Essential role of the Crk family-dosage in DiGeorge-like anomaly and metabolic homeostasis

Akira Imamoto1, Sewon Ki2, Leiming Li3, Kazunari Iwamoto4, John Devany5, Ocean Lu6, Tomomi Kanazawa7, Suxiang Zhang7, Takuji Yamada8, Akiyoshi Hirayama2, Shinji Fukuda9,10, Yutaka Suzuki11, Mariko Okada2,3,12

CRK and CRKL (CRK-like) encode adapter proteins with similar biochemical properties. Here, we show that a 50% reduction of the family-combined dosage generates developmental defects, including aspects of DiGeorge/del22q11 syndrome in mice. Like the mouse homologs of two 22q11.21 genes CRKL and TBX1, Crk and Tbx1 also genetically interact, thus suggesting that pathways shared by the three genes participate in organogenesis affected in the syndrome. We also show that Crk and Crkl are required during mesoderm development, and Crk/Crkl deficiency results in small cell size and abnormal mesenchyme behavior in primary embryonic fibroblasts. Our systems-wide analyses reveal impaired glycolysis, associated with low Hif1a protein levels as well as reduced histone H3K27 acetylation in several key glycolysis genes. Furthermore, Crk/Crkl deficiency sensitizes MEFs to 2-deoxy-D-glucose, a competitive inhibitor of glycolysis, to induce cell blebbing. Activated Rapgef1, a Crk/Crkl-downstream effector, rescues several aspects of the cell phenotype, including proliferation, cell size, focal adhesions, and phosphorylation of p70 S6k1 and ribosomal protein S6. Our investigations demonstrate that Crk/Crkl-shared pathways orchestrate metabolic homeostasis and cell behavior through widespread epigenetic controls.

DOI 10.26508/lsa.201900635 | Received 23 December 2019 | Revised 20 January 2020 | Accepted 21 January 2020 | Published online 10 February 2020

Introduction

CRK and CRKL (CRK-like), two paralogs of the CRK gene family, are localized to 17p13.3 and 22q11.21 in the human genome, respectively. CRK was first identified as the avian oncogene v-CRK, followed by the discovery of its cellular counterpart. CRKL was later identified in human chromosome 22q11 based on its sequence similarities to CRK (Feller, 2001; Birge et al., 2009). Evolutionary evidence suggests that the two genes were generated by chromosomal duplication in the common vertebrate ancestor (Shigeno-Nakazawa et al, 2016). Despite their possible redundancy, CRKL has been implicated in DiGeorge syndrome (DGS) as a dosage-sensitive gene that also shows genetic interactions with TBX1, a key 22q11.21 gene (Guris et al, 2006; Racedo et al, 2015), whereas ~90% of DGS patients have a heterozygous 3-Mb microdeletion at 22q11.21, including these two and several other genes (McDonald-McGinn et al, 2015).

Although haploinsufficiency of TBX1 has been strongly implicated in DGS, deficiency of mouse Crkl alone also affects normal development of anterior/frontal structures, including facial features, great arteries, heart, thymus, and parathyroid, as well as posterior structures, including genitourinary (GU) tissues, as collectively manifested as a condition that resembles DiGeorge anomaly (Guris et al, 2001; Racedo et al, 2015; Haller et al, 2017; Lopez-Rivera et al, 2017). CRKL point mutations have also been identified among a large cohort of patients with renal agenesis or hypoplasia (Lopez-Rivera et al, 2017). A distal region of the common deletion that includes CRKL has been linked to GU defects among 22q11.2DS patients, and haploinsufficiency of Crkl results in abnormal GU development in mice (Haller et al, 2017; Lopez-Rivera et al, 2017). Although CRKL coding mutations have not been linked to DGS without a 22q11 deletion, a recent study has identified non-coding mutations predicted to affect CRKL expression in the hemizygous region of the common 22q11 deletion with conotruncal defects (Zhao et al, 2020). Therefore, a reduction of CRKL expression below 50% may contribute to expressivity and penetrance known to be highly variable in DGS. On the other hand, CRK has not been established with a firm link to congenital disorders to date, although it is localized to the chromosomal region associated with Miller-Dieker syndrome.

References

1The Ben May Department for Cancer Research, The University of Chicago, Chicago, IL, USA 2RIKEN Integrative Medical Sciences, Tsurumi, Yokohama, Kanagawa, Japan 3Institute for Protein Research, Osaka University, Suita, Osaka, Japan 4Department of Mechanical and Aerospace Engineering, Old Dominion University, Norfolk, VA, USA 5Department of Life Science and Technology, Tokyo Institute of Technology, Meguro, Tokyo, Japan 6Department of Physics, The University of Chicago, Chicago, IL, USA 7Department of Life Science and Technology, Tokyo Institute of Technology, Meguro, Tokyo, Japan 8Institute for Advanced Biosciences, Keio University, Tsuruoka, Yamagata, Japan 9Intestinal Microbiota Project, Kanagawa Institute of Industrial Science and Technology, Kawasaki, Kanagawa, Japan 10Transborder Medical Research Center, University of Tsukuba, Tsukuba, Ibaraki, Japan 11PRESTO, Japan Science and Technology Agency, Kawaguchi, Saitama, Japan 12Department of Computer Science and Medical Sciences, Graduate School of Frontier Sciences, The University of Tokyo, Kashiwa, Chiba, Japan 13Center for Drug Design and Research, National Institutes of Biomedical Innovation, Health and Nutrition, Ibaraki, Osaka, Japan

Correspondence: aimamoto@uchicago.edu; mokada@protein.osaka-u.ac.jp
Leiming Li’s present address is AbbVie, North Chicago, IL, USA

Published Online: 10 February, 2020 | Supp Info: http://doi.org/10.26508/lsa.201900635
Downloaded from life-science-alliance.org on 5 September, 2024

© 2020 Imamoto et al. https://doi.org/10.26508/lsa.201900635 vol 3 no 2 e201900635 1 of 18
(Bruno et al, 2010). Nevertheless, mouse phenotypes from genetic ablations of either Crkl or Crk indicate that neither Crk nor Crkl alone is sufficient for normal development (Guris et al, 2001; Park et al, 2006).

CRK and CRKL encode adapter proteins, consisting of SRC homology 2 and 3 domains (SH2 and SH3, respectively) without known catalytic activities in an SH2-SH3-SH3 configuration, whereas alternative splicing generates CRK “isoform b” (commonly noted as “CRK-I” in contrast to the full length “isoform a” as “CRK-II”) that does not include the C-terminal SH3 domain (Feller, 2001; Birge et al, 2009). Most CRK/CRKL SH2-binding proteins have been identified as transmembrane proteins (such as growth-factor/cytokine receptors and integrins) and their cytosolic components (Feller, 2001; Birge et al, 2009). The task of inferring the specifics of their biological functions has been challenging due partly to co-expression and multiple aspects of the phenotypes from homozygous deletion in the CRK gene in which exon 2 is flanked by two loxP sites as CrkI2 allele (Hallier et al, 2017; Lopez-Rivera et al, 2017). We first confirmed that the CrkI-deficient embryonic phenotype generated by CrkI2/2 and Meox2Cre/+ strains recapitulated the CrkI null phenotype generated by deletion of CrkI exon 1, including arch artery and thymic defects (Fig 5A). As predicted, compound heterozygotes for CrkI and CrklI2 with Meox2Cre/+ exhibited an embryonic phenotype at E16.5, including severe edema and enlarged blood vessels, a cleft palate, IAA-B, and right-sided aortic arch accompanied by ventricular septal defect and similar thymic lobes (Fig 1E–H). IAA-B was reproducibly observed in CrkI/CrklI compound heterozygous embryos (Fig 1I). This phenotype was similar in multiple aspects to the phenotypes from homozygous deficiency of either Crk or Crkl (Figs 1A–D and S3). Furthermore, compound heterozygotes between CrkI and Tbx1 showed embryonic phenotypes at E16.5 with greater penetrance and expressivity than that of either CrkI or Tbx1 single heterozygotes (Table S1). As these phenotypes share a constellation of DGS-like defects, our observations raise the hypothesis that DiGeorge and related syndrome may result from genetic and environmental assaults on a part of the network sensitive to and commonly dependent on the CRK family genes as well as Tbx1.

The mesoderm requires at least two copies of the Crk family-combined gene dosage

To further investigate shared roles that Crk and Crkl may play in development, we generated CrkI and Crkl deficiency in the mesoderm using MesptCre (Saga et al, 1999). Some mice survived 50% family-combined gene dosages reduced in the mesoderm lineages without an overt phenotype in three genotypes: CrkI/+,MesptCre/+, CrkI2/2,MesptCre/+, and CrkI2/-,Crklf2/2,MesptCre/+. However, further dosage reduction leaving only one copy of either CrkI or Crkl in the mesoderm (CrkI/+,Crklf2/2,MesptCre/+, and CrkI2/-,Crklf2/2,MesptCre/+, respectively) resulted in abnormal embryos, associated with an enlarged heart that failed to undergo looping when examined at E9.5 (Fig 1J and K). In addition, they also had smaller numbers of somites with a large proportion of the paraxial mesoderm left unsegmented compared with that of control embryos. Although vasculogenesis was initiated in the yolk sac mesoderm, the vascular plexus failed to undergo remodeling in CrkI2/-,Crklf2/2,MesptCre/+ embryos recovered at E9.5 (Fig 1M). It is also noteworthy that

**Results**

**Deficiency of Crk, the paralog of Crkl, targets the heart and arch-derived tissues**

To probe the functional significance of the Crk family members, we targeted the mouse CrkI gene with a conditional approach by inserting loxP sites upstream and downstream of Exon 1 (CrkI allele; Fig S1). A germ-line CrkI null allele (CrkIf2 allele) was generated by Cre-mediated recombination in the epiblast using a Meox2 Cre knock-in strain (Tallquist & Soriano, 2000), followed by backcrosses with wild-type C57BL/6 mice to segregate out Meox2Cre. In addition to the developmental defects previously reported in another Crk-deficient mutant (Park et al, 2006), we noted that homozygous CrkIf2/2 embryos displayed some aspects reminiscent of DiGeorge anomaly despite the fact that CrkI is not a 22q11 gene in humans (Figs 1A–D and 1A–D’). Among three CrkIf2/2 embryos histologically examined, all three cases displayed ventricular septal defects (VSD) (Fig 1D), whereas one case accompanied an interrupted arch of aorta (IAA-B, Fig 1D), another case a right aortic arch, one case a d-transposition of the great arteries (Fig 1D), two cases with a double-outlet right ventricle (Fig 1D), two cases with a cleft palate (Fig 1A), and two cases with cervical thymic lobes outside of the thoracic cavity (Fig 1B).

