Supplemental Materials

Molecular Biology of the Cell

Course et al.

Supplemental Information

Phosphorylation of MCAD selectively rescues *PINK1* deficiencies in behavior and metabolism

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Supplemental Figure Legends

Figure S1. Schematic overview of PINK1 phosphopeptide analysis. Related to Figure 1.

Figure S2. Phosphopeptide abundance of MCAD S347 in *Drosophila*.Related to Figure 1.

Extracted ion chromatograms (XICs) for the monoisotopic peak of the phosphopeptide harboring (A) MCAD serine 347, (B) an MCAD control peptide, and (C) Marf serine 38.
(A) XIC of the doubly charged ion of RNpSYYASIAK at m/z 626.79
(B) XIC of the doubly charged peak of LSAWEIDQGR at m/z 587.8
(C) XIC of the doubly charged peak of SGPGpSPLSR at m/z 469.21.

Figure S3. Fragment ion spectrum of the doubly charged precursor ion at m/z 626.79 identifying the phosphopeptide RNpSYYASIAK.Related to Figure 1.

Phosphorylation at serine 3 is identified due to the presence of a neutral loss of H_3PO_4 from the b_6 ion, as well as the presence of the unmodified y_4 ion.

Figure S4. Expression of MCAD transgenes. Related to Figures 1 and 3.

(A) Fly lysates were immunoblotted as indicated to verify that an MCAD antibody generated against mammalian MCAD also recognizes endogenous fly MCAD.

(B) (Left) Representative Western blot of whole body lysate from *PINK1*^{RV} (control) and *PINK1*^{B9} (null) male flies with and without transgenes; probed with anti-tubulin as a loading control and anti-V5 to identify transgenic expression. (Right) Quantification of V5 expression normalized to tubulin expression. n = 5 flies lysed per sample for each experiment, 3 biological replicates per genotype. One-way ANOVA, p = 0.977, n.s. means “not significant.”

(C) Fly lysates were immunoblotted as indicated to compare expression levels of transgenic MCAD driven by Actin5C-GAL4 to expression levels of endogenous MCAD. Note that endogenous MCAD migrates faster than V5-tagged MCAD in the anti-MCAD blot.

Figure S5. Acylcarnitine analysis in *PINK1* null flies carrying MCAD transgenes. Related to Figure 3.

Acylcarnitines analysis in 6-7-day-old male flies. n=6-8 samples, 5 flies per sample, 2 technical replicates averaged per sample. One-way ANOVAs, n.s. means “not significant.”

Supplemental Table Legends

Table S1. Phos (STY) site probabilities. Related to Figure 1.

Table S2. Acylcarnitine profile of *PINK1* null flies. Related to Figures 3 and 4.

Student's t-tests, n = 3-8 samples, 5 flies per sample.

Table S3. Amino acid profile of *PINK1* null flies. Related to Figure 4.

Student's t-tests, n = 3-4 samples, 5 flies per sample.

Supplemental Experimental Procedures

Transgenic Flies

The UAS-MCAD transgene was generated by PCR amplifying *CG12262* from pOT2-CG12262 (DGRC LD22634) and ligating it into pUASTattB (Bischof et al., 2007) with an added V5 tag. UAS-MCADS347A, UAS-MCADS347D, and UAS-MCADS347DD were generated by applying site-directed mutagenesis to this UAS-MCAD plasmid (QuikChange Lightning Site-Directed Mutagenesis Kit, Agilent 210518). All constructs were verified by sequencing, then transgenes were injected into *y^1 v^1; P{CaryP}attP40* (BDSC 36304) flies and incorporated using the PhiC31 integrase system by The BestGene, Inc.

CRISPR/Cas9

The $MCAD^{mut}$ fly was generated following the protocol outlined by the O'Connor-Giles, Wildonger, and Harrison labs for defined deletions (Gratz et al., 2013, 2014, 2015). Briefly, the flyCRISPR Optimal Target Finder (http://tools.flycrispr.molbio.wisc.edu/targetFinder/) was used to identify two target sites in *CG12262*, one in exon 2 and one in exon 3, which were compared to fly sequences to rule out targets in regions with SNPs. Oligos based on these targets were annealed in the presence of T4 PNK (NEB M0201), then ligated into the pU6-BbsI-chiRNA plasmid (Addgene 45946) previously digested by the BbsI restriction enzyme (NEB R0539), using T4 DNA Ligase (NEB M0202). The resulting targeting gRNAs were transformed and maxi-prepped, then sequenced with T7 and T3 oligos. They were then injected into flies expressing Cas9 in the ovary under the control of vas regulatory sequences (BDRC 51323) by The BestGene Inc. at 250 ng/µl per plasmid.

