LRRK2 regulates endoplasmic reticulum-–mitochondrial tethering through the PERK-mediated ubiquination ubiquitination pathway

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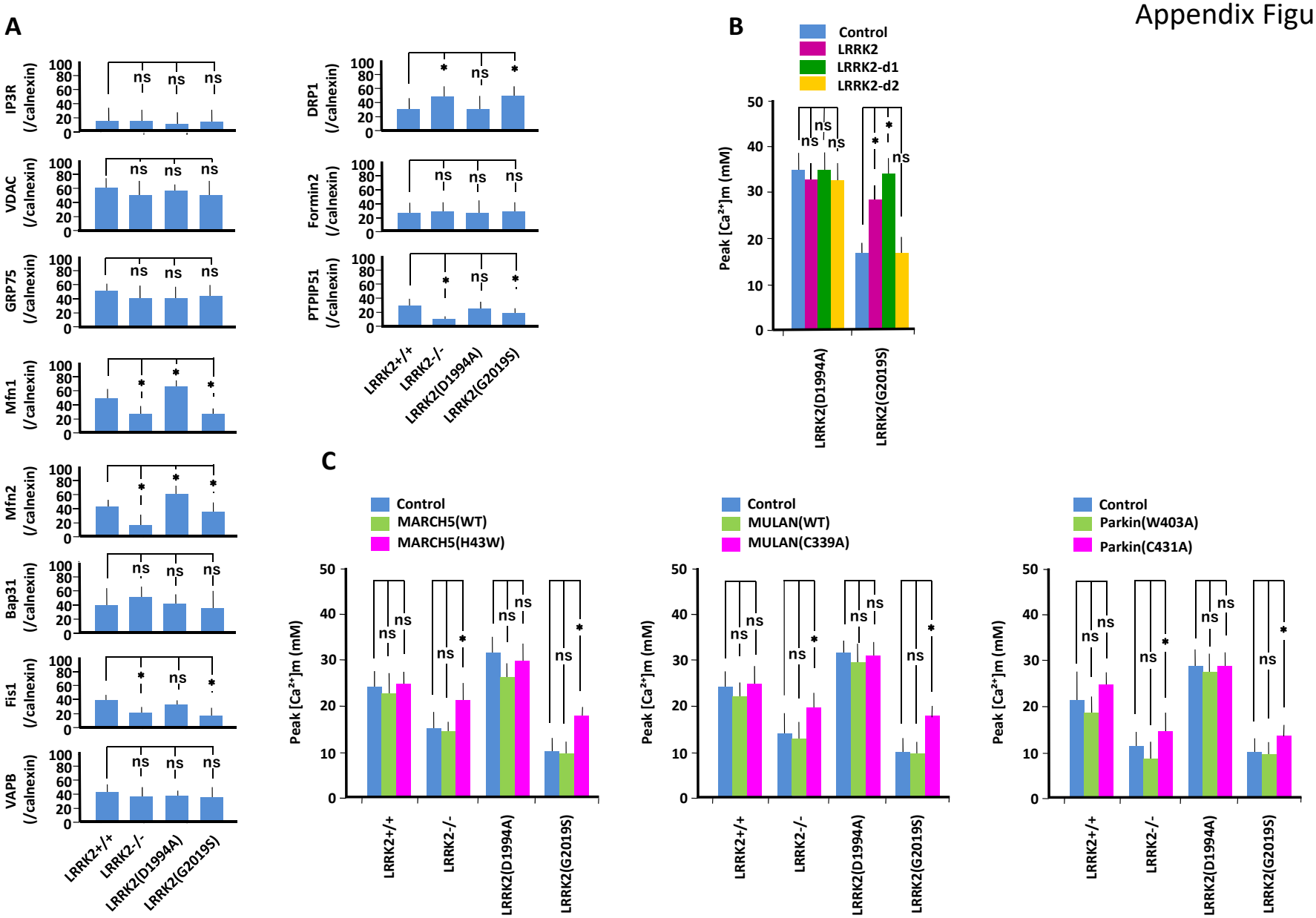
Appendix Figure S1



Appendix Figure S1. ER–-mitochondria tethering and Ca^{2+} transients in LRRK2 mutant MEFs

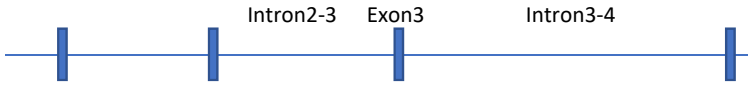
(A) Quantification of mitochondrial tethering proteins, as determined by by immunoblotting. Data represent the ratio of endogenous protein to calnexin levels. Error Error bars represent œ SD from four independent experiments. *P < 0.05 vs. $LRRK2^{+/+}$ MEFs.