**Compound heterozygosity of Crk and Crkl is sufficient to generate an embryonic phenotype**

CrkI and Crkl were expressed in largely overlapping patterns at E10.5, and the CrkI-deficient phenotype was similar to that of Crkl (Figs S2 and S3) (Guris et al, 2001). Therefore, we hypothesized that their phenotypes may be attributed to a dosage-sensitive reduction in their common functions. In addition to the CrkI conditional allele, we used a mouse strain that we previously generated with a conditional mutation in the CrkI gene in which exon 2 is flanked by two loxP sites as CrkI2 allele (Haller et al, 2017; Lopez-Rivera et al, 2017). We first confirmed that the CrkI-deficient embryonic phenotype generated by CrkI2/2 and Meox2Cre/+ strains recapitulated the CrkI null phenotype generated by deletion of CrkI exon 1, including arch artery and thymic defects (Fig 5A). As predicted, compound heterozygotes for CrkI and CrklI2 with Meox2Cre/+ exhibited an embryonic phenotype at E16.5, including severe edema and enlarged blood vessels, a cleft palate, IAA-B, and right-sided aortic arch accompanied by ventricular septal defect and similar thymic lobes (Fig 1E–H). IAA-B was reproducibly observed in CrkI/CrklI compound heterozygous embryos (Fig 1I). This phenotype was similar in multiple aspects to the phenotypes from homozygous deficiency of either CrkI or Crkl (Figs 1A–D and S3). Furthermore, compound heterozygotes between CrkI and Tbx1 showed embryonic phenotypes at E16.5 with greater penetrance and expressivity than that of either CrkI or Tbx1 single heterozygotes (Table S1). As these phenotypes shared a constellation of DGS-like defects, our observations raise the hypothesis that DiGeorge and related syndrome may result from genetic and environmental assaults on a part of the network sensitive to and commonly dependent on the CRK family genes as well as Tbx1.
embryos with only one copy of either Crh or Crh (Crh+/CrhP2/; MespCre+/ or Crh P2/;CrhP2/;MespCre+/, respectively) showed similar morphological defects. These results indicate that a developmental threshold requires at least 50% of the family-combined gene dosage during heart and somite development as well as in the yolk sac mesoderm, through their shared functions. We also identified Crh/Crh double-deficient embryos (Crh+/CrhP2/; MespCre+/) in the genetic crosses. They were much smaller than either Crh+/CrhP2/;MespCre+ or Crh+/CrhP2/;MespCre+ embryos, at E9.5 (Fig 1K). When isolated at E8.5, double-deficient embryos resembled the size and appearance of E7.5 embryos (Fig 1I). Because normal onset of gastrulation is marked by the emergence of Mesp1-positive mesoderm starting around E6.5 in mice (Saga et al, 1999), these results indicate that Crh and Crh are absolutely required immediately after mesoderm induction.

Morphological and behavioral phenotypes in primary MEFs

The results above suggest that mesodermal cells may provide a useful system to investigate the shared functions of Crh and Crh. To this end, we isolated primary MEFs at E11.5 as a model for mesodermal cells. Using this end, we isolated primary MEFs at E11.5 as a model for meso-

Figure 1. Embryonic phenotypes from deficiencies of Crh and Crh in mice. (A, B, C, D) Histologic sections from an E16.5 embryo lacking Crh (Crh+/CrhP7/; MesPcr+/-) showed defects, including a cleft palate (arrow in panel A), cervical/extra-thoracic thymic lobes (red arrows in panel B), ts in panel C), d-transposition of aorta and pulmonary trunk associated with double-outlet right ventricle (C) and ventricular septal defect (arrow in panel D). We also noted a condition known as an interrupted aortic arch type B (AA-B) in panel (D) and other sections (not shown). Asterisk in panel (A) indicates a dilated blood vessel. Accompanied panels (A‘, B‘, C‘, and D‘) show sections from a wild-type littermate corresponding to sections (A, B, C, and D, respectively). Abbreviations used in the panels are as follows: ns, nasal septum; ps, palatal shelf; to, tongue; rcc, right common carotid artery; lcc, left common carotid artery; icc, left common carotid artery; ic, left internal carotid artery; rec, right external carotid artery; lec, left external carotid artery; cv, cervical vertebra; tv1, thoracic vertebra 1; tv2, thoracic vertebra 2; es, esophagus; trachea; ts, thymus; st, stop of the sternum (manubrium); co, ribs (costae); ao, aorta; pt, pulmonary trunk; rt, right ventricle; and lv, left ventricle. (E, F, G, H, I) Compound heterozygosity for Crh and Crh deficiency resulted in an embryonic phenotype at E16.5. Timed mating was set up between Mespcre+/- and Crh+/CrhP2/; Crh+/CrhP2/ parents to drive cre-dependent recombination in the epiblast. Compound heterozygotes (Crh+/CrhP2/; Mespcre+/- showed severe edema and subcutaneous hemorrhage at E16.5 (left, E), associated with a cleft palate (F) and abnormal great arteries and heart (G, H). Ink injection into the right ventricle revealed an abnormal pattern of the great arteries such as enlarged aorta without forming a left-sided arch of aorta (ao, G) as well as a ventricular septal defect (G), as ink flowed into the left ventricles from the right ventricle (dotted ellipses, G). When viewed from the left side (panel H), pulmonary trunk abnormally branched into the left common carotid artery via the ductus arteriosus connected to the descending aorta. A similar case of interrupted arch of aorta type B was found in another compound heterozygote in the same litter (I). Asterisk indicates an abnormal outflow tract externally suspected to be a persistent truncus arteriosus. The compound heterozygote also exhibited a small cervical thymic lobe, which was removed before examination of the great arteries. cp, cleft palate; pl, palate (closed); rcc, right common carotid artery; lcc, left common carotid artery; ao, aorta; rt, right ventricle; lv, left ventricle; vsd, ventricular septal defect; da, descending aorta. (J, K, L, M) Early developmental defects were observed in E8.5 and E9.5 mouse embryos when combined Crh and Crh deficiency was induced in the mesendoderm driven by Mesp1cre. The genotypes of the individual embryos shown (numbered from 1 through 11) are indicated below the panels (K, L). Panels (J, K) show lateral views of embryos isolated at E9.5. Panel (L) shows dorsal views of two E8.5 embryos. Note that Crh+/CrhP2/;MespCre+/ and CrhP2/;MespCre+/ embryos were phenotypically similar (embryo 1 compared with embryos 2, 3, 7, and 8). Asterisks indicate enlarged hearts without proper looping and chamber development. Arrowheads indicate the position of the posterior most somite visually identifiable, thereby indicating a delay in somitogenesis in Crh+/CrhP2/;MespCre+/ and CrhP2/;MespCre+/ embryos compared with cre-negative control embryos. ht, heart; al, allantois. Panel (M) shows embryos 6, 7, and 8 in yolk sac. Note a delay in vascular remodeling in embryos 7 and 8, compared with the cre-negative control embryo (embryo 6).
Figure 2. Crk/Crkl deficiency induces multiple defects in MEFs.

(A) Crk and Crkl protein levels were determined in time course in immunoblots upon induction of Crk/Crkl deficiency. Crk \( ^{f/f} \), Crkl \( ^{f/f} \), R26\text{creERT2}/+ MEFs were induced for Crk/Crkl deficiency by 4-hydroxytamoxifen (4OHT) treatment for 24 h, whereas a control group was treated with vehicle only (CTRL). Cell lysates were isolated from MEFs at each time point indicated above each lane (0 h was the time right before 4OHT/vehicle addition).

(B) Crk/Crkl double deficiency results in slow cell growth and altered population morphology. Pictures were taken posttreatment day 2 for control and day 4 for Crk/Crkl deficient MEFs. Note that the cell density of Crk/Crkl-deficient MEFs on day 4 is similar to that of control group on day 2, and that population morphology is distinguishable between the 4OHT and control groups.

(C) Time lapse images show cell division from a single MEF (identified at 0:00 time) to two daughter cells in each group. In the control, cells migrated away from each other after division and were no
impaired metabolic state. Upon induction of Crk/Crkl double deficiency, the cell morphology changed more drastically from a typical fibroblastic appearance to a compact/condensed appearance (Fig 2B). To explore the basis of this collective morphology, we took time-lapse videos of dividing cells (Fig 2C). Normally, fibroblast-like cells show repulsive movements upon cell–cell contacts, known as contact inhibition of locomotion (CIL) (Roycroft & Mayor, 2016). Likewise, two daughter cells moved apart in the control group upon cell division (Fig 2C). In contrast, the daughter cells in the Crk/Crkl-deficient group did not separate despite their cell–cell contacts, thus exhibiting a failure related to CIL (Fig 2C).

The cell junctional marker β-catenin showed a greater accumulation to cell–cell junctions in the deficiency-induced group than control, thus indicating elevated cell–cell adhesion in the deficiency-induced MEFs (Fig 2D). A failure of post-mitotic CIL and increased cell–cell contacts may explain the abnormal population morphology in Fig 2B, thus demonstrating that Crk and Crkl play a pivotal role in cell–cell haptic communication and behavior.

