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(57) Abstract: Disclosed are methods of increasing mitochondrial respiration to treat obesity-related diseases and conditions, such as atherosclerosis, hypertension, diabetes, especially type 2 diabetes (NIDDM (non-insulin dependent diabetes mellitus)), impaired glucose tolerance, dyslipidemia, coronary heart disease, gallbladder disease, osteoarthritis and various types of cancer, such as endometrial, breast, prostate and colon cancers and the risk for premature death as well as other conditions, such as diseases and disorders, which conditions are improved by an increase in mitochondrial respiration. Also disclosed are methods of promoting weight gain, which is achieved by a decrease in mitochondrial respiration. Also disclosed are methods of identifying compounds useful for increasing mitochondrial respiration to treat obesity-related diseases and conditions.



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Target for Modulating Body Mass

RELATED APPLICATIONS

This application claims the benefit of priority to U.S. Provisional Patent Application serial number 63/073,155, filed September 1, 2020; the contents of which are hereby incorporated herein by reference in their entirety.

BACKGROUND

Obesity is a well-known risk factor for the development of many very common diseases such as atherosclerosis, hypertension, type 2 diabetes (non-insulin dependent diabetes mellitus (NIDDM)), dyslipidemia, coronary heart disease, and osteoarthritis and various malignancies. It also causes considerable problems through reduced motility and decreased quality of life. The incidence of obese people and thereby these diseases is increasing throughout the entire industrialized world. Accordingly, there is a great need to identify new methods to treat obesity.

SUMMARY OF THE INVENTION

In one aspect the present disclosure provides methods of treating an obesity-related disease, comprising administering to a subject in need thereof an effective amount of an inhibitor of MFSD7C or any one of its partners shown in Figure 17. Numerous embodiments are further provided that can be applied to any aspect of the present invention described herein. For example, in some embodiments, the inhibitor of MFSD7C or any one of its partners in Figure 17 inhibits binding of MFSD7C or any one of its partners in Figure 17 to electron transport chain (ETC) components, such as mitochondrial complex III, IV, or V. In some embodiments, the inhibitor of MFSD7C inhibits binding of MFSD7C or any one of its partners in Figure 17 to SERCA2b, results in uncoupled mitochondrial respiration, increases oxygen consumption rate and thermogenesis, or decreases mitochondrial membrane potential (MMP) and cellular ATP level. In some embodiments, the inhibitor of MFSD7C is heme, siRNA, or a CRISPR based inhibitor. In some embodiments, the CRISPR based inhibitor comprises MFSD7C gRNA. For example, the gRNA comprises the sequence of any one of SEQ ID NOs: 1-3. In some embodiments, any one of MFSD7C partners in Figure 17 is Mfsd7c, Hmox1, Tfrc, CYC1, NDUFA4, COX4I1, Atp5h, Atp5c1, Slc25a4, Slc25a5, Atp2a2, Elovl1, Mthfd1l, Cds2, Asph, Dnaja1, Immt, TIM50, Afg3l2, Phb, Dnaja3, Tmx3, Rdh13, Sqrld, Tspo, Fcer1g, Fcgr3, Fcgr1, or Itgb2. In some embodiments, the obesity-related disease is obesity, atherosclerosis, hypertension, diabetes, type 2 diabetes, impaired glucose tolerance, dyslipidemia, coronary

heart disease, gallbladder disease, osteoarthritis, or cancer. For example, the cancer is endometrial cancer, breast cancer, prostate cancer, or colon cancer.

In another aspect the present disclosure provides methods of treating an obesity-related disease comprising administering to a subject in need thereof an effective amount of an activator of SERCA2b. Numerous embodiments are further provided that can be applied to any aspect of the present invention described herein. For example, in some embodiments, the activator of SERCA2b inhibits binding of MFSD7C to SERCA2b, results in uncoupled mitochondrial respiration, increases oxygen consumption rate and thermogenesis, or decreases mitochondrial membrane potential (MMP) and cellular ATP level. In some embodiments, the activator of SERCA2b is heme. In some embodiments, the obesity-related disease is obesity, atherosclerosis, hypertension, diabetes, type 2 diabetes, impaired glucose tolerance, dyslipidemia, coronary heart disease, gallbladder disease, osteoarthritis, or cancer. For example, the cancer is endometrial cancer, breast cancer, prostate cancer, or colon cancer.

In another aspect the present disclosure provides methods of identifying an inhibitor of MFSD7C, comprising contacting a cell with a candidate agent; measuring MFSD7C activity in the cell contacted with the candidate agent; and optionally comparing MFSD7C activity in the presence of the candidate agent with the MFSD7C activity in the absence of the candidate agent, wherein a decrease in MFSD7C activity in the presence of the candidate agent is indicative of inhibition of MFSD7C. Numerous embodiments are further provided that can be applied to any aspect of the present invention described herein. For example, in some embodiments, the inhibitor of MFSD7C or any one of its partners in Figure 17 inhibits binding of MFSD7C to electron transport chain (ETC) components, such as mitochondrial complex III, IV, or V. In some embodiments, the inhibitor of MFSD7C inhibits binding of MFSD7C to SERCA2b, results in uncoupled mitochondrial respiration, increases oxygen consumption rate and thermogenesis, or decreases mitochondrial membrane potential (MMP) and cellular ATP level. In some embodiments, MFSD7C activity is measured using an ATP assay, a luciferase-based assay, a fluorescent-based assay, a β -galactosidase assay, flow cytometry, or mitochondrial membrane potential assay. In some embodiments, MFSD7C activity is measured by a method selected from the group consisting of Western blotting, ELISA, and radioimmunoassay (RIA).

In another aspect the present disclosure provides methods of identifying an activator of SERCA2b, comprising: contacting a cell with a candidate agent; measuring SERCA2b activity in the cell contacted with the candidate agent; and optionally comparing the cell's SERCA2b activity in the presence of the candidate agent with the cell's SERCA2b activity in the absence

of the candidate agent, wherein an increase in SERCA2b activity in the presence of the candidate agent is indicative of activation of SERCA2b. Numerous embodiments are further provided that can be applied to any aspect of the present invention described herein. For example, in some embodiments, the activator of SERCA2b inhibits binding of MFSD7C to SERCA2b, results in uncoupled mitochondrial respiration, increases oxygen consumption rate and thermogenesis, or decreases mitochondrial membrane potential (MMP) and cellular ATP level. In some embodiments, SERCA2b activity is measured using an ATP assay, a luciferase-based assay, a fluorescent-based assay, a β -galactosidase assay, flow cytometry, or a mitochondrial membrane potential assay. In some embodiments, SERCA2b activity is measured by a method selected from the group consisting of Western blotting, ELISA, and radioimmunoassay (RIA).

In one aspect the present disclosure provides methods of promoting weight gain comprising administering to a subject in need thereof an effective amount of an activator of MFSD7C or any one of its partners in Figure 17. Numerous embodiments are further provided that can be applied to any aspect of the present invention described herein. For example, in some embodiments, the activator of MFSD7C promotes binding of MFSD7C or any one of its partners in Figure 17 to electron transport chain (ETC) components, such as mitochondrial complex III, IV, or V. In some embodiments, the activator of MFSD7C or any one of its partners in Figure 17 promotes binding of MFSD7C or any one of its partners in Figure 17 to SERCA2b, results in coupled mitochondrial respiration, decreases oxygen consumption rate and thermogenesis, or increases mitochondrial membrane potential (MMP) and cellular ATP level. In some embodiments, the activator of MFSD7C is a CRISPR based activator. In some embodiments, any one of MFSD7C partners in Figure 17 is Mfsd7c, Hmox1, Tfrc, CYC1, NDUFA4, COX4I1, Atp5h, Atp5c1, Slc25a4, Slc25a5, Atp2a2, Elovl1, Mthfd1l, Cds2, Asph, Dnaja1, Immt, TIM50, Afg3l2, Phb, Dnaja3, Tmx3, Rdh13, Sqrdl, Tspo, Fcer1g, Fcgr3, Fcgr1, or Itgb2. In some embodiments, the subject is a human or livestock, such as pig, cattle, chicken, turkey, lamb, or fish.

In another aspect the present disclosure provides methods of promoting weight gain comprising administering to a subject in need thereof an effective amount of an inhibitor of SERCA2b. Numerous embodiments are further provided that can be applied to any aspect of the present invention described herein. For example, in some embodiments, the inhibitor of SERCA2b promotes binding of MFSD7C to SERCA2b, results in coupled mitochondrial respiration, decreases oxygen consumption rate and thermogenesis, or increases mitochondrial membrane potential (MMP) and cellular ATP level. In some embodiments, the inhibitor of

SERCA2b is a CRISPR based inhibitor, or siRNA. In some embodiments, the subject is a human or livestock, such as pig, cattle, chicken, turkey, lamb, or fish.

In another aspect the present disclosure provides methods of identifying an activator of MFSD7C, comprising contacting a cell with a candidate agent; measuring MFSD7C activity in the cell contacted with the candidate agent; and optionally comparing MFSD7C activity in the presence of the candidate agent with the MFSD7C activity in the absence of the candidate agent, wherein an increase in MFSD7C activity in the presence of the candidate agent is indicative of activation of MFSD7C. Numerous embodiments are further provided that can be applied to any aspect of the present invention described herein. For example, in some embodiments, the activator of MFSD7C promotes binding of MFSD7C to electron transport chain (ETC) components, such as mitochondrial complex III, IV, or V. In some embodiments, the activator of MFSD7C promotes binding of MFSD7C to SERCA2b, results in coupled mitochondrial respiration, decreases oxygen consumption rate and thermogenesis, or increases mitochondrial membrane potential (MMP) and cellular ATP level. In some embodiments, MFSD7C activity is measured using an ATP assay, a luciferase-based assay, a fluorescent-based assay, a β -galactosidase assay, flow cytometry, or mitochondrial membrane potential assay. In some embodiments, MFSD7C activity is measured by a method selected from the group consisting of Western blotting, ELISA, and radioimmunoassay (RIA).

In another aspect the present disclosure provides methods of identifying an inhibitor of SERCA2b, comprising: contacting a cell with a candidate agent; measuring SERCA2b activity in the cell contacted with the candidate agent; and optionally comparing the cell's SERCA2b activity in the presence of the candidate agent with the cell's SERCA2b activity in the absence of the candidate agent, wherein a decrease in SERCA2b activity in the presence of the candidate agent is indicative of inhibition of SERCA2b. Numerous embodiments are further provided that can be applied to any aspect of the present invention described herein. For example, in some embodiments, the inhibitor of SERCA2b promotes binding of MFSD7C to SERCA2b. In some embodiments, the inhibitor of SERCA2b results in coupled mitochondrial respiration, decreases oxygen consumption rate and thermogenesis, or increases mitochondrial membrane potential (MMP) and cellular ATP level. In some embodiments, SERCA2b activity is measured using an ATP assay, a luciferase-based assay, a fluorescent-based assay, a β -galactosidase assay, flow cytometry, or a mitochondrial membrane potential assay. In some embodiments, SERCA2b activity is measured by a method selected from the group consisting of Western blotting, ELISA, and radioimmunoassay (RIA).

BRIEF DESCRIPTION OF THE DRAWINGS

Figures 1A-1G show that MFSD7C interacts with heme and ETC components in the mitochondria. **Figure 1A** shows superdex 75 gel filtration chromatograms of human NTD and heme. NTD was incubated with heme and run on Superdex 75 gel filtration column. The flow through was measured for absorbance at 230 nm (gray), 380 nm (blue), and 415 nm (green). Absorbance intensity was normalized to maximum value. **Figure 1B** shows changes in absorption spectrum intensity of heme incubated with different concentrations of wild-type (red) or mutant (grey) NTD (see color scale). Heme (100 μ M) absorption was set to zero. **Figure 1C** shows changes in absorption spectrum intensity of heme (100 μ M) incubated with different concentrations of wildtype (blue) or mutant (grey) HP motif peptide (see color scale). Wildtype (WT) and mutant (Mut) peptide sequences are shown. **Figure 1D** shows Co-IP and immunoblotting analysis of HA-tagged MFSD7C and FLAG-tagged CYC1, NDUFA4, COX4I1, ATP5h, ATP5c1, or HMOX1. Shown are representative data from five separate experiments. **Figure 1E** shows Co-IP and immunoblotting analysis of endogenous MFSD7C with ATP5h, SERCA2b and HMOX1 in bone marrow-derived macrophages from *Mfsd7c^{fl/fl}* or *Mfsd7c^{-/-}* C57BL/6 mice (see Methods for details). Shown are immunoblots of MFSD7C, ATP5h, SERCA2b, and HMOX1 on whole lysates or immunoblots of MFSD7C on anti-ATP5h, anti-SERCA2b and anti-HMOX1 immunoprecipitates. Representative data from one from three experiments are shown. **Figure 1F** shows that mouse whole brain extract was fractionated using differential centrifugation to enrich for mitochondria and analyzed by immunoblotting against the indicated proteins. Shown are representative data from three separate experiments. WCE: whole cell extract, Sup: supernatant, Mito: 10,000g mitochondrial fraction. **Figure 1G** shows immunofluorescent localization of MFSD7C in mitochondria. THP-1 cells were stained with MitoTracker (green), fixed and permeabilized, and then stained with rabbit polyclonal antibody specific for the C-terminus of MFSD7C, followed with Alexa Fluor® 594-labelled goat anti-rabbit antibody (red). Nuclei were labeled using DAPI (blue). Co-localization between MFSD7C and MitoTracker appears as yellow in the merged images. Scale bar in **Figure 1D** and **Figure 1F**: 10 μ m.

Figures 2A-2L show MFSD7C and heme regulate coupling of mitochondrial respiration. **Figures 2A-2F** show comparison of mitochondrial respiratory activities between parental THP-1 cells and four *Mfsd7c* knockout clones (A11, B11, 3D12, and 4B8). Parental and knockout THP-1 cells were cultured in the presence of either vehicle or 40 μ M heme for one hour. **Figure 2A** shows representative OCR measurements of THP-1 cells and 4B8 knockout clone under the indicated conditions. Comparison of basal OCR (**Figure 2B**),

maximal OCR (**Figure 2C**), and ECAR (**Figure 2D**), between THP-1 cells and 7CKO clones from three separate experiments. Each dot represents a technical replicate (**Figures 2B-2D**, n=18 independent experiments). **Figure 2E** shows MMP was measured using TMRE (200 nM) by flow cytometry (n=3 independent experiments). **Figure 2F** shows Cellular ATP/ADP ratio (n=5 independent experiments). **Figures 2G-2H** show comparison of thermogenesis between parental THP-1 cells and two 7CKO clones (A11 and 4B8) by microscopy using FPT. Green channel detects the FPT intensity and the red channel distinguishes knockout cells, which expressed mCherry, from the parental THP-1 cells. Shown are representative images (**Figure 2G**) and FPT intensity of A11 and 4B8 relative to THP-1 cells from three separate experiments (**Figure 2F**). **Figure 2I** shows comparison of thermogenesis between THP-1 and 7CKO cells by flow cytometry. FPT fluorescence intensity was quantified by flow cytometry. Shown are representative plots from three separate experiments. **Figure 2J** shows comparison of temperature changes in THP1 (n=4 independent samples) and 7CKO (n=3 independent samples) culture media (ΔT) with or without heme treatment as measured by thermocouples. **Figures 2K-2L** show that cells were not treated or treated with heme for one hour before lysis. Cell lysates were precipitated with anti-FLAG antibody, eluted with FLAG peptide, then precipitated with anti-HA antibody, and eluted with HA peptide, and finally subjected to immunoblotting with anti-HA and anti-FLAG antibodies. Input is the total cell lysate. Shown are representative co-IP/immunoblots (**Figure 2K**) and average band intensities with standard deviation from three independent experiments (**Figure 2L**). P-values were calculated using two-way ANOVA (*p<0.05, **p<0.01, ***p<0.005). Data are presented as mean value $\pm$ standard deviation.

Figures 3A-3F show that loss of *Mfsd7c* stimulates OCR, ECAR and thermogenesis in BMDM. Bone marrow-derived macrophages (BMDM) were derived from exon 2 floxed (*Mfsd7c^{fl/fl}*) C57BL/6 mice and C57BL/6 mice with exon 2 deletion in macrophages (*Mfsd7c^{-/-}*). **Figures 3A-3B** show representative OCR analysis from *Mfsd7c^{fl/fl}* and *Mfsd7c^{-/-}* macrophages as measured by Seahorse XF96e Analyzer and their responses to oligomycin (oligo), FCCP, and rotenone plus antimycin A (Rot/AA) (n=3 technical replicates) (**Figure 3A**), and their average basal and maximal OCR (**Figure 3B**) (n=9 independent experiments from 3 biological replicates). **Figure 3C** shows representative ECAR analysis of *Mfsd7c^{fl/fl}* and *Mfsd7c^{-/-}* macrophages and their responses to glucose, Rot/AA, and 2-deoxyglucose (2-DG) (n=3 technical replicates). **Figure 3D** shows mitochondrial membrane potential of *Mfsd7c^{fl/fl}* and *Mfsd7c^{-/-}* macrophages analyzed with TMRE staining followed by flow cytometry (1 representative histogram picked from 3 biological replicates). **Figure 3E** shows cellular

ATP/ADP ratio, (n=4 biological replicates from average of 5 technical replicates). **Figure 3F** shows estimates of cellular temperature of *Mfsd7c^{fl/fl}* and *Mfsd7c^{-/-}* macrophages as measured by fluorescent thermoprobe (n=3 biological replicates). P-values were calculated using unpaired t-test (*p<0.05, **p<0.01, ***p<0.005). Data are presented as mean value $\pm$ standard deviation.

Figures 4A-4H show NTD of MFSD7C mediates heme effect on mitochondrial respiration. **Figures 4A-4C** show comparison of basal OCR (**Figure 4A**), maximal OCR (**Figure 4B**) and ECAR (**Figure 4C**) of THP-1, 4B8, and 4B8^{FL} and 4B8^{ΔN} cells treated with vehicle or heme for 1 hour (n=18 separate experiments). **Figure 4D** shows comparison of FPT intensity of THP-1, 4B8, 4B8^{FL} and 4B8^{ΔN} cells. Cells were incubated with FPT for 6 hrs, washed and reseeded in poly-lysine coated glass bottom dishes. After cells attached to the glass, medium containing 40 μ M heme was added. Cells in the same field were imaged immediately and again 1 hour later. Relative FPT fluorescent intensities, normalized to THP-1, from three experiments are shown. Representative images are shown in Fig. 15e. (n=3 independent experiments). **Figure 4E** shows restoration of thermogenesis in 7CKO cells by expression of MFSD7C^{FL} and MFSD7C^{ΔN}. THP-1 cells were co-cultured with 4B8, 4B8^{FL}, or 4B8^{ΔN} in FPT solution for 6 hrs, washed and reseeded in poly-lysine coated glass bottom dishes. Cells were imaged by confocal laser-scanning microscopy. Green channel shows the FPT intensity and the red channel (mCherry) distinguishes knockout cells from the parental THP-1 cells. Representative images from three experiments are shown. Scale bar: 10 μ m. **Figure 4F** shows heme treatment reduces MMP in 4B8 cells complemented with MFSD7C^{FL} but not MFSD7C^{ΔN}. THP-1, 4B8, 4B8^{FL} and 4B8^{ΔN} cells were treated with vehicle or heme for 1 hour and MMP was measured by using JC-10 Mitochondrial Membrane Potential Assay Kit followed by flow cytometry. Representative mitochondrial staining profiles from three experiments are shown. **Figures 4G-4H** show that heme does not disrupt MFSD7C^{ΔN} interactions with ETC components. Co-IP was performed as in Fig. 2k, except HA-tagged MFSD7C^{ΔN} was used. Shown are representative Western blotting data (**Figure 4G**) and quantification of band intensities from n=3 independent experiments (**Figure 4H**). P-values were calculated using two-way ANOVA (*p<0.05, **p<0.01, ***p<0.005). Data are presented as mean value $\pm$ standard deviation.

Figures 5A-5F show MFSD7C and heme regulate thermogenesis through SERCA2b in THP-1 cells. **Figure 5A** shows that 293FT cells were transfected with HA-tagged murine MFSD7C and FLAG-tagged murine SERCA2b. 30 hours later, MG132 was added into half of

the cells and the other half was not treated. Another 35 hours later, some cells were treated with 40 μ M heme for 1 hour before lysis. Cell lysates were immunoprecipitated with anti-FLAG antibody, and eluted, followed by anti-HA immunoprecipitation and elution. Total cell lysate and elute were subjected to Western blotting and probed with anti-SERCA2b or anti-MFSD7C antibodies. Shown are representative data from one of three experiments. **Figure 5B** shows that 293T cells were co-transfected with HA-MFSD7C and FLAG-SERCA2b. 24 hrs later, cells were either not treated or treated with 10 μ M MG132 for either 6 or 12 hours. The cells were lysed and subjected to FLAG pull-down and blotted with anti-MFSD7C, anti-SERCA2b and anti-ubiquitin antibodies. Shown are representative data from one of three experiments. **Figure 5C** shows SERCA2b protein levels in parental THP-1 cells, 7CKO cells (3D12, A11, B11 and 4B8) or *SERCA2b*^{-/-} (#1 and #3) cells. Parental THP-1 cells were either not treated or treated with heme before lysis. Shown are representative data from one of four experiments. **Figure 5D** shows that THP-1, 4B8, 4B8^{FL}, and 4B8^{AN} cells were either not treated or treated with 40 μ M heme for one hour, lysed and subjected to Western blotting with anti-MFSD7C (top), anti-SERCA2b (middle), and anti- β -tubulin (bottom). Shown are representative data from one of two experiments. **Figure 5E** shows that THP-1 cells were incubated with FPT for 6 hrs, washed, treated with or without 4 μ M thapsigargin for 2 hours. Cells were washed and treated with 40 μ M heme for one hour before flow cytometry. Representative FPT histograms from one of three experiments are shown. **Figure 5F** shows that parental and *Serca2b*^{-/-} THP-1 cells were incubated with FPT for 6 hrs, washed and treated with or without 40 μ M heme for 1 hour, followed by flow cytometry. Shown are representative FPT histograms from one of three experiments.

Figures 6A-6B. Proposed MFSD7C-regulated cellular thermogenesis model. **Figures 6A-6B** show proposed models of MFSD7C regulation of mitochondrial respiration in response to heme, with MFSD7C residing in the inner (**Figure 6A**) or the outer (**Figure 6B**) mitochondrial membrane. When heme level is low, MFSD7C interacts with ETC components and SERCA2b, leading to SERCA2b ubiquitination and degradation and coupled mitochondrial respiration: increased ATP synthesis and reduced thermogenesis. When heme level is high, heme binding to the NTD of MFSD7C disrupts its interactions with ETC components and SERCA2b, leading to SERCA2b stabilization and uncoupled mitochondrial respiration: increased thermogenesis and reduced ATP synthesis.

Figures 7A-7G show that The N-Terminal domain of MFSD7C contains heme-binding motifs. **Figure 7A** shows NTD motif logo generated from the alignment of 29 mammalian

sequences. Two putative heme-binding motifs containing histidine-proline residues (HP motif) are marked. **Figure 7B** shows SDS-PAGE analysis of purified human NTD, stained with Coomassie blue. **Figure 7C** shows superdex 75 gel filtration chromatograms of NTD alone at absorbance at 230 nm, 380 nm and 415 nm. **Figure 7D** shows SDS-PAGE analysis of the peak gel filtration fractions from Figure 1A stained with Coomassie Blue. **Figure 7E** shows plot of 415 nm Soret band absorbance versus molar ratio of NTD or HP motif peptide to heme (n=3 independent experiments, error bars represent standard deviation). **Figure 7F** shows isothermal titration calorimetry analysis of NTD binding to heme: NTD titrated into heme (left), NTD titrated into blank buffer (middle), and 3-site sequential binding model fit to isotherm of NTD binding to heme subtracted background (right). **Figure 7G** shows isothermal titration calorimetry analysis of the HP motif binding to heme (left) and one-site binding model fit (right).

Figures 8A-8E show that MFSD7C interacts with mitochondrial ETC components. **Figure 8A** shows scheme for the isolation and identification of the MFSD7C-interacting proteins by IP-MS. **Figure 8B** shows schematics of control (GFP) and experimental (GFP-MFSD7C-Myc-FLAG) vectors. **Figure 8C** shows fold changes in GAPDH-normalized MFSD7C transcript levels in freshly purified murine CD11c⁺ Siglec-F⁺ CD11b^{lo} alveolar macrophages (n=3 mice) and murine alveolar macrophage cell line MH-S (n=3 biologically independent samples). Data are presented as mean value $\pm$ standard deviation. *p<0.01 by unpaired t-test. **Figure 8D** shows that polyclonal antibodies to the C-terminal domain of MFSD7C are specific. Parental THP-1 cells, clone 3D12 of *Mfsd7c* knockout in THP-1 cells, and 3D12 reconstituted with a full length of MFSD7C (3D12R) were stained with polyclonal antibodies specific to the C-terminal domain of MFSD7C and DAPI. Shown are images of endogenous MFSD7C (top row) and merged images with DAPI (bottom row) in parental THP-1 cells (left column), 3D12 clone (middle column), and 3D12R (right column). Representative data from three separate experiments are shown. Scale bar, 10 μ m. **Figure 8E** shows localization of MFSD7C. MFSD7C-GFP fusion protein was expressed in 293T cells and cells were stained with anti-HLA antibody, MitoTracker and DAPI and visualized by confocal microscopy. Most GFP signals co-localized with MitoTracker signals, but some GFP signals also co-localized with anti-HLA staining on the plasma membrane. Shown are representative data from three separate experiments. Scale bar represents 10 μ m, and merged images are a 2x magnification of the selected area.

Figures 9A-9E show **construction of MFSD7C knockout THP1 cells by CRISPR-Cas9-mediated gene editing**. **Figure 9A** shows Western blotting analysis of MFSD7C and UCP1 proteins in THP-1 cells and PAZ6 cells, an immortalized human brown pre-adipocyte line. The cell lysates were blotted with a polyclonal antibody to the C-terminus of MFSD7C and a monoclonal antibody to UCP1 or α -tubulin. **Figure 9B** shows scheme of lentiviral vectors. **Figure 9C** shows scheme for constructing MFSD7C knockout THP-1 cells, showing two rounds of CRISPR-Cas9-mediated gene editing. Two MFSD7C specific guide RNA (gRNA) sequences were cloned into Lenti-CRISPR-V2-mCherry vector. The plasmids and lentivirus packaging plasmids were transfected into 293FT cells to produce lentiviruses expressing Cas9 and guide RNA (gRNA). THP-1 cells were transduced with these lentiviruses and cloned by sorting for mCherry expressing cells. The first round of editing produced clones 3D12 and 4B8, and the second round produced clone A11 from 3D12 and clone B11 from 4B8 by lentivirus expressing gRNA-3 sequences, Cas9 and the puromycin resistance protein. **Figure 9D** shows sequences of gRNA and PCR primers used. The sequences of gRNA-1, gRNA-2, and gRNA-3 are SEQ ID NOs: 1-3, respectively. **Figure 9E** shows illustration of deletions in genomic DNA of different clones as determined by PCR amplification and sequencing.

Figures 10A-10C show MFSD7C knockout stimulates OCR and thermogenesis. **Figure 10A** shows representative graphs of OCR output of THP-1, A11, B11, and 3D12 cells with or without hematin treatment for 1 hour and their responses to oligomycin, FCCP, and rotenone plus antimycin A from XF96e analyzer. **Figure 10B** shows targeted metabolomic analysis of ATP, ADP, and AMP levels. **Figure 10C** shows ATP/ADP ratio of parental THP-1 and 7CKO clone B11 cells (100,000 cells each, n=3 independent experiments). Data are presented as mean value $\pm$ standard deviation. P-values were calculated using unpaired t-test (*p<0.05).

Figures 11A-11C show measuring thermogenesis using fluorescent polymeric thermometer (FPT). **Figures 11A-11B** show scheme of FPT synthesis (see Methods for detail). **Figure 11C** show FPT fluorescence intensity at different temperature in THP-1 cells (left). Plot of fluorescence intensity versus temperature with curve fitting equation (right).

Figures 12A-12F show that heme and *Mfsd7c* knockout in MCF7 and 293T cells stimulate OCR and thermogenesis. **Figure 12A** shows Western blotting analysis of MFSD7C in parental MCF7 and 293T cells and their respective *Mfsd7c* knockout cells. **Figures 12B-12E** show that parental and knockout cells were cultured in the presence of either vehicle or 40 μ M heme for 1 hour and OCR was measured using a Seahorse XF96e Analyzer. Shown are

representative graphs of OCR output of the indicated cells with or without hematin treatment and their responses to oligomycin, FCCP, and rotenone plus antimycin A (**Figure 12B**), basal OCR (**Figure 12C**), maximal OCR (**Figure 12D**), and ECAR (**Figure 12E**) from two separate experiments with n=10 biologically independent samples. Data are presented as mean value $\pm$ standard deviation. Each dot represents one technical replicate. **Figure 12F** shows comparison of thermogenesis between parental and *Mfsd7c* knockout cells by flow cytometry. Parental and knockout cells were incubated with no probe or FPT for 6 hours, and then washed twice. A portion of FPT-treated cells was treated with heme for 1 hour. FPT fluorescence intensity was quantified by flow cytometry. Shown are representative plots from two separate experiments. P-values were calculated using two-way ANOVA (**p<0.001).

Figures 13A-13G show construction of macrophage-specific *Mfsd7c* knockout mice, characterization and analysis of macrophages. **Figure 13A** shows schematic representation of wild-type (*Mfsd7c*^{wt}), exon 2-floxed (*Mfsd7c*^{fl}), and exon 2-deleted (*Mfsd7c*⁻) alleles. Primer set #1 and #2 and their respective PCR products used to distinguish between different alleles are shown. **Figure 13B** shows PCR analysis of DNA from tails of wild-type (*Mfsd7c*^{wt/wt}), floxed (*Mfsd7c*^{fl/fl}) and heterozygous (*Mfsd7c*^{wt/fl}) mice using primer set #1. Shown are representative data from ten separate experiments. **Figure 13C** shows schematic diagram showing construction of myeloid-specific *Mfsd7c* knockout mice. **Figure 13D** shows comparison of F4/80 and CD11b expression by *Mfsd7c*^{fl/fl} and *Mfsd7c*^{-/-} bone marrow-derived macrophages (BMDM) by flow cytometry gating on live cells. **Figure 13E** shows PCR analysis of genomic DNA isolated from tails and BMDM from floxed (*Mfsd7c*^{fl/fl}) mice with and without LysM-Cre using primer set #2. Shown are representative data from five separate experiments. **Figure 13F** shows quantitative RT-PCR analysis of *Mfsd7c* exons 1 and 2 with RNA isolated from *Mfsd7c*^{fl/fl} and *Mfsd7c*^{-/-} BMDM. Data are presented as mean value $\pm$ standard deviation (n=3 biologically independent samples). P-values were calculated using unpaired t-test (**p<0.001). **Figure 13G** shows Western blot analysis of *Mfsd7c*^{fl/fl} and *Mfsd7c*^{-/-} BMDM lysates using antibodies against the C-terminus of MFSD7C and β -tubulin. Shown are representative data from three separate experiments.

Figures 14A-14F show measurement of cellular temperature in mouse BMDM. **Figure 14A** shows schematic representation of the protocol used to analyze dye loading efficiency into BMDM and generation of the cellular temperature standard curve using the temperature-sensitive fluorescent properties of the thermoprobe dye. **Figures 14B-14C** show representative thermoprobe dye loading efficiency into *Mfsd7c*^{fl/fl} and *Mfsd7c*^{-/-} BMDM as analyzed by flow cytometry (**Figure 14B**) and average of three biological replicates (**Figure 14C**, error bars

represent standard deviation). P-values were calculated using unpaired t-test. **Figures 14D-14E** show comparison of thermoprobe fluorescence intensities in *Mfsd7c^{fl/fl}* (**Figure 14D**) and *Mfsd7c^{-/-}* (**Figure 14E**) BMDM at indicated incubation temperatures by flow cytometry. **Figure 14F** show plot of incubation temperature versus mean fluorescence intensities (MFI) of thermoprobe loaded into *Mfsd7c^{fl/fl}* and *Mfsd7c^{-/-}* BMDM. Linear trendline (black dashed line) was used to convert MFI to relative cellular temperature of BMDM as shown in Figure 3f (showing one out of three independent biological replicates).

Figures 15A-15E show complementation of 7CKO cells with MFSD7C^{FL} or MFSD7C^{ΔN}. **Figure 15A** shows scheme showing complementation of 7CKO clone 4B8 with full-length MFSD7C (MFSD7C^{FL}) or N-terminal truncated MFSD7C (MFSD7C^{ΔN}). The complemented 4B8 cells are termed 4B8^{FL} or 4B8^{ΔN}, respectively. **Figure 15B** shows that complementation with either MFSD7C^{FL} or MFSD7C^{ΔN} restores MFSD7C expression. MFSD7C was detected with a polyclonal anti-MFSD7C antibody recognizing the C-terminus of MFSD7C. Shown are representative data from five separate experiments. **Figure 15C** shows localization of MFSD7C^{FL} and MFSD7C^{ΔN} in mitochondria. Immunofluorescent labelling of MFSD7C (red) and MitoTracker (green) in 4B8, 4B8^{FL} and 4B8^{ΔN} cells. Co-localization between MFSD7C and MitoTracker appears as yellow on merged images (Merge). Nuclei (gray) were labeled using DAPI. Shown are representative data from three separate experiments. **Figure 15D** shows representative graphs of OCR outputs from the XF96 analyzer of THP-1, 4B8^{FL} and 4B8^{ΔN} cells with or without heme treatment. **Figure 15E** shows THP-1, 4B8, 4B8^{FL} and 4B8^{ΔN} cells were incubated with FPT for 6 hours, and then washed and reseeded in 35 mm glass bottom dish. Cells in the same field were imaged before and after hematin treatment. Shown are representative data from three separate experiments. Fluorescent intensity was quantified and shown in Figure 4d.

Figures 16A-16F show Co-localization of MFSD7C and SERCA2b. **Figure 16A** shows confocal analysis of MFSD7C and SERCA2b co-localization in THP-1 cells. Shown are representative images of THP-1 cells stained for MFSD7C (red), SERCA2b (green), nuclei (blue) and merged image. **Figures 16B-16C** show that heme disrupts MFSD7C-SERCA2b co-localization. THP-1 cells were not treated or treated with 40 μM of heme for 1 hour and then stained for MFSD7C, SERCA2b and nuclei as in (**Figure 16A**). Co-localization between MFSD7C and SERCA2b was quantified. Shown are representative merged images (**Figure 16B**) and comparison of Pearson's correlation coefficients values with or without heme treatment (**Figure 16C**) (in **Figure 16C**, n=7 biologically independent samples). P-values were

calculated using unpaired t-test (***) $p < 0.05$). **Figures 16D-16E** show that MFSD7C localizes at the ER-mitochondrial junction. THP-1 cells were stained for MFSD7C (red), ER (green), mitochondria (blue), and DNA (grey). Shown are representative single and merged images (**Figure 16D**). Enlarged image of the boxed area is shown in the last panel (bottom-right). Pearson's rank correlation values between the indicated comparisons are shown (**Figure 16E**). (n=3 biologically independent samples). **Figure 16F** shows that Thapsigargin inhibits heme-induced thermogenesis in THP-1 cells. THP-1 cells were incubated with FPT for 6 hours, washed, treated with or without 4 μ M thapsigargin for 2 hours. The cells were washed and reseeded in poly-lysine coated glass bottom dishes. After cells attached to the glass, medium containing 40 μ M heme was added. Cells in the same field were imaged immediately (0 min) and again 1 hour later. Representative images from one of two experiments are shown. Data in (**Figure 16D**) and (**Figure 16D**) are presented as mean values $\pm$ standard deviation. Scale bar: 10 μ m.

