Rapid manipulation of mitochondrial morphology in a living cell with iCMM

Graphical abstract

Highlights

- iCMM can induce mitochondrial morphological changes on the minute timescale
- We develop three iCMM effectors that can alter mitochondrial morphology
- iCMM alters mitochondrial morphology with a negligible effect on membrane potential

Authors

Takafumi Miyamoto, Hideki Uosaki, Yuhei Mizunoe, ..., Naoya Yahagi, Koichi Ito, Hitoshi Shimano

Correspondence
takmi565@md.tsukuba.ac.jp

In brief

The biological significance of mitochondrial morphology in human health and diseases is not fully elucidated. Miyamoto et al. develop iCMM, a genetically encoded synthetic protein device, to alter mitochondrial morphology on a minute timescale and describe the effects of iCMM-induced mitochondrial morphological changes in living cells.
Rapid manipulation of mitochondrial morphology in a living cell with iCMM

Takafumi Miyamoto,1,9,10,* Hideki Uosaki,2 Yuhei Mizunoe,1 Song-Iee Han,1 Satoi Goto,1 Daisuke Yamanaka,3 Masato Masuda,4 Yosuke Yoneyama,9 Hideki Nakamura,5,7 Naoko Hattori,8 Yoshinori Takeuchi,1 Hiroshi Ohno,1 Motohiro Sekiya,1 Takashi Matsuzaka,1 Fumihiko Hakuno,4 Shin-Ichiro Takahashi,4 Naoya Yahagi,1 Koichi Ito,3 and Hitoshi Shimano1

1Department of Internal Medicine (Endocrinology and Metabolism), Faculty of Medicine, University of Tsukuba, Tsukuba, Ibaraki 305-8575, Japan
2Division of Regenerative Medicine, Center for Molecular Medicine, Jichi Medical University, Shimotsuke, Tochigi 329-0498, Japan
3Department of Veterinary Medical Sciences, Graduate School of Agriculture and Life Sciences, The University of Tokyo, Kasama, Ibaraki 319-0206, Japan
4Departments of Animal Sciences and Applied Biological Chemistry, Graduate School of Agriculture and Life Sciences, The University of Tokyo, Bunkyo-ku, Tokyo 113-8557, Japan
5Institute of Research, Division of Advanced Research, Tokyo Medical and Dental University (TMDU), Bunkyo-ku, Tokyo 113-8510, Japan
6Johns Hopkins University School of Medicine, Department of Cell Biology and Center for Cell Dynamics, MD 21205, USA
7Kyoto University Graduate School of Engineering, Department of Synthetic Chemistry and Biological Chemistry, Graduate School of Engineering, Kyoto University, Kyoto, Kyoto 606-8501, Japan
8Division of Epigenomics, National Cancer Center Research Institute, Chuo-ku, Tokyo 104-0045, Japan
9Transborder Medical Research Center, University of Tsukuba, Tsukuba, Ibaraki 305-8577, Japan
10Lead contact
*Correspondence: takmi565@md.tsukuba.ac.jp
https://doi.org/10.1016/j.crmeth.2021.100052

SUMMARY
Engineered synthetic biomolecular devices that integrate elaborate information processing and precisely regulate living cell behavior have potential in various applications. Although devices that directly regulate key biomolecules constituting inherent biological systems exist, no devices have been developed to control intracellular membrane architecture, contributing to the spatiotemporal functions of these biomolecules. This study developed a synthetic biomolecular device, termed inducible counter mitochondrial morphology (iCMM), to manipulate mitochondrial morphology, an emerging informative property for understanding physiopathological cellular behaviors, on a minute timescale by using a chemically inducible dimerization system. Using iCMM, we determined cellular changes by altering mitochondrial morphology in an unprecedented manner. This approach serves as a platform for developing more sophisticated synthetic biomolecular devices to regulate biological systems by extending manipulation targets from conventional biomolecules to mitochondria. Furthermore, iCMM might serve as a tool for uncovering the biological significance of mitochondrial morphology in various physiopathological cellular processes.

INTRODUCTION
A fascinating advance in the field of synthetic biology, in which biological functions that do not exist in nature are engineered, is the creation of synthetic biomolecular devices to manipulate living cells at will (Grozinger et al., 2019; Khalil and Collins, 2010). Indeed, with the advent of more sophisticated biological tools, both nucleic acid- and protein-based synthetic biomolecular devices to control cellular functions have been generated and are being applied to clinical medicine and other fields...
In general, these synthetic biomolecular devices regulate cellular functions by manipulating the expression levels, localization, and activities of key responsible biomolecules. However, organelle morphology, which also plays a critical role in the spatiotemporal regulation of biomolecule functions and subsequent execution of cellular functions, remains virtually unexplored as a target for synthetic biomolecular devices because of the paucity of applicable techniques. Meanwhile, concomitant with recent research, there is increasing interest in organelle morphology, which contributes to versatile physiopathological cellular behaviors that have not generally been thought to involve organelle morphology (Archer, 2013; Lomakin et al., 2020; Tao et al., 2020; Venturini et al., 2020). One of these organelles is the mitochondrion.

Mitochondria are organelles in eukaryotic cells that evolved from endosymbiotic α-proteobacteria (Gray et al., 2001). They are involved in numerous anabolic and catabolic reactions, including but not limited to oxidative phosphorylation, the tricarboxylic acid cycle, the β-oxidation of fatty acids, and heme biosynthesis (Smeitink et al., 2006). The products of these reactions are used in various cellular processes, including cellular structuring, chemical reactions, and signal transduction, indicating that mitochondria are unequivocally indispensable to host cells.

As their name implies (from Greek: mitos = thread; chondrion = granule), mitochondria are highly dynamic organelles that can alter their size, shape, and subcellular distribution through repetitive, coordinated fusion and fission cycles over the course of a few minutes (Detmer and Chan, 2007; Jakobs et al., 2003), a phenomenon termed mitochondrial dynamics. Accumulating evidence has revealed that disturbances in mitochondrial dynamics followed by the disruption of mitochondrial morphology occur in various diseases, including cancer and metabolic diseases (Archer, 2013; Yu-Wai-Man and Chinnery, 2012), leading to an increasing appreciation of the pivotal role of mitochondrial morphology in physiopathological cellular behaviors. However, the disorganization of mitochondrial morphology is just now emerging as a mechanism of these cellular behaviors; the biological significance of mitochondrial morphology in health and disease remains to be completely elucidated.

Over recent decades, considerable efforts have been made to elucidate the physiopathological significance of mitochondrial morphology. In most of these studies, mitochondrial morphology has been altered either chemically or by modifying genes (Casidy-Stone et al., 2008; Leonard et al., 2015; Picard et al., 2013). However, as these approaches have caused concomitant mitochondrial dysfunction, whether subsequent cell changes are due to mitochondrial morphology changes, mitochondrial dysfunction, or both remains to be clarified. Thus, a tool for the inducible, rapid, and specific manipulation of mitochondrial morphology without abrogating mitochondrial functions is required (Picard et al., 2013; Wai and Langer, 2016). Furthermore, if this tool allows us to reorganize intracellular status as intended through the rapid manipulation of mitochondrial morphology, the mitochondrion would be a promising target for synthetic biomolecular devices.

Chemically inducible dimerization (CID) systems allow manipulating target protein localization in a spatiotemporally confined subcellular compartment by forming a small molecule-induced ternary complex of two different proteins (Derose et al., 2013). Notably, CID systems are applied to synthetic biomolecular devices to control cellular outputs by spatiotemporally regulating protein functions (Fegan et al., 2010; Miyamoto et al., 2013; Putyrski and Schultz, 2012). Compared with the hours-long genetic circuit-based manipulation of cellular outputs, CID systems allow for faster control of cellular outputs (seconds to minutes) (Miyamoto et al., 2012). Therefore, they are suitable for rapidly inducing morphological changes in mitochondria at a specific time point. CID systems have been widely applied in mitochondrial studies, for example, to promote the mitochondria-endo-plasmic reticulum (ER) interaction (Csordás et al., 2010) and to suppress AMP-activated protein kinase activity in mitochondria (Miyamoto et al., 2015). However, a sophisticated platform for manipulating mitochondrial morphology into various patterns using CID systems is lacking.

In the present study, we developed inducible counter mitochondrial morphology (ICMM), a CID system-based synthetic protein device, to manipulate mitochondrial morphology in living cells on a minute timescale. Using ICMM, we determined the type of alterations that occur immediately after morphological changes, which would otherwise be difficult to study. This study illustrates that ICMM is a potentially powerful tool that, in addition to conventional methods, can reveal the biological significance of mitochondrial morphology in various physiopathological cellular behaviors. Furthermore, the ICMM-induced perturbation of mitochondrial morphology evokes reorganization of the intracellular transcriptome and amino acid profile, suggesting that it could help regulate cells as intended. Expanding the target of manipulation by synthetic devices from biomolecules to mitochondria is promising for developing more proficient strategies to control cellular functions in various applications.

RESULTS

Development of a genetically encoded synthetic protein device: ICMM

ICMM, a Boolean YES logic gate-based synthetic protein device, consists of a functional ICMM effector (FiCE) to perturb mitochondrial morphology and a mitochondrial outer membrane-specific anchor protein that tethers the effector to the mitochondria in the presence of a chemical dimerizer (Figure 1A). Considering the versatility and translocation speed of effector proteins, we adopted a rapamycin-based CID system, in which peptidyl-prolyl cis-trans isomerase FKBP12 (termed FKBP) and the FKBP12-rapamycin-binding domain in the mammalian target of rapamycin (mTOR) (termed FRB) served as an effector and anchor, respectively, by using rapamycin as a chemical dimerizer (Figure 1B).

We first used a reported fundamental fusion protein consisting of enhanced yellow fluorescent protein (EYFP) and FKBP (termed YF) as a FiCE (Figure 1B). YF has been used as a control effector in various experimental settings (Inoue et al., 2005; Nakamura et al., 2020). However, we found that once it had substantially translocated to the outer surfaces of mitochondria, which were circumscribed by anchor proteins consisting of mitochondrial targeting sequences derived from Tom20, enhanced
cyan fluorescent protein (ECFP), and FRB (termed Tom20-CR, Figure 1B), the mitochondrial morphology changed from a network structure to large punctate structures (Figures 1C, S1F, and Video S1). Correlative light and electron microscopy (CLEM) and confocal microscopy indicated that the large punctate structure of mitochondria induced by YF translocation was not a single mitochondrion but consisted of multiple mitochondria (Figures 1F and S2B). mYF containing monomeric EYFP{D207W} (mEYFP) and FKBP (Figure S1A) did not change mitochondrial morphology, although mYF substantially accumulated in the mitochondrial outer membrane (Figures S1C, S1E, S2A, and Video S2), suggesting that the inherent homodimerization activity of EYFP (Lambert, 2019) was at least partially required for the YF-induced change in mitochondrial morphology. We used mYF as a control effector (CiCE) in subsequent experiments. In addition, we termed a synthetic device consisting of CiCE and a mitochondrial outer membrane-specific anchor protein (Tom20-CR, in this study) as Negative iCMM (NiCMM) (Figure S1B).

Excessive mitochondrial tethering with other membrane structures leads to the disruption of mitochondrial morphology (Kakimoto et al., 2018). Thus, we next focused on caveolin-1, an integral membrane protein that localizes to the caveola and multiple interior compartments through vesicle trafficking (Parton and Simons, 2007). As fluorescent protein-fused caveolin-1 exhibits a punctate distribution pattern in the cell similar to that of endogenous caveolin-1 (Schlegel and Lisanti, 2000), we hypothesized that tethering mitochondria to fluorescent protein-fused caveolin-1 via the CIDs system would induce a change in mitochondrial morphology. To test this idea, we constructed the second FiCE consisting of YF and amino acids (aa) 61–178 of caveolin-1 (termed YF-Cav1s; Figure 1B), the smallest fusion protein that showed a small punctate distribution pattern throughout the cell (Figure S3A). As expected, immunocytochemistry revealed that YF-Cav1s colocalized with endogenous caveolin-1 (Figure S3B). Most of the YF-Cav1s localized to certain regions within the cell and did not diffuse throughout the cell (Video S3). After the iCMM harboring YF-Cav1s was turned on, the mitochondrial surfaces were partially interlinked with YF-Cav1s, resulting in a change in mitochondrial morphology from a network structure to punctate structures of various sizes in the cells (Figures 1D, 1G, S1G, and S2C, and Video S4).

