Identification of a Mitochondrial Target of Thiazolidinediones (mTOT)

William G. McDonald[1], Gregory S. Cavey[2], Serena L. Cole[1], Danielle D. Holewa[1], Angela S. Brightwell-Conrad[1], Cindy L. Wolfe[1], Jean S. Wheeler[1], Kristin R. Coulter[1], Rolf F. Kletzien[1], Jerry R. Colca[1]
[1]Metabolic Solutions Development Company, Kalamazoo, MI, USA.
[2]Southwest Michigan Innovation Center Core Life Science Lab, Kalamazoo, MI, USA.

Abstract

Thiazolidinediones (TZDs) are insulin sensitizing compounds with proven clinical efficacy but with an incompletely understood pleiotropic pharmacology and significant side effects that have limited their clinical utility. Here, we use a selective, photo-catalyzable affinity probe and mass spectrometry-based proteomics to identify a previously unrecognized mitochondrial target of insulin sensitizing agents. This newly identified mitochondrial target (mitochondrial Target of TZDs, mTOT) is part of a phylogenetically conserved complex in the mitochondrial membrane, which is responsible for binding of these agents to mitochondrial membranes. To determine the degree to which this complex is involved in the actions of insulin sensitizers, we have studied the ability of various analogs, including PPARγ-independent TZDs MSDC-0160 and MSDC-0602, to enhance differentiation of brown adipose tissue (BAT) progenitor cells from genetically modified mice. Ablation of the gene encoding mTOT results in embryonic lethality in mice and loss of drug-enhanced differentiation of BAT cells *in vitro*. Conversely, a targeted mutation that deletes the first 16 amino acids from the amino terminal sequence results in marked enhancement of differentiation in response to the active compounds. Insulin sensitizers of other chemical classes including MRL-24, also signal through this complex. The active compounds elicit a metabolic signal resulting in post-translational changes including activation of GSK3β associated with mitochondria. This process is necessary for differentiation of BAT cells since enforcement of Wnt signaling with LiCl or a specific inhibitor of GSK-3β (Chi-99021) completely blocked drug action. Elucidation of the mitochondrial TZD target and signaling mechanism provides an alternative approach to discovery of novel anti-diabetic agents that may avoid the side effects associated with direct activators of nuclear transcription factors

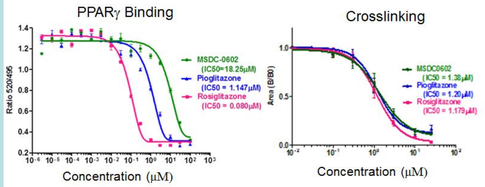
Background

- Insulin sensitizing thiazolidinediones were discovered empirically without regard to mechanism.
- It is generally thought that both the dose limiting side effects and the positive pleiotropic pharmacology occur through the adipocyte master regulator PPARγ.
- TZDs have PPARγ-independent effects (Chen *et al*, J. Biol. Chem. published 23 May 2012, 10.1074/jbc.M112.363960 http://www.jbc.org/cgi/content/abstract/M112.363960v1).
- We propose that significant insulin sensitizing pharmacology occurs through modulation of a mitochondrial target (mTOT) that has remained to be identified.

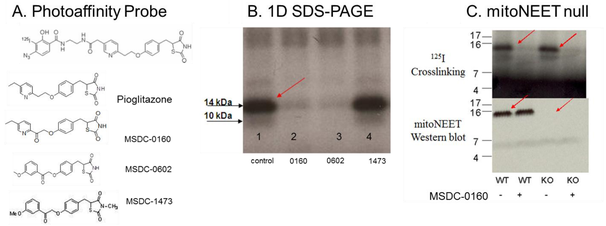
Overview

- We used drug analog photoaffinity crosslinking and MS proteomics to identify a phylogenetically conserved protein in the inner mitochondrial membrane as part of the TZD recognition complex.
- Proof of identity has been accomplished by expression and knockdown.
- Two previously uncharacterized protein family members play a role.
- Two papers published online by two separate groups in the May 24, 2012 ***Science Express*** have coincidentally found that these two proteins, which they renamed Mpc1 and Mpc2, are part of a pyruvate carrier mechanism.
- Together these findings place the TZD recognition complex (mTOT) at the "crossroads of metabolism" and provide a new way to look at insulin sensitizers.

