A Protein Scaffold Coordinates SRC-Mediated JNK Activation in Response to Metabolic Stress

Shashi Kant
*University of Massachusetts Medical School*

*Et al.*

Let us know how access to this document benefits you.

Follow this and additional works at: https://escholarship.umassmed.edu/metnet_pubs

Part of the Biochemistry Commons, Cell Biology Commons, Cellular and Molecular Physiology Commons, Endocrinology Commons, and the Molecular Biology Commons

Repository Citation

Creative Commons License
This work is licensed under a Creative Commons Attribution-Noncommercial-No Derivative Works 4.0 License. This material is brought to you by eScholarship@UMassChan. It has been accepted for inclusion in UMass Metabolic Network Publications by an authorized administrator of eScholarship@UMassChan. For more information, please contact Lisa.Palmer@umassmed.edu.
A Protein Scaffold Coordinates SRC-Mediated JNK Activation in Response to Metabolic Stress

Graphical Abstract

Highlights
- JIP1 is required for saturated-fatty-acid-stimulated JNK activation
- JIP1 is a SRC substrate and recruits the SRC tyrosine kinase to lipid rafts
- Tyrosine-phosphorylated JIP1 binds the SH2 domain of the RAC exchange factor VAV
- SRC-phosphorylated VAV bound to JIP1 activates JNK by the RAC/MLK pathway

Authors
Shashi Kant, Claire L. Standen, Caroline Morel, ..., Wojciech Swat, Richard A. Flavell, Roger J. Davis

Correspondence
shashi.kant@umassmed.edu (S.K.), roger.davis@umassmed.edu (R.J.D.)

In Brief
Kant et al. demonstrate that scaffold protein JIP1 is required for palmitate-stimulated redistribution of SRC to lipid rafts. Phosphorylation of JIP1 on tyrosine mediates SH2 domain interactions with both SRC and the RAC exchange factor VAV. This signaling complex causes RAC-dependent activation of the MLK pathway that activates JNK.
A Protein Scaffold Coordinates SRC-Mediated JNK Activation in Response to Metabolic Stress

Shashi Kant, Claire L. Standen, Caroline Morel, Dae Young Jung, Jason K. Kim, Wojciech Swat, Richard A. Flavell, and Roger J. Davis

1Program in Molecular Medicine
2Division of Cardiovascular Medicine, Department of Medicine
3Division of Endocrinology, Metabolism and Diabetes, Department of Medicine
University of Massachusetts Medical School, Worcester, MA 01605, USA
4Department of Pathology and Immunology, Washington University School of Medicine, St. Louis, MO 63110, USA
5Howard Hughes Medical Institute and Department of Immunobiology, Yale University School of Medicine, New Haven, CT 06520, USA
6Howard Hughes Medical Institute, Worcester, MA 01605, USA
7These authors contributed equally
8Lead Contact
Correspondence: shashi.kant@umassmed.edu (S.K.), roger.davis@umassmed.edu (R.J.D.)
http://dx.doi.org/10.1016/j.celrep.2017.08.025

SUMMARY

Obesity is a major risk factor for the development of metabolic syndrome and type 2 diabetes. How obesity contributes to metabolic syndrome is unclear. Free fatty acid (FFA) activation of a non-receptor tyrosine kinase (SRC)-dependent cJun NH2-terminal kinase (JNK) signaling pathway is implicated in this process. However, the mechanism that mediates SRC-dependent JNK activation is unclear. Here, we identify a role for the scaffold protein JIP1 in SRC-dependent JNK activation. SRC phosphorylation of JIP1 creates phosphotyrosine interaction motifs that bind the SH2 domains of SRC and the guanine nucleotide exchange factor VAV. These interactions are required for SRC-induced activation of VAV and the subsequent engagement of a JIP1-tethered JNK signaling module. The JIP1 scaffold protein, therefore, plays a dual role in FFA signaling by coordinating upstream SRC functions together with downstream effector signaling by the JNK pathway.

INTRODUCTION

Human obesity is a worldwide health problem that is associated with metabolic syndrome and the development of insulin resistance and type 2 diabetes (Flegal et al., 2013). Effects of obesity on metabolic syndrome are mediated, in part, by increased amounts of saturated free fatty acid (FFA) in the blood (Kahn et al., 2006). A key signaling mechanism that is activated by FFA is the cJun NH2-terminal kinase (JNK) stress response pathway (Davis, 1994, 2000). Studies using JNK-deficient mice demonstrate that JNK signaling is required for the development of obesity and insulin resistance (Sabio and Davis, 2010). Consequently, components of the JNK signaling pathway represent potential targets for the design of drugs that may be useful for the treatment of metabolic syndrome (Sabio and Davis, 2010).