**Essential roles of Crk and Crkl in spreading and cell size**

We next determined the effects of Crk and Crkl deficiency, individually or combined, on cell spreading (Fig 2E). In our modified spreading assay, we measured the surface area that each attached cell occupied over time on gelatin-coated plates rather than counting the number of spreading cells at each time point. We found that compared with control MEFs, the process of spreading was slower in deficiency of Crk or Crkl individually, and further reduced in Crk/Crkl double deficiency as indicated by the slope of spreading curves (Fig 2E).

During tissue culture, we became aware that the same number of Crk/Crkl double-deficient primary MEFs made visibly smaller pellets than that of control MEFs when harvested by dissociation and centrifugation. The observation suggested the possibility that individual Crk/Crkl double-deficient MEFs may be smaller than that of control cells. Normally, cells undergo a controlled cell-size oscillation during cell cycle to maintain their sizes in a population of control cells. Normally, cells undergo a controlled cell-size oscillation during cell cycle to maintain their sizes in a population of control cells. Roycroft & Mayor, 2016. Therefore, we estimated the size of primary MEFs in the G1 phase by light scatter measurements in FACS analysis (Fig 2F). As anticipated, induction of Crk/Crkl double deficiency resulted in a size distribution shift smaller than that of control primary MEFs, whereas Crk/Crkl double-deficient MEFs cells appeared to stay in the G1 phase for a longer time than the control group (Fig S5). We also noted that the cell size was smaller when kept confluent for 4 d, compared with the groups that were split on Day 2 to avoid overcrowding (all groups received daily media change). Therefore, these results demonstrate that Crk and Crkl are essential for cell size homeostasis in G1, whereas additional cell density–dependence mechanism may also operate in parallel.

**Transcriptome pathways dependent on Crk and Crkl**

The complex phenotypes in development and in MEFs suggested involvement of Crk and Crkl in multiple pathways. To gain insight into the impaired network from a vantage view point, we conducted a systems-level analysis by RNA-Seq in the primary MEFs in which deficiency of each or both Crk and Crkl can be induced by 4OHT (Fig 3). Differential expression (DE) was determined between deficiency-induced and uninduced groups of primary MEFs in pair per single embryo, using four independent embryos for each genotype with an average levels for the FACS analysis. Each treatment group was subdivided into two subgroups with or without a split on day 2 posttreatment to adjust and maintain low cell density until harvest on day 3 posttreatment.
Figure 3. "Crk and Crkl deficiencies affect numerous pathways."

(A) The heat map shows a list of the top 30 pathways based on comparison analysis of the Crk and/or Crkl single and double deficiency groups in Ingenuity Pathway Analysis (QIAGEN; see Supplemental Data 1). RNA-Seq experiments were performed on RNA isolated from four independent primary MEF populations for each genotype as described in the Materials and Methods section as well as in Table S2 and Supplemental Data 1. Differentially expressed genes (DE genes) were identified by Benjamini–Hochberg adjusted P-values (p.adj) smaller than 0.05 using DESeq2.

(B) Deficiencies for Crk and Crkl genes, separately or combined, resulted in overlapping lists of DE genes, categorized into subsets a-g as shown in the Venn’s diagram. Subsets a, b, c, and d are referred to subsets “red,” “orange,” “yellow,” and “green” hereafter (Supplemental Data 2). The number in each subset indicates the number of DE genes identified in the gene deficiency. The numbers in parentheses separated by colon show the numbers of genes up-regulated versus down-regulated. Note that deficiency of either Crk or Crkl was sufficient to disrupt normal expression of the genes in subset “red,” whereas the genes in subset “green” tolerated single gene disruption of either Crk or Crkl.

(C) The DE genes in subsets “red,” “orange,” “yellow,” and “green” were analyzed for their enrichment into pathways using KEGG (Kyoto Encyclopedia of Genes and Genomes). The node circles and annotations are color-coded as appeared in panel (B). Nodes are labeled only for the KEGG modules and pathways with Storey’s q-values smaller than 0.0005 (shown as FDRs), whereas node circles are shown for the pathways/modules with a q-value < 0.05. The diameter of node circle is proportional to −log10(q-value).
**KEGG analysis**

To further explore the dysregulated pathways, we categorized the DE genes either "down-regulated" or "up-regulated" in each subset (Supplemental Data 2). In subset "red," we noted that down-regulated DE genes were enriched in several KEGG "pathways" and "modules," including glycolysis, aminoacyl-tRNA biosynthesis, HIF-1 signaling, regulation of actin cytoskeleton, and focal adhesion (Fig 3C, red circles). On the other hand, up-regulated DE genes in subset "red" did not show significant enrichment in a KEGG pathway or module. Down-regulated DE genes in subset "orange" were associated with ribosome biogenesis and RNA transport, whereas the up-regulated genes were mapped to the glucoronate pathway and cytochrome P450-mediated drug metabolism (Fig 3C, orange circles). Down-regulated genes in subset "yellow" were enriched in CS-isoprenoid biosynthesis/mevalonate pathway, suggesting a specific role for Crk in biosynthesis of cholesterol and other isoprenoids, whereas no enrichment was identified in the pathways or modules for up-regulated genes (Fig 3C, yellow circles). Subset "green" included many DE genes in down-regulated pathways, including oxidative phosphorylation, purine/pyrimidine metabolism, spliceosome, ribosome, DNA repair and replication, and cell cycle (Fig 3C, green circles). A few pathways, of which most noticeable was NOD-like receptor signaling, appeared to be up-regulated in subset "green," thus implicating a redundancy between Crk and Crkl in regulating inflammasomes (Strowig et al, 2012; Wen et al, 2013).

**Validating the role of Crk and Crkl in glycolysis**

The transcriptome analysis above implicated shared family-critical roles for Crk and Crkl in glycolysis and other metabolic pathways (Fig 3). Using capillary electrophoresis time-of-flight mass spectrometry (CE-TOFMS), we found that several metabolites in the central glucose metabolism pathway were decreased (Fig 4A, squares filled with shades of blue; Supplemental Data 3), consistent with reduced transcript levels of several genes encoding glycolysis enzymes along the same pathway (Fig 4A, small circles filled with shades of blue). Several metabolites and glycolytic enzymes were affected not only in Crk/Crkl-double deficiency but also in MEFs deficient for either Crk or Crkl (Fig 4A, squares and circles enclosed by magenta-colored line). Reduced mRNA levels of several glycolysis enzymes initially identified by RNA-Seq were validated by quantitative real-time RT-PCR (Fig 4B). Furthermore, chromatin immunoprecipitation (ChIP) followed by quantitative/real-time PCR demonstrated that association of RNA polymerase II phospho-S5 C-terminal domain (CTD) repeats was reduced in Gapdh, Pglk1, and Ldha upon deficiency induction (Fig 4C). As they belong to subset "red," these glycolysis enzyme genes are sensitive to a shared function of Crk and Crkl for their transcription.

**A role for Crk and Crkl in CoCl2-stabilized Hif1a protein pool**

Several glycolysis enzymes have been identified as targets of the transcription factor Hif1a (Fig 4A, labels in orange color) (Benita et al, 2009). Although Hif alpha proteins (Hif1a and Hif2a) are rapidly degraded under the ambient air oxygen level of 21% by the von Hippel-Lindau tumor suppressor VHL and E3 ubiquitin ligase, the degradation process is controlled under physiological O2 levels of 2–9% in tissues and embryonic environment (Simon & Keith, 2008; Semenza, 2017). To investigate possible effects of Crk/Crkl deficiency on Hif pathway, we used CoCl2 to stabilize Hif1a proteins by inhibiting VHL (Yuan et al, 2003). As anticipated, Hif1a levels increased in the nucleus in the presence of CoCl2 in both Crk/Crkl deficiency-induced and uninduced MEFs (Fig 4D). However, the CoCl2-induced increase was much smaller in Crk/Crkl deficiency-induced MEFs than that of uninduced control MEFs (p.adj < 2 × 10^-16). Although the oxygen-rich environment under the standard tissue culture condition masks Hif1a protein levels, a small difference was also detectable between Crk/Crkl deficiency-induced MEFs than that of uninduced control MEFs (p.adj < 2 × 10^-16). These results demonstrate that normal Hif1a protein production relies on Crk and Crkl.

**Crk/Crkl deficiency affects chromatin-level gene regulations**

To explore the mechanism by which glycolysis enzyme expression was down-regulated, we conducted genome-wide ChIP-Seq analysis with an active chromatin marker, acetylated histone H3 lysine-27 along with RNA Polymerase II phospho-S5 CTD repeats (H3K27Ac and Pol2, respectively). Association of H3K27Ac and Pol2 with transcription start site (TSS)–proximal regions is a global feature of actively transcribed genes, as H3K27Ac positively enhances the search kinetics of transcription activators as well as the transition of Pol2 from initiation to elongation by accelerating its promoter escape (Stasevich et al, 2014).

H3K27Ac or Pol2 ChIP-Seq showed a positive correlation with mRNA DE for the genes in subset "red" as Crk/Crkl-common and Crk/Crkl-sensitive targets (Fig 5A). In particular, the down-regulated glycolysis genes in subset "red" were identified within the lower left quadrant in the scatterplots (Fig 5A). Furthermore, the ChIP-Seq reads for H3K27Ac and Pol2 were reduced globally in down-regulated genes, compared with the up-regulated gene group (Fig 5B). Interestingly, the ChIP-Seq reads for H3K27Ac and Pol2 were not increased for subset "red" up-regulated DE genes with their median values in the negative range. Therefore, reduced mRNA levels of the glycolysis genes were attributable largely to diminished transcription in Crk/Crkl-deficiency, whereas a separate mechanism may drive increased steady mRNA levels for the up-regulated DE genes in subset "red."

