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Design of a Genetically Encoded Tool to Study the Function of Mitochondria–Lysosome Interactions

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ABSTRACT: Membrane contact sites are regions of close proximity between organelles that mediate crucial organelle functions. However, the list of effective biological tools to study membrane contact sites function still needs expansion. Mitochondria and lysosomes are organelles whose dysfunctions are closely associated with Parkinson's disease pathology, and some Parkinson's disease-associated genes have been shown to play a role in mitochondria-lysosome contact regulation. A novel biosynthetic system for reversible induction of interactions between mitochondria and lysosomes is presented, leveraging abscisic-acid-mediated heterodimerization of ABI and PYL protein domains from *Arabidopsis thaliana*. Combinations of genetically encoded protein heterodimer constructs each containing the ABI or PYL domain, a fluorescent protein tag (EGFP, mCherry, iRFP, or BFP), and a transmembrane sequence targeting the lysosomal membrane or outer mitochondrial membrane were engineered. Using confocal microscopy to visualize organelle colocalization, the efficient and reversible induction of mitochondria-lysosome contacts is shown with the abscisic-acid-responsive system. Findings indicate that mitochondrial constructs tagged with mCherry and iRFP effectively mediate abscisic-acid-induced mitochondria-lysosome colocalization when paired with lysosomal constructs tagged with EGFP/BFP or lacking a fluorescent tag. In live-cell imaging experiments, mitochondria-lysosome contact durations are tunable with this system, increasing proportionally with abscisic-acid concentration used. Overall, the system of abscisic-acid-induced reversible organelle interactions is positioned as a powerful biological tool for studying organelle dynamics and membrane contact sites functions, with relevance to therapeutic strategies in neurodegenerative disorders and organelle dysfunction.

INTRODUCTION

Membrane contact sites (MCS) are areas of close proximity between two membrane-bound organelles that fulfill a specific function and that are distinct from incidental membrane association or fusion events. The involvement of organelle contacts has been implicated in a variety of cellular functions, including organelle fission, trafficking, and cellular metabolism. Importantly, misregulation of MCS is a feature of several neurodegenerative diseases, though the precise contribution of disrupted MCS to disease pathology remains an important question.[1,2] While the scientific field realizes the significance of studying organelle contacts, developing effective molecular tools to probe their functions remains a limitation. In this paper, the design of a biosynthetic system that can be used to study MCS function via the reversible induction of mitochondria-lysosome contacts is presented.

Mitochondria are sites of essential cellular processes such as adenosine triphosphate production, oxidative phosphorylation, apoptosis, calcium homeostasis, and lipid storage, which makes them central sites of cellular metabolism.[3] Mitochondrial dysfunction and dysregulation are also

hallmarks of aging, neurodegeneration, cardiovascular problems, and even cancer.[4] Likewise, lysosomes are versatile organelles critical to metabolic regulation and cell homeostasis.[5] They are prominently understood to function in the recycling of proteins and lipids, but an underappreciated functional corollary is their role in storing metabolites and signaling nutrient status to the cell in order to direct metabolic programming.[6,7] Importantly, impairment of normal lysosomal function is oftentimes associated with neurodegenerative diseases.[8,9]

Mitochondria-lysosomal contacts are more transient than other organelle contacts, but they still share similar characteristics, such as metabolite transfer and dedicated tether proteins.[10,11] Recent research suggests that mitochondria-lysosomal contacts play an important role in the regulation of localized protein synthesis in neurons, which raises the importance of this type of organelle contact in the context of neurodegenerative disease and pathology.[1] In particular, genes associated with Parkinson's disease (PD) appear to cluster around mitochondrial and lysosomal processes, and mitochondria-lysosomal contacts are often disrupted by mutations in these genes, which suggests that the crosstalk between mitochondria and lysosomes may feature prominently in PD pathogenesis.[12]

Many biological techniques have been used to characterize and study membrane contact sites. The most popular used in MCS studies are electron microscopy (EM), proximity ligation assay (PLA), live-cell confocal microscopy, and single molecule imaging involving fluorescence resonance energy transfer (FRET), or structured illumination microscopy (SIM).[13,14] These techniques have unique advantages in the study of MCS, however, each technique faces limitations. For example, SIM, FRET, and live-cell confocal microscopy can be used on live samples but demand specialized equipment, while EM and PLA require tissue fixation and dehydration procedures that may disrupt the original architecture of the contact sites, which in turn prevents the study of dynamics and function in living cells. Moreover, to examine the causal effect of contacts on other cellular functions, there is a need for a biological system that will allow us to directly manipulate the induction of mitochondria-lysosome contacts instead of passively observing them. This issue is addressed by proposing a biosynthetic inducible system of reversible organelle interactions, where the small molecule abscisic acid (ABA) is used as a mediator of mitochondria-lysosome colocalization.