(B) Peak values of Ca^{2+} transients in MEFs transfected with LRRK2 deletion constructs. Peak values of Ca^{2+} transients in LRRK2(D1994A) transfected with any of truncated LRRK2 were similar levels, while Peak values of Ca^{2+} transientswhereas the peak value of Ca^{2+} transients in LRRK2(G2019S) transfected with full-length LRRK2 and LRRK2-d1 were was higher than that in LRRK2(G2019S) transfected with empty vector (Control).

(C) Peak values of Ca^{2+} transients in MEFs transfected with shRNAs (shR) ˆD4‘specific fortargeting E3 ubiquitin ligases. Peak values of Ca^{2+} transients in $LRRK2^{-/-}$ and LRRK2(G2019S) MEFs transfected with shRNAs specific for each of E3 ubiquitin ligases were higher than that that in $LRRK2^{+/+}$ MEFs transfected with empty vector (Control). Error bars represent œ SD from six independent experiments. *P < 0.05 vs. $LRRK2^{+/+}$ MEFs transfected with empty vector (Control).

Appendix Figure S2 ↑

LRRKK2-/-



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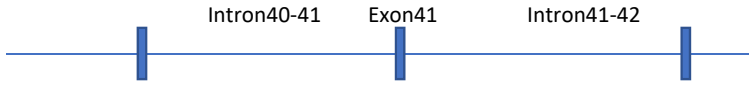
Exon3 91679778-91697887
GCGGGGTGGTCACTTCTGTGCAAATTAATAGAAGTCTGTCCAGGGACATTGCAAAGCTTAATAGGACCCCAGGATATTGGAAATGATTGGGAAGTCCTTGGTATTCACCG
Intron3-4 91683092-91697888
Gtaagtgtgatagatacgtgaagataaactgcattgaaaaattatatgt……(13kb)……ccttcgctttgctgtatgaagttcattgtcatgtgattttgtgatttttattccag

Genomic target CCCAGGATATTGGAAATGAT

Kinase inactive Lrrk2(D1994A) and Lrrk2(G2019S)



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Exon41 91786966-91787126
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GCTGCAGGATGGGAATAAAGACATCAGAGGGCACCCCAG
Intron41-42 91787127-91795996
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Lrrk2(D1994A)
Genomic target aagacctcctgtgtactctc Mutation GAC to GCG

Lrrk2(G2019S)
Genomic target CGCACAGTACTGCTGCAGGA Mutation GGG to AGC

Appendix Figure S2. Generation of genome-engineered MEFs

(A) Guide RNA for LRRK2 knock-out

LRRK2 genomic target sequence (underlined) was chosen immediately upstream of the protospacer-adjacent motif (PAM, red).

(B) Guide RNA and HDR dsDNA for LRRK2(D1994A)

LRRK2 genomic target sequence (underlined) was chosen due to the close proximity of the cut site to the desired mutation (D1994). HDR dsDNA contains the target mutation (D1994A), a PAM site mutation, and an *Afe*I restriction site to facilitate identification of clones that have undergone HDR.

(C) Guide RNA and HDR dsDNA for LRRK2(G2019S)

LRRK2 genomic target sequence (underlined) was chosen due to the close proximity of the cut site to the desired mutation (G2019). HDR dsDNA contains the target mutation (G2019S), a PAM site mutation, and a *Sfa*NI restriction site to facilitate identification of clones that have undergone HDR.

A

IP3R shRNA

IP3R-shR1-top
TGCTG-ACCGAGAGGAGACGCTGTTTA-GTTTTGGCCACTGACTGAC-TAAACAGCGTCTCCTCTCGGT
IP3R-shR1-bottom
CCTG-ACCGAGAGGAGACGCTGTTTA-GTCAGTCAGTGGCCAAAAC-TAAACAGCGTCTCCTCTCGGT-C

IP3R-shR2-top
TGCTG-CTTATGTGAACTTCGTGAATC-GTTTTGGCCACTGACTGAC-GATTCACGAAGTTCACATAAG
IP3R-shR2-bottom
CCTG-CTTATGTGAACTTCGTGAATC-GTCAGTCAGTGGCCAAAAC-GATTCACGAAGTTCACATAAG -C