In TSS-flanking regions, we observed generally diminished ChIP-Seq peaks for both H3K27Ac and Pol2 in Crk/Crkl-deficient MEFs compared with the control group (Fig 5C, KO versus CTRL in orange and green lines, respectively). Consistent with the result shown in Fig 5B, H3K27Ac and Pol2 signals were not increased for the up-regulated genes in Crk/Crkl-deficient MEFs. To quantify the changes, boxplots were generated for the peak height of the ChIP-Seq signals in the TSS ± 2 kb region (Figs 5D and S7). We noted significant differences in H3K27Ac signals between deficiency-induced and uninduced MEFs (p.adj < 1 × 10^-10 and p.adj = 8.97 × 10^-16 in the down-regulated and up-regulated gene categories, respectively). Pol2 ChIP-Seq signals were also highly different between deficiency-induced and uninduced MEFs in the down-regulated gene categories.
category (p.adj < 1 x 10^-10), but not in the up-regulated gene category (p.adj = 0.964). Pol2 elongation from the TSS downstream beyond +2 kb was also greater for the down-regulated genes in control MEFs than that of the Crk/Crkl deficiency induced MEFS (Fig 5C, the green versus orange lines in the Pol2 plots). Therefore, down-regulated mRNA levels (found in RNA-Seq) were generally attributable to reduced Pol2 transcription initiation and elongation. On the other hand, up-regulated mRNA expression did not result from elevated promoter activity. These results demonstrate that Crk/Crkl deficiency led to widespread H3K27Ac depression in the epigenome, leading to marked reduction in de novo transcription of numerous genes down-regulated as common targets of Crk and Crkl.

Crk/Crkl deficiency impairs the effect of glucose on S6K and S6 activation

The results above demonstrated impaired glycolysis in Crk/Crkl deficiency accompanied by reduced Hif1a protein production. Signaling pathways known to influence cell size via p70 S6 kinase (S6k encoded by Rps6kb1 and Rps6kb2) and the ribosomal protein S6 (Rps6) are also important for Hif1a translation (Fingar et al, 2002; Semenza, 2010; Chauvin et al, 2014). We found that glucose availability was essential for maintaining active signaling cascades through Akt, Tsc2, S6k, and S6 in a dose-dependent manner, whereas 5 mM glucose appeared optimal for Akt S473 phosphorylation as well as Tsc2 T1462 phosphorylation (Figs 6A and S6B). Upon induction of Crk/Crkl-deficiency double deficiency, glucose resulted in much muted activation of the cascade compared with that of control MEFS (Fig 6A). Although Akt S473 phosphorylation was reduced in the Crk/Crkl-deficient MEFS, glucose-induced Tsc2 T1462 phosphorylation (considered as an Akt-specific phosphorylation site) was greater in the deficiency-induced MEFS than that of the control groups. Because the phosphorylation readout was reduced on both p70 S6k and S6 proteins, these results suggest that intersecting pathways surrounding Akt and Tsc2 may be dysregulated in Crk/Crkl deficiency in response to glucose availability.

Crk/Crkl deficiency and glucose restriction lead to cell membrane blebbing

To provide further evidence for a role that the Crk family may play in glucose metabolism, we evaluated the effects of 2-deoxy-D-glucose...
(2DG), a competitive inhibitor of glucose metabolism, on Crk/Crkl deficient or control MEFs (Gu et al., 2017). We noted that when cultured in a glucose-controlled condition (5 mM glucose with 10% dialyzed FBS), a range of 2DG concentrations induced blebbing cell morphology identified by cell staining with CellMask, DAPI, and anti-vinculin (Fig S9). “Blebbing” is a feature characterized by several plasma membrane protrusions resembling small beads that decorate the cell edge boundary, as a stage of apoptotic or nonapoptotic processes (Coleman et al., 2001; Fackler & Grosse, 2008). To minimize the subjective nature of categorical judgment on cell morphology, we applied an automated computational analysis. Using a few parameters standardized for blebbing identification (Fig S9), we found that Crk/Crkl deficiency exacerbated the blebbing phenotype induced by 2DG (Fig 6B), thus consistent with their possible involvement in glucose metabolism. Although the results do not distinguish apoptotic versus nonapoptotic blebbing, it is more likely that 2DG-induced blebbing may be an indication of apoptosis based on the observation that 2DG treatments reduced the total cell counts in our experimental condition (see the “n” numbers on top of each bar in Fig 6B).

A role for Crk and Crkl in Igf1-induced S6K/S6 activation

Insulin-like growth factor 1 (Igf1) is one of the growth factors required for normal development and known to control cell size through Akt (Lloyd, 2013; Manning & Toker, 2017). Igf1 signaling was implicated in our global analysis of the RNA-Seq results (Fig 3A), and Igf1-induced Akt S473 phosphorylation, suggesting that Igf1 was able to activate MTORC2 complex. In addition, overexpression of Crk or Crkl by itself increased both phosphorylation and protein levels of S6 in

Figure 5. Chromatin immunoprecipitation (ChIP)-Seq analysis.

(A) Scatterplots indicate ChIP-Seq signals for H3K27Ac and Pol2 (hosphor-55 CTD repeats) in the x-axis and mRNA levels (data from RNA-Seq) in the y-axis, in which values are shown in log2 fold change (log2 FC) as differentials between Crk/Crkl deficiency–induced and uninduced MEFs. Each dot represents a single gene identified in subset “red” (Fig 3B). Red or light-blue dots indicate down-regulated or up-regulated genes based on their expression identified by RNA-Seq, respectively. Glycolytic genes are labeled for their gene symbols. ChIP-Seq signals in these panels are based on peak heights within transcription start site (TSS) ± 2 kb. Spearman’s rank correlation coefficient p was calculated for the entire distribution. (B) Boxplots indicate distributions of the ChIP-Seq signal differentials within TSS ± 2 kb for H3K27Ac and Pol2 in either down-regulated or up-regulated categories of subset “red” genes. Whiskers were drawn between the highest and lowest data points within 1.5× interquartile range (IQR) from the upper or lower quartile. Data points outside the IQR are indicated as outliers. The P-values were calculated by Mann–Whitney U tests between down-regulated and up-regulated categories. (C) XY plots indicate the average ChIP-Seq signals as reads per genome content (RPGC) in the y-axis and the distance from TSS in the x-axis in Crk/Crkl deficiency–induced and uninduced MEFs (KO and CTRL) in orange and green lines, respectively. Note that when ChIP-Seq signals are compared in CTRL MEFs between the down-regulated and up-regulated gene groups, the down-regulated group shows greater average peak heights in both H3K27Ac and Pol2 ChIP-Seq signals. (D) Boxplots indicate the distribution of the ChIP-Seq signals (RPGC) within TSS ± 2 kb in Crk/Crkl deficiency–induced and uninduced MEFs (KO and CTRL) for genes down-regulated or up-regulated in subset “red.” The y-axis is in a log2 scale of RPGC. ANOVA and Tukey post hoc tests were performed on log2-transformed RPGC values to bring the data distributions closer to Gaussian distributions. See Fig S7 for boxplots using untransformed data and square-root transformed data.

The CRK family controls glucose metabolism and cell size  Imamoto et al.  https://doi.org/10.26508/lsa.201900635  vol 3 | no 2 | e201900635  9 of 18
The CRK family controls glucose metabolism and cell size

Cooperative signaling between Igf1 and integrins

Crk and Crkl are involved in a broad range of signaling pathways associated with tyrosine kinases (Feller, 2001; Birge et al, 2009). Among them are pathways mediated by extracellular matrix (ECM) proteins and integrins. We, therefore, investigated pathway integration between Igf1 and the ECM protein fibronectin (FN) for activating p70S6K and S6 in MEFs (Fig 6E). Among the deficiency-uninduced control groups (Fig 6E left half, the CTRL lanes), Igf1 induced phospho-Akt S473 in 15 min at comparable levels between the poly-L-lysine or FN groups (PLL or FN, respectively), whereas neither PLL nor FN induced Akt phosphorylation without Igf1. We noted that without Igf1, phosphorylation of S6 (S240/244) and S6K (T389) was increased by plating on FN compared with that of PLL, thus the ability of FN to increase phosphorylation of S6 and S6K appears to be independent of Akt. When Igf1 stimulated the MEFs plated on FN, the phosphorylation levels on S6 and S6K was highest among the uninduced groups. Among the deficiency-uninduced groups (Fig 6E right half, the 4OHT lanes), we observed that although the general trend is similar to the deficiency-uninduced control groups (the CTRL lanes), the levels of S6 and S6K phosphorylation decreased in the 4OHT groups for their responses to Igf1 and FN, independently or combined. These results demonstrate an important role of Crk and Crkl in mediating cooperative signals to S6K and S6 activation from Igf1 and FN.