Figures 17 shows a list of MFSD7C-interacting proteins identified by IP-MS and categorized by their function and localization.

DETAILED DESCRIPTION OF THE INVENTION

ATP synthesis and thermogenesis are two critical outputs of mitochondrial respiration. How these outputs are regulated to balance the cellular requirement for energy and heat is largely unknown. Described herein is that major facilitator superfamily domain containing 7C (MFSD7C), a member of the 12-transmembrane solute carrier family, uncouples mitochondrial respiration to switch ATP synthesis to thermogenesis in response to heme. When heme levels are low, MFSD7C promotes ATP synthesis by interacting with components of the electron transport chain (ETC) complexes III, IV and V, and destabilizing sarcoendoplasmic reticulum Ca^{2+} -ATPase 2b (SERCA2b). Upon heme binding to the N-terminal domain, MFSD7C dissociates from ETC components and SERCA2b, resulting in SERCA2b stabilization and thermogenesis. The novel heme-regulated switch between ATP synthesis and thermogenesis enables cells to match outputs of mitochondrial respiration to their metabolic state and nutrient supply, and represents a novel cell intrinsic mechanism to regulate mitochondrial energy metabolism.

Energy released from oxidation of carbohydrates and lipids generates a proton gradient across the mitochondrial inner membrane that can be used for ATP synthesis, thermogenesis, and transmembrane transport. While most attention has been focused on the role of mitochondrial respiration in ATP production, it is estimated that in endotherms majority of the

proton-motive force is used for heat generation to maintain a stable body temperature. Uncoupling proteins UCP1, UCP2, and UCP3 are involved in cellular thermogenesis by transporting protons from the intermembrane space into the matrix of mitochondria. In particular, UCP1 is required for heat production by adipocytes of brown adipose tissue (BAT), where it is highly expressed. Apart from UCP1, Sarcoendoplasmic reticulum Ca^{2+} -ATPase 2b (SERCA2b), which hydrolyzes ATP to pump Ca^{2+} from cytosol into the endoplasmic reticulum (ER) promotes thermogenesis in thermogenic organs of certain species of fish. SERCA2b was shown to be required for thermogenesis in beige adipocytes of *UCP1*^{-/-} mice and in pigs, which lack a functional copy of *Ucp1*, while *SERCA1* may stimulate thermogenic activity in white adipocytes in mice. Despite these findings, molecular mechanisms that regulate whether the energy stored in the mitochondrial proton gradient is used for ATP synthesis or thermogenesis to meet dynamic cellular requirements are largely unknown.

Heme, an iron-containing cyclic tetrapyrrole, belongs to an ancient class of co-factors that support diverse cellular processes. Heme is a co-factor for proteins involved in O_2 and CO_2 transport, mitochondrial respiration, redox reactions, circadian rhythm, transcription and translation. Particularly relevant to energy metabolism, heme is a co-factor for several electron transport chain (ETC) components, where it mediates electron transfer reactions that are coupled to formation of the mitochondrial proton gradients. These observations highlight the critical function of heme in energy metabolism, but whether it plays any role in regulating mitochondrial respiration has not been examined.

Major facilitator superfamily domain containing 7C (MFSD7C), also known as feline leukemia virus subgroup C receptor-related protein 2 (FLVCR2) and solute carrier family 49 member 2 (SLC49A2), is a member of the 12-transmembrane solute carrier family, implicated in proliferative vasculopathy and hydranencephaly-hydrocephaly or Fowler syndrome. Truncation and missense mutations in *Mfsd7c* are associated with this autosomal recessive prenatal lethal disorder characterized by multi-organ defects involving brain, kidney and muscle. MFSD7C was reported to be a heme transporter based on its binding to heme-conjugated agarose beads and the increased heme uptake by MFSD7C-transfected cells, however a direct role in heme transport has been questioned. To date, the cellular function of MFSD7C and the mechanism by which its mutations cause Fowler syndrome are unknown.

The cellular function of MFSD7C described herein is: i) MFSD7C resides in the mitochondria and interacts with components of ETC complexes III, IV and V as well as SERCA2b. ii) Knockout of *Mfsd7c* results in uncoupled mitochondrial respiration characterized by increased oxygen consumption rate (OCR) and thermogenesis, a phenotype

that is phenocopied by treating parental cells with heme. iii) The knockout phenotype is corrected by expression of both a full-length and an N-terminal domain (NTD)-truncated MFSD7C, but only the former corrects response to heme. iv) Mechanistically, binding of heme to the NTD dissociates MFSD7C from ETC components and SERCA2b, leading to stabilization of SERCA2b and increased cellular thermogenesis. Our study identifies that MFSD7C switches ATP synthesis and thermogenesis in response to heme, therefore linking the outputs of mitochondrial respiration to the cell's metabolic state and nutrient supply.

MFSD7C is identified as a heme-regulated switch that controls coupling of mitochondrial respiration. Biochemical analyses support MFSD7C interacting with heme, components of ETC complexes, and SERCA2b. At least for the recombinant NTD, the findings are consistent with three molecules of heme binding, two with high affinity and one with much lower affinity, consistent with the NTD of human MFSD7C containing two and half heme-binding HP motifs. The fact that only five proteins from the ETC complexes were co-precipitated with MFSD7C in the proteomic analysis suggests there is selectivity of MFSD7C interactions with ETC components. The data suggest that MFSD7C interacts with SERCA2b at the mitochondrial-ER contact junction. Importantly, MFSD7C interactions with ETC components and SERCA2b are disrupted by heme and in particular heme stabilizes SERCA2b, which is ubiquitinated and degraded in the presence of MFSD7C. These dynamic interactions shed light on the mechanism by which MFSD7C regulates coupling of mitochondrial respiration in response to heme: when heme levels are low, MFSD7C interacts with ETC components and SERCA2b, leading to SERCA2b degradation and coupled mitochondrial respiration; upon binding of heme to the N-terminal domain of MFSD7C the interactions are disrupted, leading to the stabilization of SERCA2b and uncoupled mitochondrial respiration (Figures 6A-6B). It is also possible that the interactions of MFSD7C with ETC components could promote assembly of supercomplexes and therefore coupled mitochondrial respiration.

The Examples herein suggest heme is an endogenous metabolite that is sensed by MFSD7C to regulate mitochondrial respiration. The observation that the NTD of MFSD7C binds to 2-3 heme molecules could enable MFSD7C to respond to a range of heme concentrations. As a metabolite, heme is well suited as a proxy for monitoring the metabolic state and nutrient supply of the cell. First, heme is a co-factor for several ETC components and directly mediates electron transport reactions so that the mitochondrial heme level likely reflects the ETC capacity. Second, heme biosynthesis starts and finishes in the lumen of mitochondria. The rate limiting first step uses succinyl-CoA and glycine, which are,

respectively, intermediates of tricarboxylic acid cycle and one-carbon metabolism, two important outputs of mitochondrial metabolism. The level of heme therefore reflects the metabolic state of the cell. Third, heme contains an iron and may reflect iron availability, and because it is absorbed from food, it might also reflect nutritional status at the organismal level. In fact, increased thermogenesis after a meal has been known since ancient times and food has been classified based on their thermogenic properties. Not surprisingly, meat, especially heme-abundant red meat, is the most thermogenic. However, the molecular basis underlying the thermic effect of food is largely unknown. Our findings suggest a possible new mechanism: heme absorbed from food stimulates thermogenesis by uncoupling mitochondrial respiration. Thus, the MFSD7C-mediated switch between ATP synthesis and thermogenesis in response to heme links the outputs of mitochondrial respiration to the cell's metabolic state and nutrient supply.

Our findings shed light on how dysfunctional mutations in *Mfsd7c* may cause Fowler syndrome. Accumulating evidence suggests that defects in energy metabolism play a critical role in neurodegeneration. For example, the late-onset Alzheimer's disease is associated with rare variants of TREM2. TREM2-deficient microglia exhibit impaired mTOR activation and phagocytosis, which can be corrected with provision of an ATP precursor cyclocreatine. Similarly, DNAJC30 interacts with ATP6, a component of ATP synthase, and its mutation leads to reduced ATP production and William syndrome. Consistent with our findings, Castro-Gago et al. reported defects in ETC complexes III and IV in three patients from the same family with Fowler syndrome. It is possible that reduced ATP synthesis and increased thermogenesis due to dysfunctional mutations in *Mfsd7c* could induce chronic cellular stress and compromise neuronal cell survival. Identification of MFSD7C as a heme-regulated switch between ATP synthesis and thermogenesis provides a basis to test this hypothesis.

Our study raises many new questions for future investigation. First, we show that MFSD7C predominantly resides in the mitochondria by subcellular fractionation and confocal microscopy, but whether it resides in the inner or outer mitochondrial membrane is currently unknown. This is an important question because the orientation of MFSD7C on the inner or outer membrane determines whether it senses heme in the lumen of mitochondria or in the cytosol. If MFSD7C resides in the inner membrane, MFSD7C likely senses heme in the lumen of mitochondria (Figure 6A). In contrast, if MFSD7C resides in the outer membrane, it is likely involved in sensing cytosolic heme (Figure 6B). Second, MFSD7C is a member of the solute carrier family and has been reported to transport heme based on some indirect evidence. Although our study did not address this issue directly, our results that the NTD of MFSD7C

binds to heme *in vitro* and is required to mediate the effect of heme *in vivo* are not incompatible with heme transport. However, there are several heme-regulated potassium channels that likewise sense heme via their N-terminal domains, raising the possibility that MFSD7C is involved in the transport of calcium as initially suggested, especially in light of its interaction with SERCA2b. Third, heme treatment of parental cells phenocopies the *Mfsd7c* knockout phenotype in uncoupling mitochondrial respiration. In our study, cells were treated with heme for one hour prior to assaying for OCR, thermogenesis and co-IP, suggesting rapid and robust effect of heme. Although the concentration of heme used (5 to 40 μ M) was likely higher than the labile heme concentration in the cytosol (<1 μ M), a significant fraction of the exogenously added heme was likely bound by serum albumin and other proteins in the culture medium. Whether and how heme gains access to the cytosol or the lumen of the mitochondria to uncouple mitochondrial respiration remains to be examined. Fourth, binding of heme to the NTD disrupts MFSD7C interactions with ETC components and SERCA2b, leading to the stabilization of SERCA2b. Mechanistically, how this is achieved remains to be determined. Finally, we showed that MFSD7C regulates the uncoupling of mitochondrial respiration in response to heme in multiple cell types, including human monocytic cells, breast cancer cells, embryonic kidney cells, and mouse bone marrow-derived macrophages. MFSD7C transcript is detected at different levels in many cell types and tissues (<http://www.immgen.org>) and cellular heme is maintained through coordinated regulation of biosynthesis, uptake and degradation. The combination of two variables, both MFSD7C and heme levels, could allow different cell types to dynamically regulate the outputs of mitochondrial respiration in order to meet their physiological need for ATP versus heat.

In one aspect the present disclosure provides methods of treating an obesity-related disease comprising administering to a subject in need thereof an effective amount of an inhibitor of MFSD7C or any one of its partners in Figure 17.

In another aspect the present disclosure provides methods of treating an obesity-related disease comprising administering to a subject in need thereof an effective amount of an activator of SERCA2b.

In another aspect the present disclosure provides methods of identifying an inhibitor of MFSD7C, comprising contacting a cell with a candidate agent; measuring MFSD7C activity in the cell contacted with the candidate agent; and comparing MFSD7C activity in the presence of the candidate agent with the MFSD7C activity in the absence of the candidate agent, wherein

a decrease in MFSD7C activity in the presence of the candidate agent is indicative of an inhibitor of MFSD7C.

In another aspect the present disclosure provides methods of identifying an activator of SERCA2b, comprising: contacting a cell with a candidate agent; measuring SERCA2b activity in the cell contacted with the candidate agent; and comparing SERCA2b activity in the presence of the candidate agent with the SERCA2b activity in the absence of the candidate agent, wherein an increase in SERCA2b activity in the presence of the candidate agent is indicative of an activator of SERCA2b.

In one aspect the present disclosure provides methods of promoting weight gain comprising administering to a subject in need thereof an effective amount of an activator of MFSD7C or any one of its partners in Figure 17.

In another aspect the present disclosure provides methods of promoting weight gain comprising administering to a subject in need thereof an effective amount of an inhibitor of SERCA2b.

In another aspect the present disclosure provides methods of identifying an activator of MFSD7C, comprising contacting a cell with a candidate agent; measuring MFSD7C activity in the cell contacted with the candidate agent; and optionally comparing MFSD7C activity in the presence of the candidate agent with the MFSD7C activity in the absence of the candidate agent, wherein an increase in MFSD7C activity in the presence of the candidate agent is indicative of activation of MFSD7C.

In another aspect the present disclosure provides methods of identifying an inhibitor of SERCA2b, comprising: contacting a cell with a candidate agent; measuring SERCA2b activity in the cell contacted with the candidate agent; and optionally comparing the cell's SERCA2b activity in the presence of the candidate agent with the cell's SERCA2b activity in the absence of the candidate agent, wherein a decrease in SERCA2b activity in the presence of the candidate agent is indicative of inhibition of SERCA2b.

Definitions

Unless otherwise defined herein, scientific and technical terms used in this application shall have the meanings that are commonly understood by those of ordinary skill in the art. Generally, nomenclature used in connection with, and techniques of, chemistry, cell and tissue culture, molecular biology, cell and cancer biology, neurobiology, neurochemistry, virology,

immunology, microbiology, pharmacology, genetics and protein and nucleic acid chemistry, described herein, are those well-known and commonly used in the art.

The methods and techniques of the present disclosure are generally performed, unless otherwise indicated, according to conventional methods well known in the art and as described in various general and more specific references that are cited and discussed throughout this specification. See, e.g. "Principles of Neural Science", McGraw-Hill Medical, New York, N.Y. (2000); Motulsky, "Intuitive Biostatistics", Oxford University Press, Inc. (1995); Lodish et al., "Molecular Cell Biology, 4th ed.", W. H. Freeman & Co., New York (2000); Griffiths et al., "Introduction to Genetic Analysis, 7th ed.", W. H. Freeman & Co., N.Y. (1999); and Gilbert et al., "Developmental Biology, 6th ed.", Sinauer Associates, Inc., Sunderland, MA (2000).

The term "agent" is used herein to denote a chemical compound (such as an organic or inorganic compound, a mixture of chemical compounds), a biological macromolecule (such as a nucleic acid, an antibody, including parts thereof as well as humanized, chimeric and human antibodies and monoclonal antibodies, a protein or portion thereof, e.g., a peptide, a lipid, a carbohydrate), or an extract made from biological materials such as bacteria, plants, fungi, or animal (particularly mammalian) cells or tissues. Agents include, for example, agents whose structure is known, and those whose structure is not known.

The terms "decrease", "reduced", "reduction", or "inhibit" are all used herein to mean a decrease by a statistically significant amount. In some embodiments, "reduce," "reduction" or "decrease" or "inhibit" typically means a decrease by at least 10% as compared to a reference level (e.g., the absence of a given ligand) and can include, for example, a decrease by at least about 10%, at least about 20%, at least about 25%, at least about 30%, at least about 35%, at least about 40%, at least about 45%, at least about 50%, at least about 55%, at least about 60%, at least about 65%, at least about 70%, at least about 75%, at least about 80%, at least about 85%, at least about 90%, at least about 95%, at least about 98%, at least about 99%, or more. As used herein, "reduction" or "inhibition" does not encompass a complete inhibition or reduction as compared to a reference level. "Complete inhibition" is a 100% inhibition as compared to a reference level.

The terms "increased", "increase" or "enhance" or "activate" are all used herein to generally mean an increase by a statically significant amount; for the avoidance of any doubt, the terms "increased", "increase" or "enhance" or "activate" means an increase of at least 10% as compared to a reference level, for example an increase of at least about 20%, or at least about 30%, or at least about 40%, or at least about 50%, or at least about 60%, or at least about 70%, or at least about 80%, or at least about 90% or up to and including a 100% increase or

any increase between 10-100% as compared to a reference level, or at least about a 2-fold, or at least about a 3-fold, or at least about a 4-fold, or at least about a 5-fold or at least about a 10-fold increase, at least about a 20-fold increase, at least about a 50-fold increase, at least about a 100-fold increase, at least about a 1000-fold increase or more as compared to a reference level.

A “patient,” “subject,” or “individual” are used interchangeably and refer to either a human or a non-human animal. These terms include mammals, such as humans, primates, livestock animals (including bovines, porcines, etc.), companion animals (e.g., canines, felines, etc.) and rodents (e.g., mice and rats).

“Treating” a condition or patient refers to taking steps to obtain beneficial or desired results, including clinical results. As used herein, and as well understood in the art, “treatment” is an approach for obtaining beneficial or desired results, including clinical results. Beneficial or desired clinical results can include, but are not limited to, alleviation or amelioration of one or more symptoms or conditions, diminishment of extent of disease, stabilized (i.e. not worsening) state of disease, preventing spread of disease, delay or slowing of disease progression, amelioration or palliation of the disease state, and remission (whether partial or total), whether detectable or undetectable. “Treatment” can also mean prolonging survival as compared to expected survival if not receiving treatment.

The term “preventing” is art-recognized, and when used in relation to a condition, such as a local recurrence (e.g., pain), a disease such as cancer, a syndrome complex such as heart failure or any other medical condition, is well understood in the art, and includes administration of a composition which reduces the frequency of, or delays the onset of, symptoms of a medical condition in a subject relative to a subject which does not receive the composition. Thus, prevention of cancer includes, for example, reducing the number of detectable cancerous growths in a population of patients receiving a prophylactic treatment relative to an untreated control population, and/or delaying the appearance of detectable cancerous growths in a treated population versus an untreated control population, e.g., by a statistically and/or clinically significant amount.

“Administering” or “administration of” a substance, a compound or an agent to a subject can be carried out using one of a variety of methods known to those skilled in the art. For example, a compound or an agent can be administered, intravenously, arterially, intradermally, intramuscularly, intraperitoneally, subcutaneously, ocularly, sublingually, orally (by ingestion), intranasally (by inhalation), intraspinally, intracerebrally, and transdermally (by absorption, e.g., through a skin duct). A compound or agent can also

appropriately be introduced by rechargeable or biodegradable polymeric devices or other devices, e.g., patches and pumps, or formulations, which provide for the extended, slow or controlled release of the compound or agent. Administering can also be performed, for example, once, a plurality of times, and/or over one or more extended periods.

Appropriate methods of administering a substance, a compound or an agent to a subject will also depend, for example, on the age and/or the physical condition of the subject and the chemical and biological properties of the compound or agent (e.g., solubility, digestibility, bioavailability, stability and toxicity). In some embodiments, a compound or an agent is administered orally, e.g., to a subject by ingestion. In some embodiments, the orally administered compound or agent is in an extended release or slow release formulation, or administered using a device for such slow or extended release.

A “therapeutically effective amount” or a “therapeutically effective dose” of a drug or agent is an amount of a drug or an agent that, when administered to a subject will have the intended therapeutic effect. The full therapeutic effect does not necessarily occur by administration of one dose, and may occur only after administration of a series of doses. Thus, a therapeutically effective amount may be administered in one or more administrations. The precise effective amount needed for a subject will depend upon, for example, the subject’s size, health and age, and the nature and extent of the condition being treated, such as cancer or MDS. The skilled worker can readily determine the effective amount for a given situation by routine experimentation.

Screening Assays

The present disclosure provides methods of identifying an inhibitor of MFSD7C, comprising contacting a cell with a candidate agent; measuring MFSD7C activity in the cell contacted with the candidate agent; and optionally comparing the cell’s MFSD7C activity in the presence of the candidate agent with the cell’s MFSD7C activity in the absence of the candidate agent, wherein a decrease in MFSD7C activity in the presence of the candidate agent is indicative of inhibition of MFSD7C.

In another aspect the present disclosure provides methods of identifying an activator of SERCA2b, comprising: contacting a cell with a candidate agent; measuring SERCA2b activity in the cell contacted with the candidate agent; and optionally comparing the cell’s SERCA2b activity in the presence of the candidate agent with the cell’s SERCA2b activity in the absence of the candidate agent, wherein an increase in SERCA2b activity in the presence of the candidate agent is indicative of activation of SERCA2b.

As used herein, the term "test compound" or "candidate agent" refers to an agent or collection of agents (e.g., compounds) that are to be screened for their ability to have an effect on the cell. Test compounds can include a wide variety of different compounds, including chemical compounds, mixtures of chemical compounds, e.g., polysaccharides, small organic or inorganic molecules (e.g., molecules having a molecular weight less than 2000 Daltons, less than 1000 Daltons, less than 1500 Dalton, less than 1000 Daltons, or less than 500 Daltons), biological macromolecules, e.g., peptides, proteins, peptide analogs, and analogs and derivatives thereof, peptidomimetics, nucleic acids, nucleic acid analogs and derivatives, an extract made from biological materials such as bacteria, plants, fungi, or animal cells or tissues, naturally occurring or synthetic compositions.

Depending upon the particular embodiment being practiced, the test compounds can be provided free in solution, or can be attached to a carrier, or a solid support, e.g., beads. A number of suitable solid supports can be employed for immobilization of the test compounds. Examples of suitable solid supports include agarose, cellulose, dextran (commercially available as, i.e., Sephadex, Sepharose) carboxymethyl cellulose, polystyrene, polyethylene glycol (PEG), filter paper, nitrocellulose, ion exchange resins, plastic films, polyaminemethylvinylether maleic acid copolymer, glass beads, amino acid copolymer, ethylene-maleic acid copolymer, nylon, silk, etc. Additionally, for the methods described herein, test compounds can be screened individually, or in groups. Group screening is particularly useful where hit rates for effective test compounds are expected to be low such that one would not expect more than one positive result for a given group.

A number of small molecule libraries are known in the art and commercially available. These small molecule libraries can be screened using the screening methods described herein. A chemical library or compound library is a collection of stored chemicals that can be used in conjunction with the methods described herein to screen candidate agents for a particular effect. A chemical library comprises information regarding the chemical structure, purity, quantity, and physiochemical characteristics of each compound. Compound libraries can be obtained commercially, for example, from Enzo Life Sciences.TM., Aurora Fine Chemicals.TM., Exclusive Chemistry Ltd..TM., ChemDiv, ChemBridge.TM., TimTec Inc..TM., AsisChem.TM., and Princeton Biomolecular Research.TM., among others.

Without limitation, the compounds can be tested at any concentration that can exert an effect on the cells relative to a control over an appropriate time period. In some embodiments, compounds are tested at concentrations in the range of about 0.01 nM to about 100 nM, about

0.1 nM to about 500 microM, about 0.1 microM to about 20 microM, about 0.1 microM to about 10 microM, or about 0.1 microM to about 5 microM.

The compound screening assay can be used in a high throughput screen. High throughput screening is a process in which libraries of compounds are tested for a given activity. High throughput screening seeks to screen large numbers of compounds rapidly and in parallel. For example, using microtiter plates and automated assay equipment, a laboratory can perform as many as 100,000 assays per day, or more, in parallel.

The compound screening assays described herein can involve more than one measurement of the cell or reporter function (e.g., measurement of more than one parameter and/or measurement of one or more parameters at multiple points over the course of the assay). Multiple measurements can allow for following the biological activity over incubation time with the test compound. In one embodiment, the reporter function is measured at a plurality of times to allow monitoring of the effects of the test compound at different incubation times.

The screening assay can be followed by a subsequent assay to further identify whether the identified test compound has properties desirable for the intended use. For example, the screening assay can be followed by a second assay selected from the group consisting of measurement of any of: bioavailability, toxicity, or pharmacokinetics, but is not limited to these methods.

EXAMPLES

The invention now being generally described, it will be more readily understood by reference to the following examples which are included merely for purposes of illustration of certain aspects and embodiments of the present invention, and are not intended to limit the invention.

Example 1: Experimental Procedures

Purification of MFSD7C N-terminal domain (NTD). Plasmid harboring GST-His-tag-SUMO-NTD (amino acids 1-84) was transformed into *Escherichia coli* Lemo21(DE3) strain (New England Biolabs), and grown overnight in 50 mL of LB supplemented with ampicillin (100 µg/mL) at 37°C. The following day, 5 mL of overnight culture was diluted in 5 L of terrific broth media (TB) supplemented with ampicillin (100 µg/mL) at 37°C and grown to O.D.₆₀₀=0.6. The expression of GST-His-tag-SUMO-NTD was induced with 0.5 mM IPTG for 16 hours at 37°C. Cells were harvested by centrifugation and washed once with ice-cold Milli-Q water. The washed pellet was resuspended in 50 mL of ice-cold buffer A (50 mM Tris-HCl

pH 8.0, 500 mM NaCl, 0.5% Triton X-100) containing Protease Inhibitor Cocktail VII (RPI International), and the resuspension was frozen at -80°C for no longer than one week. The resuspension was thawed at room temperature, and cells were lysed by sonication in the cold room. The lysate was cleared by centrifugation (20,000g for 45 min) and the supernatant was incubated with 5 mL of Complete His-tag Purification Resin (Roche) pre-equilibrated with buffer A for 3 hours while rotating in the cold room. The flow-through was discarded and the resin was washed twice with 25 mL of ice-cold buffer B (10 mM Tris-HCl pH 8.0, 100 mM NaCl). GST-His-tag-SUMO-NTD was eluted from the resin with 20 mL of buffer B containing 300 mM imidazole. Eluted fraction was supplemented with β -mercaptoethanol (f.c. 2 mM), and NTD was cleaved off from the rest of the protein with catalytic subunit of yeast Ulp1 (f.c. 1 μ g/mL for 1 hour in the cold room). After incubation with Ulp1, the mixture was flash-frozen in liquid nitrogen, lyophilized overnight, and resuspended in 10 mL of pure HPLC-grade water. This step eliminated volatile molecules such as β -mercaptoethanol, and selectively precipitated GST-His-tag-SUMO, Ulp1, and other contaminating proteins while it had no effect on the stability of water-soluble and intrinsically disordered NTD. Precipitate was cleared by centrifugation (20,000g for 15 min) and the supernatant containing the NTD was transferred to fresh tubes. NTD was precipitated with isopropanol (f.c. 50% v/v) at -20°C for 2 hours, and centrifuged. The supernatant was discarded and the precipitate was dried in air at room temperature for 10 min. The precipitated NTD was resuspended in 5 mL of pure HPLC-grade water. Finally, the resuspended sample was applied to pre-equilibrated Superdex 75 10/300 gel-filtration column with filter-sterilized HPLC-grade water. Peak fractions containing NTD were pooled, lyophilized and resuspended in HPLC-grade water to desired concentration. 5 L of cells typically yield 15 mg of NTD, at least 95% pure according to SDS-PAGE and MALDI-TOF analysis. Mutant NTD carrying His30Ala/His36Ala/His48Ala/His54Ala/His66Ala mutations was purified the same way as the wild-type NTD.

Peptide synthesis. Wild-type HP motif peptide (HPSALAQPSGLAHP) and mutant HP motif peptide (APSALAQPSGLAAP) were synthesized using solid-phase synthesis and purified using HPLC by the Biopolymers & Proteomics Core at the Koch Institute for Integrative Cancer Research.

Heme absorbance-shift assay. Hemin was prepared fresh before each experiment. Approximately 20 mg of hemin (Sigma) was placed in a fresh tube and resuspended with 1 mL of DMSO. The hemin solution was slowly diluted while mixing with 1 mL of 2X buffer C (25 mM HEPES-NaOH pH 7.8, 10 mM NaCl), and aggregated hemin was eliminated by passing the solution through a 0.2 μ m filter unit. Hemin concentration was determined by diluting the

filtered solution with 1X buffer C 1:100 and using the extinction coefficient of $58,400 \text{ cm}^{-1}\text{M}^{-1}$ at 385 nm. For each reaction, the 200 μL reaction mix containing 100 μM hemin, 25 mM HEPES-NaOH pH 7.8, 10 mM NaCl, and various concentrations of wild-type or mutant NTD protein, was placed in a transparent 96-well plate. Absorbance intensity was measured using Tecan Infinite M200 Pro microplate reader, between 330 and 550 nm using 5 nm steps. The absorbance intensity for dissolved hemin was subtracted from absorbance intensity from hemin incubated with various concentrations of protein.

Gel-filtration assay. Purified NTD (200 μM) was incubated with freshly prepared heme (150 μM) in a 1 mL reaction containing 5% DMSO, 25 mM HEPES-NaOH pH 7.8, and 10 mM NaCl. The solution was run over Superdex 75 (24 mL) gel-filtration column at 0.3 mL/min rate using AKTA-FPLC. Absorbance was monitored at 230, 380, and 415 nm. 2 mL injection volume was subtracted from the final elution volume. Solution containing dissolved hemin in the reaction buffer without the NTD protein aggregated on top of the Superdex 75 column, thus this control was avoided in our experiments.

Isothermal titration calorimetry (ITC). ITC was performed using Microcal VP-ITC (Malvern). Freshly prepared 25 μM hemin solution (described above) in 10% DMSO, 25 mM HEPES-NaOH pH 7.8, was placed in the sample cell using bubble-free technique. The reference cell was filled with buffer containing 10% DMSO, 25 mM HEPES-NaOH pH 7.8 without hemin. The titration syringe was filled with 110 μM NTD protein in the matching buffer. The experiment was run following the manufacturer's instructions using the following parameters: temperature was set to 25°C, 27 total injections (the first injection was 1 μL with subsequent injections of 10 μL over 20 sec), differential power was set to 10, delay was 60 sec, and syringe was rotating at 307 rpm.

Antibodies, cell lines and flow cytometry. Antibodies specific for MFSD7C (Catalog No. HPA037984) for Western blotting or immunofluorescence were purchased from Sigma. Antibodies specific for SERCA2b (Catalog No. ab2861) for immunofluorescence were purchased from Abcam. Antibodies specific for SERCA2b (Catalog No. 4388) for Western blotting were purchased from Cell Signaling Technology. Anti-Calnexin (Catalog No. ab13504) for ER localization was purchased from Abcam. Anti-Myc (Catalog No. 5605), anti-HA (Catalog No. 2367) and anti-FLAG (Catalog No. 2368) antibodies were purchased from Cell Signaling Technology. Human SERCA2b (Catalog No. 75188) plasmid was purchased from Addgene. Cell lines THP-1 (ATCC TIB-202), MH-S (ATCC CRL-2019), and 293FT

were cultured following vendor instructions (37°C, 5% CO₂). FPT labeled cells were analyzed on BD-LSRII, collecting 20,000 live cells per sample. The data were analyzed using FlowJo.

Mouse whole brain cellular fractionation analysis. Whole brain from C57BL/6 mice was isolated, resuspended in PBS supplemented with 10 mM EDTA, and passed through 40 µm Falcon cell strainer (VWR). The resuspension was centrifuged at 1,200g and washed twice more with PBS/10 mM EDTA. The pellet was resuspended in 35 mL of cold 1x MS Buffer (210 mM mannitol, 70 mM sucrose, 5 mM Tris-HCl pH 7.5, 1 mM EDTA) supplemented with 1% fatty acid-free BSA (Sigma). Cells were lysed using Dounce homogenizer with 10-15 strokes of the pestle, and the lysate was transferred to a 50 mL centrifuge tube and centrifuged at 1,300g for 5 min at 4°C to precipitate nuclei and unbroken cells. The supernatant was transferred to a fresh 50 mL centrifuge tube and the nuclear precipitation step was repeated two more times. The supernatant was then centrifuged at 10,000g for 15 min at 4°C to precipitate mitochondria. The supernatant was saved for analysis (Sup), while the crude mitochondrial pellet was washed once more by resuspending in 35 mL of ice-cold 1x MS Buffer plus 1% BSA followed by centrifugation at 10,000g for 15 min at 4°C. Crude mitochondrial pellet was resuspended in 5 mL of 1x MS buffer, and mitochondria were used immediately for Western blot analysis (Mito). Western blot analysis was performed using the following antibodies: anti-MFSD7C (Sigma, Catalog No. HPA037984), anti-VDAC (Cell Signaling Technologies, Catalog No. 4661), anti-COX4I1 (Cell Signaling Technologies, Catalog No. 4850), anti-GAPDH (Cell Signaling Technologies, Catalog No. 5174), anti-NPM1 (Novus Biologicals, Catalog No. NB110-61646SS), anti-Calreticulin (Cell Signaling Technologies, Catalog No. 12238), anti-SERCA2b (Cell Signaling Technologies, Catalog No. 3010), and anti-LC3 (Cell Signaling Technologies, Catalog No. 2775).

DNA plasmids for IP-MS, localization, co-IP, genome editing. To construct MFSD7C tagged with FLAG and Myc epitopes for immunoprecipitation-mass spectrometry (IP-MS), GFP-P2A fragment was amplified with the primers Bgl II-NHEI-GFP-F and BamHI-P2A-GFP-R. The fragment was digested using Bgl II/Bam HI and inserted to the Bam HI site of the pLKO.1 vector (Addgene Catalog No. 10878) to obtain pLKO.1-GFP-P2A-Puro vector. The MFSD7C-FLAG-Myc fragment was amplified from the murine MFSD7C plasmid (Origene Catalog No. MR208748) using the primers MFSD7C-SgfI-F and AC-Myc-DDK-MluI-KpnI-R. The fragment was digested with SgfI and then with KpnI. The fragment was cloned into the SgfI and KpnI sites of pLKO.1-GFP vector to yield pLKO.1-GFP-P2A-MFSD7C-FLAG-Myc (Figure 8A-8B).

To construct MFSD7C-GFP fusion for localization study, MFSD7C fragment was amplified from the murine MFSD7C plasmid (Origene Catalog No. MR208748) with primers MFSD7C-SgfI-F and AC-Myc-DDK-MluI-KpnI-R and inserted into SgfI and MluI sites of pCMV6-AC-GFP vector (Origene Catalog No. PS100010) so that GFP is fused to the C-terminus of MFSD7C (Figure 8A-8B).

To construct various vectors for co-immunoprecipitation, MFSD7C fragment was amplified from the murine MFSD7C plasmid (Origene Catalog No. MR208748) with the primers MFSD7C-SgfI-F and AC-Myc-DDK-MluI-KpnI-R and inserted in SgfI and MluI sites of pCMV6-AN-3HA (Origene Catalog No. PS100066) so that HA tag is introduced into N-terminus of MFSD7C. Murine Hmox1 (Catalog No. MR203944), Cyc1 (Catalog No. MR204721), Cox4i1 (Catalog No. MR218332), Ndufa4 (Catalog No. MR216909), ATP5h (Catalog No. MR201260), ATP5c1 (Catalog No. MR204152) genes were purchased from Origene and were tagged with FLAG at the C-terminus.

