Supplementary Information

Identification of 14-3-3γ as a Mieap-interacting protein and its role in mitochondrial quality control

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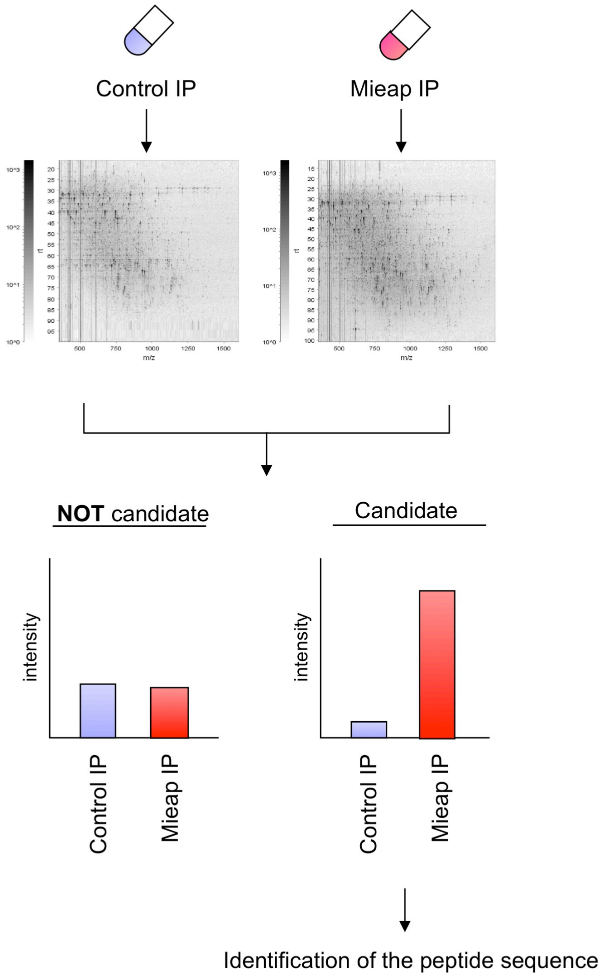
Figure S1. Schematic representation of the IP-2DICAL



The diagram shows the sequential IP-2DICAL steps for purifying and identifying Mieap-interacting proteins (see Materials and Methods for details).

Figure S2. MS/MS analysis of the 540 *m/z* (34.9 min) peak and 822 *m/z* (44.3 min) peak

14-3-3 γ
540 m/z, 34.9 min

MS/MS Fragmentation of **YLAEVATGEK**
Found in **1433G_HUMAN**, 14-3-3 protein gamma OS=Homo sapiens GN=YWHAG PE=1 SV=2

Match to Query 2406: 1079.531898 from(540.773225,2+)
Title: 459.0
Data file all.txt

Monoisotopic mass of neutral peptide Mr(calc): 1079.5499
Ions Score: 49 Expect: 0.0011
Matches (Bold Red): 12/72 fragment ions using 45 most intense peaks

#	a	a++	b	b++	Seq.	y	y++	y*	y*++	#
1	**136.0757**	68.5415	164.0706	82.5389	Y					10
2	**249.1598**	125.0835	**277.1547**	139.0810	L	**917.4938**	459.2506	900.4673	450.7373	9
3	320.1969	160.6021	348.1918	174.5995	A	**804.4098**	402.7085	787.3832	394.1953	8
4	449.2395	225.1234	477.2344	239.1208	E	**733.3727**	367.1900	716.3461	358.6767	7
5	548.3079	274.6576	576.3028	288.6550	V	**604.3301**	302.6687	587.3035	294.1554	6
6	619.3450	310.1761	647.3399	324.1736	A	**505.2617**	253.1345	488.2351	244.6212	5
7	720.3927	360.7000	748.3876	374.6974	T	**434.2245**	217.6159	417.1980	209.1026	4
8	777.4141	389.2107	**805.4090**	403.2082	G	**333.1769**	167.0921	316.1503	158.5788	3
9	906.4567	453.7320	934.4516	467.7295	E	276.1554	138.5813	259.1288	130.0681	2
10					K	**147.1128**	74.0600	130.0863	65.5468	1