All active TZDs bind to a mitochondrial target, however the ability to bind to and activate PPARγ varies greatly.

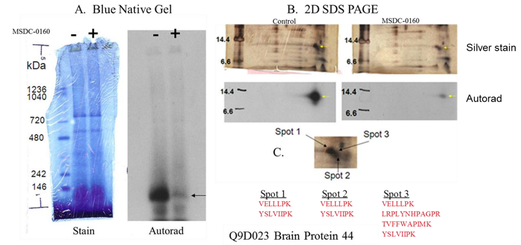


Photoaffinity probe identifies unique protein in mitochondrial membranes



- The TZD photoaffinity probe crosslinks a specific protein at about 14kDa.
- Crosslinking is specific as it is reduced by competition with biologically active TZDs (including pioglitazone) but not the inactive (e.g., 1473) analogs.
- This protein is not mitoNEET and remains to be identified.

Purification and Identification



maaagarglratyhrlmdkvelllpkklrplynhpagprtvffwapimkwglvcagladmarpaeklstaqstvlmatgfiwsryslviipknwslfavnffvgsagasqlfriwryrynqelkskgiq

- The labeled complex ran at about 150 kDa on Blue Native Gels (A).
- Separation of this complex on a second dimension SDS gel focused 3 spots (B).
- MS/MS identified the 4 peptides shown in red suggesting the crosslinked protein is BRP44.

A Mitochondrial Pyruvate Carrier Required for Pyruvate Uptake in Yeast, *Drosophila*, and Humans

Daniel K. Bricker,[1*] Eric B. Taylor,[2*] John C. Schell,[2*] Thomas Orsak,[2*] Audrey Boutron,[2] Yu-Chan Chen,[2] James E. Cox,[3] Caleb M. Cardon,[2] Jonathan G. Van Vranken,[2] Noah Dephoure,[4] Claire Redin,[4] Sihem Boudina,[1] Steven P. Gygi,[4] Michèle Brivet,[5] Carl S. Thummel,[2] Jared Rutter[2†]

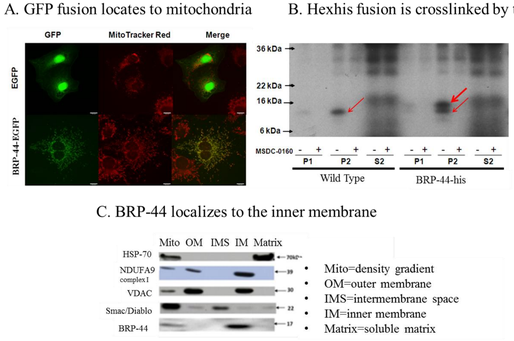
Sciencexpress / http://www.sciencemag.org/content/early/recent
24 May 2012 / Page 1/ 10.1126/science.1218099

Identification and Functional Expression of the Mitochondrial Pyruvate Carrier

Sébastien Herzig,[1] Etienne Raemy,[1] Sylvie Montessuit,[1] Jean-Luc Veuthey,[1] Nicola Zamboni,[2] Benedikt Westermann,[3] Edmund R.S. Kunji,[4] Jean-Claude Martinou[1*]

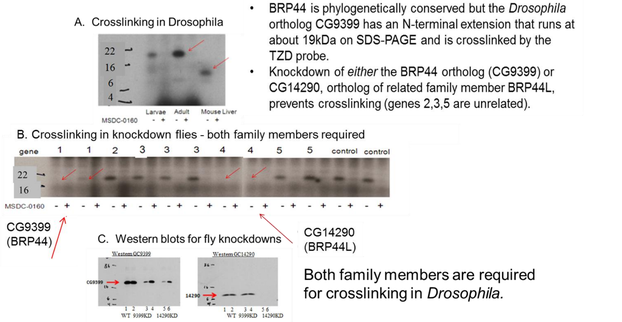
Sciencexpress / http://www.sciencemag.org/content/early/recent
24 May 2012 / Page 1/ 10.1126/science.1218530

Expressed BRP44 localizes to mitochondria and is crosslinked by the TZD probe



A and C, courtesy of Sandy Wiley and Anne Murphy, UCSD.