Finally, we optimized a previously designed interspecies fusion protein consisting of amino acids 30–262 of Listeria monocytogenes actin assembly inducing protein (ActA) that was codon-optimized for usage in mammalian cells (Nakamura et al., 2020) and amino acids 2–380 of human zyxin (termed mActZ), which is structurally and functionally similar to full-length ActA (Golsteyn et al., 1997). As ActA converts actin polymerization into a motile force (Smith and Porthnoy, 1997), we hypothesized that the generated propulsive force could alter mitochondrial morphology. mActZ was fused to FKBP and EYFP (termed mActZ-FY; Figure 1B) to produce the third FiCE. Similar to the mitochondrial morphology induced by ActuAtor (a fusion protein consisting of codon-optimized ActA, FKBP, and mCherry), a protein functionally homologous to mActZ-FY, when iCMM harboring mActZ-FY were turned on, cytoplasmic mActZ-FY was recruited to the mitochondria, and then the morphology of mitochondria rapidly and obviously changed to small dot-like structures (Figures 1E, 1H, S1H, and S2D, and Video S5). Of note, the expression level of mActZ-FY was lower than that of the other FiCEs, probably due to the instability of mActZ-FY, but significant changes in mitochondrial morphology were observed after the translocation of mActZ-FY in mitochondria.

Together, these results indicate that each of the three FiCEs developed here can induce characteristic changes in mitochondrial morphology at any given point in time on a minute timescale.

**Figure 1.** iCMM as a synthetic protein device for rapid manipulation of mitochondrial morphology in live cells
(A) Schematic diagram of the iCMM system. (B) Structural features of FiCEs and anchor. (C–E) Time-lapse imaging (2 min/frame) of HeLa cells expressing Tom20-CR (slate blue) and the indicated effectors (yellow). Mitochondria (coral) were stained with the MitoTracker Red CMXRos dye. Rapamycin (50 nM) was added after image acquisition at frame 6 (indicated as “0 min”). Scale bar: 10 μm. Insets show a magnified view of the boxed area. Scale bar in inset, 1 μm. Lookup tables (LUTs) (effector): 100–2,000, YF (C) and YF-Cav1s (D); 100–500, mActZ-FY (E). (F–H) Mitochondrial structures in HeLa cells expressing the indicated effectors and Tom20-CR were examined with CLEM after 30 min of iCMM actuation (50 nM rapamycin addition). Merged fluorescence images (blue, Tom20-CR; green, indicated effector, inset) and SEM images are shown. Scale bar, 10 μm in insets and 1 μm in SEM images. (I–K) Mitochondrial roundness in HeLa cells expressing indicated iCMM (positive, P) and in peripheral iCMM-negative cells (negative, N) were analyzed before (0 min) and after (30 min) activation of the iCMM system (50 nM rapamycin addition). (Left) Data represent mean ± standard deviation. N.S., statistically non-significant (Student’s t test); (right) gray, analyzed single cell; lavender, average. n = number of cells analyzed, from three independent experiments. P = paired t test. N.S., statistically non-significant. See also Figures S1–S5 and S9, and Videos S1, S3, S4, and S5.
had been turned on and thus did not induce morphological changes in ER, Golgi apparatus, and lysosomes over a 120-min observation window (Figure S4).

One of the CID system’s strengths is its versatility (Miyamoto et al., 2013). To determine its versatility, iCMM was transduced into Hep 3B human liver cancer cells and U-2 OS (human osteosarcoma) cells. Consistent with the results obtained from HeLa human ovarian cancer cells (Figure 1), each FICE induced a characteristic change in mitochondrial morphology in both cell types on similar timescales (Figure S5).

Together, these results indicate that iCMM is a powerful tool for specifically disrupting mitochondrial morphology in target cells.

**iCMM alters mitochondrial morphology without loss of mitochondrial membrane potential**

The mitochondrial membrane potential (MMP), which results from redox transformations, plays decisive roles in various cellular functions, including adenosine triphosphate (ATP) synthesis and innate immune responses (Ichinohe et al., 2013; Koshiba et al., 2011; Zorova et al., 2018). Remarkably, MMP loss is a major factor in the disintegration of mitochondrial morphology (Zhang et al., 2014). Thus, we examined whether iCMM-induced morphological changes in mitochondria were attributable to a loss of MMP by using tetramethylrhodamine ethyl ester (TMRE). Although TMRE is appreciated as a useful MMP indicator, it is also recognized as a potent photosensitizer that can cause mitochondrial damage by sequential fluorescent illumination (Hsieh et al., 2015; Yang and Yang, 2011). Thus, to measure MMP, the TMRE fluorescence intensity was assessed at each time point instead of prolonged time-lapse imaging. We found that, regardless of the FICE used, the MMP did not change before or after the change in mitochondrial morphology caused by iCMM over a 120-min time window (Figures 3A–3D). These results suggest that iCMM induces mitochondrial morphological changes irrespective of MMP and also indicates that such changes do not cause a loss of MMP under the experimental conditions.

We next examined whether changes in mitochondrial morphology induced by iCMM altered the susceptibility of mitochondria to carbonyl cyanide 4- (trifluoromethoxy) phenylhydrazone (FCCP), an ionophore uncoupler of oxidative phosphorylation (Figure 3I, left). Time-lapse imaging revealed that FCCP treatment resulted in a comparable loss of MMP irrespective of mitochondrial morphology (Figures 3E–3I). Together, these results indicate that iCMM alters mitochondrial morphology with a negligible effect on MMP.

We further confirmed that iCMM-induced mitochondrial morphological changes did not promote reactive oxygen species (ROS) production in mitochondria as measured by MitoSOX (Figure 4).

**Disruption of mitochondrial morphology by mActZ-FY, but not other effectors, suppresses oxygen consumption by mitochondria**

In mammalian cells, mitochondria are responsible for the majority of cellular oxygen consumption to generate ATP. To maintain bioenergetic activity in this process, mitochondria go through repeated fusion and fission cycles (Chen et al., 2005; Rapaport et al., 1998; Twig et al., 2008). To examine iCMM-induced mitochondrial morphology disruption on mitochondrial respiration capacity, we established cell lines stably expressing iCMM. Compared with control cells that stably expressed NiCMM (NiCMM cells), cells stably expressing iCMM (iCMMYF, iCMM Cav1s, and iCMM mActZ, respectively) showed no noticeable difference in cell proliferation or cell morphology (Figures S6A and S6B). As expected, cells stably expressing iCMM, not NiCMM, showed mitochondrial morphological changes in a switch-dependent manner, as were cells transiently expressing iCMM (Figure S6C).

We then examined mitochondrial respiration activity in cells stably expressing iCMM by measuring the oxygen consumption rate (OCR). Before OCR measurements, cells were treated with rapamycin to actuate iCMM/NiCMM or with DMSO as a counter-part for 30 or 120 min. Although rapamycin can affect the OCR under certain conditions (Lemer et al., 2013; Rosario et al., 2019), no significant difference in OCR was observed between NiCMM cells treated with DMSO and those treated with rapamycin, indicating that rapamycin, at least under the experimental conditions (50 nM, a 120-min time window), did not affect the OCR (Figures S6A and S6D). Similarly, disruption of mitochondrial morphology by YF and YF-Cav1s did not affect the OCR over a 120-min time window (Figures S6B, S6C, S6E, and S6F), indicating that YF and YF-Cav1s could induce changes in mitochondrial morphology without altering the OCR. In contrast, induction of mitochondrial morphological changes by mActZ-FY resulted in a significant decrease in OCR within 30 min (Figures S6D and S6G). Of note, there was no statistically significant difference in the maximum OCR in iCMM/mActZ cells at 120 min, but the maximum OCR exhibited a downward trend when the iCMM harboring mActZ-FY was turned on (Figure S6G). We also found that disruption of mitochondrial morphology by mActZ-FY reduced the basal extracellular acidification rate (ECAR), an indicator of glycolysis, at 120 min, but the difference at 30 min was
|          | mYF (CICE) | YF (FICE) | YF-Cav1s (FICE) | mActZ-FY (FICE) |
|----------|------------|-----------|-----------------|----------------|
| Time post NiCMM ON | 0 min | 30 min | 120 min | 0 min | 30 min | 120 min | 0 min | 30 min | 120 min |

![Images of fluorescence microscopy](Image)

|          | mYF (CICE) | YF (FICE) | YF-Cav1s (FICE) | mActZ-FY (FICE) |
|----------|------------|-----------|-----------------|----------------|
| Time post iCMM ON | 0 min | 30 min | 120 min | 0 min | 30 min | 120 min | 0 min | 30 min | 120 min |

![Images of fluorescence microscopy](Image)

**E**  
E: mYF (CICE)  
F: YF (FICE)  
G: YF-Cav1s (FICE)  
H: mActZ-FY (FICE)

|          | mYF (CICE) | YF (FICE) | YF-Cav1s (FICE) | mActZ-FY (FICE) |
|----------|------------|-----------|-----------------|----------------|
| Time post iCMM ON | 0 min | 30 min | 120 min | 0 min | 30 min | 120 min | 0 min | 30 min | 120 min |

![Images of fluorescence microscopy](Image)

**I**  
I: (Frame 1) 0 min 500 nM FCCP (after Frame 1)  
II: (Frame 2) 5 min  
III: (Frame 3) 10 min 40 μM FCCP (after Frame 3)  
IV: (Frame 4) 15 min

![Images of fluorescence microscopy](Image)

### (legend on next page)
not a detectable change in intracellular ATP levels in the cells. Morphology by mActZ-FY led to a decrease in OCR and ECAR but does not directly modulate genes involved in mitochondrial dynamics. However, similar to those in other stable cell lines, mitochondrial morphology by mActZ-FY would decrease intracellular ATP levels. Hence, it allowed us to examine whether iCMM-induced mitochondrial morphological changes do not render HeLa cells susceptible to staurosporine-induced apoptosis. Although the use of a previous, conventional genetic approach (modification of genes involved in mitochondrial dynamics) revealed that mitochondrial morphology changes do not promote apoptosis, we utilized iCMM to examine the biological significance of mitochondrial morphology from unconventional perspectives.

We next examined whether iCMM-induced mitochondrial morphology changes would affect susceptibility to apoptosis induction. In this experiment, we set the concentration of staurosporine (STS), a potent apoptosis inducer, to induce apoptosis in less than 40% of DMSO-treated iCMM/NiCMM cells to prepare for cell death (Elmore, 2007). One hallmark of this reorganization is mitochondrial fragmentation caused by an imbalance in the mitochondrial fusion-fission cycle (Karbowksi and Youle, 2003; Wang and Youle, 2009). Using cells stably expressing iCMM, we found that the artificial disruption of mitochondrial morphology by iCMM did not commit cells to apoptosis, as shown by cleaved caspase-3 levels (Figure 6A, lane 1 and 2) and nuclear morphology (Figure 6B, upper graph). These results suggest that changes in mitochondrial morphology per se do not trigger intrinsic apoptosis.