Kinase Assay

Purified *Tribolium castaneum* PINK1 tagged with MBP (Boston Biochem AP-180) (Woodroof et al., 2011) was incubated with purified MCAD (Origene TP720903) at a 1:9 molar ratio in 60 µl of 1x kinase assay buffer (10x is 50 mM Tris–HCl pH 7.5, 0.1 mM EGTA, 10 mM MgCl2, 2 mM dithiothreitol, and 10 mM maltose) and 3 µl of 100 mM ATP at 30°C for 2 hrs. Reactions that were not to be later treated with calf intestinal alkaline phosphatase (CIP; NEB M0508) also included 15 µl Phosphatase Inhibitor Cocktail Set III (Millipore 524627). For CIP reactions, either 5 µl or 20 µl of CIP were used, and samples were placed at 37°C for 1 hr. All reactions were terminated by the addition of SDS sample buffer (300 mM Tris/HCl pH 6.8, 25% glycerol, 10% SDS, 0.1% bromophenol blue, and 14.4 mM 2-mercaptoethanol). Reactions were run on both 10% regular SDS-PAGE gels, and 10% SDS-PAGE gels containing 30 µM Phos-tag (Wako, 300-93523) following

manufacturer's instructions. Resulting Western blots were immunoblotted with anti-MCAD (Abcam ab110296) at 1:500 and anti-MBP (CST 2396) at 1:400, followed by HRP-conjugated goat anti-mouse IgG (Jackson ImmunoResearch 115035166) at 1:10,000 for chemiluminescent imaging. See below for imaging information.

Western Blots

To observe the localization patterns of tagged constructs, 25 13-14-day-old male and female flies per sample were gently homogenized in 500 ml mitochondrial isolation buffer (sucrose, mannitol, 1M TRIS-HCl pH 7.5, 0.2M EDTA/TRIS pH 7.4) at 4°C. Lysate was then centrifuged 600g for 10 min at 4°C to remove cell debris and nuclei, then 7,000g for 10min at 4°C to separate mitochondrial and cytosolic fractions. The mitochondrial pellet was then washed three times in mitochondrial isolation buffer and re-suspended in RIPA buffer for Western blotting. To validate the human MCAD antibody in flies, 10 0-5-day-old male flies per sample were homogenized; to observe transgenic expression in *PINK1* null flies, five 13-14-day-old male flies per sample were homogenized; and to observe transgenic expression levels in $MCAD^{mut}$ flies, three 2-3-day-old male and female flies per sample were homogenized

Flies were homogenized in RIPA buffer at a ratio of one fly to 10 µl of buffer, and then centrifuged at 13,000 rpm at 4°C for 30 min to remove debris. Lysates were run on SDS-PAGE gels and immunoblotted with the primary antibodies anti-MCAD (Abcam ab110296) at 1:500, anti-V5 (Invitrogen MA5-15253) at 1:500, anti-mitofusin 2 (which recognizes OPA1 in *Drosophila*; Sigma M6319) at 1:500, and/or anti-tubulin (Invitrogen 62204) at 1:1000, as needed. Secondary antibodies used were Cy5-conjugated goat anti-mouse (Amersham PA45009V) at 1:5,000 for fluorescent imaging, and HRP-conjugated goat anti-mouse IgG (Jackson ImmunoResearch 115035166) and HRP-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch 111035144) at 1:10,000 for chemiluminescent imaging.

For representative chemiluminescent images, immunoblots were imaged on an SRX-101A film processor (Konica Minolta). For quantification, fluorescent immunoblots were imaged on a Storm 860 Imager (GE Amersham). Quantification was performed using ImageJ 1.46r (NIH).

Phosphopeptide Quantification

Mitochondrial pellets were re-suspended in Laemmli buffer (Laemmli, 1970), incubated 10 min at 95°C, cooled to room temperature and alkylated using acrylamide (1% final concentration) for 30 min. Proteins were separated using SDS-PAGE, the gel stained overnight using Coomassie Brilliant Blue, washed using MilliQ water 3 times for 5 min on a shaker, and destained for 4 h. Each gel lane was partitioned to 4 pieces, each piece cut to cubes of approximately 1 mm^2, and transferred to microtubes. Gel pieces were de-stained, dehydrated and in-gel digestion was performed overnight at 37° C using trypsin in 0.1M NH_4HCO_3 as described (Winter and Steen, 2011). Peptides were extracted from the gel by subsequent incubations with 50% acetonitrile (ACN) 0.1% trifluoroacetic (TFA) acid, 0.1M NH_4HCO_3 and ACN (for details, see (Winter and Steen, 2011)), and the combined fractions were dried using a vacuum centrifuge. Dried samples were re-suspended in 50% ACN, 0.1% TFA and phospho-peptide enrichment was performed following the SIMAC approach

(Thingholm et al., 2007). Briefly, peptides were incubated with IMAC Fe-beads (PHOS-Select Iron Affinity Gel, Sigma Aldrich) for 30 min on a vibrating shaker, the beads were centrifuged down, and the supernatant was discarded. Beads were washed using 50% ACN and 0.1% TFA, centrifuged, and the supernatant was discarded. After incubation with 20% ACN and 1% TFA, beads were centrifuged again, the supernatant was transferred to a new tube (eluate fraction 1) and the beads eluted with 0.5% NH_3 (eluate fraction 2). After another round of centrifugation, eluate fraction 2 was separated from the beads and acidified using formic acid (FA). Both eluate fractions were dried using a vacuum centrifuge, re-suspended in 80% ACN 5% TFA, and incubated with TiO_2 beads (GL Sciences) for 30 min on a vibrating shaker, centrifuged, and the supernatant was discarded. After one wash with 80% ACN and 1% TFA, beads were eluted using 0.5% NH_3, eluate fractions were acidified using FA, and dried using a vacuum centrifuge.