The photoprobe also specifically crosslinks a fly protein of larger size



- BRP44 is phylogenetically conserved but the *Drosophila* ortholog CG9399 has an N-terminal extension that runs at about 19kDa on SDS-PAGE and is crosslinked by the TZD probe.
- Knockdown of *either* the BRP44 ortholog (CG9399) or CG14290, ortholog of related family member BRP44L, prevents crosslinking (genes 2,3,5 are unrelated).

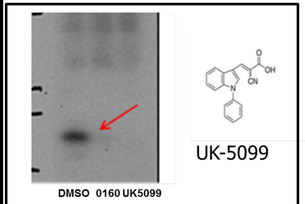
Both family members are required for crosslinking in *Drosophila*.

Collaboration with Medros, St. Louis (See also PO-2363).

Mammals	Fly	Yeast	New Term
BRP44	CG9399	YHR162W	MPC2
BRP44L	CG14290	YGL080W	MPC1

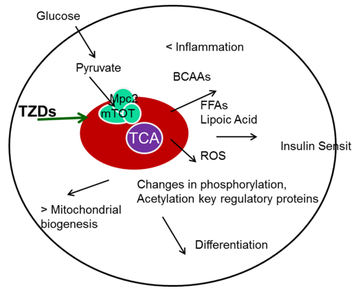
Common findings with emerging data for pyruvate carrier family

- The proteins migrate in a complex that runs at about 150 kDa on Blue Native gels.
- The complex contains both BRP44 (Mpc2) and BRP44L (Mpc1) and these proteins can be co-immunoprecipitated.
- These proteins are localized to the inner mitochondrial membrane.
- UK-5099, an inhibitor of the pyruvate carrier activity also blocks the crosslinking of the TZD probe.



UK-5099

MSDC-0160 directly inhibits pyruvate oxidation in permeabilized cells. See PO-2484.



The mTOT complex is involved in the regulation of pyruvate utilization.

Conclusions

- We have utilized drug analog photoaffinity crosslinking to identify a previously uncharacterized complex of proteins in the inner mitochondrial membrane.
- The protein crosslinked by this process is BRP44. This protein has been renamed Mpc2 by recent independent publications that show BRP44 is a key member in a protein family that constitutes the mitochondrial pyruvate carrier.
- The identity of the protein was confirmed by knockdown of expression as well as the expression of C-terminally tagged proteins.
- Modulation of the complex that regulates pyruvate entry into mitochondria places drug action at the crossroads of metabolism. These findings provide new ground toward understanding the pleiotropic effects of insulin sensitizers and how changes in metabolism can predict onset and corrections of diabetes.

See also Phase 2B data with prototype mTOT Modulator™- P-966; and 2363-PO and 2484-PO.

Identification of a Mitochondrial Target of Thiazolidinediones (mTOT)

WILLIAM G. MCDONALD[1], GREGORY S. CAVEY[2], SERENA L. COLE[1], DANIELLE D. HOLEWA[1], ANGELA S. BRIGHTWELL-CONRAD[1], CINDY WOLFE[1], JEAN WHEELER[1], KRISTIN COULTER[1], ROLF F. KLETZIEN[1], JERRY R. COLCA[1]