ABA is a plant stress hormone that mediates heterodimeric complexation of proteins containing ABI (ABA insensitive 1) and PYL (pyrabactin resistance-like) domains.[15] To induce colocalization between mitochondria and lysosomes, protein constructs were engineered on the membranes of each organelle to include these compatible domains. Fluorescent protein tags were included to visualize mitochondria-lysosome colocalization via confocal microscopy. Interactions between organelles were induced by introducing ABA to cells that express these domains on the organelle membranes. Organelle contact was reversed via washout of ABA from the cells to relax the association of heterodimer complex. Different mitochondrial and lysosomal membrane construct variants are tested to identify more compatible pairs with additional biological assays.

METHODS

Plasmids

The plasmids were designed and cloned manually. Addgene Plasmid #171013 was used as a backbone for designing each plasmid. Plasmids were designed that contained the following coding sequences: 3xMyc-PYL-mCherry; 3xMyc-PYL-iRFP; 3xMyc-PYL; TMEM192-ABI-EGFP-3xHA; TMEM192-ABI-BFP-3xHA; and TMEM192-ABI-3xHA.

Cloning of constructs

ABI and PYL domain sequences were generated by synthetic gene fragment synthesis, featuring mammalian codon optimization of the domains present in Addgene plasmid #38247. Lentiviral plasmids containing the mitochondria and lysosomal targeting domains were linearized by restriction digestion of Addgene plasmid 171013 in-frame with the targeting domains. Polymerase chain reaction (PCR) was used to generate products containing the ABI/PYL domains and products containing fluorophores and epitope tags. Primers in these PCR reactions were designed to have overlapping homology regions. Then, Gibson ligation reactions were used to combine PCR reactions into the digested backbones. Finally, plasmids were transformed into E. coli and individual bacterial clones were screened for successfully combined plasmids, which were then purified from bacterial DNA preparations for preparation of lentivirus.

Transfection of lysosomal and mitochondrial plasmids

Lentivirus generation and a stable transduction of constructs into HeLa cells was performed.

Cell culture and passaging of HeLa cells

Cells were regularly passaged to maintain log-phase growth in FBS-supplemented DMEM. When cells were nearly confluent, they were passaged with trypsin to lift and separate cells from the surface of the dish to which they were adhered, then the cells were pelleted in a centrifuge, resuspended in DMEM, and appropriate dilutions of cells were added to a fresh plate.

Treatment with abscisic acid and mitochondrial stressors

After aspirating old cell culture media, new media with either 1 μM, 500 nM, or 300 nM ABA was added to the cells. HeLa cells were then incubated for 90 minutes in the course of one ABA treatment. For treatment of HeLa cells with mitochondrial stressors, the following drug concentrations were used with their respective incubation times: 5 μM MitoParaquat for 60 minutes, 5 μM Rotenone for 90 minutes, 5 μM Oligomycin for 120 minutes, 5 μM CCCP for 150 minutes, and 5 μM Antimycin A for 180 minutes.

Immunoblotting

Proteins from cell lysates were prepared and resolved in 4-20% SDS-PAGE gradient gel at 140V for 1 hour and transferred on a PVDF membrane for 15 minutes via semi-dry transfer. Membranes were blocked with 1% BSA-TBST for 1 hour. Primary antibody stain was applied in 1% BSA-TBST and membranes were incubated overnight at 4C. The following primary antibodies and concentrations were used: 1:500 of HA (Rb, Cell Sig. 3724S), and 1:1000 of Myc (Rb, Cell Sig. 2272S). Membranes were then washed 4 times for 20 minutes total with TBST solution and incubated with 1:10,000 of Goat anti-Rabbit HRP (Jackson 115-035-144) secondary antibody for 1 hour in 1% BSA-TBST. The membranes were washed again 4 times for 20 minutes total with TBST solution and stored in DI water until they were imaged with horseradish peroxidase substrate. Images were edited using FIJI image analysis software.

Immunofluorescence

In preparation for immunofluorescence, cells were grown on number 1.5 thickness slide covers. After corresponding ABA treatment, media was aspirated from all samples and formaldehyde was added to each sample. Samples were then washed with PBS and transferred into the blocking solution for 30 minutes. Afterwards, samples were treated with 1:500 of HA (Rb, Cells Sig. 3724S) and 1:500 Myc (Mo, Cell Sig. 2276S) as primary antibodies and incubated at room temperature for 1 hour. Next, samples were washed with PBS again, treated with 1:1000 of Rb-488 (Invitrogen A21206 or A11034) and 1:1000 of Mo-568 (Invitrogen A11031) secondary antibodies, and incubated at room temperature for 30 minutes. Samples were washed with PBS again, inverted, and adhered to slides with liquid mountant.