14-3-3 γ
822 m/z, 44.3 min

MS/MS Fragmentation of **NVTELNEPLSNEER**
Found in **1433G_HUMAN**, 14-3-3 protein gamma OS=Homo sapiens GN=YWHAG PE=1 SV=2

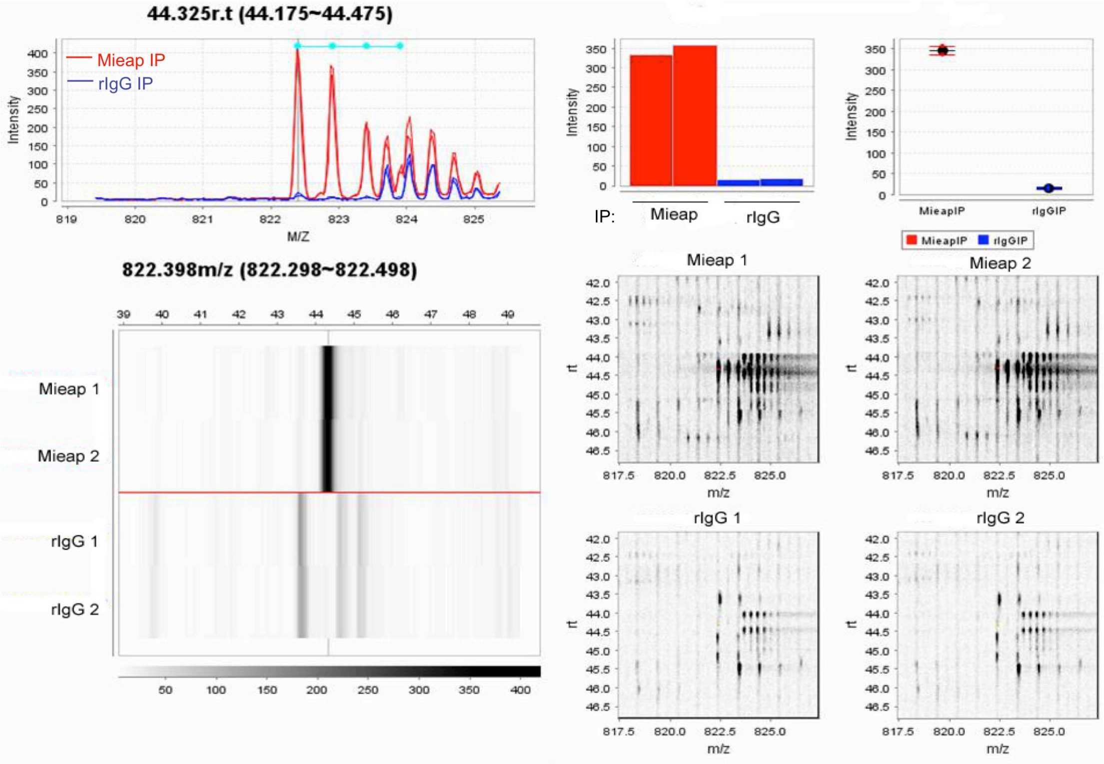
Match to Query 4956: 1642.798226 from(822.406389,2+)
Title: 228.0
Data file all.txt

Monoisotopic mass of neutral peptide Mr(calc): 1642.7798
Ions Score: 57 Expect: 0.00011
Matches (Bold Red): 29/156 fragment ions using 81 most intense peaks