[1]METABOLIC SOLUTIONS DEVELOPMENT COMPANY, LLC, KALAMAZOO, MI, USA

[2]SOUTHWEST MICHIGAN INNOVATION CENTER, KALAMAZOO, MI , USA

metabolic solutions development company

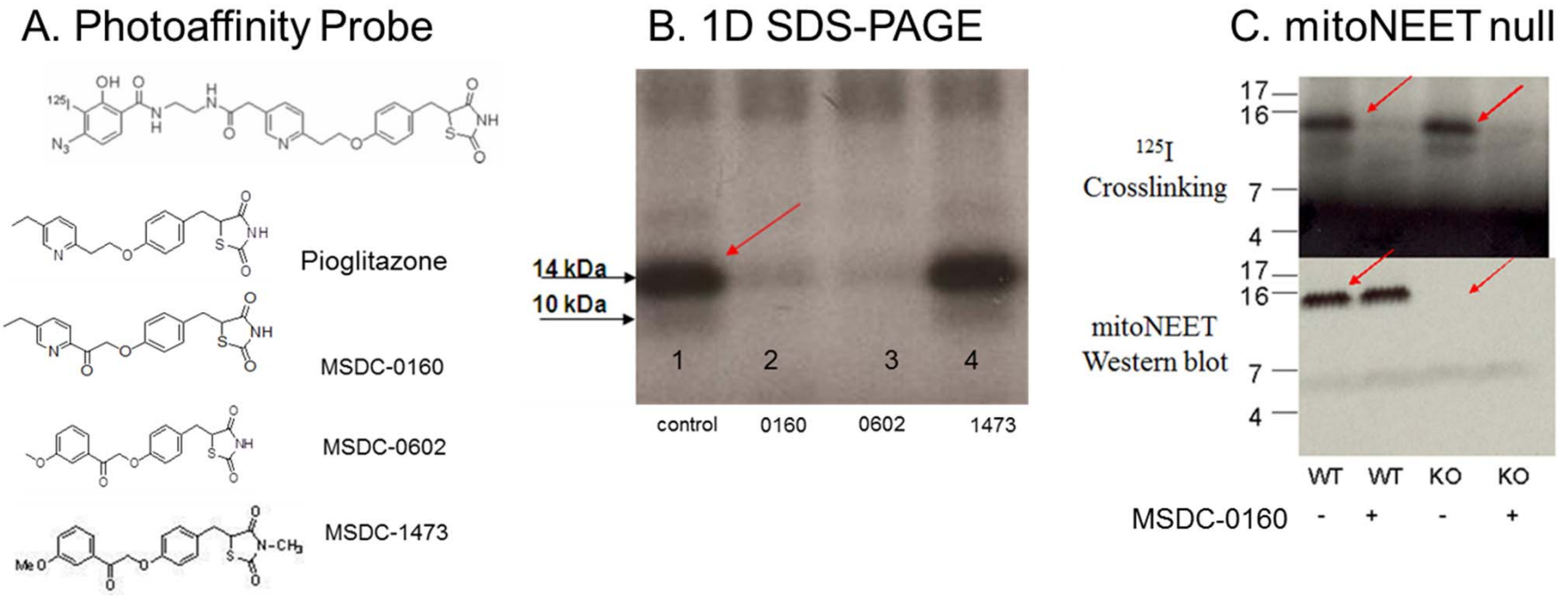
Background

- Insulin sensitizing thiazolidinediones were discovered empirically without regard to mechanism.
- It is generally thought that both the dose limiting side effects and the positive pleiotropic pharmacology occur through the adipocyte master regulator PPARγ.
- TZDs have PPARγ-independent effects (Chen *et al*, J. Biol. Chem. published 23 May 2012, 10.1074/jbc.M112.363960 http://www.jbc.org/cgi/content/abstract/M112.363960v1).
- We propose that significant insulin sensitizing pharmacology occurs through modulation of a mitochondrial target (mTOT) that has remained to be identified.