Confocal Microscopy

Microscopy was performed using a Nikon AXR confocal microscope with a 4-laser system (408, 488, 561, 640 nm), with corresponding emission filters and multispectral detectors. The system was equipped with PerfectFocus system and CO2 and a heat-controlled Tokai Hit chamber for live-cell imaging. Nikon elements imaging software was used. Image analysis was conducted using Cell Profiler and FIJI ImageJ. ANOVA p-test with post hoc Tukey test were conducted to determine significance between signal reading before ABA treatment, with ABA treatment, and after washout of ABA.

Live Cell Microscopy

Imaging parameters were 1024x1024 pixels, encompassing a space of 73.66 um by 73.66 um. This calculates to a resolution of 72 nm per pixel, though the true resolution limit for conventional light microscopy is ~200 nm. Image series were taken at 3 second intervals over a span of 3 minutes, for a total of 61 images per times series. Over the course of imaging, 5% CO_2 and 37°C were maintained. Post-hoc, 1 nm radius rolling-ball method background subtraction was applied using FIJI to facilitate analysis. Blinded analysis was performed on the images. During image series analysis, any lysosomal fluorescence signal that completely overlapped or visually touched the surface of mitochondrial signal on the first image in the series was considered to be in contact with mitochondria. Next, the proximity of such lysosomes with mitochondria was tracked with each consecutive image. When a tracked lysosome dissociated from mitochondrial signal and proceeded to move away for more than 2 images (6 seconds), the number of the last image that featured mitochondrial-lysosomal contact was recorded as the duration of contact in seconds. About 15 lysosomes per image series were tracked for contact duration in different locations inside a given cell.

RESULTS

In the initial design of the inducible system for mitochondria-lysosome interactions, lysosomal transmembrane protein 192 (TMEM192) was modified with C-terminal fusion of an ABI domain, a green fluorescent protein (EGFP), and triplicate HA epitope tag (for antibody-based recognition). Additionally, the transmembrane domain of the mitochondrial protein SYNJ2BP was modified with N-terminal fusion of the fluorescent protein mCherry, the PYL domain, and a triplicate Myc epitope tag (Figure 1A). When HeLa cells expressing these constructs are treated with ABA, ABA links ABI and PYL domains together, which forms a synthetic tether between the two organelles. To test if this interaction is enough to induce mitochondria-lysosome contacts in human cells, confocal microscopy and immunofluorescence were used to measure the colocalization between mitochondria and lysosomes.

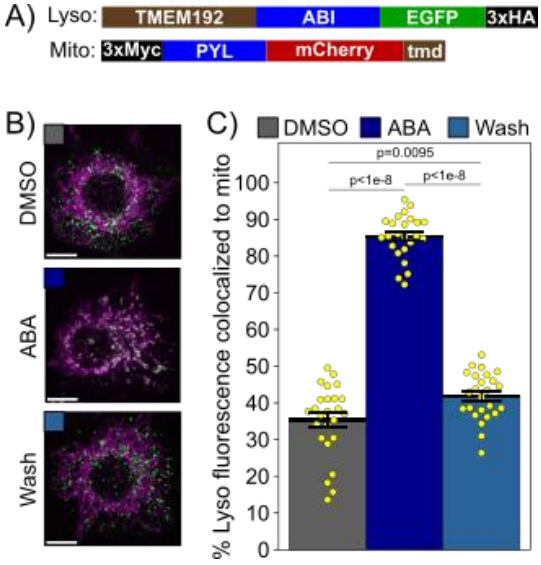


Figure 1. A system to reversibly induce mitochondria-lysosome contacts.

A) ABA mitochondrial and lysosomal heterodimer constructs. ABI/PYL are the ABA responsive domains; tmd – SYNJ2BP trans-membrane domain.

B) Fluorescence microscopy imaging of construct colocalization in HeLa cells with or without 1 μM ABA.

C) Quantification of mitochondria-lysosome association in individual cells for each treatment. Scale bars in (B) are 10 μm. ABA treatment duration is 30 minutes, and the duration of the washout is 90 minutes.

To test the system, HeLa cells expressing mitochondrial and lysosomal constructs were treated with DMSO (null treatment) or 1 μM ABA, followed by fixation of the samples. Fluorescence microscopy protocols were implemented to image fluorescence signals from lysosomes and mitochondria. The percentage of lysosomal fluorescence intensity was quantified in pixels of overlapping mitochondrial and lysosomal signal to define the extent of mitochondria-lysosome colocalization. While organelles contacts are classically defined as distances (10-80 nm) below the resolution limit of conventional light microscopy (~200 nm), the protein heterodimerization mediated by ABA would indeed tether the two organelles at distances within the classically defined range.[2] Thus, while not an absolute measure of contacts, changes in mitochondria-lysosome colocalization due to ABA treatment will strongly correlate with changes in contacts (Figure 1A).