To construct vectors for MFSD7C knockout in cell lines, mCherry fragment was amplified using the primers Bam HI-P2A-mCherry-F and mCherry-WRPE-R. WRPE fragment was amplified with the primers mCherry-WRPE-F and PmeI-R. The mCherry-WRPE fragment was amplified with primers Bam HI-P2A-F and PmeI-R using the mixture of mCherry fragment and WRPE fragment as templates. The mCherry-WRPE fragment and Lenti-CRISPR-V2 (Addgene Catalog No. 52961) were digested with Bam HI and PmeI and ligated to generate Lenti-CRISPR-V2-mCherry (Figure 9B). gRNA-1 and gRNA-2, specific for human MFSD7C, were inserted in BsmBI site of the Lenti-CRISPR-V2-mCherry vector. gRNA-3, specific for human MFSD7C, was inserted into the BsmBI site of Lenti-CRISPR-V2 vector (Addgene Catalog No. 52961).

To construct MFSD7C full length to complement 7CKO 4B8 cells, 3 MFSD7C fragments was amplified from the murine MFSD7C plasmid (Origene Catalog No. MR208748) by primer pairs MFSD7C-AsiSI-F/MFSD7C-KpnI-R, MFSD7C-KpnI-F/ MFSD7C-XmaI-R, MFSD7C-XmaI-F/ MFSD7C-MluI-R. The three fragments were Gibson assembled to pLKO.1-GFP-P2A-MFSD7C-Myc-DDK that was linearized by AsiSI and MluI. The correct constructs (pLKO.1-GFP-P2A-FL-MFSD7C-Myc-DDK) were validated by Sanger sequencing and used for Figures 4A-4F. To construct N-terminus deletion of MFSD7C for complementation of 7CKO 4B8 cells, 3 MFSD7C fragments was amplified from the murine MFSD7C plasmid (Origene Catalog No. MR208748) by primer pairs Δ NTD-MFSD7C-AsiSI-F/MFSD7C-KpnI-R, MFSD7C-KpnI-F/ MFSD7C-XmaI-R, MFSD7C-XmaI-F/ MFSD7C-MluI-R. The three fragments were Gibson assembled to pLKO.1-GFP-P2A-MFSD7C-Myc-

DDK that was linearized by AsiSI and MluI. The correct constructs (pLKO.1-GFP-P2A- Δ N-MFSD7C-Myc-DDK) were validated by Sanger sequencing and used for Figures 4A-4F (Figure 8A-8B).

All of the final constructs were confirmed by sequencing. See Table 1 for a list of primers, and Table 2 for a list of plasmids used in this study.

Generation of lentiviral vectors and stable cell lines. The protocols for lentiviral production and transduction were as described (<http://www.addgene.org/tools/protocols/plko/>). Briefly, the plasmids of lentivector, psPAX2 (packaging, Addgene Catalog No. 12260), and pMD2.G (envelope, Addgene Catalog No. 12259) were transfected into 293T cells for lentiviral production. The lentivirus was titered and used to transduce the target cells. Transduced cells were purified by flow cytometry using the encoded fluorescence proteins in the lentivectors or were selected by puromycin using the resistance gene encoded in the lentivector. Murine alveolar macrophage cell line MH-S was transduced with pLKO.1-GFP-P2A-Puro and pLKO.1-GFP-P2A-MFSD7C-FLAG-Myc. GFP positive cells were sorted and expanded. Cell lysates were then used for immunoprecipitation, followed with mass spectrometry.

THP-1 cells were transduced with lentiviruses expressing mCherry, Cas9, and MFSD7C guide RNA-1 or -2 (Figures 9B, 9D). mCherry-positive cells were cloned by single cell sorting into a 96-well plate. Deletion of MFSD7C in the clones was determined by PCR analysis followed by sequencing. Specifically, the genomic DNA of the targeted regions was amplified with the specific primers F1/R1, F2/R2, F3/R3 for sequencing, respectively (Figure 9D). Two clones 3D12 and 4B8, one each derived from gRNA-1 and gRNA-2, were identified to have deletions in MFSD7C genomic DNA with no detectable RNA transcript. To ensure the complete MFSD7C knockout, these clones were subject to another round of CRISPR-Cas9-mediated gene editing using lentivirus expressing puromycin resistant gene, Cas9 and MFSD7C gRNA-3 (Figures 9C, 9D). Puromycin-resistant cells were again cloned by single cell sorting. A total of 4 clones were identified to have the genomic deletion and no detectable wildtype genomic DNA and transcript (Figure 9E). The four clones are collectively referred to as 7CKO clones/cells. CRISPR-Cas9-mediated MFSD7C targeting in MCF7 and 293T cells were done in the same manner.

Immunoprecipitation and LC-MS/MS. Murine alveolar macrophage cell line MH-S, which expresses MFSD7C (Figure 8C), was transduced with lentivirus expressing GFP alone or GFP plus murine MFSD7C tagged with Myc and FLAG epitopes. The GFP positive cells were sorted and expanded. Two hundred million cells per sample were lysed in 5 mL of cold

Lysis Buffer, containing 20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.1% NP-40, 10% glycerol, proteinase inhibitor (Sigma Catalog No. 4693132001), and phosphatase inhibitors (Sigma Catalog No. 4906845001), and homogenized. The lysates were centrifuged at 30,000g for 10 minutes, and the supernatants were further centrifuged at 30,000g for 20 minutes. The clear supernatants were incubated with M2 magnetic beads conjugated with anti-FLAG antibody (Sigma Catalog No. M8823) for 2 hours and eluted by 3X FLAG peptide. The elutes were incubated with magnetic beads conjugated to anti-Myc antibody (Cell Signaling Technology Catalog No. 5698) for 2 hours. The beads were washed in Lysis Buffer and balanced by PBS. The immunoprecipitates were washed three times with 100 mM NH_4HCO_3 . Proteins were reduced (10 mM dithiothreitol, 56 °C for 45 min) and alkylated (50 mM iodoacetamide, room temperature in the dark for 1 h). Proteins were subsequently digested with trypsin (sequencing grade, Promega), at an enzyme/substrate ratio of 1:50, at room temperature overnight in 100 mM ammonium acetate, pH 8.9. Trypsin activity was quenched by adding formic acid to a final concentration of 5%. Peptides were desalted using C18 SpinTips (Protea) then lyophilized and stored at -80°C .

Peptides were loaded on a pre-column and separated by reverse phase HPLC (Thermo Easy nLC1000) over a 140-minute gradient before nanoelectrospray using a QExactive mass spectrometer (Thermo). The mass spectrometer was operated in a data-dependent mode. The parameters for the full scan MS were: resolution of 70,000 across 350-2000 m/z , AGC 3×10^6 , and maximum IT 50 ms. The full MS scan was followed by MS/MS for the top 10 precursor ions in each cycle with a NCE of 28 and dynamic exclusion of 30 s. Raw mass spectral data files (.raw) were searched using Proteome Discoverer (Thermo) and Mascot version 2.4.1 (Matrix Science). Mascot search parameters were: 10 ppm mass tolerance for precursor ions; 0.8 Da for fragment ion mass tolerance; 2 missed cleavages of trypsin; fixed modification was carbamidomethylation of cysteine; variable modification was methionine oxidation. Only peptides with a Mascot score greater than or equal to 25 and an isolation interference less than or equal to 30 were included in the data analysis. Potential interacting proteins are identified in the experimental sample after removal of proteins in the control sample and common contaminating proteins.

The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD021016 (<http://www.ebi.ac.uk/pride/archive/projects/PXD021016>).

Co-transfection and immunoprecipitation. For co-IP, HA-tagged MFSD7C was co-transfected with FLAG-tagged HMOX1 (Origene Catalog No. MR203944), CYC1, COX4I1, NDUFA4, ATP5h, ATP5c1 into 293FT cells using TransIT®-LT1 Transfection Reagent (Mirus). Thirty-six hours after transfection, the cells were lysed using cold Lysis Buffer containing 20 mM Tris-HCl (pH 7.4), 150 mM NaCl, 0.1% NP-40, 10% glycerol, proteinase inhibitor (Sigma Catalog No. 4693132001), and phosphatase inhibitors (Sigma Catalog No. 4906845001). The clear supernatants from the lysate were incubated with M2-magnetic beads conjugated with anti-FLAG antibody (Sigma Catalog No. M8823) for 2 hours at 4°C. Then the beads were washed twice and eluted by the 3X FLAG peptides (Sigma Catalog No. F4799).

To determine the effect of heme on MFSD7C interactions with ETC components, HMOX1 or SERCA2b, 293FT cells were transiently transfected with HA-tagged murine MFSD7C and FLAG-tagged murine CYC1, NDUFA4, COX4i1, ATP5h, ATP5c1, HMOX1 or SERCA2b. 35 hrs later, co-transfected cells were incubated with DMSO (vehicle) or 10 μ M or 40 μ M of heme for one hour before lysis. Cell lysates were precipitated with anti-HA antibody, eluted with HA peptide, further precipitated with anti-FLAG antibody, eluted with FLAG peptide, and then subjected to Western blotting with anti-HA and anti-FLAG antibodies. Cells were treated with proteasome inhibitor MG132 for co-IP between MFSD7C and SERCA2b.

Endogenous protein extraction. THP-1 cells were lysed in RIPA buffer (25 mM Tris•HCl pH 7.4, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS) with proteinase and phosphatase inhibitors (Sigma Catalog No. 4693132001 and 4906845001). The clear supernatants were used for Western blotting.

Imaging analysis of MFSD7C localization. For MFSD7C localization, 293FT cells over-expressing GFP- or mCherry-tagged MFSD7C were grown on coverslips in tissue culture and stained for mitochondria using 100 nM MitoTracker® Deep Red FM (Thermo-Fisher Catalog No. M22426) for 20 min in serum-free medium, per manufacturer's protocol. Cells were fixed using 3.5% paraformaldehyde (in 1X PBS, pH 6.7) for 10 minutes and permeabilized with 0.5% Triton-X in 1X TBS-BSA (10 mM Tris HCl pH 7.5, 150 mM NaCl, 1% BSA, 0.1% NaN₃) for another 10 minutes. Anti-human HLA-A, B, C (Biolegend W6/32) was added at a 1:1000 dilution in 1X TBS-BSA + 0.1% Triton-X for 1 hour at room temperature. Anti-mouse Alexa 647 (Life Technologies Catalog No. A31571) at a 1:2000 dilution was added to DAPI in 1X TBS-BSA and incubated with the cells for 1 hour.

Coverslips were attached to glass slides using ProLong® Diamond Antifade Mountant with DAPI (Thermo-Fisher, Catalog No. P36962) and imaged using a Nikon A1R Ultra-Fast

Spectral Scanning Confocal Microscope using Elements software. Images were taken in z-stacks of 0.2 μm and flattened using the max projection function in ImageJ.

Measurements of OCR, ECAR, and MMP. Oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) were measured using XF96e Seahorse Extracellular Flux Analyzer per manufacturer's protocol. To increase adherence of suspension cells, Seahorse plates were coated with Corning® Cell TAK (Catalog No. 354240). THP-1 and 7CKO cells were then attached to the plate according to the manufacturer's instructions. Cells were incubated in complete RPMI media with or without 40 μM heme. Changes in oxygen consumption were measured following treatment with oligomycin (5 μM), FCCP (2 μM), and rotenone (1 μM) plus antimycin A (1 μM). For BMDM, 5×10^5 cells/well were plated in 100 μL of BMDM media, 24 hours before the start of the assay. For OCR measurements, BMDM media was replaced with 180 μL of Seahorse XF Base Medium supplemented with 10 mM D-glucose, 1 mM sodium pyruvate, and 1 mM L-glutamine. For ECAR measurements, BMDM media was replaced with 180 μL of Seahorse XF RPMI Medium pH 7.4 supplemented with 2 mM L-glutamine. The Glycolysis Stress Test was performed using D-glucose, rotenone/antimycin A, and 2-deoxy-D-glucose at 10 mM, 0.5 μM , and 50 mM final concentration, respectively.

Mitochondrial membrane potential was measured using Abcam kit (Catalog No. ab113852). Briefly, BMDM, THP-1 and 7CKO cells were not treated or treated with 40 μM heme for 1 hour. The cells were incubated with mitochondrial membrane potential indicator, 200 nM TMRE (tetramethylrhodamine, ethyl ester) for 20 minutes. The mean fluorescence intensity of TMRE were determined by flow cytometry.

Measurement of cellular energy charge (ATP/ADP ratio). ATP/ADP ratio was measured using ADP Assay Kit (Sigma Catalog No. MAK133-1KT) following manufacturer's protocol. For THP-1 cells, 24 hours before the experiment, cells were resuspended in fresh complete RPMI media. For heme treatment, 1 mL of treated cell suspension was incubated for 1 hour at 37°C cell culture incubator, then centrifuged and resuspended in 1 mL of fresh warm complete RPMI media in order to wash off excess heme, which interferes with the assays. 10 μL of cell suspension per well (approximately 10,000 cells total) was placed in a white 96-well plate, lysed with 90 μL of ATP Buffer, and incubated with gentle shaking at room temperature for 10 min. Relative ATP amount was directly measured using luminescence. Then, 5 μL of ADP Enzyme mix was added to each well and the plate was incubated with light shaking for 3 minutes. Relative amount of ADP+ATP was measured using luminescence. ADP amount was calculated by subtracting the ATP signal from ADP+ATP signal. To get ATP/ADP ratio, ATP

signal was divided by the calculated ADP signal. For BMDMs, the assay was performed following the same protocol but using 20,000 cells per well.

Alternatively, ATP, ADP, and AMP levels were measured using targeted metabolomic analysis at the Whitehead Institute Metabolite Profiling Core Facility. Briefly, 100,000 cells (parental THP-1 cells and 7CKO clone B11, 3 independent experiments each) were centrifuged, and resuspended in 500 μ L of cold 0.9% NaCl. Metabolites were extracted with the addition of 600 μ L LC/MS-grade cold methanol containing internal standards, and the mixture was vortexed for 2 min. Then 300 μ L LC/MS grade water was added to each tube, followed by 400 μ L cold chloroform. The mixture was vortexed again in the cold room and then spun at 16,000g in a microcentrifuge. The top layer containing polar metabolites was transferred to a clean tube and the sample was dried using speedvac. Ultra-pressure liquid chromatography was performed using pHILIC column on Dionex UltiMate 3000 and mass-spectrometry was performed using Thermo Scientific QExactive Orbitrap instruments.

Synthesis of fluorescent polymeric thermometer. 4-N,N-Dimethylaminosulfonyl -7-fluoro-2,1,3-benz-oxadiazole (DBD-F) was purchased from TCI Chemicals. N-n-propylacrylamide (NNPAM) and N-ethylacrylamide (NEAM) were purchased from AstaTech. N, N'-dimethylethylenediamine, acryloyl chloride, triethylamine (TEA), (3-acrylamidopropyl) trimethylammonium chloride (APTMA Cl), azobisisobutyronitrile (AIBN) and other solvents were purchased from MilliporeSigma. All commercial acrylamide monomers were passed through a basic alumina column to remove inhibitors before polymerization. Other reagents were used as purchased.

The synthesis was slightly modified from the protocol in the literature. Briefly, 100 mg DBD-F is dissolved in 5 mL anhydrous acetonitrile, then added dropwise into a stirring vial containing 1.3 mL N, N'-dimethylethylenediamine. The mixture was allowed to react for 15 minutes at room temperature. The reaction mixture was condensed with rotatory evaporation and purified with silica gel liquid chromatography using eluent dichloromethane:methanol from 10:1 to 5:1, fractions were collected and monitored with thin layer chromatography. DBD-NMe(CH₂)₂NHMe was obtained as an orange liquid.

130 mg DBD-NMe(CH₂)₂NHMe was dissolved in 5 mL of anhydrous acetonitrile mixed with 58 μ L of TEA and cooled on ice. 44 μ L acryloyl chloride was dissolved in 8 mL of anhydrous acetonitrile, cooled on ice and then added dropwise into the reaction mixture. The reaction was allowed to proceed for 1.5 hours at room temperature then condensed with rotatory evaporation and purified with silica gel liquid chromatography using eluent

ethylacetate:hexane 3:1. Fractions were collected and monitored with thin layer chromatography. DBD-AA was obtained as an orange powder (Figure 10A).

The polymer synthesis was modified from the protocols described in the literature. Briefly, 4.1 mg AIBN, 20 mg DBD-AA, 41 mg APTMA Cl, 565 mg NNPAM (for fluorescent polymeric thermometer) or 495 mg NEAM (for control polymer that is not temperature sensitive), 5 mL DMF and a stir bar were added into a clean schlenk flask. The flask was sealed and purged with nitrogen for 30 mins at room temperature to remove dissolved oxygen. The flask was then immersed in 60°C oil bath to initiate the polymerization. After 12 hours of reaction, the reaction mixture was precipitated in cold ethyl ether (0°C) and redissolved in DMF for three times, then dried *in vacuo* overnight for use (Figure 10B).

Measurements of cellular thermogenesis. Three methods were used to measure cell thermogenesis. In the first approach, we used thermocouple. Three million THP-1 and 7CKO cells were re-suspended in 100 μ l media (RT, room temperature) and transferred to PCR tubes tightly fitted in a thermally insulating enclosure. Then the temperature change rate of media ($\Delta T_m/\Delta t$) was monitored real-time by type T-Type Thermocouples (Omega, Catalog No. 5SC-TT-T-3036) immersed in the liquid media. An analog to digital converter (National Instruments 24-bit Thermocouple ADC) was used to obtain and store the thermocouple data via a custom Labview interface (sampling rate: 500ms). Every measurement was compared to the free liquid media.

In the second approach, we used fluorescent polymeric thermometer (FPT), which we synthesized in-house (see above). Parental and knockout THP-1 cells were mixed together, washed and incubated with FPT in 5% w/v glucose solution for 6 hrs. The cells were washed with PBS twice and reseeded in dishes with glass bottom that was coated with poly-lysine. The cells were imaged under Confocal Laser-Scanning Microscopy at 37°C. Alternatively, THP-1, MCF7, 293T, 7CKO, *SERCA2b*^{-/-} cells were incubated with FPT and control polymer overnight and followed with vehicle or hematin treatment for 1 hour. Then the fluorescence intensities were determined by a flow cytometer.

In the third approach, we used commercial cellular fluorescent thermoprobe dye (Funakoshi Catalog Number: FDV-0005). Briefly, one day before the assay, 2x10⁵ BMDMs were plated in a 48-well non tissue-culture treated polystyrene plate in 200 μ L of BMDM media. BMDM media were aspirated and cells were washed once with Loading Solution (5% (w/v) aqueous glucose solution supplemented with 10 mM EDTA). Next, 200 μ L of Loading Solution supplemented with 50 ng/ μ L Cellular Thermoprobe Dye was added to the well and loading of the dye was performed in a 33°C, 5% CO₂ cell culture incubator for 7.5 minutes,

which simultaneously lifts BMDMs from the well. In the next 2.5 minutes, the plate was removed from the incubator, cells were resuspended by pipetting and moved to a PCR tube and combined with 22 μ L of 10x PBS/DAPI buffer. The mixture in the PCR tube was then placed in a thermocycler with a preset temperature, incubated for 5 min, and immediately analyzed using flow cytometry (BD LSR II). Cells are gated based on size (singlets) and DAPI (live cells). 15,000 FITC-positive cells were collected for the analysis. Special care was taken with regards to timing because the loading of the Cellular Thermoprobe Dye is dependent on the amount of time the cells are incubated with the dye. Different filters were used to measure loading and temperature sensitivity due to properties of Cellular Thermoprobe Dye. For loading measurements, excitation at 488 nm with 515/20 emission filter was used, while thermosensitive analysis was performed using excitation at 488 nm with 530/30 emission filter.

*Generation of *Mfsd7c* mutant mice.* C57BL/6N ES cell clone with floxed exon 2 of *Mfsd7c* was purchased from EuMMCR (European Mouse Mutant Cell Repository, ES cell Clone ID: HEPD0572_8_F01). The ES cells were transfected with plasmids encoding FLP to remove neomycin resistance cassette. The G418 sensitive ES cells with properly floxed exon 2 of *Mfsd7c* were confirmed using PCR and Sanger Sequencing. The ES cells were injected into blastocysts and then transferred into pseudopregnant mice at the Koch Institute Swanson Biotechnology Center. Germline mutant mice were identified based on the coat color and *Mfsd7c*^{wt/fl} heterozygous mice were interbred to generate homozygous *Mfsd7c*^{fl/fl} mice. *Mfsd7c*^{fl/fl} mice were bred with *LysM-Cre* mice (the Jackson Laboratory, Stock No: 004781) to generate myeloid-specific *Mfsd7c* knockout. Mice were maintained in the animal facility at the Massachusetts Institute of Technology (MIT). All animal studies and procedures were carried out following federal, state, and local guidelines under an IACUC-approved animal protocol by Committee of Animal Care at MIT.

Mouse genomic DNA extraction, genotyping, and qPCR. A small piece of mouse tail was cut using scissors, placed in 500 μ L of Genomic DNA Extraction Buffer (100 mM Tris-HCl pH 8.0, 200 mM NaCl, 5 mM EDTA, 0.2% SDS, 0.5 mg/mL Proteinase K) and digested overnight at 55°C. Digested tissue was centrifuged at 13,000g for 5 min, and the cleared supernatant was transferred to a fresh 1.5 mL tube. Genomic DNA was precipitated from the supernatant with isopropanol (50% v/v), and centrifuged at 13,000g for 2 min. Supernatant was discarded, and the precipitate was washed with 1 mL of 70% ethanol. Ethanol was discarded and the precipitate was left to dry at room temperature for 5 min. DNA was resuspended in 200 μ L of nuclease-free ddH₂O. Genomic DNA from bone marrow-derived macrophages was isolated from 5x10⁵ cells using the same protocol. Primer set #1 and #2 were used to genotype

wild-type, floxed and deleted alleles, and LysM-Cre primers were used to detect the presence of Cre recombinase (see Table 1 for primer sequences).

For qPCR analysis, 10^6 bone marrow-derived macrophages were rinsed with PBS, lysed in RLT buffer (Qiagen), and flash-frozen in liquid nitrogen. After thawing on ice and passing through a 27G needle multiple times, RNA was isolated using Qiagen's RNEasy Mini kit. cDNA was synthesized using Superscript IV (Invitrogen) and random hexamers, followed by RNA removal using *E. coli* RNase H for 20 minutes at 37°C. cDNA was diluted tenfold and qPCR was performed in triplicate using 4 μ L diluted cDNA, 0.5 μ L 5 μ M forward primer, 0.5 μ L 5 μ M reverse primer, and 5 μ L 2x SYBR Green Master Mix (Roche) per well in a 96-well plate (see Table 2 for primer sequences). A Roche Lightcycler 480 instrument was used measuring amplification for 45 cycles using Roche's SYBR Green protocol, after which melting temperatures and crossing points were assessed and quantified.

Differentiation of bone marrow-derived macrophage (BMDM). *Mfsd7*^{fl/fl} and *Mfsd7*^{-/-} (*Mfsd7*^{fl/fl} *LysMCre*^{+/+}) C57BL/6J mice were euthanized using CO₂ asphyxiation. Femoral bones were removed and cleaned and the bone marrow was flushed out using 5 mL of cold DMEM media. Bone marrow cells were collected by centrifugation (1,200g for 5 min at 4°C) and resuspended in 4 mL of ACK lysis buffer. After incubation at room temperature for 5 min, ACK buffer was neutralized by the addition of 11 mL of cold DMEM media and cell suspension was centrifuged at 1,200g for 5 min at 4°C. Cells were resuspended in DMEM media containing 10% FBS, 2 mM L-glutamine, 2 mM pyruvate, non-essential amino acids (100 μ M each), 0.55 mM 2-mercaptoethanol, penicillin/streptomycin), passed through a 40 μ m Falcon cell strainer (VWR) to remove large aggregates, and counted. Bone marrow cells were seeded in 10 cm non tissue-culture treated plates at 10 million cells per plate in 10 mL of BMDM media. Two days later, additional 10 mL of BMDM media was added. On day 4 and day 6, old media was removed and 10 mL of fresh media was added. On day 7, fully differentiated BMDM were lifted in phosphate-buffered saline supplemented with 10 mM EDTA for 5 min at 37°C, centrifuged and resuspended in BMDM media to a proper density for further experimentation.

Table 1. Primers used in this study.

Figure	Name	Forward (5'-3')	Reverse (5'-3')
12	Set #1	gaactgtgtatcagtcaagttgtcaagg	gagctcattggccagccagc
12	Set #2	gaactgtgtatcagtcaagttgtcaagg	gacagggtagtagtctggctgc
12	LysM-Cre	cccagaaatgccagattacg	ctggggctgccagaatttctc
12	<i>Mfsd7c</i> Exon1 qPCR	cagcgtgatcaaggtgagcaag	attgtgaccgggaagaggtgag
12	<i>Mfsd7c</i> Exon2 qPCR	aagcttgctaccacatcag	gcttgggcttctcctgaata
8B	BglIII-NHEI-GFP-F/ BamHI-2A-GFP-R	gaattcAGATCTGCTAGCATGGT GAGCAAGGGCGAGG	GGTGGATCCAGGTCCAGG GTTCTCCTCCACGTCTCCA GCCTGCTTCAGCAGGCTG AAGTTAGTAGCTCCGCTTC CCTTGACAGCTCGTCCAT GCC
8B	<i>Mfsd7c</i> -Sgfl-F/ AC-Myc-DDK-KpnI-R	cggaattcgcgatcgcCATGGTGAA TGAAAGTCTCAAC	cggaattcggtagccgtttaaaccttacc gtcgtcatcc
9B	BamHI-P2A-mCherry-F/ mCherry-WRPE-R	CGATAAGGGATCCGGCGCAA CAAACCTTCTCTGCTGAAAC AAGCCGAGATGTCGAAGAG AATCCTGGACCGATGGTGAG CAAGGGCGAGGA	TGATTGTCGACTTAACGCG TTCACCTGTACAGCTCGTC CATGC
9B	mCherry-WRPE-F/ PmeI-R	GCATGGACGAGCTGTACAAG TGAACGCGTTAAGTCGACAAT CA	tcgaggctgatcagcgggtt
9B	BamHI-P2A-F/ PmeI-R	CGATAAGGGATCCGGCGCAA	tcgaggctgatcagcgggtt
9B	gRNA-1-Forward/ gRNA- 1-Reverse	caccgCTCGTCCCGGTCTTCAA TGT	aaacACATTGAAGACCGGG ACGAGc
9B	gRNA-2-Forward/ gRNA- 2-Reverse	caccgCACCATGCGATTGAGAA GAG	aaacCTCTTCTGAATCGCAT GGTGc
9B	gRNA-3-Forward/ gRNA- 3-Reverse	caccgCTATAGCTTGAATTGC GAT	aaacATCGCAATTCCAAGCT ATAGc
9B	F1/R1 for genomic test of MFSD7c KO-1	CCAGATATGGGAGTAGAGGA	ACAAAGTGCATAGGACCA GC
9B	F2/R2 for genomic test of MFSD7c KO-2	TGTCCAGGACTTCTACTGAC	GCTCAGAAACACTAACTAG C
9B	F3/R3 for genomic test of MFSD7c KO-3	CTGGGTGACAGAGCGAGACT	ACCAACAGGCATTTGTCAG A

Figure	Name	Forward (5'-3')	Reverse (5'-3')
8C	mu-GAPDH-qPCR-F/ mu-GAPDH-qPCR-R	CAAGAAGGTGGTGAAGCAGG	TTGTCATTGAGAGCAATGC C
8C	mu-Mfsd7c-qPCR-F/ mu-Mfsd7c-qPCR-R	TGCCTTAGCGACCACTGATG CT	GGATAAGGCGTAGAAAGC ACCAG
4A-4F	MFSD7C-AsiSI- F/MFSD7C-KpnI-R	GAGAACCCTGGACCTGCGAT CGCCATGGTGAATGAAGGTC CCAA	gCGGTCcTCgATaTTaGGTA CCAAAAC
4A-4F	MFSD7C-KpnI-F/ MFSD7C-XmaI-R	tAaTATcGAgGACCGcGACGAG CTTG	cCGgTTCAGgAGgGTaGAC AAGGCAT
4A-4F	MFSD7C-XmaI-F/ MFSD7C-MluI-R	tACcCTcCTGAACgATGGTG ATCT	CTGCTCGAGCGGCCGCGT ACGCGTGAGATGATCCTCT GACACAG
4A-4F	ΔNTD-MFSD7C-AsiSI- F/MFSD7C-KpnI-R	GAGAACCCTGGACCTGCGAT CGCCCGTTGGGCCGTGGTCC TGGT	CGGTCcTCgATaTTaGGTAC CAAAAC

Table 2. Plasmids used in this study.

Figure	Name	Description	Originator
1, 7	GST-His-tag-SUMO-NTD	NTD expression in E.coli	N.A.I
1, 7	GST-His-tag-SUMO-Mutant NTD	Mutant NTD expression in E.coli	N.A.I
1D	pCMV6-AC-DDK-HMOX1	HMOX1 expression in mammalian cells	Origene MR203944
1D	pCMV6-AC-DDK-Cyc1	Cyc1 expression in mammalian cells	Origene MR204721
1D	pCMV6-AC-DDK-Cox4i1	Cox4i1 expression in mammalian cells	Origene MR218332
1D	pCMV6-AC-DDK-Ndufa4	Ndufa4 expression in mammalian cells	Origene MR216909
1D	pCMV6-AC-DDK-ATP5c1	ATP5c1 expression in mammalian cells	Origene MR204152
1D	pCMV6-AC-DDK-ATP5h	ATP5h expression in mammalian cells	Origene MR201260
1D	pCMV6-AN-3HA	Empty backbone	Origene PS100066
1D and 8B	pCMV6-AC-DDK-MFSD7c	MFSD7c expression in mammalian cells	Origene MR208748

Figure	Name	Description	Originator
1D	pCMV6-AN-3HA-MFSD7C	MFSD7c expression in mammalian cells	Y.L
4A-4Fa-f	pLKO.1-GFP-P2A-FL-MFSD7C-Myc-DDK	MFSD7c expression in mammalian cells	Y.L and T.D
4A-4F	pLKO.1-GFP-P2A-ΔN-MFSD7C-Myc-DDK	MFSD7c expression in mammalian cells	Y.L and T.D
8E	pCMV6-AC-GFP	Empty backbone	Origene PS100010
8E	pCMV6-AC-GFP-MFSD7C	MFSD7c expression in mammalian cells	Y.L
8B	pLKO.1 GFP shRNA	shRNA backbone	Addgene 30323
8B	pLKO.1-GFP-P2A-MFSD7C-Myc-DDK	MFSD7c expression in mammalian cells	Y.L
9B-9E	LentiCRISPR v2	CRISPR backbone	Addgene 52961
9B-9E	pLentiCRISPR-v2-mCherry-hu7CKO-1	Knockout MFSD7c in mammalian cells	Y.L
9B-9E	pLentiCRISPR-v2-mCherry-hu7CKO-2	Knockout MFSD7c in mammalian cells	Y.L
9B-9E	pLentiCRISPR-v2-mCherry-hu7CKO-3	Knockout MFSD7c in mammalian cells	Y.L

Example 2: MFSD7C Binds Heme Through the N-terminal Domain

Sequence and structural analyses predict that MFSD7C belongs to the 12-transmembrane solute carrier family. The NTD of human and mouse MFSD7C contains five regularly spaced histidine-proline (HP) repeats, a feature conserved in many mammalian species (Figure 7A). Histidine is an axial ligand to the central heme-iron in many heme-binding proteins and MFSD7C was reported to precipitate with hemin agarose. To test whether the NTD directly binds to heme, the 84 amino acid NTD of human MFSD7C was recombinantly expressed and purified (Figure 7B). In a gel-filtration chromatography, heme (616 Da) co-eluted with the NTD (8.6 kDa) as indicated by heme-specific absorbance at 380 nm and 415 nm and NTD-specific absorbance at 230 nm of the protein-containing fractions (Figure 1A and Figures 7C,7D). When heme was incubated with the NTD, a concentration-dependent increase in the intensities of the Soret band (415 nm) and the Q band (535 nm) was detected (Figure 1B), and the rate of increase of the Soret band intensities with respect to NTD concentration suggests two or more heme binding sites per NTD (Figure 7E). The absorbance shift was abolished when all five histidine residues in the HP repeats were mutated to alanine (Figure

1B). The isothermal titration calorimetry analysis showed that the NTD binds three heme molecules with two strong binding sites ($K_D \sim 1 \mu\text{M}$) and one weaker site ($K_D \sim 220 \mu\text{M}$) (Figure 7F). Similarly, when incubated with a synthetic 14-amino acid HP motif peptide, heme absorption spectrum also showed a concentration-dependent increase in Soret band and Q band peaks at a rate consistent with one heme bound to one HP motif peptide at a K_D of $\sim 1 \mu\text{M}$ (Figure 1C and Figures 7E,7G). The absorbance shift was abolished when both histidine residues in the HP motif peptide were mutated to alanine (Figure 1C). These results show that the NTD of human MFSD7C is capable of binding to 2-3 heme molecules.

Example 3: MFSD7C interacts with components of ETC complexes III, IV and V

We used immunoprecipitation (IP) and mass spectrometry (MS) to identify proteins that interact with MFSD7C. Because an anti-MFSD7C monoclonal antibody is not available, we used MFSD7C tagged with both Myc and FLAG epitopes in a MFSD7C-expressing murine alveolar macrophage cell line MH-S (Figures 8A-8C and see Methods and PRIDE (<http://www.ebi.ac.uk/pride/archive/projects/PXD021016>), for details). A total of 58 proteins were identified excluding MFSD7C and common contaminants of IP experiments such as keratin, actin, and ribosomal proteins (Table 1). Twenty-six of the 58 proteins are annotated as mitochondrial proteins and 4 (three Fc receptors and one integrin) are known to reside in the cytoplasmic membrane. The 58 proteins were classified into 10 different functional categories based on gene ontology.

To validate the IP-MS results, we tested interactions between MFSD7C and heme oxygenase-1 (HMOX1) and all five ETC proteins: CYC1 (complex III), NDUFA4 and COX4I1 (complex IV), ATP5h and ATP5c1 (complex V/ATP synthase) by co-IP. Plasmids encoding HA-tagged MFSD7C and FLAG-tagged candidate proteins were co-transfected into HEK 293T cells and cell lysates were precipitated with anti-FLAG and anti-HA antibodies sequentially, followed by Western blotting with anti-HA or anti-FLAG antibodies. MFSD7C co-precipitated with all six proteins tested (Figure 1D). To determine whether the observed interactions occur between endogenous proteins, we performed co-IP between MFSD7C and ATP5h, SERCA2b (ATP2a2) and HMOX1 using cell lysates of bone marrow-derived macrophages from C57BL/6 mice with exon 2 of *Mfsd7c* floxed (*Mfsd7c^{fl/fl}*) or deleted (*Mfsd7c^{-/-}*) specifically in macrophages (see Methods for details). The endogenous MFSD7C co-immunoprecipitated with endogenous ATP5h, SERCA2b and HMOX1 in *Mfsd7c^{fl/fl}* macrophages but not in *Mfsd7c^{-/-}* macrophages (Figure 1E). Thus, MFSD7C likely interacts with mitochondrial ETC components and ER proteins SERCA2b and HMOX1.