The mechanism of JNK activation caused by FFA is unclear. Recent studies have identified the non-receptor tyrosine kinase SRC (Holzer et al., 2011), the small GTPase RAC1 (Sharma et al., 2012), and the mixed-lineage protein kinase (MLK) family of MAP kinase kinase kinase (MAP3K) (Jaeschke and Davis, 2007; Kant et al., 2013; Sharma et al., 2012) as components of an FFA-stimulated signaling pathway that activates JNK. However, the mechanism that mediates signaling has not been established. Here, we report that the scaffold protein JIP1 can serve to link SRC, RAC1, and MLK in an FFA-stimulated signaling pathway.

RESULTS

JIP1 Is Required for FFA-Stimulated SRC Activation and Redistribution to Lipid Rafts

The scaffold protein JIP1 binds SRC family members (Kennedy et al., 2007; Nihalani et al., 2007) and is implicated in the MLK pathway that leads to JNK activation (Jaeschke et al., 2004; Morel et al., 2010; Whithmarsh et al., 1998, 2001). It is established that FFA causes SRC redistribution to Triton-insoluble lipid rafts (Holzer et al., 2011). To test whether JIP1 might contribute to SRC function, we examined whether FFA treatment caused a similar redistribution of JIP1. This analysis demonstrated increased amounts of JIP1, SRC, and activated pY416-SRC in the Triton-insoluble fraction of cells exposed to FFA (Figure 1A). Moreover, immunofluorescence analysis of Triton-permeabilized cells demonstrated co-localization of Triton-permeabilized cells demonstrated co-localization of JIP1 and SRC in FFA-treated cells (Figure 1B).

The co-regulation of SRC and JIP1 (Figures 1A and 1B) indicated that JIP1 (encoded by the Mapk8ip1 gene) may contribute to FFA signaling. We, therefore, compared the response of wild-type (WT) and Mapk8ip1−/− cells to FFA. Treatment of WT primary murine embryo fibroblasts (MEFs) with FFA caused JNK activation and the redistribution of SRC and activated
The requirement of JIP1 for FFA-stimulated JNK activation (Figure 1C) may represent a general role of JIP1 in JNK signaling. Together, these data demonstrate that JIP1 is required for the regulation of both SRC and JNK by FFA.

The JIP1 JNK-Binding Domain Is Required for HFD-Induced Insulin Resistance

To test whether JIP1-mediated JNK activation is relevant to the metabolic stress response in vivo, we established mice lacking pY416-SRC to lipid rafts (Figures 1C and 1D). In contrast, Mapk8ip1−/− MEFs were resistant to FFA-stimulated JNK activation and SRC redistribution to Triton-insoluble lipid rafts. Together, these data demonstrate that JIP1 is required for the regulation of both SRC and JNK by FFA.
the JNK-binding domain of JIP1 (Mapk8ip1^D/D^JBD mice, designated as JIP1^D/D^JBD mice). The core of the JNK-binding domain (Leu160-Asn161-Leu162) binds a hydrophobic pocket on JNK (Heo et al., 2004; Whitmarsh et al., 1998) and is required for JIP1-mediated JNK activation (Whitmarsh et al., 1998). This core motif was replaced with Gly160-Arg161-Gly162 in JIP1^D/D^JBD mice (Figure S2). Control studies demonstrated that MEF derived from JIP1^D/D^JBD mice were resistant to FFA-stimulated JNK activation (Figure 2A). Comparison of WT and JIP1^D/D^JBD mice fed a chow diet (ND) or a high-fat diet (HFD) demonstrated that the HFD-induced insulin intolerance detected in WT mice was suppressed in JIP1^D/D^JBD mice (Figure 2B). The improved insulin tolerance of HFD-fed JIP1^D/D^JBD mice was associated with markedly decreased HFD-induced hyperinsulinemia, compared with WT mice (Figure 2C).