#	a	a++	a*	a*++	b	b++	b*	b*++	Seq.	y	y++	y*	y*++	#
1	87.0553	44.0313	70.0287	35.5180	115.0502	58.0287	98.0237	49.5155	N					14
2	**186.1237**	93.5655	169.0972	85.0522	**214.1186**	107.5629	197.0921	99.0497	V	1529.7442	765.3757	1512.7176	756.8625	13
3	287.1714	144.0893	270.1448	135.5761	315.1663	158.0868	**298.1397**	149.5735	T	**1430.6758**	**715.8415**	1413.6492	707.3283	12
4	416.2140	208.6106	399.1874	200.0974	444.2089	222.6081	**427.1823**	**214.0948**	E	**1329.6281**	665.3177	1312.6015	656.8044	11
5	529.2980	265.1527	512.2715	256.6394	**557.2930**	279.1501	**540.2664**	270.6368	L	**1200.5855**	600.7964	1183.5590	592.2831	10
6	643.3410	322.1741	626.3144	313.6608	**671.3359**	336.1716	654.3093	327.6583	N	**1087.5014**	544.2544	1070.4749	535.7411	9
7	772.3836	386.6954	755.3570	378.1821	**800.3785**	400.6929	**783.3519**	392.1796	E	**973.4585**	487.2329	**956.4320**	478.7196	8
8	869.4363	435.2218	852.4098	**426.7085**	**897.4312**	449.2193	880.4047	440.7060	P	**844.4159**	422.7116	**827.3894**	414.1983	7
9	982.5204	491.7638	965.4938	483.2506	1010.5153	505.7613	993.4888	497.2480	L	747.3632	374.1852	730.3366	365.6719	6
10	**1069.5524**	535.2798	1052.5259	526.7666	1097.5473	549.2773	1080.5208	**540.7640**	S	**634.2791**	317.6432	617.2525	309.1299	5
11	1183.5953	592.3013	1166.5688	583.7880	1211.5903	606.2988	1194.5637	597.7855	N	**547.2471**	274.1272	530.2205	265.6139	4
12	1312.6379	656.8226	1295.6114	648.3093	1340.6328	**670.8201**	1323.6063	662.3068	E	**433.2041**	217.1057	416.1776	208.5924	3
13	1441.6805	721.3439	1424.6540	712.8306	1469.6754	735.3414	1452.6489	726.8281	E	**304.1615**	152.5844	287.1350	144.0711	2
14									R	**175.1190**	88.0631	158.0924	79.5498	1

The tandem mass spectrometry spectra (*top*) and peptide fragmentation table (*bottom*) of the 540 *m/z* (34.9 min) peak and 822 *m/z* (44.3 min) peak are shown. All identified molecular weights in the spectra are shown in the table (refer to http://www.matrixscience.com/help/fragmentation_help.html). The red numbers indicate the corresponding molecular weight of the detected fragments. Two different peptides (YLAEVATGEK and NVTELNEPLSNEER) from 14-3-3γ were identified as fragments of the Mieap-interacting protein.

Figure S3. The peak at 822 *m/z* and 44.3 min displayed in various combinations of axes



The immunoprecipitates generated using anti-Mieap antibody are indicated in *red,* and the immunoprecipitates generated using rIgG are indicated in *blue*. *Upper left*, *m/z* and intensity axes with indicators of isotopic mass (*light blue line* and *dot*). *Lower left*, a gray-scale intensity pattern for RT (*x* axis) and the sample (*y* axis). *Upper right*, the sample and intensity axes (*left*) and a *box-and-whisker diagram* of the immunoprecipitates generated using anti-Mieap antibody and rIgG (*right*). *Lower right*, the *m/z* and RT axes with high (*upper*) and low (*lower*) intensities are indicated by a *red dot*.

Figure S4. Sequence alignment of human 14-3-3 isotypes.

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   BETA MTMDKSELVQKAKLAEQAERYDDMAAAMKAVTEQGHELSNEERNLLSVAYKNVVGARRSSWRVISSIEQKT
  GAMMA -MVDREQLVQKARLAEQAERYDDMAAAMKNVTELNEPLSNEERNLLSVAYKNVVGARRSSWRVISSIEQKT
EPSILON -MDDREDLVYQAKLAEQAERYDEMVESMKKVAGMDVELTVEERNLLSVAYKNVIGARRASWRIISSIEQKE
    ETA -MGDREQLLQRARLAEQAERYDDMASAMKAVTELNEPLSNEDRNLLSVAYKNVVGARRSSWRVISSIEQKT
  SIGMA --MERASLIQKAKLAEQAERYEDMAAFMKGAVEKGEELSCEERNLLSVAYKNVVGGQRAAWRVLSSIEQKS
  THETA --MEKTELIQKAKLAEQAERYDDMATCMKAVTEQGAELSNEERNLLSVAYKNVVGGRRSAWRVISSIEQKT
   ZETA --MDKNELVQKAKLAEQAERYDDMAACMKSVTEQGAELSNEERNLLSVAYKNVVGARRSSWRVVSSIEQKT

