Visualization of Endogenous Mitophagy Complexes In Situ in Human Pancreatic Beta Cells Utilizing Proximity Ligation Assay

Gemma Pearson¹ and Scott A. Soleimanpour^{1,2}

¹Department of Internal Medicine, Division of Metabolism, Endocrinology and Diabetes, University of Michigan, Ann Arbor

²VA Ann Arbor Healthcare System

Abstract

Mitophagy is an essential mitochondrial quality control pathway, which is crucial for pancreatic islet beta cell bioenergetics to fuel glucose- stimulated insulin release. Assessment of mitophagy is challenging and often requires genetic reporters or multiple complementary techniques not easily utilized in tissue samples, such as primary human pancreatic islets. Here we demonstrate a robust approach to visualize and quantify formation of key endogenous mitophagy complexes in primary human pancreatic islets. Utilizing the sensitive proximity ligation assay technique to detect interaction of the mitophagy regulators NRDP1 and USP8, we are able to specifically quantify formation of essential mitophagy complexes in situ. By coupling this approach to counterstaining for the transcription factor PDX1, we can quantify mitophagy complexes, and the factors that can impair mitophagy, specifically within beta cells. The methodology we describe overcomes the need for large quantities of cellular extracts required for other protein-protein interaction studies, such as immunoprecipitation (IP) or mass spectrometry, and is ideal for precious human islet samples generally not available in sufficient quantities for these approaches. Further, this methodology obviates the need for flow sorting techniques to purify beta cells from a heterogeneous islet population for downstream protein applications. Thus, we describe a valuable protocol for visualization of mitophagy highly compatible for use in heterogeneous and limited cell populations.

Keywords

Medicine; Issue 147; Pancreatic beta cells; mitophagy; proximity ligation assay; human islets; protein-interaction; mitochondria

Correspondence to: Scott A. Soleimanpour at ssol@med.umich.edu.

Disclosures

The authors have nothing to disclose.

Video Link

The video component of this article can be found at <https://www.jove.com/video/59398/>

Introduction

Pancreatic beta cells produce the insulin required to maintain normal glucose homeostasis, and their failure results in the development of all forms of diabetes. Beta cells retain a robust mitochondrial capacity to generate the energy required to couple glucose metabolism with insulin release. Recently, it has become apparent that the maintenance of functional mitochondrial mass is of pivotal importance for optimal beta cell function^{1,2,3}. In order to sustain functional mitochondrial mass, beta cells rely on quality control mechanisms to remove dysfunctional, damaged, or aging mitochondria⁴. We and others have previously demonstrated that beta cells rely on a specialized form of mitochondrial turnover, called mitochondrial autophagy (or mitophagy), to maintain mitochondrial quality control in both rodent and human islets^{1,2,5}. Unfortunately, however, there was no simple method to detect mitophagy, or endogenously expressed mitophagy components, in human pancreatic beta cells.

We have recently shown that upstream regulation of mitophagy in beta cells relies on formation of a protein complex comprising the E3 ligases CLEC16A and NRDP1 and the deubiquitinase USP8¹. NRDP1 and USP8 have been shown independently to affect mitophagy through action on the key mitophagy initiator PARKIN^{6,7}. NRDP1 targets PARKIN for ubiquitination and degradation to switch off mitophagy⁶, and USP8 specifically deubiquitinates K6-linked PARKIN to promote its translocation to mitochondria⁷. Proximity ligation assay (PLA) technology has been a recent advance in the field of protein interaction biology⁸, allowing visualization of endogenous protein interactions in situ in single cells, and is not limited by scarce sample material. This methodology is particularly enticing for human islet/beta cell biology, due to the sparsity of sample availability, coupled to the need for understanding physiologically relevant protein complexes within heterogeneous cell types.

Utilizing the PLA approach, we are able to observe key endogenous mitophagy complexes in primary human pancreatic beta cells and neuronal cell lines, and demonstrate the effects of a diabetogenic environment on the mitophagy pathway¹. In summary, the overarching goal of this protocol is to analyze specific mitophagy protein complexes in tissues lacking abundant material, or where conventional protein-interaction studies are not possible.