Dried samples were re-suspended in a 50 mM citrate solution (Winter et al., 2009), and loaded directly on the analytical column at a flow rate of 1 µl/min 100% solvent A (water with 0.1% FA) using an EASY-nLC 1000 ultra-high performance liquid chromatography system (Thermo Scientific). Analytical columns were manufactured in-house by packing spray tips generated from 360 µm outer diameter 100µm inner diameter fused silica glass capillaries using a P2000 laser puller (Sutter Instrument) with 5 µm C_{18}-AQ material (Dr. Maisch GmbH). After loading and washing for16 min at 1 µl/min 100% solvent A, peptides were eluted with a 30 min linear gradient from 100% solvent A, 0% solvent B (ACN with 0.1% FA) to 65% solvent A, 35% solvent B at 400 nl/min. Eluting peptides were ionized in the positive ion mode at 1.6 kV in the nanosource of an Orbitrap Velos mass spectrometer (Thermo Scientific). After one survey scan from m/z 400 to 1200 in the Orbitrap part of the instrument at a resolution of 30,000, the top 10 most abundant ions were selected for fragmentation in the ion trap part of the instrument in the multi-stage activation (MSA) mode. MSA was triggered by neutral losses of 98, 49, 32.7 and 24.5 relative to the precursor ion. Dynamic exclusion was set to 60 s with an exclusion list size of 500.

Thermo raw data were analyzed using Maxquant version 1.3.0.5 with the following parameters: oxidation at methionine, acetylation of protein N-termini and phosphorylation at serine, threonine and tyrosine as variable modification; propionamide at cysteine as fixed modification; tryptic cleavage with two missed cleavage sites; MSMS ion tolerance of 0.5 Da. Data were searched against a FASTA file containing *Drosophila melanogaster* proteins contained in the 2014_01 release of Uniprot (www.uniprot.org), as well as common contaminants as defined in the cRAP database (www.thegpm.org/crap). Identified peptides were exported at a false discovery rate (FDR) of 1% and further analyzed using MS Excel. MS raw data were manually analyzed using Xcalibur 2.2 (Thermo Scientific). For quantification of peptides, extracted ion chromatograms (XICs) of the monoisotopic peptide peak were generated, the area under the curve quantified and the ratio calculated. This protocol has also been summarized (**Figure S1**).

Acylcarnitine Analysis

Sample analysis was based on the clinical assay using tandem mass spectrometry to measure butylated acylcarnitine species as described previously (Matern, 2008). Five flies per sample were homogenized in 50 µl of 3:1 methanol (Sigma) and acetonitrile (Burdick Jackson), and centrifuged to remove debris. High, low, abnormal, and negative controls were included with each run using 20

µl of sample. 300 µl of isotope-labeled internal standards (Cambridge Isotope) in acetonitrile and 0.4% formic acid (Sigma) were added directly to each sample. Samples were vortexed and centrifuged for 5 min at 13,000 rpm. Supernatant was decanted into glass reaction vials and dried for at least 45 min under nitrogen. Acylcarnitines were re-suspended and derivatized in 100 µl of 3N HCl in butanol (Regis Technologies Inc.) and incubated at 65°C for 15 min. Samples were dried again under nitrogen for at least 1 h. Finally, samples were re-suspended in 100 µl of 80% acetonitrile and transferred to glass injection vials for analysis on a tandem mass spectrometer (ABSciex 4500 ESI-MS/MS). Control samples were re-suspended in 200 µl of 80% acetonitrile. Injection volume was set at 10 µl. The acylcarnitine profile was collected in positive mode using precursor scanning of *m/z* 85. Data were acquired using Analyst 1.6.2 and quantified using ChemoView version 2.0.3.

Quantitative Free Amino Acids

Five flies per sample were homogenized in 50 µl of extraction solution (3:1 methanol: acetonitrile with 1% formic acid) and centrifuged to remove debris. Then, 2.5 µl of extract were combined with 2.5 µl of deionized water and 140 µl of amino acid-internal standard solution in a mass spec glass insert vial. Data were collected on a tandem mass spectrometer (Agilent 6460 Tandem Mass Spectrometer) following previously described methods (Le et al., 2014). Peaks were integrated and compared to calibration curves using MassHunter Quantitative Analysis software.

Free Carnitine

Five flies per sample were homogenized in 50 µl of extraction solution (3:1 methanol: acetonitrile with 1% formic acid) and centrifuged to remove debris. Then, 5 µl of fly extract were combined with 45 µl of HPLC pure water (Sigma) and 50 µl of D_3-carnitine (Cambridge Isotopes) internal standard in a Nanosep 3k MWCO spin cup (Pall). Samples were centrifuged at 13,000 rpm for 15 min. 20 µl of filtrate were combined with 400 µl of Mobile Phase A (ammonium formate, pH 8.0). 10 µl of 1M potassium hydroxide (Fisher Scientific) was added to the remaining filtrate, vortexed, and then incubated at 65°C for 15 min. Base was neutralized with 10 µl of 1M hydrochloric acid (JT Baker). Samples were vortexed and 20 µl of the hydrolyzed filtrate was combined with 400 µl of Mobile Phase A in a 96 deep well sample block. Samples were analyzed by tandem mass spectrometry (ABSciex 4500). Carnitine was detected using MRM 162.2>85.1 and 162.2>103.2 with 85 used as the qualifier ion and carnitine quantified from 103.2 m/z. The internal standard was detected at 165.2>85.1 and 165.2>105.2. Carnitine was separated on a Gemini 3x100 mm column (Phenomenex) using a 3-minute elution program. Two microliters of sample were injected into the mass spectrometer and washed with 10% mobile phase B (acetonitrile with 0.4% formic acid) for 0.8 min followed by a gradient of 10-30% mobile phase B over 1 min. The column was washed with 30%-99% mobile phase B over 0.5 min following by re-equilibration at 10% mobile phase B. Carnitine elutes at 1.24 minutes.