Figure 4. iCMM-induced changes in mitochondrial morphology do not increase mitochondrial ROS

HeLa cells transiently expressing Tom20-CR and the indicated effector were treated with either DMSO or 50 nM rapamycin for 2 h. For no device, HeLa cells not expressing NiCMM/iCMM were treated with either DMSO or 40 mM FCCP for 2 h. After the treatment, mitochondrial ROS was measured by MitoSOX. Box plot, center lines show the medians; box limits indicate the 25th and 75th percentiles as determined by R software; whiskers extend 1.5 times the interquartile range from the 25th and 75th percentiles, outliers are represented by dots. $n$ = number of cells analyzed, from two independent experiments. $p$ = Welch’s $t$ test. N.S., statistically non-significant.
Figure 5. mActZ-FY-induced disruption of mitochondrial morphology affects OCR and ECAR
(A–D) OCR and ECAR in NiCMM (A), iCMMYF (B), iCMMCav1s (C), and iCMMmActZ cells (D). Before measurement, all cell lines were treated with DMSO or 50 nM rapamycin for 30 min. Quantification was performed on three independent experiments. All data are presented as mean ± standard deviation. *p < 0.05, N.S., statistically non-significant (paired t test).

(E–H) Intracellular ATP levels in NiCMM (E), iCMMYF (F), iCMMCav1s (G), and iCMMmActZ cells (H). Each cell line was treated with DMSO (D) or 50 nM rapamycin (R) for 1 h, followed by 2-DG treatment for 4 h. Quantification was performed on three independent experiments. All data are presented as mean ± standard deviation. N.S., statistically non-significant (Student’s t test). See also Figure S6.
Figure 6. iCMM-induced changes in mitochondrial morphology do not affect STS-induced apoptosis.

(A) HeLa cells stably expressing Tom20-CR and the indicated effector were treated with DMSO (Rapa [−]) or 50 nM rapamycin (Rapa [+] for 1 h, followed by treatment with 266 nM STS for 6 h. The cells were then subjected to western blotting.

(B) HeLa cells stably expressing Tom20-CR and the indicated effector were treated as in (A), and normal and apoptotic nuclei in the cells were counted. Quantification was performed on three independent experiments. All data are presented as mean ± standard deviation. Cells stably expressing Tom20-CR and either mYF (Rapa/STS = −/− (RS00): n = 539, Rapa/STS = +/+ (RS11): n = 974), YF (RS00, n = 673; RS10, n = 643; RS01, n = 727; RS11, n = 695), YF-Cav1s (RS00, n = 1,182; RS10, n = 1,182; RS01, n = 864; RS11, n = 684), or mActZ-FY (RS00, n = 634; RS10, n = 630; RS01, n = 545; RS11, n = 568) were analyzed. N.S., statistically non-significant (Student’s t test).

(C) HeLa cells transiently expressing Tom20-CR and the indicated effector were treated as in (A), and normal and apoptotic nuclei in cells expressing NiCMM/iCMM (device-positive cells) or not expressing NiCMM/iCMM (device-negative cells) were counted. Quantification was performed on three independent experiments. All data are presented as mean ± standard deviation. Cells transiently expressing Tom20-CR and either mYF (RS00, n = 242; RS10, n = 175; RS01, n = 223; RS11, n = 211), YF (RS00, n = 164; RS10, n = 202; RS01, n = 222; RS11, n = 198), YF-Cav1s (RS00, n = 101; RS10, n = 108; RS01, n = 194; RS11, n = 261) or mActZ-FY (RS00, n = 113; RS10, n = 131; RS01, n = 190; RS11, n = 208) and cells that did not express the device in the vicinity of cells expressing Tom20-CR and either mYF (RS00, n = 393; RS10, n = 292; RS01, n = 1,002; RS11, n = 554), YF-Cav1s (RS00, n = 294; RS10, n = 203; RS01, n = 794; RS11, n = 735), YF-Cav1s (RS00, n = 198; RS10, n = 209; RS01, n = 967; RS11, n = 995), or mActZ-FY (RS00, n = 246; RS10, n = 229; RS01, n = 851; RS11, n = 810) were analyzed. N.S., statistically non-significant (Student’s t test). See also Figure S7.
fragmentation could increase susceptibility to apoptosis-inducible input (Lee et al., 2004; Sugioka et al., 2004), we found that disruption of mitochondrial morphology by iCMM did not promote STS-induced apoptosis compared with cells stably expressing NiCMM, as shown by cleaved caspase-3 levels (Figure 6A, lane 3,4) and nuclear morphology (Figure 6B, lower graph). Similarly, when cells transiently expressing iCMM and NiCMM were compared, iCMM-induced mitochondrial morphological changes did not enhance STS-induced apoptosis (Figure 6C). Of note, cells transiently expressing iCMM/NiCMM were more susceptible to STS than the surrounding cells that did not express iCMM/NiCMM (Figure 6C), possibly because of damage caused by transient gene expression.

Intriguingly, we found that rapamycin, an allosteric mTORC1 inhibitor, failed to completely attenuate mTORC1 activity in iCMMmActZ cells compared with other cell lines, as shown by the phosphorylation level of the mTORC1 substrate p70 S6 kinase (S6K) and the downward mobility shift of non-phosphorylated S6K (Figure 6A). However, STS-induced suppression of mTORC1 activity (Tee and Proud, 2001) in iCMMmActZ cells was similar to that in other cells (Figure 6A). In addition, Torin 1, an ATP-competitive mTOR inhibitor, also completely suppressed mTORC1 activity in all cell types examined (Figure S7). These results suggest that rapamycin’s inhibitory effect through its binding of FKBP12 and mTORC1 can be slightly suppressed by iCMM harboring mActZ-FY.

Rapid mitochondrial morphology changes induced by iCMM alter the transcriptome

An attractive aspect of synthetic biology is that it allows us to manipulate cellular behaviors by using rationally designed synthetic biomolecular devices. Although considerable efforts have been made to understand how biomolecules derived from inherent biological or artificially designed systems can be applied to devices, the applicability of organelle morphology to these synthetic biomolecular devices has not been explored because of technical limitations. To expand the possible range of applications of these devices, we examined how iCMM-induced changes in mitochondrial morphology modify gene expression in cells.

Gene set enrichment analysis (GSEA) of 50 “hallmark” gene sets representing major biological processes (Liberzon et al., 2015; Subramanian et al., 2005) revealed that changes in mitochondrial morphology induced by FICE promoted the downregulation of MYC target expression irrespective of the mitochondrial morphology induced (Figure 7A). Gene ontology (GO) analysis demonstrated that iCMM-induced changes in mitochondrial morphology led to changes in various cellular functions, most notably decreased ribosomal functions (Figure 7B). Furthermore, GSEA and GO analysis did not report apoptosis as an iCMM-mediated cellular function (Figures 7A and 7B), supporting the notion that iCMM-induced changes in mitochondrial morphology per se did not affect apoptosis (Figure 6).

To further examine the effect of iCMM-induced changes in mitochondrial morphology on the transcriptome, we identified differentially expressed genes (DEGs) whose expression levels were significantly changed (defined as a fold change >2 and false discovery rate [FDR]-adjusted p < 0.05) (Figure S8 and Table S1). DEGs analysis revealed that the translocation of FICE and CiC to mitochondria per se did not affect the expression of genes involved in the mitochondrial fusion-fission cycle, as shown by the measurement of DRP1, FIS1, RAB32, OPA1, and MFN1/2 (Table S1). Consistent with a previous report (Minster et al., 2016), CREBRF was identified as a DEG in rapamycin-treated cell lines expressing iCMM and NiCMM, highlighting the fidelity of the analysis (Table S1).

Among DEGs, 18, 31, 68, and 111 effector-specific DEGs were extracted from the use of mYF, YF, YF-Cav1s, and mActZ-FY, respectively (Table S1). GO analysis of the effector-specific DEGs demonstrated that the actuation of iCMM triggered diverse cellular functions depending on which effector was employed (Figure 7C). Notably, YF-Cav1s tended to increase the expression of genes related to actin-myosin interactions, whereas mActZ-FY appeared to decrease the expression of genes involved in mitochondrial translation (Figure 7C). These results suggest that the actuation of iCMM or NiCMM in target cells can provoke alteration of gene expression in an effector-dependent manner.

Rapid changes in mitochondrial morphology induced by iCMM alter the amino acid profile

All 20 amino acids, except for histidine, alanine, and cysteine, require mitochondrial enzymes for their metabolism (Guda et al., 2007). We thus hypothesized that iCMM-induced disruption of mitochondrial morphology would alter the intracellular amino acid profile. Although amino acids are well recognized

Figure 7. iCMM-induced mitochondrial morphology changes result in reorganization of the cellular transcriptome and amino acid profiles

Cells stably expressing Tom20-CR and the indicated effector were treated with either DMSO or 50 nM rapamycin for the indicated time and subsequently subjected to transcriptome analysis and amino acid profile analysis. (A and B) Hallmark gene sets (A) and GO terms (B) enriched upon mitochondrial morphology changes in cells stably expressing Tom20-CR and the indicated effector were analyzed to yield heatmaps of –log10 (FDR). FDR values were calculated by comparing DMSO- and rapamycin-treated cells for each effector at the indicated time points. Up/down indicates the direction of a transcriptomic change in rapamycin-treated cells compared with DMSO-treated cells. A maximum of 30 gene sets with FDR <0.01 in each direction and category are shown. (C) Top 10 GO terms (p < 0.05) enriched in the effector-specific DEGs. (D) Total amount of amino acids in cells stably expressing Tom20-CR and the indicated effector were analyzed after adding either DMSO or 50 nM rapamycin at the indicated time. Quantification was performed on three independent experiments. All data are presented as mean ± standard deviation. N.S., statistically non-significant (Student’s t test). (E) Amino acids with significantly altered proportions (criteria: p < 0.05, more than 10% change). White: amino acids that do not meet this criterion. (F) Network graphs based on the correlation coefficient calculated for each amino acid. The color of each node represents the correlation with each amino acid based on serine, whereas the color of each edge represents the correlation between amino acids. Blue, low correlation coefficient; red, high correlation. The thickness of the edge represents the absolute value of the correlation coefficient. See also Figure S8.
as cues that modulate various signaling pathways, recent mathematical analysis has revealed that the comprehensive amino acid profile, not a single amino acid, plays a pivotal role in modulating cellular functions (Nishi et al., 2018). Therefore, we focused on the effect of iCMM on the proportion of individual amino acids among total amino acids. Although the total amount of amino acids did not differ significantly (Figure 7D), the proportion of various individual amino acids to the total amino acids was significantly altered in a FiCE-dependent manner after iCMM was turned on (Figure 7E). Interestingly, the reorganization of amino acid profiles was also observed in NiCMM cells (Figure 7E). However, histidine, alanine, cysteine, tryptophan, lysine, and valine are not associated with mitochondria, and did not show differential levels when any effector was activated (Figure 7E). These results suggest that the changes in amino acid profiles were caused at least partially by effector accumulation in mitochondria.

All amino acid metabolism is coordinately regulated in the cell to create an appropriate amino acid profile in accordance with the circumstances. To further analyze the interplay between iCMM and amino acids, we examined correlations among amino acids. Network architectures reflecting amino acid correlations were similar in NICMM and iCMM(Cav1s) cells and in iCMM(FY) and iCMM(mActZ) cells (Figure 7F). In NiCMM and iCMM(Cav1s) cells, glutamate, aspartate, and proline, which are regulated by p53-mediated amino acid metabolism in the vicinity of mitochondria, presented a low correlation with serine that is critical for mitochondrial dynamics and functions (Gao et al., 2018) (Figure 7F). However, in iCMM(FY) cells, nearly all amino acids presented a strong correlation with serine, whereas correlations among amino acids in iCMM(mActZ) cells disappeared over time (Figure 7F). These results suggest that iCMM and NiCMM actuation led to alteration of the coordination of amino acid metabolism.