Under baseline conditions, such as when HeLa cells are treated with DMSO control, only some lysosomes colocalized with mitochondria. However, treatment of cells with 1μM ABA was visually striking, with lysosomes colocalizing with the mitochondrial membrane to produce pixels of vivid, bright white overlap between fluorescent signals (Figure 1B). When ABA is washed out, the cells returned to baseline distribution of the organelles, with most lysosomes dissociated from mitochondria (Figure 1B). After quantifying mitochondria-lysosome colocalization, only 35% of the lysosomal intensity colocalized with mitochondria in DMSO (Figure 1C). Upon 1μM ABA treatment, the extent of mitochondria-lysosome colocalization significantly increased to 85%, consistent with qualitative observations. This substantial increase in colocalization suggests a strong inducible effect of the ABI-PYL domain system. The colocalization percentage returned to 40% after washing out ABA, indicating the reversibility of contact induction with ABA. Significant statistical differences were found between the colocalization signals in each group (Figure 1B), which confirms that colocalization between two organelles was induced and reversed after ABA wash-out. These results demonstrate that the ABI-PYL heterodimer works well for inducing and reversing proximity between organelle membranes expressing these domains.

After the system was seen for reversible organelle contacts working with EGFP-tagged lysosomes and mCherry-tagged mitochondria, the next question was what other fluorescent protein pairs can effectively visualize

mitochondria-lysosome colocalization. EGFP and mCherry are among the brightest and most stable fluorophores and are thus suitable choices for experiments in which only lysosomes and/or mitochondria are being imaged. However, other biological assays, such as those which measure mitochondrial reactive oxidant species (ROS), mitochondrial membrane potential, or lysosomal pH, often feature red or green fluorescent probes. These probes usually have significant spectral overlaps with EGFP and mCherry, requiring use of alternative fluorophores to visualize these probes simultaneously with the lysosomal and mitochondrial protein constructs. Hence, further investigation focused on identifying fluorophore tags that would be effective for contact induction with the ABA-induced system and compatible with assays that use green or red fluorescent indicators.

Mitochondrial mCherry construct performance was compared to the same construct lacking the mCherry fluorophore and the construct with infra-red fluorescent protein (iRFP670 or “iRFP”) instead of mCherry.

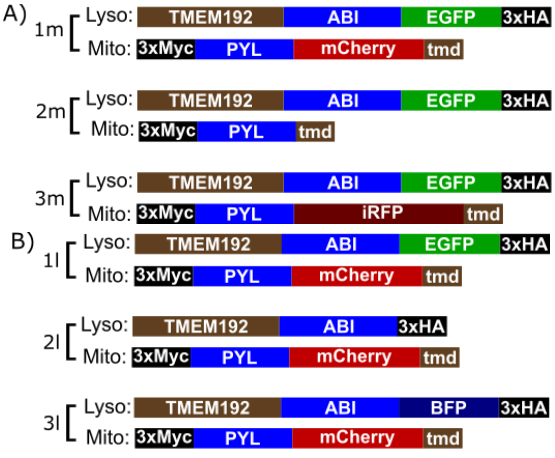
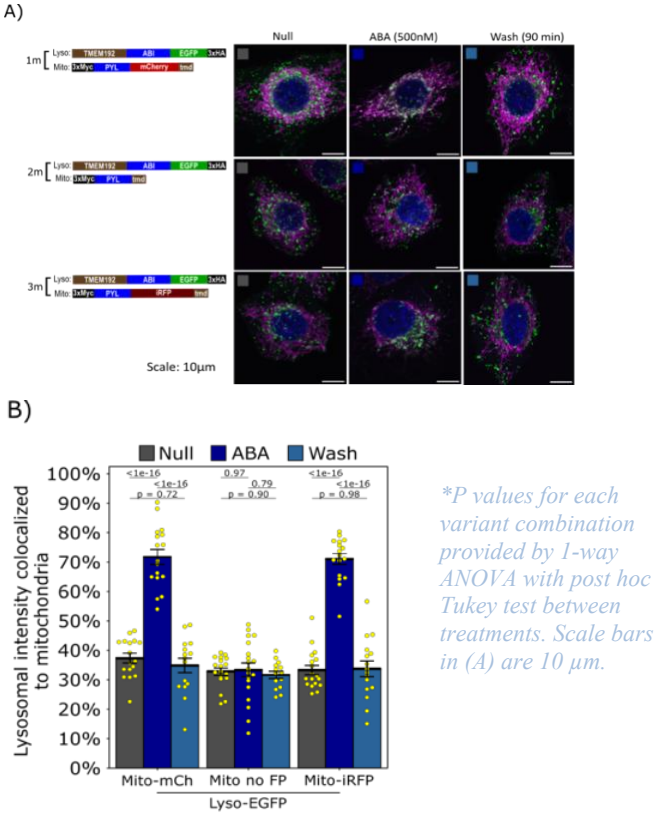


Figure 2. Mitochondrial and lysosomal variants.