Concentration of carnitine was calculated from a six-point calibration curve made in charcoal-stripped serum and handled the same as specimens: 400, 200, 100, 50, 10, and 1 µM. Concentrations were calculated using MultiQuant 3.0.2.

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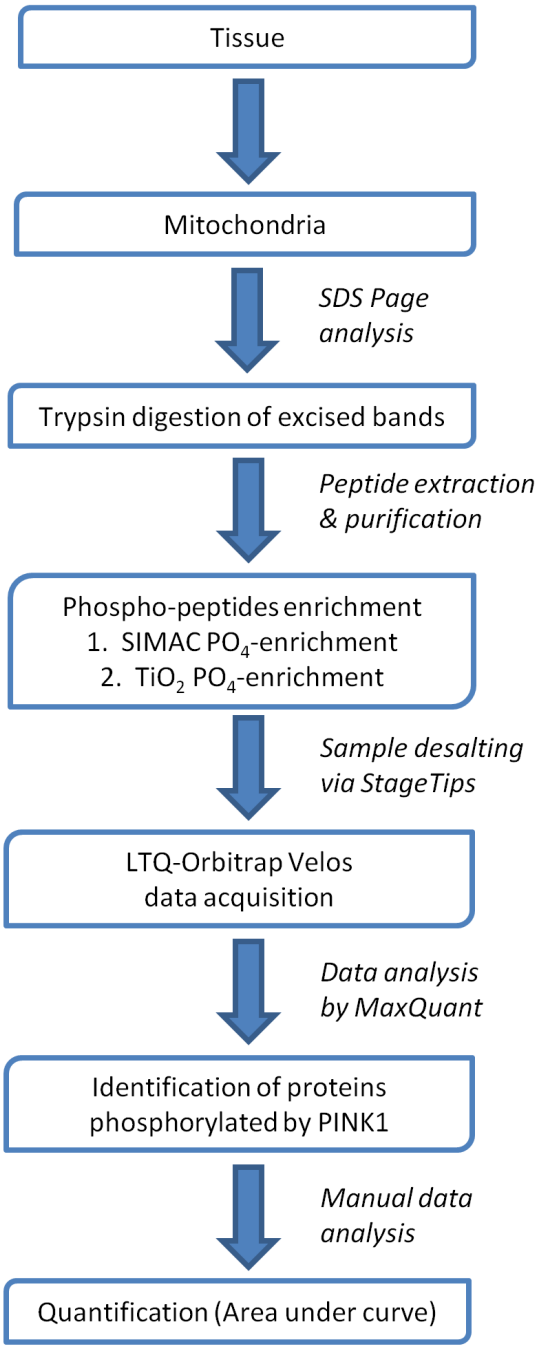
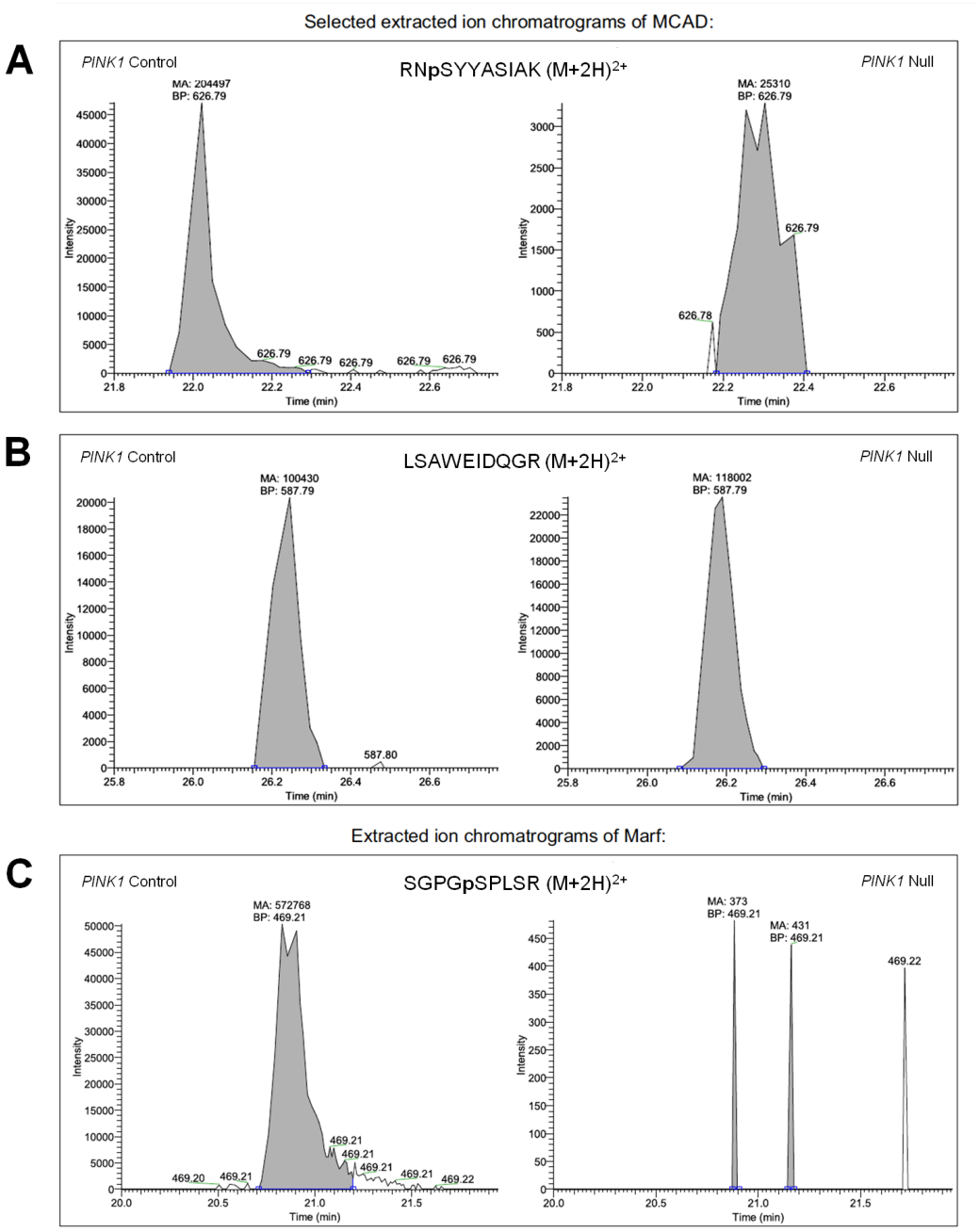