**DISCUSSION**

Recent advances in life sciences have transformed cells into objects of not only understanding but also manipulation. A comprehensive understanding of intrinsic biological systems through reductionistic approaches (e.g., molecular biology) is necessary to develop techniques for manipulating cells. On the other hand, the manipulation of cells by using constructive approaches (e.g., synthetic biology) can facilitate the understanding of biological systems. The harmonization of insights obtained from reductionistic approaches (e.g., molecular biology) is necessary to understand the detailed mechanisms, we believe that mActZ-FY could be useful in elucidating the relationship between actin skeleton architecture and the metabolic phenotype defined by OCR and ECAR. Furthermore, our results suggest that mActZ-FY is a useful FiCE for manipulating OCR and ECAR at any given time point.

Mitochondrial fragmentation is a major hallmark of apoptosis, but why mammalian cells have internalized the process of apoptosis during evolution and the biological significance of this internalization remains controversial. We expected iCM-M-induced changes in mitochondrial morphology to alter the sensitivity of mitochondria to apoptosis inducers, but, contrary to our expectations, iCM did not alter the sensitivity of HeLa cells to STS, at least under our experimental conditions. These results suggest that the process that leads to a certain mitochondrial morphology, rather than the morphology per se, might be more critical in determining sensitivity to
apoptosis inducers. For example, overexpression of the human cytomegalovirus-encoded protein viral mitochondria-localized inhibitor of apoptosis (vMIA) causes mitochondrial fragmentation. However, vMIA also exhibits anti-apoptotic effects as it binds and inhibits proapoptotic Bcl-2 family members (Norris and Youle, 2008). Future studies using various cell lines and apoptosis inducers will provide a comprehensive understanding of the relationship between mitochondrial morphology and apoptosis. We are confident that iCMM will prove to be a valuable tool in these studies with the development of appropriate effectors.

Notably, we found that the effect of rapamycin on mTORC1 was only observed in a subset of cells stably expressing mActZ-FY and Tom20-CR. Because the activity of mTORC1 was completely suppressed by rapamycin in other cells stably expressing iCMM/NiCMM, exogenously expressed FKBP and FRB are unlikely to be the main cause of the inhibitory effect of rapamycin on mTORC1 activity in iCMMmActZ cells. Although it is well appreciated that mTORC1 mainly localizes to lysosomes, several reports indicate that mTORC1 also localizes to mitochondria (Betz and Hall, 2013). Meanwhile, although the localization of endogenous FKBP12 has not been clarified, the results from tagging with fluorescent proteins (mYF and YF in this study) indicate that it is diffusely localized throughout the cell. Therefore, even if changes in mitochondrial morphology induced by mActZ-FY alter the localization of mTORC1, this change does not appear to affect rapamycin-mediated suppression of mTORC1 via FKBP12. However, it is conceivable that reorganization of the actin skeleton architecture around mitochondria might have interfered with the rapamycin-regulated binding of mTORC1 and FKBP12. Given that rapamycin and its derivatives are promising therapeutics for cancers, further studies to elucidate the mechanism by which mActZ-FY attenuates the effects of rapamycin on mTORC1 activity would be useful for both basic and clinical research. In addition, the development of strategies to completely inhibit the effects of rapamycin on endogenous mTORC1 activity in a target cell will lead to the development of more complex and sophisticated rapamycin-based synthetic biomolecular devices.

Mitochondria transfer their genetic information to the host cell nucleus in the course of an endosymbiotic relationship. Therefore, retrograde signaling, a communication system from mitochondria to the nucleus, is a prerequisite to coordinate mitochondrial functions with the nucleic transcription machinery under various physiopathological conditions. In this study, we found that the induction of mitochondrial morphological changes by iCMM reduced the expression of MYC targets and ribosome-related genes. There is no reliable evidence to mechanistically explain why the changes in mitochondrial morphology led to the impairment of MYC target and ribosome-related gene expression. However, a potential molecule to bridge this gap is MYC. The MYC family, an oncogene family, is dysregulated in >50% of human cancers, regulates the transcription of at least 15% of the entire genome (Dang et al., 2006), indicating the multifunctionality of MYC in biological systems. Remarkably, MYC plays a pivotal role in regulating ribosome biogenesis (Van Riggelen et al., 2010). In addition, over several decades, many studies have suggested an intimate relationship between MYC and mitochondria (Morrish and Hockenbery, 2014; Zhang et al., 2017). Our results also provide additional support for the relationship between MYC and mitochondria. Although the molecular puzzle underlying the MYC-mitochondria-ribosome axis remains unsolved, the finding that iCMM-induced changes in mitochondrial morphology reduced the expression of MYC- and ribosome-related genes provides one part of the solution.

We also found that the FiCes and CiCE developed in this study induced characteristic gene expression and amino acid profiles. These results show that perturbations in mitochondria (not only morphological changes but also the accumulation of extrinsic effector proteins in mitochondria) could alter cellular gene expression and amino acid profiles. Further studies, including comparisons with conventional methods, will provide detailed mechanistic insights into the mitochondrial signaling evoked by changes in mitochondrial morphology and resulting changes in the transcriptome and amino acid profile.

In summary, the iCMM system developed in this study allows the effective and rapid manipulation of mitochondrial morphology. In combination with conventional genetic approaches, iCMM might provide new insights into the physiopathological functions of mitochondrial morphology in health and disease. In this study, we mainly used HeLa cells (human cervical adenocarcinoma) for our experiments, but further comprehensive studies using other cancer cell lines and normal cells are required to understand the effects of iCMM-induced mitochondrial morphological changes on cells. We envision that one promising application of iCMM might involve the treatment of a variety of diseases. If we could alter the properties of cells (e.g., omics information) by inducing mitochondrial morphological changes at appropriate time points, this would lead to the development of new therapeutic strategies for diseases. It is also possible to study how the phenotype changes by disrupting giant mitochondria observed in various pathological tissues (such as the liver, heart, and nerve tissue) with iCMM. In life science research, the high spatial and temporal resolution of iCMM might be applied to the manipulation of local communication between the mitochondria and other organelles. Although we have a long way to go to grasp the biological significance of the ever-changing mitochondrial morphology in a cell, there is no doubt that mitochondrial morphology is more than just a phenotype.

Limitations of study

Although iCMM can artificially change the morphology of the mitochondria without losing MMP, transferring extrinsic genes and addition of chemical dimerizer (or laser irradiation) into the target cells are necessary to activate iCMM. Therefore, when comparing the effects of the change in artificial mitochondrial morphology by iCMM with the effects of the change in conventional physiological mitochondrial morphology (e.g., down-regulation of DRP1 or OPA1), it is necessary to interpret the results by considering the characteristics of both methods. For example, Li et al. (2020) reported that knockdown of OPA1 in Huh7 cells altered the expression of metabolism-related genes. However, no such changes were observed when mitochondrial morphological changes were induced by iCMM in HeLa cells (Figure 7). Although differences in cell
type might be one reason, we recognize that the mitochondrial morphological changes caused by iCMM that have developed in this study do not produce exactly the same results as physiological mitochondrial morphological changes. The development of iCMM that can induce changes in intracellular information similar to those induced by physiological mitochondrial morphological changes is an important obstacle to overcome in the future. However, it should be noted that when physiological mitochondrial morphology change is achieved by regulating gene expression, the expression levels of genes other than the target gene might also change in conjunction. Moreover, changes in mitochondrial morphology in various pathological conditions are thought to be the result of incredibly complicated changes in intracellular information. Accordingly, it might be challenging to obtain the same output caused by physiological mitochondrial morphological changes with iCMM alone. Although the term mitochondrial morphological is simple, it should be considered that it encompasses a wide variety of biological information, including the spatiotemporal distribution and heterogeneous activity of mitochondria and their crosstalk with other organelles. We believe that it is useful to integrate conventional genetic methods with artificial morphological manipulation methods such as iCMM in order to solve the fundamental question of why the mitochondria show different morphologies depending on the conditions.

STAR METHODS

Detailed methods are provided in the online version of this paper and include the following:

- **KEY RESOURCES TABLE**
- **RESOURCE AVAILABILITY**
  - Lead contact
  - Materials availability
  - Data and code availability
- **EXPERIMENTAL MODEL AND SUBJECT DETAILS**
  - Cell culture and media
- **METHOD DETAILS**
  - Plasmid construction
  - Transient transfection
  - Real-time reverse-transcription polymerase chain reaction
  - Lentivirus production
  - Establishment of HeLa cells stably expressing the iCMM system
  - Live-cell imaging
  - CLEM
  - Immunocytochemistry
  - Mitochondrial ROS measurement
  - Mitochondrial membrane potential analysis
  - Extracellular flux analysis
  - ATP measurement
  - Cell proliferation assay
  - RNA-seq
  - Amino acid profile analysis
  - Apoptosis analysis
  - Western blot analysis

- **QUANTIFICATION AND STATISTICAL ANALYSIS**
  - Mitochondrial morphology analysis
  - Transcriptome analysis
  - Amino acid correlation network analysis
  - Statistical analysis

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.crmeth.2021.100052.

ACKNOWLEDGMENTS

We thank Katsuyuki Suzuki, Akira Takebe (JEOL Ltd), and Katsuko Okubo (University of Tsukuba) for technical assistance; Keita Takahashi (NikonInstitute Co., Ltd); Hiroyuki Soejima (Tokyo Science Co., Ltd.); and Yasuko Maruyama (University of Tsukuba) for generous support. We would like to thank Editage (www.editage.com) for English language editing. This study was supported in part by grant-in-aid for Scientific Research on Innovative Areas (18H04854), Leading Initiative for Excellent Young Researchers, JST CREST (JPMJCR1927), and JST COI-NEXT (JPMJPF2017).

AUTHOR CONTRIBUTIONS

T. Miyamoto conceived the project. T. Miyamoto designed the experiments. T. Miyamoto, H.U., Y.M., S.I.H., S.G., D.Y., M.M., Y.Y., H.N., N.H., Y.T., M.S., T. Matsuzaka, F.H., H.O., S.I.T., N.Y., K.I., and H.S. conducted the experiments. T. Miyamoto and H.U. wrote the article.

DECLARATION OF INTERESTS

The authors declare no competing interests.

Received: December 3, 2020
Revised: March 12, 2021
Accepted: June 22, 2021
Published: July 22, 2021

REFERENCES

Archer, S.L. (2013). Mitochondrial dynamics–mitochondrial fission and fusion in human diseases. N. Engl. J. Med. 369, 2236–2251.

Betz, C., and Hall, M.N. (2013). Where is mTOR and what is it doing there? J. Cell Biol. 203, 563–574.

Buck, M.D.D., O’Sullivan, D., Klein Geltink, R.I., Curtis, J.D.D., Chang, C.H., Sanin, D.E.E., Qiu, J., Kretz, O., Braas, D., van der Windt, G.J.J.W., et al. (2016). Mitochondrial dynamics controls T cell fate through metabolic programming. Cell 166, 63–76.

Casalena, G., Bottinger, E., and Daehn, I. (2016). TGFβ-induced actin cytoskeleton rearrangement in podocytes is associated with compensatory adaptation of mitochondrial energy metabolism. Nephron 131, 278–284.

Cassidy-Stone, A., Chipuk, J.E., Ingerman, E., Song, C., Yoo, C., Kuwana, T., Kurth, M.J., Shaw, J.T., Hinshaw, J.E., Green, D.R., et al. (2008). Chemical inhibition of the mitochondrial division dynamin reveals its role in Bax/Bak-dependent mitochondrial outer membrane permeabilization. Dev. Cell 14, 193–204.

Chen, H., Chomyn, A., and Chan, D.C. (2005). Disruption of fusion results in mitochondrial heterogeneity and dysfunction. J. Biol. Chem. 280, 26185–26192.

Clarke, L., and Kitney, R. (2020). Developing synthetic biology for industrial biotechnology applications. Biochem. Soc. Trans. 48, 113–122.