A) Constructs of PYL with mCherry/no-FP/iRFP on mitochondrial tmd, paired to lysosomal construct with ABI and EGFP.

B) Constructs of ABI with EGFP/no-FP/BFP on lysosomal TMEM192PYL, paired to mitochondrial construct with PYL and mCherry.

An additional aim was to determine if there would be an advantage in removing EGFP from the lysosomal membrane construct and if blue fluorescent protein BFP would provide a similarly effective signal to EGFP. Hence, six constructs with different fluorescent protein tags on mitochondrial and lysosomal membranes were design. The following combinations of fluorophore tags were tested: lysosomal-EGFP against mitochondrial-mCherry/no-FP (no-fluorescent protein)/iRFP as well as mitochondrial-mCherry with lysosomal EGFP/no-FP/BFP (Figure 2A construct pairs 1m, 2m, 3m; Figure 2B construct pairs 1l, 2l, 3l).



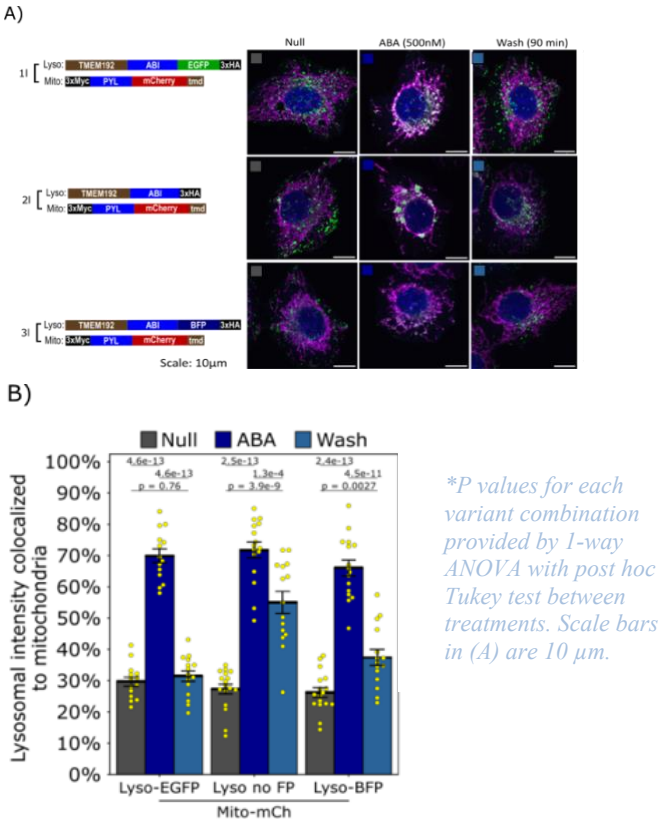
**P values for each variant combination provided by 1-way ANOVA with post hoc Tukey test between treatments. Scale bars in (A) are 10 µm.*

Figure 3. Testing ABA-induced lysosome-mitochondria colocalization of mitochondrial construct variants.

A) Immunofluorescent imaging of construct colocalization in HeLa cells with or without 30-minute treatment of 500 nM ABA.

B) Quantification of mitochondria-lysosome association in individual cells.

HeLa cells that expressed the different combinations of construct pairs were treated with 500 nM ABA or DMSO (null treatment) for 30 minutes before fixation or with ABA treatment and 90 minutes of washout before fixation (Figure 3A). Lysosomal colocalization was quantified for each treatment (Figure 3B). To keep quantification consistent across samples, immunofluorescence was performed against the HA and Myc tags present in each construct, rather than taking the direct measurement of the fluorophores. Constructs expressing mitochondrial mCherry and iRFP (Figure 3A, construct pairs 1m and 3m respectively) produced the same profile of induced and reversible contacts. On microscopy images, HeLa cells were seen expressing both 1m and 3m construct pairs showed a similar lysosomal colocalization to mitochondria upon ABA treatment and the same relaxation of contacts after the washout (Figure 3A, construct pairs 1m and 3m), which correlates with an increase in organelle colocalization from 40% to 70% (Figure 3B, Mito-mCh and Mito-iRFP). Similarly, both construct variants exhibited return of mitochondria-lysosome colocalization to the baseline of about 40% colocalization (Figure 3B, Mito-mCh and Mito-iRFP). In contrast, it appears that cells expressing mitochondrial no-FP variant were unable to mediate mitochondria-lysosome contact induction, as there are no lysosomes colocalizing with the mitochondria upon ABA treatment on microscopy (Figure 3A, construct pair 2m). Furthermore, there is no change in extent of colocalization qualitatively (Figure 3B, Mito no-FP). It is possible that removing the fluorophore from the mitochondrial construct variant situates the PYL domain too close to the mitochondrial membrane which sterically impedes the ABI-PYL complex from forming. This leads to absence of contact induction upon ABA treatment.