Figure S1. Schematic overview of PINK1 phosphopeptide analysis.



Relative quantification of peptides

Protein	Peptide	*PINK1* Control	*PINK1* null
MCAD	RN**p**SYYASIAK	204,497	25,310
MCAD	LSAWEIDQGR	100,430	118,002
Marf	SGPG**p**SPLSR	572,768	804

Figure S2. Phosphopeptide abundance of MCAD S347 in *Drosophila*.

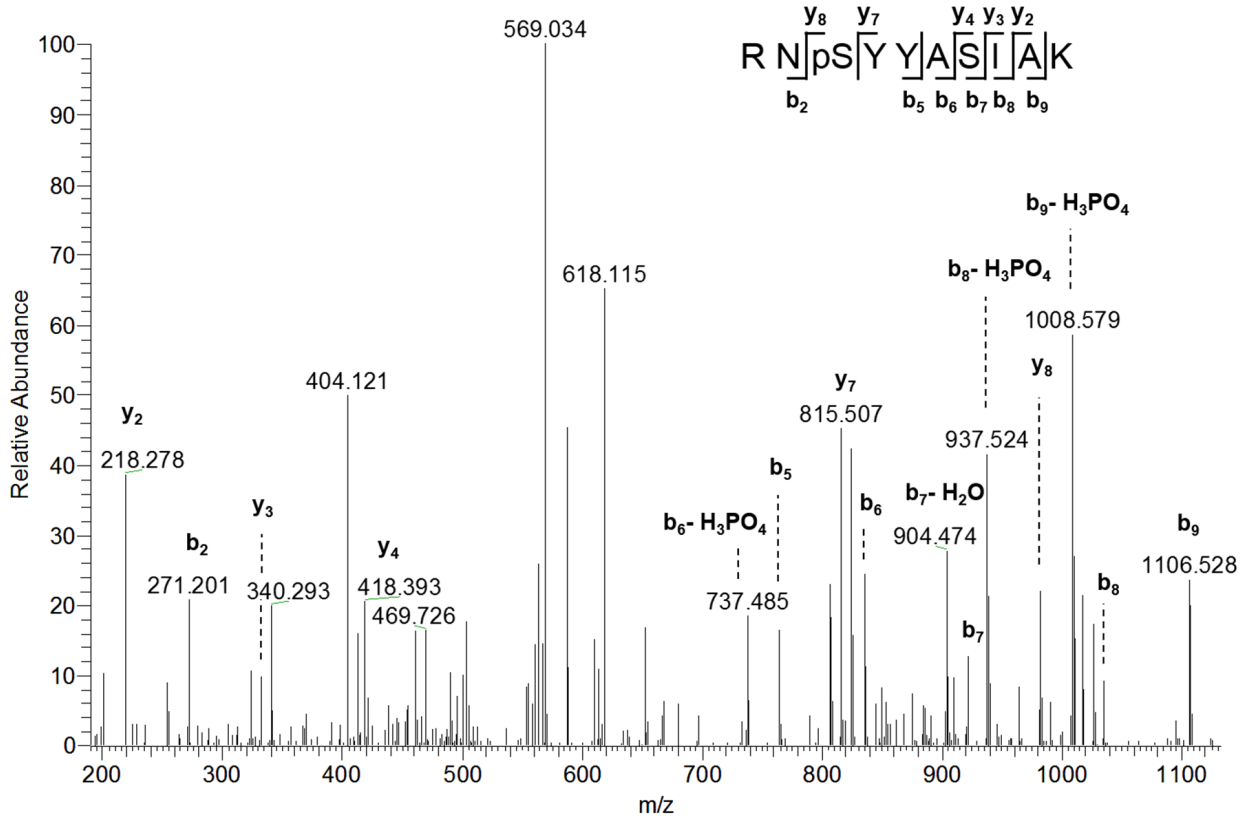


Figure S3. Fragment ion spectrum of the doubly charged precursor ion at m/z 626.79 identifying the phosphopeptide RNpSYYASIAK.

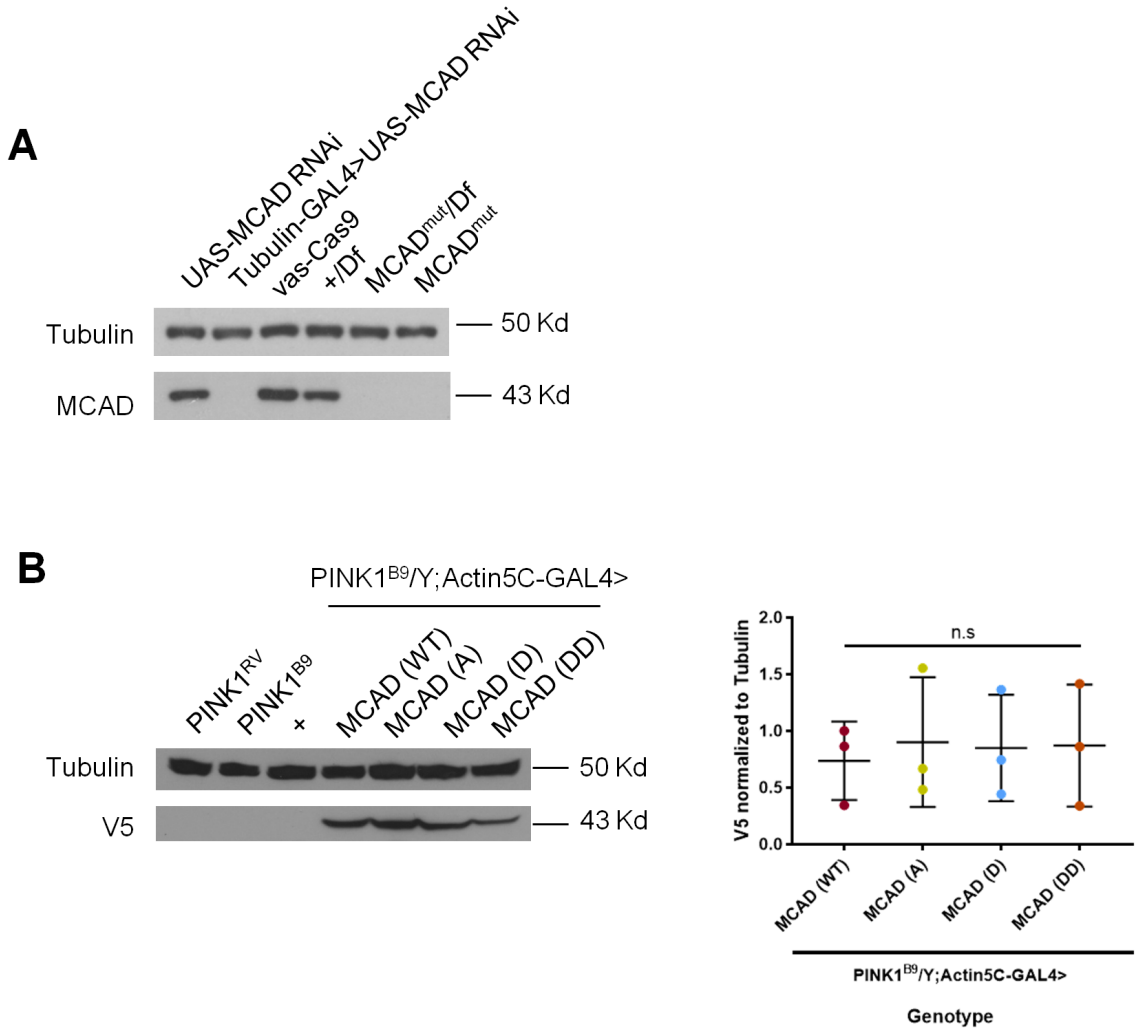


Figure S4. Expression of MCAD transgenes.