Csordás, G., Vármai, P., Golenár, T., Roy, S., Purkins, G., Schneider, T.G., Balla, T., and Hajnoczy, G. (2010). Imaging interorganelle contacts and local calcium dynamics at the ER-mitochondrial interface. Mol. Cell 39, 121–132.
Dang, C.V., O’Donnell, K.A., Zeiler, K.I., Nguyen, T., Osthus, R.C., and Li, F. (2006). The c-Myc target gene network. Semin. Cancer Biol. 16, 253–264.

Derose, R., Miyamoto, T., and Inoue, T. (2013). Manipulating signaling at will: chemically-inducible dimerization (CID) techniques resolve problems in cell biology. Pflugers Arch. Eur. J. Physiol. 465, 409–417.

Detmer, S.A., and Chan, D.C. (2007). Functions and dysfunctions of mitochondrial dynamics. Nat. Rev. Mol. Cell Biol. 8, 870–879.

Dobin, A., Davis, C.A., Schlesinger, F., Drenkow, J., Zaleski, C., Jha, S., Batut, P., Chaisson, M., and Gingeras, T.R. (2013). STAR: ultrafast universal RNA-seq aligner. Bioinformatics 29, 15–21.

Elmore, S. (2007). Apoptosis: a review of programmed cell death. Toxicol. Pathol. 35, 495–516.

Fegan, A., White, B., Carlson, J.C.T., and Wagner, C.R. (2010). Chemically controlled protein assembly: techniques and applications. Chem. Rev. 110, 3315–3336.

Folcher, M., and Fussenegger, M. (2012). Synthetic biology advancing clinical applications. Curr. Opin. Chem. Biol. 16, 345–354.

Gao, X., Lee, K., Reid, M.A., Sanderson, S.M., Qiu, C., Li, S., Liu, J., and Locascio, J.W. (2018). Serine availability influences mitochondrial dynamics and function through lipid metabolism. Cell Rep. 22, 3507–3520.

Golsteyn, R.M., Beckerle, M.C., Koay, T., and Friederich, E. (1997). Structural and functional similarities between the human cytoskeletal protein zyxin and the Acta protein of Listeria monocytogenes. J. Cell Sci. 110, 1893–1906.

Gray, M.W., Burger, G., and Franz Lang, B. (2001). The origin and early evolution of mitochondria. Genome Biol. 2, 1–5.

Grozinger, L., Amos, M., Gorochowski, T.E., Carbonell, P., Oyarzun, D.A., Stoof, R., Fellermann, H., Zuliani, P., Tas, H., and Gorli-Moreno, A. (2019). Pathways to cellular supremacy in biocomputing. Nat. Comm. 10, 1–11.

Hsieh, C.W., Chu, C.H., Lee, H.M., and Yuan Yang, W. (2015). Triggering mitophagy with far-red fluorescent photosensitizers. Sci. Rep. 5, 1–12.

Ichinohe, T., Yamazaki, T., Koshiba, T., and Yanagi, Y. (2013). Mitochondrial protein mitofusin 2 is required for NLRP3 inflammasome activation after RNA virus infection. Proc. Natl. Acad. Sci. U S A 110, 17963–17968.

Inoue, T., Heo, W. Do, Grimley, J.S., Wandless, T.J., and Meyer, T. (2005). An inducible translocation strategy to rapidly activate and inhibit small GTPase activity facilitates mitochondrial retrograde signaling and increases life span in normal human fibroblasts. Aging Cell 11, 870–880.

Kakimoto, Y., Tashiro, S., Kojima, R., Morozumi, Y., Endo, T., and Tamura, Y. (2014). Visualizing multiple inter-organelle contact sites using the organelle-masking and functional similarities between the human cytoskeletal protein zyxin and the Acta protein of Listeria monocytogenes. J. Cell Sci. 117, 1903–1906.

Khalil, A.S., and Collins, J.J. (2010). Synthetic biology: applications come of age. Nat. Rev. Genet. 11, 367–379.

Koshiba, T., Yasukawa, K., Yanagi, Y., and Kawabata, S. (2011). Mitochondrial membrane potential is required for MAVS-mediated antiviral signaling. Sci. Signa. 4, 1–8.

Lambert, T.J. (2019). FPbase: a community-editable fluorescent protein database. Nat. Methods 16, 277–278.

Lee, Y., Jeong, S.-Y., Karpowski, M., Smith, C.L., and Youle, R.J. (2004). Roles of the mammalian mitochondrial fission and fusion mediators Fis1, Drp1, and Opa1 in apoptosis. Mol. Biol. Cell 15, 5001–5011.

Leonard, A.P., Cameron, R.B., Speiser, J.L., Wolf, B.J., Peterson, Y.K., Schnellmann, R.G., Beeson, C.C., and Rohrer, B. (2015). Quantitative analysis of mitochondrial morphodynamics and membrane potential in living cells using high-content imaging, machine learning, and morphological binning. Biochim. Biophys. Acta Mol. Cell Res. 1853, 348–360.

Lerner, C., Bitto, A., Pulliam, D., Nacarelli, T., Konigsberg, M., Van Remmen, H., Torres, C., and Sell, C. (2013). Reduced mammalian target of rapamycin activity facilitates mitochondrial retrograde signaling and increases life span in normal human fibroblasts. Aging Cell 12, 966–977.

Li, M., Wang, L., Wang, Y., Zhang, S., Zhou, G., Lieshout, R., Ma, B., Liu, J., Qu, C., Verstegen, M.M.A., et al. (2020). Mitochondrial fusion via OPA1 and MFN1 supports liver tumor cell morphology and growth. Cells 9, 1–16.

Liang, F.S., Ho, W.Q., and Crabtree, G.R. (2011). Engineering the ABA plant stress pathway for regulation of induced proximity. Sci. Signal. 4, 1–10.

Liao, Y., Smyth, G.K., and Shi, W. (2014). FeatureCounts: an efficient general purpose program for assigning sequence reads to genomic features. Bioinformatics 30, 923–930.

Liberzon, A., Subramanian, A., Pinchback, R., Thorvaldsdottir, H., Tamayo, P., and Mesirov, J.P. (2011). Molecular signatures database (MSigDB) 3.0. Bioinformatics 27, 1739–1740.

Liberzon, A., Birger, C., Thorvaldsdottir, H., Ghandi, M., Mesirov, J.P., and Tamayo, P. (2015). The molecular signatures database hallmark gene set collection. Cell Syst. 1, 417–425.

Liu, P., Calderon, A., Konstantinidis, G., Hou, J., Voss, S., Chen, X., Li, F., Banerjee, S., Hoffmann, J.E., Theiss, C., et al. (2014). A bioorthogonal small-molecule-switch system for controlling protein function in live cells. Angew. Chem. Int. Ed. 53, 10049–10055.

Lomakin, A.J., Cattin, C.J., Cuvelier, D., Alraies, Z., Molina, M., Nader, G.P.F., Srivastava, N., Saez, P.J., Garcia-Arcos, J.M., Zhiltynk, I.Y., et al. (2020). The nucleus acts as a ruler tailoring cell responses to spatial constraints. Science 370, eaab2894.

McCarthy, D.J., Chen, Y., and Smyth, G.K. (2012). Differential expression analysis of multifactor RNA-seq experiments with respect to biological variation. Nucleic Acids Res. 40, 4288–4297.

Minster, R.L., Hawley, N.L., Su, C.T., Sun, G., Kershaw, E.E., Cheng, H., Buhrle, O.D., Lin, J., Reupena, M.S., Viali, S., et al. (2016). A thrifty variant in CREBFR strongly influences body mass index in Samoans. Nat. Genet. 48, 1049–1054.

Miyamoto, T., DeRose, R., Suarez, A., Ueno, T., Chen, M., Sun, T.P., Wolfgang, M.J., Mukherjee, C., Meyers, D.J., and Inoue, T. (2012). Rapid and orthogonal logic gating with a gibberellin-induced dimerization system. Nat. Chem. Biol. 8, 465–470.

Miyamoto, T., Razavi, S., Derose, R., and Inoue, T. (2013). Synthesizing biomolecule-based boolean logic gates. ACS Synth. Biol. 2, 72–82.

Miyamoto, T., Rho, E., Sample, V., Akano, H., Magari, M., Ueno, T., Gorskho, K., Chen, M., Tokumitsu, H., Zhang, J., et al. (2015). Compartmentalized AMPK signaling illuminated by genetically encoded molecular sensors and actuators. Cell Rep. 17, 657–670.

Morrish, F., and Hockenbery, D. (2014). MYC and mitochondrial biogenesis. Cold Spring Harb. Perspect. Med. 4, a014225.

Nakamura, H., Rho, E., Deng, D., Razavi, S., Matsubayashi, H.T., and Inoue, T. (2020). ActuAtor, a molecular tool for generating force in living cells: controlled deformation of intracellular structures. BioRxiv, 2020.03.30.016360.

Nishii, H., Yamanaka, D., Kamei, H., Goda, Y., Kuman, M., Toyoshima, Y., Takenaka, A., Masuda, M., Nakabayashi, Y., Shioya, R., et al. (2018). Importance of serum amino acid profile for induction of hepatic steatosis under protein malnutrition. Sci. Rep. 8, 6232–6243.

Norris, K.L., and Youle, R.J. (2008). Cytomegalovirus proteins vMIA and m38.5 target mitochondrial morphology to Bcl-2 family proteins. J. Virol. 82, 6232–6243.

Park, J.S., Burckhardt, C.J., Lazzcano, R., Solis, L.M., Isogai, T., Li, L., Chen, C.S., Gao, B., Minna, J.D., Bachoo, R., et al. (2020). Mechanical regulation of glycolysis via cytoskeleton architecture. Nature 578, 621–626.
Parton, R.G., and Simons, K. (2007). The multiple faces of caveolae. Nat. Rev. Mol. Cell Biol. 8, 185–194.

Picard, M., Shirihai, O.S., Gentil, B.J., and Burelle, Y. (2013). Mitochondrial morphology transitions and functions: implications for retrograde signaling? Am. J. Physiol. Regul. Integr. Comp. Physiol. 304, R393–R406.

Pützrski, M., and Schultz, C. (2012). Protein translocation as a tool: the current rapamycin story. FEBS Lett. 586, 2097–2105.

R Core Team (2017). (2017). R: A Language and Environment for Statistical Computing. https://www.R-project.org/.

Rapaport, D., Brunner, M., Neupert, W., and Westermann, B. (1998). Fzo1p is a mitochondrial outer membrane protein essential for the biogenesis of functional mitochondria in Saccharomyces cerevisiae. J. Biol. Chem. 273, 20150–20155.

Van Riggelen, J., Yetil, A., and Felsher, D.W. (2010). MYC as a regulator of ribosome biogenesis and protein synthesis. Nat. Rev. Cancer 10, 301–309.

Robinson, M.D., McCarthy, D.J., and Smyth, G.K. (2009). edgeR: a Bioconductor package for differential expression analysis of digital gene expression data. Bioinformatics 26, 139–140.

Rosario, F.J., Gupta, M.B., Myatt, L., Powell, T.L., Glenn, J.P., Cox, L., and Jansson, T. (2019). Mechanistic target of rapamycin complex 1 promotes the expression of genes encoding electron transport chain proteins and stimulates oxidative phosphorylation in primary human trophoblast cells by regulating mitochondrial biogenesis. Sci. Rep. 9, 1–14.

Schindelin, J., Arganda-Carreras, I., Frise, E., Kaynig, V., Longair, M., Pietzsch, T., Preibisch, S., Rueden, C., Sabaft, S., Schmid, B., et al. (2012). Fiji: an open-source platform for biological-image analysis. Nat. Methods 9, 676–682.

Schlegel, A., and Lisanti, M.P. (2000). A molecular dissection of caveolin-1 membrane attachment and oligomerization. Two separate regions of the caveolin-1 c-terminal domain mediate membrane binding and oligomer/oligomer interactions in vivo. J. Biol. Chem. 275, 21605–21617.