*P values for each variant combination provided by 1-way ANOVA with post hoc Tukey test between treatments. Scale bars in (A) are 10 µm.

Figure 4. Testing ABA-induced lysosome-mitochondria colocalization of lysosomal construct variants.

A) Immunofluorescent imaging of construct colocalization in HeLa cells with or without 30-minute treatment of 500 nM ABA.

B) Quantification of mitochondria-lysosome association in individual cells for each construct combination in each treatment.

A similar approach was taken to test HeLa cells expressing varying lysosomal constructs. Cells expressing all lysosomal EGFP, no-FP, and BFP constructs produced visually the same contact induction profile with 70% mitochondria-lysosome colocalization (Figure 4). After ABA treatment, cells expressing the lysosomal no-FP construct variant showed increased mitochondria-lysosome colocalization, but most lysosomes remained in contact with mitochondria after the washout with about 55% colocalization post-wash compared to EGFP baseline value of 30% (Figure 4A construct pair 2l, Figure 4B Lyso no-FP,

Lyso EGFP). As expected, The design of the the lysosomal no-FP construct did not change the distance between ABI and the organelle membrane (Figure 2A, construct pair 2l). Thus, in this case, removing the fluorophore should not have had as strong of an impact on contact induction as in the case of the mitochondrial no-FP construct. Interestingly, changes in lysosomal morphology were present during contact induction in cells expressing lysosomal no-FP construct, where lysosomes clustered more in cells expressing lysosomal no-FP construct than in cells expressing other constructs (Figure 4A, construct pair 2l). The lack of fluorophore in the lysosomal construct may allow for tighter packing of the heterodimer complex, which could explain this morphological change as well as the reduced recovery after washout. Some lysosomes remained in contact with mitochondria post-washout in cells expressing lysosomal BFP (Figure 4A, construct pair 3l), which correlated with a slightly diminished post-washout lysosomal colocalization of about 40% (Figure 4B, Lyso BFP).

If a construct variant is expressed at a higher level, there may be increased binding opportunities at contact sites, leading to more stable tethers that make relaxation of contacts upon ABA washout more difficult. Thus, it was hypothesized that differences in expression levels of the heterodimer constructs might underlie small differences in the reduction of mitochondria-lysosome colocalization after washout. To address this question, immunoblotting on the whole-cell lysate of the HeLa cell lines expressing the construct variants was performed. Toward this end, blotting was carried out for the lysosomal HA and mitochondrial Myc tags, incorporated in the lysosomal and mitochondrial construct designs (Figure 2, Figure 5). Cell lines with lysosomal-EGFP and a mitochondrial variant had similar HA expression across construct variants.

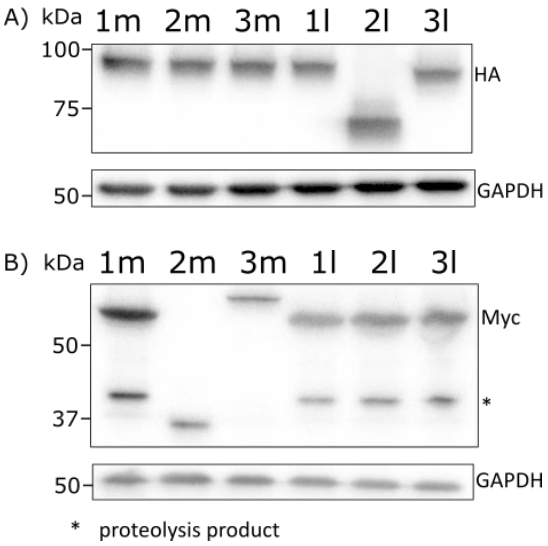
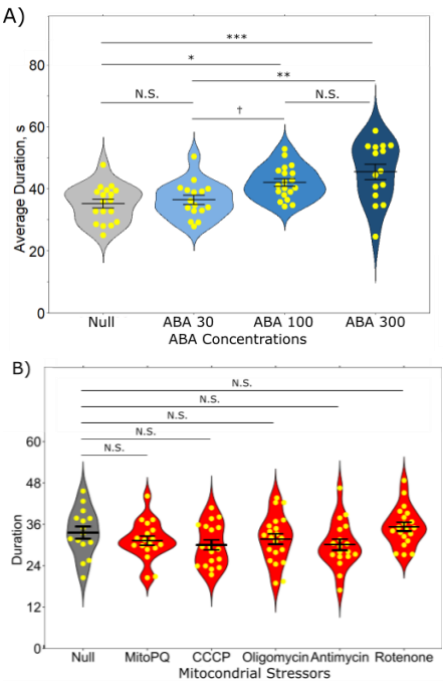


Figure 5. Immunoblotting of the whole-cell lysate to examine construct level expression.