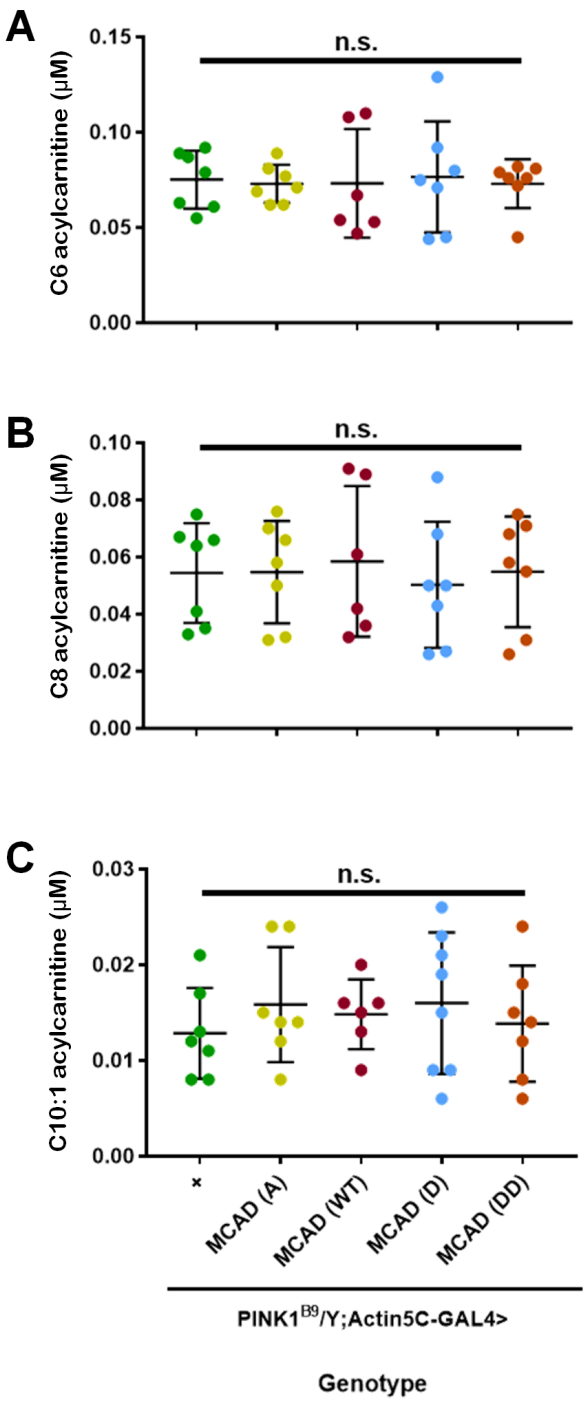


Figure S5. Acylcarnitine analysis in *PINK1* null flies carrying MCAD transgenes.

Table S1. Phos (STY) site probabilities

Proteins	Genes	Amino acid position	Location score	Modified sequence	Phos (STY) Probabilities
Q9VSA3	CG12262 (MCAD)	347	110.08	RN**p**SYYASIAK	RNS(**0.806**)Y(0.161)Y(0.032)ASIAK
Q7YU24	Marf (mitofusin)	38	67.334	SGPG**p**SPLSR	SGPGS(**0.612**)PLS(0.388)R

Table S2. Acylcarnitine profile of *PINK1* null flies

	Mean		Standard Deviation			Significant change
Acylcarnitine	**PINK1^{RV}**	**PINK1^{B9}**	**PINK1^{RV}**	**PINK1^{B9}**	**P-value**	
Free carnitine, c0	49.1	17.7	3.63	2.14	<0.0001	down
Acetyl-, c2	35.3	21.4	11.7	5.20	0.0161	down
Propienyl-, c3:1	0.0223	0.0164	0.0125	0.00416	0.262	
Proprionyl-, c3	5.41	2.62	2.16	0.574	0.0071	down
Isobutryl-, c4	0.321	0.344	0.114	0.0593	0.654	
Tiglyl-, c5:1	0.0113	0.00814	0.00742	0.00280	0.312	
Isovaleryl-, c5	0.0483	0.0513	0.0299	0.0202	0.836	
Hydroxybutyryl-, c4-OH	0.0485	0.0454	0.00959	0.0131	0.644	
Hexanoyl-, c6	0.0467	0.0701	0.0120	0.0144	0.0093	up
3-OH-Isovaleryl-, c5-OH	0.0170	0.0213	0.0141	0.00640	0.483	
Heptanoyl-, c7	0.0163	0.0235	0.00288	0.00105	0.0002	up
3-OH-Hexanoyl-, c6-OH	0.0278	0.0220	0.0122	0.00535	0.274	
Octenoyl-, c8:1	0.0144	0.0231	0.00699	0.00747	0.0674	
Octanoyl-, c8	0.0287	0.0456	0.00367	0.0105	0.0034	up
Malonyl-, c3-DC	0.270	0.244	0.176	0.204	0.810	
Decadienoyl-, c10:2	0.0227	0.0243	0.0306	0.0218	0.914	
Decenoyl-, c10:1	0.0115	0.0120	0.00485	0.00545	0.866	
Decanoyl-, c10	0.0342	0.0404	0.0103	0.0142	0.389	
Methymalonyl-, c4-DC	0.0438	0.0297	0.0397	0.0184	0.416	
3-OH-Decenoyl-, c10:1-OH	0.0222	0.0183	0.0125	0.00905	0.531	
Glutaryl-, c5-DC	0.0482	0.0381	0.0142	0.00652	0.121	
Dodecenoyl-, c12:1	0.0272	0.0490	0.00256	0.0130	0.0020	up
Dodecanoyl-, c12	0.0458	0.0800	0.00649	0.0283	0.0151	up
3-OH-Dodecenoyl-, c12:1-OH	0.0272	0.0333	0.0110	0.00905	0.294	
3-OH-Dodecanoyl-, c12-OH	0.0235	0.0384	0.00942	0.0116	0.0288	up
Tetradecadienoyl-, c14:2	0.0173	0.0231	0.00437	0.00534	0.0573	
Tetradecenoyl-, c14:1	0.0517	0.105	0.0362	0.0335	0.0193	up
Tetradecanoyl-, c14	0.0900	0.152	0.0374	0.0448	0.0217	up
3-OH-Tetradecenoyl-, c14:1-OH	0.0937	0.0993	0.0190	0.0313	0.710	
3-OH-Tetradecanoyl-, c14-OH	0.0400	0.0571	0.0114	0.0128	0.0281	up
Palmitoleyl-, c16:1	0.0845	0.198	0.0226	0.0726	0.0037	up
Palmitoyl-, c16	0.0762	0.231	0.0149	0.0746	0.0004	up
3-OH-Palmitoleyl-, c16:1-OH	0.119	0.144	0.0242	0.0411	0.215	
3-OH-Palmitoyl-, c16-OH	0.0507	0.0589	0.0144	0.0170	0.373	
Linoleyl-, c18:2	0.0250	0.0520	0.00478	0.0128	0.0005	up
Oleyl-, c18:1	0.146	0.296	0.0404	0.102	0.0061	up
Stearoyl-, c18	0.122	0.152	0.0166	0.0197	0.0142	up
3-OH-Linoleyl-, c18:2-OH	0.0263	0.0296	0.00812	0.00688	0.452	
3-OH-Oleyl-, c18:1-OH	0.0285	0.0524	0.00912	0.0159	0.0078	up
3-OH-Stearoyl-, c18-OH	0.0163	0.0193	0.00596	0.00591	0.390	