Hyperinsulinemic-euglycemic clamp analysis of glucose infusion rates confirmed that HFD-fed JIP1^D/D^JBD mice were more insulin sensitive than HFD-fed WT mice (Figure 2D). Moreover, the HFD-fed JIP1^D/D^JBD mice exhibited decreased hepatic glucose production (Figure 2E), increased hepatic insulin action (Figure 2F), and increased whole-body glucose turnover (Figure 2G), compared with HFD-fed WT mice. These phenotypes were associated with increased energy expenditure and decreased obesity (Figure S3), reduced adipose tissue hypertrophy (Figures S4A and S4B), and reduced hepatic steatosis (Figure S4C). This protection of HFD-fed JIP1^D/D^JBD mice against obesity and insulin resistance is similar to that detected in JNK-deficient mice (Sabio and Davis, 2010). Together, these data establish that JIP1-mediated JNK activation contributes to the HFD-induced metabolic stress response in vivo.

**VAV, RAC1, MLK, and MKK7 Contribute to JIP1-Mediated JNK Activation**

To examine the mechanism of JIP1-mediated JNK activation caused by exposure of cells to saturated FFA, we tested the role of JNK pathway components that interact with JIP1, including the MAP2K isoform MKK7 and the MLK family of MAP3K (Whitmarsh et al., 1998). Analysis of WT and Map2k7^−/− Map3k10^−/− Map3k11^−/− MEFs demonstrated that MKK7 is required for FFA-stimulated JNK activation (Figure 3A). In contrast, we found that MLK protein kinases were not required for FFA-stimulated SRC and activated SRC redistribution to lipid rafts (Figure 3C). This analysis identifies SRC as an upstream component of the JIP1-mediated MLK-MKK7-JNK signaling pathway that is activated by FFA.

MLK isoforms can be activated by the RHO family GTPase RAC1 by binding to a conserved MLK CRIB domain (Teramoto et al., 2011). Therefore, we tested whether MLKs contribute to JIP1-mediated JNK activation. Comparison of WT and Map2k7^−/− Map3k10^−/− Map3k11^−/− MEFs demonstrated that MLKs are required for FFA-stimulated JNK activation (Figure 3A). Moreover, the HFD-fed JIP1^D/D^JBD mice exhibited decreased hepatic glucose production (Figure 2E), increased hepatic insulin action (Figure 2F), and increased whole-body glucose turnover (Figure 2G), compared with HFD-fed WT mice. These phenotypes were associated with increased energy expenditure and decreased obesity (Figure S3), reduced adipose tissue hypertrophy (Figures S4A and S4B), and reduced hepatic steatosis (Figure S4C). This protection of HFD-fed JIP1^D/D^JBD mice against obesity and insulin resistance is similar to that detected in JNK-deficient mice (Sabio and Davis, 2010). Together, these data establish that JIP1-mediated JNK activation contributes to the HFD-induced metabolic stress response in vivo.
Figure 3. VAV, RAC1, MLK, and MKK7 Contribute to JIP1-Mediated JNK Activation

(A and B) WT and Map2k7+/− MEFs (A) or Map3k10−/− Map3k11−/− MEFs (B) were treated (for 16 hr) without (BSA) or with palmitate/BSA (FFA) and subsequently examined by immunoblot analysis.

(C) The lipid raft fraction of WT and Map3k10−/− Map3k11−/− MEFs treated (for 4 hr) without (BSA) or with palmitate/BSA (FFA) was examined by immunoblot analysis.

(D) WT and Map3k10−/− Map3k11−/− Map3k10−/− Map3k11−/− MEFs were treated with BSA or FFA (for 16 hr) and examined by immunoblot analysis.

(E) WT and Vav1−/− Vav2−/− Vav3−/− (Vav−−) cells were treated with BSA or FFA (for 16 hr) and examined by immunoblot analysis.

(F) WT and Map3k10−/− Map3k11−/− MEFs were treated with BSA or FFA, JIP1 immunoprecipitates (F) and isolated lipid rafts (G) were examined by immunoblot analysis.

(H) WT and Vav1−/− Vav2−/− Vav3−/− (Vav−−) cells were transduced with a retrovirus expressing EE-tagged JIP1. The cells were treated with BSA or FFA/BSA and examined by immunoblot analysis of cell lysate (I), JIP1 immunoprecipitates (J), or isolated lipid rafts (K).