Overview

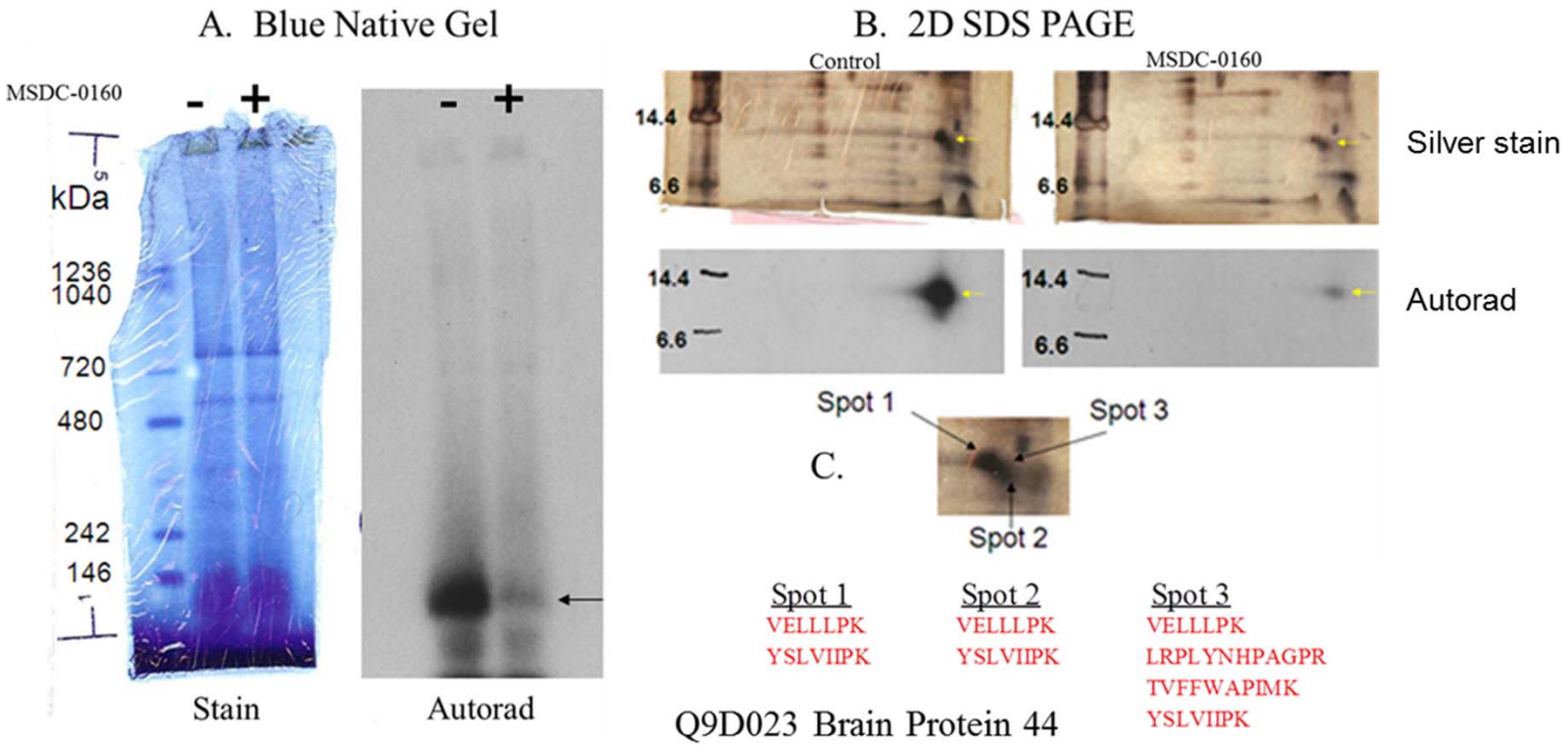
- We used drug analog photoaffinity crosslinking and MS proteomics to identify a phylogenetically conserved protein in the inner mitochondrial membrane as part of the TZD recognition complex.
- Proof of identity has been accomplished by expression and knockdown.
- Two previously uncharacterized protein family members play a role.
- Two papers published online by two separate groups in the May 24, 2012 ***Science Express*** have coincidentally found that these two proteins, which they renamed Mpc1 and Mpc2, are part of a pyruvate carrier mechanism.
- Together these findings place the TZD recognition complex (mTOT) at the “crossroads of metabolism” and provide a new way to look at insulin sensitizers.

Photoaffinity probe identifies unique protein in mitochondrial membranes



- The TZD photoaffinity probe crosslinks a specific protein at about 14kDa.
- Crosslinking is specific as it is reduced by competition with biologically active TZDs (including pioglitazone) but not the inactive (e.g., 1473) analogs.
- This protein is not mitoNEET and remains to be identified.