*Western blots against HA (A) and Myc (B) in whole-cell lysate of HeLa cells expressing tested construct variants. Proteolysis product is indicated with an asterisk * and the band in 2m line (B) is a full length and a lower weight construct variant product, but not a proteolysis product. This data is a result of a single immunoblotting experiment (n=1).*

However, Myc expression levels were lower in cells with 2m construct, which correlates with the lack of contact induction in 2m cells observed during microscopy experiments (Figure 3B, Mito no FP). In cell lines with mitochondrial mCherry and a lysosomal variant, there were similar HA expression across all lysates. There was a higher Myc expression in cells with 2l and 3l construct pair variants, which aligns with reduced washout efficiency in cells with these constructs during microscopy (Figure 4B). These results suggest that the expression of the lysosomal construct is a limiting factor in the stability of the induced mitochondria-lysosome contacts in the ABA-directed system. As the immunoblotting data was derived from a single experiment, further replicates are required to draw definitive conclusions.



** P values are provided by 1-way ANOVA with post hoc Tukey test between treatments.*

P values are provided by Dunnett's test (95% family-wise confidence level). "*" $p < 0.001$, "**" $p < 0.01$, "*" $p < 0.05$, "†" $p < 0.1$,*

Figure 6: Quantification of mitochondria-lysosome contact duration.

A) Average contact durations when HeLa cells are treated with 0 nM, 30 nM, 100 nM, and 300 nM ABA.

B) Average contact durations when HeLa are treated with MitoPQ, CCCP, Oligomycin, Antimycin A, and Rotenone.

After extensively testing ABA as a tool to induce mitochondria-lysosome contacts, this system was used to assess whether the extent of contact induction could be modulated by varying the concentration of ABA. In addition to quantifying lysosome-mitochondria overlap, the contact duration is another important measure for defining the in-duction of bona fide organelle contacts.[10] Organelle contacts are highly dynamic processes that involve multiple native or artificial tethers, such as the ones induced with ABA. Thus, the duration of organelle contacts reflects the equilibrium of the number of active tethers, which are expected to increase with increasing ABA. To test this hypothesis, HeLa cells were transduced with mito-mCherry/lyso-EGFP constructs and treated with ABA. There were statistically significant increases in average contact duration in cells treated with increasing ABA concentrations (Figure 6A). These results confirm that it is possible to tune the extent of contact induction in ABA-induced systems by manipulating abscisic acid concentrations.

In addition to studying induced mitochondria-lysosome contacts, the fluorophores on mitochondria and lysosome construct pairs can be used to track changes in contacts produced by other sources of cellular stress. Live cell imaging and contact quantification were used explore the effect of mitochondrial stressors on the duration of mitochondria-lysosome contacts. Altered contacts induced by these stressors could be indicators of cellular responses involving lysosomes, such as calcium exchange, metabolite transfer, or quality control of damaged proteins or membranes. Cells were treated with five drugs (mi-to-paraquat, CCCP, oligomycin, antimycin A, and rotenone) that induce mitochondrial stress through various mechanisms. Contact duration in each treatment averaged close to 100 seconds, but there was no statistically significant differences between null treatment and mitochondrial stressors with respect to contact duration (Figure 6B).
None of mitoparaquat, CCCP, oligomycin, antimycin A, or rotenone have any effect on the duration of mitochondria-lysosome contact duration at the concentrations and treatment times.

DISCUSSION

The system for ABA-mediated heterodimerization represents a novel tool for the reversible induction of organelle contacts. In immunofluorescence and live-cell confocal microscopy imaging, the system demonstrated tunable control of mitochondria-lysosome contacts, representing a powerful biosynthetic tool for studying dynamic organelle interactions in human cells.

To maximize the applicability of this tool, it was investigated whether it was compatible

with various fluorescent protein tags. mCherry and iRFP as mitochondrial tags were found to be equally effective for visualizing organelle colocalization in the ABA-induced system. Additionally, BFP in the lysosomal construct shows comparable results to EGFP, suggesting that the fluorescent protein tag on each construct can be altered without sacrificing the efficacy of reversible organelle contact induction. While the lysosomal construct could also tolerate the removal of the fluorescent protein tag, excision of the mitochondrial fluorophore tag abolished ABA-inducible contact formation. This may be due to steric effects from shortening the distance between the transmembrane and PYL dimerization domains. Optimizing geometric parameters of constructs, such as lengths of linker regions between functional domains, is an important aspect of protein engineering. With additional design iteration, it should be possible to generate a mitochondrial construct without fluorophores that properly mediates organelle contacts in the presence of ABA.