To determine MFSD7C subcellular localization, we performed subcellular fractionation followed by Western blotting on whole mouse brain where MFSD7C is strongly expressed³⁴. MFSD7C was only detectable in the mitochondrial fraction (10,000g precipitate), along with mitochondrial markers VDAC and COX4I1 (Figure 1F). In contrast, known cytoplasmic proteins GAPDH and nucleophosmin 1 (NPM1), ER protein calreticulin (CalR) and SERCA2b, and autophagosomal protein LC3 were predominantly present in the supernatant fraction. Furthermore, staining of human monocytic THP-1 cells with a polyclonal antibody specific for the C-terminus of MFSD7C, MitoTracker, and DAPI showed co-localization of MFSD7C and MitoTracker signals (Figure 1G), with a Pearson's correlation coefficient value of 0.6. In contrast, the polyclonal antibodies did not stain *Mfsd7c* knockout cells (Figure 8D), suggesting their specificity to MFSD7C. Similarly, when MFSD7C-GFP fusion protein was expressed in HEK 293T cells, most of the GFP signal co-localized with MitoTracker (Figure 8E).

Together, these data suggest that MFSD7C primarily resides in mitochondria where it interacts with components of the ETC complexes III, IV and V.

Example 4: MFSD7C regulates coupling mitochondrial respiration

To investigate the function of MFSD7C, we generated four independent *Mfsd7c* knockout clones (A11, B11, 3D12 and 4B8, collectively referred to as 7CKO cells) using CRISPR-Cas9 genome editing in THP-1 cells, which had readily detectable levels of MFSD7C but not UCP1 (Figure 9). We assayed oxygen consumption rate (OCR), extracellular acidification rate (ECAR), mitochondrial membrane potential (MMP), and energy charge (ATP/ADP ratio) in the parental THP-1 and 7CKO cells in the absence or presence of heme. Both the basal and maximal OCR were 1.5-2-fold higher in 7CKO cells than in the parental THP-1 cells (Figures 2A-2C and Figure 10A). Heme treatment increased both the basal and maximal OCR of the parental THP-1 cells but not the 7CKO cells. ECAR was significantly higher in 7CKO cells than in the parental THP-1 cells (Figure 2D). In contrast, MMP and ATP/ADP ratio were ~10-25% lower in 7CKO cells than in THP-1 cells and heme treatment reduced MMP and ATP/ADP ratio of THP-1 cells to the similar levels as in 7CKO cells (Figures 2E,2F). By targeted metabolomic analysis, the relative amounts of ATP, ADP, and AMP were different between THP-1 and 7CKO clone B11, and the ATP/ADP ratio was significantly reduced in B11 compared to parental THP-1 cells (Figures 10B,10C). These results show that heme and *Mfsd7c* knockout have similar effects on OCR, MMP and cellular energy charge. The observation of increased OCR and ECAR without increase of MMP or

energy charge is consistent with inefficient ATP production resulting from uncoupled mitochondrial respiration.

We measured the effect of *Mfsd7c* knockout and heme on thermogenesis using a temperature-sensitive dye: fluorescent polymeric thermometer (FPT, see Methods and Figures 11A, 11B for details). As previously reported, FPT fluorescent intensities increased when cells were incubated at increasing temperatures (Figure 11C). To compare thermogenesis between the parental THP-1 cells and 7CKO cells under the same condition, we co-cultured THP-1 cells with either A11 or 4B8 7CKO cells, loaded the cells with FPT, and imaged FPT intensity in the same culture well. The FPT fluorescent intensity was visibly higher in mCherry-positive A11 and 4B8 cells than the parental THP-1 cells in the same microscopy field (Figure 2G). Quantification of the fluorescent intensity showed a 2-4-fold increase in 7CKO cells compared to THP-1 cells (Figure 2H). Consistently, only a small fraction of THP-1 cells had an increased FPT fluorescence by flow cytometry, whereas almost all cells had dramatically increased fluorescence following heme treatment for one hour (Figure 2I). In contrast, 7CKO clones A11 and 4B8 had similarly high FPT fluorescence without heme treatment. As a complementary approach, we measured the temperature of culture media using thermal couples (See Methods for details). The temperature of the 7CKO culture was 0.15-0.3°C higher than the THP-1 culture (Figure 2J). Heme treatment increased the temperature of THP-1 culture by 0.12°C but not 7CKO cell cultures.

To validate the observed effects in different cell types, we knocked out *Mfsd7c* in human breast cancer MCF7 cells and human embryonic kidney 293T cells using CRISPR-Cas9 genome editing (Figure 11A). Compared to the parental MCF7 and 293T cells, *Mfsd7c* knockout cells exhibited significantly higher OCR and FTP intensity (Figures 12B-12F). Similarly, heme treatment of parental cells significantly stimulated OCR and FTP intensity. Collectively, these data show that both *Mfsd7c* knockout and heme treatment of THP-1, MCF7 and 293T cells promote uncoupled mitochondrial respiration.

To test whether loss of *Mfsd7c* uncouples mitochondrial respiration in primary cells, we created a C57BL/6 mouse strain with *loxP* sites flanking exon 2 of *Mfsd7c* (*Mfsd7c^{fl/fl}*). *Mfsd7c^{fl/fl}* mice were crossed with *LysMcre* mice to deplete *Mfsd7c* (*Mfsd7c^{-/-}*) specifically in myeloid cells (Figures 13A-13C). We generated bone marrow-derived macrophages (BMDM) by culturing bone marrow cells from *Mfsd7c^{fl/fl}* and *Mfsd7c^{-/-}* mice for 7 days (see Methods for details). BMDM expressed the macrophage markers F4/80 and CD11b (Figure 13D), and were confirmed for deletion of *Mfsd7c* locus and near complete loss of both *Mfsd7c* transcript and protein (Figures 13E-13G). Compared to *Mfsd7c^{fl/fl}* BMDM, *Mfsd7c^{-/-}* BMDM had

significantly higher levels of OCR and ECAR (Figures 3A-3C). Although MMP was not significantly different, ATP/ADP ratio was significantly decreased in *Mfsd7c*^{-/-} BMDM (Figure 3D-3E). We measured thermogenesis of *Mfsd7c*^{fl/fl} and *Mfsd7c*^{-/-} BMDM using a commercial cellular thermoprobe dye (Funakoshi). The uptake of the thermoprobe polymer was similar between *Mfsd7c*^{-/-} and *Mfsd7c*^{fl/fl} BMDM (Figures 14A-14C), but the temperature sensitive fluorescence was significantly higher at several different incubation temperatures (Figure 14D-14F). We estimated that *Mfsd7c*^{-/-} BMDM were on average 4°C hotter than *Mfsd7c*^{fl/fl} BMDM (Figure 3F). These results show that loss of *Mfsd7c* in macrophages results in increased thermogenesis, as was the case with THP-1 7CKO cell lines.

The fact that heme treatment phenocopies the effect of *Mfsd7c* knockout suggests that heme may work by disrupting MFSD7C interactions with ETC components. To test this hypothesis, we performed co-IP between MFSD7C and the ETC components with or without treating the cells with 10 µM or 40 µM heme for one hour before cell lysis. As expected, CYC1, NDUFA4, COX4i1, ATP5h, and ATP5c1 co-precipitated with MFSD7C without heme treatment (Figures 2D,2L). With increasing level of heme treatment, less or very little ETC components co-precipitated with MFSD7C. In contrast, interaction between HMOX1 and MFSD7C was enhanced by heme treatment. Similarly, heme also disrupted interactions between endogenous MFSD7C and ATP5h but enhanced interactions between endogenous MFSD7C and HMOX1 in bone marrow-derived macrophages (Figure 1E). These results suggest that MFSD7C normally inhibits OCR and thermogenesis by interacting with ETC components and that heme stimulates OCR and thermogenesis by disrupting MFSD7C interactions with ETC components, thus phenocopying the effects of *Mfsd7c* knockout.

Example 5: N-terminal domain of MFSD7C mediates response to heme

To delineate the relationship between the heme-binding by the NTD *in vitro* and the effect of heme on OCR and thermogenesis *in vivo*, we complemented 7CKO clone 4B8 with either the full length MFSD7C (MFSD7C^{FL}) or MFSD7C lacking the first 80 amino acid residues of the NTD (MFSD7C^{ΔN}) to generate 4B8^{FL} and 4B8^{ΔN} cells, respectively (Figure 15A). Expression of the full length and truncated MFSD7C were confirmed, as well as their localization to mitochondria (Figures 15B,15C). Expression of MFSD7C^{FL} or MFSD7C^{ΔN} fully rescued *Mfsd7c* knockout phenotype as evident from decreased OCR, ECAR and FPT intensity, and increased MMP (Figures 4A-4F and Figures 15D,15E). Heme treatment of 4B8^{FL} cells phenocopied the parental THP-1 response to heme, including increased OCR, ECAR, FPT intensity but decreased MMP. In contrast, 4B8^{ΔN} cells showed little to no response to heme.

Furthermore, heme failed to disrupt the interactions between MFSD7C^{ΔN} and CYC1, NDUFA4, COX4i1, ATP5h and ATP5c1 (Figures 4F,4H). Notably, MFSD7C^{ΔN} did not co-precipitate with HMOX1 either with or without heme treatment, suggesting that the NTD is required for MFSD7C interaction with HMOX1. These results show that heme regulates mitochondrial respiration specifically through the NTD of MFSD7C *in vivo*, consistent with direct binding of the NTD to heme *in vitro*. The NTD is not required for MFSD7C interactions with ETC components, providing a molecular explanation for the restoration of mitochondrial respiration by MFSD7C^{ΔN} without restoring the response to heme.

Example 6: MFSD7C regulates thermogenesis by degradation of SERCA2b

How does *Mfsd7c* knockout or heme treatment induce thermogenesis? We noticed that SERCA2b (a.k.a. ATP2a2) was identified as a MFSD7C-interacting protein by IP-MS (Table 1). We validated the interaction by co-IP in 293T cells. In the absence of proteasome inhibitor MG132, a low level of SERCA2b was detected but none was co-precipitated with MFSD7C (Figure 5A). In the presence of MG132, a significantly higher level of SERCA2b was detected, and an appreciable level was co-precipitated with MFSD7C. The observed co-IP was abolished when cells were treated with heme for one hour. Similarly, co-IP of endogenous MFSD7C and SERCA2b was observed in bone marrow-derived wild-type but not *Mfsd7c*^{-/-} macrophages, and the co-IP was diminished following heme treatment (Figure 1E). Consistently, MFSD7C co-localized with SERCA2b with a Pearson's correlation coefficient value of 0.89, and this value was reduced to 0.67 following heme treatment (Figures 16A-16C). A fraction of MFSD7C was also co-localized with the ER marker at the mitochondrial-ER contact junction (Figures 16D,16E), a known contact point between the two organelles. These results suggest that MFSD7C interacts with SERCA2b and that this interaction is disrupted by heme.

Stabilization of SERCA2b by proteasome inhibitor MG132 suggests that MFSD7C may promote SERCA2b degradation through ubiquitination and subsequent proteasomal degradation. To test this hypothesis, 293T cells were transfected with FLAG-tagged SERCA2b and treated with MG132 for either 6 or 12 hours, lysed, immunoprecipitated with anti-FLAG, and Western blotted with anti-MFSD7C, anti-SERCA2b and anti-ubiquitin antibodies. With longer MG132 treatment, more full-length and ubiquitinated SERCA2b was precipitated (Figure 5B). Furthermore, SERCA2b level was significantly higher in THP-1 cells following heme treatment and in 7CKO cells than in parental THP-1 cells (Figure 5C). Expression of both MFSD7C^{FL} and MFSD7C^{ΔN} in 4B8 cells led to a reduction of SERCA2b, which was restored by heme treatment in 4B8^{FL} but not 4B8^{ΔN} cells (Figure 5D). These results show that

interactions between MFSD7C and SERCA2b leads to ubiquitin-mediated degradation of SERCA2b, and heme disrupts the interaction and therefore stabilizes SERCA2b.

To investigate the role of SERCA2b in MFSD7C/heme-regulated thermogenesis, we tested if heme-stimulated thermogenesis is inhibited by thapsigargin, a known SERCA2b inhibitor. Indeed, heme-stimulated thermogenesis in THP-1 cells was mostly inhibited by thapsigargin (Figure 5E and Figure 16F). Consistently, thermogenesis of *Serca2b*^{-/-} THP-1 cells was significantly diminished following heme treatment (Figure 5F). These results support a critical role for SERCA2b in mediating MFSD7C- and heme-regulated thermogenesis.

INCORPORATION BY REFERENCE

All publications and patents mentioned herein are hereby incorporated by reference in their entirety as if each individual publication or patent was specifically and individually indicated to be incorporated by reference. In case of conflict, the present application, including any definitions herein, will control.

EQUIVALENTS

While specific embodiments of the subject invention have been discussed, the above specification is illustrative and not restrictive. Many variations of the invention will become apparent to those skilled in the art upon review of this specification and the claims below. The full scope of the invention should be determined by reference to the claims, along with their full scope of equivalents, and the specification, along with such variations.

CLAIMS

We claim:

1. A method of treating an obesity-related disease, comprising administering to a subject in need thereof an effective amount of an inhibitor of MFSD7C or any one of its partners in Figure 17.
2. The method of claim 1, wherein the inhibitor of MFSD7C inhibits binding of MFSD7C or any one of its partners in Figure 17 to electron transport chain (ETC) components.
3. The method of claim 2, wherein the ETC component is mitochondrial complex III, IV, or V.
4. The method of claim 1, wherein the inhibitor of MFSD7C inhibits binding of MFSD7C or any one of its partners in Figure 17 to SERCA2b.
5. The method of any one of claims 1-4, wherein the inhibitor of MFSD7C or any one of its partners in Figure 17 results in uncoupled mitochondrial respiration.
6. The method of any one of claims 1-5, wherein the inhibitor of MFSD7C or any one of its partners in Figure 17 increases oxygen consumption rate and thermogenesis.
7. The method of any one of claims 1-6, wherein the inhibitor of MFSD7C or any one of its partners in Figure 17 decreases mitochondrial membrane potential (MMP) and cellular ATP level.
8. The method of any one of claims 1-7, wherein the inhibitor of MFSD7C is heme.
9. The method of any one of claims 1-7, wherein the inhibitor of MFSD7C is siRNA.
10. The method of any one of claims 1-7, wherein the inhibitor of MFSD7C is a CRISPR based inhibitor.
11. The method of claim 10, wherein the CRISPR based inhibitor comprises MFSD7C gRNA.
12. The method of claim 11, wherein the gRNA comprises the sequence of any one of SEQ ID NOs: 1-3.
13. The method of any one of claims 1-12, wherein any one of its partners in Figure 17 is Mfsd7c, Hmox1, Tfrc, CYC1, NDUFA4, COX4I1, Atp5h, Atp5c1, Slc25a4, Slc25a5,

Atp2a2, Elovl1, Mthfd1l, Cds2, Asph, Dnaja1, Immt, TIM50, Afg3l2, Phb, Dnaja3, Tmx3, Rdh13, Sqrdl, Tspo, Fcgr1g, Fcgr3, Fcgr1, or Itgb2.

14. The method of any one of claims 1-13, wherein the obesity-related disease is obesity, atherosclerosis, hypertension, diabetes, type 2 diabetes, impaired glucose tolerance, dyslipidemia, coronary heart disease, gallbladder disease, osteoarthritis, or cancer.

15. The method of claim 14, wherein the cancer is endometrial cancer, breast cancer, prostate cancer, or colon cancer.

16. A method of treating an obesity-related disease, comprising administering to a subject in need thereof an effective amount of an activator of SERCA2b.

17. The method of claim 16, wherein the activator of SERCA2b inhibits binding of MFSD7C to SERCA2b.

18. The method of any one of claims 16-17, wherein the activator of SERCA2b results in uncoupled mitochondrial respiration.

19. The method of any one of claims 16-18, wherein the activator of SERCA2b increases oxygen consumption rate and thermogenesis.

20. The method of any one of claims 16-19, wherein the activator of SERCA2b decreases mitochondrial membrane potential (MMP) and cellular ATP level.

21. The method of any one of claims 16-20, wherein the activator of SERCA2b is heme.

22. The method of any one of claims 16-21, wherein the obesity-related disease is obesity, atherosclerosis, hypertension, diabetes, type 2 diabetes, impaired glucose tolerance, dyslipidemia, coronary heart disease, gallbladder disease, osteoarthritis, or cancer.

23. The method of claim 22, wherein the cancer is endometrial cancer, breast cancer, prostate cancer, or colon cancer.

24. A method of identifying an inhibitor of MFSD7C, comprising:

contacting a cell with a candidate agent;

measuring MFSD7C activity in the cell contacted with the candidate agent; and

optionally comparing the cell's MFSD7C activity in the presence of the candidate agent with the cell's MFSD7C activity in the absence of the candidate agent, wherein a decrease in MFSD7C activity in the presence of the candidate agent is indicative of inhibition of MFSD7C.

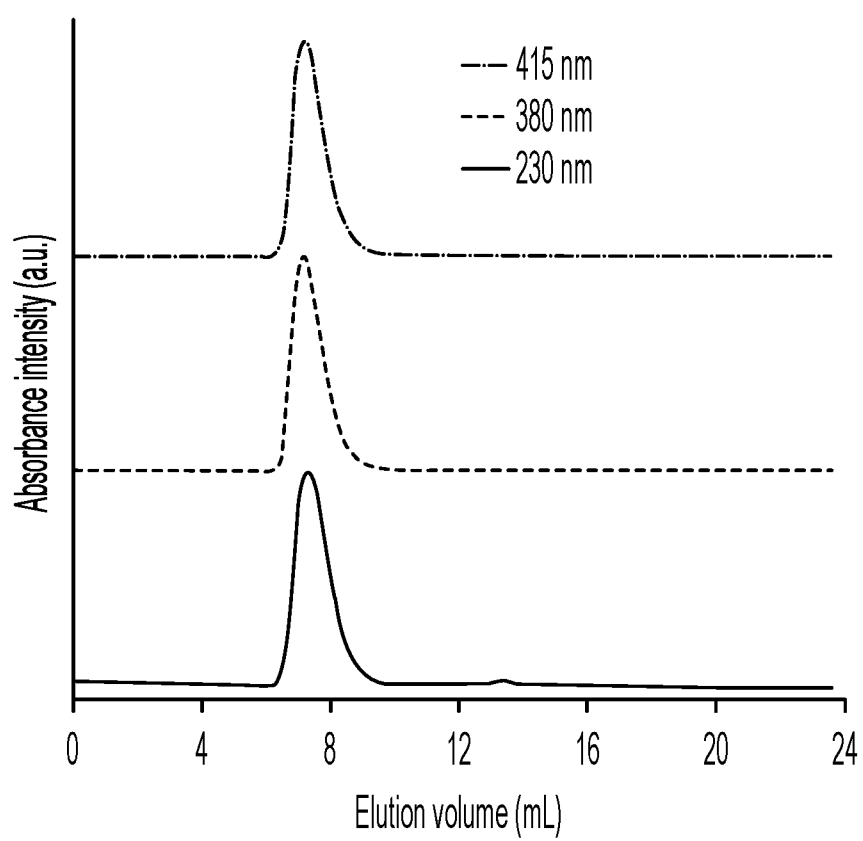
25. The method of claim 24, wherein the inhibitor of MFSD7C inhibits binding of MFSD7C to electron transport chain (ETC) components.
26. The method of claim 25, wherein the ETC component is mitochondrial complex III, IV, or V.
27. The method of claim 25, wherein the inhibitor of MFSD7C inhibits binding of MFSD7C to SERCA2b.
28. The method of any one of claims 24-27, wherein the inhibitor of MFSD7C results in uncoupled mitochondrial respiration.
29. The method of any one of claims 24-28, wherein the inhibitor of MFSD7C increases oxygen consumption rate and thermogenesis.
30. The method of any one of claims 24-29, wherein the inhibitor of MFSD7C decreases mitochondrial membrane potential (MMP) and cellular ATP level.
31. The method of any one of claims 24-30, wherein MFSD7C activity is measured using an ATP assay, a luciferase-based assay, a fluorescent-based assay, a β -galactosidase assay, flow cytometry, or mitochondrial membrane potential assay.
32. The method of any of claims 24-30, wherein MFSD7C activity is measured by a method selected from the group consisting of Western blotting, ELISA, and radioimmunoassay (RIA).
33. A method of identifying an activator of SERCA2b, comprising:
contacting a cell with a candidate agent;
measuring SERCA2b activity in the cell contacted with the candidate agent; and
optionally comparing the cell's SERCA2b activity in the presence of the candidate agent with the cell's SERCA2b activity in the absence of the candidate agent, wherein an increase in SERCA2b activity in the presence of the candidate agent is indicative of activation of SERCA2b.
34. The method of claim 33, wherein the activator of SERCA2b inhibits binding of MFSD7C to SERCA2b.
35. The method of any one of claims 33-34, wherein the activator of SERCA2b results in uncoupled mitochondrial respiration.

36. The method of any one of claims 33-35, wherein the activator of SERCA2b increases oxygen consumption rate and thermogenesis.
37. The method of any one of claims 33-36, wherein the activator of SERCA2b decreases mitochondrial membrane potential (MMP) and cellular ATP level.
38. The method of any one of claims 33-37, wherein SERCA2b activity is measured using an ATP assay, a luciferase-based assay, a fluorescent-based assay, a β -galactosidase assay, flow cytometry, or a mitochondrial membrane potential assay.
39. The method of any of claims 33-38, wherein SERCA2b activity is measured by a method selected from the group consisting of Western blotting, ELISA, and radioimmunoassay (RIA).
40. A method of promoting weight gain comprising administering to a subject in need thereof an effective amount of an activator of MFSD7C or any one of its partners in Figure 17.
41. The method of claim 40, wherein the activator of MFSD7C promotes binding of MFSD7C or any one of its partners in Figure 17 to electron transport chain (ETC) components.
42. The method of claim 41, wherein the ETC component is mitochondrial complex III, IV, or V.
43. The method of claim 40, wherein the activator of MFSD7C promotes binding of MFSD7C or any one of its partners in Figure 17 to SERCA2b.
44. The method of any one of claims 40-43, wherein the activator of MFSD7C or any one of its partners in Figure 17 results in coupled mitochondrial respiration.
45. The method of any one of claims 40-44, wherein the activator of MFSD7C or any one of its partners in Figure 17 decreases oxygen consumption rate and thermogenesis.
46. The method of any one of claims 40-45, wherein the activator of MFSD7C or any one of its partners in Figure 17 increases mitochondrial membrane potential (MMP) and cellular ATP level.
47. The method of any one of claims 40-46, wherein the activator of MFSD7C is a CRISPR based activator.

48. The method of any one of claims 40-47, wherein any one of its partners in Figure 17 is MfSD7c, Hmox1, Tfrc, CYC1, NDUFA4, COX4I1, Atp5h, Atp5c1, Slc25a4, Slc25a5, Atp2a2, Elovl1, Mthfd1l, Cds2, Asph, Dnaja1, Immt, TIM50, Afg3l2, Phb, Dnaja3, Tmx3, Rdh13, Sqrdl, Tspo, Fcgr1g, Fcgr3, Fcgr1, or Itgb2.
49. The method of any one of claims 40-48, wherein the subject is a human or livestock.
50. The method of claim 49, wherein the livestock is pig, cattle, chicken, turkey, lamb, or fish.
51. A method of promoting weight gain, comprising administering to a subject in need thereof an effective amount of an inhibitor of SERCA2b.
52. The method of claim 51, wherein the inhibitor of SERCA2b promotes binding of MFSD7C to SERCA2b.
53. The method of any one of claims 51-52, wherein the inhibitor of SERCA2b results in coupled mitochondrial respiration.
54. The method of any one of claims 51-53, wherein the inhibitor of SERCA2b decreases oxygen consumption rate and thermogenesis.
55. The method of any one of claims 51-54, wherein the inhibitor of SERCA2b increases mitochondrial membrane potential (MMP) and cellular ATP level.
56. The method of any one of claims 51-55, wherein the inhibitor of SERCA2b is a CRISPR based inhibitor.
57. The method of any one of claims 51-55, wherein the inhibitor of SERCA2b is siRNA.
58. The method of any one of claims 51-57, wherein the subject is a human or livestock.
59. The method of claim 58, wherein the livestock is pig, cattle, chicken, turkey, lamb, or fish.
60. A method of identifying an activator of MFSD7C, comprising:
contacting a cell with a candidate agent;
measuring MFSD7C activity in the cell contacted with the candidate agent; and
optionally comparing the cell's MFSD7C activity in the presence of the candidate agent with the cell's MFSD7C activity in the absence of the candidate agent, wherein an increase in MFSD7C activity in the presence of the candidate agent is indicative of activation of MFSD7C.

61. The method of claim 60, wherein the activator of MFSD7C promotes binding of MFSD7C to electron transport chain (ETC) components.
62. The method of claim 61, wherein the ETC component is mitochondrial complex III, IV, or V.
63. The method of claim 61, wherein the activator of MFSD7C promotes binding of MFSD7C to SERCA2b.
64. The method of any one of claims 60-63, wherein the activator of MFSD7C results in coupled mitochondrial respiration.
65. The method of any one of claims 60-64, wherein the activator of MFSD7C decreases oxygen consumption rate and thermogenesis.
66. The method of any one of claims 60-65, wherein the activator of MFSD7C increases mitochondrial membrane potential (MMP) and cellular ATP level.
67. The method of any one of claims 60-66, wherein MFSD7C activity is measured using an ATP assay, a luciferase-based assay, a fluorescent-based assay, a β -galactosidase assay, flow cytometry, or mitochondrial membrane potential assay.
68. The method of any of claims 60-66, wherein MFSD7C activity is measured by a method selected from the group consisting of Western blotting, ELISA, and radioimmunoassay (RIA).
69. A method of identifying an inhibitor of SERCA2b, comprising:
contacting a cell with a candidate agent;
measuring SERCA2b activity in the cell contacted with the candidate agent; and
optionally comparing the cell's SERCA2b activity in the presence of the candidate agent with the cell's SERCA2b activity in the absence of the candidate agent, wherein a decrease in SERCA2b activity in the presence of the candidate agent is indicative of inhibition of SERCA2b.
70. The method of claim 6, wherein the inhibitor of SERCA2b promotes binding of MFSD7C to SERCA2b.
71. The method of any one of claims 69-70, wherein the inhibitor of SERCA2b results in coupled mitochondrial respiration.

72. The method of any one of claims 69-71, wherein the inhibitor of SERCA2b decreases oxygen consumption rate and thermogenesis.
73. The method of any one of claims 69-72, wherein the inhibitor of SERCA2b increases mitochondrial membrane potential (MMP) and cellular ATP level.
74. The method of any one of claims 69-73, wherein SERCA2b activity is measured using an ATP assay, a luciferase-based assay, a fluorescent-based assay, a β -galactosidase assay, flow cytometry, or a mitochondrial membrane potential assay.
75. The method of any of claims 69-74, wherein SERCA2b activity is measured by a method selected from the group consisting of Western blotting, ELISA, and radioimmunoassay (RIA).

**Figure 1A**

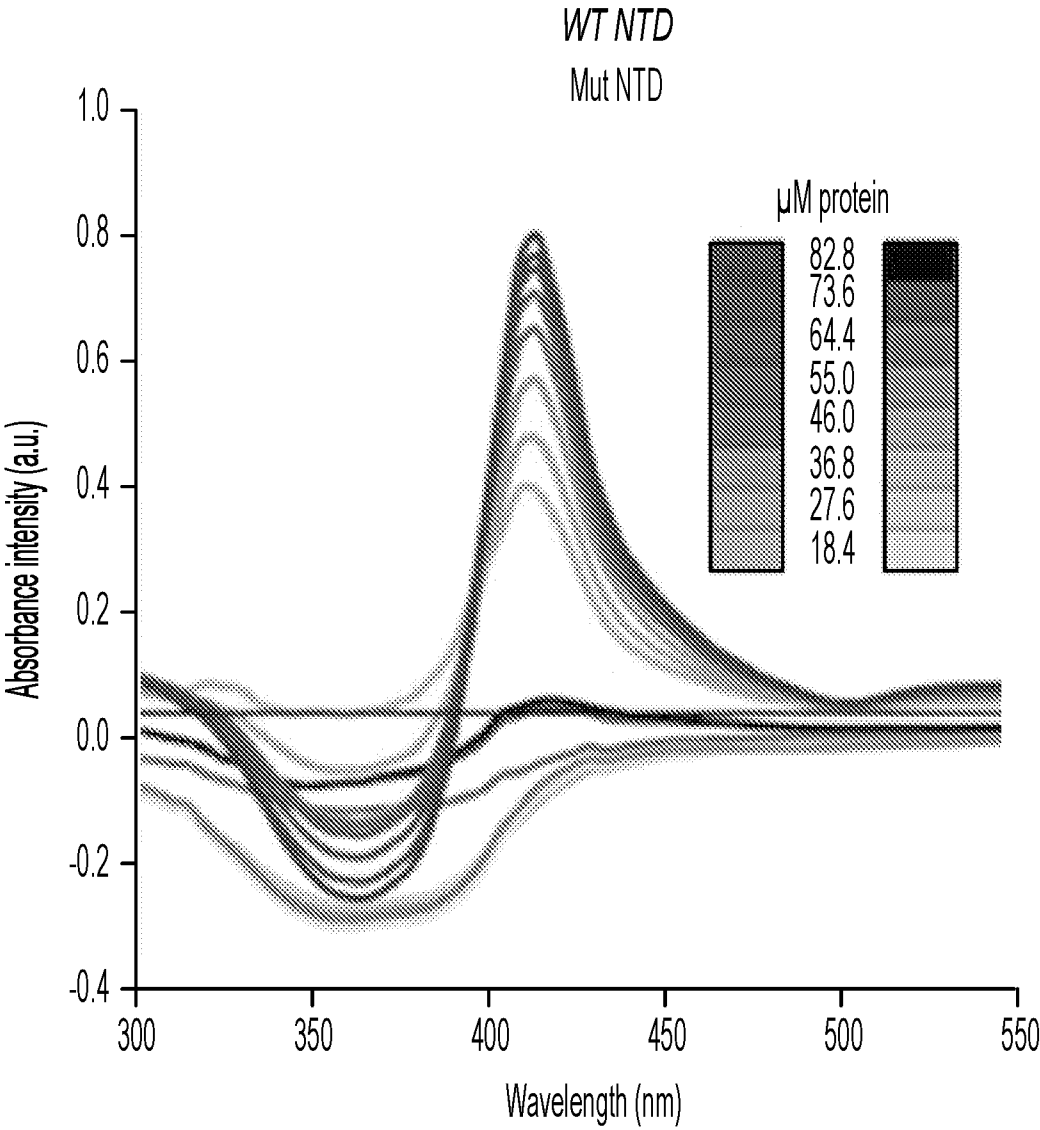
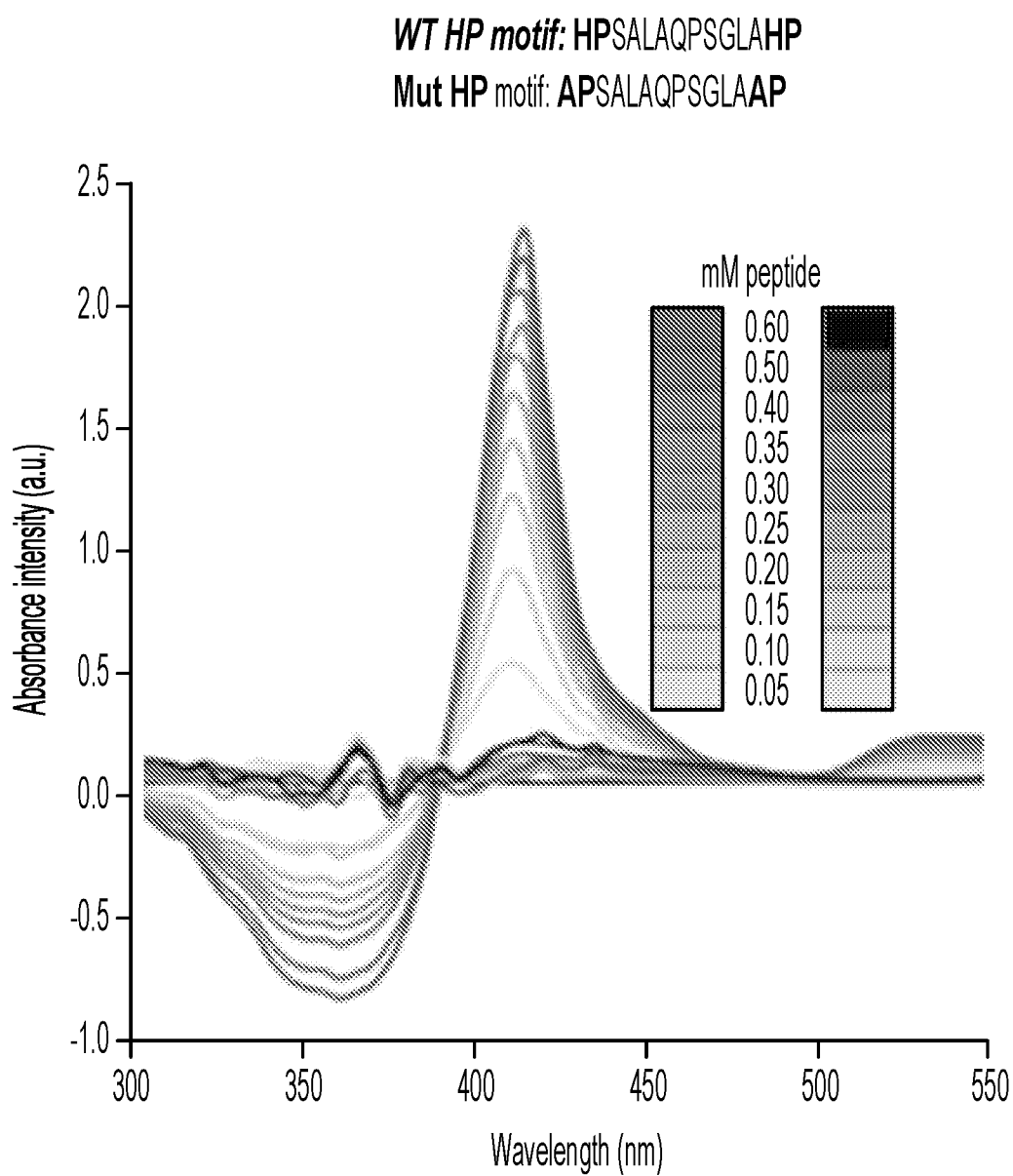


