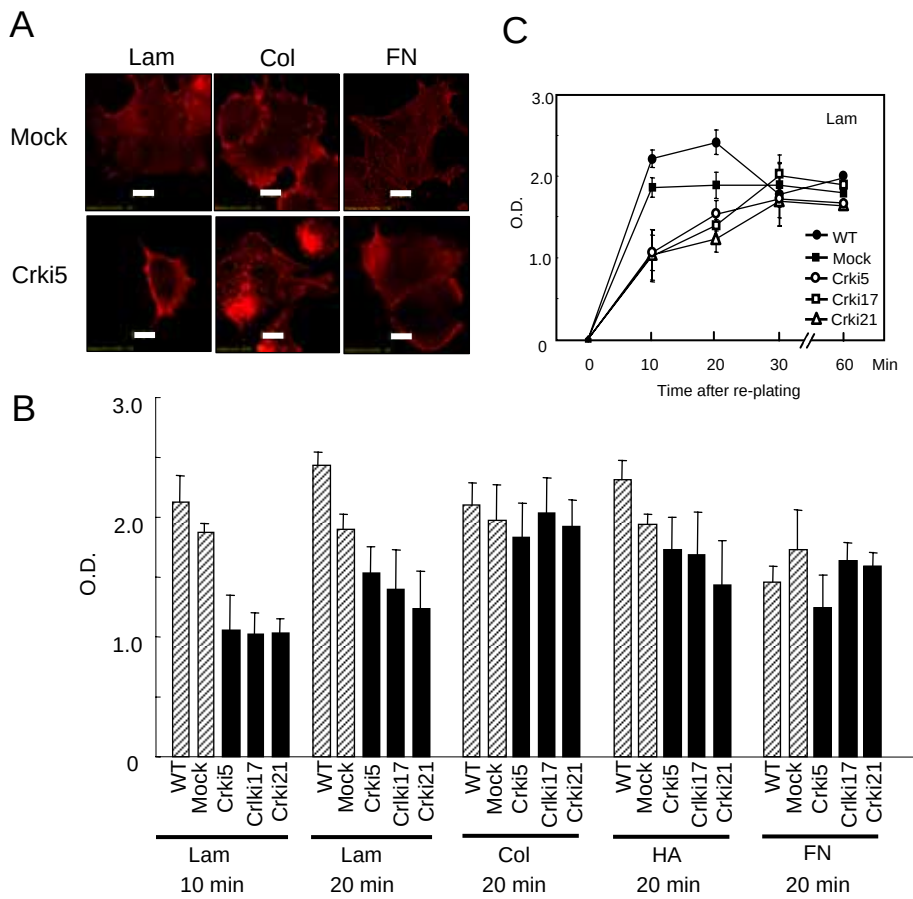
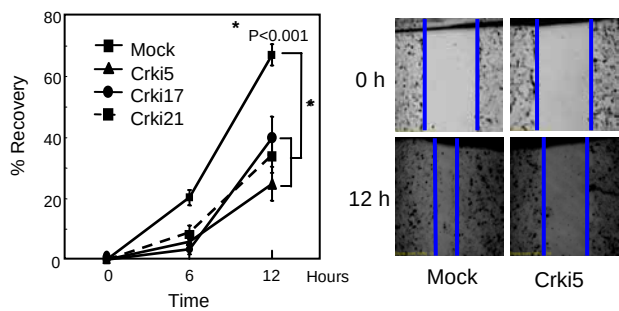


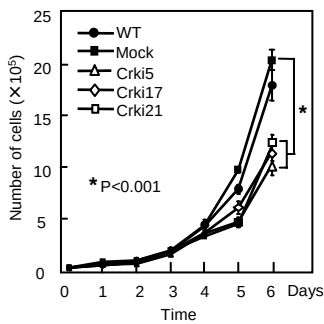
Figure4

Fig. 4

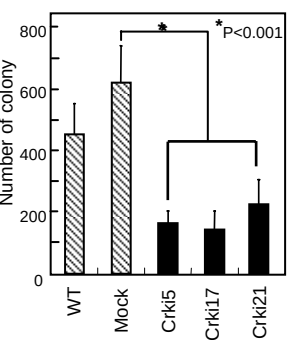
A



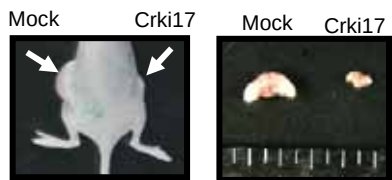
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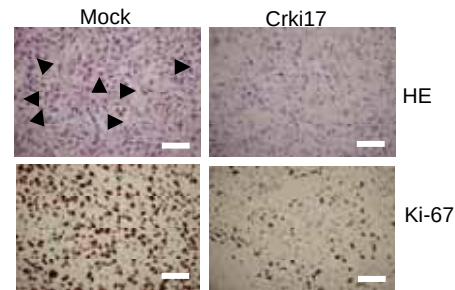
C



D



E



Supplementary figure 1