Protocol

Use of de-identified donor human pancreatic islets is via an Institutional Review Board (IRB) exemption and in compliance with University of Michigan IRB policy. Human pancreatic islets were provided by the NIH/NIDDK-sponsored Integrated Islet Distribution Program (IIDP).

1. Human islet sample preparation

1. Single cell dissociation
 1. Culture human islet samples (4000–6000 islet equivalents/10 mL media) for at least 1 day at 37 °C in pancreatic islet media (PIM(S)) media supplemented with 1 mM glutamine (PIM(G)), 100 units/mL

antimycotic-antibiotic, 1 mM sodium pyruvate and 10 % fetal bovine serum (FBS) or human AB serum.

2. Use a dissection light microscope at 3x magnification to count individual human islets from culture. Pick 40 islets per treatment/condition of interest (such as glucolipotoxicity) into 1.5 mL tubes in islet media.
 3. Centrifuge islets at $400 \times g$ for 1 min at 10 °C to sediment.
 4. Wash samples, by brief inversion of tubes at room temperature, twice with 1 mL phosphate buffered saline (PBS) containing 50 μ M PR619 (a deubiquitinase inhibitor; to preserve ubiquitin dependent protein interactions), with centrifugation between each wash at $400 \times g$ for 1 min at 10 °C.
 5. Dissociate islets into single cells with 125 μ L of 0.25% trypsin containing 50 μ M PR619 by incubating prewarmed trypsin (37 °C) for 3 min with islet pellets. Gently disperse islets during this time with periodic gentle pipetting using a 200 μ L pipette.
 6. Quench the trypsin with 1 mL warmed (37 °C) PIM(S) media containing 50 μ M PR619, then sediment cells by centrifuging at $400 \times g$ for 1 min.
 7. Wash cells by centrifugation at $400 \times g$ for 1 min, twice, with PBS + 50 μ M PR619.
 8. Finally, resuspend cells in 150 μ L PBS containing 50 μ M PR619.
2. Single cell adherence and fixation
1. After resuspension, spin the cell solution onto frosted, charged, microscope slides using a cytocentrifuge at $28 \times g$ for 10 min.
 2. Following cytocentrifugation, outline the cellular area with a hydrophobic pen (see the **Table of Materials**) to minimize antibody/PLA solution volumes needed. Fix cells with 4% paraformaldehyde in PBS for 15 min at room temperature.

CAUTION: PFA is hazardous and must be handled with care.
 3. For best results, carry out staining/PLA immediately, or within 24 h. If necessary, store samples in PBS at 4 °C until staining for no longer than 48 h.

2. Immunohistochemistry

1. Blocking
 1. Wash cells twice with 1 $\times$ PBS for 5 min at room temperature.

2. Block cell solution, to eliminate background staining, with 10% donkey serum in PBS containing 0.3% detergent (see the **Table of Materials**) for 1 h at room temperature.
2. Staining
1. Incubate cells with primary mouse or rabbit antibodies against USP8 (1:250) and NRDP1 (1:250) respectively (see the **Table of Materials**) diluted in phosphate buffered saline with detergent (PBT, see **Table of Materials**), at 4 °C overnight, to detect mitophagy complex signaling via PLA.
 2. Co-incubate cells with a marker specific for beta cell identification that was not raised in mouse or rabbit so as to not interfere with PLA signal. In this case incubate cells with anti-goat PDX1 (1:500) in PBT. Incubate all primary antibodies overnight at 4 °C, using plastic film on top of the solution to prevent evaporation.