IP3R-shR3-top
TGCTG-CCCACGGCCATCACCATTAAA-GTTTTGGCCACTGACTGAC-TTTAATGGTGATGGCCGTGGG
IP3R-shR3-bottom
CCTG-CCCACGGCCATCACCATTAAA-GTCAGTCAGTGGCCAAAAC-TTTAATGGTGATGGCCGTGGG-C

PERK shRNA

PERK-shR1-top
TGCTG-CCTCTACTGTTCACTCAGAAA-GTTTTGGCCACTGACTGAC-TTTCTGAGTGAACAGTAGAGG
PERK-shR1-bottom
CCTG-CCTCTACTGTTCACTCAGAAA-GTCAGTCAGTGGCCAAAAC-TTTCTGAGTGAACAGTAGAGG -C

PERK-shR2-top
TGCTG-CCACTTTGAACTTCGGTATA-GTTTTGGCCACTGACTGAC-TATACCGAAGTTCAAAGTGGC
PERK-shR2-bottom
CCTG-CCACTTTGAACTTCGGTATA-GTCAGTCAGTGGCCAAAAC-TATACCGAAGTTCAAAGTGGC-C

PERK shR3-top
TGCTG-CCATGAGTTCATCTGGAACAA-GTTTTGGCCACTGACTGAC-TTGTTCCAGATGAACTCATGG
PERK-shR3-bottom
CCTG-CCATGAGTTCATCTGGAACAA-GTCAGTCAGTGGCCAAAAC-TTGTTCCAGATGAACTCATGG-C

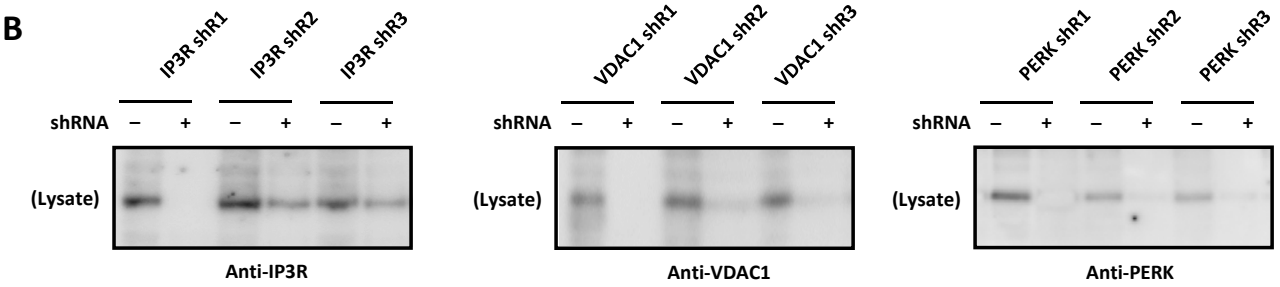
VDAC1 shRNA

VDAC1 shR-top
TGCTG-ACCAGGTATCAAACTGACGTT-GTTTTGGCCACTGACTGAC-AACGTCAGTTTGATACCTGGT
VDAC1 shR1-bottom
CCTG-ACCAGGTATCAAACTGACGTT-GTCAGTCAGTGGCCAAAAC-AACGTCAGTTTGATACCTGGT-C

VDAC2 shR-top
TGCTG-GCTACGGCTTTGGCTTAATAA-GTTTTGGCCACTGACTGAC-TTATTAAGCCAAAGCCGTAGC
VDAC1 shR2-bottom
CCTG-GCTACGGCTTTGGCTTAATAA-GTCAGTCAGTGGCCAAAAC-TTATTAAGCCAAAGCCGTAGC-C

VDAC1-shR3-top
TGCTG-GAGTTGATAAATACCACGTTA-GTTTTGGCCACTGACTGAC-TAACGTGGTATTTATCAACTC
VDAC1 shR3-bottom
CCTG-GAGTTGATAAATACCACGTTA-GTCAGTCAGTGGCCAAAAC-TAACGTGGTATTTATCAACTC-C

B



Appendix Figure S3. Design of pre-miRNA sequence for the BLOCK-iT pol II miR RNAi Expression Vector

(A) Nucleotide sequences of shRNAs specific for IP3R, VDAC1, and PERK. shRNA for pcDNA6.2-GW-miR (Clontech Laboratories) was designed by theaccording to the manufacturer's instructions.