Rescue of Crk/Crkl deficiency by an activated Rapgeff

Rapgeff (also known as C3G) encodes a guanine-nucleotide exchange factor for the small G-protein Rap1 (encoded by Rap1a and Rap1b) as one of the major proteins to which the SH3n domain of
Crk and Crkl can associate (Feller, 2001; Birge et al, 2009). Rapgef1 is ubiquitously expressed during early-mid gestation mouse embryos and its genetic ablation results in an early embryonic phenotype at E7.5, whereas a hypomorphic mutation generates a vascular phenotype around E11.5-E14.5 (Ohsba et al, 2001; Voss et al, 2003). These reports also demonstrated that Rapgef1 is an important mediator of cell adhesion to ECM proteins associated with reduced numbers of focal adhesions in MEFs isolated from the mutant embryos. We found that an activated Rapgef1 (C3GF) conferred MEFs resistance to Crk/Crl deficiency for cell size (Fig 7A). C3GF also rescued Crk/Crl-deficient MEFs for cell proliferation (Fig 7B) and restored expression of some glycolysis enzyme genes that were down-regulated in Crk/Crl deficiency (Fig 7C). Crk/Crl deficiency-induced reduction of fructose-1,6-bisphosphate (F1,6P2) was also restored by C3GF (Fig 7D). C3GF increased S6 and S6K phosphorylation, accompanied by elevated Akt phosphorylation (Fig 7E). Interestingly, C3GF by itself elevated tyrosine phosphorylation and protein levels of p130Cas/Bcar1, events thought to be upstream of Rapgef1, compared with that of the vector control groups. Likewise, C3GF enhanced focal adhesions as identified by subcellular localization of total phosphotyrosine, phosphorylated p130Cas/Bcar1, and the Fak. On the other hand, Crk/Crl-deficient MEFs in the vector control group appeared to have fewer focal adhesions (Fig 7F). As cells exhibited varying numbers of focal adhesions in each group, evaluating a small number of cells may introduce unintended bias. To objectively quantify focal adhesions in a large number of cells, we adopted an automated image analysis (Fig S11). As anticipated, whereas the number of focal adhesions was reduced by Crk/Crl deficiency, C3GF expression normalized focal adhesion counts (Fig 7G). These results confirmed not only the role of Rapgef1 in mediating positive-feedback signals from Crk and Crkl but also its important functions in glucose metabolism and cell size/adhesion homeostasis.

**Discussion**

Our present study has demonstrated that compound heterozygosity of Crk and Crkl (loss of shared functions) as well as individual gene disruption can generate developmental defects in mice, part of which resemble DiGeorge anomaly in multiple aspects, despite the fact that Crk is not a 22q11 gene. Furthermore, Tbx1x genetic interaction with not only Crk but also with Crkl provides evidence for a possible functional interaction among these genes. We have demonstrated that normal mesoderm requires at least 50% of the Crk family-combined dosage (Fig 1). It is noteworthy that Tbx1 is essential in the mesoderm for normal heart and outflow tract development, whereas Tbx1x expression is also required in the epithelia of ectoderm or endoderm origins for normal fourth arch artery and thymic development (Zhang et al, 2006). Tbx1x knockout in a cardiomyocyte-differentiating P19 subline as well as Tbx1x-mutant embryos show abnormal histone H3 monomethyl-K4 profiles (Fulcoli et al, 2016). It is also noteworthy that Tbx1 deficiency causes DE in mTOR signaling pathway, VEGF signaling pathway, phosphatidylinositol signaling pathway and focal adhesion (Fulcoli et al, 2016), which we have also identified as Crk/Crkl-shared pathways in this study (Fig 3A and C). In fact, Tbx1 knockout results in a reduced number/size of focal adhesions in C2C12 cells (Alfano et al, 2019), in similar ways to Crk/Crl-deficient MEFs, we analyzed in this study (Fig 7). Taken together, Crk, Crkl, and Tbx1 may regulate the gene regulatory network by modulating global epigenetic landscape, which directly or indirectly control cell behavior through cell–matrix adhesion and metabolism.

Our results have implicated Crk and Crkl in glucose metabolism through the transcription factor Hif1a. Whereas hypoxic conditions are known to increase Hif1 protein levels by stabilization, Hif1a is essential for developmental processes under physiological oxygen levels of 2–9% O2 in mouse embryos (Carmeliet et al, 1998; Iyer et al, 1998; Ryan et al, 1998). Furthermore, Hif1a is required for normal expression of several glycolytic enzyme genes such as Glut1, Pfk, Aldoa, Tpi1, Gapdh, Pgtk, and Ldha under the ambient oxygen level as well as in 1% O2 in mouse embryonic stem (ES) cells (Iyer et al, 1998; Ryan et al, 1998). Therefore, impaired Hif1a protein production may be attributable to reduced glycolysis gene expression in Crk/Crl-deficient MEFs, although investigated in the ambient oxygen level (Fig 4). Many MTORC1-inducible genes have been identified with Hif1a- and Myc-binding sites, whereas Hif1a is essential for MTORC1-dependent glycolytic gene expression (Düvel et al, 2010). It was also reported that Myc stabilizes Hif1a post-translationally and that Myc-induced transcription requires Hif1a in the human immortalized mammary cell line IMEC in normoxia (Doe et al, 2012). We noted that Myc was one of the down-regulated genes in subset “red,” and our ChIP-Seq results also indicated reduced association of H3K27Ac and Pol2 markers with Myc in Crk/Crl-deficiency induced MEFs (Supplemental Data 2 and Fig S12).

Vascular endothelial growth factor A (VEGFA) is one of the targets of Hif1a (Forsythe et al, 1996). It has been reported that IGF1 can stimulate VEGFA mRNA expression by stabilizing HIF1A protein in human colon cancer cell line HCT116 (Fukuda et al, 2002). We noted that VEGFA was down-regulated in subset “red,” thus commonly affected by Crk and Crkl (Supplemental Data 2). Analysis of the ChIP-Seq signals around the VEGFA gene revealed that its promoter-proximal region was poorly associated with H3K27Ac and Pol2 CTD phospho-SS, thus indicating that VEGFA promoter activity was suppressed in Crk/Crl-deficient MEFs (Fig S12). Although IGF deficiency has not been linked to DiGeorge-like anomaly in humans or in animal models, a positive role for Igf1 has been demonstrated in promoting mesoderm development and vasculogenesis in mouse embryoid bodies (Piecwicz et al, 2012). VEGFA is known as a dosagesensitive gene for normal development and VEGF164 isoform deficiency results in DiGeorge-like anomaly in mice (Carmeliet et al, 1996; Ferrara et al, 1996; Stalmans et al, 2003). Reduced vegfa also shows genetic interactions with Tbx1x knockdown in zebrafish (Stalmans et al, 2003). Therefore, reduction of VEGFA expression may also contribute to an impaired genetic network in which Crk and Crkl may have common intersection with Tbx1.
Figure 7. An activated Rapgef1 partially rescues aspects of Crk/Crl deficiency in MEFs.
(A) Overexpression of C3GF partially blocked Crk/Crl-deficiency-induced cell size changes. The histograms show distributions of cell sizes as estimated by FSC-H measurements in FACS analysis of ~6,000–7,000 cells in the G1 phase in each group. To compare the distributions, a boxplot was generated below the histograms. Human RAPGEF1 fused to a farnesylation sequence (C3GF) or empty vector was introduced into MEFs before Crk/Crl deficiency induction by 4OHT. Two-way ANOVA followed by Tukey post hoc tests were performed for statistical comparisons.
(B) Overexpression of C3GF rescued Crk/Crl deficiency–induced inhibition of cell proliferation. Cell
investigate precise mechanisms that underlie the abnormal cell contact behavior (Fig 2B–D), a recent study reported that normal CIL relies on Fak and Src for coordinated redistribution of cell–matrix contacts and intracellular force in frog neural crest cells (Roycroft et al, 2018). Because Crk and Crkl mediate signals partly from Fak and Src (Shin et al, 2004; Birge et al, 2009; Watanabe et al, 2009), it is plausible that Crk and/or Crkl are also required for traction force redistribution, a key mechanism for repulsive locomotion as an essential feature of mesenchymal cells. In this regard, it is noteworthy that Crk/Crkl deficiency inhibits focal adhesions functionally and structurally (Figs 3A and C and 7F and G). Focal adhesions include several mechanosensor proteins such as the Crk/Crkl SH2-binding protein p130Cas (Bcar1), and the small G-protein Rap1 has been identified as a critical participant in mechanotransduction and mechanosensing (Sawada et al, 2006; Lakshmikanthan et al, 2015; Freeman et al, 2017). Because an activated mutant of the Crk/Crlk SH3-binding protein Rapgefi (C3G) rescues Crk/Crlk deficiency for glycolysis, cell proliferation, and focal adhesions in MEFs (Fig 7), the Crk/Crlk-Rapgefi-Rap1 axis is a modular pathway which senses multiple extracellular signals such as Igf1 and integrin-dependent mechanotransduction. While Crk/Crlk deficiency exacerbated the blebbing phenotype induced by 2DG, the phenotype was different from the focal adhesion phenotype in Crk/Crlk-deficient MEFs (Figs 6B and 7F and G). Because activation of Rapgefi-rescued cell size/glucose metabolism as well as focal adhesions in Crk/Crlk deficiency, it is tempting to hypothesize that glucose metabolism may be regulated downstream of focal adhesions/cell–matrix adhesion.

It is also noteworthy that delayed postnatal growth is common among DGS/22q11.2DS patients (McDonald-McGinn et al, 2015). A recent study has reported that a 22q11.2DS patient with a small stature had growth-hormone and IGF1 deficiency (Bossi et al, 2016). Therefore, impaired responses to IGF1 that we found in MEFs may also have clinical relevance. In addition, although the number of reported cases is relatively few, maternal diabetes has been linked to thymic and kidney defects associated with tetralogy of Fallot and other congenital disorders in infants without a deletion in 22q11.21 (Novak & Robinson, 1994; Digilio et al, 1995; Cirillo et al, 2017; Taliana et al, 2017). We speculate that maternal glucose metabolism may be a possible contributing factor that could partly explain large variations of penetrance and expressivity observed among 22q11.2DS patients. Future studies are warranted to investigate the mechanisms by which the cell-adhesion signaling axis involving Crk/Crlk, and Rapgefi regulates the epigenetic network important for metabolism and proper development of tissues affected in DiGeorge/22q11.2DS patients.