3. Proximity ligation assay

1. PLA probe and counterstain
 1. Prepare the PLA probe solution according to manufacturer's instructions, i.e., make 20 µL of probe solution per sample by preparing a final concentration of 1:5 solution of both anti-mouse and anti-rabbit probe solution in PBT, and incubating for 20 min at room temperature.
 2. After overnight incubation, wash cells twice with 1x PBS for 5 min each, on a rocker.
 3. Just before incubation with probe solution, add anti-goat Cy5 secondary at a final concentration of 1:600 to the probe solution (for detection of endogenous PDX1). Add 20 µL probe solution to each cell condition, cover gently with plastic film and incubate at 37 °C for 1 h.
2. Ligation
 1. Wash cells twice, at room temperature, with Buffer A (see the **Table of Materials** for the recipe) for 5 min each, on a rocker.
 2. Prepare ligation solution (part of the detection reagents, see **Table of Materials**) according to manufacturer's instructions. For this, dilute ligation stock (5x) 1:5 in diethyl pyrocarbonate (DEPC)-treated water. Immediately before incubation add 0.025 U/mL ligase. Add 20 µL ligation solution to cells. Cover with plastic film and incubate at 37 °C for 30 min.
3. Amplification
 1. Wash cells twice at room temperature with Buffer A for 2 min each.
 2. Make amplification solution (part of the detection reagents, see **Table of Materials**) according to manufacturer's instructions. Dilute

amplification buffer (5x) 1:5 in DEPC-treated water and keep in the dark until use. Add a 1:80 dilution of polymerase (part of the detection reagents, see **Table of Materials**) to the solution immediately prior to adding the solution to the cells.

3. Add 20 μ L of amplification solution to the cells. Cover with plastic film and place in the dark at 37 °C for between 1 h 40 min to 2 h for maximum signal.
4. Preparation for imaging
 1. Wash cells twice with Buffer B (see the **Table of Materials** for recipe) for 10 min at room temperature, on a rocker.
 2. Finally, wash cells once in 0.01 $\times$ Buffer B, for 2 min at room temperature on a rocker.
 3. Mount samples by adding a drop of mounting media containing 4',6-diamidino-2-phenylindole (DAPI) and carefully placing a coverslip over the samples using a scalpel blade to press out any bubbles formed. Seal coverslips using clear nail polish around the edges.
 4. Image samples on a microscope capable of capturing multiple focal planes, such as a laser scanning confocal microscope, or an inverted fluorescence microscope with deconvolution capabilities, taking at least 9 different focal plane images.
 1. Capture images at 100x magnification, with approximately 0.45 mm z-stack height. Capture PLA at 550 nm excitation, 570 nm emission; counter-stain at 650 nm excitation, 670 nm emission; and DAPI at 405 nm excitation, 450 nm emission.
 5. To ensure fluorescence image background signals and stray light are minimized for downstream analysis of PLA events (especially on widefield microscopes), process images utilizing a two-dimensional deconvolution (nearest neighbor) algorithm through a standard image processing software of choice.

NOTE: For Olympus use CellSens, Nikon use NIS-Elements, Leica use LAS X, Zeiss - ZEN, Metamorph, MATLAB, Huygens Software, as well as several Plugins available through Image-J. For analysis, the total number of interactions over all the z-stacks should be analyzed using Image-J software, ensuring only Pdx-1 positive cells are analyzed (section 3.5).
5. Quantification of PLA interactions
 1. Open the free Image-J software application, and open the PLA image. Start from the first sharply in-focus PLA image.
 2. Click the **image** tab, and select **adjust**, then **threshold** (Figure 1A). Adjust the threshold to remove all non-specific PLA signal, make a note

of the threshold adjustment and try to keep it consistent throughout analysis.

3. Click the **process** tab, and select **binary**, then **make binary** (Figure 1B). Use the binary image to measure particles, by clicking on the “**analyze**” tab and pressing **analyze particles** (Figure 1C).
4. For analysis make sure the settings are as follows: **Size** = 0–Infinity, **Circularity** = 0–1.0, **Show** = Outlines, check boxes for **Display results**, and **Clear results** (Figure 1D). Click **OK**. Take note of the number of particles analyzed in a spreadsheet denoting sample, and z-stack position.
5. Repeat steps 3.5.2–3.5.4 until all in-focus z-stacks for the sample have been analyzed. Sum the total number of particles for the sample in the spreadsheet to quantify total number of interactions.