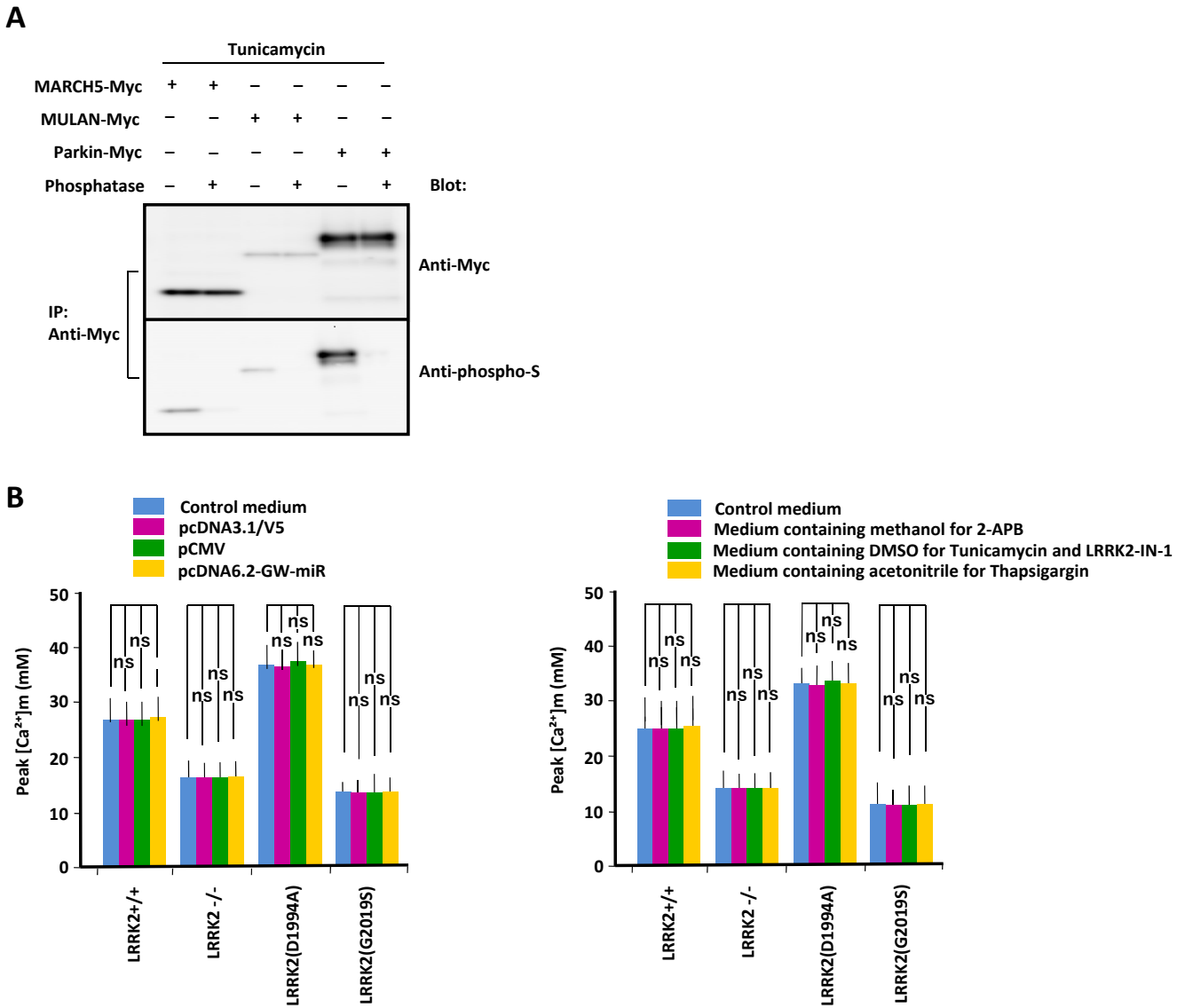
(B) Immunoblot of endogenous proteins in transfected MEFs transfected with the indicated shRNAs. Endogenous proteins were effectively decreased knocked down in MEFs by the corresponding shRNAs specific for target protein. Of three different shRNAs, one shRNA, which that suppressed endogenous protein more than 90% , was selected and used in this studyfor use in subsequent experiments..