   BETA E--RNEKKQQMGKEYREKIEAELQDICNDVLELLDKYLIP--NATQPESKVFYLKMKGDYFRYLSEVASGD
  GAMMA SADGNEKKIEMVRAYREKIEKELEAVCQDVLSLLDNYLIKNCSETQYESKVFYLKMKGDYYRYLAEVATGE
EPSILON ENKGGEDKLKMIREYRQMVETELKLICCDILDVLDKHLIP--AANTGESKVFYYKMKGDYHRYLAEFATGN
    ETA MADGNEKKLEKVKAYREKIEKELETVCNDVLSLLDKFLIKNCNDFQYESKVFYLKMKGDYYRYLAEVASGE
  SIGMA NEEGSEEKGPEVREYREKVETELQGVCDTVLGLLDSHLIK--EAGDAESRVFYLKMKGDYYRYLAEVATGD
  THETA D--TSDKKLQLIKDYREKVESELRSICTTVLELLDKYLIA--NATNPESKVFYL-MKGDYFRYLAEVACGD
   ZETA E--GAEKKQQMAREYREKIETELRDICNDVLSLLEKFLIP--NASQAESKVFYLKMKGDYYRYLAEVAAGD

   BETA NKQTTVSNSQQAYQEAFEISKKEMQPTHPIRLGLALNFSVFYYEILNSPEKACSLAKTAFDEAIAELDTLN
  GAMMA KRATVVESSEKAYSEAHEISKEHMQPTHPIRLGLALNYSVFYYEIQNAPEQACHLAKTAFDDAIAELDTLN
EPSILON DRKEAAENSLVAYKAASDIAMTELPPTHPIRLGLALNFSVFYYEILNSPDRACRLAKAAFDDAIAELDTLS
    ETA KKNSVVEASEAAYKEAFEISKEQMQPTHPIRLGLALNFSVFYYEIQNAPEQACLLAKQAFDDAIAELDTLN
  SIGMA DKKRIIDSARSAYQEAMDISKKEMPPTNPIRLGLALNFSVFHYEIANSPEEAISLAKTTFDEAMADLHTLS
  THETA DRKQTIDNSQGAYQEAFDISKKEMQPTHPIRLGLALNFSVFYYEILNNPELACTLAKTAFDEAIAELDTLN
   ZETA DKKGIVDQSQQAYQEAFEISKKEMQPTHPIRLGLALNFSVFYYEILNSPEKACSLAKTAFDEAIAELDTLS