Purification and Identification

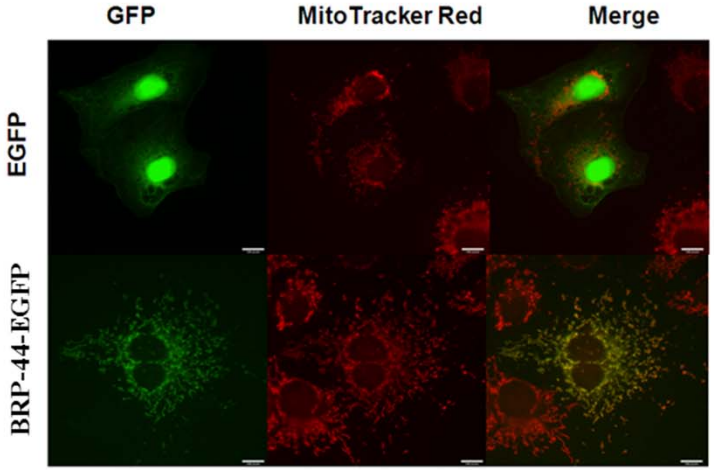


maaagarglratyhrlmdkvelllpkklrplynhpagprtvffwapimkwglvcagladmarpaeklstaqstvlmatgfiws ryslviipknwslfavnffvgsagasqlfriwrynqelkskgiq

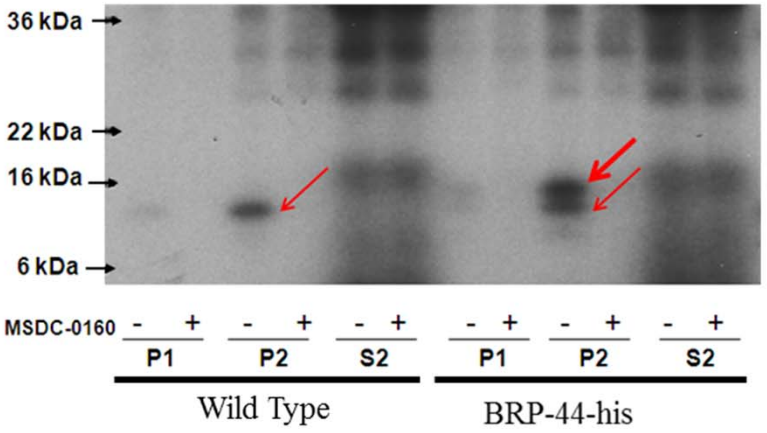
- The labeled complex ran at about 150 kDa on Blue Native Gels (A).
- Separation of this complex on a second dimension SDS gel focused 3 spots (B).
- MS/MS identified the 4 peptides shown in red suggesting the crosslinked protein is BRP44.

Expressed BRP44 localizes to mitochondria and is crosslinked by the TZD probe

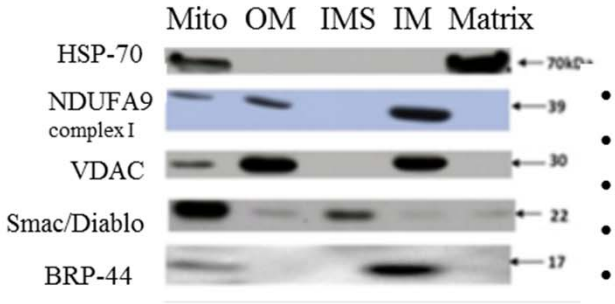
A. GFP fusion locates to mitochondria



B. Hexhis fusion is crosslinked by the probe



C. BRP-44 localizes to the inner membrane

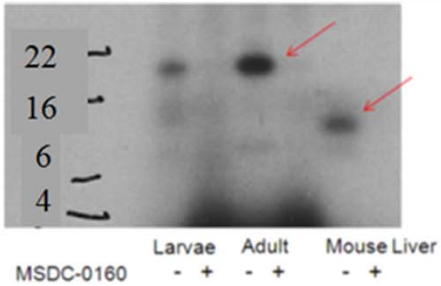


- Mito=density gradient
- OM=outer membrane
- IMS=intermembrane space
- IM=inner membrane
- Matrix=soluble matrix

A and C, courtesy of Sandy Wiley and Anne Murphy, UCSD.