(L) Constitutively activated SRC (SRCY529F) and SRC expression plasmids were used in transfection assays with WT and Map3k10−/− Map3k11−/− MEFs. Cell lysates were prepared at 24 hr post-transfection and examined by immunoblot analysis.
et al., 1996). To test whether this CRIB-mediated mechanism contributes to FFA-stimulated JNK activation, we examined the effect of an inactivating mutation in the MLK3 CRIB domain (Ile192 Ser498 replaced with Ala192 Ala498) in the context of deficiency of the redundant isoform MLK2 (Kant et al., 2011). Comparison of WT and Map3k10−/−Map3k11−/−CRIB/CRIB MEFs demonstrated that the MLK CRIB domain is essential for FFA-stimulated JNK activation (Figure 3D). This mechanism is consistent with the observation that RAC1 activation is required for the regulation of JNK activity by FFA (Sharma et al., 2012).

The exchange factor VAV has been implicated in JNK activation (Crespo et al., 1996; Kant et al., 2011). To test whether VAV contributes to FFA-stimulated JNK activation, we compared JNK activity in WT and VAV-deficient (Vav1−/−Vav2−/−Vav3−/−) cells. This analysis demonstrated that VAV is required for FFA-stimulated JNK activation (Figure 3E). Moreover, VAV treatment caused an interaction between VAV and JIP1 that was detected by co-immunoprecipitation analysis (Figure 3F). VAV treatment also caused JIP1-dependent redistribution of VAV to lipid rafts (Figure 3G), although VAV was not required for lipid rafts association of JIP1 (Figure 3H).

FFA Causes SRC-Mediated Tyrosine Phosphorylation of JIP1 and VAV

The interaction between VAV and JIP1 was associated with FFA-stimulated tyrosine phosphorylation of both proteins (Figure 3F). The role of JIP1 tyrosine phosphorylation is unclear, but it is established that tyrosine phosphorylation of VAV increases GTP/GDP exchange activity on RAC1 (Crespo et al., 1997). To examine the mechanism of FFA-stimulated tyrosine phosphorylation of JIP1 and VAV, we tested the potential role of SRC family tyrosine kinases. These studies were performed using Src−/−Fyn−/−Yes−/−(SFY) cells. Treatment of SFY cells with FFA did not cause JNK activation (Figure 3I) or tyrosine phosphorylation of JIP1 and VAV (Figure 3J). In contrast, complementation analysis demonstrated that the expression of SRC restored both JNK activation and tyrosine phosphorylation of both JIP1 and VAV in FFA-treated cells (Figures 3I and 3J). SRC was required for JIP1 redistribution to lipid rafts (Figure 3K). SRC family protein kinases, therefore, function as components of the JNK signaling pathway activated by treatment of cells with FFA.

To test whether SRC-mediated activation of JNK is mediated by the MLK pathway, we expressed constitutively activated SRC (SRC325E) in WT and Map3k10−/−Map3k11−/−MEFs. Activated SRC caused JNK activation selectively in the WT MEFS (Figure 3L). These data demonstrate that the MLK pathway is an essential mediator of SRC-induced JNK activation.

Previous studies of JIP1 tyrosine phosphorylation have implicated roles for both ABL (Dajas-Bailador et al., 2008) and SRC (Kennedy et al., 2007; Nihalani et al., 2007). To confirm the role of SRC in FFA-stimulated tyrosine phosphorylation of JIP1, we examined the effects of drugs that selectively inhibit ABL and SRC. This analysis demonstrated that the SRC-selective inhibitor AZD0530, but not the ABL-selective inhibitor Gleevec, prevented FFA-stimulated tyrosine phosphorylation of JIP1 (Figure 4A). These data confirm that SRC family tyrosine kinases contribute to FFA-stimulated tyrosine phosphorylation of JIP1 (Figure 4A).

Tyrosine Phosphorylation of JIP1 Is Required for FFA-Stimulated JNK Activation

JIP1 is extensively phosphorylated (D’Ambrosio et al., 2006), including on sites of tyrosine (Y278, Y409, and Y427) phosphorylation (Dajas-Bailador et al., 2008; Kennedy et al., 2007; Nihalani et al., 2007). This phosphorylation may create sites of interaction for signaling proteins with SH2 domains. Indeed, phosphotyrosine-dependent binding of JIP1 to the SH2 domain of SRC family proteins has been identified (Kennedy et al., 2007; Nihalani et al., 2007). A similar interaction might account for the binding of JIP1 to VAV. To test SH2 domain binding to JIP1, we examined the interaction of JIP1 with the SH2 domains of SRC and VAV2. This analysis demonstrated that JIP1 from FFA-treated cells bound to SRC-SH2 and VAV2-SH2 (Figures 4B and 4C). In contrast, JIP1 from control cells did not bind VAV2-SH2, but a low level of interaction with SRC-SH2 was detected (Figures 4B and 4C).