Smetintk, J.A., Zeviani, M., Turnbull, D.M., and Jacobs, H.T. (2006). Mitochondrial medicine: a metabolic perspective on the pathology of oxidative phosphorylation disorders. Cell Metab. 3, 9–13.

Smith, G.A., and Portnoy, D.A. (1997). How the Listeria monocytogenes ActA protein converts actin polymerization into a motile force. Trends Microbiol. 5, 272–276.

Subramanian, A., Tamayo, P., Mootha, V.K., Mukherjee, S., Ebert, B.L., Gillette, M.A., Paulovich, A., Pomeroy, S.L., Golub, T.R., Lander, E.S., et al. (2005). Gene set enrichment analysis: a knowledge-based approach for interpreting genome-wide expression profiles. Proc. Natl. Acad. Sci. U S A 102, 15545–15550.

Sugioka, R., Shimizu, S., and Tsuji moto, Y. (2004). Fzo1, a protein involved in mitochondrial fusion, inhibits apoptosis. J. Biol. Chem. 279, 52726–52734.

Sun, H., Chen, L., Cao, S., Liang, Y., and Xu, Y. (2019). Warburg effects in cancer and normal proliferating cells: two tales of the same name. Genomics Proteomics Bioinformatics 17, 273–286.

Tao, Y., Yang, Y., Zhou, R., and Gong, T. (2020). Golgi apparatus: an emerging platform for innate immunity. Trends Cell Biol 30, 487–477.

Tee, A.R., and Proud, C.G. (2001). Staurosporine inhibits phosphorylation of translational regulators linked to mTOR. Cell Death Differ. 8, 841–849.

Twy, G., Hyde, B., and Shirihai, O.S. (2008). Mitochondrial fusion, fission and autophagy as a quality control axis: the bioenergetic view. Biochim. Biophys. Acta Bioenerg. 1777, 1092–1097.

Venturini, V., Pezzano, F., Catala Castro, F., Hämkinen, H.-M., Jiménez-Delgado, S., Colomer-Rosell, M., Marro, M., Tolosa-Ramon, Q., Paz-López, S., Valverde, M.A., et al. (2020). The nucleus measures shape changes for cellular proprioception to control dynamic cell behavior. Science 370, eaab2644.

Wai, T., and Langer, T. (2016). Mitochondrial dynamics and metabolic regulation. Trends Endocrinol. Metab. 27, 105–117.

Wang, C., and Youle, R.J. (2009). The role of mitochondria in apoptosis. Annu. Rev. Genet. 43, 95–118.

Westermann, B. (2012). Bioenergetic role of mitochondrial fusion and fission. Biochim. Biophys. Acta Bioenerg. 1817, 1833–1838.

Yang, J.Y., and Yang, W.Y. (2011). Spatiotemporally controlled initiation of Parkin-mediated mitophagy within single cells. Autophagy 7, 1230–1238.

Yu-Wai-Man, P., and Chinnery, P.F. (2012). Dysfunctional mitochondrial main-tenance: what breaks the circle of life? Brain 135, 9–11.

Zhang, K., Li, H., and Song, Z. (2014). Membrane depolarization activates the mitochondrial protease OMA1 by stimulating self-cleavage. EMBO Rep. 15, 576–585.

Zhang, X., Mofer, A., Hydbring, P., Olofsson, M.H., Guo, J., Linder, S., and D’Arcy, P. (2017). MYC is downregulated by a mitochondrial checkpoint mechanism. Oncotarget 8, 90225–90237.

Zorova, L.D., Popkov, V.A., Plotnikov, E.Y., Silachev, D.N., Pevzner, I.B., Janas-kauskas, S.S., Babenko, V.A., Zorov, S.D., Balakireva, A.V., Juhaszova, M., et al. (2018). Mitochondrial membrane potential. Anal. Biochem. 552, 50–59.
# STAR METHODS

## KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| **Antibodies**      |        |            |
| Caveolin-1          | Cell Signaling Technology | Cat# 3267T; RRID: AB_2275453 |
| Alexa Fluor 555-conjugated anti-rabbit IgG | Cell Signaling Technology | Cat# 4413; RRID: AB_10694110 |
| Caspase-3           | Cell Signaling Technology | Cat# 14220; RRID: AB_2798429 |
| Phospho-p70 S6 kinase (Thr389) | Cell Signaling Technology | Cat# 9234; RRID: AB_2269803 |
| p70 S6 kinase       | Cell Signaling Technology | Cat# 2708; RRID: AB_390722 |
| β-actin             | Cell Signaling Technology | Cat# 4970; RRID: AB_2223172 |
| Horseradish peroxidase-conjugated goat anti-rabbit IgG | Cell Signaling Technology | Cat# 7074; RRID: AB_2099233 |
| **Bacterial and virus strains** |        |            |
| pAAVS1-P-CAG-Tom20-CR | This paper | N/A |
| pLenti-EF-IRE-blast-YF | This paper | N/A |
| pLenti-EF-IRE-blast-mActZ-FY | This paper | N/A |
| **Chemicals, peptides, and recombinant proteins** |        |            |
| Dulbecco’s modified Eagle’s medium (DMEM) | Thermo Fisher Scientific | Cat# 11965118 |
| Fetal bovine serum (FBS) | Thermo Fisher Scientific | Cat# 10270-106 |
| Zell Shield | Minerva Biolabs GmbH | Cat# 13-0050 |
| Torin 1             | Merck   | Cat# 702591 |
| L-glutamine         | Thermo Fisher Scientific | Cat# 25030081 |
| Phosphate-buffered saline (PBS) | Thermo Fisher Scientific | Cat# 10010023 |
| FuGENE HD           | Promega | Cat# E2311 |
| TransIT-2020 Reagent | TaKaRa   | Cat# MIR5404 |
| Puromycin Dihydrochloride | Thermo Fisher Scientific | Cat# A1113803 |
| Polybrene Solution(10mg/ml) | Nacalai Tesque | Cat# 12996-81 |
| Blasticidin S(solution) | Nacalai Tesque | Cat#03759-00 |
| Lipofectamine RNAiMax | Thermo Fisher Scientific | Cat# 13778-030 |
| Phenol red-free DMEM | Thermo Fisher Scientific | Cat# 31053028 |
| Penicillin-streptomycin | Sigma-Aldrich | Cat# P4333 |
| Rapamycin           | Calbiochem | Cat# 553211-1MGNC |
| MitoTracker Red CMXRos | Thermo Fisher Scientific | Cat# M7513 |
| TMRE                | Thermo Fisher Scientific | Cat# T669 |
| Formaldehyde        | NEM     | Cat# 3153 |
| Glutaraldehyde      | NEM     | Cat# 304 |
| Parafomaldehyde phosphate buffer solution | Wako | Cat# 163-20145 |
| 2-Deoxy-D-glucose (2-DG) | Nacalai Tesque | Cat# 10722-11 |
| Nitroculturlose membrane | Bio-Rad | Cat# 1620115 |
| Trypan Blue Stain   | Thermo Fisher Scientific | Cat# 15250-061 |
| InSolution™ Staurusporine, Streptomyces sp. (STS) | Merck | Cat# 569396-100UGCN |
| Hoechst 33342, Trihydrochloride, Trihydrate | Thermo Fisher Scientific | Cat# H1399 |
| NP40 cell lysis buffer | Thermo Fisher Scientific | Cat# FNN0021 |
| Protease/phosphatase inhibitor cocktail | Cell Signaling Technology | Cat# 5872 |
| Immobilon           | Millipore | Cat# WBKLS0500 |

(Continued on next page)
| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| Bullet CBB Stain One Super | Nacalai Tesque | Cat# 13542-65 |
| 0.05% Trypsin-EDTA solution | Thermo Fisher Scientific | Cat# 25300-054 |
| Sepazol | Nacalai Tesque | Cat# 09379-55 |
| Primescript RT Master Mix | TaKaRa | Cat# RR036A |
| TB Green Premix Ex Taq II | TaKaRa | Cat# RR820A |
| Opti-MEM | Thermo Fisher Scientific | Cat# 11058-021 |
| CELLBANKER | Nippon Zenyaku Kogyo | Cat# CB023 |

Critical commercial assays

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| Q5 Site-Directed Mutagenesis Kit | New England Biolabs | Cat# E05545 |
| In-Fusion cloning kit | TaKaRa | Cat# 639648 |
| MitoSOX™ Red Mitochondrial Superoxide Indicator | Thermo Fisher Scientific | Cat# M36008 |
| Protein Assay BCA Kit (Extracellular flux analysis) | Nacalai Tesque | Cat# 06385-00 |
| The CellTiter-Glo® Luminescent Cell Viability Assay | Promega | Cat# G7570 |
| BCA assay (Western blot analysis) | Thermo Fisher Scientific | Cat# 23227 |

Deposited data

Sequence data, analysis, and resources related to the transcriptome analysis of iCMM

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| This paper | NCBI Sequence Read Archive (SRA) under BioProject PRJNA704503 |

Experimental models: Cell lines

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| Human cervical adenocarcinoma HeLa cells | ATCC | CCL-2 |
| Human hepatocellular carcinoma Hep 3B cells | ATCC | HB-8064 |
| Human osteosarcoma U-2 OS cells | ATCC | HTB-96 |
| Human embryonic kidney HEK293T cells | ATCC | CRL-3216 |

Oligonucleotides

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| Control siRNA (si-Cont) | Merck | Cat# SIC001 |
| si-DRP1 | Merck | siRNA ID SASI_Hs02_00340087 |
| Primer: Drp1 Forward: CTGTCATAAACAAGCTCCAGGAC | This paper | N/A |
| Primer: Drp1 Reverse: ACCAGGCTTTCTAGCAGTGA | This paper | N/A |
| Primer: OPA1 Forward: CGCTATCTCATGTAGATCGGC | This paper | N/A |
| Primer: OPA1 Reverse: CACAATGTGCAGGCACAATCC | This paper | N/A |
| Primer: β-Actin Forward: AGCCATGTACGTAGCCAT ACCGTCTCCAGGAC | This paper | N/A |
| Primer: β-Actin Reverse: TCTCCGAGTCCATCACAATG | This paper | N/A |

Recombinant DNA

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| mYF | This paper | Addgene Plasmid Cat# 171460 |
| YF | This paper | Addgene Plasmid Cat# 171469 |
| YF-Cav1s | This paper | Addgene Plasmid Cat# 171462 |
| mActZ-FY | This paper | Addgene Plasmid Cat# 171463 |
| Tom20-CR | Miyamoto et al., 2015 | Addgene Plasmid Cat# 171461 |
| rat cytochrome b5 (ER-specific marker) -mCherry | This paper | N/A |

(Continued on next page)
Continued

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|----------------------|--------|------------|
| human golgin subfamily B member 1 (Golgi apparatus-specific marker)-mCherry | This paper | N/A |
| mCherry-human LAMP1 (Lysosome-specific marker) | This paper | N/A |

Software and algorithms

| Name | Source | Identifier |
|------|--------|------------|
| NIS-Elements AR 5.30 | Nikon | https://www.nsl.nikon.com/ |
| Fiji | Schindelin et al., 2012 | https://imagej.net/Fiji |
| R | R Core Team 2017, 2017 | https://www.R-project.org/ |
| edgeR | McCarthy et al., 2012; Robinson et al., 2009 | https://bioconductor.org/packages/release/bioc/html/edgeR.html |
| DAVID 6.8 | NIAID/NIH | http://david.ncifcrf.gov/ |
| python 3.7 | Python software foundation | https://www.python.org/downloads/release/python-37/ |

NetworkX version 2.3

| Name | Source | Identifier |
|------|--------|------------|
| NetworkX developers | | https://networkx.org/ |