The immunoblotting experiments highlighted that the extent of contact formation between organelles shows some dependence on expression levels of the protein constructs, especially regarding changes in the lysosomal construct organization. Additionally, increasing ABA doses highlighted the tunability of the system. Knowing that contact levels and their duration can be directly manipulated by changing abscisic acid dosage can be useful in future experiments that aim to balance the effect size of other genetic tools or chemical perturbations to mitochondria-lysosome contacts.

While the investigation into the effect of mitochondrial stress on contact duration did not yield positive results, there is no definitive conclusion that mitochondrial stress does not affect mitochondria-lysosome contact induction. The drugs used in the experiment induced mitochondrial stress via disruption of the electron transport chain (ETC) activity or by generating reactive oxidant species. Additional types of mitochondrial stress, along with different dosages and treatment durations, should be investigated in the future to study their effect on mitochondria-lysosome contact induction. Future studies could investigate how lysosomal stress influences the formation of mitochondria–lysosome contacts, as it remains unclear which organelle provides the primary signal initiating this interaction.

CONCLUSION

In this study, ABA-responsive heterodimerization was demonstrated to be an effective biosynthetic approach to induce reversible mitochondria-lysosome interactions. Data indicates that the developed system is quantifiable and modular with respect to the choice of fluorophore tags.

Substantial significance is emphasized because the ability to manipulate organelle proximity through the ABI-PYL system provides new ways to investigate the functional roles of mitochondria-lysosome contacts. Developing effective molecular proximity tools for MCS detection is an ongoing challenge in the field and has limited the ability to examine the effects of these contacts on organelle dynamics and metabolic processes. By providing means to induce and reverse interactions dynamically, the system offers a valuable model to test hypotheses about how these organelles communicate in normal physiological function or in the context of disease. Importantly, although only the induction of mitochondria-lysosome contacts was demonstrated, the paradigm of ABI-PYL membrane protein heterodimers should be applicable to studies of other organelle contacts.

The impaired function of mitochondria and lysosomes have been studied separately from each other for a long time in the context of

neurodegenerative diseases like PD, Charcot-Marie-Tooth disease, and others. However, it is becoming increasingly clear that processes that emerge from their dynamic association must be studied.[1] Thus, tools like the presented ABA-induced system for reversible mitochondria-lysosome contacts provide new avenues for investigating the pathology of these disorders. With additional engineering, the design of ABA-induced protein heterodimer formation can also be modified to study contact sites of other organelles. In the context of neurodegeneration, recent literature highlights the importance of exploring ER-mitochondria, ER-endolysosome, ER-plasma membrane, and ER-lipid droplet interactions.[14] More research will be performed to elucidate the applicability of the ABA-induced system to these types of MCS.

To study the function of mitochondria-lysosome association in the context of PD, it would be helpful to apply this system in iPSC-derived dopaminergic neurons—a cell line more relevant to Parkison's. To do this, CRISPR donor plasmids for each of the constructs are being designed. These plasmids will leverage the gene-editing technology of CRISPR to integrate the constructs into iPSC lines, including those of individuals with PD-causal mutations, allowing for the identification of which PD-associated genes are impacted by alterations to mitochondria-lysosome contacts. This integrated approach may shed light on cell type-specific molecular mechanisms of neurodegeneration and could lead to the development of new or targeted therapeutic strategies.

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Author Contributions

Yuliia Kohut performed microscopy, immunoblotting, cloning experiments, and data analysis. She also wrote the manuscript and generated most of the figures. Dr. Robert Coukos provided guidance and direction on conceiving and performing experiments during the project, including editing the manuscript. He also performed transfection of HeLa cells with lentivirus, cell culture work, and live-cell imaging. Dr. Dimitri Krainc also provided direction and support during the project.

Abbreviations

ABA - Abscisic acid

ABI - ABA insensitive 1

BFP - Blue fluorescent protein

CRISPR - Clustered regularly interspaced short palindromic repeats

EGFP - Extra green fluorescent protein

EM - Electron microscopy

ER - Endoplasmic reticulum

FRET - Fluorescence resonance energy transfer

iPSC - Induced pluripotent stem cells

iRFP - Infra-red fluorescent protein

MCS - Membrane contact sites

no-FP - No-fluorescent protein

PCR - Polymerase chain reaction

PLA – Proximity ligation assay

PYL - Pyrabactin resistance-like

SIM - Structured illumination microscopy

TMEM192 - Transmembrane protein 192

tmd - SYNJ2BP transmembrane domain

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