To examine the role of JIP1 tyrosine phosphorylation in JNK activation caused by saturated FFA, we performed complementation analysis using Mapk8ip1−/−MEFs expressing WT or mutated JIP1 proteins. Two sites of tyrosine phosphorylation on JIP1 (pY409DNC and pY427EEA) are similar to the optimal sequence (pYEEI) for phosphotyrosine binding by the SRC-SH2 domain (Songyang et al., 1993). Mutational analysis indicated that Y409 and Y427 serve partially redundant functions. We, therefore, examined the function of JIP1 with dual mutation at Y409 plus Y427. This analysis demonstrated that tyrosine phosphorylation of JIP1 was increased when cells were treated with FFA (Figure 4D). In contrast, phosphoryrosine was not detected on the mutated JIP1Y409/427F protein (Figure 4D). Co-immunoprecipitation analysis demonstrated that the interaction of SRC with JIP1 in control and FFA-treated cells was suppressed in studies with the mutated JIP1Y409/427F protein (Figure 4D). Furthermore, the FFA-stimulated redistribution of JIP1 and SRC to lipid rafts depends on these sites of JIP1 tyrosine phosphorylation (Figure 4E). These data indicate that Y409 and Y427 are required for FFA-stimulated JIP1 tyrosine phosphorylation and SRC binding.

One site of tyrosine phosphorylation on JIP1 (pY278LTP) is similar to the optimal sequence (pYMEP) for phosphotyrosine binding by the VAV SH2 domain (Songyang et al., 1994). Comparison of Mapk8ip1−/−MEFs expressing WT and JIP1Y278F proteins demonstrated that treatment with FFA promoted the co-immunoprecipitation of VAV with JIP1 and that this interaction was dependent on Y278 (Figure 4F). The phosphorylation site Y278 is, therefore, required for the FFA-induced interaction of JIP1 and VAV.

To test the requirement of JIP1 tyrosine phosphorylation for FFA-stimulated JNK activation, we compared Mapk8ip1−/−MEFs expressing WT JIP1 and mutated JIP proteins (JIP1Y278F or JIP1Y409/427F). This analysis demonstrated that expression of WT JIP1 in Mapk8ip1−/−MEFs restored FFA-stimulated JNK activation (Figure 4G). In contrast, FFA-stimulated JNK activation was not detected in Mapk8ip1−/−MEFs expressing JIP1Y278F or JIP1Y409/427F (Figure 4G). Together, these data indicate that the tyrosine-phosphorylation-dependent interaction of JIP1 with SRC and VAV is required for FFA-stimulated JNK activation (Figure 4H).
DISCUSSION

An understanding of the physiological mechanism of FFA-stimulated JNK activation has remained elusive, because several potential pathways have been implicated. Indeed, it is likely that FFA-stimulated JNK activation may occur by more than one mechanism in vivo, possibly in a cell-type- and context-specific manner. These potential mechanisms include: (1) the FFA-induced endoplasmic reticulum unfolded protein response (Fu et al., 2012); (2) FFA ligand binding to G-protein-coupled receptors (Alvarez-Curto and Milligan, 2016; Moran et al., 2016); (3) FFA metabolism and accumulation of signaling lipids, including diacylglycerol, ceramide, and sphingosine-1-phosphate (Hu et al., 2009; Montell et al., 2001; Schmitz-Peiffer et al., 1999); (4) FFA-stimulated NBR1-MEK signaling (Hernandez et al., 2014); and (5) FFA-stimulated lipid raft signaling (Holzer et al., 2011). It is also possible that FFA causes JNK activation as part of a generalized lipotoxic stress response (Neuschwander-Tetri, 2010).

Here, we examined the lipid raft signaling mechanism of FFA-stimulated JNK activation (Holzer et al., 2011). Previous studies
have demonstrated roles for SRC (Holzer et al., 2011), RAC1 (Sharma et al., 2012), and the MLK group of MAP3K (Jaeschke and Davis, 2007; Kant et al., 2013). While VAV can load GTP on RAC1 and activate MLK (Teramoto et al., 1996), the role of SRC in this signaling pathway has not been established (Holzer et al., 2011).