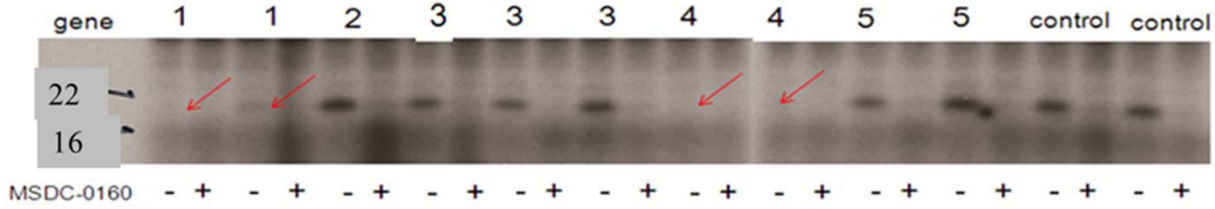
The photoprobe also specifically crosslinks a fly protein of larger size

A. Crosslinking in Drosophila



- BRP44 is phylogenetically conserved but the *Drosophila* ortholog CG9399 has an N-terminal extension that runs at about 19kDa on SDS-PAGE and is crosslinked by the TZD probe.
- Knockdown of *either* the BRP44 ortholog (CG9399) or CG14290, ortholog of related family member BRP44L, prevents crosslinking (genes 2,3,5 are unrelated).

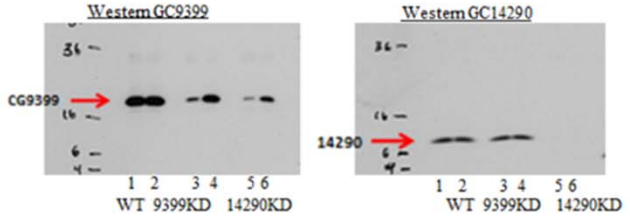
B. Crosslinking in knockdown flies - both family members required



CG9399 (BRP44)

CG14290 (BRP44L)

C. Western blots for fly knockdowns



Both family members are required for crosslinking in *Drosophila.*

Collaboration with Medros, St. Louis (See also PO-2363).

New Publications Confirm Findings and Suggest Mechanism

A Mitochondrial Pyruvate Carrier Required for Pyruvate Uptake in Yeast, *Drosophila*, and Humans

Daniel K. Bricker,[1*] Eric B. Taylor,[2*] John C. Schell,[2*] Thomas Orsak,[2*] Audrey Boutron,[3] Yu-Chan Chen,[2] James E. Cox,[4] Caleb M. Cardon,[2] Jonathan G. Van Vranken,[2] Noah Dephoure,[5] Claire Redin,[6] Sihem Boudina,[7] Steven P. Gygi,[5] Michèle Brivet,[3] Carl S. Thummel,[1] Jared Rutter[2]†

Sciencexpress / http://www.sciencemag.org/content/early/recent
24 May 2012 / Page 1/ 10.1126/science.1218099

Identification and Functional Expression of the Mitochondrial Pyruvate Carrier

Sébastien Herzig,[1] Etienne Raemy,[1] Sylvie Montessuit,[1] Jean-Luc Veuthey,[2] Nicola Zamboni,[3] Benedikt Westermann,[4] Edmund R.S. Kunji,[5] Jean-Claude Martinou[1*]

Sciencexpress / http://www.sciencemag.org/content/early/recent
24 May 2012 / Page 1/ 10.1126/science.1218530

Two recently published reports have identified BRP44 and BRP44L as components of a pyruvate carrier system that are phylogenetically conserved in yeast, drosophila, and mammals.

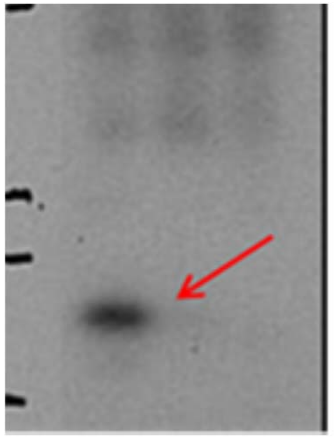
Mammals	Fly	Yeast	New Term
BRP44	CG9399	YHR162W	MPC2
BRP44L	CG14290	YGL080W	MPC1

Note: gene duplication for MPC2 in yeast resulted in MPC3 (YGR243W).