Other

| Name | Source | Identifier |
|------|--------|------------|
| 3.5-cm poly-lysine-coated glass bottom dish | Matsunami | Cat# D1131H |
| 3.2-mm sterile cloning disks | Merck | Cat# Z374431 |
| 10-cm cell culture dish | CORNING | Cat# 430167 |
| 3-kDa filters (Amicon Ultra 3K device) | Merck | Cat# UFC500324 |
| NEPA21 electroporation | NEPAGENE | http://www.nepagene.jp/products_nepagene_0001.html |
| Eclipse Ti2-E microscope and Intensilight mercury-fiber illuminator | Nikon | https://www.microscope.healthcare.nikon.com/products/inverted-microscopes/eclipse-ti2-series/eclipse-ti2-e |
| CFP-A-Basic-NTE filter | Semrock | http://www.opto-line.co.jp/sem/detail_sets.html?pn=CFP-A-Basic |
| YFP-A-Basic-NTE filter | Semrock | http://www.opto-line.co.jp/sem/detail_sets.html?pn=YFP-A-Basic |
| mCherry-B-NTE-ZERO filter | Semrock | http://www.opto-line.co.jp/sem/detail_sets.html?pn=mCherry-C |
| Plan Apochromat Lambda Series, 40× objective | Nikon | https://www.microscope.healthcare.nikon.com/products/optics/cfi-plan-apochromat-lambda-series |
| Zyla 4.2 PLUS sCMOS camera | Oxford Instruments | https://andor.oxinst.jp/products/scmos-camera-series/zyla-4-2-scms |
| A1R-HD25 | Nikon | https://www.microscope.healthcare.nikon.com/products/confocal-microscopes/a1hd25-a1rh25 |
| SR HP Plan APO λ S 100xC Sil | Nikon | https://www.microscope.healthcare.nikon.com/selectors/objective-comparison/-1917 |
| 405, 488, and 561 laser | Nikon | https://www.microscope.healthcare.nikon.com/products/light-sources/lu-nv-laser-unit |
| STX stage top incubator | Tokai Hit | https://www.tokaihit.com/about-stage-top-incubator/ |
| LightCycler 96 system | Roche Applied Science | https://lifescience.roche.com/global_en/products/lightcycler-381711.html |
| Synergy HTX Multi-Mode Reader | BioTek | https://www.biotek.com/products/detection-multi-mode-microplate-readers/synergy-htx-multi-mode-reader/ |
| LUNA cell counter | Logos Biosystems | https://logosbio.com/automated-cell-counters/brightfield/luna |
RESOURCE AVAILABILITY

Lead contact
Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Dr. Takafumi Miyamoto (takmi565@md.tsukuba.ac.jp).

Materials availability
Most of the materials used in this study are commercially available. Plasmids generated in this study have been deposited to Addgene.

Data and code availability
The article includes all data generated or analyzed during this study. All raw fastq files generated in this study are available from the NCBI Sequence Read Archive (SRA) under BioProject PRJNA704503. Original source data for Figures in the paper are available upon request to the Lead Contact author.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Cell culture and media
Human cervical adenocarcinoma HeLa cells (CCL-2), human hepatocellular carcinoma Hep 3B cells (HB-8064), and human osteosarcoma U-2 OS cells (HTB-96) were purchased from the American Type Culture Collection and were cultured in Dulbecco’s modified Eagle’s medium (DMEM; Thermo Fisher, 11965118) supplemented with 10% fetal bovine serum (FBS; Thermo Fisher, 10270-106) and 1% Zell Shield (Minerva Biolabs GmbH, 13-0050) at 37°C in 5% CO2.

METHOD DETAILS

Plasmid construction
The mYF effector was produced via point mutation of EYFP by replacing Ala207 with Lys in the YF vector (Addgene, #20175), using the Q5 Site-Directed Mutagenesis Kit (New England Biolabs, E0554S). The YF-Cav1s effector was produced by subcloning the sequence coding aa 61–178 of human caveolin 1 (UniprotKB-Q03135) into a YF vector at the C-terminus between HindIII and SalI. The mActZ-FY effector was produced by subcloning sequences coding aa 30–262 of codon-optimized Listeria monocytogenes serovar 1/2a ActA (UniProtKB-P33379) and aa 2–380 of human zyxin (UniProtKB-Q15942) into an FY vector containing the FKBP12Y mutant and EYFP, at the N-terminus between the Xhol and EcoRI sites. The ActA nucleic acid sequence was optimized for mammalian cells (Nakamura et al., 2020). The Tom20-CR anchor was produced by inserting a stop codon encoding Tom20-CR (Miyamoto et al., 2015) after the Xhol site in the vector, using the Q5 Site-Directed Mutagenesis Kit. ER and Golgi apparatus-specific marker proteins were generated by subcloning sequences coding aa 100–134 of rat cytochrome b5 (UniProtKB-P00173) and aa 3131–3259 of human golgin subfamily B member 1 (UniProtKB-Q14789) into the mCherry vector at the C-terminus between the EcoRI and SalI sites, respectively. To generate lysosome-specific marker proteins, the sequence coding aa 1–417 of human LAMP1 (UniProtKB-P11279) harboring V119A and L170P mutations was subcloned into the mCherry vector at the N-terminus, between the NheI and AgeI sites. To obtain CRISPR-Cas9 expression targeting the AAVST locus, donor vectors containing Tom20-CR driven by the CAG promoter were constructed using pAAVS1-P-CAG-mCh (Addgene, #80492). The pAAVS1-P-CAG-mCh vector was inversely amplified using primers that excluded the mCherry sequence flanked by the EcoRI sites (termed pAAVS1-P-CAG-Tom20-CR). The insert coding Tom20-CR was amplified, followed by the assembly of the vector and insert using In-Fusion cloning kit (TaKaRa). For lentiviral expression of effector constructs, each effector was amplified and then subcloned into the EcoRI and SalI sites of the pLenti-EF-ires-blast vector (a gift from Yutaka Hata). All constructs were verified by sequencing following subcloning.

Transient transfection
For transient transfection of the NiCMM/iCMM system, 2.4 × 10^5 cells were plated on a poly-lysine-coated glass-bottom dish (Matsumaru, D1131H) and incubated for 4 h at 37°C in 5% CO2. Following incubation, the cells were transfected with the plasmid using FuGENE HD (Promega, E2311). Indicated experiments were carried out 20–32 h after transfection. For siRNA transfection, 1.4 × 10^5 cells were plated on a poly-lysine-coated glass-bottom dish and incubated for 4 h at 37°C in 5% CO2. Following incubation, the cells were transfected with 10 nM of siRNA using Lipofectamine RNAiMax (Thermo Fisher Scientific, 13778-030) in accordance with the manufacturer’s protocol. 24 h after the siRNA transfection, the cells were transfected with the indicated plasmids using FuGENE HD. Indicated experiments were performed 24–30 h after the plasmid transfection. All siRNAs were purchased from MERCK (control siRNA: cat# SIC001, si-DRP1: siRNA ID SASI_Hs02_00340087).

Real-time reverse-transcription polymerase chain reaction
Cells were homogenized in Sepazol, and total RNA was extracted in accordance with the manufacturer’s instructions (Nacalai Tesque, 09379-55). Complementary DNA was synthesized from total RNA using Primescript RT Master Mix (TaKaRa, RR036A).
Real-time polymerase chain reactions (PCRs) were performed to amplify fragments representing the indicated messenger RNA (mRNA) expression using LightCycler 96 system (Roche Applied Science) and TB Green Premix Ex Taq II (TaKaRa, RR820A). For PCR amplification, the specific primers including 5′-GTGACATACAAAGTCCAGGAC-3′ and 5′-ACGAGGTTTTAGCAG-3′ for Drp1, 5′-CGCTACATCTATAGGATGGG-3′ and 5′-CAACATGTCCAGGACAATCC-3′ for OPA1, and 5′-AGCCATG TAGTAAAGCAT-3′ and 5′-TCTCCGGAGTCCATCAATG-3′ for β-actin were used.

**Lentivirus production**

HEK293T cells were transiently transfected with pLenti-EF-blast vectors together with pCAG-HIVgp and pCMV-VSV-G-RSV-Rev (provided by RIKEN BRC, Ibaraki, Japan) using TransIT-2020 Reagent (TaKaRa). The medium containing lentivirus was collected.

**Establishment of HeLa cells stably expressing the iCMM system**

First, a HeLa cell line that stably expressed Tom20-CR was established. For gene targeting at the AAVS1 locus, pXAT2 and pAAVS1-P-CAG-Tom20-CR were transfected into 1 × 10⁵ cells in a single-cell suspension using NEPA21 electroporation. Two days after electroporation, 2 μg/ml of puromycin was added daily, feeding over 7 days to select for targeted cells. Serial dilution cloning was performed to isolate single-cell-derived clones. The clone was incubated with lentivirus encoding an iCMM effector in the presence of 5 μg/ml polybrene. After 48 h, the clones were cultured in the presence of 5 μg/ml blasticidin for 10 days to select for cells expressing the iCMM effector. Following the selection process, cloning was performed using 3.2-mm sterile cloning disks (Merck, Z374431).

**Live-cell imaging**

For epifluorescence microscopy images, ECFP, EYFP, and mcherry excitation were carried out using an Intensilight mercury-fiber illuminator (Nikon). Data were processed through a CFP-A-Basic-NTE filter (Semrock), YFP-A-Basic-NTE filter (Semrock), and mcherry-B-NTE-ZERO filter (Semrock) for ECFP, EYFP, and mcherry imaging, respectively. Cells were viewed using a 40× objective (Plan Apochromat Lambda Series, Nikon) mounted on an inverted Eclipse Ti2-E microscope (Nikon) and imaged using a Zyla 4.2 PLUS sCMOS camera (Oxford Instruments). Imaging data were processed using the NIS-Elements AR imaging software (Nikon). For confocal microscopy images, the cells were imaged using a cell imaging was selected using an A1R-HD25 equipped with an SR HP Plan APO λ S 100× C Si (Nikon). A 405, a 488, and a 561 laser was used for ECFP, EYFP, mCherry excitation, respectively. All imaging experiments were completed at 37°C in 5% CO₂ using an STX stage top incubator (Tokai-Hit). For all live-cell imaging, cells were cultured in phenol red-free DMEM (Thermo Fisher, 31053028) supplemented with 10% FBS, 4 mM L-glutamine (Thermo Fisher, 25030081), and 1% penicillin-streptomycin (Sigma-Aldrich, P4333). Time was measured from the first frame, and 50 nM rapamycin (Calbiochem) was added at the indicated times. The following representative images taken by epifluorescence microscope images were processes by Clarify.ai and Denoise.ai using NIS-Elements AR 5.30. Notably, as the expression level of mActZ-FY was lower than that of other effectors, its fluorescence intensity was modified by changing the LUTs.

**CLEM**

CLEM was carried out at Japan Electron Optics Laboratory (Tokyo, Japan). Briefly, HeLa cells (cultured on a glass-bottom dish) that transiently expressed the iCMM system were washed twice with 1 ml of Phosphate buffer serin (PBS). The cells were then fixed for 10 min in a fixation mixture containing 4% formaldehyde (NEM, 3153) and 0.1% glutaraldehyde (NEM, 304) in PBS at room temperature. After three washes with PBS, the cells were imaged using a Nikon A1 plus equipped with an Apo λ S 40× C WI (Nikon), as well as a 405 and a 488 laser for ECFP and EYFP excitation, respectively. For SEM imaging, cells were subjected to post-fixation (1% OsO₄ and 1% tannic acid), Bloc contrast staining (1% uranyl acetate and lead aspartate), and dehydration, followed by Epon embedding. SEM imaging was carried out using a JSM-7900F (JEOL).