Our analysis demonstrates that the scaffold protein JIP1 plays a dual role in FFA signaling by co-ordinating upstream SRC functions together with downstream effector signaling by the JNK pathway (Figure 4H). SRC phosphorylation of JIP1 creates phosphotyrosine interaction motifs that bind the SH2 domains of SRC and the guanine nucleotide exchange factor VAV. These interactions are required for SRC-induced tyrosine phosphorylation and activation of VAV and the subsequent engagement of a JIP1-tethered JNK signaling module (Figure 4H). It is likely that this mechanism contributes to physiological regulation, because defects in JIP1 (Jaeschke et al., 2004; Morel et al., 2010), including disruption of the JIP1-JNK-binding site (Figure 2), strongly suppress the consequences of HFD consumption by mice.

The interaction of JIP1 with signaling molecules provides an opportunity for the design of small molecules to disrupt FFA-stimulated JNK activation. This approach has been used to identify drugs that target the JNK/JIP1 interaction (Chen et al., 2009; Stebbins et al., 2008) but could be extended to the new interactions identified by our study. Since JNK has many physiological functions, drugs that target the JIP1 scaffold protein may provide greater selectivity for suppression of JNK activation caused by metabolic stress. Such drugs may be useful for the treatment of obesity-induced metabolic stress, including metabolic syndrome and type 2 diabetes (Keneto et al., 2004).

**EXPERIMENTAL PROCEDURES**

**Mice**

C57BL/6J mice (stock number 000664) and Rosa-CreERT mice (stock number 00487) (Bades et al., 2003) were obtained from The Jackson Laboratory. We have previously described Mapk8ip1−/− mice (Whitmarsh et al., 2001), Map2k7LoxP/LoxP mice (Hübner et al., 2012), Map3k10−/− mice (Kant et al., 2011), Map3k11−/− mice (Branco et al., 2005), Map3k11Lcrb1Δcrb1 mice (Kant et al., 2011), and Vav1−/−; Vav2−/−; Vav3−/− mice (Fujikawa et al., 2003) on a C57BL/6J strain background.

Mice with a defect in the JNK-binding domain of JIP1 (replacement of Leu160-Asn161-Leu162 with Gly160-Arg161-Gly162) were established using homologous recombination in embryonic stem cells (ESCs) using standard electroporation with this vector and selected with 200 μM gancyclovir (Syntex). ESC clones with the floxed JBD mutation and also the introduction of an M gancyclovir cassette was excised using Southern blot analysis (Figures S2A and S2B). The targeting vector was also designed to introduce a dual role in FFA signaling by co-ordinating upstream SRC function and the guanine nucleotide exchange factor VAV. These interactions were required for SRC-induced tyrosine phosphorylation and activation of VAV and the subsequent engagement of a JIP1-tethered JNK signaling module (Figure 4H). It is likely that this mechanism contributes to physiological regulation, because defects in JIP1 (Jaeschke et al., 2004; Morel et al., 2010), including disruption of the JIP1-JNK-binding site (Figure 2), strongly suppress the consequences of HFD consumption by mice.

The interaction of JIP1 with signaling molecules provides an opportunity for the design of small molecules to disrupt FFA-stimulated JNK activation. This approach has been used to identify drugs that target the JNK/JIP1 interaction (Chen et al., 2009; Stebbins et al., 2008) but could be extended to the new interactions identified by our study. Since JNK has many physiological functions, drugs that target the JIP1 scaffold protein may provide greater selectivity for suppression of JNK activation caused by metabolic stress. Such drugs may be useful for the treatment of obesity-induced metabolic stress, including metabolic syndrome and type 2 diabetes (Keneto et al., 2004).

**Cell Culture**

SFY fibroblasts, complemented without and with SRC (Klinghoffer et al., 1999), were obtained from the American Type Culture Collection (CRL2459 and CRL2448). RIN56 cells expressing Glu-Glu-tagged JIP1b have been described previously (Standen et al., 2009). Primary WT MEFs, Mapk8ip1−/− MEFS, Mapk8ip1Lcrb1Δcrb1MEFS, Mapk3k10−/−Mapk8ip1−/− MEFS, and Mapk3k10−/−Map3k11Lcrb1Δcrb1 MEFS were isolated and cultured in vitro (Kant et al., 2011). Map2k7−/− MEFS were prepared by treating Rosa-CreERT Map2k7Lcrb1Δcrb1MEFS with 1 μM 4-hydroxytamoxifen (24 hr), MEF transduced with pBABE-puro retroviral vectors were selected by incubation (24 hr) with 10 μg/mL puromycin (Lamb et al., 2003). Studies of primary MEFs were performed using cells between passages 3 and 5. Cells were transferred to DMEM supplemented with 0.5% fetal bovine serum (Life Technologies) and incubated (1 hr) prior to treatment with 0.5% BSA or 0.75 mM palmitate/0.5% (w/v) BSA. Cells were treated with 1 μM matinib (Gleevec) or 10 nM Saracatinib (AZD0530) (Selleck Chemicals). Plasmid transfection assays were performed using Lipofectamine (Life Technologies).