Figure 1B



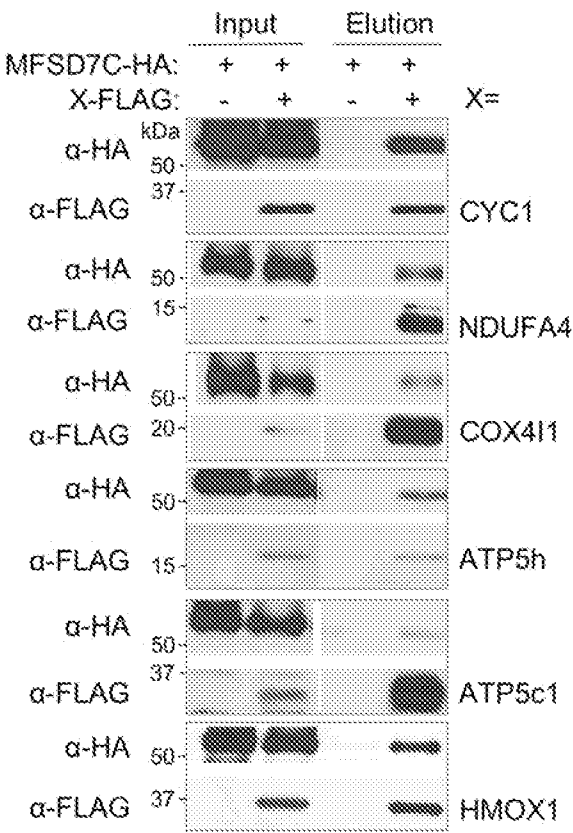
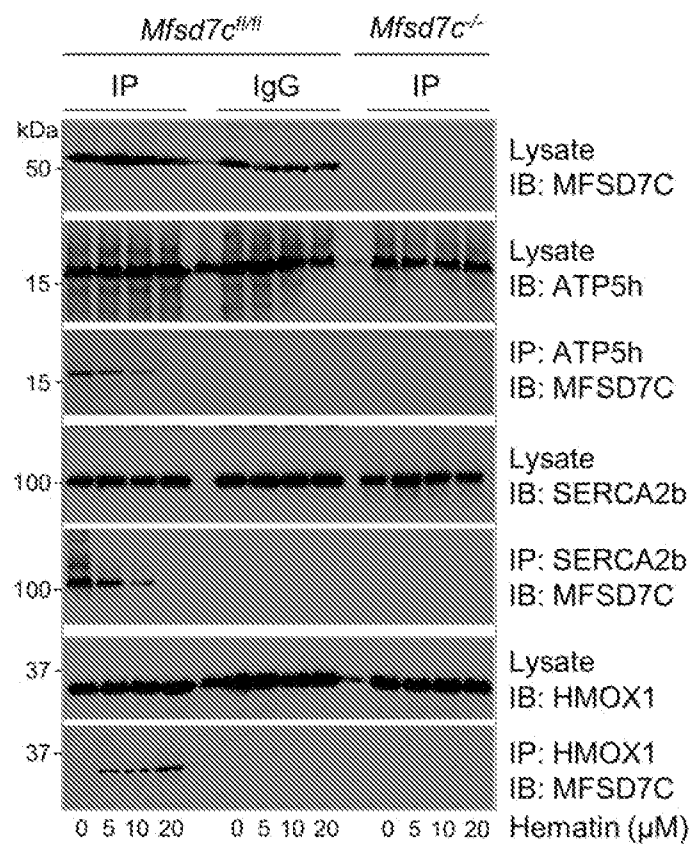


Figure 1D

**Figure 1E**

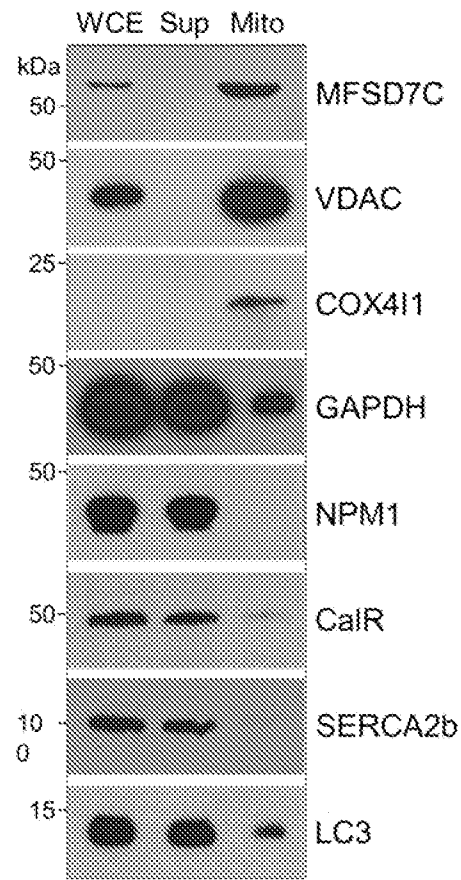


Figure 1F

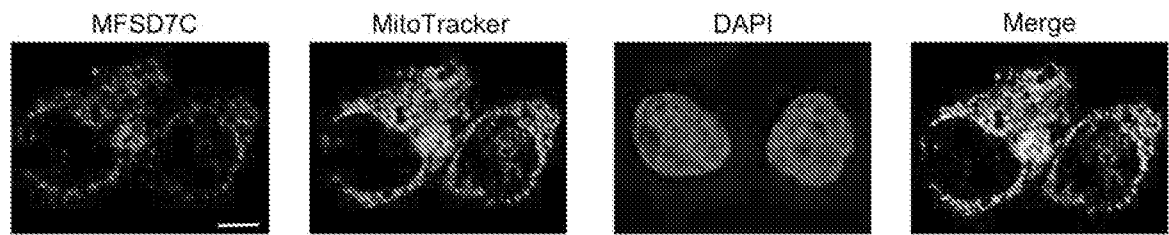
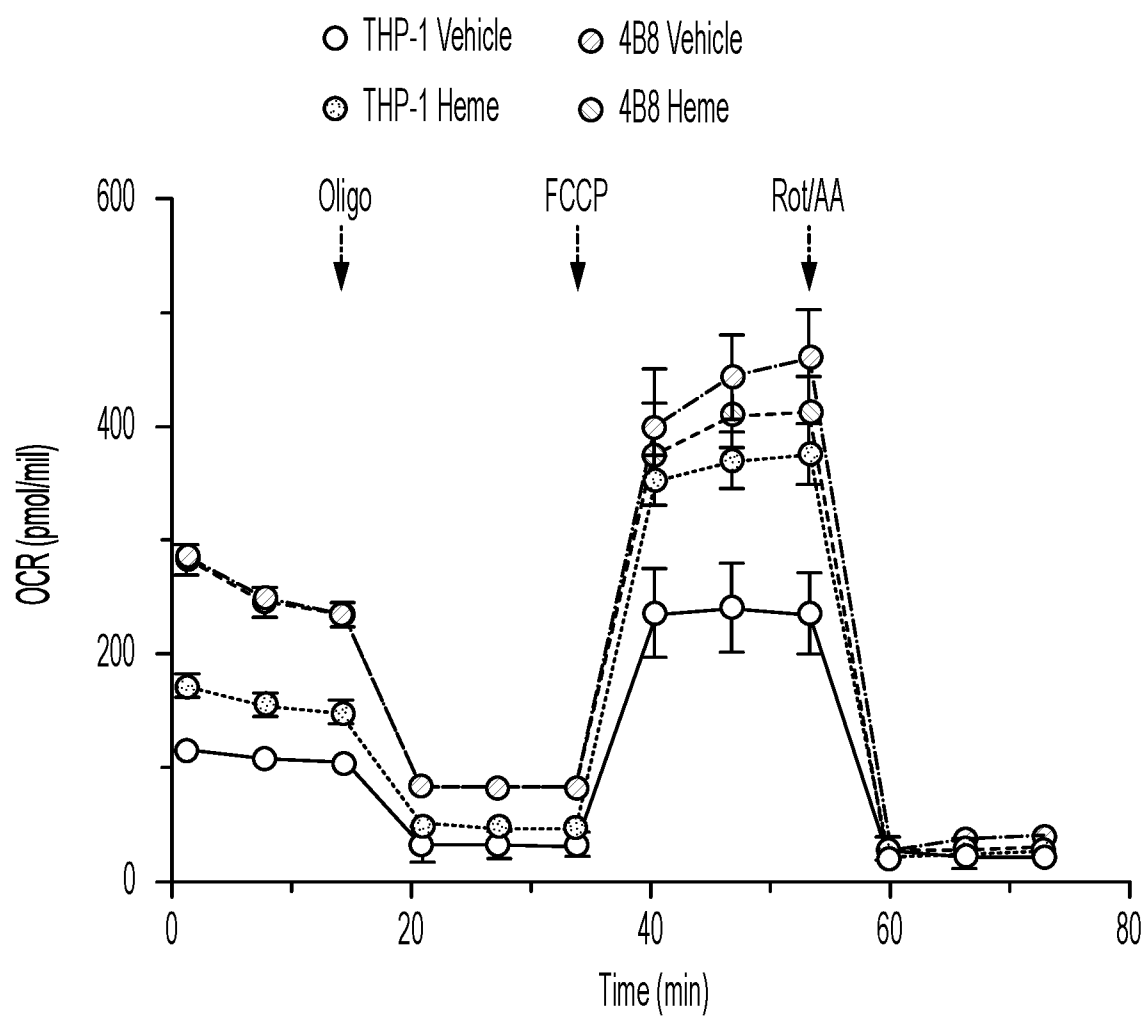
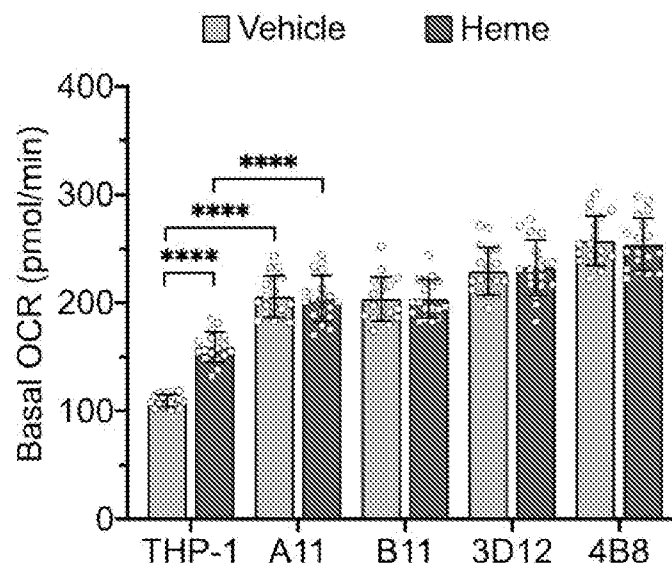


Figure 1G

**Figure 2A**

**Figure 2B**

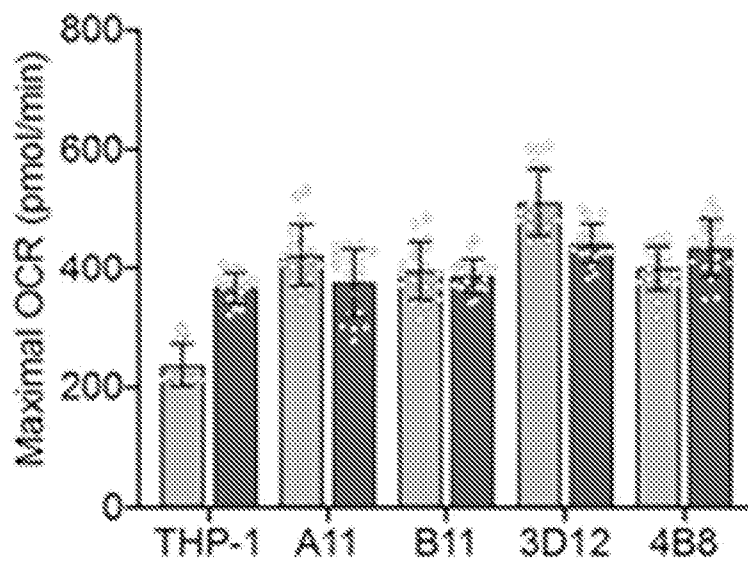


Figure 2C

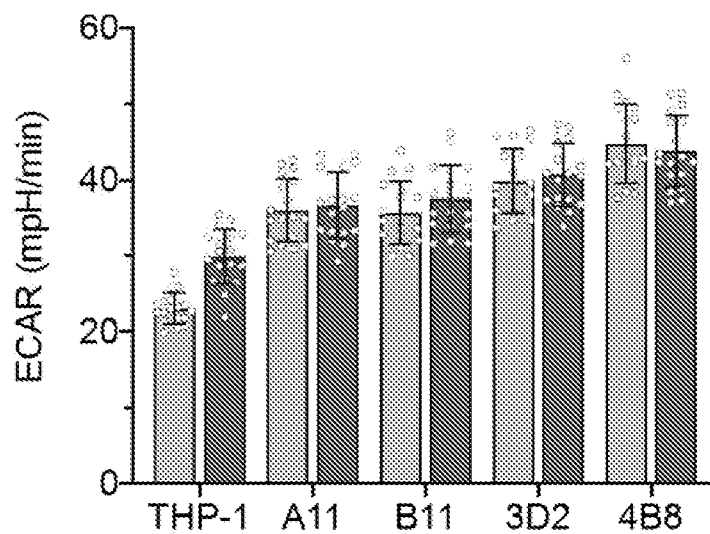


Figure 2D

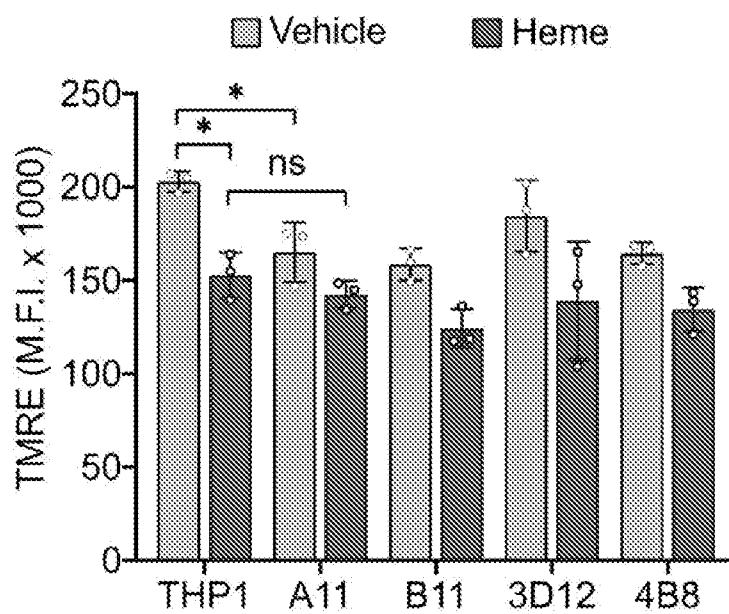


Figure 2E

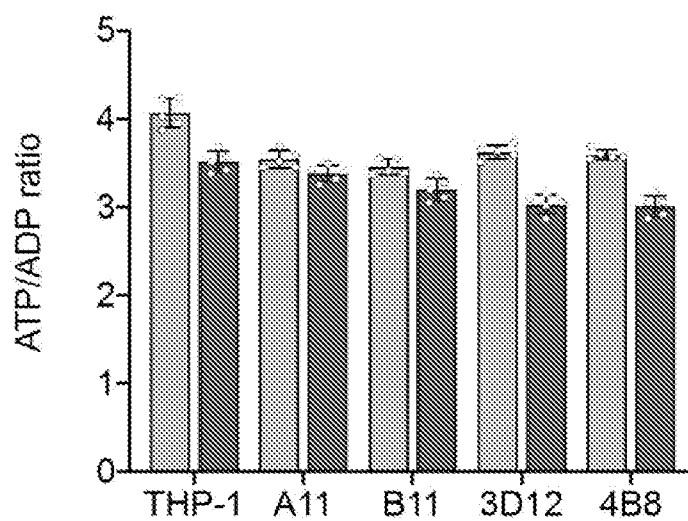
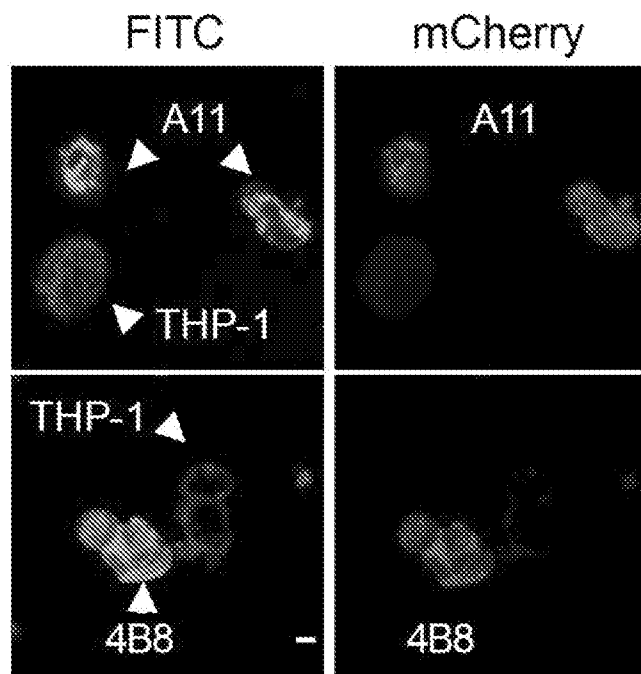
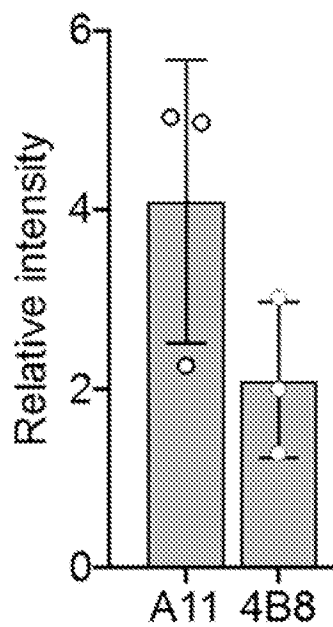
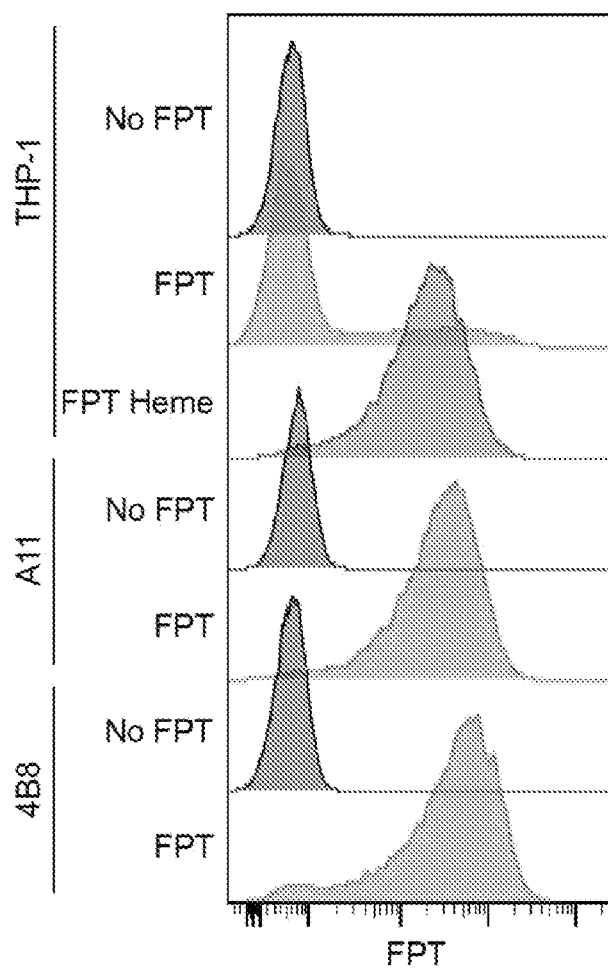
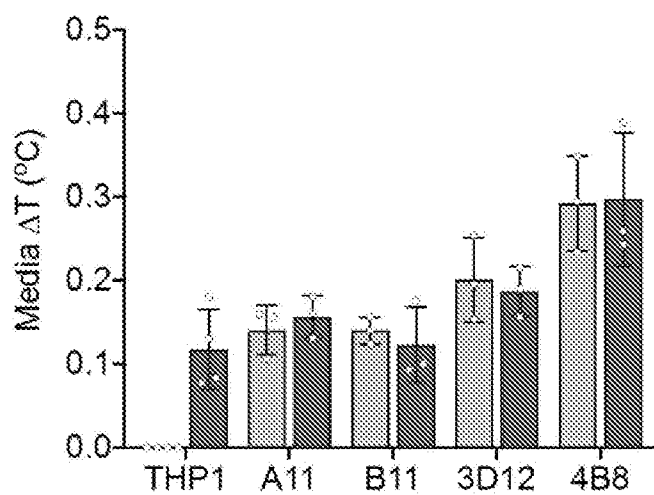


Figure 2F

**Figure 2G****Figure 2H**

**Figure 2I****Figure 2J**

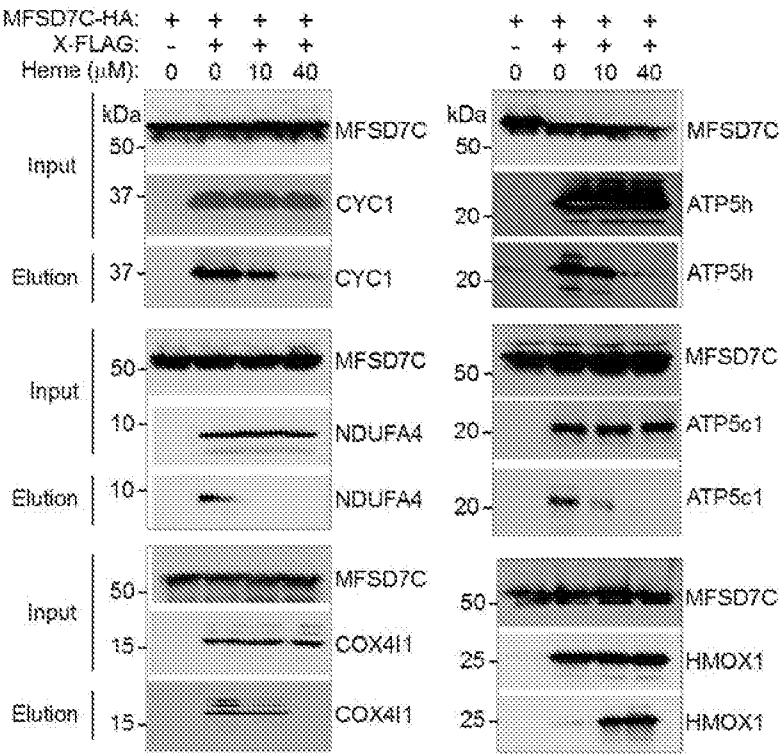


Figure 2K

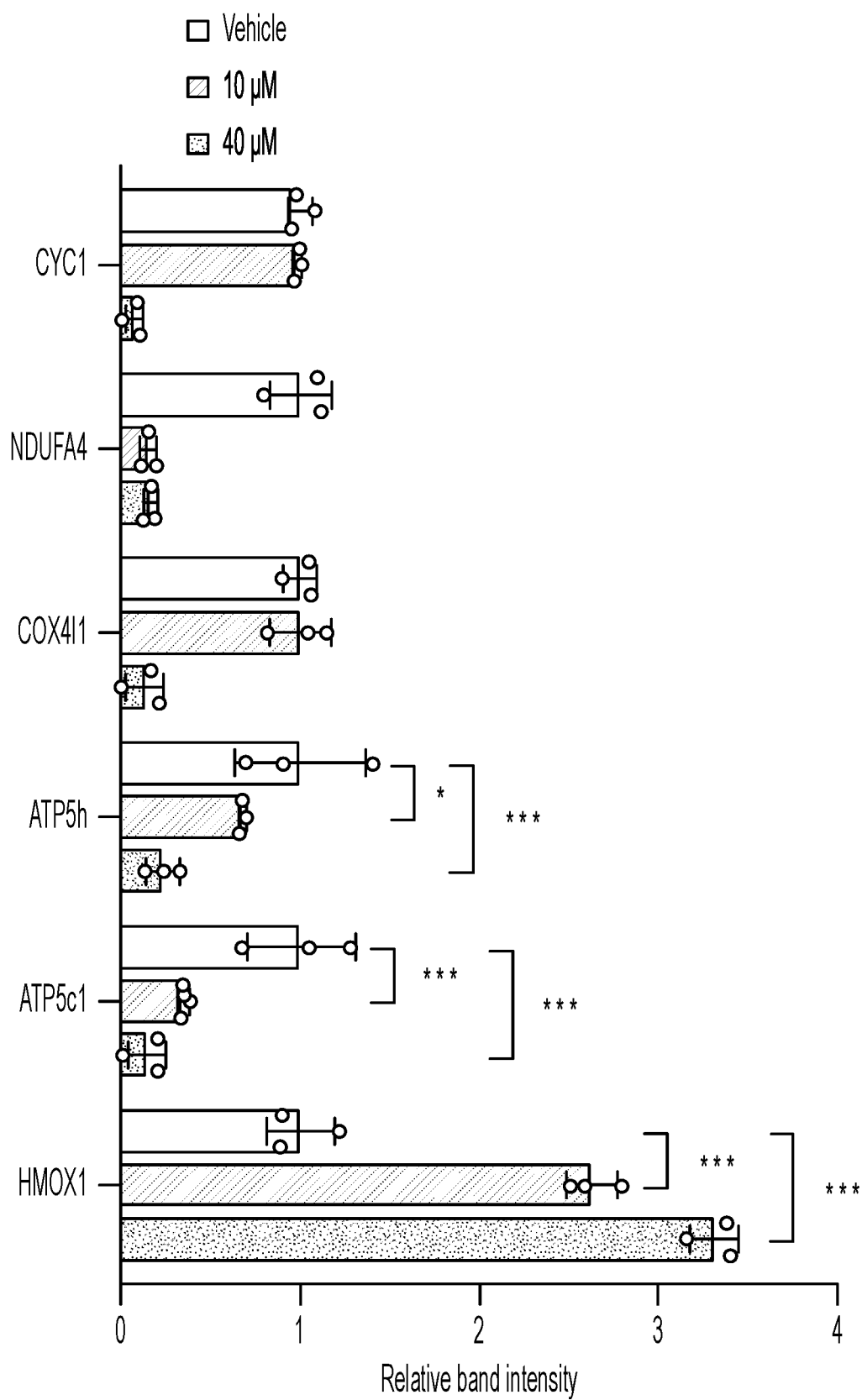
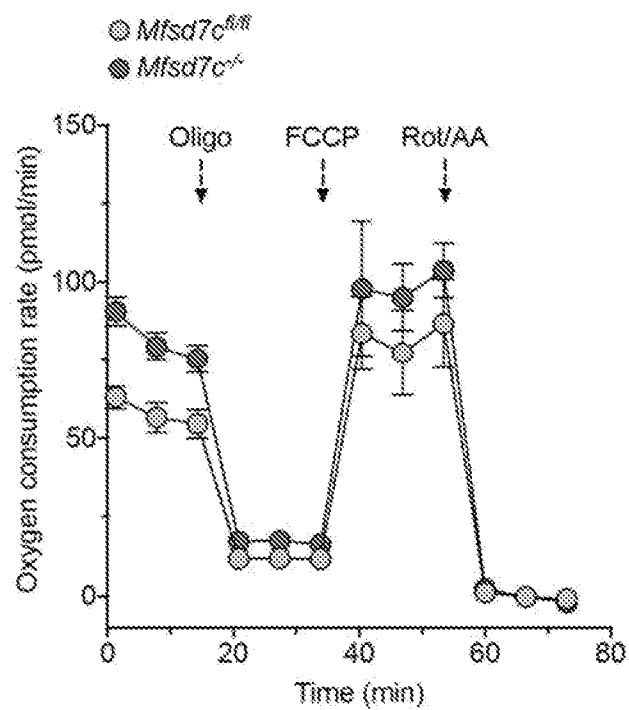
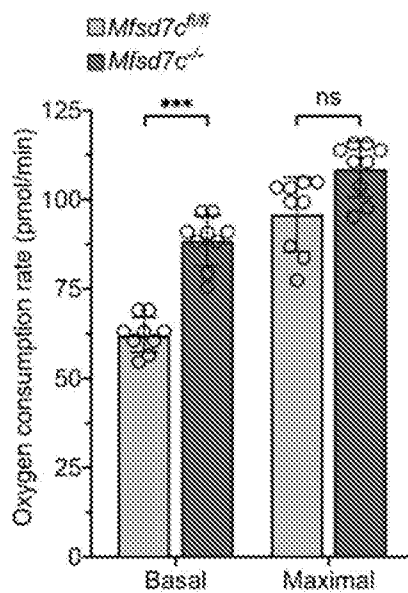
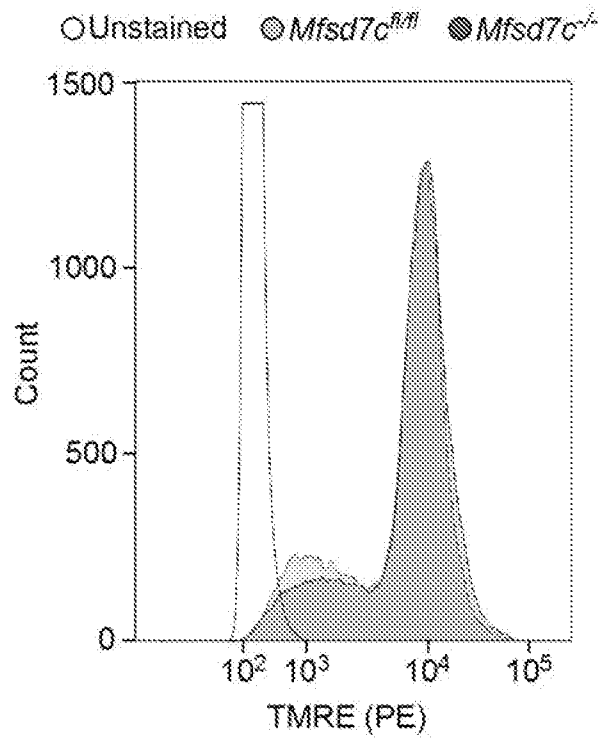
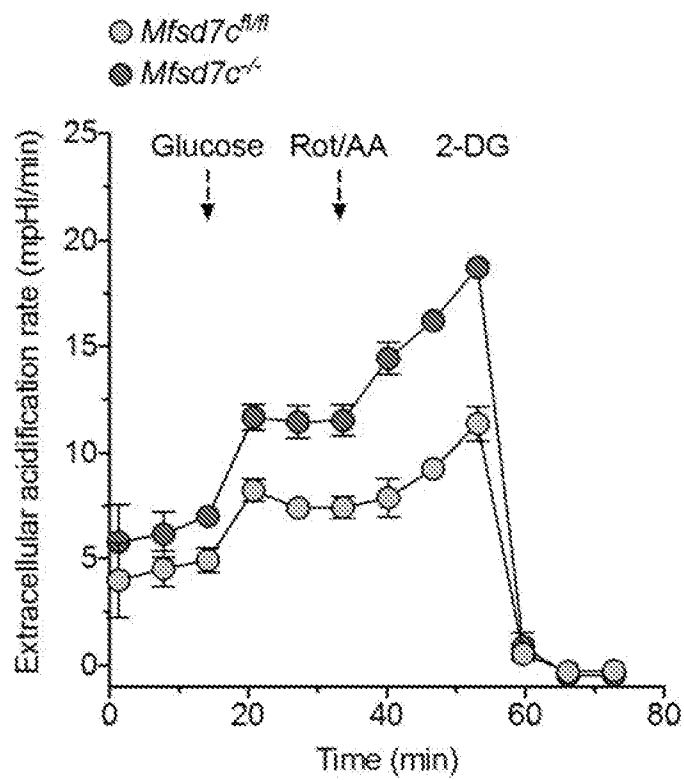


Figure 2L

**Figure 3A****Figure 3B**

**Figure 3C****Figure 3D**

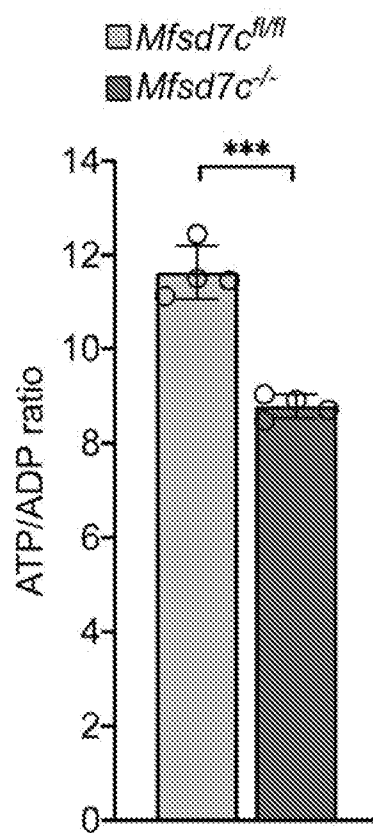
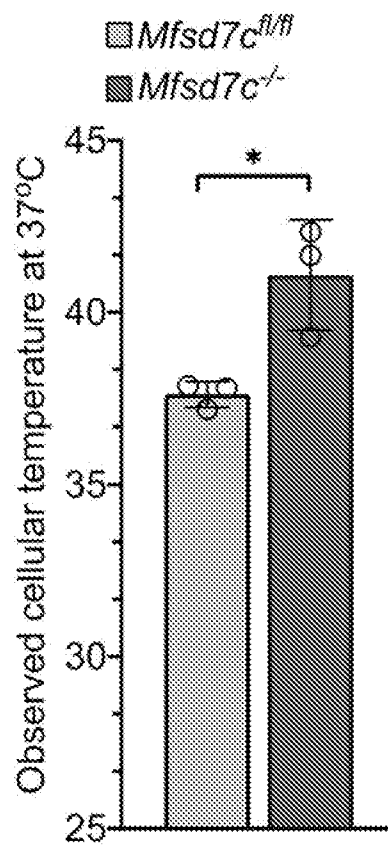