Materials and Methods

Generation of Crk conditional knockout mice

The mouse Crk gene was targeted in an 129S6-derived ES cell line using a homologous recombination vector assembled using genomic fragments isolated from an 129-derived genomic library as well as FRT-PGKneo–FRT (FneoF) and IoxP sequences as illustrated in Fig S1. Targeted ES cells were injected into C57BL/6 blastocysts to generate chimeric mice via standard technique in the Transgenic and ES Cell Technology Mouse Core Facility at the University of Chicago. Highy chimeric animals were then backcrossed with C57BL/6J. The PGKneo cassette was removed by a cross with the FLPerr mouse (B6.129S4-Gt(ROSA)26Sortm1(FigI)Dym). Mice heterozygous for Crk and FLPerr were then backcrossed with C57BL/6J to segregate out FLPerr. Crk heterozygous mice without neo or flp were then selected as a knockout-ready strain, Crk (1–Crk-flxed exon 7; B6.129S4-CrklloxPtm1(Tgneo))). We previously generated and reported a Crkl conditional knockout (B6;129S4–CrklflloxPtm1(CreERT2)Shahi/J)) (Haller et al, 2017; Lopez-Rivera et al, 2017). To make distinction easier from Crk1–, we call the Crk knockout-ready strain Crhl2– because Crhl exon 2 is flanked by two IoxP sites. After more than five generations of backcross with B6, some Crk fl– and Crhl fl–/– mice were crossed with R26 Cre-ER22 strain (B6.129-Gt(Rosa)26Sortm1(cre/ERT2)Wtsi/J) or with Mespcre (Saga et al, 1999) to set up 4-hydroxymetamoxifen (4OHT)-inducible or mesoderm-specific knockout, respectively. For some experiments, Crhl2– or Crkl2– (deletion of Crk exon 1 or Crk exon 2, respectively) was generated as a knockout allele by crossing the knockout-ready strains and a global-deletion strain, Meox2creFl (B6.129S4–Meox2creFl1(Sor)) (Tallquist & Soriano, 2000). Meox2creFl was then segregated out by backcross with C57BL/6, and Crkl2– and Crkld2– heterozygous mice were maintained by continued backcross with C57BL/6. In some experiments, Crkl2– were crossed with Tbx1– heterozygotes (a gift from Virginia Papaioannou) which had been maintained by continued backcross with C57BL/6 more than 11 generations. Mouse embryos were isolated at various stages of development by timed mating. Mice and embryos were genotyped using PCR primers listed in Table S3. All mouse works were carried out in strict accordance with the protocols approved by the Institutional Animal Care and Use Committee of the University of Chicago.

---

**Numbers were counted in tissue culture plates for 3 d after plating. Bars indicate standard deviations from triplicate determinations (n = 3). Two-way ANOVA followed by Tukey post hoc tests were performed for statistical comparisons. (C) C3GF restored expression of the glycolytic enzyme genes. Expression of glycolytic genes were determined in real-time/quantitative RT-PCR. Bars indicate standard deviations. Two-way ANOVA followed by Tukey post hoc tests were performed on raw Ct values (n = 3). (D) C3GF blocked Crk/Crlk deficiency from reducing the level of fructose-1,6-bisphosphate (F1,6P2). Bars indicate standard deviations from triplicate determinations (n = 3). Two-way ANOVA followed by Tukey post hoc tests were performed for statistical comparisons. (B) Immunoblots show C3GF-dependent rescues on S6, S6K, and Akt phosphorylation associated with elevated phosphorylation of the focal adhesion protein p130Cas (Bcar1). (F) C3GF restored phosphorylated Bcar1, phosphotyrosine, and Fak localization at focal adhesions. Representative fluorescent microscopy images are shown. (G) Violin plots show quantitative results of focal adhesions. Focal adhesions were identified by localization of Fak in immunostained MEFs followed by automated image acquisition and analysis as described in the Materials and Methods section (also see Fig S1). The sample size (the number of cells per group) was 1,481, 516, 1,666, and 1,374 in a 2X2 experimental design (Uninduced/Vector Only, 4OHT-Induced/Vector Only, Uninduced/C3GF, and 4OHT-induced/C3GF groups, respectively) after applying the cutoffs indicated in Fig S1 to minimize the possibility of counting artifacts in staining and segmentation. However, inclusion of all cells without cutoffs did not affect the statistical outcome. Statistical analysis was performed by a global pseudo rank method with Tukey tests adjusted for multiple comparisons using the mcp function in the npcorpc package written in the programming language R (konetschke et al, 2015). Similar statistical outcome was obtained by two-way ANOVA after log2 transformation (Fig S1D). White circles in each violin plot indicate the position of the median.**
RNA in situ hybridization

Anti-sense RNA probes were generated from pBluescript plasmids that included ~700-bp fragments isolated from the 3’ UTR of mouse 
Crk and Crkl cDNAs. The full-length cDNAs were synthesized by RT-
PCR using oligo(dT) and gene-specific 5’ UTR primers using total 
RNA isolated from C57BL/6J E10.5 embryos. The cDNA sequences 
were confirmed by low-throughput Sanger sequencing from 
plasmid DNA. RNA in situ hybridization was carried out in E10.5 
mouse embryos isolated from C57BL/6J mice as previously de-
scribed (Guris et al, 2006).

MEFs

Primary MEFs were isolated from individual embryos at E11.5 and 
cultured in DMEM high-glucose formula supplemented with 0.1 mM 
2-mercaptoethanol and 10% fetal bovine serum (HyClone) as previ-
ously described (Li et al, 2002). Embryos and MEFs were dissociated 
using Accutase or TrypLE (Thermo Fisher Scientific). MEFs were split 1:3 
for maintenance every 3 d. Cre-mediated gene deficiency was induced by 0.25 μM (Z)-4-hydroxytamoxifen (Sigma-Aldrich) for 24 h in MEFs 
having a genetic background of R26 Cre-ER<sup>2</sup>, and then washed and 
replated 1:3 into new plates. Cells were harvested 48 h after removal of 
4OHT as deficiency-induced MEFs for experiments, unless otherwise 
indicated. In some experiments, glass coverslips or culture plates were 
coated with 0.1% gelatin (porcine skin, Sigma-Aldrich), bronectin 
(bovine plasma; Sigma-Aldrich), or poly-L-lysine (Sigma-Aldrich) be-
fore experimental replating.

For some experiments, MEFs were stimulated with recombinant 
human IGF1 (291-G1; R&D Systems) for 15 min after a short serum 
starvation period of 3 h (longer serum-free starvation caused ap-
optosis in Crk/Crkl double-deficient cells). To determine the effect 
of medium glucose concentrations, MEFs were incubated with glucose-
free DMEM supplemented with various concentrations of glucose 
and 10% dialyzed FBS after glucose deprivation down to 0.1% for 24 h. 
The starvation concentration of glucose was determined as shown in 
Fig S8.

For measurements of cell spreading, MEFs were dissociated with 
Accutase and suspended in serum-free DMEM, then plated on gelatin-
coated plates at a low density so that most cells do not contact each 
other. Cells were fixed at each time point, and only adherent cells were 
pictured under a 10× objective after wash. The number of pixels that 
each cell occupied were determined in eight most spread cells se-
lected per 
each cell occupied were determined in eight most spread cells se-
lected per time point per group using ImageJ (thus, each data point 
represents a collection of data from a total of 72 cells).

To estimate cell size, light scatter (FSC-H, FSC-A, SSC-H, and SSC-
A) were measured in a fluorescence-activated cell sorting machine 
(FACS Canto II; BD Bioscience), after fixing cells with ethanol and 
stained using PI/RNase staining buffer (S50825; BD Pharmingen). 
~6,000 or more cells were measured in each group.

Transfection and viral transduction

To transfect or infect MEFs for transducing exogenous transgene 
expression, primary MEFs were kept on the 3T3 protocol until their 
proliferation was easily maintained and, therefore, considered 
spontaneously immortalized (passage 15 or greater). To generate 
MEFs that express an activated RAPGEF1 (C3G), a full coding se-
quence of human C3G fused to the RAS farnesylation site (C3GF, a 
gift from Michiyuki Matsuda) was subcloned into pMX-ires-GFP vector for retrovirus production (pMX-C3GF-ires-GFP). Ectropic 
retrovirus was generated in Plat-E packaging cells and used to 
infected immortalized MEFs per standard protocols. Control MEFs 
were generated with pMX-ires-GFP without C3GF. GFP-positive cells 
were then selected by FACS and maintained for experiments. In 
some experiments, in-frame fusions of EGFP and human CRK or 
CRKL was constructed using pEGFPC2 plasmid (Clontech-TAKARA). 
Human embryonic kidney 293 cells were transfected with the plasmid 
to overexpress EGFP-CRK or CRKL using Lipofectamine LTX (Invitrogen) 
as recommended in the manufacturer’s protocol.

Immunofluorescence staining

For detection of Hif1α proteins, MEFs induced for Crk/Crkl deficiency 
were replated in 96-well plates at a density of 4 × 10<sup>4</sup> cells/well 24 h 
before harvest (the time of harvest was 72 h from the time 4OHT was 
added as described above). Some cells were treated with CoCl<sub>2</sub> at a 
final concentration of 0.5 mM for 4 h before fixation with 2% 
paraformaldehyde. Cells were permeabilized with 0.1% Triton X-100 
for 5 min and blocked with 10% FBS and Blocking One (Nacalai). 
Hif1α was detected with mouse monoclonal anti-HIFα antibody 
clone H1Alpha67 (NB100-123; Novas) and goat anti-mouse IgG 
conjugated with Dylight 549 (Thermo Fisher Scientific). Nuclei 
were counter-stained with DAPI. Fluorescent signals were detected in 
IN Cell Analyzer 2000 (GE Healthcare).