**Lipid Raft Isolation**

Methods for detergent-dependent lipid raft isolation were described earlier (Holzer et al., 2011; Lingwood and Simons, 2007). Briefly, cells were prepared using Triton lysis buffer (20 mM Tris [pH 7.4], 1% Triton X-100, 10% glycerol, 137 mM NaCl, 2 mM EDTA, 25 mM β-glycerophosphate, 1 mM sodium orthovanadate, 1 mM PMSF, and 10 μg/mL of aprotinin plus leupeptin), sheared with a 25G needle and incubated at 4°C (30 min). Optiprep (Sigma) 40%-30%-5% step gradients were prepared.
prior to flotation of the cell lysate on the step gradient and centrifugation (16 hr at 260,000 x g). Raft and non-raft fractions were collected and examined by protein immunoblot analysis.

**Immunoblot Analysis**

Cell extracts were prepared using Triton lysis buffer (20 mM Tris [pH 7.4], 1% Triton X-100, 10% glycerol, 137 mM NaCl, 2 mM EDTA, 25 mM β-glycerophosphate, 1 mM sodium orthovanadate, 1 mM PMSF, 10 μg/mL aprotinin and leupeptin) for 20 min at ice. Triton-soluble and -insoluble fractions were prepared by centrifugation at 13,000 rpm (10 min) and examined by protein immunoblot analysis. Primary antibodies were obtained from Cell Signaling Technology (MLK3, pJNK, SRC, and pY416-SRC), Covance (Glu-Glu tag), Life Technologies (JNK, Flotillin1, and JIP1), Millipore (T7 tag), Santa Cruz Biotechnology (VAV2 and Flotillin2), and Sigma (α-Tubulin). Immune complexes were detected by enhanced chemiluminescence (New England Nuclear) using an ImageQuant LAS 4000 (General Electric).

**Immunoprecipitation**

Cell extracts were prepared using NP-40 lysis buffer (150 mM NaCl, 50 mM Tris [pH 8.0], 5 mM EDTA, 1% NP-40, 1 mM sodium orthovanadate, 1 mM PMSF, 10 mg/mL aprotinin and leupeptin) and incubated (5 hr at 4°C) with 10 μg control nonimmune rabbit immunoglobulin G (IgG) (Santa Cruz Biotechnology) or 10 μg rabbit antibodies to the Glu-Glu tag (Covance) or JIP1 (Yasuda et al., 1999). Immune complexes isolated using protein G sepharose (Covance) and SRC (Cell Signaling Technology) were used. The antibodies were diluted in blocking buffer at RT and then incubated (1 hr) with primary antibodies (anti-mouse or anti-rabbit immunoglobulin G conjugated to Alexa Fluor 488 or 546 (Life Technologies). Fluorescence was detected by incubation with anti-mouse or anti-rabbit immunoglobulin G conjugated to Alexa Fluor 488 or 546 (Life Technologies). Fluorescence was visualized using a Leica TCS SP2 confocal microscope equipped with a 405-nm diode laser.

**SUPPLEMENTAL INFORMATION**

Supplemental Information includes Supplemental Experimental Procedures and four figures and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2017.08.025.

**AUTHOR CONTRIBUTIONS**


**ACKNOWLEDGMENTS**

We thank Vicky Benoit and Heather Leanard for technical assistance and Kathy Gemme for administrative assistance. These studies were supported by NIH grants DK107220 and DK112698 (to R.J.D.) and American Heart Association grant 16SDG29660007 (to S.K.). The UMASS Mouse Metabolic Phenotyping Center is supported by NIH grant DK093000 (to J.K.K.), R.A.F., and R.J.D. are investigators of the Howard Hughes Medical Institute.

Received: May 10, 2016
Revised: June 30, 2017
Accepted: July 29, 2017
Published: September 19, 2017

**REFERENCES**