   BETA EESYKDSTLIMQLLRDNLTLWTSENQGDEGDAGEGEN--------
  GAMMA EDSYKDSTLIMQLLRDNLTLWTSDQQDDDGGEGNN----------
EPSILON EESYKDSTLIMQLLRDNLTLWTSDMQGDGEEQNKEALQDVEDENQ
    ETA EDSYKDSTLIMQLLRDNLTLWTSDQQDEEAGEGN-----------
  SIGMA EDSYKDSTLIMQLLRDNLTLWTADNAGEEGGEAPQEPQS------
  THETA EDSYKDSTLIMQLLRDNLTLWTSDSAGEECDAAEGAEN-------
   ZETA EESYKDSTLIMQLLRDNLTLWTSDTQGDEAEAGEGGEN-------
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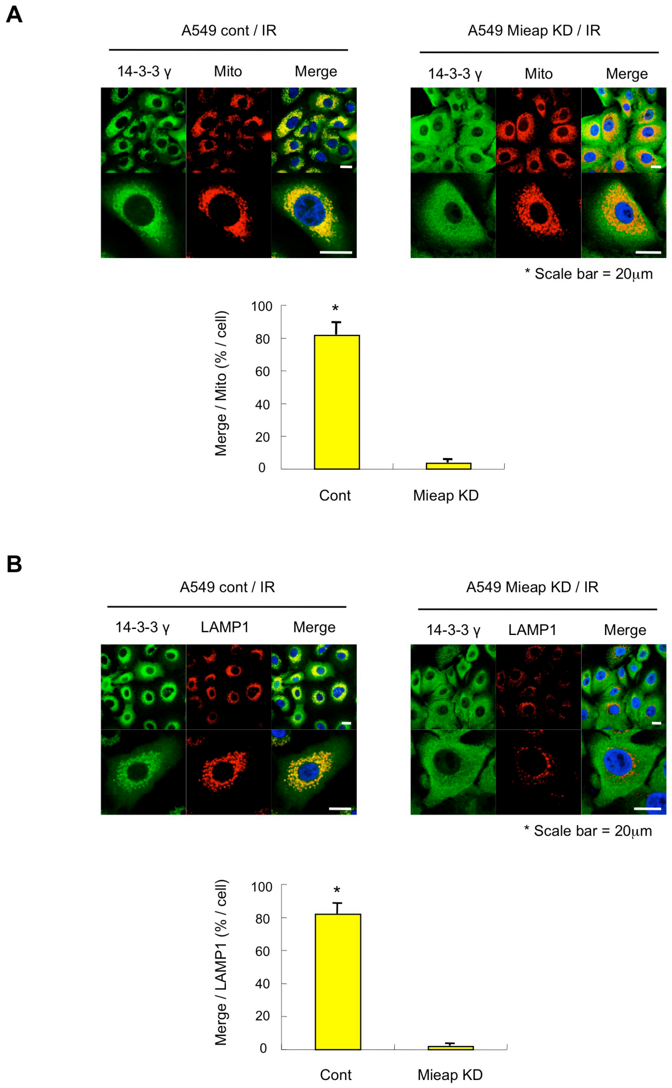
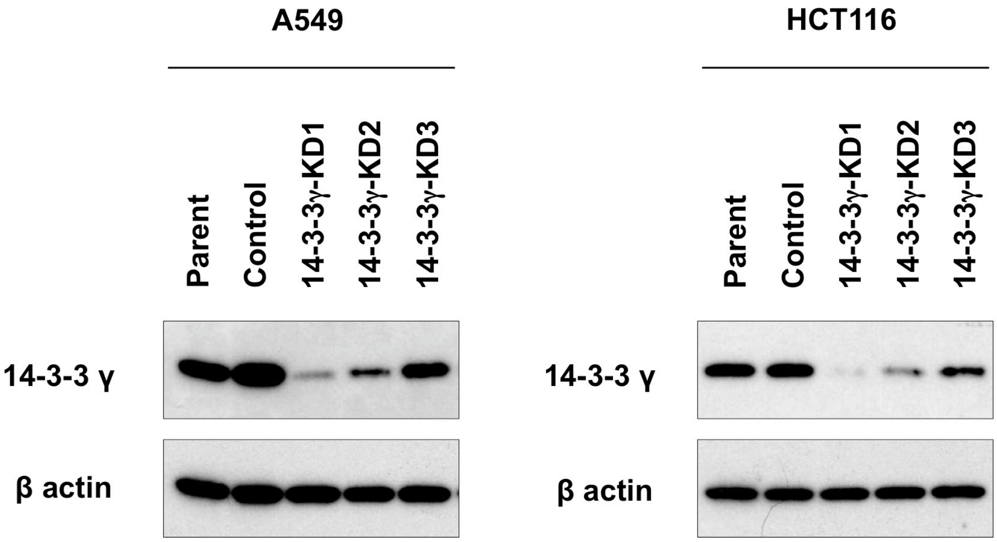
The residues conserved in at least six of the seven 14-3-3 isotypes are shown in red letters. The peptide sequences identified by IP-2DICAL are shaded gray.