Figure 3E

**Figure 3F**

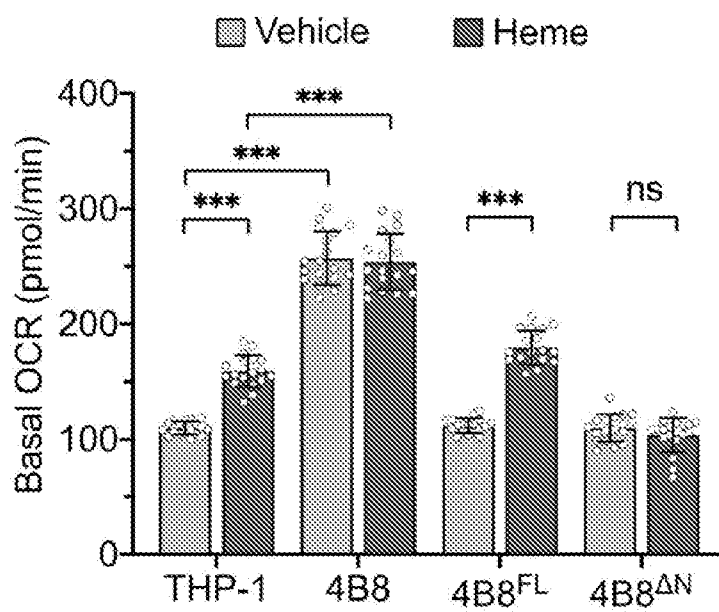


Figure 4A

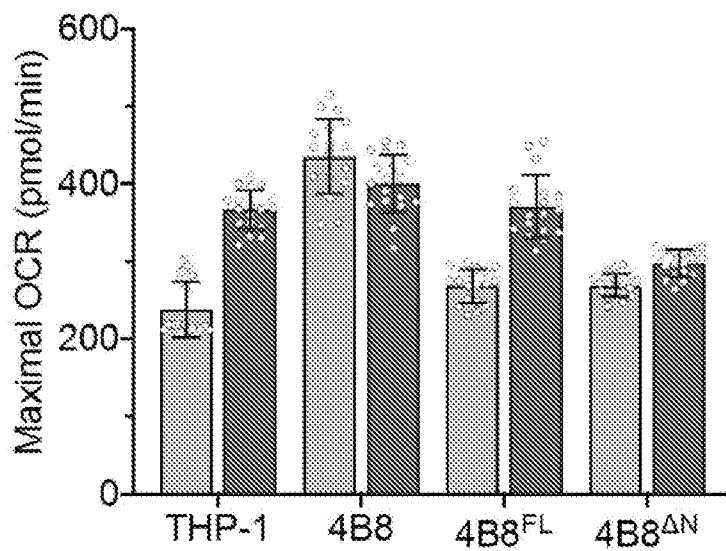


Figure 4B

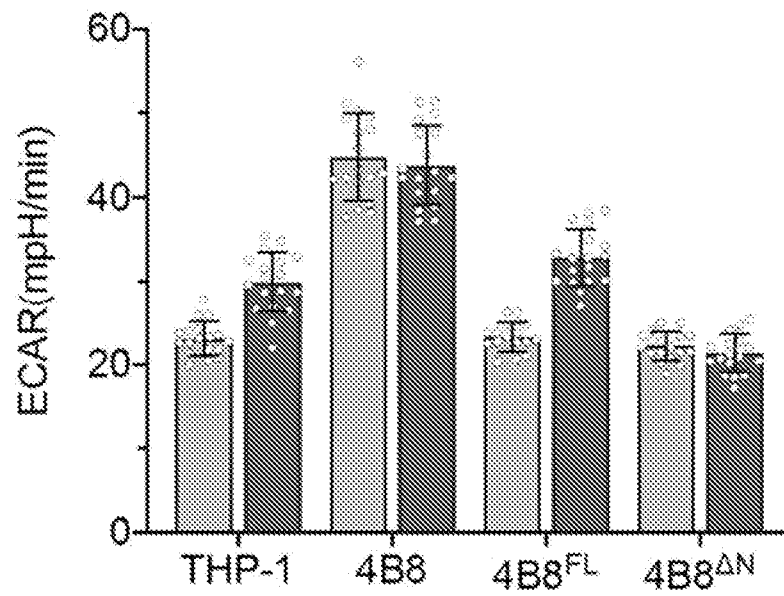


Figure 4C

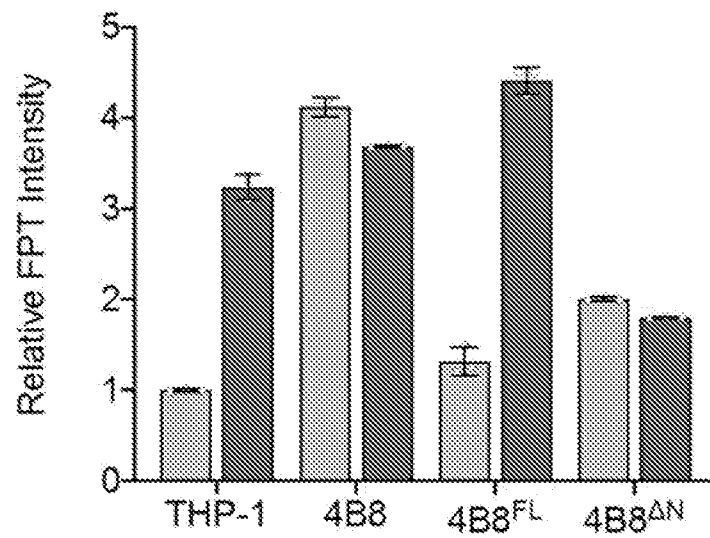


Figure 4D

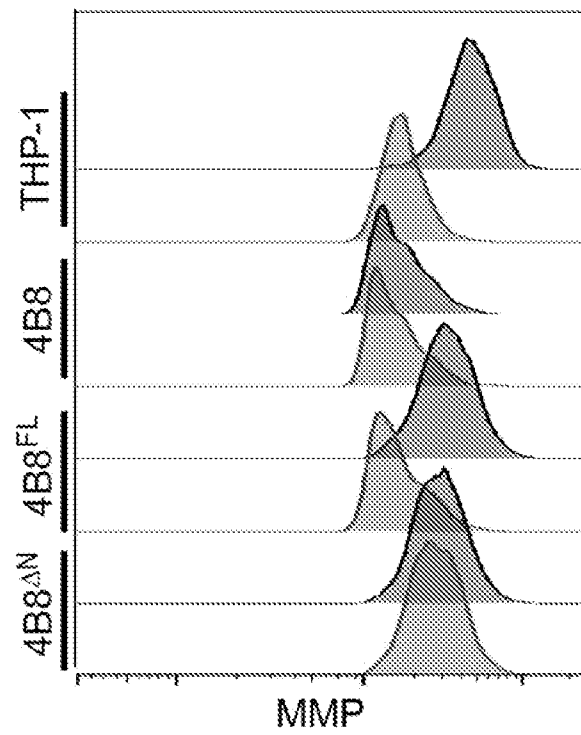


Figure 4E

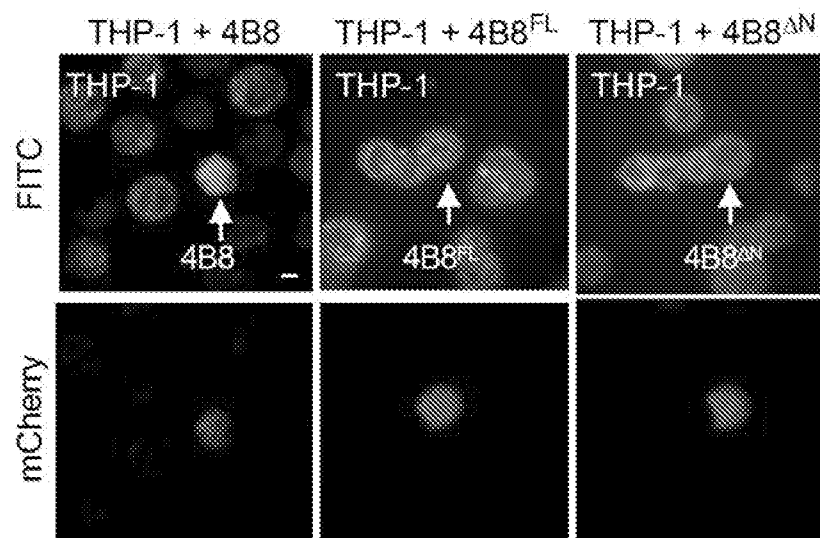


Figure 4F

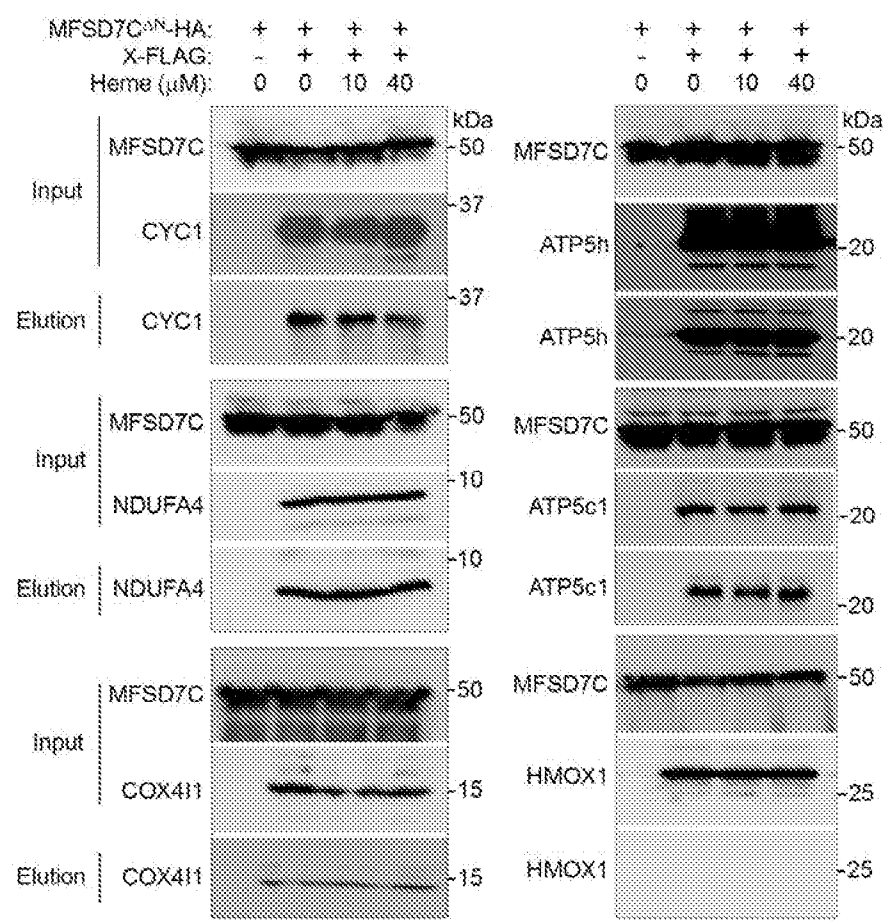


Figure 4G

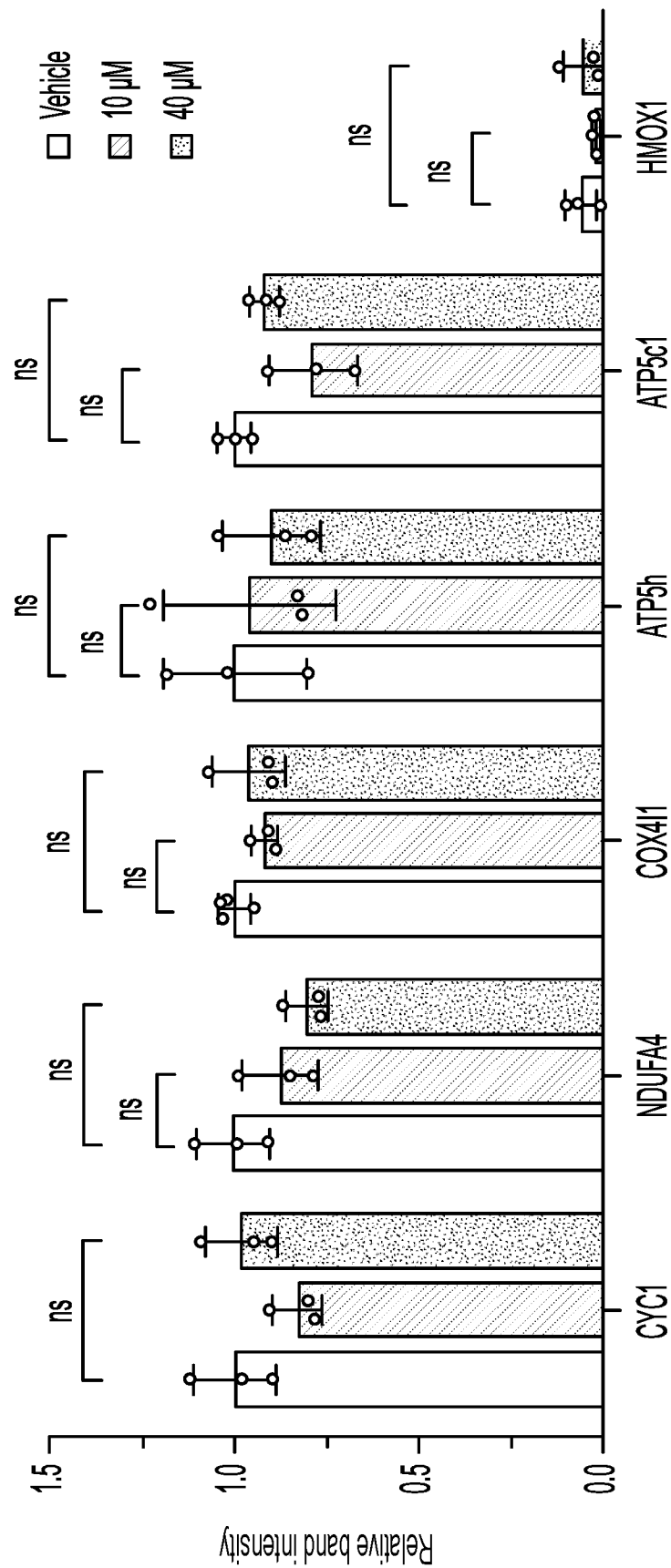
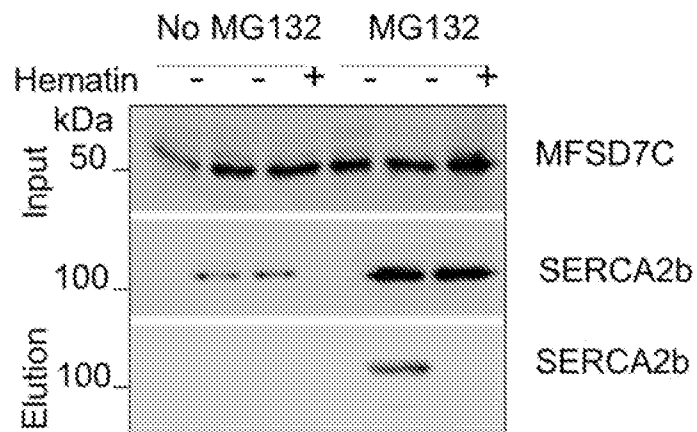
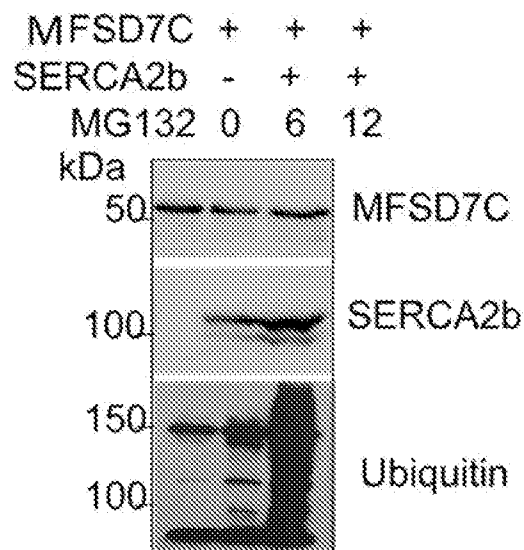


Figure 4H

**Figure 5A****Figure 5B**

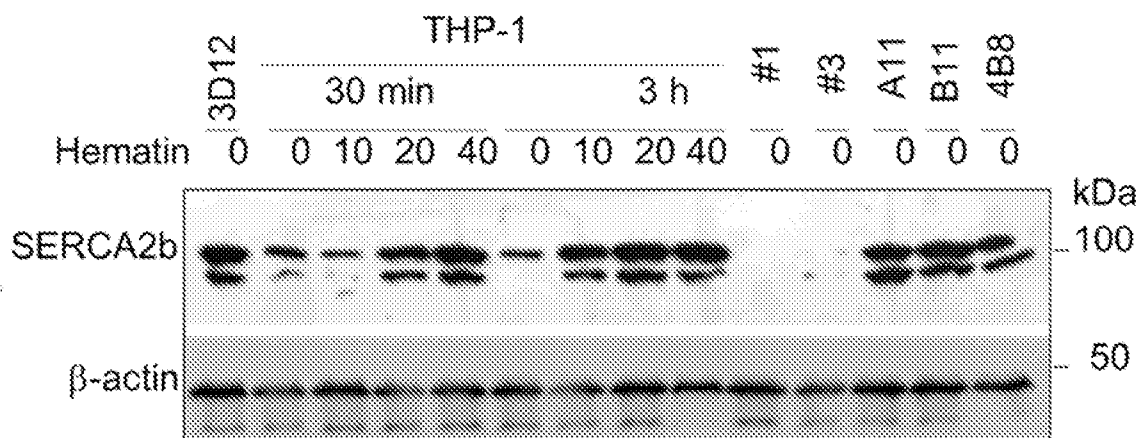


Figure 5C

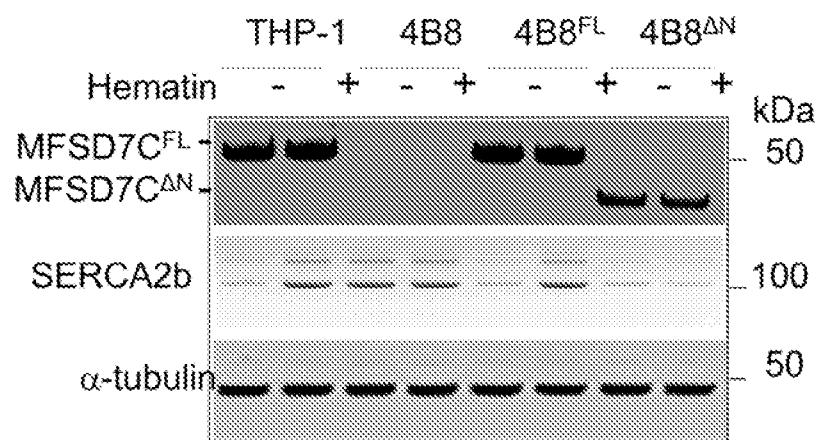
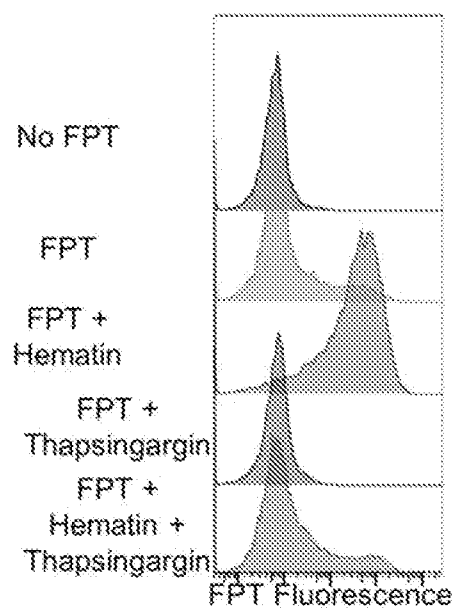
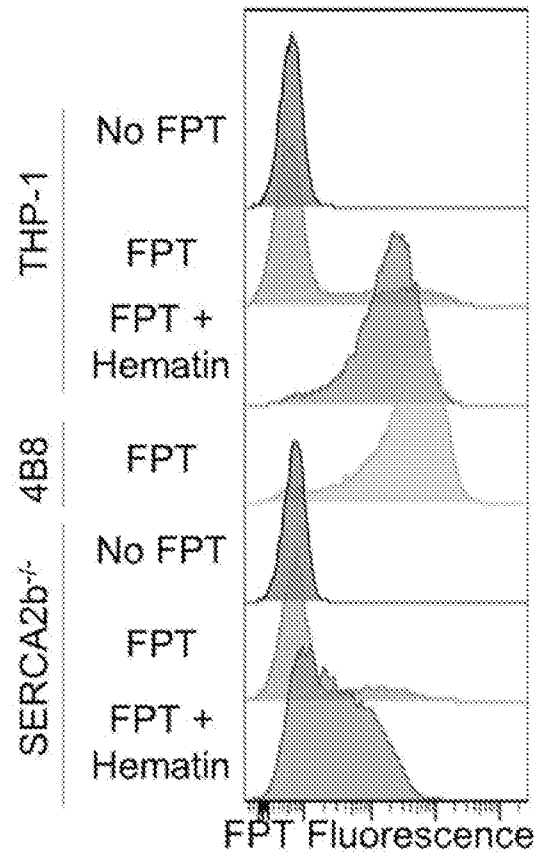


Figure 5D

**Figure 5E**

**Figure 5F**

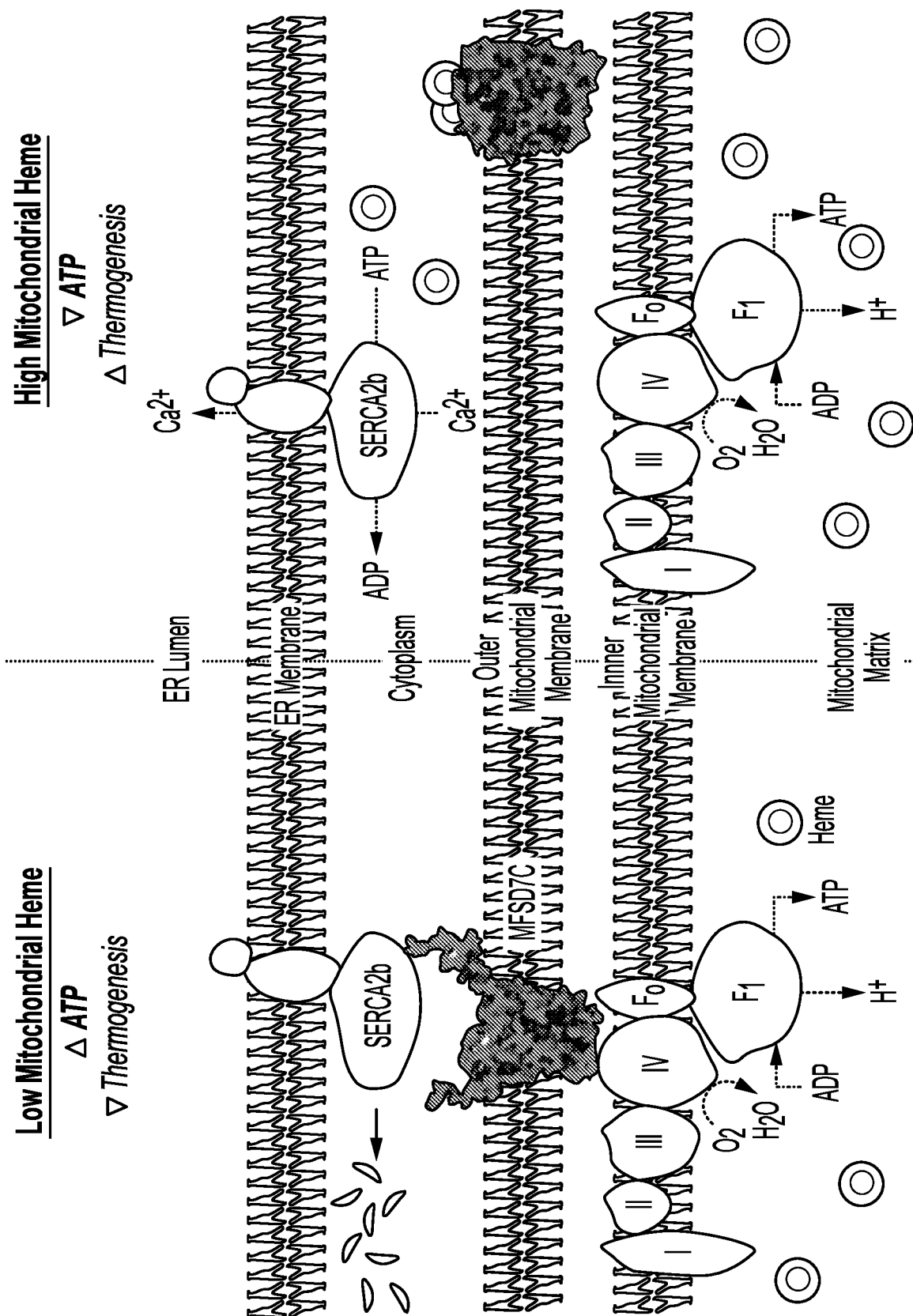


Figure 6A

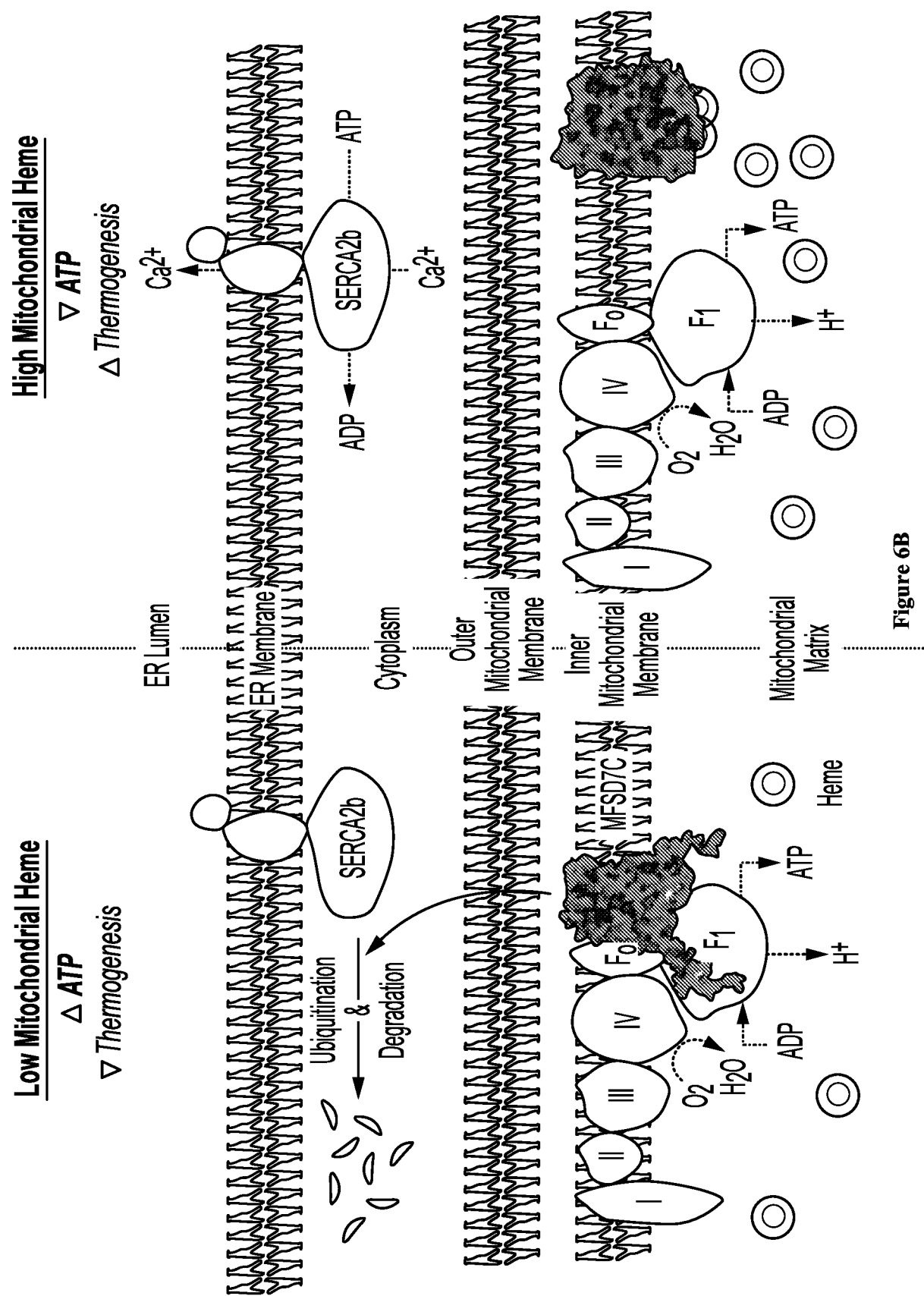


Figure 6B



Figure 7A

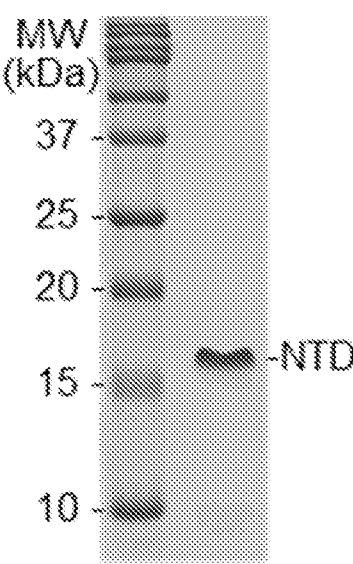
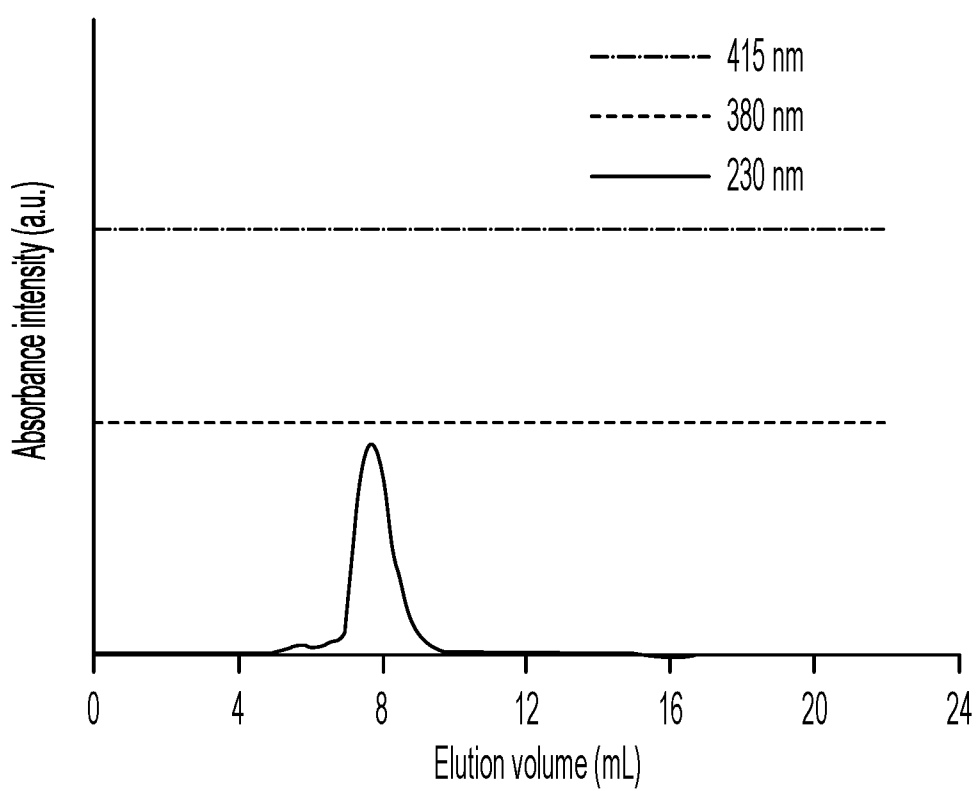
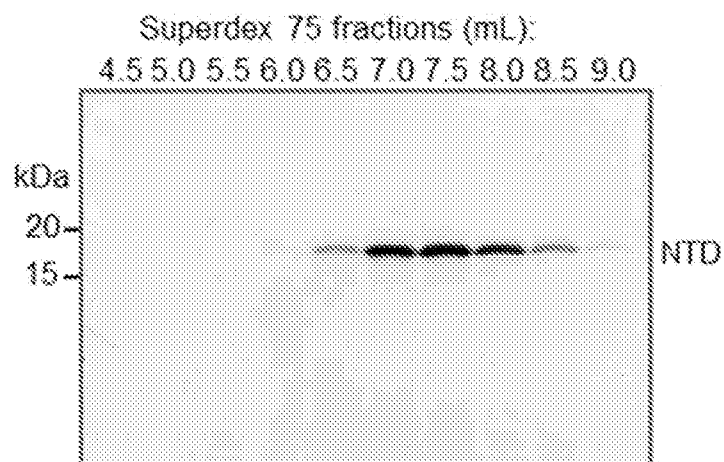
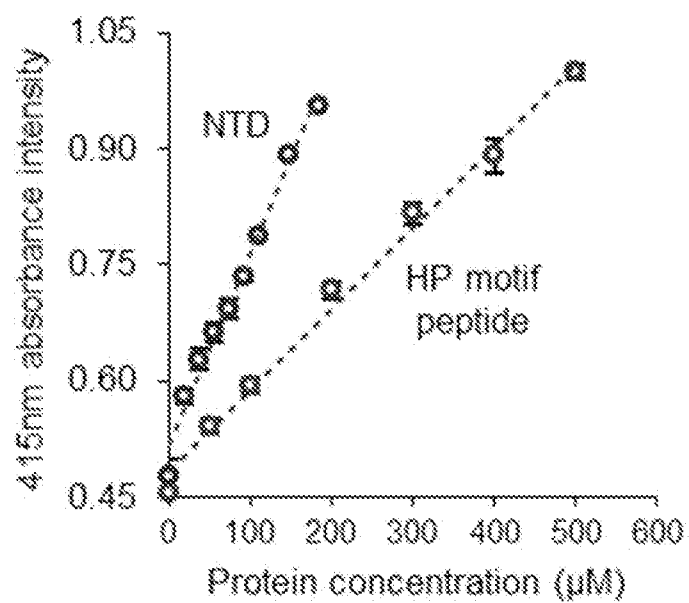
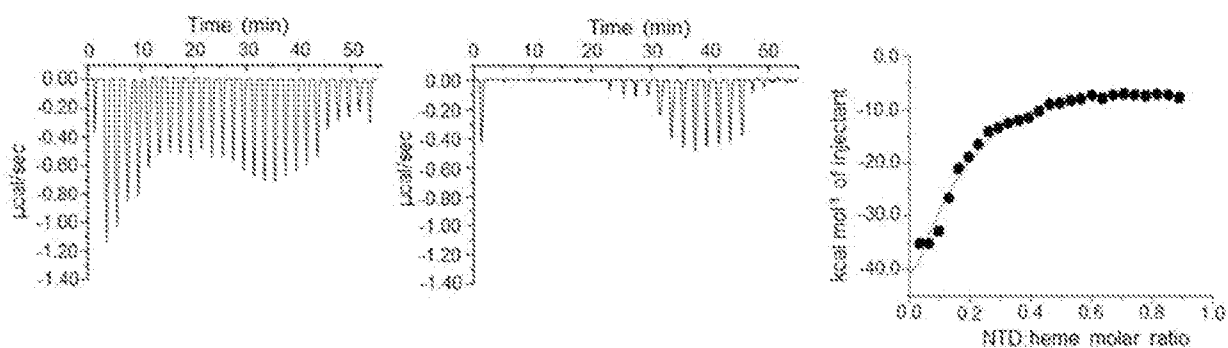
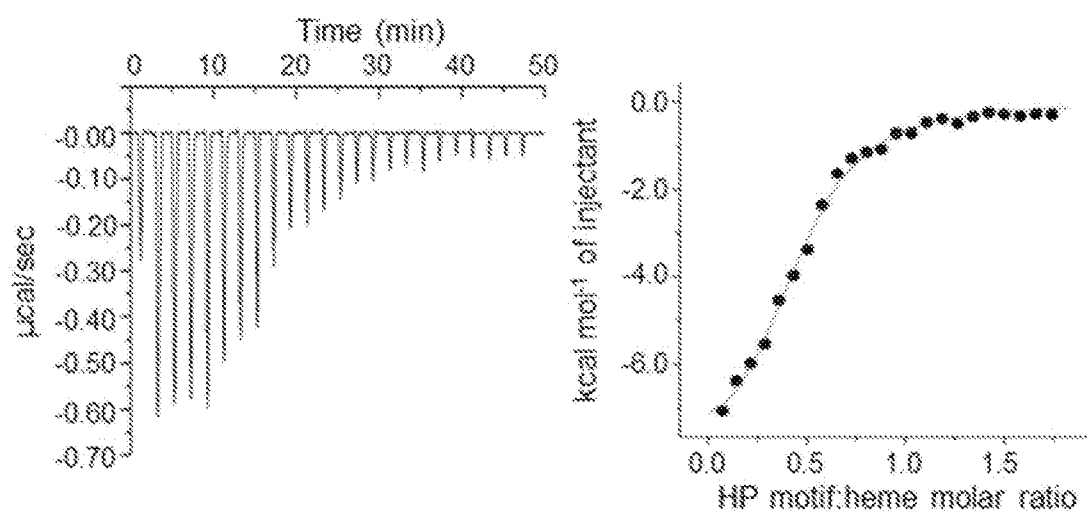
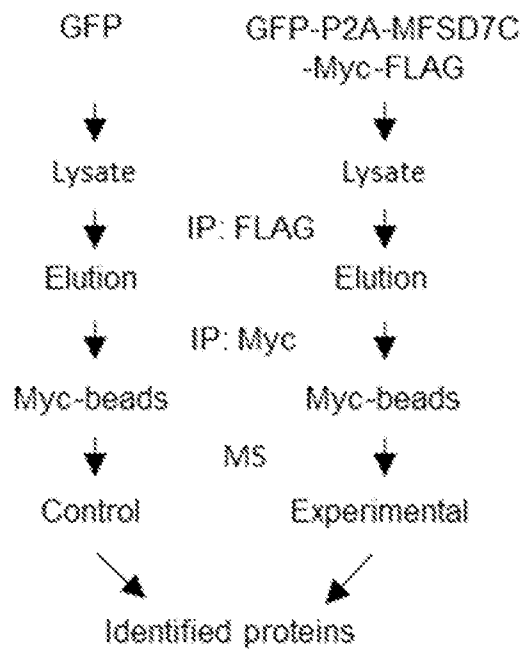
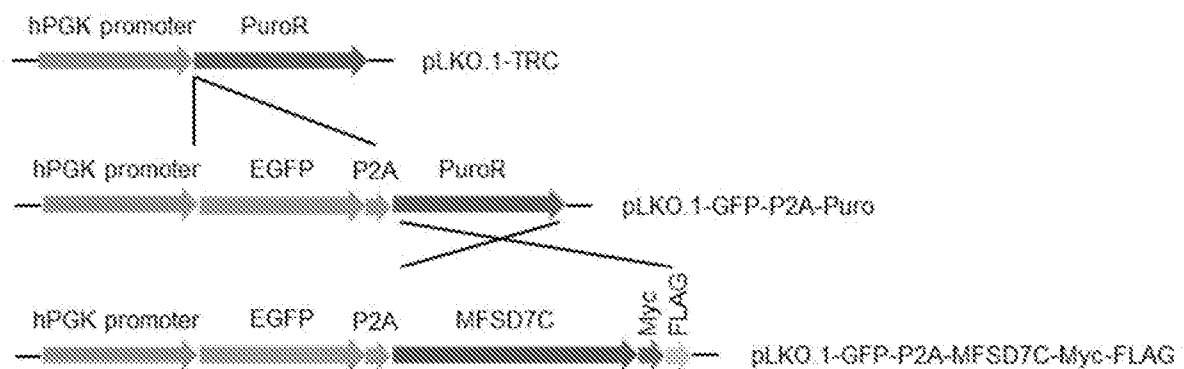