Representative Results

We conducted initial experiments in the MIN6 pancreatic beta cell line and the SH-SY5Y neuroblastoma cell line SH-SY5Y, to optimize and confirm both the specificity of the antibodies and the visualized protein interactions. MIN6 cells were plated onto coverslips at 30,000 cells/mL and left to adhere for 48 h, SH-SY5Y cells were plated onto coverslips at 15,000 cells/mL and left to adhere for 24 h. The PLA protocol was then followed as above, beginning at Step 1.2.3. To ensure the specificity of PLA signals, we first performed this approach in the presence of (i) no primary antibody (Figure 2A, Figure 3A), (ii) NRDP1 antibody alone (Figure 2B, Figure 3B), (iii) USP8 antibody alone (Figure 2C, Figure 3C), and (iv) both NRDP1 and USP8 antibodies (Figure 2D, Figure 3D). For ease of sample set-up, Table 1 denotes how to properly layout experimental controls. Notably, we observe no punctate PLA signal in single primary antibody or no primary antibody control conditions, thus confirming that in situ interactions are specifically observed between NRDP1 and USP8 in the presence of both primary antibodies, in both MIN6 and SH-SY5Y cells.

We next adapted this approach for use with human islets to analyze mitophagy complex interactions specifically within primary human beta cells. Human islets are composed of a heterogenous population of functionally distinct cells, including alpha, beta, delta, and PP-cells⁹. In contrast to mouse islets where beta cells account for ~80% of islet mass, beta cells comprise a proportionally smaller range within human islets (between 28–75%)^{10,11,12}. Thus, we sought to use PLA technology to allow us to observe the presence of NRDP1-USP8 mitophagy complexes specifically within beta cells and ensure we did not observe confounding observations from other islet cell types (which could occur from co-immunoprecipitation studies of islet lysates). Thus, it is important to ensure adequate and uniform islet dispersion to such a level that single cells can be easily discriminated and further analyzed. As seen in Figure 4, the dispersion protocol (section 1) is highly efficient at ensuring single cells in the microscope field of view for downstream analysis. To identify beta cells, we performed co-staining with PDX1 specific anti-sera during the PLA process. PDX1 is a vital beta cell specific transcription factor, which is found in mature insulin-

producing beta cells primarily within the nucleus¹³. PDX1 staining was retained following NRDP1:USP8 PLA in primary human islets (Figure 4). Importantly, we used goat PDX1 antisera to avoid cross-reactivity with primary antibodies used for PLA (rabbit anti-NRDP1 and mouse anti-USP8, respectively). Thus, the addition of PDX1 counterstaining allows for the specific assessment of endogenous mitophagy complexes within primary human beta cells.

Utilizing these approaches, we can compile a snapshot of the retention of the NRDP1:USP8 mitophagy complex to infer competence of the mitophagy pathway within beta cells. To expand this approach for use within environmental conditions emulating those of type 2 diabetes¹⁴, we treated beta cell lines and primary human islets with palmitate and high glucose to induce glucolipotoxicity. Indeed, we found that the interaction of NRDP1 and USP8 was decreased following a 48 h exposure to palmitate and high glucose in both beta cell lines as well as primary human beta cells by PLA (Figure 5 and reference¹). This result highlights the feasibility of this assay to assess key endogenous mitophagy factors following diabetogenic stimuli.

Discussion

Here we describe a simple and efficient approach to use NRDP1 :USP8 PLA in tissues/cells of interest to quantify formation of upstream mitophagy complexes. We previously confirmed the formation of the CLEC16A-NRDP1-USP8 mitophagy complex in pancreatic beta cells by several methodologies, including co-immunoprecipitation experiments, cell-free interaction studies, and in vitro as well as cell-based ubiquitination assays, and demonstrated how this complex drives regulated mitophagic flux^{1,15}. The PLA studies we report and describe here allow for a rapid, quantitative, and cell-specific view of the mitophagy pathway that is highly adaptable to primary human beta cells. Additionally, we have shown that NRDP1:USP8 PLA can be adapted for use outside of beta cell biology in SH-SY5Y neuroblastoma cells, highlighting the potential feasibility of this technique to monitor mitophagy complexes in neuronal systems as well.

PLA is a straightforward approach; however, certain steps within the protocol are of utmost importance for ensuring clear and specific results. These include: (1) ensuring the dispersal procedure for human islets is mild enough to prevent cell lysis/damage to optimize images, (2) addition of the deubiquitinase inhibitor, PR619, immediately prior to fixation and during washes to preserve the ubiquitination state (and thus binding) of the NRDP1-USP8 complex for maximal signal observation by PLA, and (3) the objective quantification of PLA events by ImageJ to ensure an unbiased assessment of mitophagy complexes and also allow for determination of conditions whereby the changes on mitophagy may not be complete but still statistically significant/biologically relevant.