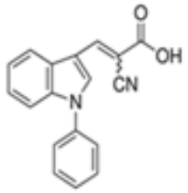
- These proteins are now referred to as MPC1 and MPC2 for Mitochondrial Pyruvate Carrier protein family 1 and 2, in the order found by Bricker *et al.,* 2012.
- We will refer to the TZD recognition complex as mTOT (mitochondrial target of thiazolidinediones). This complex contains MPC2 and MPC1, as well as other proteins that appear to be tissue specific.

Common findings with emerging data for pyruvate carrier family

- The proteins migrate in a complex that runs at about 150 kDa on Blue Native gels.
- The complex contains both BRP44 (Mpc2) and BRP44L (Mpc1) and these proteins can be co-immunoprecipitated.
- These proteins are localized to the inner mitochondrial membrane.
- UK-5099, an inhibitor of the pyruvate carrier activity also blocks the crosslinking of the TZD probe.

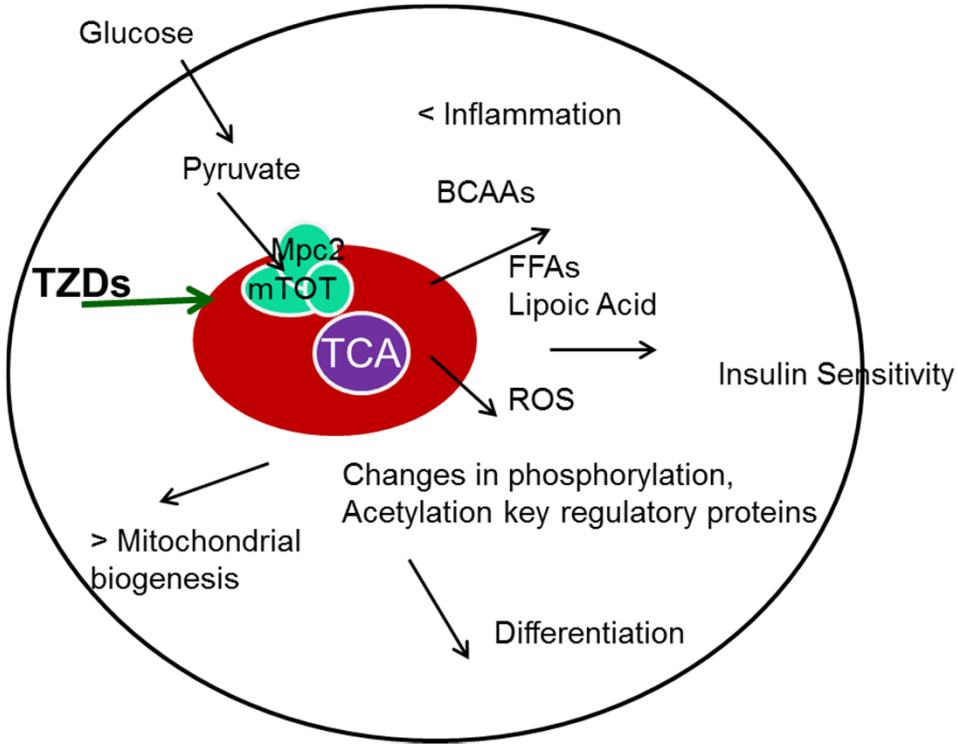


DMSO 0160 UK5099



UK-5099

MSDC-0160 directly inhibits pyruvate oxidation in permeabilized cells. See PO-2484.



The mTOT complex is involved in the regulation of pyruvate utilization.

Conclusions

- We have utilized drug analog photoaffinity crosslinking to identify a previously uncharacterized complex of proteins in the inner mitochondrial membrane.
- The protein crosslinked by this process is BRP44. This protein has been renamed Mpc2 by recent independent publications that show BRP44 is a key member in a protein family that constitutes the mitochondrial pyruvate carrier.
- The identity of the protein was confirmed by knockdown of expression as well as the expression of C-terminally tagged proteins.
- Modulation of the complex that regulates pyruvate entry into mitochondria places drug action at the crossroads of metabolism. These findings provide new ground toward understanding the pleiotropic effects of insulin sensitizers and how changes in metabolism can predict onset and corrections of diabetes.

See also Phase 2B data with prototype mTOT Modulator™- P-966; and 2363-PO and 2484-PO.