Figure S5. Mitochondrial localization of 14-3-3γ during MALM



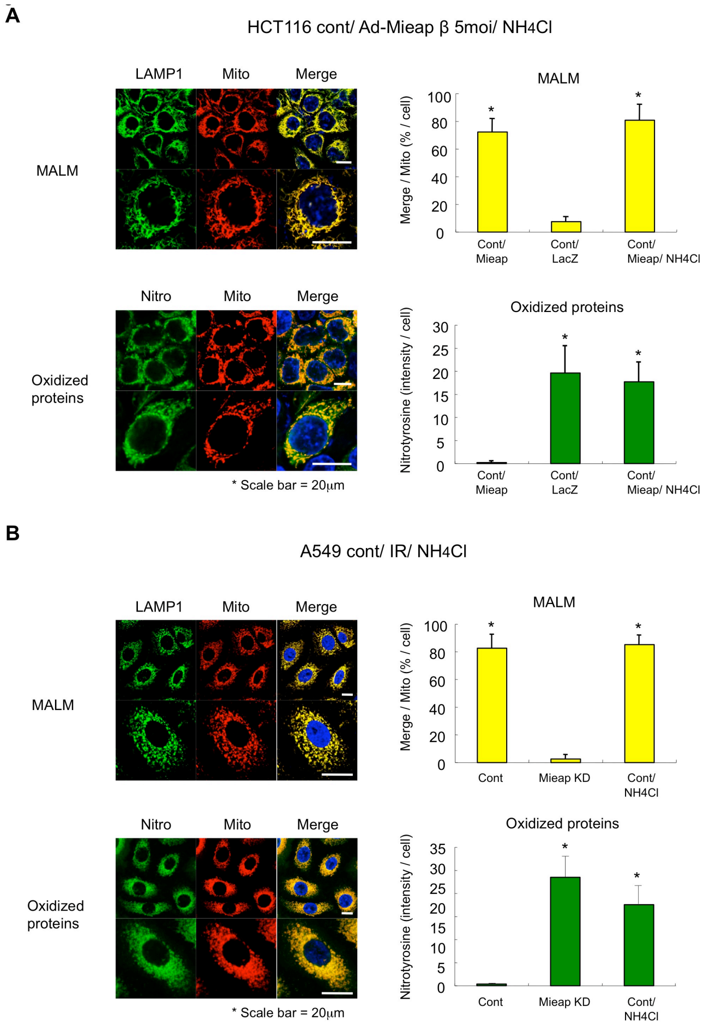
The control and Mieap-KD A549 cells were subjected to IF analysis on day 3 after the IR. The 14-3-3γ protein was stained with anti-14-3-3γ antibody (14-3-3γ: green). The lysosomes were stained with anti-LAMP1 antibody (LAMP1: red). The mitochondria are indicated by the DsRed-Mito protein signal (Mito: red). The yellow area indicates overlapping between 14-3-3γ and either mitochondria or lysosomes. A quantitative analysis of the yellow and red area was performed using 300–400 cells. The average values for the ratio of yellow to red (merged/mitochondria or lysosomes; yellow bar graph) are shown, with error bars indicating 1 SD. $P < 0.01$ (*) was considered statistically significant. Scale bar = 20 μm.

Figure S6. 14-3-3γ expression was inhibited by two shRNAs, 14-3-3γ-KD1 and 14-3-3γ-KD2



The indicated cells were subjected to Western blot analysis with anti-14-3-3γ antibody. Parent: A549 and HCT116 cells, Control: A549 and HCT116 cells infected with an empty retroviral vector, 14-3-3γ-KD1: A549 and HCT116 cells infected with the retrovirus vector expressing shRNA 14-3-3γ-KD1, 14-3-3γ-KD2: A549 and HCT116 cells infected with the retrovirus vector expressing shRNA 14-3-3γ-KD2, and 14-3-3γ-KD3: A549 and HCT116 cells infected with a retrovirus vector expressing shRNA 14-3-3γ-KD3. The A549 and HCT116 cells infected with the retrovirus vector expressing shRNA 14-3-3γ-KD1 were used as the 14-3-3γ KD cells in all of the experiments in the manuscript.

Figure S7. Neutralizing the acidic status of lysosomes with NH_4Cl causes the accumulation of oxidized mitochondrial proteins



(A and B) An IF analysis of MALM and oxidized proteins. The Ad-Mieap-β- and Ad-LacZ-infected controlHCT116 cells (A) or the control and Mieap-KD A549 cells were γ-irradiated; 3 days after the IR, an IF analysis was performed using anti-LAMP1 antibody (LAMP1) to detect MALM (MALM) or using anti-nitrotyrosine antibody (Nitro) to detect nitrotyrosine-oxidized proteins (Oxidized proteins). To evaluate the role of lysosomes, NH_4Cl was added to the Ad-Mieap-β-infected HCT116 control cells and A549 control cells on day 2 after the IR. The mitochondria are indicated by the DsRed-mito protein signal (Mito). Representative images are shown (upper panel of A or B). Quantitative analyses of the MALM and nitrotyrosine intensities were performed using 300-400 cells. The average intensities of the MALM and nitrotyrosine-oxidized proteins per cell are shown, with error bars indicating 1 standard deviation (SD; lower panel). $P < 0.01$ (*) was considered statistically significant. Scale bar = 20 μm.