Appendix Figure S4 ↑

anti-actin	Abcam	ab1801	https://www.abcam.com/actin-antibody-loading-control-ab1801.html
anti-Bap31	Abcam	ab 37120	https://www.abcam.co.jp/bap31-antibody-ab37120.html
anti-calnexin	Abcam	ab22595	https://www.abcam.co.jp/calnexin-antibody-ab22595.html
anti-DRP1	Abcam	ab184247	https://www.abcam.co.jp/drp1-antibody-epr19274-ab184247.html
anti-Fis1	Merck Millipore	MABN391	http://www.merckmillipore.com/JP/ja/product/Anti-FIS1-Antibody-Clone-4D1.1,MM_NF-MABN391
Anti-INF2	Proteintec	11259-1-AP	https://www.ptglab.com/Products/FMN2-Antibody-11259-1-AP.htm
anti-GRP75	Abcam	ab2799	https://www.abcam.co.jp/grp75--mot-antibody-jg1-ab2799.html
anti-IP3R	Abcam	ab125076	https://www.abcam.co.jp/ip3-receptor-antibody-epr4536-ab125076.html
anti-LC3	Merck Millipore	ABC929	http://www.merckmillipore.com/JP/ja/product/Anti-LC3-I-II-Antibody,MM_NF-ABC929
anti-LRRK2	Abcam	ab133475	https://www.abcam.co.jp/lrrk2-antibody-mjff3-c69-6-ab133475.html
anti-MARCH5	Abcam	ab77585	https://www.abcam.co.jp/march5-antibody-ab77585.html
anti-mitofusin1	Abcam	ab57602	https://www.abcam.co.jp/mitofusin-1-antibody-3c9-ab57602.html
anti-mitofusin2	Abcam	ab56889	https://www.abcam.co.jp/mitofusin-2-antibody-6a8-ab56889.html
anti-MULAN	ProteinTec	16133-1-AP	https://www.biocompare.com/9776-Antibodies/966931-MUL1/?pda=9776|966931_0_0||10|MULAN
anti-Parkin	Abcam	ab77924	https://www.abcam.co.jp/parkin-antibody-prk8-ab77924.html
anti-PTPIP51	GeneTex	GTX54674	https://www.biocompare.com/9776-Antibodies/7588248-PTPIP51-antibody/?pda=9776|7588248_0_0||3|PTPIP51
anti-p62	abcam	ab56416	https://www.abcam.co.jp/sqstm1-p62-antibody-ab56416.html
anti-PERK	Cell Signaling	3192	https://www.cellsignal.com/products/primary-antibodies/perk-c33e10-rabbit-mab/3192
anti-Phospho-PERK	Cell Signaling	3179	https://www.cellsignal.jp/products/primary-antibodies/phospho-perk-thr980-16f8-rabbit-mab/3179
anti-VAPB	Abcam	ab103638	https://www.abcam.co.jp/vapb-antibody-ab103638.html
anti-VDAC1	Abcam	ab14734	https://www.abcam.co.jp/vdac1-porin-antibody-20b12af2-ab14734.html
anti-FLAG	Sigma-Aldrich	F1804	https://www.sigmaaldrich.com/catalog/product/sigma/f1804?lang=ja&region=JP
anti-HA	MBL	561	http://ruo.mbl.co.jp/bio/dtl/A/?pcd=561
anti-Myc	MBL	M192-3	http://ruo.mbl.co.jp/bio/sch/?gtos=8&kw=myc
anti-phosphoserine	Cell Signaling	9631	https://www.cellsignal.com/products/primary-antibodies/phospho-ser-thr-phe-antibody/9631
anti-V5	Thermo Fisher Scientific	R960-25	https://www.thermofisher.com/antibody/product/V5-Tag-Antibody-Monoclonal/R960-25

Appendix Figure S4. Lists of antibodies used in this study

Appendix Figure S5 ↑



Appendix Figure S5. Specificity of anti-phospho-serine antibody and Ca^{2+} transients in MEFs transfected with control vectors or treated with vehicle

(A) Immunoblot of phosphorylated E3 ubiquitin ligases from transfected MEFs under treated with tunicamycin. Treatment of lysates with phosphatase decreased intensities of bands stained with anti-–phospho-serine antibody, indicating confirming the specificity of this antibody against for phosphorylated proteins.

(B) Peak values of Ca^{2+} transients in MEFs of the indicated genotypes, transfected with expression plasmids (pcDNA3, CMV) or shRNA plasmid (pcDNA6.2-GW-miR) or incubated with the vehicle used for 2-APB, LRRK2-IN-1, and tThapsigargin. All None of the empty vectors and or vehicles had no significant effects on Ca^{2+} transients in MEFs.