Table S3. Amino acid profile of *PINK1* null flies

	Mean		Standard Deviation			Significant change
Amino Acid	**PINK1^{RV}**	**PINK1^{B9}**	**PINK1^{RV}**	**PINK1^{B9}**	**P-value**	
Essential Amino Acids						
Histidine	396	232	74.6	55.3	0.0196	down
Isoleucine	30.5	26.1	8.66	4.03	0.401	
Leucine	38.0	37.8	11.2	6.56	0.983	
Lysine	67.8	247	18.7	27.5	0.0002	up
Methionine	15.0	18.5	4.16	1.67	0.176	
Phenylalanine	12.1	16.3	3.00	2.09	0.0781	
Threonine	84.7	68.8	31.6	13.4	0.397	
Tryptophan	5.88	6.25	1.47	0.813	0.691	
Valine	61.9	68.2	18.4	12.8	0.613	
Non-Essential Amino Acids						
Alanine	633	529	231	76.6	0.426	
Asparagine	19.3	13.6	5.60	1.61	0.107	
Aspartic Acid	127	116	38.2	18.5	0.617	
Glutamic Acid	533	319	137	26.8	0.0254	down
Conditional Amino Acids						
Arginine	461	280	102	50.5	0.0258	down
Cystine	0.323	0.469	0.0191	0.0274	0.0006	up
Glutamine	668	381	216	56.8	0.0471	down
Glycine	177	213	38.9	26.2	0.197	
Ornithine	13.5	14.0	4.82	0.867	0.838	
Proline	1271	746	354	86.0	0.0320	down
Serine	135	156	50.9	24.1	0.493	
Tyrosine	33.3	39.2	9.15	5.12	0.322	
Other Amino Acids						
1-MethylHistidine	3.79	0.646	0.735	0.239	0.0004	down
2-AminoAdipic Acid	0.357	0.770	0.0904	0.140	0.0069	up
3-Aminoisobutyric acid	6.23	6.74	0.916	0.429	0.363	
3-MethylHistidine	22.3	2.55	7.47	2.19	0.0036	down
4-Aminobutyric Acid	55.2	35.2	15.0	3.74	0.0454	down
Allo-Isoleucine	0.408	0.422	0.0284	0.0562	0.718	
Argininosuccinic acid	0.117	1.55	0.106	0.292	0.0005	up
β-Alanine	1299	237	268	52.3	0.0005	down
Citrulline	0.968	0.547	0.972	0.125	0.417	
Creatine	0.00359	0.00502	0.00161	0.00419	0.606	
Creatinine	0.251	0.232	0.0337	0.0359	0.492	
Cystathionine	0.816	4.70	0.269	0.670	0.0002	up
Ethanolamine	10.9	4.62	7.25	1.02	0.135	
Guanidineacetic Acid	0.153	0.133	0.0444	0.0235	0.465	
Homocystine	0.167	0.179	0.0228	0.0137	0.400	
Hydroxy Lysine	0.171	0.136	0.0881	0.0533	0.541	
Hydroxy Proline	0.331	0.0778	0.154	0.0181	0.0198	down
Sarcosine	5.18	2.58	1.92	1.38	0.0891	
Taurine	514	475	152	42.4	0.631	