A well-known challenge within human islet studies is the concern for the heterogeneous population of both beta cells and non-beta cells. Our use of the PDX1 counterstain allows for assessment of mitophagy complex formation within beta cells (as well as PDX1 negative non-beta cells). One potential concern utilizing PDX1 as a counterstain is its expression in pancreatic delta cells^{16,17,18}, albeit to a far lower degree than in beta cells, as such other

markers (i.e., NKX6.1) could be employed to target beta cells more specifically. By dispersing intact islets into single cells immediately prior to cytocentrifugation and fixation, we are also able to perform single cell mitophagy studies with only minimal disruption of the islet microenvironment during the course of drug treatments and/or glucolipotoxic exposure. However, we cannot exclude the possibility that islet dispersion could interfere with mitophagic complexes within cells independently of relevant treatments.

While more traditional protein-protein interaction studies can be employed in human pancreatic islets, such as co-immunoprecipitation and/or proteomic approaches, these procedures generally require a large amount of islet lysate, which can prove challenging given the scarcity of human islet material available. Additionally, the concern of cell type-specificity during these traditional techniques becomes more apparent or requires additional optimization of flow cytometry methods to sort pure beta cell populations^{19,20,21,22}. Thus, our PLA approach provides an attractive and practical alternative for protein-protein interaction studies of the mitophagy pathway in human beta cells. Notably, the ability to use as few as 40 total islets/condition per experiment to quantify mitophagy complex formation in thousands of individual beta-cells allows islet researchers the ability to conserve their islet preparations for other assessments from the same donor.

As NRDP1 and USP8 are ubiquitously expressed, we speculate this technique may have value for assessment of mitophagy in cell types beyond beta cells. The possibilities include other islet cell types (with relevant counterstains for alpha cells or delta cells) or cell types which heavily rely on mitophagy, such as neurons. To this end, our observation of the transferability of this technique to SH-SY5Y neuroblastoma cells (Figure 2) or PDX1-negative islet cells (Figure 4) could suggest the utility of our technique for the mitophagy pathway in other cell types.

Despite the ease and simplicity of the NRDP1 :USP8 PLA approach, there are challenges that could confound the results of this assay. While PLA is able to ascertain protein-protein interactions in very close proximity (40 nm), it does not rule out the possibility that certain experimental conditions could maintain NRDP1-USP8 proximity while disrupting interaction, which would be missed by the PLA approach. Further, while we have demonstrated that NRDP1 :USP8 complex formation is a valid readout for the canonical CLEC16A/PARKIN-mitophagy pathway in pancreatic beta cells, recent studies have observed roles for PARKIN-independent mitophagy in mitochondrial quality control^{23,24}. We do not yet know how NRDP1:USP8 complex formation is modified by the disruption of PARKIN-independent mitophagy. Thus, use of a complementary approach beyond those described here to assay mitophagy in beta cells would be advisable. Finally, the stability of this mitophagy complex is dependent on ubiquitination of the component proteins, such that the addition of a broad spectrum deubiquitinase inhibitor PR619 is critical to maintain ubiquitination during sample preparation. Again, manipulations of beta cells that may indirectly disrupt ubiquitination of components of the NRDP1-USP8 complex need to be considered when analyzing NRDP1:USP8 as a readout for mitophagy complex formation.

As the role of mitochondrial quality control is increasingly appreciated in not only beta cell function but also other highly metabolic cell types, the rigorous assessment of mitophagy

can prove challenging and time consuming to groups wishing for a more rapid assessment of crucial mitophagy components. The PLA approach we describe is a relatively low-cost, molecular-level visualization technique that can provide quantitative analysis of mitophagy complex formation with single cell resolution in small quantity human islet samples and could be broadly applied to a variety of cell types, treatments, or disease conditions.

Acknowledgments

The authors acknowledge funding support from JDRF (CDA-2016-189 and SRA-2018-539), the National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health (R01 -DK-108921), the Brehm family, and the Anthony family. The JDRF Career Development Award to S.A.S. is partly supported by the Danish Diabetes Academy, which is supported by the Novo Nordisk Foundation.