**Immunocytochemistry**

Cells expressing YF-Cav1s plated on a poly-lysine-coated glass-bottom dish were fixed with 4% paraformaldehyde phosphate buffer solution (Wako, 163-20145) for 10 min at 25°C, permeabilized PBS containing 0.5% Triton-100 for 15 min at 25°C, blocked in PBS containing 3% bovine serum albumin for 30 min at 25°C, stained with primary antibody against endogenous caveolin-1 (Cell Signaling, 3267T) for 16 h at 4°C, and incubated with Alexa Fluor 555-conjugated anti-rabbit IgG for 1 h at 25°C. The cells were washed three times with PBS after each step except for blocking. Imaging was performed using an Eclipse Ti2-E microscope equipped with NIS-Elements AR imaging software. The fluorescence intensity was measured by NIS-Elements AR imaging software.

**Mitochondrial ROS measurement**

Mitochondrial ROS was measured by MitosOX™ Red Mitochondrial Superoxide Indicator (Thermo Fisher, M36008) according to manufacturers’ instruction. Briefly, cells transiently expressing NiCMM/iCMM were treated with either DMSO or 50 nM rapamycin for 2 h at 37°C in 5% CO₂. Subsequently, the cells were incubated with 5 μM MitosOX for 10 min at 37°C in 5% CO₂, washed...
with 1 ml of warmed imaging medium, then observed under an Eclipse Ti2-E microscope equipped with NIS-Elements AR imaging software. The intensity of fluorescence was analyzed by the NIS-Elements AR imaging software.

**Mitochondrial membrane potential analysis**

Cells were cultured for 15 min in the presence of 50 nM TMRE at 37°C in 5% CO₂. They were then washed with an imaging medium twice, and TMRE fluorescence in mitochondria was recorded with NIS software (Nikon). Fluorescence obtained from whole cell area was recorded before (F₀) and 15 min after FCCP addition (F_FCPC). Background fluorescence obtained from cell-free region was subtracted from both F₀ and F_FCPC. TMRE intensity was calculated as F₀ − F_FCPC and normalized to the fluorescence signal recorded at 0 min.

**Extracellular flux analysis**

The OCR and ECAR were measured using a Seahorse XF24 extracellular flux analyzer (Seahorse Bioscience). HeLa cells stably expressing the iCMM system were seeded in a 24-well culture plate (Seahorse Bioscience) at 5 × 10⁴ cells per well in 250 μL of culture medium and were incubated at 37°C and 5% CO₂ for 24 h. The cells were treated with DMSO or 50 nM rapamycin for the indicated time, and the culture medium was replaced with 525 μL of XF Base Medium pH 7.4 (Seahorse Bioscience) supplemented with 4 mM GlutaMAX and 25 mM d-glucose. The cells were incubated at 37°C in a non-CO₂ incubator for 30 min. Meanwhile, an XF24 sensor cartridge (hydrated overnight; Seahorse Bioscience) was loaded with the appropriate volumes of oligomycin (final concentration, 1 μM), protonophore FCCP (final concentration, 0.125 μM), and rotenone/antimycin A (final concentration, 0.5 μM). Three basal oxygen consumption measurements were recorded (each for 8 min) before the addition of oligomycin, FCCP, and rotenone/antimycin A. The effects of these chemicals on mitochondrial oxygen consumption were also measured three times, each for 8 min. Data were normalized to the protein concentration in each group, determined using the Protein Assay BCA Kit (Nacalai, Japan). Basal mitochondrial OCR was measured by subtracting the OCR values after treatment with rotenone/antimycin A from the initial measurements. ATP-coupled respiration (ATP production) was determined by treatment with oligomycin by subtracting oligomycin values from that corresponding to basal respiration. The maximal mitochondrial OCR was determined by subtracting the OCR after treatment with rotenone/antimycin A (as detailed above) from that measured after FCCP treatment. Basal ECAR was the initial rate measured by the extracellular flux analyzer.

**ATP measurement**

Intracellular ATP level was measured using The CellTiter-Glo® Luminescent Cell Viability Assay (Promega, G7570) in accordance with the manufacturer’s instructions. Briefly, HeLa cells stably expressing NiCMM/iCMM were plated onto a 96-well plate. After incubation for 24 h, the cells were treated with DMSO or 50 nM rapamycin for 1 h, followed by 2-DG treatment for 4 h. Luminescence was measured using the Synergy HTX Multi-Mode Reader (BioTek).

**Cell proliferation assay**

Cells (1 × 10⁶) were cultured in 10 cm culture dish for 48 h at 37°C in 5% CO₂. Trypan blue (Thermo Fisher, 15250-061) stained cells were counted using a LUNA cell counter (Logos Biosystems).

**RNA-seq**

Cells stably expressing the indicated iCMM system were harvested at 2 h or 6 h after rapamycin treatment, and RNA was isolated using Direct-zol 96 (Zymo Research). RNA-seq libraries were prepared from 500 ng of RNA using Quant-seq 3’ FWD (Lexogen) following the manufacturer’s instructions. An equal amount of each Quant-seq library was pooled and diluted to 4 pM. The library was denatured, and 2.3 nM of the denatured library was subjected to RNA-seq in an Illumina Next-seq 500 instrument using high-output flow cells and the 75 single-end mode. Using BBDuk, low-quality bases were trimmed, and poly-A or -T sequences, adapter sequences, and 11-base and one-base reads (from the left and right sides of reads, respectively) were mapped to the human genome (GRCh38.p12) using STAR-aligner (Dobin et al., 2013). Read counts were obtained using featureCounts (Liao et al., 2014). Samples that generated at least one million mapped reads were used for further analyses.

**Amino acid profile analysis**

Cells growing on 10-cm dishes were washed twice with ice-cold PBS, and intracellular metabolites were extracted by briefly incubating the cells with 1 ml of methanol containing internal control substances (50 μM 2-morpholinoethanesulfonic acid and 50 μM methionine sulfoxone) on ice. Cell debris was removed by centrifugation (14,000 × g for 10 min at 4°C), and 600 μl of supernatant was mixed with 300 μl of ultrapure water and 450 μl of chloroform. Following centrifugation (16,000 × g for 3 min at 4°C), 800 μl of supernatant was mixed with 400 μl of ultrapure water and centrifuged again. The supernatant (1 ml) was evaporated for 40 min to reduce the organic solvent content. The samples were subjected to ultrafiltration using 3-kDa filters (Amicon Ultra 3K device, Merck). After lyophilization, the samples were dissolved in 50 μl of ultrapure water. Amino acid profile analysis was conducted by LC-MS/MS (LCMS-8030, Shimadzu, Kyoto, Japan) using the primary metabolite method package version 2 (Shimadzu) according to the manufacturer’s protocol.
Apoptosis analysis
Cells were treated with DMSO or rapamycin for 1 h and then incubated with 266 nM STS for 6 h. Then, the cells were incubated with Hoechst 33342 (Thermo Fisher, H1399) for 10 min for nuclear staining. After fixation with a 4% paraformaldehyde phosphate buffer solution (Wako, 163-20145), the cells were scored as possessing normal or apoptotic nuclei in several fields. Three independent experiments were conducted. Data are reported as the percentage of cells with apoptotic nuclei among total cells.

Western blot analysis
Total cell lysates were prepared using NP40 cell lysis buffer (Thermo Fisher, FNN0021) containing a protease/phosphatase inhibitor cocktail (Cell Signaling Technology, #5872), and protein concentrations were determined using a BCA assay (Thermo Fisher). For western blotting, protein samples were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes (Bio-Rad, 1620115). The membranes were blocked in Tris-buffered saline-Tween 20 containing 5% non-fat milk at room temperature for 1 h. The membranes were then incubated with the following primary antibodies for 18 h at 4°C: anti-caspase-3 (#14220), anti-phospho-p70 S6 kinase (Thr389) (#9234), anti-p70 S6 kinase (#2708), and anti-β-actin (#4970) (Cell Signaling Technology). The membranes were subsequently incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG (Cell Signaling Technology) and visualized using chemiluminescence detection (Immobilon, Millipore). Coomassie brilliant blue staining was conducted using Bullet CBB Stain One Super (Nacalai, 13542-65) according to the manufacturer’s protocol. Torin 1 was purchased from Merck (#475991).

QUANTIFICATION AND STATISTICAL ANALYSIS
Mitochondrial morphology analysis
Quantification of mitochondrial morphology (roundness) in Figures 1I–1K, 2D, 2E, S1D, S9D, and S9E was performed as follows: cells were cultured for 30 min in the presence of 0.5 μM MitoTracker Red CMXRos at 37°C in 5% CO2. The cells were washed with an imaging medium twice, and the fluorescence in mitochondria was recorded using NIS software (Nikon). Fluorescence images were processed by Unsharp Mask (Power: 1.0; Area: 41) and Rolling Ball Correction using NIS-Elements AR 5.01. Subsequently, the image was processed by contrast limited adaptive histogram equalization, followed by thresholding using the open-source software Fiji (Schindelin et al., 2012). The mean value for all mitochondria’ roundness in the cell was analyzed using Fiji’s “Analyze Particle” function.

Transcriptome analysis
In Figures 7A–7C and S8, downstream analysis was performed using R [R Core Team (2017)]. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL https://www.R-project.org/]. DEGs were identified using edgeR (McCarthy et al., 2012; Robinson et al., 2009). To determine DEGs, we employed a generalized linear model and the gene-wise likelihood ratio test. DEGs with fold change > 2 and FDR-adjusted P < 0.05 were considered significant. Competitive gene set tests were performed to account for inter-gene correlation using the camera function in the limma package as well as three-gene set collections (hallmark and C5 GO) from the Molecular Signatures Database (MSigDB) (Liberzon et al., 2011, 2015; Subramanian et al., 2005). An FDR < 0.01 was considered significant, and up to 30 gene sets in each direction (up- or downregulated) of a gene set collection were reported. If both directions occurred in two or more comparisons, the FDR was set as 1 for the different direction (for example, GO_Actomyosin was enriched in upregulated genes in mActZ, but it was also enriched in downregulated genes in other comparisons. In the plot showing upregulated genes, the FDRs for comparisons other than mActZ were 1). Functional enrichment analysis of iCMM effector-specific DEGs was performed using DAVID online tools (version DAVID 6.8; http://david.ncifcrf.gov/). Both upregulated and downregulated DEGs were included in the analysis, and a P-value < 0.05 was considered statistically significant.

Amino acid correlation network analysis
In Figure 7F, amino acid correlation network analysis was performed as follows:

The network graph was drawn using the Fruchterman–Reingold algorithm. This uses a force-directed graph-drawing algorithm to determine the position of each node from the relationship between the nodes and the edges. The nodes receive the force and moves. A force that brings the node closer (attractive force) and a force that moves the node away (repulsive force) work simultaneously. The attractive force \( f_a \) and the repulsive force \( f_r \) were defined by the following equations:

\[
f_a = \frac{d^2}{k} \quad \text{(Equation 1)}
\]

\[
f_r = -\frac{k^2}{d} \quad \text{(Equation 2)}
\]
where $d$ was the distance between nodes, area was the area of the drawing space, and $|V|$ was the number of nodes. The force applied to the node was $f_a + f_r$, and is shown in the graph below ($k = 10$).

In addition, the temperature $t$ that limits the amount of movement of the node was lowered step by step. The moving direction vector is given by Equation 4.

$$v = \frac{v}{|v|} \times \min (|v|, t)$$  

(Equation 4)

The initial position of each node was randomly assigned, the positions of the nodes were updated using the force and temperature parameters, and this calculation was repeated a certain number of times. The development environment was python 3.7, and the graph was created using NetworkX version 2.3. The edge weights were given by the correlation coefficient of each amino acid. The $k$ value of the graph-drawing parameters of NetworkX was set to 1.5, and the other parameters used default values.

**Statistical analysis**

Statistical analysis was performed using R and Excel for Windows (Microsoft). Details for statistical analysis can be found in the figure legends.