Figure S8. Full-length blots

Figure 3b

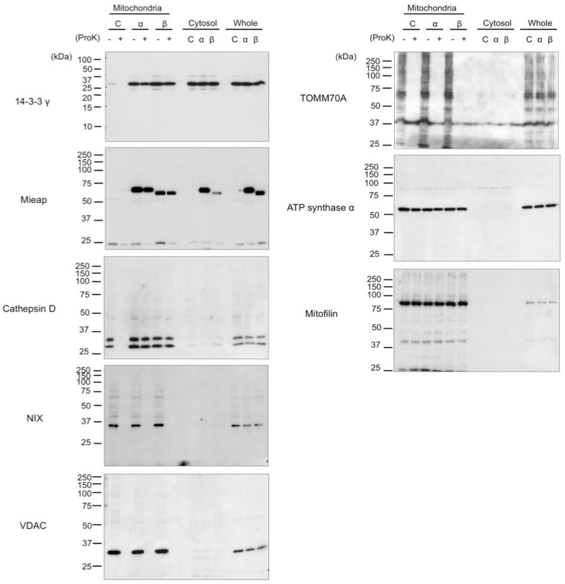


Figure 3c

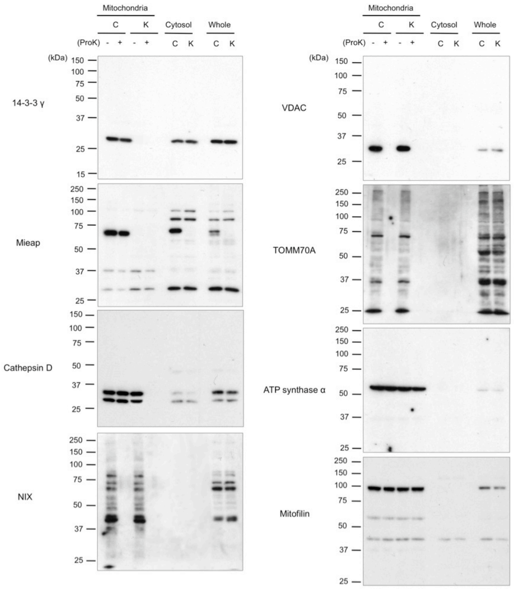


Figure 4a

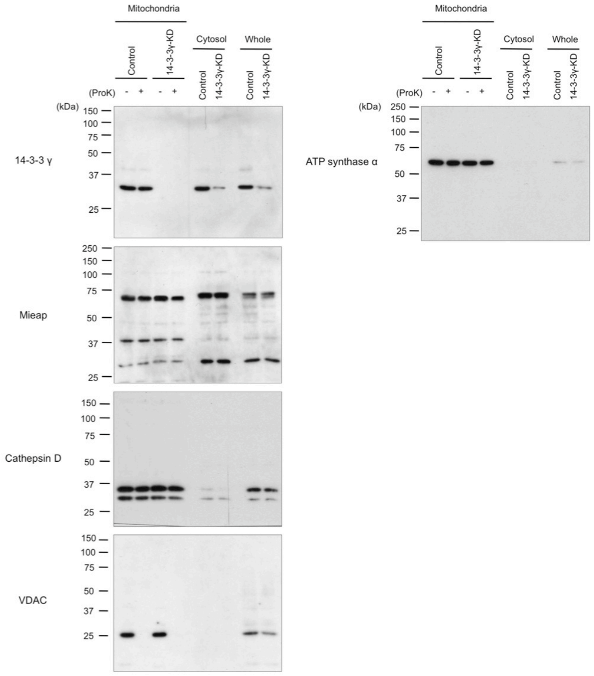


Figure 4b