References

1. Pearson G et al. Clec16a, Nrdp1, and USP8 Form a Ubiquitin-Dependent Tripartite Complex That Regulates beta-Cell Mitophagy. *Diabetes*. 67 (2), 265–277, (2018). [PubMed: 29180353]
2. Soleimanpour SA et al. The diabetes susceptibility gene Clec16a regulates mitophagy. *Cell*. 157 (7), 1577–1590, (2014). [PubMed: 24949970]
3. Kaufman BA, Li C, Soleimanpour SA Mitochondrial regulation of p-cell function: Maintaining the momentum for insulin release. *Molecular Aspects of Medicine*. (0), (2015).
4. Hattori N, Saiki S, Imai Y Regulation by mitophagy. *The International Journal of Biochemistry & Cell Biology*. 53 147–150, (2014). [PubMed: 24842103]
5. Soleimanpour SA et al. Diabetes Susceptibility Genes Pdx1 and Clec16a Function in a Pathway Regulating Mitophagy in beta-Cells. *Diabetes*. 64 (10), 3475–3484, (2015). [PubMed: 26085571]
6. Zhong L, Tan Y, Zhou A, Yu Q, Zhou J RING Finger Ubiquitin-Protein Isopeptide Ligase Nrdp1/FLRF Regulates Parkin Stability and Activity. *Journal of Biological Chemistry*. 280 (10), 9425–9430, (2005). [PubMed: 15632191]
7. Durcan TM et al. USP8 regulates mitophagy by removing K6-linked ubiquitin conjugates from parkin. *The EMBO Journal*. 33 (21), 2473–2491, (2014). [PubMed: 25216678]
8. Gauthier T, Claude-Taupin A, Delage-Mourroux R, Boyer-Guittaut M, Hervouet E Proximity Ligation In situ Assay is a Powerful Tool to Monitor Specific ATG Protein Interactions following Autophagy Induction. *PLoS ONE*. 10 (6), e0128701, (2015).
9. Da Silva Xavier G The Cells of the Islets of Langerhans. *Journal of Clinical Medicine*. 7 (3), 54, (2018).
10. Steiner DJ, Kim A, Miller K, Hara M Pancreatic islet plasticity: Interspecies comparison of islet architecture and composition. *Islets*. 2 (3), 135–145, (2010). [PubMed: 20657742]
11. Cabrera O et al. The unique cytoarchitecture of human pancreatic islets has implications for islet cell function. *Proceedings of the National Academy of Sciences*. 103 (7), 2334, (2006).
12. Brissova M et al. Assessment of Human Pancreatic Islet Architecture and Composition by Laser Scanning Confocal Microscopy. *Journal of Histochemistry and Cytochemistry*. 53 (9), 1087–1097, (2005). [PubMed: 15923354]
13. Babu DA, Deering TG, Mirmira RG A feat of metabolic proportions: Pdx1 orchestrates islet development and function in the maintenance of glucose homeostasis. *Molecular Genetics and Metabolism*. 92 (1), 43–55, (2007). [PubMed: 17659992]
14. Poirout V et al. Glucolipotoxicity of the pancreatic beta cell. *Biochimica Biophysica Acta*. 1801 (3), 289–298, (2010).
15. Pearson G, Soleimanpour SA A ubiquitin-dependent mitophagy complex maintains mitochondrial function and insulin secretion in beta cells. *Autophagy*. 14 (7), 1160–1161, (2018). [PubMed: 29799764]
16. Li J et al. Single-cell transcriptomes reveal characteristic features of human pancreatic islet cell types. *EMBO Reports*. 17 (2), 178–187, (2016). [PubMed: 26691212]