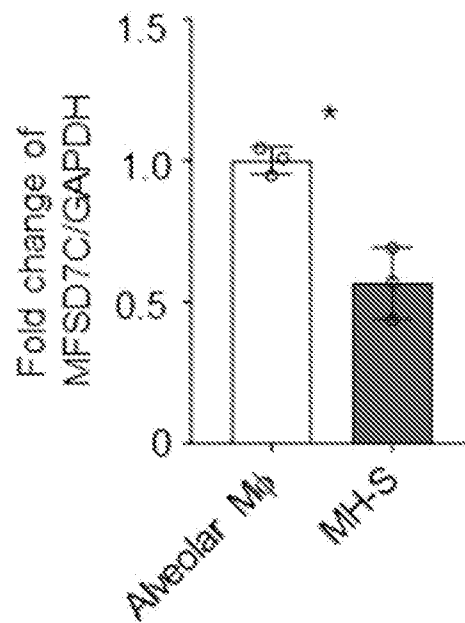
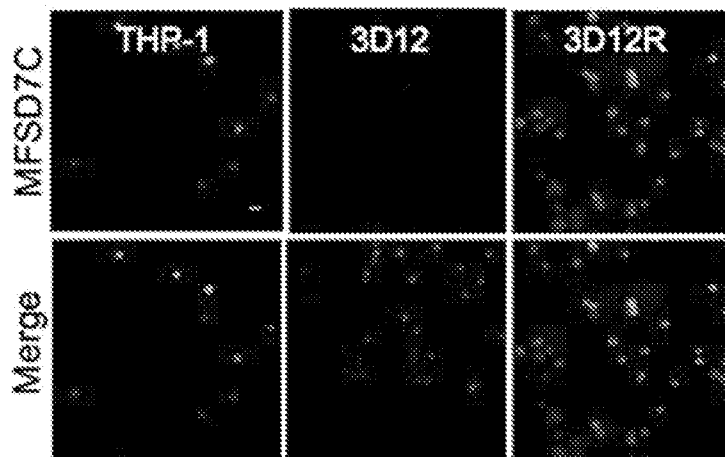
Figure 7B

**Figure 7C**

**Figure 7D****Figure 7E**

**Figure 7F****Figure 7G**

**Figure 8A****Figure 8B**

**Figure 8C****Figure 8D**

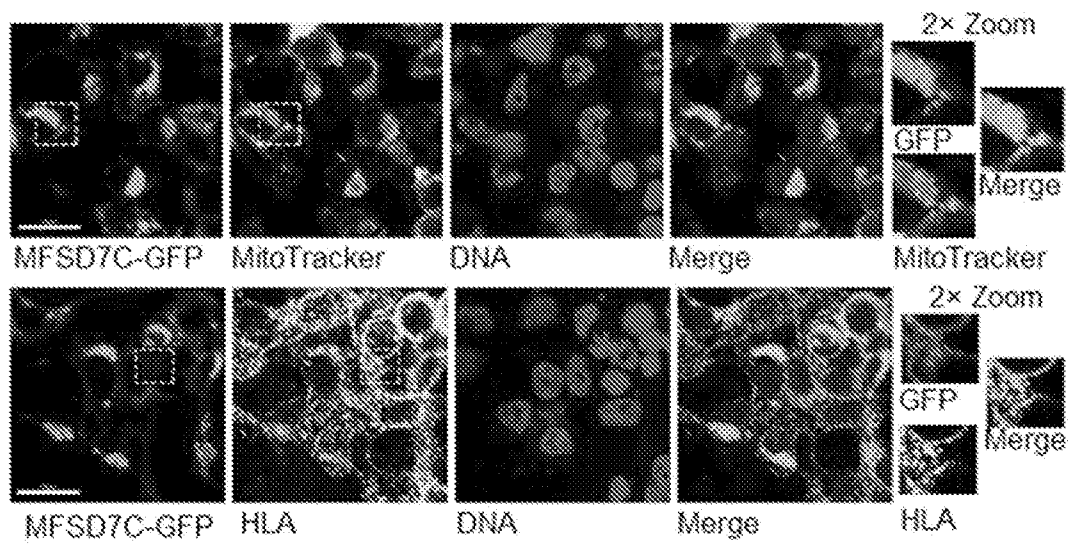


Figure 8E

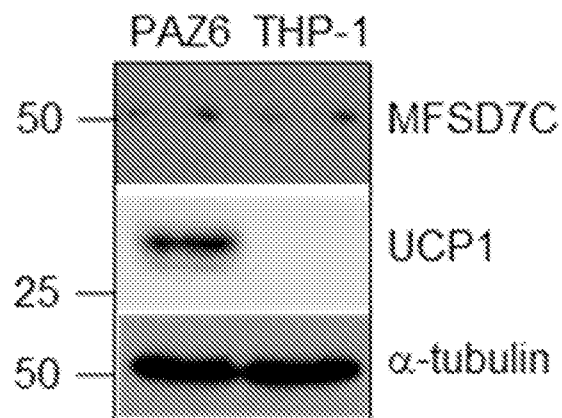


Figure 9A

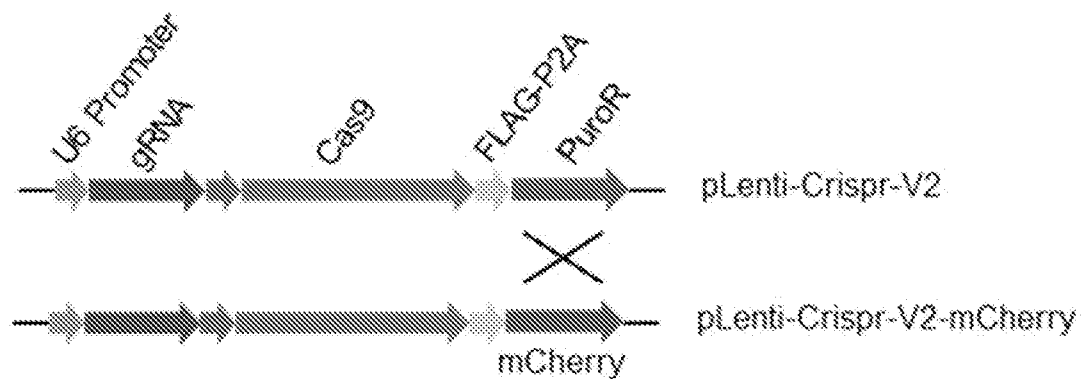


Figure 9B

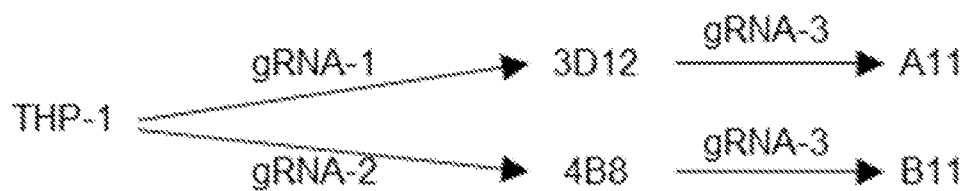
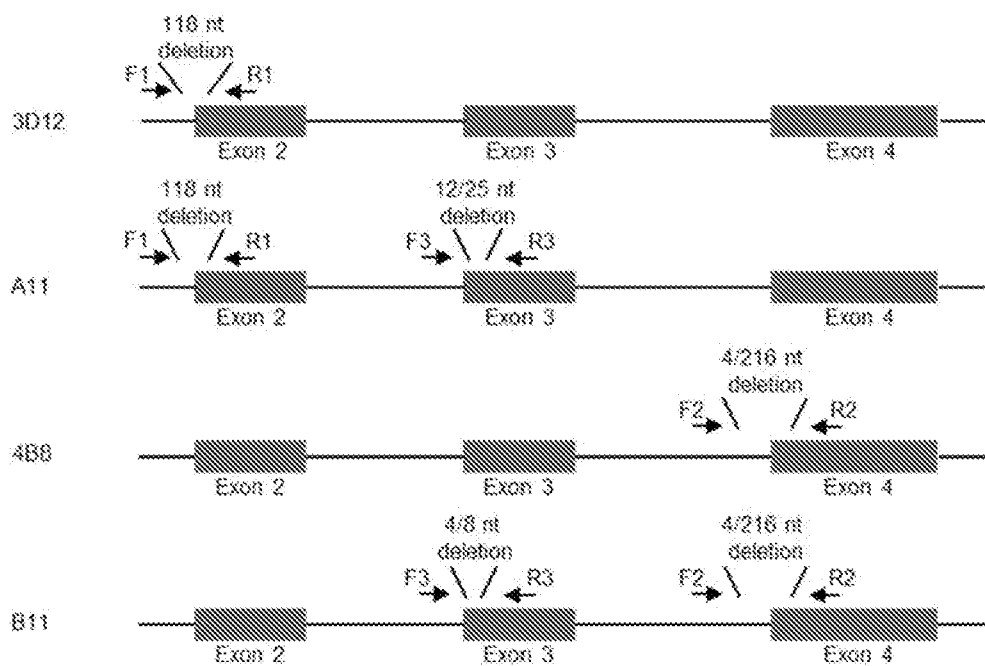


Figure 9C

Clone name	Sequences of gRNA	Sequences of PCR primers
3D12	gRNA-1: CTCGTCCCGGTCTTCAATGT	F1: CCAGATATGGGAGTAGAGGA R1: ACAAAGTGCATAGGACCAGC
4B8	gRNA-2: CTCTTCTGAATCGCATGGTG	F2: TGTCCAGGACTTCTACTGAC R2: GCTCAGAAACACTAACTAGC
A11/B11	gRNA-3: GAACCTAAGTATGAGGCATC	F3: CTGGGTGACAGAGCCGAGACT R3: ACCAACAGGCATTTGTCAGA

Figure 9D**Figure 9E**

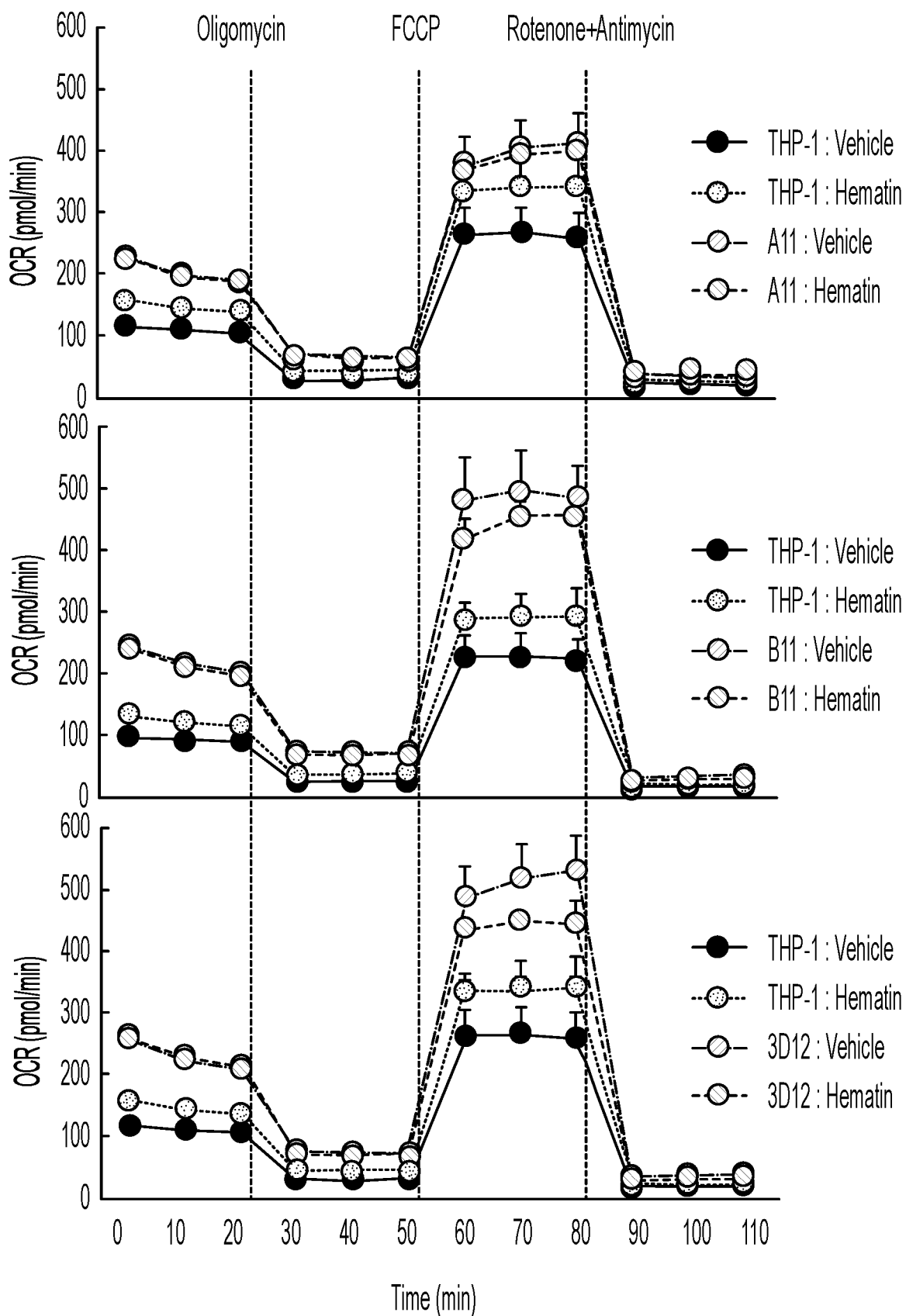


Figure 10A

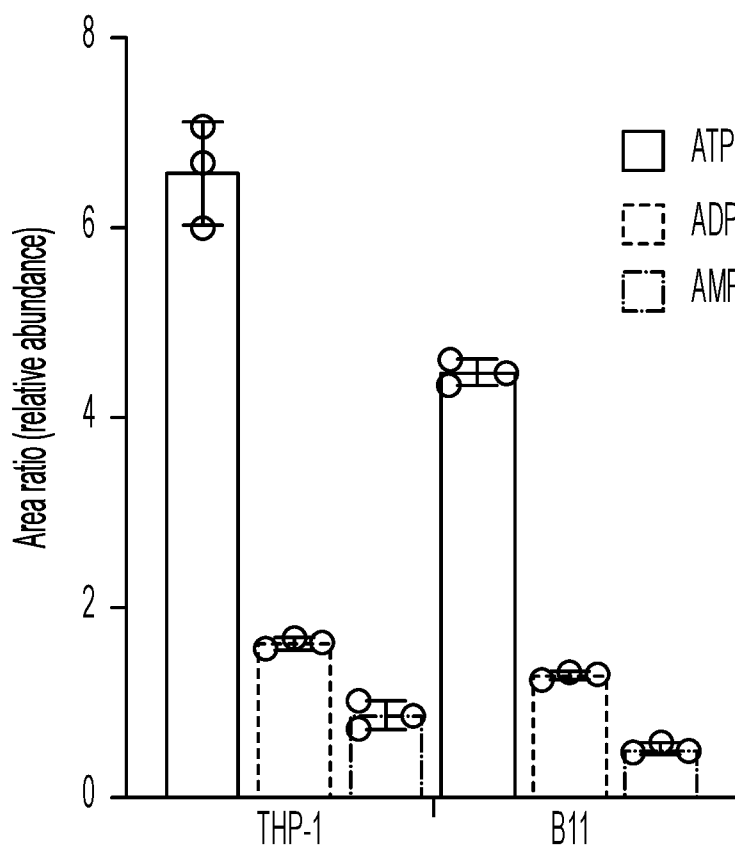


Figure 10B

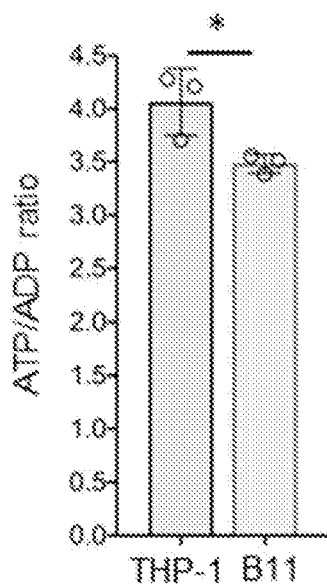


Figure 10C

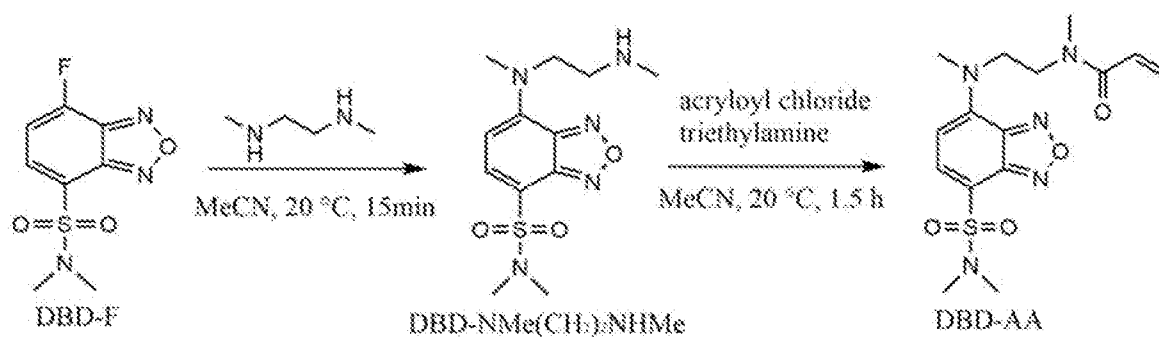


Figure 11A

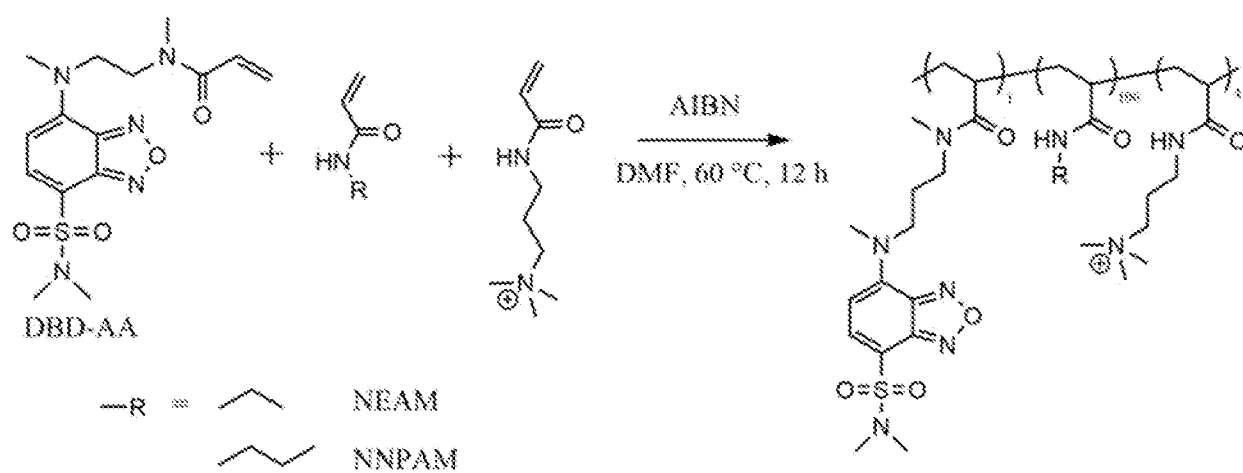


Figure 11B

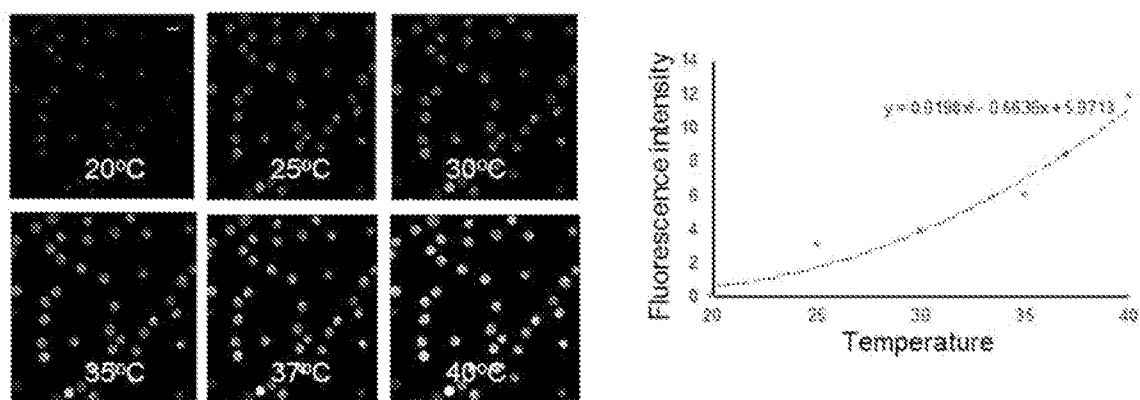


Figure 11C

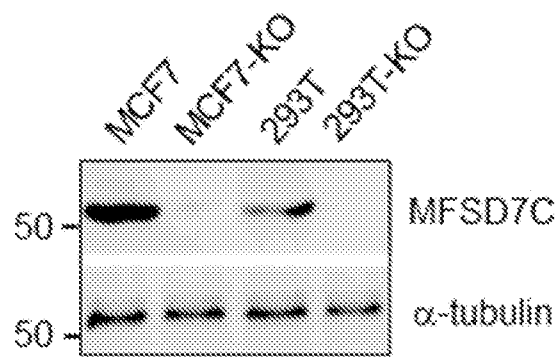
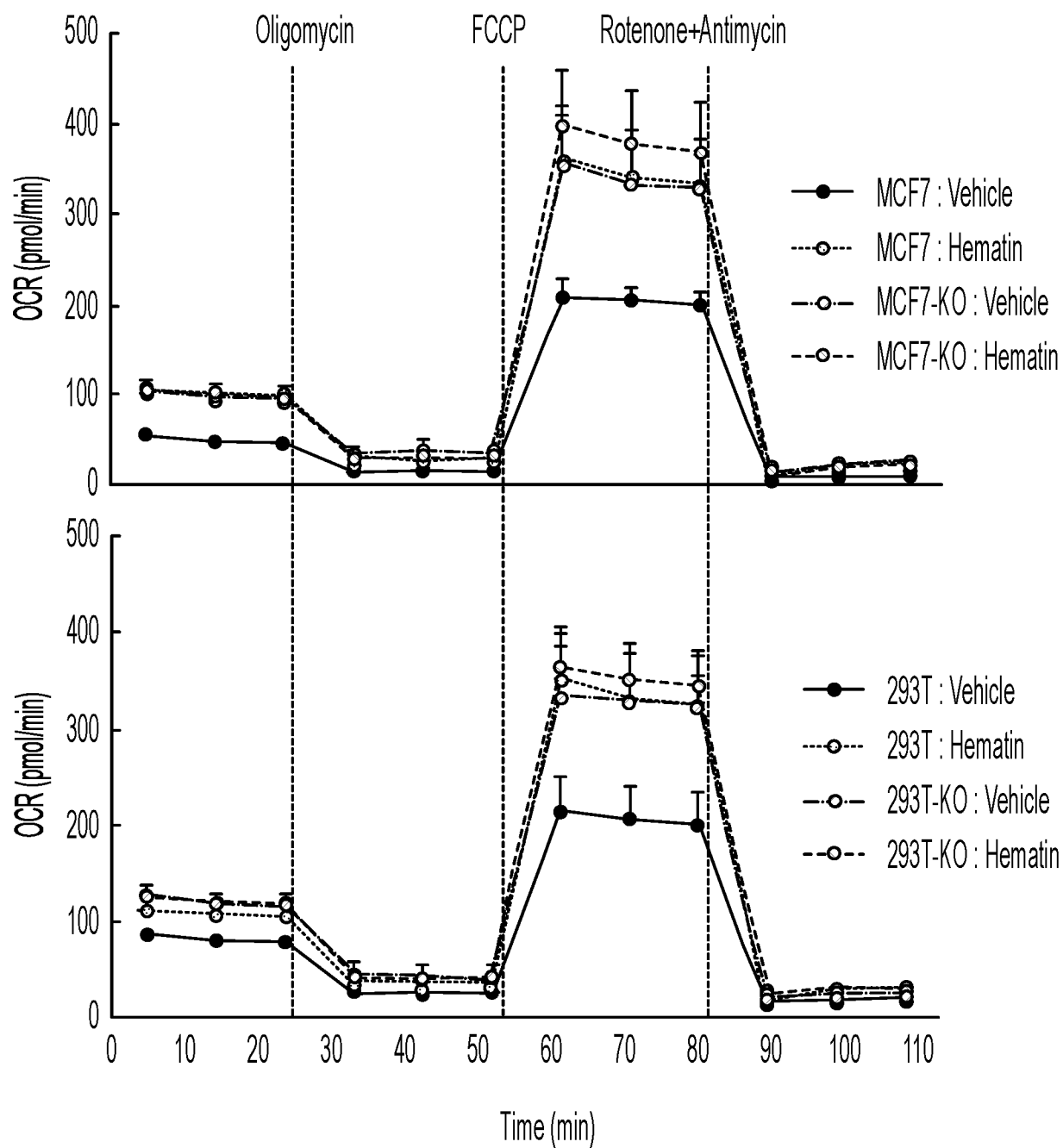


Figure 12A

**Figure 12B**

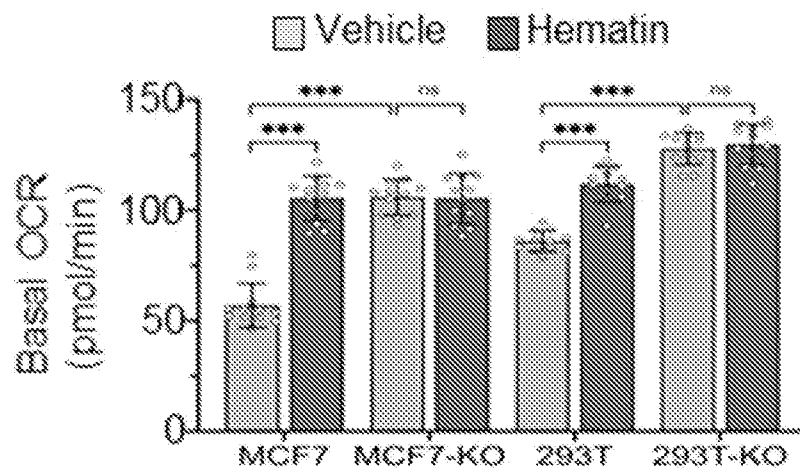


Figure 12C

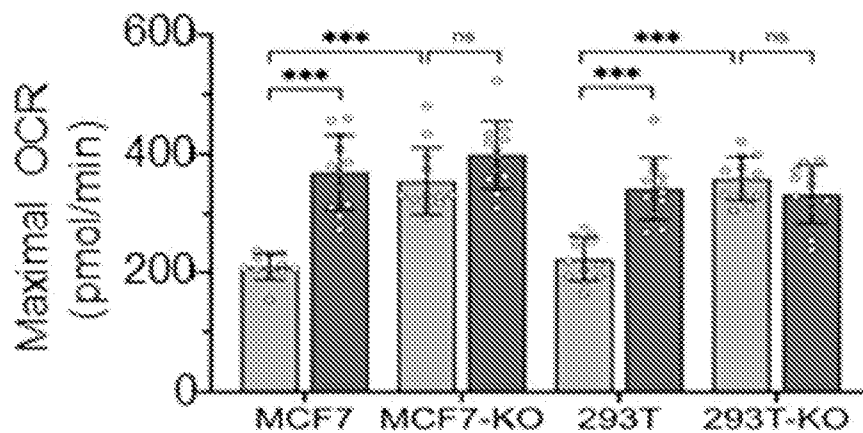
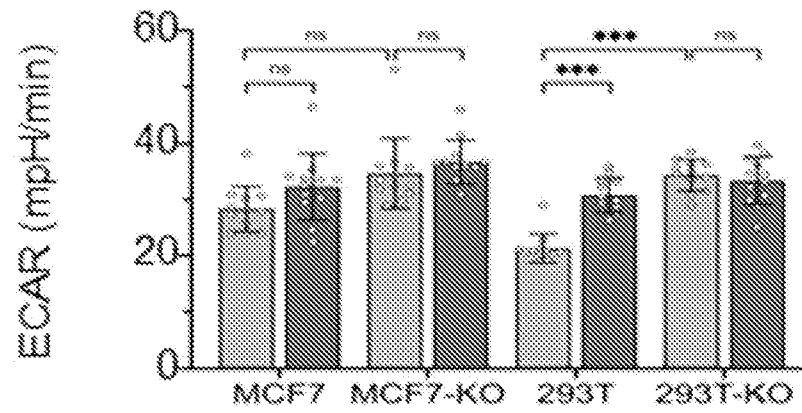
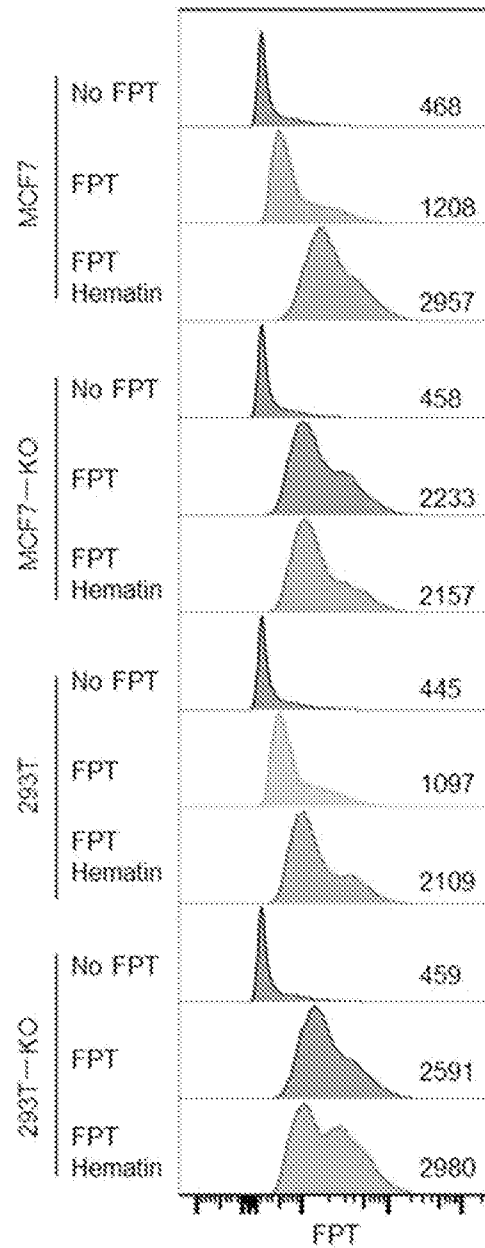