For staining other cellular proteins, MEFs were replated on glass 
coverslips coated with 0.1% gelatin. 24 h after replating, MEFs were 
fixed for 15 min with 4% paraformaldehyde, 1.5% BSA fraction V, and 
0.5% Triton X-100 in 1× CB cytoskeletal buffer (10 mM MES, pH 6.8, 3 
mM MgCl<sub>2</sub>, 138 mM KCl, and 2 mM EGTA). After three washes, the 
cells were incubated with the primary antibody (mouse monoclonal 
anti-FAK, clone 4-47, 05-537; EMD-Millipore; rabbit anti-p130CAS 
phospho-Y249, #4014; Cell Signaling Technology; or mouse mono-
clonal anti-phosphotyrosine, 4G10, 05-321; EMD-Millipore) diluted 1: 
200 in 1× CB buffer containing 1.5% BSA and 0.5% Triton X-100 for 1 h. 
After three washes, the cells were incubated with a Dylight 550– 
conjugated secondary antibody (Thermo Fisher Scientific) that matches 
the species specificity of the primary antibody diluted 1:1,000 in 1× 
CB buffer containing 1.5% BSA and 0.5% Triton X-100 for 1 h. F-actin 
and nuclei/DNA was stained with Alexa Fluor 647 phalloidin and 
DAPI (Thermo Fisher Scientific) according to a standard staining 
method. Stained cells were mounted in Prolong Gold Antifade 
(Thermo Fisher Scientific) and observed under a 60× oil objective 
len in DeltaVision Elite deconvolution microscope system (GE 
Healthcare). In some experiments, MEFs were replated with or 
without induction of Crk/Crkl deficiency in a gelatin-coated 96-well 
plate and stained as above for high-throughput image acquisitions 
in IN Cell Analyzer 2500HS (GE Healthcare). For such experiments, 
additional counterstaining was performed using HCS CellMask 
Deep Red (Invitrogen/Thermo Fisher Scientific) for identification 
and segmentation of the cell body (see also the Automated Image 
Analysis section below).

https://doi.org/10.26508/lsa.201900635 vol 3 | no 2 | e201900635
Immunoblot

Cell lysates were prepared with lysis buffer containing 1% NP-40 (or IGEPA) CA-630, 50 mM Tris pH 7.5, 10% glycerol, 0.2 M NaCl, 2 mM MgCl2, Complete protease inhibitor cocktail (Roche) and PhosSTOP (Roche). Immunoblots were prepared on Immobilon-P membrane (EMD-Millipore) after electrophoresis in SDS-polyacrylamide gel (7.5–15% gradient) using a standard protocol. Proteins were then detected using the following primary antibodies: anti-phospho-S6 S240/244 (EMD-Millipore), anti-phospho-p70 S6k T389 (CST#9205), anti-phospho-akt S473 (CST#4060), anti-pan AKT (CST#2920), anti-phospho-TSC2 T1462 (CST#617), anti-TSC2 (CST#3990), anti-phospho-p130CAS Y247 (CST#4014), anti-p130CAS (BD 610272), anti-CRK (BD 610035), anti-phospho-p70 S6K T389 (CST#9205), anti-p70 S6k (CST#9202), anti-phospho-AKT S473 (CST#4060), anti-pan AKT (CST#2920), anti-phospho-TSC2 T1462 (CST#617), anti-TSC2 (CST#3990), anti-phospho-p130CAS Y247 (CST#4014), anti-p130CAS (BD 610272), anti-CRK (BD 610035), anti-CRKL (05-414; EMD Millipore), and anti-C3G (sc-15359; Santa Cruz Biotechnology). Using a horseradish peroxidase-conjugated secondary antibody matching the species of the primary antibody, chemiluminescence was detected on the immunoblot in Image-Quant LAS4000 (GE Healthcare).

RNA-Seq

RNA-seq analysis was conducted using total RNA isolated from primary MEFs. Four embryos were isolated for each genotype (Crk+/+, Crkl+/+, Crk f/f, Crkl f2/f2, or wild-type; all compound heterozygous for R26creERT2) as four independent samples per genotype, with an exception that we isolated only two wild-type embryos as negative control samples. When cells were subconfluent, each cell lot was then induced or uninduced for deficiency with 4-hydroxytamoxifen for 24 h, then replated on to new plates without 4OHT to expand for 48 h before harvest. Total RNA was isolated using a Qiaquick RNA isolation kit as described in the manufacturer’s protocol. The quality of isolated RNA was checked in a 2100 Bioanalyzer (Agilent Technologies). RNA sequencing was performed in an Illumina HiSeq 2500 with paired end reads. The average inner fragment size was ~250 bp. The sequence reads were filtered by PRINSEQ version 0.20.4 for sequence data quality control (Schmieder & Edwards, 2011), then mapped to the mouse genome sequence in GRCm38,p3 using TopHat2 (version 2.1.0) with the following parameters: -mate-inner-dist 250 -mate-std-dev 40 (Kim et al, 2013). Aligned read counts assigned to RefSeq annotations were obtained by the featureCounts (version 1.4.6) function of Rsubread (Liao et al, 2014) and analyzed by DESeq2 version 1.12.4 (Love et al, 2014). As each lot of primary MEFs were traceable with or without 4OHT treatment, pairwise comparisons were performed in each individual MEF for evaluating DE with or without the effects of cre-induced recombination.

Pathway analysis

We used the DE genes identified in RNA-Seq analysis above (FDR < 0.05) using Ingenuity Pathway Analysis (QIAGEN) or KEGG. KEGG annotations were added using the R package clusterProfiler (Yu et al, 2012). Mapped KEGG enrichments were visualized using FuncTree (Uchiyama et al, 2015) available at https://bioviz.tokyo/functree/.

Metabolome analysis

Cellular metabolites were analyzed by CE-TOFMS using primary MEFs for Crk or Crkl single gene deficiency and Crk/Crkl double deficiency as well as their uninduced controls as previously described (Uetaki et al, 2015). Results obtained from three independent samples were compared for each genotype between deficiency-induced and uninduced MEF groups using Welch’s t test for each metabolite (P < 0.05).

ChiP and ChiP-Seq

MEFs induced Crk/Crkl deficiency were harvested 30 h after removal of 4OHT, along with control MEFs treated with vehicle instead of 4OHT. Samples were prepared using SimpleChiP Plus Enzymatic Chromatin IP Kit (#9005; Cell Signaling Technology) as recommended in the manufacturer’s protocol. Antibodies used were anti-histone H3 acetylated lysine 27 rabbit polyclonal antibody (ab4729; Abcam) and anti-RNA polymerase 2 CDT repeat YSPTPS (phospho-SS) mouse monoclonal antibody (clone 4H8, ab5408; Abcam). For immunoprecipitation, Dynabeads protein G or M-280 sheep anti-mouse IgG (Thermo Fisher Scientific) was used to best match the species range and specificity for each primary antibody. For quantitative analysis of selected glycolysis genes, Pol2 ChiP samples were used for real-time PCR using SYBR Green with the primer pairs listed on Table S3. For ChiP-Seq experiments, high-throughput sequencing was conducted in an Illumina HiSeq 2500 for 36-bp single end reads. All ChiP-Seq data were first processed with Cutadapt and FastQc under the wrapper software Trim Galore v0.4.4 with the “--q 30” option in Cutadapt to trim off low quality ends (https://www.bioinformatics.babraham.ac.uk/projects/trim_galore/). The sequence output was then aligned to the mouse reference genome GRCm38.p3 using Bowtie v1.2.1 with the “--m 1” option (Langmead et al, 2009). Duplicates were removed from aligned reads using PICARD v1.14 (https://broadinstitute.github.io/picard/). The mapped reads were then standardized for each experiment to an effective mouse genome size of 2,652,783,500 bases as “reads per genome coverage or content” (RPGC) using the utility package deepTools v2.1.1 (Ramirez et al, 2016). Utilities in deepTools were also used for downstream analysis of normalized ChiP-Seq results. ChiP-Seq results were normalized against the background signals obtained from whole cell extracts for corresponding cell groups.

Automated image quantification

Image segmentation was performed using a custom MATLAB script (available upon request; MATLAB is a programming language available from Mathworks). First, we acquired a set of images of CellMask, DAPI, and anti-vinculin (or anti-FAK) staining to segment nuclei and focal adhesions (Asghari & Jalali, 2015), while performing empirical optimizations of the input parameters. To segment the cells, we first smoothed the images and used a threshold based on the average intensity of the dimmest
20% of pixels in the CellMask channel corresponding to background pixels. We used the DAPI staining to determine if any segmented regions contained multiple nuclei corresponding to under-segmented regions. These regions were re-segmented with a higher threshold and expanded by region growing. The intersection of these expanded regions was used to segment this larger region into single cells. After this step, we removed any remaining regions with 0 or multiple nuclei.

We noticed that blebbing cells consistently showed rough cell boundaries having bead-like bulging membrane protrusions with high curvatures, small cell/nuclear area ratio, and a high cytoplasmic intensity of vinculin signals relative to that of the nucleus. To estimate the boundary curvature for each single cell, we first performed smoothing edge boundaries by Savitzky–Golay filter (Diederick, 2019). An instantaneous curvature was estimated for each set of neighboring pixels using a code deposited at the MATLAB Central File Exchange (Mjaavatten, 2019), where the curvature is defined as 1/r (r is the radius for a point, P). To standardize threshold parameters, we analyzed five randomly selected images from each group (2-by-2 groups: with or without 3 mM 2DG with or without Crk/Crkl deficiency induction) and manually identified blebbing and non-blebbing cells among the segmented cells (Fig S9). In automated analysis, the cells were then ruled “blebbing” when they have a combination of the three standardized threshold parameters: an average single-cell curvature larger than 0.029 pixel\(^{-1}\), a ratio of nuclear to cytoplasmic vinculin intensity <1.15, and a cell/nuclear area size ratio <4.5 (Fig S9). Using these standardized parameters, “blebbing” cells were then identified in a total of 7,409 MEFs segmented (Fig 6B). As some cells appear to be outliers (blue arrows in Fig S9B), we filtered out the cells having the DAPI-positive area size smaller than 1,000 pixels or with the cell area greater than 150,000 pixels, whereas the median nuclear and cell area sizes were 8,638 and 35,665 pixels, respectively. The filtering process removed 809 cells and 148 cells from analysis, respectively.