17. DiGrucio MR et al. Comprehensive alpha, beta and delta cell transcriptomes reveal that ghrelin selectively activates delta cells and promotes somatostatin release from pancreatic islets. *Molecular Metabolism*. 5 (7), 449–458, (2016). [PubMed: 27408771]
18. Lawlor N et al. Single-cell transcriptomes identify human islet cell signatures and reveal cell-type-specific expression changes in type 2 diabetes. *Genome Research*. 27 (2), 208–222, (2017). [PubMed: 27864352]
19. Dorrell C et al. Human islets contain four distinct subtypes of p cells. *Nature Communications*. 7 11756, (2016).
20. Dorrell C et al. Isolation of major pancreatic cell types and long-term culture-initiating cells using novel human surface markers. *Stem Cell Research*. 1 (3), 183–194, (2008). [PubMed: 19383399]
21. Jayaraman S A novel method for the detection of viable human pancreatic beta cells by flow cytometry using fluorophores that selectively detect labile zinc, mitochondrial membrane potential and protein thiols. *Cytometry Part A*. 73A (7), 615–625, (2008).
22. Arda HE et al. Age-Dependent Pancreatic Gene Regulation Reveals Mechanisms Governing Human IJ-Cell Function. *Cell Metabolism*. 23 (5), 909–920, (2016). [PubMed: 27133132]
23. Allen GFG, Toth R, James J, Ganley IG Loss of iron triggers PINK1/Parkin-independent mitophagy. *EMBO Reports*. 14 (12), 1127–1135, (2013). [PubMed: 24176932]
24. Villa E, Marchetti S, Ricci J-E No Parkin Zone: Mitophagy without Parkin. *Trends in Cell Biology*. (2018).

Figure 1: Workflow of Image J analysis for quantification of PLA interactions.

The important steps of analysis are highlighted here. (A) Adjustment of the image threshold to ensure specific PLA signal analysis. (B) Conversion of image to binary data to allow easy quantification. (C-D) How to finalize analysis by analyzing the particles recognized by ImageJ software.

Figure 2: NRDP1 and USP8 specifically interact in pancreatic beta cells.

High magnification (100x) images of the mouse pancreatic cell line, MIN6, are shown. (A-D) PLA signal for NRDP1:USP8 interaction is shown in red, and nuclei are delineated by DAPI in blue. (A-C) No specific punctate signal for NRDP1:USP8 interaction is seen if both (A) or either one (B, C) of the primary protein antibodies is omitted during the PLA process. However, interaction of both proteins is visible by PLA in pancreatic beta cells (D) when both antibodies are included.

Figure 3: NRDP1 and USP8 specifically interact in neuroblastoma cells.

High magnification (100x) images of the human neuroblastoma cell line, SH-SY5Y, are shown. (A-D) PLA signal for NRDP1:USP8 interaction is shown in red, and nuclei are delineated by DAPI in blue. (A-C) No specific punctate signal for NRDP1:USP8 interaction is seen if both (A) or either one (B, C) of the primary protein antibodies is omitted during the PLA process. However, interaction of both proteins is visible by PLA (D) when both antibodies are included.

Figure 4: Beta cell mitophagy complexes can be identified in primary human pancreatic islets in situ by PLA.

High magnification (100x) images of dispersed human islets are shown. Single cells are delineated by nuclear staining with DAPI (blue), beta cells are observed with PDX1 staining (magenta), and mitophagy complexes can be seen in both beta cells and non-beta cells (red).

Figure 5: The NRDP1:USP8 mitophagy complex is destabilized in beta cells by glucolipotoxicity. High magnification (100x) images of MIN6 cells treated for 48 h with either control (25 mM glucose + 0.82% BSA) or high glucose + palmitate (25 mM glucose + 0.4 mM palmitate/ 0.82% BSA) are shown. (**A-B**) PLA (NRDP1:USP8) signal is seen in both treatment groups. PLA signal decreases in MIN6 beta cells after glucolipotoxicity (**B**) when compared to controls (**A**).

Table 1:

Sample experimental design setup.

SAMPLE	ANTIBODY 1 (mouse anti-NRDP1)	ANTIBODY 2 (rabbit anti-USP8)	ANTIBODY 3 (goat anti-PDX1) (Optional counterstain)
CONTROL 1: No primary antibody (40 islets)	-	-	+
CONTROL 2: NRDP1 alone (40 islets)	+	-	+
CONTROL 3: USP8 alone (40 islets)	-	+	+
EXPERIMENTAL 1-x: all experimental conditions (40 islets each)	+	+	+