Figure 12D

**Figure 12E**

**Figure 12F**

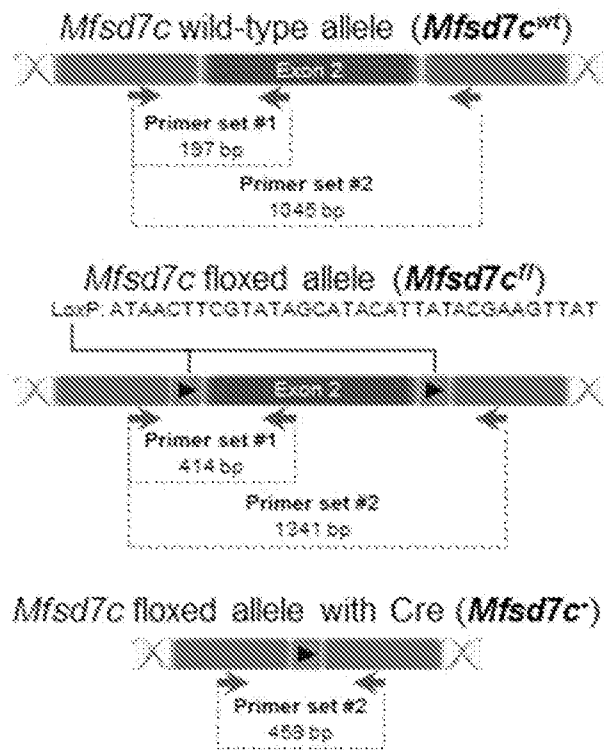


Figure 13A

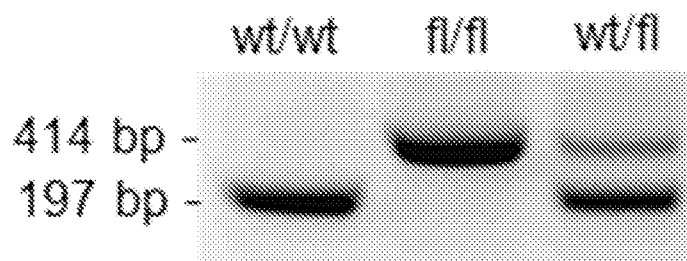


Figure 13B

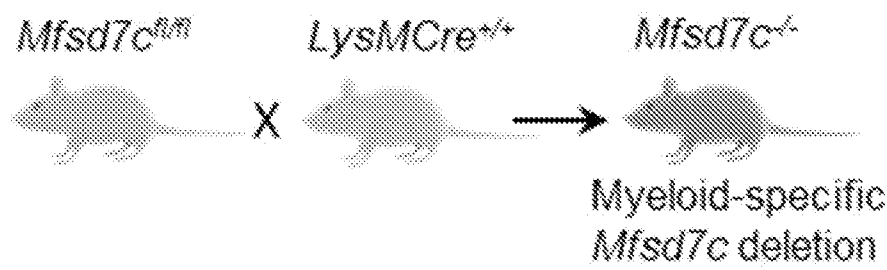


Figure 13C

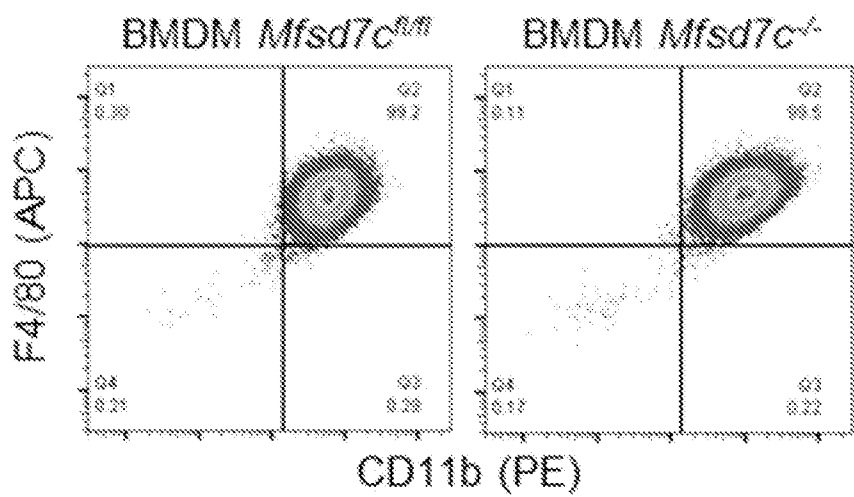


Figure 13D

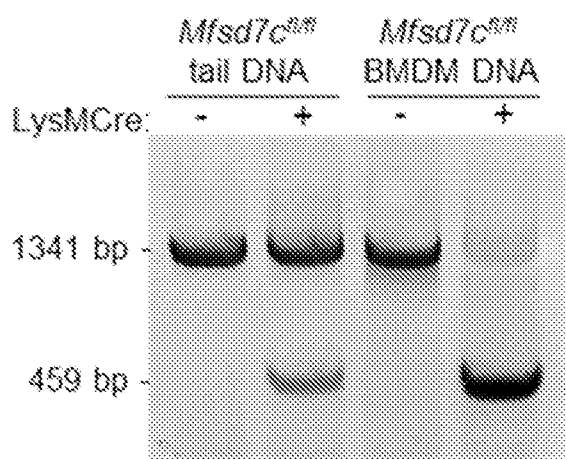
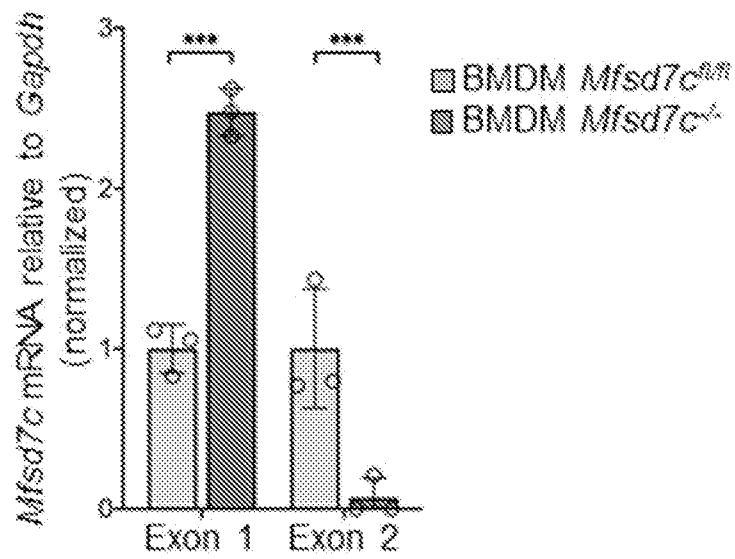
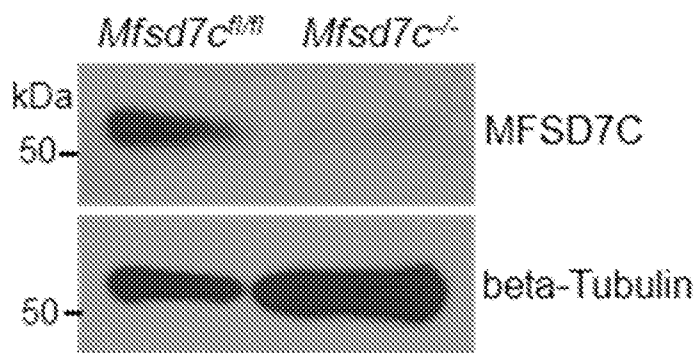
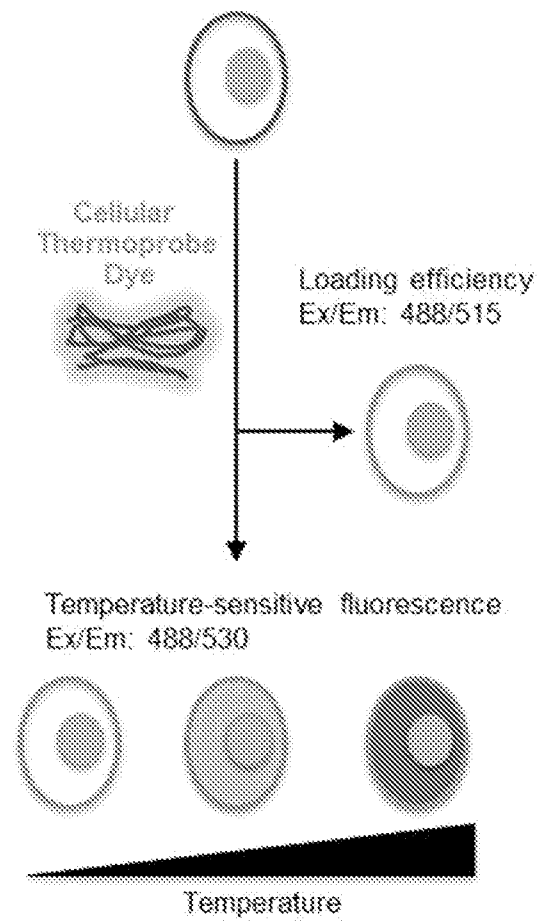
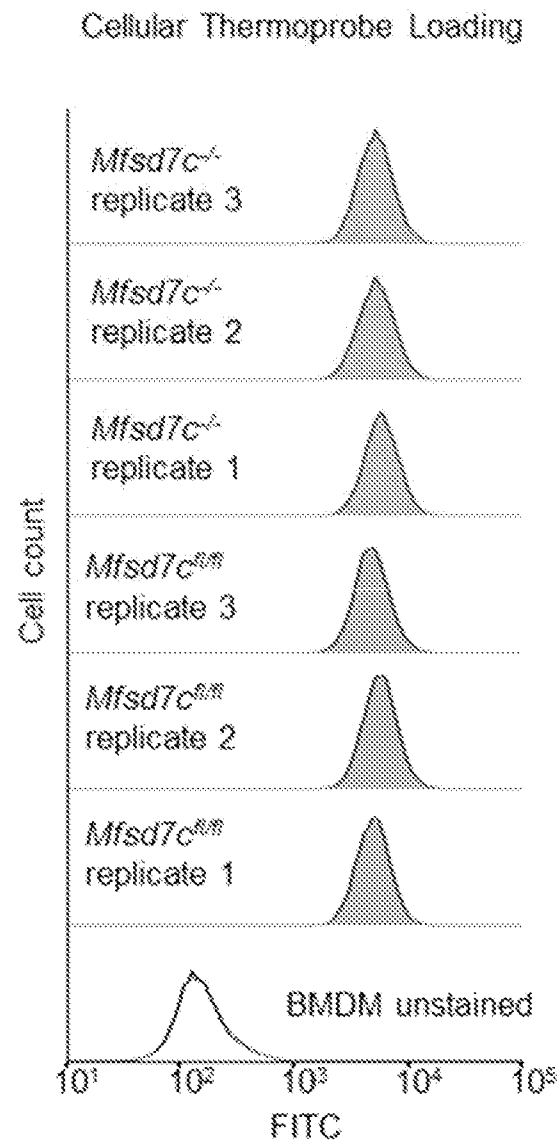


Figure 13E

**Figure 13F****Figure 13G**

**Figure 14A**

**Figure 14B**

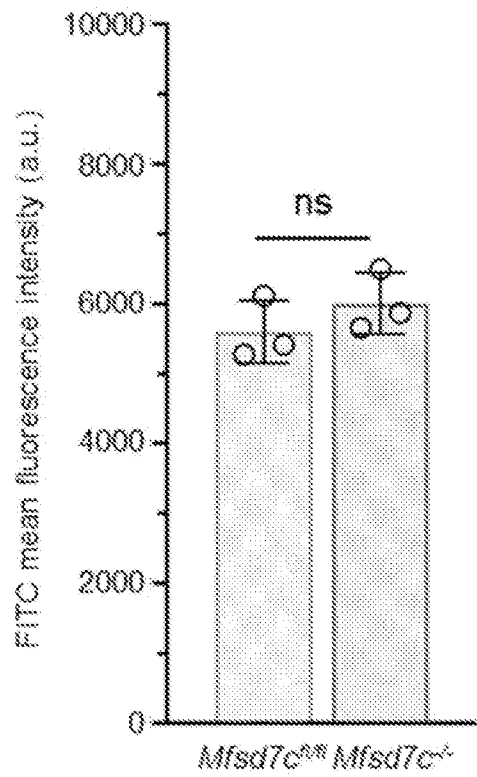


Figure 14C

Temperature-sensitive fluorescence

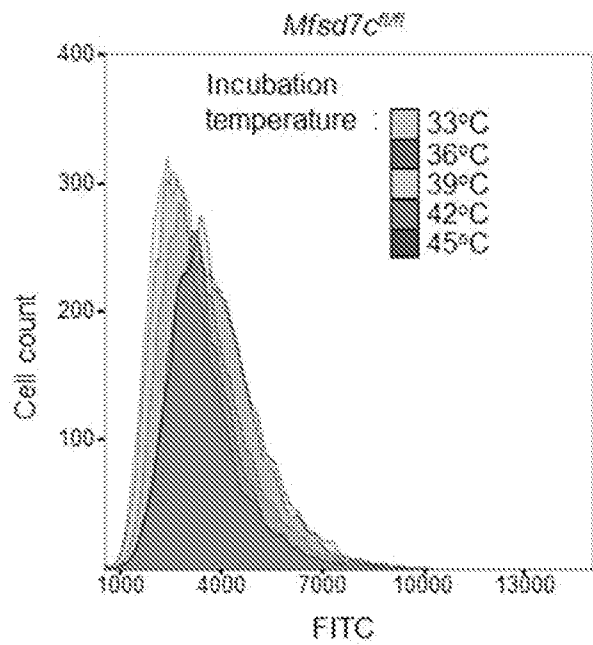
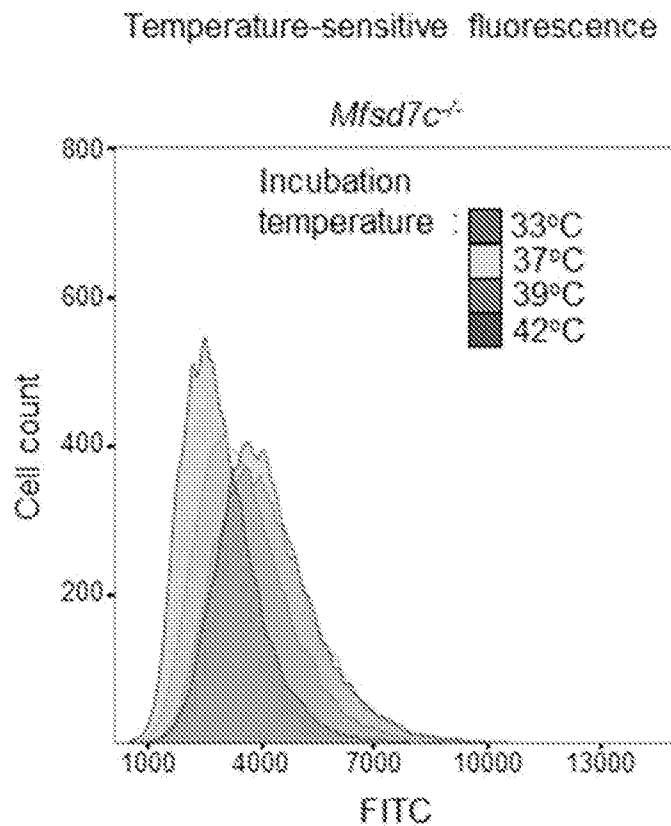
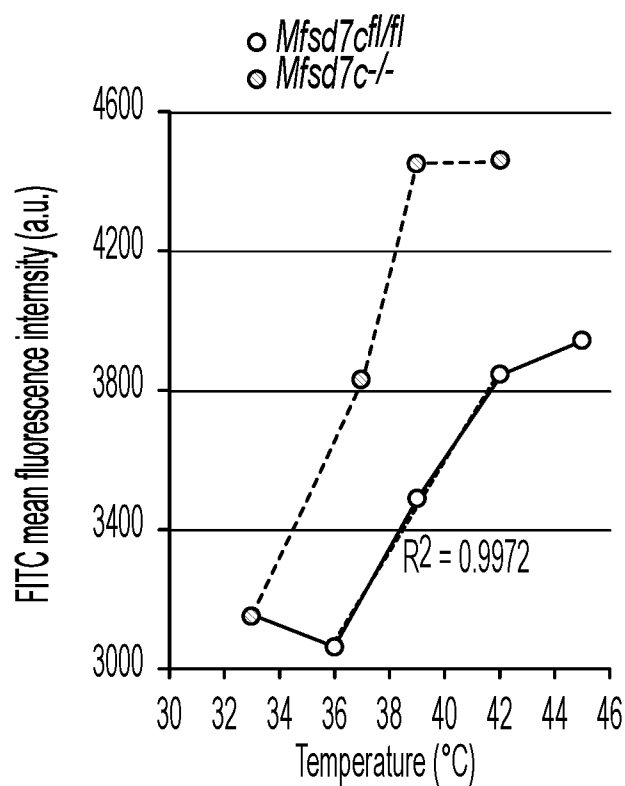


Figure 14D

**Figure 14E****Figure 14F**

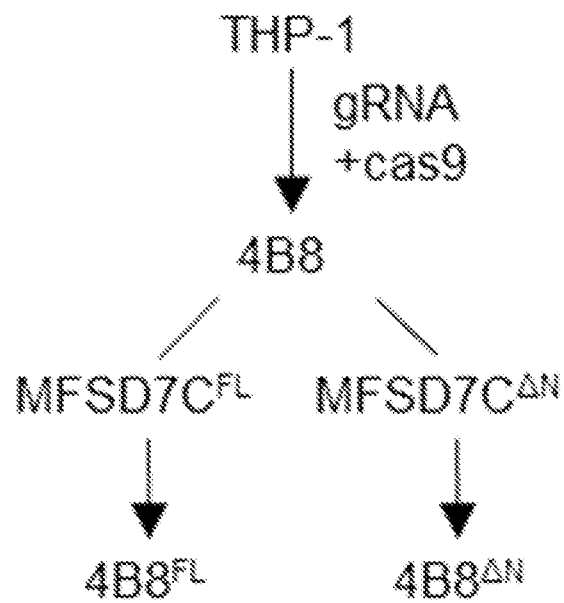


Figure 15A

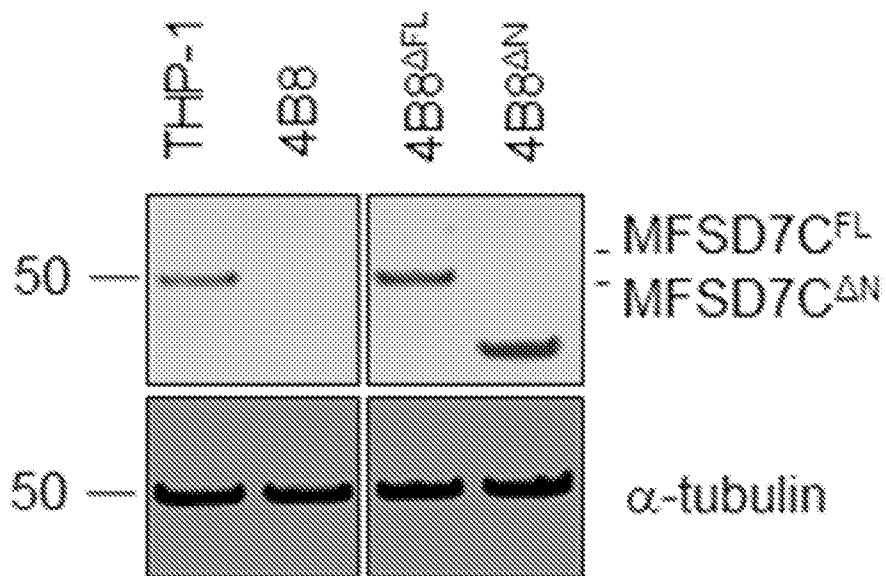


Figure 15B

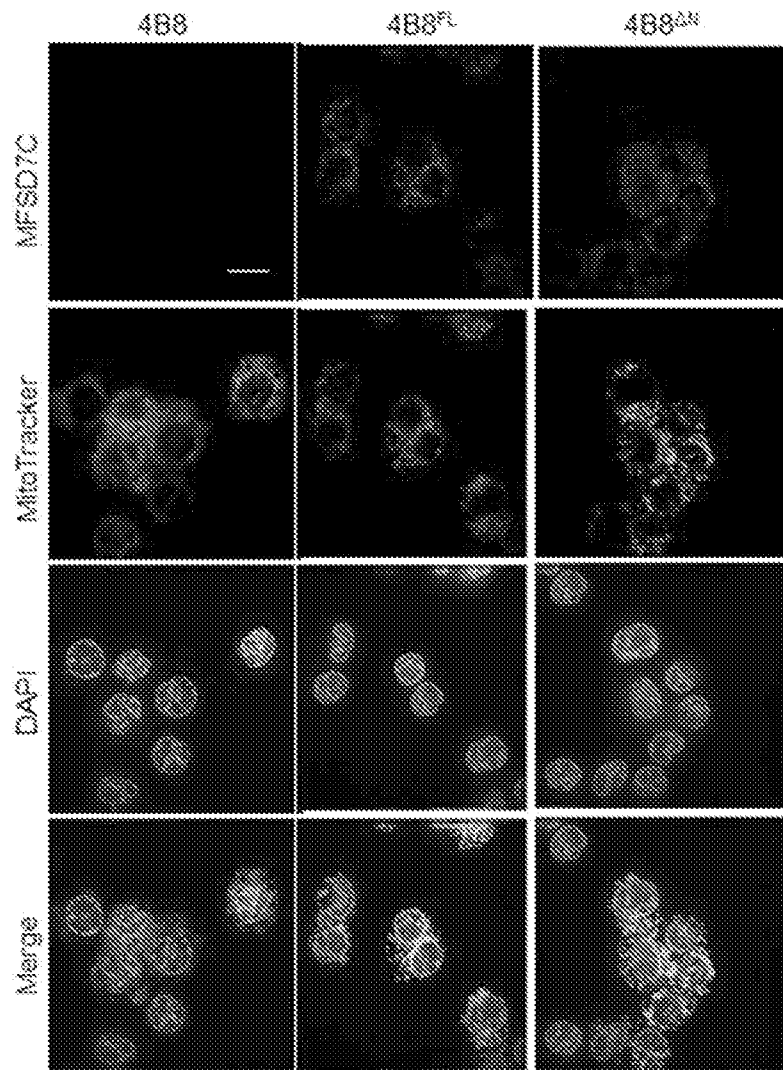
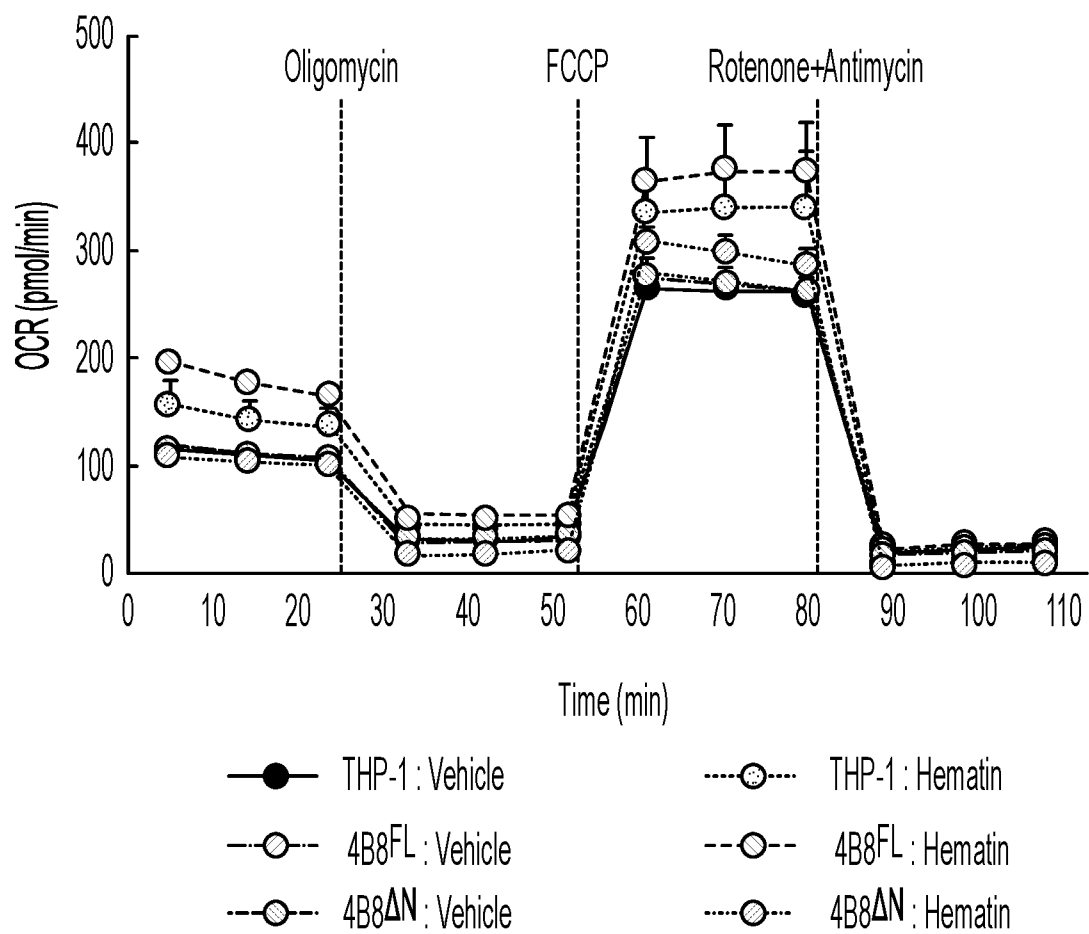
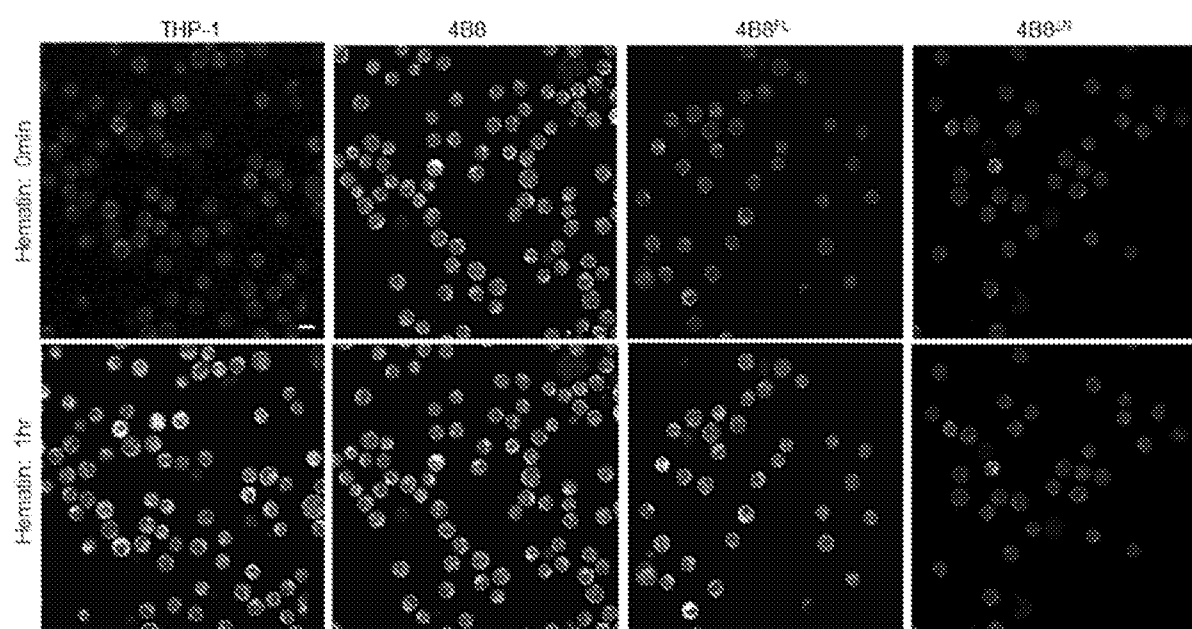
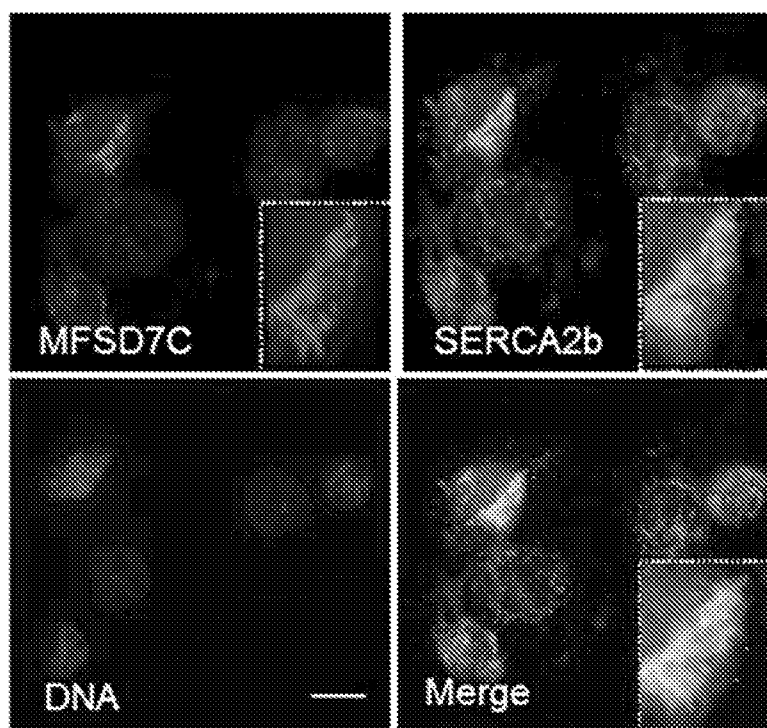
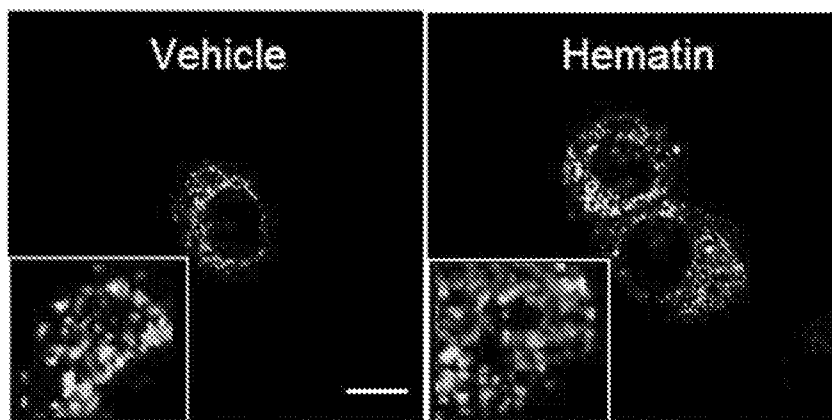
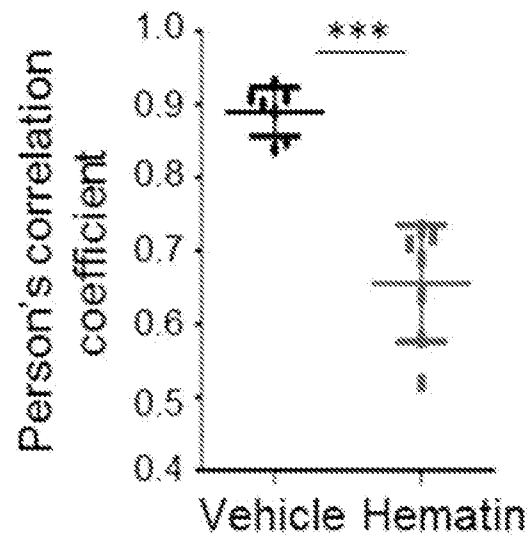
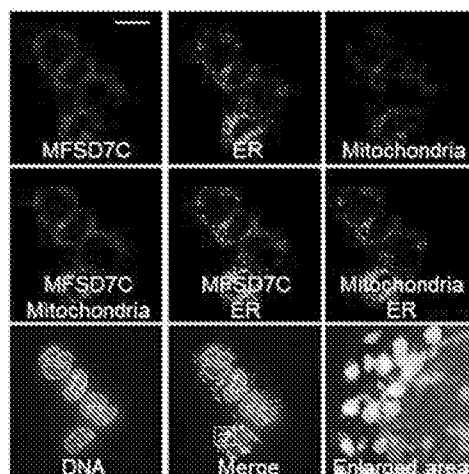


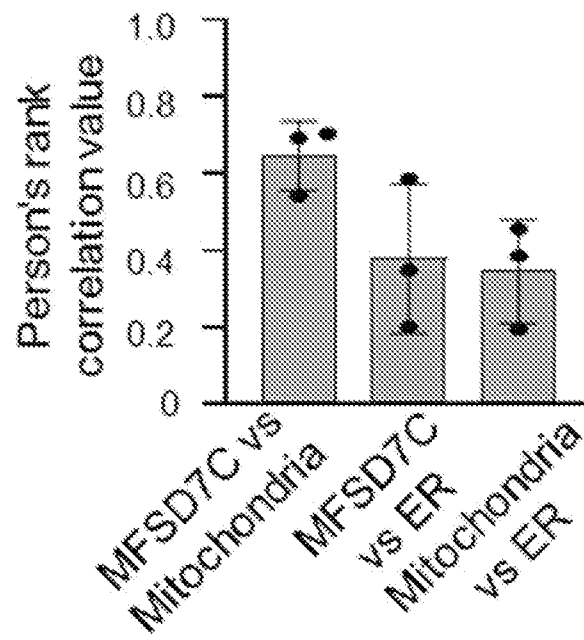
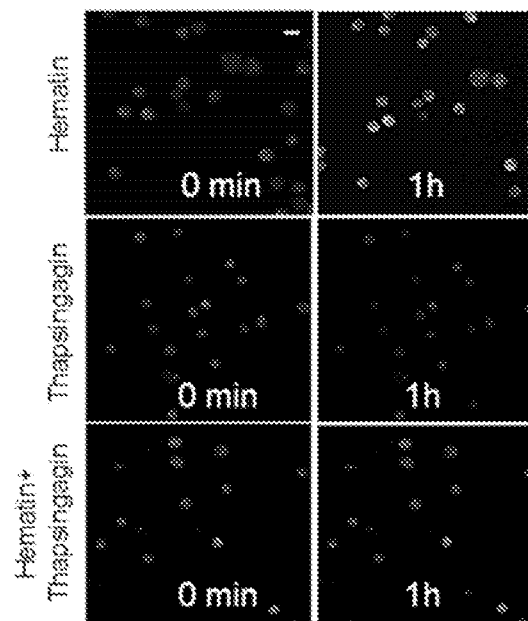
Figure 15C

**Figure 15D**

**Figure 15E**

**Figure 16A****Figure 16B**

**Figure 16C****Figure 16D**

**Figure 16E****Figure 16F**

Classification	Protein	Localization
Heme metabolism	Mfsd7c, Hmox1, Tfrc	N/A
Mitochondrial respiration	CYC1, NDUFA4, COX4l1, Atp5h, Atp5c1	Mitochondria
Adenine nucleotide translocase	Slc25a4, Slc25a5	Mitochondria
Calcium signaling	Atp2a2, Canx, Ser4, Hax1, Slc3a2	N/A
Metabolism	Acsf5, Elovl1, Mlhfd1l, Cds2, Asph	Mitochondria
Other mitochondrial proteins	Dnaja1, Immt, TIM50, Afg3l2, Phb, Dnaja3, Tmx3, Rdh13, Sqrdl, Tspo	Mitochondria
Fc receptor and Integrin	Fcgr1g, Fcgr3, Fcgr1, Itgb2	Plasma membrane

Figure 17