For quantification of focal adhesions, we used the cell and nuclear segmentations to remove noise from our focal adhesion segmentation by ruling out segmentation outside the cells or inside the nucleus. We further refined the focal adhesion selection by removing areas smaller than 30 pixels or lower intensity than the cell average staining intensity. We confirmed that differences in the number of focal adhesions segmented in each condition could not be attributed to systematic differences in rates of segmentation errors for different image sets (Figs 7G and S11).

### Resources

The knockout-ready Crk conditional strain will be available through the Jackson Laboratory (JAX Stock #032874). The RNA-Seq and ChIP-Seq data have been deposited to the DDBJ (www.ddbj.nig.ac.jp) and have been assigned the accession numbers DRA007302 and DRA007305, respectively. The deposited read data will be available via the BioProject page at NCBI as the BioProjects PRJDB4241 and PRJDB4413, respectively (www.ncbi.nlm.nih.gov/bioproject/).

### Supplementary Information

Supplementary Information is available at https://doi.org/10.26508/lsa.201900635.

---

### Acknowledgements

The authors thank VE Papaioannou for the Tbx1 null strain, P Soriano for the Meox2\(^{−/−}\) and R26\(^{flox}\) strains, Y Saga for the Mesp1\(^{−/−}\) strain, M Matsuda for the Csg-F plasmid, L Degenstein and The Transgenic and ES Cell Technology Core for assisting generation of the Crk conditional mutant strain. This work was supported in part by research grants from JSPS (17H06299, 17H06302, and 18H04031), the Nagase Science Technology Foundation, and Astellas Foundation for Research on Metabolic Disorders to M Okada; from JSPS (17H06299) to Y Suzuki, from JST PRESTO (JPMJPR1507) and Japan AMED (17ek0109187h0002) to T Yamada, and from JSPS (15H01522, 16H04901, 17H05654, and 18H04805) and JST PRESTO (JPMJPR1557) to S Fukuda.

### Author Contributions

A Imamoto: conceptualization, data curation, formal analysis, supervision, investigation, and writing—original draft, review, and editing.

S Ki: investigation.

L Li: investigation.

K Iwamoto: data curation and formal analysis.

Y Maruthamuthu: investigation and methodology.

J Devany: software, investigation, and methodology.

O Lu: investigation.

T Kanazawa: investigation.

S Zhang: investigation.

T Yamada: data curation, software, formal analysis, and visualization.

A Hirayama: investigation.

S Fukuda: investigation and methodology.

Y Suzuki: investigation and methodology.

M Okada: conceptualization, supervision, funding acquisition, and writing—review and editing.

### Conflict of Interest Statement

The authors declare that they have no conflict of interest.

### References

Alfano D, Almontone A, Cortes C, Bilio M, Kelly RG, Baldini A (2019) Tbx1 regulates extracellular matrix-cell interactions in the second heart field. Hum Mol Genet 28: 2295–2308. doi:10.1093/hmg/ddz058

Asghari MH, Jalali B (2015) Edge detection in digital images using dispersive phase stretch transform. Int J Biomed Imaging 2015: 1–6. doi:10.1155/2015/687819

Benita Y, Kikuchi H, Smith AD, Zhang MQ, Chung DC, Xavier RJ (2009) An integrative genomics approach identifies Hypoxia Inducible Factor-1 (HIF-1)-target genes that form the core response to hypoxia. Nucleic Acids Res 37: 4587–4602. doi:10.1093/nar/gkp425

Birge RB, Kalodimos C, Inagaki F, Tanaka S (2009) Crk and Crkl adaptor proteins: Networks for physiological and pathological signaling. Cell Commun Signal 7: 13. doi:10.1186/1478-811x-7-13

Bossi G, Gertosio C, Meazza C, Farello G, Bozzola M (2016) Failure to thrive as presentation in a patient with 22q11.2 microdeletion. Ital J Pediatr 42: 14. doi:10.1186/s13052-016-0224-0

Bruno DL, Anderlid BM, Lindstrand A, van Ravenswaaij-Arts C, Ganesamoorthy D, Lundin J, Martin CL, Douglas J, Nowak C, Adam MP, et al (2010) Further molecular and clinical delineation of co-locating...
17p13.3 microdeletions and microduplications that show distinctive phenotypes. J Med Genet 47: 299–311. doi:10.1136/jmg.2009.069906

Carmeliet P, Ferreira V, Breier G, Polleyef S, Kieckens L, Gertsenstein M, Fahrig M, Vandenhoek A, Harpal K, Eberhardt C, et al (1996) Abnormal blood vessel development and lethality in embryos lacking a single VEGF allele. Nature 380: 435–439. doi:10.1038/380435a0

Carmeliet P, Dor Y, Herbet JM, Fukumura D, Brusselmans K, Dewerchin M, Carmeliet P, Ferreira V, Breier G, Pollefeyt S, Kieckens L, Gertsenstein M, Fahrig Ferrara N, Carver-Moore K, Chen H, Dowd M, Lu L, O Fingar DC, Salama S, Tsou C, Harlow E, Blenis J (2002) Mammalian cell size is controlled by mTOR and its downstream targets S6K1 and 4EBP1: evidence in vivo by targeting chromatin. Nat Commun 7: 11688. doi:10.1038/ncomms11688

Ginzberg MB, Kafri R, Kirschner M (2015) Cell biology. On being the right (cell) size. Science 348: 1245075. doi:10.1126/science.1245075

Gu L, Yi Z, Zhang Y, Ma Z, Zhu Y, Gao J (2017) Low dose of 2-deoxy-D-glucose kills acute lymphoblastic leukemia cells and reverses glucocorticoid resistance via N-linked glycosylation inhibition under normoxia. Oncotarget 8: 30978–30991. doi:10.18632/oncotarget.16046

Guris DL, Fantes J, Tara D, Druker BJ, Imamoto A (2001) Mice lacking the homologue of the human 22q11.2 gene CRKL phenocopy neurocristopathies of DiGeorge syndrome. Nat Genet 27: 293–298. doi:10.1038/ng655

Guris DL, Duester G, Papaioannou VE, Imamoto A (2006) Dose-dependent interaction of Tbx1 and Crkl and locally aberrant RA signaling in a model of del22q11 syndrome. Dev Cell 10: 81–92. doi:10.1016/j.devcel.2005.12.002

Hallier M, Mo Q, Imamoto A, Lamb DJ (2017) Murine model indicates 22q11 signaling adaptor CRKL is a dosage-sensitive regulator of genitourinary development. Proc Natl Acad Sci U S A 114: 4981–4986. doi:10.1073/pnas.1619522114

Iyer NV, Kotch LE, Agani F, Leung SW, Laughner E, Wenger RH, Gassmann M, Gearhart JD, Lawer AM, Yu AF, et al (1998) Cellular and developmental control of O2 homeostasis by hypoxia-inducible factor 1α. Genes Dev 12: 169–182. doi:10.1101/gad.12.2.169

Kim D, Pertea G, Trapnell C, Pimentel H, Kelley R, Salzberg SL (2014) TopHat2: Accurate alignment of transcriptomes in the presence of insertions, deletions and gene fusions. Genome Biol 14: R36. doi:10.1186/gb-2013-14-4-r36

Konietschke F, Placek M, Schaarschmidt F, Hothorn LA (2015) pnrcomp: An R software package for nonparametric multiple comparisons and simultaneous confidence intervals. J Stat Softw 64: 1–17. doi:10.18637/jss.v064.i09

Lakshmikanthan S, Zheng X, Nishijima Y, Sobczak M, Szabo A, Vasquez-Vivar J, Zhang DX, Chrzanowska-Wodnicka M (2015) Rap1 promotes endothelial mechanosensing complex formation, NO release and normal endothelial function. EMBO Rep 16: 628–637. doi:10.15252/embr.201439846

Langmead B, Trapnell C, Pop M, Salzberg SL (2009) Ultrafast and memory-efficient alignment of short DNA sequences to the human genome. Genome Biol 10: R25. doi:10.1186/gb-2009-10-3-r25

Li L, Okura M, Imamoto A (2002) Focal adhesions require catalytic activity of hypoxia-inducible factor 1. Mol Cell Biol 22: 1203–1217. doi:10.1128/mcb.22.4.1203-1217.2002

Liao Y, Smyth GK, Shi W (2014) FeatureCounts: An efficient exact mapping program for assigning sequence reads to genomic features. Bioinformatics 30: 923–930. doi:10.1093/bioinformatics/btt253

Lloyd AC (2013) The regulation of cell size. Cell 154: 1194–1205. doi:10.1016/j.cell.2013.08.053

Lopez-Rivera E, Liu YP, Verbitsky M, Anderson BR, Capone VP, Otto EA, Yan Z, Mitrotti A, Martino J, Steers NJ, et al (2017) Genetic drivers of kidney defects in the DiGeorge syndrome. N Engl J Med 376: 742–754. doi:10.1056/nejmoa169009

Love MI, Huber W, Anders S (2014) Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. Genome Biol 15: 550. doi:10.1186/s13059-014-0550-8

Manning BD, Toker A (2017) AKT/PKB signaling: Navigating the network. Mol Cell 67: 1633–1647. doi:10.1016/j.molcel.2017.09.014

McDonald-McGinn DM, Sullivan KE, Marino B, Philip N, Swillen A, Vorstman JAZ, Zakai EH, Emanuel BS, Vermeesch JR, Morrow BE, et al. (2015) 22q11 deletion syndrome. Nat Rev Dis Primers 1: 15071. doi:10.1038/nrdp.2015.71

The CRK family controls glucose metabolism and cell size

Imamoto et al. https://doi.org/10.26508/lsa.201900635 vol 3 | no 2 | e201900635

17 of 18
The CRK family controls glucose metabolism and cell size. Imamoto et al. 2019. https://doi.org/10.26508/lsa.201900635 vol 3 | no 2 | e201900635